contain them. It is notable, though, that the artichoke preparations described here had very little hydrolytic action upon sucrose, which was present in concentrations up to 1.5% (w/w fresh tuber) in all the tubers examined.

SUMMARY

1. Aqueous extracts of tubers of the Jerusalem artichoke (*Helianthus tuberosus*) show, on incubation, an increase in reducing substances and a decrease in optical rotation.

2. The enzyme or enzymes responsible have been freed from the tuber carbohydrates and concentrated twenty-fold by fractional precipitation with ammonium sulphate.

3. By the use of paper partition chromatography such preparations have been shown to liberate fructose from various substrates, including sucrose, inulin, a levan, and irisin 'B'; glucose was detected among the hydrolysis products of inulin.

4. The liberation of reducing sugars from the tuber carbohydrates and from inulin virtually ceased before complete hydrolysis had taken place. The residual combined sugar was in the form of a disaccharide, probably sucrose.

5. From these observations the main hydrolytic activity of tuber preparations has been characterized as that of a fructofuranosidase, with a moderate degree of specificity for such residues linked to position 1 of another fructose residue.

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The Metabolism of Pyruvate by *Lactobacillus plantarum*

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When pantothenate is added to suspensions of *Lactobacillus plantarum* deficient in that factor the yield of acetylcholine is much increased (Stephenson & Rowatt, 1947). Hills (1943) showed that the rate of oxidation of pyruvate by pantothenate-deficient suspensions of *Proteus morganii* is increased by the addition of pantothenate. It was thought on the

basis of these observations that pantothenate might be required for the metabolism of pyruvate in *Lb. plantarum*, and an investigation of the latter was planned. Pyruvate was found to be broken down to acetoin and carbon dioxide without the participation of pantothenate. This finding and various other aspects of pyruvate metabolism in this organism are described in the present paper.

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METHODS

Growth of organisms. (1) *Lb. plantarum*. The organism used in the main part of this work was that isolated by Stephenson & Rowatt (1947). It was maintained on medium 1 of that paper at 25°. Cells for use as washed suspensions were grown at 25° in basal medium 5 of the same paper (basal medium 5: acid digest of casein supplemented with tryptophan, cysteine, glucose, sodium acetate, salts and growth factors including pantothenate and aneurin). The following alterations in composition were made: $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (10 mg./100 ml.) and $\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$ (0.025 mg./100 ml.) were autoclaved separately and added to the medium after sterilization; calcium pantothenate was sterilized by filtration through a Seitz filter and added to the sterile medium at a concentration of 10 μg ./100 ml.; yeast extract was sterilized by filtration through a Seitz filter and 3 ml. added to each 100 ml. of sterile medium. The yeast extract was prepared by heating 500 g. baker's yeast in 500 ml. water for 15 min. in a boiling-water bath and filtering. When added to the medium at this concentration it increased by approximately 7 times the crop of cells obtained after 15 hr. incubation. The medium with these modifications will be referred to as medium *P* and was used for growth in all cases except where otherwise stated.

A medium deficient in pantothenate was obtained by omitting the pantothenate supplement and using yeast extract which had been autoclaved for 15 min. at 15 lb. pressure with 0.1 vol. 10N-HCl. Growth with the acid-treated extract in the absence of added pantothenate was poor (0.06 mg. cells/ml. instead of 0.8 mg./ml. after 48 hr. incubation), but when pantothenate was added the same stimulation was found with acid-treated as with the untreated extract. As the crop of cells obtained from the pantothenate-deficient medium was small, one or more litres of medium were inoculated for each experiment. Each litre flask was inoculated with a 24 hr. culture in 100 ml. medium *P* containing 0.01 μg . calcium pantothenate/ml.

Aneurin-deficient cells were grown in medium *P* omitting both the aneurin supplement and the yeast extract.

Cultures were grown in 100 ml. lots of medium *P* in flat-bottomed flasks filled to the neck, as a smaller crop of less active cells was obtained under aerobic conditions and the yield was not increased by strict anaerobiosis. When larger quantities of cells were required, for the experiments at pH 6 for example, 500 ml. or more of medium were inoculated with 0.1 vol. of a 24 hr. culture in the same medium. Cells for washed suspensions were harvested after 15–18 hr. growth, because, although growth continued until the 45th hour, the younger cells were more active in washed suspensions.

Suspensions of washed cells in saline lost the capacity to form CO_2 from pyruvate on storage even at 4°, and they were generally used as soon as possible and never more than 5 hr. after removal from the medium.

(2) *Aerobacter aerogenes* and *Staphylococcus aureus*. *A. aerogenes* strain 240 (National Collection of Type Cultures no. 418) and *Staph. aureus*, strain E (McIlwain & Hughes, 1948) were grown for 15 hr. at 25–30° in 100 ml. medium containing: 10 ml. Stephenson's inorganic medium (Stephenson, 1939), 0.3% Pronutrin (Herts Pharmaceuticals Ltd.), 0.1 M-glucose and 3 ml. yeast extract (prepared as described above). The pH of the medium was adjusted to 6.5 before incubation.

