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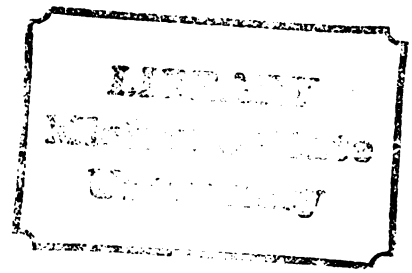

This is to certify that the

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Purification of Cytoplasmic Hexokinase from Rat Brain and Comparison with the Mitochondrial Enzyme

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PURIFICATION OF CYTOPLASMIC HEXOKINASE FROM RAT BRAIN AND COMPARISON WITH THE MITOCHONDRIAL ENZYME

By

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ABSTRACT

PURIFICATION OF CYTOPLASMIC HEXOKINASE FROM RAT BRAIN
AND COMPARISON WITH THE MITOCHONDRIAL ENZYME

By

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Hexokinase isolated from the particulate (mitochondrial) and soluble (cytoplasmic) fractions of a brain homogenate are identical in most properties, but the crude or partially purified enzymes have been reported to differ in a number of catalytic properties and in the ability to bind to mitochondria. These differences, along with an apparent lack of correlation in relative levels of soluble and particulate hexokinase in different brain regions or during development, have been interpreted as evidence for a distinct cytoplasmic form of hexokinase which is incapable of binding to mitochondria *in vivo*.

In order to resolve the nature of these differences hexokinase was purified from the soluble fraction of a rat brain homogenate and compared with the previously purified mitochondrial enzyme. It was purified by NaCl elution from DEAE-cellulose and Blue Dextran-Sepharose followed by affinity elution from the latter by glucose 6-phosphate.

The purified enzymes were compared in terms of the effect of pH and quercetin on their activity, kinetic properties (obtained from analysis of substrate depletion progress curves), and peptide maps. The crude or partially purified enzymes were examined with regard to isoelectric focusing patterns and the ability to bind to mitochondria.

Purified mitochondrial and cytoplasmic hexokinase were identical in all properties examined. Similar to the mitochondrial enzyme, freshly prepared cytoplasmic hexokinase was capable of binding extensively to mitochondria and focused as a single isoelectric form (pI = 6.35), whereas after storage the enzyme bound poorly to mitochondria and the isoelectric focusing pattern became heterogeneous. These results indicate that the observed differences are artifactual, resulting either from the impure nature of the enzymes when examined or from changes which occur in vitro.

Variations in the particulate-soluble distribution of hexokinase have been attributed to different relative levels of a non-bindable enzyme. However, most of this variation can be accounted for by differences in synaptosomal content of the homogenates rather than the amount of enzyme bound to mitochondria. The binding efficacy of the mitochondria and levels of metabolites which are known to effect hexokinase binding to mitochondria in vitro may also contribute to the observed distribution.

To Thea Davis Needels, born July 16, 1982.

Thanks for waiting.

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LIST OF ABBREVIATIONS

Abbreviations used without definition were taken from the list in the Journal of Neurochemistry Instructions to Authors - 1982. Additional abbreviations used are listed below.

BSA	bovine serum albumin
DEAE	diethylaminoethyl
Glc	glucose
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IgG	immunoglobulin G
PMSF	phenylmethylsulfonyl fluoride
SDS	sodium dodecyl sulfate

INTRODUCTION

Hexokinase catalyzes the first reaction in the glycolytic degradation of glucose, phosphorylation to produce glucose 6-phosphate. This step is a highly regulated control point for glycolysis (62,90). It has been estimated that in normally functioning brain, the glycolytic flux operates at only 3% of the maximal hexokinase rate (62). In the adult brain, which primarily uses glucose as a substrate for energy metabolism (95), net energy consumption is reflected in the rate of the hexokinase reaction.

This property has been put to advantage to estimate local neural activity in the brain (97). The glucose analog 2-deoxyglucose is transported across cell membranes and phosphorylated by hexokinase. Since the phosphorylated sugar is charged and is not further metabolized or dephosphorylated, it accumulates wherever it was phosphorylated. Autoradiography of brain sections can be used to measure the buildup of the phosphorylated sugar after systemic injection of radioactive 2-deoxyglucose. Under appropriate conditions, the amount of label in a localized brain region is proportional to the degree of glucose utilization by that region. This in turn is directly related to the functional activity of that part of the brain. The ability to estimate local neural functional activity under a variety of experimental conditions has been a boon to neuroscience, as is evidenced by the wide variety of systems to which this technique has

been applied (reviewed in 78,96). The spatial resolution of the technique has recently been increased to the cellular level (55,77).

A hallmark of hexokinase is its ability to reversibly interact with mitochondria through an integral binding protein located in the outer mitochondrial membrane (29). Several kinetic changes occur upon binding of the enzyme to mitochondria which make the enzyme more active at physiological metabolite concentrations, including a decrease in the K_m for ATP and an increase in the K_i for glucose 6-phosphate (see ref. 116). In addition, bound hexokinase may have preferred access to ATP generated within the mitochondria (52). Both glucose 6-phosphate and ATP solubilize the enzyme from mitochondria, whereas P_i inhibits the glucose 6-phosphate solubilization. All of these processes tend to favor the bound form of the enzyme under conditions (low ATP, high phosphate, low glucose 6-phosphate) in which a high glycolytic rate is desired. These observations were taken as evidence that hexokinase may be regulated by means of its distribution between the soluble and a more active mitochondrially-bound form (116). The term "ambiquitous" was subsequently coined to describe this type of regulation through changes in intracellular distribution (121).

In a brain homogenate, 15-20% of the total hexokinase is found in the soluble fraction after a classical differential centrifugation (40). The enzyme activity in this fraction is called cytoplasmic hexokinase throughout this dissertation, although presence there is not necessarily proof that an enzyme is located cytoplasmically in vivo. Electron microscopic immunolocalization of hexokinase (43) indicates that some of the enzyme is cytoplasmic rather than being associated with mitochondria, but redistribution might occur during tissue

fixation and processing. A certain amount of "mitochondrial" hexokinase should be soluble because of the equilibrium nature of the binding to mitochondria (87). The question has been raised, however, of whether most of the cytoplasmic hexokinase might differ in some way from the solubilized mitochondrial enzyme (123).

The enzymes isolated from the two locations are identical with regard to electrophoretic mobility, sedimentation and chromatographic behavior, K_m values for ATP and glucose, and antigenicity, as reviewed below. Although they appear to be the product of a single gene, a number of differences have been reported. These include differences in ability to bind to mitochondria, pH-activity profiles, inhibition characteristics, and kinetics. Variations in particulate-soluble ratios during development and between white and gray matter have also been taken as evidence for distinct mitochondrial and cytoplasmic forms of hexokinase.

Covalent modification of hexokinase to a form that does not bind to mitochondria could have important regulatory implications, especially if this modification was restricted to certain cell types (e.g. glial cells). On the other hand, the knowledge that all of the hexokinase present in a cell is participating in binding to mitochondria would simplify the interpretation of experiments designed to test the ambiquitous nature of hexokinase in vivo. In order to clarify the situation, type I cytoplasmic hexokinase was purified from rat brain and compared to the previously purified mitochondrial enzyme.

It is important to compare the purified enzymes to confirm or deny the reality of differences reported between the crude or partially purified forms, because the presence of other proteins might

specifically or nonspecifically affect the properties of the enzyme. Also, direct comparison of the proteins (e.g. by peptide mapping) is more feasible with purified proteins. The reported differences are shown to either disappear upon purification or to be related to artifactual changes that are common to both forms of the enzyme. Alternatives are proposed to explain the developmental and regional differences in particulate-soluble distribution.

The dissertation is divided into three chapters, corresponding to three general areas of research. Chapter 1 describes the purification of cytoplasmic hexokinase, as well as a number of related experiments involving chromatography of the enzyme. Chapter 2 presents the comparison between cytoplasmic and mitochondrial hexokinase. All experiments which involve the hexokinase-mitochondria binding interaction are included in Chapter 3.

LITERATURE REVIEW

Hexokinase Isoenzymes

Katzen and Schimke (44) reported that rat tissues contain varying proportions of four hexokinase isoenzymes which they termed types I-IV. The four types are separable by DEAE-cellulose chromatography or electrophoresis in starch, and each has distinctive properties which do not vary from tissue to tissue (44). Type IV hexokinase is the high K_m form of the enzyme (also called glucokinase) and is found predominantly in liver (44). The other three isoenzymes are widely distributed in various tissues. The major isoenzyme in the brain is type I, which accounts for almost all of the hexokinase activity in this organ (36,123).

Purification of Type I Hexokinase

Crane and Sols (20) purified hexokinase from brain by treating mitochondria with lipase and deoxycholate. The resulting enzyme was still particulate, but was devoid of enzymatic activities which could interfere with the assay of hexokinase. In order to further purify the enzyme, it must first be removed (solubilized) from the mitochondria. Schwartz and Basford (92) were the first to purify brain hexokinase to homogeneity, although there were earlier reports of substantial purification (39,70). Their procedure involved a rather complicated

treatment of mitochondria with deoxycholate, chymotrypsin, and Triton X-100 followed by purification of the resulting soluble enzyme.

Other treatments have also been used to solublilize hexokinase from mitochondria, including elastase with freeze/thaw cycles (41), cold acetone followed by $(NH_4)_2SO_4$ extraction (69,70), NaCl (83), Triton X-100 plus KCl (103), NaCl or ATP with and without Triton X-100 (9) and glucose 6-phosphate (12,75). Most of these procedures extract a large number of other proteins as well as hexokinase. The most specific solubilization is obtained with glucose 6-phosphate, which requires only a single DEAE-cellulose step to purify the hexokinase to homogeneity (12). The soluble (cytoplasmic) hexokinase in a brain is only a small fraction of the total activity (40) and the specific activity is quite low. Nevertheless, a number of workers have partially purified the cytoplasmic enzyme (36,42,69,103), either to avoid difficulties associated with the solubilization of mitochondrial hexokinase or in order to compare the two forms.

Type I hexokinase has been purified to near homogeneity from the brain of rat (12), rabbit (99), cow (69,82,92), and ox (41,103) as well as from other tissues including rabbit (66) and human (84) erythrocyte, pig (24) and human (72) heart, and rat kidney (127). The purification procedures rely heavily on $(NH_4)_2SO_4$ fractionation (41,66,69,72,82,84,99,103) and DEAE-cellulose chromatography (12,24,41,69,72,82,84,92,99,103,127). Other methods that have been used include chromatography on DEAE-Sephadex (66,84), phosphocellulose (24), hydroxyapatite (41,69,127), and a glucose affinity column (66,84,99,127); as well as gel filtration (24,72,84), heat treatment (69), and preparative gel electrophoresis (66,99).

The more recent purification schemes display a shift to the use of affinity methods in combination with traditional techniques. Wright et al. (127) noted that type I hexokinase from kidney was not efficiently bound to a glucose affinity column with a 6 carbon spacer arm, but did bind to a column with an 8 carbon spacer arm. Working with a 6 carbon glucose affinity column, Rijksen and Staal (84) found that type I erythrocyte hexokinase was retarded but not bound by passage through the column, whereas Magnani et al. (66) reported complete binding to the same kind of column. Type II hexokinase has been purified by a specific affinity elution from phosphocellulose with glucose 6-phosphate (81). In addition, hexokinase has been reported to bind to ADP- and ATP-Sepharose (57,108) and Cibacron Blue-Sepharose (120).

Immobilized Dye Affinity Chromatography

A number of proteins complex with Blue Dextran under low ionic strength conditions through an interaction between the Cibacron Blue F3GA chromophore and a nucleotide or other type of binding site on the protein (for references see 6). This group-specific interaction has been used to purify a wide variety of proteins (25). Stellwagen and coworkers proposed that the dinucleotide fold (a tertiary feature of the nucleotide binding sites of a number of proteins) interacts specifically with Cibacron Blue, either free in solution (106) or bound to dextran (105); and that this interaction is diagnostic for the feature. Wilson (120) found that free Cibacron Blue bound to every nucleotide-requiring enzyme that he examined, although enzymes lacking the dinucleotide fold interacted much more weakly. The coupling of dextran to the dye molecule decreased binding affinities by a factor of

10 for all enzymes containing the dinucleotide fold and effectively eliminated interaction with the others (120). Wilson concluded that the bulky dextran molecule might prevent binding of the dye to a relatively inaccessible nucleotide binding site, and that tight binding of Blue Dextran would be indicative of a freely accessible binding site (not necessarily just the dinucleotide fold). The effect of steric constraints was examined by coupling the dye tetraiodofluorescein to agarose, either directly or with 6 or 9 atom spacer arms (109). Less than 6% of the directly coupled dye is accessible to protein, and the affinity of proteins for the immobilized dye increases when spacer arms are used.

Glazer (33) found that a large number of aromatic dyes bound to hydrophobic regions of proteins that generally overlapped with substrate, coenzyme, or prosthetic group binding sites. The ability of neutral salts to elute protein from Blue Dextran Sepharose has been correlated with their ability to disrupt hydrophobic interactions (85). By comparing the binding of Cibacron Blue and some of its structural isomers to proteins, Beissner and Rudolph (5,6) concluded that the anthraquinone ring structure is required for binding, but that the aminobenzene sulfonate is not involved. The same conclusion was drawn from X-ray diffraction studies of the structure of protein-dye crystals (7). The anthraquinone ring binds in the adenine ring portion of the NAD^+ binding site of alcohol dehydrogenase, whereas the aminobenzene sulfonate ring does not interact with the nicotinamide ribose binding site. Circular dichroism measurements of Cibacron Blue binding to proteins indicate that the dye molecule is very flexible and will arrange itself to maximize favorable and minimize unfavorable

interactions (26). Cibacron Blue bound to a number of dinucleotide fold-containing enzymes shows similar but not identical circular dichroism spectra, indicating that there is no single dye conformation specific for binding to the dinucleotide fold. Overall, these and other studies indicate that Cibacron Blue binds primarily to hydrophobic pockets on proteins (which often correspond to nucleotide binding sites), but that the binding is not specific for the dinucleotide fold.

Differences in the topography of binding sites between proteins are accomodated by changes in the conformation of the dye molecule. Alterations in the dye structure might result in a molecule that is not flexible enough to fit all nucleotide sites; for instance Procion Red HE-3B shows enhanced specificity for $NADP^+$-linked over NAD^+-linked dehydrogenases (111). Clonis and Lowe (17) found that several Procion dyes are able to differentiate between the multiple binding sites of enzymes that use more than one kind of nucleotide as substrates or effectors. A number of immobilized dyes have been synthesized and tested for the ability to bind specific proteins (6,33,61,109). Several advances have been made recently in the use of immobilized dye chromatography: chromatography of proteins in the presence of nonionic detergents (86), high performance liquid affinity chromatography on silica-immobilized dyes (94), and the ability to chemically modify dyes after immobilization (15).

Cibacron Blue contains a reactive Cl group which acts as a leaving group when the dye reacts covalently (23) with a hydroxyl group (e.g. on agarose). This same reactive group is involved in the active site directed inhibition of a protein kinase by Cibacron Blue (124).

Dichloro dyes are even more effective in inhibiting nucleotide-requiring enzymes (16). These reagents should prove useful for mapping the active sites of enzymes.

Yon (128) has computer simulated affinity elution of proteins from columns to which they are bound by a combination of specific and nonspecific interactions. His model can be applied to situations ranging from pure affinity chromatography to elution by a ligand-induced perturbation of a nonspecific binding interaction (81). Any ligand that reduces the affinity of the ligand-protein complex for the adsorbant will result in desorption of the protein. Immobilized dye chromatography probably involves hydrophobic binding to both the nucleotide binding site and nonspecific regions of the protein. Normally the eluting ligand acts by competing directly at the nucleotide binding site. Alternatively, allosteric effectors or substrates may elute the protein or expediate elution by the nucleotide by indirectly altering the binding affinity. Examples of this have been reported by Tucker et al. (109).

Rat brain hexokinase has been reported to bind strongly to Cibacron Blue-Sepharose but only weakly to Blue Dextran-Sepharose, presumably because it does not possess the dinucleotide fold (120). Tetraiodofluorescein acts as a competitive (vs. ATP) inhibitor of hexokinase, apparently by binding at the ATP site (122). Affinity columns made from this (109) or any of the other dyes could be useful in the purification of hexokinase.

Comparison of the Physical Properties of Mitochondrial and Cytoplasmic Hexokinase

Grossbard and Schimke (36) partially purified and characterized hexokinase isoenzymes from the soluble fraction of a number of tissues. The four types are distinguishable in terms of their electrophoretic mobility, chromatographic properties, and K_m values for glucose and ATP, but were similar in molecular weight and pH optima. More recently, these and other properties of the soluble (cytoplasmic) and mitochondrial enzymes from the brain have been compared to determine whether or not the enzymes obtained from the two locations are a single species.

Electrophoretic mobility upon electrophoresis in starch was the original criterion for the classification of hexokinase isozymes (44). Both Wilson (115) and Ouchi et al. (74) found that mitochondrial and cytoplasmic rat brain hexokinase migrate as a single band corresponding to type I hexokinase, whereas Bigl et al. (9) found that cytoplasmic hexokinase also contained small amounts of type II and III when electrophoresed in agarose. Bachelard found type I, II and III in the cytoplasmic fraction of a guinea-pig brain, but only type I and II in the solubilized mitochondrial hexokinase (2). In all cases there is no difference in the electrophoretic mobility of the major component (type I hexokinase) obtained from the particulate or soluble fractions, although there may be some heterogeneity in the other types.

Chromatographic behavior on DEAE-cellulose has also been used in the comparison of mitochondrial and cytoplasmic hexokinase. Most workers have found that type I mitochondrial and cytoplasmic hexokinase from both bovine (103) and rat (9) brain show identical elution

patterns from DEAE-cellulose, although Moore (69) reported that the two bovine brain enzymes are eluted at different salt concentrations. The consensus seems to be that mitochondrial and cytoplasmic hexokinase have virtually identical chromatographic properties (103,123).

Grossbard and Schimke (36) reported the molecular weight of partially purified cytoplasmic hexokinase from rat brain to be 96,000 daltons using sedimentation behavior in sucrose density gradients. This agrees very well with the 98,000 value determined for the pure mitochondrial enzyme by analytical ultracentrifugation and sucrose density gradient centrifugation (12). Both mitochondrial and cytoplasmic hexokinase elute in the void volume of a Sephadex G-200 column, indicating a very large molecular weight (22). However, a number of proteins behave anomalously in the buffer system used, so that elution in the void volume is not a valid indicator of high molecular weight (119). Cytoplasmic hexokinase has also been reported to show heterogeneity and a higher molecular weight than the solubilized mitochondrial enzyme on sucrose density gradients (101). This was not reproduced by Wilson (118), who attributed the heterogeneity to aggregation of the enzyme at low ionic strength. Both cytoplasmic (118) and glucose 6-phosphate solubilized mitochondrial hexokinase (119) have been shown to sediment at a rate compatible with a molecular weight of approximately 100,000. There is no unchallenged evidence that the molecular weight of either form of the rat brain enzyme is other than this value.

Antisera raised against purified mitochondrial hexokinase has been tested for crossreactivity with the cytoplasmic enzyme (21,75,114). A line of identity is obtained upon soluble immunodiffusion analysis with

both rat brain (114) and bovine brain (21) hexokinase. Rat brain mitochondrial and cytoplasmic hexokinase are inhibited to the same extent by antiserum raised against the mitochondrial enzyme (75). These antisera do not crossreact with type II, III, or IV hexokinase (21,75).

Immediately after solubilization by glucose 6-phosphate, at least 70% of mitochondrial hexokinase will rebind to mitochondria if Mg^{+2} is added (116). In contrast, only 25-45% of the cytoplasmic enzyme will bind to exogenously added mitochondria in the presence of Mg^{+2} (47,51). If this difference pertains in vivo, it would partially explain the cytoplasmic location of a fraction of the total hexokinase.

Most of the physical characteristics which have been examined for both mitochondrial and cytoplasmic hexokinase (electrophoretic mobility, chromatographic or sedimentation behavior, and antigenicity) indicate that the two proteins are very similar, if not identical. The one major difference is in the ability of the two enzymes to bind to mitochondria.

Comparison of the Catalytic Properties of Mitochondrial and Cytoplasmic Hexokinase.

Hexokinase exhibits a pH optimum around pH 8.5 (11,36,64,69). Chou (11) found that the relative activity of crude rat brain cytoplasmic hexokinase at pH 6.5 (normalized to pH 8.5) is 30% higher than that of glucose 6-phosphate solubilized mitochondrial hexokinase. This was confirmed by Lusk et al. (64), who found 43% higher activity. The partially purified bovine cytoplasmic enzyme was 16% more active

than the pure mitochondrial form at pH 6.5 (69). Although the overall profiles are similar, the crude cytoplasmic enzyme is considerably more active than the mitochondrial enzyme at low pH values.

