THE GASEOUS METABOLISM OF L. PENTOACETICUS WITH REFERENCE TO SEVERAL REPRESENTATIVE MEMBERS OF THE LACTOBACILLUS GROUP

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Of the natural biological fermentations, two have received a great deal of attention and study—the alcoholic and lactic acid fermentations. Of these two processes, the fermentation by organisms of the Lactobacillus group would seem to be ideal for the study of the mechanism of the breakdown of carbohydrates to lactic acid. The quantity of lactic acid produced is large, while the amounts of other fermentation by-products are small and the process is apparently simple.

Much of the work done in the study of other biological processes has revealed striking similarity in the mechanisms involved. The carbohydrates containing hexose units are broken down in nearly every instance by animal and plant tissue such as muscle, yeast, mycelial fungi and bacteria to intermediate products containing three carbon atoms. The work of Neuberg has shown this to be methylglyoxal or some closely related substance. Suffice it to say that most tissues possess the property of converting this substance to lactic acid. Lactic acid bacteria are no exception.

In a study of lactic acid fermentation by members of the Lactobacillus group, Fred, Peterson and co-workers have made important contributions to our knowledge of fermentation mechanisms in general, by their painstaking analyses of bacterial cultures, and particularly in their study of the fermentation of carbohydrates by *L. pentoaceticus*.

Many members of the Lactobacillus group resemble one another culturally so closely that it is often difficult to find differential characteristics. However, *L. pentoaceticus* stands out distinctly

as an organism producing large amounts of acetic acid, in addition to the lactic acid formed by all members of the group.

A study of the amounts of the two acids produced (acetic and lactic) by *L. pentoaceticus* shows that the ratio depends upon the age of the culture, the supply of carbohydrate and the ease with which the organism is able to utilize the sugar. This was demonstrated by Fred, Peterson and Davenport (1920) when they obtained a ratio which varied from 100:520 (volatile:non-volatile acid) at fifteen days, 100:305 at thirty days and 100:115 at seventy-five days, in the fermentation of glucose. In the fermentation of mannose the ratio was 100:179 at fourteen days and 100:66 at thirty-five days. With levulose, ratios of 100:70 at fourteen days, 100:62 at twenty-eight days and 100:84 at forty-two days were obtained.

What is true of *L. pentoaceticus* is doubtless true of the other lactobacilli, but to a much more limited extent. Though the ratio of volatile acid to non-volatile acid produced increases as the age of the culture increases, this increase is much less marked.

The equation given by Peterson and Fred (1920) for the fermentation of glucose by *L. pentoaceticus* is as follows:

$$C_6H_{12}O_6 \rightarrow CH_3CHOHCOOH + CH_3CH_2OH + CO_2 \qquad (1)$$

However, they observed that pentose-fermenting organisms of the Lactobacillus type were able to convert sodium lactate to acetic acid and carbon dioxide. Hence, the ability of the organism to utilize a lactate determines the amount of volatile acid formed as an end product of the fermentation.

The conversion of glucose to lactic acid is an anaerobic process, and as such should not require the presence of oxygen nor produce carbon dioxide. The change of lactic acid to acetic acid is an oxidation, and one may expect one molecule of oxygen to be utilized and one molecule of carbon dioxide to be produced, as shown in equation (2).

$$CH_3CHOHCOOH + O_2 \rightarrow CH_3COOH + CO_2 + H_2O \qquad (2)$$

That acetaldehyde is formed as an intermediate stage of this oxidation is indicated by its accumulation in fermentations in the

presence of sulfites. Though sulfite and bisulfite salts are relatively toxic for the lactobacilli (most organisms of this group will grow in the presence of 0.01 per cent of a sulfite-bisulfite mixture having a pH of 6.5 to 7.0, but will not develop in 0.1 per cent concentration of these salts), the organisms develop in 0.01 per cent concentration of sulfite-bisulfite salts and the latter combine rapidly with volatile substances analyzing as aldehydes. However, the amount of aldehyde fixed is so small that the fermentation is not markedly modified.

With the formation of acetaldehyde in the fermentation system one could expect several side reactions to take place. A Cannizzaro reaction between an aldehyde and a ketone (equation 3) or simply between two molecules of acetaldehyde (equation 4) would account for the formation of ethyl alcohol (Neuberg and Gorr, 1925).

$$CH_3COCHO + CH_3CHO + H_2O \rightarrow CH_3COCOOH + CH_3CH_2OH \quad (3)$$

$$2\ CH_3CHO + H_2O \rightarrow CH_3COOH + CH_3CH_2OH \quad (4)$$

Inasmuch as the oxidation side-reactions involve the use of oxygen and the production of carbon dioxide, it was thought advisable to study a variety of lactobacilli in a microrespirometer such as that developed by Warburg and others for the determination of respiratory quotients of living cells (Warburg, 1931).