Preparation of washed suspensions. After incubation, cells were centrifuged from the medium, washed once with and

resuspended in 0.9% NaCl to give a suspension containing 0.5–3.0 mg. cells/ml. for the experiments done at pH 4, and 10–30 mg./ml. for those done at pH 6. Cells grown in pantothenate-deficient media tended to flocculate, and these were washed and resuspended in 0.01 M-phosphate buffer, pH 7.

The concentration of cell suspensions was estimated either by drying to constant weight at 100° or by measuring the turbidity with a Spekker absorptiometer, using neutral filters. The dry weight was related to the turbidity by means of an empirical calibration curve. All figures on cell concentrations given in this paper refer to dry weights.

Incubation of washed suspensions and measurement of gas changes. The experiments were carried out in conical Warburg manometer flasks of about 20, or, in some cases, 100 ml. capacity. A solution of pyruvate, together with the buffer and any other substrate, was placed in the main compartment and the unbuffered washed cell suspension in the side arm. Buffers were omitted from the cell suspensions because at the acid pH the enzymes concerned were inactivated on incubation without substrate. CO_2 output and O_2 uptake were measured manometrically by the method of Warburg & Yabuseo (1924). All gas changes are expressed in μl . and, for convenience of comparison, quantities of pyruvate, acetoin and glucose are also expressed in μl ., 22.4 μl . being taken as equivalent to 1 μmol . Reaction rates are expressed in terms of *Q* values (μl . metabolite/mg. dry wt. cells/hr.).

Taking and preservation of samples. Suspensions were incubated at 25° (pH 6) or 37° (pH 4) until the rate of gas evolution indicated that the reaction had almost ceased or, in the case of slow reactions, until quantities of metabolites sufficient to be measured had accumulated. If acetoin alone was to be determined, samples were pipetted into 1 ml. 10N- H_2SO_4 immediately after incubation. Samples for the estimation of pyruvic acid were acidified by the addition of 0.1 vol. 2N-HCl from the side arm of the manometer, and were then incubated at 60° for 15 min. to inactivate the bacterial enzymes. Samples for the estimation of lactic acid or 2:3-butyleneglycol were measured into the CuSO_4 solution used in the copper-lime precipitation, and stored in the ice chest. Samples for the estimation of volatile acid were treated with 4 ml. 1.3% (w/v) 2:4-dinitrophenylhydrazine in 2N- H_2SO_4 to precipitate residual pyruvic acid (see Speck, Moulder & Evans, 1946). When acetaldehyde was estimated, the manometer cups, on removal from the bath, were stoppered and cooled in ice for 15 min.; the samples were then pipetted into the aeration tubes used in the determination.

Estimation of acetoin. Acetoin was oxidized to diacetyl according to Westerfeld (1945). After 15 min. treatment with the reagent, the solution was cooled and subjected to steam distillation in a micro-Kjeldahl apparatus. The diacetyl in 10 ml. distillate was estimated by the method of Eggleton, Elsdon & Gough (1943) with a Spekker absorptiometer.

Estimation of lactic acid. Acetoin is not removed by treatment with copper-lime and, as it is volatile and bound by bisulphite, it interferes with the determination of lactic acid by the method of Friedemann & Graesser (1933). It is not sufficiently volatile to be removed by preliminary boiling, as is suggested by Friedemann & Graesser (1933) for the removal of other volatile bisulphite-binding substances. In order to remove the acetoin before estimation of lactic acid by the method of these authors, steam was passed through the solution in the Kjeldahl flask in which the determination was to be carried out. Distillation was continued for 15–20 min.,

until about 100 ml. water had been collected, the volume in the flask remaining approximately constant. These conditions were found to be sufficient for quantitative removal of the acetoin present.

Pyruvic acid was estimated by the carboxylase method (see Krebs & Eggleston, 1945).

Acetylcholine was assayed by means of the frog rectus abdominalis method (see Stephenson & Rowatt, 1947).

Volatile acid was estimated by the method of Friedemann (1938).

Acetaldehyde was transferred by a stream of air into a solution of bisulphite, as described by Rees (1946) for the estimation of threonine and serine, except that buffer was not added.

2:3-Butylene glycol was estimated by a method similar to that described by Johnson (1944), based on the oxidation of the substance to acetaldehyde by periodate. The solutions were precipitated with copper-lime and the filtrate treated with periodate without preliminary ether extraction. Acetoin and lactic acid are also oxidized to acetaldehyde by periodate. Acetoin was determined separately by the method given above; lactic acid was shown to be present in small amounts, about 5% of the pyruvate used. In calculating butylene glycol values, corrections were made for the acetoin and lactic acid present.

Succinate was estimated manometrically with succinic dehydrogenase (Krebs, 1937*b*).

Glucose was estimated by the fermentation method of Winzler (1944).

Preparation of pyruvic acid. Pyruvic acid was redistilled several times, immediately diluted with water to 2*N* strength and stored at 4°. Before use the pH of the solution was adjusted to 4 by the addition of *N*-NaOH.

