Pyruvate Metabolism in *Lactobacillus brevis*

By J. R. L. WALKER

The Dairy Research Institute (N.Z.), Palmerston North, New Zealand

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The pyruvate metabolism of *Lactobacillus plantarum* and *Lactobacillus arabinosus*, which according to Bergey (1948) are identical organisms, has been studied by Rowatt (1951) and Nossal (1952). Both these homofermentative lactobacilli were shown to form 3-hydroxybutan-2-one (acetoin) as the main product of their pyruvate metabolism. No one appears to have studied the metabolism of pyruvate by any of the heterofermentative lactobacilli, of which *Lactobacillus brevis* is a typical example. This species frequently occurs in cheddar cheese, in the ripening of which it may play a significant part. It was considered of interest therefore to compare the pyruvate metabolism of *L. brevis* with that of the homofermentative species.

MATERIALS AND METHODS

Growth of cells. The organism used throughout these studies was *L. brevis* strain 1-2, originally isolated from New Zealand cheddar cheese by Sherwood (1939). Throughout this work stock cultures were maintained in sterile skim milk containing 0.2% of yeast extract (Oxoid, Oxo Ltd.). Cells for experimental use were grown in the lactose-yeast-phosphate broth of Hunter (1946) for 48 hr. at 30°. After centrifuging, the cells were washed twice and finally resuspended in sterile 1% (w/v) KCl. Individual cell crops showed random variation in yield and activity; a similar phenomenon was reported by Nossal (1952) with *L. arabinosus*. This fact, together with the slow growth rate of *L. brevis*, made it difficult to obtain exactly reproducible quantitative results; for example, the final yields of CO₂ sometimes fell short of the theoretical quantity by amounts between 10 and 20%. The overall qualitative picture, however, was not affected.

Reagents. Pyruvic acid and 2-oxobutyric acid were redistilled *in vacuo*. Solutions of their sodium salts were prepared by suitable dilution and neutralization (with NaOH). Lithium lactate (British Drug Houses Ltd., Poole, Dorset, England) was purified by dissolving in a minimum of hot water, filtering, precipitating with ethanol and drying over CaCl₂. Other compounds were commercial products.

Measurement of gas changes. Oxygen uptake and CO₂ production were measured by conventional Warburg techniques at 30°. Carbon dioxide was absorbed in KOH for O₂-uptake determinations and the values so obtained were used to correct the values for CO₂ evolution.

Each vessel contained 0.5 ml. of cell suspension and 1.5 ml. of 0.2M-phosphate-citrate buffer (McIlvaine, 1921), pH 4.8, unless otherwise stated. Except in cofactor experiments, 0.1 ml. of 0.01M-thiamine and 0.1 ml. of

0.01M-MnSO₄ soln. were added to all flasks. Substrate(s) was added from the side arm after equilibration. The total volume of fluid per flask was adjusted to 3 ml. in all experiments.

Analytical methods

At the conclusion of an experiment the Warburg flask contents were centrifuged to remove cells and the clear supernatants stored in the refrigerator before analysis.

Acetoin. This was estimated by a quantitative Voges-Proskauer reaction according to Westfeld (1945).

α -Acetolactic acid. This compound has been shown by Juni (1952) to be decarboxylated to acetoin in acid solutions and this method was used to test for the presence of this compound.

Diacetyl. This was assayed by the specific method of White, Krampitz & Werkman (1946).

Keto acids. These were estimated as 2:4-dinitrophenylhydrazones by the method of Friedemann & Haugen (1943).

Lactic acid. This was estimated by the method of Barker & Summerson (1941). A control tube containing 10 μ moles of pyruvate did not give significant colour development under the conditions used.

Volatile fatty acids. These were estimated by the method of McLendon (1944), modified by using 1 ml. of 10% (w/v) orthophosphoric acid, instead of 0.5 ml. of syrupy phosphoric acid, and using the apparatus of Markham (1942). It was found more convenient to use a direct-reading pH meter, rather than an indicator, for following the titration of the distillate. Volatile fatty acids were identified by paper chromatography with the method of Reid & Lederer (1952).

Glucose. The disappearance of glucose was followed with the anthrone method as modified by Trevelyan & Harrison (1952).

RESULTS

Effect of pH on the rate of evolution of carbon dioxide from pyruvate. When washed suspensions of *L. brevis* were incubated with pyruvate a gas, identified as CO₂ by its absorption in 20% (w/v) KOH, was evolved. With 0.1M-phosphate-citrate buffers the initial rate of CO₂ evolution was determined at several pH values in the range 3.5-6.5, with air and with N₂ as gas phases. The results shown in Fig. 1 reveal a pH optimum at 4.8 for both aerobic and anaerobic conditions. Subsequent experiments were therefore carried out at this pH.

