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INHIBITION OF GERMINATION BY GLUCOSE ANALOGUES THAT ARE HEXOKINASE SUBSTRATES

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Key Word Index—*Vigna radiata* mung beans; *Trigonella foenum-graecum* fenugreek; *Sinapis alba* mustard; *Triticum aestivum* wheat; hexokinase; fructokinase; germination; D-mannose; 2-deoxy-D-glucose.

Abstract—K_{is} values for the 6-phosphates of D-mannose and 2-deoxy-D-glucose with a plant hexokinase against ATP were high (52 and 62 mM) and there was negligible inhibition against D-glucose. The degree of reduction of germination and seedling growth of fenugreek, mung beans, white mustard and wheat seeds was related to the phosphorylation coefficient and concentration of the added analogue, and varied with plant species. The significance of these results in inhibition is discussed. 2,5-Anhydro-D-mannitol and D-psicose were exceptions. The bisphosphate of the former potently inhibits fructose bisphosphatase and the latter is a substrate for fructo-6-kinase, consistent with a role for fructo-6-kinase in plants independent of hexokinase. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

A number of D-glucose analogues, such as D-mannose, 2-deoxy-D-glucose (2d-Glc) and D-glucosamine, that are also substrates for hexokinase [EC 2.7.1.1] inhibit the growth of plants [1] as well as microorganisms, insects and animals, unless enzymes that can further metabolize the phosphates formed are available—as with Man in some legumes and yeast, where there is sufficient phosphomanno-isomerase (PMI) to convert Man-6-P to Fru-6-P. In plants and algae unable to metabolize the analogue phosphates, various processes, such as organ growth, ion uptake and transport, starch accumulation, photosynthesis, fruit ripening, germination, respiration and translocation, are affected [1–5].

The phosphorylation and transport of hexose are closely linked and the relative $V_{max}:K_m$ ratios for Glc, Man, 2d-Glc and D-glucosamine are generally of a similar order of magnitude [6–19]. In plants [10–24] phosphorylation coefficients [$V_{max}:K_m$ (analogue)$\div V_{max}:K_m$ (Glc)] for Man and 2d-Glc have varied from 0.4 to 0.8, allowing these to react significantly in the presence of Glc. They act at the same site, both as substrates and competitive inhibitors of Glc [8, 9, 19, 25]. K_i (Man) values (versus Glc) for brain and honey locust (*Gleditsia triacanthos*) hexokinase of 0.038 mM [8] and 0.031 mM [19] have been found. Analogues with less structural resemblance to Glc like 1,5-anhydro-D-glucitol (1-deoxy-D-glucopyranose), 1,5-anhydro-D-mannitol and D-allose are phosphorylated with lower phosphorylation coefficients [6]. Whereas the product Glc-6-P competitively inhibits mammalian hexokinase (vs ATP) at very low concentrations (K_i 0.04 mM) [7, 8] in yeasts mixed inhibition occurs and only at much higher levels of Glc-6P (K_i 20–30 mM) [25]. In plants Glc-6-P non-competitively inhibited wheat germ hexokinase (to Mg-ATP) (K_i 16.2 mM) [11] and with a hexokinase from potato tubers [18] it inhibited non-competitively against Glc (K_i 4.1 mM). The cellular concentration of Glc-6-P indicated a possible moderate physiological effect. Analogue phosphates also inhibit [6, 26]. Hexokinase from beef heart [27] with 2d-Glc as substrate was inhibited competitively by 2d-Glc-6-P against ATP and non-competitively to 2d-Glc with a K_i of 1.4 mM. With yeast hexokinase [27] and Glc as substrate, Man-6-P showed mixed inhibition vs ATP, with a K_{is} value of about 2–3 mM. When Man- or 2d-Glc are deleterious in yeast or mammals, their 6-phosphates accumulate [3, 26, 28–32]. If plant hexokinases had similar inhibition constants for accumulated analogue phosphates to those of mammals or yeast they could cause effective inhibition.

Large decreases in concentrations of some metabolites (ATP, P_n, Glc-6-P, and PEP) result from analogue feeding; after minutes for some tissues (e.g.

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ascites tumour cells and maize scutellum slices) to hours for others [1–5, 28–34]. The fractional reductions in [ADP] and [P_i] are less during the same periods.

