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LYOTROPIC SALTS AND THE HEXOKINASE MEMBRANE INTERACTION: PURIFICATION, RECONSTITUTION AND CHARACTERISTICS OF THE OUTER MITOCHONDRIAL MEMBRANE BINDING SITE FOR HEXOKINASE

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LYOTROPIC SALTS AND THE HEXOKINASE MEMBRANE INTERACTION: PURIFICATION, RECONSTITUTION AND CHARACTERISTICS OF THE OUTER MITOCHONDRIAL MEMBRANE BINDING SITE FOR HEXOKINASE

By

Philip L. Felgner

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ABSTRACT

LYOTROPIC SALTS AND THE HEXOKINASE MEMBRANE INTERACTION: PURIFICATION, RECONSTITUTION AND CHARACTERISTICS OF THE OUTER MITOCHONDRIAL MEMBRANE BINDING SITE FOR HEXOKINASE

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Lyotropic Salts and the Hexokinase/Membrane Interaction--Neutral salts can be ranked according to their relative abilities to disrupt or stabilize the tertiary structure of macromolecules. This ranking has been called the Hofmeister or lyotropic series, with the more lyotropic salts being more effective at disrupting the native structure of macromolecules than non-lyotropic salts. Some salts in this series, in order of increasing lyotropicity, are Na_2SO_4 < NaF < NaCl < NaBr < NaI < $NaClO_4$ < NaSCN.

A molecular interpretation of the effect of neutral salts on macromolecular structure was developed that accommodates two general properties of neutral salts on macromoleculars in aqueous solution. (1) Salts can disrupt ionic linkages (including hydrogen bonds) by binding to charged (or partially charged) moieties. The binding constants, though small, can be measured. (2) Salts can promote hydrophobic interactions by changing the solution properties of water such that hydrophobic molecules are less

readily hydrated. These two effects oppose one another with respect to whether or not the tertiary structure of a macromolecule will be stabilized or destabilized; i.e., breakage of ionic linkages destabilizes the tertiary structure, and promoting hydrophobic interactions tends to stabilize tertiary structure. Lyotropic salts have a large destabilizing component and a small stabilizing component. Conversely, non-lyotropic salts have a relatively smaller destabilizing component and a large stabilizing component.

A system of equations has recently been derived by Melander and Horvath (1) which allow the quantitation of the relative contributions of electrostatic and hydrophobic forces between two interacting molecules. Their development was applied to the hexokinase/membrane interaction and it was estimated that about half of the surface of the enzyme molecule is involved in hydrophobic interactions with its binding site on the membrane surface.

Purification, Reconstitution and Characteristics of the Mitochondrial Binding Site for Hexokinase--Very pure outer mitochondrial membranes (OMM) have been obtained from rat liver that contain a binding site for rat brain hexokinase. The specific activity for this membrane binding site (units of hexokinase bound/mg membrane protein) is 40 fold higher than in either microsomes, erythrocytes, or inner mitochondrial membranes. Glucose-6-P at low concentrations (1 mM) specifically elutes the enzyme from

these

liver

these membranes as it does from intact mitochondria from either liver or brain.

As a means of gaining insight into the nature of the OMM binding site, numerous attempts were made to chemically or enzymatically modify the binding site. All these approaches were met with equivocal results. The enzyme would not bind to any liposome preparations in the glucose-6-P sensitive manner that is characteristic of the intact binding site. The OMM proteins can be depleted of their lipid content by treatment with low concentrations of detergent. This treatment modifies the binding site in a partially reversible manner.

Treatment of the OMM with the non-ionic detergent octyl glucoside preferentially extracts a single protein of mol. wt. 31,000 that has been tentatively identified as the hexokinase binding protein. Removal of the detergent from this solubilized membrane protein by dialysis results in the formation of lipid containing membrane vesicles that contain a glucose-6-P sensitive hexokinase binding site.

ACKNOWLEDGMENTS

I am certainly pleased to finally be able to acknowledge the very capable technical (as well as intellectual) help of Jan Messer and Suzanne Murrmann. I would also like to acknowledge John Wilson who is primarily responsible for training these two individuals and who's ability at preparing responsible laboratory help I have always admired. Thanks are extended to David Alessi who's data appear on Table 13 of this thesis and to Pat Kelly who provided much of the data on Table 12. Judy Kao's expert electron microscopy skills and willingness to help are also very much appreciated. And thanks are extended to Clarence Suelter who's enzymology class prompted my thinking about salt effects and hydrophobic effects on macromolecules.

I acknowledge the assistance of my committee members who are Dr. Wilson, Dr. Suelter, Dr. Ferguson-Miller, Dr. K. Schubert and Dr. Tien. I owe special thanks to Dr. Schubert for his very thorough reading of my thesis and to Dr. Ferguson-Miller for stimulating discussions which I am sure will continue past the publication of this thesis. And I am grateful to John Boezi, Dave McConnell and John Wilson for supporting me through some difficult times. Obvious additional appreciation is due to Dr. Wilson for providing an admirable personal and professional example over the past several years.

suppor

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This thesis is dedicated to my parents who provided constant support, to my brothers who were always good examples and especially to Jean, who would rather I became a minstrel.

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PREFACE

The requirements of the Graduate School limited the length of the Abstract that appears in the front of this dissertation. This preface is intended to be an expanded version of that Abstract. It is hoped that this will aid in the reading of this material.

Chapter I

Lyotropic Salts and the Hexokinase/Membrane Interaction--Neutral salts can be ranked according to their relative abilities to disrupt or stabilize the tertiary structure of macromolecules. This ranking has been called the Hofmeister or lyotropic series, with the more lyotropic salts being more effective at disrupting the native structure of macromolecules than nonlyotropic salts. Some salts in this series, in order of increasing lyotropicity, are Na_2SO_4 < NaF < NaCl < NaBr < NaI < $NaClO_4$ < NaSCN.

A molecular interpretation of the effect of neutral salts on macromolecular structure was developed that accommodates two general properties of neutral salts on macromolecules in aqueous solution. (1) Salts can disrupt ionic linkages (including hydrogen bonds) by binding to charged (or partially charged) moieties. The binding constants, though small, can be measured. (2) Salts

can promote hydrophobic interactions by changing the solution properties of water such that hydrophobic molecules are less readily hydrated. These two effects oppose one another with respect to whether or not the tertiary structure of a macromolecule will be stabilized or destabilized; i.e., breakage of ionic linkages destabilizes the tertiary structure, and promoting hydrophobic interactions tends to stabilize tertiary structure.

With respect to the first point, several observations have been made. (1) Salts bind to the peptide bond dipole. (2) The binding affinity of the salts for the dipole varies according to the position of the salt in the lyotropic series, such that the more lyotropic salts bind more tightly. (3) Vicinal hydrophobic groups around the dipole are required to produce the observed variations in the binding affinities of different salts to the dipole. (4) Hydrophobic groups are surrounded by interfacial water that is different from bulk water. (5) Surface tension theory predicts that salts with a deeper hydration sphere, are repelled from interfacial water more than salts with smaller hydration spheres. (6) The depth of the hydration sphere around anions decreases as the lyotropicity increases. Considering all these points together, the general conclusion is that, the smaller the hydration sphere around the anion, the more tightly it will bind to peptide bond dipoles containing vicinal hydrophobic groups. Binding to such a dipole could lead to destabilization of the tertiary structure of a macromolecule by disrupting the α-helix or other secondary structures.

The second point regarding stabilization of hydrophobic interactions by salts is also supported by several observations. (1) To disrupt a hydrophobic interaction, a new hydrocarbon/water interface must be created that has characteristics similar to the air/water interface. (2) Since the value of the surface tension can be described as the extent to which a given solution prefers to minimize its interfacial area, anything which increases the surface tension will tend to decrease the interfacial area, favoring hydrophobic interactions. (3) The extent to which a given salt will increase the surface tension of an aqueous solution (the surface tension increment) is dependent on the salt used, and non-lyotropic salts increase the surface tension more than lyotropic salts. From consideration of these three points the general conclusion is that neutral salts tend to stabilize hydrophobic interactions with non-lyotropic salts being more effective than lyotropic salts.

So, to summarize, a given salt will either stabilize or destabilize the tertiary structure of a macromolecule depending on the interplay between two opposing forces, one tending to stabilize the overall structure and the other tending to destabilize it. Lyotropic salts have a large destabilizing component and a small stabilizing component. Conversely, non-lyotropic salts have a relatively smaller destabilizing component and a large stabilizing component.

A system of equations has recently been derived by Melander and Horvath (1) which allow the quantitation of the relative contributions of electrostatic and hydrophobic forces between two

interacting molecules. Their development was applied to the hexokinase/membrane interaction and it was estimated that about half of the surface of the enzyme molecule is involved in hydrophobic interactions with its binding site on the membrane surface.

Chapter II

Purification, Reconstitution and Characteristics of the Mitochondrial Binding Site for Hexokinase--Purified outer mitochondrial membranes (OMM) have been obtained from rat liver mitochondria that contain a binding site for rat brain hexokinase. The effective concentration for this membrane binding site (units of hexokinase bound/mg membrane protein) is 40 fold higher than in either microsomes, erythrocytes, or inner mitochondrial membranes. Glucose-6-P at low concentrations (1 mM) specifically elutes the enzyme from these membranes as it does from intact mitochondria of either liver or brain. Binding appears to be weaker in the OMM, however. Exogenous protein in the crude mitochondrial preparation, which is absent in the purified OMM, appears to play a role in strengthening the binding.

The enzyme can be bound to positively charged liposomes, and to negatively charged liposomes that contain tightly bound lysozyme, but in neither case is the binding sensitive to glucose-6-P. Hexokinase binding to the liposomes that contain bound lysozyme is $MgCl_2$ dependent as it is in OMM, but glucose-6-P causes tighter instead of weaker binding of hexokinase. Negatively charged

liposomes or liposomes that have been prepared from total lipid extracts of either OMM or microsomes do not bind any hexokinase.

SDS gels of chymotrypsin or trypsin treated OMM indicate that proteolysis modifies several proteins in the membrane, but the binding properties of the protease treated membranes are not appreciably modified. Periodate treatment of OMM removes 30% of the binding sites for hexokinase and the enzyme bound to this modified binding site does not show the glucose-6-P specific elution effect that is characteristic of intact OMM. While it was clear that periodate inactivated the binding site, no inferences about the involvement of carbohydrate residues in the binding site could be made, since it was demonstrated that periodate readily inactivates a protein (hexokinase) which contains no carbohydrate. Incubation of OMM at 37°C leads to losses in the number of binding sites as well as in the glucose-6-P sensitivity. Paradoxically, Mg^{++} and Ca^{++}, as well as EDTA, have stabilizing effects. Phospholipase A_2, intrinsic to the OMM, may play a role in this loss of the binding properties.

Treatment of OMM with the non-ionic detergent Emulgen 913, extracts more than 90% of the phospholipids from the OMM while solubilizing less than 40% of the membrane protein. The insoluble, lipid depleted, membrane proteins remaining after Emulgen treatment, contain a hexokinase binding site but this binding site is not glucose-6-P sensitive. The addition of phospholipids extracted from OMM or microsomes to the lipid depleted proteins, with sonication, restores the glucose-6-P dependent solubilization effect.

The reason for this unusual ability of detergent to preferentially extract phospholipids from the OMM without solubilizing the proteins may be that the OMM proteins are unusually difficult to solubilize by detergents. Consistent with this view, it was observed that at least a 10 fold higher concentration of either cholate or Emulgen 913 was required to completely solubilize the OMM than was required to solubilize microsomes.

Treatment of OMM with the non-ionic detergent octyl glucoside under the appropriate conditions solubilizes primarily two proteins which have molecular weights on SDS gels of 61,500 and 31,000. On the basis of its molecular weight the 61,500 M.W. protein has been tentatively identified as monoamine oxidase. When this solubilized material is dialyzed to remove the detergent, membranous vesicles are formed which retain about 50% of the original glucose-6-P sensitive hexokinase binding properties. Electron microscopy confirms the membranous character of the reconstituted material. Resolubilization of these reconstituted OMM with octyl glucoside, followed by dialysis results in the formation of twice reconstituted vesicles which still contain 25-50% of the original glucose-6-P sensitive hexokinase binding sites. These twice reconstituted membranes contain primarily a single protein of molecular weight 31,000. When the octyl glucoside-solubilized membranes are treated with trypsin, the resulting hydrolysate cannot be reconstituted into membranes containing an intact, glucose-6-P sensitive binding site for hexokinase. SDS gels of the octyl glucoside-solubilized, protease-treated membranes

confirmed that the amount of the 31,000 M.W. protein was substantially reduced. These results strongly suggest that hexokinase binding is dependent on a single protein (M.Wt. 31,000) of the OMM, and that this protein can be selectively purified by solubilization of the OMM with octyl glucoside followed by reconstitution of membrane vesicles.

CHAPTER I

LYOTROPIC SALTS AND THE HEXOKINASE MEMBRANE INTERACTION

Introduction

The effect of salts on macromolecules has been studied for nearly one hundred years and yet after all this time no clear explanation, in molecular terms, has appeared, despite a truly remarkable abundance of data on the subject. In this first chapter I will develop a working hypothesis, based on the literature and my own data, that describes, in molecular terms, the basis for the action of neutral salts on macromolecules.

Is there any physiological relevance for studies of macromolecular structure at high ionic strength, with neutral salts like KSCN or $NaClO_4$ which are rarely found in vivo? In view of my bias, it's obvious that my answer to this question will be affirmative. We have found, for instance, that hexokinase in the presence of mitochondria in vitro can be predominantly either soluble or membrane-bound depending on the ionic strength in the range 0-0.25M. The physiological ionic strength is within this range. The fact that the intracellular salt concentration is (presumably) a fixed value does not argue against performing

experiments outside that range. For example, one way to shed light on the role of KCl at intracellular concentrations (~ 0.15M) is by observing its influence at concentrations above and below intracellular levels. Likewise, a means of determining specific roles for KCl, is by comparing its effects with other salts.

Another point that deserves some attention here is that *in vivo* solute concentrations are far different from those typically used for *in vitro* experiments. For example, one rarely sees *in vitro* experiments done with the protein concentration as high as 10%, as it is *in vivo*. It seems likely that some important basic principles about protein structure and function *in vivo* may be gleaned from studying proteins in concentrated protein solutions as well as in concentrated solutions of pure solutes such as neutral salts. The effect of neutral salts on macromolecules may be entirely different at high protein concentrations (10%). Some data that begin to address these points are presented in Chapter II and in reference 3 (see appendix).

Apart from the physiological relevancy, there are other reasons for studying salt effects on macromolecules. As will be shown later, salts can be used as a probe to semiquantitatively measure the extent of hydrophobic vs. electrostatic bonding in a given macromolecular interaction. In addition, insights into the molecular nature of the hydrophobic effect can be gained through the study of neutral salt effects on macromolecules.

The Molecular Basis for Neutral Salt Effects on Macromolecules

Previous explanations of the molecular basis for neutral salt effects (4-7) all included the concept of "water structure." The following description, which is based on some recent developments, is intended to provide a more lucid molecular description of the effect of salt on macromolecules, without depending as much on the rather ill defined "water structure" concept (8).

Most of the effects of salts on proteins can be explained in terms of direct binding by the salts to the peptide bond dipole, and, by inference, to other charged groups on the protein molecule (3). The relative influence of different salts can be interpreted on the basis of their relative binding affinities for the dipole and the differences in their binding affinities can be explained in terms of their relative tendencies to be excluded from a hydrocarbon/water interface.

Description of the Hofmeister (or Lyotropic) Series

Neutral salts can be ranked with respect to their relative abilities to solubilize macromolecules. If the salts tend to solubilize proteins, they are called "salting-in" type salts. If they tend to force macromolecules out of solution, they are called "salting-out" type salts. This ranking, called the Hofmeister or lyotropic series, was first established by Hofmeister in 1888 (9) in a study of the relative effectiveness of various salts at salting-out euglobins from aqueous solution. [This

series is sometimes termed, somewhat erroneously, the "chaotropic" series. The term chaotropic is commonly used in reference to the "disorganization" of water molecules in bulk solution by various solutes (7, 8). Since the chaotropic properties of different neutral salts (as measured by various physical methods) are not always correlated with their relative tendencies to salt-out proteins, it has been suggested that this terminology be dropped (W. P. Jencks, personal communication).] Table 1 is a comprehensive list of neutral salts in the order of increasing effectiveness at salting-out (i.e. decreasing lyotropicity) of the peptide analogue, acetyltetraglycine ethyl ester (10). A similar ranking has been shown for the effect of neutral salts on the collagen-gelatin phase transition, ribonuclease denaturation, DNA unfolding, the polyvinylmethoxazolidinone cloud point (precipitation) and other phenomena (8, 11).

Some aspects of the working hypothesis which will be presented in the next sections are unpublished. Other aspects of the hypothesis are derived from the work of Tanford (12), von Hippel and Schleich (8), Kuntz and Kauzmann (13), Robinson and Jencks (10), Felgner and Wilson (3), Roseman and Jencks (14) and Kauzmann (29). Basically the hypothesis represents a synthesis between the work of von Hippel et al. (6, 15, 16, 17) and Melander and Horvath (1).

Table 1.--Approximate Salting-out Constants for Acetyltetraglycine Ethyl Ester at 25.0°[a]

Compound	K_s[b]	Compound	K_s[b]
Lithium-3,5-diiodosalicylate	-1.3	NaBr	0.00
Phenol	-0.48	$MgCl_2$	0.00
$NaClO_4$	-0.33	$(CH_3)_4NBr$	0.018
Sodium tosylate	-0.31	LiCl	0.021
$C_6H_5NH_3Cl$	-0.31	NH_4Cl	0.035
LiI	-0.28	KCl	0.046
$Cl_3CCOONa$	-0.27	NaCl	0.046
NaSCN	-0.25	CsCl	0.054
NaI	-0.23	$NaBrO_3$	0.090
KI	-0.21	$(CH_3)_3CCOONa$	0.15
LiBr	-0.17	Glycine	0.16
C_6H_5COONa	-0.14	$NaHSO_3$	0.16
$C_6H_{11}NH_3Cl$	-0.13	KF	0.23
NH_4Br	-0.11	CH_3COONa	0.23
$(CH_3CH_2)_4NBr$	-0.11	$Na_2S_2O_3$	0.35
$BaCl_2$	-0.11	NaH_2PO_4	0.36
$CaCl_2$	-0.09	$(NH_4)_2SO_4$	0.45
$NaNO_3$	-0.075	Na_2SO_4	0.48
KBr	-0.023	Na_3 citrate	0.90

[a] From reference 10.

[b] Log $S^o/S = K_sM$, where S^o is the solubility in water, S is the solubility in other solvent, and M is the concentration of salt moles per liter. Values of K_s were estimated from solubility measurements at salt concentration 0-0.5 M. "Salting-in" type salts have large negative values of K_s and "salting-out" type salts have large positive values of K_s.

There is an enormous amount of data regarding the effect of neutral salts on numerous solvent parameters such as water activity, viscosity, heat capacity, entropy of solution, ionic mobility, self-diffusion of water, internal pressure and spectral properties (I.R., NMR and Raman) and the literature is likewise replete with data on the effects of neutral salts on macromolecular structure (8, 10). All of these data must eventually be included in a coherent framework describing the effects of solvent and salts on macromolecular structure. In this chapter, however, I will concentrate on only two prominent factors that, when considered together, can explain much of the data as well as provide a molecular interpretation of the Hofmeister series.

These facts are that (1) neutral salt binding affinities to the peptide bond dipole increase in the same order as the Hofmeister series (15,16) and (2) the surface tension increment (i.e. that amount by which the surface tension of pure water is changed due to the addition of a mole of solute) increases in the same order as the Hofmeister series (1). These two points will be dealt with separately and then considered together in order to draw a molecular picture of the effects of salts on macromolecules.

Neutral Salt Binding Affinities to the Peptide Bond Dipole

Von Hippel and his colleagues (15, 17) have been able to determine relative binding constants of various

neutral salts to polyacrylamide columns (-CH_2-$\underset{H}{\overset{O=C-NH_2}{C}}$ -). Comparative measurements on a polystyrene column (-CH_2-$\underset{H}{\overset{CH_2\phi}{C}}$-) indicate that the bindings occurs only to the amide moieties and not to the hydrophobic backbone or to the hydration shell surrounding the backbone of the polyacrylamide. Ions that bind tighter than water to the dipole, tend to destabilize macromolecular conformations, i.e. are more lyotropic. Likewise, those salts that tend to stabilize macromolecular conformations bind less tightly than water to the acrylamide column. In fact, the lyotropic series as it relates to conformational stability of macromolecules is fully developed in the acrylamide binding experiments so that the ions with the greatest structure destabilizing characteristics bind tightest and those that tend to stabilize macromolecular structure, bind the weakest. From these studies then, von Hippel infers that the destabilizing characteristics of salts are the result of direct binding to the macromolecule (6, 15, 16, 17). The stabilizing characteristics probably arise because of some other effect that salts have on the solvent properties of water favoring hydrophobic interactions. Insight into the nature of this structure stabilizing effect comes from the effect of salts on the surface tension of water which will be

dealt with in more detail later. For the present I want to emphasize that the overall effect of a particular salt on macromolecular structure depends on the relative magnitudes, for that salt, of two opposing effects: the first is a structure destabilizing component which arises from direct binding of the salt to polar moieties in the macromolecule, and the second is a structure stabilizing component which results from the tendency of the salt to stabilize hydrophobic interactions by changing the solvent properties of water. (At this point the phrase "solvent properties of water" may still seem vague. This will, hopefully, become somewhat clearer later.)

Von Hippel and his colleagues (11, 13) have also demonstrated that the affinity of the various salts for an ion retardation column varies with the solvent used to elute the salt. Elution by water was compared to the elution by various other solvents that were considered to be peptide bond analogues, such as formamide, acetamide, N-methyl acetamide, N-methyl formamide, and N,N-dimethyl formamide. The relative affinities of the salts for these solvents varied with the amount of methyl substitution around the dipole, such that the more methyl groups around the dipole, the lower the affinity of the salt for the dipole. The decrease in affinity for the dipole that results from added methyl groups is most marked for NaCl; then comes NaBr, NaI and $NaClO_4$, in that order (consistent with the lyotropic series). All salts bind approximately as well to formamide which represents

an "ideal" peptide bond dipole with no vicinal non-polar groups. Thus it appears that the Hofmeister specificity of binding to the peptide bond dipole is dependent on the nonpolar environment in the immediate vicinity of the dipole.

There are at least two ways to explain these data (8, 17): either the methyl groups around the dipole cause an inductive effect on the dipole which introduces the Hofmeister specificity, or the methyl groups perturb the water structure in the vicinity of the dipole and this leads to the Hofmeister specificity. The first hypothesis doesn't seem likely because methyl groups substituted in different positions around the dipole give approximately the same effect (i.e. salts bind about as tightly to N-methylacetamide as to N,N dimethyl formamide). If an inductive effect were operating, one would expect substitution at the carbonyl moiety to give substantially different results than substitution at the amide nitrogen. Furthermore, data on the effect of salts on the surface tension of water suggest another hypothesis that accounts for the observed Hofmeister binding specificity (see below).

The Effect of Neutral Salts on the Surface Tension Increment

Recently Melander and Horvath (1) reported a relationship between the extent to which a given salt influences the surface tension of pure water and its position in the lyotropic (Hofmeister) series. The expression which approximately describes the surface tension of many inorganic salt solutions is

$$\gamma = \gamma^{o} + \sigma m$$

where γ^{o} is the surface tension of pure water, m is the molality of the salt, and, for a given salt, γ is a constant called the molal surface tension increment (1, 18). In Table II is a list of inorganic salts and their surface tension increments. Notice that all salts give positive values of σ which means that they all increase the surface tension of water.

In Table 1 are listed the salting-out constants for many of the salts listed in Table 2. A comparison of these two tables indicates that, in general, the larger the salting-out constant, the larger the surface tension increment. The plot of σ vs. K_s (Figure 1) emphasizes this relationship. In their paper, Melander and Horvath (1) similarly demonstrated that the concentration dependence of salt-induced protein flocculation for different salts bears a similar relationship to the surface tension increment of the salt used. That is, salts that induce flocculation (precipitation) of proteins at lower concentrations have higher surface tension increments.

Physical Description of the Surface Tension Increment

According to Moore (19) and others (20, 21), surface tension is a reflection of the internal cohesiveness of the bulk solution. If the solution properties are such that molecules in the bulk phase have a high affinity for one another, this will be reflected in a high value for the surface tension. If the molecules in

Table 2.--Molal Surface Tension Increments of Various Salts

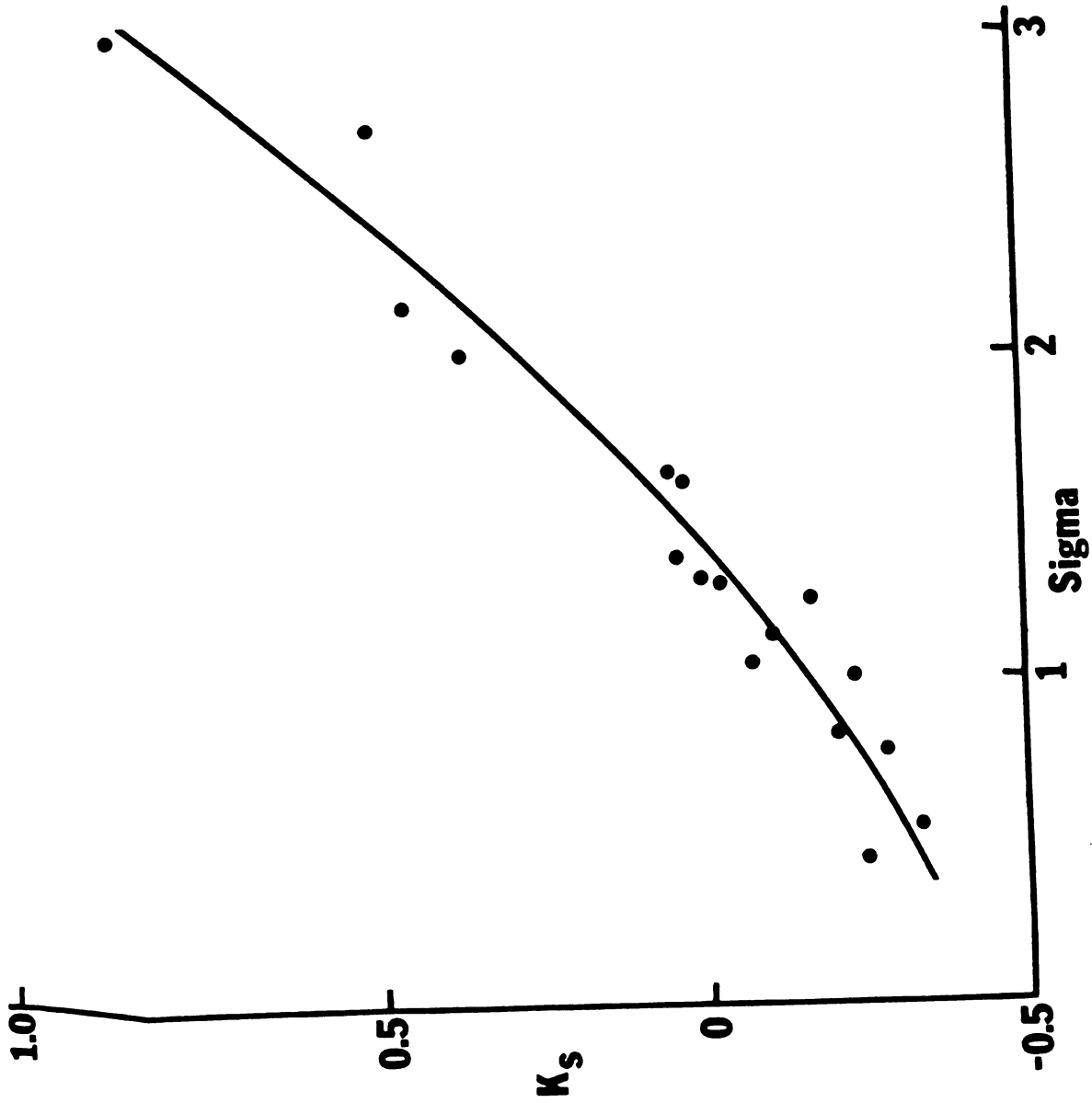
($\times 10^3$ $\frac{dyn\ g}{cm\ mol}$)	Salt	($\times 10^3$ $\frac{dyn\ g}{cm\ mol}$)	Salt
0.45	KSCN	1.96	K_2-tartarate
0.55	$NaClO_3$	2.0	$Ba(NO_3)_2$
0.74	NH_4I	2.0	LiF
0.79	LiI	2.02	Na_2HPO_4
0.84	KI	2.10	$NiSO_4$
0.85	NH_4NO_3	2.10	$MgSO_4$
0.86	$KClO_3$	2.10	$MnSO_4$
1.02	NaI	2.15	$CuSO_4$
1.06	$NaNO_3$	2.16	$(NH_4)_2SO_4$
1.14	NH_4Br	2.27	$ZnSO_4$
1.16	$LiNO_3$	2.35	Na_2-tartarate
1.26	LiBr	2.58	K_2SO_4
1.31	KBr	2.66	Na_3PO_4
1.32	NaBr	2.73	Na_2SO_4
1.39	CsI	2.78	Li_2SO_4
1.39	NH_4Cl	2.78	$FeCl_3$
1.4	$KClO_4$	2.93	$BaCl_2$
1.55	$FeSO_4$	3.12	K_3-citrate
1.63	LiCl	3.16	$MgCl_2$
1.64	NaCl	3.66	$CaCl_2$
1.57	$CsNO_3$	3.9	$K_4Fe(CN)^6$
1.82	$CuSO_4$	4.3	$K_3FE(CN)^6$

[a]Taken from reference 1.

Figure 1

The Relationships Between the
Solubility Coefficent and
the Surface Tension
Increment

The values for K_s and σ were obtained from Tables 1 and 2, respectively.


1.0
0.5
0
-0.5
K_s
1
2
3
Sigma

solution have a low affinity for one another, the solution will spread out easily and one will observe a low value for the surface tension. Water molecules exhibit particularly strong intermolecular interactions via hydrogen binding. It is this special property that, in large part, leads to the high values for the surface tension of pure water relative to other liquids at comparable temperature (19, 20). It is misleading to think that great cohesive energy between the surface molecules is the only factor producing surface tension. The surface molecules do not hold bulk water in a ball like a rubber balloon holds air, but rather water prefers the bulk phase so that it seeks to minimize the surface area. This is reflected in high surface tension. Putting it in thermodynamic terms, the free energy of the water molecules in the bulk phase is less than the free energy of the surface molecules. The solution seeks to minimize the free energy by minimizing the surface area. Another useful way to state the point is that it takes work to move a molecule of water from the bulk phase and place it on the surface (19-22). The more work it takes to do this, the greater will be the surface tension.

The increase in the surface tension that results from added salt can be understood from the definition of surface tension. Surface tension is defined as the amount of work, dw, required to increase the surface area by an amount dx (21). To do this one must remove water molecules from the bulk phase and put them on the surface. Therefore, anything that makes it more difficult to

extract molecules from the bulk phase will increase the surface tension. By this interpretation, salts increase the surface tension of water because they (somehow) increase the cohesiveness of bulk water. It takes more work to extract a water molecule from a salt water solution and put it on the surface, than from pure water; therefore, one observes an increase in the surface tension. Data on the enthalpy of hydration for the different anions should presumably bear on the validity of this interpretation, because enthalpy is generally considered to be a reflection of bonding energy (21, 23). Thus, ions that bind water molecules tightly would be expected to give high values for the hydration enthalpy and would also be expected to make those water molecules less available to create a new interface. This decreased ability of water to escape from the bulk phase could lead to an increase in the surface tension. The enthalpies of hydration have been determined for the halides and for perchlorate. For these five anions the enthalpy of hydration decreases in the expected order, i.e., $F^- > Cl^- > Br^- > I^- > ClO_4^-$ (24).

What is the Relationship Between Surface Tension, σ, and the Hydrophobic Effect?

Surface tension is a reflection of the tendency for a liquid to form spherical drops (19, 20, 21) and thereby minimize the air/water interfacial surface area which, therefore, minimizes the free energy of the drop. A hydrocarbon in an aqueous solution creates a hydrocarbon/water interface, similar to the air/water

interface (10, 25, 26). Consequently, hydrocarbon molecules in aqueous solution tend to associate in an attempt to minimize the area of the interface. It follows then that anything one does to increase the interfacial tension will lead to an increased tendency for hydrocarbon moieties to associate, so that the interfacial area will be minimized.

As mentioned earlier, the relative tendencies of different salts to salt-out proteins depends on the relative magnitudes of a salting-in component (which depends on direct binding of the salt to the macromolecule) and a salting-out component which involves the solvent properties of the solution and the ability of the solvent to accommodate hydrophobic surfaces. It is consistent with the previous interpretation that the surface tension increment is a direct measure of the solvating properties of salt solutions, such that, for a larger surface tension increment, the solution will have a smaller tendency to solvate hydrophobic groups. In other words, salts with large surface tension increments will tend to promote hydrophobic interactions.

