JOURNAL OF BACTERIOLOGY, Jan. 1971, p. 226-231

Vol. 105, No. 1
Printed in U.S.A.

Catabolism of Fructose and Mannitol in *Clostridium thermocellum*: Presence of Phosphoenolpyruvate: Fructose Phosphotransferase, Fructose 1-Phosphate Kinase, Phosphoenolpyruvate: Mannitol Phosphotransferase, and Mannitol 1-Phosphate Dehydrogenase in Cell Extracts[1]

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Received for publication 7 October 1970

Fructose and mannitol are fermented by *Clostridium thermocellum* in a medium containing salts and 0.5% yeast extract. The initial reaction in the catabolism of fructose was found to be the formation of fructose 1-phosphate by phosphoenolpyruvate (PEP): fructose phosphotransferase which resembles the Kundig-Roseman phosphotransferase system. The phosphorylation of fructose 1-phosphate to form fructose-1,6-diphosphate is catalyzed by fructose 1-phosphate kinase. Fructose-1,6-diphosphate can be further metabolized by the Embden-Meyerhof pathway. The formation of both PEP: fructose phosphotransferase and fructose 1-phosphate kinase is induced by growth in fructose medium. Mannitol catabolism was found to proceed by the phosphorylation of mannitol by PEP: mannitol phosphotransferase to form mannitol 1-phosphate. Mannitol 1-phosphate is converted to fructose 6-phosphate by a nicotinamide adenine dinucleotide-specific mannitol 1-phosphate dehydrogenase. The fructose 6-phosphate formed in the reaction can enter the glycolytic scheme. The formation of both PEP: mannitol phosphotransferase and mannitol 1-phosphate dehydrogenase is induced by growth in mannitol medium. Evidence is presented for the induction by mannitol of PEP: mannitol phosphotransferase and mannitol 1-phosphate dehydrogenase in suspensions of fructose-grown cells.

The ability of *Clostridium thermocellum* to utilize D-fructose was reported by Vidrine and Quinn (Bacteriol. Proc., p. 135, 1969). We have confirmed that this organism grows in a medium containing fructose and, furthermore, it has been found that growth is obtained with D-mannitol (12). In the present study we determined the metabolic pathways for the catabolism of fructose and mannitol in *C. thermocellum*.

MATERIALS AND METHODS

D-Fructose and D-mannitol were purchased from Pfanstiehl Laboratories. D-Fructose 1-phosphate (fructose-1-P), D-mannitol 1-phosphate (mannitol-1-P), and sorbitol 6-phosphate (sorbitol-6-P) were products of C. F. Boeringer and Soehne. Sorbitol (D-glucitol) was a product of Mann Research Laboratories. The source of commercial enzymes and other reagents has been listed (12). All sugars were of the D-configuration.

The growth and maintainance of *C. thermocellum* strain 651 which was used in this study has been described (12). Cell extracts were prepared as before (12), except that sometimes the cells were disrupted in 20 mM tris(hydroxymethyl)aminomethane (Tris), *p*H 7.6, and in certain cases the sonic treatment lasted for 60 sec instead of 90 sec. Protein was routinely determined as before (12).

Spectrophotometric assays were conducted as described previously (12) at 27 C in cuvettes of 1.0-cm light path. Unless otherwise noted, the enzyme activities were proportional to the amount of protein.

[1] This paper was presented in part at the 160th National meeting of the American Chemical Society, in Chicago, Ill., 13-18 September 1970.

Fructose 1-P kinase activity was determined by an adaptation of a procedure described for the determination of fructose 6-phosphate kinase (11). The reaction mixture in a volume of 1.0 ml contained 50 mM glycylglycine (*p*H 8.2), 4.8 mM $MgCl_2$, 0.08 mg of albumin, 2.3 mM reduced nicotinamide adenine dinucleotide (NADH), 25 mM cysteine, 0.1 mM fructose-1-P, 0.5 mM adenosine triphosphate (ATP), 10 μg of glycerophosphate dehydrogenase (EC 1.1.1.8)-triose phosphate isomerase (EC 5.3.1.1) mixture, 20 μg of aldolase (EC 4.1.2.13), and crude extract (0.075 mg of protein). The specific activity is expressed as the micromoles of fructose-1-P phosphorylated per minute per milligram of protein at 27 C.

