CLIV. THE SPECIFICITY OF THE ACTION OF LACTIC ACID BACTERIA ON THE PHOSPHOGLYCERIC ACIDS

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THE specificity of the form of the 2- and 3-phosphoglyceric acids decomposed by lactic acid bacteria has not yet been demonstrated, although the production of phosphoglycerate from glucose was recently proved by Stone & Werkman [1937] with *Lactobacillus pentoaceticus* and *Lactobacillus plantarum*, and Neuberg & Kobel [1933] ascertained the decomposition of phosphoglycerate into pyruvic acid by *Bacillus delbrückii*.

In the present work experiments were carried out with various strains of lactic acid bacteria, in order to examine whether any selective decomposition of phosphoglycerates occurred and whether the racemization of phosphoglycerates by racemase took place.

Methods

Ba *dl*-2- and Ba *dl*-3-phosphoglycerates were prepared by the methods described by Kiessling [1935] and by Neuberg *et al.* [1928] respectively, and the purifications of these Ba salts were in each case repeated several times until their Ba and P contents had attained nearly the theoretical values.

The Ba salts were dissolved in acetic acid, the Ba removed by 10% Na_2SO_4 and the pH brought to 6.3 by addition of 33% NaOH.

Lactobacillus plantarum (*dl*-acid-former), *Leuconostoc mesenteroides* var. *sake* (*l*-acid-former) and *Lactobacillus sake* (*d*-acid-former) isolated in our laboratory were used in the experiments.

The bacteria were cultivated at 30° for 4 days in flasks containing a medium consisting of 600 ml. koji extract containing 24–30 g. sugar and 30 g. CaCO_3 . The bacterial cells were collected and washed with water on the centrifuge, after being separated from CaCO_3 by decantation. The fresh cells (nearly 1.8 g. dry matter) were suspended in about 50 ml. water.

The bacterial suspension was added to about 80 ml. Na phosphoglycerate solution prepared from 2–3 g. Ba salt, toluene was added and the mixture diluted with water up to 150–200 ml.

After being kept at about 30° for 0.5–3 hr., 50 ml. 40% trichloroacetic acid were added and the mixture centrifuged. The supernatant liquid was concentrated under reduced pressure and neutralized by NaOH after the addition of 5 g. Ba acetate; the phosphoglycerate was then recovered from the solution as crude Ba salt by the addition of two volumes of alcohol.

The crude Ba salt was dissolved in dil. HCl, acidified with 2N H_2SO_4 and filtered. The inorganic phosphate in the solution was precipitated by the addition of NH_4OH and then magnesia mixture. The dephosphorylated solution was again acidified to Congo red by HNO_3 and 25% Ba acetate was added. The filtrate was made weakly acid to litmus with NaOH and the Ba salt precipitated by alcohol.

The Ba salt thus obtained was purified 2–3 times until crystalline precipitates of nearly 90–98 % purity were obtained, by dissolving in dil. HCl which gave a solution faintly acid to Congo red and then adding alcohol drop by drop. The yield recovered was calculated by weighing these crystalline salts, $C_3H_5O_7PBa \cdot 3H_2O$, and the rotatory power (calculated as $[\alpha]_D^{20}$) was determined with its solution in *N* HCl in the 2 dm. tube.

In the case of yeast maceration extract prepared from dried beer yeast, 150 ml. of the extract were first neutralized with $M/2 Na_2CO_3$ and then added to 50 ml. 6 % Na phosphoglycerate, and the experiments were carried out in the presence of toluene.

dl-Phosphoglycerates

By the action of yeast maceration extract upon Na *dl*-2- or -3-phosphoglycerate for 2 hr. at 28° and by the action of fresh cells of various strains of lactic acid bacteria on the 2-phosphoglycerate for 3 hr. at 28° and on the 3-phosphoglycerate for 2.5 hr. at 27°, part of the phosphoglycerate was always decomposed as will be seen in the yields of recovery of the acids given in Table I, and the production of pyruvic acid was observed in all the reacting solutions by the method described by Case [1932].

