CHARACTERIZATION OF THE HEXOKINASE MEMBRANE INTERACTION: RAPID PHOTOLYSIS OF DANSYL TYROSINE; PARTIAL CHARACTERIZATION OF BINDABLE AND NON - BINDABLE HEXOKINASE ISOZYMES

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ABSTRACT

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By

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Two of the three dansyl chloride derivatives of tyrosine have been identified and characterized by three independent methods. These two products are the mono- and di-dansylated forms of tyrosine. The mono-dansylated species has the dansyl group attached to the primary amine and the di-dansylated product is labeled at the phenolic oxygen and at the primary amine. N-ethyl morpholine, the standard solvent for protein dansylation as suggested by Gray (Gray, W. R., Methods in Enzymology, 25, 121 [1972]) was found to give an unexpectedly high yield of mono-dansylated tyrosine; therefore sodium bicarbonate was used as buffer since this was found to favor complete conversion to the di-dansyl product. Under these circumstances, the N-terminal amino acid of rat brain hexokinase (ATP:D-hexose-6-phosphotransferase, E.C.2.7.1.1) was found to be tyrosine, not glycine, as previously reported (Chou, A. C., Wilson, J. E., Arch. Biochem. Biophys., 151, 48 [1972]). Chymotrypsin treatment of this enzyme exposed two new N-terminals, phenylalanine and lysine, completely removing tyrosine. And, finally, it has been shown that di-dansyl tyrosine has an unusually

high photolysis rate under U.V. light, a property which can easily lead to complications in interpretation of experimental results unless measures are taken to restrict photolytic degradation.

Type I rat brain hexokinase purified by the method of Chou and Wilson (Chou, A. C., Wilson, J. E., Arch. Biochem. Biophys., 151, 48 [1972]) has been shown to consist of at least two isozymes. One of these isozymes (type I_b) binds to mitochondrial membranes and the other (type I_n) does not. Recently discovered peculiarities in the binding assay have obscured this discovery for some time. (i) Hexokinase binds to the polypropylene microcentrifuge tubes routinely used for the binding assay. (ii) There is an unexpected inhibition of binding by relatively low concentrations of salt. (iii) The routinely used rat liver mitochondria bind less hexokinase and are more susceptible to the salt effect than rat brain mitochondria.

A peptide(s) can be removed from pure hexokinase, at least part of which comes from the N-terminal end and may be involved in the binding process. This peptide(s) awaits further characterization.

The phosphate-induced reversal of solubilization first demonstrated by Rose and Warms (Rose, I. A., and Warms, J. V. B., J. Biol. Chem., 242, 1635 [1967]) can also be done with potassium chloride. This salt induced rebinding suggests a hypothesis for the binding mechanism. Further experiments will be required to ascertain whether or not the salt-induced rebinding is due to identical factors necessary for phosphate-induced rebinding.

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ABBREVIATIONS

ATP	Adenosine triphosphate
BSA	Bovine serum albumin
Dansyl-Cl	1-dimethylaminonaphthalene-5-sulfonyl chloride
DEAE-cellulose	Diethylaminoethyl-cellulose
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
EDTA	Ethylenediamine tetraacetate
G-6-P	Glucose-6-phosphate
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HK	Hexokinase
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
SDS	Sodium dodecyl sulfate
Tris	Tris-(hydroxymethyl) aminomethane

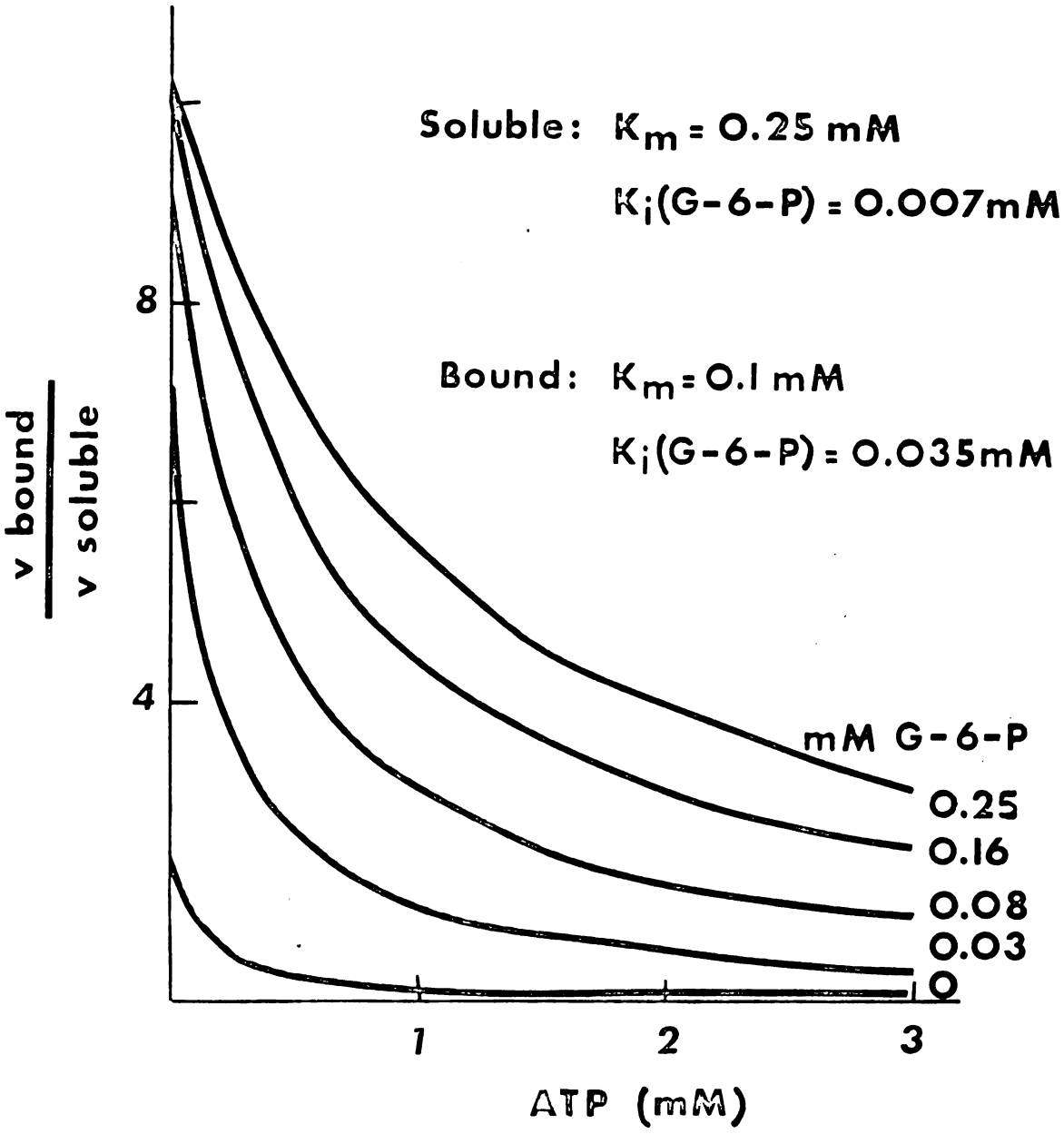
BACKGROUND AND PURPOSE OF THE PROJECT

Background

In 1953 Crane and Sols (1) observed that a major portion of the hexokinase activity in mammalian brain was associated with a particulate fraction. Johnson's report that the particulate enzyme was bound to mitochondria (2) was subsequently confirmed by other workers (3-8). It was later reported by Kropp and Wilson (9) for liver and by Craven and Basford (6) for brain that the enzyme was located on the outer-mitochondrial membrane.

Wilson and others found that in the presence of glucose-6-phosphate (1 mM), ATP (10 mM), or high ionic strength (200 mM) the enzyme could be solubilized (1, 8, 10). Neither ADP, other 6-phosphosugars, nor low salt could solubilize the enzyme. Because of this specificity and because glucose-6-phosphate and ATP are end products of hexokinase and glycolysis, respectively, it was suggested the ATP/G6P-induced soluble/particulate equilibrium played a regulatory role *in vivo* (8, 10). In support of this view, it was determined that the bound enzyme was less susceptible to inhibition by glucose-6-phosphate and had a greater affinity for ATP (11). Calculations based on the Michaelis-Menten equation (see Figure 1) indicated that a shift from soluble to bound states under "physiological" substrate concentrations (12, 13) would result in a 3-4 fold increase in the hexokinase catalytic rate. This compared

Figure 1.--Relative activity of soluble and bound forms of brain hexokinase. The ordinate gives the velocity of the reaction catalyzed by the bound (mitochondrial) form relative to the velocity for the soluble form of hexokinase as a function of concentration of the substrate ATP (indicated on the abscissa) and of the inhibitory product, glucose-6-phosphate. Values for K_m (ATP) and K_i (G6P) were obtained from Tuttle and Wilson (11).


Soluble: K_m = 0.25 mM
K_i(G-6-P) = 0.007mM
Bound: K_m = 0.1 mM
K_i(G-6-P) = 0.035mM
v bound / v soluble
8
4
1
2
3
ATP (mM)
mM G-6-P
0.25
0.16
0.08
0.03
0

favorably with the estimated 3-7 fold increase in the glycolytic flux during ischemia (12) or convulsions (14). It was also calculated that it is precisely under "physiological" substrate concentrations where the difference between the velocities of the two forms is greatest (see Figure 2). Furthermore, Knull et al. (15, 16) have shown that disturbances of energy metabolism in chick brain (caused by ischemia, insulin shock, or high blood galactose levels) result in altered glucose-6-phosphate levels and soluble/particulate hexokinase distribution in accord with the hypothesis (10). All these data taken together provide considerable support for the proposed regulatory role of the ATP/G6P-induced soluble/particulate equilibrium.

Purpose

The objective of this project has been to describe in greater detail the nature of the interaction between rat brain hexokinase and mitochondria. While progressing toward this goal, two new hexokinase isozymes were isolated in pure form; type I_b binds to mitochondria and type I_n does not. This topic is covered in Chapter II.

Chapter I is concerned with technical difficulties in N-terminal determination by the dansyl chloride method.

Figure 2.--Difference between the catalytic activities of soluble and bound forms of brain hexokinase. The ordinate gives the difference between the velocities of the reactions catalyzed by the bound (B) and soluble (S) forms of brain hexokinase, i.e., $\Delta v = v_B - v_S$. This difference is expressed in terms of the maximum attainable velocity (v_T), which would be observed at saturating levels of ATP (at which the Δv would be zero). ATP concentration is given on the abscissa, and the curves are drawn for three different glucose-6-phosphate concentrations, indicated on the curves. Maximal differences between the soluble and bound forms are seen to occur at ATP and glucose-6-phosphate concentrations in the physiological range (2.5 mM and 0.16 mM, respectively [12, 13]), which would be consistent with variation in soluble/particulate distribution providing a sensitive mechanism for control of hexokinase activity.

CHAPTER I

RAPID PHOTOLYSIS OF DANSYL TYROSINE

Introduction

The problems covered in this chapter concern some peripheral discoveries regarding technical difficulties in N-terminal determination by the dansyl chloride method. This problem was initiated to determine from which end of hexokinase a peptide was clipped by the action of chymotrypsin. (See Figure 8, Chapter II, for the effect of chymotrypsin on bindable hexokinase.) Since published data on the identity of the N-terminal of pure hexokinase was being contradicted (25), an exhaustive study was necessary.

Materials and Methods

Chemicals

Dansyl chloride, L-tyrosine and standard dansyl amino acids were obtained from Sigma Chemical Company. Sodium dodecyl sulfate (sequenation grade), N-ethyl morpholine (sequanal grade), and dimethylformamide (silylation grade) were purchased from Pierce Chemical Company (Rockford, Illinois). U-[^{14}C]-L-tyrosine and G-[^{3}H]dansyl chloride were products of Amersham/Searle Corporation. 1-nitroso-2-naphthol came from Mallinckrodt Chemical (St. Louis, Missouri) and Cheng-Ching polyamide layer sheets were obtained from Gallard-Schlesinger Chemical Mfg. Corp. (Carle Place, New York). All chemicals were reagent grade if not otherwise specified.

