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PARTIAL PURIFICATION AND PROPERTIES OF A SPECIFIC GLUCOKINASE FROM *STREPTOCOCCUS MUTANS* SL-1 *

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Summary

The presence of glucokinase (ATP:D-glucose 6-phosphotransferase, EC 2.7.1.2) activity in seven strains of oral streptococci is demonstrated. The glucokinase purified from *Streptococcus mutans* SL-1 cells is shown to be a highly specific enzyme, phosphorylating only glucose (eight sugars tested). The enzyme is a true glucokinase: formation of the product, shown here to be glucose 6-phosphate, is dependent on the presence of glucose, ATP, divalent metal ion and enzyme. The K_m for glucose is 1.40 mM, the pH optimum for the enzyme is a broad plateau from pH 7.1 to 9.5 and the molecular weight is estimated to be 40 000. The finding of a glucokinase in oral streptococci indicates the existence of an intracellular mechanism of glucose phosphorylation. The implications of this observation are discussed.

Introduction

Most anaerobic and facultative anaerobic bacteria that are capable of glycolysis transport hexoses via a phospho*enol*pyruvate-dependent phosphotransferase system [1]. With few exceptions, strictly aerobic bacteria use an active transport system and subsequently utilize intracellular kinases for phosphorylation. Among the anaerobes, specific phospho*enol*pyruvate-dependent phosphotransferase systems exist for each sugar transported, and transport occurs with concomitant phosphorylation [2]. These phospho*enol*pyruvate-dependent phosphotransferase systems use a common cytoplasmic mechanism to generate

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Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

a high-energy phosphoprotein whose donation of its phosphate group to the incoming hexose is mediated by sugar-specific membrane components [2]. Metabolism of carbohydrates by *Streptococcus mutans* is of interest because the organism has been implicated in the etiology of dental caries [3]. *S. mutans* possesses phospho*enol*pyruvate-dependent phosphotransferase systems for glucose and sucrose [4—6]. The product of glucose transport is glucose 6-phosphate while the product of sucrose transport is sucrose 6-phosphate which is thought to be hydrolyzed to glucose 6-phosphate and fructose [5,6].

Both a sucrose permease [7] and an intracellular invertase [8] in *S. mutans* have been demonstrated. The action of intracellular invertase on sucrose in *S. mutans* [8] would lead to accumulation of glucose and fructose. Hamilton and Ellwood [9] have demonstrated that at high substrate concentration and low pH, the activity of the glucose phospho*enol*pyruvate-dependent phosphotransferase system of *S. mutans* is markedly reduced. In the absence of a phospho*enol*pyruvate-dependent phosphotransferase activity for glucose or sucrose, an ATP-dependent kinase would be required for the formation of intracellular glucose 6-phosphate. In addition, hydrolysis of maltose phosphate or lactose phosphate transported via phospho*enol*pyruvate-dependent phosphotransferase systems could lead to the accumulation of intracellular glucose [10].

Preliminary work in this laboratory indicated the presence of a glucokinase (ATP:D-glucose 6-phosphotransferase, EC 2.7.1.2) activity in six strains of oral microorganisms [11]. Because transport of certain disaccharides results in accumulation of intracellular glucose, and since Hamilton's work indicated a requirement for an alternate mechanism to produce glucose 6-phosphate, we undertook a study of glucokinase in *S. mutans*. This paper reports the purification, characterization and properties of a highly specific glucokinase from *S. mutans* SL-1 cells.

Materials and Methods

Maintenance and growth of bacterial strains. Stock cultures of *S. mutans* E49, BHT, OMZ70, SL-1 (ATCC 27607), LM7, *S. sanguis* ATCC 10558 and *S. salivarius* ATCC 25975 were maintained in a modified fluid thioglycollate medium [12]. Fermentations were conducted in Jordan's medium [12] or in modified Jordan's medium supplemented with 5 g/l $K_2HPO_4 \cdot 3\ H_2O$.

Reagents. Fisher (Silver Spring, MD) certified and Sigma (Saint Louis, MO) reagents and enzymes were used throughout. Yeast extract and trypticase were obtained from BBL (Cockysville, MD). All isotopically labelled sugars were purchased from New England Nuclear (Boston, MA). DEAE-cellulose and Ultrogel AcA 54 were purchased from Whatman (Clifton, NJ) and LKB (Rockville, MD), respectively.