For this purpose both growing cultures and suspensions of living organisms were placed in the flasks described by Warburg and attached to a Barcroft open-end manometer, and the flask shaken in a water bath at 37° or 32°, as the optimum temperature of the organism required. Oxygen consumption was determined by removing CO_2 by KOH solution, and the carbon dioxide was estimated at the end of the experiment by releasing the carbonate formed with an excess of sulfuric acid.

Several varieties of the Lactobacillus group were employed:

L. delbrücki
L. leichmanni
L. odontolyticus (3 strains)
L. pentoaceticus (3 strains)

For the study of growing cultures, a medium of the following basic composition was employed:

	per cent
Carbohydrate	1
Peptone	1
Yeast extract (Savita)*	0.3
Phosphate	1
Water to volume	pH adjusted to 6.5 to 7.0

* Savita obtained from Battle Creek Food Company, Battle Creek, Michigan.

This medium was inoculated heavily with a culture of the lactobacillus and 2.5 cc. added to the respirometer flask.

Washed cell suspensions were prepared by growing the organisms in broth culture, centrifuging and washing in saline three or four times and finally resuspending in 1 per cent phosphate (pH 6.5 to 7.0) and aerating for three to six hours with a current of air. Cells were counted in a counting chamber and by plating.

Results are expressed in total cubic millimeters of the gas formed or utilized during the time of experiment. No attempt has been made to determine the rate of change per hour per unit of cell substance, since the cell suspensions of the lactobacilli changed in their activity upon standing. The determinations were made using fresh suspensions of young cultures and comparisons made of data obtained at one time.

COMPARISONS OF GROWING CULTURES OF LACTOBACILLI

Six cultures of lactobacilli were inoculated into yeast-extract broth (1.0 cc. culture per 100 cc. medium) and the changes in pressure due to utilization of oxygen were observed over a period of eight hours. All the cultures grew readily in the medium and the turbidity due to the cells was comparable in each case at the end of the period of observation. The volume of oxygen used by each organism is shown by the curves of charts 1 and 2. It is evident that all the strains used for this study utilized some oxygen, but *L. pentoaceticus* is distinctive in that it consumed over five times the amount used by the next most active strain, *L. odontolyticus*. The five least active lactobacilli differ but slightly

in their rates of oxygen consumption. *Lactobacillus delbrücki* and *L. leichmanni* utilize almost none. As such they might be termed

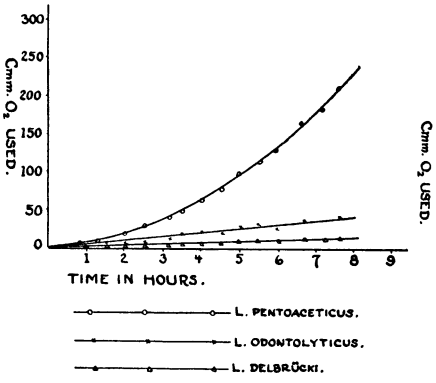

CHART 1

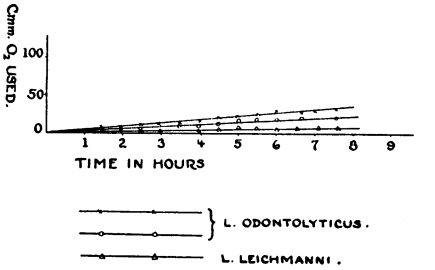

CHART 2

CHART 1. OXYGEN CONSUMPTION OF THREE CULTURES OF LACTOBACILLI
CHART 2. OXYGEN CONSUMPTION OF THREE CULTURES OF LACTOBACILLI

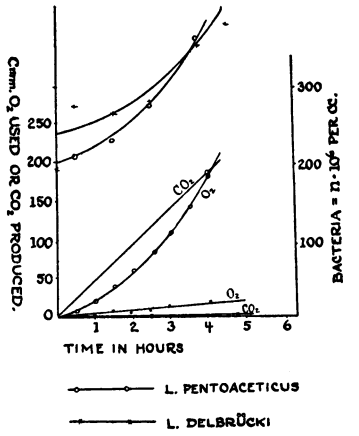

CHART 3

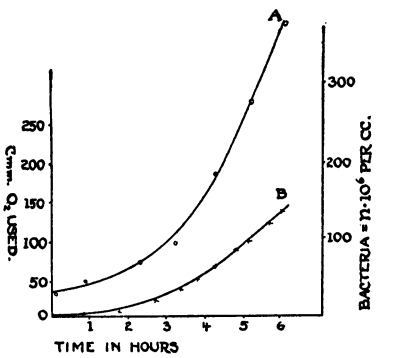

CHART 4

CHART 3. COMPARATIVE STUDY OF L. PENTOACETICUS AND L. DELBRÜCKI FOR GROWTH AND GASEOUS METABOLISM
CHART 4. RELATION OF OXYGEN CONSUMPTION TO CELL NUMBERS, USING L. PENTOACETICUS
Curve A: Plate count.
Curve B: Oxygen consumption.

a unique type of anaerobe, for though they live in a medium in equilibrium with 20 per cent oxygen, the latter neither interferes

nor takes part markedly in their metabolism. *Lactobacillus pentoaceticus* presents a different picture. From the first it begins to use oxygen and in comparatively large amounts.

