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Aerobic Formation of Acetate from Pyruvate by *Lactobacillus bulgaricus*

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Abstract

The pathway of formation of acetate from pyruvate in the homofermentative organism *L. bulgaricus* was studied. Three pathways for the formation of acetate were investigated. These were the formation of acetyl CoA by the pyruvate dehydrogenase (lipoate) system, the formation of acetaldehyde by pyruvate decarboxylase, and the formation of acetyl phosphate by pyruvate oxidase. The first two pathways were eliminated when it was found that the formation of acetate was not inhibited by arsenite and that acetaldehyde was not converted to acetate by *L. bulgaricus*. The formation of acetyl phosphate and acetate by dialysed cell-free extracts indicated the presence of pyruvate oxidase in *L. bulgaricus*. The pyruvate oxidase system, unlike the pyruvate dehydrogenase (lipoate) system, was not inhibited by unsaturated fatty acids. The organism was shown to possess both acetate kinase and phosphate acetyltransferase which suggested that acetyl phosphate could be converted to acetate or acetyl CoA.

Introduction

Lactobacillus bulgaricus is used extensively in the manufacture of cultured dairy products, notably yoghurt and cheese. It is a homofermentative organism which employs glycolysis for energy production. Glucose is fermented largely to lactic acid although small amounts of acetic acid and CO_2 are also produced (Dirar and Collins 1972). The mechanism of acetate formation in L. *bulgaricus* is unknown.

Three mechanisms have been identified in bacteria by which pyruvate can be oxidized to acetate and CO_2. The initial reactions for each of these mechanisms are given below.

(1) Via the pyruvate dehydrogenase (lipoate) complex (EC 1.2.4.1):

$$\text{pyruvate}+\tfrac{1}{2}O_2+\text{HSCoA}\rightarrow\text{acetyl CoA}+CO_2+H_2O$$

(2) Via pyruvate decarboxylase (EC 4.1.1.1):

$$\text{pyruvate}\rightarrow\text{acetaldehyde}+CO_2$$

(3) Via pyruvate oxidase (EC 1.2.3.3):

$$2\ \text{pyruvate}+O_2+P_i\rightarrow\text{acetyl phosphate}+\text{acetate}+2CO_2+H_2O.$$

The aim of the present investigation was to determine which of these mechanisms was present in *L. bulgaricus* and responsible for the formation of acetate in this organism.

Materials and Methods

Micro-organisms

The organisms used were *Streptococcus lactis* C10 and *Lactobacillus bulgaricus* strains NCDO 1373 (LB1), NCDO 1489 (LB5) and LB2.

Preparation of Cells and Cell-free Extracts

L. bulgaricus strains were grown for 18 h at 37°C in MRS broth (Oxoid, initial pH 6·2), either as static cultures or in a batch fermenter (Pont and Holloway 1968) using 5 M NaOH to maintain the pH at 6·0. *S. lactis* C10 was grown at pH 6·3 for 16 h at 30°C in tryptone–yeast extract–lactose broth which contained tryptone (Oxoid), 30 g; yeast extract (Difco), 10 g; lactose, 5 g; KH_2PO_4, 4 g; 'Lab-Lemco' powder (Oxoid), 2 g and water to 1 litre. The cells were harvested by centrifugation at 5500 *g* for 10 min at 4°C, washed once with 0·9% (w/v) NaCl and resuspended in the same medium at a concentration of 20 mg (dry weight)/ml for respiratory studies or 1 g (wet weight)/ml for the preparation of cell-free extracts and acetone-dried cells.

Cell-free extracts were prepared by extrusion of the cell suspension through a French pressure cell (French and Milner 1955) at a constant pressure of 155 MPa (1575 kg/cm^2) or by sonication of a cell suspension for 90 s in a Rapidis Model 150 ultrasonic disintegrator (Ultrasonic Ltd, Shipley, Yorkshire, England). The cell debris was removed by centrifugation at 27 000 *g* at 4°C for 30 min and the supernatant (cell-free extract) was used either without further treatment or after dialysis against 20 mM KH_2PO_4, pH 7·0.

Acetone-dried cells were prepared as described by Gunsalus (1955).

Respiratory Studies

The volumes of carbon dioxide released and oxygen taken up by suspensions of whole cells were measured in a conventional Warburg apparatus at 37°C. The reaction mixture (total volume 3·0 ml) contained (in micromoles): KH_2PO_4, 300 (adjusted to the appropriate pH with 0·1 M NaOH); $MgCl_2$, 5; thiamine pyrophosphate (TPP) 0·1; sodium pyruvate, 60 (unless stated otherwise) and 10 mg (dry weight) of cells. The reaction was initiated by the addition of the pyruvate from the side-arm of the reaction flasks. For anaerobic studies the air in the flasks was replaced with oxygen-free nitrogen.

