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HEXOKINASE

Studies on Hexokinase of Ox Brain

A Thesis
submitted to the
UNIVERSITY OF POONA
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A C K N O W L E D G E M E N T

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C O N T E N T S

LIST OF ABBREVIATIONS

ATP	Adenosinetriphosphate
CTP	Cytidinetriphosphate
ITP	Inosinetriphosphate
GTP	Guanosinetriphosphate
UTP	Uridinetriphosphate
ADP	Adenosinediphosphate
AMP	Adenosinemonophosphate
DPN	Diphosphopyridine nucleotide
DPNH	Reduced diphosphopyridine nucleotide
TPN	Triphosphopyridine nucleotide
TPNH	Reduced triphosphopyridine nucleotide
DEAE-cellulose	Diethylaminoethylcellulose
CM-cellulose	Carboxymethylcellulose
Pi	Inorganic phosphate
PPi	Pyrophosphate
Glucose-6-P	Glucose-6-phosphate
P-enolpyruvate	Phosphoenolpyruvate
Tris	Tris(hydroxymethyl)aminomethane
EDTA	Ethylenediaminetetraacetate
p-CMB	para-Chloromercuribenzoate
-SH group	Sulphydryl group
°	degree centigrade
hr	hour, hours
min	minute, minutes
g	gram, grams
mg	milligram, milligrams
µg	microgram, micrograms
x g	acceleration of gravity
S	Sedimentation coefficient
$S_{20, w}$	Sedimentation coefficient in water at 20°
O.D.	Optical density
$\triangle$ O.D.	Optical density change

CHAPTER 1

INTRODUCTION

INTRODUCTION

Hexokinase catalyses the transfer of the terminal phosphate group from a nucleotide triphosphate (usually ATP) to a hexose. After its discovery in 1927 by Meyerhof, it was shown to be widely distributed in microorganisms, plants and animal tissues. It has been partially purified from several different sources and the enzyme from yeast has been obtained in crystalline form. However, the hexokinase of animal tissues has been studied mainly in crude extracts and little progress has been made in obtaining it in highly purified form because of its attachment to insoluble particles or its instability. The present work deals with new methods for the solubilization and stabilization of ox brain hexokinase, its isolation in highly purified form and the study of its properties.

SECTION I

Historical and Scope of the Literature Survey

Historical

The presence of hexokinase was first demonstrated when a greatly increased rate of utilization of glucose was observed by the addition of an alcohol precipitated fraction of yeast autolysate to the extracts of aged muscle of frog or rabbit (Meyerhof, 1927). The presence of hexokinase in the lactic acid forming system during muscular contraction and in erythrocyte was also shown by Meyerhof (1930, 1932).

von Euler and Adler reported in 1935 that hexose monophosphate dehydrogenase system oxidized non-phosphorylated hexose when supplemented with ATP. In that system ATP could not be replaced by any other substance or combination of substances such as AMP, phosphagen, hexose diphosphate or PPi. In a yeast preparation which was active in fructose dehydrogenation, two components were shown to be present. One of these was a dehydrogenase which, when coupled with a flavin enzyme and coenzyme II, dehydrogenated hexose monophosphates but not fructose. With the other, the presence of ATP was required for the dehydrogenation of hexose. The second enzyme was found to be activated by Mg^{++} and was named "heterophosphatese". Its function was recognised as the transfer of the labile phosphate group of ATP to the 6-position of the monosaccharide. Lätwak-Mann

and Mann (1935) also showed that yeast contains an enzyme which phosphorylated glucose in the presence of Mg^{++} and ATP.

In 1935 it was shown by Meyerhof that Euler's "heterophosphatese" was identical with his hexokinase. Glucose and ATP in the presence of hexokinase gave hexose monophosphate and AMP. The reaction was given as:

$$2 \text{ Glucose} + \text{ATP} \longrightarrow 2 \text{ Glucose-6-P} + \text{AMP}$$

When this incubation mixture was boiled and a muscle extract added, lactic acid formation was observed. "Heterophosphatese" gave the same results as hexokinase. The presence of the enzyme in brain was also demonstrated.

Bauer described some of the properties of the "heterophosphatese" in 1936. Fructose, glucose and mannose were phosphorylated but not galactose or ribose. The optimum concentration of magnesium was found to be between 1 to 10 mM. The enzyme was active over a wide range of glucose concentrations and there was a broad pH optimum between 5.3 and 7.9. von Euler and his colleagues (1937) reported that brain extracts transferred labile phosphate groups of ATP to glucose in the same manner as yeast cells.

Colowick and Kalckar further clarified the action of hexokinase in 1941 when they demonstrated that only the terminal phosphate group of ATP was transferred to hexose and that the primary products of the reaction were ADP and glucose-6-P. The formation of AMP and the phosphorylation of the second molecule of

glucose by ATP, which was observed by Meyerhof, was shown to be due to the presence of myokinase in the enzyme preparation.

$$2\ ADP \rightleftharpoons ATP + AMP$$

The hexokinase reaction may, therefore, be represented as follows:

$$Glucose + ATP \xrightarrow[Mg^{++}]{} Glucose\text{-}6\text{-}P + ADP$$

or more accurately,

$$R.OH + ATP^{4-} \underset{Mg^{++}}{\rightleftharpoons} R.O.PO_3^{2-} + ADP^{3-} + H^+$$

where R.OH is the hexose. It will be seen that a proton is liberated in the reaction when the hexose is phosphorylated.

Subsequent progress in the study of the enzyme has been rapid. In 1945 the isolation of crystalline hexokinase from yeast was reported by Berger et al. and by Kunitz and McDonald. The availability of the highly purified enzyme from yeast led to detailed investigations on its properties, specificity, kinetics and mechanism of action. Hexokinase was shown to be present in a wide variety of microorganisms, plants and animal tissues. A particulate preparation was partially purified from ox brain and separated from interfering enzymes for studies on its kinetics (Crane and Sols, 1953). In addition to enzymes which phosphorylate monosaccharides in the 6-position other transphosphorylating enzymes which catalyse the phosphorylation of galactose and fructose in the 1-position have also been described. Enzymes have also been reported which catalyse the phosphorylation of hexoses with

phosphate donors other than ATP, such as inorganic polyphosphate, acetyl phosphate, and P-enolpyruvate and phosphoramidate. The presence of particulate and soluble forms as well as different types of hexokinase in animal tissues has been shown and the glucokinase of liver has been highly purified. The effect of hormones on hexokinase has been the subject of many studies which have mostly been inconclusive. A survey of the literature on hexokinase is presented in the following sections.

Scope of the Literature Survey

The name for the group of enzymes included under the term hexokinase (recommended by the International Union of Biochemistry) is Adenosinetriphosphate:D-hexose 6-phosphotransferase (EC 2:7:1:1).

The trivial name hexokinase will be used to designate this group of enzymes which catalyse phosphorylation of glucose and other D-hexoses in the presence of ATP to form hexose-6-phosphate and ADP. Kinases which phosphorylate hexoses such as fructose and galactose in the presence of ATP to form the corresponding hexose 1-phosphates will not be discussed. Only brief reference will be made to enzymes which are active with non-nucleotide phosphate donors such as acetyl phosphate, P-enolpyruvate, etc. Limitations of space also preclude detailed discussion of the enzyme from microorganisms (other than yeast) and plants or of the vast literature on hormonal effects on animal tissue hexokinases. This survey deals mainly with studies on the enzyme from yeast and animal tissues with special reference to those aspects (purification, stability, properties and kinetics) which form the subject of this thesis.

SECTION II

Microorganisms and Plants

Yeast

Isolation: Berger et al. described the preparation of crystalline hexokinase from yeast by a method which involved autolysis of yeast in the presence of toluene, precipitation with alcohol, removal of inert proteins at pH 4.7, two fractionations with ethanol at 0°, aluminium hydroxide gel adsorption and two fractionations with ethanol at 0° and -7°. The enzyme was crystallized from ammonium sulphate solutions according to the method of Kunitz and McDonald.

Kunitz and McDonald (1946) also used a yeast autolysate which was filtered and fractionated with ammonium sulphate and then with alcohol at pH 5.4. The enzyme was finally crystallized from ammonium sulphate solution. The yield of crystalline enzyme was very low in both the procedures (1 to 3 per cent). In both methods glucose (0.056 M) was added to stabilize the enzyme in the later stages of purification.

Different methods for the preparation of hexokinase from yeast were also reported by Bailey and Webb (1948), Sols and de la Fuente (1958), Ågren et al. (1960, 1963), Darrow and Colowick (1962) and Lazarus et al. (1966). According to method of Bailey and Webb, an autolysate of yeast was precipitated with ammonium sulphate and dialysed. The enzyme was then purified by calcium phosphate gel adsorption, ammonium sulphate fractionation in the presence of glucose, readsorption on calcium phosphate gel and finally ethanol precipitation

at -10° followed by crystallization according to the procedure of Kunitz and McDonald. The method of Sols and de la Fuente is primarily useful for the rapid isolation of a stable preparation free from interfering enzymes. Ågren et al. (1960, 1963) have also described a method for the purification of hexokinase by chromatography on CM-cellulose and DEAE-cellulose. The purified enzyme was found to be homogeneous by ultracentrifugal studies.

Darrow and Colowick have described an improved method for the isolation of crystalline yeast hexokinase. The enzyme was extracted from dry yeast with phosphate buffer and fractionated with ammonium sulphate. It was then adsorbed on bentonite and eluted with Tris-EDTA solutions. It was precipitated with ammonium sulphate and finally crystallized from ammonium sulphate solution. This method is relatively rapid and simple and gives a high yield (15 per cent) of enzyme and has been of value in making available large amounts of pure hexokinase.

Lazarus et al.(1966) purified the enzyme from yeast autolysates by precipitation of impurities at pH 4.5, fractionation with ammonium sulphate and chromatography on DEAE-cellulose and twice on TEAE-cellulose. The final yield of enzyme was about 14 per cent. It was claimed that there were two hexokinases -a and b- which were different with regard to electrophoretic mobility and substrate specificity. Hexokinase 'a' had a lower purity whereas hexokinase 'b' had a specific activity higher than that reported by other workers.

The crystalline enzyme was found to be stable for months on storage as a suspension in ammonium sulphate solution. In dilute

solution it could be kept for two to three days at 5° or less. Maximum stability was observed at pH 5 near its isoelectric point. The stability of a very dilute solution (0.0025 per cent) of hexokinase at 26° was markedly increased in the presence of certain sugars as well as glycine. Glucose and mannose showed maximum effectiveness as stabilisers, sucrose was much less effective whereas galactose, maltose and fructose had very little protective action. Glucose was also found to be an effective protective agent against inactivation by proteolytic enzymes such as trypsin. Sulphydryl compounds such as cysteine and glutathione showed no protection of the enzyme against inactivation and also showed no activation (Berger et al. 1946). It was concluded that yeast hexokinase does not contain reactive -SH groups which are essential for its activity.

Mg^{++} is essential for hexokinase activity. Trayser and Colowick (1961) found several elements such as Al^{+++}, Ca^{++}, etc. in the first crystals of yeast hexokinase but the amounts of these elements were negligible after repeated recrystallization. The enzyme was not inhibited by fluoride either in the presence of absence of phosphate. No requirement of phosphate or any other cofactor except Mg^{++} has been demonstrated for yeast hexokinase. Mg^{++} can be replaced by Mn^{++} but not by Ca^{++}. More information is needed on the effect of other metals. The report by Brintzinger et al. (1959) that yeast hexokinase shows remarkable activation with Co^{++}, Ni^{++}, Mn^{++}, Zn^{++} and Cd^{++} requires confirmation

Strsuss and Moat (1958) reported that biotin stimulates the fermentation of glucose and fructose by yeast and that the hexokinase

activity of biotin deficient dry yeast cells and extracts could be increased by the addition of biotin. However Trayser and Colowick (1961) found no biotin in any of the samples of several times recrystallized yeast hexokinase. The largest sample of hexokinase tested by them would have contained five hundred times the amount of biotin required for maximum growth of the assay organism assuming that one molecule of biotin was present per molecule of hexokinase. Since the enzyme was fully active and contained no biotin, it was suggested that the results of Strauss and Moat could possibly be due to the presence of a naturally occurring hexokinase inhibitor the effect of which could be reversed by biotin. However, a point to be noted in the work of Trayser and Colowick is the possible presence of biotin in the glucose used for the assay of hexokinase. Unless special precautions had been taken to remove traces of biotin from the test system and especially from glucose, it is not possible to determine whether hexokinase is fully active without biotin.

It was also shown by Trayser and Colowick (1961) that hexokinase does not contain any phosphorus. Kunitz and McDonald (1946) found that the purified enzyme contained phosphorus which was not dialysed out in 24 hr. Trayser and Colowick dialysed the enzyme for nearly 96 hr. It is, therefore, unlikely that yeast hexokinase contains covalently linked phosphate. Trayser and Colowick, however, observed that a small amount of carbohydrate was present in the purified enzyme even after several recrystallizations. The amount present was only 0.2 to 0.5 mole of hexose per 100,000 g of protein. A part of this carbohydrate has been identified as glucose. More

recent work has shown that the glucose content can be reduced to 0.05 mole per mole of hexokinase (Kaji and Colowick, 1965). There is no conclusive evidence that this glucose is enzyme linked and not merely adsorbed on the enzyme.

The ultraviolet spectrum of the several times recrystallized enzyme is that of a typical protein and shows no evidence for a prosthetic group. On irradiation at 290 mμ at pH 8 hexokinase showed fluorescence at 340 mμ which has been ascribed to tryptophan. This fluorescence was found in all preparations of enzyme.

On treatment of hexokinase with perchloric acid or on boiling, a soluble fragment was obtained which showed ultraviolet absorption with a maximum at 270 mμ and a minimum at 240 mμ. It was slowly dialysable and was probably a tyrosine-containing fragment. It was present in all samples of hexokinase even after repeated crystallization, but it had no activity or effect on hexokinase activity. Its function and relation to hexokinase are unknown.

The enzyme phosphorylates several D-hexoses. The relative maximal rates of phosphorylation for some of the hexoses and their derivatives are: fructose, 1.8; glucose 1; 2-deoxyglucose, 1; mannose 0.8; glucosamine, 0.7; glucosone, 0.2; allose, 0.1; arabinose, 0.02; 1,5-sorbitan, 0.01 and galactose, 0.002. The different substrates compete for the same enzyme and the rate of phosphorylation with two substrates is not greater than that with either of the individual carbohydrates. The rate for the following compounds was less than one-thousandth of that with glucose. Methyl-α-glucoside,

mannoheptulose, 1-methyl fructose, N-acetylglucosamine, 3-O-methyl glucose, 3-O-methyl fructose, 1,4-sorbitan, L-sorbose and xylose. It should however be noted that further studies are necessary to determine whether the observed low rates of phosphorylation with these substrates are really due to these compounds or due to traces of impurities of other sugars such as glucose. In some experiments with purified brain hexokinase, which will be reported later, very low rates of phosphorylation were observed with some compounds such as methylglucosides. However this activity was found to be due to the presence of small amounts of glucose as determined by the actual estimation of glucose-6-P in the reaction mixture. Similar studies may be necessary to determine the true activities of yeast hexokinase with the different carbohydrates. The K_m values for some of the substrates are: fructose, 7×10^{-4}M; glucose, 1×10^{-4}M; 2-deoxyglucose, 3×10^{-4}M; mannose, 5×10^{-5}M; glucosamine, 1.5×10^{-3}M; glucosone, 2×10^{-5}M; allose > 0.1; arabinose > 0.1 M. The K_m values for the carbohydrates are however dependent also on the phosphate donor and the relative rates of phosphorylation with ATP and ITP were different for different sugars. The K_m for glucose and mannose with ITP were 3.7 and 2.9×10^{-3}M respectively (Martinez, 1961). An esterifiable hydroxyl group at C atom 6 is obviously essential. Both the α- and β -pyranose forms of d-glucose and d-mannose and furanose form of d-fructose are acted upon by hexokinase (Gottschalk, 1947).

The relative rates (in μmoles of glucose phosphorylated per min per mg protein) for different nucleotides are: ATP 800; deoxy-ATP 400; ITP 23; UTP 3; CTP 1; GTP 0.6, deoxy-CTP 0.002; deoxy-GTP 0.002.

The possibility that the low rates observed with some of the nucleotide triphosphates is due to the presence of small amounts of ATP requires consideration. PPi, polyphosphate and adenosinetetraphosphate show no activity. The K_m value for ATP with glucose as the substrate was reported to be 9.5×10^{-5}M (McDonald, 1952) and more recently by Fromm et al.(1962a) and Zewe et al.(1964) as 1.6×10^{-4}M with Mg^{++} and 1.3×10^{-4}M in the presence of Mn^{++}. There was no activity with ADP. The temperature coefficient of activity of the enzyme between 0 to 30° was reported to be 1.9 to 2.2.

Earlier studies by Kunitz and McDonald showed that the isoelectric point was 4.8 and that the enzyme was homogeneous at pH 5.6 though at pH 6 a double boundary appeared. The diffusion constant D°_{20} at 1° in acetate buffer, pH 5.5 was 2.9×10^{-7} $cm^2 \times sec^{-1}$ and the sedimentation constant at the same pH and temperature was 3.1 S. The molecular weight of the enzyme (assuming a specific volume of 0.74) was reported to be 96,000. The turnover number of the enzyme with glucose at pH 7.5 at 30° was 13,000 moles per min per 100,000 g of protein. The value for fructose was about twice as great and for mannose about half this value.

There is, however, a considerable variation in the specific activities of the crystalline enzyme reported by different authors. Using a direct titration method Kunitz and McDonald reported a maximum specific activity of 1400 units per mg, one unit being equivalent to the formation of 1×10^{-8} hydrogen equivalent of acid per min at 5° and pH 7.5. It was stated that one unit is also equivalent to one µl

of carbon dioxide liberated from bicarbonate per min at 25° when the acid formed is determined by the usual manometric method. Berger et al. reported an activity of 3000 µl of carbon dioxide liberated in the manometric assay. Darrow and Colowick reported a specific activity of 600 units per mg at 30°, one unit being equivalent to the formation of one µmole of acid per min at 30°. They used a method based on the estimation of the acid base indicator such as cresol red. The specific activity reported by Darrow and Colowick is equivalent to 13,500 µl of carbon dioxide liberated per min per mg protein which is more than four times greater than that reported by Berger et al. But the activity was only 3000 to 4000 µl carbon dioxide per min per mg protein in a manometric assay in bicarbonate buffer. It was suggested that the low activity in the manometric assay may be due to the instability of the enzyme, but this has not been definitely established and there is no evidence for progressive loss of activity with time in this assay. The reason for the variation in specific activities is not clear. Lazarus et al. have recently claimed that their purest preparation of yeast hexokinase has a specific activity of 800 µmoles glucose phosphorylated per min per mg protein at 25° when assayed by the measurement of the acid produced spectrophotometrically. This corresponds to a specific activity nearly 50 per cent higher than that obtained by Darrow and Colowick when corrected for the difference in temperature. On the basis of the studies reported by Trayser and Colowick the turnover number of the enzyme is 57,000 per min for glucose at 30° for a molecular weight of 95,000.

In the hexokinase reaction one proton is involved and the equilibrium, therefore, varies with the pH. The equilibrium constant $\frac{[ADP][Glucose\text{-}6\text{-}P]}{[ATP][Glucose]}$ was determined at different pHs and Mg^{++} concentrations (Robbins and Boyer, 1957). At pHs 6, 7 and 8 at 30° with excess Mg^{++} the values of K were 155, 1550 and 15,500 respectively. These correspond to free energy (standard) changes of -3.2, -4.7 and -6.2 kcal. respectively. The phosphorylation of glucose is virtually irreversible at higher pHs, though it is possible to demonstrate the reversal of the reaction if the ADP and glucose-6-P concentrations are high and the pH is 6.0 or less.

Several compounds have been reported to be inhibitors of yeast hexokinase. Suramin, sodiumtripolyphosphate, ADP and sorbose-1-P inhibit yeast hexokinase and the inhibition by ADP and sodiumtripolyphosphate is reversed by ATP but not by glucose. Glucose-6-P was reported to be inhibitory by Wajzer (1953) and non-inhibitory by two groups of workers (Weil-Malherbe and Bones, 1951 & Sols and Crane, 1953). Glucose-6-P is an extremely potent inhibitor of animal tissue hexokinases but not of yeast hexokinase. The discovery that sorbose-1-P inhibits yeast hexokinase is of considerable interest. L-glyceraldehyde has long been known to be an inhibitor of alcoholic fermentation. This inhibition was shown by Lardy and his coworkers (1949) to be due to condensation of L-glyceraldehyde and dihydroxyacetone phosphate in the presence of aldolase to form sorbose-1-P which is a very potent inhibitor of yeast hexokinase.

Several carbohydrates inhibit yeast hexokinase and the K_i values for some of them are: methyl-α-glucoside > 0.1, mannoheptulose 1.5×10^{-4}; 1-methylfructose > 0.1, N-acetylglucosamine 1×10^{-3},

2-C-hydroxymethylglucose 1.5 x 10^{-3}; 3-O-methylglucose > 0.1; 3-O-methylfructose > 0.1; 1,4-sorbitan > 0.1; L-sorbose > 0.1; 6-deoxy-6-fluoroglucose 5 x 10^{-3} and xylose 1 x 10^{-2}. Yeast hexokinase was markedly inhibited by 10^{-3} M myristic, palmitic, stearic and ricinoleic acids and by higher concentration of oleic and lauric acids. Digitonin, saponin, deoxycholate and different Tweens had no effect. The inhibition by palmitic acid was irreversible (Bargoni, 1959).

As indicated previously earlier studies by Berger et al. showed that hexokinase does not appear to contain any reactive -SH groups. It was however found to be irreversibly inactivated by treatment with mustard gas or nitrogen mustards. This was clarified by subsequent work by Bernard and Ramel (1962) and by Colowick and coworkers (Kenkare and Colowick, 1965; Kaji, 1966). Bernard and Ramel found that though no -SH groups could be detected by p-CMB titration in water at 25° in 5.4 M guanidine approximately four -SH groups per mole (47,000) could be titrated. Four cysteine groups per mole, were also shown to be present by amino acid analysis. The reason for the discrepancy between different authors with regard to the molecular weight of the enzyme and the number of -SH groups per mole is not clear. Though the enzyme was not directly inhibited by p-CMB or mercuric chloride, at 35° and pH 8 it was completely inactivated by 5 x 10^{-5} M methylmercurinitrate and this was reversed by 0.01 M cysteine. Similarly sodium bromoacetate irreversibly inactivated the enzyme at appreciable rates only at temperatures above 30° with a very steep increase in the inactivation rate over the range 30° to 37°. The inactivation at 35°

was not pH dependent below 7 but between 7 to 8 the time required for half inactivation decreased from 31 min to 3 min. By the use of C^{14} labeled bromoacetate 3.9±0.4 carboxymethyl groups were found to be introduced per mole of enzyme. Glucose protected the enzyme considerably against bromoacetate inactivation. These results suggest that the -SH groups of hexokinase are not accessible to sulphydryl reagents but conformational changes occurring at higher temperatures and pHs expose these groups to the action of sulphydryl reagents. Ramel et al.(1961) have also shown that the enzyme is split into inactive chains of molecular weight of 20,000 by sodium dodecylsulphate.

Fasella and Hammes (1963) concluded that all the six -SH groups of hexokinase could be titrated with p-CMB in the presence of 10^{-2}M glucose without loss in activity suggesting that the -SH groups are not required for enzyme activity but that they may be required to stabilise the active enzyme configuration. These results were further extended by the work of Kaji (1966) who has shown the presence of six -SH groups per molecule of hexokinase on the basis of spectrophotometric assay with p-CMB. In the presence of glucose the reaction of enzyme with p-CMB is slower and at pH 7 there is no loss of activity when four groups have reacted and even after all the groups have reacted there was an appreciable amount of activity left. The inactivation is reversible by cysteine, but on prolonged incubation at high concentrations of the enzyme and p-CMB, the activity is irretrievably lost. The enzyme then shows two molecular species

one of which has the same sedimentation constant as the normal enzyme whereas the other sediments at twice the velocity.

Recent physico-chemical studies by Kenkare and Colowick (1965) have elucidated some aspects of the structure of yeast hexokinase. It was found to have a molecular weight of 95,000 as measured by hydrodynamic and light scattering methods. At high or low pHs it dissociates into subunits of molecular weight 24,000 and this dissociation is accelerated in the presence of a salt. It is accompanied by the conversion of the globular, compact and highly ordered particles to subunits, which have lost a large part of their helical structure as measured by optical rotatory dispersion but not of its tertiary structure as shown by changes in viscosity. Under specific conditions this dissociation is reversible and a considerable amount of activity is regained on neutralisation. The loss of activity in the case of alkali treated enzyme is markedly high in air and is not restored by neutralisation though the addition of mercaptoethanol leads to restoration of activity indicating the involvement of -SH groups. The reversibility is not decreased with time though high salt or protein concentration led to low restoration of activity due to aggregation of protein. EDTA and mercaptoethanol favour reactivation. Under optimum conditions nearly 80 per cent of the original activity can be reobtained and after recrystallization, the reactivated enzyme is indistinguishable from the native molecule. It has been suggested that in its native atate the enzyme exists as a reversible mixture of whole and half molecules and that dissociation into the half molecules is possible at values above pH 5.5 in the presence of salt, especially phosphate.

These half molecules are presumed to be the form present under assay conditions. Gooding and Colowick (1966) have also reported six cysteine residues per 96,000 molecular weight which become directly titratable by p-CMB while reversibly inactivated at pH 10.5 at 25°. Inactivation is appreciable even at pH 8 in the presence of 0.04 to 0.1 M mercaptoethanol. Mercaptoethanol can be replaced by cysteine and dithiothreitol but not glutathione or sulphite. The activity is restored by adjusting the pH to 6.5 to 7. Glucose protects against inactivation by mercaptoethanol. There is no evidence for sulphur-sulphur linkages in the enzyme.

The mechanism of action of yeast hexokinase and other hexokinases will be discussed in a later section.

Other Microorganisms and Plants

Hexokinase has been reported to be present in several microorganisms other than yeast and also in several plant tissues. It is unfortunately not possible to discuss in detail the hexokinases of microorganisms and plant tissues or the enzymes which phosphorylate sugars with phosphorus donors other than ATP without making this introduction unduly lengthy. Only brief reference will be made to the work on these hexokinases. Hexokinase has been reported to be present in the following: Escherichia coli (Cardini, 1957), Salmonella typhosa (Perova, 1961), Pseudomonas putrefaciens (Klein and Doudoroff, 1950), Azotobacter vinelandii (Mortenson and Wilson, 1954), Spirochaeta recurrentis (Smith, 1960), Plasmodium gallinaceum (Speck and Evans, 1945) Leishmania donovani (Chatterjee et al.1958), Aspergillus niger (Tiwari and Krishnan, 1961), Trypanosoma gambiense (Seed and Baquero, 1965) and in the worms Ascaris lumbricoides (Rathbone and Rees, 1954) and Aeromonas formicans (Pivnic and Sabina, 1957). The enzyme from A.niger is of interest since it was claimed that it requires Zn^{++} for its activity. Hexokinase has been partially purified from Neurospora crassa, Aspergillus parasiticus, Aspergillus oryzae, Aerobacter aerogenes and Brevibacterium fusicum. The enzyme from the last two organisms was reported to be a glucokinase. The purification of these enzymes was however only partial and there is no evidence that homogeneous preparations have been obtained from any of these sources. The properties of some of these enzymes have been summarised in Table 1a and 1b.

Plants

Hexokinases have been reported to be present in potato tuber, soybean, wheat germ, sugar beet and corn scutellum but little progress has been made in the study of the hexokinases of plants.

Phosphorylation with phosphate donors other than ATP

Hexokinases which act with phosphate donors other than ATP have been reported from several microorganisms. Corynebacterium diphtheriae and Mycobacterium phlei contain enzymes which transfer phosphate from inorganic polyphosphate to glucose and glucosamine. The enzyme from Aerobacter aerogenes can utilise acetyl phosphate as a phosphate donor. The enzyme from E.coli which utilises phosphoramidate has been partially purified. An enzyme which phosphorylates glucose in the presence of P-enolpyruvate occurs in E.coli. Some of the properties of these enzymes are shown in Table 2.

TABLE 1a

PROPERTIES OF HEXOKINASE FROM MICROORGANISMS

Source	Specific activity	Optimum pH	K_m values M	Inhibitors	Miscellaneous properties	Reference
N.crassa	7	8.3 to 9.4	Glucose 5.4×10^{-4}	Glucose-6-P Ki 2×10^{-3} M N-acetylglucosamine Ki 5.4×10^{-4} M	Activity depends on the presence of Zn^{++} in medium during growth. Zn^{++} protects against action of p-CMB.	Medina & Nicholas (1957)
A.parasiticus	7.5	7.5(glucose) 8.0(galactose)	-	-	Reacts also with D-galactosamine and D-galactose	Davidson (1960)
A.oryzae	0.9	8.0	Glucose 5×10^{-5} Mannose 7.5×10^{-5} Fructose 3×10^{-2}	ADP	-	Ruiz-Amil & Sols (1961)
A.niger	-	-	-	-	Activity depends on the presence of Zn^{++} in the medium during growth. Mn^{++} and Fe enhance Zn-dependent activity. Incapable of using poly- or metaphosphate for phosphorylation.	Tiwari & Krishnan (1960, 1961)

A.
A.

TABLE 1b

PROPERTIES OF GLUCOKINASE FROM MICROORGANISMS

Source	Specific activity	Optimum pH	K_m values	Inhibitors	Miscellaneous properties	Reference
A.aerogenes	68	7.5	ATP 8×10^{-4} Glucose, 8×10^{-5}	ADP Ki 4×10^{-4} M D-glucosamine D-xylose	No inhibition by glucose-6-P. Stable at room temperature for several days, unstable at -20°.	Kamel, Allison & Anderson (1966)
B.fusicum	55	8.0 to 8.5	Glucose, 1×10^{-3} ATP, 8×10^{-4}	Glucose-6-P, NaF, cysteine, cystine, glutathione, heavy metals	-	Siato (1965)

Specific activity expressed as μmoles phosphorylated per min per mg protein

TABLE 2

PHOSPHORYLATION BY PHOSPHATE DONORS OTHER THAN ATP

Source	Specific activity	Optimum pH	K_m values M	Inhibitors	Miscellaneous properties	Reference
A) Phosphorylation by inorganic polyphosphate						
C.diphtheriae	-	-	-	-	Glucose is phosphorylated by either ATP or inorganic polyphosphate	Szymona & Szymona (1960)
M.phlei	-	8.9	-	-	Requires Mg^{++}; glucose and glucosamine are phosphorylated by inorganic polyphosphate. No ATP is required.	Szymona (1962) Szymona & Ostrowski (1964)
B) Acyl phosphate; Hexose phosphotransferase						
A.aerogenes	1.3	-	Glucose $1x10^{-4}$ Acetyl phosphate $2x10^{-3}$	-	Only glucose is phosphorylated and not mannose or fructose; no activity with ATP and P-enolpyruvate.	Kamel & Anderson (1964)
C) Phosphoramidate; Hexose transphosphorylase						
C) Phosphoramidate; Hexose transphosphorylase						
E.coli	-	-	-	-	Phosphorylation of glucose, fructose, sorbose, glucosamine, and galactose by N-phosphoramidate which is not replaced by ATP; no requirement of divalent ion.	Smith (1959)
E.coli	-	-	-	-	Phosphoramidate, N-phosphorylglycine or monophosphoglycerylhistidine act as phosphate donors; reaction product is glucose-1-P.	Smith and Fujimoto (1960)
E.coli	23	-	Glucose $1.3x10^{-2}$ Phosphoramidate $5.4x10^{-3}$	Fluoride	Phosphorylates glucose, 2-deoxyglucose, mannose, etc.	Smith and Theisen (1966)
D) P-enolpyruvate:Hexose phosphotransferase						
E.coli	Two enzymes I 119 II 98	7.4	P-enolpyruvate 6×10^{-4}	Cu^{++}, Fe^{++} and Ca^{++}	Enzyme I is stable for a week at 0°, Enzyme II is stable for 1 to 2 days at 0°; requires Mg^{++}, Mn^{++}, Zn^{++} or Co^{++}.	Kundig, Ghosh, Roseman (1966)

Specific activity expressed as μmoles phosphorylated per min per mg protein.

SECTION III

Animal Tissues (other than brain)

Animal tissue hexokinases have not yet been obtained in homogeneous form and most of the information available about them is derived from studies with homogenates, crude extracts or partially purified preparations. Owing to the effect of other interfering substances the interpretation of such data often presents considerable difficulties. In comparison with the wealth of data available about yeast hexokinase the information available about animal tissue enzymes is relatively meagre. As stated earlier many of these enzymes are either highly unstable or attached to insoluble particles, which render them difficult to purify. However, in recent years considerable progress has been made in the study of these enzymes. Some of the soluble preparations and also the particulate hexokinase of brain have been partially purified and methods have been developed for the solubilization of some of the enzymes. Isozymes of hexokinase in different tissues, particularly in liver, have been characterized. A unique feature of most of the animal tissue enzymes is their inhibition by glucose-6-P at low concentrations. This inhibition of hexokinase, which is one of the rate-limiting steps of glycolysis, and the reversal of the inhibition by Pi are undoubtedly of importance in the regulation of glucose metabolism. Some of the work on animal tissue hexokinases (a few references to the enzyme from insects, etc. have also been included here for convenience) especially on those enzymes which have been partly

purified, is discussed in the following sections. Glucokinases and isozymes will be referred to only briefly and in a separate section. For convenience one unit of hexokinase activity is defined as the amount of enzyme required to phosphorylate one μmole of glucose per min and the results in the earlier literature have been recalculated in terms of this unit.

Hexokinase has been shown to be present in several animal tissues. Its occurrence has been noted in brain, heart, skeletal muscle, liver, adipose tissue, erythrocyte, pancreas, intestine, caecum, kidney, testis, uterus, skin, retina, placenta, lung, spleen, mammary gland, ascites cells etc. Brain was found to contain the maximum amount of hexokinase whereas lung and liver had relatively little activity. It is of interest to note that whereas the hexokinase activity of dry yeast is about 300 units per g the activity of brain is only about 10 units per g. Animal tissues generally contain less hexokinase (in terms of μmoles of substrate phosphorylated per min per g of tissue) than the most of the other glycolytic enzymes. The relatively low abundance of the enzyme also makes it more difficult to purify than enzymes which occur in relatively large amounts such as aldolase or glyceraldehyde phosphate dehydrogenase.

The existence of soluble and insoluble hexokinase in animal tissues was recognised early in the study of animal tissue hexokinase. Crane and Sols (1953) estimated the relative amounts of particulate and soluble hexokinase in different tissues by centrifuging homogenates of the different tissues in mannitol or sucrose solutions at 18,000 x g for 60 min and determining the relative amounts of activity in the

sediment and in the supernatant. There was wide variation in the relative amounts of the two forms of enzyme in different tissues. Rat brain had almost 90 per cent of the enzyme in the particulate fraction, whereas all the hexokinase of red blood corpuscles was in the supernatant. The percentage of activity in the sediment was 52 for heart and about 35 for kidney, liver and stomach. Glucokinases, which occur in a few tissues, were all found to be soluble.

The K_m values for glucose, ATP and Mg^{++} and the K_i values for ADP and glucose-6-P for different tissues are summarised in Table 3. It will be seen that the K_m values for different substrates vary widely for the different tissues.

The properties of hexokinase from different animal tissues are described below.

Heart

Heart muscle contains both soluble and insoluble hexokinase in approximately equal amounts. Pigeon ventricle muscle was also found to contain 50 per cent of the enzyme in a form sedimentable at 18,000 x g (Bargoni, 1958).

Methods for the partial purification of both the soluble and insoluble hexokinase of heart have been described (Crane and Sols, 1955). Homogenates in phosphate buffer, pH 7.4, containing KCl and EDTA were centrifuged at 1500 x g to remove inactive debris and the particulate enzyme was sedimented by centrifugation again at 18,000 xg.

TABLE 3

K_m VALUES OF SUBSTRATES AND K_i VALUES OF INHIBITORS OF HEXOKINASE FROM DIFFERENT ANIMAL TISSUES

Source	K_m glucose M	K_m ATP M	K_m Mg^{++} M	K_i ADP M	K_i glucose-6-P M	Reference
Rat skeletal muscle	1.8×10^{-4}	4.6×10^{-4}	-	-	-	Hanson and Fromm (1965)
Rat skeletal muscle	1.1×10^{-4}	8.0×10^{-4}	-	8.0×10^{-4}	-	Toews (1966)
Rat muscle(soluble)	2.3×10^{-4}	7.8×10^{-4}	-	2.2×10^{-3} (for ATP)	2.1×10^{-5} (for ATP)	Grossbard and Schimke (1966)
Adipose tissue	3.0×10^{-5}	5.0×10^{-3}	-	-	-	Sols and Hernandez (1966)
Adipose tissue (soluble)	2.8×10^{-4}	7.5×10^{-4}	-	-	-	Grossbard and Schimke (1966)
Adipose tissue	7.0×10^{-5}	-	-	-	-	DiPietro (1963)
Rat intestinal mucosa	2.0×10^{-4}	-	-	-	6.0×10^{-3}	Sols (1956)
Rat intestine	6.5×10^{-5}	-	-	-	-	Lange and Kohn (1964)
Rat kidney	4.8×10^{-5}	-	-	-	-	Lange and Kohn (1964)
Rat kidney(soluble)	4.9×10^{-5}	4.4×10^{-4}	-	-	-	Grossbard and Schimke (1966)
Rat liver	4.0×10^{-5}	-	-	-	-	Lange and Kohn (1964)
Rat liver(soluble)	7.0×10^{-6}	9.8×10^{-4}	-	8.8×10^{-4} (for ATP)	7.4×10^{-5} (for ATP)	Grossbard and Schimke (1966)
Islets of Langerhans of teleost fish	1.16×10^{-6}	-	-	-	-	Renold (1961)
Ascites cells	2.8×10^{-5}	1.7×10^{-4}	1.0×10^{-3}	-	4.0×10^{-4} (glucose)	McComb and Yushok (1959)
Ascites cells	-	4.3×10^{-4}	-	5.1×10^{-5}	-	Uyeda and Racker (1965)
Ox brain particulate	8.0×10^{-6}	1.3×10^{-4}	8.0×10^{-4}	1.3×10^{-4}	4.0×10^{-4}	Crane and Sols (1953)
Ox brain particulate	6.4×10^{-5}	3.4×10^{-4}	-	-	-	Fromm and Zewe (1962b)
Rat brain(soluble)	4.5×10^{-5}	4.0×10^{-4}	-	6.2×10^{-4} (for ATP)	2.6×10^{-5} (for ATP)	Grossbard and Schimke (1966)

It was "solubilized" by 0.1 per cent Triton X-100 and centrifugation at 50,000 x g gave enzyme free from several interfering enzymes such as ATPase and phosphofructokinase. The 18,000 x g supernatant which contained the soluble enzyme was partially purified by removal of proteins insoluble at pH 5.5 followed by fractionation with ammonium sulphate. This enzyme was also free from phosphofructokinase activity and its specific activity was 1.5 units per mg.

