SODIUM-DEPENDENT AMINO ACID TRANSPORT IN RECONSTITUTED PLASMA MEMBRANE VESICLES FROM EHRLICH ASCITES CELL PLASMA MEMBRANE.

by
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ABSTRACT

Plasma membranes from Ehrlich Ascites Cells were solubilized in 2% cholate - 4M urea. The 150,000 g supernatant was free of vesicular structure and able to penetrate a 5% polyacrylamide gel. Addition of lipids (protein/exogenous lipids 1:1) followed by detergent removal by dialysis led to reformation of vesicles with proteins embedded in the membrane as visualized by electron microscope. The reconstituted vesicles have a chemical composition similar to that of the native vesicles and are sensitive to osmotic strength. They exhibit sugar transport activity, temperature sensitive amino acid transport activity and ouabain sensitive ($Na^+ + K^+$) ATPase activity. The transport of amino acid which is Na^+ dependent and can be stimulated by a Na^+ gradient, leads to accumulation against a concentration gradient. The amino acid transport system is reversible and a reversed gradient (Na^+ inside > Na^+ outside) can stimulate AIB efflux. Further stimulation occurs by increasing the membrane potential (positive inside) by valinomycin.

RESUME

Les membranes plasmiques des cellules Ehrlich Ascitiques ont été solubilisées par le mélange 2% cholate - 4M urée. Le surnageant, après ultracentrifugation à 150,000 g, ne contient plus de structures vésiculaires et peut pénétrer dans un gel de polyacrylamide à 5%. L'addition de lipides (rapport protéines/lipides éxogènes de 1:1) suivie de l'élimination du détergent par dialyse conduit à la formation de vésicules dont les protéines sont incorporées dans la membrane, ainsi que le montre l'analyse au microscope électronique. Les vésicules reconstituées ont une composition chimique semblable à celle des vésicules natives et sont sensibles à la pression osmotique. Elles possèdent l'activité de transport des sucres, l'activité de transport des acides aminés sensible à la température et l'activité ATPasique stimulée par Na^+ et K^+ et inhibibée par l'ouabaine. Le transport des acides aminés, qui dépend de la présence de l'ion Na^+ et qui peut être stimulée par un gradient de Na^+, conduit à l'accumulation contre un gradient de concentration. Le système de transport des acides aminés est reversible, et un gradient de Na^+ inversé (Na^+ interne > Na^+ externe) peut stimuler l'efflux de AIB. Une stimulation supplémentaire peut être obtenue en accroissant le potentiel de membrane (potentiel interne positif) par l'addition de valinomycine.

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INTRODUCTION

CHAPTER I: Membrane Composition

The plasma membrane forms a barrier between the outside and the inside of the cell. It is involved in the maintenance of a specific intracellular composition and participates in most of the fundamental processes associated with living: secretion, vision, cellular interactions, muscular contraction, neuronal transmission, hormonal control, immunological response, and respiration in microorganisms. The plasma membrane plays all these roles as a result of its particular composition and structure, that is, the nature and organization of its protein and lipid components.

Most membranes are about 75 Å thick and composed essentially of lipids and proteins. In animal cells, carbohydrates are found mostly in covalent association with proteins. The lipid - protein ratio is a characteristic of each type of membrane (1,2). In many instances with mammalian cells, proteins form 60 to 70% of the membrane mass, with the exception of myelin where the proteins form only 20% of the membrane mass.

Membrane Proteins

Membranes from a variety of sources have been isolated and solubilized, and their proteins have been analyzed by SDS polyacrylamide gel electrophoresis. The number of proteins found varies from membrane to membrane. Some membranes show as little as 15 bands of distinct sizes of proteins and others show as many as 60 or more discrete bands with molecular weights ranging from 10,000 to 200,000 daltons.

Some membrane proteins have been purified (rhodopsin, myelin basic protein, cytochrome c ...). Physico-chemical analysis shows that their overall content of hydrophobic amino acids does not render them a special

class of proteins. Membrane proteins exhibit a mean residue hydrophobicity and polarity index similar to that of many soluble proteins (3). What distinguishes membrane proteins from soluble proteins (and this has only been shown in a few cases) is their primary as well as their tertiary structure, where segregation of polar and apolar domains can be observed (4). It is the conformation of the protein that is adapted to the lipid environment of the membrane. And since the conformation is a function of the amino acid sequence, segregation is expected.

Many membrane proteins bear sugar residues covalently linked to them. The sugars most commonly found are: D-galactose, D-mannose, D-fucose, N-acetyl-glucosamine, N-acetylgalactosamine and N-acetylneuraminic acid (or sialic acid). The average number of sugar residues per protein is seven. The sugars are oriented toward the external milieu and contribute to the negative polarity of the membrane. These glycoproteins have been found to be involved in cellular interactions, hormonal and immunological responses (5).

Membrane proteins have also been classified as extrinsic and intrinsic, or peripheral and integral proteins (6). Extrinsic or peripheral proteins can be removed from the membrane by mild treatments like chelating agents, changes in pH, or changes in ionic strength (7). Their purification is greatly facilitated by their solubility in water and by the fact that many of them which exhibit enzymatic activities are not much modified after their detachment from the membrane (8). A number of them have been isolated, purified and characterized (2,7,9). Most of these are acidic and bear a negative charge at neutral pH; a few of them are basic, as for example cytochrome c (2) and myelin basic protein (9).

However, the majority (70 to 80%) of membrane proteins are very tightly bound to the membrane. These so-called integral or intrinsic proteins require much more drastic conditions for their extraction from the membrane, as for example chaotropic agents or detergents (10,11). The isolated proteins are often aggregated or associated to lipids, insoluble in water, or denatured. This makes their isolation, purification and characterization a much more complicated task.

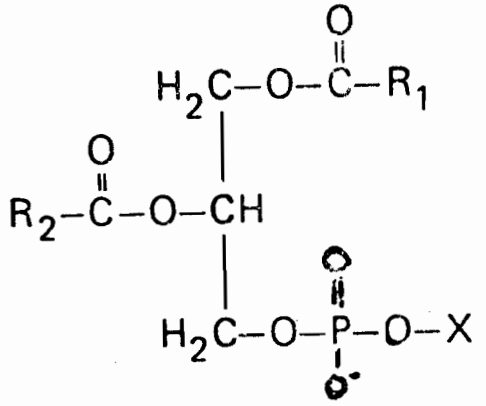
The variation in size, composition, solubility and structure of the membrane proteins is a reflexion of the numerous functions assigned to the membrane.

Membrane Lipids

The lipids found in biomembranes consist mainly of phospholipids, glycolipids and sterols. The proportion of these different classes varies from membrane to membrane (12). Of cell membranes the plasma membranes of higher organisms are the richest in cholesterol (0.8 to 1 mole of cholesterol per mole of phospholipid) whereas microsomes from the same cells are poor in cholesterol (0.2 mole of cholesterol per mole of phospholipid) and mitochondria contain practically no cholesterol (13).

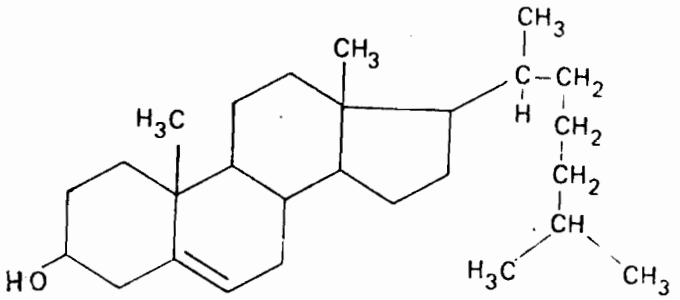
The general formula for phospholipids and sterols is shown in Figure 1. The fatty acid chains are commonly C_{12} to C_{20} saturated or unsaturated alkyl chains.

Phospholipids are amphipathic molecules, that is the molecule is composed of a polar region and an apolar region: the polar region consists of the phosphorylated head groups; the apolar region is represented by the fatty acid chains. Each lipid has a characteristic gel → liquid state transition temperature (14,15), which is primarily a function of the length and of the degree of unsaturation of the fatty acid chains,



R_1, R_2: fatty acid chain

PHOSPHOLIPID



CHOLESTEROL

Figure 1. General formula of phospholipids and sterols

but can also be affected by the environment of the lipid: pH, metal ions... (16,17).

In aqueous media, the polar head groups tend to associate with water, while the apolar regions tend to associate with each other in some micellar or bilamellar array away from the water molecules. The phospholipid level at which molecular aggregation occurs, critical micellar concentration (CMC), is extremely low: 10^{-9} - 10^{-10} (18,19). Sterols also have an extremely low monomer solubility in aqueous media and form rod-shaped micelles in water (20). Stim et al., (21) using differential thermal analysis with Mycoplasma laidlawii, established the existence of two types of membrane lipids. The majority of the membrane lipids showed an equivalent endothermic phase transition when present in the intact cells, in the isolated plasma membrane, or in the extracted lipids. When extracted lipids interacted with a basic protein, the phase transition was shifted (22). This suggested that these lipids, called the bulk lipids, are loosely bound and do not interact with membrane proteins. The fraction of lipids which forms the bulk lipids has been determined by several methods reviewed by Träuble and Overath (23). The bulk lipids account for 80% of the total membrane lipids. The question of whether all the classes of lipids are represented in the bulk lipids has not yet been resolved.

As much as 20% of the lipids bound to a membrane do not undergo a phase transition equivalent to that observed with the isolated lipids. These lipids, called the boundary lipids, appear to be involved in the interactions with membrane proteins, resulting in a restriction of their mobility. This fraction of lipids is not extracted from the membranes as pure phospholipids. Acidic phospholipids seem to be the principal constituents of this class of lipids (24,25).

CHAPTER II: Protein - Lipid Interaction

Forces Involved in Lipid-Protein Interaction (26).

Various forces participate in lipid-protein association.

Electrostatic Forces

Electrostatic forces are due to the attraction between charges on lipids and proteins. For example these forces are important in the binding of cytochrome c to the acidic mitochondrial lipids.

Induction Forces

Induction forces are observed when a negative charge can polarize a nearby apolar group. However these forces are too small to be important in lipid-protein interactions.

London-Van der Walls Dispersion Forces

The London-Van der Walls forces operate between all groups of proteins and lipids. They are the only forces involved in the interaction between non-polar residues. They depend strongly on distance and steric factors.

Hydrophobic Interactions

The fundamental driving force for any process is the tendency to move to a state of lowest free energy. Therefore apolar groups will tend to cluster together, thereby excluding water, increasing the entropy of the system as a whole and decreasing its free energy. Thus a mixture of monomeric membrane components should, under appropriate conditions, assemble spontaneously to form native membrane domains. For example, if the structure of the proteins in the membrane is such as to expose large hydrophobic regions, the proteins will either aggregate through

apolar associations, or rearrange with hydrophobic residues concentrated in the interior and polar groups at the surface of the molecules. They rearrange to form structures which are thermodynamically stable in aqueous media.

Methods to Investigate Protein-Lipid Interactions

Many techniques can be used to study protein-lipid interactions: I.R. and Raman spectroscopy, light scattering, ESR, NMR, fluorescence, X-ray diffraction, differential calorimetry, freeze fracture electron microscopy, all have been used for such studies. By detecting diffracted X-rays, it has even been possible to follow the kinetics of some slow conformational changes due to lipid-protein interactions(27,30).

Interactions of Proteins with Lipid Bilayer

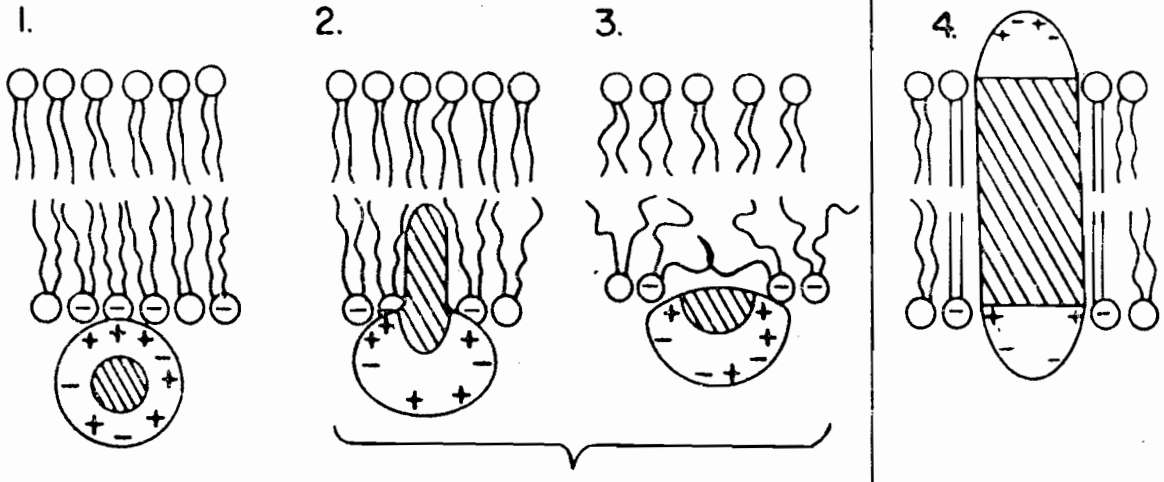
Some general conclusions have been presented by Kimelberg (31) on the interactions between proteins and lipid bilayers. Lipid bilayers are largely impermeable to charge or polar solutes. Association of the bilayer with proteins alters the permeability barrier in the following generalized way: (cf. Figure 2).

1) An extrinsic protein, whose hydrophobic amino acids are predominantly buried inside the protein molecule, interacts with the surface of the bilayer by charge interaction only. No changes in the permeability of the bilayer are observed.

2) and 3) Proteins like cytochrome c can interact initially by charge interactions. This electrostatic binding leads to an alteration in protein conformation resulting either in penetration of the hydrophobic portion into the bilayer or in an increase of hydrophobic groups at the surface of the protein. These interactions result in increased permeability

"Extrinsic"

"Intrinsic"



Effects on Lipids:			
None	More Fluid		Domain Lipid, More or Less Fluid. Tc Remainder :- Unaltered Increased Permeability
	Tc Lowered		
	Increased Permeability		

of the bilayer. A lowering in transition temperature of the bilayer after protein binding has also been measured by differential scanning calorimetry. This lowering in Tc is equivalent to an increased lipid fluidity.

4) Integral proteins do not usually require initial charge interactions. Hydrophobic binding is dominant (the permeability of the bilayer is increased by interacting with the protein). This fourth picture shows a protein which spans the entire membrane. A portion of the phospholipids are removed from the bulk lipids and do not participate in a phase transition. This lipid constitutes a microenvironment specific for the protein and corresponds to what is called the boundary lipid.

Phospholipid Dependence of Membrane Structure and Function

A great number of membrane bound enzymes have been shown to require phospholipids for stability, deinhibition or maximal activity (14,32,33). The problem of phospholipid dependence can only be studied if one is able to control the lipid composition of the natural or model membrane. Different approaches can be used to study this problem:

a) Effect of delipidation on enzyme activity.

b) Delipidation followed by addition of specific lipids.

c) Substitution of the native lipid in the membrane using phospholipid exchange proteins.

d) Growth in media of different compositions to vary the fatty acid components of the phospholipids.

Delipidation by detergents, solvents or phospholipases often leads to loss of activity in membrane bound enzymes. This does not necessarily mean lipid dependence, because inactivation can be due to specific

denaturation unrelated to lipids, or inhibitory action of the products of the modification (32). Reactivation of the inactive enzyme by added lipids is an essential criterion for establishing lipid dependency. Fleisher et al. (34) suggested that there should be a correlation between lipid removal and loss of activity and a correlation between restoration of activity and rebinding of lipid. In a few cases (NADH coenzyme Q reductase, acyl CoA synthetase, $(Na^+ + K^+)$ - ATPase), the addition of lipid causes stimulation of the original enzyme activity (35,37).

A list of some of the lipid dependent enzymes is given(Table I). In an extensive summary of the $Na^+ + K^+$ - ATPase, it can be seen that the results do not always agree with the action of phospholipids on the enzyme activity. But as suggested by Kimelberg (31), this can be due to the manner in which the lipid is removed, the amount of lipid removed, or the purity of the phospholipids used for reactivation. Despite much controversy, most investigators seem to agree that phosphatidylserine is the lipid required to obtain maximum activity of the $(Na^+ + K^+)$ - ATPase.

Not only is the nature of the polar head group of the phospholipid important, but the fatty acyl chain length and degree of unsaturation (32,38,39) play a role in maintaining enzyme activity). Studies with fatty acid auxotrophs (40,41) of Escherichia coli indicate that discontinuities in the Arrhenius plots for membrane enzymes reflect temperature dependent phase transitions of membrane phospholipids. Thus, the fluidity of the phospholipid fatty acyl chains may contribute to the control of the membrane protein function.

Many more examples of phospholipid dependence for enzyme activity are available in the literature (31,42-49).

TABLE I

Lipid-dependent membrane proteins

	Enzyme (source)	Lipid activator
(a)	Amino peptidase (brush border)	Phosphatidylcholine (1)
	Glucose-6-phosphatase (brain)	Various phospholipids (2)
	Acetylcholinesterase (erythrocytes)	Endogenous cardiolipin (3)
	Glycophorin (erythrocytes)	Phosphatidylcholine, mixed lipids (4)
	Ca^{++}-ATPase (sarcoplasmic reticulum)	Phosphatidylcholine (5) Phosphatidylethanolamine (6)
(b)	$(Na^+ + K^+)$-ATPase (beef brain)	Lysophosphatidylethanolamine (7)
		Lysophosphatidylcholine and lysophosphatidylserine (8)
		Phosphatidylserine and phosphatidylinositol (9)
	$(Na^+ + K^+)$-ATPase (erythrocytes)	Phosphatidylserine (10)

(a) from the article by Sanderman H. Jr. Biochim. Biophys. Acta 515 209 (1978)

(b) from the article by Kimelberg H.K. Mol. Cell. Biochem. 10 171 (1976)

TABLE I (continued)

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CHAPTER III: Membrane Structure

Membrane Models

Since 1925, numerous models have appeared purporting to represent membrane structure (6,50-57). The model which today has the greatest appeal is the fluid lipid - globular protein mosaïc model first proposed by Singer and Nicholson (8). According to these authors the membrane is a solution of proteins dispersed in a fluid lipid matrix. The model can be represented by the three - dimensional and cross-sectional view of figure 3. The hydrocarbon chains of the fatty acid are oriented toward the interior of the membrane and under physiological conditions are in a disordered conformation, thus creating a fluid matrix. The exact arrangement of the lipids depends on the environment and on such properties as the length of the hydrocarbon chains and their degree of unsaturation, and branching as well as the size and shape of the polar head groups.

The proteins are either at the surface of the membrane, being held through ionic or polar interactions with other proteins, or through divalent cation bridges. Such proteins would represent the extrinsic proteins. Other proteins are embedded partly or totally, or even span the lipid bilayer. These are the intrinsic proteins.

The association of the proteins with the bilayer involves predominantly hydrophobic interactions between the apolar fatty acid chains and the apolar amino acid residues. The hydrophilic portions of the proteins are either exposed to the outside of the membrane or clustered by forming an α helix which becomes a polar transmembrane channel. Helicity is not an absolute requirement, unordered or β structure can also provide the apolar parameter required for penetration through the bilayer (58).

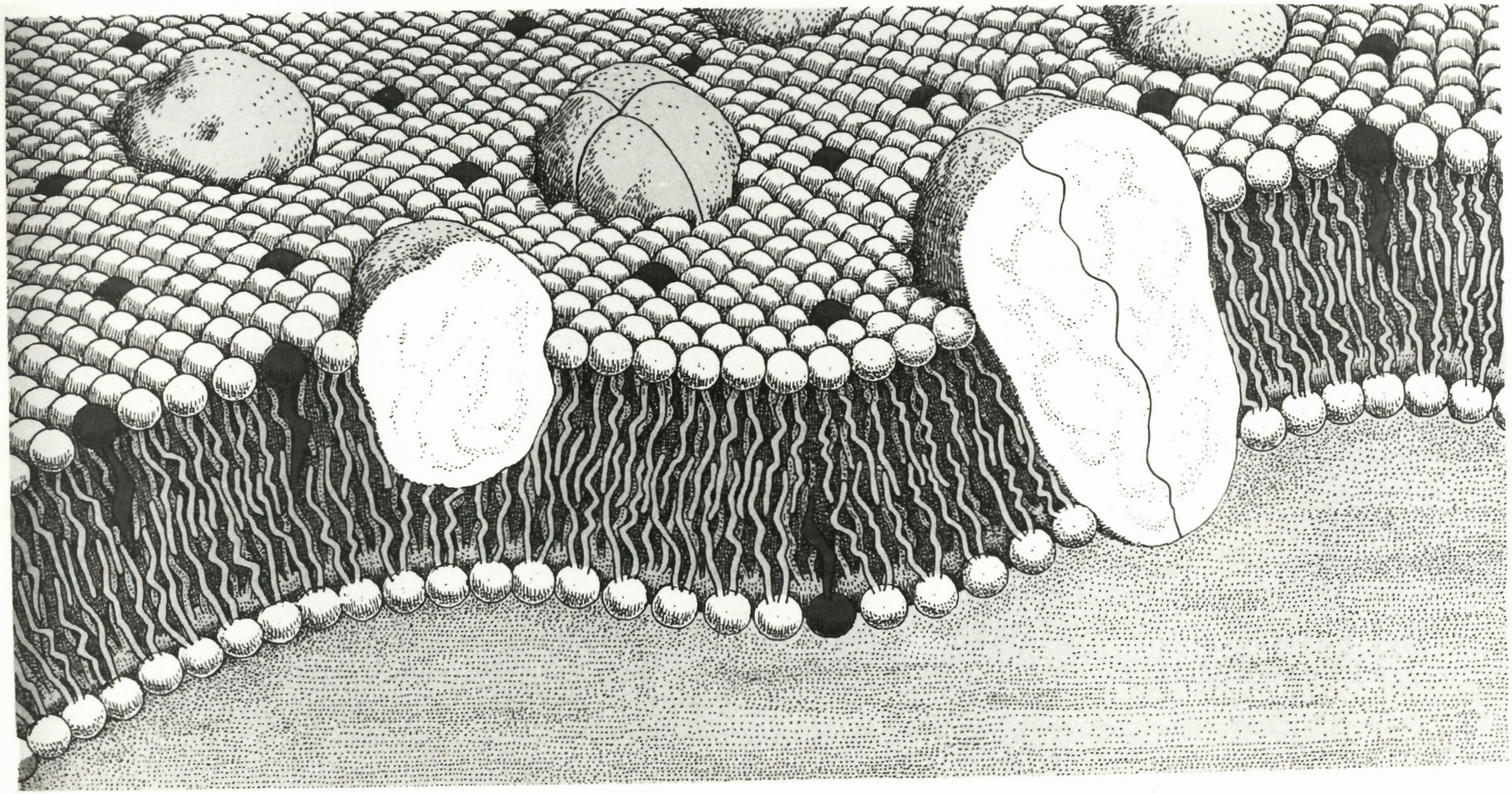


Figure 3. The lipid-globular protein mosaic model of membrane structure

from Cell Membranes Weissman G. and Clairborne R., Eds, H P Publishing Co, Inc, New-York.

Some proteins are simply embedded on one side or the other, while others pass entirely through the bilayer of lipids and cholesterol (black molecules).

Experiments using labelled sugars indicate that glycoproteins and glycolipids are located on the outer monolayer, the sugar residues facing the external medium (59).

Experimental Observations Leading to the Formulation of the Singer-Nicholson Model

The Singer-Nicholson model is deduced from a number of considerations and experiments.

Thermodynamic Considerations (8)

In the phospholipid bilayer structure, the non-polar fatty acid chains are sequestered away from contact with water, thereby maximizing hydrophobic interactions. The ionic groups are in direct contact with the aqueous phase at the exterior faces of the bilayer, maximizing hydrophilic interactions. The extent to which a protein is embedded in the membrane is determined by its amino acid sequence.

The sequence allows the protein to adopt either an amphipathic structure (intrinsic protein) or a structure in which the distribution of ionic groups is nearly symmetrical (extrinsic protein). The interactions of the protein with its environment are such that the free energy of the system is at a minimum.

Freeze Fracture Electron Microscopy (50-63)

Freeze fracture splits membranes in planes parallel to their surfaces, exposing the membrane interior. Analysis of biological membranes by freeze fracture shows that the fracture faces are populated with particles which represent proteins or protein complexes on one face and pits on the opposite face. This is consistent with the existence of proteins embedded in the bilayer.

Physico Chemical Studies (23,64-66)

Physicochemical studies of intact membranes suggest autonomous domains of lipid and protein. Despite the presence of protein as a major membrane component, a large proportion of membrane is arranged in a bilayer, as evidenced by X-ray, fluorescence, ESR.

Salt Effects

High salt concentrations do not dissociate a large fraction of proteins from the membrane (6). This implies that forces other than polar or electrostatic forces are involved in the maintenance of the membrane structure.

Enzyme Digestion

An earlier model of membrane structure (Davson-Danielli model (50)) suggested that proteins covered the lipid bilayer. Experiments with hydrolytic enzymes which do not penetrate the membrane bilayer have shown that in some cases phospholipids are directly accessible to phospholipases whereas in other instances treatment with proteolytic enzymes or other hydrolases is required to render all phospholipids susceptible to enzymatic digestion by phospholipases. This suggests that in some membranes, proteins cover or render inaccessible all or part of the phospholipid head groups.

When treated at the external surface only a few proteins are digested by exposure to proteases (67).

In the red blood cell membranes, as much as 70% of the lipids can be hydrolyzed without causing a change in protein configuration (55). This result indicates that there is little binding between the phospholipids and the proteins.

Sialic acid from sealed RBC is released by neuraminidase, suggesting that sugar residues are located on the outer monolayer (68).

Labelling Experiments (69-74)

Iodination of the proteins of human red blood cells, ghosts and inverted vesicles (74) have shown that some proteins are labelled at either one or the other surface, while others can be labelled from both surfaces, suggesting that these proteins span the lipid bilayer.

Further Experimental Evidence for the Basic Singer-Nicholson Model

Membrane Asymmetry

The model described by Singer and Nicholson proposes that the membrane components are arranged asymmetrically. Experimental evidence is available which is consistent with this model (75-79).

Asymmetry of the phospholipid distribution: S.E. Gordeski (80) has summarized the evidence for phospholipid asymmetry in the human erythrocyte membrane. Using chemical probes for phospholipids (1, fluoro 2,4-dinitrobenzene (FDNB) as a permeant probe and 2,4,6-trinitrobenzene sulfonic acid as a impermeant probe), enzyme treatments (phospholipase A_2, sphingomyelinase), and phospholipid-exchange reactions, it was concluded that:

1° phosphatidylcholine and sphingomyelin are located on the outer surface.

2° phosphatidylserine and 27% of phosphatidyl-ethanolamine are refractory to labelling by FDNB, suggesting that in these phospholipids the reactive groups are inaccessible presumably because they are involved in a tight binding with the proteins.

The significance of this asymmetry is unknown, but it would clearly alter the net charge distribution at the two surfaces.

Asymmetry of the protein distribution: Labelling experiments have been done extensively to locate proteins at each membrane surface. The clearest experiments are obtained with red blood cell membranes and show that some proteins are only accessible from one side or another of the membrane, while some are accessible from either side (74). With most plasma membranes, more membrane proteins are accessible at the cytoplasmic surface than are accessible at the external surface.

Asymmetry of membrane function: Several functions of the cell membrane have been discussed by Bretscher with respect to functional asymmetry (81). One example is the $(Na^+ + K^+)$ - ATPase of the erythrocyte membrane, which shows that the enzyme is asymmetric in its arrangement across the membrane. The enzyme is stimulated by K^+ on the outside and by Na^+ on the inside of the cell. Only internal ATP is hydrolyzed (82) and the catalytic activity is not abolished by treatment of the intact cell with trypsin (83), whereas treatment of unsealed ghosts with trypsin inactivates the ATPase (84).

Membrane Fluidity

According to the Singer-Nicholson model (8), the fatty acid chains of the phospholipids are in disordered conformation in the core of the bilayer and form a fluid matrix. On the basis of this fluidity, movement of lipids and proteins in the plane of the membrane is expected to be relatively free.

The assessment of the correctness of this conclusion requires the use of spin labels attached to the phospholipid residues and fluorescent

labels for the proteins. But the results should be taken with caution because of the lack of specificity of the labels and the perturbations that they can induce (85).

Lateral diffusion of lipids: Experimental evidence shows that most of the lipids exhibit rapid diffusion in the plane of the membrane as predicted by the model (86-90).

Transmembrane diffusion of lipids: Thermodynamically, transmembrane diffusion of the lipids is an unfavorable process, because it implies the passage of the polar head group through the hydrophobic membrane interior.

Studies from synthetic phosphatidylcholine vesicles containing a spin-label show no evidence for transmembrane diffusion (91). However, studies with natural membrane containing a spin label suggest a very rapid movement (92). Obviously the data from the two systems are not in agreement. The issue has not been resolved as there may be experimental artefacts leading to erroneous conclusions, e.g. it is not certain where the spin label is located in the membrane when introduced into a pre-existing membrane.

Furthermore a rapid rate of transmembrane diffusion would be inconsistent with the evidence of phospholipid asymmetry in the membrane.

Lateral diffusion of proteins (93-94): The capping phenomenon and patch formation on lymphocyte membranes, as shown by the techniques of immuno-fluorescence and freeze fracture are good examples of the mobility of the proteins in the plane of the membrane. Aggregation and disaggregation of membrane particles can also be induced by changes in temperature, pH and by bifunctional agents.

Rotational motion of proteins: It does not appear to be possible to generalize on the rotational motion of proteins in a membrane environment. Some proteins seem highly mobile as for example rhodopsin, cytochrome a_3-CO complex (95,98), while others appear highly restrained (bacteriorhodopsin).

Transmembrane diffusion of proteins (99): There is no evidence for transmembrane diffusion of proteins. Mobile protein carriers as models for transport systems are no longer considered likely, because of the thermodynamic restrictions to the passage of a protein through the membrane.

Lipid Segregation

Membrane proteins have binding sites for lipid molecules and these sites have different affinities for different species of lipid. In the membrane the proteins are able to segregate specific lipids from the lipid pools and thereby acquire a local lipid environment which is compatible with the function exhibited by the protein in question. It has been mentioned above that some 20% of the membrane lipids form the so-called boundary lipids. In red blood cell membranes, it has been shown that phosphatidylserine and phosphatidylethanolamine are involved in a tight association with the membrane proteins (100-102).

Role of Cholesterol

Cholesterol does not bind to membrane proteins, but has high affinity for phosphatides.

Cholesterol acts as a buffer of membrane fluidity. Studies done with pure phospholipids show that cholesterol increases the fluidity of the fatty acid chains which in absence of cholesterol would be in an ordered crystalline state, and increases the crystalline structures

of those chains which are in a liquid state at the physiological temperature. Thus in the membrane cholesterol creates a fluid liquid crystalline phase in lipid mixtures which would, in the absence of cholesterol, form separate crystalline and liquid domains (103-106).

Cholesterol might play an essential role in the insertion of lipids and proteins into the membrane and in controlling the activity of the different membrane enzymes.

A review of the function of sterols in membranes has been published recently by Demel and Kruyff (107).

CHAPTER IV: Transport

Due to its composition and structure, the membrane is highly impermeable to most polar and charged molecules. But as quoted by Racker (108) "with the privilege of isolation comes the reponsibility of communication". To cope with this responsibility the membrane is equipped with specific transport systems for the passage of solutes from one side of the membrane to the other, as well as surface receptor mechanisms to translate information from the external milieu to the internal environment.

Different Types of Transport

Transport of low molecular weight solutes across mammalian cell plasma membranes can be subdivided into three categories as follows:

Facilitated Diffusion

Facilitated diffusion is a process independent of metabolic energy. It does not lead to accumulation of the solute against an electrochemical potential, but to the equilibration of the solute across the membrane. This process is differentiated from simple diffusion by the fact that it is highly specific, temperature sensitive and much more rapid than a process of simple physical diffusion. It shows saturability and competition with specific structural analogs.

Exchange

Exchange is a special case of facilitated diffusion. The flow of one solute down its concentration gradient is linked to the flow of another solute (transported by the same system) in the opposite direction.

This type of transport can produce transient accumulation, but without any additional energy requirement other than the flow of one solute down its concentration gradient.

Active Transport

This definition of transport is used to described those systems where a substance is accumulated against its electrochemical potential gradient and where metabolic energy is associated in some manner with the translocation of the solute. The other characteristics of translocation are similar to those of facilitated diffusion. Chemical modification of the solute or of the transport system could also be involved in this type of mechanism.

For the purpose of this discussion, the term transport will be limited to a specific translocation of solute from one side of the membrane to the other, without any chemical intermediates of the solute formed during the translocation process. The discussion will also be limited to a description of the transport of organic solutes across eukaryotic plasma cell membranes.

Mechanism of Transport

How does a solute traverse from one side of the membrane to the other? A few models have been proposed but so far no one is able to describe the translocation process in molecular terms. Historically the first idea of metabolically linked translocation was phosphorylation of the solute during passage across the membrane as in the bacterial phosphotransferase system (109). Then it was shown by use of sugar analogs(110) that active transport does not involve chemical modification of the solute. Two models have been proposed to account for translocation without chemical alteration: the carrier model and the pore model.

The Carrier Model

Some of the earliest attempts to explain translocation considered that the carrier would be a small amphiphilic molecule which would bind the solute on one side of the membrane, diffuse through the bilayer and release the solute on the other side of the membrane (111).

This idea was reinforced by the discovery of the ionophores and their mechanism of action and specificity, as for example K^+ accumulation in the presence of valinomycin (111).

The fact that transport systems often show enzyme-like properties of high specificity and saturation kinetics has given rise to the idea that proteins are the carrier substances. Thus it was proposed that the carrier was a reversibly modified protein (111) and that between acceptance and release of the solute there was a transmembrane rotation of the whole molecule. Considering the structure of the membrane this process would require a prohibitive level of energy of activation.

The Pore Model

According to the pore model, the protein extending through the membrane forms a pore or channel. The surface of the pore provides points for sequential binding to complementary chemical groups of the migrating solute molecule in a highly specific manner, whether for passive or active transport. Accumulation would occur if the binding sites form an asymmetric chain or if energy is applied to the system in an asymmetric manner (112).

Recent Findings

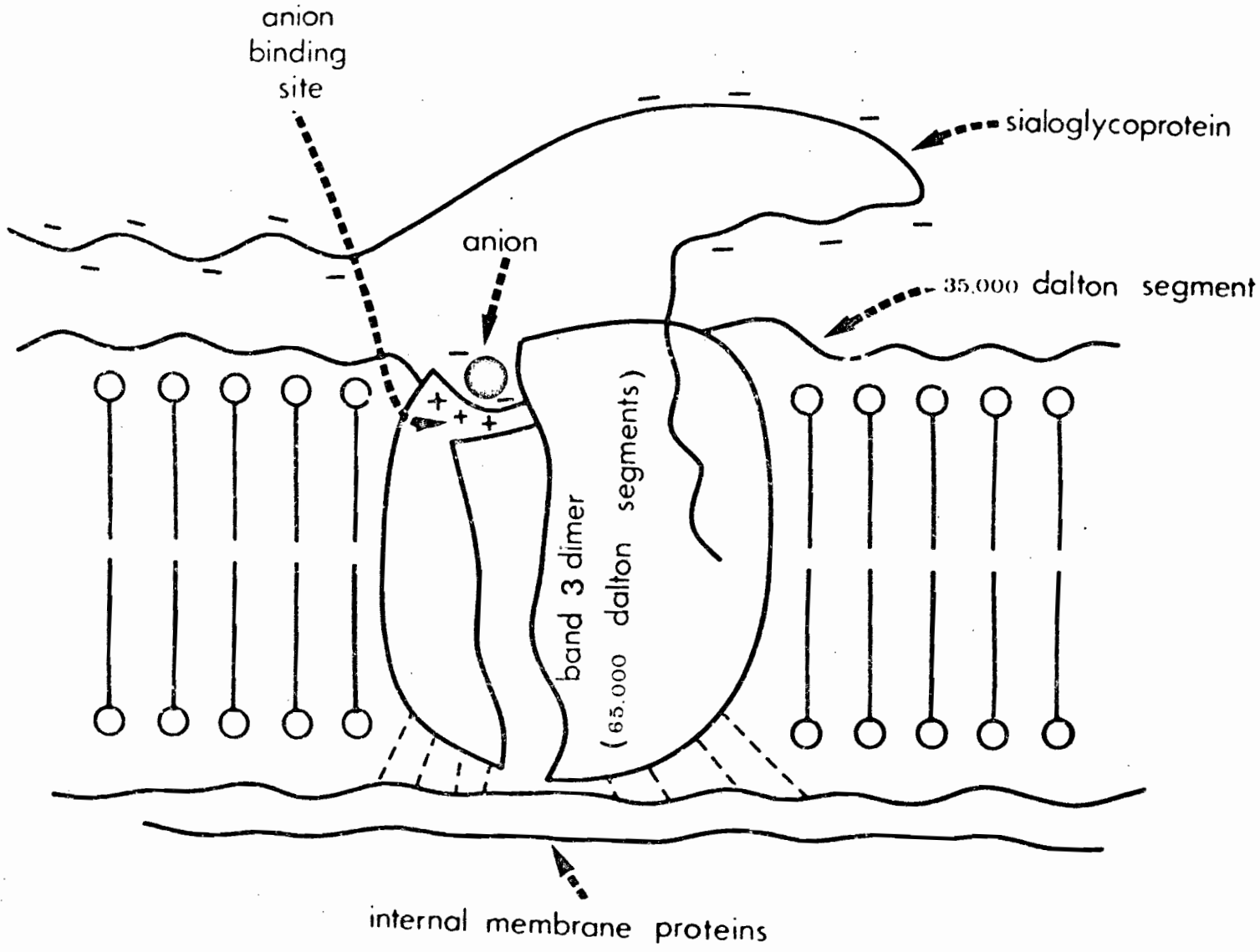
The answer to the question on how proteins carry out transport may lie between the two extreme models of the mobile carrier and the fixed pore.

A number of membrane transport systems have been isolated and characterized: these include the ($Na^+ + K^+$) ATPase of the plasma membrane from the kidney of dog (113), the rectal gland of the dog fish (114) or the electric organ of the electric eel (115), the (Ca^{++}) ATPase of Sarcoplasmic reticulum (116), the anion exchange protein of the red blood cell (117), the glucose transport system of the red blood cell (118), rhodopsin from vertebrate rods (119).

The existence of these isolated transport systems provides more information on solute translocation. These proteins all have the same fundamental characteristics (120). They are transmembrane glycoproteins They are embedded in a fixed asymmetric orientation in the bilayer. The glycosylation appears to be a special feature of eukaryotic cells. The best available model is the anion transport system of the human red blood cell (extensively studied by Rothstein et al. (117)), where transport is believed to be catalyzed through conformational changes in the protein.

The transport function is associated with the band 3 protein (so-called from the protein electrophoretic pattern on SDS gels). This protein acts as an anion permeation channel through the bilayer as visualized on Figure 4 . Near the outside surface of the membrane, the protein possesses the anion binding site which can undergo a local conformational change allowing a one for one anion exchange across the diffusion barrier.

Although the term mobile carrier is still used, the carrier is now believed to be part of a protein and its mobility is restricted to small distances within a fixed protein structure.


anion
binding
site
sialoglycoprotein
anion
35,000 dalton segment
band 3 dimer
(65,000 dalton segments)
internal membrane proteins

Energization of Active Transport

The characteristic of active transport is that osmotic work is done. Accumulation of a solute means transmembrane asymmetric distribution of the solute. In order to produce and maintain this asymmetry, metabolic energy is required. Different methods can be visualized to transduce this energy to the transport system. The energy can be applied by acting on the solute or on the carrier mechanism itself.

Energy Transduction to the Solute or Group Translocation

The solute is modified as it is transported, thus preventing its exit via the same solute specific carrier. This type of mechanism is widespread in the bacterial world and has first been described by Kundig et al. for E. coli (121). The best example is the phosphoenolpyruvate sugar phosphotransferase system, which has been extensively studied (109) and results in accumulation of phosphorylated derivatives at the expense of phosphoenolpyruvate, involving at least three membrane enzymes.

Energy Transduction by Carrier Modification

Several systems have been described in which the energy transduction occurs via a modification of the carrier itself. The best examples are the ($Na^+ + K^+$) ATPases of a variety of animal cells (113,115) and the (Ca^{++}) ATPase of sarcoplasmic reticulum (116).

In these systems, ion accumulation occurs as the result of a reversible phosphorylation of the carrier by ATP. The carrier exists in two forms, a phosphorylated and a dephosphorylated form, each of which has different ion binding affinities.

This hypothesis is referred to as the covalent hypothesis, ATP being used directly to produce an energized carrier.

Indirect Coupling or Coupled Flow Hypothesis

Energy and transport can be coupled via indirect mechanisms involving neither solute nor carrier modification.

This hypothesis was originally proposed by Crane (122). Studying active transport of glucose in the intestinal epithelical cell, Crane made the following observations. The process requires energy, and, in the presence of Na^+, free sugar accumulates in the cell. This accumulation is dependent on a component that pumps Na^+ out of the cell and which uses ATP. He concluded that "sugar absorption is coupled to a second and different energy dependent transport process such as Na^+ transport, and does not itself require a direct energy input".

It then appeared that this proposition affects a large number of systems for the transfer of organic solutes, the flow of solutes being coupled to Na^+ or H^+ depending on the type of cell. Na^+ and H^+ transport are directly linked to a primary metabolic event.

The Na^+-Gradient Hypothesis

As early as 1900, the effect of Na^+ on sugar uptake in small intestine was noted by Reid (123,124). In 1958, Riklis and Quastel (125) made the first report of a functional role for Na^+: Na^+ is essential for sugar transport. Its replacement by K^+ abolishes all active transport of glucose. Sugars which will accumulate required the presence of Na^+. Structural modifications leading to a loss of accumulation were also associated with a loss of the Na^+ requirements.

Subsequently Crane (126) proposed that Na^+ dependence was associated with the molecular basis of active sugar transport, and he ruled out chemical transformation of solute as a necessary part of the entry mechanism by using transported but non phosphorylatable sugars. He suggested the

formation of a transient complex, Na^+-sugar-carrier, which can traverse the membrane to the cell interior where dissociation occurs. Na^+ can then be extracted by an energy dependent process and the sugar would be trapped in the cell.

Dependence on Na^+ and dependence on active Na^+ transport appeared to be widespread phenomena for the transport of organic solutes (127) (amino acids, sugars...) and as stated by Schultz and Curran (128) may be characteristic of all organisms that require Na^+ in the extracellular medium.

Considering the asymmetric distribution of Na^+ between the inside (low Na^+ concentration) and the outside (high Na^+ concentration) of the cell, it was suggested that Na^+ asymmetry (129) could provide the energy required for the active transport of solutes. Furthermore a number of experiments showed that Na^+ transport was creating a potential difference varying directly with the Na^+ concentration (130) and that electro-neutrality was maintained by a passive Cl^- flux (130). On the other hand, active glucose transport was shown to stimulate Na^+ transport in the intestine and to be important in the determination of the transmural potential difference (131). Thus a link exists between Na^+ and glucose entry.

