ASYMMETRY OF THE MITOCHONDRIAL INNER MEMBRANE

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By

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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Master of Science

McMaster University

September, 1982

MASTER OF SCIENCE (1982)
(Biochemistry)

McMaster University
Hamilton, Ontario

TITLE: Asymmetry of the Mitochondrial Inner Membrane

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NUMBER OF PAGES: xiv, 107

ACKNOWLEDGEMENT

I would like to thank Dr. G.E. Gerber for his guidance, encouragement and patience throughout the course of this research and the preparation of this thesis.

Further thanks are extended to Mrs. N. Quilliam for her help and discussion and to Mr. P. Leblanc and Mr. R. Morton for their assistance.

I would like to thank the members of my supervisory committee, Dr. K.B. Freeman and Dr. H.P. Ghosh for their suggestions.

I would also like to thank Mrs. B. Sweet and Mrs. D. Tomlinson for the typing of the manuscript.

Special thanks are extended to my husband Edward for his love and support.

ABSTRACT

The mitochondrial inner membrane is highly selective with regard to permeability to solutes and the movement of a large number of large or charged molecules across it therefore requires specific transport processes provided by specific membrane proteins.

In order to study the spatial arrangement of one such protein the adenine nucleotide translocator protein which transports ADP and ATP across the mitochondrial inner membrane, a number of chemical labelling studies of the mitochondrial inner membrane were carried out.

Mitochondrial inner membrane preparations of normal (mitoplasts) and inverted (submitochondrial particles) configuration with respect to mitochondria have been isolated and the external phosphatidylethanolamine and proteins modified by ^{3}H isethionyl acetimidate. An upper limit of 40-46% of the total PE in mitoplasts was found to be located in the external monolayer.

Differences in protein labelling patterns of isethionyl acetimidate modified mitochondria and SMP was observed. IAI was found to penetrate the outer membrane but not the inner membrane of intact mitochondria.

A tritiated photoreactive phospholipid, 1 palmitoyl-2-(m-diazirinophenoxynonanoyl) phosphatidylcholine (DAP-PC) was incorporated into mitoplasts and submitochondrial particles symmetrically into both monolayers by sonication and asymmetrically using phospholipid exchange protein isolated from beef heart. Photolysis yielded the translocator

as a major crosslinked product in both types of particles and with both methods of incorporation.

It was shown that the adenine nucleotide translocator can be asymmetrically labelled by modification of membrane particles of opposite orientation by water soluble and membrane soluble probes.

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ABBREVIATIONS

ACS	aqueous counting scintillant (Amersham)
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
ATR	atractyloside
BKA	bongkrekic acid
BSA	bovine serum albumin
CAT	carboxyatractyloside
CFC	cycloheptaamylose fluorescamine complex
CL	cardiolipin
CM cellulose	carboxymethyl cellulose
CNBr	cyanogen bromide
CoA	coenzyme A
Cyt c	cytochrome c
c-side	cytoplasmic side of mitochondrial inner membrane
DAP-PC	1-palmitoyl-2-(m-diazirinophenoxy nonanoyl) phosphatidyl-choline
EDTA	ethylene diamine tetraacetic acid
FeCy	ferricyanide
HAP	hydroxylapatite
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HPLC	high performance liquid chromatography
IAI	isethionyl acetimidate

ISO	inside out
MLV	multilamellar vesicles
MOPS	morpholinopropane sulphonic acid
m-side	matrix side of mitochondrial inner membrane
NADH	nicotinamide adenine dinucleotide
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PLEP	phospholipid exchange protein
PS	phosphatidylserine
RSO	right side out
SDS	sodium dodecyl sulphate
SMP	submitochondrial particles
Succ DH	succinate dehydrogenase
THF	tetrahydrofuran
Tris	tris(hydroxymethyl)amino methane
UQ	ubiquinone

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I INTRODUCTION

Biological membranes consist of a phospholipid bilayer with proteins either loosely associated with the surface or embedded in the hydrophobic core of the bilayer (Singer and Nicolson, 1972). These membranes are highly selective with regards to permeability to solutes in order to maintain a controlled intra-cellular environment. Thus, small, uncharged molecules are able to transverse membranes by passively diffusing in response to their concentration gradients while larger molecules or charged molecules are not able to freely diffuse across the membrane. The movement of the latter across the membrane requires specific transport proteins. Some such proteins act as pores, facilitating the transport of certain solutes across the membrane through an aqueous channel. Other membrane proteins act as carriers binding solute at one side of the cell membrane, diffusing across the membrane, releasing the solute at the other side and returning for more. In some cases, metabolic energy is required to move solutes from one side of the membrane to the other. This is called active transport.

The highly selective permeability of plasma membranes to solutes has also been illustrated in the membranes of mitochondria and erythrocytes (for a review see Guidotti, 1976).

Many bulky, highly charged and hydrophilic molecules which are in the cell cytosol are required within the mitochondrial matrix for various purposes and other such molecules which originate in the mitochondria are required elsewhere in the cell. The mitochondrial outer

membrane is permeable to molecules up to a molecular weight of about 4,000. The mitochondrial inner membrane, however, is impermeable to such molecules and, therefore, specific transport mechanisms are required to carry them through this membrane. This transport is provided by specific proteins within the mitochondrial inner membrane called carrier proteins.

ADP and ATP are bulky, highly charged and hydrophilic molecules which must be shuttled through the mitochondrial inner membrane. ATP is generated in the mitochondrial matrix by oxidative phosphorylation but utilized in the cytosol and must, therefore, be transported through the mitochondrial inner membrane. The ADP which is generated by the energy consuming reactions in the cytosol must be transported through the mitochondrial inner membrane to be re-phosphorylated in the mitochondrial matrix to form ATP. This transport of ATP and ADP through the mitochondrial inner membrane is provided by an integral membrane protein called the adenine nucleotide translocator protein (Fig. 1). It is one of the most intensively studied carrier proteins and the elucidation of its mechanism of transport is a matter of great interest. It is possible that the majority of membrane carrier proteins catalyze the transport of their specific substrates by similar mechanisms so that the elucidation of the mechanisms of action of one carrier protein would provide insight into the mechanisms of several others.

1. ADENINE NUCLEOTIDE TRANSLOCATOR AS A MODEL FOR CARRIER PROTEINS

The adenine nucleotide translocator protein is ideally suited for investigations of the mechanism of action of carrier proteins. It is the most abundant protein in heart and liver mitochondria (Klingenberg et al. 1978; Klingenberg, 1979b). The isolation of the adenine nucleotide

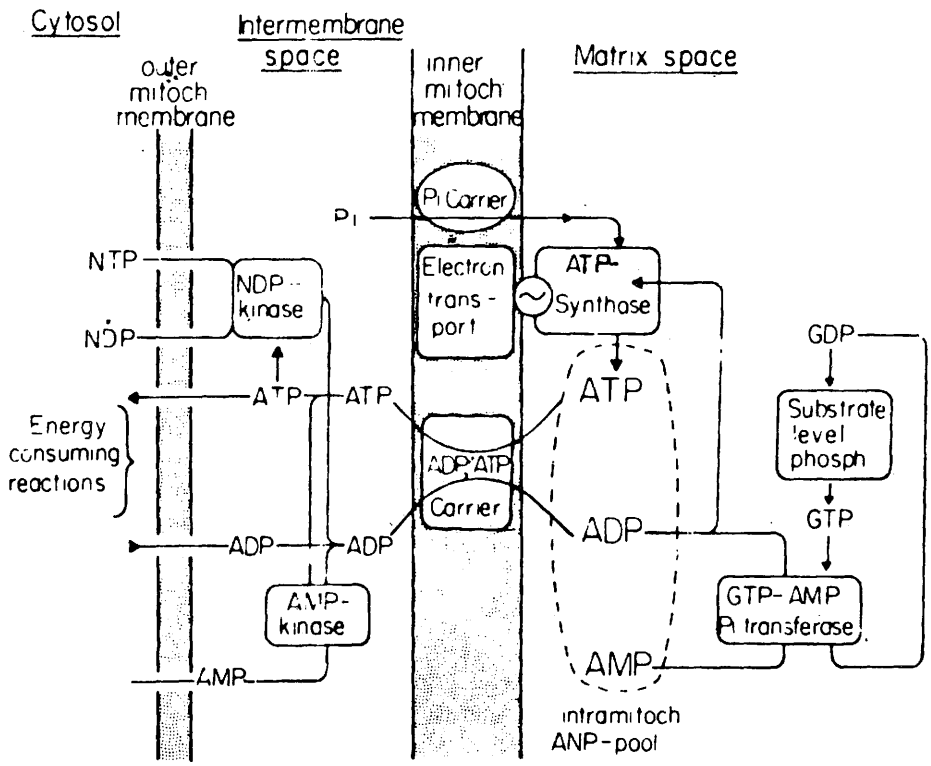


Figure 1. The role of the ADP-ATP carrier in mitochondrial phosphate transfer reactions. The function of the intramitochondrial ANP pool as an intermediate in the synthesis of the extramitochondrial ATP: Localization of transport systems on the impermeable inner mitochondrial membrane and diffusion through the permeable outer membrane. Exclusion of AMP by the inner mitochondrial membrane and localization of AMP reutilizing phosphate transferases in the perimitochondrial and intramitochondrial space. (Klingenberg 1976)

translocator is facilitated by the use of the specific inhibitors atractyloside, carboxyatractyloside (Vignais et al. 1973) and bongkrekic acid (Henderson & Lardy, 1970) which protect the translocator from denaturation during subsequent solubilization and isolation (Riccio et al. 1975a;1975b). These inhibitors fix the translocator in one of two conformational states, with its binding site facing the cytosol or the matrix. Inner membrane particles of opposite orientation can be prepared: mitoplasts have the same orientation as mitochondria while submitochondrial particles have an inverted orientation relative to mitochondria. It is thus possible to study the adenine nucleotide translocator from either side of the membrane in either of its two conformations. It is possible to solubilize the translocator from mitochondria and reconstitute it into liposomes (Shertzer & Racker, 1976; Krämer et al. 1977; Kramer & Klingenberg, 1977). This reconstituted protein exhibits similar properties to the translocator in mitochondria. Reconstitution experiments can provide valuable information regarding mechanism of action of carrier proteins.

2. THE ADENINE NUCLEOTIDE TRANSLOCATOR

A. Transport

The adenine nucleotide translocator is highly specific with regards to substrate, transporting ADP and ATP but virtually excluding AMP. This specificity of transport has been summarized by Klingenberg, as shown in Table I (Klingenberg, 1976). The specificity of the adenine nucleotide translocator is higher than for ATPase and many kinases (Klingenberg, 1976).

The adenine nucleotide translocator operates by an electrogenic

Table I Specificity of Transport for Exogenous Nucleotides

Nucleotides[a]	Uptake Activity (%)
ADP	100
ATP	≃70
AMP	>2
dADP	15
dATP	15
AMP-PNP	19
AMP-PCP	10
Formycin DP	15
G-, C-, U-IDP	≃0
3',5'-ADP	0

[a] 18°C, 200 µM nucleotide, in uncoupled state (+ FCCP); rat liver mitochondria.

(Klingenberg, 1976)

exchange of anions. This type of transport involves a counter exchange of two differently charged anions and is an energy requiring process (LaNoue & Schoolwerth, 1979).

In the transport catalyzed by the adenine nucleotide translocator protein, ATP carrying four negative charges is exchanged for ADP carrying three negative charges. It is a one to one exchange between intra-mitochondrial and extramitochondrial ATP and ADP. This results in charge separation across the membrane (LaNoue & Schoolwerth, 1979). The driving force of ADP-ATP translocation in coupled or energized mitochondria is the membrane potential (Klingenberg et al. 1969; Klingenberg, 1970b)which is negative inside and positive outside, thus favouring inward transport of ADP and outward transport of ATP. Evidence for the electrogenic nature of the adenine nucleotide translocator has been provided (LaNoue et al. 1978; LaNoue & Schoolwerth, 1979). The transport catalyzed by the adenine nucleotide translocator is rapid, but there is evidence that the exchange of ADP for ATP may be the rate limiting step in cellular respiration (Davis & Luming, 1975; Akerboom et al. 1977).

The adenine nucleotide translocator exhibits Michaelis-Menten type saturation kinetics (Pfaff & Klingenberg, 1968; Duée & Vignais, 1969). The apparent Km for external ADP and external ATP is dependent on the energy state of the mitochondria (Vignais, 1976) varying from 1-10 μM for ADP (Duée & Vignais, 1969; Pfaff et al. 1969) and 1-150 μM for ATP (Souverijn et al. 1973). In uncoupled mitochondria the rates for the uptake and extrusion of ADP or ATP are virtually the same. In coupled mitochondria, however, these rates vary greatly and the uptake of ADP and extrusion of ATP is favoured more than 20-fold over any of the other three modes of exchange (Klingenberg, 1979b).

B. Inhibitors

Study of the adenine nucleotide translocator has been facilitated by the existence of three highly specific and effective inhibitors. These are atractyloside (Pfaff & Klingenberg, 1968) and carboxyatractyloside (CAT) (Vignais et al. 1973), two plant toxins from Atractyles Gummifera and bongkrekic acid (BKA) (Henderson & Lardy, 1970), a bacterial toxin (Fig. 2).

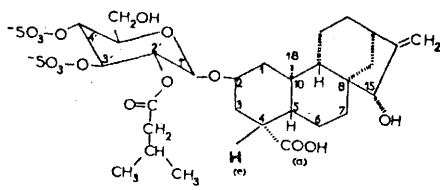
In addition to the plant and bacterial toxins CAT, ATR and BKA there are two other classes of inhibitors of the translocator. Long chain acyl CoA derivatives inhibit non-specifically (Pande & Blanchaer, 1971) and N-ethylmaleimide inhibits the translocator in the presence of ADP.

Atractyloside binds to the translocator only on the cytosol side of the membrane (Klingenberg et al. 1974). It is a nonpenetrant, competitive inhibitor (Vignais & Vignais, 1964; LaNoue & Schoolwerth, 1979), with a high specificity and a fairly high affinity with Kd = 10^{-8}M (Klingenberg et al. 1979b).

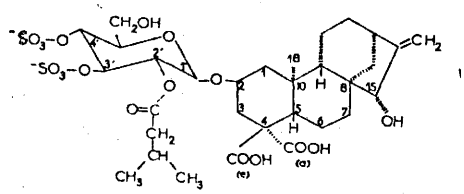
Carboxyatractyloside also binds to the translocator only on the cytosol side of the membrane (Klingenberg et al. 1974). It is a nonpenetrant, noncompetitive inhibitor (LaNoue & Schoolwerth, 1979) with a high specificity and a high affinity with Kd = 10^{-11}M (Vignais et al. 1973; Klingenberg et al. 1979b).

Bongkrekic acid binds to the translocator only on the matrix side of the inner mitochondrial membrane (Klingenberg et al.1974). It is a penetrant and uncompetitive inhibitor (LaNoue & Schoolwerth, 1979).

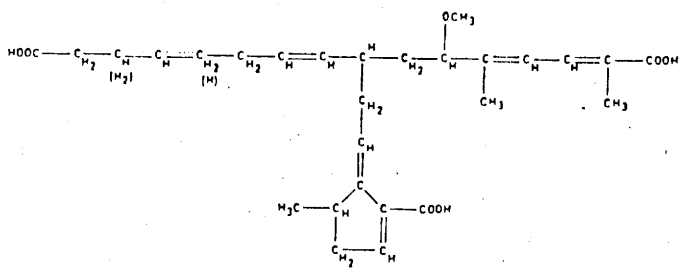
The differences in binding between the atractyloside compounds



ATRACTYLOSIDE



CARBOXYATRACTYLOSIDE



Bongkrekic Acid

Figure 2. The structure of the inhibitors of the adenine nucleotide translocator: Atractyloside(Vignais et al.1973), Carboxyatractyloside (Vignais et al.1973) and Bongkrekic acid (Henderson & Lardy 1970).

and bongkrekic acid have provided evidence that the adenine nucleotide translocator can be fixed in two different conformational states. CAT or ATR fixes the adenine nucleotide translocator in the cytosol state or "c state" and BKA fixes it in the matrix state or "m state" (Erdelt et al. 1972; Klingenberg & Buchholz, 1973). The translocator cannot simultaneously bind the two inhibitors CAT/ATR and BKA. In order to bring the adenine nucleotide translocator from one state to the other a conformational change brought about by the translocation of ADP or ATP is required (Klingenberg et al. 1980). The unliganded protein cannot move (Klingenberg, 1976; 1979b).

C. Evidence for Two Conformational States

In addition to the differences in binding of the two types of inhibitors, further evidence of a conformational change in the translocator in going from the "c state" to the "m state" has been provided by a number of studies.

Iodination of the adenine nucleotide translocator in intact mitochondria and submitochondrial particles by non-permeant iodine surface labels indicates conformational changes. In both types of particles the addition of CAT enhances the iodination while the addition of BKA decreases it. The tyrosine groups on both the c side and the m side of the matrix are more accessible to iodination when the translocator is fixed in the c-state than when it is fixed in the m-state (Klingenberg et al. 1980).

The use of proteases with intact membrane preparations and isolated translocator can also yield information concerning conformational changes. In the presence of CAT, translocator in intact mitochondria is

protected from degradation by a number of proteases. BKA, however, has the opposite effect. When BKA is present, these proteases are able to degrade the translocator (Klingenberg et al. 1980). Similar results have been obtained with isolated translocator protein (Aquila et al. 1978). These results indicate conformational changes with lysine and/or arginine more accessible when the translocator is fixed in the "m state" (Klingenberg et al. 1980).

Immunological evidence for different conformation states of the adenine nucleotide translocator has also been provided (Buchanan et al. 1976). An antibody produced against the translocator isolated as the CAT binding protein was found to be highly specific for the protein isolated in the c-state, having no reactivity for the protein isolated in the m-state. The antibody was shown not to cover the CAT binding site as the bound CAT could exchange with free CAT. Similarly, antibody produced against the translocator isolated as the BKA binding protein complex reacted specifically with the translocator in the m-state, but not in the c-state (Buchanan et al. 1976).

D. Isolation of the Adenine Nucleotide Translocator

The adenine nucleotide translocator has been isolated from the mitochondrial inner membrane as will be discussed in a subsequent chapter (Riccio et al. 1975a;1975b). Chromatography on Sepharose 6B or Agarose 1.5M has indicated that the translocator has a molecular weight of 60,000 while electrophoresis in sodium dodecyl sulfate (SDS) on polyacrylamide gels indicated a molecular weight of 30,000 (Riccio et al. 1975b; Aquila et al. 1978; Klingenberg et al. 1978). It was concluded, therefore, that the adenine nucleotide translocator exists in the

mitochondrial membrane as a dimer. Both subunits have the same molecular weight but it is not known whether they are absolutely identical (Brandolin et al. 1974; Klingenberg et al. 1974; Bojanovski et al. 1976; Klingenberg et al. 1978).

E. Model of translocation mechanism

Both the existence of the adenine nucleotide translocator in two distinct conformational states and its existence in the inner mitochondrial membrane as a dimer suggest a specific mechanism by which the translocator may operate.

It has been proposed that the adenine nucleotide translocator and other carrier proteins have hydrophobic and hydrophilic surfaces. The association of these proteins into dimers or oligomers which span the membrane and orient themselves in such a way that their hydrophobic regions interact with the lipid bilayer,and their hydrophilic portions form a channel or pore through which a hydrophilic molecule can be transported, has been suggested.

Klingenberg et al. (1976; 1977) have postulated a mechanism for the adenine nucleotide translocator which they called the "gated pore mechanism". According to this mechanism ADP interacts with the translocator in the c state, triggering a conformational change. The ADP is then squeezed through the hydrophilic pore and released into the matrix. ATP then interacts with the translocator which is now in the m state and is transported back across the membrane. The unliganded translocator is unable to move ensuring a 1:1 exchange. CAT (ATR) and BKA bind to the translocator fixing it in the c state and m state, respectively, and preventing the translocation step. The gated pore mechanism is illustrated in Figure 3 (Klingenberg, 1979b).

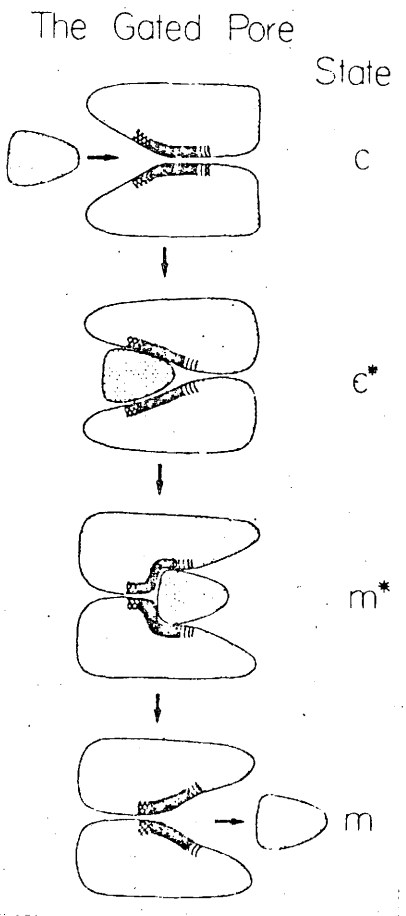


Figure 3. In a "gated pore" the asymmetric substrate (such as ADP) is accepted and released such that the same binding site is significantly different when opening to the outside or the inside. Therefore, it changes specificity to inhibitors from the 'c' to 'm' state. (Klingenberg 1979b).

F. Structure of the adenine nucleotide translocator

The complete primary structure of the adenine nucleotide translocator from beef heart mitochondria has been determined (Babel et al. 1981; Aquila et al. 1982). The amino acid sequence, derived by fragmentation of purified translocator by a variety of chemical and enzymatic methods followed by sequencing and overlapping of fragments, is shown in Figure 4. The methionine residues are all located at the carboxy terminal third of the protein. Fragmentation by cyanogen bromide yields a large fragment of about 22,000 MW, several small fragments with molecular weights of about 3,200, 2,300, 1,600, 1,400 and 1,000 and two homoserine residues (Figure 5).

The carboxy terminus region of the translocator is predominantly polar indicating interaction with the aqueous medium surrounding the mitochondrial inner membrane. The amino terminus region and the remainder of the protein have a lower percentage of polar amino acids which would indicate that these regions are in contact with the lipid environment rather than the aqueous medium surrounding the inner membrane.

The sequence contains one ASP PRO bond which has been demonstrated to be sensitive to cleavage by acid in other proteins (McDowall & Smith, 1965; Tsung & Frankel-Conrat, 1965; Piszkiewicz et al. 1970; Jaurequi-Adell & Marti, 1975) and in translocator (Babel et al. 1981).