The PEP:fructose phosphotransferase reaction mixtures in a volume of 1.0 ml contained 20 mM Tris (*p*H 7.6), 10 mM phosphoenolpyruvate (PEP), 5 mM fructose, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and crude extract (2 to 3 mg of protein). After a 15-min incubation period at 37 C, the reaction was stopped by boiling for 5 min, and the reaction mixture was centrifuged to remove the coagulated protein. The amount of fructose-1-P formed was estimated after transferring an 0.05-ml sample to the fructose 1-phosphate kinase assay system. The fructose 1-phosphate kinase assay system was identical to the one described above except that the commercial fructose-1-P was deleted and the added crude extract from fructose-grown cells, which served as a source of fructose 1-phosphate kinase, contained about 0.8 mg of protein. No PEP:fructose phosphotransferase activity was observed under the conditions of the fructose-1-P assays (see 0 min control in Table 2).

Mannitol 1-phosphate dehydrogenase (EC 1.1.1.17) was assayed by measuring the oxidation of mannitol-1-P or by measuring the reduction of fructose-6-P (5). The reaction mixture for the oxidation of mannitol-1-P contained 70 mM Tris, 2.0 mM mannitol-1-P, 1.0 mM nicotinamide adenine dinucleotide (NAD), and crude extract in a volume of 1.0 ml. For the reduction of fructose-6-P, the reaction mixture contained 70 mM Tris, 2.0 mM fructose-6-P, 1.0 mM NADH, and crude extract in a volume of 1.0 ml. The rate of oxidation of NADH between 30 and 90 sec was used to determine enzyme activity.

The PEP:mannitol phosphotransferase reaction mixture contained 20 mM Tris (*p*H 7.6), 10 mM PEP, 5 mM mannitol, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and crude extract (3 to 6 mg of protein) in a volume of 1.0 ml. After incubating for 15 min at 37 C, the reaction was stopped by boiling for 5 min, and the coagulated protein was removed by centrifugation. The amount of mannitol-1-P formed in 0.1-ml samples of the reaction mixture was estimated by the mannitol 1-phosphate dehydrogenase procedure described above. In the mannitol-1-P assays, which were carried out at *p*H 9.0, commercial mannitol-1-P was deleted, and crude extracts (about 0.9 mg of protein) from mannitol-grown cells were used as a source of mannitol 1-phosphate dehydrogenase. No PEP:mannitol phosphotransferase activity could be detected under the conditions of the mannitol-1-P assays. The activities are expressed as micromoles of mannitol-1-P produced per minute per milligram of protein at 37 C.

Induction of PEP:mannitol phosphotransferase and mannitol 1-phosphate dehydrogenase in cell suspensions was carried out by the same procedure used for glucokinase induction (12).

RESULTS

Fructose 1-phosphate kinase. Fructose 6-P formation from fructose and ATP or PEP could not be detected in fructose-grown cells. However, an active fructose 1-phosphate kinase is found in extracts from fructose-grown cells (Table 1). Little or no fructose 1-phosphate kinase is present in extracts from cells grown in glucose, mannose, or mannitol medium, which indicates that synthesis of the enzyme is induced during growth in the presence of fructose.

PEP:fructose phosphotransferase. The presence of fructose 1-phosphate kinase in fructose-grown cells suggests that the initial step in fructose metabolism is the formation of fructose-1-P. Fructose and ATP or PEP were incubated with extracts from fructose-grown cells and the reaction mixtures were assayed for fructose-1-P formation. The results (Table 2) indicate that fructose-1-P formation occurs only when PEP is added as the phosphoryl donor. Our results were confirmed with purified fructose 1-phosphate kinase (14) by R. W. Walter and R. L. Anderson of Michigan State University (Table 2). Little or no fructose-1-P was converted to fructose-1,6-diphosphate in these assays.

The actual rate of PEP:fructose phosphotransferase may be higher than the values reported here, since we have not determined whether the enzyme is saturated or whether the rate is proportional to the amount of protein.