RESULTS

Products of pyruvate decomposition by Lactobacillus plantarum

Effect of pH on products. On addition of pyruvate to suspensions of *Lb. plantarum* a gas was evolved which was identified as CO₂ by the fact that it was absorbed by alkali. The rate of CO₂ formation with 0.008*M*-pyruvate in citrate buffer (Kolthoff, 1932) with one suspension was 10 µl./mg. cells/hr. at pH 6, 113 at pH 5 and 272 at pH 4. At each of these pH values, acetoin was formed; the molecular ratio of CO₂ to acetoin was higher at pH 6 than at pH 5 or 4. Between pH 5.5 and 4 (citrate buffer) the ratio was 1.9; at pH 6.0 it varied between 4 and 6. The amount of CO₂ formed at pH 6.0 varied from 0.5 to 1 mol./mol. of pyruvate used, and the acetoin accounted for 20–32% of the pyruvate used, if it is assumed that 2 mol. of pyruvate yield 1 of acetoin and 2 of CO₂. At this pH, therefore, CO₂ and acetoin do not account for all the pyruvate metabolized. Volatile acid was formed which, calculated as acetic acid, accounted for about 6% of the pyruvate used. Lactic acid also accounted for about 6%. Acetaldehyde, 2:3-butyleneglycol and succinate were not found.

At pH 4, the ratio of CO₂ to acetoin formed remained constant throughout the reaction (Fig. 1)

and was approximately the same as that found by Silverman & Werkman (1941) in *A. aerogenes*. The rate of CO₂ formation was most rapid between pH 4.1 and 4.7 (Fig. 2). Further experiments on pyruvate metabolism were carried out at pH 4 for reasons to be stated below.

Fig. 1. Formation of acetoin and CO₂ from pyruvate. Cells grown in medium P with 10% boiled yeast extract (instead of 3% acid-treated yeast extract) for 16 hr. Cups contained 2.1 mg. cells, 0.02*M*-citrate buffer (pH 3.5), 0.02*M*-KHPO₄ and 280 µl. pyruvic acid (0.005*M*) in 2.5 ml., 37°. Cups removed from the bath at 10 min. intervals for estimation of acetoin.

Fig. 2. Effect of pH on Q_{CO_2} . 1.4 mg. cells incubated in 2.5 ml. 0.02*M*-citrate; 0.008*M*-pyruvate; 0.004*M*-glucose. 37°; N₂. Q_{CO_2} calc. for first 20 min.

Effect of oxygen. According to Silverman & Werkman (1941), the formation of acetoin from pyruvate by *Aerobacter* is independent of O₂. The rate of CO₂ formation from pyruvate by *Lb. plantarum* in citrate buffer was greater when O₂ was present than when N₂ was the gas phase, and more acetoin was formed, although the ratio of CO₂ to acetoin formed was more than 2 (2.8). In barbiturate buffer, more CO₂ was formed aerobically than anaerobically, but the quantity of acetoin formed was the same in each case. As the presence of O₂ appeared to complicate the reaction, experiments were carried out anaerobically.

Optical rotation and identification of acetoin formed. Hirsch (1922) obtained optically active acetoin from a fermentation of pyruvic acid in a culture of top yeast. The acetoin formed by yeast juice was

not optically active, and Dirscherl (1930) suggested that this was formed non-enzymically, only the decarboxylation of pyruvic acid to a 2-carbon unit being enzymic. The optical activity of the acetoin formed by *Lb. plantarum* was therefore tested.

A sufficient quantity of acetoin could be obtained only in the presence of glucose. It was therefore necessary to extract the acetoin from the suspension medium before measuring the optical activity. 1.62 g. cells were incubated at 37° for 5 hr. in 750 ml. 0.02M-citrate buffer (pH 3.9) containing 25 ml. 2M-pyruvate and 0.54 g. glucose. The suspension was concentrated according to the method of Fulmer, Underkoffler & Bantz (1943), clarified with kieselguhr, and further concentrated by saturation with anhydrous sodium sulphate at 40° and removal of the hydrated sulphate after cooling. The saturation of the solution with sodium sulphate facilitated extraction of acetoin. The solution was extracted with ether for 20 hr. in Kutscher-Steudel extractors. A few ml. water were added to the extract and the ether removed under reduced pressure. The fraction boiling at 29–30° at 19 mm. Hg pressure was collected. The 2:4-dinitrophenylosazone prepared from this fraction had a melting point of 317.5° (decomp.) (uncorr.); that of an authentic specimen of the osazone of acetoin was 317° (decomp.) (uncorr.). Of the substances present in the original solution which could be extracted by ether, pyruvic and citric acids are not optically active and lactic acid does not distil under the conditions used.

The distillate, when diluted to contain 62 mg. acetoin/ml., gave an α_D^{19} of -8.77° in a 2 dm. polarimeter tube. Thus $[\alpha]_D^{19}$ was -70.6° . Silverman & Werkman (1941) report a value of -100° for acetoin formed from pyruvate by *A. aerogenes*. In their experiments, as no other optically active substance was present in the fermentation liquor, the rotation was measured directly, with no attempt at purification.