Rose and Warms (1965) made the interesting observation that several mitochondrial hexokinases could be released in soluble form by glucose-6-P. Detailed studies by Hernandez and Crane (1966) on the effect of pH, monovalent and divalent metal salts and glucose-6-P in releasing bound heart hexokinase in soluble form are of considerable significance and throw light on the attachment of the enzyme to the insoluble particles. They determined the amount of enzyme obtained in soluble form at different pHs and ionic strengths. It was observed that at pH 7 the solubilization was about 35 per cent with 0.4 M KCl or 0.2 M ammonium sulphate and 90 per cent with 0.2 M $MgCl_2$. At pH 5.5 the solubilization was 70 per cent with KCl and 80 per cent with $MgCl_2$. On the basis of the change in solubilization with pH it was suggested that imidazole was involved in the binding of hexokinase to the particulate forms which is dependent on the ionic strength and pH. Low concentrations of glucose-6-P also give rise to appreciable solubilization of the enzyme. At pH 7.5 the solubilization was 19 per cent at 0.1 mM, 31 per cent at 1 mM but only 19 per cent at 5 mM. In the case of the monovalent cations the removal of the salts

from the solubilized enzyme resulted in rebinding of the enzyme to the particles which was not due to aggregation. With divalent ions the solubilization was irreversible.

Skeletal Muscle

The enzyme is present mostly in soluble form in this tissue. Methods for the partial purification of skeletal muscle hexokinase have been described by Crane and Sols (1955), Teews (1966) and by Hanson and Fromm (1965, 1967). The specific activities obtained by these procedures were however very low. More recently Grossbard and Schimke (1966) have purified rat skeletal muscle hexokinase by chromatography on DEAE-cellulose, fractionation with ammonium sulphate, adsorption on hydroxylapatite, fractionation with ammonium sulphate and chromatography on Sephadex G-200 to a final specific activity of about 13 units per mg at 37°.

A study of the metal requirement of rabbit muscle hexokinase was made by Walaas and Walaas (1962) using a partially purified enzyme. They observed maximum activity when the Mg^{++} to ATP ratio was 1. Activation was also found with Ca^{++}, Co^{++}, Mn^{++} and Zn^{++}. Maximum activity was found when the ratio of metal to ATP was less than 1. With Co^{++} and Mn^{++} the concentration at which half maximal activity occurred was about 10 times lower than that of Mg^{++}, but higher amounts of Co^{++} and Mn^{++} were strongly inhibitory. Since hexokinase activation by Ca^{++} has hitherto not been reported, it would be of considerable importance to confirm this observation with a purified enzyme from skeletal muscle.

The hexokinase of the muscle of L.migratoria was studied (Kerly and Leaback, 1957) using the 18,000 x g supernatant of an aqueous extract of the tissue. The enzyme was similar to brain hexokinase in the relative rates of phosphorylation of glucose, mannose, fructose and glucosamine and in its higher affinity for glucose and mannose than for fructose. It was reported to be inhibited by 5 mM glucose-6-P or fructose-6-P, inhibition by the latter being presumably due to the presence of phosphoglucoisomerase in the crude extract.

Liver

A method for preparing active extracts from liver has been described (Crane and Sols, 1955). Rat liver was homogenised with KCl-EDTA-$MgCl_2$ solution and centrifuged at 100,000 x g and the enzyme was precipitated from the supernatant with ammonium sulphate at pH 7.5. After dialysis against Tris-EDTA-$MgCl_2$ buffer the enzyme could be stored for several weeks at -15°. However, subsequent work has shown the presence of glucokinase in addition to a hexokinase in liver and it is not clear whether the enzyme obtained by this procedure is a glucokinase or hexokinase. An insoluble hexokinase is also present in liver.

Grossbard and Schimke (1966) have presented evidence for the occurrence of three types (I, II and III) of soluble hexokinase in animal tissues and have purified some of them. They separated Type-III enzyme of liver from Types I and II and partially purified it, but the final specific activity was only 1.4 units per mg at 37°.

In liver cells of rabbit embryo hexokinase activity was observed to be higher than in mature cells and it was reported to decrease during development of the embryo. In regenerating liver also hexokinase was found to be higher than in normal liver cells (Ilyin, 1964).

Adipose Tissue

Adipose tissue contains a glucokinase as well as a hexokinase. The hexokinase was studied by DiPietro (1963), Sols and Hernandez (1963) and Grossbard and Schimke (1966). DiPietro's studies with a dialysed KCl-$MgCl_2$-EDTA extract showed activation by Mg^{++} and Mn^{++} and activity only with ATP and not with ITP or CTP. This is in contrast to brain hexokinase which is active with ITP, though at a lower rate than with ATP.

The enzyme from the epididymal fat pad was partly soluble, since 60 per cent of it was not sedimented at 100,000 x g in 1 hr. This soluble fraction was purified by Sols and Hernandez by fractionation with ammonium sulphate in the presence of EDTA. It resembled brain hexokinase in its inhibition by glucose-6-P and ADP. Its activities with mannose and deoxyglucose were, however, 1.5 to 1.6 times higher than with glucose. This is in contrast to enzymes from other tissues like brain which show greater activity with glucose than with mannose. The increase in activity between 30° and 40° was about 1.4-fold.

Grossbard and Schimke (1966) have described the purification of rat epididymal fat pad Type-II hexokinase to a specific activity

of about 13 units per mg protein at 37° by a procedure similar to that used with the muscle enzyme. The properties of this enzyme along with those of other enzymes purified by these workers are reported in Table 3.

Erythrocytes

Little progress has been made in obtaining even a partially purified preparation from erythrocytes though a preliminary separation of the enzyme from hemoglobin by DEAE-cellulose chromatography has been reported (Henessey et al. 1962). This enzyme, which occurs almost entirely in soluble form, was found to be partially stabilised by glucose and glutathione. The observation that the hexokinase activity of red blood cells is increased in the presence of 2 mM sodium fluoride is unusual and requires further study (Grignani and Löhr, 1960). Older cells were reported to have a lower activity than younger ones (Brewer and Powell, 1963).

Pancreas

The hexokinase of dog pancreas was found to be present mainly in the soluble fraction. It was prepared by centrifuging the extract at 23,000 x g for 1 hr (Villar-Palasi, 1957) followed by precipitation with 0.5 saturated ammonium sulphate. The enzyme was found to phosphorylate glucose, fructose, mannose, 2-deoxy-glucose, allose and 1,5-sorbitan. The hexokinase activity of normal and alloxan diabetic dogs showed no significant difference. This is in contrast with the lower activity of diabetic heart tissue compared to normal tissue, though this lowering of activity was not

observed in the Houssay animal (Park et al. 1961).

The hexokinase of the islets of Langerhans (Renold, 1961) of teleost fish (Opsanus tau) showed K_m values of glucose, fructose and mannose similar to those of several other tissues.

Intestine

Cell-free extracts of rat intestine, kidney and liver were used for a comparative study of the phosphorylation of different sugars (Lange and Kohn, 1962). The K_m values for glucose, deoxyglucose and glucosamine were 6.5×10^{-5}M, 9×10^{-5}M, 3.3×10^{-4}M respectively for the hexokinase of intestine. However allose and tallose were phosphorylated by intestine whereas liver tissue phosphorylated only allose and gulose.

The hexokinase in crude homogenates of intestinal mucosa was studied by Sols (1956). The enzyme was very unstable though it was partially stabilised by EDTA and mannitol. Its substrate specificity, K_m for glucose and glucose-6-P inhibition were similar to those of the enzyme from brain.

Retina

The hexokinase of retina was also found to be very unstable and glucose (1 per cent), cysteine (0.1 M) and sodium sulphide had no effect on the stability of the enzyme. The optimum pH was about 7.4 to 8.6. It was noted that Co^{++} was also effective in activating the enzyme in addition to Mg^{++} and Mn^{++} (Hoare and Kerly, 1954).

Kidney

Grossbard and Schimke (1966) have also purified Type-I hexokinase of rat kidney to a specific activity of 6.8 units per mg protein at 37°. The properties of the enzyme are different from those of Type-II and Type-III with respect to K_m values (Table 3).

The interesting observation was made that kidney cells in culture responded to the addition of different sugars at 5 mM by an increased synthesis of hexokinase. In the presence of glucose, fructose and galactose, hexokinase activity increased 3.7, 2.3 and 1.2-fold respectively. This was partly due to increased enzyme synthesis, since this increase was inhibited by p-difluorophenyl-alanine (Lieberman et al. 1964). It is unfortunately not known whether this increase in activity was due to a glucokinase or hexo-kinase, since there are few reports of enhancement of activity of hexokinase in response to variations in environmental factors or nutritional status.

Testis

The claim of Akaeda (1956) that rabbit testis contains glucokinase which gives glucose-1-P as a reaction product needs confirmation.

Skin

Hexokinase activity was also shown to be present in aqueous extracts of the skin of rabbit, mice and guineapigs. The enzyme had a pH optimum of 8 and was inactive below pH 6. This is in contrast to the broad pH optimum between 6 and 8 of the brain enzyme (Smirnova, 1959).

Miscellaneous

Hexokinase has also been reported to be present in the embryo of snail (Horstmann, 1960) in the sphinter muscle of fresh water mussels (Karpiak, 1960) in the nephredial canal of Astacus leptodactylus (Keller,1965), in mammary gland (McLean, 1958) and in choroid plexus and leptomeninges (Coltorti, 1954).

In human placenta the activity was 0.16 units per g at 4 months and 0.12 at term (Marazzini and Tessari, 1957). The variation in the hexokinase of corneal epithelium stroma and lens capsule, cortex and nucleus of rats at 0 to 100 days age was determined and it was observed that the hexokinase content was higher in earlier development stages than at more mature stages (Kuhlman and Resnik, 1958). A survey of the enzyme in aortic and pulmonary artery tissue in human beings from infancy to old age showed virtually the same activity (about 0.16 units per g wet tissue) at all ages (Brandstrup et al. 1957). Krahl et al. (1954) reported that there was no change in hexokinase activity on fertilization of Arbacia punctulata.

Ascites Cells

The hexokinase of ascites cells has been studied by three different groups of workers. Homogenates of washed ascites cells in sucrose-EDTA were centrifuged at 700 x g. The supernatant was recentrifuged at 4,000 x g. The sediment which contained the activity was suspended in a solution of glucose, EDTA, deoxycholate and salts and centrifuged at 12,000 x g to obtain a particulate preparation

which was stable in the cold for several weeks. Its properties were similar to those of brain hexokinase (McComb and Yushok, 1959).

It was noted earlier that the hexokinase of the mitochondria of ascites cells was released by incubation with glucose-6-P or anhydro glucitol-6-P (Rose and Warms, 1965). Mannose-6-P had no effect in bringing the enzyme into solution. In the presence of Mg^{++} or Ca^{++} the enzyme could be bound again to the particles.

Uyeda and Racker (1965) purified ascites hexokinase about 100-fold. The enzyme was extracted from an acetone powder of the ascites cells with EDTA-thioethanol-phosphate buffer, treated with protamine sulphate and precipitated with ammonium sulphate. It was then subjected to digestion with trypsin and chymotrypsin followed by inactivation of the proteolytic enzymes with diisopropylfluorophosphate. Further fractionation with ammonium sulphate and chromatography on Sephadex G-75 and DEAE-cellulose gave a final specific activity of 9.1 units per mg.

It is of interest that the purified enzyme was unstable in the absence of glucose at 0 to -70° whereas 10 mM glucose was sufficient to keep the enzyme stable for at least two months at 0°. The enzyme also had a higher stability at neutral pHs than at pH 5.5 or 9. It was inhibited by ADP and the inhibition was reversed by ATP. The inhibition of the enzyme by glucose-6-P and its reversal by Pi are discussed in a later section.

As reported earlier the reaction catalysed by yeast hexokinase can be shown to be reversible at low pH values and the

equilibrium constant was actually determined by studying the reverse reaction. Uyeda and Racker reported that the reversal of the hexokinase reaction could not be demonstrated with the purified ascites enzyme even when the glucose-6-P concentration was kept low to minimise the inhibition by this compound. The reason for this is obscure and deserves further study, since it may throw light on the mechanism of the reaction which is in contrast to that of yeast hexokinase.

Insects, etc.

Hexokinase has been reported to be present in the house-fly (Musca domestica) (Chefurka,1954). The enzyme from honey-bee was partially purified by fractionation with ammonium sulphate and DEAE-cellulose chromatography and was obtained as a stable preparation. Several of its properties such as broad pH optimum, competitive inhibition by ADP which was reversed by ATP and non-competitive inhibition by glucose-6-P were similar to those of the enzyme from brain (Ruiz-Amil, 1962).

The unusual claim was made that the tape worm (Echinococcus granulosus) contained four hexokinases which were specific for glucose, fructose, mannose and glucosamine respectively. These enzymes showed no action on deoxyglucose. Glucose-6-P inhibited the enzyme which acted on glucose, fructose and mannose. Glucokinase was inhibited by p-CMB and the inhibition was reversed by cysteine. Since this is one of the very few reports of the occurrence of four hexokinases specific for each of the four hexoses, confirmation of these results and isolation of the pure enzymes would be of considerable interest (Agosin and Aravena, 1959).

SECTION IV

Brain

The hexokinase of brain has been extensively investigated for nearly thirty years (Euler et al. 1937; Geiger, 1950; Ochoa,1941; Meyerhof, 1947). Utter and his coworkers (1945) observed that the metabolism of glucose by brain extracts decreased on centrifugation and suggested that this was due to the removal of insoluble brain hexokinase. They also observed that the cerebrum had the highest rate of glucose metabolism among different parts of the brain. At low hexose concentrations the rate of phosphorylation of fructose was half that of glucose (Meyerhof and Wilson, 1948). Weil-Malherbe and Bone (1951) found that glucose-6-P was highly inhibitory to the enzyme and that the inhibition was independent of glucose and ATP concentrations. They also stated that a heat-stable, non-dialysable activator of hexokinase was present in erythrocytes and muscle. Colowick et al.(1947) described a method for the preparation of partially pure brain hexokinase by fractionation of an aqueous extract of the gray matter of brain with ammonium sulphate. Using an enzyme purified by this procedure Wiebelhouse and Lardy (1949) reported that the enzyme phosphorylates glucose, fructose and mannose and not galactose, ribose, gluconic acid and several other sugars. An observation made with this enzyme that sodium salts inhibited phosphorylation of glucose but not of fructose does not appear to have been further studied by other investigators.

Though brain tissue is generally reported to contain most of the enzyme in particulate form, there is some variation in the results reported by different authors. Biesold and Canzler (1965) reported on the basis of gradient centrifugation that 52 per cent of the activity was present in mitochondria, 11 per cent in cytoplasm and 2 per cent in microsomes. They suggested that hexokinase is bound to a membrane containing nerve endings. Johnson (1960) observed that 75 per cent of the hexokinase of rat brain homogenates in 0.3 M sucrose was present in the mitochondrial fraction and that 15 per cent was not sedimentable. Crane and Sols (1953) reported that about 90 per cent of the activity of brain was present in the insoluble form.

A detailed study of the hexokinase activity of different parts of rat brain was carried out by Bennet et al.(1962). They found that the cortex and cerebellum contained significantly higher activity compared to that of other parts. The difference between the areas with the highest (cortex) and lowest (medulla) activity was nearly two-fold. The relative activities of different parts of the brain were: visual cortex, 106; cerebellum, 100; remainder of dorsal cortex, 93; hypothalamus, 79; ventral cortex, 86; caudate, 73; olfactory, 70; medulla and pons 57. The maximum activity reported for whole brain was rather high (16 units per g). They also observed an increase (upto 125 per cent) in the hexokinase activity of homogenates prepared in Tris and mercaptoethanol or glucose when stored at 0° at pH 6.3 to 7.7. The high hexokinase activity of brain may be related to the fact that unlike other tissues the main energy source for this tissue is glucose.

An interesting observation on the difference in the glucose affinity of sheep and rat tissues was noted by Raggi and Kronfeld (1966). Though the K_m values for the enzymes of liver, muscle and adipose tissue were not different in the two species, the K_m value of the sheep brain enzyme was only half of that with the rat brain enzyme. Since the blood glucose levels of sheep (50 mg per 100 ml) are much lower than those of rat or dog, it was suggested that the lower K_m value of the sheep brain enzyme may be related to the need to maintain adequate glucose levels in the brain in spite of lower blood sugar concentrations.

An important development in the study of brain hexokinase was the preparation of purified particulate calf brain hexokinase by Crane and Sols (1953). By centrifugation of a phosphate buffer homogenate of brain cortex at 800 x g to remove insoluble debris and recentrifugation at 3500 x g they obtained a seven-fold purification. Further purification by treatment with lipase and centrifugation in the presence of deoxycholate gave a preparation which was free from a large number of interfering enzymes such as phosphofructokinase, phosphomannoseisomerase, phosphoglucomutase, adenylic kinase, phosphoglucokinase and hydrolytic enzymes which act on ATP, phosphomonoesters and PPi. Only traces of phosphoglucoisomerase were present in the preparation. A total purification of about 45 to 50-fold was obtained relative to the initial homogenate and the specific activity increased from about 0.1 to 4.7 units per mg protein at 30°. This enzyme, which had a higher specific activity than any reported till recently, has been used for studies on the kinetics, specificity and other properties. The method of Crane

and Sols (but without treatment with lipase or deoxycholate) for the preparation of insoluble brain hexokinase has been used in the present work for the preparation of the soluble enzyme.

The following K_m values were obtained for the Crane and Sols preparation with different carbohydrates: glucose (8×10^{-6}M); mannose (5×10^{-6}M); 2-deoxyglucose (2.7×10^{-5}M); glucosamine (8×10^{-5}M); fructose (1.6×10^{-3}M); 1,5-sorbitan (3×10^{-2}M). Glucose, 2-deoxyglucose and 1,5-sorbitan had nearly the same relative maximum activities with the enzyme, and mannose and glucosamine had about half the activity and fructose about 1.4 times the activity with glucose. It is of interest that yeast hexokinase also shows maximum activity with fructose and minimum activity with mannose. The K_m values for these sugars are much greater for yeast hexokinase than for brain hexokinase. The enzyme is competitively inhibited by N-acetylglucosamine, 6-deoxy D-glucose, D-xylose and D-lyxose. The K_m value for ATP was 1.3×10^{-4}M in the presence of 5 mM $MgCl_2$ and the K_m for $MgCl_2$ was 8×10^{-4}M in the presence of 0.01 M ATP. The brain enzyme was found to have maximum activity over a wide pH range between 6 and 8. It showed maximum stability at pH 6. It was stable to heating and required 15 min at 55° for total inactivation. The activity of the enzyme increased 2.2-fold over the range 30 to 40°.

Moore and Strecker (1963) described in a preliminary report the solubilization and purification of brain hexokinase by acetone treatment of the particulate enzyme. They purified it by ammonium sulphate fractionation, heat treatment and adsorption on DEAE-cellulose and calcium phosphate gel. Details of this work are awaited.

A study of the kinetics of brain hexokinase prepared according to the method of Crane and Sols has recently been carried out by Fromm and Zewe (1962 b). They observed an apparent K_m value of glucose ranging from 2.6×10^{-5}M at the lowest ATP concentration to 6×10^{-5}M at the highest ATP concentration. Similarly the K_m values for ATP varied from 1.1×10^{-4}M to 2.9×10^{-4}M at the lowest and highest glucose concentrations respectively. The work of Fromm and his colleagues on the mechanism of action of yeast and animal tissue hexokinase based on kinetic studies will be described in a later section.

Grossbard and Schimke (1966) have purified the soluble hexokinase of brain (Type-I) by a procedure similar to that used by them for the purification of the soluble hexokinases of muscle, kidney, etc. The activity of the soluble enzyme was only 1.2 units per g of brain at 37° which is a small fraction of the total hexokinase activity of rat brain (calculated from other data to be about 7 to 10 units per g). The final specific activity obtained by these authors was about 11 units per mg at 37° which is not appreciably higher than that reported by Crane and Sols for the particulate enzyme after correction for the temperature. Its properties were generally similar to those of the particulate enzyme.

SECTION V

Inhibitors of Animal Tissue Hexokinase

Sulphydryl agents such as o-iodosobenzoate inhibit brain hexokinase. p-CMB was found to inhibit the enzyme markedly and this inhibition could be prevented by cysteine (Crane and Sols, 1955). Disulfiram inhibition could also be prevented by reduced glutathione or cysteamine (Stromme, 1963). These results indicate that brain hexokinase contains essential -SH groups in contrast to the enzyme from yeast, which has less readily accessible -SH groups. Xanthurenic acid (10^{-3}M) was reported to inhibit dog muscle hexokinase and this inhibition was reversed by kynurenic acid and cysteine (Kotake et al. 1957). It was suggested that this was probably due to interaction either with Mg^{++} or -SH groups. Liver hexokinase is sensitive to dihydroascorbic acid and alloxan (Bhattacharya, 1958) and skeletal muscle hexokinase, which is inhibited by alloxan, can be reactivated by cysteine (Griffiths, 1949).

Deoxycorticosterone (10^{-3}M) was found to inhibit rabbit muscle hexokinase by 50 per cent (Rosenkrantz, 1959). The inhibition of muscle hexokinase by sodium acetoacetate (3.5 mM) was reported to be prevented by insulin (Motlag and Nath, 1961). Brain hexokinase activity was found to be decreased by 50 per cent by continued inhalation of chloroprene (Mkhitaryan and Khachatryan, 1964). The claim that rat erythrocyte hexokinase activity is increased by sodium acetate requires confirmation (Ovchinnikov, 1965). The activation of rabbit brain hexokinase by butylamine and γ-aminobutyrate and its

inhibition by N-acetyl-γ-aminobutyrate are of interest (Mori, 1958). It was also reported that rabbit heart hexokinase activity was increased by δ-aminovalerate and γ-aminobutyrate by 105 and 69 per cent respectively. It was suggested that NH_2-$(CH_2)_3$- groups are required for enzyme activity.

Competitive inhibition by several sugars, such as inhibition of glucose phosphorylation by mannose, has been noted by several authors. Of greater interest and physiological significance is the effect of glucose-6-P, ADP, ATP and Pi on animal tissue hexokinase activity.

ADP has been reported to inhibit brain, heart and skeletal muscle enzymes and the K_i values have been given in Table 3. Inhibition by ADP was stated to be competitive with respect to ATP by Crane and Sols (1954). Froman and Zewe (1962 b), however, reported that inhibition by ADP was noncompetitive with respect to ATP for brain hexokinase. More recent studies with a purified ascites enzyme indicate that ADP inhibition is competitive with respect to ATP (Uyeda and Racker, 1965). Further clarification of these conflicting reports is needed.

In addition to glucose-6-P five other phosphate esters (3-deoxy-D-glucose-6-P, α-D-glucose 1,6-diP, 1,5-anhydro-D-glucitol 6-P, L-sorbose-1-P and D-allose-6-P) caused inhibition. Alterations in the 2 or 4-positions of the hexose apparently render the compound ineffective as an inhibitor. It is of special interest that fructose- and mannose-phosphates are not inhibitory and only glucose-6-P shows marked inhibition. Glucose-6-P inhibition was reported to be non-competitive with respect to glucose and ATP (Weil-Malherbe and Bone, 1951),

Crane and Sols, 1954). It was suggested on the basis of these results that there were different binding sites for these compounds. However Frosm and Zewe (1962 b) presented data which indicate that inhibition by glucose-6-P is competitive with respect to ATP and suggested that there was no need to postulate separate binding sites for ATP and glucose-6-P. These results were obtained with particulate brain hexokinase. The reason for the discrepancy in these results, as in the case of ATP and ADP, is not clear.

It may be noted in this connection that in most of the earlier work with crude enzyme preparations and especially when the method was based on the estimation of the disappearance of hexose or of labile phosphates, the observed activity of hexokinase would be influenced by phosphofructokinase, the removal of fructose-6-P by the latter enzyme would reduce the glucose-6-P levels and thereby favour further phosphorylation of glucose. Recent work has shown that phosphofructokinase activity is influenced by a large number of compounds such as ATP, ADP, fructose-6-P, fructose 1,6-diP, citrate, etc. It will be obvious that all substances which affect phosphofructokinase activity will indirectly have an effect on hexokinase activity. The clarification of the effect of different compounds on the two enzyme systems is simpler and less ambiguous with purified enzymes.

The recent observation that glucose-6-P inhibition is reversible by Pi is of considerable physiological interest.

Rose and colleagues (1964) first observed an increase in the utilization of glucose by human red blood cells in the presence of Pi. In the presence of15 mM Pi the apparent inhibition constant of glucose-6-P was increased from 35 μM to 150 μM. It was also reported that the alteration of glucose-6-P inhibition by Pi could be

demonstrated with particulate and soluble enzymes of ascites cells. This observation on the reversal of glucose-6-P inhibition by Pi was also extended to the mitochondrial and soluble hexokinases of heart (Mayer et al. 1966) and the particulate enzyme of brain (Tiedemann and Born, 1959). It was however stated by Rapoport and Gerber (1965) that the effect was probably due to the effect of Pi on phosphofructokinase, since partially purified human red cell hexokinase was unaffected by Pi. Lowry and Passonneau (1964) also observed that the inhibition of the hexokinase of brain homogenates by glucose-6-P was not affected by Pi.

Uyeda and Racker (1965) made a detailed study of the effect of glucose-6-P and Pi on the partially purified particulate hexokinase of brain and the soluble hexokinase of ascites cells. They observed inhibition by glucose-6-P with both the tissues and the reversal of the inhibition by Pi. Inhibition by glucose-6-P was remarkably stable and was unaffected by heating at 58° in the presence of different substrates, incubation with mercury or silver or digestion with proteolytic enzymes in the presence or absence of glucose. The ascites and brain hexokinases were less inhibited by glucose-6-P and the inhibition was more readily reversible by Pi than in the case of red blood cells haemolysates. The crude ascites enzyme was also inhibited less by glucose-6-P than the purified enzyme, but this was possibly due to the effect of other compounds which release Pi or metabolise glucose-6-P.

SECTION VI

Multiple Forms of Hexokinase

The existence of soluble and insoluble forms of hexokinase in animal tissues has already been noted. In addition to these the occurrence of multiple forms of hexokinase in several animal tissues has been described in recent years. The discovery of a glucokinase in liver, which is markedly different in its property, kinetics and specificity from other hexokinase, is an important development in this field but due to limitations of space the literature on multiple forms of hexokinase can only be briefly summarized.

Glucokinase

The presence of a glucokinase which differs from hexokinase in having a markedly higher K_m value for glucose was reported by three different groups of workers (DiPietro and Weinhouse, 1960; Walker, 1962; Vinuela et al. 1963). The enzyme is soluble and has been purified by Sols et al. (1965), McLean and Brown (1966) and Parry and Walker (1966). The preparation obtained by Walker and his coworkers from rat liver was purified about 850 times relative to the original extract (maximum specific activity was about 9 to 11 units per mg protein at 28°) but it was far from homogeneous. An interesting feature of the purification procedure is the use of a high concentration of potassium salts (0.1 M), which was essential for the stability of the enzyme. Liver glucokinase differs from hexokinase in several important respects. Its K_m value for glucose was 2×10^{-2} M as compared to a K_m value of 10^{-4} or 10^{-5} M

for the hexokinases. It was active with glucose, mannose and 2-deoxyglucose, the relative rates being 1, 0.9 and 0.5. The activity with fructose was very low and the apparent K_m value for fructose was 2 M. This is in marked contrast to the wider specificity and the high rate of phosphorylation of fructose by hexokinases. Another notable feature of this enzyme is the lack of inhibition by glucose-6-P upto 10 mM. It however resembled hexokinase in being inhibited competitively by ADP. The optimum pH was 7.7 to 8 and the enzyme was activated by sulphydryl compounds and inhibited by p-CMB. Though glucokinase occurs predominantly in liver preliminary reports on its presence in a few other tissues such as adipose tissue, have been presented.

The occurrence of glucokinase in several mammals has been noted, though it has been reported to be absent in human liver. The adaptive nature of this enzyme and its response to a variety of environmental conditions and hormones are of considerable physiological interest. Glucokinase content is low in alloxan diabetes, in starvation and on high fat diet, whereas treatment with insulin or high carbohydrate diets leads to rapid restoration of normal enzyme levels. The absence of the enzyme in several tissues which respond to insulin and its absence from some species makes it doubtful whether the observed effects of insulin can be attributed entirely to its effect on glucokinase. However, this enzyme has undoubtedly an important role in carbohydrate metabolism and the observed hormonal effects are undoubtedly of great physiological interest.

Other types of Hexokinase

In addition to glucokinase multiple forms of hexokinase have been reported to be present in several tissues. González et al.(1964) separated rat liver extracts on DEAE-cellulose columns and showed the presence of three isozymes A, B and C (which had a low K_m value for glucose) in addition to glucokinase. Under different physiological conditions major changes were found to occur mainly in the levels of glucokinase. Katzen and Schimke (1965) also noted the existence of four different types of hexokinase in liver on the basis of starch-gel electrophoresis and chromatography on DEAE-cellulose or hydroxyl-apatite. Kidney was found to contain three types. Adipose tissue, skeletal muscle, heart and brain contained only two types of hexo-kinase. Two isozymes were stated to be present in human white blood cells and seven in red blood cell hemolysates (Eaton et al. 1966). Grossbard et al. (1966) have also reported on the electrophoretic mobilities of the different forms of hexokinase in several species (rat, mice, hamster, guineapig, rabbit, monkey and cow). It was claimed that the electrophoretic mobilities of one or more the hexokinases vary sufficiently from species to species to allow iden-tification of species by this method. This work was, however, carried out entirely with 100,000 x g supernatants of the tissues, which were examined by starch gel electrophoresis and hence represent studies of only the soluble and not of the particulate forms. In all species brain showed only two forms, the major fraction of which had a lower mobility. Skeletal muscle and adipose tissue also showed only two types in all the animals, whereas the livers of animals showed several types of hexokinase varying from three in the case of mouse and rabbit

to five for human beings. Kidney extracts contained all the types present in liver except the one with maximum mobility. One of these was presumably a glucokinase. The K_m values of these hexokinases for glucose varied from 10^{-4}M to 10^{-6}M. Bachelard (1967) claims to have demonstrated by starch-gel electrophoresis three bands in the soluble fraction of cerebral cortex extracts of guineapig and two bands in the mitochondrial fraction after solubilization.

Karpatkin (1966) studied the particulate and soluble fractions of hexokinase in frog sartorius muscle and separated them by centrifugation at 105,000 x g. It was observed that the enzyme was divided equally between the soluble and insoluble fractions. The K_m for glucose and ATP, the optimum concentration of Mg^{++}, the change in activity with temperature and inhibition by p-CMB were identical for the two fractions. Though both enzymes were inhibited by 1-deoxyglucose-6-phosphate, the inhibition could be reversed by Pi only for the particulate enzyme. The soluble enzyme was also more stable to heat inactivation. It was suggested that the differences in the properties of the two enzymes, especially the reversal of hexose-6-P inhibition by Pi, may play a role in regulating glucose metabolism in muscle. These observations are of interest in view of a recent preliminary note by Fromm et al. (1967) on the existence of two hexokinases in muscle (see section on Mechanism of Action of Hexokinase).

Grossbard and Schimke (1966) have purified the soluble enzymes from rat kidney, muscle, adipose tissue, liver and brain. Some of their work has been referred to in earlier sections. The

specific activities of the purified enzymes ranged from about 1.4 units per mg for liver to about 13 units per mg for muscle and adipose tissue enzymes. The purified enzyme preparations were similar with respect to their specificity, pH optimum and molecular weight. Three different hexokinase types were characterized on the basis of differences in K_m values for glucose and ATP, K_i values for ADP and glucose-6-P and stability to heat and proteolytic enzymes. The three types also differ in their mobility on starch gel electrophoresis. It was claimed that the observed differences were not due to the action of proteolytic enzymes on a single hexokinase to produce the different types. Since all types had the same molecular weight of 96,000, the observed differences could not be due to a difference in the degree of aggregation of subunits.

These results are undoubtedly of interest, but some features of the work on multiple forms of hexokinase require careful examination. Except for the work of Grossbard and Schimke most of the work has been carried out with crude enzyme preparations. It is of interest that Colowick and coworkers (1961) originally demonstrated six different forms of yeast hexokinase even in the crystalline enzyme and suggested that they were not formed by the action of proteolytic enzymes but were originally present in yeast. Subsequent work by Schulze et al.(1966) has, however, shown that these multiple forms of hexokinase were artifacts arising from the method of isolation of the enzyme. Care is, therefore, required in assessing the results obtained with crude tissue extracts which are likely to contain proteolytic enzymes. Another point to be noted is that most of the work has been carried out with soluble hexokinase whereas in

many tissues hexokinase occurs mainly in particulate form. During the disruption of the cells the particles to which hexokinase is attached may be broken in such a way that enzyme molecules attached to different smaller fragments are present in the extracts. It is possible that the observed differences are due not to the enzyme but to the different constituents attached to the same enzyme. Undue importance need not be attached to differences in electrophoretic mobility of the enzyme on starch gel in the absence of further characterization of these enzyme types. It should also be emphasized that characterization of the differences in the properties of the hexokinase types in terms of physiological function, as in the case of the lactate dehydrogenase isozymes, has not so far been possible and the observed differences in properties cannot yet be related to differences in structure or function. The work of Grossbard and Schimke was no doubt carried out with purified soluble hexokinases but it is to be noted that the enzymes of highest purity obtained by them from kidney and skeletal muscle when corrected for temperature had only a specific activity of 8 units per mg which may be compared to a specific activity of about 5 for the enzyme of Crane and Sols and a specific activity of 63 units per mg for the purified ox brain hexokinase obtained in this Laboratory. The preparation of these "isozymes" of hexokinase in homogeneous form and the study of their structure and properties are required definitely to establish that they actually occur in animal tissues and that the observed differences in properties are of physiological significance.

SECTION VII

The Effect of Hormones on Animal Tissue Hexokinases

Price *et al.* reported in 1945 that hexokinase from animal tissues can be inhibited by anterior pituitary extract either by injecting the rats with the hormone before the preparation of the tissue extracts or even *in vitro* in a cell-free system. This inhibition was reversed by insulin both in the whole organism and in cell-free extracts. Similar results were obtained with alloxan diabetic rat tissue. Yeast hexokinase showed no inhibition by the pituitary extract. Though brain extracts showed no inhibition when the rats were injected with alloxan or pituitary extract, it was claimed that the brain extracts could be inactivated by the anterior pituitary hormone *in vitro*. Insulin had no effect directly on hexokinase activity, though it reversed the inhibitory effect of the pituitary hormone. The reversal of the inhibition was proportional to insulin within a specified range. Muscle extracts of diabetic rats were also found to show normal hexokinase activity when injected with insulin before the preparation of muscle extracts. Reduced insulin showed no reversal of the inhibition by the pituitary hormone. Similar results were also observed with adrenal cortex extracts which had no direct effect on the hexokinase of animal extracts but intensified inhibition by anterior pituitary extracts. The effect of adrenal cortex could also be reversed by insulin. The inhibitory effect of the adrenal fractions was absent from the crystalline compounds (a, b and c) obtained at that time and was found in the amorphous fraction (Price *et al.* 1946).

These interesting observations have been the subject of a voluminous literature during the past twenty years. No attempt will be made here to summarize the findings of different groups of workers in this highly controversial field. Even the demonstration of the primary effects of the hormones is a subject for controversy and not merely the interpretation of the results. Only a few points relevant to the subject of this thesis will be noted here. All the work in this field has hitherto been carried out with very crude preparations of hexokinase. The high instability and insolubility of the hexokinase preparations and the presence of several interfering enzymes preclude any definite conclusion regarding the effect of the adrenal and pituitary or insulin directly on hexokinase activity. Similarly several claims have been made (e.g. Weil-Malherbe et al. 1951; Long, 1955, etc.) that specific inhibitors of hexokinase are present in erythrocytes, muscle, etc. These studies were also carried out only with crude preparations. As has been noted earlier, it would be difficult to distinguish between the effects of different substances on phosphofructokinase and hexokinase in crude systems, since any alteration of the activity of the former would lead to an alteration of glucose-6-P, ATP or Pi levels (by further glycolysis) and hence indirectly of the apparent hexokinase activity. It is sufficient to state here that no definite evidence exists for a specific naturally occurring hexokinase inhibitor. It is also generally held that no unequivocal in vitro effect of hormones on animal tissue hexokinases has been demonstrated. The contrary view which categorically holds that hormones do not have an effect in vitro on hexokinase cannot also be said to have been definitely established

(Krahl, 1961). Work with purified enzymes and with assay systems which have not generally been used for such studies will be needed to investigate the effect of hormones on hexokinases for the following reasons.

