Regulation of Hexose Phosphate Metabolism in *Acetobacter xylinum*

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The metabolism of glucose and fructose was studied in resting succinate-grown cells of *Acetobacter xylinum*. From fructose only cellulose and CO₂ were formed by the cells, whereas from glucose, gluconate was formed much more rapidly than these two products. The molar ratio of sugar converted into cellulose to sugar converted into CO₂ was significantly greater than unity for both hexoses. The pattern of label retention in the cellulose formed by the cells from specifically ¹⁴C-labelled glucose, fructose or gluconate corresponded to that of hexose phosphate in a pentose cycle. On the other hand, the isotopic configuration of cellulose arising from variously singly ¹⁴C-labelled pyruvate did not agree with the operation of a pentose cycle on gluconeogenic hexose phosphate. Readily oxidizable tricarboxylic acid-cycle intermediates such as acetate, pyruvate or succinate promoted cellulose synthesis from fructose and gluconate although retarding their oxidation to CO₂. The incorporation into cellulose of C-1 of fructose was greatly increased in the presence of these non-sugar substrates, although its oxidation to CO₂ was greatly diminished. It is suggested that the flow of hexose phosphate carbon towards cellulose or through the pentose cycle in *A. xylinum* is regulated by an energy-linked control mechanism.

Hexose phosphate is a common intermediate in *Acetobacter xylinum* of cellulose synthesis and the oxidative dissimilation of carbohydrate via the pentose phosphate and tricarboxylic acid cycles (Schramm *et al.*, 1957; Gromet *et al.*, 1957). Hexose phosphate arises in this organism directly by phosphorylation of exogenous hexose (Benziman & Rivetz, 1972), and indirectly via the pentose phosphate cycle and the gluconeogenic pathway (Benziman & Eizen, 1971; Benziman & Burger-Rachamimov, 1962). *A. xylinum* was found to possess distinct NAD- and NADP-specific glucose 6-phosphate dehydrogenases (Benziman & Mazover, 1973). The inhibition by ATP of the NAD-linked dehydrogenase suggests that modulation of this enzymic activity is a key regulatory process for controlling carbohydrate metabolism at the hexose phosphate branch point.

In the present study the pattern of hexose phosphate utilization by *A. xylinum* resting cells under various physiological conditions was investigated. The results obtained suggest that *in vivo* the flow of hexose phosphate carbon through the pentose cycle or towards cellulose synthesis is regulated by an energy-linked control mechanism. A preliminary report of this work has been published (Weinhouse & Benziman, 1971).

Materials and Methods

Cells

The cellulose-synthesizing strain of *A. xylinum* was the same as that employed in earlier investigations reported from this laboratory (Schramm & Hestrin, 1954). The culture medium prepared with glass-distilled water was as follows: succinic acid, 2% (w/v); yeast extract (Difco Laboratories, Detroit, Mich., U.S.A.), 0.5% (w/v); Bacto peptone (Difco), 0.5% (w/v) and monopotassium phosphate, 0.3% (w/v). The final pH was adjusted to 4.0 with NaOH. Cells were grown at 30°C under static conditions and harvested after 24 h as previously described (Benziman, 1969).

Incubation procedure for sugar utilization

Ehrlenmeyer flasks (100 ml) containing a centre well and stoppered with rubber caps served as reaction vessels. Reaction mixtures (10 ml) contained 50 mM-potassium phosphate, an appropriate cell suspension, and substrates at pH 5.0, with O₂ as the gas phase. CO₂ formed was collected in small test tubes containing 1.0 ml of freshly prepared 40% (w/v) KOH (low in carbonate) placed in the centre

well. The mixtures were shaken at 30°C in a Gyrorotatory incubator shaker (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.) at 100 oscillations/min. After incubation, the reaction was terminated by injection of H₂SO₄ into the main compartment. Incubation was then continued for another 20 min to ensure complete trapping of evolved CO₂. The contents of the centre-well tubes were diluted with CO₂-free water and kept for analysis. The cells and synthesized cellulose were separated by centrifugation at 9000g for 15 min and washed repeatedly. Supernatants were combined and kept for analysis.

