Metabolism of the propionic acid bacteria

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METABOLISM OF THE PROPIONIC ACID BACTERIA

by

George Hugo Nelson

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Physiological Bacteriology

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>REVIEW OF LITERATURE</td>
<td>2</td>
</tr>
<tr>
<td>Lactic Acid</td>
<td>2</td>
</tr>
<tr>
<td>Occurrence</td>
<td>2</td>
</tr>
<tr>
<td>Utilization</td>
<td>10</td>
</tr>
<tr>
<td>Isomer specificity</td>
<td>10</td>
</tr>
<tr>
<td>Products of metabolism</td>
<td>10</td>
</tr>
<tr>
<td>Propionate</td>
<td>11</td>
</tr>
<tr>
<td>Succinate</td>
<td>12</td>
</tr>
<tr>
<td>Acetate</td>
<td>14</td>
</tr>
<tr>
<td>Carbon dioxide</td>
<td>14</td>
</tr>
<tr>
<td>Aerobiosis vs. anaerobiosis</td>
<td>14</td>
</tr>
<tr>
<td>Oxidation and reduction</td>
<td>18</td>
</tr>
<tr>
<td>Essential components</td>
<td>19</td>
</tr>
<tr>
<td>Tracer experiments</td>
<td>20</td>
</tr>
<tr>
<td>Pyruvic Acid</td>
<td>22</td>
</tr>
<tr>
<td>Occurrence</td>
<td>22</td>
</tr>
<tr>
<td>Anaerobic utilization</td>
<td>23</td>
</tr>
<tr>
<td>Aerobic utilization</td>
<td>30</td>
</tr>
<tr>
<td>Oxalacetic Acid</td>
<td>31</td>
</tr>
<tr>
<td>Malic Acid</td>
<td>32</td>
</tr>
</tbody>
</table>

T15794
Fumaric Acid .................................................. 33
Succinic Acid .................................................. 35
  Occurrence .................................................. 35
  Utilization .................................................. 39
  Mechanism of utilization of succinate .................. 44
  Inhibitors .................................................. 46
MATERIALS AND METHODS .................................... 47
  Preparation of Cells and Cell-free Extracts ........ 47
  Special Reagents ......................................... 51
  Analytical Methods ....................................... 51
  Qualitative determinations .............................. 52
  Quantitative determinations ............................. 52
EXPERIMENTAL .................................................. 57
  Effect of Growth Conditions on Enzyme Activity .... 57
  Succinic Decarboxylase Activity among the Species ... 57
  Properties and Synthesis of Succinic Decarboxylase . 72
  Succinic Dehydrogenase and the Dicarboxylic Acid
  Cycle ....................................................... 81
  Utilization of Lactate and Pyruvate ................... 89
    Effect of pH on formation of carbon
dioxide from lactate and pyruvate .................... 89
    Effect of fluoride on the utilization
    of lactate and pyruvate .............................. 107
<table>
<thead>
<tr>
<th>Topic</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactic Dehydrogenase</td>
<td>116</td>
</tr>
<tr>
<td>pH optimum</td>
<td>116</td>
</tr>
<tr>
<td>Effect of concentration of lactate on reduction of methylene blue</td>
<td>118</td>
</tr>
<tr>
<td>Effect of pyruvate on reduction of methylene blue by lactate</td>
<td>121</td>
</tr>
<tr>
<td>Effect of concentrations of methylene blue on reduction by lactate</td>
<td>121</td>
</tr>
<tr>
<td>Effect of inhibitors on reduction of methylene blue</td>
<td>125</td>
</tr>
<tr>
<td>Essential components</td>
<td>126</td>
</tr>
<tr>
<td>Purification of the extract</td>
<td>128</td>
</tr>
<tr>
<td>Lactic dehydrogenase and inhibition by fluoride</td>
<td>128</td>
</tr>
<tr>
<td>Substrate hydrogen acceptors</td>
<td>132</td>
</tr>
<tr>
<td>Coupled Reaction between Fumarate and Lactate</td>
<td>135</td>
</tr>
<tr>
<td>Effect of pH on reaction between fumarate and lactate</td>
<td>138</td>
</tr>
<tr>
<td>Stoichiometry of reaction at pH 5.6</td>
<td>141</td>
</tr>
<tr>
<td>Essential components of the dismutation reaction</td>
<td>144</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>149</td>
</tr>
<tr>
<td>CONCLUSIONS AND SUMMARY</td>
<td>157</td>
</tr>
<tr>
<td>LITERATURE CITED</td>
<td>160</td>
</tr>
<tr>
<td>ACKNOWLEDGMENT</td>
<td>179</td>
</tr>
</tbody>
</table>
DEDICATION

To Diana

"... the hidden person of the heart with the imperishable jewel of a gentle and quiet spirit, which in God's sight is very precious." -- I Peter 3:4
INTRODUCTION

Perusal of the data contained in various reports on the propionic acid fermentation reveals the consistent occurrence of lactate in dissimilations of various substrates. Little is known about the dissimilation of lactate per se. It is the purpose of this investigation to contribute further to the information relating to the fermentation of lactate by the propionic acid bacteria. Formation and utilization of related substances, pyruvate and the dicarboxylic acids, are also the subject of investigation.
REVIEW OF LITERATURE

Lactic Acid

Occurrence

The propionic acid bacteria produce lactate in fermentations of various substances, among which are starch (66), pentoses (55, 56, 120), glucose (62, 66, 120, 162, 174, 187, 188, 189, 195, 198), glycerol (119, 194, 196), succinate (62), pyruvate (7, 62, 65, 204) and inositol (180). Lactate is formed from glucose by Plectridium tertium, an organism responsible for a propionic-like fermentation (112). Despite the failure of some workers to detect lactate, it is given a place of importance as an intermediate in proposed fermentation schemes (66, 162, 190). Lactate was considered at first to be a stabilization product in fermentations, but Wood, Stone and Werkman (187, 188) reasoned that "since this acid is readily fermented by all species of propionic acid bacteria it is logical to assume that it functions as an intermediate." The same authors indicated further that "in order to show that a compound may be an intermediary in a fermentation, it is necessary to isolate the compound and demonstrate that it is fermented as rapidly as its precursor."
A closer relationship of lactate to the products of the propionic acid fermentation was established with the demonstration of formation of lactate from various substrates followed by disappearance of lactate and appearance of volatile acids. Disaccharides and monosaccharides are utilized relatively slowly by the propionic acid bacteria (115, 134, 181). Phelps, Johnson and Peterson (120), in serial analyses of fermentations of glucose and arabinose by *Propionibacterium pentosaceum*, observed an increasing concentration of lactate up to a maximum at seven days. Beyond this point, lactate levels decreased progressively to a zero value at the termination of the fermentations. In the words of the authors, this finding "tends to substantiate the theory that lactate is an intermediate which is formed during the early stages of the fermentation and later utilized."

In glycerol fermentations, lactate levels were observed to increase at first and then decrease, the decrease being marked by an increase of volatile acids (119). Wood, Stone and Werkman (187) and Fromageot and Bost (62) reported similar data derived from fermentations of glucose.

The occurrence of lactate in various fermentations is quite general, although the amounts vary from small to appreciable. Many of the reports of negative lactate values are based on analyses conducted on completed long term
fermentations in which lactate undoubtedly could be consumed by the time the fermentation is complete. The amount of lactate formed varies with the pH, nutrients and other factors (120, 136, 137). Addition of growth factors does not increase yields of lactate, although potato extract is reported to increase yields of lactate from glucose. Sherman and Shaw (134) demonstrated that addition of Lactobacillus casei, a homofermentative microorganism converting lactose into lactate almost entirely, increases by a multiple of six the total volatile acids (propionate and acetate) produced but does not change the ratio of the products. The authors claim that the increase in products is not due to conversion of lactose to a more readily available form (lactate). Using a mixed culture of Lactobacillus casei and Propionibacterium pentosaceum, Leviton and Hargrove (105) found that the former converted lactose into lactate. Significantly, the authors reported that:

Although available to the propionic acid bacteria, lactic acid was not dissimilated until all of the lactose had been consumed. Then, rapidly, . . . the greater part of the lactic acid was dissimilated within the next 24 hours.

Katagiri and Ichikawa (86) found the initial accumulation of lactate was followed by its utilization in the fermentation of both arabinose and glucose by propionic acid bacteria. Strecker (152), using unidentified microorganisms, found
lactate to be an intermediate in the formation of propionate from sugars.

In cheeses, lactic acid bacteria convert lactose and other sugars into lactate, lowering the oxidation-reduction potential and the pH in the process (92), and then the propionic acid bacteria convert lactate into the products of the propionic acid fermentation (43). Similar observations were made by von Freudenreich and Orla-Jensen (59). Van Niel (115) pointed out that the propionic acid bacteria may ferment some of the milk sugars but not as readily as do the lactic acid bacteria. Kiure (92) noted that the propionic acid fermentation in cheese is modified by the type of lactose conversion occurring immediately before. Heterofermentative lactic microorganisms convert lactose into several products. The amount of lactate formed is less than that produced by the homofermentative bacteria. The amount of volatile acids formed by the propionic acid bacteria in cheese with heterofermentative lactic acid bacteria is less than that formed in the presence of homofermentative lactic acid bacteria. Kiuri (91) also noted that in cheese decreased levels of lactate are accompanied by increased propionate-to-acetate ratios whereas increased levels of lactate are accompanied by decreased ratios, suggesting a close relationship between propionate and lactate.
In fermentations of carbohydrates by sheep rumen microorganisms (47, 48, 121), the soluble sugars are broken down by one group of "non-propionic bacteria" and then the lactate formed by this group is fermented to the volatile fatty acids by the propionic acid bacteria. Apparently the formation of lactate by "non-propionic bacteria" accelerates the process of making lactate available for utilization by the propionic acid bacteria.

Lactate accumulates in the utilization of glucose moreso under anaerobic than aerobic conditions (187), indicating that lactate may function as a stabilization product (189) resulting from pyruvate serving as an oxidant for glucose (7, 151, 189).

Wiggert and Werkman (182) grew Propionibacterium pentosaceum in a medium containing glucose and 0.02 molar fluoride. Cells harvested from this culture were tested for enzymatic action on glucose and phosphoglycerate in the presence of 0.02 molar fluoride. Utilization of glucose is inhibited only slightly, if at all, by the fluoride. Phosphoglycerate, a precursor of pyruvate in the glycolytic system, is not dissimilated significantly. Werkman, Stone and Wood (174), in analyses of fermentations of glucose carried out in the presence of fluoride, found the usual products, among them lactate. Wood and Werkman (197) reported the formation of considerable amounts of lactate from glucose in 0.02 molar fluoride as compared to the relatively small amounts found
in normal fermentations. "Fluoride fermentations" apparently
do not proceed via hexosediphosphate and phosphoglycerate
(174). Volk (164) found that cells of P. pentosaceum grown
in 0.05 molar fluoride cannot utilize phosphorylated com-
ounds such as glucose-6-phosphate and 3-phosphoglycerate,
whereas non-phosphorylated compounds, such as glucose, glu-
conate, glycerol and lactate are utilized readily. Phospha-
tase poisons (tungstate and molybdate) have the same effect
as fluoride. Apparently cell membrane phosphatases are in-
hibited by fluoride. Inhibition of these enzymes prevents
utilization of the phosphorylated compounds which may have
to be dephosphorylated by the membrane phosphatases before
entering the cell for utilization. The ability of fluoride-
grown cells to utilize glucose even in the presence of
fluoride (in contrast to cells grown in the absence of
fluoride) was shown to be due to modification of the per-
meability of the cell membrane to fluoride, which is unable
to enter cells grown in the presence of the poison. Volk
(164) points out that these findings still do not rule out
the presence of a second metabolic pathway in the propionic
acid bacteria.

The second pathway may be that proceeding via pentoses.
Pentoses are utilized readily with the formation of the
usual products (119, 129, 173) and in some instances large
amounts of lactate are produced (55). In studies on fermentation of glucose by *Lactobacillus mesenteroides*, a heterofermentative microorganism, Gibbs and Demoss (63) showed the formation of equimolar quantities of lactate, ethanol and carbon dioxide. Data obtained with C\(^{14}\)-l-glucose were consistent with the concept of oxidative decarboxylation to pentose, since the carbon dioxide was labeled whereas little or no activity appeared in either ethanol or lactate. D-Xylose (67, 102) and L-arabinose (130), each with C\(^{14}\) in the one-position, are fermented by *Lactobacillus pentosus* and *L. pentosaceticus* respectively, with the formation of a ketopentose or phosphorylated derivative. Nutting and Carson (117) demonstrated the formation of lactate from xylose by *Escherichia coli*. Krebs and Eggleston (98) demonstrated that pentoses are fermented readily by the propionic acid bacteria, even in the presence of fluoride, with the formation of the usual products. Barker and Lipmann (7) demonstrated that *Propionibacterium pentosaceum* esterifies arabinose and converts it to phosphoglycerate. Erythritol was shown to be esterified and oxidized, a fact supporting the hypothesis that pentose phosphate can be degraded by oxidation and decarboxylation by way of tetrose phosphate. Rappoport and Barker (129) reported the utilization of L-arabinose with the formation of propionate, acetate and
carbon dioxide according to the following equation:

\[
2 \text{Arabinose} \rightarrow 2 \text{Propionate} + \text{1 Acetate} + \text{1 Carbon dioxide}
\]

Formation of lactate from propionate as a secondary product of fermentation was suggested by studies using methylene blue and other oxidation-reduction indicators (126, 150, 193). Quastel (124) demonstrated in studies with *Escherichia coli* that lactate is a better hydrogen donor than propionate, so that if the propionate-to-lactate conversion does occur, it occurs to a much smaller degree than the oxidation of lactate. Aerobically, at pH 5.0, propionate is utilized with uptake of oxygen by *P. pentosaceum*. No evidence for the operation of this mechanism under anaerobic conditions is available. Huennekens, Mahler and Nordmann (77) demonstrated the aerobic utilization of propionate by liver and kidney preparations via the proposed pathway:

\[
\text{Propionate} \rightarrow \text{Acrylate} \rightarrow \text{Acetate} \rightarrow \text{Pyruvate} \rightarrow \text{Lactic acid}
\]

It would seem, however, that formation of lactate from propionate is of little significance in the anaerobic formation of lactate in dissimilations by the propionic acid bacteria (150).

The mechanism of formation of lactate still remains to be solved. None-the-less, lactate does occur quite consistently in the dissimilation of various substrates by the
propionic acid bacteria. Of greater interest is the mode of utilization of lactate.

**Utilization**

With the exception of *Propionibacterium acnes* (46) all propionic acid bacteria ferment lactate.

**Isomer specificity**

Von Freudenreich and Orla-Jensen (59) reported the utilization of both isomers and the racemate of lactate. This report was verified by Kiuri (92) and by Phelps, Johnson and Peterson (120). The latter report indicated a slight difference in the rates of utilization. No racemase occurs in the propionic acid bacteria at pH 6.8 and below (120). This finding is unusual in that the same authors isolated from fermentations of glucose and arabinose only the L (+) isomer of lactate.

**Products of metabolism**

The pioneer work of Fitz (54), carried out with impure cultures, demonstrated the formation of propionate, acetate
and carbon dioxide from lactate. Other substances reported as occurring are succinate (98, 109, 163, 198), pyruvate (50, 183, 188, 189), acetoin (157, 158) and diacetyl (157, 158). The two last-mentioned substances were reported in fractions of a milligram in fermentations of lactate in gram quantities. They may be secondary products of the fermentation.

**Propionate**

The mechanism of formation of propionate from lactate is unknown except for indications of possibilities. Strecker (152) proposed the formation of propionate from various sugars via lactate. Stone and Werkman (148) speculated that the sequence of formation may be direct reduction of lactate to propionate. Maurer (109) and von Freudenreich and Orla-Jensen (59) suggested such a possibility. Foubert and Douglas (58) proposed this mechanism for Micrococcus lactilyticus. The direct reduction of lactate to propionate by Veillonella gazogenes was disproved since cells grown on D-tartrate do not attack lactate, yet form propionate (81).

Wood, Stone and Werkman (187) state that:

> With regard to lactic acid as a precursor of propionic acid, it is probable that enough energy is not available in the somewhat artificial
system to bring about the difficult reduction of lactic to propionic acid.

It may be that propionate arises from lactate by a dismutation of lactate, according to the equation:

\[ 2 \text{Lactate} = \text{1 Pyruvate} + \text{1 Propionate} + \text{1 H}_2\text{O} \]

This reaction has been suggested as the mechanism involved in propionate formation (6, 26, 62).

The inability of lactate to serve as an oxidant of glucose in the presence of fluoride, in contrast to its ability to serve as a reducing substance in the presence of fluoride, suggested to Barker and Lipmann (6) that a fluoride-sensitive mechanism for the reduction of lactate to propionate is operative and that lactate is not a necessary intermediate between pyruvate and propionate. With respect to this contention, Carson, Anthony, Kuna, Bachman and Long (19) isolated an intermediate between pyruvate and propionate. The substance remained unidentified because of impurities. Evidence indicated that the intermediate is formed by the C₁-C₃ condensation, followed by decarboxylation of succinate to propionate.

**Succinate**

Little is known about the formation of succinate with lactate as substrate. Krebs and Eggleston (98) found that
formation of succinate from lactate proceeds at the same rate as its formation from glycerol, oxalacetate, fumarate and malate. However, the final amount of succinate formed was smaller than that from the above-mentioned substances. The pathway of formation therefore may be via the C$_1$-C$_3$ condensation reaction. Or, it may be via a C$_2$ condensation (See section on succinic acid). The original study of carbon dioxide fixation (194) involved glycerol as substrate. Later, glucose was used (196). Fixation of carbon dioxide with pyruvate, lactate and arabinose as substrates was insignificant on the basis of carbon balance studies (197, 198). Conflicting reports as to the need for carbon dioxide in dissimilation of lactate have been made (81, 82). However, fixation of carbon dioxide into pyruvate with the formation of dicarboxylic acids and propionate does occur (See section on succinic acid). Involvement of succinate and its decarboxylation in the formation of propionate from lactate is open to question. Results of tracer lactate fermentations (185) indicate the functioning of succinate decarboxylation in dissimilation of lactate. Cardon and Barker (17) found that Clostridium propionicum does not form succinate and yet forms the usual products of the propionic acid fermentation with lactate as substrate. The fermentation proceeds at pH 7.4, a pH level completely outside the reported range
of activity of succinic dehydrogenase. Lactate, as an energy source, inhibits formation of carbon dioxide from succinate (82).

Whichever pathway is followed, formation of succinate from lactate depends on preliminary formation of pyruvate from which dicarboxylic acids or acetate may be formed.

**Acetate**

Formation of acetate from lactate presupposes the formation of pyruvate initially, pyruvate undergoing dismutation (6, 171) or decarboxylation with acetate as one of the products (See section on pyruvic acid).

**Carbon dioxide**

Formation of carbon dioxide, too, depends on the formation of pyruvate initially since the only known reactions which yield carbon dioxide occur with pyruvate and succinate as substrates.

**Aerobiosis vs. anaerobiosis**

Lactate is utilized readily under both aerobic and anaerobic conditions (147). As pointed out by Erb, Wood and
Werkman (50), the mechanism of aerobic utilization of lactate appears to be the same as that of anaerobic utilization except that oxygen serves as a hydrogen acceptor and, accordingly, the amount of propionate is reduced. Virtanen (162) reported that aeration had only slight influence on the fermentation of lactate. Van Niel (115) found increased yields of acetate with aeration.

Comparison of the aerobic and anaerobic fermentations is shown in the two generally accepted equations:

(a) Aerobic

\[ 3 \text{ Lactate} = 1 \text{ Propionate} + 2 \text{ Acetate} + 2 \text{ Carbon dioxide} \]

(b) Anaerobic

\[ 3 \text{ Lactate} = 2 \text{ Propionate} + 1 \text{ Acetate} + 1 \text{ Carbon dioxide} + H_2O \]

Equation (a) was proposed by van Niel (115) and Equation (b) by Fitz (54).

The type of fermentation shown by equation (b) is not limited to members of the genus Propionibacterium. Clostridium propionicum (17), an anaerobic micrococcus isolated from sheep rumina (81), and Veillonella gazogenes, now classified as Micrococcus lactilyticus (58, 81), carry out fermentations similar to that shown in equation (b).

For each mole of lactate fermented under anaerobic conditions, 0.25-0.30 mole of carbon dioxide is formed (98).
Anaerobic formation of carbon dioxide is inhibited completely by 0.02 molar fluoride (6, 25, 27).

For each mole of lactate utilized under aerobic conditions, 0.62-0.68 mole of oxygen is utilized (50, 63). Uptake of oxygen is quite rapid initially, far exceeding the rate of formation of carbon dioxide. The latter increases after a lag period and proceeds at a constant level to the termination of the reaction (50). Aerobic utilization, as evidenced by oxygen-to-carbon dioxide ratios, is unaffected by methylene blue or cyanide (63), but is decreased by one-half in an atmosphere of 95 per cent carbon monoxide and 5 per cent oxygen (26). The latter finding indicates the presence of a carbon monoxide-sensitive system, the latter being called a cytochrome oxidase by the authors (26).

Aerobic utilization of lactate is unaffected by concentrations of fluoride up to 0.04 molar (25). The pH optimum for formation of carbon dioxide and uptake of oxygen lies in the range 5.3-5.5 (50, 63). There is no significant fermentation of lactate below pH 5.0 (50). Aerobically, with increasing pH, formation of carbon dioxide decreases more than does consumption of oxygen (50). A broad pH range of hydrogen transfer activity is indicated. The ratio of carbon dioxide-to-oxygen varies with pH (63). The closer the pH to the optimum for formation of carbon dioxide and uptake of
oxygen, the closer the carbon dioxide-to-oxygen ratio to unity. Failure of the formation of carbon dioxide to equal uptake of oxygen suggests the formation of an oxidized 3-carbon compound (50).