The amino acid sequence also contains numerous glutamic acid residues some of which may be sensitive to cleavage by staphylococcus aureus V8 protease.

It is possible, therefore, to study the spatial arrangement of

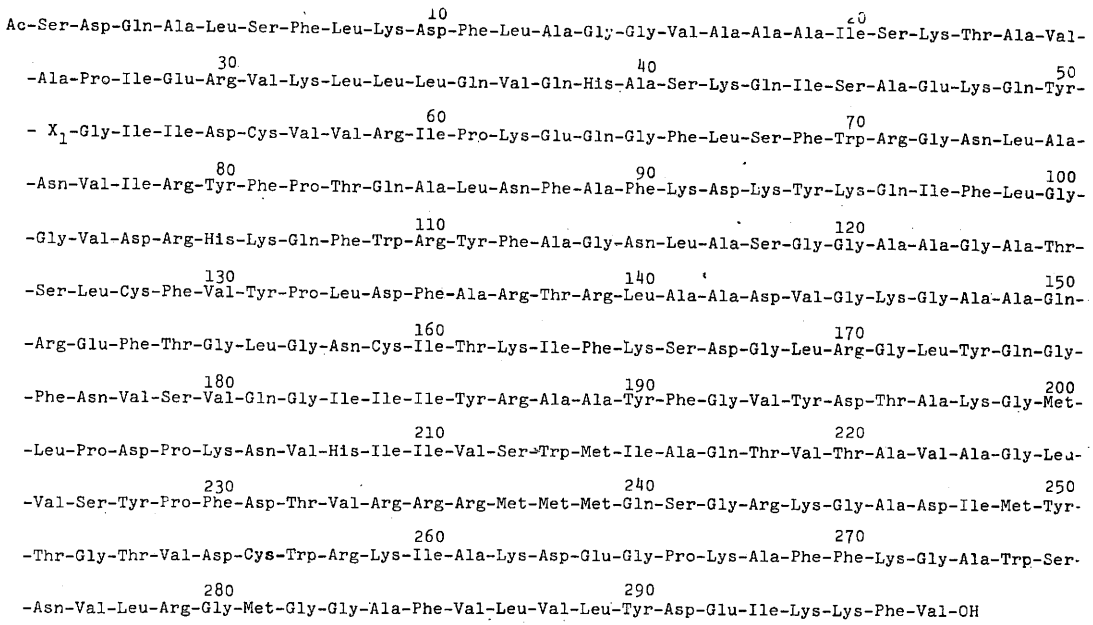


Figure 4. The complete amino acid sequence of the adenine nucleotide translocator isolated from beef heart mitochondria.

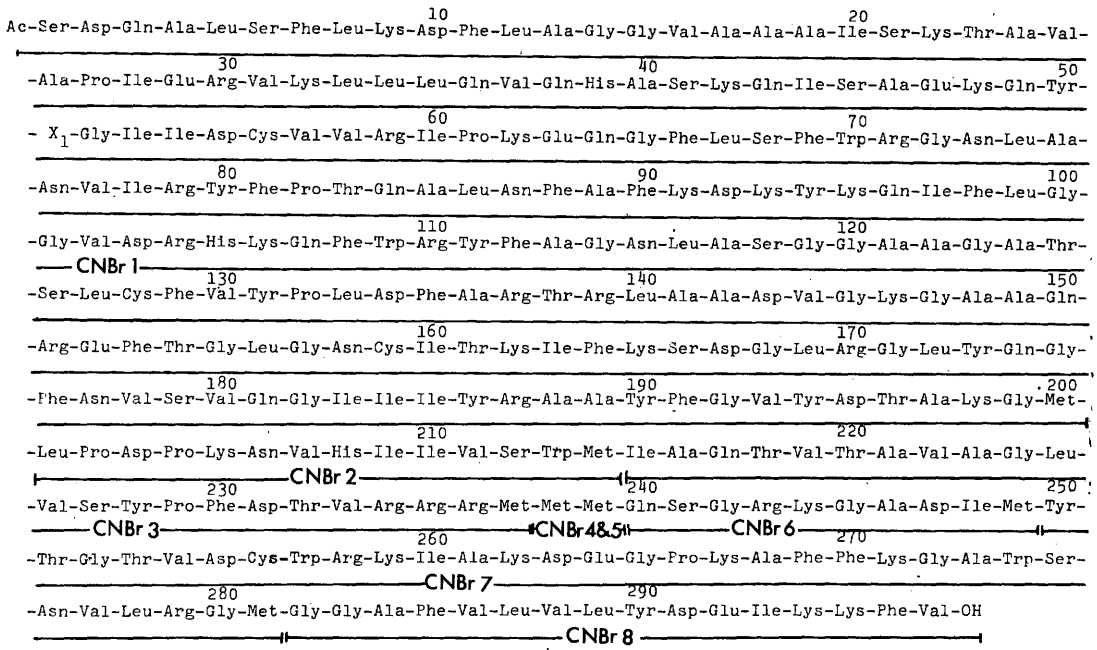


Figure 5. Sites of cyanogen bromide fragmentation of adenine nucleotide translocator.

the adenine nucleotide translocator by asymmetrically labelling it, as will be subsequently discussed, and fragmenting it by a variety of chemical and enzymatic methods.

The adenine nucleotide translocator has a large temperature dependence (Pfaff et al. 1969), being strongly inhibited at low temperatures (Heldt et al. 1965). The translocation process has an unusually high activation energy which has been interpreted to mean that a lipid-protein interaction is important. The importance of lipid-protein interaction would indicate that the use of DAP-PC as a membrane soluble probe will provide valuable information regarding the spatial arrangement of the translocator protein in the inner mitochondrial membrane.

As seen from the amino acid sequence of the adenine nucleotide translocator (Fig. 4) there are two hydrophobic domains. This hydrophobicity has also been indicated by the ability of the translocator to bind 130 molecules of triton per dimer (Klingenberg, 1979,b). The hydrophobic domains consist of amino acids which are α-helix formers. Circular dichroism measurements indicate that the translocator protein is 40% helical (Klingenberg, 1979 b). Therefore these hydrophobic segments exist as transmembrane α-helices.

3. STUDY OF THE THREE DIMENSIONAL STRUCTURE OF MEMBRANE PROTEINS

In order to fully understand the mechanism by which the adenine nucleotide translocator transports its substrates, a knowledge of its three-dimensional structure within the mitochondrial inner membrane is essential. Generally, to elucidate the three-dimensional structure of a protein, the protein is crystallized and X-ray crystallography is carried out on it. With membrane proteins, however, the formation of

good crystals has not been possible and alternate techniques must be utilized.

Two types of approaches have been useful in elucidating the spatial arrangement of membrane proteins. One of these involves the use of electron microscopy to obtain a picture of the three-dimensional structure of the protein. This technique is particularly useful with bacteriorhodopsin, the protein which comprises 75% of the purple membrane of Halobacterium halobium (Oesterhelt & Stoeckenius, 1971), and functions as a light driven proton pump (Oesterhelt & Stoeckenius, 1971; 1973; Danon & Stoeckenius, 1974). Although electron microscopy destroys unit cells with the passage of few electrons, bacteriorhodopsin, unlike most membrane proteins, forms a highly regular two-dimensional array in the membrane (Blaurock & Stoeckenius, 1971); therefore the large number of unit cells in the purple membrane make it possible to obtain a picture of the average unit cell. Henderson & Unwin (1975) obtained a 7Å resolution map of the purple membrane using electron microscopy of tilted unstained samples and found that bacteriorhodopsin has seven segments of α-helices, each of which spans the membrane.

Bacteriorhodopsin in purple membrane forms a two-dimensional hexagonal crystal which can be isolated (Michel et al. 1980). Solubilization of purple membrane by Triton X-100 followed by removal of the detergent by dialysis yields an alternate two-dimensional crystal which has been useful in the determination of the polypeptide boundary of a single bacteriorhodopsin molecule and of which a 6.5Å resolution map has been obtained by electron microscopy exhibiting identical molecular structure to the original crystal form (Michel et al. 1980).

It is possible that this technique, which was developed with bacteriorhodopsin, will be useful in studying other membrane proteins.

The three-dimensional structure of bacteriorhodopsin in the membrane has been studied by selective proteolysis of the purple membrane in sheets, reconstituted vesicles and whole cells (Gerber et al. 1977). Using a number of proteases, Gerber et al. (1977) deduced that most of the protein is embedded in the purple membrane with only a small segment at the carboxyl terminus accessible to proteolysis, and that the carboxyl terminus is on the cytoplasmic side of the membrane. Bacteriorhodopsin has been isolated from purple membrane and fragmented by a variety of chemical and enzymatic means (Gerber et al. 1979; Khorana et al. 1979). Gerber et al. (1979) have developed methods for sequencing integral membrane proteins which, due to their hydrophobic nature, cannot be sequenced by methods used for water soluble proteins. These methods, which include separation of fragments by gel permeation in organic solvents and reverse phase high-pressure liquid chromatography and a combination of gas chromatographic mass spectrometry and automated Edman degradation for sequencing, have been used to determine the primary structure of bacteriorhodopsin (Gerber et al. 1979; Khorana et al. 1979). By a combination of all the techniques discussed above, the three-dimensional structure of bacteriorhodopsin in the purple membrane has been clearly established.

The other approach that has been useful in elucidating the spatial arrangement of membrane proteins involves obtaining as many points of reference as possible using a variety of techniques such as chemical crosslinking, limited proteolysis in the membrane and asymmetric

labelling with non-permeant probes followed by fragmentation and sequencing and identification of the sites of labelling. A good example of this approach is Band 3 protein in the erythrocyte membrane. It is the predominant protein in this membrane and is involved in anion transport (Bretscher, 1973; Cabantchik & Rothstein, 1974; Ho & Guidotti, 1975).

Limited proteolysis at the cytoplasmic and extracellular sides of the erythrocyte membrane by a number of enzymes has led to a knowledge of the order in which the fragments of Band 3 occur along the polypeptide chain and their orientation with respect to the membrane (Steck et al. 1976; Drickamer, 1976; 1977; Jenkins & Tanner, 1977; Reithmeier, 1979). Surface labelling from either side of the membrane and subsequent identification of the labelled regions has indicated the portions of the Band 3 protein in the membrane which are accessible to external non-penetrating reagents (Drickamer, 1977; 1978).

The Band 3 protein in the erythrocyte membrane has been shown to exist as a non-covalent dimer. This was indicated by chemical cross-linking studies (Steck, 1972; Peters & Richards, 1977; Kiehm & Ji, 1977), gel filtration and sedimentation velocity studies (Appell & Low, 1981) and confirmed by an approach based on the measurement of the rotational diffusion of the protein in the membrane (Nigg & Cherry, 1979).

The second approach outlined above will be used to study the spatial arrangement of the adenine nucleotide translocator in the mitochondrial inner membrane.

4. ASYMMETRIC MODIFICATION OF MEMBRANES

The spatial arrangement of proteins within membranes can be

studied by means of a detailed analysis of the labelling patterns obtained with a variety of radioactive chemical reagents. In order for the conclusions to be valid, the integrity and orientation of the membranes as well as the specific region modified by each reagent must be clearly established.

A. Isolation and characterization of mitochondria, mitoplasts and submitochondrial particles

The mitochondrial inner membrane can be prepared in reasonably intact form in both the normal (mitoplast) as well as the inverted orientation (submitochondrial)particles. The integrity and sidedness of these membranes can be established by means of specific enzyme assays. This system thus affords the opportunity to selectively modify either the cytoplasmic or matrix face of the inner membrane; both types of particles have therefore been prepared, characterized and used for labelling in this study.

ENZYMATIC ASSAYS OF INTACTNESS AND SIDEDNESS

(i) Monoamine oxidase and malate dehydrogenase assays

Removal of outer membranes in mitoplast preparations can be monitored by measuring the release of monoamine oxidase, an enzyme known to reside in the outer membrane of mitochondria. The release of malate dehydrogenase, a matrix enzyme, can be used to test for leakiness of the inner membranes of mitoplasts.

(ii) Sidedness assay - succinate catalyzed reduction of ferricyanide

The ferricyanide method was developed by Klingenberg (1970a,1979a) to determine the "sidedness" of membrane fractions. It is based on the knowledge that succinate dehydrogenase is located on the matrix side of the

inner membrane and that the inner membrane is impermeable to ferricyanide. The reduction of ferricyanide, using succinate as a substrate, can proceed by two pathways (see Figure 6):

(a) indirectly through ubiquinone-cytochrome b_1 c_1 - cytochrome c - ferricyanide or

(b) directly through succinate.

The two pathways can be distinguished by testing their sensitivity to antimycin A as it will specifically inhibit only the indirect pathway (a). In normally oriented mitochondria and mitoplasts with intact inner membranes only the indirect route is operational and the reduction of ferricyanide should be completely blocked by antimycin A. In inverted vesicles or ones containing leaky inner membranes, the ferricyanide will be reduced directly by the succinate dehydrogenase and this reaction will be insensitive to antimycin A. Therefore, in this system the rate of reduction of ferricyanide in the presence of antimycin A expressed as a percentage of the rate in the absence of antimycin A gives an estimate of the % inside-out character of membranes (or the percentage which are leaky).

B. Modification of membrane preparations

The reagents used to modify specific regions in membrane preparations can be considered to be of two types:

(i) membrane soluble to label regions of the protein located within the bilayer;

(ii) water soluble to label hydrophilic portions of proteins; if the reagent is shown to be "non permeant", the labelling occurs only on the "outside" surface of closed membrane systems.

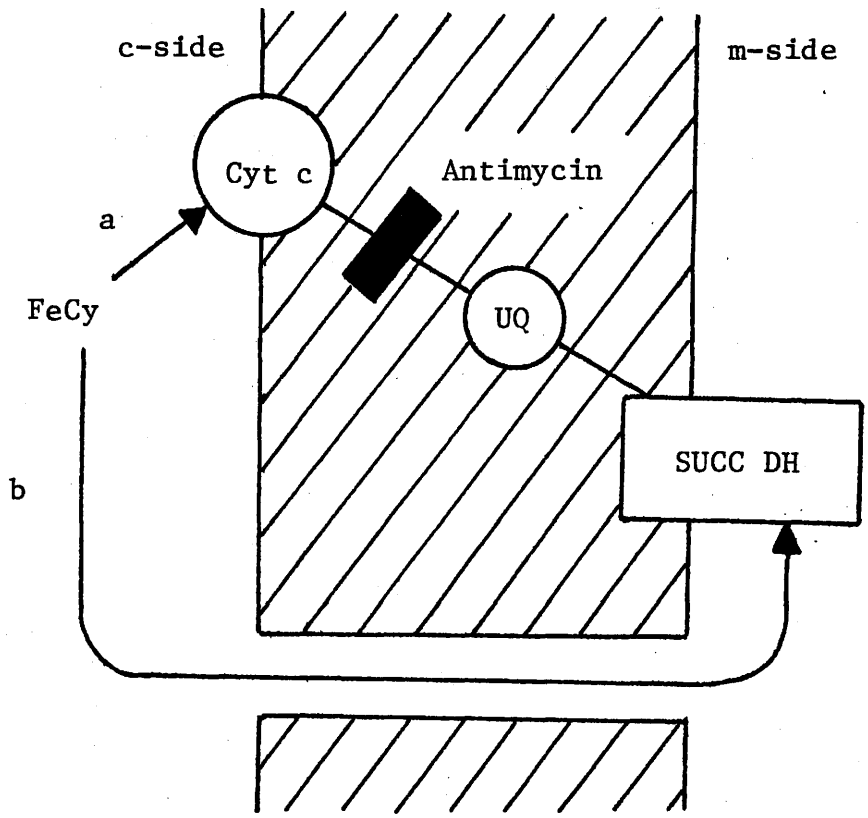


Figure 6. Succinate catalyzed reduction of ferricyanide: The reduction of ferricyanide using succinate as a substrate can proceed by two pathways;

(a) indirectly through ubiquinone-cytochrome b_1c_1-cytochrome c-ferricyanide

or

(b) directly by succinate dehydrogenase

Only the indirect pathway (a) is specifically inhibited by antimycin A.

(i) Membrane soluble reagents

Lipid soluble photochemical probes can be used to label those regions of proteins buried within the membrane. The general absence of chemically reactive groups within the hydrocarbon region dictates the use of photochemically generated reactive groups.

a) Photochemical labelling

The use of photogenerated reagents was introduced by Westheimer and his co-workers (for recent reviews see Bayley & Knowles, 1977; Peters & Richards, 1977; Chowdhry& Westheimer, 1979). A photoreactive group is itself chemically inert but upon irradiation with ultraviolet light, generates a highly chemically reactive species capable of reacting even with the hydrophobic amino acids which predominate in the bilayer. Such a highly reactive group would be used up by the solvent and must, therefore, be generated by irradiation at the time and location chosen by the experimenter.

The photogenerated species is required to be highly reactive while its precursor must be extremely stable (Bayley & Knowles, 1977). Precursors of two photochemically derived highly reactive species have been extensively used. These photogenerated reagents are nitrenes and carbenes. Some of the precursors of these two reagents are reviewed by Bayley & Knowles (1977).

Carbenes are a highly reactive species. They react by insertion into multiple bonds and single bonds by coordination to nucleophilic centers and by hydrogen abstraction (Bayley & Knowles, 1977).

Aromatic nitrenes are significantly less reactive and more discriminating than carbenes. They are also electrophilic and react

more readily with an O-H bond than a C-H bond (Bayley & Knowles, 1977).

Nitrenes were shown to be unsatisfactory for the labelling of lipids or hydrophobic portions of proteins due mainly to their long half life and electrophilic character (Bayley & Knowles, 1978a). Carbenes generated from diazirines have been shown to be much better reagents for the labelling of lipids (Bayley & Knowles, 1978b).

Gupta et al. (1979) have shown that carbenes that are generated from trifluorodiazopropionyl (Chowdhry et al. 1976) and diazirinophenoxy (Smith & Knowles, 1975) groups incorporated into phospholipids yield crosslinked products formed by incorporation into C-H bonds. Extensive intermolecular crosslinking occurred when vesicles formed from these phospholipids were photolyzed. Nitrenes, however, were shown to be unsuitable for forming intermolecular crosslinks by insertion into C-H bonds (Gupta et al. 1979).

It is the proposal of this study to incorporate a radioactively labelled photoreactive derivative of phosphatidylcholine into mitoplasts and submitochondrial particles either uniformly by sonication into both sides of the bilayer or asymmetrically into the external monolayer only utilizing phospholipid exchange protein.

b) Phospholipid exchange protein

Phospholipid exchange proteins are water-soluble proteins which catalyze the exchange of phospholipids between biological membranes and phospholipid bilayer vesicles (Wirtz, 1974; Dawidowicz & Rothman, 1976; Rothman et al. 1976; Rousselet et al. 1976a; Zilversmit & Hughs, 1976). Only the outer layer of membranes and vesicles is accessible to the phospholipid exchange proteins (Johnson et al. 1975; Rothman & Dawidowicz, 1975; Rothman et al. 1976; Rousselet et al. 1976a). Phospholipid exchange

proteins have been isolated from many sources (Wirtz, 1974; Zilversmit & Hughs, 1976). These proteins have varying degrees of specificities for the various phospholipid head groups (Zilversmit & Hughs, 1976). A phospholipid exchange protein highly specific for phosphatidylcholine has been isolated from beef heart (Wirtz et al. 1972; Kamp et al. 1973).

Phospholipid exchange proteins have been used as probes of phospholipid distribution and flip-flop in a variety of biological membranes (Bloj & Zilversmit, 1976; Rothman et al. 1976; Rousselet et al. 1976a, 1976b; Zilversmit & Hughs, 1977).

c) Incorporation of photoreactive phospholipids

In this study phospholipid exchange protein from beef heart was used to effect an asymmetric incorporation of photoreactive phospholipid into the outer monolayer only of mitoplasts. The photoreactive group, a carbene precursor, resides towards the end of the fatty acid in the 2 position of the phosphatidylcholine. Methods have been developed in this lab to vary the length of the fatty acid to alter the site of interaction on the translocator protein (Leblanc et al.,submitted). Photolysis of the membrane preparation containing this photoreactive phospholipid leads to the crosslinking of the phospholipid with lipid and protein portions adjacent to the photoreactive group. Isolation and chemical or enzymatic fragmentation of the translocator protein will then provide information on which fragments are accessible from each side of the membrane and how deeply imbedded in the membrane the various portions are.

(ii) Water-soluble reagents

A number of water-soluble reagents have been reported to modify

phospholipids and proteins only at the "outside" surface of membranes (Whiteley & Berg,1974; Roseman et al. 1975; Krebs et al. 1979). Some of these have therefore been investigated to study their suitability for our present aims. The question of asymmetric phospholipid distribution has been studied by a number of such reagents as well as by phospholipase treatment. This study was undertaken to demonstrate that incorporation of photoreactive phospholipids into inner membrane preparations using phospholipid exchange protein is restricted to the external monolayer of the particles. In the case of reagents which label phosphatidyl-ethanolamine, if this restriction indeed holds, none of the unlabelled phosphatidylethanolamine would be crosslinked to the photoreactive phospholipid. It is therefore essential for this purpose that labelling of the external monolayer of the membrane preparations be exhaustive, without any labelling of the internal monolayer.

A study by Krebs et al. (1979) indicated that the phospholipids in beef heart mitochondria are asymmetrically distributed across the inner membrane. These results were obtained using partially complementary techniques; chemical labelling with cycloheptaamylose fluorescamine complex, phospholipase A_2 treatment and immunoreaction with cardiolipin antibodies. The results using the first two methods appeared very clear cut and unambiguous regarding the distribution of phosphatidylcholine and phosphatidylethanolamine. It was thought that these two methods might be useful in demonstrating whether or not the incorporation of photoreactive phospholipids into mitochondrial inner membrane particles utilizing phospholipid exchange protein is asymmetric.

A second reason for labelling inner membrane preparations

asymmetrically is to label segments of proteins which are in contact with the external medium in either mitoplasts or submitochondrial particles. This will give an indication of the localization of various membrane proteins and in addition, labelled protein can be isolated and fragmented and an indication of its spatial arrangement in the membrane obtained.

Two surface labels which react specifically with primary amino groups were investigated, the cycloheptaamylose fluorescamine complex which had been used by Krebs et al. (1979) with great success, and isethionyl acetimidate which had been shown to be nonpermeant in red blood cells (Whiteley & Berg, 1974).

a) Cycloheptaamylose complex of fluorescamine (CFC)

In order to study membrane topology it is necessary to use reagents which are water-soluble as the addition of organic solvents may cause perturbation of the membrane.