In order to follow this comparison more closely, the experiment was repeated with *L. pentoaceticus* and *L. delbrücki*. The medium was heavily inoculated with an actively-growing eighteen to twenty-four-hour culture and the O_2 consumption and CO_2 production of each determined. At intervals the cell numbers were determined by plating from control flasks. The curves are shown in chart 3. It will be seen from chart 3 that though the two cultures are comparable as to cell numbers, the metabolism differs markedly in degree. The *L. pentoaceticus* utilizes nearly ten times as much oxygen as does *L. delbrücki* under the same conditions. Nearly 20 times as much CO_2 is produced by the former as by the latter, but it is evident from the curves that approximately the same volumes of CO_2 are produced as of O_2 utilized.

The relation of cell numbers to oxygen utilization by *L. pentoaceticus* may be followed more closely by comparison of the curves shown in chart 4. For this, a sample of yeast-extract broth was inoculated with *L. pentoaceticus* and oxygen consumption recorded by one manometer, while cell numbers were determined by plating samples taken from a duplicate flask. The two curves show that O_2 consumption is directly related to cell numbers.

OBSERVATIONS WITH WASHED CELL SUSPENSIONS

One of the characteristics of *L. pentoaceticus* is its fermentation of xylose with the formation of lactic and acetic acids, but with the production of much more volatile acid per molecule of carbohydrate fermented, than from glucose. Hence, it was of interest to compare the action of a suspension of the washed cells upon the two carbohydrates.

Suspensions of *L. pentoaceticus* were prepared as previously described, and flasks containing the following reagents were attached to the manometers: the reaction flask contained 1.0 cc. of a phosphate-buffered cell suspension, 1.0 cc. of the carbohydrate substrate (10 per cent solution), 0.4 cc. of 0.85 per cent NaCl solution, 0.2 cc. of a 10 per cent solution of H_2SO_4 (in the

side chamber) and 0.10 cc. of a 10 per cent KOH solution (in inset). An oxidation control was used consisting of all reagents in the absence of the cell suspension. In most instances this latter correction was insignificant. The cells of the suspension were estimated at the beginning of the experiment by plating and by a Petroff-Hauser bacteria-counting chamber. The curves are shown in chart 5. It will be seen from chart 5 that more oxygen is utilized in a glucose medium than in a xylose medium, but in either case the O_2 used is equal in volume to the CO_2

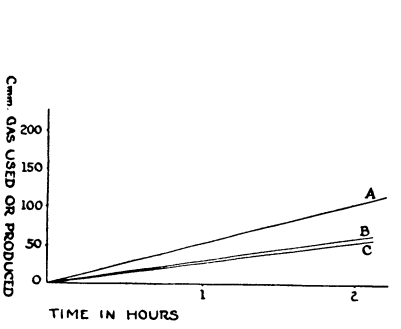

CHART 5

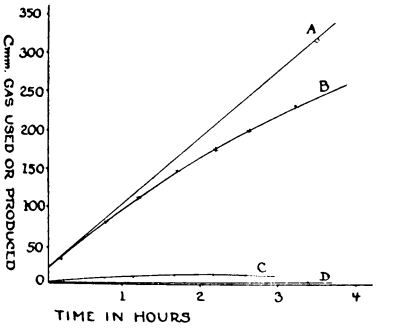

CHART 6

CHART 5. ACTION UPON GLUCOSE AND XYLOSE, OF A WASHED CELL SUSPENSION OF L. PENTOACETICUS PREPARED FROM XYLOSE BROTH CULTURE

Curve A: Oxygen used and CO_2 produced (glucose).
Curve B: Carbon dioxide produced (xylose).
Curve C: Oxygen used (xylose).

CHART 6. ACTION UPON GLUCOSE AND XYLOSE, OF A WASHED CELL SUSPENSION OF L. PENTOACETICUS PREPARED FROM GLUCOSE BROTH CULTURE

Curve A: Carbon dioxide produced (glucose).
Curve B: Oxygen used (glucose).
Curve C: Oxygen used (xylose).
Curve D: Carbon dioxide produced (xylose).

produced. It should be mentioned that care must be taken in the interpretation of data obtained from experiments in which washed cells are employed. In determining the activity of a suspension upon a substrate, the organism should be grown with that substrate in the medium from which the suspension is prepared. The strain of *L. pentoaceticus* (FA) had been carried for two years or more in a stock culture containing glucose. The suspension obtained from a glucose-broth medium utilized xylose scarcely at all, while glucose was acted upon without difficulty.