Analytical methods

Cellulose samples were freed from protein by treatment with 2M-KOH for 5 min at 100°C, washed repeatedly and then dried. Protein-free samples were acetolysed and hydrolysed and the released glucose was determined by the use of a phenol-H₂SO₄ reagent as described previously (Benziman & Burger-Rachamimov, 1962). Trapped CO₂ was determined manometrically (Umbreit *et al.*, 1957). Radioactivity was assayed in a Packard Tri-Carb scintillation counter. ¹⁴C-labelled material was counted in 10 ml of Bray's (1960) scintillation fluid. Values were corrected for quenching by the use of internal standards. Glucose was determined by use of the glucose oxidase (EC 1.1.3.4)-peroxidase (EC 1.11.1.7) system (Bergmeyer & Bernt, 1963). Fructose was assayed colorimetrically by the method of Roe *et al.* (1949) or enzymically (Klotzch & Bergmeyer, 1963). Gluconate was determined as described by Lien (1959).

Chemicals

Enzyme cofactors and most of the enzymes (analytical grade) were obtained from Sigma Chemical Co.,

(St. Louis, Mo., U.S.A.) or from C. F. Boehringer and Soehne G.m.b.H. (Mannheim, Germany). Radioactive chemicals were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). [1-¹⁴C]Fructose was prepared by the epimerization of [1-¹⁴C]glucose (Hers *et al.*, 1954). A sealed ampoule containing a 2.5% (w/v) solution of [1-¹⁴C]glucose in 0.2M-sodium phosphate buffer, pH 7.5, was heated in a boiling-water bath for 3 h. The fructose formed was completely separated from glucose and mannose by high-voltage paper electrophoresis in 0.8M-sodium borate buffer, pH 6.0, on Whatman no. 3 filter paper at 24 V/cm for 3.5 h. Strips containing fructose were cut and repeatedly eluted with hot water in the presence of unlabelled fructose carrier. The eluates were evaporated to dryness in a Rinco-flash evaporator. Methanol was added and the solution was evaporated at 40°C under reduced pressure to remove the borate. Methanol distillation was repeated five times. The residual material was then dissolved in water. It was checked chromatographically and electrophoretically and was found to be free from glucose and mannose.

Results

Products of the metabolism of glucose and fructose

Glucose utilization by resting succinate-grown cells of *A. xylinum* was accompanied by the formation of gluconate, cellulose and CO₂. Gluconate accounted for more than 90% of the glucose utilized. With fructose as substrate, cellulose and CO₂ were the only products detected (Table 1). Although the rate of cellulose formation from fructose was somewhat lower than that from glucose, the molar ratio of cellulose formed to sugar oxidized to CO₂ was approximately the same for the two hexoses at any of the concentrations tested. As seen from Table 1, there was an excellent agreement between the

Table 1. *Metabolic changes during the utilization of ¹⁴C-labelled glucose and fructose*

Cells (18.4 mg dry weight) were incubated with U-¹⁴C-labelled substrates (specific radioactivity 3×10^3 and 4×10^3 c.p.m./ μ mol of glucose and fructose respectively), at the concentrations signified, for 60 min as described in the Methods and Materials section. Results in the table are expressed in μ mol.

Substrate	Concentration (mM)	Cellulose formed*	Substrate converted into cellulose†	Substrate converted into CO ₂ †	Gluconate formed	Substrate utilized
Glucose	5	6.3	6.4	3.45	39	49
	10	8.1	8.4	4.45	79	99
	20	12.1	11.9	7.60	181	200
	40	13.0	12.5	8.00	320	360
Fructose	5	3.7	3.9	2.73	0	7
	10	6.0	6.3	3.68	0	10
	20	7.7	8.0	4.24	0	15
	40	10.7	10.3	5.55	0	18

* Determined chemically; expressed as glucose.

† Calculated from total ¹⁴C incorporation and the specific radioactivity of the substrate.

Table 2. Retention of ^{14}C in cellulose formed from specifically labelled substrates

Cells (10–30mg dry wt.) were incubated with 40mm of each ^{14}C -labelled substrate (specific radioactivities 3×10^3 – 5×10^3 c.p.m./ μmol) for 30–90 min as described in the Methods and Materials section.

Substrate	Specific molar radioactivity of cellulose monomer/ specific molar radioactivity of substrate
[U- ^{14}C]Glucose	0.96
[1- ^{14}C]Glucose	0.20
[2- ^{14}C]Glucose	0.90
[U- ^{14}C]Fructose	0.96
[1- ^{14}C]Fructose	0.49
[2- ^{14}C]Fructose	0.92
[U- ^{14}C]Gluconate	0.95
[1- ^{14}C]Gluconate	0
[U- ^{14}C]Pyruvate	2.2
[2- ^{14}C]Pyruvate	2.1
[2- ^{14}C]Pyruvate	2.3
[U- ^{14}C]Succinate	1.5
[2,3- ^{14}C]Succinate	2.3

amounts of cellulose determined chemically and the amounts of label incorporated into cellulose from ^{14}C -labelled fructose or glucose. This indicates that the cellulose was formed exclusively from these substrates without dilution.