Fluorescamine can be used in the study of membrane topology as it reacts specifically with primary amino groups as in phosphatidylethanolamine and the amino terminus and lysine side chains of proteins. However, fluorescamine is not stable in aqueous solution and must, therefore, be dissolved in organic solvents which could cause perturbations in the membrane. Nakaya et al. (1975) incorporated fluorescamine into cycloheptaamylose and the resulting cycloheptaamylose fluorescamine complex (CFC) was found to be useful as a fluorescent label, soluble in water and non-penetrating. CFC itself and its hydrolysis products are not fluorescent.

Cycloheptaamylose fluorescamine complex has been used to label red blood cells (Nakaya et al. 1975), sarcoplasmic reticulum membranes (Hidalgo & Ikemoto, 1977) and mitoplasts and submitochondrial particles (Krebs et al. 1979). In the first two cases exhaustive labelling of the external surface of the membrane was not required for the study. In the third study using beef heart mitoplasts and submitochondrial particles, phospholipid asymmetry was being investigated and exhaustive labelling of the external monolayer was therefore essential. It was shown that the labelling of phosphatidylethanolamine in mitoplasts and submitochondrial particles levelled off at 38% and 63% respectively of the PE modified (Krebs et al. 1979). These results are in very good agreement with one another.

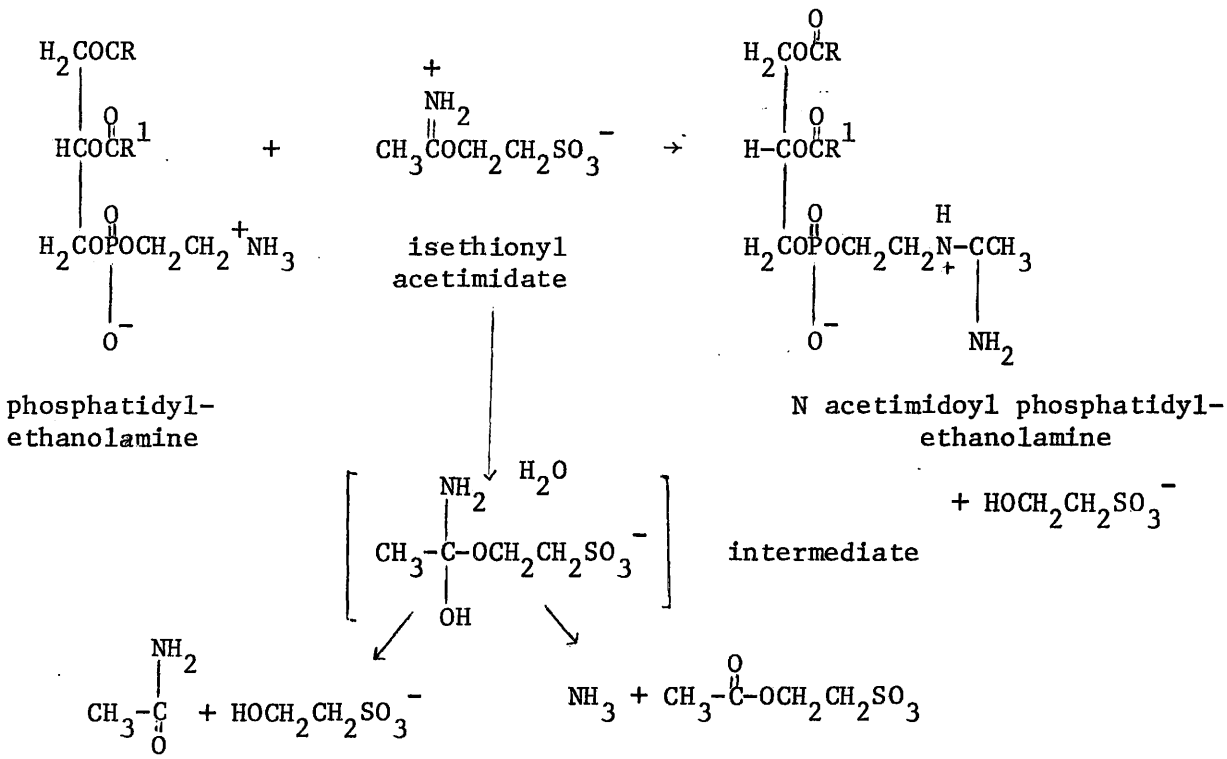
Cycloheptaamylose fluorescamine complex was used in this study in an attempt to exhaustively label the outer monolayer only of rat liver mitoplasts.

b) Isethionyl acetimidate (IAI)

Isethionyl acetimidate is a water-soluble imido ester which reacts with primary amino groups such as those found in PE, and the amino terminus and lysine side chains of proteins. Isethionyl acetimidate has been shown to be impermeant to red blood cells (Whiteley & Berg, 1974) and single walled bilayer vesicles (Roseman et al. 1975). Isethionyl acetimidate reacts under mild physiological conditions to form amidines. It does not seriously perturb the membrane or impair membrane function as the amidine formed has the same charge as the original amino group and is not much larger (Whiteley & Berg, 1974). The amidines formed are stable (Whiteley & Berg, 1974).

Imido esters hydrolyze rapidly to form ammonia and esters (Whiteley & Berg, 1974; Crain & Marinetti, 1979).

This study is concerned partially with the reaction of isethionyl acetimidate with phosphatidylethanolamine.



Isethionyl acetimidate can be radioactively labelled by incorporating either ^{14}C or ^{3}H into the methyl group.

Studies have also been done using IAI to label phosphatidyl-ethanolamine in mitoplasts (Crain & Marinetti,1979). Unlike the results found for red blood cells (Whiteley & Berg, 1974) and liposomes (Roseman et al. 1975) it was shown that only 30-36% of the total PE could be reacted with IAI in mitoplasts which had been disrupted or solubilized by a variety of methods. These results indicated that the limited reaction of IAI was not due to an asymmetric arrangement of PE but to some membrane property which makes the bulk of PE unavailable to IAI (Crain & Marinetti, 1979).

The reaction of isethionyl acetimidate with free amino groups results in an increase in the pH of the reaction mixture. As a high pH may damage the integrity of the membrane the reaction mixture is titrated to maintain the pH at 8.0. Browne & Kent (1975) found that the treatment of horse liver alcohol dehydrogenase with isethionyl acetimidate at pH 8.0 led initially to the production of protein N-alkyl imidates which then partition approximately equally between reaction with ammonia to form amidines and hydrolysis to yield free amine. This results in only partial amidination. At pH 10 the N alkyl imidate is not formed to an appreciable extent and amidination is nearly complete (Browne & Kent, 1975).

As incubation at pH 10 may perturb the mitoplast membrane, incubation at pH 8.0 is preferable. In order to achieve complete amidination at this pH, Browne & Kent (1975) made multiple additions of isethionyl acetimidate. Liver alcohol dehydrogenase treated in this way was aimdinated to the same extent as it was when subjected to a single addition of isethionyl acetimidate at pH 10.0.

These two reagents, CFC and IAI, can be used with mitoplasts and submitochondrial particles. Subsequent fragmentation and analysis of the labelling patterns can yield information concerning which portions of the translocator are in contact with the aqueous environment on either side of the membrane.

(iii) Phospholipase A_2 treatment

Phospholipases have been used extensively in studying phospholipid topography in biological membranes (for a review see Etemadi, 1980).

Phospholipase A_2 specifically removes the fatty acid from the 2 position of phosphoglycerides.

Phospholipase A_2 isolated and purified from Naja naja naja cobra venom according to the method of Deems & Dennis (1975) was shown by Krebs et al. (1979) to selectively cleave fatty acids from the 2 position of phospholipids on the external monolayer only of beef heart mitoplasts and submitochondrial particles. However, phospholipase A_2 isolated and purified according to the procedure of Cremona & Kearney (1964) has been shown to penetrate the membrane (Lankisch et al. 1971; Martin et al. 1975; Higgins et al. 1977; Krebs et al. 1979). This "leakiness" of the membrane is thought to be caused by the presence of a "direct lytic factor" described by Lankisch et al. (1971) which can be removed from the phospholipase by chromatography on CM cellulose, a step included in the purification procedure of Deems & Dennis (1975) but not in the purification procedure of Cremona & Kearney (1964).

It is, therefore, necessary for this study that phospholipase A_2 be isolated and purified from Naja naja naja cobra venom according to the procedures of Deems & Dennis (1975).

(iv) Proteolysis of intact inner membrane preparations

It is possible that a portion of the adenine nucleotide translocator could protrude from the membrane on either the matrix side or the cytosol side. This portion could be sensitive to proteolytic degradation by a number of proteolytic enzymes in intact mitoplasts and submitochondrial particles. Isolation and fragmentation of the translocator protein would indicate which, if any, portion protruded from the membrane. The use of this technique to study the translocator has not been reported in the literature. In this study, a variety of proteolytic enzymes were tried but identification of fragments was not possible. If the treated inner

membrane particles were applied directly to polyacrylamide gels, fragments from the adenine nucleotide translocator could not be distinguished from fragments from other proteins. The translocator could not be isolated, by standard procedures, from inner membrane preparations which had been treated with proteolytic enzymes, indicating that the protein had been cleaved. It was not therefore possible to use this technique in this study.

Rationale

Transport proteins are of great importance in the regulation of cellular processes. A knowledge of the mechanism of action of these proteins is essential for a complete understanding of how they exert their regulatory effects.

As previously discussed, the adenine nucleotide translocator protein is ideally suited for investigations of the mechanism of action of carrier proteins due to its abundance in the mitocondrial inner membrane, facilitation of its isolation by the inhibitors, CAT, ATR and BKA, availability of inner membrane preparations of opposite orientations, and the ability to reconstitute it into liposomes. It was therefore of interest to study the spatial arrangement of this protein in the mitochondrial inner membrane as this could yield insight into its mechanism of action.

The spatial arrangement of the adenine nucleotide translocator can be investigated using an approach which involves the use of several independent methods, which are at least partially complementary, to obtain as many points of reference as possible. The methods include surface

labelling with non-penetrating soluble probes, labelling with a membrane soluble probe, a photoreactive phospholipid, and fragmentation of labelled isolated protein.

II METHODS

Isolation of mitochondria

Rat liver mitochondria were prepared essentially according to the method of Schnaitman & Greenawalt (1968) and Greenawalt (1974).

Male rats ranging in weight from 150 to 250 g were fasted overnight then killed by a blow to the head. The livers were immediately removed and placed in ice cold H medium (70 mM sucrose, 220 mM D-mannitol, 2.0 mM HEPES, 0.5 mg BSA (fatty acid free)/ml pH 7.4). All fat and connective tissue was removed and the livers were then cut into small pieces and rinsed three times with fresh H medium. One volume of mince was homogenized with two volumes of H medium using the Thomas C homogenizer with the serially radiated pestle and Caframo motor. Four to five strokes at a setting of three were usually required. The homogenate was diluted one to three with H medium and centrifuged at 600 x g for 10 mins at 4°C to sediment the nuclei and cell debris. The supernatant was again centrifuged at 600 x g for 10 min. The supernatant was then centrifuged at 7,000 x g for 15 mins at 4°C to sediment the mitochondria. The supernatant was discarded and the mitochondria were gently resuspended, using a cold rubber policeman, to one half their original volume. The suspension was again centrifuged at 7,000 x g for 15 min, the supernatant discarded and the mitochondria resuspended in H medium to one quarter their original volume. The mitochondrial suspension was centrifuged at 7,000 x g for 15 min, the supernatant discarded and the mitochondria resuspended in a minimum volume of H medium.

Preparation of mitoplasts

Mitoplasts were prepared according to the method of Schnaitman and Greenawalt (1968) and Greenawalt (1974).

Rat liver mitochondria (100 mg protein/ml) were mixed with an equal volume of 5% to 20% digitonin in H medium. The H medium without BSA requires warming almost to boiling in order to dissolve the digitonin. The solution was slowly cooled to 4°C, then the BSA was added.

The mitochondrial solution was incubated on ice for 15 mins with frequent gentle vortexing. The reaction was stopped by the addition of three volumes of ice cold H medium. The mitoplasts were sedimented by centrifugation at 11,000 x g for 15 min, washed once then resuspended in a minimum volume of ice cold H medium.

In some isolations of mitochondria and mitoplasts 0.25 M sucrose, 2 mM EDTA pH 7.2 was used instead of H medium.

Preparation of submitochondrial particles

Submitochondrial particles were prepared essentially according to the method of Hackenbrock & Hammond (1975) as modified by Wehrle et al. (1978).

Mitoplasts were suspended in ice cold H_2O to give a protein concentration of 2 mg/ml. This was centrifuged for 10 min at 10,000 x g. The pellet was resuspended in isolation medium to give a protein concentration of 10 mg/ml. The mitoplasts were sonicated in the pulse mode (30 sec sonication, 30 sec cooling on ice) using a Branson probe sonifier with the large probe and 80% power. The total sonication time was 4 min. The membrane preparation was centrifuged at 10,000 x g for 10 min to sediment any unbroken mitoplasts. The supernatant was

centrifuged at 105,000 x g for 30 min. The supernatant of this centrifugation was discarded and the pellet was washed once with isolation medium. The submitochondrial particles were resuspended in isolation medium.

Enzymes assays used to evaluate mitochondria, mitoplasts and submitochondrial particles

1) Ferricyanide method for the elucidation of membrane sidedness

Membrane sidedness was assayed according to the procedure of Klingenberg (1970; 1979).

Assay Medium

Succinate (disodium salt-hexahydrate)	40 mM
KCN	1 mM
$K[Fe(CN)_6]$	1.5 mM
KCl	10 mM

This was made up in 0.22 M mannitol. 0.07 M sucrose, 0.01 M HEPES, 0.0001 M EDTA pH 7.4

Antimycin A:

1 mg/ml in ethanol.

Procedure:

To 3 ml of assay medium was added 0.3 to 1.0 mg mitochondria or mitoplasts. This was mixed and the reduction in absorbance at 420 nm was monitored. Ten microlitres of antimycin A solution was added and the reduction in absorbance at 420 nm again monitored.

The percentage of particles with inside out or damaged membranes is the ratio of the reduction in A_{420} in the presence of antimycin A to the reduction in A_{420} in the absence of antimycin A multiplied by 100

$$\text{i.e. \% ISO (or damaged)} = \frac{(\Delta A_{420} + \text{antimycin A})}{(\Delta A_{420} - \text{antimycin A})} (100)$$

The percentage of particles with their membrane right side out is

$$\% \text{ RSO} = 100 - \% \text{ ISO}$$

2) Assay for monoamine oxidase

Monoamine oxidase was assayed according to the method of Tabor et al. (1951) as modified by Salach (1978).

Stock Solutions

Potassium phosphate	0.2 M, pH 7.2
Benzylamine	0.1 M neutralized to pH 7.0 with 6 N HCl
Triton X-100	4.0% v/v

Reaction Mixture

Potassium phosphate	0.2 M, pH 7.2, 1.00 ml
Triton X-100	0.15 ml
Enzyme and double distilled H_2O	1.75 ml

The above was mixed and the absorbance at 250 nm was monitored until it was stable. The reaction was then started by the addition of 0.100 ml benzylamine and the increase in absorbance at 250 nm due to

$$\text{Benzylamine} \rightleftarrows \text{Benzaldehyde}$$

was followed.

Calculations based on ε of 12,080 for benzaldehyde units/ml = µmol produced/min/ml.

$$= (\Delta A_{250}/\text{min}) \ (0.2483) \ (^{1}/\text{aliquot size})$$

3) Assay for malate dehydrogenase

Malate dehydrogenase was assayed according to the method of Ochoa (1957) with some modifications from Comte and Gautheron (1979).

Stock Solutions

Potassium phosphate	0.25 M, pH 7.4
NADH	0.0015 M, made up fresh in phosphate buffer
Oxaloacetate	0.0076 M, pH 7.4, made up fresh
Triton X-100	0.30 % v/v
Rotenone	1.2 mM in ethanol

Reaction Mixture

Potassium phosphate	0.25 M pH 7.4,	0.30 ml
Triton X-100		0.15 ml
Rotenone		0.01 ml
NADH		0.10 ml
Enzyme and double distilled H_2O		2.35 ml

The above was mixed and the absorbance monitored at 340 nm until it was stable. The reaction was started by the addition of 0.10 ml oxaloacetate and the decrease in absorbance at 340 nm due to

$$\text{oxaloacetate} + \text{NADH} + H^+ \rightleftharpoons \text{malate} + \text{NADH}$$

was followed.

Calculations

(using ε of NADH of 6.2×10^3), units/ml = μmol oxidized/min/ml

$$= (\Delta A_{340}/\text{min})\ (0.484)\ (^1/\text{aliquot size})$$

Isolation and purification of phospholipid exchange protein from beef heart

Phospholipid exchange protein (PLEP) was isolated and purified from beef heart according to the method of Johnson & Zilversmit (1975) and Zilversmit & Johnson (1975).

Beef hearts were trimmed of connective tissue and fat, cut into small pieces and passed through a precooled meat grinder. This was then

homogenized in a Waring blender in lots of 400 g with 1200 ml 0.25 M sucrose, 0.01 M K_2HPO_4 pH 7.4 for 30 sec. The pH was maintained between 7.2 and 7.4 by the addition of 6 N KOH. The homogenate was centrifuged at 1600 x g for 15 min to remove the debris. The supernatant was filtered through two layers of cheesecloth and centrifuged at 10,000 x g for 20 min to sediment the mitochondria. The supernatant was then adjusted to a pH of 5.1 with 4 N HCl and stirred for one hour at 4°C to flocculate protein. The flocculated protein was removed by centrifugation at 10,000 x g for 30 min. The pH of the supernatant was readjusted to 7.4 with 10 N NaOH. To 900 ml of the supernatant was slowly added 596 g solid ammonium sulphate to reach 90% saturation and the mixture was stirred overnight then centrifuged for 50 min at 13,000 x g. The pellet was suspended in 50 ml of water and dialyzed for 40 hours against 40 volumes of water with three changes of medium. The dialyzate was filtered and the filtrate was applied to a Sephadex G-75 column 5 cm x 83 cm equilibrated in 0.042 M Tris-acetate pH 7.4 containing 5 mM β-mercapto-ethanol. The sample was eluted with the same buffer at a flow rate of 2 $ml/cm^2/hr$ (0.6 ml/min).

Assays of phospholipid exchange protein activity

The following buffers were used:

Liposome buffer; 0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl pH 7.4.

Enzyme buffer; 25 mM sodium chloride, 10 mM β-mercaptoethanol, 5 mM sodium phosphate pH 7.2.

Multilamellar vesicle buffer (MLV); 5 mM EDTA, 0 or 0.25 mM sucrose as indicated, 50 mM Tris-HCl pH 7.4.

The lipids used were egg phosphatidylcholine, pig brain phosphatidylserine, beef heart cardiolipin, egg phosphatidylethanolamine and 1 palmitoyl-2-(m-diazirinophenoxynonanoyl) phosphatidylcholine.

Preparation of liposomes

The appropriate phospholipids in either chloroform or ethanol were combined in a sonication tube and mixed thoroughly. The solvent was evaporated under a stream of nitrogen while the tube was rotated in warm water to obtain a maximum and uniform film of phospholipid. The lipid mixture was then further dried under vacuum for 15 min. The vacuum was released with nitrogen and the lipid mixture was again flushed with nitrogen for 15 min. Liposome buffer was added to the lipid film while still flushing with nitrogen to give a final concentration of 2 mg lipid/ml.

The lipids were sonicated at 40°C in a bath sonicator for 30 min or until clear. The liposomes were centrifuged for 10 min at 12,000 x g to sediment any large or aggregated lispsomes. The supernatant was stored at room temperature for one hour prior to use.

Preparation of multilamellar vesicles

Multilamellar vesicles were prepared according to Bangham et al. (1965) as modified by Dicorleto & Zilversmit (1977). Lipids were combined in a round bottom flask with 0.1 mole percent butylated hydroxytoluene as antioxidant. These were mixed well and the solvent was removed by rotary evaporation. The lipid was redissolved in diethyl ether. This was removed by rotary evaporation and a thin lipid film on the walls of the flask was obtained. The film was taken up in MLV buffer and the flask swirled by hand and vortexed very gently until all lipid was freed from

the sides of the flask and all large lipid aggregates were dispersed. The solution was stored at room temperature for two hours prior to use.

Assays of transfer activity of phospholipid exchange protein

Three different types of exchange assays were used.

A. Assay with liposomes a donor particles and mitochondria as acceptor particles

Liposomes, mitochondria and phospholipid exchange protein were mixed together and incubated at 25°C for one hour. They were made 1 M in sucrose where indicated and centrifuged for 5 min at 12,000 x g to sediment the mitochondria. The supernatant containing the liposomes was discarded and the pellet was washed with 1 M sucrose in liposome buffer, centrifuged and washed again. The pellet was suspended in water and counted for 1 min in ACS.

B. Assay with multilamellar vesicles as donor particles and submitochondrial particles as acceptor particles

Submitochondrial particles were diluted to 1 mg protein/ml in 0.01 M Tris-HCl pH 7.8, 0.25 M sucrose. These were centrifuged for 6 min at 12,000 x g just prior to use and the supernatant was used for the assay. Multilamellar vesicles were centrifuged for 6 min at 12,000 x g and the pellet resuspended in MLV buffer. Multilamellar vesicles, submitochondrial particles and phospholipid exchange protein were mixed together and incubated at 37°C for one hour. After this time the sample was centrifuged at 12,000 x g to sediment the MLV and the supernatant was counted in ACS.

C. Exchange assay using phospholipid exchange protein as a reagent rather than a catalyst and mitochondria as acceptor particles

Liposomes were prepared as outlined in 0.05 M Tris-HCl pH 7.4, 0.05 M NaCl, 0.02% NaN_3. These were incubated with purified phospholipid exchange protein in 50% glycerol for 30 min at 37°C. This was then applied to a Sepharose 6B column (1.5 x 48 cm), equilibrated in the above buffer and previously loaded with 100 mg egg PC.

Mitoplasts were incubated with photoreactive phospholipid-phospholipid exchange protein complex at 25°C for 1 hour then sedimented by centrifugation at 12,000 x g for 10 min. The mitoplasts were washed once with isolation medium then resuspended in isolation medium and counted in ACS.

Labelling of mitoplasts and submitochondrial particles with 1-palmitoyl-2-(m-diazirinophenoxynonanoyl) phosphatidylcholine

Liposomes were prepared as described previously and incubated with phospholipid exchange protein in 50% glycerol at 37°C for 30 min. This was then incubated with mitoplasts and submitochondrial particles at 25°C or 37°C. Controls were also done in the absence of phospholipid exchange protein . At appropriate times the mixture was photolyzed for 30 sec using a Schoeffel Model LPS 255 HR photolysis setup with two Corning 7-51 filters, the one closest to the light source immersed in cold water. Samples were photolyzed under an atmosphere of nitrogen.

