Physiology of the lactic acid bacteria

Milton Ephriam Nelson

Iowa State College

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Physiology of the Lactic Acid Bacteria

By

Milton Ephriam Nelson

A thesis submitted to the Graduate Faculty for the Degree of
Doctor of Philosophy
Major Subject: Physiological Bacteriology

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1938
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>2</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>5</td>
</tr>
<tr>
<td>PART I</td>
<td></td>
</tr>
<tr>
<td>LITERATURE REVIEW</td>
<td>6</td>
</tr>
<tr>
<td><strong>CHAPTER ONE</strong></td>
<td></td>
</tr>
<tr>
<td>Discovery of Lactic Acid and Lactic Acid Forming Bacteria</td>
<td>6</td>
</tr>
<tr>
<td>Discovery of lactic acid</td>
<td>6</td>
</tr>
<tr>
<td>Discovery of lactic acid forming bacteria</td>
<td>8</td>
</tr>
<tr>
<td><strong>CHAPTER TWO</strong></td>
<td></td>
</tr>
<tr>
<td>Classification of the Lactic Acid Bacteria</td>
<td>11</td>
</tr>
<tr>
<td><strong>CHAPTER THREE</strong></td>
<td></td>
</tr>
<tr>
<td>Dissimilation of the Lactic Acid Bacteria</td>
<td>29</td>
</tr>
<tr>
<td>The heterofermentative lactic acid bacteria</td>
<td>29</td>
</tr>
<tr>
<td>The homofermentative lactic acid bacteria</td>
<td>41</td>
</tr>
<tr>
<td><strong>PART II</strong></td>
<td></td>
</tr>
<tr>
<td>EXPERIMENTAL</td>
<td>48</td>
</tr>
<tr>
<td><strong>CHAPTER ONE</strong></td>
<td></td>
</tr>
<tr>
<td>Methods</td>
<td>48</td>
</tr>
<tr>
<td>General considerations</td>
<td>48</td>
</tr>
<tr>
<td>Methods of analysis</td>
<td>50</td>
</tr>
<tr>
<td>Bacteria used</td>
<td>59</td>
</tr>
<tr>
<td>Medium</td>
<td>60</td>
</tr>
</tbody>
</table>
CHAPTER TWO

Quantitative Studies on the Dissimilation of Glucose ........................................ 61
The homofermentative lactic acid bacteria ............................................................... 61
The heterofermentative lactic acid bacteria .............................................................. 71
Serial analysis of the dissimilation of glucose ......................................................... 76

CHAPTER THREE

Intermediary Reactions in the Dissimilation of Glucose ........................................ 89
Homofermentative lactic acid bacteria ........................................................................ 89
Heterofermentative lactic acid bacteria ...................................................................... 93

CHAPTER FOUR

Diversion of the Normal Dissimilation of Glucose by the Addition of Hydrogen Acceptors 100
The addition of acetaldehyde ..................................................................................... 100
The addition of acetylmethylcarbinol ........................................................................ 105

CHAPTER FIVE

The Dissimilation of Levulose by Lactic Acid Bacteria ....................................... 110

CHAPTER SIX

Discussion .................................................................................................................... 122
Summary and Conclusions ....................................................................................... 130

LITERATURE CITED .................................................................................................. 132
INTRODUCTION

The lactic acid bacteria constitute a large and ubiquitous group of microorganisms which play an important part in both industry and agriculture. Study of their dissimilation of carbohydrates should prove of direct value to both in a practical sense and at the same time be of substantial assistance in providing information of inestimable value in problems of classification. Relatively little is known regarding the physiology of these bacteria as pertains to their mechanism of carbohydrate dissimilation.

Representatives of the lactic acid bacteria are found widespread in nature under varying environmental conditions. They are found in the soil, and are abundant on grains; they grow luxuriantly in dairy products and are present in dental caries. Representatives of the group are contaminants of industrial fermentation processes, and are themselves used in the commercial production of lactic acid.
PART I
LITERATURE REVIEW
CHAPTER ONE

Discovery of Lactic Acid and Lactic Acid Forming Bacteria.

Discovery of lactic acid

The discovery of lactic acid by Scheele (1780) from sour milk introduced a large field for investigation and opened up many opportunities for scientific discovery and industrial exploitation. Bouillon-Lagrange (1803) obtained an acid from milk which resembled very much that isolated by Scheele. A different method, however, was used for isolation of the acid. It was claimed that Scheele's acid was not pure lactic, but contained a mixture of acetic and muriatic acids as well as a little iron and some animal matter. Berzelius (1830) pointed out that the reason Bouillon-Lagrange obtained the different acids was that hydrochloric acid and ammonium acetate had been used and on distillation acetic acid was found in the distillate.

Braconnot (1813) studied fermentations of rice and isolated an acid which he characterized by its zinc salt. Either ignoring or unaware of the acid described by Scheele, Braconnot considered the compound as a new acid which he called "acid nanseique." for Nancy, the place where the compound was first studied. Later it was established that Braconnot was working with lactic acid.
Gay-Lussac and Pelouze (1833) studied the chemical structure of lactic acid. It was prepared from a 2-months' fermentation of beet juice. It is of interest to note that mannitol was formed, which led them to believe that sucrose from the beet had been hydrolyzed, since mannitol can be formed only from levulose. Analysis of the lactic acid obtained revealed two acids differing by the quantity of water they contained. The formulae given for the two were as follows:

- Liquid acid: \( C_6H_{12}O_6 \) or \( C_6H_4O_4 + 2\text{H}_2\text{O} \)
- The acid of the salts: \( C_6H_{12}O_8 \) or \( C_6H_4O_4 + \text{H}_2\text{O} \)
- The solid acid: \( C_6H_4O_4 \)

It is probable that the authors were dealing with anhydrides of lactic acid which would account for the differences that were obtained in the water content.

Many conflicting views have been proposed to account for the chemical structure of lactic acid. Strecker (1852, 1854) contended that the acid was a 12-carbon dibasic compound.

Kolbe (1859) on the other hand, believed lactic acid to be oxypropionic acid, then thought to be a 6-carbon monobasic compound. Wurtz and Friedel (1860, 1861) agreed to the oxypropionic acid suggestion, but contended that it contained only three carbons.

The latter work was supported by Wislicenus (1863) who described lactic acid as a 3-carbon monobasic acid and accepted the formula of Wurtz and Friedel. Later studies of chemical structure have established lactic acid with its present formula:
Discovery of lactic acid bacteria

Long before the chemical structure of lactic acid had been determined, many theories had been proposed to account for its formation. Boutron and Fresmy (1841) pointed out that all experiments which had been made demonstrated that the phenomena accompanying fermentation are produced by the influence of a particular "force of decomposition" brought about by substances of animal nature. Fermentation is not just a detached fact which is applicable only to the decomposition of sugar in the presence of yeast, but must be general for animal and plant tissues. The authors suggested the specificity of ferments pointing out that the products obtained depended much on the state of the animal matter. The view was held that air was necessary to change the animal matter into a ferment, but once fermentation had been instigated it could continue without air. Certain vegetable matters also were able to bring about the formation of lactic acid. A temperature of 100° was shown to stop the fermentation. In describing the souring of milk, casein was purported to be the active agent for the formation of lactic acid. As the milk sours the acid enters into combination with the casein of the milk rendering the ferment inactive. Upon the addition of calcium carbonate the acid is neutralized and the casein liberated to continue its fermentation until the lactose is completely fermented. Many similar theories were put forth and generally accepted until Pasteur
(1857, 1858) introduced his views on the existence of organized bodies, microscopic in size as responsible for the fermentation. These bodies he called "lactique levures" and were described as short rods or globules much smaller than yeast. A small quantity of the extract of a lactic fermentation added to a solution of sugar and chalk would bring about another fermentation in which lactic acid was formed. The nitrogenous material to which has been attributed the ability to bring about the breakdown of carbohydrates was asserted to serve as food for the microorganisms. The fermentations carried out by Pasteur apparently were not brought about by one ferment inasmuch as butyric acid was also obtained as one of the products. To Pasteur, however, must go the credit of assigning the role of lactic acid formation to microorganisms.

Béchamp (1866) found living microorganisms accompanying lactic and butyric acid fermentations which he named Mycrozyms. He supposed that these microorganisms occurred in the chalk added to neutralize the acids. It was intimated that they may have some connection with the fermentation but their role was not established.

Tréoul (1869) observed microorganisms in the must of beer. They occurred isolated and in chains and were about 0.1 mm. long with fine granulations. The medium in which they were grown became acid and the microorganisms conformed to Pasteur's description of the "lactique levures." The microorganisms isolated were reported to be bacteria. Perhaps Lister (1873) did the
first pure culture work with the lactic acid bacteria. He isolated and described a species which he named *Bacterium lactis* which was shown to be responsible for the souring of milk. Hueppe (1884), using Lister's technic, isolated another species which was named *Bacillus acidi lactici*. This microorganism differed from that of Lister in being able to produce gas. In Hueppe's study it was observed that the products varied with the species and condition of the culture. This work was followed by the isolation of lactic acid bacteria from milk, cheese, intestines, must of beer and spoiled wine, among other sources, by many investigators. As a result many species of lactic acid bacteria were isolated.
CHAPTER TWO

Classification of the Lactic Acid Bacteria.

The pure culture technic introduced by Lister (1873) led to an extensive program of isolation of new species and varieties. There was no uniformly accepted basis for describing and naming bacteria and as a result the descriptions were inadequate. Microorganisms when grown on different media often produce cells of varying size and arrangement. In cases the products are changed. The lactic acid bacteria in particular vary with their environment and in many cases the same organisms have been described as different species because of the failure to use a common medium. This lack of uniformity has made it difficult to correlate the properties and characteristics of the microorganisms, and allocate them to suitable systematic groups. To eliminate the chaos it would be necessary to study original cultures and make comparisons under controlled conditions. Such a procedure is impossible. Orla-Jensen (1919) suggested that the old descriptions be discarded and a new classification be worked out. From one point of view this might be satisfactory, but it would discard much work of value. An intelligent use should be made of the early investigations and descriptions.

The question arises as to the properties which should be
general description of bacteria which are given the
parasite's description of "tactique touissant" was
response to the letter (1878) on the parasitic
and are introduced with them in this classification. The macro-
resemblance of parasites of "tactique touissant" is
and should be associated with the bacterium of "touissant" or"touissant"
which they can be grouped. The tactique and "touissant" can-
parasite's food and the certain relations existing
in the classification of bacteria, in the grouping of species and the grouping in
The tactique of new species of bacteria to the proper group.
allocate one which has been treated to the proper group may be much less difficult to describe a new organism than to
terminate which may appear to be of minor significance. In
have been described as "tactique touissant" of bacteria
considered of sufficient importance to be used in different
probably the first time the gram stain was used to differentiate bacteria. The lactic acid bacteria were included in the group with *Bacillus aerogenes* and described as non-motile, medium sized, mostly plump, non-sporeforming, gram negative organisms. The lactic acid bacteria differed from other members of this group in their property of staining by the gram method.

Lehmann and Neumann (1896) and Migula (1897) placed the lactic acid bacteria in the genus *Bacterium*.

Conn (1899) found in his investigations in Dairy Bacteriology that the number of organisms with which he was confronted was rapidly increasing. He classified these bacteria into ten groups, basing his differentiation largely on morphology and pigment formation. Many of the organisms included in Conn's classification are now recognized as not being true lactic acid bacteria.

Weigmann (1899) realized the confusion existing with regard to bacteria encountered in the dairy industry. It was observed that the kind of medium employed greatly influenced the morphology of the bacteria. Sometimes organisms considered to be cocci under one set of conditions appeared as rods under another. This made classification difficult. It was thought best to consider those organisms subject to such variations as rods. It was observed that part of the organisms grew on the surface of the agar while others had a tendency to grow under the surface. Weigmann referred to the former group as aerobic and the
latter anaerobic. To the anaerobes he observed, belonged most of the organisms causing souring of milk. The bacteria were then divided into six groups. The manner of separation can best be shown by listing these groups. Much significance was attached to the optical rotation of the lactic acid formed.

Group I  **Bacterium lactis acidi** group. Short oval cocci occurring singly and in chains, facultative aerobes producing dextro-lactic acid. This group of bacteria contains most of those that acidify milk at room temperature.

Group II  **Bacillus acidi lactici** group. Short oval rods sometimes appearing as cocci, aerobic and produce dextro-lactic acid.

Group III  Levo-lactic acid bacteria. Each species differs in morphology and physiology. All form levo-lactic acid.

Group IV  Gelatin liquefying lactic acid bacteria. An aerobic micrococcus group.

Group V  Aerobic group which forms no gas. All other aerobic forms produce gas.

Group VI  True lactic acid bacteria having variable properties and not belonging to the above groups.

This grouping like that of Conn was not used as a basis of generic or species differentiation but was used in an attempt to place closely related organisms in common groupings. Weigmann (1905) extended his study of the lactic acid forming bacteria and made a general separation on the basis of their re-
the gram stain and the other on hydrogen formation.

the true tetlock method. One separation was on the basis of
that of Perlmutter suggested the cocoid-anaerobes occur in forms
bacteriologic Peet. Cocoid anaerobes. These differentiation like
soiled with gram positive. The former he treated with
soiled with gram negative and Lactobacillus Bacillus soiled
Kuhne (1920) differentiated between hydros, Peptococcus salt.

than the cocoid.

method was formed from lactose much more abundantly by the rods
lack of motility, smell of odors and absence of acetate. Men-
ordered the general characteristics of many acid production,
called Peptococcus. To Peptococcus and Peptococcus he as-
used the anaerobic group formed on hydrogen and was

the rods were shaped like small, diphtheroids. The rods were
name Peptococcus. The rods were shaped like small, diphtheroids.
The rods were shaped like small, diphtheroids. The rods were
two compounds in milk. The active, fermenting, product about the
made a systematic study of the forms not generated by
beside or lactate and formic acid bacteria from which.
Pratt reported to the milk organisms. These substances isolated a num-
studies on the lactate and baceteria had been continued
until the work of Given and Dubose (1899) and Kerster

as Peptococcus Peptococcus Peptococcus Peptococcus Peptococcus
the more anaerobic forms were referred to

15-
Lühnis (1907) attempting to further clarify the classification of the lactic acid bacteria made an extensive review of the literature pertaining to descriptions of these forms. Previous work had established the organisms as non-sporeforming cocci and rods which form white to yellow colonies, do not liquefy gelatin, but produce acid and gas and acidify milk. This general group was subdivided into four classes each represented by a general type.

**Group I** [**Bact. pneumoniae**. (**Bact. acidi lactici**)]

Plump rods, some form long threads, non-motile, gram-negative and grow both aerobically and anaerobically. This group is identical with Beijerinck's *Aerobacter*.

**Group II** [**Streptococcus pyogenes**. Oval, non-motile, capsule forming, non-sporeforming, gram positive organisms. Chiefly anaerobic. Identical with *Lactococcus*.

**Group III** [**Bacterium caucasicum**. Long rods, forming neither capsules nor spores, non-motile, gram positive, often forming long chains and mostly anaerobic. This group corresponds to Beijerinck's *Lactobacillus*.

**Group IV** [**Micrococcus pyogenes**. Spheres, non-motile, non-sporeforming, gram positive, mostly anaerobic, do not occur in chains.

The lactic acid bacteria had narrowed down to essentially a group of anaerobic non-motile, non-sporeforming rods and cocci. The tendency was to separate the gram negative hydrogen formers from this group and to leave what is now accepted as
KEY FOR THE DIFFERENTIATION OF THE RODS AND COCOIDS:  
without the following is the case ...  
new meaning following the Orfe-Jensen's ...  
pseudo monophosphates should not be used without some pretix of  
seaweed... it was stated, "The old generic names which are based  
lead to each other than some of the rods and cocoes are more to  
"assertion that can be made, "coherent rods and cocoes are more re-  
out that the above-differentiated in the only monophosphates  
solubility bacteria are non-motile and non-sporulating. It was pointed  
the primary differentiation separated rods and cocoes. Since the latter  
"would refer the confusion of the containing characteristics  
and described the monoregulated by new names which he espoused  
described all previous known names and many specific names  
"described the general accepted names of monoregulated le  
detailed study and classification of these and the  
"detailed work, (1979) Orfe-Jensen (1979) made a  
descriptions with previous characteristics and understand  
ctal characteristics  
characteristic suffixes in view of the differentiation in monophosphates  
Hes characterization of the bacteria are quite complete, but this  
made evident on the tenet of the cells and on chain formation  
product was taeate, "a characterization of those bacteria was  
non-sporulating rods which produced no hydrogen and whose other  
terea from many sources and accepted on the exam positive,  
the true lacto acid bacteria. Hennepen (1990) tested the  
-17-
Key for the differentiation of the rod forms.

I. Without catalase, reduction of nitrate or surface growth.

A. Produce only trace of by-products besides lactic acid.

   Long rods growth at 50°C. No acid in pentoses.
   a. Inactive lactic acid.
   b. Acid in maltose.
      1. Tbm. helveticum.
      bb. No acid in maltose.
      2. Tbm. Jugurt
      aa. Levo-lactic acid.
      b. Acid in milk.
          c. Acid in sucrose and maltose.
      3. Tbm. lactis.
         cc. No action on sucrose and maltose.
      4. Tbm. bulgaricum.
         bb. Do not curdle milk.
          c. Acid in sucrose and maltose.
      5. Tbm. cereale.

2. Genus: Streptobacterium.
   Inactive or dextro-lactic acid.
   a. Inactive lactic acid. Prefer maltose and sucrose to lactose.

5. Sbm. plantarum.
   aa. Dextro-lactic acid. Prefer lactose to maltose and sucrose.
7. *Bbm. casei*.
AA. Produce by-products other than lactic acid.
   a. Prefer arabinose. No action on disaccharides.
8. *Bbm. caucasicum*.
   aa. Ferment arabinose.
9. *Bbm. breve*.
   aaa. Never ferment arabinose, but sometimes ferment xylose.
10. *Bbm. longum*.
II. Usually produce catalase, reduce nitrate and give surface growth.
   11. *Bbm. lacticum*.
   12. *Bbm. mesentericum*.
   13. *Bbm. flavus*.

Key for the differentiation of the cocci.
I. Without catalase, reduction of nitrate or surface growth.
   A. Produce only trace of by-products besides lactic acid.
   1. Genus: *Streptococcus*.
      Always form dextro-lactic acid. Grow well in milk, not so well in yeast extract. As a rule only divide in one plane.
      a. Mostly shorter or longer chains. Never ferment pentoses.
      b. Ferment sucrose.
-20-

c. Do not ferment maltose, dextrin and salicin.  
   Do not break down casein
1. **Sc. thermophilus.**
cc. Ferment maltose, dextrin and salicin.  
   Do not break down casein.
d. Curdle milk.
2. **Sc. mastitides.**
   dd. Do not curdle milk.
3. **Sc. pyogenes.**
   bb. Do not ferment sucrose, maltose or dextrin,  
      frequently not salicin. As a rule break  
      down casein.
4. **Sc. cremoris.**  
   aa. Diplococci as well as longer chains, mostly  
      pentose fermentation. Always ferment maltose,  
      dextrin and salicin, as a rule also sucrose.  
   b. Ferment sorbitol and glycerol.
   c. Break down casein and liquefy gelatin.
5. **Sc. liquefaciens.**
   cc. Do not break down casein or liquefy gelatin.
6. **Sc. glycinerinaceous.**
   bb. Do not ferment sorbitol and glycerol.  
      Ferment raffinose, inulin and starch.
   c. Do not break down casein. Ferment xylose.
7. **Sc. inulinaceous.**
   cc. Break down casein.
8. **Sc. bovis.**
aa. Mostly diplococci. Always ferment maltose, dextrin and salicin, mostly pentoses too. 
b. Always ferment arabinose, as a rule sucrose too and frequently raffinose and rhamnose. Do not break down casein.

