LXXXIX. ACTIVATION OF THE LOWER FATTY ACIDS BY PROPIONIC ACID BACTERIA.[1]

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SUCCINIC acid arising in the dissimilation of glucose by propionic acid bacteria has been regarded as resulting either from a 4- and 2-carbon splitting of the sugar molecule [Virtanen, 1923] or from the yeast extract in the medium [Van Niel, 1928]. Recently Wood *et al.* [1935] suggested its formation by condensation of two molecules of acetic acid followed by dehydrogenation. Assuming the latter reaction, resting cells of *Propionibacterium* should "activate" acetic acid, *i.e.* (cause a donation of hydrogen to a suitable acceptor, such as methylene blue [see Thunberg, 1920; Quastel and Whetham, 1924]. This study deals with the activation of acetic and related acids by *Propionibacterium.*

EXPERIMENTAL.

The resting cell technique of Quastel and Whetham was followed but, instead of timing the colour change, the progress of the reaction was followed by the redox potential [Yudkin, 1935]. The organisms were grown in a medium consisting of 0·5 % yeast extract (Difco), 0·5 % peptone, 1 % glucose and 0·2 % K_2HPO_4. After 72 hours' incubation at 30° the cells were washed three times with sterile 0·85 % NaCl by centrifuging. One ml. of the paste-like centrifugate was diluted to 40 ml. with saline solution and stored at 6–8° not longer than 24 hours before use.

The experiments were carried out in pyrex tubes with side arms (Fig. 1), fitted with rubber stoppers, each carrying a KCl-agar electrode, a bright platinum electrode, a gas inlet tube and a gas outlet. A constant temperature water-bath at 37° was equipped to hold 16 of the redox tubes. After addition of the organisms (in the side cup), buffer and solutions, a stream of nitrogen, purified by passing over hot copper gauze and through alkaline pyrogallate, was bubbled rapidly through each tube for at least five minutes.

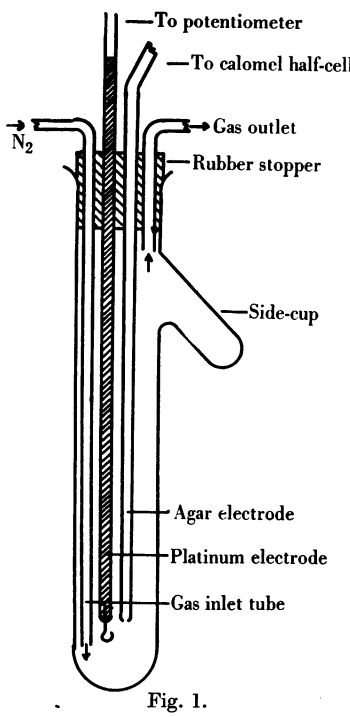

Fig. 1.

Reading of the initial potential was taken on the substrate-buffer mixture by use of the vacuum-tube amplifier described by Werkman, Johnson, and Coile [1933]; the organisms were then mixed with the contents of the tube and the mixture agitated. During the experiment a constant nitrogen pressure was

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maintained in the tubes by means of a mercury trap. The course of the potentials was followed by constructing time-potential curves.

The tubes and electrodes were thoroughly cleaned with chromic acid. Electrodes were calibrated against each other by placing them in one large cell; only those reading within 2 mv. of the mean were used.

The substrate and buffer solutions were prepared with boiled redistilled water and the p_H taken with a glass electrode. During the experiment the p_H rarely changed more than 0·05 of a unit owing to the use of a relatively large quantity of buffer.

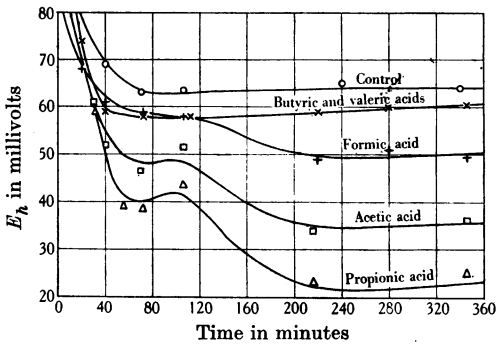

Fig. 2.

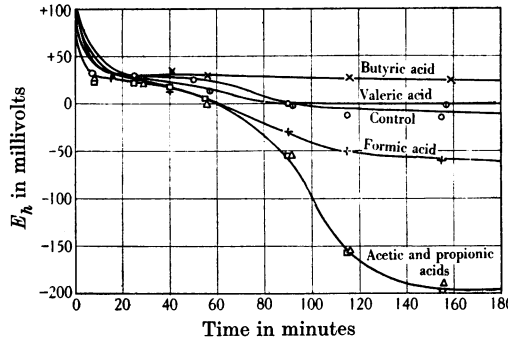

Fig. 3.