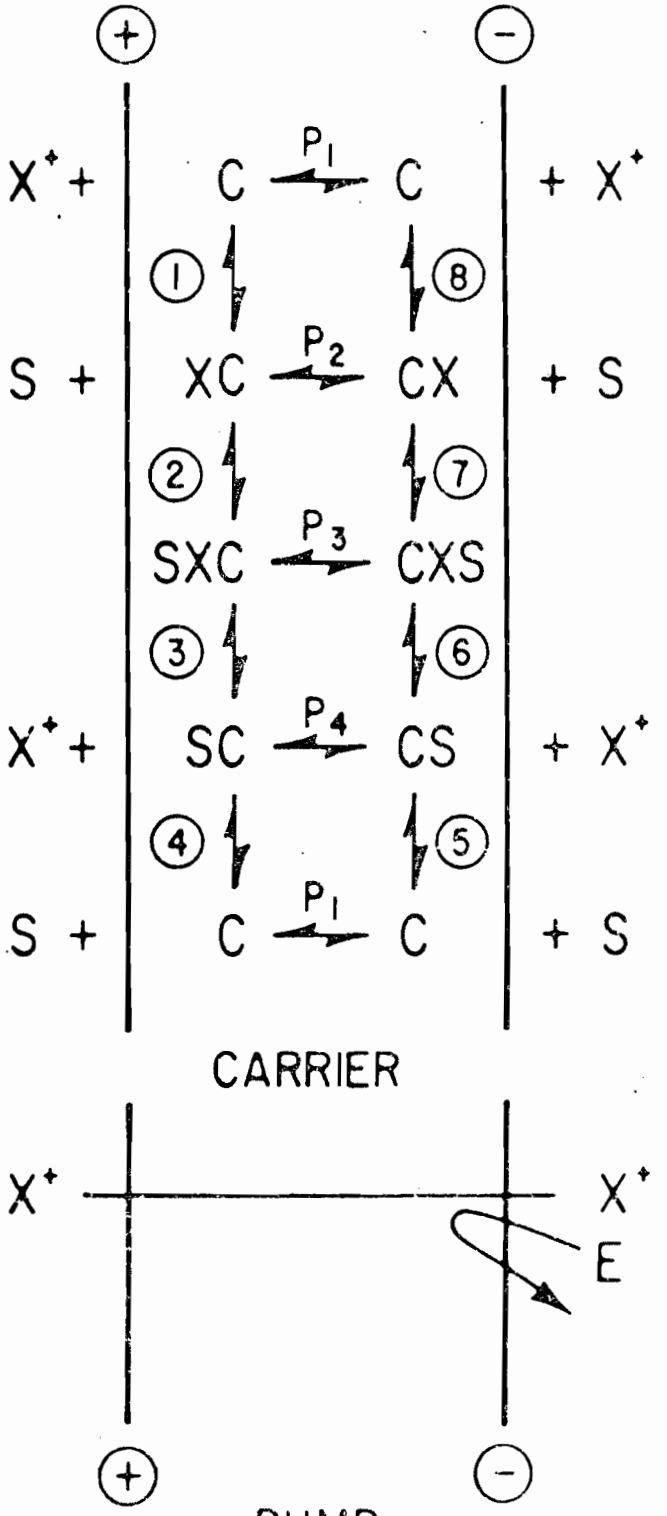
Experiments by Csaky (132) on the inhibitory effects of phloridzin and ouabain on both sugar transport and the (Na^+ + K^+) ATPase lead him to propose that "it is not the carrier which requires Na^+ for functioning, but part of the transport system which is responsible for the conversion of chemical energy into pumping energy"(133) and hence that the (Na^+ + K^+) ATPase could be considered the link between Na^+ and non electrolyte pumps (134). These conclusions of direct coupling between

the Na^+ pump and active sugar transport were in disagreement with Crane's propositions (122) in which a direct requirement for Na^+ by the solute carrier was predicted. The discovery that the Na^+ pump is located at the serosal boundary of the cell while the active sugar carrier is located at the mucosal boundary eliminated the possibility of direct coupling between the two functions. This fact strenghtened Crane's hypothesis of a Na^+ dependent carrier and indirect coupling between intestinal Na^+ and sugar transport.

Description of the Na^+ Gradient Hypothesis

The Na^+ gradient hypothesis is now accepted as a fundamental principle by which some cells are able to couple organic solute transport to the energy derived from the electrochemical potential gradient established by the Na^+ pump. In 1970, Schultz and Curran (128) proposed a refined model taking in account the effects of the movement of the carrier in any of its forms. This model is depicted in figure 5 .

The carrier C is totally unrestricted. It may translocate empty, with Na^+ ion or solute S or with both together. Effective coupling of the flow of S and Na^+ is achieved only through the mediation of the carrier form $C\text{-}S\text{-}Na^+$. If the flow is restricted to the form $C\text{-}S\text{-}Na^+$, then the coupling coefficient is 1 and the coupling efficiency is 100%. The carrier mediated entry of solute will continue until the degree of saturation of the carrier with solute at the inner membrane surfaces is equal, at which point solute influx and efflux rates equalize and a steady state cellular solute concentration is maintained. This model implies that the system is symmetrical, the only asymmetry being the solute and Na^+ concentrations on each side of the membrane.


+
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X⁺ +
C
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XC
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CXS
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6
X⁺ +
SC
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CS
+ X⁺
4
5
S +
C
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+ S
CARRIER
X⁺
X⁺
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PUMP

The second part of the figure represents the ATP driven Na^+ pump which maintains a Na^+ gradient. The role of the Na^+ ion is twofold; Na^+ is the cofactor and Na^+ gradient is the driving face of the system.

The Na^+ Ion as a Cofactor

The interaction of the Na^+ ion with the carrier can affect the affinity of the carrier for the solute, the mobility of the ternary carrier complex or both the affinity and the mobility. Therefore three types of gradient coupled carriers have been described and found experimentally: affinity (Km) type, velocity (Vmax) type and the mixed type (135).

The affinity type model in which the primary effect of Na^+ is to increase carrier affinity with unaltered mobility is the model of sugar transport in hamster (136) and rat (137) jejunum and rabbit kidney vesicles (138), and of amino acid transport for several systems: alanine transport in rabbit ileum (139), glycine transport in pigeon red blood cells (140), phenylalanine transport in rabbit kidney vesicles (141).

For the velocity type model, the order of binding of S and Na^+ is inconsequential, and the binding of either S or Na^+ does not increase the affinity for Na^+ or S. But translocation of the ternary complex or of the free carrier across the membrane barrier is much more rapid than translocation of either of the binary complexes. A transport system of this type is for example the transport of 3-O-methylglucose in rabbit ileum (142).

In the mixed type model, both affinity and mobility changes occur as, for example, glycine uptake in red blood cells (143), glycine transport in Ehrlich Ascites tumor cells (144).

The Role of the Na^+ Gradient

Active transport is dependent on the Na^+ gradient as the driving force. The transmembrane Na^+ gradient is the sum of the chemical potential for Na^+ and of the electrical potential difference $\Delta \psi$

$$\Delta \tilde{\mu}_{Na^+} = \Delta \psi + RT \ln \frac{[Na^+]_i}{[Na^+]_o}$$

The mobility of the Na^+-dependent carrier will be influenced by the magnitude of the Na^+ gradient, and, if charged, by the electrical difference.

The Role of the Na^+ Pump

Mammalian cells are equipped with a Na^+ pump which continuously pumps Na^+ out of the cell at the expense of ATP, thus maintaining a continuous flow of Na^+ outward.

Assuming the degree of coupling between the solute transport and the electrochemical potential for Na^+ to be 100%, the rate of solute transport will depend on the magnitude of the Na^+ gradient.

Predictions from this Model

In the ideal case, the following predictions can be made:

The unidirectional fluxes of both S and Na^+ are a function of the values of [S] and [Na^+] and of the potential difference $\Delta \psi$.

If both fluxes are completely linked and no other systems exist for translocation other than the coupled mechanism, Na^+ and S should translocate in the same stoichiometric ratio and no translocation of either should occur in the absence of the other.

When the Na^+ pump is working, the transmembrane electrochemical potential gradient for Na^+ can be maintained. The final state which the organic solute achieves, is a steady state, the level of which is a

function of $[Na^+]$ out, $[Na^+]$ in, and of the magnitude of the potential difference at steady state.

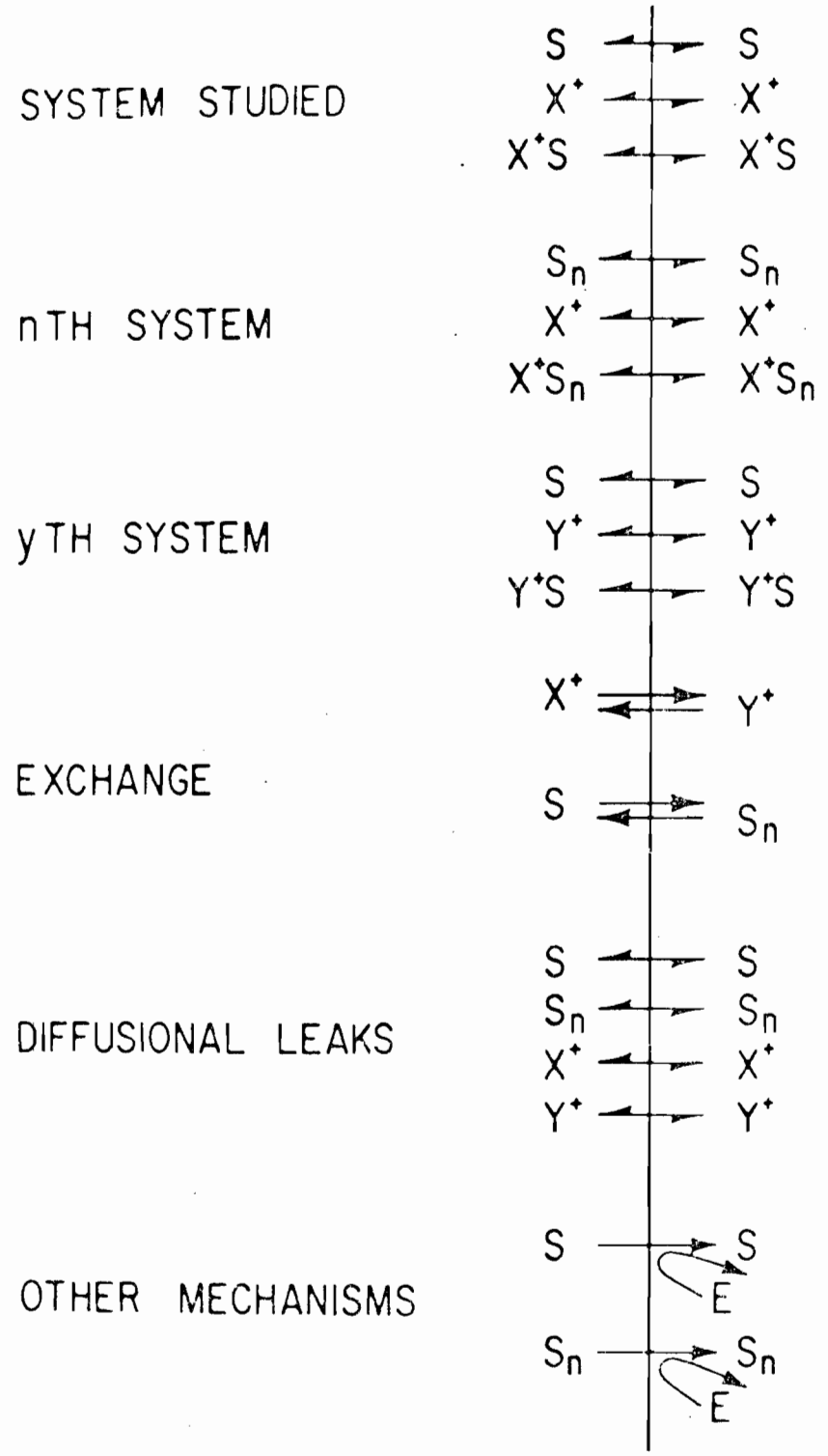
When the Na^+ pump is off, the cellular Na^+ gradient cannot be maintained, the final state is an equilibrium.

Coupling and Energy Transduction

The steady state accumulation achieved by a gradient coupled system depends on the degree of asymmetry of the system, the tightness of coupling and the leaks in the system. Crane (145) defined two types of leaks, the inner leaks whose importance depends on the proportions of the various forms of the carrier and their mobility, and the outer leaks due to the presence and interactions of other solutes and ions with the carrier and to the existence of other membrane pathways.

The outer leaks are described in figure 6 : the membrane has more than one gradient coupled system, the solute S may translocate by exchange diffusion, diffusion or by other active transport processes with energy transduction mechanisms other than gradient coupling. In the alanine transport system of rabbit ileum (145) the outer leak of Na^+ is 7 times the flux of Na^+ on the alanine carrier. A similar flow ratio for Na^+ has been established for the Ehrlich Ascites cell (146). Leaks caused by other Na^+ dependent transport systems have also been shown for glycine in rabbit reticulocytes (145) and pigeon red blood cells (147). Na^+ independent leaks occur by exchange diffusion as for example phenylalanine, cycloleucine or methionine leaks in the Ehrlich Ascites cells (148).

For all the reasons mentioned above, the coupling efficiency is unlikely to be 100%, and the coupling coefficient will be greater than 0, but smaller than 1.


SYSTEM STUDIED
S
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nTH SYSTEM
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EXCHANGE
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DIFFUSIONAL LEAKS
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OTHER MECHANISMS
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Energy Requirement

The energy adequacy of any driving device depends on two conditions: enough energy in the "driving system" should be available and the coupling between the driving process and the driven process should be tight enough to warrant sufficient transfer of this energy.

The degree of tightness for Na^+ coupled glycine transport has been examined by Heinz et al. (146) using the analytical principles of non equilibrium thermodynamics. They were able to show a fairly tight coupling between amino acid influx and Na^+ influx in intact cells (degree of coupling between 0.5 and 0.6).

The area that needs quantification is the amount of energy available for Na^+ coupled transport. Is the Na^+ gradient adequate or must there be an additional energy input?

For discussion purposes the Na^+ gradient can be presumed to consist of a chemical potential and of an electrical potential. A number of observations suggest that the chemical component of the Na^+ gradient is not sufficient.

1) the energy available from the chemical component of the Na^+ gradient can be calculated and cannot exceed the "ceiling value (149)" of $\frac{RT}{F} \ln \frac{[Na^+]_i}{[Na^+]_o}$, $[Na^+]_i$ and $[Na^+]_o$ representing the activities of Na^+ in the internal and external medium, the other letters having their usual meaning. Assuming a 1:1 stoichiometry between solute and Na^+ for AlB accumulation in the Ehrlich Ascites cells, the energy calculated from the above relationship is inadequate to account for the observed AlB accumulation.

2) Active transport can take place during complete inhibition of metabolism, as long as appropriate gradients of Na^+ and K^+ can be maintained (150), but ATP depleted cells are 3 times less effective than metabolically active cells despite very comparable chemical gradients of Na^+ (150,151).

3) Metabolically active cells can continue to accumulate amino acids while the chemical gradients of both Na^+ and K^+ are absent or even reversed (151,153).

4) Ouabain inhibits amino acid and sugar transport before there is any marked change in cellular Na^+ and K^+ concentrations (153,155).

The apparent inconsistency of the above data with the Na^+ gradient hypothesis can be resolved if there is an electrical component to the Na^+ gradient which is part of the driving force for organic solute accumulation. If the Na^+ pump is electrogenic, its contribution to charge separation will increase when pump activity increases, i.e. when cellular Na^+ is elevated. Therefore conditions when chemical gradients are reversed may be precisely the time of large electrical difference across the membrane. The rapid action of ouabain would also be consistent because it would be anticipated that electrical difference would decrease more rapidly than change in the bulk of cellular ions. Moreover Na^+ coupled transports are electrogenic, i.e. they alter the membrane potential and are sensitive to changes in the membrane potential.

A number of experimental observations are available which show that 1) the pump is electrogenic, 2) the Na^+ dependent transport of solute is electrogenic and 3) the membrane potential contributes to the driving force for solute transport:

1- When the cellular concentration of Na^+ is elevated (for example by a cold storage), the pump activity is increased, the electrical

potential is also increased and uptake of AIB in the Ehrlich Ascites cells is accelerated, even in the absence of a Na^+ chemical gradient (156).

2-In Ehrlich cells with inverted Na^+ and K^+ gradients, the pump operates at a more vigorous rate than normal, until physiological Na^+ and K^+ concentrations are restored. This results in a transient hyperpolarization of the cell interior as followed by the distribution of Tetraphenyl-phosphonium (TPP^+), a lipid soluble cation used to monitor potential difference changes (156).

3- Laris et al. (161) monitoring membrane potential with 3-3' dipropylthiocarbocyanine iodide, showed that amino acids which require Na^+ for transport cause a depolarization of the Ehrlich Ascites cells. When the cells contain normal Na^+ concentration, the Na^+ pump has no effect on the membrane potential and the addition of ouabain has no effect. But if the cellular Na^+ concentration is raised, the cells become hyperpolarized. The observed hyperpolarization requires external K^+ and is inhibited by ouabain (162).

4- Na^+ coupled sugar and amino acid influx across the luminal membrane of small intestine and renal proximal tubule bring about a transmembrane depolarization. By acting as Na^+ ionophores, Na^+ coupled flows tend to shunt the electrical potential difference (157,160).

5- Using a cyanine dye as a fluorescent probe of transmembrane potential difference, Philo and Eddy (163), showed that in the Ehrlich Ascites cells, valinomycin (which increases K^+ permeability) causes hyperpolarization of the potential difference and that in the presence of Na^+, glycine causes a decrease in the potential difference.

6- Treatment of K^+-loaded vesicles from membrane of small intestine (164,165) and proximal tubule (166) with valinomycin markedly accelerates

the rate of Na^+ coupled sugar and amino acid entry.

7- Similarly, treatment of K^+ loaded ATP depleted Ehrlich Ascites cells with valinomycin results in an increase in methionine accumulation (166). This effect is not inhibited by ouabain, but gramicidin which affects both Na^+ and K^+ permeabilities (167) abolished the increased uptake of methionine due to valinomycin by short-circuiting the membrane potential (166).

From all this experimental evidence, it can be concluded that the rate of transport and the steady level of sugar and amino acid accumulation are influenced by the electrochemical potential difference for Na^+ across the membrane. The contribution of the electrogenic Na^+ pump explains the effect of ouabain and the fact that optimal solute accumulation depends on the presence of ATP.

The energy available from the Na^+ electrochemical gradient can be expressed by the following equation (162):

$$E = \frac{RT}{F} \ln \frac{P_K [K^+]_o + (P_{Na^+} + P_c [S]_o)[Na^+]_o}{P_K [K^+]_i + (P_{Na^+} + P_c [S]_i)[Na^+]_i + A_{Na^+}}$$

P: permeability constants

$P [S] [Na^+]$ accounts for the increased Na^+ movement with solute

A_{Na^+} represents the electrogenic movement of Na^+ through the pump.

It is not clear yet, even after considering the contribution of the electrical membrane potential that the amount of energy available for Na^+ dependent transport of organic solutes is sufficient. Other energy sources might exist.

Recent studies of marker enzymes have indicated the presence of NADH dehydrogenase activity in plasma membrane preparations of liver and fat cells (169). Garcia-Sancho et al. (168) also observed that the electron donor phenazine methosulfate plus sodium ascorbate was able

to restore uptake of 2 methylamino isobutyric acid (Me- AIB), in ATP depleted cells in the absence of ion gradients. Quinacrine, which inhibits NADH dehydrogenase, completely suppresses the uptake of Me-AIB, even if ATP levels are maintained and ion gradients not depressed. This led the authors to conclude that "an oxidoreduction system in the plasma membrane can participate in energization of both Na^+-dependent and Na^+ independent amino acid transport, allowing the utilization of reducing equivalents which can be made available by shuttle from the mitochondria."

Organic Solute Transport in Plasma Membrane Vesicles

The use of plasma membrane vesicles has been introduced by Kaback (170) and is now very commonly used to study transport. In these simplified systems, ion sequestration is unlikely, little metabolism is expected, the interactions of ions and solutes with the cytoplasm are eliminated. It becomes possible to vary the composition of ions and solutes on each side of the membrane. But in these systems there is no ATP, and hence there is no active Na^+ pump. The only driving force is the applied Na^+ gradient

Intestinal Brush Border Membrane Vesicles

Hopfer et al. (171) were the first to obtain a membrane vesicle preparation from brush border membranes of small intestine, which showed D-glucose transport activity specifically stimulated by Na^+, inhibited by phlorizin and D-galactose. The vesicles also contain a Na^+-dependent L-alanine (165) and phenylalanine (164) transport system. Both systems are electrogenic.

As the vesicles lack an active Na^+ pump only equilibrium is expected.

However it was possible to observe a transient active uptake by imposing an artificial Na^+ gradient (172). An electrochemical potential difference for Na^+ was established by applying an inward downhill gradient of SCN^-, by increasing the conductance for K^+ by valinomycin from K^+ preloaded vesicles or by increasing the conductance for H^+ by carbonylcyanide p. trifluoromethoxyphenylhydrazone (CF-CCP) from H^+ preloaded vesicles. In each case the inside of the vesicle would become negative with respect to the outside and a transient overshoot of solute entry above the equilibrium level was observed.

More recently Mürer et al. (173) were able to isolate separately the luminal and the contraluminal plasma membranes. The luminal membrane vesicles possess the Na^+-dependent transport systems described above, whereas the contraluminal membrane vesicles possess a facilitated diffusion transport system for glucose.

Renal Brush Border Membrane Vesicles

Characteristics similar to those of the isolated intestinal brush border membrane vesicles have been described for the kidney brush border membrane vesicles. Electrogenic transport systems have been demonstrated for D-glucose (138,174), L-alanine (175), β-alanine (176), L-proline (177), L-phenylalanine (141), glycine (178).

Uptake of D-glucose is inhibited by the simultaneous flow of L-alanine. This inhibition is dependent on Na^+. The addition of L-alanine to membrane vesicles preincubated with D-glucose causes an efflux of D-glucose. Monactin and valinomycin prevent these interactions (179). This suggests that amino acid and sugar transport are coupled electrically.

Pigeon Red Cell Membrane Vesicles

Lee et al. (180) prepared membrane vesicles from pigeon red cells showing glycine uptake similar to that observed in intact cells. Glycine uptake requires Na^+ and is dependent on glycine concentration.

Ehrlich Ascites Tumor Cell Membrane Vesicles.

Colombini and Johnstone (181) prepared plasma membrane vesicles from the Ehrlich Ascites cells. This preparation contains an exchange diffusion system for amino acids and a Na^+ dependent amino acid uptake system (182). Influx of AIB can be stimulated by an inward Na^+ chemical gradient or a valinomycin induced K^+ efflux (183). Gramicidin, which dissipates the Na^+ gradient inhibits the uptake of AIB. Evidence of a two fold accumulation of AIB in the absence of Na^+ gradient and the inability to stimulate AIB efflux by reversed Na^+ gradient, suggests another source of energy than the Na^+ gradient alone.

Ghosts from Sheep Reticulocytes

Benderoff et al. (184,185) identified a Na^+ insensitive, ATP independent transport system for histidine, methionine, leucine and phenylalanine, and a Na^+ dependent transport system for glycine which was sensitive to the membrane potential. They also showed that upon maturation both activities were lost.

Vesicles from Virus-Transformed Fibroblasts

A number of reports have described the uptake of amino acids in simian virus 40 transformed fibroblast vesicles.

Quinlan et al (186) demonstrated a Na^+ stimulated uptake of L-leucine and AIB. L-leucine uptake was shown to be saturable and strongly inhibited by L-valine; L-alanine and glycine had very little effect.

Hamilton et al. (187) studied AIB uptake and their results indicated two kinetically independent transport systems for AIB: one with a low apparent affinity for AIB which is Na^+ independent, and the other with a high affinity for AIB which is Na^+ dependent. The action of Na^+ was shown to be an alteration of the Km value.

More recently Lever (188) made an extensive study of the effect of Na^+ and membrane potential on amino acid uptake (alanine, glycine, AIB). Accumulation of amino acids could be driven either by an electrical potential difference imposed across the membrane in the presence of Na^+ or by an imposed chemical difference in Na^+ concentration alone. An electrochemical Na^+ gradient and an interior negative membrane potential contribute additively to the driving force for amino acid accumulation.

CHAPTER V: Approach to the Identification of the Membrane Components Involved in Transport

Several approaches have been used to detect the membrane protein components involved in transport. Although numerous examples are found in the literature, only a few of them are cited here:

- Protein labelling procedure (69-74,189). If the transport system under study is the prominent function of the membrane, one can assume that this transport system is represented by a large number of protein molecules. In this situation, iodination is a good and easy method.

- Differential protein (190-193) labelling. Labelling is performed in the presence and absence of the substrate to be transported. By analysis of the labelled proteins in each situation, it is possible to determine the protein substrate "carrier".

- Affinity labelling (194-197). Using a derivative of the substrate which does not translocate, but which binds to the carrier, attempts have been made to identify the membrane components involved in transport. These derivatives become particularly useful if a covalent bond can be made between the protein and the derivative or if the dissociation constant is very low. The dansyl derivatives used by Kaback (198) are an example of this type of approach.

- Extraction of the membrane with various reagents like enzymes (199), chaotropic agents, solvents, detergents, protein perturbants, anhydrides...

After treatment of the membrane with the reagent, the activity of the remaining material is tested. This method has been very successful in helping to identify the carbohydrate transport protein in red blood cells (228).

- Reconstitution experiments. These experiments involve complete solubilization of the membranes followed by reconstitution of the membrane from all or part of the solubilized components into a liposome. We have initiated studies on identification of the transport components by determining whether reconstitution is a viable approach with amino acid transport systems of Ehrlich Ascites cells.

Membrane Solubilization

As already mentioned, the membrane is composed largely of lipids and proteins. Its structure is believed to be a lipid bilayer with proteins partly or totally embedded in it. Solubilization means disruption of the membrane by breaking the electrostatic and hydrophobic bonds, and dispersion of the different components.

Criteria of Solubilization (200)

Complete solubilization means dissociation of the membrane into its basic units. The criteria usually used to define solubilization are operational rather than absolute. Optically clear solutions and one sharp Schlieren peak on analytical centrifugation, do not necessarily mean true dispersion. Other operational criteria have to be introduced as follows:

- a) No sediment is formed on centrifugation at 100,000 g for at least 1 hour.
- b) No membranous structure is detectable under the electron microscope.
- c) The soluble material is also retained by Sepharose 4 B after equilibration of the resin with detergent or solubilizing agent.
- d) The soluble material penetrates a 5% polyacrylamide gel

equilibrated with the solubilizing agent.

Solubilizing Methods

Mechanical and chemical methods have been used to solubilize membranes.

Mechanical methods(7): Sonication is a method often used for membrane disruption, but this method does not lead to the formation of a homogenous solution. It is a difficult method to control and reproduce and often leads to a loss in enzyme activity.

Chemical methods:

Organic solvents (201): The behaviour of organic solvents towards membranes is dependent largely on their balance of polar and non-polar characteristics. If a solvent is relatively polar (e.g. glycerol) there is little possibility of non-polar interactions. Likewise highly non-polar solvents (e.g. hydrocarbons) are incapable of polar interactions and normally not useful for membrane dissolution. The most effective solvents, such as chlorinated or hydroxylated hydrocarbons, lie between the two extremes. Some solvent extraction procedures are often most effective at slightly alkaline or acid pH's. But extraction of the most polar lipids (cardiolipin or polyphosphoinositides) sometimes require extremes of pH.

Solvents usually separate the lipids from the proteins, the lipids entering the solvent phase. As the lipids are removed, and because of the low dielectric constant of the solvent, electrostatic interactions between the proteins are enhanced and conformational changes occur often, leading to inactivation. Thus the usefulness of solvents for isolation of membrane components is limited.

Chaotropic agents: Chaotropic agents are able to break the water structure and in doing so increase the water solubility of non-electrolytes (202,203). Chaotropes cause protein release from the membrane and leave a lipoprotein residue. Among the chaotropic agents which have been used are: SCN^-, ClO_4^-, I^-, haloacetates, urea, guanidine. For example, succinate dehydrogenase has been extracted from beef heart mitochondria with $NaClO_4$, 0.4 M (204).

Detergents: With all the agents described above, the environment of the proteins is perturbed so that the chances of denaturation are very high. A new class of solubilizing agents which is more efficient and less likely to denature, the mild detergent, has been introduced for membrane solubilization.

Other agents: Many other agents can be used to try to solubilize membranes (7,32,205-207): chelating agents, which deplete the membranes of the bridging ions, alkali and salts which weaken the electrostatic bonds, solutions of low ionic strength which also deplete the membrane of the bridging ions, solutions of high ionic strength which lead to a decrease in electrostatic stabilization, changes in pH which lead to changes in ionization and hydrogen bonding. But these agents are only able to bring the peripheral proteins into solution. The bulk proteins are not solubilized.

Detergents (208)

If solubilization must be accomplished without disruption of the native structure, the solvent medium must be able to simulate the native environment of the proteins at the lipid/aqueous interface. Like lipids, detergents are amphipathic molecules. They possess a non-polar structure

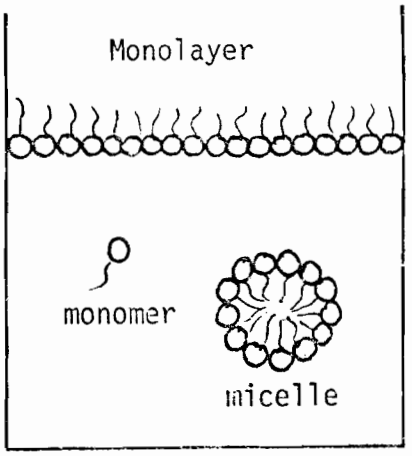
with some polar groups. Consequently they are more likely to preserve membrane component integrity than organic solvents or ions.

Classification of detergents: Detergents fall into two classes depending on the charge of their polar heads.

Ionic detergents: The ionic detergents can be represented by sodium dodecylsulfate (SDS) or the bile salts like sodium cholate. An example of a cationic detergent is given by tetradecylammonium bromide.

Nonionic detergents: A few examples of these detergents are given in table II.(208)

Physical properties of detergents: When small quantities of detergents are added to water, part dissolve as monomers and part form monolayers at the air/water interphase. When the monomer reaches a critical value, added detergent begins to associate to form micelles. The driving force of the aggregation is hydrophobicity. The interior of the micelle consists of the hydrophobic groups which are sequestered from the water by the polar groups covering the surface of the micelle. Micelles are thermodynamically stable colloidal aggregates.

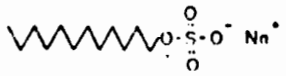


The concentration at which micelle formation occurs is the critical micellar concentration (CMC).

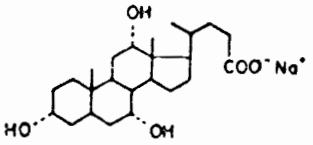
The CMC values decrease as the hydrophobic character of the detergent increases. The ionic detergents thus have higher CMC values than non-ionic detergents. The CMC values of both ionic and non-ionic detergents can be

TABLE II

Anionic detergents

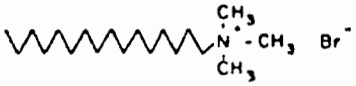


Sodium dodecylsulphate



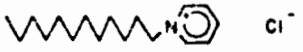
Sodium cholate
(trihydroxy bile salt)

Cationic detergents



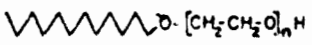
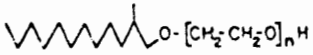
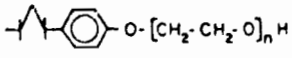
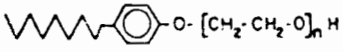
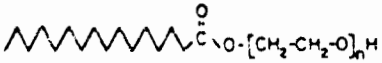
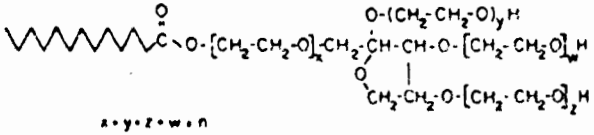
Cetyltrimethylammonium bromide

Tetradecylammonium bromide



Dodecylpyrimidinium chloride

Non-ionic surfactants

 Polyoxyethylene alcohol	**Brij series, Lubrol W, AL series**
 Polyoxyethylene isoalcohol	Sterox AJ, AP series Emulphogen BC series Renex 30 series
 Polyoxyethylene p-t-octyl phenol	Triton X series Igepal CA series Nonidet P 40
 Polyoxyethylene nonylphenol	Triton N series Igepal CO series Surfonic N series
 Polyoxyethylene esters of fatty acids	Sterox CO series Myrj series
 Polyoxyethylene sorbitol esters*	Span series Tween series Emasol series

decreased by increasing the salt concentration (reduction of electrostatic repulsion between the head groups). pH has little effect on micellar properties of non-ionic and ionic detergents with strongly acidic or basic groups. However lowering the pH to 6.5 precipitates the bile salts. Micelle formation will occur only above the critical micellar temperature which coincides roughly with the melting temperature of the hydrocarbon chains. Micelles can be considered homogeneous in size. The mean micellar size and shape depend on the characteristics of the detergent molecule and on the experimental conditions. Usually low CMC values correspond to high micellar weight whereas high CMC values correspond to low micellar weight, with the exception of bile salts which form small micelles.

Another parameter assigned to detergent is the hydrophilic lipophilic balance (HLB). This parameter represents the ratio of hydrophilicity and lipophilicity in a detergent. It derives from the distribution ratio of the molecule between hydrocarbon oil and water. If the HLB is higher than 7, the detergent is more soluble in water than in oil.

Mechanism of solubilization: It is generally believed that the monomeric form and not the micellar form of the detergent binds to the membrane. The binding of detergent monomers to the membrane prevents the formation of pure detergent micelles by reducing the free detergent concentration below the CMC, so that micelles of detergents do not occur together in the presence of intact membranes.

The extent of solubilization depends mainly on the amount of detergent bound and can therefore be correlated with the proportion of detergent bound to the membrane. The lower the CMC of the detergent and the higher the concentration of the membrane, the closer will be the

total detergent to membrane ratio to the bound detergent to membrane ratio. Consequently for evaluation and reproduction of solubilization it is necessary to indicate both the detergent and the membrane concentration.

The following figure (figure 7) (11) describes what happens when increasing concentrations of detergent are added to a membrane.

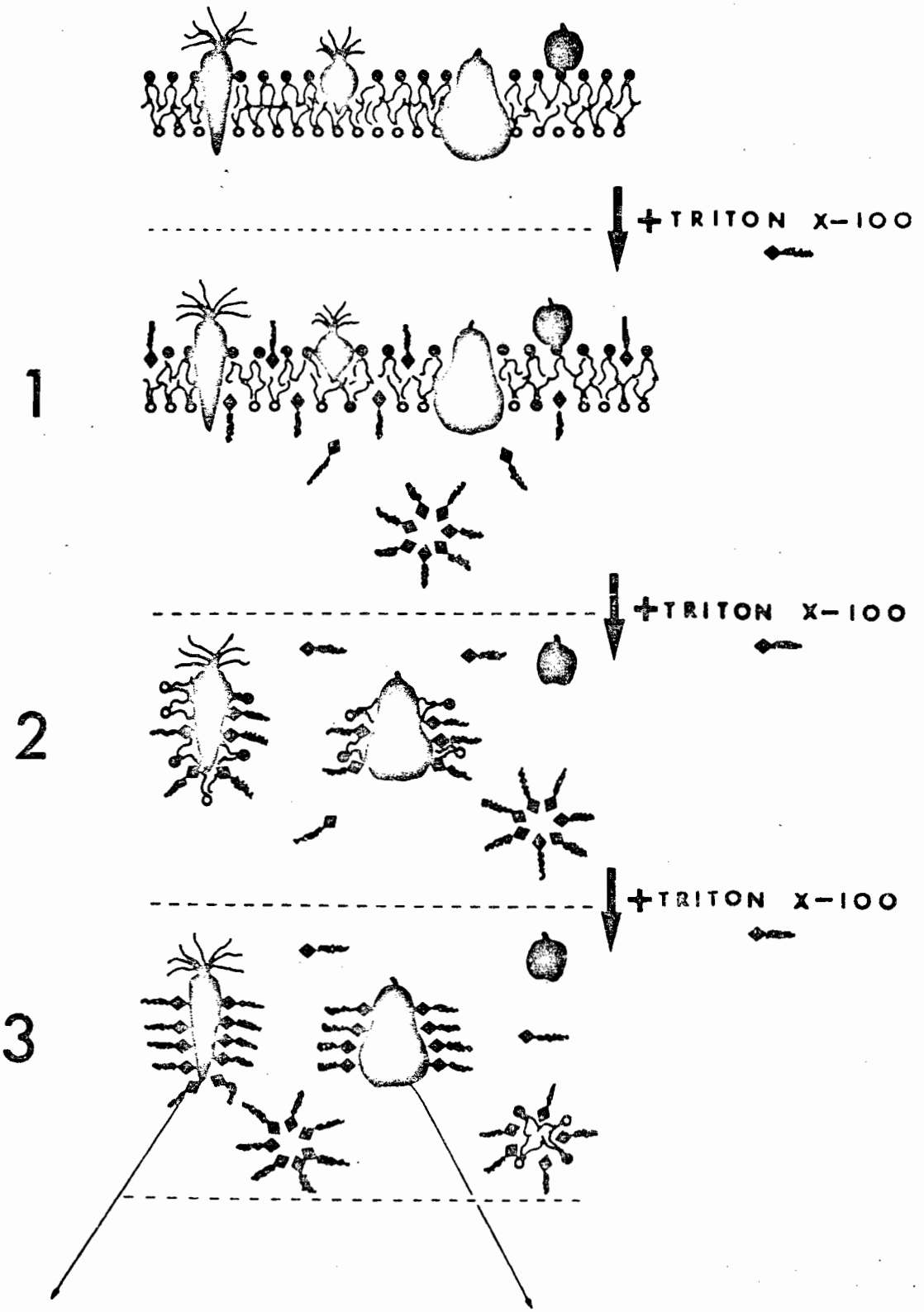
Step 1: when a small amount of detergent is present, the molecules of detergent are incorporated into the membrane, without breaking it.

Step 2: when more detergent is added, the membrane is solubilized into the micellar solution containing mixed protein-lipid detergent micelles in equilibrium with detergent micelles and free detergent molecules.

Step 3: when enough detergent is added, pure protein detergent micelles are obtained in equilibrium with detergent lipid and detergent micelles. The membrane is completely solubilized into its individual components.

Interaction of membrane proteins with detergents: Helenius and Simons recently (208) reviewed the interactions between proteins and detergents. Detergents can be classified as "mild" and "denaturing".

Ionic detergents are frequently considered denaturing agents, the best example being SDS (sodium dodecyl-sulfate). Different levels of affinity characterize the binding of SDS to proteins. Native protein molecules have a small number of high affinity binding sites for all detergents, but when the concentration of SDS is increased and all the high affinity binding sites are saturated, binding to other sites occurs. This binding is cooperative and involves a conformation change of the protein by which bur ied hydrophobic sites become exposed. Thus, with SDS two types of SDS protein - complexes can form: one containing 0.4 g SDS per g of protein at a free SDS concentration between 5.10^{-4} and 8.10^{-4} M,


+TRITON X-100
1
+TRITON X-100
2
+TRITON X-100
3

and one containing 1.4 g SDS per g of protein at a free SDS concentration above 8.10^{-4} M. Most membrane proteins are denatured by SDS, and it is possible by increasing the ionic strength to reduce the CMC for SDS, thus reducing the possibility of denaturation. Some enzymes have been shown to be resistant to denaturation by SDS (alkaline phosphatase from liver cell plasma membrane, phospholipase A of E. coli cell membranes).

Non ionic detergents and bile salts do not induce cooperative modes of binding with accompanying denaturation. Triton-X-100 and DOC (deoxycholate) only bind to high affinity binding sites; in fact they are very inefficient in breaking protein-protein interactions. Most proteins preserve their quaternary structure in the presence of high concentration of Triton X-100 or DOC. Most activities are usually maintained, the exceptions being $(Na^+ + K^+)$ ATPase, Ca^{2+}-ATPase, cytochrome oxidase, glucose-6-phosphatase, and hormone responsiveness of adenylate cyclase.

Choice of a detergent: A few rules can be drawn from all the above considerations, remembering that a prime consideration is the restoration of activity when the detergent is removed:

- If phospholipid simulation is not important, there are practical advantages is choosing a detergent that forms small micelles since the amount of detergent bound to the protein will be small and will not interfere with the physical characterization of the protein as for example molecular weight determinations.

- Ionic detergents are more likely to denature proteins than non ionic detergent or bile salts.

- If the CMC of the detergent is low, the binding of the detergent to the proteins may be very strong. This accounts for the observation that ionic detergents are easier to remove than non-ionic.

Study of Protein in Detergents

Isolation of a membrane protein involves the separation of a given protein-detergent micelle from another protein-detergent and lipid-detergent micelle. Methods for separation are based on density differences(density gradient centrifugation), charge differences(ion exchange columns) and micelle dimensions (gel filtration). The most selective method is affinity chromatography, which is widely used for the isolation of membrane receptors. A review on characterization of membrane proteins in detergent solutions has been written recently by Tanford and Reynolds (209).

Membrane Reconstitution

Reconstitution of a single enzyme function provides a method for the investigation of the lipid requirement of the afore mentioned enzyme in situ and the details of the interaction of the enzyme with its membrane environment at the molecular level. It is a method which can yield information on the insertion of proteins into preexisting membranes. It is a unique approach to deduce the mechanism of action and control of ion pumps, and transport systems at the molecular level. A number of methods for reconstitution have appeared in the literature and are summarized below.

Reconstitution is carried out with the solubilized membranes or components isolated from membranes to which it may be necessary to add exogen ous lipids or to reincorporate the proteins into a preexisting vesicle or bilayer. If detergents are used to prepare the proteins, reconstitution is not complete until detergent is largely removed.

Reconstitution by Detergent Removal Technique

This is usually accomplished by dialysis or gel filtration.

Dialysis is not applicable to non ionic detergents, because they form very large micelles and are tightly bound to the proteins. Gel filtration using Biobeads or Biogel A-50 m has become the method of choice. The membrane components appear in the void volume and the detergent remains on the column. But gel filtration is not applicable if the starting material consists of isolated membrane proteins in detergent, because lipids and divalent cations have to be added for the formation of functional membranes.