In contrast to the activity in extracts from fructose-grown cells, no activity was found in extracts from mannitol-grown cells, an indication that synthesis of the enzyme is induced by fructose.

Mannitol 1-phosphate dehydrogenase. In studying the metabolism of mannitol, we assumed that the initial step would entail either a phosphorylation or an oxidation of mannitol. No mannitol dehydrogenase activity could be detected in cell extracts from mannitol-grown cells. Likewise, no phosphorylation of mannitol with ATP was detected in our initial experiments. An

TABLE 1. *Fructose-1-phosphate kinase activity in extracts from cells grown on different carbohydrates*

Growth substrate (0.9%)	Specific activity[a]
Fructose	0.280
Glucose	0.001
Mannose	0.001
Mannitol	0.0035

[a] Expressed in micromoles per minute per milligram of protein.

TABLE 2. *PEP:fructose phosphotransferase activity[a] in extracts from fructose-grown cells*

Reaction mixture	Fructose-1-P[b]	Fructose-1,6-di P[b]
Complete (0 min) .	0.000 (0.000)[c]	0.000 (0.000)
Complete (15 min)	0.065 (0.077)	0.005 (0.006)
Minus PEP	0.003	0.001
Minus PEP, plus ATP (10 mM) ...	0.005 (0.000)	0.001 (0.001)
Complete[d]	0.000	

[a] Based on the rate of fructose 1-P formation.

[b] Results are expressed as micromoles per minute per milligram of protein at 37 C. For the 0-min reaction mixture, results are expressed as micromoles per milligram of protein.

[c] Figures in parentheses were obtained by R. W. Walter and R. L. Anderson.

[d] Extract was prepared from mannitol-grown cells.

TABLE 3. *Mannitol 1-phosphate dehydrogenase activities[a] in crude cell extracts*

Source of extract	*p*H of reaction mixture	Mannitol-1-P oxidation	Fructose-6-P reduction
Mannitol-grown cells ..	8.0	1.28	
	8.6	1.92	
	9.0	1.92	0.364
	9.5	1.28	
Fructose-grown cells ...	9.0	0.135	0.027
Glucose-grown cells ...	9.0	0.016	
Mannose-grown cells ..	9.0	0.018	

[a] Results are expressed as micromoles of pyridine nucleotide oxidized or reduced per minute per milligram of protein at 27 C.

active mannitol 1-phosphate dehydrogenase was observed, however, in extracts from mannitol-grown cells (Table 3). The enzyme can be measured either by the oxidation of mannitol-1-P or by the reduction of fructose-6-P. Although in some systems fructose-6-P may be converted to sorbitol-6-P instead of mannitol-1-P, this possibility seemed unlikely in that mannitol 1-phosphate dehydrogenase is highly active only in mannitol-grown cells, and *C. thermocellum* is unable to ferment sorbitol. Moreover, no activity was found with sorbitol-6-P and NAD.

The *p*H optimum for the oxidation of mannitol-1-P appears to be between 8.6 and 9.0 in 70 mM Tris buffer (Table 3). At *p*H 9.0, the rate of oxidation of mannitol-1-P is about fivefold more than the rate of fructose 6-P reduction. The ratio of these activities is similar in extracts from mannitol-grown cells or from fructose-grown cells. The specific activity for mannitol 1-phosphate dehydrogenase is much higher than for any other enzyme found in this organism (12). Fructose-grown cells contain more of the enzyme than cells grown in glucose or mannose, but they contain less of the enzyme than mannitol-grown cells. No activity was detected when NADP was substituted for NAD in the reduction of mannitol-1-P.

PEP:mannitol phosphotransferase. Finding mannitol 1-phosphate dehydrogenase led us to search for a mannitol phosphorylation reaction. After cell extracts from mannitol-grown cells were incubated with mannitol and ATP or PEP, samples were withdrawn and assayed for mannitol-1-P. Mannitol-1-P was found only in the PEP-containing reaction mixture (Table 4). Little or no activity was found when PEP, mannitol, $MgCl_2$, or cell extracts were deleted. Likewise, no activity was detected in extracts from fructose-grown cells.