Isolation and purification of phospholipase A_2 from Naja naja naja cobra venom

Phospholipase A_2 was isolated and purified from Naja naja naja cobra venom according to the method of Deems & Dennis (1975). The lyophilized venom was dissolved in water and centrifuged to remove any insoluble material. The clear supernatant was stirred with perchloric acid overnight, the precipitate collected by centrifugation and dissolved in a minimum amount of H_2O and adjusted to pH 8.0. This was centrifuged to remove a white precipitate which exists between pH 5 and 9. The clear supernatant was applied to a CM-52 column. The protein containing fractions from this column were pooled and lyophilized. The lyophilized protein was taken up in H_2O and applied to a Sephadex G-100 column. The second protein peak from this column was the phospholipase A_2. These fractions were pooled and stored at -20°C.

Preparation of cycloheptaamylose fluorescamine complex

Cycloheptamylose fluorescamine complex was prepared according to the method of Nakaya et al. (1975). A solution of 100 mg fluorescamine in 4 ml acetone was added dropwise to 80 ml 2% aqueous cycloheptaamylose solution (w/v) at room temperature with stirring over a period of 15 min. The mixture was left standing in ice for 30 min then the precipitate was collected by centrifugation at 10,000 x g for 15 min and dried over P_2O_5 under vacuum. The cycloheptaamylose fluorescamine complex was stored over P_2O_5.

Modification of mitoplasts with cycloheptaamylose fluorescamine complex

CFC was added to buffer (220 mM mannitol, 70 mM sucrose, 10 mM $NaHCO_3$ pH 8.0) at a concentration of 2.5 mg/ml. This was warmed at 50°C

for one min then incubated at room temperature for 5 min with frequent vortexing. Any undissolved CFC was sedimented by centrifugation and the supernatant was used immediately.

Mitoplasts were treated with CFC according to the method of Krebs et al. (1979). Mitoplasts were incubated with CFC for 50 min at 25°C then sedimented by centrifugation. The phospholipids were extracted from the mitoplasts according to the method of Bligh & Dyer (1959), and separated by thin layer chromatography using $CHCl_3/CH_3COCH_3/CH_3OH/CH_3COOH/H_2O$ 6:8:2:2:1 as a developing solvent. The fluorescent spots were visualized using long wavelength UV light and the phospholipid was visualized with iodine vapour. The spots were scraped and the amount of phosphorous was determined according to the method of Bartlett (1959).

Synthesis of tritiated isethionyl acetimidate

Tritiated isethionyl acetimidate was synthesized according to the method of Whiteley & Berg (1974).

Sodium isethionate was recrystallized from ethanol and stored over P_2O_5. HPLC grade tetrahydrofuran was checked for the presence of peroxides using KI and stored under nitrogen. HPLC grade acetonitrile was used. Tritiated acetonitrile was obtained from New England Nuclear. Just prior to use, hydrogen chloride gas was bubbled through concentrated sulphuric acid to remove any water and then into the tetrahydrofuran to a concentration of 35% w/w.

149.7 mg acetonitrile was added to 1637 mg 35% (w/w) HCl in THF. The ampoule containing tritiated acetonitrile was frozen in liquid nitrogen. The top was broken off the ampoule and half of the acetonitrile in HCl/THF (35% w/w) was added. This was then transferred to a tube

containing 550 mg sodium isethionate. The rest of the acetonitrile in HCl/THF was used to rinse the ampoule then this too was transferred to the tube. The tube was sealed, placed inside another sealed jar and stirred at 4°C for 13 days.

The solid was filtered on a 0.5 µm teflon Millipore filter and washed three times with THF. The powder was dried slightly with a stream of nitrogen gas, transferred to a tube and dried to constant weight on a vacuum pump (∿ 30 min). The tritiated isethionyl acetimidate was stored at -20°C over dessicant. Yield=74%.

Modification of mitoplasts with tritiated isethionyl acetimidate (IAI)

Isethionyl acetimidate was dissolved in buffer and the pH was immediately adjusted to 8.0. This was immediately added to ^{3}H IAI to give a final concentration of 100 mM IAI, 10% of which was tritiated. The IAI solution was used immediately. In some cases ^{3}H IAI was not used.

The IAI solution was added to mitoplasts in H medium (pH 8.0). The reaction was allowed to proceed at room temperature with or without titration with 0.1 N HCl to maintain the pH at 8.0. The reaction was stopped by the addition of 0.17M ethanolamine and the phospholipids extracted according to the method of Bligh & Dyer (1959). The extracted phospholipids were separated by thin layer chromatography with $CHCl_3/CH_3OH/4$ N NH_4OH 65:35:5 as the developing solvent. The phospholipids were visualized with iodine vapour. The spots were scraped and the phosphorous determined according to the method of Bartlett (1959) or, if ^{3}H IAI was used, the spots were scraped and counted in 16 ml aqueous counting scintillant.

In some experiments fresh IAI solution was added at 30 min intervals.

Isolation and purification of adenine nucleotide translocator protein

Buffers used:

Elution buffer	0.1 M NaCl
	0.5% Triton X-100
	10 mM MOPS
	0.5 mM EDTA
	pH 7.2
Preincubation buffer	0.25 M sucrose
	0.5 mM EDTA
	2.5 mM $MgCl_2$
	20 mM MOPS
	pH 6.8
Pre-extraction buffer	60 mM NaCl
	20 mM MOPS
	1% Triton X-100
	0.25 mM EDTA
	pH 7.2
Solubilization buffer	0.5 M NaCl
	5% Triton X-100
	20 mM MOPS
	5 mM EDTA
	pH 7.2

The adenine nucleotide translocator was isolated according to the procedure of Riccio *et al.* (1975a;1975b).

Mitochondria were washed twice with excess preincubation buffer, then suspended in preincubation buffer and 1 mM CAT to a concentration of 0.1 mM CAT and 17 mg protein/ml. The mitochondria were incubated at 0°C for 15 min then sedimented by centrifugation at 7,000 x g for 15 min. The mitochondria were then solubilized in solubilization buffer at a concentration of 17 mg protein/ml. Unsolubilized material was sedimented by centrifugation at 7,000 x g for 15 min. The solubilized mitochondria were applied to a hydroxylapatite column equilibrated in elution buffer at a ratio of 1 mg protein/ml of hydroxylapatite. The protein was eluted with the same buffer.

Fractions containing protein were lyophilized, dissolved in 88% formic acid and chromatographed on Sephadex LH60 in 88% formic acid/ethanol (30:70) to remove the Triton X-100.

An alternate procedure for the isolation of the adenine nucleotide translocator was sometimes used. This was a modification of a procedure according to von Jagow et al. (1978).

Mitochondria were washed twice with excess preincubation buffer. They were then suspended in preincubation buffer and 1 mM CAT to a concentration of 0.1 mM CAT and 17 mg protein/ml. The mitochondria were incubated at 0°C for 15 min then sedimented by centrifugation at 7,000 x g for 15 min. They were then suspended in pre-extraction buffer to a concentration of 17 mg protein/ml. They were incubated at 0°C for 30 min then sedimented by centrifugation at 120,000 x g for one hour. The mitochondria were resuspended in solubilization buffer to a concentration of 17 mg protein/ml and incubated at 0°C for 15 min. Unsolubilized material was sedimented by centrifugation at 15,000 x g for 15 min. The supernatant was then treated as in the previous procedure.

MISCELLANEOUS METHODS

A. SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis was done according to the procedure of Laemmli (1970) or Swank & Munkres (1971). Fluorography of gels was done according to the procedure of Bonner & Laskey (1974).

B. Protein determination

For determination of protein in the microgram range a modification of the procedure according to Lowry *et al*. (1951) was used. For determination of protein in the milligram range the Buiret protein determination according to the procedure of Gornall *et al*. (1949) was used.

C. Phosphorous determination

For the determination of phosphorous in phospholipid spots on TLC plates, the spot was scraped into an acid-washed pyrex test tube and the phosphorous determined essentially according to the procedure of Bartlett (1959).

D. Phospholipid extraction

Phospholipids were extracted essentially according to the procedure of Bligh & Dyer (1959).

III RESULTS AND DISCUSSION

1. Preparation of mitochondria, mitoplasts and submitochondrial particles

It has been generally accepted that mitoplasts prepared by standard techniques are necessarily right-side-out oriented (Schnaitman & Greenawalt, 1968) and that submitochondrial particles, most commonly prepared by sonication, are inverted with respect to the normal mitochondrial orientation (Racker, 1970). Recent work has indicated, however, that it may be difficult even to isolate mitochondria in a highly intact homogenous form. In particular, results from a number of laboratories (for a review see Harmon et al. 1974) have indicated that mitochondria isolated from beef heart can be as high as 48-78% inverted. In this study, even heavy beef heart mitochondria were shown to be at least 30% inverted. If the isolated mitochondria are not almost completely right-side-out, it is impossible to prepare intact, right-side-out mitoplasts from them.

Since mitochondria can be isolated from rat liver under more gentle conditions than those used for the isolation of beef heart mitochondria, they can be obtained in a more intact and homogenous form.

In this study, rat liver mitochondria could be isolated in an extremely intact state as indicated by sidedness determinations consistently greater than 95% RSO.

Figure 7 shows the plots of the % of monoamine oxidase released, the % of malate dehydrogenase released and the % of right-side-out membranes in the mitoplasts obtained as a function of different digitonin to mitochondrial protein ratios. Much less malate dehydrogenase was

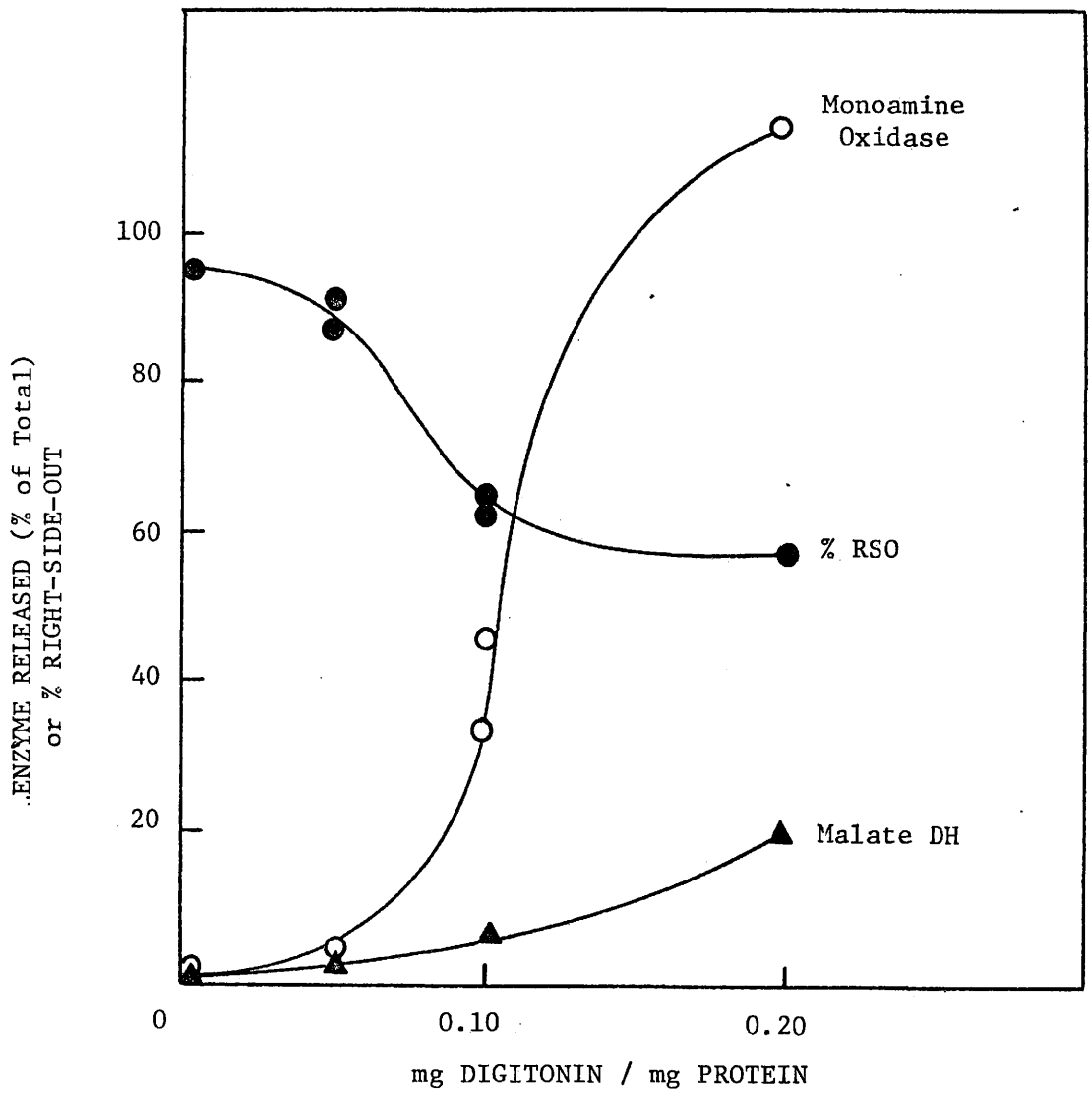


Figure 7. The effect of different digitonin concentrations in the production of rat liver mitoplasts. The activities of monoamine oxidase (outer membrane marker) and malate dehydrogenase (matrix marker) released into supernatants after centrifugation of the mitoplasts at 12,000 x g are expressed as percentages of the original total activities and plotted versus digitonin concentrarion. The percent right-side-out character of the resulting mitoplasts as determined by the sidedness assay is also shown.

released into the supernatants at all concentrations, indicating that, at least by this criterion, the inner membranes obtained were not very leaky. However, the % right-side-out character of the mitoplasts, as indicated by the sidedness assay, decreased considerably with increasing digitonin concentrations. Removal of 100% of the monoamine oxidase resulted in 57% right-side-out mitoplasts. The malate dehydrogenase and sidedness data are somewhat contradictory and it may be that high digitonin concentrations introduce only small holes in the inner membrane which allow ferricyanide ions to penetrate but not the malate dehydrogenase to leak out. Still, it would seem possible with this method to obtain mitoplasts which have had their outer membranes opened up sufficiently to allow the outside of the inner membranes to be fully accessible to reagents but which are still 65-75% right-side-out and very intact.

Ambiguous results were obtained in the preparation of submitochondrial particles however. It is possible with sonication to generate an inhomogenous membrane preparation with some particles resealed in an inverted configuration and some in the native configuration (Astle & Cooper, 1974), some particles resealed with part in the native configuration and part in an inverted configuration (Tracy et al. 1978), some particles where certain proteins have been "scrambled" to non-physiological positions (Eytan et al. 1975) and some non-sealed membrane sheets (for a review see Harmon et al. 1974).

Therefore, if the particles are all inside-out but the cytochrome c has been "scrambled" and some is accessible to the ferricyanide in the assay for sidedness, it will appear that a population of the particles is right-side-out.

In most preparations of submitochondrial particles isolated for this research, 60-85% inside-out character was achieved according to the ferricyanide method for the elucidation of membrane sidedness. Due to the limitations just outlined, however, these values may not be accurate.

2. Surface labelling

An investigation of surface labelling of mitoplasts and submitochondrial particles was undertaken for two reasons:

i) to identify regions of proteins in contact with the aqueous environment on either surface of the mitochondrial inner membrane.

ii) to achieve asymmetric modification of phospholipids.

Asymmetric modification of phospholipids was required:

1) To study the asymmetric distribution of phospholipids in the mitochondrial inner membrane.

2) To establish the non-permeant nature of the soluble probes studied.

3) To provide complementary evidence to confirm the asymmetric insertion of phospholipid by phospholipid exchange protein.

It must be established, when using surface labelling to look at which regions of proteins are accessible from either side of the membrane, that the probes used do not penetrate the membrane. This can be achieved by studying asymmetric modification of phospholipids. If the membrane is indeed non-permeable to the probe, all of the external phospholipids will be labelled without any labelling of the phospholipids in the inner bilayer.

By labelling mitoplasts and submitochondrial particles with reagents which react with groups in phospholipids such as the free amino

groups in PE, differences in the amount of phospholipid labelled in the two types of particles yields information concerning the transverse distribution of phospholipids across the inner mitochondrial membrane.

As will be discussed later, phospholipid exchange protein has been utilized to effect an asymmetric incorporation of photoreactive phospholipid into mitoplasts and submitochondrial particles. The use of surface labels could provide complementary evidence that this incorporation is restricted to the external monolayer. It is essential for this purpose that the probe exhaustively label the reactive groups on the external monolayer of the inner membrane preparation without labelling any reactive groups on the inner monolayer. Thus an absence of unmodified crosslinked phospholipid would verify asymmetric incorporation of photoreactive phospholipid.

In this study two water-soluble probes, cycloheptaamylose fluorescamine complex and isethionyl acetimidate, were used. Both of these reagents react specifically with free amino groups such as are found in phosphatidylethanolamine and the amino terminus and lysine side chains in proteins.

A. Cycloheptaamylose fluorescamine complex (CFC)

Cycloheptaamylose fluorescamine complex was synthesized according to the procedure of Nakaya et al. (1975) and stored over P_2O_5. Various aspects of its behaviour were studied.

i) Chacterization of behaviour of CFC

Various aspects of the CFC were studied by monitoring the fluorescence of its reaction with free amino groups using a Perkin Elmer MPF-44 Fluorescence Spectrophotometer with a Perkin Elmer 56 Recorder. The excitation wavelength was 390 nm and the emission wavelength was 465 nm.

(a) The CFC was reacted with ten-fold excess tris to determine the kinetics of the reaction. The CFC was found to be reactive and the reaction levelled off after 26 min (Fig. 8, Curve A).

(b) The stability of the CFC in solution was determined. It was found that incubation of CFC in buffer at room temperature for 90 min caused a decrease in activity of 60% (Fig. 8, Curve B).

(c) As CFC is sparingly soluble in water, sonication was tried to increase solubility. Sonciation was found to significantly reduce the activity of CFC (Fig. 9).

ii) Labelling of mitoplasts with CFC

CFC has previously been shown to be non-penetrating in red blood cells (Nakaya et al. 1976), sarcoplasmic reticulum membrane (Hidelgo & Ikemoto, 1977) and mitoplasts and submitochondrial particles (Krebs et al. 1979). Krebs et al. (1979) found that the labelling of phosphatidyl-ethanolamine in mitoplasts and submitochondrial particles levelled off at 38% and 63% respectively of the PE modified using 0.5 mg CFC/mg mitochondrial protein.

In this study (Fig. 10), the modification of PE by CFC is almost complete, presumably due to increased flip-flop rate as CFC would not be expected to actually penetrate the membrane. There is however a discontinuity in the experimental points which could indicate some degree of levelling off at 2 mg CFC/mg protein. This is suggestive evidence that approximately 30% of the total PE in mitoplasts may be in the external monolayer.

There could be differences in the stability of the rat liver and beef heart inner mitochondrial membrane which lead to this discrepancy.

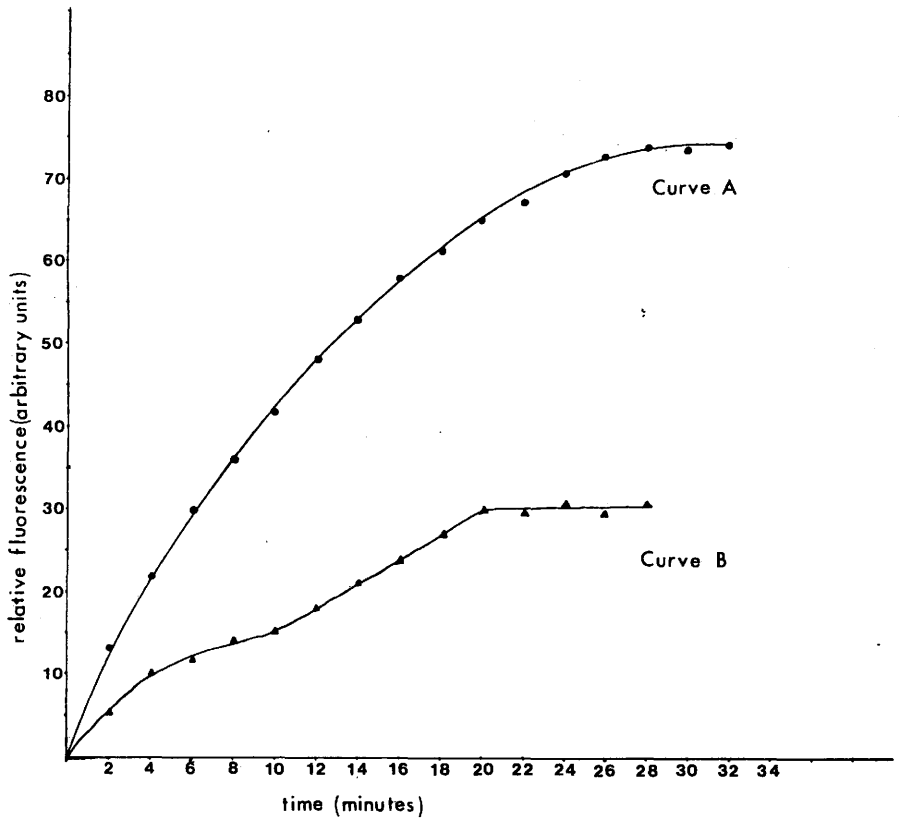


Figure 8. Stability of CFC in aqueous solution: CFC was dissolved in 220mM mannitol, 70mM sucrose, 10 mM $NaHCO_3$ pH 7.8 to a concentration of 1 mg/ml and reacted with excess tris. Curve A (●——●): tris added immediately after CFC was dissolved in buffer; Curve B (▲——▲); tris added after incubating the CFC in buffer for 90 minutes. Both reactions were carried out at room temperature and fluorescince measured using a Perkin Elmer MPF-44 Fluorescence Spectrophotometer with a Perkin Elmer 56 Recorder, using an excitation wavelength of 390 nm and an emission wavelength of 465 nm.