*Rate of decomposition of pyruvate by
Lactobacillus plantarum*

Effect of buffers. Citrate interferes with the determination of lactic acid by the method of Friedemann & Graesser (1933), and it was necessary

to use another buffer when this estimation was to be carried out. In acetate buffer the rate of CO₂ formation was less than that found in citrate: in 0.04M-acetate with 0.008M-pyruvate the Q_{CO_2} was 84 % of that in 0.02M-citrate; in 0.1M-acetate it was 52 % and in 0.2M 12 %. In 0.02, 0.06 and 0.1M-citrate, the Q_{CO_2} values were, respectively, 243, 248 and 219. The rate of CO₂ formation from pyruvate in succinate buffer is shown in Table 1. At 0.04M, the rate of the reaction was considerably lower than in citrate and was very little higher when the cells were incubated in a mixture of the two buffers. The effect, therefore, was not due to the absence of citrate. The addition of 0.008M-succinate to cells incubated in citrate buffer did not affect the rate of CO₂ formation from pyruvate.

Table 1. *Inhibition by succinate of CO₂ formation from pyruvate by Lactobacillus plantarum*

(*Lb. plantarum*, 0.9 mg., incubated in 2.5 ml. solution containing 0.008M-pyruvate and 0.002M-glucose where added; citrate or succinate buffer pH 4 as stated.)

Addition	Q_{CO_2} (10–20 min.)	
	Pyruvate	Pyruvate and glucose
Succinate, 0.04M	56	70
Citrate, 0.04M	333	453
Succinate, 0.02M, and citrate, 0.02M	70	81
Citrate, 0.02M	190	443
Citrate, 0.02M, and succinate, 0.0008M	203	427

When cells were incubated in 0.01M-barbituric acid-NaOH buffer the rate of CO₂ formation from pyruvate and glucose (see below) was approximately the same as in citrate buffer; with pyruvate alone CO₂ formation was initially slower than in citrate buffer, but after 15 min. there was little difference between the two systems (Table 2). Consequently, as acetate would interfere with estimations of this substance, barbiturate was used as buffer when lactate was to be estimated and in some other experiments. It is noteworthy that no other buffer tested gave as high a rate of CO₂ formation as citrate. The Q_{CO_2} in 0.04M-lactate with pyruvate was

Table 2. *Effect of barbiturate on rate of CO₂ formation from pyruvate by Lactobacillus plantarum*

(*Lb. plantarum*, 0.9 mg., incubated in 2.5 ml. citrate or barbituric acid-NaOH buffer with 0.008M-pyruvate.)

Additions	Q_{CO_2}			
	5–10 min.	10–15 min.	15–20 min.	20–25 min.
Citrate, 0.02M	180	202	202	157
Barbiturate, 0.02M	67	67	67	67
Barbiturate, 0.01M	66	131	153	88
Glucose, 0.002M, and citrate, 0.02M	577	533	512	422
Glucose, 0.002M, and barbiturate, 0.02M	133	333	488	355
Glucose, 0.002M, and barbiturate, 0.01M	402	594	764	529

13 % and that with pyruvate and glucose 3 % of that found in 0.02M-citrate buffer.

Utilization of pyruvate. At 37°, the rate of CO₂ formation from pyruvate gradually decreased (Fig. 3). This fall in the rate of the reaction was not due to a change in pH. Although the conversion of 400 μ l. pyruvate into acetoin may raise the pH from 4.0 to 4.7 in the presence of 0.02M-citrate buffer,

Fig. 3. Course of CO₂ formation from pyruvate. 0.34 mg. cells incubated at 37°, N₂, in 2.5 ml. 0.06M-citrate buffer (pH 3.9); 0.008M-pyruvate; 0.008M-glucose.

such a change in pH does not cause a major change in rate: at pH 4.7, the Q_{CO_2} was 160, and at pH 4.0 it was 175 (conditions as in Fig. 2). In later experiments, 0.06M-citrate buffer was used, and, in this buffer, the pH did not change appreciably during the utilization of 400 μ l. pyruvate.

The yield of acetoin and CO₂ accounted for all the pyruvate used at pH 4 (Table 3, Exp. a). When

cells were incubated with a higher concentration of pyruvate, more CO₂ and acetoin were formed, but the quantity of pyruvate unused was greater than with the lower concentration of pyruvate. The decrease in the concentration of pyruvate, therefore, was not the factor stopping the reaction.

Fig. 4. Effect of pH on the course of CO₂ evolution. 1.4 mg. cells incubated in 2.5 ml. 0.02M-citrate buffer at pH indicated; 0.008M-pyruvate, 37°, N₂.

When different concentrations of the same suspensions of cells were incubated with the same concentration of pyruvate the final quantity of pyruvate utilized was approximately proportional to the weight of cells (Table 3, Exp. b). At 20° the rate of CO₂ formation from pyruvate was constant for 60 min. (Q_{CO_2} = 113). At 15° the rate of CO₂ formation by the same suspension was constant for 3.5 hr. (Q_{CO_2} = 92). The quantity of CO₂ formed by the cells

Table 3. *Factors influencing the metabolism of pyruvate at pH 4*

(Cells incubated in 2.5 ml. solution containing either 0.06M-citrate, 0.01M-barbiturate or 0.016M-acetate.)

