Glucokinase of *Dictyostelium discoideum**

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ABSTRACT: Glucokinase from the slime mold *Dictyostelium discoideum* was purified 50-fold by DEAE-cellulose chromatography. The enzyme had a pH optimum of about 7.5. Mannose, fructose, and 2-deoxyglucose did not act as substrates or inhibitors of the enzyme. Only adenosine triphosphate served as the phosphate donor. No phosphorylation of glucose was observed with uridine triphosphate, cytidine triphosphate, inosine triphosphate, or guanosine triphosphate. The K_m for glucose was 1.2×10^{-4} M and the K_m for adenosine triphosphate was 1.1×10^{-3} M. Variations in the concentration of either substrate did not affect the K_m of the corresponding substrate. Glucose 6-phosphate was a competitive inhibitor with respect to glucose and a noncompetitive inhibitor with respect to adenosine triphosphate. Adenosine diphosphate gave a mixed inhibition with respect to glucose and a noncompetitive inhibition with respect to adenosine triphosphate.

No significant reduction of enzyme activity was observed with guanosine diphosphate, inosine diphosphate, cytosine diphosphate, uridine diphosphate, adenosine monophosphate, deoxyadenosine monophosphate, 3′,5′-adenosine monophosphate, inorganic phosphate, or pyrophosphate. Inorganic phosphate did not alter the inhibition by glucose 6-phosphate and adenosine diphosphate. The possible significance of the glucokinase reaction in *D. discoideum* metabolism is discussed.

The slime mold *Dictyostelium discoideum* multiplies as a free living amoeba. Upon exhaustion of the food supply the amoebae aggregate and differentiate to form a multicellular fruiting body containing spore cells. During differentiation there is a decrease in soluble glycogen, protein, and RNA, and a rapid synthesis of cell wall materials (cellulose, insoluble glycogen, and mucopolysaccharide) as well as trehalose (for a review, see Wright *et al.*, 1968). The work of Cleland (1969) indicates that during differentiation gluconeogenesis from amino acids is a minor source of glucose. This as well as other data (Baumann and Wright, 1968, 1969; Cleland and Coe, 1968) suggests that the hexose units of soluble glycogen are directly converted into the carbohydrate end products of differentiation. Various lines of evidence suggest that during this transformation, proteins (Wright *et al.*, 1968) as well as lipids (Cleland, 1969) serve as energy sources.

Glycolysis does not appear to be of great importance during the differentiation of *D. discoideum* (Cleland, 1969). A study of the phosphofructokinase from this organism showed that this enzyme is weakly regulated in a manner which is inconsistent with a central role in the control of energy-yielding metabolism (Baumann and Wright, 1968). Cellular levels of fructose phosphates (Cleland, 1969) as well as ATP and ADP (Pannbacker, 1967) suggest that the availability of F-6-P determines the rate of the phosphofructokinase reaction. The level of G-6-P may be controlled by the action of hexokinase on free glucose, which is present in high concentrations during differentiation (Wright *et al.*, 1964), as well as by the action of phosphorylase on glycogen.

The above observations suggest an important role for hexokinase during growth and differentiation. Cleland and Coe (1968) determined the levels of this enzyme in *D. discoideum*. Only a slight difference in activity was shown in extracts obtained from amoebae as compared with the preculmination stage. The substrate specificity of the slime mold enzyme indicates that it should properly be called a glucokinase (ATP:D-glucose 6-phosphotransferase, EC 2.7.1.2).

Materials and Methods

Materials. Glucose, fructose, mannose, 2-deoxyglucose, G-6-P (sodium salt), PEP[1] (sodium salt), nucleotides (acid form), NADP, NADH, dithiothreitol, and the enzymes used in the assays were obtained from Calbiochem. Streptomycin sulfate was obtained from Sigma. All other chemicals were obtained from Fisher Scientific Co.

