THE PURIFICATION AND PROPERTIES OF A HEXOKINASE FROM THE CORN SCUTELLUM

By

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INTRODUCTION

The properties of plant enzymes, especially those of higher plants, are less well known than those of similar enzymes isolated from animal tissues. It has been assumed that the properties associated with the characterized animal and lower plant (yeast) enzymes are the same for higher plants. This may be the case, but it is necessary that the plant enzymes be characterized in order to gain a better understanding of the similarities and differences in metabolism between plants and animals.

The enzyme, hexokinase, which catalyzes the phosphorylation of glucose in the presence of adenosine-5'-triphosphate and magnesium ion, occupies an important position in the metabolism of sugars in both plants and animals. Humphreys and Garrard (47) have presented evidence which suggests that the hexokinase reaction may be important in controlling the rate of glucose uptake by the corn scutellum; an organ positioned between the root-shoot axis and endosperm of the corn seed, where glucose absorbed from the endosperm is converted to sucrose which is subsequently translocated to the developing seedling during germination (28). Their data indicates that glucose-6-phosphate competitively inhibits an enzymatic step associated with net glucose uptake in scutellum slices, and they suggest that the step might be the hexokinase-catalyzed phosphorylation of glucose. Since it has been demonstrated that brain hexokinase (15, 16) and to a small extent, yeast hexokinase (33), is inhibited by glucose-6-phosphate, although

noncompetitively, it seemed desirable to investigate the properties of corn scutellum hexokinase.

REVIEW OF LITERATURE

Historical

In 1927, Meyerhof (73) gave the name, hexokinase, to an alcohol precipitable fraction of autolyzed yeast which when added to extracts of aged frog or rabbit muscle greatly enhanced glycolysis. Independently, in 1935, Euler and Adler (29) and Lutwak-Mann and Mann (66), isolated an enzyme from yeast--heterophosphatase--which catalyzed the following reaction in the presence of Mg^{++} :

Meyerhof (74), in a description of the hexokinase reaction the same year, reported that the enzyme described by the two groups was the same one responsible for the activity of his original preparation.

Kalckar (51), in 1939, demonstrated that kidney extracts phosphorylated glucose and fructose and, in 1940, Geiger (36) observed that extracts from brain tissue phosphorylated fructose, mannose and glucose. Belitzer and Golovskaya (5), the same year, showed that muscle tissue contained hexokinase, which in the presence of glucose and creatine catalyzed the phosphorylation of glucose to hexose-6-phosphate. In 1941, Ochoa (78) showed the presence of hexokinase in acetone powder of rat brain. The acetone partially inactivated adenosinetriphosphatase (ATPase). The hexokinase required Mg^{++} and transferred phosphate from ATP to glucose without the liberation of free orthophosphate.

Colowick et al. (12), in 1941, demonstrated that phosphorylation of glucose preceded the oxidation of glucose in heart muscle and kidney extracts and that hexokinase catalyzed phosphorylation of mannose to mannose-6-phosphate (M6P). The M6P was in turn converted to fructose-6-phosphate (F6P) by an isomerase.

In 1946, Berger et al. (6) and Kunitz and MacDonald (57) succeeded in crystallizing hexokinase from yeast, the only organism from which it has been crystallized. The hexokinases from various sources have been recently reviewed by Crane (20).

Hexokinases of Various Animal Tissues

Mammalian brain hexokinases. Colowick et al. (14) prepared purified beef brain hexokinase by eluting acetone powder from beef brain with water and fractional precipitation of the eluate with ammonium sulfate. The hexokinase was precipitated between 30 and 50 per cent saturation with a two-fold increase in specific activity. The 30 to 50 per cent fraction could be purified further by refractionation between 45 and 50 per cent saturation with ammonium sulfate. The product of the beef brain hexokinase reaction was shown to be glucose-6-phosphate (G6P).

Meyerhof and Wilson (75) observed that brain extracts catalyzed the phosphorylation of glucose and fructose at about the same rate at 0.020M concentrations of the substrates, while at concentrations of 0.0015M to 0.003M the rate was the same for glucose and much lower for fructose. They observed the same difference relative to the ATP concentration and there was a greater ATPase activity in the presence of fructose than glucose. They suggested that there are actually two separate enzymes in brain extracts--a glucokinase and a fructokinase.

Wiebelhous and Lardy (104) noted that dialyzed extracts of beef brain showed different hexokinase activities with respect to glucose and fructose in the presence of inhibitory concentrations of sodium salts. Sodium salts inhibited phosphorylation of glucose, but not of fructose, indicating the possibility of two different hexokinases. Their beef brain extracts promoted the phosphorylation of glucose, fructose and to a lesser extent, mannose, but were not active with L-glucose, galactose, L-sorbose, D-gluconate, 2-keto-0-gluconate, D-ribose, D-arabinose, L-rhamnose and D-xylose. Inorganic pyrophosphate inhibited activity 46 to 80 per cent depending on its concentration. Magnesium ion, up to two times the concentration of the pyrophosphate, did not reverse inhibition. Orthophosphate was only slightly inhibitory.

Long (63) found that of five rat tissues--brain, liver, kidney, skeletal muscle, and intestine--brain had the greatest amount of hexokinase activity, while liver had the least.

Crane and Sols (16, 17, 86) prepared particulate hexokinase from brain, which was free of interfering enzymes, by fractional centrifugation and solubilization of the enzyme with lipase and/or deoxycholate. They obtained 35 per cent recovery. With this preparation, it was found (as Weil-Malherbe and Bone (102) had reported in 1951) that G6P inhibited hexose phosphorylation noncompetitively (15), while ADP behaved as a competitive inhibitor (85). They (86) examined the specificity of brain hexokinase toward thirty-five compounds structurally related to glucose in order to determine which groups of the glucose molecule were possibly involved in formation of a glucose-brain hexokinase complex. Sixteen of the analogs served as substrates

(Table 1), five behaved as competitive inhibitors, and the remainder were inactive. They estimated the relative influence of each hydroxyl group of glucose by comparing the Michaelis constants (K_m) and the relative maximal rates (V_{max}) of phosphorylation (Table 1). The conclusion was that the formation of a glucose-enzyme complex involved the ring structure and hydroxyl groups at carbon atoms 1, 3, 4 and 6, and that each hydroxyl group had a specific quantitative influence on enzyme-substrate affinity.

Crane and Sols (16) investigated the specificity for inhibition of the brain hexokinase reaction by G6P and related compounds. They tested some twenty-five compounds and only six were inhibitory (Table 2). They concluded that the inhibitor complex involved the pyranose ring structure, the hydroxyl groups at carbon atoms 2 and 4 and the phosphate group at carbon atom 6. They interpreted the lack of influence by carbon atoms 1 and 3, and the influence by carbon atom 2 as indicating that the enzyme possessed a third, specific binding site for G6P in addition to the two for ATP and glucose and that the data supported their hypothesis that inhibition by G6P, when present, was a part of an intrinsic cellular mechanism for the control of the rate of the hexokinase reaction. They found inhibition by the hexose phosphates to be reversible, independent of either glucose or ATP concentration and, therefore, noncompetitive.

Maley and Lardy (67) tested the effects of a variety of N-substituted glucosamines on brain hexokinase. All were powerful inhibitors ($K_i=10^{-3}M$ to $10^{-6}M$). They interpreted their data (and from studying molecular models) as indicating that the substituted glucosamines

TABLE 1

MICHAELIS CONSTANTS (K_m) AND RELATIVE MAXIMAL RATES FOR BRAIN AND YEAST HEXOKINASE

Data from references (86) and (23)

(1) Modified at Carbon	(2) Compound	(3)	(4)	(5)	(6)
		Brain K_m (M/L)	Rel. Max. Vel.	Yeast K_m (M/L)	Rel. Max. Vel.
	glucose	8×10^{-6}	1.0	1×10^{-4}	1.0
1	1,5-sorbitan	3×10^{-2}	1.0	3×10^{-3}	0.01
1,2	1,5-mannitan	2×10^{-2}	0.9		
1	glucoheptulose	2×10^{-4}	0.006		
1,2	mannoheptulose	5×10^{-5}	0.015	2×10^{-4} *	0.001
1,2	fructose	2×10^{-3}	1.5	7×10^{-4}	1.8
1,2	2,5-sorbitan	2×10^{-1}	0.08		
1,2	arabinose	2	0.1	$> 1 \times 10^{-1}$	0.02
1	methyl- α -glucoside	+	+	$> 1 \times 10^{-1}$ *	< 0.001
1	methyl- β -glucoside	+	+		
1	1-thioglucoase	+	+		
1,2	glucal	+	+		
1	α -glucose-1-P	+	+		
1	glucono-1,5-lactone	+	+		
2	mannose	5×10^{-6}	0.4	5×10^{-5}	0.8
2	2-deoxyglucose	3×10^{-5}	1.0	3×10^{-4}	1.0
2	glucosamine	8×10^{-5} ***	0.6	2×10^{-3}	0.7
2	glucosone	1×10^{-5}	0.08	2×10^{-5}	0.2
2	N-acetylglucosamine	8×10^{-5} **	+	1×10^{-3} *	< 0.001
2	N-methylglucosamine	2×10^{-4} **	+		

(TABLE 1 continued next page)

TABLE 1 (continued)

(1) Modified at Carbon	(2) Compound	(3) Brain		(5) Yeast		(6)
		Km (M/L)	Rel. Max. Vel.	Km (M/L)	Rel. Max. Vel.	
2	2-O-methylglucose	+	+			
3	allose	7×10^{-3}	0.5	$> 1 \times 10^{-1}$		0.1
3,2	altrose	3×10^{-3}	0.11			
3	3-deoxyglucose	2×10^{-2}	0.2			
3	3-O-methylglucose	+	+	$> 1 \times 10^{-1*}$		< 0.001
4	galactose	1×10^{-1}	0.02	5×10^{-2}		0.002
4,3	gulose	+	+			
5,4	1,4-sorbitan	+	+	$> 1 \times 10^{-1*}$		< 0.001
5,1	L-sorbose	+	+	$> 1 \times 10^{-1*}$		< 0.001
6	6-deoxyglucose	$2 \times 10^{-3**}$	+			
6	xylose	$2 \times 10^{-3**}$	+	$1 \times 10^{-2*}$		< 0.001
6,2	lyxose	$1 \times 10^{-3**}$	+			
6,3	ribose	+	+			
6	6-deoxy-6-fluoroglucose			$5 \times 10^{-3*}$		< 0.001
1,2	1-methylfructose			$> 1 \times 10^{-1*}$		< 0.001
2	2-C-hydroxymethylglucose			$2 \times 10^{-3*}$		< 0.001
1,2,3	3-O-methylfructose			$> 1 \times 10^{-1*}$		< 0.001
	sorbitol	+	+			
	glucoguloheptose	+	+			
	i-inositol	+	+			

*Inhibition constant (K_i)

**Determined by competitive inhibition of fructose

***Determined at pH 7.5

+Undetectable

P=phosphate

TABLE 2

EFFECT OF GLUCOSE-6-P AND RELATED COMPOUNDS ON PHOSPHORYLATIONS BY
BRAIN HEXOKINASE

Data from reference (16)