With detergents which can be removed by dialysis, the method of choice is to add, to the proteins dissolved in the detergent, lipids also suspended in the same detergent, and to dialyze away the detergent in a buffer containing the divalent cations. The advantage of the dialysis method is a high degree of reproducibility suitable for comparative studies as for example, the effect of phospholipid composition. The disadvantage is that dialysis is slow (10-20 hours) and that inactivation might occur during this long process. Many systems have been reconstituted by this method: NADH-CoQ reductase (210), site 3 of phosphorylation (211), cytochrome oxidase (212,213), proton pump with bacteriorhodopsin (214), light stimulated uptake by vesicles containing cyt c oxidase and bacteriorhodopsin (215), Pi-ATP exchange (216,217), ($Na^+ + K^+$) - ATPase showing active K^+ transport coupled to Na^+ transport, from the rectal gland of Squalus acanthias (218), alanine carrier from thermophilic bacterium PS3 (219), Ca^{++}-ATPase from solubilized sarcoplasmic reticulum (220,221), glucose transport system from adipocyte plasma membrane (222).

Reconstitution by Sonication

The method as described by Racker (223,224) consists of suspending dried phospholipids in a salt solution containing detergent extracted membrane proteins, and exposing the mixture to sonic oscillation. Major advantages are: the reconstitution by sonication is performed in the absence of detergent and the speed of reconstitution. The vesicles often have higher activity than those obtained by dialysis. The ATP-dependent Na^+ pump reconstituted by sonication of the ATPase from the electric eel and pure phospholipids shows a rate of translocation of Na^+ 10 to 20 times the rate obtained from vesicles reformed by the dialysis method (225).

The major disadvantage of the method is the variability due to the difficulties of controlling sonication. Moreover, optimal sonication times depend on the phospholipid composition, so that comparative studies are limited. Some proteins, like cytochrome oxidase, are quite sensitive to sonic oscillation. Among the systems reconstituted by the sonication method are: Pi-ATP exchange by the oligomycin sensitive ATPase (223), the Ca^{++} pump from the sarcoplasmic reticulum (223,226), the proton pump with bacteriorhodopsin (223), Na^+ dependent D-glucose transport from brush border membranes of rabbit kidney cortex (227), D-glucose transport catalyzed by a protein fraction from human erythrocytes (228,229) and from hamster small intestine (230), adenine nucleotide transporter from bovine heart (231).

Reconstitution by Cholate Dilution Procedure

This method has also been introduced by Racker and it avoids both sonication of the membrane and dialysis (232) to reform the vesicles. Phospholipids are sonicated either in the presence or absence of cholate

and mixed with the membrane protein at a final concentration of about 0.7% of cholate. After an incubation period, the sample is diluted at least 25-fold.

By this method, Racker and his coworkers successfully reconstituted the Ca^{++} pump with rates twice those obtained from vesicles reformed by the dialysis method, and the Pi/ATP exchange (232).

This method is very rapid and reproducible and high activities are obtained. However systems requiring drastic conditions of dissociation may not be responsive to this reconstitution procedure. This method also shows that detergent removal does not necessarily need to be complete, and in a more extreme case, cytochrome oxidase from mitochondria solubilized by Triton X-100 is reconstituted by decreasing the ionic strength without any change in the detergent concentration.

A functional disadvantage of the 3 proposed procedures is that there is no possibility of controlling the orientation of the proteins during the reconstitution process.

Incorporation of Proteins into Preformed Liposomes

In earlier work, Eytan et al. (233) used liposomes containing lysolecithin (10% of total phospholipids). Lysolecithin was used to facilitate the incorporation of various mitochondrial proteins into the liposomes. By this method the authors reconstituted cytochrome oxidase activity, complex III., Pi-ATP exchange, but attempts to incorporate Ca^{++}-ATPase from sarcoplasmic reticulum were not successful. The disadvantage of this method is the fact that it involves high concentrations of lysolecithin which may affect the properties of the lipid bilayer.

The same group pioneered the incorporation of membrane proteins in liposomes containing acidic phospholipids (234) instead of lysolecithin (cardiolipin, phosphatidylinositol or phosphatidylserine). This procedure leads to unidirectional incorporation of the enzyme tested (cytochrome oxidase, aQH_2-cytochrome c reductase, oligomycin-sensitive ATPase). Phospholipid specificity and requirement can be easily studied. The effect of the presence of one protein in the liposome on the incorporation of additional proteins has also been investigated, as well as the selectivity of the process by incubating the proteins with a mixture of two liposome populations differing in either lipid composition or protein content. The presence of another membrane protein in the liposomes either increases or decreases the affinity of a protein to the liposomes (ATPase is preferentially incorporated into cytochrome oxidase vesicles, while cytochrome oxidase is preferentially incorporated into protein free liposomes).

Thus there are many considerations in selecting an approach for reconstitution. The choice of the technique for reconstitution will depend on the starting material, whole membranes or isolated proteins, stability of the protein, lipid requirements etc. Depending on the technique used, the amount of phospholipid used and reincorporated will vary and the activity of the system reattained will vary with the method used.

Mechanism of Reconstitution

When membrane vesicles are reconstituted from their constituents, one observes a formation of membrane de novo (235-237). When the detergent is removed, reconstitution of a membrane is spontaneous because of the low CMC of the phospholipids and because the bilayer configuration is

energetically favored in aqueous solutions. The problem is to restore the original configuration. The binding of the proteins to the lipids may be expected to be the same as in the native membrane if no alteration of the protein occurs in the solubilization process. What directs the proper orientation is unknown. More experiments on incorporation of proteins into preformed liposomes may shed some light on this question. In most of the cases to date removal of detergent by dialysis or gel filtration, or sonication methods to reform vesicles, a mixed population of inside out and right side out vesicles is obtained.

PURPOSE OF THE WORK

In order to understand the process of transport, the essential components involved must be characterized. Transport systems are located in the cell membrane, but if the general composition of the membrane is now well documented, very little is yet known about the exact arrangement of its components.

It is thus necessary to develop an assay for the transport proteins which can be used along the purification procedure and the characterization of the system.

As transport of organic solutes in mammalian cells does not involve any chemical transformation of either the solute or the carrier, only the translocation of solute from one side of a membrane to the other can be observed. To measure transport activity, the isolated transport proteins have to be incorporated in a system which mimic s the original membrane vesicles.

Transport of amino acids is only one of the numerous activities of the plasma membrane of these cells,the proteins involved represent only a small percentage of the membrane proteins.

Thus labelling techniques cannot be visualized. Binding assays are generally not successful because binding affinity constants are generally too low ($Kd > 10^{-6}$ M).

The purpose of this research is to solubilize plasma membranes from the Ehrlich Ascites tumor cells and to reconstitute transport activities similar to that of the original membranes from the solubilized material.

The purpose is to show that the components are stable enough to withstand dissolution and that characteristic functional activity can be

obtained. This is a first step to identify the specific components and elucidate the molecular basis for transport.

METHODS

Maintenance of the Ehrlich Ascites Tumor Cells

The cell line was obtained from the Institut du Cancer de l'Hopital Notre-Dame in Montreal. The cell line was maintained by weekly intraperitoneal injection of cell suspension into male swiss white mice using 0.4 ml of ascitic fluid per mouse.

Preparation of Plasma Membranes from Ehrlich Ascites Tumor Cells

The method described here is a modification of the method of Colombini and Johnstone (181). All centrifugations were done at 4°C. Seven days after injection of the tumor, 20 mice were killed by cervical dislocation. The cells were immediately aspirated from the mouse and collected into two centrifuge bottles containing 200 ml of 0.9% NaCl and a drop of heparin to prevent clotting. The cells were centrifuged at 900 g for 2 minutes in the Sorvall RC-3 and then washed at least 4 times in 0.9% NaCl using centrifugation periods of 1 to 2 minutes. Before the last centrifugation, a known aliquot of the cell suspension was centrifuged in a graduated tube at 1,400 g to determine the packed cell volume. To the washed cells was added ten cell volumes of 1 mM $ZnCl_2$. The cells were kept for 10 minutes at room temperature followed by 10 additional minutes in an ice bath to obtain swollen cells. Under phase contrast the plasma membrane appeared dark and well differentiated while the cytoplasm appeared condensed around the nucleus. The cells were ruptured using a polytron homogenizer PT-10-20 at a setting of 3. Lysis was followed by examining aliquots of the cell suspension every minute under phase contrast microscopy to determine the percentage of

broken cells. When 80 to 90% of the cells were lysed the homogenization was stopped. Further homogenization caused nuclear damage and led to contaminated membranes. The bulk of nuclei and unbroken cells were removed by centrifuging the cell homogenate for 1 minute at least 3 times at 900 g in the Sorvall RC-3. The resulting supernatant was then layered on top of 4 sucrose step gradients each consisting of: 250 ml of sucrose 30% (w/w), 100 ml of sucrose 35% (w/w), 100 ml of 40% (w/w) sucrose 200 ml of sucrose 45% (w/w) and 50 ml of sucrose 60% (w/w). All sucrose solutions contained 0.5 mM $ZnCl_2$ and 0.5 mM phosphate buffer, pH 7.4. The gradients were centrifuged for 35 minutes at 3,800 g in Sorvall RC-3. During this centrifugation the remaining nuclei and unbroken cells were pelleted to the bottom of the gradients. After centrifugation, the layers were scanned using phase contrast microscopy. The membranes were found mostly in the 45 and 40% sucrose layers. The membrane fractions were collected, pooled and diluted with cold distilled water to 1.5 times their volume. EDTA, 0.1 M, was added to a final concentration of 0.66 mM to chelate the zinc ions. The membrane suspension was then transferred to 250 ml polycarbonate centrifuge bottles and centrifuged in a Sorvall RC-2B using the GSA rotor at 16,300 g (10,000 rpm) for 30 minutes. The pellets were suspended in 15% DMSO. The pellets were scanned for remaining nuclei by examining samples under phase contrast. The nuclei were removed by centrifugation on a table top Sorvall SS-34 rotor with an input voltage of 50 (5,000 rpm) for 1.5 minutes. The centrifugations were repeated until less than four nuclei were detected in 100 fields scanned. The membranes were finally centrifuged at 12,000 g for 15 minutes in the SS-34 rotor of the Sorvall RC-2B at 4°C. The membranes were suspended in a known volume of 15% DMSO and stored at -18°C, where they can retain

their activity for about two months.

Solubilization of Plasma Membranes

1- Cholate 2% - urea 4 M mixtures.

This method is a modification of Kagawa and Racker's procedure for solubilizing membrane components (216). The reaction mixture contained: membranes 1-2 mg of protein/ml, Na-cholate (pH 7.4) 2%, urea 4 M, EDTA (pH 7.4) 0.1 mM, Tris-HCl (pH 7.4) 5 mM, NaCl 100 mM. The membranes were suspended in a solution containing all the above components except Na^+-cholate. Sodium cholate was added last, drop by drop, to avoid any local high concentration of cholate at any time. The mixture was stirred for 30 minutes at 4°C. During this period the turbidity of the suspension almost completely disappeared. After solubilization, the mixture was centrifuged at 145,000 g for 2 hours in a Beckman L5-50 ultracentrifuge using the type 65 rotor. A small yellowish pellet of insoluble material formed. The supernatant was designated as the "solubilized fraction".

2- Triton X-100 - urea 2 M.

The procedure of Garewal and Wasserman (238) was used. The reaction mixture contained the following components: Tris-HCl (pH 7.4) 5 mM, NaCl 100 mM, EDTA (pH 7.4) 0.1 mM, Urea 2 M, Triton X-100 2% and membranes whose concentration was adjusted so that the ratio Triton/protein (w/w) was equal to 4. The mixture was stirred at 4°C for 60 minutes and then ultracentrifuged as described above.

Preparation of Phospholipids for Reconstitution

The lipids (40 mg/ml) were suspended by sonication under N_2 at 4°C in cholate, 2%, urea, 4 M, EDTA, 0.1 mM until clear (circa 5 minutes). The suspension was then centrifuged in an Eppendorf microfuge for 20 minutes

to pellet any undispersed lipids and the metal particles from the sonication probe.

Reconstitution of Membrane Vesicles

1- Recovery of vesicles from membranes solubilized in cholate-urea.

A suspension of lipids was added to the solubilized plasma membranes to give a final concentration of exogenous lipids of 1 mg lipid per mg of protein. The mixture was placed in a dialyzing tube (1 cm dry flat width). Dialysis continued for 22 hours at 4°C with at least 100 volumes excess of buffer (100 mM NaCl, 5 mM Tris-HCl pH 7.4, 0.1 mM $MgCl_2$, 0.1 mM $CaCl_2$) containing 10 mM phenylmethylsulfonylfluoride, a protease inhibitor. The buffer was changed once, after the first 4 hours of dialysis. During the dialysis period, the removal of the detergent led to the formation of insoluble material which was first centrifuged at 12,000 g for 30 minutes. Subsequently the reformed membranes were centrifuged in the Eppendorf microfuge for 3 minutes.

2- Recovery of vesicles from membranes dissolved in Triton X-100-urea 2 M.

Two approaches have been tried to obtain functional vesicles from Triton dissolved membranes: a) Triton X-100 was first removed from the solubilized membranes by treatment with Biobeads SM-2 for two hours in the cold with constant stirring. As much as 70 mg of Triton can be removed per gm of Biobeads. The biobeads were then removed by centrifugation at 2000 rpm. Lipids (1 mg/ml final concentration) were added to the supernatant which contained the membrane proteins. The mixture was sonicated at 4°C for 10 minutes. After centrifugation in the Eppendorf microfuge for 5 minutes, a white pellet was obtained.

b) Lipids dispersed in Triton X-100 - 2 M urea were added to the solubilized membranes. Then the mixture was added to the Biobeads to remove the Triton.

Uptake Measurements

Native and reconstituted membrane vesicles were handled in the same way to measure uptake and efflux.

Sealing: To seal the vesicles and to preload them with the desired solute, the vesicles were preincubated at 37°C for 30 minutes in a medium containing: 100 mM NaCl, 5 mM Tris-HCl, pH 7.4, 0.1 mM $MgCl_2$, 0.1 mM $CaCl_2$ and a protein concentration of 10 mg of proteins per ml of native membranes or 5 mg per ml of reconstituted membranes. After 30 minutes, the vesicles were transferred to 20°C to measure uptake.

Incubation: The reaction was started by the addition of the radioactive solute. 5 µl of radioactive solute (circa 1.6×10^6 cpm/µl) was added per 100 µl of incubation medium. 50 µl samples were taken at intervals, injected into 2 mls of ice cold Na^+ standard medium, pH 7.4, filtered under vacuum on a glass fiber filter, grade 934 AH (Reeve Angel), washed with 10 mls of ice cold standard Na^+ medium, dried under an infra-red lamp and finally counted in glass vials containing 10 mls of scintillant (5 gm PPO, and 50 mg of dimethyl-POPOP per liter of toluene).

A correction for non-specific retention was always determined by injecting an aliquot of preincubated membranes (50 µl) into a tube containing two mls of ice cold standard sodium buffer and 2.5 µl of radioactive solute. Immediately after mixing, the content of the tube was filtered and washed as described above.

Calculations: Transport activity is expressed either as moles of substrate taken up per milligram of membrane protein per unit time or as microliter equivalents of medium taken up per milligram of protein per unit time. The latter expression represents the microliters of medium cleared of substrate and is a convenient way of normalizing the data from different experiments using a variety of ^{14}C solute concentrations of different specific activities. It is calculated by the following equation: $\mu liter/mg = \frac{\text{cpm per mg protein on the filter}}{\text{cpm per } \mu\text{liter medium}}$

The amount of substrate taken up is calculated by multiplying the microliter equivalents of medium by the concentration of the substrate in the particular experiment.

Efflux Measurements (reconstituted vesicles)

Efflux experiments were conducted at room temperature. After loading of the vesicles with the radioactive solute, a sample was taken to determine the content of label in the vesicles. Then aliquots of 50 µl of membranes were diluted into 2 mls of medium containing: 100 mM chloride salt, 5 mM Tris-HCl, 0.1 mM $MgCl_2$, 0.1 mM $CaCl_2$, at room temperature. After different periods of time, the contents of the tubes were filtered, washed with the basic sodium medium, and handled as described above.

Results are expressed as percentage of initial solute remaining in the vesicles.

Space Measurements

$^{22}Na^+$ was used as a marker of intravesicular volume. This solute is readily taken up and in the absence of ATP there should be no active transport of Na^+. Therefore the distribution of Na^+ in the vesicle

could be carried out with solutes containing two different labels.

SDS Polyacrylamide Gel Electrophoresis (SDS PAGE)

SDS PAGE was performed according to Laemmli (239). The proteins were heated for 5 minutes at 100°C in 1% SDS. They were then applied on top of a 3% stacking gel over a running gel of 7.5%. The current applied was 3 mA/tube. The electrode buffer was maintained at 10-12°C during the electrophoresis to prevent curvature of the protein bands. When the tracking dye reached the last 1/2 cm of the running gel, the electrophoresis was stopped. The gels were fixed, stained with Coomassie Blue and destained according to Fairbanks, Steck and Wallach (240). Stained gels were scanned at 540 nm using a Gilford Linear Transport, a Beckman DU Spectrophotometer and a Fisher Recordall Recorder.

Analytical Polyacrylamide Gel Electrophoresis: 4 M urea, 2% cholate, pH 8.3.

A sample of solubilized membranes was layered on top of a 3%, 2.7% crosslinked stacking gel containing: 0.375 M Tris-HCl, pH 8.3, 2% cholate, 4 M urea and 1 µl/ml TEMED. The running gel was of 5% polyacrylamide containing the same components and the same degree of crosslinking as the stacking gel. The electrode buffer contained: urea 4 M, 2% cholate, 10 mM Tris, 77 mM glycine at pH 8.3. The electrophoresis was conducted at room temperature for 20 hours with a current of 6 mA/tube.

The gels were fixed, stained with Coomassie Blue and destained according to Fairbanks, Steck and Wallach.

Electron Microscopy

1- Thin sectioning.

Small aliquots of membranes were fixed in suspension with an equal volume of half - strength Karnovsky fixative (241) and pelleted. The pellets were fixed overnight at 4°C with the same fixative, then post-fixed for 1 hour in 1.33% osmium buffered with collidine and finally block stained for 1/2 hour in saturated aqueous uranyl acetate.

The pellets were dehydrated in graded ethanol and embedded in Vestophal W (242). Ultrathin sections were cut on an LKB Ultratone III using glass or diamond knives. Sections were mounted on copper grids with carbon coated collodion support film. Reynold's lead citrate (243) was used for 1 minute to stain the sections.

2- Negative staining.

The method used is based on the procedure of Brenner and Morne (244). Droplets of membranes in suspension were deposited on collodion and carbon coated grids and allowed to adsorb for 1 to 5 minutes. Excess material was removed by touching the droplet with the edge of a filter paper. A drop of 1-2% phosphotungstic acid pH 7.2 neutralized with 1 N KOH was then put on the grid and stained for 1 minute, and the excess stain was again removed as above.

3- Freeze-etching.

A sample of reconstituted vesicles was centrifuged at 50,000 g at 4°C using a SW 39 rotor. A hard pellet was formed, which was dehydrated in 25% glycerol for 2 hours. The pellet was cut and small specimens were placed on gold disks. These disks were subjected to rapid freezing in liquid Freon, then in liquid N_2. Fracturing of the frozen specimen

was done at -150°C under vacuum, etching was carried out for 1 minute at -100°C. The exposed surface was shadowed with a platinum and carbon layer (20 Å) and coated with a 200 Å layer of carbon. The organic material was digested away by floatation in hypochlorite solution; the replicas were washed by floating on water and placed on grids.

All the grids from the above preparations were examined in a Phillips 300 Electron Microscope operated at 60 KV using a 20 or 30 nm objective aperture. Micrographs were recorded on Kodak Electron image plates. Magnification was calibrated using an E.F. Fullam carbon grating replica of both 28,000 LPI or 54,684 LPI cat ≠ 1000 or ≠ 1002 respectively.

Preparation of ^{32}P Labelled Intact Plasma Membranes

^{32}P(0.17 mC/mouse) was injected into the peritoneal cavity of mice 48 and 24 hours before harvesting the cells. Membranes from these ^{32}P labelled cells were prepared in the usual way.

Chemical Determinations

1- Proteins.

Proteins were measured according to the method of Lowry et al. (245). When the urea concentration was above 1% urea, the standard curve had to be prepared in the presence of urea at the same concentration as in the samples, since it is known that high urea levels give some reaction with the Lowry reagents. To measure proteins solubilized in Triton X-100, Biuret method (246) was the method of choice, since the phenoxy groups of the Triton interfere with the Phenol reagent.

2- Phospholipids.

Lipids were extracted in methanol chloroform (2:1) (v/v) according to Bligh and Dyer (247). They were then hydrolyzed overnight at

150-160°C in 10 N H_2SO_4. Two drops of H_2O_2 were added and the heating was continued for another 1.5 hour until the solution was clear. The phosphorus released was measured according to Bartlett's procedure using the Fiske-Subbarow reagent (248).

3- Sialic acid.

Sialic acid was measured by the thiobarbituric acid method of Warren (249). Total sialic acid was measured after hydrolysis for 1 hour at 80°C in 0.1 N H_2SO_4. Neuraminidase-accessible sialic acid was measured according to the procedure of Cassidy et al. (250).

ATPase

The ouabain sensitive Na-K ATPase is determined according to Colombini and Johnstone using 250 µM $^{32}_{\gamma}$P-ATP (181).

Materials

Cholic acid was purchased from Eastman Kodak, N.Y. Prior to its use it was recrystallized from ethanol 70% according to Kagawa and Racker (216).

Purified phospholipids were obtained from Gibco (Grand Island, N.Y.) and Calbiochem (La Jolla, Calif.).

L-α-Lecithin from soybean was obtained from Sigma Biochemicals (St-Louis, Missouri) and asolectin from Associated Concentrates (Woodside, N.Y.). Prior to use, these phospholipid mixtures were subjected to a purification step according to Kagawa and Racker (216). They were then kept at 4°C under N_2.

Radioactive materials were purchased at NEN (Boston, Mass.). All other chemicals came from local dealers (Fisher Scientific mainly)

or from Sigma Biochemicals (St-Louis, Missouri).

Abbreviations

AIB	α-amino isobutyric acid
TEMED	N,N,N',N'-tetramethylethylenediamine
EDTA	Ethylene-diamine-tetracetic-acid
PAGE	Polyacrylamide gel electrophoresis
ATP	Adenosine triphosphate
EAC	Ehrlich Ascites cells
SDS	Sodium dodecyl sulfate
ATPase	Adenosine triphosphatase
NEM	N-ethylmaleimide
3-OMG	3-O-methylglucose
BSA	Bovine serum albumin
PC MBS	P. chloromercuribenzene sulfonic acid
IR	Infrared spectroscopy
NMR	Nuclear magnetic resonance
ESR	Electron spin resonance

RESULTS

CHAPTER I: Assessment of Solubilization

The first attempt to solubilize the membranes using 2% cholate brought only 40% of the proteins into solution. The addition of urea at 4 M doubled the amount of protein that was brought into solution. Since the degree of dispersion is not known, solubilized material was defined as material not sedimented by centrifugation at 145,000 g for 2 hours.

Based on the intact membranes, it was found that 80 to 90% of the proteins, 70% of the phospholipids and 100% of the sialic acid were present in the supernatant after ultracentrifugation for 2 hours at 145,000 g.

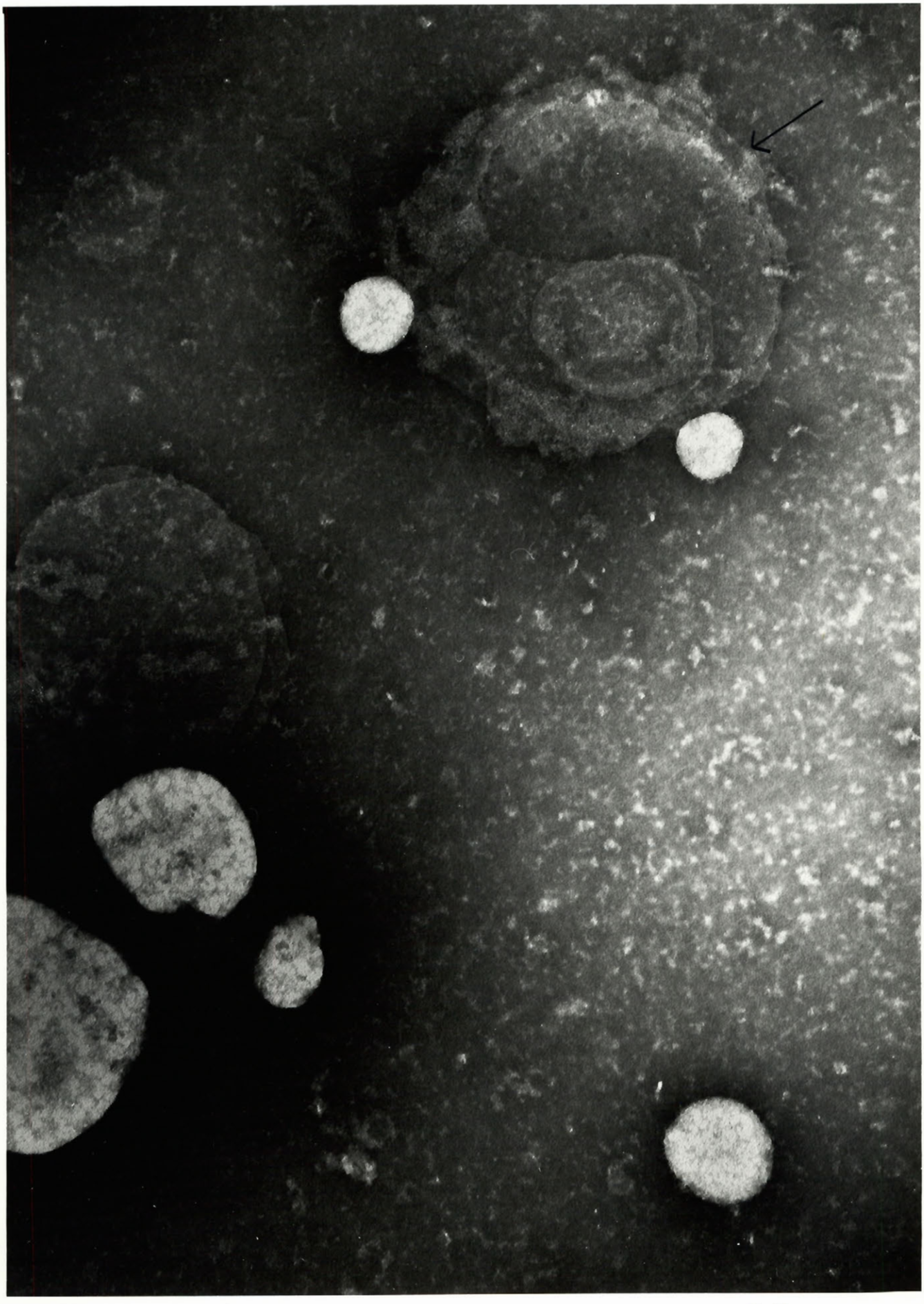
As noted above, the material which comprises the supernatant of the 145,000 ultracentrifugation may still contain large aggregates of material. The lipids can act as "life preservers keeping the proteins afloat" (236). Therefore additional tests were carried out to estimate whether large complexes ($> 10^6$) remained in the supernatant.

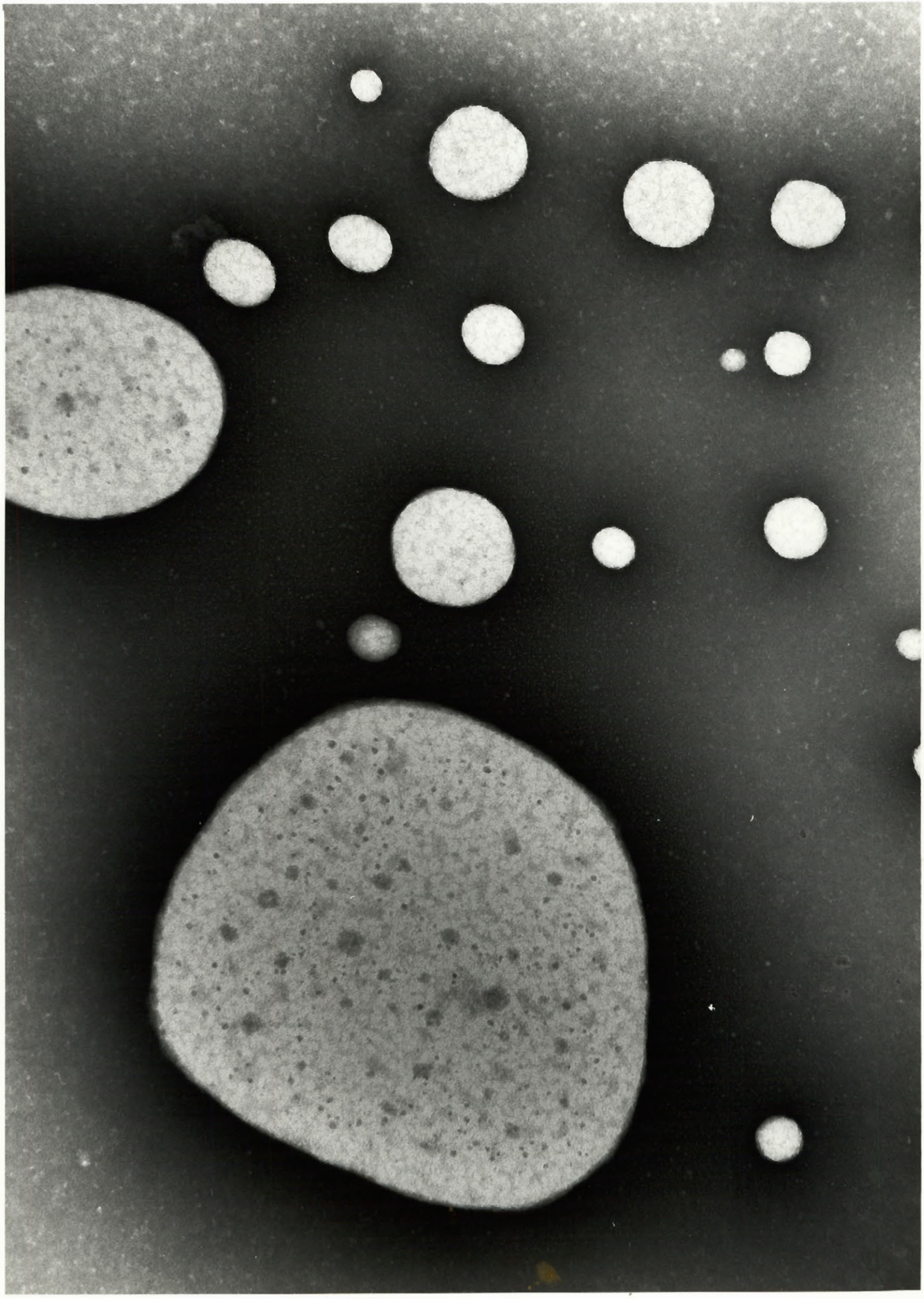
Negative Staining

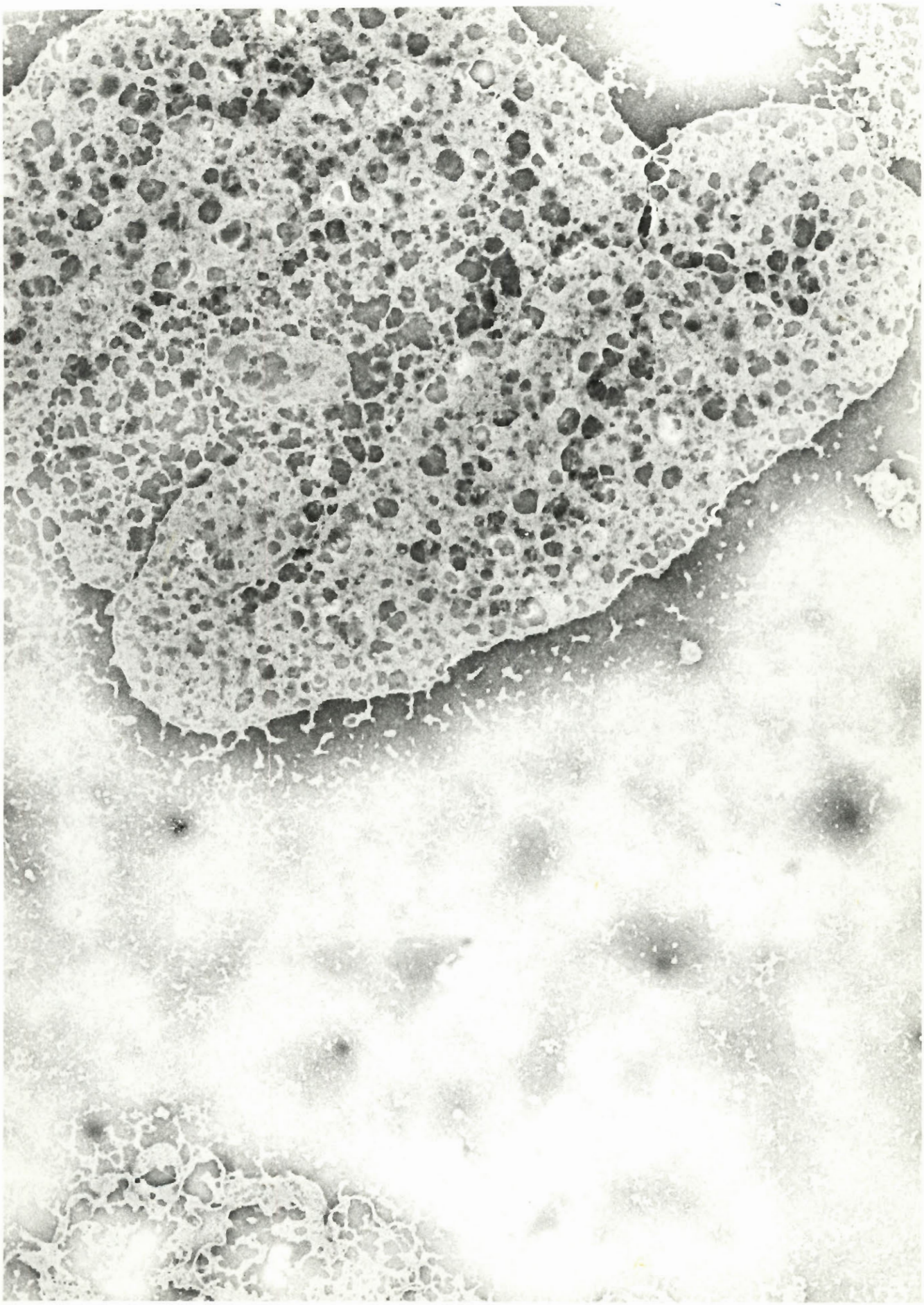
Examination of the supernatant under the electron microscope after negative staining failed to show any structural material . The non centrifuged material show white very homogeneous disks which probably represent lipid or lipoprotein droplets (Figure 8). The appearance of this material is very different from that of the vesicles. If the sample of solubilized material was kept in the cold or frozen, the size of these droplets increased enormously (Figure 9) but a freshly prepared sample showed only very small droplets. For comparison, intact membranes negatively stained are also presented (Figure 10).

Figure 8. Negative staining of non-centrifuged material

Membranes were treated with 2% cholate-4M urea for 30 minutes at 4°C. The arrow points to the residual membrane vesicle. The other vesicles represent liposomes. Magnification is 32,700X.







Penetration into Acrylamide Gels

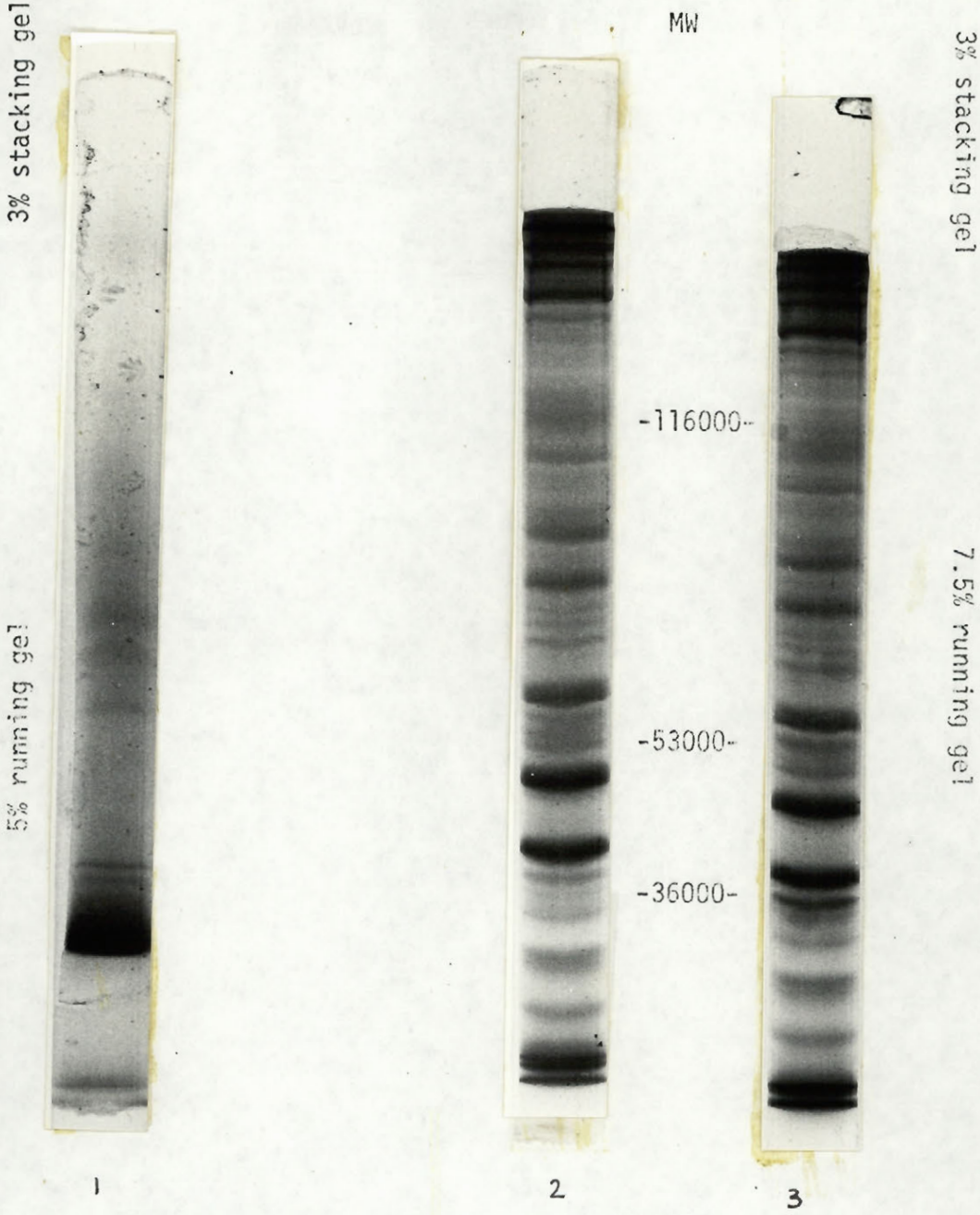
Another criterion of the degree of solubilization is the ability to penetrate a 5% polyacrylamide gel in cholate-urea. First runs were done in the cold room (4°C) for a few hours (7 hours, until the dye reached the bottom of the tubes). Only a fraction of the material was able to penetrate the gel. Subsequent electrophoresis was carried out at room temperature for 20 hours in a PAGE containing 2% cholate 4 M urea. As shown in figure 11 most of the supernatant had penetrated the 5% gel. Only a very faint band appeared on top of the stacking gel. In the 5% gel several bands are detectable. Near the interface in the 5% gel, little resolution had occured. It was necessary to run these gels for extended times because little material penetrated the 5% gel in the first three to four hours, presumably because there was little net charge in the proteins at pH 8.3 in absence of SDS. It is possible that in the 20 hours run some of the low molecular weight membrane proteins were lost. Attempts to estimate the degree of disaggregation by carrying out the electrophoresis in the cold or for shorter periods of time were not successful. In the cold, little material penetrated the gel presumably because lipid globules seen under the electron microscope reformed and caused clogging.

The ability of the cholate-urea proteins to enter the 5% gel suggests that the particle molecular weight is below 10^6 daltons.

In the studies conducted, the proteins were derived from cholate-urea solubilized membranes. However, the PAGE protein scans were done of membranes directly dissolved in SDS. Since there may be some loses of specific proteins by the cholate-urea procedure, the proteins solubilized by cholate-urea were subjected to SDS electrophoresis and compared with

membranes directly solubilized. It was apparent that the distribution of the protein bands is nearly identical (figure 11).

FIGURE 11



Gel 1: analytical PAGE in 2% cholate - 4 M urea, pH 8.3, at 20°C, of the solubilized membranes. The gel contains 120 µg of protein. The gel was stained with Coomassie Blue.

Gel 2 and 3: SDS PAGE of native (1) and reconstituted (2) membrane vesicles. The membranes were dissolved in 1% SDS. Each gel contains 170 µg of protein. The gels were stained with Coomassie Blue.

CHAPTER II: Reconstitution of Osmotically Active Membrane Vesicles

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Reconsitution procedure

1- Membranes solubilized in cholate-urea.

A sonicated dispersion of lipids in cholate-urea was added to the solubilized fraction of the membranes. The detergent was removed by dialysis in the cold and insoluble material reformed spontaneously. It is interesting to note that during a period when the membrane preparation was badly contaminated with mitochondria, reconstitution of vesicles was poor. Any insoluble material that reformed was difficult to centrifuge.

To examine the possibility that some uptake of amino acid might be due to contamination with microorganisms the reformed vesicles were plated on agar. No colonies were formed, indicating the absence of bacterial contamination. In addition the presence of penicillin in the reconstitution medium did not alter the reformation of the vesicles nor their properties.

To minimize oxidation of lipids and proteins during reconstitution, dithiothreitol or mercaptoethanol was added. Neither sulfhydryl compound altered the reformation of vesicles. Tests with ^{14}C cholate showed that over 99% of the cholate was removed during dialysis. The content of cholate in the reconstituted vesicles was estimated to be 0.008%.

2- Membranes dissolved in Triton X-100-urea 2M.

With membranes dissolved in Triton X-100_urea 2M, reconstitution cannot be obtained by the dialysis method, since it is difficult to

remove Triton by dialysis. This is probably due to its high molecular weight and low critical micellar concentration. It was not possible to obtain material able to take up AIB.

Characteristics of the reconstituted material

1- Chemical composition.

The reconstituted vesicles have protein/lipid ratios similar to those of the original membranes, namely 68/32 (w/w) for the intact membranes and 66/34 (w/w) for the reconstituted membranes.

To determine what percentage of the cells' own phospholipids were reincorporated into the vesicles, mice were injected with ^{32}P and used for preparation of membranes. The data showed that 60% of the phospholipids of the reconstituted vesicles came from the original phospholipids. All the sialic acid appeared to be reincorporated into the reconstituted vesicles. They have a higher content of sialic acid than the original membranes, 70±16 nmoles of sialic acid per mg of protein for the reformed vesicles as compared to 18±2 for the original membranes.

2- Protein profile of native and reconstituted vesicles.

Since only a fraction of the original proteins is reincorporated into the vesicles, the question arises whether the protein distribution in the reformed vesicles was identical to that of the original material or whether some proteins were enriched, others deleted, in the reconstituted vesicles.