Assays. All assays were performed within 8 min at 20° using a Beckman DU spectrophotometer with a Gilford recorder. The reaction mixture (1.0 ml) consisted of 13 mM $MgCl_2$, 5 mM $(NH_4)_2SO_4$, 2 mM dithiothreitol, 2 mM glucose, 2 mM ATP, 50 mM imidazole-HCl buffer (pH 7.0), and, depending on the product assayed, 0.5 mM NADP or NADH. Glucokinase was measured by two methods, both dependent upon changes of optical density at 340 nm. In the first, G-6-P formation was coupled to NADP reduction with glucose 6-phosphate dehydrogenase (Slein *et al.*, 1950). In the second, ADP formation was coupled to NADH oxidation with pyruvate kinase and lactic dehydrogenase, the assay being performed in the presence of 0.3 mM PEP (Kornberg and Pricer, 1951). A unit of enzyme was defined as the amount catalyzing the formation of 1 μmole of G-6-P/min, at pH 7.0 and 20°. In experiments designed to measure the stoichiometry of the glucokinase reaction, glucose and

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[1] Abbreviations used are: PEP, phosphoenolpyruvate; NADP, nicotinamide-adenine dinucleotide phosphate; NADH, reduced nicotinamide-adenine dinucleotide.

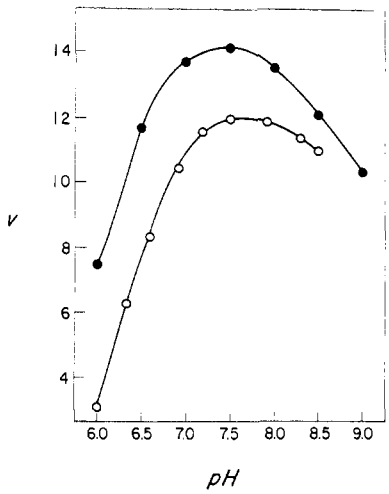

FIGURE 1: Effect of pH on the reaction rate. v = nanomoles per minute. (●) 50 mM imidazole and 50 mM Tris; (○) 50 mM β-glycerophosphate (sodium salt). Both buffers were adjusted to the appropriate pH with HCl. Reaction mixture was the same as described in Methods.

ATP were measured by coupling to NADP reduction with hexokinase and glucose-6-phosphate dehydrogenase. Protein was assayed by the method of Lowry *et al.* (1951).

Enzyme Preparation. D. discoideum was grown as described by Ward and Wright (1965). Unless otherwise stated, cells were harvested at the stage of late aggregation in 0.05 M Tris-HCl buffer (pH 8.0), containing 0.1 mM EDTA. This buffer was used in all the subsequent steps. The harvested cells were washed twice in buffer, collected by centrifugation, and stored at −15°. When stored at this temperature, cell preparations did not lose enzyme activity for at least 3 weeks. The harvesting and all steps in the enzyme purification were performed at 4°. Within 2 weeks of harvesting the cells were suspended in the buffer and centrifuged at 30,000*g* for 30 min. Less than 5% of the hexokinase activity was present in the pellet. Table I summarizes the enzyme purification. The supernatant solution was diluted with buffer until the protein concentration was 40–50 mg/ml (fraction A). Nucleic acids were removed by addition of 0.4 ml of streptomycin sulfate (0.4 g/ml) per 10 ml of crude extract, over a period of 5 min, with stirring. After 30 min of agitation the mixture was centrifuged at 30,000*g* for 30 min, and the supernatant fraction was dialyzed overnight against the described buffer (fraction B). This fraction was applied to a column of Whatman DEAE-cellulose (2.5 × 50 cm) equilibrated in buffer. After washing with 100–150 ml of buffer the column was developed with a linear gradient of NaCl in buffer, the final concentration being 1 M. The total volume of the developing solution was 200 ml. Fractions of 2.0 to 3.0 ml were collected. The enzyme eluted in a single peak at 0.8 M NaCl. The fractions having the highest specific activity were combined and dialyzed overnight against buffer (fraction DEAE), and used in kinetic studies. Routinely an overall purification of 45- to 55-fold was obtained by this procedure. When stored at −15° the enzyme was stable for at least 2 weeks. The enzyme preparation did not exhibit ATPase, myokinase, G-6-P dehydrogenase, or 6-phosphogluconic acid dehydrogenase activity. The concentration of substrates used to test for these enzymes was comparable with those used in the experiments with glucokinase.

TABLE I: Enzyme Purification.[a]

Fraction	Vol (ml)	Protein (mg/ml)	Sp Act.[b]	Recov (%)	Purifcn Factor
A	50	49	0.011	100	1
B	52.5	39	0.013	98	1.2
DEAE	4.2	2.9	0.51	23	46.4

[a] All assays were performed as described in Methods. [b] Units per milligram of protein.