Virtanen (163) found the optimum pH for fermentation of lactate to be 6.2.

Chaix and Fromageot (25) and Chaix-Audemard (27) demonstrated that anaerobic utilization of lactate is inhibited by 0.2 molar fluoride. Barker and Lipmann (6) extended these studies to verify the inhibition and to demonstrate removal of the inhibition by fumarate. The lactate disappearing in the fluoride-fumarate system is almost equivalent to the amount of pyruvate formed. According to the authors, 2 systems for the utilization of lactate are operative and are represented by the following equations:

(a) Lactate (O) + Lactate (R) = Propionate + Pyruvate + H₂O

(b) Lactate (O) + (Pyruvate + H₂O) (R) = Propionate + Acetate + Carbon dioxide + H₂O

System (O), in which lactate acts as an oxidant, is represented as follows:

Lactate + 2 H = Propionate + H₂O.

System (R), in which lactate acts as a reducing agent, is represented as follows:

Lactate = Pyruvate + 2 H.
The oxidizing system, system (0), is considered to be the fluoride-sensitive one.

**Oxidation and reduction**

Wood, Stone and Werkman (187) demonstrated that lactate is a poor acceptor of hydrogen in the formation of phospho-glycerate from glucose in the presence of fluoride. Barker and Lipmann (6) verified this finding, suggesting that in the absence of fluoride lactate may act as an oxidant. Phelps, Johnson and Peterson (120) demonstrated the reducing capacity of lactate. Desnuelle, Wookey and Fromageot (44) provided supplementary evidence for this capacity of lactate. The pH optimum for dehydrogenation of lactate by the propionic acid bacteria occurs at pH 6.0 using whole cells (120).

The reaction, lactate → pyruvate, proceeds at a faster rate than does the reverse reaction, pyruvate → lactate (6). With respect to oxidation of lactate, pyruvate does not inhibit the reaction (6). This finding is inconsistent with the finding that keto acids inhibit oxidation of hydroxy acids (69). Under aerobic conditions lactate inhibits utilization of pyruvate by thiamine-deficient cells (126). Quastel and Webley (126) presented evidence indicating the formation and accumulation of pyruvate from lactate under
aerobic conditions at pH 7.4 by thiamine-deficient cells. The fluoride-sensitivity demonstrated by the experiments of Barker and Lipmann (6) probably is concerned in a lactate-to-pyruvate transformation and therefore is possibly a matter of inhibition of a hydrogen transfer system (38).

**Essential components**

Information on this phase of the metabolism of lactate is meager. Barker and Lipmann (6) pointed out that most oxidations of hydroxy acids are catalyzed by pyridine enzymes. The enzyme involved in the oxidation of lactate has a normal potential higher than that of the pyridine coenzymes and in this respect is analogous to the lactic dehydrogenase of yeast (2).

Foote, Fred and Peterson (55) speculated on the role of catalase in the utilization of lactate. It was pointed out that an organism which is incapable of forming catalase should be unable to utilize lactate. Such an idea seemed plausible when it was recalled that the lactic acid bacteria, which synthesize lactate, are catalase negative. This consideration suggests the possibility of catalase being involved in the dissimilation of lactate by the catalase positive propionic acid bacteria. Rollman and Sjöström
(131) pointed out that there seemed to be a relationship between the ability to form gas and catalase activity, the catalase activity being strongest in cultures with strong gas production. However, Erb, Wood and Werkman (50) reported the absence of hydrogen peroxide in the dissimilation of lactate. Hitchner (76) and Claybaugh (30) reported that the catalase property varies with the species and strain of propionic acid bacteria. The variation in this property tends to minimize the importance of catalase in the utilization of lactate.

A report by Fromageot and Tatum (66) indicates that potato extract, which stimulates utilization of glucose, does not stimulate utilization of lactate. The report failed to identify the active principle. Aerobic utilization of lactate by thiamine-deficient cells results in the accumulation of pyruvate, indicating that a part, if not all, of lactate is dissimilated via pyruvate. Lactate utilization per se is not dependent on thiamine. Virtanen (162, 163) pointed out that toluene treatment of whole cells destroys the enzyme system responsible for the fermentation of lactate.

**Tracer experiments**

Utilization of lactate in the presence of $^{13}$C-carbon
dioxide yields propionate with labeling in the carboxyl group. This finding suggests formation of propionate by de-carboxylation of succinate (31). Leaver (103) allowed *Propionibacterium arabinosum* to ferment C\(^{14}\)-methyl-labeled lactate and found that the resulting propionate contained labeling randomized in the alpha- and beta-carbons. Similar data were reported by Leaver and Wood (104) in a more extensive treatise. In the latter report, lactate with the alpha-carbon labeled, yielded propionate with the labeling randomized. It was pointed out that possibly a recycling of labeled carbon had occurred during these long time fermentations. Evidence for this interpretation was presented by Wood, Leaver and Stjernholm (185). Fermentation of unlabeled lactate in the presence of 3-C\(^{14}\)-propionate yielded propionate with labeling equally distributed between the 2- and 3-carbons of propionate. A similar labeling resulted when 1,3-C\(^{14}\)-propionate was introduced into the fermentation of unlabeled lactate, but in this case there was no equivalent loss of activity in the carboxyl group, suggesting that recycling occurs in the alpha- and beta-carbons only.
Pyruvic Acid

Occurrence

Van Niel (115) first postulated the formation of pyruvate from glucose but failed to demonstrate its occurrence or role as an intermediate. Wood and Werkman (190) reported finding an unidentified keto acid in a fermentation and later (189, 191) isolated and identified the acid as pyruvate. Pyruvate is formed in large amounts in aerobic utilization of lactate (50, 183), in anaerobic fermentations of glucose, lactate, glycerol and mannitol (6, 13, 50, 137, 183, 198) and in dissimulation of phosphoglycerate (148, 175). One report indicated the absence of pyruvate in fermentations of glycerol (196) whereas another (23) recorded the occurrence of inactive pyruvate in the dissimulation of glycerol with $^{11}$C-carbon dioxide present. This "cardinal intermediate" keto acid occurs in the fermentation of other compounds, too. Phosphopyruvate has not been detected (7).

The mechanism of formation of pyruvate from glucose was surmised to be the glycolytic or Embden-Meyerhof-Parnas system (189). The role of pyruvate as an oxidant for glucose and hexosediphosphate (7, 151, 187) and its formation from phosphoglycerate (148, 175) tend to favor the glycolytic mechanism of formation. Cells grown in fluoride, which pre-
vents utilization of phosphoglycerate in the glycolytic system, dissimilate glucose in the presence of fluoride just as if no fluoride were present (182). This evidence indicates possibly that pyruvate is not the "cardinal intermediate" arising via the glycolytic system but rather a substance arising by another route.

**Anaerobic utilization**

The products of anaerobic utilization of pyruvate are propionate, acetate, lactate, succinate and carbon dioxide. Lactate and succinate occur only in small amounts (93, 184, 204). Acetaldehyde does not occur (187), and acetyl phosphate has not been detected (7). The overall utilization is represented by the equation (109, 162):

\[ 3 \text{Pyruvate} \rightarrow 1 \text{Propionate} + 2 \text{Acetate} + 2 \text{Carbon dioxide} \]

This equation resembles that for aerobic utilization of lactate (See section on lactic acid). The products have been verified experimentally in the propionic acid bacteria (21, 38, 115) and in another organism carrying out the propionic acid fermentation, *Clostridium propionicum* (17), which does not synthesize succinate. Anaerobic formation of mainly acetate and carbon dioxide occurs (171) in the propionic acid bacteria (196), in *Rhodospirillum rubrum* (93) and in
Micrococcus lactilyticus (73, 81), the latter carrying out a propionate-like fermentation with the formation of molecular hydrogen. Characteristically, M. lactilyticus does not ferment sugars which the propionic acid bacteria utilize (58) indicating the presence of only the terminal portion of the enzyme sequence involved in the usual fermentation mechanisms.

Werkman (171) speculated that "the propionic acid bacteria bring about a dismutation of pyruvic acid through lactic acid and acetic acid and carbon dioxide." In evidence for this statement, references are made to van Niel (115) and Wood and Werkman (190).

Pyruvate may be utilized by serving as an oxidant for glucose or hexosediphosphate at pH 6.8 in the presence of fluoride. Phosphoglycerate (originating from the hexoses) and lactate (originating from pyruvate) accumulate (7, 151, 187). The amount of lactate formed in this system or from pyruvate alone is very small (6), indicating further utilization of lactate. In this respect, it is pointed out that the reaction, pyruvate → lactate, is much slower than the reverse reaction, lactate → pyruvate. This situation contrasts sharply with that in most organisms in which the reverse is true. Phosphopyruvates not only serves as an oxidant for glucose and other substrates but also transfers
phosphate enzymatically to these compounds (7), resulting in esterification of 1.5-2.0 moles of phosphate per mole of glucose. The latter finding is consistent with the known process of esterification of glucose to form hexosediphosphate in the glycolytic system. Utilization of pyruvate in the presence of fluoride involves a small amount of phosphate esterification or none at all, the ratio of the amount of phosphate esterified to the amount of pyruvate decomposed being 0.09-0.33. In the absence of fluoride, no inorganic phosphate disappears (7) indicating utilization without phosphorylation. Pyruvate, as an oxidant, may inhibit formation of carbon dioxide from succinate by oxidizing succinate to fumarate. Evidence for this possibility is seen in the inhibition of formation of carbon dioxide from succinate with pyruvate as a source of energy (82). No other mode of formation of lactate from pyruvate has received experimental support. This situation does not negate the existence of other mechanisms.

The formation of succinate from pyruvate by the propionic acid bacteria may occur via formation of acetate followed by condensation of two acetate molecules or by a C1-C3 synthesis, as suggested by Wood and Werkman (See section on succinic acid). Formation of acetate from pyruvate has not been studied very extensively. The formation of succinate
from pyruvate is a limited reaction, the amount of succinate formed being less than that from glycerol, oxalacetate, fumarate or malate even though the rates of formation are the same (98).

Besides succinate (shown to have Cl\textsubscript{14}O\textsubscript{2} fixed during the fermentation of pyruvate) and propionate, a carbonyl compound, precipitable along with pyruvate and oxalacetate as 2,4-dinitrophenylhydrazones and capable of being decarboxylated by yeast carboxylase, has been reported (21, 22). A supposed intermediate between pyruvate and propionate, not pure enough to identify, has been isolated (19). Acrylate, alanine, cysteine, phosphoserine, glycerate and phosphoglycerate are not the intermediate reported (6). It may be that, in the case of Clostridium propionicum, acrylate is an intermediate since, in contrast to the propionic acid bacteria, C. propionicum utilizes acrylate with the formation of products and proportions thereof resembling those occurring in the utilization of lactate (17).

Maurer (109) suggested that two molecules of pyruvate yield two of acetate and two of carbon dioxide with four hydrogen atoms unaccounted for. Fromageot and Bost (62) suggested formation of one mole each of propionate, acetate and carbon dioxide in a reaction between one mole of pyruvate and one of lactate. Barker and Lipmann (6), Fromageot and Chaix (62),
and Fromageot and Safavi (65) concurred in this suggestion. The latter introduced the concept of "dismutation" of pyruvate into lactate, acetate and carbon dioxide. Carson, Anthony, Kuna, Bachmann and Long (19) proposed the same scheme of pyruvate utilization, as represented by the following equation:

\[ 3 \text{Pyruvate} + \text{H}_2\text{O} = \text{1 Propionate} + 2 \text{Acetate} + 2 \text{Carbon dioxide} \]

A trace of succinate occurs in the reaction. Carson (18) presented a working scheme and proposals for mechanisms of the propionic acid fermentation but did not attempt verification with experimental evidence. Johns (81) emphasized the formation of propionate and carbon dioxide via the fixation of carbon dioxide into pyruvate, yielding dicarboxylic acids with subsequent decarboxylation of succinate.

In the presence of tracer carbon dioxide, pyruvate is fermented with the formation of randomly-labeled propionate (104). This finding has been shown to be due to the recycling of labeled carbons (135). Labeling nevertheless does occur in propionate, succinate (evidence for the C₁-C₉ mode of formation of succinate from pyruvate), and in the unidentified carbonyl compound discussed earlier (22).

In contrast to its oxidizing property, pyruvate is a poor reducing substance for the propionic acid bacteria (44, 98).
The mechanism of formation of carbon dioxide from pyruvate remains an enigma. Formation of carbon dioxide occurs over a pH range of 5.0-8.0 and increases with decreasing pH (6). The optimum occurs at pH 5.2 (6, 38) or thereabouts (184). Formation of carbon dioxide is curtailed sharply below pH 5.0 (184). The pH optimum corresponds to that for formation of carbon dioxide from lactate (184). Utilization of pyruvate, as measured by formation of molecular hydrogen by *Veillonella gazogenes*, occurs optimally at pH 6.2 (31).

The mode of formation of carbon dioxide from pyruvate differs from that from lactate in sensitivity to fluoride. Formation of carbon dioxide from pyruvate is much less sensitive to fluoride than is that from lactate (6, 25). The ready fermentation of pyruvate in the presence of a concentration of fluoride which inhibits completely the decomposition of lactate indicates that lactate is not a necessary intermediate between pyruvate and propionate (27). Stone and Werkman (148) reported that "in a normal propionic fermentation pyruvic acid is reduced, first to lactic acid and then to propionic acid."

Fermentation of pyruvate in the presence of concentrations of fluoride great enough to stop utilization of lactate does not result in the accumulation of lactate (6), even
though the main product in either case is propionate (6).

Fumarate removes fluoride inhibition of formation of carbon dioxide from lactate under anaerobic conditions (6). Formation of carbon dioxide occurs more rapidly in the system, lactate-fumarate-fluoride, than in the system, pyruvate-fluoride (6), indicating again two separate mechanisms of formation of carbon dioxide.

Dissimilation of phosphopyruvate requires a much greater concentration of fluoride for inhibition than does the dissimilation of phosphoglycerate (197).

Lactate inhibits aerobic utilization of pyruvate by thiamine-deficient propionic acid bacteria (126).

Cocarboxylase is involved in the utilization of pyruvate. Thiamine-deficient cells show greatly decreased formation of carbon dioxide from pyruvate. Addition of thiamine or cocarboxylase stimulates the formation of carbon dioxide to normal levels (135, 137, 138, 139, 140). One report discusses involvement of cocarboxylase in a "dismutation" of pyruvate but no evidence is presented in support of this mechanism (135). The same report suggests the possibility of involvement of thiamine in the transfer of hydrogen.

Repeated washings of whole cells with alkaline phosphate solution does not decrease the activity of propionic acid bacteria on pyruvate (136). This type of washing effectively
removes cocarboxylase from dried yeast (107).

**Aerobic utilization**

The amount of carbon dioxide formed from pyruvate is the same under aerobic or anaerobic conditions (63). Measured by uptake of oxygen and formation of carbon dioxide, pyruvate is utilized less rapidly than lactate (6).

The pH optimum for formation of carbon dioxide and uptake of oxygen occurs at pH 5.2 (184). These activities decrease markedly below pH 5.0 (184). The ratio of carbon dioxide-to-oxygen increases with increasing pH over a range of 5.2-6.9 (184). At any given pH within this range, the ratio decreases with the progress of the reaction in contrast to the ratio for lactate which increases with time (50). The final ratio for pyruvate is approximately 0.8 (63).

Cyanide (0.002 N) and fluoride (63) have no effect on the aerobic utilization of pyruvate.

Acetate, propionate, succinate, lactate and carbon dioxide are the products of aerobic utilization of pyruvate (184). Succinate and lactate are formed in insignificant amounts.

At pH 7.4, thiamine-deficient cells utilize glucose, lactate and glyceral with an accumulation of pyruvate (126).
Addition of cocarboxylase minimizes the accumulation, indicating involvement of cocarboxylase in aerobic utilization of pyruvate (126). Utilization of pyruvate is unaffected by 0.08 molar concentrations of malonate, formate, acetate, propionate and alpha-glycerophosphate (126). Lactate, succinate, fumarate and glycerol inhibit utilization of pyruvate at pH 7.4 (126). The role of thiamine still is obscure (156).

The presence of a tricarboxylic acid system in the propionic acid bacteria has been demonstrated with pyruvate as a substrate (20, 41). The enzyme systems responsible for dissimilation of pyruvate are considered to be constitutive in nature (81).

Oxalacetic Acid

Utilization of oxalacetate yields succinate as a product and the latter probably arises via the oxalacetate → fumarate system which is a hydrogen carrier in the propionic acid bacteria (96). Oxalacetate, probably functioning as a hydrogen acceptor, catalyzes the fermentation of glycerol, arabinose, erythritol, inositol and mannitol (96, 98). Arising from oxalacetate in these catalyzed fermentations are malate and fumarate which persist in the early stages of the
fermentations and then disappear (93). Succinate (up to 40 micromoles for each 100 micromoles of oxalacetate added) and carbon dioxide (up to 74 micromoles for each 100 micromoles of oxalacetate added) are formed (93). These reactions occur in bicarbonate buffer with an atmosphere of carbon dioxide. An overall reaction equation was proposed (77):

$$3\text{ Oxalacetate} = 1\text{ Succinate} + 2\text{ Acetate} + 2\text{ Bicarbonate} + 2\text{ Carbon dioxide}$$

Fermentations of glucose, lactate and pyruvate are not stimulated by oxalacetate (93). Aerobically, oxalacetate takes part in a tricarboxylic acid system which forms citrate (20, 40, 41).

Malic Acid

Utilization of malate by the propionic acid bacteria was studied first by Fitz (54), who proposed the reaction mechanism:

$$3\text{ Malate} = 2\text{ Propionate} + 1\text{ Acetate} + 4\text{ Carbon dioxide} + 1\text{ H}_2\text{O}$$

The D (+) isomer is not utilized (93). The L(-) isomer is utilized with the formation of carbon dioxide (147) and succinate (96, 98), which may be formed via the dicarboxylic acid series, the first step being mediated by fumarase (51, 98) which is very prominent in the propionic acid bacteria.
The fumarase system is not affected by treatment of whole cells with toluene (51). In the presence of semicarbazide at pH 5.2, carbon dioxide is formed slowly from malate (33). Malate donates hydrogen only slightly to methylene blue and ortho-chlorophenol indophenol (147, 149) in the pH range 5.5-7.0. Redox potentials in the utilization of malate have been determined (147).

Aerobically, malate is utilized with only a slight uptake of oxygen (41, 98, 147).

Utilization of L-malate by *Veillonella gazogenes* (53) yields molecular hydrogen and acetate in equivalent amounts (81). This organism does not utilize sugars fermented by the propionic acid bacteria. Rumen microorganisms metabolize malate with the formation of propionate, acetate and carbon dioxide (78).

Glycerol is utilized at an increased rate by addition of malate to the fermentation. Malate apparently acts as a carrier of hydrogen (98).

Anaerobically, at pH 5.8 and in the presence of C^{14}-carbon dioxide, the dissimilation of malate yields succinate which contains only part of the C^{14} which appears in succinate incubated with C^{14}-carbon dioxide (5).

Fumaric Acid

Formation of fumarate from oxalacetate (98) and glycerol
Fumarate is utilized with the formation of malate at pH 6.8 (98), and succinate (7, 96, 98). In an atmosphere of carbon dioxide and in bicarbonate buffer, 95 per cent of fumarate added is converted to succinate (98). In the presence of semicarbazide at pH 5.2, fumarate is utilized with the formation of carbon dioxide (38). At pH 6.8, cell-free extracts of 40-hour-old cells of Propionibacterium pentosaceum form more carbon dioxide than extracts from 24-hour-old cells (160). Whole cells form carbon dioxide from fumarate as well.

Fumarate donates hydrogen to methylene blue and ortho-chlorophenol indophenol (147, 149) in the pH range, 5.5-7.0, resembling malate in this respect. The system, fumarate oxalacetate, acts catalytically (96, 93) and the system, fumarate \(\rightarrow \) malate has been demonstrated polarimetrically (98).

In fermentations of glycerol, catalytic amounts of fumarate increase the amount of acid (including succinate) and carbon dioxide formed (96, 93). The reaction, fumarate \(\rightarrow \) succinate, is insensitive to fluoride (7). In the presence of fluoride, fumarate accepts hydrogens from the oxidation of glucose, and phosphoglycerate accumulates along with succinate, the amount of the latter formed from fumarate being approximately equivalent to the inorganic phosphate.
esterified in the reaction (7). The system, fumarate \(\rightarrow\) succinate, has been established as a carrier of hydrogen (See section on succinic acid).

At pH 5.03 and under anaerobic conditions, utilization of fumarate in the presence of \(\text{C}^{14}\)-carbon dioxide yields succinate containing no \(\text{C}^{14}\) activity (5).

Fumarate inhibits utilization of pyruvate aerobically in the presence of thiamine (126). Under aerobic conditions, oxygen is taken up (41, 147) by whole cells and by cell-free extracts (160).

Rumen microorganisms ferment fumarate with the formation of propionate, acetate, carbon dioxide and molecular hydrogen (78). Veillonella gazogenes utilizes fumarate with the formation of equivalent amounts of acetate and molecular hydrogen (81).

**Succinic Acid**

**Occurrence**

Formation of succinate in the propionic acid fermentation of glucose was first reported by Virtanen (162). Since then, succinate has been identified in and isolated from fermentations of glucose, glycerol, pyruvate, lactate (90), pentoses, starch and maize wort. Foote, Fred and Peterson (55), Krebs
and Eggleston (98), van Niel (115), and others reported fermentations in which succinate is found. In only a few instances (the report of Maurer (109) is an example) is succinate reported completely absent in the fermentation.

