The Mechanism of Propionic Acid Formation by Propionibacteria

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SUMMARY: The main mechanism for propionic acid formation by organisms of the genus *Propionibacterium* is by the decarboxylation of succinic acid. The varying ratios of propionic to acetic acid reported in the literature as a result of the fermentation of glucose are attributed to differences in carbon dioxide tension and in the initial and final pH values of the cultures.

The method of formation of propionate from lactate and pyruvate by propionibacteria has remained uncertain. Werkman & Wood (1942) reviewed the various mechanisms suggested for the formation of propionic acid and made two proposals. The first was that water is removed from lactate to form acrylic acid which is then reduced to propionic acid. They regarded this as the most probable mechanism, though the reduction of acrylic acid by propionibacteria has not been demonstrated. The second possibility suggested was that the formation of propionic acid is by the decarboxylation of a symmetrical dicarboxylic acid. This was based on their experiments with isotopes and on unpublished evidence that the decarboxylation of succinate can occur by a reaction with a pH optimum of 5.2. Wood & Werkman (1942) conclude by saying: 'Frankly, sufficient data is not available with which to formulate a defensible scheme.'

In the present paper evidence is presented that the formation of propionic acid by the decarboxylation of succinate does occur in *Propionibacterium* as has been demonstrated with *Veillonella gazogenes* (Johns, 1951). Similar evidence, based on work with *Propionibacterium pentosaceum*, has been published by Delwiche (1948) since the completion of this work (Johns, 1948).

EXPERIMENTAL

Methods

Succinic acid was estimated by the procedure of Umbreit, Burris & Stauffer (1945); glucose by the method of Hassid (1937); volatile acids by the chromatographic method of Elsdon (1946). The accuracy of the latter method for mixtures of acetic and propionic acids was found to be $\pm 3\%$ with a satisfactory preparation of the silica gel. Lactic acid was estimated by the method of Friedemann & Graeser (1933). The general purposes salt mixture of Stephenson (1948) with 0.4% Difco yeast extract was used as the basal medium for growing bacteria.

Organisms used. The strains of propionic acid bacteria used were *P. shermanii* E11.1 and *P. zeae* E8.1 kindly supplied by Prof. C. B. van Niel. A dried preparation of *P. shermanii* was made according to the method of Barker & Lipmann (1944).

RESULTS

P. zeae was grown at 30° for 40 hr. in the basal medium with 1 % (v/v) Na lactate solution (c. 50 %) added. The cells were spun down, washed twice with distilled water and taken up in phosphate buffer of pH 5.3. The results of the action of this suspension are shown in Table 1, which shows that there was an evolution of CO₂ (identified by using manometers with 20 % NaOH in the centre well) with succinate, but that the rate of evolution decreased rapidly with time.

Table 1. *Decarboxylation of succinate by washed suspensions of Propionibacterium zeae grown 40 hr. at 30°*

Bacterial suspension in buffer, 2 ml. (12 mg. dry weight/ml.), 0.2 ml. 0.1M succinate; 0.5 ml. 0.1M-phosphate buffer (pH 5.3) in side bulb with succinate.

Manometers ...	1	2	3	4
Period (min.)	Succinate (μl. CO ₂)		Blank (μl. CO ₂)	
5	15	16	7	8
20	39	41	20	20
60	64	68	22	23

Q_{CO_2} for first 5 min., 4.5; for first 20 min., 2.5; for first 60 min., 1.75.

Attempts were made to increase the rate of decarboxylation. It was thought that if cell permeability were a controlling factor dried cells might show an increased permeability to succinate. A dried preparation of *P. shermanii* was made from 10 l. of bacteria grown in 1 % (v/v) Na lactate + basal medium at 30° for 5 days. These cells were active with pyruvate, having a Q_{CO_2} of 2.5 compared with a Q_{CO_2} for fresh cells of 5.2, and kept well in a vacuum desiccator at 4°.

An experiment to determine the pH optimum of the decarboxylation reaction using 20 mg. dried bacteria/ml. is shown in Fig. 1. The pH optimum for CO₂ evolution was about pH 5 and the rate fell off sharply below this pH and above pH 6. The Q_{CO_2} at pH 5 was 0.35 as compared with a Q_{CO_2} of 0.48 with fresh cells.

The effect of detergents on the rate of succinate decarboxylation was tried with fresh cells. Neither an anionic (Aerosol O.T.) nor a cationic (CTAB) detergent over a wide range of concentrations increased decarboxylation but rather inhibited it. A third possibility for increasing the Q_{CO_2} when using succinate was the provision of a source of energy for the transference of succinate more rapidly across the cell wall; but rather than an increase in the rate of succinate utilization in the presence of pyruvate or lactate, there was a significant decrease. All efforts to prepare a cell-free extract which would decarboxylate succinate, from fresh or dried cells of *P. shermanii*, were unsuccessful.