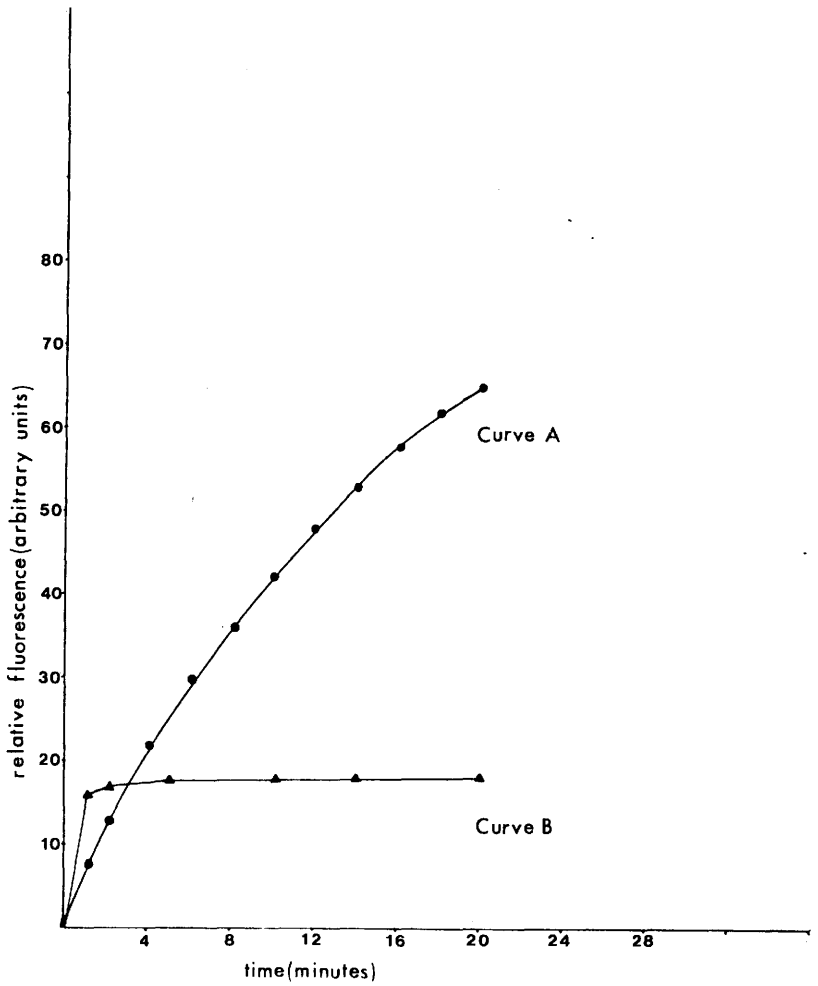


Figure 9. Effect of sonication on CFC activity: CFC was dissolved in 220 mM mannitol, 70 mM sucrose, 10 mM $NaHCO_3$ pH 7.8 to a concentration of 1 mg/ml and reacted with excess tris. Curve A (●——●); CFC was dissolved in buffer then incubated with tris at room temperature. Curve B (▲——▲); CFC was dissolved in buffer, sonicated 5 min, then incubated with tris at room temperature. Fluorescence was measured using a Perkin Elmer MPF-44 Fluorescence Spectrophotometer with a Perkin Elmer 56 Recorder using an excition wavelength of 390 nm and an emission wavelength of 465nm.

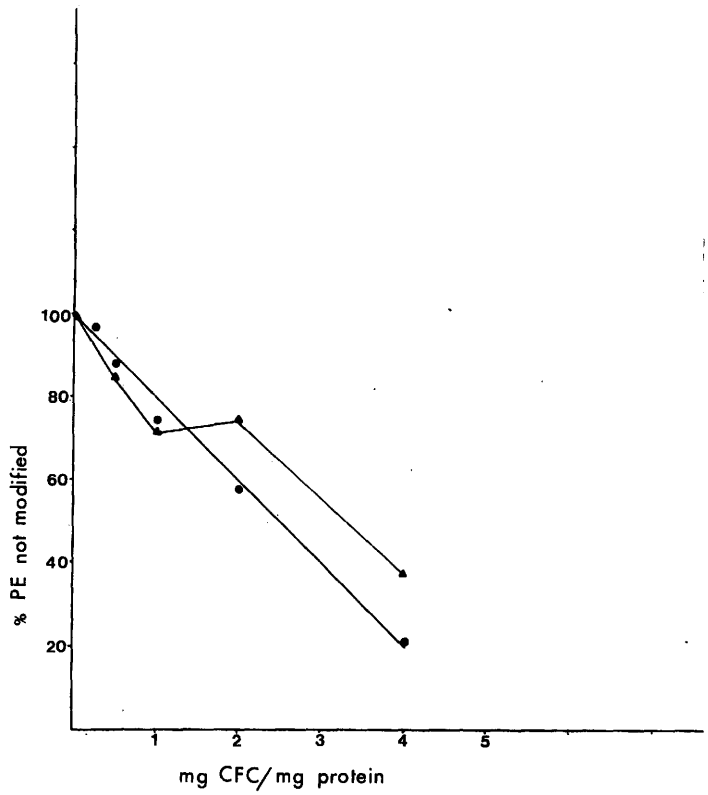


Figure 10. Reaction of intact ▲——▲ and solubilized (4.5 % sodium deoxycholate) ●——● rat liver mitoplasts with increasing concentrations of CFC. Samples were incubated at 25°C for 50 min at a concentration of 1.2 mg protein/ml.

It may be possible to choose conditions under which asymmetric labelling can apparently be achieved but in view of the results of this study, this is not necessarily reproducible.

In this study, therefore, it can be concluded that exhaustive labelling of the outer monolayer in rat liver mitoplasts cannot be achieved without perturbation of the membrane and the resulting labelling of the inner monolayer.

B. Isethionyl acetimidate

Isethionyl acetimidate is a water-soluble imido ester which has been shown to be impermeant to red blood cells (Whiteley & Berg, 1974) and single walled bilayer vesicles (Roseman et al. 1975). It reacts with primary amino groups and therefore can be used to label proteins and phosphatidylethanolamine.

i) Synthesis of ^{3}H IAI

By introducing a radioactive isotope into a surface label, the product of the reaction can more easily be studied. Therefore, tritiated isethionyl acetimidate was synthesized.according to the procedure of Whiteley & Berg (1974). This was stored at -20°C over dessicant.

ii) Labelling of mitoplasts with IAI - analysis of phospholipid labelling

In this study rat liver mitoplasts were incubated with a range of IAI concentrations at 0°C and 25°C to determine if a levelling off of labelling of the phosphatidylethanolamine could be achieved. Fig. 11 shows the results of such an experiment. At 25°C 100 mM IAI causes modification of 90% of the PE indicating penetration of the membrane. There is no levelling off of labelling at this temperature. As seen in

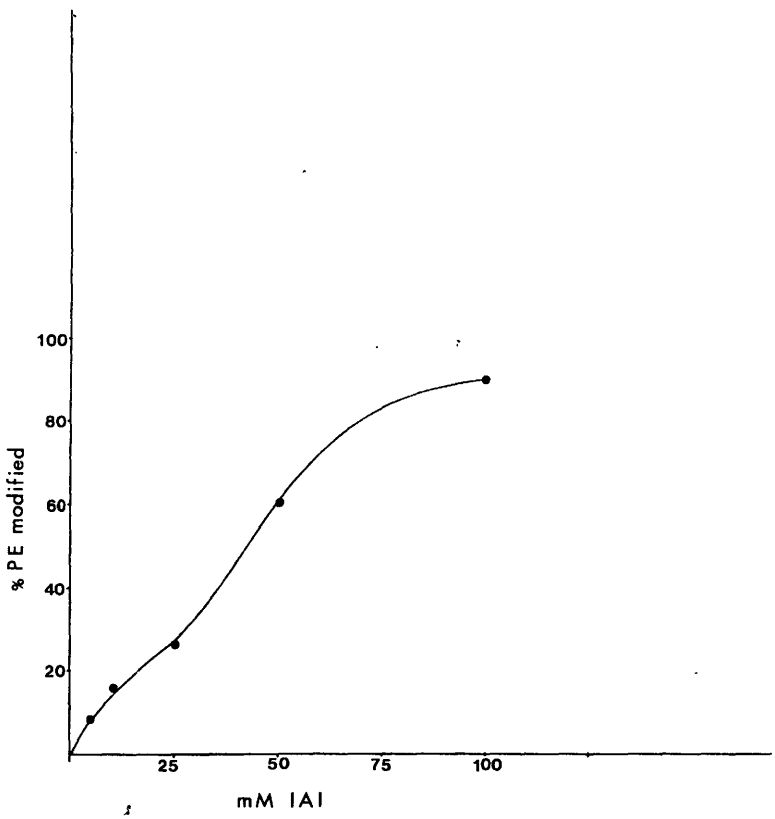


Figure 11. Labelling of intact rat liver mitoplasts (2 mg protein/ml) with increasing concentrations of IAI in 0.7 M sucrose, 0.22 M mannitol, 0.02 M borate pH 8.0 at 25°C for 30 min.

Fig. 12 the amount of PE labelled by 100 mM IAI at 0°C is much lower (50%) but there is not a levelling off of labelling in this case either.

Figure 13 shows the kinetics of labelling of PE in mitoplasts by 50 mM IAI. In this case the reaction seems to be complete by about 10 min. The levelling off could be a result of complete modification of the external PE or of the loss of reagent due to labelling of PE and proteins and hydrolysis of the IAI to non-reactive products.

The kinetics of labelling in mitoplasts were studied for a range of IAI concentrations at 25°C. It is observed in Figure 14 that with increasing amounts of IAI used the percent PE labelled increases. There is, however, some degree of levelling off at 50 mM.

As has been previously discussed, the reaction of isethionyl acetimidate with free amino groups results in an increase in the pH of the reaction mixture. As a high pH may damage the integrity of the membrane, the reaction mixture was titrated to maintain the pH at 8.0. Figure 15 shows the kinetics of labelling of PE in mitoplasts with a range of IAI concentrations with titration to pH 8.0. The level of modification in the titrated samples is considerably lower than the level in the untitrated samples.

As was previously discussed,Browne & Kent (1975) observed that the treatment of horse liver alcohol dehydrogenase with isethionyl acetimidate at pH 8.0 led initially to the production of protein N-alkyl imidates which then partition approximately equally between reaction with ammonia to form amidines and hydrolysis to yield free amine. At pH 10, however, the N-alkyl imidate is not formed to an appreciable extent and amidination is nearly complete. It is to be expected, therefore, that

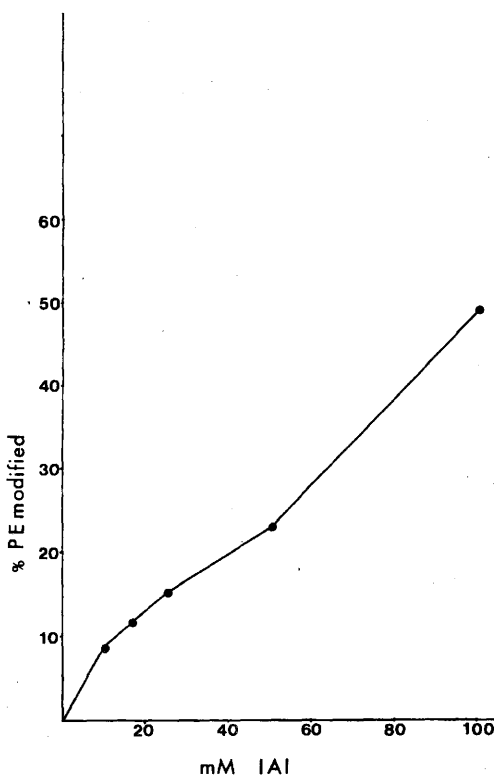


Figure 12

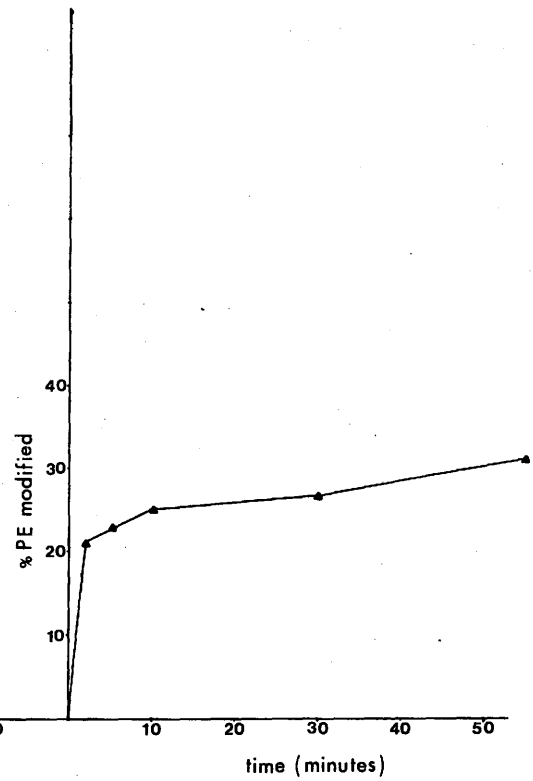


Figure 13

Figure 12. Labelling of intact rat liver mitoplasts (2 mg protein/ml) with increasing concentrations of IAI in 0.07 M sucrose, 0.22 M mannitol, 0.02 M borate pH 8.0 at 0°C for 30 min.

Figure 13. Kinetics of reaction of intact rat liver mitoplasts (2 mg protein/ml) with 50 mM IAI in 0.07 M sucrose, 0.22 M mannitol, 0.02 M borate pH 8.0 at 0°C.

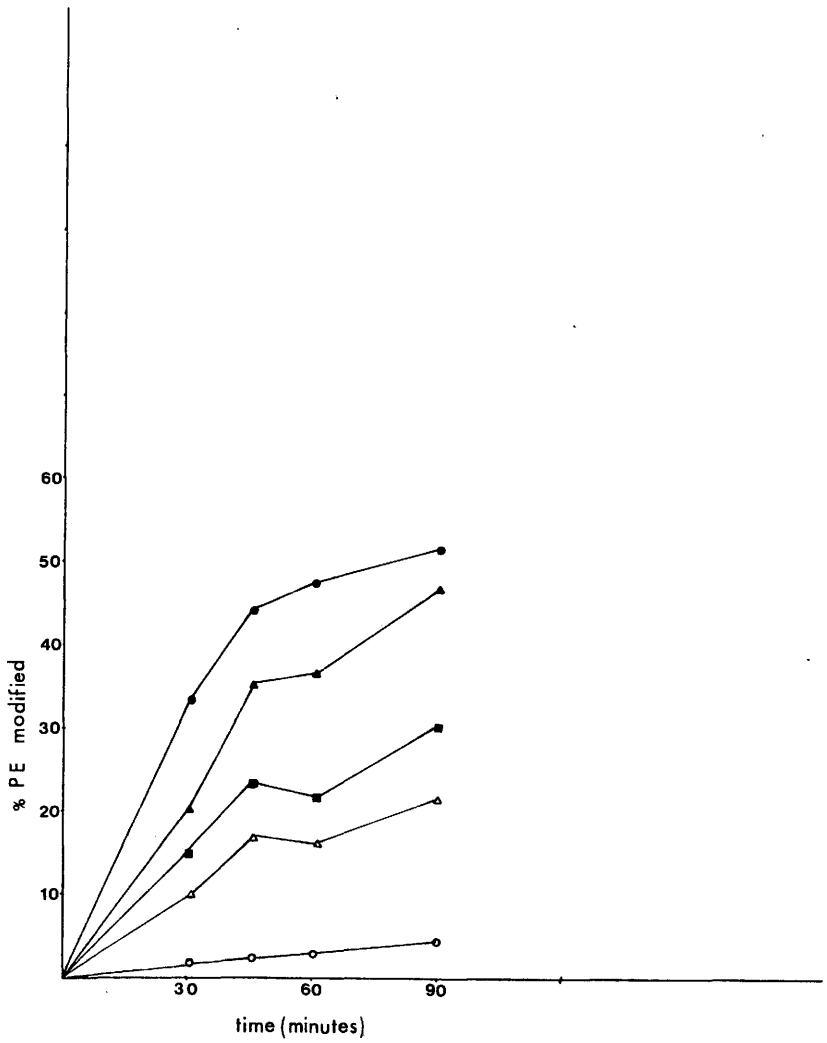


Figure 14. Kinetics of reaction of intact rat liver mitoplasts (5 mg protein/ml) with 10 mM (o——o), 30 mM (△——△), 40mM (■——■), 50 mM (▲——▲) and 70 mM (●——●) IAI in 220 mM mannitol, 70 mM sucrose, 2 mM Hepes, 0.5 mg BSA/ml pH 8.0 at 25°C.

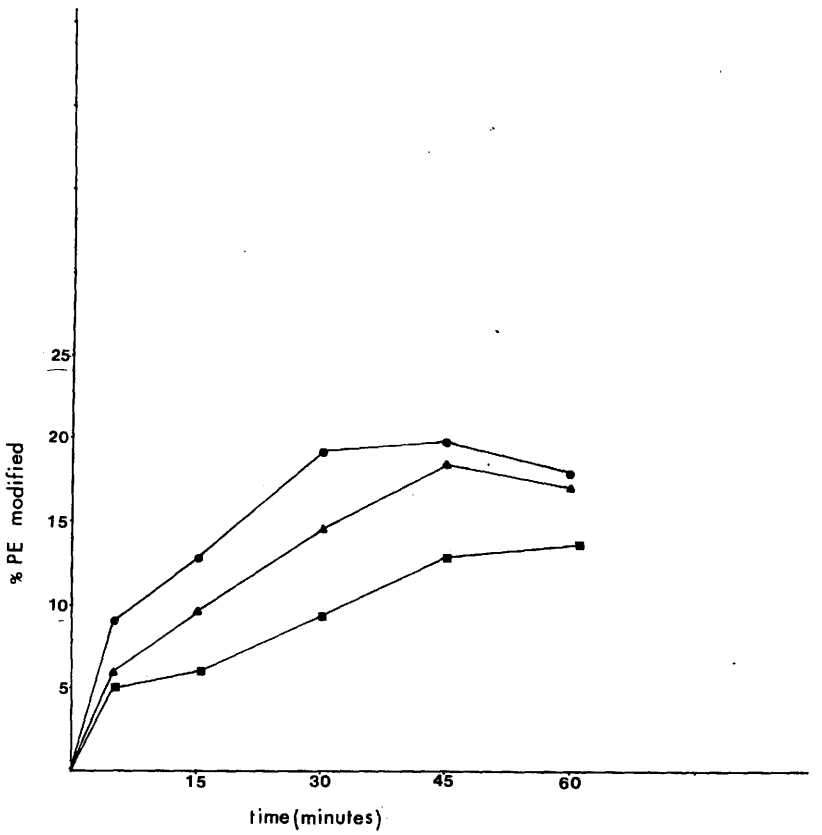


Figure 15. Reaction of intact rat liver mitoplasts (5 mg protein/ml) with 40 mM (■——■), 50 mM (▲——▲) and 60 mM (●——●) IAI in 220 mM mannitol, 70 mM sucrose, 2 mM Hepes, 0.5 mg BSA/ml pH 8.0 at 25°C with titration with 0.1 N HCl to maintain the pH at 8.0.

for the same amount of IAI used, the level of labelling would be lower when the pH is maintained at 8.0 than when it is allowed to rise freely to 9.0 - 10.0.

Since high concentrations of IAI appear to penetrate the inner mitochondrial membrane, a high pH of reaction damages the integrity of the membrane and at low IAI concentration and pH 8.0 the reaction is incomplete, it was decided that multiple additions of a fairly low concentration of IAI would be used with the pH maintained at 8.0. Browne & Kent (1975) have shown that multiple additions of a low concentration of fresh IAI at pH 8.0 result in the same level of labelling of horse liver alcohol dehydrogenase as a single addition at pH 10.0.

Figure 16 shows the results of the modification of solubilized and intact mitoplasts by multiple additions of IAI. It can be seen from the experiment using solubilized mitoplasts that complete modification of phosphatidylethanolamine has been achieved after the second addition of IAI. In the experiment with intact mitoplasts there is a levelling off of modification after each addition of IAI. There is some modification of PE even after the fourth addition of IAI, although the increment is significantly lower than that after the first three additions.

This could be interpreted to indicate that about 40 to 46% of the PE is on the outside of mitoplasts. Krebs et al. (1979) found that 38% of the PE in beef heart mitoplasts is located on the outside. These results do not differ greatly and the difference could be due to the different origins of the mitoplasts.

In view of the fact that the fourth addition of IAI leads to a small increment in the level of labelling, it is probable that prolonged

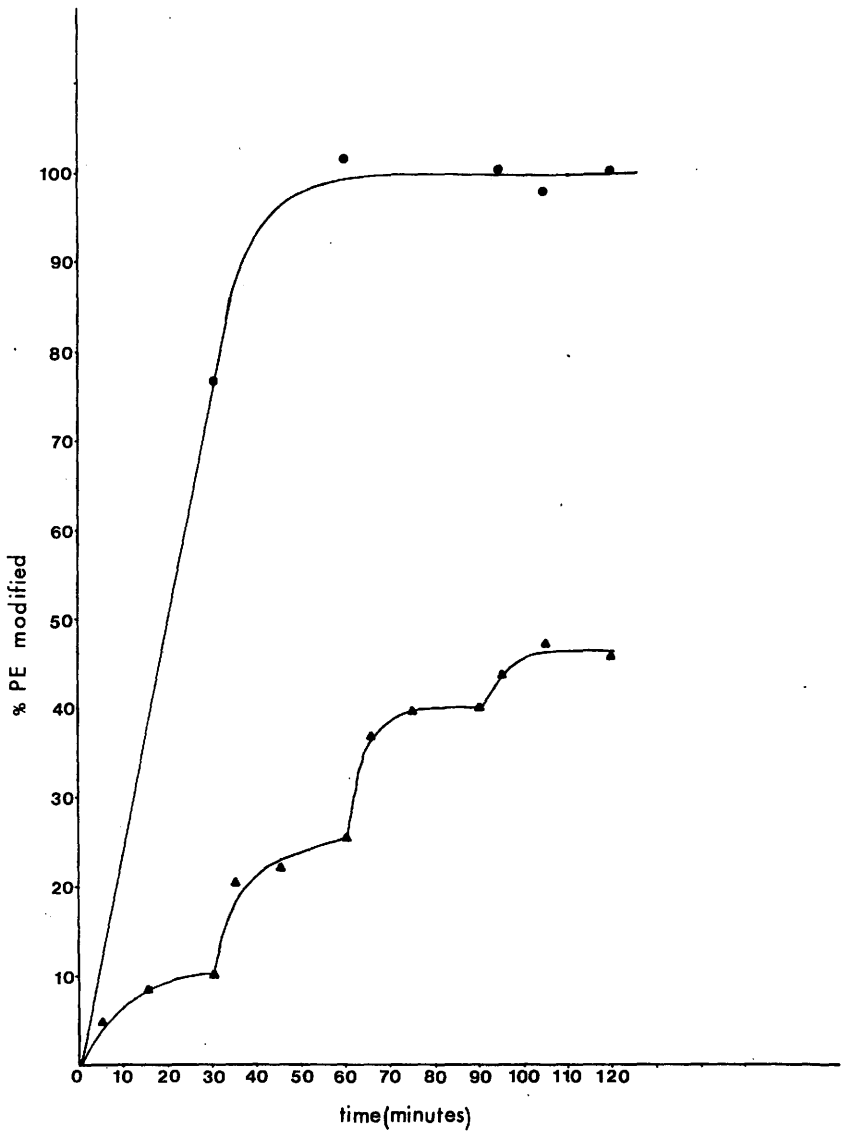


Figure 16. Kinetics of labelling of intact (▲——▲) and solubilized (●——●) (1.3 % sodium deoxycholate) rat liver mitoplasts (5 mg protein/ ml) with IAI with titration with 0.1 N HCl to maintain the pH at 8.0 at 25°C. Freshly prepared IAI (25 mM) was added at 0, 30, 60, and 90 minutes of incubation to both the intact and solubilized mitoplasts.

exposure of mitoplasts to the reagent leads to penetration of the inner mitochondrial membrane. As the reaction is carried out at room temperature it is possible that with increasing time the membrane becomes somewhat permeable to the IAI. It is more likely, therefore, that the labelling of 46% PE in mitoplasts includes some amount of internal PE and is an upper limit and that the amount of PE on the external monolayer of mitoplasts is very much lower, perhaps closer to 30% as was suggested from CFC studies.