Cofactor requirements. The effect of various likely cofactors on the rate of CO₂ evolution was tested in a series of experiments (Table 1). It is evident that both thiamine and Mn²⁺ ions were necessary

for maximum rate of CO_2 evolution when *L. brevis* was incubated with pyruvate. Replacement of Mn^{2+} by Mg^{2+} ions resulted in an 11% lowering of the rate of CO_2 evolution. In subsequent experiments thiamine and Mn^{2+} ions (as MnSO_4) were added to all reaction mixtures.

Aerobic metabolism of pyruvate. When *L. brevis* was incubated with pyruvate there was no absorption of O_2 until an amount of CO_2 corresponding to 0.5 μmole of $\text{CO}_2/\mu\text{mole}$ of pyruvate had been evolved. After this initial rapid reaction O_2 was absorbed and a further corresponding amount of CO_2 liberated, as can be seen from Fig. 2. The Warburg flasks at the conclusion of the experiment,

Fig. 1. Effect of pH on the rate of CO_2 evolution from pyruvate. Reaction mixture (3 ml.) contained 10 μmoles of pyruvate, thiamine, MnSO_4 , phosphate-citrate buffer and 11 mg. of cells dry wt. Gas phase was air (O) or N_2 (●).

Table 1. Effect of various cofactors on the rate of evolution of carbon dioxide from pyruvate

Warburg flasks contained 10 μmoles of pyruvate, cofactors (0.1 ml. of each, 0.01M) as stated below, 7 mg. of cells (dry wt.) and the volume was made up finally to 3 ml. with phosphate-citrate buffer, pH 4.8. Gas phase was nitrogen.

Cofactors added	Rate of CO_2 liberation (μl. in first 30 min.)
None	38
Thiamine	62
MnSO_4	63
MgSO_4	55
Thiamine + MnSO_4	74
Thiamine + MnSO_4 but no pyruvate	5

and immediately after the primary rapid reaction, were examined for acetoin and α -acetolactic acid since, on the basis of similar earlier work by Juni (1952), these were considered to be likely intermediates. Neither compound was detected.

In order to attempt to establish the pathway of pyruvate breakdown in this micro-organism, Warburg flask contents were examined at suitable intervals for lactic acid, volatile fatty acids and residual pyruvate. From Fig. 2 it can be seen that all the pyruvate was utilized before the commencement of the O_2 -absorbing reaction and that the lactate concentration reached a maximum at this point. The final flask contents, when acidified and steam-distilled, yielded amounts of volatile acid approximately equivalent to the initial concentration of pyruvate. The volatile acid was subsequently identified as acetic acid by paper chromatography.

The possibility that acetate was present in the form of acetyl phosphate in the final reaction mixture was visualized. Tests by the method of

Fig. 2. Aerobic metabolism of pyruvate. Upper diagram: O, O_2 uptake; ●, CO_2 evolved. Cells (10 mg. dry wt.) were incubated in 3 ml. of solution containing phosphate-citrate buffer, pH 4.8, thiamine, MnSO_4 and the following substrates: 10 μmoles of pyruvate (O); 5 μmoles of lactate (Δ). ---, No substrate. The lower diagram records the changes in concentration of pyruvate, lactate and acetate throughout the experiment.

Lipmann & Tuttle (1945), however, gave negative results.

Added DL-lactate was slowly oxidized at a rate compatible with the slow O_2 -absorbing reaction.

Anaerobic experiments. Experiments carried out in atmospheres of N_2 or $N_2 + CO_2$ (95:5) with different amounts of pyruvate (see Fig. 3) gave values for CO_2 evolution of 0.8 molecule of CO_2 /2 molecules of pyruvate. This value agrees approximately with that for the initial CO_2 -evolving reaction (before the onset of O_2 absorption) of the aerobic experiments detailed above. The final flask contents were examined for acetoin, lactate and volatile acid. From an initial 10 μ moles of pyruvate, 4.1 μ moles of acetate and 4.0 μ moles of lactate, but no acetoin, were recovered.

