Some Properties of a Zinc-Dependent Hexokinase from *Neurospora crassa*

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Hexokinase which phosphorylates glucose, fructose and mannose at the relative rates of 100:140:30 has been crystallized from yeast (Berger, Slein, Colowick & Cori, 1946; Bailey & Webb, 1948; Kunitz & McDonald, 1946) and its activity determined in animal tissues (Colowick, Cori & Slein, 1947; Slein, Cori & Cori, 1950; Colowick, 1951; Crane & Sols, 1953; Weil-Malherbe & Bone, 1951). A glucokinase which phosphorylates glucose but not fructose or mannose has been reported in extracts of muscle and liver (Cori & Slein, 1947; Colowick, 1951). A specific fructokinase has been found in pea seeds (Medina & Sols, 1956).

Few purified kinases have been prepared from bacteria. A glucokinase specific for the conversion of glucose into glucose 6-phosphate (G 6-P) has been found in *Staphylococcus aureus*, *Micrococcus pyogenes* var. *aureus* and in *Escherichia coli* (Cardini, 1951). Hexokinase activity was shown in extracts of *Leuconostoc mesenteroides* (De Moss, 1953), *Streptococcus faecalis* (Sokatch & Gunsalus, 1954), *Lactobacillus bulgaricus* (Rutter & Hansen, 1953) and *Clostridium butyricum* (Gavard, 1954).

In this paper some properties of a hexokinase from *Neurospora crassa* are described.

MATERIALS AND METHODS

Organism and methods of culture. *N. crassa* microconidial Wild type (146) was kindly supplied by Dr William D. McElroy, McCollum-Pratt Institute, Johns Hopkins University, U.S.A. The fungus was grown in the following culture solution: glucose, 20 g.; NH_4NO_3 , 3 g.; sodium tartrate, 1 g.; KH_2PO_4 , 3 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; NaCl, 0.1 g.; CaCl_2 , 0.1 g.; biotin, 5 μg ; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.96 mg.; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 8.8 mg.; $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 0.27 mg.; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 72 μg ; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 12 μg ; water, 1 l. The culture solution was dispensed in 100 ml. amounts into 500 ml. Erlenmeyer flasks; these, after sterilizing by autoclaving at 10 lb./in.² for 15 min. and cooling, were inoculated with a mycelial suspension of the fungus in glass-distilled water. The fungus was grown in the dark for 4 days at 30°. The cultures were shaken periodically to prevent sporulation. The organism, which was maintained on nutrient-agar slopes, was subcultured weekly to ensure active growth (Nicholas, 1952).

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Removal of trace metals from the culture solutions. The methods used to remove iron, copper, zinc, manganese and molybdenum from a solution of the macronutrients have been described previously (Nicholas, 1952). Special precautions were taken to avoid metal contamination; these included the use of inverted beakers instead of cotton wool to close the necks of the flasks (Nicholas, 1952).

Harvesting the fungus and preparation of extract. The mycelial felts were collected in a Büchner funnel and washed well with glass-distilled water. Surplus water was squeezed out of the felts and they were frozen for a minimum of 3 hr. at -17°. The felts were homogenized in a mortar with four times their weight of cold glass-distilled water containing 5 mm-sodium ethylenediaminetetra-acetate (sodium-EDTA) and then in a 50 ml. capacity Ten Broeck glass macerator (made to our specification by Jencons Ltd., London) at 0°. The homogenate was centrifuged at 2000 g for 5 min. Over 90% of the hexokinase activity of the homogenates was in the supernatant.

Measurement of enzyme activities. Hexokinase was determined by the method of Crane & Sols (1953). The enzyme extracts were incubated for a defined period, usually 15 min. at 30° with 6 μmoles of sugar, 15 μmoles of adenosine triphosphate (ATP; disodium salt, 98% pure, obtained from Sigma Chemical Co., St Louis, U.S.A.), 7.5 μmoles of MgSO_4 and 20 μmoles each of NaF, K_2HPO_4 and 2-amino-2-hydroxy-methylpropane-1:3-diol (tris buffer) at pH 8; final volume was 0.3 ml. The phosphorylation was measured by determining residual sugar by the method of Somogyi (1952). Fructose and fructose 6-phosphate (F 6-P) were determined by the procedure of Roe (1949). The units are defined as the number of μmoles of glucose phosphorylated/15 min.; specific activity is defined as units/mg. of protein.