In plant hexokinases [11–14, 18, 23, 24], as found for mammalian [7, 8] and yeast enzymes [27], Mg-ADP inhibits. In a wheat enzyme [11] a K_i value of 1 mM, with non-competitive inhibition against Glc was determined. Mg-ADP was a competitive inhibitor with respect to Mg-ATP in enzymes from potato tubers [18] and *Dendrophthoe falcata* leaves [12] (K_i values 0.04–1.2 mM); in potato it was considered that, whereas it would be unlikely to be an effective inhibitor under aerobic conditions when high ATP : ADP ratios are expected, if this ratio fell (under anaerobic conditions) inhibition would become significant.

Further possible effects of analogues have been suggested. 2d-Glc-6-P can be converted to UDP 2d-Glc which may modify polysaccharide synthesis. The analogue 6-phosphates could inhibit the four enzymes that use Glc-6-P as substrate; PGI in glycolysis, PGM in nucleotide sugar synthesis, Glc-6-PDH in the pentose-P pathway and *myo*-inositol 1-P synthase in inositol phosphate formation. Man-6-P has been reported to inhibit PGI, and 2d-Glc both Glc-6-P DH and PGI. Catabolite repression has also been described for plant cells [35].

Another aspect of hexose-6-kinase activity in plants is the occurrence of specific (soluble) D-fructo 6-kinases [EC 2.7.1.4] e.g. in peas [15], potato tubers [36] and honey locust cotyledons [19]. Whether sucrose is depolymerised via sucrose synthase or invertase, the fructosyl fragment requires phosphorylation prior to metabolism. Fru is a poor substrate for hexokinase.

The extent of inhibition of a plant hexokinase with two analogue 6-phosphates and the effects of a number of analogue substrates of hexokinase on four species of plant seeds are now reported.

RESULTS AND DISCUSSION

Inhibition of plant hexokinase by analogue phosphates

Inhibition by analogue phosphates of plant hexokinase was studied with a solubilized particulate enzyme from honey locust cotyledons [19]. Phosphate transfer from ATP and phosphorylation of Glc were measured in the presence of either Man-6-P or 2d-Glc-6-P from 0–25 mM (Figs 1 and 2). K_{is} Values with regard to ATP of 52 mM for Man-6-P and 62 mM for 2d-Glc-6-P were estimated for both compounds. Linear plots of slope vs concentration of inhibitor gave the equations:

$$\text{slope} = 0.105\ [\text{mM Man-6-P}] + 5.45;\ r^2 = 0.88 \text{ and}$$
$$\text{slope} = 0.082\ [\text{mM 2d-Glc-6-P}] + 5.11;\ r^2 = 0.48$$

Negligible inhibition was found against Glc. These K_{is} values are much higher than K_m (Glc) and the K_i (analogue) values for mammals and microorganisms.

Comparative effects of different analogues on seed germination and early seedling growth

The four species of seeds differ in their carbohydrate reserves and capacity to metabolize Man-6-P. Fenugreek has galactomannan in an endosperm and contains PMI. Mung beans contain starch in their cotyledons and also have PMI. Mustard has a tri-acyl glycerol reserve and no PMI, and wheat no PMI and a starchy endosperm. Mung beans deplete starch by phosphorolysis to Glc-1-P. Fenugreek depolymerises galactomannan hydrolytically to D-Gal and Man, which are then taken up by the embryo. In mustard, gluconeogenesis provides carbohydrate from hydrolysed tri-acyl glycerol, and, in wheat, endospermic starch is hydrolysed to give ultimately Glc.

Seeds were germinated and kept for 72 h in the dark at constant temperature. The combined root and shoot lengths were measured. With wheat, the mean of the lengths of the three roots formed, were added to the coleoptile length. The fraction due to the coleoptile was noted. The percentage of seeds that germinated was also recorded. When growth was limited by an added sugar, the development of root hairs was reduced and the root tips were often translucent. The responses to each of the various sugars differed among the species and there was varying behaviour within a single species to different sugars (Figs 3–5). 2d-Glc reduced growth (Fig. 3A–D) very effectively in all, with 20–25 mM stopping germination of mustard, fenugreek and wheat and a higher concentration reducing germination and growth in mung beans. In Figs 3–5, where the germination was reduced this is shown as a percentage (the whole numbers) beside the plotted value. In wheat the proportion of coleoptile length, relative to the total length of coleoptile plus the average of three roots, is shown as a fraction beside the plotted value.

