ISOLATION OF HEXOKINASE FROM BAKER'S YEAST*

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Hexokinase was first described by Meyerhof (2), who prepared the enzyme from baker's yeast and demonstrated its stimulatory effect on the fermentation of hexoses by muscle extracts. Through the experiments of von Euler and Adler (3), and Meyerhof (4), it was established that this enzyme catalyzes a transfer of phosphate from adenosinetriphosphate (ATP) to hexoses, thus initiating the fermentation or oxidation of the latter. Colowick and Kalckar (5) showed that only the terminal phosphate group of ATP was transferred to hexose, and demonstrated that the primary products of the reaction were adenosinediphosphate (ADP) and hexose-6-phosphate. The reaction catalyzed by yeast hexokinase may thus be written as follows:—

$$\text{ATP} + \text{hexose} \rightarrow \text{ADP} + \text{hexose-6-phosphate}$$

In the present paper a method is described for the isolation of hexokinase in highly purified form from baker's yeast. Glucose, fructose, and mannose are all acted upon by the enzyme, but the rate of phosphorylation varies with the sugar used. The turnover number, when the phosphate acceptor is glucose, is 13,000 moles of substrate per 10^5 gm. of protein per minute at 30° and pH 7.5 and is not changed by repeated crystallization of the enzyme. The specific activity is approximately 30 times that of the crude yeast extract, indicating that hexokinase constitutes about 3 per cent of the extracted protein.

Measurement of Hexokinase Activity

The manometric method described by Colowick and Kalckar (5) was adopted. The method is based on the fact that one acid equivalent is liberated per mole of phosphate transferred from ATP to glucose.

A Warburg vessel, the side bulb of which contained:
0.2 cc. 0.04 M ATP[1] (sodium salt, pH 7.5)

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[1] The rate of the hexokinase reaction depends to a considerable extent on the degree of purity of the ATP sample used. When a commercial sample of ATP was used, the

0.05 cc. 0.1 M $NaHCO_3$, 0.2 M $MgCl_2$

and the main compartment of which contained:

0.5 cc. 0.054 M $NaHCO_3$

0.1 cc. 0.5 M glucose

0.75 cc. hexokinase solution

was filled with 5 per cent CO_2-95 per cent N_2 (pH 7.5 at 30°). The amount of protein used varied from 100 γ for crude extracts to as little as 2 γ for the pure enzyme. After equilibration for 10 minutes at 30°, the stopcock was closed and the contents of the side bulb and main compartment were mixed. CO_2 evolution in the absence of glucose (due to adenylpyrophosphatase action) was negligible except with crude hexokinase preparations, in which cases it was necessary to apply a small correction.

The validity of the manometric method was checked by comparing the CO_2 evolution with the amount of phosphate transferred from ATP to glucose. Samples were incubated, without and with glucose, and fixed with trichloro-

TABLE I

Comparison of CO_2 Liberated with P Transferred to Glucose in Hexokinase Test System

Experiment No.	P transferred to glucose	CO_2 liberated
	micromoles	*micromoles*
1	4.77	4.70
2	4.60	4.70
3	4.65	5.00
4	5.20	5.30
5	2.45	2.36
6	2.97	3.10

acetic acid at the end of the manometric measurement. The filtrates were analyzed for the acid-labile P of ATP by determining orthophosphate (6) before and after 11 minute hydrolysis in N H_2SO_4 at 100°. The difference in labile P in the two samples was a measure of the amount of phosphate transferred from ATP to glucose, since the hexose-6-phosphate formed is not appreciably hydrolyzed under the above conditions. From Table I it can be seen that 1 mole of CO_2 was evolved in the manometric test per mole of phosphate transferred.

