ERRATA

Fig. 6.2. p. 143, at the end of 1st paragraph

read: though the swelling and shrinking step is omitted.

In Bibliography, after Klingenberg, M. (1970)

read:

Koch, G.L.E. (1979) in British Society for Cell Biology, p.24, Glasgow, Scotland.

In Bibliography read:

First authors with surname beginning with letter 'V' before those beginning with 'W'.

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Topography and Organisation of Proteins and Glycoproteins in Rat Liver Mitochondrial Membranes

A thesis submitted to the
University of Glasgow
for the Degree of Doctor of Philosophy
in the Faculty of Science

by

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And the end of all our exploring
will be to arrive where we started
and know the place for the first time.

T.S. Elliot.

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ABBREVIATIONS

Abbreviations used in this thesis are those recommended in the Biochemical Journal Instructions to Authors (revised 1978), with the following additions :

ATPase	adenosine triphosphatase
ATR	Atractyloside
BIS-MSB	p-BIS (o-methyl-steryl) benzene
BSA	Bovine serum albumin
CAT	Carboxyatractyloside
Con A	Concanavalin A
DMSO	Dimethyl sulphoxide
DOC	7-Deoxycholic acid
F_0	integral membrane non-catalytic portion of mitochondrial ATPase complex
F_1	soluble catalytic portion of mitochondrial ATPase complex
HLA	Human Major Histocompatability Complex
IEF	Isoelectric focusing
Ig	Immunoglobulin
ISO	Inside-out
M_1	Standard mitochondrial fraction after differential centrifugation
MOPS	mannitol, sucrose, morpholinopropane sulphonate buffer
OSCP	oligomycin sensitivity conferring protein

PAS	Periodic acid-Schiff reagent
PBS	Phosphate buffered saline
Pi	Phosphate
PM_1	Mitochondrial fraction, further purified on a discontinuous density gradient
PMSF	Phenylmethylsulphonyl fluoridine
PPO	2,5 diphenyloxazole
RSO	Right side-out
SBA	Soya bean agglutinin
SDS	Sodium dodecyl sulphate
SMP	Submitochondrial particle
TCA	Trichloroacetic acid
T.D.	Tracker dye
TEMED	NNN' N' tetramethylethylenediamine
TKM	Tris-potassium-magnesium buffer
WGA	Wheat germ agglutinin

SUMMARY

The basic objective of this thesis is to develop our understanding of the topography, organisation and function of mitochondrial membrane proteins/glycoproteins to a level comparable to the red blood cell plasma membrane.

Initial characterisation of the isolated standard mitochondrial fraction (M_1) or sucrose density purified material (PM_1), involves assessment of the extent of contamination with other subcellular fractions. M_1 preparations contain 5.0% acid phosphatase (lysosomes), 5.4% glucose-6-phosphate (microsomes) and 4.4% 5'-nucleotidase (plasma membrane), while analogous values for the PM_1 fractions are 1.1%, 1.7% and 0.6% respectively.

Integrity of the outer membrane in isolated mitochondria is monitored by an assay using exogeneous cytochrome c as substrate for cytochrome c oxidase, an inner membrane protein complex. Estimates of the degree of damage to the outer membrane reveal 3-5% breakage in the M_1 mitochondria and 11-17% in PM_1 mitochondria.

Optimum procedures for subfractionation of mitochondria into inner and outer membranes, and a soluble fraction are developed employing sonication and sucrose density gradient analysis. Minimal cross-contamination (less than 10%) of individual fractions is achieved as assessed by specific marker enzymes, namely cytochrome c oxidase for the inner membrane, monoamine oxidase for the outer membrane and adenylate kinase for the soluble fraction.

Analysis of rat liver mitochondria pre-labelled *in vivo* with D-[6-^{3}H]-glucosamine confirm that 70-80% of the carbohydrate is released in a soluble form on disruption of the organelle, the remainder being distributed equally between inner and outer membranes. The bulk of the carbohydrate is associated with protein rather than lipid.

Treatment of rat liver mitochondria with low levels of digitonin (0.01mg/mg protein) releases approx. 50% of the glucosamine-labelled mitochondrial glycoprotein, without destroying the integrity of the outer membrane. Immunoprecipitation of these extrinsic components with Con A and anti-Con A serum reveals 4 components, including a major doublet of 40,000 and 48,000 mol. wt.

Direct agglutination and binding studies with [^{125}I]-WGA demonstrate the presence of integral carbohydrate on the external surfaces of mitochondrial inner and outer membranes. The number of WGA receptors on the inner membrane is approx. twice those on the outer surface.

Specific surface-labelling studies employing lactoperoxidase-catalysed iodination reveal the presence of 7 major polypeptides, designated bands 1 to 7, located externally on the outer membrane. Similar studies on mitoplasts (outer membrane removed) show that bands 1a, 1b, 2b, 5a, 6a and 6b are exclusively located on the inner membrane. Components 1c, 2a and 6c are available for iodination only when the outer membrane is partially removed, while bands 1 and 5 are equally accessible in both intact mitochondria and mitoplasts.

Direct immunoprecipitation of these surface components demonstrate that all of these species, except band 2 appear to act as receptors for WGA or Con A indicating their glycoprotein nature or specific association with membrane glycoproteins. Band 6 appears to interact with WGA but not Con A.

Comparison of the glycosylated components exposed on the outer surfaces of mitochondrial membranes with the total glycosylated complement of the membranes is achieved by the lectin overlay method. With $[^{125}I]$ -Con A, at least 20 glycoproteins can be detected. In contrast, with WGA only 5 components (1, 1a, 1b, 2a and 2b) are visualised.

Lectin affinity procedures are developed to fractionate mixed populations of inner membrane vesicles. ISO vesicles are not absorbed or precipitated by WGA and anti-WGA-IgG, indicating the absence of accessible WGA binding sites in this population. The polarity of these membrane fragments is also assessed by monitoring the crypticity of cytochrome c oxidase with exogeneous cytochrome c, which normally interacts with the outer surface of the inner membrane. By this criterion, ISO vesicles greater than 90% purity can be isolated.

Identification of the transmembrane proteins of the inner membrane by vectorial labelling of RSO vesicles (mitoplasts) and ISO vesicles show that bands 1, 2b, 5, 5a and 6a span the lipid bilayer. Bands 1 and 5 also traverse the outer membrane.

The role of glycoproteins on the two main energy linked functions in mitochondria : ATP synthesis and Ca^{2+} uptake is

investigated by the use of lectins as blocking agents. The data indicates that SBA specifically inhibits Ca^{2+} transport in intact mitochondria. Both SBA and WGA have a similar effect when the outer membrane is partially disrupted to allow permeation of lectin. SBA also affects P:O ratios in intact mitochondria. No respiratory control is observed in mitoplasts treated with WGA, SBA or Con A.

Preliminary 2-dimensional separation of mitochondrial membrane proteins indicates the presence of 70-80 discrete protein spots as detected by Coomassie Blue staining. This emphasizes the potential of the technique which could provide high resolution peptide maps for further study on individual mitochondrial enzyme complexes.

CONTENTS

CHAPTER 1

Introduction

1.1. General review of membranes

The membranes of eukaryotic cells divide the cell into compartments that are distinctive in both morphology and metabolism. Membranes, as well as surrounding the cell as a whole, or an organelle within the cell, e.g. the mitochondrion, also provide an internal framework consisting of rough endoplasmic reticulum, smooth endoplasmic reticulum and Golgi apparatus on which many ordered macromolecular functions take place.

Biological membranes are not simple mechanical barriers, but a highly ordered array of molecules which display a variety of functions. Eukaryotic plasma cell membranes are composed of approx. 40% (w/w) lipid, and 60% (w/w) protein as well as 2-10% (w/w) carbohydrate covalently associated with either membrane protein or lipid.

Functionally, the plasma membrane provides a selective permeability barrier that controls the amount and nature of substances that can pass between the cell and its environment. In addition, the compartmentalisation of metabolic processes within membrane-bound organelles enables a high concentration of reactants to be maintained at discrete sites and has important implications for metabolic control e.g. the tricarboxylic acid and urea cycles are located in the mitochondrial matrix. The processes which compete for common metabolite may be separated by having enzymic systems

in different compartments, and their relative rates controlled by varying the rate of entry of the metabolite. This integrates the metabolic functions of the cell in a coherent fashion while simultaneously regulating the individual metabolic pathways.

The plasma membrane also provides the structural framework to which functional entities are firmly bound. Many important receptors are located on cell surfaces. These include : receptors which recognise hormones such as insulin and catecholamine; energy receptors, which convert light and heat into electrical impulses; and surface components, which are species specific, such as the histocompatibility antigens and blood group determinants that are involved in mediating cell immunity.

1.1.1. Types of membranes that are of biochemical interest

The chemical composition of different membranes depends on their particular functions. The mature red blood cell, whose major function is transport of oxygen, has neither nucleus, nor an internal membrane system. These factors, together with the ease in obtaining large quantities of starting material, have made the erythrocyte plasma membrane one of the best studied to date. The lymphocyte membrane is another example of a well-studied system, because of its immunological importance, in that it contains many surface antigens of known specificity such as the histocompatibility antigens and IgG and IgM. However, much less is known about the membranes of intracellular organelles, due to the difficulties encountered in their purification. An important

exception in this category is the mitochondrial inner membrane which has been thoroughly investigated with respect to electron transport and ATP synthesis.

Certain specialised plasma membranes have been studied in great detail because of unique ultrastructural or biochemical features. One of these, the retinal outer segment membrane is composed of approx. 80% (w/w) rhodopsin. The membrane has a disc-like appearance under the electron microscope owing to invaginations of the plasma membrane, which increase the surface area and thus maximise the amount of light absorbed. Another example of a specialised plasma membrane is the myelin sheath, which surrounds certain axons. This is comprised of about 75-80% (w/w) lipid arranged in a multilayered lipid structure (as observed by X-ray diffraction and electron microscopy), making it suitable for its function of insulation.

In recent years, the purple membrane of Halobacterium halobium has been the subject of intensive investigation, both from a biophysical and biochemical standpoint. Its unique structure containing 75% (w/w) of the simple protein, bacteriorhodopsin present in semi-crystalline arrays, has proved a most advantageous system for X-ray diffraction studies. Similarly its apparent role as a light-induced proton pump involved in ATP synthesis has excited the interest of bioenergicists

1.1.2. Composition of membranes

The protein and lipid components of biological membranes associate with one another through non-covalent forces, and the manner in which they interact depends on the amphipathic nature of the individual constituents.

Table 1.1.

PROTEIN AND LIPID CONTENT OF MEMBRANES FROM RAT TISSUES

Membrane	Approximate protein/lipid ratio (w/w)	Approximate cholesterol/polar lipid ratio
PLASMA MEMBRANES		
Myelin	0.25	0.95
Erythrocyte	1.1	1.0
Rat liver cells	1.5	0.50
MITOCHONDRIAL MEMBRANES		
Inner membrane	3.6	0- 0.02
Outer membrane	1.2	0.04

(reproduced from Molecular Biol. of Cell Membranes, P.J. Quinn (1976) p.31)

(a) Lipids

The lipids that are found in membranes are usually amphipathic, possessing hydrophobic regions in the form of long hydrocarbon chains and a polar region that consists of one or more ionisable groups.

The four main lipid classes present in eukaryotic cells are :

(i) Glycerophosphatides, which consist of a glycerol moiety to which long fatty acid chains are acylated.

(ii) Sphingolipids, which are similar to glycerophosphatides except that sphingosine replaces glycerol

These two classes of phospholipids are the principal polar lipid constituents of most animal cells.

(iii) Glycolipids, which consist of mono or oligo-saccharide units glycosidically linked to the terminal hydroxy-group of either glycerol, sphinganine (fatty acid amide of sphinogsine) or a sterol.

(iv) sterols, e.g. cholesterol

(b) Distribution of Lipids

The composition of lipids within the different membranes varies and is dependent on a membrane's function. The proportion of protein to lipid and cholesterol to polar lipids in the various membranes of rat liver is shown in Table I.1. Plasma membranes generally contain most of the glycolipids of the cell and are usually richer in cholesterol than other intracellular membranes. Notably, the outer mitochondrial membrane contains far more cholesterol than the inner membrane

and this fact provides the basis for the preferential disruption of the former with digitonin (Schnaitman et al., 1967). In a similar vein, the proportion of diphoshatidyl-glycerol (cardiolipin) in the inner membrane is much higher than in other mammalian membranes.

(c) Proteins

The protein content of a membrane is roughly proportional to its metabolic activity. This is demonstrated by the myelin sheath which contains only 20% (w/w) protein and by the fact that its main function is that of insulation. On the other hand the inner mitochondrial membrane which is involved in ATP synthesis contains 75-80% (w/w) protein.

Proteins of the plasma membrane can be broadly classified into 2 groups by the way they interact with the membrane lipid. They can be either loosely associated with the lipid, termed "extrinsic" or they can be firmly integrated into the membrane, termed "intrinsic" (Capaldi and Green, 1972). Extrinsic proteins can be easily dissociated from the membrane by relatively mild treatment. For example, cytochrome c can be extracted from the membrane of mitochondria by washing in 0.15M NaCl (Jacobs and Sanadi, 1960). Intrinsic membrane proteins on the other hand are firmly associated with the membrane : their solubilisation requires agents such as SDS, Triton X-100 or chaotrophic agents like lithium di-iodo salicylate or guanidinium chloride that destroy the structural integrity of the membrane. Further, other proteins can be distinguished depending on whether they contain carbohydrate or not. The amount and type of sugar varies in the different

membranes. Basically there are two types of oligosaccharide linkages to the polypeptide chain :

(a) α-O-glycosidic linkages between N-acetyl galactosamine and the OH group of serine or threonine, which are alkali-labile.

(b) β-N-glycosidic bonds between N-acetylglucosamine and the amino group of asparagine residues, which are stable to hydrolysis by alkali.

1.1.3. Molecular organisation of membranes

Current concepts of membrane organisation still incorporate the lipid bilayer, originally proposed by Gorter and Grendal (1925), as its central feature. In this model the essential structural repeating units are the phospholipid molecules. These are arranged with their hydrocarbon regions towards the inside and the polar head groups facing the aqueous environment.

To explain the arrangement of protein in the membrane several models were proposed, including one by Danielli and Davson (1935) which suggested that globular proteins were attached to the polar head groups on either side of the membrane. This model accounted for the low surface tension of biological membranes as compared with model lipid systems. It was later modified by Robertson (1959) in the light of new information obtained from the electron microscopic studies and X-ray diffraction data of myelin. At this stage proteins were thought to occur in the extended β-configuration, rather than in a globular arrangement, and, further, their distribution with respect to lipid was asymmetric. The model was not thermodynamically favourable because a significant proportion

of the non-polar amino residues of the protein would be exposed to the aqueous environment thereby increasing the entropy of the system. However, Danielli (1958) did modify the model to include protein-lined pores which spanned the membrane, thus providing a functional basis for proteins such as the active transport of ions. Later experiments by Engleman (1969) showed that most membranes contained insufficient lipid to form a continuous bilayer over the entire surface, and evidence from optical rotatory dispersion and circular dichroism techniques (Glaser and Singer, 1971) showed that 30-60% of the protein had an α-helical conformation, suggesting a globular shape for them. Additionally, it was found that drastic membrane disrupting procedures were required before all the proteins could be extracted with minimum lipid contamination. This suggested that hydrophobic interactions, in addition to electrostatic forces, were present between the proteins and lipids.

In order to explain these observed phenomenon, Singer and Nicolson (1972) proposed the Fluid Mosaic model. In the model the membrane was envisaged as an alternating mosaic of proteins and lipids in which proteins are buried to varying extents in a fluid lipid bilayer. The model distinguished between 2 classes of membrane proteins : extrinsic proteins which are associated with the polar lipid head groups via electrostatic or ionic interactions, and intrinsic proteins which are embedded in the bilayer and associated hydrophobically with the lipid. Other proteins span the bilayer completely. In comparison with the Davson-Danielli model, as well as

being thermodynamically stable, and accounting for the 30-60% α-helical content of proteins, the fluid mosaic model decreased the total surface area covered by lipid thus explaining the previous discrepancies. Two important features of this model, with particular reference to the erythrocyte plasma membrane, were the asymmetric distribution of all membrane components and fluidity of lipid bilayer. Exposed proteins surface labelled with impermeable reagents, or peptides cleaved from the surface of intact cells with proteases, were different on both sides of the membrane (Bretscher, 1971b). All the carbohydrate associated with the membrane was located on the non-cytoplasmic side of the membrane (Singer and Nicolson,1974).

Lipid asymmetry has been demonstrated by a variety of experiments. In an experiment using ^{31}P n.m.r., the resonances from phospholipids on either side of the bilayer in sonicated vesicles were found to be distinguishable (Michaelson et al., 1973). Paramagnetic ions (such as Mn^{2+}) which cause the broadening of nearby phosphorus resonance signals have been used to show the arrangement of the latter in the bilayer. In this way phosphatidylcholine and sphingomyelin have been found to show a preference for the outer surface, whereas phosphatidylethanolamine prefers the inner surface (Michaelson et al., 1973). Additionally, chemical-labelling reagents that specifically react with amino groups, such as $[^{35}S]$-formylmethionyl methyl phosphate (FMMP) have shown that the bulk of the phosphatidylethanolamine is labelled in human erythrocyte membrane preparations as opposed to the intact cell (Bretscher, 1972). Other

experiments using phospholipases A_2 and C have also confirmed lipid asymmetry (Roelofsen, 1971).

The second important feature of this model is its dynamic nature. Lipids have been observed to be constantly moving on a lateral plane in their own half of the bilayer with a exchange rate of 10^7 per sec (Kornberg and McConnell, 1971). In contrast,the "flip-flop" of lipid molecules across the bilayer was very slow, 0.07/h at 30°C (Rothman and Lenard, 1977). Similarly, Frye and Edidin (1970) have shown the lateral motion of intrinsic membrane proteins by a fluorescent antibody technique, after the formation of human-mouse heterokaryons. Further evidence of this motion has come from experiments showing the redistribution of lymphocyte surface antigens, "patching and capping" on the addition of antisera to surface components (de Petris and Raff, 1973).

1.2. Erythrocyte Plasma Membrane

Many of our current concepts about the molecular organisation of the membrane bilayer as incorporated in the Fluid Mosaic model (Singer and Nicolson, 1972) stem directly from research on the red blood cell. The red blood cell system has always been the focal point of much research because of the technical advantages which allow large quantities of purified membrane to be prepared. Further, the fact that distinct marker enzymic activities are associated with a particular face of the membrane, (for example acetylcholinesterase (EC. 3.1.1.7) with the external face and glyceraldehyde-3-phosphate dehydrogenase (EC. 1.1.1.49)with th cytoplasmic face) has been exploited, particularly in the

preparation of a population of vesicles of defined orientation (Steck and Kant, 1974). The purity of the vesicles, either "right side-out" (RSO) or "inside-out" (ISO), can be assessed by measuring the latency of the corresponding marker enzyme. Subsequent surface-labelling studies of these vesicles has provided valuable information on the arrangement of proteins within the membrane bilayer.

1.2.1. Organisation of the Erythrocyte Plasma Membrane

Erythrocyte membrane ghosts can be prepared according to the procedure of Steck *et al*. (1970). Under these conditions, all the lipid and carbohydrate remains associated with the membrane. Rosenburg and Guidotti (1968) have shown that the membrane is composed of 49.2% protein, 43.6% lipid and 7.2% carbohydrate, all by weight, the majority of the carbohydrate being covalently associated with the protein (Winzler, 1971).

The proteins of the human erythrocyte membrane can be separated by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis according to the method of Fairbanks *et al.* (1971). In this system, the major protein bands which are stained by Coomassie Brilliant Blue are numbered 1 to 7 in order of decreasing mol. wt. Sialic acid and galactose containing containing proteins can be visualised by the periodic-acid schiff (PAS) stain. One major band termed PAS-1 or glycophorin and several minor bands are observed. Only one of the Coomassie blue bands (component 3) appears to be a glycoprotein. This is not stained by the PAS reagent, due to the lack of appropriate saccharide residues (Tanner and Boxer, 1972). A knowledge of the organisation of the

erythrocyte membrane has accumulated from labelling studies of intact cells, permeable unsealed ghosts or vesicles of defined orientation. Basically there are two types of surface labelling probes :

(1) Low mol. wt. impermeable molecules which react directly with accessible surface groups. Carraway (1975) lists 7 low mol. wt. membrane labelling reagents which have been used on animal cells. Most of these reagents are bulky anionic moieties that penetrate the membrane only very slowly and react primarily with exposed amino groups (Rothstein et al., 1976). A typical reagent $[^{35}S]$-FMMP used by Bretscher (1971a), has demonstrated that PAS-1 and component 3 the 2 major glycoproteins in the erythrocyte membrane have a transmembrane orientation, (for review see, Carraway, 1975).

(2) Enzymes which catalyse or promote the transfer of a radio-label to accessible sites on the cell surface, e.g. lactoperoxidase (EC. 1.11.1.7) catalysed radio-iodination of accessible tyrosine and possibly histidine residues in the presence of hydrogen peroxide (Carraway, 1975). Gahmberg and Hakomori (1973) have also reported a method for specific surface labelling of carbohydrate residues on cell surfaces by using galactose oxidase, (EC. 1.1.3.9) followed by reduction with $[^{3}H]$-$NaBH_4$.

The erythrocyte membrane is asymmetric with respect to the arrangement of its protein components. The extrinsic proteins are all located on the cytoplasmic face of the membrane, as in spectrin (Marchesi and Steers, 1968). Spectrin comprises bands 1 and 2 which are two myosin-like

proteins and can be extracted at low ionic strength under mildly alkaline conditions in the presence of chelating agents. Bands 1, 2 and 5 an actin-like protein, together comprise the microfilament system, and are thought to be structurally significant, for their removal results in breakdown and vesiculation of the membrane (Steck, 1972). Nicolson (1973) has shown also, that by aggregating spectrin in ghosts with anti-spectrin γ-globulin, a concurrent aggregation of surface glycoproteins is observed. Therefore spectrin can act as the underlying microfilament system exerting a form of control on membrane topography. Glyceraldehyde-3-phosphate dehydrogenase (band 6), the cytoplasmic surface marker, is also an extrinsic protein.

The intrinsic membrane proteins are firmly embedded into the bilayer, the majority of the carbohydrate being associated with these proteins and all exposed at the external surface of the membrane. Evidence for the asymmetric distribution of the carbohydrate comes from the work of Nicolson and Singer (1974) who show that all the carbohydrate binding sites for lectins (proteins which bind to specific carbohydrate residues) are exclusively located on the outer surface of the membrane. Earlier evidence from Eylar et al. (1962) demonstrated that all the sialic acid can be released from intact human erythrocytes by neuraminidase (EC. 3.2.1.18) treatment. Finally, the use of the carbohydrate specific probe galactose oxidase (Gahmberg,1976) has confirmed the conclusion that all the carbohydrate of the erythrocyte is present at the external surface of the cell, thus confirming the morphological studies of Winzler (1971).

The two main glycoproteins of the red blood cell membrane, PAS-1 and band 3 have been reported to span the entire bilayer to the cytoplasmic surface. These conclusions were drawn from the work of Bretscher (1971b). By 2-dimensional gel analysis of peptides derived from the glycoproteins labelled in intact cells and unsealed ghosts, he revealed that 2 additional peptides were heavily labelled in ghosts but not at all in intact cells, thus showing that the protein part of the glycoprotein extends across the membrane. Other evidence that proteins are embedded within the bilayer comes from freeze fracture studies under the electron microscope, where the proteins are visualised as globular particles amidst the lipid matrix (Pinto da Silva and Nicolson, 1974). Thus the fact that the glycoproteins are exposed to both the external and internal environments, provides a means of communication across the bilayer and implies a function for these macromolecules.

1.3. Lymphocyte Plasma Membrane

After the erythrocyte plasma membrane, the lymphocyte cell membrane has provided a lot of interest from an immunological and biochemical standpoint. The lymphocyte membrane provides a framework for the location of a number of important receptors such as Ia, (immune-associated) HLA-A, B,C histocompatibility antigens, IgM and IgD, (Walsh and Crumpton, 1977).

A variety of tissues have been used as a source of lymphocytes, including the pig lymph node, the pig and human thymus and the lymphoblastoid cell line B R1 8 (Kennel and Lerner, 1973). As in the case of the erythrocyte plasma

membrane, the first step in elucidating the organisation of proteins within the bilayer is to obtain a good yield of highly purified preparations of undamaged plasma membrane. This in general is prepared by differential centrifugation and purification through a sucrose density gradient. The purified membrane consists predominantly of vesicles within the range 100-800nm diameter. According to various morphological, biochemical and immunological criteria, the plasma membrane fraction is not contaminated by significant amounts of endoplasmic reticulum or Golgi membrane (Allan and Crumpton, 1970).

The lymphocyte plasma membrane is composed of equal amounts of protein and lipid. On SDS-polyacrylamide gel electrophoresis, the membrane can be resolved into more than 30 components (Ragland et al., 1972). Of these at least 10 of the bands are reported to stain for carbohydrate (Hayman and Crumpton, 1972) and can be separated on columns of Lens culinaris(lentil)lectin (Hayman and Crumpton, 1972). Factors that have facilitated the study of this membrane are initially the existence of marker proteins which are associated with a particular face of the membrane. These include 5'-nucleotidase (EC. 3.1.3.5) and more recently the histocompatability antigens HLA-A, B, C (Snary et al., 1974). Further, the fact that the plasma membrane carbohydrate is most probably located exclusively on the outside surface (Hirano et al., 1972; Nicolson and Singer, 1974) has been exploited to separate populations of "inside-out" vesicles, which have no accessible carbohydrate on columns of immobilised Concanavalin A(Con A) (Walsh et al., 1976). By using vesicles

of defined orientation, through lactoperoxidase-catalysed iodination, it is possible to see which proteins are transmembrane, as these would be the favoured candidates for the transfer of information or nutrients across the membrane as in the case of the erythrocyte membrane (Steck, 1974).

Walsh and Crumpton (1977) have reported that in BR1 8 lymphocytes at least 10 polypeptides are transmembrane in that they are iodinated in "right side-out" and "inside-out" vesicles. Further, by use of antisera directed against specific surface receptors, they have identified 2 of these proteins. Both are integral membrane glycoproteins which span the lipid bilayer and include the HLA-A, B, C antigens of mol. wt. 43,000 and the Ia antigens which comprises 2 polypeptides of mol. wt. 28,000 and 30,000. Further work on this system has demonstrated that cross linking of the surface receptors, namely the histocompatibility antigens (Bourguignon and Singer, 1977) and Ig (Flanagan and Koch, 1978) by the capping and patching phenomenon, induces a specific association with the microfilament system. This work further stresses the importance of transmembrane proteins, which in general appear to be glycosylated, in the transmission of signals across the bilayer.

1.4. Lectins

Lectins are oligomeric proteins which bind to specific carbohydrate determinants (for reviews see, Sharon and Lis, 1972; Nicolson, 1974; Liener, 1976). They were initially discovered in the seeds of leguminous plants, but are known

Table 1.2.

SUMMARY OF LECTIN PROPERTIES USED IN THIS STUDY

PROPERTIES	CONCANAVALIN A	WHEAT GERM AGGLUTININ	SOYA BEAN AGGLUTININ
SOURCE	Jack bean (*Canavalia ensiformis*)	Wheat germ (*Triticum vulgare*)	Soya bean (*Glycine max*)
MOL. WT.	tetramer - 110,000, pH 7.0 dimer - 55,000, pH 5.0	monomer - 18,000, pH 2.0 dimer - 36,000, pH 7.0	tetramer - 120,000 over a wide range of pH.
METAL ION DEPENDENCE	Ca^{2+}, Mn^{2+}, or a similar transition metal ion. (Summer and Howell, 1936)	not known	Mn^{2+}, Ca^{2+} (Jaffe *et al.*, 1977)
SUGAR SPECIFICITY	-glucopyranoside -mannopyranoside optimum binding requires the c-3, c-4, c-6 hydroxyl groups to bind to the lectin (Goldstein *et al.*, 1965b).	N-acetyl-D-glucosamine optimum binding requires a sequence of 3 (1 4) linked N-acetyl 'D-glucosamine units (Goldstein *et al.*, 1975)	N-acetyl-D-galactosamine optimum binding requires an equatorial 2-acetamido group and an axial 4-hydroxy group. (Lis *et al.* (1970).

to be present in invertebrates such as Limulus Polyphemus (Roche and Monsigny, 1974) as well as in mammals. Hudgin et al. (1974) have reported a lectin located in the plasma membrane of rabbit liver that functions by binding itself to serum asialoglycoproteins. Other mammalian lectins that have been reported include a bovine liver lectin specific for N-acetyl glucosamine (Bowles and Kauss, 1976) and a lectin in bovine heart and lung specific for galactose containing oligosaccharides sequences (De Waard et al., 1976). Finally, evidence has also been presented for lectin binding proteins in the lymphocyte membrane (Keida et al., 1978).

The interaction of lectins with specific carbohydrate sequences, provides a valuable tool for the study of glycoproteins. These interactions can be reversed by use of an appropriate hapten as an inhibitor (Goldstein, 1975). In addition to their capacity to bind carbohydrate residues, lectins have also been reported to bind to cell constituents in a manner which does not involve carbohydrate recognition sites. Thus Con A can engage in non-specific interactions which are not reversed by hapten treatment (Goldstein, 1975). A summary of the properties of lectins used in this thesis is tabulated in Table 1.2.

1.4.1. Factors affecting glycoprotein binding to lectins

Several variables appear to be important in the binding of glycoproteins to lectins and their elution by specific carbohydrate residues. These include ionic strength (Bishayee and Bachhwat, 1974) : application of samples in 0.5M NaCl minimised the electrostatic interactions between

Con A and other proteins. It has also been reported by Lotan et al. (1977) that the proportion of fetuin binding to Con A and wheat germ agglutinin (WGA) Sepharose increased two-fold, and three-fold in the case of Soya bean agglutinin (SBA) when NaCl was present.

The phenomenon of microheterogenity in glycoproteins suggests that the oligosaccharide chains may be in various stages of completion due to the lack of absolute specificity of the glycosyl transferases. Thus it is possible that, within limits, a glycoprotein could have a variety of oligosaccharide chain structures. This phenomenon has been observed by Beeley (1974). He has shown fractionation of sialic acid free ovomucoid species into components differing in neutral sugar content on columns of immobilised Con A, on application of a linear gradient of α-methyl glucoside.

Adair and Kornfeld (1974) have pointed out the importance of column loads in affinity chromatography. On overloaded columns they have suggested that high affinity species bind themselves preferentially to the immobilised lectin, while low affinity species pass through the column.

The effect of detergents on lectins must be considered, as for the most part glycoproteins are membrane-bound and their isolation and purification requires the use of buffered detergents on lectin activity by assaying haemagglutination of formalin-fixed erythrocytes. They have concluded that cationic and non-ionic detergents are the least harmful to lectins, in that their biological activity is still preserved.

A detailed study of the application of lectins to membrane biochemistry will be considered in Chapter 7.

1.5. Mitochondria

1.5.1. Identification of Mitochondria

Mitochondria were first observed under the microscope in the late 19th century as characteristically arranged granules in the sarcoplasm. The granules are frequently seen as long filamentous threads, but also occur as rods and spheres. The morphological form of mitochondria in living cells is not stable and Lewis and Lewis (1914) found mitochondria to undergo changes with time in shape, size, and location, which suggest they have plasticity of structure and play a dynamic role. Similarly, mitochondria can vary from cell to cell, depending on the physiological status of the tissue. Classically, they were distinguished from other intracellular organelles by their cytological characteristics. Michaelis (1900) showed that mitochondria could be specifically stained with the dye, Janus Green B. When observed for a sufficient length of time after staining the mitochondria were seen first to decolourise and then to go red. This series of changes with the dye suggested that oxidation-reduction changes were taking place within the organelle.

These observed phenomena led to the concept of the respiratory chain which arose soon after the discovery of the cytochromes and their sequential arrangement by Keilin (1925). It was suggested that the elctron carriers were arranged in a

chain of increasing oxidation-reduction potentials, with the electrons flowing down the chain towards the more electropositive oxygen. Englehardt (1930) conceived the idea that the free energy from the oxidative processes was coupled to phosphorylation. This was confirmed by Ochoa (1943) who established that for each atom of oxygen three molecules of phosphate were esterified. These facts, coupled with the initial observation of Kennedy and Lehninger (1948) that the mitochondrial fraction of cells could catalyse fatty acid and Krebs citric acid cycle oxidations linked to energy production, led to the development of methods for isolation of these organelles under conditions in which the morphological and cytological integrity was maintained. Isolation of these organelles, which carried out their biochemical function *in vitro*, stimulated a tremendous amount of interest. Soon Lehninger (1951) discovered that NADH was the primary hydrogen donor for the respiratory chain. Since then, knowledge about the phosphorylation process has progressed exponentially. Nonetheless, there is still much controversy regarding the molecular mechanism by which the energy of electron flow is conserved as phosphate bond energy.

1.5.2. Isolation of Rat Liver Mitochondria

To date the most common procedure for isolating mitochondria is by differential centrifugation. Although Bensley and Hoerr (1934) are acknowledged as the first to develop a method for the isolation of mitochondria, they did not immediately publish the details of their procedure. In the meantime, Claude (1946) developed and published systematic procedures

whereby subcellular particles such as mitochondria could be isolated from tissues. In earlier fractionations distilled water or isotonic saline were used to isolate the organelles. These media respectively destroyed morphological integrity and caused extensive aggregation of the mitochondria as assessed by the classical staining methods (Hogeboom et al., 1946). However, when mitochondria were isolated in 0.88M sucrose they showed cytological and staining properties characteristic of normal intracellular mitochondria (Hogeboom et al., 1948). To date mitochondria are isolated essentially by the procedure of Hogeboom et al. (1948), that is, in buffered sucrose with the addition of EGTA. This is used to chelate Ca^{2+} ions and consequently improves the stability and integrity of the preparation.

An important matter relative to the preparation of mitochondria is the integrity of the outer membrane and purity of the preparation. In the former case, the intactness can be estimated by measuring cytochrome c oxidase activity (EC. 1.9.3.1), an inner membrane protein complex using exogenous cytochrome c as substrate (Wojtczak et al., 1972). Previous investigation has shown that the outer membrane in rat liver mitochondria is impermeable to externally added cytochrome c (Wojtczak and Zaluska, 1969), and consequently entry of the latter into mitochondria to its binding site on the external face of the inner membrane provides a criterion to assess their integrity. By this method Wojtczak et al. (1972) assessed the breakage in rat liver mitochondria to be between 5-10%. When mitochondria were isolated from other

organs e.g. heart, the degree of breakage of the outer membrane was much higher (44%), and varied from one preparation to another, even when the same procedure was employed. Thus, it appears that the ability to isolate mitochondria in a high state of integrity is most easily achieved in rat liver.

The purity of mitochondria is estimated by assaying marker enzyme activities for various contaminants. Under suitable conditions, contamination with nuclei or nuclear fragments, as judged by staining reactions for DNA, is non-existent, (Schneider, 1946). Cytoplasmic contaminants such as lysosomes, microsomes and peroxisomes can be assessed by alkaline phosphatase (EC. 3.1.3.1.) glucose-6-phosphatase (EC. 3.1.3.9) and uricase (EC. 1.7.3.3), respectively. Plasma membrane activity can be assayed by 5'-nucleotidase activity (EC. 3.1.3.5). However, it is uncertain whether these enzymes are really absent from mitochondria, as a minimum amount of activity is always isolated with the organelle even after purification. The isolated preparation would therefore be more appropriately termed as the mitochondrial fraction.

1.5.3. Structure of Mitochondria

Mitochondria are spherical or rod-like organelles of dimensions 0.3-5 μm long and 0.2-0.5 μm wide (Palade, 1952) and are shown to vary in size in response to external and internal stimuli, (Lehninger, 1959).

Surrounding the lumen or matrix is a double membrane system. The outer membrane which is approximately 6 μm wide comprises of 7-15% by weight of the total protein. It is

smooth and reported to be permeable to low mol. wt. solutes such as adenine nucleotides and coenzyme A (Werkheiser and Bartley, 1957), but is impermeable to cytochrome c (Wojtczak and Zaluska, 1969). The outer membrane is separated from the inner membrane by a space of about 8μm although contact points between the two membrane have been reported (Hackenbrock, 1968a).

The rat liver mitochondrial inner membrane comprises of 25-29% of the total protein by weight (Allmann et al., 1968), and has many invaginations or cristae which are reported to increase the surface area of the membrane (Palade, 1952). The surface of the cristae have characteristic spheres 8.5μm in diameter which project into the matrix and are connected to the membrane by a narrow stalk (Fernandez-Moran, 1962). In these mitochondria the inner membrane is seen to enlarge upon swelling through incorporation of much of the cristal mass (Hackenbrock, 1968b). On contraction the inner and cristal membranes condense together to form a tightly packed matrix (Green et al., 1968). This suggests that the inner membrane can undergo conformational changes independent of the outer membrane (Hackenbrock, 1968b). These changes have been correlated with the energy state of the mitochondrion and may represent a form of energy coupling in the mitochondrial membrane, (Green and Ji, 1972).

Enclosed within the inner membrane is a space called the matrix which comprises 65% (w/w) of the total protein (Schnaitman and Greenawalt, 1960). The matrix contains the enzymes for fatty acid oxidation and the Krebs cycle

intermediates; malate dehydrogenase (EC. 1.1.1.37) activity is often used to mark the presence of this compartment. Matrix enzymes can be readily extracted in the soluble form when mitochondrial membranes are disrupted, as in the case of hypotonic lysis (Caplan and Greenawalt, 1966).

1.5.4. Outer Membrane

The outer membrane is morphologically and biochemically distinct from the inner membrane. Sottocasa et al. (1967) have shown that when mitochondria were subjected to swelling in hypotonic medium and subsequent shrinking in the presence of ATP, they obtained separation of a particulate "light subfraction". This has been isolated by density gradient centrifugation and identified as outer membrane.