Suspensions prepared from xylose broth utilized both carbohydrates without difficulty. The oxygen consumption of the two suspensions is shown in charts 5 and 6.

The action of suspensions of *L. pentoaceticus* upon lactates is shown in chart 7. In this instance a sample of lactic acid solution containing approximately 10 per cent acid was neutralized with sodium hydroxide and an excess of NaOH solution added. This alkaline solution was boiled for ten minutes to hydrolyze the lactides present and the excess NaOH neutralized with HCl. It will be seen from the curves of chart 7 that the lactate is acted upon in the same manner as the xylose and glucose, namely, nearly equal volumes of O_2 and CO_2 are utilized and produced,

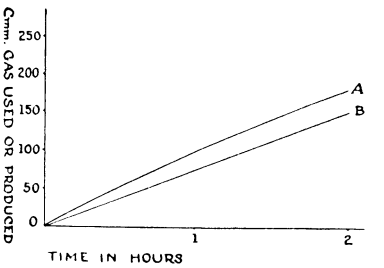

CHART 7. ACTION OF A SUSPENSION OF L. PENTOACETICUS UPON SODIUM LACTATE
Curve A: Oxygen used.
Curve B: Carbon dioxide produced.

respectively. Hence, whether or not the lactobacillus is able to utilize pyruvic acid, methylglyoxal and similar possible intermediate compounds, oxidation of lactic acid is to some extent, a measure of the ability of the organism to produce volatile acids, and can account for the O_2 consumption and CO_2 formation.

Many biological oxidations may be accelerated by the use of some substances capable of being oxidized by molecular oxygen and reduced by the biological system, the oxidation and reduction of the accelerating agent being reversible. Methylene blue has been much used for this purpose in M/5000 concentration. More recently Friedheim and Michaelis (1931) demonstrated that pyocyanine, the chloroform-soluble pigment of *Ps. pyocyanea*, may be reversibly oxidized and reduced, and acts in a manner similar to methylene blue with washed cell suspensions of bacteria.

While methylene blue and pyocyanine may act as accelerators of oxidation reactions, other reagents such as potassium cyanide and carbon monoxide have been found to inhibit these reactions, particularly those induced by the respiratory enzymes of the cell. A third group of reagents have been shown to permit respiration of a cell, though they inhibit the fermentation or glycolytic reactions. The substances of the latter type most commonly used are fluorides, and more recently Lundsgaard (1930) has employed monoiodoacetic acid.

The lactobacilli have been described as a group dependent upon carbohydrates for growth. Some organisms of the group have been shown to grow in carbohydrate-free media, but growth when it occurs is relatively slow. Some workers have stated that lactic acid is sometimes formed from polypeptides (Sherman, 1920). This would indicate that these organisms may utilize some of the constituents of the peptone for energy, though generally the process is slow. An organism of the Lactobacillus group obtaining its energy chiefly by the conversion of the carbohydrate to lactic acid should be but slightly inhibited by potassium cyanide (pH 7.0 to 7.2). On the other hand, if substances such as sodium fluoride or monoiodoacetic acid (neutralized by sodium hydroxide) inhibit glycolysis but permit respiration, they should be toxic for the lactobacilli in high dilution. Furthermore, if the lactic acid organisms are able to substitute molecular oxygen for the carbohydrate, the toxicity of the fluoride and monoiodoacetic acid should be less in aerobic than in anaerobic conditions.

The glucose-savita medium previously described was used, and dilutions of KCN, NaF and CH_2ICOOH were prepared from neutral stock solutions of the reagents sterilized by filtration through a Chamberlain L 5 candle. The tubes were then inoculated with 0.1 cc. of active culture of the organism. The KCN was employed in concentrations of from 0.1 to 0.01 per cent, but none of the strains of the lactobacilli were markedly inhibited in growth or acid production in these concentrations. There was a slight inhibition of growth in 0.1 per cent concentration with *L. delbrücki.*

Lactobacillus pentoaceticus was inoculated in aerobic and anae-

robic conditions (CO_2 displacement of air) in broth containing 0.0 N, 0.1 N, 0.01 N, 0.001 N and 0.0001 N NaF (pH 6.8 to 7.0) and incubated at 30° for twenty-four hours. The results follow:

	0.1 N	0.01 N	0.001 N	0.0001 N	0.0 N	CONTROL
Aerobic:						
Growth	±	+++	++++	++++	++++	−
pH	6.0	5.2	4.6	4.6	4.6	6.5
Anaerobic:						
Growth	−	±	++	++++	++++	−
pH	6.0	5.6	5.2	4.6	4.6	6.0

Similarly, *L. pentoaceticus*, *L. delbrücki* and *L. leichmanni* were inoculated into tubes of broth containing 0.0 N, 0.1 N, 0.01 N, 0.001 N, 0.0001 N and 0.00001 N CH_2ICOOH (pH 6.8 to 7.0) and incubated twenty-four hours under aerobic and anaerobic conditions. The results obtained with *L. pentoaceticus* are typical and are given below:

	0.1 N	0.01 N	0.001 N	0.0001 N	0.00001 N	0.0 N	CONTROL
Aerobic:							
Growth	−	−	−	++	+++	++++	−
pH	6.5	6.5	6.5	6.3	6.0	5.0	6.5
Anaerobic:							
Growth	−	−	−	±	++	++++	−
pH	6.0	6.0	6.0	6.0	5.8	4.8	6.0

From these results it would seem that, though KCN has little or no effect upon the metabolism of members of the Lactobacillus group, such substances as NaF and CH_2ICOOH have a marked inhibitive action, especially under anaerobic conditions. The iodoacetic acid is especially toxic for the organisms studied. In each instance (NaF or CH_2ICOOH) the inhibitive action is greater under anaerobic conditions than in the presence of oxygen, but the difference is not marked over a wide range of dilutions. This may indicate that *L. pentoaceticus*, as well as the other lacto-

bacilli studied, are able to utilize the energy of an oxidation reaction to some extent when the normal fermentation mechanism is inhibited.

These preliminary test-tube experiments were followed by determinations of oxygen consumption of washed cell suspensions.

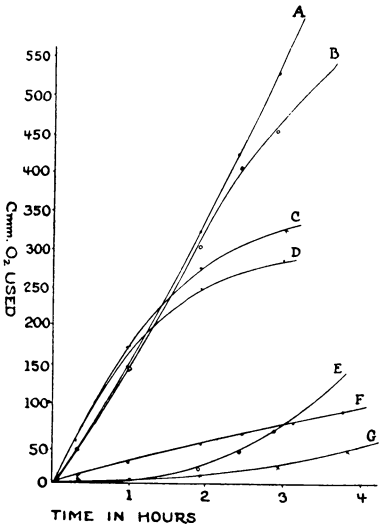

CHART 8

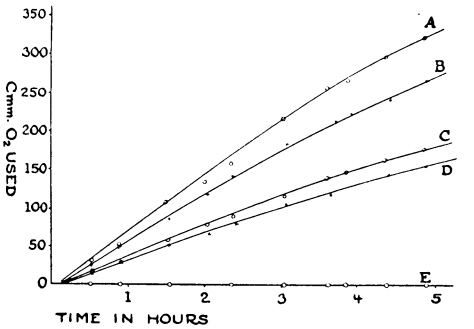

CHART 9

CHART 8. INFLUENCE OF GLUCOSE, PYOCYANINE AND CYANIDE UPON THE OXYGEN CONSUMPTION OF WASHED CELL SUSPENSIONS OF PS. PYOCYANEA

Curve A: Suspension, glucose and pyocyanine.
Curve B: Suspension and glucose.
Curve C: Suspension and pyocyanine.
Curve D: Suspension alone.
Curve E: Suspension and KCN.
Curve F: Suspension, glucose, pyocyanine and KCN.
Curve G: Suspension, pyocyanine and KCN.

CHART 9. INFLUENCE OF GLUCOSE, METHYLENE BLUE AND CYANIDE UPON THE OXYGEN CONSUMPTION OF WASHED CELL SUSPENSIONS OF L. PENTOACETICUS

Curve A: Suspension, methylene blue and glucose.
Curve B: Suspension, methylene blue, glucose and KCN.
Curve C: Suspension and glucose.
Curve D: Suspension, glucose and KCN.
Curve E: Suspension alone, suspension plus methylene blue, or suspension plus methylene blue plus KCN.

Pyocyanine (prepared as described by Friedheim and Michaelis, (1931) in M/5000 concentration was found to influence the O_2 consumption of *Ps. pyocyanea* and *L. pentoaceticus* in the same

manner as M/5000 methylene blue. Inasmuch as methylene blue is more readily available and was nearly as active as pyocyanine, it was used more extensively in this work. Potassium cyanide was used in N/500 concentration and the stock solution was neutralized with HCl just before use, the reaction being 7.0 to 7.2 (Dixon and Elliott, 1929). The effect of pyocyanine and KCN upon pyocyanea suspensions is shown in chart 8, and the effect of methylene blue and KCN upon *L. pentoaceticus* is shown in Chart 9. Similar results were obtained in each case with either pyocyanine or methylene blue when used in M/5000 concentration. The results of this work show a marked difference in the metabolism of *Ps. pyocyanea* and *L. pentoaceticus*. The former organism possesses an oxidative mechanism which functions in the suspension itself, utilizing large amounts of oxygen. The oxygen consumption is stimulated by the addition of pyocyanine or glucose, and especially by the addition of both. Potassium cyanide markedly inhibits the utilization of oxygen, whether glucose or pyocyanine has been added or not. *L. pentoceticus*, however, does not utilize oxygen without the presence of an added carbohydrate. It lacks this characteristic, whether an activator such as pyocyanine or methylene blue is added or not. When a suitable carbohydrate is added, the addition of N/500 KCN inhibits oxygen consumption slightly, while the methylene blue accelerates O_2 utilization. The KCN inhibition is not marked, as in the case of *Ps. pyocyanea*.