Survey of strains for the presence of hexokinase activity. Streptococci were adapted to Jordan's medium by growth overnight at 37°C in Jordan's medium containing 5 mM glucose. 500-ml flasks of modified Jordan's medium at pH 7.2 containing 30 mM sucrose or 60 mM glucose were inoculated with a 1% (v/v) inoculum. At the cessation of growth, cells were collected by centrifugation, washed, and suspended in 10 ml cold 0.1 M potassium phosphate buffer, pH 6.5 (buffer 1). The bacterial suspension was subjected to ultrasonic disrup-

tion at 4°C for two 1.5-min intervals. The crude extract was cleared by centrifugation at 48 000 × *g* for 30 min and subsequently dialyzed overnight in 5 mM buffer 1. The dialysate was centrifuged at 105 000 × *g* for 45 min and the supernatant fluid was concentrated 10-fold in a Minicon B15 (Amicon) ultrafiltration cell. These preparations were assayed for enzyme activity.

Determination of glucokinase activity. Glucokinase activity was determined by a spectrophotometric assay using glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:$NADP^+$ 1-oxidoreductase, EC 1.1.1.49) [13]. The amount of product (glucose 6-phosphate) formed is directly proportional to the amount of reduced NADP formed as measured by the change in absorbance at 340 nm. The assay is linear with time for at least 30 min and proportional to the amount of enzyme present in the range of 0.001—0.1 IU/assay. Production of NADPH is stoichiometric with production of glucose 6-phosphate. The standard assay mixture contained 10 mM ATP, 10 mM $MgCl_2$, 10 mM NADP, glucose (usually 50 mM), 200 mM potassium phosphate buffer, pH 7.5, 1—3 IU glucose-6-phosphate dehydrogenase, and enzyme preparation in a final volume of 0.5 ml. Enzyme activity is expressed as IU (μmol of product formed/min at 22°C); specific activity is expressed as IU/mg protein. Protein was determined by the method of Lowry et al. [14].

Purification of glucokinase. S. mutans SL-1 was grown in Jordan's medium containing 60 mM glucose in a 6 l fermentation vessel maintained at pH 6.5 by the addition of 2 N KOH. At the end of growth, as measured by cessation of acid production, cells were collected by centrifugation at 16 300 × *g* for 30 min and washed three times with 200 ml cold 0.1 M buffer 1. The wet weight of the cells was determined, and cold 0.1 M buffer 1 was added to achieve 150 ml of a 20% (w/v) cell slurry. The slurry was subjected to sonic disruption at 4°C for two 3-min intervals. The slurry of broken cells was centrifuged at 48 000 × *g* for 30 min and the supernatant fluid dialyzed overnight in 5 mM buffer 1. The dialysate was centrifuged at 105 000 × *g* for 45 min to obtain the crude enzyme preparation, which was concentrated 10-fold in an Amicon cell fitted with a PM 10 membrane. The concentrated preparation was applied to a 2.5 × 40 cm DEAE-cellulose column with a flow rate of 1.2 ml/min. A 16 h linear gradient was used with 0.1 M buffer 1 as the initial buffer and 0.1 M buffer 1 containing 0.35 M KCl as the final buffer. Fractions containing glucokinase activity were pooled, concentrated, and applied to an Ultrogel AcA 54 column equilibrated with 0.1 M buffer 1 at a flow rate of 30 ml/h. Standards applied to the Ultrogel column for molecular weight calibration were blue dextran (M_r = 1 500 000), bovine serum albumin (M_r = 68 000), ovalbumin (M_r = 45 000), chymotrypsinogen (M_r = 25 000) and ribonuclease (M_r = 13 900). A curve relating log M_r to elution volume was used to estimate molecular weight as described by Andrews [15].