9. **Sc. faecium.**
bb. Never ferment sucrose, raffinose or rhamnose. Frequently break down casein.

10. **Sc. lactis.**

**AA:** Produce by-products other than lactic acid.

2. Genus: **Bacillus.**

Form levo-lactic acid or inactive lactic acid. Thrive well in yeast extract, but not in milk. Never ferment inulin and starch but ferment raffinose.

a. Ferment arabinose with predilection, frequently xylose. Diplococci or chains.

1. **Bc. arabinaceus**

aa. Never ferment arabinose, but frequently xylose. Often irregular forms dividing in two planes.

2. **Bc. bovis.**

II. With catalase, reduction of nitrate and surface growth.

3. Genus: **Tetracoccus.**

Form dextro-lactic acid. Division in two or three planes.

a. Ferment arabinose. Do not break down casein.

b. Ferment raffinose and salicin. Do not liquefy gelatin.
differentiation at home and heterogeneity of the acto-

Kluyver and monker (1926) referred to these as phyto-

sideres, were also made on the base of products formed from 

glucose. The acto sideres were used in the sterilization 

and served out directly and other remained in suspension. 

This be-

observed that some of them after a short time would fix the 

van Steenberge (1930) in a study of the acto acid bacteria. 

and on carbonhydrate fermentation. 

made primarily on the aerobic condition of the acto acid bacteria. 

and in a scheme of classification the Scottish classification 

however was the first to incorporate the phyto sideres reductanes. 

This desaturation is often used as the present time. 

One journey worked only at zero acid were called the true acto acid bacteria. 

bacit was first suggested by Dunst (1901). Those which pro-

of products other than lacto acid. The separation on the 

acid bacteria. The second separation is made on the formation 

include catalase positive organisms with the group of lacto 

These on the bases of catalase production. Besides that all 

orla-Jensen's (1919) differentiation of rod forms is made 

2. 2. Heterogeneity. 

and Heterogeneity. 

as do not ferment xylulose, break down ossein 

2. Fermentation. 

Heterogeneity. 

Fermentation. 

Fermentation. 

3. Do not ferment xylulose or reduction. 

1. Heterogeneity.
bacteria. The former is that group which produces only traces of products other than lactic acid while the latter produces, in addition, appreciable quantities of acetic acid, carbon dioxide, ethyl alcohol and glycerol. Van Steenberge suggested a separation of the bacteria on the basis of mannitol formation from levulose, a characteristic peculiar to the hetero-fermentative lactic acid bacteria. Beijerinck (1901) attributed the ability to produce mannitol from levulose to both genera, Lactobacillus and Lactococcus. The latter genus formed only small quantities of mannitol.

In 1917, a committee under the chairmanship of Dr. C.-E. A. Winslow of the Society of American Bacteriologists prepared a classification of the Bacteria into families and genera. The genus name Lactobacillus was accepted for the rod forms of lactic acid bacteria. The spherical forms were included in the genus Streptococcus.

Most of the spherical lactic acid bacteria encountered in the dairy industry have been placed in the species Streptococcus lactis. Owing to certain characteristics brought about by strains of this species in milk, Hammer and Baker (1926) have classified these strains as varieties of the species S. lactis. Hammer (1920) isolated two species of lactic acid bacteria from "starter" which he named Streptococcus citrovorus and Streptococcus paracitrovorus. Bergey (1923) placed a small group of heterofermentative lactic in the genus Leuconostoc which corresponds to the genus Betacoccus of Orla-Jensen (1919). The
genus Leuconostoc was studied systematically by Husker and Pederson (1930). Hammes's two species, S. citrovorus and S. paracitrovorus, were included as species of this genus.

A number of investigators have classified the lactic acid and Baker bacteria encountered in their particular fields. Hammes/1926 separated members of the genus S. lactis into varieties. Pederson (1929) gives a key for the differentiation of species encountered in spoiled tomato products. Rahe (1918) studied a group of lactics encountered in dental caries. Hunt and Rettger (1930) made a systematic study of the lactic acid organisms isolated from grain and soil. Serological and physiological reactions were not in agreement. It was concluded that the former reaction could not be used for differentiating species of lactic acid bacteria.

Henneberg (1926) enlarged on his old classification and accepted in large part Orla-Jensen's nomenclature.

In an attempt to provide a better and more satisfactory classification, Pederson (1934) presented a key to the species of the genus Lactobacillus. The primary separation was made into homo- and heterofermentative lactic acid bacteria. The second division was based on habitat. The latter division was less satisfactory than the former. It is difficult to point out definitely the natural habitat of many microorganisms. It is difficult to determine whether an organism isolated from soil is of plant, animal or soil origin. Species differentiations were made by Pederson on carbohydrate fermentations. In the key
many species have been grouped. In view of the incomplete descriptions given in the literature this procedure is probably advisable until further study is made.

The following key was presented by Pederson:
A. Produce only traces of by-products other than lactic acid.
   a. Usually of animal origin.
      b. Acid in sucrose.
         1. Lactobacillus caucasicus. B. Isbenis, Bact. mazuni.
         2. L. lactis.
         3. L. acidophilus.
         4. L. lactis acid.
         5. L. casei.
         6. L. thermophilus.
      bb. No action on sucrose.
         7. L. helveticus. Bacillus E.
      bbb. Sucrose unknown.
         9. L. boas-oppleri
   aa. Usually of plant or soil origin.
      b. Acid in lactose.
         c. Acid in arabinose.
         10. L. plantarum.
         11. L. cucumeris.
         12. L. wortmanni.
         13. L. busaeasiaticus.
25. *L. maninitoicus*.
26. *L. pastorianus*.
27. *L. wehmeri*.
28. *L. buchneri*.
29. *L. hayduckii*.

cc. No action on raffinose.

30. *L. pentoaceticus*.
31. *L. fermentatus*.
32. *L. panis*.
33. *L. lycopersici*.

bb. No action on arabinose.

34. *L. intermedius*.
35. *L. lindneri*.
36. *L. gayoni*.
37. *L. gracilis*.

bbb. Arabinose unknown.

38. *L. fermenti*.
39. *L. sovae*.

Morphological characteristics on which most of the previous classifications have been based must be supplemented by physiological behavior.

Fred, Peterson and Anderson (1921) in discussing the lactic acid bacteria state, "In general the organisms described by Hayser, Grimbert, Bertrand, Bendix, Gayon and Dubourg, Müller-Thurgau and Osterwalder and Henneberg are not described in sufficient detail to follow in identification of unknown forms of
lactic acid bacteria." Before a satisfactory classification can be made, basic study of the physiological reactions is essential. Such a study should show the natural relationships existing between the organisms and assist greatly in providing a satisfactory classification.
CHAPTER THREE

Dissimilation of the Lactic Acid Bacteria.

The heterofermentative lactic acid bacteria

Before the discovery of lactic acid bacteria many facts regarding their dissimilation of carbohydrates had been observed. Gay-Lussac and Pelouze (1833) conducted a two months' fermentation of beet juice and obtained, in addition to lactic acid, appreciable quantities of mannitol. It had already been observed that levulose was the only 6-carbon sugar that yielded mannitol. It was suggested that in the fermentation of beet juice the sucrose had been hydrolyzed and the resulting levulose reduced to mannitol. It was suggested that the sugar was converted into mannitol before being broken down to lactic acid. Fremy (1839) extended work on the lactic acid fermentation to mannitol and dextrin. Mannitol was shown to be fermented to lactic acid in the presence of calves' stomach tissue. It was concluded that this work supported the theory of Gay-Lussac and Pelouze that sugar passed through mannitol in its breakdown to lactic acid. In experiments on lactose it was found in some cases that nearly 100 per cent of the sugar could be accounted for as lactic acid.

Flügge (1894) pointed out that equation 1 held for fermentations in which the hexose was converted quantitatively into
lactic acid. In fermentations having additional products, the equation did not hold.

Gayon and Dubourge (1894, 1901) isolated a number of microorganisms from wine which were referred to as "ferment mannitique." The descriptions given for the organisms are incomplete, but the study made on their products of dissimilation places them definitely as heterofermentative lactic acid bacteria. This work appears to be the first quantitative study made of the dissimilation of carbohydrates by the heterofermentative lactic acid bacteria. Determinations were made of the fermentation products of glucose, levulose, galactose, mannose, sucrose, lactose, raffinose and xylose. In all experiments, a large percentage of the sugar fermented was accounted for by the products. Lactic, acetic and carbonic acids, glycerol and ethyl alcohol were formed from all except xylose which was fermented to acetic and lactic acids only. In addition to the above compounds mannitol was formed from levulose. Table 1, taken from Gayon and Dubourge (1901) shows the results of fermentation of glucose, levulose and xylose. The data are calculated as millimoles per 100 millimoles of sugar fermented.
In an anaerobic fermentation of glucose, which is not

the bacteria the oxidation-reduction index or redox index

is defined as the sum of the oxidation-reduction

values of the products and the sum of the

value of 1. To use the values given in Table 1, we

have an oxidation value or if glucose is given in

oxidation

standard

form (O = 0) and a reduction value of 1.

have a value of if glucose is given in the

standard

form of glucose. Two atoms

accompany with one-half the excess hydrogen atoms.

Under these conditions, if the compound is given in a reduction value of 1.0 found in water is considered negligible if the ratio is

and

Table 1. Oxidation of Glucose, Lactose, and

Redox Index

<table>
<thead>
<tr>
<th></th>
<th>Redox Index</th>
<th>Redox Index</th>
<th>Redox Index</th>
<th>Redox Index</th>
<th>Redox Index</th>
<th>Redox Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>glucose</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>lactate</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>acetate</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

(Data from Cahan and Dubrow, 1907)

* OXIDATION OF GLUCOSE, LACTOSE, AND

Table 1: Oxidation of Glucose, Lactose, and

-21-
values or redox index should equal one.

Since protein constituents of the medium may act as hydrogen acceptors and donors, perfect redox indexes can hardly be expected in fermentations requiring a complex nitrogen source. Fairly good agreement between redox indexes and carbon balances was obtained in most of the experiments carried out by Gayon and Dubourg (1901). It will be observed that only a small percentage of the compounds formed were either oxidized or reduced. A small error in the determinations of these compounds will markedly influence the redox index, but would have less effect on the carbon balance.

On the basis of their experiments Gayon and Dubourg (1901) proposed the following equations for the formation of the products:

2. For lactic acid \( C_6H_{12}O_6 \rightarrow 2 C_3H_6O_3 \)

3. For acetic acid \( C_6H_{12}O_6 \rightarrow 3 C_3H_4O_3 \)

4. For mannitol \( 15 C_6H_{12}O_6 + 6H_2O \rightarrow 12 C_6H_{14}O_6 + 6 CO_2 \)

It was suggested that succinic acid and glycerol were probably residues of microbial life.

For the fermentation of xylose, equation 5 was given.

5. \( C_8H_{10}O_5 \rightarrow C_3H_6O_3 + C_2H_4O_2 \)

Each compound is formed by an independent reaction which involves no definite quantitative relationship among the products.

Gayon and Dubourg (1901) conducted an experiment in which the fermentation was extended two months after the glucose had been completely used. Upon completion of the utilization of glucose, the ratio of lactic to acetic acid was 5.0. One month
later the ratio was 3:1; after two months, 1.8. It was con-
cluded from this experiment that the lactic acid was fermented
to acetic acid as represented by equation 6.

6. \[2 \text{C}_2\text{H}_5\text{O}_2 \rightarrow 3 \text{C}_2\text{H}_4\text{O}_2\]

In studying the formation of acetic acid in the lactic acid
fermentation Barthel (1900) pointed out that equation 7 could
not be correct for the lactic acid fermentation of lactose be-

7. \[\text{C}_{12}\text{H}_{22}\text{O}_{11} + \text{H}_2\text{O} \rightarrow 4 \text{C}_2\text{H}_4\text{O}_2\]

cause of other products that were formed. It was observed that
air and high temperatures markedly increased the formation of
acetic acid and it was concluded that acetic acid was formed
as the result of conditions unfavorable to the bacteria.

Mazé and Perrier (1903) made a study of the dissimilation
of glucose and levulose by microorganisms isolated from wine.
Glycerol and succinic acid were not determined. The products
from glucose were lactic, acetic and carbonic acids and ethyl
alcohol and, in addition, mannitol from levulose.

In the presence of air some microorganisms were able to
oxidize ethyl alcohol to acetic acid. Inasmuch as the fermen-
tations in these experiments were anaerobic equation 8 was pro-
posed for the formation of acetic acid. Ethyl alcohol is formed
intermediately in the reaction.

8. \[\text{C}_2\text{H}_5\text{O}_2 + 2 \text{H}_2\text{O} \rightarrow 2 \text{C}_2\text{H}_4\text{O}_2 + 4 \text{H}_2 + 2 \text{CO}_2\]

No free hydrogen was produced in these experiments. It
was therefore explained that the hydrogen formed reduces levu-
lose to mannitol. The formation of mannitol was represented by
equation 9.

\[ C_6H_5OH + 2 C_6H_12O_6 + H_2O \rightarrow C_6H_4O_2 + 2 C_6H_14O_6 \]

This reaction requires the formation of two molecules of mannitol for each of acetic acid. The dissimilation of levulose involves the same reactions as that of glucose. Ethyl alcohol reacts with levulose as shown by equation 9. To support this view, it was pointed out that by deducting the mannitol from sugar and the acetic acid accompanying the mannitol from the total acetic acid, the products were obtained in the same percentage relationship as they were from glucose. Equation 10 represents the general reaction proposed by Mazé and Perrier for the formation of mannitol from levulose. The intermediary

\[ 15 C_6H_{12}O_6 + 6 H_2O \rightarrow 6 C_6H_4O_2 + 12 C_6H_14O_6 + 2 CO_2 \]

formation of ethyl alcohol is assumed in the reaction. Lactic acid is formed from the sugar by an independent reaction.

Kayser (1904) extended the study of microorganisms isolated from wine. In general the products of fermentation were the same as those determined by Gayon and Dubourg (1894). In some instances formic and propionic acids were found. Acetic, formic and propionic acids were never found in the same fermentation. It is probable that Kayser was dealing with propionic acid bacteria as well as the lactic or his determination of propionic acid was at fault. The presence of air stimulated the formation of volatile acids as well as reduced the quantity of mannitol. Mannitol was shown to be fermented aerobically.

Laborde (1904) confirmed Kayser's contention that mannitol disappears after the levulose has been fermented.
Smit (1913) made a detailed study of *Lactobacillus fermentum*. After characterizing the organism, complete analyses were made of the products of different sugars. Formic acid in small quantities was added to the list of compounds formed from glucose by the heterofermentative lactic acid bacteria. This work is of sufficient importance in its completeness and its bearing on a scheme of dissimilation proposed later by Kuyver and Donker (1924) to present Table 2 showing the dissimilation products of glucose and levulose.

Table 2. Dissimilation of Glucose and Levulose by *L. fermentum*.

(Data from Smit (1913))

<table>
<thead>
<tr>
<th>Sugar fermented</th>
<th>100</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>mL</td>
<td>mL</td>
</tr>
<tr>
<td>Levulose</td>
<td>mL</td>
<td>mL</td>
</tr>
<tr>
<td>Ethyl alcohol</td>
<td>65.9</td>
<td>trace</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>11.1</td>
<td>25.6</td>
</tr>
<tr>
<td>Carbon dioxide</td>
<td>55.3</td>
<td>28.5</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>95.0</td>
<td>21.2</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>1.1</td>
<td>3.6</td>
</tr>
<tr>
<td>Glycerol</td>
<td>12.1</td>
<td>0</td>
</tr>
<tr>
<td>Mannitol</td>
<td>0</td>
<td>55.5</td>
</tr>
<tr>
<td>Formic acid</td>
<td>trace</td>
<td>trace</td>
</tr>
<tr>
<td>Carbon recovery</td>
<td>89.7</td>
<td>81.8</td>
</tr>
<tr>
<td>Redox index</td>
<td>0.769</td>
<td>1.026</td>
</tr>
</tbody>
</table>

In the table given by Smit, the weight of the microorganisms was included to account for the sugar utilized. In Table 2 as in Table 1 there is a marked deficiency in oxidized products.

Kuyver and Donker (1924) proposed that glucose is split into two 3-carbon compounds and proceeded according to the scheme
in figure 1.

\[ \text{C}_6\text{H}_{12}\text{O}_6 \]
\[ \text{Hexosephosphate} \]
\[ \text{Glyceraldehyde} \]
\[ \text{Methylglyoxal} \quad \rightarrow \quad \text{Lactic acid} \]
\[ \text{CH}_3\text{CHO} + \text{HCOOH} \rightarrow \text{CO}_2 + 2\text{H} \]
\[ \text{CH}_3\text{CH}_2\text{OH} \quad \text{CH}_2\text{COOH} \]

Figure 1. Dissimilation of Glucose by Heterofermentative Lactic Acid Bacteria. From Kluyver and Donker (1924).

If the scheme for the dissimilation of glucose is correct, the data in Tables 1 and 2 should show carbon dioxide equivalent to the sum of acetic acid and ethyl alcohol. Neither the data obtained by Smit nor that by Gayon and Dubourg support this relationship. Inasmuch as the redox index is low in both tables it is probable that complete recovery of carbon dioxide has not been obtained. In the fermentation of levulose good agreement was obtained between the one and two carbon compounds (Table 2). In Table 1, however, a large difference exists. Further discussion of this scheme will be given later.

Fred, Peterson and Davenport (1919) studied the fermentation of xylose by lactic acid bacteria. As Gayon and Dubourg (1901) had observed, only lactic and acetic acids were obtained from the fermentation by homofermentative lactic acid bacteria, which accounted for about 95 per cent of the sugar fermented. In all
cases the ratio of acetic to lactic acid was only slightly greater than 1. The highest values for the ratio were obtained in aerobic fermentations. In view of observations made on the fermentation of lactic acid, the ratio greater than one may be due to a secondary fermentation of lactic acid. The hetero-fermns produced, in addition, carbon dioxide and ethyl alcohol. The acetic acid was formed in a much higher concentration than in the glucose fermentation.