Concentration of radioactivity in cellulose formed from specifically labelled substrates

The degree of retention of label in the cellulose formed by washed cells from specifically ^{14}C -labelled compounds is shown in Table 2. The labelling of cellulose with 1- ^{14}C -labelled glucose or fructose was much lower than with the 2- ^{14}C -labelled sugars. This is most probably due to the intermediary cycling of the hexose phosphate precursor of cellulose in the pentose cycle, which would extensively dilute the radioactivity of its C-1. The absence of incorporation of gluconate C-1 into cellulose is consistent with this reasoning, since the carbon of gluconate can be introduced into the hexose phosphate pool only via the reactions of the pentose cycle. Further, the rapid oxidation of glucose to gluconate could thus account for the larger dilution of radioactivity observed when [1- ^{14}C]glucose replaced [1- ^{14}C]fructose as the exogenous substrate.

The relative specific radioactivities of cellulose formed from U- ^{14}C -labelled pyruvate or succinate were in accordance with the expected stoichiometric relationship of 1 mol of glucose arising from 2 mol of gluconeogenic substrate. However, the degree of retention of radioisotope in the cellulose derived from singly ^{14}C -labelled pyruvates are not consistent

with those that would have been expected had the hexose phosphate formed from pyruvate been cycled in the pentose cycle before its conversion into cellulose. As shown previously (Benziman & Burger-Rachamimov, 1962) most of the labelling of the cellulose monomer formed from [3- ^{14}C]pyruvate and [2- ^{14}C]pyruvate is shared equally by the pairs C-1, C-6 and C-2, C-5 respectively. Thus with the operation of a pentose cycle the cellulose formed from [3- ^{14}C]pyruvate should have a much lower specific radioactivity than the cellulose formed from [2- ^{14}C]pyruvate. The data obtained here, however, do not agree with such a prediction.

Effect of tricarboxylic acid-cycle intermediates on the pattern of sugar metabolism

A. xylinum cells readily oxidized succinate, pyruvate and acetate to CO_2 , and synthesized cellulose from succinate and pyruvate when these were present at relatively high concentrations. To test the effect of such compounds on the flow of hexose phosphate carbon towards cellulose or towards oxidation via the pentose cycle, cells were incubated with either U- ^{14}C - or 1- ^{14}C -labelled fructose and unlabelled succinate, pyruvate or acetate, or alternatively with ^{14}C -labelled succinate, pyruvate or acetate and unlabelled fructose. The incorporation of ^{14}C from [1- ^{14}C]fructose into cellulose was taken as a measure of cellulose formation from non-cycled hexose phosphate derived from exogenous hexose. On the other hand, hexose phosphate entry into the pentose cycle was characterized by $^{14}\text{CO}_2$ production from cells utilizing [1- ^{14}C]fructose. Fructose was chosen as the sugar substrate in these experiments, because it does not appear to be subject to a direct oxidation, whereas glucose is rapidly oxidized to gluconate which in turn gives rise to cellulose. Consequently the bulk of glucose carbon is introduced into the hexose phosphate pool via a pentose cycle operating on gluconate.

When 40mm-pyruvate was added to fructose, both the total mass of cellulose formed and the percentage of fructose incorporated into cellulose increased. As shown by the chemical and radioisotopic data of Table 3, the added pyruvate caused a twofold enhancement of cellulose synthesis from fructose, a fivefold increase in the incorporation of fructose C-1 into cellulose and a 70% decrease in conversion of C-1 to CO_2 .

The metabolic fate of fructose C-1 was similarly affected by succinate (Table 3). Addition of succinate caused a 2.3-fold increase in C-1 incorporation into cellulose accompanied by an 80% decrease in C-1 conversion into CO_2 . It should be noted that succinate exerted these effects under conditions in which the cells approached a maximum rate of cellulose synthesis.

Table 3. *Effect of pyruvate and succinate on fructose metabolism*

Cells (25 mg dry wt.) were incubated for 60 min as described in the Methods and Materials section. Substrates: fructose, 5 mM; pyruvate and succinate, 40 mM. Specific radioactivities (c.p.m./ μ mol of substrate): [U- 14 C]fructose, 3×10^3 ; [1- 14 C]fructose, 2.5×10^3 ; [U- 14 C]pyruvate and [U- 14 C]succinate, 2×10^3 .

