Glucose Metabolism by *Lactobacillus divergens*

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(Received 12 April 1988)

Earlier studies on the fermentation of D-[1-¹⁴C]- and D-[3,4-¹⁴C]glucose by *Lactobacillus divergens* showed that lactate was the major fermentation product and that it was probably produced by glycolysis. It was therefore recommended that *L. divergens* be reclassified as a homofermentative organism. In the present investigation, products of D-[1-¹⁴C]-, D-[2-¹⁴C]- and D-[3,4-¹⁴C]glucose fermented by *L. divergens* were isolated, and their specific radioactivities and the distribution patterns of radioactivity in their C-atoms were determined. The positional labelling patterns of the fermentation products, their specific radioactivities and their concentrations confirmed that glucose is degraded via the glycolytic pathway. Some secondary decarboxylation/dissimilation of pyruvate to acetate, formate and CO₂ was also observed. These results provide conclusive proof that *L. divergens* is indeed a homofermentative organism. Results obtained with D-[U-¹⁴C]glucose showed that approximately three-quarters of the lactate but less than 10% each of the formate and acetate were produced from glucose. The remainder was presumably derived to a varying degree from endogenous non-glucose sources such as fructose and/or amino acids.

INTRODUCTION

Lactobacillus divergens, isolated from vacuum-packaged meat, was initially classified as an atypical heterofermentative organism (Holzapfel & Gerber, 1983). Recent radioactivity incorporation studies with D-[1-¹⁴C]- and D-[3,4-¹⁴C]glucose as substrates have, however, suggested that *L. divergens* metabolizes glucose principally by the glycolytic pathway (De Bruyn *et al.*, 1987), and it was recommended that the organism be reclassified as a homofermentative organism.

These studies have now been extended to provide information on the distribution of the ¹⁴C-label in the fermentation products of D-[1-¹⁴C]-, D-[2-¹⁴C]- and D-[3,4-¹⁴C]glucose. The results allow definitive conclusions to be drawn on the principal route by which *L. divergens* metabolizes glucose.

METHODS

Strain and materials. *Lactobacillus divergens*, isolate 66 (DSM 20623), from our collection (Holzapfel & Gerber, 1983) was used. D-[1-¹⁴C]Glucose [specific activity 53.4 mCi mmol⁻¹ (1 mCi = 37 MBq)], D-[2-¹⁴C]glucose (49.3 mCi mmol⁻¹) and D-[3,4-¹⁴C]glucose (10.32 mCi mmol⁻¹) were obtained from New England Nuclear. D-[U-¹⁴C]Glucose (specific activity 286 mCi mmol⁻¹) was from ICN Radiochemicals. Insta-gel II was obtained from Packard Instrument Co. All reagents for HPLC were of HPLC-grade (Merck). Water was twice-distilled in glass and deionized in a Milli-Q water system (Millipore).

Radioactivity measurements. These were made with a Packard Tricarb model 4430 liquid scintillation counter with automatic external standardization. Samples were counted either to a confidence limit of 95.5% and an uncertainty of 1%, or for 300 min. Only radioactivity values higher than twice the background were used.

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Fermentation experiments. The fermentation of differently labelled glucose, and the extraction and isolation of fermentation products by HPLC, were done as described earlier (De Bruyn *et al.*, 1987). *L. divergens* was pre-grown in modified MRS broth at pH 8.5 (containing sucrose instead of glucose and without acetate) for 14 h at 25 °C. The cells were harvested during the exponential growth phase by centrifugation, washed twice with 0.047 M-sodium/potassium phosphate buffer pH 7.8, and suspended in 4 ml of the same buffer. Fermentation was carried out in a Gilson respirometer by adding 1 ml cell suspension to about 55 µmol glucose in 2 ml 0.047 M-phosphate buffer. Labelled glucose was added to the final specific radioactivities given in Table 1. After 2.5 h at 25 °C, fermentation was terminated on ice and the filter paper plus KOH from the centre well of the Gilson flask was placed in a glass scintillation vial. Radiolabelled CO₂, as KH¹⁴CO₃, was determined by liquid scintillation counting after addition of 12 ml Insta-gel II and standing for 15 h to allow for the decay of chemiluminescence. A separate flask containing 0.2 ml water in the centre well instead of 2% KOH was included to determine the amount of CO₂ produced.

