FERMENTATION OF C^{14}-LABELED GLUCOSE BY *CLOSTRIDIUM PERFRINGENS*[1]

L. M. PAEGE[2], MARTIN GIBBS, AND R. C. BARD[3]

Frankford Arsenal, Philadelphia, Pennsylvania; Department of Biology, Brookhaven National Laboratory, Upton, Long Island, New York, and Department of Bacteriology, Indiana University, Bloomington, Indiana

Received for publication December 6, 1955

Limited data suggest that members of the genus *Clostridium* possess a glycolytic system similar to the Embden-Myerhof-Parnas (E-M-P) pathway (Elsden, 1952). Moreover, more recent data appear to support such a hypothesis. Evidence for the existence in *Clostridium perfringens* of such glycolytic enzymes as hexokinase, phosphohexose isomerase, phosphohexokinase, phosphofructokinase, aldolase, adenosine triphosphate-phosphoglyceryl transphosphorylase, glyceraldehyde phosphate dehydrogenase, ethanol dehydrogenase, lactate dehydrogenase, and triosephosphate isomerase has been obtained; the presence of phosphoglyceromutase and enolase is not clearly established (Bard and Gunsalus, 1950; Shankar and Bard, 1955).

Recently another technique has been employed to determine the pathway of glucose dissimilation in microorganisms; this involves the isolation and degradation of fermentation products derived from glucose specifically labeled with C^{14}. Since each pathway of glucose dissimilation yields a definite tracer pattern, this technique provides a rapid method for the determination of glucose breakdown. In the present investigation, carbon dioxide, ethyl alcohol, and acetic acid derived from glucose-1-C^{14}, glucose-2-C^{14}, glucose-3,4-C^{14}, and glucose-6-C^{14}, have been isolated and degraded in order to determine the pathway of glucose fermentation in *Clostridium perfringens*.

MATERIALS AND METHODS

Clostridium perfringens strain BP6K from the Indiana University collection was maintained by transfer in heart infusion broth, and in medium A (Bard and Gunsalus, 1950). Cultures in medium A were incubated for 5 hrs at 37 C, harvested by centrifugation, washed once with saline and suspended in 10 ml water.

Production of gas was measured by the usual Warburg technique at 37 C; the gas phase was nitrogen. Each vessel contained 1.0 ml of cell suspension in 0.03 M phosphate buffer, pH 6.5. In a typical experiment approximately 35 mg of dry weight of cells was used. The cells were permitted to ferment until gas evolution ceased, at which time 0.2 ml 3N H_2SO_4 was added. Approximately 80 per cent of the glucose introduced was utilized.

Separation and degradation of the fermentation products was carried out in the following manner. The carbon dioxide was collected and counted according to a method described by Gibbs and Gastel (1953). After collection of the carbon dioxide, the cells were removed by centrifugation. The supernatant liquid was made alkaline to phenol red with sodium hydroxide and distilled to remove ethanol. The residue was adjusted to pH 1.0 with H_2SO_4 and steam-distilled to remove acetic and butyric acids. These steam-volatile acids were separated and measured by the celite method of Beuding and Yale (1951). The location of tracer in the acetic acid was determined by two procedures: (1) the method of Phares (1951) which yielded the individual carbon atoms, and (2) total oxidation (Osburn and Werkman, 1932). The difference between the activity of the total oxidation and that of the carboxyl carbon from the Phares degradation procedure was used as the activity of the methyl carbon. The tracer pattern in the ethanol was determined by converting it to acetic acid (Gibbs *et al.*, 1954) and degraded as acetic acid. All C^{14} samples were assayed for activity as barium carbonate in a methane flow beta proportional counter. Specific activity is expressed as millimicrocuries per milligram carbon (mμc/mg C).

Glucose-1-C^{14}, glucose-2-C^{14}, and glucose-6-C^{14} were supplied by Dr. H. Isbell of the National

[1] Research carried out at Brookhaven National Laboratory under the auspices of the United States Atomic Energy Commission.