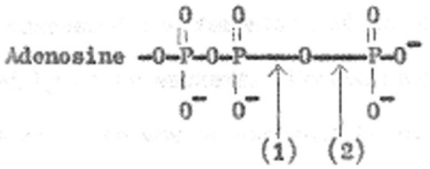
Recent observations on the inhibition of hexokinase by glucose-6-P and the release of this inhibition by Pi suggest the possibility that some of the observed effects on hexokinase may be due to the effect of glucose-6-P or Pi on hexokinase. The possibility exists that hormones may alter the binding of glucose-6-P or Pi to hexokinase thereby increasing or decreasing its activity. Such studies, however, are obviously not possible with any test system in which glucose-6-P is removed by conversion to 6-phosphogluconate or to fructose-1,6-diP. Since the enzyme is inhibited 50 per cent by 10^{-4} to 10^{-5}M glucose-6-P the study of the effect of the latter would be difficult with an assay system based on the measurement of the decrease in glucose or labile phosphorus, which would lack the required sensitivity. Even with other assays (measurement of ADP enzymically or of the liberated H^+ colorimetrically) inhibition by glucose-6-P the concentration of which changes during the course of the reaction, makes the study of the reaction more complicated, especially with highly purified preparations which are more sensitive to glucose-6-P than the crude enzyme. Most of the test systems used for the study of hormonal effects on hexokinase were unsuitable for elucidating the possible role of the hormones in modifying the effect of glucose-6-P on the enzyme. The exchange of labelled glucose with glucose-6-P in the presence of different concentrations of glucose-6-P and Pi (Uyeda and Racker, 1965) or a method similar to

the one described later in this thesis will be needed to evaluate the effect of hormones on hexokinase in the presence of glucose-6-P, Pi and possibly other related compounds which have an effect on the enzyme. A purified enzyme free from interfering enzymes and impurities will be needed but care must be taken that the hexokinase is not altered during the solubilization and purification since allosteric properties of an enzyme may be lost due to heating, etc. without loss of activity. The effect of insoluble fragments and their binding to hexokinase may also influence the effect of hormones on the enzymes in the presence of glucose-6-P, etc. To summarize, a study by a suitable assay of the effect of hormones on a complex system consisting of purified animal tissue hexokinase, glucose-6-P, Pi and other factors is needed to establish conclusively whether or not the hormones have an effect <u>in vitro</u> on the enzyme.

SECTION VIII

Mechanism of Action of Hexokinase

Several studies have been made on the mechanism of yeast hexokinase whereas relatively little work has been carried out with animal tissue hexokinases. The mechanism of action of yeast hexokinase was investigated by Cohn (1958) using O^{18}. The enzyme was incubated with glucose, ATP and Mg^{++} in Tris buffer with H_2O^{18}. After two hours the reaction was stopped by trichloracetic acid and ADP and glucose-6-P were separated by barium precipitation. The resulting hexose-6-P was hydrolysed enzymically and the phosphate analysed for O^{18}. ADP was also hydrolysed by 1N hydrochloric acid to determine the O^{18} of the terminal phosphate. Neither ADP nor glucose-6-P contained excess O^{18}. It was, therefore, concluded that cleavage occurs between oxygen and the terminal phosphate of ATP.



If cleavage occurs at (1) ADP will be labeled. Cleavage occurs, therefore, at (2). The O atom of glucose-6-P, which links C^6 of glucose with P, is derived from glucose and not from ATP

Paramagnetic resonance studies were carried out with yeast hexokinase using Mn^{++} instead of Mg^{++} since manganous ion is

paramagnetic (Cohn,1965). The effect of Mn^{++} on the relaxation rate of the nuclear magnetic resonance of the protons of water is sensitive to the type of metal complex formed. Use was made of this property to study the formation of complexes of the enzyme with ATP, Mg^{++} and glucose, singly or together. In the case of crystalline yeast hexokinase there was no enhancement of the relaxation rate with manganese and enzyme, manganese and nucleotide alone or with enzyme, manganese and ATP or ADP unless glucose was also present. It was concluded that a quaternary complex is required for the enhancement of the relaxation rate. It was suggested that there is a compulsory order of addition, the metal nucleotide being bound only to the glucose-enzyme complex.

The possibility of the formation of a covalent enzyme-phosphate complex by the interaction of enzyme and ATP followed by the transfer of phosphate from the enzyme-phosphate complex to glucose to form enzyme and glucose-6-P has been investigated by several workers. Ågren and Engström (1956) isolated phosphoserine from the acid hydrolysate of crude yeast hexokinase which was incubated with P^{32} labeled ATP and glucose or with glucose-6-P. However, these results, which suggested the formation of an enzyme-phosphate, could not be confirmed by other workers. Preliminary results by Najjar (1958) showed that there was no exchange between C^{14}-labeled glucose and glucose-6-P in the absence of the adenine nucleotides. Such an exchange should occur if phosphoenzyme were to react with glucose to form glucose-6-P and dephospho-enzyme. More extensive studies were carried out by Boyer and by Colowick. Boyer (1961) observed that when yeast hexokinase was incubated with P^{32} labeled ATP, the specific activity of the isolated enzyme was less than 1/500 of that of ATP.

Further studies by Colowick and Trayser (1961) also ruled out the possibility of formation of a phosphoenzyme complex. When an excess of hexokinase is incubated with ATP in the presence of P-enolpyruvate, pyruvic kinase and lactic dehydrogenase and DPNH there should be a burst of ADP formation if an enzyme phosphate complex is formed and this can be detected by the oxidation of DPNH. No such reaction was observed. A slow formation of ADP was noted but this was ascribed to ATPase activity and to the presence of traces of glucose in the enzyme. Ågren et al (1963) have also reported that with highly purified yeast hexokinase they found no phosphoenzyme intermediate on incubation with ATP. It may, therefore, be concluded that the formation of a phosphoenzyme appears improbable at least in the case of yeast hexokinase. Similar studies have not been carried out with animal tissue hexokinases.

A second mechanism which was suggested by Najjar (1958) is the formation of an enzyme-glucose complex which then reacts with ATP to form a glucose-6-P-enzyme and ADP. The glucose-6-P-enzyme complex then reacts with glucose to form glucose-enzyme and glucose-6-P. However studies by Colowick and Trayser (1961) failed to furnish any support for this hypothesis. If the enzyme exists as an enzyme-glucose complex, in the absence of added glucose it should react with ATP labeled with P^{32} to form an enzyme glucose-6-P complex. No such complex formation was detected and no exchange between ADP and ATP was observed in the absence of added glucose-6-P or glucose.

It has been mentioned earlier that crystalline yeast hexokinase contained small amounts of glucose. The amount of glucose present was only 0.25 mole per mole of enzyme of molecular

weight 96,000. The dissociation constant for different sugars and glucose-6-P could not be measured by direct equilibrium dialysis methods. An indirect method based on the protection of hexokinase against inactivation by trypsin showed that the dissociation constants (Trayser and Colowick, 1961) for glucose and fructose were 2.4×10^{-4}M and 0.8×10^{-3}M respectively, which correspond to the K_m values for glucose and fructose. The fact that hexokinase in yeast extract is readily attacked by trypsin unless glucose is added also suggests that most of the enzyme was not present in combination with glucose. These studies indicate that there is no definite evidence to support the formation of covalent enzyme-glucose or enzyme-glucose-6-P complex as an intermediate of the yeast hexokinase reaction.

Crystalline hexokinase was found to have ATPase activity in the absence of hexose as described above. ATPase activity was found to parallel hexokinase activity and it was suggested that both activities were due to the same enzyme. Both activities were inhibited to the same extent by hexokinase inhibitors (N-acetylglucosamine, sorbose-1-P) and silver ions caused inactivation of both the activities to the same extent. Mg^{++} was also required for ATPase activity and the K_m value for ATP was 5 mM. The relation of ATPase activity to hexokinase activity is obscure unless it is an intrinsic property of hexokinase and the enzyme can transfer the phosphoryl group not only to the hydroxyl of glucose but also to water at a much lower rate.

The mechanism of action of yeast hexokinase has also been investigated by Fromm and his colleagues by kinetic studies. Fromm

and Zewe (1962 a) found that the substrates glucose and ATP can be added to the enzyme in any sequence without altering the equilibrium kinetics of the reaction. This suggests that neither an enzyme-phosphate nor an enzyme-glucose intermediate is formed in the yeast hexokinase reaction. The formation of a quaternary complex of enzyme, ATP, Mg^{++} and glucose is also supported by the kinetic studies of Hammes and Kochavi (1962).

It may be concluded that there is no definite evidence for the formation of enzyme-glucose or enzyme-phosphate as intermediates in the yeast hexokinase reaction. The formation of a quaternary complex of enzyme, Mg^{++}, ATP and glucose as suggested by paramagnetic resonance studies appears probable. The only definite information available about the reaction is from the O^{18} studies of Cohn, which show that cleavage occurs between the oxygen and the terminal phosphate of ATP.

The mechanism of action of animal tissue hexokinases has been investigated mainly by Fromm and his colleagues (Fromm and Zewe, 1962 b; Hanson and Fromm, 1965). The kinetics of particulate brain hexokinase and partially purified soluble muscle hexokinase and the effect of mannose, mannose-6-P, glucose-6-P and ADP were studied. They concluded that the animal tissue hexokinases differ from yeast hexokinase and that with these enzymes interaction with one substrate and dissociation of a product occur before the second substrate combines with the enzyme. This differs from the quaternary complex postulated for the enzyme from yeast. Since a glucose-glucose-6-P exchange did not take place with the skeletal muscle

enzyme in the absence of adeninenucleotide, an enzyme-phosphate intermediate was ruled out. The initial formation of an enzyme-glucose intermediate was suggested. More recently (Fromm et al. 1966) skeletal muscle hexokinase was found to catalyse an ADP-ATP exchange in the absence of hexose. However N-acetylglucosamine which shows half maximum inhibition of hexokinase at 0.1 mM did not inhibit the exchange reaction even at 25 mM. It was claimed on the basis of initial reaction rates at different glucose concentrations that two hexokinases were present in this preparation. Uncertainty about the hexose or hexose-6-P content of these enzymes and their low purity render the interpretation and assessment of these results difficult. Further work with purified preparations is required to clarify and extend these interesting observations.

SECTION IX

The Estimation of Hexokinase Activity

$$\text{Hexose} + \text{ATP} \longrightarrow \text{Hexose-6-P} + \text{ADP} + H^+$$

The course of the above reaction can obviously be followed by estimating the disappearance of hexose or ATP or the formation of hexose-6-P or ADP or the liberation of hydrogen ions. All these methods have been used for the assay of the enzyme.

(1) The amount of ATP utilized can be estimated after hydrolysis by 1N acid in 7 min at 100°. The disappearance of hexose can also be followed by any conventional procedure such as that of Nelson after precipitation of hexose-6-P and nucleotides by zinc hydroxide. These methods are,however, of low sensitivity.

(2) Methods based on the estimation of the protons liberated are sensitive and are of value in the absence of enzymes which hydrolyse the adenine nucleotides or glucose-6-P. The hydrogen ions formed can be estimated manometrically in the presence of bicarbonate by measuring carbon dioxide evolution (Berger et al.1946). The acid produced can also be directly titrated with alkali in the presence of an indicator such as phenol red (Kunitz and McDonald,1946). A third method for measuring the protons liberated is based on the use of an indicator with the same pKa as the buffer and the change in colour can be followed spectrophotometrically as suggested by Wajzer. Darrow and Colowick have used cresol red in glycylglycine buffer at 25° and measured the change in optical density at 560 mμ,

which was proportional within limits to the acid produced. Variations in temperature must be avoided, but the method is otherwise sensitive and rapid and does not require the use of other enzymes.

(3) ADP formation can be measured in the presence of pyruvic kinase lactic dehydrogenase and P-enolpyruvate, Mg^{++}, K^{+} and DPNH.

$$\text{P-enolpyruvate} + \text{ADP} \xrightarrow[\text{Pyruvic kinase}]{K^{+}\ Mg^{++}} \text{Pyruvate} + \text{ATP}$$

$$\text{Pyruvate} + \text{DPNH} \xrightarrow[\text{dehydrogenase}]{\text{Lactic}} \text{Lactate} + \text{DPN}$$

As is evident from the above two reactions, in the presence of an excess of the two enzymes one mole of ADP is equivalent to one of pyruvate and will cause the oxidation of one mole of DPNH which can be measured spectrophotometrically at 340 mμ. This method is sensitive and accurate provided ATPase and myokinase are absent from the test system.

(4) Glucose-6-P formation can also be measured spectrophotometrically in the presence of TPN and an excess of glucose-6-phosphate dehydrogenase. The rate of TPN reduction measured spectrophotometrically at 340 mμ is a measure of glucose-6-P formation and hence of hexokinase activity. The details of the above methods, especially of methods 3 and 4, are given in the experimental section. It should however be noted that the choice of the method is dependent on the enzyme and the properties to be investigated. Whereas yeast hexokinase can be estimated by any of the methods, animal tissue hexokinases, which are markedly inhibited by glucose-6-P, are less conveniently assayed by methods 2 and 3 if glucose is used as a substrate than by method 4.

(5) Uyeda and Racker (1965) have measured the exchange of labelled glucose with glucose-6-P for studying hexokinase activity especially in the presence of glucose-6-P.

SECTION X

Present Work

The work reported in this thesis deals with the preparation of soluble hexokinase from the particulate enzyme of ox brain, its purification and the study of its properties and kinetics.

This work is based on preliminary studies (Jagannathan, 1963) which indicated that the hexokinase of ox brain can be obtained in soluble form by treatment with crystalline elastase. This method has been studied in detail and the conditions for obtaining the enzyme in good yield in soluble form were determined. The enzyme after treatment with crystalline elastase was not sedimented at 100,000 x g in one hour. However it was not possible to fractionate it at this stage by ammonium sulphate precipitation or calcium phosphate gel adsorption. But on freezing and thawing several times and treating it with protamine sulphate, a water-clear supernatant was obtained which could be fractionated by conventional methods.

The use of several methods of purification was studied and adsorption on calcium phosphate gel followed by elution with phosphate buffer was found to give significant purification. Further purification of this enzyme was possible by several different methods but the enzyme thus obtained lost its activity very rapidly. It was clear at this stage that no progress was possible unless conditions for the stabilization of the enzyme were determined. After extensive studies it was discovered that the enzyme could be stabilized in the presence of thioethanol, Pi and high concentrations (0.5 to 1 M) of sucrose or

glucose. An enzyme preparation which lost its activity even at -20° in a few days, was so stable in the presence of sucrose-phosphate and thioethanol that it could be heated at 50° for 5 minutes without significant loss in activity.

By the use of these stabilisers the enzyme was then fractionated further on DEAE-cellulose columns. The purification procedure is described by Joshi and Jagannathan (1966). The maximum specific activity obtained by this procedure was 63 µmoles of glucose phosphorylated per minute per mg of protein at 30°. At this level of purity the enzyme was found by ultracentrifugation to consist of one major component which contained hexokinase activity and about 90 per cent of the total protein and two minor components with a higher sedimentation rate.

The properties and kinetics of this enzyme which include the effect of pH, temperature and concentrations of glucose, magnesium and ATP on activity, the stability of the enzyme and its specificity towards different sugars, nucleotides and metals were studied. The inhibition of the enzyme by glucose-6-P and the reversal of the inhibition by Pi were investigated and the effect of anterior pituitary hormones, insulin and other compounds on hexokinase, either directly or in the presence of glucose-6-P and Pi, was determined.

Chapter II of the thesis deals with the materials and experimental methods used in these studies.

Chapter III deals with the preparation of soluble hexokinase from the particulate enzyme of ox brain and the purification of soluble hexokinase.

Chapter IV deals with the properties and kinetics of purified hexokinase.

Chapter V contains a discussion of the results of these studies.

Chapter VI contains a summary of the results and conclusions of this work.

CHAPTER 2

MATERIALS AND METHODS

Materials and Methods

Materials

The following chemicals were of analytical grade: potassium dihydrogen phosphate, dipotassium hydrogen phosphate, sodium pyrophosphate, ammonium sulphate, magnesium chloride, Tris, sodium acetate, acetic acid, potassium chloride, EDTA and 2-mercaptoethanol. The following sugars were also of high purity: glucose, sucrose, D- and L-arabinose, mannose, trehalose, fructose, galactose, D-xylose, rhamnose, mellibiose, N-acetylglucosamine, D-ribose, raffinose, L-xylose, sorbitol, l-sorbose, dulcitol, mannitol, cellobiose, glucosamine and α-methylglucoside. TPN, DPNH and ATP (crystalline disodium salt) were obtained from Sigma Chemical Co. Glucose-6-P was obtained as the barium salt $7.H_2O$ from Reanal or C.F.Boehringer and converted to the potassium salt according to Swanson (1955). P-enol pyruvate was prepared according to Clark and Kirby (1963). Protamine sulphate (ex-herring) was obtained from Sigma or Koch-Light Co. and salmine sulphate from the British Drug Houses. Protamine sulphate and salmine sulphate were used interchangeably in hexokinase purification. Baker's active dry yeast was obtained from Shaw Wallace and Co.

Calcium phytate from L. Light & Co. was purified according to Posternak (1921). Celite 535 was obtained from Johns Manville & Co. Whatman standard grade of cellulose powder was used. Calcium phosphate gel was prepared according to the procedure of Swingle and Tiselius (1951).

The gel was finally suspended in water (60 mg per ml dry weight). DEAE cellulose was either prepared according to Peterson and Sober (1956) (100-200 mesh 0.5 meq/g) or was obtained from Bio-Rad Laboratories ("Cellex-D", 0.62 meq/g). Amberlite IRC-50 (XE-64) (mesh 200 to 400) was obtained from Rohm and Haas. Carboxymethylcellulose and cellulose phosphate were obtained from Bio-Rad Laboratories.

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Pyruvate kinase, Type II, crystalline (specific activity 300 units per mg) from rabbit muscle and lactic dehydrogenase crystalline Type II from rabbit muscle (specific activity 580 units per mg) were obtained from Sigma Chemical Co. Twice crystallized elastase from pancreas was obtained from Sigma Chemical Co. or prepared from 1-300 Trypsin (Nutritional Biochemical Corporation) by the procedure of Lewis et al. (1956). Glucose-6-phosphate dehydrogenase, Type V, specific activity 140 units per mg was obtained from Sigma Chemical Co. It was also prepared from yeast by the following method.

Preparation of glucose-6-phosphate dehydrogenase from Baker's yeast

Dried baker's yeast (75 g) was mixed with 300 ml of 0.1 M sodium bicarbonate and allowed to autolyse for 3 hr at 37°. The autolysate was cooled to 0° and centrifuged at 14,000 x g for 40 min. The supernatant was filtered on Whatman No.1 filter paper to remove suspended material. To 130 ml of filtrate 8 ml of 2M Tris base was added to adjust the pH to 8 to 8.1. It was then heated for 70 min

at 46° and left at -20°. It was allowed to thaw the next day and heated again at 46° for 70 min and centrifuged at 0° at 14,000 x g for 40 min. The time required for heating the enzyme varied from batch to batch and was empirically determined by heating the autolysate for different periods and determining the ratio of glucose-6-phosphate dehydrogenase to hexokinase. The heating was continued till the ratio of the two activities was between 50 to 100.

The heated enzyme was brought to 50 per cent ammonium sulphate saturation (29.07 g for 100 ml), kept for 20 min and centrifuged at 14,000 x g for 30 min. The supernatant was then precipitated at 75 per cent ammonium sulphate saturation by the addition of 15.82 g for 100 ml. It was then kept for 20 min and centrifuged as above. The precipitate was dissolved in a small volume of 0.05 M Tris-HCl buffer, pH 7.6, and dialysed against three changes of the same buffer for 24 hr at 0 to 5°.

The dialysed enzyme (vol.25.5 ml) was diluted with 127 ml of 0.05 M Tris-HCl buffer, pH 7.6, so that it had 4.5 to 5 units per ml and its pH was adjusted to 4.7 by the addition of 30.6 ml of 0.2 M acetic acid and 16.3 ml 2M acetate buffer pH 4.7. After 20 min at 0° it was centrifuged at 14,000 x g for 30 min and the precipitate was carefully freed of as much supernatant as possible. It was then dissolved in 0.05 M Tris buffer, pH 7.6, centrifuged to remove turbidity and stored at -20°. If the ratio of glucose-6-phosphate dehydrogenase to hexokinase was less than 1000, precipitation at pH 4.7 was repeated once or twice as described above till the ratio of the two enzymes was over 1000. The results of a typical purification run are shown in Table 4.

TABLE 4

PURIFICATION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE FROM BAKER'S YEAST

Fraction	Volume	Glucose-6-phosphate dehydrogenase		Hexokinase		Ratio glucose-6-phosphate dehydrogenase / Hexokinase
	ml	units/ml	Total units	units/ml	Total units	
1) Autolysate from 75 g dried yeast	138	18.6	2566	4.6	635	4
2) 140 min heated at 46°	124	7.2	893	0.09	11	81
3) 50-75% ammonium sulphate fraction	25.5	26.4	673	0.22	5.6	120
4) Ist pH 4.7 precipitation	7	40.8	285	0.04	0.28	1020

It may be noted that the main objective was not to obtain enzyme of high specific activity but mainly to remove interfering enzymes. All glucose-6-phosphate dehydrogenase preparations were tested for the following interfering enzymes: hexokinase, TPNH-oxidase, 6-phosphogluconic dehydrogenase, ATPase, glucose-6-phosphatase, TPNase and glucose-TPN reductase. When the enzyme containing 1 unit of glucose-6-phosphate dehydrogenase was used for the assay no significant activity was observed in 1 hr for any of the above enzymes. There was a slight blank for glucose-TPN-reductase when the glucose concentration was 0.1 M. The slight hexokinase activity and the glucose-TPN-reductase activities were corrected for during all assays for brain hexokinase.

Methods

Spectrophotometric determinations were carried out in a Model DU Beckman spectrophotometer with silica cuvettes of 10 mm light path. The temperature was 30° unless otherwise specified. The extinction coefficients for DPNH and TPN were taken as 6.22×10^6 cm^2 per mole for the calculation of enzyme activities (Horecker and Kornberg, 1948). pH estimations were carried out with the glass electrode. The pH of ammonium sulphate solutions was determined after diluting four times with water.

Ammonium sulphate saturations refer to 0° and were calculated according to Jagannathan et al. (1956). Solid ammonium sulphate was slowly added over a period of about 10 to 20 min with gentle stirring but without allowing the liquid to froth. The liquid was

kept for about 20 min and then centrifuged at 14,000 x g for 30 min. The precipitates were dissolved in a known volume of buffer and the final volume was then noted. The increase in volume was assumed to be due to ammonium sulphate saturation at which the precipitate was obtained and a correction was made for the ammonium sulphate concentration in the enzyme solution.

Centrifugations were carried out at 0° in an International Centrifuge (Models PR-1 & PR-2) and Sorvall (Model SS-1). Ultracentrifuge measurements were carried out in a Spinco (Model E) analytical ultracentrifuge. Chromatographic fractions were collected on a Technicon Automatic fraction collector.

All glassware was routinely washed with sodium carbonate and then with nitric acid, rinsed successively with tap water, distilled water and glass distilled water and dried. The use of chromic acid or detergents was avoided. As described in a later section it was necessary to avoid contact of solutions used for enzyme purification with plastic or rubber tubes and stoppers or glassware previously kept in contact with chromic acid, phosphomolybdate or phosphotungstate. No grease or silicone was used for groundglass joints and stop-cocks.

Glass distilled water was used for preparation of all solutions.

Phosphate was estimated according to Fiske and Subbarow (1925). Protein was routinely determined by the following methods.

(i) A modification of the method of Warburg and Christian (1941) was used. A correction for nucleic acid and other ultraviolet

absorbing impurities was made by the following empirical equation (Jagannathan et al. 1956). It was assumed that a 0.1 per cent protein solution has an optical density of 1 at 280 mμ.

$$\frac{4}{7}\left[2.3\ (O.D._{280\ m\mu} - O.D._{340\ m\mu}) - (O.D._{260\ m\mu} - O.D._{340\ m\mu})\right]$$

= mg protein per ml.

Enzyme solutions were diluted with 0.1 M phosphate, pH 7.5, and the optical densities at 340, 280 and 260 mμ were determined. A solution of the same composition as that of the enzyme (e.g. with respect to phosphate, glucose, thioethanol, etc.) was prepared and its optical densities were also determined and these blank values were subtracted from the readings of the enzyme solutions. These corrections were appreciable for thioethanol containing solutions and were essential for determining the protein content of DEAE-cellulose eluates.

(ii) Protein was also estimated by the method of Lowry et al. (1951). Crystalline bovine serum albumin was used as the standard. The concentration of serum albumin was calculated from its extinction coefficient at 280 mμ according to Long (1961). Phosphate, ammonium sulphate, thioethanol and glucose interfere with this estimation and it was essential to dialyse enzyme preparations till the enzyme was free of these substances before carrying out the estimations. A blank of the final dialysate was carried out so as to correct for any small residual interfering substances in the enzyme. Corrections for change in volume of the enzyme during dialysis were also made. Since the enzyme lost activity during dialysis against water or buffer it was not possible to know the activity of the enzyme after

dialysis. Protein estimations on the final purified enzyme were carried out either by the spectrophotometric or Folin's method. The results with the latter method, which required only small amounts of enzyme and was carried out with dialysed preparations, were considered more accurate. The protein values by Folin's method were lower than by the other method, especially for dilute enzyme solutions for which the corrections for thioethanol in the spectrophotometric method were high.

Definition of unit of activity and specific activity

All enzyme activities (hexokinase, pyruvic kinase, glucose-6-phosphate dehydrogenase and lactic dehydrogenase, etc.) are expressed as conversion of 1 μmole of substrate in 1 min unless otherwise stated.

The unit of hexokinase activity is defined as the amount of enzyme required to phosphorylate 1 μmole of glucose in 1 min at 30° under the experimental conditions given in the text. The specific activity of the enzyme is defined as the activity per mg of protein.

The results of some activity determinations, especially on enzyme kinetics, are presented as changes in optical density (O.D.) instead of μmoles of glucose-6-P or ADP formed in the hexokinase assays, when only comparative data are required.

Dilution of enzyme for estimation

Crude hexokinase was diluted at 0° with 0.05 M phosphate buffer, pH 7.5 and the purified enzyme in 0.05 M phosphate buffer, pH 7.5 containing 0.5 M glucose or sucrose, 0.001 M thioethanol and

0.01 per cent serum albumin unless otherwise mentioned.

Estimation of hexokinase from brain

The following three methods were used for the estimation of hexokinase activity. A few of the precautions and controls and other salient features of each method are also noted here in order to avoid repeated reference to these details for each experiment in the section on enzyme kinetics.

1) Glucose-6-Phosphate Dehydrogenase Assay

(Slein, Cori and Cori, 1950)

This method has been used routinely for assaying hexokinase during the purification of the enzyme and for some kinetic studies. The details of the method are as follows except when otherwise stated:

The assay system consisted of Tris-HCl buffer, pH 7.5 (50 µmoles), $MgCl_2$ (20 µmoles), glucose (15 µmoles), EDTA (0.1 µmole), ATP (10 µmoles), TPN (0.13 µmole), 0.1 unit of glucose-6-phosphate dehydrogenase and hexokinase in a total volume of 1.0 ml. The final pH of the reaction mixture was 7.5 and the temperature 30°. The reaction was initiated by adding ATP and the rate of the reaction was followed by measuring the increase in O.D. per min at 340 mµ. The amount of enzyme added was such that the ΔO.D. was between 0.005 to 0.020 per min and was measured from the second to the tenth min. The reaction was linear during this period and the average ΔO.D. per min was taken as a measure of the initial velocity of the reaction. The quantity of TPNH formed per min was calculated from ΔO.D. Suitable blanks were run by measuring TPN reduction in controls without ATP and without brain hexokinase to correct for

glucose-TPN reductase and hexokinase activities respectively of the glucose-6-phosphate dehydrogenase. There was no need to correct for 6-phosphogluconic dehydrogenase activity since it was negligible in the yeast glucose-6-phosphate dehydrogenase and in brain hexokinase except in homogenates.

For each variation in experimental conditions controls were run to ensure that an excess of glucose-6-phosphate dehydrogenase and TPN were present and that these were not rate limiting. The blank for each variation in experimental condition was run in the absence of brain hexokinase and all results are corrected for these blanks. For instance there was a small blank at high glucose concentrations due to a glucose-TPN reductase present in the glucose-6-phosphate dehydrogenase. This blank was not appreciable at lower glucose concentrations which were generally used for enzyme assay but was important in studies on the effect of glucose concentration on activity. Similarly at different Mg^{++} and salt concentrations, care was taken that glucose-6-phosphate dehydrogenase was present in adequate amounts. This method will be referred to as Assay-I.

2) Direct Hexokinase Assay

For experiments in which the presence of other enzymes was undesirable (e.g. where the pH optimum or other properties of the second enzyme were not suitable for the assay) the following procedure was used. The hexokinase reaction was run in a reaction mixture containing Tris buffer, $MgCl_2$, glucose, EDTA, enzyme and ATP at a definite temperature. The reaction was terminated

after a definite period by the addition of acetic acid. The solution was then neutralized by the addition of sodium hydroxide to pH 7.5 and the glucose-6-P formed was determined by the addition of TPN and glucose-6-phosphate dehydrogenase. The details of the method are as follows except for changes (e.g. temperature) which are noted for each experiment.

The reaction mixture contained Tris-HCl buffer, pH 7.5 (17 μmoles), $MgCl_2$ (6.6 μmoles), glucose (5 μmoles), EDTA (0.03 μmole), ATP (3.3 μmoles) and enzyme in a final volume of 0.34 ml. After temperature equilibration ATP was added last to start the reaction. The reaction was stopped by the addition of 240 umoles of acetic acid and the reaction mixture was then neutralized by the addition of NaOH. The total glucose-6-P formed was estimated by the addition of Tris-HCl buffer pH 7.5 (50 μmoles), $MgCl_2$ (10 μmoles) and glucose-6-phosphate dehydrogenase 0.3 unit and finally TPN (0.52 μmole) in a final volume of 1.0 ml. The O.D. was noted before and after the addition of TPN and the reaction was allowed to proceed till the O.D. reached a maximum, the difference in O.D. being taken as the measure of total glucose-6-P formed in the reaction mixture. A blank was run in which enzyme was added after the addition of acetic acid.

It may be noted in this connection that the glucose-6-phosphate dehydrogenase was markedly inhibited by high concentrations of several salts such as NaCl, Na_2SO_4, etc. After several trials it was found that acidification with acetic acid to stop the reaction was preferable to the use of other acids. Otherwise a very large

excess of glucose-6-phosphate dehydrogenase was required to complete the glucose-6-P estimation in a short time. Care was taken to ensure that hexokinase was totally destroyed by the acetic acid added. This was done in all experiments by allowing aliquots of the acidified and neutralized reaction mixtures to stand for 1 to 2 hr before redetermining glucose-6-P. If the hexokinase is not totally inactivated by the acid, there will be a marked increase in glucose-6-P on incubation of the neutralized liquid for a few hours before the estimation of glucose-6-P.

This method has the disadvantage that the glucose-6-P formed markedly inhibits the enzyme so that the reaction is not linear with respect to time. In studies such as on the effect of temperature on enzyme activity, care was taken to ensure that the amount of glucose-6-P formed during the course of the reaction was not sufficient to cause appreciable inhibition and no correction was, therefore, necessary for glucose-6-P inhibition. This method will be referred to as Assay-II or direct hexokinase assay.

3) Pyruvate Kinase-Lactic Dehydrogenase Assay

(Ochoa, 1961)

In the third method ADP formed was estimated by coupling with pyruvate kinase and lactic dehydrogenase. For studies in the presence of glucose-6-P this method is obviously to be preferred. Enzyme concentration was however not proportional to activity with glucose as substrate since there was marked inhibition of the purified enzyme even by 3×10^{-5} M glucose-6-P. The study of glucose-6-P inhibition using glucose as substrate was difficult since the glucose-6-P

concentration was markedly changing during the course of reaction. It was, therefore, necessary to use mannose as the substrate for kinetic studies on glucose-6-P inhibition and reversal of this inhibition by Pi. With mannose as substrate, inhibition by mannose-6-P was not significant under the experimental conditions used so that there was proportionality between enzyme concentration or time and $\triangle$ O.D. over a wide range. This method will be referred to as Assay-III or pyruvate kinase-lactic dehydrogenase method. The details of the method are given below:

The reaction mixture consisted of Tris-HCl buffer, pH 7.5 (50 µmoles), $MgCl_2$ (10 µmoles); EDTA (0.1 µmole), KCl (10 µmoles), mannose (5 µmoles), ATP (5 µmoles), P-enolpyruvate (4 µmoles), pyruvate kinase (1.2 units), lactic dehydrogenase (1.5 units), DPNH (0.25 µmole) and brain hexokinase in a total volume of 1.0 ml, final pH 7.5 at 30°. The blank cuvette contained Tris-HCl buffer, 0.005 M pH 7.5 and 0.1 µmole DPNH. Pyruvate kinase and lactic dehydrogenase were diluted in 0.05 M Tris-HCl buffer, pH 7.5. All the components except brain hexokinase were mixed and left for 3 to 5 min till a constant reading was reached when the small amount of ADP in the ATP was used up. The reaction was initiated by the addition of brain hexokinase. The amount of enzyme was sufficient to give $\triangle$ O.D. of 0.020 to 0.030 per min. Blanks were run without brain hexokinase or mannose. The optical densityr was measured at intervals of one minute and readings were noted from the second to fifteenth minute.

CHAPTER 3

EXPERIMENTAL AND RESULTS

SECTION I

Solubilization of Hexokinase

(a) Heart mitochondrial hexokinase

Several preliminary experiments were carried out on the solubilization of the hexokinase of beef heart mitochondria. Beef heart mitochondria were prepared and sonicated according to Sanadi and Fluharty (1963). After removing material which sedimented at 25,000 x g in 10 min the supernatant was centrifuged at 100,000 x g for 30 min and the sediment was suspended in 0.25 M phosphate buffer, 0.25 M sucrose, pH 7.5. The hexokinase activity was about 0.5 unit per ml.

The effect of different proteolytic enzymes such as papain, trypsin, etc. on solubilizing the enzyme was determined. These experiments will not be described in detail since they resulted either in the destruction of hexokinase or were ineffective in obtaining the enzyme in soluble form. But when the particles were treated with pancreatic elastase it was observed that there was no loss in hexokinase activity even after digesting for 24 to 48 hr at 0°. On thawing these preparations which were left overnight in a freezer they became highly viscous. Freezing and thawing were repeated four to five times over a period of a week. There was no appreciable loss in hexokinase activity during this period but on centrifugation at 100,000 x g for 2 hr the supernatant contained about 50 per cent of the hexokinase activity. The supernatant was also highly viscous and attempts to fractionate this liquid with

ammonium sulphate or by adsorption on calcium phosphate gel gave no significant purification. After trying several different procedures it was observed that on treatment with protamine sulphate, the enzyme preparation was no longer viscous. Subsequent fractionation with ammonium sulphate and adsorption on calcium phosphate gel followed by elution with phosphate buffer gave 3 to 4-fold purification. Moreover, the enzyme could be stored at this stage for several weeks without significant loss in activity.

These results indicated that treatment with crystalline elastase followed by freezing and thawing and treatment with protamine sulphate gave hexokinase in a soluble form which was highly stable and suitable for further purification by conventional methods. These preliminary experiments carried out with heart hexokinase formed the basis for further detailed studies. Since the hexokinase content of brain is much higher than that of heart (Crane and Sols, 1953) all further work was carried out only with brain hexokinase, after preliminary studies showed that it was possible to solubilize brain hexokinase by the above procedure.

(b) Brain hexokinase

(i) Preparation of particulate brain hexokinase

The following procedure for the preparation of the particulate enzyme from ox brain is based on that of Crane and Sols (1953) with slight modifications. Fresh ox brains (3 to 4) were washed with cold distilled water, the connective tissue was removed and the cortex was scraped free from the white matter. 400 g of cortex were homogenised in two lots with 800 ml of 0.1 M phosphate buffer, pH 6.8,

for 3 min in a Waring blendor. The temperature rose to 10° to 12° during homogenization. 400 ml of the same buffer were added to the homogenate and it was then centrifuged at 800 x g for 20 min. The supernate was collected and the residue was mixed with 1200 ml of the same buffer and recentrifuged as before. Both the supernates were combined and centrifuged at 3500 x g for 30 min. The sediment was suspended in 150 ml of 0.05 M phosphate buffer, pH 6.2, and centrifuged at 6000 x g for 25 min. The residue was dispersed by the use of a Potter-Elvehjem homogenizer in 0.05 M phosphate buffer, pH 7.2, to give a final volume of 90 ml.

(ii) Preparation of soluble hexokinase

On the basis of several preliminary trials the following procedure was adopted for preparing the soluble enzyme. To 90 ml of the particulate brain hexokinase 18 ml of sodium pyrophosphate-HCl buffer, 0.09 M, pH 8.4 and 2.6 mg of twice crystallized pancreatic elastase were added. It was left at 0° for 24 hr with occasional stirring (elastase is almost completely insoluble at this pH and tends to settle down and occasional stirring is, therefore, essential). The enzyme was kept at -20° to -30° for a day and then allowed to thaw slowly at room temperature with occasional stirring, care being taken to avoid a local rise in temperature above 0-5°. The liquid was again frozen and thawed in the same manner six or seven times. The enzyme at this stage became highly viscous and jelly-like. It was centrifuged at 14,000 x g for 1 hr. Approximately 40 to 60 per cent of the activity was obtained in the supernatant.

The residue obtained after centrifugation was in some experiments treated again with elastase as before and frozen and thawed five to six times and centrifuged as before. An additional 20 to 30 per cent of the enzyme was obtained in solution. A total of 60-80 per cent solubilization was obtained by treatment with elastase twice. The second treatment with elastase was not generally adopted, since it was simpler to use fresh brains for preparing the enzyme and the danger of bacterial contamination was also less. Hence all further work was carried out with only one elastase treatment with a total recovery of 40 to 60 per cent of enzyme. The soluble enzyme was not sedimented on centrifugation for 1 hr at 100,000 x g. The results of forty different preparations of the soluble enzyme are summarized in Table 5. In a few experiments low yields may have been due to the use of elastase of lower specific activity.

The enzyme at this stage could be stored for a few days, but there was a loss of activity of 40 to 50 per cent in three to four weeks. It was, therefore, desirable to carry out treatment with protamine sulphate soon after the solubilization of the enzyme.