Studies of protein labelling, which will subsequently be discussed have also indicated that, under the conditions used for IAI modification, considerable penetration of the mitochondrial inner membrane occurs.

Crain & Marinetti (1979) observed a low level of modification by isethionyl acetimidate of phosphatidylethanolamine in both intact and solubilized mitoplasts. The reason for the discrepancy of the results presented here and those observed by Crain & Marinetti (1979) could be explained by differences in the method of addition of IAI. Crain & Marinetti (1979) made a single addition while in this study four consecutive additions were made. A single addition of IAI, as stated earlier, has been shown to result in incomplete amidination of free amino groups (Browne & Kent, 1975).

Crain & Marinetti (1979) concluded from other methods that 30-40% of the total PE in the mitoplast membrane is located on the outer membrane surface. The results of this study show that 40-46% of the PE in intact mitoplasts can be modified by isethionyl acetimidate. Due to the penetration of the membrane at prolonged exposure to reagent and prolonged incubation at room temperature, this value is probably much higher than the actual value for externally located PE.

It can be concluded, therefore, that less than 40 to 46% of the total PE in rat liver mitoplasts is located in the external bilayer. It also seems that exhaustive labelling of the external monolayer of mitoplasts with IAI can not be achieved in the absence of labelling of the inner monolayer.

3. Phospholipase A_2

A number of studies have been done using specific phospholipases to investigate the transverse localization of phospholipids across biological membranes (for a review see Etemadi, 1980). Several groups using specific phospholipases have established an asymmetric distribution of phospholipids across the erythrocyte membrane (Colley et al. 1973; Verkleij et al. 1973; Zwaal et al. 1973).

Phospholipase A_2 has been used also to study the transverse distribution of phospholipids across the mitochondrial inner membrane (Nilsson & Dallner, 1977; Krebs et al. 1979). Nilsson & Dallner (1977) used phospholipase A_2, isolated from Naja naja naja cobra venom according to the procedure of Cremona & Kearny (1963) as modified by Verkleij et al. (1973), to modify rat liver mitoplasts. They found that more than 90% of the PE and over 50% of the PC was hydrolyzed under conditions where about 56% of the total phospholipid was hydrolyzed. It must be noted that some of the phospholipids, notably cardiolipin and sphingomyelin are very poor substrates for phospholipase A_2 so that the hydrolysis of 56% of the total phospholipid cannot be taken to mean that this proportion is external while 44% is located on the inner monolayer.

Krebs et al. (1979) using phospholipase A_2 isolated from Naja naja naja cobra venom according to the procedure of Deems & Dennis (1975) to

study the transverse distribution of phospholipids across the inner membrane of beef heart mitochondria observed hydrolysis of 73% of the total PC and 39% of the total PE in mitoplasts and 30% of the total PC and 63% of the total PE in submitochondrial particles. These results differ significantly from the results obtained by Nilsson & Dallner (1977). A number of factors could be responsible for the differences. Krebs et al. (1979) were investigating beef heart mitoplasts while Nilsson & Dallner (1977) looked at rat liver mitoplasts. It is possible that some differences in phospholipid distribution exist in the mitochondria of different species, although it does not seem likely that such a difference would be as large as the experimentally found difference. Krebs et al. (1979) found that phospholipase A_2 isolated according to the procedure of Cremona & Kearny (1964) had a tendency to make the membrane leaky. This had also been observed by Salach et al. (1968), Lankisch et al. (1971) and Higgins & Dawson (1977). It would appear, therefore, that the results observed by Nilsson & Dallner (1977) do not represent the true distribution of the phospholipids across the rat liver mitoplast membrane, due to the tendency of the phospholipase A_2 preparation to make the membrane leaky.

In this study, therefore, phospholipase A_2, isolated according to the method of Deems & Dennis (1975), was utilized in order to generate asymmetric membranes.

A. Isolation and purification of phospholipase A_2 from Naja naja naja cobra venom

Phospholipase A_2 was isolated according to the method of Deems & Dennis (1975). Crude Naja naja naja cobra venom and purified phospholipase

A_2 were subjected to SDS polyacrylamide gel electrophoresis as shown in Fig. 17.

B. Treatment of mitochondria, mitoplasts and SMP with phospholipase A_2

The results of various phospholipase A_2 experiments are shown in Table II. BSA is used in the experiments to bind released fatty acids which could otherwise disrupt the membrane. The concentration of BSA was increased when hydrolysis failed to level off. $CaCl_2$ is essential for the activity of phospholipase A_2. Dennis (1973) found optimal activity of phospholipase A_2 with 5 mM $CaCl_2$ and a levelling off of activity with 2.5 mM $CaCl_2$. The concentration of $CaCl_2$ was therefore increased over the amount used by Krebs et al. (1979). $CaCl_2$ also increases the stability of the membrane. The temperature was decreased from the 37°C used by Krebs et al. (1979) to further increase the stability of the membrane.

As can be seen from Table II, the results of Krebs et al.(1979) could not be reproduced. The results of the various experiments using beef heart mitoplasts and submitochondrial particles and rat liver mitoplasts and mitochondria indicated that hydrolysis with phospholipase A_2 caused perturbations in these membranes and therefore accessibility of phospholipids on the internal monolayer. This would not be surprising as the phospholipase A_2 generates lysophospholipids and fatty acids, both of which act as detergents.

It can be concluded that phospholipase A_2 isolated according to the procedure of Deems & Dennis (1975) causes perturbation of the inner membrane of rat liver mitochondria under all the conditions tried in this study. This perturbation may be penetration of the membrane, increased rate of flip-flop or both and is caused by the liberation of free fatty acids and lysophospholipids, both of which act as detergents.

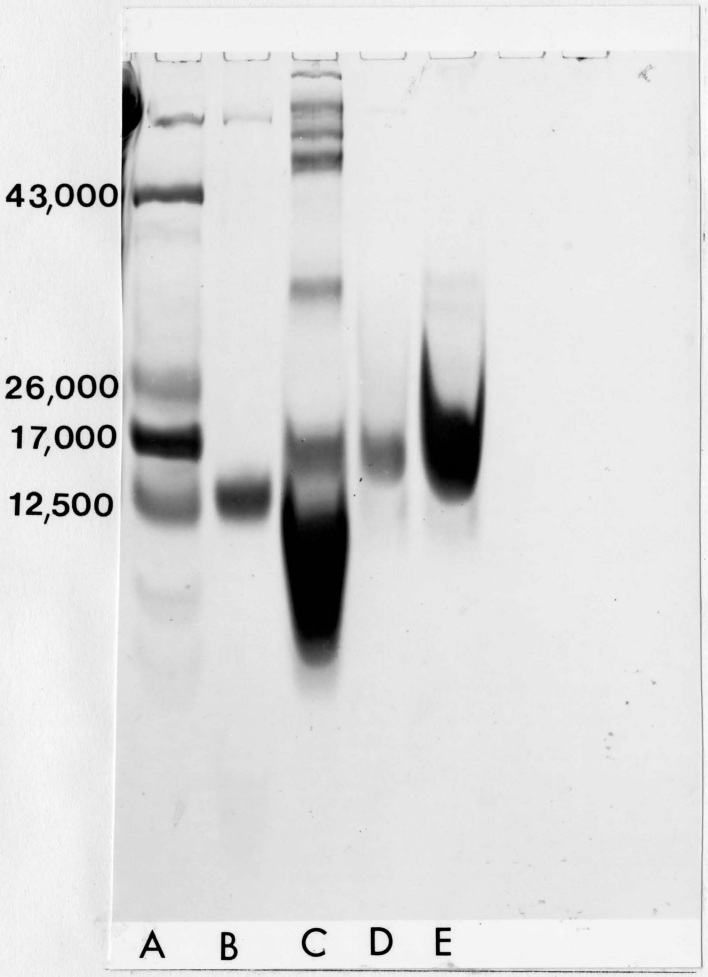


Figure 17. Purification of Phospholipase A_2; stained gel

A 10 µg each of ovalbumin (M_r=43,000), bacteriorhodopsin (M_r= 26,000), sperm whale myoglobin (M_r=17,000) and cytochrome c (M_r=12,500)

B 10 µg cytochrome c (M_r=12,500)

C 200 µg crude Naja naja naja cobra venom

D 10 µg purified phospholipase A_2

E 100 µg purified phospholipase A_2

TABLE II

Summary of Conditions and Results of Phospholipase A_2 Experiments

Phospholipase /5 mg protein	[BSA]	[$CaCl_2$]	Temp.	Type of particles	Results
0.7, 0.07	0.5 mg/ml	0.25 mM	37°C	Beef Heart SMP	Failed to level off in 60 min.
				Beef Heart Mitoplasts	" " "
0.7	0.5 mg/ml	0.25 mM	37°C	Beef Heart SMP	Essentially levelled off by 120 min at 92% PE and 73% PC hydrolyzed
0.7	0.5 mg/ml	0.25 mM	37°C	Rat Liver SMP	Failed to level off in 60 min
				Rat Liver Mitoplasts	" " "
0.5	5 mg/ml	0 mM	25°C	Beef Heart SMP	Rate of reaction decreased but did not level off after 60 min with 94% PE and 81% PC hydrolyzed
0.5	5 mg/ml	0.25 mM	25°C	Beef Heart SMP	Rate of reaction decreased but did not level off after 60 min with 94% PE and 76% PC hydrolyzed
0.5	5 mg/ml	2.5 mM	25°C	Beef Heart SMP	Rate of reaction decreased but did not level off after 60 min with 93% PE and 72% PC hydrolyzed
0.5	5 mg/ml	10 mM	25°C	Beef Heart SMP	Rate of reaction decreased but did not level off after 60 min with 96% PE and 85% PC hydrolyzed
25	5 mg/ml	2.5 mM	6°C	Rat Liver Mitoplasts	Levelled off by 60 min with 85% PE and 78% PC hydrolyzed
0.25	5 mg/ml	2.5 mM	0°C	Rat Liver Mitoplasts	Failed to level off after 60 min

TABLE II (continued)

g Phospholipase $_2$/5 mg protein	[BSA]	[$CaCl_2$]	Temp.	Type of particles	Results
2.5	5 mg/ml	2.5 mM	0°C	Rat Liver Mitochondria	Rate of reaction decreased but did not level off after 60 min with 79% PE and 65% PC hydrolyzed
2.5	0.5 mg/ml	2.5 mM	0°C	Rat Liver Mitochondria	Essentially levelled off after 60 min with 91% PE and 68% PC hydrolyzed
2.5	5 mg/ml	0.25 mM	0°C	Rat Liver Mitochondria	Rate of reaction decreased but did not level off after 60 min with 71% PE and 60% PC hydrolyzed
2.5	0.5 mg/ml	0.25 mM	0°C	Rat Liver Mitochondria	Rate of reaction decreased but did not level off after 60 min with 8% PE and 67% PC hydrolyzed

In this study it was not possible to find conditions under which a levelling off of hydrolysis of phospholipids, as demonstrated by Krebs et al. (1979), was observed.

The results of the investigation of asymmetric modification of phospholipids in mitochondria, mitoplasts and SMP indicate that less than 40 to 46% of the total PE in mitoplasts is located in the external monolayer. This upper limit was obtained by labelling mitoplasts with IAI. Results obtained by labelling mitoplasts with CFC suggested that 30% of the total PE might be externally located.

Under none of the conditions used in this study could exhaustive modification of phospholipids in the external monolayer of membrane preparations by IAI, CFC or phospholipase A_2 be achieved in the absence of modification of phospholipids located on the internal monolayer.

4. Analysis of Protein Labelling by IAI

As was discussed earlier, it appears that treatment of mitochondrial inner membrane preparations with IAI results in a considerable level of penetration, though this is not complete. This was indicated in the study of phospholipid labelling by IAI Analysis of protein labelling by IAI was also done and the results of such an experiment are shown in Figure 18. It can be seen that there are no significant differences between the labelling patterns of the mitoplasts (track C) and the submitochondrial particles (track G). This would suggest that, as in earlier observations, under the conditions used in this experiment, either the mitoplast membrane is disrupted or the mitoplast preparation is contaminated with SMP, causing proteins on the matrix surface to be accessible to labelling by IAI.

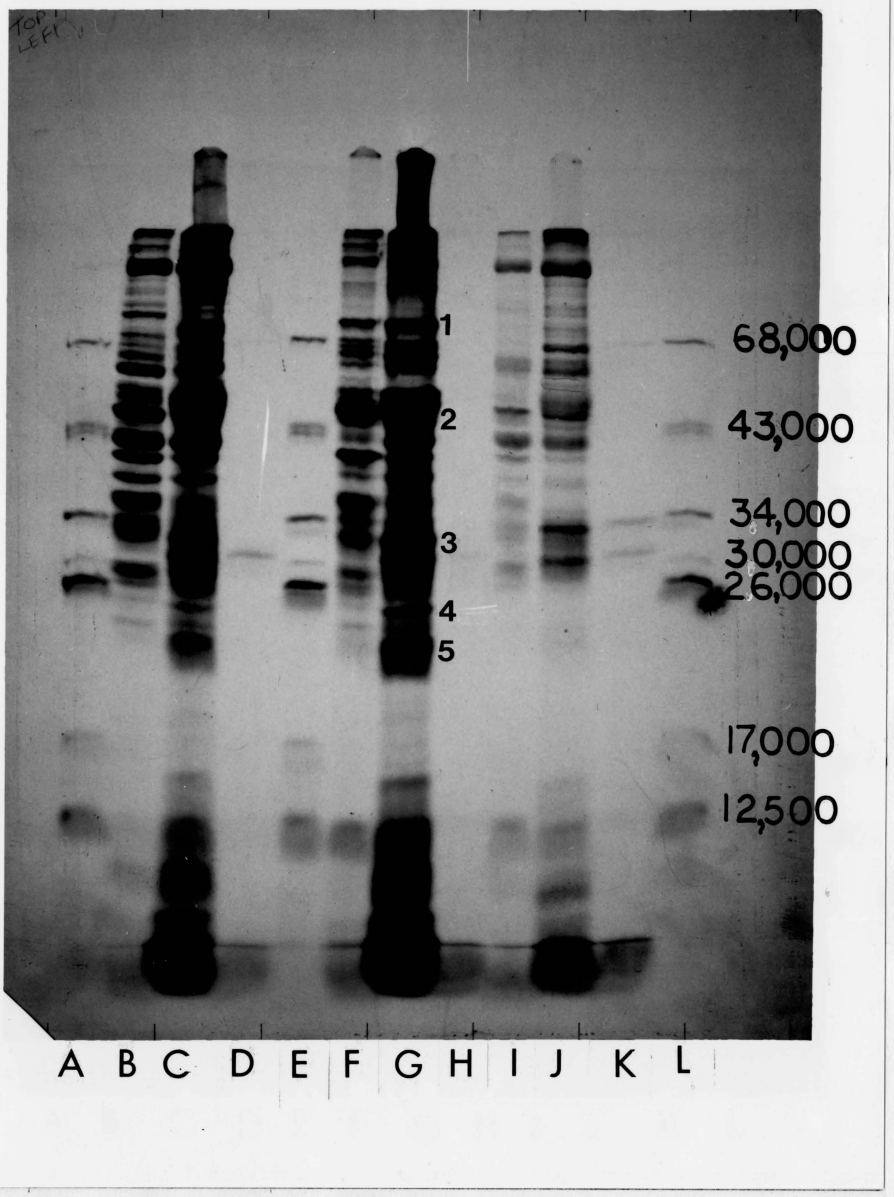


Figure 18. Labelling of rat liver mitochondria (track J), mitoplasts (track C), and submitochondrial particles (track G) at a concentration of 5 mg protein/ml with 25 mM IAI in 0.25 M sucrose, 0.5 mM EDTA, 2.5 mM $MgCl_2$, 20 mM MOPS pH 8.0 for 30 min at 25°C. Samples (0.5 mg protein) were analyzed by SDS polyacrylamide gel electrophoresis followed by fluorography. Tracks K, D and H show adenine nucleotide translocator isolated by chromatography on HAP from 0.5 mg labelled mitochondria, mitoplasts and SMP respectively. Tracks A, E and L show BSA (M_r=68,000), ovalbumin (M_r=43,000), ATCase (M_r=34,000 & 17,000), carbonic anhydrase (M_r=30,000) chymotrypsinogen (M_r=26,000) and cytochrome c (M_r=12,500).

The labelling patterns of mitochondria and SMP by IAI are significantly different. It can be observed that several proteins are labelled when SMP are treated IAI which are not labelled in IAI treated mitochondria. These proteins, 1 to 5 are located on the matrix surface of the mitochondrial inner membrane. There are also several high molecular weight proteins which are labelled much more heavily in SMP than in mitochondria. It is evident that IAI is able to penetrate the mitochondrial outer membrane as the adenine nucleotide translocator is labelled in intact mitochondria as well as in mitoplasts and SMP. The labelled translocator protein can be isolated from all three types of particles as shown in tracks D, H and K of Figure 18. The level of labelling of the translocator is much lower in SMP than in mitochondria. This could indicate that there is not a translocator reactive site for IAI on the matrix surface of the inner membrane, the small amount of labelling which is observed being due to contamination of SMP with right side out oriented particles. In order to determine whether or not an internal reactive site exists a double labelling experiment can be done, exhaustively labelling intact mitochondria with multiple additions of ^{3}H IAI then sonicating an aliquot to form SMP and solubilizing an aliquot. Subsequent labelling of these preparations with ^{14}C IAI would incidate whether any internal portions of the translocator protein are available. If there is an absence of ^{14}C labelling of the adenine nucleotide translocator, it can be concluded that there is not an internal site of IAI labelling.

In order to obtain mitoplasts which are asymmetrically modified by IAI on the external monolayer only, mitochondria can be exhaustively

labelled by multiple additions of ^{3}H IAI, then treated with digitonin to effect removal of the outer membrane. The labelling pattern of the resulting mitoplasts can then be studied and compared to the labelling pattern obtained with SMP.

As can be seen in Figure 18 track K, a higher molecular weight (Mr = 34,000), IAI labelled protein is isolated from mitochondria in addition to the adenine nucleotide translocator. The protein is not isolated by HAP chromatography from either mitoplasts or SMP indicating that it could be a protein of the mitochondrial outer membrane, in the intermembrane space or even loosely associated with the cytoplasmic surface of the inner membrane. It should be possible to purify this protein by chromatography of the digitonin wash, resulting from the formation of mitoplasts, on HAP.

5. Labelling of Mitochondrial Inner Membrane with Photoreactive Phospholipid

Photoreactive phospholipids are useful as membrane soluble probes to study the spatial arrangement of proteins in the mitochondrial inner membrane. They can be asymmetrically incorporated into the outer monolayer only of mitoplasts and submitochondrial particles by use of phospholipid exchange proteins - water soluble proteins which catalyse a 1:1 exchange of phospholipids between the outer monolayer of membranes.

In this study a radioactively labelled photoreactive derivative of phosphatidylcholine, 1-palmitoyl-2(m-diazirinophenoxynonanoyl) phosphatidylcholine (DAP-PC) was used. This photoreactive phospholipid, the structure of which is shown in Figure 19 was synthesized according

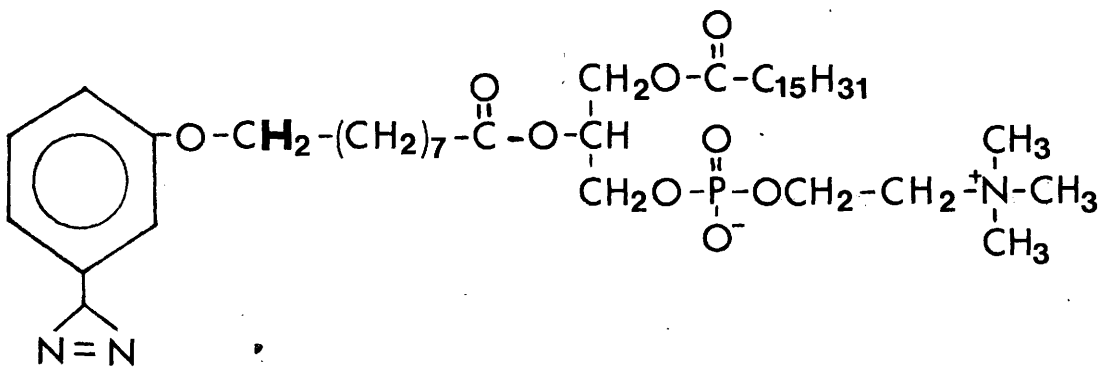


Figure 19. Structure of 1-palmitoyl-2-(m-diazirinophenoxynonanoyl) phosphatidylcholine-DAP-PC. **H** denotes tritium.

to the procedure of Gupta et al. (1977) by G.E. Gerber, and incorporated into mitoplasts and submitochondrial particles either symmetrically by sonication or asymmetrically using phospholipid exchange protein isolated from beef heart.

In the preparation of liposomes the lipid mixtures are sonicated at temperatures close to or above their transition temperatures in order to form vesicles. The T_m for dipalmitoyl PC is about 42°C and the T_m for DAP-PC is considerably lower at about 6°C (Curatolo et al., 1981). In this study sonication of all lipid mixtures was done at 40°C which is sufficient to allow vesicle formation with egg PC and DAP-PC mixtures and DAP-PC alone.