If *L. pentoaceticus* requires a suitable carbohydrate for growth, then, as was stated before, Na Fand CH_2ICOOH should markedly inhibit the oxygen consumption of cell suspensions in the presence of glucose, for though this organism promotes the oxidation of lactic acid, the latter must first be produced from glucose by a glycolytic process. The oxygen consumption of *Ps. pyocyanea* and *L. pentoaceticus* was determined in the presence of 0.1 N, 0.01 N and 0.001 N NaF, using washed cell suspensions. The results are expressed in chart 10. The curves in chart 10 indicate that, though *L. pentoaceticus* is inhibited by 0.1 N and 0.01 N NaF, 0.001 N concentration inhibits but slightly. On the other hand, *Ps. pyocyanea* is not inhibited by 0.01 N NaF, though determina-

tions not shown in the above chart indicate that it is inhibited by 0.1 N concentrations of the salt. The curve for the oxygen consumption of *Ps. pyocyanea* with 0.1 N NaF coincides with the curve above for *L. pentoaceticus* in the presence of 0.1 N NaF.

Similar experiments with suspensions of *L. pentoaceticus* and CH_2ICOOH show more marked inhibition of oxygen consumption by 0.1 N, 0.01 N and 0.001 N concentrations. This is shown in chart 11. From the data presented above it appears that the for-

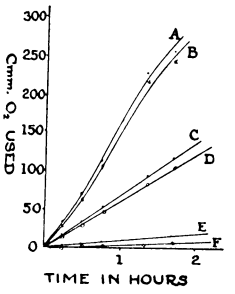

CHART 10

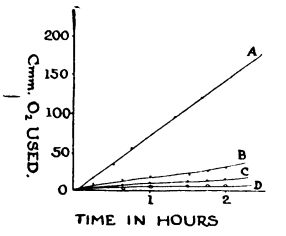

CHART 11

CHART 10. COMPARATIVE ACTION OF SODIUM FLUORIDE UPON WASHED CELL SUSPENSIONS OF PS. PYOCYANEA AND L. PENTOACETICUS

Curve A: *Ps. pyocyanea* suspension alone.
Curve B: *Ps. pyocyanea* suspension and 0.01 N NaF.
Curve C: *L. pentoaceticus* suspension and glucose.
Curve D: *L. pentoaceticus* suspension and glucose and 0.001 N NaF.
Curve E: *L. pentoaceticus* suspension and glucose and 0.01 N NaF.
Curve F: *L. pentoaceticus* suspension and glucose and 0.1 N NaF.

CHART 11. INFLUENCE OF VARIOUS CONCENTRATIONS OF MONOIODOACETIC ACID UPON THE OXYGEN CONSUMPTION OF WASHED CELL SUSPENSIONS OF L. PENTOACETICUS

Curve A: Suspension and glucose.
Curve B: Suspension and glucose and 0.001 N iodoacetic acid.
Curve C: Suspension and glucose and 0.01 N iodoacetic acid.
Curve D: Suspension and glucose and 0.1 N iodoacetic acid.

mation of volatile acid by a strain of lactobacillus is correlated with O_2 consumption and CO_2 production. It has been stated by many workers that growth under aerobic or anaerobic conditions did not modify the ratio of volatile acid to non-volatile acids (acetic to lactic acids) in the case of *L. pentoaceticus* (Fred, Peterson and Davenport, 1919, and Weinstein and Rettger, 1932). Other authors have stated that less volatile acid was formed by *L. leichmanni* and *L. delbrücki*, when grown under anaerobic con-

ditions (Kayser, 1894), than under aerobic. If this is true, then *L. pentoaceticus* must employ a mechanism for the breakdown of glucose different from that of *L. leichmanni* and *L. delbrücki*. However, if oxygen is utilized by *L. pentoaceticus*, as is indicated by the respirometer findings, then it is difficult to understand how removal of oxygen could fail to influence the amount of volatile acid produced, especially if the acetic acid is produced at the expense of lactic acid.