After incubation for 180 min at 37°C the reaction was stopped by rapidly cooling the flasks in an ice-bath. The contents of each flask were centrifuged at 5000 *g* for 5 min and the supernatant stored at −20°C until analysed for residual pyruvate and for products arising from the pyruvate metabolized.

Estimation of Products

Pyruvate was estimated enzymically using a Sigma diagnostic kit (Sigma Chemical Co., St Louis, Missouri, U.S.A., Cat. No. 726-UV).

Acetate was measured as a volatile acid as described by Anders and Jago (1970).

Acetaldehyde, ethanol, acetoin, diacetyl and *2,3-butylene glycol* were separated and estimated by gas chromatography on Chromosorb 101 by the method of E. H. Ramshaw (personal communication).

Acetyl phosphate was estimated by the method of Lipmann and Tuttle (1945).

Assay of Enzymic Activities

Acetate kinase (EC 2.7.2.1) activity was estimated as described by Lees and Jago (1976*a*).

Phosphate acetyltransferase (EC 2.3.1.8) activity was estimated by the method of Stadtman (1955) and defined as the arsenate-dependent hydrolysis of acetyl phosphate.

Pyruvate oxidase activity was determined using the assay conditions described by Hager *et al.* (1954).

Preparation of Fatty Acids

Fatty acids were added to the reaction mixtures in solution as described by Anders and Jago (1970).

Results

As shown in Table 1 when whole cells of *L. bulgaricus*, strains LB1, LB2 and LB5, were incubated with pyruvate, TPP and $MgCl_2$, acetate and CO_2 were the only products formed. For each micromole of pyruvate consumed 1 μmol of acetate and CO_2 was produced. As expected, approximately 2 molecules of acetate were formed for each molecule of O_2 utilized. The same ratio of products formed to pyruvate and O_2 utilized was obtained when acetone-dried cells replaced whole cells in the reaction mixture.

Table 1. Formation of acetate from pyruvate by *L. bulgaricus*

The incubation mixture (pH 5·5) and assays used are described under Methods

Strain	Pyruvate consumed (μmol)	O_2 uptake (μmol)	CO_2 evolved (μmol)	Acetate formed (μmol)
LB1	56·0	24·7	55·5	51·6
LB1[A]	32·5	14·0	26·7	31·5
LB2	34·0	19·0	40·5	37·5
LB5	25·6	11·0	21·2	21·8

[A] Acetone-dried cells.

As shown in Table 2 increasing the pyruvate concentration in the reaction mixture above 40 mM appeared to saturate the acetate-forming pathway. At a pyruvate concentration of 80 mM the amount of acetate formed did not account for all the pyruvate consumed. Some pyruvate must therefore have been converted into other compounds, possibly lactic acid, as no volatile compounds other than acetate were detected by gas chromatography.

Table 2. Effect of substrate concentration on the metabolism of pyruvate by *L. bulgaricus* LB1

The incubation mixture (pH 5·5) and assays used are described under Methods

Initial pyruvate concn (mM)	Pyruvate consumed (μmol)	O_2 uptake (μmol)	CO_2 evolved (μmol)	Acetate formed (μmol)
20	56·0	24·7	55·5	51·6
40	87·8	37·6	84·2	82·8
80	118·4	33·2	72·9	80·6

The effect of pH on the utilization of pyruvate by *L. bulgaricus*, strain LB1, is shown in Fig. 1. In contrast to the group N streptococci (Anders and Jago 1970) whose utilization of pyruvate was markedly dependent on pH, the utilization of pyruvate by *L. bulgaricus* appeared to be relatively insensitive to a change in pH. The ratio of products formed to pyruvate utilized appeared to be fairly constant over the pH range 4·5–6·5.

It has been shown that acetate production by group N streptococci is completely inhibited by 5 mM oleic acid (Anders and Jago 1970) or 5 mM linoleic acid (Broome 1976). This inhibition of acetate production by unsaturated fatty acids is due to inhibition of the pyruvate dehydrogenase (lipoate) complex in the group N streptococci (Broome 1976). As shown in Table 3, 5 mM linoleic acid inhibited acetate production by *L. bulgaricus* by only *c.* 35%, which suggested that acetate was not produced in this organism via the pyruvate dehydrogenase (lipoate) complex. The