The somewhat greater reduction in growth by 2d-Glc (in wheat and mustard (Fig. 3B and D)) relative to Man may, in part, be due to the higher proportion of the reacting β-anomer in the equilibrium mixture (49–35%), its ability to inhibit PGI and Glc-6-PDH and the capacity to interfere with polysaccharide synthesis. The lesser effect on mung beans (Fig. 3A) may arise from their ability to obtain Glc-1-P via phosphorolysis of starch. Within a species the level of reduction of growth increased as the concentration of analogue increased.

Man, with a similar phosphorylation coefficient to 2d-Glc, reduced growth effectively in mustard (Fig. 3B). Rapeseed, another oilseed, was similarly affected. The requirement for the production of hexose-6-P via sucrose through gluconeogenesis, transport to the embryo, scission and phosphorylation, combined with the absence of PMI would lead to this high sensitivity. Growth of mung beans and fenugreek (Fig. 3A and C) which contain PMI, was only slightly modified even at 75 mM. Wheat (Fig. 3D) showed an intermediate effect, which may be due to a high level of

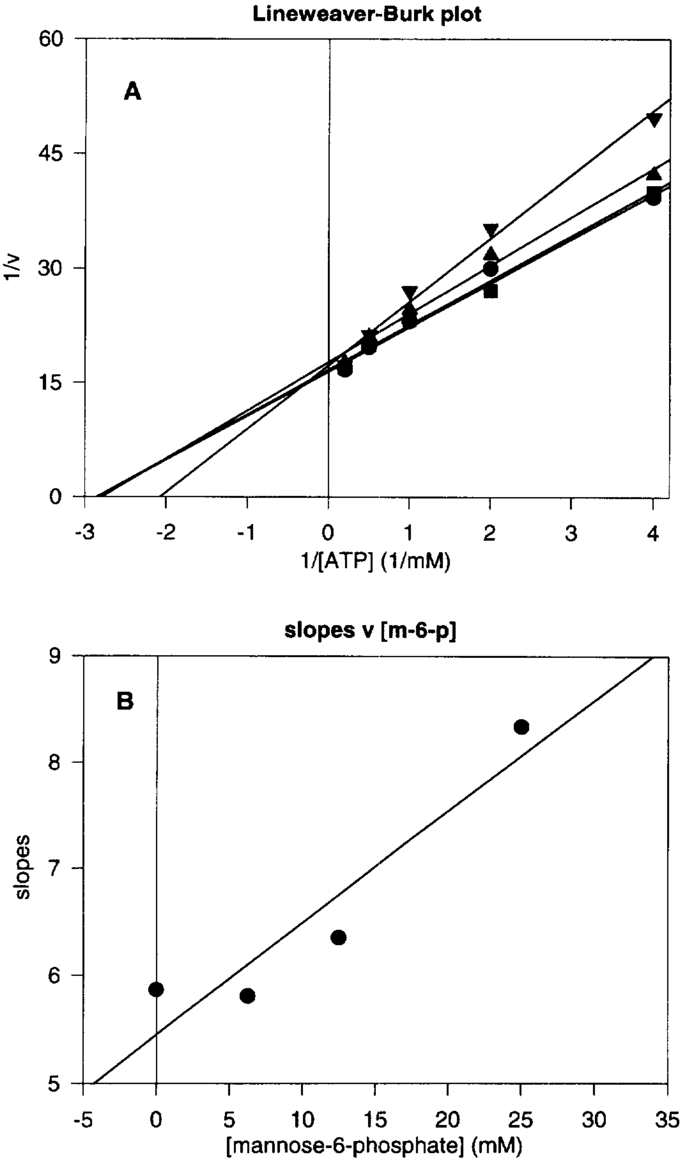

Fig. 1. Lineweaver-Burk plot (A) and a secondary plot (B) of inhibition of phosphoryl transfer from ATP to 1 mM D-glucose by Man-6-P: ● 0 mM, ■ 6.25 mM, ▲ 12.5 mM, ▼ 25 mM Man-6-P.