Exp.	Dry wt. of cells (mg.)	Buffer	Additions	Time (min.)	Temp. (°)	Initial pyruvate (μ l.)	CO ₂ formed (μ l.)	Acetoin formed (μ l.)	Residual pyruvate (μ l.)	Recovery (%)
a	0.3	Citrate	—	190	37	0	10	—	—	—
						312	71	34	243	100
						547	159	88	400	113
b	0.35	Citrate	—	140	37	400	16	—	—	—
	1.9			140	37	400	69	—	—	—
c	1.8	Barbiturate	—	100	37	400	213	114	177	97
			Glucose (112 μ l.)	100	37	400	345	190	38	96
d	0.9	Acetate	—	150	25	—	318	177	—	—
			Acetaldehyde (45 μ l.)	150	25	—	259	133	—	—
			Glucose (224 μ l.)	150	25	—	704	380	—	—
			Acetaldehyde + glucose	150	25	—	539	277	—	—

before the rate fell off was approximately the same in these two cases.

The rates of reaction fell off more rapidly at a lower pH (Fig. 4). The reason for this effect of pH was not discovered.

Effect of glucose. In the presence of glucose more pyruvate was decomposed than without glucose, but the acetoin and CO_2 formed again accounted for the pyruvate used (Table 3, Exp. c). There was no stoicheiometric relation between the glucose added and the effect produced, e.g. 20 μl . was sufficient to enable 0.4 mg. cells to decompose 300 μl . pyruvate. Glucose increased not only the quantity of pyruvate used, but also the rate of the reaction (Fig. 3). Using citrate buffer, the highest Q_{CO_2} values were obtained with 0.0004–0.001M-glucose (Table 4). Glucose had no effect on the ratio CO_2 : acetoin formed.

Table 4. *Effect of glucose on CO_2 formation from pyruvate by Lactobacillus plantarum*

(2.5 ml. solution containing 1.6 mg. cells, 0.02M-citrate buffer, pH 4.0, 0.008M-pyruvate, incubated at 37° in N_2 ; Q_{CO_2} from 10th to 20th min.)

Glucose added		Q_{CO_2}
(μl .)	(mm)	
0	0	177
4.5	0.08	312
11.2	0.20	374
22.4	0.40	488
44.8	0.80	565

At pH 6.5 glucose is converted by the organism chiefly (80%) to lactic acid (Stephenson & Rowatt, 1947). Lactic acid was also formed at pH 4 during the formation of acetoin from pyruvate in the presence of glucose. In an experiment in which 1.6 mg. cells formed 376 μl . CO_2 from 400 μl . pyruvate, 114 μl . lactic acid were formed from 114 μl . glucose and approximately 54 μl . glucose were recovered. In a parallel experiment, when glucose alone was added, neither glucose utilization nor lactate formation occurred. Barbiturate buffer was used in these experiments. The effect of glucose on the pH activity curve is shown in Fig. 2. Glucose sharpened the peak of the curve. The time course of

the CO_2 evolution varied with the pH (Fig. 5). Adenosinetriphosphate (0.0016M) reduced, and glutathione (0.04% w/v) or sodium glycerophosphate (0.02M) had no effect on the decomposition of pyruvate at pH 4.

Fig. 5. Effect of pH on course of CO_2 formation from pyruvate in presence of glucose. 2.5 ml. solution containing 1.4 mg. cells, 0.02M-citrate buffer, 0.008M-pyruvate and 0.004M-glucose incubated at 37° in N_2 . The Q_{CO_2} recorded at each time is that obtaining over the previous 5 min.

The addition of phosphate to suspensions containing pyruvate alone did not affect the rate of CO_2 formation, but, in the presence of glucose and pyruvate, phosphate increased the maximum rate and delayed the fall in rate (Table 5). The quantity of acetoin formed was similarly increased.

Incubation of cells at 37° in citrate buffer at pH 3.9 without substrate reduced their ability to form acetoin and CO_2 from pyruvate at pH values below 4. At higher pH values little CO_2 was formed from pyruvate alone, but, in the presence of glucose and pyruvate, the rate of CO_2 formation by these pre-treated cells gradually increased. The reactivation was very sensitive to pH; the Q_{CO_2} at pH 4.16 was, in the experiment shown in Table 6, twenty times greater than the Q_{CO_2} at pH 3.94.

Table 5. *Effect of phosphate on rate of CO_2 formation from pyruvate in presence of glucose by Lactobacillus plantarum*

(2.5 ml. solution containing 1.2 mg. cells, 0.04M-citrate buffer (pH 4), 0.008M-pyruvate incubated at 37° in N_2 .)

Addition	Q_{CO_2}			
	5–15 min.	15–25 min.	25–35 min.	35–45 min.
None	240	124	54	—
0.02M-Potassium phosphate, pH 4	252	133	86	—
0.008M-Glucose	286	258	274	262
0.008M-Glucose + 0.02M-phosphate	313	346	522	417

Table 6. *Effect of glucose and pH on activity of pretreated cells*

(5 mg. cells incubated 20 min. at 37° in 12 ml. 0.02M-citrate buffer (pH 3.8); centrifuged from medium, resuspended 0.9% NaCl, incubated 0.06M-citrate buffer. Initial pH estimated on buffer, final pH estimated on suspension medium.)