It was difficult to carry out a balance experiment on the decarboxylation of succinate as the reaction proceeded so slowly that a long period of incubation or a large quantity of bacteria would be needed to obtain a reasonable con-

version. It was decided that the easiest and most satisfactory method would be to carry out a growth experiment in which glucose and succinate together were fermented, with a fermentation of glucose alone as a control. In this case growth would result from the fermentation of glucose, the pH would fall and if the organisms were able to decarboxylate succinate they should do so at the acid pH.

Two identical manometers were used with 100 ml. flasks. The medium in both flasks was adjusted to pH 6.5 (by glass electrode); glucose was sterilized separately and identical amounts added to each flask. Both flasks were inoculated with 5 ml. of a 5-day culture of *P. shermanii* grown on sodium lactate. Samples were removed with sterile precautions for the initial determinations of glucose and succinate. The whole fermentation was carried out under sterile conditions in an atmosphere of nitrogen at 37°. Manometer readings were taken twice daily until gas production ceased. The two sets of manometer readings corrected to N.T.P. are plotted in Fig. 2.

Fig. 1. Rate of decarboxylation of succinic acid by dried cells of *P. shermanii* at different pH values.

Fig. 2. The effect of succinate on the production of CO₂ by *P. shermanii* grown with glucose.

When the fermentation had ceased the dissolved CO₂ in the fermentation liquid was determined by the method described by Peters & van Slyke (1932) for urine. The results are given in Table 2. The final acid figures include the amounts added in the inoculum; these were the same in both flasks. The 'initial succinate' in the glucose control *B* was that added with the inoculum. Neglecting the slight difference (0.02 mmol.) in the amount of glucose fermented and the acetic acid produced (0.01 mmol.), 0.48 mmol. of succinate disappeared to give rise to 0.51 mmol. of propionic acid and 0.46 mmol. of CO₂. The final pH was 4.4.

In spite of the large inoculum used it will be noticed from Fig. 2 that the complete fermentation of glucose took 6 days. This emphasizes the slow rate of

Table 2. *A comparison of the amount of CO₂ and propionic acid produced by Propionibacterium shermanii in the fermentation of: A, glucose + succinate; B, glucose alone*

Manometer *A*, 50 ml. basal medium (pH 6.5) and glucose + 1.5 ml. 0.1 M sodium succinate. Manometer *B*, the same except for 1.5 ml. distilled water in place of sodium succinate. Temp. 37°, gas phase N₂.

	<i>A</i> (mmol.)	<i>B</i> (mmol.)
Glucose fermented	1.32	1.30
Succinate fermented	0.49	0.01
Final propionate	2.76	2.25
Final acetate	0.76	0.75
Total CO ₂	1.40	0.94
Excess propionate	0.51	—
Excess acetate	0.01	—
Excess CO ₂	0.46	—

growth of the propionibacteria, and emphasizes the fact that it would not require a high rate of succinate decarboxylation to account for all the propionic acid produced. This experiment confirms the indications given by the washed suspension experiments that propionibacteria can decarboxylate succinate to propionic acid and carbon dioxide at pH values below 6.

The theoretical equation for the fermentation of glucose by propionibacteria is

giving a propionic acid:acetic acid ratio of 2:1. This ratio has rarely been observed. In a previous paper (Johns, 1950) it was demonstrated that the CO₂ concentration in the medium influenced the acetic to propionic acid ratio during the fermentation of lactate by *Veillonella gazogenes*. These experiments were repeated with *Propionibacterium shermanii* using three different concentrations of CO₂: *A* was carried out in a sealed tube with added sodium carbonate so that the fermentation finished up with CO₂ under pressure; *B* was similar to *A* but without sodium carbonate, not sealed, and incubated

Table 3. *Effect of CO₂ concentration on the fermentation of lactate by Propionibacterium shermanii*

Incubation time 14 days at 30°; pH 7.2. Lactate 6 mmol. in each tube; 20 ml. basal medium. Acid production in mmol.

Tube	...	<i>A</i> , 2 mmol. Na ₂ CO ₃ tube sealed	<i>B</i> , unsealed tube	<i>C</i> , CO ₂ absorbed
Propionic acid (P)		3.45	3.38	3.38
Acetic acid (A)		1.61	1.72	1.78
Ratio P/A		2.14	1.96	1.90

anaerobically; *C* was carried out in a 50 ml. volumetric flask to which was connected a second flask containing 20 ml. of alkaline pyrogallol to absorb the CO₂ produced during the experiment and to maintain anaerobic conditions. The results are shown in Table 3. As can be seen (from the results), there is

a slightly higher ratio with the fermentation carried out in excess CO_2 as compared with that in which the CO_2 was absorbed, but the difference could hardly be considered convincing. In this experiment there was little change in acidity. Three molecules of lactic acid give rise to three molecules of volatile acid (two of propionic acid, one of acetic acid).