A. Isolation of Phospholipid Exchange Protein

Phospholipid exchange protein was isolated from beef heart according to the procedure of Johnson and Zilversmit (1975) and Zilversmit and Johnson (1975). Partial purification was achieved by chromatography on Sephadex G-75. Figure 20 shows the protein profile and transfer activity profile. Further purification was achieved by chromatography on carboxymethyl cellulose. Figure 21 shows a profile of the transfer activity.

B. Assay of Transfer of DAP-PC by Phospholipid Exchange Protein

As seen in Table III, several sets of conditions were employed to assay the phospholipid exchange protein for transfer activity. The standard conditions shown in assay number 1 resulted in a high background. The background radioactivity is believed to be due to either fusion of the liposomes with the mitochondria or "sticking" of the liposomes to

Table III Summary of Conditions for Exchange Assay

Assay Number	Exchange Protein	Donor Particle	Acceptor Particle	BSA	Background[c]	Transfer above Background	Spun through Sucrose
1	5.1 supernatant	egg PC/PS/DAP-PC 800:89:1 liposomes	mitochondria	No	13%	13%	No
2	5.1 supernatant	egg PC/PS/DAP-PC 800:89:1 liposomes	mitochondria	No	8%	13%	Yes
3	5.1 supernatant	*PC$\bar{v}$ liposomes	mitochondria	No	13%	4%	Yes
4	5.1 supernatant	egg PC/DAP-PC 1:1, 4% PS^- liposomes	mitochondria	No	2%	1%	Yes
5	5.1 supernatant	egg PC/PS/DAP-PC 1000:111:11 liposomes	mitochondria	No	25%	10%	Yes
6	5.1 supernatant	egg PC/DAP-PC 10:1, 2% PS liposomes	mitochondria heated 60°C, 30 min	No	46%	3%	Yes
7	5.1 supernatant	egg PC/DAP-PC 10:1, 2% PS	mitochondria	No	32%	0%	Yes

... continued

... Table III continued

Assay Number	Exchange Protein	Donor Particle	Acceptor Particle	BSA	Background[c]	Transfer above Background	Spun through Sucrose
8	5.1 supernatant	egg PC/PE/CL/DAP-PC 75:25:5:0.7 multilamellar vesicles	SMP	NO	MLV did not spin down as required		No
9	5.1 supernatant	same as 8 but in 0.25 M sucrose	SMP	No	Acceptor and Donor membranes could not be separated even by sucrose gradient		Yes
10	5.1 supernatant	egg PC/DAP-PC 100:1, 4% PS liposomes	mitochondria	2%	8%	1%	Yes
11	5.1 supernatant	same as 10 except freeze thawed 6X	mitochondria	No	18%	8%	Yes
12	5.1 supernatant	egg PC/PS/DAP-PC 800:89:1 liposomes	mitochondria	2 mg/ml	16%	13%	Yes
13	purified exchange protein	loaded exchange	mitochondria	No	4%[d]	32%	Yes

[c]Amount of radioactivity found in acceptor particles in the absence of exchange proteins.
[d]This is the background in the absence of SMP. The level of fusion in the presence of inner membrane particles must be established.

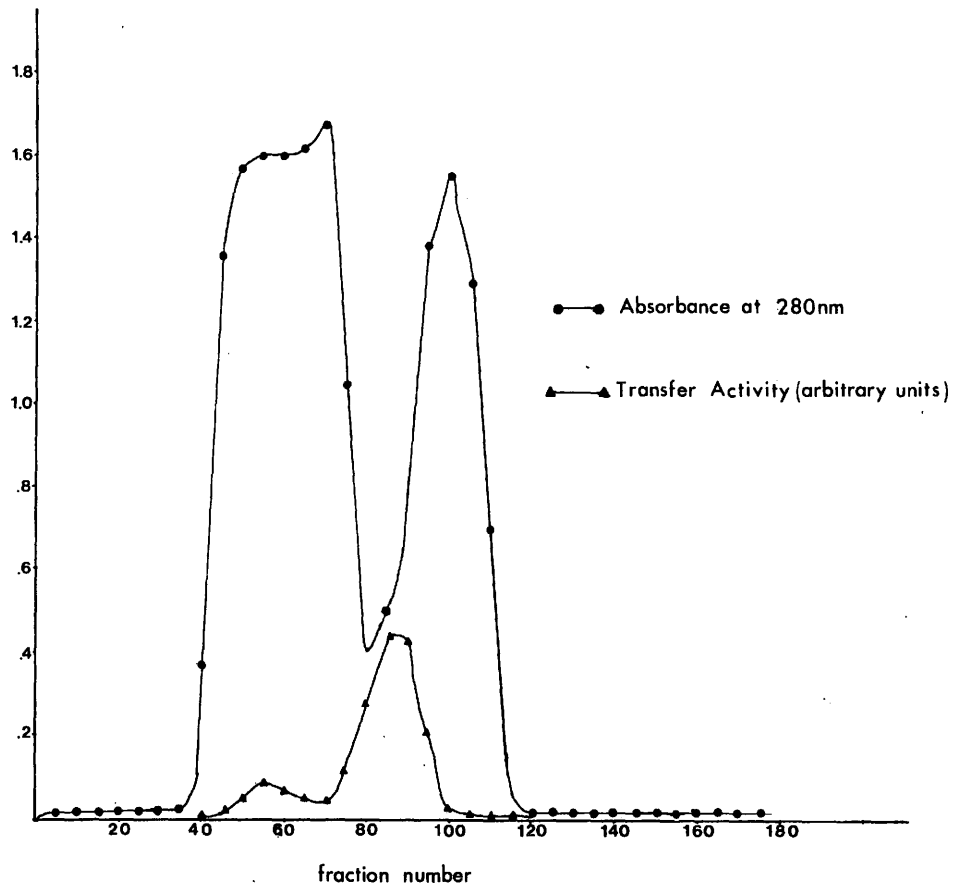


Figure 20. Isolation of phospholipid exchange protein from beef heart: Chromatography of partially purified protein on Sephadex G-75; protein profile ●——● and transfer activity profile ▲——▲.

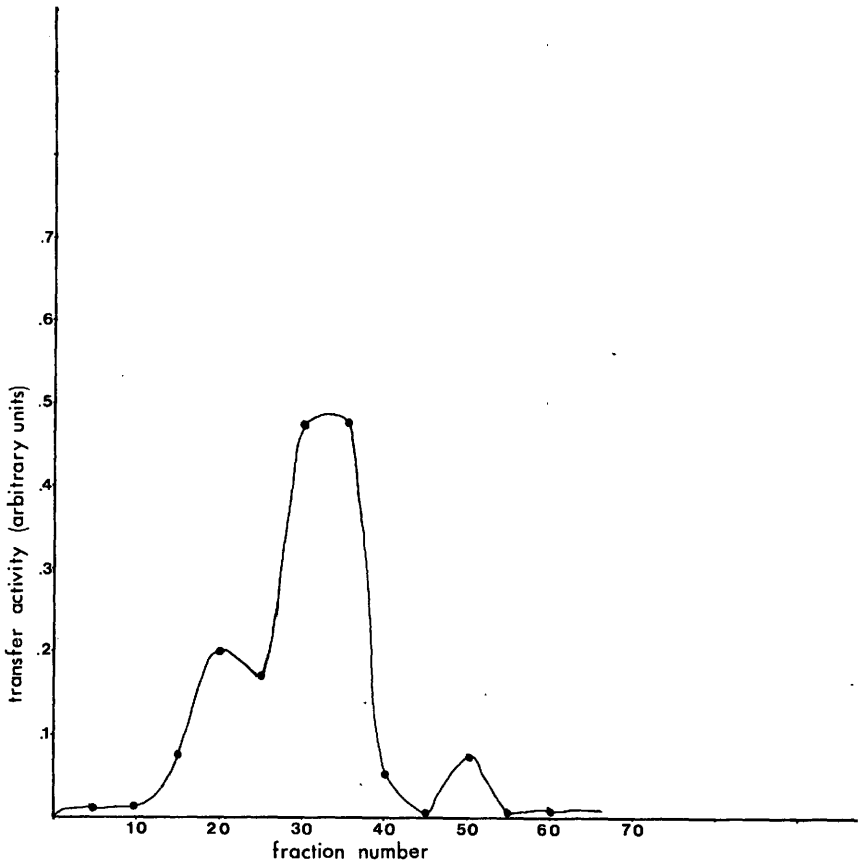


Figure 21. Chromatography of purified phospholipid exchange protein on carboxymethyl cellulose: transfer activity profile.

the mitochondria or a combination of both. The background was reduced somewhat by centrifugation of the samples through 0.5 M sucrose.

In general, increasing the amount of negative or acidic lipid (eg. phosphatidyl serine) stimulates the PLEP catalyzed transfer of lipid but also increases the co-sedimentation of liposomes with mitochondria (Wirtz et al., 1976; Dicorleto et al., 1977). It is best therefore to incorporate a small percentage of negative or acidic phospholipid into donor liposomes in order to stimulate PLEP catalyzed transfer but also keep fusion of liposomes to mitochondria at a minimal level.

In order to further reduce the fusion or sticking, liposomes of differing compositions were used. As seen in Table III, assays 3 to 7, none of these gave improved results over assay 2. Previous studies (Dicorleto et al., 1977) had heated mitochondria prior to use to destroy lipolytic activity. This was tried but gave a very high background.

Due to the difficulty in eliminating the high background, a different system was used employing multilamellar vesicles (Dicorleto et al., 1977) as donor particles and SMP as acceptor particles, but because of the great difficulty of separating donor and acceptor particles, this system was discarded. In addition, it had been observed that sonication increases the liposomal PC pool that participates in exchange (Wirtz et al., 1976). Therefore, different variations in the original system were again tried.

Previous studies (Rousselet et al., 1976a;Dicorleto et al., 1977) had indicated that the addition of BSA to the system significantly reduced fusion. No improvement over the conditions in assay 2 was observed.

It was reported that larger size liposomes reduced the level of background radioactivity. Two types of larger size liposomes were prepared, one by freeze-thawing and the other by ether vaporization (Deaner & Bangham, 1976). Neither of these methods gave improvement over assay 2.

Although some of the variations of conditions shown in Table III reduced background there was also an accompanying reduction in transfer so that there was no overall improvement over the conditions in assay 2.

As it seems to be very difficult to eliminate the fusion or sticking of donor and acceptor particles, different conditions using the exchange protein as a reagent rather than a catalyst, were tried.

The exchange protein was incubated with liposomes as outlined previously to load it with DAP-PC. No negative lipid was used in the liposomes as it has been previously observed to cause sticking of PLEP to liposomes (Wirtz et al., 1976; Dicorleto et al., 1977; Krebs et al., 1979).

The loaded exchange protein purified by Sepharose 6B chromatography was assayed for transfer activity - Assay 13, Table III. This appears to be a feasible method for the asymmetric incorporation of DAP-PC into sided particles.

It is preferable to avoid the purification by Sepharose 6B chromatography because, in the absence of excess PC preloaded on the column, some of the loaded exchange protein sticks to the column and in the presence of preloaded egg PC, some of the photoreactive PC is exchanged for egg PC.

Therefore, phospholipid exchange protein was preincubated with liposomes then incubated with mitoplasts and submitochondrial particles without prior removal of the liposomes. The preincubation of phospholipid exchange protein with liposomes allows for shorter incubation times with the membrane particles thus reducing the possibility of fusion.

Other groups studying the phospholipid exchange protein have incorporated trace amounts of ^{3}H leucine (Wirtz & Zilversmit, 1968) trace ^{14}C triolein (Zilversmit, 1971) or trace ^{3}H triolein (Dicorleto et al., 1977) into ^{32}P labelled microsomes or liposomes made with ^{32}P labelled PC, as a nontransferable marker in the donor membranes. The amount of donor membrane contaminating the acceptor membranes after separation of the two types of particles can then easily be quantitated and corrected for. This type of control however fails to distinguish between fusion and sticking of liposomes to inner membrane preparations and therefore is not an adequeate control in this study.

C. Incorporation of DAP-PC into Inner Membrane Particles and Analysis of Protein Labelling

In order to draw any valid conclusions concerning the distribution and spatial arrangement of proteins in the inner mitochondrial membrane, asymmetry of incorporation of the probe, in this case DAP-PC, must first be established. In this study DAP-PC is incorporated into inner membrane particles uniformly by sonication or asymmetrically using phospholipid exchange protein. This asymmetric incorporation is accomplished in the presence of liposomes and therefore sticking and fusion of liposomes to mitochondria are distinct possibilities.

If sticking only is occurring then, upon photolysis, the photoreactive phospholipid in the liposomes would merely crosslink within the liposomes. If, however, fusion is occurring, the photoreactive phospholipid is incorporated on the inner monolayer of the mitoplasts as well as the outer monolayer and photolysis will lead to labelling of portions of proteins in the inner monolayer of the mitochondrial inner membrane as well as the outer monolayer. This would lead to generally invalid conclusions regarding the distribution and spatial arrangement of proteins in the mitochondrial inner membrane. To determine if fusion is occurring to an appreciable extent during the transfer reaction the samples are all done with controls, one with phospholipid exchange protein included and one without.

The kinetics of fusion and transfer of radioactive photoreactive phospholipid from liposomes to mitoplasts were studied using two types of liposomes, egg PC/DAP-PC 10:1 and DAP-PC alone, and at two temperatures, 25°C and 37°C. The results of the experiments at 37°C are shown in Figure 22. With both types of liposomes, both fusion and PLEP catalysed transfer of DAP-PC were higher at 37°C than at 25°C (data not shown). The level of labelling is comparable with DAP-PC liposomes and egg PC/DAP-PC 10:1 liposomes but the level of fusion is significantly higher with egg PC/DAP-PC liposomes as seen in Figure 22. In all cases, fusion is reasonably low and follows the same trend as transfer. In view of this minimal level of fusion, phospholipid exchange protein can be used as previously described to asymmetrically label proteins in the outer monolayer only of mitoplasts and submitochondrial particles.

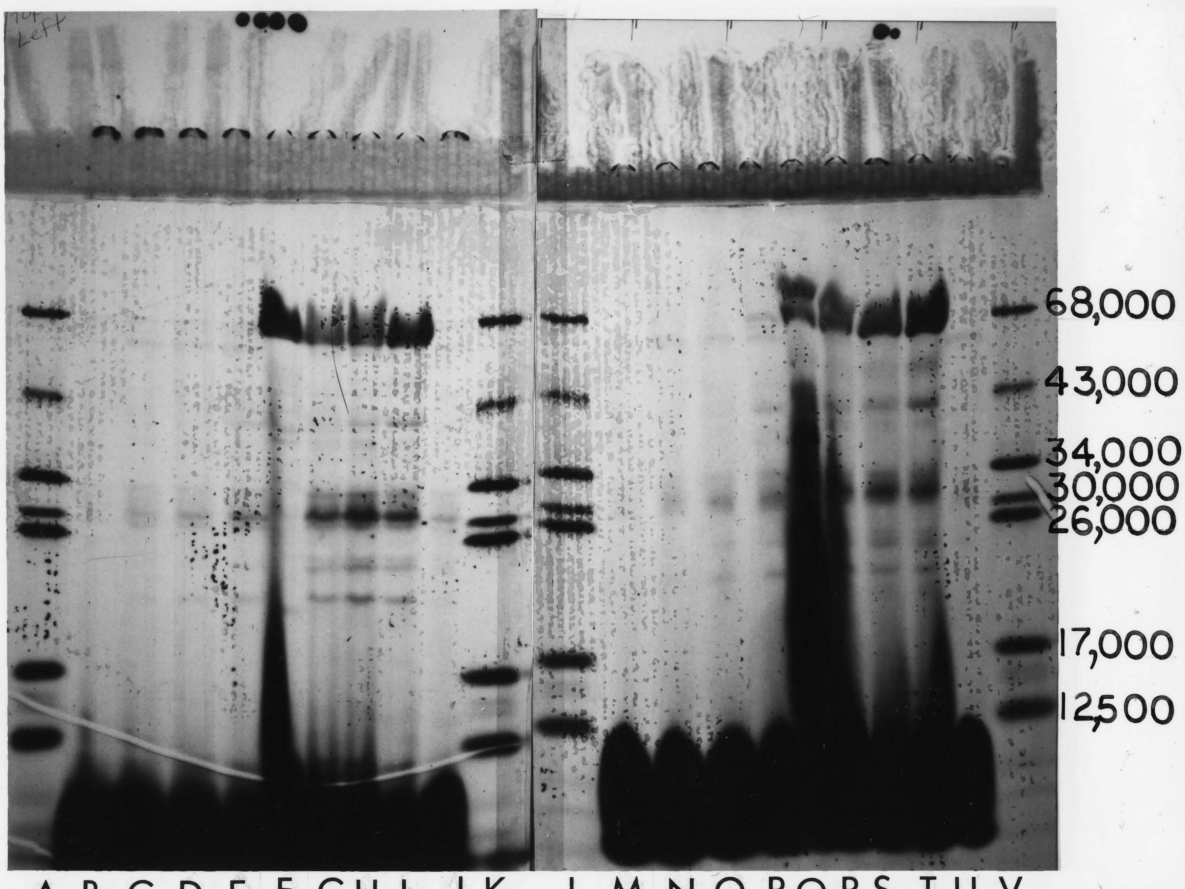


Figure 22. Kinetics of transfer of DAP-PC fron liposomes into mitoplasts, catalyzed by phospholipid exchange protein, and fusion controls: Tracks F to I show the kinetics of transfer of DAP-PC from DAP-PC liposomes into mitoplasts (0.5 mg protein/track) catalyzed by PLEP and tracks Q to T show the kinetics of transfer of DAP-PC from egg PC/DAP-PC 10:1 liposomes into mitoplasts (0.5 mg protein/track) catalyzed by PLEP. Liposomes (42 µg DAP-PC/ml) and phospholipid exchange protein (3.0 mg/ml) were preincubated for 30 min at 37°C. Mitoplasts were added to a concentration of 2.9 mg protein/ml and the kinetics studied at 25°C at 0', 10', 30', and 60'. All samples were immediately photolyzed for 30 seconds under an atmosphere of nitrogen.

Tracks B to E and M to P show 0', 10', 30', and 60' fusion controls at 37°C (mitoplasts; 0.5 mg protein/track). Liposomes (42 µg DAP-PC/ml) and mitoplasts (2.9 mg protein/ml) were incubated together at 37°C in the absence of phospholipid exchange protein. All samples were immediately photolyzed under an atmosphere of nitrogen.

Tracks A, K, L and V show BSA (M_r=68,000), ovalbumin (M_r=43,000), ATCase (M_r=34,000 & 17,000), carbonic anhydrase (M_r=30,000) chymotrypsinogen (M_r=26,000) and cytochrome c (M_r=12,500).

Samples were analysed by SDS polyacrylamide gel electrophoresis followed by fluorography.

Figure 23 shows transfer kinetics of DAP-PC from DAP-PC liposomes into mitoplasts catalyzed by PLEP, tracks E to H, 0', 10', 30' and 60' respectively and a sample in which mitoplasts and liposomes have been sonicated together in order to effect a uniform incorporation of DAP-PC into both monolayers of the mitochondrial inner membrane. The labelling pattern of the sonicated sample is significantly different from that of the samples involving asymmetric incorporation using PLEP. The major band, 1 in the sonicated sample is much less intense in all of the transfer kinetics samples. This band could span the membrane and thus be more accessible to labelling in the sonication control or could be internally located and labelled to an extent in the kinetic experiment due to somewhat disrupted mitoplasts or contamination of the mitoplast preparation with inverted particles. Another protein, band 2 in the sonicated sample, is completely absent in the transfer kinetics samples indicating its internal localization. Band 3 in the sonicated samples is gradually labelled in the kinetic experiment indicating that it is somewhat protected but this protection can be removed by sonication or longer incubation. It is possible that this protein, like band 1 is internally located. There are also several high molecular weight bands which are labelled in the sonicated control but not in the kinetics experiment. These are likely to be located on the internal monolayer of the mitochondrial inner membrane.

In the fusion controls, tracks C and D in Figure 23, it is observed that Band 1 is prominant relative to other bands under these conditions, and is much less prominent under the short term PLEP catalyzed transfer conditions. It appears therefore to be on the matrix face as

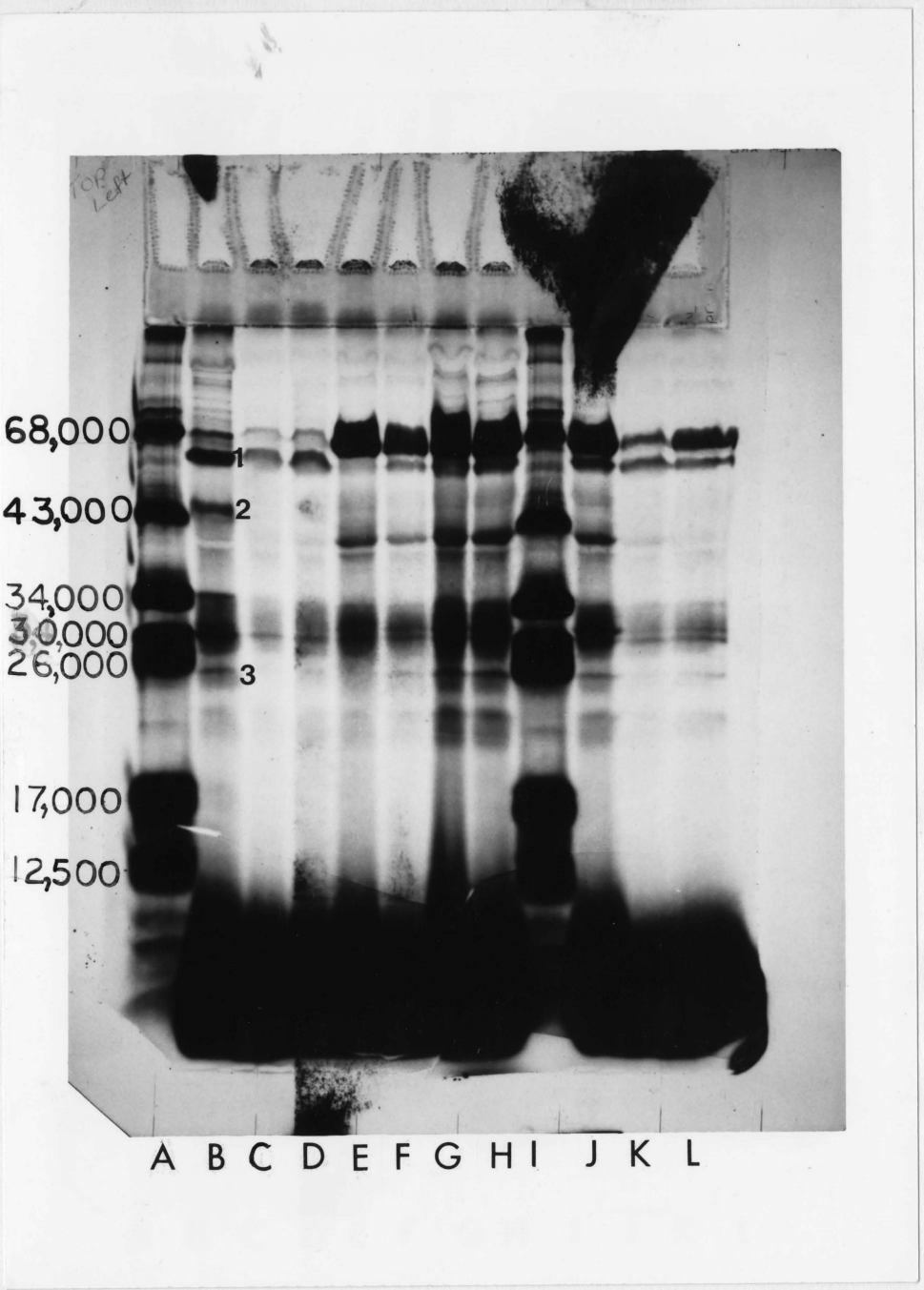


Figure 23. Kinetics of transfer of DAP-PC from DAP-PC liposomes preincubated with PLEP into mitoplasts. DAP-PC (74 µg DAP-PC/ml) and phospholipid exchange protein (2.1 mg/ml) were preincubated for 30 min at 37°C. Mitoplasts were added to a concentration of 5.2 mg protein/ml and the kinetics studied at 25°C at 0', 10', 30', and 60' as shown in tracks E to H (0.5 mg mitochondrial protein/track). Samples were immediately photolyzed for 30 seconds under nitrogen. Track B shows a sonication control (0.5 mg protein/track). Liposomes (74 µg DAP-PC/ml) and mitoplasts (5.2 mg protein/ml) were sonicated for 15 minutes at 25°C then photolyzed for 30 seconds under nitrogen. Track J shows an identical experiment to track G except that 0.1 mM CAT was included. Tracks C and D show 30' and 60' fusion controls. Track L shows an identical experiment to track G except the preincubation of PLEP and liposomes was omitted.