Adenosine triphosphatase (ATPase) was determined by measuring the release of inorganic P from ATP as described by Gilmour (1955).

Phosphoglucose isomerase was determined by the method of Slein (1955).

Phosphorylated intermediates. After 30 min. incubation the hexokinase reaction mixture was treated with 5% (w/v) trichloroacetic acid and the esters were precipitated as barium salts, as described by Sols (1956). The barium salts were treated with Amberlite IR 120 (H) (British Drug Houses, Ltd., London) as recommended by Wade & Morgan (1955). The resulting clear solution, containing the esters, was spotted on acid-washed filter paper (Whatman no. 1) and run for 12 hr. in a mixture of 80 ml. of *tert*-butyl alcohol, 20 ml. of water and 4 g. of picric acid, as described by Hanes & Isherwood (1949). The paper was air-dried and sprayed with a mixture of 5 ml. of 60% (w/v) perchloric acid, 10 ml. of N-HCl and 25 ml. of 4% (w/v) ammonium molybdate and water to 100 ml., and dried at 85° for 7 min.

The paper was suspended for 10 min. in a glass jar containing water saturated with H_2S . The mixture of esters was also analysed chemically by the Roe (1949) method for F 6-P and for total sugar by the Somogyi (1952) method. The difference between the two assays is considered to be due to G 6-P. The presence of G 6-P in the reaction mixture was confirmed by an enzymic method: crystalline G 6-P dehydrogenase, prepared from yeast by the method of Kornberg & Horecker (1955), was added to the hexokinase reaction mixture together with 0.3 μ mole of triphosphopyridine nucleotide (TPN; 98% pure, obtained from Sigma Chemical Co.). G 6-P, which inhibits hexokinase activity, is thus oxidized to phosphogluconic acid and hexokinase is no longer inhibited.

Determination of SH groups. These were determined in the purified enzyme by the method of Grunert & Phillips (1951).

Protein. The protein content of the enzyme extracts was determined by the Folin method used by Lowry, Rosebrough, Farr & Randall (1951).

Determination of zinc and copper in protein fractions of the fungus. Protein fractions were digested in redistilled nitric acid and A.R. perchloric acid as described by Nicholas (1948). Zinc and copper were determined in the digests by the dithizone method of Holmes (1945).

Reagents. The following pure D-sugars were obtained from Kerfoot and Co. Ltd., Lancs.: glucose, fructose, glucosamine, mannose, sorbose and galactose. G 6-P and F 6-P were obtained as the barium salts from Sigma Chemical Co. The esters were made soluble by using Amberlite IR 120 (H) to adsorb the barium, as described by Wade & Morgan (1955). *N*-Acetyl-D-glucosamine and *p*-chloromercuribenzoate were supplied by L. Light and Co. Ltd., Colnbrook, England. Glutathione was obtained from Schwarz Laboratories, New York, U.S.A.

RESULTS

Purification of hexokinase. The supernatant prepared by centrifuging the homogenate was fractionated in the cold with a saturated solution of $(NH_4)_2SO_4$, and the 20–75% fraction was dissolved in water containing 5 mM-sodium-EDTA and dialysed for 12 hr. at 4° against 0.5 mM-sodium-EDTA and tris buffer, pH 8. The dialysed preparation was further fractionated and the 30–65% fraction collected. The results are summarized in Table 1. The over-all purification achieved (fraction 3) was about 60-fold; about 50% of the total

activity in the supernatant was recovered. The enzyme is strongly adsorbed by alumina C_γ gel, $Ca_3(PO_4)_2$ gel or by Norit carbon (Norit Vereeniging, Verkoop Centrale, Amsterdam), but attempts to elute the enzyme from them with pyrophosphate or tris buffers, of various molarities and at different pH values, failed to increase the specific activity of the enzyme.