Dansylation

Free tyrosine was dansylated essentially according to the method of Gros and Labouesse (17) with some minor modifications. 10 µl of 1 mM tyrosine (raised to pH 10 to promote solubilization) was added to 20 µl of 100 mM sodium bicarbonate, pH 9.5. The reaction was started by adding 30 µl of 10 mM dansyl chloride in acetone. After one hour at room temperature the reaction was stopped with 50 µl 0.1 N NaOH and the solution acidified with 20 µl 6 N HCl. After removal of acetone under a stream of nitrogen, the samples were completely dried on a rotary evaporator at 35°C, suspended in 10 µl 40% acetic acid-acetone and then chromatographed on 5 x 5 cm polyamide layer plates according to Weiner et al. (18). In some experiments, 100 mM sodium borate or 100 mM sodium phosphate at various pHs replaced the bicarbonate.

The method outlined by Gray (19) for dansylation of proteins was routinely followed with three modifications: N-ethyl morpholine was replaced by 500 mM sodium bicarbonate, pH 9.5; protein samples were not performic acid oxidized, the dansylated protein sample was washed more extensively. The procedure was as follows: 50-250 µg of lyophilized protein was placed in a 6 x 50 mm culture tube and dissolved in 50 µl of 1% SDS by heating in a boiling water bath for about five minutes. If the protein sample contained buffer or salts it was dialysed overnight against 2 liters of water before lyophilization. After cooling, 50 µl of 500 mM sodium bicarbonate, pH 9.5, followed by 75 µl of a 25 mg/ml dansyl chloride solution in dimethyl formamide were added. The test tube was vortexed, covered

with parafilm and incubated at room temperature for at least one hour. Since the dansyl chloride was not entirely soluble in this aqueous solution the reaction mixture had a cloudy appearance that cleared as the reaction went to completion, but this did not affect the reaction. One volume of 20% TCA (w/v) was added to stop the reaction and precipitate the protein. The precipitated protein was washed once with 0.2 ml 1N HCl and once with 80% acetone:water. To the dried precipitate was added 50 µl constant boiling HCl, and the culture tube was sealed in vacuo and placed in a boiling toluene bath at 110°C for 18 hours. The dried hydrolysate was dissolved in 10 µl 2:3, acetic acid:acetone, spotted on 5 x 5 cm polyamide layer sheets and chromatographed according to Weiner et al. (4).

Photolysis of Di-Dansyl Tyrosine on Thin Layer Plates

Radioactive di-dansyl tyrosine was prepared from commercial tyrosine and radioactive dansyl chloride as described above. The dansylated tyrosine was chromatographed in two dimensions and the well resolved di-dansyl tyrosine spot was irradiated with an ultraviolet light source (100 watt mercury spot lamp from General Electric with a long wave length filter) for various time intervals. The plates were placed exactly 20 cm from the lamp, and centered in the most intense portion of the beam. At this distance from the source, the temperature was 35°C which was 16°C above ambient temperature.

Results

Characterization of Two Dansylated Forms of Tyrosine

From Figure 3, it is clear that commercially obtained di-dansyl tyrosine (top two chromatograms) can contain numerous chromatographically distinct fluorescent species. Further characterization of some of these was considered necessary because published data on their identity was either scarce or absent. Based on the literature, compound II (Figure 3) was di-dansyl tyrosine and compound I was N-dansyl tyrosine (20). To confirm this, three experiments were performed.

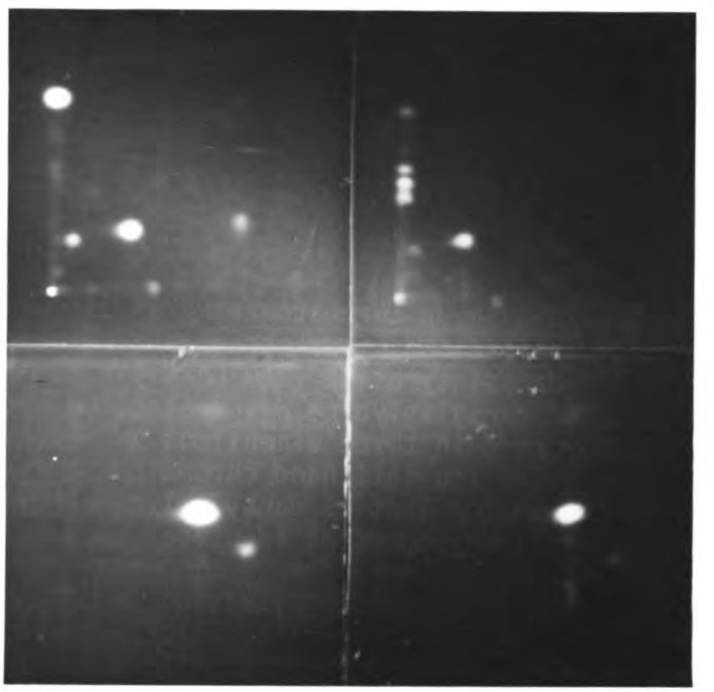
(i) Since dansyl chloride reacts with unprotonated amines and with unprotonated phenols, the reactivity of either group depends both on the pH of the reaction mixture and on the pK of the reactive group (17, 20). Since the pK of the phenolic hydroxyl on tyrosine is 10.1 and the pK of the amino group is 9.1, the amino group should be more reactive at lower pHs than the phenolic hydroxyl. Table 1 indicates that as the pH of the reaction was decreased, relatively more compound I was formed than compound II. This was consistent with the proposal that compound II was di-dansyl tyrosine and compound I was N-dansyl tyrosine.

(ii) 1-nitroso-2-naphthol in the presence of nitric acid has been shown to react specifically with the phenolic group on tyrosine to give a red colored product (21). When they were sprayed with this reagent, compound I reacted to give a red color and compound II did not. This strongly indicated that the phenolic group on compound II was blocked and on compound I was not.

Figure 3.--Thin layer chromatography of dansylated tyrosines and dansyl glycine, before and after photolysis. Approximately 0.5 µl of commercial di-dansyl tyrosine (250 µg/ml in 40% acetic acid: acetone) or 0.5 µl of dansyl-glycine was spotted on a thin layer plate and chromatographed in three dimensions as described by Weiner et al. (18). The top left hand chromatogram is didansyl-tyrosine (A) and the bottom left is dansyl glycine (C). The chromatograms on the right represent identical amounts of each amino acid derivative, photolysed for 20 min after the second dimension as described in the methods section and then chromatographed in the third solvent in order to resolve the amino acid derivative from its degradation products. Plate B is photolysed tyrosine; plate D is photolysed glycine. The drawing that accompanies the photograph identifies all the spots on plates A and B.

1. Di-dansyl tyrosine (compound II)
2. N-dansyl tyrosine (compound I)
3. O-dansyl tyrosine (not visible on these plates)
4. Dansyl amine
5. Dansyl sulfonic acid
6. Photolysis products

The identities of O-dansyl tyrosine, dansyl amine and dansyl sulfonic acid are well supported in the literature (18, 19, 20).



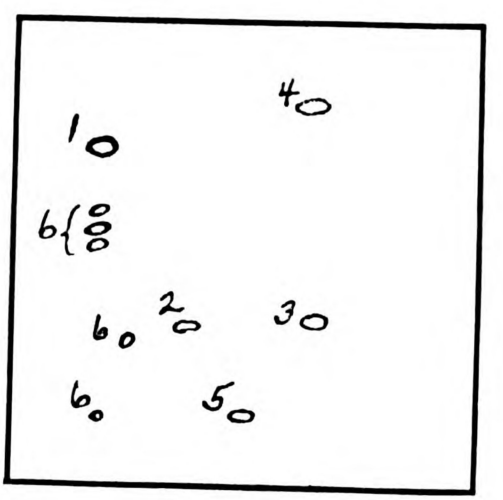

4₀
1₀
6{0 0 0
6₀ 2₀ 3₀
6₀ 5₀

TABLE 1.--The relative amounts of the three dansyl tyrosine derivatives produced at various pHs. Dansylation was carried out as described in the methods section except that 1 μCi of [^{3}H]dansyl chloride was mixed with 0.5 ml cold dansyl chloride before adding it to buffered tyrosine. Samples were chromatographed as in Figure 1 and the appropriate spots scraped off, placed into scintillation solution (4 gm PPO; 0.05 gm POPOP; 1 liter toluene), mixed and counted in a Packard Tri-Carb liquid scintillation counter. (n.d. = not detected.)

Buffer		% di-dansyl tyrosine	% N-dansyl tyrosine	% O-dansyl tyrosine
33 mM Na-phosphate	pH 7.2	50	50	n.d.
33 mM Na-phosphate	pH 8.0	62	38	n.d.
33 mM Na-bicarbonate	pH 8.3	70	30	n.d.
33 mM Na-bicarbonate	pH 9.5	99	1	n.d.
33 mM Na-bicarbonate	pH 10.5	99	1	n.d.
33 mM Na-Borate	pH 8.5	99	1	n.d.
33 mM Na-Borate	pH 9.5	99	1	n.d.

(iii) An experiment was performed to determine whether compound II in fact contained twice as many dansyl groups as compound I. [^{14}C]Tyrosine was dansylated with [^{3}H]dansyl chloride at a low pH so that both dansylated products were generated. Under these circumstances, the tritium to carbon-14 ratio should be twice as large for the di-dansyl tyrosine as for N-dansyl tyrosine. The data in Table 2 indicate that this was nearly the case.

The data from all three experiments indicate that the identity of derivates I and II is as given in Figure 3.

TABLE 2.--The determination of the ratio of dansyl groups to tyrosine on compounds I and II. Dansylation was carried out as described in the methods section except that 10 mM [^{3}H]dansyl chloride (1 μCi/0.2 ml) and 1 mM [^{14}C]tyrosine (1 μCi/0.1 ml) were used. Samples were chromatographed as in Figure 3 and the appropriate spots scraped off, placed into scintillation solution (3300 ml; 200 gm naphthalene; 20 gm PPO; 1.6 gm dimethyl POPOP; 2000 ml xylenes; 1,100 Triton X-114), mixed and counted in a Beckman LS 230 liquid scintillation counter. Appropriate corrections were made from standard curves (provided by Dr. A. J. Morris) to account for counting efficiency and carbon-14 spill into the tritium window. Two additional experiments were performed with essentially similar results.

Compound	DPM		
	^{3}H	^{14}C	^{3}H/C^{14}
Di-dansyl tyrosine	2566	3569	0.72
Amino-dansyl tyrosine	1964	4672	0.42
Dansyl amine	315	12	41
Dansyl sulfonic acid	16032	390	26
Random scrapings	21	9	--

Effect of N-Ethyl Morpholine as Buffer in the Dansylation Reaction

To buffer the dansylation reaction mixture, Gray (19) used 2.3 M N-ethyl morpholine. This buffer has detergent-like characteristics to help solubilize protein and it keeps the H_2O concentration low. Table 3 indicates, however, that the use of N-ethyl morpholine during dansylation of free tyrosine can lead to very high levels of N-dansyl tyrosine because as the buffer concentration is increased more N-dansyl tyrosine is produced. Table 4 illustrates that this same phenomena, though not as pronounced, is observed

TABLE 3.--The relative amounts of the three dansyl tyrosine derivatives at various concentrations of N-ethyl morpholine. The experiment was performed exactly as described under Table 1 with the indicated concentrations of N-ethyl morpholine. (n.d. = none detected.)

Buffer	% di-dansyl tyrosine	% N-dansyl tyrosine	% O-dansyl tyrosine
33 mM N-ethyl morpholine pH 10.3	44	56	n.d.
133 mM N-ethyl morpholine pH 10.3	42	58	n.d.
533 mM N-ethyl morpholine pH 10.3	29	71	n.d.
2230 mM N-ethyl morpholine pH 10.3	1	99	n.d.