available Glc from amylolysis of endospermic starch. 2,5-Anhydro-D-talitol (an-Tal), which is a poor substrate for hexokinase, gave a much smaller reduction in growth (Fig. 4A–D) with all species. Analogues with intermediate rates of phosophorylation [6] gave intermediate levels of inhibition. 1,5-Anhydro-D-mannitol (1-deoxy-D-mannopyranose) at 10 mM reduced the growth of fenugreek by 20% compared with 50% by 10 mM-2d-Glc and 10% by 25 mM an-Tal. With mustard, 25 mM 1,5-anhydro-D-glucitol (1-deoxy-D-glucopyranose) (Table 1) gave 40% reduction compared to complete inhibition by 10 mM 2d-Glc or Man and 10% by 25 mM an-Tal. 50 mM D-Mannitol and 25 mM D-sorbose (neither substrates for hexokinase) produced no reduction in the growth of mustard. 10 mM Glc or sucrose, or 25 mM D-Gal also did not reduce the growth of mustard (Table 1) when 10 mM Man or 2d-Glc gave reductions of 65% and 100%, indicating that inhibition by Man and 2d-Glc was not due to catabolite repression. The slower, continuing decrease in growth of fenugreek and mung beans at higher levels of Man (40–80 mM) may have been a consequence of this effect. 10 mM *myo*-Inositol was without effect and did not lessen the reduction caused by 10 mM Man (Table 1).

The reduction in growth produced by 10 mM Man with mustard was not relieved by co-feeding 10 mM P_i (Table 1). P_i alone increased growth by 21%, probably due to buffering, but when Man was present the percentage reduction was similar to that in water.

The relationship between reduction in growth and the phosphorylation coefficient of an analogue, and

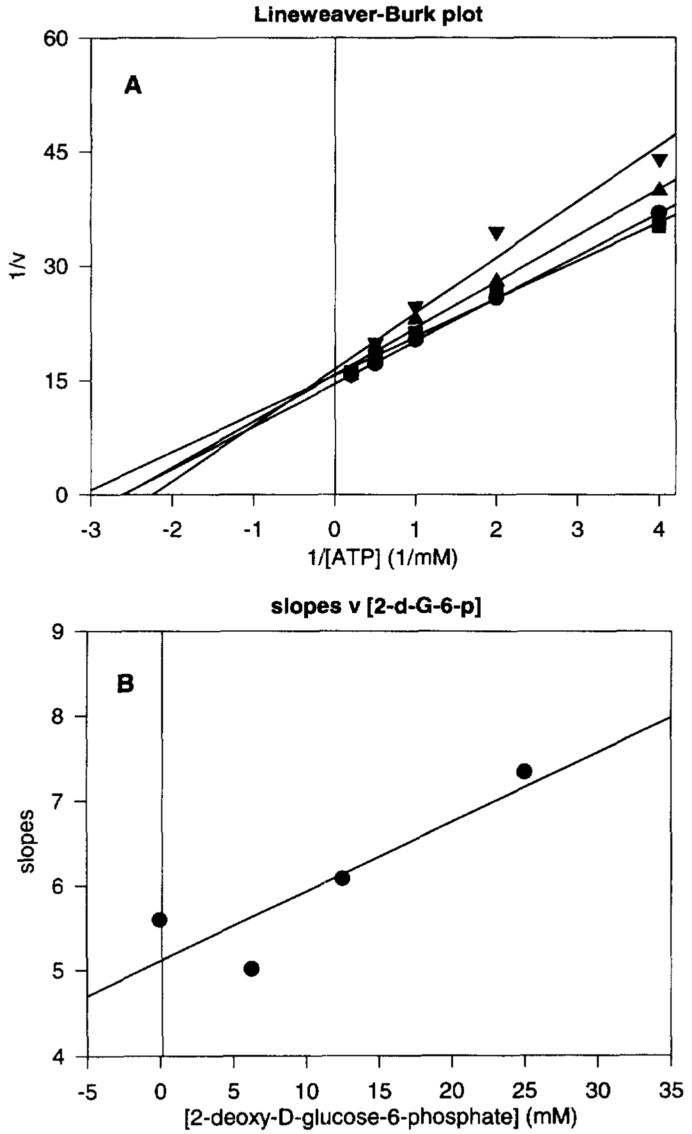

Fig. 2. Lineweaver-Burk plot (A) and a secondary plot (B) of inhibition of phosphoryl transfer from ATP to 1 mM D-glucose by 2d-Glc-6-P: ● 0 mM, ■ 6.25 mM, ▲ 12.5 mM, ▼ 25 mM 2d-Glc 6-P.