For this purpose, the original and reconstituted vesicles were dissolved in 1 % SDS and then subjected to SDS PAGE using a 7.5% gel. Figure 12 and 13 show the spectrophotometric trace of the stained proteins. It is apparent from the scans that the major protein bands

Figure 12. Polyacrylamide gel electropherogram of SDS dissolved membrane vesicles

The gel was scanned at 540 nm using a Gilford linear transport attached to a Beckman UV spectrophotometer. For preparartion of gels see figure 11.

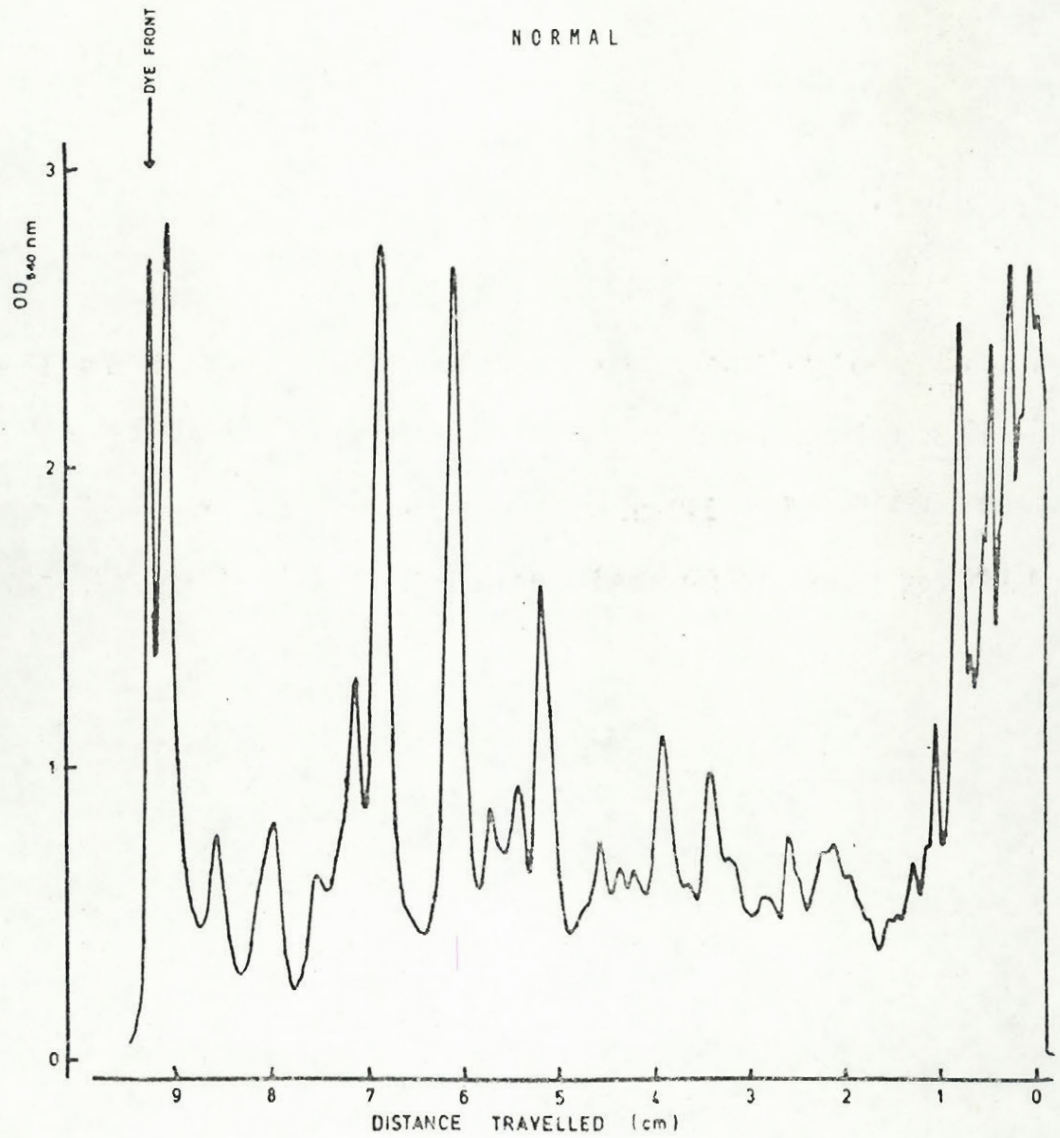
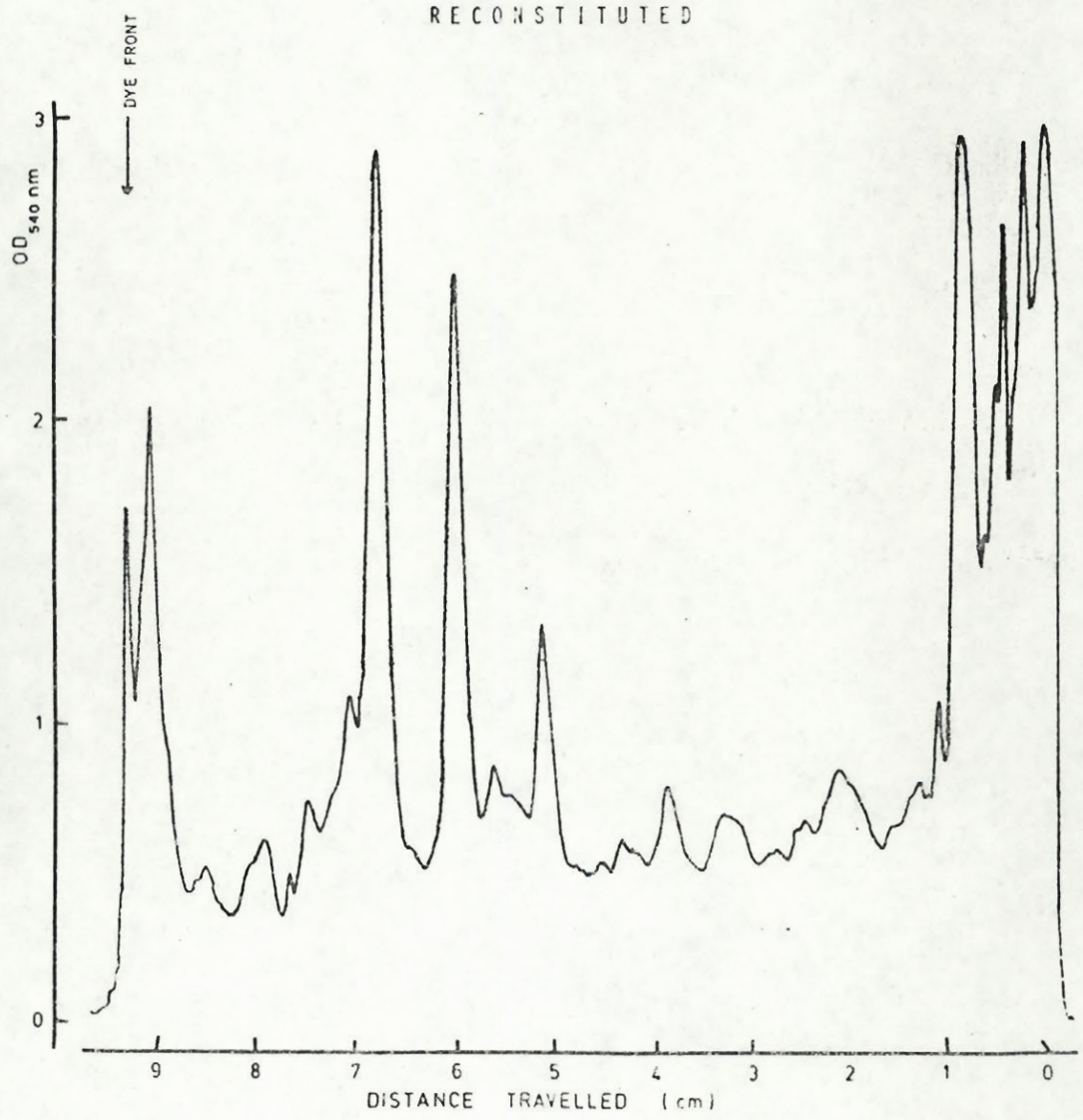

NORMAL
DYE FRONT
$OD_{840\,nm}$
3
2
1
0
9
8
7
6
5
4
3
2
1
0
DISTANCE TRAVELLED (cm)

Figure 13. Polyacrylamide gel electropherogram of SDS dissolved membrane vesicles

See figures 11 and 12 for conditions.


RECONSTITUTED
DYE FRONT
$OD_{540\,nm}$
3
2
1
0
9
8
7
6
5
4
3
2
1
0
DISTANCE TRAVELLED (cm)

were reincorporated in the vesicles. The high molecular weight and low molecular weight proteins appear to be nearly completely retained, while the intermediate molecular weight are reduced in the reconstituted vesicles.

3- Electron microscope studies. Evidence that proteins are incorporated in the bilayer

Thin sectioning: An electron micrograph of the original membrane vesicles is shown in figure 14 and is compared to the appearance of the reconstituted vesicles (Figure 15). It is evident that the reconstituted material appears vesicular and that the vesicles are of fairly uniform size, but smaller than the original vesicles. The diameter of the reconstituted vesicles is 0.3μ as compared to 1μ for the original vesicles. The solid black lines outlining the vesicle membrane are believed to be due to proteins. If proteins were trapped inside the vesicles rather than incorporated in the bilayer, the vesicles would not look empty in this section micrograph as these vesicles do.

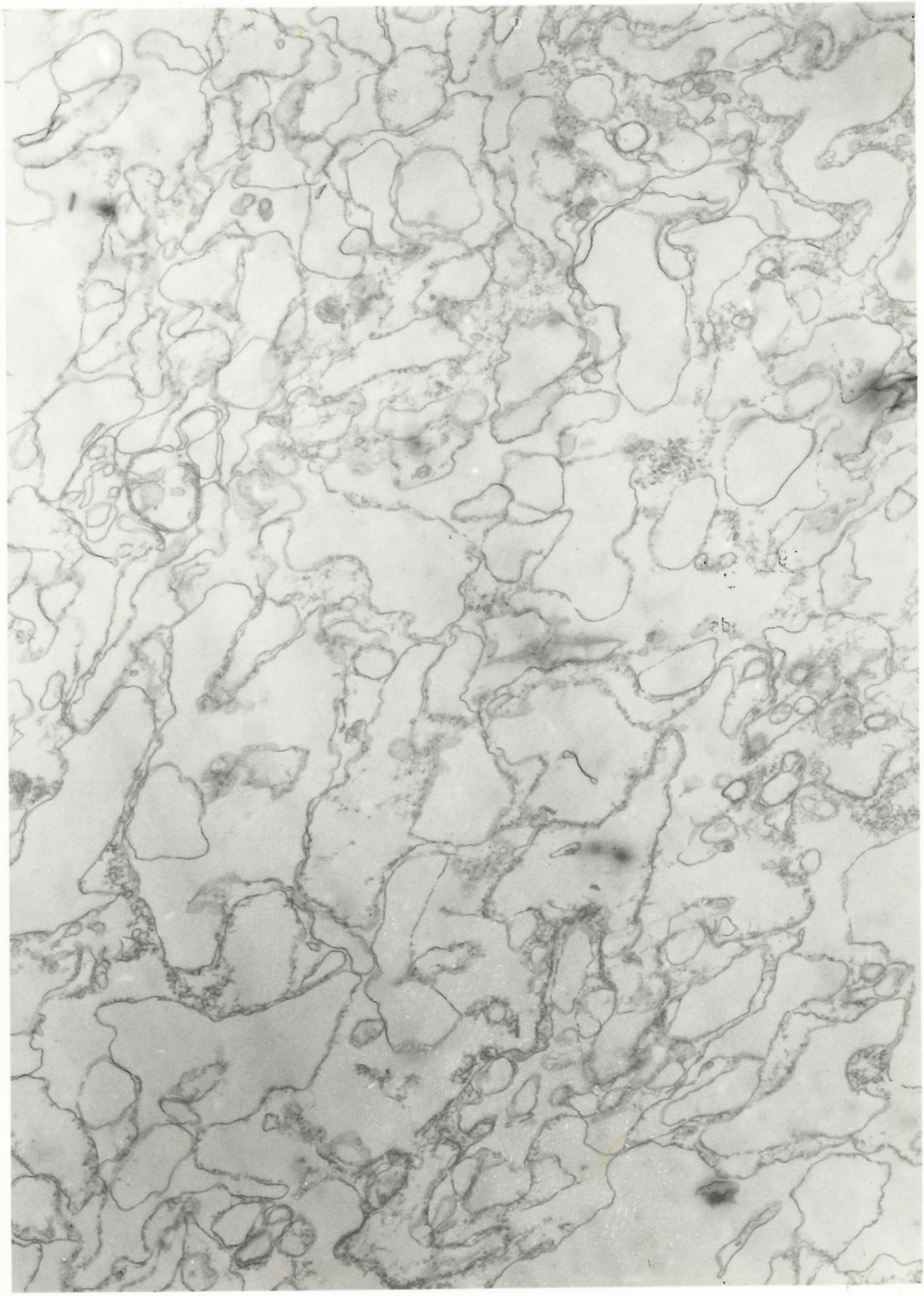
Freeze fracture: Another approach to localizing the proteins in the reformed vesicles is by freeze fracture. When a membrane is fractured at very low temperature, the lipid bilayer opens and the proteins either go to one surface or the other one, so that when the fracture face is shadowed, convex or concave shadows are seen. Freeze-fractured micrographs of the reconstituted vesicles are shown exhibiting the characteristic graining due to the presence of proteins (Figure 16).

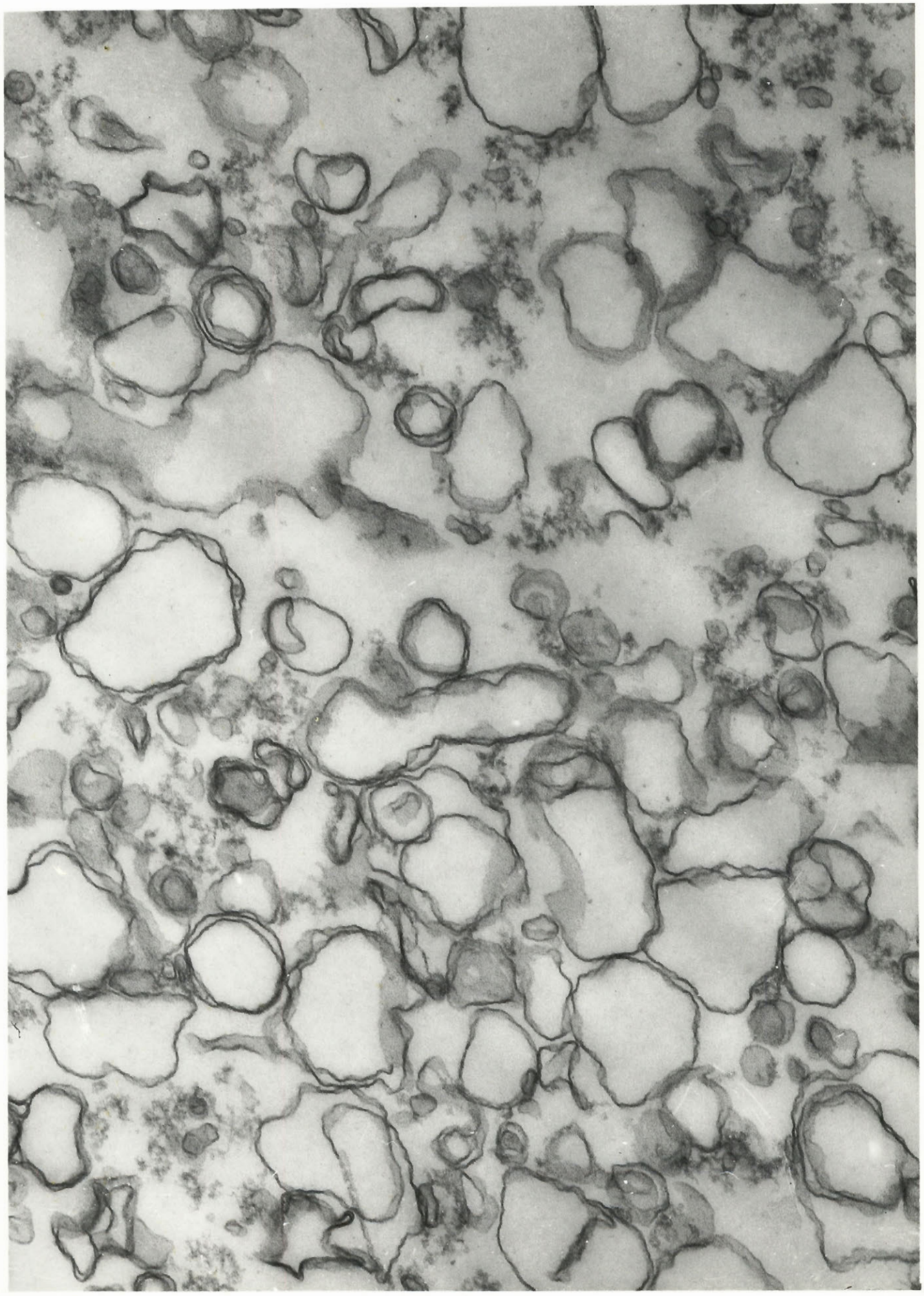
4- Sidedness of the reconstituted vesicles

A potential variable in vesicle formation is the sidedness of the reformed vesicles. Within experimental error, close to all the sialic

Figure 14. Thin-sectioning of fixed and stained native membrane vesicles

See Methods for details. Magnification is 58,500.





acid residues are incorporated into the membranes: facing interior, exterior, or randonly distributed. In the original membrane vesicles, if one assumes that the vesicles are all sealed to neuraminidase, virtually all the available sialic acid is released by neuraminidase, suggesting that sialic acid is exposed to the outside giving a homogeneous population of right-side out vesicles. In reconstituted membrane vesicles, despite the use of a large excess of neuraminidase and an extended incubation time period, only a fraction of the total sialic acid is released by neuraminidase. Moreover, if replicate samples are dialysed simultaneously to reform vesicles, the degree of accessibility to neuraminidase is still variable. On the average less than 40% of the total sialic acid is accessible to neuraminidase. These data suggest two possibilities: a) the reconstituted vesicles form an heterogeneous population of inside-out and right-side-out vesicles, b) the sialic acid is not accessible because it is located on the inner tracks of some multilayered liposome vesicles.
Another indication of the inaccessibility of the sugar residues from the outside was given by the fact that no vesicles were retained on a Con A Sepharose 4B column.

Factors affecting the formation of vesicles

Many parameters can influence the reconstitution, namely temperature, ions, lipids, and proteins, etc. Before examining each of these parameters it was necessary to define the criteria to be used to determine the degree of vesicle formation and the functional state of the vesicles. The criteria chosen to assess vesicle formation and function were:

a. Formation of a pellet capable of precipitating at 12,000 g for 30'

b. Presence of a vesicular structure under the electron microscope.

c. Time-dependent uptake of amino-acids (since objective of the work is oriented toward amino-acid transport). It is necessary to demonstrate that the reconstituted vesicles were sealed to small molecules, that is to show that they were able to take up amino acids in a time dependent way. If the vesicles were very leaky, it would be anticipated that the washout would remove most of the intravesicular solute. Only sealed vesicles could retain solute in the intravesicular space. The amino acid used in most experiments was AIB (α-amino isobutyric acid), a non-metabolizable aminoacid which can be transported, but does not undergo accelerated exchange with other amino acids in the intact Ehrlich cells.

d. To distinguish between non-specific and specific uptake, and at the same time to measure the intravesicular volume, ^{22}Na was used as a marker and incubated with the membranes at the same time as ^{14}C-AIB. Without ATP it is unlikely that there is Na^+ transport. The diffusion of Na^+ should give a measure of the intravesicular volume if incubation is carried out to an equilibrium position. It was repeatedly found that, after 30 minutes at 20°C, the value of μl AIB/μl Na^+ was greater than 1. This does not necessarily mean that AIB was accumulated inside the vesicles against a concentration difference, but that AIB was moving in faster than Na^+, and that a steady state had not yet been achieved after 30 minutes incubation at room temperature. However, incubation for longer periods of time did not increase the uptake of AIB or Na^+. In fact, decreases were observed. This was probably due to the instability of the transport system of the reconstituted vesicles. Therefore attempts to get at a true intravesicular volume were not entirely successful.

e. Even if the reformed membranes had a vesicle like appearance under the electron microscope, it was essential to ascertain that retention of solutes by the vesicles was due to the filling of a space and not to binding. To distinguish between time-dependent filling and time dependent binding, we examined the effect of osmotic strength on the uptakes of AIB and Na^+. Based upon studies done in the original membranes and with intact cells, sucrose was used as a non-permeant molecule. When the sucrose concentration was increased, the vesicles should shrink. Binding would be unaffected, but reduction of the intravesicular space would be expected to reduce the amount of amino acid and Na^+ retained. The data for original membranes vesicles and reconstituted vesicles are shown in Figure 17. The effects of increasing sucrose concentrations from 0 to 200 mM on the intravesicular space, as measured by the amount of AIB and Na^+ retained, are given. It can be seen, for the intact vesicles, as well as for the reconstituted ones, that both AIB and Na^+ values were reduced when the sucrose concentration was increased, consistent with the reduction of volume of the intravesicular space.

1- Temperature of reconstitution.

Meissner and Fleischer reported that the reconstituted Ca^{2+}-ATPase from sarcoplasmic reticulum showed higher activity if dialysis for reconstitution was carried out at 20°C rather than 4°C (220). However, for vesicles reconstituted from membranes of the Ehrlich Ascites Tumor Cells, higher activity was recovered after dialysis at 4°C, as shown on figure 18. Both Na^+ uptake and AIB uptake were reduced when reconstitution occured at 20°C. In particular the uptake of AIB was reduced to a greater extent than Na^+ uptake with vesicles reformed at 20°C.

Figure 17. Effect of osmolarity on AIB and Na^+ uptake

Vesicles, reconstituted with asolectin, were sealed at 37°C in the standard Na^+ medium: 100 mM NaCl, 5 mM Tris-HCl pH 7.4, 0,1 mM$CaCl_2$ 0,1 mM $MgCl_2$. After 30 minutes the vesicles were centrifuged and incubated at room temperature for additional 30 minutes in the standard Na^+ medium containing the appropriate concentration of sucrose along with 0.5 mM [1-^{14}C] AIB (specific activity 9 mCi/mmole) and $^{22}Na^+$.

effect of osmolarity on AIB and Na^+ uptake

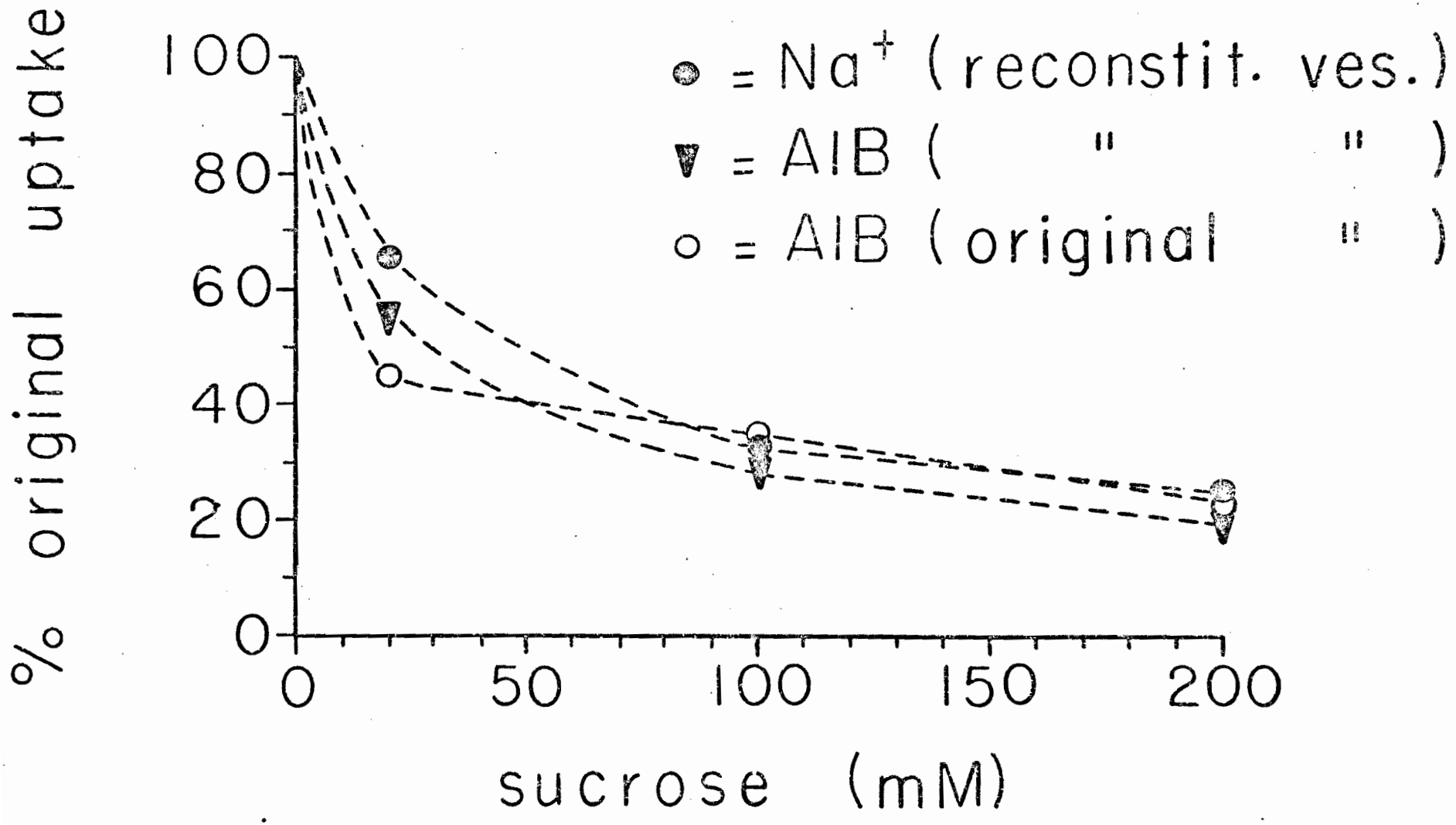
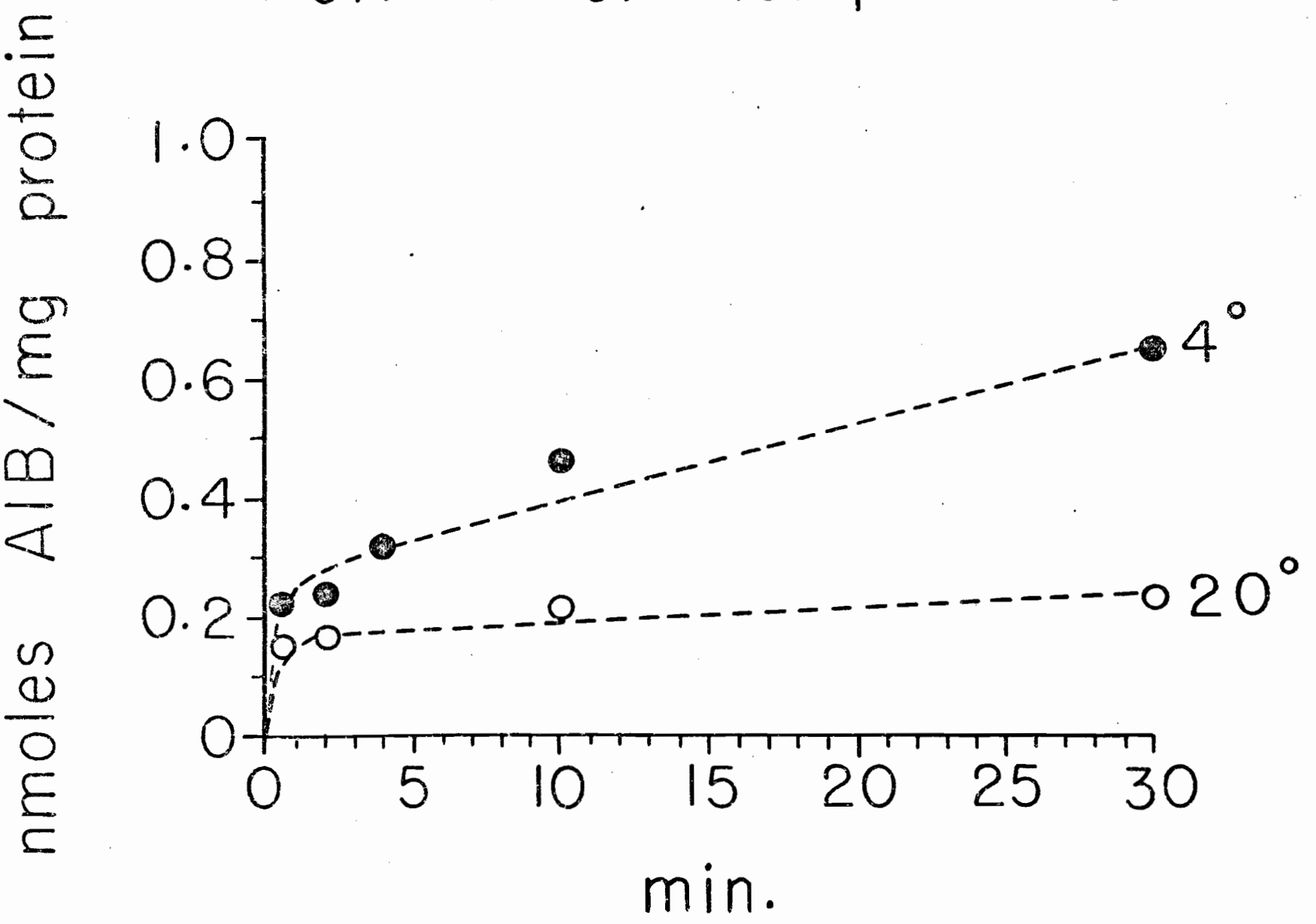


Figure 18. Effect of the temperature of reconstitution on AIB uptake

Vesicles, reformed with asolectin, were sealed at 37°C in the standard Na^+ medium (see figure 17). After 30 minutes the vesicles were transferred to room temperature. [1-^{14}C] AIB (specific activity 9mCi/mmole) was addedto a final concentration of 0.5 mM. 100 µl samples were taken at specified times as described under Uptake measurements.


effect of temperature
nmoles AIB / mg protein
1·0
0·8
0·6
0·4
0·2
0
0
5
10
15
20
25
30
min.
4°
20°

If the uptake of Na^+ at thirty minutes can be regarded as a measure of the vesicular volume, the data suggest that the AIB transport system is adversely affected by reconstitution at 20°C. Consequently all subsequent reconstitutions were done at 4°C in the cold room. As pointed out earlier, conditions which tend to maximize the difference between AIB and Na^+ uptake were considered indicative of restoration of transport function.

2- Ion requirement.

Razin (235) emphasized the necessity for high Mg^{++} concentrations (at least 10 mM) in the dialysis medium for better reconstitution. The effect of Mg^{++} and Ca^{++} concentrations on vesicle formation and activity has been studied for membranes reconstituted with soybean lecithin. The results are shown in Table III: Ca^{++} or Mg^{++} concentration was increased while the other divalent cation concentration was kept constant. Two conclusions may be drawn from these experiments:

a. increasing the divalent cation concentrations resulted in an increase in the amount of protein in the reconstituted material; nearly 5 times when the Ca^{++} concentration was increased from 0.1 to 2.0 mM and 6.4 times when the Mg^{++} concentration was increased from 0.1 to 10 mM.

b. less AIB was taken up per mg of protein and the ratio AIB/Na^+ taken up was smaller than the ratio obtained with vesicles reconstituted in the presence of low Ca^{++} and Mg^{++} concentrations.

Contrary to what was reported for other systems increasing the Ca^{++} and Mg^{++} concentrations did not improve the transport activity of reconstituted vesicles from membranes of EAC, optimal restoration of activity occurred in a medium containing low (0.1 mM) concentration of divalent cations.

TABLE III

Effect of the concentration of divalent cations during reconstitution on AIB and Na^+ uptake.

	Divalent cation concentrations during dialysis								
	Mg^{++} 0.1 mM Ca^{++} 0.1 mM			Mg^{++} 0.1 mM Ca^{++} 2 mM			Mg^{++} 10 mM Ca^{++} 0.1 mM		
Time	Na^+	AIB	AIB/Na^+	Na^+	AIB	AIB/Na^+	Na^+	AIB	AIB/Na^+
1'	1.7	2.3	1.35	1.4	2.1	1.5	1.6	1.7	0.88
5'	2.5	3.2	1.28	2.0	3.4	1.7	2.2	1.9	0.86
10'	2.6	4.0	1.54	2.9	3.4	1.17	2.2	1.9	0.86
30'	3.6	4.4	1.22	2.5	2.7	1.08	2.7	2.2	0.81

The vesicles were reconstituted with soybean lecithin, with different concentrations of divalent cations in the dialysis medium. The experimental conditions are described in Figure 18. Na^+ and AIB are expressed as µl/mg of protein.

3- Protein specificity.

Experiments have been conducted to demonstrate that restoration of transport activity was associated with reincorporation of native membrane proteins. The results are summarized in Table IV:

a. When the solubilized membrane proteins were dialyzed without addition of exogenous lipids, a small precipitate of insoluble material was obtained which did not show time dependent uptake of AIB and Na^+.

b. Without proteins, that is when asolectin or soybean lecithin alone in cholate 2%-urea 4M was dialyzed, no material was obtained at the end of the dialysis period after a 30 minute centrifugation at 12,000 g.

c. Heating the solubilized membranes at 100°C for 10 minutes prior to using the material for reconstitution with asolectin resulted in formation of a very small pellet of insoluble material. No uptake was observed.

d. Using BSA at 1 mg/ml instead of membrane proteins resulted in the formation of a minute pellet after dialysis which showed no ability to retain AIB.

Vesicles showing time dependent uptake of AIB and Na^+ with a ratio AIB/Na^+ greater than 1 after a 30-minute incubation required the presence of native membrane proteins as well as the addition of exogenous lipids.

4- Phospholipid specificity.

The phospholipid specificity has been investigated since it has been reported that some membrane proteins required specific phospholipids form maximum activity (14, 32, 33). Different natural and synthetic

TABLE IV

Requirements for the formation of functional vesicles.

	Increment in AIB uptake between 0 and 30 min
Asolectin + solubilized membranes (Control)	1.67
Asolectin + heated solubilized membranes	0
Asolectin + BSA	0
Solubilized membranes only	0
Asolectin only	0

For the control, 1-^{14}C-AIB uptake was measured as described in Figure 18, the ratio $(AIB/Na)_{30}$, was 1.6.

phospholipids have been tested for their ability to support the formation of functional vesicles. In Table V are listed the different lipids assayed in two groups corresponding to two different reconstitution experiments. The criteria used as a measure of functionality were:

1) Time dependent uptake of AIB

2) Volume of medium cleared of solute, and

3) The ratio AIB/Na^+ taken up.

The percentage of original protein which recombined with the lipids to form reconstituted membranes is also given. It can be seen that with the following phospholipids; sphingomyelin, L-αdipalmitoylphophatidylethanolamine, β dipalmitoylphosphatidylethanolamine, phosphatidylethanolamine saturated ether, phosphatidylserine, there was no time dependent uptake of AIB, although 10 to 20% of the original proteins were found in the reformed material. With the other phospholipids: (phosphatidylcholine from egg or soybean, dipalmitoylphosphatidylcholine, phosphatidylethanolamine and asolectin) there was evidence for time dependent uptake of AIB and Na^+.

Fifteen to 40% of the original proteins were found in the reconstituted membranes. It can be seen that there was no correlation between the amount of proteins reformed into vesicles and the specific activity of transport as defined by the ratio AIB/Na^+ taken up.

Recently cardiolipin has been tested for its ability to form active vesicles. 55% of the original proteins were found in the reformed material using cardiolipin. The vesicles formed with cardiolipin were able to take up more AIB per mg of protein than vesicles formed with asolectin. The Na^+ uptake was also higher in the vesicles formed with cardiolipin. Since cardiolipin is an acidic phospholipid, it was impossible

TABLE V

Restoration of transport activity with various phospholipids.

	Lipid added	Percent protein reincorporated	$(AIB/Na^+)_{30'}$
1.	L-α-Phosphatidyl choline (egg)	7	1.24
	L-α-Lecithin Type II (soybean)	6	1.22
	L-α-Dipalmitoylphosphatidyl choline (synthetic)	39	2.12
	Phosphatidylinositol	37	1.43
	Phosphatidylserine	13	0
2.	L-α-Phosphatidylethanolamine (E. coli) (Calbiochem)	19	1.26
	Phosphatidylethanolamine (E. coli) (Gibco)	15	1.65
	L-α-Dipalmitoylphosphatidylethanolamine (synthetic)	12	1.26
	L-α-Dipalmitoylphosphatidyl choline (synthetic)	45	2.38
	L-α-Dipalmitoylphosphatidyl choline (synthetic) + cholesterol	47	2.11
	Asolectin	30	1.6
	L-β-Dipalmitoylphosphatidylethanolamine (synthetic)	18	0
	Phosphatidylethanolamine, dihexadecyl; saturated ether	13	0
	Sphingo myelin	11	0

In column 2 is given the percentage of the protein reincorporated into vesicles. Uptake of [1-^{14}C]AIB and ^{22}Na was followed in all cases. The accumulation of AIB represented by the ratio $(AIB/Na^+)_{30'}$ is given in column 3. Experimental conditions are described in Figure 18.

to conclude from these results whether the Na^+ values represented a measure of the intravesicular space, or if this value was the sum of the intravesicular Na^+ taken up plus the amount of Na^+ electrostatically bound to the negative charges of the lipid. Clearly this lipid requires further study.

The addition of cholesterol, which has been shown to regulate the fluidity of the membrane and thereby may act as a modulator of enzyme activity, did not appear to influence the extent of vesicle formation or the activity of the reformed vesicles. Mixtures of phosphatidyl-ethanolamine and phosphatidylcholine with or without cholesterol have also been used with no improvement of transport activity.

The most consistent results were obtained with asolectin, with which about 30% of the solubilized proteins were found in the vesicles, although membranes with dipalmitoylphosphatidylcholine have a higher AIB/Na^+ ratio than vesicles formed with asolectin. Two problems were encountered with the dipalmitoylphospholipid:

a. the vesicles obtained were very difficult to handle, because they tended to form a jelly.

b. large liposomes even formed when the dipalmitoylphosphatidyl-choline-cholate 1%-urea 4M was dialyzed, causing high non-specific uptake.

In summary, the apparent lack of phospholipid specificity may be due to the fact that the solubilized membranes had not been delipidated before reconstitution. Alternatively there may not be much specific phospholipid requirement for the transport systems or the lipids from the cell membranes avoided the specific requirements. For pratical purposes asolectin has been the lipid most commonly used in these experiments.

5- Protein-lipid ratio for reconstitution.

Another variable in reconstitution and one about which there is a lot of controversy in the amount of exogenous lipid required for reconstitution of vesicles. It has been shown for many mitochondrial enzymes and for the purified Ca^{++}-ATPase of sarcoplasmic reticulum (251), that a ratio lipid/protein of 20/1 to 100/1 is desirable. But it has also been shown that this ratio depends on the enzymatic (236) system studied and on the method of reconstitution (compare 220 and 252). Obviously if the reconstitution is done with non-delipidated solubilized membranes, the amount of lipid to be added will be less than in the case of purified system. In the early part of this work, dipalmitoyphosphatidylcholine was used for reconstitution, and considerable variation was observed in the amount of AIB and Na^+ taken up. This variation could have been due to several factors:

a. the sonication step was difficult to control and to duplicate from experiment to experiment.

b. the membrane proteins were unstable.

c. there was variation in the lipid to protein ratio used for reconstitution. The latter variable has been examined and the results are shown in Table VI. There was a fairly good correlation between the amount of AIB and Na^+ taken up by the vesicles and the protein to lipid ratio; the largest vesicles (as measured by Na^+ uptake) were obtained with lower protein to lipid ratios, that is, with excess lipid. Subsequent experiments showed that the most active vesicles (ratios of $AIB/Na^+ > 1$) were obtained when equivalent weights of exogenous phospholipids and proteins (ratio 1:1) were used for the

TABLE VI

Effect of the protein/lipid ratio during reconstitution on AIB uptake.

P/L	$Na^+_{30'}$	$AIB_{30'}$	$(AIB/Na^+)_{30'}$
0.35	2.6	5.5	2.1
0.57	3.2	5.0	1.7
1	0.9	2.0	2.4
2	1.5	2.3	1.6
2.4	0.5	1.4	2.8
2.8	0.6	1.3	2.1

Different amounts of soybean lecithin per mg of solubilized protein have been used for reconstitution. Each protein/lipid ratio was tested at least two to three times. The experimental conditions for uptake are described in Figure 18. $Na^+_{30'}$, and $AIB_{30'}$ represent the increment in Na^+ and AIB uptake between 0 and 30'. The data given are representative of a typical experiment and are expressed as µl/mg of protein.

reconstitution, and this ratio was used in all subsequent experiments.

Proportionality between the amount of AIB taken up by the reconstituted vesicles and the amount of vesicles in the assay

Incubations were carried out with different amounts of reconstituted vesicles. AIB uptake in a Na^+ medium was followed. The rate of uptake was calculated from the uptake value at 30 seconds of incubation, and the steady state value was given by the uptake after 30 minute incubation. The background retention was determined at every protein concentration and subtracted from each of the values given on the graph.

Figure 19 shows that the uptake of solute, both the rate of uptake and the steady state value, varied directly with the amount of vesicles.

Conclusion

It has been demonstrated that it was possible to reconstitute membrane vesicles from solubilized membrane proteins and exogenous phospholipids. These vesicles were osmotically active and were capable of taking up aminoacids and Na^+. The conditions for optimum reconstitution were described.