(1) Modified at Carbon No.	(2) Ester	(3) Substrate	(4) Ester concen- tration	(5) Per cent inhibi- tion	(6) K _i (M/L)
1	1,5-Sorbitan-6-P	2-Deoxyglucose	0.67	38	1×10^{-3}
1	α -Glucose-1,6-diP	2-Deoxyglucose	0.6	47	7×10^{-4}
1	β -Glucose-1,6-diP	2-Deoxyglucose	1.0	0	
1	Glucoheptulose-7-P	2-Deoxyglucose	8.6	0	
1	Methyl-glucoside-6-P	2-Deoxyglucose	3.8	0	
2	Mannose-6-P	Mannose	8.5	0	
2	2-Deoxyglucose-6-P	Glucose	1.0	0	
2	Glucosamine-6-P	Glucose	1.5	0	
2,1	Fructose-6-P	2-Deoxyglucose	2.5	0	
2,1	2,5-Sorbitan-6-P	2,5-Sorbitan	13	0	
2,1	Fructose-1,6-diP	2-Deoxyglucose	1.8	0	
2,1	1,5-Mannitan-6-P	1,5-Mannitan	4.0	0	
2,1	Mannoheptulose-7-P	Mannoheptulose	2.0	0	
2,1	Arabinose-5-P	Arabinose	5.5	0	
2,3	Altrose-6-P	2-Deoxyglucose	7.5	0	
2,3,1	Ribose-5-P	2-Deoxyglucose	2.0	0	
2,3,4,1	Xylose-5-P	2-Deoxyglucose	3.3	0	
3	Allose-6-P	2-Deoxyglucose	9.2	58	7×10^{-3}
3	3-Deoxyglucose-6-P	Mannose	3.2	14	2×10^{-2}
4	Galactose-6-P	Mannose	20	0	

(TABLE 2 continued next page)

TABLE 2 (continued)

(1) Modified at Carbon No.	(2) Ester	(3) Substrate	(4) Ester concen- tration	(5) Per cent inhibi- tion	(6) K _i (M/L)
5,1	L-Sorbose-1-P	2-Deoxyglucose	0.45	40	7×10^{-4}
6,1	α -Glucose-1-P	2-Deoxyglucose	2.5	0	
No ring	Gluconate-6-P	2-Deoxyglucose	1	0	
No P	Glucuronate	2-Deoxyglucose	2	0	

combined with the active enzyme site and that the inhibition might be through blocking of the site on the enzyme to ATP, since the substituted groups did not overlap the carbon 6 position on the sugar.

By kinetics studies of the mechanism of the brain hexokinase reaction, Fromm (31) and Fromm and Zewe (32) found that G6P acted as a competitive inhibitor of ATP and as an uncompetitive inhibitor with respect to glucose. Inhibition by ADP was of a more complex nature, but appeared to be noncompetitive with respect to ATP and uncompetitive with respect to glucose. They concluded that their results were consistent with a mechanism involving either a phospho- or a gluco-enzyme complex, but were at variance with any mechanism in which both substrates need to be present simultaneously on the enzyme for the reaction to occur. They also concluded that there seemed to be no reason for G6P occupying a third site on the enzyme. They presented the following compulsory pathway type mechanism for the brain hexokinase reaction:

- (1) Enzyme + ATP $\longrightarrow$ Enzyme-X complex + ADP
- (2) Enzyme-X complex + glucose $\longrightarrow$ Enzyme-Y complex
- (3) Enzyme-Y complex $\longrightarrow$ Enzyme + G6P

The nature of the Enzyme-X and Enzyme-Y complexes was unknown. The mechanism is similar to that presented by Hammes and Kochavi (42) for the yeast hexokinase reaction.

Kerly and Leaback (54) measured the specificity of hexokinase of the brain of several nonmammalian animals. They found that extracts of brain from pigeon, four-day-old chick, two elasmobranchs, two teleost fishes, frog and squid catalyzed the phosphorylation of

glucose and fructose. With pigeon brain extract, phosphorylation of fructose was inhibited by mannose and N-acetylglucosamine, while phosphorylation of both fructose and glucose was inhibited by G6P and F6P. The affinity of pigeon brain hexokinase for fructose was similar to that of the beef brain enzyme, except beef brain hexokinase was not inhibited by F6P (16).

Muscle. Crane and Sols (17) partially purified soluble skeletal muscle hexokinase by fractional precipitation with cold acetone and drying in vacuo. The enzyme was precipitated by 33 per cent acetone (v/v) with 50 per cent recovery and a specific activity of one. Skeletal muscle hexokinase catalyzed the phosphorylation of glucose, mannose, fructose, glucosamine and 2-deoxy-D-glucose (2 DOG). The K_m for glucose and mannose of the muscle enzyme was about ten times higher than that for the brain enzyme. The K_m for fructose was only slightly higher than that of brain hexokinase. Skeletal muscle hexokinase activity was optimum at pH 8.0.

Crane and Sols (17) partially purified particulate heart muscle hexokinase by fractional centrifugation and solubilization of the enzyme from the particulate fraction with 0.1 per cent Triton X-100. ADP inhibited heart muscle and skeletal muscle hexokinase competitively ($K_{iADP} = K_{mATP}$) and G6P inhibited noncompetitively. The inhibition constants (K_i) of G6P, 1,5-sorbitan-6-P, and L-sorbose-1-P for heart muscle hexokinase were 25 per cent, or less, of those for the brain enzyme.

Strickland (90) observed that glycolysis by a muscle extract in the presence of added hexokinase could be inhibited by 0.003M

glyceraldehyde, but the inhibition could be reversed by a small excess of hexokinase. He concluded that glyceraldehyde inhibition could be pin-pointed as inhibition of glucose phosphorylation by hexokinase.

Walaas and Walaas (99) extracted an acetone powder of rat skeletal muscle with tris-EDTA solution and obtained a five-fold increase in specific activity by fractionating the eluate with cold ethanol. The ethanol precipitate was dried in vacuo. Fractionation of either the acetone powder eluate or the ethanol-precipitable fraction with ammonium sulfate decreased recovery. EDTA, when added to the crude homogenate from which the acetone powder was prepared, increased recovery. Added glucose or ATP only slightly stabilized the enzyme. Prolonged contact with 0.05M $MgCl_2$ completely inhibited the enzyme. Chloride ion was without effect. Orthophosphate provided slight protection and 0.03M pyrophosphate was very effective in stabilizing muscle hexokinase. However, 0.005M pyrophosphate strongly inhibited hexokinase during incubation of the reaction mixture. Potassium chloride, in a narrow range of 0.02 to 0.05M, slightly activated the hexokinase during incubation while orthophosphate inhibited. The hexokinase had a pH optimum at 8.0 to 8.2 and one-third maximum activity at pH 7. Maximum activity of hexokinase was observed at a molar ratio $Mg/ATP = 1$ for several concentrations of ATP. Magnesium ion in excess of ATP, except at relatively high concentrations, was not inhibitory. At a molar ratio ATP/Mg greater than 4, ATP inhibited the reaction. The K_m for both ATP and Mg was $1.7 \times 10^{-3}M$. Inorganic pyrophosphate inhibited the hexokinase reaction competitively with respect to $MgATP$ when the molar ratio was 1. At inhibitory concentrations of Mg^{++} ,

pyrophosphate had a small activating effect on the enzyme, which they interpreted as a release of Mg^{++} inhibition. They suggested that inorganic pyrophosphate inhibited hexokinase by forming a Mg^{++} -pyrophosphate complex which excluded $MgATP$ from the enzyme-substrate complex and furthermore, that the $MgATP$ complex was the actual substrate (other than glucose) for muscle hexokinase. Muscle hexokinase was also activated by Ca^{++} , Co^{++} , Mn^{++} and, to a very small extent by Zn^{++} . The maximum activities for these activators were lower than that for Mg^{++} and the K_m for each was lower than that for Mg^{++} . The molar ratio (metal/ATP) for maximum activation by the ions was less than 1. Strong inhibition occurred as the concentrations of the metals were increased above those giving maximum activation. They explained the differences between these activators and Mg^{++} as being due to the different metal ions combining with different ligand centers on the enzyme.

Walaas and Walaas (99) also reported that inosinetriphosphate (ITP), guanidinetriphosphate (GTP) and uridinetriphosphate (UTP) would not substitute for ATP in the muscle hexokinase reaction.

Griffiths (40) found that 0.005M alloxan inhibited muscle hexokinase completely and 0.005M ninhydrin inhibited the enzyme 80 per cent. Inhibition by 0.003M alloxan was reversed by 0.005M cysteine, but 0.01M cysteine only partially reversed inhibition by 0.0025M ninhydrin. He interpreted the results as indicating that the inhibition probably amounted to more than just thiol destruction since reversal required a molar ratio of cysteine to alloxan that was greater than 1.

Liver hexokinase. Long (63) measured levels of hexokinase in several rat tissues and found that liver had the lowest level of hexokinase.

Crane and Sols (17) described a purification procedure for the soluble hexokinase of rat liver. They concentrated a 100,000XG supernatant fraction of liver extract with ammonium sulfate up to 50 per cent saturation. Their liver preparation catalyzed phosphorylation of glucose, mannose, fructose, glucosamine and 2DOG. There was some evidence that fructose and glucose might have been phosphorylated by separate enzymes. Lange and Kohn (58) found that allose, talose and gulose were also substrates of rat liver hexokinase. They studied hexokinase in rat intestine, kidney and liver extracts and their data indicated that while the hexokinases from the three sources were similar, they were not identical. The K_m for glucose, 2DOG and glucosamine were 4×10^{-5} , 9×10^{-5} and $3.7 \times 10^{-4} M$, respectively, and the relative maximal velocities were in the same order.

Vinuela et al. (98) detected two enzymes in rat liver extracts which catalyzed phosphorylation of glucose. One enzyme precipitated between 20 and 50 per cent saturation with ammonium sulfate, while the second enzyme precipitated between 60 and 70 per cent saturation. The first enzyme had a low K_m for glucose, was inhibited by G6P, and only moderately by N-acetylglucosamine. The latter enzyme had a higher K_m for glucose, was not inhibited by G6P, was strongly inhibited by N-acetylglucosamine, and had a low K_m for mannose. They interpreted the characteristics of the first enzyme as being those of a typical animal hexokinase. It was relatively stable and was present in small amounts in normal liver. They designated the second enzyme, a glucokinase. It showed a maximal rate for glucose phosphorylation of approximately 1.0 micromole per minute per gram liver at 21 to 23°C or

2 micromoles at 37°C which was comparable to the rate of glycogen synthesis from glucose in rat liver (0.4 to 1 micromole per minute per g liver). The activity also approached the maximum rate of glycogen synthetase (3 micromoles per minute per g at 37°C). The glucokinase disappeared in starved and diabetic animals. They suggested that the physiological instability of the glucokinase accounted for the inability of liver to synthesize glycogen from glucose and mannose in diabetic animals, while fructose and galactose, which were incorporated into glycogen, were phosphorylated by other hexokinases specific for those sugars. The K_m for glucose of the hexokinase and of the glucokinase were found to be $1 \times 10^{-5} \text{M}$ and $1 \times 10^{-2} \text{M}$, respectively. The glucokinase was not active on fructose to any extent. Their conclusion was that the glucokinase was associated with the glycogen synthesizing system of the liver since: (1) its activity was comparable with the rate of glycogen synthesis, (2) it was not inhibited by its product and (3) UDPG-glycogen glucosyltransferase is dependent on high levels of G6P for maximum activity.