In characterising the mitochondrial outer membrane, Parsons et al. (1967) and Sottocasa et al. (1967) found an electron transfer system composed of NADH cytochrome b_5 reductase and cytochrome b_5 (EC. 1.6.2.2) to be exclusively located in the membrane. This system is insensitive to the respiratory inhibitors rotenone and antimycin A; thus distinct from the inner membrane. However, the NADH b_5 reductase enzyme appears very similar to the microsomal one (NADH-cytochrome c reductase)(Sottocasa, 1967), although Parsons et al., (1967) have noted minor differences in the absorption spectra. The outer mitochondrial membrane is similar in composition and enzymic activities to the microsomal membrane. However, both Parson et al. (1967) and Sottocasa et al. (1967) have shown that marker enzymes for microsomes such as NADPH-cytochrome b_5 reductase (EC. 1.6.2.3) and glucose-6-phosphatase

do not concentrate in the outer mitochondrial membrane fraction. Further, Schnaitman et al. (1967) have shown that monoamine oxidase (EC. 3.6.1.3) activity is specifically associated with the outer membrane.

The outer mitochondrial membrane appears very similar to the mammalian plasma membrane in its lipid composition. Distribution of lipids between the two membranes is very different; phosphatidylinositol and cholesterol are relatively abundant in the outer membrane, whereas cardiolipin is almost entirely confined to the inner membrane (Parson et al., 1967; Parsons and Yano, 1967).

Functionally, the outer membrane does not seem to play any role, although it could be involved in interactions with the cytoplasm. It has been proposed that it could act as a permeability barrier from the extramitochondrial environment. However, it must be stressed that the structure is freely permeable to solutes of mol. wt. less than 4,000 (Pfaff et al., 1968).

1.5.5. Inner Membrane

Since the demonstration by Kennedy and Lehninger (1948) that electron transport coupled to energy production in the form of ATP occurs in the mitochondrial fraction of cells, it has been well established that the respiratory chain is situated in the inner mitochondrial membrane.

The respiratory chain is composed of 3 main components : pyridine-linked dehydrogenases, the flavoproteins and the cytochromes. These components are arranged sequentially in

order of increasing redox potential and accept electrons from the NAD and FAD-linked dehydrogenases of the Krebs and fatty acid oxidation cycle. The electrons flow down the chain towards the terminal electropositive acceptor ; oxygen.

The composition and organisation of the respiratory chain was elucidated by 2 different approaches :

(1) Chance and Williams (1956) by use of a combination of steady state measurements in the individual cytochromes during active respiration, and rapid kinetic studies on pulsing anaerobi mitochondria in the presence of oxygen, have deciphered the sequence of components in the respiratory chain. These studies have been aided by the use of inhibitors that block specific electron carriers, e.g. when respiration is blocked by the antibiotic antimycin A, it was found that pyridine nucleotides, flavoprotein and cytochrome b become fully reduced, whereas cytochrome c, a and a_3 became fully oxidised. This phenomenon is referred to as "crossover point". The data obtained from these studies is consistent with the normal mid-point potentials of the carriers going from substrate to oxygen (Wilson et al., 1972).

(2) The other approach which contributed to elucidating the organisation of the electron transfer chain was by isolation and reconstitution studies. The respiratory chain can be separated into 5 distinct electron transfer complexes which together carry out the oxidation of NAD^+ and FAD^+ linked substrates. The complexes, which have been separated by treatment with detergent and ammonium sulphate fractionation are extracted with phospholipid and include :

Complex 1- NADH - Coenzyme Q (Co Q) reductase (EC. 1.6.99.5)

Complex 2- Succinate - Co Q reductase (EC 1.3.99.1)

Complex 3- Co Q - cytochrome c reductase (EC. 1.6.99.1)

Complex 4- Cytochrome c oxidase

(Green et al., 1967; Hatefi et al., 1966)

Complex 5- ATP synthetase (Hatefi et al., 1974) (EC. 3.6.1.3)

The presence of "mobile carriers" which are not integral constituents of the respiratory complexes such as, coenzyme Q which transfers reducing equivalents between complexes 1, 2 and 3, and cytochrome c which links electron transport between complexes 3 and 4 has also been demonstrated. However, the number of carriers and their sequential arrangement within the respiratory chain is still an area of active research and constantly under revision as new components are discovered, e.g. the use of low temperature electron paramagnetic resonance shows the involvement of at least 10-12 iron-sulphur proteins in the respiratory chain (Ohnishi, 1975), although no definitive information is available on the location or orientation of any of these centres.

1.5.6. Organisation of the respiratory components

After the proposal by Mitchell (1966) that the electron chain existed in "loops" crossing the inner membrane from the matrix to the cytoplasmic side, a new field of research was established. As well as considering the linear relationship between the different carriers, it became necessary to examine their topography and organisation within the inner membrane. Much work has been carried out on this aspect of the respiratory chain on mitochondria, depleted of their outer membrane and

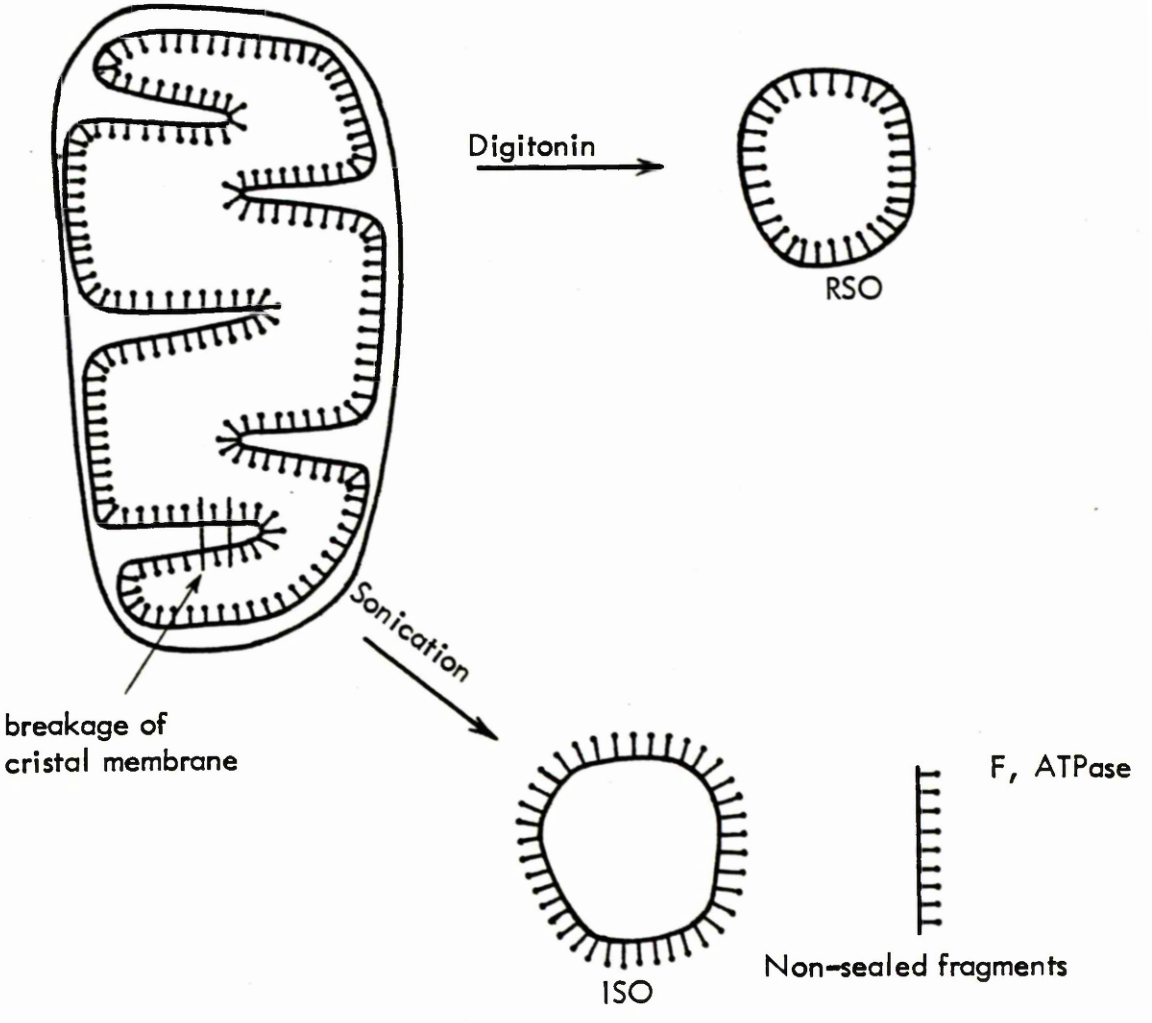


Fig. 1.1.

Preparation of SMPs with a normal or inverted membrane orientation.

submitochondrial particles (SMP), prepared by sonication of mitochondria. SMP have the characteristic inner membrane spheres (F_1) on the outer surface, in contrast to the inside in mitochondria and mitoplasts, (see Fig. 1.1 for illustration). Because of this location of the inner membrane spheres, and also several other considerations of altered functions, e.g. proton translocation, SMP particles were recognised as being "inside-out" (Lee and Ernster, 1966).

Two approaches were used to explore the organisation of proteins in the mitochondrial respiratory chain. The first was to raise a specific antiserum against a particular protein and test the two types of particles (mitoplasts, SMP) for inhibition of electron transport. The other was to specifically label surface proteins using a membrane impermeable reagent; [^{35}S]-diamino benzensulphonic acid (DABS) or lactoperoxidase catalysed radio-iodination.

The antibody studies from these experiments have demonstrated that externally added antibody against cytochrome c inhibits respiration in mitoplasts but not in SMP (diJeso et al., 1969), suggesting that cytochrome c is exposed on the cytoplasmic surface. Further confirmation of this comes from the fact cytochrome c can be easily extracted from intact mitochondria but not SMP. A combination of both the techniques have shown that cytochrome c_1 is exposed on the cytoplasmic surface, but it appears to be an integral protein, unlike cytochrome c, in that its extraction requires detergents. Cytochrome c oxidase and the ATPase have been shown to be transmembrane protein complexes (Eytan and Schatz, 1975; Tzagoloff, 1972).

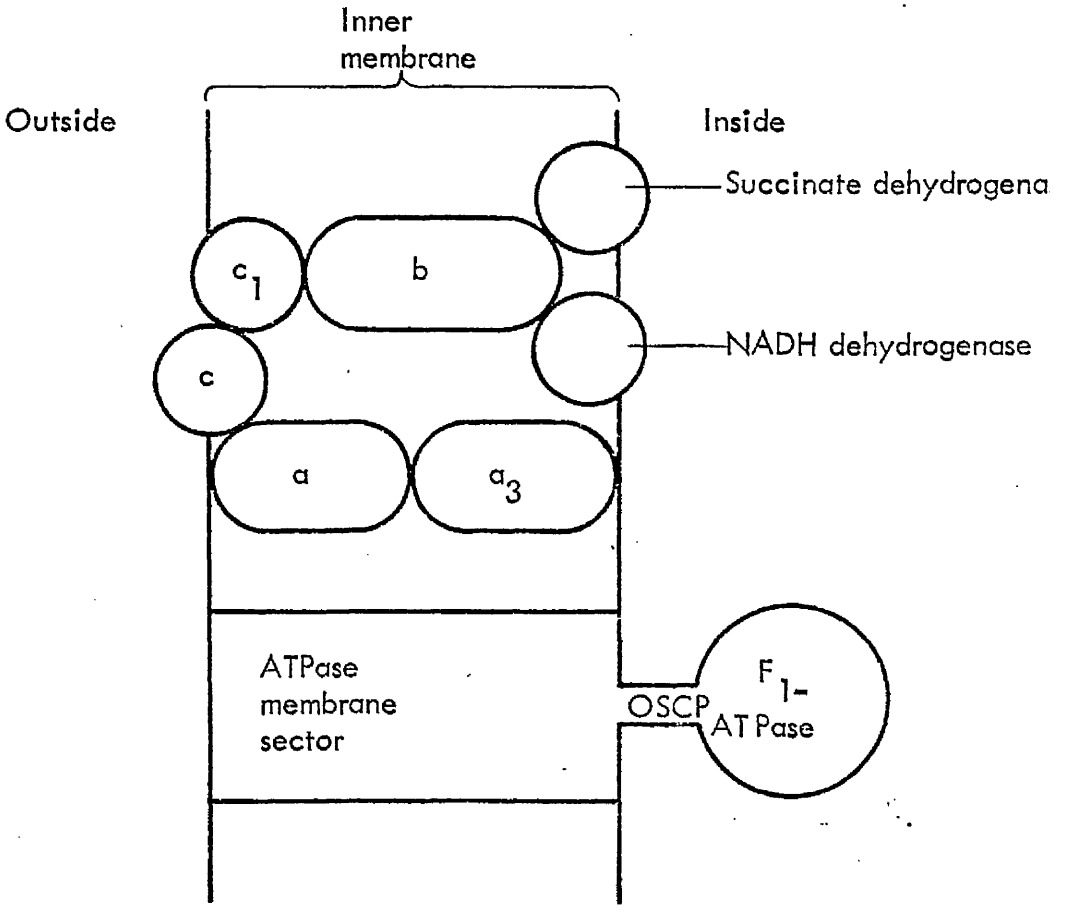


Fig. 1.2

Structural organisation of the oxidative phosphorylation chain.

(reproduced from Mitochondria : structure, function and assembly, P.A. Whittaker and S.M. Danks (1978) p. 64).

Cytochrome b is inaccessible to all probes suggesting that it is embedded within the bilayer. Finally, succinate and NADH dehydrogenase are both membrane bound on the matrix side. Thus, the conclusions from these experiments suggest that the proteins in the respiratory chain are organised in a loop that spans the membrane from the cytoplasmic to the matrix side (see Fig. 1.2). The detailed topography of these trans-membrane complexes will be considered in the next section.

1.6. Topography and assembly of the inner membrane of mitochondria

It is a well known fact that not all mitochondrial proteins are coded for by its DNA (Borst, 1972). Mitochondrial DNA consists of a homogeneous population of small duplex circles between 4.4 to 5.9μm length in animals though it is much longer in plants, fungi and unicellular organisms (Borst, 1972; Schatz and Mason, 1974). In addition Nass (1969) reported that mitochondria possess DNA-synthesising enzymes, ribosomes, rRNA, tRNA and aminoacyl tRNA synthetase.

The process of mitochondrial biogenesis and assembly is under the control of two separate genetic systems : mitochondrial and nuclear, both of which are involved in the assembly of the organelle. It is now well established that the great majority of the mitochondrial proteins are coded by nuclear genes and synthesised on cytoplasmic ribosomes. These proteins are subsequently imported into the organelle and assembled in a coherent manner with the 8-10 hydrophobic proteins synthesised on mitochondrial ribosomes (for review see, Schatz and Mason, 1974). Hence, a mechanism must exist in the cell to control

both the expression of these 2 genetic systems as well as the synthesis of proteins at intracellular sites, such that the different enzyme complexes of the inner membrane are assembled in a co-ordinated manner.

In an attempt to understand the assembly process of mitochondria, attention has been focused on a few well defined enzyme complexes, associated with the inner mitochondrial membrane : cytochrome c oxidase, the oligomycin-sensitive ATPase and the cytochrome bc_1 complex (Tzagoloff et al., 1973; Schatz and Mason, 1974). All three complexes are involved in respiratory-linked energy functions and are composed of mitochondrially as well as cytoplasmically synthesised polypeptides. In order to identify the origin of a particular subunit, that is whether it is mitochondrial or cytoplasmic in origin the same general experimental protocol was used, summarised below :

(1) Yeast (Saccharomyces cerevisiae) or Neurospora crassa are labelled in vivo, with a radioactive amino acid in the presence of either cycloheximide, the cytoribosomal inhibitor or chloramphenicol, the mitoribosomal inhibitor.

(2) The enzymic subunits are then isolated by specific immunoprecipation with antiserum to the purified subunit (Tzagoloff and Meagher, 1972; Mason and Schatz, 1973; Ebner et al., 1973b).

(3) The labelled proteins are subsequently identified by SDS polyacrylamide gel electrophoresis.

The assembly of these complexes involves several intricate controls of which little is known and consequently the subject of much investigation. The progress to date on the

organisation and assembly of 3 oligomeric enzyme complexes will be briefly considered.

1.6.1 Cytochrome c oxidase :

Yeast cytochrome c oxidase can be resolved into 7 polypeptides of mol. wt. ranging from 40.000 to 4.500. The three largest polypeptides which are coded for by mitochondrial DNA and synthesised on mitochondrial ribosomes appear to be hydrophobic in nature on purification (Mason and Schatz, 1973), (Poyton and Schatz, 1975). The 4 smaller polypeptides are coded for by nuclear DNA and synthesised on cytoplasmic ribosomes (Schatz et al., 1972).

(a) Topography of the subunits :

Using membrane impermeant probes which included enzymatic iodination with lactoperoxidase, or coupling with $[^{35}S]$ DABS, the accessibility of cytochrome c oxidase subunits in intact mitochondria and inverted SMP from yeast was compared (Eytan and Schatz, 1975). Cytochrome c oxidase is shown to span the inner membrane in an asymmetric manner. Subunits 2, 3, 6 and 7 are only accessible from the outer side, subunit 4 is accessible from the inner side and 1 and 5 are inaccessible.

Further affinity labelling studies of Birchmeier et al. (1976) have shown that subunit 3 can be "affinity labelled" by activated cytochrome c. Subunit 3 is thus at the very least close to the cytochrome c binding site and, by implication, on the same side of the mitochondrial inner membrane as cytochrome c. Similar studies on the topography of cytochrome c oxidase in bovine heart have been reported (Downer et al., 1976; Eytan et al., 1975; Ludwig et al., 1979).

(b) Effect of mutations on the assembly of cytochrome c oxidase

Yeast, nuclear mutants of cytochrome c oxidase were isolated and assessed for residual activity (Ebner et al., 1973a,b). The results have shown that each of the 3 nuclear mutants lacked at least one cytochrome c oxidase component, in particular the 23,000 mol. wt. polypeptide. All 4 of the cytoplasmically synthesised subunits were present. From these results Ebner et al. (1973 a,b) concluded that the 23,000 mol. wt. polypeptide is essential for functional cytochrome c oxidase activity. Also, it appears that all 7 cytochrome c oxidase subunits form a tight complex with each other as antiserum which cross-reacts with only a single subunit precipates all 7 from the crude mitochondrial extract (Poyton and Schatz, 1975).

1.6.2. F_1 ATPase complex :

The oligomycin-sensitive ATPase from yeast mitochondria has been resolved into 3 functional components. The basic catalytic unit of the complex is a water soluble F_1 ATPase (Pullman et al., 1960) comprising of 5 polypeptides of mol. wt. ranging from 55,000 to less than 8,000 (Tzagoloff, 1971). The oligomycin sensitivity conferring protein (OSCP) involved in the binding of F_1 to the membrane (Tzagoloff, 1970) accounts for another polypeptide. The third component is the membrane lipo-protein complex which consists of at least 4 hydrophobic polypeptides (F_0), (Tzagoloff, et al., 1973).

Preliminary evidence has suggested that the beef heart mitochondrial ATPase is glycosylated (Andreu et al., 1977).

The same authors have confirmed the glycoprotein nature of the F_1 ATPase from bacteria and chloroplasts (Andreu et al., 1978) and hypothesise further the glycoprotein nature of several coupling factors, suggesting that this is a general property of proteins involved in phosphorylation.

(a) Assembly of the complex

The F_1 ATPase is synthesised on cytoplasmic ribosomes and is labelled in intact cells in the presence of chloramphenicol (Tzagoloff and Meagher, 1972). The OSCP is also a cytoplasmic product formed in chloramphenicol inhibited cells (Tzagoloff, 1970). The 4 hydrophobic proteins in contrast, are made in the presence of cycloheximide (Tzagoloff, 1972).

If synthesis of the 4 mitochondrially-synthesised ATPase subunits is prevented by growth of cells in the presence of chloramphenicol, or in 'petite' mutants which have a defective respiratory system and are incapable of mitochondrial protein synthesis (Schatz and Satzgaber, 1969), F_1 is still synthesised (Schatz, 1968). However, it becomes oligomycin-resistant and can easily be detached from the mitochondrial membrane by mild sonication. As mentioned before for cytochrome c oxidase, it seems that mitochondrially synthesised subunits ensure the proper binding of F_1 to the membrane.

1.6.3. Cytochrome bc_1 complex of yeast mitochondria

The isolated bc_1 complex of yeast mitochondria consists of at least 7 non-identical subunits of mol. wt. ranging from 44,000, 40,000, 32,000, 32,000, 17,000, 14,000, 11,000, (Katan et al., 1976a). Of these subunits only the cytochrome b apoprotein is coded for by the mitochondrial genome. The remaining being under the control of nuclear DNA (Katan et al., 1976b).

(a) Topography of the subunits

The transmembrane distribution of the subunits in the cytochrome bc_1 complex from beef heart mitochondria was investigated using the non-penetrating reagent [^{35}S]-DABS (Bell et al., 1978). The results have shown that the complex is composed of 9 polypeptides in addition to 2 hemes, a c_1 heme and a non-iron heme moiety all of which asymmetrically span the membrane (for review see, Reiske, 1976). Polypeptides 1 and 7 were exposed on the cytoplasmic side of the membrane, whereas 2 and 3 were located on the matrix side. Polypeptides 8 and 9 were not visibly labelled from either side of the membrane. These results are in general agreement with those of Mendel-Hartvig and Nelson (1978).

(b) Assembly of the complex

Incubation of yeast cells in the presence of cycloheximide results in incorporation of radio-labelled methionine into only one band of mol. wt. 32,000. Cells labelled in the presence of chloramphenicol, incorporate radioactivity into 4 of the 6 bands of the complex no label being found in the bands of 40,000 and 17,000, (Katan et al., 1976b). This observation suggests that integration of labelled polypeptides of mol. wt. 40,000 and 17,000 into the completed subunit can only occur when the cytochrome b_1 apoprotein, that is coded for by the mitochondrial genome is synthesised simultaneously. This situation is reminiscent of that found previously, for the biosynthesis of cytochrome c oxidase (Ebner et al., 1973b) and the ATPase complexes (Schatz, 1968).

1.7. Carbohydrate in mitochondria

Isolated mitochondria are reported to contain 1-2% carbohydrate by weight, (de Bernard *et al.*, 1971; Yamashina *et al.*, 1965) and are capable of glycosylating endogenous proteins *in vitro*, suggesting the presence of a mechanism independent from the rough endoplasmic reticulum and Golgi apparatus. (Bernacki and Bosmann, 1970).

Evidence for the presence of carbohydrate on mitochondrial surfaces has come from direct agglutination studies using multivalent lectins. Nicolson and Singer (1971) have found that isolated bovine mitochondria were agglutinated by Con A, WGA, and ricin agglutin from *Ricinus communis* (RCA), which is specific for D-galactose. They inferred from this that saccharide residues specific to the above mentioned lectins were exposed on the external mitochondrial surface. Contrary results using the same lectins were reported for rat liver mitochondria by Henning and Uhlenbruck (1973). These authors did not observe any agglutination with WGA and RCA and the results for Con A were inconsistent, though agglutination increased strongly after pronase treatment.

It has also been reported by Bosmann *et al.* (1972) that rat liver mitochondria, mitoplasts and outer mitochondrial membranes contained 2.0, 1.1 and 4.1 nmoles of sialic acid/g of protein respectively. The sialic acid apparently contributes to the negatively charged nature of the mitochondrial surface.

Attempts to define the location of the mitochondrial carbohydrate have proved vague and inconclusive. Most of the mitochondrial hexosamine (77%) and sialic acid (80%) is released

in the soluble form on disruption of the organelle (de Bernard et al., 1971; Sottocasa et al., 1971). It is reported that this material is located in the intermembrane space, the remaining sialoglycoproteins being mainly associated with the outer membrane (de Bernard et al., 1971).

Further evidence for the distribution of carbohydrate in mitochondria comes from studies by Glew et al. (1973). Their data suggest that 40% of the Con A binding sites are located in the outer membrane. Of these, only 15% are firmly associated with the membrane, the remaining 25% is thought to be either loosely associated with the outer membrane, or reside in the inter-membrane space with the appropriate lectin receptors exposed on the external surface. Despite all the data that has been reported on the location of carbohydrate on the outer surface of an "intact" mitochondria, none of the workers have checked for intactness of the outer membrane in the organelle. This can often get badly disrupted during sucrose density gradient purification. Hence, the results are not completely valid as it is possible that specific surface probes such as lectins could permeate through a broken membrane.

Other evidence for the presence of glycoprotein and glycolipid in mitochondria is the ability of these organelles to incorporate monosaccharides into endogenous protein receptors. It was first shown by Bosmann and Martin (1969) that monosaccharides from nucleotide diphosphates were incorporated into glycoproteins located in the inner membrane of rat liver mitochondria. This observation was later extended to cerebral

cortex mitochondria (Bosmann and Hemsworth, 1970). The enzymatic characteristics of the mitochondrial glycoprotein : glucosyl transferase and mannosyl transferase were described and have been reported to be located in the inner membrane where endogenous acceptor proteins are also localised (Martin and Bosmann, 1971).

Thus, although there is definitive evidence for the presence of carbohydrate in mitochondria and its incorporation into endogenous protein acceptors, very little is known about the topography of these macromolecules, their function and whether they are constituents of the isolated mitochondrial complexes.

1.8. Transport in Mitochondria

The permeability properties of the mitochondrial double membrane system have been well established. The outer membrane is freely permeable to all small molecules but is not penetrated by larger molecules e.g. inulin (Werkheiser and Bartley, 1957). The inner membrane on the other hand poses a permeability barrier even to certain anions such as chloride, but allows phosphate and acetate to pass through. This in part can be explained by the high cardiolipin content of the membrane as it has been shown that incorporation of cardiolipin into artificial phospholipid micelles reduces the permeability of anions (McGivan and Chapell, 1967).

Many of the most important functions of the mitochondrion (Krebs cycle oxidations, fatty acid oxidation, ATP synthesis) occur in the matrix and substrates and co-factors for these

reactions are screened from the enzymes by the two membrane barriers. This compartmentalisation of reactions in mitochondria suggests that a mechanism must exist for the specific transport of the substrates, and products across the inner membrane in a manner in which the membrane potential and neutrality is maintained. Thus, the functions of mitochondria are such that permeases must exist so that substrates and products can be transported across mitochondrial membranes as, at neutral pH most of these metabolites are hydrophilic ions and as such would not be expected to penetrate a lipid bilayer in the absence of a specific carrier system (Klingenberg, 1970).

1.8.1. Types of transporters

There are two main types of transporters in mitochondria : uniporters where a single ion is transported into the matrix and antiporters, where a single ion is moved into the matrix in exchange for one of similar charge. Also present are symporters where the transport of the anion is electroneutral, as it is accompanied by a proton to achieve electrical balance, e.g. phosphate or glutamate transport. This makes it difficult to distinguish symporters from antiporters.

It was proposed by Mitchell and Moyle. (1969) as a consequence of his chemiosmotic hypothesis that anions accumulate in the mitochondrial matrix by electroneutral exchange with OH^- ions. The entry of anions in exchange for OH^- would be facilitated by H^+ on the outside of the membrane, whereas the reverse exchange would be inhibited by OH^- inside.

Table 1.3.

Mitochondrial Transporting Systems

(reproduced from Mitochondria : structure, function and assembly. P.A. Whittaker and D.H. Danks (1978) pp. 96-97)

Transporter	Probable *in vivo* function (*Out* → *In*)	Inhibitors	Probable biological importance
Phosphate	Phosphate → / ← OH^-	*N*. ethyl maleimide, mersalyl	1. Mitochondrial ATP synthesis 2. Allowing flux of dicarboxylates and therefore tricarboxylates
Adenine nucleotide	ADP^{3-} → / ← ATP^{4-}	Atractyloside, CO-atractyloside, Bongkrekic acid	In mitochondrial ATP synthesis
Pyruvate	Pyruvate → / ← OH^-	α-Cyano (4.OH) cinnamate	The link between glycolysis and the TCA cycle
Dicarboxylate	Malate → / ← Phosphate or Succinate	n-Butyl malonate, 2-Phenyl succinate	1. Transferring reducing equivalents into and out of the matrix 2. Provides C-skeleton for PEP
(Malate and Succinate)	Succinate or Phosphate → / ← Malate		
Tricarboxylate	Citrate → / ← Malate or Isomalate	Citrate analogues	1. Transfer of acetyl CoA into cytosol 2. Control of PFK 3. Provision of NADPH in cytosol
(Citrate and isocitrate)	Citrate or Isocitrate → / ← Isocitrate		
Oxoglutarate	Oxoglutarate → / ← Malate or Malonate	Aspartate	1. Transfer of reducing equivalents across the membrane 2. Transport of amino groups into the matrix
Glutamate	Glutamate → / ← OH^-	*N*. ethyl maleimide	Glutamate in the matrix gives rise to NH_3 for urea formation
Glu/Asp	Glutamate $^-H^+$ → / ← Aspartate	Glisoxepide	1. Transfer of reducing equivalents 2. Aspartate involved in urea synthesis in cytosol 3. In gluconeogenesis
Glu/GluN	Glutamine → / ← Glutamate	–	NH_3 production in the kidney
Orn/Cit. (liver only)	?	-	In operation of the urea cycle
Carnitine/ acyl carnitine (heart only)	Acyl Carnitine → / ← Carnitine	Carnitine derivatives	Movement of fatty acids across the membrane
Ca^{++}	Ca^{++} →	Ruthenium red La^{++}	Storage of Ca^{++} in the matrix?

Out is the cytosol and *In* is the matrix. ○ represents the carrier.

Prior to the proposal of Mitchell, concerning the driving force for anion accumulation, a series of papers appeared from Chapell's laboratory (Chapell, 1969; Chapell and Haarhoff, 1966), in which it was shown that many of the anion transport systems are operated by very specific processes. By using techniques such as ammonium swelling (Chapell and Crofts, 1966) or kinetic measurements of the reduction state of mitochondrial pyridine nucleotides a number of separate carrier systems were identified. These are summarised in Table 1.3.

The original proposal, that all anions are accumulated by means of a direct proton symport or hydroxyl ion exchange, has been modified as a result of various studies of the different carrier systems. It now appears that the di and tri-carboxylates are accumulated in the matrix space in a manner proportional to the Δ pH due to indirect coupling with the transport of phosphate, which is coupled directly to proton movements (Palmieri et al., 1970; Papa et al., 1971).

The study of transport in mitochondria is at an advantage with respect to the elucidation of carrier systems. The high rate of metabolite transport indicates that these carriers are present in the membrane at high concentrations which should facilitate their identification and molecular characterisation. It is possible that in order to facilitate transport of ions, the proteins involved traverse the inner and perhaps the outer membrane too. In fact, the contact points that have been observed between inner and outer membranes (Hackenbrock, 1968a)could be junctions which

facilitate the transmembrane orientation of proteins. The only carriers that have been well defined to date, include the adenine nucleotide and Ca^{2+} translocators. Also, the increasing interest in mitochondrial biogenesis has provided much information on the import of cytoplasmically synthesised proteins into mitochondria. These three transport systems will now be considered.

1.9. Functional aspects of mitochondria

1.9.1 Ca^{2+} Transport

It has been well established that mitochondria from most eukaryotic cells have the ability to accumulate Ca^{2+} against a concentration gradient (Carafoli and Lehninger, 1971). Three separate classes of Ca^{2+} binding sites have been reported for mitochondria :

(a) Low affinity binding sites which are distributed between the two membranes (Rossi *et al.*, 1967)

(b) High affinity binding sites which are isolated with the Ca^{2+} binding protein and show the following characteristics.

1. requires energy
2. shows saturation kinetics
3. inhibited by lanthinum and ruthenium red (Vasington *et al.*, 1972)
4. absent from some mitochondrial types for example blow fly muscle (Carafoli and Lehninger, 1971).

(c) High affinity binding sites which are located in the inner membrane and involved in the oxidation of α-glycerophosphate dehydrogenase (Carafoli and Sacktor, 1972).

Isolation of the Ca^{2+} binding protein

Efforts to extract the high affinity binding sites from the mitochondrial membrane have led to the isolation of 2 factors capable of binding Ca^{2+}:

(1) An insoluble Ca^{2+} binding factor that was extracted with distilled water and reported to be a glycoprotein of mol. wt. 67,000 (Gomez-Puyou et al., 1972). It binds Ca^{2+} to two classes of sites, one of which has a very high affinity for the cation and is inhibited by La^{3+} at the same low concentrations which abolish energy linked uptake of the cation into mitochondria (Gomez-Puyou et al., 1972). However, this glycoprotein has not been well characterised as regards its location in mitochondria.

(2) A soluble glycoprotein has been isolated by Carafoli, Sottocasa and their associates (1972) from mitochondria by mild osmotic shock. Sonication of the osmotically treated mitochondria results in release of an additional portion of this glycoprotein. This suggests that part of the glycoprotein is firmly embedded in the inner membrane and is released by sonication. The remaining is loosely associated with the inner membrane and/or free in the intermembrane space, (Carafoli et al., 1972).

Studies on the isolated glycoprotein have shown that it has a mol. wt. of 30-33,000, contains about 10% carbohydrate by weight, and does not bind to Con A. This is consistent with the fact that the most abundant sugar present is galactosamine. It also contains a variable amount of bound phospholipid. Among its interesting properties, is the ability to bind Ca^{2+} at 2 classes of sites in a reaction that is inhibited by La^{3+} and ruthenium red. Also of interest is the absence of high affinity binding sites in glycoproteins isolated from mitochondria which do not have

the ability to actively transport Ca^{2+} (Carafoli and Lehninger, 1971). Final definitive evidence that the isolated glycoprotein does in fact represent the high affinity binding sites of isolated mitochondria is by the work of Panfili et al. (1976). They prepared the antibody to the Ca^{2+} binding glycoprotein and showed that it specifically inhibited active Ca^{2+} transport without interfering with other mitochondrial activities, such as electron transport.

1.9.2. Adenine Nucleotide Translocation

One of the most studied transport systems in the last decade is the exchange between extra and intra-mitochondrial ADP and ATP. This is of vital importance to the cell as it transfers the ADP originating from energy consuming reactions in the cytosol, for ATP generated by oxidative phosphorylation in mitochondria. The carrier catalyses a one to one trans-membrane exchange of intramitochondrial ADP or ATP with ADP or ATP added to mitochondria. The overall transfer is a net negative charge out of the matrix. The carrier specifically transports only adenine nucleotides, in particular ADP and ATP (Pfaff et al., 1965), although deoxy and phosphonic acid analogues of adenine are transported slowly (Duee and Vignais, 1969).

(a) Properties of the carrier

The ADP/ATP transmembrane exchange obeys saturation kinetics. Two specific inhibitors of the translocation process have also been identified : Atractyloside (ATR), a competitive inhibitor capable of removing the ADP specifically bound to the carrier sites (Pfaff et al., 1965), and

carboxyatractyloside (CAT), its derivative which is a non-competitive inhibitor whose presence enhances ADP binding to the carrier molecule (Erdelt et al., 1972). Both inhibitors differ in their structure and their pH dependence for the inhibitory effect. ATR and its derivative are amphilic molecules which have been shown to bind specifically to the outer membrane and not penetrate it (Vignais et al., 1973). In contrast, bongkrekic acid a tricarboxylic acid is inhibitory only at pH values below 7; this pH requirement was not necessary for ATR. When the carboxyl groups of bongkrekic acid are protonated it is thought to penetrate the inner membrane to its binding sites which are located facing the matrix (Erdelt et al., 1972).

It was these properties of the ADP/ATP transport that led to the hypothesis that a definite protein moiety must be involved in the transport process. The events which led to the isolation of the translocator are summarised below.

(b) Isolation of the carrier protein

The isolation of the carrier protein is based on the high affinity binding of the latter to CAT under saturating conditions. On solubilisation with Triton X-100, the carrier CAT binding is retained. In fact, CAT is thought to protect the protein against denaturation in the detergent (Riccio et al., 1975a). The Triton X-100 extract is then purified by passing down a hydroxyapatite column and further purification on Sepharose or agarose columns (Riccio et al., 1975b). On polyacrylamide gel electrophoresis a single peak of mol. wt. 29,000 is obtained and the protein is estimated to account for

6% (w/w) of the total protein of beef heart mitochondria.

Further work on the characterisation of this protein has come from work by Boxer and co-workers (1977). They demonstrate that the only major protein which can be labelled on the external surface of beef heart mitochondria is the CAT-binding protein. This has been verified by mol. wt. analysis and double immunodiffusion tests using antibodies prepared to the purified CAT-binding protein.

In a similar vein Aquila and co-workers (1978) have isolated the ADP/ATP translocator from beef heart mitochondria as the bongkrekate protein complex. They present data to show that the isolated bongkrekate and CAT complexes represent two different conformational states of the translocator protein, namely 'm' and 'c'. The 'm' state is when the transporter is facing into the matrix in this conformation bongkrekic acid binds irreversibly to it and the protein cannot alter its configuration. In the 'c' state the transporter faces the inter-membrane space and will bind atractyloside and carboxyatractyloside. Both states are inter-convertible in the presence of ADP.

These results indicate that ADP/ATP transport is indeed mediated by a specific carrier-protein, which is not known to exist in 2 different conformational states; a transition between the two states occurring in the presence of either ADP/ATP.

1.9.3. Transport of proteins into mitochondria

In discussing protein transport into mitochondria three different categories must be recognised : Proteins of the

mitochondrial matrix which are translocated across the two membranes, proteins of the inner membrane which have to pass through the outer membrane, and proteins of the outer membrane which need not be translocated.