The ability of the lyotropic salts to salt-in proteins is explained by their ability to bind with greater affinities to hydrophobically shielded, charged sites as indicated by the data of von Hippel. The reasons for these different binding affinities can also be interpreted in terms of the surface tension increment. Von Hippel determined that all salts bind with equal affinity to an ideal peptide bond dipole containing no hydrophobic groups around it. In line with the present hypothesis, the hydrocarbon

moieties around the dipole form an interface with a layer of water at the interface. As will be discussed below, this water layer acts like a barrier that interferes with the approaching salt molecules, lowering their effective concentration in the vicinity of the dipole and decreasing their apparent binding affinities. This "barrier" is more difficult for non-lyotropic salts to penetrate, which leads to a decreased binding affinity for the charge site when compared to more lyotropic salts.

In thermodynamic terms the data of von Hippel can be described in the following way. There is an intrinsic association constant, Keq, defined as,

$$Keq = \frac{[\text{dipole-salt}]}{[\text{dipole}]\ [\text{salt}]_{int}}$$

which is characteristic of the interaction between any salt and an ideal peptide bond dipole with no vicinal hydrophobic groups. In the present situation, $[salt]_{int}$ specifically refers to the concentration of salt at the interface with the aqueous phase immediately adjacent to the dipole; this is signified by the subscript "int." In the absence of secondary effects resulting from the presence of vicinal hydrophobic groups, $[salt]_{int}$ is equal to the concentration in the bulk solution $[salt]_{bulk}$. When hydrophobic groups are placed in the vicinity of the dipole, the effective concentration of the salt around the dipole is reduced by a factor, f, such that,

$$f[salt]_{bulk} = [salt]_{int}.$$

The expression for the apparent association constant, Keq_{app}, then becomes,

$$Keq_{app} = \frac{[\text{dipole-salt}]}{[\text{dipole}]\ [\text{salt}]_{bulk}} = f\ Keq$$

where f is close to 1 for the most lyotropic salts, and approaches zero for the less lyotropic salts.

Further evidence that salts are excluded from the hydrocarbon/water interface comes from the thermodynamic treatment of the variation of surface tension with composition, as derived by J. W. Gibbs (20, 22). His model describes the change in the surface tension, $d\gamma$, at an interface of undefined thickness, between the bulk phases (such as air and water or hydrocarbon and water) as,

$$d\gamma = -SdT - \sum_{i=1}^{\infty} \Gamma_i d\mu_i \ . \quad (1)$$

The quantity S, the surface excess entropy (22), is the amount by which the entropy per unit area of surface exceeds the entropy of the bulk phase. The quantity Γ_i, is the moles of component i at the interface region (of depth x cm) in moles per cm^2 surface area (Figure 2). Γ_i is the chemical potential of component i. For a two component system at constant temperature and pressure and by applying a Gibbs-Duhem expression (20, 22), one gets,

$$-d\gamma/d\mu_2 = [\Gamma_2-(N_2/N_1)\Gamma_1] = \Gamma_{2(1)}.$$

Now the surface excess of component 2, i.e. $\Gamma_{2(1)}$, is the amount (in moles/cm^2 surface) by which Γ_2 exceeds the quantity of component 2 that would be associated with Γ_1 of component 1 in the bulk phase.

Figure 2

The Relationship Between Γ and δ

s = solute molecules (the empty space contains solvent)

x = the depth of the interface

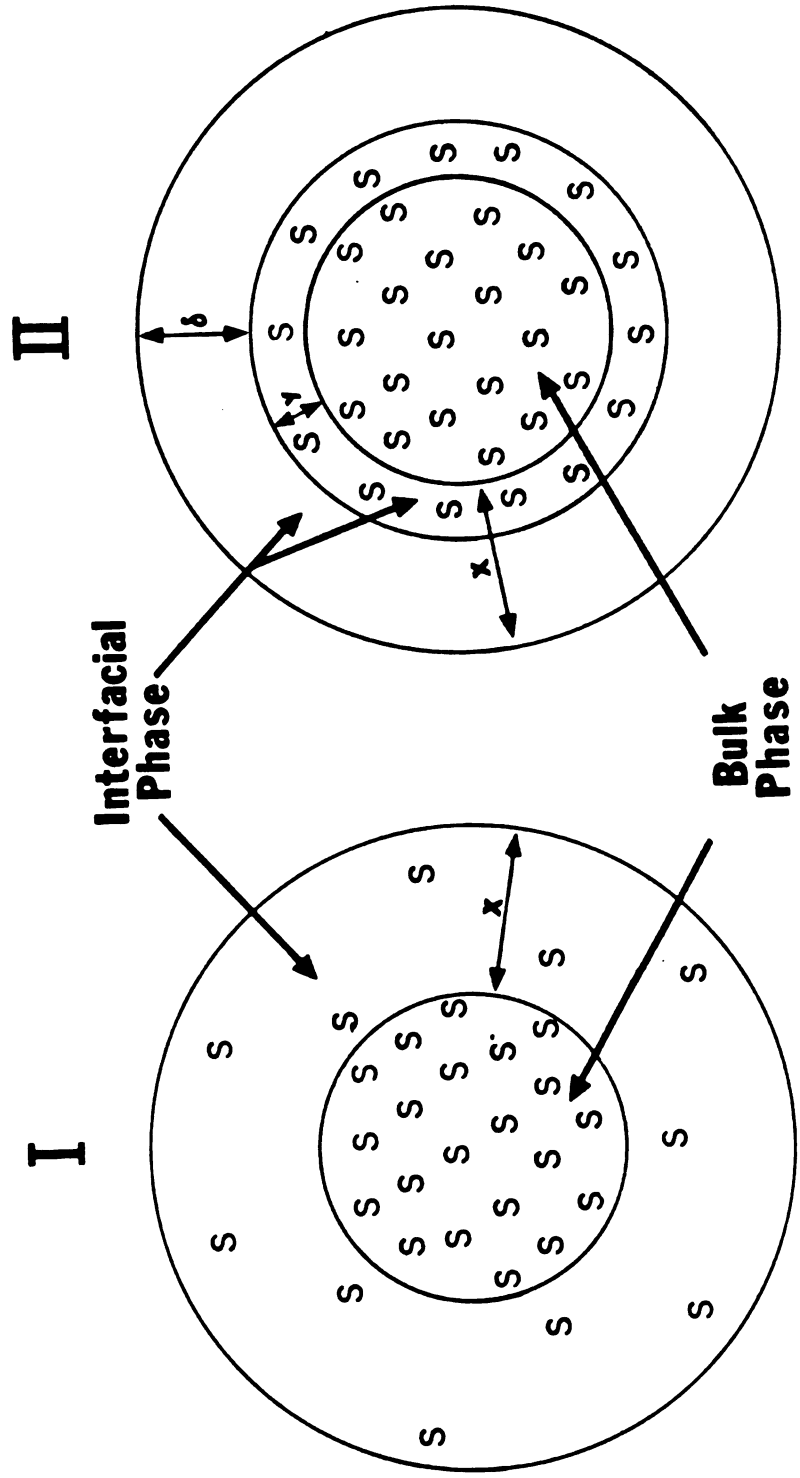
$\Gamma_{1(2)}$ = moles of S/cm^2 surface at depth x that is less than the moles of solute in a similar volume in the bulk phase ($\Gamma_{1(2)}$ is a negative number).

Likewise,

$\Gamma_{2(1)}$ = moles of excess solvent in the interfacial phase relative to the bulk phase

$\Gamma_{1(2)/x}$ = the interfacial concentration of solute

To get a difference in the solute concentration between a surface phase (defined at depth, x) and a bulk phase, the solute molecules may be organized in either of two ways. Either the solute molecules are distributed uniformly throughout each phase (I) or nonuniformly as in diagram II. In diagram II the value of Γ is the result of a surface layer, of thickness, δ, that is solute free (the ion free layer). The concentration of solute in the layer defined by the distance, y, is the same as in the bulk phase.


II
b
x
Interfacial Phase
Bulk Phase
I
x

N_1 and N_2 are the mole fractions of the respective components in the bulk phase.

The important point to glean from this relationship is that a difference in the solute concentration at the surface of a solution relative to the bulk phase can contribute to the interfacial tension. If component 2 is the solvent (water) and component 1 a solute (salt) then a surface excess of component 2 (positive $\Gamma_{2(1)}$) will give rise to an increase in the surface tension. Likewise, a surface decrement in component 2 ($\Gamma_{2(1)}$ negative) gives rise to a decrease in the surface tension. Direct measurements of surface excess quantities and $\Delta\gamma$ have verified the Gibbs equation (20).

In order to get some idea what the actual concentration differences are between solutes in the bulk phase vs. solutes at an interface it is necessary to come up with some means of defining the depth of the interface. One way that this can be done is by assuming that the surface excess of solvent ($\Gamma_{2(1)}$) at the interface is due to a surface layer of solvent that is completely solute free and that the molecules of solute present in the interfacial layer are surrounded by the same number of solvent molecules as in the bulk phase. The amount of excess solvent ($\Gamma_{2(1)}$) in the interfacial phase will then be given by the depth of the solute free layer. For an aqueous solution of salt this distance, δ, is called the ion free layer, given by,

$$\delta \text{ cm} = \Gamma_{2(1)} \left(\frac{\text{moles}}{\text{cm}^2}\right) \times 18 \frac{\text{cm}^3}{\text{mole}}$$

where 18 cm^3/mole is the molar volume of water (see Figure 2). It has been demonstrated that a relationship similar to that used to define σ can be written for δ such that,

$$\gamma = \gamma_0 + \delta m \nu Q K \qquad (4)$$

where m is the molality of added salt, ν is the moles of ions per mole of solute and Q, the osmotic coefficient of the solution (22). Plots of $\Delta\gamma$ vs. $m\nu K$ yield straight lines with slope, $K\delta$ where K is a constant that includes the temperature in °K, the gas constant and the molar volume of water. The order of increasing δ for different anions is $SCN^- < ClO_3^- < Cl^- < SO_4^= < FeCN_6^{\equiv}$ again consistent with the Hofmeister series (22).

Since the enthalpies of hydration of the anions show an obvious relationship to δ, (22) the inference is that the larger values of δ are the reflection of a larger hydration sphere around the anions. This larger hydration sphere leads to larger values of δ because the anion is limited in its approach to the surface by the depth of its hydration sphere. There are other methods for calculating the depth of the hydration sphere around ions. The approaches have been reviewed by Marcus and Kertes (27). These values for different anions have been tabulated in Table III. And again we see that the depth of the hydration layer follows the

Table 3.--The Hydration Radii of Several Ions

	Å r_e	Å[a] r_{hi}	Å hyd sphere	Å[b] r_{hi}	Å hyd sphere	Å δ
F^-	1.23	3.52	2.29	3.06	1.83	
$SO_4^=$	2.18	3.79	1.61	3.64	1.46	5.10
Cl^-	2.02	3.32	1.30	2.55	0.53	3.46
Br^-	2.21	3.30	1.09	2.42	0.21	
NO_3^-	2.34	3.35	1.01	2.23	-0.11	
I^-	2.48	3.31	0.83	2.04	-0.44	
ClO_4^-	2.64	3.38	0.74	2.43	-0.21	1.67
SCN^-	2.57	-	-	2.21	-0.36	0.80

r_e = electronic radius

r_{hi} = hydrated radius

hyd sphere - $r_{hi} - r_e$

The two different values of r_{hi} were obtained from two different experimental approaches (27). The values of δ (the ion free layer) are from refernence 22.

[a]Calculated from the partial molal ionic volume of the salt solution (27).

[b]Calculated using ionic mobilities and Stoke's law (27).

lyotropic series, which is related to the surface tension increment and δ, the ion free layer.

Summary

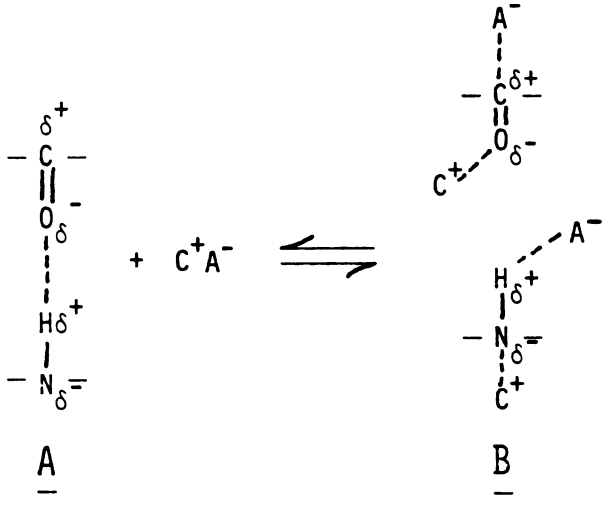
The following picture then emerges for the molecular basis of the binding data of von Hippel et al.: (1) All salts bind equally well to an ideal peptide bond dipole with no hydrophobic groups in the immediate vicinity of the dipole. As hydrophobic groups are placed around the dipole, a water/hydrocarbon interface is created similar to the air/water interface. The less lyotropic salts bind less tightly to the dipole because they are excluded from the interfacial water around the hydrophobically shielded dipole. The extent of exclusion from this interface is determined by the depth of the hydration layer of the ion.

(2) If the salts have a high binding affinity for the protein, they will tend to salt it into solution. If they do not have a high binding affinity, they will increase the interfacial tension until additional hydrophobic associations are favored and the protein will be salted-out. Or, in other words, the increased interfacial tension produced by added salt, results in a greater tendency for the solution to minimize the amount of hydrocarbon/water interface and several proteins will contact each other at their hydrophobic surfaces leading to large molecular weight aggregates and salting-out.

The salting-in phenomena can be described by the following equilibrium.

protein + salt ⟷ protein-salt
(insoluble) (soluble)

Binding of salt to regions of the macromolecule that are not readily hydrated by water, makes that molecule more soluble by making it more readily hydrated and hence more soluble. Take, for example, an α-helical segment of a protein with its peptide bond involved in intrahelical hydrogen bonds:



The segment of the protein molecule labeled A is expected to be less water soluble than segment B. This is because, due to intrahelical hydrogen bonding, the amide group is not accessible for hydrogen bonding with water. Binding of salt to the amide group (segment B) breaks the helix and allows interaction of the amide moiety with water through hydrogen bonding, as well as allowing ionic interactions with other ions in the bulk solution. Disruption of α-helical structure by neutral salts has been demonstrated (8). This is somewhat analogous to the situation with

some small molecules like short chain fatty acids that are relatively water insoluble at low pH's (when they are uncharged) but are more water soluble at high pH's as the salt.

Theoretical Treatment of the Salt Effects Data on the Hexokinase/Membrane Interaction

Melander and Horvath (1) have derived, from thermodynamic principles, an equation which can numerically quantitate the relative importance of electrostatic and hydrophobic forces in the interaction of small molecules or macromolecules with hydrophobic affinity columns. The equation is of the form

$$\ln {}^{k}/_{k_o} = - \beta - \Lambda m + \Omega \sigma m \quad (1)$$

where k_o, the capacity factor in the absence of salt, is the retention volume of the solute minus the holdup volume of an unretained solute, k is the capacity factor in the presence of added salt, m is the molality of added salt, σ the surface tension increment for a given salt, β an electrostatic "salting-in" component proportional to the dipole moments of the interacting groups and Ω is proportional to the square angstrom contact area between the hydrophobic groups of a macromolecule and the hydrophobic affinity column. As indicated by this equation, at low salt concentrations ($m \approx o$) the capacity factor (k) is largely determined by the constant, β; at higher salt concentrations the other terms become predominant.

To convert the above equation into a form applicable to the hexokinase/membrane interaction, we note that the capacity factor, is defined (28) by the equation,

$$\ln k = \ln K_{assoc} - \varepsilon \qquad (2)$$

where K_{assoc} is the association constant for the equilibrium expression,

$$S + L \rightleftharpoons SL \ . \qquad (3)$$

S is the concentration of free solute applied to the affinity column, L is the concentration of free hydrophobic binding sites on the affinity column, and SL the concentration of bound solute. ε is a constant characteristic of each individual column. The hexokinase/membrane interaction can be similarly written

$$E + M \rightleftharpoons EM \qquad (4)$$

where E, is the soluble hexokinase, M, the membrane binding site and EM, the amount of bound enzyme. For this expression an association constant can be written,

$$K_{eq} = \frac{[EM]}{[E]\ [M]} \qquad (5)$$

and equation (1) becomes

$$\ln {}^{K_{eq}}/_{K_{eq_o}} = -\beta - \Lambda m + \Omega\sigma m \qquad (6)$$

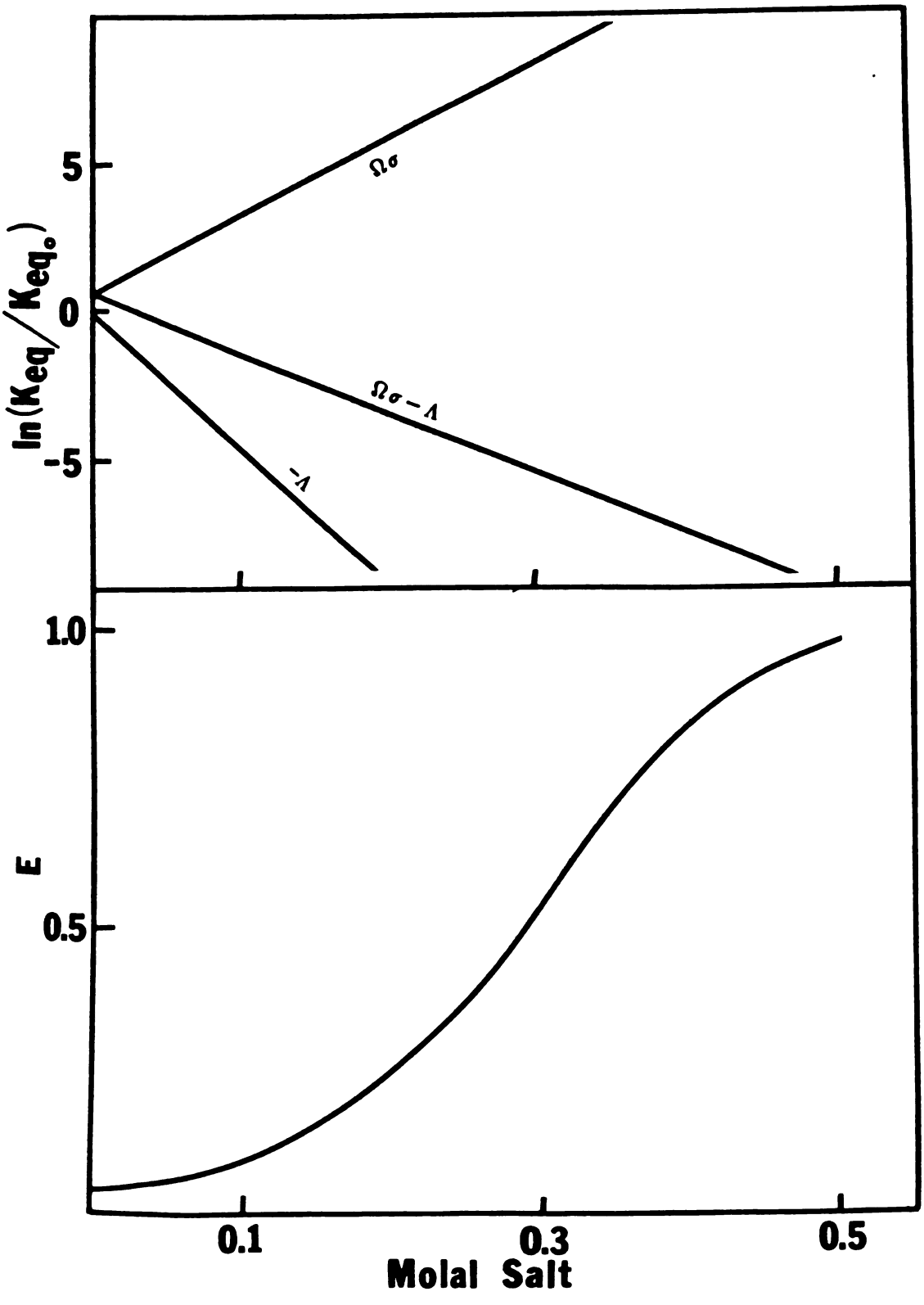
where K_{eq_o} is the equilibrium constant in the absence of added salt. According to this equation plots of $\ln K_{eq}/K_{eq_o}$ vs. m give straight lines with slope equal to $(\Omega\sigma-\Lambda)$ and y intercept equal to $-\beta$ as in figure 3A. This figure (as well as equation 6) illustrates that the salt dependence for solubilization is the net result of two components, one of which, Λ, tends to solubilize the enzyme and the other, $\Omega\sigma$, which tends to hold the enzyme on the membrane. Since the slope, Λ is greater than $\Omega\sigma$, the overall effect is to solubilize the enzyme. β is a constant that describes the effect of low salt on the equilibrium constant. In this example (figure 3A) the positive value of β indicates that low salt increases the association constant of the enzyme for the membrane. The slope Λ is a constant characteristic of the interaction and is related to the dipole moments of the interacting species. Likewise, Ω is a constant, but the slope $\Omega\sigma$ depends on the value of σ which is an empirically determined characteristic of the salt used (Table 2). Ω can be related by a complex equation (given in reference 1) to the square angstrom contact area between interacting hydrophobic groups.

The experimental data for the solubilization of hexokinase by salts as in reference 3 (appendix) was usually presented by plotting the fraction of enzyme solubilized versus the molality of the salt added. If, E, is the fraction solubilized, such that $E + EM = 1.0$, then $K_{eq} = (1 - E)/E^2$. Likewise, $K_{eq_o} = (1-E_o)/E_o^2$ where E_o is the fraction soluble at zero salt. By solving equation

Figure 3

Plots using Equation 6 with Assumed Values for All Constants

The constants used to generate these curves were Ω = 15.7, Λ = 45.3, and β = -0.5. The salt used was NaCl which has a value of σ = 1.64. E_o was equal to 0.05. The values were chosen to fit the experimental data (see Figure 4 and text).


5
0
-5
ln(Keq/Keq°)
Ωσ
Ωσ − Λ
−Λ
1.0
0.5
E
0.1
0.3
0.5
Molal Salt

6 for E at different salt concentrations, plots such as figure 3B are obtained. (The values of β, Λ, Ω and K_{eq_o} were the same as for figure 3A.) On the x asis is the molality of salt added to the membrane bound enzyme and on the y axis is the fraction solubilized. The addition of salt gradually solubilizes the enzyme until virtually all is solubilized. Also notice that the curve is sigmoidal indicating that low concentrations of salt have little solubilizing effect.

Figure 4 shows several curves constructed in a fashion similar to Figure 3B with the same values for Λ, Ω and β. Notice that the curves shift depending on the salt used. This is because each salt has a characteristic σ which changes the slope, $\Omega\sigma$, while Λ, Ω, and β stay constant (figure 3A).

The constants (Ω, Λ, and β) used to generate the curves on figures 3 and 4 were chosen to fit experimental data (3). The points plotted on Figure 4 are the experimental data points obtained for the hexokinase-membrane interaction. Notice that these data points fit the theoretical curves fairly well. This means that the constants Λ, β, and Ω are characteristic values for the hexokinase/membrane interaction.

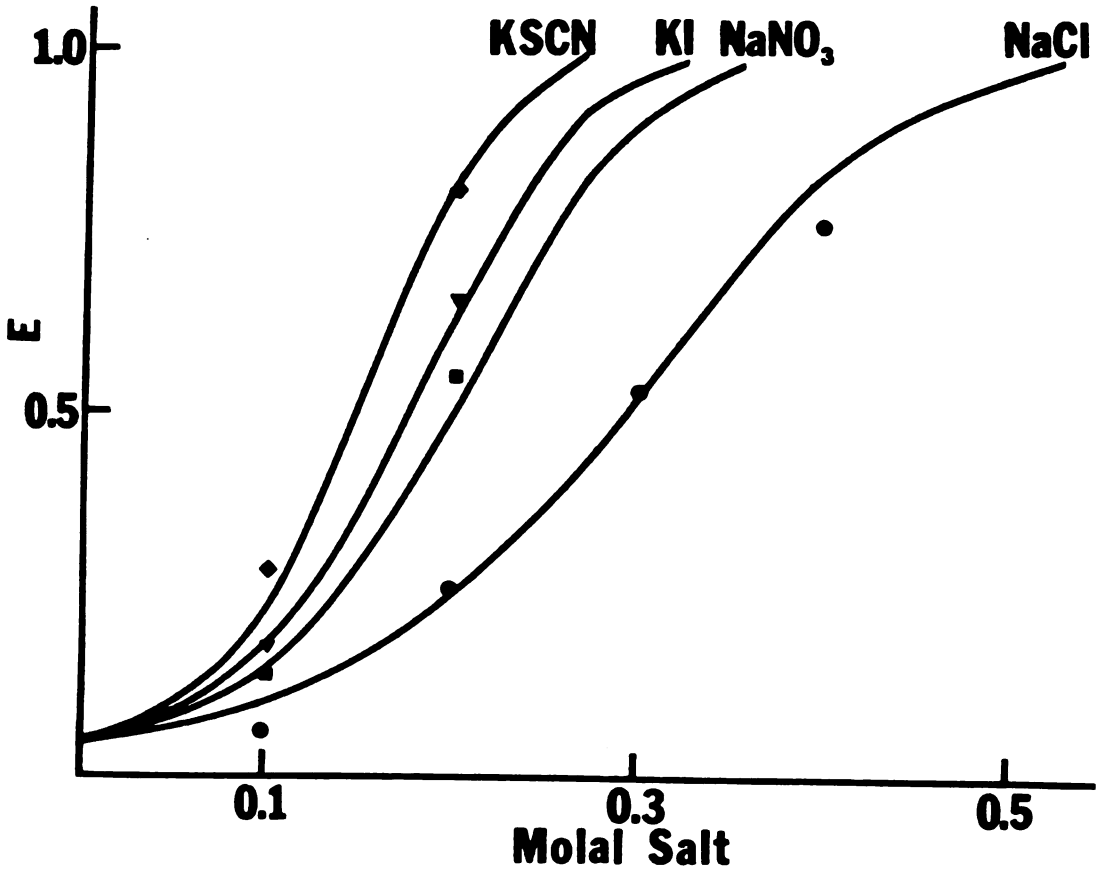
To calculate the nonpolar contact area between enzyme and membrane we can substitute the value for Ω (from Figure 4) into the equation

$$\Phi = 411\Omega - 12$$

Figure 4

Plots Using Equation 6 with Assumed Values for All Constants; the Effect of Different Salts

All constants were the same as in Figure 3. The values for σ, for KSCN, KI, $NaNO_3$ and NaCl, were 0.45, 0.84, 1.06 and 1.64 respectively (Table 2). The data for KSCN (■), KI (▼), $NaNO_3$ (◆) and NaCl (●) were obtained from reference 3. The lines are theoretically calculated with equation 6.


1.0
0.5
E
KSCN
KI
NaNO3
NaCl
0.1
0.3
0.5
Molal Salt

where Φ is the nonpolar contact area in square angstroms (1). Ω from Figure 4 is 15.7, so that Φ is equal to 6,440 A^2. Assuming a value of 0.74 gm/cm^3 and a molecular weight of 100,000 for the hexokinase molecule it can be calculated that the total surface area of hexokinase (if the molecule is spherical) is about 12,000 A^2. This means that about half of the total surface area is in contact with hydrophobic groups on the membrane. In other words, half of the molecule is embedded in the hydrophobic portion of the membrane. While this calculation may be extending the implications of the theory to the extreme, it is nevertheless interesting that it gives a somewhat reasonable value for the nonpolar contact area.

The value of this development is that numbers can be calculated which affix relative importance to hydrophobic vs. electrostatic effects in any given interaction where experimental data can be obtained on the effect of salts. The equations used to generate these numbers evolve out of a theoretical framework using principles of thermodynamics, so that the numbers have physical chemical meaning. It will be very interesting to see this approach applied to other systems so that the magnitudes of Ω and Λ can be compared.

CHAPTER II

SOLUBILIZATION, RECONSTITUTION AND CHARACTERISTICS OF THE OUTER MITOCHONDRIAL MEMBRANE BINDING SITE FOR HEXOKINASE

Introduction

The mitochondrial hexokinase from brain can be solubilized by low concentrations of glucose-6-P (30, 31). In the presence of $MgCl_2$, the enzyme rebinds to the outer mitochondrial membrane. Investigations dealing with the effect of neutral salts on the association between hexokinase and the mitochondrion, have provided the basis for a hypothesis describing the interactions between the outer mitochondrial membrane and hexokinase (3, appendix). These interactions appear to be primarily electrostatic in nature, in accord with Teichgraber and Biesold (32) and include both repulsive and attractive components.

As indicated in the first chapter of this thesis, there also appears to be a substantial hydrophobic component to the interaction. The extent of this hydrophobic component can be roughly approximated in terms of the square angstrom surface area of interacting hydrophobic groups. In order to reconcile the presence of a hydrophobic effect with the proposal that the interaction is primarily electrostatic we have suggested that the electrostatic interactions occur in a hydrophobic milieu. When compared to "ideal"

ionic bonds (i.e. ionic bonds not surrounded by hydrophobic groups), they are more difficult to dissociate because they are protected from attack by water and by salts. Lyotropic salts are more effective at disrupting the interaction because they can penetrate into a hydrophobic milieu more readily than non-lyotropic salts. The basis for these conclusions can be found in reference 3 included in the appendix.

So it develops that from the effects of neutral salts on the hexokinase membrane interaction, we were able to draw a more lucid picture of the hexokinase/membrane interaction. But after all this work was done, several key questions about the nature of the binding site still remained unanswered. What is the role of protein in the binding site? Is there a specific binding protein? What is the role of lipid? Is there a specific lipid or phospholipid ratio that is required to give the proper binding specificity and solubilization characteristics?

In this Chapter I outline several approaches directed at these questions. The experiments fall into three general categories. I will now briefly summarize and give the conclusions of each approach. Unlike the studies outlined in the first chapter and in reference 3 (appendix) which utilized the rat brain enzyme and hexokinase binding sites from the rat brain particulate fraction, the studies presented in this chapter utilized rat liver outer mitochondrial membranes (OMM) which could be obtained in a very pure form and in high yields. The OMM were found to contain

binding sites for hexokinase that had very similar properties to those of brain mitochondria (33).

1. Specificity of the outer mitochondrial membrane binding site--Outer mitochondrial membranes (OMM) from rat liver were purified and the specific activity of the binding site (in units of hexokinase bound/mg membrane protein) determined. When compared to various other membranes, the OMM had a specific activity of binding at least 40 fold greater, thus indicating that some specific factor(s) resides in the OMM that is required for binding. Some liposomes and liposome/protein mixtures could be shown to bind hexokinase but none of these showed the glucose-6-P dependent solubilization effect that is characteristic of the OMM. This result once again pointed to some specific characteristic(s) of the OMM that is (are) required to give the appropriate binding properties.

2. The role of lipid in the binding site--I discovered that treatment of the OMM with the non-ionic detergent, Emulgen 913, under the appropriate conditions would extract the phospholipid and most of the cholesterol from the OMM, without solubilizing the proteins. This treatment resulted in the loss of the glucose-6-P dependent solubilization effect (3, 31). When chloroform/methanol extracted lipids from the OMM were added back to the membrane proteins, the glucose-6-P dependent solubilization effect was restored. (See the Methods section for an explanation of what is meant experimentally by the "glucose-6-P dependent solubilization

effect.") Since lipids extracted from microsomes (which do not contain a binding site for hexokinase) also reconstituted the glucose-6-P dependent solubilization effect, it was concluded that the reason for the glucose-6-P sensitive, specific binding of hexokinase did not reside in the outer mitochondrial lipids alone.

3. The role of protein in the binding site--Several experiments aimed at chemically or enzymatically inactivating a proteinacious binding site in intact OMM gave negative results and it was consequently not possible by this approach to either confirm or rule out the hypothesis that protein was involved.

Another more direct approach toward determining the nature of the binding site involved solubilization, fractionation and reconstitution of a putative binding protein. The success of this approach was obviously contingent on the existence of a binding protein, for which there was no direct experimental evidence. After many fruitless attempts using different detergents and different methods of reconstitution, it finally became possible to prepare reconstituted membranous vesicles, greatly enriched in a single protein, that gave glucose-6-P-sensitve binding of hexokinase.

Materials and Methods

Chemicals

Biochemicals and HEPES buffer were obtained from Sigma Chemical Company. 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 was assayed spectrophotometrically by the glucose-6-P dehydrogenase method as previously described (34).

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 (4-6 strokes). The homogenate was centrifuged at 600 x g for 10 min and the pellet discarded. The supernatant was centrifuged at 6,500 x g for 15 min and the supernatant discarded. The 6,500 x g pellet was resuspended in 10 volumes sucrose and centrifuged at 6,500 x g for 15 min. The final pellet was resuspended in 2 volumes (based on original liver weight) of 0.25 M sucrose and 1 ml aliquots were stored at -20°C.