Substrate	Initial pH	Final pH	Q_{CO_2}	
			5-10 min.	30-35 min.
0.008M-Pyruvate	4.16	4.17	123	25
0.008M-Pyruvate + 0.008M-glucose	3.94	3.90	100	10
	4.16	4.17	133	208

The pyruvate concentration used in the above experiments was not the optimal concentration; the rate of CO_2 formation increased with substrate concentration up to 0.01 or 0.03M (Fig. 6) varying according to pH. Above this concentration the rate fell, and at 0.1M very little CO_2 was formed. At this concentration the experimental error in the determination of pyruvate was too great to show whether

Fig. 6. Effect of substrate concentration on the rate of CO_2 formation from pyruvate. 1.6 mg. cells incubated in 2.5 ml. 0.06M-citrate buffer (pH 4), 0.008M-glucose, pyruvate concentration as stated. Q_{CO_2} with pyruvate from 5th to 10th minute; Q_{CO_2} pyruvate and glucose from 15th to 20th minute.

any pyruvate had been used. The rate of CO_2 formation from pyruvate in presence of glucose was similarly inhibited at high concentrations of pyruvate, but the inhibitory effect appeared at a lower concentration of pyruvate. High concentrations of pyruvate had the same effect on the formation of CO_2 from pyruvate by the strain of *A. aerogenes* used. With this organism, concentrations above 0.01M were inhibitory.

Effect of inhibitors and acetaldehyde. Acetoin formation by cells incubated in citrate buffer with

pyruvate was not inhibited by 0.001M-cyanide. 0.004M-Fluoride caused 61% inhibition when glucose was absent and 84% when it was present. 0.005M-Azide inhibited the reaction by 97% when glucose was absent and 95% when it was present.

Acetaldehyde did not increase, but slightly inhibited, the formation of acetoin when it was added to cells incubated with pyruvate or with pyruvate and glucose (Table 3, Exp. d). The ratio of CO_2 to acetoin formed was virtually unaltered by acetaldehyde (1.8 when it was added and 1.9 when it was not).

Effect of dicarboxylic acids on the decomposition of pyruvate by Lactobacillus plantarum, Staphylococcus aureus and Aerobacter aerogenes

Effect of fumarate on Lb. plantarum. The reduced rate of CO_2 formation from pyruvate in the presence of succinate suggested that fumarate might be concerned in the breakdown of pyruvate. This compound, however, when added to suspensions of cells

Fig. 7. The effect of varying concentrations of fumarate on the rate of CO_2 formation from pyruvate by *Lb. plantarum*. Cells (1.2 mg.) incubated in 2.5 ml. 0.06M-citrate buffer (pH 4) containing (a) 0.016M-pyruvate, $\blacktriangle$ — $\blacktriangle$; (b) 0.016M-pyruvate + 0.008M-glucose, $\bullet$ — $\bullet$.

incubated with pyruvate in citrate buffer, caused a strong inhibition of pyruvate utilization; for example, cells (1.1 mg.) incubated with 500 μ l. pyruvate formed 454 μ l. CO_2 , while 459 μ l. pyruvate were used; when 1120 μ l. fumarate were added,

10 μ l. CO_2 were formed and 20 μ l. pyruvate used. The inhibition affected CO_2 and acetoin formation equally: 0.0025 M-fumarate inhibited CO_2 formation by 92 % and acetoin formation by 96 %; the figures with 0.0002 M-fumarate were 64 and 69 % respectively. The inhibition was progressive (Fig. 7) and reached over 90 % at 10^{-3} M-fumarate after 25 min. incubation. A significant inhibition was caused by only 1×10^{-4} M-fumarate. In the presence of glucose the degree of inhibition was greater.

Effect of fumarate on acetoin formation by Staph. aureus and A. aerogenes. In order to find whether fumarate generally inhibited this type of pyruvate breakdown, its effect on other organisms forming acetoin was tested. Lemoigne (1913) showed that Staphylococci formed acetoin when growing in media containing glucose. Washed cells of a strain of *Staph. aureus* were incubated with pyruvate under the conditions used for *Lb. plantarum* (pH 4.0). Acetoin and CO_2 were formed in the same proportions (2:1) as in *Lb. plantarum*, the Q_{CO_2} being about 900; the formation of each was inhibited by fumarate (Table 7). The inhibition was again progressive and reached 80 % in the presence of 0.005 M-fumarate after 11 min.

Table 7. *Inhibition by fumarate of formation of CO_2 and acetoin from pyruvate by Staphylococcus aureus and Aerobacter aerogenes*

(*Staph. aureus* (1.4 mg.) incubated 17 min. or *A. aerogenes* (0.8 mg.) incubated 20 min., each in 2.5 ml. 0.06 M-citrate (buffer pH 4).)

(buffer pH 4.)			
Additions	Pyruvate used (μ l.)	CO ₂ formed (μ l.)	Acetoin formed (μ l.)
<i>Staph. aureus</i>			
Pyruvate (0.008M)	—	250	112
Fumarate (0.005M)	—	40	1
Pyruvate (0.008M)	—	87	36
+ Fumarate (0.005M)			
<i>A. aerogenes</i>			
Pyruvate (0.006M)	240	260	81
Fumarate (0.005M)	—	16	0
Pyruvate (0.006M)	67	71	24
+ Fumarate (0.005M)			

Acetoin and CO_2 formation from pyruvate by *A. aerogenes* (see Silverman & Werkman, 1941) at pH 4 was also inhibited by fumarate (Table 7), but the concentration of fumarate required was higher than in the case of *Lb. plantarum*; only 30 % inhibition was caused by 0.004 M-fumarate. Further differences were that the ratio CO_2 :acetoin exceeded 2 with some suspensions of this organism, and glucose did not increase the rate of CO_2 formation.