The mode of formation of succinate in fermentations has been considered rather extensively. Van Niel (115) suggested that succinate is formed from aspartate and nitrogen sources in the dried yeast used in the culture medium. This suggestion was questioned by Foote, Fred and Peterson (55) who felt that the relatively large amounts of the acid formed in fermentations made formation of the acid from small amounts of yeast extract hardly possible. Wood and Werkman (195) showed conclusively that the amount of succinate formed is much in excess of the nitrogen content of yeast extract and that therefore the yeast extract is not the main source of succinate.

Wood and Werkman (195, 198) suggested that succinate is formed by synthesis from acetate. This suggestion was based on inconclusive data. A subsequent report (187) provided further evidence for the condensation. Contrary to this concept, Krebs and Eggleston (98) stated that the reaction, fumarate $\rightarrow$ succinate, is not a hydrogen carrier under anaerobic conditions and that "since succinic cannot be oxidized under anaerobic conditions, it cannot be an intermedi-
ate in the oxidation of acetic acid". Krebs (96) had pro-
pounded this argument earlier. In a private communication
to Carson and co-workers (21) and later in valid publication
(97), Krebs reported that the set of reversible reactions
between oxalacetate and succinate does occur in the propionic
acid bacteria and that these bacteria can reduce oxalacetate
to succinate reversibly.

Krebs and Eggleston (98) increased the yields of succin-
ate in the fermentation of glycerol by adding acetate to the
system. Barban and Ajl (5) incubated succinate with 2-c^{14}-
acetate but found the equilibrium succinate inactive. No
external hydrogen acceptors were present. The implication
is that acetate as such can be condensed to succinate only
in the presence of a suitable hydrogen acceptor (substrate).
In a report appearing ten year earlier, Krebs and Eggleston
(98) pointed out that synthesis of succinate is increased by
the addition of acetate during the fermentation of oxalacetate.
It was suggested that possibly acetate donates hydrogens to
the reduction of oxalacetate, but evidence for the anaerobic
oxidation of acetate per se to carbon dioxide was lacking.

The question of condensation of acetate remains unans-
swered. However, the finding of succinyl phosphate and the
implication of coenzyme A in its metabolism (177, 178, 179)
may tie in with the coenzyme A-acetyl complex shown to be
involved in the utilization of acetate. It may be that the complex or some associated form of acetate is essential to condensation.

A third concept of the formation of succinate originated with Virtanen (163) who proposed a split of the glucose molecule into a 4-carbon and a 2-carbon fragment, the 4-carbon fragment being succinate. Wood, Stone and Werkman (196) and Wood and Werkman (195) at first favored the idea but later withdrew support (192). "There is in reality no substantial proof of the often proposed 4- and 2-carbon cleavage of glucose" (193).

Wood and Werkman (192) introduced a further concept of the formation of succinate when it was emphasized that succinate, a 4-carbon compound, is formed from glycerol, a 3-carbon compound, and that therefore the process must involve a synthesis. A report appearing in the same year (193) dissociated the formation of succinate from utilization of carbon dioxide, but later extensive reports (82, 120, 176, 194, 195, 198) demonstrated a close relationship between utilization of carbon dioxide and the formation of succinate. The concept of fixation of carbon dioxide onto a 3-carbon moiety with the formation of succinate (possibly via the dicarboxylic acid series) (98) evolved. Later reports (119) verified these findings and substantiated the C1-C3 concept.
Tracer experiments (22, 23) showed almost uniform distribution of labeled carbon atoms in succinate and propionate and placed the carbon dioxide fixation reaction in doubt. Subsequent reports demonstrated that the method of degradation of products used to locate the tracer atoms was non-specific (199, 203). Location of the labeled carbon dioxide carbon solely in the carboxyl groups of succinate and propionate was demonstrated and confirmed (21, 200, 201, 202).

More recent evidence (1, 19) derived from tracer experimentation indicates the existence in the propionic acid bacteria of enzymatic systems carrying out (a) the Thunberg-Wieland condensation of acetate and (b) the fixation of carbon dioxide leading to the formation of succinate.

Utilization

Succinic decarboxylase has been classified as a constitutive enzyme since it occurs whether cells are grown in lactate, glucose or D-tartrate (81). Enzymes responsible for the utilization of dicarboxylic acids have been reported (81) as being the constitutive type since their activity does not vary with the growth substrate.

Shaw and Sherman (133) were the first to report utilization of succinate with the formation of propionate and ace-
Decarboxylation of succinate resulting in the formation of propionate was suggested on several occasions (49, 62, 147, 184, 186), the reaction occurring more readily at lower pH levels and under anaerobic conditions (187). The close relationship of succinate and propionate was demonstrated by Poote, Fred and Peterson (55) when they found that the greater the amount of succinate formed, the lower the relative value of propionate. Hitchner (76) pointed out that in the presence of fermentable carbohydrates, succinate is utilized whereas no utilization occurs at pH 7.0. A similar report (52) verified these findings. Krebs and Eggleston (98) found no utilization of succinate at alkaline pH levels under anaerobic conditions. In the pH range, 4.4-6.5, they found that whereas utilization of glucose yields the usual products qualitatively and quantitatively, addition of succinate increases the relative amounts of propionate and carbon dioxide. In experiments using constant pH levels (79), it was found that when the pH remains above 6.5, the rate of decarboxylation is not increased by the addition of glucose. Decarboxylation of succinate occurs only if the pH of the medium falls below 6.5 during the fermentation. Similar results were obtained by Katagiri and Ichikawa (87) who set the maximum pH for activity at 6.4. They reported acceleration of decarboxylation by addition,
at pH 5.2, of glucose and yeast extract in combination but not individually. With lactate as a carbon source, there is no change of acidity during the fermentation and, with an initial pH of 7.0, added succinate is not attacked (79). Johns (82) reported that with lactate or pyruvate as energy sources, formation of carbon dioxide from succinate is inhibited. This report may be similar to the others in that the pH level of the fermentation is above the activity range of succinic dehydrogenase.

Other evidence indicates almost complete conversion under anaerobic conditions of succinate to propionate and carbon dioxide (38, 78, 79, 142, 175, 184, 186) with the pH optimum around 5.2 (38, 79, 82, 177, 178, 187). One report places the pH optimum at 5.9-6.0 (81). Activity of the enzyme is nil above pH 6.8 (38, 79) although pH 6.4 is said to be the top limit (87). A contrast to these reports is the one (78) showing conversion of succinate almost quantitatively (95 per cent conversion) to propionate and carbon dioxide at pH 7.4 by an anaerobic micrococcus from rumina. This finding is of interest since the permeability of cells to succinate is considered to be a possible rate-determining factor in the utilization of succinate (21, 38, 82). "Evidently succinic acid can pass out of the bacterial cell at pH values above approximately 6.5 but cannot reenter until the pH
values above approximately 6.5 but cannot reenter until the pH drops below that level" (82).

C₁¹-carboxyl-labeled succinate, fermented in the presence of glycerol and bicarbonate, was reported (22) to yield propionate with randomized labeling of the carbons. Subsequent reports (119, 203) showed that the method of degradation of products was non-specific and that with proper degradation the labeling is found only in the carboxyl groups of propionate and in the carbon dioxide formed. These findings are further evidence for the decarboxylation of succinate with the formation of propionate and carbon dioxide. Low interconversion values for the reaction, succinate $\rightarrow$ propionate + carbon dioxide, have been demonstrated using tracer compounds (5, 21). Hartelius (74) presented evidence favoring the formation of succinate by combination of carbon dioxide and propionate.

Propionic acid bacteria grown in glucose or glycerol yield increased amounts of propionate and carbon dioxide from glucose in the presence of fluoride even though the utilization of glucose is decreased. In fermentations of glycerol in the presence of fluoride, the amount of substrate utilized is decreased even though the formation of propionate is stimulated (198). These findings resemble those of Barker and Lipmann (6) in which formation of prop-
ionate from pyruvate is increased in the presence of fluoride. The amount of succinate formed from glucose or glycerol in the presence of fluoride is decreased below the usual level (198). Increased formation of succinate in the absence of fluoride is accompanied by an almost equivalent decrease in the yield of carbon dioxide (198).

Little evidence concerning the utilization of succinate by aerobic or oxidative processes is available. Krebs (96, 97, 98) has contributed most to the information on the aerobic dicarboxylic acid cycle in the propionic acid bacteria (see section on dicarboxylic acids). *Micrococcus lactilyticus* cleaves succinate with the formation of acetate (178).

Formation of propionate and carbon dioxide from succinate is not limited to the members of the genus *Propionibacterium*. *Veillonella gazogenes* (later named *Micrococcus lactilyticus* (57)) carries out a quantitative conversion (81) at the pH optimum, 5.9-6.0, with no inhibition by 0.01 molar malonate, as reported for *Propionibacterium pentosaceum* by Delwiche (38). No acetate is formed in the reaction. *Clostridium propionicum* does not form succinate in its fermentations (17) although under anaerobic conditions it produces a propionic-like fermentation at pH 7.2 and 37°C. It may be inferred that propionate is formed in this instance by a mechanism not involving decarboxylation of succinate.
Hartelius (74), in studies on the fermentation of glycerol, presented evidence for the occurrence of propionate in the absence of succinate.

**Mechanism of utilization of succinate**

Perhaps the first attempt at showing cofactors essential to the decarboxylation of succinate was that of Barban and Ajl (5). At pH 5.8, under anaerobic conditions, interconversion of propionate and labeled carbon dioxide was demonstrated. The involvement of adenosinetriphosphate in the reaction was pointed up. Formation of a phosphorylated succinyl compound was indicated in the findings of Barker and Lipmann (7). In the fermentation of glycerol, the quantity of succinate formed is equivalent to the inorganic phosphate esterified. Delwiche, Phares and Carson (42), using cell-free extracts, showed the involvement of adenosine triphosphate and coenzyme A in the reversible decarboxylation of succinate. The data indicate that a 1-carbon compound, derived from succinate, combines reversibly with a 3-carbon derivative of propionate. The 1-carbon compound apparently is not carbon dioxide. Phares, Carson and Delwiche (118) demonstrated that the 1-carbon fragment, originating from the gamma-carboxyl of succinyl-coenzyme A, may (a) combine
reversibly with propionate to form succinate, (b) form carbon dioxide, (c) be incorporated into malate carboxyl groups without going through fumarate or carbon dioxide. Whitely (177) demonstrated that the essential components involved in decarboxylation of succinate by cell-free extracts of *Micrococcus lactilyticus* are coenzyme A, adenosine triphosphate, cocrboxylase and magnesium. Van Niel (116) reported this work in symposium.

Krampitz (116) reported that cells incubated in an atmosphere of hydrogen suffer a reduced (60-70 per cent) activity of succinic decarboxylase whereas incubation in an atmosphere of nitrogen or helium has no effect. Methyl viologen, with an oxidation-reduction potential of 0.412 volt, increases the inhibition to 100 per cent. Krampitz pointed out that hydrogen probably reduces the 3-carbon precursor of propionate through the action of hydrogenases and consequently oxidation of succinate, which is the first step in decarboxylation of succinate, does not occur.

Whitely (178, 179), using cell-free preparations of *Micrococcus lactilyticus*, demonstrated total conversion of succinate to propionate and carbon dioxide at pH 5.5. Required for full activity of the enzyme, which remains in solution even after centrifugation at 60,000 x G., are a reducing agent (such as cysteine), magnesium, coenzyme A
and adenosine triphosphate. Biotin is not involved. The latter finding is not in agreement with that of Delwiche (28, 39) using propionic acid bacteria.

Whitely presented evidence (179) favoring the formation of succinyl-phosphate prior to decarboxylation. The overall mechanism is indicated as follows:

```
\[
\text{Succinate} \xrightarrow{\text{Adenosine triphosphate}} \text{Succinyl-Coenzyme A} \\
\text{Succinyl-Coenzyme A} \xrightarrow{\text{Propionyl-Coenzyme A}} \text{Propionate} \\
\text{Carbon dioxide} \xrightarrow{\text{Propionate}} \text{Propionyl-Coenzyme A} \xrightarrow{\text{Succinate}}
\]
```

**Inhibitors**

Malonate (0.3 molar) inhibits formation of carbon dioxide from succinate and prevents formation of propionate from pyruvate (38). Malonate (0.01 molar) does not inhibit decarboxylation of succinate at pH 5.9–6.0 by Veillonella gazogenes (31). Semicarbazide (0.01 molar) does not prevent formation of propionate and carbon dioxide from succinate (38).
Preparation of Cells and Cell-free Extracts

The organisms used in this investigation were *Propionibacterium pentosaceum*, *P. petersonii*, *P. thöni*, *P. jensenii*, *P. zeae*, *P. fraudenreichii*, and *P. shermanii*.

Unless otherwise indicated, cells were grown under micro-aerophilic conditions for 36 hours at 30°C with occasional swirling. Six liters of medium were inoculated with 200 ml of a 36 hour culture. The culture medium contained 1.5 per cent carbohydrate carbon source, 0.5 per cent yeast extract (Difco), 0.3 per cent tryptone (Difco), 0.15 per cent ammonium sulfate, 0.005 per cent magnesium sulfate, and 0.02 molar phosphate buffer (pH 7.1). The pH of the medium following autoclaving was approximately 6.8.

Cells were harvested in a Sharples high speed centrifuge and washed twice with ice cold demineralized water in a chilled Waring blender.

Where fresh whole cells were used in experiments, a given wet weight of cells was suspended in buffer of the appropriate pH and molarity and the density of the suspension adjusted using a Klett-Summmerson photometer with a 600 mμ filter.
Dried cells were prepared by a modification of the technic of Barker and Lipmann (6) in which sodium hydroxide pellets were included in the vacuum jar along with phosphorus pentoxide. Dried cells retained their activity over a period of two months when stored in dry stoppered vials at temperatures below 0° C.

Thiamine-deficient cells were prepared by the method described by Silverman and Werkman (140) using a basal medium devised by Tatum, Wood and Peterson (154).

Cell-free extracts were prepared by sonic treatment (9,000 cycles for 30 minutes at approximately 0° C.) of washed fresh cells suspended in ice-cold demineralized water or phosphate buffer at the appropriate pH. Sonic treatment was followed by centrifugation for 30 minutes at 35,000 gravities (relative) at temperatures just above 0° C., unless otherwise specified.

Fractionation of the cell-free extracts was achieved by means of addition of various specified amounts of ammonium sulfate in a neutralized saturated solution. During the drop-wise addition of the precipitant, all solutions and vessels were kept chilled to approximately 0° C. Calculations of the amounts of ammonium sulfate to be added for obtaining a given per cent of saturation were made using the formula:

\[
(\text{ml. extract}) \times (\text{actual saturation}) - 100 \times x = (\text{ml. extract} - x) \times (\text{desired saturation}),
\]

where \(x\) is the amount of ammonium sulfate to add.
It was found that considerable amounts of enzymatically inactive protein may be removed conveniently by preliminary use of the method described by Kaufman, Korkes and del Campillo (39) which employs manganese chloride as a precipitating agent.

Several methods of dialysis were tried. Of these, the method using internal stirring of the extract during dialysis proved to be the most effective (See Fig. 1). Less successful methods were: (1) the same arrangement as above but without internal stirring and with the Visking casing sealed on both ends; (2) the same arrangement as in (1) with the Visking casing and its contents agitated in an up-and-down fashion by means of a string attached to a rotating wheel; (4) the Visking casing and its contents suspended in 12 liter volumes of water or buffer and attached to a stirring rod which is rotated by a motor; (5) the Visking casing drawn tightly over a glass stirring rod bent into an approximate rectangular outline. The casing is fastened at the bottom and top, air being excluded before sealing. The rod is then rotated in the water or buffer.

In those cases where internal stirring was not employed, the extract tended to layer out, the heavier portions of the material settling out in the casing. Also, where internal stirring was absent, longer periods of time of dialysis were
Fig. 1 Apparatus for dialysis using internal stirring.
required to achieve satisfactory results.

**Special Reagents**

Sodium pyruvate was prepared by the procedure outlined by Watt (163). Each sample was tested for chemical purity and biological availability. Lithium lactate was prepared by the method described in Hawk, Oser and Summerson (75). Dicarboxylic acids were recrystallized from one normal hydrochloric acid followed by drying in a hot air oven and storage in vacuo over pellets of sodium hydroxide.

**Analytical Methods**

Dehydrogenase activities were studied by means of the Thunberg methylene blue technic described by Umbreit, Burris and Stauffer (159).

Manometric studies were carried out using the conventional Barcroft-Warburg respirometer apparatus. Where required, anaerobic conditions were obtained by flushing the system with tank nitrogen passed over hot copper turnings to remove any oxygen. Calculations were made according to the recommendations of Umbreit, Burris, and Stauffer (159).

The Beckman pH meter was used for hydrogen ion concentration determinations.
Spectrophotometric determinations were made on the Beckmann spectrophotometer. (Model DU).

Qualitative determinations

Organic acids were detected chromatographically using a modification of the method of Stark, Goodban and Owens (145). Solvents used were those recommended by Berry, Sutton, Cain and Berry (12). Volatile fatty acids were detected chromatographically by the method of Fink and Fink (52). Keto acids were detected chromatographically by the method of Cavallini, Frontali and Toschi (24) as modified by Warburton, Eagles and Campbell (167).

Quantitative determinations

Total volatile fatty acids were determined by steam distillation in an all-glass apparatus of deproteinized reaction mixtures in the presence of 0.4 molar sulfuric acid and magnesium sulfate, as recommended by Neish (112). Deproteinization of reaction mixtures was accomplished best by treatment with sulfuric acid in concentrations indicated in the text. Where pyruvate and lactate were present in the reaction mixture, steam distillation was
conducted in the presence of mercuric sulfate. Formate, pyruvate and lactate are destroyed by this treatment (60).

Distillates were titrated using standardized carbonate-free sodium hydroxide solutions with one per cent phenolphthalein in 95 per cent ethyl alcohol as an indicator. The resulting solution of salts was used in the determination of individual volatile fatty acids. The neutralized distillates were evaporated down to dryness and the individual volatile fatty acids determined by a modification of the method of Ramsey and Patterson (123) using the column shown in Fig. 2.

Pyruvate was determined by the method of Bueding and Wortis (16). To facilitate the procedure, a manifold apparatus (Fig. 3) was constructed. Each pipette of the apparatus served as a gassing tube for mixing the solvents by bubbling gas through. In addition, by applying negative pressure to the system, each pipette served as a sample remover. When the mixture of solutions had been carried into the pipette, the system was closed and the solvent layers allowed to separate. After a suitable period of time, the system was opened and the lower layer of solvent was drained off for further use or for discard. As pointed out by Friedeman and Haugen (61), toluene is a more specific solvent for the extraction of the phenylhydrazone of pyru-
Fig. 2 Apparatus for column separation of volatile fatty acids.
Fig. 3 Manifold apparatus for determination of pyruvate.
vate but, since standards are run using the same solvent, ethyl acetate may be used as the solvent.

Biological availability of pyruvate was determined by the method described by Umbreit, Burris and Stauffer (159).

Lactate was determined by the method of Parker and Summerson (8). When pyruvate was present in the reaction mixtures, suitable dilutions were made to avoid errors due to excessive amounts of pyruvate.

Succinate and fumarate were determined by the manometric method described by Umbreit, Burris and Stauffer (159) using freshly prepared crude preparations of succinoxidase derived from pigeon breast muscle. Oxalacetate was determined using the aniline-citrate method (159).

The protein content of cell-free extracts was determined by the method of Weichselbaum (170), using Armour's bovine plasma albumin (crystallized) as a standard.
Effect of Growth Conditions on Enzyme Activity

Conflicting evidence related to the metabolism of lactate by the propionic acid bacteria may be explained by heterogeneous enzyme composition due to diverse conditions of growth. Investigation into the effect of the type of substrate and age of cells on the enzymatic composition of cells yielded results which indicate significant differences in enzyme composition.

*Propionibacterium pentosaceum* was grown for various periods of time in separate media containing glucose, glycerol, lactate, and a combination of fumarate and lactate. The yield of cells, pH of the growth medium at harvest, and dehydrogenase and decarboxylase activities of the freshly prepared cells was determined. Results are shown in Tables 1 and 2.

Of the substrates tested, glucose is utilized most readily for growth. Yields of cells from glucose media are approximately twice that from lactate media. These findings are in agreement with those of Leviton and Hargrove (105). Multiplication does not occur to any significant degree beyond 24 hours except in the lactate medium. Leviton and
Table 1

Effect of growth conditions on dehydrogenase activity of *Propionibacterium pentosaceum*<sup>a</sup>

<table>
<thead>
<tr>
<th>Growth medium</th>
<th>Incubation time (hrs.)</th>
<th>Yield (grams)</th>
<th>Final pH</th>
<th>Time (minutes) for 90 per cent methylene blue reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Malate</td>
</tr>
<tr>
<td>Glucose</td>
<td>24</td>
<td>6.2</td>
<td>4.9</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>6.4</td>
<td>4.6</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>6.5</td>
<td>4.8</td>
<td>20</td>
</tr>
<tr>
<td>Glycerol</td>
<td>24</td>
<td>4.1</td>
<td>5.9</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>4.3</td>
<td>5.2</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>4.3</td>
<td>4.6</td>
<td>27</td>
</tr>
<tr>
<td>Lactate</td>
<td>24</td>
<td>3.2</td>
<td>6.9</td>
<td>*&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>3.8</td>
<td>6.8</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>4.2</td>
<td>6.8</td>
<td>*</td>
</tr>
<tr>
<td>Lactate</td>
<td>24</td>
<td>3.0</td>
<td>5.9</td>
<td>15</td>
</tr>
<tr>
<td>Fumarate</td>
<td>36</td>
<td>3.2</td>
<td>5.1</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>3.2</td>
<td>4.8</td>
<td>*&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Each Thumberg tube contained 10 mg. cells (wet weight), 3 ml. 0.1 M phosphate buffer (pH 6.4), 1 ml. 1: 10,000 methylene blue, 40 μMoles substrate (pH adjusted to pH 6.4), and demineralized H₂O to total volume of 6.0 ml. The Thumberg technic was conducted using naked eye observation at an equilibration temperature of 30° C.