(iii) Protamine sulphate treatment

The supernatant obtained after elastase treatment was mixed with 0.1 volume of 1 M phosphate buffer, pH 7.5, followed by 0.2 volume of a 5 per cent solution of protamine sulphate or salmine sulphate with rapid stirring. The final pH was 7.2 to 7.3. It was allowed to stand for about 10 min and centrifuged at 14,000 x g for 30 min and the precipitate was discarded. The

TABLE 5

SOLUBILIZATION OF BRAIN HEXOKINASE

	Particulate preparation				Elastase treated enzyme			
	Volume ml	Activity units/ml	Total activity units	Brain tissue activity units/g	Volume ml	Activity units/ml	Total activity units	Solubilization per cent
Maximum values	90	8.6	774	1.9	70	7.0	490	63
Minimum values	90	8.2	738	1.8	76	3.5	273	37
Average values	90	8.0	720	1.8	68	5.7	387	53

highly viscous and slightly opalescent supernatant obtained after elastase treatment became water clear and non-viscous after protamine sulphate treatment. The ratio of optical density at 280 mμ to that at 260 mμ increases from 0.70-0.75 to 0.9-1 after protamine sulphate treatment. Table 6 shows that the recovery of the enzyme was nearly quantitative but there was no significant increase in the purity of the enzyme. Protamine sulphate and salmine sulphate gave similar results but other preparations were not tested. The protamine sulphate-treated enzyme could be stored for several weeks at -20° without significant loss of activity.

TABLE 6

SALMINE SULPHATE TREATMENT OF HEXOKINASE

	Volume	Activity	Total activity	Protein	Total protein	Specific activity	O.D. 280 mμ / O.D. 260 mμ	Recovery of enzyme
	ml	units/ml	units	mg/ml	mg			per cent
Elastase treated enzyme	74	3.76	278	4.9	362	0.75	0.72	
Salmine sulphate treated enzyme	96	2.84	273	2.4	230	1.2	0.91	98

SECTION II

Purification of Hexokinase

Several preliminary experiments were carried out to determine which methods were suitable for the purification of the enzyme. It was observed that at pH 7.5 or 5.5 the enzyme was completely destroyed in the presence of alcohol or acetone. Fractionation with ammonium sulphate gave good recovery of enzyme but very little purification. A detailed study was, therefore, made of the use of different adsorbents for purifying the enzyme. All operations were carried out at 0 to 4°.

1) Calcium Phosphate Gel

The amount of gel required for adsorption was determined in a preliminary experiment. About 15 to 16 mg of gel were required for the adsorption of 1 unit of enzyme from 0.05 M phosphate, pH 7.5, and this proportion of gel to enzyme was used in all subsequent experiments. The adsorbed enzyme was then eluted with 0.1 to 0.2 M phosphate buffer, pH 7.5. It was observed that generally very little enzyme was eluted when the concentration was less than 0.1 M phosphate whereas appreciable amounts of enzyme were eluted from 0.15 to 0.2 M phosphate. The amount of enzyme eluted per ml was not significantly different when the concentration of phosphate was higher than 0.2 M. On the basis of these results the enzyme was adsorbed on gel from 0.05 M phosphate, pH 7.5, washed successively thrice with 50 ml of 0.1 M phosphate buffer and once with 30 ml of 0.13 M buffer and finally eluted with 75 ml of 0.2 M phosphate. The results are presented in Table 7.

Several other methods of elution from calcium phosphate gel were tried in order to determine whether a higher specific activity could be obtained. In one experiment a mixture of citrate and phosphate was used for elution. The enzyme was adsorbed as before and then eluted with 0.1 M and 0.15 M citrate phosphate buffer, pH 7.1. It was observed that the enzyme was eluted even at 0.1 M citrate-phosphate buffer. In one experiment, 6 ml of enzyme containing 13.2 units were adsorbed on gel which was washed twice with 6 ml of 0.05 M citrate phosphate buffer, pH 7.1. The enzyme was eluted with 6 ml lots of 0.1 M citrate-phosphate buffer of the

TABLE 7

PURIFICATION OF HEXOKINASE ON CALCIUM PHOSPHATE GEL

(elution with phosphate buffer, pH 7.5)

Fraction	Volume	Activity	Total activity	Protein	Total protein	Specific activity	Recovery of enzyme
	ml	units/ml	units	mg/ml	mg		per cent
Elastase and protamine sulphate treated enzyme	55	1.64	90	3.60	108	0.8	
0.2 M phosphate eluate	75	0.63	47	0.09	6.7	7.0	54

same pH. Table 8 shows that there was excellent recovery of enzyme with a final specific activity of 8 to 11. However these results were not readily reproducible and the enzyme obtained by this procedure rapidly lost its activity on storage even at -20°. On the other hand, the enzyme obtained by the elution of phosphate alone was very stable and could be stored for several weeks without any appreciable loss in activity.

The use of glucose and thioethanol along with phosphate buffer or of other salts, singly or in combination with phosphate, or variation of the pH did not give higher purification than by elution by 0.2 M phosphate.

Owing to the marked increase in purity obtained by successive washings with increasingly high phosphate concentrations an attempt was made to chromatograph the enzyme on cellulose-calcium phosphate gel columns prepared as follows. 20 g of Schleicher-Schüll chromatography grade cellulose powder (No.123) were suspended in glass-distilled water and fines were removed by sedimentation for 10 min. The process was repeated four to five times. It was then mixed with 70 ml of calcium phosphate gel containing 57 mg of dry weight per ml and de-aerated in vacuo for 15 min. A 2 x 20-cm column was prepared with a cellulose pad at the bottom over a sintered glass disc. The column was equilibrated by passing 250 ml of 0.05 M phosphate pH 7.5. About 10 units of enzyme were loaded on the column and the sides of column rinsed down with 6 ml of 0.5 M phosphate and gradient elution was then carried out. 200 ml of 0.5 M potassium phosphate buffer pH 7.5 was placed in a separating funnel and 200 ml of 0.05 M phosphate buffer of the same pH in the mixing

TABLE 8

CALCIUM PHOSPHATE GEL TREATMENT OF BRAIN HEXOKINASE

(elution of gel with citrate-phosphate buffer)

Fraction	Volume	Activity	Total activity	Protein	Total protein	Specific activity	Recovery
	ml	units/ml	units	mg/ml	mg		per cent
Protamine sulphate treated	6.0	2.2	13.2	1.77	10.6	1.2	
Supernatant after adsorption on gel	11.0	0.01	0.1	0.54	5.9		
0.1 M citrate-phosphate buffer, pH 7.1, Ist eluate	6.0	1.2	7.2	0.15	0.9	8.0	96
0.1 M citrate-phosphate buffer, pH 7.1, IInd eluate	6.0	0.73	4.4	0.06	0.36	12.0	

chamber. The flow rate from the column was 1 drop per 20 sec. 1.6 to 1.8 ml fractions were collected in a fraction collector and assayed for activity. The total recovery of the enzyme was however only 5.4 units and the maximum purity was 2 units per mg protein. A second experiment was simultaneously carried out using step-wise increase in the concentration of the potassium phosphate buffer instead of gradient elution. The phosphate concentration was successively increased from 0.13 to 0.17 and 0.25 M. Further details of this experiment will not be given since the total recovery of the enzyme was only about 8 units out of 37 units. Two similar columns were run but in all experiments the recovery of the enzyme was very low and the purity obtained was also not significantly higher than that obtained in batch elutions. The addition of thioethanol (0.005 M) or glucose (0.1 M) was ineffective in giving better results. The reason for this low recovery of the enzyme compared to the fairly good recovery obtained in batch elution was not clear at this stage. As indicated in a later section it is possibly due to the fact that the enzyme is irreversibly adsorbed on cellulose or inactivated by it.

It may however be stated at this stage that subsequent experiments showed that the enzyme is markedly sensitive to a wide variety of commonly used materials. For instance the enzyme lost activity rapidly when kept in contact with rubber or several varieties of plastic tubing. It was also found that phosphate buffer (0.1 M, pH 7.5) kept in contact with these materials was also found to be inhibitory to the enzyme when added to the assay system at a level of 0.1 ml per ml of reaction mixture. Use of

silicone and similar materials also led to loss in activity. In all subsequent experiments the use of any material such as rubber, plastic tubing, grease or glass which had been previously kept in contact with chromic acid, molybdate or tungstate (especially glassware which had been used for phosphorus estimations) was rigorously excluded during purification of the enzyme. Only glassware previously washed with sodium carbonate followed by rinsing with nitric acid was used and only standard glass joints without lubricant were used for column chromatography.

On the basis of these experiments the following batch-wise procedure for calcium phosphate gel adsorption and elution was used. The enzyme obtained by this method was used for subsequent studies on further purification.

Procedure I

The salmine sulphate treated enzyme was adsorbed on calcium phosphate gel (15 mg dry weight of gel per unit) at a final concentration of phosphate of 0.05M, pH 7.5. (The buffer pH was 7.5 unless otherwise stated). After keeping 30 to 40 min for adsorption with occasional stirring, it was centrifuged and the gel was washed two or three times with 50 ml lots of 0.1 M phosphate and once with 30 ml of 0.13 M phosphate. The enzyme was then eluted with 75 ml of 0.2 M phosphate buffer. For the uniform dispersion of the gel during washing or elution, a Potter-Elvehjem glass homogenizer was used. The results have already been presented in Table 7.

With some batches of calcium phosphate gel, especially if very old, some difficulty was experienced in carrying out this

procedure. In such experiments it was observed that some enzyme was eluted even at 0.1 M phosphate concentration. The following modified procedure (Procedure II) was used for several of the experiments for obtaining enzyme of maximum purity.

Procedure II

The elastase and salmine sulphate treated enzyme was fractionated with ammonium sulphate between 0.4 and 0.9 saturation. To every 100 ml of enzyme solution 22.5 g of powdered ammonium sulphate were added with stirring and after 20 to 30 min at 0° the solution was centrifuged at 14,000 x g for 30 min. To every 100 ml of the supernatant 33.4 g of ammonium sulphate were added to increase the saturation from 0.4 to 0.9. After 20 to 30 min standing the precipitate was collected by centrifugation at 14,000 x g for 30 min. The precipitate was dissolved in and dialysed against 0.04 M phosphate buffer containing 0.001 M thioethanol for 3 to 4 hr with three changes of buffer. The dialysed enzyme solution was clarified by centrifugation. It was then mixed with calcium phosphate gel and suitably diluted so that it contained 2 units per ml and 15 mg per ml (dry weight) per unit of enzyme and the phosphate concentration was 0.05 M. The enzyme was left for 30 to 40 min for adsorption with occasional stirring. Then it was centrifuged and the supernatant was discarded. The gel was then washed three to four times with 100 ml lots 0.05 M phosphate buffer and the enzyme was finally eluted first with 100 ml and then with 50 ml of 0.2 M phosphate buffer. To every 100 ml of the combined eluates 3.6 g glucose and 100 μmoles of thioethanol were added (for a final concentration of 0.2 M glucose and 0.001 M thioethanol)

and 60.2 g ammonium sulphate (0.9 saturation). After 30 min the precipitate was collected by centrifugation at 14,000 x g for 1 hr. The precipitate was dissolved in 0.005 M phosphate buffer containing 0.001 M thioethanol and stored at -20°. The results are presented in Table 9.

Ammonium sulphate precipitations were carried out by adding small amounts of solid at a time without too rapid stirring. Care is necessary to avoid loss of enzyme during transfers, since the precipitate tends to float to the surface in concentrated ammonium sulphate.

2) Celite 535

The following adsorbents were also tested for their suitability for purifying hexokinase. Celite 535 was thoroughly washed with water and then mixed either with the protamine sulphate treated enzyme or with enzyme obtained after calcium phosphate gel adsorption and elution. The conditions for the experiment are given below. 3 ml (7.2 units) of protamine sulphate-treated enzyme were treated with 1.8 g of celite in a final volume of 6 ml in 0.06 M phosphate, pH 7.5. The enzyme was found to be completely adsorbed, since the supernatant obtained on centrifugation had no significant activity. It was then washed with 20 ml of 0.06 M phosphate, pH 7.5, and the supernatant obtained on centrifugation was discarded since it had no activity. It was then eluted successively twice with 10 ml each of 0.17 M phosphate and then with 10 ml 0.25 M phosphate, pH 7.5. The results are shown in

TABLE 9

CALCIUM PHOSPHATE GEL TREATMENT OF HEXOKINASE

(elution with phosphate buffer)

Fraction	Volume	Activity	Total activity	Protein	Total protein	Specific activity	Recovery
	ml	units/ml	units	mg/ml	mg		per cent
Salmine sulphate treated enzyme	104	2.84	296	2.4	249	1.2	
0.4 to 0.9 ammonium sulphate fraction dialysed	12	24	288	9.43	113	2.5	97
First supernatant after adsorption on gel	115	-	-	0.24	27.6	-	
First washing	100	-	-	0.12	12.0	-	
Second washing	100	-	-	0.08	8.0	-	
Third washing	100	-	-	0.06	6.0	-	
First eluate	100	1.6	160	0.32	32	5.0	64
Second eluate	50	0.77	39	0.19	19	4.0	
Ammonium sulphate precipitated	4.8	40	172	5.8	25.5	6.9	58

Table 10. A similar experiment was then carried out with 6 units of enzyme obtained after calcium phosphate gel treatment. The results are shown in Table 11. It was observed that there was no significant increase in purity by celite 535 adsorption after calcium phosphate gel treatment. Some ultraviolet absorbing material was eluted out of washed celite 535 so that the protein values were not accurate. No further experiments were carried out with celite.

3) Bentonite

Bentonite was also used for adsorption of hexokinase, but no purification was obtained.

4) Calcium Phytate

2 ml of protamine sulphate treated enzyme (8 units) were mixed with 250 mg (dry) calcium phytate and centrifuged after 20 min. The phytate was then washed with water and then eluted with 0.1 M phosphate and 0.2 M phosphate, pH 7.5. The results are shown in Table 12. An increase in specific activity was also observed when calcium phytate was used for purifying the enzyme obtained after calcium phosphate gel treatment. The enzyme, obtained after calcium phosphate gel treatment and precipitated with ammonium sulphate was dissolved in a small amount of potassium phosphate buffer and dialysed against 0.005 M phosphate, pH 7.5 containing 0.001 M thioethanol. It was then adsorbed on 850 mg calcium phytate in a final volume of 3.8 ml. After washing with

TABLE 10

ADSORPTION OF BRAIN HEXOKINASE ON CELITE-535

(using protamine-sulfate treated enzyme)

Fraction	Volume	Activity	Total activity	Protein	Total protein	Specific activity	Recovery
	ml	units/ml	units	mg/ml	mg		per cent
Protamine sulphate treated	3.0	2.4	7.2	2.6	7.8	0.9	
Supernatant after adsorption on Celite-535	5.0	0.04	0.2	1.06	5.3	0.04	
0.04 M phosphate buffer washing	20.0	0.01	0.2	0.24	4.8	0.04	
Ist 0.17 M phosphate buffer eluate	10.0	0.13	1.3	0.15	1.5	0.9	
IInd 0.17 M phosphate buffer eluate	10.0	0.13	1.3	0.10	1.0	1.3	57
IIIrd 0.25 M phosphate buffer eluate	10.0	0.15	1.5	0.08	0.8	2.0	

eluates from Celite contained some ultraviolet absorbing material

TABLE 11

ADSORPTION OF BRAIN HEXOKINASE ON CELITE-535

(using calcium phosphate gel eluted enzyme)

Fraction	Volume	Activity	Total activity	Protein	Total protein	Specific activity	Recovery
	ml	units/ml	units	mg/ml	mg		per cent
Calcium phosphate gel eluate	15.0	0.40	6.0	0.071	1.06	5.6	
Supernatant after adsorption on Celite-535	4.3	0.021	0.9	0.042	0.18	0.5	
0.04 M phosphate buffer washing	20.0	0.013	0.26	0.023	0.46		
Ist 0.17 M phosphate buffer eluate	10.0	0.15	1.5	0.04	0.4	3.8	
IInd 0.17 M phosphate buffer eluate	10.0	0.10	1.0	0.046	0.46	2.1	53
IIIrd 0.25 M phosphate buffer eluate	10.0	0.07	0.7	0.042	0.42	1.7	

10 ml and 18 ml lots of 0.025 M phosphate it was successively eluted with 0.05 M, 0.1 M and 0.4 M phosphate, pH 7.5. The results are shown in Table 13. Though the purity increased from about 6 to about 11 to 15 the total recovery of enzyme was only 39 per cent and the purity of the pooled active fractions was not significantly higher. Further experiments on calcium phytate were discontinued.

5) Cellulose

A few preliminary experiments indicated that the enzyme was adsorbed on cellulose. 14 units of enzyme (9 ml) obtained after calcium phosphate gel treatment were mixed with 950 mg of cellulose. The supernatant obtained on centrifugation had no activity. The cellulose was washed twice with 10 ml of water and aliquots were eluted with phosphate buffers of 0.1 to 0.6 M or 0.1 M buffer containing 0.2 M glucose. The total activity in the eluates was in no case more than 0.1 unit. It is not known whether the enzyme was irreverisbly bound to the cellulose or whether there were impurities in the cellulose which caused inhibition or inactivation of the enzyme.

6) Sephadex

In one experiment the enzyme was fractionated on a Sephadex G-75 column. The enzyme was obtained by calcium phosphate gel treatment followed by ammonium sulphate precipitation. It was dissolved and dialysed against 0.01 M phosphate buffer,

TABLE 12

ADSORPTION OF BRAIN HEXOKINASE ON CALCIUM PHYTATE

(using salmine sulphate treated enzyme)

Fraction	Volume	Activity	Total activity	Protein	Total protein	Specific activity	Recovery
	ml	units/ml	units	mg/ml	mg		per cent
Salmine sulphate treated	2.0	4.0	8.0	2.15	4.3	1.8	
Supernatant after calcium phytate adsorption	2.0	0.03	0.06	0.90	1.8	0.03	
0.1 M phosphate buffer eluate	3.0	0.6	1.8	0.4	1.2	1.5	55
0.2 M phosphate buffer eluate	3.0	0.86	2.58	0.25	0.75	3.4	

TABLE 13

ADSORPTION OF BRAIN HEXOKINASE ON CALCIUM PHYTATE

(using calcium phosphate gel eluted, ammonium sulphate precipitated enzyme)

Fraction	Volume	Activity	Total activity	Protein	Total protein	Specific activity	Recovery
	ml	units/ml	units	mg/ml	mg		per cent
Calcium phosphate gel eluted-ammonium sulphate precipitated, dialysed	0.8	17.6	14.0	2.93	2.34	6.0	
Supernatant after adsorption on calcium phytate	3.8	0.02	0.08	0.24	0.9	0.1	
IInd 0.025 M phosphate buffer washing	18.0	0.063	1.1	0.045	0.81	1.3	
0.05 M phosphate buffer eluate	12.0	0.23	2.8	0.026	0.3	8.8	
0.1 M phosphate buffer eluate	10.0	0.34	3.4	0.03	0.3	11.3	39
0.4 M phosphate buffer eluate	5.0	0.42	2.1	0.028	0.14	15.0	

pH 7.6 containing 0.001 M thioethanol. 2.6 ml containing 9.4 units with a purity of 3 units per mg was used. The column (2.5 x 21 cm) was equilibrated with buffer of the same composition. After loading the enzyme 150 ml of the same buffer was passed through the column. The maximum purity obtained was only 4.7 units per mg in any of the fractions and hence further fractionation on sephadex was not tried.

7) Carboxymethylcellulose and phosphorylated cellulose

Carboxymethylcellulose and phosphorylated cellulose were washed (Peterson and Sober, 1962) equilibrated with 0.005 M acetate buffer or phosphate buffer, pH 5.5. The quantities of the adsorbents used and the concentrations of the eluting buffers were determined in preliminary experiments which will not be described in detail. The enzyme was obtained by calcium phosphate gel treatment and ammonium sulphate precipitation and was dissolved in a small amount of acetate buffer 0.005 M, phosphate buffer, 0.005 M, pH 5.5 and dialysed against the same buffer. It was mixed with 7 ml of 0.01 M acetate, 0.005 M phosphate, pH 5.5 and 60 mg carboxymethylcellulose or 80 mg cellulose phosphate in a final volume of 10 ml. After keeping for 15 min with occasional shaking the adsorbent was removed by centrifugation and washed with 10 ml of 0.01 M acetate containing 0.005 M phosphate at pH 5.5. The washed adsorbent was divided into two equal portions in each case and one half was eluted with 0.2 M acetate-0.005 M potassium phosphate, pH 5.5. The other half of the washed adsorbent was eluted with 0.2 M potassium phosphate pH 7.2. The results are shown in Tables 14 and 15. It will be seen that there was an appreciable increase

TABLE 14

ADSORPTION OF BRAIN HEXOKINASE ON CARBOXYMETHYLCELLULOSE

Fraction	Volume	Activity	Total activity	Protein	Total protein	Specific activity	Recovery
	ml	units/ml	units	mg/ml	mg		per cent
Calcium phosphate gel eluted, ammonium sulphate precipitated, dialysed	1.8	4.3	7.7	0.76	1.37	6.0	
Supernatant after adsorption on carboxymethylcellulose	10.0	0.01	0.1	0.025	0.25	0.4	
0.2 M acetate-0.005 M phosphate pH 5.5, eluate	8.0	0.11	0.9	0.007	0.05	15.5	23
0.2 M phosphate, pH 7.2 eluate	12.0	0.11	1.3	0.04	0.48	2.7	33

TABLE 15

ADSORPTION OF BRAIN HEXOKINASE ON CELLULOSE PHOSPHATE

Fraction	Volume	Activity	Total activity	Protein	Total protein	Specific activity	Recovery
	ml	units/ml	units	mg/ml	mg		per cent
Calcium phosphate gel eluted and ammonium sulphate precipitated, dialysed	1.8	4.3	7.7	0.76	1.37	6.0	
Supernatant after adsorption on cellulose phosphate	10.0	0.01	0.1	0.004	0.04	3.2	
0.2 M acetate 0.005 M phosphate, pH 5.5 eluate	8.0	0.17	1.4	0.01	0.08	17.0	35
0.2 M phosphate pH 7.2 eluate	8.0	0.20	1.6	0.02	0.16	10.0	41

in purity but the total recovery of the enzyme of maximum specific activity (15 to 17) was poor. Slightly better results were obtained by elution with acetate-phosphate buffers than with 0.2 M phosphate. But further experiments with these adsorbents were not continued in view of the low recovery of the enzyme.

8) Adsorption on IRC-50

The cation exchange resin IRC-50 (XE-64) was washed as described by Hirs et al.(1953). On the basis of preliminary results of adsorption and elution at different pHs and phosphate concentrations, more detailed studies were carried out at pH 6.8. The resin was equilibrated with 0.01 M phosphate and 0.001 M thioethanol, pH 6.8. 3 ml of enzyme containing 12 units of specific activity 4 units per mg (obtained by calcium phosphate gel elution and ammonium sulphate precipitation followed by dialysis against buffer of the same composition) was mixed with 5.5 g of the resin in 4.5 ml of 0.01 M phosphate buffer, pH 6.8 containing 0.001 M thioethanol. After 40 min it was centrifuged and washed with 7 ml of 0.01 M phosphate, pH 6.8. This and all the subsequent buffers used for elution contained 0.001 M thioethanol. It was then eluted successively with 4 ml and 5 ml of 0.1 M potassium phosphate, pH 7.5. The results are shown in Table 16. It will be seen that there was a 2 to 3-fold increase in the purity in the different fractions with 52 per cent recovery of the enzyme. On the basis of these results chromatography of the enzyme on an IRC-50 column was carried out. The enzyme obtained by calcium phosphate gel treatment and ammonium sulphate

TABLE 16

ADSORPTION OF BRAIN HEXOKINASE ON IRC-50

Fraction	Volume	Activity	Total activity	Protein	Total protein	Specific activity	Recovery
	ml	units/ml	units	mg/ml	mg		per cent
Calcium phosphate gel eluted, ammonium sulphate precipitated dialysed enzyme	3.0	4.0	12	0.08	2.04	4.1	
Supernatant after adsorption on IRC-50	7.5	0.23	1.7	0.11	0.82	2.1	
0.1 M phosphate, pH 7.5, 0.001 M thioethanol, E_1	4.0	1.1	4.4	0.10	0.40	11.0	52
0.1 M phosphate, pH 7.5 0.001 M thioethanol, E_2	5.0	0.4	2.0	0.045	0.22	9.0	

precipitation followed by dialysis against 0.01 M phosphate, pH 7.5 (17 units of a specific activity 10 units per mg). It was loaded on a 1 x 8 cm column of the resin which was equilibrated with 0.01 M phosphate pH 6.8 (all solutions contained 0.001 M thioethamol). After loading on the column the following solutions were successively passed through the column: 19 ml of 0.01 M phosphate, pH 6.8, 22 ml of 0.1 M phosphate, pH 7.5 and 13 ml of 0.5 M, pH 7.5. 2 ml fractions were collected. Tubes 1 to 23 contained very little or no activity (less than 0.006 units per ml), tubes 23 to 25 contained 1.4 units per ml and tube 26 had 0.68 units per ml and tubes 27 and 28 had 0.3 units per ml. After pooling the active fractions and precipitation with ammonium sulphate a total of 12 units of specific activity of 18 to 20 were obtained.

Though there was a significant purification by this procedure the experiments were not reproducible. On the basis of the data obtained subsequently the variability in results was probably not due to differences in the fractionation procedure but due to the low stability of the highly purified enzyme. Though further work was not carried out with IRC-50 resin, it is possible that by using conditions which were later established for stabilizing the enzyme, the enzyme can be obtained in a highly purified form by using this resin.

9) DEAE-Cellulose

DEAE-cellulose was washed according to Peterson and Sober (1962) and equilibrated with 0.005 M phosphate pH 7.5. This solution and all the other solutions used for adsorption and elution contained 0.001 M thioethanol. The enzyme used was calcium phosphate gel eluate precipitated with ammonium sulphate and dialysed against 0.005 M phosphate, pH 7.5. On the basis of preliminary experiments it was observed that about 15 to 20 mg of cellulose were required for the adsorption of 1 unit of enzyme under these conditions. 8 units of enzyme were then adsorbed by the addition of 140 mg of DEAE-cellulose in a final volume of 13 ml and the phosphate concentration was 0.005 M. (The pH of all solutions was 7.5 unless otherwise stated.) After 30 min it was filtered and then washed successively twice with 20 ml of 0.007 M phosphate once with 5 ml of 0.01 M phosphate and once with 7 ml of 0.03 M phosphate. The enzyme in the last two washes had only 0.001 and 0.05 units per ml respectively. The DEAE-cellulose was then divided into two equal portions one of which was eluted with 5.4 ml of 0.05 M phosphate and the other with 5.0 ml of 0.03 M phosphate at pH 6.5. The specific activities of the eluates were 25 and 9 units per mg respectively for the pH 7.5 and 6.5 eluates. The recovery of the enzyme was only about 37 and 25 per cent respectively. The results are shown in Table 17. On the basis of these preliminary trials several experiments were carried

TABLE 17

PURIFICATION OF BRAIN HEXOKINASE ON DEAE-CELLULOSE

Fraction	Volume	Activity	Total activity	Protein	Total protein	Specific activity	Recovery
	ml	units/ml	units	mg/ml	mg		per cent
Calcium phosphate gel eluted, ammonium sulphate precipitated enzyme, dialysed	1.0	8.0	8.0	1.1	1.1	7.3	
Supernatant after adsorption on DEAE cellulose	12.5	-	-	0.01	0.12	-	
0.03 M phosphate buffer, pH 7.5 washing	7.0	0.05	0.35	0.01	0.07	5.0	
0.05 M phosphate buffer, pH 7.5 eluate	5.4	0.27	1.46	0.01	0.06	24.5	37
0.03 M phosphate buffer, pH 6.5 eluate	5.0	0.20	1.00	0.022	0.11	9.0	25

out on the optimum conditions for DEAE-cellulose adsorption and elution. No significant improvement in the purification or recovery of enzyme was observed by adsorption of enzyme and elution at other pHs (5.5, 6.5 and 8.5) or by the addition of varying amounts of thioethanol, ATP, glucose (upto 0.1 M) or ammonium sulphate solution (30 per cent). Adsorption at a high pH followed by elution with buffers of lower pH was also not significantly different from the experiments cited above. On the basis of these results, column chromatographic experiments were also carried out. The recovery of the enzyme varied from 20 to 60 per cent in most of the experiments and the losses were considerable during precipitation of the enzyme with ammonium sulphate at 90 per cent saturation. When column chromatography on DEAE-cellulose was carried out both the recovery and purity of the enzyme obtained were poor. Only about 30 to 40 per cent of the enzyme was recovered and the specific activity of the active fractions was only 10 to 15.

The purified enzyme obtained after DEAE-cellulose chromatography was also found to lose activity very rapidly on storage. In several experiments the enzyme lost almost all the activity even at -20° in 48 hr. Similar observations were made with enzyme obtained after purification by other procedures. It was obvious at this stage that no significant progress could be made in this work unless suitable methods could be devised for stabilizing the enzyme. The loss of activity at 0° was also very marked and no kinetic and other studies could be made even with the partially purified enzyme unless the enzyme was

stabilized. The instability as well as the insolubility of several animal tissue hexokinases has been a major difficulty in the purification of this enzyme till now. A solution to the problem of stabilizing the enzyme was, therefore, essential for further purification and study of brain hexokinase.

Stabilization of Hexokinase

Further work on purification was, therefore, suspended and detailed studies were made on the stability of the enzyme under a variety of conditions. The enzyme obtained after calcium phosphate gel fractionation was remarkably stable and could even be heated at 50° with no loss of activity, but further purification caused extremely rapid inactivation as noted above. For studies on the stabilization the enzyme obtained after calcium phosphate gel fractionation was further purified by DEAE-cellulose chromatography to obtain enzyme of specific activity 15 to 20 units per mg. These studies, which extended for over a year, will not be discussed in detail especially since it will be clear from the following section that several different factors were necessary for stabilizing the enzyme so that the study of any one of them gave only a negative result or limited success. For instance, the effect of varying the concentration of either phosphate or thioethanol/enzyme ~~concentration~~ gave only partial stabilization.

High ammonium sulphate concentrations (0.5 to 0.7 saturation at pH 7.5) tend to stabilize the enzyme, but this was of little practical value for further purification of the

enzyme especially by the use of adsorbents. ATP, glucose (0.1 M), DPN, TPN, histidine, glycine, fatty acids and several anions and cations (carbonate, acetate, Mg^{++}) had little effect on enzyme stability. Storage of the enzyme under anaerobic conditions and combinations of different substances gave only partial improvement and it was obvious that none of these methods was of practical value.

During one of the experiments on the preparation of concentrated enzyme for determining its stability at high concentrations the DEAE-cellulose eluate was "dialysed" against finely powdered solid sucrose. The 0.07 M phosphate eluate from DEAE-cellulose (about 60 ml) was kept in a dialysis bag and the bag was left in the cold covered with an excess of sucrose for about 3 hr. The volume of the enzyme was reduced to about 4 ml. This concentrated enzyme was tested in connection with other experiments after 15 days storage at -20°. It was found to retain all its activity. Since the activity of this concentrated enzyme was only 1.6 units per ml it was thought that it was the sucrose content of the enzyme which was responsible for the stability of the enzyme. Subsequent work showed that high concentrations of sucrose were extremely effective in stabilizing the enzyme. It was also observed that not only was the enzyme stable at -20° in the presence of 0.5 to 1 M sucrose, but it was also stable at 0° for several days and could also be heated at 55° for a few minutes with no loss of activity. This remarkable effect of sucrose on the enzyme made possible subsequent work on purification.

The effect of other sugars on the stability of the enzyme was then determined by using calcium phosphate gel eluate and

heating it in the presence of the sugars for different periods of time. The results are shown in Table 18. Though these experiments were carried out with calcium phosphate gel eluate the relative effectiveness of the sugars and the effect of different concentrations of sugars on stability will be apparent from these results. Fructose was not as effective as glucose and sucrose in stabilizing the enzyme. Glucose was more effective than sucrose in protecting the enzyme though with highly purified enzyme the two sugars were nearly equally effective. Even 0.2 M glucose shows a significant stabilizing effect at 65° for 5 min but with more purified enzyme and for prolonged storage 0.5 M to 1 M glucose or sucrose was essential.

However with more purified preparations loss of activity was observed even in the presence of 0.5 M to 1 M sucrose unless thioethanol and Pi were added.

Even with calcium phosphate gel eluate the effect of thioethanol could be demonstrated when the enzyme was diluted to about 0.07 units per ml and stored at -20° for 4 days with and without 0.005 M thioethanol. The loss in activity without thioethanol was 80 per cent whereas with the thioethanol it was 50 per cent. Similar results were observed with purified enzyme even at high concentration if no thioethanol was added. An optimum concentration of 0.005 to 0.01 M thioethanol was generally desirable for prolonged storage of the enzyme. However for short term experiments such as DEAE-cellulose column chromatography lower concentrations of thioethanol (0.001 M) or sugar (0.2 M) could be used.

TABLE 18

EFFECT OF DIFFERENT SUGARS ON HEATING OF BRAIN HEXOKINASE

(calcium phosphate gel eluted enzyme used for heating)

	Sugar	Quantity M	Temp °	Time min	Loss in activity per cent
1	Sucrose	0.8	55	10	0
	Sucrose	0.8	55	30	0
	Sucrose	0.8	65	5	90
2	Fructose	1.5	55	5	4
	Fructose	1.5	65	5	17
3	Glucose	1.5	55	5	0
		1.5	65	5	0
		1.5	75	5	80
		0.75	65	5	0
		0.4	65	5	0
		0.2	65	5	0
		1.3	80	5	90
		3.0	80	5	40

A third factor which was also essential for the stability of the enzyme was Pi. It was observed that dialysis of the calcium phosphate gel eluate against Tris or buffers other than phosphate resulted in very rapid loss of activity. When the purified enzyme was stored in the absence of phosphate the loss of activity was high even in the presence of a high concentration of sucrose. About 0.01 M phosphate was the minimum amount required for stability of the enzyme for short periods though generally the enzyme was kept in 0.1 M phosphate, pH 7.5. The protective effect was especially high at pHs lower than 6.

The following experiments show that high concentrations of glucose protect the enzyme not only against denaturation on storage but also due to high acidity or organic solvents. To 3 ml (2.4 units) of calcium phosphate gel eluate, 1.2 ml acetic acid (1 M) was slowly added with stirring at 0° to give a final pH of 4.5. It was kept at room temperature for 10 min and then neutralized with 1.5 ml of Tris base and its activity determined. Similarly 3 ml (2.4 units) of enzyme were mixed with 1.5 ml of 1.5 M glucose and 3 ml of 1 M of acetic acid to give a final pH of 4. After 10 min at room temperature it was neutralized with (3.7 ml of Tris base and the activity determined. Table 19 shows that in the absence of glucose 70 per cent of the enzyme was destroyed at pH 4.5 in 10 min at room temperature whereas in the presence of glucose there was no loss of activity even at pH 4.

TABLE 19

EFFECT OF pH ON BRAIN HEXOKINASE

Fraction at pH	Glucose M	Temp °	Time min	Volume ml	Activity units/ml	Total activity units	Recovery per cent
Calcium phosphate gel, eluate, pH 7.5	0			3.0	0.80	2.4	
4.5	0	28	10	5.7	0.13	0.7	30
4.0	0.3	28	10	11.2	0.22	2.4	100

Calcium phosphate gel eluate precipitated by ammonium sulphate was dialysed against 0.05 M Tris buffer, pH 7.5 containing 0.001 M thioethanol. 2 ml of the enzyme (24 units) containing glucose (0.22 M) were treated with acetic acid to give a pH of 5.4. 43 mg of magnesium acetate were added followed by 5 ml of alcohol at -10°. After 20 min at -10° it was centrifuged for 10 min and the precipitate was dissolved in a small volume of Tris buffer, pH 7.5, containing 0.4 M glucose. The recovery of enzyme was 33 per cent of the initial activity and the increase in purity 2.4-fold (Table 20). In the absence of glucose the enzyme was totally destroyed by precipitation with alcohol. This experiment was carried out before the requirement of phosphate for the stability of the enzyme was known and hence the precipitation was carried out in the absence of phosphate. Fractionations with alcohol and acetone at pH 7.5 in the presence of glucose were also carried out. The recovery of enzyme was about 40 per cent. But further work on increasing the yield of enzyme or on purification of the enzyme by solvent fractionation was not carried out.

A fourth factor which was also important for the stability of the enzyme was as in the case of several other enzymes, the rapid loss of activity at high dilutions. Though there was no significant loss of the enzyme at a concentration of 0.5 unit per ml in the presence of 0.5 M glucose or sucrose, 0.1 M phosphate and 0.001 M thioethanol for about 24 hr at -20°, prolonged storage of dilute enzyme under these conditions resulted in loss of activity. It was, therefore, considered desirable to keep the enzyme concentration at not less than 1 to 2 units per ml

TABLE 20

ALCOHOL PRECIPITATION OF BRAIN HEXOKINASE IN PRESENCE OF GLUCOSE

Fraction	Volume ml	Activity units/ml	Total activity units	Protein mg/ml	Total protein mg	Specific activity	Recovery per cent
Calcium phosphate gel eluted, ammonium sulphate precipitated	2.0	12.0	24	2.0	4.0	6	
Alcohol precipitated	0.8	10.0	8	0.7	0.55	14	33

in a buffer of the above composition for storage for a few days whereas for prolonged storage the enzyme was kept at a concentration of at least 50 to 100 units per ml. In the presence of ammonium sulphate (0.4 to 0.5 saturation) in a solution containing sucrose, thioethanol and phosphate of the same composition even enzyme of the highest purity could be maintained for several weeks at 0° with no significant loss in activity.

On the basis of the above findings on the stability of the enzyme in the presence of high concentrations of glucose or sucrose, phosphate and thioethanol it was possible to proceed further with the purification of the enzyme. The results of chromatography of the enzyme on DEAE-cellulose are described below.

The following procedure was generally used for DEAE-cellulose chromatography.

Washed DEAE-cellulose was equilibrated with phosphate buffer by suspending it in 0.5 M phosphate buffer, pH 7.5, and after 30 min filtering it and washing 4 to 5 times with water. The suspension was de-aerated under vacuum for 15 to 20 min. It was finally suspended in 0.005 M phosphate buffer, pH 7.5, 0.2 M glucose and 0.001 M thioethanol. The suspension containing 25 g DEAE-cellulose was poured into a 5 x 9-cm column with a layer of acid-washed glass-wool at the bottom. After settling down, the column was finally equilibrated by passing 400 ml of the same buffer under gentle pressure to pack the column.