A number of reaction mechanisms (most recently rapid-equilibrium random) have been proposed for hexokinase based on kinetic data (3). Table 1 is a tabulation of Michaelis constants for ATP and glucose (K_m^{ATP} and K_m^{Glc}) extracted from the literature. Most are apparent K_m values determined in the presence of saturating amounts of the other substrate. All enzyme preparations were cytoplasmic or solubilized mitochondrial hexokinase except for one particulate preparation which was included for comparison (32). Most of the K_m values fall within a fairly small range except for three rather high values for K_m^{ATP} (18,22,92) and one for K_m^{Glc} (69). Most authors who directly compared the solubilized mitochondrial and cytoplasmic enzymes concluded that they had the same K_m values. In contrast, Moore (69) reported the K_m^{Glc} to be ten fold higher in the cytoplasmic enzyme. Bachelard (2) found a two fold difference in the opposite direction. On the other hand, Thompson and Bachelard (104) found no difference in the K_m^{Glc} but did find a two fold differences in K_m^{ATP} (mitochondrial enzyme higher). Overall, there are no consistent differences in the reported K_m values for cytoplasmic and solubilized mitochondrial hexokinase when measured with saturating levels of the second substrate.

When initial rates are measured with various subsaturating concentrations of the second substrate, however, the apparent K_m values remain constant for the cytoplasmic enzyme but progressively change for the mitochondrial enzyme (3,64,104). When the data are

Table 1. Literature K_m values for brain hexokinase

Reference	Species	Source	K_m (μM) ATP	K_m (μM) Glucose
Fromm and Zewe (1966)	bovine	mitochondria	292	26
Schwartz and Basford (1967)	bovine	mitochondria	4890	50
Copley and Fromm (1967)	bovine	mitochondria	1700	52
Bachelard and Goldfarb (1969)	bovine	cytoplasm	350	-
Moore (1968)	bovine	mitochondria	220	26
Moore (1968)	bovine	cytoplasm	150	267
Thompson and Bachelard (1970)	bovine	mitochondria	600	42
Thompson and Bachelard (1970)	bovine	cytoplasm	560	48
Bachelard et al. (1971)	bovine	mitochondria	550	50
Bachelard et al. (1971)	bovine	cytoplasm	400	60
Thompson and Bachelard (1977)	bovine	mitochondria	710	66
Craven and Basford (1972)	rat	mitochondria	1800	-
Grossbard and Schimke (1966)	rat	cytoplasm	400	45
Tuttle and Wilson (1970)	rat	cytoplasm	240	29
Purich and Fromm (1971)	rat	cytoplasm	510	-
Kamikashi, et al. (1974)	rat	cytoplasm	550	51
Bigl, et al. (1971)	rat	mitochondria	240	46
Bigl, et al. (1971)	rat	cytoplasm	210	32
Ouchi, et al. (1975)	rat	mitochondria	520	44
Ouchi, et al. (1975)	rat	cytoplasm	590	50
Lusk, et al. (1980)	rat	mitochondria	-	30
Lusk, et al. (1980)	rat	cytoplasm	-	56
Bachelard (1967)	guinea-pig	mitochondria	400	136
Bachelard (1967)	guinea-pig	cytoplasm	400	74

plotted in the Lineweaver-Burk format (60) with glucose as the varied substrate, the family of lines generated by carrying out assays at different fixed ATP concentrations meet on the abscissa when the cytoplasmic enzyme is assayed (3,64), but below the axis with the mitochondrial enzyme (3,64,104).

A number of agents activate or inhibit hexokinase activity. The increase in activity observed upon the treatment of particulate hexokinase with Triton X-100 has been shown to be due to the disruption of synaptosomes, vesicular structures which contain a large fraction of the hexokinase in a brain homogenate (117). Triton X-100 has been variously reported to have no effect on cytoplasmic hexokinase (74,115), to activate both cytoplasmic and solubilized mitochondrial hexokinase (102), and to activate mitochondrial but not cytoplasmic hexokinase (103). Crude cytoplasmic hexokinase is less sensitive to inhibition by quercetin than is the solubilized mitochondrial enzyme (35). Also, mitochondrial and cytoplasmic enzymes differ in their response to inhibition by p-chloromercuribenzene sulfonate; inhibition of mitochondrial hexokinase is "noncompetitive", whereas inhibition of cytoplasmic hexokinase is "competitive" (74). The p-chloromercuribenzene sulfonate reagent covalently reacts with sulfhydryl groups to inhibit hexokinase. Since the equations describing competitive and noncompetitive inhibition were derived using the assumption of reversible inhibition, the meaning of these results is unclear.

Although the catalytic properties of partially purified mitochondrial and cytoplasmic hexokinase are broadly similar, subtle differences do occur in the interaction of substrates and inhibitors

with the enzymes as well as in the effect of pH on their activity. These differences may be caused by a minor postranslational modification of one form, or they may be artifactual in nature.

Binding of Hexokinase to Mitochondria

Ascites tumor hexokinase binds to mitochondria in a reversible equilibrium which is sensitive to glucose 6-phosphate, ATP, P_i, and ionic strength (87). The situation with rat brain hexokinase appears to be the same (116). The effect of salts on the binding interaction was thoroughly investigated by Felgner and Wilson (31). The more specific solubilization of hexokinase from mitochondria by glucose 6-phosphate has been put to use in the purification of mitochondrial hexokinase (12,75).

A protein has been isolated from the outer membrane of liver mitochondria which appears to be responsible for the specific binding of hexokinase (29), even though liver mitochondria normally contain no bound hexokinase. Presumably, brain mitochondria contain a similar or identical protein. Brain mitochondria, compared to liver mitochondria, bind a greater percentage of the enzyme when hexokinase is titrated with mitochondria (30) and have more binding sites per mg protein when mitochondria are titrated with hexokinase (51). This may reflect differences in the amount or binding efficiency of the hexokinase-binding protein.

Intracellular Distribution of Hexokinase

The distribution of an enzyme after differential centrifugation has long been used to determine the intracellular distribution of that enzyme, but artifactual solubilization or adsorption during

homogenization or centrifugation can lead to misleading results. Because of the equilibrium nature of hexokinase binding, some "mitochondrial" hexokinase will actually be soluble, as will any enzyme that is permanently soluble. Most of the hexokinase in a brain homogenate is particulate (19,40), although some of the activity can only be assayed after the addition of Triton X-100 (115). Wilson (117) showed that this latency was due to the trapping of hexokinase within the vesicular structures called synaptosomes, which are pinched off nerve endings formed during homogenization. The distribution of hexokinase in brain homogenates is therefore somewhat complicated. The particulate fraction contains a mixture of mitochondrial and synaptosomal hexokinase, and the soluble fraction would be a mixture of solubilized mitochondrial and permanently soluble hexokinase (if any).

A number of workers have determined the distribution of hexokinase during development of the rat brain (45,56,65). Kellogg et al. (45) found that total particulate hexokinase levels increased four fold during the second and third week postnatally, whereas soluble levels remained virtually constant. This was interpreted as evidence for a distinct cytoplasmic form of the enzyme, because artifactual redistribution after homogenization should result in approximately parallel changes in particulate and soluble levels. MacDonald and Greengard (65) also found very large increases in particulate hexokinase during development, but the soluble enzyme also increased somewhat. They concluded that most or all of the increase in particulate hexokinase was due to enzyme trapped within synaptosomes. Clark and colleagues have measured the amount of hexokinase bound to purified mitochondria and calculated bound-soluble distributions during the development of

rat (56) and guinea pig (10) brain. They purified non-synaptosomal mitochondria and extrapolated to total mitochondria using citrate synthase recoveries, after correction for cytoplasmic inclusion using lactate dehydrogenase. Soluble hexokinase was calculated as the difference between total and mitochondrial activity. These workers found that mitochondrial hexokinase was preferentially increased during development of the rat (56), but not the guinea pig (10) brain.

Bigl et al. (8) noted that homogenates of gray matter have a much higher particulate-soluble ratio than do homogenates of white matter. This led to the suggestion (8) that a mitochondrially-bound form predominated in neurons and a cytoplasmic form in glia. Consistent with this, over 80% of the hexokinase found in cultured astrocytes is in the soluble fraction and has properties similar to those reported for cytoplasmic hexokinase in whole brain homogenates (64,88).

There is also evidence from electron microscopic immunolocalization (43) that the hexokinase mitochondrial-soluble distribution varies widely from region to region and even within various portions of a single cell (e.g. the soma, dendrites, and nerve endings of a neuron). Although the distribution is consistent for a cell type or region, there is no apparent division of cells into those containing solely mitochondrial or cytoplasmic hexokinase.

The distribution of hexokinase is altered under a number of experimental conditions. Wells and coworkers found that ischemia (47) galactose overload (47), and hyperinsulinemia (48) all cause a shift in the distribution of hexokinase toward a particulate form in chick brain, which is reversed by glucose injection in the latter two cases. On the other hand, anesthetics induce a reversible solubilization of

hexokinase (50). Ouchi et al. reported that the soluble hexokinase of rat brain increases with no concomitant decrease in the particulate form during streptozotocin-induced diabetes, and that this is rapidly reversed by glucose injection (74). This apparent activation of soluble hexokinase was not reproduced by Wilson (see 123). From these experiments it seems probable that the intracellular distribution of hexokinase is sensitive to metabolic status.

Variations in the Ability of Hexokinase to Bind to Mitochondria

After solubilization from mitochondria by glucose 6-phosphate, 70% of the hexokinase will rebind to mitochondria upon addition of Mg^{++} (116). This value increases to around 90% if additional mitochondria are added (P. Polakis, unpublished data). On the other hand, only 25-45% of the cytoplasmic hexokinase will bind to exogenous mitochondria in the presence of Mg^{++} (47,51). This has been taken as evidence that the two forms are fundamentally different (45,51).

Rose and Warms found that both a lysosomal protease contaminant of liver mitochondria and chymotrypsin will modify hexokinase so that it cannot bind to mitochondria, but with no effect on catalytic activity (87). A similar process occurs to a variable extent during the purification of mitochondrial hexokinase (30). DEAE-cellulose chromatography partially resolves the enzyme into bindable and nonbindable forms which show some variation in isoelectric focusing patterns (30). Immediately after solubilization by glucose 6-phosphate, the hexokinase exhibits a single band (pI = 6.35) upon isoelectric focusing (P. Polakis, unpublished data). With time (or during subsequent purification) a major band with pI = 6.45 and a

number of minor bands are generated in amounts that roughly correlate with the loss of bindability (P. Polakis, unpublished data). Both the shift in isoelectric focusing pattern and the loss of binding ability are retarded by the cathepsin D inhibitor, pepstatin (68), indicating that both may be protease mediated.

The apparently artifactual nature of the loss of bindability during purification raises the possibility that the observed differences between mitochondrial and cytoplasmic hexokinase may also be artifactual. Since there do appear to be endogenous proteases which can cause the changes in bindability and isoelectric focusing (P. Polakis, unpublished data), the key question is whether they act before or after homogenization.

Summary

Cytoplasmic and mitochondrial hexokinase are similar with regard to electrophoretic mobility, sedimentation and chromatographic behavior, K_m values for ATP and glucose, and antigenicity. They differ in their ability to bind to mitochondria, pH-activity profiles, inhibition characteristics, and kinetics. Variations in particulate-soluble distribution have also been taken as evidence that distinct mitochondrial and cytoplasmic forms of hexokinase exist, perhaps segregated by cell type.

In order to more directly compare the two enzymes, type I cytoplasmic hexokinase must be purified. A combination of traditional and affinity techniques can be used, including immobilized dye chromatography. The possible artifactual nature of each reported difference must be carefully evaluated.

MATERIALS AND METHODS

Materials

Glass beads (0.25-0.32 mm) were purchased from Arthur H. Thomas Co. (Philadelphia, PA), ampholine from LKB (Rockville, MD), S. aureus V8 protease from Miles Research Laboratories (Elkhart, IN), nitrocellulose and sodium dodecyl sulfate from Bio-Rad (Richmond, CA), acrylamide from Bethesda Research Laboratories (Gaithersburg, MD), triethanolamine from J.T. Baker, Co. (Philadelphia, PA), S-ethyl-trifluorothioacetate and 8-amino-octanoic acid from Aldrich Chemical Co. (Milwaukee, WI), DEAE-cellulose from Gallard-Schlesinger (Carle Place, NY), Celite from Aloe Scientific (St. Louis, MO), ATP-agarose from P-L Biochemicals (Milwaukee, WI), Blue Dextran from Pharmacia (Piscataway, NJ), and goat serum from Grand Island Biological Co. (Grand Island, NY). Cyanogen bromide-activated Sepharose and other biochemicals and reagents were obtained from Sigma (St. Louis, MO). Other chemicals were bought from standard sources.

Sprague Dawley-derived rats were bred in the departmental animal facility. Glucose 6-phosphate-solubilized mitochondrial hexokinase and the purified mitochondrial enzyme were prepared as described (12). The preparation of antiserum against pure hexokinase was described previously (114).

Chromatography Buffers

All chromatography buffers contained 50 mM HEPES, 0.5 mM EDTA, 10 mM glucose, 10 mM thioglcerol and 10% (v/v) glycerol unless otherwise noted. The HEPES buffer was added as the desiccated free acid and sodium salt in proportions calculated to give the nominal pH at 4°C using pKa = 7.77 (34). Because of oxidation upon storage, the thioglycerol was added to degassed buffer immediately before use.

Synthesis of Affinity Matrices

Blue Dextran-Sepharose was prepared as described (89) by reacting Blue Dextran with cyanogen bromide-activated Sepharose 4B. Based on the decrease in A_{610} during the reaction, 0.034 g Blue Dextran/g Sephadex was coupled. This corresponds to a dye content of 0.77 µmol Cibacron Blue/ml packed gel, using an extinction coefficient of 13.6 mM^{-1} cm^{-1} (106). A glucose affinity column was prepared by the synthesis of N(8-amino-octanoyl)2-amino-2-deoxy-D-glucose (127) which was then coupled to cyanogen bromide-activated Sepharose 4B.

Enzyme and Protein Assays

Hexokinase was assayed as previously described (12) except that the pH of the assay medium was increased to 8.5 to avoid possible inhibition by Al^{+3} contamination of commercially obtained ATP (125). Under these conditions, the addition of citrate (a chelator of Al^{+3}) has no effect on the measured hexokinase rate. Lactate dehydrogenase (49) was assayed according to a published procedure. Overt and latent enzyme activities in particulate fractions are defined as the activity observed in the absence of Triton X-100, and the

activity which is rendered assayable by presence of Triton X-100, respectively. One unit of enzyme activity will catalyze the formation of 1 μmol of product per minute under the specified conditions; both enzyme activities were measured at 25°C. Protein was measured by a modified Lowry assay that involves prior precipitation with trichloroacetic acid (76).

Purification of Cytoplasmic Hexokinase

Particulate and soluble hexokinase were separated as described by Chou and Wilson (12). The postmitochondrial supernatant from 150 g frozen rat brains (1.3 l, 250 units) was made 20 mM in HEPES, pH 7.1, 10 mM thioglycerol, 10 mM glucose, and 5 mM $MgCl_2$ and centrifuged at 100,000 x g x 30 min. The resulting supernatant was stirred for 30 min with 160 g (weight after just sucking dry on a Buchner funnel) of DEAE-cellulose equilibrated with pH 7.1 chromatography buffer, and filtered on a Buchner funnel. After washing with 250 ml pH 7.1 buffer containing 20 mM NaCl and suspending in 350 ml of the same, the suspension was packed into a 3.0 cm diameter column and washed overnight at 4 psi air pressure with 1 l buffer (final column height 30 cm). The enzyme was eluted with a 1.3 l, 20-150 mM NaCl linear gradient at 50 ml/h, collecting 14 ml fractions. Tubes containing hexokinase(units/ml)/A_{280} ratios of 0.1 or greater were pooled and concentrated to 30 ml.

After dialysis or dilution to a NaCl concentration of less than 4 mM with pH 7.5 chromatography buffer, the enzyme was ultracentrifuged (100,000 x g x 30 min) and loaded onto a 2.0 x 15 cm Blue Dextran Sepharose column equilibrated with pH 7.5 buffer. The column was

washed with 900 ml buffer and the hexokinase was eluted with a 500 ml, 0-200 mM NaCl gradient at 50 ml/h, collecting 11 ml fractions. The tubes containing hexokinase activity were pooled and the solution diluted to less than 10 mM NaCl. The enzyme was concentrated, ultracentrifuged, and reloaded onto the same column (after cleaning with 2 M NaCl/3 M urea and re-equilibrating with pH 7.5 buffer). After washing with 350 ml of buffer, the hexokinase was specifically eluted with 1 mM glucose 6-phosphate.

At this stage the hexokinase was substantially pure (90-95%, based on staining pattern in SDS gels). Occasionally a third chromatographic step on Blue Dextran Sepharose (with glucose 6-phosphate elution) or chromatography on ATP-agarose was used to further remove minor impurities. Up to 100 units of hexokinase were dialyzed against pH 8.0 chromatography buffer (minus glucose) and loaded onto a 0.8 x 1 cm ATP-agarose column. The column was washed with 30 ml buffer and the enzyme eluted with 1 mM ATP (2.1 ml fractions).

Other Purification Procedures

Reverse $(NH_4)_2SO_4$ gradient solubilization of proteins has been reported from Celite (46) and agarose (39). Crude cytoplasmic hexokinase (75 ml, 5.9 units) was stirred with 3.75 g Celite and enough $(NH_4)_2SO_4$ was slowly added to bring the final concentration to 3.5 M, maintaining the pH at 7.0. After 45 min the mixture was filtered on a fritted glass filter. The cake was resuspended in 35 ml 3.0 M $(NH_4)_2SO_4$ in 50 mM phosphate, pH 7.0, 10 mM thioglycerol, 10 mM glucose, and 0.5 mM EDTA and poured into a 2.0 cm column with 0.5 cm sand at the bottom. After packing under 0.5 psi air pressure for

1.5 h, the height was 4.5 cm. The column was washed with 3 M $(NH_4)_2SO_4$ at 16 ml/h collecting 1.2 ml fractions. After 8 fractions a 100 ml, 3-0 M $(NH_4)_2SO_4$ reverse gradient was started.

Glucose affinity chromatography was carried out according to Wright et al. (127). Approximately 2 units of partially purified cytoplasmic hexokinase in 20 mM triethanolamine, pH 7.0, 10 mM KCl, 4 mM EDTA, 7.5 mM $MgCl_2$, and 10 mM thioglycerol was loaded onto a 0.8 x 10 cm glucose-Sepharose column. After washing with buffer at 16 ml/h for 10 fractions (1.6 ml each), the hexokinase was eluted by the addition of 1 M glucose to the buffer.

Isolation of Mitochondria

Both liver and brain mitochondria were used to assess the ability of hexokinase to bind to mitochondria. Rat liver mitochondria were prepared as described previously (98). When hexokinase is incubated with mitochondria isolated in this manner in the absence of Mg^{++}, it loses the ability to bind when Mg^{++} is subsequently added because of a contaminating lysosomal protease (87). Mitochondria were partially separated from this protease by centrifugation through a 0.25 M/ 1.2 M sucrose step gradient in an SW-27 rotor at 82,500 x g x 1.5 h.

Stripped, crude, brain mitochondria were obtained as the final pellet during the preparation of glucose 6-phosphate-solubilized hexokinase (12). The mitochondria from 5 g rat brain (in 20 ml 0.25 M sucrose) were layered onto 18.5 ml of 1.2 M sucrose and centrifuged in an SW 27 rotor at 82,500 x g x 2h. The resulting pellet was

homogenized in 133 ml 2 mM glucose 6-phosphate in 0.25 M sucrose and incubated for 1 h at room temperature to further solubilize residual hexokinase. After centrifugation at 40,000 x g x 20 min the pellet was rehomogenized in a total volume of 13 ml 0.25 M sucrose, 10 mM glucose, 10 mM thioglycerol, pH 7.0. The resulting purified brain mitochondria preparation contained about 1 unit of hexokinase (1% of the enzyme present in the original homogenate) bound to an unknown proportion of the total brain mitochondria.

Hexokinase-Mitochondria Binding Assays

Purified liver mitochondria were routinely used in mitochondrial binding assays. Each 10 X 77 mm tube contained 25 munits hexokinase, about 650 µg mitochondrial protein, 4 mM $MgCl_2$, 10 mM glucose, and 0.25 M sucrose, pH 7.0 in a total volume of 0.2 ml. After incubation on ice for 10 min, the tubes were centrifuged at 40,000 x g x 10 min. The pellets were resuspended in 0.2 ml of 0.5% Triton X-100 in 10 mM glucose/0.25 M sucrose, pH 7.0 with the aid of glass beads. The percent hexokinase bound was calculated as the enzyme activity in the pellet divided by the total recovered activity.

For the titration of a brain homogenate the normal binding assay was modified to include 0.1 µM pepstatin, various amounts of liver mitochondria, and 0.20 units of hexokinase in a total volume of 0.25 ml. The percent of the hexokinase that was particulate was calculated as the difference between the soluble and total activity of the homogenate (measured in the presence of 0.5% Triton X-100).

Electrophoresis

Slab gels for SDS polyacrylamide gel electrophoresis were poured using a Bio-Rad 220 apparatus. The running buffer contained 0.1% SDS, 25 mM Tris (3.03 g/l) and 192 mM glycine (14.42 g/l), pH 8.3 (53). Both the separating gel (6.5-20% linear acrylamide gradient in 370 mM Tris, pH 8.8) and the stacking gel (5% acrylamide in 125 mM Tris, pH 6.8) were prepared from stock solution containing 35.7% acrylamide and 0.62% N,N'-methylene bis acrylamide. Samples (50 µl in 6 x 50 mm glass tubes) were denatured by placing in boiling water for 3 min in the presence of 1% SDS, 5% mercaptoethanol, and 125 mM Tris, pH 6.8. For the mapping of partial proteolytic degradation fragments (14), the samples were first boiled in the absence of mercaptoethanol. After 30 min of incubation at 37°C with an appropriate protease concentration, mercaptoethanol was added and the sample boiled again for 3 min. Nondenaturing gradient acrylamide gels were poured and run according to Lambin and Fine (54) using the 89 mM Tris (10.75 g/l), 82 mM boric acid (5.04 g/l), and 25 mM EDTA-Na_2 (0.93 g/l) buffer, pH 8.2. A 3-20% linear gradient gel (1.5 mm x 12 cm x 15 cm) was poured using a stock solution containing 28.86% acrylamide and 1.14% N,N'-methylene bisacrylamide.