TABLE 4.--Comparison of Na-bicarbonate and N-ethyl morpholine as buffers for N-terminal determination of hexokinase. Dansylation was performed as described in the methods section except that 10 μCi of [^{3}H]dansyl chloride was added to the reaction mixture. Spots were cut out and counted as described under Table 1.

Buffer	% di-dansyl tyrosine	% N-dansyl tyrosine	% O-dansyl tyrosine
215 mM Bicarbonate pH 9.5	99	1	--
230 mM N-ethyl morpholine pH 10.3	37	63	--

with hexokinase and demonstrates conditions under which 99% di-dansyl tyrosine can be generated.

Photolysis of Tyrosine on Thin Layer Plates

Figure 4 shows the rapid U.V. light catalysed photodegradation of di-dansyl tyrosine on thin layer plates. The half time for photolysis under these conditions was 3-4 minutes. Figure 3 indicates that di-dansyl tyrosine is photolysed more rapidly than either dansyl glycine or N-dansyl tyrosine, suggesting that dansyl groups attached to amines are more stable than those attached to phenols.

Hexokinase N-Terminal Determination

The reliability of the revised method for N-terminal determination was evaluated by checking N-terminals on several commercial standard proteins. Lysozyme, cytochrome-c, RNAse, hemoglobin, aldolase, and KDPG aldolase (from W. A. Wood) all unambiguously gave the expected N-terminal amino acid.

Using this method, it was determined that hexokinase, purified by the method of Chou and Wilson (25), contained mostly tyrosine, not glycine, as reported previously (25). Quantitation of the dansylated amino acids obtained using tritiated dansyl chloride gave 85% tyrosine, 10% phenylalanine and 5% leucine. The leucine and phenylalanine spots have consistently appeared in many preparations but never exceed 5% and 10%, respectively. This result was verified using the Edman procedure (kindly performed by

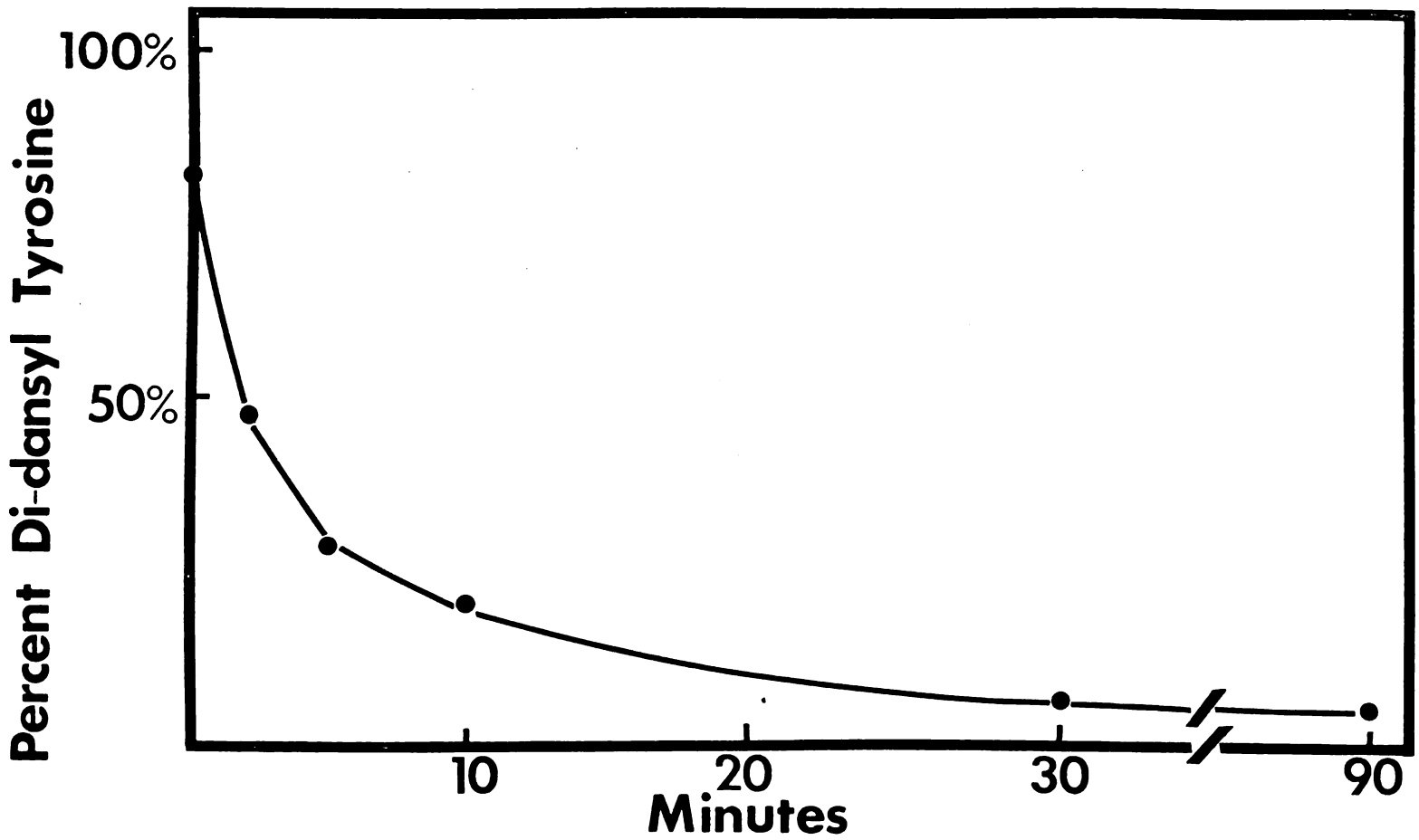


Figure 4.--Time course for the photolysis of di-dansyl tyrosine. Radioactive dansyl tyrosine was produced exactly as described under Table 1 and photolysed for various time intervals as described in the methods section. The ratio of di-dansyl tyrosine remaining to its photolysis products (see Figure 3) was used to calculate the % di-dansyl tyrosine remaining (ordinate).

Dr. Dean Ersfeld, working in the laboratory of Dr. W. A. Wood). By this method the N-terminal was predominantly tyrosine, with faintly observable traces of phenylalanine and leucine.

The N-terminal of chymotrypsin treated pure hexokinase (see Figure 15, Chapter II of this thesis) was found to be about 50% phenylalanine and 50% lysine. The tyrosine was completely removed.

Discussion

In order to avoid hydrolysis of dansyl chloride and to help solubilize the protein, Gray (19) suggests N-ethyl morpholine to buffer the dansylation reaction. Table 3, however, indicates that such high concentrations (greater than 2 molar) of N-ethyl morpholine interact with tyrosine to inhibit the reaction of dansyl chloride with the phenolic hydroxyl. Because of its low pK_a (7.70), low concentrations of N-ethyl morpholine cannot successfully buffer the reaction around the optimum pH of 9.5, also leading to multiple tyrosine derivatives (Table 1). The N-ethyl morpholine effect is seen both with the free amino acid and protein (Tables 3 and 4), so it seems advisable to use pH 9.5 sodium bicarbonate which gives only the di-dansylated product. The increased water content of the reaction mixture did not give any obvious problems with the technique.

All the relevant dansylated amino acids except N-dansyl tyrosine can be purchased as pure standards from various chemical companies (e.g., Sigma Chemical Company), suggesting that a method is needed to synthesize N-dansyl tyrosine. These data indicate

that N-ethyl morpholine could be used to devise a simple preparation of pure N-dansyl tyrosine in excellent yields.

Pouchan and Passeron (22) found that dansyl glycine and dansyl proline (both mono-dansylated in the N-position) underwent photodegradation on thin layer plates at identical rates. The relatively mild photolysis conditions in Figure 4 (22, 23) were sufficient to degrade most of the di-dansyl tyrosine in 10 minutes while leaving dansyl glycine largely intact (Figure 3). These data indicate that in a dansyl protein hydrolysate exposed to excessive U.V. light, an N-terminal amino acid such as glycine, present in relatively low amounts, could be erroneously assigned the N-terminal in preference to tyrosine. Or in a similar sample, an N-terminal tyrosine might be entirely lost due to excessive exposure to U.V. light, leaving one to conclude that the N-terminal is blocked. In addition, the photolysis products of tyrosine degradation lead to numerous spots that complicate interpretation of any chromatogram. Figure 3 shows that this problem is more obvious with tyrosine than glycine.

All of the previously mentioned difficulties have been experienced with rat brain hexokinase and the N-terminal was found to be tyrosine, not glycine as reported by Chou and Wilson (25). A complete explanation for their result is not available, but I have found that the Woods and Wang (24) method that was used does not remove some trace amino acid contaminants from the dansylation reaction that show up in the dansyl protein hydrolysate. Among these contaminants is a prominent glycine which could have been

confused for the N-terminal. And in view of all the difficulties I have uncovered regarding the rapid photolysis and consequent loss of dansyl tyrosine (Figures 3 and 4), it is not hard to imagine why this amino acid was not observed.

Summary

Two of the three dansyl chloride derivatives of tyrosine have been identified and characterized by three independent methods. These two products are the mono- and di-dansylated forms of tyrosine. The mono-dansylated species has the dansyl group attached to the primary amine and the di-dansylated product is labeled at the phenolic oxygen and at the primary amine. N-ethyl morpholine, the standard solvent for protein dansylation as suggested by Gray (19), was found to give an unexpectedly high yield of mono-dansylated tyrosine; therefore, sodium bicarbonate was used as buffer since this was found to favor complete conversion to the di-dansyl product. Under these circumstances, the N-terminal amino acid of rat brain hexokinase was found to be tyrosine, not glycine, as previously reported (25). Chymotrypsin treatment of this enzyme exposed two new N-terminals, phenylalanine and lysine, completely removing tyrosine. And finally, it has been shown that di-dansyl tyrosine has an unusually high photolysis rate under U.V. light, a property which can easily lead to complications in interpretation of experimental results unless measures are taken to restrict photolytic degradation.

CHAPTER II

PARTIAL CHARACTERIZATION OF BINDABLE AND NON-BINDABLE HEXOKINASE ISOZYMES

Introduction

The main thrust of this research topic has been to describe the nature of the interaction between rat brain hexokinase and mitochondria. To completely satisfy this query, it will be necessary to purify both membrane and enzyme components required in the binding process. This chapter deals with the purification of enzyme that can bind to mitochondria and the partial characterization of some requisite binding components on the enzyme.

Materials and Methods

Chemicals

Biochemicals and HEPES buffer were obtained from Sigma Chemical Company. DEAE-cellulose was purchased from Gallard-Schlesinger. Polypropylene microcentrifuge tubes were the product of Brinkmann Instruments. All other chemicals were reagent grade, obtained from commercial sources.

Adult male and female rats (ranging from 150-500 gm) of the Sprague-Dawley type were obtained from Spartan Research (Haslett, Michigan) and maintained on a common laboratory diet and water *ad libitum*.

Hexokinase Assay

Hexokinase activity was determined spectrophotometrically at 25°C ± 0.5 in an assay mixture containing 3.3 mM glucose, 6.7 mM ATP, 6.7 mM Mg Cl_2, 40 mM HEPES, 10 mM 1-thioglycerol, 0.64 mM NADP, and 2 units of glucose-6-phosphate dehydrogenase in a total volume of 1.0 ml (pH 7.5). The enzyme sample was added to an assay mixture containing all components except ATP, and oxidation of any endogenous glucose-6-phosphate was permitted to go to completion if necessary (up to approximately 3 min). Subsequently, the hexokinase assay was initiated by addition of 0.030 ml of 220 mM ATP solution (pH 7.3). NADPH formation was followed at 340 nm with a Turner Model 330 Spectrophotometer connected to a Sargent SRL Recorder. One unit is defined as the amount of enzyme which catalyzes the formation of 1 μmole of glucose-6-phosphate per minute.