The question of protein transport into mitochondria is a topic of much speculation and several hypothesis have been put forward to clarify the situation. Butow et al. (1975) have shown that isolated yeast mitochondria contain bound cytoplasmic ribosomes. These observations raised the possibility that cytoplasmically synthesised mitochondrial proteins are made on a special class of cytoplasmic ribosomes attached to the mitochondrial surface. The newly synthesised polypeptides are then transferred by direct insertion of nascent chains into the membranes.

However, conflicting evidence for this hypothesis has been presented (Hallermayer et al., 1977a, b). Investigations of the transport of cytoplasmically synthesised proteins into mitochondria of intact Neurospora crassa cells, suggest that newly synthesised mitochondrial proteins exist in a precursor form in the cytosol from which they are imported into the organelle. Later work by Zimmerman et al. (1978) has reported on the synthesis and transfer of 2 mitochondrial proteins : cytochrome c and the carboxyatractyloside binding protein, both of which are synthesised on cytoplasmic ribosomes, and exist in an extra mitochondrial pool prior to transfer into the organelle. This work however, has failed to demonstrate the existence of larger precursor forms of these proteins.

Confirmatory evidence for this same hypothesis has come from Schatz and co-workers (1979). They have shown that in the case of yeast F_1 ATPase, the cytoplasmically coded ATPase subunits are synthesised as a larger precursor in the cytoplasm. Transport of these precursors into mitochondria is accompanied by proteolytic cleavage of this signal sequence (Blobel and Dobberstein, 1975) and is thought to be initiated by the interaction of the precursor with a specific receptor in the membrane, followed by the opening of a polar trans-membrane channel.

1.10 Rationale for Investigation

The basic underlying theme of this research programme is to develop our understanding of the topography, organisation and function of the mitochondrial membrane proteins/glycoproteins to a level comparable with our knowledge of the red blood cell plasma membrane.

In several respects, studies on mitochondrial membrane lack the technical advantages which are available for experimenting on the red blood cell. Isolation of morphologically intact organelles and preparation of membranes of defined purity is more difficult. Additionally, the polypeptide composition of the inner and outer membranes as examined by SDS electrophoresis is considerably more complex. No techniques exist for isolation of homogeneous populations of vesicles of defined orientation by simple manipulation of ionic conditions, as in the case of the erythrocyte plasma membrane (Steck and Kant, 1974). Furthermore, the exact nature, distribution and function of membrane-associated glycoproteins is still vague and a matter for speculation.

However, mitochondria provide a suitable system for the study of many membrane-associated activities linked to ATP synthesis including ion and substrate transport. The outer membrane provides a protective barrier for inner membrane proteins during surface labelling studies. Similarly, specific labelling of inner membrane proteins can be achieved by selective removal of the outer membrane

under defined conditions (Clarke, 1976b).

Although much work has been reported on the inner membrane, particularly as regards isolation and topography of the respiratory chain complexes, and identification of the various inner membrane transport systems, e.g. phosphate transporter, knowledge is lacking at a molecular level. This is owing to the absence of refined methodology necessary for the detailed investigation.

In the last five years, the use of lectins, which have specificities for a variety of monosaccharide moieties have provided an important tool for investigation of mitochondrial molecular architecture and isolation of membrane glycoproteins. This capacity of lectins to bind to glycoproteins exposed at the external surface can be employed to monitor their effects on various transport and energy-linked functions in the mitochondrion. Any specific inhibition by lectin treatment will implicate membrane glycoprotein for an active role in that particular function. In a similar manner, the associations between the surface components and the cytoskeletal system may be modulated by changes in the valency of the surface receptors owing to cross-linking by lectins (Koch, 1979). Lectins have also been exploited as a means of purifying "inside-out" vesicles from a mixed membrane population (Walsh et al., 1976). Selective labelling of the outer and inner surfaces in membrane vesicles has then allowed identification of proteins which span the lipid bilayer (Walsh and Crumpton, 1977).

Since one of the most important functions of the plasma membrane is to transmit information across the lipid bilayer into the cell, transmembrane proteins are attractive candidates for this role, as they are exposed on both the external and cytoplasmic surfaces. The results of several studies in particular on the red blood cell and lymphocyte membranes have documented that transmembrane proteins are glycosylated with the carbohydrate moieties preferentially located on the external surface of the membrane (Steck, 1974; Walsh and Crumpton, 1977). These studies have led to the proposal by Rothman and Lenard (1977) that all membrane glycoproteins have a transmembrane orientation and further, all transport processes carried out across eukaryotic membranes should be catalysed by oligomeric glycoproteins (Guidotti, 1976). The application of these lectin techniques to the mitochondrial membrane system should be useful in elucidating information about the topography and functions of proteins/glycoproteins associated with the membranes.

The question of mitochondrial biogenesis is also one of considerable importance. It is well established that the majority of the mitochondrial proteins are synthesised on the cytoplasmic ribosomes (Schatz and Mason, 1974). The mode of insertion of these proteins across the double membrane system is one of much controversy and speculation (Butow et al., 1975; Maccecchini et al., 1979). Finally, the mechanism by which mitochondrial proteins are glycosylated is also an area which requires much investigation.

To date a detailed examination of mitochondria in relation to :

(1) Nature and distribution of the carbohydrate.

(2) Topography of the proteins in the two membranes.

(3) Isolation and characterisation of glycoproteins.

(4) Identification of transmembrane proteins.

(5) Functional studies by use of lectins.

(6) Biosynthesis, glycosylation and import of proteins.

has not yet been undertaken. This thesis encompasses the efforts towards an understanding of some of these questions.

CHAPTER 2

Materials and Methods

MATERIALS

M 1. Biological Materials

1. Female rats; Wistar strain
2. Rabbits - either Dutch or half lops strain
3. *Staphylococcus aureus* (Cowan 1 strain)
4. Lectin sources

   Wheat germ - Health shop, Byres Road, Glasgow, Scotland.

   Soya bean meal - BDH Chemicals, Poole, Dorset

M 2. Chemicals

1. Radiochemicals

   All radiochemicals are supplied by the Radiochemical Centre, Amersham, Bucks.

	Specific activity
D-[6-^{3}H]-glucosamine hydrochloride	20Ci/mmol.
2-[^{14}C]-ethanol-1-2-amine	38Ci/mmol.
N-succinimidyl 3-(4-hydroxy, 5 [^{125}I]-iodophenyl) propionate (Bolton Hunter reagent)	1,600Ci/mmol.
[^{125}I]-NaI supplied by Western Infirmary, Glasgow.	carrier free

2. Photographic materials

Kodak DX-80 developer	Kodak London Ltd., London, England.

Kodak FX-40 fixer	Kodak London Ltd., London, England.
Kodak X-omat RH royal film	Kodak Canada Ltd., Toronto, Ontario.
Kodirex film	Kodak London Ltd., London, England.
Fuji RX X-ray film	Hanimex X-ray, Swindon, Wilts.
2,5-diphenyloxazole (PPO)	Koch-Light, Colnbrook, Bucks.

3. Chromatographic Materials

Sephadex G-50 (fine)	Pharmacia (G.B.) Ltd., London.
DEAE-cellulose (DE-52, DE-32)	Whatman, W. and Balston, R., Maidstone, Kent.
Con A covalently coupled to Sepharose 4B	Pharmacia (G.B.) Ltd., London.

4. Electrophoresis materials

Acrylamide	BDH Chemicals, Poole, Dorset.
NN'methylenebis-acrylamide	" " "

Urea - Aristar grade	BDH Chemicals, Poole, Dorset.
Ammonium persulphate	" " " "
NNN' N' tetramethyle-thylenediamine (TEMED)	Koch-Light, Colnbrook, Bucks.
Riboflavin	Sigma (London) Chemical Co., Poole, Dorset.
Ampholine carrier ampholytes	LKB, South Croydon, Surrey.

5. Enzymes

Horse radish peroxidase (Type VI)	Sigma (London) Chemical Co., Poole, Dorset.
Hexokinase (EC. 2.7.1.1)	" " " "
Glucose-6-phosphate dehydrogenase (EC. 1.1.1.49)	" " " "
Trypsin (bovine pancreas) (EC. 3.4.21.4)	" " " "
β-galactosidase (EC. 3.2.1.23)	" " " "
Chymotrypsinogen (bovine pancreas)	" " " "
Lactoperoxidase (B grade) (EC. 1.11.1.7)	Calbiochem Ltd., Bishops Stortford, Herts.
Pronase (EC. 3.4.24.4)	" "
Carboxypeptidase A (EC. 3.4.12.2)	Sigma (London) Chemical Co., Poole, Dorset.

RNA polymerase (EC. 2.7.7.6)	Sigma (London) Chemical Co., Poole, Dorset.

6. Other proteins

Bovine serum albumin (essentially fatty acid free) (BSA)	"	"	"	"
Cytochrome c (horse heart)	"	"	"	"
Ferritin	"	"	"	"
Ovalbumin	"	"	"	"

7. Specific sugars

D-mannitol	"	"	"	"
2-Acetamido-2-Deoxy-D-Galactose (N-acetyl-D-glucosamine)	"	"	"	"
1-O-Methyl-D-Glucopyranoside (α-Methyl-D-glucoside)	"	"	"	"
β-glycerophosphate (disodium salt)	"	"	"	"
Glucose-6-phosphate	"	"	"	"

8. Detergents

Triton X-100	Sigma (London) Chemical Co., Poole, Dorset.
7-Deoxycholic acid (Na salt) (DOC)	" " " "
Digitonin	" " " "
Nonidet P-40	BDH Chemicals, Poole, Dorset
Sodium lauryl sulphate (SDS)	" " " "
Triton WR-1339	Micro-Bio Laboratories Ltd., London, England.

9. Liquid scintillation spectrophotometry

Toluene, A.R. Grade	Koch Light, Colnbrook, Bucks.
Triton X-114	Rohm and Haas, Croydon, England.
p-BIS (O-methyl-steryl) benzene (BIS-MSB)	Eastman Kodak Ltd., Liverpool, Lancs.

10. Other chemicals

Adenosine diphosphate	Sigma (London) Chemical Co., Poole, Dorset.
Agarose (type II)	" " " "
Aquacide (type II)	Calbiochem. Ltd., Bishops Stortford, Herts.
3-amino-1,2,4,-triazole	Aldrich Chemical Co., Gillingham, Dorset.

L-ascorbic acid	Sigma (London) Chemical Co., Poole, Dorset.
Benzylamine hydrochloride	Hopkins and Williams, Chadwell Heath, Essex.
Chloramine-T	BDH Chemicals, Poole, Dorset.
Coomassie Brilliant Blue - G-250	Sigma (London) Chemical Co., Poole,Dorset.
2'-7'-Dichlorofluorescein diacetate	Eastman Kodak Ltd., Liverpool, Lancs.
Dimethyl sulphoxide (DMSO)	Sigma (London) Chemical Co., Poole, Dorset
2,4-dinitrophenol	" " " "
EGTA	" " " "
Folin Ciocalteu's phenol reagent	BDH Chemicals, Poole, Dorset.
Freund's complete adjuvant	Difco Laboratories, West Molesey, Surrey.
" incomplete adjuvant	" " "
Lanthanum nitrate	BDH Chemicals, Poole, Dorset
2-mercaptoethanol	" " " "
Morpholinopropane sulphonic acid (MOPS)	Sigma (London) Chemical Co., Poole, Dorset.

β-nicotinamide adenine dinucleotide (from yeast)	Sigma (London) Chemical Co., Poole, Dorset.
5'-nucleotidase kit (colourimetric method)	BDH Chemicals, Poole, Dorset
Orcinol	" " " "
Phenylmethyl sulfonyl-fluoridine (PMSF)	Calbiochem Ltd., Bishops Stortford, Herts.
Rotenone	Sigma (London) Chemical Co., Poole, Dorset.
Trichloroacetic acid (TCA)	Koch-Light Colnbrook, Bucks.

All other chemicals were purchased from BDH or Sigma and were 'Analar' grade.

M 3 Composition of standard solutions

M 3.1 Isolation and subfractionation of mitochondria

1. Isolation Medium

0.225M-mannitol, 0.150M-sucrose, 400µM-EGTA, pH 7.2, 2mM-MOPS, pH 7.2.

2. Swelling of mitochondria

10mM-Tris-phosphate buffer, pH 7.5.

3. Shrinking of mitochondria

1.8M-sucrose containing 2mM-ATP, 2mM-$MgSO_4$.

M 3.2 Solutions for enzymic assays

1. Monoamine oxidase

Method (a)

0.05M-phosphate buffer, pH 7.6, 2.50mM-benzylamine hydrochloride.

Method (b)

0.60ml, 0.25mM-leuco 2'-7'-Dichlorofluorescein diacetate in 0.01M NaOH, 1.80ml, 0.83mg/10ml horse radish peroxidase in 0.1M-Na phosphate buffer, pH 7.15, 0.15ml H_2O, 0.05ml, 0.1M-benzylamine.

2. Malate dehydrogenase

0.3ml, 0.25M-glycylglycine buffer, pH 7.4, 0.1ml, 7.6mM-oxaloacetate, pH 7.4 (freshly prepared), 0.1ml, 1.5mM-NADH, 2.5ml H_2O.

3. Adenylate kinase

50mM-Tris-HCl buffer, pH 7.5, 5mM-ADP,
10mM-glucose, 10 units hexokinase*
10 units glucose-6-phosphate dehydrogenase**
5mM-$MgSO_4$, 200μM-NADP.

* One unit will catalyse the phosphorylation of 1.0μmol of glucose/min., pH 8.5, 25°C.

** One unit will oxidise 1.0μmol of glucose-6-phosphate to 6-phosphogluconate/min., pH 7.4, 25°C in the presence of NADP.

4. Cytochrome oxidase

Method (a)

60mM-KCl, 250μM-sucrose, 10mM-Tris-HCl, pH 7.4, 0.75μM-Na ascorbate, 10μM-2,4-dinitrophenol, 100μM-cytochrome c, 0.3% (w/v) DOC (final concentration) where applicable.

Method (b)

3ml, 17μM-cytochrome c in0.03M-phosphate buffer, pH 7.4, reduced by addition of 100μl of 1.2M-$Na_2S_2O_4$, freshly prepared.

5. Acid phosphatase

0.05M-β-glycerophosphate, 0.05M-sodium acetate, pH 5.0, 1.00mM-NaF.

6. Glucose-6-phosphatase

0.04M-glucose-6-phosphate, 7.0mM-histidine, 1.0mM-EDTA, pH 6.5.

7. 5'-Nucleotidase

Reagents are obtained from aBDH kit and solutions made up as per instructions.

M 3.3. Solutions for chemical assays

1. Inorganic phosphate

5M-sulphuric acid, 50:50 isobutanol/benzene, 3.2% (w/v) concentrated H_2SO_4 in ethanol, 10% (w/v) $SnCl_2$ in concentrated HCl, freshly prepared and diluted 20 times in 0.5M-H_2SO_4.

2. Uronic acid

Orcinol reagent :

155.0ml concentrated HCl, 10.0ml, 1.5% (w/v) Ferric chloride in 0.01M-HCl.

M 3.4. Solutions for polyacrylamide gel electrophoresis of proteins

M 3.4.1 Slab gels 10% (w/v)

1. Solution A

30.0% (w/v) acrylamide, 0.8% (w/v) NN'-methylenebisacrylamide.

2. Solution B

0.75M-Tris-HCl, pH 8.8, 0.50% (w/v) SDS 0.05% (v/v) TEMED.

3. Solution C

0.17M-Tris-HCl, 0.137% (w/v) SDS, 0.034% (w/v) TEMED.

4. Running gel solution

8ml solution A, 12ml solution B, 4ml, 0.15% (w/v) ammonium persulphate.

5. Stacking gel solution

0.9ml solution A, 4.1ml solution B, 1.0ml, 5.0% (w/v) ammonium persulphate.

6. Electrode buffer

0.025M-Tris, 0.192M-glycine, 0.40% (v/v) SDS, pH 8.8.

M 3.4.2 Cylindrical gels (5.6% w/v)

1. 10x buffer

40ml 1.0M-Tris, 10ml 2.0M-Na acetate, 10ml 0.2M-EDTA, glacial acetic acid to pH 7.4.

2. Con Ac Bis.

40.0% (v/v) acrylamide, 1.5% (v/v) NN'-methylene-bisacrylamide.

3. Gel mixture

5.6ml Con Ac Bis, 4.0ml 10x buffer, 2.0ml 20% (v/v) SDS, 28.4ml H_2O, 4.0ml 1.5% (w/v) ammonium persulphate, 2.0ml 0.5% (v/v) TEMED.

4. Electrophoresis buffer

1% (v/v) SDS in 10x buffer.

5. Layering buffer

5% (v/v) ethanol, 1% (v/v) SDS.

M 3.4.3 Isoelectric focusing gels

1. Acrylamide stock

28.38% (w/v) acrylamide, 1.62% (w/v) NN'-methylenebisacrylamide.

2. Nonidet P-40

10% (v/v) in H_2O.

3. Riboflavin

0.14mg/ml riboflavin, 1% (v/v) TEMED.

4. IEF gel mixture

2.75g urea, 0.67ml acrylamide stock, 1.00ml 10% (v/v) Nonidet P-40, 0.99ml H_2O, 0.20ml riboflavin, 0.10ml ampholine (pH 3.5-10.0 unless otherwise stated).

Electrophoresis buffers

5. Cathode electrode solution

0.02M-NaOH, degassed

6. Anode electrode solution

0.01M-H_3PO_4, degassed

7. Layering buffer

8M-urea

8. Sample dilution buffer

9.5M-urea, 2% (v/v) ampholine, pH 3.5-10.0, 8% (v/v) Nonidet P-40.

9. SDS-sample buffer

5.0% (v/v) 2-mercaptoethanol, 10.0% (v/v) glycerol, 2.3% (w/v) SDS, 0.0625M-Tris-HCl, pH 6.8.

M 3.5 Staining solutions

M 3.5.1 SDS gels

1. Overnight stain

0.04% (w/v) Coomassie Brilliant Blue, G-250, 25.0 (v/v) isopropanol, 10.0% (v/v) glacial acetic acid.

2. Second stain (6-9h)

0.0025% (w/v) Coomassie Brilliant Blue, G-250, 10.0% (v/v) isopropanol, 10.0% (v/v) glacial acetic acid.

3. Destainer

10% (v/v) glacial acetic acid.

4. Fixing of radioactive gels

25% (v/v) isopropanol, 10% (v/v) glacial acetic acid.

M 3.5.2 Ouchterlony plates

1. 15 min stain

45.0% (v/v) ethanol, 45.0% (v/v) H_2O, 10.0% (v/v) glacial acetic acid, 0.1% (w/v) Coomassie Brilliant Blue, G-250.

2. Destainer

30% (v/v) ethanol, 65% (v/v) H_2O, 5% (v/v) glacial acetic acid.

M 3.5.3 IEF gels

1. Fixer

10% (w/v) TCA, followed by several changes of distilled H_2O until the opaque gel goes transparent.

2. 30 min stain at 60°C

100mg Coomassie Brilliant Blue G-250 in 200ml H_2O, 15g urea, 7.5ml, 70% (v/v) perchloric acid, added with vigorous mechanical stirring.

3. Destainer

10% (v/v) ethyl acetate, 78% (v/v) H_2O,
5% (v/v) glacial acetic acid, 7% (v/v) ethanol.

M 3.6 Solutions for Iodination

M 3.6.1 Iodination of mitochondria and mitoplasts

1. Iodination buffer

10.0mM-Tris, 0.225M-mannitol, 0.15M-sucrose,
2.0mM-MOPS.

M 3.6.2 Iodination of lectins

1. 0.05M sodium phosphate buffer, pH 7.2 (M 3.12.1), 10mg/ml chloramine-T in sodium phosphate buffer, pH 7.2, 10mg/ml sodium metabisulphite in sodium phosphate buffer, pH 7.2., 200µCi ^{125}I -NaI.

M 3.7 Solutions for Immunological Experiments

M 3.7.1 Immunoprecipitation

1. TKM buffer, pH 8.2

100mM-KCl, 100mM-Tris-HCl, pH 8.2, 5mM-$MgCl_2$,
10% (v/v) DOC when detergent is included.

2. Phenylmethylsulfonyl chloride (PMSF)

100mM PMSF in 0.5ml DMSO.

3. 2-Detergent TKM

1ml 10% (v/v) Nonidet P-40, 1ml 10% (v/v) Triton X-100, 1ml 10% (v/v) TKM buffer, pH 8.2, 7ml H_2O.

4. Con A Immunoprecipitate washing buffer

TKM buffer, pH 8.2, 0.1mM-KI, 0.1% (v/v) Nonidet P-40, 0.1% (v/v) Triton X-100, 1mg/ml BSA.

5. WGA Immunoprecipitate washing buffer

Phosphate buffered saline, pH 7.2 (M 3.12.1), 0.1mM-KI, 0.1% (v/v) DOC , 1mg/ml BSA.

M 3.7.2 Ouchterlony Plates

1. 20x concentrated borate buffer

0.1M-boric acid, 25.0mM-sodium tetraborate, 75.0mM-NaCl.

2. Borate-saline

0.85% (w/v) NaCl in borate buffer.

3. Borate-saline mixture

1% (w/v) agarose in borate-saline

M 3.8 Solutions for functional studies on mitochondria

1. MS-MOPS-Pi

0.20M-mannitol, 0.05M-sucrose, 20.0mM-MOPS, 10mM-NaH_2PO_4, pH 7.2.

M 3.9 Scintillation solutions

1. Triton-Toluene

64.45% (v/v) toluene, 35.00% (v/v) Triton X-100, 0.05% (w/v) BIS-MSB, 0.5% (w/v) PPO.

M 3.10 Purification of lectins

M 3.10.1 Affinity chromatography of WGA on chitin

1. Column washing buffer

10mM-Tris-HCl, pH 8.5.

2. Column eluting buffer

0.05M-HCl.

M 3.10.2 Affinity chromatography of SBA on Sepharose-N-caproylgalactosamine

1. Column washing buffer (PBS)

0.12M-NaCl, 0.01M-Na_2HPO_4, 3.20mM, KH_2PO_4. final pH 7.2.

2. Column eluting buffer

0.1M-galactose in PBS.

M 3.11 Isolation of mitochondrial glycoproteins

1. Column washing buffer

20mM-MOPS, pH 8.2, 0.15M-KCl, 1mM-$MnCl_2$, 1mM-$CaCl_2$, 0.4% (w/v) NaN_3, 1.0% (w/v) Nonidet P-40, 1.0% (w/v) Triton X-100.

2. Column eluting buffer

20mM-MOPS, pH 8.2, 0.15M-KCl, 1.0% (w/v) Nonidet P-40, 1.0% (w/v) Triton X-100, 0.2M α-Methyl-D-glucoside.

M 3.12 Buffers

1. Sodium phosphate buffer, pH 7.2

0.52M-NaH_2PO_4, 2.00mM-Na_2HPO_4.

2. Phosphate buffered saline, pH 7.2 (PBS)

0.15M-NaCl, 0.01M-NaH_2PO_4, 0.04mM-Na_2HPO_4.

3. High salt buffer

0.12M-KCl, 20mM-MOPS, pH 7.2.

METHODS

M 4 Preparation of Mitochondrial fraction

M 4.1 Isolation of Mitochondria (M_1)

Mitochondria are prepared by a modification of the method of Hogeboom et al., (1948). Female rats of the Wistar strain (180 - 200g) are starved overnight to eliminate glycogen storage particles. Animals are killed by stunning on the head and decapitation. The livers are excised and rinsed twice in ice-cold isolation medium (M3.1.1), before chopping finely with scissors. The livers are homogenised with a tight fitting Teflon pestle (setting 5, clearance 0.004-0.006) in a Potter-Elvenhjem homogeniser for 5 passes to cause cell disruption. The suspension is then centrifuged at 800g in an MSE-18 Centrifuge (8 x 50ml rotor) for 7 min to sediment the nuclei and cell debris. The supernatant fraction is retained and re-centrifuged at 6,500g for 15 min. The precipitate is resuspended in isolation medium and centrifuged again at 6,500g for 7 min to exclude contaminating microsomes. Broken mitochondria are discarded and the precipitate resuspended in isolation medium and centrifuged at 800g for 7 min to remove any residual blood cells. Finally, the supernatant is centrifuged at 6,500g for 7 min to pellet the mitochondrial fraction. The isolated sample is resuspended in 5-6ml of

isolation medium to a concentration of approx. 20mg/ protein/ml.

Labelling of mitochondrial glycoproteins *in vivo* is achieved by injecting the rat with 200µCi of D-[6^3-H]-glucosamine 16 h prior to sacrifice. Mitochondria are then isolated in the usual manner.

M 4.2 Purification of Mitochondria (PM_1)

Isolated rat liver mitochondria are purified on a discontinuous sucrose density gradient made up as follows :

2.0ml 54% (w/v) sucrose

6.5ml 45% (w/v) sucrose

6.5ml 39% (w/v) sucrose

5.5ml 20% (w/v) sucrose

The gradient is centrifuged at 49,000g for 2 h at 4°C in the 3 x 20ml rotor of an MSE-50 superspeed ultracentrifuge.

After centrifugation 3 distinct bands are visible. The upper band of density 1.16g/cm^3 represents principally microsomes and plasma membrane. The middle band of density 1.19g/cm^3 is purified mitochondria. The third band of density 1.22g/cm^3 represents lysosomes. The mitochondria are harvested from the density gradient, diluted slowly in isolation medium and centrifuged at 6,500g for 10 min to remove the sucrose and pellet the mitochondria.

An improved separation of mitochondria from

contaminating organelles particularly lysosomes is effected by injecting the rat with the detergent Triton-WR-1339 (1mg/g of body wt), 3 days prior to sacrifice. The detergent is reported to make lysosomes light by accumulating in the organelles (Wattiaux et al., 1963), which subsequently band at a density of 1.16g/cm^3.

M 4.3 Subfractionation of mitochondria

M 4.3.1 Separation of Inner, Outer and Soluble fractions of rat liver mitochondria

Mitochondria containing approx. 30mg protein are resuspended in 7.5ml of Tris-phosphate buffer, pH 7.5 (M 3.1.2) by means of a Teflon pestle and homogeniser. After standing at 4°C for 5 min, during which time the mitochondria undergo swelling, 2.5ml of the shrinking solution (M 3.1.3) is added. A visible increase in turbidity appears owing to energisation of the inner membrane in the presence of ATP and subsequent contraction of the mitochondria. After another 5 min at 4°C the suspension is sonicated for 2 x 20 s at 4°C. The sonicated suspension consisting of 10ml of the mitochondrial sample in 0.45M-sucrose is layered onto a 3 layer sucrose discontinuous gradient of :

6ml 1.32M-sucrose

6ml 1.14M-sucrose

5ml 0.76M-sucrose

The gradient is centrifuged in the 3 x 20ml head of the MSE-50 superspeed ultracentrifuge at 64,000g for 3 h at 4°C. This method is a slight modification of that described by Sottocasa et al., (1967).

After centrifugation 4 subfractions could be distinguished. A tightly packed reddish brown pellet at the bottom of the tube (inner membrane). The two bands at the interfaces of sucrose layers between 1.32M and 1.14M and 1.14M and 0.76M represent a mixture of inner and outer membrane and outer membrane respectively. A clear yellow supernate representing the soluble fraction is present in the 0.45M-sucrose layer. The membranes are harvested from the gradient using a syringe and a J-shaped meedle. Inner and outer membranes are washed twice in 100mM-Na phosphate buffer, pH 8.0 (100,000g at 4°C for 40 min in the 10 x 10ml rotor of MSE-50 superspeed) to remove any contaminating matrix proteins.

M 4.3.2 Treatment of mitochondria with digitonin

Mitoplasts are prepared by stripping off the outer mitochondrial membrane using the detergent digitonin as described by Schnaitman et al., (1967). In all cases, purified digitonin is used. This is prepared by recrystallising the commercial digitonin from hot absolute ethanol.

Isolated mitochondria (M 4.1) are treated as

described (M 4.3.1) up to the shrinking stage. The mitochondria are pelleted by centrifugation at 10,000g resuspended in high salt buffer (M 3.12.3) to a known protein concentration and incubated with digitonin on ice for 15 min. The reaction is stopped by addition of 3 vol. of the high salt buffer and the mitochondria re-pelleted. The ratio of digitonin to protein varied from 0.01 (low digitonin) to 0.22 (high digitonin) according to the experimental requirement.

M 5 Enzyme assays

M 5.1 Monoamine oxidase activity is estimated by 2 methods

(a) By the method of Tabor et al., (1954) as modified by Schnaitman et al., (1967). Benzyaldehyde formation is followed at A_{250} using 1ml of benzylamine hydrochloride and 2ml phosphate buffer, pH 7.6 (M 3.2.1a). The reaction is started by the addition of 10µl of the mitochondrial sample.

(b) Using the spectrophotometric assay of Kochli and von Wartburg (1978). The method is based on a coupled indicator reaction measuring the monoamine oxidase-dependent production of hydrogen peroxide. In this reaction the hydrogen peroxide-dependent oxidation of leuco-2',7'-dichlorofluorescein to 2',7'-dichlorofluorescein catalysed by horse radish peroxidase is followed at A_{502} and 25°C using benzylamine as substrate. (M 3.2.1b).

M 5.2 Cytochrome c oxidase activity is measured by 2 methods

(a) According to the method of Cooperstein and Lazarow (1951), cytochrome c is reduced by addition of sodium dithionite (M 3.2.4b). To 3.0ml of the reduced cytochrome c in a cuvette, is added 10-20µl of mitochondrial sample. The reaction is monitored at A_{550}.

(b) The other method for assessing cytochrome c oxidase is according to Wojtczak et al., (1972). Into a cuvette of the oxygen electrode is put 3ml of assay buffer (M 3.2.4a). Respiratory inhibitors, ascorbate and cytochrome c are added. The reaction is started by addition of mitochondrial protein (20-50µl). Oxygen uptake is monitored in the mitochondrial samples ± DOC, at 25°C.

M 5.3 Adenylate kinase

Adenylate kinase is assayed spectrophotometrically at A_{340} and 30°C, by coupling the reaction to glucose-6-phosphate dehydrogenase. To 1ml of reaction mixture (M 3.2.3) is added 10µl of the mitochondrial sample.

M 5.4 Malate dehydrogenase

The reaction mixture contains 0.3ml of buffer, NADH, oxaloacetate and water to a final volume of 3.0ml (M 3.2.2). The reaction is started by

addition of 10µl of mitochondrial sample and the decrease in absorbance of the NADH monitored at A_{340} and 20°C.

M 5.5 Estimation of acid phosphatase activity

To 0.9ml of the reaction mixture (M 3.2.5) is added 50 µl-100 µl of the prepared mitochondrial sample. The reaction is incubated at 37°C for 10 min and stopped by addition of 1.0ml of 10% (w/v) TCA. The precipitate is centrifuged down at 800g in an MSE Major Centrifuge and the supernatant fraction assayed for inorganic phosphate (M 6.1).

M 5.6 Estimation of Glucose-6-phosphatase activity

To 1.0ml of the reaction mixture (M 3.2.6) is added 50µl-100µl of the prepared sample. The reaction is incubated at 37°C for 10 min and stopped by addition of 1.0ml of 10% (w/v) TCA. The precipitate is centrifuged down at 800g in an MSE Major Centrifuge and the supernatant fraction assayed for inorganic phosphate (M 6.1).

M 5.7 Estimation of 5'-nucleotidase activity

The assay involves the measurement of liberated ammonia which is proportional to the 5'-nucleotidase activity and is measured by the indophenol reaction. In the reaction, the adenosine liberated by 5'-nucleotidase from the substrate adenosine

5'-monophosphoric acid is quantitatively determined using the enzyme adenosine deaminase which catalyses the following reaction :

$$\text{Adenosine} + H_2O \longrightarrow \text{Inosine} + NH_3.$$

The assay is set up as per instructions in the BDH Kit (M 3.2.7).

M 6 Chemical assays

M 6.1 Estimation of inorganic phosphate

To 2.0ml of the supernatant fraction under test is added 1.0ml of distilled water, 0.5ml sulphuric acid, 5.0ml isobutanol/benzene mixture and 0.5ml ammonium molybdate (M 3.3.1). The reagents are vortexed for 2 x 15 s intervals. To a 1.0ml aliquot of the organic layer is added 4.0ml of the sulphuric acid in ethanol followed by 0.5ml of the diluted $SnCl_2$. The resulting blue colour is read within 10 min at A_{730}. A standard curve of phosphate 0 - 3.0µmol is constructed.

M 6.2 Determination of uronic acid

To a 1.0ml sample containing 0.02 - 0.15µmol of uronic acid is added 3.0ml of the orcinol reagent (M 3.3.2). The tubes are capped, and the solution heated for 40 min at 100°C. The resulting green colour is then measured at A_{660} after the solution has cooled.

M 6.3 Estimation of protein

The concentration of protein is estimated by the method of Lowry *et al.* (1951) using BSA (1.876mg/ml) as a standard.

M 6.4 Amino acid analysis

Amino acid analysis of $[^3H]$ -glucosamine labelled mitochondria is carried out in 4M-methane sulphonic acid at 100°C for 70 h in evacuated, sealed and previously acid-washed pyrex tubes.

The membrane sample, suspended in H_2O (0.5ml) is made up to a volume of 1.0ml with 8M-methane sulphonic acid (final concentration 4M in methane sulphonic acid). DL-Norleucine (50nmol) is added as an internal standard. After hydrolysis, 3.5M-NaOH (0.5ml) and 1M-NaOH (0.2ml) is added. This is followed by further small additions of alkali until the pH became 2 - 2.5.

The resulting solution is analysed in an amino acid analyser JLC-JAH (Japanese Electron Optics Laboratory Co. Ltd., Tokyo, Japan) using 10cm and 50cm columns of LCRI resin.

M 7.1 Iodination of mitochondria and mitoplasts : by lactoperoxidase catalysed iodination

Mitochondria (M_1) are isolated as per usual (M 4.1) except that the rat is given an injection of 3-amino-1,2,4-triazole (1mg/g of body wt.), 1 h prior

to sacrifice to reduce endogenous catalyse activity (Clarke, 1976). Surface iodination of mitochondria and mitoplasts are performed essentially according to Boxer (1975).

To no more than 0.5mg of protein resuspended in 0.5ml of iodination buffer (M 3.6.1.1) is added 100µl of 10mM-Tris-HCl, pH 7.8, 200µCi of [^{125}I]-NaI and 100µl of lactoperoxidase in iodination buffer (0.5mg/ml). The reaction is initiated by addition of 25µl aliquots of 2mM-H_2O_2. The additions are made a total of 8 times, at 2 min intervals. The iodinated material is washed 4 times in iodination buffer containing 2mM-KI.

M 7.2 Iodination of Lectins

Lectins are iodinated essentially by the method of Tanner and Anstee (1976).

(a) Con A

2mg of Con A are dissolved in 0.5ml of sodium phosphate buffer, pH 7.2 (M 3.12.1) containing 0.3M α-methyl-D-glucoside. The protein is iodinated with 200µCi of [^{125}I] -NaI and 200 g of chloramine-T. The reaction is terminated after 4 min by addition of 100 g Na metabisulphite, dissolved in sodium phosphate buffer, pH 7.2 (M 3.6.2). The radioactive lectin is exhaustively dialysed against sodium phosphate buffer, pH 7.2 containing 0.01M-KI at 4°C for 3 changes.

The labelled protein is repurified by application to a column (10cm x 1.5cm) of Sephadex G-50, equilibrated in sodium phosphate buffer and eluted with 0.2M α-methyl-D-glucoside in the same buffer.

(b) WGA

The lectin is labelled in the presence of 0.3M N-acetyl-D-galactosamine as described for Con A (M 7.2a), though the protein is not repurified after labelling.

M 7.3 Iodination of mitochondrial proteins using the Bolton Hunter reagent

Mitochondria (M_1) are isolated as in (M 4.1) and resuspended in 0.1M-borate buffer, pH 7.4. 200µl of the iodinating reagent is dispensed into a vial. The benzene is evaporated off by passage of a stream of dry nitrogen. To this is added 200µg of mitochondrial protein in 20µl of the borate buffer and incubated by agitation on ice for 10 mins. The mitochondria are washed four times in the same buffer containing 0.01M-KI.

M 8 SDS-polyacrylamide gel electrophoresis

M 8.1 (5.6% w/v) cylindrical gels

Electrophoresis of samples is performed as described by Fairbanks et al. (1971). Gels are set up in cylindrical tubes of dimension 12.0 x 0.6cm.

The tubes are sealed by parafilm and the gel mixture (M 3.4.2.3) is poured in and layered with buffer (M 3.4.2.5). The polymerised gel is made to be 10cm long. Gels are slotted into a cylindrical gel apparatus, which could hold a maximum of 12 gels. The apparatus is filled up with electrophoresis buffer (M 3.4.2.4) and the gels electrophoresed at 7mA per gel.

Electrophoresis is stopped when the tracking dye was about 1cm from the end of the gel. The position of the tracking dye is marked in each gel by pricking it with a needle dipped in indian ink.

M 8.2 (10% w/v) slab gels

SDS-polyacrylamide slab gels are run using a discontinuous buffer system as described by Laemmli (1970). The gel apparatus consists of 2 glass plates of dimensions 14 x 8cm, separated by a perspex spacer of 0.15cm thickness. The glass plates are fixed into place with insulating tape and sealed by immersing in paraffin wax.

The main running gel (M 3.4.1.4) is poured and layered with buffer (M 3.4.2.5). After polymerisation, which took about 1 h the buffer is decanted and the top surface of the gel of final length 12cm rinsed twice with distilled water. The stacking gel (M 3.4.1.5) is then poured. Sample wells are made by inserting a teflon comb into the gel solution.

Polymerisation is complete in 10 min leaving a stacking gel 0.5cm in height. The prepared slab gels are gently eased into the slots of the Pharmacia slab gel apparatus. The apparatus is filled up with electrode buffer (M 3.4.1.6) and electrophoresis carried out at room temperature at a constant current of 20mA per gel.

M 8.3 2-Dimensional gels

A combination of the methods of O'Farrell (1975) and Ames and Nikaido (1976) is incorporated into this technique.