The fermentation studies of \textit{L. pentosaceticus} (typical hetero-fermentative organism) was extended to glucose, levulose and other sugars by Peterson, Fred and co-workers (1920, a, b, 1922). Glucose fermentations formed acetic acid, lactic acid, carbon dioxide and ethyl alcohol. Glycerol and succinic acid were not determined. In every experiment lactic acid and ethyl alcohol were formed in almost equimolar concentrations. This relationship led the authors to formulate the following equation for the dissimilation of glucose.

\[ C_6H_{12}O_6 \rightarrow CH_3CH(OH)COOH + CH_3CH_2OH + CO_2 \]

It was also observed that lactic acid was fermented to acetic acid. The failure of lactic acid to equal the ethyl alcohol and the formation of acetic acid were thus explained. An examination of the data showed that the sum of the milli-moles of acetic and lactic acids is greater than the ethyl alcohol. In nearly all cases the sum of ethyl alcohol and acetic acid equals the carbon dioxide, supporting the primary breakdown of the glucose into two 3-carbon compounds which are subsequently fermented to the 2- and 1-carbon compounds after the
manner of Kluvyer and Donker (1924). The redox index was greater than one and the carbon recovery was about 80 per cent. These balances show that some compound reduced with respect to glucose had not been determined. Glycerol, which had been found by previous investigators, would satisfy the requirements for a balanced fermentation.

From levulose, Peterson and Fred (1920) obtained mannitol to account for 70 per cent of the sugar. No ethyl alcohol was obtained. Acetic acid production was greater than from glucose. The results are in agreement with those of previous workers. The breakdown of levulose was represented by equation 12.

12. \[ C_6H_{12}O_6 + H_2O \rightarrow C_3H_6O_2 + 2H_2 + C_4H_6O_5 \]
levulose acetic acid malic acid
\[ C_4H_6O_5 \rightarrow C_3H_6O_2 + CO_2 \]
malic acid lactic acid
\[ 2C_6H_{12}O_6 + 2H_2 \rightarrow 2C_6H_{14}O_6 \]
levulose mannitol

The data in Table 2 substantiate this theory of the breakdown of levulose; those in Table 1 do not. Pederson's (1929a) experiments show that the organisms which ferment mannitol form a larger quantity of lactic than acetic acid from levulose. The data in Table 1 and those of Masé and Perrier (1903) show greater quantities of acetic acid than lactic acid from levulose. Appreciable quantities of ethyl alcohol were also obtained. The ability of the organisms to ferment mannitol and lactic acid may account for many of the different results.
Peterson and Fred (1920a) studied the intermediate products of glucose dissimilation by *L. pentosaceticus*. Sodium and calcium sulfite were added to the medium as fixatives. Sensitivity of these organisms to alkali resulted in little growth. Only traces of acetaldehyde were obtained. It was suggested that acetaldehyde is formed intermediately in the dissimilation of glucose.

Pederson (1929) isolated a number of lactic acid bacteria from spoiled tomato products and studied their dissimilation. The relationship obtained between ethyl alcohol and lactic acid is definitely in disagreement with the theory of their formation from glucose in equimolar concentrations as proposed by Peterson and Fred (1920). The study involving five different species showed the volatile acid to be pure acetic. Fermentation of arabinose was also carried out. Unlike the xylose fermentation, larger quantities of lactic than acetic acid were formed. Small quantities of carbon dioxide were also obtained.

The gaseous metabolism of *L. pentosaceticus* was investigated by Hunt (1933). Using a Warburg-Barcroft respirometer, the respiration of a suspension of the bacteria was studied. Comparisons were made between hetero- and homofermentative bacteria. All cells in glucose media utilized some oxygen, but *pentosaceticus* utilized a greater quantity than the homo-lactic organisms. The volume of oxygen consumed was practically equal to the carbon dioxide formed. Almost the same quantities of oxygen were consumed and carbon dioxide formed from glucose as from lactic acid.
From these facts it was concluded that the breakdown of glucose to lactic acid is an anaerobic process and respiration results from the breakdown of lactic acid. Aerobic dissimilation, however, resulted in little more acetic acid than did anaerobic.

Charleton, Nelson and Werkman (1934) isolated a new species of heterofermentative lactic acid bacteria from spoiled salad dressing. The products of fermentation of glucose and of levulose were the same as those obtained by Peterson, Fred and coworkers.

In discussing the heterofermentative lactic acid bacteria, Kluver (1935) suggested that succinic and formic acids obtained by Smit (1913) probably were not formed from the sugar. It was proposed by Kluver that the lactic acid formed during the fermentation is broken down by a secondary fermentation to acetic acid and carbon dioxide. This reaction, it was pointed out, involves a dehydrogenation. A hydrogenated product must, therefore, be accounted for. Glycerol arising from intermediary glyceric aldehyde could accept the hydrogen. The same contention with supporting data was put forth independently by Nelson and Werkman (1935).

From the review of studies of dissimilation the following conclusions may be drawn.

1. No studies have been made on the mechanism of the primary breakdown of carbohydrates. The initial breakdown represented by Kluver and Donker was patterned after that of other fermentations.
2. In most instances the schemes have been based on those of other fermentations and on the quantitative relationship of the final products of fermentation. In cases the data do not support the scheme.

3. Acetaldehyde is the only intermediate compound isolated. The role this compound plays in the fermentation has not been determined.

4. In the majority of cases the conditions of fermentation were not defined making the results quite indefinite.

Much work is necessary to clarify the basic reactions involved in the dissimilation of carbohydrates by the heterofermentative lactic acid bacteria.

The homofermentative lactic acid bacteria

Unlike the heterofermentative lactic acid bacteria, the homofermentative group ferments the carbohydrates essentially to lactic acid. The fermentation is generally accepted as the simplest known. The dissimilation by the homo-lactics involves the splitting of glucose into 3-carbon compounds and a molecular rearrangement. Von Euler and Svanberg (1917) pointed out the similarity of lactic and alcoholic fermentation to muscle glycolysis. It was suggested that the initial phases of the breakdown may be identical for the three types of metabolism. In view of the similarity of muscle glycolysis to the lactic fermentations the investigations dealing with muscle metabolism may throw additional light on the course of dissimilation by lactic acid bacteria.

Studies on the initial breakdown of glucose by homofermentative lactics were begun by Virtanen and co-workers in 1924. Using
suspensions of dried as well as living *Lactobacillus casei* (E, *casei* c) Virtanen (1924, 1924a, 1924b) showed that inorganic phosphate in the medium decreased to a minimum after which they began to increase. When the sugar had completely disappeared the phosphate concentration was as high as at the beginning of the fermentation. Studies with *Streptococcus lactis* failed to show any phosphorus uptake. The reaction with *L. casei* is very similar to that shown by Harden and Young (1908) in the yeast fermentation from which a hexosephosphate ester was isolated and assigned an intermediary role. The work of Virtanen demonstrates an intermediate phosphorylation which was suggested as the formation of a hexosephosphate ester. Virtanen and Karström (1928) showed that by grinding the microorganisms an extract could be obtained that would bring about a phosphorylation independently of the cells. The interesting observation was made that the dried bacteria transformed only about 50 per cent of the glucose fermented, into lactic acid. Virtanen and Tikka (1930) investigated this behavior and found that the dried bacteria had converted part of the sugar into two different phosphoric acid esters. One ester was difficultly soluble and the others easily soluble in water. Analysis of the soluble ester showed it to be a 12-carbon monophosphoric acid ester. The formation of a disaccharide monophosphate ester had been shown by Neuberg and Leibowitz (1928) when hexosebiphosphate was fermented by lactic acid bacteria. The Virtanen and Tikka ester was different in that no reducing sugar was formed from it by hydrolysis.
The diffusely soluble ester gave indications of being a six-carbon monophosphate ester. The latter compound hydrolyzed with difficulty and leads one to speculate as to the possibility of this ester being the same as Lohmann's (1930) diffusely hydrolyzed ester. Lohmann's ester on first consideration was thought to be a hexose ester but was later established as a mixture of two triose esters. An intermediate hexosemonophosphate ester corresponding to the Robison ester was isolated by Virtanen and Tikka from both dried and living bacteria. The following scheme (figure 2) was proposed for the dissimilation of glucose by living and dried suspensions of *L. casei*.

![Scheme for Dissimilation of Glucose by L. casei](image)

<table>
<thead>
<tr>
<th>Living Bacteria</th>
<th>Dried Bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>Glucose</td>
</tr>
<tr>
<td>→</td>
<td>→</td>
</tr>
<tr>
<td>Hexosemonophosphate</td>
<td>Hexosemonophosphate</td>
</tr>
<tr>
<td>→</td>
<td>→</td>
</tr>
<tr>
<td>Lactic acid (100%)</td>
<td>New stable phosphate ester (50%)</td>
</tr>
<tr>
<td></td>
<td>Lactic acid (50%)</td>
</tr>
</tbody>
</table>

**Figure 2. Scheme for Dissimilation of Glucose by *L. casei***

Tychowski and Kobel (1928) showed the hexosebiphosphate ester to be fermented to lactic acid by *L. delbrückii*.

A series of investigations by Neuberg and co-workers has thrown much light on the breakdown of glucose by the homo-lactic acid bacteria. The dismutation of methylglyoxal to lactic acid was shown for several microorganisms. Neuberg and Gorr (1925) demonstrated the formation of lactic acid from methylglyoxal by *L. delbrückii*. The compound was suggested as intermediary in the lactic acid fermentation. Virtanen, Karström and Bach (1926) and
Virtanen and Karström (1926) attempted to confirm the work of Neuberg and Gorr using different species of lactic acid bacteria, but were forced to conclude that methylglyoxal was not converted to lactic acid by the true lactic acid bacteria. Negative results were obtained by using the same species as Neuberg and Gorr used. Similar observations were made by Schrader (1931).

Kostytschew and Soldatenkov (1927) reported the isolation of methylglyoxal from the dissimilation of glucose by members of the homofermentative lactic group. In carrying out the isolation, semicarbazide was used as a fixing agent. Neuberg and Kobel (1928) questioned this work showing that hydrazo-di-carbon-amid, a compound very similar to methylglyoxal in its properties was formed from semicarbazide itself. Neuberg and Kobel (1929) isolated methylglyoxal from a fermentation of the hexosebiphosphate. About 85 per cent of the ester was recovered as methylglyoxal. The formation of methylglyoxal was represented by equation 15.

13. \[ \text{C}_6\text{H}_{12}\text{O}_6 \longrightarrow \text{CH}_3\text{COCHO} + \text{H}_2\text{O} \text{ or CH}_3\text{COCH(\text{CH})}_2 \]

Simon (1931) offered new evidence for the formation of lactic acid from methylglyoxal by lactic acid bacteria.

The methylglyoxal scheme of dissimilation was questioned by Lohmann (1932). He showed that reduced glutathion was necessary for the formation of lactic acid from methylglyoxal by muscle tissue, but glycogen and also glucose could be fermented to lactic acid in the absence of the co-enzyme. Synthetic methylglyoxal was converted into lactic acid in the presence of the co-enzyme.
Embden, Deuticke and Kraft (1933) and Meyerhof (1933) proposed schemes of carbohydrate dissimilation by muscle tissue and yeast not involving methylglyoxal as an intermediary. The following reactions were proposed for muscle glycolysis:

1. The hexose is phosphorylated with the fermentation of a hexose-biphosphate ester.
2. The hexose ester is split into two molecules of dihydroxyacetonephosphate (in equilibrium with glycerinaldehydephosphate).
3. One molecule of the 3-carbon ester undergoes an oxidation to phosphoglyceric acid with the simultaneous reduction of a second molecule to glycerophosphoric acid.
4. The phosphoglyceric acid is hydrolyzed and converted to pyruvic acid.
5. The pyruvic acid accepts hydrogen from glycerophosphoric to form lactic acid, oxidizing the glycerophosphoric acid to dihydroxyacetonephosphate.

Dissimilation by yeast differs slightly from muscle glycolysis. The formation of glycerophosphoric acid is necessary only to start the fermentation. Pyruvic acid is decarboxylated instead of being reduced. Acetaldehyde formed by the decarboxylation is reduced with the simultaneous oxidation of the 3-carbon ester to phosphoglyceric acid. Inasmuch as the intermediary formation of methylglyoxal is questionable and in view of the recent isolation of phosphoglyceric acid from fermentations by Citrobacter freundii (Werkman, Zoellner, Gilman and Reynolds, 1936) the principles of the Embden-Meyerhof scheme may be valuable in outlining the breakdown of carbohydrates in bacterial fermentations.
Kostytschew, Gwaladzi and Eliasberg (1930) demonstrated the formation of pyruvic acid by lactic acid bacteria. It was suggested that pyruvic acid itself may not be an intermediate compound but may exist as a radical of the nature CH₃CO-CO- which by the addition of fixatives becomes stabilized as pyruvic acid or methylglyoxal.

Simon (1932) disagreed with Kostytschew's theory and pointed out that pyruvic acid had been formed in yeast fermentations without the aid of fixatives. He further showed that pyruvic acid could be obtained from lactic acid and suggested Kostytschew, Gwaladzi and Eliasberg may have obtained their pyruvic acid from the secondary fermentation of lactic acid.

Fromageot and Roux (1931, 1933, 1933a) studied the relative rates of fermentation of different sugars by L. bulgaricus. The relative velocities were in the following order: levulose, mannose, glucose and galactose. In the presence of air, hydrogen peroxide was formed from glucose. Am-hexose represents the form of hexose that undergoes fermentation.

Bertho and Glick (1932) demonstrated the formation of hydrogen peroxide and reported it to be a typical hydrogenation process
in which oxygen acts as a hydrogen acceptor. The reaction involved was reported to be the conversion of glucose to carbon dioxide and water. Davis (1933, 1933a) confirmed the hydrogen peroxide formation. Certain species (L. casei), it was observed used no oxygen and in saturated solutions of air formed no hydrogen peroxide. Methylene blue had no action; the aerobic and anaerobic reactions were almost identical. L. delbrückii on the other hand fermented glucose with the formation of hydrogen peroxide. Hydrogen peroxide was formed from lactic acid also (equation 14).

\[ \text{CH}_3\text{CHOHCOOH} + 3\text{H}_2\text{O} + 6\text{O}_2 \rightarrow 3\text{CO}_2 + 6\text{H}_2\text{O} \]

The respiratory quotient was found to be 0.6; according to the equation it should be 0.5.

No hydrogen peroxide was obtained by Davis from pyruvic acid. It was suggested that pyruvic acid was broken down to acetic acid and carbon dioxide (equation 15). The respiratory quotient was 2 in agreement with the equation.

\[ \text{CH}_3\text{CO COOH} + 0 \rightarrow \text{CH}_3\text{COOH} + \text{CO}_2 \]

Methylene blue stimulated the fermentation of glucose and lactic acid but not that of pyruvic acid. The fermentation of glucose to \( \text{CO}_2 \) and water passed through lactic acid but probably not pyruvic acid. Pyruvic acid was isolated from aerobic fermentations by fixing with bisulfite.

From a review of the work on the mechanism of dissimilation of carbohydrates by lactic acid bacteria, it is evident that only fragmentary knowledge has been gained. With respect to the
heterofermentative group, acetaldehyde is the only intermediate compound that has been isolated. Otherwise, the quantitative relationships of the final products and the observation of the fermentation of lactic to acetic acid constitute the only basis for a clear understanding of the dissimilative behavior.

The similarity between the products of dissimilation of the homofermentative lactic acid bacteria and muscle tissues has led to a more complete study of this group of bacteria. The phosphorus uptake and formation of hexosephosphate has been established by Virtanen and co-workers. Although methylglyoxal has been isolated from hexosebiphosphate and subsequently fermented, this compound is losing favor as a normal intermediary. Pyruvic acid has been isolated and fermented, but its rôle in the course of dissimilation is still a matter of controversy and speculation. Much work is necessary before an understanding of the nature of the dissimilation can be attained.
PART II

EXPERIMENTAL

CHAPTER ONE

Methods

General considerations

A study of the basic reactions involved in the dissimilation of carbohydrates by lactic acid bacteria is essential to an understanding of their relationships to other microorganisms as well as to develop further the fields in which they play an important rôle. In the present investigation the work has been confined primarily to a study of the course of dissimilation by certain species of lactic acid bacteria represented by identified and widely known cultures.

In studying the sequence of reactions involved in the dissimilation of carbohydrates the following methods may be employed:

1. The carbohydrates may be fermented and the products analyzed quantitatively. Quantitative relationships of the compounds formed indicate certain reactions in the course of dissimilation.

2. The normal course of fermentation may be disturbed in some manner (addition of fixing agents, change in pH, etc.) to bring about the stabilization of intermediate compounds to the
extent that they can be isolated and identified.

3. The fermentation of probable intermediate compounds alone and in combination with carbohydrates often does much to clarify the breakdown of such compounds in the course of fermentation.

4. Certain hydrogen acceptors and donators added to carbohydrate fermentations affect the quantitative relationships of products as to throw light on intermediary reactions.

5. Serial analysis of the liquor during fermentation may lead to certain conclusions with respect to the course of dissimilation.

With a view of ascertaining the physiological behavior and intermediate compounds in the breakdown of carbohydrates by known species, the investigation was carried out as follows:

1. Quantitative studies were made on the dissimilation of glucose.

2. Analyses were made on the medium at intervals during the period of fermentation.

3. Hydrogen acceptors, acetaldehyde and acetyl-methylcarbinol, were added to glucose fermentations.

4. Certain fixatives were added to fermentations with the view to isolating intermediary compounds.

5. Probable intermediate compounds, pyruvic acid and lactic acid, the latter with and without hydrogen acceptors were fermented.

6. Levulose was fermented. Quantitative analyses of the products were made at intervals during fermentation.
Methods of analysis

It is evident that a relatively large number of products are formed whose determinations are complicated. The complex nitrogen source required for growth increases the difficulty. There is little doubt that yeast extract and peptone, used as nitrogen sources in these investigations influence the course of dissimilation. In fact Parnas, Ostern and Mann (1934) have demonstrated the necessity of certain nitrogen compounds in the dissimilation of glycogen by muscle tissue. In the present experiments control fermentations, in which no carbohydrates were present, were run. In general the fermentation was slight and the analyses were little different from unfermented media. In later experiments unfermented media were analyzed and the values obtained used as controls.

The fermentation of glucose yielded the following compounds: lactic acid, acetic acid, ethyl alcohol, carbon dioxide, glycerol, succinic acid and formic acid. Other experiments involved the quantitative determination of acetaldehyde, pyruvic acid, acetylmethylcarbinol, 2,3-butyleneglycol, levulose and mannitol.

Carbon dioxide. A stream of oxygen-free nitrogen was passed through the medium to insure anaerobic conditions as well as to carry the carbon dioxide into Bowen potash bulbs where it was determined gravimetrically. The carbon dioxide remaining in solution was removed by acidifying the medium to congo red with sulfuric acid and heating under a reflux condenser.
Ethyl alcohol. An aliquot part of the medium (usually about 300-400 ml.) was distilled directly until about two-thirds had distilled over. In the presence of unfermented carbohydrates the distillation was carried out in a slightly acid solution. The distillate was made alkaline with sodium hydroxide and two-thirds again distilled over. Ethyl alcohol was determined in an aliquot part of the second distillate by oxidation to acetic acid according to Stahly, Osburn and Werkman (1934). Stahly (1935) showed that when ethyl alcohol alone was oxidized a yield of acid equivalent to only 94 per cent was obtained. When acetylmethylcarbinol is present, it also is oxidized. One molecule of acetylmethylcarbinol yields two of acetic acid. Corrections were made.