Effect of other acids on CO_2 formation by Lb. plantarum. Malate and maleate when added to suspensions of cells in citrate buffer caused approxi-

mately the same degree of inhibition as succinate; *cis*- and *trans*-aconitate and aspartate had a smaller effect (Table 8). Malonate at a concentration of 0.04 M caused 47 % inhibition. In this case the cells were incubated in acetate buffer (0.04 M) instead of citrate. All these compounds inhibited acetoin formation to approximately the same extent as CO_2 formation.

Table 8. *Effect of substances related to fumarate on CO_2 formation from pyruvate by Lactobacillus plantarum*

(Cells incubated with 0.06 M-citrate buffer, pH 4, 0.016 M-pyruvate. Inhibition measured as percentage drop in Q_{CO_2} after 20–25 min. incubation.)

Concentration of inhibitor	Inhibition (%)	
	0.004 M	0.015 M
Inhibitor		
Maleate	10	64
DL-Malate	16	69
<i>cis</i> -Aconitate	5	31
<i>trans</i> -Aconitate	0	15
DL-Aspartate	—	11

Table 9. *Inhibition by phthalate of CO_2 formation from pyruvate by Lactobacillus plantarum*

(Cells (1.9 mg.) incubated in 2.5 ml. 0.06 M-citrate buffer (pH 4) with 0.016 M-pyruvate for 130 min.)

Additions	Q_{CO_2}		Total CO_2 formed (μ l.)
	5–10 min.	20–25 min.	
None	208	174	362
Phthalate, 0.004 M	39	20	37
Phthalate, 0.002 M	119	89	153
Phthalate, 0.001 M	149	129	217
Glucose, 0.008 M	231	508	782
Glucose, 0.008 M, and phthalate, 0.004 M	40	25	46
Glucose, 0.008 M, and phthalate, 0.002 M	113	89	177
Glucose, 0.008 M, and phthalate, 0.001 M	135	153	705

With the exception of fumarate, phthalate had the highest inhibitory activity of the substances tested. The inhibition of Q_{CO_2} by 0.001 M-phthalate was 30 % in the presence of pyruvate alone and 70 % in the presence of pyruvate and glucose (Table 9). At the two higher phthalate concentrations shown in this table, the rate of CO_2 formation was the same whether glucose was added or not. Phthalate did not alter the ratio CO_2 :acetoin formed. The inhibition by lactate and acetate, and especially succinate and barbiturate, may be of the same type as those mentioned in this section.

Effect of aneurin on pyruvate decomposition by Lactobacillus plantarum

Comparison of normal and aneurin-deficient cells. That cocarboxylase is required for the formation of acetoin and CO_2 from pyruvate has been shown by

Table 10. *Effect of aneurin*

(Cells grown in medium *P* without yeast extract, with or without 0.1 μ g. aneurin/ml. Cells incubated in 2.5 ml. 0.06 M-citrate pH 4.1, 0.008 M-pyruvate, at 37° in N₂.)

Additions	Aneurin-deficient cells (0.6 mg.)		Aneurin-rich cells (0.7 mg.)	
	Q _{CO₂} 15-20 min.	CO ₂ /acetoin	Q _{CO₂} 15-20 min.	CO ₂ /acetoin
None	55	—	166	2.5
0.008 M-Glucose	96	4	554	1.9
Aneurin, 8 μ g./ml.	47	—	180	2.1
Glucose + aneurin	664	1.9	567	2.1

Silverman & Werkman (1941) with *A. aerogenes* and by Green, Westerfeld, Vennesland & Knox (1942) with heart muscle. When *Lb. plantarum* was grown in a medium deficient in aneurin, the crop of cells was little less than in the presence of aneurin (e.g. 5.6 and 4.8 mg./100 ml.), but the deficient cells, when incubated with pyruvate, or pyruvate and glucose, formed very little CO₂ or acetoin. Addition of aneurin alone did not increase the CO₂ formation from pyruvate, but on incubation with pyruvate, glucose and aneurin, deficient cells formed CO₂ and acetoin at the same rate as cells grown in a medium containing aneurin. The quantity of CO₂ formed by the deficient cells was 1 mol./mol. of pyruvate used, whether aneurin was added or not. Aneurin did not affect the rate of CO₂ evolution by cells grown in a normal medium (Table 10).

Fig. 8. The effect of aneurin on the course of CO₂ formation from pyruvate in presence of glucose. Conditions as in Table 10. Cells grown in aneurin-deficient medium (1) incubated with glucose, ○—○; (2) incubated with glucose + aneurin, ●—●. Cells grown in aneurin-rich medium (1) incubated with glucose, △—△; (2) incubated with glucose + aneurin ▲—▲.