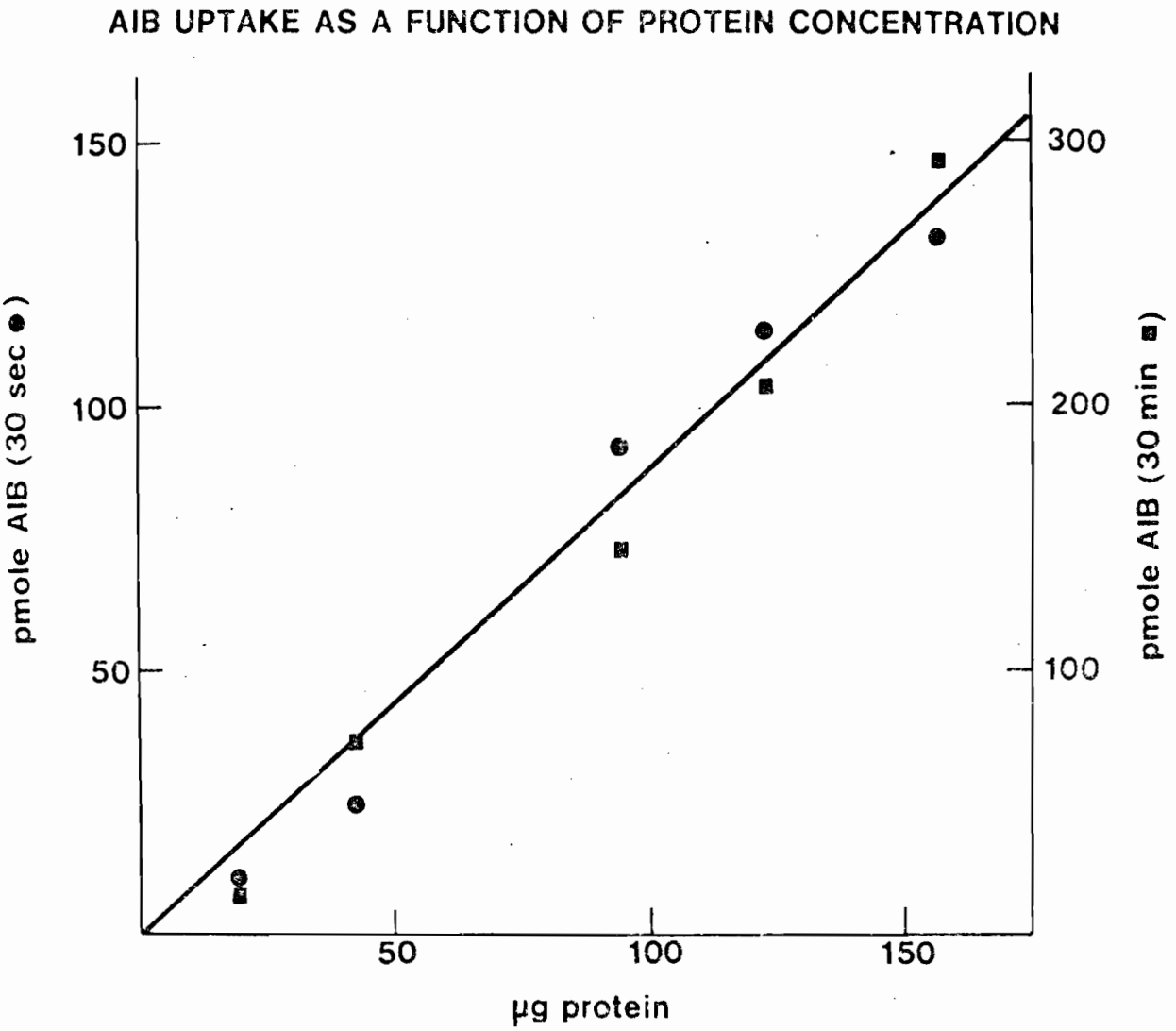


Figure 19. Uptake of AIB as a function of protein concentration

Conditions for the experiments are described in figure 18.

CHAPTER III: Amino Acid Exchange in Reconstituted Vesicles

The Ehrlich Ascites Tumor Cells possess an amino acid exchange system which is a simpler mechanism for amino acid transport than the net transport system, since it is not energy dependent and does not require the presence of Na^+ (252).

As no fractionation of the solubilized plasma membranes was carried out before reconstitution, it seemed reasonable to expect that the exchange system might be reincorporated into the reconstituted vesicles. Exchange diffusion, a one to one exchange of internal for external solute, is usually assayed by showing that the presence of a specific solute on the trans side of the membrane accelerates the movement of the solute from the cis side or vice versa.

For these experiments phenylalanine was a substrate of choice, because it could be obtained at a high specific activity, the background retention on the filter was low, and it is known to be a good substrate for exchange in the intact cells.

The effect of nonradioactive phenylalanine in the medium on the rate of loss of intravesicular phenylalanine was examined. The data are presented on figure 20. The vesicles were loaded with ^{14}C-phenylalanine, then the ^{14}C-phenylalanine exodus was followed under two conditions:

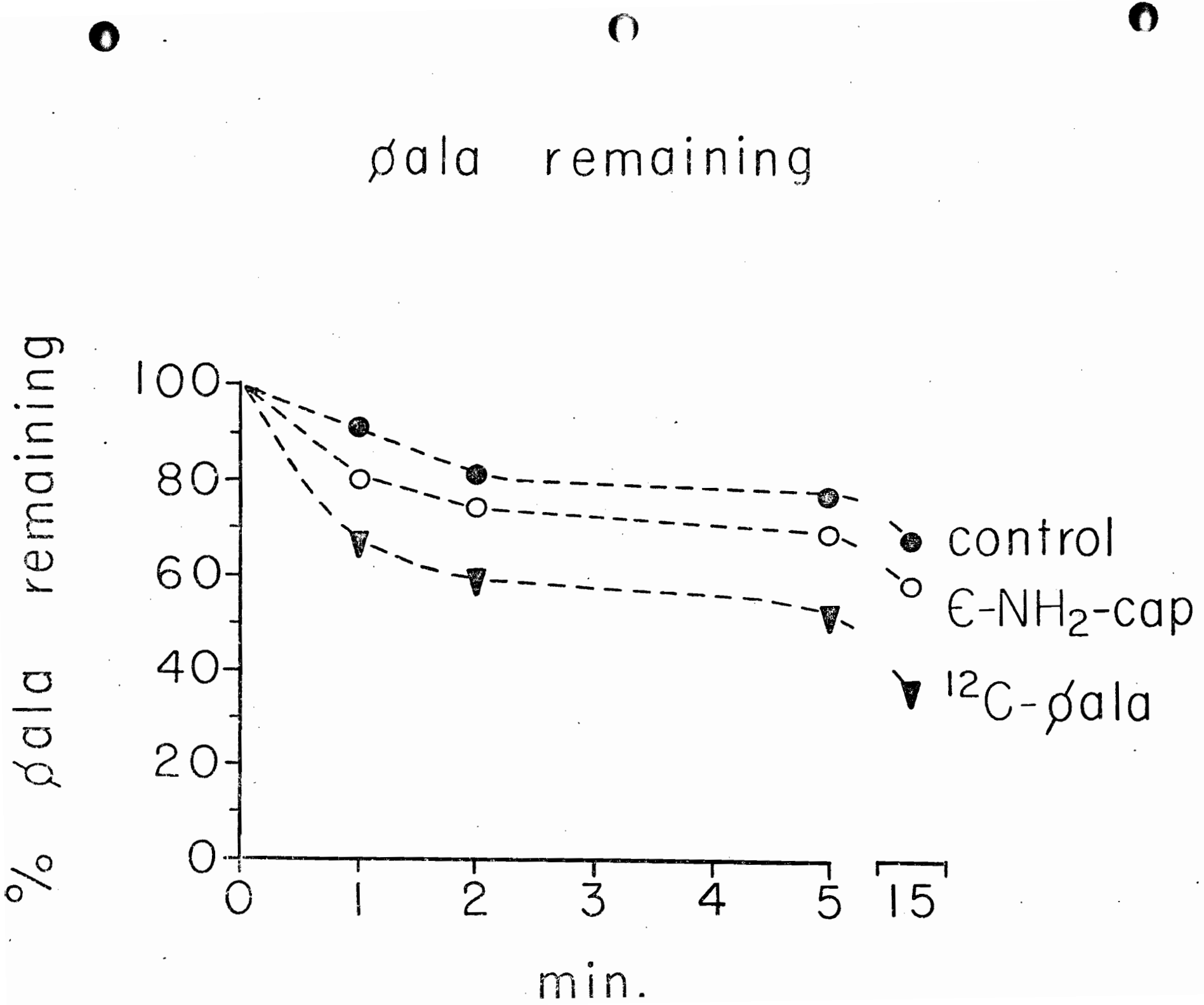
a. in a medium free from aminoacids

b. in a medium containing 10 mM ^{12}C-phenylalanine.

It can be seen that the presence of ^{12}C-phenylalanine in the medium accelerated the exodus of ^{14}C-phenylalanine from the vesicles.

Figure 20. Accelerated counterflow of phenylalanine from reconstituted vesicles

After preincubation, 0.012 mM L-[U-^{14}C] phenylalanine (specific activity 414 mCi/mmole) was added and incubation continued at room temperature for an additional 30 minutes. 50 or 100 µl of the ^{14}C-loaded vesicles were distributed into 2ml of nonradioactive medium containing the standard incubation medium at room temperature, (control); the standard medium with 10 mM ϵ aminocaproic acid; the standard medium with 10 mM L-[U-^{12}C] phenylalanine. After given intervals the 2 ml samples were filtered. The filters were washed, dried and counted. The data are representative of four similar experiments in vesicles reconstituted with asolectin or soybean lecithin.


øala remaining
% øala remaining
100
80
60
40
20
0
0
1
2
3
4
5
15
min.
control
ε-NH2-cap
12C-øala

To confirm that this stimulation of efflux was observed only when an "exchangeable" amino acid was on the trans side, efflux was followed in the presence of a non-transported amino acid, ε-aminocaproic acid. Figure 20 shows that ε-aminocaproic acid has very little effect on phenylalanine efflux.

It is unlikely that the presence of 10 mM ^{12}C-phenylalanine on the trans side caused shrinkage of the vesicles, thereby reducing the intravesicular volume and hence the content of ^{14}C-phenylalanine, since ε-aminocaproic acid at the same concentration (10 mM) on the trans side did not alter appreciably the rate of loss of vesicular phenylalanine.

It is also unlikely that the increase in efflux was an artefact due to an inhibition of reuptake of ^{14}C-phenylalanine by the presence of excess ^{12}C-phenylalanine, because the reuptake would be neglibible due to the dilution of the ^{14}C-phenylalanine in the efflux medium.

However to verify the reuptake did not play a part in the stimulated phenylalanine efflux the same experiment was repeated with AIB which is transported but does not participate in exchange. Figure 21 shows that extravesicular ^{12}C-AIB did not increase the exodus of intravesicular ^{14}C-AIB.

These results indicate that the system behaves in a manner consistent with the presence of an exchange system for phenylalanine in the reconstituted vesicles.

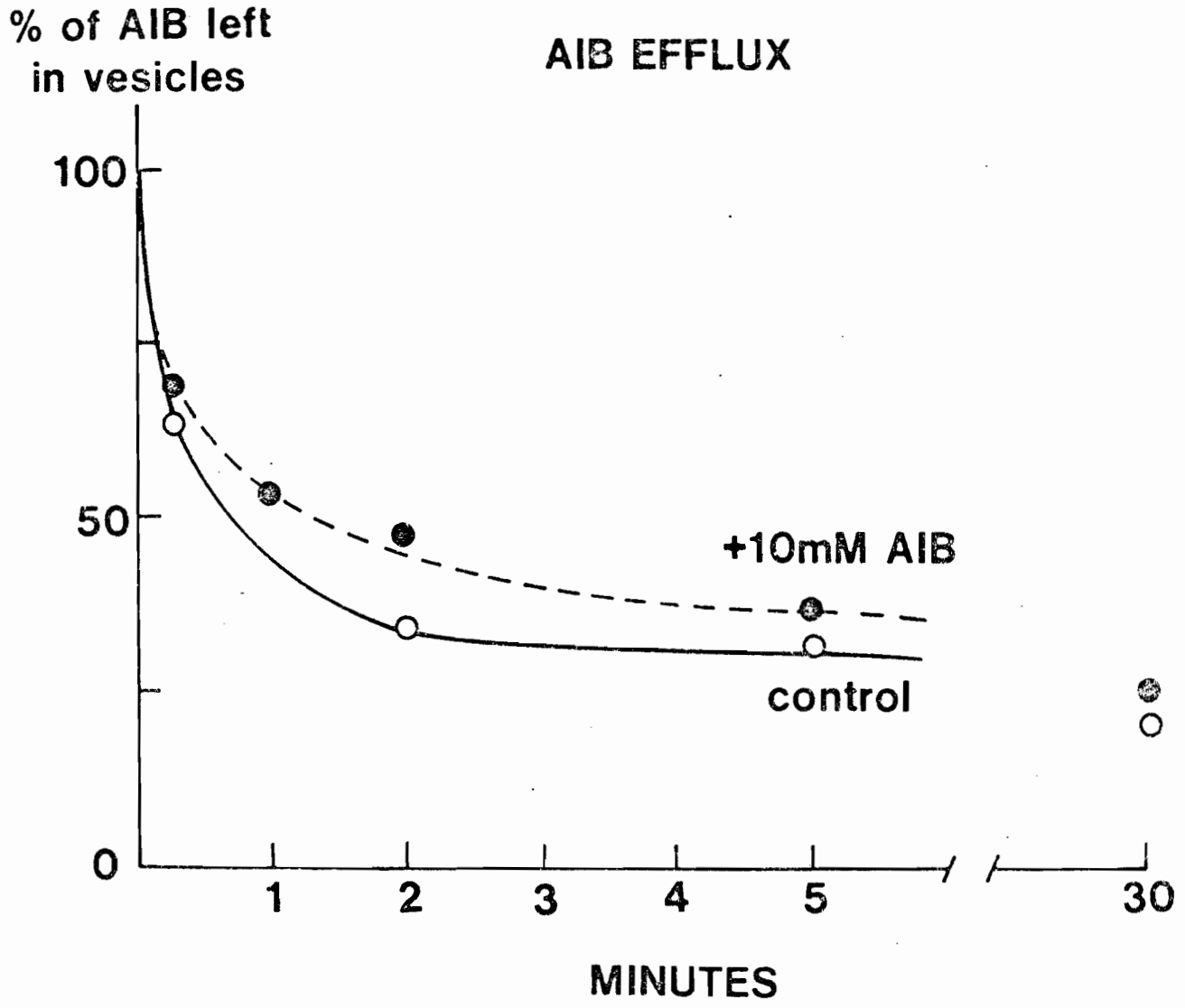


Figure 21. Absence of accelerated counterflow of AIB from reconstituted vesicles

The experimental conditions are identical to those described in figure 20, except that 0.5 mM [1-^{14}C] AIB was used to fill the vesicles. Efflux was measured with and without [1-^{12}C] AIB in the medium.

CHAPTER IV: Reconstituted Membrane Vesicles Showing Amino-Acid Transport

Transport of various amino-acids

Although AIB has been used as the test amino-acid for assessment of reconstitution of transport activity, it is not the only amino acid transported by the Ehrlich cells. Many other amino acids are transported into these cells via several different transport systems.

With vesicles reconstituted with asolectin the uptake of a variety of amino acids was observed as shown in figure 22. The uptakes observed were time dependent, but the rates of uptake were different for different amino acids. For example, ε-aminocaproic acid, which is a non-transported amino-acid was taken up much more slowly than methionine or phenylalanine. Leucine, which is transported mainly by the exchange L system in the intact cells, was taken up slowly, probably because there was no intravesicular amino acid with which to exchange. The amino-acids which are all actively transported in the intact cells, AIB, glycine, phenylalanine, methionine, reached the same steady state uptake. Thus it is clear that the expression of transport with AIB is not an isolated example.

Effect of temperature

It is well known that carrier-mediated transport processes are markedly affected by the temperature. It is for this reason that uptake is stopped by cooling, as, for example, injecting the sample into ice cold dilution medium. Like every enzymatic or chemical system, transport activity increases with increasing temperature until denaturation.

Figure 22. Uptake of various amino acids by reconstituted vesicles

After preincubation in the standard Na^+ medium, the vesicles were transferred to room temperature. Then the amino acids listed below were added and 100 µl samples were taken at specified times.

The amino acids were used at the following specific activities and concentrations:

[1-^{14}C] AIB	specific activity	9 mCi/mmole, 0.5 mM
L-[methyl-^{14}C] methionine	specific activity	55 mCi/mmole, 0.1 mM
L-[U-^{14}C] phenylalanine	specific activity	414 mCi/mmole, 0.012 mM
ε-amino[1-^{14}C] caproic acid	specific activity	38 mCi/mmole, 0,16 mM
L-[4,5-^{3}H] leucine	specific activity	5 Ci/mmole, 0.01 mM

The data are representative of two different experiments.

amino acid uptake in reconstituted vesicles

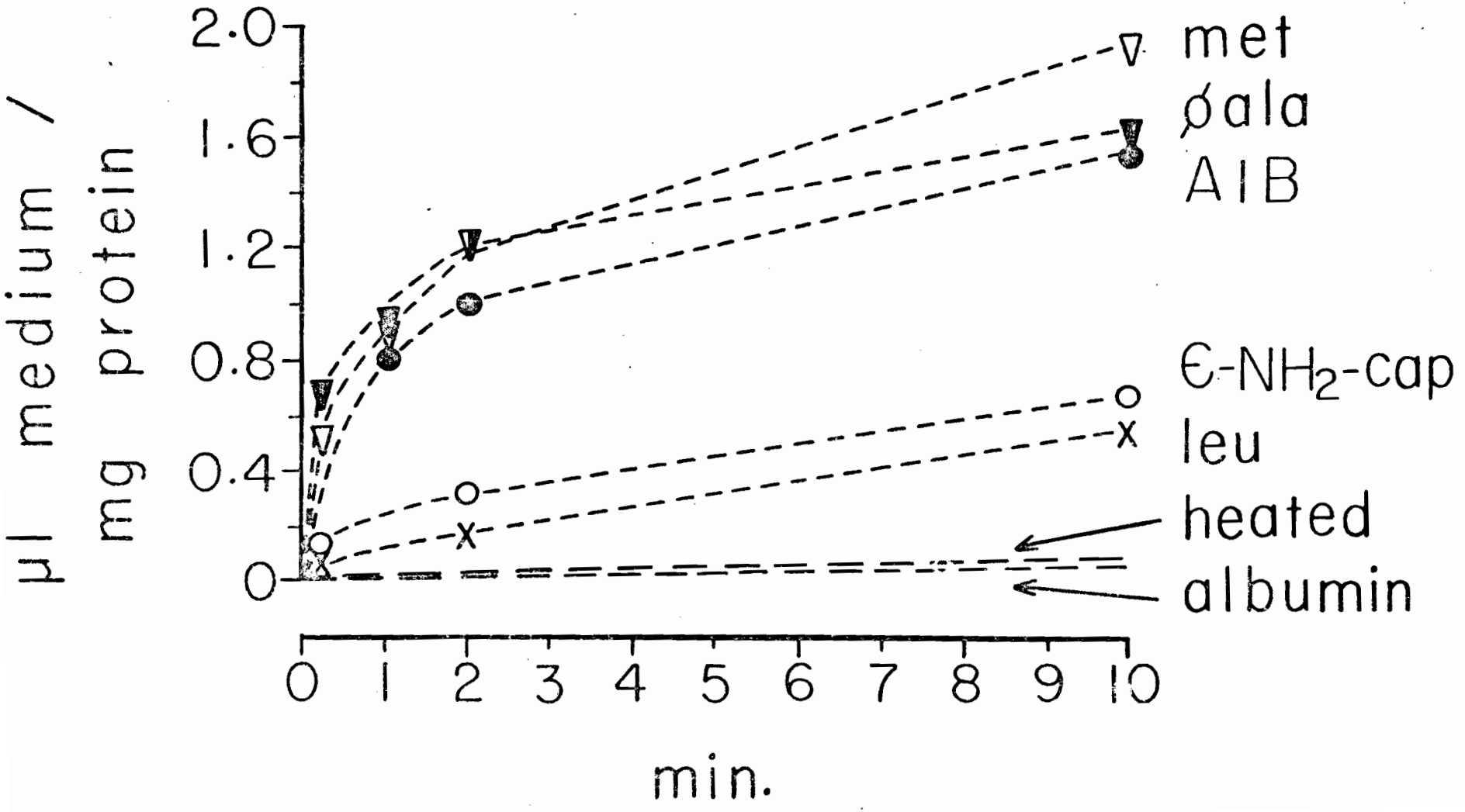


Figure 23 shows that AIB transport in reconstituted membrane vesicles was also temperature dependent, the uptake increasing as temperature increased. This increase in uptake cannot be attributed to increases in binding, since binding usually decreases when the temperature is elevated (thermal motion increases).

Saturability of the transport system

Since there is a finite number of transport sites on the membrane, it is clear that the amount of solute transported cannot exceed some limiting value if the uptake depends on these sites. In other words the system should be saturable. If the transport system in reformed vesicles has not been altered, a Km value close the Km observed in intact cells or native membrane vesicles should be obtained. Figures 24 and 25 show initial (1') and near steady state (30') uptake in a Na^+ medium as a function of AIB concentration plotted according to Lineweaver-Burk. The linear relationship supports the existence of mediated transport in the vesicles as opposed to simple diffusion. A Km of 3 mM is obtained, which is in very good agreement with the Km observed in the intact cells and membrane vesicles(182).

The same experiment has been repeated in a Na^+ free medium. As shown in figures 24 and 25(inset)there is no evidence of saturation, suggesting that the uptake is either by diffusion or that the Km value is too high to see evidence of saturation in the concentration range of AIB examined. Thus, the Km for the system in the reconstituted vesicles is comparable to that in the original cell and native vesicles.

Figure 23. Effect of temperature on AIB uptake in reconstituted vesicles

The conditions for the experiment are given in figure 18. Incubation for AIB uptake was carried out at 0°C, 20°C and 37°C. The data are an average of two experiments.

temperature dependent AIB uptake

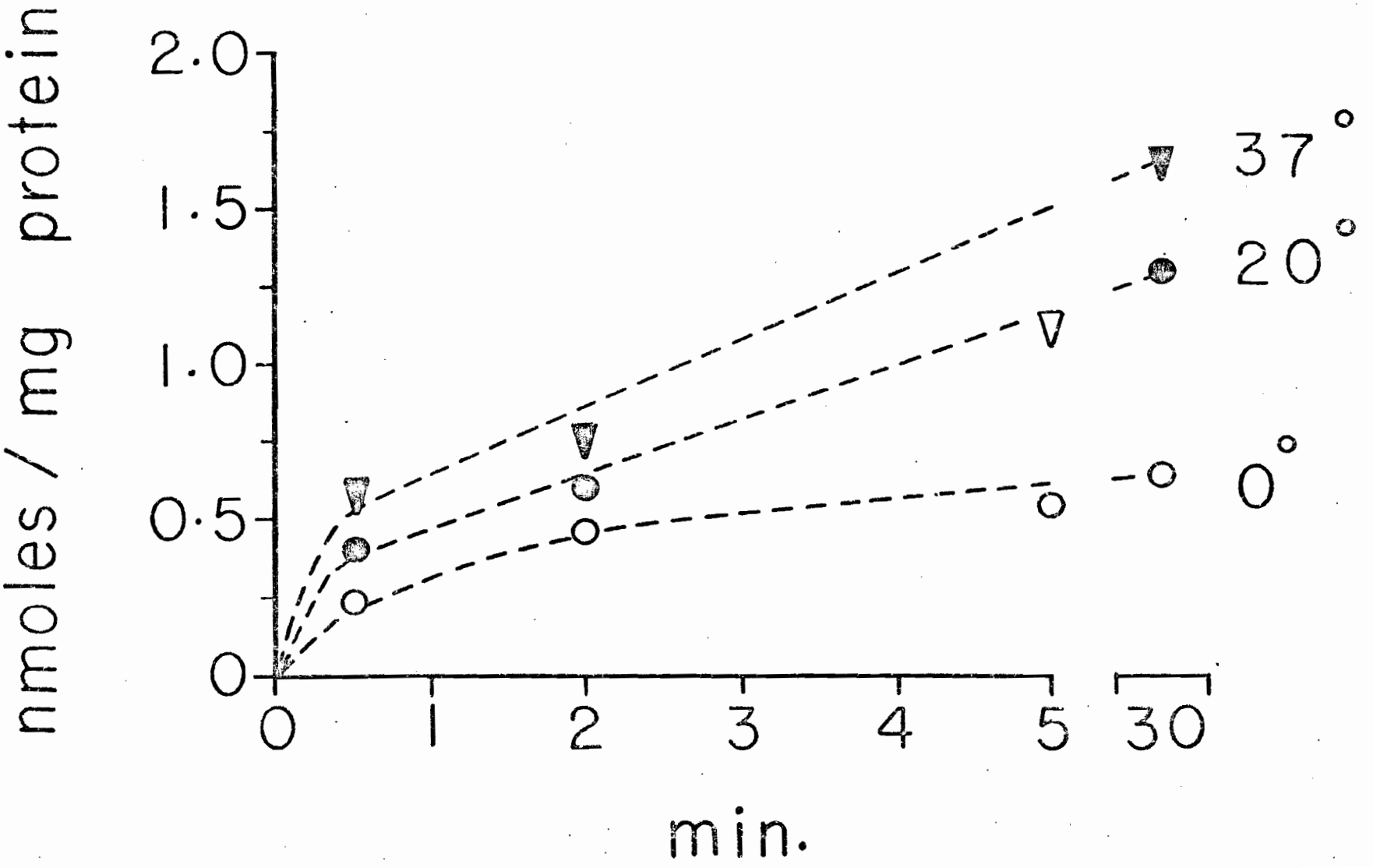


Figure 24. Saturation of AIB uptake in reconstituted vesicles (1 minute uptake)

Conditions for the experiment are described in figure 18. Uptake was measured in choline chloride and NaCl media. Asolectin was used for reconstitution. The data are the mean of two separate experiments.

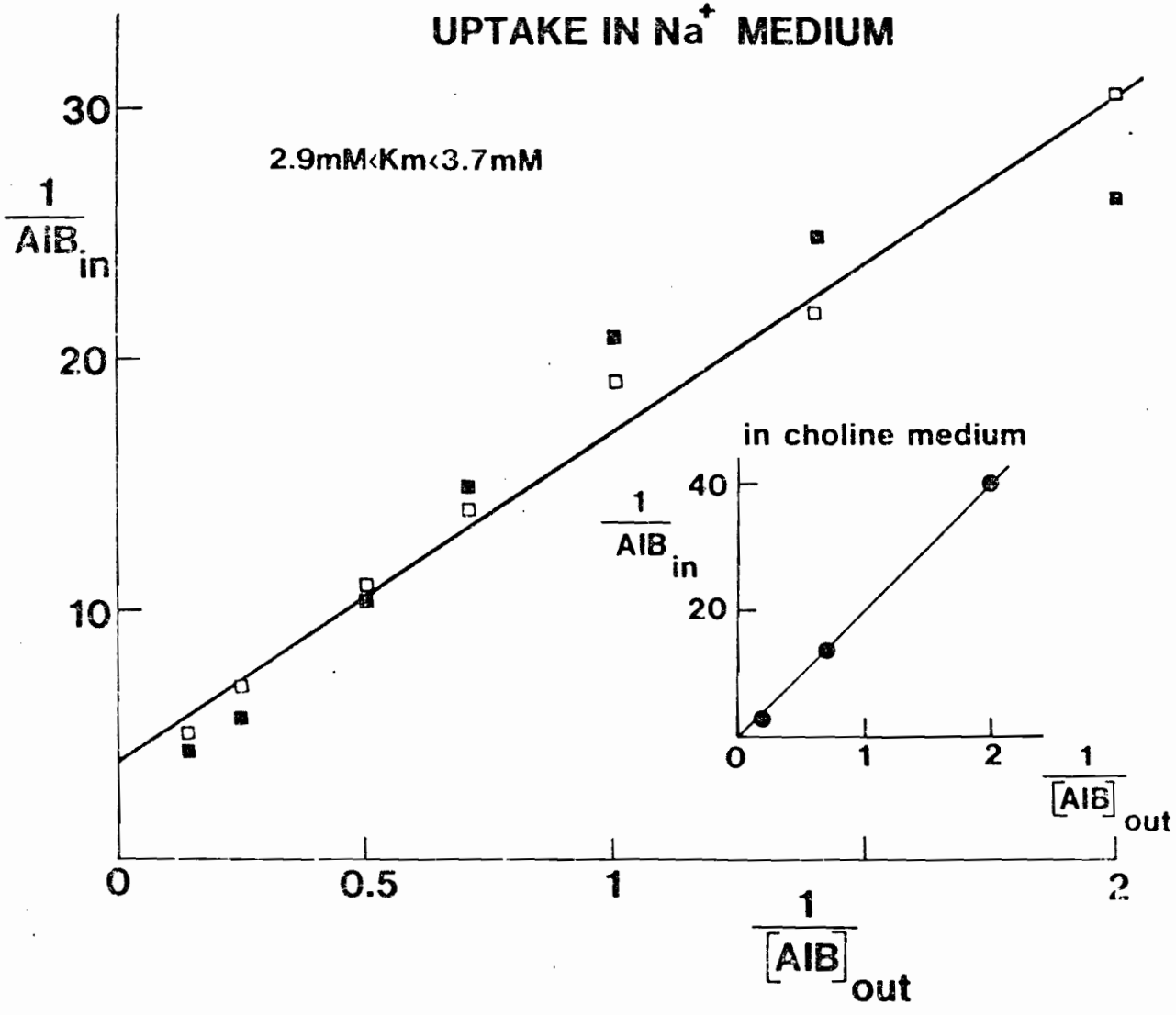
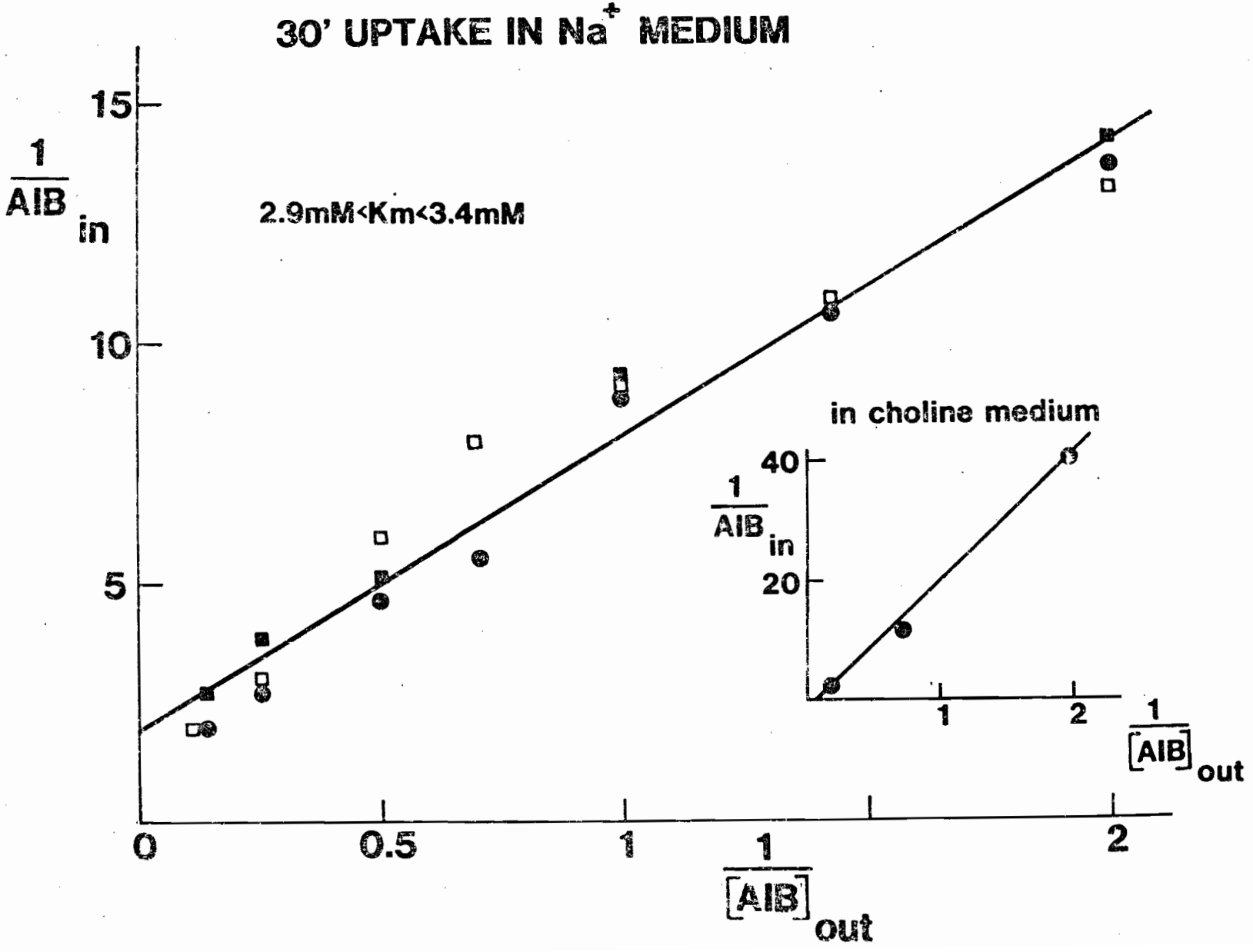

UPTAKE IN Na+ MEDIUM
2.9mM<Km<3.7mM
30
20
10
1/AIB in
0
0.5
1
2
1/[AIB]out
in choline medium
40
20
0
1
2
1/AIB in
1/[AIB]out

Figure 25. Saturation of AIB uptake in reconstituted vesicles (30 minute uptake)

Same experiment as in figure 24. Uptake was measured after 30 minutes of incubation.


30' UPTAKE IN Na+ MEDIUM
2.9mM<Km<3.4mM
1/AIB in
15
10
5
0
0.5
1
2
1/[AIB] out
in choline medium
40
20
1
2
1/AIB in
1/[AIB] out

Interaction between amino-acids

There have been many studies on the uptake of amino-acids in the Ehrlich Cells and considerable information is available about the nature of the interactions (252). Up to the present time, no kinetic studies on the type of interaction, K_i determinations, etc. have been done with intact and reconstituted membrane vesicles from Ehrlich Ascites Cell membranes. Therefore we report data obtained with the original and reconstituted membranes.

1- Native membrane vesicles.

Interactions between aminoacids have been investigated first in the intact membrane vesicles. AIB, glycine and methionine uptakes have been followed under different conditions as follows:

- The action of methionine and leucine on AIB uptake has been studied in a Na^+ medium with and without a Na^+ gradient. The results are shown on figures 26 and 27. In a Na^+ medium there was little effect of methionine on AIB uptake. However when a Na^+ gradient was applied, methionine was a potent inhibitor, while leucine had no effect.

- Glycine uptake has been followed in a choline medium, in a Na^+ medium with and without a Na^+ gradient, and in presence and absence of phenylalanine, methionine or leucine. In a choline medium no inhibition of uptake was observed by any of the amino acids. In a Na^+ medium, methionine and phenylalanine inhibited glycine uptake while leucine had no effect (figure 28). When a Na^+ gradient was applied, the same results were obtained, methionine and phenylalanine were inhibitory, but methionine was a more potent inhibitor than phenylalanine (figure 29).

Figure 26. Uptake of AIB in the presence of methionine in native membrane vesicles

The procedures used to assay transport in native membrane vesicles were identical to those used for reconstituted vesicles (see figure 18). After preincubation in 100 mM Na^+ or K^+ medium, the vesicles were incubated at room temperature with 0.5 mM [1-^{14}C] AIB (specific activity 9 mCi/mmole) in the absence or presence of 2 mM methionine in the standard Na^+ medium. The control represents the uptake of AIB after 30 minute incubation in the Na^+ medium in the absence of methionine (●). The variation observed in this experiment is less than 5%.

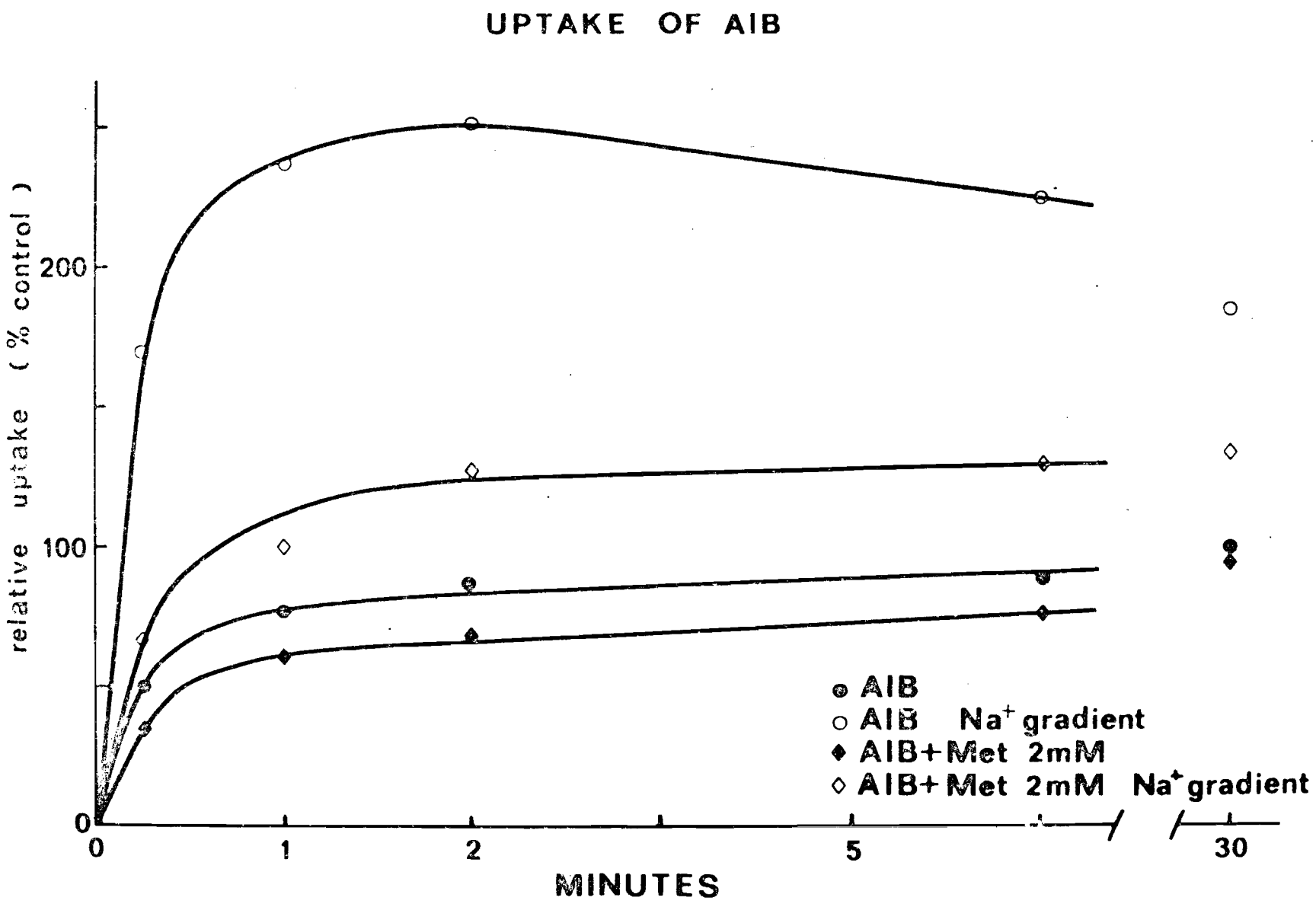

UPTAKE OF AIB
relative uptake (% control)
200
100
0
0
1
2
5
30
MINUTES
AIB
AIB Na+ gradient
AIB+Met 2mM
AIB+Met 2mM Na+ gradient

Figure 27. Uptake of AIB in the presence of leucine in native membrane vesicles

The conditions of the experiment are described in figure 26. The data are the mean of two separate experiments.

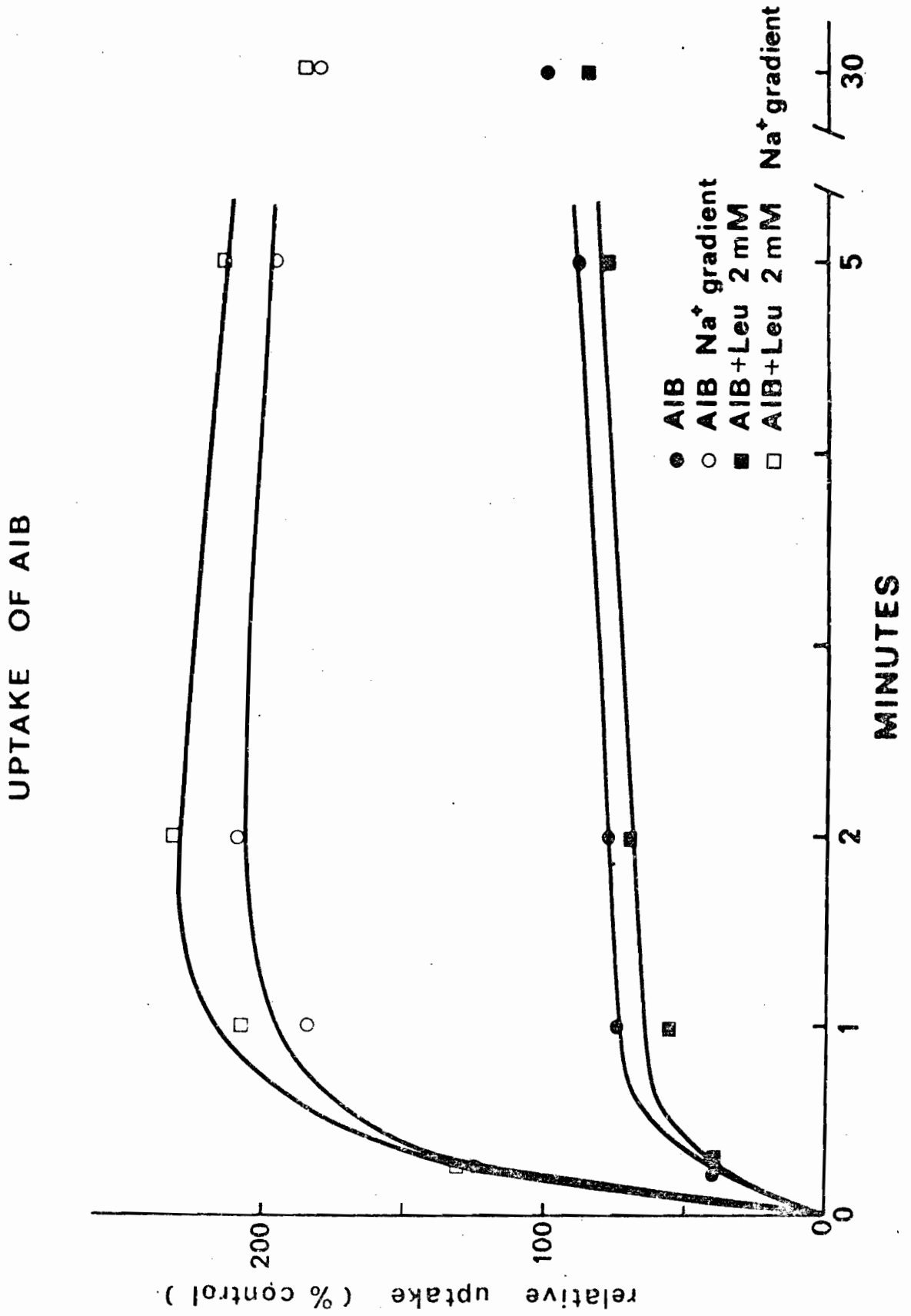

UPTAKE OF AIB
relative uptake (% control)
200
100
0
0
1
2
5
30
MINUTES
AIB
AIB Na+ gradient
AIB+Leu 2 mM
AIB+Leu 2 mM Na+ gradient

Figure 28. Na^+-dependent uptake of glycine in the presence of leucine, methionine and phenylalanine in native membrane vesicles

The conditions for the experiment are described in figure 26. The vesicles were preincubated and incubated in a Na^+ medium. [U-^{14}C] glycine (specific activity: 102 mCi/mmole) was used at a concentration of 0.05 mM.