Walker and Rao (100) examined the effects of 2DOG, glucosamine and N-acetylglucosamine on the hexokinase and glucokinase of rat liver. The three compounds inhibited both enzymes competitively, but the K_i for 2DOG was much higher for the glucokinase than for the other two compounds. The K_i for 2DOG was comparable to the K_m for glucose for the enzyme.

Kidney hexokinase. Kalckar (51) showed that kidney extracts catalyzed phosphorylation of glucose and fructose. Colowick *et al.* (14) demonstrated phosphorylation of mannose by kidney extracts. Mannose isomerase in the extract converted M6P to F6P.

Lange and Kohn (58) found the K_m for glucose, 2DOG and glucosamine to be $4.8 \times 10^{-5}M$, $9 \times 10^{-5}M$ and $0.1M$, respectively. Allose and talose were also phosphorylated, while altrose and N-acetylglucosamine were not. It appeared that kidney hexokinase differed from hexokinases of other rat tissues (and other animal and plant hexokinases) in its specificity towards modification at carbon atom 2 since glucosamine was such a poor substrate.

Intestine hexokinase. Sols (87) examined the hexokinase of rat intestinal mucosa, and found fructose was phosphorylated at rates less than glucose, while galactose, 3-methylglucose, L-sorbose, mannoheptulose, N-acetylglucosamine, xylose, ribose and L-arabinose were not phosphorylated. G6P at $6 \times 10^{-3}M$ inhibited hexokinase activity by 50 per cent, which was ten times the concentration required to inhibit the brain enzyme to the same extent. All the sugars were phosphorylated by the same enzyme. The K_m for glucose and fructose were $2 \times 10^{-4}M$ and $4 \times 10^{-3}M$, respectively. These results failed to support the hypothesis that the rate of phosphorylation of sugars limits the rate of sugar absorption by intestinal mucosa since galactose and 3-methyl glucose, which were not phosphorylated, were absorbed at faster rates than other sugars (except glucose). Fructose, whose rate of absorption was intermediate, was phosphorylated at a rate greater than any of the other sugars.

Lange and Kohn (58) found the K_m for glucose, 2DOG and glucosamine of rat intestine hexokinase to be 6.5×10^{-5} , 9×10^{-5} and $3.3 \times 10^{-4}M$, respectively. Allose and talose, as with kidney hexokinase, were also active. Changes at carbon atom 3 of glucose (allose) had considerably

greater effect on activity than changes at carbon atom 2 (2DOG or glucosamine). Changes at either carbon atoms 2 and 3 (altrose) or 3 and 4 (gulose) resulted in loss of all activity. They concluded that all the active substrates of kidney, liver and intestine hexokinase were similar in at least two respects: (1) They had an available hydroxyl at carbon atom 6 and (2) they had a hydroxyl available at the anomeric position (carbon atom 1).

Erythrocyte hexokinase. Christensen et al. (11) found that the hexokinase of rat erythrocyte phosphorylated glucose, mannose and fructose at the relative rates 1.0:0.77:0.36, respectively. Galactose was not active. The activity of normal or diabetic rats was not affected by insulin or adrenal cortical extracts.

Retina hexokinase. Hoare and Kerly (44) showed that extracts of rat retina phosphorylated glucose, fructose, mannose and glucosamine. Magnesium ion, manganese ion and to a lesser extent, cobalt ion activated dialyzed extracts. Glucose and glucosamine inhibited phosphorylation of fructose.

Krebs - 2 ascites tumor hexokinase. McComb and Yushok (69) partially purified the hexokinase of Krebs-2 ascites tumor. The hexokinase had a pH maximum between 5.6 and 7.8. The K_m for glucose and Mg^{++} were 1.7×10^{-4} and $1 \times 10^{-3}M$, respectively. The K_i for G6P inhibition was 4×10^{-4} , while M6F, 2DOG6P and F6P were not inhibitory. Adenosine diphosphate was inhibitory. Glucosone ($K_m = 8 \times 10^{-6}M$) was also phosphorylated by the tumor enzyme. The hexokinase activity was localized in the mitochondria of both tumor and normal tissues.

Domestic fowl hexokinases. Makata *et al.* (71) examined tissue extracts from several organs of domestic fowl (white leghorn hen) and found that the heart and gizzard hexokinases were active with both glucose and fructose, while the enzyme of liver phosphorylated fructose but not glucose.

Honey bee hexokinase. Ruiz-Amil (81) partially purified hexokinase from honey bee by fractional precipitation of the enzyme between 50 and 70 per cent saturation with ammonium sulfate and column-chromatography on DEAE-cellulose. The yield was 28 per cent of the original hexokinase in the crude extract. The thorax contained the largest amount of hexokinase activity (75 per cent). The enzyme showed optimum activity between pH 7.5 and 8.8. Glucose-6-phosphate behaved as a competitive inhibitor of glucose, while 2DOG6P and L-glycerophosphate did not inhibit the enzyme to any extent. The K_m for ATP and the K_i for its competitive inhibitor, ADP, were $7.5 \times 10^{-4} M$ and $9 \times 10^{-4} M$, respectively. The K_m for fructose ($3 \times 10^{-3} M$) was similar to that of the mammalian brain enzyme, while those for glucose ($4 \times 10^{-5} M$) and mannose ($1 \times 10^{-5} M$) were similar to those of yeast hexokinase. Ruiz-Amil concluded that the enzyme from honey bee was similar to the hexokinase of other animals in most respects.

Locust hexokinase. Kerly and Leaback (53) studied the hexokinase in the thorax muscle and the salivary gland of locust (Locusta migratoria). The properties of the enzyme were similar to those of the mammalian brain enzyme with respect to substrates and inhibitors. Glucose had a K_m of $5.5 \times 10^{-3} M$. Both G6P and F6P, at a concentration of $5 \times 10^{-3} M$, completely inhibited hexokinase activity.

House fly hexokinase. Chefurka (10) observed hexokinase activity in a soluble extract of house fly (Musca domestic L.).

Sea urchin egg hexokinase. Krah1 et al. (56) studied hexokinase in egg and embryo homogenates of sea urchin (Arbacia punctulata). The enzyme was shown to have a pH optimum of 7. The substrate specificity of the enzyme was similar to that of the other animal hexokinases. The Km for the substrates that were examined fell in about the same range (2 to $8 \times 10^{-5} M$).

Bivalve hexokinase. Mekata et al. (71) observed that the hexokinase of the fresh-water mussel (Hyopsis schlegeli) phosphorylated fructose but not glucose.

Worm hexokinase. Bueding and MacKinnon (7) purified the hexokinase of the parasitic worm, Schistosoma mansoni, 15- to 20-fold with the aid of calcium phosphate and alumina C γ gels. They found that the worm contained four distinct hexokinases: (1) glucokinase, (2) fructokinase, (3) mannokinase and (4) glucosaminekinase. The glucokinase was inhibited by ADP, G6P, sorbose-1-phosphate (SIP), and glucosamine-6-phosphate. The inhibition of the glucokinase by ADP was noncompetitive with respect to ATP. The Km for glucose, Mg^{++} and ATP were higher for worm glucokinase than for those of mammalian brain hexokinase. Worm fructokinase did not phosphorylate L-sorbose in contrast to fructokinase of mammalian liver and muscle. Worm glucokinase was inhibited by sulfhydryl inhibitors and such inhibition was reversed by glutathione.

Bacterial Hexokinases

Echinococcus hexokinases. Agosin and Aravena (1) partially purified the hexokinases of hydrated cyst scolices of Echinococcus

granulosus. They isolated four separate hexokinases: (1) glucokinase, (2) fructokinase, (3) mannokinase and (4) glucosaminekinase.

2-Deoxy-D-glucose was not phosphorylated by any of the hexokinases.

Glucose-, manno- and fructokinase were inhibited by G6P, while M6P inhibited glucose- and mannokinase. Phosphorylation of fructose by fructokinase was competitively inhibited by ADP. Glucokinase was inhibited by p-chloromercuric benzoate (PCMB) and the inhibition was reversed by cysteine.

Spirochaeta hexokinase. Smith (84) examined a hexokinase of the particulate system of Spirochaeta recurrentis. The enzyme showed maximum specificity toward glucose and mannose, while fructose and L-sorbose were phosphorylated to a lesser extent. Galactose was not active. Glucosamine and N-acetylglucosamine inhibited glucose phosphorylation. The enzyme was quite sensitive to low concentrations of ADP.

Pseudomonas hexokinases. Klein (55) observed that extracts of Pseudomonas putrefaciens catalyzed phosphorylation of glucose and glucosamine. The hexokinase of the extracts showed a very low affinity for fructose (0 to 10 per cent relative to glucose) and mannose (0 to 5 per cent). Phosphorylation was not inhibited by 0.012M G6P. Sulfhydryl (SH) poisons inhibited the hexokinase and the inhibition was reversed by cysteine.

Hochster and Watson (45) reported that extracts of Pseudomonas hydrophila had a distinct pentokinase (a xylokinase) that catalyzed phosphorylation of xylose ($K_m = 2.3 \times 10^{-3}M$).

Escherichia hexokinase. Cardini (8) partially purified the hexokinases of Escherichia coli. Extracts of E. coli phosphorylated glucose, fructose and mannose at relative rates of 1.00:0.14:0.29, respectively. He observed that acetone or alcohol precipitation removed the phosphorylating ability of the extracts for fructose, while that for glucose and mannose remained constant, indicating that a separate kinase was responsible for fructose phosphorylation in the extracts.

Staphylococcus hexokinase. Cardini (8) found the hexokinase of Staphylococcus aureus was active only with glucose as the substrate. He observed that normal cells of E. coli and S. aureus could be induced to phosphorylate galactose in the carbon 1 position by growing the cells on lactose.

Mycobacterium phlei inorganic polyphosphate glucokinase. Szymona and Ostrowski (92) isolated an inorganic polyphosphate glucokinase from Mycobacterium phlei which appeared to phosphorylate glucose by a direct transfer of phosphate from inorganic polyphosphates containing more than four phosphate residues. The apparent K_m for inorganic polyphosphate and glucose were $1.75 \times 10^{-4} M$ and $2.8 \times 10^{-4} M$, respectively.

Plant Hexokinases

Yeast hexokinase. Berger et al. (6) and Kunitz and MacDonald (57) reported methods for crystallizing hexokinase of yeast at the same time. Both methods were based mainly on fractional precipitation of the enzyme with ammonium sulfate and ethanol from a toluene treated extract. The enzyme was crystallized from an ammonium sulfate solution and recrystallized up to six times.

Darrow and Colowick (23) reported a simpler method that gave a good yield and high purity. Their method employed fractional precipitation of the enzyme with ammonium sulfate, absorption and elution from bentonite gel and repeated crystallization from ammonium sulfate. The method gave about 150-fold increase in specific activity over the crude homogenate.