Determination of Specific Activity.—One unit of hexokinase is defined as the amount of enzyme causing an initial rate of CO_2 evolution of 1 c.mm. per minute under the specified condition of the test. The initial rate was readily determined by measuring CO_2 evolution during the first 8 minutes, since,

rate was only about 65 per cent of that observed with samples prepared in this laboratory. Values for specific activity recorded in this paper were obtained with ATP which had been carefully purified and which did not contain an appreciable amount of ADP.

within certain limits of enzyme concentration, the rate was always linear during this period.

The level of purity of a given enzyme preparation is expressed in terms of the specific acitivty; *i.e.*, the number of units per mg. of protein. Protein was determined colorimetrically by means of the biuret reaction (7) after precipitation with trichloroacetic acid.

The degree of accuracy of the specific activity determination is illustrated by the data in Table II. The activity of a crude hexokinase preparation was tested over an eightfold range of protein concentration. The number of units of activity found was proportional to the amount of protein added, so that the specific activity (units per mg.) was independent of protein concentration, the average value found being 103 units per mg., with a maximum deviation from the mean of ±5 units.

TABLE II

Proportionality between Enzyme Concentration and Activity in Hexokinase Test System

Crude yeast hexokinase	CO_2 liberated (0–8 min.)	Specific activity
mg. protein per test	*c.mm.*	*units per mg. protein*
0.0109	9	103
0.0218	19	109
0.0436	34	98
0.0872	71	102

Purification

The procedure used may be outlined briefly as follows:—

(1) Autolysis of yeast with toluol, extraction with water, and precipitation of extracted proteins with 50 per cent of alcohol, essentially as described by Meyerhof (2). (2) Removal of an isoelectric precipitate. (3) Two fractionations with alcohol at 0°, the material soluble in 25 to 29 per cent alcohol but precipitated by 45 to 48 per cent alcohol being collected. (4) Adsorption of the active material on $Al(OH)_3$ gel, and elution with phosphate buffer. (5) Fractionation with alcohol at 0°, the material soluble in 40 per cent alcohol but precipitated by 57 per cent alcohol being collected. (6) Fractionation of a more concentrated protein solution with alcohol at −7°, resulting in removal of amorphous material with 20 per cent alcohol, removal of an as yet unidentified crystalline protein fraction with 24 per cent alcohol, and removal of more amorphous material with 29 per cent alcohol. (7) Crystallization from ammonium sulfate solution.

Several points in this outline deserve comment. In the early work, the extraction of the autolyzed yeast was carried out at 35°, the temperature recommended by Meyerhof (2). In later experiments the extraction was carried out

at 5°, as recommended by Kunitz and McDonald,[2] who found a greater yield of hexokinase at this extraction temperature.

It was found desirable to carry out the extraction and early fractionation procedures in the presence of glucose, since, as first reported by van Heyningen[2], crude preparations of hexokinase lose activity rapidly on standing unless glucose is present. The protective action of glucose or fructose is illustrated in Fig. 1. Incubation of a partially purified hexokinase preparation (300 units per mg. protein) in $NaHCO_3$-CO_2 buffer, pH 7.5, at 30° for 30 minutes resulted in the loss of

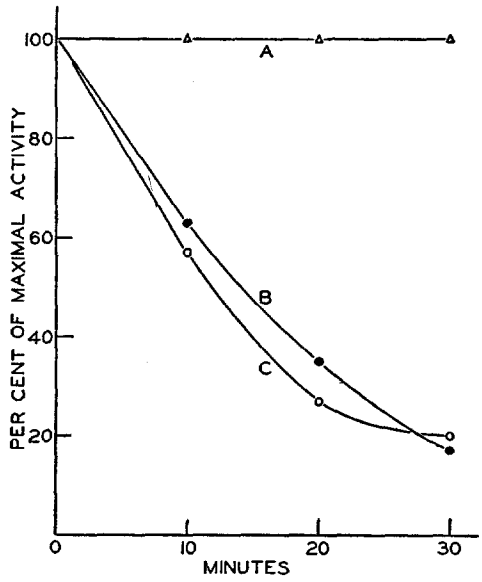

FIG. 1. Protective action of glucose or fructose on crude yeast hexokinase. 22 γ of protein per cc. (300 units per mg.) incubated at 30°, pH 7.5, and tested for hexokinase activity after 10, 20, and 30 minutes. Curve A shows the stability of the enzyme when either glucose or fructose (0.037 M) was present during the incubation period. Curves B and C show the loss in activity toward fructose and glucose, respectively, when these sugars were absent during the incubation period.