Samples entered the stacking gel at 40V, and electrophoresis was carried out at 80V for 12-18 h (native gels) or until the Pyronin B tracking dye reached the bottom of the gel (about 6 hours, SDS gels). Protein bands were visualized with Coomassie Blue (0.33 mg/ml in 25% isopropanol/10% acetic acid) and destained in 10% isopropanol/10% acetic acid and then 10% acetic acid. Proteins used as molecular weight markers were cytochrome c (14,000 daltons), ovalbumin (45,000

daltons), BSA (68,000 daltons), and rat brain mitochondrial hexokinase (98,000 daltons).

Isoelectric focusing was carried out in agarose gels using an LKB 2117 Multiphor apparatus with an ampholyte range of pH 5-8. Approximately 7.5 munits of hexokinase was loaded into each lane and focused at 1000 volts for 0.5 h. The gels were stained for hexokinase activity by coupling to nitro blue tetrazolium reduction through glucose 6-phosphate dehydrogenase. The reaction medium contained all components of a normal hexokinase assay except thioglycerol, with pH adjusted to 9.5 and addition of 0.02 mg/ml phenazine methosulfate and 0.3 mg/ml nitro blue tetrazolium. Triton X-100 (1%) was added to increase the sensitivity of the staining reaction (73).

Transfer of Proteins to Nitrocellulose

Protein transfer from SDS acrylamide gels (107) was carried out for 16 h at a constant current of 400 ma (13-7 V/cm) in a cold room using a Bio-Rad Trans-Blot apparatus. The transfer buffer contained 20% methanol, 25 mM Tris, 192 mM glycine, pH 8.3. Whole sheets of nitrocellulose (15 x 9.2 cm) were stained in a 10 x 20 cm baking dish using 25 ml of antibody solutions and 75 ml for the washes. Strips cut from the nitrocellulose (1-1.5 cm wide) were stained in 13 x 100 mm test tubes with 4 ml incubations and 8 ml washes. All solutions contained 0.9% NaCl in 10 mM Tris, pH 7.5; antibody solutions also contained 3% BSA and 1% normal goat serum. Excess protein binding sites were blocked by a 1 h incubation in 3% BSA, 1% goat serum. After rinsing with saline, the replica was incubated for 2 h with a 1:75 dilution of rabbit anti-hexokinase serum and rinsed 5 times over 30 min.

At this point the replica was treated with goat anti-rabbit IgG coupled to horseradish peroxidase to detect all rabbit IgG's (107). The anti-rabbit IgG was used at a dilution of 1:750 for 1 h. After rinsing 5 times over 30 min, bands were visualized by incubation with 50 ml 0.3 mg/ml diaminobenzidine, 0.005% H_2O_2, 10 mM Tris, pH 7.4 for 30 min in the dark. The reaction was stopped by rinsing extensively with water. When desired, untreated nitrocellulose replicas were stained for protein by placing in 0.1% Napthol Blue Black in 45% methanol/10% acetic acid for 5 min and destaining in 3 changes of 90% methanol/2% acetic acid. The untransferred protein remaining in the gel was stained as usual.

Kinetic Measurement

Kinetic data were obtained by analysis of substrate depletion progress curves (129) with glucose as the varied substrate. Subsaturating ATP concentrations were maintained with a regenerating system. A Gilford 2600 microprocessor-controlled spectrophotometer was used to collect absorbance data (120 data points per run), from which substrate concentrations (129) and velocities (59) were calculated as previously described. Data were analyzed using a program written for a Hewlett-Packard 9815A calculator interfaced to the Gilford 2600. The program facilitated the input of parameters and carried out all necessary calculations, curve fitting by weighted least squares, and data plotting as in (59).

The assay mixture for kinetic experiments contained 50 mM HEPES, pH 8.5, 10 mM thioglycerol, 6.7 mM $MgCl_2$, 0.1-6.7 mM ATP, 150 μM glucose, 0.64 mM $NADP^+$, 50 mM KCl, 1 mM phosphoenolpyruvate, 2

unit/ml pyruvate kinase, and 1 unit/ml glucose 6-phosphate dehydrogenase. The amount of hexokinase added was such that the rate never exceeded 0.2 OD/min and the reaction went to completion in 7-30 minutes. To avoid pre-steady state events, data collection was started after a delay of at least 1 minute.

CHAPTER 1

PURIFICATION OF CYTOPLASMIC HEXOKINASE

The final procedure used to purify cytoplasmic hexokinase (as described in detail in Methods) involved chromatography on DEAE-cellulose, Blue Dextran-Sepharose (with NaCl and affinity elution), and sometimes ATP agarose. Besides the results of a purification, this chapter includes a number of experiments which involve the binding and elution of hexokinase from Blue Dextran-Sepharose as well as two techniques which were investigated but not incorporated into the final procedure.

RESULTS

Purification

Chromatographic profiles from a purification are presented in Figures 1-4. Hexokinase was eluted from DEAE-cellulose at 68 mM NaCl (Figure 1) which is quite similar to the elution of the mitochondrial enzyme under slightly different conditions (12). Blue Dextran-Sepharose binds a wide variety of nucleotide-requiring enzymes (25), but hexokinase has been reported to not bind well (120). However, I found that >90% of DEAE-cellulose-purified hexokinase bound and was eluted by NaCl (Figure 2). A more specific elution was obtained using 1 mM glucose 6-phosphate (Figure 3), which is a potent inhibitor of

Figure 1. DEAE-cellulose chromatography. Crude cytoplasmic hexokinase (260 units) was chromatographed on a 3.0 x 27.8 cm DEAE-cellulose column collecting 14.4 ml fractions. The activity was eluted by a NaCl gradient with the peak at 70 mM NaCl. Fractions 40-64 were pooled and contained 187 units hexokinase. Symbols: ● hexokinase, o A_{280}, Δ NaCl.

Figure 2. Blue Dextran-Sepharose chromatography, NaCl elution. DEAE-cellulose-purified cytoplasmic hexokinase (157 units) was chromatographed on a 2.0 x 15.2 cm Blue Dextran-Sepharose column, collecting 10.2 ml fractions. The breakthrough fractions (2-10) contained 18 units of hexokinase. The remainder of the activity was eluted by a NaCl gradient with the peak at 29 mM NaCl. Fractions 84-93 were pooled and contained 105 units hexokinase. Symbols: o hexokinase, o A_{280}, Δ NaCl.

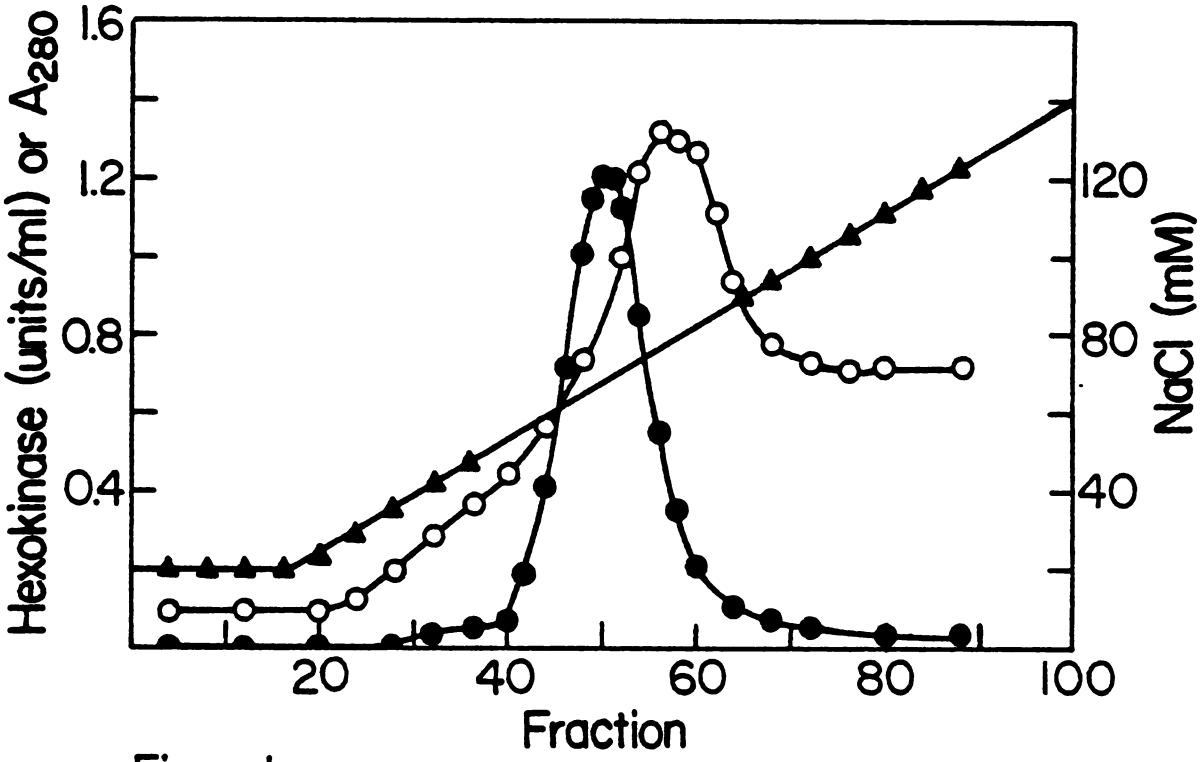

Figure 1

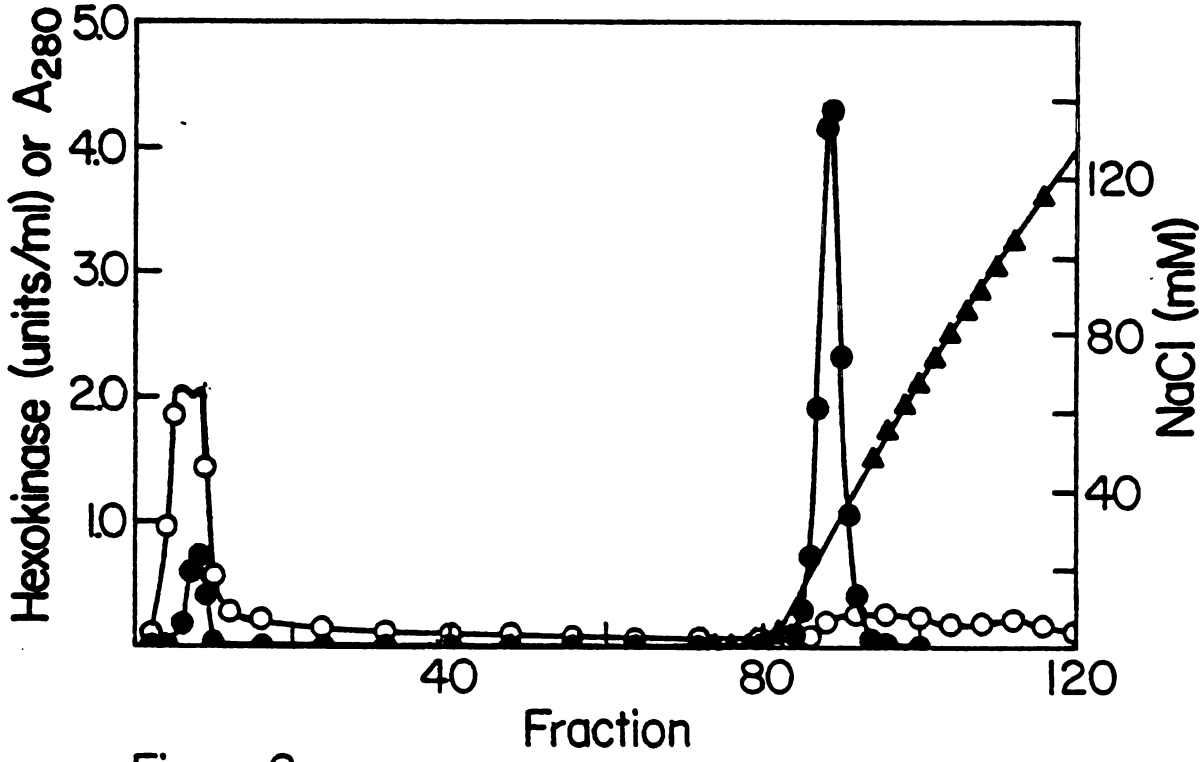

Figure 2

Figure 3. Blue Dextran-Sepharose chromatography, glucose 6-phosphate elution. After elution from Blue Dextran-Sepharose by NaCl, cytoplasmic hexokinase (132 units) was rechromatographed on a 2.0 x 15.2 cm Blue Dextran-Sepharose column, collecting 10.5 ml fractions. The addition of 1 mM glucose 6-phosphate to the buffer (at the arrow) resulted in the elution of 97 units in fractions 36-54. Symbols: • hexokinase, o A_{280}

Figure 4. ATP-agarose chromatography. Purified (>90% pure) cytoplasmic hexokinase (78 units) was chromatographed on a 0.8 x 11 cm ATP-agarose column, collecting 2.1 ml fractions. The breakthrough fractions (2-4) contained 3.6 units. The addition of 1 mM ATP (at the arrow) resulted in the elution of 47 units in fractions 15-19.

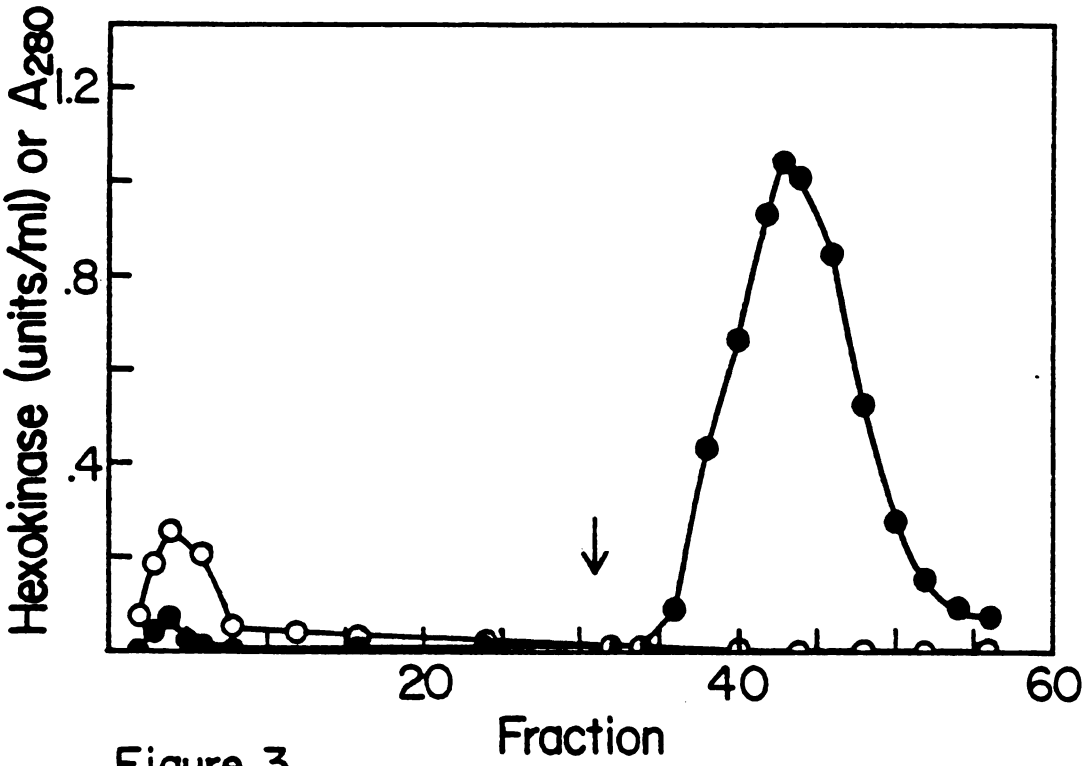

Figure 3

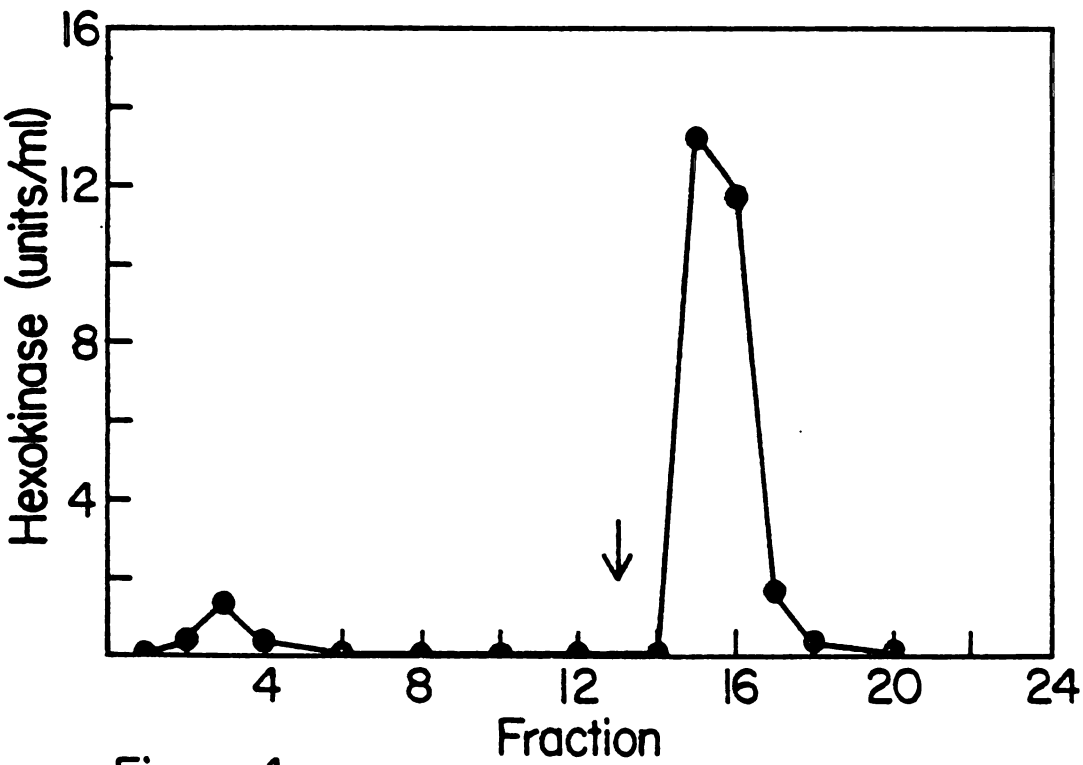

Figure 4

Table 2. Purification of cytoplasmic hexokinase

Fraction	Volume (ml)	Protein (mg)	Activity (units)	% Recovery	Specific activity (units/mg)
Crude	1235	4710	242	(100)	0.051
DEAE-cellulose eluate	330	446	174	72	0.390
Blue Dextran-Sepharose/ NaCl eluate	22.5	18.2	144	60	7.90
Blue Dextran-Sepharose/ glucose 6-phosphate eluate	10.2	1.81	77.8	32	43.1
ATP-agarose eluate	5.1	1.01	49.9	21	49.4

Figure 5. Purification of cytoplasmic hexokinase. Hexokinase from each stage of the purification (see Table 2) was subjected to SDS electrophoresis in a 6.5-20% gradient gel. A. Crude cytoplasmic hexokinase (46 μg). B. After DEAE-cellulose chromatography (50 μg). C. After NaCl elution from Blue Dextran-Sepharose (16 μg). D. After glucose 6-phosphate elution from Blue Dextran-Sepharose (6 μg). E. After ATP-agarose chromatography (5 μg). Arrows mark the position of standards with the indicated molecular weight in daltons.

