DISSIMILATION OF GLUCOSE BY HETEROFERMENTATIVE LACTIC ACID BACTERIA[1]

M. E. NELSON AND C. H. WERKMAN

Department of Bacteriology, Iowa State College, Ames

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It is convenient to divide the true lactic acid bacteria into two natural groups for study of their dissimilative properties. Orla-Jensen (1919) suggested that those bacteria which convert carbohydrates essentially into lactic acid be distinguished from those producing, in addition, substantial quantities of volatile acid and carbon dioxide. Kluyver and Donker (1924) proposed the terms homofermentative and heterofermentative respectively for the two groups.

Study of the intermediary dissimilation by the lactic acid bacteria has been confined for the most part to the homofermentative group and little attention has been given to the heterolactic forms.

In the present paper a scheme for the dissimilation of glucose by the heterofermentative bacteria is proposed based on the results of quantitative investigations carried out with several representative species.

Gayon and Dubourg (1901) have studied the dissimilation of glucose by the heterofermentative lactic acid bacteria. Although descriptions of the organisms were given, no attempt was made to classify or name them. Acetic, lactic, succinic and carbonic acids, ethyl alcohol and glycerol were identified as final products of fermentation. Equations were presented for the formation of the products from glucose. A secondary breakdown of lactic to acetic acid was also suggested. Kayser (1904), Laborde (1904), and Smit (1913) identified the same products found by Gayon

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and Dubourg in the fermentation of glucose by members of the heterofermentative group. Fred, Peterson and Davenport (1920) and Peterson and Fred (1920) determined products of the dissimilation of glucose by *Lactobacillus pentoaceticus*. They confirmed the reports of a secondary breakdown of intermediately-formed lactic acid but did not report the presence of succinic acid or glycerol. Pederson (1929a) quantitatively studied the products formed by several species of *Lactobacillus* and *Leuconostoc*. Interpretation of his results is made difficult by the failure to determine carbon dioxide and the other products on the same fermentation. Charleton, Nelson and Werkman (1934) presented a carbon balance of the dissimilation of glucose and of levulose by *Lactobacillus gracilis* and a new species which they named *Lactobacillus fructivorans*.

METHODS

Lactobacillus lycopersici, *Lactobacillus mannitopoeus* and *Lactobacillus acidophil-aerogenes* used in this experiment, were kindly furnished by Dr. C. S. Pederson of the New York Agricultural Experiment Station, Geneva, New York and were chosen as typical of the heterofermentative lactic acid bacteria. Detailed descriptions of these strains of *L. lycopersici* and *L. mannitopoeus* are given by Pederson (1929). The culture of *Lactobacillus acidophil-aerogenes* is a transplant of the original strain isolated from feces and described by Torrey and Rahe (1915). At the time of isolation the organism was described as producing hydrogen. In the present work no free hydrogen was detected.

The medium (table 1) consisted of 2 per cent glucose, 0.3 per cent yeast extract (Difco), 1.0 per cent peptone and 0.2 per cent di-basic potassium phosphate. The glucose was sterilized separately for twenty minutes at 20 pounds and added to the basal medium at the time of inoculation. One liter of medium was placed in each 2-liter flask and inoculated with 25 cc. of a three-day culture grown in medium of the same composition.

In later experiments (table 2) the phosphate content was changed to 0.6 per cent K_2HPO_4 and 0.6 per cent KH_2PO_4 and the pH was adjusted to 6.2. More rapid fermentation occurred

in this medium. The solutions of (*a*) glucose, (*b*) yeast extract and peptone, and (*c*) phosphates were sterilized separately for twenty minutes at 20 pounds pressure and combined just prior to inoculation.

Strict anaerobiosis was maintained by continuously bubbling oxygen-free nitrogen through the medium.

Methods of analysis

The fermented media were analyzed after incubation for three weeks at 30°C.