In experiments to which acetaldehyde had been added, the second distillation was made into a solution of 2,4-dinitrophenylhydrazine. The hydrazone was filtered from the solution and washed. The combined distillate and washings were neutralized and distilled. Ethyl alcohol was determined on the distillate.

Acetylmethylcarbinol. This compound was determined on a 50 to 100 ml. fraction by the method of van Niel (1927) as modified by Stahly and Werkman (1936).

Acetaldehyde. Owing to the volatility of acetaldehyde it was necessary to determine loss carried over by the stream of nitrogen by placing a bead tower containing sodium bisulfite between the fermentation flask and Bowen potash bulbs. To prevent sulfur dioxide, formed by the breakdown of sodium bisulfite,
from interfering with the carbon dioxide determination, an acid permanganate solution was placed between the bisulfite tower and potash bulbs. The acetaldehyde carried over was determined on an aliquot part of the bisulfite solution. The uncombined sodium bisulfite was removed with iodine; the acetaldehyde was then liberated from bisulfite by means of sodium bicarbonate and the bisulfite titrated with standard iodine solution.

The acetaldehyde in the fermentation liquor was determined on an aliquot part by distilling the aldehyde into a sodium bisulfite solution and determining as described above.

**2,3-Butylene Glycol.** 2,3-Butylene glycol was determined on a separate aliquot part of the fermentation by the method of Brockmann and Werkman (1933). Corrections were made for acetyl-methylcarbinol according to Stahly and Werkman (1936). Before distillation, however, the unfermented sugar was removed by copper sulfate and lime (Hewitt, 1931) to avoid the decomposition of the sugar which would interfere with determinations made on the residue of the distillation.

**Volatile acids.** The residues of the first and second distillations in the determination of ethyl alcohol were combined, acidified to Congo red with sulfuric acid and determined by the method of Osburn, Wood and Werkman (1933).

In the presence of pyruvic acid which is slightly volatile the first distillate was neutralized, evaporated to about 300 ml. and subjected to a second steam distillation. The volatile
acids were determined on the second distillate.

When possible separate aliquot parts of the fermented liquor were used for determination of the non-volatile acids. When the quantity of medium was limited, determinations were made on the residues of steam distillation. About 100 ml. of the medium were evaporated to 10-20 ml. and acidified to congo red with 25 per cent sulfuric acid. The acidified liquor was taken up in anhydrous sodium sulfate and extracted with ethyl ether.

**Lactic acid.** The lactic acid was determined on an aliquot part of the ether extract by Nelson's (1933) modification of the Friedemann, Gorton and Schaffer (1927) method. In fermentations in which 2,3-butylene glycol was present an aliquot part of the residue of the distillation for 2,3-butylene glycol was evaporated to 20 ml., taken up in anhydrous sodium sulfate and determined as above.

**Succinic acid.** Succinic acid was determined as the silver salt by a modification of Moyle's (1924) method. A detailed discussion of this method is given by Reynolds (1935) and Stahly (1935).

**Pyruvic acid.** After deproteinating an aliquot part of the carbohydrate-free medium, the pyruvic acid was determined according to Wendel (1931).

**Glucose and levulose.** An aliquot part of the medium was deproteinated with phosphotungstic acid (25 per cent in 5 per cent sulfuric acid solution and the sugar determined by the
Munson and Walker (1906) method. Acetaldehyde was removed by evaporation before the glucose was determined. Corrections were made for acetyl methylcarbinol according to Stahly and Werkman (1936).

Glycerol. Glycerol was determined by a modification of Wagenaar's (1911) method. This method consists of adding solutions of sodium hydroxide and copper sulfate in equal quantities to a solution containing glycerol. The copper hydroxide precipitate was removed by centrifuging and an aliquot part of the supernatant liquor acidified with sulfuric acid. Potassium iodide was added to the acidified solution and the liberated iodine titrated with sodium thiosulfate. An empirical relationship was set up between the mgm. of glycerol and ml. of standard sodium thiosulfate. Table 1 shows the relative accuracy with which determinations of solutions of pure glycerol can be duplicated.

Table 1. Determination of Glycerol in Pure Solutions.

<table>
<thead>
<tr>
<th>Mgm. glycerol: 0; 80; 160; 320; 480; 800; 1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ml. of 0.1N: 0.2; 1.5; 3.1; 7.0; 11.3; 21.4; 27.5</td>
</tr>
<tr>
<td>sodium thiosulfate: 0.3; 1.5; 3.2; 6.9; 11.2; 21.2; 27.0</td>
</tr>
<tr>
<td>sulfate: 0.3; 1.5; 3.2; 7.2; 11.0; 21.3; 27.4</td>
</tr>
</tbody>
</table>
Figure 1. Nomogram for Determination of Glycerol.
The curve by which the mgm. of glycerol in the 100 ml. of copper solution are read directly is shown in figure 1. It will be noted that the curve begins a little above the origin. This condition results from the titration induced by the solutions used in the determination.

Lactic acid, sugars and certain proteins have been found to affect the determination. It was necessary, therefore, to eliminate these. Pure glycerol was found to be extracted slowly with ethyl ether from a neutral solution taken up in anhydrous calcium sulfate. (The technical sodium sulfate used was too acid for a neutral extraction.) After 72 hours the glycerol was completely extracted. In later experiments in which glucose, yeast extract and peptone were present, the quantity extracted was quite variable. In general complete extraction was obtained but often the results were low. To overcome this difficulty as well as to shorten the period of extraction, acetone was used as a solvent. It was observed, however, that some of the carbohydrates and proteins were extracted. This was overcome by removing the proteins with phosphotungstic acid and the sugar with copper sulfate and lime according to Hewitt (1931).

Typical results carried out with known quantities of glycerol are shown in Table 2. Yeast extract, peptone and sugar were present in quantities of the same magnitude as occurred in the media.
Table 2. Effect of Proteins and Carbohydrates on the Determination of Glycerol.

<table>
<thead>
<tr>
<th>Glycerol added:</th>
<th>Glycerol found</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mgm.</td>
<td>Ml. of 0.1N Na₂S₀₄</td>
<td>Percent</td>
</tr>
<tr>
<td>80</td>
<td>1.5</td>
<td>100.0</td>
</tr>
<tr>
<td>200</td>
<td>3.9</td>
<td>92.3</td>
</tr>
<tr>
<td>400</td>
<td>8.9</td>
<td>97.5</td>
</tr>
<tr>
<td>80</td>
<td>1.5</td>
<td>100.0</td>
</tr>
<tr>
<td>200</td>
<td>4.3</td>
<td>102.3</td>
</tr>
<tr>
<td>400</td>
<td>8.8</td>
<td>96.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>1.6</td>
<td>106.8</td>
</tr>
<tr>
<td>200</td>
<td>4.0</td>
<td>95.3</td>
</tr>
<tr>
<td>400</td>
<td>9.0</td>
<td>98.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Determination of glycerol in the fermentations was made as follows: The residue of acid steam distillation was evaporated to about 20 ml., neutralized with sodium hydroxide and taken up in anhydrous calcium sulfate. The resulting mixture was allowed to stand two or three hours to become dry, pulverized, placed in extraction thimbles and extracted continuously for eight hours with acetone. The acetone was distilled from the extract. About 25 ml. of water were added when the volume of acetone containing the extract reached 10 to 20 ml. and the remainder of acetone removed by heating on steam bath. To the extract was added about 5 ml. of phosphotungstic acid solution, the volume made to 100 ml. and filtered. An aliquot part of the filtrate (about 80 ml.) was placed in 200 ml. volumetric
flasks and 40 ml. each of a 5 per cent lime suspension and a 10 per cent copper sulfate solution were added. The volume was made to 200 ml. and the solution shaken intermittently for thirty minutes. The precipitate was removed by centrifuging and a 25 to 40 ml. aliquot part of the supernatant liquid neutralized and placed in a 100 ml. volumetric flask. Twenty-five ml. of 4 N sodium hydroxide and 25 ml. of a 12.5 per cent copper sulfate solution were added. After shaking intermittently for ten minutes the mixture was centrifuged. To 25 ml. of supernatant liquid, 10 ml. of 25 per cent sulfuric acid and 10 ml. of 30 per cent potassium iodide were added. The iodine liberated was titrated with sodium thiosulfate and the mgm. of glycerol read from the chart (figure 1).

**Mannitol.** Mannitol and glycerol undergo the same reactions with copper under the conditions used for the determination of glycerol. Smit (1914) made a nomogram for the determination of mannitol using the same conditions as have been described for glycerol. Smit's method was followed in the present work. The same procedure was used in preparing the mannitol solution for analysis as described for glycerol. Ethyl alcohol, instead of acetone, was used as a solvent. In the presence of mannitol, glycerol was extracted with ethyl ether and determined. The titration corresponding to the glycerol determination was subtracted from that of the mannitol determination to correct for glycerol. The mgm. of mannitol were read from Smit's nomogram.
Bacteria used

In this investigation it was attempted to employ physiologically different groups of lactic acid bacteria. Identified cultures of the following species were used. For complete cultural descriptions the reader is referred to Pederson (1929), Charleton, Nelson and Werkman (1934); also Bergey (1934).

Homofermentative group

\textit{Lactobacillus plantarum}

\textit{Lactobacillus cucumeris}

Heterofermentative group

\textit{Lactobacillus lycopersici}

\textit{Lactobacillus acidophil-aerogenes}

\textit{Lactobacillus manntopoecus}

\textit{Lactobacillus fructivorans}

\textit{Lactobacillus gracilis}

\textit{Leuconostoc dextranicus}

All cultures except \textit{L. fructivorans} and \textit{L. gracilis} were obtained from Dr. C. S. Pederson. \textit{L. gracilis} was received from Dr. A. Osterwalder. \textit{L. fructivorans} was isolated from spoiled salad dressing and described by Charleton, Nelson and Werkman (1934). \textit{L. fructivorans} and \textit{L. gracilis} were carefully studied and compared morphologically and culturally by the latter investigators. They concluded that two distinct species should be recognized. Morphologically \textit{L. fructivorans} differs from \textit{L. gracilis} in size, the latter being much smaller. The colonies
of *L. gracilis* are also smaller. Oxygen and temperature relationships and nutritive requirements are the same for both organisms. Trehalose and α-methylglucoside are fermented by *L. gracilis*, but not by *L. fructivorans*.

All species were gram positive, non-motile, non-sporeforming, microaerophilic and catalase negative.

**Medium**

The composition of the medium used in different experiments varied. In general the following basal medium was employed to which the substrate was added:

- **Yeast extract (Difco)** 0.2 per cent
- **Peptone (Difco)** 1.0 per cent
- **K₂HPO₄** 0.6 per cent
- **KH₂PO₄** 0.6 per cent

It was observed that equal quantities (by weight) of the phosphate salts brought about the desired pH of 6.2 after autoclaving. In early experiments 0.2 per cent of each of the phosphate salts were used. By increasing the quantity of buffer to 0.6 per cent of each, the substrate was more rapidly and completely fermented.

The solutions of (1) substrate, (2) yeast extract and peptone and (3) phosphates were sterilized separately and combined aseptically at the time of inoculation.
CHAPTER TWO

Quantitative Studies on the Dissimilation of Glucose.

The homofermentative lactic acid bacteria

The homofermentative lactic acid bacteria produce only traces of compounds other than lactic acid from glucose. Schrader (1931) pointed out that in the industrial preparation of lactic acid small amounts of acetic acid were always formed. In reviewing the work of Dunst (1923) Schrader pointed out that the nitrogen source influenced appreciably the quantity of acetic acid obtained. It was suggested that the volatile acids come from the nitrogen source. Peptone alone gave appreciable quantities of volatile acid. Acetic acid was not obtained by fermenting lactic acid or ethyl alcohol. A quantity of volatile acid not exceeding 0.2 per cent of the sugar was obtained in fermentations by L. delbrückii.

In many reports on the lactic acid fermentation, yields of 85 to 95 per cent of the sugar fermented have been obtained as lactic acid.

An experiment was arranged in which the products were determined quantitatively. The basal medium with two per cent glucose was inoculated with 25 ml. of a three-day culture. The fermentations were carried out anaerobically. After 21 days'
Schroeder (1837)

To determine the quantity of volatile extraneous matter that may have been removed from the yeast extract by the previous fermentation, the following experiment was performed:

1. A sample of yeast extract was fermented for 24 hours at 30°C.
2. The fermented extract was then heated to 80°C for 15 minutes to denature the proteins.
3. The denatured extract was then fermented for an additional 24 hours at 30°C.
4. The quantity of volatile extraneous matter was determined by analyzing the gas produced during fermentation.

The results showed that approximately 10% of the initial volatile extraneous matter was removed during the first fermentation. When the extract was further denatured and refed, an additional 5% of volatile extraneous matter was removed.

It is noteworthy that the removal of volatile extraneous matter was not complete, as residual quantities were still present after the second fermentation.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Concentration of Volatile Extraneous Matter (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>10</td>
</tr>
<tr>
<td>50</td>
<td>5</td>
</tr>
</tbody>
</table>

From these results, it can be concluded that a significant amount of volatile extraneous matter can be removed from yeast extracts by controlled fermentation conditions.
It has been suggested that acetic acid may arise from the proteins. The quantitative relationship between acetic acid and carbon dioxide suggests their formation from a 3-carbon compound. The formation of these two compounds involves an oxidation which in an anaerobic fermentation must be accompanied by a reduction of one or more compounds formed from the dissimilation of glucose. Glycerol was the only reduction product. To satisfy the oxidation-reduction relationships, glycerol should be equivalent to twice the acetic acid. This relationship does not hold for all experiments in Table 3. Either some reduced compound other than glycerol was formed or the determination of glycerol or acetic acid was in error. The agreement between acetic acid and carbon dioxide supports the accuracy of their determinations.

In Table 4 a comparison is made between phosphate and calcium carbonate buffers. The phosphates in the basal medium were replaced by 1.0 per cent calcium carbonate which increased the rate of fermentation. Table 4 shows less carbon dioxide and acetic acid formed in the medium containing calcium carbonate than in the phosphate buffered medium. In many of the experiments the carbon dioxide obtained from fermentations to which calcium carbonate had been added was less than could be obtained from calcium carbonate alone. At the time of analysis the failure to obtain complete recovery of the carbon dioxide was attributed to the method of determination. Soda-lime was used to determine the carbon dioxide. In view of the recent report of Wood and Werkman (1935) that propionic acid bacteria are able to utilize
Table 3. The Dissimilation of Glucose by Homofermentative Lactic Acid Bacteria.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Lactic acid</th>
<th>Acetic acid</th>
<th>Carbon dioxide</th>
<th>Glycerol</th>
<th>Percent</th>
<th>Redox index</th>
<th>% of carbon</th>
<th>% of recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobacillus</td>
<td>180.0</td>
<td>90.0</td>
<td>16.4</td>
<td>5.4</td>
<td>16.5</td>
<td>2.7</td>
<td>9.7</td>
<td>4.8</td>
</tr>
<tr>
<td>plantarum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Streptobacterium</td>
<td>164.2</td>
<td>82.1</td>
<td>17.2</td>
<td>5.7</td>
<td>33.7</td>
<td>5.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>plantarum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactobacillus</td>
<td>178.5</td>
<td>89.2</td>
<td>9.3</td>
<td>3.1</td>
<td>6.1</td>
<td>3.0</td>
<td>19.2</td>
<td>9.6</td>
</tr>
<tr>
<td>cucumeris</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4. Effect of Calcium Carbonate on the Dissimilation of Glucose by Homofermentative Lactic Acid Bacteria

<table>
<thead>
<tr>
<th>Organism</th>
<th>Lactic acid</th>
<th>Acetic acid</th>
<th>Carbon dioxide</th>
<th>Ethyl alcohol</th>
<th>Per cent</th>
<th>Redox recovered</th>
<th>index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobacillus plantarum</td>
<td>CaCO₃</td>
<td>160.7</td>
<td>84.8</td>
<td>4.0</td>
<td>1.3</td>
<td>4.7</td>
<td>0.8</td>
</tr>
<tr>
<td>Phosphate</td>
<td>170.8</td>
<td>85.4</td>
<td>10.2</td>
<td>3.4</td>
<td>5.6</td>
<td>0.9</td>
<td>0.5</td>
</tr>
<tr>
<td>Lactobacillus cusceriae</td>
<td>CaCO₃</td>
<td>191.4</td>
<td>95.7</td>
<td>8.4</td>
<td>2.8</td>
<td>3.2</td>
<td>0.5</td>
</tr>
<tr>
<td>Phosphate</td>
<td>191.0</td>
<td>82.5</td>
<td>17.4</td>
<td>5.7</td>
<td>10.5</td>
<td>1.9</td>
<td>0.5</td>
</tr>
</tbody>
</table>
carbon dioxide the question arises as to whether the lactic acid bacteria may be able to do likewise. At present the relationship of other products formed do not warrant such a theory.

It has often been observed that upon extending the period of incubation, lactic acid is broken down to acetic acid and carbon dioxide. In most of these reports the conditions of fermentation were not defined.

Lactic acid was found to be fermented aerobically; no anaerobic breakdown of added acid was obtained. Aerobically, oxygen will act as a hydrogen acceptor but anaerobically no acceptor is present, except probably in the proteins. When glucose is fermented hydrogen acceptors may be reduced with the simultaneous oxidation of lactic acid.

To study the quantitative relationship between the products at intervals during fermentation a five-liter fermentation of glucose was set up and samples withdrawn on the second, fourth, eighth and eleventh days of incubation at 30° C. The medium was inoculated with 50 ml. of a three-day culture of L. plantarum. Anaerobic conditions were maintained by continuously bubbling oxygen-free nitrogen slowly through the medium. The results are shown in Table 5, calculated as mM per 100 mM of glucose fermented.

Certain relationships are shown by the data. Acetic acid and carbon dioxide were formed in equimolar quantities, and increased continuously during fermentation. It is not probable that the two compounds would be formed in this relationship from
the proteins. The acetic acid and carbon dioxide are probably formed from an intermediary three-carbon compound. The carbon balance was low throughout the fermentation, but increased as the fermentation progressed. The question arose as to whether an intermediary compound, not determined by analysis, was formed which was slowly fermented to acetic acid and carbon dioxide.

In view of the report of Wood and Werkman (1934) of the formation of a non-reducing sugar by the propionic acid bacteria as well as the communication by Virtanen and Tikka (1931) showing the formation of a disaccharide phosphate ester, a second fermentation by L. plantarum was run. The sugar was determined on each sample before and after hydrolyzing with 10 per cent hydrochloric acid. The results of the analyses are shown in Table 6.

In agreement with the previous experiment Table 6 shows a simultaneous increase in acetic acid and carbon dioxide. The lactic acid, however, decreased during the period of fermentation. No ethyl alcohol was formed. The determinations of glucose on hydrolyzed and non-hydrolyzed portions agree within the limits of experimental error. It is evident that no non-reducing carbohydrate that can be hydrolyzed by 10 per cent hydrochloric acid was formed. The disaccharide phosphate ester obtained by Virtanen and Tikka (1931) would not reduce Fehling's solution after hydrolysis. The compound was not obtained, however, from proliferating bacteria.