Further evidence that mannitol-1-P is formed in the phosphotransferase reaction was obtained by adding phosphoglucose isomerase (EC 5.3.1.9), glucose 6-phosphate dehydrogenase (EC 1.1.1.49), and NADP to the mannitol-1-P assay system. The results (Table 5), which show that the increase in optical density at 340 nm is doubled by the addition of NADP and the auxiliary enzymes, are interpreted to mean that fructose-6-P is indeed formed in the mannitol-1-P assay system. The fructose-6-P is converted to glucose-6-P and 6-phosphogluconate by the auxiliary enzymes.

Further information on the identity of the product of the PEP:mannitol phosphotransferase reaction was obtained in the following experiment. Boiled samples of a previously incubated PEP:mannitol phosphotransferase reaction mixture were added to mannitol 1-phosphate dehydrogenase reaction mixtures that contained known amounts of a standard mannitol-1-P. If mannitol-1-P is produced in the PEP:mannitol phosphotransferase reaction, no change in the rate of mannitol 1-phosphate dehydrogenase activity would be expected upon the addition of the boiled reaction mixture to a system containing saturating amounts of standard mannitol-1-P. With less than saturating amounts of standard

TABLE 4. *Activity of PEP:mannitol phosphotransferase in extracts from mannitol-grown cells*

Reaction mixture	Specific activity[a]
Complete	0.0059
Minus PEP	0.0002
Minus PEP, plus ATP (10 mM)	0.0005
Minus mannitol	0.0001
Minus extract	0.0001
Minus $MgCl_2$	0.0001

[a] Expressed as micromoles per minute per milligram of protein.

mannitol-1-P, however, an increase in the rate of mannitol 1-phosphate dehydrogenase activity would be expected upon the addition of the boiled reaction mixture. The results (Table 6) show that the boiled reaction mixture does not enhance the rate obtained with the saturated enzyme, but it does cause an increase in rate with the unsaturated enzyme. Thus, these data further indicate that mannitol-1-P is formed in the PEP:mannitol phosphotransferase reaction.

The results in Fig. 1 indicate that the rate of the PEP:mannitol phosphotransferase reaction is linear during the first 15 min. From these data, the specific activity was found to be 0.0061 μmole per min per mg of protein at 37 C.

Induction of PEP:mannitol phosphotransferase and mannitol 1-phosphate dehydrogenase. The relatively high activities of PEP:mannitol phosphotransferase and mannitol 1-phosphate dehydrogenase in mannitol-grown cells in relation to the activities in cells grown with other sugars are typical of situations involving inducible enzymes. The following experiment was conducted to determine whether PEP:mannitol phosphotransferase and mannitol 1-phosphate dehydrogenase are inducible in suspensions of cells grown without mannitol. The activity of PEP:mannitol phosphotransferase and mannitol 1-phosphate dehydrogenase after incubation of fructose-grown cells for 90 min in the presence of mannitol is shown in Table 7. After induction, the specific activity of both enzymes is about 40% of the activity in extracts from mannitol-grown cells. Before induction, little or no PEP:mannitol phosphotransferase is present, and the initial amount of mannitol 1-phosphate dehydrogenase is less than 10% of the amount in mannitol-grown cells. Thus it appears that mannitol is able to induce the synthesis of these enzymes. No increase in the amount of protein could be detected by the biuret method (3) in either the cell extracts or the cell debris as a result of the induction treatment.

TABLE 5. *Sequential formation of fructose-6-P and 6-phosphogluconate from the mannitol-1-P produced in the PEP:mannitol phosphotransferase reaction*[a]

Incubation time of phosphotransferase reaction (min)	Fructose-6-P[b] formation	6-Phosphogluconate[c] formation
0	0.037	0.040
6	0.136	0.140
15	0.388	0.341

[a] The conditions were the same as in the complete reaction mixture in Table 4.

[b] The formation of fructose-6-P was determined with mannitol-1-phosphate dehydrogenase. Results are expressed as micromoles per milliliter of PEP:mannitol reaction mixture.