Characterization of reaction products. Purified preparations of glucokinase were incubated with isotopically labelled sugars in a reaction mixture containing 100 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) buffer, pH 7.5, 10 mM ATP, 10 mM $MgCl_2$, 10 mM sugar, an amount of enzyme calculated to yield 50% completion of the reaction in 30 min, and the following ^{14}C- (1 μCi/ mM) or ^3H-labelled (4.5 μCi/ mM) sugars in a volume of 1 ml: D-[1-^{14}C]glucose, D-[U-^{14}C]fructose, D-[U-^{14}C]sorbitol, D-[1-^{14}C]ribose,

D-[1-^{14}C]mannitol, D-[G-^{3}H]galactose, D-[1-^{14}C]mannose and L-[1-^{14}C]-arabinose. After 30 min incubation at 37°C, reactions were stopped by boiling for 5 min and 50 µl of the reaction mixture were spotted on paper strips (3 × 60 cm, Whatman 3 MM) together with appropriate standards. The chromatograms were developed overnight in butanol/acetic acid/water (5 : 2 : 3). Radioactivity profiles, determined with a Packard radiochromatogram scanner, were correlated with standards following visualization of the strips with a phosphate-detecting reagent [16]. Concurrently run chromatograms spotted with appropriate standards were sprayed with 1% aniline phthalate in ethanol to detect aldoses and ketoses.

Disc gel electrophoresis. Electrophoresis of samples in 7.5% polyacrylamide using a pH 9.3 Tris/glycine buffer system was carried out according to the method of Ornstein [17] and Davis [18]. Protein was detected in the gels using the Coomassie blue G-250 (Gallard Schlesinger) staining method of Diezel et al. [19]. Glucokinase activity in the gels was localized using an activity stain based on the precipitation of nitroblue tetrazolium in its reduced form [20]. The activity stain contained 50 mM Hepes buffer at pH 7.5, 10 mM ATP, 10 mM $MgCl_2$, 1 mg/ml NADP, 50 mM glucose, 3 IU glucose-6-phosphate dehydrogenase, 0.002% phenazine methosulfate and 0.2 mg/ml nitroblue tetrazolium. Activity staining solutions were prepared just prior to use. After electrophoresis, gels were rinsed in 0.1 M Hepes buffer, pH 7.5, immersed in the activity staining solution, and allowed to develop in the dark at ambient temperature. To avoid nonspecific binding of the formazan, gels were removed from the staining mixture as soon as activity bands (purple color) were seen. The gels were stored in 5% acetic acid.

Results

Identification of glucokinase activity in strains of oral streptococci

Dialyzed crude extracts of various oral streptococci were assayed for glucokinase activity. In extracts from glucose-cultured cells, the activities observed ranged from a barely detectable 0.011 IU/mg protein in *S. mutans* E49 (see Table I) to 12.4 IU/mg protein in *S. sanguis* 10558. When strains were cultured

TABLE I

SPECIFIC ACTIVITY OF GLUCOKINASE IN VARIOUS STRAINS OF ORAL STREPTOCOCCI

Specific activity is expressed as I.U./mg protein. For details of the spectrophotometric assay see Materials and Methods. Glucose cells were harvested from cultures grown in medium containing 60 mM glucose. Sucrose cells were harvested from cultures grown in medium containing 30 mM sucrose.

Strain (serotype)	Specific activity	
	Glucose	Sucrose
S. mutans E49 (a)	0.011	0.133
S. mutans BHT (b)	0.714	1.21
S. mutans OMZ70 (c)	2.16	1.37
S. mutans SL-1 (d)	0.637	1.61
S. mutans LM7 (E)	3.34	1.49
S. sanguis 10558	12.4	6.58
S. salivarius 25975	0.032	0.083

with sucrose as the growth carbohydrate, differences in glucokinase content were observed (Table I), but no clear-cut pattern emerged. For example, when cultured on sucrose, *S. mutans* E49 had 10-fold higher levels of glucokinase, although it was still the strain with the lowest activity overall. Under these same conditions of sucrose growth, *S. mutans* BHT and SL-1 as well as *S. salvarius* glucokinase activities were 2—3-fold higher, while the activities observed in *S. mutans* OMZ70, LM7 and *S. sanguis* were approximately half those observed after growth on glucose. The reaction was completely dependent on ATP, $MgCl_2$, glucose, glucose-6-phosphate dehydrogenase and NADP. However, with fructose and glucosephosphate isomerase (EC 5.3.1.9) added instead of glucose, the rate was 11% of that observed with glucose. Addition of mannose and mannosephosphate isomerase (EC 5.3.1.8) produced similar results. Other sugars, as will be shown below, were without activity.