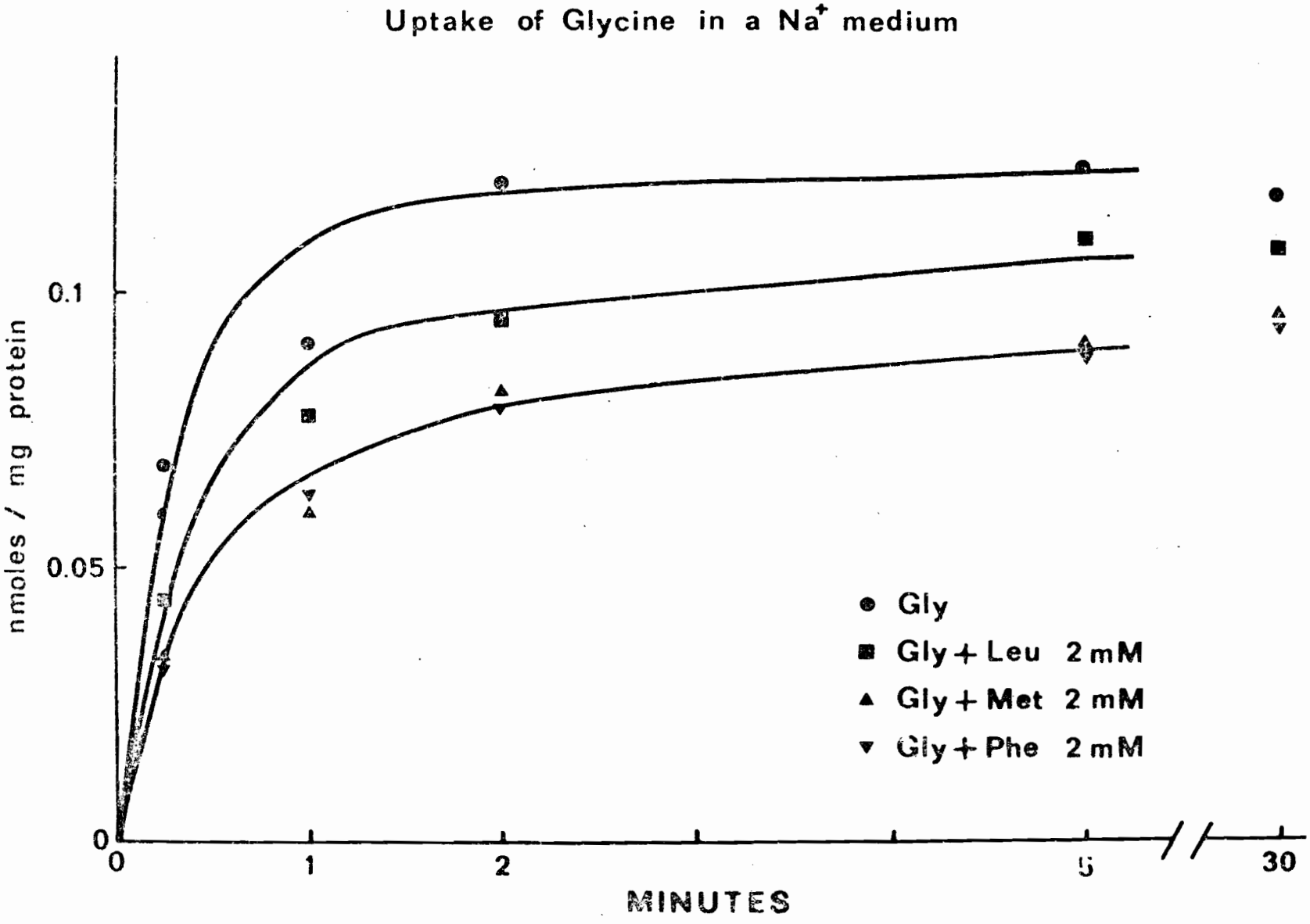
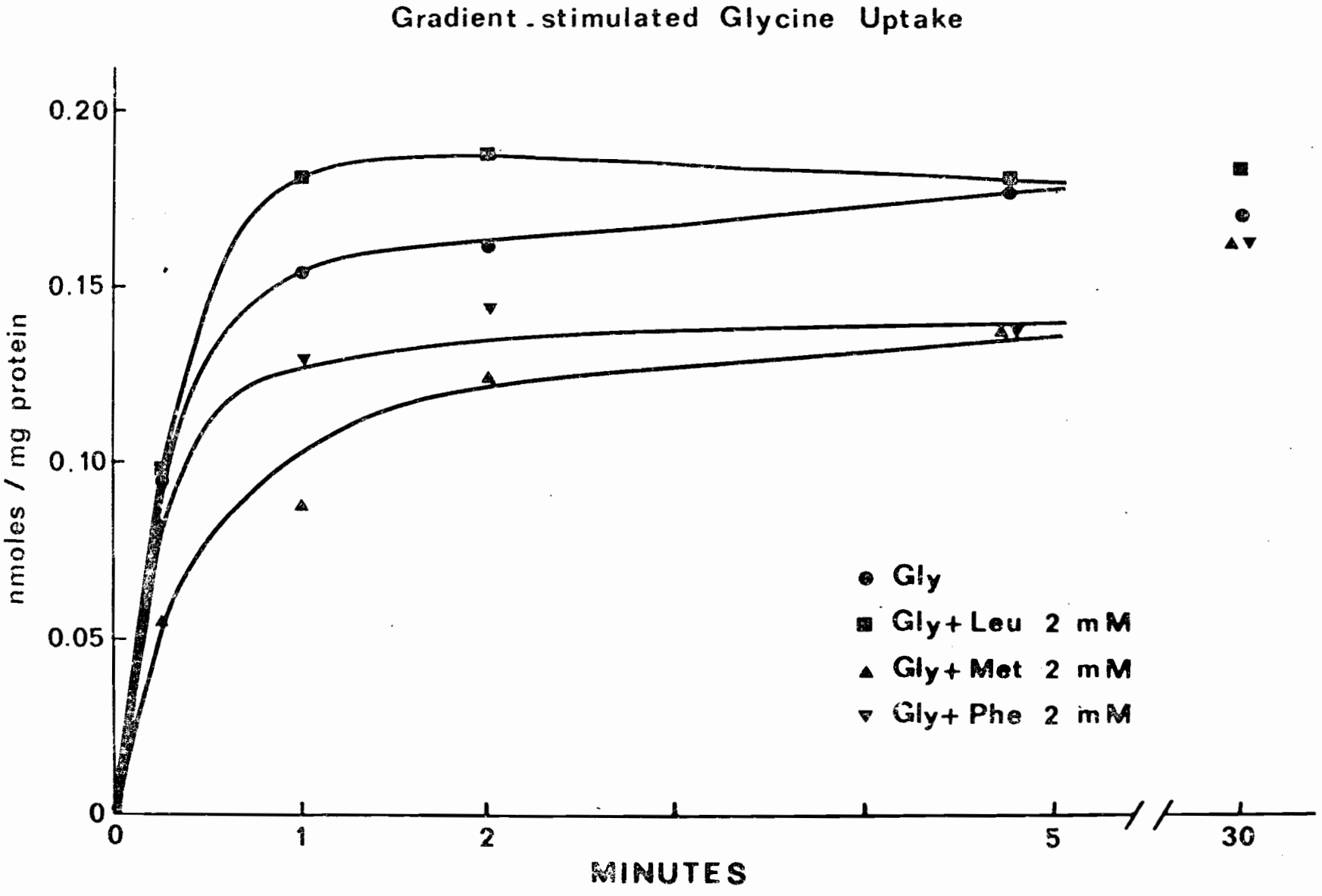

Uptake of Glycine in a Na+ medium
nmoles / mg protein
0.1
0.05
0
0
1
2
5
30
MINUTES
Gly
Gly + Leu 2 mM
Gly + Met 2 mM
Gly + Phe 2 mM

Figure 29. Na^+-stimulated uptake of glycine in the presence of leucine, methionine and phenylalanine in native membrane vesicles. The conditions for the experiment are described in figure 28. After preincubation in a K^+ medium, the vesicles were transferred to the standard Na^+ medium for incubation at 20°C.


Gradient - stimulated Glycine Uptake
nmoles / mg protein
0.20
0.15
0.10
0.05
0
0
1
2
5
30
MINUTES
Gly
Gly+Leu 2 mM
Gly+Met 2 mM
Gly+Phe 2 mM

- Methionine uptake in a Na^+ medium was slightly inhibited by phenylalanine and more significantly by leucine. When a Na^+ gradient was applied, the same results were obtained. Glycine had no effect on methionine uptake (figures 20 and 31). This pattern of inhibitory action of amino acids uptake is analogous to the results with intact cells as reviewed by Johnstone *et al.* (252).

2- Reconstituted membrane vesicles.

Studies of the interaction between amino acids with reconstituted membrane vesicles have been done using AIB as the substrate and methionine, leucine and ε-aminocaproic acid as inhibitors, in a Na^+ medium only. The results presented in figure 32 show that AIB uptake was inhib ited by methionine, and was not affected by leucine or ε-aminocaproic acid, a non-transported amino-acid.

The inhibition by 10 mM methionine cannot be due to shrinkage of the vesicles since leucine and ε-aminocaproic acid at the same concentration had no effect. These results are similar to those described above with the native membrane vesicles.

Inhibition of transport

In the course of this project, it became evident that in order to be able to show that the reconstituted vesicles were able to transport amino-acids, the simplest way was to find a specific inhibitor of the transport systems. Different agents have been tested on the intact membrane vesicles and the results can be summarized as follows:

1. Iodoacetate, iodoacetamide, NEM, and Harmaline had no effect on Na^+ and AIB uptake.

2. $HgCl_2$ 2 mM affected Na^+ and AIB in a similar way (Figure 33).

3. PCMBS showed an early effect on Na^+ uptake followed by a decrease in AIB uptake (figure 34). This effect could be due either to

Figure 30. Na^+-dependent uptake of methionine in the presence of glycine, phenylalanine and leucine in native membrane vesicles

The conditions are described in figure 28. L-[methyl-^{14}C] methionine was used at a concentration of 0.1 mM (specific activity 55 mCi/mmole).

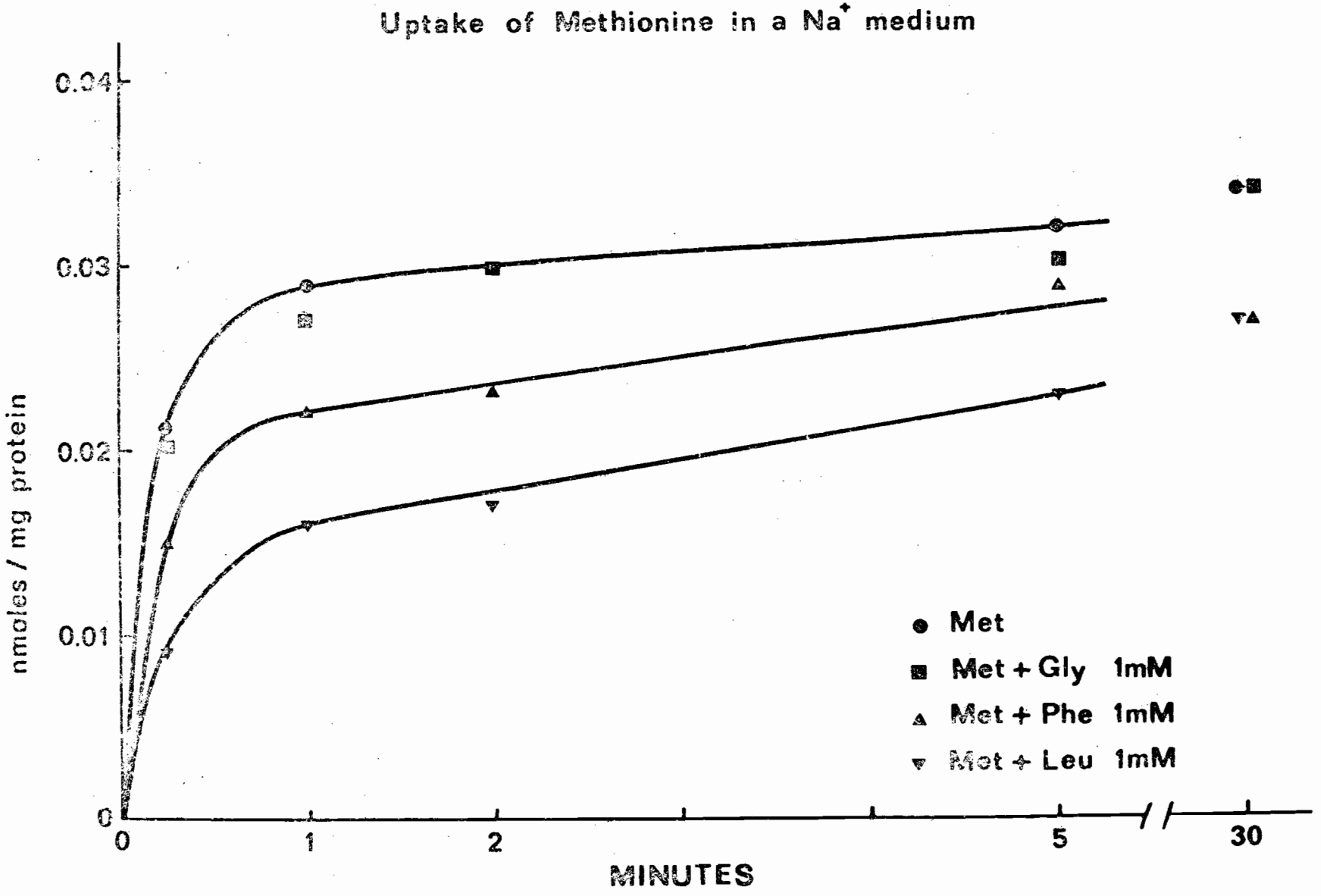

Uptake of Methionine in a Na+ medium
0.04
0.03
0.02
0.01
0
nmoles / mg protein
0
1
2
5
30
MINUTES
Met
Met + Gly 1mM
Met + Phe 1mM
Met + Leu 1mM

Figure 31. Na^+-stimulated uptake of methionine in the presence of phenylalanine and leucine in native membrane vesicles

The conditions are described in figure 30. The vesicles were preincubated in a K^+ medium.

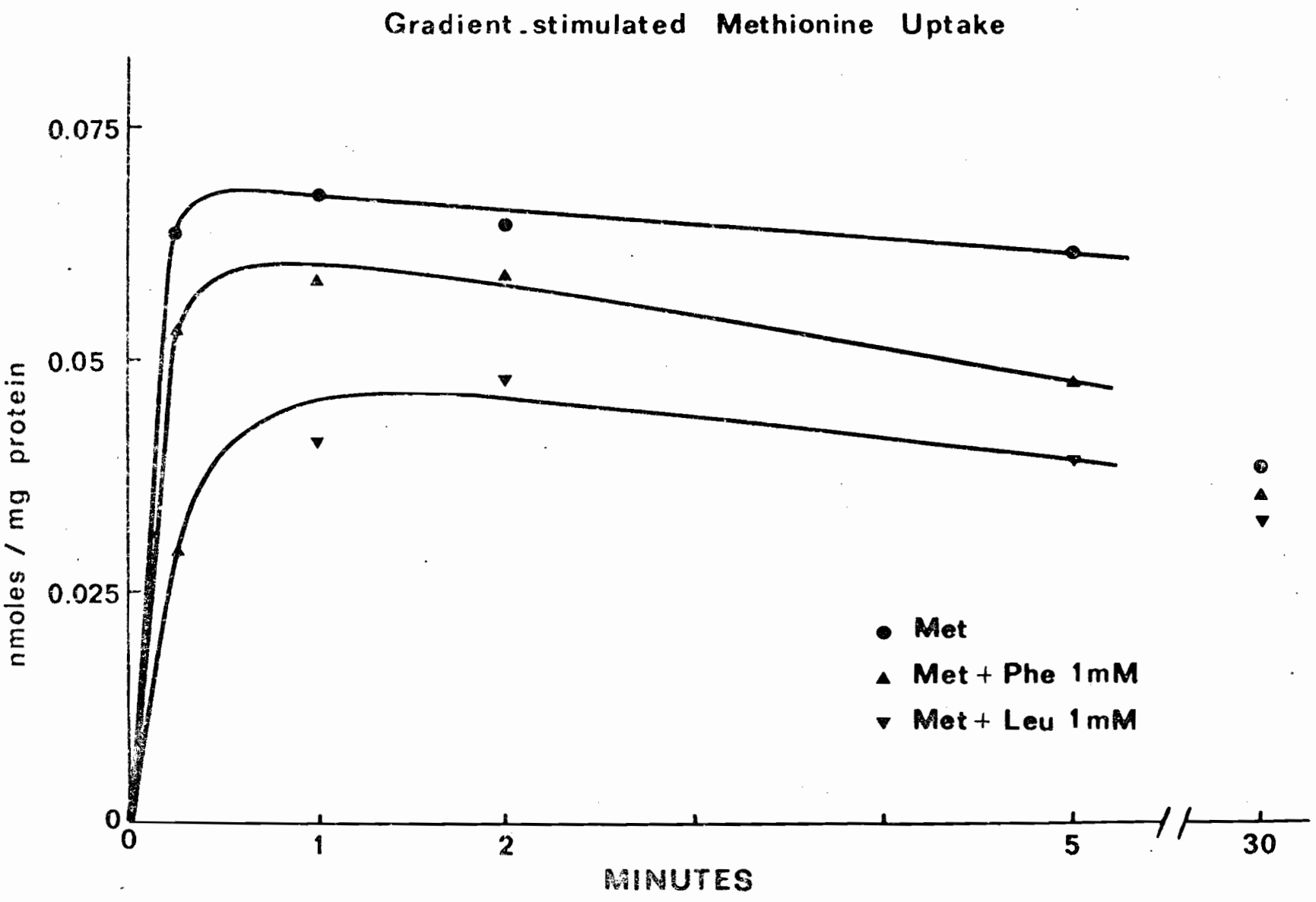

Gradient-stimulated Methionine Uptake
0.075
0.05
0.025
0
nmoles / mg protein
0
1
2
5
30
MINUTES
Met
Met + Phe 1mM
Met + Leu 1mM

Figure 32. Uptake of AIB in the presence of leucine and methionine in reconstituted membrane vesicles

The conditions are described in figure 18. The data are the mean of three separate experiments.

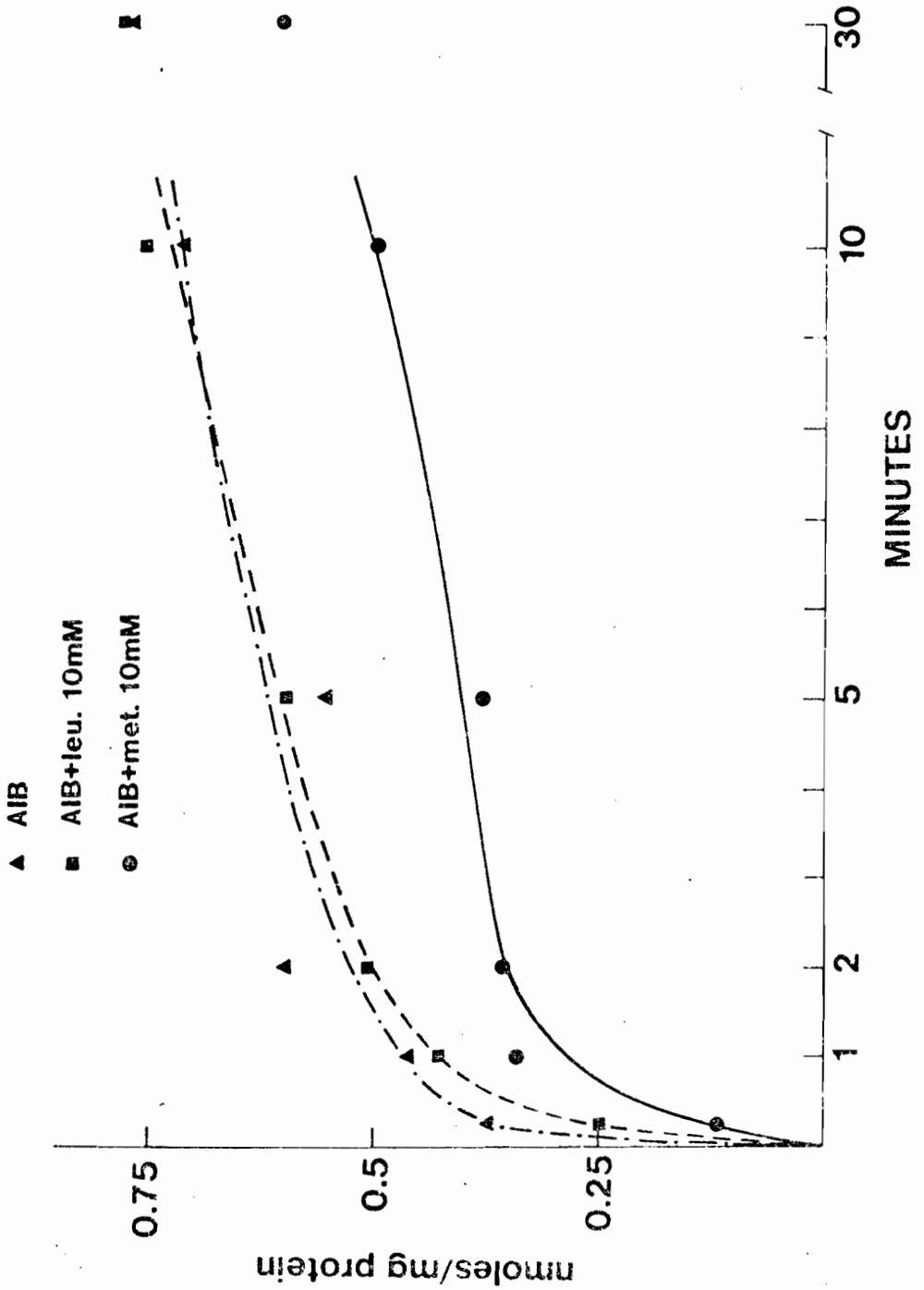

AIB
AIB+leu. 10mM
AIB+met. 10mM
nmoles/mg protein
0.75
0.5
0.25
1
2
5
10
30
MINUTES

Figure 33. Effect of $HgCl_2$ on AIB and Na^+ uptakes in native membrane vesicles

The conditions are described in figure 26. The control represents the 30 minute uptake of AIB in the absence of $HgCl_2$ (●). The data are the mean of four separate experiments.

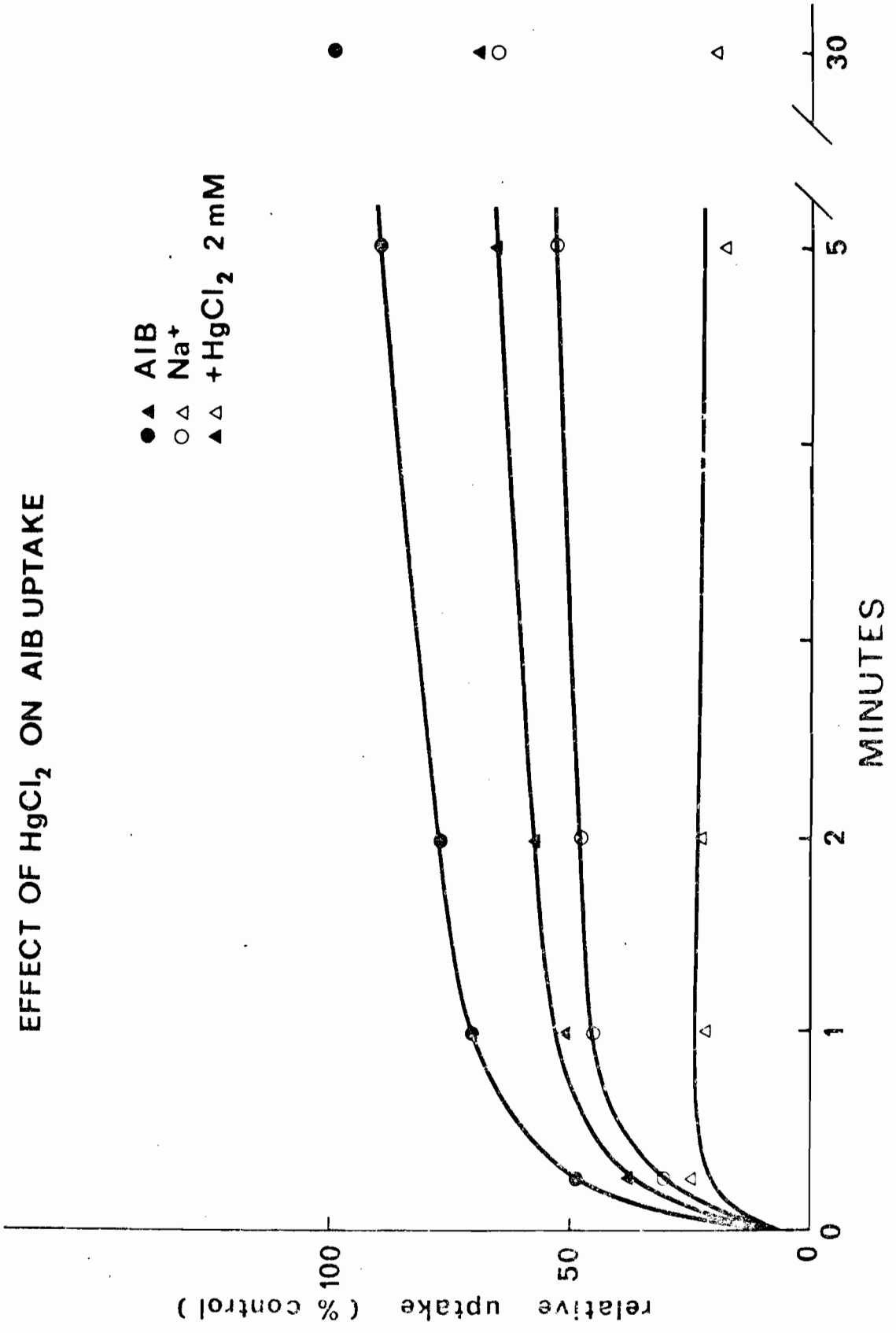
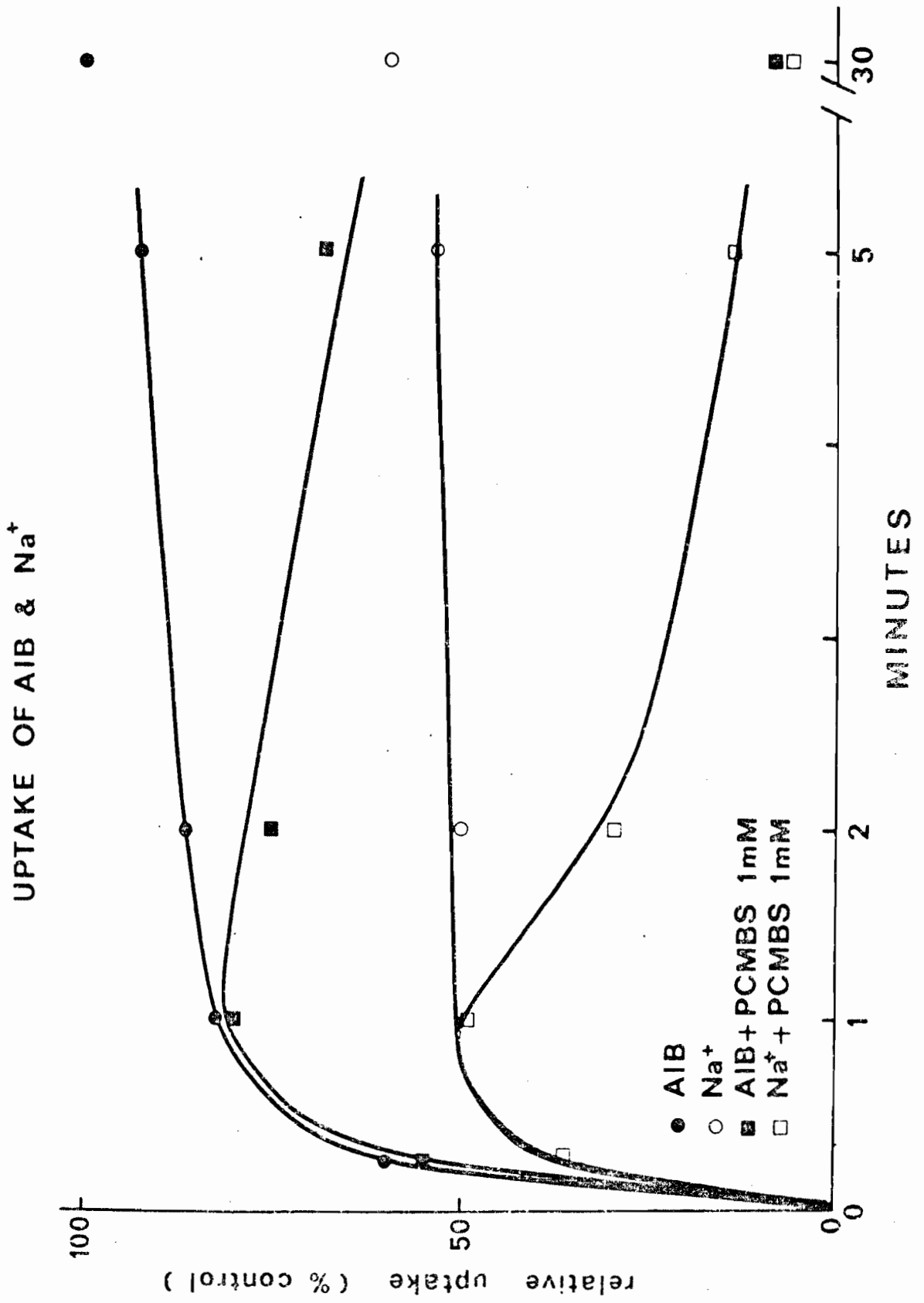

EFFECT OF $HgCl_2$ ON AIB UPTAKE
● ▲ AIB
○ △ Na^+
▲ △ + $HgCl_2$ 2 mM
relative uptake (% control)
100
50
0
0
1
2
5
30
MINUTES

Figure 34. Effect of PCMBS on AIB and Na^+ uptakes in native membrane vesicles

The conditions are described in figure 26. The control represents the 30 minute uptake of AIB in the absence of PCMBS (●).


UPTAKE OF AIB & Na^+
relative uptake (% control)
100
50
0
0
1
2
5
30
MINUTES
AIB
Na^+
AIB+PCMBS 1mM
Na^+ + PCMBS 1mM

the presence of the ion Na^+, or to a decrease in intravesicular volume. These two possibilities have been investigated:

a) The reduction of AIB uptake observed in a non-Na^+ medium was the same as the one observed in a Na^+ medium.

b) The effect of PCMBS on L-glucose (a non-transported sugar) and on 3-OMG (a sugar transported but not accumulated in the cell) uptakes has been studied. The results (figure 35) show that L-glucose and 3-OMG uptakes were affected by PCMBS in a similar way to AIB uptake.

The simplest explanation of these data is that PCMBS is causing shrinkage of vesicles with concomitant reduction of solute uptake. PCMBS also affected AIB and Na^+ exodus in a similar manner to its effect on uptake as shown in figure 36.

Since it was not possible to find an inhibitor specific for Na^+ dependent amino acid uptake except other amino acids, no studies of the effects of these compounds on the reconstituted vesicles have been undertaken.

Figure 35. Effect of PCMBS on Na^+, L-glucose and 3-O-methylglucose uptake in native membrane vesicles.

The conditions are described in figure 26. L-[1-^{14}C] glucose (specific activity 51.6 mCi/mmole) was used at a concentration of 0.1 mM. 3-O[methyl-^{14}C] glucose (specific activity 52.7 mCi/mmole) was used at a concentration of 0.09 mM. The control represents the 30 minute uptake of L-glucose or of 3-OMG in the absence of PCMBS (▲ and ▼).

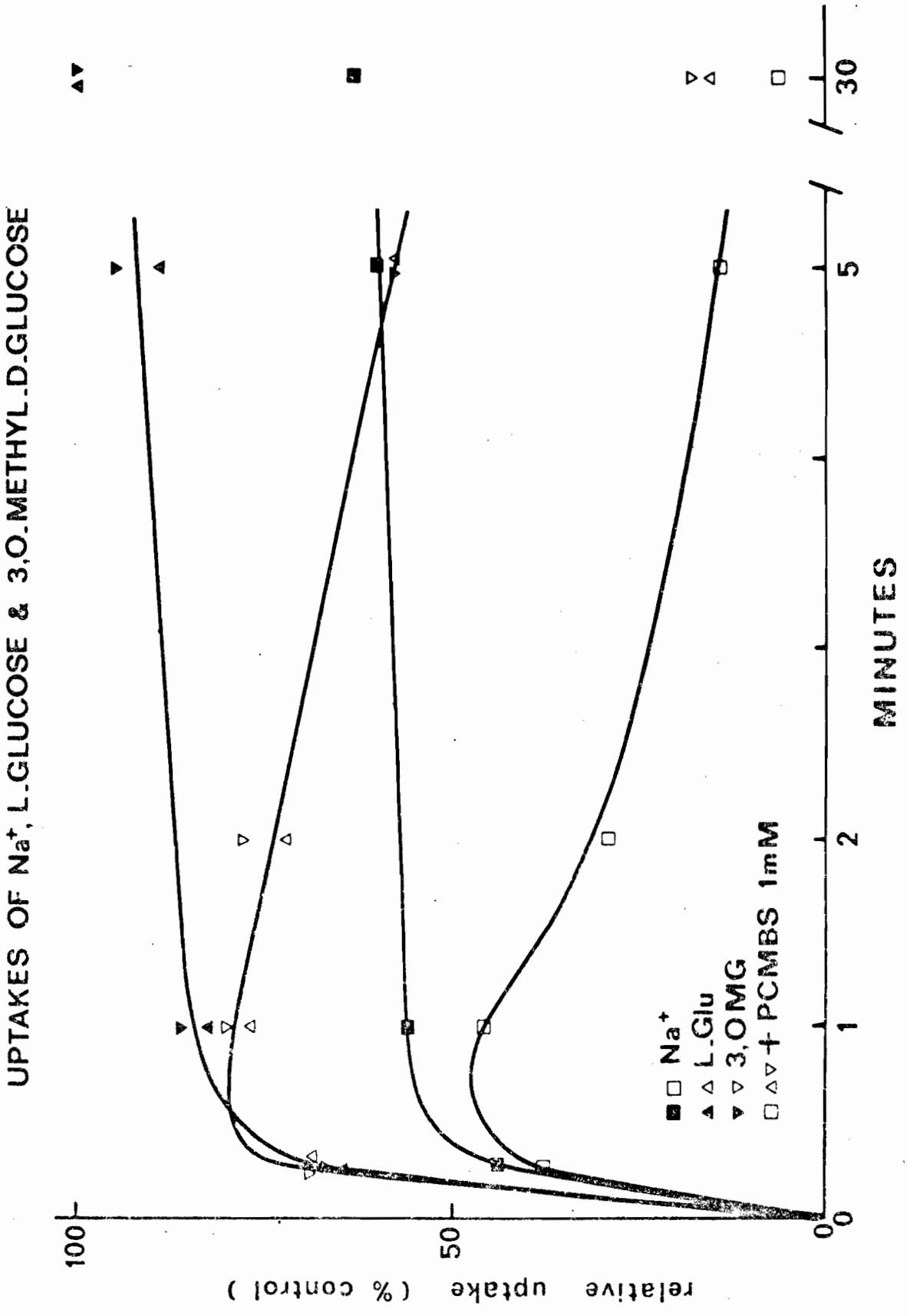
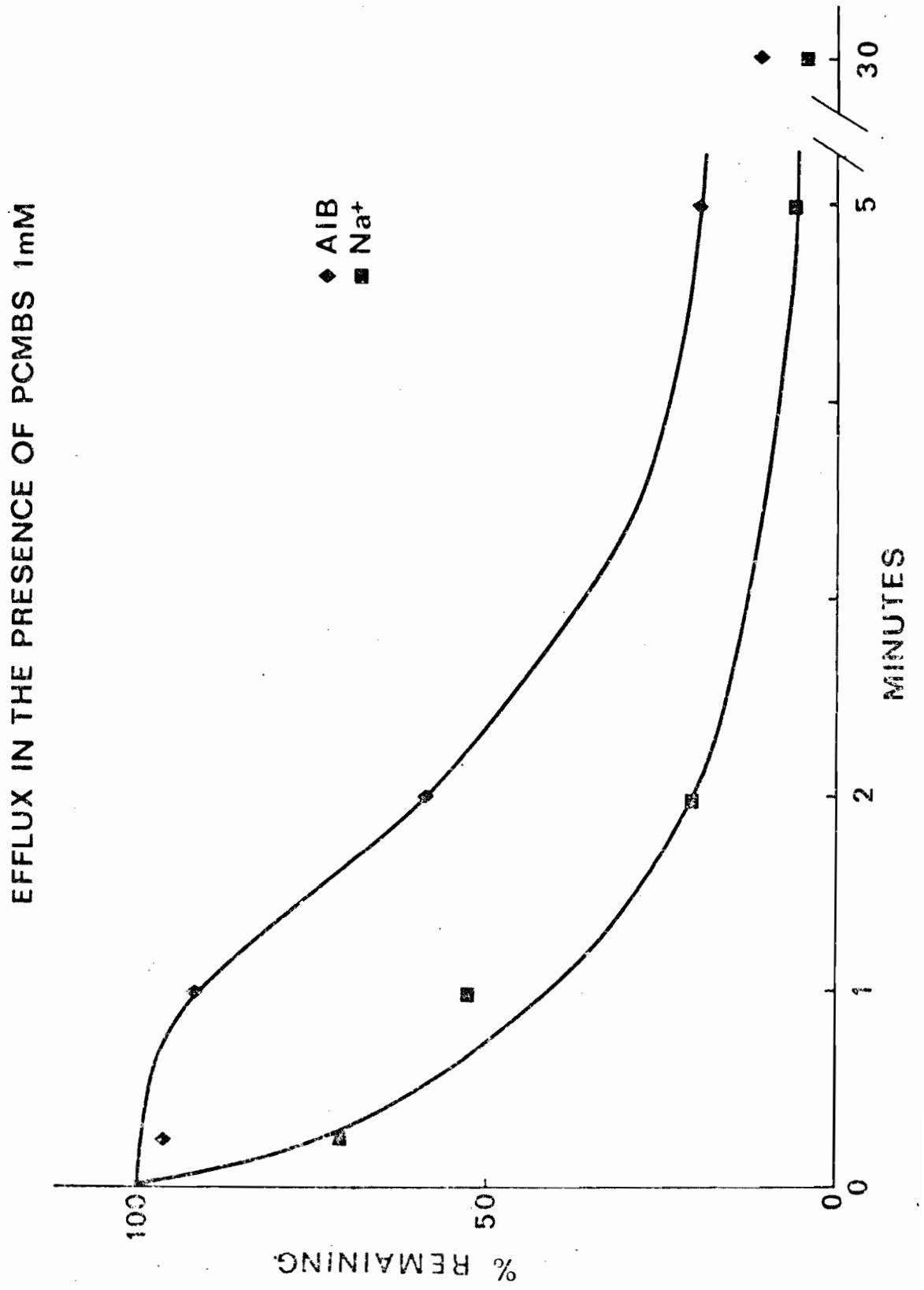

UPTAKES OF Na+, L.GLUCOSE & 3,O.METHYL.D.GLUCOSE
relative uptake (% control)
100
50
0
0
1
2
5
30
MINUTES
Na+
L.Glu
3,OMG
+ PCMBS 1mM

Figure 36. Effect of PCMBS on AIB and Na^+ efflux from native membrane vesicles

Efflux measurements from native membrane vesicles loaded with [1-^{14}C] AIB and $^{22}Na^+$ are performed at 0°C. Other experimental conditions are described in figure 20.


EFFLUX IN THE PRESENCE OF PCMBS 1mM
◆ AIB
■ Na+
% REMAINING
100
50
0
0
1
2
5
30
MINUTES

CHAPTER V: Na^+ Dependent Transport of Amino-Acid In Reconstituted Membrane Vesicles

Na^+ dependent transport in reconstituted membrane vesicles

As has been reported in the introduction, many transport systems in eukaryotic cells require Na^+ as a modifer and cosubstrate for organic solute transport. Na^+ can affect Km, Vmax, or both Km and Vmax for transport. In the previous chapter it has been shown that the reconstituted vesicles take up amino acids.

Since the Na^+ dependent transport system is a major transport system in the Ehrlich Ascites cells, it is very important to demonstrate the restoration of the effects of Na^+ on amino-acid uptake in the reformed vesicles.

The Na^+ dependence of AIB transport is demonstrated in figure 37: the uptake in a K^+ medium and in a choline medium is plotted as a percentage of the steady state uptake in a Na^+ medium; the control uptake in a Na^+ medium is also indicated. In both cases it is apparent that the removal of Na^+ reduced the uptake of AIB by about 60%.