Fig. 2. o Control; × butyric and valeric acids; + formic acid; □ acetic acid; △ propionic acid.

Fig. 3. × Butyric acid; ⊙ valeric acid; o control; + formic acid; □ acetic acid; △ propionic acid.

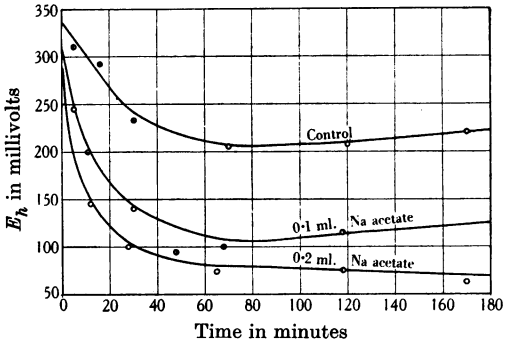

Fig. 4.

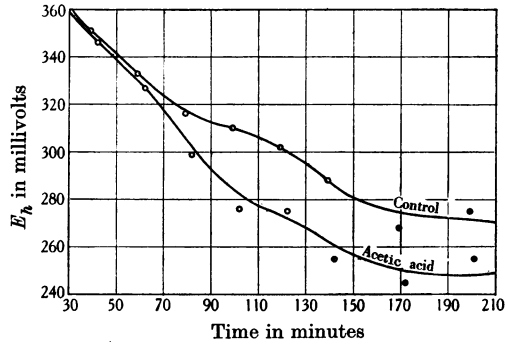

Fig. 5.

Fig. 4. 1st curve (top) control; 2nd curve (middle) 0·1 ml. Na acetate; 3rd curve (bottom) 0·2 ml. Na acetate.

Fig. 5. Upper curve control; lower curve acetic acid.

Studies were carried out at 37° on formic, acetic, propionic, butyric and valeric acids using methylene blue and in some cases $NaNO_3$, or *o*-chlorophenol-indophenol as hydrogen acceptor. Three species of *Propionibacterium* have been employed: *P. arabinosum* (34W), *P. pentosaceum* (49W), and *P. shermanii* (52W). These are described by Werkman and Brown [1933]. In a typical experiment 10 ml. of 1% phosphate buffer, 1 ml. of methylene blue (0·005 *M*), 0·2 ml. of 0·05 *M* substrate (Na salt) and 3·8 ml. of H_2O were placed in the redox

tube and 1 ml. of bacterial suspension in the side-cup. For the control tube 4·0 ml. of H_2O were added and the substrate omitted. Bacteria effecting dehydrogenation of the substrate bring about a reduction of methylene blue and a corresponding lowering of E_h. Representative time-potential curves are shown in Figs. 2–5, and additional results are condensed in Table I.

Table I. *Activation of lower fatty acids by* Propionibacterium *at 37°.*

			p_H 5·0–6·0			p_H 6·0–6·5			p_H 6·5–7·0			Total		
Substrate	H-Accep.	Species	+	−	?	+	−	?	+	−	?	+	−	?
Formic acid	M.B.	34W	1	0	0	0	0	1	3	1	1			
	,,	49W	0	1	0	1	0	0	2	2	0	8	5	2
	,,	52W	.	.	.	0	1	0	1	0	0			
Acetic acid	M.B.	34W	2	0	0	1	0	0	5	0	1			
	,,	49W	2	0	0	2	1	0	2	1	1	18	2	3
	,,	52W	.	.	.	3	0	1	1	0	0			
Acetic acid	$NaNO_3$	34W	1	0	0	.	.	.	.	.	.			
	$NaNO_3$	49W	3	0	0	1	0	0	2	1	0	7	1	0
	OCI	34W	.	.	.	2	0	0	2	0	0	4	0	0
Propionic acid	M.B.	34W	1	0	0	1	0	0	5	0	0			
	,,	49W	1	0	1	0	1	0	3	0	0	14	3	1
	,,	52W	.	.	.	1	2	0	2	0	0			
Butyric acid	M.B.	34W	0	1	0	0	0	1	0	1	0			
	,,	49W	0	1	0	0	0	0	0	0	1	0	3	2
Valeric acid	M.B.	49W	0	1	0	0	0	1	0	1	0	0	2	1

H-Accep. = Hydrogen acceptor. OCI = *o*-Chlorophenolindophenol. M.B. = Methylene blue.

NOTE. Figures in the columns marked + indicate the number of experiments showing activation under the conditions stated; − indicates no activation. Results showing slight or questionable activation are placed in the ? column.

DISCUSSION.