The carbon dioxide evolved by the fermentation was forced through Bowen potash bulbs by a stream of oxygen-free nitrogen and was determined gravimetrically. The residual carbon dioxide was determined by acidifying an aliquot part of the medium and heating under a reflux condenser with a stream of carbon-dioxide-free air passing through the liquid to carry the carbon dioxide into Bowen potash bulbs for gravimetric analysis.

The volatile acids were determined by steam-distilling two liters of distillate from 400 cc. of the fermented medium. The acids present in the distillate were determined by the partition method of Osburn, Wood and Werkman (1933). The non-volatile acids were determined on a 100 cc. aliquot part of the fermented medium. The acids were extracted with ethyl ether and the lactic acid determined by the Friedemann, Cotonio and Shaffer (1927) method. The succinic acid was determined on an aliquot part of the extract by preparation of the silver salts.

The ethyl alcohol was determined on 300 cc. of the medium by the method of Stahly, Osburn and Werkman (1934).

The sugar determinations were made on the medium at the time of inoculation and at the conclusion of the fermentation by the method of Munsen and Walker (1906) after deproteinating with basic lead acetate.

Accurate determination of glycerol is difficult because of the necessity of separating it from interfering substances. An aliquot part of the medium was evaporated to 15 cc. and enough NaOH added to insure alkaline extraction. This solution was taken up in sodium sulfate. A three-day extraction with ethyl

ether was necessary to obtain quantitative separation of glycerol from the medium. The ether extract was made up to a volume of 100 cc and glycerol determined by Wagenaar's (1911) method. Rather than allow the alkaline copper sulfate solution to stand several hours, as described in the original method, the precipitate was centrifuged and the analysis made on the supernatant liquid.

Purity of the cultures was insured by microscopic and cultural examination at the time of inoculation and just before analysis.

EXPERIMENTAL

In tables 1 and 2 may be found seven typical experiments showing the quantitative dissimilation of glucose by *L. mannitopoeus*, *L. lycopersici* and *L. acidophil-aerogenes*. Reliability of the experimental data is satisfactory in view of the difficulty encountered in making quantitative determinations of the end products. The general agreement between the carbon balances and the oxidation-reduction ratios is satisfactory. The data, therefore, should lend themselves to the formulation of a scheme for the dissimilation of glucose by typical lactic acid bacteria of the hetero-type.

In table 1, glycerol was not experimentally determined, and the results showed a low carbon recovery and an excess of oxidized products. From these data it was evident that some reduced product had not been determined. The work of Gayon and Dubourg (1901) and of Smit (1913) suggested that this reduced compound was glycerol. It has been found (table 2) under our conditions that the quantity of glycerol formed is equivalent to twice the acetic acid. If glycerol is calculated on this basis for the experiment shown in table 1, the carbon balances and oxidation-reduction ratios show excellent agreement. In a similar manner, if glycerol is calculated in the experiments of Peterson and Fred (1920) a more satisfactory agreement of their carbon and oxidation-reduction balances is obtained. Similarly calculation of glycerol from the data of Charleton, Nelson and Werkman (1934) improves the carbon and oxidation-reduction balances.

The small quantity of succinic acid present (table 2) suggests its origin from protein. *L. lycopersici* (table 2) shows a carbon

TABLE 1

Anaerobic dissimilation of glucose by L. mannitopoeus and L. lycopersici

PRODUCT	L. MANNITOPOEUS		L. LYCOPERSICI	
	mM *per liter*	mM *carbon*	mM *per liter*	mM *carbon*
Glucose fermented	64.4	386.4	51.0	306.0
Ethyl alcohol	42.8	85.6	30.25	60.5
Acetic acid	13.8	27.6	9.3	18.6
Carbon dioxide	58.0	58.0	38.0	38.0
Lactic acid	54.2	162.6	48.05	144.15
Glycerol*	27.6	82.8	18.6	55.8
Total		416.6		316.8
Percentage of carbon		107.4		103.5
$\frac{\text{Oxidation}}{\text{Reduction}}$ ratio		1.02		0.974

* Calculated.