Apparently carbon dioxide and acetic acid are formed from some 3-carbon compound. The decrease in lactic acid in Table 6
Table 5. Serial Analysis of the Dissimilation of Glucose by *L. plantarum.*

<table>
<thead>
<tr>
<th>Period of fermentation: Days</th>
<th>Lactic acid (mM)</th>
<th>Acetic acid (mM)</th>
<th>Carbon dioxide (mM)</th>
<th>Ethyl alcohol (mM)</th>
<th>Glycerol (mM)</th>
<th>Carbon (mM)</th>
<th>Sugar (mM per liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>120.5</td>
<td>60.2</td>
<td>1.8</td>
<td>0.6</td>
<td>2.3</td>
<td>0.4</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>171.5</td>
<td>85.6</td>
<td>2.2</td>
<td>0.7</td>
<td>4.3</td>
<td>0.7</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>158.0</td>
<td>84.9</td>
<td>4.7</td>
<td>1.6</td>
<td>4.9</td>
<td>0.9</td>
<td>0.74</td>
</tr>
<tr>
<td>11</td>
<td>176.5</td>
<td>88.3</td>
<td>5.9</td>
<td>2.0</td>
<td>5.7</td>
<td>1.0</td>
<td>0.89</td>
</tr>
</tbody>
</table>

*Calculated as millimoles per 100 millimoles of glucose fermented.
suggests a secondary fermentation of lactic acid to acetic acid and carbon dioxide. If an intermediary precursor of lactic acid were being dissimilated to acetic acid and carbon dioxide the same effect would be obtained, i.e., an increase in acetic acid and carbon dioxide and a decrease in lactic acid.
Table 6. Serial Analysis of the Dissimilation of Glucose by *L. plantarum.*

<table>
<thead>
<tr>
<th>Period of fermentation</th>
<th>Lactic acid</th>
<th>Acetic acid</th>
<th>Carbon dioxide</th>
<th>Glycerol</th>
<th>Carbon</th>
<th>Glucose fermented balance</th>
<th>mM per liter</th>
<th>Not hydrolyzed</th>
<th>Hydrolyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days</td>
<td>mM</td>
<td>cent</td>
<td>mM</td>
<td>cent</td>
<td>mM</td>
<td>cent</td>
<td>mM</td>
<td>cent</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>186.2</td>
<td>93.1</td>
<td>3.5</td>
<td>1.2</td>
<td>0.4</td>
<td>0.1</td>
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<td>0.4</td>
<td>4.5</td>
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</tr>
<tr>
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<td>171.8</td>
<td>85.9</td>
<td>4.6</td>
<td>1.5</td>
<td>4.6</td>
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<td>2.8</td>
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<td>5.1</td>
<td>1.7</td>
<td>5.4</td>
<td>0.9</td>
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<td>1.9</td>
<td>5.8</td>
<td>0.9</td>
<td>3.8</td>
<td>1.9</td>
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<td>1.9</td>
<td>5.9</td>
<td>1.0</td>
<td>6.2</td>
<td>3.1</td>
<td>88.2</td>
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<td>81.0</td>
<td>5.4</td>
<td>1.8</td>
<td>5.7</td>
<td>0.9</td>
<td>3.8</td>
<td>1.9</td>
<td>83.8</td>
</tr>
<tr>
<td>85</td>
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<td>6.1</td>
<td>1.0</td>
<td>5.3</td>
<td>2.7</td>
<td>87.4</td>
</tr>
</tbody>
</table>

*Calculated as millimoles per 100 millimoles of glucose fermented.*
The heterofermentative lactic acid bacteria

The heterofermentative lactics are closely related to the homofermentative group. They form appreciable quantities of ethyl alcohol, acetic acid, carbon dioxide and glycerol from glucose.

Quantitative determinations were made of the products of the anaerobic dissimilation of glucose. Twenty grams of glucose were added to each liter of basal medium. In the first experiment only 0.2 per cent $K_2HPO_4$ and 0.2 per cent $KH_2PO_4$ were used. However, it was found that increasing the percentage of phosphate buffers to 0.6 per cent of each the glucose was fermented at a greater rate. The medium was inoculated with 25 ml. of a 3-day culture. Nitrogen was passed continuously through the medium to insure anaerobic conditions. In view of the similarity between the products of glucose fermentation by species of Lactobacillus and Leuconostoc studies were made with both genera.

In Tables 7 and 8 are typical results. Reliability of the data is satisfactory in view of the difficulty encountered in making quantitative determinations of the products. The general agreement between carbon balances and redox indexes is satisfactory. The relationships of the products should throw some light on the intermediary mechanism of dissimilation.

In Table 7 glycerol was not experimentally determined. The results showed a low carbon recovery and an excess of oxidized products. It is evident that some reduced product had not been
### Table 7. Dissimilation of Glucose by Heterofermentative Lactic Acid Bacteria.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Lactic acid</th>
<th>Acetic acid</th>
<th>Carbon dioxide</th>
<th>Ethyl alcohol</th>
<th>Glycerol</th>
<th>Carbon balance</th>
<th>Redox index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobacillus</td>
<td>mM</td>
<td>mg</td>
<td>mM</td>
<td>mg</td>
<td>mM</td>
<td>mg</td>
<td>mM</td>
</tr>
<tr>
<td>mannitoposus</td>
<td>84.2</td>
<td>42.1</td>
<td>21.4</td>
<td>7.1</td>
<td>90.1</td>
<td>150</td>
<td>66.4</td>
</tr>
<tr>
<td>Lactobacillus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysopersici</td>
<td>94.1</td>
<td>47.5</td>
<td>18.2</td>
<td>6.1</td>
<td>74.6</td>
<td>123</td>
<td>59.3</td>
</tr>
<tr>
<td>Leuconostoe</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mesenteroides</td>
<td>90.0</td>
<td>45.0</td>
<td>18.4</td>
<td>6.1</td>
<td>98.7</td>
<td>163</td>
<td>87.3</td>
</tr>
<tr>
<td>Leuconostoe</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mesenteroides</td>
<td>85.7</td>
<td>42.8</td>
<td>10.0</td>
<td>3.0</td>
<td>93.8</td>
<td>15687.0</td>
<td>29.0</td>
</tr>
</tbody>
</table>

*Calculated.*
determined. Glycerol was found in fermentations run by Gayon and Dubourg (1901) and Smit (1913). In the second experiment (Table 8) glycerol was determined. The data show that under the conditions of the experiments the glycerol formed was equivalent to twice the acetic acid. If glycerol is calculated on this basis for the experiments in Table 7 the carbon balances and redox indexes show good agreement.

Succinic acid also was determined in the experiments in Table 3. Only traces of the compound were found. It is probable that succinic acid arises from the proteins as suggested by Kluyver (1935) and Nelson and Werkman (1935).

Several important relationships are shown by the data. The percentage of sugar fermented that can be accounted for by each product is variable. Lactic acid accounts for as much as 59.6 per cent of the glucose fermented by L. mannotop ears in Table 8 and as low as 42.1 per cent in Table 7. The difference in the quantity of buffer present in the two media probably had some effect on the quantities of products formed. Different conditions affect different relationships among the products.

To determine whether results could be duplicated under conditions maintained as nearly identical as possible (1) glucose, (2) yeast extract and peptone and (3) phosphate solutions were sterilized separately. At the time of inoculation identical aliquot parts were placed, aseptically, in four 1-liter flasks and the pH adjusted to 6.2. Inoculations were made with 10 ml. of a 3-day culture of L. lycopersici. Anaerobic conditions were
<table>
<thead>
<tr>
<th>Organism</th>
<th>Lactic acid</th>
<th>Acetic acid</th>
<th>Carbon dioxide</th>
<th>Ethanol</th>
<th>Glycerol</th>
<th>Carbon balance:Redox</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobacillus mannitopoeus</td>
<td>119.2:59.6:25.4</td>
<td>6.5:65.2:10.9:50.3:16.7:22.5:14.2:107.9</td>
<td>1.010</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. acidophil-aerogenes</td>
<td>121.5:60.7:11.5</td>
<td>3.8:49.5</td>
<td>8.3:44.5:14.8:24.5:12.2</td>
<td>99.8</td>
<td>.873</td>
<td></td>
</tr>
<tr>
<td>L. lycopersici</td>
<td>81.5:40.7:15.1</td>
<td>5.0:80.5:13.4:78.9:26.3:31.2:15.6</td>
<td>101.0</td>
<td>.851</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. lycopersici</td>
<td>73.5:36.8:18.0</td>
<td>3.0:86.8:14.5</td>
<td>75.6:25.2:39.3:18.6</td>
<td>101.9</td>
<td>.912</td>
<td></td>
</tr>
<tr>
<td>L. lycopersici</td>
<td>81.2:40.6:15.1</td>
<td>2.5:79.2:13.2:72.5:24.1:31.9:15.8</td>
<td>99.0</td>
<td>.884</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. lycopersici</td>
<td>80.0:40.0:17.4</td>
<td>2.9:84.7:14.3:70.1:23.3:38.7:19.8</td>
<td>103.0</td>
<td>.943</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
maintained during twenty-one days' incubation at 30° C. The results (Table 7) were quite consistent. Lactic acid accounts for 36.8 to 40.7 per cent of the sugar fermented. Three of the determinations are almost identical. The variations among the other products are somewhat greater although not so large as in fermentations carried out under different conditions. It appears that under similar conditions, the relationships between the products of fermentation may be duplicated with a fair degree of consistency.

Much larger quantities of acetic acid, carbon dioxide and ethyl alcohol were formed by the heterofermentative lactics than by the homofermentative group. The data in Tables 7 and 8 show that the millimoles of carbon dioxide nearly equal the sum of acetic acid and ethyl alcohol. It appears that both acetic acid and ethyl alcohol are formed by the breakdown of 3-carbon intermediary compounds; each molecule of acid and alcohol being accompanied by one of carbon dioxide.

The glycerol was equal to twice the acetic acid. The oxidation of a 3-carbon intermediary to acetic acid and carbon dioxide must be accompanied by a reduction. If an intermediary such as glyceric aldehyde were reduced, the quantitative relationship of glycerol to acetic acid would be as was found, i.e., two molecules of glycerol for each molecule of lactic acid.

In view of observations that lactic acid may be fermented aerobically to acetic acid and carbon dioxide, it is possible that lactic acid formed from glucose may undergo slow secondary
fermentation. Other explanations are discussed later.

**Serial analysis of the dissimilation of glucose**

As a fermentation progresses, many changes take place in the conditions under which the microorganisms are growing. It is probable that a change in the conditions may affect a change in the relationships between the products. By analyzing a medium at intervals such changes in the relationships may be observed. Results thus obtained may throw light on the intermediary mechanism of dissimilation.

Five-liter portions of basal medium to which was added glucose (2 per cent) were inoculated with 50 ml. of 3-day cultures of *L. pentosaceus*, *Leuco. dextranicus* and *L. lycoopersici*. Nitrogen was passed intermittently through the medium. The data are calculated (Table 9) as millimoles per liter and also (Table 10) as millimoles per 100 millimoles of glucose fermented.

The sugar fermented rapidly at first reaching a value of 63.8 mM in four days. In the next five days only 4.5 mM were fermented. The relationships existing among the products varied. Although the quantity of lactic acid was not decreased (Table 9) the percentage of sugar fermented to lactic acid became less (Table 10). Ethyl alcohol, carbon dioxide and glycerol increased. There was some increase in acetic acid. The relationships can be better visualized by plotting the millimoles of product per 100 millimoles of glucose fermented against time (figure 2). If the product when plotted in this manner forms a straight line paral-
parallel to the horizontal axis, the percentage of sugar going to
this compound is constant and can be expressed by a simple chem-
ical equation. If the slope of the line is concave upward the
percentage of sugar being fermented to this compound is increas-
ing, if downward the percentage is decreasing, showing (a) a
change in the rate of the various reactions or (b) a secondary
fermentation of one or more of the products.

In figure 2 ethyl alcohol and acetic acid are substantially
constant. Carbon dioxide and glycerol show an increase. Lactic
acid on the other hand is decreasing as the fermentation pro-
gresses and may be undergoing a secondary fermentation to acetic
acid and carbon dioxide with a simultaneous formation of glycerol.
The results may be explained also by a shifting in the equilib-
rium of the system such that the rate of formation of acetic acid
and carbon dioxide as well as glycerol from intermediary compounds
is increased, resulting in a decrease in the percentage of lactic
acid formed from the sugar.

The data for L. lycopersici are shown in Tables 11 and 12.
This fermentation and that by Leuconostoc dextranicus were slow
in getting started. Little sugar had disappeared in the first
ten days of fermentation. In view of the small quantities of
products formed much of the variation during the first three or
four analyses may be due to the experimental conditions.

As in the experiment with L. pentaceticus, the percentage
of sugar fermented to lactic acid gradually decreased. Acetic
acid remained more or less constant. Carbon dioxide and ethyl
Table 9. Periodic Analysis of the Dissimilation of Glucose by L. pentosaceus.*

<table>
<thead>
<tr>
<th>Period of fermentation</th>
<th>Lactic</th>
<th>Acetic</th>
<th>Carbon dioxide</th>
<th>Ethyl alcohol</th>
<th>Glycerol</th>
<th>Glucose</th>
<th>fermented</th>
<th>balance</th>
<th>Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days</td>
<td>Car-</td>
<td>Car-</td>
<td>Car-</td>
<td>Car-</td>
<td>Car-</td>
<td>Car-</td>
<td>Car-</td>
<td>Car-</td>
<td>Car-</td>
</tr>
<tr>
<td>2</td>
<td>76.0:228.0</td>
<td>6.5:13.0</td>
<td>6.2:6.2:4.4:8.8:2.7:8.1:48.5:291.0</td>
<td>100.7:1.07</td>
<td>8.1:48.5:291.0</td>
<td>100.7:1.07</td>
<td>8.1:48.5:291.0</td>
<td>100.7:1.07</td>
<td>8.1:48.5:291.0</td>
</tr>
<tr>
<td>4</td>
<td>110.7:332.1</td>
<td>9.0:18.0:11.0:11.0</td>
<td>5.4:10.8:8.7:26.1:63.8:382.8</td>
<td>104.0:1.12</td>
<td>8.7:26.1:63.8:382.8</td>
<td>104.0:1.12</td>
<td>8.7:26.1:63.8:382.8</td>
<td>104.0:1.12</td>
<td>8.7:26.1:63.8:382.8</td>
</tr>
<tr>
<td>8</td>
<td>111.0:333.0</td>
<td>9.8:19.6:14.0:14.0</td>
<td>5.9:11.8:19.6:58.8:68.0:408.0</td>
<td>107.1:0.89</td>
<td>5.9:11.8:19.6:58.8:68.0:408.0</td>
<td>107.1:0.89</td>
<td>5.9:11.8:19.6:58.8:68.0:408.0</td>
<td>107.1:0.89</td>
<td>5.9:11.8:19.6:58.8:68.0:408.0</td>
</tr>
</tbody>
</table>

*Calculated as millimoles per liter.
Table 10. Periodic Analysis of the Dissimilation of Glucose by *L. pentosaceicu*. *

<table>
<thead>
<tr>
<th>Period of fermentation</th>
<th>Lactic acid</th>
<th>Acetic acid</th>
<th>Carbon dioxide</th>
<th>Ethyl alcohol</th>
<th>Glycerol</th>
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<tbody>
<tr>
<td>Days</td>
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<td>mM</td>
<td>mM</td>
<td>mM</td>
<td>mM</td>
<td>mM</td>
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<td>78.3</td>
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<td>4.5</td>
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<td>2.1</td>
</tr>
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<td>4.7</td>
<td>17.2</td>
<td>3.0</td>
</tr>
<tr>
<td>8</td>
<td>163.2</td>
<td>81.6</td>
<td>14.4</td>
<td>4.8</td>
<td>20.6</td>
<td>3.4</td>
</tr>
<tr>
<td>11</td>
<td>162.6</td>
<td>81.5</td>
<td>16.7</td>
<td>5.5</td>
<td>24.2</td>
<td>4.0</td>
</tr>
</tbody>
</table>

*Calculated as millimoles per 100 millimoles of glucose fermented.
Figure 2. Serial Analysis of the Products of Glucose Dissimilation by *L. pentosaceus*. 
alcohol on the other hand showed increases. Instead of equaling the sum of ethyl alcohol and acetic acid as has been shown in previous experiments with *L. lycopersici*, the carbon dioxide nearly equals the ethyl alcohol. In determining the carbon dioxide an error made on one of the first determinations would be included in the remainder of the determinations of this compound. Such an error may account for the low yield of carbon dioxide in this fermentation.

The data for the fermentation of glucose by *L. lycopersici*, plotted in figure 3, show a decrease in lactic acid and an increase in ethyl alcohol and carbon dioxide. It appears that lactic acid may be undergoing a decarboxylation to ethyl alcohol and carbon dioxide. Since the decarboxylation of lactic acid by lactic acid bacteria has never been demonstrated it is probable that ethyl alcohol is formed by the dissimilation of an intermediary 3-carbon compound.

The results of the experiment with *Leuconostoc dextranicus* are assembled in Tables 13 and 14. In this experiment the third and fourth determinations of carbon dioxide were low, due to an accident which resulted in the error being carried forward in the remainder of the determinations. In spite of this error there is a definite increase in the percentage of glucose fermented to carbon dioxide. Ethyl alcohol was also increased. The percentage of glucose going to lactic acid, on the other hand, decreased. In this experiment, also, the percentage of glucose fermented to acetic acid remained more
Table II. Periodic Analysis of the Dissimilation of Glucose by L. lycopersici.*

<table>
<thead>
<tr>
<th>Period of fermentation</th>
<th>Glucose</th>
<th>Lactic</th>
<th>Acetic</th>
<th>Carbon Dioxide</th>
<th>Ethyl Alcohol</th>
<th>Glycerol</th>
<th>Carbon</th>
<th>Redox Balance</th>
<th>Index</th>
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</thead>
<tbody>
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<td>mm</td>
<td>mm</td>
<td>mm</td>
<td>mm</td>
<td>mm</td>
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<td>0.6</td>
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<td>9.2</td>
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<td>8.3</td>
<td>8.3</td>
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<td>156.0</td>
<td>16.9</td>
<td>50.7</td>
<td>8.8</td>
<td>17.6</td>
<td>16.3</td>
<td>16.3</td>
<td>13.8</td>
</tr>
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<td>37.2</td>
<td>223.2</td>
<td>31.9</td>
<td>95.7</td>
<td>9.2</td>
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</tr>
<tr>
<td>32</td>
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<td>502.2</td>
<td>58.5</td>
<td>175.5</td>
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<td>33.4</td>
<td>46.5</td>
<td>46.5</td>
<td>47.6</td>
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<tr>
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<td>654.0</td>
<td>85.1</td>
<td>255.3</td>
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<td>37.4</td>
<td>75.7</td>
<td>75.7</td>
<td>81.0</td>
</tr>
<tr>
<td>86</td>
<td>120.0</td>
<td>720.0</td>
<td>91.6</td>
<td>274.8</td>
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<td>56.0</td>
<td>87.6</td>
<td>87.6</td>
<td>199.2</td>
</tr>
</tbody>
</table>

*Calculated as millimoles per liter.
Table 12. Serial Analysis of the Dissimilation of Glucose by *L. lycopersici.*

<table>
<thead>
<tr>
<th>Period of fermentation</th>
<th>Days</th>
<th>Lactic acid</th>
<th>Acetic acid</th>
<th>Carbon dioxide</th>
<th>Ethyl alcohol</th>
<th>Glycerol</th>
<th>Carbon balance</th>
<th>Redox index</th>
</tr>
</thead>
<tbody>
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<td>50.0</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>140.0</td>
<td>70.0:51.0:59.0:75.0:13.0:40.5:13.5:48.7:24.3:149.8</td>
<td>1.15</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>68.7</td>
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<td>0.31</td>
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</tr>
<tr>
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<td>92.5</td>
<td>46.2:31.7:10.6:57.2:9.8:57.2:19.1: -: -: 85.4</td>
<td>1.00</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>64.2</td>
<td>32.1:33.4:11.1:61.9:10.3:52.5:17.5:46.0:25.0:95.3</td>
<td>0.82</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>88.8</td>
<td>42.9:24.7:8.2:57.0:9.8:80.0:26.6:34.2:17.1:103.2</td>
<td>0.59</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>80.9</td>
<td>40.4:23.4:7.3:50.3:8.4:54.5:21.5:21.1:10.5:99.5</td>
<td>0.67</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>78.2</td>
<td>39.1:17.1:5.7:69.5:11.6:74.3:24.8: -: -: 82.8</td>
<td>0.90</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>86</td>
<td>76.4</td>
<td>33.2:24.6:5.2:73.0:12.2:82.6:27.5:23.2:11.6:101.6</td>
<td>0.77</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Calculated as millimoles per 100 millimoles of glucose fermented.
or less constant. The quantity of lactic acid present in the fermentation was not decreased. The relationships between the products are shown graphically in figure 4.