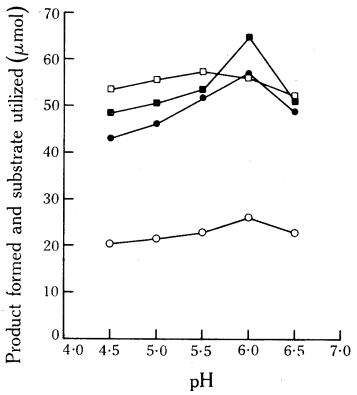


Fig. 1. Effect of pH on the metabolism of pyruvate by resting cells of *L. bulgaricus* LB1. The incubation mixture at pH values indicated and the assays used are described under Methods.
○ O_2 uptake.
● Acetate formed.
□ Pyruvate utilized.
■ CO_2 evolved.

effect of arsenite, a specific inhibitor of the pyruvate dehydrogenase (lipoate) complex, on the production of acetate by *L. bulgaricus* was examined. Since oxygen uptake is directly related to acetate production in group N streptococci and in *L. bulgaricus*, inhibition of the pyruvate dehydrogenase (lipoate) complex by arsenite was determined by measuring oxygen uptake. As shown in Table 4, oxygen uptake in *S. lactis* C10 was markedly inhibited by arsenite, whereas arsenite had no effect on oxygen uptake by *L. bulgaricus*. Thus it was clearly evident that *L. bulgaricus* did not form acetate via the pyruvate dehydrogenase (lipoate) complex.

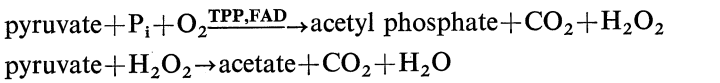
Table 3. Effect of linoleic acid on the metabolism of pyruvate by *L. bulgaricus*
The incubation mixture (pH 5·5) and assays used are described under Methods

Strain	Concn of linoleic acid (mM)	Pyruvate consumed (μmol)	O_2 uptake (μmol)	CO_2 evolved (μmol)	Acetate formed (μmol)
LB1	0	48·0	17·0	46·3	40·8
LB1	5	32·0	12·0	25·1	26·6
LB2	0	34·0	19·0	40·5	37·5
LB2	5	28·0	10·0	21·3	25·0

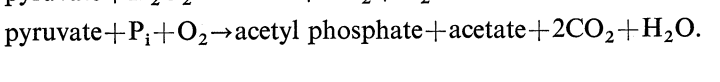
Other pathways by which acetate can be formed were therefore investigated. The first of these concerned the direct formation of acetaldehyde from pyruvate by pyruvate decarboxylase. The formation of acetate from acetaldehyde would depend on the presence of an aldehyde dehydrogenase. Although both whole cells and acetone-dried cells of *L. bulgaricus* LB1 were able to produce acetate from pyruvate (Table 1), they were unable to produce acetate from acetaldehyde. In addition, Lees and Jago (1976*a*) have shown that aldehyde dehydrogenase activity in strain

LB1 is negligible. Thus, *L. bulgaricus* did not appear to use this pathway for the production of acetate.

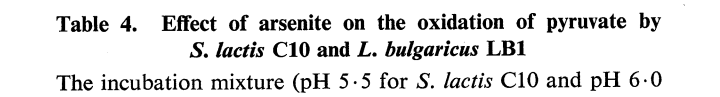
The next system to be investigated was the pyruvate oxidase system described in *L. delbrueckii* by Hager *et al.* (1954). The reaction sequence is as follows:



$$\text{pyruvate}+P_i+O_2 \xrightarrow{\text{TPP,FAD}} \text{acetyl phosphate}+CO_2+H_2O_2 \quad (1)$$



$$\text{pyruvate}+H_2O_2 \rightarrow \text{acetate}+CO_2+H_2O \quad (2)$$



$$\text{pyruvate}+P_i+O_2 \rightarrow \text{acetyl phosphate}+\text{acetate}+2CO_2+H_2O. \quad (3)$$

Table 4. Effect of arsenite on the oxidation of pyruvate by *S. lactis* C10 and *L. bulgaricus* LB1

The incubation mixture (pH 5·5 for *S. lactis* C10 and pH 6·0 for *L. bulgaricus* LB1) and assays used are described under Methods

Organism	Sodium arsenite (mM)	O_2 uptake (μmol)	CO_2 evolved (μmol)
S. lactis C10	—	7·76	32·76
	1·0	0·79	9·54
	2·5	0·82	9·06
L. bulgaricus LB1	—	4·77	8·51
	1·0	5·01	7·85
	2·5	5·35	9·14