the dependence on the concentration of analogue, as well as the changes in ATP level and the ADP/ATP ratio observed in other tissues [2–4, 28, 29, 33, 34, 36], suggests that initiation of inhibition may be due to competition for hexokinase and inhibition of Glc phosphorylation by the analogues, leading to reduced levels of Glc-6-P for glycolysis and lowered levels of ATP [2–4, 28, 29, 33]. A decrease in the ATP/ADP ratio [4, 33, 34, 36] would then inhibit hexokinase and further reduce ATP. ATP is also required in many other processes, such as polynucleotide metabolism, protein synthesis, transport and phosphoprotein conversion. Although plant cells can obtain Glc-1-P from sucrose via sucrose synthase without a requirement for ATP, this is unlikely to occur with translocated sucrose in seeds. Acid invertase occurs in large amounts in rapidly growing plant tissue, where it is associated with cell wall fractions. Sucrose would be presented to growing cells as Glc and Fru, both requiring ATP for further metabolism.

Later, if a sufficiently high concentration of analogue phosphate were reached, this would also affect enzyme rate.

*Growth reduction by 2,5-anhydro-*D*-mannitol and* D*-psicose*

2,5-Anhydro-D-mannitol (an-Man) strongly reduced growth in all seeds at low concentration (Fig. 4A–D) with no growth in any species at 25 mM, even though it is a poor substrate for hexokinase (Table 2). An-Tal, which is almost as effective a substrate for hexokinase (Table 2) had a much more limited effect. Also, the 6-phosphate only inhibited honey locust