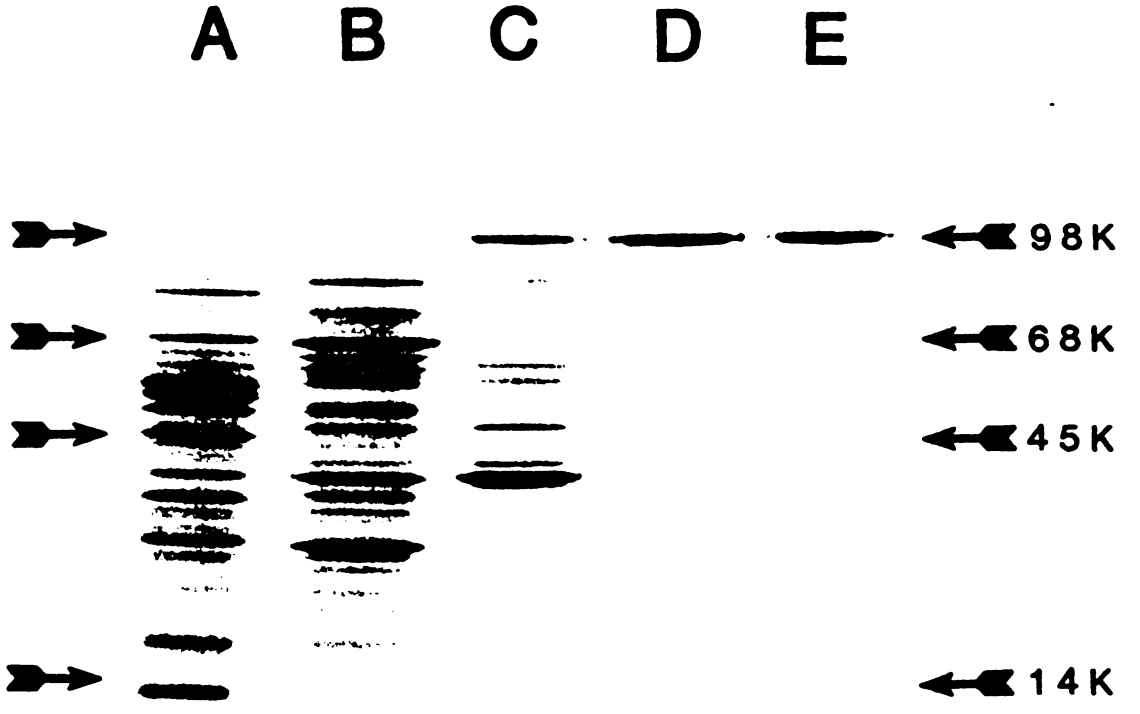

Figure 5

hexokinase with respect to ATP (20,32). Either glucose 6-phosphate or ATP could be used to remove hexokinase from the ATP affinity column, but ATP resulted in a sharper elution. When glucose was included in the chromatography buffer the ability of ATP-agarose to bind hexokinase was rapidly decreased, presumably due to phosphorylation of the glucose by the matrix-bound ATP. Even in the absence of glucose, the ATP-agarose slowly lost its capacity to bind hexokinase.

The results of a purification are summarized in Table 2 and Figure 5. The specific activity of the cytoplasmic enzyme was comparable to that of mitochondrial hexokinase using the same protein assay (40-50 units/mg). Close examination of gels revealed that the minor impurities present in mitochondrial and cytoplasmic hexokinase showed a very similar pattern of molecular weights, even though the enzymes were purified from different subcellular fractions by different procedures. This suggests that the impurities may in fact be proteolytic degradation products of hexokinase. Further evidence bearing on this point is presented in Chapter 2.

Blue Dextran-Sepharose Chromatography

Since hexokinase had been reported to bind poorly to Blue Dextran-Sepharose (120), this affinity matrix was originally used in an attempt to selectively remove protein impurities with a high affinity for the matrix before purification of hexokinase on a Cibacron Blue-Sepharose column. The first time hexokinase was applied to a newly synthesized Blue Dextran-Sepharose column, the enzyme was retarded, but not bound (Figure 6A). This resulted in a substantial

Figure 6. Anomalous chromatography of hexokinase on Blue Dextran-Sepharose. Cytoplasmic hexokinase (after DEAE-cellulose chromatography) was chromatographed on Blue Dextran-Sepharose equilibrated with 10 mM phosphate buffer, pH 7.0. At the arrow, 1 M NaCl was added to the buffer to elute bound proteins. A. First use. Hexokinase (42 units) was chromatographed on a 1.2 x 10.8 cm column, collecting 5.3 ml fractions. Fractions 7-17 and 18-21 contained 31 and 4.9 units hexokinase respectively. B. Subsequent use. Hexokinase (1.9 units) was chromatographed on a 0.5 x 5.1 cm column, collecting 0.3 ml fractions. Symbols: ● hexokinase, o A_{280}.

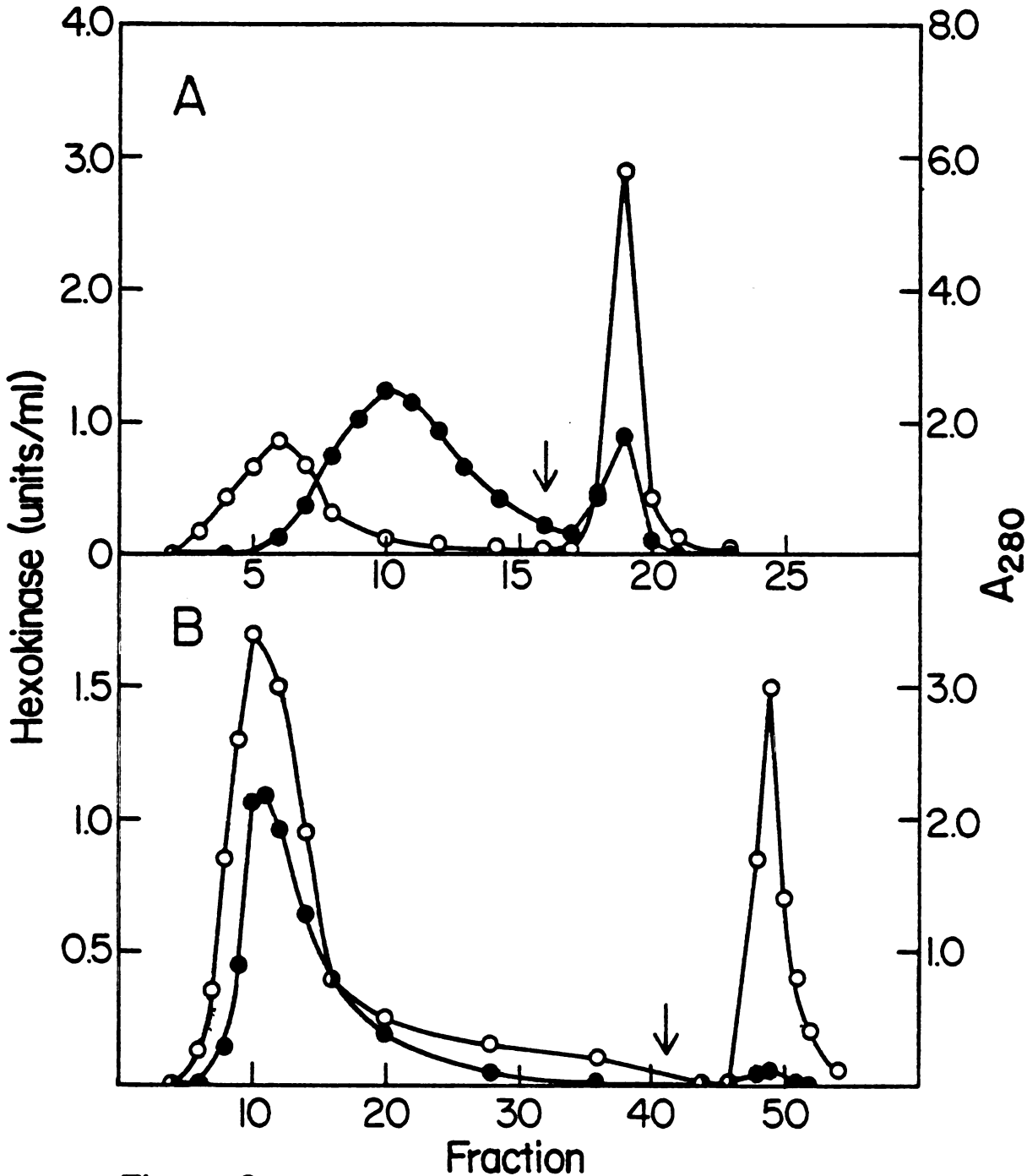
A
B
Hexokinase (units/ml)
A_{280}
Fraction
4.0
3.0
2.0
1.0
0
8.0
6.0
4.0
2.0
5
10
15
20
25
1.5
1.0
0.5
3.0
2.0
1.0
10
20
30
40
50

Figure 6

purification, but was never observed again. Normally the ionic strength of 10 mM phosphate was sufficient to prevent even retardation of the enzyme by the column (Figure 6B).

While investigating this effect, I noticed that 90-98% of the hexokinase loaded onto a Blue Dextran-Sepharose column at low ionic strength bound, and was eluted by NaCl (Figure 7A). When rechromatographed on a smaller column, the enzyme that did not bind to the first column was also not bound by the second column (Figure 7B) whereas the eluted enzyme did bind (Figure 7C). Similar results were obtained with chromatography on ATP-agarose (not shown), indicating that some sort of permanent change occured which prevented hexokinase from being able to bind ATP or Blue Dextran. These observations may explain the earlier report (120) that only 55-60% of the hexokinase loaded onto a Blue Dextran-Sepharose column was bound.

Glucose appears to slow down the change since when hexokinase was chromatographed in the absence of glucose the enzyme slowly leaked off of the column for the duration of the wash. Hexokinase was stored for 1 or 8 days with and without glucose and then chromatographed (with or without glucose) on Blue Dextran-Sepharose (Table 3). The absence of glucose resulted in a larger fraction of the loaded hexokinase washing off the column, especially in the later fractions. The total recovery of the enzyme was also considerably lower.

The activity of the non-binding hexokinase assayed at 0.5 mM ATP was only 37% relative to the activity at 6.6 mM ATP, compared to 73% for the enzyme which bound to Blue Dextran-Sepharose. Apparently, the K_m values for Blue Dextran-Sepharose and ATP are higher for the non-binding hexokinase. Kinetic measurements on the non-binding enzyme

Figure 7. Rechromatography of hexokinase on Blue Dextran-Sepharose. Cytoplasmic hexokinase (after DEAE-cellulose chromatography) was chromatographed and rechromatographed on Blue Dextran-Sepharose equilibrated with pH 7.5 column buffer. A. Chromatography. Hexokinase (78 units) was loaded onto a 2.0 x 15.2 cm column (collecting 10.7 ml fractions) and eluted by addition of 2 mM glucose 6-phosphate at the arrow. B. Rechromatography of breakthrough enzyme. After concentration, 3.1 units of fractions 5-14 was loaded onto a 0.55 x 7 cm column and washed with 12.3 ml buffer (W). The enzyme was eluted with 12.3 ml 1 M NaCl (E). C. Rechromatography of eluted enzyme. After dialysis, 3.6 units of fractions 65-72 was loaded, washed, and eluted as in (B).

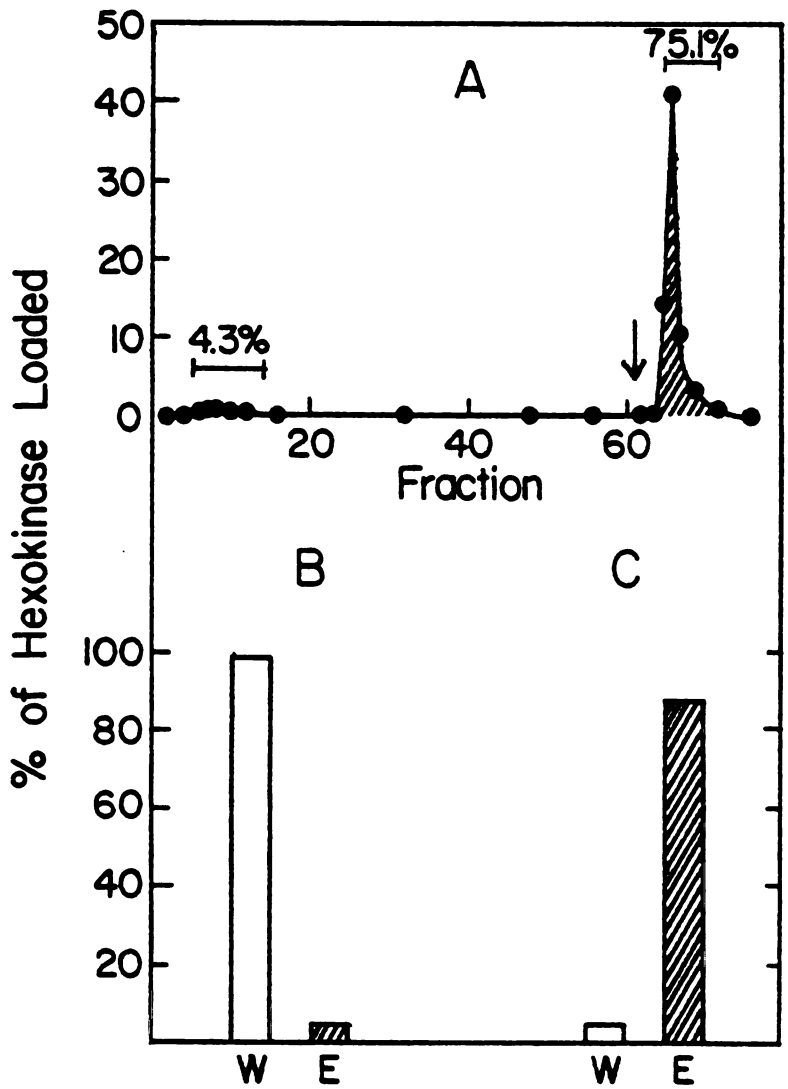
50
40
30
20
10
0
A
75.1%
4.3%
20
40
60
Fraction
% of Hexokinase Loaded
B
C
100
80
60
40
20
W
E
W
E

Figure 7

Table 3. Effect of glucose on Blue Dextran-Sepharose chromatography

	% of hexokinase loaded			
	10 mM glucose		0 mM glucose	
Fraction	Day 1	Day 8	Day 1	Day 8
W-1	4.5	7.7	5.7	11.1
W-2	0.7	0.6	2.2	4.7
W-3	0.4	0.6	2.0	2.7
E-1	86.3	84.6	63.6	60.1
E-2	0.7	3.4	4.8	5.1
E-3	0.1	0.5	0.2	0.2

Cytoplasmic hexokinase was purified by DEAE-cellulose chromatography and glucose 6-phosphate elution from Blue Dextran-Sepharose, then dialyzed against pH 7.5 buffer containing either 0 or 10 mM glucose. After 1 or 8 days, 3.6 units was loaded onto each of several 0.55 x 7 cm Blue Dextran-Sepharose columns (equilibrated with buffer containing 0 or 10 mM glucose) and eluted with 1 M NaCl. Wash (W) and elution (E) fractions (4.1 ml each) were collected and assayed for hexokinase.

using the substrate depletion method (detailed in Methods and Chapter 2) revealed a pronounced curvature in Lineweaver-Burk plots with glucose as the varied substrate (data not shown). The affinity for ATP appeared to be lower, although exact determination of the K_m was impossible because of the curvature. Storage of pure mitochondrial hexokinase for 2 days with and without glucose and thioglycerol produced a similar pattern (Figure 8). The curvature was most pronounced when both glucose and thioglycerol were missing, but also occured in the presence of glucose without thioglycerol.

The specificity of the elution of hexokinase from Blue Dextran-Sepharose by glucose 6-phosphate was tested (Figure 9). Glucose 6-phosphate, which is a potent inhibitor (vs. ATP) of hexokinase (20), resulted in sharp elution of hexokinase when present at a concentration 1 mM (Figure 9A). Galactose 6-phosphate, which is not an effective inhibitor (20), resulted in a much broader elution, even when present at 2 mM (Figure 9B).

Other Purification Techniques

Precipitation of hexokinase by $(NH_4)_2SO_4$ occured over an unusually wide range of salt concentrations; only 60% of the activity was precipitated between 1.0 M and 2.2 M $(NH_4)_2\ SO_4$. In order to increase the resolution of the technique, hexokinase which had been precipitated onto Celite by $(NH_4)_2SO_4$ was solubilized by a reverse $(NH_4)_2SO_4$ gradient (Figure 10), as described with other proteins (46). Two peaks of hexokinase activity were resolved, which explains the wide range of $(NH_4)_2SO_4$ concentrations required to fractionate the enzyme. Very little purification was effected by this

Figure 8. Effect of glucose and thioglycerol on kinetic pattern. Pure mitochondrial hexokinase was stored for 2 days with or without 100 mM glucose or thioglycerol. Lineweaver-Burk plots were prepared from substrate depletion kinetic data. Glucose concentrations ranged from 5-100 μM. Each line represents data collected at a different constant ATP concentration (0.15, 0.20, 0.33, 0.67, and 2.00 mM). A. With glucose, with thioglycerol. B. Without glucose, with thioglycerol. C. With glucose, without thioglycerol. D. Without glucose, without thioglycerol.

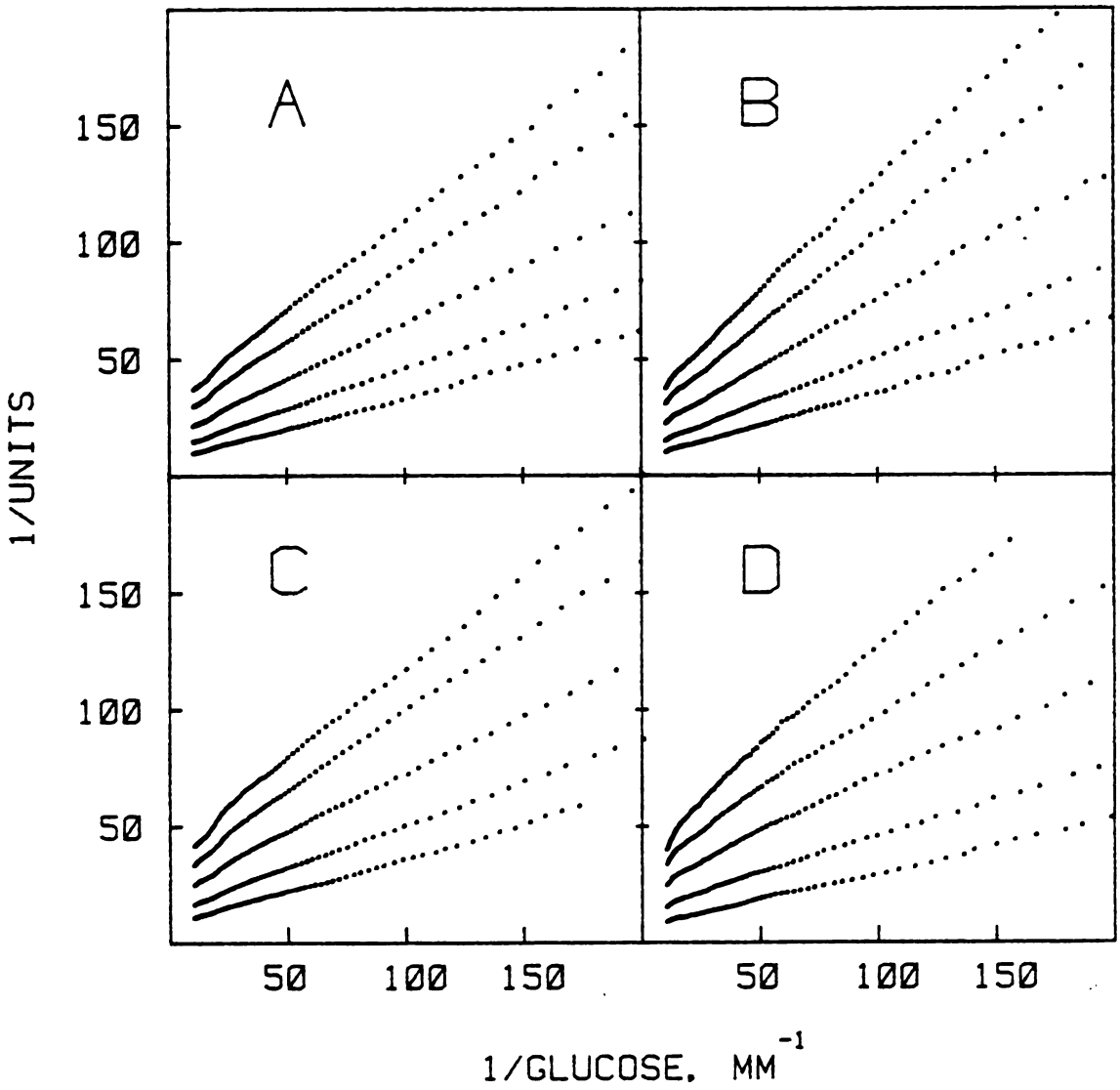
A
B
C
D
150
100
50
150
100
50
50
100
150
50
100
150
1/UNITS
1/GLUCOSE, MM^{-1}

FIGURE 8

Figure 9. Glucose 6-phosphate vs. galactose 6-phosphate elution from Blue Dextran-Sepharose. DEAE-cellulose-purified cytoplasmic hexokinase (2.6 units) was loaded onto a 0.5 x 7.6 cm Blue Dextran-Sepharose column equilibrated in pH 7.5 column buffer (collecting 0.8 ml fractions). A. Glucose 6-phosphate elution. At the arrow 1 mM glucose 6-phosphate was added to the buffer. B. Galactose 6-phosphate elution. At the first arrow 2 mM galactose 6-phosphate was added to the buffer and was replaced with 2 mM glucose 6-phosphate at the second arrow.