Preparation of Mitochondria

Rat liver mitochondria were prepared by homogenizing the liver from a starved rat (15-17 hr, with water ad libitum) in 10 volumes (10 ml/gm tissue) cold 0.25 M sucrose with a Teflon-glass homogenizer. The homogenate was centrifuged at 600g for 10 min and the pellet discarded. The supernatant was centrifuged at 6,500g for 15 min and the supernatant discarded. The 6,500g pellet was resuspended in 10 volumes sucrose and centrifuged. The final pellet was resuspended in 2 volumes of 0.25 M sucrose and 1 ml aliquots stored at -20°C.

Crude rat brain mitochondria were prepared by homogenizing frozen brains (stored in liquid N_2) in 10 volumes of 0.25 M sucrose with a Teflon-glass homogenizer. The homogenate was centrifuged at 40,000g for 15 min and the supernatant discarded. The pellet was washed three times by rehomogenization in 10 volumes 0.25 M sucrose and centrifugation. The final pellet was suspended in 4 volumes 0.25 M sucrose and frozen at -20°C in 1 ml aliquots.

Glucose-6-Phosphate Solubilized Enzyme

Rat brains, frozen in liquid N_2, were thawed and homogenized in 0.25 M sucrose (10 ml/gm). The homogenate was centrifuged at 1000g for 10 min, and the pellet discarded. The 1000g supernatant was centrifuged at 40,000g for 10 min and the resulting pellet washed several times, depending on the experiment, by rehomogenization in 10 volumes 0.25 M sucrose and centrifugation. The washed pellet was resuspended in 10 volumes 0.25 M sucrose containing 1.2 mM glucose-6-phosphate and incubated for 30 min at 25°C. The solubilized enzyme was obtained in the supernatant after centrifugation at 40,000g for 10 min.

Purification of Rat Brain Hexokinase

The three times washed particulate fraction from 195 gms rat brain was prepared exactly according to Chou and Wilson (25). The resuspended particles were incubated with 1 mM glucose-6-phosphate at 25°C for 1 hour and centrifuged at 40,000g for 40 min. The supernatant, which contained solubilized hexokinase, was

decanted and potassium phosphate buffer (0.5 M, pH 7.0), glucose (0.5 M), Na_2EDTA (25 mM), and thioglycerol (12 M) were added to final concentrations of 0.01 M, 0.01 M, 0.5 mM, and 10 mM, respectively. The supernatant fluid was then concentrated by use of an Amicon ultrafiltration device with a PM-10 membrane, to about 300 ml and centrifuged a final time at 105,000g for 1 hour. This supernatant was applied to a DEAE-cellulose column and washed exactly according to Chou and Wilson (25). The enzyme was eluted from the column with a 600 ml linear gradient from 0 to 0.2 M KCl in column buffer, collecting 3.8 ml fractions. Overall recovery of glucose-6-phosphate solutilized hexokinase was 83%.

1% Sodium Dodecyl Sulfate-Polyacrylamide Disc Gel Electrophoresis

The method of Fairbanks et al. (26) was only slightly modified. Samples (1-5 mg/ml) were prepared in 1% sodium dodecyl sulfate, 5-10% sucrose, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA and 2% mercaptoethanol. They were then heated at 100°C for 15 min, 0.2% pyronin B tracking dye was added, and the samples were layered on 5.6% polyacrylamide gels (5 mm x 100 mm) prepared in tubes which had been coated with dimethyl dichlorosilane. Electrophoresis was performed at constant current of 4 ma/gel with a running time of about 4 hours. Gels were fixed and washed by agitating each gel in a 30 ml capacity test tube with 10% TCA/33% isopropanol. The washing solution was changed every two hours for three washes. The dehydrated gels were placed into 10% TCA until they regained their

original size and then were stained with xylene brilliant cyanin-G (K + K Laboratories, Inc., Plainview, N.Y.) according to published procedures (27, 28). The rather extensive gel wash removes SDS that interferes with the stain.

Mitochondrial Binding Assay

All assays were done in polypropylene microcentrifuge tubes coated with 2% BSA. The coating was done by dipping each tube in 2% BSA and drying in an oven at about 60°C. If the tubes were left uncoated, artifacts were introduced as explained below.

For the assay, aliquots of hexokinase and either liver or brain mitochondria were mixed in a microcentrifuge tube and 3 mM $MgCl_2$ was added. Tubes were incubated at 0°C for 15 min and spun at room temperature in an Eppendorf 3200 microcentrifuge for 2 min. Hexokinase activity in the supernatant was measured directly. Pellets were assayed after suspending them in a known volume of 0.5% Triton X-100 - 0.25 M sucrose by vortexing in the presence of glass beads (Sargent, No. S-61740, size A-7) until homogeneous. With each new mitochondrial preparation the number of binding sites per aliquot was approximated by titrating in a fixed volume of mitochondria with increasing hexokinase. In this way, it was always possible to determine when the binding sites were in excess for a given amount of hexokinase.

When purified enzyme, containing column buffer, was assayed for bindability to mitochondria, a 1 to 20 dilution into a 0.25 M sucrose-50 mM glucose was first made in order to lower the salt concentration.

Sephadex Column Chromatography of Chymotrypsin Treated Pure Hexokinase

In order to remove any small molecular weight fragments, before use pure hexokinase and alpha-chymotrypsin (Worthington iochemical Corporation) were individually passed over a Sephadex G-25 column that was equilibrated with DEAE column buffer (10 mM potassium phosphate pH 7.0, 10 mM glucose, 0.5 mM Na_2EDTA, 10 mM thioglycerol). Chymotrypsin (4 mg) was incubated at 25°C for 10 min with 1 mg hexokinase in a total volume of 15 mls. The sample was frozen and lyophilized down to 1.5 mls and 70% of the hexokinase activity was recovered. This mixture was sephadexed on a G-50 column that was equilibrated with DEAE column buffer. From this column, 100% of the remaining activity was recovered (total recovery equals 70%).

Results

Binding of Hexokinase to Polypropylene Centrifuge Tubes

Figure 5 shows that adding G6P-solubilized hexokinase to polypropylene centrifuge tubes decreases the activity. As the G6P-solubilized enzyme becomes more pure,[1] exposure to polypropylene leads to a greater activity loss. Figure 6 indicates that successive assays taken out of the same tube contain less hexokinase activity. Both of these problems are eliminated by coating the

[1]Albert Chou has shown that as mitochondria are washed more times the solubilized enzume obtained from them has a higher specific activity. (Personal communication.)

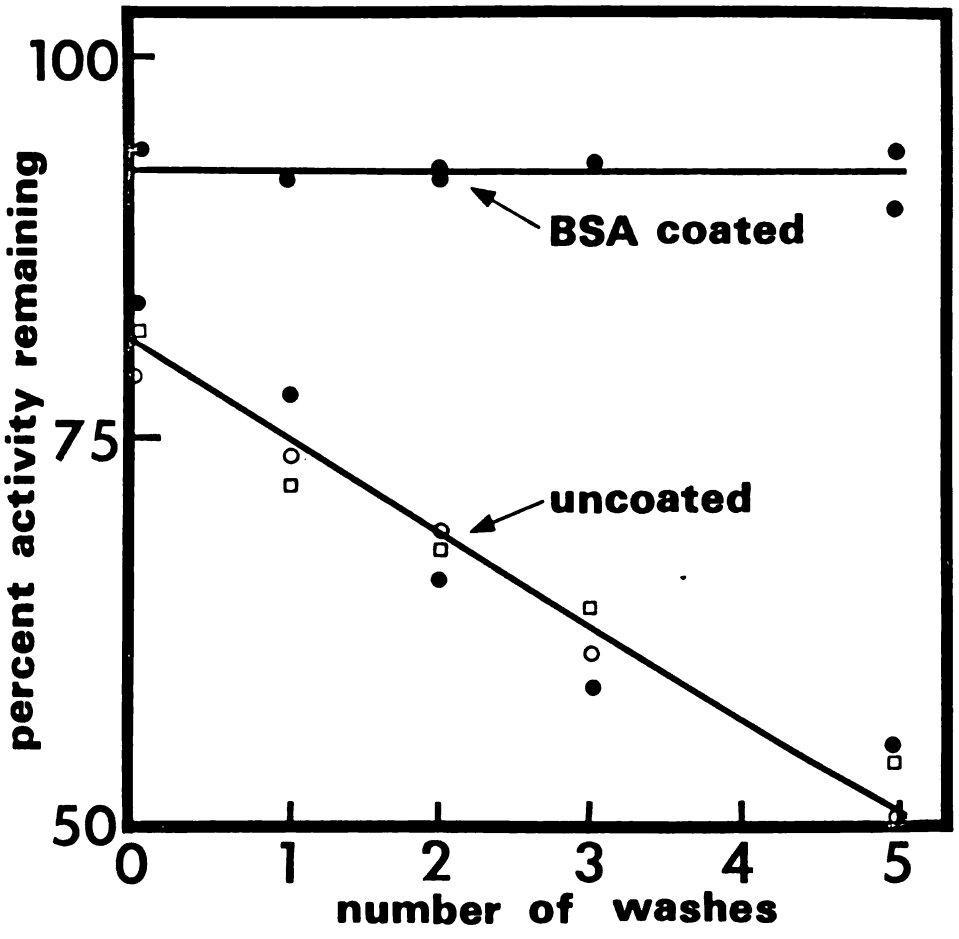


Figure 5.--Increased binding to polypropylene with increasing purity of hexokinase. G6P-solubilized hexokinase was obtained from brain particles that were washed various times as described under methods. Activities ranged from 0.56 units/ml (0 times washed) to 0.37 units/ml (5 times washed). As mitochondria are washed more often, the solubilized enzyme becomes more pure (see footnote 1). About 0.3 ml of the enzyme was added to and vortexed in BSA coated and uncoated tubes. Freshly prepared (●), 18 hours refrigerated (○), and 42 hours refrigerated (□) enzymes all responded the same.

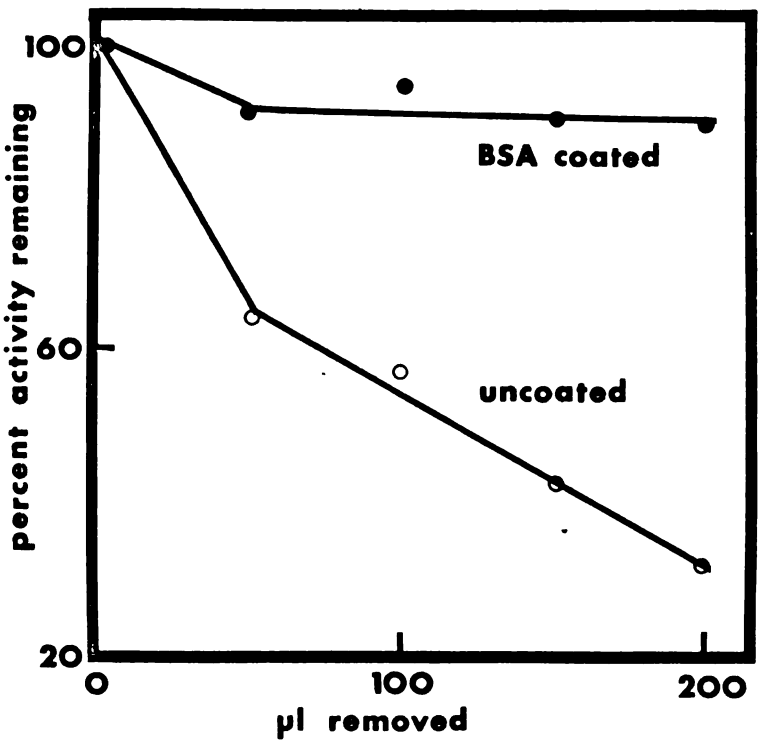


Figure 6.--Assaying successive aliquots from a polypropylene tube. 0.2 ml of hexokinase (0.43 units/ml) was put in a polypropolene tube and vortexed. 50 µl was removed for the first assay, 50 µl for the second point, and so on.

tubes with 2% BSA. Washing uncoated tubes with detergent, 1:1 chloroform:methanol, 6N HCl or 1% dichlorodimethyl silane in benzene, has no effect. The activity loss was too fast to determine any time dependence (faster than 1 min).