Formic acid shows activation under certain conditions; however, the reduction is not as rapid as in the cases of acetic and propionic acids. Clear-cut hydrogen donations are most frequently shown at p_H values near 7·0 (Fig. 3). The species of *Propionibacterium* show no marked individual differences in their behaviour towards formic acid. It may be pointed out that of the 5 negative results, 1 was obtained with organisms older than 72 hours, and 3 others had potentials higher than the control. We have found that aging causes inhibition of the dehydrogenases of *Propionibacterium*. It is possible that the high potentials may be explained by the sodium formate either inhibiting dehydrogenase activity or acting to some extent as a hydrogen acceptor. Apparently the propionic acid bacteria show a moderate attack on formic acid. This is of interest in relation to the observation of Wood and Werkman [1936] that CO_2 is utilised by the propionic acid bacteria in their dissimilation of glycerol. It is possible that the CO_2 is reduced to formic acid and that the formic acid is in turn assimilated.

Activation of acetic acid.

Propionibacterium generally activates acetic acid to donate hydrogen to methylene blue. Nitrates and *o*-chlorophenolindophenol also act as hydrogen acceptors (Figs. 4 and 5). The results with nitrate show a strong activation at p_H 5·0–6·0. At p_H values near 7·0 the reduction is not as marked. In experiments using $NaNO_3$ as a hydrogen acceptor, the potentials were not as stable as when a dye was added. However, though no added reversible system was present, a

readable potential was set up, owing perhaps to some reversible system in the bacterial suspension.

The donation of hydrogen by acetic acid in the presence of *Propionibacterium* is of particular interest in view of the suggestion of Wood *et al.* [1935, see also Grey, 1924] that two molecules of acetic acid may condense to form one molecule of succinic acid along with two atoms of hydrogen. The hydrogen may, in the dissimilation of glucose, be accepted by pyruvic acid [Wood and Werkman, 1934] or some other hydrogen acceptor. The fact that the hydrogen donation to nitrates is particularly marked at an acid reaction is interesting when we consider that acetic acid tends to accumulate in the early period of the propionic fermentation (when the medium is less acid) and then decreases [Wood and Werkman, 1934].

Activation of propionic acid.

The propionic acid bacteria cause a marked activation of propionic acid, particularly at p_H values near 7·0. Failures to activate occurred at lower p_H values. In general the rate of reduction of the dye by propionic acid approximates to that by acetic acid (Figs. 2 and 3). The significance of these observations cannot be determined definitely at the present time. Possibly activation is to be expected, since the organism produces the compound, and enzymic catalysis is by definition reversible. The conversion of lactic into propionic acid is readily brought about by *Propionibacterium* but the exact mechanism is not understood. If we assume that the reverse process takes place (*i.e.* propionic acid to lactic acid), we may postulate two mechanisms:

(1) Dehydrogenation followed by hydration.

$$\underset{\text{Propionic acid}}{CH_3.CH_2.COOH} \rightarrow \underset{\text{Acrylic acid}}{CH_2{=}CH{-}COOH} + 2H \xrightarrow{+H_2O} \underset{\text{Lactic acid}}{CH_3.CHOH.COOH} + 2H$$

(2) Enolisation followed by hydration and dehydrogenation.

$$\underset{\text{Propionic acid}}{CH_3.CH_2.COOH} \rightarrow \underset{\text{Enol}}{CH_3{-}CH{=}C(OH)_2} \xrightarrow{+H_2O} CH_3.CHOH.CH(OH)_2 \longrightarrow \underset{\text{Lactic acid}}{CH_3.CHOH.COOH} + 2H$$

In each of these reactions two hydrogen atoms are available and the reduction of methylene blue or other acceptor is accounted for. Thus in the fermentation of glucose, whether the dissimilation of propionic acid occurs would depend on the presence of a suitable hydrogen acceptor. Such a breakdown of the acid might go unnoticed if it proceeded *via* either of these reactions, for the resulting lactic acid may be isolated as an intermediary and is readily fermented. The possibility of propionic acid occurring as an intermediate product has apparently not been previously suggested.

Activation of butyric and valeric acids.

Neither butyric nor valeric acid showed any substantial donation of hydrogen to methylene blue. In the case of butyric acid, two of the results showed higher potentials than the controls (see Fig. 3). This may be the result of an inhibition of dehydrogenases by the acid.

SUMMARY.

A study with resting cells of *Propionibacterium* has been carried out on the lower fatty acids with three hydrogen acceptors by determining the E_h of the systems. The following observations have been made.

1. Acetic and propionic acids are shown to be activated by *Propionibacterium* to reduce methylene blue at p_H 5·0–7·0.

2. Formic acid is able to donate hydrogen to methylene blue to a small extent, whilst butyric and valeric acids show no such ability.

3. Acetic acid may donate hydrogen to nitrates and *o*-chlorophenol-indophenol.

These results are discussed in view of their significance to the propionic fermentation.

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