Fructose-1,6-Diphosphatase and Acid Hexose Phosphatase of *Escherichia coli*

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**Abstract**

Fraenkel, D. G. (Albert Einstein College of Medicine, New York, N.Y.), and B. L. Horecker. Fructose-1,6-diphosphatase and acid hexose phosphatase of *Escherichia coli*. J. Bacteriol. 90:837-842. 1965.—The conversion of fructose-1,6-diphosphate to fructose-6-phosphate (fructose-1,6-diphosphatase activity) is essential for growth of *Escherichia coli* on glycerol, acetate, or succinate, but is unnecessary for growth on hexoses or pentoses. It has sometimes been assumed that fructose-1,6-diphosphatase activity is due to a nonspecific acid hexose phosphatase. We have now obtained a number of one-step mutants which have lost the ability to grow on glycerol, succinate, or acetate, but which grow normally on hexoses; these mutants are deficient in a fructose-1,6-diphosphatase which can be assayed spectrophotometrically in the presence of Mg²⁺ and low concentrations of substrate. These mutants still possess the nonspecific acid hexose phosphatase, which does not require Mg²⁺ and is active only at much higher concentrations of fructose-1,6-diphosphate. Evidence is presented to support the hypothesis that the newly described activity is the physiological fructose-1,6-diphosphatase. The acid hexose phosphatase is a different enzyme whose function remains unknown.

Fructose-1,6-diphosphatase catalyzes the dephosphorylation of fructose-1,6-diphosphate to yield fructose-6-phosphate and inorganic phosphate (Gomori, 1943). The formation of fructose-1,6-diphosphate from fructose-6-phosphate requires adenosine triphosphate (ATP) and is catalyzed by phosphofructokinase (Ostern, Guthke, and Terszakowec, 1936; Krebs and Kornberg, 1957). The relative rate of these two reactions determines whether carbohydrate metabolism is glycolytic or gluconeogenic, and there has been much work on the physiological mechanism of control of these enzyme activities (Mansour, 1963; Passonneau and Lowry, 1962; McGilvery and Pogell, 1964).

Fructose-1,6-diphosphatases have been purified from spinach leaves (Racker and Schroeder, 1958), rabbit liver (Pogell, 1962; Pontremoli et al., in press), rat liver (Bonsignore et al., 1963), pig kidney (Medicino and Vassarhely, 1963), *Euglena gracilis* (Smillie, 1964; App and Jagendorf, 1964), and *Pseudomonas saccharophila* (Fossitt and Bernstein, 1963). The purified enzymes have been found to be highly specific for fructose-1,6-diphosphate (although the enzyme from mammalian liver also splits sedoheptulose-1,7-diphosphate), to have pH optima above 7 and *Kₘ* values of $10^{-4}$ M or lower, and, in general, to require magnesium or manganese ion.

In prototrophic bacteria like *Escherichia coli*, fructose-1,6-diphosphatase would presumably be essential for cells during growth on carbon sources such as succinate, acetate, or glycerol, since the formation of hexose monophosphate, which is necessary for the biosynthesis of pentose, aromatic amino acids, and polysaccharide, would require the hydrolysis of fructose diphasphate. Fructose-1,6-diphosphatase should not be necessary for growth on hexoses or pentoses. With this hypothesis in mind, Gotto and Pogell (1962) examined the ability of sonic extracts of *E. coli* B to dephosphorylate fructose diphasphate and found that the rate of hydrolysis was 10-fold greater in acetate-grown cells than in glucose-grown cells. For optimal activity this “fructose-1,6-diphosphatase” required $10^{-2}$ M fructose-1,6-diphosphate and a pH of 5. The activity was not specific for fructose-1,6-diphosphate; hexose monophosphates were split at about the same rate, and they were unable to account for the apparent lack of specificity of the induced enzyme activity.

The activity described by Gotto and Pogell (1962) differed from the specific fructose-1,6-diphosphatases of other sources and resembled more the “acid phosphatase” of *E. coli*, as de-
scribed by Rogers and Reithel (1960) and by Neu and Heppel (1964). The latter authors found the "acid phosphatase" to be one of the enzymes selectively removed from whole cells by successive treatment with succrose-ethylenediaminetetraacetate (EDTA) and cold water. Other enzymes removed by this treatment included alkaline phosphatase, cyclic phosphodiesterase, 3'-nucleotidase, a ribonuclease, and a deoxyribonuclease. These enzymes are possibly involved in the provision of inorganic phosphate and the breakdown of exogenous nucleic acids; none is known to have a role in normal intermediary metabolism.