Crude rat brain mitochondria were prepared as described in reference 3.

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

1000 x g for 10 min, and the pellet discarded. The 1000 x g supernatant was centrifuged at 40,000 x g for 10 min and the resulting pellet washed once 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-P and incubated for 30 min at 25°C. The solubilized enzyme was obtained in the supernatant after centrifugation at 40,000 x g for 30 min and stored frozen at -20°C in 10 ml aliquots. Just prior to use the thawed aliquots were centrifuged at 160,000 x g for 30 min to remove particulate material.

Preparation of Purified Outer Mitochondrial Membranes

Rat liver mitochondria were prepared from 5 or 6 rats (85-120 gms of liver) by differential centrifugation from a 10% (w/v) homogenate (3 strokes w/homogenizer) in 0.25 M sucrose. After sedimentation of the nuclear fraction at 600 x g (2,250 rpm, 9RA rotor) for 15 minutes, mitochondria were sedimented from the supernatant by centrifugation at 6500 x g (7,500 rpm, 9RA rotor) for 20 minutes. The pellet was washed twice, with one half and one fourth of the initial volume of sucrose. The fluffy layer on top of the pellet was carefully removed after the last wash by aspirating with a Pasteur pipet. This procedure was slightly different from the one used when only crude liver mitochondria were required (see above).

The final pellets were suspended in 90 ml of ice-cold 10 mM Tris-phosphate (10 mM Tris + phosphoric acid to pH 7.5) buffer,

pH 7.5, by means of a Teflon pestle fitted into the centrifuge tube. After standing at 0° for 5 minutes (during which time the mitochondria underwent swelling) 30 ml of a solution containing 1.8 M sucrose, 2 mM ATP, and 2 mM $MgSO_4$ was added to the suspension. A visible increase in turbidity immediately appeared due to contraction of the mitochondria. After another 5 minutes at 0°, the suspension was subjected, in aliquots of 20 ml, to sonic oscillation at 3 amperes with a Branson Sonifier for 20 seconds at 0°.

The sonicated mitochondria were divided equally among freshly prepared sucrose density step gradients. Each gradient contained 5 ml of 1.32 M, 5 ml of 1.00 M and 5 ml of 0.76 M sucrose, and 20 mls of the sonicated mitochondria. The gradients were centrifuged at 25,000 rpm in the Beckman SW 27.1 rotor for 4.5 hrs. The purest outer membrane fraction sediments to the interface between 0.76 M and 1.0 M sucrose. This fraction was diluted with approximately 4 volumes of water and pelleted at 100,000 x g for 90 min. The pelleted OMM were taken up in 0.25 M sucrose to a concentration of 2-4 mg protein/ml and frozen in 0.5 ml aliquots at -20°.

1% Sodium Dodecyl Sulfate-Polyacrylamide Disc Gel Electrophoresis

The method of Fairbanks et al. (55) was somewhat modified. Samples (1-15 mg/ml) were prepared in 1% sodium dodecyl sulfate, 5-10% sucrose, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA and 2%

β-mercaptoethanol. They were then heated at 100° 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 overnight by gently agitating each gel in a 30 ml capacity test tube with two changes of 10% TCA/33% isopropanol. 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 (35, 36). The rather extensive washing of the gels removes SDS that interferes with the staining.

Mitochondrial Binding Assay

For the assay, aliquots of glucose-6-P solubilized hexokinase (prepared as above) and either liver mitochondria, brain mitochondria, purified outer mitochondrial membranes (OMM) or reconstituted OMM were mixed in polycarbonate centrifuge tubes and 3 m$\underline{M}$ $MgCl_2$ was added. The aliquots were incubated at 25°C for 10-15 min and spun at 40,000 x g for 10 min at 4°C or at 160,000 x g for 30 min if purified OMM or reconstituted OMM were used. 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 $\underline{M}$ sucrose by vortexing in the presence of glass beads (Sargent, No. S-61740, size A-7) until

homogeneous. With each new membrane preparation the concentration of binding sites was determined by titrating a fixed volume of the membrane suspension with increasing amounts of hexokinase. In this way, it was always possible to determine when the binding sites were in excess for a given amount of hexokinase.

Determination of Glucose-6-P Dependent Solubilization of Hexokinase from Purified Outer Mitochondrial Membranes

Binding of hexokinase to purified outer membranes was done by incubating an aliquot of OMM (50 µl, 2.0-4.0 mg/ml) with enough glucose-6-P solubilized hexokinase to saturate all available binding sites (5 ml, 0.7 u/ml) and 3 mM $MgCl_2$ for 10 min at 25°C. This suspension was centrifuged at 100,000 x g x 30 min. The pellet, containing bound hexokinase, was homogenized in a convenient volume (0.6 ml) of phosphate buffer (2 mM Na-phosphate; 2 mM thioglycerol; 0.1 mM EDTA; 2 mM glucose; 0.25 M sucrose; pH 6.6). 150 µl aliquots of the resuspended pellet were added to each of three tubes and 1.0 mM of glucose-6-P or galactose-6-P was added to two of the tubes. After incubation at 25°C for 15 min the tubes were spun for 5 min at top speed in a Beckman Airfuge and the supernatants were assayed. The data were usually presented as the percent of the total hexokinase activity that was in the supernatant after incubation and centrifugation. The amount of enzyme solubilized in the presence of glucose-6-P divided by the amount solubilized in the presence of galactose-6-P (the G6P/Gal6P

ratio) was also used as an indicator of the magnitude of the glucose-6-P dependent solubilization effect (see below).

Explanation of the Glucose-6-P/ Galactose-6-P Ratio and Rationale For Using This Ratio as a Means of Assessing the Intactness of the Hexokinase Binding Site

In many of the studies presented in this chapter the amount of enzyme solubilized with glucose-6-P divided by the amount solubilized with galactose-6-P was used to reflect the degree of intactness of the hexokinase binding site (see Tables 5-8, 9, 10, and 12-16, and Figures 13 and 13). When this ratio exceeded 1.3, it was routinely concluded that some intact binding receptor was present in the preparation. Values of 1.1 or less were judged to contain negligible amounts of intact binding sites. Mere binding (adsorption) of hexokinase to a given membrane (or protein) preparation alone, was not considered to adequately reflect the intactness of a native binding site (see Table 11). In this thesis the G6P/Gal6P ratios greater than 1.3 are often referred to as indicating "glucose-6-P dependent solubilization," "a glucose-6-P dependent solubilization effect," "glucose-6-P sensitive binding" or a glucose-6-P effect."

In experiments where various agents were used to perturb the native binding site, decreases in the G6P/Gal6P ratios were sometimes observed. There are two possible factors that can give rise to such decreases in the G6P/Gal6P ratio, i.e. either the

amount of enzyme solubilized in the presence of glucose-6-P decreases or the amount solubilized in the presence of galactose-6-P increases. When decreases in the G6P/Gal6P ratio were observed, this was sometimes referred to as a "decrease in the glucose-6-P effect." This terminology may be somewhat misleading since the reason for the decreased G6P/Gal6P ratio was often due to an increase in the amount of enzyme solubilized in the presence of galactose-6-P without any marked effect on the amount solubilized in the presence of glucose-6-P. In these cases then it might have been more appropriate to describe the effect as a galactose-6-P effect. I did not, however, change the terminology in the body of the thesis, because no ambiguity arises as long as it is understood that when I refer to the "glucose-6-P dependent binding," I am referring to the G6P/Gal6P ratio.

For reasons which remain as yet unclear, the percent of solubilized enzyme in the presence of glucose-6-P was found to vary from one experiment to the next as did the amount of enzyme released in the presence of galactose-6-P, sometimes without any deliberate change in the conditions of the experiment or without any appreciable change in the G6P/Gal6P ratio. Due to this variability in the percent solubilized by glucose-6-P and galactose-6-P it was considered difficult to make firm conclusions about the precise effect that a given perturbant had on the binding site with respect to glucose-6-P or galactose-6-P solubilization. Despite this variability, the G6P/Gal6P ratio was considered, for the

purposes of this thesis to be a sufficiently sensitive and quantitative as well as convenient indicator of the intactness of the native hexokinase binding site.

Results

Specificity of Hexokinase Binding to the Outer Mitochondrial Membrane

The data in Table 4 indicate that the specific activity for hexokinase binding by the purified outer mitochondrial membrane (OMM), is at least 40 fold higher than in erythrocytes, microsomes or inner mitochondrial membranes. SDS gel electrophoresis of purified outer mitochondrial membranes, with and without hexokinase, indicates that the binding is specific for hexokinase (Figure 5) since appreciable amounts of other proteins in the crude hexokinase preparation are not adsorbed.

Glucose-6-P Dependent Solubilization of Hexokinase from Purified Outer Mitochondrial Membranes

The data in Table 5 indicates that glucose-6-P solubilized enzyme, rebound to the outer mitochondrial membrane, can be specifically eluted with glucose-6-P and not with galactose-6-P. This specific elution is similar to that observed in the crude particulate fraction from a brain homogenate, as reported previously (3), except that the conditions for solubilization are somewhat different. With OMM the pH must be maintained at a relatively low value (pH 6.6) in order to get an observable effect and for reasons

Table 4.--Specificity of Hexokinase Binding to Outer Mitochondrial Membranes

Membrane	Units Hexokinase Bound / mg Membrane Protein
Outer Mitochondrial	4.1
Inner Mitochondrial	0.061
Microsomes	0.063
EDTA-Washed Microsomes	0.104
Erythrocytes (right side out)	0.021
Erythrocytes (inside out)	0.027

Microsomes were prepared from rat liver by centrifuging a rat liver homogenate (10 volumes, 0.25 M sucrose) at 40,000 x g for 10 min. This supernatant was centrifuged at 100,000 x g for 60 min. and the pellet was resuspended in 0.25 M sucrose to a concentration of 30 mg/ml protein. Part of the microsomal fraction was washed with 20 mM EDTA, pH 7.4, to remove loosely bound protein and ribosomes (37). Inner mitochondrial membranes were obtained as a pellet in the sucrose density gradient during the preparation of outer membranes (see Methods). Right side out and inside out human erythrocyte membranes were prepared according to Steck and Kant (38). The specific activity of hexokinase binding sites in each membrane preparation was expressed as the total number of units of hexokinase bound (in the presence of $MgCl_2$ and excess hexokinase) per mg of membrane protein (see Methods for more details of hexokinase binding determination).

Figure 5

SDS Gels of Purified Outer Mitochondrial Membranes With and Without Added Hexokinase

A. 30 µl of an outer membrane preparation containing 3 mg/ml of protein was prepared for SDS electrophoresis according to methods.

B. As in A except that prior to preparation for electrophoresis hexokinase was bound to the outer membrane, by the procedure described in Methods. The two bands at the top of the gel on scan B come from the crude glucose-6-P solubilized hexokinase preparation.

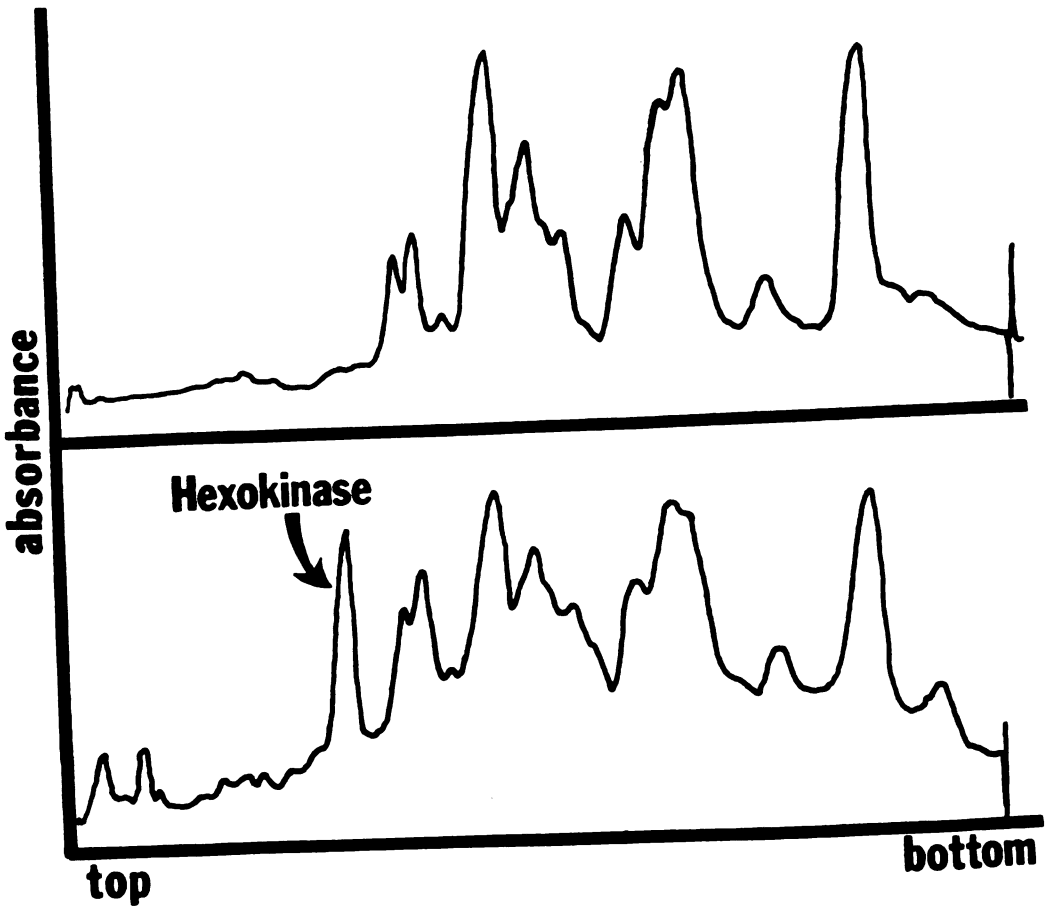

absorbance
Hexokinase
top
bottom

Table 5.--Glucose-6P Dependent Solubilization of Hexokinase from Purified Outer Mitochondrial Membranes.

	Units Solubilized	% Solubilized	G6P/ Gal6P
No Additions	.031	20	
Galactose-6-P	.031	20	4.7
Glucose-6-P	.142	94	

This experiment was done as described in the Methods section. The total units of hexokinase bound per 0.15 ml of the resuspended OMM was 0.152 units.

which remain unclear the spontaneous release from OMM is more sensitive to pH changes than that seen with intact mitochondria. At pH 8.0 all of the enzyme is released spontaneously from OMM (see below), whereas, crude particulate hexokinase remains 60-70% bound at pH 8.0 (3 , and appendix). Part of the explanation for this difference is apparently due to the removal of exogenous protein in the purified membrane preparation (see below).

The Effect of Exogenous Protein and Polyethylene Glycol on the Hexokinase Membrane Interaction

One readily apparent difference between the crude particulate enzyme and enzyme bound to purified outer membranes is that the amount of exogenous protein in the purified OMM is much lower. I, therefore, decided to determine the influence of added protein on the binding affinity.

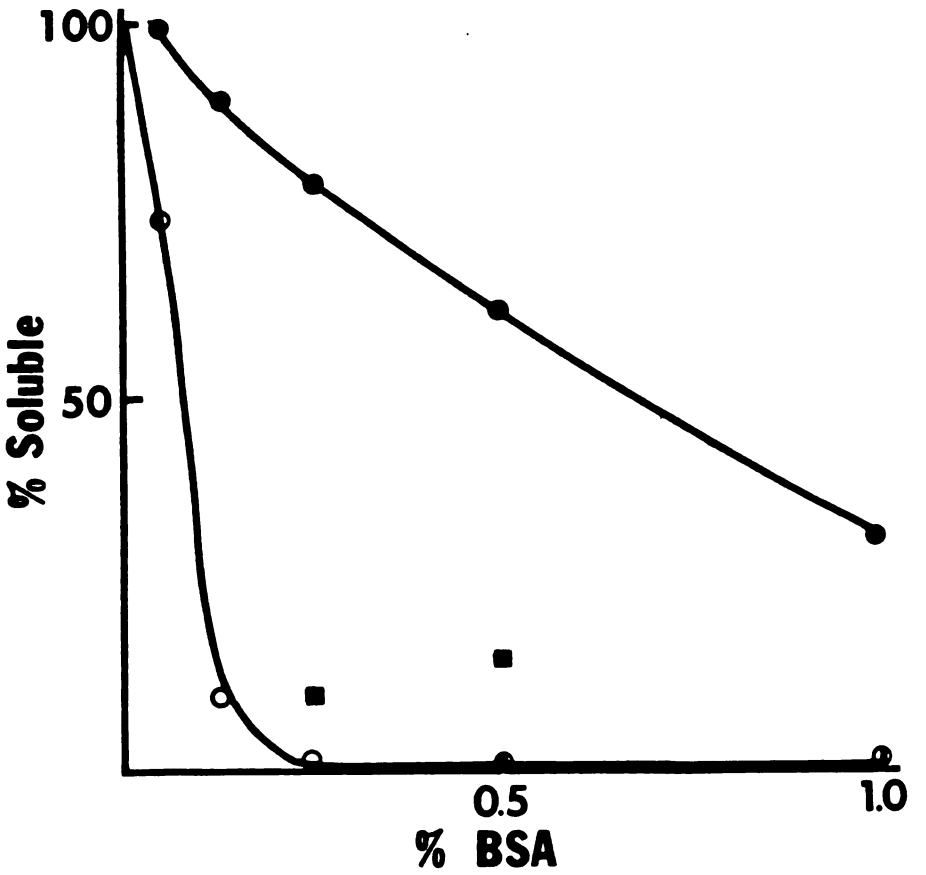
From Figure 6 it is apparent that the addition of exogenous protein (bovine serum albumin, BSA) results in a marked decrease in the amount of spontaneous release from purified outer mitochondrial membranes at pH 8.0. Glucose-6-P, however, still causes extensive solubilization.

A possible explanation for the enhanced binding affinity observed upon addition of BSA is that, because of the requirement for BSA to be hydrated when it is in solution, it causes a reduction in the free water concentration and leaving less water available to bind to the sites between the enzyme and membrane. When the hexokinase molecule is not in contact with its binding site, there

Figure 6

The Effect of BSA on the Hexokinase/OMM Association

Aliquots of OMM containing bound hexokinase were prepared as in Methods. To each of the several pellets was added phosphate buffer (see Methods), adjusted to pH 8.0, containing the indicated concentrations of bovine serum albumin (BSA). The BSA had been dialysed extensively [3 days, three changes against 15 volumes of water] and lyophilized prior to preparation of the 10% stock solution. The BSA stock solution was adjusted to pH 8.0 with NaOH.) To each resuspended pellet was added 1 mM glucose-6-P(●), 1 mM galactose-6-P (○) or 3 mM NaCl (■) and the samples were incubated at 25°C for 15 min. After centribugation at 160,000 x g for 30 min the supernatants were assayed to determine the percent of the total activity that was solubilized.


100
50
% Soluble
0.5
1.0
% BSA

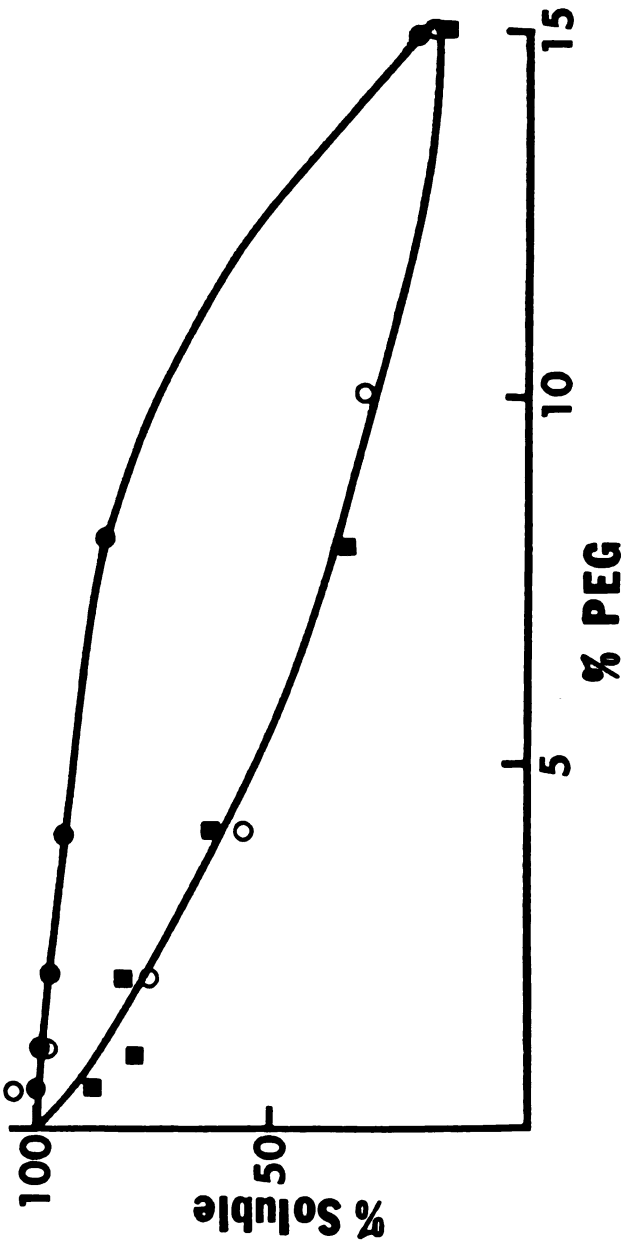
must be some water molecules occupying the points between the enzyme and the membranes that are in contact when the enzyme is bound. Hence, it takes more water to hydrate the solubilized hexokinase in the presence of the binding site, then it does to hydrate the enzyme when it is bound to its binding site. A reduction in the water availability might, therefore shift the equilibrium, between the enzyme and its binding site, more toward the bound form. To test this hypothesis I added increasing concentrations of polyethylene glycol (which like BSA must be extensively hydrated in solution) (Figure 7) to OMM bound hexokinase to determine its influence on the binding affinity. The effect of added polyethylene glycol-6000 (PEG) was similar to BSA, i.e. there was a marked reduction in the amount of spontaneous release with a relatively smaller reduction in the amount of glucose-6-P releasable enzyme. At 15% PEG the apparent affinity of hexokinase for the binding site was so high that glucose-6-P did not cuase any specific elution.

An equally plausible explanation for the effect of BSA on the OMM/hexokinase interaction, is that the BSA is binding to the OMM, thereby inducing some type of change in the enzyme binding site to give the effects observed in Figure 6. This explanation is especially tenable in light of the recent report that BSA binds specifically to the outer side of the outer mitochondrial membrane (39). BSA binding to negatively charged regions of the OMM may decrease the repulsive forces (3) between the negatively charged enzyme and negatively charged membrane, and thereby, give rise to a higher binding affinity. Experiments

Figure 7

The Effect of Polyethylene Glycol on the Hexokinase/OMM Association

The binding of hexokinase was done as in Figure 6 but the pellets were resuspended in phosphate buffer containing the indicated amounts of polyethylene glycol 6000. After addition of glucose-6-P (●) galactose-6-P (■), or no additions (o) the samples were incubated at 25°C for 15 min and centrifuged as in Figure 6, and the supernatants assayed.


% Soluble
100
50
0
5
10
15
% PEG

aimed at resolving this question have not yet been done. The effect of polyethylene glycol could conceivably be analogous to this BSA binding effect.

Hexokinase Binding to Liposomes

In Table 6 are the results of several experiments designed to determine the binding properties of various liposome preparations. Liposomes with a net negative or neutral charge did not bind any enzyme even in the presence of $MgCl_2$. Positively charged liposomes bound enzyme tightly but this enzyme could not be released with glucose-6-P. Presumably the positively charged liposomes bound the negatively charged enzyme molecule via attractive electrostatic interactions. There was no $MgCl_2$ requirement for this effect. Negatively charged liposomes that had the positively charged protein lysozyme added to them, bound hexokinase. $MgCl_2$ was required to facilitate this interaction, (Figure 8) as is observed with mitochondria or purified OMM (3, 31, 33). The liposome bound enzyme could not, however, be specifically eluted with glucose-6-P. In fact, glucose-6-P caused decreased solubilization when compared with galactose-6-P. Presumably this indicates that glucose-6-P causes a conformation change in the enzyme that leads to a higher affinity of the enzyme for this type of liposome.

Liposomes prepared from a chloroform/methanol extract of outer mitochondrial membranes gave results similar to those obtained with negatively charged liposomes, that is, no enzyme bound to them. These liposomes when sonicated extensively to produce

Table 6.--Hexokinase Binding to Liposomes

	pH During Solubilization	Units Bound	% Solubilized Control	% Solubilized Gal6P	% Solubilized G6P	G6P / Gal6P
Neutral liposome	6.6	ns				
	8.0	ns				
Negative liposome	6.6	ns				
	8.0	ns				
Positive liposome	6.6	0.257	ns	ns	ns	
	8.0	0.241	ns	ns	ns	
Lysozyme/Neg. liposome	6.6	0.190	45	52	37	0.71
	8.0	0.148	91	91	82	0.90

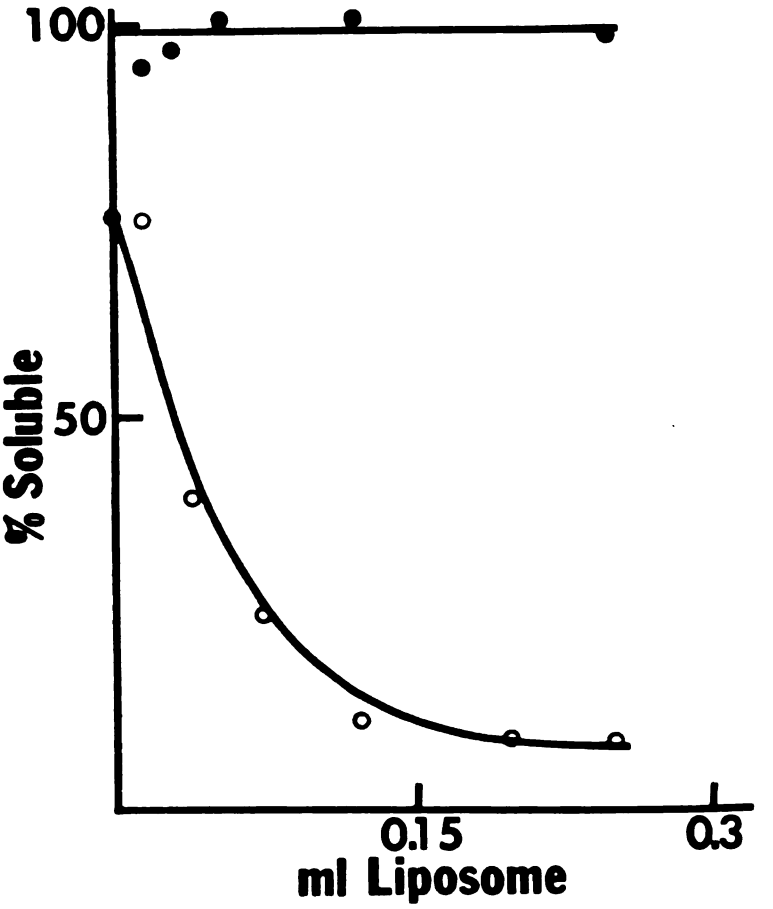
ns = not significant

Liposomes with a net negative or a net positive charge were prepared according to Sessa and Weissman (40) in 10 mM Na-phosphate pH 7.5 at a concentration of 5 mg/ml of lecithin with the appropriate amounts of cholesterol, and dicetylphosphate or stearyl amine. Liposomes coated with lysozyme were prepared by mixing 5 ml of the negatively charged liposomes with 1 mg lysozyme. Hexokinase binding and solubilization was done similarly to the outer membranes (see Methods) using 0.25 mls of each liposome preparation. The composition of the solubilization buffer was the same as in Methods, except that half of the solubilization was done with the buffer adjusted to pH 8.0.

Figure 8

Magnesium Requirement for Binding to
Lysozyme Coated Liposomes

The indicated volume of lysozyme coated liposomes (prepared as described under Figure 8) and 3 ml of glucose-6-P solubilized hexokinase (~0.6 units/ml) were incubated at 25°C for 10 min with (o) and without (●) 3 mM $MgCl_2$. The aliquots were centrifuged at 40,000 x g for 10 min and the supernatants assayed for hexokinase activity. The 25% precipitation of activity that is seen when $MgCl_2$ is added to the enzyme (with zero liposomes) is normal for the crude glucose-6-P solubilized enzyme that has not been centrifuged at 160,000 x g. The high speed centrifugation removes some particulate material from the solubilized enzyme that can presumably bind hexokinase in the presence of $MgCl_2$.


100
50
% Soluble
0.15
0.3
ml Liposome

unilamellar liposomes, still would not bind enzyme. Taken together all of the previous data indicate that there is (are) some specific characteristic(s) of the outer mitochondrial membrane that enables it to specifically bind hexokinase in a glucose-6-P dependent manner.

The Role of Protein in the Binding Site; Attempts to Modify the Binding Properties Chemically and Enzymatically

Protease digestion.--Extensive protease digestion of OMM led to substantial modifications in the banding pattern as determined by SDS gel electrophoresis (Figure 9). Several proteins were removed or reduced as the result of treatment with chymotrypsin or trypsin. Only two of the major bands in intact membranes remained substantially unmodified. Despite these marked changes in the protein composition of the treated membranes, however, the effects on their binding properties when compared to control membranes were minimal (Table 7). There was an increase (50%) in the specific activity of the binding site in chymotrypsin treated membranes and a slight decrease (15%) in the trypsin treated membranes, and the G6P/Gal-6P ratios in both protease treated preparations were only slightly decreased (10%); the latter differences were, however somewhat variable and were not considered large enough to make any inferences about the involvement of protein in the binding site for hexokinase on the membrane.

Figure 9

SDS Gels of Protease Treated Membranes

Membranes were treated as described in the legend to Table 7 and SDS gels were run as described in the Methods.

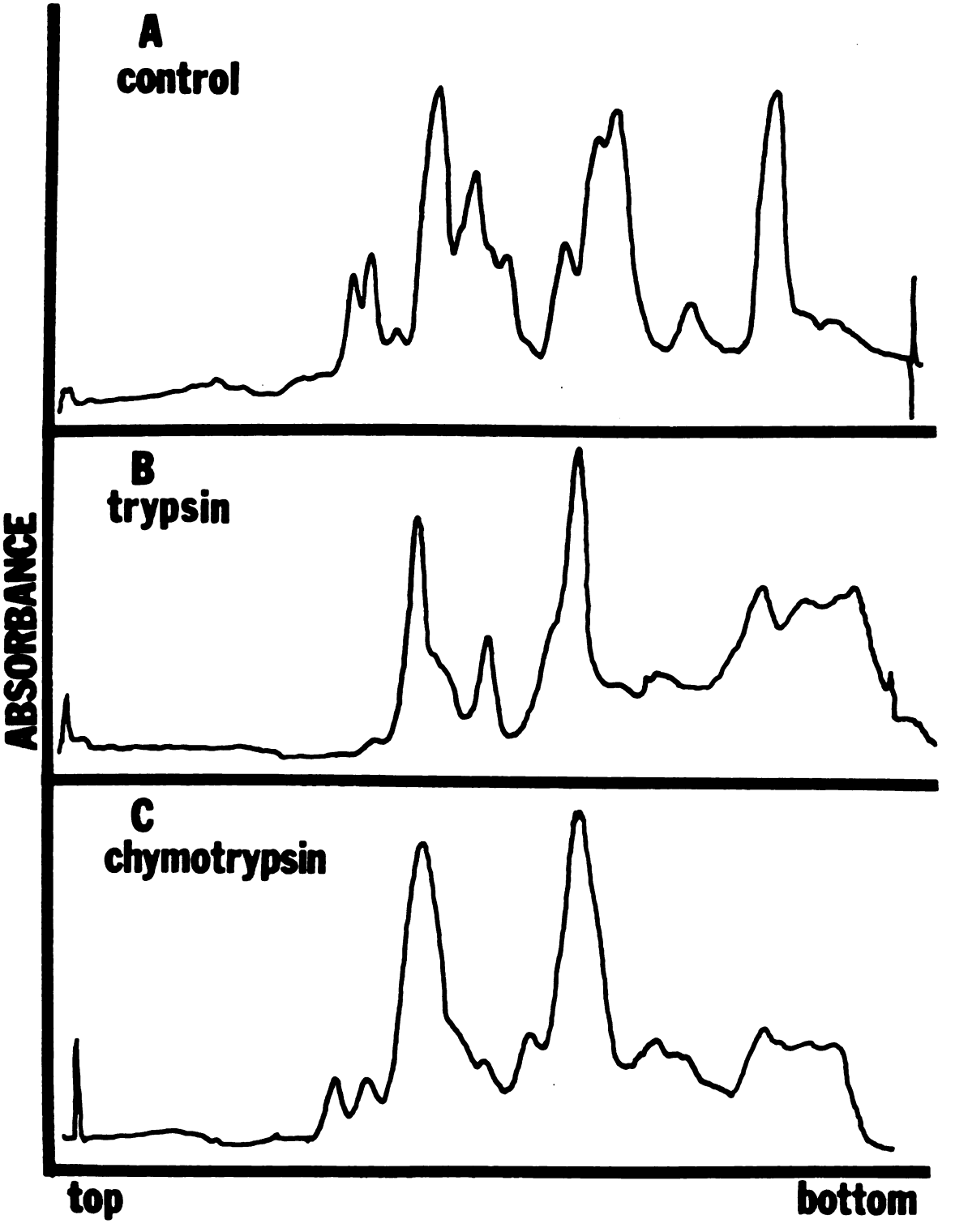

A
control
B
trypsin
C
chymotrypsin
ABSORBANCE
top
bottom

Table 7.--The Effect of Protease Treatment on the Outer Membrane Binding Site for Hexokinase

Outer Membrane Treatment	G6P / Gal6P	Units Bound/ mg membrane protein
Control	2.90	1.8
Chymotrypsin	2.76	3.0
Trypsin	2.65	1.47
Papain	2.90	3.1

Outer membranes at 1 mg protein/ml in buffer (50 mM tris pH 7.5; 150 mM NaCl; 2 mM $CaCl_2$) were incubated with 1 mg/ml of three different proteases for 1 hr at 37°C. In this experiment the control was left on ice. The specific activity of binding and the glucose-6P, galactose-6P solubilization ratios were determined for each of the protease treated outer membrane preparations, as in Methods. After proteolysis and centrifugation of the OMM to remove protease solubilized protein the mg membrane protein was determined.