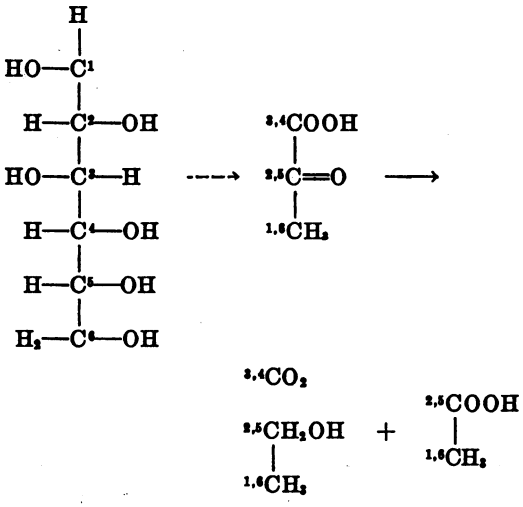
[2] Guest at Brookhaven National Laboratory.

[3] Present address: Smith, Kline and French Laboratories, Philadelphia, Pennsylvania.

Bureau of Standards. Glucose-3,4-C^{14} was prepared from glycogen isolated from a rat injected with $NaHC^{14}O_3$.

RESULTS AND DISCUSSION

The Embden-Myerhof-Parnas pathway predicts that the glucose molecule (aldehyde carbon is carbon atom 1) should be metabolized in the following manner:



First consideration of the data listed in table 1 indicates fermentation by the E-M-P pathway since this scheme predicts that glucose-3,4-C^{14} yields labeled carbon dioxide and unlabeled acetate and ethyl alcohol. Not only was this found, but also the specific activity of the carbon dioxide was essentially that of the carbon in the glucose-3,4-C^{14}, indicating that carbon atoms 3 and 4 of glucose were the sole source of CO_2 carbon.

In experiments in which glucose-1-C^{14} and glucose-6-C^{14} were fermented, the distribution of tracer carbon in the methyl groups of ethanol and acetic acid and the lack of activity in the carbon dioxide again indicate the E-M-P pathway. The presence of activity in the carboxyl carbon of acetate and the carbinol carbon of ethanol when glucose-2-C^{14} was fermented supports the contention.

The results do not support the E-M-P scheme as being the only pathway functioning. When 1- or 6-labeled glucose was fermented, the specific activity of the methyl carbon of acetate was approximately two times the specific activity of the methyl group of ethanol. Similar data were obtained with respect to the carboxyl carbon of acetate and the carbinol carbon of ethanol when glucose-2-C^{14} was fermented. The E-M-P pathway requires that acetic acid and ethanol arise from a common triose precursor, in which case the specific activity of the methyl groups of acetic acid and ethanol would be the same.

It is interesting to note that in each case the specific activity of the acetate carbon atoms was approximately that predicted by the E-M-P scheme. For example, glucose carbon atom 1 with a specific activity of 46.8 mμc/mg carbon should give rise to methyl carbons with an activity of 23.4. The specific activity of acetate methyl carbon was found to be 19 while that of the ethanol methyl carbon was 10.5. As seen in table 1, similar data were obtained with glucose-6-C^{14}.

TABLE 1

Distribution of tracer in products formed during fermentation by Clostridium perfringens

	Specific activity, mμc/mg C						
	Glucose-3,4-C^{14}		Glucose-1-C^{14}		Glucose-2-C^{14}	Glucose-6-C^{14}	
	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 1	Expt 2
Tracer carbon of glucose	37.8	39.6	46.8	46.8	106.8	408.0	43.2
Carbon dioxide	35.9	34.5	0.0	0.0	0.0	0.0	0.0
Ethanol CH_3—	1.6	0.0	11.8	9.2	0.3	92.8	7.2
—CH_2OH	0.8	0.6	0.2	0.4	22.7	0.9	0.2
Acetic acid CH_3—	0.4	0.0	19.4	18.6	0.3	143.4	14.4
—COOH			0.1	0.1	49.9	0.0	0.1

In each experiment, each Warburg vessel contained 1.0 ml cell suspension (35 mg dry weight), 3.0 ml 0.05 M phosphate buffer, pH 6.5. The reaction was begun by tipping 450 μmoles glucose. The total volume was 6.0 ml. The gas phase was nitrogen. The temperature was 37 C. After gas evolution had ceased 0.2 ml 3N H_2SO_4 was added. Separation and degradation of the fermentation products are described under Materials and Methods.

Data expressed as mμc/mg C = millimicrocuries per milligram of carbon.

With glucose-2-C^{14} as substrate, the carboxyl carbon of acetate (49.9) showed a close relationship to carbon atom 2 of glucose (106.8).

If ethanol carbon does arise from pyruvic acid, as predicted by the E-M-P scheme, then some mechanism must be supplying inactive ethanol to dilute its activity. This unlabeled ethanol cannot be supplied by the endogenous metabolism of the cells since this value was determined and found to be insignificant.