We have now obtained evidence for a true fructose-1,6-diphosphatase in E. coli K-12 and have isolated mutants lacking this enzyme. These mutants are unable to grow on acetate, glycerol, or succinate, but grow on hexoses and pentoses. They still possess the activity described by Gotto and Pogell (1962), which we will call the acid hexose phosphatase. The fructose-1,6-diphosphatase is not removed from the cells by the succrose-EDTA and water treatment of Neu and Heppel (1964), which does extract the acid hexose phosphatase.

MATERIALS AND METHODS

Acid hexose phosphatase was assayed by essentially the same procedure employed by Gotto and Pogell (1962) for "fructose-1,6-diphosphatase" and by Neu and Heppel (1964) for "acid phosphatase." The reaction mixture (0.25 ml) contained 0.12 M sodium acetate buffer (pH 5), 0.008 M substrate (sodium fructose diphosphate or sodium glucose-6-phosphate, both from the Sigma Chemical Co., St. Louis, Mo.), and E. coli extract. The mixture was incubated at 37 °C for 20 to 60 min, and the reaction was stopped by the addition of 0.25 ml of 0.5 M trichloroacetic acid; the precipitates were removed by centrifugation, and inorganic phosphate in the supernatant solution was measured by the method of Fiske and SubbaRow (1925). Fructose-1,6-diphosphatase was measured spectrophotometrically. (These conditions were employed by O. Rosen of this laboratory.) The reaction mixture (1.0 ml) contained 0.045 M tris(hydroxymethyl)aminomethane (Tris)-HCl buffer (pH 7.8); 2 × 10⁻⁴ M nicotinamide adenine dinucleotide phosphate (NADP), 10⁻³ M MgCl₂, 5 × 10⁻⁴ M EDTA, 1 μg of glucose-6-phosphate dehydrogenase (C. F. Boehringer & Soehne GmbH., Mannheim-Waldhof, Germany), 1 μg of phosphohexose isomerase (C. F. Boehringer & Soehne), and 1.2 × 10⁻⁴ M sodium fructose diphosphate. The reduction of NADP at room temperature was followed in a Gilford recording spectrophotometer at 340 μm. There was generally an increase in rate during the first few minutes as hexose-monophosphate accumulated. The values reported were calculated from the maximal rates, usually attained at about 12 min. β-Galactosidase was measured by following the hydrolysis of ß-nitrophenylgalactoside (ONPG; Lederberg, 1950). The cuvettes contained 0.022 M potassium phosphate (pH 7.6), 5 × 10⁻⁴ M ONPG, and extract. The change in absorbance at 420 μm was followed, and the rates were calculated by use of the molar absorbancy coefficient for ß-nitrophenol of 3,120.

The primary strain used in these experiments was E. coli Hfr 3,300 (obtained from J. M. Monod, Institut Pasteur, Paris, France), a K-12 strain constitutive for β-galactosidase, but otherwise possessing the wild-type phenotype (Fraenkel, Falcoz-Kelly, and Horecker, 1964). Cells were grown in minimal medium buffered with 0.1 M sodium phosphate (Fraenkel and Horecker, 1964), and supplemented with 4 mg/ml of the appropriate carbon source and with 1 μg/ml of thiamine.

Extracts were prepared from cells washed once with 0.9% NaCl by grinding frozen pellets with twice their weight of alumina (Special Alumina A-305, Alcoa Aluminum Co. of America), and extracting the paste with 0.01 M Tris-HCl buffer (pH 8.0). The mixtures were centrifuged at 20,000 × g for 15 min, and the residue was discarded. Protein was measured by the method of Lowry et al. (1951).

Selection of fructose-1,6-diphosphatase-less mutants. An overnight culture of E. coli Hfr 3,300 was diluted to 2 × 10⁸ cells per milliliter in minimal medium containing 0.9 mg/ml of the mutagen N-methyl-N′-nitro-N-nitroso-N-nitroguanidine (MNNNG) and 10⁻² M sodium acetate, purchased from Neu and Soehne (1960), obtained from K & K Laboratories, Plainfield, N.J., and incubated for 30 min at 37 °C. This treatment reduced the number of colonies formed on nutrient agar to 10⁸ cells per milliliter. The treated cells were washed in 0.9% saline and allowed to grow out in minimal medium containing glucose as sole carbon source. Full turbidity was reached in 40 hr. The cells were plated onto minimal glucose-agar plates, and the resulting colonies were replicated directly to sucinate-agar, glycerol-agar, and glucose-agar. Of 526 colonies which grew on glucose, 12 failed to grow on glycerol but grew on succinate, 2 grew on glycerol, but failed to grow on sucinate, and 3 failed to grow on either glycerol or succinate (Q11 is one of the last type). In another experiment, a penicillin selection step (Gorini and Kaufmann, 1960) was included before the first plating on glucose agar. The survivors of the nitrosoguanidine treatment were subcultured for 4 hr in a medium containing glycerol, succinate, and acetate, treated with 2,000 units per ml of penicillin, and 2 hr later plated on glucose medium.