[c] After fructose-6-P had been determined, excess phosphoglucose isomerase (10 μg), glucose 6-phosphate dehydrogenase (10 μg), and NADP (0.2 mM) were added, and the amount of 6-phosphogluconate was estimated by the additional increase in optical density at 340 nm. Results are expressed as micromoles per milliliter of PEP:mannitol phosphotransferase reaction mixture.

TABLE 6. *Effect of the product of PEP:mannitol phosphotransferase reaction on the rate of mannitol 1-phosphate dehydrogenase at various concentrations of mannitol-1-P*

Standard mannitol-1-P		Standard mannitol-1-P plus reaction mixture[a]			Increase[b] (%)
Mannitol-1-P concn (mM)	ΔOD[c]	Mannitol-1-P concn (mM)	Phospho-transferase reaction mixture[a] (ml)	ΔOD[c]	
0.04	0.075	0.04	0.1	0.120	60
0.1	0.150	0.1	0.1	0.160	6.7
2.0	0.240	2.0	0.1	0.240	0

[a] Boiled PEP:mannitol phosphotransferase reaction mixture as described in Table 4 was incubated for 15 min at 37 C and estimated to contain 0.47 μmole of mannitol-1-P per ml.

[b] Per cent increase in the rate of mannitol 1-phosphate dehydrogenase due to the addition of a sample of the PEP:mannitol phosphotransferase reaction mixture.

[c] Optical density (OD) at 340 nm per min.

DISCUSSION

The present investigation indicates that the initial step in the catabolism of fructose in *C. thermocellum* is the formation of fructose-1-P by PEP:fructose phosphotransferase, followed by conversion of fructose-1-P to fructose 1,6-diphosphate by fructose 1-phosphate kinase. Fructose 1,6-diphosphate enters the Embden-Meyerhof pathway, which was found in this organism (12).

Bacterial catabolism of mannitol may be initiated by a phosphorylation reaction or by the oxidation of mannitol (16). According to Tanaka et al. (16), ATP never has been shown conclusively to act as a phosphoryl donor for mannitol phosphorylation, although a mannitol kinase has been reported (7). The current studies indicate that mannitol catabolism in *C. thermocellum* is initiated by the formation of mannitol-1-P in a reaction in which PEP acts as the phosphoryl donor. Mannitol-1-P is oxidized by mannitol 1-phosphate dehydrogenase to form fructose-6-P, which is further metabolized by the Embden-Meyerhof pathway.

Although the components of the phosphotransferase systems in *C. thermocellum* have not been delineated, it appears to be similar to the system described by Kundig et al. (10). Since

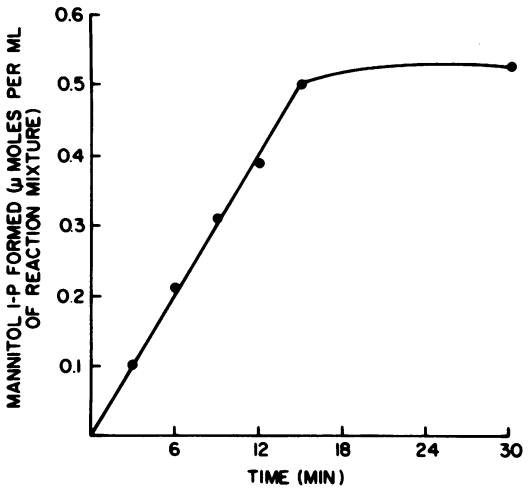

FIG. 1. *Rate of the PEP:mannitol phosphotransferase reaction. The experimental conditions were the same as in Table 4 except that the reaction mixture contained 5.5 mg of protein per ml.*

TABLE 7. *Induction of PEP:mannitol phosphotransferase and mannitol 1-phosphate dehydrogenase in fructose-grown cells*

Growth substrate	Additions to buffered cells[a]	PEP:mannitol phosphotransferase[b]	Mannitol 1-phosphate dehydrogenase[c]
Fructose	None	0.0001	0.134
Fructose	Mannitol	0.0026	0.740
Mannitol[d]		0.0059	1.92

[a] Induction medium contained 5 mM mannitol, 100 mM potassium phosphate (*p*H 7.0), and about 0.1 g of cells (wet weight) per ml.