The K_m for substrates and K_i for inhibitors, as well as relative maximum velocities (compared to glucose) reported by Darrow and Colowick are given in Table 1. The specificity of the enzyme for substrates and inhibitors, except for G6P, was similar to that of brain hexokinase. However, the K_m 's were a magnitude smaller and yeast hexokinase failed to phosphorylate mannoheptulose. Gottschalk (39) presented evidence that indicated that the furanose ring configuration of fructose might be the form which was active with yeast hexokinase.

The K_m for ATP and Mg^{++} were found to be 10×10^{-5} (68) and $260 \times 10^{-5} M$ (6), respectively. The enzyme was specific for ATP. Deoxy-ATP, ITP, UTP, CTP (cytosinetriphosphate), CTP, deoxy-CTP, deoxy-GTP and adenosine tetraphosphate were ineffective as substitutes for ATP (23) in the reaction. The requirement for Mg^{++} was not replaced or antagonized by Ca^{++} (2).

Kaji et al. (50) showed that crystalline hexokinase, which was prepared by the method of Darrow and Colowick (23), had a weak ATPase activity which paralleled the hexokinase activity through six crystallizations. Hexokinase inhibitors such as N-acetylglucosamine

and sorbose-1-phosphate inhibited the ATPase activity of the hexokinase preparations. They observed parallel inactivation of the ATPase and hexokinase activities by various amounts of silver nitrate. Chromatography of the crystalline hexokinase on DEAE-cellulose gave fractions in which the ATPase activity/hexokinase activity was identical. The K_m of the ATPase for ATP was $5 \times 10^{-3}M$ compared to $1 \times 10^{-4}M$ for that of the hexokinase. The hexokinase and ATPase were found to have distinctly different specificity towards nucleotides.

Berger et al. (6) reported that thiol compounds provided no protective action for their crystalline enzyme, while Bailey and Webb (3) reported that all SH poisons, including Lewisite, were powerful inhibitors of crystalline yeast hexokinase. Dixon and Needham (25) reported inhibition of yeast hexokinase by mustard gas, while Stromme (91) demonstrated that disulfiram was a potent inhibitor of the enzyme, as was diethyldithiocarbamate when oxidized to disulfiram by cytochrome C. Barnard and Ramel (4) obtained results that indicated one to four -SH groups per molecule were required for the active center(s) of yeast hexokinase and that these groups were not normally available for reaction with -SH reagents, but probably became available after a time-dependent structural change occurring at $30^{\circ}C$. Berger et al. (6) found that fluoride at $0.125M$ did not inhibit crystalline hexokinase when Mg^{++} and phosphate were present in concentrations of 6.5 and $1 \times 10^{-3}M$, respectively, but Bailey and Webb (3) observed that $0.04M$ NaF inhibited hexokinase activity 46 per cent.

Glucose-6-phosphate has been reported to be both inhibitory to (101) and without effect (13, 102) on yeast hexokinase. Fromm and

Zewe (33) presented data that was interpreted as indicating that G6P acts as a competitive inhibitor of both forward reaction substrates. They suggested that the concentrations of ATP used by other investigators may have been too high to see inhibition. Trayser and Colowick (96) reported the dissociation constant of G6P to be $4 \times 10^{-3} \text{M}$, while Hammes and Kochavi (42) found it to be $1 \times 10^{-2} \text{M}$.

Strauss and Moat (89) reported that biotin stimulated fermentation of glucose and fructose by air-dried yeast grown in a medium deficient in the vitamin. Biotin also stimulated hexokinase activity in extracts from cells grown on deficient medium. Trayser and Colowick (94) found fully active yeast hexokinase did not contain biotin. The results indicated that the enzyme was not a metalloprotein and did not contain a readily demonstrable prosthetic group.

Crystalline yeast hexokinase has been reported to be a protein of the albumin type with a maximum molecular weight (MW) of 96,000 (3, 57) and a minimum MW of 30,000 (57). Its isoelectric point and greatest stability is at pH 4.8 (3, 57). It has a pH optimum at pH 7.5 (88). It has a turnover number (TN) of 13,000 moles per 10^5 g protein per minute at 30°C and pH 7.5 (6). The Q_{10} of the reaction between 0 and 30°C has been reported to be approximately 1.9 (6) and is rapidly inactivated at temperatures above $55\text{--}60^\circ\text{C}$ (88).

Six-times crystallized hexokinase was separated into at least two major components by either starch gel electrophoresis or DEAE-cellulose column chromatography (23). The forms, A and B, obtained by column chromatography were reported by Darrow and Colowick (22) to be indistinguishable in specific activity, in K_m values for substrates

and in sensitivity to inhibition by various substrates. However, Trayser and Colowick (97) reported that the crystalline enzyme was separated into six molecular forms (isozymes) of equal specific activity by DEAE chromatography. The isozymes showed different catalytic properties with respect to K_m , V_{max} , pH optima, ATPase activity and sensitivity to inhibitors.

Berger et al. (6) found that the crystalline enzyme showed a loss of activity when highly dilute, which could be prevented by diluting the enzyme in the presence of small amounts of insulin (6 micrograms per ml) or serum albumin (60 micrograms per ml). Glucose, fructose and to some extent mannose, prevented inactivation of the enzyme in crude preparations and by trypsin. Insulin also protected against inactivation by dilute alkali.

Several investigators (35, 52, 80), have demonstrated the reversibility of the hexokinase reaction by measuring exchange of either C^{14} -labeled glucose between G6P and glucose or P^{32} -labeled ADP between ADP and ATP. However, the equilibrium greatly favored G6P synthesis.

Agren and Engstrom (2) isolated phosphoserine from an acid hydrolyzate of purified yeast hexokinase incubated in P^{32} -labeled ATP or G6P. They suggested that the hexokinase reaction involves the formation of a stable phosphoenzyme intermediate. Najjar and McCoy (77) ruled out the phosphoenzyme hypothesis for yeast hexokinase when they found no exchange of phosphorus between C^{14} -labeled glucose and C^{12} -labeled G6P (or vice versa) in the presence of the enzyme. They then postulated the following mechanism for the reaction:

They argued that the formulation accounted for: (a) the lack of exchange of phosphorous between glucose and G6P, (b) labeling of the enzyme with G6P³² or ATP³² on the assumption that labeling of phosphoserine might have been due to the transfer of phosphorous during acid hydrolysis of the protein, (c) the marked inhibition of the enzyme by G6P, and (d) C¹⁴-glucose labeled the enzyme. They contended that when G6P accumulates, the greater part of the enzyme would exist in the phosphoglucose-enzyme form, thereby bringing about a corresponding reduction in the glucose-enzyme concentration which would retard the forward rate of the first reaction and consequently inhibit the overall reaction. They reported that C¹⁴-labeled glucose labeled 30-60 per cent of the enzyme. One flaw in their formulation is that there is no report in the literature that G6P markedly inhibits yeast hexokinase.

Fromm and Zewe (33) examined the kinetics of the yeast hexokinase reaction and they interpreted their results as being consistent with the "classical" mechanism in which glucose and ATP add randomly to the enzyme and equilibrium kinetics prevail. Their data indicated that participation of either a phosphoenzyme complex or a glucoenzyme complex was unlikely. Their results showed that mannose and ADP behaved as competitive inhibitors of glucose and ATP, respectively. Inhibition by AMP was competitive with respect to ATP and noncompetitive with respect to glucose.

Trayser and Colowick (95, 96) come to essentially the same conclusions as Fromm and Zewe (33) since they could not detect either a phosphoenzyme complex or a glucoenzyme complex in their kinetic studies of the yeast hexokinase reaction.

Hammes and Kochavi (41, 42, 43) did a detailed study of the kinetics of the yeast hexokinase reaction. They derived from their data what they considered to be the most probable mechanism for the reaction. It involved the combination of MgATP and a glucoenzyme complex to form two quaternary intermediates which in turn decomposed to MgADP and a dissociable 6-phosphoglucoenzyme complex. They presented the following compulsory pathway mechanism which is similar to that postulated for brain hexokinase:

- (1) $Mg^{++} + ATP \longrightarrow MgATP$
- (2) $E + G \longrightarrow E - G \text{ complex}$
- (3) $E - G + MgATP \longrightarrow X_1 \longrightarrow X_2 \longrightarrow E - G_6P + MgADP$
- (4) $E - G_6P \longrightarrow E + G_6P$
- (5) $MgADP \longrightarrow Mg^{++} + ADP$

where E, G, X_1 , X_2 and G_6P are enzyme, glucose, first quaternary complex, second quaternary complex, and glucose-6-phosphate, respectively. They did point out, however, that it is possible that both substrates may have to be present at the same time in order for the enzyme to have the correct conformation for phosphate transfer. Their data revealed that Mg^{++} may not be a very important factor for binding of the substrate to the enzyme, but that the primary role of Mg^{++} most likely is to polarize the oxygen-phosphate bond that is being broken, while anchoring the phosphate group of ATP to the enzyme. Nuclear

magnetic resonance studies indicated that binding of MgATP to the enzyme might occur through the adenine and/or ribose portion of ATP.

Hammes and Kochavi (42) observed a large difference in the binding constants for glucose and G6P by the enzyme and interpreted it as indicating that either the hydroxyl group at carbon atom 6 of glucose was important in the binding process or that the electrostatic effect of the charged phosphate group inhibited binding of G6P to the enzyme. They further concluded that G6P broke binding of MgATP very effectively. It can be seen from the foregoing that the mechanism of the yeast hexokinase reaction is unclear, and Fromm and Zewe (33) point out that the differences may be in the assumptions made and in the interpretation of data.

Aspergillus hexokinase. Davidson (24) purified the hexokinase of Aspergillus parasiticus 225-fold. He employed fractional precipitation with ammonium sulfate (50-70%), followed by fractionation with cold acetone (38-46%), negative absorption to alumina C_γ gel, and refractionation with ammonium sulfate (60-80%). The purified enzyme phosphorylated D-glucose, D-galactose, D-glucosamine, D-galactosamine, D-mannose and D-fructose at the relative rates (based on glucose) of 1.00:0.86:0.58:0.40:0.67:0.36, respectively. L-sorbose and L-arabinose were active to a very small extent. The enzyme phosphorylated galactose and galactosamine to yield the respective 6-phosphates. The K_m for glucose and galactose were almost identical ($1.6 \times 10^{-4}M$ compared to $4.3 \times 10^{-4}M$). They interpreted their data as indicating that all the substrates were phosphorylated by the same enzyme and that the substrate specificity suggested that the hexokinase was different from that of either the yeast or brain enzyme.

Neurospora hexokinase. Medina and Nicholas (70) purified the hexokinase of Neurospora crassa 60-fold. The enzyme phosphorylated glucose and at a lower rate, mannose, fructose and glucosamine. The enzyme was noncompetitively inhibited by G6P and was competitively inhibited by N-acetylglucosamine. Iodoacetate, EDTA and PCMB were also inhibitors of the enzyme.

Higher plants. Saltman (82) demonstrated the occurrence of hexokinase in several plants and purified the soluble hexokinase of wheat germ 5-fold. The hexokinases of the plants that were examined were distributed between insoluble and soluble fractions. The distribution depended on the tissue and the method of preparation of the tissue.