M 8.3.1 Isoelectric focusing

The IEF gel reagents are dissolved and the gel mixture (M 3.4.3.4) degassed before mixing in the solubilised membrane sample (200µg protein/gel). The mixture is pipetted into cylindrical gel tubes of dimension 10.0 x 0.25cm. Gels are layered with 8M-urea and fluorescent light used to catalyse the polymerisation. Gels are electrophorsed in the cylindrical gel apparatus which contained anode electrode solution in the upper chamber (M 3.4.3.6) and the cathode electrode solution in the lower chamber (M 3.4.3.5). Bands are focused at 300v for 18 h and at 400v for a further 1 h to sharpen the bands. IEF gels are treated in one of 2 ways :

(1) Gels are stained with Coomassie Brilliant Blue

G-250 according to Vesterburg et al. (1977) (M 3.5.3), subsequent to the proteins being fixed overnight.

(2) Gels are equilibrated for 2h in SDS sample buffer (M 3.4.3.9) in preparation for electrophoresis in the 2nd dimension.

M 8.3.2 SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide slab gels of dimension 8 x 8cm are made up as described in (M 8.2). The gel mixture is filled to 1cm from the top edge so as to exclude a stacker gel. The equilibrated IEF gel is joined to the slab gel by a 1% agarose solution in SDS-sample buffer (M 3.4.3.9). After electrophoresis, protein spots are visualised by Coomassie blue staining (M 3.5.1).

M 8.3.3 Measurement of pH gradient on IEF gels

In order to measure pH, the freshly extruded IEF gels are rinsed twice in distilled H_2O so as to remove any traces of electrophoresis buffer. Millivolt measurements at 0.5cm intervals along the gel are made by direct contact with an antimony micro-electrode and a calomel reference electrode (Beeley et al., 1972). The antimony electrode is previously calibrated using standard buffer solution of known pH.

M 8.4 Solubilisation of samples

M 8.4.1 SDS gels

Proteins are denatured by solubilisation in 2% (w/v) SDS, and 2-mercaptoethanol 2% (v/v) final concentration. Samples are boiled for 3 min prior to addition of sucrose to give a final concentration of 20% (v/v). Pyronin y marks the leading edge of the gel. No more than 20-30µl of sample is applied to each well.

M 8.4.2 IEF gels

To 3mg of mitochondrial membrane protein is added 20µl, 0.5M-Tris-HCl, pH 6.8, 20µl, 20% (w/v) SDS, 1µl, 0.1M-$MgCl_2$ and water to 200µl. The tube is capped with parafilm and incubated at 70°C for 30 min. The solubilised protein is diluted with 2 vol. sample dilution buffer (M 3.4.3.8).

M 8.5 Processing of gels

M 8.5.1 Slicing and counting of cylindrical gels

Gels on which radioactive samples are electrophoresed are fixed in acetic acid (M 3.5.1.4). The gels are then frozen in a solid CO_2/methanol mixture and sliced into 1mm thick slices using the Mickle gel slicer (Mickle Laboratory Engineering Co., Gernshall, Surrey). To count the [^{125}I] isotope, slices are put into disposable Durham tubes which are then inserted into Beckmann bio-vials. The

isotope is counted in the Beckmann Biogamma T.M. counter (Beckmann RIIC Ltd., Glenrothes, Fife).

M 8.5.2 Fluorography (Slab gels)

Fluorographs are prepared as described by Bonner and Laskey (1974). Gels are immersed in 3 successive baths of DMSO for a total of 80 min with shaking. The gel is then incubated by immersion in a 20% (w/v) PPO in DMSO for 40 min. The PPO is precipitated in the gel by washing extensively in 2-3 changes of distilled water. The gel is dried under vacuum onto Whatman 3mM paper.

A fluorograph is obtained by placing the dried gel in contact with either Kodak X-omat RH royal film or Fuji RX X-ray film in between glass plates and exposed at -70°C for the appropriate time. In general iodinated samples are exposed for 1-5 days, whereas immune-precipitates are exposed for 7-14 days.

M 8.5.3 Autoradiography (Slab gels)

After electrophoresis the gels are stained overnight with Coomassie Brilliant Blue G-250 (M 3.5.1) to visualise the polypeptides before drying under vacuum onto Whatman 3mM filter paper.

Radioactive polypeptides are visualised by exposing the dried gel to Kodirex film in a light tight box at 20°C for the appropriate time.

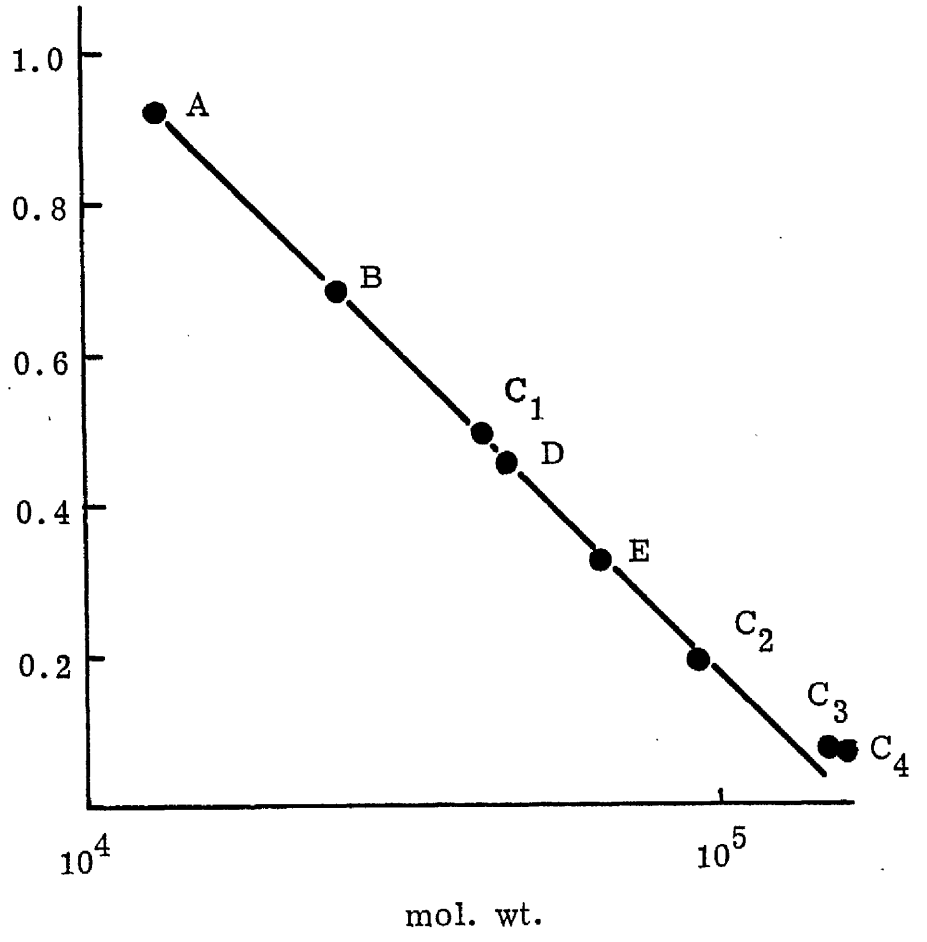


Fig. 2.1

Calibration curve of polypeptide mol. wt. versus electrophoretic mobility on a 10% (w/v) SDS-polyacrylamide slab gel.

Mol. wt. standard markers are denatured in SDS (M 8.4.1) and electrophoresed on a 10% (w/v) slab gel as described in Methods (M 8.2). The gel is stained with Coomassie Brilliant Blue (M 3.5.1) and dried onto 3mM Whatman filter paper. The migration distances from the top of the main gel are measured and plotted against mol. wt. on a logarithmic scale.

Proteins used are (20µg) :
A cytochrome c (12,700) ; B chymotrypsinogen A (24,500) ; C_{1-4} RNA polymerase; 42,000, α, β, β_1 ; D ovalbumin ; E BSA (65,000).

M 8.5.4 Developing of Exposed films

Exposed fluorographs or autoradiographs are developed in Kodak DX-80 developer for 5 min. Films are fixed in Kodak FX-40 liquid fixer for a further 5 min before washing in distilled H_2O and drying.

M 8.5.5 Determination of mol. wt.

Mol. wt. of the polypeptides are estimated by simultaneous electrophoresis of protein standards.

These included RNA polymerase (165,000, 155,000, 90,000. 42,000), β-galactosidase (130,000) BSA (65,000) ovalbumin (45,000) chymotrypsinogen A (24,500) and cytochrome c (12,700). Gels are stained with Coomassie Brilliant Blue G-250 as described in (M 3.5.1). A graph of R_f values versus mol. wt. is plotted on a semi-logarithmic paper. Mol. wts. are estimated by reading off the calibration curve (See Fig. 2.1).

M 8.5.6 Liquid scintillation spectrophometry

The amount of soluble radioactivity present in samples is determined by taking 0.5ml aliquot of the sample and mixing with 4.5ml of the Triton-toluene scilliant (M 3.9). Samples are counted in the Beckmann LS 8100 liquid scintillation counter (Beckmann RIIC Ltd., Glenrothes, Fife).

M 9 Immunological techniques

M 9.1 Preparation of antisera

Antisera is prepared in rabbits by injecting 0.5mg antigen in 0.90% saline (1ml) mixed with an equal volume of Freund's complete adjuvant at multiple sub-cutaneous sites. The immune response is boosted by two further injections of the antigen mixed in Freund's incomplete adjuvant at a 30 day and 10 day interval after the initial injection. Antisera is prepared against the following antigens :

(a) Con A

(b) WGA

(c) total mitochondrial glycoprotein complement as prepared in (M10.3.1).

Rabbits are bled from the ear vein and the blood allowed to clot. The serum is collected and stored in 5ml aliquots at -20°C.

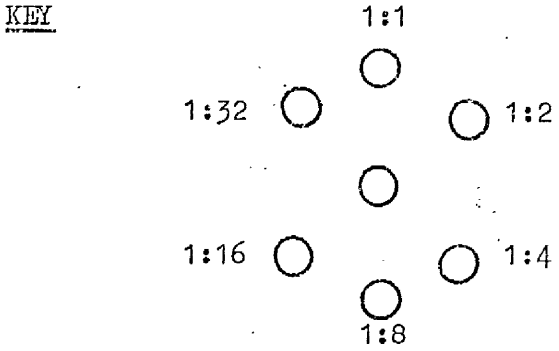
M 9.2 Antibody titre of antiserum raised against lectins, Ouchterlony plates

The antibody titre of antiserum raised by inoculation of rabbits with purified lectins : Con A and WGA is examined by a modification of the method of Ouchterlony (1964).

Into sterile petri dishes is poured 8ml of borate-saline mixture (M 3.7.2.3). The agar is allowed to gel, holes punched into the gel, and

Fig. 2.2

Immunodiffusion patterns of anti-lectin sera against lectins by double diffusion precipitation



The antibody titre of anti-lectin serum raised against purified lectins is examined by the method of Ouchterlony (1964) as described in Methods (M 9.2). Lectins of fixed concentration is applied to the centre well. Anti-lectin serum in a two-fold serial dilution is applied to the side wells.

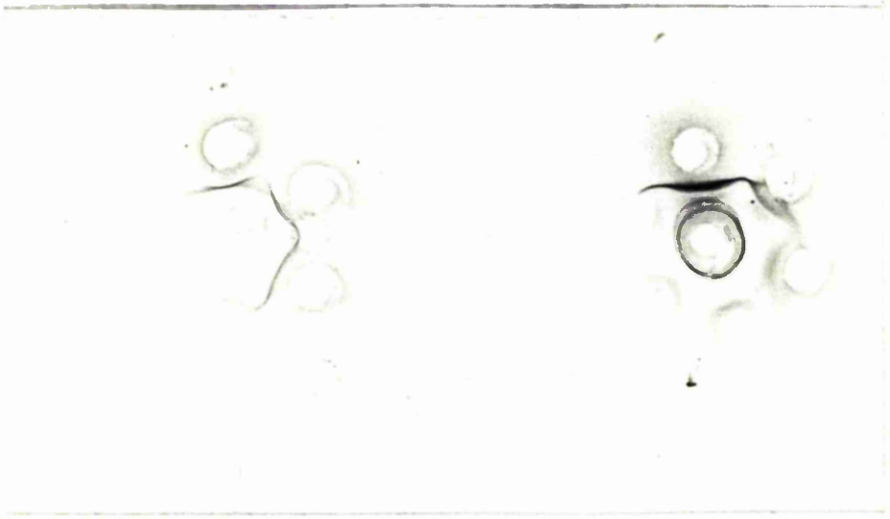
(a) Con A, 0.1mg/ml; anti-Con A, 1:1-1:32 serial dilution

(b) Con A, 1.0 mg/ml; anti-Con A, " " " "

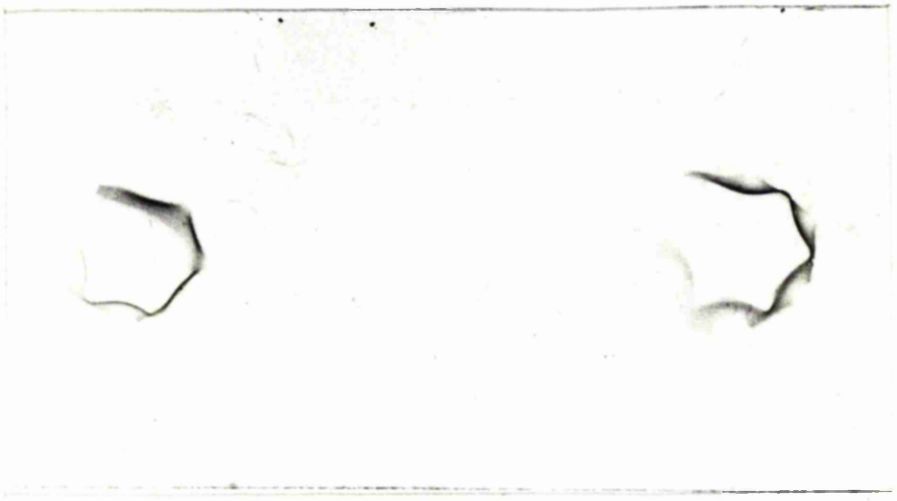
(c) WGA, 0.1mg/ml ; anti-WGA " " " "

(d) WGA, 1.0mg/ml ; anti-WGA " " " "

Immunoprecipitation lines are visualised by staining the agar gels for protein as described in (M 3.5.2). A saline blank included as a control showed no lines.



(a) (b)



(c) (d)

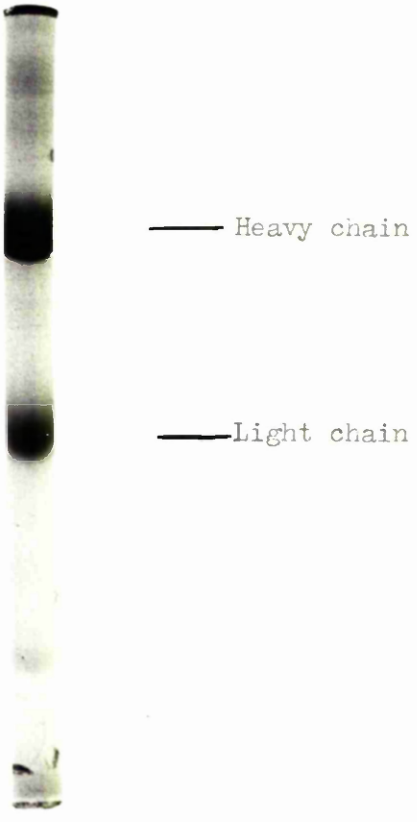


Fig. 2.3

Purification of IgG from anti-WGA serum.

Anti-WGA is prepared by injecting a rabbit with purified lectin and subsequently collecting the serum as described in (M 9.1).

Anti-WGA-IgG is purified by equilibrating the serum in 10mM-phosphate buffer, pH 7.0 and purification on a DEAE-cellulose (DE-52) column (M 9.3).

the agar sucked out with a pasteur pipette attached to vacuum. Into each well is put 5µl of sample. The experiment is incubated at 4°C. Excess protein is washed from the plates with several changes of PBS pH 7.2 (M 3.12.2) and left overnight in PBS. The gel is stained and destained as described in (M 3.5.2) and dried at 25°C, with moist filter paper over it to prevent cracking, prior to photographing.

Immunisation with relatively pure lectins gave rise to potent specific antisera as evidence by sharp, single immunoprecipitation lines and lack of cross reactivity, (see Fig 2.2). Immunoprecipitates are observed between Con A and anti Con A serum diluted up to 1:8 and between WGA and anti WGA diluted up to 1:16.

M 9.3 Preparation of IgG

IgG is purified from the antiserum pre-equilibrated in 10mM-Na phosphate buffer, pH 7.0. The IgG is separated on a column of DEAE-cellulose (DE-52) and eluted with 10mM-phosphate buffer, pH 7.0. Aliquots of the eluant are collected and monitored at A_{280} for protein absorbance. The relevant fractions are combined and concentrated with Aquacide. The purified protein is photographed in Fig. 2.3.

M 9.4 Titration of lectin with anti-lectin serum

The volume of anti-lectin serum required to cause maximum precipitation of a fixed amount of lectin is assessed by incubating increasing quantities of anti-lectin serum with 10µg of lectin at 37°C, and then overnight at 4°C. The immune-complexes are centrifuged at 1000g for 10 min, washed once in PBS; the pellets are dissolved in 0.1ml of 0.2M-NaOH, and the protein estimated by the method of Lowry et al. (1951)(M 6.3). Anti-Con A serum gave an equivalence zone at approximately 210µl, while anti-WGA serum gave an equivalence zone at about 100µl, (see Fig 2.4). The quoted antisera volumes are thereafter used in all the immunoprecipitation experiments.

M 10. Purification of lectins

M 10. 1 Purification of WGA using affinity chromatography on chitin

The procedure of Bloch and Burger (1974) is essentially followed. The agglutinin is purified on a chitin column pre-equilibrated in column washing buffer (M 3.10.1) and finally eluted with 0.05M-HCl. The purified protein is photographed in Fig 2.5a.

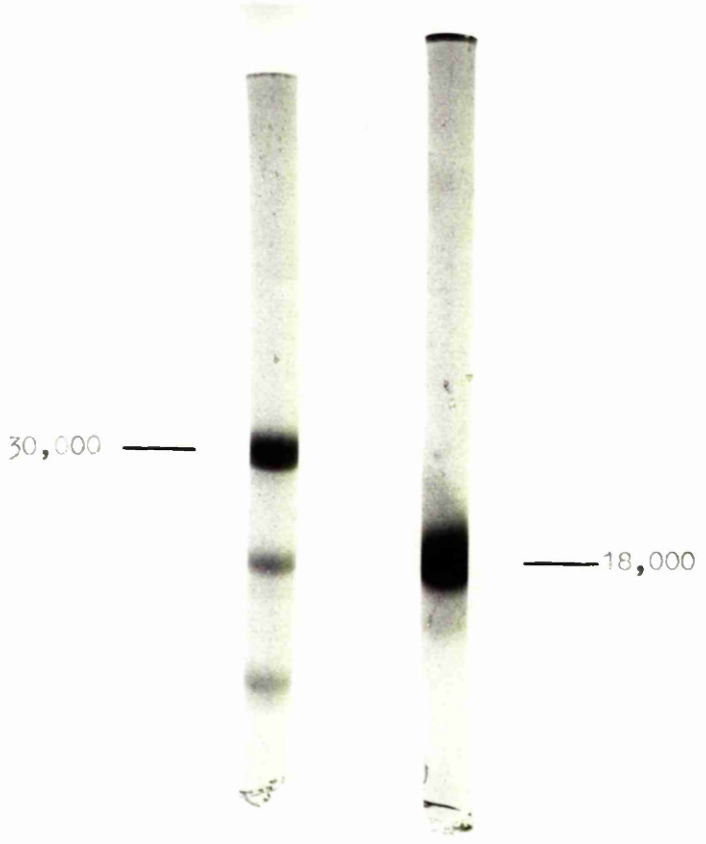


Fig. 2.5.

Purification of WGA and SBA.

(a) SBA is purified on Sepharose-N-caproylgalactosamine as described in (M 10.2).

(b) WGA is purified on chitin columns as described in (M 10.1).

The purified proteins (50µg) are electrophoresed on 5.6% (w/v) gels and proteins visualised by Coomassie Blue staining (M 3.5.1).

Fig. 2.4.

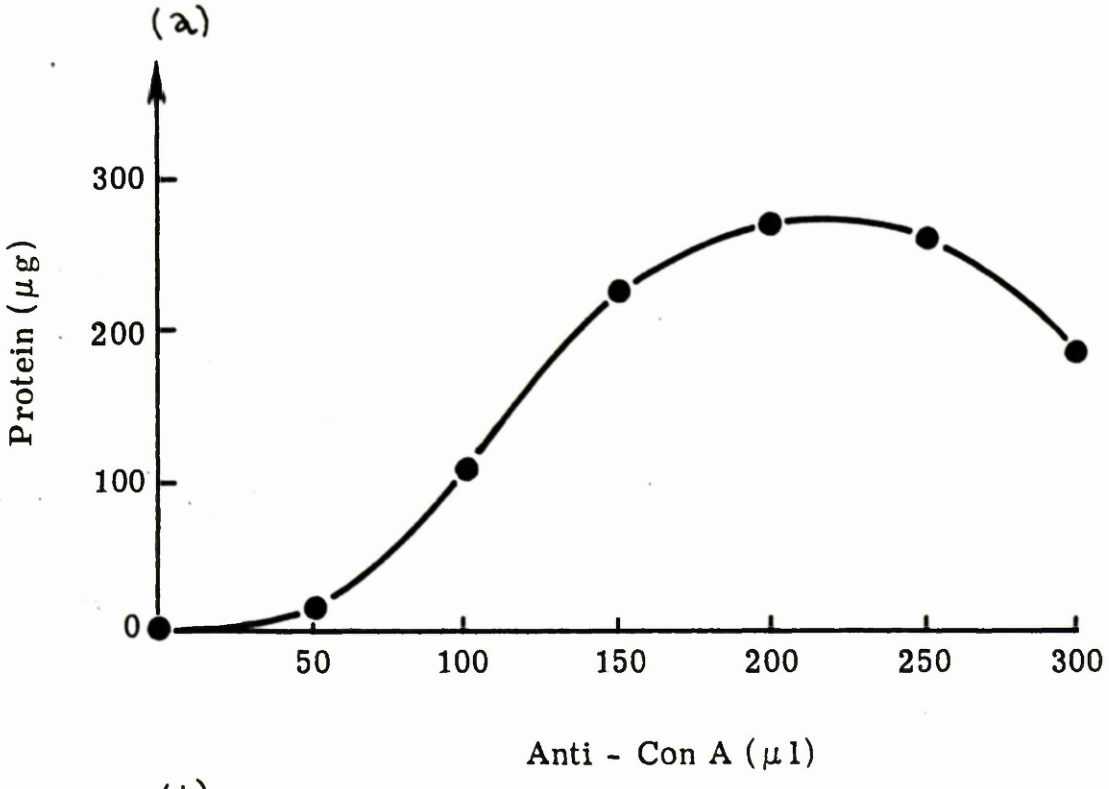
Lectin immunoprecipitation curves.
Titration of lectin with anti-lectin serum.

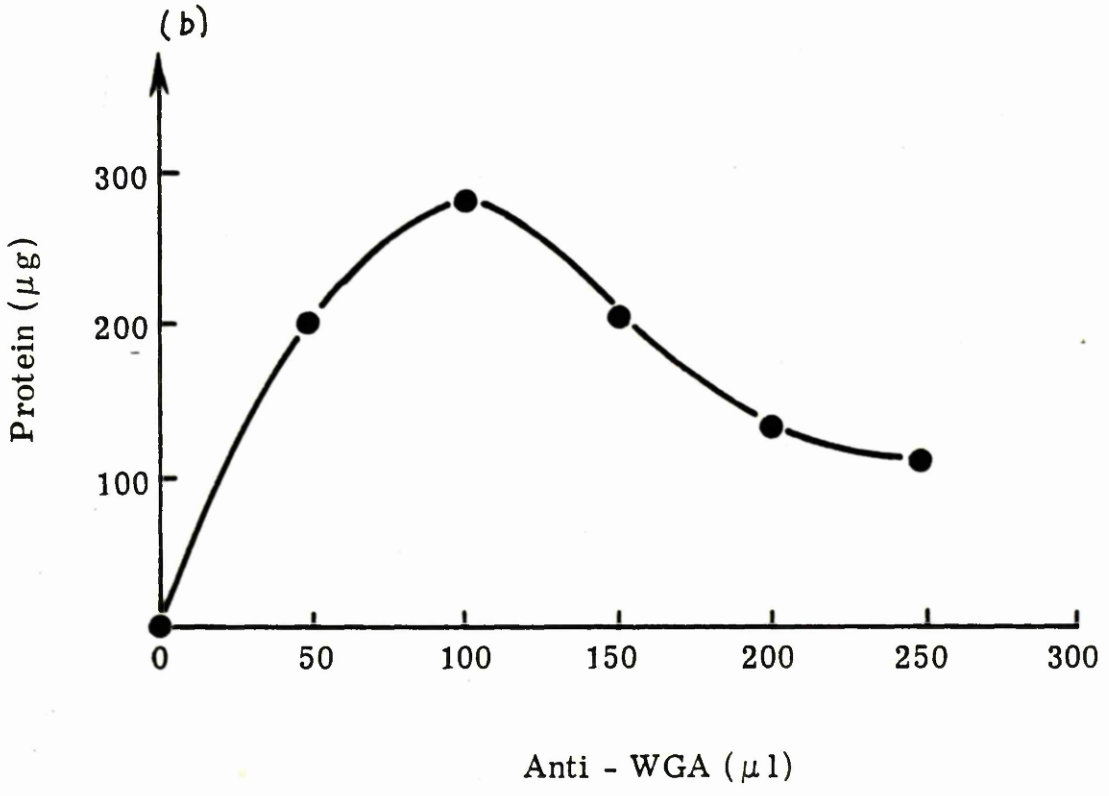
The equivalence zone for each lectin anti-lectin pair is established by immune-precipitating 10µg of lectin with increasing volumes (0-250µl) of anti-serum as described in (M 9.4).

The resulting immunoprecipitates are centrifuged at 1,000g after 10 min and washed (x4) in PBS, pH 7.2 (M 13.2.2), prior to assaying for protein (M 6.3).

(a) Con A incubated with anti-Con A serum

(b) WGA incubated with anti-WGA serum


(a)
Protein (μg)
300
200
100
0
50
100
150
200
250
300
Anti - Con A (μl)


(b)
Protein (μg)
300
200
100
0
50
100
150
200
250
300
Anti - WGA (μl)

M 10.2 Purification of SBA by affinity chromatography on Sepharose-N-caproylgalactosamine conjugate

The method of Allen and Neuberger (1975) is followed. The agglutinin is purified on a column of sepharose-N-caproylagalactosamine equilibrated in column washing buffer (M 3.10.2) and eluted in the same buffer containing 0.1M-galactose. (See Fig. 2.5b).

M 10.3 Lectin techniques

M 10.3.1 Isolation of soluble mitochondrial glycoproteins by affinity chromatography in Con A sepharose-4B

Isolated mitochondria (PM_1) from 12 livers (M 4.2) are solubilised by boiling in 2% (w/v) SDS and diluted in column washing buffer (M 3.11.1) to a final SDS concentration of 0.1% (w/v). A Pharmacia glass column (8.0 x 1.5cm) is packed with the prepared sepharose-4B bound Con A and the column is pre-equilibrated overnight in washing buffer (M 3.11.1). The sample is loaded onto the column and 3 volumes of buffer passed through to remove unbound material. The bound glycoproteins are eluted in buffer containing 0.2M α-Methyl-D-glucoside (M 3.11.2) and concentrated by precipitating with 5 volumes of ice cold acetone. Routinely 3-4mg of glycoprotein are purified.

M 10.3.2 Direct demonstration of the lectin binding components of mitochondrial membranes

Samples of the inner, outer and soluble fractions of mitochondria are separated by SDS-polyacrylamide gel electrophoresis of 10% (w/v) acrylamide slab gels. The polypeptide bands are fixed overnight in acetic acid (M 3.5.1.4). The gels are then equilibrated for 2-3h in 200ml of PBS, pH 7.2 (M 3.12.2), the buffer being changed 3 times. Equilibrated gels are incubated in 50ml of PBS pH 7.2 containing 2mg bovine haemoglobin carrier/ml, 0.05% sodium azide and $[^{125}I]$ -lectin equivalent to 1.0 x 10^6 cpm of $[^{125}I]$. After gentle shaking overnight the solution is decanted and the gels are washed exhaustively in PBS pH 7.2 containing 0.05% sodium azide until no further labelled lectin could be detected in the washing; about 2 days. The gels are then processed for fluorography and dried under vacuum before exposing to Kodirex X-ray film (M 8.5.2). Control experiments are performed by including 0.2M of the appropriate hapten sugar during the lectin incubation and washings.

M 10.3.3 Immunoprecipation of lectin binding receptors

Surface labelled glycoproteins in mitochondria and mitoplasts are immunoprecipated by a combination of the methods of Juliano and Li (1978) and Henkart and Fisher (1975).

Intact mitochondria and mitoplasts are surface labelled using lactoperoxidase catalysed iodination as described in (M 7.1).

(a) Immunoprecipation of Con A binding receptors

[^{125}I] radiolabelled mitochondria and mitoplasts are solubilised in 0.5ml TKM buffer, pH 8.2 (M 3.7.1.1), pH 8.2 containing 1% (w/v) DOC, and incubated on ice for 1h. 10µl of PMSF is added to inhibit protease activity.

Unsolubilised material is pelleted by centrifugation at 100,000g for 45 min at 4°C in the 10 x 10ml rotor of the MSE-50 superspeed ultracentrifuge. To the supernatant fraction is added 1.2ml of 2-detergent TKM (M 3.7.1.4) and the mixture incubated with 10µg of Con A for 1h at 37°C. The Con A-glycoprotein receptor complex is immunoprecipitated with 210µl of anti-Con A serum by incubating for 1h at 37°C and leaving overnight at 4°C. PMSF is again added to a final concentration of 2mM. Immune complexes are centrifuged at 1000g and the precipitates washed 4 times in Con A immunoprecipitate washing

buffer (M 3.7.1.4). The immunoprecipitate is finally washed in TKM, pH 8.2.

(b) Immunoprecipitate of WGA binding receptors

The same procedure as summarised for Con A is used, except that in all cases 0.1M-Tris-HCl-DOC pH 7.8 is substituted for TKM pH 8.2.

The WGA binding proteins are immunoprecipitated using 100µl of anti-WGA serum. Immune complexes are washed 4 times in WGA immunoprecipitate washing buffer (M 3.7.1.5) and finally in PBS (M 3.12.2). A control experiment by addition of 0.2M of the appropriate hapten inhibitor prior to addition of the lectin is also performed.

M 11 Separation techniques

M 11.1 Gel filtration

Sephadex G-50 (fine) in a column of dimensions 10.0 x 0.8cm is used to separate the high and low mol. wt. species of the extramitochondrial material released by digitonin and high ionic strength buffer. The elution buffer is 10mM-Tris, pH 8.2 containing 0.15M-KCl.

M 11.2 Ion exchange chromatography

Extramitochondrial material released by digitonin and high ionic strength buffer is separated on DEAE-cellulose as described by Kraemer (1971). The DEAE-cellulose (DE-32)

is soaked overnight in 0.1M-ammonium acetate. A column (10.0 x 1.5cm) is packed and equilibrated in 0.01M-ammonium acetate. The radiolabelled sample is loaded on and eluted with a linear gradient from 0.01M to 2.0M-ammonium acetate. Fractions are collected (3.0ml) and 0.5ml aliquots treated for liquid scintillation spectrometry (M 8.5.6).

CHAPTER 3

Biochemical assessment of the mitochondrial fraction from rat liver.

3.1 Introduction

Mitochondria provide a suitable system for study of the topography, organisation and function of membrane-associated proteins and glycoproteins. These organelles perform a variety of membrane activities linked to oxidative phosphorylation and contain many specialised transport and exchange diffusion systems, such as the phosphate, dicarboxylic and tricarboxylic acid carriers and the adenine nucleotide translocase. In addition, the available methodology for isolation of the organelles in a high state of morphological integrity, and the existence of distinct marker enzymic activities associated with various mitochondrial subfractions has provided us with the necessary impetus to undertake this study.

To date, very little is known about the detailed topography and organisation of the proteins within the two membranes, although the inner mitochondrial membrane has always been the subject of much investigation particularly with respect to its role in electron transport and ATP production. The first chapter is consequently dedicated towards carefully defining the mitochondrial fraction in terms of degree of damage to the outer and inner membranes during isolation of the organelle, which is important for surface labelling studies that involve membrane impermeant probes. The purity of the fractions has also been carefully

catalogued, particularly with respect to contaminating lysosomes and microsomes which tend to co-purify with mitochondria.

3.2 Purification of rat liver mitochondria on a discontinuous sucrose gradient.

The subcellular fractions that are isolated together with mitochondria are mainly lysosomes, microsomes and plasma membrane. Particular marker enzymic activities are associated with each of these fractions and their respective densities on a sucrose gradient can be determined by these criteria.

Mature female rats are injected with Triton WR 1339, 3 days prior to sacrifice. The detergent is taken up by pinocytosis and accumulates in lysosomes which consequently band at a lighter density, resulting in an improved purification of the mitochondria (Wattiaux et al., 1963). In this experiment, the discontinuous sucrose gradient on which mitochondria are purified is fractionated into 1ml aliquots and assayed for glucose-6-phosphatase, acid phosphatase, 5'-nucleotidase and cytochrome c oxidase activity.

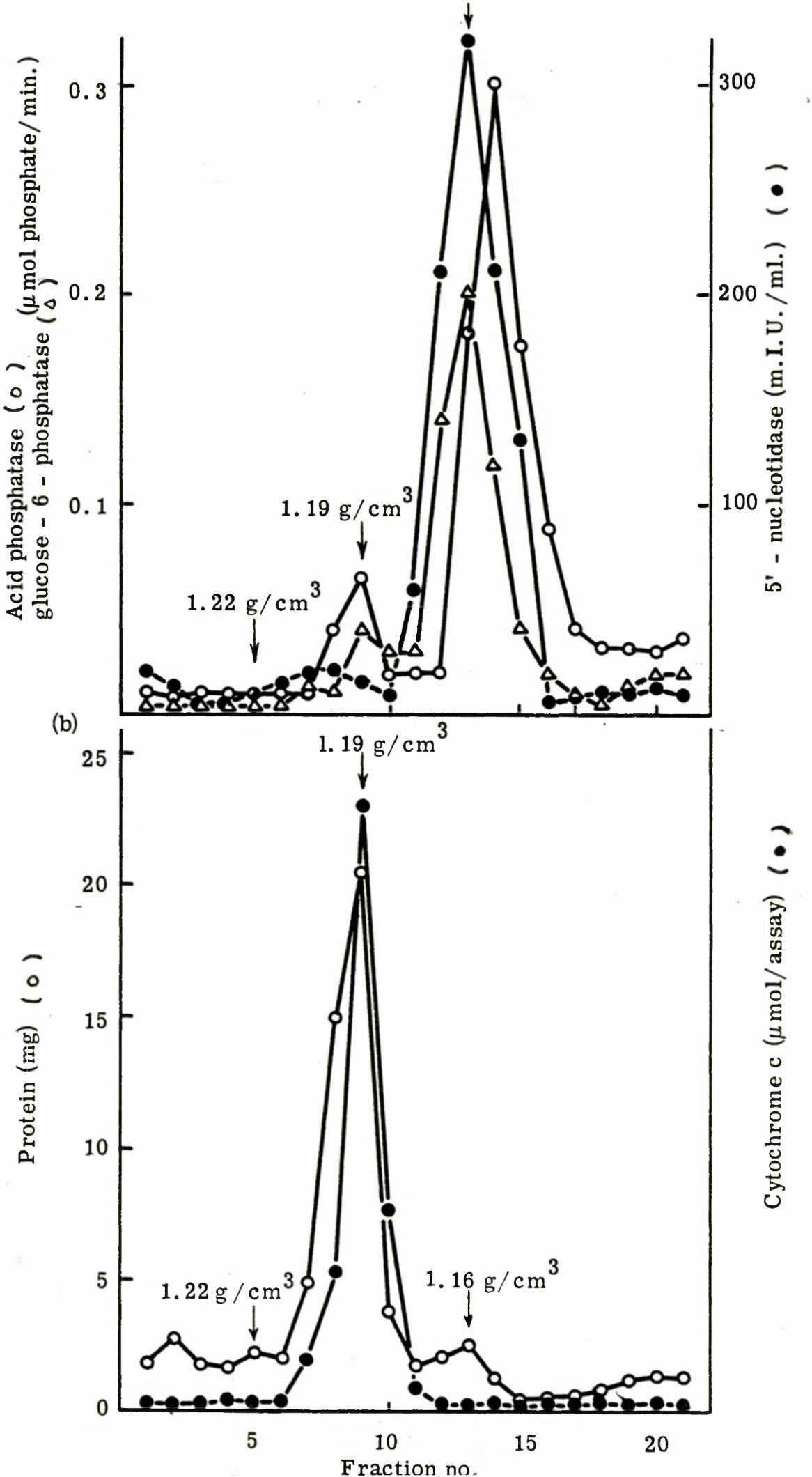
The results plotted in Fig 3.1a. demonstrate that glucose-6-phosphatase and 5'-nucleotidase activity band principally on the 1.16g/cm^3 shelf. Lysosomes which normally band at a density of 1.22g/cm^3 become lighter owing to ingestion of the detergent. Acid phosphatase activity is mainly located at a density of 1.16g/cm^3, remote from the mitochondria which band at a density of 1.19g/cm^3 as assessed by cytochrome c oxidase activity.