In the experiment shown in Table 10 the time taken by normal cells to reach the maximum rate of CO₂ formation with glucose was the same as that taken by aneurin-deficient cells to which aneurin and glucose had been added (Fig. 8).

Effect of pantothenate on metabolism of pyruvate by Lactobacillus plantarum

Cells grown in pantothenate-deficient medium, when incubated at pH 4, formed acetoin at the normal rate, and this was not affected by the addition of pantothenate (Table 11). The same cells formed little acetylcholine unless pantothenate was added to the suspension medium. Nor had pantothenate a definite effect on the rate of pyruvate decomposition at pH 6, nor on the O₂ uptake.

Table 11. *Effect of pantothenate on the formation of acetylcholine and acetoin by pantothenate-deficient cells of Lactobacillus plantarum*

(Cells grown in 1 l. medium without pantothenate 20 hr., washed and made up in 0.01 M-phosphate buffer (pH 7). Acetylcholine formation tested in suspensions containing 1 mg. cells in 5 ml. 0.3 M-acetate buffer (pH 6.5), 0.001 M-glucose, 0.05% choline HCl; 210 min. incubation at 25° in N₂. Acetoin formation tested in suspensions containing 0.6 mg. cells in 2.5 ml. 0.02 M-citrate buffer (pH 4), 0.01 M-glucose, 0.04 M-pyruvate; 120 min. incubation. Pantothenate concentrations 0.16–0.2 μ g./ml.)

Metabolite formed	Control	Calcium pantothenate added
Acetylcholine (μ g. in 5 ml.)	0.6	12.2
Acetoin (μ l. in 2.5 ml.)	123	128
Q _{CO₂} (0.004 M-pyruvate)	190	240
Q _{CO₂} (0.004 M-pyruvate + 0.01 M-glucose)	330	342

DISCUSSION

A quantitative formation of acetoin from pyruvate has previously been found in *Aerobacter* by Silverman & Werkman (1941), but has not been described in *Lb. plantarum* and related organisms, although small quantities of this compound are formed during the growth of many bacteria (Stahly & Werkman, 1942). The following four factors were found to affect the rate of acetoin formation from pyruvate: pH; glucose in catalytic quantities; aneurin; phosphate. The effects of aneurin and phosphate were observed by previous workers in other organisms. Pantothenate, which is required by the organism for the synthesis of acetylcholine, is not necessary for the formation of acetoin.

The mechanism of acetoin formation is not fully understood, and at the present stage of knowledge no explanation of the glucose effect can be offered. The fact that the acetoin formed is optically active indicates that it is formed by an enzymic process (see Dirscherl, 1930).

No explanation can be offered for the fact that low concentrations of fumarate inhibit the formation of acetoin from pyruvate by *Lb. plantarum*, *A. aerogenes* and *Staph. aureus*. It is noteworthy that in *Staph. aureus* at pH 7.0, when pyruvate is broken down by a different route, the anaerobic decomposition of pyruvate is stimulated by fumarate (Krebs, 1937a). The effects of other dicarboxylic acids such as phthalate, malate, maleate, malonate and succinate are probably based on the same type of reaction as the inhibition by fumarate.

The formation of acetoin from pyruvate is one of the few relatively rapid reactions known to occur in *Lb. plantarum*.

SUMMARY

1. *Lactobacillus plantarum* converted pyruvate quantitatively into laevorotatory acetoin and carbon dioxide at pH 4. At pH 6 pyruvate decomposition was slower and the amount of pyruvate was greater than the sum of acetoin, carbon dioxide, lactic and acetic acids formed.

2. The initial rate of carbon dioxide formation from pyruvate gradually fell, especially when the pH was below 4. Addition of glucose in catalytic amounts raised the Q_{CO_2} and delayed its fall.

3. Potassium phosphate further increased the Q_{CO_2} and delayed its fall in the presence of glucose but not in its absence.

4. At high concentrations of pyruvate (0.1M), carbon dioxide formation was strongly inhibited.

5. Preliminary incubation of cells at pH 3.8 in buffer solutions without substrate reduced the capacity of the cells to decompose pyruvate. At pH values above 4 the capacity could be restored by glucose.

6. The decomposition of pyruvate by *Lactobacillus plantarum* at pH 4 is inhibited by low concentrations of fumarate. At all concentrations of fumarate, the degree of inhibition of acetoin and carbon dioxide formation is approximately the same. The degree of inhibition by a given concentration of fumarate is greater when glucose is present than when it is not.

7. The formation of carbon dioxide and acetoin from pyruvate by strains of *Aerobacter aerogenes* and *Staphylococcus aureus* is also inhibited by fumarate, but with these organisms a higher concentration of fumarate is required.

8. The formation of carbon dioxide and acetoin by *Lactobacillus plantarum* is inhibited to a lesser degree by phthalate, succinate, DL-malate, maleate, malonate, barbiturate, *cis*- and *trans*-aconitate and DL-aspartate.

9. The concentration of pantothenate in the suspension medium did not affect the rate of pyruvate decomposition.

10. Cells grown in a medium deficient in aneurin formed very little carbon dioxide or acetoin unless both aneurin and glucose were added to the suspension.

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