TABLE II: Stoichiometry of the Glucokinase Reaction.[a]

Reaction Component	Init Concn (μmoles/ml)	Final Concn (μmoles/ml)	Net Changes (μmoles)
Glucose	0.98	0.12	−0.86
ATP	1.03	0.21	−0.82
G-6-P	0.00	0.84	+0.84
ADP	0.00	0.79	+0.79

[a] Reaction was terminated after 10 min and the reaction components were assayed as described in Methods.

Results

Stoichiometry of the glucokinase reaction is shown in Table II. The reaction mixture contained 13 mM $MgCl_2$, 5 mM $(NH_4)_2SO_4$, 2 mM dithiothreitol, and 50 mM imidazole-HCl buffer (pH 7.0). At zero time and after a 10-min incubation at 20°, an equal volume of cold 0.7 M $HClO_4$ was added. The mixture was brought to pH 7.0 with 5 N KOH and the $KClO_4$ was removed by centrifugation. To different aliquots of the supernatant solution appropriate purine nucleotides, substrates, and assay enzymes were added in order to measure the four compounds involved in this reaction. A control mixture without enzyme was incubated and assayed as described; no significant change in substrate concentration was detected.

Substrate Specificity. The ability of fructose, mannose, and deoxyglucose to act as substrates for the slime mold glucokinase was tested using the ADP assay. Each sugar was tested at 2, 5, and 10 mM with 2 mM ATP. No reaction was observed, indicating the inability of slime mold glucokinase to phosphorylate these compounds. Addition of 10 mM fructose, mannose, or deoxyglucose to a reaction mixture containing 1 mM glucose and 2 mM ATP did not change the reaction rate when the G-6-P assay was used. Since this assay is specific for G-6-P, these results indicate that fructose, mannose, and deoxyglucose do not inhibit the rate of glucose phosphorylation. UTP, CTP, ITP, and GTP, when tested at 2 and 4 mM in the presence of 2 mM ATP, were unable to phosphorylate glucose. The reaction was completely depen-

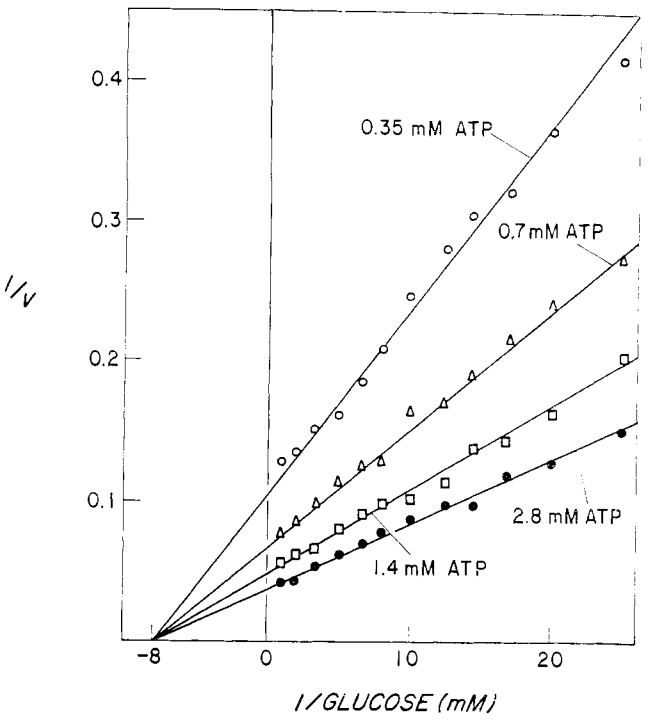

FIGURE 2: Effect of ATP on the kinetics of glucose utilization. v = nanomoles per minute. Other components of the reaction mixture are described in Methods.