Fig. 3.1

Purification of rat liver mitochondria on a discontinuous sucrose gradient

Rat liver mitochondria are isolated as described in (M 4.1) after an injection of Triton WR-1339 (1mg/g of body weight) 3 days prior to sacrifice.

The mitochondria are purified on a discontinuous sucrose gradient (M 4.2) and the gradient harvested into 1ml fractions. Aliquots (50-100µl) are assayed for glucose-6-phosphatase (M 5.6), acid phosphatase (M 5.5) and 5'-nucleotidase (M5.7) activity (Fig. 3.1(a)). In Fig 3.1(b), aliquots from the same fraction are assayed for cytochrome c oxidase activity (M 5.2(a)) and protein content (M 6.3).


Acid phosphatase (o)
glucose - 6 - phosphatase (Δ) (μmol phosphate/min.)
5' - nucleotidase (m.I.U./ml.) (•)
1.19 g/cm3
1.22 g/cm3
(b)
Protein (mg) (o)
Cytochrome c (μmol/assay) (•)
1.19 g/cm3
1.22 g/cm3
1.16 g/cm3
Fraction no.

Approximately 80% of the protein as estimated by the method of Lowry et al. (1951) appears to be associated with this fraction too, see Fig. 3.1b.

3.3 Estimation of the various activities that co-purify with mitochondria.

The extent of subcellular contamination in the mitochondrial fraction at the various stages of purification, starting from the crude liver homogenate is quantitated as described in Fig. 3.1. Estimation of the various enzymic activities, during the step-wise purification of mitochondria is tabulated in Table 3.1.

The data show that although 85.7% of the original cytochrome c oxidase activity in the mitochondrial fraction after differential centrifugation (M_1) is recovered, only 52.3% of the total enzymic activity remains after purification of mitochondria on the sucrose gradient (PM_1). Contamination levels of acid phosphatase, glucose-6-phosphatase and 5'-nucleotidase activities in the M_1 fraction are between 4-6% of the total enzymic activity, and decreased to less than 2% in the PM_1 fraction. Total recoveries of protein and all the enzymic activities within experimental error are good, and in general approached 90% of their original values.

3.4 Assay for the integrity of the outer membrane in isolated rat liver mitochondria

The degree of damage of the outer mitochondrial membrane is estimated by measuring the activity of cytochrome c oxidase, as inner membrane protein complex using exogenous

Table 3.1.

Distribution of enzymic activities in sub-cellular fractions of rat liver obtained after differential and sucrose gradient centrifugation.

Rat liver mitochondria are isolated as described in (M 4.1). Cytochrome c oxidase (M 5.2(a)), glucose-6-phosphatase (M 5.6) and 5'nucleotidase (M 5.7) activities are assessed in the various subcellular fractions. Contamination of the final mitochondrial preparation (M_1 and PM_1) by lysosomes, microsomes or plasma membrane is calculated on the basis of recovered marker activities. Percentage of contamination refers to the degree of contamination of the final mitochondrial preparations by the marker enzymes indicated as compared to the original homogenate.

The experiment is performed in duplicate and the % of enzymic activities calculated are an average of 2 values.

Table 3.1

Fraction	Protein (mg)	Enzymic activity (total units) n mol product min^{-1}			
		cytochrome c oxidase	Acid phos-phatase	glucose-6-phos-phatase	5'-nucleo tidase.
crude homogenate	1214.0	66,000.0	2575.0	4780.0	18089.0
mitochondrial pellet 6900 g (M_1)	113.7	56,532.0	128.8	258.1	795.9
1.19g/ml shelf of sucrose gradient (PM_1)	79.2	34,552.0	27.6	82.9	116.1
1.08, 1.16, 1.22g/ml shelf of sucrose gradient	24.6	527.0	93.4	156.3	646.8
post mitochondrial supernatant	1076.0	1700.0	2185.0	4005.0	13838.0
% recovery	97.9	88.2	89.8	89.1	83.8
% recovery/ contamination (M_1)	-	85.7	5.0	5.4	4.4
% recovery/ contamination (PM_1)	-	52.3	1.1	1.7	0.6

cytochrome c as substrate (Wojtczak et al., 1972). Under normal circumstances cytochrome c does not penetrate the outer membrane, (Wojtczak and Zaluska, 1969) hence, the observed cytochrome c oxidase activity can be correlated with damage to the outer membrane.

The dependence of the rate of cytochrome c oxidation by mitochondria on the concentration of added cytochrome c is shown in Fig 3.2. When mitochondria are solubilised in 0.3% (w/v) DOC, the reaction rate attains its maximum at 100μM cytochrome c, cytochrome c oxidase being stimulated 8-10 times, as compared to untreated mitochondria where the oxidation rate increases linearly with increasing levels of cytochrome c. The percentage of the activity in untreated mitochondria with respect to solubilised mitochondria remains essentially independent of cytochrome c concentration up to 100μM and then slowly increases with increasing concentrations of cytochrome c. Therefore, by measuring O_2 uptake in intact and detergent-treated mitochondrial fractions M_1 and PM_1, the degree of damage to the outer mitochondrial membrane is estimated, (see Table 3.2).

3.5 Discussion

The first step, prior to biochemical investigation at a molecular level, is to define carefully the system being studied, particularly with respect to purity of the initial starting material. We have monitored the mitochondrial preparation for the extent of contamination by other cellular fractions including lysosomes and microsomes which tend to co-purify with the mitochondria. In addition,

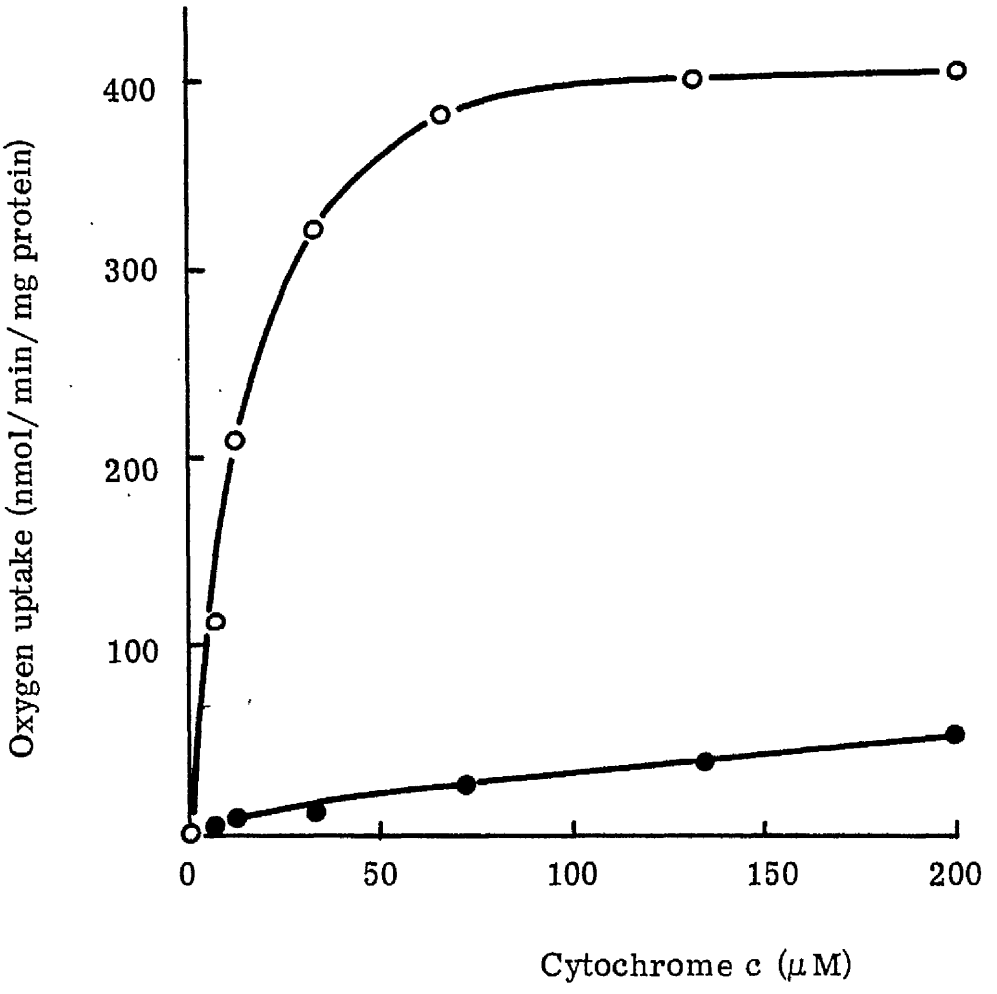


Fig. 3.2.

Effect of concentration of cytochrome c on the rate of its oxidation by preparations of rat liver mitochondria.

Rat liver mitochondria are isolated and resuspended in isolation medium at 25mg of protein/ml. Cytochrome c oxidase activity is measured as a function of cytochrome c concentration in an oxygen electrode by monitoring oxygen consumption. The assay medium is described in (M 5.2(b)) and the reaction initiated by addition of mitochondrial protein (0.5mg).

(●) "intact" mitochondria

(o) mitochondria solubilised in 0.3% DOC (w/v).

Table 3.2

Estimation of the integrity of the outer mitochondrial membrane in the M_1 and PM_1 fractions.

Rat liver mitochondria are isolated after differential centrifugation (M 4.1) and further purification on a sucrose density gradient (M 4.2). The mitochondrial fractions are resuspended to approx. 25mg of protein/ml and aliquots assayed for cytochrome c oxidase activity as described in Fig. 3.2.

The % breakage of the outer membrane is estimated by comparing the enzymic activities obtained in the presence of DOC to that in the intact preparation.

Table 3.2.

Mitochondrial Fraction	O_2 uptake nmol O_2/min/mg protein		% breakage
	-	+ 0.3% (w/v) DOC	
M_1	15.1	485.8	3.1
PM_1	62.2	398.6	15.6

as mitochondria have only 1-2% carbohydrate co-valently associated with the organelle, (Bernacki and Bosmann, 1970) it is important to establish that glycoproteins detected by use of lectins are mitochondrial in origin and not due to contaminating subcellular fractions.

The step-wise purification of the mitochondrial fraction starting from the crude liver homogenate is tabulated in Table 3.1. The relatively low recovery (52.3%) of cytochrome c oxidase activity in the PM_1 fraction can be attributed to inhibition of the enzyme by the high sucrose concentration employed in the assay (J.G. Lindsay personal communication). This is confirmed by the lower specific activity of the enzyme in purified mitochondria. By injecting the rats with the detergent Triton WR-1339 according to the method of Wattiaux et al. (1963), we have succeeded in further purifying the mitochondrial fraction as the detergent accumulates in lysosomes, decreasing their density from $1.22 g/cm^3$ to $1.16 g/cm^3$. However, one disadvantage of the detergent-filled lysosomes is that they are more fragile and are readily disrupted by osmotic or mechanical shock, releasing a variety of degradative enzymes to which the exposed mitochondrial polypeptides would be particularly susceptible. This constitutes a major disadvantage in surface labelling studies of these exposed proteins.

The outer mitochondrial membrane is often exposed to damage during isolation or as a result of mitochondrial swelling in the isolation medium (Wlodawer et al., 1966). In particular, for the studies reported that are concerned with detecting exposed carbohydrate (Glew et al., 1973 ;

Henning and Uhlenbruck, 1973), it is imperative that the damage to the outer mitochondrial membrane should be carefully assessed in order to distinguish between carbohydrate exposed on the outer and inner membrane. Therefore, as this study is concerned with elucidating the organisation of exposed proteins and glycoproteins using membrane impermeant probes, we felt it is necessary to develop a rapid quantitative method for assessing the morphological integrity of the mitochondria prior to biochemical investigation.

Using the oxygen electrode method of Wojtczak et al. (1972) for estimating cytochrome c oxidase activity, we have found that our mitochondria are between 3-5% broken. Further purification on a discontinuous sucrose gradient resulted in additional breakage routinely estimated to be between 11-17%. One inaccuracy of this method may be the limited rate of cytochrome c diffusion into the inter-membrane and intracristal spaces. If the rupture of the outer membrane is too small, the rate of diffusion may be lower than the rate of cytochrome c oxidation.

In conclusion, we have established the parameters of our mitochondrial system in terms of contaminating sub-fractions and integrity of the outer membrane. It appears that although the M_1 fraction had slightly higher levels of contaminating cellular organelles (4-6%), the breakage incurred to the outer membrane during the isolation procedure is minimal (3-5%). In contrast, the PM_1 fraction is contaminated to a lesser degree with lysosomes and microsomes but the mitochondria are much more disrupted

probably owing to exposure of the organelles to hyper-osmotic sucrose solutions. In addition, the disadvantage of increased susceptibility to protease activity from fragile lysosomes has prompted us for the major part to use the M_1 mitochondrial fraction in our continuing investigations.

CHAPTER 4

Location of carbohydrate in rat liver mitochondria.

4.1 Introduction

Isolated mitochondria are reported to contain 1-2% carbohydrate by weight, and are capable of incorporating sugar nucleotides *in vitro* into endogeneous glycoprotein receptors (Bernacki and Bosmann, 1970). Most of the mitochondrial hexosamine and sialic acid is released in the soluble form on disruption of the organelle (de Bernard *et al.*, 1971). However, the nature and distribution of the carbohydrate has not been fully investigated, and this chapter is directed towards clarifying its location within the organelle.

4.2 Distribution of enzymic activities in the mitochondrial subfractions.

Mitochondria are separated after sonication and sucrose gradient purification into inner and outer membranes and a soluble fraction. Optimal conditions for sonication of the mitochondrial sample are determined such that the degree of cross contamination between the various components is minimal.

Distribution of enzymic activities characteristic for the subfractions is summarised in Fig. 4.1b. Sonication for 2 x 20s appears to give the best subfractionation as assessed by marker enzymic criteria. In this case the majority of the cytochrome c oxidase activity is associated with the inner membrane, only 10-11% appearing in the outer membrane fraction. In comparison 8 and 15% of the monoamine

Fig. 4.1.

Distribution of marker enzymic activities of the inner and outer membranes and soluble fractions of rat liver mitochondria.

Rat liver mitochondria are isolated (M 4.1) and further purified on a discontinuous sucrose density gradient (M 4.2).

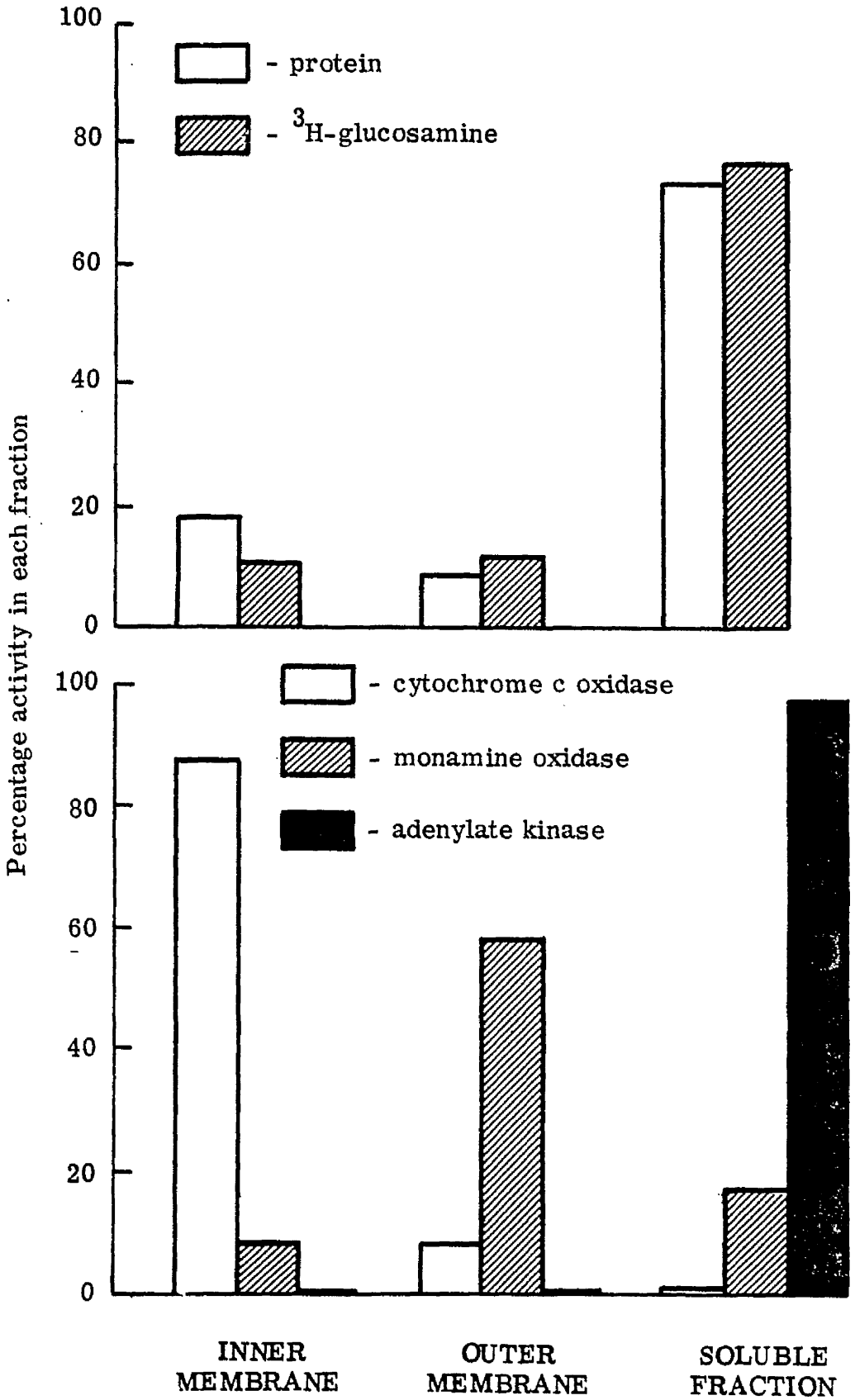
Purified mitochondria (15mg of protein) are resuspended in 7.5ml of 10mM-Tris-phosphate buffer, pH 7.5 to swell the organelles and then shrunk by addition of 2.5ml of shrinking solution (M 3.1.3). The suspension is sonicated and separated on a discontinuous sucrose gradient (M 4.3.1). Bands of density ($1.22g/cm^3$), ($1.19g/cm^3$) and the soluble fraction ($1.16g/cm^3$) are harvested from the gradient with the aid of a 'J' shaped needle.

In (a) aliquots (0.5ml) of the three subfractions are prepared for liquid scintillation spectrometry(M 8.5.6) and counted for $[^3H]$-glucosamine activity.

In (b) Monoamine oxidase (M 5.1(a)), cytochrome c oxidase (M 5.2(a)) and adenylate kinase (M 5.3) activities are assayed in each of the three subfractions. The protein concentrated is estimated as described in (M 6.3).

The experiment is performed in duplicate and the % of radioactivity and enzymic activity is an average of 2 values $\pm$ 2%.

(a)


100
80
60
40
20
0
- protein
- ^{3}H-glucosamine
100
80
60
40
20
0
- cytochrome c oxidase
- monamine oxidase
- adenylate kinase
Percentage activity in each fraction
INNER MEMBRANE
OUTER MEMBRANE
SOLUBLE FRACTION

(b)

oxidase activity is distributed between the inner membrane and soluble fraction respectively, the majority being associated with the outer membrane. Adenylate kinase, an inter-membrane protein, is virtually all recovered in the soluble fraction with very little or none appearing in the inner and outer membranes. A number of nicotinamide nucleotide-linked dehydrogenase activities, including the malate, glutamate, and $NADP^+$-specific isocitrate dehydrogenase which are originally enclosed within the matrix have been reported to subfractionate with the inner membrane (Sottocasa et al., 1967). However, in our case, negligible malate dehydrogenase activity is associated with either mitochondrial membrane (data not shown).

4.3 Distribution of D-[6- ^{3}H]-glucosamine label in the mitochondrial subfractions.

This experiment is performed so as to determine the distribution of carbohydrate in the inner, outer and soluble components of isolated mitochondria.

[^{3}H]-glucosamine labelled mitochondria are sub-fractionated by sonication and sucrose density gradient separation as described in Fig. 4.1. The data shows that 77% of the total label is released in the soluble form on disruption of the organelle, the majority of the protein being associated with this fraction. The remaining [^{3}H]-glucosamine label (23%) is almost equally distributed between the inner and outer membrane(see Fig. 4.1a). However, on a protein basis it appears that the outer

membrane incorporates approx. twice as much glucosamine as the inner membrane.

4.4 Separation of protein and lipid components of solubilised mitochondrial membranes on Sephadex G-100.

In order to determine the relative distribution of [^{3}H]-glucosamine in membrane glycoprotein or glycolipid, mitochondria are labelled with either [^{3}H] -leucine or [^{14}C]-ethanolamine. The labelled organelles are then solubilised in DOC and protein and lipid components separated on a Sephadex G-100 column.

The data plotted in Fig. 4.2 shows that the majority of the [^{3}H]- leucine label is incorporated into macromolecular proteins and elutes at the void volume of the column, distinctly separate from the smaller [^{14}C]-ethanolamine labelled DOC-lipid micelles. In a comparative experiment, where mitochondria are labelled with [^{3}H] -glucosamine, subsequent separation of the solubilised components reveals that the majority of the label elutes between fraction 20-40 coinciding with the protein elution peak, and suggesting that most of the carbohydrate is associated with protein rather than lipid.

4.5 Amino Acid Analysis of rat liver mitochondria labelled with [^{3}H]-glucosamine.

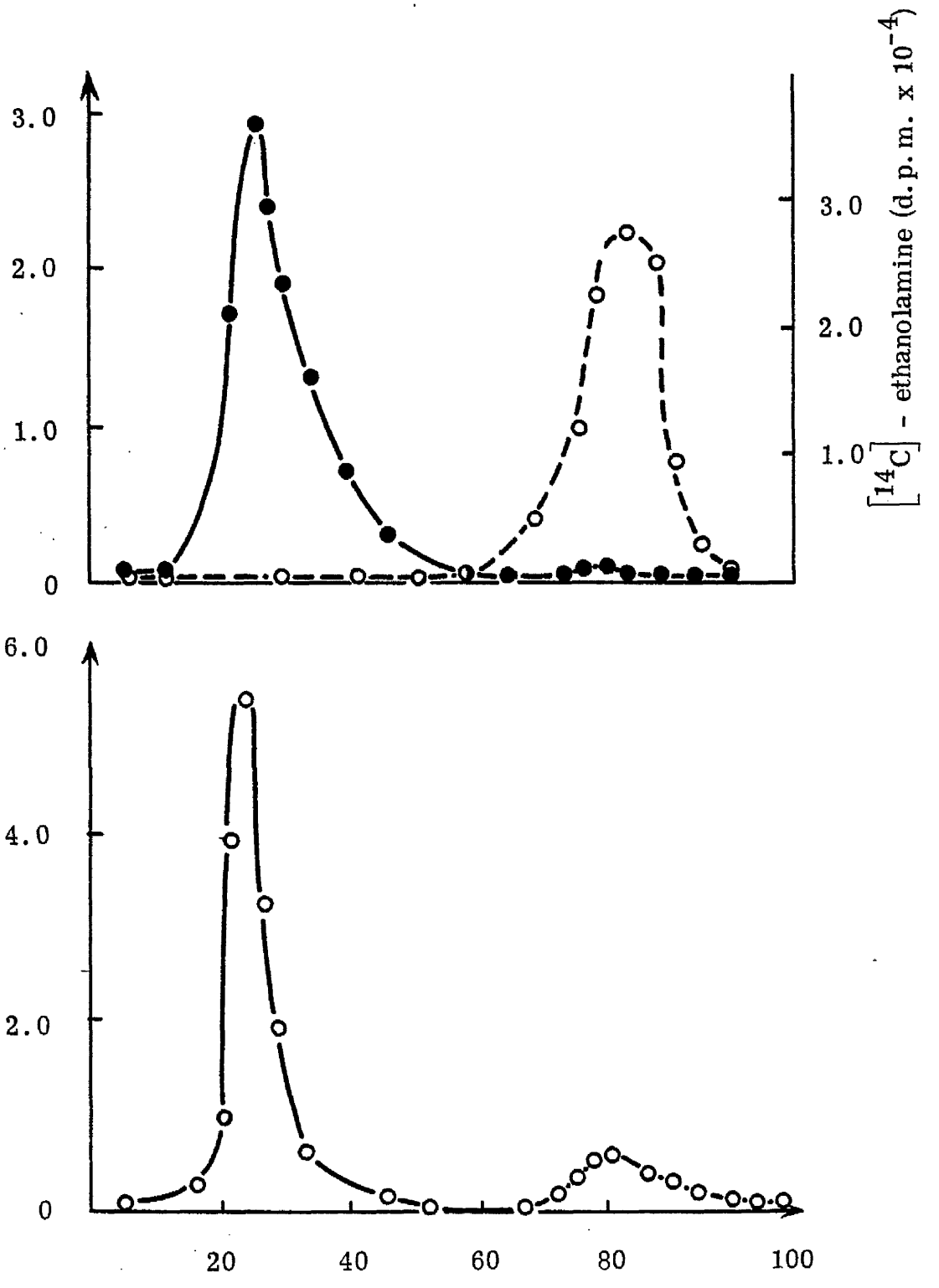
Amino acid analysis is performed to confirm that the [^{3}H]-glucosamine label detected in mitochondria is primarily incorporated into carbohydrate moieties and not metabolised into amino acids after a 16h period.

Fig. 4.2.

Elution profile of DOC-solubilised [^{3}H]-leucine, [^{14}C]-ethanolamine or [^{3}H]-glucosamine labelled mitochondrial membranes from Sephadex G-100 columns.

In three separate experiments, a female rat is injected with either 50μCi of [^{3}H]-leucine (-●-), 20μCi of [^{14}C]-ethanolamine (-○-), or 250μCi of [^{3}H]-glucosamine (-o-) 16h prior to sacrifice. Mitochondria are then isolated as usual (M 4.1).

The isolated mitochondria are solubilised in DOC (2% w/v) and applied to an upward flow column of Sephadex G-100 (60.0 x 1.5cm). Labelled mitochondrial proteins and lipids are eluted in 1% (w/v) DOC, 0.1M-KCl, 10mM-Tris-HCl buffer pH 8.2. Fractions are collected (2ml) and aliquots (0.5ml) counted by liquid scintillation spectrometry (M 8.5.6).


3.0
2.0
1.0
0
3.0
2.0
1.0
[14C] - ethanolamine (d.p.m. x 10-4)
6.0
4.0
2.0
0
20
40
60
80
100
Fraction no.

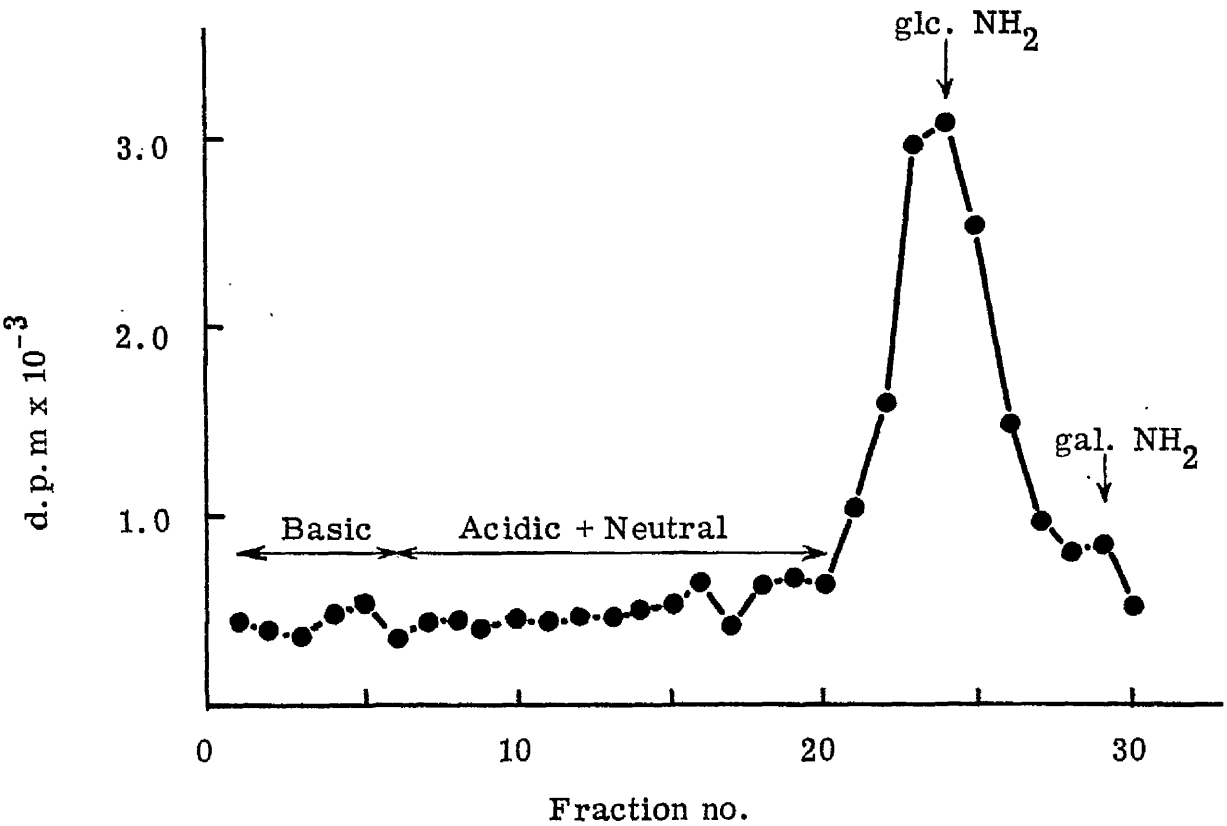


Fig. 4.3.

Amino acid analysis of [^{3}H]-glucosamine labelled mitochondria.

Rat liver mitochondria are purified from starved Wistar rats, injected intraperitoneally 16h previously with 500µCi of D-[6-^{3}H]-glucosamine (M 4.1).

An aliquot of the mitochondrial protein (0.5mg) is hydrolysed as described in the Methods (M 6.4). Fractions (1ml) are collected from the analyser and counted for the isotope by liquid scintillation spectrometry (M 8.5.6).

[^{3}H]-glucosamine labelled mitochondria are isolated (M 4.1) and hydrolysed as described in the Methods (M 6.4). Amino acid analysis is then performed on the sample and fractions collected from the analyser and monitored for the presence of the isotope. The elution profile of tritium label is shown in Fig. 4.3. A peak of radioactivity is found coincidentally at the normal elution point for glucosamine. No significant label is detected in amino acids which elute before the amino sugars.

4.6 Discussion

At present, although mitochondria are reported to contain 1-2% membrane-associated carbohydrate (Bernacki and Bosmann, 1970), and there is evidence to demonstrate the *in vivo* incorporation of glucosamine into protein constituents of mitochondria (Wu *et al.*, 1969; Molnar, 1967) no precise data is available on the carbohydrate location.

The procedure we have adopted for looking at the distribution of carbohydrate in mitochondria is to sub-fractionate the organelle into inner and outer membranes and a soluble fraction. We have optimised the method by varying the length of time for which the mitochondrial sample is sonicated, such that a minimum of cross-contamination between the different subfractions is obtained, as estimated by marker enzymic activities. Sonicating for 40s produced the best results, whereas longer periods resulted in solubilisation of cytochrome c oxidase activity. Conversely, with shorter sonication

periods, increasing quantities of the outer membrane appear to contaminate the inner membrane. The histogram in Fig. 4.1a which summarises the results obtained shows that the inner membrane is always contaminated to a small extent with monoamine oxidase activity, the converse also being true. This cross contamination can be explained on the basis of contact points between the two membranes as described by Hackenbrock (1968a). If this fact is correct, then it would be virtually impossible to prepare pure inner and outer membrane fractions.

Our experiments to determine the location of the glycoprotein material in mitochondria are in agreement with those of Sottocasa et al. (1971). Their data suggests that approximately 80% of the total carbohydrate containing material does not represent an integral part of the membrane, or is very loosely bound to it. In contrast, however, we do not consider that this material is confined to the inter-membrane space, but could exist in loose association with inner or outer mitochondrial membranes as will be discussed later.

In direct contrast to these results, Bosmann et al. (1972) suggest that only 25% of the total sialic acid is released from rat liver mitochondria. These apparent discrepancies can be partly reconciled if the differences in the methods of solubilisation used by the different groups are considered. Thus, the results of Bosmann et al. (1972), are obtained by comparing the sialic acid content of whole mitochondria with that recovered in preparations of outer

membranes and of the intact submitochondrial particle. In the studies of Sottocasa et al. (1971), the glycoproteins are solubilised during swelling of intact mitochondria in hypotonic medium, under conditions in which the leakage of matrix components is minimised. No decision can be made on the authenticity of either result. However, it seems likely that the hypotonic incubation conditions used by de Bernard et al. (1971) and Sottocasa et al. (1971) may have removed glycoprotein material from attachment to the inner membrane, which may have contributed to the higher yields of carbohydrate in the soluble extract.

Further experiments to determine the distribution of [^{3}H]-glucosamine in membrane constituents is performed by separating protein and lipid on a column of Sephadex G-100. Sodium deoxycholate is used to solubilise the mitochondria. At the concentration employed (2% w/v), the detergent effects a complete disruption of protein-lipid interactions (Allan and Crumpton, 1971), resulting in a clear separation of the larger DOC-protein aggregates from the smaller lipid-DOC micelles (Helenius and Simons, 1975). The fact that most of the [^{3}H]-glucosamine label is associated with protein in mitochondria (Fig. 4.2) is similar to the situation in the plasma membrane of the red blood cell, where the majority of the carbohydrate is co-valently associated with the protein components rather than the lipid fraction (Steck, 1974). It is appropriate to mention at this point that the labelling of glycoprotein and glycolipid depends on the turnover rate, and although lipids are reported to have a

faster turnover rate (Bailey et al., 1966; Omura et al., 1967), we found that the proportion of [^{3}H]-glucosamine label in protein and lipid after 3h and 16h revealed no major differences. This agrees with the distribution data of Sottocasa et al. (1971) where the hexosamine content is estimated in freshly-isolated mitochondria.

Finally, amino acid analysis of the [^{3}H]-glucosamine label in mitochondria indicates that virtually all the label is incorporated into glucosamine rather than protein after a 16h period. This confirms that the [^{3}H]-glucosamine label used in this study is incorporated mainly into the oligosaccharides of mitochondrial glycoproteins and to a lesser extent glycolipids.

CHAPTER 5(a)

Identification of the carbohydrate material located on the outer surface of rat liver mitochondria.

5.1 Introduction

In the previous chapter we have shown that approx. 77% of the total mitochondrial hexosamine can be released in the soluble form on disruption of the organelle. By use of the detergent digitonin which specifically interacts with cholesterol (Fieser and Fieser, 1959), we have further investigated these observations. The results show that a low digitonin to protein ratio (0.01mg digitonin/mg protein), results in selective release of 50% of the carbohydrate containing species from the organelle, without disruption of the outer membrane. This chapter is dedicated to resolving these observations.

5.2 Effect of digitonin on the outer mitochondrial membrane. Release of radioactively labelled glycoprotein and lipid.

Investigation into the exact location of the [^{3}H]-glucosamine containing carbohydrate material released by sonication from mitochondria is undertaken by use of the detergent digitonin.

Purified [^{3}H]-glucosamine-labelled mitochondria are treated with increasing concentrations of digitonin, and aliquots of the supernatant fractions are counted for release of soluble label as described in Fig. 5.1. The data plotted in Fig. 5.1. show the effect of digitonin on mitochondria. At low levels

(0.01-0.02 mg/mg protein) approx. 50% of the total radioactivity is dissociated from the organelle in a soluble form. Simultaneously, the integrity of the outer membrane is preserved as assessed by measuring cytochrome c oxidase activity using exogenous cytochrome c as substrate. The second phase of glycoprotein/lipid release closely parallels the disruption of the outer membrane, at approx. 0.11mg of digitonin per mg of protein. This is indicated by the concomitant 8-10 fold increase in cytochrome c oxidase activity.

If the supernatant fractions are pooled and applied to a Sephadex G-50 column, equilibrated as described in the Materials and Methods (M 11.1), most of the $[^{3}H]$ -glucosamine label is eluted in the void volume of the column (data not shown) suggesting it is high mol. wt. in nature: possibly glycoprotein or mucopolysaccharide.

In a comparative experiment (Fig. 5.1.) where mitochondria are labelled with $[^{14}C]$- ethanolamine, the release of labelled phospholipid parallels cytochrome c oxidase activity. This confirms that the integrity of the outer membrane is preserved at low detergent levels. At a digitonin concentration of 0.24mg/mg protein which completely strips off the outer membrane, 32% of the total phospholipid is released. This value correlates well with the fact that the outer membrane comprises only one third of the total membrane mass. Simultaneous assays to measure release of malate dehydrogenase activity into the supernatant fluid revealed no detectable activity, even at maximum digitonin levels. This suggests that the matrix compartment remains intact under these conditions.

Fig. 5.1

Effect of digitonin on the integrity of the outer membrane and the release of radioactively labelled glycoprotein and lipid from rat liver mitochondria.

Purified rat liver mitochondria are isolated from starved Wistar rats (M 4.1), injected intraperitoneally 16h previously with 200µCi of D-[6-^{3}H]-glucosamine or 20µCi of [^{14}C]-ethanolamine. The mitochondria are finally resuspended in isolation medium (M 3.1.1) at 20mg/ml and incubated at 4^{o}C for 15 min with increasing amounts of digitonin, before centrifugation at 6,800g for 10 min.

Radioactivity in 0.5ml aliquots of the supernatant fractions is measured by liquid scintillation spectrometry (M 8.5.6). The pellets are resuspended in 1.0ml of the same medium and cytochrome c oxidase activity determined by the oxygen electrode method (M 5.2(b)). Maximal (100%) activity is that obtained on treatment of samples with 0.3% (w/v) DOC.