Figure 7 demonstrates that an aliquot vortexed in successive tubes progressively loses activity, suggesting that hexokinase binds to polypropylene. BSA coated tubes showed a markedly diminished effect.

It was demonstrated that hexokinase activity remains tightly bound in spite of washing. A tube containing hexokinase was rinsed several times with 0.25 M sucrose and hexokinase assay mix was added to the tube. Table 5 shows that less glucose-6-phosphate accumulated in the BSA coated tubes than in the uncoated tubes. These data prove that hexokinase can bind tightly to polypropylene and that BSA substantially reduces the effect.

TABLE 5.--Assay of the activity that remains bound to polypropylene tubes. G6P-solubilized hexokinase from twice washed particles (0.52 units/ml) was added to BSA coated or uncoated tubes, drawn out and the tubes then washed with 0.25 M sucrose. Hexokinase assay mix containing everything but glucose-6-phosphate dehydrogenase was added to the tubes and incubated at 25°C for 15 min. The "control" was an uncoated tube that did not have any hexokinase added.

	μMole G6P Formed in 15 min
Control	0.0
Uncoated tube	0.080
BSA coated	0.035

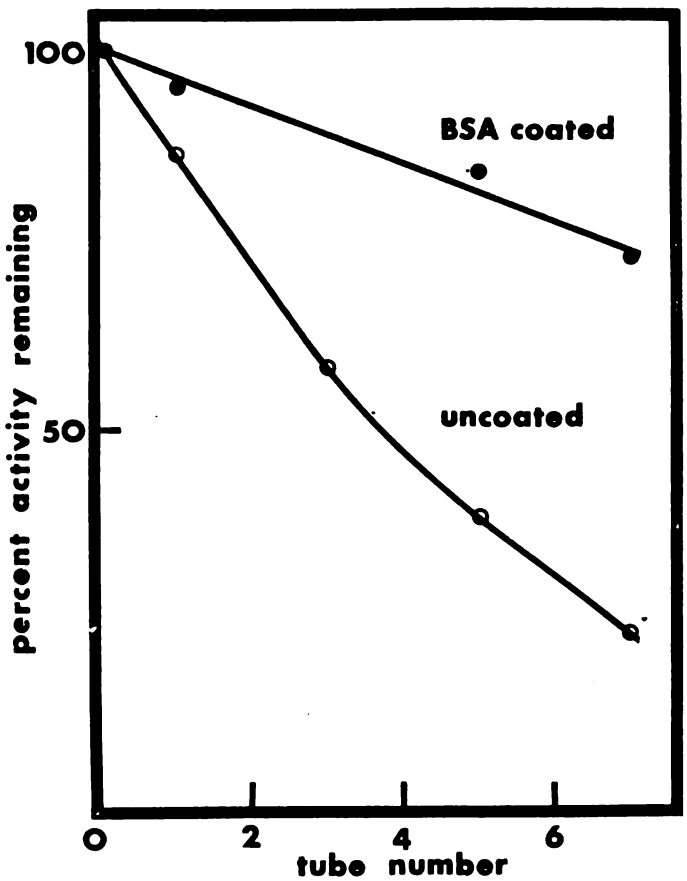


Figure 7.--Hexokinase binding to successive polypropylene tubes. G6P-solubilized hexokinase from twice washed particles (0.52 units/ml) was added to BSA coated (●) or uncoated (o) polypropylene tubes, drawn out and added to other tubes for the indicated number of times, and finally assayed.

The Effect of Salt on $MgCl_2$-Induced Rebinding of Hexokinase to Mitochondria

From Figure 8, it is clear that low ionic strength severely inhibits binding of G6P-solubilized hexokinase to liver mitochondria. Fifty percent inhibition occurs at 20-30 mM ionic strength. Figure 9 shows that the salt effect is not as pronounced with rat brain mitochondria.

Binding Assay With Pure Hexokinase

Figure 10 shows the results of a binding assay on pure rat brain hexokinase using low salt, BSA coated centrifuge tubes and rat brain mitochondria. In this experiment, the enzyme was more than 50% bindable. This is the first time that purified type I mammalian hexokinase has been reported to bind to mitochondria.

Hexokinase Isozymes

It was noticed that pure hexokinase as eluted from a DEAE-cellulose column, according to Chou and Wilson (25), sometimes yielded a slightly skewed curve. When a shallower salt gradient was used to elute the enzyme, the results in Figure 11 were obtained. The elution pattern clearly indicates two enzyme types represented by the shoulder eluting at 0.065 M KCl and the peak at 0.075 M KCl. An even shallower gradient resolves two distinct peaks (Figure 12). The relative amounts of the two peaks have varied in several experiments from about 75%:25% to 25%:75%; the reasons for this remain under investigation. 1% SDS gel

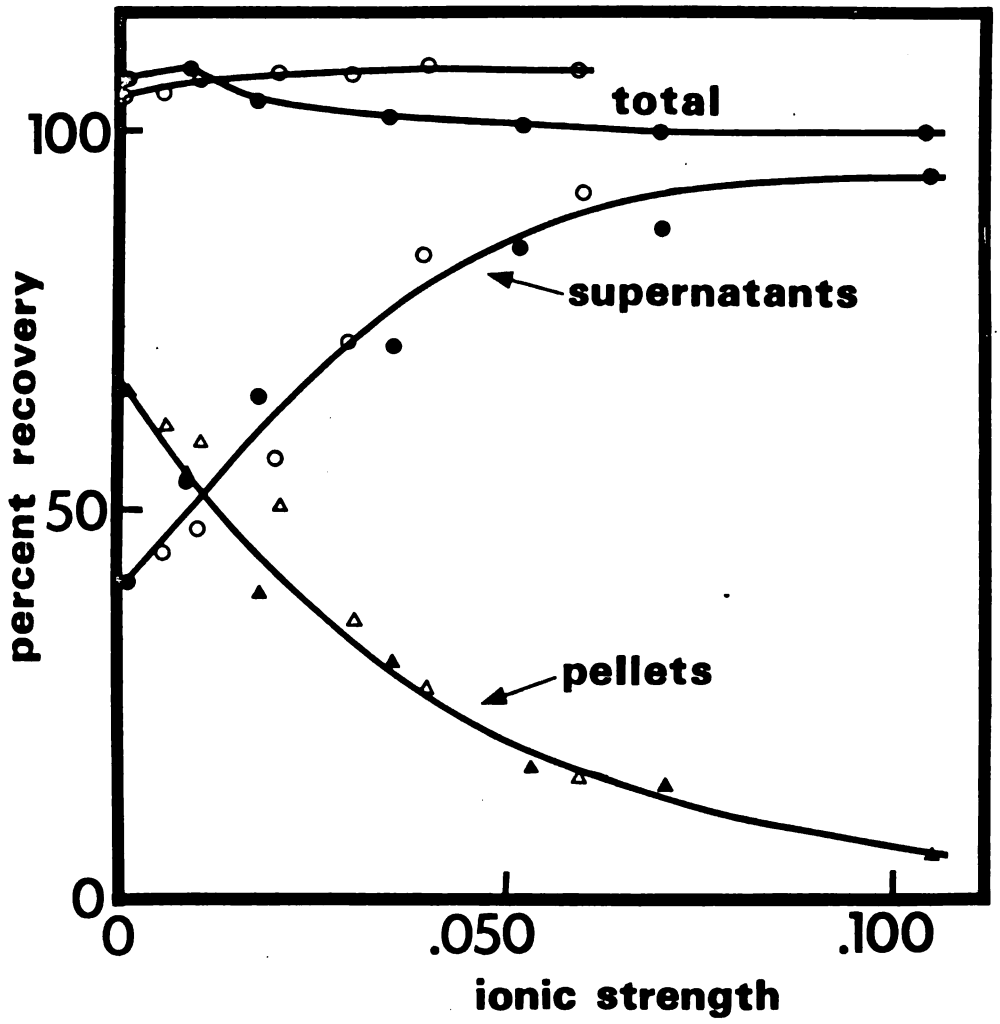


Figure 8.--The inhibition of rebinding by increasing ionic strength. Binding assays were performed as described in the text with G6P-solubilized hexokinase from 1X washed mitochondria (0.58 units/ml), with liver mitochondrial binding sites in excess. Appropriate salt concentrations were added to the tubes before adding $MgCl_2$. Open dots (o , ▵) represent KCl and closed dots (● , ▲) potassium phosphate.

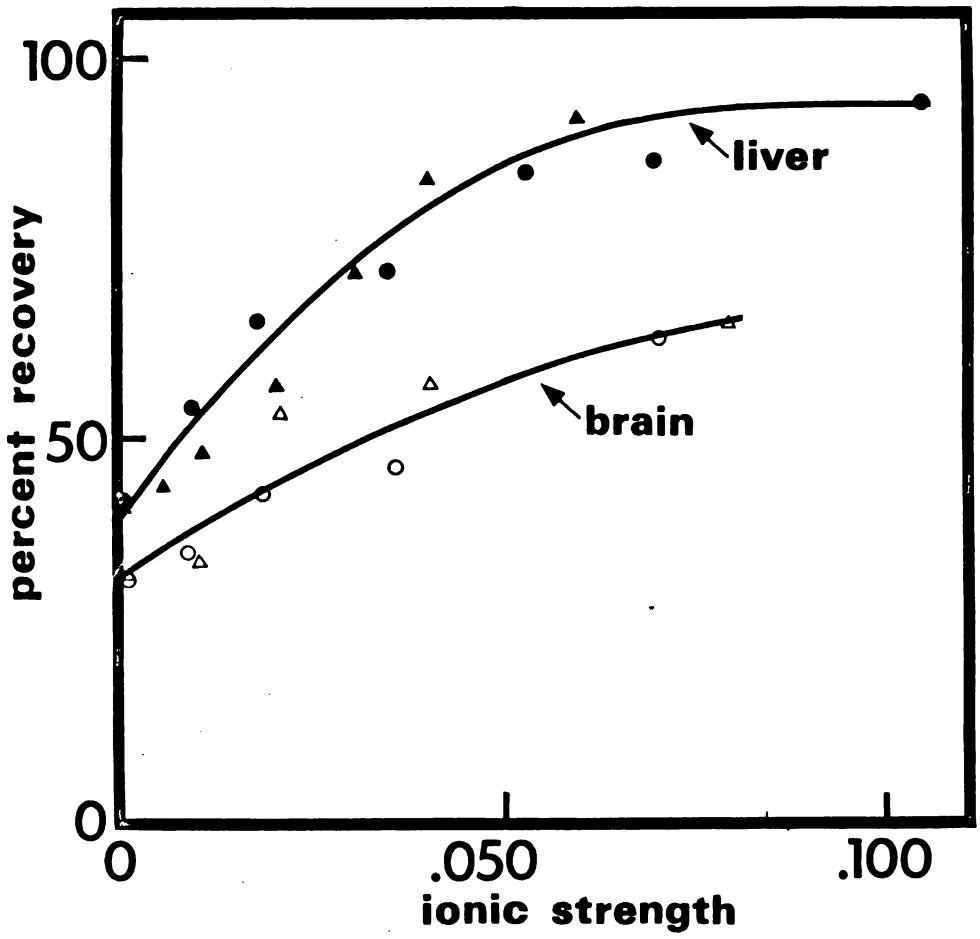


Figure 9.--Salt-induced inhibition of binding; comparison between liver and brain mitochondria. This figure compares the difference in the salt effects between rat liver and brain mitochondria. Experimental details were the same as under Figure 8.