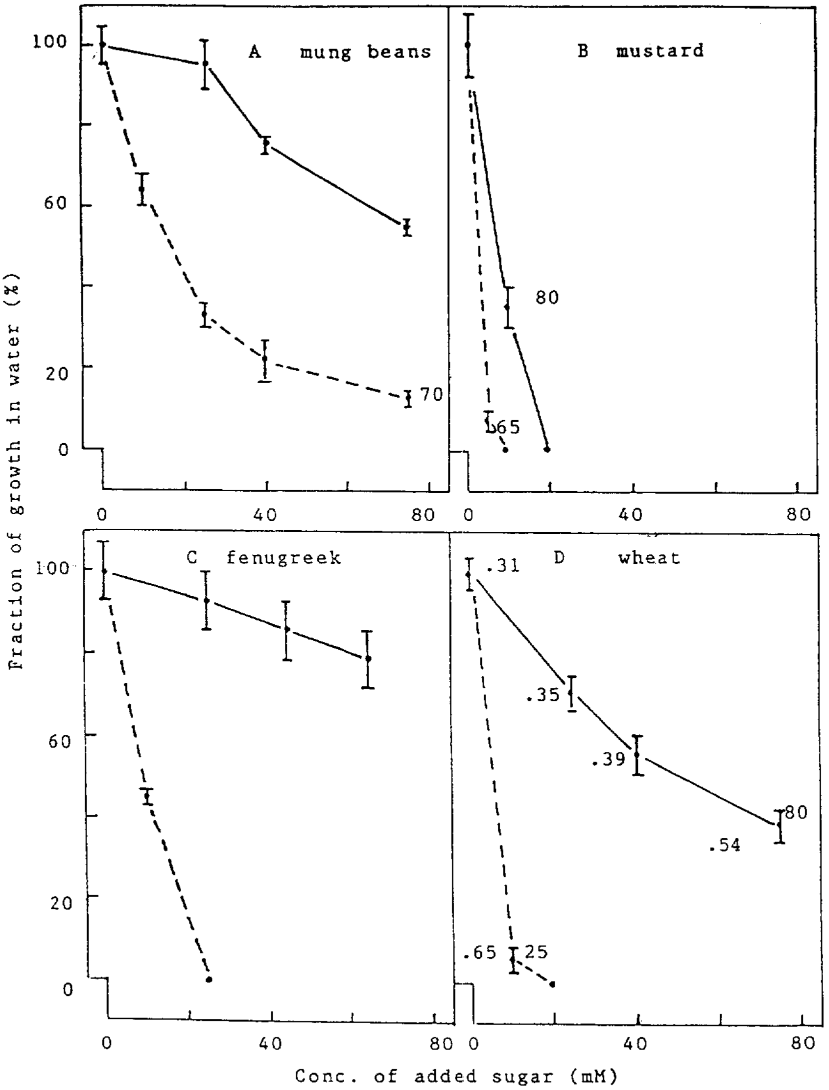

Fig. 3. Effect of D-mannose and 2-deoxy-D-glucose on germination and seedling growth, after 72 h, of mung bean, mustard, fenugreek and wheat seeds: —— D-mannose; – – – 2-deoxy-D-glucose.

The % germination, where germination was reduced, is given in whole numbers beside a point. The fractions describe the proportion of coleoptile length in wheat relative to the total length of coleoptile plus the average of three roots. The bars show s.e. values.

Table 1. Effects of various sugars and Pi on the germination and growth of mustard after 72 h

Germination solution	Compound added	Percentage of growth in water (and s.e.)
Water	10 mM Man	34±4
Water	10 mM Glc	98±4
Water	10 mM sucrose	96±8
Water	25 mM D-galactose	95±5
Water	10 mM *myo*-inositol	98±9
10 mM Man	10 mM *myo*-inositol	34±6
Water	25 mM 1,5-anhydro-D-glucitol	60±2
Water	10 mM Pi*	121±6
10 mM Man	10 mM Pi*	40±4

* $KH_2PO_4 + K_2HPO_4$, pH 6.5

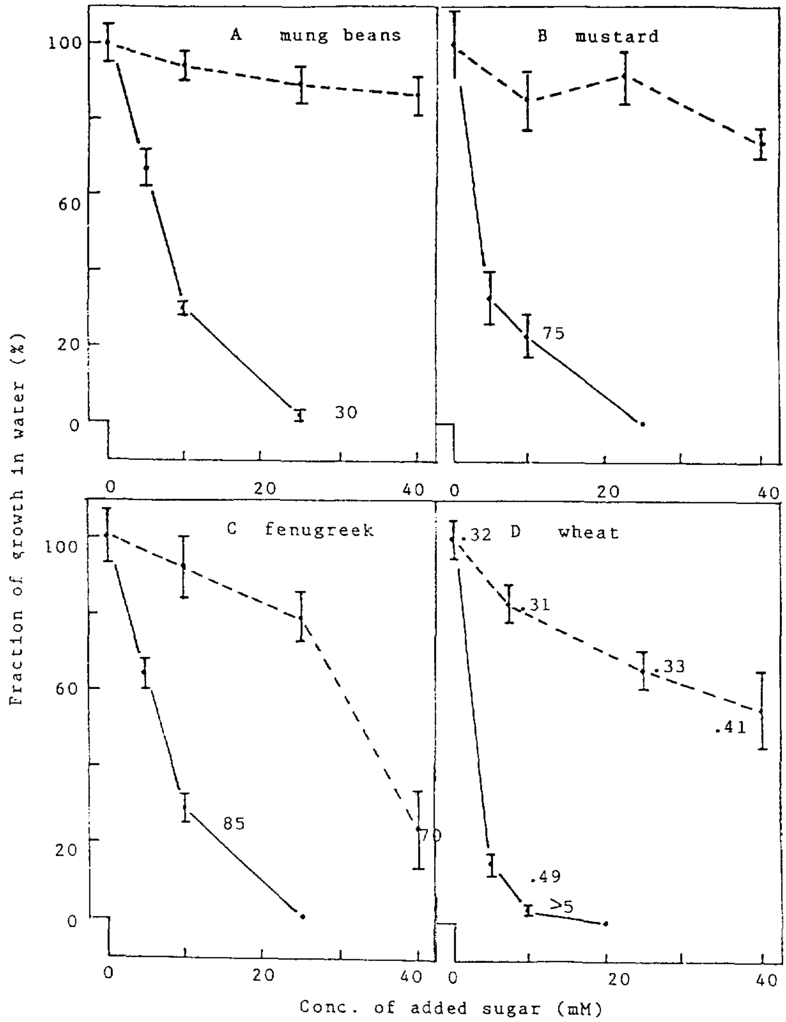

Fig. 4. Effect of 2,5 anhydro D-mannitol and 2.5 anhydro D-talitol on germination and seedling growth, after 72 h, of mung bean, mustard, fenugreek and wheat seeds: —— 2,5-anhydro-D-mannitol; - - - 2,5-anhydro D-talitol.