Recovery and analysis of fermentation products. The fermentation products were quantitatively recovered (≥ 98%) by four successive extractions of the contents of the Gilson flasks according to Guerrant *et al.* (1982). The extracts were freeze-dried, dissolved in water and the fermentation products separated by HPLC using an Aminex HPX-87H cation-exchange column (Bio-Rad) with 10.8% (v/v) acetonitrile in 0.0035 M-H₂SO₄ as eluent (Guerrant *et al.*, 1982). The concentration of lactate was determined by enzymic assay according to Noll (1974). The concentrations of acetate and formate were determined from HPLC peak heights, unless indicated otherwise.

Endogenous end-product dilution, caused by the metabolism of other unknown substrates (Dawes, 1980), was ascertained from the specific activity of the end-products of fermentation of 10 µCi D-[U-¹⁴C]glucose by *L. divergens* using the method described above.

The specific radioactivity of glucose was determined after its extraction from a zero-time Gilson flask according to Williams *et al.* (1971). Glucose was purified from the extract by HPLC on a Radial-Pak Silica Cartridge with Radial Compression Module (Waters Associates) with 75% acetonitrile and 0.1% tetraethylene pentamine in water as eluant at a flowrate of 3 ml min⁻¹. The concentration of glucose was determined by enzymic assay (Bernt & Bergmeyer, 1974) and its radioactivity by liquid scintillation counting.

The chemical degradation of the respective organic acids was essentially according to Williams *et al.* (1971) except that about 25 mg unlabelled lactic acid or acetic acid was added to each of their respective radioactive counterparts (isolated by HPLC). CO₂ resulting from the oxidation was trapped as BaCO₃, the suspension was transferred to a clean and pre-weighed glass centrifuge tube, and the BaCO₃ precipitate was recovered by centrifugation. The supernatant was carefully removed and discarded. The BaCO₃ precipitate was washed three times with 5 ml hot water to remove any remaining Ba(OH)₂, dried at 100 °C for 36 h, cooled to room temperature in a desiccator and weighed. A 20 mg portion of the dried BaCO₃ precipitate was transferred to a 20 ml glass counting vial and 6 ml water was added. The vials were placed in a Bransonic model 42 sonic bath for a few minutes until a homogeneous colloidal suspension was formed. Insta-gel II (8 ml) was added and the mixture was vortexed. The radioactivity of the resulting gel suspension of BaCO₃ was measured. A vial containing 6 ml water and 8 ml Insta-gel II was used as a blank. Counting efficiencies were determined by both external and internal standardization. Only 95% of the actual counts in BaCO₃ was accounted for by the external standardization method. The remaining 5% was presumably lost by self-absorption, as was also reported by Cluley (1962). All results obtained for BaCO₃ were corrected for this self-absorption.

RESULTS AND DISCUSSION

Labelling patterns from differently labelled glucose precursors

In Table 1 it is shown that lactate was the only major radioactive product produced from the differently labelled glucose precursors, confirming our previous findings (De Bruyn *et al.*, 1987) and agreeing with the expected symmetric fermentation pathway of glycolysis (Fig. 1a). The low incorporation of radioactivity into CO₂ produced from D-[1-¹⁴C]glucose, or into acetate produced from D-[3,4-¹⁴C]glucose, indicates that only a small amount of glucose was fermented by the hexose monophosphate (Fig. 1b) and/or Entner-Doudoroff (Fig. 1c) pathways.

End-product dilution

The specific radioactivity of lactate produced from D-[1-¹⁴C]-, D-[2-¹⁴C]- and D-[3,4-¹⁴C]glucose came to only 38.6%, 38.7% and 38.0% of that of the respective glucose precursors (Table 1). The corresponding values for CO₂, acetate and formate were even lower. If glycolysis is the major pathway of glucose fermentation and if glucose is the only fermentable substrate, the specific radioactivity of all of the end-products is expected to be half that of glucose (Fig. 1a).

Fig. 1. Distribution of C-atoms in products obtained from the fermentation of glucose by lactobacilli (Abo-Elnaga & Kandler, 1965). (a) Glycolytic pathway, (b) hexose monophosphate pathway, (c) Entner-Doudoroff pathway.

Uniformly labelled D-[U- ^{14}C]glucose was therefore used as substrate in order to establish whether end-product dilution had occurred, and to what extent.