TABLE 2

Anaerobic dissimilation of glucose by L. acidophilaerogenes and L. lycopersici

PRODUCTS	D. ACIDO-PHILAERO-GENES	L. LYCOPERSICI			
		I	II	III	IV
	mM *per liter*	mM *per liter*	mM *per liter*	mM *per liter*	mM *per liter*
Glucose fermented	88.9	80.0	92.1	102.2	112.2
Ethyl alcohol	39.5	63.0	69.6	74.1	78.9
Acetic acid	10.2	12.1	16.5	15.5	19.5
Carbon dioxide	43.9	64.3	80.0	81.0	95.1
Lactic acid	108.2	65.2	67.6	83.1	89.7
Glycerol	21.7	25.0	36.2	32.6	43.5
Succinic acid	0.8	3.8			
Percentage of carbon recovered:					
With succinic acid	100.2	104.2			
Without succinc acid	100.0	101.0	101.9	99.0	103.0
$\frac{\text{Oxidation}}{\text{Reduction}}$ ratio	.873	.851	.912	.884	.943

balance of 104 per cent when succinic acid is included and of 101 per cent when excluded. *L. acidophil-aerogenes* shows the carbon

recovered inclusive of succinic acid to be 100.2 per cent and, excluding succinic acid, to be almost exactly 100 per cent.

Both experiments show a small deficiency of oxidized products, although the error is within the experimental limits of the analysis of such a mixture.

In view of the lack of evidence to support any scheme of initial breakdown of glucose by the heterofermentative lactic acid bacteria, the scheme presented here will deal only with the final reactions of the dissimilation.

Peterson and Fred (1920a) have presented evidence for the formation of acetaldehyde as an intermediate compound in the dissimilation of glucose by *L. pentoaceticus*. The authors have been unable to secure acetaldehyde by fixation methods in sufficient quantity to characterize by its derivatives. It is not improbable that acetaldehyde formed is so readily utilized that the fixatives are unable to act or the organisms may be able to utilize the acetaldehyde in the presence of fixatives.

It is difficult to account for the formation of ethyl alcohol, assuming a splitting of the glucose into two 3-carbon compounds, and not postulate acetaldehyde as an intermediate precursor. The ethyl alcohol might arise: (*a*) from the acetaldehyde acting solely as a hydrogen acceptor, (*b*) from a Cannizzaro reaction between two molecules of acetaldehyde, forming both ethyl alcohol and acetic acid, or (*c*) from a mixed Cannizzaro reaction between acetaldehyde and some other aldehyde. In view of the absence of an oxidation product typical of a Cannizzaro reaction and equivalent in quantity to ethyl alcohol, it is highly probable that the latter is formed by direct reduction of acetaldehyde.

Peterson and Fred (1920) have shown that a sufficient quantity of acetic acid is formed by a slow secondary breakdown of lactic acid to account for that which occurs in the normal fermentation of glucose. It has been found in this laboratory that pyruvic acid is formed as an intermediate product in the dissimilation of lactic acid. Nelson and Werkman (1935) have further shown that pyruvic acid is fermented anaerobically by *L. lycopersici* with the production of acetic acid, carbon dioxide and lactic acid in equimolar quantities. One molecule of pyruvic acid is broken down to

acetic acid, carbon dioxide and active hydrogen while a second is reduced to lactic acid. These facts indicate that the acetic acid found in the breakdown of glucose probably arises from a secondary fermentation of lactic through pyruvic to acetic acid, carbon dioxide and active hydrogen. The evidence is that hydrogen acceptors which are not present when pyruvic acid alone is fermented are formed from glucose and reduced by the active hydrogen.