about 80 per cent of the activity, the loss of activity toward fructose and glucose occurring at about the same rate. The presence of glucose or fructose (0.037 M, the concentration used in the manometric system) completely prevented this inactivation. The concentration of glucose present during the various stages in the isolation procedure was about 1 per cent (0.055 M).

Fractional precipitation with alcohol was always carried out at acid reaction (pH 5.5) at 0° or below. Under these conditions, the total recovery of hexo-

[2] This information was made available to us through confidential reports circulated by the National Defense Research Committee.

kinase in the various fractions obtained from a given solution was practically quantitative. Attempts to fractionate with alcohol at pH 7 resulted in a complete loss of hexokinase activity.

Close temperature control was essential in carrying out fractional precipitation with alcohol. It will be noted that in some cases fractionation was carried out at 0°, in other cases at −5° to −7°. Slight variations in temperature in this range result in marked changes in protein solubility. For example, protein fractions completely soluble in 20 per cent alcohol at 0° could be largely precipitated by lowering the temperature to −5°.

Purification was achieved almost entirely by means of fractional precipitation with alcohol. The only other procedure used which resulted in marked purification was the adsorption of the enzyme on aluminum hydroxide. The chief

TABLE III

The Purification of Yeast Hexokinase

Fraction	Total protein	Total units	Recovery of units	Specific activity
	gm.		*per cent*	*units per mg. protein*
Crude extract from 11 kg. yeast	40.50	4,050,000		100
1	29.40	2,646,000	65	90
2	21.50	2,365,000	58	110
3	2.93	998,000	25	340
3*a*	2.00	800,000	20	400
4	1.18	755,000	19	640
5	0.23	368,000	9	1600
6	0.060	167,000	4	2780
6*a*	0.043	129,000	3	3000
Crystals				3000

value of this procedure lies in the fact that a large amount of gummy material of non-protein nature remains unadsorbed and can be discarded. The enzyme activity can then be recovered quantitatively, and with a considerable increase in specific activity, by elution with phosphate buffer. It will be noted that, after this purification step, the concentration of alcohol required to precipitate the enzyme is considerably higher than that required in the earlier stages.

The following is a detailed protocol for one experiment in which 43 mg. of hexokinase were isolated from 40 gm. of yeast proteins, extracted from 11 kg. of yeast. The results are summarized in Table III.

Preparation of Crude Extract.—Eleven kg. of fresh, starch-free, Anheuser-Busch[3] baker's yeast were warmed from 15° to 39° over a period of 2 hours. 750 cc. of toluol

[3] We are grateful to the staff of the Anheuser-Busch yeast plant for their cooperation in providing the facilities necessary for the large scale preparation of the crude yeast extracts.

were added and thoroughly mixed with the yeast. After 45 minutes at 37–40°, the yeast had partially liquified. 500 cc. of 50 per cent glucose were added and the mixture was stirred for an additional 45 minutes. Eleven kg. of shaved ice and 250 cc. of 50 per cent glucose were added, and the mixture was kept at 5° for 18 hours. The suspension was centrifuged at 5° in 250 cc. bottles. The slightly cloudy supernatant fluid (11.5 liters, pH 6.2) was stored at 0° overnight. This solution is designated "crude extract" in Table III.