Enzyme stability and pH optimum. The enzyme, fraction 3, was stable for about 10 days at -15° at about pH 8; its maximum activity was at pH 8.3–9.45.

Substrate saturation and identification of product of reaction. The dissociation constant (K_m) for the enzyme–glucose complex determined from the saturation curve is 0.54 mM. A number of related substrates have been tested for phosphorylation by the purified enzyme. The effect of inhibitors on hexokinase was also examined. The results, which are given in Table 2 and in Fig. 1, show that the enzyme is inhibited competitively by *N*-acetyl-D-glucosamine, an effect which is characteristic of hexokinase (Sols, 1956). The enzyme has the highest activity for glucose and lesser rates for glucosamine, mannose and fructose. Very low rates are recorded for sorbose and galactose, which have a different configuration from the other sugars at C_4 . The product of the enzyme reaction inhibited its activity and G 6-P was shown to inhibit the enzyme non-competitively, in agreement with the data of Crane & Sols (1954) for brain hexokinase. The progressive inhibition of hexokinase activity was prevented by adding TPN and crystalline G 6-P dehydrogenase, as shown in Table 3. This is evidence that the reaction product is G 6-P.

The product of the hexokinase reaction with glucose was identified as G 6-P by means of paper chromatography, pure esters being used as standards. F 6-P was also detected in the reaction mixture, which suggested the presence of phosphoglucose isomerase.

The total sugar phosphorylated determined in the reaction mixture (see Methods) was 51 μ moles, and the amount of F 6-P was 16 μ moles; thus the

Table 1. *Purification of hexokinase of Neurospora crassa*

The mycelium was homogenized and the supernatant from centrifuging the homogenate was fractionated as described in the text. Ammonium sulphate fractions were dialysed against 0.5 mM-sodium-EDTA in 2 mM-tris buffer (pH 8) before assay and further fractionation. Mycelia weighing 60 g. (fresh wt.) from about twenty flasks were used for purification of the enzyme. Activity of the enzyme was measured with the usual reaction mixture (see Methods).

Fraction	Total units	Total protein (mg.)	Specific activity (units/mg. of protein)	Recovery (%)
(1) Supernatant	425	234	1.81	—
(2) First $(NH_4)_2SO_4$ fractionation: fraction obtained between 20 and 75% saturation	305	10.4	29.4	71
(3) Second $(NH_4)_2SO_4$ fractionation: fraction obtained between 30 and 65% saturation	211	1.97	107	49

amount of G 6-P was 35 μ moles. The equilibrium is, therefore, 69 % G 6-P $\rightleftharpoons$ 31 % F 6-P, which is in agreement with the observed ratio (68:32) for the pure isomerase enzyme as determined by Slein (1955).

The activities of hexokinase and phosphoglucose isomerase in the preparation were: hexokinase, 63 μ moles of glucose esterified/ml./15 min.; phosphoglucose isomerase, 24 μ moles of G 6-P converted into F 6-P/ml./15 min. (determined as in Methods).

Effect of metal deficiencies. The effects of individual deficiencies of magnesium, iron, copper, zinc,

manganese and molybdenum on hexokinase are shown in Table 4. Deficiencies of magnesium, iron, copper or manganese did not markedly affect the enzyme activity. When molybdenum was deficient hexokinase increased, whereas a zinc deficiency depressed it. The effect on hexokinase activity in the felts of varying the concentration of Zn^{2+} ions in the culture solution is illustrated in Fig. 2.

Table 2. *Substrate specificity of hexokinase (fraction 3)*

(A) All sugars were tested independently as substrates with the reaction mixture described in Methods. (B) D-Glucose (3 μ moles) as substrate; 6 μ moles of the other sugars as inhibitors were added singly to the reaction mixture to determine their effect on the phosphorylation of D-glucose (see Fig. 1). K_i was calculated from the equations of Lineweaver & Burk (1934).