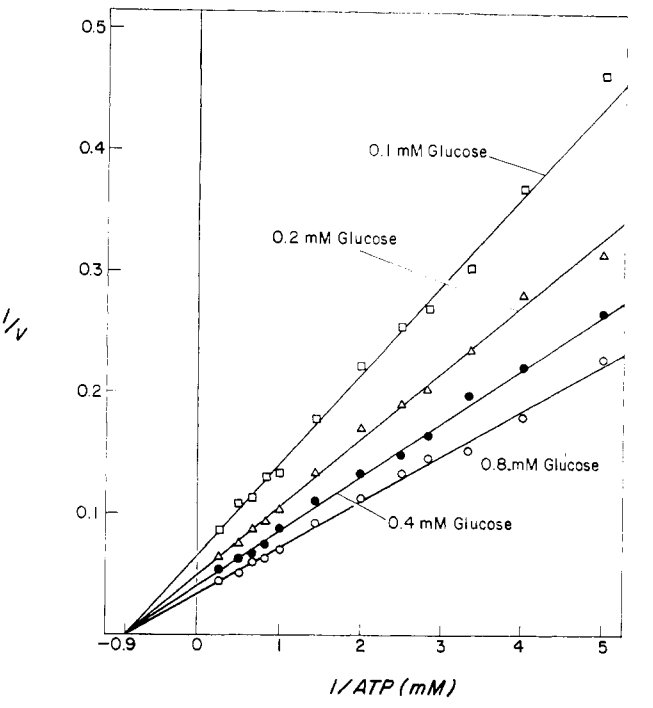

FIGURE 3: Effect of glucose on kinetics of ATP utilization. v = nanomoles per minute. Other components of the reaction mixture are described in Methods.

dent on the presence of Mg^{2+} and the same rate was observed when the concentration of this ion was 5, 10, 15, or 20 mM. The pH optimum of the enzyme was about 7.5 (Figure 1).

Rat liver contains two types of enzyme able to phosphorylate glucose in the C-6 position (González *et al.*, 1964). These enzymes differ in their K_m for this sugar, one type having a low K_m (below 0.1 mM) and the other a high K_m (approximately 20 mM). On DEAE-cellulose these enzymes are readily separable and can be distinguished by assaying the fractions with 2 and 100 mM glucose. To test for the presence of similar types of enzymes in *D. discoideum*, extracts were prepared from cells at the stage of late aggregation and early culmination and chromatographed on DEAE-cellulose. Only one peak of enzyme activity, identical in position and height, was obtained when the fractions were assayed at 2 or 100 mM glucose. These results suggest that *D. discoideum* has only a low K_m glucokinase, which chromatographs as a single peak under the conditions employed.

Kinetics with Respect to Glucose and ATP. Plots of $1/v$ *vs.* 1/[glucose] at different ATP concentrations gave lines convergent on the negative side of the 1/[glucose] axis (Figure 2). These results indicate that the K_m for glucose remains constant while the V_{max} varies with different concentration of ATP. The K_m for glucose was 1.2×10^{-4} M and varied within 10% in subsequent determinations. Similar results were obtained when the K_m for ATP was determined at various concentrations of glucose (Figure 3). The K_m for ATP was 1.1×10^{-3} M and varied within 8% with subsequent determinations.

Inhibition by G-6-P. G-6-P is a competitive inhibitor with respect to glucose (Figure 4), and a noncompetitive inhibitor with respect to ATP (Figure 5). This inhibition is of doubtful physiological significance, since high concentrations of G-6-P are required. In a reaction mixture containing 1 mM glucose and 1 mM ATP, 5 mM G-6-P inhibited activity 13%, while 10 mM G-6-P inhibited 20%.

Inhibition by ADP. ADP gave a mixed inhibition with respect to glucose (Figure 6), and a noncompetitive inhibition with respect to ATP (Figure 7). Although ADP appears to be a better inhibitor than G-6-P, the high concentration required makes this inhibition of doubtful physiological

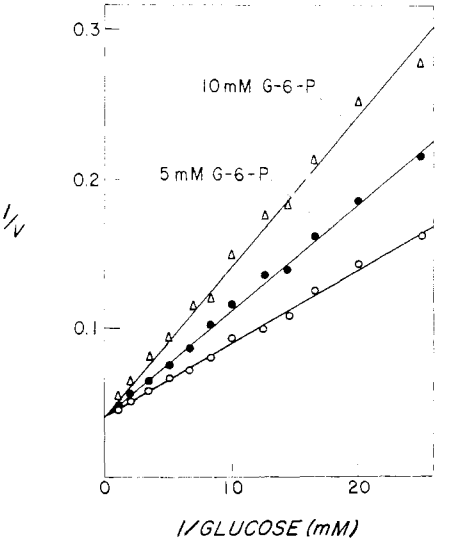

FIGURE 4: Effect of G-6-P on kinetics of glucose utilization. v = nanomoles per minute, (O) no G-6-P added. Assayed by the ADP method in the presence of 1 mM glucose and 2 mM ATP. Other components of the reaction mixture are described in Methods.