Effect of added glucose. Rowatt (1951) and Nossal (1952) both found that for their systems the rate of CO_2 evolution from pyruvate was increased in the presence of glucose. Fig. 4 indicates that suspensions of *L. brevis* incubated with glucose as sole substrate showed an immediate uptake of O_2 with a corresponding evolution of CO_2 . When pyruvate and glucose were both present O_2 was not absorbed until the rapid CO_2 -liberating reaction had occurred; thereafter there was an uptake of O_2 and a corresponding further CO_2 evolution. This suggested that glucose oxidation was inhibited in the presence of pyruvate and this was confirmed by measuring the disappearance of glucose after suit-

able time intervals. From an initial 2 μ moles of glucose, in the presence of 10 μ moles of pyruvate (see Fig. 4), 1.8 μ moles of glucose was still present after 30 min., but no glucose remained at the conclusion of the experiment.

Effect of various inhibitors. With reaction mixtures containing pyruvate, thiamine and Mn^{2+} ions, the effects of the following inhibitors were studied: 0.01M-fluoride, 0.01M-azide, 0.01M-arsenite, 0.01M-arsenate and 0.001M-fluoropyruvate. Fluoride, azide and arsenate did not inhibit pyruvate metabolism at these concentrations.

The effect of arsenite is shown in Table 2 and Fig. 5. This compound caused a slight lowering of the initial rate of CO_2 evolution but the O_2 -absorbing reaction was markedly inhibited. Examination of the flask contents revealed an accumulation of acetoin and lactate in those flasks containing arsenite.

Fig. 5 also shows that fluoropyruvate caused an inhibition of the O_2 -absorbing reaction similar to

Fig. 3. Relationship between pyruvate concentration and CO_2 production. Cells (10 mg. dry wt.) were incubated in 3 ml. of solution containing phosphate-citrate buffer, pH 4.8, thiamine, $MnSO_4$ and different amounts of pyruvate, in an atmosphere of nitrogen.

Fig. 4. Aerobic metabolism of pyruvate and glucose. Cells (12 mg. dry wt.) were incubated in 3 ml. of solution containing phosphate-citrate buffer, pH 4.8, thiamine, $MnSO_4$ and the following substrates: 10 μ moles of pyruvate (●); 10 μ moles of pyruvate + 2 μ moles of glucose (■); 2 μ moles of glucose (▲). ---, No substrate.

that caused by arsenite but at one-tenth of the concentration. Fluoropyruvate poisoning likewise resulted in an accumulation of acetoin and lactate.

Substrate specificity. The substrate specificity of the keto acid-utilizing enzymes present in *L. brevis* was investigated by using 2-oxobutyrate or 2-oxoglutarate in place of pyruvate in Warburg experiments. It was found that 2-oxobutyrate was readily utilized but that 2-oxoglutarate was unaffected by suspensions of *L. brevis*. In one such experiment 10 μ moles of 2-oxobutyrate yielded 8 μ moles of volatile acid with the corresponding

evolution of 8.4 μ moles of CO_2 and with an absorption of 4.4 μ moles of O_2 . In this experiment the volatile acid was identified as propionic acid. By contrast with pyruvate, 2-oxobutyrate did not give rise to an acyloin compound in the presence of arsenite, as evidenced by a negative Voges-Proskauer test.

Confirmatory experiments with *Lactobacillus brevis* 6107. Experiments showed that *L. brevis* strain no. 6107, obtained from The National Institute for Research in Dairying, Reading, behaved in a similar manner to *L. brevis* strain 1.2 in Warburg experiments with pyruvate as substrate. Only when arsenite was added to the reaction mixture did acetoin accumulate. The reactions described in this paper are therefore not specific to *L. brevis* strain 1.2 but are probably characteristic of the *brevis* species.

DISCUSSION

The experiments reported here suggest that the metabolism of pyruvate in *L. brevis* occurs in two stages. On the basis of the results shown in Fig. 2 it is suggested that the initial reaction (i) corresponds to a dismutation

Under anaerobic conditions only reaction (i) normally occurs but in aerobic experiments reaction (i) was followed by a slower reaction (ii) involving oxidation of the lactate molecule to acetate. These reactions are in marked contrast with the reaction

already established as the main pathway for pyruvate metabolism in the homofermentative lactobacilli (Rowatt, 1951; Nossal, 1952).

Hunt & Nossal (1954) showed that the stimulatory effect of glucose on the rate of evolution of

Fig. 5. Inhibition of pyruvate oxidation by arsenite and fluoropyruvate. Open symbols denote values for O_2 uptake, filled-in symbols values for CO_2 evolved. Cells (10 mg. dry wt.) were incubated in 3 ml. of solution containing phosphate-citrate buffer, pH 4.8, thiamine, MnSO_4 , 6 μ moles of pyruvate ($\circ$, $\bullet$) or 6 μ moles of pyruvate + 0.01M-arsenite ($\triangle$, $\blacktriangle$) or 6 μ moles of pyruvate + 0.001M-fluoropyruvate ($\square$, $\blacksquare$). ---, Endogenous respiration.