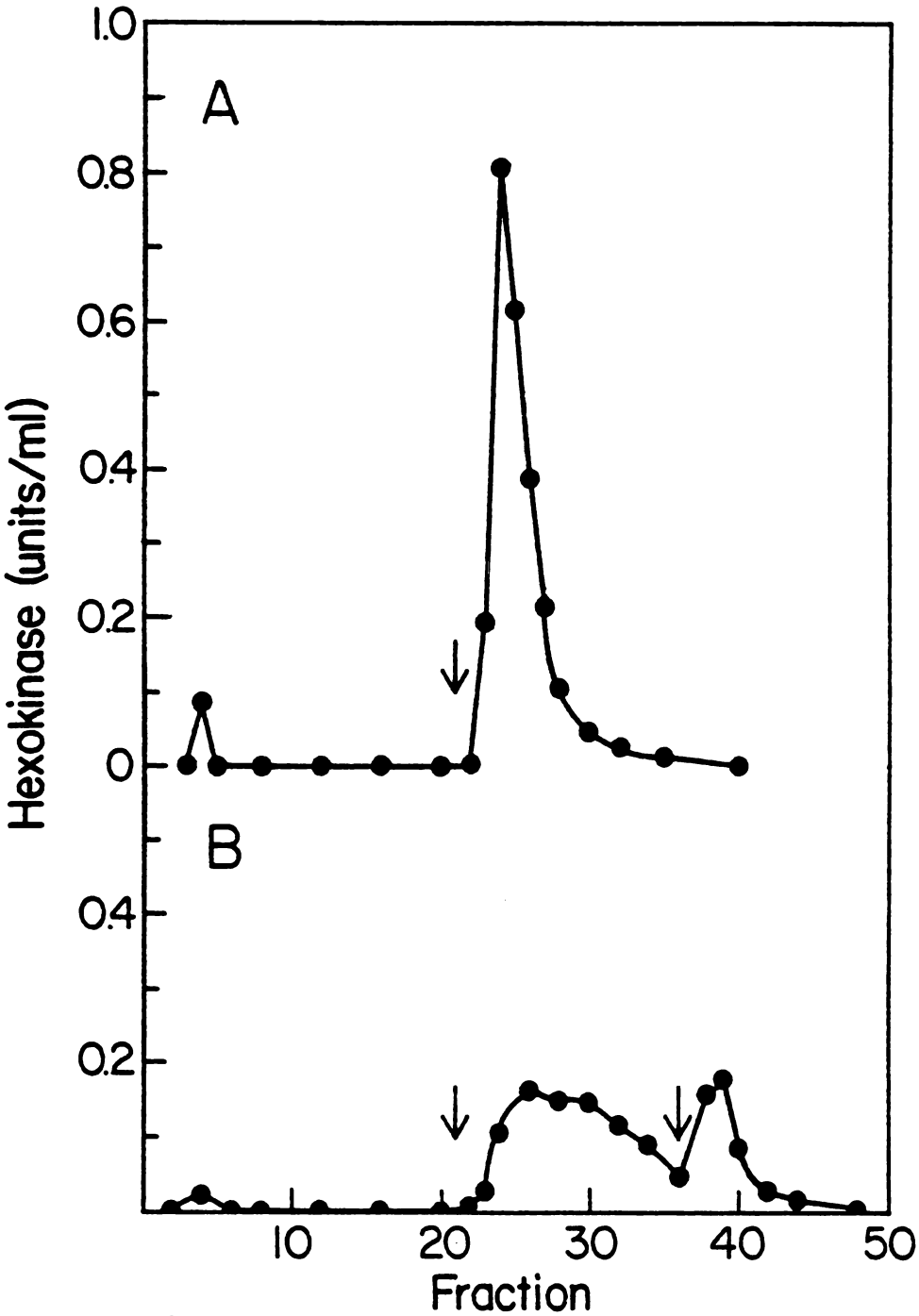
1.0
0.8
0.6
0.4
0.2
0
0.4
0.2
A
B
Hexokinase (units/ml)
10
20
30
40
50
Fraction

Figure 9

Figure 10. Reverse $(NH_4)_2SO_4$ gradient solubilization of hexokinase. Crude cytoplasmic hexokinase (5.9 units) was precipitated onto Celite with 3.0 M $(NH_4)_2SO_4$ and poured into a column (2.0 x 4.5 cm). The enzyme was eluted with a 3.0-0 M reverse $(NH_4)_2SO_4$ gradient collecting 1.2 ml fractions. Two peaks of activity were eluted at 1.7 M and 1.0 M $(NH_4)_2SO_4$ respectively. Fractions 38-51 contained 1.1 units and fractions 52-74 contained 1.6 units. Symbols: ● hexokinase, o A_{280}, Δ $(NH_4)_2SO_4$.

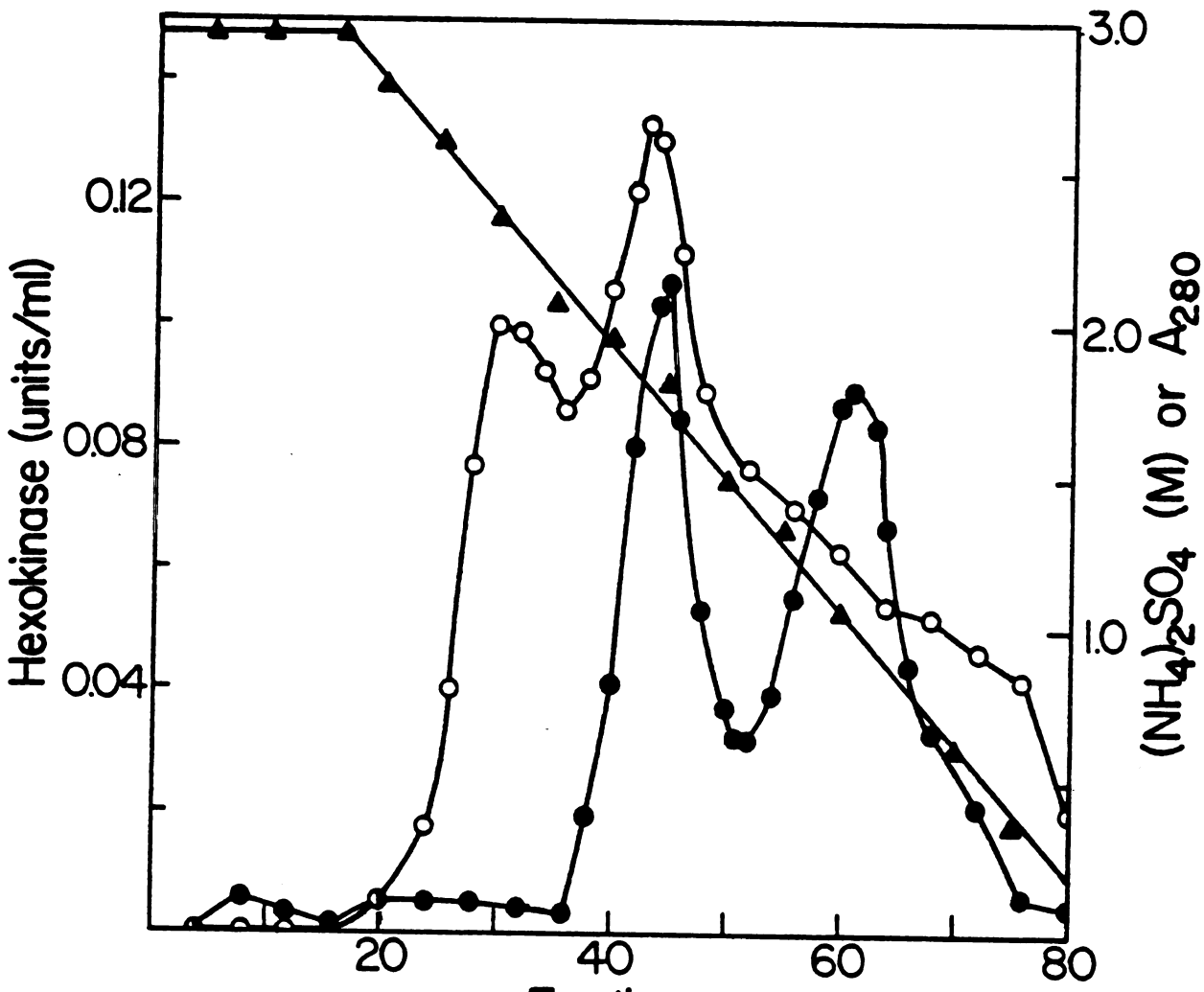
Hexokinase (units/ml)
0.12
0.08
0.04
3.0
2.0
1.0
$(NH_4)_2SO_4$ (M) or A_{280}
20
40
60
80
Fraction

Figure 10

procedure, so it was not used or followed up.

A glucose-Sepharose affinity column with an 8 carbon arm was synthesized (127) and used to purify cytoplasmic hexokinase. Although the affinity column was initially capable of specifically binding pure mitochondrial hexokinase (not shown) and DEAE-cellulose-purified cytoplasmic hexokinase (Figure 11A), it started losing this capacity almost immediately. By the sixth use of the column, cytoplasmic hexokinase which had been purified by DEAE-cellulose and ATP-agarose chromatography was not bound by the column (Figure 11B). Washing the column with 3 M KCl/3 M urea and treatment with pronase as described (127) did not salvage the function of the column. The great time investment involved in the synthesis and the short functional lifetime of this column precluded its further use.

DISCUSSION

Cytoplasmic rat brain hexokinase has been purified to near homogeneity. The key to the purification scheme was the affinity elution of the enzyme from Blue Dextran-Sepharose by glucose 6-phosphate. This and other immobilized dyes (33,61) are extremely useful tools in protein purification because of their ease of synthesis, wide applicability, and long term stability. This particular column was used more than 40 times over a two year period with no detectable deterioration in function, in great contrast to the ATP-agarose and glucose-Sepharose columns.

The binding of hexokinase to Blue Dextran-Sepharose was weak, but sufficient to allow total retention of the enzyme at low ionic

Figure 11. Loss of glucose affinity column function. Cytoplasmic hexokinase was loaded onto a 0.8 x 10 cm glucose affinity column collecting 1.6 ml fractions. Bound hexokinase was eluted by the addition of 1 M glucose at the arrow. A. First use. DEAE-cellulose-purified enzyme (2.0 units) was used. B. Sixth use. DEAE-cellulose-purified enzyme was further purified by ATP-agarose chromatography before 2.5 units was applied to the glucose affinity column. Symbols: ● hexokinase, o A_{280}.

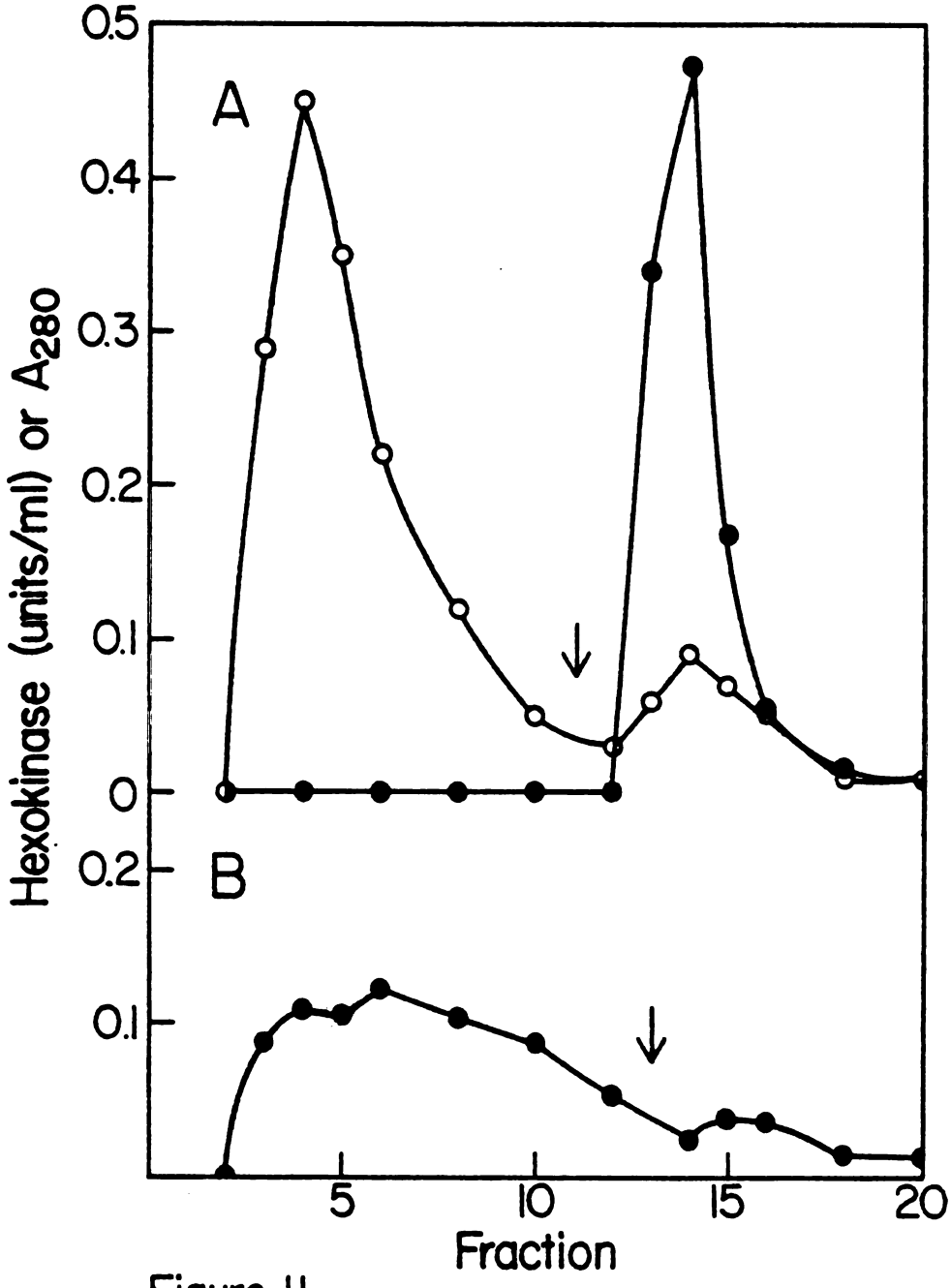
0.5
0.4
0.3
0.2
0.1
0
0.2
0.1
A
B
Hexokinase (units/ml) or A_{280}
5
10
15
20
Fraction

Figure 11

strength. Since both ATP (data not shown) and glucose 6-phosphate were effective at elution, the interaction appears to be specific to the ATP binding site of the enzyme. This is consistent with previous work which has shown that the Cibacron Blue dye moiety of Blue Dextran binds to the nucleotide sites of a number of enzymes (106) and acts as a competitive (vs. ATP) inhibitor of brain hexokinase (120). Glucose 6-phosphate prevents the binding of ATP to hexokinase in a competitive manner, possibly through direct competition between the phosphate group and the γ-phosphate of ATP for a common binding site (27). By analogy, glucose 6-phosphate may block the binding of Cibacron Blue by preventing interaction of sulfate groups on the dye with the enzyme. Alternatively, it has been suggested that the detrimental effect of glucose 6-phosphate on nucleotide binding is indirect, resulting from binding of glucose 6-phosphate at a discrete allosteric site on the enzyme (58). This too would be consistent with the elution of hexokinase from Blue Dextran-Sepharose by glucose 6-phosphate. In light of the minimal involvement of electrostatic effects in the elution of proteins from Blue Dextran-Sepharose by salts (85), I believe the allosteric mechanism to be more probable. Other examples of non-nucleotide substrates or effectors altering the binding of proteins to immobilized dyes have recently been reported (109).

The anomalous retardation of hexokinase in phosphate buffer by Blue Dextran-Sepharose (Figure 6A) may have been due to a higher initial ligand concentration. Even though the newly synthesized adsorbant was first washed extensively with 1 M KCl, a distinct blue color was visible in the later fractions of Figure 6A. Perhaps the loss of Blue Dextran from the matrix at this stage was sufficient to

cause the observed change in hexokinase binding behavior.

The removal of glucose and thioglycerol from a hexokinase solution resulted in a decreased affinity of the enzyme for both ATP and Blue Dextran-Sepharose, presumably due to oxidation of sulfhydryl groups. A similar process occured to some extent even in buffers containing thioglycerol and was surely a contributor to the lack of quantitative recovery during the purification procedure. It does not appear to be directly related to the inactivation of hexokinase by sulfhydryl reagents (13,83) or the air oxidation of rabbit red blood cell hexokinase (67) because both of these processes involve total inactivation of the enzyme when assayed under normal conditions. Variation in the degree of sulfhydryl modification may, however, be responsible for the observed differences in inhibition pattern observed with p-chloromercuribenzene sulfonate (74).

The curvature noted in the Lineweaver-Burk plots from Figure 8 seems to be related to the loss in the ability of hexokinase to bind to Blue Dextran-Sepharose. The curvature could be due either to the presence of a mixture of kinetic forms or to an unusual reaction mechanism of some sort. Elucidation of the cause of this change is not within the scope of this project.

The cause of the heterogeneity observed upon reverse $(NH_4)_2SO_4$ gradient solubilization of hexokinase is unknown. One possibility is that the two peaks correspond to the two major isoelectric focusing bands. This separation has not been repeated since the analytical isoelectric focusing method was worked out in our laboratory, nor has it been used with purified hexokinase.

CHAPTER 2

COMPARISON OF MITOCHONDRIAL AND CYTOPLASMIC HEXOKINASE

Cytoplasmic hexokinase, purified as outlined in Chapter 1, was compared to the pure mitochondrial enzyme with respect to the properties in which the impure enzyme have been reported to differ. These included inhibition by quercetin, the effects of pH on activity, and kinetics; the reported difference in ability to bind to mitochondria is considered in Chapter 3. Proteolytic peptide maps of the pure enzymes and some experiments dealing with "impurities" that may be proteolytic degradation products are also included in this chapter.

RESULTS

Inhibition of Hexokinase by Quercetin

Both cytoplasmic brain hexokinase (J. Lusk, unpublished data) and soluble Ehrlich ascites tumor hexokinase (35) are less sensitive than the respective glucose 6-phosphate solubilized mitochondrial enzymes to inhibition by quercetin. The inhibition of hexokinase by quercetin was affected by the amount of protein present in the cuvette during the assay. Glucose 6-phosphate solubilized mitochondrial hexokinase (5.1 munits, 2.0 μg protein) was inhibited 66 ± 5% by 25 μg/ml quercetin. As increasingly pure cytoplasmic hexokinase was assayed the degree of

inhibition steadily increased from virtually no inhibition of the crude enzyme to about 80% inhibition of the pure enzyme (Figure 12). The inhibition of the pure enzyme was reversed by the addition of increasing amounts of BSA (Figure 12), although this protein was not as effective at reversal on a μg for μg basis as were the proteins removed during the purification procedure. Since the purified mitochondrial and cytoplasmic enzymes were inhibited to the same degree (75 ± 3% and 79 ± 4% respectively), the difference in sensitivity of the impure enzymes appears to be due more to the relative purity of the enzymes than their source.

Effect of pH on Activity

Both Chou (11) and Lusk et al. (64) noted differences in the effect of pH on impure mitochondrial and cytoplasmic hexokinase from rat brain, the cytoplasmic enzyme being more active at pH 5-6. This was not the case when the purified enzymes were compared (Table 4). If anything the cytoplasmic enzyme actually tended to be less active at all pH values except 9.5, but these differences were minimal. Since inhibition of hexokinase by Al^{+3} is pH dependent (125), I considered the possibility that the pH profiles reported with the crude enzymes may reflect differences in the ability to overcome Al^{+3} inhibition. This is not likely, however, because under the assay conditions used (6.7 mM ATP), the magnitude of the inhibition was not great enough, even in the presence of exogenously added Al^{+3}, to account for a two-fold difference in activity (not shown).

Figure 12. Effect of protein on the inhibition of hexokinase by quercetin. Cytoplasmic hexokinase (2.6 munits of crude, 5 munits of all others) was assayed in triplicate in the presence and absence of 25 μg/ml quercetin and the degree of inhibition plotted against the amount of protein present in the cuvette. The open symbols represent hexokinase at various stages of purification (crude, and after DEAE-cellulose chromatography, NaCl elution from Blue Dextran-Sepharose, and glucose 6-phosphate elution from Blue Dextran-Sepharose). The closed symbols represent the pure cytoplasmic hexokinase with BSA added to the indicated levels.

