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The Mechanism of Propionic Acid Formation by *Clostridium propionicum*

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SUMMARY: *Clostridium propionicum* evidently possesses a mechanism for the conversion of lactate to propionate different from that found in the previously studied propionic acid producing bacteria. Reactions common to certain propionibacteria and *Veillonella gazogenes* which could not be demonstrated with *Cl. propionicum* include: (a) the decarboxylation of succinic acid; (b) the fermentation of malate and fumarate; (c) the variation in the ratio of acetic to propionic acid according to the concentration of CO₂; (d) the fixation of CO₂ in propionic acid.

Two mechanisms have been proposed for the formation of propionic acid from lactate by propionibacteria (Werkman & Wood, 1942): (a) the dehydration of lactate to acrylate which is then reduced to propionate; (b) the formation of succinic acid and its subsequent decarboxylation, i.e. lactate → pyruvate ⇌ oxalacetate ⇌ malate ⇌ fumarate → succinate → propionate + CO₂. The propionibacteria are unable to ferment acrylic acid, so the first mechanism is untenable for these organisms. Evidence has been presented for strains of *Propionibacterium* (Delwiche, 1948; Johns, 1951*b*) and for *Veillonella gazogenes* (Johns, 1951*a*), which indicated that (b) represents the probable pathway for propionate formation in these bacteria. Cardon & Barker (1947) have shown that *Cl. propionicum*, an organism isolated from marine mud, decomposes lactate, acrylate and pyruvate to give acetic and propionic acids and carbon dioxide. The present paper gives results of experiments which attempted to demonstrate with *Cl. propionicum* reactions common to propionibacteria and *V. gazogenes*.

EXPERIMENTAL

Methods

The methods were essentially the same as those used previously (Johns, 1951*a*). The Van Slyke-Folch wet oxidation as used by Barker (1949*a*) was employed instead of the Pregl micro-combustion for the conversion of propionate and acetate to carbonate.

The strain of *Cl. propionicum* used was isolated by the enrichment culture method (Cardon & Barker, 1946) from a heap of decomposing grass clippings. The organism obtained readily fermented alanine, lactate, pyruvate, serine and threonine, failed to ferment sugars, and appeared in every way identical with the strain described by Cardon & Barker (1946).

The medium used for growth and the method of preparing washed cell suspensions were those of Cardon & Barker (1947). Cultures were harvested after 16 hr. growth at 37°.

RESULTS

Succinic acid decarboxylation

Washed suspensions of *Cl. propionicum* were tested by the Warburg technique for their ability to ferment succinic acid. The Warburg cups contained 1 ml. bacterial suspension (c. 20 mg. dry wt./ml.), 1.3 ml. M/15 phosphate buffer, 0.2 ml. 0.2M sodium succinate. The gas phase was nitrogen and the temperature of incubation 37°.

Over the range pH 5.3–7.6 there was no change of gas pressure in the manometers. The experiment was repeated with 0.1 ml. of 0.1M succinate in the presence of an equimolar quantity of alanine. These combined substrates gave no greater gas production than that given by alanine alone. By the same technique it was shown that DL-malate and fumarate, at pH values between 5.0 and 7.6, were not fermented by washed suspensions of the organism grown on either alanine or lactate.

The effect of sodium fluoride on fermentation of lactate and pyruvate

Barker & Lipmann (1944), using *Propionibacterium pentosaceum*, found that in presence of sufficient fluoride entirely to prevent lactate reduction, pyruvic acid was reduced to propionic acid. The influence of sodium fluoride on the fermentation of lactate and pyruvate by washed suspensions of *Cl. propionicum* was determined by the effect on the rate of CO₂ production. The rates of both dissimilations were progressively inhibited to a similar extent by increasing concentrations of fluoride (Table 1).

Table 1. *Fluoride inhibition of lactate and pyruvate fermentations by Clostridium propionicum*

Manometer flasks contained 1.5 ml. of bacteria (23 mg./ml.) in 0.1M phosphate buffer (pH 6.8); substrate 0.1 ml. 0.1M sodium pyruvate or 0.2 ml. 0.1M sodium lactate; NaF as indicated; 0.2 ml. 2N-H₂SO₄; total volume 2.5 ml. Gas phase N₂. Temp. 37°. Acid tip after 20 min.

NaF concentration (1 %)	Percentage inhibition	
	Lactate	Pyruvate
0	0	0
0.01	19	23
0.02	39	42
0.04	76	80
0.10	87	92
0.20	92	95

Since the effect of fluoride on the fermentation of lactate and pyruvate by washed suspensions of *V. gazogenes* had not previously been determined the experiment was repeated using a washed suspension of this organism (Johns, 1951*a*). The gas produced in this case was hydrogen and carbon dioxide; the results are shown in Table 2. Although the fermentation of lactate is more sensitive to fluoride than is that of pyruvate, complete inhibition was not achieved even with 0.2M-NaF.

Table 2. *Fluoride inhibition of lactate and pyruvate fermentations by Veillonella gazogenes*

Manometer flasks contained 1.5 ml. of bacteria (27 mg./ml.) in 0.1 M phosphate buffer (pH 6.8); substrate 0.1 ml. 0.1 M sodium pyruvate or 0.2 ml. 0.1 M sodium lactate; NaF as indicated; 0.2 ml. 2N-H₂SO₄; total volume 2.5 ml. Gas phase N₂. Temp. 37°. Tip after 10 min.