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The data in Table 8 indicate (as do the data in Table 7) that chymotrypsin and trypsin do not inactivate the hexokinase binding site. This Table also shows that more binding sites were lost as a result of the 30 minute incubation in the control than in the protease treated membrane preparation. This protease induced stabilization effect, though small, was observed in three other experiments. It also appears from Table 8 that the protease treatment stabilizes the OMM against decreases in the G6P/Gal6P ratio.

A general approach to test for the involvement of carbohydrate in the binding site is to determine the effect of periodate treatment on the binding properties, since periodate characteristically oxidizes carbohydrate residues. The data in Table 9 indicate that this treatment did in fact have a marked affect on the binding site since about 30% of the binding sites were lost and the glucose-6-P dependent solubilization effect was completely lost (i.e. the G6P/Gal6P ratio was reduced to 1.0).

However, Figure 10 demonstrates that purified hexokinase, which is not a glycoprotein, is rapidly inactivated at a 40 fold lower concentration of periodate than was used to inactivate the binding site, indicating that protein, like carbohydrate, is also markedly susceptible to inactivation by periodate. This point has, in fact, already been demonstrated by Clamp and Hough (45) in a thorough study of the relative rates of oxidation of amino acids by periodate. Therefore, while it is clear from the data in Table 8 that periodate is reacting with something in the membrane that is

Table 8.--Proteoloysis of Intact OMM with Trypsin and Chymotrypsin

		% Solubilized	G6P/Gal6P	Units HK / mg prot.
Control	Control	28		
	Gal6P	40	2.22	2.4
	G6P	88		
Trypsin	Control	25		
	Gal6P	30	2.53	2.8
	G6P	77		
Chymotrypsin	Control	28		
	Gal6P	36	2.49	3.0
	G6P	91		
Unincubated Control	Control	21		
	Gal6P	27	2.81	3.6
	G6P	76		

To 0.15 ml of OMM (4.0 mg/ml) were added 75 μg of chymotrypsin or trypsin and the aliquots (including a control with no additions) were incubated for 30 min. at 25 C. The proteolysis was stopped by the addition of 2 m$\underline{M}$ phenylmenthyl-sulfonyl fluride (PMSF) from a 0.3$\underline{M}$ stock in ethanol; obtained from Sigma Chemical Co.). The aliquots were centrifuged at 160,000 x g for 30 min and the pellets resuspended in 0.25 $\underline{M}$ sucrose (0.4 ml). Hexokinase was added to each aliquot and the G6P/Gal6P ratio was determined as in Methods. In this experiment the amount of membrane protein was calculated from the initial amount of membrane protein present before protease treatment. The amount of membrane protein present after proteolysis was not determined.

Figure 10

Inactivation of Purified Hexokinase
by Periodate

Purified hexokinase (in 50 m$\underline{M}$ Hepes pH 7.5; 0.5 m$\underline{M}$ EDTA) was incubated at 25°C with 1 m$\underline{M}$ $NaIO_4$. Aliquots were taken out at various time intervals and assayed for hexokinase activity.

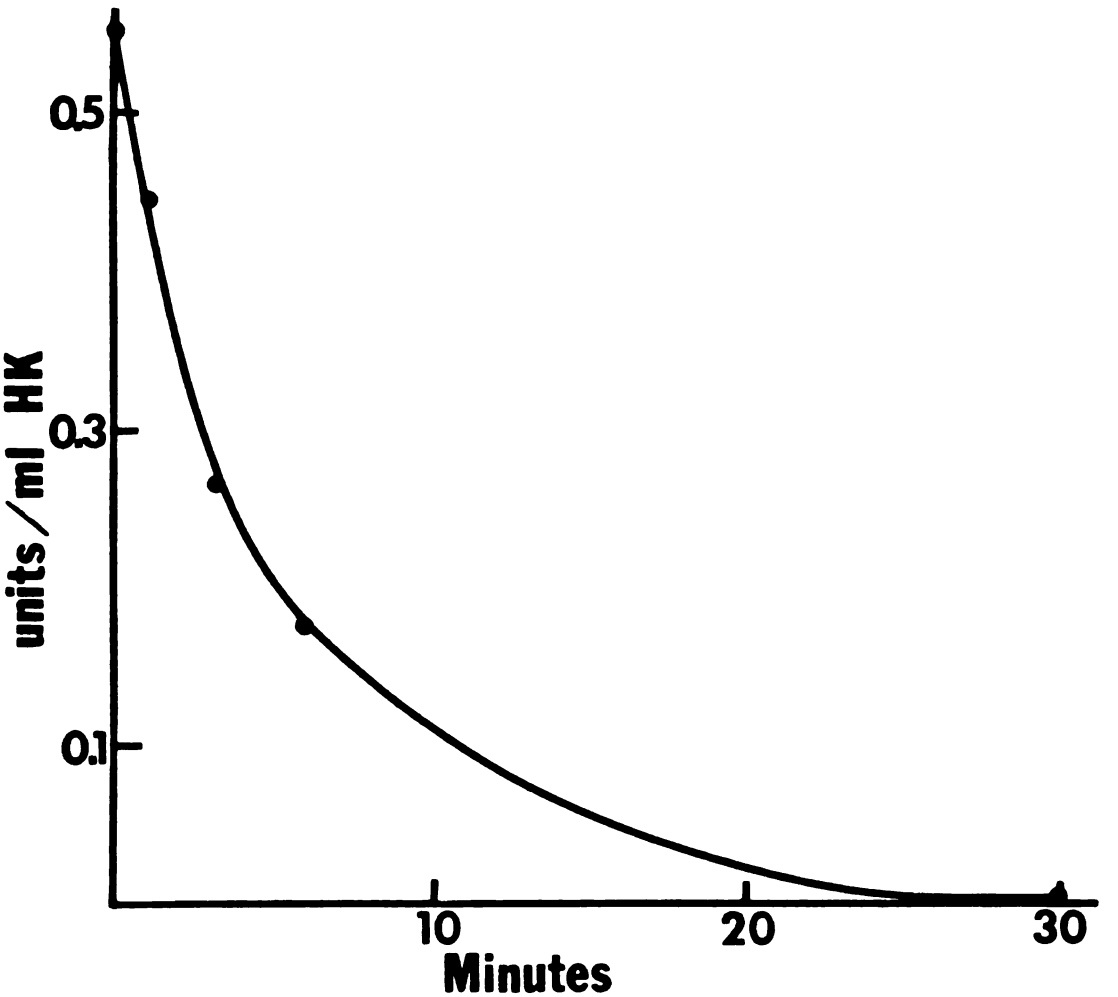

0.5
0.3
0.1
units/ml HK
10
20
30
Minutes

Table 9.--Effect of Periodate on Hexokinase Binding

	Units Bound		% Soluble	G6P/ Gal-6P
Control	0.32	No Additions	21	
		Galactose-6P	27	2.6
		Glucose-6P	71	
Periodate	0.20	No Additions	69	
		Galactose-6P	67	1.0
		Glucose-6P	67	

Intact liver mitochondria in 50 mM Na-phosphate pH 7.5 and 150 mM NaCl were incubated with and without 40 mM Na-periodate at 25° for 20 min. The mitochondria were pelleted and hexokinase binding and glucose-6P solubilization determined as described in Methods. Note that the 0.25 M sucrose in the resuspension media will quench any residual, unreacted periodate before the binding determination is begun. Formaldehyde (40 mM) which is a potential product of periodate oxidation was added to some binding assays as a control and found to have no effect on the binding properties.

essential to the binding process, no inferences can be made about whether that group is a protein or a carbohydrate.

Heat inactivation of the Glucose-6-P dependent solubilization effect.--Incubation of purified outer mitochondrial membranes at 37°C sites on the membranes as well as a decrease in the G6P/Gal6P ratio. Since a Ca^{++} activated phospholipase A is present in the outer membrane (41), the effect of added calcium and EDTA in the incubation medium were tested for their effects on inactivation. The data in Table 10 indicate that while EDTA partially stabilizes the binding site during incubation at 37°C (consistent with the phospholipase A hypothesis), calcium does not lead to destabilization (inconsistent with the lipase hypothesis). Table 10 also shows that thioglycerol does not stabilize the binding, suggesting that the mode of destabilization of this binding site is not oxidation of sulfhydryl groups. The data in Table 11 suggest that divalent metal ions tend to stabilize the binding properties against heat inactivation. One interpretation of these results might be that phospholipase A activity is the cause of the heat inactivation. The failure of calcium to cause increased destabilization of the binding site may be explained by a divalent ion stabilizing effect.

Miscellaneous.--Thiocyanate has been reported to solubilize membrane proteins (7). I, therefore, thought it would be of interest to determine the effect of this salt on the binding properties of the membrane. Extensive washing of intact mitochondria with

Table 10.--Heat Inactivation of the Glucose-6-P Dependent Solubilization Effect

Condition		Total Units Bound	% Soluble	G6P/Gal6P
phosphate	control	.979	44	1.70
	Gal6P		40	
	G6P		68	
phos + EDTA	control	.933	27	2.31
	Gal6P		26	
	G6P		60	
phos + Ca^{++}	control	.844	39	1.94
	Gal6P		38	
	G6P		74	
phos + T.G.	control	.732	54	1.30
	Gal6P		54	
	G6P		70	
sucrose	control	.563	59	1.37
	Gal6P		54	
	G6P		74	
No Incubation	control	1.367	21	3.00
	Gal6P		18	
	G6P		54	

OMM (3 mg/ml) were diluted with ten volumes of 0.25 M sucrose or with 10 mM Na-phosphate (pH 7.5) containing either 5 mM EDTA, 5 mM $CaCl_2$ or 10 mM thioglycerol. These aliquots were incubated at 37°C for 1.5 hours and subsequently pelleted by centrifugation at 160,000 g for 30 min. The G6P/Gal6P ratios were determined as described in Table 5.

Table 11.--Heat Inactivation of the Glucose-6-P Dependent Solubilization Effect

Condition	Total Units Bound		% Soluble	G6P/Gal6P
10 mM phosphate pH 7.5	0.74	Control Gal6P G6P	48 45 70	1.56
5 mM EDTA	0.707	control Gal6P G6P	24 26 59	2.27
5 mM $MgCl_2$	0.587	control Gal6P G6P	30 30 58	1.93
10 mM $MgCl_2$	0.474	control Gal6P G6P	38 35 68	1.94
100 mM NaCl	0.209	control Gal6P G6P	65 65 77	1.18
500 mM NaCl	0.346	control Gal6P G6P	34 34 53	1.56
100 mM NaCl 10 mM $MgCl_2$	0.306	control Gal6P G6P	42 43 63	1.47
0.25 M Sucrose (no phosphate)	0.289	control Gal6P G6P	49 45 51	1.13

This experiment was exactly analogous to Table 10 with the indicated additions of salt. All tubes contained 10 mM Na-phosphate, pH 7.5, except the 0.25 M sucrose control.

2 M KSCN still left the glucose-6-P solubilization effect intact. Consequently, if a protein in the membrane is involved in the binding site, it is not removed by salt washing. Treatment with 1 M NaCl was also found to leave the binding properties as well as the banding pattern on SDS gels, substantially intact.

Another way to approach the question of whether a protein is involved in the binding site is by treating the membranes with sulfhydryl reagents and determining their effect on the binding properties. If a sulfhydryl group on a protein is important in the binding site, this treatment would be expected to modify the binding properties. When intact mitochondria were exhaustively treated with DTNB, no change in the number of binding sites was observed. The effect on glucose-6-P solubilization was not checked.

Neuraminidase cleaves sialic acid residues from glycoproteins. Consequently, if membrane bound sialic acid is important in the binding site, neuraminidase treatment should modify the binding properties. When intact mitochondria were treated with neuraminidase, about 50% of the sialic acid was released but no change in the number of binding sites or in the concentration dependence of $MgCl_2$ for binding was observed.

Lectins bind to carbohydrate residues associated with membranes. If such residues are intrinsic to the binding site, then lectins would be expected to bind at the site and block

hexokinase binding. Pretreatment of intact mitochondria with concanavalin-A or wheat germ agglutinin did not decrease the amount of hexokinase binding on intact liver mitochondria. The G6P/Gal-6P ratio was not determined.

Lipid extraction from outer membranes with Emulgen 913.-- While attempting to solubilize outer membranes with the non-ionic detergent, Emulgen 913, I noticed that the membranes seemed unusually diffiuclt to solubilize. With relatively high concentrations of detergent (i.e. 1-2%) a large insoluble pellet remained after ultracentrifugation. A hexokinase binding site was found in this pellet but no glucose-6-P dependent solubilization could be detected. I suspected that the lipid had been preferentially removed from the membrane protein and subsequent assays proved that this was the case. Readdition of phospholipid back to the lipid depleted membranes, with sonication, substantially restored the glucose-6-P dependent solubilization effect. The experiments reveal a role for phospholipid in the binding site.

The data in Table 12 show that treatment with Emulgen 913 substantially removes the phospholipid from the outer membranes. The cholesterol level is also reduced but to a lesser extent. The thin-layer plates (Figure 11) likewise indicate a marked reduction in phospholipid upon Emulgen treatment. Figure 11 also

Table 12.--Lipid Extraction of OMM with Emulgen 913

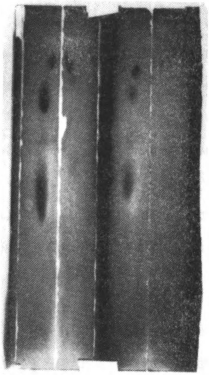
	Before Extraction	After Extraction	% Reduction Due to Emulgen Treatment
Protein	4.6 mg/ml	2.88 mg/ml	
Phospholipid	4110 nmol/ml - 893 nmole/mg prot.	160 nmole/ml - 55 nmole/mg prot.	94%
Cholesterol	129 nmole/ml - 28 nmole/mg prot.	31 nmole/ml - 11 nmole/mg prot.	61%

Outer membranes were diluted with 5 volume of cold 10 mM Na-Phosphate pH 7.5 and 1% Emulgen 913 was added. The membranes were immediately centrifuged at 160,000 x g for 30 min and the pellets were resuspended in a volume of 0.25 M sucrose equivalent to the volume of membranes used initially. Assays for protein (42), chloroform/methanol extractable phosphate (43) and cholesterol were done on the membranes before and after Emulgen treatment. Cholesterol was determined by reaction with cholesterol oxidase and flourometric measurement of H_2O_2 (Pat Kelly, personal communication). A second extraction of the OMM with Emulgen reduced their cholesterol content by an additional 25%. The % reduction of lipid due to Emulgen treatment was based on the amount of protein remaining after Emulgen treatment.

Figure 11

Thin-layer Plates of the Chloroform/Methanol Extracts of OMM, Emulgen Extracted OMM, and Reconstituted Emulgen Extracted OMM

Chloroform/methanol extracts of intact OMM (track #1) and Emulgen extracted OMM (track #2) (prepared as in Table 12) were spotted on silica gel plates and developed with chloroform-methanol-acetic acid-water, 25:15:4:2 (v/v). A portion of the Emulgen extracted material was sonicated in the presence of an excess (1 mg phospholipid/1 mg protein) of liposomes prepared from microsomal lipids and the resulting "reconstituted" vesicles were pelleted. An aliquot of this material comparable to that used on track #1 and #2 was extracted with chloroform/methanol and spotted on track #3.


1
2
3

demonstrates that upon sonication in the presence of phospholipid (extracted from microsomes), the phospholipid is extensively reincorporated back into the proteins.

The effect of lipid extraction on glucose-6-P dependent solubilization.--From Figure 12 it is clear that treatment with increasing concentrations of Emulgen leads to a progressive decrease in the glucose-6-P dependent solubilization effect (i.e., a decrease in the G6P/Gal6P ratio). In this experiment it appeared as though the reason for the reduction in the G6P/Gal6P ratio resulted primarily from an increase in the amount of release that occurred in the presence of galactose-6-P. However a relatively smaller decrease in the amount of glucose-6-P dependent solubilization was also observed. (These data are included in the addendum.)

Restoration of glucose-6-P dependent solubilization by adding back OMM lipids to Emulgen extracted membranes.--From Figure 13 it is clear that adding back chloroform/methanol extracted lipid from OMM to Emulgen treated membranes restores the glucose-6-P dependent solubilization effect. In this experiment, the total lipid extract from a 2x aliquot of membranes was required to give optimal restoration of the glucose-6-P effect from a 1x aliquot of Emulgen extracted OMM.

David Alessi has obtained data on the restoration of the glucose-6-P dependent solubilization effect from Emulgen extracted OMM using lipids extracted from microsomes. These data (Table 13)

Figure 12

The Effect of Emulgen Treatment of OMM on Glucose-6-P Dependent Solubilization

0.2 ml aliquots of OMM (~3 mg protein/ml) and 0.8 mls of 50% Buffer A (Buffer A: 0.3 M NaCl, 50 mM Na-Phosphate, 1 mM EDTA, pH 7.5) were incubated for 10 min at 4°C with the indicated amounts of Emulgen 913. The samples were centrifuged at 150,000 x g for 30 min (4°C) and the pellets were assayed for the G6P/Gal6P ratio as in Methods.

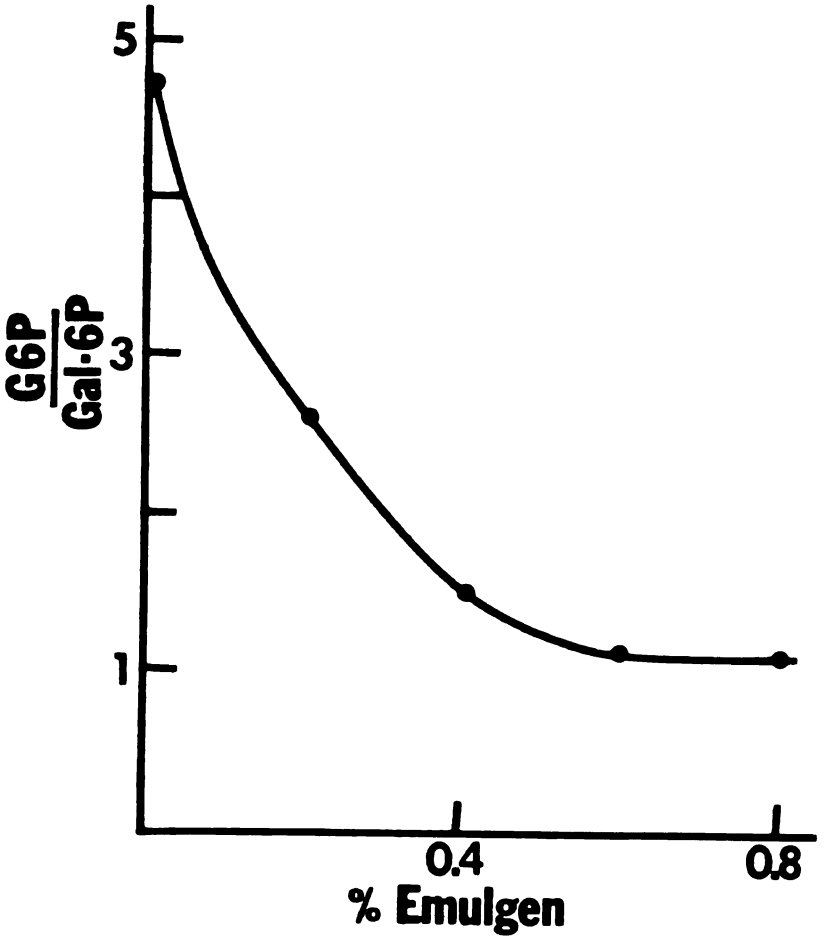

5
3
1
G6P / Gal-6P
0.4
0.8
% Emulgen

Table 13.--Partial Restoration of the Glucose-6-P Dependent Solubilization Effect to Emulgen Treated OMM, Using Lipids Extracted from Microsomes

		Experiment #1		Experiment #2		Experiment #3	
		% Solubilized	G6P Gal6P	% Solubilized	G6P Gal6P	% Solubilized	G6P Gal6P
Control	Control	44		18		56	
	Gal6P	48	1.75	17	3.82	40	2.32
	G6P	84		56		93	
Delipidated	Control	97		77		96	
	Gal6P	103	1.00	75	0.89	85	0.92
	G6P	103		67		78	
Reconstituted	Control	51		63		59	
	Gal6P	67	1.40	60	1.50	59	1.24
	G6P	92		91		70	

Table 13.--Continued

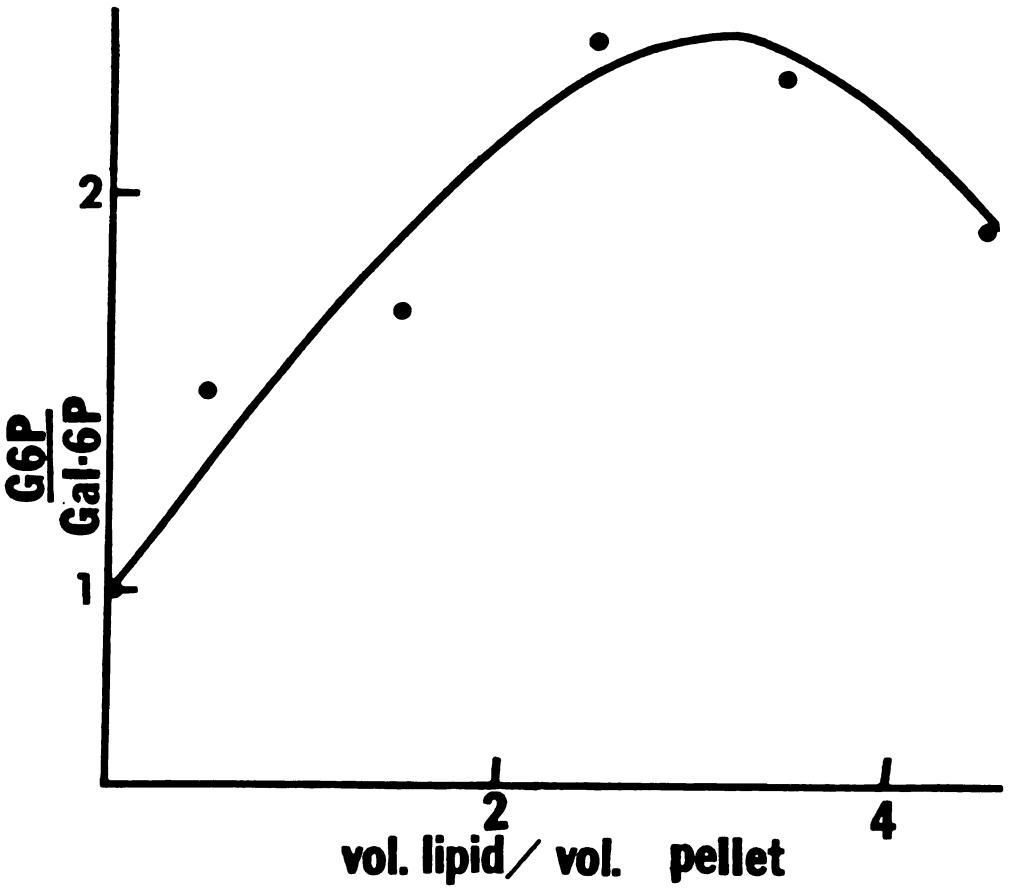
The Emulgen extracted OMM were obtained exactly as described in Figure 12. The Emulgen treated pellet from each 0.2 ml aliquot of OMM was homogenized in 0.5 ml, 0.01 M Na-phosphate pH 7.0. To this rehomogenized pellet was added 0.05 ml microsomal lipid (prepared as described below) and the sample was briefly (~30 sec.) sonicated in a bath type sonicator. Hexokinase was added directly to these "reconstituted" OMM and the G6P/Gal6P ratios determined as described in Methods.

The microsomal lipids were prepared by following a Folch type extraction procedure (44) on intact microsomes (30 mg microsomal protein/ml) prepared as described in the legend to Table 4. After drying this total lipid extract under a stream of nitrogen, the lipids were resuspended in a volume of 0.01M Na-phosphate pH 7.0 that was equivalent to the original volume of microsomes used.

Figure 13

Restoration of Glucose-6-P Dependent Solubilization from Emulgen Extracted OMM by the Addition of OMM Lipids

OMM were treated with Emulgen exactly as in Table 13 using 0.8% Emulgen. The Emulgen extracted pellet was suspended in a volume of 0.01M Na phosphate (pH 7.0) equivalent to the original volume of OMM used. To aliquots of the pellet were added increasing amounts of liposomes prepared from the chloroform/methanol extract of OMMs. The liposomes were prepared by sonicating the chloroform/methanol extracted (44) lipids in a volume of 0.01 M Na phosphate (pH 7.0), equivalent to the original volume of OMM from which the lipids had been extracted, until they were clear. On the y-axis is the G6P/Gal6P ratio, determined as in Methods. On the x-axis is the amount of liposomes that were added back to the Emulgen extracted pellet, expressed as the volume of the liposomes added per volume of the resuspended Emulgen extracted pellet. In this experiment an amount of lipid extracted from a 2 x volume of OMM was required to optimally restore the G6P/Gal6P ratio to a 1 x volume of Emulgen extracted OMM.


G6P / Gal-6P
2
1
2
4
vol. lipid / vol. pellet

indicate that microsomal lipids partially restore the glucose-6-P dependent solubilization effect to Emulgen treated OMM (as do lipids extracted from OMM; see Figure 12). Decreases in the G6P/Gal6P ratio due to Emulgen treatment appear to result primarily from increases in the amount of enzyme solubilized in the presence of galactose-6-P; however, decreases in the amount solubilized with glucose-6-P are also sometimes observed. Restoration of the glucose-6-P dependent solubilization effect by the addition of lipid seems, reproducibly, to imply decreases in the amount of enzyme released in the presence of galactose-6-P. However, increases, as well as decreases, in the amount solubilized in the presence of glucose-6-P are observed. The amount of enzyme solubilized when no hexose-phosphate is added, is about the same as that which is solubilized with galactose-6-P.

The scatter in the data on the extent of solubilization that occurs in the delipidated and reconstituted membranes by the hexose-phosphates, prohibits any firm conclusions on the exact role that lipid plays in the binding site, with respect to glucose-6-P or galactose-6-P solubilization. It is, nevertheless, clear that the G6P/Gal6P ratio is a sensitive enough indicator of the intactness of the hexokinase binding site to support the conclusions presented here. These conclusions are that removal of lipid from OMM by Emulgen causes a reproducible decrease in the G6P/Gal6P ratio, to approximately a value of 1.0, so that no glucose-6-P dependent solubilization effect is apparent. Since this property

can be restored by the readdition of lipid from either OMM or microsomes (which do not contain a hexokinase binding site), then the lipid requirement for recovery of the binding site is not a specific property of the OMM lipids alone. There must, therefore, be some other factor(s) present in the OMM (presumably protein) that gives rise to the specificity of binding and to the glucose-6-P dependent solubilization effect. Subsequent experiments (see below) have fully supported this contention that factors in addition to lipid (i.e. a hexokinase binding protein) are required for a fully intact hexokinase binding site.

I consider this experimental approach of delipidation of OMM by Emulgen an adequate one for demonstrating that lipids play some kind of role in forming a native binding site for hexokinase. I don't, however, think this approach is adequate for determining precisely what that role is since the data on the extent of solubilization by galactose-6-P and glucose-6-P were too scattered. One reason for this scatter may be that when the lipids are extracted from the OMM, the remaining insoluble proteins form large, partially denatured aggregates which are, subsequent to Emulgen treatment, pelleted at high speed. The extent of recovery of this binding site into its native conformation upon readdition of lipids, may depend on the method of resuspension of the pellet or on the extent of sonication, two factors which are difficult to control precisely.

A better approach for solving this problem would avoid the formation of such high molecular weight aggregates as well as avoid

sonication. We have found that the non-ionic detergent octyl glucoside solubilizes, without irreversibly denaturing, the hexokinase binding protein (see below). We have also noticed that phospholipids in the presence of octyl glucoside, can be dialysed away from OMM protein (data not shown). These observations suggest that a much better approach for determining the role of lipid in the binding site would be to dialyse octyl glucoside-solubilized OMM against octyl glucoside to remove the phospholipids. Purified lipids could be added back to this lipid depleted OMM protein to determine which lipids are required to restore the binding site to its native conformation. Hopefully this approach, since it would not involve formation of large delipidated, protein aggregates and sonication, would lead to more consistent results so that the precise role of lipid, with respect to glucose-6-P or galactose-6-P solubilization, might be more clearly resolved.

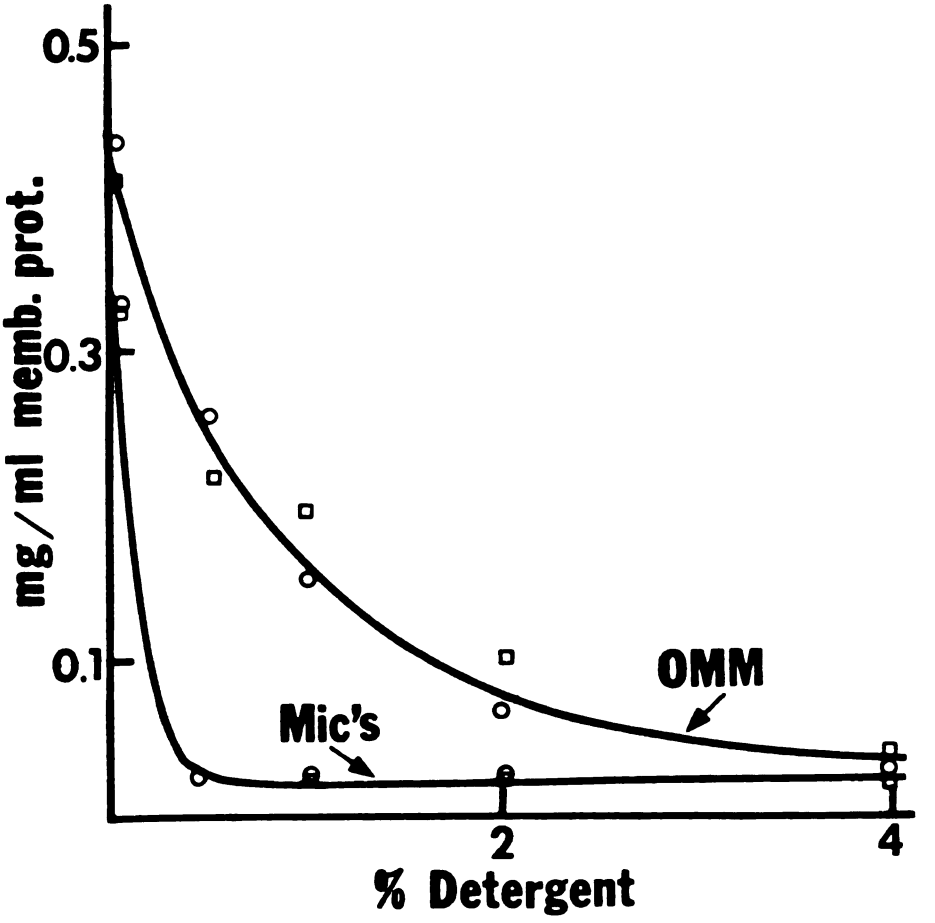
Comparison between outer mitochondrial membranes and microsomes with respect to detergent solubilization.--Figure 14 indicates that, compared to microsomes, outer mitochondrial membranes are strikingly resistant to detergent solubilization. Compared to the microsomes it takes at least a 10 fold higher concentration of either cholate or Emulgen to solubilize a comparable percentage of the outer membrane protein.