The results obtained with D-[U- ^{14}C]glucose (Table 2) show that only 76.7%, 42.8%, 8.7% and 6.3%, respectively, of the expected lactate, CO_2 , acetate and formate were produced from glucose under the experimental conditions employed. The remainder was derived to a varying degree from unknown endogenous sources. It is significant that unequal dilution of the fermentation products had occurred. About 23% of the lactate was derived from endogenous substrates other than radioactive glucose, whereas almost 60% of the CO_2 and more than 90% of each of the acetate and formate came from other sources. If identical metabolic pathways were responsible for the production of lactate as well as that of CO_2 , acetate and formate, the extent of dilution would have been the same. The results clearly indicate that the latter two products were mainly formed by metabolic pathways other than those by which lactate was produced.

Although stock cultures of *L. divergens* were pre-grown in a medium containing sucrose (see Methods), it is unlikely that the remaining intracellular sucrose (or glucose and fructose derived from it) could have contributed significantly to the end-product dilution observed after D-[U- ^{14}C]glucose fermentation by the washed cells. This is particularly so in view of the relatively high concentration (about 55 μmol) of labelled glucose in the medium. Some bacteria ferment amino acids to NH_3 , CO_2 , acetate, formate, butyrate and H_2 by the Stickland reaction (Gottschalk, 1979; Stams *et al.*, 1985). *L. divergens* was originally isolated from raw, minced meat (Holzapfel & Gerber, 1983). It is conceivable that the organism could have produced these fermentation products from intracellular amino acids, accumulated during pre-growth in the peptone-containing medium (see Methods).

Table 1. *Distribution of radioactivity from differently labelled glucose precursors in fermentation products*

About 55 $\mu\text{mol D-[1-}^{14}\text{C]glucose}$ [specific activity $43\,330 \pm 2.1\%$ (6) d.p.m. μmol^{-1}] or $\text{D-[2-}^{14}\text{C]glucose}$ [$47\,500 \pm 0.63\%$ (3) d.p.m. μmol^{-1}] or $\text{D-[3,4-}^{14}\text{C]glucose}$ [$15\,090 \pm 3.6\%$ (4) d.p.m. μmol^{-1}] was fermented, and the products were extracted with diethyl ether ($\text{pH} \leq 1.0$) and re-extracted with water ($\text{pH} \geq 9.0$). Products were separated by HPLC, quantified from peak heights and their radioactivities were determined by liquid scintillation counting as described in Methods. Results are the means of up to three fermentations. The coefficient of variation was determined where appropriate (number of assays in parentheses).

Labelled glucose	Total radioactivity (d.p.m.)	Fermentation products							
		Percentage of total radioactivity in:				Percentage of specific radio-activity* recovered in:			
		CO ₂	Lactate	Acetate	Formate	CO ₂	Lactate	Acetate	Formate
D-[1- ¹⁴ C]	329904 ± 5.7% (3)	9.7 (1)	82.9 ± 5.5% (4)	7.5 ± 0.5% (3)	0	14.6 ± 0.9% (3)	38.6 ± 1.7% (8)	4.4 ± 0.6% (3)	0
D-[2- ¹⁴ C]	398805 ± 8.1% (3)	0.43 (1)	93.5 ± 0.3% (4)	6.5 ± 6.30% (4)	0	0.73 (1)	38.7 ± 0.7% (4)	2.9 (2)	0
D-[3,4- ¹⁴ C]	140910 ± 3.2% (3)	5.9 (2)	91.3 ± 2.2% (8)	1.0 ± 15.1% (8)	1.6 ± 20.4% (6)	9.2 (2)	38.0 ± 1.3% (8)	0.85 ± 0.2%† (4)	4.0 ± 0.7% (3)

* Expressed as a percentage of the specific radioactivity in glucose.

† Specific radioactivity of acetate from enzymic assay (Bergmeyer & Möllering, 1974) and liquid scintillation counting.

Table 2. *Radioactivity recovered per C-atom of the fermentation products from D-[U-¹⁴C]glucose*

Results are from a duplicate fermentation. Mean values $\pm$ coefficient of variation are shown where appropriate, with the number of assays in parentheses. (See Table 1 for conditions).