It is evident from the foregoing experiments that a change in experimental conditions resulted in different relationships between the products. Ethyl alcohol was formed at the expense of lactic acid. Either lactic acid is being decarboxylated to ethyl alcohol and carbon dioxide or an intermediary precursor of lactic acid is being broken down.

The ability of lactic acid bacteria to ferment lactic acid to acetic acid and carbon dioxide aerobically suggests lactic acid as a possible precursor of acetic acid. In the fermentation of glucose, other compounds intermediary in the dissimulation may give rise to acetic acid and carbon dioxide. Glycerol may result from the reduction of intermediary glyceric aldehyde or some closely related compound or even from the hydrolysis of glycerophosphoric acid which has been postulated as an intermediary in the Embden-Meyerhof scheme of muscle and yeast dissimilation. A discussion of the various schemes of dissimilation will be given later.
Figure 3. Serial Analysis of the Dissimilation of Glucose by L. lycopersici.
Table 15. Products of Dissimilation of Glucose by Leucoscentoc dextranicus.

<table>
<thead>
<tr>
<th>Period of fermentation</th>
<th>Lactic acid</th>
<th>Acetic acid</th>
<th>Carbon dioxide</th>
<th>Alcohol</th>
<th>Glycerol</th>
<th>Carbon balance</th>
<th>Redox index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days:</td>
<td>mM</td>
<td>mM</td>
<td>mM</td>
<td>mM</td>
<td>mM</td>
<td>mM</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>17.4:4.1:2.6:1.0:1.0:0.7:1.4:5.6:10.2:181.5:0.40</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>6.2:37.2:4.4:13.2:8.8:17.6:2.4:2.4:1.8:3.6:2.1:6.3:116.7:0.84</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>10.7:64.2:14.3:42.9:3.7:7.4:5.2:5.2:5.2:6.4:11.2:33.6:148.9:0.57</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>43.4:250.4:42.8:128.4:6.8:13.6:12.1:12.1:20.7:41.4:--:--:75.1:0.58</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>62.8:376.3:62.0:186.0:6.1:12.2:18.6:18.6:46.3:92.6:--:--:82.1:0.40</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>75.5:453.0:53.2:189.6:8.2:16.4:37.9:37.9:60.3:120.6:18.1:54.3:88.0:0.54</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>93.3:529.2:37.9:203.7:8.5:17.0:43.0:43.0:67.8:135.6:--:--:75.5:0.64</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>89.3:535.3:52.2:156.6:8.5:17.0:64.7:64.7:63.3:126.6:18.5:55.5:80.0:0.88</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>86</td>
<td>103.9:623.4:81.1:243.3:12.5:25.0:69.7:69.7:90.5:181.0:18.2:54.6:92.0:0.69</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Calculated as millimoles per liter.
<table>
<thead>
<tr>
<th>Period of fermentation</th>
<th>Lactic acid</th>
<th>Acetic acid</th>
<th>Carbon dioxide</th>
<th>Ethyl alcohol</th>
<th>Glycerol</th>
<th>Carbon : Redox balance : index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days</td>
<td>mM</td>
<td>cent</td>
<td>mM</td>
<td>cent</td>
<td>mM</td>
<td>cent</td>
</tr>
<tr>
<td>2</td>
<td>86.7</td>
<td>44.8</td>
<td>157.2</td>
<td>52.4</td>
<td>58.4</td>
<td>6.4</td>
</tr>
<tr>
<td>4</td>
<td>71.0</td>
<td>35.5</td>
<td>142.0</td>
<td>47.0</td>
<td>38.7</td>
<td>6.4</td>
</tr>
<tr>
<td>7</td>
<td>134.5</td>
<td>67.2</td>
<td>34.5</td>
<td>11.5</td>
<td>48.6</td>
<td>8.1</td>
</tr>
<tr>
<td>10</td>
<td>98.9</td>
<td>49.4</td>
<td>15.8</td>
<td>5.3</td>
<td>27.8</td>
<td>4.6</td>
</tr>
<tr>
<td>14</td>
<td>98.6</td>
<td>49.4</td>
<td>9.7</td>
<td>3.2</td>
<td>29.6</td>
<td>4.9</td>
</tr>
<tr>
<td>19</td>
<td>83.8</td>
<td>41.9</td>
<td>10.3</td>
<td>3.6</td>
<td>50.2</td>
<td>8.4</td>
</tr>
<tr>
<td>32</td>
<td>76.8</td>
<td>38.4</td>
<td>9.6</td>
<td>3.2</td>
<td>48.7</td>
<td>8.1</td>
</tr>
<tr>
<td>46</td>
<td>53.4</td>
<td>29.2</td>
<td>9.5</td>
<td>3.2</td>
<td>72.5</td>
<td>12.1</td>
</tr>
<tr>
<td>86</td>
<td>78.1</td>
<td>39.1</td>
<td>12.0</td>
<td>4.0</td>
<td>67.0</td>
<td>11.2</td>
</tr>
</tbody>
</table>

*Calculated as millimoles per 100 millimoles of glucose fermented.*
Figure 4. Serial Analysis of Glucose dissimilation by Leuconostoc dextranicus.
CHAPTER THREE

Intermediary Reactions in the Dissimilation of Glucose.

Homofermentative lactic acid bacteria

The breakdown of glucose by homofermentative lactic acid bacteria was described in Part I. Virtanen and co-workers showed the phosphorylation of the hexose molecule with the formation of a hexosephosphate ester. Neuberg and co-workers showed the formation of methylglyoxal from hexosebiphosphate and the conversion of methylglyoxal into lactic acid. Meyerhof and Lohmann have discounted methylglyoxal as an intermediary inasmuch as a certain co-enzyme was necessary to bring about the conversion but was not necessary for the formation of lactic acid from glucose or glycogen. Instead of methylglyoxal, phosphoglyceric acid and pyruvic acid were put forth as intermediaries.

Fermentation of pyruvic acid. Pyruvic acid has been repeatedly isolated from fermentations of glucose by muscle tissue and yeast. Kostyschew, Gwaladzi and Eliasberg (1930) isolated pyruvic acid from fermentations by homofermentative lactic acid bacteria. Simon (1932) showed pyruvic acid to be formed by the dissimilation of lactic acid. It is not improbable that pyruvic may be intermediary in the breakdown of both glucose and lactic acid.
In view of the important role assigned to pyruvic acid in yeast and muscle fermentation, a study was made of the dissimilation of pyruvic acid by homofermentative lactic acid bacteria. In Table 15 are shown the results of fermentations of pyruvic acid by *L. plantarum* and *L. curvatus*. About 0.6 per cent pyruvic acid was added to the basal medium. Pyruvic acid, from Eastman Kodak Co., was distilled under vacuum, neutralized with sodium hydroxide, sterilized by Seitz filtration and added to the sterile basal medium. Three-day cultures of bacteria, grown on pyruvic acid medium, were used for inoculation.

Table 15. Dissimilation of Pyruvic Acid by Homofermentative Lactic Acid Bacteria.

<table>
<thead>
<tr>
<th>Product</th>
<th><em>L. plantarum</em></th>
<th><em>L. curvatus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mM</td>
<td>mM</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>19.5</td>
<td>25.1</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>19.4</td>
<td>16.6</td>
</tr>
<tr>
<td>Carbon dioxide</td>
<td>--</td>
<td>19.7</td>
</tr>
</tbody>
</table>

Although the quantity of pyruvic acid used was not determined it is shown in Table 15 that lactic acid is formed from pyruvic acid. Acetic acid and carbon dioxide are formed in quantities equal to the lactic acid. Pyruvic acid may be a precursor of both lactic and acetic acid. It is probable in these experiments that one molecule of pyruvic acid was oxidized with the simultaneous reduction of a second. In the normal fermentation of glucose other hydrogen donators and acceptors may be
present which alter materially the relationships.

Fixation experiments. An attempt was made to isolate pyruvic acid from glucose fermentations. Glucose medium containing 0.02 to 0.5 per cent calcium sulfite, sodium sulfite and sodium bisulfite was inoculated with three-day cultures of L. plantarum and L. cucumeris and incubated thirty days at 30°C. Growth under these conditions was very slow. Colorimetric tests and precipitation tests for pyruvic acid were negative. The procedure was varied and repeated several times, but no pyruvic acid was isolated.

In view of the negative results with sulfite fixatives the method used by Kostyschew, Gwaladzi and Eljasberg (1930) was followed. To a glucose medium was added 0.15 per cent semicarbazide hydrochloride (neutralized to pH 6.2 with NaOH). To one set of flasks was added 0.6 per cent CaCO₃. The other set contained a phosphate buffer. The flasks were inoculated with three-day cultures of L. plantarum and L. cucumeris. Rapid fermentation occurred in all flasks. A precipitate with 2,4-dinitrophenylhydrazine was obtained in the medium after a short period of incubation. A volatile carbonyl compound was obtained whose properties conformed to those of acetaldehyde. The melting points and mixed melting points of the derivatives are given in Table 16.
Table 16. Melting Points of Acetaldehyde Isolated from Fermentation of Glucose by L. plantarum.

<table>
<thead>
<tr>
<th>Derivative</th>
<th>Melting points °C.</th>
<th>Unknown</th>
<th>Mixed</th>
<th>Acetaldehyde</th>
<th>Literature</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-Dinitrophenyl-hydrazone</td>
<td></td>
<td>160</td>
<td>161</td>
<td>164</td>
<td>165</td>
</tr>
<tr>
<td>Para-nitrophenyl-hydrazone</td>
<td></td>
<td>119</td>
<td>119.5</td>
<td>119</td>
<td>123.5</td>
</tr>
<tr>
<td>Dimedon derivative</td>
<td></td>
<td>137</td>
<td>137</td>
<td>138</td>
<td>139-140</td>
</tr>
</tbody>
</table>

The melting points leave little doubt that the compound was acetaldehyde. In fermentations by members of the hetero-fermentative group no acetaldehyde was obtained by this method.

In chapter two it was shown that acetic acid was formed by the fermentation of glucose. It is probable that acetaldehyde may precede acetic acid in the dissimilation (equations 1 and 2).

1. \( \text{C}_3 \text{ compound} \rightarrow \text{CH}_3\text{CHO} + \text{CO}_2 + 2\text{H} \)

2. \( \text{CH}_3\text{CHO} + \text{H}_2\text{O} \rightarrow \text{CH}_3\text{CH}_2\text{OH} \rightarrow \text{CH}_3\text{COOH} + 2\text{H} \)

Reynolds (1935) showed that pyruvic acid was formed by Citrobacter freundii without the addition of fixatives if the medium was buffered with sodium bicarbonate. Attempts to obtain pyruvic acid in fermentations by lactic acid bacteria by buffering with sodium bicarbonate met with failure. The failure to fix pyruvic acid is by no means proof that it is not intermediary in the dissimilation of glucose by lactic acid bacteria. The compound may have been fermented in the presence of fixatives.
or the breakdown may take place so rapidly that the fixatives were unable to act.

**Fermentation of lactic acid.** Inasmuch as lactic acid has been considered as a source of acetic acid in the fermentation of glucose, attempts were made to ferment lactic acid with cultures of *L. plantarum* and *L. cuumeria*. All anaerobic experiments met with failure. It is possible, that in the presence of glucose, which may provide satisfactory hydrogen acceptors, lactic acid may be oxidized to acetic acid and carbon dioxide. In view of Simon's (1932) isolation of pyruvic acid from fermentations of lactic acid by *L. delbrückii* it is probable that pyruvic acid may be intermediary in the dissimilation of lactic acid.

**Heterofermentative lactic acid bacteria**

**Fermentation of pyruvic acid.** In view of the intermediary role assigned pyruvic acid in bacterial muscle and yeast fermentation, the mechanism of its breakdown by heterofermentative lactic acid bacteria is of importance. *Lactobacillus lycopersici*, *Leuconostoe dextranicus* and *Lactobacillus maninitoposus* were used in a study of pyruvic acid dissimilation. The medium consisted of pyruvic acid, 0.6 per cent, (purified by vacuum distillation and neutralized with sodium hydroxide) added to the basal medium. The flasks were inoculated with 25 ml. of a three-day culture grown on pyruvic acid medium. Oxygen-free nitrogen was bubbled continuously through the medium to insure anaerobic
conditions. Pyruvic acid was determined at the beginning and at the conclusion of fermentation. The results of two experiments with *L. lycopersici* and one with *Leuconostoc dextranicus* and one with *L. maninitococcus* are assembled in Table IV. Good carbon balances and redox indexes lend assurance to the reliability of the data.

The three compounds resulting from the fermentations were found to occur in equimolar quantities, suggesting a dissimilation involving the oxidation of one molecule of pyruvic acid with the simultaneous reduction of a second. These results correspond to those obtained by the dissimilation of pyruvic acid by the homofermentative lactic. Attempts to isolate acetaldehyde from pyruvic acid fermentations with sulfite and dimedon fixatives were repeatedly unsuccessful. Van Niel (1928) and Wood and Wherckman (1954) suggested that the propionic acid bacteria brought about a hydration of pyruvic acid followed by a dehydrogenation resulting in the formation of acetic acid and carbon dioxide (equation 3). The lactic acid bacteria may bring about the same reaction. The active hydrogen formed may serve to reduce a second molecule of pyruvic acid (equation 4).

\[
\text{3. } \text{CH}_3\text{COCOOH} + \text{H}_2\text{O} \rightarrow \text{CH}_3\text{C}^\text{O}\text{C}^\text{O} \text{CH} \rightarrow \text{CH}_3\text{COOH} + \text{CO}_2 + 2\text{H}^\text{OH}
\]

\[
\text{4. } \text{CH}_3\text{COCOOH} + 2\text{H} \rightarrow \text{CH}_3\text{CHOHCOOH}
\]

Peterson and Fred (1920a) fermented sodium pyruvate by *L. pentosaceticus* and measured the ability of the bacteria to ferment
Table 17. Dissimilation of Pyruvic Acid by Heterofermentative Lactic Acid Bacteria.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Pyruvic acid</th>
<th>Lactic acid</th>
<th>Acetic acid</th>
<th>Carbon dioxide</th>
<th>Carbon</th>
<th>Redox</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. lycopersici</td>
<td>70.5:211.5</td>
<td>37.7:113.1:</td>
<td>35.1:70.2</td>
<td>38.8:38.3</td>
<td>105.2</td>
<td>1.10</td>
</tr>
<tr>
<td>L. lycopersici</td>
<td>46.7:140.0</td>
<td>22.9:69.4:</td>
<td>22.6:45.2:</td>
<td>23.8:23.8</td>
<td>95.1</td>
<td>1.02</td>
</tr>
<tr>
<td>L. manitopoeus</td>
<td>37.7:263.1</td>
<td>46.9:140.7:</td>
<td>54.8:109.6</td>
<td>43.1:43.1</td>
<td>111.1</td>
<td>0.98</td>
</tr>
<tr>
<td>Leuconostoc</td>
<td>72.1:216.3</td>
<td>35.8:107.4:</td>
<td>35.5:71.0</td>
<td>32.3:32.3</td>
<td>97.4</td>
<td>0.90</td>
</tr>
<tr>
<td>dextranicus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
the compound by the increase in alkalinity, on the theory that pyruvic acid was broken down to acetaldehyde and carbon dioxide; the latter being liberated as a gas. The change in the reaction of the medium was very small from which it was concluded that pyruvic acid was not readily fermented. In later experiments in which pyruvic acid was added to the medium at intervals and titrated, little pyruvic acid was used.

In view of the results shown in Table 17 it is evident that there would be very little change in pH. Two molecules of sodium pyruvate yield one of sodium acetate and one of sodium lactate. In a slightly acid medium the carbon dioxide would be evolved as a gas, producing no change in pH.

Although the relationships between lactic acid and acetic acid formed by the fermentation of glucose are not the same as the relationships when formed from pyruvic acid it does not disprove pyruvic acid as an intermediary in the dissimilative mechanism. Hydrogen donators in addition to pyruvic acid may be formed from glucose and reduce pyruvic to lactic acid in the normal fermentation.

Fermentation of lactic acid. Repeated attempts to bring about the anaerobic breakdown of lactic acid by \textit{L. lycopersici} \textit{L. pentosaceicus} and \textit{L. mannotoposus} met with failure.

It is not improbable that hydrogen acceptors formed from glucose have the ability to accept active hydrogen formed by the oxidation of lactic acid. To determine whether lactic acid is oxidized anaerobically in the presence of hydrogen acceptors,
acetaldehyde was added to a medium containing lactic acid and subjected to fermentation. One per cent lactic acid was added to the basal medium and one liter placed in each of four 1.5-liter flasks. Acetaldehyde was added to two flasks on the third, sixth and tenth days. The aldehyde was sterilized by Seitz filtration. The media were inoculated with \textit{L. lycopersici} centrifuged from 50 ml. of a three-day culture.

Table 18. The Effect of Acetaldehyde on the Dissimilation of Lactic Acid by \textit{L. lycopersici}.

<table>
<thead>
<tr>
<th></th>
<th>Lactic acid</th>
<th>Acetic acid</th>
<th>Ethyl alcohol</th>
<th>Carbon dioxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flask I</td>
<td>40.5</td>
<td>20.1</td>
<td>15.9</td>
<td>22.9</td>
</tr>
<tr>
<td>Flask II</td>
<td>41.5</td>
<td>13.3</td>
<td>9.5</td>
<td>10.0</td>
</tr>
</tbody>
</table>

Although no carbon balance nor redox index can be made, several points are established by the data (Table 18). The presence of hydrogen acceptors enables the bacteria to dissimilate lactic acid anaerobically. The acetaldehyde added was reduced to ethyl alcohol and the lactic acid oxidized to acetic acid and carbon dioxide. In similar experiments Wiggert, working in this laboratory, has shown lactic acid to be oxidized to acetic acid and carbon dioxide in the presence of acetilmethylcarbinol which was reduced to 2,3-butylene glycol.
Isolation of pyruvic acid. Inasmuch as lactic acid is fermented to acetic acid and carbon dioxide aerobically by heterofermentative lactics an attempt was made to isolate pyruvic from a lactic acid fermentation by \textit{L. gracilis} and \textit{L. fructivorans}.