Tracks A and I show BSA (M_r=68,000), ovalbumin (M_r=43,000), ATCase (M_r=34,000 & 17,000), cabonic anhydrase (M_r=30,000), chymotrypsinogen (M_r=26,000) and cytochrome c (M_r=12,500).

Samples were analysed by SDS polyacrylamide gel electrophoresis followed by fluorography.

it is most accessible in uniformly labelled mitoplasts (i.e., sonication sample) and fusion controls.

SMP, uniformly labelled with DAP-PC by sonication with DAP-PC liposomes followed by photolysis have been solubilized and the adenine nucleotide translocator isolated and shown to be modified with DAP-PC (Figure 24). Mitoplasts and submitochondrial particles, asymmetrically labelled with DAP-PC using PLEP exhibit a high level of labelling of a Mr = 30,000 protein presumably the adeninine nucleotide translocator. Figure 23, track J shows the labelling of mitoplasts in the presence of 0.1 mM CAT with DAP-PC from DAP-PC liposomes preincubated with PLEP. In this case also it appears that the adenine nucleotide translocator is modified and can be isolated by chromatography on hydroxylapatite, as will be subsequently discussed. Track L (Figure 23) shows the labelling of mitoplasts by DAP-PC from DAP-PC liposomes catalyzed by PLEP but omitting the preincubation of PLEP with the liposomes. The sample was incubated for 30 minutes at 25°C prior to photolysis. It can be seen that the level of labelling without the preincubation is much lower than that of the 30 minute sample in which the liposomes and PLEP had been preincubated (track G). Preincubating, therefore, loads the exchange protein with DAP-PC and thus minimizes the length of incubation of mitoplasts with the loaded exchange protein-liposome mixture. It is necessary to keep this incubation time to a minimum as prolonged incubation of inner membrane particles at 25°C or 37°C damages the integrity of the membrane.

From the results obtained from both IAI and DAP-PC labelling it appears to be possible to effect an asymmetric modification of the

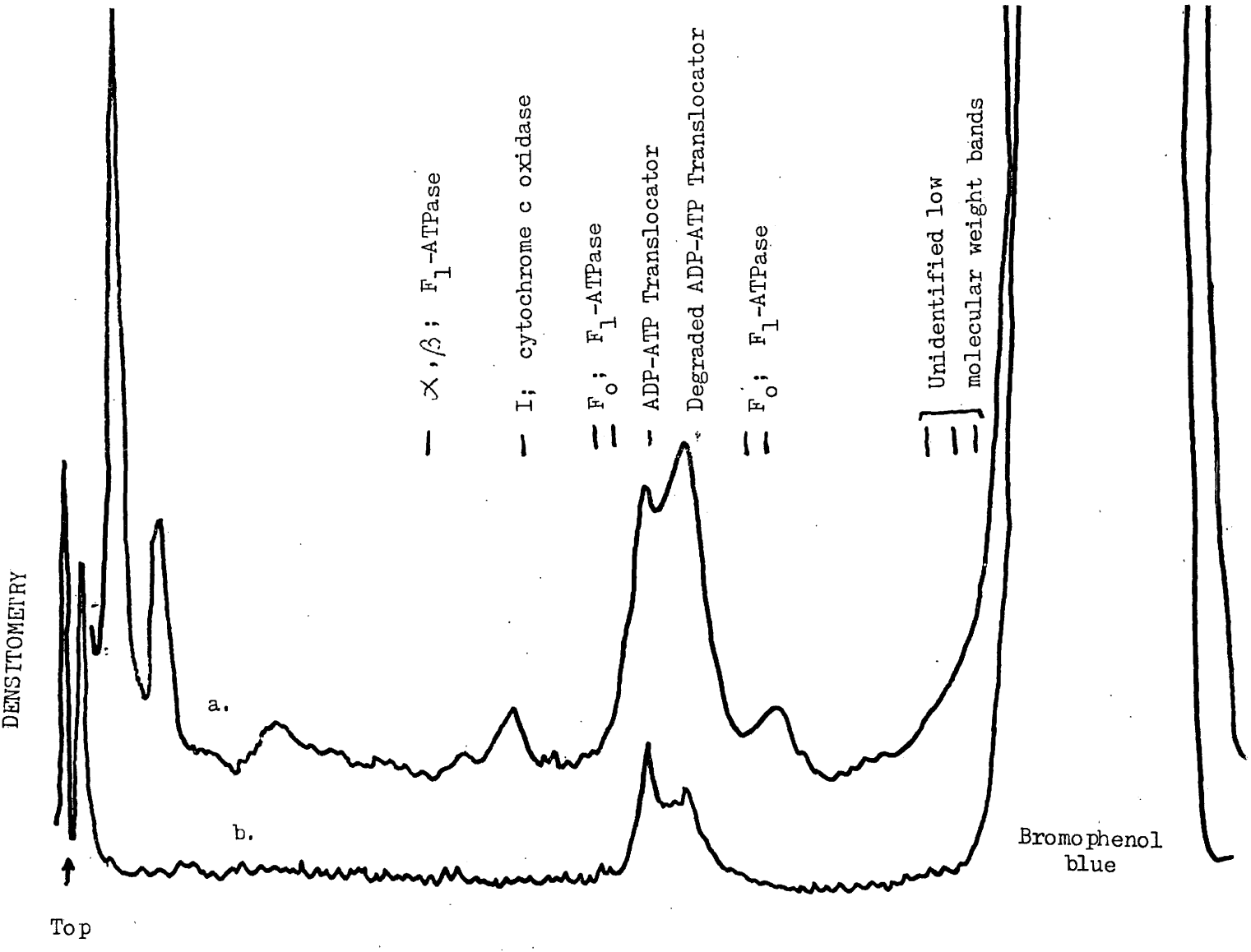


Figure 24. Phospholipid labelling of the inner mitochondrial membrane: identification of the adenine nucleotide translocator as a major crosslinked photolysis product: Beef heart SMP (1 mg/ml) were sonicated under an atmotsphere of nitrogen (15 min, bath sonicator) in the presence of DAP-PC. The sample was photolyzed for 30 min using a Bausch and Lomb Xe/Hg lamp. Samples were analysed by SDS polyacrylamide gel electrophoresis followed by fluorography and densitometry.

a. Total photolyzed sample

b. One of the protein-containing fractions obtained by chromatography of a. on hydroxylapatite

adenine nucleotide translocator from either side of the membrane.

6. Isolation and Purification of Adenine Nucleotide Translocator

The isolation and purification of the adenine nucleotide translocator is greatly facilitated by the highly specific inhibitor carboxyatractyloside. This inhbitor binds tightly to the translocator preventing denaturation upon subsequent solubilization by Triton X-100. The undenatured translocator does not adsorb to the hydroxylapatite thus greatly facilitating its isolation as most other mitochondrial proteins adsorb to the hydroxylapatite.

The rat liver mitochondrial translocator containing fraction eluted from HAP, however, is generally not completely pure although a very clean preparation can be obtained from beef heart mitochondria and submitochondrial particles (Figure 25). In particular, two contaminants are observed on polyacrylamide gels, differing in intensity with different preparations. A discrete band at 34,000 MW is believed to be the phosphate carrier. A general fuzziness is also observed at times directly below the translocator band. This is possibly due to proteolysis of the translocator by endogenous proteolytic enzymes. Some of these enzymes may be from lysosomes which could have co-sedimented with the mitochondria during their isolation. Two changes were therefore made in the procedure. During the isolation of the mitochondria, the mitochondria were sedimented at a lower speed to minimize co-sedimentation of lysosozmes. Many of the enzymes in lysosomes are pepsin-like enzymes and can be inhibited by pepstatin. The isolation of the adenine nucleotide translocator was therefore carried out in the presence of pepstatin and also

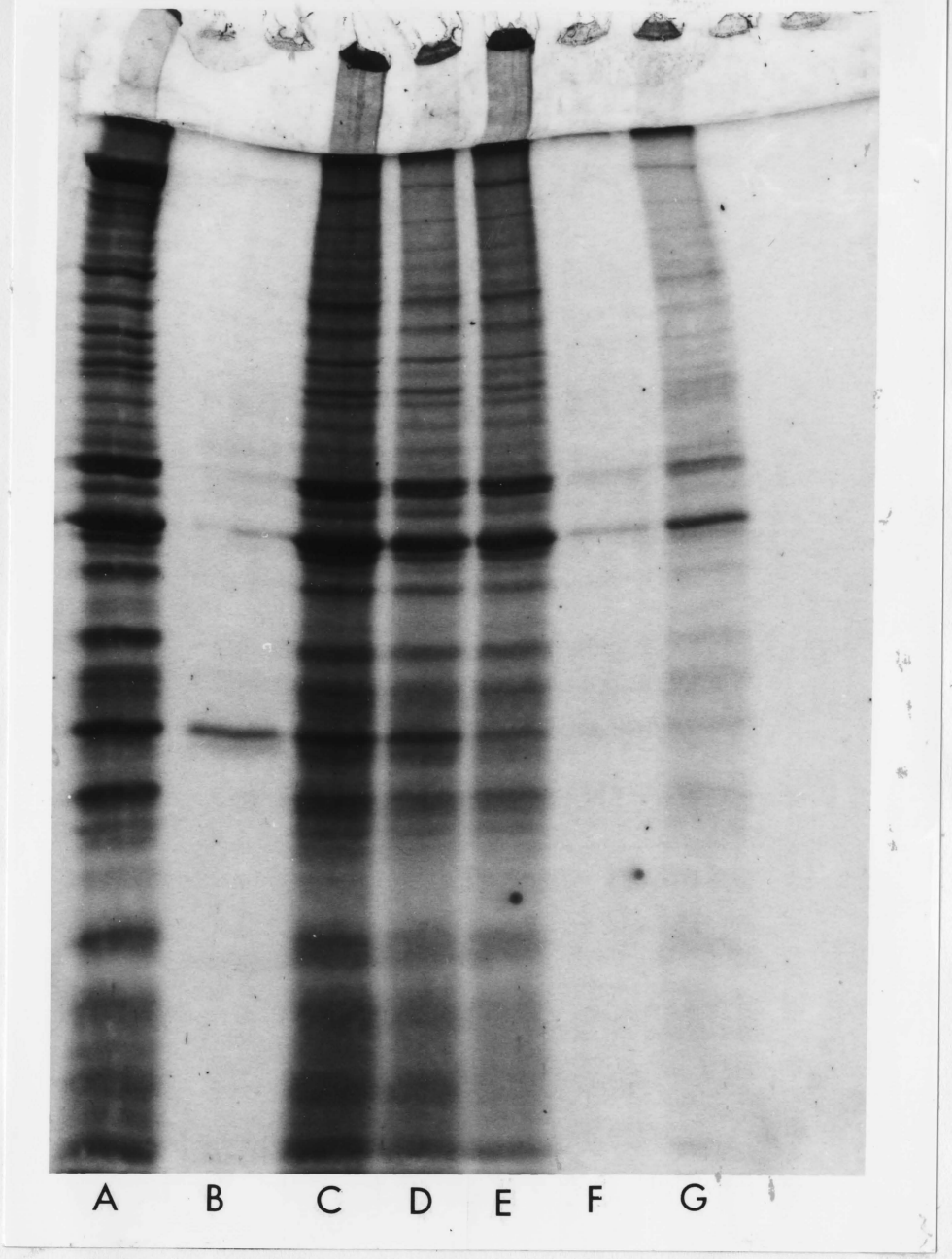


Figure 25. Hydroxylapatite isolation of the adenine nucleotide translocator from sonicated submitochondrial particles: The SMP were sonicated in the presence of 25 μM CAT. Aliquots were then treated as follows:

A. solubilized in 3% SDS and applied to the gel;

B. solubilized in 3% triton X-100, chromatographed on HAP and the eluate applied to the gel as in A;

C. solubilized in 3% triton X-100 and applied to the gel as in A;

D. solubilized in 3% triton X-100, centrifuged at 12,000 x g for 5 min and the supernatant applied to the gel as in A;

E. the pellet from D. was solubilized in 3% SDS and applied to the gel;

F. the pellet as in D. was resuspended in 3% triton X-100, centrifuged as in D. and the supernatant applied to the gel;

G. the pellet from F. was solubilized in 3% SDS and applied to the gel.

phenylmethylsulphonyl fluoride and p-chloromercuric benzoic acid to inhibit chymotrypsin and trypsin and sulfhydryl requiring enzymes.

A very clean preparation of translocator had been achieved using previously frozen mitoplasts. It was thought that enzymes either associated with the outer membrane or in the matrix which would be released on digitonin treatment or freeze-thawing could be responsible for the fuzziness. Therefore high and low digitonin mitoplasts were prepared and the translocator isolated from these.

None of the above procedures gave consistently good results although some improvement was observed. The major problem with the protease inhibitors was their insolubility in aqueous solution.

The use of mitoplasts and mitochondria sedimented at low speed decreased but did not eliminate the fuzziness observed below the translocator band on polyacrylamide gel electrophoresis.

An alternate procedure for the isolation of the adenine nucleotide translocator protein was therefore required.

von Jagow et al. (1978) used a procedure involving removal of adenine nucleotide translocator protein by chromatography on hydroxylapatite for isolation of complex III from beef heart. This procedure used a pre-extraction step with 1% Triton X-100 to remove matrix proteins. The mitochondria were then sedimented and solubilized in 5.5% Triton X-100, undissolved material removed by centrifugation and the supernatant chromatographed on hydroxylapatite.

A modification of this procedure as described in Chapter II was employed for the isolation of adenine nucleotide translocator from rat

liver. Much improvement was observed with this procedure with respect to elimination both of the higher molecular weight species and of the fuzziness observed below the translocator band on SDS PAGE. This procedure was routinely used for the isolation of adenine nucleotide translocator from mitochondria, mitoplasts and SMP.

Chromatography in organic solvents on Sephadex LH60 was employed to effect the removal of the Triton X-100 from the adenine nucleotide translocator. The translocator protein elutes at the void volume with 88% formic acid/ethanol 30:70 and the Triton X-100 is retarded. A very good separation was routinely achieved as shown in Figure 26.

Current Work as an Extension of the Thesis

In order to study the sites of labelling on the adenine nucleotide translocator by asymmetric modification with IAI and DAP-PC, procedures have been worked out to fragment the isolated, labelled protein.

Cyanogen bromide fragmentation of the translocator has been done and separation of fragment by chromatography in organic solvents (88% formic acid/ethanol 30:70) on Sephadex LH20 has been achieved. Amino acid analysis will be carried out on these fragments in order to assign their positions in the primary structure.

Other fragmentation procedures including Staphylococcus aureus V8 protease, n-bromosuccinimide and acid cleavage have been investigated, and fragmentation patterns obtained.

By asymmetrically labelling the translocator protein from the matrix side or the cytoplasmic side of the membrane, fragmenting the labelled protein and assigning the fragments along the sequence of the

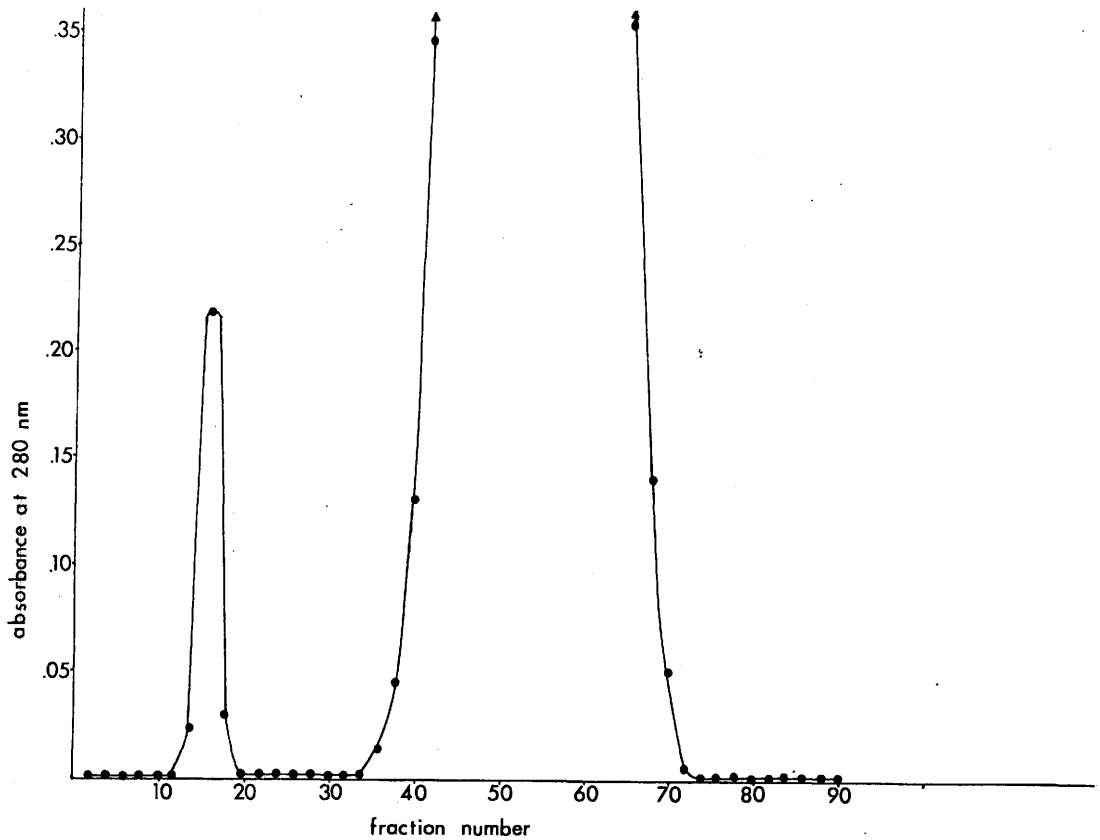


Figure 26. Separation of triton X-100 from the adenine nucleotide translocator by chromatography in organic solvents on Sephadex LH60: Lyophilyzed translocator, isolated by hydroxylapatite, was taken up in a minimum volume of 88% formic acid and applied to a Sephadex LH60 column. Fractions were eluted with 88% formic acid/ethanol 30:70. The protein elutes at the void volume while the triton X-100 begins to elute at two thirds the bed volume.

protein, its spatial arrangement in the mitochondrial inner membrane can be partially elucidated.

7. Summary

Mitochondrial inner membrane particles of opposite configuration, mitoplasts and submitochondrial particles, have been prepared in reasonably pure and intact form. These inner membrane preparations were used in the investigation of the transverse distribution of phospholipids in the mitochondrial inner membrane, the asymmetric distribution of proteins across this membrane, and the spatial arrangement of the adenine nucleotide translocator in the mitochondrial inner membrane.

In studies involving the asymmetric modification of mitoplasts with the surface probe, cycloheptaamylose fluorescamine complex, suggestive evidence indicated that approximately 30% of the total PE in mitoplasts may be located in the external monolayer.

With both, IAI and phospholipase A_2 extensive perturbation of the membrane was observed.

In studies involving the asymmetric modification of mitoplasts and submitochondrial particles with isethionyl acetimidate, similar protein labelling patterns were observed suggesting that, in the mitoplasts at least, perturbation of the membrane was occuring under the conditions used.

The protein labelling pattern of IAI modified mitochondria differed significantly from that of IAI modified SMP. Several proteins were labelled in SMP that were not labelled in mitochondria indicating that the IAI was not penetrating the mitochondrial inner membrane.

Labelling of the adenine nucleotide translocator in intact mitochondria demonstrated penetration of the outer membrane by IAI.

DAP-PC was shown to label the adenine nucleotide translocator in mitoplasts and submitochondrial particles. Fusion of liposomes and inner membrane particles is minimal under the conditions used, indicating that PLEP catalyzed incorporation of DAP-PC into mitoplasts is essentially restricted to the cytoplasmic surface. Further indication of the asymmetry of the PLEP catalyzed incorporation of DAP-PC was supplied by the significant differences in the labelling patterns between the PLEP catalyzed incorporation and the sonication sample. A number of proteins labelled in the sonication sample were not labelled in the PLEP catalyzed sample indicating that the matrix surface of the inner membrane was inaccessible.

The adenine nucleotide translocator has been isolated from beef heart and rat liver mitochondria, mitoplasts and submitochondrial particles in essentially pure form. Triton X-100 has been removed by chromatography of lyophylized protein on Sephadex LH60 in organic solvents.

It can be concluded that the adenine nucleotide translocator can be asymmetrically labelled from either side of the membrane by IAI and DAP-PC. Isolation and fragmentation of labelled protein followed by analysis of the fragments will yield information concerning the spatial arrangement of the adenine nucleotide translocator in the mitochondrial inner membrane.

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