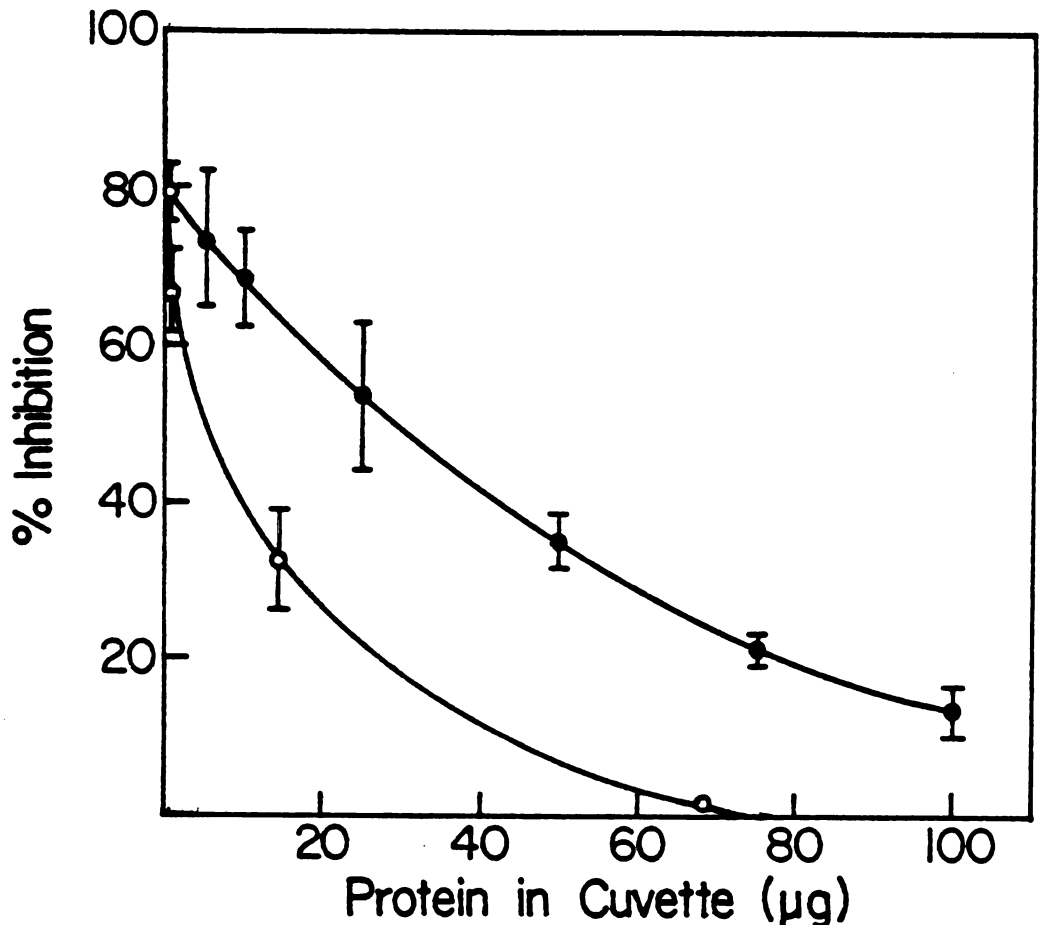
100
80
60
40
20
% Inhibition
20
40
60
80
100
Protein in Cuvette (µg)

Figure 12

Table 4. Effect of pH on hexokinase activity

	Hexokinase activity, % of value at pH 8.5	
pH	Mitochondrial	Cytoplasmic
5.5	21 ± 1	20 ± 1
6.5	64 ± 2	61 ± 2
7.5	86 ± 3	81 ± 2
8.5	100 ± 2	100 ± 1
9.5	101 ± 3	108 ± 3

Hexokinase was assayed as described in Methods with the indicated change in pH, except that the glucose 6-phosphate dehydrogenase was doubled at pH 5.5. The buffers used (all at 50 mM) were N-morpholinoethane sulfonic acid (pH 5.5), HEPES (pH 6.5-8.5) and triethanolamine (pH 9.5). The tabulated values are the mean ± S.D. (n=6), normalized to the value at pH 8.5.

Kinetic Results

The kinetic differences between mitochondrial and cytoplasmic hexokinase reported by Thompson and Bachelard (3,104) are rather subtle and require very good data to be detected. Because of the great time commitment required and difficulties experienced in reproducibly demonstrating these differences with initial rate kinetics, the substrate depletion method was devised.

Data from substrate depletion progress curves were collected over a considerable period of time and range of absorbance values. It must be verified that the assay system produces valid absorbance changes and that the spectrophotometer accurately measures them. The linearity of the photometer response was evaluated by the incremental addition of small aliquots of NADPH to a cuvette. As can be seen in Figure 13, the photometer response was linear over the required absorbance range of 0-1.0, although slight curvature is evident at higher absorbance values.

An unexplained lag in the rate was observed after initiation of each assay. When both substrates were saturating, the rate increased steadily from its initial value of 70-80% maximum to the maximum value in 30-45 seconds. This lag occured whether the reaction was started with glucose or with ATP and was not eliminated by an increase in the coupling enzyme concentration. To avoid these pre-steady state events only data collected after the first minute of reaction were used for kinetic analysis.

The possibility of hexokinase inactivation during the assay was tested by running kinetic assays using several enzyme concentrations, as suggested (129). Since the time required to deplete the substrate

Figure 13. Linearity of photometer response. Increments of NADPH were added to a cuvette in a Gilford 2600 spectrophotometer and the resulting absorbance values at 340 nm (■) were plotted against the number of increments. The change in absorbance or delta A (□) values were also plotted. The line is the best fit through the first ten points.

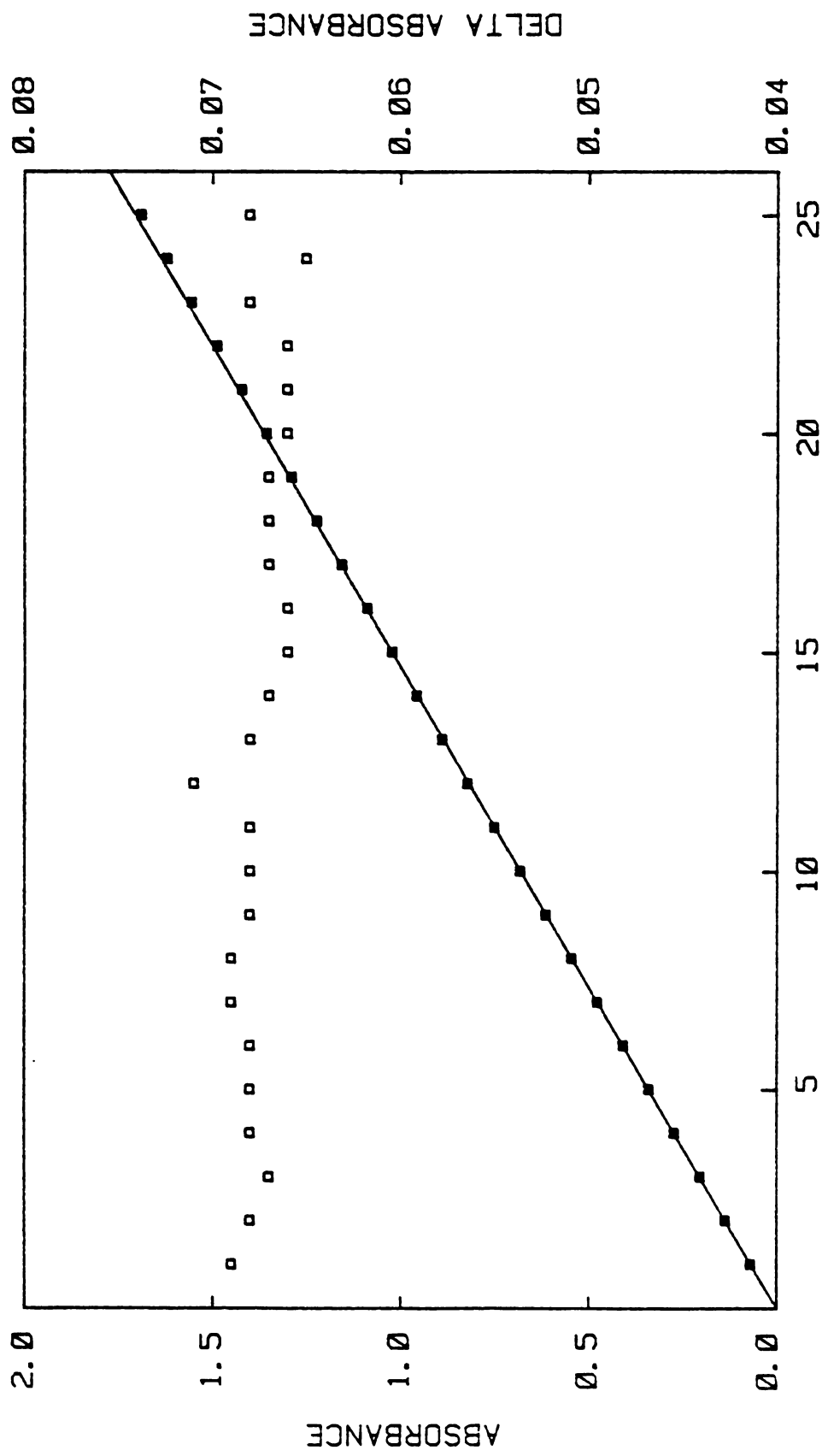
DELTA ABSORBANCE
0.08
0.07
0.06
0.05
0.04
ABSORBANCE
2.0
1.5
1.0
0.5
0.0
5
10
15
20
25
INCREMENT NADPH

FIGURE 13

depends on the amount of enzyme, any time-dependent events (e.g. inactivation) would occur to different extents over a given substrate range. Identical results were obtained, indicating that no appreciable inactivation occured. The whole system (enzyme plus assay plus spectrophotometer) was tested by measuring the hexokinase activity under the conditions used in kinetic assays except with both ATP and glucose present in saturating concentrations. The rate measured after the reaction had proceeded long enough to change the absorbance by 1.0 absorbance unit is only 2-3% lower than the maximal rate, a discrepancy that should pose no problems.

The Lineweaver-Burk kinetic patterns obtained with purified mitochondrial and cytoplasmic hexokinase are shown in Figure 14. In agreement with previous reports (3,64,104), the mitochondrial enzyme yielded a family of lines that intersected below the abscissa (Figure 14A). In contrast to the pattern observed with the crude or partially purified cytoplasmic enzyme (3,64), the purified enzyme also yielded the same pattern (Figure 14B).

Peptide Mapping

Similarities between protein chains can be analyzed by comparison of the fragments generated by partial proteolytic degradation of the denatured protein (14). Figure 5 is a comparison of the maps obtained from the digestion of mitochondrial and cytoplasmic hexokinase with three different proteases. The undigested protein migrated identically in all of the lanes, indicating that the intact mitochondrial and cytoplasmic hexokinases have the same molecular weight. More than 25 bands were visible in each lane of the original gel, and most of the

Figure 14. Kinetic patterns of mitochondrial and cytoplasmic hexokinase. Lineweaver-Burk plots were prepared from substrate depletion kinetic data. Glucose concentrations ranged from 10-80 μM. Each line represents data collected at a different constant ATP concentration (0.15, 0.20, 0.33, and 1.00 mM). A. Mitochondrial hexokinase. B. Cytoplasmic hexokinase.

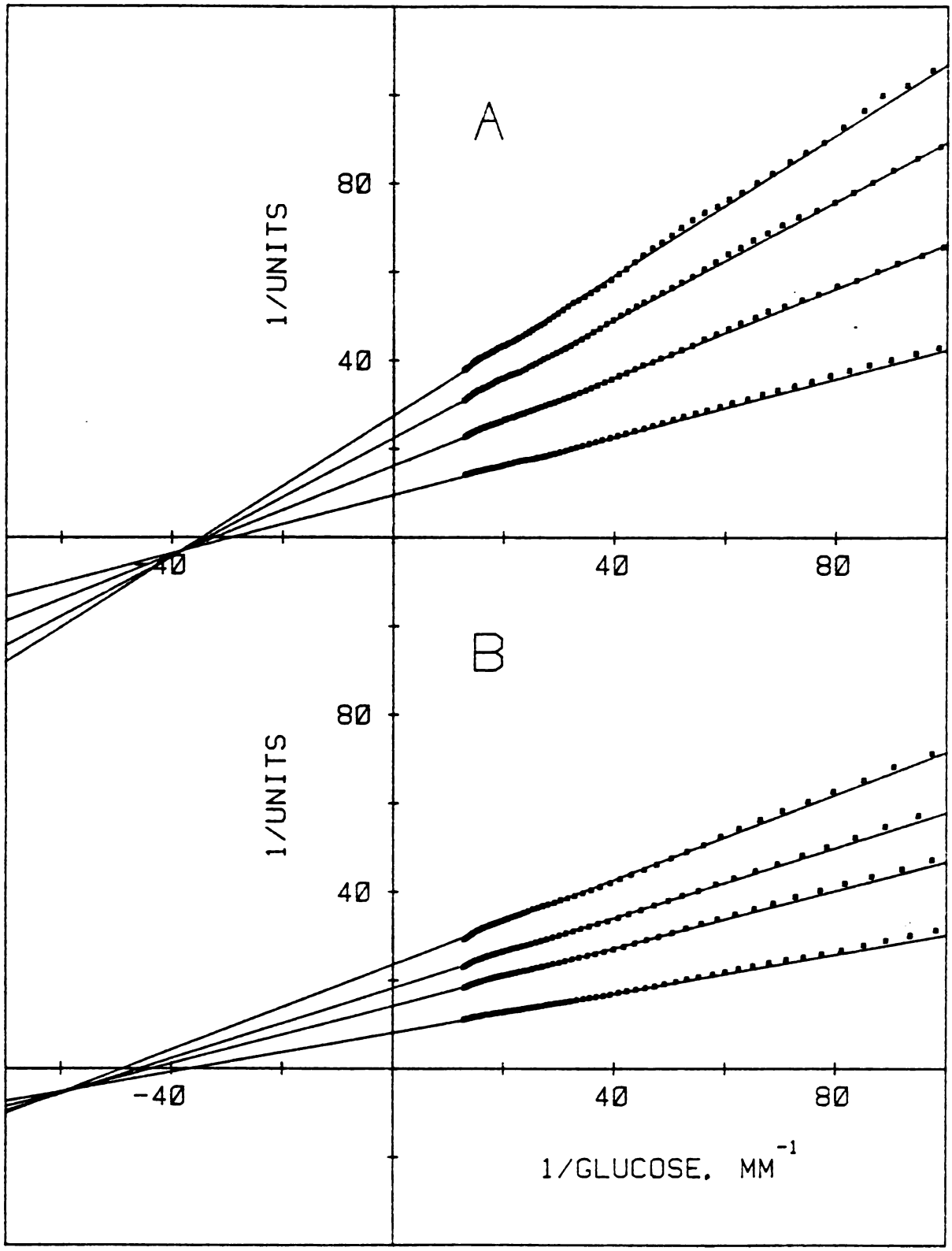

FIGURE 14

Figure 15. Comparative peptide mapping of mitchondrial and cytoplasmic hexokinase. Mitochondrial (M) and cytoplasmic (C) hexokinase (36 µg) were digested with the indicated proteases for 30 min at 37°C in the presence of 1% SDS, and the resulting fragments were separated on a 6.5-20% gradient SDS acrylamide gel. The protease concentrations used were: S. aureus protease, 2 µg/ml; chymotrypsin, 2 µg/ul; and trypsin, 50 µg/ml. Arrows mark the positions of standards with the indicated molecular weights in daltons.

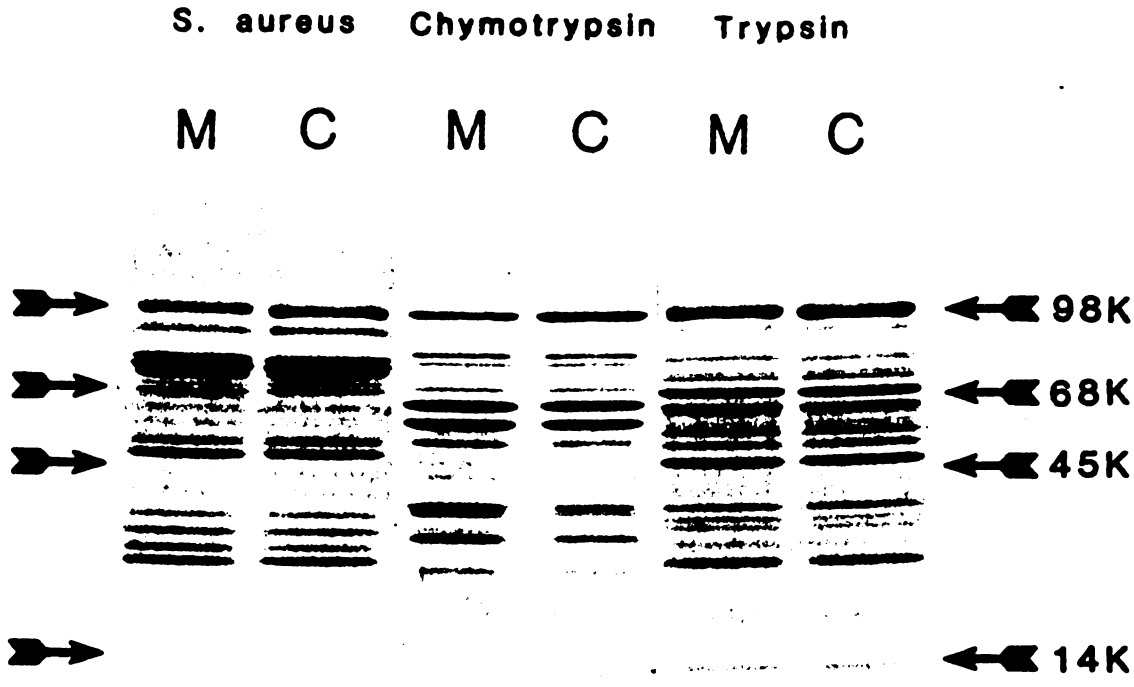

Figure 15

differences noted between the two forms were minor quantitative ones. A few very minor bands were visible in the digests of one form but not the other, but they were at the limit of detection and of uncertain significance. Clearly, any differences between mitochondrial and cytoplasmic hexokinase, including those responsible for the multiple isoelectric forms (see Chapter 3), must be minor since they do not significantly alter the peptide maps obtained with these three proteases.

Endogenous Protease

When SDS gels were overloaded with samples of pure mitochondrial and cytoplasmic hexokinase there were a number of bands with molecular weights of less than 100,000 which appeared to be common to both proteins (Figure 16). Because of the strikingly different purification histories of the two enzymes, it is unlikely that the same impurities would copurify with both proteins to similar degrees. Copurification of a tightly bound protease is one possible explanation of the presence of common "impurities" in both the mitochondrial and cytoplasmic enzymes in which case the lower molecular weight bands would be generated from the 100,000 dalton hexokinase by proteolysis.

Purified hexokinase was incubated at 37°C for various times in the presence of 1% SDS and examined for changes in the pattern of lower molecular weight bands. Both mitochondrial (Figure 16A) and cytoplasmic (Figure 16B) hexokinase showed a time dependent increase in some, but not all, of the bands. This apparent proteolytic degradation was stopped by boiling the enzyme for 3 minutes in SDS, but not by treatment with 1 μM pepstatin, 1 mM PMSF, or 1 mM 1,10-phenanthroline (data not shown).

Figure 16. Endogenous protease in pure hexokinase. Purified hexokinase (33 μg) was incubated at 37°C in 1% SDS for the indicated periods of time (in hours) before SDS electrophoresis on a 6.5-20% gradient acrylamide gel. Arrows mark the positions of standards with the indicated molecular weights in daltons. A. Mitochondrial hexokinase, B. Cytoplasmic hexokinase.

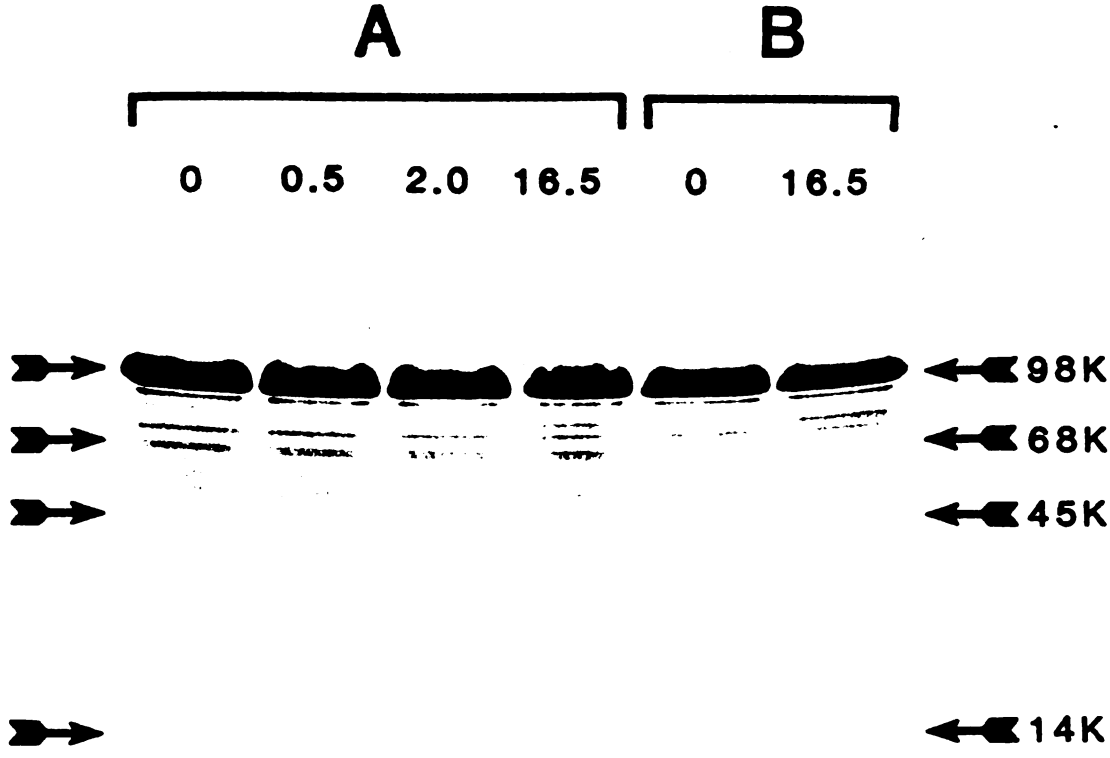

Figure 16

Transfer of Proteins to Nitrocellulose

If the "impurities" seen on SDS gels of hexokinase are proteolytic degradation products, some or most of them should contain antigenic sites in common with hexokinase. Binding of antibodies to proteins separated on SDS gels is most readily detected after transfer of the proteins to nitrocellulose (107). The efficiency of protein transfer under the conditions described in Methods was determined by comparing the staining of protein in gel before and after transfer (Figure 17). Crude cytoplasmic hexokinase (1.3 mg protein) was loaded onto one half of an SDS slab gel and an S. aureus protease digest of pure mitochondrial hexokinase (290 μg incubated for 30 minutes at 37°C with 1 μg/ml S. aureus protease) was loaded onto the other side. After electrophoresis, the ends of the gel were cut off and stained for protein. The remainder of the gel was used in the transfer procedure and then the residual proteins in the gel were stained. A photograph of the gel is included in Figure 17A/B, and the resulting scans in Figure 17C and D. Most proteins were transferred completely under the conditions used, but some (including intact hexokinase and some high molecular weight proteins) were transferred less than quantitatively. Size does not seem to be the only factor determining efficiency of transfer because some medium molecular weight bands were also incompletely transferred.