Table 2. Final analyses of contents of Warburg flasks

Flasks contained cells incubated in 3 ml. of solution containing 0.1M-phosphate-citrate buffer, pH 4.8, thiamine, MnSO_4 , pyruvate and inhibitor as stated. Reaction time was 180 min. This table records the results of two different experiments.

Initial pyruvate (μ moles)	Gas atmosphere	Inhibitor	CO_2 evolved (μ moles)	O_2 absorbed (μ moles)	Acetate (μ moles)	Lactate (μ moles)	Residual pyruvate (μ mole)	Acetoin (μ mole)
10	Air	None	8.8	3.9	8.8	0.8	0.1	None
10	N_2	None	4.0	0.0	4.1	4.0	0.2	None
6	Air	None	4.9	1.9	4.7	0.6	0.1	None
6	Air	0.01M-Arsenite	2.7	0.05	2.1	2.3	0.2	0.65
6	Air	0.001M-Fluoropyruvate	2.7	0.0	2.6	2.7	0.1	0.50

carbon dioxide from pyruvate by *L. arabinosus* was concomitant with its glycolysis to lactic acid. In the present system added glucose did not stimulate the rate of evolution of carbon dioxide and was not oxidized until reaction (i) was completed. This difference may be correlated with the finding of Buyze, Van de Hamer & De Haan (1958) that heterofermentative lactobacilli lack aldolase and operate the hexose monophosphate-shunt mechanism for glucose oxidation.

The action of arsenite in this system is unusual and difficult to explain since arsenite is usually considered as an inhibitor of oxidative decarboxylation. It is possible that both reactions (i) and (ii) involve oxidative decarboxylation but the latter is very much more sensitive to the poison than the former. The inhibitory action of fluoropyruvate may be due to a blockage of an enzyme system by a 'lethal synthesis' of a fluoro-substituted compound, or (a more acceptable hypothesis) to the greater sensitivity of the second reaction (ii) to the poison than the acetoin-forming system. Dolin & Gunsalus (1951), in the course of their studies with *Streptococcus faecalis*, reported the presence of two pathways for pyruvate metabolism in this organism. Acetate was found to be the main product of pyruvate breakdown by intact cells of *S. faecalis*, but an acetoin-synthesizing system, not apparent during whole-cell studies, was demonstrated in cell-free extracts. These workers also found that under anaerobic conditions *S. faecalis* formed acetate and lactate from pyruvate by the reaction:

A latent acetoin-forming system appears to be present also in *L. brevis*, demonstrable only in the presence of suitable inhibitors of the alternative pathways.

When *L. brevis* was incubated with 2-oxobutyrate in the presence of arsenite, no acyloin compounds (propionin) were formed. Therefore it may be concluded that the acyloin-forming system is specific for pyruvate. A similar high degree of specificity for an acyloin-condensation system in *Aerobacter aerogenes* has been reported by Juni (1952).

The main conclusions reached therefore are that at least two pathways for pyruvate metabolism exist in *L. brevis*. The main route involves an anaerobic dismutation of 2 molecules of pyruvate to form lactate, acetate and carbon dioxide followed by oxidation of the lactate to acetate and carbon dioxide, a mechanism not exhibited by the homofermentative lactobacilli. These species synthesize acetoin from pyruvate, a reaction that is also available to *L. brevis* but is not of primary metabolic importance to this species.

SUMMARY

1. In contrast with the homofermentative lactobacilli, which are known to form acetoin as the main product of pyruvate metabolism, suspensions of *Lactobacillus brevis* metabolized pyruvate, to form ultimately acetate and carbon dioxide, in two distinct stages.

2. The first stage involved a dismutation reaction of pyruvate to form acetate, lactate and carbon dioxide. Under aerobic conditions the lactate was subsequently oxidized to acetate and carbon dioxide.

3. The rate of carbon dioxide evolution was maximum at pH 4.8. Thiamine and manganese (Mn^{2+} ions) stimulated the rate of carbon dioxide formation from pyruvate.

4. Oxidation of glucose, added to the above system, was suppressed until the dismutation reaction was completed.

5. When arsenite or fluoropyruvate was present in the system the second-stage oxygen-consuming reaction was suppressed and acetoin accumulated.

6. *Lactobacillus brevis* produced propionate from 2-oxobutyrate by a similar mechanism, but propionin accumulation could not be demonstrated in the presence of arsenite.

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