The medium consisted of 50 ml. of filtered tomato juice, 20 grams of sodium lactate and two grams of $K_2HPO_4$ per liter. Calcium sulfite solution (freshly prepared) was added to the medium in concentrations varying from 0.01 to 0.10 per cent. The medium without sulfite added was inoculated with bacteria centrifuged from 50 ml. of three-day cultures grown on a glucose medium. The sulfite was added after three days of fermentation. After thirty days' incubation at 30$^\circ$ C., 10 grams of calcium carbonate were added to the medium which was distilled into an iced condenser. No precipitate was obtained in the distillate with 2,4-dinitrophenylhydrazine. The residue of distillation was filtered and treated with a 2,4-dinitrophenylhydrazine solution. A copious yellow precipitate formed in the liquor from flasks containing 0.02 to 0.05 per cent calcium sulfite. The precipitate was soluble in a sodium carbonate solution and reprecipitated upon acidification. The precipitate, after recrystallizing from ethyl alcohol, gave melting points of the corresponding pyruvic acid hydrazone. The para-nitrophenylhydrazones also was prepared. The melting points are listed in Table 19.
Table 19. Melting Points of Derivatives of Pyruvic Acid Isolated from Glucose Fermentations by *L. fructivorans*.

<table>
<thead>
<tr>
<th>Derivative</th>
<th>Melting points C°</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unknown Pyruvic acid</td>
<td></td>
</tr>
<tr>
<td>Mixed melt-:Lactic acid:</td>
<td></td>
</tr>
<tr>
<td>Sing point: Nature</td>
<td></td>
</tr>
<tr>
<td>2,4-Dinitrophenyl-</td>
<td></td>
</tr>
<tr>
<td>hydrazone: 211-212: 214-214.5: 213.5</td>
<td></td>
</tr>
<tr>
<td>Para-nitrophenyl-</td>
<td></td>
</tr>
</tbody>
</table>

The compound formed was evidently pyruvic acid and may be assumed to be intermediary in the breakdown of lactic acid.

From investigations with pyruvic acid and lactic acid it is seen that pyruvic acid may be converted into lactic acid and that lactic acid may be fermented to pyruvic acid under suitable conditions. It may be justifiable to assume a reversible reaction between pyruvic and lactic acid in the dissimilation of glucose.
CHAPTER FOUR

Diversion of the Normal Dissimilation of Glucose by the Addition of Hydrogen Acceptors.

The addition of acetaldehyde

It was pointed out in chapter one that the addition of hydrogen acceptors and donors to carbohydrate fermentations may affect the relationship of the products in a manner to throw light on the intermediary mechanism of dissimilation. An understanding of the behavior of added hydrogen acceptors would be of value in formulating schemes of dissimilation or in determining the suitability of schemes already proposed.

Peterson and Fred (1920a) have found evidence for the occurrence of acetaldehyde as an intermediary. Positive tests for acetaldehyde were reported in chapter three. Since acetaldehyde may play a role as an intermediary precursor of ethyl alcohol in the lactic acid fermentation acetaldehyde was chosen as a hydrogen acceptor to be added to the fermentation.

The medium used in this investigation consisted of 2 percent glucose in the basal medium. One liter of medium (pH 6.2) was placed in each of three 1500 ml. Erlemeyer flasks and inoculated with 25 ml. of a three-day culture of L. lycopersici. The acetaldehyde was sterilized by Seitz filtration and added to the medium. Quantities of 0.36 gram and 0.72 gram of acetal-
dehydrate were added to experiments II and III respectively on the third, sixth and tenth days of fermentation. Experiment I had no acetaldehyde added. Typical experiments showing the effect of the addition of acetaldehyde to a glucose fermentation are shown in Table 20.

A comparison of representative data in experiments I, II and III shows: (1) The difference in quantities of ethyl alcohol in the two latter experiments is equivalent to the difference in the quantities of acetaldehyde fermented. In experiment II there were 27.3 mM of acetaldehyde fermented and 55.8 mM in experiment III. (2) The difference in the quantities of acetic acid and carbon dioxide formed in experiments II and III is equal to one-half the difference found for ethyl alcohol. (3) There was less lactic acid formed in experiment III than in experiment II; the difference was equivalent to the increase in the quantity of acetic acid formed in experiment III.

Acetaldehyde may undergo a number of reactions in a fermentation. Oxidation of one molecule with the simultaneous reduction of a second may occur, giving rise to one molecule of acetic acid and one of ethyl alcohol. This reaction may take place between a molecule of acetaldehyde added to the fermentation and one formed in the dissimilation of glucose or it may take place between two molecules of the aldehyde added to the fermentation. The former reaction would be accompanied by carbon dioxide equivalent (in millimoles) to the acetic acid or ethyl alcohol formed, while the latter reaction would produce only acetic acid
and ethyl alcohol in equimolar quantities. It will be noted in Table 20 that the increase in carbon dioxide owing to the addition of acetaldehyde is only one-half the increase in acetic acid. It does not appear that either of the above reactions has taken place.

Secondly, two molecules of acetaldehyde may condense to form acetylmethylcarbinol. The failure to find the carbinol or its reduction product, 2,3-butyleneeglycol, eliminates this possibility.

The third possible reaction is the complete reduction of acetaldehyde to ethyl alcohol. The occurrence of this reaction is supported by the data in Table 20. There were 27.5 mM of acetaldehyde fermented in experiment II and 55.8 mM in experiment III; a difference of 28.3 mM. The ethyl alcohol formed was 30.8 and 108.4 mM in experiments II and III respectively, a difference of 77.6 mM. It appears that acetaldehyde added to the fermentation of glucose has been transformed to ethyl alcohol. In order to reduce acetaldehyde to ethyl alcohol some compound formed from glucose must be oxidized. An examination of the data (Table 20) shows a decrease in lactic acid corresponding to the increase in acetic acid. Either the lactic acid itself was oxidized or some intermediary precursor of lactic acid has been diverted from its normal course to donate hydrogen to acetaldehyde and at the same time be oxidized to acetic acid and carbon dioxide. The oxidation of lactic to acetic acid and carbon dioxide involves the formation of four atoms of active hydrogen
Table 20. The Effect of the Addition of Acetaldehyde on the Dissimilation of Glucose by L. lycopersici.

<table>
<thead>
<tr>
<th></th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>Difference (III-II)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose fermented</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Acetaldehyde fermented</td>
<td>0</td>
<td>27.3</td>
<td>55.8</td>
<td>+28.5</td>
</tr>
<tr>
<td>Ethyl alcohol</td>
<td>78.8</td>
<td>80.8</td>
<td>103.4</td>
<td>+27.6</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>16.1</td>
<td>24.1</td>
<td>36.8</td>
<td>+12.7</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>81.5</td>
<td>91.5</td>
<td>86.8</td>
<td>-5.7</td>
</tr>
<tr>
<td>Glycerol</td>
<td>31.3</td>
<td>14.8</td>
<td>16.2</td>
<td>+1.4</td>
</tr>
<tr>
<td>Carbon dioxide</td>
<td>80.3</td>
<td>71.5</td>
<td>79.5</td>
<td>+8.0</td>
</tr>
<tr>
<td>Percentage of carbon recovered</td>
<td>101.0</td>
<td>92.6</td>
<td>96.8</td>
<td></td>
</tr>
<tr>
<td>Oxidation-reduction index</td>
<td>10.851</td>
<td>10.959</td>
<td>0.893</td>
<td></td>
</tr>
</tbody>
</table>
for each molecule of acetic acid. If the active hydrogen formed by the oxidation of lactic acid is accepted by the added acetaldehyde, there will be two molecules of acetaldehyde reduced for each molecule of acetic acid formed. This relationship corresponds to that existing between the products in experiments II and III, Table 20.

In chapter two it was shown that as glucose was being fermented the percentage being dissimilated to lactic acid decreased while the percentages as ethyl alcohol, acetic acid and carbon dioxide was increased. The actual quantity of lactic acid per liter was not decreased. It is possible that acetic acid and carbon dioxide may have been formed from an intermediary compound which would result in a decrease in the percentage of glucose going to lactic acid. Experiments in chapter three showed that acetic acid and carbon dioxide could be formed from pyruvic acid. In view of the important role assigned to pyruvic acid in the intermediary dissimulation of glucose by muscle tissue and yeast it is not improbable that pyruvic acid may be intermediary in the dissimulation by heterofermentative lactics and provide the active hydrogen for reduction of the added acetaldehyde.

Pyruvic acid may undergo an oxidation to acetic acid and carbon dioxide with the simultaneous reduction of the added acetaldehyde to ethyl alcohol, equation 5.

5. \( \text{CH}_2\text{COCOOH} + \text{CH}_3\text{CHO} + \text{H}_2\text{O} \rightarrow \text{CH}_3\text{CH}_2\text{OH} + \text{CH}_3\text{COOH} + \text{CO}_2 \)

This reaction forms one molecule of acetic acid and carbon dioxide with each molecule of ethyl alcohol, while the data in Table 20
require two molecules of ethyl alcohol for each of acetic acid or carbon dioxide. However, if the formation of pyruvic acid involves the principles of the Embden-Meyerhof scheme, hydrogen donators are formed which may provide adequate active hydrogen to bring about the relationships between acetic acid and ethyl alcohol obtained in Table 20. These relationships will be discussed in a later chapter.

The addition of acetylmethylcarbinol

The addition of acetylmethylcarbinol to glucose fermentations is of interest not only from the standpoint of its role as a hydrogen acceptor, but also owing to the importance of the carbinol in the production of favorable flavor and aroma in butter. Hammer, Stahly, Werkman and Michaelian (1935) have shown the reduction of acetylmethylcarbinol to 2,3-butylene glycol by organisms isolated from butter cultures.

In the present investigations 0.5 per cent acetylmethylcarbinol was added to the basal medium containing 2.0 per cent glucose. The acetylmethylcarbinol was purified by washing with ethyl ether, dissolved in water, sterilised by Seitz filtration and added to the medium at the time of inoculation. Inoculations were made with L. lycopersici, L. plantarum and Leuconostoc dextranicus. Three-day cultures were used for inoculation. The medium was analyzed after twelve days' incubation at 30° C.

Acetylmethylcarbinol did not influence the fermentation of glucose by the homofermentative organism, L. plantarum. No change
took place in the acetylmalonylcarbinol added and the products were the same as from the normal dissimilation of glucose. The results of the fermentation by *L. lycopersici* are shown in Table 21, for *Leuconostoc dextranicus* in Table 22. In experiment I is shown the data for the normal dissimilation of glucose. In experiment II is shown the effect of acetylmalonylcarbinol on the dissimilation of glucose.

The acetylmalonylcarbinol was reduced quantitatively to 2,3-butyleneglycol. The quantities of ethyl alcohol, lactic acid and glycerol were decreased with a corresponding increase in acetic acid and carbon dioxide. Table 21 shows an increase in acetic acid of 37.4 mM. The increase in carbon dioxide was only 19.1 mM. Since ethyl alcohol was decreased 19.3 mM and each molecule of alcohol is accompanied by a molecule of carbon dioxide, the sum of the increase in carbon dioxide and decrease in ethyl alcohol (40.7 mM) can be accounted for by the increase in acetic acid. The same relationships are shown by *Leuconostoc dextranicus*, Table 22.

It will be seen in Tables 20, 21 and 22 that lactic acid and glycerol as well as ethyl alcohol are decreased by the addition of hydrogen acceptors. One would expect hydrogen acceptors added to a fermentation to compete with hydrogen acceptors present in the system from the breakdown of glucose, resulting in a decrease in the products formed by the reduction of intermediary hydrogen acceptors. Following this view the data indicate that lactic acid, ethyl alcohol and glycerol are formed from the reduction
Table 21. The Effect of the Addition of Acetylmethylcarbinol on the Dissimilation of Glucose by L. lycopersici.

<table>
<thead>
<tr>
<th></th>
<th>I</th>
<th>II</th>
<th>Difference (II - I)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mM</td>
<td>mM</td>
<td>mM</td>
</tr>
<tr>
<td>Glucose fermented</td>
<td>102.2</td>
<td>101.7</td>
<td>- 0.5</td>
</tr>
<tr>
<td>Acetylmethylcarbinol fermented</td>
<td>0</td>
<td>54.1</td>
<td>+ 54.1</td>
</tr>
<tr>
<td>2,3-Butylene glycol</td>
<td>74.1</td>
<td>54.8</td>
<td>- 19.3</td>
</tr>
<tr>
<td>Ethyl alcohol</td>
<td>15.3</td>
<td>52.9</td>
<td>+ 37.4</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>81.0</td>
<td>100.1</td>
<td>+ 19.1</td>
</tr>
<tr>
<td>Carbon dioxide</td>
<td>83.1</td>
<td>61.7</td>
<td>- 21.4</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>32.6</td>
<td>26.3</td>
<td>- 7.3</td>
</tr>
<tr>
<td>Glycerol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage of C recovered</td>
<td>99.0</td>
<td>97.1</td>
<td></td>
</tr>
<tr>
<td>Oxidation-reduction index</td>
<td>0.884</td>
<td>1.002</td>
<td></td>
</tr>
</tbody>
</table>
of intermediary hydrogen acceptors. This view will be discussed later in connection with the Embden-Meyerhof theory.

If *Leuconostoc dextranicus* is the same as the citric acid fermenter isolated from starter it supports the work of Hammer, Stahly, Werkman and Michaelian (1935) with respect to the reduction of acetylmethylocarbinol to 2,3-butylene glycol in milk.
Table 22. The effect of the Addition of Acetylmethylcarbinol on the Dissimilation of Glucose by \textit{Leuconostoc dextranicus}.

<table>
<thead>
<tr>
<th></th>
<th>I</th>
<th>II</th>
<th>Difference (II - I)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose fermented</td>
<td>100</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Acetylmethylcarbinol</td>
<td>0</td>
<td>60</td>
<td>+60.0</td>
</tr>
<tr>
<td>2,3-Butyleneglycol</td>
<td>0</td>
<td>58.8</td>
<td>+58.8</td>
</tr>
<tr>
<td>Ethyl alcohol</td>
<td>81.2</td>
<td>67.4</td>
<td>-13.8</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>10.8</td>
<td>38.9</td>
<td>+28.1</td>
</tr>
<tr>
<td>Carbon dioxide</td>
<td>86.5</td>
<td>103.1</td>
<td>+16.6</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>85.8</td>
<td>78.0</td>
<td>-5.8</td>
</tr>
<tr>
<td>Glycerol</td>
<td>24.0</td>
<td>18.4</td>
<td>-5.6</td>
</tr>
<tr>
<td>Carbon balance</td>
<td>98.9</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>Redox Index</td>
<td>0.930</td>
<td>0.975</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER FIVE

The Dissimilation of Levulose by Lactic Acid Bacteria.

It was pointed out in part I that levulose has the properties of a hydrogen acceptor, being reduced to mannitol. It also has the ability to supply energy by being broken down to lactic acid, acetic acid and carbon dioxide. If the breakdown of levulose takes place in the same manner as the breakdown of glucose, the action of levulose as a hydrogen acceptor should have the same effect as the addition of acetaldehyde and acetylmethylcarbinol to the glucose fermentation.

Levulose (2 per cent) was added to the basal medium, inoculated with three-day cultures of \textit{L. lycopersici}, \textit{Leuconostoc dextranicus}, \textit{L. plantarum}, \textit{L. fructivorans} and \textit{L. gracilis}. After twenty-one days' incubation at 30° C. the medium was analyzed. Typical results are assembled in Table 23.

The products of fermentation of levulose by \textit{L. plantarum}, a homofermentative lactic acid organism, were formed in the same relationship as they were from glucose, forming only traces of products other than lactic acid.

The data for the heterofermentative lactics show carbon dioxide equal to the sum of acetic acid and ethyl alcohol. The percentage of levulose going to all products is markedly less than from glucose, due to the large percentage being reduced.
### Table 23. Dissimilation of Levulose by Lactic Acid Bacteria.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Lactic acid</th>
<th>Acetic acid</th>
<th>Carbon dioxide</th>
<th>Ethyl alcohol</th>
<th>Glycerol</th>
<th>Mannitol</th>
<th>Carbon index</th>
<th>Redox balance</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. fructivorans</td>
<td>11.1:5.6:39.5:13.2:46.3:7.7:6.0:2.0:---</td>
<td>74.1:74.1:102.6:1.07</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. gracilis</td>
<td>8.2:4.1:52.6:17.5:39.9:6.7:15.0:5.0:---</td>
<td>68.8:68.8:102.6:0.81</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. mannitopoeus</td>
<td>188.9:84.5:4.5:1.5:6.5:1.1:0.0:1.8:0.9:0.0:0.0</td>
<td>86.0:3.60</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. lycopersici</td>
<td>33.1:16.5:40.3:19.4:44.7:7.4:0.8:0.3:3.8:1.9</td>
<td>48.9:48.9:94.4:1.64</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leuconostoc dextranicus</td>
<td>53.4:26.7:34.9:11.3:51.2:17.1:2.1:1.5</td>
<td>33.2:33.2:101.4:1.19</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
A very small quantity of ethyl alcohol was formed. An exception was shown by *Leuconostoc dextranicus* which formed much less mannitol. This organism also produced large quantities of ethyl alcohol; this is not characteristic of other organisms used in this experiment.

If the quantity of mannitol formed is subtracted from the levulose fermented and the products calculated as mM per 100 mM of levulose fermented the relationships shown between these calculations and a normal glucose fermentation should be similar to the relationships between normal glucose fermentations and glucose fermentations to which hydrogen acceptors have been added. Such calculations have been made for the fermentation of levulose by *Lactobacillus lycopersici* and *Leuconostoc dextranicus*.

The data in Table 24 show that very little ethyl alcohol and glycerol have been formed by *L. lycopersici*. The lactic acid on the other hand has remained practically constant. Large quantities of acetic acid have been formed. The results differ from those to which acetyl-methylcarbinol has been added in that (1) there has been no decrease in lactic acid and (2) the formation of ethyl alcohol was entirely suppressed. It appears that levulose is competing with hydrogen acceptors formed by the breakdown of levulose resulting in the formation of less ethyl alcohol and glycerol than is formed from glucose. The competition with the precursor of lactic acid if it is a hydrogen acceptor, for the active hydrogen by levulose is not as strong as by acetyl-methylcarbinol or acetaldehyde. The fact that acetic acid has
increased without a decrease in lactic acid is evidence that
the former is not formed by a secondary fermentation of lactic
acid, but probably arises from the oxidation of some intermediary
compound.