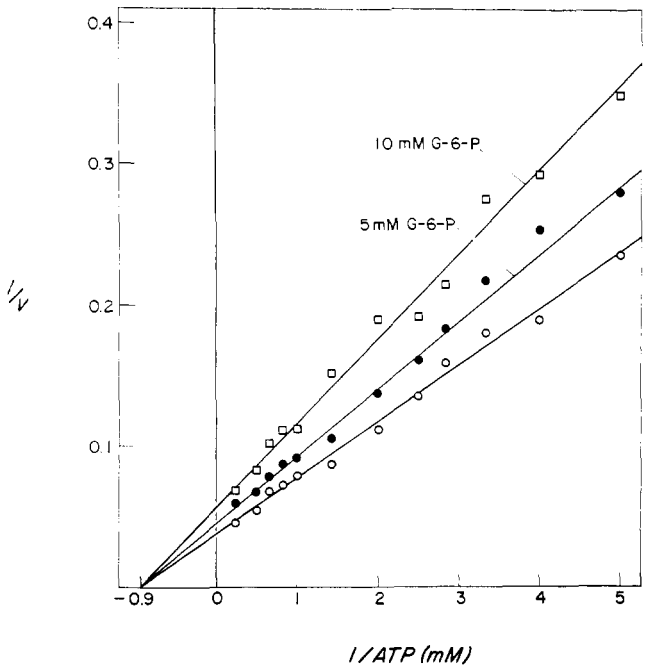

FIGURE 5: Effect of G-6-P on kinetics of ATP utilization. v = nanomoles per minute, (O) no G-6-P added. Assayed by the ADP method in the presence of 1 mM glucose and 1 mM ATP. Other components of the reaction mixture are described in Methods.

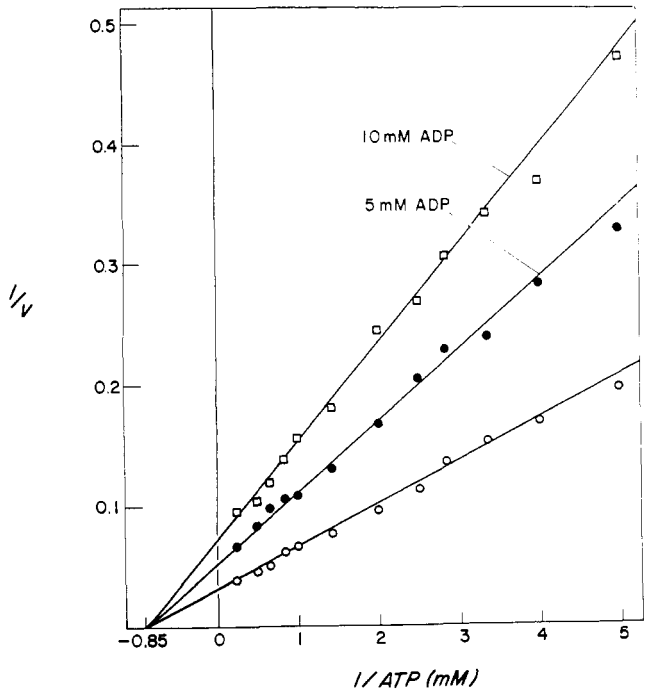

FIGURE 7: Effect of ADP on kinetics of ATP utilization. v = nanomoles per minute, (O) no ADP added. Assayed in the presence of 1 mM glucose and 1 mM ATP. Other components of the reaction mixture are described in Methods.

relevance. In a reaction mixture of 1 mM glucose and 1 mM ATP, 5 mM ADP inhibited activity 35%, while 10 mM ADP inhibited 53%.

Effect of Other Compounds on Enzyme Activity. When tested in a reaction mixture containing 1 mM glucose and 1 mM ATP, the following compounds at 2, 5, and 10 mM gave less than 10% inhibition: AMP, d-AMP, 3′,5′-AMP, GDP, IDP, CDP, UDP, P_i, and PP_i. PEP tested at 2 and 5 mM gave less than 3% inhibition. P_i at 2 and 5 mM did not significantly alter the inhibition of hexokinase activity by G-6-P and ADP, when the latter compounds were present at 2, 5, or 10 mM. Glucose tested at 5, 10, and 15 mM did not inhibit the reaction. ATP at the same concentrations was not inhibitory.