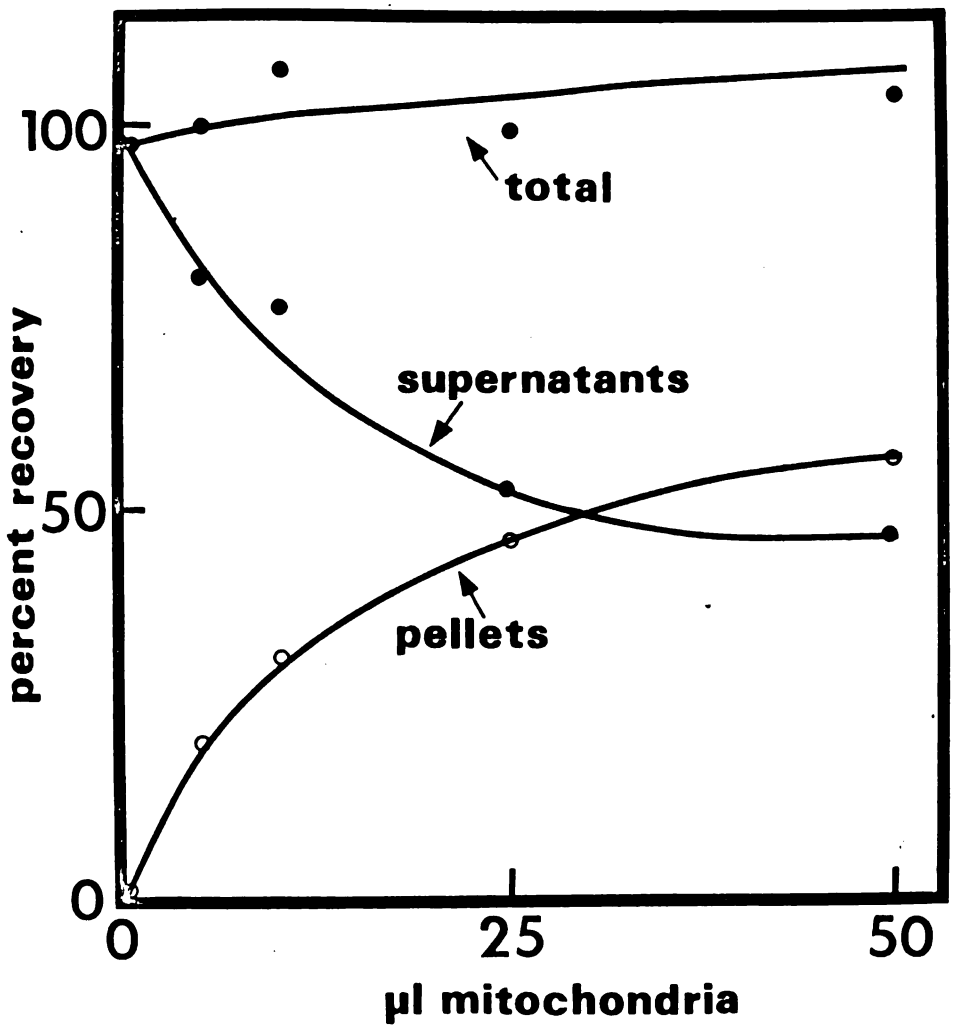


Figure 10.--Bindability of pure hexokinase. The binding assay was done as described in the methods section for pure enzyme using rat brain mitochondria, low salt and BSA coated tubes. 100% recovery (ordinate) in this experiment was equivalent to 0.26 units/ml.

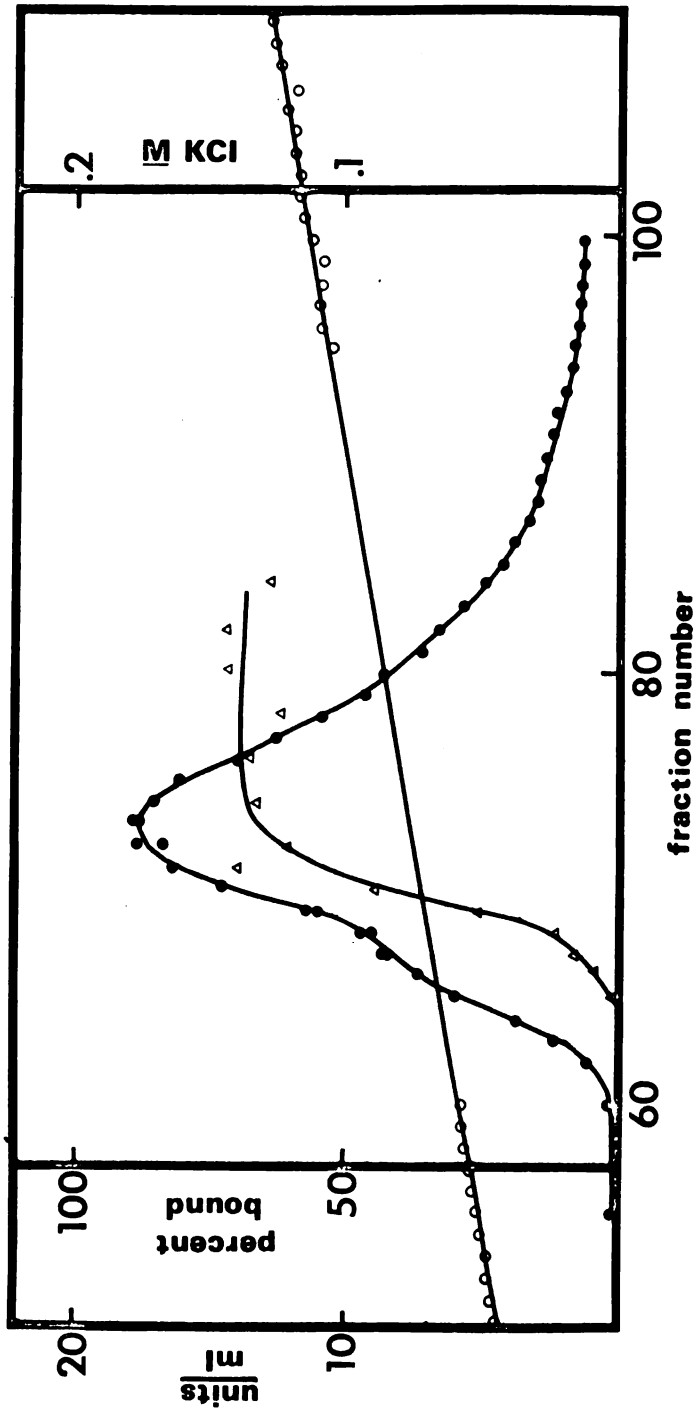


Figure 11.--DEAE-cellulose column chromatograph of rat brain hexokinase. Chromatography was performed as described in the methods section. The KCl concentration (o) was determined by conductivity measurements on the fractions. Hexokinase activity (●) and percent bound were measured as described in the methods section.

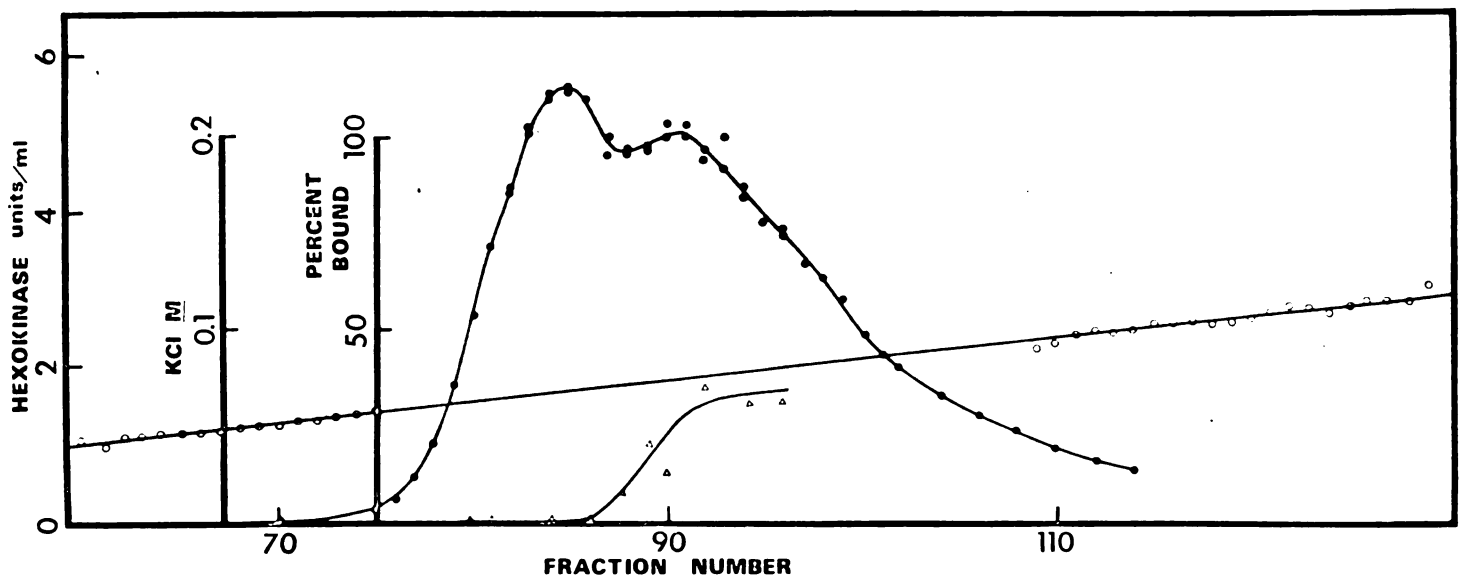


Figure 12.--DEAE-cellulose column chromatography of rat brain hexokinase; elution with a shallower gradient. The details of this experiment were essentially the same as for Figure 11, but since some new techniques were being attempted (a zonal rotor was tried for centrifugation), the enzyme recovery was only about 40%. The enzyme was eluted with a 600 ml linear gradient from 0.0 to 0.15 M KCl in column buffer, collecting 3.8 ml fractions. The binding ability is once again observed only in the high salt peak (0.075 M KCl) but in this experiment the percent bound was only about 35%, compared to 70% in the experiment of Figure 11.

electrophoresis of each peak as well as mixtures of both revealed no differences with respect to purity or molecular weight (Figure 13).

The curve underneath the elution pattern in Figure 11 indicates that the enzyme in the high salt fraction is bindable to brain particles, while the low salt fraction is completely non-bindable. This justifies the naming of new new isozymes of type I hexokinase called type I_b for the bindable enzyme, and type I_n for the non-bindable. The irregularity of the eluted peaks together with preliminary isoelectric focusing experiments suggest that type I_n and type I_b are composed of more isozymes (at least three total).

Chymotrypsin Treatment of the Pure Type I_b Hexokinase

Figure 14 indicates that like the crude G6P-solubilized enzyme, bindability of the pure hexokinase is destroyed by treatment with low levels of chymotrypsin while having no effect on the activity. Lower concentrations of chymotrypsin are required for the pure enzyme because of less extraneous substrate.

When chymotrypsin treated pure enzyme was passed over a Sephadex G-50 column, the elution pattern in Figure 15 was obtained. The protein eluting ahead of glucose may represent the peptide(s) responsible for the loss of hexokinase bindability. A control needs to be run with just chymotrypsin and one with just hexokinase to show that the peptide is not simply generated during incubation in column buffer of either protein alone. Further characterization of this fraction is obviously required.

Figure 13.--1% SDS-polyacrylamide gel electrophoresis of purified rat brain hexokinase. A sample (40 μg) of the purified type I_b hexokinase (from the preparation in Figure 11) was electrophoresed, and the gel was stained as described in the text. The densimetric tracing was obtained by scanning the gel at 550 nm in a Gilford spectrophotometer equipped with a gel-scanning attachment. This scan indicates that the protein is at least 95% pure but is not homogeneous.