In the experiment presented in Figure 14 a rather high ionic strength was used (0.2M). Preliminary results suggest that, at low ionic strength, detergents are even less effective at

Figure 14

Solubilization of Outer Membranes and Microsomes with Emulgen 913 and Cholate

Outer membranes and microsomes (approx. 0.4 mg/ml) in 50 mM Na-phosphate pH 7.5, 0.3 M NaCl and 1 mM EDTA, were incubated for 15 min on ice with increasing concentrations of detergent either Na-cholate (o) or Emulgen 913 (□) (x-axis). After centrifugation at 150,000 x g for 45 min the pellets were resuspended in an equivalent volume of buffer and assayed for protein (y-axis) (42). From this figure it is apparent that relative to microsomes, outer membranes of mitochondria are markedly resistant to solubilization by either cholate or Emulgen 913.


0.5
0.3
0.1
mg/ml memb. prot.
OMM
Mic's
2
4
% Detergent

solubilizing the OMM. Taking these observations into consideration, the results in Figure 14 are taken to be an indication that the outer membrane proteins have an unusually high affinity for one another through ionic bonds as well as through hydrophobic attractions.

Solubilization, Reconstitution and Purification of a Putative Hexokinase Binding Protein from the Outer Mitochondrial Membrane

Solubilization of the OMM with octyl glucoside and reconstitution of the hexokinase binding properties by dialysis.--Addition of octyl glucoside (1-4%) to OMM in 50% buffer A (see figure 12) followed by centrifugation at 160,000 g x 30 min yields a clear supernatant and a small pellet. When this supernatant is dialyzed against 0.01 M Na-phosphate pH 7.0 for 4 hours to remove the detergent, membrane-like material forms which sediments out during centrifugation at 160,000 x g for 40 min. The data in Table 14 indicate that these particles are capable of binding hexokinase in a glucose-6-P sensitive manner. The G6P/Gal6P ratios approach or exceed 2. Solubilization with 2-4% octyl glucoside is required for optimal recovery of binding sites and the G6P/Gal6P ratios.

When octyl glucoside is added to a cold OMM sample, the membranes clarify immediately. If this sample is kept on ice, it stays clear for at least 30 min. If, however, the sample is warmed up to 25°C, cloudiness develops within minutes. The 160,000 x g

Table 14.--Solubilization and Reconstitution of the OMM Binding Site for Hexokinase; Detergent Concentration Dependence

% Octyl-Glucoside		Units Bound	% Soluble	G6P / Gal6P
	control		58	
1%	Gal6P	.023	58	1.34
	G6P		78	
	control		26	
2%	Gal6P	.130	27	2.07
	G6P		56	
	control		35	
4%	Gal6P	.101	35	1.94
	G6P		68	

OMM (~4 mg/ml) were mixed 1:1 with buffer A (0.3 M NaCl; 50 mM Na phos pH 7.5; EDTA 1 mM) on ice and the indicated concentration of octyl glucoside was added from a 20% stock solution. The solubilized membranes were immediately centrifuged for 30 min at 160,000 x g and the supernatants were dialyzed for 4 hours against 500 volumes of 0.01 M Na phosphate, pH 7.0. An amount of dialyzed material equivalent to 0.1 ml of the original membranes was used in the standard binding and solubilization assay as described under Methods.

pellet from a warmed sample is noticably larger than the pellet from a sample that has been kept cold. The data in Table 15 indicate that warming the sample prior to centrifugation and reconstitution does not appreciably reduce the recovery of binding sites or the G6P/Gal6P ratio. The procedure for solubilization and reconstitution as described in Table 15 with the 30 min incubation at 25°C prior to centrifugation was adopted for all subsequent experiments (unless otherwise indicated).

A comparison of the 1% SDS gels of intact OMM (Figure 15, A) with the reconstituted membranes (Figure 15, C), indicates that the reconstitution procedure results in a substantial reduction in the number of bands. Of the 15 bands that are present in intact membranes, primarily two, a 31,000 MW band and a 61,500 MW band, remain after reconstitution. SDS gels of the octyl glucoside insoluble material indicates that most of the membrane proteins are insoluble in the detergent and pellet out in the 160,000 x g centrifugation step (Figure 15, B).

When the reconstituted membranes are solubilized a second time, centrifuged, and dialysed, the reconstituted membranes obtained give the SDS gel banding pattern observed in Figure 16, C. From this figure, it is clear that 2x reconstituted membranes contain primarily the 31,000 M.W. protein. The data in Table 16 indicate that the 2x reconstituted membranes retain a substantial number of binding sites for hexokinase that are sensitive to glucose-6-P solubilization, although there are decreases in both the binding sites and the G6P/Gal6P ratio.

Table 15.--Solubilization and Reconstitution of the OMM Binding Site; the Effect of Preincubation of Solubilized Membranes at 25°C Prior to Centrifugation

	G6P/Gal6P	% Recovery of Binding Sites
Untreated OMM	1.99	≡ 100%
Control Reconstituted	1.85	52%
10 min; 25°C	2.15	40%
30 min; 25°C	1.75	42%

The membranes were treated identically to Table 14 with 2.6% octyl glucoside except that the solubilized membranes were either kepton ice (control reconstituted) or incubated at 25°C for 10 minutes or 30 minutes, prior to centrifugation. The recovery of binding sites in 1x reconstituted OMM is consistently around 50%. The most likely reason for this persistent result is that half of the binding sites in the reconstituted membranes are located on the inner side of the reformed vesicle and are, therefore, made inaccessible for hexokinase binding. While much precedence for this type of randomization of membrane sidedness during reconstitution exists (57), there could be other reasons for the 50% loss in binding sites observed in Table 15.

Figure 15

SDS Gel Electrophoresis of
Reconstituted OMM

OMM were solubilized and reconstituted exactly as in Table 15 with the 30 min preincubation at 25°C. The octyl glucoside insoluble material, the reconstituted membranes and intact OMM were applied to SDS gels exactly as described under Figure 5 and in the Methods section.

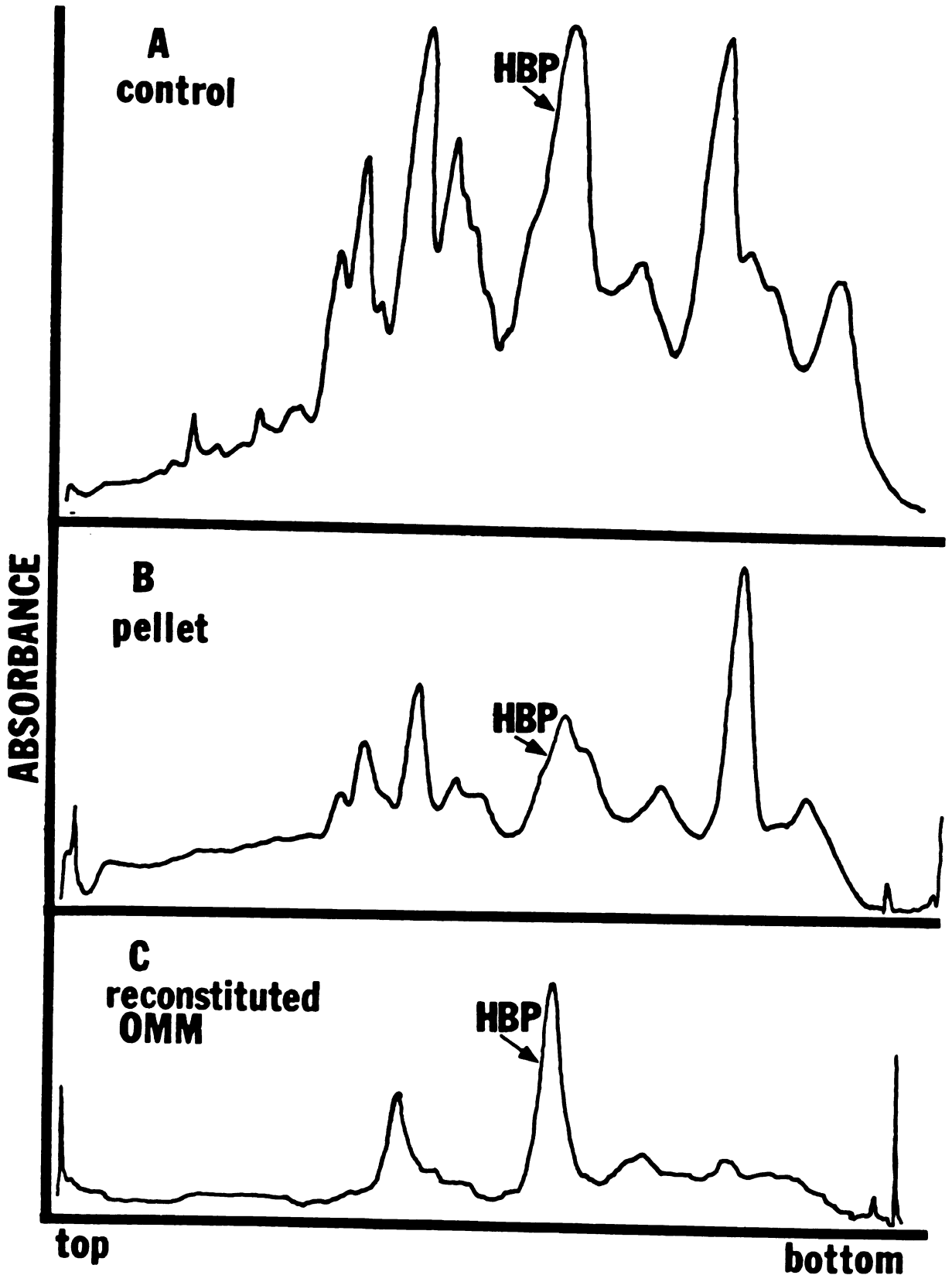

A
control
HBP
B
pellet
HBP
C
reconstituted
OMM
HBP
ABSORBANCE
top
bottom

Table 16.--Hexokinase Binding Properties of 1X and 2X reconstituted OMM

	G6P/Gal6P	% Recovery of Binding Sites
Intact OMM	2.22	≡ 100%
1X reconstituted	3.35	46%
2X reconstituted	1.51	22%

1X reconstituted OMM were prepared exactly as described under Table 15 and a portion of these membranes were used to determine the binding capacity and the G6P/Gal6P ratio. Another portion of the 1X reconstituted membranes were pelleted, and resuspended in a volume of 0.25 M sucrose equivalent to the original volume of the intact membranes. These membranes were resolubilized, incubated, centrifuged and dialyzed exactly as in Table 15 to give the 2X reconstituted membranes. The increase in the G6P/Gal6P ratio observed in this experiment for the 1X reconstituted membranes is not always seen. One usually sees a decrease in the G6P/Gal6P ratio after reconstitution.

Figure 16

SDS Gels of 1X and 2X Reconstituted OMM;
Comparison of Gels With and Without
Bound Hexokinase

Aliquots of intact OMM (A,B) 1X reconstituted OMM (C,D), and 2X reconstituted OMM (E,F), were prepared as in Table 16 and run on SDS gels according to the procedure described under Figure 5. For comparison, the same membrane preparations with hexokinase bound to them (as in Figure 5) were also run.

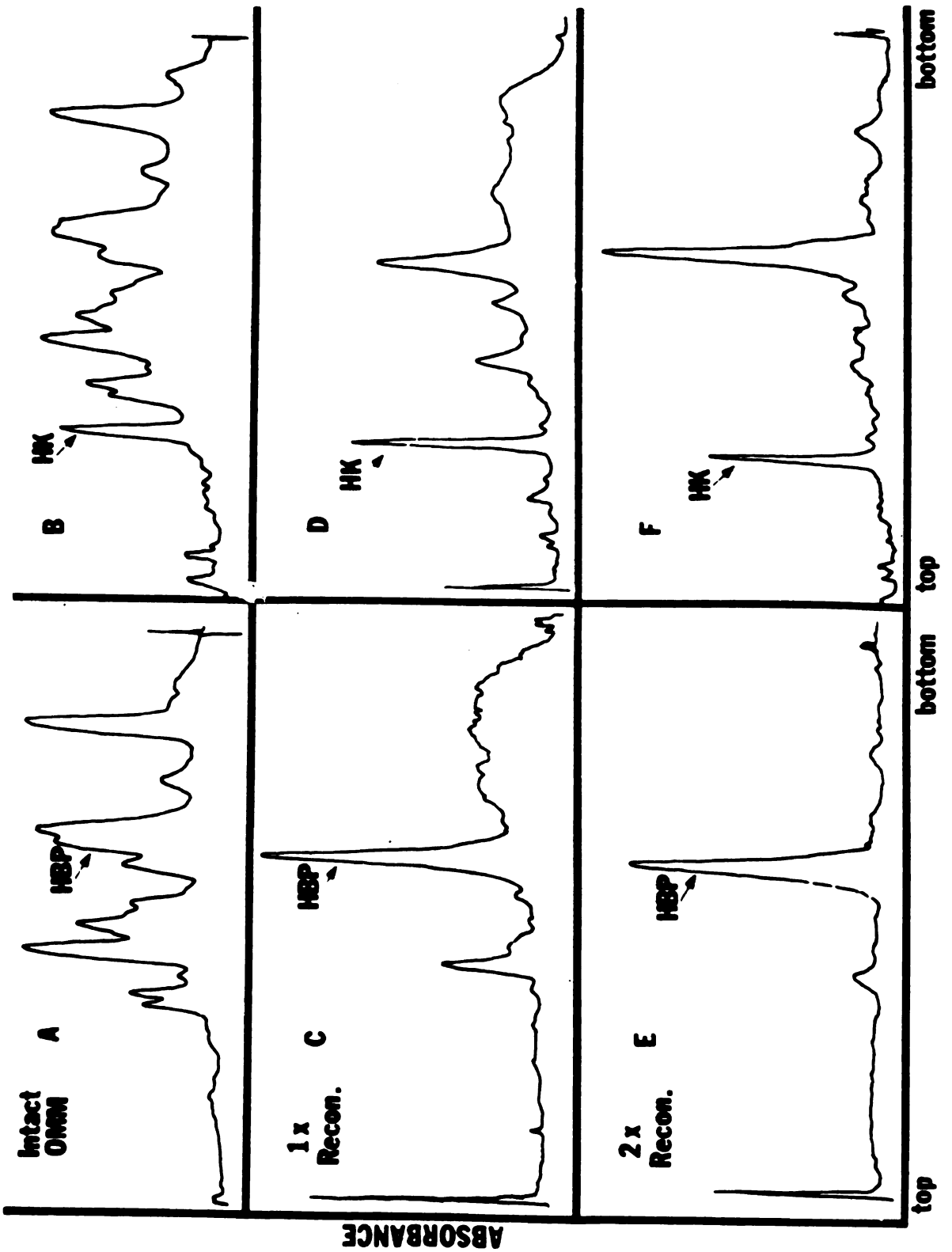

Intact
ONNM
A
HBP
B
HK
1x
Recon.
C
HBP
D
HK
2x
Recon.
E
HBP
F
HK
top
bottom
top
bottom
ABSORBANCE

Figure 16 also gives a comparison of the SDS gels of intact membranes, 1x reconstituted and 2x reconstituted membranes, with and without bound hexokinase. One finds that the amount of hexokinase bound is in each case somewhat less (based on the staining intensity of the bands) than the amount of the 31,000 M.W. protein.

Electron microscopy of the reconstituted vesicles.--Negative staining of 1x reconstituted OMM reveals the obvious formation of large vesicles (Figure 17, b) some of them comparable in size to the intact OMM (Figure 17, a). The vesicle diameter for both intact and reconstituted membranes ranges between 500 and 7000 Å but in the reconstituted preparation a larger fraction of the vesicles are of the small variety. The membrane thickness in both preparations appears to be about 70-140 Å, consistent with the view that the vesicles are unilamellar.

Thin-sections of OMM and reconstituted membranes after pelleting and fixation again revealed a similarity in the membrane thickness and vesicle diameter of the two preparations (Figure 18, 19). However, the reconstituted vesicles apparently collapse around one another during the pelleting leading to figures that often appear multilamellar. Close examination of the photographs, keeping in mind the differences in plane of sectioning through various types of collapsed vesicles, leads to the conclusion that the vesicles are primarily unilamellar not multilamellar. The differences in the electron micrographs of the two preparations is apparently a consequence of differences in the flexibility

Figure 17

Negative Staining, Electron Microscopy
of Intact and Reconstituted OMM

Intact (A) and reconstituted (B) OMM were prepared and stained briefly with 2% phosphotungstic acid on a Formvar coated electron microscopy grid.

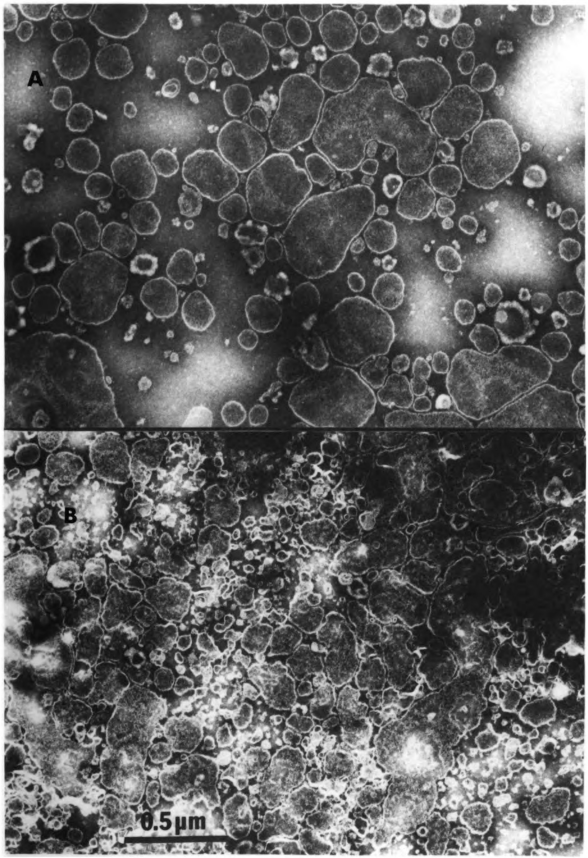

A
B
0.5 µm

Figure 18

Thin Sectioning, Electron Microscopy of
Intact and Reconstituted OMM

Intact (A) and reconstituted (B) OMM preparations were fixed in cold 2% glutaraldehyde in 0.01 M phosphate buffer (pH 7.0) for 2 hr. The pellets were then washed in three changes of the same buffer, and postfixed in 1% buffered osmium tetraoxide for 30 min. They were dehydrated in an ascending ethanol series and propylene oxide, and embedded in an Epon-Araldite mixture. Ultrathin sections, 500-600 Å in thickness, were then cut and examined with a Philips EM 201 electron microscope.

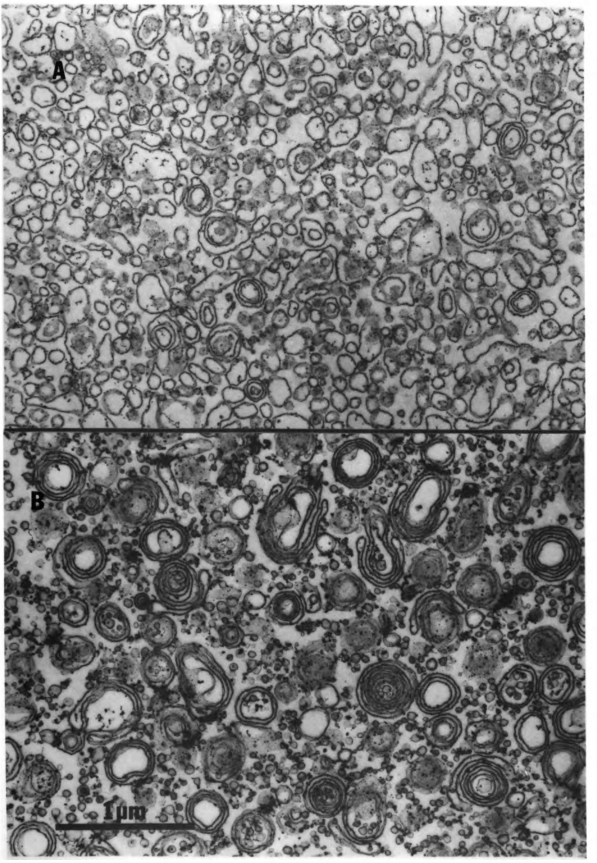
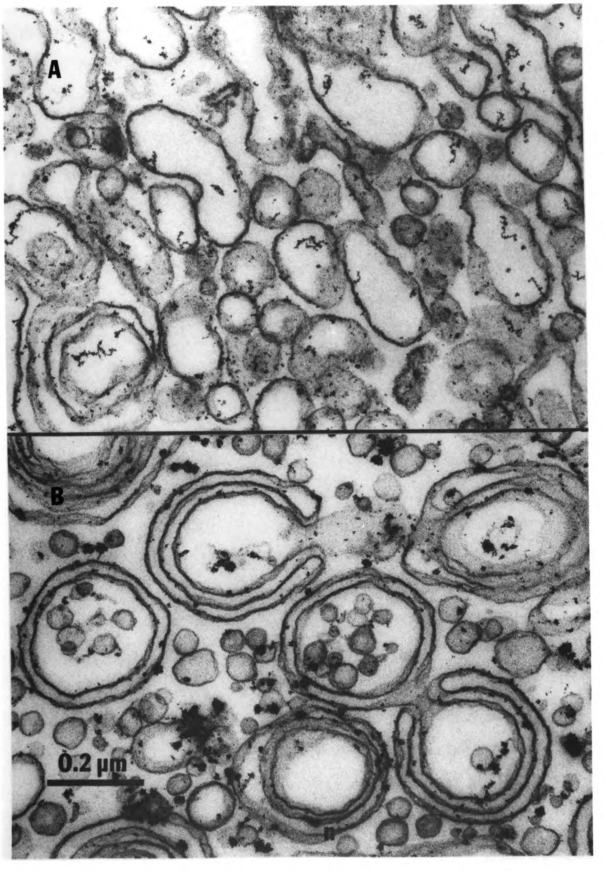

A
B
1 µm

Figure 19

Thin Sectioning, Electron Microscopy of
Intact and Reconstituted OMM

The intact (A) and reconstituted (B) OMM were processed in the same manner as in Figure 18.


A
B
0.2 µm

and/or the osmotic sensitivity of the two preparations, the intact OMM being more rigid.

Protease treatment of OMM followed by reconstitution.--The data in Table 17 indicate that prior treatment of OMM with either chymotrypsin or trypsin does not destroy the ability of the protease treated OMM to be reconstituted. The % recovery of binding sites as well as the G6P/Gal6P ratio is very good in all preparations. Since protease treatment alone apparently led to a purification of the binding protein (Figure 9) it seemed that combined protease treatment and reconstitution would lead to a greater purification. The gel scans (Figure 20), which compare protease treated/ reconstituted membranes to the ordinary reconstituted membranes, indicate that a somewhat more purified preparation was obtained from the protease treated membranes, but the differences weren't considered substantial enough to adopt protease treatment (with its possible artifacts) as a step in the standard purification procedure. The data in Tables 7 and 8 which give the binding characteristics of the various preparations may indicate that chymotrypsin treatment actually improves the recovery of intact binding sites, although the differences are small.

The data in Table 18 indicate that trypsin treatment of octyl glucoside-solubilized OMM prior to reconstitution destroys the binding site. The reconstituted membrane material obtained from the trypsin treated, solubilized membranes lost about 70% of the binding sites and the binding that remained was practically

Table 17.--Chymotrypsin and Trypsin Treatment of OMM Prior to Reconstitution

	G6P/Gal-6P	% Recovery of Binding Sites
Untreated OMM	3.73	100%
Reconstituted OMM	3.40	49%
Chymotrypsin/Reconstituted	4.08	54%
Trypsin/Reconstituted	2.41	47%

Aliquots of OMM were diluted 1:1 with buffer A containing 2 mM $CaCl_2$ and 1 mg/ml chymotrypsin or trypsin were added. The samples were incubated at 25°C for 45 min, 1 mM PMSF in ethanol was added and the samples were centrifuged at 160,000 x g for 45 min. The resulting pellets were resuspended in 0.25 M sucrose to their original volume and carried through the ordinary reconstitution procedure (Table 14 and 15) without the 30 min preincubation. The resulting membranes were checked for hexokinase binding ability and the G6P/Gal6P ratio was determined in Methods.

Figure 20

SDS Gels of Protease Treated/
Reconstituted OMM

Aliquots of the reconstituted membranes from Table 17 were applied to SDS gels as in Figure 5.

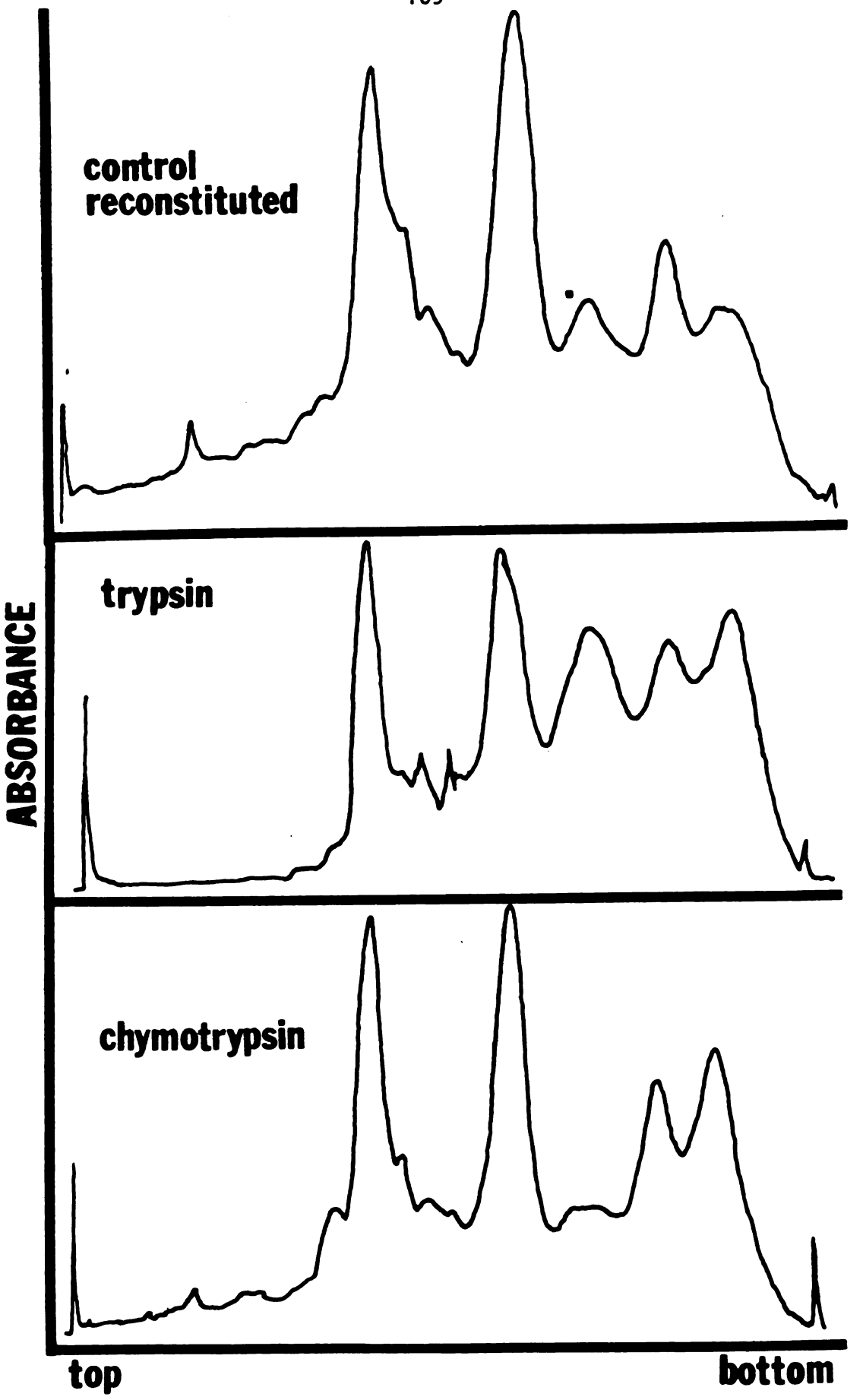

control
reconstituted
trypsin
chymotrypsin
ABSORBANCE
top
bottom

Table 18.--Trypsin Treatment of Octyl Glucoside Solubilized OMM, Followed by Reconstitution

	G6P/Gal6P	Relative Recovery of Binding Sites
Control Reconstituted	1.73	1.0
Trypsin Treated Reconstituted	1.09	0.29

Octyl glucoside solubilized OMM (Table 15) were centrifuged at 160,000 g for 30 min and the supernatant was treated at 25°C with 0.5 mg/ml trypsin for 30 min. 1 mM PMSF was added and the sample was dialyzed against 0.01 M phosphate pH 7.0 (as in Table 15). Binding assays on the trypsin treated and control samples were done as in Methods.

insensitive to glucose-6-P. The gels of the trypsin treated/reconstituted membranes (Figure 21) indicate that the reconstituted membranes were substantially modified and the binding protein was greatly reduced.

To summarize the current status of our understanding of the hexokinase binding site, the evidence implicating the involvement of a protein in the binding site is:

1. Lipids alone do not give a glucose-6-P sensitive hexokinase binding site.

2. Other isolated membrane preparations, including some with a lipid composition similar to OMM, show no significant ability to bind hexokinase.

3. The reconstitutability of octyl glucoside solubilized OMM is protease sensitive.

The evidence favoring the 31,000 M.W. protein as the hexokinase binding site is that:

1. Reconstituted OMM which retain substantial amounts of the 31,000 M.W. protein also retain 25-50% of their original hexokinase binding capacity. Other proteins in some of these reconstituted membrane preparations have been reduced by 95% or more without comparable losses in the binding capacity.

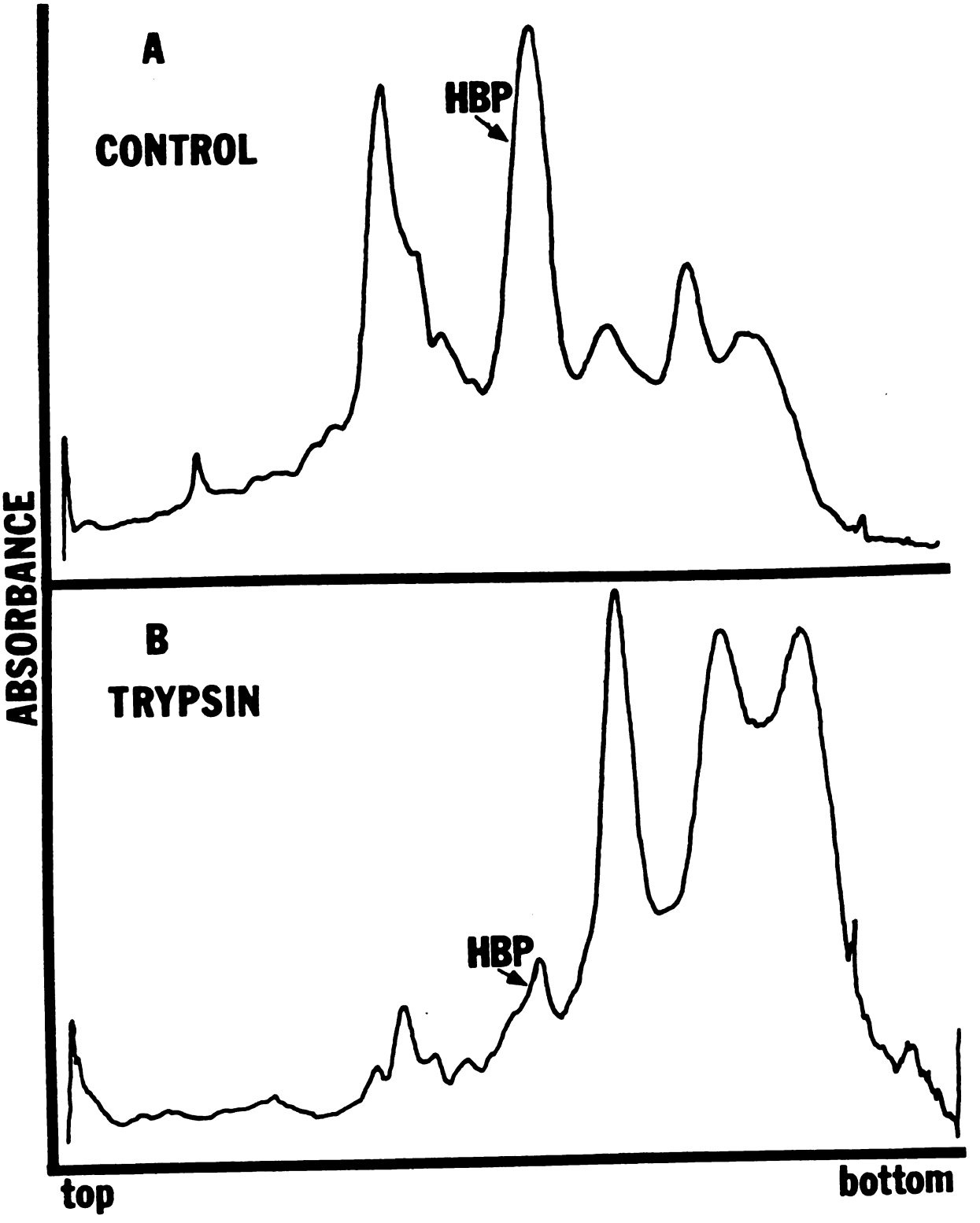
2. Hexokinase associates with intact or reconstituted OMM in amounts (gm/gm) that are roughly comparable to the 31,000 M.W. protein. The amount of hexokinase binding does not correlate with the amounts of any other protein present in intact or reconstituted OMM.