1. Precipitation with Alcohol.—The protein of the crude extract was precipitated by the addition of an equal volume of cold 95 per cent ethanol. Care was taken to prevent the temperature from rising above 5° during the addition of the alcohol. The mixture was allowed to stand for 1 hour before centrifuging in the cold in 250 cc. bottles at 2000 R.P.M. The precipitate was suspended in 1 per cent glucose and mechanically stirred overnight at 5°. It was then centrifuged in the cold yielding about 2 liters of supernatant fluid (fraction 1).

2. Isoelectric Precipitation of Inert Proteins.—The pH of fraction 1 was adjusted at 0° to pH 4.7 by the slow addition of 75 cc. of M acetic acid. The precipitate which formed was centrifuged off in the cold, and the supernatant fluid (fraction 2) was adjusted to pH 5.6 by the addition of 30 cc. of 1.5 N NaOH. A flocculent precipitate formed but was not removed.

3. Fractionation with Ethanol at 0°.—The temperature was maintained at 0° while 900 cc. of ethanol were slowly added (final concentration of alcohol was 29 per cent). The resulting precipitate was discarded after centrifugation. The ethanol content of the supernatant fluid was increased to 45 per cent and the suspension was centrifuged in the cold. The syrupy precipitate was dissolved in 1 per cent glucose (410 cc., fraction 3).

Fraction 3 was diluted with 400 cc. of 0.1 M acetate buffer, pH 5.4, and 270 cc. of cold ethanol were slowly added (25 per cent final alcohol concentration). The precipitate obtained after centrifugation was discarded and the ethanol content of the supernatant fluid was increased to 48 per cent at 0°. The syrupy precipitate was centrifuged down and dissolved in 1 per cent glucose (200 cc., fraction 3*a*).

4. Adsorption of Hexokinase on $Al(OH)_3$.—Fraction 3*a* was diluted with an equal volume of 0.02 M acetate buffer, pH 5.4, containing 1 per cent glucose. 160 cc. of $Al(OH)_3$ gel[4] were added to the solution at 0°. The suspension was centrifuged in the cold, the supernatant discarded, and the residue washed three times with 500 cc. portions of 0.01 M acetate buffer, pH 5.4, containing 1 per cent glucose. The washed residue was eluted three times with 200 cc. portions of cold 0.1 M phosphate buffer, pH 7.2. The eluates were combined (600 cc., fraction 4).

5. Fractionation with Ethanol at 0°.—Fraction 4 was adjusted to pH 5.3 by the addition of 42 cc. of M acetic acid, and cold ethanol was slowly added until the alcohol concentration was 40 per cent. The suspension was centrifuged and the precipitate was discarded. The ethanol concentration of the supernatant fluid was increased to 57 per cent and the precipitate obtained by centrifugation in the cold was dissolved in 1 per cent glucose. A small amount of insoluble material was removed by centrifugation and the pH of the supernatant was readjusted to pH 5.4 by the addition of acetate buffer (fraction 5, 150 cc.).

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[4] Alumina C_γ, prepared as described by Willstätter (8).

6. Fractionation with Ethanol at −7°.—Fraction 5 was treated in the cold with 180 cc. of ethanol and the precipitate was suspended in 20 cc. of 0.01 M acetate buffer, pH 5.4, containing 1 per cent glucose. A small amount of insoluble material was removed by centrifugation.

The supernatant solution (23 cc.) was dialyzed in the cold for 2 hours against 2 liters of 0.01 M acetate buffer, pH 5.4, containing 1 per cent glucose. The dialyzed solution (41 cc.) was treated with 10 cc. of ethanol at 0°, cooled to −7°, and the precipitate was discarded. The alcohol content was increased to 24 per cent at −7°, and crystals (hexagonal plates) appeared within 2 days. After several more days, the crystalline material was removed by centrifugation at −5°. This crystalline protein fraction has not as yet been identified. 40 cc. of supernatant fluid were obtained (fraction 6).