(●) % release of glucosamine-labelled material

(o) % release of ethanolamine-labelled lipid

(Δ) % of maximal cytochrome c oxidase activity

The experiment is performed in duplicate and the % of [^{3}H]-glucosamine released is an average of 2 values ± 5%.

Release of radioactivity (% of maximum)
0
20
40
60
80
100
.02
.04
.06
.08
.12
.18
.24
Digitonin concn. (mg/mg) protein
Enzymic activity (% of maximum)
0
20
40
60
80
100

5.3 Effect of different washing treatments on release of D-[6-^{3}H]-glucosamine labelled mitochondria.

In order to test the specificity of digitonin on release of this soluble glycosylated fraction, the effect of various washing treatments on [^{3}H]-glucosamine labelled mitochondria is monitored in Table 5.1. The data indicates the 42.5% of the [^{3}H]-glucosamine label is removed by washing in high salt buffer (0.12M-KCl, 20mM-MOPS, pH 7.2). On treatment with low digitonin (0.01mg/mg protein) only an additional 15.7% is released. In both cases the integrity of the outer membrane is preserved, as assessed by cytochrome c oxidase activity using cytochrome c as substrate.

These results demonstrate that release of the bulk of this carbohydrate containing material is not exclusive to digitonin, and further, confirms that this fraction is loosely associated with the external mitochondrial surface. In addition, this fraction can be released without disruption of the outer membrane,thus behaving like extrinsic protein(s). The use of 10mM-Tris-phosphate, pH 7.5 which causes the mitochondria to swell releases 77.5% of the total label indicating an additional loss of soluble carbohydrate containing material, presumably from the intermembrane space. Evidence which suggests that the outer membrane is damaged in this case, is the high percentage of cytochrome c oxidase activity (65.0%). If the mitochondria are shrunk after swelling, only 58.6% of the total label is released. This can be explained on the assumption that shrinking of the organelles, traps the soluble inter-membrane glycoproteins,

Table 5.1.

Effect of different buffers on the integrity of the outer membrane and the release of [^{3}H]-glucosamine labelled carbohydrate material from rat liver mitochondria

Two female rats are injected with 250µCi of D-[6-^{3}H]-glucosamine 16h prior to sacrifice. Mitochondria are isolated and resuspended in 4ml of MS-MOPS, pH 7.2 (M 3.8.1).

Aliquots of the mitochondrial suspension (4mg) are taken and incubated in 1.5ml of the various buffers listed in Table 5.1. for 15 min at 4°C. The mitochondria are pelleted by centrifugation at 6,800g for 10 min and 0.5ml aliquots of the supernatant fractions prepared for liquid scintillation spectrometry (M 8.5.6). Mitochondrial pellets are subsequently assayed for breakage of the outer membrane by monitoring cytochrome c oxidase activity (M 5.2(b)).

The experiment is performed in duplicate and the % of counts released is an average of 2 values ± 5%.

Table 5.1

Experimental conditions	% $[^3H]$-glucosamine counts released	% breakage
1. MS-MOPS, pH 7.2	11.3	12.6
2. 0.12M-KCl, 20mM-MOPS, pH 7.2	42.5	10.3
3. MS-MOPS, pH 7.2 containing 0.01mg digitonin/mg protein	58.2	13.9
4. 10mM-Tris-phosphate, pH 7.5	77.5	65.0
5. 10mM-Tris-phosphate, pH 7.5 containing 1.8M-sucrose, 2mM-ATP, 2mM-$MgSO_4$.	58.6	43.9
6. MS-MOPS, pH 7.2 containing 0.22mg digitonin/mg protein.	78.9	86.9
7. MS-MOPS, pH 7.2 containing 0.1M-xylose and 0.1M-galactose.	15.5	-

preventing their release. Finally, attempts to release the soluble carbohydrate material from the mitochondrial surface with xylose and galactose, two constituents of digitonin, show a negligible release of label in comparison to the control.

5.4 Effect of trypsin on glucosamine-labelled rat liver mitochondria

Confirmation of the presence of carbohydrate on the outer mitochondrial surface is achieved by testing its susceptibility to trypsin. The time course for release of labelled glycopeptides from mitochondria is shown in Fig. 5.2. The release of labelled glucosamine starts to plateau after 45 min. At the end of 60 min, 47% of the total label is released.

In a control experiment performed in the absence of trypsin, 25% of the total glucosamine label is released. Simultaneous assays to assess the integrity of the outer membrane by measuring the latency of cytochrome c oxidase show only a small increase in activity which suggests that trypsin did not damage the outer membrane, (see Table 5.2.)

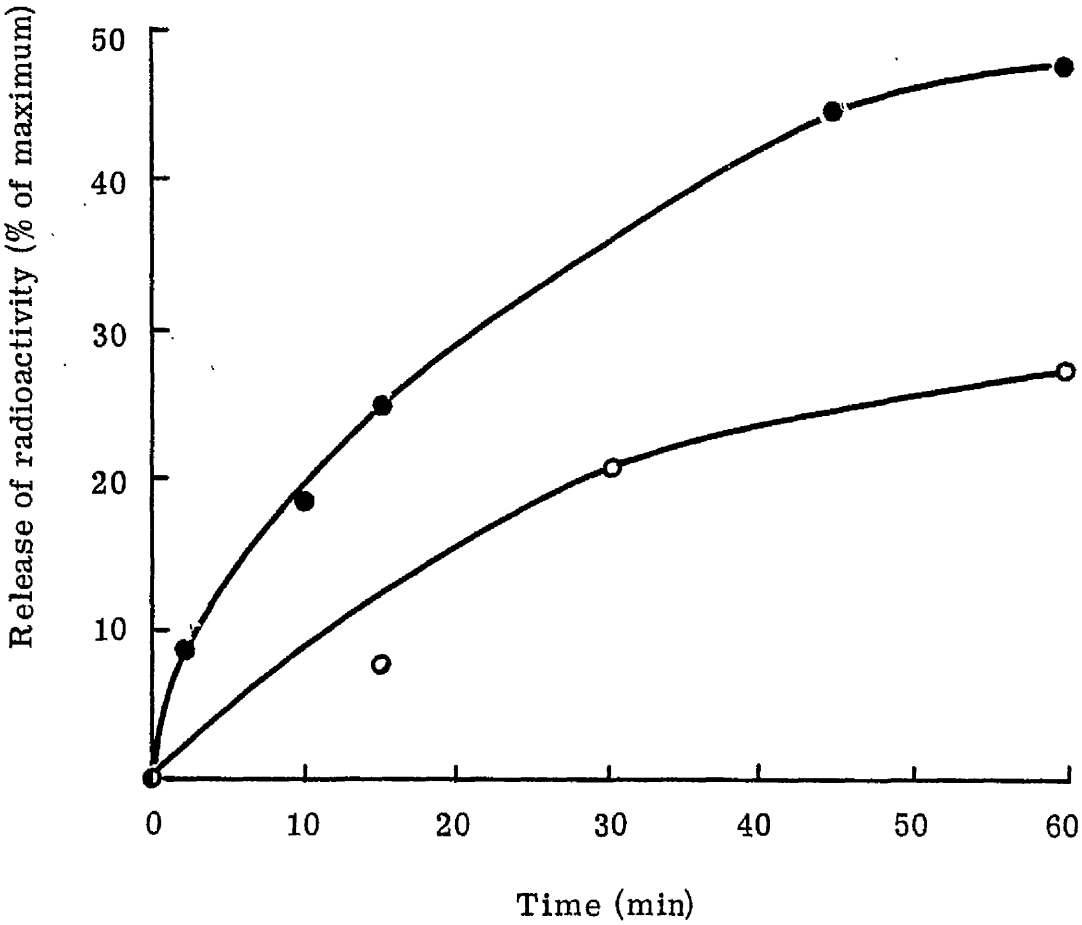


Fig. 5.2.

Release of [^{3}H]-glucosamine labelled glycopeptides from rat liver mitochondria by trypsin treatment.

[^{3}H]-glucosamine labelled mitochondria are isolated as described in Legend 5. Aliquots of mitochondria (2.66mg) resuspended in isolation medium (M 3.1.1) are incubated with 100µg of trypsin for increasing time at 25°C, with shaking. The mitochondrial suspension is diluted 2-fold in isolation medium and centrifuged at 6,800g for 10 min. An aliquot (0.5ml) of the supernatant fraction is mixed with liquid scintillant (M 8.5.6) and counted for release of the radioactivity. A comparative experiment in which trypsin is omitted is simultaneously performed.

(●) Incubation of mitochondria in the presence of trypsin

(o) Control experiment, no trypsin added.

Table 5.2

Rat liver Mitochondria	Time of incubation (min)	O_2 uptake nmol(O_2)/min/mg protein		% breakage
		-DOC	+ 0.3%(w/v) DOC	
Control	2	13.6	490.4	2.8
	60	26.9	472.8	5.7
Trypsin treated	2	13.7	490.4	2.8
	60	36.9	462.4	8.0

Release of [^{3}H]-glucosamine labelled glycopeptides from rat liver mitochondria by trypsin treatment

[^{3}H]-glucosamine labelled mitochondria are isolated and treated with trypsin as described in Fig. 5.2. The mitochondrial pellets obtained by centrifugation at 6,800g for 10 min after trypsin treatment, are then monitored for breakage of the outer membrane by estimating cytochrome c oxidase activity as described in Fig. 5.2 (b).

CHAPTER 5(b)

5.5 Investigation of the high mol. wt. material released by digitonin and KCl treatment of D-[6-^{3}H]-glucosamine labelled mitochondria

The nature of the carbohydrate containing material that is released by digitonin and KCl treatment as described in Section 5.3, is further investigated by ion exchange chromotography on a column of DEAE-cellulose.

The supernatant fraction containing the high mol. wt. material released by digitonin treatment (0.01mg/mg protein) is dialysed against ammonium acetate to remove free glucosamine, prior to pronase digestion. On application of the sample to a DEAE-cellulose column (DE-32) pre-equilibrated in 0.01M-ammonium acetate, and elution using a linear gradient of $CH_3COOONH_4$ between 0.01M and 2.0M, one major peak is eluted at a concentration of 0.34M, (see Fig. 5.3).

In order to identify the nature of this peak a mixture of protein, hyaluronic acid and chondroitin sulphate that is pre-digested with pronase is separated on a similar column. On application of a linear $CH_3COOONH_4$ gradient, the protein elutes at a concentration of 0.32M, as measured by absorbance at 280mn, suggesting that the [^{3}H]-glucosamine containing peak is glycoprotein in nature. Another protein peak also elutes prior to application of the salt gradient. This is probably owing to the fact that the peptides derived from BSA after pronase digestion bind to the DEAE-cellulose with varying affinity. Hyaluronic acid and chondroitin sulphate elute at concentrations of 0.72M and 1.12M respectively, see Fig. 5.3. A comparative experiment that is performed on the supernatant fraction obtained after KCl treatment reveals an identical result (data not shown).

Fig. 5.3.

Investigation into the glycosylated nature of the high mol. wt. material released by digitonin treatment of D-[6-^{3}H]-glucosamine labelled mitochondria.

Rat liver mitochondria are purified from starved Wistar rats, injected intraperitoneally 16h previously with 500μCi of D-[6-^{3}H]-glucosamine (M 4.1). [^{3}H]-glucosamine-labelled mitochondria are pelleted at 6,800 for 10 min and the supernatant fraction dialysed against 0.01M-ammonium acetate, prior to pronase digestion (1mg/ml) at 37°C for 2h. The sample is then applied to a DEAE-cellulose column and eluted by means of a linear gradient of ammonium acetate (0.01M-2.0M). Fractions are collected (3ml) and aliquots (0.5ml) taken for liquid scintillation counting (M 8.5.6).

The elution pattern for a control mixture containing BSA (1mg), hyaluronic acid (1mg), and chondroitin sulphate (1mg) dissolved in 1ml of 0.01M ammonium acetate, digested with pronase and separated by ion exchange chromatography as described above is also performed. The samples that are eluted from the column on application of the linear salt gradient are assayed for protein (M 6.3) and uronic acid (M 6.2).

(O) release of glucosamine label

(Δ) protein absorbance

(●) uronic acid absorbance

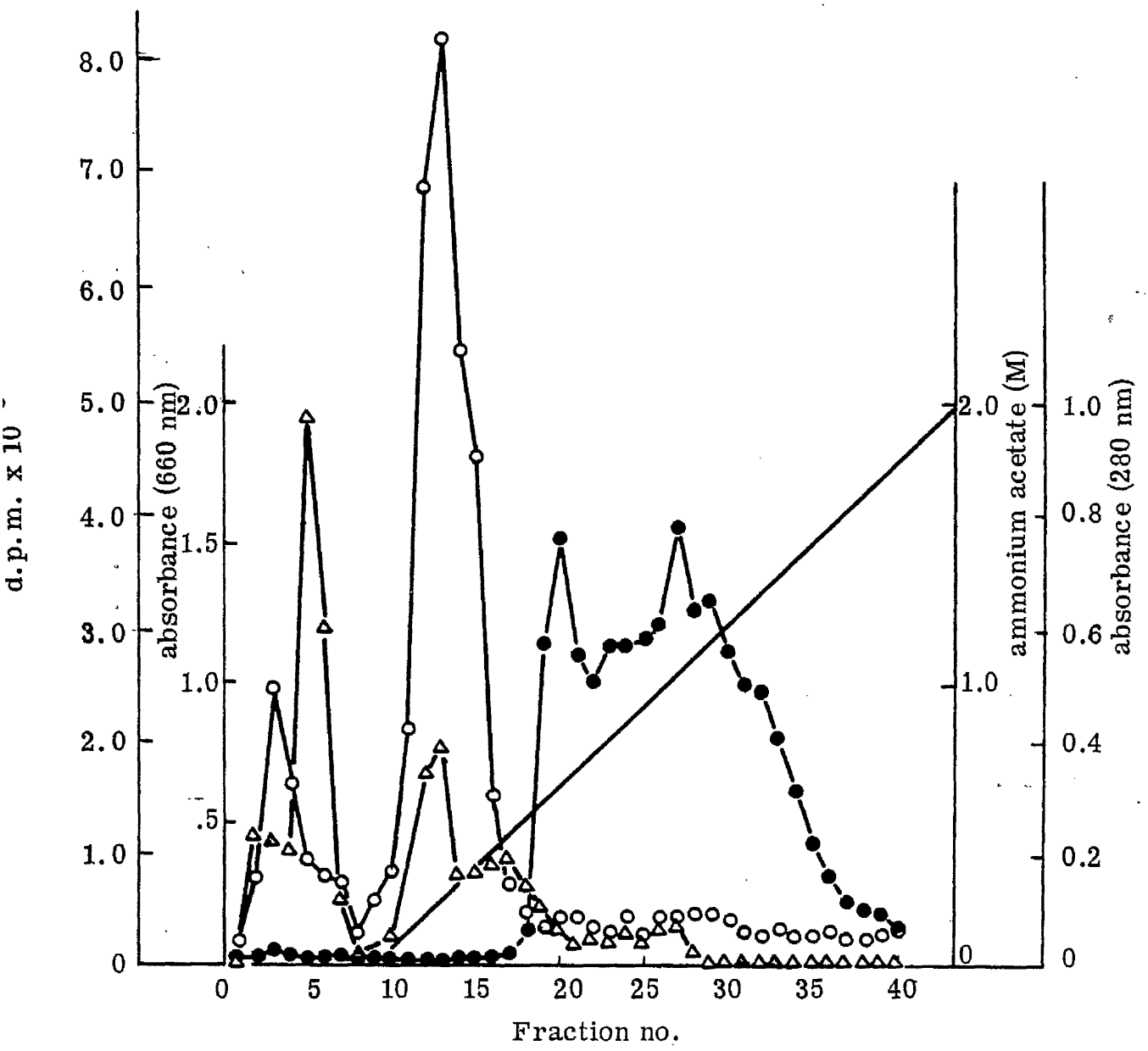

d.p.m. x 10⁻³
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
absorbance (660 nm)
2.0
1.5
1.0
.5
0
ammonium acetate (M)
2.0
1.0
0
absorbance (280 nm)
1.0
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
30
35
40
Fraction no.

Fig. 5.4

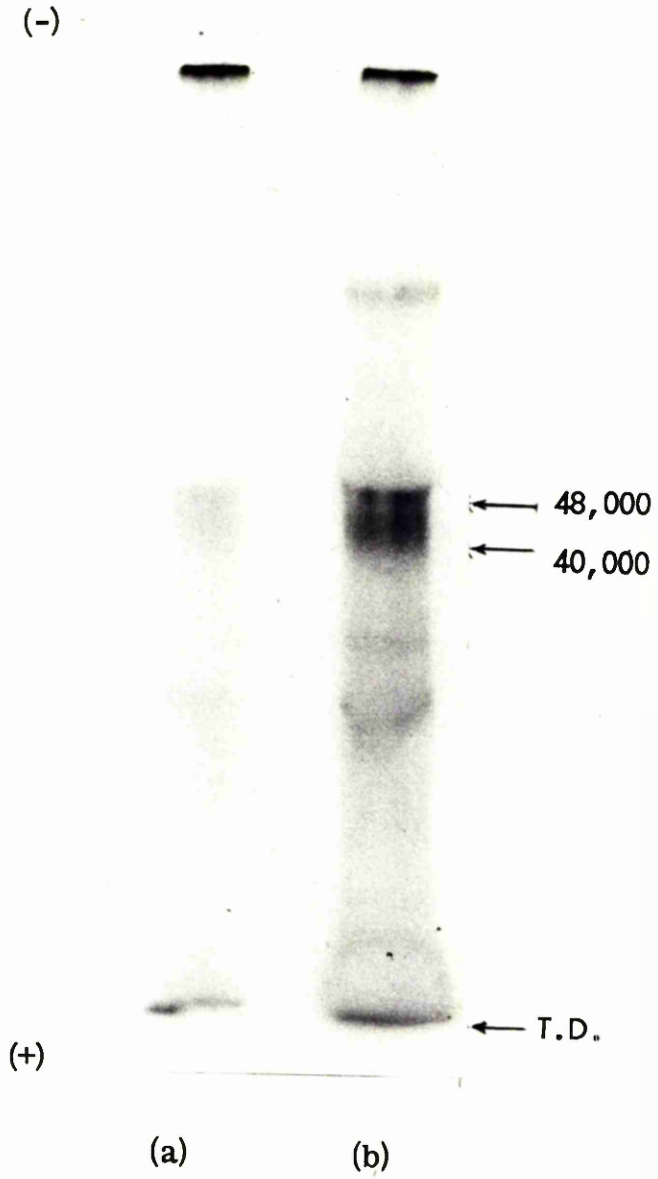
Identification by SDS-polyacrylamide gel electrophoresis of the glycoprotein material released from mitochondria by treatment with a low digitonin concentration

Rat liver mitochondria are isolated as described in (M 4.1). The mitochondria resuspended in isolation medium (M 3.1.1) are treated with digitonin (0.01mg/mg protein) as described in (M 4.3.2) though the swelling and shrinking stage is omitted. The supernatant fraction is then iodinated by the Chloramine T Method (M 7.2), dialysed exhaustively against TKM buffer (M 3.7.1.1) and subsequently immunoprecipitated with Con A (10µg) and anti-Con A serum (200µl) (M 10.3.3(a)). Labelled bands are visualised by fluorography (5 day exposure) after SDS-polyacrylamide gel electrophoresis (M 8.2) of samples containing equal amounts of protein.

An identical experiment using the same protocol is performed, except that addition of digitonin is omitted.

(a) Control experiment in the absence of digitonin

(b) Con A immunoprecipitate of supernatant fraction after digitonin treatment (0.01mg/mg protein).


(-)
48,000
40,000
T.D.
(+)
(a)
(b)

5.6 Analysis of the material released by digitonin on SDS-polyacrylamide gel electrophoresis.

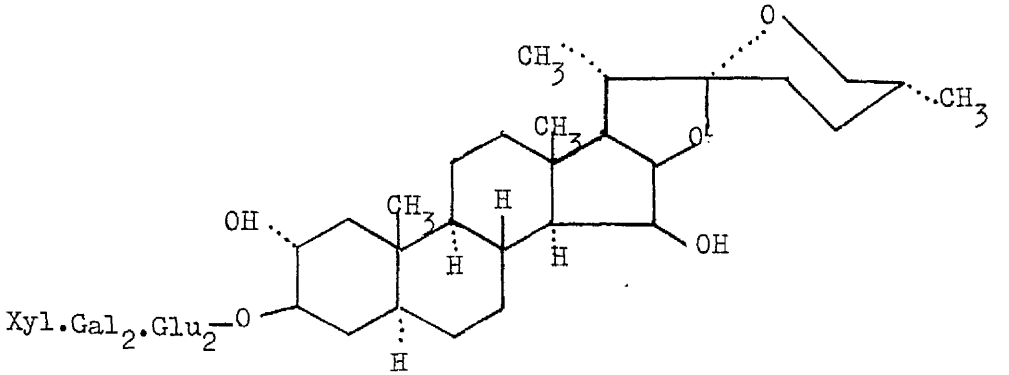
On the basis of the previous experiment the extra-mitochondrial carbohydrate containing material, is considered to be glycoprotein in nature. In order to define this macromolecular species more clearly, the supernatant fraction released on digitonin treatment (0.01mg/mg protein) is iodinated and subsequently immune-precipitated with Con A and anti-Con A serum (M 10.3.3(a)).

On identification of the labelled bands by fluorography after SDS-gel electrophoresis, a doublet of approximate mol. wt. 48,000 and 40,000 is detected. In addition, 3 other bands are observed: one of high mol. wt. approx. 80,000, another in the 20,000 range and finally a low mol. wt. species. The result is photographed in Fig. 5.4.; where (a) represents the control experiment performed in the absence of digitonin and (b) shows the position of the glycosylated polypeptides.

5.7 Discussion

The precise mechanism by which the digitonin interacts with membranes is still unknown. However, it is clear that it combines with free cholesterol, the 3β hydroxy group being essential to form a digitonide which leads to solubilisation or disruption of the membrane (Schnaitman et al., 1967)

Structure of Digitonin



The higher cholesterol content of the outer membrane, as compared to the inner, has been used as a tool in the selective removal of the former by digitonin. This method provides an important means for preparing homogeneous right-side-out mitoplasts (Malviya, 1968), which are particularly useful in the studies of the topography of inner membrane proteins (Racker et al., 1965). These results demonstrate that it is possible to release approx. 50% of the titrated glucosamine label in the soluble form at low levels of digitonin. This is achieved without disruption of the outer membrane as assayed by cytochrome c oxidase activity, using exogenous cytochrome c as substrate, (Wojtczak et al., 1972).

In addition to digitonin, we have also found that washing the mitochondrial fraction in high ionic strength buffer (0.12M-KCl, 20mM-MOPS, pH 7.2), causes release of extra-mitochondrial carbohydrate, suggesting that the effect is not specific to digitonin. Tris-phosphate buffer which causes the mitochondria to swell has a similar effect, though additional carbohydrate containing material is released in this case, since the outer membrane is disrupted to a large extent.

We have tried to correlate the release of this soluble carbohydrate material with loss of specific "lectin receptors", on the premise that the carbohydrate moieties present in digitonin (xylose, galactose and glucose) act as competitive hapten sugars. Recently, evidence has been presented that plant mitochondria have lectin binding proteins which do not appear to be integral membrane components, and can be released with the appropriate monosaccharides (Bowles et al., 1976). However, attempts to release the "lectin receptors" with

xylose and galactose have proved unsuccessful, dismissing this hypothesis for digitonin action on rat liver mitochondria. It also indicates that the glycosylated material is bound to the membrane by electrostatic and/or hydrophobic forces.

Release of 40-50% of the labelled carbohydrate by trypsin confirms that the bulk of the material is exposed on the external surface of mitochondria. Confirmation into the nature of the macromolecular [^{3}H]-glucosamine containing material released by KCl and digitonin treatment is obtained by ion exchange chromatography on a DEAE-cellulose column. One major peak elutes which coincides with protein rather than hyaluronic acid or chondroitin sulphate (Stein et al., 1975; Kramer, 1971). On the basis of this experiment the extra-mitochondrial material appears to be glycoprotein.

Attempts to specifically define the nature of the glyco-protein material by Con A immunoprecipitation reveals a major doublet of mol. wt. 48,000 and 40,000 respectively. These mol. wts. are identical with those reported for the hepatic binding glycoprotein in rabbit liver plasma membrane, which functions by removing serum asialoglycoproteins from the circulation (Hudgin et al., 1974). More recently, the same group of people (Pricer and Ashwell, 1976) have presented evidence for a similar component in rat liver mitochondria that binds to desialylated serum glycoproteins. The correlation to the macromolecular species in our system is strong. However, as regards our data in which we show that the glycoprotein material is loosely associated with the membrane, the studies of Pricer and Ashwell (1976) are

questionable, as the existence of a precursor pool of the rabbit liver protein within the cell has been demonstrated (Hudgin et al., 1974). In this context, it can be speculated that the protein detected in mitochondria is not a genuine component, but binds adventitiously to the organelle during isolation.

Thus, it appears that this extramitochondrial carbohydrate material is bound to the membrane by both electrostatic and hydrophobic interactions. Release of this material can be achieved by both digitonin and ionic strength under controlled conditions which do not destroy the integrity of the outer membrane, to an extent which allows cytochrome c to penetrate to its binding site.

Hence, it seems likely that the effect of digitonin on mitochondria is not related to its specific interaction with cholesterol, an may be mimicked by KCl and other detergents too.

CHAPTER 6

The asymmetric distribution of proteins in the inner and outer membranes of rat liver mitochondria.

6.1 Introduction

One of the most popular methods for identification of proteins that are exposed on the outer surface of biological membranes, involves co-valent modification utilizing radioactive membrane impermeant probes. These studies are of particular importance as, at a molecular level, membrane function ultimately depends on the arrangement of these components within the membrane.

Although the topography of membrane proteins in certain cells has been well established, especially in the plasma membrane of the red blood cell (Carraway, 1975), comparable information for mitochondria is lacking, partly due to the technical difficulties experienced in isolating organelles with an intact outer membrane. This is particularly evident when proteolytic enzymes are used to soften the tissue, such as in rat heart, where mitochondria have an excessively damaged outer membrane (Wojtczak et al., 1972).

We have performed our studies on rat liver mitochondria which have provided us with a most suitable system, mainly because the organelles can be isolated with minimum disruption of the outer membrane (3-5% broken), an important aspect overlooked by other workers (Huber and Morrison, 1973). Secondly, the double membrane system of mitochondria allows selective iodination of the individual membranes, as the outer membrane limits the access of macromolecular reagents such as lactoperoxidase (Morrison and Bayse, 1970). Specific

"tagging" of inner membrane proteins can be achieved by employing the detergent digitonin, which allows removal of the outer membrane to varying degrees (Schnaitman et al., 1967), resulting in an intact mitoplast with the same orientation as in mitochondria. The detergent has no apparent effect on the integrity of the inner membrane which can be monitored by checking for iodination of carbamyl phosphate synthetase, a protein enclosed within the matrix (Clarke, 1976a) which is easily detectable on SDS-polyacrylamide gels.

Thus, by lactoperoxidase-catalysed radio-iodination of exposed tyrosine or possibly histidine residues, we have compared the surface labelling patterns of intact mitochondria and mitoplasts, in which the outer membrane has been removed to varying degrees. The evidence presented here suggests that there are 4 classes of proteins. One of these consists of polypeptides that are exposed on the outer mitochondrial surface. A second class consists of proteins which appear only when approx. 50% of the outer membrane is released, and thus appear to be located in the inter-membrane space, or on the inner side of the outer membrane. A third class comprises of proteins exposed on the inner membrane, which become increasingly more prominent as the outer membrane is removed. Finally, the last class is representative of proteins which appear to traverse the outer membrane, as well as being labelled on the outer surface of the inner membrane.

6.2 Preliminary investigations of the surface labelled polypeptides in mitochondria and mitoplasts by SDS-polyacrylamide gel electrophoresis.

Early attempts to resolve the proteins which are exposed on the outer surface of mitochondrial membranes, employed SDS-gel electrophoresis of solubilised polypeptides according to the method of Fairbanks et al. (1971).

Intact mitochondria and mitoplasts (digitonin/protein ratio: 0.01, 0.11, 0.20) are iodinated by the lactoperoxidase method and denatured in SDS prior to separation on 5.6% (w/v) gels. Radioactive bands are subsequently detected by gel slicing and counting. The data is expressed in Fig. 6.1. Surface labelling of intact mitochondria reveals at least 9 components of apparent mol. wt. 82,000, 74,000, 59,000, 45,000, 28,000, 25,000, 21,000, 15,000 and 9,000 as assessed by co-electrophoresing mol. wt. standards, indicating a heterogenous population of accessible species on the surface of the organelle. When mitochondria have 50% and 90% of their outer membrane removed, corresponding to a digitonin/protein ratio of 0.11 and 0.18 respectively, there is a marked reduction of incorporated label into all these species. At the same time one new species appears, a major component of apparent mol. wt. 32,000, presumably located on the outer surface of the inner membrane. Treatment of mitochondria with a very low concentration of digitonin (0.01mg/mg protein) reveals no difference in the iodinated profile as compared to intact mitochondria (data not shown). This suggests that none of the major polypeptides are removed at this concentration of detergent.

Fig. 6.1.

SDS-polyacrylamide gel analysis of [^{125}I]-labelled proteins on the external surface mitochondrial outer and inner membranes

Rat liver mitochondria are isolated as described in (M 4.1) after injecting the rat with 3-amino-1, 2,4-triazole (1mg/g body wt.) 1h before sacrifice. Mitochondria are resuspended to a final protein concentration of 20mg/ml in iodination buffer (M 3.6.1.1).

Lactoperoxidase-catalysed iodination (M 7.1) are carried out simultaneously, on intact rat liver mitochondria (●) or mitochondria treated with 0.11mg of digitonin (o) or 0.18mg of digitonin/mg of protein (Δ) subfractionated as described in (M 4.3.2). SDS-polyacrylamide gel analysis (5.6% w/v) is carried out by the method described in (M 8.1) after samples containing equal amounts of radioactivity are applied to the gels.

The gels are sliced and counted in a Beckmann Biogamma counter (M 8.5.1) after equilibration as described in (M 3.5.1.4) Mol. wts. are estimated by co-electrophoresing standard protein mixtures as in (M 8.5.5).

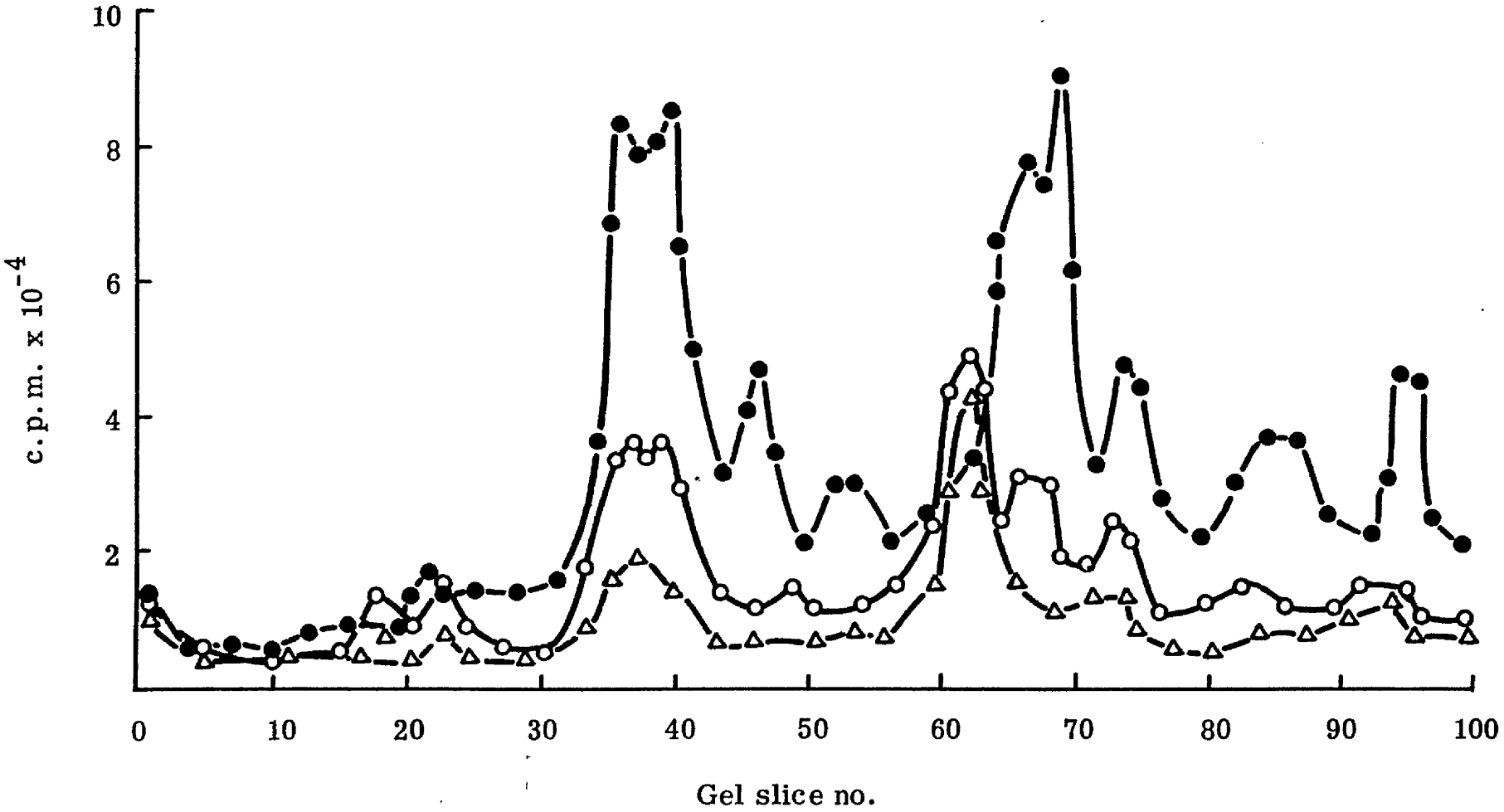
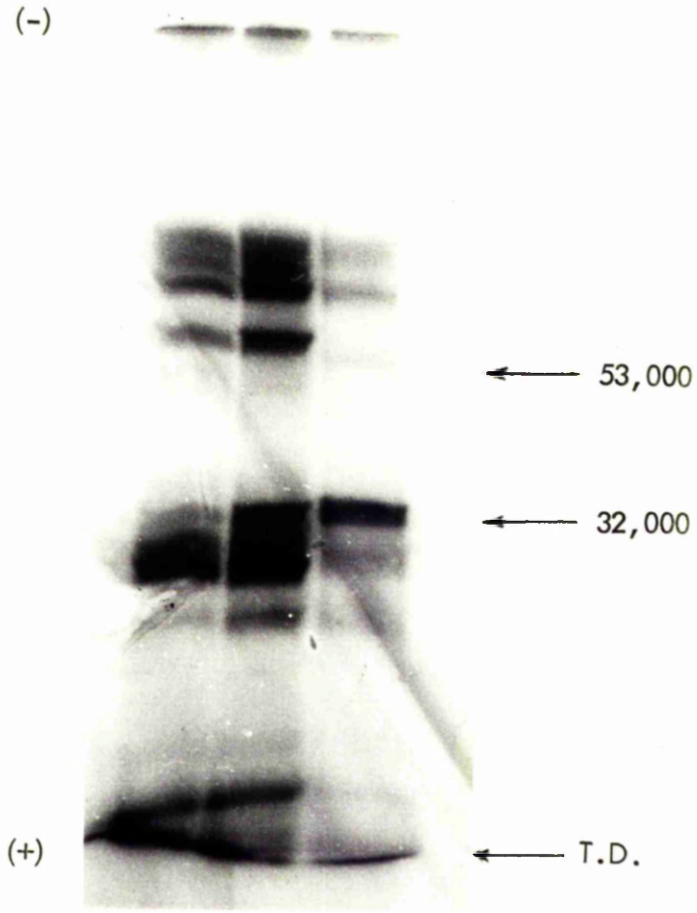

10
8
6
4
2
c.p.m. x 10^{-4}
0
10
20
30
40
50
60
70
80
90
100
Gel slice no.

Fig. 6.2.

Comparison of [^{125}I]-labelling patterns on the external surface of intact mitochondria and mitoplasts prepared by digitonin fractionation

A female rat is injected with 3-amino-1, 2,4-triazole (1mg/g body wt.) 1h prior to sacrifice. Mitochondria are isolated as in (M 4.1) and resuspended in iodination buffer (M 3.6.1.1). Aliquots of the mitochondrial fraction are treated with 0.11mg of digitonin or 0.20mg of digitonin/mg of protein as described in (M 4.3.2).

Intact mitochondria (a) or mitochondria where the outer membrane is partially (b) or almost wholly removed (c) are iodinated by the lactoperoxidase method (M 7.1). SDS-polyacrylamide gel (10% (w/v))analysis is carried out as in (M 8.2) after application of samples containing equal amounts of radioactivity (100,000cpm). Gels are processed for fluorography (M 8.5.2) and exposed for 4 days. Mol. wts. are estimated by co-electrophoresing standard protein mixtures (M 8.5.5).


(-)
53,000
32,000
(+)
T.D.
(a)
(b)
(c)

6.3 SDS-polyacrylamide gel electrophoresis of labelled polypeptides in mitochondria and mitoplasts.

In this experiment, we demonstrate the improved resolution and sensitivity of the autoradiographic/fluorographic method for detection of labelled polypeptides as compared to the gel slicing technique.

Labelled mitochondria and mitoplasts (digitonin/protein ratio: 0.11, 0.20) are electrophoresed on 10% (w/v) SDS slab gels, according to the method of Laemmli (1970). The result is photographed in Fig. 6.2. Detection of the labelled bands by autoradiography reveals at least 9 components in intact mitochondria of mol. wt. similar to that described in section 6.2. In mitoplasts, where 50% of the outer membrane has been removed, the 32,000 mol. wt. component is clearly visible. In addition, removal of 90% of the outer membrane reveals labelling of a minor species of mol. wt. 53,000.