Substrate	A	B
	Relative rates at 6 mm. substrate	K_m/K_i
D-Glucose	1	1
D-Glucosamine	0.29	—
D-Mannose	0.23	—
D-Galactose	<0.05	—
D-Fructose	0.23	0.133
D-Sorbose	<0.05	0.044
D-Glucose 6-phosphate	—	0.208
N-Acetyl-D-glucosamine	—	0.540

Fig. 1. Effect of different inhibitors on the phosphorylation of D-glucose. D-Glucose phosphorylation, O; competitive inhibition by N-acetylglucosamine, Δ ; non-competitive inhibition by fructose, $\bullet$, by sorbose, $\blacktriangle$, by glucose 6-phosphate, $\square$. V, Amount of substrate (glucose) phosphorylated (μ moles/ml. of enzyme/15 min.); S, concentration of substrate (moles/l.) in the reaction mixture (see Methods).

Table 3. *Enzymic identification of glucose 6-phosphate as the reaction product of hexokinase*

Activity of the enzyme was measured: (A) with the usual reaction mixture (see Methods); (B) with the further addition of 0.3 μ mole of TPN and 10 μ g. of cryst. G 6-P dehydrogenase (final vol., 0.5 ml.). Figures represent μ moles of glucose phosphorylated/ml. of enzyme/15 min.

Incubation time (min.) ...	Expt. 1				Expt. 2		
	15	30	45	60	15	60	120
	A	B	A	B	A	B	A
A	10.2	6.78	4.82	3.84	5.8	4.2	2.85
B	10.12	9.67	9.78	9.82	4.3	5.3	4.07

Table 4. *Effect of metal deficiencies on hexokinase from Neurospora crassa*

Enzyme preparation, 0–100% $(NH_4)_2SO_4$ saturated, of fraction 1, was dissolved in 5 ml. of 5 mm-sodium EDTA in water and dialysed for 12 hr. against 0.5 mm-sodium-EDTA in 2 mm tris buffer, pH 8. Activity of the enzyme was measured with the reaction mixture described in Methods. Results are expressed as specific activity (μ moles of glucose esterified/15 min./mg. of protein).

Treatment of culture solns.	Mg*		Fe		Cu		Zn		Mn		Mo	
	Control	deficient	Control	absent	Control	absent	Control	absent	Control	absent	Control	absent
Expt. 1	2.33	2.49	3.25	3.3	3.17	2.80	3.09	1.47	2.60	2.20	3.17	4.13
Expt. 2	—	—	1.84	1.81	2.82	2.71	1.84	0.95	2.48	2.17	2.82	4.06
Expt. 3	—	—	—	—	2.97	2.92	2.40	1.19	—	—	2.97	3.40

* One-hundredth of the magnesium content of the complete medium.

An increase in the zinc content of the media was paralleled by an increase in hexokinase in the felts. Experiments with mixtures of enzymes prepared from normal and zinc-deficient felts showed that there was neither an inhibitor in the deficient felts nor an activator in the normal ones. ATPase activity was similar in the normal and in the zinc-deficient felts.

After 4 days' growth, zinc-deficient felts were supplied aseptically with 200 μg . of zinc per flask, and triplicate cultures were harvested at intervals within a further 24 hr. incubation period. The results are illustrated in Fig. 3. The hexokinase in the zinc-deficient mats was increased to about 90 % of that in the normal felts within 12 hr. of adding the Zn^{2+} ions. There was an initial rapid increase of activity of hexokinase, i.e. 27 % of the recovery to the normal level in the control felts within 1 hr. of adding the metal. The addition of other trace metals was without effect. The addition of a similar amount of zinc to the normal felts did not affect the enzyme.

The effect of adding graded amounts of ZnSO_4 *in vitro* to the enzyme prepared from normal and zinc-deficient felts is illustrated in Table 5. The hexokinase prepared from the zinc-deficient felts was stimulated over the range 0.59–1.19 μg . of zinc/ μmole of glucose phosphorylated, but there was no effect on the enzyme prepared from the control felts. The addition of Zn^{2+} ions to the partially purified enzyme (fraction 3) increased its activity by approximately 25 %.

Fig. 2. Hexokinase activity of *Neurospora crassa* grown with varying amounts of Zn^{2+} ions.

