PRODUCT LABELING OF GLUCOSE-1-C^{14} FERMENTATION BY HOMOFERMENTATIVE AND HETEROFERMENTATIVE LACTIC ACID BACTERIA[1]

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Lactobacillus casei, a homofermentative lactic acid bacterium, was shown by Gibbs *et al.* (1950) to ferment glucose-1-C^{14} to products labeled as predicted for the classical Embden-Meyerhof glycolytic scheme, whereas *Leuconostoc mesenteroides* strain 39, a heterofermentative lactic acid coccus, gave product labeling incompatible with an Embden-Meyerhof pathway and was interpreted as indicative of the occurrence of an anaerobic hexosemonophosphate pathway (Gunsalus and Gibbs, 1952). When glucose-1-C^{14} was fermented by the homofermentative organism, about 97 per cent of the tracer appeared in the methyl carbon of lactic acid. In contrast, the heterofermentative organism gave unlabeled lactic acid, with carbon atom 1 (aldehyde carbon) of the glucose giving rise exclusively to carbon dioxide. Although the fermentation of glucose-3,4-C^{14} by *Leuconostoc mesenteroides* yielded further evidence of the nature of the heterofermentative pathway, as had previous enzymatic data (DeMoss *et al.*, 1951), the fermentation of glucose-1-C^{14} was sufficient to differentiate the Embden-Meyerhof from the heterolactic pathway. The purpose of the present study was to determine whether other homofermentative and heterofermentative lactic acid bacteria widely used in metabolic studies possess the fermentation patterns outlined with *Lactobacillus casei* and *Leuconostoc mesenteroides*.

METHODS

Cultures. For fermentation, the strains were maintained and cells were grown as described previously by Gunsalus and Gibbs (1952) and by Gibbs and DeMoss (1954). The strains selected, from among those used in many previous fermentative and metabolic studies, were received from the following investigators, to whom we wish to express our appreciation for these cultures.

Heterofermentative organisms. Leuconostoc mesenteroides strain 535, originally isolated by Dr. C. S. Pederson, was supplied to us through the kindness of Dr. C. F. Niven. *Leuconostoc dextranicum* strain elai, shown by Whiteside-Carlson and Carlson (1949*a*, 1949*b*) to convert the glucose half of sucrose quantitatively to dextran, was kindly furnished by Dr. Virginia Whiteside-Carlson of the University of Alabama Medical School. *Lactobacillus pentoaceticus* strain 118-8, sent to us by Dr. McCoy from the Wisconsin collection, was isolated by Fred *et al.* (1919), was characterized by Fred *et al.* (1921), and its fermentative pattern and properties were studied by Peterson and Fred (1920). *Lactobacillus fermenti* strain ATCC 9338, strain 36 of the Wisconsin collection, was isolated and its fermentative properties were studied by Stiles *et al.* (1925). *Lactobacillus* species strain S40A was isolated as a causal organism of greening of bologna and kindly supplied to us by Dr. C. F. Niven; for its cultural and fermentative properties, see Niven *et al.* (1949) and Evans and Niven (1951).

Homofermentative organisms. Lactobacillus pentosus strain 124-2, kindly supplied to us through the courtesy of Dr. Howard Gest of the Western Reserve University Medical School, was isolated and characterized by Fred *et al.* (1919, 1921) and used in their studies of pentose fermentation. This strain has also been used (Lampen and Peterjohn, 1951) in studies of induced pathways of hexose and pentose fermentation and in studies of fermentative pattern of xylose-1-C^{14} (Lampen *et al.*, 1951). The homofermentative coccus *Streptococcus faecalis* strain 10C1, from the Cornell culture collection, has been characterized by Sherman (1938), Gunsalus and Sherman (1943),

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and Campbell and Gunsalus (1944), and used in metabolic studies by Gunsalus (1949, 1953).

Chemicals. In our fermentation experiments with the heterofermentative strains, only the CO_2 was collected. These fermentations were carried out in Warburg vessels, the CO_2 was absorbed in KOH in the second side arm after fermentation was complete, and the CO_2 was recovered and assayed as previously described by Gunsalus and Gibbs (1952).

The *Lactobacillus pentosus* fermentation was carried out in a CO_2-filled Erlenmeyer flask and the lactic acid was isolated, degraded and assayed as previously described by Gibbs *et al.* (1950). The fermentation of *S. faecalis* strain 10Cl was carried on in like manner.