The % germination, where germination was reduced, is given in whole numbers beside a point. The fractions describe the proportion of coleoptile length in wheat relative to the total length of coleoptile plus the average of three roots. The bars show s.e. values.

Table 2. Relative rates of phosphorylation (compared to 1 mM Glc) of analogues by solubilized, particulate hexokinase from honey locust cotyledons

Compound (and conc.)	Relative rate (%) (and s.e.)
25 mM 2,5-Anhydro-D-mannitol	8±0.9
19 mM 2,5-Anhydro-D-talitol	4±0.3
1 mM 2d-Glc	69±1
25 mM D-Psicose	4±0.8

hexokinase slightly—25 mM an-Man-6-P in 1 mM Glc had 86% of the rate with Glc alone. 2,5-an-Man, which is a Fru analogue (2-deoxy-β-D-fructofuranose), inhibits gluconeogenesis in cells. The monophosphate formed by hexokinase (2,5-an-Man-1-P ≡ 2,5-an-Man-6-P) is a substrate for 6-phosphofructokinase [37]. The 1,6-bis-phosphate is an extremely sensitive inhibitor of fructose-bis-phosphatase, with a K_i value in the 10^{-2} μM range [38]. The stereochemistry of an-Tal-P precludes phosphorylation by 6-phosphofructokinase. The dependence of mustard on gluconeogenesis makes it very vulnerable to inhibition of fructose-bisphosphatase by an-Man-1,6-bis-P. Fructose-bisphosphatase is also required for the phosphate-triose-phosphate shuttle.

D-Psicose (Psi) is a poor substrate for honey locust hexokinase (Table 2) with no known capacity for further metabolism, yet a considerable reduction in growth was apparent in all species (Fig. 5A–D). Although its low rate of phosphorylation by hexokinase is similar to that of an-Tal (Table 2) more reduction in growth occurred (Figs 4A–D and 5A–D). Plants contain D-fructo 6-kinase (EC 2.7.1.4) [15,