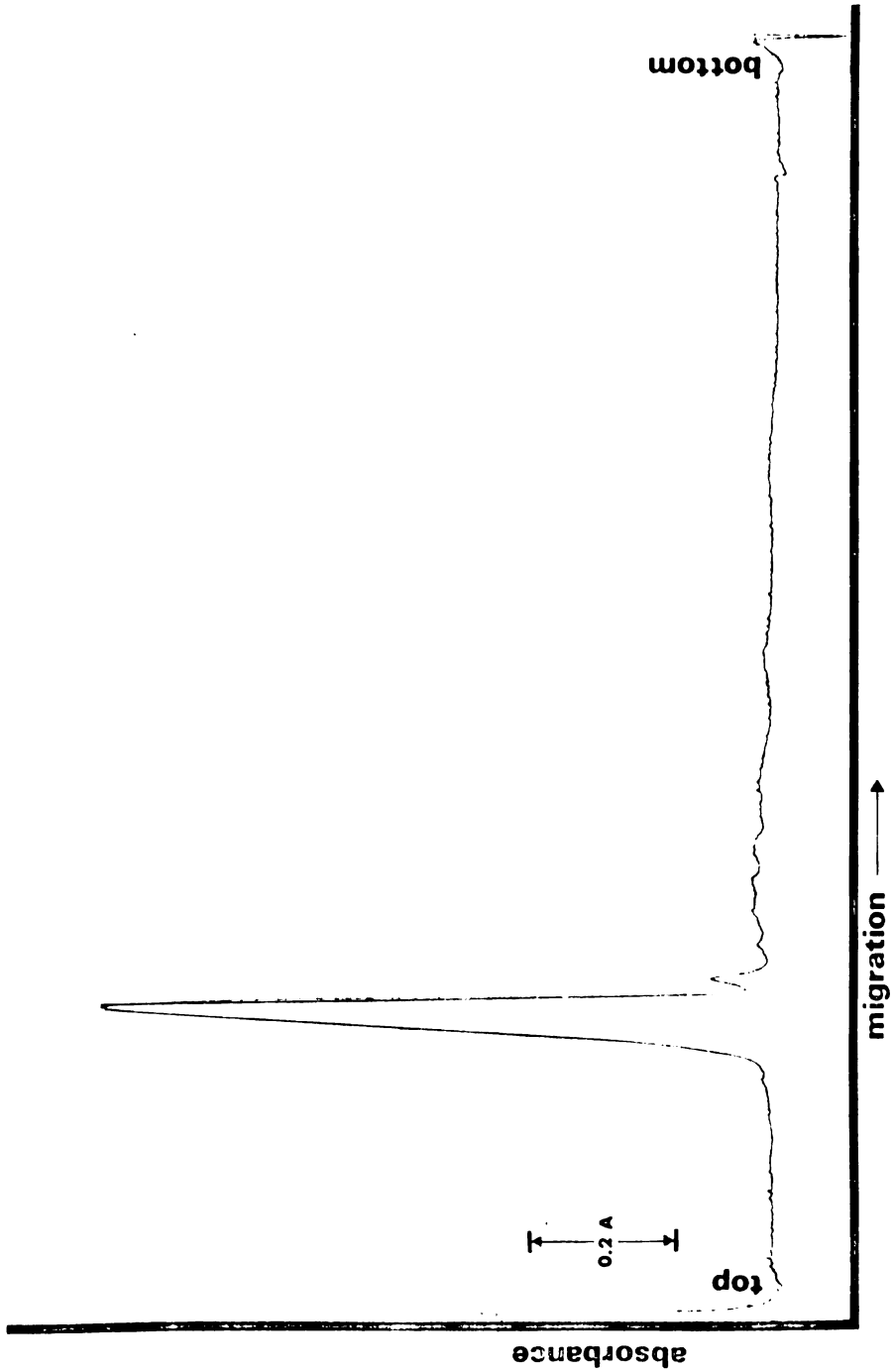

bottom
top
0.2 A
migration
absorbance

Figure 14.--Inhibition of binding by chymotrypsin treatment of crude and pure hexokinases. For the crude hexokinase, 0.5 mls of hexokinase (0.61 units/ml) was incubated for 10 min at 25°C with the indicated amounts of chymotrypsin. At the end of the incubation period, all tubes were chilled on ice and .5 mM PMSF (phenylmethylsulfonylfluoride) was added to stop protease digestion. Binding assays were performed by the standard procedure. Mitochondria treated under identical conditions did not destroy binding ability. Chymotrypsin treatment did not affect the hexokinase activity.

The same procedure was used for pure hexokinase except the reaction was stopped after the 10 min incubation period by adding 2% BSA (1:1) and chilling on ice. Rat brain mitochrondria were used instead of liver.

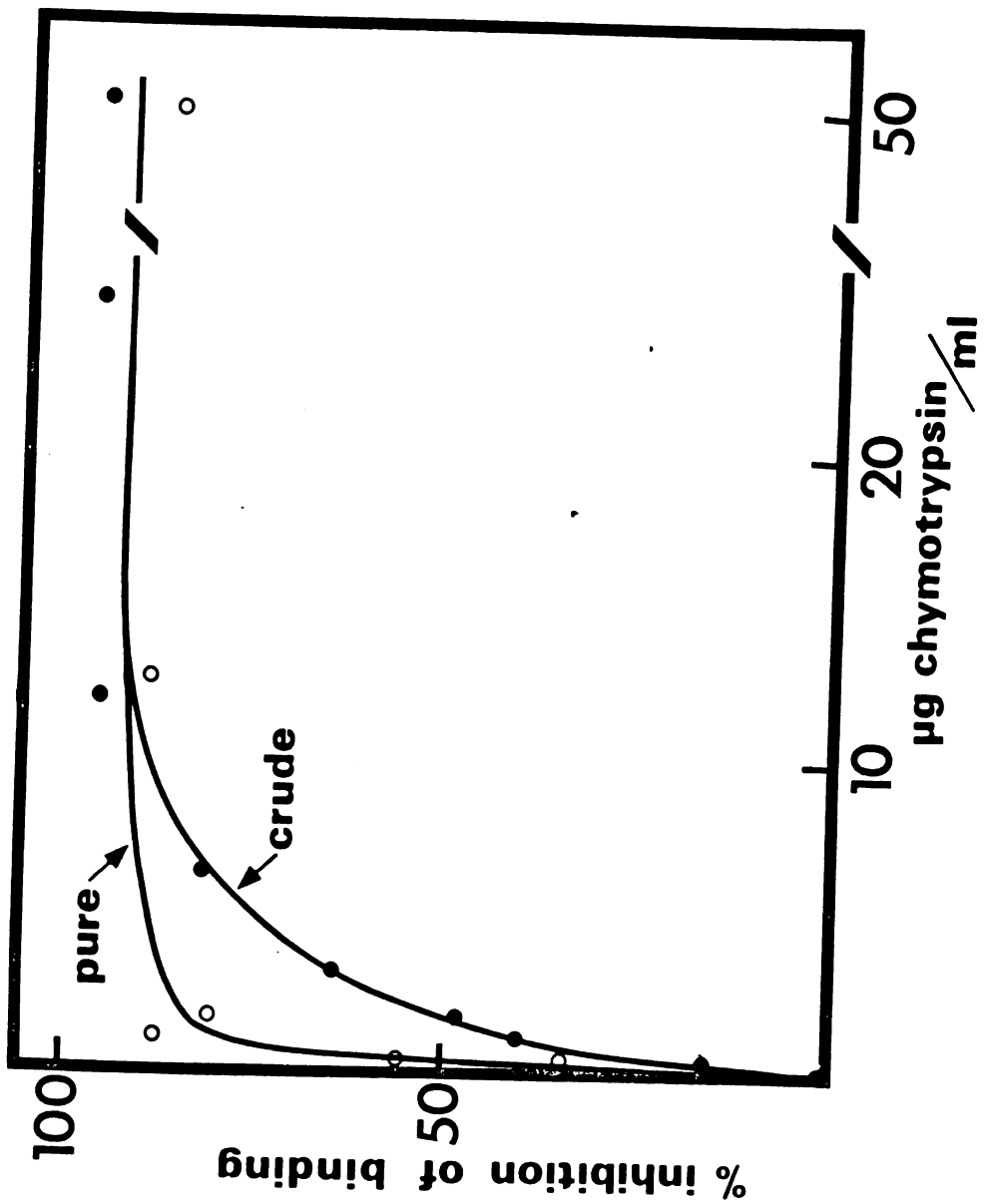
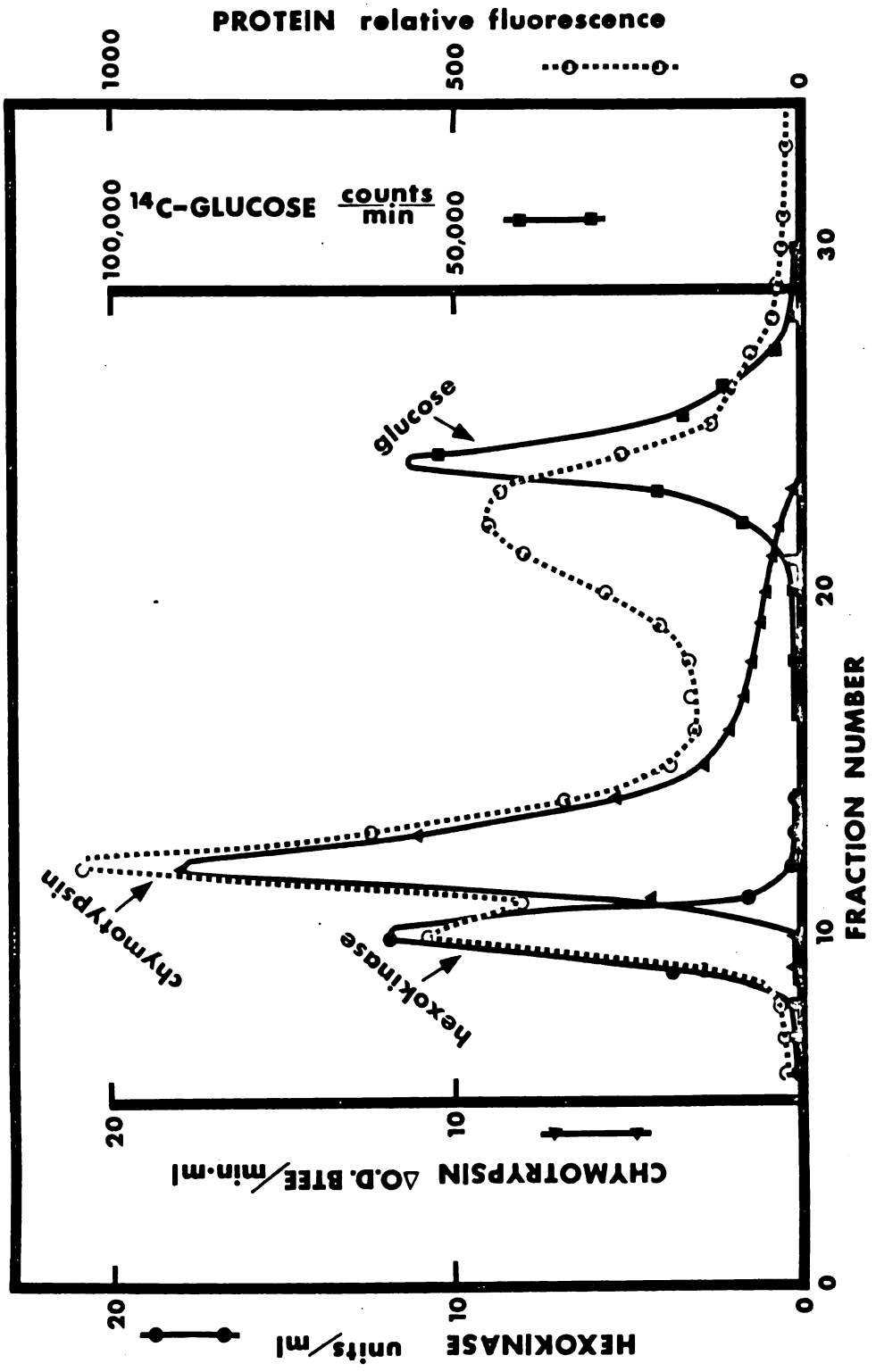

% inhibition of binding
100
50
pure
crude
10
20
50
µg chymotrypsin/ml

Figure 15.--Sephadex G-50 column chromatography of chymotrypsin treated hexokinase. The sample was treated, chromatographed, and assayed for hexokinase as described under methods. Chymotrypsin was measured at 25°C using BTEE (N-benzoyl-L-tyrosine ethyl ester) in the presence of 0.1 M $CaCl_2$, according to published procedures (46). Radioactivity was counted in a Packard Liquid scintillation counter in a toluene based cocktail (4 gm PPO; 0.05 gm POPOP; 1 liter toluene). Protein was determined fluorometrically using "Fluram" (Hoffmann-LaRoche, Inc.) according to the manufacturer's instructions. Relative fluorescence was determined on a Bowman Spectrophotofluorometer.