Subsequent operations were carried out without applying any pressure. The pH of all solutions was 7.5.

The enzyme (calcium phosphate gel eluate precipitated with ammonium sulphate) was dialysed against 0.005 M phosphate and 0.001 M thioethanol. About 5 to 10 ml of enzyme (20 to 25 mg protein) were slowly loaded on the column and the sides of the column were rinsed with about 5 ml of the buffer. The column was washed with 400 ml of 0.01 M phosphate buffer - 0.2 M glucose-0.001 M thioethanol. The enzyme was then eluted with 0.07 M phosphate buffer - 0.2 M glucose - 0.001 M thioethanol. 15 to 20 ml fractions were collected on an automatic fraction collector every 10 min and tested for activity. The active fractions were pooled and precipitated with ammonium sulphate (0.9 saturation) by adding 60.2 g solid ammonium sulphate for every 100 ml of eluate. After standing for 1 hr the precipitate was collected by centrifugation at 14,000 x g for 1 hr. The precipitate was dissolved in 0.1 M phosphate buffer- 0.5 M glucose - 0.01 M thioethanol. It was centrifuged to remove any insoluble material and stored at -20°. The results of a typical chromatography are shown in Table 21 and the elution pattern is represented in Fig. 1.

In a few runs in the beginning the enzyme in all the active fractions was pooled together and precipitated with ammonium sulphate. In subsequent experiments the contents of two to three tubes containing enzyme were pooled and precipitated separately and the specific activities determined. Those fractions which had specific activities less than 25 units per mg were discarded and the rest were combined. Generally the first two or three tubes

TABLE 21

PURIFICATION OF BRAIN HEXOKINASE ON DEAE-CELLULOSE COLUMN

Fraction No.	Volume	Activity	Total activity	Protein	Total protein	Specific activity	Recovery
	ml	units/ml	units	mg/ml	mg		per cent
Enzyme loaded on the column	5	24.0	120.0	4.2	21.0	5.7	
1-7	152						
8	19	0.20	3.8				
9	19	0.60	11.5				
10	19	0.77	14.6				
11	19	0.85	16.1				
12	19	0.67	12.7				
13	19	0.60	11.4				
14+15	38	0.56	21.2				
16+17	38	0.30	11.4				
Fractions 9 to 17 pooled	171		99.0				82
Ammonium sulphate precipitate	2.9	30.0	87.0	0.7	2.0	43	72

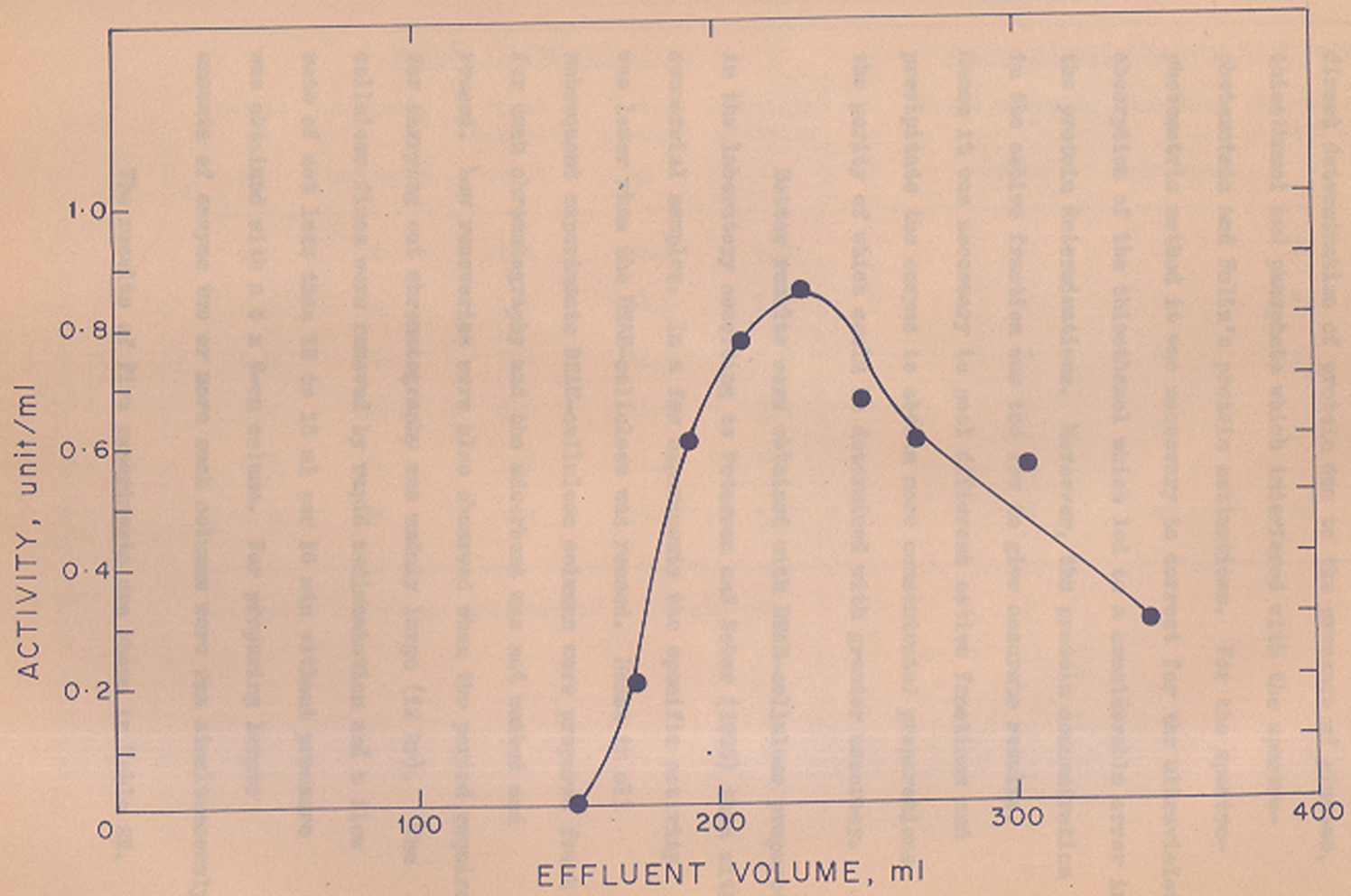


FIG. 1. ELUTION PATTERN OF HEXOKINASE ON DEAE-CELLULOSE COLUMN (5 x 9-cm)

had to be discarded. The activity of the eluates could be determined with accuracy, but there was considerable error in the direct determination of protein due to the presence of glucose, thioethanol and phosphate which interfered with the spectrophotometric and Folin's protein estimations. For the spectrophotometric method it was necessary to correct for the ultraviolet absorption of the thioethanol which led to a considerable error in the protein determinations. Moreover, the protein concentration in the active fraction was too low to give accurate results. Hence it was necessary to pool different active fractions and precipitate the enzyme to obtain more concentrated preparations the purity of which could be determined with greater accuracy.

Better results were obtained with DEAE-cellulose prepared in the laboratory according to Peterson and Sober (1956) than with commercial samples. In a few experiments the specific activity was lower when the DEAE-cellulose was reused. Hence in all subsequent experiments DEAE-cellulose columns were prepared fresh for each chromatography and the adsorbent was not washed and reused. Low recoveries were also observed when the period required for carrying out chromatography was unduly large (12 hr). The cellulose fines were removed by rapid sedimentation and a flow rate of not less than 12 to 15 ml per 10 min without pressure was obtained with a 5 x 9-cm column. For preparing larger amounts of enzyme two or more such columns were run simultaneously.

The results of five experiments are shown in Table 22.

TABLE 22

DEAE-CELLULOSE COLUMN CHROMATOGRAPHY

Enzyme loaded on column					Enzyme eluted from the DEAE column		
Volume	Activity	Total activity	Total protein	Specific activity	Total activity	Recovery	Specific activity
ml	units/ml	units	mg		units	per cent	
8.0	13	104	15.2	6.8	52	50	33
8.0	24	192	24.8	7.7	86	44	46
5.0	24	120	21.0	5.0	60	50	47
5.0	24	120	21.0	5.7	120	50	52
5.0	24	120	21.0	5.7			
3.4	24	82	14.3	5.7	35	43	57

Column: 5 x 9-cm in presence of 0.2 M glucose

On the basis of the above findings the following was the final procedure for the purification of hexokinase from ox brain. All operations were carried out at 0° to 4°.

1. Preparation of particulate brain hexokinase

Fresh ox brains (3 or 4 nos.) were washed with cold distilled water, the connective tissue was removed and the cortex scraped free from white matter. 400 g of cortex were homogenized in two lots with 800 ml of 0.1 M phosphate buffer, pH 6.8, for 30 min in a Waring blendor. The temperature rose to 10° to 12° during homogenization. The homogenate was mixed well with 400 ml of the same buffer (total volume 2100 ml) and centrifuged at 800 x g for 20 min. The supernatant was collected and the residue was mixed with 1200 ml of the same buffer and recentrifuged at 800 x g for 20 min. The combined supernatants were centrifuged at 3500 x g for 30 min. The sediment was suspended in 150 ml of 0.05 M phosphate buffer, pH 6.2 and centrifuged at 6000 x g for 25 min. The residue was finally dispersed in 0.05 M phosphate buffer, pH 7.2 with a glass homogenizer. The final volume was 90 ml.

2. Elastase treatment

The particulate enzyme was mixed with 18 ml of 0.09 M sodium pyrophosphate-HCl buffer, pH 8.4, and 2.6 mg of twice crystallized pancreatic elastase and kept at 0° for 24 hr with occasional stirring. The enzyme was frozen and thawed five to six times by keeping at -20° for 16 to 24 hr and then thawing

slowly at 5°. The enzyme was centrifuged at 14,000 x g for 1 hr to obtain soluble hexokinase. The volume of the supernatant was about 84 ml.

3. Salmine sulphate treatment

The soluble enzyme was mixed with 0.1 volume of 1 M phosphate buffer, pH 7.5, and 0.2 volume of 5 per cent salmine sulphate solution and centrifuged at 14,000 x g for 30 min. The supernatant (109 ml) was stored at -20°.

4. Ammonium sulphate precipitation

The enzyme was then precipitated by ammonium sulphate at 0.4 saturation by adding 22.5 g ammonium sulphate for every 100 ml. After 30 min it was centrifuged at 14,000 x g for 30 min. The supernatant was raised to 0.9 saturation by adding 33.4 g ammonium sulphate for every 100 ml. The precipitate was collected after 30 min by centrifugation at 14,000 x g for 30 min, dissolved in 0.04 M phosphate buffer, pH 7.5, containing 0.001 M thioethanol. It was dialysed against the same buffer for 3 to 4 hr with three changes of buffer. The dialysed enzyme was centrifuged to remove turbidity and the clear supernatant (15 ml) was stored at -20°.

5. Calcium phosphate gel treatment

In all subsequent steps the pH was 7.5. For adsorption of the enzyme on gel 15 mg (dry weight) of calcium phosphate gel was added for 1 unit of hexokinase and the final concentration of enzyme was 2 units per ml in 0.05 M phosphate. The enzyme was

kept for 30 to 40 min with occasional stirring, centrifuged and the supernatant discarded. The sides of the centrifuge tube wiped clean with filter paper and the gel was washed successively three to four times with 100 ml lots of 0.05 M phosphate buffer. Then the enzyme was eluted twice with 0.2 M phosphate buffer first with 100 ml and then with 40 ml. Glucose and thioethanol were added to the eluate to give final concentrations of 0.2 M and 0.001 M respectively and 60.2 g of ammonium sulphate were then added (0.9 saturation). After 30 min it was centrifuged at 14,000 x g for 60 min. The precipitate was dissolved in 0.005 M phosphate buffer - 0.001 M thioethanol and dialysed against the same buffer for 4 to 5 hr with three changes of buffer. The dialysed enzyme was centrifuged to remove any precipitate formed during dialysis and the supernatant (10 ml) was stored at -20°.

6. DEAE-cellulose column chromatography

DEAE-cellulose was equilibrated with 0.005 M phosphate buffer - 0.2 M glucose - 0.001 M thioethanol and a 5 x 9-cm column (25 g adsorbent) was prepared with a layer of acid-washed glass-wool at the bottom. The column was equilibrated with 400 ml of 0.005 M phosphate buffer - 0.2 M glucose - 0.001 M thioethanol under gentle pressure to pack the column. About 10 ml of enzyme (25 mg protein) was loaded on the column. The column was washed with 400 ml of 0.01 M phosphate buffer - 0.2 M glucose - 0.001 M thioethanol and then eluted with 0.07 M phosphate buffer - 0.2 M - glucose - 0.001 M thioethanol. 20 ml fractions were collected every 10 min and tested for activity. Two to three of the active

fractions were pooled and precipitated with ammonium sulphate by adding 60.2 g solid ammonium sulphate for every 100 ml. After 1 hr the precipitate was collected by centrifugation at 14,000 x g for 1 hr and dissolved in 0.1 M phosphate buffer (pH 7.5) - 0.5 M glucose - 0.01 M thioethanol. The fractions were assayed for protein and enzyme and those with the highest specific activity were retained. The purified enzyme was centrifuged to remove any insoluble material and stored at -20°. The results of a typical purification procedure are presented in Table 23.

The first six steps of the above purification procedure have been repeated about 50 times and DEAE-cellulose fractionations 12 times though the procedure was not identical in all cases. The first six steps were reproducible and the specific activities after DEAE-cellulose chromatography varied from 25 to 55.

Fractionation with Ammonium Sulphate

Two to three lots of DEAE-cellulose eluted and ammonium sulphate precipitated enzyme from 2 to 3 columns were mixed together. 8.6 ml of enzyme (266 units) of specific activity 50 was further fractionated with ammonium sulphate. To 8.6 ml of enzyme 1.8 g of solid ammonium sulphate was added (0.38 saturation). The liquid was allowed to stand for 1 hr and centrifuged to remove the precipitate (precipitate I). The supernatant was further precipitated with 1.6 g ammonium sulphate (0.70 saturation) and centrifuged the next day (precipitate II). Both the precipitates were dissolved in 0.5 M glucose - 0.1 M phosphate buffer (pH 7.5) - 0.01 M thioethanol and tested for activity and protein. Precipitate I had very little

TABLE 23

PURIFICATION PROCEDURE OF BRAIN HEXOKINASE

Fraction	Volume	Activity	Total activity	Protein	Total protein	Specific activity	Recovery
	ml	units/ml	units	mg/ml	mg		per cent
1) Crude extract	2100	1.05	2205	17.50	36750	ca-0.06	-
2) Particulate preparation	90	7.00	630	23.30	2097	0.30	100
3) Elastase treated	84	4.30	361	5.37	451	0.80	57
4) Salmine sulphate treated	109	3.23	352	3.00	327	1.1	56
5) Ammonium sulphate precipitation 40-90 per cent fraction	15	22.40	336	11.20	168	2.0	53
6) Calcium phosphate gel treated, ammonium sulphate precipitated	10	18.84	188	2.52	25	7.5	30
7) DEAE-cellulose eluted, ammonium sulphate precipitated	3.5	40.00	140	1.2	4.2	33.3	22

activity and the second precipitate had a specific activity of 63 units per mg protein. This was the highest purity obtained. No further studies on the purification of the enzyme were carried out.

CHAPTER 4

PROPERTIES AND KINETICS

SECTION I

PROPERTIES

Solutions of purified enzyme were water clear and pale-brown in colour at concentrations greater than 5 to 6 mg per ml. It is not known whether this colour was due to an impurity or to the enzyme itself.

The ultraviolet absorption spectrum of hexokinase is shown in Fig.2. A solution of 0.5 mg of brain hexokinase of maximum purity in 1 ml of 0.1 M Tris-HCl buffer, pH 7.5, containing 0.2 M glucose was used; the blank cuvette contained glucose - Tris-HCl buffer of the same composition (light path 10 mm). The enzyme shows the characteristic ultraviolet spectrum of a protein with a maximum at 280 mμ and a minimum at 250 mμ. There is no evidence for the presence of nucleotides or other ultraviolet absorbing material in the enzyme. The absorption of a 1 mg per ml solution of enzyme at 280 mμ was 1.084, which is similar to that of most proteins.

Ultracentrifugal Studies

Sedimentation velocity runs with purified brain hexokinase were carried out in a Spinco model E Analytical Ultracentrifuge equipped with a phase plate and a temperature control system capable of maintaining a constant temperature during an experiment. Velocity runs were carried out at 59,780 r.p.m. in a

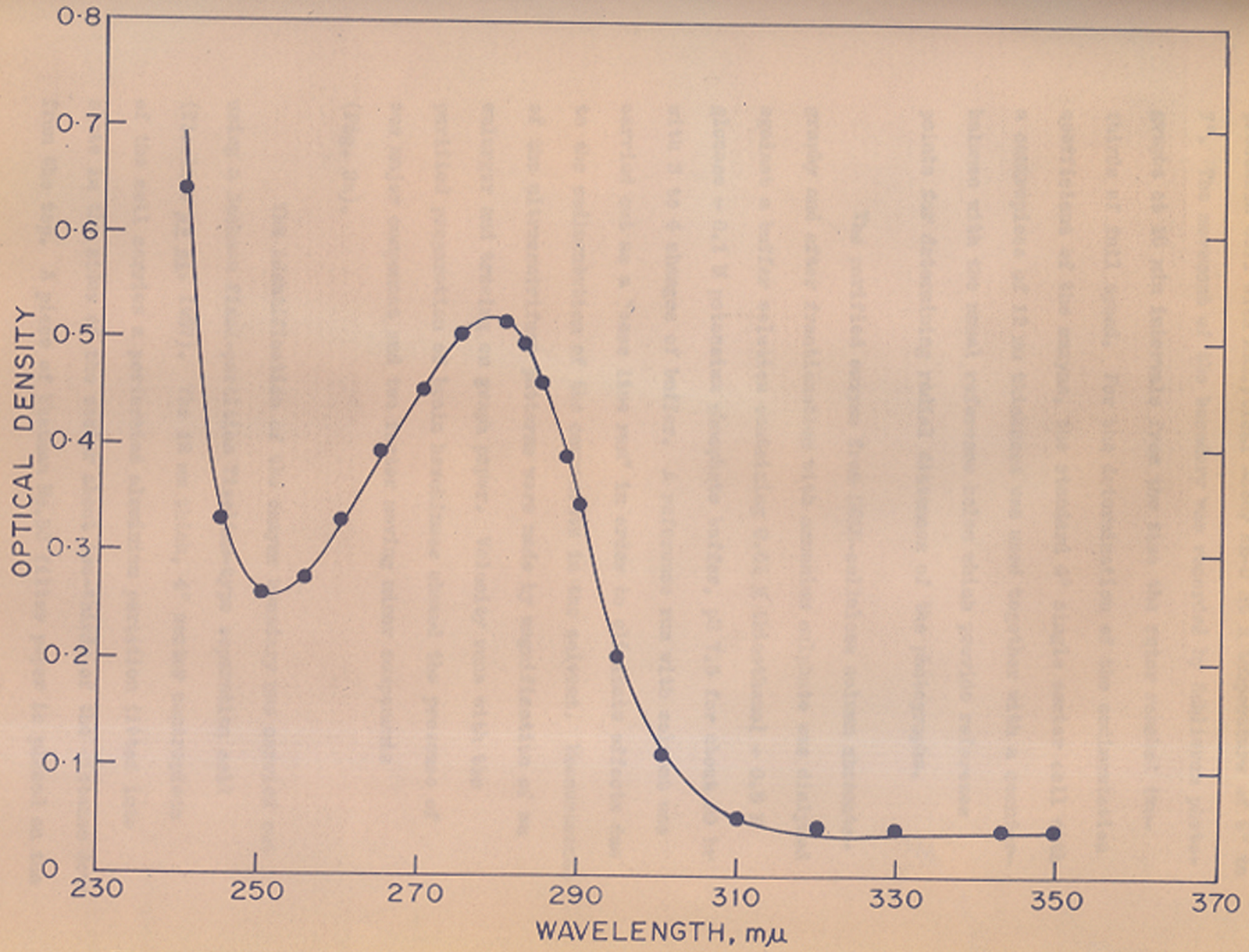
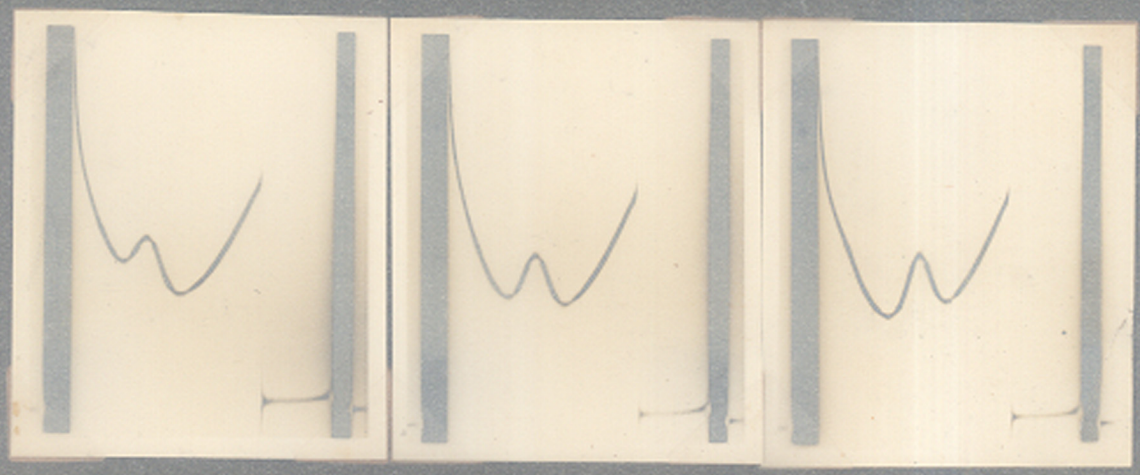


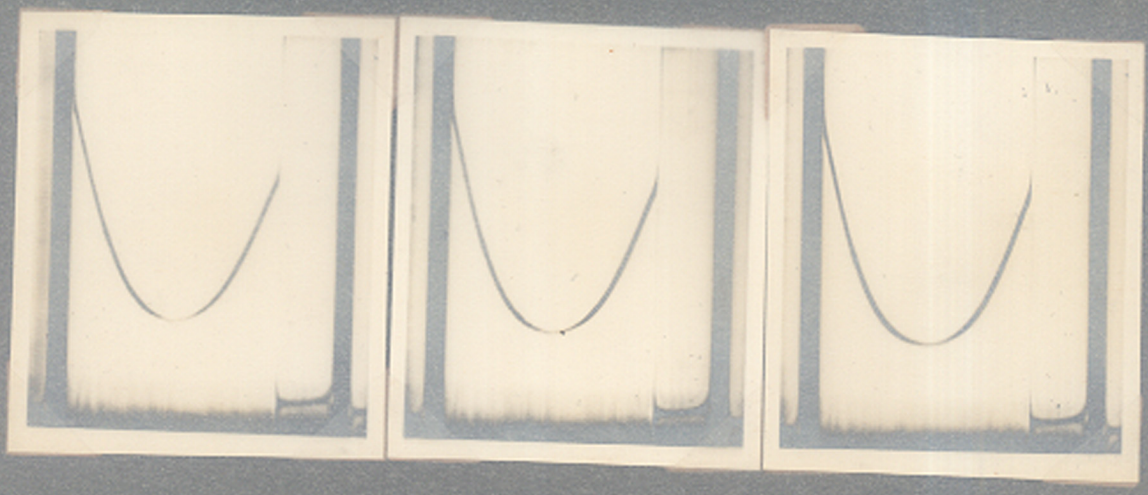
FIG.2. ULTRAVIOLET ABSORPTION SPECTRUM OF BRAIN HEXOKINASE (PROTEIN 0.5 mg per ml)

(i)



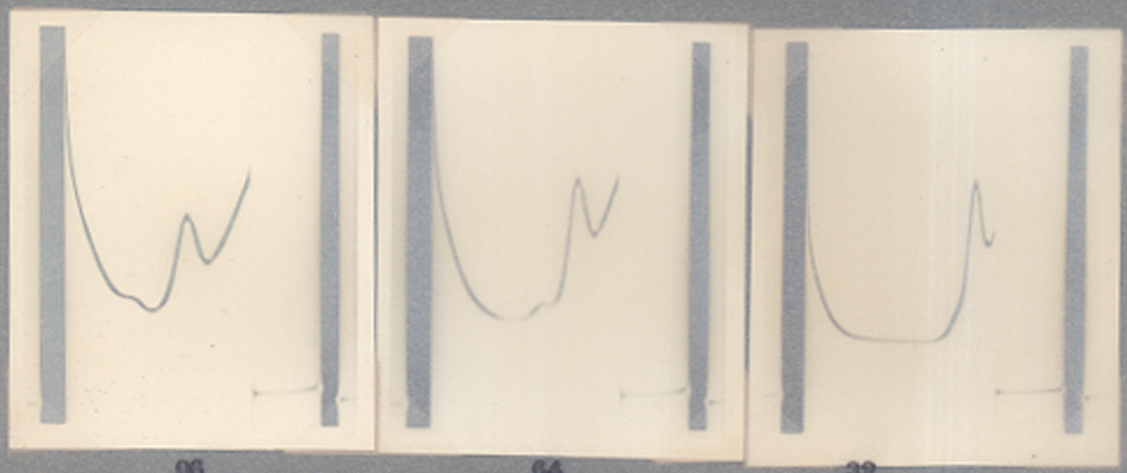
Time (min)	160	144	128
Bar angle	50°	50°	50°

(ii)



SEDIMENTATION

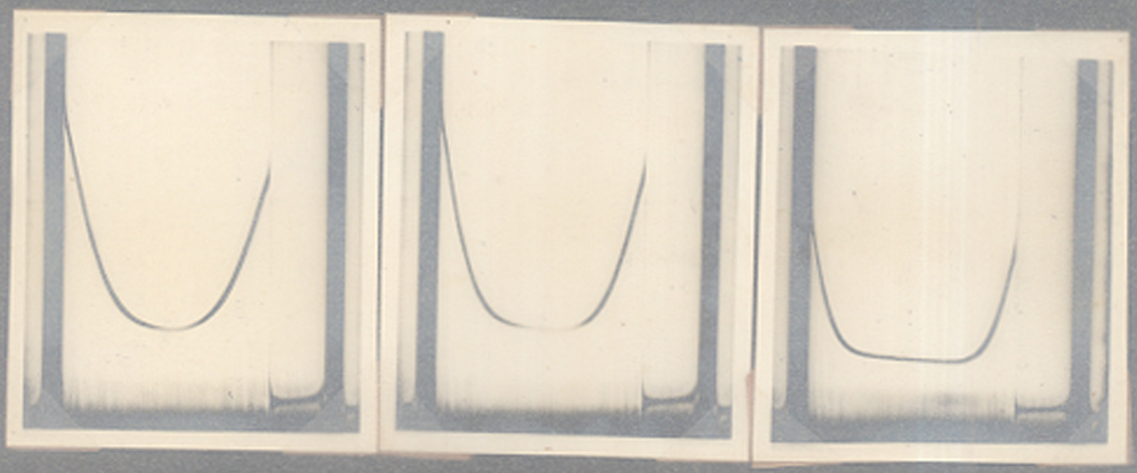
FIG. 3a. Schlieren photographs of purified brain hexokinase; speed, 59,780 r. p. m. temperature, 6.5°. The direction of sedimentation and order of the pictures read from right to left.



06
50°

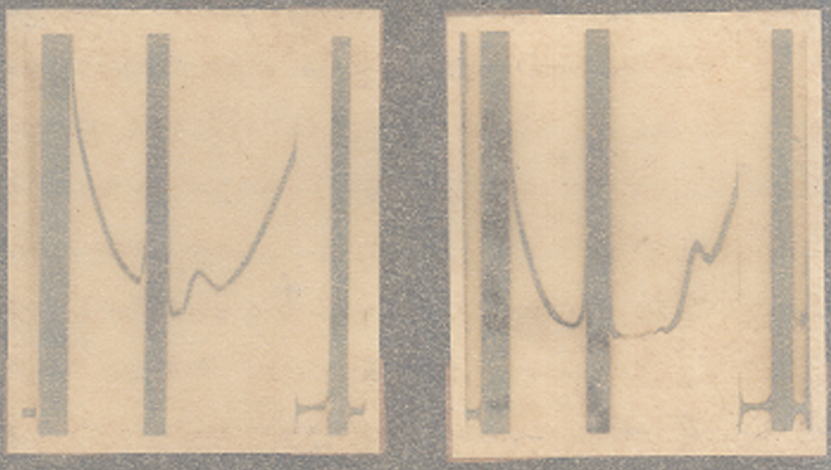
64
50°

32
60°



VELOCITY RUNS

i) Protein concentration 0.4 mg per ml in 0.1 M phosphate buffer, pH 7.5 - 0.5 M glucose - 0.01 M thioethanol. ii)"Base line run"with the buffer system as mentioned in (i).



Time (min)	140	60
Bar angle	45°	30°

FIG. 3b. The ultracentrifugal run of purified brain hexokinase performed in the fixed partition type separation cell. Protein concentration 0.6 mg per ml in 0.1 M phosphate buffer, pH 7.5,- 0.5 M glucose - 0.01 M thioethanol. Sedimentation from right to left. Speed, 59,780 r.p.m.; temperature 6.7°.

top of the partition so that the upper fraction can be drawn off after the run without mixing with the lower fraction. The run was terminated after the two fast moving components had passed below the partition and the slowest moving boundary was still above the partition (Fig. 3b, picture No.2). Samples were drawn off separately from the two sections of the cell and tested for activity. Both upper and lower fractions showed hexokinase activity. The specific activity of the major fraction was about 60 units per mg, but the protein was not estimated after dialysis to remove thioethanol and glucose. The purity of the lower fraction containing the two faster moving components (which would also contain some of the slow moving component) could not unfortunately be determined with accuracy. However, since the major slowest moving component, which was isolated after the two faster fractions had passed below the partition, contained hexokinase activity and had virtually the same specific activity as the enzyme taken initially the major fraction is definitely hexokinase. No conclusions can, however, be drawn regarding the two minor components especially whether they are also hexokinase of higher molecular weight.

By enlargement and area determination by trapezoidal analysis the proportion of the area under the major active peak was found to be 90 per cent of the total protein (Fig. 3a, picture No. 3).

Calculation of sedimentation coefficient

The sedimentation coefficients of the major component at two different protein concentrations (6.4 and 4.3 mg per ml) were

determined according to the procedure described by Schachman (1957). Log x was plotted against time t, where x is the distance in cm of the boundary from the axis of rotation and t is the time in minutes. The slope of this plot, d log x /dt, was substituted in the equation

$$S_{obs} = \frac{2.303 \ d \log x / dt}{(\omega^2) \ (60)}$$

where S_{obs} is the observed sedimentation coefficient under the conditions of the experiment and ω, the angular velocity in radians per second, which is calculated from the equation $\omega = 2\pi x$ revolutions per second (Svedberg, 1940). The observed sedimentation coefficient S_{obs} at the different protein concentrations are converted to $S_{20,w}$ the value the material would have in a solvent with the density and viscosity of water at 20°. The observed values are corrected to this standard state according to the equation

$$S_{20,w} = S_{obs.}\left(\frac{\eta_t}{\eta_{20}}\right)\left(\frac{\eta}{\eta_0}\right)\left(\frac{1 - \bar{V}\rho_{20,\omega}}{1 - \bar{V}\rho_t}\right)$$

where (η_t/η_{20}) is the correction factor corresponding to the viscosity of water at t° relative to that at 20°, (η/η_{20}) is the relative viscosity of the solvent to that of water, and $\rho_{20,w}$ and ρ_t are respectively the densities of water at 20° and of the solvent at t°(Table 24a).

Sedimentation of brain hexokinase at two concentrations is shown in Table 24b. It was unfortunately not possible to carry out more extensive studies on the dependence of $S_{20,w}$ on enzyme concentration.

TABLE 24a

VALUES FOR TEMPERATURE OF 6.5°

$(\eta_t/\eta_{20}) = 1.43$ $\rho_t = 1.049$

$(\eta/\eta_o) = 1.45$ $\rho_{20,w} = 0.998$

$\bar{V}$, the partial specific volume, was assumed to be 0.740 ml/g (Kunitz and McDonald, 1946).

TABLE 24b

Run no.	Protein concentration mg/ml	$S_{20,w}$ expressed in S units
1	6.40	5.86
2	4.27	5.90

Stability of the enzyme

As indicated in a previous section the stability of the enzyme varies considerably with its purity. The particulate enzyme was stable for several weeks in the frozen condition. The soluble enzyme was less stable in the presence of elastase, but after treatment with protamine sulphate and calcium phosphate gel, it could be stored at -20° in 0.1 M phosphate pH 7.5 for several weeks without loss of activity.

On further purification the enzyme became highly unstable. Some of the studies on partially purified preparations have been reported in Chapter III. The results of experiments on the stability of enzyme of high purity in concentrated and dilute solution are presented below, though it appears to be somewhat repetitive.

The DEAE-cellulose eluted enzyme, when kept with 0.02 M histidine, showed a loss of 35 per cent of activity in two days at -20°. With histidine (0.1 M) and thioethanol (0.001 M) it showed 84 per cent loss in six days and with histidine (0.1 M), glucose (0.15 M) and thioethanol (0.001 M) 36 per cent loss was observed. With DPN (0.5 mg/ml) there was 75 per cent loss. In the presence of fatty acids such as stearic acid (3 μmoles/ml) and thioethanol (0.001 M) there was 90 per cent loss. However in the presence of bovine plasma albumin (0.2 mg/ml) there was no loss of activity during the same period in the frozen condition. In the presence of 1 M sucrose the enzyme showed no loss in sixteen days

at -20°. On further purification the enzyme became progressively more unstable and at the highest degree of purity, it required 0.5 to 1.0 M glucose or sucrose, 0.05 to 0.1 M phosphate, pH 7.5 and 0.001 to 0.010 M thioethanol for storage at -20°. The enzyme was also very stable in this buffer at 0° for several weeks in the presence of ammonium sulphate at about 0.4 to 0.6 saturation.

The above results apply to enzyme at a protein concentration of 0.1 per cent or higher. When the enzyme was highly diluted the activity losses were appreciable even in a short time. For kinetic studies requiring enzyme concentrations of about 1 to 2 µg per ml it was necessary to determine its stability over a wide range of conditions. This was particularly necessary for studies on the effect of the concentration of glucose and Pi on activity which require dilution with buffers containing no glucose or Pi. The loss of activity in the absence of glucose or Pi was so rapid that kinetic studies under these conditions were not possible with dilute enzyme solutions even for a few hours without corrections for loss of activity. The stability of the enzyme in dilute solutions was, therefore, determined under different conditions. The enzyme was diluted with solutions of different compositions, kept at 0° for different periods and tested for activity. It will be seen from Table 25 that even in the presence of phosphate, glucose and thioethanol the enzyme is quite unstable at a concentration of about 1.8 µg/ml. The loss in activity was also appreciable in the absence of phosphate. Marked protection was obtained in the presence of insulin or bovine plasma albumin at concentrations of 0.04 mg per ml and 0.1 mg per ml

TABLE 25

STABILITY OF HEXOKINASE ON DILUTION

(Assay-I)

	Enzyme diluted in solution containing							Period at 0°	Loss in activity
	Phosphate buffer pH 7.5	Tris-HCl buffer pH 7.5	Glucose	Sucrose	Thioethanol	Bovine albumin	Insulin		
	M	M	M	M	M	mg/ml	mg/ml	hr	per cent
1	0.05	-	0.5	-	0.01	-	-	8	50
2	0.05	-	-	-	0.01	0.01	-	8	0
3	0.05	-	-	-	0.01	-	-	7	40
4	-	-	-	-	0.01	-	-	1	33
5	-	-	-	-	0.01	-	-	8	82
6	-	-	-	-	0.01	-	0.04	8	40
7	0.05	-	-	-	0.01	-	0.04	8	0
8	-	-	0.4	-	0.01	-	0.04	8	42
9	0.05	-	0.4	-	0.001	-	0.04	8	0
10	-	-	-	0.5	0.01	10 (pH 6.0)	-	5	33
11	-	0.1	-	0.4	0.01	8	-	11	0

respectively. No studies were made on the effect of different concentrations of these proteins. It will be noted that phosphate was essential for stabilization even in the presence of the protein since there was no loss of activity in the presence of phosphate, thioethanol and insulin whereas in the presence of glucose, thioethanol and insulin there was a 42 per cent loss in activity. In the presence of higher concentrations of albumin (8 mg/ml) the enzyme was stable even in the absence of phosphate at pH 7.5 (experiment 10). But at lower pHs the enzyme was highly unstable and even at this level of albumin and in the presence of sucrose (0.5 M) and thioethanol (0.01 M) the loss of activity at pH 6.0 within three to four hr was appreciable (33 per cent). The stability of the enzyme at different pHs was however not determined. It may be concluded from these studies that in the presence of a protective protein such as albumin (0.1 mg/ml), hexokinase is stable in phosphate-thioethanol-sucrose solutions. The stability is markedly less in the absence of phosphate especially at lower pHs but the enzyme can be stabilized at pH 7.5 by the addition of more albumin (8 mg/ml).

The stability of the enzyme of maximum purity at high temperatures and the effect of glucose concentration, phosphate, thioethanol and pH are shown in Table 26. It will be noted that the stability of the enzyme is markedly enhanced by increase in glucose concentration. In 10 min at 65° the losses in activity were 95, 54 and 41 per cent at glucose concentrations of 0.25 M, 0.4 M and 1 M respectively. In the absence of thioethanol even in 1 M glucose and 0.1 M phosphate the loss in activity in 10 min

TABLE 26

STABILITY OF HEXOKINASE ON HEATING

(Assay-I)

	Enzyme concentration	Enzyme heated in the presence of				Temp.	Time	Loss in activity
		Glucose	Phosphate	Thioethanol	pH			
	unit/ml	M	M	M		°	min	per cent
1	0.3	1.0	0.1	0.01	7.5	50	5	9
						65	5	29
						65	10	41
						70	5	80
2	0.3	0.4	0.1	0.01	7.5	65	10	54
3	0.2	0.25	0.1	0.01	7.5	65	10	95
4	0.2	1.0	0.1	-	7.5	65	10	80
5	0.2	1.0	0.1	0.01	5.5	65	10	38
6	0.2	1.0	-	0.01	6.0	65	10	70

at 65° was 80 per cent. Though the enzyme is markedly unstable at high dilutions at lower pHs in the absence of phosphate, in the presence of phosphate there is no significant difference in stability at 65° in 10 min in the presence of 1 M glucose and 0.01 M thioethanol at pH 7.5 or 5.5. In the absence of phosphate the loss in activity was markedly higher and was about 70 per cent in 10 min at 65°.