An insoluble hexokinase preparation was used for characterization of the enzyme. Saltman's results, however, showed that the soluble and insoluble enzymes were almost identical in their properties.

The enzyme phosphorylated glucose, fructose, mannose and glucosamine, in the presence of ATP and Mg^{++} , at the relative rates 1.00:0.62:0.68:0.52, respectively. The K_m for glucose was $4.4 \times 10^{-4} M$ and that for ATP was $8.7 \times 10^{-4} M$, while the K_m for glucose by the soluble enzyme was $4.6 \times 10^{-4} M$. Galactose, ribose, arabinose, ribulose, adenosine, glyceraldehyde, dihydroxyacetone, mannitol and glucose-1-phosphate (GIP) were inactive as substrates. Inosinetriphosphate (ITP) was 35 per cent as effective as ATP. Magnesium ion activation was optimum at a concentration of the ion equal to the concentration of ATP. Activation by Mn^{++} was 80 per cent as effective as Mg^{++} at 0.01M, while Co^{++} was ineffective. Cupric, zinc and mercuric ions strongly inhibited the

enzyme. Potassium, sodium and ammonium ions neither activated nor inhibited the enzyme. Substances which influence -SH groups had little effect on either the soluble or insoluble hexokinase. Zinc ion inhibited the enzyme noncompetitively. Dinitrophenol ($5 \times 10^{-3}M$) inhibited phosphorylation 70 per cent, while G6P ($5 \times 10^{-3}M$) only inhibited 17 per cent. The wheat germ hexokinase was found to be similar to yeast hexokinase.

Mekata et al. (71) demonstrated the occurrences of hexokinase in a variety of higher plants.

Itoh (48) partially purified hexokinase from homogenates of soybeans, Ozuki beans and mung bean by fractional precipitation of the crude homogenates with ammonium sulfate. The hexokinase of soybean precipitated at about 60 per cent saturation. The highest activity of soybean hexokinase appeared three days after germination at $26^{\circ}C$.

Itoh and Inouye (49) separated three different hexokinases of soybean by fractional precipitation with ammonium sulfate. They identified: (1) a glucokinase, specific for glucose and glucosamine, (2) a fructokinase and (3) a galactokinase, specific for galactose and galactosamine.

Comparison of Animal and Plant Hexokinases

It appears that animal and plant hexokinases are similar in substrate specificity and, in many cases, K_m . The principal differences appear to be the following:

- (1) Hexose-6-phosphates produce a more marked inhibition of animal than of plant hexokinases.

- (2) ADP inhibition of plant hexokinases appears to be competitive with respect to ATP, while in animals it appears to be of a more complex nature.
- (3) The hexokinase reaction catalyzed by brain hexokinase from animals seems to follow a compulsory pathway mechanism involving a stable phospho- or glucoenzyme complex(es). Although there is disagreement, it appears that the enzyme in plants might promote a random interaction of glucose and ATP in the presence of Mg^{++} , without the formation of stable phospho- or glucoenzyme complexes (33, 95).

Hexokinase and Sugar Uptake

The subject of sugar uptake by various animal tissues has been thoroughly reviewed (18, 72) and will not be gone into in detail, except to point out a few findings concerning the role of hexokinase in sugar uptake.

Lundsgaard (65) first proposed that the active absorption of sugars depended upon the sequential phosphorylation and dephosphorylation of the actively absorbed sugar. He later abandoned the hypothesis. Drabkin (26) revived the hypothesis and proposed that the driving force of active absorption involved the phosphorylation of the sugar outside the cell by hexokinase and dephosphorylation inside the cell by glucose-6-phosphatase.

Sols (87) and Crane and Krane (22) have studied the specificity of intestinal hexokinase and sugar absorption, respectively, and have demonstrated the specificity of the enzyme to be contrary to that of absorption. Dratz and Handler (27) have reported that the labeling of

the sugar-phosphate pool with P^{32} is inconsistent with the phosphorylation-dephosphorylation hypothesis. Landau and Wilson (61) concluded from their data that absorbed glucose does not pass through the G6P pool of hamster intestine.

Crane (18) points out that, in the intestine, all actively absorbed sugars have the common structure:

Sugars that are not actively absorbed have a large (3-O-butyl-D-glucose) or ionized substituent on some part of the structure or lack one of the essential features of the glucose molecule (2-deoxy-D-glucose or fructose). It also appears that the pyranose form is essential; specifically the C1 chair form. He also points out that the configuration specificity at carbon atom 2 doesn't necessarily hold for other animal tissues. He observed that the process of sugar absorption by the intestine follows Michaelis-Menten kinetics, requires the presence of the free sugar, and doesn't depend on the gross metabolism of the substrate since 3-O-methyl-D-glucose, which is absorbed at a great rate, is not metabolized.

Crane et al. (19) and Crane (21) observed that sugar uptake by the intestine depends upon the presence of Na^+ ions and that the extent of accumulation depends on the external concentration of Na^+ . They proposed that glucose, on entering the brush border cells of the intestine, combines with a carrier- Na^+ complex. The complex moves through the diffusion barrier to the interior of the cell where the Na^+ portion

of the complex is extruded back through the diffusion barrier in the reverse direction by an energy requiring process, which is inhibited by strophanthidin, leaving the glucose "trapped" in the cytoplasm (e.g. the so-called sodium "pump" involving the membrane ATPase system).

Cirillo (9) has shown that the yeast cell transports various nonfermentable sugars across the cell membrane and that both nonfermentable and fermentable sugars appear to share a common membrane transport mechanism.

Morgan et al. (76) have demonstrated, from their studies of glucose transport and phosphorylation in the perfused heart of normal and diabetic rats, that glucose uptake is controlled by the combined operation of two sequential steps, membrane transport and intracellular phosphorylation, and that in the steady state the net rate of membrane transport and intracellular phosphorylation are equal. In the absence of insulin, glucose uptake is limited by membrane transport. In the presence of insulin, glucose uptake is accelerated and glucose phosphorylation is increased. Phosphorylation becomes increasingly limiting as the external concentration of glucose is raised and provides the major limitation to glucose uptake. They point out that the apparent K_m for glucose phosphorylation by hexokinase in diabetic heart muscle is at least 7 times higher than in normal tissue. They found insulin in vitro to have no large, immediate effect on glucose phosphorylation.

Randle (79) reported that the uptake of glucose and the accumulation of D-xylose in isolated rat diaphragm are accelerated by inorganic phosphate, G6P, G1P, F6P, FDP, AMP and ATP. He points out that the effect is not marked and that perhaps it is the phosphate

group which is responsible for the acceleration. Anoxia and 2,4-dinitrophenol were also found to accelerate glucose uptake, but such treatment also increased the rate of uptake of substances (sorbitol) which are not ordinarily absorbed by diaphragm tissue. He suggests that uptake might be regulated by phosphorylation and dephosphorylation of a membrane carrier.

Horecker et al. (46) observed that a mutant strain of E. coli (MM6), which utilized galactose much more rapidly than glucose, in contrast to the wild type, has a galactokinase that has a much higher affinity for ATP than the glucokinase of the cell. The galactokinase is also inducible in the wild type cells. When ATP is limiting in extracts of either strain, galactose is a strong inhibitor of glucose phosphorylation. The inhibition also occurs in vivo in the mutant, but doesn't in the wild type.

The findings of Humphreys and Garrard (47) with respect to glucose uptake by corn scutellum slices are reviewed in the introduction and discussion sections of this dissertation.

Glasziou (37, 38) has reported detailed studies of sugar uptake and transformation in immature sugar cane internodal disks. His results do not indicate a role for hexokinase in sugar uptake by that tissue.

MATERIALS AND METHODS

Plant Materials

Corn grains (Zea mays L., var. Funk's G-76) were soaked twenty-four hours in running tap water and planted, scutellum-side up, on four layers of moist filter paper on trays. The trays were covered with aluminum foil and placed in the dark at 23°C for five days. The scutella were removed from the germinated grains and placed in ice-cold glass distilled water until all the scutella were harvested. All subsequent steps were carried out in the cold or in an ice-water bath.

Preparation of the Enzyme

Extraction. The scutella were weighed and then ground in a chilled Waring Blendor for one and a half minutes in four volumes (w/v) of ice-cold 0.005M ethylenediaminetetraacetate (EDTA), 0.005M magnesium chloride ($MgCl_2$) and 0.01M potassium chloride (KCl), pH 7.0. The use of either two volumes or six volumes of extracting solution decreased recovery of the hexokinase. Cysteine (0.005M) or glutathione (0.01M) did not increase recovery.

The homogenate was squeezed through two layers of cheesecloth and the filtrate was centrifuged at 0°C and 2,000XG for one hour. The supernatant fraction was filtered through glass wool to remove the fatty layer and was then centrifuged at 0°C and 32,000XG for one hour. The supernatant fraction was filtered through glass wool and saved. This fraction was designated "crude homogenate."

Ammonium sulfate fractionation. The crude homogenate was made 50 per cent saturated in ammonium sulfate ($(\text{NH}_4)_2\text{SO}_4$) by slow addition of the salt to the magnetically stirred solution. The solution was allowed to equilibrate, with stirring, for fifteen minutes after the addition of the salt. The solution was centrifuged at 12,000XG for twenty minutes at 0°C. The precipitate was dissolved in a minimum amount of 0.05M potassium phosphate buffer, pH 7.0, and was dialyzed against several four liter changes of the same buffer for forty-eight hours. This fraction was designated "F-1".

The 50 per cent saturated supernatant fraction from the above centrifugation was made 75 per cent saturated in ammonium sulfate and handled in the same manner as F-1. After dialysis the dissolved protein fraction was designated "F-2" and the supernatant fraction, "F-3". The F-2 fraction contained most of the recoverable activity (Table 3) and was further purified by three successive treatments with alumina C γ gel. The first two treatments absorbed substantial amounts of the ATPase (Table 4) and the final treatment absorbed the hexokinase which was used for this research. The following paragraph describes the procedure that was used to purify the enzyme with alumina C γ gel.

Absorption and elution from alumina C γ gel. The F-2 fraction was treated with solid alumina C γ gel (7.4 per cent solids, Sigma Chemical Company) as follows: (1) The F-2 fraction was treated with 0.0137g gel per ml (equivalent to 2.4 mg solids per ml). The gel was dispersed by stirring with a ground-glass homogenizer and was allowed to stand, with occasional stirring, for about thirty minutes. The

mixture was centrifuged at 0°C and 12,000XG for twenty minutes. The supernatant fraction was decanted and saved for further treatment. The precipitate was dispersed and eluted for four hours with a volume of 0.2M ammonium sulfate equal to one-third the volume of the F-2 fraction. The mixture was centrifuged at 0°C and 12,000XG for twenty minutes. The supernatant fraction was decanted, adjusted to pH 7-7.5 with solid tris(hydroxymethyl)aminomethane base (tris base), portioned among several plastic centrifuge tubes and frozen at -20°C until needed. This adjustment was necessary because the enzyme was inactivated during storage at pH's below approximately 7. This eluate was designated "A". The precipitate was eluted a second time with the same volume of 0.4M ammonium sulfate and treated in the same manner as the previous eluate. It was designated "A-1". (2) More gel (0.063 g per ml, equivalent to 4.7 mg solids per ml) was added to the supernatant fraction from the prior gel treatment. The mixture was handled as before and the two eluates designated "B" and "B-1". (3) To the supernatant fraction of the second gel treatment was added 0.0951 g per ml of gel (equivalent to 7.1 mg solids per ml) and the mixture centrifuged and eluted as before. The two eluates were designated "C" and "C-1". Fractions C and C-1 were the preparations used to obtain the results reported in this paper. Both preparations had identical Km for glucose, ATP and Mg^{++} and were considered to be the same enzyme. They contained some adenosinetriphosphatase (ATPase) and phosphoenolpyruvic phosphatase (PEPase) activity. Sodium molybdate ($Na_2Mo_2O_7$), 0.002M, completely inhibited the PEPase. Fractionation of C and C-1 with solid ammonium sulfate, which resulted in 80 to

90 per cent recovery, did not remove the two interfering enzymes and only succeeded in concentrating them along with the hexokinase.