In this experiment, of 66 survivors, 2 were succinate-negative, 1 was glycerol-negative, and 16 (including Q17) were negative for both succinate and glycerol.

RESULTS

Growth of mutants. The mutants selected for the simultaneous loss of ability to grow on
glycerol or succinate had the growth pattern expected of fructose-1,6-diphosphatase-less cells. For example, when tested for growth on solid or in liquid media, Q11 grew, as did the parent strain, on glucose, fructose, galactose, glucose, and xylose, but failed to grow on glycerol, succinate, pyruvate, or acetate.

**Enzyme levels in wild-type and mutant strains.**

After growth on glucose, strains 3,300, Q11, and Q17 contained similar activities of acid hexose phosphatase, but both Q11 and Q17 were deficient in fructose-1,6-diphosphatase (Table 1). The low activities in the mutant extracts were at the limit of sensitivity of the fructose-1,6-diphosphatase assay and are of doubtful significance. Fructose-1,6-diphosphatase in the parent strain 3,300 was dependent on magnesium ion; the effect of EDTA varied from one extract to another, but generally it stimulated about two-fold. Thus, for example, with 350 μg of an extract of succinate-grown cells, the rates (millimicro moles per minute per milligram of protein) were: complete system, 4.1; minus EDTA, 2.2; minus magnesium chloride, 0.3; and minus substrate, 0.2.

The mutant strains Q11 and Q17, like their parent strain, were constitutive for β-galactosidase (Table 1).

**Enzyme levels in various growth conditions.** In agreement with the results of Gotto and Pogell (1962), we found that the level of acid hexose phosphatase depended on the carbon source used for growth. Activity was high in succinate-grown cells and low in glucose-grown cells (Table 2). The level of fructose-1,6-diphosphatase, in contrast, did not vary significantly. The level of β-galactosidase in the constitutive strain 3,300 also depended on the carbon source, as was first shown by Brown (1961). The mutants could not be grown on succinate, but, in minimal medium containing 1% nutrient broth as carbon source, some limited growth was achieved (probably due to pentose and hexose in the nutrient broth); the cells grown under these conditions contained high levels of acid hexose phosphatase, but still lacked fructose-1,6-diphosphatase (Table 2).

**Separation of acid hexose phosphatase from fructose-1,6-diphosphatase.** A separation of the two activities was accomplished by the succrose-EDTA and cold water treatment of Neu and Heppel (1964; Table 3). All the acid hexose phosphatase activity was found in the water supernatant fraction after cold-water treatment, whereas the fructose-1,6-diphosphatase remained in the cells.

**Mapping of fructose-1,6-diphosphatase.** Yu, Kaney, and Atwood (1965) employed a different procedure to isolate a series of mutants of E. coli K-12 which possess the same growth pattern as Coli K-12 isolated by Brown.

### Table 1. Enzyme levels in parent and mutant strains*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Acid hexose phosphatase</th>
<th>Fructose-1,6-diphosphatase</th>
<th>β-Galactosidase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FDP</td>
<td>G6P</td>
<td></td>
</tr>
<tr>
<td>3,300</td>
<td>72</td>
<td>60</td>
<td>10.6</td>
</tr>
<tr>
<td>Q11</td>
<td>55</td>
<td>32</td>
<td>0.15</td>
</tr>
<tr>
<td>Q17</td>
<td>43</td>
<td>30</td>
<td>0.32</td>
</tr>
</tbody>
</table>

*The strains were grown on glucose, and extracts were prepared by grinding with alumina. Acid hexose phosphatase was tested with fructose-1,6-diphosphate (FDP) or glucose-6-phosphate (G6P). Results are expressed as millimicro moles per minute per milligram of protein.

### Table 2. Enzyme levels in various growth conditions*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Carbon source and growth phase</th>
<th>Acid hexose phosphatase</th>
<th>Fructose-1,6-diphosphatase</th>
<th>β-Galactosidase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>FDP</td>
<td>G6P</td>
<td></td>
</tr>
<tr>
<td>Wild type (3,300)</td>
<td>Glucose Logarithmic</td>
<td>39</td>
<td>39</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>Stationary</td>
<td>72</td>
<td>60</td>
<td>10.6</td>
</tr>
<tr>
<td></td>
<td>Succinate Logarithmic</td>
<td>218</td>
<td>133</td>
<td>5.2</td>
</tr>
<tr>
<td></td>
<td>Stationary</td>
<td>340</td>
<td>283</td>
<td>4.0</td>
</tr>
<tr>
<td>Mutant (361)</td>
<td>Glucose Stationary</td>
<td>7</td>
<td>7</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Nutrient broth Stationary</td>
<td>206</td>
<td>178</td>
<td>0.5</td>
</tr>
</tbody>
</table>

*Results are expressed as millimicro moles per minute per milligram of protein. FDP = fructose-1,6-diphosphate; G6P = glucose-6-phosphate.