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RESULTS AND DISCUSSION

The recovery of carbon 1 of glucose-1-C^{14} during fermentation by five heterofermentative lactic acid bacteria is shown in table 1. In each case, within the experimental accuracy of the methods, 1 CO_2 was formed per mole of the hexose fermented, and contained the total label of the glucose-1-C^{14} at a specific activity 6 times that of the glucose fermented. This confirms the data with *Leuconostoc mesenteroides* strain 39 (Gunsalus and Gibbs, 1952), namely, complete recovery of the position 1 of glucose without dilution, indicating that this is the sole source of CO_2 in the fermentative products and that all of position 1 gives rise to CO_2. These observations indicate that the fermentative pattern of the two Leuconostoc strains and the three heterofermentative Lactobacilli conforms to the present concept of an anaerobic hexosemonophosphate pathway as the mechanism of the heterolactic fermentation (Gunsalus and Gibbs, 1952; DeMoss *et al.*, 1951).

These data do not include complete fermentation balances to determine if glycerol is formed in the resting cell fermentation as previously found with growing cultures (Stiles *et al.*, 1925; Nelson and Werkman, 1935). Other enzymatic data indicate that glycerol is formed through reduction of dihydroxyacetone phosphate to α-glycerol phosphate by a DPN-linked α-glycerol phosphate dehydrogenase (Schlenk, 1951) and hydrolysis of the latter by a specific phosphatase (Schlenk, 1951). Since these strains are presumed to lack aldolase (DeMoss *et al.*, 1951) and because of the nonmixing of the two halves of glucose (data in table 1) the mechanism of glycerol formation during the heterolactic fermentation remains to be determined.

Further studies will be required to elucidate the mechanism in terms of the oxidation reduction balance. Present evidence would indicate that

TABLE 1

Glucose-1-C^{14} fermentation by heterofermentative lactic acid bacteria

Per Warburg cup: 0.05 ml M/1 phosphate buffer, pH 6.0; 1.0 ml cell suspension (≈ 10 mg dry weight); 0.1 ml glucose (μM and C^{14} as indicated in table); water to 3.0 ml; atmosphere N_2; 30 C. After fermentation, CO_2 absorbed in KOH and transferred to flask containing 94 μM Na_2CO_3 as carrier.

Organism	Glucose Added			CO_2 Formed*					
				Absorbed		Total activity		Specific activity	
	μM	Total activity	Specific activity	μM	%§	mμc	%¶	mμc/mg C	CO_2/glucose
		mμc†	mμc/mg C‡						
Leuconostoc mesenteroides strain 535	7.0	6.0	13.1	6.6	94	5.8	97	72.7	5.6
Leuconostoc dextranicum strain elai.	7.0	6.0	13.1	6.8	97	5.7	95	69.1	5.1
Lactobacillus fermenti strain 9338..	10.0	14.0	20.0	11.6	116	14.7	105	133.0	6.7
Lactobacillus pentoaceticus strain 118.8	10.0	14.0	20.0	10.9	109	13.7	98	140.0	7.0
Lactobacillus sp. strain S40A	7.0	6.0	13.1	7.1	102	5.4	90	64.6	4.9

* Each value is average of two experiments.

† mμc = millimicrocuries = 1×10^{-3} microcuries.

‡ mμc/mg C = millimicrocuries per milligram of carbon.

§ Calculated on basis of 1 CO_2 formed/glucose.

¶ Calculated on complete conversion of C-1 to CO_2.

TABLE 2

C^{14} distribution in lactic acid from glucose-1-C^{14} fermentation by homofermentative lactic acid bacteria

Protocol:

Lactobacillus pentosus. $NaHCO_3$-CO_2 as buffer, pH 6.4, 270 μM glucose-1-C^{14} ($\simeq$ 2 mμc/mg C) 400 mg cells (wet weight) water to 10 ml. Temperature 30 C.

Streptococcus faecalis. $NaHCO_3$-CO_2 as buffer, pH 7.0, 268 μM glucose-1-C^{14} ($\simeq$ 2.3 mμc/mg C) 500 mg cells (wet weight) water to 20 ml. Temperature 30 C.

Carbon Atom of Lactic Acid	*L. pentosus*, 124-2			*S. faecalis*, 10Cl		
	Specific activity		Total activity	Specific activity		Total activity
	mμc/mg C*	*S.A. lactate/ S.A. glucose*	%	mμc/mg C	*S.A. lactate C/ S.A. glucose*	%
COOH	0.2	0.1	3	0.06	0.03	1
CHOH	0.4	0.2	6	0.6	0.3	9
CH_3	6.4	3.2	91	5.9	2.6	90

* mμc/mg C = millimicrocuries per milligram of carbon.

glycerol formation (Johnson *et al.*, 1931) would be accompanied by the production of a half mole of acetate replacing a similar amount of ethanol (Nelson and Werkman, 1935). Acetate, like the ethanol, would presumably be formed from carbons 2 and 3 of glucose with the carboxyl from carbon 3 and a methyl group from carbon 2. This mechanism is further substantiated by Gunsalus and Gibbs (1952), who demonstrated the reduction of labeled acetate to ethanol during glucose fermentation by *Leuconostoc mesenteroides*.