Purification of glucokinase from S. mutans SL-1

Glucose-grown *S. mutans* SL-1 was chosen for isolation of glucokinase since enzyme levels observed under controlled fermentation conditions were among the highest found at the cessation of growth. Glucokinase from the concentrated, dialyzed extract of cells harvested from 6 l of 60 mM glucose-cultured *S. mutans* SL-1 was purified by adsorption to a DEAE-cellulose ion-exchange column and elution with a linear increasing gradient of KCl. By pooling fractions 56—65 (see Fig. 1), 30.5% of the applied activity was recovered in the form of a 122-fold purified enzyme. This preparation no longer phosphorylated fructose or mannose as described above; analysis of other fractions indicated resolution of glucokinase activity (fractions 56—65) from fructokinase activity (fractions 67—75, Fig. 1) had been effected. Intracellular invertase was localized in fractions 47—55. The pooled fractions containing glucokinase were concentrated to 6 ml and subjected to gel filtration on Ultrogel AcA 54. This step resulted in an essentially complete recovery of a 352-fold purified enzyme (29.3% overall yield). The molecular weight estimated from the gel filtration was 40 000. A summary of the results of the purification is presented in Table II. The purity of the glucokinase preparation as judged from a densito-

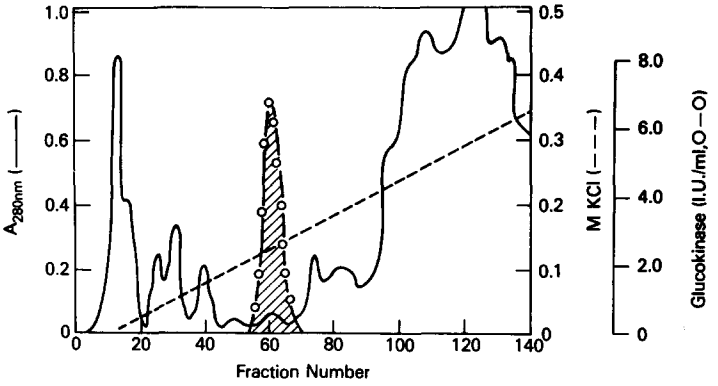

Fig. 1. DE52 chromatography of the crude extract of *S. mutans* SL-1 cells. Protein is indicated by absorbance at 280 nm. - - - - - -, KCl gradient.

TABLE II

PURIFICATION OF GLUCOKINASE FROM GLUCOSE-GROWN *S. MUTANS* SL-1 CELLS

Preparation	Volume (ml)	I.U./ml	Total I.U.	mg protein/ ml	I.U./mg protein	Yield (%)	Purification (-fold)
Crude extract	132	1.32	1740	16.1	0.82	100	1.00
DE52	6	88.7	532	0.889	99.8	30.5	122
Ultrogel AcA 54	1	510	510	1.77	288	29.3	352

metric scan of a Coomassie blue-stained disc gel (Fig. 2), was at least 80%. Activity staining corresponded with the two major protein bands seen in Fig. 2. Attempts at further purification by preparative electrophoresis, isoelectric focusing or hydroxyapatite chromatography lead to complete loss of activity.