<sup>b</sup> No reduction within 60 minutes

<sup>c</sup> Only partial reduction
Table 2

Effect of growth conditions on formation of carbon dioxide from several substrates by Propionibacterium pentosaceum\(^a\)

<table>
<thead>
<tr>
<th>Growth medium</th>
<th>Incubation time (hrs.)</th>
<th>Final pH</th>
<th>Malate</th>
<th>Succinate</th>
<th>Lactate</th>
<th>Pyruvate</th>
<th>Fumarate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>24</td>
<td>4.9</td>
<td>20</td>
<td>99</td>
<td>86</td>
<td>147</td>
<td>32</td>
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<tr>
<td></td>
<td>36</td>
<td>4.6</td>
<td>18</td>
<td>102</td>
<td>102</td>
<td>188</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>4.8</td>
<td>16</td>
<td>129</td>
<td>174</td>
<td>271</td>
<td>46</td>
</tr>
<tr>
<td>Glycerol</td>
<td>24</td>
<td>5.9</td>
<td>17</td>
<td>86</td>
<td>141</td>
<td>241</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>5.2</td>
<td>15</td>
<td>81</td>
<td>152</td>
<td>257</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>4.6</td>
<td>14</td>
<td>84</td>
<td>163</td>
<td>309</td>
<td>19</td>
</tr>
<tr>
<td>Lactate</td>
<td>24</td>
<td>6.9</td>
<td>15</td>
<td>49</td>
<td>168</td>
<td>229</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>6.8</td>
<td>16</td>
<td>41</td>
<td>132</td>
<td>238</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>6.8</td>
<td>14</td>
<td>39</td>
<td>206</td>
<td>374</td>
<td>21</td>
</tr>
<tr>
<td>Lactate plus</td>
<td>24</td>
<td>5.9</td>
<td>28</td>
<td>55</td>
<td>164</td>
<td>208</td>
<td>24</td>
</tr>
<tr>
<td>fumarate</td>
<td>36</td>
<td>5.1</td>
<td>23</td>
<td>61</td>
<td>132</td>
<td>212</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>4.8</td>
<td>15</td>
<td>71</td>
<td>141</td>
<td>239</td>
<td>31</td>
</tr>
</tbody>
</table>

\(^a\)Each flask contained 40 mg. cells (wet weight), 1 ml. 0.1 M phosphate buffer (pH 6.4), 40 \(\mu\) moles substrate (pH adjusted to pH 6.4), 0.2 ml. 6N H\(_2\)SO\(_4\) (for stopping reaction and releasing bound carbon dioxide), and demineralized H\(_2\)O to total volume of 2.0 ml. Each flask was flushed with O\(_2\)-free tank N\(_2\) and equilibrated to 30.4\(^\circ\) C.
Hargrove (105) reported that their fermentations were complete within 40 hours, in contrast to the five or seven days required for completion of fermentations, as reported by earlier workers. Low pH values in the media probably limit growth in all except the lactate medium where the pH does not change to any marked degree.

The effect of age of cells and carbon source on the dehydrogenase properties of cells is indicated in Table 1. The Thunberg methylene blue technic was used in these determinations. The relatively great amount of lactic dehydrogenase in all types of cells suggests a universal constitutive intermediary function in the propionic acid bacteria. Malic dehydrogenase is relatively inactive in all cells, no activity being detected in lactate-grown cells and in the 72 hour lactate-fumarate cells. Succinic dehydrogenase activity in glucose- and lactate-fumarate-grown cells is much greater than in the C3-grown cells. Dehydrogenase activities with the volatile organic acids as substrates also are weak with the exception of those cells grown in glucose and lactate-fumarate media.

Dehydrogenase activities apparently decrease with age. In any one age, the type of substrate determines the extent of dehydrogenase activity. Glucose- and lactate-fumarate-grown cells apparently synthesize greater amounts of dicarb-
oxylic acid dehydrogenases than those cells grown on C₃-compounds. Stone, Wood and Werkman (150) found that fatty acid dehydrogenases are inhibited by aging.

Reduction of methylene blue in the presence of glucose, pyruvate and molecular hydrogen is incomplete, as it is with oxalacetate as substrate. Addition of the latter to the reaction mixture is accompanied by frothing, probably due to decarboxylation of oxalacetate. Methylene blue is not reduced by lactate-grown cells with fumarate as substrate.

A similar series of experiments was conducted manometrically to determine decarboxylation activities of cells with the same diversity of growth conditions. Results are shown in Table 2.

Propionate, acetate and formate yield values of carbon dioxide approximately equivalent to the endogenous values and may be considered nil. Formation of carbon dioxide from the dicarboxylic acids is moderately weak in all cases. The activity of succinic decarboxylase is weak in lactate-grown cells. Addition of 2 x 10⁻⁴ molar manganese chloride to the malate reaction failed to stimulate formation of carbon dioxide, indicating the absence of a significantly active malic decarboxylase. Formation of carbon dioxide from lactate and pyruvate is quite prominent, glucose- and lactate-fumarate-grown cells showing the least activity. Aging
seems to increase decarboxylation of pyruvate and lactate. Utter, Kalnitsky and Werkman (160) found a similar situation using extracts of 24- and 40-hour cells of Propionibacterium pentosaceum.

Enzymatic activities of cells vary with the growth conditions. Discrepancies in reported characteristics and enzymatic capacities of the propionic acid bacteria most probably are due to the lack of uniformity of cultural conditions to which the bacteria were exposed. Of particular interest is the finding of comparatively low activities of succinic decarboxylase in cells grown in lactate. Delwiche (38) favored decarboxylation of succinate as the principle pathway by which propionate is formed. The role of succinate in the formation of propionate has been included in most reviews and discussions on the mechanism of formation of the products of the propionic acid fermentation. Since such importance is attached to this particular reaction, it was felt that possibly the cultural conditions used here may have affected the enzyme system negatively. The nature of this effect was investigated.

Delwiche (38) achieved success in obtaining cells with a high activity of succinic decarboxylase. The cells used in his experiments were grown in glucose medium consistently. In the experiments (see above) on the effect of cultura-
tion of Propionibacterium pentosaceum in various media on
the enzymatic properties of the organism, stock cultures
were carried in glucose as were the inocula used to start
the various growth cultures. In effect, the cells, before
inoculation into the various media, had been exposed to
only one type of cultural environment. Following growth
in various media for a maximum of 72 hours, enzymatic com-
position showed variations as shown in Tables 1 and 2. The
effect of exposure of the cells to the diverse cultural en-
vironments for greater periods of time was investigated.
Succinic decarboxylase was selected for study since it
apparently was considered the main enzyme system responsible
for the formation of propionate.

Exposure to the several media was accomplished by weekly transfers of cells in glucose, glucose-succinate, glyc-
erol, lactate and lactate-fumarate broths. Growth in lactate characteristically is ropy, in contrast to the usual
granular growth. Growth in acetate was attempted. Growth
decreases slightly with each transfer through the third
week, at which time growth returns to its usual level. How-
ever, growth in acetate is sparse in comparison to growths
in the other media and therefore was discontinued at this
point. No growth was observed in succinate, formate and
propionate after the third transfer. Following growth for
seven days, a one ml. inoculum was removed for transfer to fresh media. The remaining cells were harvested, washed twice with demineralized water and used in manometric experiments to determine the degree of activity of succinic decarboxylase in each type of cell. The pH used was 5.2. Values shown are those for 40 mg. (wet weight) of cells. Results are shown in Fig. 4.

The activity of succinic decarboxylase remains relatively constant in glucose-grown cells, whereas cells grown in the other media exhibit progressively weaker activities which approach a constant level after several transfers.

Following growth for seven days, all of the media show low pH values (4.4-4.8) except the lactate medium, in which a relatively high pH level (6.8) is maintained. Johns (79) found no change in pH with fermentations of lactate, as did Utter, Kalnitsky and Werkman (160) using cell-free preparations.

It may be that pH determines in part the enzyme composition of cells. Krebs and Eggleston (98) found that lactate-grown cells (no significant pH change in the growth medium) ferment glycerol slowly, whereas glycerol-grown cells (low pH in growth medium) ferment glycerol rapidly. Their results may be explained by pH effects on enzyme composition as well as by substrate differences. The effect of growth
Fig. 4 Effect of growth substrate on activity of succinic decarboxylase in Propionibacterium pentosaceum.
pH on succinic decarboxylase activity was noted by Delwiche (38) who reported the absence of succinic decarboxylase activity in cells grown in glucose media and harvested before the pH of the growth medium dropped below 6.5. In contrast to this report, Johns (73) noted the quantitative decarboxylation of succinate at pH 7.4 by an anaerobic micrococcus from sheep rumina responsible for a propionic acid fermentation.

The data reported here indicate that synthesis and activity of succinic decarboxylase may be only partially dependent on the pH of the growth medium. The final pH levels of the glucose and glycerol media were the same yet there is a marked difference in the activities of succinic decarboxylase of the two types of cells. It may be that the type of substrate also determines the enzyme activity of cells. Variation in activity of enzymes is not limited to succinic decarboxylase. Decarboxylation activity with pyruvate and lactate as substrates is greater in lactate-grown cells than in glucose-grown cells. These findings indicate that lactate may be dissimilated by a mechanism involving succinate in a minor role and pyruvate in a major role.
Succinic Decarboxylase Activity among the Species

In the previous experiments, the same species of microorganism was used but growth substrates, age and growth pH were variables. With the last three mentioned factors constant, differences in activity of succinic decarboxylase among the species were determined. Cells were grown for 48 hours at 30° C. under microaerophilic conditions in glucose broth inoculated from stock cultures carried in glucose agar deep cultures. Results are shown in Table 3.

The pH optimum for succinic decarboxylase was found to be pH 5.2, as reported by various investigators. *Propionibacterium pentosaceum*, *P. shermanii* and *P. zeae* were used in this determination. The appropriate concentrations of enzyme and substrate were determined and subsequent experiments were conducted using 40 mg. (wet weight) of cells and 40 micromoles of substrate (See Fig. 5, 6, and 7).

Fresh whole cells were utilized since lyophilization, drying over phosphorus pentoxide in vacuo, and acetone drying of cells destroys a part or all of the succinic decarboxylase. The enzyme system involved in formation of carbon dioxide from lactate is affected similarly except that in the case of drying over phosphorus pentoxide the activity remains high. The enzyme system concerned in forma-
tion of carbon dioxide from pyruvate is affected only slightly by these treatments. Heating to 100°C for ten minutes destroys succinic decarboxylase.

Table 3
Activity of succinic decarboxylase in several species of the genus *Propionibacterium*

<table>
<thead>
<tr>
<th></th>
<th>Succinate</th>
<th>Lactate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μMoles</td>
<td>% Theor.</td>
</tr>
<tr>
<td><em>F. pentosaceum</em></td>
<td>7.1</td>
<td>17.8</td>
</tr>
<tr>
<td><em>F. thoenii</em></td>
<td>4.0</td>
<td>10.0</td>
</tr>
<tr>
<td><em>F. lensenii</em></td>
<td>5.8</td>
<td>14.5</td>
</tr>
<tr>
<td><em>F. petersenii</em></td>
<td>7.0</td>
<td>17.5</td>
</tr>
<tr>
<td><em>F. zee</em></td>
<td>3.2</td>
<td>8.0</td>
</tr>
<tr>
<td><em>F. freudenreichii</em></td>
<td>5.0</td>
<td>12.5</td>
</tr>
<tr>
<td><em>F. shermanii</em></td>
<td>5.4</td>
<td>13.5</td>
</tr>
</tbody>
</table>

*Each flask contained 40 mg. (wet weight) of cells, 0.25 M phosphate buffer (pH 5.2), 40 μMoles of substrate, 0.2 ml. 6N H₂SO₄ (to release bound CO₂ at end of reaction), and demineralized H₂O to a total volume of 2.0 ml. The flasks were flushed with O₂-free tank N₂ and equilibrated to 30.4°C.*

Activity of the decarboxylase within the species is not uniform despite common cultural environments. The relatively higher levels of decarboxylase activity with lactate as substrate would seem to indicate the constitutive nature of the enzymes responsible for utilization of lactate (and pyruvate).
Fig. 5 Activity of succinic decarboxylase as influenced by pH.
Fig. 6 Effect of concentration of enzyme on decarboxylation of succinate by Propionibacterium plantaeceum.

\[ \mu \text{L CO}_2 / 30 \text{ MINUTES} \]

mg (WET WT.) CELLS

0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0
Fig. 7 Effect of concentration of substrate on decarboxylation of succinate by *Propionibacterium pentosaceum.*
Leviton and Hargrove (106) noted that "all species of the genus when cultivated under the same conditions will produce active substances, yet in different quantities."

Properties and Synthesis of Succinic Decarboxylase

The activity of succinic decarboxylase demonstrated here does not compare favorably with the activity reported by Delwiche (38). Cells (40 mg. wet weight) grown in glucose showed a rate of formation of carbon dioxide amounting to approximately 125 microliters in 60 minutes. Delwiche achieved a rate of formation equal to 180 microliters in 60 minutes using 35 mg. (wet weight) of cells. Delwiche included in the reaction mixture 0.01 molar semicarbazide "as a ketone-trapping agent to eliminate the possibility of reversion to pyruvate in the cases of the dicarboxylic acids". This addition serves as a precautionary measure since "separate experiments established that the rates of decomposition of succinate, fumarate, and malate remain unchanged upon the addition of this inhibitor". Comparison of the activities of decarboxylation using succinate, pyruvate, and lactate with and without semicarbazide is shown in Table 4 and Figure 8, the latter showing the progress curves without semicarbazide in the reaction mixture.
Effect of semicarbazide on formation of CO₂
by Propionibacterium pentosaceum

<table>
<thead>
<tr>
<th></th>
<th>Total µMoles CO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cin.</td>
</tr>
<tr>
<td>F. pentosaceum</td>
<td>7.3</td>
</tr>
<tr>
<td>F. petersenii</td>
<td>4.8</td>
</tr>
<tr>
<td>F. thoenii</td>
<td>4.0</td>
</tr>
<tr>
<td>F. jensenii</td>
<td>5.8</td>
</tr>
<tr>
<td>F. zee</td>
<td>3.2</td>
</tr>
<tr>
<td>F. freudenreichii</td>
<td>5.0</td>
</tr>
<tr>
<td>F. shermanii</td>
<td>5.4</td>
</tr>
</tbody>
</table>

Each flask contained 40 mg. (wet weight) of cells, 0.025 M phosphate (pH 5.2), 0.2 ml. 6N H₂SO₄ (to release bound CO₂ at end of reaction), and demineralized H₂O to total volume of 2.0 ml. The flasks were gassed with O₂-free tank N₂ and equilibrated to 30.4° C.

bNot determined.

No significant inhibition of formation of carbon dioxide from succinate and lactate occurs with semicarbazide present. Evolution of carbon dioxide from pyruvate is inhibited only partially by 0.01 molar semicarbazide. Separate experiments indicated that this concentration of semicarbazide has no effect on the formation of carbon dioxide from fumarate and malate at pH 5.2.
Fig. 8 Progress of formation of carbon dioxide from lactate, pyruvate and succinate by Propionibacterium pentosaceum.
Since semicarbazide reportedly (38) ties up oxalacetate and prevents its decarboxylation to pyruvate, it may be assumed that evolution from malate, fumarate and succinate in the presence of semicarbazide occurs via decarboxylation of succinate. Since approximately the same values are obtained with and without semicarbazide, it may be suggested further that under anaerobic conditions at pH 5.2, utilization of dicarboxylic acids tends to proceed toward succinate and its decarboxylation. The presence of suitable hydrogen donors and enzymes involved in the transfer of hydrogen probably stimulates the production of succinate.

Malonate (0.3 molar) was reported (38) to inhibit formation of carbon dioxide from succinate and to inhibit the formation of propionate from pyruvate to the same extent. These findings have been verified here. The effect of increasing concentrations of malonate on formation of carbon dioxide from succinate, pyruvate and lactate is shown in Table 5. The general occurrence of malonate inhibition of the formation of carbon dioxide from succinate and lactate is shown in the results in Table 6.

Malonate affects the formation of carbon dioxide from not only succinate but also from pyruvate and lactate. With lactate as the substrate, inhibition is just as intense as when succinate is the substrate. Only partial inhibition
of formation of carbon dioxide from pyruvate occurs. The effect of malonate on succinic decarboxylase has been verified by Sirotnek, Doltach, Robinson and Shaw (141). In their work, decarboxylation of succinate was inhibited 80-90 per cent by 0.3 molar malonate using rumen bacteria.

Table 5
Effect of malonate on formation of CO₂ from succinate, pyruvate and lactate by Propionibacterium pentosaceum

<table>
<thead>
<tr>
<th>Molarity of malonate</th>
<th>μMoles CO₂</th>
<th>Succinate</th>
<th>Pyruvate</th>
<th>Lactate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7.1</td>
<td>13.5</td>
<td>7.6</td>
<td></td>
</tr>
<tr>
<td>0.0003</td>
<td>6.7</td>
<td>12.3</td>
<td>7.3</td>
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<tr>
<td>0.003</td>
<td>6.0</td>
<td>11.3</td>
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<td></td>
</tr>
<tr>
<td>0.03</td>
<td>4.1</td>
<td>7.2</td>
<td>5.6</td>
<td></td>
</tr>
<tr>
<td>0.3</td>
<td>1.3</td>
<td>4.7</td>
<td>1.2</td>
<td></td>
</tr>
</tbody>
</table>

*Each flask contained 40 mg. (wet weight) of cells, 0.25 M phosphate (pH 5.2), 40 μMoles substrate, 0.2 ml. H₂SO₄ (stopping reagent), inhibitor solns, as indicated, demineralized H₂O to total volume of 2.0 ml. Flasks were gassed with O₂-free tank N₂ and equilibrated to 30.4°C.*
Table 6

Effect of 0.3 M malonate on formation of CO₂ from succinate and lactate by propionic acid bacteria

<table>
<thead>
<tr>
<th></th>
<th>Succinate</th>
<th>Malonate</th>
<th>Lactate</th>
<th>Malonate</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. pentosaceum</td>
<td>7.1</td>
<td>1.3</td>
<td>7.6</td>
<td>1.2</td>
</tr>
<tr>
<td>P. thoenii</td>
<td>4.0</td>
<td>0.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P. jensenii</td>
<td>5.3</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P. petersenii</td>
<td>4.8</td>
<td>2.4</td>
<td>5.8</td>
<td>0</td>
</tr>
<tr>
<td>F. zeae</td>
<td>3.2</td>
<td>0.8</td>
<td>4.5</td>
<td>0</td>
</tr>
<tr>
<td>F. freundreichii</td>
<td>5.0</td>
<td>2.8</td>
<td>4.9</td>
<td>1.1</td>
</tr>
<tr>
<td>F. shermanii</td>
<td>5.4</td>
<td>1.5</td>
<td>11.3</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Each flask contained 40 mg. (wet weight) of cells, 0.25 M phosphate (pH 5.2), 40 μMoles substrates, 0.2 ml. 6N H₂SO₄ (stopping reagent), inhibitor solution as indicated, demineralized H₂O to total volume of 2.0 ml. Flasks were gassed with O₂-free tank N₂ and equilibrated to 30.4°C.

Not determined.

The relatively high concentration of malonate required for inhibition suggested a salt effect. Accordingly, the effect of increasing concentrations of sodium chloride on decarboxylation activities was determined. Concentrations varying from zero to three molar failed to inhibit the activity in the slightest.
At pH 7.4, malonate acts as an inhibitor of succinic dehydrogenase and fumaric hydrogenase (100). At a low pH (5.2) it acts as an inhibitor of succinic decarboxylase. Since malonate functions as a competitive inhibitor for the dehydrogenase, it may function in the same manner with regard to succinic decarboxylase.

Failure of cells to exhibit full succinic decarboxylase activity suggested the possibility of the need for some growth factor in the synthesis of the enzyme. Addition of 0.005 molar magnesium chloride to the medium increased activity only slightly. Leviton and Hargrove (105) stated that strong aeration leads to destruction of preformed vitamin-active substances. Although strong aeration was not used here, the possibility of air affecting the synthesis prompted introduction of 0.01 molar l-cysteine, as a reducing agent, into the medium. No increased activity was noted as a result of this addition. Delwiche (39) and Chambers and Delwiche (28) demonstrated involvement of biotin in the functioning of succinic decarboxylase. In the present studies, growth of cells in biotin-deficient media resulted in reduced activity of succinic decarboxylase (a decrease of some 80 per cent), but addition of biotin to the manometric reaction mixtures failed to restore the activity to its original level. Whitely (179) found that
biotin is not concerned in the activity of succinic decarboxylase in cell-free extracts of Micrococcus lactilyticus. It may be that biotin is essential for the synthesis of the enzyme rather than for its function. Addition of biotin to the normal culture medium failed to raise the level of succinic decarboxylase in the cells. Whitely (179) included in the growth medium thiamine and unnamed salts. It may be that one of these is essential for the synthesis of succinic decarboxylase.

Treatment of cells following growth may influence enzyme activity. Cells were washed as a routine procedure in ice cold demineralized water in an iced Waring blender. Substitution of this method by one using agitation with a stirring rod and by a method of shaking cell suspensions in a stoppered vessel failed to improve activities. Addition of l-cysteine to the wash water increased activities only slightly.