6.4 Assessment of mitoplasts prepared by various methods

In the previous experiments, exposed proteins in mitochondria and mitoplasts are iodinated by use of the membrane impermeant probe, lactoperoxidase. The pattern of labelled polypeptides in both cases is very similar, although there is a reduction in the amount of label incorporated into the exposed polypeptides in mitoplasts. It is possible that the similarity of the iodinated profiles of mitoplasts and mitochondria is due to incomplete removal of the outer membrane in mitoplasts. We have further investigated this by employing a variety of methods to prepare mitoplasts from mitochondria that have been washed in KCl containing buffer, which removes

Table 6.1

Assessment of mitoplasts prepared by various methods.

Rat liver mitochondria are isolated from a single female rat as described in (M 4.1). The mitochondria are further washed in 0.12M-KCl, 20mM-MOPS, pH 7.2 and resuspended in the same buffer to 20mg of protein/ml.

Aliquots of the mitochondrial suspension (1ml) are then incubated in 1.5ml of the various buffers listed in Table 6.1 for 15 min at 4°C. The mitochondria are pelleted by centrifugation at 6,800g for 10 min and resuspended in 1ml of the KCl buffer. The mitochondrial fractions (0.1ml) are then monited for breakage of the outer membrane by assessing cytochrome c oxidase activity (M 5.2 (b)).

Table 6.1

Preparation of mitoplasts	cytochrome c oxidase n mol O_2/min/mg protein		% breakage
	- DOC	+ DOC 0.3% (w/v)	
0.12M-KCl, 20mM-MOPS, pH 7.2	19.0	557.4	3.4
0.12M-KCl, 20mM-MOPS, pH 7.2, 0.22mg digitonin/ mg protein.	429.2	557.4	77.0
10mM-Tris-phosphate, pH 7.2	403.5	620.76	65.0
10mM-Tris -phosphate, pH 7.2, 1.8M-sucrose, 2mM-$MgSO_4$, 0.22mg digitonin/ mg protein	658.1	685.5	96.0

any loosely-bound material from the external surface (see section 5.3). Cytochrome c oxidase activity is simultaneously assessed so as to determine the breakage of the outer membrane.

The data in Table 6.1 which monitors the breakage of the outer membrane shows that the best method for removing the outer mitochondrial membrane is by swelling in Tris-phosphate and shrinking in the presence of ATP and Mg^{2+} followed by digitonin treatment. In this case the % breakage is estimated at 96.0. If the swelling and shrinking step is omitted, mitochondria are only 77.0% broken, suggesting that a significant amount of outer membrane is still associated with the mitoplast. The monoamine oxidase activity (M 5.1(b)) remaining in the mitochondrial pellet after Tris-phosphate, ATP and digitonin treatment is approx. 3.0%, indicating a minimal contamination of the outer membrane.

6.5 SDS-polyacrylamide gel electrophoresis of iodinated mitochondria and mitoplasts.

Autoradiographic visualisation of labelled polypeptides.

In this experiment we have employed the refined methodology developed for preparing mitoplasts, together with the more sensitive fluorographic procedure for detecting labelled bands, in an attempt to obtain a comprehensive picture of the topography of proteins in mitochondrial membranes.

Mitochondria and mitoplasts are iodinated using lactoperoxidase as catalyst, and solubilised polypeptides separated on SDS-polyacrylamide slab gels prior to visualisation of the labelled bands by fluorography. The result is photographed

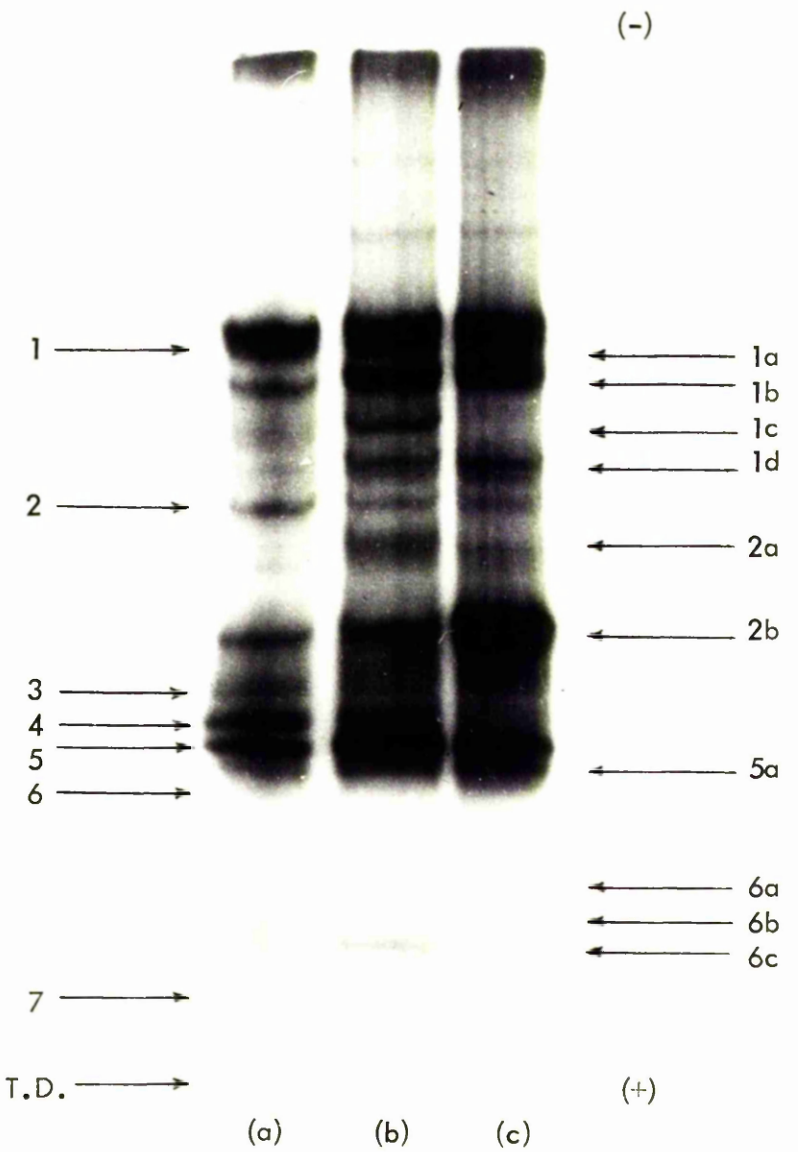
Fig. 6.3a.

SDS-polyacrylamide slab gel analysis of surface iodinated proteins on the external surface of rat liver mitochondria and mitoplasts.

A female rat is injected with 3-amino-1,2,4-triazole (1mg/g body wt.) 1h prior to sacrifice. Mitochondria are then isolated as usual (M 4.1), and resuspended in 0.12mM-KCl, 20mM-MOPS, pH 7.2. An aliquot of the mitochondrial suspension (20mg) is swollen in Tris-phosphate (M 3.1.2) and shrunk in the presence of ATP and Mg^{2+} (M 3.1.3) as described in (M 4.3.1) prior to centrifugation at 6,800g for 10 min.

Lactoperoxidase-catalysed iodinations are carried out simultaneously on intact rat liver mitochondria (a) or swollen and shrunk mitochondria treated with 0.10mg of digitonin (b) or 0.20mg of digitonin/mg of protein (c) (M 4.3.2). Samples are maintained in high salt buffer (M 3.12.3) throughout the procedure before washing (x4) in the same buffer containing 1mM-KI.

SDS-polyacrylamide gel electrophoresis is carried out on slab gels (M 8.2) after application of solubilised samples (M 8.4.1) each containing 300,000cpm. Gels are processed for fluorography (M 8.5.2) and labelled polypeptides visualised after a 3 day exposure. Mol. wts. are estimated by co-electrophoresing standard proteins mixtures as described in (M 8.5.5).

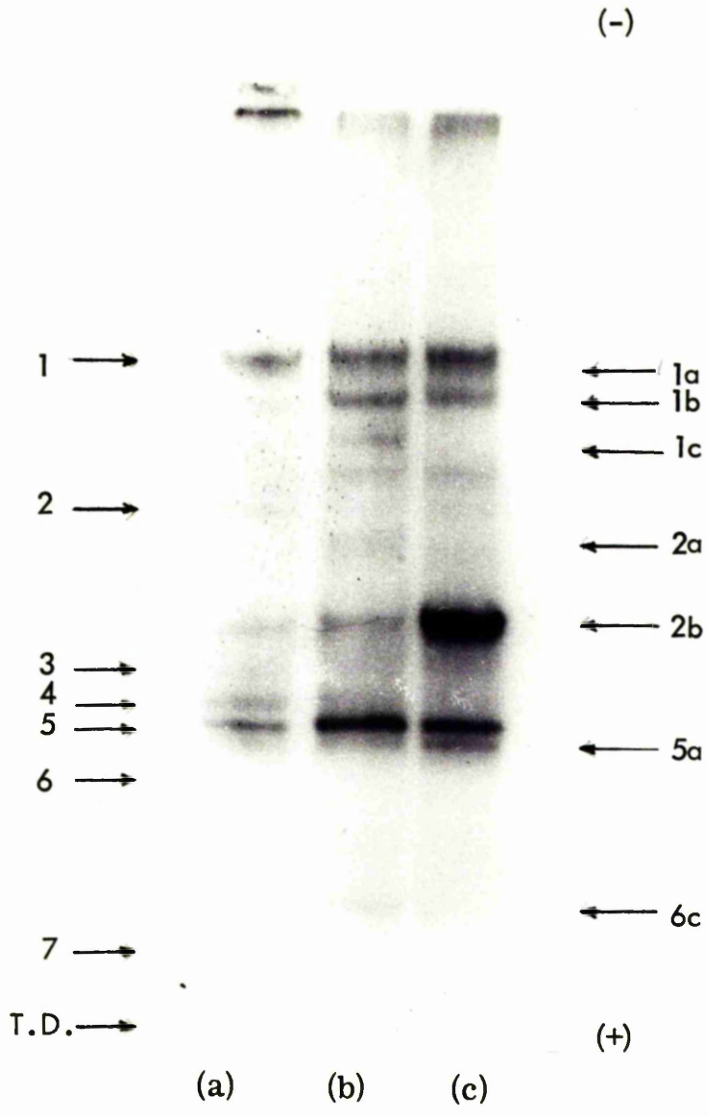

(-)
1
2
3
4
5
6
7
T.D.
1a
1b
1c
1d
2a
2b
5a
6a
6b
6c
(+)
(a)
(b)
(c)

in Fig. 6.3(a) and (b) after a 3 day and 1 day exposure respectively. The data summarised in Table 6.2. shows that 7 polypeptides are exposed at the exterior surface of the outer membrane because they are iodinated by the membrane impermeable labelling system in intact mitochondrial : 1, 2, 3, 4, 5, 6 and 7. The mol. wts. of these designated bands are : 83,000, 49,000, 28,000, 26,000, 23,000, 15,000 and 11,000 respectively. In mitoplasts, where 97% of the outer membrane is removed, 8 species are labelled. Apart from bands 1 and 5, the remaining bands appear to be inner membrane components : 1a, 1b, 2b, 5a, 6a and 6b, and are assigned mol. wts. of 82,000, 74,000, 31,000, 22,000, 19,000 and 16,500 respectively. Polypeptides 1b, 2b and 5a are also labelled in intact mitochondria where one might have expected that the outer membrane would prevent access of the lactoperoxidase catalyst. However, since the amount of label in these components is small, and increases between 4-7 fold with increasing amounts of digitonin, it is possible that they represent inner membrane proteins, which become labelled in mitochondria that have a disrupted outer membrane. Similarly, band 1d which is thought to be located on the inside of the inner membrane, becomes labelled in mitoplasts, possibly due to disruption of the inner membrane (see Chapter 8). If the outer membrane has only been partially removed (50%), bands 1c, 2a and 6c (mol. wt. 62,000, 43,000 and 14,000 respectively) become exposed, suggesting that the polypeptides are located on the inside of the outer membrane.

Fig. 6.3b

SDS-polyacrylamide slab gel analysis of surface iodinated proteins on the external surface of rat liver mitochondria and mitoplasts

An identical procedure to that described in Legend 6.3(a) is followed, except that labelled polypeptides are visualised after a 24h exposure.


(-)
1
2
3
4
5
6
7
T.D.
1a
1b
1c
2a
2b
5a
6c
(+)
(a)
(b)
(c)

A comparative densitometric scan of the individual sample tracks, together with peak integration values for the labelled bands allowed us to assign the detected polypeptides to one of 4 categories. Referring back to the data in Table 6.2. bands 1 and 5 appear to traverse the outer membrane as well as being labelled equally in mitoplasts and are included in category 4. Bands 2, 3, 4 and 7 are exclusive outer membrane components and assigned to category 1.

Similarly, bands 1a, 1b, 2b, 5a, 6a and 6b appear to be inner membrane components belonging to category 3. In category 2 are components that become labelled only when the outer membrane is partially removed, suggesting a possible inter-membrane location for bands 1c, 2a and 6c.

6.6 Polyacrylamide gel electrophoresis of [^{125}I]-lactoperoxidase.

One of the problems experienced with lactoperoxidase catalysed iodination is its ability of "self-iodination" (David and Reisfeld, 1974). It is probable that one of the high mol. wt. bands identified as being a mitochondrial polypeptide corresponds to the autolabelled enzyme. This experiment is performed to clarify the situation.

Lactoperoxidase is "self-iodinated" by excluding mitochondria in the iodination mixture until the washing stage (see Materials and Methods M7.1). [^{125}I]-lactoperoxidase, electrostatically bound to mitochondria, is then co-electrophoresed with labelled mitoplasts so as to identify the banding position of the enzyme. On visualisation of the labelled

Table 6.2.

Summary of information derived from [^{125}I] surface labelling studies of rat liver mitochondria and mitoplasts by digitonin fractionation.

The information obtained from Fig. 6.3a and 6.3b is summarised in this Table. The major proteins detectable on the surface of intact rat liver mitochondria are assigned numbers (1-7) in order of decreasing mol. wt. Components which are allocated a letter of the alphabet in addition to the numeral denote species in the appropriate mol. wt. range that only become labelled as the outer membrane is removed.

A densitometric scan (Joyce Loebl Chromoscan, Mark II, Gateshead, Northumberland) of the fluorographs has allowed us to quantitate the degree of labelling of individual components in mitochondria and mitoplasts (integration values). These have been employed to assign the bands into a category (1-4) suggesting a location for these species.

Table 6.2

BAND DESIGNATION						
Intact mitochondria		Mitoplasts		Mitoplasts		
Band designation	Peak Integration	Digitonin/protein: 0.12	Peak Integration	Digitonin/protein: 0.18	Peak Integration	Assigned category
1		1		1		4
-	47	1a	62		70	3
1b(?)	11	$1b^{++}$	31	$1b^{++}$	43	3
-	-	1c	26	-	-	2
-	-	1d(?)	29	1d(?)	46	3(?)
2	19	-	14	-	-	1
-	-	2a	37	-	-	2
2b(?)	22	$2b^{+}$	49	$2b^{++}$	150	3
3	23	-	-	-	-	1
4	27	-	-	-	-	1
5	42	5	73	5	73	4
5a(?)	10	5a	35	5a	46	3
6	11	-	-	-	-	1
-	-	6a	11	6a	16	3
-	-	-	-	6b	29	3
-	-	6c	31	6c	13	2
7	14	-	-	-	-	1

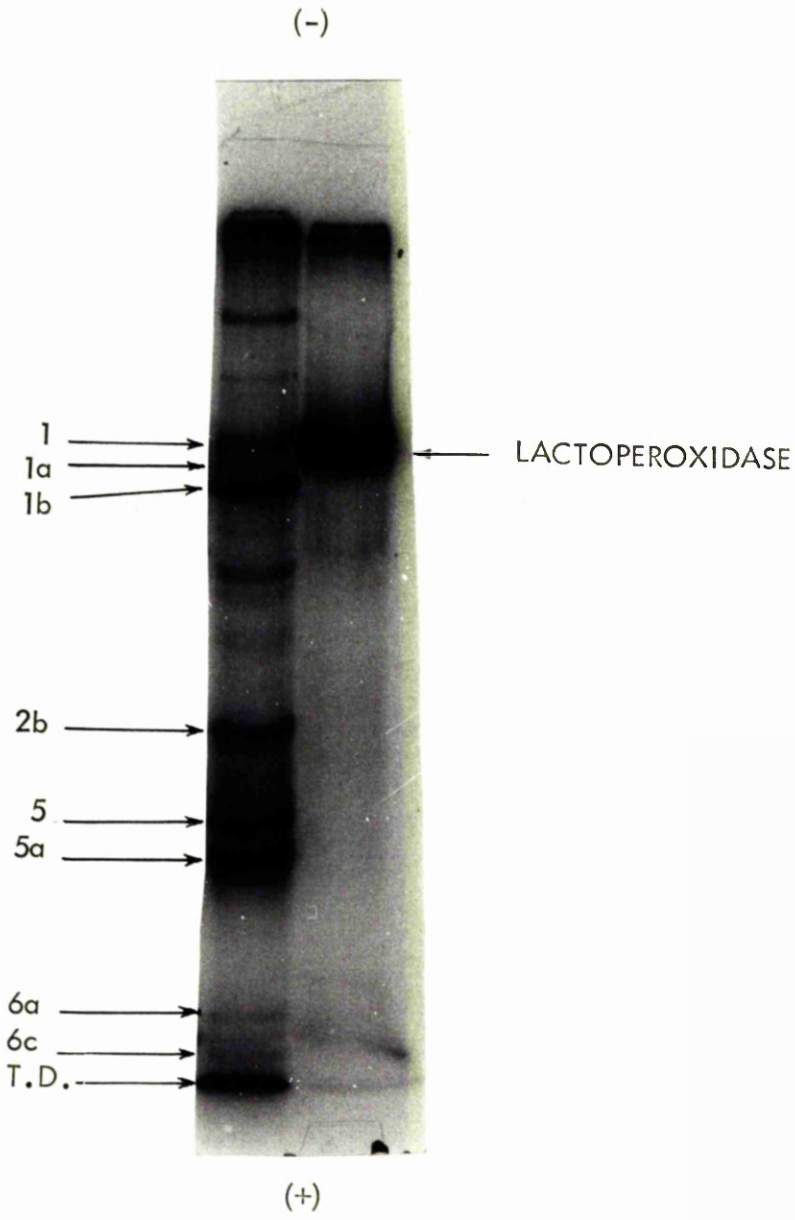
KEY: Category 1: outer membrane; Category 2: intermembrane; Category 3: innermembrane
Category 4: transmembrane.

Fig. 6.4.

Identification of auto-labelled lacto-peroxidase bound to rat liver mitochondria by SDS-polyacrylamide gel electrophoresis.

Rat liver mitoplasts are prepared and iodinated as described in Legend 6.2. Lactoperoxidase is auto-labelled by following the identical procedure to that described in the legend except for the omission of mitoplasts (200-400µg) which are added after completion of iodination. The samples are then treated in the usual manner.

Labelled mitoplasts (a) and lactoperoxidase-bound mitoplasts (b) are denatured in SDS and 2-mercaptoethanol (M 8.4.1) and solubilised samples containing equal counts (180,000cpm) are electrophoresed as in (M 8.2). Gels are processed for fluorography (M 8.5.2) and labelled bands detected after a 9h exposure.


(-)
1
1a
1b
LACTOPEROXIDASE
2b
5
5a
6a
6c
T.D.
(+)

bands, lactoperoxidase appears to coincide with band 1 (Fig. 6.4). However, later on in the discussion we suggest why we consider band 1 to be a mitochondrial polypeptide rather than autolabelled lactoperoxidase.

6.7 Total labelling of mitochondrial proteins

In order to check the validity of lactoperoxidase as a membrane impermeant labelling reagent, a comparative labelling experiment using Bolton-Hunter reagent is performed.

Bolton-Hunter reagent offers the additional advantage of labelling all amino groups, as revealed by the much larger heterogeneous population of labelled species, including carbamyl phosphate synthetase, the matrix component. Fig. 6.5. (a) and (b) compare a Coomassie Brilliant Blue G-250 stain of mitochondrial polypeptides and the subsequent autoradiograph of the components labelled with the reagent. The result shows that most of the proteins visible by Coomassie blue staining, are also labelled with Bolton-Hunter reagent, indicating the membrane permeability of the reagent.

6.8 Discussion

In assessing the validity of surface labelling studies, two important criteria must always be considered : the extent of disruption of the preparation during the labelling procedure, and the "sidedness" of the inner membrane vesicles prepared by digitonin fractionation of mitochondria.

We have carefully quantitated the integrity of the outer mitochondrial membrane by measuring the latency of cytochrome c

Fig. 6.7.

Iodination of intact rat liver mitochondria with N-succinimidyl 3-4-hydroxy, 5 [^{125}I] - iodophenyl propionate (Bolton Hunter reagent).

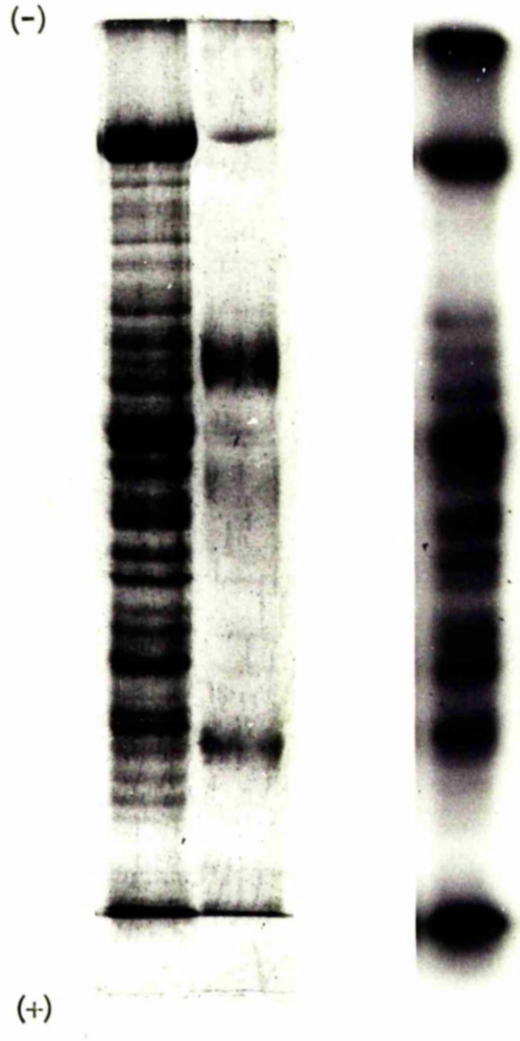
Rat liver mitochondria are isolated (M 4.1) and finally resuspended in 0.2M-mannitol, 0.15M-sucrose, 10mM-Borate, pH 8.0. An aliquot (200µg) is iodinated with 200µCi of Bolton Hunter reagent as described in (M 7.3) and the sample washed (x4) in iodination buffer containing 1mM-KI (M 3.6.1.1).

The iodinated mitochondria are denatured in SDS (M 4.1) and an aliquot of the sample containing 1 x 10^6 cpm is electrophoresed on a 10% (w/v) acrylamide slab gel as described in (M 8.2). The gel is stained with Coomassie Brilliant Blue to visualise the polypeptides (M 3.5.1), prior to drying on to 3mM Whatman paper. Labelled bands are detected by autoradiography after a 5h exposure (M 8.5.3). Mol. wt. standards proteins are co-electrophoresed as described in (M 8.5.5).

(a) Coomassie Blue stain

(b) mol. wt. standards (RNA polymerase and cytochrome c)

(c) Autoradiograph corresponding to (a)


(-)
(+)
(a)
(b)
(c)

oxidase, an inner membrane protein complex, using exogenous cytochrome c as substrate. All labelling studies are subsequently performed on the M_1 mitochondrial fraction, rather than on the purified organelles (PM_1), as in the first case the outer membrane is damaged between 3-5%, as compared to 10-15% after sucrose gradient purification. In mitoplasts disruption of the inner membrane is monitored by measuring the label incorporated into a protein that appears to be totally contained within the inner membrane, carbamyl phosphate synthetase. This accounts for 15-20% of the total mitochondrial protein and migrates with a mol. wt. of 165,000 on dodecyl sulphate gel electrophoresis (Clarke, 1976a). Both these controls are extremely important, because it is necessary to assign a component to its correct location, and complications can arise if the membranes are excessively disrupted, and leaky to the membrane impermeant probes. Further, digitonin prepared mitoplasts may not be uniformly sealed or RSO (Astle and Cooper, 1974).

The experimental conditions used in these studies are adjusted so that asymmetric labelling occurs, based on the fact that the outer mitochondrial membrane is reported to be impermeable to solutes of mol. wt. greater than 4,000 (Pfaff et al., 1968). By this criterion lactoperoxidase (mol. wt. 80,000) should not permeate the membrane, and consequently the proteins iodinated limited to those exposed on the external surface. The lack of label in carbamyl phosphate synthetase justifies this assumption. However, component 1d is labelled to a small extent, possibly in a population of disrupted mitoplasts (see Chapter 8). The enzymatic method

for the radio-iodination of proteins has been found to minimise damage to labelled proteins maintaining their structural integrity, (Miyachi and Chrambach, 1972) resulting in preparations with a high retention of immunological and other biological activities.

In our iodination experiments we have always injected the rat with 3-amino-1,2,4-triazole 1h prior to sacrifice (Price et al., 1962). Mitochondria that are subsequently isolated have greatly reduced levels of catalase which competes for hydrogen peroxide, the substrate of lactoperoxidase. In this manner, we have obtained proteins that have high specific radioactivity. The use of butylated hydroxy toluene in the iodination mixture which is thought to prevent lipid peroxidation and subsequent membrane damage (Welton and Aust, 1972) has no special protective effect, hence we have omitted its further use in this study.

The data obtained from our surface labelling studies suggest that mitochondrial polypeptides can be divided into 4 classes. Bands designated 2, 3, 4, 6 and 7 appear to be exclusively located on the external surface of the outer membrane. Other proteins such as bands 1b and 2a only become labelled when the outer membrane is partially removed, suggesting that they are located on the inner surface of the outer membrane or possibly in the inter-membrane space. Bands 1a, 2b and 6a appear to be associated with the inner membrane because they are labelled in mitoplasts where 97% of the outer membrane is removed (0.20mg/mg of protein). These polypeptides are also labelled in mitoplasts where the

outer membrane is partially removed as well as in intact mitochondria (1a, 2b). However, since the amount of label in these components is small and increases with increasing amount of digitonin the possibility exists that these proteins are not exposed to the exterior surface of the mitochondria. Their labelling arises due to the exposure of these proteins in a small population of disrupted organelles. The maximum label incorporated into inner membrane components (1a, 2b) on removal of the outer membrane is only 3-7-fold more, rather than the expected 20-fold. This indicates that the outer mitochondrial membrane may be exposed to further damage during the iodination procedure. Alternatively, labelling of these species could be due to the participation in the reaction of free activated iodide, such as I_2 or ICl, rather than a membrane impermeable lactoperoxidase tyrosine-iodide complex (Morrison and Bayse, 1970).

In a fourth category we have designated, belong polypeptides that appear equally labelled in both the outer and inner membranes and include bands 1 and 5. Three explanations could account for this; these polypeptides may represent single molecules which traverse both membranes. Second, two polypeptides of similar mol. wt. could be present on both the inner and outer membrane or, third, it could be due to the presence of contact points that are present between the inner and outer membrane (Hackenbrock, 1968a). If this is the case, then it would be impossible to prepare outer membrane free mitoplasts. The latter point has also been considered by Huber and Morrison (1973) as an explanation for the distinct similarity in labelling

patterns of the external surfaces of the inner and outer mitochondrial membranes.

A major limitation of the lactoperoxidase iodination method is the problem of accessible tyrosines, these being the amino residues into which the iodine atom is inserted. Therefore, proteins with no accessible tyrosine residues escape substitution. In this regard, we have assumed that if a protein is not labelled on a particular membrane face it is due to its absence, rather than lack of appropriate amino residues.

We have also attempted to see if any of the labelled bands correspond to autolabelled lactoperoxidase. On co-electrophoresis of [^{125}I] lactoperoxidase with solubilised mitoplasts (Fig. 6.4) it appears that the enzyme may correspond to band 1. However, the fact that WGA specifically binds to band 1 in the absence of lactoperoxidase, argues against this possibility (see Chapter 7).

Also, in later experiments mitochondria are always washed in ionic strength buffer after iodination; this could aid removal of adventitiously bound lactoperoxidase. A corollary of these findings is the appearance of lactoperoxidase as a single band. This indicates there is no proteolytic degradation of the sample during the time course for iodination and all the bands correspond to genuine components.

Other authors have also used lactoperoxidase to study the location of mitochondrial polypeptides (Huber and Morrison, 1973). However no attempt has been made to

correlate the autolabelled enzyme with a specific band on acrylamide gels. Furthermore, although small amounts of the intermembrane marker enzyme, adenylate kinase are released in their preparations of mitochondria, they did not consider that the breakage of the outer membrane in a small population of mitochondria would result in the labelling of inner membrane proteins.

These results are in broad agreement with these of Clarke (1976b) who has reported at least 6 components that are exposed at the exterior surface of the inner membrane, as well as being labelled in intact mitochondria, but to a lesser extent. The author however has failed to quantitate the extent of disruption of the outer mitochondrial membrane.

It is known that several components of the respiratory chain are exposed on the cytoplasmic surface of the inner membrane and it may be possible to correlate these with labelled polypeptides identified in this study. However, before a definite function can be assigned to a component it is necessary to have some criteria with which it can be specifically associated, e.g. atractyloside is a competitive inhibitor of the adenine nucleotide translocator and thus allows its identification by specifically binding to the translocase. By this criterion Boxer et al. (1977) have identified the 29,000 mol. wt. protein in beef heart mitochondria as the adenine nucleotidase transporter. In their system, this protein appears to be the major labelled component on the inner membrane and constitutes 10% of the

total membrane protein. It correlates very strongly with component 2b in our study which is the major labelled species on the cytoplasmic surface of the inner membrane. Also, Coty and Pederson (1975) have identified 5 components of phosphate transport in rat liver mitochondria. Two of these correspond in mol. wt. to components identified here of mol. wt. 53,000 and 32,000 on the inner mitochondrial surface. However, identification and functional correlation of these proteins is an area of much potential and requires further study.

CHAPTER 7

Investigation of mitochondrial glycoproteins by use of lectins.

7.1 Introduction

In the previous chapter we have identified the major protein components that are exposed on the external surfaces of the outer and inner mitochondrial membranes. In this Chapter we attempt to distinguish between the glycosylated, and non-glycosylated components exposed on the external membrane surfaces by using the technique of lectin immunoprecipitation, in conjunction with lactoperoxidase-catalysed surface iodination of mitochondria and mitoplasts.

The study of cell surface glycoproteins has been considerably facilitated by the use of lectins, which have carbohydrate binding sites displaying a variety of specificities for individual monosaccharides. These interactions can be easily reversed by using appropriate sugars as haptenic inhibitors (for review, see Lis and Sharon, 1973). It is these properties of lectins that have been exploited for identification and characterisation of the different surface receptors.

7.2 Applications of lectins to Membrane Biochemistry

1. Direct agglutination and binding studies

Lectins are cell-agglutinating and sugar specific proteins which are useful tools for studying polysaccharides, glycoproteins, and the presence of carbohydrate on cell surfaces. For the formation of precipitates with complementary molecules or for the

agglutination of cells, a lectin must possess at least 2 binding sites. Many lectins agglutinate the erythrocytes of all human blood groups, indicating that they bind to saccharide receptors which are present on the surface of the red blood cell, e.g. WGA binding indicates the presence of GlcNAc on the cell surface. By using radio-iodinated lectins, we have exploited this property of lectins to demonstrate the presence of carbohydrate located externally on the outer and inner mitochondrial membranes.

2. Affinity chromatography.

This method employs lectins covalently bound to Sepharose 4B to isolate lectin receptor proteins from solubilised extracts. In the past, isolation of binding receptors has been impeded by the insolubility of membrane proteins in orthodox biochemical solvents, while more drastic solubilisation procedures destroy the biochemical activity.

However, glycoproteins specifically associated with the membrane can be solubilised by treatment in 1% (w/v) DOC (Allan and Crumpton, 1971), or non-ionic detergents (Lotan et al., 1977) without loss of lectin binding activity. The lectin binding proteins may be subsequently isolated on a column of Con A-Sepharose, as we have done for mitochondria.

We have further exploited the idea to isolate homogeneous populations of ISO vesicles from a mixed vesicle population of the inner membrane. This is

based on the premise that the carbohydrate on mitochondria is asymmetrically distributed on the membranes. Thus, using lectins as an external ligand for accessible carbohydrate, we have selectively purified ISO vesicles (Walsh et al., 1976).

3. Immune-precipitation

This approach begins with the solubilisation of lectin receptors from radio-labelled membrane by an ionic or non-ionic detergent. The solubilised membrane components are then allowed to react with a specific lectin, and the lectin-glycoprotein complex precipitated by an antibody directed against the lectin. The precipitates are then analysed by SDS-polyacrylamide gel electrophoresis (Juliano and Li, 1976; Henkart and Fisher, 1975)

Using this technique we have attempted to identify the different surface receptors for Con A and WGA in mitochondria and mitoplasts.

4. Lectin Overlays.

This procedure is particularly useful as it allows rapid analysis of several samples simultaneously, and has been employed by several groups e.g. Tanner and Anstee (1976) who have identified the lectin binding receptors in the human erythrocyte membrane.

This technique for identifying glycoprotein components of membranes involves running the sample under denaturing and reducing conditions on SDS-

polyacrylamide gels. After fixation, the gel is incubated with radio-iodinated lectin and bands to which the lectin is bound can be visualised and compared by autoradiography.

We have adopted the method to identify the total lectin binding complement in mitochondrial membranes using [^{125}I]-Con A and WGA.

7.3 Lectin agglutination and binding studies to rat liver mitochondria and mitoplasts.

The presence of carbohydrate on the mitochondrial membranes has been demonstrated by several groups of workers (Glew et al., 1973; Henning and Uhlenbruck, 1973). In earlier data, we have shown that approx. 50% of the total glycoprotein complement is only loosely-associated with the organelle, and can be released under controlled conditions which do not disrupt the integrity of the outer membrane. These experiments are performed to investigate if any carbohydrate remains exposed on the external surfaces of mitochondrial membranes after treatments which remove these extrinsic proteins.

(a) Agglutination studies on mitochondria pre-treated with KCl or digitonin.

Isolated mitochondria are resuspended in MS-MOPS and aliquots of the suspension incubated in ionic strength buffer or MS-MOPS buffer containing digitonin as described in Fig. 7.1. The mitochondria/mitoplast fractions are then treated with increasing quantities of WGA. After low speed centrifugation

Fig. 7.1.

Agglutination by WGA of rat liver mitochondria and mitoplasts pretreated with KCl or digitonin

Mitochondria are isolated from the livers of 2 female rats and finally resuspended in 4ml of MS-MOPS, pH 7.2 (M 3.8.1). Aliquots of the mitochondrial suspension (20mg protein) are washed twice in one of the buffers listed below by centrifugation at 6,800g for 20 min and finally resuspended in 1ml of MS-MOPS, pH 7.2.

(i) MS-MOPS, pH 7.2 (M 3.8.1) (●)

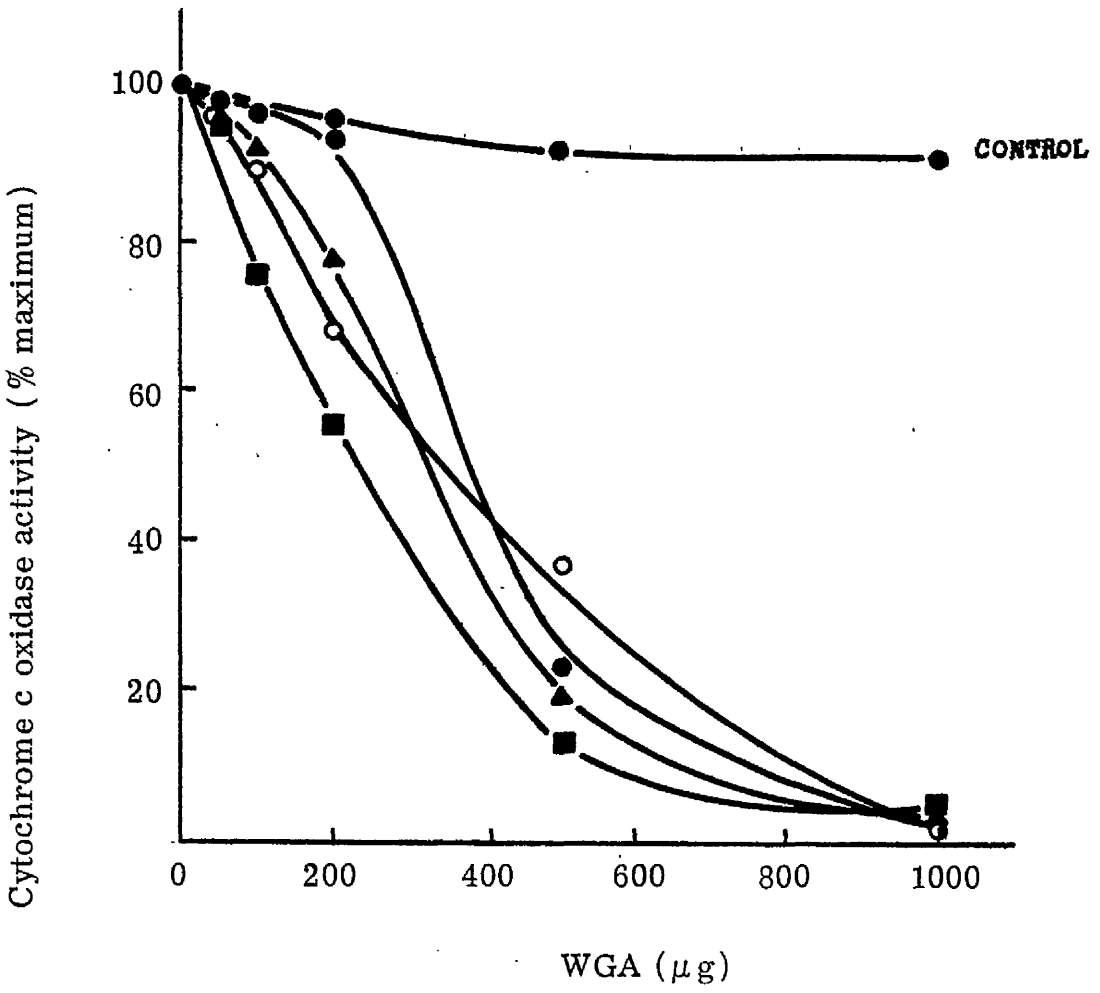
(ii) 0.12M-KCl, 20mM-MOPS, pH 7.2 (▲)

(iii) MS-MOPS, pH 7.2 containing 0.01mg of digitonin/mg protein (o)

(iv) MS-MOPS, pH 7.2 containing 0.22mg of digitonin/mg protein after the mitochondria have been swollen and shrunk as described in (M 4.3.2) (■)

Samples of the mitochondrial fraction containing 0.3mg of protein are then treated with increasing quantities of WGA by incubation at 25°C for 20 min. The agglutinated mitochondria are centrifuged at 600g for 10 min and the supernatant fractions assayed for residual cytochrome c oxidase activity (M 5.2(b)).