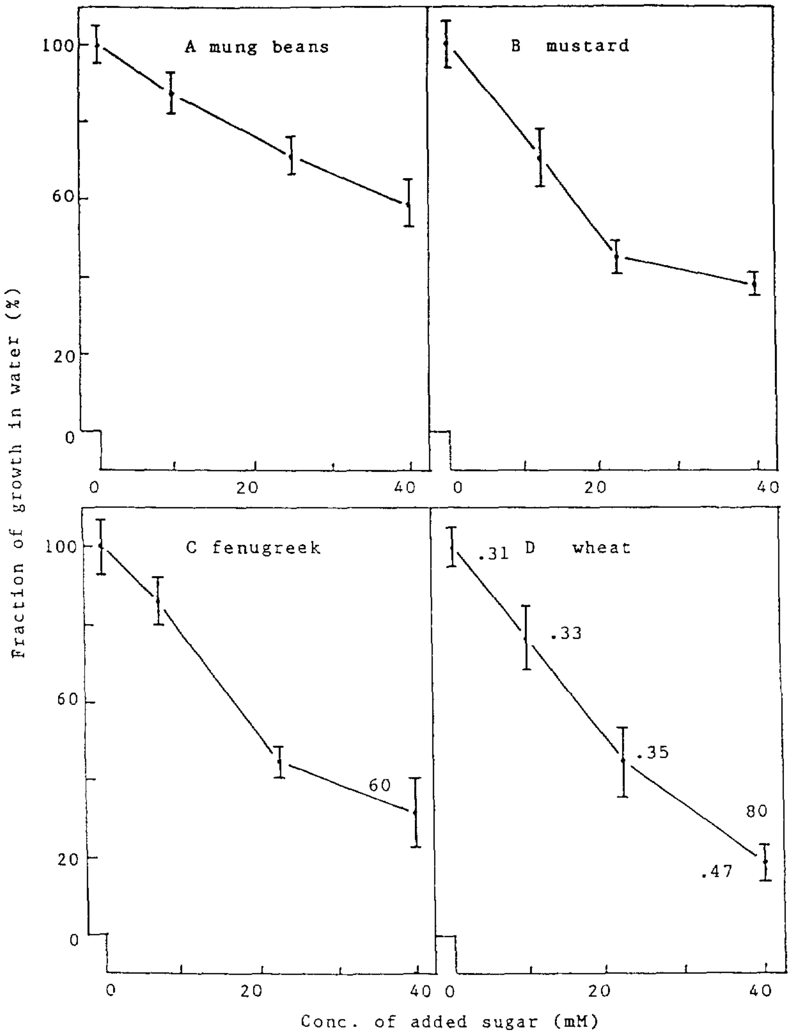

Fig. 5 Effect of D-psicose on germination and seedling growth, after 72 h, of mung beans, mustard, fenugreek and wheat seed.

The % germination, where germination was reduced, is given in whole numbers beside a point. The fractions describe the proportion of coleoptile length in wheat relative to the total length of coleoptile plus the average of three roots. The bars show s.e. values.

19, 39]. Whether sucrose is depolymerized by sucrose synthase or invertase the fructosyl fragment requires phosphorylation prior to metabolism. Psi has a higher rate of phosphorylation with honey locust fructo 6-kinase than an-Tal (15 ×)—10 mM Psi had 69% of the activity of 1 mM Fru, whereas 50 mM an-Tal had only 30% [19]. The growth reductions and kinetic behaviour indicate that phosphorylation of Fru by fructo 6-kinase has a separate (but essential) role to that of hexokinase during germination and early seedling growth.

EXPERIMENTAL

Seed sources

Mung beans (*Vigna radiata*), fenugreek (*Trigonella foenum-graecum*) and white mustard (*Sinapis alba*) were retail sprouting seeds. Wheat (*Triticum aestivum* var. Vulcan) was obtained from the University of Sydney Plant Breeding Institute. Germinated honey locust (*Gleditsia triacanthos*) cotyledons were prepared as described [19].

Sources of sugar

An-Man and an-Tal were made as previously described [19]. 2d-Glc, Man, Psi, Gal, 1,5-anhydro-D-glucitol, 1,5-anhydro-D-mannitol, 2d-Glc-6-P, and Man-6-P were obtained from Sigma and Glc from Merck. 2,5-an-Man-6-P was prepared as previously described [37] by reaction with hexokinase and ATP (Boehringer Mannheim).

Germination of seeds

Seeds (20 mung bean or 30 fenugreek, mustard or wheat) were soaked for 10 min in 0.5% NaOCl, washed 4 times with sterile H_2O and spread uniformly on Whatman no. 1 filter paper (90 cm) in Petri dishes with 4 ml of solution of analogue substrate or non-substrate or a H_2O blank for mung beans and 3 ml for the other seeds. The dishes were incubated in the dark at 30°. After 72 h the root plus shoot lengths of mung beans, fenugreek and mustard were measured and the average calculated for each dish. In wheat the average length of the three roots that formed was added to the shoot length. Triplicates of dishes were measured and means and s.e. values calculated for the averages from the three dishes.

Sources of enzymes

Solubilized, particulate hexokinase from the cotyledons of germinated honey locust seeds was prepared as described [19]. Assay enzymes were obtained from Boehringer Mannheim and buffers (PIPES and Tris base), Triton X-100 and co-factors from Boehringer Mannheim and Sigma.

Enzymic assays

These were performed and constants calculated as previously described [19]. Rates of phosphorylation of an-Man, an-Tal, 2d-Glc and Psi by solubilized, particulate locust bean hexokinase and inhibition by 2d-Glc 6-P of Glc phosphorylation were measured with the lactate dehydrogenase-pyruvate kinase linked assay. Inhibition by Man-6-P and 2,5-an-Man-6-P of Glc phosphorylation were estimated with the glucose 6-phosphate dehydrogenase linked assay. Each value in Figs 1A and 2A is a mean of triplicates.

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