The report of Whitely (179) clarified considerably the need for magnesium ions and l-cysteine in the preparation of an active succinic decarboxylase. The enzyme was studied in a cell-free extract of Micrococcus lactilyticus. An attempt was made here to secure a cell-free extract of Propionibacterium pentosaceum possessing an active succinic decarboxylase. Previous to the appearance of the Whitely re-
ports, cell-free extracts were prepared by sonic treatment of cells grown in glucose. The activity was very weak and may be explained partially by the cultural conditions in which the cells were grown. Another factor involved is the degree of disintegration of cells achieved by sonic treatment. More activity was found in the cell debris resulting from sonic disintegration than in the supernatant, suggesting either that the disintegration was only partially effective or that the enzyme is associated with particulate matter of the cell and therefore is sedimented from the treated suspension by centrifugation. Whitely (179) reported the enzyme to be soluble in nature. Subsequent studies with another enzyme system revealed a preparative technical error in sonic treatment which caused the original failures in preparation of a cell-free extract. Modifications in sonic treatment were applied to later cell-free preparations with greater success. Variable results were obtained using the alumina-grinding technic of McIlwain (110).

In later studies, the stock culture was carried in lactate medium, in contrast to earlier stock cultures which were carried in glucose. Growth for 36 hours in glucose after an extended period of cultivation in lactate apparently is insufficient to restore the activity of succinic dehydrogenase to the cells. The yield of cells from growth
in glucose is approximately the same as that in the previously reported growth experiments, but the level of succinic decarboxylase activity is as low as that in cells grown in lactate. No attempt was made to increase the level of activity of succinic decarboxylase by serial transfers in glucose. Cell-free extracts have the same low level of activity of the previously prepared extracts. Addition of the factors reported essential for decarboxylation of succinate by cell-free extracts of Micrococcus lactilyticus (179) failed to stimulate the extracts prepared from low-activity cells of Propionibacterium pentosaceum. As indicated by Whitely (179), fluoride is required to demonstrate the full activity of succinic decarboxylase since adenosine triphosphatase, present in the cell-free extracts, dephosphorylates adenosine triphosphate so that it cannot function in the phosphorylation of succinate, a step essential to subsequent decarboxylation. Conceivably, then, the results reported here may be due to the activity of the adenosine triphosphatase present in the extract.

Succinic Dehydrogenase and the Dicarboxylic Acid Cycle

The mode of utilization of succinate by the propionic acid bacteria apparently is pH-dependent. The occurrence
and optimum functioning of succinic decarboxylase at the lower pH levels suggested consideration of the mode of utilization of succinate at the higher pH levels.

Thunberg methylene blue technics were used in the study of dehydrogenase activities of the cells and extracts at various pH levels. In whole cells, the pH optimum for dehydrogenation occurs at pH 6.4. In cell-free extracts, prepared by sonic disruption of cells, it was found that the optimum occurs at pH 7.4. Malate may be dehydrogenated optimally at pH 7.4 using the extract and at pH 6.6 using whole cells. With fumarate and oxalacetate as substrates, the rates of reduction of methylene blue are much lower. In some instances, no reduction was observed over a period of hours.

It would seem that succinate is utilized by oxidative processes at the higher pH levels and by decarboxylation mechanisms at the lower pH levels.

Quastel and Whetham (127), using whole cells of Bacterium coli, found the pH optimum for succinic dehydrogenase to be 7.4. Fumarate was shown to inhibit the reduction of methylene blue by succinate. The reverse reaction,

leuco-Methylene blue + Fumarate = Methylene blue,

was shown to be operative. Succinate was not determined.

In the present report, similar evidence was obtained for the propionic acid bacteria. The functioning of an
enzyme system was verified by allowing a mixture of cell-
free extract, succinate and methylene blue to react to the
point of complete reduction of methylene blue, heating of
the mixture to 100° C, for ten minutes (gently releasing the
ground glass sidearm to equalize pressures during heating),
and addition of fumarate to the mixture after it had cooled
to test temperature. No restoration of the methylene blue
color was obtained. A like experiment without the heating of
the reaction mixture showed oxidation of leuco-methylene to
methylene blue. The two experiments indicate the transfer of
hydrogens from leuco-methylene blue to fumarate by an
enzyme system. Similarly, reduction of methylene blue by
succinate in the presence of heat-treated extract failed to
occur, indicating involvement of an enzyme system.

Dialysis of the cell-free extract against demineralized
water and phosphate buffer (0.1 molar, pH 5.4 and 7.4) does
not decrease the activity of succinic dehydrogenase signifi-
cantly. Succinic dehydrogenase of Veillonella gazogenes is
inhibited by 0.2 molar malonate (30), as shown in experi-
ments using whole cells at a pH of 7.0. No success in
securing a cell-free preparation was realized. It was found
here that succinic dehydrogenase in cell-free extracts of
Propionibacterium pentosaceum is inhibited strongly by 0.02
molar malonate. Fluoride does not inhibit the enzyme in
Bacterium coli (127). Similar results were obtained here using cell-free extracts of Propionibacterium pentosaceum.

The other dicarboxylic acids inhibit succinic dehydrogenase as follows:

Oxalacetate >> Fumarate > Malate.

Oxalacetate inhibits as strongly as malonate. Fumarate and malate have only a slight inhibitory effect on the enzyme.

Krebs and Eggleston (98) demonstrated that the propionic acid bacteria readily reduce oxalacetate to malate, fumarate and succinate at pH 7.0. Oxalacetate and fumarate were categorized as catalysts in the process. In the present study, whole cells of P. pentosaceum were shown to utilize lactate and pyruvate with the formation of considerable amounts of carbon dioxide over a broad range of pH. At pH 7.4, the dicarboxylic acids are utilized with the formation of only small amounts of carbon dioxide. Oxalacetate is decarboxylated rather rapidly, mainly by non-enzymatic mechanisms. Cell-free extracts behave in much the same way as whole cells with dicarboxylic acids as substrates. Since the amount of carbon dioxide formed is so small, it was felt that possibly the equilibrium among the dicarboxylic acids is functional but not detectable by the usual manometric procedures. Accordingly, the effect of cell-free extracts on the various dicarboxylic acids was de-
terminated chromatographically. Results are shown in Table 7. Since the dehydrogenases were shown to function optimally at pH 7.4, the reactions were carried out at that pH. The results indicate the occurrence of the equilibrium system in the cell-free extract. Lactate was included as a possible hydrogen source which might further the reduction of oxalacetate and fumarate to succinate.

With regard to utilization of oxalacetate, Krebs (95) proposed the occurrence and functioning of the equilibrium between dicarboxylic acids in *E. coli*. In the system, half of the oxalacetate is transformed into succinate and the other half into various oxidized products. The amount of carbon dioxide formed in the studies reported here would indicate that the reaction does not proceed to the same extent in cell-free extracts. However, the formation of succinate would seem to indicate the reduction of some of the oxalacetate to succinate and formation of pyruvate which may be reduced to lactate. Formation of succinate is dependent on the presence of a suitable donor of hydrogen, as evidenced by formation of succinate from oxalacetate only in the presence of lactate. On the basis of the small amounts of carbon dioxide and other dicarboxylic acids formed, it may be that the equilibrium does not function optimally at higher pH levels in the direction of
### Table 7

**Formation of organic acids by cell-free extracts of Propionibacterium pentosaceum**

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Carbon dioxide (µmoles)</th>
<th>Acids detected chromatographically</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate</td>
<td>0</td>
<td>Lactate, Fumarate and Malate (traces)</td>
</tr>
<tr>
<td>Lactate</td>
<td></td>
<td>Pyruvate, Succinate, Lactate</td>
</tr>
<tr>
<td>Oxalacetate</td>
<td>1.1</td>
<td>Pyruvate, Malate</td>
</tr>
<tr>
<td>Oxalacetate</td>
<td>2.0</td>
<td>Pyruvate, Lactate, Malate, Succinate</td>
</tr>
<tr>
<td>Lactate</td>
<td>2.5</td>
<td>Pyruvate, Malate, Succinate</td>
</tr>
<tr>
<td>Fumarate</td>
<td>2.0</td>
<td>Malate and Fumarate</td>
</tr>
<tr>
<td>Lactate</td>
<td>2.2</td>
<td>Pyruvate, Lactate, Malate, Succinate</td>
</tr>
<tr>
<td>Malate</td>
<td>3.3</td>
<td>Pyruvate, Malate, Fumarate, Succinate</td>
</tr>
</tbody>
</table>

*Each flask contained cell-free extract (0.6 mg. protein), 40 µMoles substrate, 0.25 M (pH 7.4), 0.2 ml. 6N H₂SO₄ (added at end of reaction to release bound CO₂), and demineralized H₂O to total volume of 2.0 ml. Flasks were gassed with O₂-free tank N₂ and equilibrated to 30.4°C.*
succinate. Need for suitable donors of hydrogen (or acceptors) to facilitate formation of the dicarboxylic acids is indicated.

A more detailed study of the reaction between oxalacetate and lactate was made. Results are shown in Table 8. It would seem that lactate retards formation of carbon dioxide from oxalacetate, possibly by reduction of oxalacetate to malate. The levels of pyruvate formed in the presence of lactate are far in excess of those formed in the absence of lactate, indicating that not only oxalacetate but also lactate is utilized with the formation of pyruvate. Lactate alone is not utilized with the formation of pyruvate.

In summary, the propionic acid bacteria exhibit various enzymatic properties which are dependent upon the conditions of growth and cultivation. Cells grown in glucose medium contain an active succinic dehydrogenase, succinimide, and other enzymes. By contrast, the dehydrogenase operates optimally at pH 5.2, whereas the dehydrogenase functions optimally at pH 7.4. The presence of an enzyme system mediating the equilibrium between the dicarboxylic acids is indicated. Cells grown in lactate medium exhibit low activities of succinic dehydrogenase but the same level of activity of the dehydrogenases as in glucose-grown cells. Cell-free extracts of both lactate- and glucose-grown cells
show low succinic decarboxylase activities but succinic dehydrogenase and the enzymes responsible for the equilibrium between dicarboxylic acids are readily detected.

Table 8

Utilization of oxalacetate and lactate by cell-free extracts of *Propionibacterium pentosaceum*

<table>
<thead>
<tr>
<th>Substrates</th>
<th>CO$_2^b$</th>
<th>Lactate utilized$^b$</th>
<th>Pyruvate$^b$</th>
<th>Succinate$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate</td>
<td>0</td>
<td>0.1</td>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td>Oxalacetate</td>
<td>7.1</td>
<td>- c</td>
<td>7.3</td>
<td>0.1</td>
</tr>
<tr>
<td>Lactate Oxalacetate</td>
<td>15.4</td>
<td>15.7</td>
<td>33.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Lactate Oxalacetate (Control)</td>
<td>7.7</td>
<td>0</td>
<td>7.6</td>
<td>0</td>
</tr>
</tbody>
</table>

$^a$Each flask contained one ml. cell-free extract (0.6 mg. protein), 20 μl substrate, 0.25 M (pH 7.4), 0.2 ml. 6N H$_2$SO$_4$ (added at end of reaction to release bound CO$_2$), and demineralized H$_2$O to total volume of 2.0 ml. Flasks were gassed with O$_2$-free tank N$_2$, and equilibrated to 30.4° C.

$^b$Values shown are in terms of μMoles.

$^c$Not determined.
The relatively low activity of succinic decarboxylase in cells grown in lactate is of particular interest since the enzyme reportedly is the main pathway to the formation of propionate, one of the products of the propionic acid fermentation. The mode of formation of propionate from lactate therefore becomes important since cells grown in lactate are capable of carrying out the normal propionic fermentation of lactate.

Utilization of Lactate and Pyruvate

Cells grown on lactate utilize lactate with the formation of typical products of the propionic acid fermentation without significant participation of succinic decarboxylase. Investigation of the dissimilation of lactate was carried out to elucidate the mechanisms involved. The structural similarity of lactate to pyruvate warranted parallel studies of the two compounds.

Effect of pH on formation of carbon dioxide from lactate and pyruvate

A pH range of 5.0-7.8 was selected. Results are shown in Fig. 9 and Table 9. On the basis of rate of formation of carbon dioxide, the pH optimum with lactate as substrate
Table 9

pH Optima for formation of CO₂ from pyruvate and lactate

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Maximum rate</th>
<th>Total CO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate</td>
<td>6.5</td>
<td>5.2</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>5.2</td>
<td>5.2</td>
</tr>
</tbody>
</table>

*Each flask contained 0.1 M phosphate (appropriate pH), 87 mg. cells (wet weight), 96 μMoles substrate, 2 ml. 3N H₂SO₄ (added at appropriate time to stop reaction and release bound CO₂), demineralized H₂O to total volume of 30.0 ml. Flasks were gassed with O₂-free tank N₂ and equilibrated to 30.4° C.*

is 6.4, whereas with pyruvate as substrate the pH optimum is 5.2. Barron and Miller (11), in studies using gonococci, found pH optimum differences in the aerobic utilization of pyruvate and lactate. For pyruvate the optimum was found to be 7.0 in contrast to 6.7 for lactate. On the basis of total formation of carbon dioxide, the pH optimum found here is 5.2 in both instances. These findings suggest involvement of a rate-controlling reaction which functions optimally at pH 6.4 (whole cells) in the case of lactate and which is not involved in the utilization of pyruvate.
Fig. 9 Formation of carbon dioxide from pyruvate, lactate and succinate as influenced by pH.
The effect of pH on relative amounts of carbon dioxide produced and the amount of substrate utilized was investigated. Three distinct pH levels (5.2, 6.4, 7.5) were selected for study. On the basis of separate experiments, concentrations of buffer were used so that the final pH remained the same as the initial pH. Tests for residual lactate indicated the absence of lactate in dissimilations of both pyruvate and lactate. Results are shown in Table 10.

Presence of residual pyruvate indicates that pyruvate is utilized less completely than lactate and that pyruvate occurs as an intermediate in the utilization of lactate. Volatile acids are formed in the dissimilation of both pyruvate and lactate.

Formation of carbon dioxide from lactate was investigated at pH 6.4, the pH optimum for maximum rate of formation. In anaerobic utilization of lactate by dried cells of Propionibacterium pentosaceum, one mole of carbon dioxide is evolved for every three moles of lactate utilized. Under similar experimental conditions, two moles of carbon dioxide are formed from every three moles of pyruvate utilized. Using relatively large amounts of cells (50 mg.), comparison of formation of carbon dioxide from lactate and pyruvate was made. Results are shown in Fig. 10. No tests for residual pyruvate were run. Starting amounts of substrate were 40 micromoles.
Table 10

Effect of pH on utilization of lactate and pyruvate

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>CO₂</th>
<th>Pyruvate</th>
<th>Vol. acids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Start</td>
<td>Final</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactate</td>
<td>5.20</td>
<td>5.24</td>
<td>29.2</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>6.51</td>
<td>6.49</td>
<td>26.8</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>7.53</td>
<td>7.48</td>
<td>21.6</td>
<td>9.1</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>5.20</td>
<td>5.21</td>
<td>50.3</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>6.51</td>
<td>6.53</td>
<td>48.1</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>7.53</td>
<td>7.51</td>
<td>46.1</td>
<td>7.1</td>
</tr>
</tbody>
</table>

*a Each flask contained 0.1 M phosphate (appropriate pH), 37 mg. cells (wet weight), 98 μMoles substrate, 2 ml. 3N H₂SO₄ (added at end of reaction to release bound CO₂), demineralized H₂O to total volume of 30.0 ml. Each flask was gassed with O₂-free tank N₂ and equilibrated to 30.4°C.

*b In terms of μMoles

*c In terms of μequivalents

A lag period is indicated in the curve for formation of carbon dioxide from lactate, suggesting a preliminary modification of the lactate molecule before decarboxylation. Preliminary dehydrogenation of the molecule is indicated by the change in carbon dioxide-to-oxygen ratios with progress of the reaction under aerobic conditions (Fig. 11). With lactate as the substrate, the ratio is very small, suggest-
Fig. 10 Progress of formation of carbon dioxide from pyruvate and lactate.
Fig. 11 Progress of carbon dioxide-to-oxygen ratios in the utilization of lactate and pyruvate.
ing oxidation of the molecule before decarboxylation. The ratio increases with time, suggesting subsequent increased formation of carbon dioxide and decreased uptake of oxygen.

With pyruvate as the substrate, formation of carbon dioxide is almost instantaneous and little or no uptake of oxygen is apparent at first. Subsequent uptake of oxygen decreases the ratio, suggesting the occurrence of a secondary reaction involving oxidation of a product resulting from utilization of pyruvate. Krebs (94) pointed out that the primary dismutation of pyruvate aerobically shows an induction period, probably because the pyruvate is reduced to lactate and then the latter is oxidized.

The concept of enzyme systems "wearing out" or being inhibited by the products of their metabolism was tested by successive additions of substrate to the reaction mixture. Results are shown in Table 11. Where two additions of substrate were made, the second was not added until evolution of carbon dioxide from the first addition had ceased. In this experiment, only approximately 2.5 micromoles of carbon dioxide were formed from every ten micromoles of lactate added. This value is different from the usual 3.3 micromoles formed. The difference may be due to deterioration of the dried cells, which at the time of use were relatively old. Freshly prepared dried cells showed the
Table 11

Effect of successive additions of lactate on formation of CO₂ by Propionibacterium pentosaceum

<table>
<thead>
<tr>
<th>Lactate added (μmoles)</th>
<th>Total CO₂ (μmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>First addition</td>
<td>Second addition</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>

Each flask contained 1 ml. 0.1 M phosphate, (pH 6.4), substrate as indicated, 4 mg. cells (dry weight), 0.2 ml. 6N H₂SO₄ (added at appropriate time to release bound CO₂) and demineralized H₂O to a total of 2.3 ml. Flasks were gassed with O₂-free tank N₂ and equilibrated to 30.4°C.

same additive pattern of formation of carbon dioxide but each addition yielded the full 3.3 micromoles of carbon dioxide. This finding again points up the one of many possible sources of available results reported in the references concerned with the propionic acid fermentation.

The effect of concentration of substrate on formation of carbon dioxide was determined. The rates of evolution were approximately the same. Only the total values differed. Results are shown in Table 12.
Table 12

Effect of various concentrations of lactate on formation of CO$_2$ by *Propionibacterium pentosaceum*.

<table>
<thead>
<tr>
<th>Lactate (µmoles)</th>
<th>Total CO$_2$ (µmoles)</th>
<th>Rate of formation (µlites/50 min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>3.7</td>
<td>64</td>
</tr>
<tr>
<td>20</td>
<td>7.1</td>
<td>77</td>
</tr>
<tr>
<td>40</td>
<td>16.7</td>
<td>83</td>
</tr>
<tr>
<td>60</td>
<td>24.7</td>
<td>96</td>
</tr>
<tr>
<td>80</td>
<td>30.0</td>
<td>77</td>
</tr>
<tr>
<td>100</td>
<td>35.5</td>
<td>77</td>
</tr>
<tr>
<td>5</td>
<td>1.8</td>
<td>32</td>
</tr>
<tr>
<td>2.5</td>
<td>1.0</td>
<td>15</td>
</tr>
<tr>
<td>2.0</td>
<td>1.0</td>
<td>15</td>
</tr>
</tbody>
</table>

*Each flask contained 4 mg. (dry weight) of cells, 0.1 M phosphate (pH 6.4), substrate as indicated, 0.2 ml. 6N H$_2$SO$_4$ (added at end of reaction to release bound CO$_2$), and demineralized H$_2$O to total volume of 2.3 ml. Flasks were flushed with O$_2$-free tank N$_2$ and equilibrated to 30.4°C.*

Formation of carbon dioxide was affected by the use of veronal as a buffer instead of phosphate. Veronal decreases the rate of evolution of carbon dioxide from lactate but the total amount of carbon dioxide formed was greater in veronal than in phosphate (Table 13). A typical sigmoid curve for formation of carbon dioxide was exhibited in phosphate. In veronal, the plot of formation of carbon dioxide is a straight line. In either buffer, pyruvate occurs as an intermediate.
Table 13
Effect of phosphate and veronal on formation of CO₂ from lactate by Propionibacterium pentosaceum

<table>
<thead>
<tr>
<th></th>
<th>Liters CO₂</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>First 60 minutes</td>
<td>Total</td>
</tr>
<tr>
<td>Phosphate</td>
<td>121</td>
<td>270</td>
</tr>
<tr>
<td>Veronal</td>
<td>49</td>
<td>291</td>
</tr>
</tbody>
</table>

Each flask contained 4 mg. (dry weight) of cells, 1 ml. buffer as indicated, 40 μMoles substrate, 0.2 ml. 6N H₂SO₄ (added at end of reaction to release bound CO₂), and demineralized H₂O to total volume of 2.3 ml. Flasks were flushed with O₂-free tank N₂ and equilibrated to 30.4°C.

The formation of carbon dioxide from pyruvate is the same in either buffer.

The total amount of carbon dioxide formed from pyruvate under aerobic conditions is approximately the same, as was reported by Fromageot and Chaix (63). Utilization of lactate under aerobic conditions yields carbon dioxide in an amount twice that produced under anaerobic conditions and approximately equivalent to that produced from pyruvate under anaerobic conditions. Cook and Stephenson (35) in studies with Bacterium coli and E. alkaligenes demonstrated that
complete aerobic utilization of lactate requires about two-thirds the amount of oxygen required for complete oxidation, suggesting formation of incompletely oxidized substances. The pH optimum was found to be between 6 and 7. In studies on the progress of dissimilation of lactate and pyruvate, the instantaneous and rapid uptake of oxygen by cells in the presence of lactate was very evident (Fig. 12). The only apparent difference in utilization of pyruvate and lactate seems to be in the preliminary modification of the lactate molecule before formation of carbon dioxide. As is indicated in Fig. 12, the preliminary modification is one of oxidation of the lactate molecule or some related reaction. The presence of two hydrogens on lactate and not on pyruvate also modifies the ratios of products formed from pyruvate in contrast to lactate.