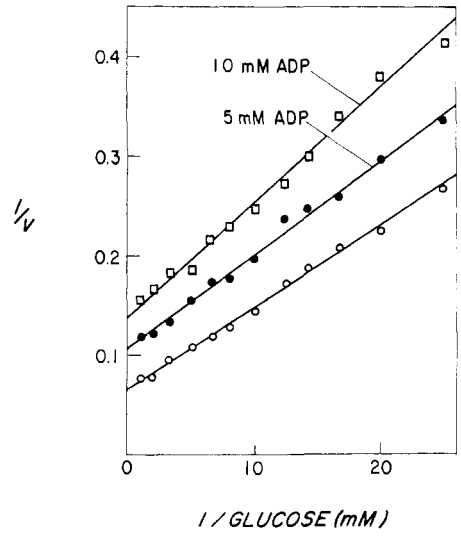

FIGURE 6: Effect of ADP on kinetics of glucose utilization. v = nanomoles per minute, (O) no ADP added. Assayed in the presence of 1 mM glucose and 2 mM ATP. Other components of the reaction mixture are described in Methods.

Discussion

The glucokinase of *D. discoideum* is unable to catalyze the phosphorylation of mannose, fructose, or 2-deoxyglucose, nor is the phosphorylation of glucose inhibited by these sugars. In these properties the enzyme resembles the glucokinase from *Aerobacter aerogenes* (Kamel *et al.*, 1966) and differs from the multiple substrate hexokinases of yeast and mammals (Crane, 1962). The slime mold appears to have only one enzyme able to phosphorylate glucose at the terminal (C-6) carbon. This differs from liver which has two types of hexokinases, one having a low and the other a high K_m for glucose (Gonzáles *et al.*, 1964; Salas *et al.*, 1965). The enzyme kinetics observed upon variation of substrates and end products were similar to those obtained for yeast hexokinase by Noat *et al.* (1968). The high levels of G-6-P and ADP necessary for inhibition of the reaction rate, compared with the levels found *in vivo* (Wright, 1965; Pannbacker, 1967), make it doubtful that these inhibitions are of physiological significance. Unlike the mammalian enzyme (Crane and Sols, 1953), and like the yeast hexokinase (Racker, 1965), the slime mold glucokinase was not inhibited by physiological concentrations of G-6-P. Determinations of the mass action ratio in the slime mold for the glucokinase reaction (Cleland, 1969) indicates that this reaction is not at equilibrium, suggesting a regulatory

role for this enzyme. A comparison of the kinetic parameters of this enzyme with cellular levels of ADP, ATP (Pannbacker, 1967), G-6-P (Wright, 1965), and glucose (Wright *et al.*, 1964) suggests that during differentiation the rate of the glucokinase reaction is controlled by the availability of ATP. The linking of glucose phosphorylation to ATP availability is consistent with a biosynthetic role for the slime mold glucokinase.

During differentiation free glucose is found in *D. discoideum* (Wright *et al.*, 1964). This organism contains a nonspecific acid phosphatase which hydrolyzes G-1-P and G-6-P (Gezelius, 1966). However, the low activity against these compounds at physiological pH as well as the probable localization of acid phosphatases in lysosomes suggests that the activity of this enzyme is not responsible for the free glucose. Gezelius (1968) has shown that *D. discoideum* does not contain a specific G-6-P phosphatase of the type found in liver; this enzyme is able to hydrolyze G-6-P as well as phosphorylate glucose using PP_i as the phosphate donor (Stetten, 1965). The source of free glucose may, therefore, involve the combined action of an amylase (Rosness, 1967) and a α-glucosidase, as well as the action of trehalase (Ceccarini, 1967). The resulting glucose could then enter central metabolism *via* hexokinase. Compared with the action of phosphorylase, such a pathway is wasteful. Nevertheless, it may be useful in providing a rapid source of hexose phosphates under conditions in which phosphorylase does not function at its maximal capacity. Such a situation may exist in early differentiation where the level of phosphate is relatively low (Gezelius and Wright, 1965).

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