3. The protease treatment of octyl glucoside solubilized OMM results in the loss of the 31,000 M.W. protein as well as the loss in the reconstitutability of the hexokinase binding site.

Figure 21

SDS Gels of Trypsin Treated
Reconstituted OMM

SDS gels were run according to Figure 5 of the control reconstituted and trypsin treated-reconstituted membranes in Table 18.


A
CONTROL
HBP
ABSORBANCE
B
TRYPSIN
HBP
top
bottom

The experiments contained in this thesis do not definitively prove that the 31,000 M.W. protein is the one and only protein required for binding of hexokinase to the native OMM. Chemical crosslinking studies which are currently in progress may eventually resolve this point. If hexokinase can be shown to specifically crosslink to the 31,000 M.W. protein in intact OMM, then this certainly will be consistent with our contention that the 31,000 M.W. protein is the native binding protein.

The preparation of antibodies against the hexokinase binding protein will be attempted in the near future. If this antibody preparation specifically modifies the binding properties of hexokinase to OMM, this will be further proof that the 31,000 M.W. protein is the native binding protein. The antibody preparation should also prove very useful for screening different tissues of the rat (including brain) to determine whether all rat mitochondria contain the binding protein. Other mammalian mitochondria (e.g. cow, human) as well as non-mammalian (e.g. yeast, plant) could be checked for the presence of the binding protein to determine whether it is ubiquitous among all organisms.

Subunit molecular weights and tentative identification of some of the protein bands on SDS gels of OMM.--Figure 22 lists the molecular weights of the 15 bands that we routinely see on polyacrylamide gels of OMM. The putative hexokinase binding protein is band 11 and has a molecular weight of 31,000. Monoamine oxidase has been recently purified and shown to have a molecular weight on SDS

of about 65,000 (46), approximately equivalent to band 6. An excellent cytochrome b_5 spectra is easily obtainable from the purified outer membrane preparations. Since the cytochrome oxidase spectrum is nearly absent (and sometimes completely undetectable) in these preparations the outer mitochondrial membrane preparation as reported herein is judged to be virtually free from inner mitochondrial membrane contaminents. Cytochrome b_5 has been reported to have a molecular weight of approximately 16,000 corresponding to band 14.

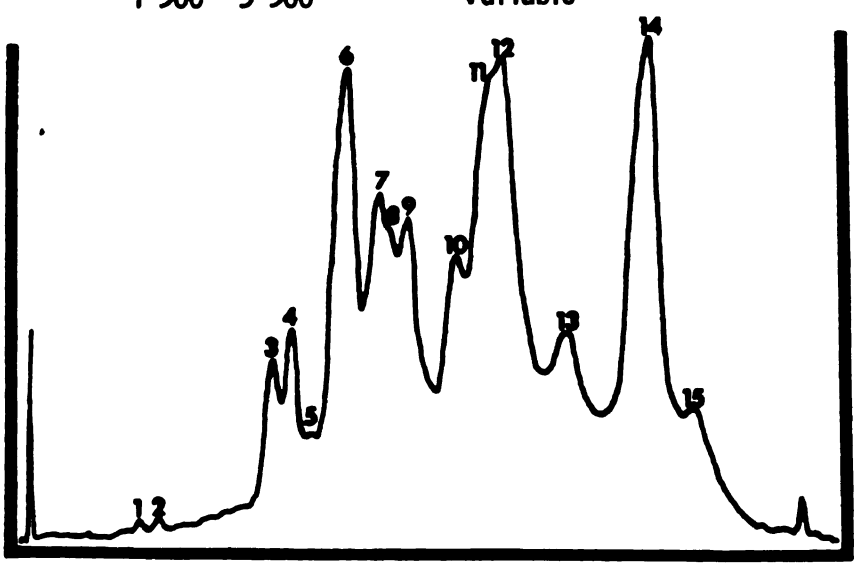
There are several other activities that have been reported to be associated with the outer mitochondrial membrane (41). Among these are a "rotenone insensitive" NADH cytochrome b_5 reductase and a phospholipase A_2. The cytochrome b_5 in the OMM has been reported to be different from the one found in microsomes (47) but the reductase has recently been shown to be the same (48). The phospholipase A_2 may be involved in the inactivation of the hexokinase binding protein (Tables 10, 11) and may also be involved in the uncoupling of isolated rat liver mitochondria (49). Bovine serum albumin (BSA) may bind to the lipase thus inactivating it and giving rise to enhanced stability of oxidative phosphorylation in isolated mitochondria (39). Te BSA binding to the phospholipase A_2 may also change the properties of the hexokinase binding site producing the effects observed on Figure 6 of this chapter. (The ability of BSA to bind free fatty acids might also give rise to some of these observed effects.)

Figure 22

Tentative Identification and Molecular Weight Determination of the Proteins in Native OMM

SDS gels of intact OMM and of the Dalton Mark VI standard mixture (Sigma) were run as in Figure 5. The molecular weights of the OMM proteins were assigned by comparison with a standard curve generated from the Dalton Mark VI mixture.

Band Number	Molecular Weight	Comments	Tentative Identification
1	135 000	Very	---
2	125 000	Faint Pair	---
3	85 000	Medium	---
4	79 000	Dark Pair	---
5	70 000	Very Faint	---
6	61 500	Broad Dark	Monoamine Oxidase
7	55 000	Faint to	---
8	50 000	Medium Dark	---
9	46 000	Group	---
10	35 000	Medium Dark	---
11	31 000	Broad Dark	Hexokinase Binding Protein
12	29 000	Dark	---
13	21 500	Faint	---
14	16 000	Dark	Cytochrome b_5
15	1 500 - 3 500	Variable	---



Recently S. J. Singer and his colleagues have presented data indicating that mitochondria in cultured cells are linked to microtubules (50). This suggests that the OMM contain a protein that binds to microtubules.

It has long been known that the OMM is markedly insensitive to changes in osmolarity when compared to the inner mitochondrial membrane, both in vitro and in vivo (51). This suggests that the OMM contains a pore that is permeable to small molecules such as sucrose. This pore is most likely formed by a protein or a complex of proteins.

The outer membrane contains saturable, high affinity bindings sites for ribosomes (52). This suggests that there is a specific binding protein(s) similar to the ribophorins I and II that have been demonstrated to be responsible for the binding of ribosomes to the rough endoplasmic reticulum (53). If this could be demonstrated it would suggest an attractive hypothesis for the mode of insertion of intramitochondrial proteins that are synthesized from extramitochondrial RNA. This hypothesis would be similar to the "signal hypothesis" proposed by G. Blobel (54).

Ultimately all of the proteins responsible for the above mentioned effects will be identified and assigned molecular weights. But at the present time it is clear from Figure 22 that the identifies of most of the proteins in the OMM are unknown. It may be possible to identify some of the remaining proteins in the OMM using techniques similar to those that were used to identify the hexokinase binding protein.

ADDENDUM

Addendum

Data for Figure 12

Vol. Lipid / Vol. Protein	Units Bound	Condition	% Soluble	G6P / Gal6P
0.0	0.24	condition Gal6P G6P	45 53 52	0.98
0.5	0.28	control Gal6P G6P	32 32 47	1.5
1.5	0.25	control Gal6P G6P	29 29 49	1.7
2.5	0.33	control Gal6P G6P	21 20 48	2.4
3.5	0.25	control Gal6P G6P	26 25 58	2.3
4.5	0.27	control Gal6P G6P	21 27 52	1.9

Addendum

Data for Figure 13

% Emulgen	Units Bound	Condition	% Soluble	$\frac{G6P}{Gal6P}$
0.0	0.61	control	20	
		Gal6P	20	4.7
		G6P	94	
0.2	0.29	control	35	
		Gal6P	38	2.6
		G6P	97	
0.4	0.31	control	51	
		Gal6P	51	1.5
		G6P	79	
0.6	0.34	control	58	
		Gal6P	70	1.1
		G6P	78	
0.8	0.35	control	56	
		Gal6P	58	1.1
		G6P	63	

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APPENDICES

APPENDIX A

Vol. 68, No. 2, 1976 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS

PURIFICATION OF NONBINDABLE AND MEMBRANE-BINDABLE MITOCHONDRIAL HEXOKINASE FROM RAT BRAIN

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SUMMARY: $MgCl_2$-induced binding of glucose-6-P solubilized rat brain hexokinase to rat liver mitochondria has been found to be markedly diminished by increasing ionic strength. Using a modified assay of binding ability, it has now been possible to demonstrate that purified preparations of brain hexokinase do retain appreciable ability to bind to mitochondria. A slight modification of the previous DEAE-cellulose chromatography procedure (4), permits resolution of the hexokinase into two major components designated as Type I_b and Type I_n based on their ability to bind and not bind, respectively, to mitochondria. I_b and I_n appear to be identical in molecular size and subunit composition, but differ slightly in net charge.

It has been demonstrated (1-3) that the Type I isozyme of hexokinase, as found in brain, selectively binds to the outer mitochondrial membrane, presumably indicating some specific component (or components) of this membrane which selectively interacts with the enzyme. In order to better understand the nature of the interaction between hexokinase and the outer mitochondrial membrane it would be advantageous to purify the enzyme and the requisite component(s) of the membrane. Although the enzyme has been purified to homogeneity (4), it was not previously possible to demonstrate that the purified enzyme retained the ability to interact with the mitochondrial membrane. It is the purpose of this communication to describe a method for resolution of purified bindable and nonbindable forms of hexokinase and to indicate some of the reasons why this was not observed earlier.

MATERIALS AND METHODS

Chemicals and rats were obtained from commercial sources given earlier (4,5). Hexokinase was assayed as described previously (4) except that the NADP and glucose-6-P dehydrogenase concentrations were doubled. Rat liver mitochondria were prepared according to the method of Sottocasa et al. (6).

Three times washed rat brain particles (5) and glucose-6-P solubilized hexokinase (7) were obtained as previously described. Rat brain hexokinase was purified according to Chou and Wilson (4) except for slight modification in the DEAE-cellulose column procedure (see Fig. 3).

Binding ability was assayed by incubation of the enzyme with excess mitochondrial binding sites. Except where noted (Fig. 1), the conditions were as described previously (5) with the following modification: a) hexokinase preparations were diluted with 0.25 M sucrose such that the total ionic strength in the incubation medium did not exceed 0.005 M, b) rat brain particles were used in place of rat liver mitochondria, and c) incubation was at 25° rather than 0°. These modifications resulted from observations that rat brain mitochondria bound an appreciably greater proportion of the hexokinase activity than did liver mitochondria, and the binding was less susceptible to inhibition by increasing ionic strength (see Fig. 1); with brain particles (but not with liver mitochondria), binding was also enhanced by increasing the temperature to 25°. Binding assays were done

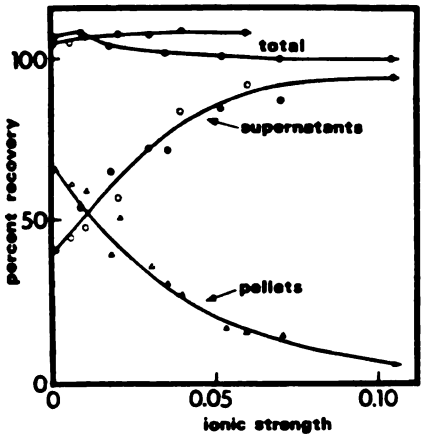

Fig. 1. Inhibition of Binding by Increasing Ionic Strength. Binding ability of glucose-6-P solubilized hexokinase was assayed as described previously (5), using rat liver mitochondria. The indicated salt concentrations were added to the tubes before initiating binding with $MgCl_2$. Open symbols (o,Δ) represent KCl and closed symbols (●,▲) potassium phosphate (pH 7.0).

in polypropylene microcentrifuge tubes (Brinkmann Instruments) which had been dipped in 2% bovine serum albumin (BSA) solution then dried at approx. 60°; coating with BSA was found to prevent artifacts due to adsorption of the hexokinase to polypropylene (unpublished observation).

RESULTS

From Fig. 1, it is clear that low ionic strength severely inhibits the $MgCl_2$ - induced binding of glucose-6-P solubilized hexokinase to liver mitochondria, which have previously been routinely used for the binding assay (5). Fifty percent inhibition occurs at 0.02-0.03 M ionic strength.

Using a modified binding assay (see Methods), it has been possible to show for the first time that purified hexokinase can bind to rat brain particles (Fig. 2). In this experiment more than 50% of the enzyme was bound when excess binding sites were present.

When the conditions for DEAE-cellulose column chromatography used by Chou and Wilson (4) are slightly modified by using a shallower gradient of KCl, elution patterns such as those shown in Fig. 3 are obtained. Under these conditions, two peaks of hexokinase, one at about 0.065 M KCl and the other at about 0.075 M KCl, have been reproducibly resolved (eight experiments).

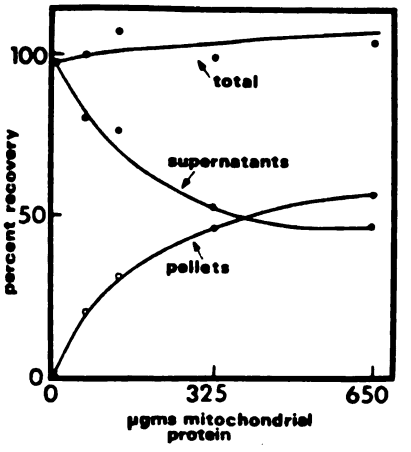

Fig. 2. Binding of Pure Hexokinase. Hexokinase (0.065 units, purified according to Chou and Wilson (4)) was incubated with brain particles as described in Methods. Total volume was 0.25 ml.

The relative amounts of activity in the two peaks have, however, varied from about 75%:25% to 25%:75%; the reasons for this remain under investigation.

Fig. 3 also indicates that the enzyme eluted at higher KCl concentrations is at least partly bindable to brain particles, while hexokinase eluted at lower salt concentrations is completely nonbindable. For this reason we refer to these two forms of the enzyme as Type I_b for the bindable enzyme, and Type I_n for nonbindable. The asymmetry of the eluted peaks, together with preliminary analytical isoelectric focusing experiments, suggest that

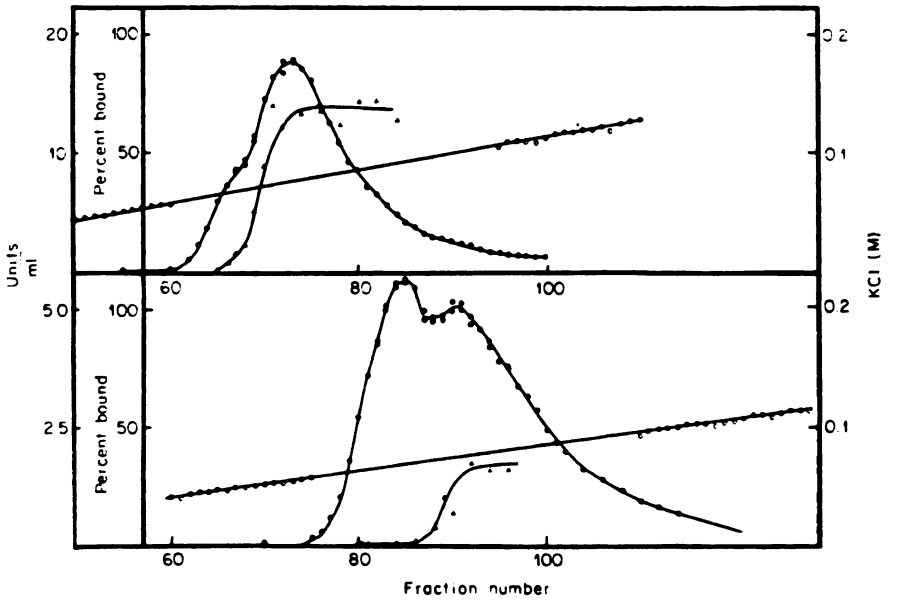

Fig. 3. DEAE-Cellulose Column Chromatography of Rat Brain Hexokinase. Chromatography was performed as previously described (4) except that shallower salt gradients were used. In the upper elution pattern a 600 ml linear gradient from 0.0 to 0.20 M KCl in column buffer was used, collecting 3.8 ml fractions. The lower elution pattern was done exactly the same except the gradient went from 0.0 to 0.15 M KCl. The KCl concentration (o) was determined by conductivity measurements on the fractions. Hexokinase activity (●) and percent bound (Δ) were measured as described in the Methods section. In both experiments bindable enzyme was found only in the high salt peak (0.075 M KCl). The proportion of the enzyme in the second peak which could be bound was, however, found to vary in different experiments e.g. in the lower profile, only 35% of the activity in the second peak was bindable, whereas in the experiment shown in the upper profile, 70% was bindable.

Type I_n and Type I_b are in turn composed of more subforms (at least six total). Variation in the percentage bindability of Type I_b (see Fig. 3) also suggests that some of these subforms are nonbindable.

DISCUSSION

It had been previously observed that high ionic strength could cause solubilization of mitochondrial hexokinase (1, 8, 9). In contrast, low ionic strengths actually appeared to slightly enhance the strength of association of the enzyme with the membrane (8). In view of this observation, it was presumed that low to moderate ionic strengths would have little or no effect on the $MgCl_2$-induced rebinding of the enzyme to mitochondria. The results presented here show that this presumption was incorrect. Furthermore, we have also found that (for reasons which remain under investigation) binding of hexokinase by brain mitochondria is less susceptible to the ionic strength effect than is binding by liver mitochondria which have, for reasons of practicality (5), previously been routinely used in assays of binding ability. Using this new information, and making appropriate modifications of the binding assay, it has now been possible to demonstrate that purified brain hexokinase can bind to mitochondria.

Furthermore, slight modification of the previously described DEAE-cellulose chromatography procedure (4) has permitted the demonstration of a previously undetected heterogeneity of the enzyme. Two major forms, designated Type I_n and Type I_b have been resolved. Although I_n and I_b are readily distinguished by the difference in their ability to interact with the mitochondrial membrane, the physical or chemical basis for this distinction remains under investigation. Since mitochondrial enzyme (presumably all Type I_b) serves as the starting point for the purification, it seems quite probable that Type I_n represents, at least to some extent, an artifactual form produced in variable amounts (see Fig. 3) during purification. The enzyme does not contain detectable carbohydrate (10, and J. E. Wilson unpublished observations) or lipid (5), so the difference between I_b and I_n

is not likely to be due to such factors. Partial proteolysis during purification of the enzyme has not been entirely ruled out, but seems unlikely in that I_b and I_n have not been found to differ in N-terminal amino acid (by the dansylation technique) or apparent molecular weight (98,000 by SDS-gel electrophoresis). [1] The latter results suggest that the principle difference between I_b and I_n lies in a modification affecting their net charge, with I_b being more negatively charged (based on its greater affinity for DEAE-cellulose) than is I_n. Such modification might include phosphorylated and dephosphorylated forms e.g. perhaps I_b is a phosphorylated form which is (enzymatically?) converted to the dephosphorylated (and thus less negative) I_n during purification with resulting loss of binding ability.

ACKNOWLEDGEMENT

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[1] These results are consistent with earlier studies using the enzyme prepared according to the original Chou and Wilson (4) procedure. These enzyme preparations probably were, in view of the present observations, mixture of I_b and I_n, but were found to be homogeneous with regard to molecular weight (e.g., SDS-gels or centrifugal methods).

APPENDIX B

Hexokinase Binding to Polypropylene Test Tubes

Artifactual Activity Losses from Protein Binding to Disposable Plastics

In the course of our studies with rat brain hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1), we have frequently used disposable polypropylene centrifuge tubes. As long as relatively crude systems with substantial amounts of extraneous protein were used, no difficulties were encountered. However, with more purified preparations, substantial apparent activity losses were incurred. Investigation of the situation disclosed that the reason for these apparent losses was adsorption of the hexokinase to polypropylene vessels, an unusual adsorption that appeared to be particularly effective at the air–water interface. Subsequently, we found similar effects with other enzymes. Although binding of some proteins, such as ribonuclease (1,2), to glassware has been reported, we do not know of a similar phenomenon being described for plastic ware. The increased use of disposable plastic laboratory ware necessitates an awareness of this potential problem. We present here the results of our study of the adsorption of hexokinase to polypropylene.

MATERIALS AND METHODS

Polypropylene microcentrifuge tubes were obtained from Brinkman Instruments. In some experiments these tubes were coated by dipping them in a 2% bovine serum albumin (BSA) solution, then drying in a 60°C oven. Glucose-6-*P*-solubilized (3) and purified hexokinase (4) were prepared as previously described. Hexokinase activity was assayed according to Chou and Wilson (4).

RESULTS AND DISCUSSION

As shown in Fig. 1, successive transfers of hexokinase between polypropylene tubes caused a progressive loss of activity. Coating the tubes with BSA before enzyme addition diminished the observed losses; washing uncoated tubes with ordinary dishwashing detergent, 1:1 chloroform:methanol, or 6 N HCl or treatment with 1% dichlorodimethyl silane in benzene had no effect. That the tubes were binding (rather than totally inactivating) enzyme was demonstrated by the presence of residual glucose phosphorylating activity in tubes which had been ex-

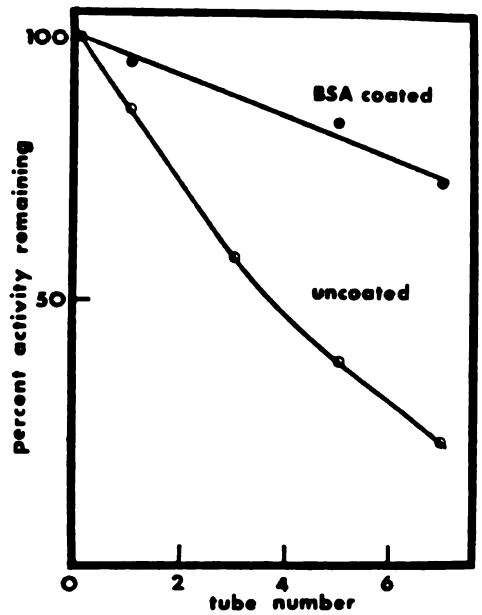

FIG. 1. Activity losses resulting from multiple transfers of hexokinase between polypropylene tubes. Glucose-6-*P*-solubilized hexokinase (1 ml, 0.52 unit) was added at 0°C to polypropylene tubes which had (●) or had not (○) been pretreated with BSA as described in the text; these initial tubes are designated as "tube 1" in the above figure. After removal of an aliquot for assay, the solution was transferred to a second tube (also with or without BSA) using a Pasteur pipet. This procedure was repeated for a total of six transfers.

posed to hexokinase solutions and subsequently washed with 0.25 M sucrose (e.g., see Fig. 3).

As shown in Fig. 2, successive aliquots taken from the same tube showed decreasing activity. This somewhat puzzling result could be explained if the enzyme were preferentially adsorbed at the air–water interface. This interpretation is supported by the experiment described in Fig. 3, which demonstrated the preferential adsorption of the enzyme to regions where a meniscus (i.e., an air–water interface) had passed. When formation of an interface between air and the enzyme solution was prevented by overlay of the enzyme solution with water, less adsorption occurred (Table 1).

Our initial reaction to these observations was that this preferential adsorption at the air–water interface seemed so unusual that it was likely to be a property restricted to hexokinase and perhaps a few other proteins. We were somewhat surprised, therefore, to find that nearly 40 years ago a strikingly similar phenomenon was studied by Langmuir *et al.* (5), who demonstrated the adsorption of proteins to a "hydrocarbon surface" (a brass plate coated with barium stearate) which was passed through a sur-

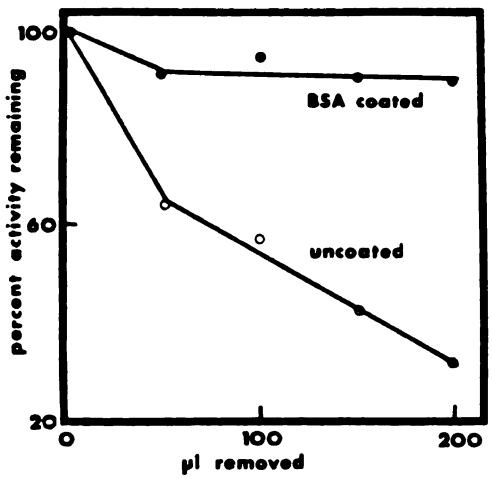

FIG. 2. Activity losses resulting from removal of successive aliquots from a single polypropylene tube. Glucose-6-*P*-solubilized hexokinase (0.2 ml, 0.43 U/ml) was placed in polypropylene centrifuge tubes at 0°C which had (●) or had not (○) been pretreated with BSA. Successive 50-µl aliquots were removed for assay.

face layer of protein formed at an air–water interface. We believe our results are analogous to those of Langmuir *et al.* (5), with the highly hydrophobic polypropylene being the equivalent of the "hydrocarbon surface" used by Langmuir *et al.* Although the earlier workers (5) produced the surface layer of protein by experimental manipulation, such surface films are reported to form spontaneously (2) in all protein solutions. Thus, in analogy with the experiments of Langmuir *et al.* (5), we would interpret results such as those shown in Fig. 2 in the following way: As the aliquot of solution is removed, the meniscus passes down the hydrophobic polypropylene surface with resulting adsorption from the surface layer of protein to the polypropylene. Subsequent spontaneous reformation of the surface layer (2) results in net removal of enzyme from the bulk solution. Repetition of this process would account for the progressive depletion of enzyme activity in the bulk solution, accompanied by adsorption of enzyme to hydrophobic surfaces which had been traversed by the air–water interface. Furthermore, we have observed that a tube containing protein differs markedly from one that did not, since after withdrawal of the protein solution the sides of the tube are observably wet, whereas a tube that never contained protein sheds water. Langmuir *et al.* similarly observed that the "hydrocarbon surface" (in our case, polypropylene) shed water unless it was coated by a protein film, while coating with the hydrophilic protein layer allowed the otherwise hydrophobic surface to bind water molecules.

Since protein surface films (2) and "monolayer adsorption" (5) are

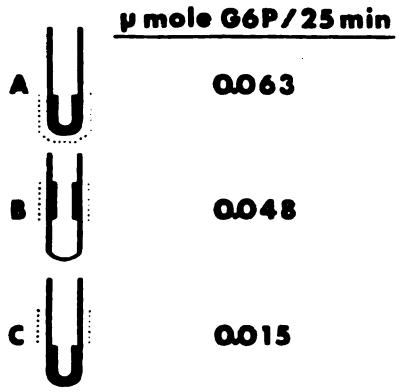

FIG. 3. Preferential binding of hexokinase at the air–water–polypropylene interface. A 0.5-ml aliquot of 0.25 M sucrose–0.1 M glucose was added, at 0°C, to each of three polypropylene tubes. Pure hexokinase (0.05 ml, 11.0 U/ml) was then injected directly into the sucrose–glucose, followed by gentle stirring. In tube A, the enzyme was slowly drawn out (over a period of 5 sec) with a Pasteur pipet, and the tube was then washed twice with 0.25 M sucrose–0.1 M glucose. Assay mix (3) (0.5 ml) minus the glucose-6-*P* dehydrogenase was added, and the tube was incubated at 25°C for 25 min; subsequently, glucose-6-*P* formation was measured spectrophotometrically after addition of glucose-6-*P* dehydrogenase. Tube B was like A except that the enzyme was raised from the bottom of the tube by underlaying it with 0.5 ml of 0.9 M sucrose. After careful removal of the enzyme solution and the 0.9 M sucrose with a Pasteur pipet, the tube was washed as above. Sucrose (0.9 M, 0.5 ml) was then placed in the tube, overlaid with 0.5 ml of assay mix, and assay for hexokinase activity done as described above. Tube C was exactly like B except that the 0.5 ml of assay mix was placed directly into the tube. In the above figure, the solid lines indicate the regions of the tubes assayed for adsorbed hexokinase, and the dotted lines indicate the regions of the tube through which an air–water interface has passed. The results of the assays are given in the column to the right.

generalized phenomena that pertain to a wide variety of proteins, it seemed reasonable that other proteins might adsorb to polypropylene tubes. Therefore, we performed experiments similar to that shown in Fig. 3 using yeast hexokinase, beef heart lactate dehydrogenase, and glucose-

TABLE 1
INJECTION OF ENZYME UNDER A WATER LAYER[a]

	Micromoles G6P produced/10 minutes
Control	.030
Experimental	.016

[a] In the control, 0.5 ml of glucose-6-*P*-solubilized enzyme in 0.25 M sucrose was added to a polypropylene tube, then drawn back out. The experimental sample was treated exactly the same way except that the enzyme was underlaid beneath a 0.3-ml water layer and drawn back out from the bottom of the tube. The tubes were then washed and assayed for adsorbed hexokinase as described in the legend to Fig. 3.

6-*P* dehydrogenase. Each of these enzymes was shown to be adsorbed (with retention of catalytic activity) in amounts comparable to those seen with rat brain hexokinase. Furthermore, since BSA diminishes hexokinase adsorption, presumably by contributing to the surface film and competing for hydrophobic binding sites, it too must be adsorbed. In addition, we have observed that plastics other than polypropylene also give adsorption artifacts with hexokinase.

Referring to protein adsorption on glass, Sobotka and Trurnit (2) mention that "many proteins are adsorbed onto solid surfaces from solution to form complete, well-adhering monolayers of undenatured molecules. This phenomenon has caused—and is still causing—errroneous results in work with highly diluted protein solution." The observations in this paper, coupled with the increasing use of plastic laboratory products, suggest that an awareness of this problem is necessary whenever a dilute protein solution is used with polypropylene (or similarly hydrophobic) vessels.

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APPENDIX C

Dansylation of Tyrosine: Hindrance by *N*-Ethylmorpholine and Photodegradation of *O*-Dansylated Derivatives

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Dansylation of free tyrosine or of rat brain hexokinase (ATP:D-hexose 6-phosphotransferase: EC 2.7.1.1), which contains an *N*-terminal tyrosine residue, yields both the didansyl and *N*-monodansyl derivatives if *N*-ethylmorpholine is used in the dansylation procedure [W. R. Gray (1972) *in* Methods in Enzymology (Hirs, C. H. W., and Timasheff, S. N., eds.), Vol. 25, pp. 121–138, Academic Press, New York]. If the *N*-ethylmorpholine is replaced by $NaHCO_3$ buffer (pH 9.5), the didansyl derivative is formed almost exclusively, and the ambiguity resulting from the formation of two derivatives from a single N-terminal residue is thereby eliminated. Therefore, a slightly modified dansylation procedure, using $NaHCO_3$ buffer is recommended: the validity of the modified procedure was demonstrated by its successful application to six different proteins having previously known N-terminal amino acids. The didansyl and *O*-monodansyl derivatives of tyrosine are remarkably photolabile as compared to the *N*-dansyl derivatives. Unless specific precautions against unnecessary irradiation are observed, photolytic degradation of the didansyl tyrosine derivatives could occur during experimental manipulations: loss of the didansylated compound and formation of photolysis products complicates the interpretation of experiments in which a single didansyl derivative (of the N-terminal residue) is expected.

Dansylation[1] is one of the most frequently employed methods for determining the N-terminal amino acid of proteins or peptides. The popularity of the method derives from its relative simplicity, sensitivity, and general reliability. While working with a protein (rat brain hexokinase; ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) found to have an N-terminal tyrosine (see below), we encountered some technical problems with the dansylation procedure which led to equivocal results. Additional studies disclosed that these technical problems were not unique to the N-terminal tyrosine of hexokinase, but were, in fact, generally

[1] Abbreviations used: Dansyl chloride, 5-dimethylamino-1-naphthalene sulfonyl chloride; SDS, sodium dodecyl sulfate; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2(5-phenyloxazoyl)]benzene. Dansylation will be used to include both the reaction with dansyl chloride, or, in reference to the determination of N-terminal amino acid residues, in the more inclusive sense of reaction with dansyl chloride followed by hydrolysis and subsequent identification of dansyl derivatives.

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encountered when working with dansyl derivatives of tyrosine. Briefly, these problems are due to (a) a marked photosensitivity of the didansylated[2] tyrosine derivative; unless special precautions are taken to prevent photolysis, a multiplicity of fluorescent photolysis products are formed which hinders the straightforward interpretation of chromatographic identification procedures; (b) a formation of appreciable (or even predominant) amounts of the N-monodansylated derivative under conditions recommended (1) for the dansylation reaction. This result contrasts with the frequent assumption that the reactivity of the amino and phenolic groups are sufficiently similar that the didansyl derivative will be the predominant (or exclusive) form resulting from N-terminal tyrosine residues. The present work reveals that this is not the case and that the extent of didansylation depends markedly on the reaction conditions. Conditions have been devised which yield almost exclusively the didansyl derivative and thus avoid the potential uncertainty which can result from formation of both monodansyl and didansyl derivatives of N-terminal tyrosine residues.