Table I. *Selective decomposition of dl-2- and -3-phosphoglycerates*

Organism	2-Phosphoglyceric acid		3-Phosphoglyceric acid	
	Yield of recovery %	$[\alpha]_D^{20}$	Yield of recovery %	$[\alpha]_D^{20}$
Yeast maceration extract	45–50	– 23.33	40–45	+ 18.66
<i>dl</i> -Acid-former	30	– 0.73	35	– 0.80
<i>l</i> -Acid-former	40	– 23.98	30	– 14.34
<i>d</i> -Acid-former	40	+ 26.45	30	+ 18.66

As will be seen from Table I, the optical forms of the recovered phosphoglyceric acids were in agreement with those of the lactic acids produced by the organisms which were employed: (–)-2-phosphoglyceric acid (theor. $[\alpha]_D^{20}$ – 24.3°) was recovered with yeast and *l*-acid-former, (+)-2-acid with *d*-acid-former; (+)-3-acid (theor. $[\alpha]_D^{20}$ + 14.5°) with yeast and *d*-acid-former, (–)-3-acid with *l*-acid-former, and with *dl*-acid-former the *dl*-forms of 2- and 3-phosphoglyceric acids were obtained.

Thus it was ascertained that yeast and *d*- and *l*-acid-formers decomposed phosphoglycerates selectively, while no optical specificity was found with the *dl*-acid-former.

Optically active 2-phosphoglycerates

The products of decomposition of *dl*-2-phosphoglycerate by a *d*- or *l*-acid-former were carefully purified. With these optically active Ba salts experiments were carried out in the same manner as was mentioned above.

The selective decomposition of the 2-phosphoglycerates by the bacterial cells is clearly shown in Table II: (–)-2-phosphoglyceric acid was decomposed by the *d*-acid-former but never by the *l*-acid-former with which only the optical antipode ((+)-isomeride) was easily attacked.

The results of the experiments shown in Table II not only showed that both forms of 2-phosphoglycerate were decomposed by the *dl*-acid-former as would be expected from the results shown in Table I, but also suggested that the bacterial cells caused racemization of the 2-phosphoglycerate, since the *dl*-form of

2-phosphoglyceric acid was always obtained from optically active acids by the actions of the *dl*-acid-former.

Table II. *Decomposition of (+)- and (-)-2-phosphoglyceric acids*

Lactic acid bacteria	Period of reaction hr.	Temp. of reaction	Yield of recovery %	$[\alpha]_D^{20}$
(-)-2-Phosphoglyceric acid				
<i>dl</i> -Acid-former	0.5	26.5	19.00	- 1.67
<i>l</i> -Acid-former	2.5	26	98.56	- 22.50
<i>d</i> -Acid-former	3.0	26	20.00	- 22.50
(+) -2-Phosphoglyceric acid				
<i>dl</i> -Acid-former	0.5	27	22.13	+ 1.67
<i>l</i> -Acid-former	2.5	26	21.21	+ 26.67
<i>d</i> -Acid-former	—	—	—	—

Optically active 3-phosphoglycerates

With the optically active 3-phosphoglycerates prepared from the *dl*-compound by the action of *d*- and *l*-acid-formers, the experiments shown in Table III were carried out.

Table III. *Decomposition of (-)- and (+)-3-phosphoglyceric acids*

Lactic acid bacteria	Period of reaction hr.	Temp. of reaction	Yield of recovery %	$[\alpha]_D^{20}$
(-)-3-Phosphoglyceric acid				
<i>dl</i> -Acid-former	0.5	30	52.43	- 0.83
<i>l</i> -Acid-former	2.5	26	93.32	- 13.33
<i>d</i> -Acid-former	0.5	25.5	79.86	- 13.33
(+) -3-Phosphoglyceric acid				
<i>dl</i> -Acid-former	0.5	30	46.88	+ 0.83
<i>l</i> -Acid-former	2.5	25.5	15.04	+ 14.17
<i>d</i> -Acid-former	2.5	26	95.96	+ 15.83

It is clearly seen from Table III that the *l*-acid-former decomposed only (+)-3-phosphoglyceric acid and the *d*-acid-former only (-)-3-phosphoglyceric acid, since (+)- and (-)-3-phosphoglyceric acids were never attacked by *d*- and *l*-acid-formers respectively.

With the *dl*-acid-former, both forms of 3-phosphoglyceric acid were decomposed and *dl*-3-phosphoglycerate was always found to be produced from any form of 3-phosphoglyceric acid.

Thus it was clearly demonstrated that the *l*-acid-former decomposed only (+)-2- and (+)-3-phosphoglyceric acids, whereas the (-)-2- and (-)-3-isomerides were selectively attacked by the *d*-acid-former.