Further confirmation of the orientation of the carbons from glucose arises from the fermentation of pentoses by both hetero- and homofermentative lactic acid bacteria. Three such studies involving *Lactobacillus pentoaceticus*, a heterofermentative rod with arabinose-1-C^{14} (Rappaport *et al.*, 1951), and *Lactobacillus pentosus*, a homofermentative rod with both xylose-1-C^{14} (Lampen *et al.*, 1951) and ribose-C^{14} (Bernstein, 1953), have shown, in agreement with older fermentation data, equimolar yields of lactate and acetate and have, in addition, demonstrated that carbon atom 1 of the pentoses is converted exclusively to the methyl group of acetate without dilution.

Another key problem in the anaerobic hexose-monophosphate mechanism is the formation of "C_2," its form, and the reactions for conversion to ethanol or acetate. Presumably the transketolase cleavage of ribulose-5-phosphate to yield a "glycolaldehyde-diphosphothiamin" (Horecker *et al.*, 1953; Racker *et al.*, 1954) would furnish such an intermediate. While no direct evidence exists, preliminary data of DeMoss (1954) favor this view. Furthermore, the order of labeling in the products, ethanol and acetate, is compatible with such a route but not with the other hexosemonophosphate pathways described to date, i. e., 6-phosphogluconate cleavage *via* 2-keto-3-deoxy-6-phosphogluconate (MacGee and Doudoroff, 1954) or 2-keto-6-phosphogluconate (Wood *et al.*, 1955). The pentose fermentation data—labeling and adaptive enzyme patterns (Lampen and Peterjohn, 1951)—are also compatible with this hypothesis.

The fermentation of glucose-1-C^{14} by the homofermentative lactic acid bacterium, *Lactobacillus pentosus* strain 124-2, and the homofermentative coccus, *Streptococcus faecalis* strain 10Cl, yield, (table 2) in agreement with the previous data, essentially only methyl-labeled lactate, i. e., greater than 90 per cent of the label from carbon 1 of glucose appears in this position, thus implying a classical Embden-Meyerhof glycolytic route in these organisms.

These data are particularly interesting in view of the fermentation of xylose-1-C^{14} to methyl-labeled acetate plus unlabeled lactate by homofermentative strain 124-2 (Lampen *et al.*, 1951). These data, in common with similar labeling during fermentation by the heterofermentative rod (Rappaport *et al.*, 1951), indicate a pentose fermentation pathway common to the homo- and heterofermentative organisms indicated both by the similar product yields and by the product labeling during pentose fermentation. Further evidence of similarities inducible in homo- and heterofermentative organisms was presented by

the demonstration of Sokatch and Gunsalus (1954) of an inducible aldonic acid fermentative pathway in *S. faecalis* strain 10Cl. Cells grown on glucose fermented only glucose, whereas those grown on gluconate fermented, without lag, gluconate and 2-ketogluconate as well as glucose. The gluconate-grown cells fermented these three substrates according to the following partially completed balances:

glucose → 2 lactate (1)

gluconate → 1.5 lactate + .5 CO_2 + ? (2)

2-ketogluconate → 1 lactate + 1 CO_2 + ? (3)

Gluconic acid-1-C^{14} fermentation by cell suspensions yields both labeled CO_2 and labeled lactic acid with approximately 50 per cent of the label appearing in CO_2 at 6 times the specific activity of the gluconate fermented

Further experiments are in progress to define completely the products of gluconate and ketogluconate fermentation, and to elucidate the steps in what appears to be an inducible monophosphate pathway beyond gluconate. A more complete picture of the fermentative pathways of homo- and heterofermentative organisms and the points of convergence should be available from these studies.

SUMMARY

Five heterofermentative strains of the *Lactobacillus* and *Leuconostoc* genera have been shown to ferment glucose by the hexosemonophosphate pathway previously described in *Leuconostoc mesenteroides* strain 39. The strains tested included *Leuconostoc mesenteroides* strain 535, *Leuconostoc dextranicum* strain elai, *Lactobacillus fermenti* strain ATCC 9338, (Wisconsin strain 36), *Lactobacillus pentoaceticus* strain 118-8, and a greening *Lactobacillus* species strain S40A.

Two homofermentative lactic acid bacteria, *Lactobacillus pentosus* strain 124-2 and *Streptococcus faecalis* strain 10Cl, ferment glucose-1-C^{14} to methyl-labeled lactate, characteristic of an Embden-Meyerhof pathway, as previously shown in *Lactobacillus casei*.

The demonstration of the Embden-Meyerhof fermentation of glucose by *Streptococcus faecalis* strain 10Cl confirms the hypothesis that the hexosemonophosphate pathway of fermentation of aldonic acids is induced by growth on gluconate.

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