SECTION II

KINETICS

Enzyme concentration and activity

It will be seen from Figs. 4a and 4b that enzyme concentration is proportional to $\triangle$ O.D. using methods I and III when the $\triangle$ O.D.s were less than 0.020 and 0.060 per min respectively.

Enzyme activity and time

Enzyme activity was also portional to time (Figs 5a and 5b). There was no fall in activity during the periods for which the reactions were studied. Assay-II gave slightly lower results than Assay-I (the same amounts of enzyme were used for the two assays in the experiment shown in Fig.5a) but the difference was negligible when the O.D. was less than 0.100.

Effect of temperature on enzyme activity

The effect of temperature on hexokinase activity was determined by Assay-II (Table 27, Fig. 6). It will be seen that there was linearity between the logarithm of initial velocity and absolute temperature between 20° and 40° with an increase of enzyme activity of 2.4 times for every 10° rise in temperature. At higher temperatures there was a fall in activity, probably due to the partial destruction of the enzyme. The energy of activation of the reaction was calculated according to the method of Arrhenius

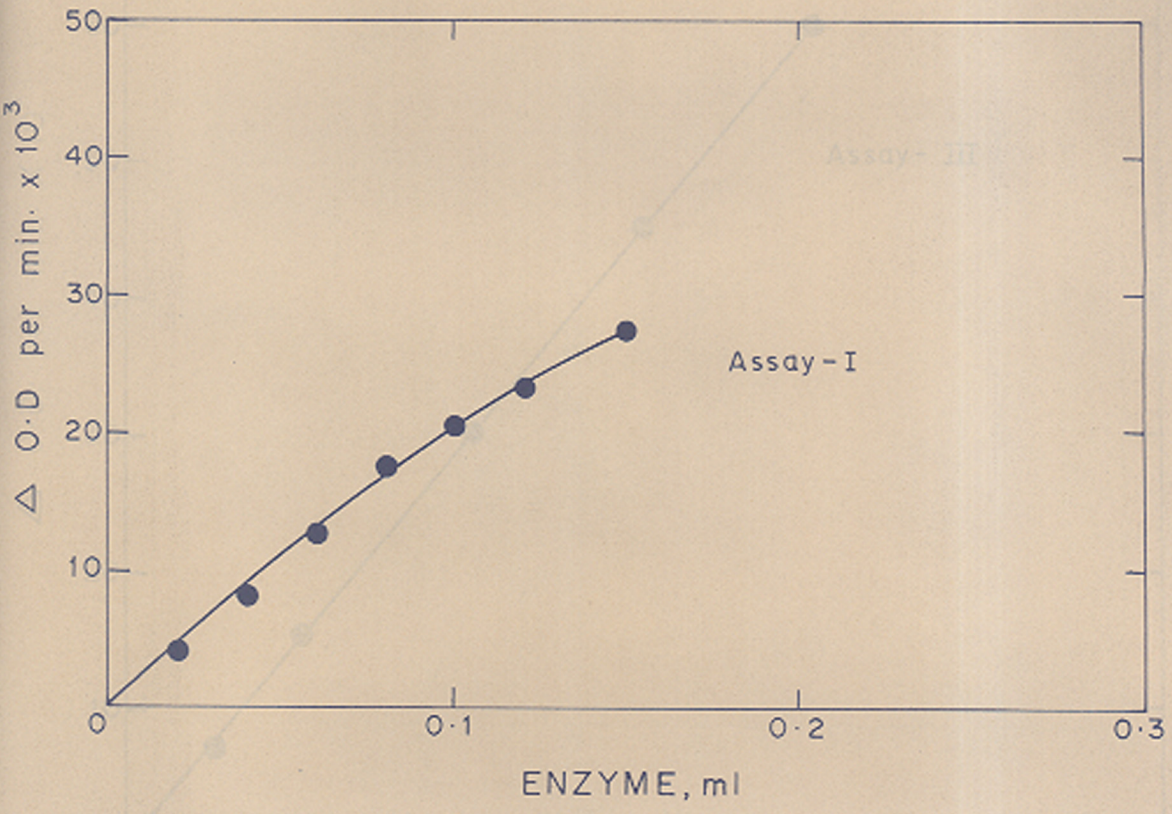


FIG. 4a. EFFECT OF ENZYME CONCENTRATION ON ACTIVITY OF BRAIN HEXOKINASE.

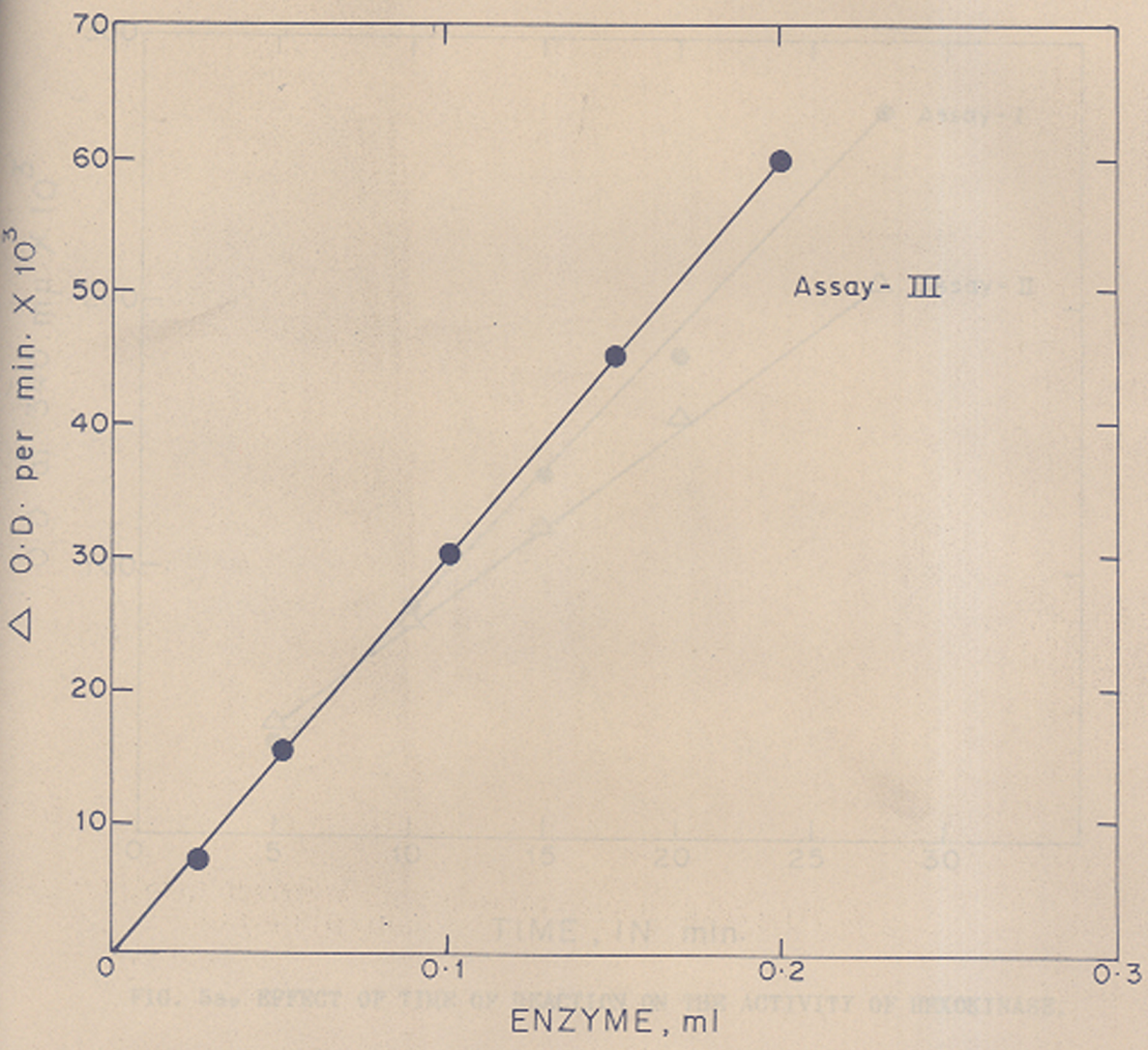


FIG. 4b. EFFECT OF ENZYME CONCENTRATION ON ACTIVITY OF BRAIN HEXOKINASE

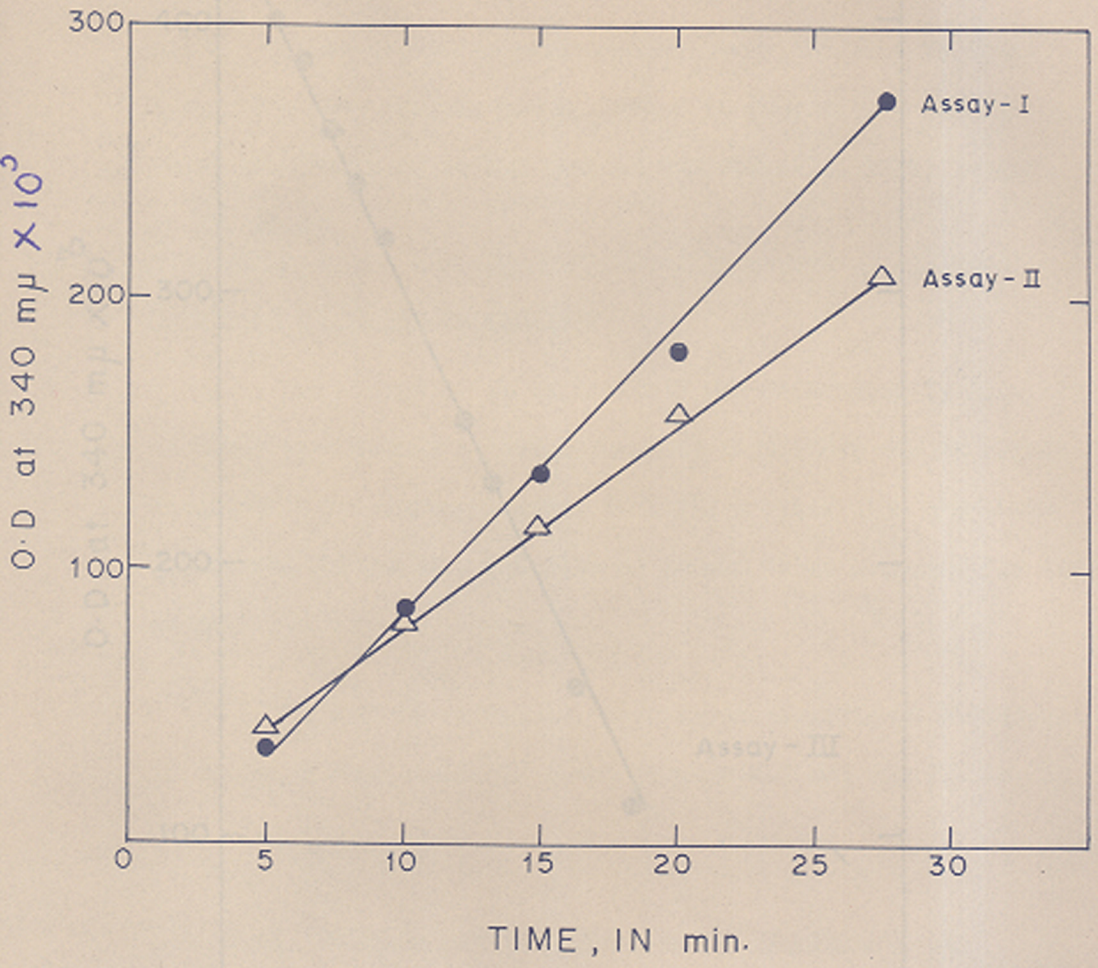


FIG. 5a. EFFECT OF TIME OF REACTION ON THE ACTIVITY OF HEXOKINASE.

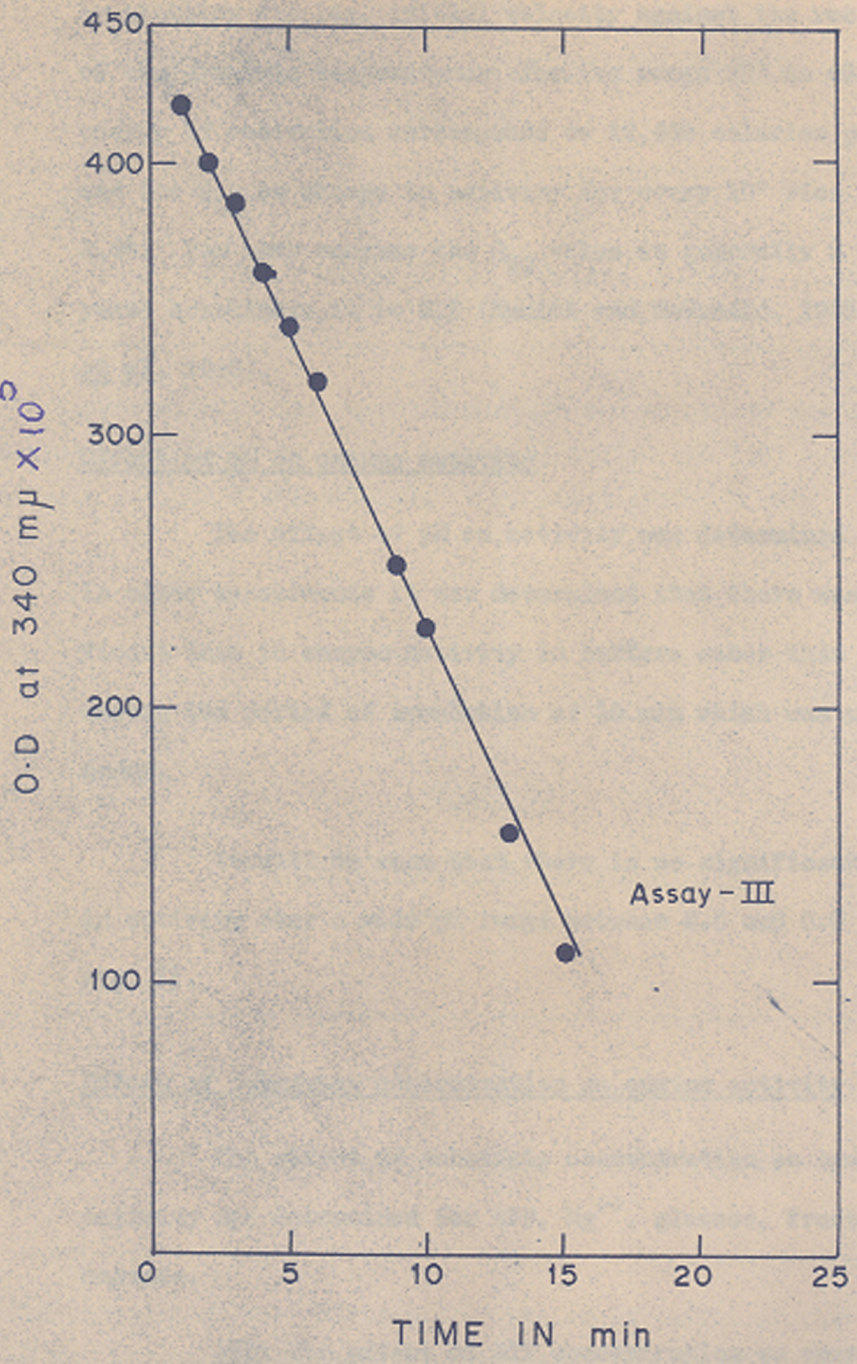


FIG. 5b. EFFECT OF TIME OF REACTION ON THE ACTIVITY OF BRAIN HEXOKINASE

by plotting the log. initial velocity against the reciprocal of the absolute temperature. For the range 20° to 40° the energy of activation corresponds to 12,400 calories per mole and the Q_{10} or change in activity for every 10° rise is about 2.43. For many enzymes the Q_{10} value is generally 2 and for yeast hexokinase it is 2.2 (Kunitz and McDonald, 1946; Berger et al. 1946).

Effect of pH on enzyme activity

The effect of pH on activity was determined by Assay-II. In other experiments it was determined that there was no significant loss in enzyme activity in buffers other than phosphate during the period of incubation of 10 min which was used for the assay.

It will be seen that there is no significant difference in activity over a wide pH range between 5.5 and 8.0 (Table 28, Fig. 7).

Effect of substrate concentration on enzyme activity

The effect of substrate concentration on enzyme activity was determined for ATP, Mg^{++}, glucose, fructose and mannose.

ATP: The effect of ATP concentration on enzyme activity was determined using Assay-I. The results are shown in Table 29 and Fig. 8. Substrate concentration $[S]$ was plotted against $[S]/v$ (where v is velocity per min) according to Lineweaver and Burk (1934). The k_m value for ATP (at 0.02 M Mg^{++}) was found to be 5×10^{-4} M.

TABLE 27

EFFECT OF TEMPERATURE ON HEXOKINASE ACTIVITY

(Assay-II)

Temperature (°)	20	30	40	50	60	65
△ O.D.	0.025	0.054	0.093	0.117	0.126	0.023

The composition of the reaction mixture was as stated for Assay-II. Enzyme was diluted in 0.05 M phosphate buffer (pH 7.5) - 0.05 mg per ml albumin - 0.001 M thioethanol. Incubation period 5 min.

TABLE 28

EFFECT OF pH ON HEXOKINASE ACTIVITY

(Assay-II)

pH	5.0	5.5	6.0	6.5	7.0	7.5	8.0
△ O.D.	0.072	0.085	0.084	0.084	0.085	0.086	0.086

The reaction mixture contained 17 µmoles of buffer of the appropriate pH and the other components as described in Assay-II.

1. Sodium acetate-acetic acid buffer for pH 5 to 6.
2. Tris-acetate buffer for 6.5 and 7.
3. Tris-HCl buffer for 7.5 to 8.0.

Incubation period 10 min.

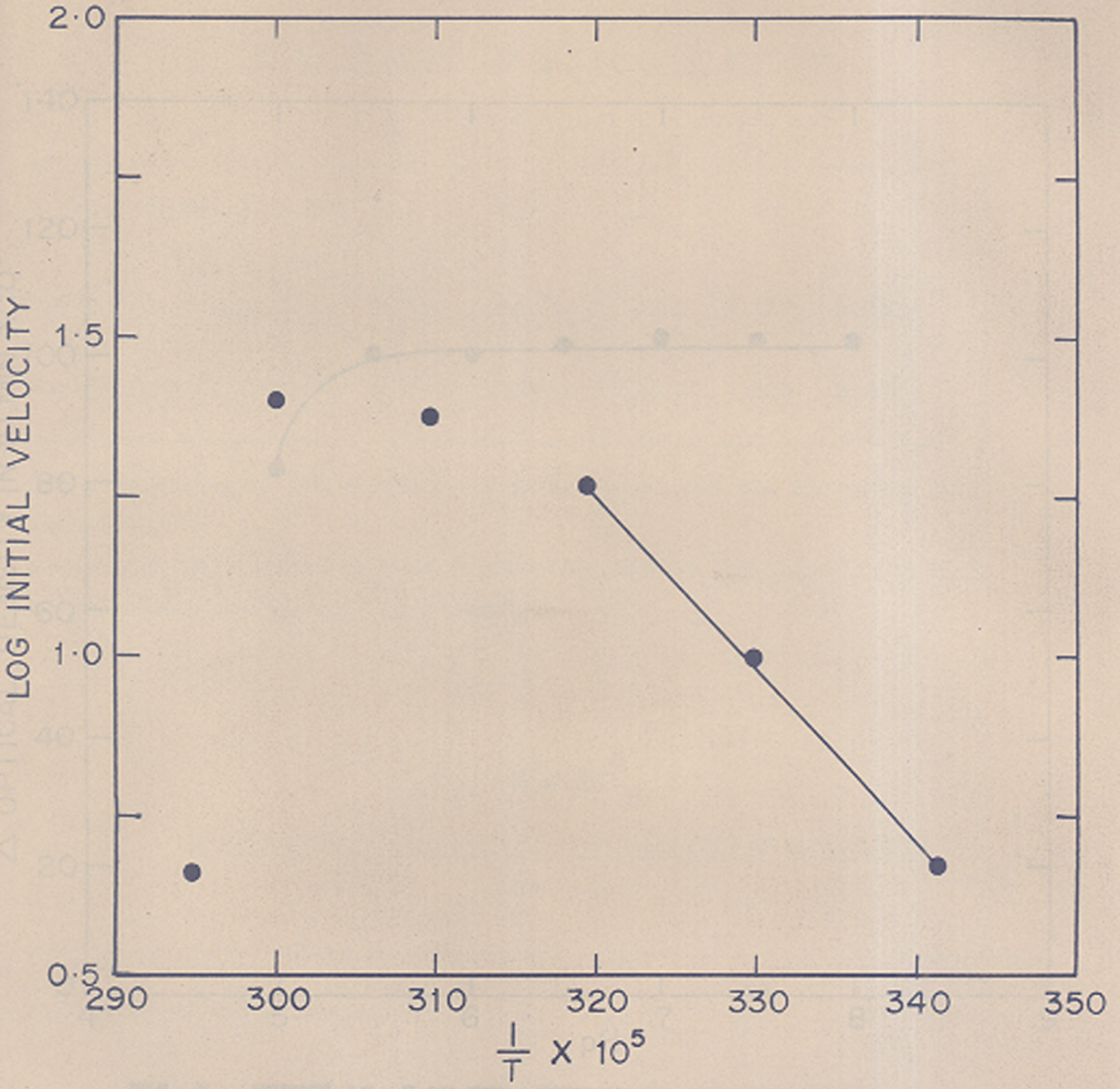


FIG. 6. ARRHENIUS PLOT FOR THE ACTIVITY OF BRAIN HEXOKINASE ASSAY-II.

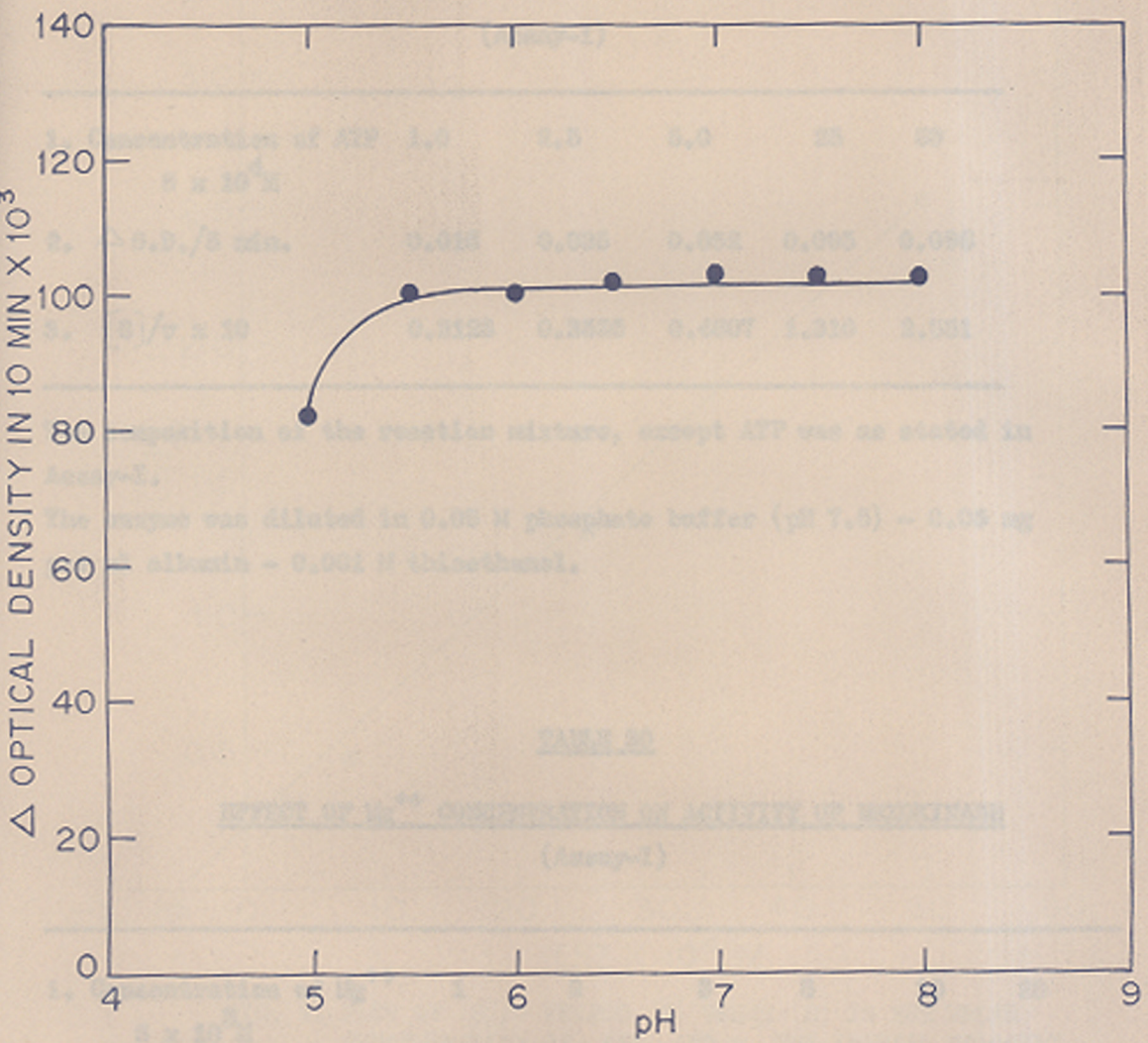


FIG. 7. EFFECT OF pH ON THE ACTIVITY OF BRAIN HEXOKINASE ASSAY-II.

TABLE 29

EFFECT OF ATP CONCENTRATION ON HEXOKINASE ACTIVITY

(Assay-I)

1. Concentration of ATP S x 10^{4}M	1.0	2.5	5.0	25	50
2. △O.D./5 min.	0.016	0.035	0.052	0.095	0.098
3. [S]/v x 10	0.3125	0.3555	0.4807	1.316	2.551

The composition of the reaction mixture, except ATP was as stated in Assay-I.

The enzyme was diluted in 0.05 M phosphate buffer (pH 7.5) - 0.05 mg per ml albumin - 0.001 M thioethanol.

TABLE 30

EFFECT OF Mg^{++} CONCENTRATION ON ACTIVITY OF HEXOKINASE

(Assay-I)

1. Concentration of Mg^{++} S x 10^{3}M	1	2	3	5	10	20
2. △O.D./5 min	0.013	0.027	0.032	0.053	0.056	0.056
3. [S]/v	0.3846	0.3703	0.4687	0.4718	0.893	1.785

The composition of the reaction mixture, except $MgCl_2$ and glucose-6-phosphate dehydrogenase, was as stated in Assay-I (0.2 unit glucose-6-phosphate dehydrogenase).

Enzyme was diluted as stated in Table 29.

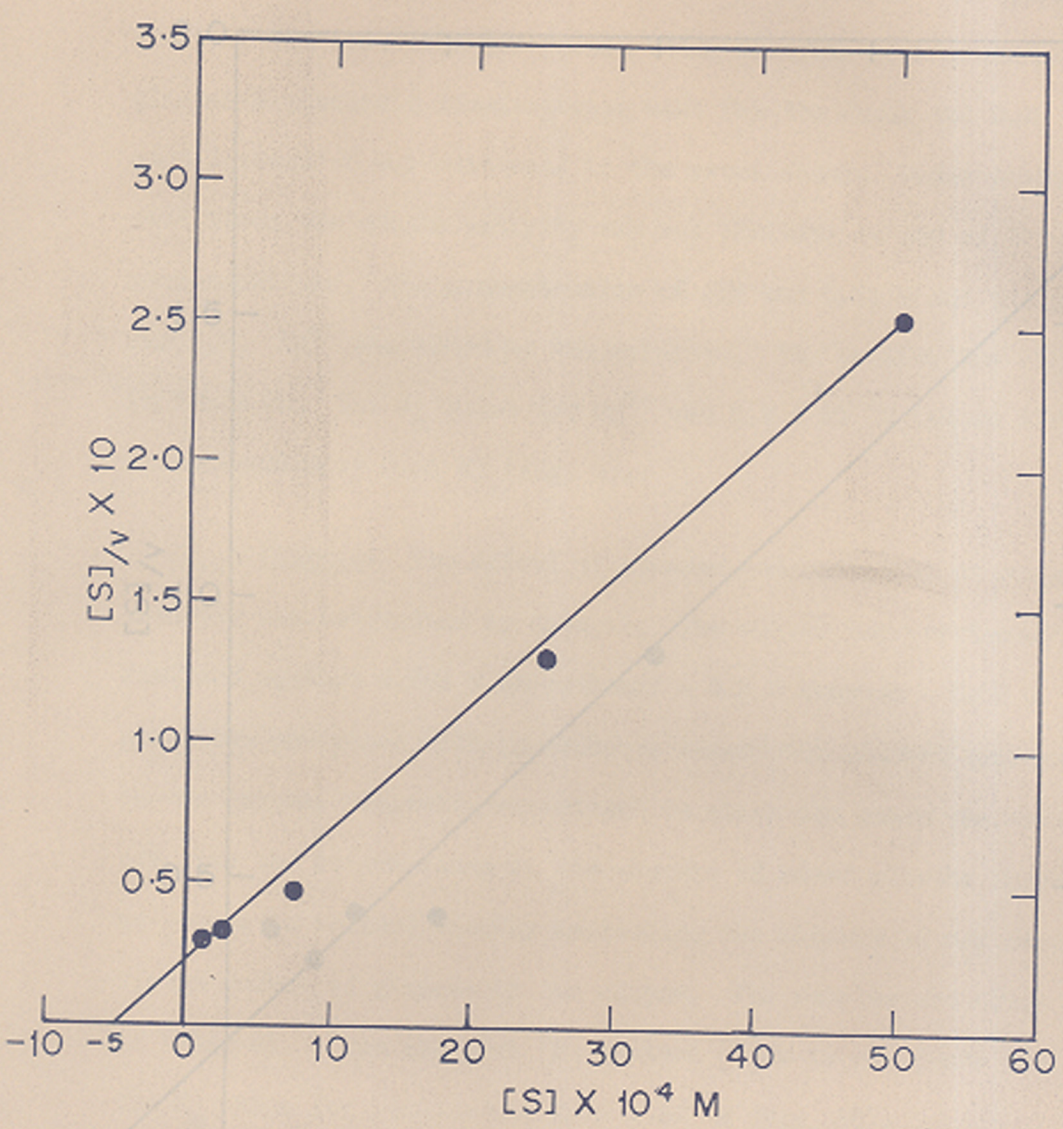


FIG. 8. PLOT OF [S]/v VERSUS [S] WHERE [S] IS THE MOLAR CONCENTRATION OF ATP AND v, THE INITIAL VELOCITY ASSAY-I.

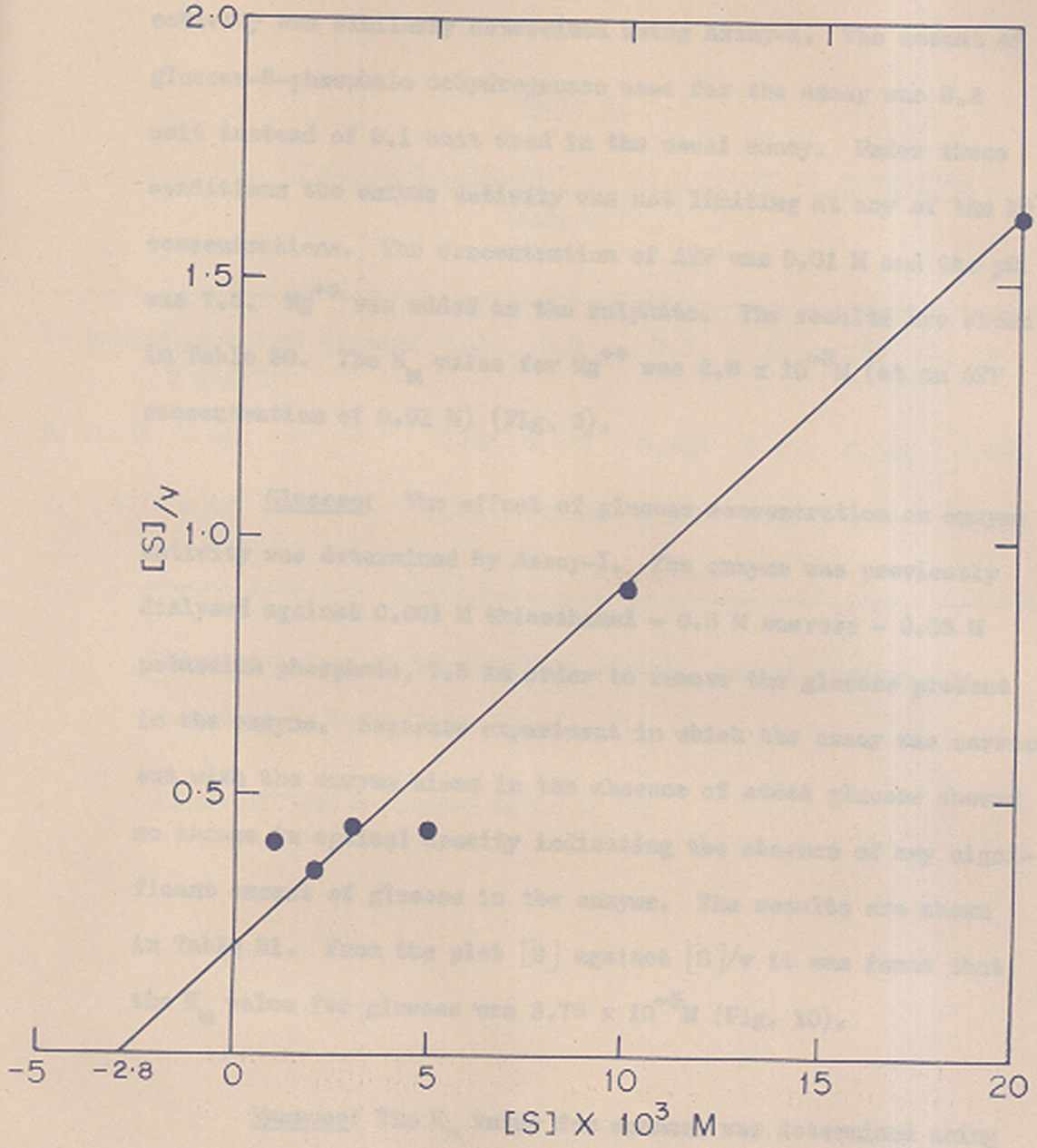


FIG. 9. PLOT OF [S] /v VERSUS [S] WHERE [S] IS THE MOLAR CONCENTRATION OF Mg^{++} AND v, THE INITIAL VELOCITY ASSAY-I.

Magnesium: The effect of Mg^{++} concentration on enzyme activity was similarly determined using Assay-I. The amount of glucose-6-phosphate dehydrogenase used for the assay was 0.2 unit instead of 0.1 unit used in the usual assay. Under these conditions the enzyme activity was not limiting at any of the Mg^{++} concentrations. The concentration of ATP was 0.01 M and the pH was 7.5. Mg^{++} was added as the sulphate. The results are shown in Table 30. The K_m value for Mg^{++} was 2.8×10^{-3}M (at an ATP concentration of 0.01 M) (Fig. 9).

Glucose: The effect of glucose concentration on enzyme activity was determined by Assay-I. The enzyme was previously dialysed against 0.001 M thioethanol - 0.5 M sucrose - 0.05 M potassium phosphate, 7.5 in order to remove the glucose present in the enzyme. Separate experiment in which the assay was carried out with the enzyme alone in the absence of added glucose showed no change in optical density indicating the absence of any significant amount of glucose in the enzyme. The results are shown in Table 31. From the plot [S] against [S]/v it was found that the K_m value for glucose was 3.75×10^{-5}M (Fig. 10).

Mannose: The K_m value for mannose was determined using the pyruvate kinase-lactic dehydrogenase method. The enzyme was dialysed against 0.5 M sucrose - 0.05 M phosphate (pH 7.5) - 0.001 M thioethanol. The results are presented in Table 32. There was no activity in the absence of mannose. From the plot [S] vs S /v , the K_m value for mannose was 3×10^{-5}M (Fig. 11).

TABLE 31

EFFECT OF GLUCOSE CONCENTRATION ON ACTIVITY OF HEXOKINASE

(Assay-I)

1. Concentration of glucose S x 10^5M	1.0	2.5	5.0	10	20	35	50
2. $\triangle$O.D./5 min	0.020	0.037	0.060	0.070	0.081	0.085	0.090
3. $[S]/v \times 10^2$	0.25	0.3246	0.4165	0.7142	1.227	2.000	2.63

The composition of the reaction mixture, except glucose, was according to Assay-I.

Enzyme diluted as stated in Table 29.