Preliminary experiments indicate that the end products of the fermentation by *L. pentoaceticus* are influenced by the presence or absence of oxygen, but the differences obtained are not as

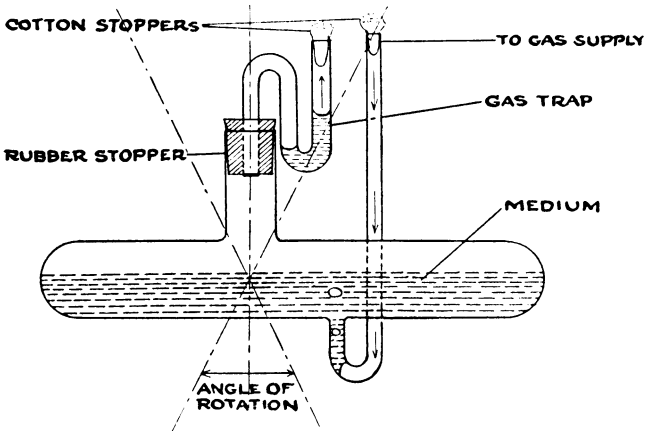

FIG. 1. APPARATUS FOR SIMULTANEOUSLY SHAKING AND INCUBATING CULTURES OF L. PENTOACETICUS UNDER AEROBIC OR ANAEROBIC CONDITIONS

striking as would be expected if the formation of acetic acid were due solely to the oxidation of lactic acid by molecular oxygen.

For the study of this question two types of fermentation systems were used. The first consisted of glucose-savita medium containing 1 per cent phosphate buffer (pH 6.5) in two 500 cc. Erlenmeyer flasks containing 150 cc. of the medium. One flask was placed in the incubator as an ordinary aerobic fermentation. The other flask was placed in an anaerobic jar and the air repeatedly exhausted and replaced with CO_2. A third flask contained uninoculated broth for a control analysis. After a week's incubation the fermentation mixtures were analyzed for volatile and non-

volatile acids, the first by distilling from the medium acidified with H_2SO_4, and the second by the Friedemann and Kendall modification of the Clausen aldehyde-fixation method (Friedemann and Kendall, 1929). The second system consisted of a tube (illustrated in figure 1) which was filled half full of the glucose-savita medium employed in the first system (50 cc.) and CO_2 or O_2 gas passed through the tube as it was rocked in a water bath at 32°. The current of gas was only sufficient to insure thorough mixing of the fluid and to maintain anaerobic or aerobic conditions—usually one or two bubbles per minute after the tube had been flushed free from air.

In each instance there was a slightly lower ratio of volatile to non-volatile acids under anaerobic than under aerobic conditions. Two typical analyses are shown in the following table:

	N/10 VOLATILE ACID PER 100 CC.	LACTIC ACID	MOLAR RATIO VOLATILE: NON-VOLATILE ACIDS
	cc.	*mgm. per 100 cc.*	
Flasks and phosphate:			
Aerobic	15.08	589	18.8:81.2
Anaerobic	10.80	486	16.6:83.4
Rocked tube:			
Aerobic	29.5	390	41:59
Anaerobic	33.4	511	37:63

The differences found in any instance are not marked in the analyses made of the paired fermentations (aerobic and anaerobic), but the total amounts of volatile acid found in a given fermentation varied considerably, depending upon the length of incubation, supply of carbohydrate and the activity of the culture used for inoculum. Though the ratios obtained for fermentation acids at different times varied considerably, the ratios observed in fermentations carried out at the same time under the same conditions were consistently slightly lower in anaerobic fermentations than in the presence of molecular oxygen.

DISCUSSION

It is evident from the data presented that two types of reactions take place in the metabolism of the lactobacilli. The first is the anaerobic breakdown of carbohydrate to lactic acid, while the second is the oxidation of lactic acid to acetic acid and carbon dioxide. With most lactobacilli commonly studied, the first reaction is predominant and the second a minor one. With *L. pentoaceticus* the oxidation of lactic acid proceeds at a much greater velocity, and comparatively large amounts of O_2 are utilized and CO_2 and acetic acid produced. This reaction is increased by the addition of methylene blue or pyocyanine, and is only slightly inhibited by KCN. Even 0.1 per cent concentration of the latter reagent is not markedly toxic.

It has been stated many times that growth of lactobacilli such as *L. delbrücki* or *L. leichmanni* under anaerobic conditions decreases the ratio of volatile to non-volatile acid. However, in the case of *L. pentoaceticus* large amounts of volatile acid are formed even under anaerobic conditions and, though removal of molecular oxygen from the system decreases the ratio of volatile to non-volatile acid, this difference is not marked. Hence, the data presented suggest that two mechanisms are responsible for the production of acetic acid, one aerobic and the other anaerobic—or, at least, taking place under anaerobic cultural conditions.

The effect of inhibitors such as KCN or CO has been the subject of much debate, but the work of Dixon (1929) and his coworkers has demonstrated that the inhibition of O_2 consumption is not complete with animal tissue prepartions or with yeast cells; there is always some utilization of oxygen due to the presence of other oxidases. Keilin and others (1929) have worked with the oxidases of cells of many types and particularly yeasts, and showed the existence of oxidizing enzymes such as cytochrome and indophenol oxidase. The latter resembles the respiratory enzyme studied by Warburg in that it is sensitive to cyanide, carbon monoxide and hydrogen sulfide. Yaoi and Tamiya (1928) studied the cytochrome in bacteria and found that aerobic bacteria such as *Ps. pyocyanea* and *B. subtilis* contained cytochrome

in large concentrations, while facultative anaerobic organisms such as *Bact. coli* contained about half the concentration found with aerobic forms, and anaerobic bacteria contained none. The work of Callow (1924) indicated that the anaerobes resemble *Strep. lacticus* in the inability of washed-cell suspensions to utilize oxygen. Fenyvessy and Scheff (1930) observed that *Spironema recurrentis* used carbohydrates without the utilization of oxygen, but with the production of carbon dioxide. They suggest that the CO_2 formation may be due to either the action of the fermentation acids upon the bicarbonates of the serum-Ringer's solution medium employed, or to a direct oxidation of the carbohydrate.