The ethanol content of fraction 6 was raised to 29 per cent at 0° and the solution was cooled slowly to −5°. The material which precipitated was amorphous and was removed. The hexokinase in the supernatant fluid (fraction 6*a*) had a specific activity of 3000 units per mg. protein which could not be increased by further fractionation with alcohol or by crystallization from ammonium sulfate.

Crystallization.—Attempts to crystallize hexokinase from fraction 6*a* with ethanol under various conditions were uniformly unsuccessful. Crystallization from ammonium sulfate, under the conditions recommended by Kunitz and McDonald,[2] was readily achieved as follows:—

The ethanol content of an aliquot of fraction 6*a* was raised to 60 per cent at −4° in order to recover the protein. The precipitate was dissolved in 0.1 M phosphate buffer, pH 7.0, and saturated ammonium sulfate was added to slight turbidity. The solution was kept at 3° and long needle-like crystals of hexokinase formed in a few days. A photograph of these crystals has already been published (1).

Properties of the Crystalline Enzyme

Effect of Magnesium Ions.—The crystalline enzyme is inactive in the absence of Mg ions. In Fig. 2, the activity is plotted against the $MgCl_2$ concentration. It can be seen that the concentration required to produce one-half of the maximal activating effect is 2.6×10^{-3} M (dissociation constant for the Mg-protein complex). The concentration used routinely in the manometric tests, 6.5×10^{-3} M, is sufficient for practically maximal activity.

Sodium fluoride, which inhibits the activity of a number of enzymes requiring Mg ions, had no effect on the activity of crystalline hexokinase when tested in concentrations ranging from 0.006 to 0.125 M. Warburg and Christian (9) showed that the inhibition of enolase by fluoride depended on the presence of orthophosphate. The inorganic phosphate concentration in the hexokinase test system was approximately 0.001 M. The effect of fluoride at higher phosphate concentrations has not been investigated.

Action on Various Hexoses.—Studies on substrate specificity showed that the crystalline enzyme acted on *d*-glucose, *d*-fructose, and *d*-mannose, the relative rates with the three sugars being 1:1.4:0.3. That a single protein is

responsible for the actions on glucose and fructose is indicated by the following observation. When the enzyme was tested in the presence of a mixture of glucose and fructose, each present in sufficient concentration to saturate the enzyme, the rate was intermediate between the rates observed with glucose and fructose alone. This result suggests a competition between glucose and fructose for the same enzyme.

Effect of Proteins on Inactivation by Dilution.—The activity of the crystalline enzyme, in contrast to the crude enzyme (Table II), was not proportional to enzyme concentration, the activity at low protein concentrations being considerably lower than that calculated from the dilution. This loss of activity

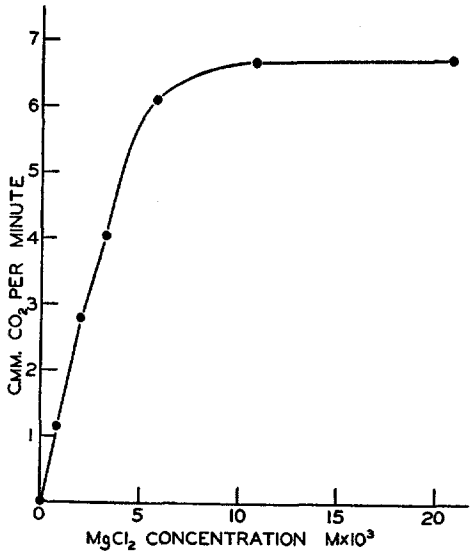

FIG. 2. Effect of Mg^{++} concentration on the activity of crystalline yeast hexokinase at 30°, pH 7.5.

on high dilution did not occur when the dilution was carried out in the presence of other proteins (*e.g.* hemoglobin, serum albumin, insulin). The amount of protein required to prevent the loss of activity on dilution varied with the different proteins.