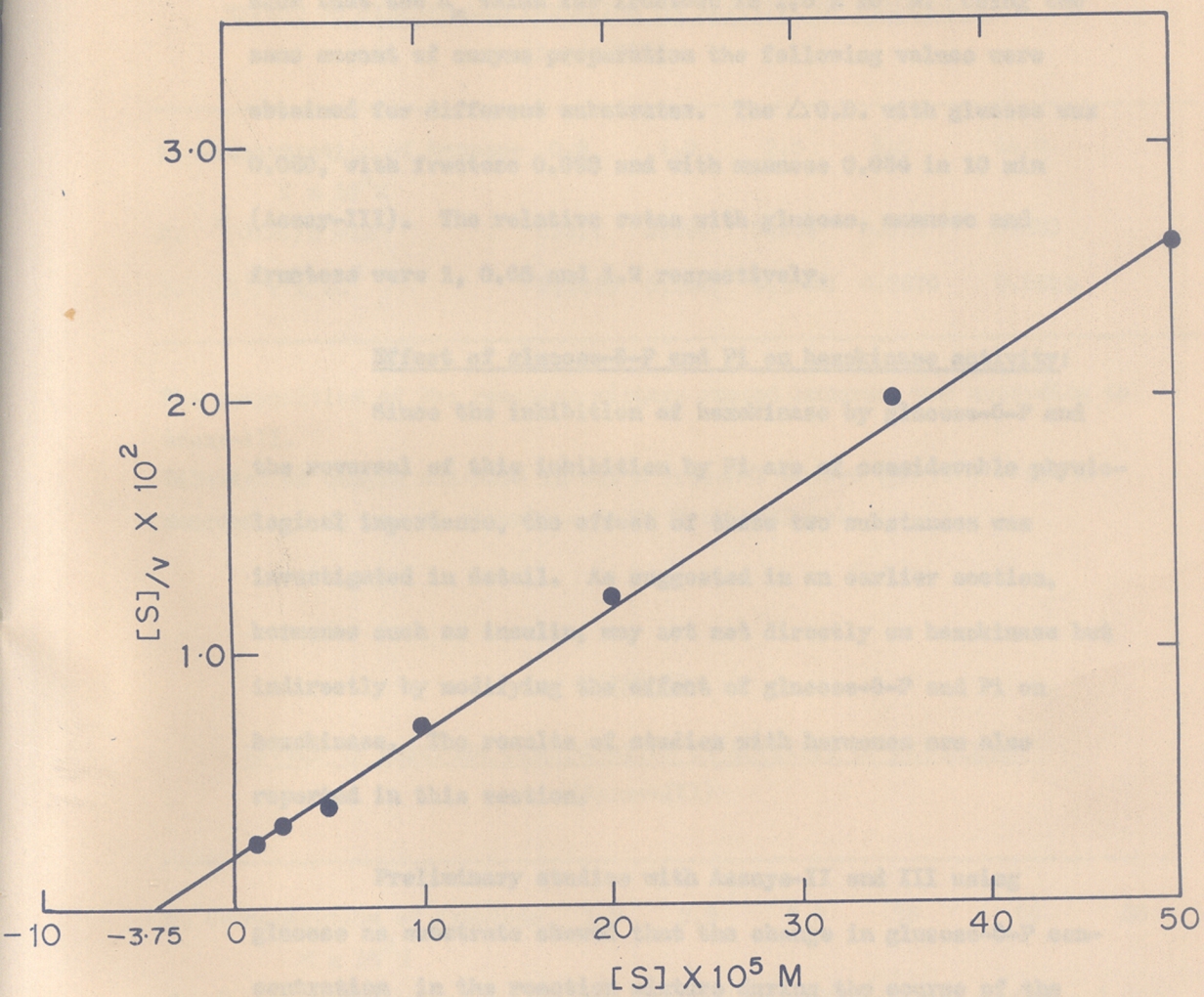


FIG. 10. PLOT OF [S]/v VERSUS [S] WHERE [S] IS THE MOLAR CONCENTRATION OF GLUCOSE AND v THE INITIAL VELOCITY ASSAY-I.

Fructose: Similar studies were carried out with fructose with Assay-III. The results presented in Table 33 and Fig. 12 show that the K_m value for fructose is 1.5×10^{-3} M. Using the same amount of enzyme preparation the following values were obtained for different substrates. The Δ O.D. with glucose was 0.080, with fructose 0.098 and with mannose 0.054 in 10 min (Assay-III). The relative rates with glucose, mannose and fructose were 1, 0.68 and 1.2 respectively.

Effect of glucose-6-P and Pi on hexokinase activity:

Since the inhibition of hexokinase by glucose-6-P and the reversal of this inhibition by Pi are of considerable physiological importance, the effect of these two substances was investigated in detail. As suggested in an earlier section, hormones such as insulin, may act not directly on hexokinase but indirectly by modifying the effect of glucose-6-P and Pi on hexokinase. The results of studies with hormones are also reported in this section.

Preliminary studies with Assays-II and III using glucose as substrate showed that the change in glucose-6-P concentration in the reaction mixture during the course of the reaction was sufficient to decrease the reaction rate and except over a very limited optical density change renders these studies difficult especially at low glucose-6-P levels. With mannose as substrate in Assay-III the reaction was proportional to time and enzyme concentration over a wide range indicating that mannose-6-P inhibition was not significant under the conditions of the assay.

TABLE 32

EFFECT OF MANNOSE CONCENTRATION ON ACTIVITY OF HEXOKINASE

(Assay-III)

1. Concentration of Mannose S x 10^5 M	0.5	1.0	2.5	5.0	10.0
2. $\triangle$ O.D./5 min	0.015	0.030	0.047	0.068	0.086
3. $[S]/v \times 10^2$	0.1613	0.1666	0.2632	0.3676	0.5814

The composition of the reaction mixture except mannose, was according to Assay-III.
Dilution of enzyme was made in 0.1 M Tris-HCl buffer (pH 7.5) - 0.4 M sucrose - 8 mg per ml albumin - 0.001 M thioethanol.

TABLE 33

EFFECT OF FRUCTOSE CONCENTRATION ON ACTIVITY OF HEXOKINASE

(Assay-III)

1. Concentration of fructose S x 10^4 M	1.0	2.0	3.0	10	20	50
2. $\triangle$ O.D./5 min	0.006	0.015	0.025	0.044	0.070	0.098
3. $[S]/v \times 10$	0.8333	0.6666	0.6000	0.1123	1.4280	2.5510

The composition of the reaction mixture except mannose was as described in Assay-III. The enzyme was diluted as described in Table 32.

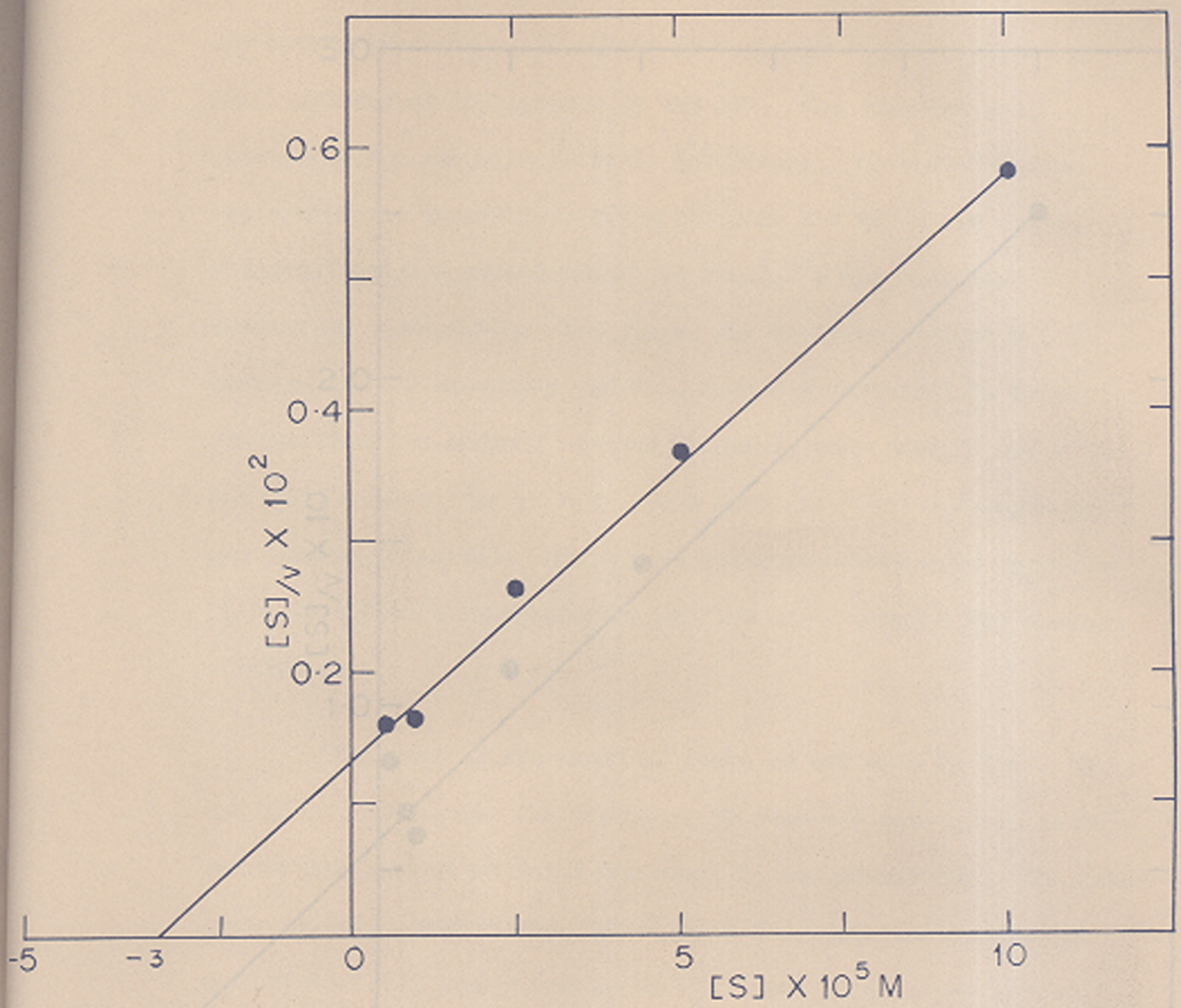


FIG. 11. PLOT OF [S]/v VERSUS [S] WHERE [S] IS THE MOLAR (MANNOSE) CONCENTRATION AND v, THE INITIAL VELOCITY. ASSAY-III.

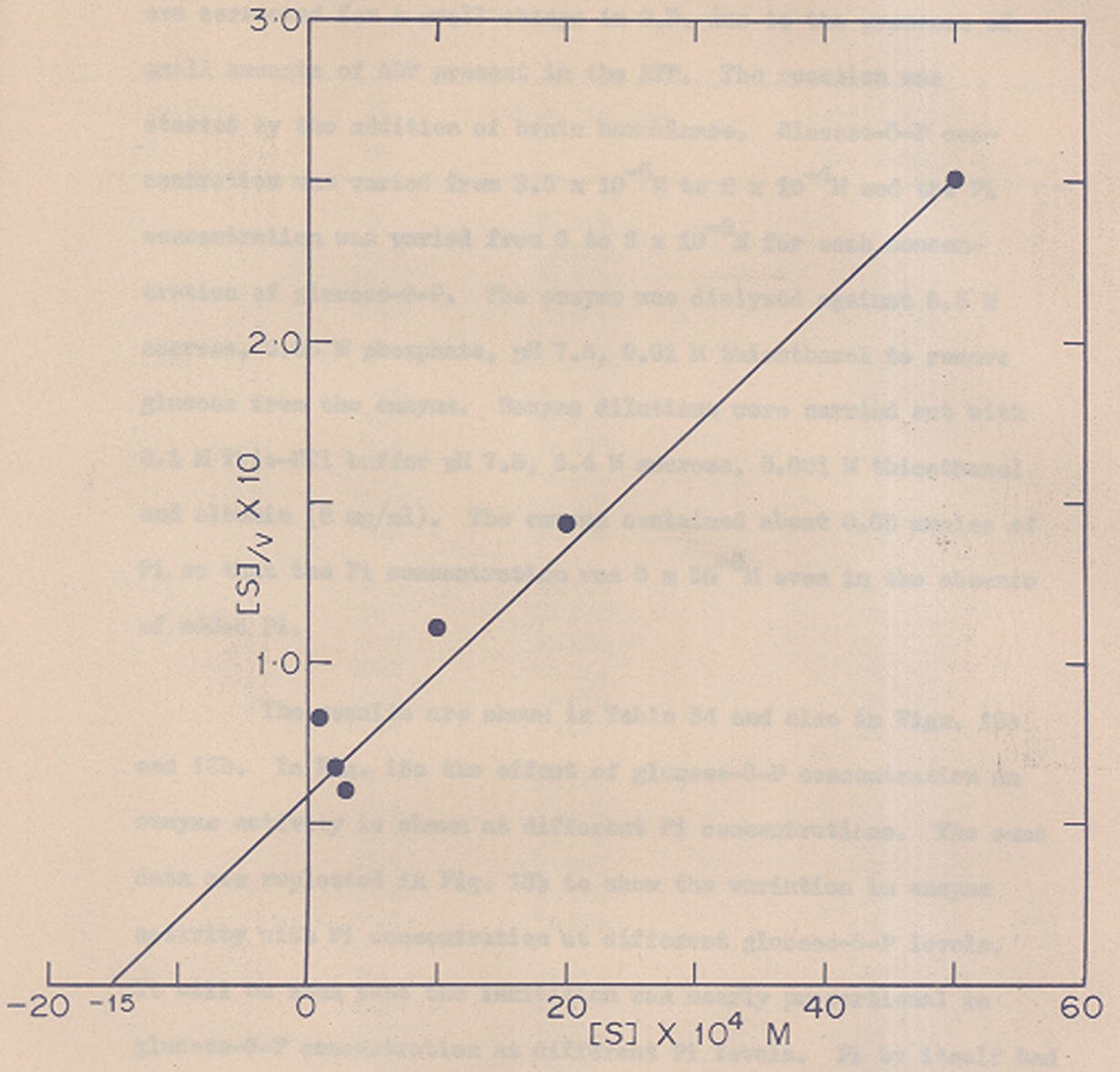


FIG. 12. PLOT OF [S]/v VERSUS [S] WHERE [S] IS THE MOLAR CONCENTRATION OF FRUCTOSE AND v, THE INITIAL VELOCITY ASSAY--III.

The linearity of the reaction during the experimental period and over a wide O.D. range was established. The results are corrected for a small change in O.D. due to the presence of small amounts of ADP present in the ATP. The reaction was started by the addition of brain hexokinase. Glucose-6-P concentration was varied from 3.5×10^{-5}M to 2×10^{-4}M and the Pi concentration was varied from 0 to 3×10^{-2}M for each concentration of glucose-6-P. The enzyme was dialysed against 0.5 M sucrose, 0.05 M phosphate, pH 7.5, 0.01 M thioethanol to remove glucose from the enzyme. Enzyme dilutions were carried out with 0.1 M Tris-HCl buffer pH 7.5, 0.4 M sucrose, 0.001 M thioethanol and albumin (8 mg/ml). The enzyme contained about 0.05 µmoles of Pi so that the Pi concentration was 5×10^{-5}M even in the absence of added Pi.

The results are shown in Table 34 and also in Figs. 13a and 13b. In Fig. 13a the effect of glucose-6-P concentration on enzyme activity is shown at different Pi concentrations. The same data are replotted in Fig. 13b to show the variation in enzyme activity with Pi concentration at different glucose-6-P levels. It will be seen that the inhibition was nearly proportional to glucose-6-P concentration at different Pi levels. Pi by itself had no effect on enzyme activity.

It may also be noted that the reversal of glucose-6-P inhibition by Pi was observed at all glucose-6-P levels, but the reversal was never complete. Thus even at a Pi concentration of 3×10^{-2}M the inhibition was 44 and 52 per cent at glucose-6-P

TABLE 34

EFFECT OF GLUCOSE-6-P AND Pi ON ACTIVITY OF BRAIN HEXOKINASE

(Assay-III)

Glucose-6-P mM	Pi mM	△O.D./10 min	Inhibition per cent
0	0	0.270	
	5.0	0.264	
	10.0	0.275	
	20.0	0.263	
0.035	0	0.208	23
	2.5	0.226	16
	5.0	0.226	16
	7.5	0.226	16
	10.0	0.236	13
	20.0	0.241	11
	30.0	0.241	11
0.05	0	0.194	28
	2.5	0.211	22
	5.0	0.224	17
	10.0	0.211	21
	20.0	0.218	19
	30.0	0.219	19
0.07	0	0.148	45
	2.5	0.178	34
	5.0	0.175	35
	10.0	0.188	30
	20.0	0.193	29
	30.0	0.208	23
0.1	0	0.118	56
	5.0	0.159	41
	10.0	0.174	37
	20.0	0.179	34
	30.0	0.180	33
0.15	0	0.087	68
	5.0	0.132	51
	10.0	0.144	47
	20.0	0.150	44
	30.0	0.150	44
0.2	0	0.066	76
	5.0	0.100	63
	10.0	0.116	57
	20.0	0.135	50
	30.0	0.130	52

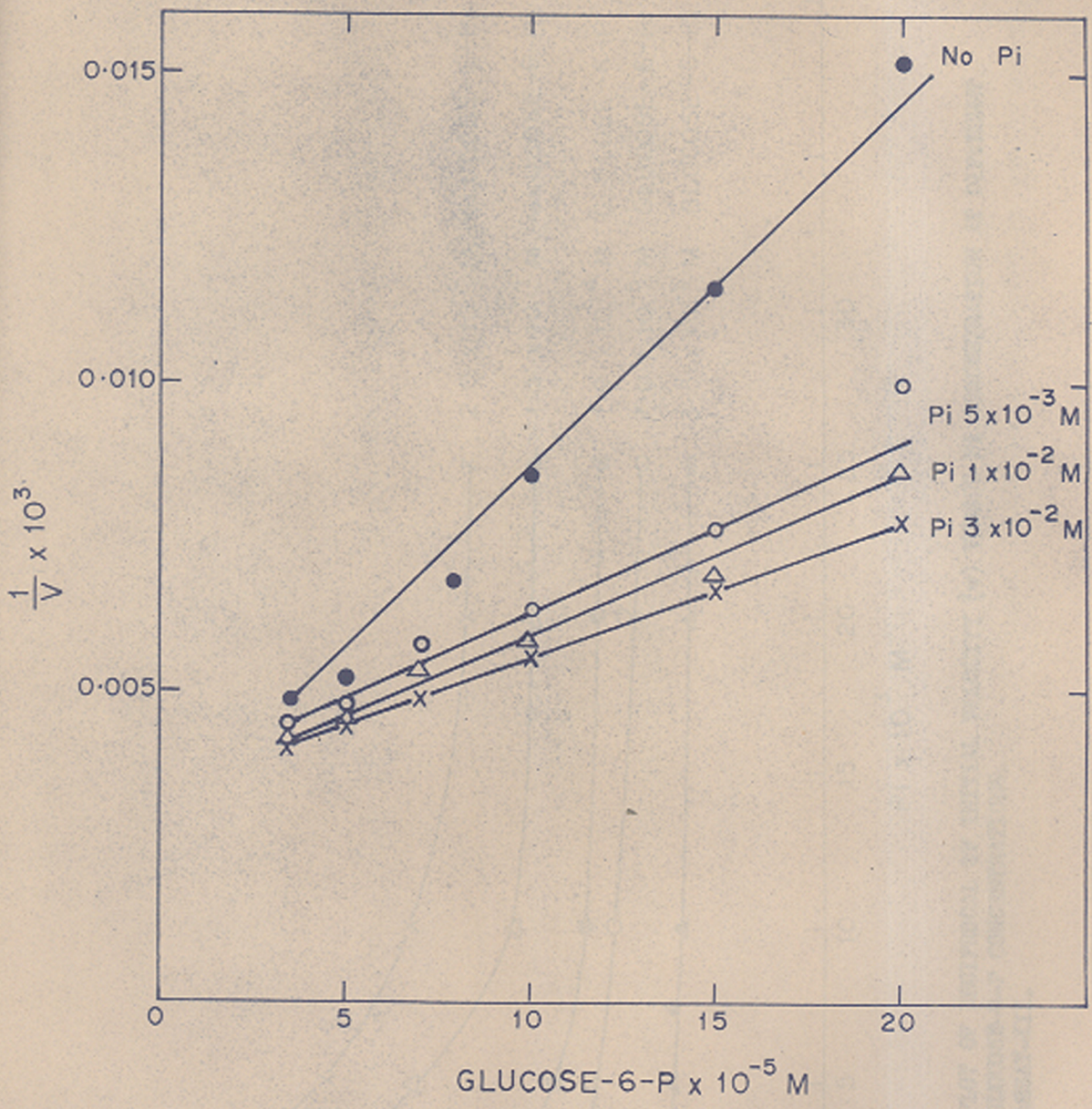


FIG. 13a. PLOT OF RECIPROCAL OF INITIAL VELOCITY (v) VERSUS GLUCOSE-6-P CONCENTRATION AT DIFFERENT Pi CONCENTRATIONS.
ASSAY-III.

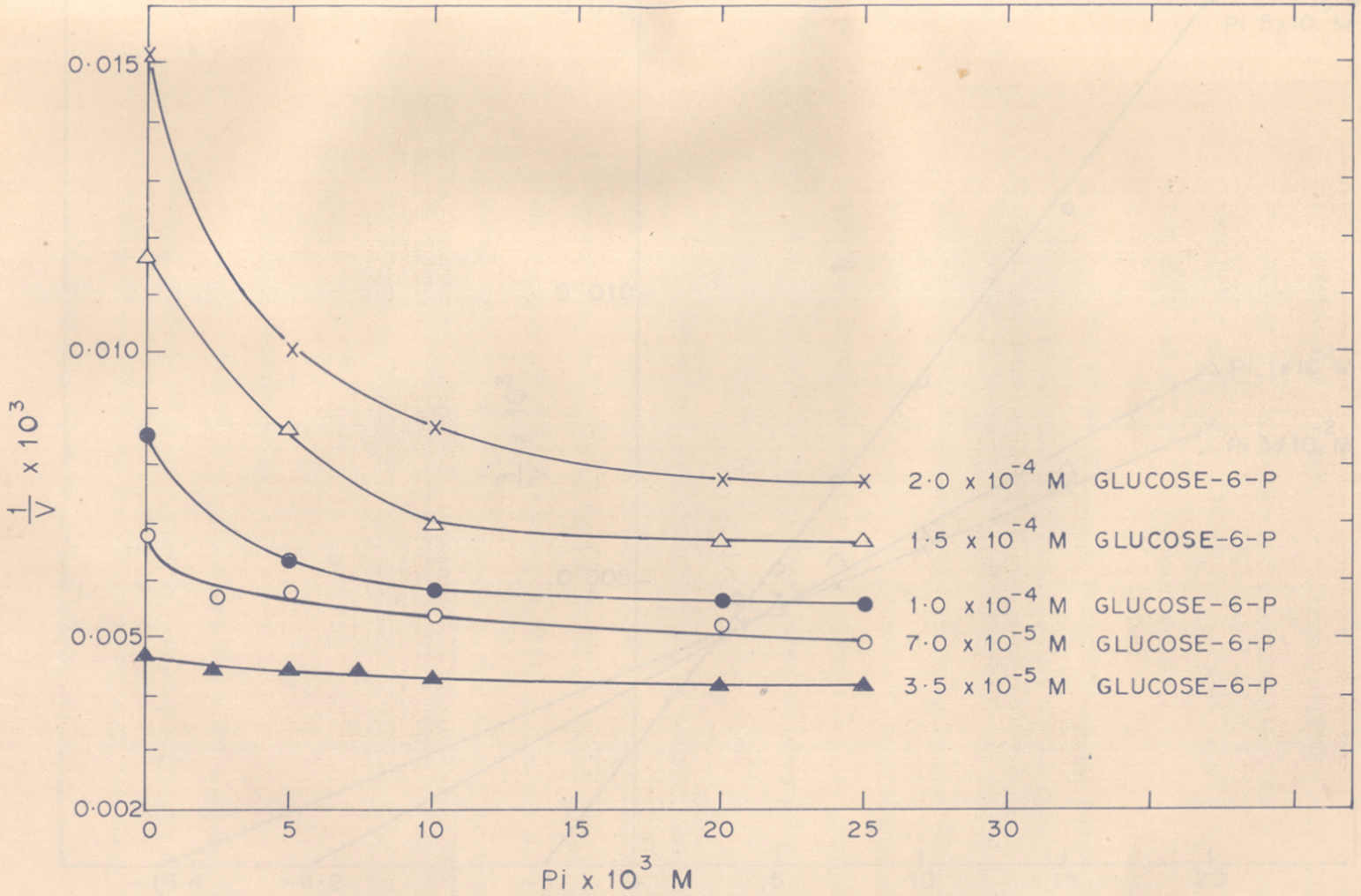


FIG. 13b. PLOT OF RECIPROCAL OF INITIAL VELOCITY (v) VERSUS Pi CONCENTRATION AT DIFFERENT GLUCOSE-6-P CONCENTRATIONS. ASSAY-III.

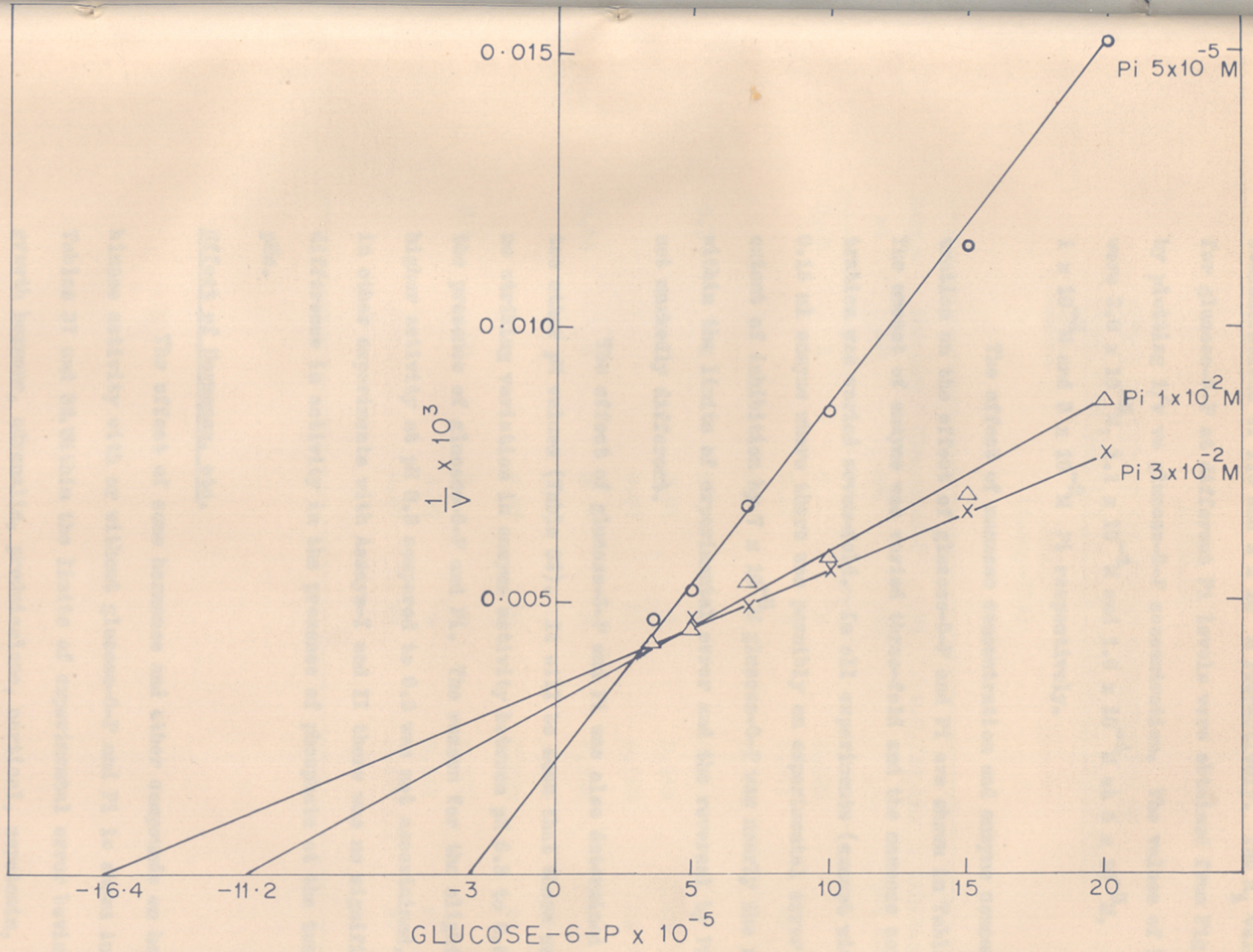


FIG. 14. PLOT OF RECIPROCAL OF INITIAL VELOCITY (v) VERSUS GLUCOSE-6-P AT DIFFERENT Pi CONCENTRATIONS. ASSAY-III.

concentrations of 1.5 and 2 x 10^{-4}M respectively. The K_i values for glucose-6-P at different Pi levels were obtained from Fig. 14 by plotting 1/v vs glucose-6-P concentration. The values of K_i were 3.0 x 10^{-5}M, 1.1 x 10^{-4}M and 1.6 x 10^{-4}M at 5 x 10^{-5}M, 1 x 10^{-2}M and 3 x 10^{-2}M Pi respectively.

The effect of mannose concentration and enzyme concentration on the effect of glucose-6-P and Pi are shown in Table 35. The amount of enzyme was varied three-fold and the mannose concentration was varied seven-fold. In all experiments (except with 0.15 ml enzyme where there was possibly an experimental error) the extent of inhibition by 7 x 10^{-5}M glucose-6-P was nearly the same within the limits of experimental error and the reversal by Pi was not markedly different.

The effect of glucose-6-P and Pi was also determined at two other pH values (Table 36). It will be seen that there was no striking variation in enzyme activity between pH 6.3 to 8.5 in the presence of glucose-6-P and Pi. The reason for the slightly higher activity at pH 8.5 compared to 6.3 was not ascertained, in other experiments with Assays-I and II there was no significant difference in activity in the presence of phosphate at the two pHs.

Effect of Hormones, etc.

The effect of some hormones and other compounds on hexokinase activity with or without glucose-6-P and Pi is shown in the Tables 37 and 38. Within the limits of experimental error bovine growth hormone, adrenalin, prednisolone, cortisol, serotonin,

TABLE 35

EFFECT OF MANNOSE AND ENZYME CONCENTRATION ON HEXOKINASE ACTIVITY IN THE PRESENCE OF GLUCOSE-6-P AND Pi

(Assay-III)

Enzyme ml	Mannose µM	Glucose-6-P mM	Pi mM	△O.D./10 min	Inhibition per cent
0.05	5	0	0	0.135	
		0.07	0	0.077	42
		0.07	10	0.103	23
0.1	5	0	0	0.270	
		0.07	0	0.148	45
		0.07	10	0.188	30
0.15	5	0	0	0.396	
		0.07	0	0.242	39
		0.07	10	0.300	24
0.1	35	0	0	0.273	
		0.07	0	0.141	47
		0.07	10	0.190	27

TABLE 36

EFFECT OF GLUCOSE-6-P AND Pi ON HEXOKINASE ACTIVITY AT DIFFERENT pH VALUES

(Assay-III)

pH	Glucose-6-P mM	Pi mM	△ O.D./10 min	Inhibition per cent
6.3	0	0	0.179	
	0.1	0	0.088	51
	0.1	20	0.128	29
7.5	0	0	0.185	
	0.1	0	0.082	56
	0.1	20	0.122	33
8.5	0	0	0.195	
	0.1	0	0.083	57
	0.1	20	0.134	31

TABLE 37

EFFECT OF HORMONES ON INHIBITION BY GLUCOSE-6-P AND REVERSAL BY Pi

(Assay-III)

Additions	Quantity	Control	+ Glucose-6-P (0.07 mM)		+Glucose-6-P (0.07 mM) + Pi (10 mM)	
		△O.D./10 min	△O.D./10 min	Inhibition	△O.D./10 min	Inhibition
	y			per cent		per cent
Enzyme		0.220	0.140	36	0.160	27
Prednisolone	1	0.210	0.130	38	0.146	30
	5	0.214	0.136	36	0.159	29
	40	0.220	0.135	39	0.162	26
Serotonin	1	0.204	0.126	38	0.154	25
	5	0.216	0.136	38	0.152	29
	45	0.220	0.122	45	0.160	27
Cortisol	1	0.210	0.134	36	0.158	25
	5	0.220	0.136	38	0.158	28
	55	0.216	0.124	42	0.160	26
Adrenalin	1	0.202	0.132	35	0.150	26
	5	0.218	0.144	32	0.160	26
	110	0.220	0.114	48	0.160	27
Bovine growth hormone	1	0.204	0.124	39	0.156	24
	5	0.220	0.134	39	0.152	31
	50	0.220	0.144	35	0.160	27
Insulin	1	0.220	0.136	38	0.159	27
	5	0.220	0.140	36	0.159	27

TABLE 38

EFFECT OF CYCLIC AMP AND GUANOSINE (2)3' MONOPHOSPHATE

(Assay-III)

Addition	Control △ O.D./10 min	+ Glucose-6-P (0.07 mM)		+ Glucose-6-P (0.07 mM) + Pi (10 mM)	
		△ O.D./ 10 min	Inhibition per cent	△ O.D./ 10 min	Inhibition per cent
Enzyme	0.320	0.192	40	0.224	30
Adenosine-3'-5'-P (0.14 µmole/ml)	0.328	0.182	40	0.243	21
Guanosine(2)3' mono-phosphate (1.5 µmole/ml)	0.315	0.190	40	0.230	27

insulin, 3',5'-adenosine monophosphate, guanosine-(2)3'-monophosphate at the concentrations stated in the tables showed no clear-cut effect on enzyme activity, on glucose-6-P inhibition or on the reversal of glucose-6-P inhibition by Pi. Variations of the order of 10 per cent in activity were not considered to be of significance.

Effect of ATP on inhibition by glucose-6-P

The effect of different concentrations of ATP on inhibition by glucose-6-P was studied by Assay-III. The results are shown in Fig.15. It will be seen that inhibition by glucose-6-P is non-competitive with respect to ATP.

Effect of ADP on hexokinase activity

The effect of ADP on the activity of hexokinase at different concentrations of ATP was studied by Assay-I. The results are shown in Fig.16. It will be seen that inhibition by ADP is non-competitive with respect to ATP. 0.02 µmole of ADP per µmole of ATP was present as impurity in the sample of ATP used.

Effect of different compounds on hexokinase activity

There was no inhibition or activation of hexokinase by the compounds listed in Table 39a at the concentrations shown in the Table. P-enolpyruvate could not also replace ATP as a phosphate donor.

Compounds which inhibited hexokinase activity are listed in Table 39b.

p-CMB inhibited 52 and 64 per cent at 1×10^{-6} M and 2×10^{-6} M respectively. Inhibition due to p-CMB (3×10^{-6} M) was prevented by the simultaneous addition of reduced glutathione (5×10^{-3} M);

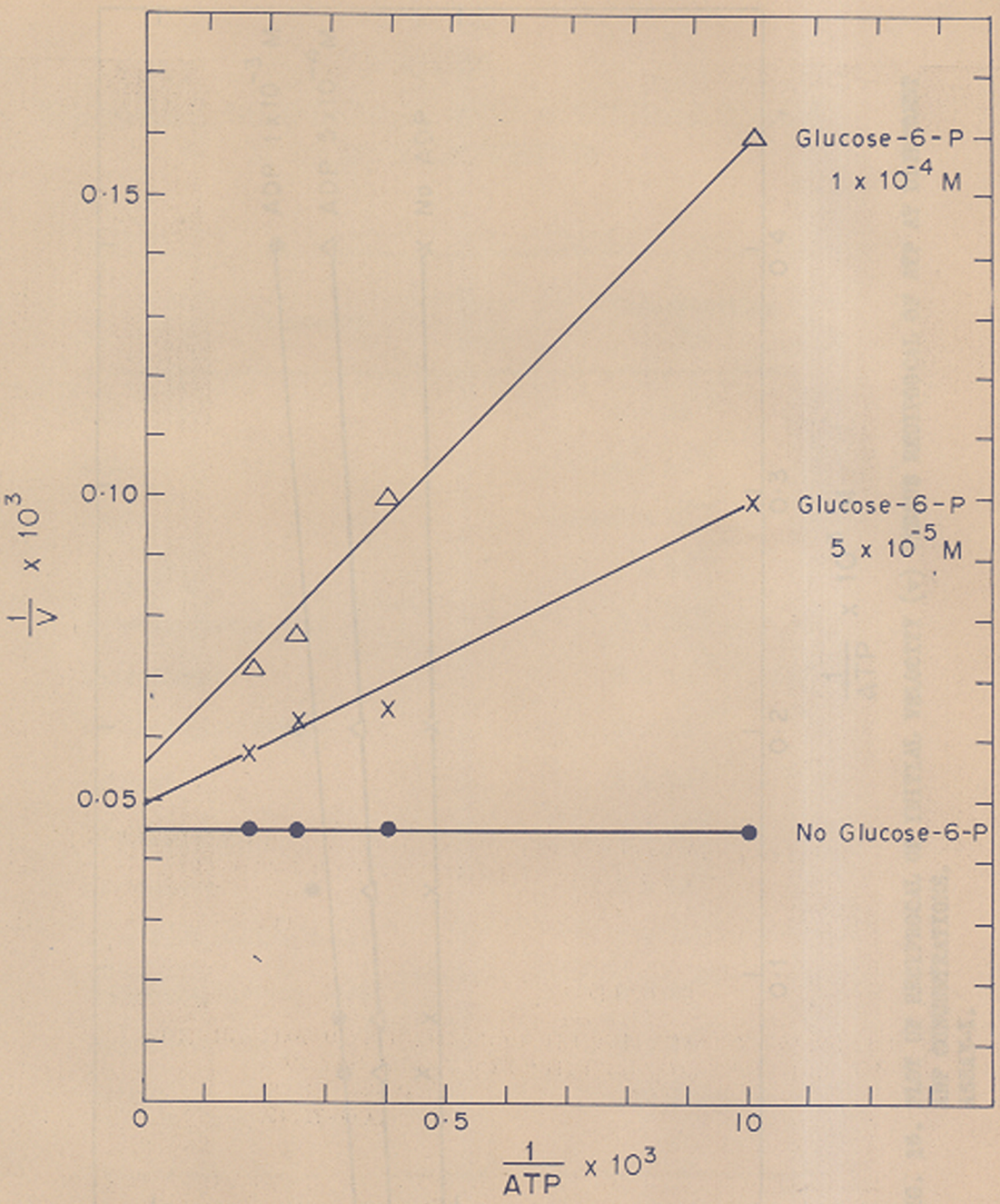


FIG. 15. PLOT OF RECIPROCAL OF INITIAL VELOCITY (v) VERSUS THE RECIPROCAL OF ATP AT DIFFERENT CONCENTRATIONS OF GLUCOSE-6-P.
ASSAY-III.