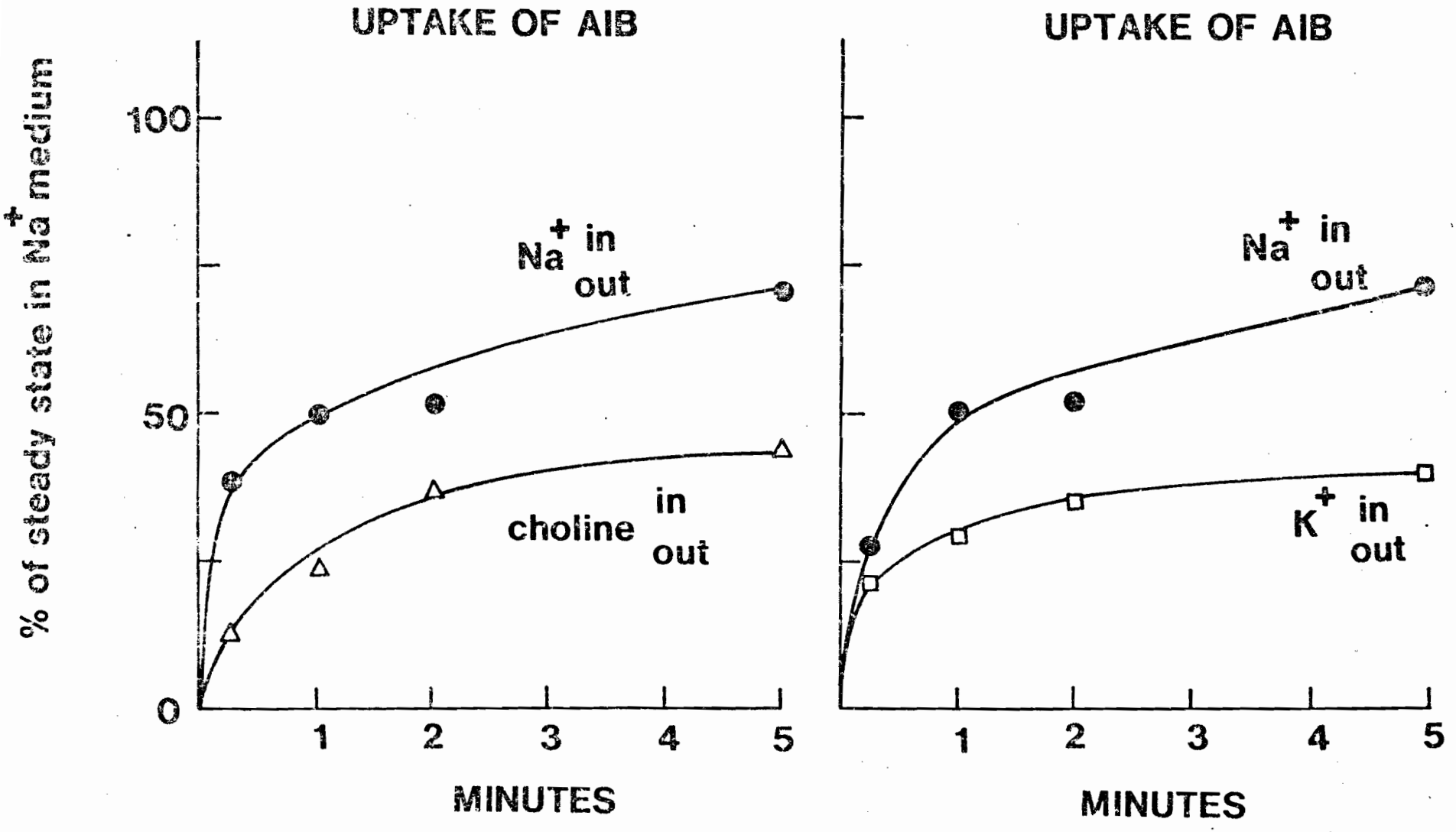
Na^+ dependence has also been verified with different amino-acids, namely phenylalanine, methionine and proline. In contrast with these amino-acids, leucine and ε-aminocaproic acid uptakes are not affected by the removal of Na^+.

Effect of a proton gradient on AIB uptake in intact membrane vesicles

It has been shown that a proton gradient is the driving force for the transport of substrates in bacterial membranes. For this

Figure 37. Uptake of AIB in Na^+ containing and Na^+ free media in reconstituted vesicles.

The experimental conditions are described in figure 18. The vesicles were preincubated in 100 mM of KCl, choline chloride or NaCl as indicated. No change of the medium after the preincubation. The data are an average of three experiments comparing Na^+ and K^+ media, and two experiments comparing choline and Na^+ media.


UPTAKE OF AIB
% of steady state in Na+ medium
100
50
0
Na+ in
out
choline in
out
1
2
3
4
5
MINUTES
UPTAKE OF AIB
Na+ in
out
K+ in
out
1
2
3
4
5
MINUTES

reason, it has been decided to see if an artifically imposed proton gradient could replace a Na^+ gradient to support AIB transport in isolated plasma membranes from the Ehrlich Ascites cells.

First it was necessary to compare the behaviour of the vesicles at the two extreme pH's chosen (6 and 8.5) to the behaviour of the vesicles at the physiological pH (7.4). The test pH values have been chosen because at pH 6 and pH 8.5 there is not likely to be an overall change in the structure of the membrane.

1- AIB and Na^+ uptake at pH 6.

At pH 6, the intravesicular volume was larger than at pH 7.4 so that the apparent greater uptake of AIB at pH 6 than at pH 7.4 was an artefact. The ratio AIB/Na^+, which was used as a measure of the specific transport system, was lower at pH 6 than at pH 7.4.

Furthermore, at pH 6, the uptake was not dependent on the presence of Na^+ gradient (high Na^+ outside). The lack of response to Na^+ at pH 6 has not yet been resolved; it is not clear if the swelling alters the transport system, or if the transport system itself is insensitive to Na^+ at pH 6. The latter possibility is clearly a contributing factor, since Na^+ dependent amino acid transport is very low at pH 6 in the intact cells (252).

Because the uptake of AIB at pH 6 did not prove to be Na^+-dependent, the study of the vesicles at this pH was abandonned.

2- AIB and Na^+ uptake at pH 8.5.

The uptake of AIB at pH 8.5 was less than at pH 7.4; the steady state level was affected but not the rate; this was due, at least in part, to a reduction of 20% in the intravesicular volume. In constrast to the results obtained at pH 6, the uptake of AIB at pH 8.5 was Na^+

dependent and could be stimulated by a Na^+ gradient (high Na^+ outside), in a similar manner to that at pH 7.4 (figure 38).

From these studies it can be concluded that the vesicular volume changes with pH: the lower the pH, the larger the intravesicular volume. Na^+ dependence is not observed when the pH is lowered to 6. When the system shows sensitivity to Na^+, the transport can be further stimulated by a Na^+ gradient.

3- Response to a proton gradient

The imposition of a proton gradient between pH 7.4 and pH 8.5 did not affect the accumulation of AIB in a Na^+ medium. Results are shown in figure 39.

Similarly, the accumulation of AIB in the presence of a Na^+ gradient was not affected by the imposition of a proton gradient between 7.4 and pH 8.5.

If a proton gradient can contribute driving force for accumulation of amino acids, it was not possible to demonstrate its effect, because the magnitude of the proton gradient imposed was not big enough or because denaturation occured.

Effect of Na^+ gradient at pH 7.4 on AIB uptake in reconstituted membrane vesicles

In general with reconstituted vesicles, the imposition of a Na^+ gradient stimulated AIB uptake. The degree of stimulation was highly variable. Moreover the response to Na^+ itself was also variable. Despite variability in the response to Na^+, uptake was never less with Na^+ than without Na^+ in over 100 experiments. In figure 40, three separate experiments are presented, which show the ratio of uptake of AIB with a Na^+ gradient versus uptake without a Na^+ gradient

Figure 38. AIB accumulation at pH 7.4 and 8.5 in native membrane vesicles

The conditions are similar to those described in figure 26. The vesicles are preincubated and incubated either at pH 7.4 or pH 8.5. The data are the mean of two separate experiments.

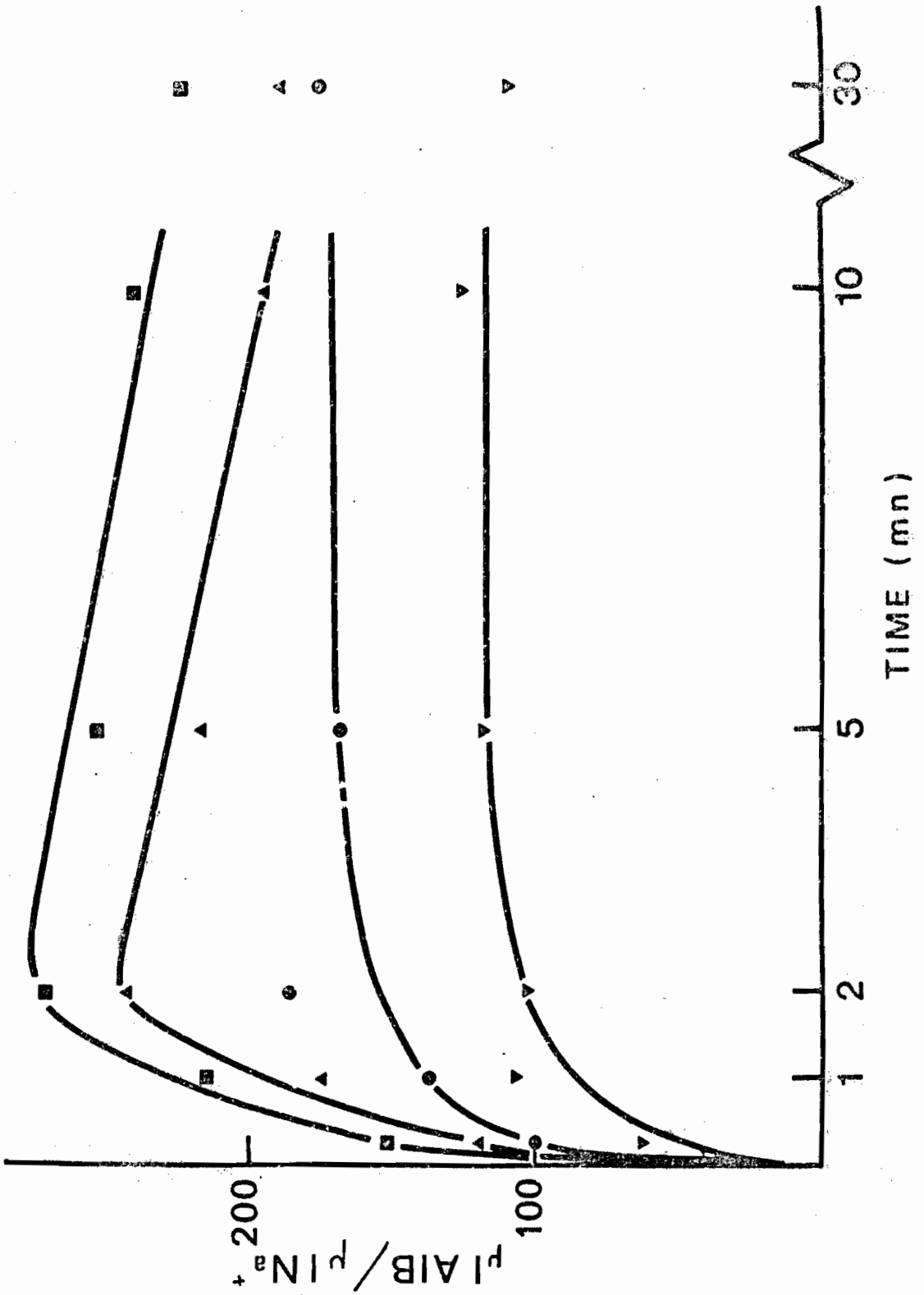

pH 7.5 Na+ med
pH 7.5 Na+ grad
pH 8.5 Na+ med
pH 8.5 Na+ grad
pI AIB/pI Na+
200
100
1
2
5
10
30
TIME (mn)

Figure 39. Effect of a proton gradient between pH 7.4 and pH 8.5 on AIB accumulation in native membrane vesicles

The vesicles were preincubated at 37°C in the standard Na^+ medium (figure 17) at pH 7.4 or pH 8.5, centrifuged and transferred to the standard Na^+ medium at pH 8.5 or pH 7.4 for incubation with [1-^{14}C] AIB and $^{22}Na^+$ at room temperature. The data are the mean of two separate experiments.

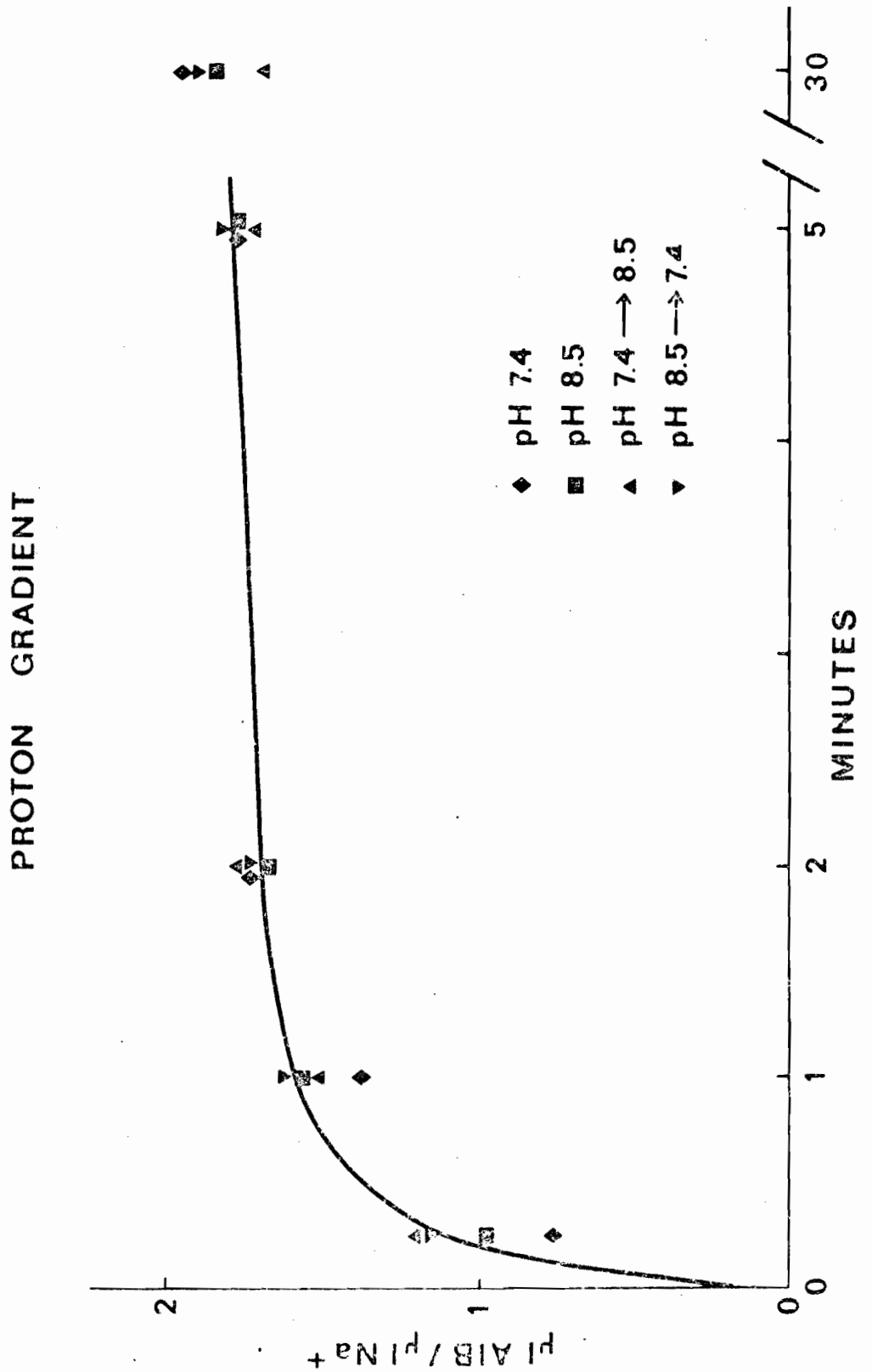
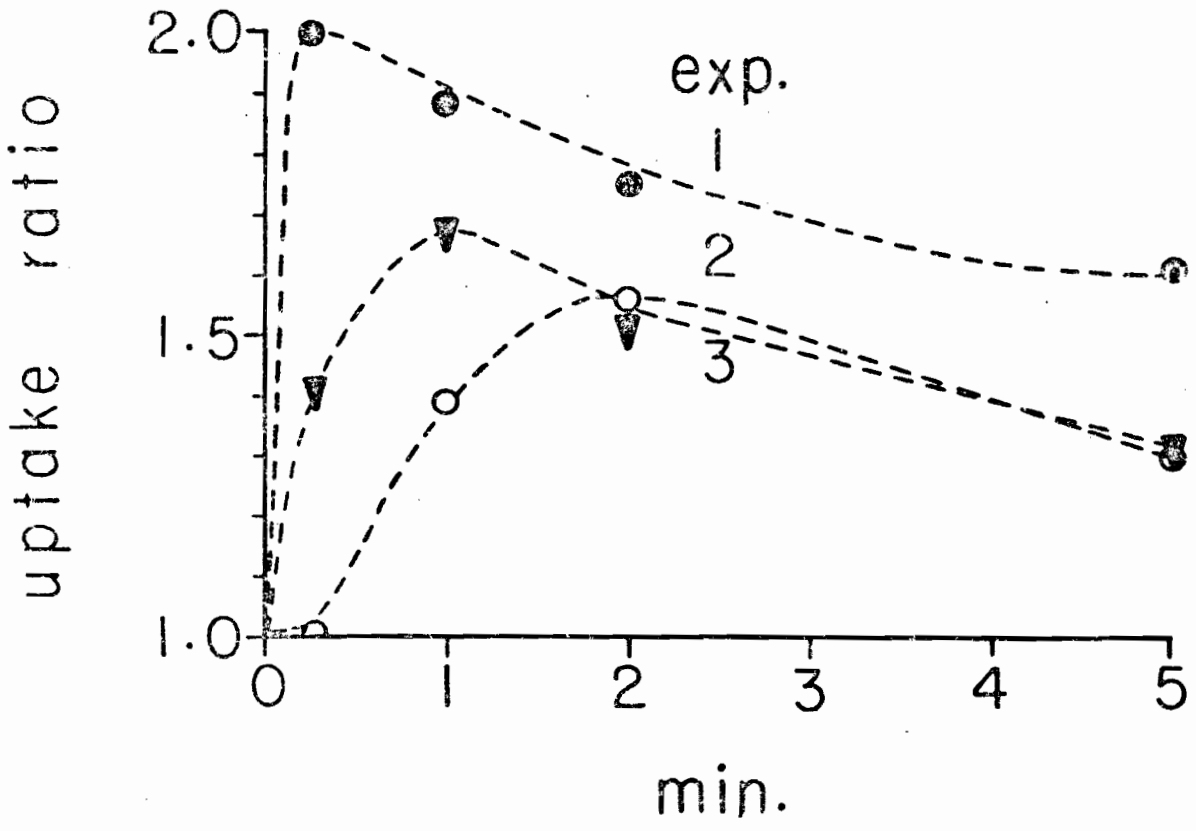

PROTON GRADIENT
pH 7.4
pH 8.5
pH 7.4 → 8.5
pH 8.5 → 7.4
MINUTES
0
1
2
5
30
pI AIB / pI Na+
0
1
2

Figure 40. Stimulation of AIB uptake at pH 7.4 in presence of a Na^+ gradient in reconstituted membrane vesicles

Vesicles were preincubated in 100 mM sodium chloride medium (no gradient) or in a medium in which 100 mM KCl replaced NaCl (Na^+ gradient). After the preincubation the vesicles were centrifuged and resuspended in the standard Na^+ medium containing [1-^{14}C] AIB. Other procedures were as described in figure 18. Three representative experiments are shown. The data are expressed as the ratios of $\frac{\text{uptake with a } Na^+ \text{gradient}}{\text{uptake without a } Na^+ \text{ gradient}}$.

RATIO of AIB UPTAKE in the presence and absence of a Na^+ GRADIENT



plotted against time of incubation. The data show the variability of the experiments with respect to the degree of stimulation, the time at which stimulatory response was seen (15 sec, 1 min, etc.). It is not useful to calculate mean values of all these experiments, because of this variability. Hence individual examples are given. This variability might be due to variabilities in the degree of sealing of the vesicles, permeability to Na^+ and sidedness of the vesicles which at present cannot be controlled.

Figure 41 shows a typical experiment of AIB uptake in a Na^+ medium and when a Na^+ gradient is applied (high Na^+ outside). The stimulation of uptake by the Na^+ gradient cannot be explained by a change in volume of the vesicles loaded with K^+, because Na^+ is taken up to the same level in a Na^+ medium or when a Na^+ gradient is applied.

Effect of a Na^+ gradient at pH 8.5 on AIB uptake in reconstituted membrane vesicles

On the basis of the results obtained with the native membrane vesicles, the effect of a Na^+ gradient at pH 8.5 has been examined in the reformed vesicles. At this pH the results are much more consistent than at pH 7.4. 75% of the experiments showed Na^+ gradient-stimulated AIB uptake. The stimulation observed was of the same order of magnitude as at pH 7.4. Figure 42 shows a mean of four different experiments.

In line with the observations in the native vesicles, it was never possible to show a stimulation of uptake by a Na^+ gradient at pH 6 with reconstituted vesicles.

Figure 41. Na^+ gradient stimulated uptake of AIB at pH 7.4 in reconstituted vesicles

The conditions are described in figure 40. The data show a typical experiment.

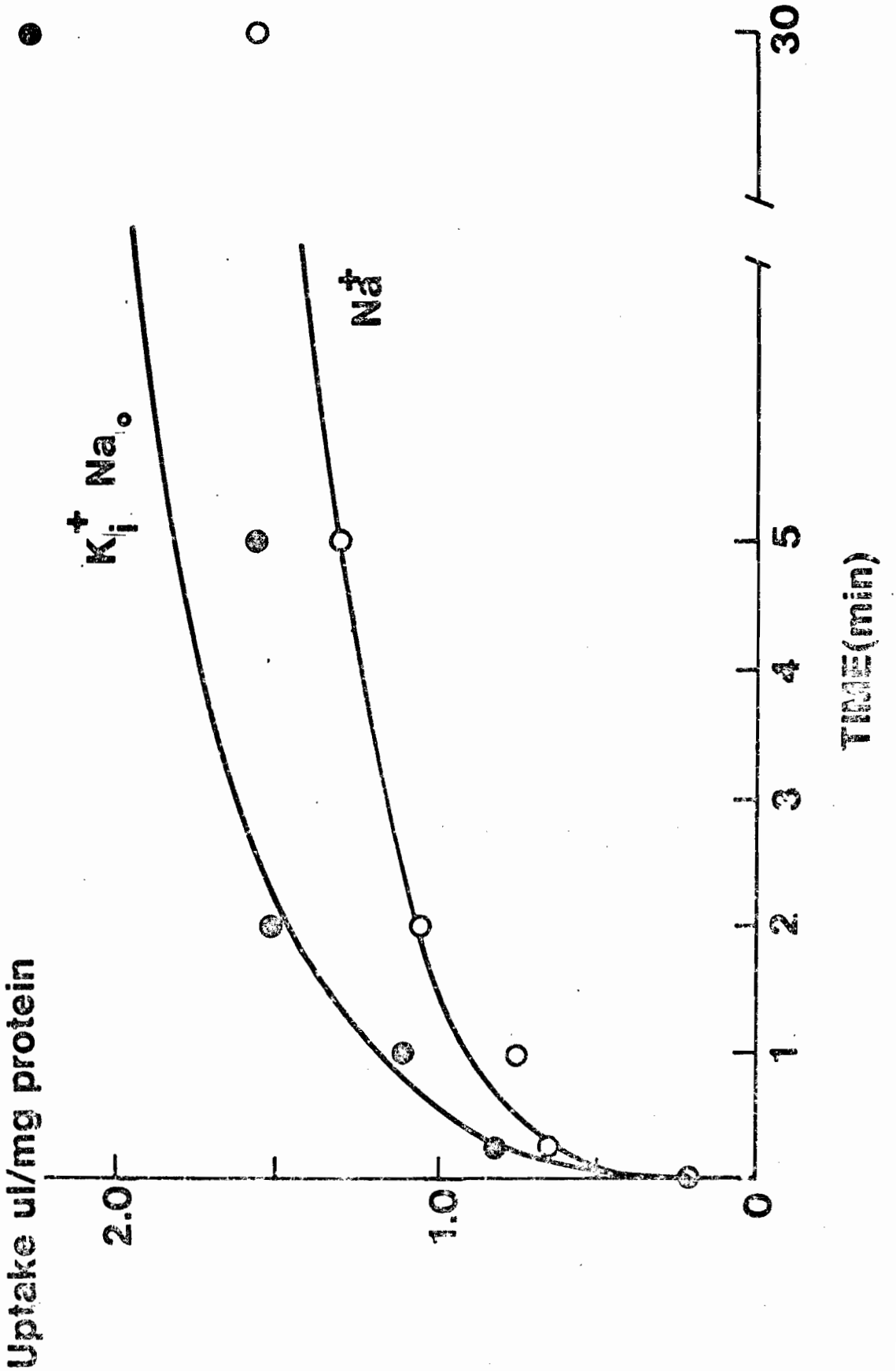
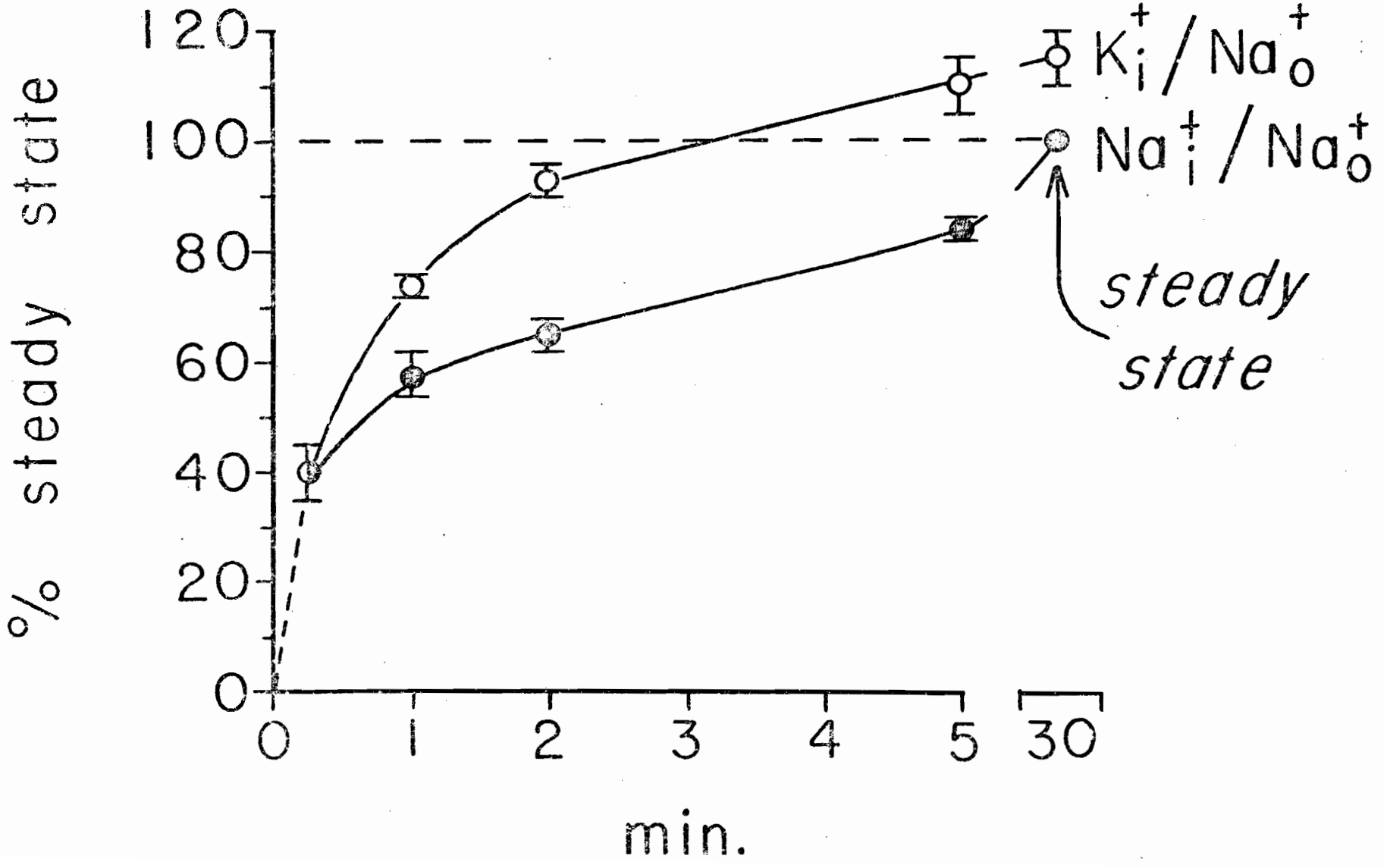

Na^+ GRADIENT AT pH 7.4
Uptake ul/mg protein
2.0
1.0
0
1
2
3
4
5
30
TIME(min)
K^+_i Na^+_o
Na^+

Figure 42. Na^+ gradient stimulated AIB uptake at pH 8.5 in reconstituted membrane vesicles

The conditions are as in figure 40, except that Tris buffer at pH 8.5 was used. The data shown are the mean of three experiments. The vertical bars are the variations from the mean value.

gradient effect on AIB uptake at pH 8·5



Evidence for amino acid accumulation against a chemical potential

To obtain additional and more direct evidence for the accumulation of AIB against its concentration gradient, the following experiments were designed: The vesicles were loaded with AIB for 30 minutes in a K^+ medium; at this time, the AIB taken up should be close to an equilibrium position. If a Na^+ gradient is imposed, that is, if the vesicles are transferred to a Na^+ medium without changing the AIB concentration, uptake of AIB should increase if movement against a concentration gradient can occur. However, if the vesicles are transferred to a fresh K^+ medium containing the same concentration of AIB, the level of AIB in the vesicles should remain unchanged if the solute was equilibrated, or continue to increase if the level before transfer was remote from the steady state position. The data (figure 43) show that when the vesicles loaded with K^+ and AIB were transferred to a medium containing Na^+ and AIB, there was a further increase, albeit small, in AIB taken up by the vesicles; this effect was consistently observed. In contrast, vesicles transferred to K^+ and AIB showed little, if any, further increase. Table VII summarizes the separate experiments.

AIB efflux from the reconstituted membrane vesicles

It is relevant to know whether by reversing the direction of the Na^+ gradient it is possible to show gradient-stimulated AIB exodus.

Vesicles were preincubated with Na^+ and ^{14}C-AIB; they were then transferred to a medium containing either Na^+, K^+ or K^++valinomycin, but no amino acid. As shown in figure 44, exodus of AIB from the vesicles was increased in a K^+ medium as compared to a Na^+ medium.

Figure 43. Uptake of AIB at pH 7.4 against a concentration gradient in reconstituted vesicles

After sealing, the vesicles were incubated for 30 minutes at 20°C in a KCl medium containing 0.5 mM [1-^{14}C] AIB. A sample was taken at the end of 30 minutes to determine the nmoles amino acid per mg protein (initial vesicular amino acid). The vesicles were centrifuged and resuspended in fresh Na^+ or K^+ medium containing [1-^{14}C] AIB at the same concentration and specific activity as in the pretreatment. Samples were taken at intervals at 20°C. The data show a typical experiment.

uptake against a concentration gradient

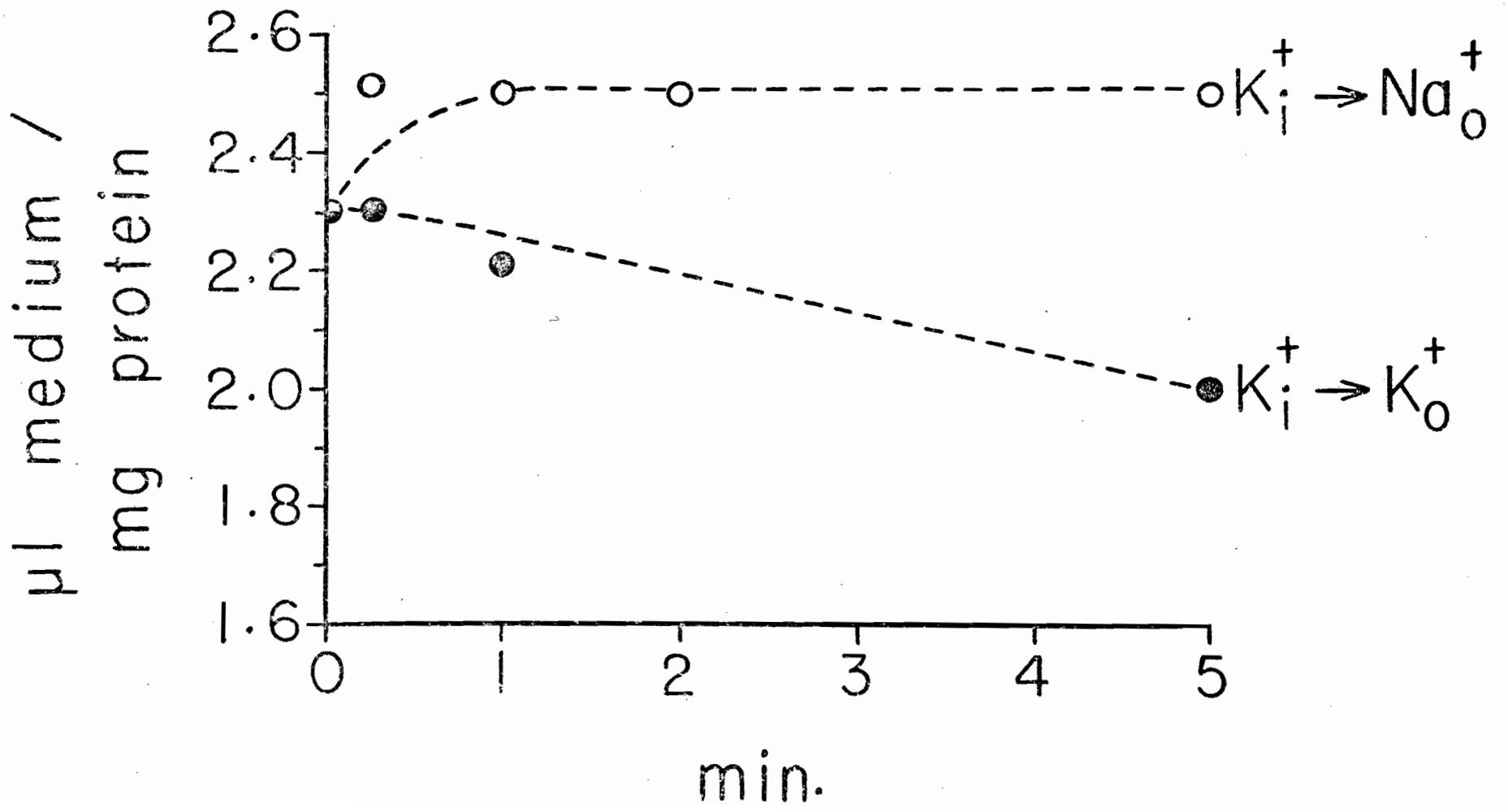


TABLE VII

Accumulation of α-aminoisobutyrate against its chemical potential.

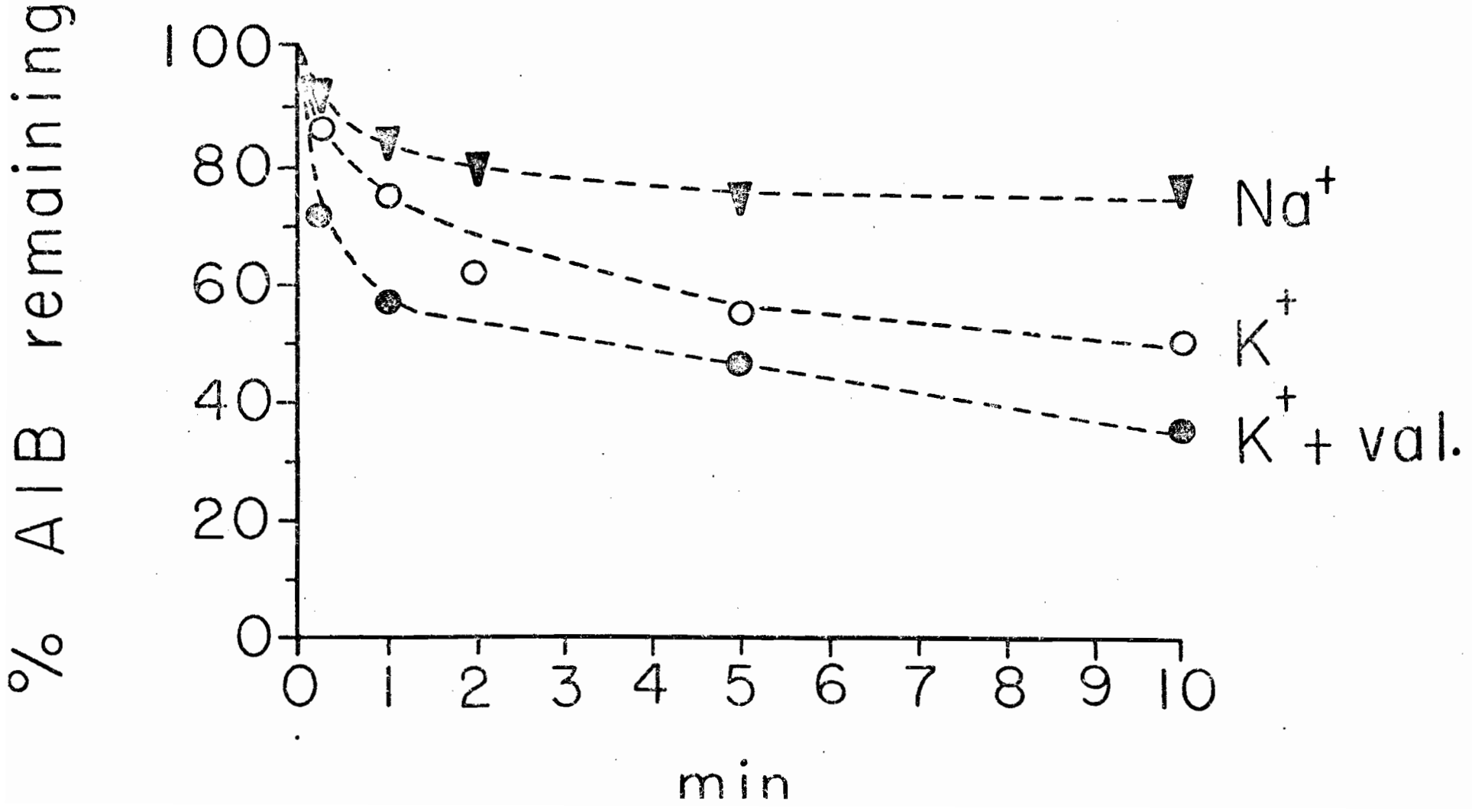
Medium:	Percent initial α-aminoisobutyrate at							
	15"		1'		2'		20'	
	Na^+	K^+	Na^+	K^+	Na^+	K^+	Na^+	K^+
Initial vesicular amino acid content per mg protein:								
I. 1.15 nmols	112	100	112	96	112	104	123	83
II. 1.04 nmols	139	119	138	123	142	100	160	101
III. 1.54 nmols	107	90	135	95	124	88	128	97
IV. 1.00 nmols	128	111	135	118	137	125	147	110
V. 1.20 nmols	117	103	116	106	116	106	112	86
Mean ratio of uptakes in Na^+/K^+	1.15±.001 S.D.		1.18±.03 S.D.		1.22±.07 S.D.		1.40±.03 S.D.	

Experimental conditions are described in Figure 43.

Figure 44. Na^+ gradient stimulated AIB efflux from reconstituted vesicles

After preincubation, the vesicles were incubated for 30 minutes at room temperature in the standard Na^+ medium containing [1-^{14}C] AIB. Then 50 or 100 µl of the suspension was added to 2 ml of NaCl or KCl medium, without or with 5 µg/ml of valinomycin. At the times noted, the vesicles were filtered, washed and dried. The data are the mean of three separate experiments.

gradient - stimulated AIB efflux



Valinomycin could further increase the loss of ^{14}C-AIB from the vesicles in a K^+ medium.

Control experiments were conducted to verify if the stimulation by valinomycin was specifically due to the increase in K^+ permeability. The results are shown in figure 45. It can be seen that valinomycin did not increase the exodus of AIB in a Na^+ medium.

Moreover it could be demonstrated (figure 46) that AIB efflux was stimulated by a Na^+ gradient only when there was high intravesicular Na^+ and no Na^+ externally. If the vesicles were loaded with K^+ and AIB and then transferred to either a K^+ or a Na^+ medium, the loss of AIB from the vesicles was the same. The increased movement of AIB from the vesicles is thus linked to the outflow of Na^+.

Figure 45. Effect of valinomycin on AIB exodus from reconstituted vesicles

Experimental conditions are described in figure 44. As valinomycin is used as an ethanolic solution, the absence of effect of the same concentration of ethanol (10 µl/ml) on AIB exodus was verified.