The above experiment was repeated with glucose as the substrate, when a considerable increase in acidity occurred. The results are presented in Table 4.

Table 4. *The effect of the CO_2 concentration on fermentation of glucose by Propionibacterium shermanii*

6 mmol. glucose in each flask; pH 6.5; temperature 30° ; acid production measured in mmol.

	2 mmol. Na_2CO_3 tube sealed	Tube open	CO_2 absorbed
Propionic acid (P)	1.14	1.00	1.04
Acetic acid (A)	0.32	0.35	0.59
Ratio P/A	3.56	2.80	1.76

It will be seen in Table 4 that the increase in CO_2 tension led to a higher propionic:acetic ratio in the fermentation of glucose. No doubt this is in part the explanation of the widely varying ratios of propionic:acetic reported in the literature. The experiments of previous investigators have been carried out under varying conditions of pH and with different types of buffer.

DISCUSSION

Lactate and pyruvate are readily converted into propionate by propionibacteria, hence it was generally assumed (Kluyver, 1931) that lactate was an intermediate in the reduction of pyruvate, i.e. pyruvate $\xrightarrow{2\text{H}}$ lactate $\xrightarrow{2\text{H}}$ propionate. Wood & Werkman (1936) introduced the concept of heterotrophic utilization of CO_2 in connexion with their studies on the fermentation of glycerol by propionibacteria. Wood & Werkman (1938) proposed that succinic acid was formed in the fermentation by the combination of a C_1 and a C_3 compound. Krebs & Eggleston (1941) showed that the set of reactions from oxaloacetate to succinate occurred in the propionibacteria (oxaloacetate $\rightleftharpoons$ malate $\rightleftharpoons$ fumarate $\rightarrow$ succinate). Carson & Ruben (1940) using C^{11} and Wood, Werkman, Hemingway & Nier (1940, 1941) using C^{13} , confirmed that fixation occurred and that the labelled CO_2 appeared in the carboxyl group of succinic acid. However, quite unexpectedly, labelled carbon was also found in the carboxyl group of propionic acid. Both Carson, Foster, Ruben & Barker (1941), and Krebs & Eggleston (1941) explained the presence of isotopic carbon in propionic acid by postulating that the fixation process proceeded by the same pathway as for the formation of succinic acid but only as far as fumaric acid, and that by a reversal of these steps propionic acid was formed by reduction of the pyruvic acid which then contained the labelled carbon in its carboxyl group. Neither groups of workers demonstrated any decarboxylation of succinate. Barker & Lipmann (1944), working with dry preparations of *P. pentosaceum*, found, on the basis of fluoride

inhibition studies, that lactate was not on the normal pathway from pyruvate to propionate.

The quantitative studies of Werkman & Wood (1942), in which they found equal concentrations of C^{13} in the carboxyl groups of the succinic and propionic acids, find their simplest interpretation in the presence of a succinic decarboxylase in the bacteria. The results presented above, and those of Delwiche (1948), demonstrate that propionibacteria are able to decarboxylate succinic acid below a pH of 6.0 at a rate sufficient to justify the assumption that this reaction is concerned in the production of propionic acid. Shaw & Sherman (1923) and Hitchner (1934) claimed to have demonstrated the fermentation of succinate with propionibacteria, but in both cases the utilization of succinate was only achieved under aerobic conditions. The experiments of Fromageot & Bost (1938) with washed suspensions of *P. pentosaceum* indicated that succinate was attacked only in the presence of glucose, but Krebs & Eggleston (1941) could not confirm this. The results of Fromageot & Bost (1938) are readily explained by the fact that the fermentation of glucose lowered the pH sufficiently to permit succinic decarboxylase activity. Krebs & Eggleston (1941) used bicarbonate buffer where a marked drop in pH does not take place. In the present work lactate or pyruvate in addition to succinate gave no increase in the rate of CO_2 evolution from succinate.

Although the equations for the fermentation of lactate (Fitz, 1878, 1879, 1880), and glucose by propionibacteria, have been established for a long time the ratio of 2:1 for propionic:acetic acid has rarely been obtained. The explanations for this variability in volatile acid ratio have previously centred round hypothetical pathways for anaerobic utilization of acetate (Wood & Werkman, 1938). Krebs & Eggleston (1941) rejected Wood & Werkman's hypothesis and suggested that acetate was oxidized to CO_2 , but produced no convincing evidence to support this. Carson (1948) obtained indications in isotope experiments with propionibacteria that acetate was metabolized through a condensation reaction which subsequently yielded CO_2 . The degree of importance of this reaction depended on the state of oxidation of the substrate. The amount of initial C^{14} acetate which was converted to $C^{14}O_2$ was 2 % with glycerol as substrate and 88 % with pyruvate.