Assay Methods

Two methods were used for the assay of hexokinase activity and for characterizing the enzyme.

Method 1. The rate of glucose-6-phosphate (G6P) formation at 25°C was measured by following nicotinamide adenine dinucleotide phosphate (NADP) reduction in the presence of excess glucose-6-phosphate dehydrogenase (G6PD) spectrophotometrically at 340 millimicrons. The standard reaction cuvette contained the following: glucose, 20 micromoles; $MgCl_2$, 20 micromoles; Adenosine-5'-triphosphate, 20 micromoles; NADP, 1 micromole; Tris buffer, pH 8.0, 180 micromoles; G6PD, 1EU; hexokinase preparation and water to 3.2 or 3.3 ml. The blank cuvette did not contain G6PD. This method was used to measure: (1) the rate of phosphorylation of glucose and fructose (coupled to phosphoglucosomerase), (2) competitive inhibition by nonsubstrates and glucose-1-phosphate (G1P), (3) nucleotide activation and inhibition, (4) metal activation, and (5) pH and temperature optima, which were examined by running the hexokinase reaction at the various pH's and temperatures in a total volume of 6 ml with twice the above ingredients, except for NADP and G6PD. The reaction was stopped at the end of 10 min by placing the reaction tube in a boiling water bath for 2 min. A 3 ml aliquot was assayed for G6P with NADP and G6PD.

Method 2. The rate of adenosine-5'-diphosphate (ADP) production at 25°C by hexokinase was determined by measuring the oxidation of reduced nicotinamide adenine dinucleotide (NADH) in the presence of

excess phosphoenolpyruvate (PEP), pyruvic kinase (PK) and lactic dehydrogenase (LD) spectrophotometrically at 340 millimicrons. The standard reaction cuvette contained the following: Tris buffer, pH 8.0, 180 micromoles; NADH, 0.282 micromoles; PEP, 10 micromoles; $\text{Na}_2\text{Mo}_2\text{O}_7$, 6 micromoles; MgCl_2 , 20 micromoles; ATP, 10 micromoles; PK, 1 EU; LD, 1 EU; hexokinase preparation and water to 3.2 or 3.3 ml. The blank cuvette contained no NADH and a control was included, which contained no sugar, to measure ATPase activity. This method was used to measure: (1) the rate of phosphorylation of mannose, 2-deoxy-D-glucose, L-glucose, L-mannose, N-acetyl-glucosamine, ribose, xylose, and galactose, and (2) inhibition by nonsubstrates and the hexose-6-phosphates, namely, G6P, 2-deoxy-D-glucose-6-phosphate (2DOG6P) and mannose-6-phosphate (M6P). Mannose-6-phosphate inhibition could not be measured by the G6PD reaction because it contained fructose-6-phosphate (F6P) which was converted to G6P by phosphoglucisomerase in the scutellum hexokinase preparation.

Activity determinations by the two methods gave identical rates for glucose after correcting for ATPase activity. With both methods the observed rates were constant over at least sixteen minutes after an initial one or two minute lag period.

Assay for phosphofructokinase, phosphoglucomutase and glucose-6-phosphatase activities. Phosphofructokinase was assayed by coupling the phosphorylation of F6P to NADH oxidation in the presence of excess aldolase, triosephosphate isomerase (TPI), and α -glycerophosphate dehydrogenase (α -GPD). Phosphoglucomutase activity was determined by Method 1 above, using G1P as the substrate. Glucose-6-phosphatase

was measured by the same method after incubation of the hexokinase preparation with a known amount of G6P for ten minutes.

Protein Determination

Protein was estimated using the Folin-Ciocalteu reagent method of Lowery et al. (64) as described by Layne (59). The colorimetric readings were made on the Beckman DU Spectrophotometer at 720 millimicrons. Crystalline bovine serum albumin was used as the standard.

Chemicals and Enzymes

All the chemicals and enzymes used in this investigation were of the highest purity obtainable commercially. The enzymes were purchased from the California Foundation for Biochemical Research. The nucleotides, NADP, NADH, PEP, G6P, M6P, F6P, 2DOG, and 2DOG6P were obtained from Sigma Chemical Company. The other chemicals were obtained either from the above firms, or from Nutritional Biochemicals Company or Fisher Scientific Company.

RESULTS

Purification

The hexokinase in the crude homogenate could not be assayed satisfactorily because of interfering enzyme activities.

Table 3 outlines the results of a typical purification of the hexokinase from the scutellum. Although the amount of hexokinase in the crude homogenate could not be estimated accurately, Table 3 shows an "apparent" 30-fold increase in specific activity of the 50 to 75 per cent saturated ammonium sulfate fraction (F-2) over the crude homogenate. From Table 3 it can be seen that almost all of the hexokinase activity was precipitated between 50 and 75 per cent saturation with ammonium sulfate. The F-2 fraction contained the following enzymes which might interfere with either one or the other assay: glucose-6-phosphatase (G6Pase), phosphoenolpyruvic phosphatase (PEPase), phosphoglucoisomerase (PGI), and adenosinetriphosphatase (ATPase), but did not contain phosphoglucumutase (PGM), phosphofructokinase (PFK), 6-phosphogluconic dehydrogenase and enzymes that destroy reduced NAD or NADP. The G6Pase activity should not have interfered seriously with the G6PD assay method because the excess G6PD in the reaction mixture should act as a trap for the G6P produced by the hexokinase.

Treatment of the F-2 fraction with alumina C_γ gel resulted in a 2.3-fold increase in specific activity of the C-1 fraction over the

TABLE 3
PURIFICATION OF HEXOKINASE

Fraction	Specific activity (micromoles/min/mg protein)	Total activity (units*)	% Recovery from F-2 fraction
Crude homogenate	0.002	2.80	---
F-1 (0-50% $(\text{NH}_4)_2\text{SO}_4$)	0.002	0.57	---
F-2 (50-75% $(\text{NH}_4)_2\text{SO}_4$)	0.060	12.60	---
A { 1st alumina C_γ }	0.026	0.64	5.1
A-1 { gel treatment }	0.012	0.11	0.9
B { 2nd alumina C_γ }	0.070	1.83	14.5
B-1 { gel treatment }	0.049	0.81	6.4
C { 3rd alumina C_γ }	0.102	3.45	27.4
C-1 { gel treatment }	0.141	3.91	31.0

*One unit = amount of enzyme that will phosphorylate 1 micromole of glucose per minute at 25°C.

F-2 fraction. This amounted to an "apparent" 70-fold increase over the crude homogenate. When the F-2 fraction was dialyzed against the extraction solution (EDTA-Mg-KCl) the ATPase activity paralleled the absorption and the elution of the hexokinase activity from the gel, but dialysis of the fraction against 0.05M potassium phosphate buffer, pH 7.0, changed the absorption and elution characteristics of the protein with respect to the gel so that the ATPase activity could be satisfactorily separated from the hexokinase activity as is shown in Table 4. Buffers such as potassium phosphate, glycylglycine and tris at several concentrations and several pH values were ineffective in eluting the hexokinase from the gel. Tris buffer in concentrations above 0.4M inhibited the hexokinase activity irreversibly. The C and C-1 fractions, which were used as the enzyme preparations in these investigations, contained PEPase activity which was completely inhibited by 0.002M sodium molybdate, a small amount of ATPase activity (Table 4) for which a correction was applied, and PGI activity. The G6Pase activity of the F-2 fraction was not absorbed onto the gel.

The ATPase activity of the F-2 fraction was neither decreased by centrifuging the crude homogenate fraction at 105,000XG for one and one-quarter hours nor was it inhibited by ouabain (0.0005M to 0.005M) or by fluoride (0.002M to 1.2M). The addition of Mg^{++} but not Na^{+} or K^{+} was required for ATPase activity. Since the content of ADP in the reaction mixture was increased by the addition of AMP, part of the "apparent ATPase" activity was due to adenylic kinase.

Further purification of the enzyme by acrylamide gel electrophoresis was attempted. The F-2 fraction separated into 11 or 12 bands

TABLE 4
ATPASE ACTIVITY OF THE HEXOKINASE PREPARATIONS

Fraction	Specific activity (micromoles/min/mg protein)	Total activity (units*)	% Recovery from F-2 fraction
F-1	0.002	0.51	---
F-2	0.096	20.10	---
A	0.076	1.33	6.6
A-1	0.233	1.79	8.9
B	0.058	1.04	5.2
B-1	0.134	1.55	7.7
C	0.025	0.58	2.9
C-1	0.020	0.38	1.9

*One unit = amount of enzyme that will produce 1 micromole of ADP per minute at 25°C.

in the positive direction, while the C and C-1 fractions separated into four bands--two large bands between two smaller bands. The four bands had Rf's corresponding to four similar bands separated from the F-2 fraction. Hexokinase activity could be detected in one of the bands, with Rf of 0.45-0.60, by slicing out the band, placing it in a reaction cuvette and following the reduction of NADP at fifteen-minute intervals up to one and one-half hours. The activity, 0.160 micromoles/cuvette/hour, was small and attempts to elute a significant amount of the hexokinase from whole gel slices or homogenized slices with several concentrations of glucose, phosphate buffer, ammonium sulfate or glycylglycine buffer were unsuccessful. Most of the ATPase activity in the gel was localized in a band with Rf of 0.77-1.0 (calculated with reference to the distance traveled by the salt front). Such a band had an activity of 0.037 micromoles/cuvette/hour. The other bands also had some ATPase activity.

Acrylamide gel electrophoresis of commercial crystalline hexokinase (CalBiochem) also gave four bands that had a separation pattern similar to that of the C and C-1 fractions with the two large bands having Rf's almost identical to those of the C or C-1 fractions. Again the recovery was small. Approximately 28 units of activity were added to each gel and at the end of the run the gel was cut into small pieces and eluted with 1 ml of 0.04M glycylglycine buffer, pH 7.5, for thirty minutes. The resulting eluate had an activity of 0.224 units/ml or about 0.9 per cent recovery.

Attempts to purify the enzyme by absorption and elution from bentonite by the method described by Darrow and Colowick (23) were

unsuccessful. The hexokinase and ATPase activities were absorbed but could not be eluted.

Fractionation of the enzyme preparations at any step in the outlined procedure with cold acetone or ethanol (-7°C) resulted in a complete loss of activity.