Peterson and Fred (1920) have shown the dissimilation of lactic to acetic and carbonic acids by *L. pentoaceticus*, but have shown no reduction product to account for the activated hydrogen in the reaction. Davis (1933) and Bertho and Glück (1932) have demonstrated the formation of hydrogen peroxide, equivalent to the oxygen used, by members of the homofermentative group. Hunt (1933) has shown by respiration experiments that a quantity of oxygen equivalent to the carbon dioxide produced was consumed by *L. pentoaceticus* in a fermentation using lactic acid as a substrate. In flask experiments he found little difference in the quantity of acetic acid formed under aerobic and anaerobic conditions. Hunt also did not account for the hydrogen formed in the reaction. It is clear that oxygen may act as an acceptor with the formation of hydrogen peroxide. Since the organisms are catalase-negative the H_2O_2 accumulates and may be determined quantitatively. Anaerobically there is some hydrogen acceptor other than oxygen functioning. Kluyver (1933) has suggested that the glycerol formed may account for the hydrogen liberated in the dissimilation of lactic acid. This view is supported by the data in Table 2. The quantity of glycerol produced is equivalent to the hydrogen that would be formed if acetic acid arose solely from the dissimilation of lactic acid. It appears that under the conditions of the experiment glyceric aldehyde or a closely related compound accepts the hydrogen formed by the secondary fermentation of the lactic acid with the formation of glycerol.

Peterson and Fred (1920) suggested equation 1 to account for the dissimilation of glucose by *L. pentoaceticus*. The deficiency in lactic acid obtained in their data was explained on the basis of

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

a secondary decomposition of lactic acid to acetic acid and carbon dioxide. While the values obtained by them support this view, the reaction cannot be considered general for the heterofermentative lactic acid bacteria—not even for *L. pentoaceticus* as will be apparent when the data of Pederson (1929a) are calculated on the basis of millimols of the various products.

Evidence indicates that a complex and unstable equilibrium exists among the products in a fermentation system, which may be shifted in one direction or another by a change in such conditions as composition of the medium, pH, or degree of aerobiosis or anaerobiosis. This shifting in the quantitative relationships of the products would not be accommodated by equation 1 since it requires the formation of lactic acid, ethyl alcohol, and carbon dioxide in equimolar concentrations. It is necessary to use a scheme that will account for the differences in the results as found by different investigators.

A tentative scheme for the dissimilation of glucose by the heterofermentative lactic acid bacteria is presented in figure 1. This scheme is flexible and will serve to explain the data presented in contributions on the dissimilation of glucose by the heterofermentative lactic acid bacteria. It is not complete in as much as the initial phases of phosphorylation are far from clear, and there is no convincing evidence as to the identity of the intermediary compounds. It is tempting to suggest the importance of phosphoglyceric acid; however, the compound has not been isolated and identified in these fermentations.

The values presented in the scheme are taken from data in table 2.

The scheme suggests two sources of carbon dioxide in the dissimilation of glucose: 1. A quantity of carbon dioxide equivalent to the ethyl alcohol is formed by the splitting of the hypothetical 3-carbon compound into acetaldehyde, carbon dioxide, and active hydrogen. 2. Carbon dioxide equivalent to acetic acid is formed from the secondary fermentation of lactic acid. The total quantity of carbon dioxide produced should, therefore, be equal to the sum of the mM of ethyl alcohol and acetic acid. The data

for *L. acidophil-aerogenes* in table 2 show 43.9 mM of carbon dioxide found as compared with the calculated value of 49.7 mM (sum of the mM of acetic acid and ethyl alcohol). The difference is only 5.8 mM. In adapting these values to the scheme, the number of mM of acetic acid determined (10.2) has