The data in Fig. 3 show that insulin (a purified amorphous preparation supplied by Eli Lilly and Co.) was the most effective of the proteins tested, complete protection being afforded by 10γ per 1.6 cc. of reaction mixture. Its effectiveness as a protective protein was not impaired by treatment with dilute alkali under conditions known to abolish its physiological activity (10). Reduction of the insulin with cysteine by the procedure of Wintersteiner (11), which also abolishes its physiological activity, caused a marked decrease in its

protective power. Human serum albumin (a 4-times crystallized sample kindly supplied by Dr. A. A. Green) was much less effective than insulin, more than 100γ being required to protect the hexokinase completely. Solutions of crude yeast hexokinase (300 units per mg.), when inactivated by heating for 5 minutes at 100°, were about as effective as serum albumin in protecting the pure enzyme from loss of activity on dilution. Heat-inactivated crystalline hexokinase was by far the least effective as a protective protein, 160γ being required for full protection. The effect of heating on the protective power has not been investigated quantitatively; 100γ of heat-treated insulin or serum

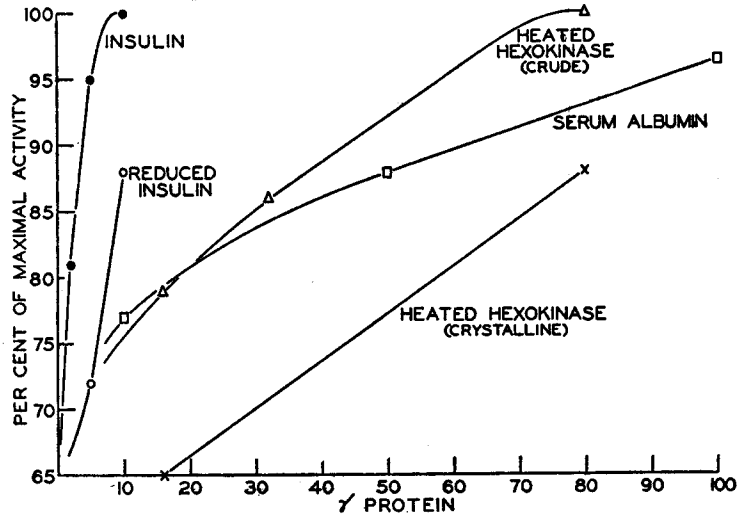

FIG. 3. Effects of various proteins on the activity of a dilute solution (6.7 γ per 1.6 cc.) of crystalline hexokinase. The activity of the enzyme in the absence of protective protein was 65 per cent of that observed when the dilution was carried out in the presence of an excess of any one of the proteins tested. Temperature 30°, pH 7.5.

albumin showed the same protective action as the unheated controls. In addition to the proteins shown in Fig. 3, crystalline horse hemoglobin and the protein of a crude aqueous extract of rabbit muscle have been tested. The former showed maximal action in a concentration of 100γ per 1.6 cc., but was not tested at lower concentrations. 10γ of the muscle protein was about as effective as 10γ of reduced insulin.

In Fig. 4, the effect of dilution on the activity of crystalline hexokinase is illustrated. In the absence of protective protein, the specific activity fell off markedly on dilution. When the dilution was carried out in the presence of an excess of insulin (100γ), the specific activity was independent of the hexoki-

nase concentration. This amount of insulin was used as a routine in testing the activity of the pure enzyme at high dilutions.