Humphreys and Garrard (47) found that slices of the corn scutellum take up glucose at the rate of 70-80 micromoles/g fresh weight/hour (approximately 1 unit per g of tissue) but do not accumulate it. Therefore, if it is assumed that all the glucose entering the slices is phosphorylated, the hexokinase content of the tissue should be much higher than that recovered. Since the F-1 and F-2 fractions would account for only about one-fifth of the total apparent hexokinase activity of scutellum, attempts were made to increase the yield. Reduced glutathione or cysteine in concentrations of 0.005M and 0.01M in the extracting solution did not increase the yield of hexokinase. Including glucose in concentrations of either 1 or 10 per cent in the extracting solution or in the crude homogenate also did not increase the yield. Detergents such as deoxycholate (0.026 per cent) and Triton X-100 (0.1 per cent) not only failed to increase the yield, but also increased the solubility of lipid in the crude homogenates so that it could not be removed by centrifugation followed by filtering through glass wool. The lipid interfered with protein precipitation in the subsequent salt fractionations by causing the protein to float to the top of the centrifuge tubes with the lipid. The floating material was difficult to collect quantitatively and the hexokinase activity in this material was lower than that obtained by the method described in

the materials and methods section. Dialysis of the floating layer did not solubilize the enzyme and treatment with cold acetone or ethanol at -7°C to remove the lipid destroyed the hexokinase activity. Itoh (48) was able to use the floating layer to study the hexokinase of soybean. Dimethylsulfoxide (DMSO) at concentrations of 0.1 and 1.0 per cent in the extracting solution did not affect the yield. Extracts of acetone powders prepared from scutella yielded smaller amounts of hexokinase activity.

The endosperm of the germinated corn grains did not contain detectable hexokinase activity.

Substrate Specificity

Sugars. The specificity of scutellum hexokinase for sixteen sugars was examined. The Michaelis constants (K_m), maximum velocities (V_{max}) and relative V_{max} with respect to glucose are given in Table 5. The enzyme phosphorylated D-glucose, D-mannose, D-fructose, 2-deoxy-D-glucose and D-glucosamine. Glucose had the lowest K_m ($6.4 \times 10^{-5} \text{M}$) and the greatest V_{max} . The substrate concentration versus rate curve for glucose and the corresponding Lineweaver-Burk plot (62) are presented in Figures 1 and 2, respectively.

Nucleoside triphosphates. The K_m for ATP was found to be $8 \times 10^{-5} \text{M}$. Figures 3 and 4 show the curves from which this value was calculated. Commercial preparations of UTP (uridinetriphosphate), GTP (guanosinetriphosphate), CTP (cytidinetriphosphate) and TTP (thymidinetriphosphate) were found to be poor substitutes for ATP at the concentrations that were examined (Table 6). The best substitute for ATP was UTP which gave 28 per cent of the rate of 0.0015M ATP at a concentration of 0.003M .

TABLE 5

SUBSTRATE SPECIFICITY OF CORN SCUTELLUM HEXOKINASE

Substrate	K _m (MX10 ⁵)	V _{max} **	Relative V _{max}
D-glucose	6.4	0.047	1.00
D-mannose	9.3	0.028	0.59
D-fructose	168	0.046	0.97
2-deoxy-D-glucose	30	0.044	0.94
D-glucosamine	37	0.030	0.63
N-acetyl-D-glucosamine	55*		
D-xylose	870*		
α-methyl-D-glucoside	--		
3-O-methyl-D-glucose	--		
L-glucose	--		
L-mannose	--		
6-deoxy-L-mannose	--		
D-galactose	--		
D-galactosamine	--		
D-ribose	--		
α-D-glucose-1-phosphate	--		

*Inhibition constant (K_i)

**O.D./2 min/cuvette at 25°C

--No detectable activity or inhibition at 0.03M

The low rates obtained by these compounds could have been due to a small amount of nucleoside diphosphokinase activity for which the preparations were not assayed. Saltman (82) observed that inosine-triphosphate (ITP) was 35 per cent as effective as ATP as a phosphate donor in the hexokinase reaction catalyzed by an insoluble hexokinase from wheat. Walaas and Walaas (99) could not detect hexokinase activity with ITP, GTP or UTP with their preparations from muscle tissue.

Metal Activators

Divalent metal cations were required for scutellum hexokinase activity. Table 7 gives the K_m and a comparison of the activation of scutellum hexokinase by Mg^{++} , Co^{++} and Mn^{++} . Figures 5, 6 and 7 show the metal concentration versus rate curves and the Lineweaver-Burk plots for the three metal cations. Magnesium ion was by far the best activator. An inhibition of magnesium activation was observed when the molar ratio of ATP/Mg exceeded approximately four. When the ratio was 7.5 micromoles/1 micromole or 7.5/2 micromoles the rate, compared to 2 micromoles/1 micromole or 2 micromoles/2 micromoles, was 20 and 10 per cent lower, respectively, and caused the Lineweaver-Burk plot for Mg^{++} to deviate from linearity. Therefore, the ratio was always maintained less than four.

Manganese ion was inhibitory at concentrations greater than the concentration of ATP (0.003M) (Table 8 and Figure 5) which indicates a maximal activation by the ion when the molar ratio ATP/Mn is one. The inhibition did not occur when such a ratio was maintained. Such inhibition is similar to that observed by Walaas and Walaas (99).

Figure 1. Effect of glucose concentration on the rate of phosphorylation. The standard reaction mixture was used except the glucose concentration was varied.

Figure 2. Lineweaver-Burk plot of the effect of glucose concentration on the rate of phosphorylation.

TABLE 6
EFFECT OF NUCLEOSIDETRIPHOSPHATES
AS SUBSTITUTES FOR ATP

Nucleosidetriphosphate	Concentration ($\text{M} \times 10^3$)	% of Rate Obtained with $1.5 \times 10^{-3} \text{M}$ ATP*
UTP	1.5	19
	3.1	28
GTP	1.5	14
	3.1	17
CTP	1.5	14
	3.1	19
TTP	1.5	15
	3.1	19

* Mg^{++} concentration constant at $6 \times 10^{-3} \text{M}$

Figure 3. Effect of ATP concentration on the rate of phosphorylation, and competitive inhibition by ADP and AMP. Reaction cuvettes contained: 180 micromoles Tris buffer, pH 8.0; 20 micromoles glucose; $MgCl_2$ in amounts equal to, or greater than, the concentration of inhibitor plus ATP; 1 micromole of NADP; 1 EU G6PD; 10 micromoles of ADP or 20 micromoles of AMP; 0.1 ml enzyme preparation and water to 3.3 ml.

Figure 4. Lineweaver-Burk plots of the effect of ATP concentration on the rate of phosphorylation, and competitive inhibition by ADP and AMP.

TABLE 7
ACTIVATION OF HEXOKINASE BY METAL IONS*

Ion	Km ($\text{MK}10^5$)	Vmax (O.D./2 min/cuvette)	% Vmax Relative to Mg
Mg^{++}	20	0.040	100
Co^{++}	70	0.025	63
Mn^{++}	100	0.020**	50**

*Ratio of ATP/metal ion was always maintained at less than 4.

**Calculated from extrapolated Lineweaver-Burk plot. Actual observed relative Vmax was 38 per cent.

Figure 5. Effects of Co^{++} and Mn^{++} concentrations on the rate of phosphorylation. The standard reaction mixture was used, except the concentration of ATP was 10 micromoles per cuvette.

Figure 6. Effect of Mg^{++} concentration on the rate of phosphorylation. The standard reaction mixture was used except: for the first two concentrations of Mg^{++} , 2.0 micromoles ATP were used and for the last four concentrations, 7.5 micromoles ATP were used.

Figure 7. Lineweaver-Burk plots of the effect of Mg^{++} , Co^{++} and Mn^{++} concentration on the rate of phosphorylation.

No inhibition by Mg^{++} and Co^{++} was seen at the concentrations employed in these experiments.

The addition of potassium ion or sodium ion did not cause activation of the enzyme.

Inhibitors

Sugars. Of the nonsubstrates listed in Table 5, N-acetylglucosamine and D-xylose were found to be competitive inhibitors of glucose with K_i of $55 \times 10^{-5}M$ and $870 \times 10^{-5}M$, respectively. Figure 8 shows the Lineweaver-Burk plots from which the K_i in Table 8 were calculated. The other nonsubstrates had no inhibitory activity.

Nucleoside di- and triphosphates. ADP and AMP were found to be competitive inhibitors of ATP with K_i of $14 \times 10^{-5}M$ and $1 \times 10^{-3}M$, respectively. Figures 3 and 4 show the curves for inhibition by $0.003M$ ADP and $0.006M$ AMP. UTP, GTP, TTP, CTP, UDP and GDP were found to have little inhibitory activity with respect to ATP at the concentrations that were examined (Table 8). The slight inhibition of hexokinase by these six compounds was not increased by increasing the concentration of the nucleotides.

Sugar phosphates. No inhibition of glucose phosphorylation was observed with G6P, F6P, M6P, G1P, galactose-6-phosphate (Gal6P) or ribose-5-phosphate (R5P) at concentrations of the sugar phosphate up to $0.033M$. The concentration of glucose in these experiments was that which resulted in about half maximal velocity ($6 \times 10^{-5}M$).

Anions. Fluoride in concentrations of 0.002 to $1.2M$ did not inhibit scutellum hexokinase. $MgCl_2$ and $MgSO_4$ were equally effective in activating the enzyme.

Figure 8. Lineweaver-Burk plots of the competitive inhibition of glucose phosphorylation by xylose (—●—) and N-acetylglucosamine (—△—). The standard reaction mixture was used except for 50 micromoles xylose or 20 micromoles N-acetylglucosamine per cuvette. Glucose alone (—○—).

TABLE 8
EFFECT OF NUCLEOSIDE DI- AND TRIPHOSPHATES AS INHIBITORS
OF ATP IN THE HEXOKINASE REACTION*

Nucleotide	Concentration** ($\text{M} \times 10^3$)	% of Rate Relative to $1.5 \times 10^{-3} \text{M}$ ATP Alone
UTP	---- 3.1	-- 95
GTP	1.5 3.1	91 89
TTP	---- 3.1	-- 80
CTP	---- 3.1	-- 96
ADP	---- 3.1	-- 42
UDP	1.5 3.1	80 80
GDP	1.5 3.1	90 90

* Mg^{++} concentration constant at $6 \times 10^{-3} \text{M}$

**ATP concentration was $1.5 \times 10^{-3} \text{M}$

pH and Temperature Optima

A pH optimum (Figure 9) was observed at pH 8.0, while the optimum temperature for scutellum hexokinase was found to be 49°C (Figure 10).

This optimum is similar to that observed for wheat germ hexokinase (82), muscle hexokinase (99) and honey bee hexokinase (81). The scutellum hexokinase lost activity rapidly when stored at pH's below 7.