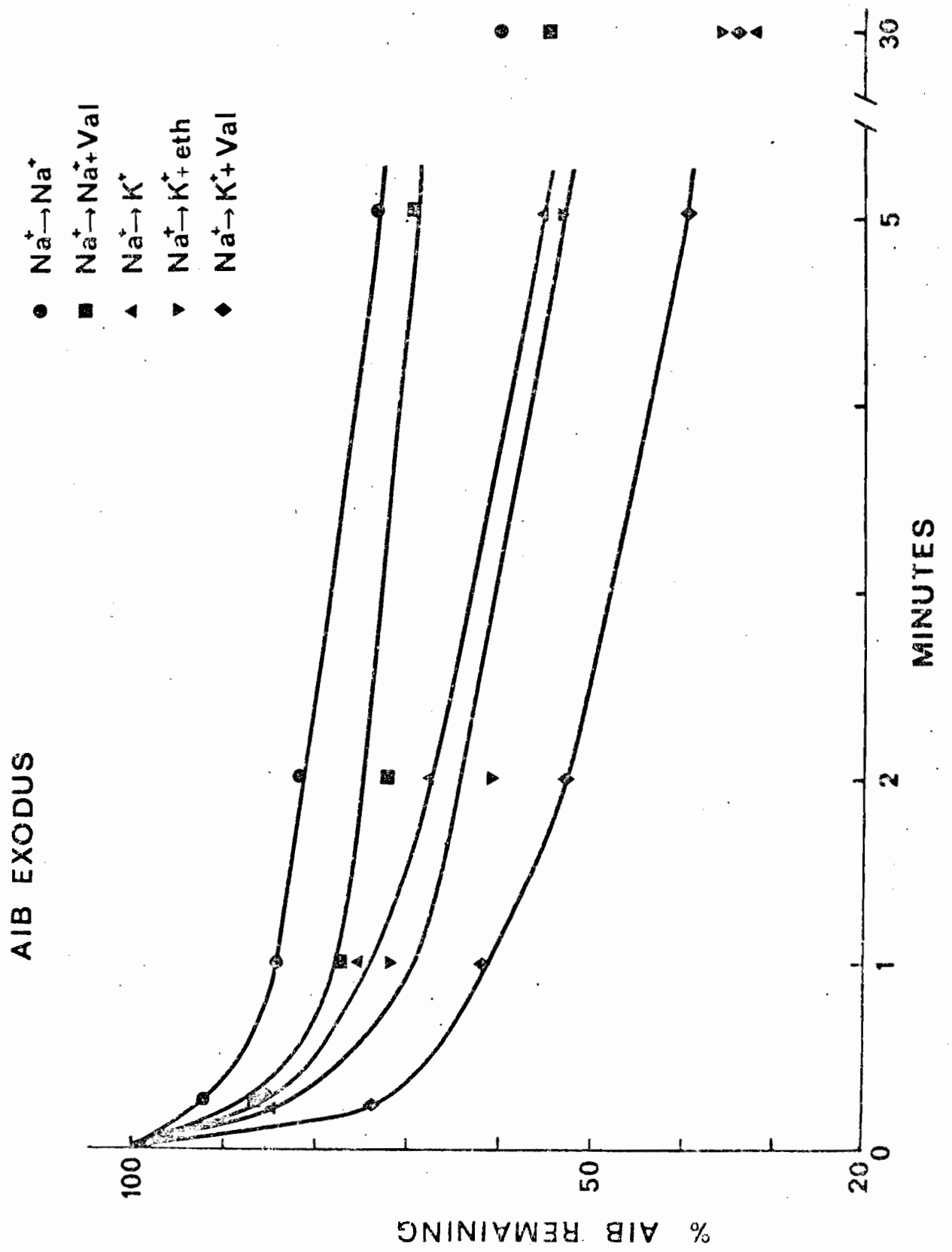
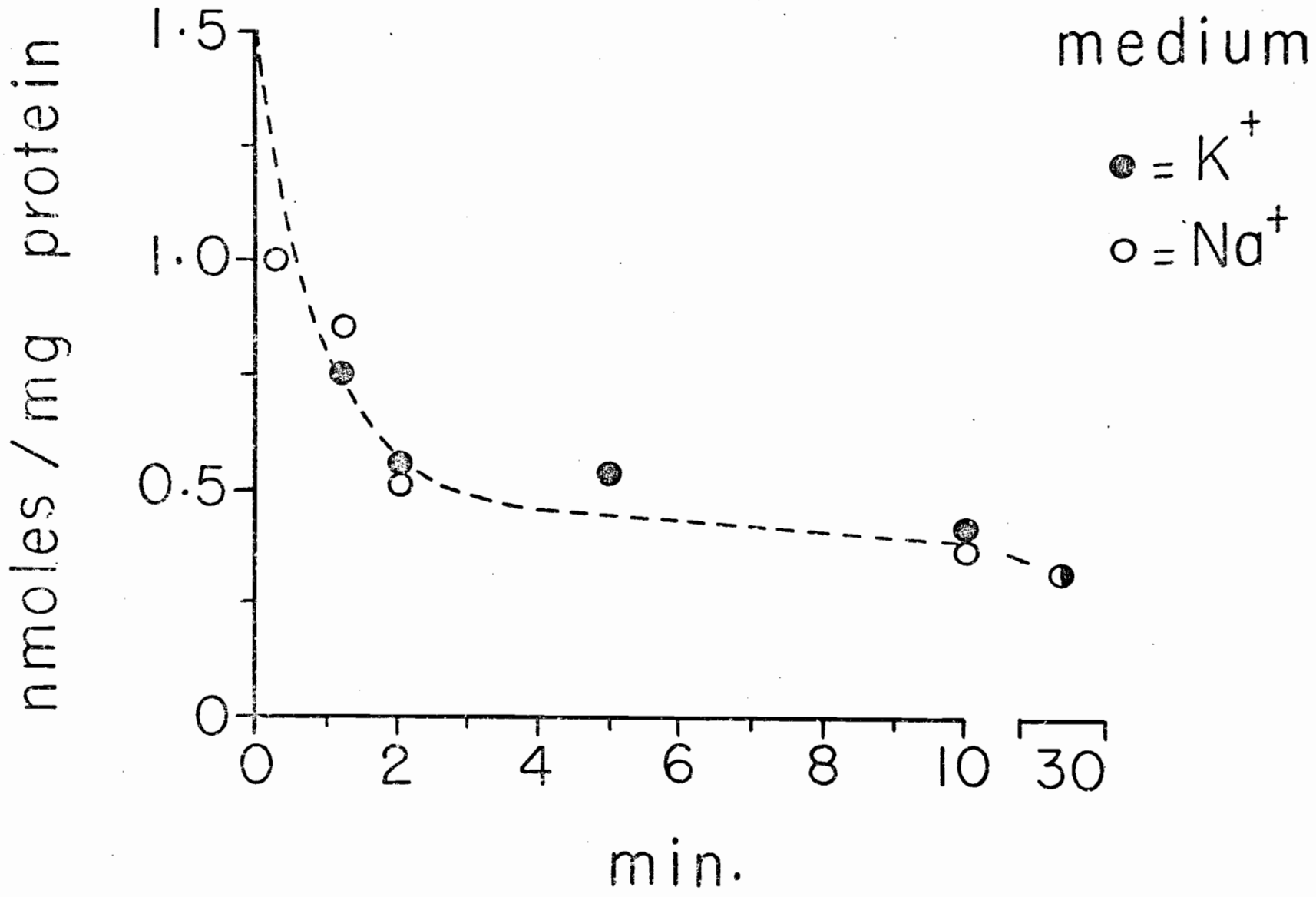

AIB EXODUS
$Na^+ \rightarrow Na^+$
$Na^+ \rightarrow Na^+ + Val$
$Na^+ \rightarrow K^+$
$Na^+ \rightarrow K^+ + eth$
$Na^+ \rightarrow K^+ + Val$
% AIB REMAINING
100
50
20
0
1
2
5
30
MINUTES

Figure 46. AIB efflux from K^+ loaded reconstituted vesicles

The experimental conditions are described in figure 44. The vesicles were preincubated in a K^+ medium and efflux was followed either in a Na^+ medium or in a K^+ medium.

AIB efflux from vesicles containing K_i^+



CHAPTER VI: Additional Membrane Functions Restored

Sugar transport activity

In the intact Ehrlich cell, 3-O methylglucose is transported by a facilitated process, but is not accumulated (261). No Na^+ dependence is observed. The same results have been obtained with the isolated membrane vesicles (183). The non metabolizable sugar, L-glucose, is not transported into the Ehrlich cell. It enters the cell by passive diffusion.

The uptake of these two sugars has been followed in the vesicles reconstituted with asolectin. The results (figure 47) show that the uptake of 3-O methylglucose is more rapid than that of L-glucose but after 30 minute incubation at 20°C the steady state is not obtained.

Furthermore previous studies using vesicles reconstituted with pure phospholipids show that the uptake of 3-O methyl glucose can be inhibited by the addition of D-glucose (30 mM) or sorbitol (30 mM) (figure 48). It is unlikely that the observed inhibition by D-glucose and D-sorbitol is due to vesicle shrinkage because these sugars are permeant and the space as measured by ^{22}Na was unchanged by the addition of these sugars.

All these considerations lead to the conclusion that at least some sugar transport activity has been restored in the reconstituted vesicles.

The (Na^+ + K^+) ATPase

The Ehrlich Ascites Tumor Cells possess a (Na^+ + K^+) activated ATPase (262). This ATPase has also been characterized in the isolated plasma membrane vesicles.

The presence of (Na^+ + K^+) ATPase activity in the reconstituted membrane vesicles has been examined. As with Na^+ stimulated transport,

Figure 47. Sugar uptake in reconstituted vesicles

Experimental conditions are described in figure 18. L-[1-^{14}C] glucose (specific activity 51.6 mCi/mmole) was used at a concentration of 0.1 mM. The data are the mean of two separate experiments. 3-O[methyl-^{14}C] glucose (specific activity 52.7 mCi/mmole) was used at a concentration of 0.09 mM. The data are the mean of two separate expriments. The control represents the 30 minute uptake of 3-OMG.

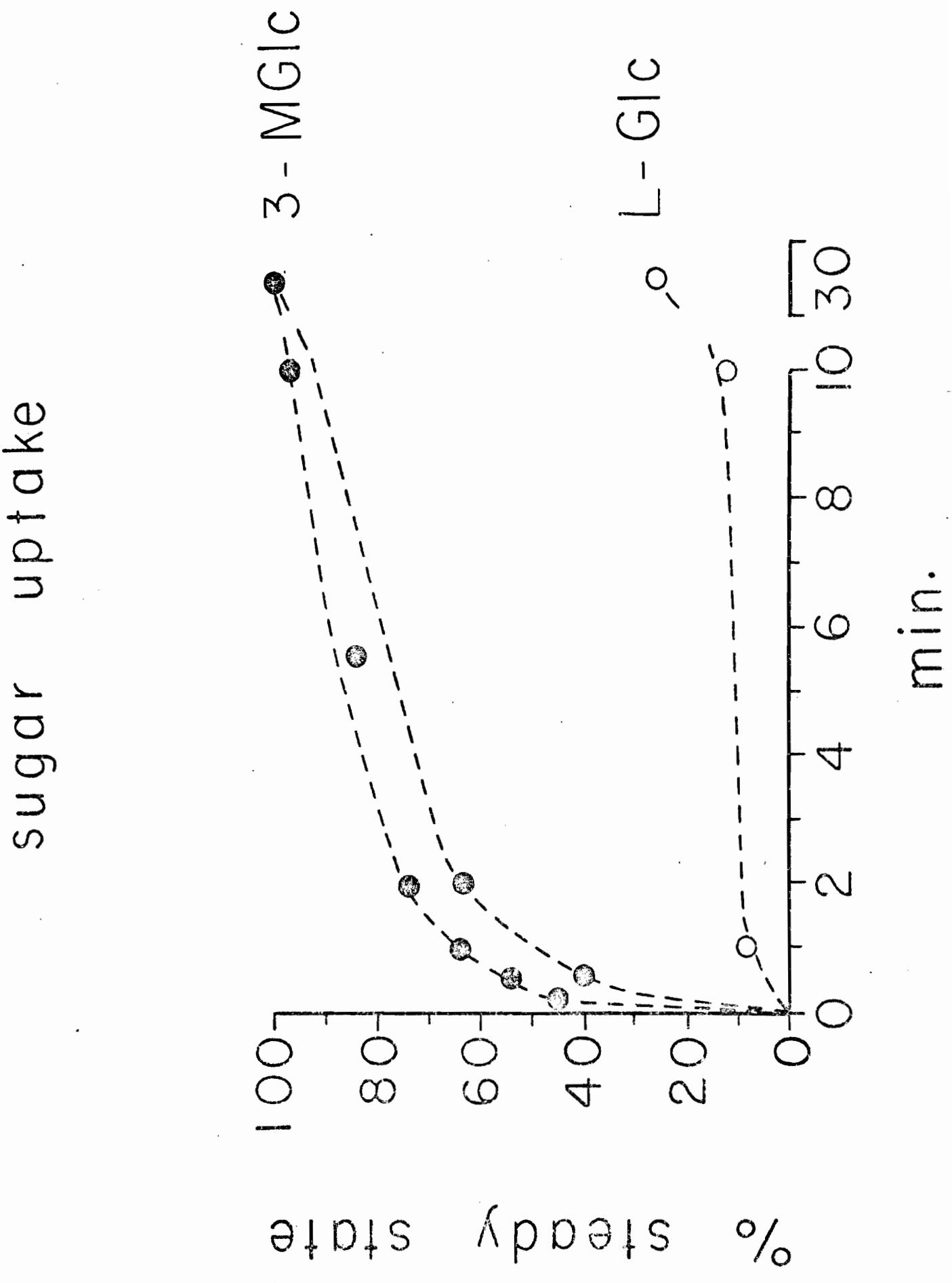
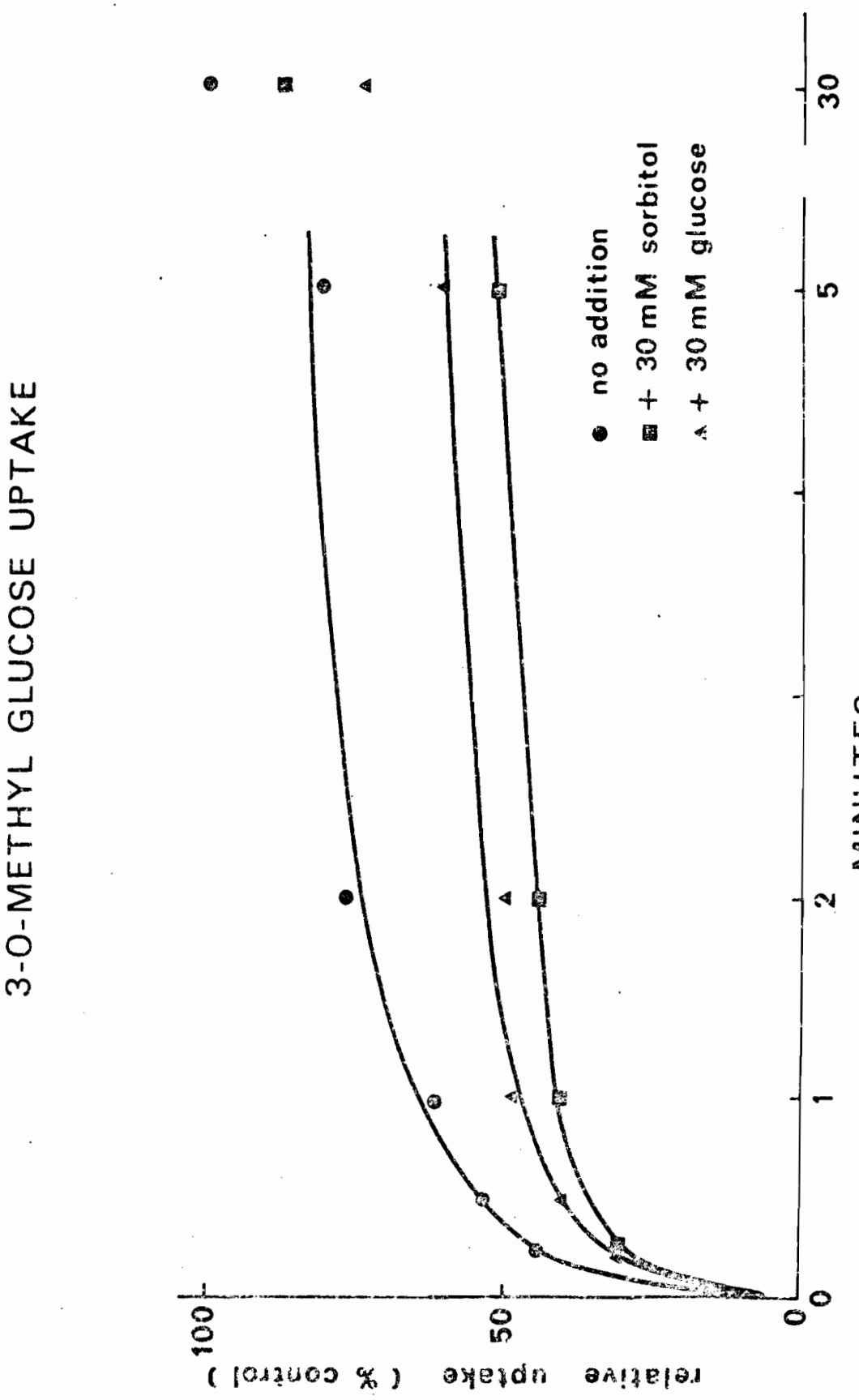

sugar uptake
3-MGlc
L-Glc
% steady state
100
80
60
40
20
0
0
2
4
6
8
10
30
min.

Figure 48. Action of D-glucose and D-sorbitol on 3-OMG uptake in reconstituted vesicles

The experimental conditions are similar to those of figure 47. The vesicles were reconstituted with phosphatidylethanolamine and phosphatidylcholine (1:1). The data are the mean of two experiments. The control represents the 30 minute uptake of 3-OMG in the absence of other sugars (●).


3-O-METHYL GLUCOSE UPTAKE
relative uptake (% control)
100
50
0
0
1
2
5
30
MINUTES
● no addition
■ + 30 mM sorbitol
▲ + 30 mM glucose

the reconstitution of the ($Na^+ + K^+$) ATPase is variable and in a sizeable percentage of experiments (40%) no measurable activiy is recovered. When the Na^+ stimulated ATPase can be demonstrated the rate of ATP hydrolysis per mg of protein is variable. Nonetheless, ATPase activity has been recovered in sufficient instances so that it is clearly not an aberration. The data in Table VIII show the results of ATPase assays with 2 different preparations of reconstituted vesicles I and II. In the case of preparation II, total ATPase activity was measured with vesicles treated with 1% Triton X-100 in order to solubilize all the enzyme, which then becomes accessible to ATP.

From these data it can be concluded that the reconstituted vesicles possess a ouabain sensitive, ($Na^+ + K^+$) activated ATPase activity, the ATPase activity measured is variable, all the ATPase activity is not detected unless the vesicles are treated with Triton-X100. These data suggest that 1) there is a mixed population of sealed vesicles, some oriented in the normal direction and some being inside-out, so that only a percentage of the total ATPase is measured. The percentage of vesicles oriented in one direction compared to the other direction is highly variable. Or 2) all the vesicles are oriented in the normal way, but the sealing is poor so that ATP leaks in, albeit slowly, giving measurable ATPase. With Triton, the rate of entry of ATP is not limiting and ATPase activity is increased. Or 3) a mixture of 1) and 2) is also possible.

TABLE VIII

ATPase ACTIVITY OF THE RECONSTITUTED VESICLES

Incubation Medium	Vesicle Preparation I	II	II + Triton X-100 1%
no Na^+	16	42	43
75 mM Na^+	103	53	135
75 mM Na^+ + 1 mM ouabain	5	31	24

The ATPase activity is expressed as the amount of ATP in nmoles hydrolyzed in 10' by 1 mg of reconstituted vesicles.

The vesicles, suspended in a medium containing 10 mM choline chloride, 5 mM Tris-HCl (pH 7.4), 0.1 mM $CaCl_2$ and 0.1 mM $MgCl_2$ were incubated for 10' in a medium containing 40 mM Tris-HCl (pH 7.4), 75 mM NaCl or choline chloride, 5 mM KCl, 0.5 mM Tris-EGTA (pH 7.4), 150 µM $MgCl_2$ and 250 µM ^{32}P-ATP. The incubation was stopped by the addition of 1 ml of ice cold 5% TCA.

20 µl samples were taken to measure the total radioactivity of the medium. 100 mg of charcoal were added to absorb non-hydrolyzed ATP. After filtration 200 µl samples were taken to measure the amount of ^{32}P liberated.

DISCUSSION

The biochemist has always been interested in the understanding of what is happening in the living cell at the molecular level. In order to satisfy his curiosity, his approach has often been the dissociation, purification and restoration of the original system.

Transport activity is one of the systems where a lot of effort have been made in attempts to describe the observed phenomena, but no information on how a solute can traverse a membrane is available.

To get to the molecular reactions involved in transport, simpler systems than whole cells have been developed such as subcellular particles in the form of membrane vesicles. But this is still not sufficient. The new approach, the biochemical one, is to see if it is possible to disaggregate the membrane, to isolate the transport proteins, and to see how the system works after its reincorporation in a vesicle like structure.

The objective of this work was to see if transport activity could withstand membrane solubilization and be restored in reconstituted vesicles.

Solubilization of Membranes

A true solubilization of membranes means being able to separate the various chemical species from each other as well as from the bulk phase lipid components. The main forces involved in association of proteins with lipids being hydrophobic, the method of dissociation must reduce hydrophobic interactions while retaining the catalytic activity of the proteins.

Sodium cholate was chosen as the solubilizing agent because it is a mild detergent with a low CMC, it has a low molecular weight and can be readily removed, and because it has been successfully used by Racker for solubilization and reconstitution of mitochondrial enzymes (216, 217 231, 262, 263). Cholate by itself (2%) did not prove to be a sufficiently powerful solubilizing agent of the plasma membranes of the Ehrlich Ascites cells, solubilizing only 40% of the membrane proteins after 30 minutes at 4°C. Urea was introduced into the medium for extraction since Garewal et al. (238) had used urea to improve solubilization of mitochondria by Triton X-100. Addition of 4 M urea to cholate increased the degree of solubilization of the membrane proteins to 90%. The pellet of non-solubilized material obtained after a 2 hour centrifugation at 150,000 g must be largely lipids, since only 40% of the phospholipids were recovered in the supernatant.

Soluble State

When is "soluble" really soluble?

As a working definition "soluble material" has been designated as that material which does not sediment at 150,000 g. This does not necessarily mean that the suspension is truly dispersed into its molecular components. Lipid-protein aggregates may still be present if the lipids associated with the proteins can keep the density sufficiently low to prevent sedimentation. In the present work, additional observations show that the molecular size of the units in the supernatant are below the size of pieces of membranes and more in line with a molecular dispersion. Thus, this solubilized material is able to penetrate a 5% polyacrylamide gel indicating a unit size of less than 10^6. No organized structure is detectable under the electron microscope. The

globular structures probably represent lipoproteins or lipid globules, since their size grow with time and when the sample is kept in the cold. None of the globules is seen in freshly prepared samples. Their formation and presence probably accounts for the fact that no material penetrated the 5% polyacrylamide gel when electrophoresis was performed at 4°C.

Reconstitution of Membrane Vesicles

Physical and chemical analyses show that the material brought down by centrifugation after removal of cholate and urea by dialysis is vesicular in nature.

Electron micrographs of fixed thin sections or negative staining procedures as well as freeze-fracture show evidence of particle-free vesicles limited by a membrane, usually multilamellar. The size of the vesicular structures (0.3 to 0.5 μ in diameter) observed after reconstitution is more homogenous than that observed with the native membrane vesicles.

Although vesicle formation, as indicated by electron microscopy, requires proteins, there is no direct evidence from the micrographs that the proteins form part of the limiting membrane structure and do not in some manner enhance the formation of liposomes which may actually trap the protein. A number of experimental observations suggest incorporation of the proteins into the limiting membrane:

1- liposomes can be formed in cold. These "vesicles" do not show in electronmicrographs the dark outline characteristic of the vesicles reformed with membrane proteins.

2- the core of the reformed vesicles appear devoid of material. If proteins were trapped inside, they would be expected to come out of

solution, aggregate and form electron dense masses.

3- Freeze fracture studies showing "graining" on the surface are consistent with proteins embedded in a lipid bilayer. Smooth areas in the photograph suggest liposomes lacking proteins in the bilayer.

4- Thermodynamically, membrane proteins will tend to associate with lipids rather than being trapped in a liposome. Nevertheless the existence of protein free liposomes is not excluded.

Experimental evidence for incorporation of the proteins into the bilayer also comes from the transport studies. Vesicles formed with lipids alone are unable to retain amino acids. Lipid vesicles would be unlikely able to show competitive interaction between amino acids nor between D and L sugars. Since the transport of solute is sensitive to osmotic strength, it is unlikely that the discrimination between solutes is due to binding. The fact that uptake is sensitive to osmotic activity also supports the conclusion that the membrane proteins have been reincorporated into the bilayer.

A second problem linked to reconstitution is the problem of orientation of proteins in the reformed vesicles. How is the proper orientation achieved? As stated by Racker (236), there are three possibilities: "the information is in the protein or in the phospholipid or in neither, i.e. there is something else in the membrane that does the directing". In the case of bacteriorhodopsin, it is the protein itself which directs the orientation (254). The curvature of the liposome can also affect the orientation of protein (255). But in most cases of reconstitution by dialysis of the detergent unindirectional orientation of the protein is not achieved. According to the sialic acid measurements and ATPase results the preparations of

reconstituted vesicles are rather heterogenous, compared to the original membrane vesicles. Racker and Kandrach used an external inhibitor such as polylysine to impose asymmetry when reconstituting vesicles with cytochrome c (256). Racker and his coworkers also propose incorporation of proteins into preexisting liposomes containing lysolecithin (233) or acidic phospholipids (234). These methods avoid sonication and dialysis, do not involve the disruption of the liposome structure, allow the study of the effects of size, composition and asymmetry of the liposomes as well as the study of the selectivity of the process. They lead to unindirectional orientation of the proteins. These and similar procedures have not yet been applied to reconstitution of amino acid transport from Ehrlich cell membrane proteins.

The reconstituted vesicles preparation obtained here also show some multilayer vesicles which may interfere with uptake measurements. Methods of preparing single layer liposomes have been described (257, 258) and it could be interesting to apply Racker's incorporation of proteins into these liposomes in order to get right side out single layer vesicles from the solubilized membranes of the Ehrlich Ascites cells.

The formation of reconstituted vesicles is dependent on a number of factors including the temperature of reconstitution, the presence of ions during the reconstitution, the presence and nature of phospholipids.

The optimum temperature for reconstitution appears to be variable depending on the nature of the particular system. Thus Meissner and Fleischer (220) found that Ca^{+}-ATPase activity of sarcoplasmic reticulum was best recovered if the cholate solubilized reticulum was dialyzed

at 20°C. In the present work, activity was obtained when reconstitution was carried out at 4°C. Moreover in most experiments with more purified systems, reconstitution appeared to be best at 4°C.

Divalent cations are necessary to keep the integrity of the membrane (235) 0.1 mM Ca^{++} and 0.1 mM Mg^{++} have been found to be the optimal cation concentration for reconstitution.

It is believed (32) that membrane proteins need a specific environment provided by the so-called boundary lipids. In the reconstitution experiments no attempt was made to assess the boundary lipids and whether they are required. The problem of phospholipid specificity and efficiency for reconstitution of vesicles has been studied in a preliminary way. It was found that the non-natural phospholipids, such as β-phosphatidyl ethanolamine dipalmitoyl, do not yield functional reconstituted vesicles. With phospholipids leading to the formation of vesicles capable of restoring AIB transport activity, it was necessary to check if the phospholipid vesicles themselves were capable of taking up AIB. If the latter happens, one would expect a mixed population of phospholipid vesicles and phospholipid-protein vesicles. Such appeared to be the case with phosphatidyl choline dipalmitoyl. Because the non-specific uptake was large, it was deemed unsatisfactory as a test system. The best results (minimal non-specific uptake relative to total uptake) were obtained with asolectin, a mixture of soybean lipids. The apparent absence of a requirement for a specific lipid may be due to the fact that there is no predelipidation of the membrane material before reconstitution. The essential phospholipids are probably present in the solubilized membranes, since 60% of the original phospholipids are found in the reconstituted vesicles. The

addition of cholesterol had no measurable effect.

Vesicles of different sizes were obtained by varying the protein/exogenous lipid ratio for reconstitution. As measured by the Na^+ uptake, the largest vesicles were obtained in the presence of excess lipids. However the best activity was obtained when a ratio protein/exogenous lipid close to 1 was used for reconstitution.

Composition of the Reconstituted Vesicles

The overall composition of the reconstituted vesicles is quite similar to that of the native membrane vesicles. The reconstituted vesicles have a protein/lipid ratio of 66/34 (w/w) compared to 68/32 (w/w) for the native vesicles. The reconstituted vesicles have a sialic acid content 3 to 4 times greater per mg of protein than the native vesicles. In reconstituted vesicles all the sialic acid is not accessible to neuraminidase in contrast with the native vesicles. While this suggests a heterogenous population of inside out and right side out vesicles, other possibilities are not excluded. If the vesicles are multilayered the sialic acid of the internal layers may not be accessible to neuraminidase. Alternatively in the native membranes, the ease with which all the neuraminic acid is removed by neuraminidase may indicate that the vesicles are not sealed to the enzyme and even internalized neuraminic acid residues from inside out vesicles is released. It has not yet been possible to determine whether the functional native vesicles are all right side out. The data, which suggest that they are, is the Km value which is close to that of intact cells. Studies examining the Km for AIB and glycine at the cytoplasmic surface show a much higher Km (264). No systematic attempt has been made to impose

a specific orientation of the proteins during reconstitution.

When the normal and reconstituted vesicles were subjected to SDS polyacrylamide gel electrophoresis, similar protein profiles are obtained although the major proteins are incorporated into the reconstituted vesicles. There is a visible loss of intermediate molecular weight proteins in the reconstituted vesicles (5.10^4 - 10^5).

All these considerations indicate that the reformed vesicles are very similar to the original vesicles. As many proteins are found in the reconstituted vesicles, a number of membrane functions may be recovered such as organic solute transport and ($Na^+ + K^+$) ATPase. These activities have been investigated and the extent and characteristics of reconstitution of function assessed.

Amino Acid Exchange in Reconstituted Vesicles

Amino acid exchange is the ability of a specific solute on one side of the membrane to accelerate the movement of a like solute in the opposite direction in a stoichiometric manner. It is a simpler transport system than active transport probably because it does not require energy or the presence of Na^+.

Phenylalanine exchange in the reconstituted vesicles has been demonstrated. This conclusion is based on the following evidence:

(1) Efflux of phenylalanine from the vesicles is stimulated by the presence of phenylalanine in the external medium.

(2) Efflux of phenylalanine is not affected by the presence of a non transported amino acid in the external medium (ε-NH_2 caproic acid).

(3) Efflux of AIB, a non exchanging amino acid is not stimulated by the presence of AIB in the external medium.

The exchange observed with phenylalanine is not an artefact due to a shrinkage of the vesicles induced by the presence of a higher concentration of phenylalanine in the external medium, because ε-NH_2 caproic acid at the same concentration has very little ifany effect.

Amino acid transport

The central work in this thesis is that the Na^+ dependent amino acid transport can be reconstituted after solubilizing the plasma membranes of Ehrlich Ascites cells. The extent to which the original characteristics of the system is recovered is a measure of the stability of the system. Moreover, in order to serve its ultimate objective, i.e. purification to study transport at the molecular level, it is essential to know that the fundamental characteristics of the system are not lost or altered during the treatments required to solubilize and reconstitute.

If the reconstituted vesicles possess transport systems for amino acid, they should be able to take up different amino acids in a manner similar to the intact cells and native vesicles and the characteristic uptake should show enzyme-like behaviour since the carrier responsible for transport is likely to be protein in nature.

The vesicles are sufficiently sealed to show a time dependent uptake of various amino acids. It is apparent from the results that the rates of uptake differ for different amino acids. The amino acids actively transported show the highest rates of uptake and after 30 or so minutes of incubation reach the same steady state position.

AIB uptake is dependent on the amount of membrane proteins whether the initial uptake or the uptake after 30 min. incubation at 20^0 C is

measured. This activity is also temperature sensitive, the rates being greater at 37° C than at 20° C.

The uptake of AIB is saturable, in accordance with the operation of a carrier mediated function. The K_m was estimated both from initial rates and from uptakes at 30 minutes of incubation. A K_m of 3 mM is obtained, similar to the K_m observed in intact cells (2 mM) and native membrane vesicles (3.7 mM). Uptake of AIB is inhibited by methionine and not by leucine and ε-NH_2 caproic acid,a result which is consistent with the observations in intact cells and native membrane vesicles.

Na^+ dependent amino acid transport

In eukaryotic cells, transport systems for organic solutes frequently require Na^+ as a cofactor.

In the reconstituted vesicles, AIB uptake is enhanced by the presence of Na^+. Uptake is relatively slow in a K^+ or in a choline medium. The presence of Na^+ does not alter the vesicular volume, since measurements of tracer ^{22}Na uptake in the presence or absence of 100 mM Na^+ in the medium gave identical results.

Other amino acids like methionine, phenylalanine or glycine show enhanced uptake in a Na^+ medium. The transport of leucine, known to be poorly transported by a Na^+ dependent route, and ε-NH_2 caproic acid which is not known to be transported are not affected by Na^+.

The response to Na^+ of the various amino acids is strictly comparable to the response in the intact cells or native vesicles (259).

Na^+ gradient stimulated amino acid uptake

Besides being a cofactor, Na^+ also plays a role in the energization of transport. It has been shown that the Na^+ electrochemical potential is a major driving force for active transport in Ehrlich Ascites cells and other tissues. When a Na^+ gradient is applied to the reconstituted vesicles at pH 7.4, the response, although variable in degree, is an enhancement of influx and a transient accumulation above the steady state level seen in the absence of the Na^+ gradient. The degree of stimulation of AIB uptake by imposition of a Na^+ gradient could vary from 20 to 100% and the peak of response was seen anywhere from 15 sec. to 2 min. No obvious reason was found for this variability. Possible reasons are a variable degree of sealing, variable permeability to Na^+ and the proportion of vesicles which were inverted. The data on the effect of a Na^+ gradient on AIB uptake suggested that accumulation against a chemical potential had occured. However, without a direct measure of vesicle volume, it was not certain whether the vesicular AIB concentration exceeded that of the medium.

To obtain direct evidence that AIB is accumulated against its concentration gradient in reconstituted vesicles, a different approach was used. If the uptake in a non Na^+ medium leads to equilibrium between the inside and the outside concentrations of AIB, and if the Na^+ gradient is able to drive AIB against its own concentration gradient, switching the vesicles from a non Na^+ medium to a Na^+ medium should lead to an increase in the uptake of AIB when a gradient is imposed. This prediction was verified experimentally, the results show a burst of uptake for the vesicles switched to a Na^+ medium with a Na^+ gradient ($Na_i > Na_o$). It is assumed that the system was at equilibrium prior to the switch, because

AIB uptake had reached a steady state before transferring to a Na^+ medium and did not increase if the transfer was made to non Na^+ medium.

AIB efflux

The transport system should be reversible and if the direction of movement of Na^+ is reversed, Na^+ dependent efflux should ensue.

This prediction was shown to hold true. With a reversed Na^+ gradient (high Na^+ inside) AIB efflux was increased. Addition of valinomycin to medium high in K^+ with low K^+ inside creates a diffusion potential inside positive. This increase in membrane potential is expected to increase AIB efflux and was shown to occur in the present experiments.

Comparison of intact and reconstituted vesicles

The uptakes of AIB in both the intact and the reconstituted membrane vesicles are summarized for comparison in table IX. It can be seen that the reconstituted vesicles show greater uptake activity per mg of protein than the intact vesicles, indicating that a considerable fraction of activity was restored.

TABLE IX

Uptake of α-aminoisobutyrate in the native and reconstituted membrane vesicles.

Medium	Uptake at	α-aminoisobutyrate taken up by	
		Native vesicles	Reconstituted vesicles
		nmols/mg protein	
100 mM NaCl	15 sec	0.20±.05 (10)	0.30±.10 (48)
	30 min	0.40±.10 (10)	1.00±.30 (48)
100 mM Choline chloride	15 sec	0.12±.02 (4)	0.20±.10 (5)
	30 min	0.25±.05 (4)	0.65±.10 (5)

Mean values ± standard deviations are given. The numbers in brackets are the numbers of individual experiments. Incubation was at 20°C. The concentration of α-aminoisobutyrate was 0.5 mM.

Conclusion

These data indicate the feasibility of reestablishing amino acid transport from isolated components. The system is sufficiently stable to withstand the potential denaturing action of the detergents. Moreover the transport must either be fairly simple or, if multiple peptides are involved, the agents used to disaggregate the membrane do not disaggregate the basic functional unit.

It would appear very unlikely that a multipeptide transport system would become reassociated in very dilute solution with restoration of function.

The reformation of a vesicle containing the transport system is spontaneous upon removal of detergent, although it has not yet been feasible to direct the orientation of the proteins in a unique manner.

Most of the basic characteristics of the Na^+dependent transport system are recovered as well as additional membrane functions such as the sugar transport system and the $(Na^+ + K^+)$ATPase. The ground has been laid to initiate purification procedures to identify and purify the amino acid transport system and to study transport at the molecular level.

Such studies may eventually disclose the mechanisms by which a cell permits the passage of a low molecular weight water soluble substance across a lipid-like membrane at a rapid rate and in a highly specific manner and furthermore how this mechanism is poised and coupled to energy so that the transport system carry out osmotic work.

CONTRIBUTION TO ORIGINAL KNOWLEDGE

1- This work represents the first solubilization of plasma membranes from Ehrlich Ascites cells. The solubilization, performed with 2% cholate 4 M urea, is able to bring into solution 80 to 90% of the membrane proteins.

2- Membrane vesicles can be reformed by adding exogenous lipids and dialyzing the detergent.

3- The reconstitution of functional vesicles depends on the divalent cation concentration and temperature of reconstitution, and on the presence of some specific phospholipids.

4- The reconstituted vesicles have a chemical composition similar to that of the native vesicles and are osmotically active.

5- The reconstituted vesicles show ouabain sensitive ($Na^+ + K^+$) activated ATPase activity.

6- Sugar transport activity is present in the reconstituted vesicles.

7- The reconstituted vesicles possess an amino acid exchange system.

8- The reconstituted vesicles also possess a Na^+ dependent transport system for amino acids. The Km for AIB uptake is similar to that measured for native membrane vesicles and intact cells.

9- In native vesicles, AIB uptake is inhibited by methionine but not by leucine. Methionine uptake is not affected by glycine, but is inhibited slightly by phenylalanine and more significantly by leucine. Glycine uptake is inhibited by methionine and phenylalanine but not by leucine.

10- In the reconstituted vesicles, AIB uptake is inhibited by methionine and unaffected by leucine.

11- The energy coupling system is restored:

- The uptake of amino acids can be stimulated by a Na^+ gradient (high Na^+ outside).

- By reversing the Na^+ gradient (low Na^+ outside) it is possible to stimulate AIB efflux from Na^+ and AIB loaded vesicles. This efflux was further stimulated by the addition of valinomycin.

- Amino acids accumulate against their chemical potential.

12- As it is possible to desintegrate and reform membrane vesicles without loosing transport activity, this work represents a viable assay for further identification of the transport system.

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Uptake of Amino Acids in Reconstituted Vesicles Derived from Plasma Membranes of Ehrlich Ascites Cells

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ABSTRACT To obtain a clearer concept of the mechanism of organic solute transport in mammalian cells, we have attempted to reconstitute a functional transport system for amino acids from plasma membranes of Ehrlich ascites cells. Purified plasma membranes were dissolved in 2% Na cholate—4 M urea, a mixture which brought over 85% of the membrane proteins into solution. After centrifugation of the solubilized material for 2 hrs at 100,000 × g, the supernatant was dialyzed in the cold for 20 hrs with additional lipid. The reformed vesicles were tested for the ability to transport amino acids. The preliminary results obtained show that the uptake of α-aminoisobutyric acid can be inhibited by L-methionine and much less by L-leucine as would be predicted from the known properties of α-aminoisobutyrate transport in the intact cells. In addition, it has been possible to show accelerated efflux of intravesicular phenylalanine when phenylalanine is added to the trans side (medium side). The data are consistent with the conclusion that there is carrier mediated transport in the reconstituted vesicles.

Although it has become accepted by most investigators that the passage of small water soluble molecules across plasma membranes is dependent on specific proteins (carriers) in the plasma membrane, there is little understanding as yet of the actual mechanism of the transport event. To find a solution to this problem, many attempts are being made to study transport in a simpler system than the intact cell. Methods have been devised to study transport in vesicles prepared from plasma membranes (Busse et al., '72; Carter et al., '72; Hopfer et al., '73; Lee et al., '73; Colombini and Johnstone, '74a; Colombini and Johnstone, '74 b; Beck and Sacktor, '75; Kinne et al., '75; Quinlan and Hochstadt, '76) and others have reconstituted transport systems in liposomes (Kagawa and Racker, '71; Meissner and Fleischer, '74; MacLennan, '75). In addition, in work with vesicles from microorganisms, considerable progress has been made (Kaback, '74; Schuldiner and Kaback, '75).

We have successfully prepared a plasma membrane fraction from Ehrlich ascites cells. These membranes form vesicles which show accumulation of amino acids against their chemical potential in the presence of a Na^+ gradient (Na^+ external). As part of a program to determine which membrane components are required for amino acid transport, we began by determining whether it is possible to restore some functional transport activity after dissolving the plasma membranes. This brief report is a summary of some of our results with the reconstitution.

METHODS AND MATERIALS

The Ehrlich ascites cell membranes were prepared as described earlier (Colombini and Johnstone, '73) with the following modifications: (a) the sucrose-Zn^{2+} gradient was buffered with 5 mM phosphate buffer, pH 7.4, and (b) a cushion of 50% sucrose was added to the gradient. Most of the nuclei and a few of the membranes penetrated the 50% sucrose layer. The isolated plasma membranes were dissolved in 2% cholate—4M urea—0.1 mM EDTA using 1–2 mg protein per ml. The mixture was stirred at 0° C until a clear solution was obtained (usually 30 min). The clarified solution was centrifuged at 10^5 g for 2 hrs at 4° C and the clear supernatant used for reconstitution. A small yellowish pellet formed on centrifugation.

Phospholipids were dispersed by sonica-

filtration technique as described before (Colombini and Johnstone, '74b). Essentially, the membrane suspension was incubated with ^{14}C amino acid and 100 μl samples are taken at intervals, diluted 20 times in cold wash medium (100 mM NaCl, 0.1 mM Mg^{2+}, 0.1 mM Ca^{2+}, and 5 mM Tris pH 7.4) filtered on glass fiber filters under suction and followed by a rapid wash of the filter with another 10 ml of wash medium. The filters were dried under an infrared lamp and counted in a toluene-based scintillation solvent.

RESULTS

The data in table 1 show that the uptake of α-amino-isobutyrate by the reformed vesicles is time dependent. If the temperature is reduced to 0°, there is little increase with time of AIB associated with the vesicles (results not shown).

The data also show that methionine significantly reduces AIB uptake by the reconstituted vesicles but L-leucine at an equivalent concentration has no effect. Similar results are obtained with the original vesicles (unpublished data) and with the original cells (Johnstone and Scholefield, '59). It should be pointed out that there is no evidence in this experiment for accumulation of α-amino-isobutyric acid against its chemical potential. Additional experiments also show that α-amino-isobutyrate uptake is a saturable process in the reconstituted system although the Km for AIB is about 12–16 mM in the reconstituted system compared to 4 mM in the original vesicles.[1]

The Ehrlich ascites cells are known to possess an amino acid exchange system in addition to a system for net amino acid uptake (Potashner and Johnstone, '70; Oxender and Christensen, '63). Therefore we wished to determine whether there was any evidence for exchange in the reconstituted vesicles. Evidence has previously been presented to show that the original plasma membrane vesicles show the phenomenon of accelerated efflux characteristic of the exchange process (Colombini and Johnstone, '74b). The results in table 2 show that efflux of intravesicular phenylalanine is accelerated by the presence of non-radioactive phenylalanine in the medium.

TABLE 1

Competition between amino acids in reconstituted vesicles

Time (min.)	Uptake of α-aminoisobutyric acid μmoles/mg protein		
	Control	+ Methionine	+ Leucine
1	0.39	0.30	0.43
2	0.50	0.33	0.47
5	0.78	0.55	0.81

Vesicles were incubated in a medium containing 100 mM Na^+, 0.1 mM Mg^{2+}, 0.1 mM Ca^{2+}, and 5 mM Tris buffer at pH 7.4. I-^{14}C-labelled α-amino-isobutyric acid was used at a concentration of 0.4 mM and specific activity of 9.0 mc/mM. L-Methionine and L-Leucine were at 10 mM. The incubation was carried out at 20° C in air. The uptake at 0 time was 0.14 μmoles per mg protein. This value has been subtracted from the values in the table above. A representative experiment is shown. These vesicles were reconstituted with asolectin 1 mg/ml. In two similar experiments of this type, methionine inhibited uptake of α-amino-isobutyrate by 25 and 30 percent respectively while ϵ-amino-caproate had no measurable effect.

TABLE 2

Counterflow of phenylalanine in reconstituted vesicles

Time (min)	% Remaining of initial ^{14}C-phenylalanine	
	Control	+ Phenylalanine (^{12}C) in medium
1	98	65
2	75	57
10	70	43

Vesicles were preincubated with 0.1 mM ^{14}C-phenylalanine, 414 mC/mmole in the medium described in table 1. After 20 min, the vesicles were divided into two equal portions, centrifuged down, and the supernatant removed. The vesicles were resuspended in fresh medium with and without the presence of non-radioactive phenylalanine (10 mM). Sampling commenced as soon as the vesicles were resuspended in the fresh medium. The data given are typical of 6 experiments where, with phenylalanine in the medium, the percent phenylalanine remaining after one minute was 60–66% compared to 98–91% in the control situation.

In summary, the results obtained are consistent with the expression of carrier mediated amino acid transport in reconstituted vesicles, showing the characteristics of time and temperature dependent uptake as well as saturability and specificity.

We are presently engaged in attempts to show that imposition of a Na^+ gradient increases amino acid uptake. Thus far, although uptake is generally highest in a

[1] With recent preparations of reconstituted vesicles, the Km for AIB uptake has been 3.4 ± 0.4 based on 1 min. fluxes.