Expt. no.	Substrates	Cellulose formed (μ mol)*	CO ₂ evolved (μ mol)	Fructose utilized (μ mol)	10 ⁻³ × Total radioactivity (c.p.m.)	
					Cellulose	CO ₂
1	[U- 14 C]Fructose	4.4	18	7.5	12.7	9.0
2	[1- 14 C]Fructose	4.4	18	7.7	4.8	13.1
3	[U- 14 C]Pyruvate	5.3	206	—	24.5	132.0
4	[U- 14 C]Succinate	20.0	277	—	59.0	146.0
5	[U- 14 C]Fructose + pyruvate	15.3	254	10.5	28.0	3.6
6	[1- 14 C]Fructose + pyruvate	15.0	250	10.0	21.9	4.5
7	[U- 14 C]Fructose + succinate	21.2	300	8.3	15.2	1.9
8	[1- 14 C]Fructose + succinate	21.2	305	7.8	11.2	2.9
9	[U- 14 C]Pyruvate + fructose	15.2	250	9.4	24.0	164.0
10	[U- 14 C]Succinate + fructose	21.7	300	8.3	47.6	150.0

* Determined chemically; expressed as glucose.

Table 4. *Effect of acetate and low concentrations of pyruvate on fructose metabolism*

Cells (22 mg dry wt.) were incubated for 60 min as described in the Methods and Materials section. Substrates: fructose, 5 mM; pyruvate, 10 mM; acetate, 40 mM. Specific radioactivities (c.p.m./ μ mol of substrate): [U- 14 C]fructose, 3×10^3 ; [1- 14 C]fructose, 2.5×10^3 ; [U- 14 C]pyruvate, 2×10^3 ; [U- 14 C]acetate, 4×10^3 .

Expt. no.	Substrates	Cellulose formed (μ mol)*	CO ₂ evolved (μ mol)	Fructose utilized (μ mol)	10 ⁻³ × Total radioactivity (c.p.m.)	
					Cellulose	CO ₂
1	[U- 14 C]Fructose	3.8	16	6.5	11.1	7.8
2	[1- 14 C]Fructose	3.8	16	6.3	4.2	11.4
3	[U- 14 C]Acetate	0	41	—	0	83.7
4	[U- 14 C]Pyruvate	0.4	94	—	1.7	62.3
5	[U- 14 C]Fructose + acetate	5.2	65	6.5	15.0	3.9
6	[1- 14 C]Fructose + acetate	5.2	65	6.8	11.4	4.4
7	[U- 14 C]Fructose + pyruvate	9.9	146	10.4	28.1	4.0
8	[1- 14 C]Fructose + pyruvate	9.6	148	10.5	21.8	4.6
9	[U- 14 C]Acetate + fructose	5.2	70	6.7	0.9	113.0
10	[U- 14 C]Pyruvate + fructose	9.8	146	10.2	2.0	91.6

* Determined chemically; expressed as glucose.

The effect of readily oxidizable substrates which afford very little cellulose is shown in Table 4. The addition of 40 mM-acetate or 10 mM-pyruvate to fructose increased the yield of cellulose by factors of 1.4 and 2.6 respectively. The radioisotopic data indicate clearly that cellulose carbon was derived almost exclusively from fructose. Thus the specific radioactivity of the cellulose carbon was 96% that of added 14 C-labelled fructose and only a minor fraction of 14 C-labelled acetate or pyruvate appeared in the cellulose. Some 80–90% of the fructose utilized in the presence of acetate or pyruvate could be accounted for as cellulose, compared with 55% in the absence of these compounds, the remaining

fructose being oxidized to CO₂. The incorporation of C-1 of fructose into cellulose was increased by factors of 2.7 and 5.6 respectively in the presence of acetate or pyruvate, the specific radioactivity of the monomer increasing thereby from 44% to 90% of that of the substrate [1- 14 C]fructose. The conversion of C-1 into CO₂ decreased by 60% in both supplemented systems.