Effect of inhibitors. In Table 6 are given data on the effect of inhibitors on the enzyme. One of the reasons for the EDTA inhibition is presumably the chelation of zinc.

KCN, NaN_3 , $\alpha\alpha'$ -dipyridyl, 8-hydroxyquinoline, each at mM and 10 mM concentration, and sulphanilamide at 2 mM, did not inhibit hexokinase.

Fig. 3. Reconstitution of hexokinase in zinc-deficient felts. ○-○, Enzyme from organism grown in complete medium; □-□, enzyme from zinc-deficient organism; ●, 200 μg . of zinc was added aseptically to zinc-deficient felts after 4 days' growth, and enzyme production measured at intervals within a further 24 hr. incubation period.

Table 5. Effect of Zn^{2+} ions on the activity of enzyme prepared from normal and zinc-deficient felts

Enzyme preparation used was fraction 1 treated as described in Table 4. The enzyme activity was measured with the reaction mixture described in Methods, plus zinc (as ZnSO_4) at the levels indicated.

Zinc added to enzyme ($\mu\text{g./}\mu\text{mole}$ of glucose phosphorylated)	Inhibition of enzyme from control felts (%)	Inhibition (I) or activation (A) of enzyme from zinc-deficient felts (%)
0.06	0	0
0.28	0	0
0.59	0	30A
1.19	0	9A
2.2	0	9I
4.8	4	22I
19.2	30	37I
42.7	60	59I

The inhibition by iodoacetate and *p*-chloromercuribenzoate, and the reversal of the *p*-chloromercuribenzoate effect by glutathione, suggest the presence of SH groups in the enzyme.

The quotients $\mu\text{m-moles of SH}/\mu\text{mole of glucose phosphorylated}$ were 0.16 and 0.53 for the enzyme prepared from normal felts and zinc-deficient felts respectively.

Protection of SH groups by zinc. The effect of zinc in protecting the enzyme from the inhibitory action of *p*-chloromercuribenzoate is shown in Fig. 4. Maximum protection from this inhibition was afforded by incubating the normal enzyme with 1.2 $\mu\text{g.}$ of zinc and the enzyme from zinc-deficient felts with 2.5 $\mu\text{g.}$ of zinc/mole of glucose phosphorylated. The addition of copper or iron at similar concentrations was without effect on the inhibition of the enzyme by *p*-chloromercuribenzoate. The results in Table 7 show that Zn^{2+} ions cannot reverse the inhibition of the enzyme by *p*-chloromercuribenzoate when this compound is added to the enzyme first.

Table 6. *Effect of inhibitors on hexokinase*

Enzyme activity was determined as in Methods. Inhibitors were incubated with the enzyme for 30 min. at 4° before the enzyme activity was determined.

Compound added	Inhibition (%)
Sodium-EDTA (0.1M)	83
Sodium-EDTA (0.01M)	47
Iodoacetate (0.01M)	47
<i>p</i> -Chloromercuribenzoate (2 mM)	90
<i>p</i> -Chloromercuribenzoate (mM)	89
<i>p</i> -Chloromercuribenzoate (mM) + glutathione (0.01M)	4.4

Fig. 4. Effect of zinc sulphate, added *in vitro*, in protecting hexokinase from inhibition by mM-*p*-chloromercuribenzoate. O, Enzyme from normal felts; ●, enzyme from zinc-deficient felts. The percentage inhibition is computed by comparison with controls, which were treated identically except for the omission of *p*-chloromercuribenzoate.

DISCUSSION

Foster (1939) and Foster & Waksman (1939) have suggested that zinc is important for the utilization of carbohydrates by fungi, because less sugar is oxidized when there is a deficiency of the metal. The accumulation of inorganic phosphorus and reducing sugars and a decrease in sucrose and starch have been observed in zinc-deficient tomatoes (Reed, 1946). Aldolase activity may be reduced when zinc is deficient in plants (Quinlan-Watson, 1953). Nason, Kaplan & Colowick (1951) have reported an increase in diphosphopyridine nucleotidase activity in *N. crassa* Wild type (5297a) when zinc was deficient, but they found no effect on hexokinase in the crude extracts. In our experiments with Wild type (146), however, when the culture solution (which contained glucose instead of sucrose) was freed from zinc, and the enzyme then concentrated before assay, zinc was shown to be required for optimum activity of hexokinase.