PROTEIN relative fluorescence
1000
500
0
100,000
14C-GLUCOSE counts/min
50,000
30
glucose
20
chymotrypsin
hexokinase
10
FRACTION NUMBER
20
10
CHYMOTRYPSIN ΔO.D. BTEE/min·ml
0
20
10
0
HEXOKINASE units/ml

Potassium Chloride-Induced Rebinding of Hexokinase to Mitochondria

Figure 16 demonstrates the phosphate-induced rebinding phenomena observed in several laboratories (29, 47, 48). Since phosphate had been shown to have the specific effect on some hexokinases of overcoming glucose-6-phosphate inhibition (29, 30), it was presumed that phosphate-induced rebinding of hexokinase was also specific. Figure 10 suggests that this is not the case because reversal of solubilization can be induced equally well with potassium chloride.

Discussion

Hexokinase Binding to Polypropylene

It has long been known that certain enzymes such as RNAse bind to glass test tubes (31), but no reference has been made regarding binding to polypropylene tubes. The forces responsible for polypropylene binding are probably analogous to those in hydrophobic affinity chromatography (32, 33). Supporting this idea, J. E. Wilson has found that hexokinase can stick to an affinity column prepared by coupling glucosamine to agarose via a long hydrophobic arm. The enzyme was eluted by high salt, but not by glucose; this would be consistent with hydrophobic interactions being required rather than binding to the glucosamine moiety (personal communication). Polypropylene $\left(-\underset{H}{\overset{H}{C}}-\underset{H}{\overset{H}{C}}- \right)_n$ is extremely hydrophobic and should make good affinity material for hexokinase.

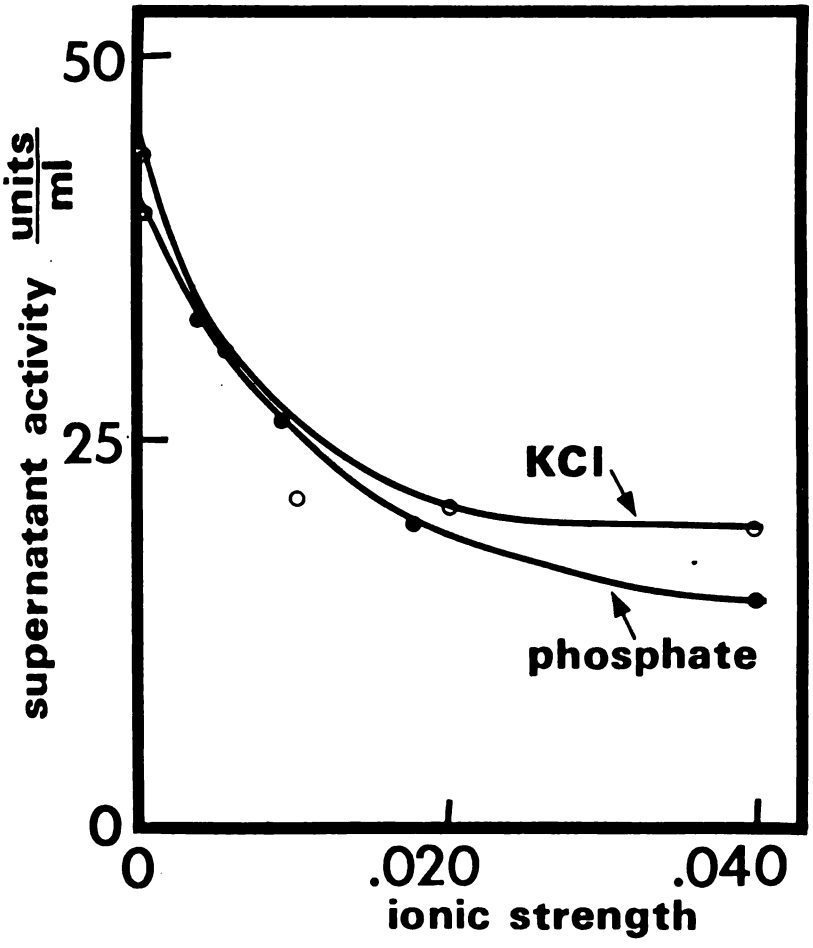


Figure 16.--Reversibility of solubilization by salt. Before removal of the particles from 2X washed G6P-solubilized hexokinase, an appropriate aliquot of 0.1 M potassium phosphate or 1.0 M KCl was added. These tubes were incubated at 25°C for 30 min, spun in an Eppendorf microcentrifuge and the supernatants assayed.

It has already been shown that serum albumins can be purified by hydrophobic affinity methods (33), so it is not unreasonable that BSA could be binding to the available sites on the polypropylene tubes in place of hexokinase. It would be interesting to try a polypropylene fiber affinity column to purify hexokinase.

Inhibition of Rebinding by Ionic Strength

Salt effects on solubilization of hexokinase have been thoroughly studied (10), but salt effects on $MgCl_2$ mediated rebinding have not. Since salt-induced solubilization was observed to be 50% maximum at 0.120 μ ionic strength, it was always assumed that low ionic strength (less than 0.05 μ) would not greatly affect rebinding. However, the data on Figure 8 indicate that 50% inhibition of rebinding occurs at 0.025μ clearly indicating that the ionic strength in the binding assay must be kept very low (less than 0.005μ).

Differences Between Liver and Brain Mitochondria

A considerably diminished salt effect was observed for the $MgCl_2$ mediated rebinding of hexokinase to rat brain particles. This marks the first time that a difference between the binding sites in mitochondrial preparations from different tissues has been observed. After this discovery, J. E. Wilson found that there is a much higher energy of activation for the binding of hexokinase to brain particles than liver mitochondria (personal communication).

Investigation into the nature of these differences is warranted. Since the brain preparation is much cruder than liver, the brain particles must first be purified to see if the differences between rat and brain depend on the purity of the organelles rather than an actual difference in the membrane binding sites. To carry out a further purification, outer mitochondrial membranes from both tissues could be isolated (9, 34) and checked for differences in binding.

Purified Bindable and Non-Bindable Isozymes

With the increased knowledge concerning rebinding and an appropriately modified assay, it has been possible to show for the first time that pure hexokinase can bind to mitochondria (Figure 10). In addition, two new isozymes have been obtained in a purified form. Type I_b is the bindable form and type I_n is non-bindable.

Investigation into the differences between these two types is obviously required. To aid in this type of study, a quick assay method for the different isozymes is necessary. For this purpose, isoelectric focusing on gels is proposed. Preliminary evidence has already indicated microheterogeneity in different preparations of purified hexokinase with at least three isozymes. This heterogeneity was not observed previously (25) because the sucrose density gradient technique employed by Chou and Wilson is inherently less sensitive (35, 36).

There are numerous possible explanations for the heterogeneity (37). Genetic heterogeneity and errors made during

synthesis of the messenger RNA or protein strand are ruled out because these effects would be constant in all preparations. In fact, different preparations of pure hexokinase have different ratios of type I_b to type I_n. The enzyme does not contain carbohydrate (38) and has only one subunit (25, 39), removing these variables from consideration. N-terminal cleavage has been tentatively ruled out because both forms contain the same N-terminal amino acid, tyrosine. The possibility that a second tyrosine is generated after proteolysis cannot be ignored, however. C-terminal cleavage is also possible.

Three promising possibilities for the heterogeneity are sulfhydryl modification, deamination and/or stable conformational isomers. Of these possibilities, the first two are documented with examples (39, 40, 41, 42). There is no proven example of the latter; however, the possibility has been raised (43, 44, 45).

The sulfhydryl modification idea is easily tested by treating the protein with DTNB and stopping the reaction at various times with a stabilizing substrate. It has already been shown by Chou (39) that different enzymes whose sulfhydryls have been variously modified can be obtained in this way. It may be found that certain sulfhydryls are required for binding to mitochondria and certain are not.

Pure Hexokinase and the "Binding Peptide"

The ability of chymotrypsin to convert hexokinase to a non-bindable form was first shown by Rose and Warms with crude ascites

tumor enzyme (8). Following the precedence of cytochrome-b_5 reductase, their result suggests that the enzyme contains a binding peptide that "holds on to" the membrane like a prehensile tail (46). Figure 14 demonstrates the chymotrypsin-induced loss in bindability of a pure preparation and Figure 15 shows that the cleaved fragment(s) can be easily isolated. N-terminal analysis of the chymotrypsin treated enzyme indicates that this fragment(s) comes, at least partially, from the N-terminus because N-terminal tyrosine is completely replaced by phenylalanine and lysine (Chapter I of this thesis). The amino acid composition of the fragment obviously needs to be determined.

An alternative explanation, of course, would be that chymotrypsin may not be removing a "binding peptide" but instead simply altering the protein conformation to make it non-bindable. In this case, the amino acid composition of the fragment(s) would not be expected to be particularly notable.

Salt-Induced Reversal of Solubilization and the Putative Binding Mechanism

Phosphate reversal of solubilization has been demonstrated in several laboratories (29, 47, 48), but in none of their work was a control run to see if the reversal was an ionic strength effect. In Figure 10 is the first demonstration that potassium chloride works as well in the binding assay as potassium phosphate.

In light of this new evidence, a hypothesis for the mechanism of binding can be proposed. This hypothesis is somewhat

analogous to a membrane reconstitution hypothesis already in the literature (49). Because of phospholipids that are in membranes, there is a negative charge envelope around each mitochondria at neutral pH (50). Since the pI of hexokinase is 6.35 (25) it, too, has a negative charge at neutral pH and consequently the two negatively charged particles repel each other. Binding of cations, especially divalent, serves to decrease the negative charge envelope around the mitochondria, allowing hexokinase to interact with the membrane binding site. Glucose-6-phosphate affects solubilization by causing a conformational change in the enzyme that makes it more easily repelled by the membrane.

This hypothesis can be readily tested with a number of obvious experiments. (i) The mitochondrial charge barrier can be gradually removed by phospholipases which should lead to a diminishing salt requirement for binding. (ii) Lowering the pH should lead to a diminished salt requirement as the enzyme and membrane become less negatively charged. (iii) The cationic portion of the salt should show some specificity; i.e., some cations should work better than others.

Summary

Type I rat brain hexokinase purified by the method of Chou and Wilson (25) has been shown to consist of at least two isozymes. One of these isozymes (type I_b) binds to mitochondrial membranes and the other (type I_n) does not. Recently discovered peculiarities in the binding assay have obscured this discovery

for some time. (i) Hexokinase binds to the polypropylene microcentrifuge tubes routinely used for the binding assay. (ii) There is an unexpected inhibition of binding by relatively low concentrations of salt. (iii) The routinely used rat liver mitochondria bind less hexokinase and are more susceptible to the salt effect than rat brain mitochondria.

A peptide(s) can be removed from pure hexokinase, at least part of which comes from the N-terminal end and may be involved in the binding process. This peptide(s) awaits further characterization.

The phosphate-induced reversal of solubilization first demonstrated by Rose and Warms (8) and confirmed by other workers (47, 48) can also be done with potassium chloride. This salt-induced rebinding suggests a hypothesis for the binding mechanism. Further experiments will be required to ascertain whether or not the salt-induced rebinding is due to identifical factors necessary for phosphate-induced rebinding.

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