Using a small quantity of cells, the lag in formation of carbon dioxide from lactate becomes apparent. Investigation of the dissimilation of lactate and pyruvate at pH 6.4 was carried out using time study analyses of duplicate reaction mixtures. It was noted that formation of carbon dioxide from pyruvate occurs at a lower rate at pH 6.4 than at pH 5.2, the optimum pH. Results of experiments using 40 mg. (dry weight) of cells are shown in Fig. 13. During the lag period in formation of carbon dioxide (period (a)),
Fig. 12 Progress of uptake of oxygen by *Propionibacterium pentosaceum* with lactate and pyruvate as substrates.
Fig. 13 Formation of carbon dioxide from pyruvate and lactate by Propionibacterium pentosaceum.
traces of pyruvate and propionate are formed. In the period
of maximum rate of formation of carbon dioxide ((b) in Fig.
13), all compounds tested for were detected. An unidentified
ketocompound in trace amounts was detected during this
period. Its phenylhydrazone has an Rf value of approximately
0.95. The report by Carson, Kuna, Bachmann and Long (19)
of the finding of an unidentified intermediate between
pyruvate and propionate is of interest in this respect.
Following formation of carbon dioxide, only propionate and
acetate are detectable. The latter finding contrasts with
that reported earlier. In the present instance, only 40 mg.
of dried cells and 20 micromoles of substrate were utilized
whereas, in the earlier work, 96 micromoles of substrate
and 87 mg. of fresh whole cells were used. Substrate utili-
zation was incomplete, as shown by residual pyruvate and by
carbon balances. In the experiments reported here, forma-
tion of carbon dioxide is approximately 100 per cent of
theoretical, suggesting complete utilization of substrate.

The occurrence of pyruvate and propionate before sig-
nificant amounts of carbon dioxide are formed (Table 14)
suggests occurrence of a reaction (not decarboxylation)
yielding pyruvate and propionate, perhaps a dismutation.
Active participation and possible precursor role of pyruvate
in the formation of carbon dioxide is indicated by the
### Table 14

Compounds formed in the dissimilation of pyruvate and lactate by *Propionibacterium pentosaceum*

<table>
<thead>
<tr>
<th></th>
<th>Lactate</th>
<th></th>
<th>Pyruvate</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a</td>
<td>b</td>
<td>c</td>
<td>a</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>+b</td>
<td>+c</td>
<td>-d</td>
<td>+</td>
</tr>
<tr>
<td>Lactate</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>*</td>
</tr>
<tr>
<td>Propionate</td>
<td>*</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Acetate</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Carbon dioxide</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

*a* Each flask contained 40 mg. (dry weight) of cells, 20 μMoles substrate, 0.1 molar phosphate (pH 6.4), 0.2 ml. 3N H₂SO₄ (added at appropriate time to stop reaction and release bound CO₂), and demineralized H₂O to total of 2.3 ml. Flasks were flushed with O₂-free tank N₂ and equilibrated to 30.4°C. Samples were deproteinized, made up to appropriate volume and aliquots taken for analyses by chromatography.

*b* Trace

*c* Present

*d* Absent
simultaneous absence of carbon dioxide.

In connection with the occurrence of propionate without formation of carbon dioxide, the report of Delwiche (38) is of interest. At pH 6.76, an aliquot of a glucose fermentation mediated by Propionibacterium pentosaceum showed the occurrence of appreciable amounts of propionate. Succinic decarboxylase, when present, functions only to a slight degree at this pH and it may be assumed that a second reaction leading to the formation of propionate occurs.

With pyruvate as substrate (Fig. 13), formation of carbon dioxide is almost instantaneous. Within the period of time corresponding to the first phase of utilization of lactate, all compounds tested for, except propionate, were detected. All compounds were present in period (b) and, following termination of formation of carbon dioxide (period (c)), only propionate and lactate were found (Table 14). The almost similar maximum rates of formation of carbon dioxide from pyruvate and lactate suggest a common pathway, perhaps lactate yielding pyruvate which is then utilized in the same fashion as added pyruvate. However, the pyruvate formed from lactate must be approximately one-half that of the added pyruvate (as shown by formation of carbon dioxide), even though equivalents of lactate and pyruvate were added initially. These findings suggest, again, one-half of lactate
being utilized in a reaction not immediately involving de-
carboxylation.

The determinations used in these experiments, with the
exception of that for carbon dioxide, were qualitative and
therefore no stoichiometry can be established. The final
products are as indicated.

Absence of significant activity of succinic decarboxy-
lase, and occurrence of propionate prior to the formation of
carbon dioxide from lactate suggest a dismutation of lactate.

A further contrast between the utilization of pyruvate
and lactate is provided in the information obtained from
manometric studies of the utilization of lactate and pyru-
vate in the presence of methylene blue. During the period
in which no carbon dioxide is formed from lactate, one ml.
of a 1:10,000 solution of methylene blue is reduced com-
pletely, indicating the functioning of an enzyme system
catalyzing transfer of hydrogen from lactate. A similar
study with pyruvate as substrate showed no reduction of
methylene blue until later in the phase of formation of
carbon dioxide at which time there is only partial reduction
of methylene blue. These findings agree with the data ob-
tained from aerobic studies on lactate and pyruvate dissimi-
lation (Fig. 11 and 12).
Effect of fluoride on the utilization of lactate and pyruvate

Barker and Lipmann (6) proposed a dismutation of lactate. That portion of the reaction involved in reduction of lactate to propionate was proposed as one sensitive to fluoride. But conceivably dismutation is not the only reaction to which lactate is subject. Lactate may be oxidized by lactic dehydrogenase with the formation of pyruvate. A suitable substrate hydrogen acceptor must be present for the reaction to proceed. It is conceivable that in washed whole cells such acceptors are present but only in trace amounts. Oxidation could not proceed without large amounts of these acceptors unless trace amounts are involved in a cycle regenerating the original acceptor.

The sensitivity to fluoride of the enzyme system involved in utilization of lactate was investigated to determine the actual area of sensitivity in the reaction sequence.

Formation of carbon dioxide from lactate and pyruvate is affected by increasing concentrations of fluoride, the lactate system being more sensitive (Table 15). The results obtained here agree with those of Barker and Lipmann (6). Not only are the rates of formation of carbon dioxide affected but also the total values. Rate studies indicate that formation of carbon dioxide from pyruvate is affected only
Table 15
Effect of sodium fluoride on rate of formation of CO₂ from lactate and pyruvate

<table>
<thead>
<tr>
<th>NaF (Molarity)</th>
<th>Pyruvate Liters CO₂/20 min.</th>
<th>Lactate Liters CO₂/20 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>290</td>
<td>125</td>
</tr>
<tr>
<td>1.25 x 10⁻³</td>
<td>260</td>
<td>120</td>
</tr>
<tr>
<td>2.5 x 10⁻³</td>
<td>230</td>
<td>110</td>
</tr>
<tr>
<td>3.15 x 10⁻³</td>
<td>15</td>
<td>90</td>
</tr>
<tr>
<td>5.0 x 10⁻³</td>
<td>-</td>
<td>35</td>
</tr>
<tr>
<td>6.3 x 10⁻³</td>
<td>190</td>
<td>5</td>
</tr>
<tr>
<td>1.25 x 10⁻²</td>
<td>175</td>
<td>-</td>
</tr>
<tr>
<td>2.5 x 10⁻²</td>
<td>160</td>
<td>-</td>
</tr>
<tr>
<td>5.0 x 10⁻²</td>
<td>128</td>
<td>-</td>
</tr>
<tr>
<td>1.0 x 10⁻¹</td>
<td>85</td>
<td>-</td>
</tr>
<tr>
<td>5.0 x 10⁻¹</td>
<td>12</td>
<td>-</td>
</tr>
</tbody>
</table>

*Each flask contained 50 mg. dried cells, 0.05 M phosphate (pH 6.4), 40 μMoles substrate, inhibitor as indicated, 0.2 ml. 6N H₂SO₄ (to stop reaction and release bound CO₂), demineralized H₂O to total volume of 2.3 ml. Flasks were flushed with O₂-free tank N₂ and equilibrated to 30.4°C.

*Not determined

partially by concentrations of fluoride which completely inhibit formation of carbon dioxide from lactate, the effect being manifested in a slight decrease in the rate of evolution and in a slight shortening of the period of maximum formation. With lactate as substrate, relatively small
quantities of fluoride ($1.25 \times 10^{-2}$ molar) are required for complete inhibition. With increasing concentrations of fluoride, the lag period in formation of carbon dioxide noted in the normal reaction is increased progressively (Fig. 14), and the period of maximum formation of carbon dioxide is decreased markedly. In greater concentrations of fluoride, the rate of evolution is decreased. The extension of the lag period in the formation of carbon dioxide due to the action of fluoride is not observed in the fluoride effect on carbon dioxide formation from pyruvate. The increased lag period suggests inhibition of a reaction preceding formation of pyruvate since pyruvate is utilized fairly readily in concentrations of fluoride which cause the lag with lactate as substrate.

The effect of fluoride on formation of products with pyruvate as substrate was investigated by Barker and Lipmann (6) with the following results:

<table>
<thead>
<tr>
<th>Moles/100 Moles pyruvate fermented</th>
<th>No NaF</th>
<th>0.025 M NaF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propionate</td>
<td>31</td>
<td>52</td>
</tr>
<tr>
<td>Acetate</td>
<td>54</td>
<td>47</td>
</tr>
<tr>
<td>Carbon dioxide</td>
<td>60</td>
<td>63</td>
</tr>
</tbody>
</table>

Apparently the concentration of fluoride which completely inhibits formation of carbon dioxide from lactate shunts hydrogens into a fluoride-insensitive system leading to the
Fig. 14 Effect of increasing concentrations of fluoride on formation of carbon dioxide from pyruvate and lactate.
formation of propionate.

In the studies reported here, it was found that, in the presence of 0.02 molar fluoride, pyruvate is detectable throughout the entire period of time required for complete utilization of an equivalent amount of lactate in the absence of fluoride, even in the period after completion of formation of carbon dioxide. It may be concluded that part of the enzyme system concerned with utilization of pyruvate is affected by fluoride.

Warburg and Christian (166) presented evidence to show that fluoride inhibits enolase by forming a protein-magnesium-fluorophosphate complex. It was felt that possible such a complex is formed in the present inhibition studies and that perhaps an excess of the inorganic ion might antagonize the inhibition produced by fluoride. Addition of quantities of manganese and magnesium in excess of the concentration of fluoride failed to remove the inhibition due to fluoride with lactate as substrate.

Growth of Propionibacterium pentosaceum in lactate medium containing 0.02 molar fluoride yields cells which show no change in pattern of utilization of pyruvate and lactate in the presence of fluoride (Table 16). These results agree with those of Volk (164).

The effect of other inhibitors on the anaerobic utilization of lactate and pyruvate was studied. Results are shown
Table 16
Effect of growth in fluoride on utilization of lactate and pyruvate by Propionibacterium pentosaceum

<table>
<thead>
<tr>
<th></th>
<th>Aerobic</th>
<th></th>
<th>Anaerobic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No NaF</td>
<td>0.02 M NaF</td>
<td>No NaF</td>
</tr>
<tr>
<td></td>
<td>O₂</td>
<td>CO₂ b</td>
<td>O₂</td>
</tr>
<tr>
<td>Lactate</td>
<td>29.3</td>
<td>24.7</td>
<td>22.3</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>7.0</td>
<td>16.4</td>
<td>4.8</td>
</tr>
</tbody>
</table>

a Each flask contained 40 mg. dried cells, 0.05 M phosphate (pH 6.4), 40 μMoles substrate, inhibitor concentration as shown, 0.2 ml. 6N H₂SO₄ (added at end of reaction to release bound CO₂), and demineralized H₂O to total of 2.3 ml. Gaseous environment achieved by methods described in "Manometric Techniques" (159).

b In terms of μMoles

in Table 17. Cyanide is utilized not only as an inhibitor of respiration but also as a keto-fixative. Green, Loomis and Auerbach (71) reported that oxalacetate reacts almost instantaneously with cyanide to form the cyanohydrin. Anaerobically, it does not affect utilization of either pyruvate or lactate. The concentration of fluoride which inhibits utilization of lactate almost completely decreases the formation of carbon dioxide from pyruvate by approximately...
Effect of inhibitors on formation of CO₂ from lactate and pyruvate by *Propionibacterium pentosaceum*\(^a\)

<table>
<thead>
<tr>
<th>Additions</th>
<th>µMoles CO₂</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lactate</td>
<td>Pyruvate</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>12.5</td>
<td>25.6</td>
<td></td>
</tr>
<tr>
<td>KCN</td>
<td>13.5</td>
<td>25.1</td>
<td></td>
</tr>
<tr>
<td>NaF</td>
<td>1.0</td>
<td>13.2</td>
<td></td>
</tr>
<tr>
<td>Semicarbazide</td>
<td>11.2</td>
<td>4.9</td>
<td></td>
</tr>
<tr>
<td>Bisulfite</td>
<td>0</td>
<td>14.4</td>
<td></td>
</tr>
</tbody>
</table>

\(\text{a}\) Each flask contained 40 µMoles substrate, 0.05 M phosphate (pH 6.4), 40 mg. dried cells, inhibitors as indicated, 0.2 ml. 6N H₂SO₄ (added at end of reaction to release bound CO₂), and demineralized H₂O to total volume of 2.3 ml. Flasks were flushed with O₂-free tank N₂ and equilibrated to 30.4°C.

\(\text{b}\) 40 µMoles of each.

Semicarbazide, another keto-fixative, affects utilization of lactate only slightly whereas utilization of pyruvate is decreased by approximately 50 per cent. Bisulfite completely inhibits utilization of lactate whereas utilization of pyruvate is decreased by approximately 50 per cent. Methylene blue (separate experiments) inhibits slightly the anaerobic formation of carbon dioxide from lactate.
With respect to the lag period in carbon dioxide formation and the effect of fluoride on utilization of lactate, the results in Table 13 were obtained. During the lag period in the formation of carbon dioxide, no gas is evolved yet pyruvate occurs in the reaction. This finding indicates the role of pyruvate as an intermediate in the formation of carbon dioxide from lactate. Fluoride not only inhibits formation of carbon dioxide but also formation of pyruvate.

Carbon dioxide is formed in the dissimilation of lactate in the presence of 0.02 molar fluoride, as reported earlier. The reaction was conducted simultaneously with an uninhibited reaction, the latter serving as an index of completeness of reaction (formation of carbon dioxide). During the first one-third (60 minutes) of the reaction period, no carbon dioxide was formed in the inhibited reaction but a gradual increase in formation of gas was noted after this time. Pyruvate was detected in this reaction and points up the close relationship between pyruvate and formation of carbon dioxide.

Reduction of methylene blue in the presence of fluoride by dried cells acting on lactate suggests operation of a dehydrogenase which is fluoride-insensitive. Failure to detect pyruvate in the reaction mixture weighed against the conclusion that the dehydrogenase is lactic dehydrogenase
Table 18

Effect of fluoride on dissimilation of lactate
by Propionibacterium pentosaceum

<table>
<thead>
<tr>
<th>Lactate</th>
<th>Fluoride</th>
<th>Lactate utilized</th>
<th>CO₂</th>
<th>Pyruvate</th>
<th>Reaction time</th>
</tr>
</thead>
<tbody>
<tr>
<td>_</td>
<td>+</td>
<td>0.3</td>
<td>0.1</td>
<td>-</td>
<td>37 minutes</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>-</td>
<td>20.7</td>
<td>5.3</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>20.7</td>
<td>-</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>4 &quot;</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>3.3</td>
<td>0</td>
<td>-</td>
<td>4 &quot;</td>
</tr>
</tbody>
</table>

Each flask contained 0.05 M phosphate (pH 6.4), 40 mg dried cells, approximately 20 μMoles substrate (as indicated), 0.02 M fluoride (as indicated), 0.2 ml 5N H₂SO₄ (to stop reaction and release bound CO₂), demineralized H₂O to total volume of 2.3 ml. Flasks were flushed with C₂-free tank N₂ and equilibrated to 30.4°C.

bAbsent  
cPresent

Specifically since a dehydrogenase acting on decomposition products of pyruvate utilization might be responsible for reduction of methylene blue (206). Accordingly, a detailed study of lactic dehydrogenase in cell-free extracts was undertaken to determine its sensitivity to fluoride and other properties.
Lactic Dehydrogenase

pH optimum

The pH optimum using whole cells and cell-free extracts was determined by the Thunberg methylene blue technic. Results are shown in Fig. 15. The pH optimum for dried cells agrees with that reported by Phelps, Johnson and Peterson (120) for whole cells. Naked eye observation of temperature-equilibrated Thunberg tubes was used in these experiments and in the experiments with cell-free extracts. Cell-free extracts were prepared by sonic disruption. The pH optimum for dehydrogenation of lactate by cell-free extracts seemed to occur at pH 7.4.

A complicating factor in experiments with intact bacterial cells is their varying degree of permeability for substrate; it may be favored by a pH value different from the optimum for the enzyme inside the cell, and a pH-activity curve obtained under these circumstances would not represent the in vitro properties of the isolated enzyme. There is ample evidence that bacterial cells can maintain a hydrogen ion concentration in their protoplasm which is quite different from that of the medium. It is obvious then that statements about the pH optimum for activity of a system should include details of the experimental conditions. (132, p. 132)

A more precise determination of the pH optimum using cell-free extracts was conducted using the Evelyn colorimeter for measuring methylene blue concentrations. Results
Fig. 15 Effect of pH on reduction of methylene blue by whole cells and cell-free extracts with lactate as substrate.
are shown in Table 19. The pH optimum for reduction of methylene blue using the overall 90 per cent reduction as an index occurs at approximately 7.5. Using the maximum rate of reduction as a measure, the pH optimum occurs at pH 5.75. The confusion arises out of the occurrence of initial lag periods during which reduction of methylene blue is not detectable. Yudkin (206) itemized the factors which may influence reduction of methylene blue. The factors which might increase reduction time are:
(a) temperature (no equilibration of reaction mixture)
(b) residual oxygen in tube
(c) gradual poisoning of enzyme by methylene blue
(d) affinity of enzyme for methylene blue

All these factors influence reduction of methylene blue within a given system and do not affect the systems merely on the basis of pH differences. Uniformity of handling of each of the tubes and mixtures eliminates these variables. It would seem then that the results obtained are an expression of a pH effect.

Effect of concentration of lactate on reduction of methylene blue

The effect of concentrations of substrate on rates of reduction of methylene blue was studied. Increasing concentrations of lactate decrease the lag period at pH 5.6.
Table 19

Effect of pH on rates of reduction of methylene blue by cell-free extracts of *Propionibacterium pentosaceum*.

<table>
<thead>
<tr>
<th>pH</th>
<th>Time (in seconds) for reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0-10 (per cent)</td>
</tr>
<tr>
<td>5.2</td>
<td>280</td>
</tr>
<tr>
<td>5.5</td>
<td>250</td>
</tr>
<tr>
<td>5.75</td>
<td>165</td>
</tr>
<tr>
<td>6.2</td>
<td>130</td>
</tr>
<tr>
<td>6.7</td>
<td>103</td>
</tr>
<tr>
<td>7.2</td>
<td>78</td>
</tr>
<tr>
<td>7.55</td>
<td>72</td>
</tr>
<tr>
<td>7.9</td>
<td>70</td>
</tr>
</tbody>
</table>

Each tube contained 0.2 ml. cell-free extract, 40 \( \mu \)moles lactate, 1 ml. of 1:10,000 methylene blue, 4 ml. 0.1 M phosphate (appropriate pH), and demineralized H\(_2\)O to total volume of 6 ml. Each tube evacuated for 10 minutes using aspirator and equilibrated to 30° C.

(See Fig. 16), whereas no effect is observed at pH 7.4. Saturation of the enzyme at 40 micromoles is apparent if we consider only the period of active reduction (10-90 per cent). A greater concentration of lactate is required to bring the reduction rate in the lag period (0-10 per cent) to the same level as that in the 10-90 per cent period. This finding suggests the existence of a competing reaction which "steals"
Fig. 16: Effect of increasing concentration of SRI on reduction of methylene blue.

REDUCTION TIME IN SECONDS
0 40 80 120 140 200

LAG (0-10 %)
REDUCTION (10-90 %)

LI LACTATE (µ MOLES)
0 20 40 60 80

280
lactate from the dehydrogenase and which produces a substance, the utilization of which subsequently may lead to reduction of methylene blue.

**Effect of pyruvate on reduction of methylene blue by lactate**

At pH 5.6, increasing concentrations of pyruvate (a) do not affect the lag period observed in the absence of pyruvate, (b) increase the maximum rate of reduction of methylene blue, and (c) prevent decolorization of methylene blue to the 90 per cent value. With respect to (c), increasing concentrations of pyruvate up to ten micromoles causes the final reading on the Evelyn colorimeter to drop from the 90 per cent value with no pyruvate present to a meter reading representing 70 per cent reduction.

At pH 7.4, no effects were noted with increasing concentrations of pyruvate.

**Effect of concentrations of methylene blue on reduction by lactate**

At pH 5.6, increased concentrations of methylene blue increase the lag period, but have no effect on the reduction rate in the 10-90 per cent range. Decreasing concentrations
of methylene blue fail to remove the lag period at pH 5.6. No effects were noted at pH 7.4.

Apparently, a reaction, which utilizes lactate and competes with the dehydrogenase for lactate, functions at the lower pH levels. One of the products of the reaction at pH 5.6 may be pyruvate since addition of pyruvate increases the reduction time and prevents complete (80 per cent) reduction of methylene blue.