[b] Expressed as micromoles per minute per milligram of protein at 37 C.

[c] Expressed as micromoles per minute per milligram of protein at 27 C.

[d] The mannitol-grown cells were not incubated with mannitol. The activities in extracts from these cells are shown for comparative purposes.

PEP:sugar phosphotransferase systems have been implicated in active transport (1), it is possible that both transport and phosphorylation are accomplished in the initial reaction of fructose and mannitol metabolism. The relatively low specific activities of the phosphotransferase reactions in relation to the specific activities of fructose 1-phosphate kinase and mannitol 1-phosphate dehydrogenase may raise doubt about the metabolic significance of the phosphotransferases. It is likely that further efforts to improve the conditions for the latter would result in higher activities. Even though the phosphotransferase systems have not been studied in detail, the specific activities we obtained compare favorably with the activities in other organisms. The rate of the PEP:fructose phosphotransferase in *Aerobacter aerogenes* was 0.01 μmole per min per mg of protein at 25 C (4), whereas the value obtained in *C. thermocellum* was 0.06 μmole per min per mg of protein at 37 C. The activity of the PEP:mannitol phosphotransferase system in *A. aerogenes* of 0.03 μmole per min per mg of protein at 25 C (16) is considerably higher than the activity in *C. thermocellum* (0.006 μmole per min per mg of protein at 37 C). At the optimum growth temperature of *C. thermocellum*, which is about 20 C higher than the incubation temperature, the activity probably would be more in line with the activity in *A. aerogenes*.

In contrast to the high activities of fructose 1-phosphate kinase of glucose-grown cells in two other anaerobes, namely *Bacteroides symbiosus* (6, 13) and *C. pasteurianum* (8, 9), *C. thermocellum* has no detectable activity when grown on glucose. The activity in *B. symbiosus is only 2.5- to 8-fold higher in fructose-grown cells than in glucose-grown cells (6). In this regard C. thermocellum* resembles two other bacteria, *Escherichia coli* (2) and *A. aerogenes* (15), in having relatively high activities of fructose 1-phosphate kinase only in fructose-grown cells.

The relatively high activities of PEP:fructose phosphotransferase and fructose 1-phosphate kinase in extracts from fructose-grown cells and the relatively high activities of PEP:mannitol phosphotransferase and mannitol 1-phosphate dehydrogenase from mannitol-grown cells indicate that these enzymes are induced by growth on fructose or mannitol. The increase in activity of PEP:mannitol phosphotransferase and mannitol 1-phosphate dehydrogenase in suspensions of fructose-grown cells during incubation with mannitol is further evidence that these enzymes are inducible. The possibility that the increase in activity is due to enzyme activation rather than induction seems unlikely in view of the evidence that an inhibitor of protein synthesis (chloramphenicol) prevents glucokinase induction in this system (12).

If the phosphotransferase systems are typical of the Kundig-Roseman phosphotransferase (10) in having HPr, enzyme I, and enzyme II, then the HPr (histidine-containing protein) and enzyme I components of the system would be expected to be constitutive. Enzyme II or some component of the enzyme II complex would be expected to be the protein which is induced (1).

The proposal for the pathways for fructose and mannitol catabolism in *C. thermocellum* is based on several lines of evidence. The enzymes involved in the catabolism of fructose are induced by growth on fructose, and the enzymes of man-

nitol catabolism are induced by growth on mannitol. The specific activities of fructose 1-phosphate kinase and mannitol 1-phosphate dehydrogenase are relatively high in relation to other enzymes of carbohydrate metabolism in this organism (12) or in other organisms. The specific activities of the phosphotransferase systems are comparable to the activities found in other bacteria. The enzymes are active in the presence of relatively low concentrations of substrates. Other known pathways for the catabolism of fructose and mannitol could not be demonstrated.

ACKNOWLEDGMENTS

We thank R. W. Walter and R. L. Anderson for their assays of some of our samples.

This investigation was supported by National Science Foundation grant GB-8446.

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