Effect of pyruvate on gluconate metabolism

Washed *A. xylinum* cells produced cellulose from gluconate at approximately one-third the rate obtained with fructose or glucose. The molar ratio

Table 5. *Effect of pyruvate on gluconate metabolism*

Cells (29 mg dry wt.) were incubated for 50 min as described in the Methods and Materials section. Substrates: gluconate, 40 mM; pyruvate, 40 mM. Specific radioactivities (c.p.m./ μ mol of substrate): [U- 14 C]gluconate, 2.5×10^3 ; [1- 14 C]gluconate, 3.2×10^3 ; [U- 14 C]pyruvate, 2×10^3 .

Expt. no.	Substrates	Cellulose formed (μ mol)*	CO ₂ evolved (μ mol)	Gluconate utilized (μ mol)	10 ⁻³ × Total radioactivity (c.p.m.)	
					Cellulose	CO ₂
1	[U- 14 C]Gluconate	5.3	78	18	13.0	34.0
2	[1- 14 C]Gluconate	5.5	74	18	0	60.5
3	[U- 14 C]Pyruvate	6.1	220	—	24.0	150.0
4	[U- 14 C]Gluconate + pyruvate	13.8	240	18	25.5	19.5
5	[1- 14 C]Gluconate + pyruvate	13.8	240	17	0	58.0
6	[U- 14 C]Pyruvate + gluconate	14.0	245	20	12.0	150.0

* Determined chemically; expressed as glucose.

of cellulose formed to substrate oxidized to CO₂ was, however, considerably smaller for gluconate than for the sugars. When pyruvate (40 mM) was added to gluconate (40 mM), the amount of cellulose formed was only slightly greater than the sum of the amounts formed separately from these substrates (Table 5). The radioisotopic distribution data, however, show that addition of pyruvate caused a 2-fold increase in gluconate incorporation into cellulose, concomitant with a 40% decrease in gluconate oxidation to CO₂. On the other hand, pyruvate incorporation into cellulose was diminished by 50% in the presence of gluconate, whereas its conversion into CO₂ was unaffected.

Hexose phosphate formation from gluconate by way of a pentose cycle involves the oxidative decarboxylation of 6-phosphogluconate as a primary step. As seen from the data of Table 5, the output of CO₂ from gluconate C1 was not altered by the presence of pyruvate. On the other hand, the complete oxidation to CO₂ of subsequently formed hexose phosphate appears to have been suppressed, in the presence of pyruvate, by almost 60%. This was calculated from the total amount of 14 CO₂ evolved from [U- 14 C]-gluconate, corrected for the 14 CO₂ evolved from [1- 14 C]gluconate. The hexose phosphate carbon not converted into CO₂ could be accounted for by the extra cellulose formed under these conditions.

Discussion

Although useful energy might be afforded by the oxidation of glucose to gluconate by *A. xylinum*, such energy does not appear to be essential for cellulose synthesis, since fructose has been shown to be able to sustain vigorous cellulose synthesis even though its metabolism does not involve gluconate formation.

The pattern of label retention observed in the cellulose formed from specifically 14 C-labelled substrates (Table 2) indicates that the metabolism of gluconate proceeds exclusively via pentose phosphates (Schramm *et al.*, 1957, 1958), the metabolism of glucose and fructose does so to a large extent, whereas gluconeogenic substrates are not metabolized via pentose phosphates at all.

From the chemical and radioisotopic data of Tables 3 and 4 it is evident that acetate, pyruvate or succinate promote cellulose synthesis from fructose, while at the same time they retard fructose oxidation to CO₂. The data on the distribution of 14 C, introduced as [1- 14 C]fructose, strongly suggest that hexose metabolism via pentose phosphates is suppressed in the presence of the above-mentioned readily-oxidizable non-sugar substrates. This is also suggested by the effect of pyruvate on the pattern of gluconate oxidation (Table 5).

Regulation of the conversion of hexose phosphate into pentose phosphates may be related to the sensitivity to inhibition by ATP of the NAD-linked glucose 6-phosphate dehydrogenase of *A. xylinum* (Benziman & Mazover, 1973). Generation of ATP brought about by the rapid oxidation of non-sugar substrates in the tricarboxylic acid cycle (Benziman & Levy, 1966) might be sufficient to inhibit this dehydrogenase and thereby restrict the subsequent influx of carbon into the pentose phosphate pathway. Such regulation may be important physiologically for cells growing on tricarboxylic acid-cycle intermediates when hexose phosphate is produced via gluconeogenesis (Benziman & Eizen, 1971). An uncontrolled oxidation of such hexose phosphate would restrict its availability for cellulose biosynthesis, which is essential for the growth of *A. xylinum* under static conditions (Schramm & Hestrin, 1954).

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