It will be noted that the activity measurements in Fig. 4 were carried out at 15° instead of at the standard temperature of 30°. This was done in order to slow down the activity with the larger amounts of crystalline hexokinase to a conveniently measurable rate. The observed specific activity at 15° (1050 units per mg.) as compared to that at 30° corresponds to a temperature coefficient

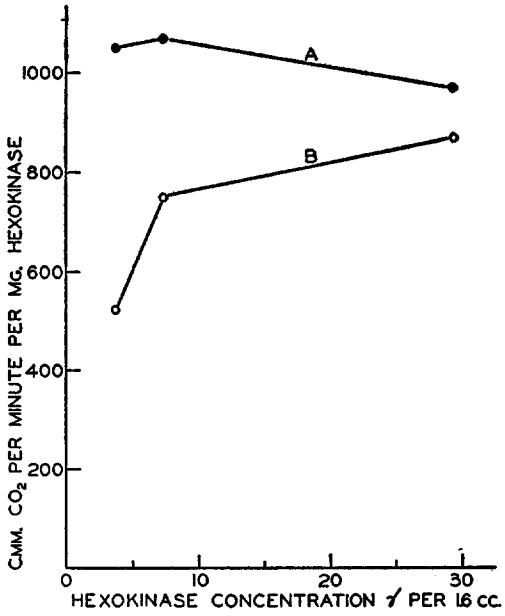

FIG. 4. Effect of dilution on the activity of crystalline hexokinase in the presence and absence of protective protein (insulin). Activity was determined at 15° at pH 7.3. Curve B shows the marked decrease in specific activity on dilution in the absence of protective protein. Curve A shows the protective action of 100 γ insulin per 1.6 cc. reaction mixture.

($Q_{10°}$) of 2.2. In another experiment in which the activity was measured at 0°, with the addition of sufficient $NaHCO_3$ (0.052 M) to maintain the pH at 7.5 at this temperature, a $Q_{10°}$ value of 1.9 was obtained for the range 0° to 30°. Even at 0°, the enzyme lost activity on high dilution, and this loss could be prevented by the presence of protective protein.

Effect of Glucose on Inactivation by Proteolytic Enzymes.—Solutions of the crystalline enzyme could be incubated in the absence of glucose without any loss of activity (Table IV), in contrast to crude preparations which lost activity rapidly unless glucose was added (Fig. 1). It seemed likely that the loss of activity in crude preparations was due to the action of proteolytic enzymes,

and if this was true, it was to be expected that glucose would protect hexokinase against proteolytic inactivation. This was tested in the experiments in Table IV. Solutions of crystalline hexokinase were almost completely (97 per cent) inactivated by incubation for 5 minutes at pH 7.6 and 20° with 100γ of crystalline trypsin,[5] when no glucose was present. When 0.17 M glucose was present, the inactivation after 5 minutes of incubation with trypsin amounted to only 17 per cent. Even after 20 minutes of incubation with this large amount of trypsin a protective action of glucose could be demonstrated. Hexokinase, when combined with glucose, appears therefore to be much more resistant to proteolytic inactivation than is the free enzyme.[6]

Effect of Reducing Agents.—Hexokinase does not appear to contain any highly reactive —SH groups with are essential to its catalytic activity. The

TABLE IV

Inactivation of Crystalline Hexokinase by Trypsin and Its Prevention by Glucose

4 samples were prepared, each containing 24 units of crystalline hexokinase in 0.1 cc. of 0.03 M potassium phosphate buffer pH 7.6, plus 0.2 cc. of the following additions: (1) 0.2 cc. H_2O; (2) 0.1 cc. 0.5 M glucose + 0.1 cc. H_2O; (3) 0.1 cc. 0.1 per cent crystalline trypsin + 0.1 cc. H_2O; (4) 0.1 cc. 0.1 per cent crystalline trypsin + 0.1 cc. 0.5 M glucose. 0.1 cc. samples were removed after 5 and 20 minutes at 20° and tested immediately for hexokinase activity by the usual procedure.

Sample No.	Units of hexokinase activity found	
	After 5 min.	After 20 min.
1	7.2	7.8
2	7.7	8.4
3	0.2	0.0
4	6.0	3.0

crystalline enzyme is optimally active without the addition of cysteine or glutathione. In no case has it been possible to demonstrate a stimulatory or protective effect of these substances on hexokinase activity. They are ineffective in preventing the loss in activity of the crystalline enzyme which occurs on high dilution, and do not protect the crude enzyme against inactivation by heat or proteolytic action.