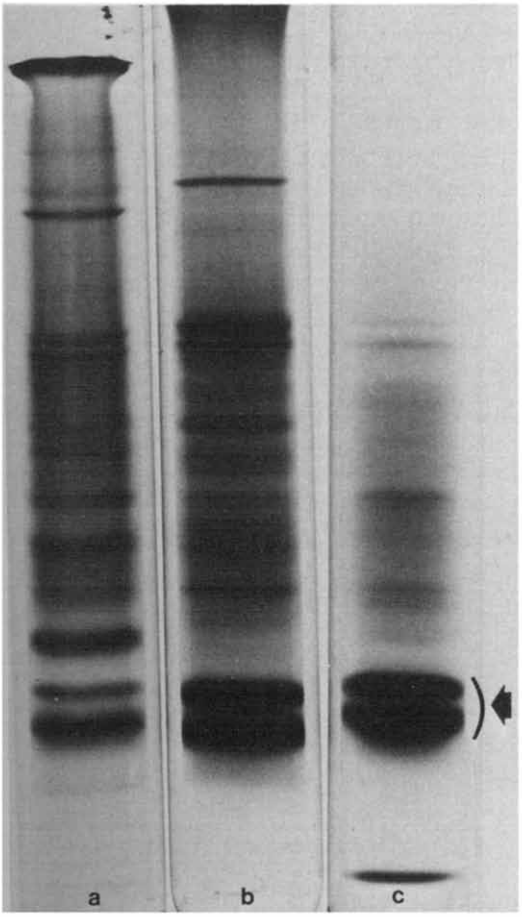

Fig. 2. Disc gel electrophoresis of fractions from purification of glucokinase from *S. mutans* SL-1 cells. (a) Crude extract; (b) pooled glucokinase fractions after chromatography on DEAE-cellulose, and (c) pooled glucokinase fractions after chromatography on Ultrogel AcA 54. The protein bands (gels stained for protein with Coomassie blue) corresponding to activity stain are indicated by the arrow. Electrophoresis was conducted on 7.5% gels in a pH 9.3 buffer system.

The partially purified glucokinase was rather unstable, because it lost all of its activity in a few weeks at −20°C. A number of stabilizing agents such as Mg^{2+}, glucose, dithiothreitol and glycerol or combinations of these agents, failed to stabilize the preparation.

Proof of product structure and enzyme specificity

Purified glucokinase was incubated with ATP and [^{14}C]glucose in the presence of 0.1 M Hepes (pH 7.5) and 10 mM $MgCl_2$. An aliquot was subjected to paper chromatography. The radioactive areas corresponded with glucose and glucose 6-phosphate standards. The mobility of glucose 6-phosphate (R_g = distance migrated/distance migrated by glucose = 0.61) was distinct from that observed for mannose 6-phosphate (R_g = 0.34), glucose 1-phosphate (R_g = 0.69), or fructose 6-phosphate (R_g = 0.65). The radioactive product reacted typically with both the aldohexose and the phosphate spray. These results, together with the known specificity of the glucose-6-phosphate dehydrogenase used in the spectrophotometric assay, prove that the product of the glucokinase reaction was glucose 6-phosphate.

A similar analysis with labelled galactose, ribose, mannose, sorbose, arabinose, fructose, sorbitol and mannitol as substrates failed to reveal any phosphorylation by the purified glucokinase. Inclusion of 10-fold molar excess of these same carbohydrates into the spectrophotometric assay failed to reveal any competitive inhibition (Table III). Both the failure of the enzyme to phosphorylate sugars other than glucose, and the absence of competitive inhibition of glucose phosphorylation by these sugars indicated that the *S. mutans* SL-1 glucokinase was highly specific for glucose. A series of glucose analogs were tested as potential competitive inhibitors of glucose phosphorylation using the standard spectrophotometric assay. The compounds tested (α-methylglucoside, 2-deoxyglucose, 3-*O*-methylglucose, 6-deoxyglucose, *N*-acetyl-D-glucosamine and glucosamine hydrochloride) had no affect on the rate of glucose phosphorylation when added to the standard assay mixture at a concentration equimolar with glucose. This finding, although not conclusive, suggests that these compounds are also not substrates for glucokinase.

TABLE III

EFFECT OF VARIOUS CARBOHYDRATES ON GLUCOKINASE ACTIVITY

Enzyme activity was assayed as outlined in Materials and Methods with 10 mM glucose in the assay mixture. The concentration of added compounds was 100 mM.

Compound added	Relative rate
None	100
Fructose	102
Rhamnose	108
Galactose	117
Ribose	108
Mannose	108
Xylose	95
Sorbose	94
Arabinose	96
Sorbitol	96
Mannitol	106

TABLE IV

EFFECT OF ADDITION OF VARIOUS KEY METABOLITES ON THE ACTIVITY OF THE GLUCOKINASE FROM *S. MUTANS* SL-1 CELLS

Enzyme activity was assayed as outlined in Materials and Methods with 20 mM additions as noted except in the case of ADP which was tested also at 2 mM.