Zinc appears to have two distinct effects on hexokinase: (a) *in vitro* activation of the purified enzyme, prepared from zinc-deficient felts, by about 30 % of the possible recovery to the level in the control felts; (b) the *in vivo* reconstitution of the enzyme in deficient felts to 90 % of that in control felts. Effect (a) is probably direct and immediate, involving a substrate-enzyme relation, whereas effect (b), which is considerably more than (a), is slower and is probably associated with the more complex processes of synthesis of the apoenzyme. Action (b) may be similar to the effect of zinc on the apoenzyme of carbonic anhydrase discussed by Wood & Sibley (1952). The fact that the partially purified enzyme was activated by Zn^{2+} ions indicates that the metal is essential for hexokinase action.

The precise role of zinc in kinase action is less clear. It is established that Zn^{2+} ions afford protection to the SH groups in the enzyme against inhibition by *p*-chloromercuribenzoate, but the inhibition by the latter cannot be reversed by Zn^{2+} ions.

Enzyme from zinc-deficient felts required 0.53 $\mu\text{m-mole}$ of SH to phosphorylate 1 μmole of glucose, whereas the value for the normal enzyme was only 0.16 $\mu\text{m-mole}$ of SH. Thus it appears that more than three times the number of SH groups are required in the zinc-deficient enzyme, as compared with the control enzyme, to phosphorylate the same amount of sugar. Zinc may be responsible for the activation of the SH sites during hexokinase action.

Yeast hexokinase (Sigma, Preparation II) is inhibited by *p*-chloromercuribenzoate only at a much higher concentration (10 mM), and then Zn^{2+} ions did not afford any protection against the inhibitor.

Table 7. *Effect of glutathione and Zn²⁺ ions on the inhibition of hexokinase by p-chloromercuribenzoate (PCMB)*

Enzyme (fraction 1) was prepared as described in Table 4 and determined as in Methods. PCMB (mM) was incubated with 0.1 ml. of enzyme at 4° for 30 min., then 0.05 mM-glutathione (GSH) or 4 µg. of zinc or both were added, followed by a further incubation for 30 min. before enzyme assay.

Inhibition (%)	Enzyme + PCMB	Enzyme + PCMB + GSH	Enzyme + PCMB + ZnSO ₄	Enzyme + PCMB + ZnSO ₄ + GSH
	68	25	60	20

Hexokinase from *N. crassa* may be another example of a zinc metallo-enzyme, and in this respect would resemble carboxypeptidase (Vallee & Neurath, 1954), yeast alcohol dehydrogenase (Vallee & Hoch, 1955) and glutamic dehydrogenase (Vallee, Adelstein & Olson, 1955).

SUMMARY

1. A hexokinase which phosphorylates glucose and, at a lower rate, mannose, fructose and glucosamine has been purified 60-fold from *Neurospora crassa*, Wild type (146).
2. The phosphorylation product from glucose is shown, by chromatographic and enzymic methods, to be glucose 6-phosphate.
3. The enzyme is competitively inhibited by *N*-acetylglucosamine.
4. The enzyme is inhibited by sodium ethylenediaminetetra-acetate, iodoacetate and *p*-chloromercuribenzoate. The inhibition by the last compound is reversed by reduced glutathione.
5. Zinc deficiency in the mycelial felts reduced hexokinase activity. The enzyme activity was brought up to 90 % of the control mats within 12 hr. after adding Zn²⁺ ions to the zinc-deficient culture medium. The enzyme prepared from zinc-deficient felts was activated to about 30 % of the normal activity, by adding Zn²⁺ ions to it *in vitro*, but not zinc ethylenediaminetetra-acetate.
6. Zinc sulphate gave some protection to the SH groups in the enzyme from inhibition by *p*-chloromercuribenzoate, but the metal did not reverse the inhibitory effect if added after the inhibition.
7. The possible role of zinc in hexokinase action is discussed.

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