Antibody dilutions for use in immunostaining of the nitrocellulose replicas were optimized using serial dilutions of hexokinase spotted onto nitrocellulose (not shown). Increasing dilution of hexokinase antiserum resulted in minor decreases in sensitivity at a constant dilution of goat anti-rabbit IgG. The 1:75 dilution was used as a

Figure 17. Efficiency of transfer to nitrocellulose. Proteins were separated by SDS electrophoresis on a 6.5-20% gradient gel and the ends of the gel were cut off. The proteins in the center portion of the gel were transferred to nitrocellulose using a constant current of 400 ma for 16 hours. The proteins remaining in the transferred and untransferred portions of the gel were then stained. A. Crude cytoplasmic hexokinase (1.3 mg) was loaded onto one half of a gel. B. Pure mitochondrial hexokinase (290 µg) was digested for 30 min at 37°C with 1 µg/ml S. aureus protease and loaded onto one half of a gel. C. The resulting scans from the transferred (lower trace) and untransferred (upper trace) portions of the gel in (A). D. The resulting scans from the transferred (lower trace) and untransferred (upper trace) portions of the gel in (B).

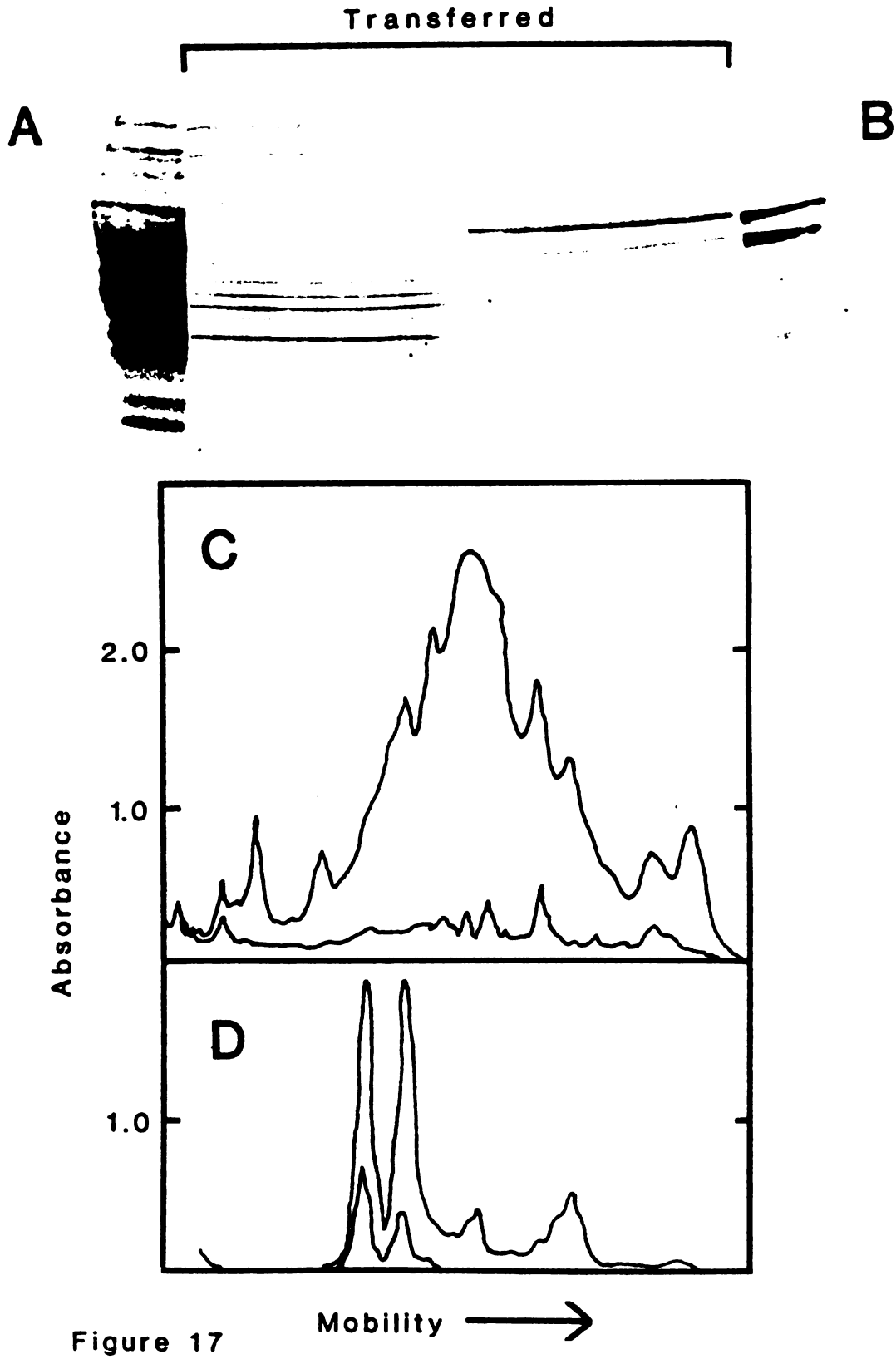

Figure 17

compromise between sensitivity and economy. High concentrations of anti-rabbit IgG resulted in darker spots and an increased background staining, but did not change the lowest level of hexokinase detectable. A dilution of 1:750 was used in the final procedure. Under these conditions, a 1 mm diameter dot containing 0.1 μg of hexokinase was easily visible, but 0.01 μg was not. The addition of 1% SDS to the enzyme before spotting resulted in a considerable decrease in sensitivity, partly due to a spreading of the spot. After transfer from SDS gels to nitrocellulose, 1 μg of hexokinase was easily visible, but 0.1 μg was not.

After transfer from an SDS gel, most of the fragments generated by partial S. aureus digestion of hexokinase were visible after immunostaining for hexokinase although the intensities of the bands did not correlate exactly with the protein staining (Figure 18A). Above some minimum molecular weight, antigenic sites were present on most fragments.

A number of bands from the soluble fraction of a brain homogenate were stained by hexokinase antiserum. The major non-hexokinase bands were also visible on replicas treated with only the anti-rabbit IgG (Figure 18B), indicating that this staining is artifactual. The reason that proteins are found in a rat brain homogenate that crossreact with anti-rabbit IgG is not clear.

At least some of the lower molecular weight proteins found in "pure" hexokinase do appear to be derived from hexokinase since they bound hexokinase antiserum (Figure 18D). In contrast to the results with the crude soluble brain proteins, horseradish peroxidase staining did not occur in the absence of hexokinase antiserum (Figure 18D).

Figure 18. Immunostaining of nitrocellulose replicas. Proteins were separated by SDS electrophoresis on a 6.5-20% gradient gel and transferred to nitrocellulose as described in Methods. The resulting replicas were stained for protein (P) or for immunoreactivity with hexokinase antiserum (I) or a control (C). The arrowheads point to some of the important bands. A. Pure mitochondrial hexokinase (58 µg per lane) after digestion for 30 min at 37°C with 1 µg/ul S. aureus protease. B. Crude cytoplasmic hexokinase (260 µg per lane). The control was incubated with buffered saline instead of hexokinase antiserum. C. Standards (2 µg each) with molecular weights of 98K, 68K, 45K, and 14K daltons. D. Pure mitochondrial hexokinase (35 µg per lane). The control was incubated with preimmune serum instead of hexokinase antiserum.

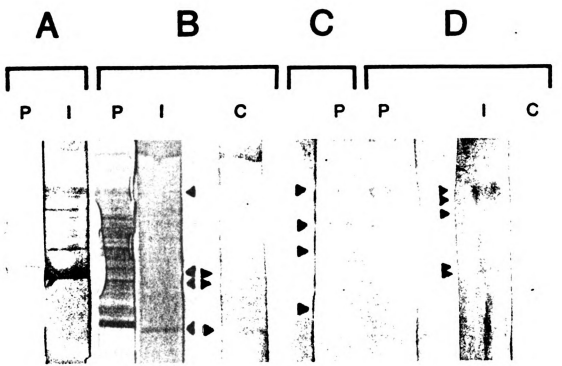

Figure 18

Apparent Molecular Weight of "Impurities" Under Native and Denaturing Conditions

The apparent subunit molecular weight of a protein is often estimated after electrophoresis in SDS polyacrylamide gels (112). Proteins can be separated by molecular weight under non-denaturing conditions by electrophoresis on native gradient polyacrylamide gels (54). When pure mitochondrial hexokinase was electrophoresed on such a native gel, most of the "impurities" observed upon SDS electrophoresis (Figure 19B) were not visible (Figure 19A). When subjected to a second dimension electrophoresis on an SDS gel the "impurities" were again visible (Figure 19C). However, instead of being distributed in a diagonal band (as would be expected if they had migrated with the same apparent molecular weight in the two gel systems) most of the "impurities" were found directly beneath the high molecular weight region of the native gel in which hexokinase migrated. In combination with the probability that these bands are proteolytic fragments of hexokinase (Figures 16 and 18), these data suggest that hexokinase may be clipped by protease(s) but that the fragments remain together in a 100,000 dalton complex under non-denaturing conditions.

DISCUSSION

By all of the criteria examined purified cytoplasmic and mitochondrial hexokinase were identical. The antagonistic effect of BSA on the inhibitory action of quercetin was previously noted by Suolinna *et al*. (100). Apparently the effective concentration of quercetin is reduced by adsorption to BSA or other proteins present in

Figure 19. Apparent molecular weight of "impurities" under native and denaturing conditions. Pure mitochondrial hexokinase was electrophoresed in a native 3-20% gradient gel, an SDS 6.5-20% gradient gel, or a two dimensional combination of the two. A. Hexokinase (36 μg) on a native gel. B. Hexokinase (36 μg) on an SDS gel. C. Two dimensional map. Hexokinase (72 μg) was run on a native gel followed by electrophoresis in the second dimension into an SDS gel. The approximate position in the SDS gel of standards with the indicated molecular weights (taken from another gel) are marked by arrows. A lane from the first dimensional native gel containing the same molecular weight markers (4 μg each) shows the placement of the first dimensional gel on top of the SDS gel.

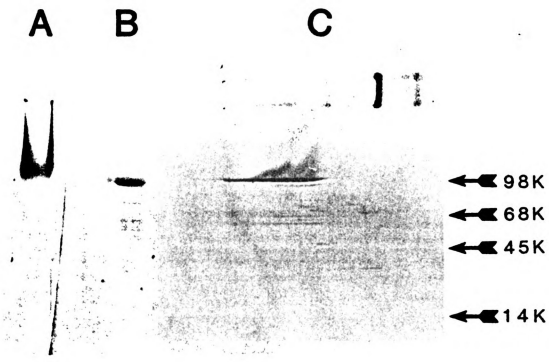

Figure 19

the crude hexokinase, causing the degree of inhibition to be dependent on the purity of the enzyme. This is a clear illustration of the importance of repeating work done with a crude system after purification of the components. Balanced against this must be an awareness of the possible pitfalls associated with the use of purified proteins, such as lack of stability, sulfhydryl oxidation, proteolysis, and other changes which might occur during or after purification of the protein.

I have no definitive explanation for the reported differences in the effect of pH on the activity of impure mitochondrial and cytoplasmic hexokinase. Despite the uncertainties concerning results obtained with impure preparations, it is clear that there was no difference in the pH profiles of the purified enzymes.

The similarity of the peptide maps obtained after partial proteolysis by three proteases is another indication of the identity of these two proteins. Although separation of the fragments by molecular weight alone might not detect small differences in the charge or amino acid composition, it should detect any substantial proteolytic differences or changes in any of the amino acids for which the proteases are specific.

Yun and Suelter (129) have shown that kinetic data obtained from the analysis of substrate depletion progress curves are in excellent agreement with initial rate data as long as product concentrations at infinite time are precisely known, product inhibition doesn't occur (or is corrected for), and the enzyme does not inactivate during the course of the assay. The method is fast, reproducible, and internally consistent. Since a linear trace is not necessary, reaction velocities can be measured at much lower substrate levels than is possible with

the initial rate method. It has the additional advantage that an enzyme which undergoes time dependent changes or becomes unstable upon removal of a substrate can be stored in the presence of that substrate until the moment of assay. The method described here is an improvement in that the tangents to the progress curve are determined by a much better algorithm (59). Also, since a computer carries out all data collection and manipulation, many more time points can be conveniently collected.

The substrate depletion method is ideally suited for the kinetic analysis required in this study. The hexokinase reaction is irreversible, both products are removed during the assay, and the enzyme is stable over the course of the assay. In order to ensure that the coupling reaction is also irreversible, the initial product (6-phosphogluconolactone) must be rapidly hydrolyzed to 6-phosphogluconate. At neutral pH the rate of hydrolysis is very slow, but at pH 8.5 it should be fast enough to prevent buildup of the lactone (63). The internal consistency and reproducibility of the method facilitate the direct comparison of kinetic patterns obtained under identical conditions. Such a direct comparison between the purified mitochondrial and cytoplasmic enzymes did not confirm the difference reported between the crude or partially purified enzymes (3,64,104).

Unfortunately the kinetics of impure cytoplasmic hexokinase could not be measured with this method due to the presence of a glucose-independent rate of NADPH production which prevented the accurate calculation of residual glucose concentrations from the absorbance data. The unsuspected existence of such a background rate

might also influence the kinetic pattern observed with an impure enzyme using the initial rate method. Moreover, the sizable lag in attaining the steady state rate of reaction after addition of substrates makes accurate measurement of initial rates difficult, especially at low substrate levels. The difficulty is overcome in the substrate depletion method by using only data collected after the first minute of reaction. Initial rate kinetic data obtained for hexokinase should probably be re-examined in light of these findings.

The lower molecular weight "impurities" visible upon SDS electrophoresis of both mitochondrial and cytoplasmic hexokinase appear to be proteolytic degradation products of hexokinase. Moreover, in native polyacrylamide gels these fragments behaved as a larger molecular weight complex that may be protease-clipped hexokinase which has not dissociated into individual fragments. These observations would explain the tenaciousness of these "impurities" if the complex had properties similar enough to native hexokinase to copurify with it. Trypsin-treated hexokinase also appears to migrate as a complex during isoelectric focusing and sucrose density gradient centrifugation, but not SDS electrophoresis (P. Polakis, unpublished data).

The presence of a contaminating protease in proteins purified from yeast is fairly common (37,79, others listed in 1). There does appear to be a protease associated with purified mitochondrial and cytoplasmic hexokinase, but the degree of proteolysis is minor compared to that seen with some of the yeast enzymes. Even after incubation overnight at 37°C in the presence of 1% SDS, >90% of the protein remained intact.

The immunodetection of proteins after electrophoretic transfer of proteins from SDS gels to nitrocellulose is subject to several difficulties. The efficiency of transfer varies drastically for different proteins and should be evaluated for each protein of interest. However, it was recently reported (28) that the inclusion of 0.1% SDS in the transfer buffer resulted in quantitative transfer of all proteins. In addition, the electric field strength must be uniform across the whole gel. There was some indication in Figure 17 of zones of good and poor transfer separated by approximately the distance between adjacent loops of the electrode.

The staining of minor bands by hexokinase antiserum might be due to the presence of antibodies to protein impurities in the hexokinase used for immunization. Monoclonal antibodies would provide greater specificity. Preliminary experiments with two anti-hexokinase clones under development in the laboratory indicated that the intensity of staining is much less than that obtained with the polyclonal antiserum. An alternative way (71) of demonstrating that a band on an SDS gel binds hexokinase antibodies is to incubate a nitrocellulose replica with an excess of hexokinase antiserum followed by pure hexokinase, which will bind to only hexokinase antibodies. The bands which contain hexokinase antigenic sites can then be indirectly located on the replica by staining for hexokinase activity.

The most serious problem is the artifactual staining of crude mixtures that occurs even in the absence of any primary antibody. This can be detected by appropriate controls, and does not seem to occur with more purified preparations. Other methods (28,71) for the detection of bound hexokinase antibodies should not have this problem.

With proper precautions, immunodetection of proteins on nitrocellulose replicas can provide valuable information on the heterogeneity of an antigen or antiserum.

CHAPTER 3

BINDING OF HEXOKINASE TO MITOCHONDRIA

Hexokinase binding to mitochondria was measured using a variety of incubation times and component concentrations. Comparisons were made of the binding of mitochondrial and cytoplasmic hexokinase at 0° and 25° C and of the effect of Mg^{++} on the binding of hexokinase to brain and liver mitochondria. The ability of freshly prepared hexokinase to bind to mitochondria was evaluated by titration of hexokinase in a brain homogenate with mitochondria immediately after homogenization. Since the isoelectric focusing pattern of hexokinase seems to be correlated with its ability to bind to mitochondria (P. Polakis, unpublished data), comparisons were also made between the focusing patterns obtained with mitochondrial and cytoplasmic hexokinase. The kinetic patterns obtained with the isoelectric forms, after separation by DEAE-cellulose chromatography, were also compared. The effect of increased osmolarity of the assay on the latency and apparent distribution of hexokinase in brain homogenates was determined. Previous studies on the soluble-particulate distribution of hexokinase, which had been considered to support the existence of discrete cytoplasmic and mitochondrial forms of the enzyme, were reinterpreted in light of the present work.

RESULTS

Conditions for Binding of Hexokinase to Mitochondria

The binding conditions described in Methods were worked out by individually varying a number of parameters (using glucose 6-phosphatesolubilized hexokinase dialyzed against 0.25 M sucrose and purified liver mitochondria). As would be expected for an equilibrium interaction, the amount of binding decreased when the mixture of hexokinase and mitochondria was diluted (not shown). At concentrations of hexokinase greater than 0.1 units/ml there was little increase in the amount of binding, so this concentration was used in the final procedure.

When the total volume was less than 0.25 ml, the variability of the results increased dramatically. Volumes or concentrations larger than these values can be used if sufficient material is available. The period of incubation before centrifugation was varied from 0 to 30 minutes. Maximal binding occured after incubation for 5 minutes or longer; 10 minutes was used in the final procedure.

Titration of Hexokinase with Mitochondria

The capacity of mitochondria to bind hexokinase was evaluated for each preparation used in binding studies. Glucose 6-phosphate-solubilized mitochondrial hexokinase was bound to liver mitochondria under the conditions described in Methods, except that the amount of liver mitochondrial protein added was varied. Typical results are presented in Figure 20. More than 1 mg of mitochondrial protein was necessary to totally saturate the bindable hexokinase. For

reasons of economy, an amount of protein (in this case 640 μg) which resulted in 90-95% maximal binding was routinely used in binding assays.

Binding of Hexokinase to Brain and Liver Mitochondria with and without Mg^{++}

While performing some experiments not described here I noticed that, in contrast to the situation with liver mitochondria, a substantial amount of hexokinase bound to brain mitochondria in the absence of Mg^{++}. Fractions containing highly bindable hexokinase were selected from a mitochondrial hexokinase preparation and dialyzed against 0.25 M sucrose containing 10 mM thioglycerol and 10 mM glucose. This enzyme was used in binding assays with an amount of purified brain or liver mitochondria sufficient for nearly maximal binding of the hexokinase in the presence of 4 mM $MgCl_2$. The Mg^{++} concentration was varied from 0-2 mM and the percent of the hexokinase that bound was determined in duplicate (Table 5). Although both liver and brain mitochondria bound the same amount of hexokinase at 2 mM Mg^{++}, the brain mitochondria were much less sensitive to decreases in the amount of Mg^{++}. Liver mitochondria bound virtually no hexokinase in the absence of Mg^{++}. The residual hexokinase which was bound to the brain mitochondria before they were added (about 10% of the total enzyme) cannot account for this difference even if it remained completely bound (i.e. did not participate in the equilibrium).

Binding of Hexokinase to Mitochondria at 0° and 25°C

Preliminary experiments suggested that the binding of mitochondrial and cytoplasmic hexokinase to mitochondria might be

differentially affected by the temperature of incubation (J. Lusk, unpublished data). Crude cytoplasmic and glucose 6-phosphate-solubilized hexokinase were prepared and dialyzed against 0.25 M sucrose. Binding assays were carried out in duplicate with a 15 minute incubation either on ice or at 25°C. As can be seen in Table 6, neither mitochondrial or cytoplasmic hexokinase were substantially affected by changes in the temperature of incubation. When impure liver mitochondria were used (not shown), a variable amount of loss in bindability was observed at 25°C, presumably due to a contaminating lysosomal protease (87).

Titration of Hexokinase in a Fresh Brain Homogenate with Mitochondria

In order to test for the possibility that the poor ability of cytoplasmic hexokinase to bind to mitochondria results from changes in vitro, 0.2 units of total brain hexokinase was titrated with liver mitochondria immediately after homogenization of a rat brain (as described in Methods). Almost all of the hexokinase was particulate in the presence of 1 mg mitochondrial protein (Figure 21). Similar results were obtained when impure brain mitochondria were substituted (not shown). These results are not consistent with the presence of a substantial amount of hexokinase in a fresh brain homogenate that is incapable of binding to mitochondria.