If the main method of propionic acid formation is by succinate decarboxylation, it would be expected that the amount of propionic acid present would be dependent on the concentration of CO_2 in the medium. Elsdon (1938) showed with *Escherichia coli* that the amount of succinic acid formed in the fermentation of pyruvate, glucose and galactose was influenced by the CO_2 concentration in the medium.

With propionibacteria a large effect of CO_2 concentration on the propionic:acetic ratio was only found with a substrate whose dissimilation caused an increase in acidity and so allowed the extra succinic acid formed in the presence of CO_2 to be decarboxylated.

There is always a tendency to obtain a higher propionic:acetic ratio with glucose than with lactate, e.g. in the present work the highest ratio with lactate was 2.14, while with glucose it was 3.56. As glucose and lactate are at

the same redox level this difference in propionic:acetic ratio cannot be explained by the difference in the amounts of acetate converted to CO_2 due to their different levels of oxidation as indicated by the experiments of Carson (1948).

It appears that the large variations reported in the literature for the ratio of propionic acid:acetic acid in fermentations of glucose and lactate by propionibacteria can be explained on differences in CO_2 concentration in the medium. Evidently succinic acid can pass out of the bacterial cell at pH values above c. 6.5 but cannot re-enter until the pH drops below that level. When the CO_2 concentration in the medium is increased, more succinic acid is formed, the excess passes out into the medium and can only be decarboxylated when the pH falls giving a high propionic:acetic ratio.

Van Niel (1928) found that the propionic:acetic ratio was always less than 2 in lactate fermentations with propionibacteria, and always greater than 2 with glucose as substrate. The explanation seems to be that he absorbed the CO_2 produced in his lactate fermentations, and added CaCO_3 to neutralize the acid produced during the glucose dissimilation.

Foote, Fred & Peterson (1930), in glucose fermentation experiments with propionibacteria, did not allow the pH to fall, but neutralized the acidity with NaOH daily. The highest propionic:acetic ratio was 2.16 and large amounts of succinic acid accumulated. In one case CaCO_3 was used in the medium instead of the daily neutralization and this gave a propionic:acetic ratio of 2.85. Wood & Werkman (1934), in an experiment on glucose fermentation with NaHCO_3 as buffer, analysed the fermentation liquid at intervals. The pH remained constant at 7.2 until just before the end of the experimental period of 14 days. The ratio of propionic to acetic acid remained constant at about 2.4 during this time, while the amount of succinic acid steadily increased. Wood & Werkman (1936) used CaCO_3 as the buffer and found that the pH dropped from 7.0 to 5.5 during the fermentation of glucose, the amount of succinic acid formed decreased and the propionic:acetic ratio steadily increased.

Delwiche (1948) made an analysis of a fermentation broth for volatile acids at varying times and pH intervals throughout the fermentation. Significant amounts of propionic acid did not appear until the pH was below 6.5 and the peak of production was at a pH below 6. The ratio of propionic to acetic acid rose steadily from 0.7:1 at 23 hr. to 2.35:1 at 41 hr. and there was no indication of a utilization of acetate of any period.

There were indications in the present work that there may be strain differences in the propionic:acetic acid ratio produced under the same conditions; this was indicated by the work of van Niel (1928).

The factors which appear to influence the propionic:acetic acid ratio in fermentation of glucose by propionibacteria may be summarized as follows: (a) the CO_2 tension in the medium; (b) the initial and final pH of the medium, the initial pH influencing the amount of succinic acid formed and the final pH determining how much will be decarboxylated; (c) the strain of *Propionibacterium*; (d) with the more oxidized substrates such as pyruvate the conversion of acetate to CO_2 evidently becomes important (Carson, 1948).

It seems quite clear therefore that in at least two bacterial species, *Propioni-*

bacterium and *Veillonella gazogenes*, the main mechanism of propionic acid production is by the decarboxylation of succinic acid. Whether there is a second mechanism for propionic acid formation in bacterial fermentations is not yet clear. Cardon & Barker (1947) studied an anaerobic alanine-fermenting bacterium, *Clostridium propionicum*, which produces acetic acid, propionic acid and CO₂ from alanine and lactate. It rapidly converts acrylate to propionate (which *Propionibacterium* does not), and is inactive against malate and fumarate. It would be of interest to determine whether this organism fixes CO₂ in propionic acid.

My thanks are due to Dr S. R. Elsdén for his help and advice in the carrying out of this work and for reading the proofs.

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(Received 1 February 1950)