†Strain 361 is a fructose-1,6-diphosphatase-less strain of Escherichia coli K-12 isolated by Yu et al. (1965).
Table 3. Separation of acid hexose phosphatase and fructose-1,6-diphosphatase*

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Enzyme</th>
<th>FDP</th>
<th>G6P</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA-sucrose supernatant solution</td>
<td>Fructose-1,6-diphosphatase &amp; Acid hexose phosphatase</td>
<td>26</td>
<td>20</td>
</tr>
<tr>
<td>Water</td>
<td>Remaining in cells</td>
<td>1,150</td>
<td>750</td>
</tr>
</tbody>
</table>

* Strain 3,300 was grown in minimal medium containing 1% nutrient broth. The method of fractionation was that of Neu and Heppel (1964). The cells were washed once with 0.01 N Tris- HCl (pH 7.8). The pellet, 1.2 g (wet weight), was suspended in 45 ml of 0.03 M Tris (pH 8.0), containing 5 × 10⁻⁴ M EDTA and 20% sucrose, and was stirred at room temperature for 15 min; the cells were collected by centrifugation. The cell pellet was frozen and extracted by alumina grinding, as usual. Results are expressed as micromoles per hour per gram (wet weight) of cells. FDP = fructose-1,6-diphosphate; G6P = glucose-6-phosphate.

† The specific activity of the fructose-1,6-diphosphatase in the “remaining in cells” fraction was 12 μmole per min per mg of protein.

The function of the acid hexose phosphatase remains unclear. It cannot replace fructose-1,6-diphosphatase, since mutants lacking fructose-1,6-diphosphatase are evidently unable to form hexose monophosphate from fructose-1,6-diphosphate, despite the presence of substantial levels of acid hexose phosphatase activity. The acid hexose phosphatase may be responsible for the growth on glucose-1-phosphate at pH 5 in a medium free from inorganic phosphate (Hofsten, 1961). It is of interest that Roberts and Wolfe (1951) showed that glucose-1-phosphate could not serve as a carbon source for E. coli, and in their relative activities with different hexose phosphate substrates; these were thought to be multiple forms of one enzyme. Neu and Heppel (1964) separated three distinct acid phosphatases from E. coli K-12: (i) a cyclic nucleotide (Anraku, 1964a, b), which may be identical with phosphatase I of Rogers and Reithel (1960); (ii) a 3'-nucleotidase; and (iii) an “acid phosphatase,” which splits phosphate from hexose-6-phosphates and hexose-α-1-phosphates (L. A. Heppel, personal communication). Hofsten and Forath (1961) also fractionated acid phosphatases from E. coli.
enter *E. coli* without hydrolysis (see also Fraenkel et al., 1964). Klungsøy and Endresen (1964) recently implicated the acid hexose phosphatase in the utilization of fructose diphosphate by resting cells of *E. coli*, but Roberts and Wolfe (1951) found that *E. coli* does not grow in a high phosphate medium on fructose diphosphate as sole carbon source, and we have confirmed this finding. Thus, there is no clear example of a phosphorylated compound whose utilization requires the acid hexose phosphatase.

Englesberg, Watson, and Hoffer (1961) found that mutants of *S. typhimurium* lacking the glucose effect possessed elevated acid hexose phosphatase levels. Hofsten (1961) suggested that this might be a consequence of impaired glucose metabolism rather than a cause of it. Neidhardt (personal communication) has come to the same conclusion regarding a strain of *Aerobacter aerogenes* which lacks the glucose effect (Neidhardt, 1960), is missing a pathway of glucose oxidation (Magasanik and Boyarski, 1960), and shows elevated acid hexose phosphatase levels. Some interesting suggestions for the function of the acid hexose phosphatase include a role in the glucose permease system (Englesberg et al., 1961), and a role in regulating the internal inorganic phosphate level (Englesberg et al., 1961; H. Rickenberg, personal communication). Hofsten (1963) has isolated mutants of *E. coli* unable to grow on succinate but capable of growth on glucose. None of these showed altered levels of the acid hexose phosphatase. One strain (E 15/2) was able to grow on hexose or pentose, but failed to grow on succinate, glycerol, or acetate. The metabolic lesion was not established. We would suggest that this mutant may be deficient in fructose-1,6-diphosphatase.

The apparent constitutive nature of the fructose-1,6-diphosphatase of *E. coli* K-12 was surprising in view of the fact that this activity might be expected to be detrimental to cells growing on hexose. It is likely that this fructose-1,6-diphosphatase, like others, is subject to some kind of metabolic control in the cell. To study this possibility, we are now engaged in purifying the enzyme from *E. coli*.

Acknowledgments

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Literature Cited


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