Isoelectric Focusing of Hexokinase

The isoelectric focusing patterns of cytoplasmic and mitochondrial hexokinase were compared in fractions eluted from DEAE-cellulose. Mitochondrial (Figure 22A) and cytoplasmic (Figure 22B) hexokinase

Figure 20. Titration of hexokinase with mitochondria. Glucose 6-phosphate-solubilized hexokinase (26 munits) was incubated with the indicated amounts of liver mitochondrial protein and centrifuged. The % bound is the fraction of the recovered hexokinase located in the pellet.

Figure 21. Titration of brain homogenate hexokinase with mitochondria. A 0.1 ml aliquot of a 1:9 brain homogenate (200 munits hexokinase) was incubated with the indicated amounts of liver mitochondrial protein and centrifuged. The % particulate is the fraction of the added hexokinase recovered in the pellet.

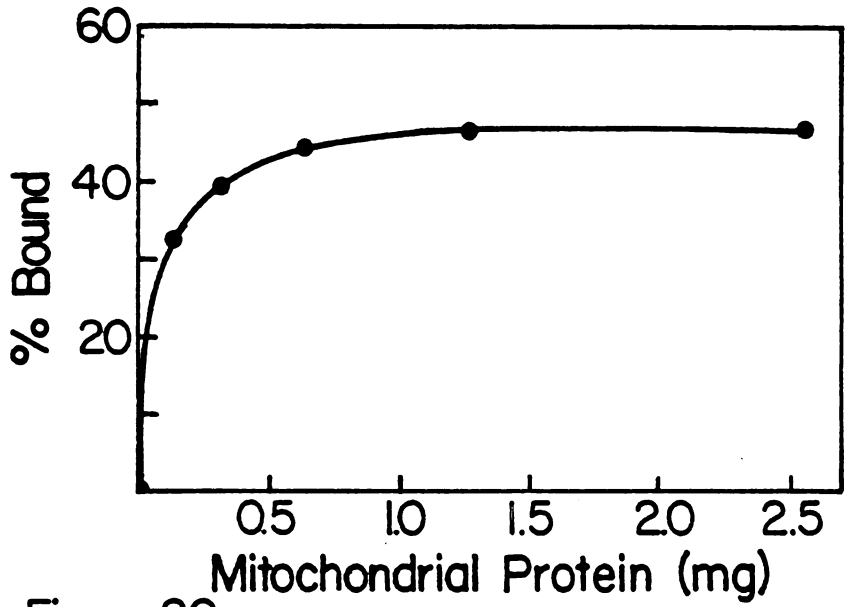

Figure 20

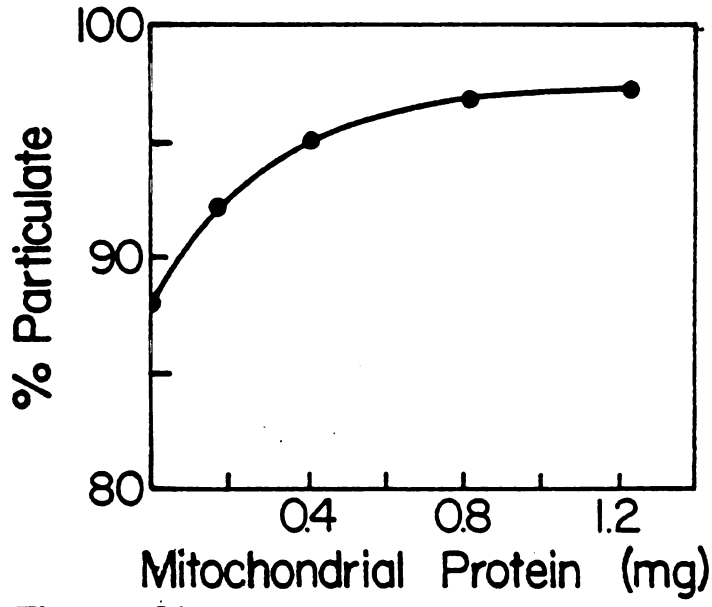

Figure 21

Table 5. Effect of Mg^{++} on the binding of hexokinase to mitochondria

	% hexokinase bound	
$[Mg^{++}]$, (mM)	Brain Mitochondria	Liver mitochondria
0	28	2.6
0.5	51	47
2.0	72	69

Highly bindable mitochondrial hexokinase (25.2 munits) was incubated with purified liver and brain mitochondria as described in Methods, using the indicated concentrations of $MgCl_2$. The tabulated values are the average % hexokinase bound in duplicate determinations.

Table 6. Binding of hexokinase to mitochondria, 0° and 25°C

	% hexokinase bound	
Hexokinase	0°C	25°C
Mitochondrial	66.0	66.5
Cytoplasmic	44.1	43.0

Crude cytoplasmic (25.7 munits) and glucose 6-phosphate-solubilized mitochondrial (25.7 munit) hexokinase was incubated with liver mitochondria as described in Methods, at either 25°C or on ice. The tabulated values are the average % hexokinase bound in duplicate determinations.

yield similar patterns, with variations only in the relative amounts of each band. It is possible that these variations as well as the difference in the binding of mitochondrial and cytoplasmic hexokinase to mitochondria are due only to the relative amount of proteolysis of each form which occurs during or after homogenization. A frozen brain was homogenized in 40 volumes of 0.25 M sucrose and an aliquot was immediately subjected to isoelectric focusing. Only a single band (pI = 6.35) was visible, indicating that the observed heterogeneity in pI was a consequence of post-homogenization events.

The isoelectric focusing and binding data indicate that the observed low binding ability of cytoplasmic hexokinase is due to an alteration of the enzyme in vitro which is qualitatively identical to the change which occurs with the mitochondrial enzyme.

Kinetic Pattern of Isoelectric Focusing Forms

Since cytoplasmic and mitochondrial hexokinase consist of different proportions of the isoelectric focusing forms, the reported difference in kinetic patterns (see Chapter 2) might be due to differences between these forms. Fraction of mitochondrial hexokinase from a DEAE-cellulose column enriched in bands with pI = 6.45 (>85%) and pI = 6.35 (>95%) were analyzed by the substrate depletion kinetic method. The resulting patterns were identical (Figure 23).

Effect of Sucrose in Assay on Enzyme Latency and Distribution

When isolated synaptosomes (113) were assayed for a soluble enzyme (lactate dehydrogenase) only 50% of the activity was latent (releasable by Triton X-100), indicating that only half of the synaptosomes were

Figure 22. Isoelectric focusing of hexokinase. Fractions from a DEAE-cellulose column were subjected to isoelectric focusing and stained for hexokinase activity. The first lane is an aliquot of the enzyme which was loaded onto the column, the other lanes were taken from successive fractions which span the peak of hexokinase activity. Approximately 7.5 munits was loaded in each lane.

A. Mitochondrial hexokinase (from the work of P. Polakis).

B. Cytoplasmic hexokinase.

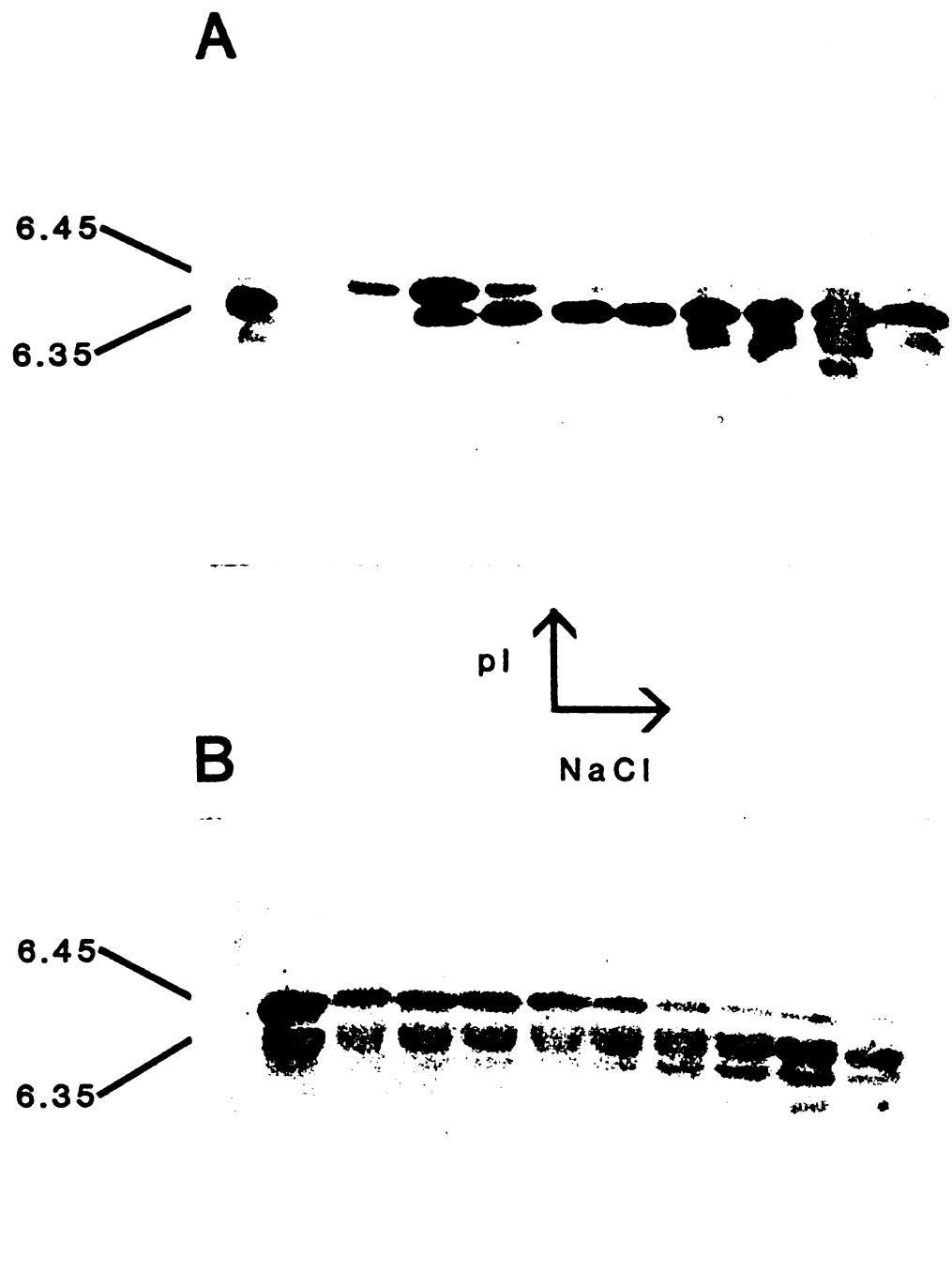

Figure 22

Figure 23. Kinetic patterns of isoelectric forms. Lineweaver-Burk plots were prepared from substrate depletion kinetic data. Glucose concentrations ranged from 10-90 μM. Each line represents data collected at a different constant ATP concentration (0.15, 0.20, 0.33, and 1.00 mM). The isoelectric forms of mitochondrial hexokinase were separated by chromatography on DEAE-cellulose.
A. Hexokinase, pI = 6.45 (> 85%). B. pI = 6.35 (> 95%).

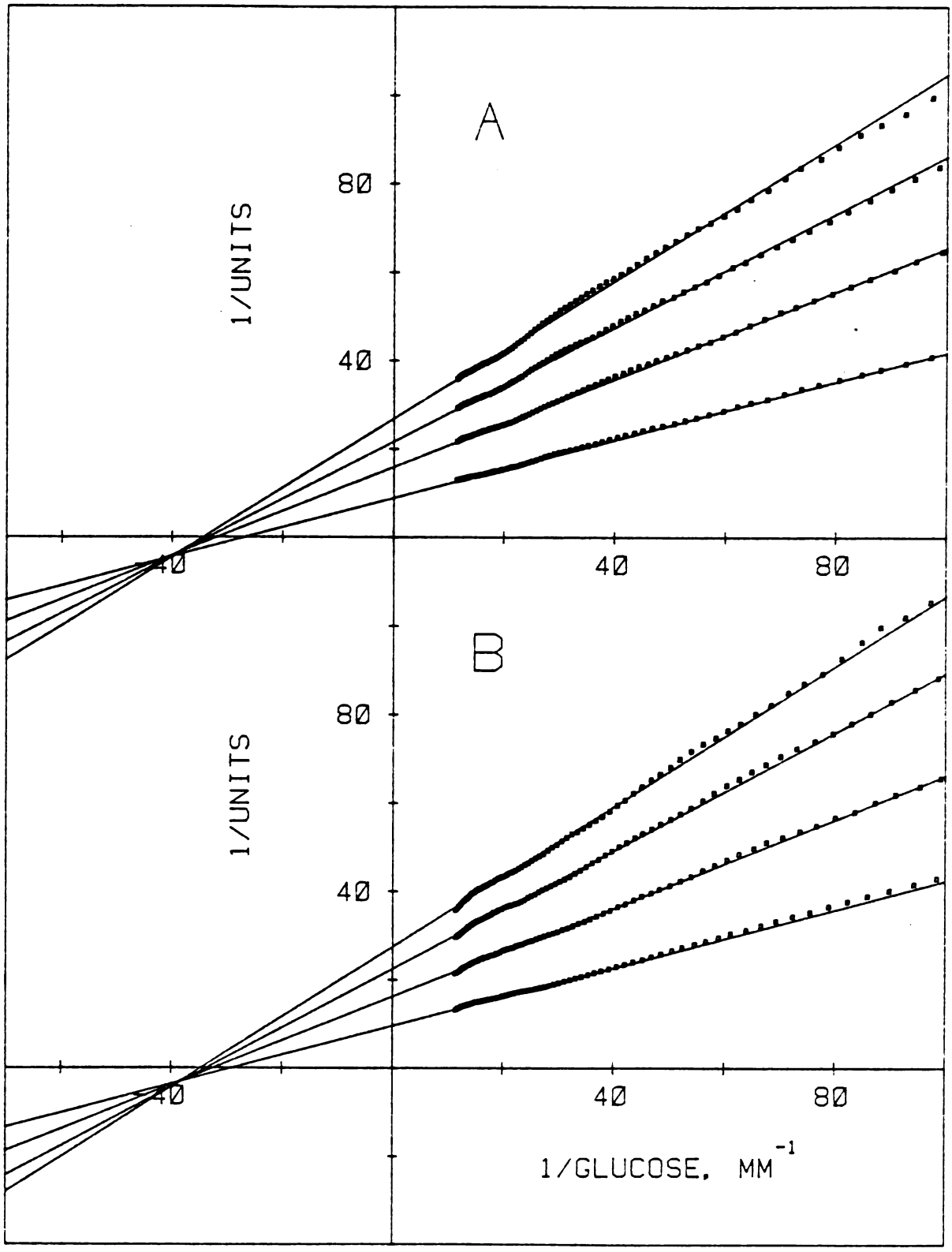

FIGURE 23

intact (not shown). However, when these same synaptosomes were centrifuged, all of the lactate dehydrogenase activity was particulate. This discrepancy may be due to breakage of the synaptosomes when they are placed in a hypo-osmolar environment such as assay mix (93) and would result in an overestimation of overt lactate dehydrogenase activity (i.e. the activity measureable in the absence of Triton X-100).

The effect of synaptosomal breakage on enzyme latency was determined by assaying in the presence or absence of 0.3 M sucrose. Lactate dehydrogenase was assayed in the particulate fraction of a brain homogenate (Table 7). In the presence of 0.3 M sucrose only 10% of the enzyme was overt, representing the upper limit of synaptosomal breakage at this sucrose concentration. When assayed under normal conditions (no sucrose), 44% of the activity was overt (i.e. over 1/3 of the synaptosomes were broken). Parallel measurements of hexokinase activity (Table 7) revealed a less drastic shift in latency when sucrose was added to the assay mix because a substantial fraction of the particulate enzyme is located outside of synaptosomes on free mitochondria.

The effect of this breakage of synaptosomes on the measured hexokinase distribution in the adult rat brain is shown in Table 8. Synaptosomal breakage resulted in a slight, but significant overestimation of free mitochondrial hexokinase if overt particulate activity is interpreted as being non-synaptosomal mitochondrial hexokinase.

Table 7. Effect of sucrose in assay on enzyme latency

	% overt activity	
Sucrose (M)	Hexokinase	Lactate dehydrogenase
0	51.2 ± 0.5	43.7 ± 1.7
0.3	40.6 ± 0.8	10.3 ± 0.4

Overt and total (measured in the presence of 0.5% Triton X-100) hexokinase and lactate dehydrogenase activity were assayed in the particulate fraction of a brain homogenate. The osmolarity of the assay medium was increased in some of the measurements by the addition of 0.3 M sucrose. The tabulated values are the average ± S.D. of the % overt activity in 3 brain homogenates.

Table 8. Effect of sucrose in assay on hexokinase distribution

	Hexokinase (units/g)				
Sucrose (M)	Overt	Latent	Particulate	Soluble	Total
0	8.64 ± 0.17	8.69 ± 0.25	17.3 ± 0.4	1.99 ± 0.21	19.3 ± 0.4
0.3	6.94 ± 0.59	9.21 ± 0.42	16.2 ± 1.0	1.99 ± 0.21	18.2 ± 1.0

The distribution of hexokinase in a brain homogenate was determined in the presence and absence of 0.3 M sucrose. The particulate activity was measured in the presence (particulate) or absence (overt) of 0.5% Triton X-100 and the latent activity was calculated as the difference. The tabulated values are the average ± S.D. of the hexokinase per g of brain found in each fraction.

DISCUSSION

The ability of hexokinase to bind to mitochondria was measured using an amount of mitochondria sufficient to provide 90-95% maximal binding. The accurate evaluation of binding constants and capacities requires a more rigourous analysis. Since the binding interaction appears to be an equilibrium, it should be possible to put titration data in a form that can be extrapolated to infinite mitochondria concentrations. Analysis is complicated by the presence of nonbindable enzyme that does not participate in the equilibrium. If the proportion of the enzyme that is nonbindable is exactly known and corrected for, binding titration data can be linearized. If the proportion is fairly small and the data good, a reiterative curve fitting procedure could be used. If the proportion is zero (all of the enzyme is bindable) the data can be analyzed directly without correction. Preliminary experiments using Schatchard analysis (91) of uncorrected binding data obtained with highly bindable mitochondrial enzyme (selectively taken from a DEAE-cellulose column) resulted in linear plots from which both equilibrium constants and binding capacities of the mitochondria under a variety of conditions could be extracted. This approach to the study of the hexokinase-mitochondria binding interaction should prove fruitful once large amounts of reproducibly bindable hexokinase are available.

The marked Mg^{++} dependence of binding to liver, but not brain mitochondria is another indication of the differences between the mitochondria from these sources (30,51). Variations in the ability of mitochondria in different locations to bind hexokinase may partially

explain variations in the intracellular distribution of hexokinase (see below).

In harmony with the results of Chapter 2 the experiments in this chapter demonstrated the basic similarity between mitochondrial and cytoplasmic hexokinase. Both enzymes initially existed as a single isoelectic form (pI = 6.35) which was subsequently converted to a heterogeneous mixture, apparently due to the action of endogenous protease(s). Both enzymes were initially able to bind extensively to mitochondria, but partially lost this ability in parallel with the time-dependent shift in isoelectric focusing patterns. In addition neither enzyme showed a temperature-dependence of binding to mitochondria.

Kellogg et al. (45) interpreted the lack of correlation between particulate and soluble hexokinase during development as indirect support for the existence of a distinct cytoplasmic enzyme. The argument neglects the fact that all of the hexokinase trapped in synaptosomes is included in the particulate fraction whether it is bound to mitochondria or not, i.e. it cannot distribute in a homogenate according to its intracellular location. Moreover, the particulate hexokinase measured in the absence of Triton X-100 is not a good measure of nonsynaptosomal hexokinase because a fairly large fraction of the synaptosomes break when placed in hypo-osmolar assay mix. Since much of the increase in particulate activity during development is probably synaptosomal (65), the actual change in mitochondrial-soluble distribution would be much less striking than the change observed by Kellogg et al. (45). Similar arguments apply to the different

hexokinase distribution observed in white and gray matter (8) which also differ in synaptosomal content.

The results presented here make it very unlikely that there is, in fact, a distinct cytoplasmic form of brain hexokinase. It is therefore concluded that differences in the distribution of hexokinase between mitochondrial and cytoplasmic locations must be governed by factors other than intrinsic differences in the enzyme that is found in these subcellular compartments. A hexokinase binding protein has been isolated from the outer membranes of liver mitochondria (29) and is presumed to be present in other mitochondria capable of binding hexokinase. It is conceivable that differences in relative amounts of this protein or in its affinity for hexokinase may exist between mitochondria in different neural cell types or cell regions (e.g. neuronal perikarya vs. nerve endings). Thus, a relative deficiency of this protein in mitochondria from astrocytes might explain the high proportion of hexokinase having a cytoplasmic location in these cells (64). Alternatively, since binding is sensitive to the levels of several function-related metabolites (116), it is apparent that the soluble-particulate distribution in different cell types or regions may reflect differences in steady state metabolite levels rather than in the enzyme or mitochondria _per se_.

In any case, it is now apparent that renewed attention must be directed at other factors governing the soluble-particulate distribution of hexokinase since the postulated existence of distinct cytoplasmic and mitochondrial forms, with intrinsic differences in binding ability, is no longer tenable.

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