MATERIALS AND METHODS

Materials. Dansyl chloride, L-tyrosine, and standard dansyl amino acids were obtained from Sigma (St. Louis, Mo.). Sodium dodecyl sulfate (sequenation grade), *N*-ethylmorpholine (sequanal grade), and dimethylformamide (silylation grade) were purchased from Pierce Chemical Company (Rockford, Ill.). L-[U-^{14}C]Tyrosine and [G-^{3}H]dansyl chloride were products of Amersham–Searle (Arlington Heights, Ill.) and 1-nitroso-2-naphthol was from Mallinckrodt (St. Louis, Mo.). Polyamide thin-layer sheets were obtained from Gallard–Schlesinger (Carle Place, N.Y.). All other chemicals were reagent grade and were obtained from commercial sources.

Thin-layer chromatography of dansyl derivatives. Thin-layer chromatography was performed as described by Weiner *et al.* (2). Samples were spotted on 5 × 5-cm polyamide plates, and chromatography was carried out in the first dimension with 1.5% formic acid in water (Solvent I). After drying, chromatography was performed in the second dimension using, successively, benzene:acetic acid, 9:1 (Solvent II) and the ethyl acetate:acetic acid:methanol, 20:1:1 (Solvent III). [The further chromatography in the second dimension employed by Weiner *et al.* (2) was not required for the present studies.]

Dansylation. Tyrosine (10 μl of a 1 mM solution, raised to pH 10 with

[2] Tyrosine contains two groups which can react with dansyl chloride, the α-amino and the phenolic hydroxyl, which would give the *N*-dansyl and *O*-dansyl derivatives, respectively. The didansyl derivative refers to the compound produced by reaction of both the amino and phenolic functions.

NaOH to promote solubilization) was added to 20 μl of 0.1 M sodium bicarbonate, pH 9.5. The reaction was started by adding 30 μl of 10 mM dansyl chloride in acetone. After 1 hr at room temperature, 50 μl of 0.1 N NaOH was added to promote hydrolysis of any remaining dansyl chloride (1), and then the solution was acidified with 20 μl of 6 N HCl. After removal of acetone under a stream of nitrogen, the samples were completely dried under vacuum at 35°C on a rotary evaporator, dissolved in 10 μl of acetic acid–acetone (40:60, v/v), and then chromatographed. This procedure yields the didansyl tyrosine derivative almost exclusively; however, in some experiments, other buffers at various pHs replaced the bicarbonate in order to generate significant amounts of the *N*-monodansyl derivative (see below).

For dansylation of proteins, the method outlined by Gray (1) was routinely followed with three modifications: *N*-ethylmorpholine was replaced by 0.5 M sodium bicarbonate, pH 9.5; protein samples were not performic acid oxidized; and the dansylated protein sample was washed more extensively. The procedure was as follows. Lyophilized protein, 50-250 μg, was placed in a 6 × 50-mm culture tube and was dissolved in 50 μl of 1% SDS by heating in a boiling water bath for about 5 min; if the protein sample contained buffer or salts, it was dialyzed overnight against distilled water before lyophilization. After cooling, 50 μl of 0.5 M sodium bicarbonate, pH 9.5, followed by 75 μl of a 25-mg/ml dansyl chloride solution in dimethyl formamide, was added. The sample was mixed with a Vortex mixer, covered with Parafilm, and incubated at room temperature for at least 1 hr. 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. One volume of 20% TCA (w/v) was added to stop the reaction and precipitate the protein. The precipitated protein was washed once with 1 N HCl and once with 80% acetone:water. To the dried precipitate was added 50 μl of constant-boiling HCl; the culture tube was sealed *in vacuo*, and the sample was hydrolyzed at 110°C for 18 hr. The HCl was removed on a rotary evaporator, and the residue was dissolved in 10 μl of 40% acetic acid:acetone and was spotted on polyamide-layer sheets for chromatography.

Photolysis of didansyl tyrosine on thin-layer plates. Radioactive didansyl tyrosine was prepared from tyrosine and [^{3}H]dansyl chloride as described above. The dansylated tyrosine was chromatographed in Solvents I and II (see Materials and Methods), and the chromatograms were then irradiated with an ultraviolet light source (Sargent–Welch Catalog No. S-44260; long wavelength: ~366 nm maximum emission, 100 W) for various time intervals. The plates were placed exactly 20 cm from the lamp and were centered in the most intense portion of the beam. At this distance from the source, the temperature was 35°C. After irradiation, further chromatography using Solvent III was done to resolve the

remaining didansyl tyrosine from photolysis products. The relevant areas were scraped from the plates, and radioactivity was determined by scintillation counting. The scintillation solution used contained 4 g of PPO and 0.05 g of POPOP per liter of toluene.

RESULTS

Characterization of Dansylated Tyrosine Derivatives

Both commercially obtained (Fig. 1) samples of "dansyl tyrosine" or samples prepared under various conditions in the laboratory (see below) can contain several fluorescent species. We are primarily concerned here

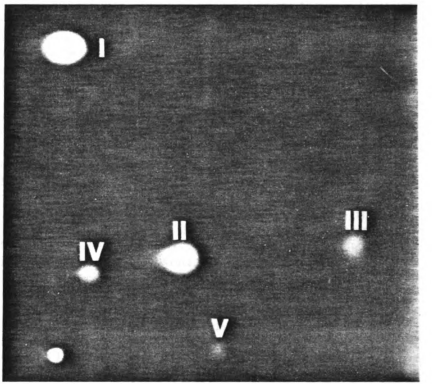

FIG. 1. Fluorescent species in commercially obtained sample of dansyl tyrosine. Approximately 0.5 μl of a solution of commercially obtained dansyl tyrosine (0.25 mg/ml in 40% acetic acid:acetone) was spotted on a polyamide sheet and was chromatographed according to Weiner *et al.* (2). Several fluorescent species (some of which may be photolytic degradation products; see text) are present in trace amounts, but the principal components observed are: (I) didansyl tyrosine: (II) *N*-dansyl tyrosine; (III) *O*-dansyl tyrosine; (IV) unknown; (V) dansylsulfonic acid (the hydrolysis product of dansyl chloride). Neither III nor IV was seen in dansyl tyrosine samples prepared in our laboratory by the procedure given in Materials and Methods.

with the species labeled I, II, and III in Fig. 1. Compound III was present in the commercially obtained sample of dansyl tyrosine, and, based on its bright yellow fluorescence and chromatographic properties, it is presumed to be the *O*-dansyl derivative of tyrosine (1–3) such as would be formed by dansylation of non-N-terminal tyrosine residues in proteins or peptides; dansylation of a tripeptide (gly–leu–tyr) with a non-N-terminal tyrosine, which is expected to yield *O*-dansyl tyrosine, did give a fluorescent derivative corresponding to Compound III (data not shown). Compound III was not produced in appreciable amounts when we directly dansylated tyrosine except at high pH (see below), and we have not attempted to characterize this species in the present work. In contrast, Compounds I and II were readily produced from both free tyrosine and N-terminal tyrosine. These species have been reported to be the didansyl (1–3) and *N*-dansyl (3) derivatives, respectively, but the experimental basis for this assignment of identities has not, to our knowledge, been published. Therefore, we further characterized these compounds in the present study.

Dansyl chloride reacts with unprotonated amines and phenols (1,4), and, therefore, the relative reactivity of these groups is markedly dependent on the pH of the reaction mixture. Since the *pK* of the phenolic hydroxyl of tyrosine is 10.1 and the *pK* of the amino group is 9.1 (5), at lower pH, the amino group should be more reactive than the phenolic hydroxyl. The results shown in Table 1 are consistent with this expectation: At pH ~7–8, substantial amounts of Compound II, proposed to be the *N*-dansyl derivative, are formed. As the pH is raised, the phenolic function becomes more reactive, and the formation of the didansyl derivative (I) is favored. At pH 10.5 in $NaHCO_3$ buffer, the phenolic hydroxyl apparently reacts sufficiently fast so that the *O*-dansyl derivative comprises an appreciable percentage of the reaction mixture.

1-Nitroso-2-naphthol, in the presence of nitric acid, has been shown to react specifically with phenolic groups to give a red-colored product (6). When sprayed with this reagent, Compound II reacted to give a red color while Compound I did not. Thus, Compound II contained a free phenolic group (consistent with its identification as the *N*-dansyl derivative), while Compound I did not (as expected for the didansyl derivative).

Finally, Table 2 shows the results of an experiment in which [^{14}C]tyrosine was dansylated with [^{3}H]dansyl chloride, and the $^{3}H/^{14}C$ ratio was determined in various reaction products after their separation by thin-layer chromatography. If Compound I is the didansyl derivative and II is the *N*-dansyl derivative of tyrosine, the $^{3}H/^{14}C$ ratio of Compound I should be twice that found for II; the observed value was 0.72/0.42 = 1.7; essentially identical results were obtained in other experiments [mean ratio of 1.7 ± 0.1 (SD) in four separate experiments]. We cannot presently explain the departure of this ratio from the expected value of 2.0, but, nevertheless, it is clear that Compound I contains approximately twice as

TABLE 1

RELATIVE AMOUNTS OF DANSYL TYROSINE DERIVATIVES PRODUCED AT VARIOUS PHs[a]

Buffer	pH	Didansyl tyrosine (%)	*N*-Dansyl tyrosine (%)	*O*-Dansyl tyrosine (%)
Phosphate	7.2	50	50	nd[b]
Phosphate	8.0	62	38	nd
Bicarbonate	8.3	70	30	nd
Bicarbonate	9.5	99	1	nd
Bicarbonate	10.5	91	1	8
Borate	8.5	99	1	nd
Borate	9.5	98	1	1

[a] Dansylation was carried out as described in Materials and Methods, except that 1 μCi of [^{3}H]dansyl chloride was mixed with the unlabeled dansyl chloride before addition to buffered tyrosine. Buffers used were all 0.1 M, had the indicated pH, and were prepared with the sodium salts. Samples were chromatographed, the appropriate spots were scraped off, and radioactivity was determined.

[b] nd, Not detected.

many dansyl groups per tyrosine residue as does II, which would support their identification as the didansyl and monodansyl derivatives, respectively.

In summary, all of these experiments are consistent with the previous identification (1–3) of Compounds I and II as the didansyl and monodansyl derivatives of tyrosine. We have also confirmed the identification of Compound I as the didansyl derivative by direct-probe mass spectrometry

TABLE 2

DETERMINATION OF THE DANSYL:TYROSINE RATIO IN COMPOUNDS I AND II[a]

Compound[b]	DP		
	^{3}H (dpm)	^{14}C (dpm)	^{3}H/C^{14} (dpm)
(I) Didansyl tyrosine	2566	3569	0.72
(II) *N*-Dansyl tyrosine	1964	4672	0.42
(V) Dansyl sulfonic acid	16032	390	41

[a] Dansylation was carried out as described in Materials and Methods using [^{3}H]dansyl chloride and [^{14}C]tyrosine; the buffer was 0.1 M sodium phosphate, pH 8.0. After chromatography, the indicated spots were scraped off, and the radioactivity was determined. In this experiment only, the scintillant used contained 200 g of naphthalene, 20 g of PPO, 1.6 g of dimethyl POPOP, 2 liter of xylene, and 1.1 liter of Triton X-114; standard curves for calculating the disintegrations per minute of ^{3}H and ^{14}C were provided by Dr. A. J. Morris (Biochemistry Department, Michigan State University, East Lansing, Mich.).

[b] Compounds I, II, and V are as designated in Fig. 1.

TABLE 3

EFFECT OF *N*-ETHYLMORPHOLINE ON DANSYLATION OF TYROSINE[a]

N-Ethylmorpholine (M)	Didansyl tyrosine (%)	*N*-Dansyl tyrosine (%)	*O*-Dansyl tyrosine (%)
0.1	44	56	nd[b]
0.4	42	58	nd
1.6	29	71	nd
7.8 (neat)[c]	1	99	nd

[a] Tyrosine was dansylated as described in Materials and Methods using the indicated concentration of *N*-ethylmorpholine in place of the 0.1 M bicarbonate; the pH of the diluted *N*-ethylmorpholine solutions was 10.3. [^{3}H]Dansyl chloride was used to permit quantitation by radioactivity determination.

[b] nd, Not detected.

[c] The final concentration of *N*-ethylmorpholine in the reaction mixture was 2.6 M (see Materials and Methods); this is comparable to the concentration (2.2 M) used in the dansylation procedure of Gray (1).

of the compound eluted from thin-layer plates: The mass spectrum was in excellent agreement with that previously reported by Seiler *et al.* (7). Unfortunately, for unknown reasons, we have not been able to obtain a mass spectrum of the similarly isolated Compound II, and, hence, mass spectrometry has not yet been useful in confirming the identity of this species as the *N*-dansyl compound.

Use of N-Ethylmorpholine as Buffer in Dansylation Reactions

Gray (1) has recommended *N*-ethylmorpholine for dansylation of proteins, stating that this compound not only serves as a base but also has a detergent-like action which facilitates reaction with proteins. When we utilized *N*-ethylmorpholine for dansylation of rat brain hexokinase (8) using the procedure of Gray (1), both didansyl and *N*-monodansyl tyrosine derivatives were obtained, the ratio of didansyl:*N*-monodansyl derivatives being approximately 2:3. In contrast, when 0.5 M sodium bicarbonate, pH 9.5, was used as buffer, only the didansyl tyrosine (>99%) derivative was formed.[3] These results clearly suggested that *N*-ethylmorpholine affects the reactivity of the tyrosine residues, and this was confirmed by experiments with free tyrosine (Table 3): Increasing the *N*-ethylmorpholine content of the reaction mixture drastically reduced the reactivity of the phenolic hydroxyl, resulting in an increasing yield of the *N*-monodansyl derivative. Conceivably, this may result from an interaction of the detergent-like *N*-ethylmorpholine (1) with the phenolic ring, thereby hindering its dansylation.

[3] We are grateful to Dr. Dean Ersfeld for confirming the presence of N-terminal tyrosine by the Edman method.

Dansylation of Proteins in Bicarbonate Buffer

In contrast to the results with *N*-ethylmorpholine, dansylation of rat brain hexokinase in bicarbonate buffer gave a single didansylated derivative of the N-terminal tyrosine. To check on the general applicability of the method, other proteins with known N-terminal amino acids were examined by this same procedure. In each case, the expected N-terminal amino acid was unambiguously identified. The proteins used and their N-terminal residues were: *P. putida* 2-keto-3-deoxy-6-phosphogluconate aldolase, threonine (9); rabbit muscle aldolase, proline; egg white lysozyme, lysine; bovine pancreatic ribonuclease, lysine; horse heart cytochrome c, glycine; bovine fetal hemoglobin, α-chain valine, β-chain methionine [see Ref. (10) for references related to the last five proteins]. We therefore conclude that this method is an acceptable alternative to that of Gray (1) and is, in fact, an improvement in cases in which an N-terminal tyrosine is involved.

Photolability of O-Dansyl Tyrosine Derivatives

As shown by the results in Fig. 2, the didansyl derivative of tyrosine is remarkably labile when irradiated with ultraviolet light. A 20-min photolysis under the conditions described in Materials and Methods resulted in virtually complete conversion of the didansyl derivative to unidentified photolysis products (compare Figs. 1 and 2). Photodegradation of the *O*-dansyl derivative (Compound III) was also evident, while, in contrast, no obvious photolytic degradation of *N*-dansyl tyrosine (again compare Figs. 1 and 2) or dansyl glycine (not shown) was observed under these conditions. Thus, it would appear that the *O*-dansyl group is much more susceptible to photolytic degradation as compared to *N*-dansyl derivatives. The rapidity of this photodegradation is emphasized by the results in Fig. 3, which demonstrate that over 50% of the didansyl tyrosine is converted to photolytic degradation products within 3–4 min under the conditions employed. It should be noted here that photodegradation was even more rapid when the distance from the light source was decreased (as would usually be the case when thin-layer plates were being examined for fluorescent compounds), or when a short-wavelength (mineralogical) ultraviolet source (e.g., Sargent–Welch Catalog No. S-44240, 254 nm maximum emission) was used.

DISCUSSION

The present results demonstrate two potential sources of difficulty that may be encountered when utilizing the dansylation procedure with proteins containing N-terminal tyrosine residues.

(a) One of these is formation of two derivatives (didansyl and *N*-dansyl) when dansylation is done by the procedure of Gray (1), employing

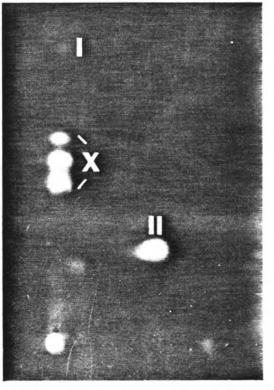

FIG. 2. Separation of didansyl tyrosine from photodegradation products. Commercially obtained dansyl tyrosine was chromatographed as in Fig. 1 (with which this figure should be compared) *except* that, prior to running in Solvent III (see Materials and Methods), the plate was irradiated for 20 min with ultraviolet light as described in Materials and Methods. Subsequent chromatography in Solvent III resolved the remaining didansyl derivative (I) from photolytic degradation products (X). Also note the marked loss of *O*-dansyl tyrosine (III, Fig. 1) as a result of irradiation, while no appreciable destruction of the *N*-dansyl derivative (II) was noted; these results clearly illustrate the much greater photolability of *O*-dansyl derivatives as compared to *N*-dansyl compounds.

N-ethylmorpholine in the solvent; the formation of two derivatives of a single N-terminal amino acid obviously is not an overwhelming problem, but, nevertheless, it does make the interpretation of thin-layer chromatograms (or analogous identification procedures) less straightforward and represents an unnecessary complication.

(b) Another source of difficulty is the remarkable photolability of the *O*-dansyl group; unless precautions against photolysis are taken, substantial amounts of photolytic degradation products may be formed, markedly diminishing the yield of the expected didansyl derivative and complicating the interpretation of experiments in which a single derivative (of the N-terminal residue) is expected. Although the photolability of

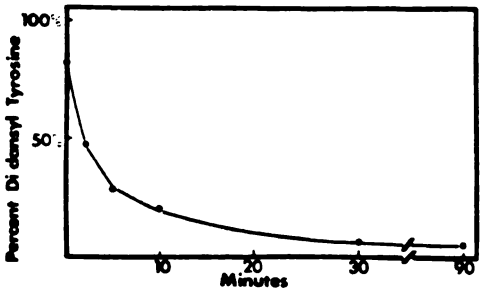

FIG. 3. Rate of photolysis of didansyl tyrosine. The experiment was conducted essentially as described in the legend to Fig. 2, except that the dansyl tyrosine was prepared by the procedure given in Materials and Methods using [^{3}H]dansyl chloride. The duration of irradiation was varied as indicated on the abscissa. After chromatography in Solvent III, the areas corresponding to didansyl tyrosine and its photolysis products (areas I and X, respectively, shown in Fig. 2) were scraped off, and their radioactivity was determined. The results are expressed as the percentage of total counts (I + X) found as undegraded didansyl derivative (I). We attribute the observation of substantial (~20%) photodegradation products at zero time of irradiation to their formation during the preceding preparative and chromatographic procedures: no special precautions were taken against light exposure, i.e., the operations were conducted under ordinary laboratory lighting conditions.

various *N*-dansyl derivatives has been previously studied (11,12), we are not aware of any results which emphasized the much greater photolability of *O*-dansyl derivatives.

The present results further provide the basis for avoiding these two potential problems.

(A) Dansylation should be done as described in Materials and Methods, using sodium bicarbonate as buffer.

(B) Obviously, prevention of photolysis requires the avoidance of significant exposure to light, especially ultraviolet, during the manipulations. Previously, it was not uncommon in our laboratory (and, we suspect, in others) to examine briefly thin-layer plates under ultraviolet light *before* chromatography to verify that sufficient sample was spotted to provide readily observable fluorescence; in the light of the present results, such "sneak previews" are obviously to be avoided.

It is likely that the technical problems described here contributed to the previous erroneous identification of glycine as the N-terminal amino acid of rat brain hexokinase (8).

Weiner *et al.* (2) have summarized some technical problems which may complicate N-terminal analysis by the dansylation procedure. In addition, the oxidative capability of dansyl chloride (13) might also cause problems with N-terminal cysteine residues. The present paper draws attention to the difficulties possible when proteins with N-terminal tyrosine are encountered. The effective use of the dansylation procedure demands an awareness of these potential sources of difficulty.

ACKNOWLEDGMENTS

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APPENDIX D

Effect of Neutral Salts on the Interaction of Rat Brain Hexokinase with the Outer Mitochondrial Membrane

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The glucose 6-phosphate (Glc-6-P)-induced solubilization of mitochondrial hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) from rat brain can be reversed by low concentrations (ionic strength $<\sim 0.02$ M) of neutral salts. When compared to the original particulate enzyme (i.e., enzyme found on the particles prior to solubilization by Glc-6-P), the rebound enzyme is similar in distribution on sucrose gradients, K_m for ATP, inhibition by antiserum to purified brain hexokinase, and resistance to removal by exhaustive washing of the particles. The effectiveness of chloride salts at promoting rebinding increases in the order $Cs^+ < Rb^+ < K^+ \leq Na^+ < Li^+ \ll Mg^{2+}$. This salt-induced rebinding is attributed to the screening of negative charges on the enzyme and/or membrane by cations, thereby decreasing repulsive forces and enhancing attractive interactions between enzyme and membrane. Solubilization of the enzyme, both in the presence and absence of Glc-6-P, is increased at alkaline pH, as would be expected due to increasing repulsive interactions between negative charges on membrane and enzyme as the pH is increased beyond the pI of the enzyme (pI = 6.3). In contrast to previous interpretations, P_i displayed no special efficacy at reversing Glc-6-P-induced solubilization, being comparable to other neutral salts on an ionic strength basis. However, P_i and its structural analog, arsenate, were shown to counteract specifically the Glc-6-P-induced inhibition and conformational change in the enzyme. At higher concentrations (ionic strength $>\sim 0.02$ M) neutral salts themselves lead to reversible dissociation of the enzyme from the mitochondria. The efficacy of the salts depends primarily on the pH and on the position of the anion in the Hofmeister series, with salts of chaotropic anions (SCN^-, I^-, Br^-) being most effective. At pH 6, both chaotropic and nonchaotropic salts solubilize the enzyme, while at pH 8.5, only the chaotropes retain this ability. Neutral salts also have a reversible effect on the conformation of the enzyme, as reflected by enzymatic activity, with chaotropic salts again being most effective; there is no pronounced influence of pH (in the range of pH 6–8.5) on the ability of the salts to cause conformational change in the enzyme. Based on a lack of correlation between salt-induced solubilization and conformational changes affecting activity, it is concluded that the latter are not directly responsible for release of the enzyme from the membrane. In the presence of KSCN, the extent of solubilization decreased with increase in temperature, indicating a negative enthalpy for solubilization. In contrast, in the absence of salt, the enthalpy for solubilization was positive. These temperature effects and the effects of neutral salts on the hexokinase–membrane interaction are interpreted in terms of a model in which electrostatic forces are considered to be of major importance. At low ionic strength, repulsive forces between negative charges on enzyme and membrane predominate; screening of these charges by cations diminishes the repulsion, effectively enhancing attractive electrostatic forces between enzyme and membrane and thus promoting their interaction. At higher ionic strengths, the attractive electrostatic forces are themselves disrupted, resulting in dissociation of the enzyme from the membrane. It is proposed that the greater effectiveness of chaotropic salts at disrupting these attractive forces is due to their increased ability to penetrate through hydrophobic regions of enzyme and membrane to relatively inaccessible sites of electrostatic interaction.

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The mitochondrial hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) from brain can be solubilized by low concentrations (~1 mM) of Glc-6-P[1] (1, 2). In the presence of $MgCl_2$, the enzyme rebinds to the outer mitochondrial membrane but not to inner membranes or microsomes (1, 3) nor to myelin and other heterogeneous membrane fragments derived from brain (this paper). This presumably indicates that some specific component (or components) of the outer mitochondrial membrane selectively interacts with the enzyme. Pure bindable enzyme has recently been obtained in this laboratory (4, 5), and an investigation of the membrane component(s) necessary for binding is currently in progress. The objective of these studies is to gain an understanding of the molecular basis for the selectivity of binding and the forces involved in the hexokinase–mitochondrial membrane interaction.

The present investigation, dealing with the effect of neutral salts on the association between hexokinase and the mitochondrion, has provided the basis for a hypothesis describing the interactions between the outer mitochondrial membrane and hexokinase. These interactions appear to be primarily electrostatic in nature, in accord with the suggestion of Teichgräber and Biesold (6) and include both repulsive and attractive components.

MATERIALS AND METHODS

Adult Sprague-Dawley rats were obtained from Spartan Research (Haslett, Michigan) and maintained on a common laboratory diet and water *ad libitum*. Brain tissue from these animals was stored under liquid N_2. Tris, ATP, NADH, phosphoenolpyruvate, lactate dehydrogenase, and pyruvate kinase were products of the Sigma Chemical Co.

Particulate hexokinase was prepared by the following procedure. Brain tissue, which had been stored frozen in liquid N_2, was thawed at room temperature in 0.25 M sucrose and, after thawing, the supernatant was decanted. The tissue was homogenized in 10 volumes of 0.25 M sucrose at 0°C using a Teflon-glass homogenizer (size C, A. H. Thomas Co.). The homogenate was centrifuged at 1000*g* × 10 min at 4°C and the pellet discarded. The supernatant was centrifuged at 40,000*g* × 10 min and the supernatant discarded. The pellet was washed by resuspending in 10 volumes of 0.25 M sucrose (0°C) followed by centrifugation at 40,000*g* × 10 min, and the particulate enzyme then resuspended in 10 volumes of 0.25 M sucrose. Total hexokinase activity was determined after treatment of the particles with 0.5% (v/v) Triton X-100 (2).

Unless noted otherwise, solubilization was studied at pH 6.6 ± 0.1 in unbuffered 0.25 M sucrose containing the indicated salt concentrations. Adjustment of pH was done with 0.02 N KOH or 0.02 N HCl, as necessary.

Except where indicated, hexokinase was assayed by the Glc-6-P dehydrogenase method as previously described (4). For studying inhibition by Glc-6-P, a coupled assay employing pyruvate kinase plus lactate dehydrogenase was used. This assay solution contained, in a total volume of 1.0 ml, 2 mM glucose, 5 mM ATP, 10 mM $MgCl_2$, 50 mM Tris–Cl buffer, pH 7.4, 0.8 IU of pyruvate kinase, 1 IU of lactate dehydrogenase, 1 mM phosphoenolpyruvate, 0.15 mM NADH, and 50 mM potassium chloride. The reaction was initiated by the addition of hexokinase, and the progress of the reaction was recorded as the decrease in absorbance at 340 nm.

RESULTS

Sucrose Density Gradient Fractionation of $MgCl_2$-Rebound Hexokinase

Brain hexokinase has been shown to be associated with mitochondria, being bound to the outer membrane of that organelle (1, 3, 7). Solubilization of the enzyme with Glc-6-P and subsequent addition of $MgCl_2$ leads to rebinding of the enzyme to the mitochondrial fraction (1, 2). Rebinding does not occur with microsomes or inner mitochondrial membranes (1, 3), indicating a specific interaction with the outer mitochondrial membrane rather than an indiscriminate adsorption of the enzyme to membranes in general. Additional support for the specificity of this interaction is provided by the results shown in Fig. 1. Fractionation of the crude mitochondrial fraction on discontinuous sucrose density gradients, according to De Robertis *et al.* (8), shows the hexokinase to be primarily located in fraction D (a heterogeneous fraction containing predominantly nerve endings and mitochondria) (Fig. 1, panel 1). These results are in excellent agreement with those previously obtained in this lab-

[1] Abbreviation used: Glc-6-P, glucose 6-phosphate.

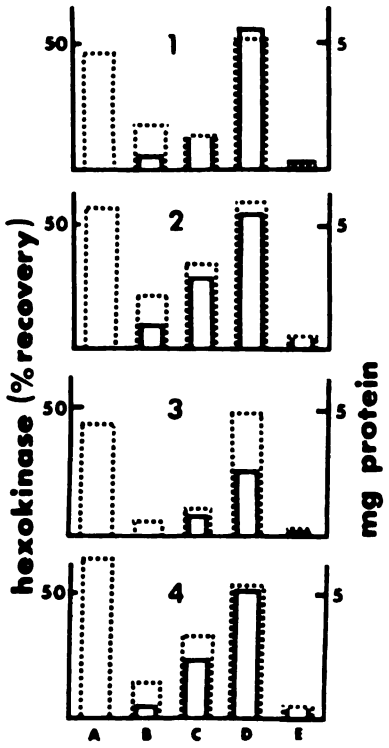

FIG. 1. Distribution of native and rebound particulate hexokinase on sucrose gradients. Particulate hexokinase was prepared as described in the text. Aliquots (5 ml) containing 5.9 units total activity were treated in the following manner: Panel 1, maintained at 0°C; panel 2, incubated at 25°C for 15 min with 3 mM $MgCl_2$; panel 3, incubated at 25°C for 30 min with 1 mM Glc-6-P; panel 4, incubated at 25°C for 30 min with 1 mM Glc-6-P followed by addition of 3 mM $MgCl_2$ and a further 15-min incubation. After the indicated treatments, all samples were pelleted at 40,000*g* × 10 min, washed once with 0.25 M sucrose, then fractionated on discontinuous sucrose gradients as previously described (9). Fractions A-E are as defined by De Robertis *et al.* (8) and are enriched in: (A) myelin fragments; (B) heterogeneous membrane fragments; (C) nerve endings; (D) nerve endings and mitochondria; and (E) mitochondria. Hexokinase activity (solid lines) is expressed as a percentage of the original 5.9 units; the milligrams of protein in each fraction are shown by the broken lines.

oratory (9) which is perhaps somewhat surprising since the present studies were done with frozen brain while in the previous work (9) fresh tissue was used; evidently freezing of the intact brain does not lead to appreciable alteration of the sedimentation properties of subcellular particles derived therefrom. The significance of the observed distribution has been previously discussed (9) and will not be reiterated here.

Treatment of the crude mitochondrial fraction with 3 mM $MgCl_2$ does not markedly affect the distribution of hexokinase on the gradient (Fig. 1, panel 2). Incubation of the crude mitochondrial fraction with Glc-6-P results in extensive solubilization of the enzyme (1, 2), with a corresponding reduction in the amount of hexokinase found in the particulate fractions (Fig. 1, panel 3). Solubilization with Glc-6-P, followed by rebinding in the presence of $MgCl_2$, restores the hexokinase distribution (Fig. 1, panel 4) to one essentially identical to that seen with the original particles (Fig. 1, panels 1 and 2). Of particular note is that, although there are substantial amounts of membranous structures [primarily myelin and heterogeneous membranous fragments (8)] in fractions A and B, there is virtually no observable interaction of the hexokinase with the membranes represented in these fractions. These results, therefore, support the view that $MgCl_2$-induced rebinding of solubilized hexokinase restores the enzyme to its original binding site on the outer mitochondrial membrane and does not result in significant nonspecific adsorption of the enzyme to other membranous components of the crude mitochondrial fraction.

Additional criteria indicating the similarity of the rebound enzyme and the original particulate hexokinase are (a) both forms have an apparent K_m for ATP that is substantially less than that of the solubilized enzyme (Fig. 2), and (b) both forms are inhibited to exactly the same extent in titrations with antiserum prepared against the purified (4) enzyme, and both are equally resistant to elution by washing of the particles with 0.25 M sucrose or 2 mM EDTA, pH 7.4 (G. P. Wilkin and D. K. Young, personal communication).