The evidence suggests that the reduction noted at lower pH levels is not that due to simple dehydrogenation of lactate since this reaction does not function significantly at the lower pH levels. Rather, a reaction dependent on the preliminary alteration of the lactate molecule (as evidenced by the lag period) followed by a transfer of hydrogen to methylene blue, and possibly yielding pyruvate as a product, may occur. The reaction which modifies the lactate molecule may be the rate-determining reaction. Subsequent dehydrogenation may depend on the existence of the modified lactate molecule. Once formed, the modified molecule may catalyze the overall reaction and increase the rate of transfer of hydrogen (as evidenced by the fairly rapid rate of reduction of methylene blue in the 10-90 per cent range). Possibly, a product of the reaction is dehydrogenated, too.
Differentiation of mutases and dehydrogenases, as set forth by Green Needham and Dewan (72), is of interest in this regard. Mutases use alpha-keto acids as oxidizing agents whereas dehydrogenases do not. Under anaerobic conditions, mutases can link up with dehydrogenases. In mutase systems, the coenzyme is reduced and oxidized only by the substrates of the enzyme. In dehydrogenase systems, the reduced coenzyme can also react with carriers such as flavin, flavoprotein, methylene blue, etc. The pathways for the two systems are as follows:

\[
\text{substrate} \xrightarrow{\text{mutase}} \text{alpha-keto-acid} \xrightarrow{\text{dehydrogenase}} \text{carrier} \xrightarrow{\text{flavoprotein, methylene blue, etc.}} \text{oxygen}
\]

Under anaerobic conditions, with a suitable alpha-keto acid present, hydrogens may be shunted to that acid to oxidize lactate, thus taking hydrogens away from the system mediating reduction of methylene blue. At pH 5.6, lactic dehydrogenase does not function significantly, therefore the keto acid cannot occur by direct oxidation of lactate. One source of the keto-compound may be the dismutation of lactate.

Preliminary manometric experiments using cell-free extracts yielded results indicating that formation of carbon dioxide does not occur significantly at pH 5.2, 6.4, or 7.4
with the following as substrates:

dicarboxylic acids, lactate, pyruvate, acetate, formate.

These extracts differ from those prepared by Utter, Kalnitsky and Werkman (160) in not showing formation of carbon dioxide. Attempts at preparation of extracts using the glass-grinding technic (85) used by these authors yielded extracts showing no decarboxylase activity. The cells used were grown in lactate whereas Utter, Kalnitsky and Werkman used cells grown in glucose.

If an enzyme system in the extract were competing with lactic dehydrogenase, its activity could not be detected by formation of carbon dioxide. Absence of formation of carbon dioxide eliminates the dismutation of pyruvate since the latter involves formation of carbon dioxide. Then, too, preliminary dehydrogenation of lactate must occur before dismutation of pyruvate can take place. At the pH of the experiment, lactic dehydrogenase does not function significantly. Also, analyses showed that pyruvate is not utilized by the cell-free extracts at this pH. Therefore, dismutation of lactate may be the competing reaction.

In summary, dehydrogenation of lactate by cell-free extracts occurs optimally at pH 7.4. A confusing reduction pattern at lower pH levels suggests the functioning of a second reaction which competes for lactate as a substrate.
Absence of complicating features at the higher pH levels and the reports of Stephenson (146) and Cook, Haldane and Mapson (34) prompts the selection of pH 7.4 as the optimum for the activity of lactic dehydrogenase. Subsequent studies using the Thunberg methylene blue technic were carried out at this pH.

The cell-free extract prepared by sonic disruption does not mediate the reduction of methylene blue by glucose, malate, fumarate, pyruvate, acetate and formate. Only succinate and lactate reduce methylene blue, the latter being ten times as active as the former.

**Effect of inhibitors on reduction of methylene blue**

No increase in time of reduction was noted using concentrations of fluoride up to 0.02 molar. Neither addition of the inhibitor together with the substrate nor pre-incubation of the extract with fluoride had any effect. The enzyme is insensitive to $7 \times 10^{-3}$ molar malonate, hyposulfite, hypophosphite and cyanide. A temporary inhibition was observed using $7 \times 10^{-3}$ molar iodacetate, indicating the presence of sulfhydryl groups in the centers of activity of the enzyme.

Contradictory reports regarding the effect of the various inhibitors on enzymes have been made. Iodacetate
reportedly (37) inhibits lactic and malic dehydrogenases, 
mutases (72), or the carrier enzyme which oxidizes the re-
duced lactic dehydrogenase (9). Fluoride supposedly inhibits 
lactic dehydrogenase (37). Lactic dehydrogenase is said to 
be the oxidizing portion of the mutase and not that respon-
sible for the reduction of keto acids (72).

All in all, the use of inhibitors contributes little 
to the study of the enzyme at this point.

Essential components

Extracts were prepared in 0.1 molar phosphate (pH 7.4) 
and dialysed against demineralized water for various periods 
of time. The undialysed extract (0.2 ml.) reduces methylene 
blue in two minutes. Dialysis for 1.5 hours increases the 
time for reduction to four minutes. Dialysis for five hours 
yields a reduction time of six minutes. Dialysis up to 40 
hours causes no further increase in reduction time. It was 
noted that the extract separates into two definite layers, 
a light amber-colored upper layer which reduces methylene 
blue only after a period of 30 minutes or more and a dark 
brown layer reducing methylene blue within several minutes. 
Apparently the lactic dehydrogenase is fairly stable to 
dialysis and is associated with the more particulate or
insoluble fractions of the extract. The latter property became more apparent following fractionation with ammonium sulfate (See later).

Dialysis against 0.1 molar phosphate (pH 7.1) increases the inactivation but layering-out occurred here too. Dialysis against phosphate resulted in more or less the same occurrences noted above. Addition of $10^{-3}$ molar solutions of manganese, magnesium and diphosphopyridine nucleotide failed to restore the activity of the dialysed extracts to that of the undialysed extract. Addition of boiled whole extract likewise failed to have any effect.

Treatment of the extract with Dowex-1 (50 - 100 and 200 - 400 mesh) does not inactivate the dehydrogenase. Addition of coenzyme A to the treated extract produces an increase in methylene blue reduction time, indicating an inhibition by this cofactor.

Using the procedure for identification of the flavin adenine dinucleotide moiety in an enzyme, as described by Warburg and Christian (165) and Sumner, Chou and Bever (153), it was found that added apoenzyme of D-amino acid oxidase or addition of flavin adenine dinucleotide does not reactivate the system. These findings do not exclude the possibility of denaturation of the protein portion of the enzyme by the treatment. Flavin adenine dinucleotide cannot be excluded as an essential component of the system.
Purification of the extract

Preliminary experiments with crude extracts showed no detectable diphosphopyridine nucleotide curves or peaks in spectrophotometric analyses. Many substances present in protein may contribute to spectrophotometric curves resembling the diphosphopyridine nucleotide curve. Purification of the lactic dehydrogenase by ammonium sulfate fractionation showed the activity to be contained in the 25 - 35 per cent fraction. The enzyme was stable to fractionation and to the dialyses involved in removal of the ammonium sulfate precipitant. Spectrophotometric analysis of the purified extract failed to show the curve or peaks shown by diphosphopyridine nucleotide.

Lactic dehydrogenase and inhibition by fluoride

Demonstration of the insensitivity of lactic dehydrogenase to fluoride prompted further study of the locus of action of fluoride on the utilization of lactate.

Barker and Lipmann (6) found that fumarate and malate remove the inhibition caused by fluoride. Since the propionic acid bacteria contain a strong fumarase (51) malate may be converted to fumarate and thereby indirectly serve in the
role of hydrogen acceptor or other capacity. Barker and Lipmann (6) found that for each mole of lactate utilized in the presence of fluoride and fumarate approximately one mole of pyruvate appears. Formation of carbon dioxide from the pyruvate formed from lactate approximates that from pyruvate added in separate experiments. In the presence of fluoride, this rate of formation of carbon dioxide is lower than the rate of formation of pyruvate from lactate in the presence of fumarate, therefore pyruvate accumulates.

The effect of increasing concentrations of fumarate on the formation of carbon dioxide from lactate in the presence of fluoride is shown in Table 20. Malate has a similar effect.

Formation of carbon dioxide from lactate is inhibited by fluoride whereas that from fumarate is not. Fumarate removes the inhibition by fluoride with lactate as substrate. The amount of fumarate added determines the extent of formation of carbon dioxide. In a substrate-saturated enzyme system, the amount of carbon dioxide formed is equivalent approximately to that from lactate in the absence of fluoride. This finding suggests that fumarate serves as a hydrogen acceptor in the presence of fluoride, permitting the conversion of lactate to pyruvate which then is utilized with the formation of carbon dioxide. If the system is a simple
<table>
<thead>
<tr>
<th>Fumarate (μMoles)</th>
<th>0</th>
<th>0.4</th>
<th>1.0</th>
<th>10.0</th>
<th>40.0</th>
<th>50.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>μMoles CO₂</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No additions</td>
<td>0.2</td>
<td>0.3</td>
<td>1.5</td>
<td>3.1</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td>Fluoride (0.02 M.)</td>
<td>0.3</td>
<td>0.3</td>
<td>1.7</td>
<td>3.4</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>Lactate (40 μMoles)</td>
<td>13.4</td>
<td>13.7</td>
<td>13.9</td>
<td>14.2</td>
<td>14.0</td>
<td></td>
</tr>
<tr>
<td>Lactate Fluoride</td>
<td>0.3</td>
<td>0.4</td>
<td>2.4</td>
<td>13.2</td>
<td>13.4</td>
<td></td>
</tr>
<tr>
<td>Pyruvate (40 μMoles)</td>
<td>26.4</td>
<td>-b</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pyruvate Fluoride</td>
<td>13.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

aEach flask contained 40 mg. dried cells, 0.1 molar phosphate (pH 6.4), substrate and fluoride concentrations as indicated, 0.2 ml. 3 N H₂SO₄ (added at end of reaction to release bound CO₂), demineralized H₂O to total volume of 2.3 ml. Flasks were flushed with O₂-free tank N₂ and equilibrated to 30.4° C.

bNot determined

hydrogen transfer, for every mole of lactate utilized one mole of pyruvate should be formed and then utilized with the formation of the amount of carbon dioxide which would be formed from pyruvate in the concentration of fluoride
used. The data (Table 20) indicate that this is the case. Data presented by Barker and Lipmann (6) verify this explanation.

Barker and Lipmann (6, p. 365), in studies of rates rather than total formation of carbon dioxide showed the following:

<table>
<thead>
<tr>
<th></th>
<th>Maximum rate carbon dioxide cmm./hr.</th>
<th>Lactate cmm.</th>
<th>Pyruvate cmm.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate</td>
<td>30</td>
<td>-20</td>
<td>48</td>
</tr>
<tr>
<td>Lactate</td>
<td>236</td>
<td>-2,000</td>
<td>1702*</td>
</tr>
<tr>
<td>Fumarate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyruvate</td>
<td>222</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endogenous</td>
<td>30</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The asterisk points out the value corrected for pyruvate formed from lactate alone. Assuming formation of pyruvate from lactate in the presence of fluoride and fumarate, the pyruvate formed should be utilized at almost the same rate as pyruvate alone in the presence of fluoride. Such is evident from the data presented. The data also suggest a fluoride-insensitive oxidation-reduction reaction between lactate and fumarate.

The formation of succinate from fumarate was reported by Barker and Lipmann (7) using glycerol or erythritol as a reducing agent instead of lactate.
By means of a cell-free extract which does not catalyze the formation of carbon dioxide from pyruvate, lactate, fumarate, succinate, and other compounds, it was possible to test the reaction proposed by Barker and Lipmann (6) for the propionic acid bacteria and by Quastel and Whetham (127) for Escherichia coli.

Since dehydrogenases function optimally at the higher pH levels, pH 7.4 was selected. Earlier experiments using dried cells were conducted at pH 6.4.

Utilization of lactate causes the reduction of methylene blue, whereas utilization of fumarate does not. Addition of a solution of lactate and fumarate together causes a rapid reduction of methylene blue followed by a partial re-oxidation of the indicator. By means of a two-arm Thunberg tube (Fig.17), lactate was added initially and methylene blue was reduced. Addition of fumarate from the second side-arm of the tube results in an instantaneous and complete re-oxidation of the indicator. This occurrence is followed by a gradual reduction which never reaches a full 90 per cent stage.

Substrate hydrogen acceptors

By means of the two-arm Thunberg tube (Fig.17), several compounds were tested for capacity to accept hydrogens
Fig. 17 Two-armed Thunberg tube.
(electrons) from leuco-methylene blue formed in the presence of the cell-free extract and lactate. Pyruvate, acetate, formate, malate, succinate glycerophosphate, phosphoglycerate, acrylate, acrolein, and methyglyoxal failed to reoxidize the reduced methylene blue. Fumarate and oxalacetate served as oxidizers for leuco-methylene blue, the second named compound providing an incomplete reoxidation.

The usual procedure for testing was as follows:

Lactate was placed in one sidearm and the re-oxidizing substance in the second sidearm. Following the usual evacuation and temperature equilibration, lactate was added to the reaction mixture in the tube. Reduction of methylene blue was followed photometrically using the Evelyn colorimeter with a 540 mμ filter. Following complete reduction, the second substance was added and the rate of reoxidation followed colorimetrically.

Characteristically, addition of fumarate yields an almost instantaneous reoxidation, whereas oxalacetate causes a much slower reaction which never reaches the full 90 per cent methylene blue oxidized condition. Addition of oxalacetate is accompanied by frothing, probably due to the decomposition by decarboxylation of oxalacetate.

It was found that 0.0125 molar fluoride does not affect the reaction.
Coupled Reaction between Fumarate and Lactate

Whether the transfer of hydrogen from lactate to fumarate or oxalacetate occurs in the absence of methylene blue was investigated. Analytical procedures were used to determine both qualitatively and quantitatively the substrates of the reaction.

The Warburg-Barcroft respirometer was used for measuring formation of gas and also for maintaining a constant temperature, anaerobic system. The effect of the extract on lactate on fumarate, and on fumarate and lactate together was determined. The results of a typical experiment are shown in Table 21.

It is apparent that the reaction is carried out by the cell-free extracts. The effect of various concentrations of fluoride which inhibit the utilization of lactate by whole cells was investigated. Results are shown in Table 22. Little or no carbon dioxide is formed. From the results it may be concluded that the oxidation-reduction reaction does occur and that it is insensitive to the action of fluoride. In effect, a portion of the reaction which may occur when lactate is utilized by whole cells has been isolated by using cell-free extracts.
<table>
<thead>
<tr>
<th>Substrate</th>
<th>CO₂</th>
<th>Lactate utilized</th>
<th>Pyruvate utilized</th>
<th>Fumarate utilized</th>
<th>Succinate utilized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate</td>
<td>0</td>
<td>0.2</td>
<td>0.1</td>
<td>-b</td>
<td>0</td>
</tr>
<tr>
<td>Fumarate</td>
<td>0.9</td>
<td>-</td>
<td>0.1</td>
<td>0.9</td>
<td>0.1</td>
</tr>
<tr>
<td>Lactate</td>
<td>2.9</td>
<td>12.8</td>
<td>13.3</td>
<td>11.2</td>
<td>7.8</td>
</tr>
<tr>
<td>Fumarate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>1.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Each flask contained 0.1 M phosphate (pH 7.4), 0.8 ml. cell-free extract (prepared in phosphate, pH 7.4), substrates indicated above (40 μMoles each), 0.4 ml. 5 per cent HPC₃ (added at end of 30 minute reaction period to release bound CO₂), and demineralized H₂O to total volume of 2.0 ml. Flasks were flushed with O₂-free tank N₂ and equilibrated to 30.4°C.

bNot determined
Table 22

Effect of 0.02 M fluoride on oxidation-reduction reaction between lactate and fumarate

<table>
<thead>
<tr>
<th>Substrate</th>
<th>0.02 M Fluoride</th>
<th>μMoles CO₂</th>
<th>μMoles pyruvate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate</td>
<td>-b</td>
<td>0</td>
<td>0.85</td>
</tr>
<tr>
<td>Lactate</td>
<td>+c</td>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td>Lactate</td>
<td></td>
<td>0.3</td>
<td>5.7</td>
</tr>
<tr>
<td>Fumarate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactate</td>
<td>+</td>
<td>1.5</td>
<td>6.5</td>
</tr>
<tr>
<td>Fumarate</td>
<td>-</td>
<td>0.6</td>
<td>0.3</td>
</tr>
<tr>
<td>Fumarate</td>
<td>+</td>
<td>0.35</td>
<td>0.85</td>
</tr>
</tbody>
</table>

Each flask contained 0.1 M phosphate (pH 7.4), 40 μMoles substrates and 0.02 M fluoride as shown, 0.4 ml. cell-free extract, 0.4 ml. five percent HPO₃ (to stop reaction and release bound CO₂), demineralized H₂O to total volume of 2.0 ml. Flasks were flushed with CO₂-free tank N₂ and equilibrated to 30.4°C. Reaction time: 30 minutes.

bAbsent

cPresent
Effect of pH on reaction between fumarate and lactate

The effect of pH on the oxidation-reduction reaction was determined with cell-free extracts, which do not utilize pyruvate. Previous studies on dehydrogenation of lactate using the Thunberg methylene blue technic indicated unexpected occurrences at the lower pH levels. To avoid complications related to the indicator technic, fumarate was used as the hydrogen acceptor instead of methylene blue. Formation of pyruvate was used as a measure of enzyme activity. Results are shown in Fig. 18. The pH optimum obtained using the analytical procedure agrees fairly well with that found using maximum rates of reduction of methylene blue as a measure.

Analysis of reaction mixtures for lactate and pyruvate showed variations with change in pH. Results are shown in Table 23. The lactate-to-pyruvate ratio (1:1) apparent at pH 7.4 is lacking at the lower pH levels. A ratio of 2:1 occurs near pH 6.9. However, maximum utilization of lactate and maximum production of pyruvate occurs at or near pH 5.6. It is interesting to note that Krebs (94) found the maximum dismutation of pyruvate occurring at pH 6.6 in gonococci and staphylococci. Analyses of aliquots of reaction mixtures using larger volumes of reaction mixtures yielded the results
Figure 18. Formation of pyruvate in reaction between lactate and pyruvate as affected by pH.
Table 23
Effect of pH on coupled reaction between lactate and fumarate

<table>
<thead>
<tr>
<th>pH</th>
<th>Lactate utilized (µmoles)</th>
<th>Pyruvate (µmoles)</th>
<th>Lactate/pyruvate</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.3</td>
<td>13.7</td>
<td>8.2</td>
<td>1.7</td>
</tr>
<tr>
<td>5.7</td>
<td>14.4</td>
<td>9.1</td>
<td>1.5</td>
</tr>
<tr>
<td>6.0</td>
<td>11.1</td>
<td>7.3</td>
<td>1.4</td>
</tr>
<tr>
<td>6.5</td>
<td>12.8</td>
<td>6.8</td>
<td>1.9</td>
</tr>
<tr>
<td>6.9</td>
<td>12.2</td>
<td>6.2</td>
<td>2.0</td>
</tr>
<tr>
<td>7.2</td>
<td>5.5</td>
<td>4.1</td>
<td>1.3</td>
</tr>
<tr>
<td>7.4</td>
<td>4.1</td>
<td>4.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

*Each flask contained 0.3 ml. cell-free extract (1.3 mg. protein), 40 µMoles substrate (each), 0.8 ml. 0.5 M phosphate (appropriate pH), 0.2 ml. 3 M H2SO4 (to stop reaction and release bound CO2), demineralized H2O to total volume of 2.0 ml. Flasks were flushed with O2-free tank N2 and equilibrated to 30.4°C. Reaction time: 30 minutes.*

shown in Table 24. Apparently fumarate serves as a substrate hydrogen acceptor to a progressively smaller degree with decreasing pH. The amount of lactate utilized at the lower pH levels is approximately three times that at pH 7.4. Therefore, dehydrogenation of lactate is not the main reaction for utilization of lactate at the lower pH levels. The ratio of lactate utilized to pyruvate appearing seems to approximate 2 : 1.
Table 24
Effect of pH on utilization of fumarate in lactate-fumarate coupled reaction

<table>
<thead>
<tr>
<th>pH</th>
<th>Lactate utilized (µmoles)</th>
<th>Fumarate utilized (µmoles)</th>
<th>Pyruvate (µmoles)</th>
<th>Succinate (µmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.4</td>
<td>8.2</td>
<td>8.6</td>
<td>8.0</td>
<td>3.2</td>
</tr>
<tr>
<td>6.5</td>
<td>25.6</td>
<td>6.0</td>
<td>13.6</td>
<td>6.2</td>
</tr>
<tr>
<td>5.3</td>
<td>27.4</td>
<td>3.4</td>
<td>16.4</td>
<td>2.6</td>
</tr>
</tbody>
</table>

Each flask contained 4.5 ml. cell-free extract (17.5 mg. protein), 12 ml. 0.3 M phosphate (pH as indicated), 80 µMoles substrate (each), 1.5 ml. 3N H₂SO₄ (to stop reaction and release bound CO₂), demineralized H₂O to total volume of 30 ml. Flasks were flushed with O₂-free tank N₂ and equilibrated to 30.4° C.

Stoichiometry of reaction at pH 5.6

Taking into consideration the amounts of lactate utilized and pyruvate appearing in the oxidation-reduction reaction with fumarate, the following results are obtained:

<table>
<thead>
<tr>
<th>pH</th>
<th>Lactate utilized</th>
<th>Pyruvate appearing</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5</td>
<td>9.8</td>
<td>3.8</td>
</tr>
<tr>
<td>5.3</td>
<td>12.0</td>
<td>6.5</td>
</tr>
</tbody>
</table>
The data indicate an unbalance due to formation of a substance other than pyruvate. From the 40 micromoles of fumarate included in the reaction only 0.1 micromole of pyruvate is formed.