Ultraviolet Absorption Spectrum.—It has been reported recently that muscle

[5] Kindly supplied by Dr. J. Northrop.

[6] By taking advantage of this finding, it has been possible to effect a considerable purification of crude yeast hexokinase merely by allowing a concentrated solution to stand in the presence of glucose until the bulk of the protein has been digested away. The hexokinase and other remaining proteins can then be salted out with ammonium sulfate. By this simple procedure, the specific activity of a crude preparation has been increased from 100 to 400 units per mg. of protein.

hexokinase requires both guanine and dihydrocozymase for its activity and that the latter is present in a bound form (12). Yeast hexokinase does not require the addition of these substances for its activity and the ultraviolet absorption spectrum (Fig. 5) does not reveal their presence either at 255 or 340 mμ.

SUMMARY

1. A method is described for the isolation of hexokinase from baker's yeast. The method is based mainly on fractionation with alcohol and results

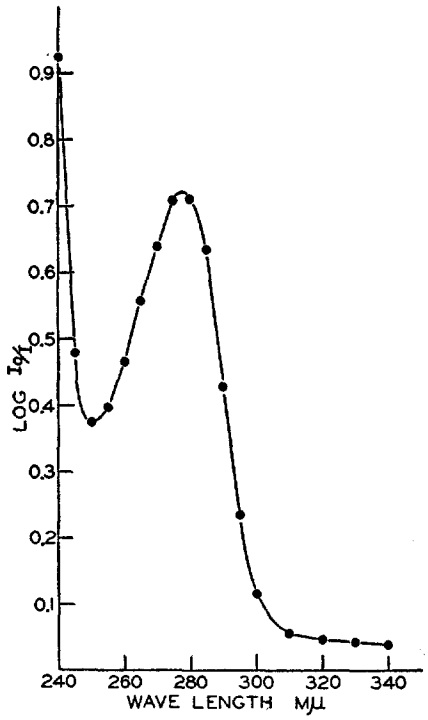

FIG. 5. Ultraviolet absorption spectrum of crystalline yeast hexokinase. Enzyme solution had been dialyzed against distilled water. Protein concentration 0.55 mg. per cc. Width of quartz cell 1 cm.

in a 30-fold increase in specific activity. The final product could be crystallized from ammonium sulfate without change in specific activity.

2. The enzyme catalyzes a transfer of phosphate from adenosinetriphosphate to glucose, fructose, or mannose, the relative rates with these three sugars being 1:1.4:0.3.

3. With glucose as substrate, the turnover number for the crystalline enzyme is 13,000 moles of substrate per 10^5 gm. of protein per minute at 30° and pH 7.5. The temperature coefficient ($Q_{10°}$) between 0 and 30° is 1.9.

4. Magnesium ions are necessary for the activity, the dissociation constant for the Mg^{++}-protein complex being 2.6×10^{-3}. Fluoride in concentrations as high as 0.125 M has no inhibitory effect on the enzyme when the Mg^{++} and orthophosphate concentrations are 6.5×10^{-3} M and 1×10^{-3} M, respectively.

5. The crystalline enzyme shows a loss in activity when highly diluted. This loss in activity can be prevented by diluting in the presence of small amounts of other proteins. Of the various protective proteins tested, insulin was the most effective, providing complete protection in a concentration of 6 micrograms per cc.; with serum albumin, a concentration of 60 micrograms per cc. was necessary. Thiol compounds (cysteine, glutathione) exerted no protective action.

6. The inactivation of the crystalline enzyme on incubation with trypsin can be prevented to a marked degree by the presence of glucose. The instability of crude preparations of yeast hexokinase may be attributed to the presence of proteolytic enzymes, since glucose or fructose has a remarkable protective effect on such preparations.

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