The ability of dialysed cell-free extracts of *L. bulgaricus* to form acetyl phosphate from pyruvate was investigated. The cell-free extracts were dialysed to remove ADP, thus preventing the conversion of acetyl phosphate to acetate by acetate kinase, a reaction which occurs when whole cells are present in the incubation mixtures (Tables 1–3). The results shown in Table 5 indicate that acetyl phosphate, acetate and CO_2 were produced in the ratio of 1:1:2 which is in agreement with the above reaction sequence catalysed by pyruvate oxidase. TPP was required for maximum

Table 5. Formation of acetyl phosphate and acetate from pyruvate by *L. bulgaricus* LB1

The incubation mixture (total volume 3 ml) contained (in micromoles): sodium pyruvate, 50; FAD, 0·1; TPP, 0·2; $MgCl_2$, 10; KH_2PO_4 (pH 6·5), 200; CoA (when present), 0·1 and cell-free extract (C.F.E.) (previously dialysed against 20 mM KH_2PO_4, pH 7·0, for 48 h), 0·5 ml (2 mg protein). Incubation was for 2 h at 37°C and the products were assayed as described under Methods

Incubation mixture	Pyruvate consumed (μmol)	O_2 uptake (μmol)	CO_2 evolved (μmol)	Acetyl phosphate formed (μmol)	Acetate formed (μmol)
Complete	28·6	13·0	24·5	11·7	11·6
−FAD	25·0	11·4	22·4	10·6	10·4
−TPP	7·0	3·5	7·2	2·1	3·9
Dowex-1 treated C.F.E.[A]	22·5	12·1	23·5	10·0	13·3
Dowex-1 treated C.F.E.+CoA	27·5	11·6	22·6	10·8	11·8

[A] To remove Co-enzyme A.

activity but since the presence of added FAD had no effect on enzyme activity it was obvious that dialysis did not remove the FAD bound to the enzyme. Hager *et al.* (1954) have shown that the FAD moiety can be removed from pyruvate oxidase with acid ammonium sulphate and that the apoenzyme requires the addition of FAD before activity can be observed.

The possibility existed that acetyl phosphate was being formed from acetaldehyde and CoA via acetyl CoA. However, no CoA-dependent aldehyde dehydrogenase activity was detected in *L. bulgaricus*. Moreover, treatment of the cell-free extract with Dowex-1 (to remove CoA) had no effect on the amount of acetyl phosphate formed and did not lead to the accumulation of acetaldehyde. Thus, acetyl phosphate appeared to be formed directly from pyruvate. The conversion of acetyl phosphate to acetate would require the presence of acetate kinase. As shown in Table 6 both acetate kinase and phosphate acetyltransferase activity were detected in *L. bulgaricus*.

Table 6. Acetate kinase and phosphate acetyltransferase activities of *L. bulgaricus*

Enzyme activities were assayed as described under Methods

Enzyme	Specific activity[A]	
	Strain LB1	Strain LB2
Acetate kinase	0·148	0·542
Phosphate acetyltransferase	0·433	0·722

[A] Micromoles of acetyl phosphate utilized per minute per milligram protein.

Discussion

The results of this investigation suggest that *L. bulgaricus* forms acetate from pyruvate by the pyruvate oxidase system previously described in *L. delbrueckii* by Hager *et al.* (1954). This reaction sequence, in contrast to the pyruvate dehydrogenase (lipoate) system in group N streptococci, does not allow the formation of acetaldehyde, ethanol, diacetyl, acetoin or 2,3-butylene glycol from carbohydrate. Thus, the acetaldehyde which accumulates in yoghurt as a major flavour compound must either be produced by *L. bulgaricus* from threonine, by the action of serine hydroxymethyltransferase (EC 2.1.2.1) (Lees and Jago 1976*b*), or by *S. thermophilus* which is also present in yoghurt.

Czulak *et al.* (1974) reported that Cheddar cheese produced from bovine milk containing a high content of linoleic acid developed no flavour unless *L. bulgaricus* (strain YB) was present. In the absence of *L. bulgaricus* YB acetate formation in the cheese was markedly depressed due to the effect of unsaturated fatty acids on the pyruvate dehydrogenase (lipoate) system in group N streptococci. The addition of *L. bulgaricus* YB to the cheese resulted in an increase in the acetate level and in the flavour of the cheese (Czulak *et al.* 1974). The failure of unsaturated fatty acids to inhibit the pyruvate oxidase in *L. bulgaricus* LB1 partly explains the increase in acetic acid.

Since *L. bulgaricus* was shown to possess both acetate kinase and phosphate acetyltransferase activities any acetyl phosphate formed via pyruvate oxidase could be converted either to acetate or acetyl CoA. The latter compound is utilized in the synthesis of lipids.

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