Substrate and products	Specific radioactivity			Percentage of original recovered†
	D.p.m. μmol^{-1}	D.p.m. per C-atom*		
D-[U- ¹⁴ C]Glucose	499 967 $\pm$ 2.1% (4)	83 328		100.0
CO ₂	35 655 (2)	35 655		42.8
Lactate	191 690 $\pm$ 3% (8)	63 897		76.7
Acetate	14 655 $\pm$ 1.5% (3)	7 328		8.7
Formate	5 274 $\pm$ 9.4% (3)	5 274		6.3

* Calculated from carbon content.

† Expressed as percentage of the specific radioactivity of a single C-atom of glucose.

Table 3. *Corrected recoveries of fermentation products from differently labelled glucose precursors*

Results are the means for up to three fermentations which were corrected for endogenous product dilution, using the recovery values in Table 2. (See Table 1 for conditions.)

Labelled glucose	Fermentation products							
	Concentration ($\mu\text{mol ml}^{-1}$)				Percentage of specific radioactivity* recovered in:			
	CO ₂	Lactate	Acetate	Formate	CO ₂	Lactate	Acetate	Formate
D-[1- ¹⁴ C]	2.1 (1)	12.5 $\pm$ 3.7% (4)	1.4 $\pm$ 10.2% (4)	0.61 $\pm$ 20% (4)	34.2 $\pm$ 5.9% (3)	50.3 $\pm$ 3% (8)	50.5 $\pm$ 13.1% (3)	0
D-[2- ¹⁴ C]	2.1 (1)	15.2 $\pm$ 9.1% (4)	1.6 $\pm$ 8.9% (4)	0.81 $\pm$ 5.8% (4)	1.7 (1)	50.5 $\pm$ 2% (4)	32.2 (2)	0
D-[3,4- ¹⁴ C]	2.7 (1)	17.2 $\pm$ 4% (8)	0.96 $\pm$ 5.5% (8)	0.23 $\pm$ 7.4% (8)	21.6 (2)	49.6 $\pm$ 3.5% (8)	9.8 $\pm$ 21% (4)	63.5 $\pm$ 16.2% (3)
D-[U- ¹⁴ C]	2.4 (1)	15.1 $\pm$ 7% (8)	1.4 $\pm$ 9.4% (6)	0.39 $\pm$ 9.2% (6)	—	—	—	—

* Expressed as a percentage of the specific radioactivity in glucose.

Table 4. *Positional distribution of radioactive label in lactate and acetate*

Lactate or acetate was oxidized and the released CO₂ was trapped as BaCO₃ and its radioactivity measured (see Methods). Results are from a typical experiment.

Labelled glucose	Specific radioactivity*	Position of label in lactate or acetate					
		-COOH		-CHOH		-CH ₃	
		Specific radioactivity*	% Distribution of radioactivity†	Specific radioactivity*	% Distribution of radioactivity†	Specific radioactivity*	% Distribution of radioactivity†
D-[1- ¹⁴ C]	Lactate 16757 Acetate 3318	0 0	0 0	0 —	0 —	15315 3137	91.4 94.5
D-[2- ¹⁴ C]	Lactate 15022 Acetate 2176	0 1993	0 91.6	14400 —	95.9 —	0 0	0 0
D-[3,4- ¹⁴ C]	Lactate 20405 Acetate 1401	19745 1333	96.8 95.1	0 —	0 —	0 0	0 0

* D.p.m. per μmol lactate, acetate or fragment.

† Radioactivity in each C-atom expressed as a percentage of the specific radioactivity in lactate or acetate.

Corrected recoveries of fermentation products derived from differently labelled glucose precursors

The observed concentrations and specific radioactivities of each fermentation product were corrected for endogenous product dilution according to the results of Table 2. From the corrected concentrations (Table 3), it is again obvious that lactate was the major product of glucose metabolism by *L. divergens*. The corrected specific radioactivities of lactate, which came to 50.3%, 50.5% and 49.6% of that of D-[1-¹⁴C]-, D-[2-¹⁴C]- or D-[3,4-¹⁴C]glucose, respectively, are close to the expected value of 50% for glucose fermented to lactate via glycolysis.