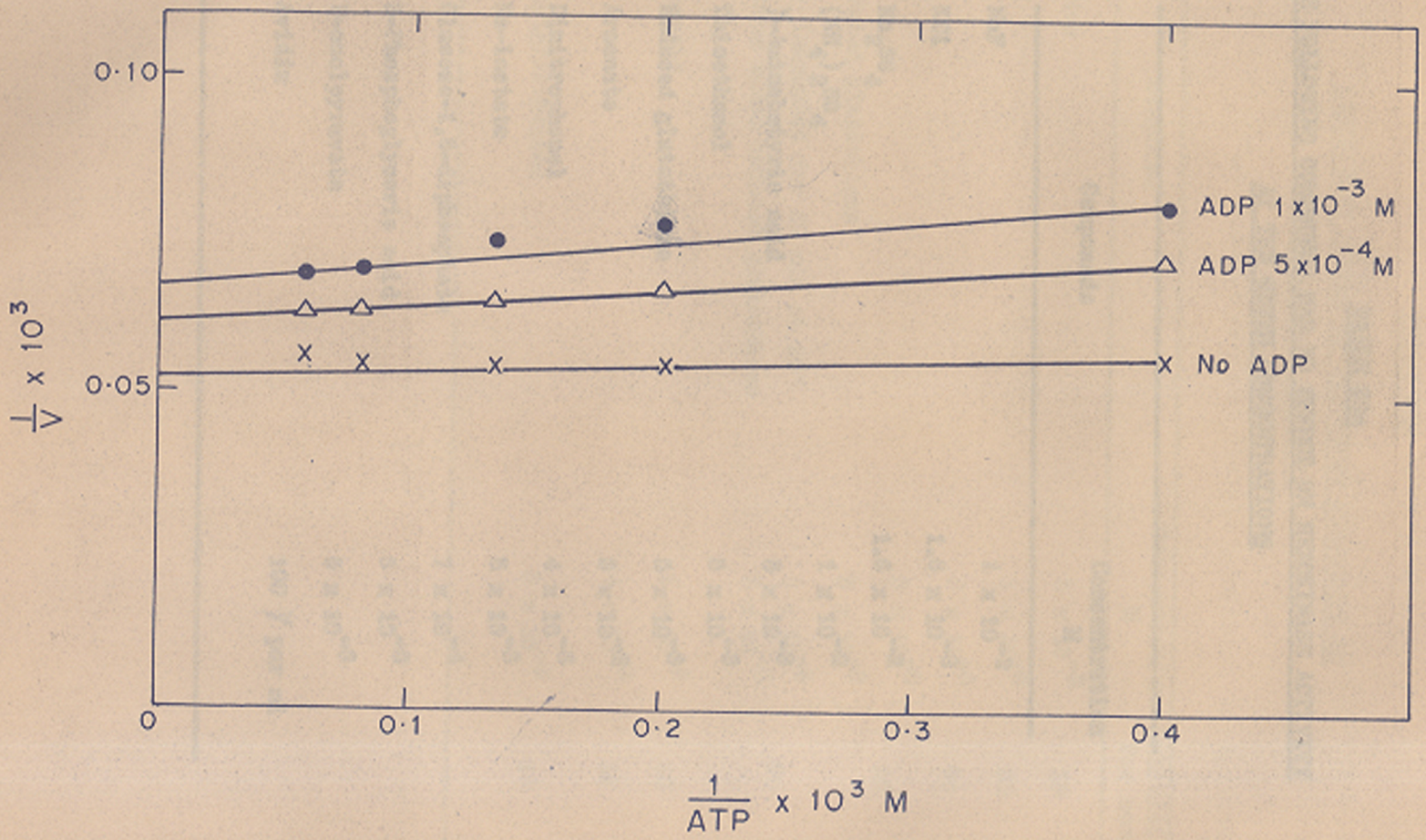


FIG. 16. PLOT OF RECIPROCAL OF INITIAL VELOCITY (v) VERSUS RECIPROCAL OF ATP AT DIFFERENT ADP CONCENTRATIONS.
ASSAY-I.

TABLE 39a

THE FOLLOWING COMPOUNDS HAD NO EFFECT ON HEXOKINASE ACTIVITY AT THE GIVEN CONCENTRATIONS

Compounds	Concentration M
NaF	1×10^{-2}
KCl	1.6×10^{-2}
Na_2SO_4	1.6×10^{-2}
$(NH_4)_2SO_4$	1×10^{-2}
γ-Aminobutyric acid	5×10^{-3}
Thioethanol	5×10^{-3}
Reduced glutathione	5×10^{-3}
Arsenate	3×10^{-2}
Dinitrophenol	4×10^{-5}
Na-lactate	5×10^{-3}
Glucose-1,6-diphosphate	7×10^{-4}
3-Phosphoglyceric acid	5×10^{-3}
P-enolpyruvate	5×10^{-3}
Avidin	100 γ per ml

TABLE 39b

INHIBITORY EFFECT OF DIFFERENT COMPOUNDS ON HEXOKINASE ACTIVITY

(Assay-I)

Compound	Concentration M	Inhibition Per cent
Cysteine	5×10^{-3}	18
p-CMB	1×10^{-6}	52
	2×10^{-6}	64
p-CMB	3×10^{-6}	10
+ Reduced glutathione	5×10^{-3}	
p-CMB; incubated for 10 min at 30°	3×10^{-6}	60
and then added reduced glutathione	5×10^{-3}	
Iodoacetate	1×10^{-3}	10
	4×10^{-3}	10
N-acetylglucosamine	5×10^{-3}	18

but it was not reversed when the enzyme was preincubated with p-CMB for 10 min at 30° and then treated with glutathione. There was only slight inhibition of the enzyme by iodoacetate even at 4×10^{-3}M.

Other substances which inhibit hexokinase are cysteine and N-acetylglucosamine.

SECTION III

Specificity of the Enzyme

1) Nucleotides

The specificity of the enzyme with respect to different nucleotides was determined with ATP, ITP, GTP and UTP. There was no activity except with ATP and ITP. The activity with ITP was about 13 per cent of that obtained with ATP. It was not determined whether ITP was completely free of ATP. There was no activity with ADP, which indicates the absence of myokinase (Table 40).

2) Metals

The enzyme was active both with Mg^{++} and Mn^{++}. There was no activity in the absence of Mg^{++} as determined by Assay-I. (Glucose-6-phosphate dehydrogenase was active in the absence of added Mg^{++}). Ca^{++} (0.011 M) could not replace Mg^{++}. There was neither activation nor inhibition by 5×10^{-3}M Ca^{++} in the presence of 2×10^{-2}M Mg^{++}. The enzyme was also active with Mn^{++} (Table 41). Higher concentration of Mn^{++} could not be used in Assay-I owing to precipitation of manganese and were not further studied. Zn^{++} and Cu^{++} (5×10^{-4}M) were also ineffective in replacing Mg^{++} (Assay-I) and were found to inhibit the enzyme 37 and 100 per cent respectively in the presence of 10^{-2}M Mg^{++}.

3) Carbohydrates

The specificity of hexokinase for carbohydrate was determined chiefly by the pyruvate kinase-lactic dehydrogenase method. The relative activities obtained with different substrates

TABLE 40

EFFECT OF NUCLEOTIDES ON HEXOKINASE ACTIVITY

(Assay-I)

Nucleotide	△ O.D./5 min	Relative rate
ATP	0.062	1.0
ITP	0.008	0.13
GTP	0	0
UTP	0	0
ADP	0	0

TABLE 41

EFFECT OF Mn^{++} ON HEXOKINASE ACTIVITY

(Assay-I)

Concentration of Mn^{++} M	0.001	0.002	0.003	0.004	0.005
△ O.D./10 min	0.033	0.040	0.085	0.094	0.130

Activity of same amount of enzyme with Mg^{++} (20 µmoles) △ O.D./10 min 0.200. The composition of the reaction mixture except Mg^{++} was as stated in Assay-I. Dilution of enzyme was made according to Table 29.

are shown in Table 42. It will be seen that the enzyme showed high activity with glucose, mannose, glucosamine and fructose. There was a small amount of activity with melibiose, mannitol, L-sorbose, galactose and appreciable activity with D-xylose, α-methylglucoside and sorbitol. There was no activity with D-arabinose, L-arabinose, L-xylose, lactose, rhamnose, trehalose, D-cellobiose, D-ribose, raffinose and dulcitol.

However it is possible that some of the carbohydrates which showed activity might have contained small amounts of glucose and other impurities such as mannose and fructose and that the observed activity was due only to these impurities. The activities with galactose, xylose, sorbitol and α-methyl glucoside were probably due only to the presence of glucose as a contaminant. This was determined as follows. The same quantities of carbohydrates as were used in the pyruvic kinase-lactic dehydrogenase assay were taken in Assay-I using glucose-6-phosphate dehydrogenase and TPN. If the observed activity in the pyruvic kinase-lactic dehydrogenase assay was due to a phosphorylation of galactose to galactose-6-P the enzyme cannot show any activity in Assay-I, since there was no galactose-6-P dehydrogenase or an enzyme which converts galactose-6-P to glucose-6-P in the purified glucose-6-phosphate dehydrogenase. Similarly none of the other carbohydrates except glucose can give activity in Assay-I. A true phosphorylation of these compounds by hexokinase would show activity in Assay-III and none in Assay-I. However it will be seen from Table 43 that galactose, D-xylose, sorbitol and α-methyl glucoside gave appreciable activities in Assay-I. Moreover, these activities are comparable to those obtained in

TABLE 42

RELATIVE ACTIVITIES OF HEXOKINASE WITH DIFFERENT SUBSTRATES
(Assay-III)

Substrate	△O.D./10 min	Relative activity
Mannose	0.212	100
Glucosamine	0.245	115
Fructose	0.380	179
D(+)-Melibiose	0.008	4
Mannitol	0.007	3
l-Sorbose	0.009	4
D(+)-Galactose	0.017	8
D(+)-Xylose	0.110	52
∝-Methylglucoside	0.190	90
Sorbitol	0.026	12
D(-)-Arabinose	-	-
Dulcitol	-	-
Lactose	-	-
L(+)-Xylose	-	-
L(+)-Arabinose	-	-
Rhamnose	-	-
Trehalose	-	-
D(+)-Cellobiose	-	-
D-Ribose	-	-
Raffinose	-	-

The composition of the reaction mixture except for substrate was as described in Assay-III. The dilution of enzyme was made as in Table 32.

TABLE 43

RELATIVE ACTIVITIES OF HEXOKINASE WITH DIFFERENT SUBSTRATES

(Assay-I)

Substrate	△ O.D./ 10 min	Relative activity
Glucose	0.234	100
D(+)-Galactose	0.014	6
D(+)-Xylose	0.079	34
Sorbitol	0.017	7
α-Methylglucoside	0.108	46
Mannitol	-	-
l-Sorbose	-	-
d(+)-Mellibiose	-	-
Fructose	-	-
Mannose	-	-

The composition of the reaction mixture except for glucose was as described for Assay-I.

The dilution of the enzyme was made as in Table 32.

the Assay-III. Hence the observed activities with these sugars are probably due to the presence of glucose as a contaminant. It is not known whether they are phosphorylated by brain hexokinase. It may however be noted that the absence of any activity with mannitol, sorbose and melibiose in the Assay-I only shows the absence of glucose as a contaminant. It does not necessarily rule out the absence of fructose or mannose as contaminants. To ascertain whether the observed activities with these compounds is due to impurities a more detailed study of their purity would be necessary. The results in this section cast doubt on some of the earlier reports on the specificity of brain hexokinase in which slight activity was reported for several carbohydrates. A detailed study of the purity of these compounds or an enzymic assay as has been reported in this section using both Assays I and III will show the presence of any sugar contaminant. The addition of phosphofructoisomerase and phosphomannoisomerase to the glucose-6-P dehydrogenase assay would make the method more sensitive and rule out the possible presence of traces of three substrates of hexokinase (glucose, fructose and mannose) in the different carbohydrates. Alternatively chromatographic analysis of the different substrates may be carried out. Unfortunately such a detailed study with different carbohydrates was not possible with the purified enzyme in the present study.

CHAPTER 5

DISCUSSION

DISCUSSION

Purification

The particulate hexokinase of ox brain was obtained in soluble form and purified till it was about 90 per cent pure as determined by ultracentrifugal studies. It is the only particulate hexokinase of animal tissues to be obtained in a stable, soluble and highly purified form. Its specific activity is also higher than that of any other hexokinase purified hitherto from animal tissues.

There have been some reports on the solubilization of animal tissue hexokinases (Moore and Strecker, 1963). But they have not been purified to any significant extent. The glucokinases of animal tissues have also not been obtained in homogeneous form. The soluble hexokinase of different animal tissues has been purified to a greater extent. The specific activities of the purified soluble enzymes of rat kidney and muscle were 6.8 and 13.3 units per mg respectively at 37° (Grossbard and Schimke, 1966) and the specific activity of the soluble enzyme purified from ascites cells by Uyeda and Racker (1965) was 9 units per mg. The maximum specific activity of the purified ox brain hexokinase obtained in the present work was 63 units per mg at 30°. Since this enzyme was about 90 per cent pure, the specific activity of homogeneous ox brain enzyme should be at least 70 units per mg.

Though the hexokinases of different animal tissues and from different sources need not have the same maximum specific activity, it is of interest to compare the specific activity of

the enzymes from brain and yeast. When tested by Assay-I, the twice recrystallized enzyme from yeast had a specific activity of about 100 units per mg at 30° for glucose. This is of the same order as that of the enzyme from ox brain.

The methods used for solubilization and stabilization of ox brain hexokinase form the basis for the present work. Some aspects of these methods are noteworthy and are discussed below.

Solubilization

This is the first report on the use of crystalline pancreatic elastase for obtaining particulate enzymes into solution. More recently ox brain acetylcholinesterase, which also occurs in particulate form has also been obtained in soluble, stable form by treatment with elastase and has been purified about a thousand-fold (Kaplay and Jagannathan, 1966 and private communication from Mr S. S. Kaplay). It is also of interest that the effect of elastase is not restricted to brain since the insoluble hexokinase of ox heart has also been obtained in soluble, stable form and partially purified in this laboratory. Since elastase has been shown to be useful for solubilizing enzymes, especially in cases where other known methods of solubilization have been ineffective, it would be of interest to explore its use for isolating other insoluble enzymes.

The yield of soluble hexokinase is high and by two treatments with elastase nearly 60 to 80 per cent of the enzyme can be obtained in soluble form. There appears also to be very little or no destruction of hexokinase on incubation with elastase.

In one experiment hexokinase from brain after being obtained in soluble form was further incubated with elastase for 24 hr. There was no appreciable loss or destruction of the hexokinase. The stability of hexokinase in the presence of elastase even in the absence of glucose, makes it possible to obtain the enzyme in good yield in the soluble state. Since elastase is highly insoluble at neutral pH, its removal, unlike that of trypsin or papain, offers no special difficulty.

It has been reported that crystalline pancreatic elastase even after recrystallization contains more than one component. One of them is an elastase while the other has an affinity to the mucoid fraction of the elastin fibre. Separation of the two components by electrophoretic or other methods gives an elastase which is no longer crystallizable (Hall, 1957). It is not known whether the preparation of the soluble enzyme from ox brain was the result of the action of elastase or of the other enzyme present in the crystalline preparation. Further studies are needed to establish which of the two enzymes is responsible for the observed effect and to elucidate the mode of action of elastase in releasing hexokinse and other enzymes from their attachment to particles.

Another feature of interest is the requirement for repeated freezing and thawing to obtain the soluble enzyme after elastase treatment. No significant solubilization could be obtained by elastase treatment or freezing and thawing alone. Freezing and thawing in addition to digestion with elastase appear to be essential for releasing the enzyme from the particles.

Whether a lipid or a lipoprotein is involved in the attachment of the hexokinase to the particles remains to be studied. A marked physical change takes place when the elastase digests are frozen and thawed and the turbid suspension becomes a highly viscous jelly-like mass. This transformation is not very noticeable when the brain particulate preparations were frozen and thawed without elastase treatment. Similar results were also obtained by freezing and thawing after elastase treatment of nuclear fractions (sedimented at 800 x g) from brain and from a mitochondrial fraction from ox heart muscle. The changes which take place during freezing and thawing after elastase treatment also remain to be clarified.

In addition to treatment with elastase and freezing and thawing, protamine sulphate treatment also appears to be essential for further purification of the enzyme. Without protamine sulphate treatment very little purification of the enzyme can be obtained by conventional methods whereas after protamine sulphate treatment the enzyme can be purified by a wide variety of methods. It may, however, be noted that in one experiment, the details of which have not been described in the experimental section, centrifugation at 100,000 x g for 2 hr after elastase treatment and freezing and thawing gave a water-clear supernatant which could be purified by calcium phosphate gel adsorption and elution. The possibility that a high molecular weight impurity is removed during protamine sulphate treatment requires consideration. Protamine sulphate also converts the highly viscous mass to a water-clear solution, which is as fluid as water. The high viscosity may be due to DNA, but this was not further studied.

After protamine sulphate treatment the hexokinase preparation which is obtained is a truly soluble enzyme capable of further purification by methods similar to those used for other soluble enzymes. These methods are conventional ones and require no special comment. The main difficulty in obtaining a highly purified enzyme was the marked instability of the enzyme as the purity of the enzyme increased.

Stability of the enzyme

It is well known that several enzymes become markedly unstable on purification and are unstable on dilution. The choice of a suitable pH, temperature, ionic strength and buffer, preservation as ammonium sulphate suspension and the use of high enzyme concentrations are of general value in the stabilization of enzymes. However these conditions vary considerably from one enzyme to another and the choice of the conditions is largely empirical. The high instability of animal tissue hexokinases is one of the main reasons why they have been difficult to purify and obtain in a homogeneous form, though the other glycolytic enzymes of animal tissues have been obtained in homogeneous form or crystallized.

The factors which influence the stability of brain hexokinase were found to be glucose or sucrose at high concentration, Pi, thioethanol and also high ammonium sulphate concentrations. At high dilutions a protective protein such as albumin and neutral pH were also essential in addition to the sulphydryl compound, sugar and Pi.

The protective action of glucose on yeast hexokinase is well known and in earlier experiments with brain hexokinase concentrations similar to those used with yeast were tried under a wide variety of conditions with other additions, but with little effect on the stability of the enzyme. The unique feature of the brain enzyme is the requirement of markedly high sucrose or glucose concentrations (i.e., 0.25 to 1 M depending on the degree of purity of the enzyme) for its stability. Glucose and sucrose were almost equally effective, whereas fructose is less effective in protecting the enzyme. Since sucrose is not a substrate for the enzyme and hexokinase activity with fructose is higher than that with glucose, there appears to be no correlation between the protective action of the sugars and their utilization as substrates by the enzyme.

It is of interest that the protective action of glucose and sucrose is not merely against progressive inactivation of the enzyme on storage or on heating. The sugar in high concentration also protects the enzyme against inactivation by solvents such as alcohol and acetone and also against inactivation at low pH. Soluble brain hexokinase was rapidly inactivated when fractionated with alcohol or acetone. This inactivation occurred both with highly purified enzyme as well as with enzyme obtained after calcium phosphate gel treatment. However, in the presence of 0.2 M or higher glucose the enzyme could be fractionated with alcohol and acetone. It is of interest that yeast hexokinase can be fractionated with alcohol only at pH 5.5, whereas it is

completely inactivated when precipitated with alcohol at neutral pH. Brain hexokinase can be fractionated both at pH 7.5 as well as 5.5 in the presence of glucose, whereas it is inactivated at either pH in the absence of sugar by alcohol. Similarly brain hexokinase is completely inactivated at pHs lower than 4.5 even at 0°, but in the presence of glucose it can be kept at a pH as low as 4.0 for 10 min at 28° without appreciable loss of activity. Further physico-chemical studies will be necessary to clarify the mode of action of the sugars and of Pi in protecting the enzyme against inactivation by heating or treatment with solvents. In the case of yeast hexokinase the work of Kenkare and Colowick shows that the enzyme dissociates into subunits at low pH and that this dissociation into subunits is reversible on neutralization.

Pi is also essential for the stability of the enzyme, though by itself it is not effective in protecting the enzyme in the absence of glucose. The effect of Pi is particularly marked at high enzyme dilutions and at pHs below 6.0. The effect of other buffers was not systematically investigated, since the main object was to determine the conditions for maximum stability of the enzyme, but Tris or acetate buffers had very little protective action.

The effect of thioethanol was not appreciable with enzyme preparations of low specific activity. In view of the reports on the effect of sulphydryl agents on particulate brain hexokinase and on purified yeast hexokinase, the effect of cysteine, glutathione and thioethanol on brain hexokinase was investigated

at different levels of purity both on its activity and its stability. No marked effect was observed in the earlier stages of purification and even with the purified enzyme there was little protection by thioethanol and phosphate in the absence of sucrose or glucose. However, enzyme preparations which contained no thioethanol progressively lost activity when stored at 0° or at -20°. No significant effect directly on enzyme activity could be shown by the addition of any of the sulphydryl compounds. The thioethanol is apparently required mainly for protecting the enzyme against inactivation and not for activation. The loss of activity in the absence of sulphydryl agents is apparently irreversible and prolonged incubation with thioethanol or cysteine did not restore the activity. It was in general noted that with increase in purity the thioethanol concentration had also to be increased. In the earlier stages 0.001 M thioethanol was used, whereas with enzyme of highest purity it was desirable to use 0.005 M to 0.015 M thioethanol for the preservation of the enzyme.

In the presence of sucrose (or glucose), thioethanol and Pi, brain hexokinase is remarkably stable and loses no activity in 5 min at 50°, whereas in the absence especially of the sugar it is rapidly inactivated within a few days at 0° or -20°.

In neutral solutions of ammonium sulphate containing glucose or sucrose, thioethanol and Pi especially when stored as a suspension in high concentrations of ammonium sulphate, brain hexokinase could be kept for several weeks at 0° without significant loss in activity. The protective action of ammonium sulphate is well known in the case of several enzymes.

The rapid inactivation of enzymes on dilution is also well known. Triosephosphate dehydrogenase, for instance, can be stored in concentrated solution at 0° without significant loss in activity for a long period. But at high dilutions it loses activity within a few minutes especially in the absence of cysteine or if EDTA was not used during the isolation of the enzyme. Even in the presence of sucrose, phosphate and thioethanol brain hexokinase was rapidly inactivated on dilution to a concentration of about 2 µg per ml or less. This inactivation occurred both at 0° and at -20° though it was more rapid at 0°. However the addition of albumin or insulin gave complete protection of the enzyme and there was no significant loss of activity in about 8 hr at 0° even in dilute solutions. There was no unique feature about the protection of the enzyme by insulin since serum albumin was equally effective. In the absence of Pi the enzyme was highly labile even when diluted with albumin, sucrose and thioethanol at a pH below 6 but at pH 7.5 the enzyme could be stabilized by adding a higher concentration of albumin. The sensitivity of the enzyme to low pH was however much less when Pi was present.

Properties

There is no evidence for any prosthetic group or activator for brain hexokinase except Mg^{++} or Mn^{++}. The ultraviolet absorption spectrum shows no indication of any nucleotide or ultraviolet absorbing compound in the enzyme. Further studies are however necessary to determine whether the purified enzyme contains any metal or other coloured substance since in concentrated solution the enzyme has a

pale brown colour. It is not known whether this colour is due to an impurity or is associated with an essential component of the enzyme. Biotin is apparently not required for enzyme activity, since avidin does not inhibit the purified enzyme.

Substrate specificity

The requirement for Mg^{++} or Mn^{++} for enzyme activity is similar to that of several other hexokinases and transphosphorylating enzymes. It was unfortunately not possible to make detailed studies on the effect of variation in the ratio of Mg^{++} to ATP on enzyme activity. Walaas and Walaas (1962) have observed activation of skeletal muscle hexokinase by Ca^{++}, Co^{++} and Zn^{++}. We have not observed any activation by Zn^{++} and Cu^{++} but Zn^{++} and Cu^{++} (5×10^{-4}M) inhibited the enzyme 37 and 100 per cent respectively.

The enzyme is specific for ATP. There was no activity with UTP or GTP but with ITP the activity was 13 per cent of that obtained with ATP. The enzyme was inactive with ADP. ADP inhibited the action of the enzyme. The specificity of the enzyme for different nucleotides is similar to that reported for particulate brain hexokinase and shows the high specificity of the enzyme for the nucleotidetriphosphate.

The specificity of the enzyme for the phosphate acceptor is also very similar to that of the insoluble enzyme. Glucose, fructose and mannose are phosphorylated and the relative rates with the three hexoses are similar to those of the insoluble brain enzyme or the crystalline yeast enzyme. As discussed in an earlier section,

detailed studies on the purity of the carbohydrates used are essential to determine the true specificity of the purified enzyme. Since only very low activities were noted with some substrates it is necessary to determine whether small amounts of glucose, fructose or mannose were present in these substrates or whether the observed rates truly represent enzyme activity with the respective substrates. In a few cases which were examined (such as galactose) small amounts of glucose were present as impurities. The use of very pure substrates is, therefore, essential with compounds which show marginal activities in order to determine the true substrate specificity of the enzyme and to establish the structural requirements in the substrate for the action of purified brain hexokinase.

Effect of Hormones

Studies on purified hexokinase showed that the activity of the enzyme was unaffected by growth hormone, adrenalin, prednisolone, cortisol, serotonin, insulin and adenosine,3'5'phosphate at the concentrations tested. These substances also showed no alteration of the inhibition of hexokinase by glucose-6-P or on the reversal of this inhibition by Pi.

A few points are however noteworthy with regard to these negative results. The studies were made with mannose as a substrate and though there is no indication that mannose behaves differently from glucose with regard to binding by the enzyme it would be desirable to study the effect of the different hormones with glucose as a substrate. Moreover, glucagon has not been studied. Secondly brain tissue is generally regarded as not particularly responsive to

insulin. Studies similar to those carried out on the effect of the hormones on brain hexokinases should be made with the enzyme from skeletal muscle, diaphragm, or adipose tissue in order to establish unequivocally whether or not these hormones have direct or indirect effect on hexokinases of hormone susceptible tissues. Thirdly it is possible that the binding of hexokinase to other cell fragments, especially to particulate elements, may be essential for demonstrating the effect of the hormones. Studies similar to those reported by Hernandez and Crane (1966) with heart will be needed to determine the mode of binding in order to determine whether a purified enzyme can be bound again to particulate elements in a reversible fashion in order to test for hormonal effects. The results presented here are essentially of a preliminary nature and serve to establish that the hormones which were studied have no effect on purified brain hexokinase.

Kinetics

Effect of temperature and pH

The increase of activity of 2.4-fold for a 10° rise in temperature between 20° and 40° is of the same order as that reported for the particulate enzyme and for yeast hexokinase. The broad pH optimum between 6 and 8 is also a feature not only of the purified enzyme but also of the hexokinase of several animal tissues.

Effect of substrate concentration

A comparison of the K_m values of the purified enzyme for glucose, ATP and Mg^{++} with those of particulate brain hexokinase and the partially purified soluble animal tissue hexokinases is of interest (see Table No. 3). The K_m value of 3.75×10^{-5} M for glucose is

comparable to those of rat brain and rat kidney enzymes and the particulate enzyme of ox brain (Fromm and Zewe,1962b). It is however markedly different from the value (8×10^{-6}M) reported by Crane and Sols for the particulate ox brain enzyme.

The K_m value for ATP for the purified enzyme (5×10^{-4}M) is similar to the values of 4.3×10^{-4}M for ascites cells, 4×10^{-4}M for rat brain (soluble enzyme), 4.4×10^{-4}M for rat kidney (soluble enzyme) and 3.4×10^{-4}M for the ox brain particulate enzyme (Fromm and Zewe) but differs from the value reported by Crane and Sols (1.3×10^{-4}M).

The K_m value for magnesium (2.8×10^{-3}M) for the purified enzyme is also higher than that reported by Crane and Sols (8×10^{-4}M) for the insoluble enzyme from brain.

With regard to glucose, ATP and Mg^{++} the K_m values for the soluble purified ox brain enzyme are similar to those of the partially purified enzymes obtained from ascites, rat brain and rat kidney and the particulate ox brain enzyme according to Fromm and Zewe. But they are markedly different from the data reported by Crane and Sols for ox brain hexokinase. The reason for this discrepancy is not known. The K_m values of the purified brain enzyme are also different from those of rat muscle and adipose tissues.

A relatively higher K_m value for fructose (1.5×10^{-3}M) compared to those for glucose and mannose (3.75×10^{-5}M and 3×10^{-5}M respectively) is a general feature of hexokinases from several tissues. Though the relative maximum rate with fructose is higher than that with glucose, at physiological concentrations of the

hexoses the phosphorylation of fructose will be much less rapid than that of glucose. It has been suggested that only the furanose form of D-fructose is acted upon by hexokinase and since only about 20 per cent of the fructose is present as fructofuranose, the K_m value for fructose is higher than that for glucose.

Inhibition by ADP

Inhibition by ADP was found to be non-competitive with respect to ATP. This confirms the results of Fromm and Zewe but differs from the conclusions of Uyeda and Racker for purified ascites enzyme and of Crane and Sols for purified particulate ox brain hexokinase.

Inhibition by glucose-6-P

The K_i value for glucose-6-P inhibition was found to be 3×10^{-5}M (at a Pi level of 5×10^{-5}M). This differs from the values reported by Crane and Sols for brain hexokinase, by Sols for the enzyme from intestinal mucosa and by McComb and Yushok for ascites cells, but is similar to that of soluble rat muscle and brain enzymes. Since the effect of Pi was not systematically investigated in the earlier work and the methods used were different, a discussion of these differences would be difficult.

Inhibition by glucose-6-P was non-competitive with respect to ATP in confirmation of the results of Crane and Sols and in contrast with the findings of Fromm and Zewe. The reason for this discrepancy, as in the case of ADP inhibition, is not clear.

Effect of Pi on glucose-6-P inhibition

Pi has no effect on hexokinase directly but purified ox brain hexokinase clearly shows reversal of glucose-6-P inhibition of Pi. These results are similar to those of Uyeda and Racker with the ox brain enzyme and ascites cells of Rose et al. (1964) with erythrocytes. As noted earlier, inhibition by glucose-6-P is not completely reversed by Pi. For instance at 5×10^{-5}M glucose-6-P the addition of Pi up to 10 mM reduces the inhibition from 28 per cent to 21 per cent. The decrease in inhibition on further increase of Pi from 10 to 30 mM is not appreciable. Similarly at the highest level of glucose-6-P tested (2×10^{-4}M) the inhibition decreases from 76 per cent to 57 per cent by increasing Pi from 0 to 10 mM. Further increase of Pi from 10 to 30 mM only causes a decrease of inhibition from 57 to 52 per cent. The reversal of inhibition is, therefore, especially marked at low Pi levels. The alteration in glucose-6-P inhibition by Pi is expressed quantitatively by the increase of K_i for glucose-6-P from 3.0×10^{-5}M at 5×10^{-5}M Pi to 1.6×10^{-4}M at 3×10^{-2}M Pi.

Inhibition by glucose-6-P and its reversal by Pi are not markedly affected by the concentration of enzyme or mannose within the limits which were investigated or by variation of pH between 6.3 to 8.5.

It may be concluded, therefore, that inhibition by glucose-6-P and its reversal by Pi is a unique feature of brain hexokinase which remains unaltered during the solubilization and purification procedures (cf. Uyeda and Racker, 1965) and is undoubtedly of importance in the regulation of glucose metabolism.

It may also be concluded that the properties of purified ox brain hexokinase (with respect to K_m values for glucose, ATP and Mg^{++}, pH optimum and variation in activity with temperature, relative rates of phosphorylation of different carbohydrates, substrate specificity for metal, nucleotide and carbohydrate, inhibition by glucose-6-P and its reversal by Pi) closely resemble those of the particulate enzyme from which it was isolated.

Inhibitors

Several compounds were tested for their effect on hexokinase and none of the salts, glycolytic intermediates and other substances listed in Table 39a showed any inhibition or activation of the enzyme. Inhibition of hexokinase by N-acetylglucosamine is similar to that reported for the enzyme from other tissues. Since there was no inhibition by thioethanol and reduced glutathione, it is uncertain whether the observed inhibition by cysteine was due to cysteine or to an impurity in cysteine.

Inhibition by p-CMB was prevented by the simultaneous addition of glutathione, but was not reversed by the latter if the enzyme was first incubated with p-CMB. These results and the lack of inhibition by iodoacetate and marked inhibition by low levels of p-CMB are similar to the observations with particulate hexokinase (Crane and Sols, 1955).

CHAPTER 6

SUMMARY AND CONCLUSIONS

SUMMARY & CONCLUSIONS

The particulate hexokinase of ox brain was obtained in soluble form. The method of solubilization consisted of digestion of the particulate enzyme by crystalline pancreatic elastase followed by repeated freezing and thawing and treatment with protamine sulphate. The soluble enzyme was not sedimented by centrifugation at 100,000 x g for one hr.

The soluble enzyme was purified by adsorption on calcium phosphate gel followed by elution with phosphate buffers and by DEAE-cellulose chromatography and ammonium sulphate fractionation. The purification was 800-fold relative to the homogenate.

The purified enzyme had a specific activity of 63 µmoles of glucose phosphorylated per min per mg protein at 30°. The enzyme was about 90 per cent pure when examined ultracentrifugally and consisted of one major slow moving component and two minor fast moving components. This enzyme is the purest hexokinase obtained hitherto from animal tissues.

The enzyme became highly unstable on purification and could not be stored even for one or two days at 0 to -20° when its specific activity was about 15 units per mg or higher. The enzyme was, however, found to be highly stable in the presence of 0.25 to 1 M sucrose or glucose - 0.005 to 0.1 M phosphate

buffer, pH 7.5, - 0.001 to 0.01 M thioethanol. In the presence of these stabilizers the enzyme could be heated for 5 min at 50° with no loss in activity. Glucose and sucrose were equally effective in stabilizing the enzyme, whereas fructose was relatively ineffective in protecting the enzyme. The enzyme was rapidly inactivated by treatment with solvents or by adjustment of the pH to 4.5 or less. In the presence of a high concentration of glucose or sucrose, the enzyme was stable even at low pH and could be fractionated with organic solvents.

The purified enzyme was also stable in ammonium sulphate (0.4 to 0.6 saturation) in the presence of sucrose, phosphate and thioethanol at 0° for several weeks. Dilute solutions required the addition of a protective protein such as serum albumin in addition to sucrose, phosphate and thioethanol. The enzyme was highly unstable in the absence of phosphate, especially at pHs below 6.

The purified enzyme showed the ultraviolet absorption of a typical protein and there was no evidence for the presence of an ultraviolet absorbing component other than protein in the enzyme. There was also no evidence of biotin requirement, since the enzyme was not inhibited by avidin.

$S_{20,w}$ of the major component of the purified enzyme, which was shown to be identical with hexokinase, was 5.90 and 5.86 S at protein concentrations of 4.3 to 6.4 mg per ml respectively.

The increase in hexokinase activity for a 10° rise between 20° to 40° was 2.43. At higher temperatures (40° and 60°) the increase in activity was less and at 65° there was a marked fall in activity. The energy of activation for the range 20° to 40° was calculated to be 12.4 kcal per mole.

The purified enzyme showed a broad pH optimum between 6 and 8.

Hexokinase required Mg^{++} for its activity. Mg^{++} could be replaced by Mn^{++} but not by Ca^{++}, Zn^{++} or Cu^{++}. The K_m value for magnesium at an ATP concentration of 10^{-2}M at pH 7.5 was 2.8×10^{-3}M.

The enzyme was active with ATP and ITP (at a rate 13 per cent of that with ATP). It showed no activity with CTP, UTP, GTP or ADP.

The K_m value of ATP was 5×10^{-4}M in the presence of 2×10^{-2}M Mg^{++} at pH 7.5.

Hexokinase phosphorylated glucose, fructose, mannose and glucosamine. The relative rates of phosphorylation of glucose, fructose and mannose were, 1:1.2:0.68. The enzyme was inactive with D- or L-ribose, L-xylose, sucrose, lactose, rhamnose, cellobiose, raffinose, trehalose. It had very low activity with a few other carbohydrates, but it is not certain whether these were genuine activities or were due to the presence of traces of impurities such as glucose, fructose and mannose.

The K_m for glucose was 3.75×10^{-5}M, for mannose 3.0×10^{-5}M and for fructose 1.5×10^{-3}M.

The enzyme was inhibited by ADP and the inhibition was non-competitive with respect to ATP.

The enzyme was inhibited by glucose-6-P. Inhibition by glucose-6-P was non-competitive with respect to ATP.

Inhibition by glucose-6-P was reversed by Pi. The reversal was partial even at low glucose-6-P concentrations over the range of Pi concentrations which was tested. The reversal of inhibition was particularly marked at low Pi concentrations. The Ki values of glucose-6-P were 3×10^{-5}M, 1.1×10^{-4}M and 1.6×10^{-4}M at Pi concentration of 5×10^{-5}M, 1×10^{-2}M and 3×10^{-2}M respectively.

Inhibition by glucose-6-P and the reversal by Pi were unaffected by variation of enzyme or mannose concentration or by variation of pH from 6.3 to 8.5.

Growth hormone, prednisolone, adrenalin, cortisol, serotonin, insulin and adenosine 3',5'-phosphate had no effect either on hexokinase activity or on the inhibition of hexokinase by glucose-6-P or on the reversal of glucose-6-P inhibition by Pi.

The enzyme was not inhibited or activated by glutathione or thioethanol. Several inorganic salts, glycolytic intermediates and other compounds which were tested had no effect on hexokinase activity.

Iodoacetate (4×10^{-3}M) showed only a slight inhibitory effect but the enzyme was inhibited 52 per cent by 10^{-6}M p-CMB. Inhibition by p-CMB could be prevented by reduced glutathione if added along with p-CMB, but enzyme which was inhibited by prior incubation with p-CMB could not be reactivated by glutathione. It was inhibited by Zn^{++}, Cu^{++} and N-acetylglucosamine.

The properties of the purified enzyme were in general similar to those of particulate brain hexokinase.

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