Salt-Induced Reversal of Solubilization

Glc-6-P is a potent inhibitor of brain hexokinase and P_i is effective at reversing this inhibition (11-13). Binding of Glc-6-P results in marked conformational changes,

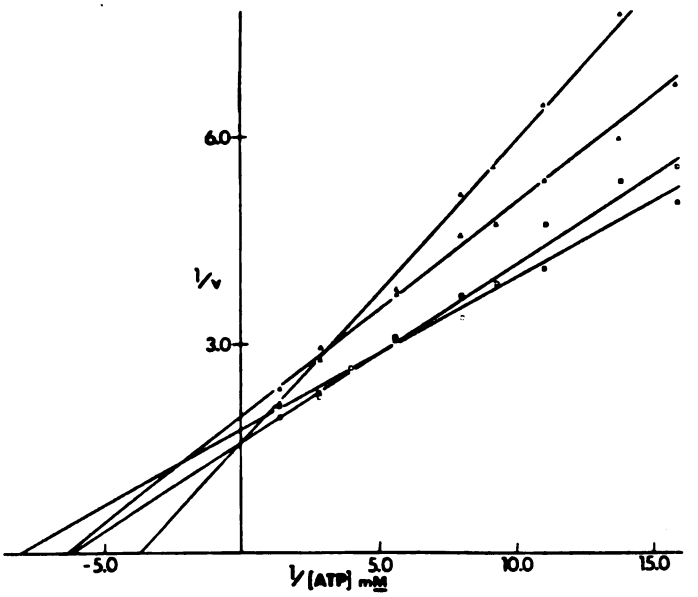

FIG. 2. Apparent K_m for ATP of the native particulate, Glc-6-P-solubilized, and rebound hexokinase. Particulate hexokinase was prepared as described in the text. Solubilized enzyme was prepared by incubation of the particulate enzyme with 1 mM Glc-6-P for 30 min at 25°C, followed by centrifugation at 40,000g × 10 min. Rebound enzyme was prepared by solubilizing the enzyme with Glc-6-P as just described, but then adding either 50 mM NaCl or 3 mM $MgCl_2$ and incubating a further 30 min at 25°C before centrifuging; the pellets, containing the rebound enzyme, were resuspended in 0.25 M sucrose. Initial velocities were determined using the Glc-6-P dehydrogenase coupled assay with the indicated ATP concentrations and other conditions as described in the text. The data were analyzed using the method of Wilkinson (10). An aliquot of the original particulate enzyme, incubated at 25°C for 60 min without addition of Glc-6-P or salts was used in this comparison to detect any effect that incubation under these conditions might have on the apparent K_m, but no such effect was observed, i.e., both freshly prepared particulate enzyme and particulate hexokinase subjected to these incubation conditions showed equivalent apparent K_m values. Similarly, controls in which the original particulate enzyme was incubated with 3 mM $MgCl_2$ or 50 mM NaCl also displayed apparent K_m values identical to the unincubated particulate enzyme. Thus the slightly increased K_m seen with "rebound" enzyme must reflect a real, albeit rather small, difference between the original and rebound particulate enzyme which is not attributable to the incubation per se. The K_m values (ATP) are: (■) particulate enzyme, 0.12 ± 0.01 mM; (△) Glc-6-P-solubilized enzyme, 0.27 ± 0.01 mM; (□) $MgCl_2$-rebound enzyme, 0.17 ± 0.01 mM; (▲) NaCl-rebound enzyme, 0.16 ± 0.01 mM.

and P_i counteracts the Glc-6-P-induced changes (14, 15). A similar antagonism between solubilization by Glc-6-P and reversal by P_i was previously interpreted in terms of ligand-induced conformational changes (14), i.e., the conformation induced by Glc-6-P was considered to have low affinity for the outer mitochondrial membrane while that induced by P_i was preferentially bound. However, as shown in Fig. 3, the effectiveness of P_i at reducing the solubilization by Glc-6-P does not appear to be attributable to any specific action of P_i since other salts are also effective at bringing about reversal. There are, in fact, two antagonistic effects of increasing ionic strength: Lower ionic strengths (<~0.02 M) bring about reversal of Glc-6-P-induced solubilization while further increase in ionic strength leads to solubilization. A similar but less marked effect of ionic strength has been seen in the absence of Glc-6-P (2). The effectiveness of various salts at reversing Glc-6-P-induced solubilization and at directly solubilizing the enzyme is related to the position of the salt in the Hofmeister series (16); "salting in"-type salts (KSCN, KI, KNO_3), are

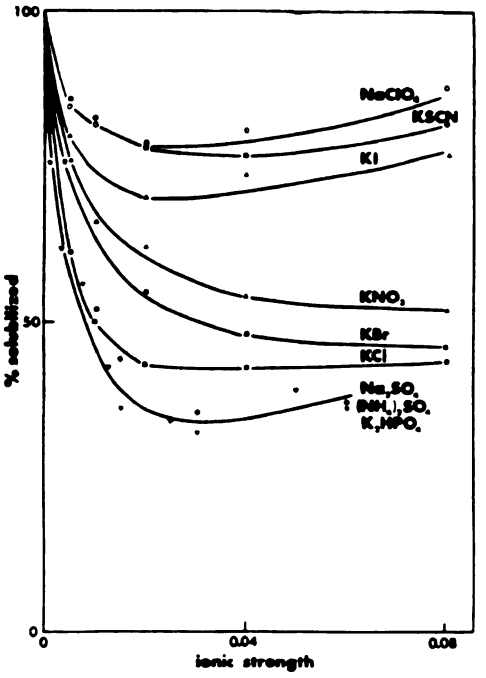

FIG. 3. Reversal of solubilization by various salts. Particulate hexokinase (0.97 unit/ml) was incubated with 1 mM Glc-6-P at 25°C for 30 min to solubilize the hexokinase. Appropriate additions of 1 M salt solutions were added to 0.34 ml aliquots and the volumes adjusted with 0.25 M sucrose so that the final volume of each sample was 0.5 ml. These samples were incubated for an additional 30 min at 25°C and the tubes then spun at 40,000g × 10 min. Supernatants were assayed for hexokinase and results expressed as a percentage of the enzyme solubilized (0.47 unit/ml) when Glc-6-P and no added salt were used. It could be shown that the remaining activity was indeed present in the pellet, i.e., the activity in supernatant plus pellet was >90% of the initial activity.

more effective at solubilizing the enzyme than are "salting out"-type salts (KH_2PO_4, Na_2SO_4). The solubilization of hexokinase by various salts is explored in greater detail in experiments described below. The relative effectiveness of the various salts at causing rebinding is inversely related to their effectiveness at bringing about solubilization, as would be expected since rebinding and solubilization represent competing processes.

Although the rebinding observed with P_i does not appear to be a specific effect resulting from interaction of P_i with the enzyme as previously thought, P_i *does* have direct effects on the activity and conformation of hexokinase which cannot be attributed to ionic strength effects per se. Of all the salts tried, only P_i or its structural analog, arsenate, cause reversal of the inhibition by Glc-6-P (Fig. 4) and the protection against chymotryptic digestion (Fig. 5) which results from Glc-6-P binding (14). These results clearly demonstrate that the specific effects of P_i on the kinetic properties and conformation of the enzyme are not directly connected to P_i-induced reversal of solubilization. Kosow *et al.* (17) reached a similar conclusion in their work with the Type II isozyme of sarcoma 37 ascites tumor, but these authors also apparently did not recognize the generality of the salt effect on solubilization, i.e., that reversal was not specific to P_i.

Effectiveness of Different Cations at Reversing Glc-6-P-Induced Solubilization

The effectiveness of monovalent cations in causing rebinding varies inversely with their crystal radius with the largest cation (Cs^+) being least effective (Fig. 6). Although the differences are relatively small, they are consistently observed

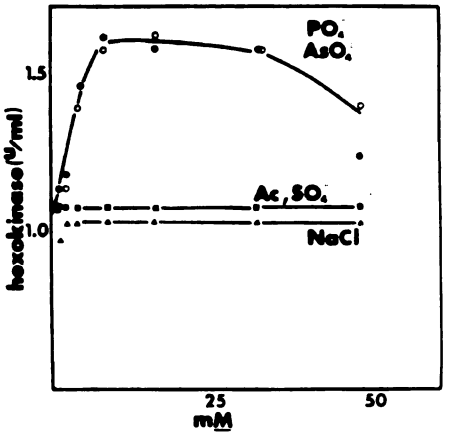

FIG. 4. Release of Glc-6-P inhibition by phosphate and arsenate. All assays were performed with pure hexokinase (4) (6.1 units/ml in the absence of Glc-6-P) and 0.1 mM Glc-6-P, using the pyruvate kinase/lactate dehydrogenase coupled assay system with the indicated additions (sodium salts were used): phosphate (●), arsenate (○), acetate or sulfate (which gave identical results) (■), and chloride (▲). The inhibition by elevated levels of phosphate has been previously reported (11).

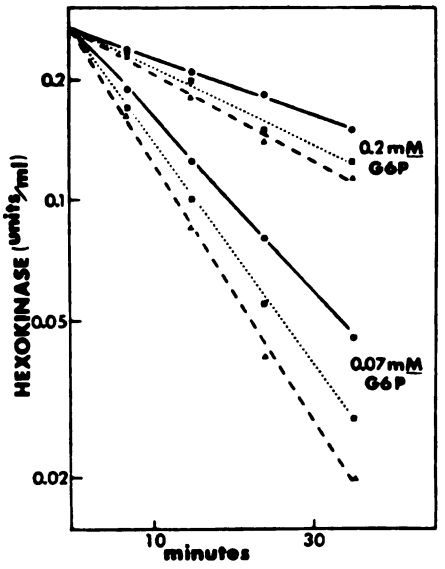

FIG. 5. Phosphate reversal of Glc-6-P protection against chymotryptic digestion. The conditions for this experiment were as previously described (14). The ordinate shows (on a logarithmic scale) the activity remaining at various times after chymotrypsin addition. Two different Glc-6-P concentrations were used as indicated, either without arsenate (●) or with 5 mM arsenate (■) or 5 mM phosphate (▲). Five millimolar concentrations of all other salts tried (NaCl, Na_2SO_4, $NaNO_3$) gave exactly the same results as with Glc-6-P alone.

when saturating levels, e.g., 25–50 mM, are used. Magnesium, as well as other divalent cations (Ca^{2+}, Be^{2+}, Mn^{2+}; data not shown), are much more effective than monovalent cations when compared on an ionic strength basis (Fig. 6).

The Effect of pH on Solubilization

It had previously been found (18) that various commonly used buffers have a marked influence on the extent of solubilization at a given pH, i.e., the proportion of hexokinase solubilized by Glc-6-P depends not only on pH but on the buffer used to maintain that pH. To avoid this complication, we have determined the pH dependence of solubilization using a pH-Stat rather than using buffers to maintain pH. The results (Fig. 7) are consistent with the existence of repulsive electrostatic interactions between membrane and enzyme. Solubilization in the absence of Glc-6-P increases at alkaline pH when negative charges on both membrane and enzyme [pI = 6.3 (4)] would be increased; Glc-6-P increases the extent of solubilization, presumably due to ligand-induced changes in conformation (14) which result in still less favorable interactions with the membrane. At pH values below the isoelectric point of the enzyme, negligible solubilization occurs either in the absence or in the presence of Glc-6-P.

Solubilization of Mitochondrial Hexokinase by Neutral Salts

Solubilization of particulate brain hexokinase at elevated concentrations of various salts has been reported previously (1, 2), but there has been no extensive comparison of the efficacy of different salts. Such a comparison is shown in Fig. 8. The cations appeared of minimal importance in this process, e.g., KCl and NaCl gave essentially identical results. In contrast it is quite apparent that different anions are

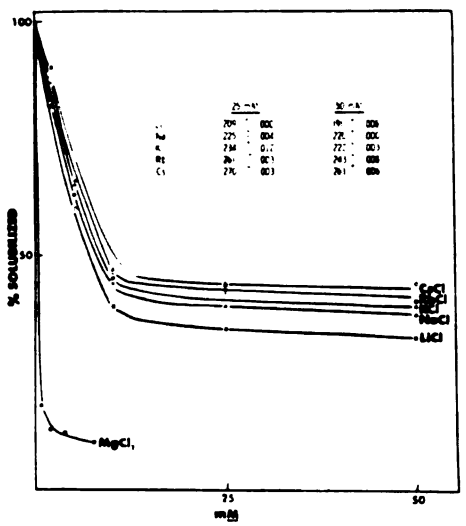

FIG. 6. Reversal of solubilization by various chloride salts. The conditions for this experiment were as described in the legend to Fig. 3. There was 0.43 unit/ml solubilized in the absence of added salts. The inset shows the results of a similar but separate experiment in which the enzyme was solubilized with Glc-6-P, then rebound in the presence of the chloride salts of the indicated cations. Each sample was run in triplicate. The values given are the mean activities ± SD (units/milliliter) remaining in the supernatant; in the absence of added salt, 0.59 unit/ml was solubilized.

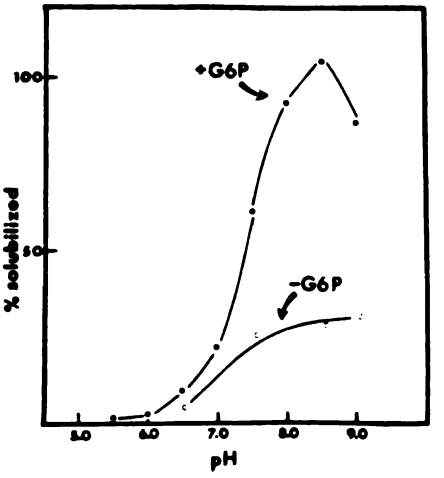

FIG. 7. The pH dependence of solubilization by Glc-6-P. Particulate hexokinase (0.95 unit/ml) was adjusted to the indicated pH in a pH Stat with either 0.02 N HCl or 0.02 N NaOH, and solubilization was initiated by addition of 1 mM Glc-6-P. After a further incubation for 25 min at 25°C, the samples were centrifuged at 40,000g × 10 min and supernatants assayed. Results are expressed as a percentage of the original activity which was found in the supernatant; the slight increase in total activity seen in the presence of Glc-6-P has been previously observed (2).

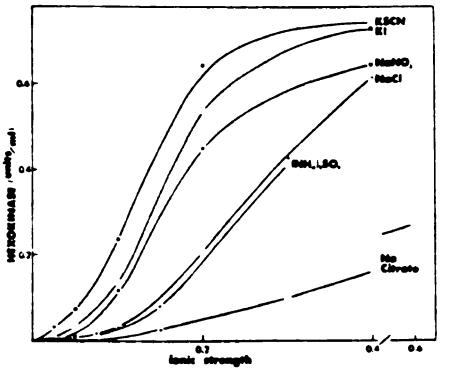

FIG. 8. Solubilization of particulate hexokinase by neutral salts. Particulate hexokinase (0.80 unit/ml) was incubated at 25°C for 30 min in the presence of the indicated salts; the samples were centrifuged at 40,000g × 10 min (4°C) and the supernatants assayed for solubilized hexokinase. The final concentration of the salts in the assay medium was always <10 mM; thus the direct effects of the salts on enzyme activity (see Fig. 9 and text) were negligible. A similar experiment, with comparable results, was done with enzyme that had first been solubilized with 1 mM Glc-6-P and then rebound by addition of 3 mM $MgCl_2$. Thus, there is no obvious difference between native particulate enzyme and rebound hexokinase in their susceptibility to solubilization by neutral salts; additional similarities between the native and rebound enzymes are described in the text.

markedly different in their solubilization abilities. Salts of chaotropic anions (16, 19), e.g., SCN^-, I^-, Br^-, are highly effective at solubilization of the enzyme, while anions at the opposite end of the Hofmeister series, e.g., chloride, sulfate, are relatively ineffective.

Solubilization by salts is readily reversible. The results of an experiment using KSCN are shown in Table I. Treatment of particulate hexokinase with 80 mM KSCN leads to extensive solubilization; subsequent dilution to a concentration of 40 mM KSCN results in rebinding, with the final soluble:particulate distribution being comparable to that seen after directly solubilizing the enzyme with 40 mM KSCN. Similar reversal is seen after dilution of the KSCN from an initial concentration of 40 mM to one of 20 mM. The slight increase in solubilization which results from dilution alone (i.e., with maintenance of the original KSCN concentration) is expected for a dissociative process such as the release of hexokinase from the membrane (1).

TABLE I

REVERSIBILITY OF KSCN-INDUCED SOLUBILIZATION OF HEXOKINASE[a]

Initial [KSCN] (mM)	Diluent	Solubilized (%)
80	None	61
	0.25 M Sucrose	34
	80 mM KSCN	68
40	None	19
	0.25 M Sucrose	13
	40 mM KSCN	28
0	None	7
	0.25 M Sucrose	7

[a] Particulate hexokinase (0.95 unit/ml) in 0.25 M sucrose was incubated for 30 min at 25°C with the indicated concentrations of KSCN. Subsequently, aliquots (1 ml) were diluted with an equal volume of KSCN or 0.25 M sucrose or left undiluted. After an additional 30 min at 25°C, the samples were centrifuged at 40,000g × 10 min, and the solubilized hexokinase was determined by assaying the supernatants.

The extent of KSCN-induced solubilization was decreased at increasing temperatures (Table II), indicating a *negative* enthalpy for the dissociation process.

The Effect of Salts on Hexokinase Activity

The solubilization of hexokinase by salts could conceivably be due to an effect of these salts on the enzyme, on the membrane, or on both. In an attempt to assess directly the effects of salts on hexokinase, the experiments shown in Fig. 9 were performed. At concentrations below ~0.1 M, all salts tested *activated* the enzyme to a comparable extent, the maximal increase in activity being about 20% over that seen in the absence of added salt. At higher salt concentrations, inhibition was observed. Analogous salt effects have previously been found to occur with pig heart fumarase (20).

As was the case with salt-induced solubilization, the cation had little effect; thus, the inhibitory action of various salts depended primarily on the nature of the anion, with highly chaotropic anions (SCN^-, I^-) being much more effective than less chaotropic anions (Cl^-, SO_4^{2-}).

The inactivation was rapid and reversible. Thus, if active enzyme was added to the assay mix containing inactivating concentrations, e.g., ~0.4 M, of KSCN, the indicated (Fig. 9) inhibition of the activity was seen from the first observable moment in the spectrophotometric assay. Conversely, if the enzyme was pretreated with inactivating concentrations of KSCN and then diluted into assay mix with no KSCN, the activity expected from the final KSCN concentration in the assay was observed immediately, with no evidence of concavity in the reaction progress curve which would be indicative of a slow renaturation process.

TABLE II

TEMPERATURE DEPENDENCE OF KSCN-INDUCED SOLUBILIZATION OF HEXOKINASE[a]

Temperature (°C)	Solubilized (%)[b]
10	64
20	59
30	53
40	50

[a] Particulate hexokinase (0.88 unit/ml) was incubated with 80 mM KSCN for 30 min at the indicated temperatures; a preliminary experiment demonstrated that 30 min was adequate to attain maximal solubilization at each of these temperatures and that no detectable loss of activity occurred, even at 40°C. After centrifugation (40,000*g* × 10 min, at a temperature equal to the incubation temperature), hexokinase activity was determined in the supernatant.

[b] Expressed as a percentage of the original activity.

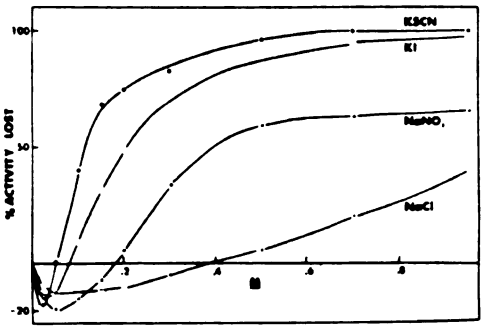

FIG. 9. Effect of neutral salts on hexokinase activity. Hexokinase was assayed as described in the methods section with addition of the indicated salts to the assay medium; the results shown were obtained at the usual assay pH [40 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer, pH 7.5], but identical effects were also observed at pH 8.5 (40 mM HEPES buffer) and at pH 6.0 [40 mM 2(*N*-morpholino)ethanesulfonic acid (MES) as buffer]. The reaction was initiated by addition of hexokinase. In the experiment shown, crude Glc-6-P-solubilized enzyme was used, but the same results were observed in experiments with pure (4) or particulate hexokinase. The coupling enzyme in the assay, Glc-6-P dehydrogenase, was also inhibited at elevated salt concentrations, but was still in excess with respect to the hexokinase; increasing the amount of coupling enzyme had *no* effect on the observed reaction rate. It is also conceivable that added anions might compete with ATP for available Mg^{2+} and thereby reduce the availability of the ATP-Mg^{2+} complex required as substrate; however, this was not an important factor in the observed inhibition since doubling the $MgCl_2$ concentration had no effect on activity.

Effect of pH on Salt-Induced Rebinding and Solubilization

As shown in Fig. 3, solubilization of mitochondrial hexokinase with Glc-6-P followed by subsequent addition of neutral

salts leads first to reversal of the Glc-6-P-induced solubilization, followed by resolubilization of the enzyme with an efficacy depending largely on the chaotropicity of the anion. The effect of pH on this process when the relatively nonchaotropic NaCl is the salt involved is shown in Fig. 10A. At pH 6.0, there is very little solubilization due to Glc-6-P and, hence, no reversal of Glc-6-P-induced solubilization to be observed, but moderate concentrations of NaCl are shown to be quite effective at extensively solubilizing the enzyme. At pH 8.5, Glc-6-P is very effective at solubilizing the enzyme, with reversal by low concentrations of NaCl being dramatically evident. However, at pH 8.5, NaCl is notably *ineffective* at bringing about resolubilization of the enzyme. At an intermediate pH (pH 7.1), both activities of NaCl, i.e., reversal of Glc-6-P-induced solubilization, and resolubilization, can be observed.

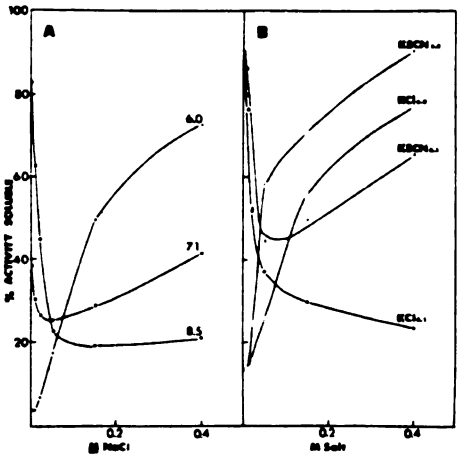

FIG. 10. Effect of pH on solubilization by a chaotropic (KSCN) and nonchaotropic (KCl) salt. (A), Particulate hexokinase (0.73 unit/ml) was incubated with 1 mM Glc-6-P for 30 min at 25°C. Portions of this mixture were adjusted to the indicated pH, and 0.8-ml aliquots were diluted to a final volume of 1.0 ml with appropriate amounts of 2 M NaCl or 0.25 M sucrose to yield the indicated final NaCl concentration. After 30 min at 25°C, the samples were centrifuged at 40,000*g* × 10 min, and hexokinase activity in the supernatant was determined. Final salt concentrations in the assay medium were such as to have negligible effect on enzyme activity. (B), The procedure was essentially as for the experiment in (A), except that KCl and KSCN were used at the indicated pH values. In both (A) and (B), the activity in the supernatant is expressed as a percentage of the total original activity. Control experiments confirmed that recovery (in supernatant and pellet) of initial activity was >90%.

In contrast to the relatively nonchaotropic KCl, KSCN was effective at solubilizing the enzyme at both pH 6.0 and 8.5 (Fig. 10B). Although both salts solubilized the hexokinase at pH 6.0, KSCN was appreciably more effective than was a comparable concentration of KCl, clearly indicating that this was not simply an ionic strength effect.

DISCUSSION

Clearly, neutral salts have two distinct effects on the interaction of hexokinase with the mitochondrial membrane. At low concentrations (<~0.02 M), salts serve to enhance this interaction while elevated concentrations have a disruptive effect. In the present discussion, we will first consider these effects separately and then suggest a model for the hexokinase-membrane interaction which accounts for these antagonistic effects of neutral salts.

We propose that the enhancement of hexokinase-membrane interactions observed at low salt concentrations is due to the shielding of negative charges on the membrane and/or enzyme, thereby decreasing repulsive electrostatic forces between the two. This enhancement cannot be explained simply as electrostatic screening in the Debye-Hückel sense since the effectiveness of various salts is not solely a function of ionic strength but, rather, varies with the salt used. Clearly this effectiveness depends directly on the charge density of the unhydrated cation (Fig. 6). This kind of relationship has also been observed for the binding of cations to pyrophosphate, AMP, ADP, and ATP (21). Ross and Scruggs (22, 23) found a similar order of effectiveness of these cations in promoting the double-helix formation in DNA and attributed their results to a shielding of negatively charged phosphate groups on the DNA by the cations, thereby diminishing repulsive interaction between the DNA strands. Ross and Scruggs also suggest that water of hydration must be released from the cations during binding. Otherwise Na^+ and K^+ would be expected to be more effective than Li^+ which has a

hydrated radius larger than that of hydrated Na^+ and K^+ ions. By analogous reasoning, it would appear that the ionic interactions leading to rebinding of hexokinase involve the unhydrated cation. Rottem *et al.* (24) demonstrated that membrane reconstitution is facilitated by low pH and the presence of cations (monovalent as well as divalent), while Okada *et al.* (25) found that divalent cations promoted adhesion of cells to protein-coated surfaces. All of these investigators offer a similar explanation for these cation effects, namely, that the cation serves to diminish repulsive electrostatic interactions and allows association to occur. Thus, there is ample precedent for the role that we have proposed for cations in enhancing the interaction of hexokinase with the mitochondrial membrane.

Elevated concentrations of neutral salts serve to solubilize the enzyme. If this were purely an ionic strength effect, one would expect comparable effectiveness of various salts compared on an ionic strength basis. Clearly this is not the case, since the present results show the solubilization to depend greatly on the nature of the anion, with salts of chaotropic anions being much more effective than salts of relatively nonchaotropic anions. The net effect of a salt on the hexokinase-membrane interactions is thus a composite one, with charge screening effects (primarily due to the cation) resulting in enhanced interactions while counteracting chaotropic effects seek to weaken the interaction. The charge-shielding action (observed at low ionic strengths) of the cations apparently outweighs their chaotropic effect: The effectiveness of the chloride salts at promoting membrane-hexokinase interactions (Fig. 6) increases in the order $Cs^+ < Rb^+ < K^+ \gtrsim Na^+ < Li^+$, which is also the order of increasing chaotropicity of the ions (16). Thus, if the chaotropic effects of the cations were predominant, their effectiveness in promoting hexokinase-membrane interactions should be the reverse of that observed. We interpret these results to mean that, at low ionic strengths, chaotropic properties of the salts are of minimal influence and the major effect of the salt is to enhance hexokinase-membrane interactions by the charge-screening effects of the cations. At higher ionic strengths, chaotropic effects primarily due to the anions became predominant, resulting in disruption of the association of enzyme with membrane.

Neutral salts also have a marked effect on the activity of the enzyme (Fig. 9), presumably reflecting alterations in the tertiary structure of the protein (20, 26). Is this alteration per se necessary for solubilization? We believe the answer to be "No," based on the following observations.

(a) KCl and NaCl are relatively ineffective at inactivating the enzyme at any pH in the range of 6.0–8.5 (Fig. 9) but, at comparable concentrations, they *are* quite effective in solubilizing the enzyme, at least in the pH range of ~6–7 (Figs. 8 and 10).

(b) At appropriate concentrations, neutral salts virtually instantaneously inactivate the enzyme; in contrast, we have found solubilization to be noticeably time dependent, being complete within 15–20 min at 25°C under the conditions used in the present study.

(c) Although the concentration dependence for solubilization (Fig. 8) and inactivation (Fig. 9) have some similarity, there are also notable differences; e.g., compare the relative effectiveness of NaCl at solubilizing (Fig. 8) or inhibiting (Fig. 9) the enzyme. We conclude that a direct effect of neutral salts on the conformation of the enzyme, as reflected by catalytic activity, is *not* sufficient to explain the observed solubilization.

At pH 6, both relatively nonchaotropic salts such as KCl as well as chaotropes such as KSCN are highly effective at solubilizing the enzyme (Fig. 10). Furthermore, this solubilizing action is apparent at salt concentrations well below those typically required to have pronounced effects on water structure and to bring about extensive disruption of hydrophobic interactions (16, 19). These observations lead us to suggest that, at pH 6, the solubilization is primarily due to the effectiveness of these salts at disrupting nonhydrophobic interactions. Certainly the most obvious possibility would be attractive electrostatic interactions between oppositely

charged groups on enzyme and membrane, as already suggested by Teichgräber and Biesold (6). But it was also observed that, even at pH 6.0, noticeable differences could be observed between chaotropic (KSCN) and relatively nonchaotropic (KCl) salts (Fig. 10). Von Hippel and his colleagues (16, 27–30) have suggested that the effectiveness of various salts in bringing about conformational change in proteins may be due to interaction of the salts with the peptide bond dipole. This effectiveness varies with the position of the salt in the Hofmeister series, i.e., its chaotropicity, reflecting the differences with which the various salts can penetrate hydrophobic regions of the protein and thereby extend their range of action. Hexokinase must exist in rather intimate contact with the outer mitochondrial membrane; e.g., it is not readily removed by washing with 0.25 M sucrose or dilute salt solution, and it is largely protected from inhibition by antihexokinase serum (31); given the high lipid content of the outer mitochondrial membrane (32), it seems likely that the enzyme must be semi-embedded in a rather hydrophobic milieu. Extrapolating from the thoughts of Von Hippel and his co-workers (16, 27–30), we suggest that the relatively greater efficacy of chaotropic salts reflects their increased proficiency in penetrating through hydrophobic regions to the interacting charges on membrane and enzyme.

At higher pH, e.g., 8.5, relatively nonchaotropic salts, e.g., KCl, are ineffective at bringing about solubilization whereas chaotropic salts retain their effectiveness. It is, of course, possible that hydrophobic interactions (which would not be disrupted by nonchaotropes) could become dominant at elevated pH. However, we feel that this is unlikely. First, there is no inherent reason to expect hydrophobic interactions to be markedly pH dependent; since they did not appear to play a prominent role at pH 6, there is no *a priori* reason to expect them to predominate at higher pH. Second, as at pH 6, the concentrations of chaotropic salts that bring about solubilization are considerably lower than those generally found to be effective at disrupting hydrophobic interactions (16, 19). As an alternative, we suggest that electrostatic forces remain the prime attractive interaction, but at higher pH, the interacting ionic groups become less accessible to disruptive exogenous ions. Decreased accessibility could be a consequence of more intimate contact between interacting groups on enzyme and membrane resulting from pH-induced changes in membrane and/or enzyme structure. By virtue of their greater ability to penetrate through hydrophobic regions and then exert disruptive influences on electrostatic interactions, chaotropes would retain their capability for bringing about solubilization whereas the effectiveness of nonchaotropes would be greatly diminished. The thoughts expressed in the above discussion are presented diagrammatically in Fig. 11.

Finally, we would like to comment briefly on the negative enthalpy seen for solubilization in the presence of KSCN (Table II). In the *absence* of salts, solubilization *increases* with increase in temperature (2, and unpublished observations), indicating a positive enthalpy. In the absence of salt, the interactions between enzyme and membrane are pictured as being primarily electrostatic in nature and occurring in a generally hydrophobic environment; under these conditions, we would anticipate a negative enthalpy for *formation* of the ionic interactions (27, 33) and conversely, a positive enthalpy for their disruption. Thus, in the absence of salts,

$$\Delta H_{\text{solubilization}} = -\Delta H_{\text{ME}} > 0,$$

where ΔH_{ME} is the enthalpy for formation of the attractive electrostatic interactions between enzyme and membrane. Solubilization in the presence of salts, however, is considered to be the result of interactions of exogenous salts with the charged groups on enzyme and membrane, with consequent rupture of the attractive interaction between the latter species. Thus, in the presence of salts:

$$\Delta H_{\text{solubilization}} = -\Delta H_{\text{ME}} + \Delta H_{\text{SE.SM}}$$

where $\Delta H_{\text{SE.SM}}$ is the enthalpy for *formation* of new electrostatic interactions be-

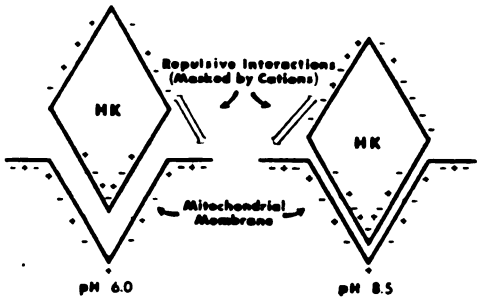

FIG. 11. A model for interaction of hexokinase with the outer mitochondrial membrane. The forces between enzyme and membrane are considered to be primarily electrostatic in nature. Repulsive interactions, which are more marked at pH 8.5, can be masked by cations. Neutral salts can disrupt the attractive interactions *if* the salts can penetrate to the region between enzyme and membrane. At pH 6.0, the association between the interactants is considered relatively "loose" in the sense that both chaotropic and nonchaotropic salts can readily enter the region of attractive electrostatic interaction between the membrane and enzyme; thus, chaotropes would possess only slight advantage in gaining access to this region via hydrophobic routes. At pH 8.5, the region of electrostatic interaction is considered to be much less accessible to nonchaotropes, while chaotropes can still gain access by penetration of hydrophobic regions. The representation of the binding site as an invagination into the membrane is diagrammatic and should not necessarily be taken to indicate a penetration of the hexokinase molecule (or portion thereof) into the hydrophobic interior of the membrane. Nor should the emphasis on electrostatic interactions be taken to exclude the possible involvement of hydrophobic interactions; the present results do not provide any direct evidence for the latter, however, and suggest that electrostatic interactions are the predominant if not the exclusive forces involved.

tween the added salts and groups on the enzyme and membrane; $\Delta H_{SE,SM}$ is expected to be negative (27, 33), and thus the ΔH for solubilization in the presence of salt is predicted to be more negative than that for solubilization in the absence of salt, as observed.

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