These specificities of the lactic acid bacteria are in disagreement with that of yeast maceration extract which was found to effect the same selective decomposition of 2- and 3-phosphoglyceric acids as that observed for muscle extract by Meyerhof & Kiessling [1935] and by Kiessling & Schuster [1938].

When the *d*-acid-former was compared with muscle extract by which *d*-lactic acid would be produced, the specificity of 2-phosphoglycerate was found to be quite different, although the same (-)-form of 3-phosphoglyceric acid was selectively decomposed by the muscle and the *d*-lactic acid-former.

It is therefore suggested that Meyerhof's scheme of lactic acid formation, based upon experiments with muscle extract, would have to be modified as regards the behaviour of the phosphoglycerates before it could be applied to the fermentations produced by lactic acid bacteria.

Action of racemase upon phosphoglyceric acids

From the experimental results mentioned above, it was concluded that all the forms of 2- and 3-phosphoglycerates were decomposed by the *dl*-acid-former and it was suggested that *dl*-phosphoglyceric acids were always obtained from any one of the optically active components of 2- and 3-phosphoglyceric acids by the action of the bacterial cells.

When 0.4 g. Ba salt of the *dl*-product obtained from (+)-3-phosphoglyceric acid by the *dl*-acid-former (see Table III) was decomposed by *d*-acid-former, 0.0318 g. Ba salt of (+)-3-phosphoglyceric acid was produced as was expected from the results shown in Table I, since the specific rotation of the active salt was observed to be $+707.5^\circ$ (theor. $[\alpha]_D^{20} + 745^\circ$) by the molybdate method described by Meyerhof & Schulz [1938].

Therefore it is suggested that the *dl*-acid-former can catalyse racemization of 2- and 3-phosphoglyceric acids.

In order to ascertain whether the racemization of these phosphoglyceric acids would be effected by racemase in the bacterial cells, as had been already observed by us [1936; 1937; 1938, 1, 2] with the lactic acids, experiments were carried out at 30° for 2.5 hr. with the resting cells and with the maceration extract of dried cells of the *dl*-acid-former which were prepared as follows: 3.5 g. dried bacteria, dehydrated under reduced pressure over H_2SO_4 , were added to 15 ml. water, kept at 30° for 3 hr. in the presence of toluene and centrifuged.

Table IV shows that the optical properties of the recovered phosphoglyceric acids were quite different in the two cases, since (–)-2- and (–)-3-phosphoglyceric acids were obtained with the maceration extract as was observed with the *l*-acid-former, whereas *dl*-phosphoglyceric acids were always obtained by the resting cells as was mentioned above.

Table IV. *Effect of racemase*

	<i>dl</i> -2-Phosphoglyceric acid		<i>dl</i> -3-Phosphoglyceric acid	
<i>dl</i> -Acid-former	Yield of recovery %	$[\alpha]_D^{20}$	Yield of recovery %	$[\alpha]_D^{20}$
Resting cells	30	– 0.73	35	– 0.8
Maceration extract	28.2	– 25.00	21.3	– 16.66

It is worthy of note that the same (–)-2-phosphoglyceric acid ($[\alpha]_D^{20} - 23.33^\circ$) was recovered when the maceration extract of the *dl*-acid-former was allowed to act at 30° for 2 hr. on (–)-2-phosphoglyceric acid, whilst the racemic product was obtained with the resting cells (see Table II).

Thus it was ascertained that the action of racemase was not limited to lactic acids, since the racemization of 2- and 3-phosphoglycerates was effected by the enzyme.

SUMMARY

1. Selective decomposition of phosphoglycerates was observed with yeast maceration extract, a *d*-acid-former (*Lactobacillus sake*) and a *l*-acid-former (*Leuconostoc*).

2. With yeast maceration extract (+)-2- and (-)-3-phosphoglyceric acids were decomposed, a *l*-acid-former decomposed (+)-2- and (+)-3-phosphoglyceric acids and with the *d*-acid-former (-)-2- and (-)-3-phosphoglyceric acids were decomposed.

3. No optical specificity was found with resting cells of a *dl*-acid-former (*Lactobacillus plantarum*) by which all the forms of 2- and 3-phosphoglyceric acids were decomposed, while the same specificity as that of the *l*-acid-former was observed with maceration extract of the *dl*-acid-former.

4. It was found that racemization of 2- and 3-phosphoglyceric acids was produced by racemiase.

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