The data in Table 25a for the fermentation by \textit{Leuconostoc
dextranicus} show the breakdown of levulose to resemble closely
the glucose fermentation to which acetyl methylcarbinol had been
added. There was a decrease in lactic acid, ethyl alcohol and
glycerol and a corresponding increase in acetic acid and carbon
dioxide. Unlike the fermentation by \textit{L. lycopersici}, the forma-
tion of ethyl alcohol was not entirely suppressed. This exper-
iment is further evidence that ethyl alcohol, lactic acid and
glycerol are formed by the reduction of intermediary hydrogen
acceptors.

A further study of the dissimilation of levulose was made
by analyzing the medium at intervals during fermentation. \textit{L.
lycopersici} and \textit{Leuconostoc dextranicus} were used. Three liters
of the basal medium containing 1.5 per cent levulose were placed
in 4-liter Erlenmeyer flasks and inoculated with 50 ml. of a
three-day culture. Oxygen-free nitrogen was passed continuously
through the flasks during fermentation.

It is clear from Table 25 that there is no actual decrease
in the quantity of lactic acid present, but as the fermentation
progressed the percentage of levulose going to lactic acid was
constantly decreasing (Table 26). Acetic acid, carbon dioxide
and mannitol, on the other hand, were constantly increasing.
### Table 24. Comparison of Levolose and Acetyl methylcarbinol as Hydrogen Acceptors. *L. lycopersici*.

<table>
<thead>
<tr>
<th>Products</th>
<th>Glucose + acetyl- methylcarbinol</th>
<th>Levulose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mM</td>
<td>mM</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>83.1</td>
<td>61.7</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>15.5</td>
<td>52.9</td>
</tr>
<tr>
<td>Ethyl alcohol</td>
<td>74.1</td>
<td>54.8</td>
</tr>
<tr>
<td>Carbon dioxide</td>
<td>81.0</td>
<td>100.1</td>
</tr>
<tr>
<td>Glycerol</td>
<td>32.6</td>
<td>25.3</td>
</tr>
</tbody>
</table>

| Carbon balance | 99.0                             | 97.1     | 98.3     |

### Table 25a. Comparison of Levolose and Acetyl methylcarbinol as Hydrogen Acceptors. *Leuconostoc dextranicus*.

<table>
<thead>
<tr>
<th>Products</th>
<th>Glucose + acetyl- methylcarbinol</th>
<th>Levulose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mM</td>
<td>mM</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>93.8</td>
<td>78.0</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>10.8</td>
<td>38.9</td>
</tr>
<tr>
<td>Carbon dioxide</td>
<td>86.5</td>
<td>103.1</td>
</tr>
<tr>
<td>Ethyl alcohol</td>
<td>81.2</td>
<td>67.4</td>
</tr>
<tr>
<td>Glycerol</td>
<td>24.0</td>
<td>18.5</td>
</tr>
</tbody>
</table>

| Carbon balance | 98.9                             | 100.0    | 104.5    |
This relationship may be more clearly visualized by the graphical representation in figure 5. It was pointed out in chapter two that if the curve is parallel to the horizontal axis the percentage of carbohydrate fermented to the compound represented by the curve is constant. If the slope of the curve is concave upward the percentage of carbohydrate going to this compound is increasing, if concave downward the percentage is decreasing.

The lactic acid decreased and the acetic acid, carbon dioxide and mannitol increased. It appears that lactic acid or an intermediary precursor of lactic acid may be broken down to acetic acid and carbon dioxide and the hydrogen formed by the oxidation may reduce levulose to mannitol.

The products of dissimilation of levulose by Leuconostoc dextranicus are shown in Table 27 as mM per liter of medium. This fermentation differed from that of L. lycopersici. Larger quantities of ethyl alcohol and smaller quantities of mannitol were formed. In Table 28 the data are calculated as millimoles per 100 millimoles of levulose fermented. The data are represented graphically in figure 6. Acetic acid and ethyl alcohol show a slight decrease. Lactic acid decreases to a minimum in thirteen days after which it increases again. Carbon dioxide and mannitol, on the other hand, increase to a maximum in thirteen days and then decrease. Ethyl alcohol and acetic acid decrease continuously. There is apparently an equilibrium existing in the dissimilative mechanism that shifts with the changing conditions of growth. As a result there is a constant shift in the relationship between the products as fermentation progresses.
Table 25. Serial Analysis of the Dissimilation of Levulose by L. lycopersici.*

<table>
<thead>
<tr>
<th>Period of fermentation</th>
<th>Lactic acid</th>
<th>Acetic acid</th>
<th>Carbon dioxide</th>
<th>Ethyl alcohol</th>
<th>Glycerol</th>
<th>Mannitol</th>
<th>Car-Redox balance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days</td>
<td>mM</td>
<td>mM</td>
<td>mM</td>
<td>mM</td>
<td>mM</td>
<td>mM</td>
<td>mM</td>
</tr>
<tr>
<td>4</td>
<td>23.9</td>
<td>145.4</td>
<td>18.3</td>
<td>54.9</td>
<td>3.5</td>
<td>6.6</td>
<td>7.1</td>
</tr>
<tr>
<td>7</td>
<td>35.7</td>
<td>356.2</td>
<td>27.3</td>
<td>81.9</td>
<td>25.5</td>
<td>50.6</td>
<td>16.5</td>
</tr>
<tr>
<td>13</td>
<td>75.2</td>
<td>451.2</td>
<td>24.9</td>
<td>74.7</td>
<td>30.3</td>
<td>60.6</td>
<td>33.6</td>
</tr>
<tr>
<td>25</td>
<td>180.5</td>
<td>493.0</td>
<td>34.7</td>
<td>104.1</td>
<td>37.9</td>
<td>75.8</td>
<td>36.4</td>
</tr>
</tbody>
</table>

*Calculated as millimoles per liter.
Table 26. Serial Analysis of the Dissimilation of Levulose by L. lycopersici

<table>
<thead>
<tr>
<th>Period</th>
<th>of fermentation</th>
<th>Lactic</th>
<th>Acetic</th>
<th>Carbon Dioxide</th>
<th>Ethyl Alcohol</th>
<th>Glycerol</th>
<th>Mannitol</th>
<th>Carbon Balance Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days</td>
<td>mM</td>
<td>;cent;</td>
<td>mM</td>
<td>;cent;</td>
<td>mM</td>
<td>;cent;</td>
<td>mM</td>
<td>;cent;</td>
</tr>
<tr>
<td>4</td>
<td>76.5:38.2:14.7:4.9:29.8:5.0:1.7:0.6:6.7:3.8:42.2:42.2:93.3:1.30</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>45.5:22.7:42.8:14.3:27.0:4.5:1.3:0.4:4.5:2.2:50.3:50.3:94.8:1.03</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
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<td>25</td>
<td>43.1:21.5:47.0:15.7:45.2:7.5:0.6:0.2:3.4:1.7:45.2:45.2:91.9:1.86</td>
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*Calculated as millimoles per 100 millimoles of levulose fermented.
Figure 5. Serial Analysis of the Dissimilation of Levulose by *L. lycopersici*.
Table 27. Serial Analysis of the Dissimilation of Levulose by Leuconostoc dextranicus. *

| Period | of fer- | Levulose | Lactic | Acetic | Carbon | Ethyl | Glycerol | Mannitol | Car-<Re-
|--------|--------|----------|--------|--------|--------|-------|----------|----------|----
| tion   |        |          |        |        |        |       |          |          | me-
|        | mM     | mM       | mM     | mM     | mM     | mM    | mM       | mM       | nta: dex
| Days   | Car-:  | Car-:    | Car-:  | Car-:  | Car-:  | Car-: | Car-:    | Car-:    | 
| 4      | 58.2: 331.2:50.0:150.0:20.5:41.0:24.2:24.2:27.4:54.8:0.9:1.2:10.8:64.8:101.0:0.74 |  |  |  |  |  |  |  |  
| 7      | 71.7:430.2:43.6:130.8:26.2:52.4:49.8:49.8:33.8:67.6:1.4:4.2:20.5:123.0:98.4:1.13 |  |  |  |  |  |  |  |  
| 13     | 76.3:457.2:40.7:122.1:26.6:58.2:58.3:58.3:39.0:78.0:2.0:6.0:25.3:161.8:102.9:1.05 |  |  |  |  |  |  |  |  

* Calculated as millimoles per liter.
Table 28. Serial Analysis of the Dissimilation of Leuvalose by *Leuconostoc dextranicus.*

<table>
<thead>
<tr>
<th>Period of fermentation</th>
<th>Lactic Acid</th>
<th>Acetic Acid</th>
<th>Carbon Dioxide</th>
<th>Ethyl Alcohol</th>
<th>Glycerol</th>
<th>Mannitol</th>
<th>Carbon</th>
<th>Redox Balance</th>
<th>Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days</td>
<td>mM</td>
<td>mM</td>
<td>mM</td>
<td>mM</td>
<td>mM</td>
<td>mM</td>
<td>mM</td>
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</tr>
<tr>
<td>4</td>
<td>91.0:45.5:37.1:12.7:45.8:7.3:49.7:16.5:1.6:0.8:19.6:19.6:101.0</td>
<td>0.74</td>
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<tr>
<td>7</td>
<td>80.9:80.4:36.5:12.2:69.4:11.6:47.1:15.7:1.9:0.9:28.6:28.6:98.4</td>
<td>1.13</td>
<td></td>
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<tr>
<td>13</td>
<td>53.4:26.7:34.9:11.6:77.5:12.9:51.2:17.0:2.6:1.3:33.1:33.1:102.9</td>
<td>1.05</td>
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<tr>
<td>25</td>
<td>73.0:36.5</td>
<td>--</td>
<td>73.5:12.3:44.3:14.7:2.6:1.3:32.3:32.3:82.1</td>
<td>1.12</td>
<td></td>
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</tr>
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</table>

*Calculated as millimoles per 100 millimoles of leuvalose fermented.*
Figure 6. Serial Analysis of the Dissociation of Levulose by Leuconostoc dextranicus.

mm per 100 mM of levulose

Time (days)

Acetic acid
Mannitol
Glycerol
Ethyl alcohol
Carbon dioxide

20

60

80

120
CHAPTER SIX

Discussion.

Although the dissimilation of glucose brought about by the homofermentative lactic acid bacteria is generally accepted as one of the simplest among the Eubacteriales, the transformation unquestionably involves a series of reactions in which even a synthetic stage is present. It is interesting to note that the formation of a methyl group not present in the glucose molecule is involved. On the other hand, the dissimilation of the hetero-lactic bacteria is characterized by greater complexity. Lactic acid is the product of the homo-lactic fermentation with essentially traces of acetic acid and CO₂, whereas the hetero-forms yield substantial quantities of acetic acid, CO₂, glycerol, and ethyl alcohol as well as lactic acid. The present work has shown particularly clearly the marked difference in behavior of the two physiologically distinct groups with regard to their ability to bring about certain reductions under anaerobic conditions. This is shown in the case of glucose fermentations with added acetaldehyde and acetyl methylcarbinol and in the case of the fermentation of levulose.
The hetero-forms bring about a rapid reduction of the hydrogen acceptor whereas, the homo-forms fail in this respect. Physiologically, at least, the true lactic acid bacteria may be sharply differentiated into the two groups. A simple differential test may be developed with acetylmethylcarbinol in a glucose medium. The O'Meara test with the hetero-lactic bacteria becomes negative while the carbinol remains unreduced with the homo-forms.

The present work may be interpreted as supporting either the generally accepted scheme of dissimilation among bacteria involving methyglyoxal as a 3-carbon intermediary or an adaptation of the Embden-Meyerhof scheme of muscle glycolysis in which phosphoglyceraldehyde acid plays an important part. It should prove of interest to examine the data of the present investigation in order to learn whether they are in support of one or the other of those schemes.

In the fermentations of glucose by both homo- and heterofermentative lactic acid bacteria, acetic acid was accompanied by equimolar concentrations of carbon dioxide. It is probable that these two compounds originate from the breakdown of the same 3-carbon intermediary. Nelson and Werkman (1935) suggested the formation of acetic acid and carbon dioxide from the secondary fermentation of lactic acid by the hetero-forms. The literature reveals lactic acid to be fermented aerobically to acetic acid and carbon dioxide by both homo- and heterofermentative bacteria. The probable precursor of the 2- and 1-carbon compounds, according to the Embden-Meyerhof scheme, is
pyruvic acid. This compound was fermented to acetic acid, carbon dioxide and lactic acid. However, pyruvic acid was isolated as an intermediary in the aerobic breakdown of lactic acid by hetero-lactic bacteria. A similar isolation was made in aerobic homo-lactic fermentations by Simon (1932). These observations may be explained in either scheme as a reversible reaction between lactic and pyruvic acids.

Ethyl alcohol, formed by the heterofermentative lactic bacteria, also was accompanied by equimolar quantities of carbon dioxide indicating that the alcohol, also, is formed from a 3-carbon compound. None of the 3-carbon compounds fermented gave ethyl alcohol as a final product. In serial analyses of glucose fermentations it was shown that the percentage of lactic acid decreased while acetic acid, carbon dioxide and ethyl alcohol increased. Ethyl alcohol, acetic acid and carbon dioxide are formed at the expense of the lactic acid. The formation of ethyl alcohol by the decarboxylation of lactic acid has never been shown, to the knowledge of the author.

With yeast, according to the Embden-Meyerhof theory, ethyl alcohol is formed by the reduction of acetaldehyde, the latter resulting from the decarboxylation of pyruvic acid. This theory may be used to advantage in explaining the changing relationships among the products during fermentation. Lactic and pyruvic acids may be assumed in equilibrium. As lactic acid accumulates the reaction is shifted in the direction of pyruvic acid which is in turn broken down to ethyl alcohol, CO₂ and acetic acid.
The addition of acetaldehyde and acetylmethylcarbinol to glucose fermentations resulted in a decrease in ethyl alcohol, lactic acid and glycerol and an increase in acetic acid and carbon dioxide. These facts may be explained by the methyl glyoxal scheme as follows. Lactic acid may be fermented to acetic acid and carbon dioxide; the active hydrogen formed reducing the added hydrogen acceptors which compete with precursors of ethyl alcohol and glycerol resulting in a decrease in the quantities of these two products. In the phosphoglyceric acid scheme lactic acid, ethyl alcohol and glycerol are assumed to be formed by the reduction of intermediary hydrogen acceptors. Acceptors added to glucose fermentations, then, may compete with hydrogen acceptors formed from glucose for the active hydrogen. There would result a decrease in ethyl alcohol, lactic acid and glycerol in agreement with the experimental data.

In view of the fact that the experimental data may be explained differently, two schemes of dissimilation are given; one (figure 7) based on the occurrence of methylglyoxal and the other (figure 8) based on the Embden-Meyerhof theory. The homofermentative dissimilation does not involve the part of the scheme in which ethyl alcohol is formed. Data from fermentation by *L. plantarum* (Table 3) are used to show dissimilation by homofermentative lactic bacteria and are underlined. The other data are from the fermentation by *L. lycopersici* (Table 8) to show the dissimilation by heterofermentative lactic bacteria. The theoretical and experimental values agree in one
scheme as well as in the other. It is evident that quantitative analyses of the final products cannot be used alone in formulating schemes of dissimilation.

The following facts support the methylglyoxal scheme of dissimilation: (1) The quantitative data agree with the theoretical values. (2) Lactic acid is fermented to acetic acid and carbon dioxide with pyruvic acid as an intermediary.

![Diagram of the Methylglyoxal Scheme of Dissimilation]

Figure 7. The Methylglyoxal Scheme of Dissimilation.
Figure 8. Scheme of Dissimilation Based on the Embden-Meyerhof Scheme.
Although these facts support the methylglyoxal scheme they may also be explained by the phosphoglyceric acid mechanism.

Mr. R. W. Stone (personal communication), working in this laboratory has recently isolated phosphoglyceric acid from fermentations by non-proliferating cells of L._pentosaceus.

In view of (1) the isolation of phosphoglyceric acid, (2) the agreement between the experimental and theoretical data applied to the scheme, (3) the effect of added hydrogen acceptors on the dissimilation of glucose, (4) the isolation and dissimilation of pyruvic acid, (5) the changing relationships of the products during dissimilation and (6) the similarity between lactic, muscle and yeast dissimilation, it appears that serious consideration of the Embden-Meyerhof ideas must be made in an attempt to reveal the function of both phosphoglyceric acid and methylglyoxal in the dissimilation of glucose by lactic acid bacteria.

It has been of interest throughout this study to compare species of Leuconostoc with heterofermentative rods. The physiological properties are strikingly similar. (1) Glucose is fermented to lactic acid, acetic acid, carbon dioxide, ethyl alcohol and glycerol. In serial analyses variation in the relationships of products are similar. (2) The same fermentation of pyruvic acid is brought about by Lactobacillus lycopersici and Leuconostoc dextranicus. (3) The effect of added hydrogen acceptors on the dissimilation of glucose is similar. Acetyl-methylcarbinol is reduced to 2,3-butylene glycol. A slight
difference was observed in the fermentation of levulose. *Leu-
conostoc dextranicus* produced more ethyl alcohol and less manni-
tol than *Lactobacillus lycopersici* in one experiment. In general
the physiological properties of the two groups of bacteria are
alike.
Summary and Conclusions

Glucose is fermented by homofermentative lactic acid bacteria with the formation of lactic acid, with essentially traces of acetic acid, carbon dioxide and glycerol; in addition to these ethyl alcohol is formed by heterofermentative forms.

Physiologically the heterofermentative species of Lactobacillus are very closely related to Leuconostoc.

Acetic acid and ethyl alcohol are probably formed from 3-carbon intermediaries, each accompanied by equimolar quantities of carbon dioxide.

Pyruvic acid and lactic acid are discussed as probable intermediary compounds. The latter is fermented aerobically to acetic acid and carbon dioxide. Pyruvic acid has been isolated as an intermediary in the breakdown of lactic acid. Acetic acid, lactic acid and carbon dioxide are formed from pyruvic acid in equimolar quantities.

Serial analyses of products of dissimilation of glucose show an increase in the rate of lactic acid formation and a decrease in the rates of formation of acetic acid, ethyl alcohol and carbon dioxide.

Acetaldehyde was isolated from the homofermentative dissimilation of glucose.

The addition of acetaldehyde and acetyl methylcarbinol to
fermentations of glucose results in an increased production of acetic acid and carbon dioxide and a decrease in the quantities of ethyl alcohol, lactic acid and glycerol formed. The added acceptors have no effect on fermentations by homo-lactic bacteria.

The dissimilation of levulose by heterofermentative forms was similar to fermentations of glucose to which hydrogen acceptors had been added. Part of the levulose acted as a hydrogen acceptor; being reduced to mannitol.

Two schemes of dissimilation are reviewed, one based on methylglyoxal as an intermediary and the other on phosphoglyceric acid. The data obtained in this study do not permit of the acceptance of one scheme to the exclusion of the other; either theory may be used to interpret the results. In view of the many mechanisms that may bring about the end-products in the same quantitative relationship and the isolation of intermediary compounds that do not satisfy a single scheme of dissimilation it is probable that no one of the present outlines of dissimilation governs fermentation under all conditions. Further study should conciliate the various theories of carbohydrate breakdown and clarify the roles played by intermediary compounds such as methylglyoxal and phosphoglyceric acid.
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Zur exakteren Studien an Mitochondrien. 

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Zum Schluß der Zeitung.


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