In experiments conducted in the absence of fumarate at pH 6.4, it was found that lactate per se is utilized to only a slight degree. Approximately 40 micromoles of lactate yielded only 0.3 micromole of pyruvate and an equivalent amount of lactate is utilized.

These findings suggest the need for some factor or factors present in the previous experiments and absent here. Accordingly, one micromole of fumarate was added and the reaction mixture analysed. It was found that the same pattern of products occurred here as in the experiments with a large excess of fumarate. Apparently, then, fumarate contributes to the operation of the system using two moles of lactate with the formation of only one mole of pyruvate.

Steam distillation of an aliquot of the reaction mixture in the presence of sulfuric acid and mercuric sulfate indicated the presence of a volatile acid which chromatographically resembles propionate. Mercuric sulfate was added to the steam distillation to destroy any pyruvate or lactate present. Previous experiments indicated that propionate and acetate are untouched by mercuric sulfate and are delivered
quantitatively whereas pyruvate and lactate are destroyed. Results were as follows:

<table>
<thead>
<tr>
<th>pH</th>
<th>Lactate Utilized</th>
<th>Pyruvate formed</th>
<th>Volatile acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.4</td>
<td>10.1</td>
<td>5.0</td>
<td>4.8</td>
</tr>
</tbody>
</table>

Values are in terms of micromoles. The results indicate the occurrence of a reaction yielding pyruvate and propionate.

The requirement of fumarate to catalyze the reaction suggests involvement of a phosphorylation or some energy-creating cycle. Catalysis of dismutations by fumarate is not without precedent. Krebs (94) demonstrated catalysis of dismutation of pyruvate by fumarate. Krebs (95) also declared that there is no anaerobic breakdown of lactate by *Escherichia coli* in the absence of fumarate. Green, Loomis and Auerbach (71) reported that fumarate acts catalytically to spark the oxidation of pyruvate. Colowick, Kalckar and Cori (31) presented evidence for the catalysis of pyruvate and glucose oxidations by the succinate-fumarate reaction, suggesting the occurrence of a phosphorylation system. The report of Krebs (95) placed the optimum pH for the dismutation reaction in *Escherichia coli* at 5.5. At pH 7.0, little or no lactate and free carbon dioxide are formed. The catalysis by fumarate (94) was quite apparent. Twenty micromoles of added fumarate were sufficient to increase the reaction more than 200 per cent over that for pyruvate alone.
Fumarate per se was not utilized significantly. Barker and Lipmann (7) demonstrated that for each mole of fumarate reduced to succinate, an almost equivalent amount of inorganic phosphate is esterified. The system investigated here may require an energy source which is "sparked" by preliminary phosphorylation or by an energy-forming reaction cycle. Solomon, Vennesland, Klemperer and Hastings (144) proposed the phosphorylation of fumarate with the formation of an intermediate, \( \text{HOOCCH}_2\text{CH(PO}_3\text{H}_2\text{)}\text{COOH} \), which is dehydrogenated to form \( \text{HOOCCH}^\text{=CO(PO}_3\text{H}_2\text{)}\text{COOH} \). The latter is decarboxylated to phosphoenolpyruvate and carbon dioxide.

**Essential components of the dismutation reaction**

The effect of energy-rich compounds on the activation of the whole and dialyzed cell-free extract was investigated. Results are shown in Table 25. Addition of adenosine triphosphate (ATP) to the coupled reaction inhibits the reaction slightly but has no stimulating effect. Dialysis of the extract fails to decrease the activity of the enzyme system significantly, suggesting the absence of dialyzable components in the enzyme system. Addition of adenosine diphosphate (ADP) shows a definite increase of activity, especially in the dialyzed extracts. It is conceivable that
Table 25

Effect of adenosine triphosphate and adenosine diphosphate on reaction between lactate and fumarate at pH 5.6a

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Whole extract</th>
<th>Dialysed extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lactate utilized</td>
<td>Pyruvate</td>
</tr>
<tr>
<td>Lactate</td>
<td>0.3</td>
<td>0.4</td>
</tr>
<tr>
<td>Fumarate</td>
<td>.b</td>
<td>0.2</td>
</tr>
<tr>
<td>Lactate</td>
<td>12.4</td>
<td>6.1</td>
</tr>
<tr>
<td>Fumarate</td>
<td>12.4</td>
<td>6.1</td>
</tr>
<tr>
<td>Lactate</td>
<td>9.5</td>
<td>4.5</td>
</tr>
<tr>
<td>Fumarate</td>
<td>12.1</td>
<td>5.8</td>
</tr>
<tr>
<td>Fumarate</td>
<td>12.1</td>
<td>5.8</td>
</tr>
</tbody>
</table>

a Each flask contained 0.8 ml cell-free extract, 40 μMoles of each substrate, 0.05 M phosphate (pH 5.6), and H2O to total volume of 2.3 ml. ATP and ADP were added in quantities of ten Moles. Each flask was flushed with O2-free tank N2 and equilibrated to 30.4°C. Results are reported in terms of Moles.

b Not determined.

a reaction involving incorporation of inorganic phosphate into an energy-rich molecule precedes the dismutation reaction and that the extent of the latter reaction is dependent upon the presence of a suitable phosphate acceptor, such as ADP.
The reports of Silverman and Werkman (137) and Quastel and Webley (125) indicate the involvement of thiamine in the utilization of pyruvate and lactate. Addition of trace amounts of thiamine increased formation of carbon dioxide from lactate by 54 per cent and from pyruvate by 126 per cent. Significantly, only approximately one-half the amount of carbon dioxide formed from pyruvate is formed from lactate. With the addition of thiamine the amount of carbon dioxide formed from pyruvate is three times that from lactate.

*Propionibacterium pentosaceum*, grown on thiamine-deficient media (154), was contrasted with normally grown cells in relation to anaerobic activity on lactate and pyruvate with and without the addition of thiamine and coenzyme III (Table 26). Apparently coenzyme III is involved in the formation of carbon dioxide from both pyruvate and lactate. It is conceivable that lactate is utilized with the formation of pyruvate which then undergoes decomposition by a coenzyme III-dependent enzyme system with the eventual formation of carbon dioxide. The word, decomposition, is used advisedly since studies on utilization of pyruvate by the propionic acid bacteria are limited and simple decarboxylation is not the only reaction to which pyruvate may be subject. Dismutation of pyruvate has been demonstrated in several bacterial species and in animal tissues (10, 94, 99, 143, 161).
## Table 26
Utilization of lactate and pyruvate as influenced by thiamine and cocarboxylase

<table>
<thead>
<tr>
<th>Type cell</th>
<th>Additions</th>
<th>$\mu$ Moles CO$_2$</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pyruvate</td>
<td>Lactate</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>None</td>
<td>25.2</td>
<td>13.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thiamine</td>
<td>25.3</td>
<td>13.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cocarboxylase</td>
<td>25.4</td>
<td>13.0</td>
<td></td>
</tr>
<tr>
<td>Thiamine-</td>
<td>None</td>
<td>0.5</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>deficient</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thiamine</td>
<td>1.0</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cocarboxylase</td>
<td>5.7</td>
<td>7.4</td>
<td></td>
</tr>
</tbody>
</table>

*a Each flask contained 40 mg. (wet weight) cells, 0.01 M phosphate (pH 6.4), 40 $\mu$Moles substrate as indicated, and H$_2$O to total volume of 2.3 ml. Flasks were flushed with O$_2$-free tank N$_2$ and equilibrated to 30.4 C.

*b Thiamine and cocarboxylase added directly with substrates. No preliminary incubation was involved. These substances were added in amounts of 10 gamma each.

Diphosphopyridine nucleotide acts as a catalyst for both the oxidation and dismutation of pyruvate (10). Smyth (143) demonstrated that thiamine may be replaced as a catalyst in the dismutation of pyruvate by staphylococci.
Oxalacetate or fumarate may replace the vitamin. The assumption was made that thiamine catalyzes the fixation of carbon dioxide onto pyruvate with the formation of oxalacetate which then is converted into the other dicarboxylic acids. Colowick, Welch and Cori (32, 33) presented evidence for the functioning of fumarate as a catalyst in the oxidation of glucose and pyruvate by extracts of kidney. Fluoride (0.02 molar) inhibits the catalysis in the case of glucose but not in the case of pyruvate. Apparently phosphorylation of glucose is dependent upon the oxidation of pyruvate. Phosphorylation precedes oxidation of glucose. Fumarate is an essential link between phosphorylation and oxidation. A number of substrates whose oxidation involves catalysis by fumarate may be concerned with the phosphorylation of glucose. Teng (155) found that anaerobic utilization of glycerol is dependent upon the simultaneous metabolism of pyruvate. The implication here is that during the dismutation of pyruvate high energy phosphate bonds are generated, resulting in the phosphorylation of glycerol.
DISCUSSION

The role of succinic decarboxylase in the formation of propionate by the propionic acid bacteria has been emphasized by earlier workers (38). Propionibacterium pentosaceum was found to possess low levels of or no succinic decarboxylase activity as a result of cultivation for long periods of time in lactate medium. These cells utilize lactate under anaerobic conditions with the formation of the products and proportions shown in the equation,

$$3 \text{Lactate} = 2 \text{Propionate} + 1 \text{Acetate} + 1 \text{Carbon dioxide}.$$  

Active lactic dehydrogenase and succinic dehydrogenase are found in cells lacking succinic decarboxylase. Cell-free extracts were used in the study of these dehydrogenases. The pH optimum for each is 7.4. The concept of a pH optimum for the function of an enzyme embodies the idea that the forward and reverse reactions (assuming that one enzyme mediates both reactions) both function optimally at the same pH. Therefore, with lactate as substrate, formation of pyruvate should occur readily at pH 7.4. Dehydrogenation of lactate has been demonstrated with the Thunberg technic. But in the absence of methylene blue, a suitable hydrogen acceptor must be present to receive the hydrogen derived from lactate. With washed cells and in cell-free extracts
this is not the case. Assuming that such a hydrogen acceptor is present, fixation of carbon dioxide, derived from pyruvate, may occur with the formation of oxalacetate. Once formed, oxalacetate may serve as a hydrogen acceptor to form malate and simultaneously another molecule of lactate is converted to pyruvate. The net result would be the formation of succinate in large amounts at pH 7.4. However, succinic decarboxylase does not function at this pH. Conceivably, in normal fermentations in which the pH level falls with progress of the fermentation, succinate is formed initially and not utilized until the pH level drops to the level at which succinic decarboxylase functions. In the case of cultivation of the organism in lactate medium, the pH never drops below 6.8. The formation and the function of succinic decarboxylase are inhibited under such conditions, as was shown in the experiments reported here. Therefore, in cells lacking succinic decarboxylase, the dehydrogenases function readily but the product, succinate, cannot be utilized by a decarboxylative reaction. The other mode of utilization would be the oxidative mechanism or dehydrogenation of succinate. Here again a suitable hydrogen acceptor must be available to allow the reaction to proceed to any appreciable extent. At pH 7.4 lactic dehydrogenase functions optimally and competes for suitable hydrogen acceptors. The net result is a stale-
mate with the only possible avenue of outlet being via pyruvate and its degradation. Propionate has been shown to occur in fermentations of glucose outside the functional pH range of succinic decarboxylase (38). Another mechanism must function in the synthesis of propionate.

In those fermentations such as that of glucose, in which the initial pH is approximately neutral, succinate may be formed and accumulate, so that with the progress of the fermentation and with decreasing pH there is a ready supply of succinate available to the succinic decarboxylase. When the pH reaches that for optimum functioning of the decarboxylase, decarboxylation of succinate becomes the main reaction by which propionate is formed. But here again the importance of succinic decarboxylase to the formation of propionate is in doubt, since after depletion of the succinate which has accumulated, the chance for formation of succinate via the dicarboxylic acid cycle is very small. The latter is true since the dehydrogenases which assist in the formation of malate and succinate are relatively inactive at the lower pH levels, so that formation of succinate by this means is at a standstill. The other possible source of succinate may be the condensation of two molecules of acetate with the formation of the four-carbon acid. But formation of succinate by condensation of acetate is dependent upon the preliminary
utilization of pyruvate with the formation of acetate, according to the equation:

\[
\begin{align*}
4 \text{ Pyruvate} + 2\text{H}_2\text{O} &\rightarrow 2 \text{ Lactate} + 2 \text{ Acetate} + 2 \text{ Carbon dioxide} \\
2 \text{ Lactate} &\rightarrow 1 \text{ Pyruvate} + 1 \text{ Propionate} + \text{H}_2\text{O} \\
\text{or} &\quad 3 \text{ Pyruvate} + \text{H}_2\text{O} = 2 \text{ Acetate} + 2 \text{ Carbon dioxide} + 1 \text{ Propionate}
\end{align*}
\]

The final equation is that used to represent the overall dissimilation of pyruvate under anaerobic conditions.

It should be noted that pyruvate is considered the intermediate of cardinal importance in the dissimilation of glucose. The dissimilation of pyruvate represented above has not been shown to be operative although it has been proposed as the reaction which probably occurs. In the dissimilation of glucose at pH levels above the range of succinic decarboxylase, this reaction may occur, contributing to the formation of propionate. This reaction may explain the finding of propionate in fermentations of glucose above the pH range in which succinic decarboxylase is active (38). The formation of propionate under these conditions is dependent upon the operation of the enzyme system responsible for the dissimilation of lactate.

The overall reaction sequence in the dissimilation is represented as follows:
4 Lactate = 2 Pyruvate + 2 Propionate + 2H₂O

2 Pyruvate + H₂O = 1 Lactate + 1 Acetate + 1 Carbon dioxide

or

3 Lactate = 2 Propionate + 1 Acetate + 1 Carbon dioxide + H₂O

The final equation is that accepted as representing the anaerobic dissimilation of lactate (based on product analysis).

Utilization of lactate under anaerobic conditions has been shown to be inhibited completely by fluoride. Since dehydrogenation of lactate (which does not occur to any great extent at the lower pH levels) is insensitive to fluoride, it may be that the dismutation reaction is the point of inhibition due to fluoride. Since utilization of pyruvate is not affected by fluoride to the same extent as utilization of lactate, any pyruvate formed in the dismutation of lactate should be metabolized with the formation of carbon dioxide. Since carbon dioxide is not formed, it may be that pyruvate is not formed and that therefore an intermediate step in the dismutation of lactate is the sensitive step. It is tempting to suggest that formation or utilization of a phosphorylated lactate derivative is affected by fluoride so that no pyruvate is formed as a result and therefore no carbon dioxide is formed.

By the same token, if the dismutation of pyruvate is operating in the propionic acid bacteria, fluoride may be
affecting the utilization of pyruvate by the same mechanism. It was found that the concentration of fluoride, which completely inhibits formation of carbon dioxide from lactate, decreases the formation of carbon dioxide by 30 to 50 per cent with pyruvate as substrate. Conceivably, that portion of the reaction which yields lactate, acetate and carbon dioxide is unaffected by fluoride but that part of the reaction which utilizes the lactate so formed is blocked by fluoride.

Barker and Lipmann (6) proposed a schematic representation of the system involved in the inhibition of the utilization of lactate by fluoride. They represented the reaction as follows:

```
Pyruvate + 2H → X + 2H → Propionate
   /           \     /           \          
  +2H            -2H   +2H
   \           /     \           /          
    Lactate      X
```

According to Barker and Lipmann (6), lactate cannot act as an oxidant in the presence of fluoride as it does in the absence of fluoride. Conceivably the formation of compound X may be inhibited by fluoride, whereas the formation of pyruvate from lactate is not. In effect, since compound X cannot be formed, the formation of propionate cannot occur, and therefore lactate cannot serve as an oxidant.
A better version of the reaction may be indicated if we consider that formation of compound X from lactate is dependent upon a phosphorylation or dephosphorylation. Fluoride affects the formation of compound X, the reduction of which is essential to the dehydrogenation of lactate to form pyruvate. Since pyruvate is not formed in any significant amount, no carbon dioxide is produced. In other words, compound X serves as the oxidant of a lactate molecule to yield pyruvate with the simultaneous formation of propionate by reduction of compound X.

Barker and Lipmann (6) demonstrated the increased formation of propionate from pyruvate with the addition of 0.02 molar fluoride. It may be that the formation of compound X from lactate is inhibited. The net effect would be the blocking of the pathway from pyruvate to lactate to compound X. The hydrogen normally donated to the formation of lactate would then be shunted to the pathway from pyruvate to compound X to propionate. The latter pathway apparently is not dependent upon phosphorylation (7).
**CONCLUSIONS AND SUMMARY**

*Propionibacterium pentosaceum*, grown in lactate medium under microaerophilic conditions, possesses low levels of activity of succinic decarboxylase. This organism carries out the anaerobic dissimilation of lactate resulting in the formation of propionate, acetate and carbon dioxide. The reaction is represented by the equation,

\[ 3 \text{ Lactate} = 2 \text{ Propionate} + 1 \text{ Acetate} + 1 \text{ Carbon dioxide} + 1 \text{ H}_2\text{O} \]

Formation of propionate in the usual quantities and proportions by cells possessing low levels of activity of succinic decarboxylase indicates the functioning of an enzyme system, other than succinic decarboxylase, in the formation of propionate.

Utilization of lactate is characterized by a preliminary modification of the molecule before formation of carbon dioxide. The modification is evident from the lag in formation of carbon dioxide. The modification also involves shifting of hydrogen, as evidenced by instantaneous uptake of oxygen under aerobic conditions. The hydrogen atoms so affected are those on the alpha carbon, since pyruvate does not show an instantaneous uptake of oxygen under aerobic conditions but rather a lag followed by consumption of oxygen.
The modification of the lactate molecule is not simply oxidation to pyruvate, since, if each molecule of lactate were converted to pyruvate, the amount of carbon dioxide formed from each should be the same. The amount of carbon dioxide formed from lactate is just half of that formed from pyruvate, indicating the formation of a three-carbon compound which is not decarboxylated, or formation of a two-carbon and a one-carbon compound (not carbon dioxide).

Studies using cell-free extracts indicate the presence of lactic and succinic dehydrogenases which function optimally at pH 7.4. The enzymes are unaffected by dialysis and the essential components are still undetermined. The equilibrium existing between the dicarboxylic acids is mediated by enzymes contained in the extract and operative at pH 7.4.

At pH 7.4, the following reactions have been demonstrated using cell-free extracts:

Lactate + Methylene blue = leuco-Methylene blue
leuco-Methylene blue + Fumarate = Methylene blue
and Lactate + Fumarate = Pyruvate + Succinate.

In cell-free extracts, the functioning of an enzyme system responsible for the dismutation of lactate has been demonstrated. The enzyme system functions optimally at pH 5.6 and carries out the reaction,

2 Lactate = 1 Propionate + 1 Pyruvate.
The essential components in this system still are indefinite although the need for fumarate or some energy-forming system is indicated. Dialysis has little effect on the enzyme system involved.

The dismutation of lactate, together with a postulated dismutation of pyruvate, is responsible for the formation of the products of the usual propionic acid fermentation.

The occurrence of these and other reactions are dependent upon the type of medium in which the cells are grown, the pH of the medium, the age of the cells, and other factors.
LITERATURE CITED


(9) Barron, E. S. G. Studies on biological oxidations. II. The oxidation of lactic acid by alpha-hydroxy-oxidase, and its mechanism. J. Biol. Chem. 100: 155-182. 1933.


(22) and M. E. Kamen. Radioactive carbon as a tracer in the synthesis of propionic acid from carbon dioxide by the propionic acid bacteria. Science 92: 433-434. 1940.


(64) ________, and E. L. Piret. Sur la nutrition azotée de quelques espèces de bactéries propioniques. Arch. Mikrobiol. 7: 551-570. 1936.


(95) ________. The role of fumarate in the respiration of Bacterium coli commune. Biochem. J. 31: 2095-2124. 1937.

(96) ________. The formation of succinic acid by propionic acid bacteria. Chem. and Indus. (London) 18: 349. 1940.


(100) and . The role of citric acid in intermediate metabolism in animal tissues. Enzymologia 4: 143-156. 1937.


(104) and H. G. Wood. Evidence from fermentation of labeled substrates which is inconsistent with present concepts of the propionic acid fermentation. J. Cell. and Comp. Physiol. 41: (suppl. 1) 225-240. 1953.


(120) and . The production and utilization of lactic acid by certain propionic acid bacteria. Biochem. J. 33: 1605-1610. 1939.


(127) and M. D. Whetham. The equilibrium existing between succinic, fumaric and malic acids in the presence of resting bacteria. Biochem. J. 18: 519-534. 1924.


(130) and W. Z. Hassid. Fermentation of L-arabinose-l-C₁⁴ by Lactobacillus pentoceticus. Arch. Biochem. and Biophys. 51: 326. 1951.

(131) Rollman, N. O. and G. Sjöström. Investigations of the behavior of some propionic acid bacteria strains in relation to sodium chloride, sodium nitrate and heating. Svenska Mykologi. 38: 199-201; 209-212. 1948. (Original not available
for examination; abstracted in Chem. Abstr. 42: 8281b. 1948.)


(139) _______ and _______. Adaptation of the propionic acid bacteria to vitamin B\textsubscript{1} synthesis including a method of assay. J. Bact. 38: 25-32. 1939.


(156) Thompson, R. C. The B-vitamin requirements of the propionibacteria. J. Bact. 46: 99-104. 1943.


(161) and C. H. Werkman. Formation and reactions of acetyl phosphate in Escherichia coli. Arch. Biochem. 5: 413-422. 1944.


(166) and . Chemischer Mechanismus der Fluoridhemmung der Garung. Naturwiss. 29: 590. 1941.


(188) and . The final oxidation-reduction phases of the propionic dissimilation. (Abstract) J. Bact. 35: 102. 1937.


(201) __________, __________, __________ and __________. Heavy carbon as a tracer in heterotrophic carbon dioxide assimilation. J. Biol. Chem. 139: 365-376. 1941.


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