The data presented in this paper suggest that the majority of lactobacilli act in the same manner as *Strep. lacticus*, the anaerobes (such as *Cl. sporogenes*) and the spirochetes in regard to oxygen utilization. The lactic acid organisms and *Spironema recurrentis* differ from the anaerobes, however, in that the former are able to grow in the presence of molecular oxygen while the latter are inhibited. It is possible that the spirochete obtains its energy by a lactic acid fermentation. *Lactobacillus pentoaceticus* differs from other lactobacilli in its oxygen utilization, but judging from the action of cyanides, none of the members of the group contain indophenol oxidase.

It is difficult to expect that the addition of such reagents as NaF or CH_2ICOOH would quantitatively limit or inhibit the action of one mechanism (fermentation) over a wide range of concentrations without affecting others, but the changes observed in the metabolism of the lactobacilli by the addition of these inhibiting agents are striking. The oxygen consumption of washed cells of *L. pentoaceticus* is greatly inhibited by 0.1 N and 0.01 N NaF, but not by 0.001 N concentration, as is shown in chart 10. *Pseudomonas pyocyanea*, however, is not inhibited by 0.01 concentration, though 0.1 N concentration inhibits this organism fully as much as it does *L. pentoaceticus*. In a similar manner, monoiodoacetic acid markedly inhibits the oxygen consumption of *L. pentoaceticus* in 0.1 N, 0.01 N and 0.001 N concentrations.

The action of monoiodoacetic acid even in high dilution is shown by its effect upon the growth of the lactobacilli in culture

media. In each instance the action of the inhibiting agent is to limit growth and acid production, the action being greater under anaerobic conditions (in the highest inhibiting concentrations) than in the presence of molecular oxygen. Inasmuch as the method of producing anaerobiosis was by evacuation and replacement of the air by CO_2, the pH of the anaerobic tubes was lower than that of the aerobic. The difference in growth and acid formation by the lactobacilli may be due to the combined action of the iodoacetic acid and CO_2, or to an ability of the organism to initiate the reaction of molecular oxygen with the substrate and to utilize the energy for growth. It should be stated that CO_2 did not inhibit growth of any of the lactobacilli, for in some of the control tubes growth was heavier in the presence of this gas than without it. In any case, the difference between aerobic and anaerobic inhibition does not occur over a wide range of dilutions, as might be expected if this oxidation reaction were easily accomplished in the presence of molecular oxygen. Experience with the cultivation of the lactobacilli in carbohydrate-free media is that growth of the organisms, if it does occur, is relatively slight.

Lactobacillus pentoaceticus resembles the other organisms of the group in its reaction towards NaF and CH_2ICOOH. This, together with the action of KCN upon these organisms, suggests that the mechanism of the first stages of the fermentation reaction is the anaerobic breakdown of the glucose to lactic acid, and that it is common to all organisms of the group. The subsequent stages depend upon the ability of the organism to break down lactic acid or possibly a product giving rise to lactic acid. *Lactobacillus pentoaceticus* acccomplishes this step very readily.

SUMMARY

The majority of lactobacilli utilize oxygen and produce carbon dioxide in the fermentation of carbohydrates in relatively small amounts. *Lactobacillus pentoaceticus* differs from other members of the group in that it utilizes large volumes of oxygen and produces CO_2 in nearly equal volume. The latter organism can utilize xylose and lactates readily with the consumption of oxygen and formation of carbon dioxide in the same volume ratio of 1:1.

None of the lactobacilli studied are inhibited by KCN in less than 0.1 per cent concentration. All of the species studied are inhibited by NaF and CH_2ICOOH in 0.1 N and 0.0001 N concentrations respectively. The inhibition is somewhat greater in anaerobic than in aerobic conditions.

Methylene blue and pyocyanine accelerate the oxygen consumption of *L. pentoaceticus*, whether KCN is present or absent.

Analyses of volatile and non-volatile fermentation acids produced by *L. pentoaceticus* indicate that slightly higher ratios of volatile to non-volatile acids are obtained under aerobic than anaerobic conditions. However, the formation of large amounts of volatile acids under anaerobic conditions suggests that two mechanisms exist for the formation of the volatile acids, one aerobic and the other anaerobic.

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