It is also evident that in some cases the corrected specific radioactivities of CO₂ and acetate came to less than 50% of that of the glucose precursors (Table 3). Substantial further dilution presumably had occurred by unlabelled end-products formed in the hexose monophosphate or Entner–Doudoroff pathways, and/or secondary transformations of pyruvate (Fig. 1 and De Bruyn *et al.*, 1987). It was not possible to draw firm conclusions about the relative contributions of the latter pathways from the distribution and recovery of radioactivity in the fermentation products alone. A more definitive answer was therefore sought by analysis of the positional distribution of radioactivity in the individual C-atoms of the fermentation products.

Positional distribution of radioactivity in lactate and acetate

Lactate produced from D-[3,4-¹⁴C]glucose was exclusively labelled in its carboxyl carbon, while acetate produced from D-[1-¹⁴C]glucose was exclusively labelled in its methyl carbon (Table 4). These results are in agreement with those expected for fermentation products formed by glycolysis (Fig. 1*a*). If the Entner–Doudoroff pathway (Fig. 1*c*) had been operative, the label in lactate produced from D-[3,4-¹⁴C]glucose would have been distributed equally between the carbons of the methyl and carboxyl groups. Acetate produced from D-[3,4-¹⁴C]- and D-[1-¹⁴C]glucose, on the other hand, would respectively have been labelled in its methyl group or unlabelled (Entner & Doudoroff, 1952). On these grounds, operation of the Entner–Doudoroff pathway could be excluded.

The small amount (1% of total radioactivity) of radioactive acetate produced from D-[3,4-¹⁴C]glucose (Table 1) was labelled in its carboxyl carbon (Table 4), which is consistent with the fermentation of some glucose by the hexose monophosphate pathway (Fig. 1*b*). In view of the 90-fold higher radioactivity found in lactate (Table 1), it is clear that the hexose monophosphate pathway could only make a minor contribution to the end-products. This is supported by consideration of the fate of the label of D-[2-¹⁴C]glucose, which was only recovered in the carboxyl carbon of acetate, and that of D-[1-¹⁴C]glucose, where the methyl group of acetate was uniquely labelled (Table 4). Both of these results are predicted for the glycolytic pathway (Fig. 1*a*).

The labelling patterns of lactate and acetate obtained from D-[2-¹⁴C]glucose (Table 4) provided additional support for the conclusion that glycolysis was the major pathway of glucose metabolism by *L. divergens*. Lactate and acetate were labelled in the hydroxylated and carboxyl carbons, respectively, as is expected for the glycolytic pathway (Fig. 1*a*). The specific radioactivities recovered in the carbon atoms after chemical oxidation of lactate and acetate were in all cases about 95% of the specific radioactivity of the respective labelled fermentation product, suggesting that a single pathway of fermentation was operative.

CONCLUSION

Lactate is the major product of glucose fermentation by *L. divergens*, since the radioactivities in CO₂, acetate and formate never exceeded 10% of the total radioactivity in the fermentation products (Table 1). *L. divergens* therefore ferments glucose predominantly by the glycolytic pathway to lactate. The extent to which product dilution had occurred suggests that unknown pathways, using substrates other than glucose, may be operative in *L. divergens*. Their principal products are CO₂, acetate and formate, since only 23% of the total lactate was produced from non-glucose sources (Tables 2 and 3). Endogenous substrates giving rise to these products could include fructose (from sucrose) and/or amino acids.

The positional distribution of the label of differently labelled glucose precursors in the fermentation products corresponds with the pattern expected for homolactic lactobacilli. It is uncertain to what extent the hexose monophosphate pathway contributed to the fermentation of glucose. At most it constituted about 11% of the glycolytic pathway, based on the incorporation of radioactivity from D-[1-¹⁴C]glucose into CO₂ (Table 1). The amount of label (Table 1) in the carboxyl carbon of acetate (Table 4) from D-[3,4-¹⁴C]glucose suggests that this pathway may constitute as little as 1% of the glycolytic pathway.

Under glucose limitation, *L. divergens* also produced CO₂, acetate and formate by secondary decarboxylation/dissimilation of pyruvate/lactate. It would therefore seem appropriate to reclassify *L. divergens* as belonging to group II of the family *Lactobacillaceae* recently described by Kandler & Weiss (1986). This group of lactobacilli had previously been classified as *Streptobacterium* (Sharpe, 1981). These results emphasize that classification of bacteria should be based on the distribution patterns of labelled C-atoms, rather than on the concentrations of the fermentation products only.

We thank Elna Ellis and Nils Dittmer for technical assistance, and the CSIR and the Department of Agriculture for financial support.

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