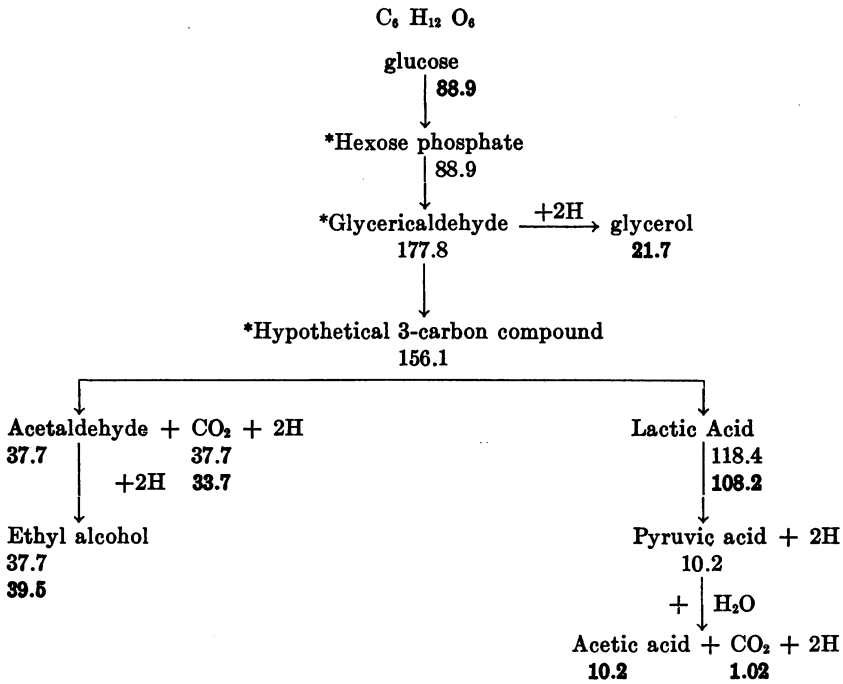

FIG. 1. SCHEME FOR THE DISSIMILATION OF GLUCOSE BY THE HETEROFERMENTATIVE LACTIC ACID BACTERIA

Figures in bold face represent mM found; other data are calculated.
* Has not been identified in heterofermentative lactic acid dissimilation.

been assigned to the carbon dioxide formed from the dissimilation of the lactic acid, since carbon dioxide and acetic acid are formed in equimolar quantities. The 5.8 mM difference between carbon dioxide determined and calculated is assigned to the carbon dioxide arising from the breakdown of the hypothetical 3-carbon compound.

It is possible to calculate the amount of glucose used from the products determined. The value thus obtained is 89.7 mM; the amount actually fermented was 88.9 mM.

By applying the experimental data to the scheme, the oxidation-reduction relationships may be more clearly pointed out. Active hydrogen may be formed from three sources: (*a*) the breakdown of the hypothetical 3-carbon compound to acetaldehyde, carbon dioxide and hydrogen, (*b*) the oxidation of lactic to pyruvic acid, and (*c*) the dissimilation of pyruvic acid to acetic acid, carbon dioxide and hydrogen. On the other hand there are only two points at which reduction takes place; the reduction of glyceric aldehyde to glycerol and of acetaldehyde to ethyl alcohol. In analyzing the data it will be seen that 20.4 mM of hydrogen were formed by the dissimilation of lactic acid and 39.5 mM from the breakdown of the hypothetical 3-carbon compound, totalling 59.9 mM of active hydrogen formed during the fermentation. It will readily be seen that 39.5 mM are used in the formation of ethyl alcohol, leaving 20.9 mM to reduce the glyceric aldehyde to (21.7 mM) glycerol. This analysis supports the view that the quantity of glycerol formed during the anaerobic fermentation of glucose by the heterfermentative lactic acid bacteria is equivalent to twice the quantity of acetic acid formed.

CONCLUSIONS

Carbon and oxidation-reduction balances for the fermentation of glucose by three species, representative of the heterofermentative lactic acid bacteria, are presented. Lactic, acetic and carbonic acids, ethyl alcohol and glycerol were found as end products.

The millimols of carbon dioxide produced were equivalent to the sum of the acetic acid and ethyl alcohol.

The glycerol produced was equivalent to twice the millimols of acetic acid.

A scheme is suggested for the dissimilation of glucose by the heterofermentative lactic acid bacteria.

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