The temperature optimum (Figure 10) is much higher than that reported for other hexokinases. Hexokinase isolated by Saltman (99) from wheat germ had a temperature optimum of 37°C but Taha and El-Towesy (93) reported that phytases from several seeds including barley and wheat, had temperature optima in the vicinity of 51°C. Berger *et al.* (6) found that the Q_{10} for yeast hexokinase was close to 2 over the range of 0-30°C. The Q_{10} of the hexokinase from the corn scutellum (Figure 10) is 2 between 20 and 30°C but falls off at temperatures above and below this range. The Q_{10} might vary with the enzyme, the components of the reaction mixture and at higher temperatures can be a reflection of the rate of denaturation and the rate of the reaction.

Figure 9. Effect of pH on the rate of phosphorylation expressed as the per cent of the maximum rate obtained. The reaction tubes contained the reactants in the same molar concentrations as for the standard reaction cuvettes with 0.2 ml of enzyme preparation in a total volume of 6 ml except they were buffered by 300 micromoles/tube of Tris or glycylglycine buffer at the various pH's and contained no G6PD or NADP. The reaction was stopped at the end of 10 minutes by placing the tube in a boiling water bath for 2 minutes. A 3 ml aliquot was assayed for G6P with G6PD and NADP.

Figure 10. Effect of temperature on the rate of phosphorylation. The reaction tubes contained the reactants in the same molar concentrations as for the standard reaction cuvette with 0.2 ml of enzyme preparation (C-1) in a total volume of 6 ml, but did not contain G6PD or NADP. The reaction was stopped at the end of 10 minutes by placing the tube in a boiling water bath for 2 minutes. A 3 ml aliquot was assayed for G6P with G6PD and NADP.

DISCUSSION

The results indicate that the pattern of substrate specificity of scutellum hexokinase is similar to that of yeast hexokinase. The K_m of the scutellum enzyme for D-glucose, D-mannose, 2-deoxy-D-glucose and ATP are of the same order of magnitude as those of the yeast enzyme (Table 1). The K_m for D-fructose is an order of magnitude larger. However, Gottschalk (39) has shown that the furanose ring configuration of fructose is the true hexokinase substrate and, at equilibrium, about 20 per cent of a fructose solution is in the furanose form. If this is the case with the scutellum enzyme, the true K_m would be about five times smaller than the value given in Table 5. The K_m for glucosamine is an order of magnitude smaller than that of yeast hexokinase.

The most significant difference between the hexokinase from the scutellum and that from brain or yeast is the relative rate of phosphorylation of the other four substrates compared to glucose. Glucose is phosphorylated at a rate greater than any of the other substrates. Apparently the scutellum enzyme shows a greater specificity with respect to differences at carbon atom 2 of the glucose molecule than does the yeast or brain hexokinases.

Sois and Crane (86) suggest that the coefficient of phosphorylation ($\frac{V_{max} \text{ substrate}}{V_{max} \text{ glucose}} \times \frac{K_m \text{ glucose}}{K_m \text{ substrate}}$) is a true measure of the physiological suitability of a substrate for hexokinase. With the scutellum enzyme the coefficients of phosphorylation for D-glucose, D-mannose,

2-deoxy-D-glucose, D-glucosamine and D-fructose are 1.00:0.41:0.30:0.11:0.04, respectively. These values indicate that D-glucose is the most suitable physiological substrate for the scutellum hexokinase. Correcting for the furanose configuration of fructose would place fructose closer to (about the same as 2-deoxy-D-glucose), but still lower than, glucose in suitability as a substrate.

The results from the limited studies of the substrate specificity and of inhibition by the various nonsubstrates are essentially in agreement with the findings of Sols et al. (88) for yeast hexokinase and of Sols and Crane (86) for brain hexokinase. The scutellum hexokinase is specific for the D-configuration since the L- forms of the substrates are neither phosphorylated nor are they inhibitory. The five-carbon sugars (ribose and xylose) are not phosphorylated but xylose is a competitive inhibitor. The enzyme also shows specificity for hydroxyls at carbon atoms 1 (G1P and α -methyl-D-glucoside) and 3 (3-O-methyl-D-glucose) and for inversion of the hydroxyl group at carbon atom 4 since galactose and galactosamine were not active as substrates or inhibitors. Inversion of the hydroxyl at carbon atom 2 might account for the low rate of phosphorylation of mannose and the inversion at carbon atom 3 would account for ribose being noninhibitory. No analogs of glucose which lack hydroxyls at carbon atom 6 were tested except for the hexose-6-phosphates and the fact that they were not inhibitory suggests that scutellum hexokinase requires a hydroxyl at that position for binding. This is supported by the fact that xylose is a weak competitive inhibitor.

Activation of the scutellum hexokinase by Mg^{++} , Co^{++} and Mn^{++} closely resembles that observed by Walaas and Walaas (99) for muscle

hexokinase, although inhibition by Mg^{++} and Co^{++} was not observed at the concentrations used in the experiments. Saltman (82) observed that Mn^{++} was 80 per cent as effective as Mg^{++} for activating the insoluble hexokinase of wheat germ while Co^{++} was completely ineffective. The K_m ($2 \times 10^{-4} M$) for activation of the scutellum hexokinase by Mg^{++} is a magnitude smaller than that reported by Walaas and Walaas (99) for the muscle enzyme and by Berger *et al.* (6) for the yeast enzyme.

The results show that ADP and AMP appear to act as competitive inhibitors of ATP. The K_m for ATP and the K_i for ADP suggests that the enzyme shows similar affinity for both nucleotides. Similar inhibition has been demonstrated for yeast hexokinase (33) and honey bee hexokinase (81). Inhibition by ADP with respect to ATP for brain hexokinase has been reported to be noncompetitive (27) or of a complex nature (32). In the experiments reported in this investigation the magnesium ion concentration was maintained at levels equal to, or greater than, the concentration of substrate (ATP) plus inhibitor (ADP or AMP) so that there would be no inhibition caused by competition for magnesium ion.

Inhibition of the scutellum hexokinase reaction by G6P or by other hexose-6-phosphates, as suggested by Humphreys and Garrard (47) from their studies of glucose-uptake by scutellum slices, was not observed in these investigations. The levels of ATP used in these experiments, 0.003 and 0.006M, may have masked inhibition, as has been suggested by Fromm and Zewe (33) for yeast hexokinase, but such inhibition must not have been very large. It may be that there is another glucose-phosphorylating enzyme in the scutellum but the results indicate that there are none.

When one considers that the role of the scutellum in the germinating seedling is essentially that of a "sucrose factory" and that it is capable of absorbing and utilizing glucose at a rapid rate (1 micromole/min/g fresh weight at 30°C) it seems reasonable that the sucrose and energy synthesizing systems of the scutellum should be efficient ones and may be analogous to the glycogen synthesizing system of rat liver studied by Vinuela *et al.* (98). They demonstrated that there was a glucokinase in the liver which was distinguishable from the typical animal hexokinase of that organ. The glucokinase had a higher K_m for glucose, was not inhibited by G6P and phosphorylated glucose at a rate which was comparable to the rate of glycogen synthesis in the liver. Leloir *et al.* (60) reported that uridine diphosphoglucose-glycogenglucosyltransferase was activated by high levels of G6P. The system might also be similar to starch synthesizing system in seeds which appear to be highly specific towards substrates (30). Also, the hexokinase of yeast, an organism which utilizes sugars as its principal source of energy, is not inhibited to any extent by its products (13, 33, 83, 102).

Humphreys and Garrard (47) observed that preincubation of scutellum slices in water prior to introduction of glucose into the bathing medium, increased the rate of glucose uptake by the slices. There was a concomitant decrease in G6P in the slices with the length of preincubation which they interpreted as indicating that G6P was acting as a competitive inhibitor of glucose phosphorylation and thereby limiting glucose-uptake. They observed similar inhibition with preincubation in mannose solutions, which could be partially reversed by washing the

mannose from the slices. The residual inhibition was attributed to the high level of M6P. The results reported in this paper suggest that G6P and M6P are not inhibitors of scutellum hexokinase, and the factors controlling glucose uptake in the scutellum must be other than inhibition of the hexokinase reaction by hexose-6-phosphates. M6P has not been found to be an inhibitor of brain (16) or yeast hexokinase (83), but might well be inhibiting some step in the utilization of G6P. 2-Deoxy-D-glucose has been shown to be a competitive inhibitor of G6P for the phosphoglucosomerase of rat kidney (103). Both the availability of ATP at the site of the hexokinase reaction and ADP inhibition could affect the rate of glucose phosphorylation and consequently the rate of glucose uptake. Preincubation might cause an increase in available ATP and removal of inhibitory ADP. At the same time, ATP availability and nucleosidediphosphate inhibition could be affecting the utilization of G6P by limiting the rate of uridinediphosphoglucose pyrophosphorylase through the nucleoside diphosphokinase reaction, and preincubation would reflect increases in high-energy phosphate compounds at these sites with an accompanying decrease in G6P. Various investigators (30, 34) have demonstrated strong interrelationships between nucleotides and between nucleotide-sugar compounds in starch and sucrose synthesis. Morgan et al. (76) have shown that glucose-uptake in the heart of rat is controlled by both transport and intracellular phosphorylation. When membrane transport is not limiting (i.e. sufficient insulin is available) the rate of phosphorylation limits the rate of glucose-uptake at high levels of exogenous glucose.

It is believed that the results presented in this paper support the conclusion that the hexokinase of the corn scutellum is more correctly a glucokinase which is not inhibited by its product, G6P.

SUMMARY

A hexokinase was extracted from the scutellum of corn and purified with an "apparent" 70-fold increase in specific activity. It was free of interfering enzyme activities and appeared to be the only soluble hexokinase of the scutellum. The enzyme showed greatest specificity towards glucose as the substrate and the data supports the conclusion that the hexokinase is more specifically a glucokinase. The enzyme phosphorylated D-glucose, D-mannose, D-fructose, D-glucosamine and 2-deoxy-D-glucose. It was specific for ATP as the phosphate donor. N-acetylglucosamine and xylose were competitive inhibitors of glucose phosphorylation, while ADP and AMP were competitive inhibitors of ATP. The divalent cations of magnesium, cobalt and manganese activated the enzyme with cobalt and manganese ion being 63 and 38 per cent as effective as magnesium, respectively. The hexokinase was not inhibited by hexose-6-phosphates at concentrations up to 500-times the glucose concentration. Optimum activity of the enzyme was observed at pH 8.0 and 49°C.

The properties of the hexokinase from the scutellum are compared with those of the brain and yeast hexokinases and are discussed in relation to glucose-uptake by the scutellum.

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BIOGRAPHICAL SKETCH

Herbert Charles Jones III was born October 6, 1936, in Leesburg, Lake County, Florida. He attended public schools in Ocala, Florida and was graduated from Ocala High School in June, 1954.

He attended the Colorado School of Mines and the University of Florida before entering the United States Air Force in 1956. While in the service he married the former Winifred Catherine Gassaway of Jacksonville, Florida. After two years of active duty with the Air Force he returned to the University of Florida and was graduated August, 1960, with the Degree of Bachelor of Science in Forestry.

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He is a member of the following honorary societies: Xi Sigma Pi, Gamma Sigma Delta, Alpha Zeta and Phi Sigma.

This dissertation was prepared under the direction of the chairman of the candidate's supervisory committee and has been approved by all members of that committee. It was submitted to the Dean of the College of Agriculture and to the Graduate Council, and was approved as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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