Two routes in addition to the E-M-P pathway have been reported to supply ethanol from glucose: (1) heterolactic fermentation postulated in *Leuconostoc mesenteroides* by DeMoss, Bard, and Gunsalus (1951) and Gunsalus and Gibbs (1952), and (2) *Pseudomonas lindneri* fermentation described by Gibbs and DeMoss (1954). Since these two pathways predict that carbon atom 1 of glucose yields carbon dioxide, they are presumably not present in *Clostridium perfringens*.

The data suggest that some mechanism as yet not described must be supplying unlabeled ethanol to dilute the activity of the total ethanol presumably from pyruvic acid. Assuming that the dehydrogenation of glyceraldehyde-3-phosphate yields a product that undergoes further metabolism via two divergent pathways to yield two trioses which are similarly decarboxylated, the dioses arising from carbons 1–2 and 5–6 can then be visualized to undergo reduction to ethanol and oxidation to acetic acid in different quantitative manners, depending upon the rates of the individual enzyme systems. Regardless of the true situation, it is clear that the glycolytic enzymes of this organism appear typical through the triose dehydrogenation stage. The absence of data concerning the fate of the oxidized trioses makes impossible the mechanistic interpretation of the isotope data presented above.

ADDENDUM

The findings of Wood *et al.* (1955) with Propionibacterium reveal similar difficulties in equating the pathways of glucose dissimilation in this genus as in Clostridia to known pathways of glucose degradation. Nevertheless, certain similarities exist between the results obtained with the proprionibacteria and those described above with *Clostridium perfringens*. Most notable is the differential labeling of the methyl group of acetate in both fermentations when glucose-1-C^{14} and glucose-6-C^{14} served as substrates.

SUMMARY

The fermentative metabolism of *Clostridium perfringens* has been studied using glucose specifically labeled with carbon 14. Evidence is presented which indicates that although the Embden-Myerhof-Parnas pathway is in part operative in this organism, a different terminal pathway exists, probably involving triose metabolism.

REFERENCES

Bard, R. C. and Gunsalus, I. C. 1950 Glucose metabolism of *Clostridium perfringens*: existence of a metallo-aldolase. J. Bacteriol., **59**, 387–400.

Beuding, E. and Yale, H. W. 1951 Production of α-methylbutyric acid by bacteria-free *Ascaris lumbricoides*. J. Biol. Chem., **193**, 411–423.

DeMoss, R. D., Bard, R. C. and Gunsalus, I. C. 1951 The mechanism of the heterolactic fermentation: a new route of ethanol formation. J. Bacteriol., **62**, 499–511.

Elsden, S. R. 1952 Bacterial fermentation. In *The enzymes*. Vol. II, part 2, pp. 791–843. Edited by J. B. Sumner, and K. Myrback. Academic Press Inc., New York, N. Y.

Gibbs, M. and DeMoss, R. D. 1954 Anaerobic dissimilation of C^{14}-labeled glucose and fructose by *Pseudomonas lindneri*. J. Biol. Chem., **207**, 689–694.

Gibbs, M. and Gastel, R. 1953 Glucose dissimilation by Rhizopus. Arch. Biochem. and Biophys., **43**, 33–38.

Gibbs, M., Cochrane, V. W., Paege, L. M. and Wolin, H. 1954 Fermentation of D-xylose-1-C^{14} in the products of glucose dissimilation by *Fusarium lini* Bolley. Arch. Biochem. and Biophys., **50**, 237–242.

Gunsalus, I. C. and Gibbs, M. 1952 The heterolactic fermentation. II. Position of C^{14} in the products of glucose dissimilation by *Leuconostoc mesenteroides*. J. Biol. Chem., **194**, 871–875.

Osburn, O. L. and Werkman, C. H. 1932 Determination of carbon in fermented liquors. Ind. Eng. Chem. Anal. Ed., **4**, 421–423.

Phares, E. F. 1951 Degradation of labeled propionic and acetic acids. Arch. Biochem. and Biophys., **33**, 173–178.

Shanker, K. and Bard, R. C. 1955 Effect of metallic ions on the growth, morphology, and metabolism of *Clostridium perfringens*. I. Magnesium. J. Bacteriol., **69**, 436–443.

Wood, Harland G., Stjernholm, Rune, and Leaver, F. W. 1955 The metabolism of labeled glucose by the propionic acid bacteria J. Bacteriol., **70**, 510–520.