Compound added	Relative rate
None	100
3-Phosphoglycerate	100
2-Phosphoglycerate	109
Pyruvate	100
Acetyl coenzyme A	99.6
L-Lactate	101
Phospho*enol*pyruvate	106
Fructose 1,6-bisphosphate	130
Cyclic 3′,5′-adenosine monophosphate	84.7
Adenosine 5′-monophosphate	48.2
Adenosine 5′-diphosphate	18.7
2 mM adenosine 5′-diphosphate	67.3

Characterization of glucokinase

It was determined from a Lineweaver-Burk plot of reaction velocity versus varying glucose concentration that the enzyme had a K_m for glucose of 1.40 mM and V of 132 IU/mg protein. The dependence of the reaction on added metal ion was complete; the reaction rate with no metal ion was zero. Equimolar amounts of Mn^{2+} gave 40% of the rate observed with Mg^{2+} and Ca^{2+} stimulated the reaction only 20% relative to Mg^{2+}. Other nucleoside triphosphates were unable to substitute for ATP. The pH optimum was somewhat unusual, in that activity dropped quickly at acid values; essentially no activity was observed at pH 5.5, but nearly equal activity was observed from pH 7.1 to 9.5. A number of glycolytic intermediates were added to assay mixtures to evaluate the possibility of regulatory control of glucokinase (Table IV). Although fructose 1,6-diphosphate gave a 30% stimulation, the other intermediates were virtually without effect. In contrast, the addition of ADP and AMP caused inhibition. The kinetics of these phenomena were not further investigated.

Discussion

The data presented here show the presence of glucokinase activity in extracts of oral streptococci. The K_m for glucose is low (1.40 mM), the reaction requires divalent metal ion and ATP, and the enzyme has a low molecular weight (40 000). Gel electrophoresis of purified preparations indicated the presence of two bands of enzyme activity. This could be the first evidence for the presence of isozymes or dissimilar active subunits of glucokinase in a bacterial species. Alternatively, these bands could represent the product of a single gene modified after protein synthesis either intracellularly or during purification. Genetic analysis would be required to resolve this point. The product has been characterized both enzymatically and chromatographically as glucose 6-phosphate. While a rapid and simple two-step purification procedure resulted in prepara-

tions that were 80% pure, such preparations were unstable and lost activity rapidly. With the exception of the observation of two forms of the enzyme, other properties such as K_m, and V, metal ion specificity and pH optimum were not markedly different from previously studied bacterial glucokinases [21–23].

S. mutans SL-1 possesses a glucose phospho*enol*pyruvate-dependent phosphotransferase system that would be expected to produce intracellular glucose 6-phosphate [6]. However, Hamilton and Ellwood have demonstrated that at low pH, glucose phospho*enol*pyruvate-dependent phosphotransferase activity decreases markedly [9]. The loss of glucose phospho*enol*pyruvate-dependent phosphotransferase activity would require the existence of an alternate pathway for glucose phosphorylation since cells are able to grow in media at reduced pH. Although the extracellular pH is reduced, the cells would be expected to maintain their intracellular pH near neutral thereby favoring glucokinase activity. The results reported here show that the quantity of glucokinase activity in *S. mutans* SL-1 is sufficient to convert all of the glucose in the fermentation medium to glucose 6-phosphate within the time taken to complete a given fermentation. All of the strains studied, with the exception of *S. mutans* E49 and *S. salivarius* 25975, have this capacity. These factors make glucokinase a potential alternative to the phospho*enol*pyruvate-dependent phosphotransferase system for the production of glucose 6-phosphate. Further work will be necessary to reveal what factors control which of these two alternative pathways of glucose activation is utilized. A clear-cut role for glucokinase would be in the fermentation of disaccharides liberating free intracellular glucose such as occurs in the hydrolysis of lactose phosphate [10], maltose phosphate and sucrose.

In conclusion, a consideration of the known pathways of carbohydrate dissimilation in oral streptococci indicated the need for a mechanism for the intracellular phosphorylation of glucose. The results shown here demonstrate that a number of oral streptococci have enough glucokinase to satisfy this requirement. With the exception of the apparent presence of two forms of the enzyme, the purified preparation is characteristic of bacterial glucokinases. An assessment of what portion of glucose carbon is metabolized by glucokinase under a given set of conditions awaits detailed genetic and physiological analysis.

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