NaF concentration (M)	Percentage inhibition	
	Lactate	Pyruvate
0	0	0
0.0004	0	—
0.002	55	—
0.004	82	—
0.01	88	25
0.02	90	38
0.1	93	44
0.2	94	45

Effect of CO₂ concentration on fermentation of lactate

The concentration of carbon dioxide influences the ratio of propionic to acetic acid formed by the fermentation of lactic acid by propionibacteria and by *V. gazogenes* (Johns, 1951*a, b*). Increased CO₂ concentration produced a greater CO₂ fixation leading to an increased amount of propionic acid. Similar experiments were carried out with *Cl. propionicum* using three tubes of the type used by Barker (1949*b*). Each contained 26.5 ml. of fluid made up of 25 ml. of Barker's medium (pH 7.18), 1 ml. sodium lactate (0.3 g./ml.) and 0.5 ml. 1 % sodium sulphide. In tube (*a*) carbon dioxide was absorbed in alkali during incubation. In tube (*b*) 1 ml. of sterile N sodium carbonate was added after autoclaving (the pH of the buffer was adjusted so that the final pH after addition was 7.17). This tube was gassed with sterile CO₂ and sealed so that pressure increased by the amount of CO₂ produced during fermentation. Tube (*c*) was similar to (*a*) except that the pH was 5.95. After inoculation the tubes were incubated for 48 hr., and the ratio of volatile acids determined chromatographically (Elsden, 1946). The ratio propionic: acetic acid was 1.96 in tube (*a*), 1.93 in tube (*b*) and 1.95 in tube (*c*), compared with a ratio of 2 as found by Barker (1949*b*). All the lactate was fermented in each case.

CO₂ fixation experiments

Wood, Werkman, Hemingway & Nier (1941) working with propionibacteria and Johns (1951*a*) with *V. gazogenes* showed that during fermentation these organisms fixed C¹³O₂ in the carboxyl group of the propionic acid produced. The same technique as used for *V. gazogenes* (Johns, 1951*a*) was used to determine whether *Cl. propionicum* could utilize C¹³O₂ in the formation of propionic acid.

The medium used was as in tube (*b*) above, with the sodium carbonate in the form of Na₂C¹³O₃ (18.1 atoms % excess C¹³). The complete experiment was carried out in duplicate. The mass spectrograph determinations of the C¹³

content of products which were separated chromatographically (Elsden, 1946) showed in no instance any excess of C^{13} over that in the control sample, indicating that there had been no fixation of carbon dioxide in either acetate or propionate.

DISCUSSION

The results indicate that *Cl. propionicum* decomposes lactate to give propionic acid by a type of fermentation different from that observed with *V. gazogenes* (Johns, 1951*a*) and propionibacteria (Krebs & Eggleston, 1941; Delwiche, 1948) which ferment malate and fumarate readily and decarboxylate succinate to acid and carbon dioxide, whereas *Cl. propionicum* does not. The ratio acetic: propionic acid, as determined in fermentations by propionibacteria, varies greatly according to conditions of growth. This depends mainly on the CO_2 tension (Johns, 1951*b*), which determines the amount of succinate formed, and on the final pH value of the medium, which determines the amount of succinate decarboxylated. With *Cl. propionicum* variation in CO_2 tension and pH of the medium have no effect on the acid ratio, in agreement with the observation that succinate appears not to be an intermediate in propionate formation by this bacterium.

Barker & Lipmann (1944) found with *P. pentosaceum* that, whereas lactate decomposition was practically stopped by 0.00625M fluoride, pyruvate decomposition was only 30 % inhibited at this concentration; even with 0.1M fluoride, pyruvate was still fermented at an appreciable rate. They also found that, with lactate reduction blocked, little or no lactate accumulated when pyruvate was decomposed. This was interpreted to mean that lactate was probably not here the pathway of pyruvate reduction to propionate. With *V. gazogenes* lactate fermentation is much more sensitive to fluoride than pyruvate fermentation (Table 2). However, lactate reduction is not completely eliminated by a high concentration of fluoride. In contrast to the dissimilation of lactate and pyruvate by propionibacteria and by *V. gazogenes*, this dissimilation by *Cl. propionicum* is progressively inhibited by increasing fluoride concentrations to very nearly the same degree, indicating that lactate may be on the pathway of pyruvate reduction to propionate by this organism. As shown by Cardon & Barker (1947) *Cl. propionicum* ferments acrylic acid, whereas *Propionibacterium pentosaceum* does not (Barker & Lipmann, 1944). The final piece of evidence for the existence of two separate mechanisms for propionic acid formation is that whereas *Cl. propionicum* does not utilize CO_2 for propionic acid production, propionibacteria (Werkman & Wood, 1942) and *V. gazogenes* do (Johns, 1951*a*). It seems probable that the route of propionic acid formation from lactate by *Cl. propionicum* is lactate $\rightarrow$ acrylate $\rightarrow$ propionate; but there is no evidence yet for the formation of acrylate during fermentation. It seems certain from the work of Carson (1948) that there is still more to be learnt about the mechanism of propionic acid formation in bacterial fermentations. He has demonstrated a conversion of acetate to propionate which is not explained by the two schemes proposed by Werkman & Wood (1942).

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