The top curve (labelled in the fig.) represents a control experiment performed in the presence of 0.2M-GlcNAc.


100
80
60
40
20
0
200
400
600
800
1000
CONTROL
Cytochrome c oxidase activity (% maximum)
WGA (μg)

of the agglutinated mitochondria/mitoplasts, the supernatant fractions are assayed for cytochrome c oxidase activity. The data in Fig. 7.1. shows the percentage of enzymic activity remaining in the supernatant fractions after lectin treatment.

The result suggests that despite treatment of mitochondria with ionic strength or low levels of digitonin which remove approx. 50% of the total carbohydrate, the organelles did not display any reduction in agglutinability with WGA. Similarly, when mitochondria are depleted partially or wholly of their outer membrane by digitonin fractionation, the resulting organelles are equally susceptible to agglutination. Thus, it can unequivocally be concluded that both the outer and inner mitochondrial membranes contain accessible carbohydrate on their external surfaces which is susceptible to direct agglutination by WGA.

The presence of approx. 90% of the total cytochrome c oxidase activity in the presence of GlcNAc shows that the complex formed between lectin and saccharide residues is specific and can be prevented in the presence of the appropriate competitive monosaccharide.

(b) Binding studies on mitochondria and mitoplasts.

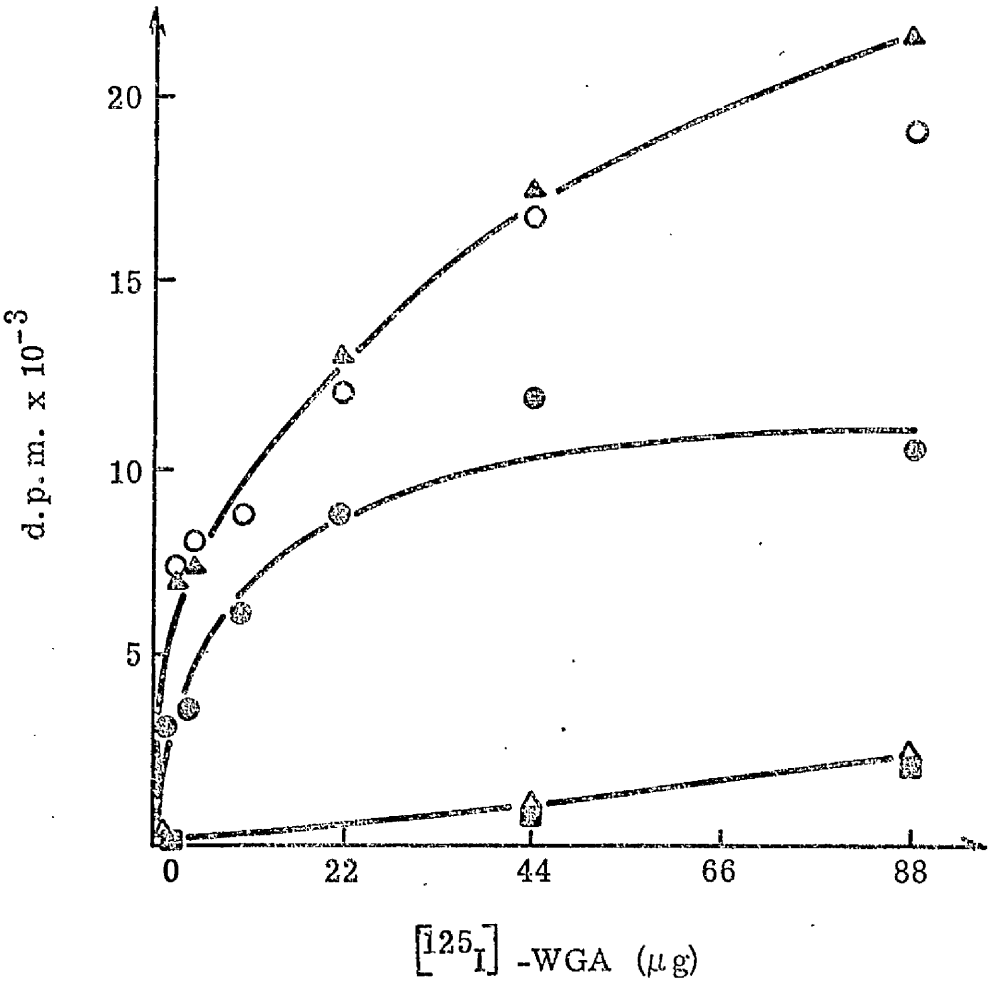
[^{125}I]-WGA binding studies are performed on mitochondria and mitoplasts washed in ionic strength buffer prior to digitonin fractionation as described in Fig. 7.2. The results show that the number of binding sites for WGA in mitochondria treated with the two digitonin concentrations (0.01, 0.22mg digitonin/mg protein) is almost double that present in intact mitochondria. Inhibition of the binding to the lectin

Fig. 7.2.

Binding studies of [^{125}I]- WGA to exposed lectin binding receptors in rat liver mitochondria and mitoplasts

Mitochondria are isolated from the liver of a single rat as described in (M 4.1), and resuspended in 0.12M-KCl, 20mM-MOPS, pH 7.2.

[^{125}I]-WGA (M 7.2(b)) binding studies are carried out simultaneously in siliconised eppendorf tubes on intact rat liver mitochondria (●) or mitochondria treated with 0.01mg of digitonin (o) or swollen and shrunk mitochondria treated with 0.22mg of digitonin/mg protein (▲) as described in (M 4.3.2). Samples are maintained in 0.12M-KCl, 20mM-MOPS, pH 7.2 throughout the procedure before washing (x4) in the same buffer containing 1mM-KI. A control experiment (■) in which 0.1M-GlcNAc is present during the lectin incubation and washing stages is also performed. Mitochondrial pellets are measured for radioactivity in a Beckmann Biogamma counter. Another control experiment adopting the identical procedure is also performed, except that addition of the mitochondrial sample is omitted. The empty, washed tubes are counted for residual [^{125}I]-activity bound to the tube (Δ).


20
15
10
5
0
22
44
66
88
d.p.m. x 10^{-3}
[^{125}I] -WGA (μg)

receptors is carried out by including 0.1M-GlcNAc in the assay.

The data suggests that treatment with low concentrations of digitonin in some manner either exposes a further population of cryptic lectin receptors, or alters the membrane topography such that the number of available binding sites in the intact organelle increases. Similarly, the increased number of binding sites in the inner membrane may be explained by assuming an equal density distribution of glycoproteins between the two membranes. Consequently, the inner membrane which has a much larger surface area owing to its intense invaginations could also contain more lectin binding receptors.

7.4 SDS-polyacrylamide gel electrophoresis of iodinated mitochondria and mitoplasts

Our previous data have established the presence of carbohydrate on both mitochondrial membranes. In these experiments we attempt to see if the detectable carbohydrate can be correlated with any of the major surface accessible proteins.

Labelled mitochondria and mitoplasts that are prepared by digitonin fractionation in which the swelling step is omitted are electrophoresed on slab gels and bands visualised by fluorography. As this experiment was performed before we refined the methodology for preparation of mitoplasts, about 10-15% of the outer membrane probably still remains associated with the mitoplast. Consequently, this result is slightly different to that described in Section 6.5. However, on correlation of the bands one new component is evident, 4*, apparent predominantly in mitoplasts.

The results photographed in Fig. 7.3. suggest that bands 1, 2, 3, 4, 4*, 5, 6 and 7 are exposed on the exterior surface of mitochondria because they are labelled in a lactoperoxidase impermeable system. A new component designated band 8 appears in both mitochondria and mitoplasts, and probably represents labelled phospholipid or doliochol phosphate intermediates. The labelling of bands 1b, 2a and 2b, inner membrane components, is thought to occur in a population of mitochondria with disrupted outer membranes. In mitoplasts, the labelling pattern is similar to previously described, (Bands 1, 1a, 1b, 1c, 2a, 2b, 5, 5a, 6a, 6b) except for the appearance of 4* and another minor component, 7a. These may represent genuine extrinsic membrane components which are easily extracted when mitochondria are washed in KCl or swollen in Tris-phosphate.

The result from this experiment serves as a control for the subsequent immunoprecipitation experiments which are performed simultaneously on the same samples.

7.5 Identification of lectin binding receptors in intact surface labelled mitochondria and mitoplasts.

In the previous experiment we have demonstrated the major surface iodinated components accessible on the outer and inner membranes of mitochondria. By use of the immunoprecipitation technique we show which of the major surface proteins are glycosylated.

(a) Con A

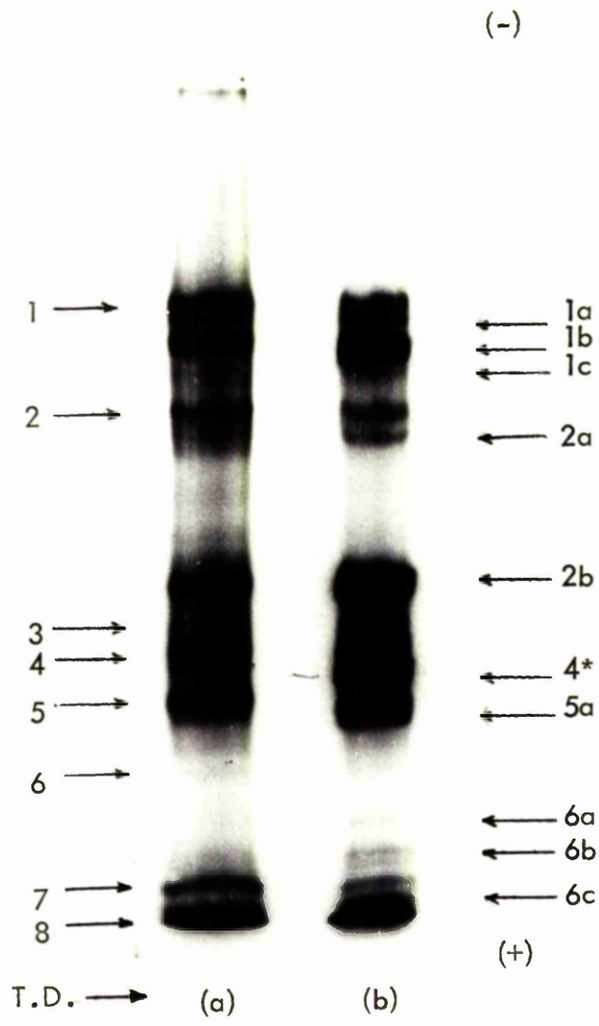
Labelled mitochondria and mitoplasts are immune-precipitated as described in the Methods (M 10.3.3a). On

Fig. 7.3

Detection of surface labelled components in rat liver mitochondria and mitoplasts on analysis by SDS-polyacrylamide gel electrophoresis.

Rat liver mitochondria (a) and mitoplasts (b) in which the outer membrane is removed are prepared as described in Legend 6.2.

Solubilised samples (M 8.4.1) containing 180,000cpm are applied to 10% (w/v) acrylamide slab gels and electrophoresed (M 8.2), prior to detecting the labelled bands by fluorography after a 2 day exposure (M 8.5.2).


(-)
1
2
3
4
5
6
7
8
T.D.
(a)
(b)
1a
1b
1c
2a
2b
4*
5a
6a
6b
6c
(+)

detection of the labelled components by fluorography after SDS-polyacrylamide gel electrophoresis, at least 6 of the major surface iodinated components appear to be glycosylated in mitochondria and include bands 1, 3, 4, 4*, 5 and 7. In mitoplasts bands 1, 1a, 1b, 1c, 2a, 2b, 4*, 5 and 7a appear to be glycosylated, all corresponding to major surface iodinated species. The appearance of bands 1b, 2a and 2b in mitochondria is attributed to labelling owing to disrupted outer membranes. Similarly, in mitoplasts the presence of bands 4 and 7 could be due to contaminating outer membrane, (see Fig. 7.4a. and Table 7.1).

In a control experiment, 0.2M α-methyl-α-D-glucoside is incubated with the lectin prior to addition to the membrane extract. Subsequent analysis of the immune-precipitate reveals no iodinated bands suggesting the lectin binds specifically to glycosylated components.

(b) WGA

Surface iodinated mitochondria are immune-precipitated with WGA as described in Methods (M 10.3.3b). Detection of the labelled bands by fluorography reveals that at least 6 of the major surface iodinated components, namely bands 1, 4, 4*, 5, 6 and 7 are glycosylated. In mitoplasts, a larger population of the bands corresponds to the major surface iodinated components and include bands 1, 1a, 1b, 1c, 2a, 2b, 4*, 5, 5a, 6b and 8, (see Fig. 7.4b). The labelling detected in components 4 and 7 in mitoplasts is attributed to contaminating outer membrane fragments that co-purify with the mitoplasts. Similarly, labelling in components 1b and 2b in

Fig 7.4a.

Immunoprecipitation of Con A binding receptors in surface iodinated rat liver mitochondria and mitoplasts.

Rat liver mitochondria are isolated as in (M 4.1) after injecting the rat with 3-amino-1, 2, 4-triazole (1mg/g body wt.) 1h before sacrifice. Mitoplasts are prepared using a digitonin/protein ratio of 0.2 (M 4.3.2), though in this case the swelling and shrinking stage is omitted.

An aliquot (200-400μg) of the mitochondrial and mitoplast fractions are surface iodinated as described in (M 7.1), prior to immune-precipitation with Con A and anti-Con A serum (M 10.3.3(a)). The precipitated proteins are separated on 10% (w/v) acrylamide slab gels (M 8.2), after application of samples containing equal amounts of radioactivity (10,000cpm). Iodinated bands are detected by fluorography (M 8.5.2) after a 7 day exposure.

(a) Immune-precipitated components of iodinated mitochondria

(b) Immune-precipitated components of iodinated mitoplasts

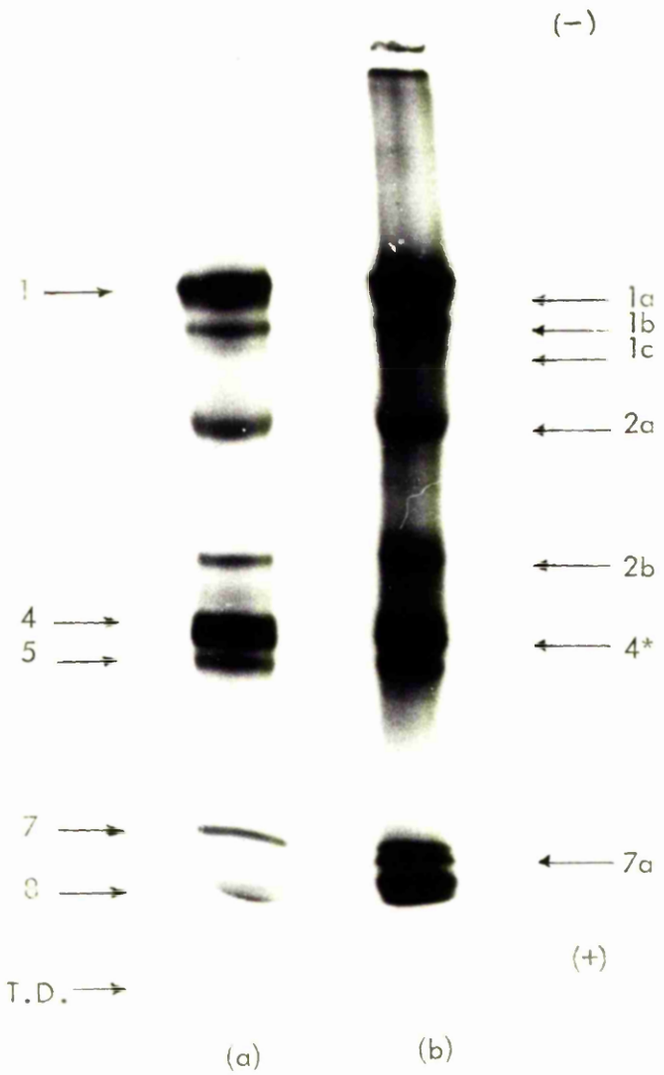

(−)
1
4
5
7
8
T.D.
1a
1b
1c
2a
2b
4*
7a
(+)
(a)
(b)

Fig. 7.4b.

Immunoprecipitation of WGA binding receptors in surface iodinated rat liver mitochondria and mitoplasts

Rat liver mitochondria and mitoplasts are prepared and iodinated as described in Legend 6.2. The iodinated samples are immunoprecipitated with WGA and anti-WGA serum (M 10.3.3(b)).

The precipitated proteins are separated on 10% (w/v) slab gels (M 8.2), after application of samples containing equal quantities of radioactivity (12,000cpm). Iodinated bands are detected by fluorography (M 8.5.2) after a 7 day exposure.

(a) Immune-precipitated components of iodinated mitochondria

(b) Immune-precipitated components of iodinated mitoplasts

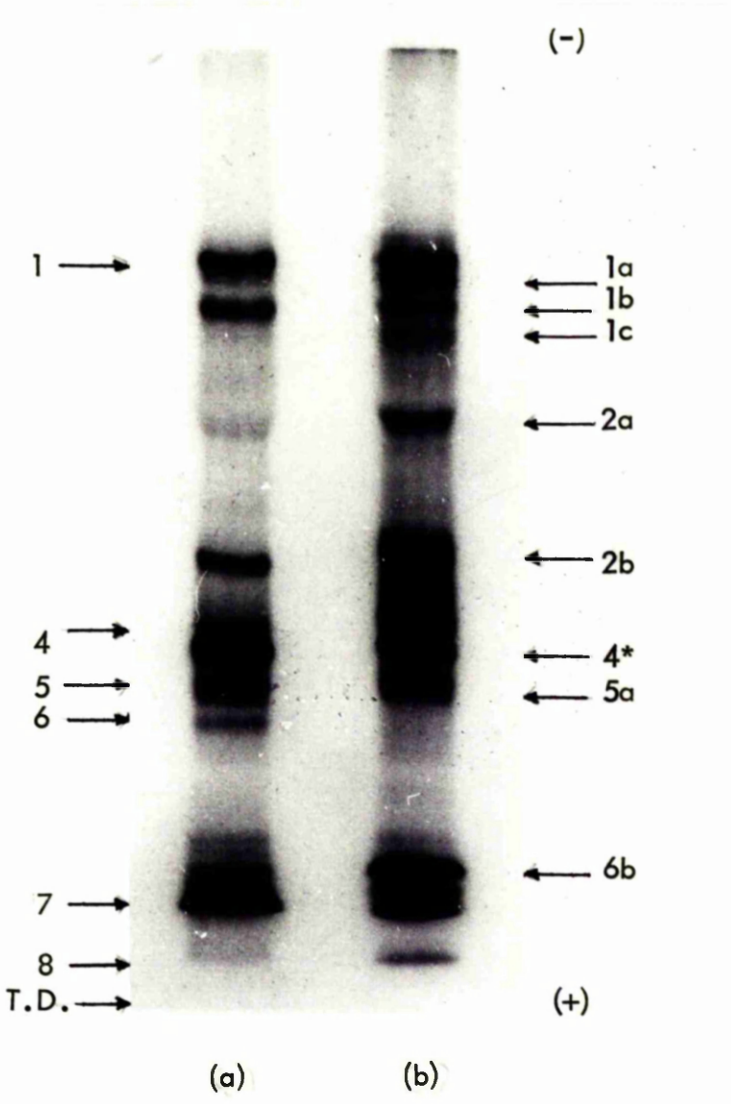

(-)
1
4
5
6
7
8
T.D.
1a
1b
1c
2a
2b
4*
5a
6b
(+)
(a)
(b)

Table 7.1

Summary of the available lectin binding receptors for Con A and WGA exposed on the external surfaces of the outer and inner mitochondrial membranes.

The information obtained from Fig. 7.4a and 7.4b is summarised in this Table. The bands visualised after fluorography are assigned numbers (1-8) in correlation to the surface iodinated components identified in Table 6.2. Components which have a letter of the alphabet in addition to the numeral, denote species that are not assigned to the external surface of the outer membrane, but which only become significantly labelled as the membrane is removed.

Table 7.1

MITOCHONDRIA			MITOPLASTS		
Major Surface iodinated components	Major surface glycosylated components		Major surface iodinated components	Major surface glycosylated components	
	Con A	WGA		Con A	WGA
1	1	1	1	1	1
-	-	-	1a	$1a^{++}$	1a
1b	1b(?)	1b(?)	$1b^{+++}$	$1b^{++}$	$1b^{++}$
-	-	-	1c	1c	1c
2	-	-	2(?)	-	-
2a(?)	2a(?)	-	$2a^{++}$	$2a^{++}$	2a
2b(?)	2b(?)	2b(?)	$2b^{++}$	$2b^{++}$	$2b^{++}$
3	3	-	-	-	-
4	4	4	4(?)	4(?)	4(?)
4*	4*	4*	4*+	4*	4*
5	5	5	5	5	5
-	-	-	5a	-	5a
6	-	6	-	-	-
-	-	-	6a	-	-
-	-	-	6b	-	6b
-	-	-	-	-	-
7	7	7	7(?)	7(?)	7(?)
-	-	-	7a	7a	-
8	-	-	8	8	-

mitochondria may be due to disruption of the outer membrane in these organelles. When the experiment is performed in the presence of 0.2M-GlcNAc, no iodinated bands can be detected by fluorography.

The data in Table 7.1. summarises the information obtained from the immunoprecipitation studies and shows which of the major surface iodinated components in mitochondria and mitoplasts are glycosylated.

7.6 Isolation of soluble mitochondrial glycoproteins on a Con A-Sepharose 4B column.

From our previous experiments it appears that the majority of the surface accessible proteins on both the inner and outer membranes are glycosylated. These results are somewhat surprising, so in order to confirm this data we have re-approached the problem using different methodology.

Purified mitochondria are solubilised by boiling in SDS so as to eliminate protease activity, and completely separate hydrophobic linkages between subunit components in enzyme complexes e.g. cytochrome c oxidase. The solubilised mitochondria are diluted in 2-detergent buffer, until the final SDS concentration is 0.1% (w/v) and applied to a Con A-Sepharose 4B column equilibrated in 2-detergent buffer as described in Methods (M 10.3.1). About 98% of the total protein elutes in the void volume of the column, while 60-70% of the total glucosamine label representing 2.0% of the protein is retained. On elution with 0.2M α-methyl-α-D-glucoside, 90% of the total bound label is recovered,(see Fig. 7.5).

Fig. 7.5.

Isolation of rat liver mitochondrial glycoproteins on a Con A-Sepharose 4B column

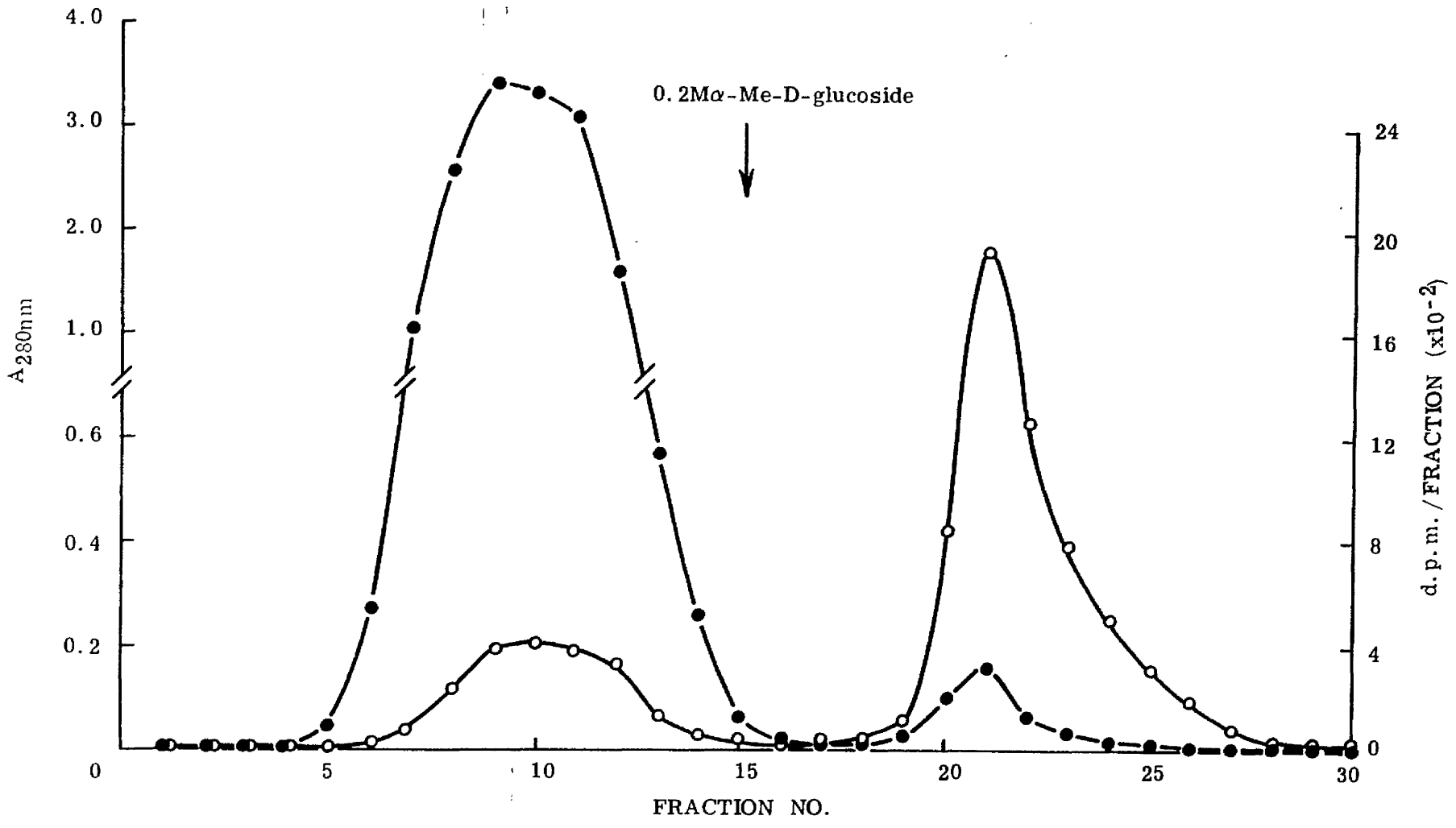
Rat liver mitochondria are isolated from 2 starved rats (M 4.1), injected intraperitoneally 16h previously with 200μCi of D-[6-^{3}H]-glucosamine. The mitochondria are further purified on a discontinuous sucrose gradient (M 4.2).

Labelled mitochondria are solubilised by boiling in SDS (2% w/v) and 2-mercaptoethanol (1% v/v) before diluting in column washing buffer (M 3.11.1). The solubilised material is applied to a Con A-Sepharose 4B column as described in the Methods (M 10.3.1) and the glycoproteins are eluted with 0.2M-methyl-α-D-glucoside containing buffer (M 3.11.2).

An identical procedure is adopted for isolating the mitochondrial glycoproteins for anti-serum preparation with the exception that unlabelled mitochondria are purified from 12 rats. The eluted glycoproteins are then concentrated by precipitation with 5 vol. of ice cold acetone.

(o) % of glucosamine labelled material (% of maximum)

(●) % of protein (% of total)


4.0
3.0
2.0
1.0
0.6
0.4
0.2
0
5
10
15
20
25
30
FRACTION NO.
A280nm
0.2Mα-Me-D-glucoside
24
20
16
12
8
4
0
d.p.m./FRACTION (x10-2)

7.7 Immune-precipitation of surface iodinated glycoproteins by specific anti-serum.

In section 7.5. by immunoprecipitation using Con A we have demonstrated the glycosylated components on the surface of the mitochondrial outer membrane. In this experiment we validate that data by using antiserum directed against mitochondrial glycoproteins isolated on an immobilised Con A column.

Surface iodinated mitochondrial glycoproteins are immune-precipitated with anti-glycoprotein serum. An aliquot of formalin-fixed S. aureus (Cowan I strain) is used to adsorb the complex prior to acrylamide gel analysis as described in Fig. 7.6. Six surface iodinated components are specifically immune-precipitated in mitochondria. A control experiment in which the anti-glycoprotein serum is omitted did not precipitate these glycosylated species, suggesting that the anti-serum is specific.

These six components must presumably represent genuine glycosylated species, as all interactions with either the cytosketal system or adjoining polypeptides have been destroyed by boiling in SDS.

7.8 Demonstration of total mitochondrial membrane glycoproteins by lectin staining.

The immune-precipitation studies have provided information on glycosylated surface proteins that are accessible to lacto-peroxidase-catalysed iodination. By the lectin overlay method, we are able to identify the total glycoprotein

Fig. 7.6.

Comparison of the surface iodinated glycoproteins in rat liver mitochondria by specific immunoprecipitation using anti-Con A serum and anti-mitochondrial glycoprotein serum.

Rat liver mitochondria are isolated as in (M 4.1) after injecting the rat with 3-amino-1,2,4-triazole (1mg/g body wt.) 1h prior to sacrifice. Three aliquots (200-400µg protein) of the mitochondria are iodinated as described in (M 7.1).

Iodinated mitochondria are immunoprecipitated with either Con A (a) as described in Fig. 7.4a or anti-mitochondrial glycoprotein serum (M 9.1) (b) as described for Con A, except that addition of lectin is omitted. Anti-mitochondrial glycoprotein serum is added and the sample incubated at 37°C for 30 min and at 4°C for not less than 4h. The immune complex is precipitated by indirect adsorption with S. aureus (Kesseler, 1975) overnight at 4°C. A control experiment (c) in which addition of the antiserum is omitted is also performed. The immune-complexes are centrifuged at 1000g after 10 min and denatured in SDS and 2-mercaptoethanol (M 8.4.1). The S. aureus is centrifuged from the solubilised samples (b and c) by pelleting at 100g for 2 min.

Samples containing equal amounts of protein are analysed on 10% (w/v) acrylamide slab gels (M 8.2) and the bands detected by fluorography after a 3 day exposure.

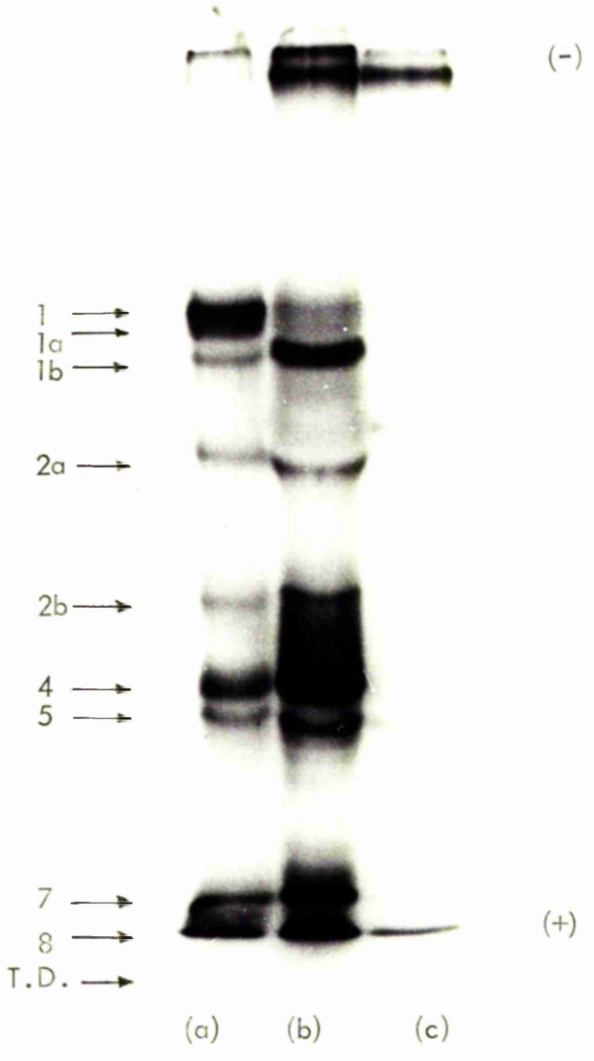

(-)
1
1a
1b
2a
2b
4
5
7
8
T.D.
(+)
(a)
(b)
(c)

Fig. 7.7a.

Binding of [^{125}I]-Con A to lectin binding receptors in rat liver mitochondrial membranes

Rat liver mitochondria are isolated (M 4.1) and sonicated for 2 x 30s, prior to separation on a discontinuous sucrose gradient (M 4.3.1). The membranes are pelleted and further washed (x2) in 100mM-phosphate buffer, pH 7.5 at 100,000g for 90 min, before resuspending in 10mM-Tris, pH 7.2. Mitochondrial inner and outer membranes (20µg) are denatured in SDS (M 4.3.1) and constituent proteins resolved by SDS-polyacrylamide gel electrophoresis (M 8.2).

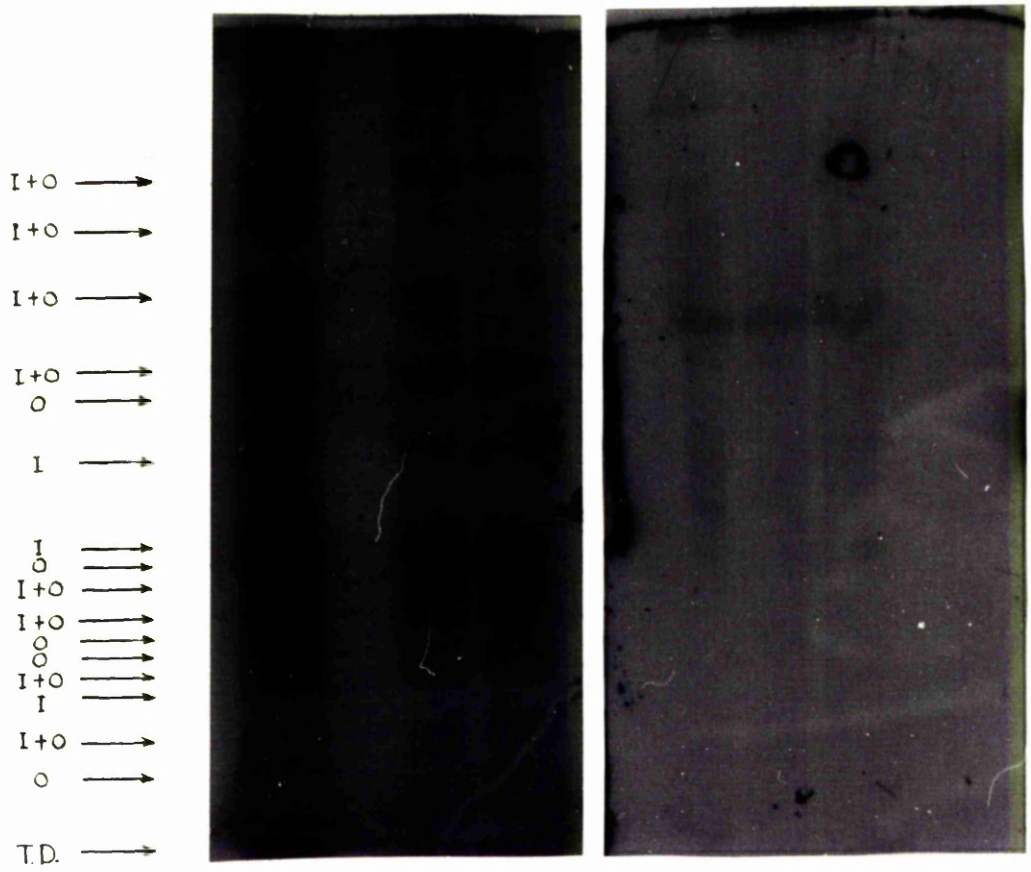
The gels are incubated with [^{125}I]-Con A (1 x 10^6cpm) as described in the Methods (M 10.3.2) and processed for fluorography (M 8.5.2). Labelled bands are visualised after a 5 day exposure.

(a) Con A receptors in an inner and outer membrane mixture (I + O)

(b) Con A receptors in the inner membrane (I)

(c) Con A receptors in the outer membrane (O)

(d) control experiment performed in the presence of 0.2M-methyl-α-D-glucoside.



(a) (b) (c) (d)

complement in mitochondrial membranes and compare the data obtained from both methods.

Solubilised mitochondrial membrane components are separated by SDS-polyacrylamide gel electrophoresis. After fixation and equilibration in phosphate buffer, the gels are incubated with radio-iodinated lectin as described in Fig. 7.7.

(a) Binding of Con A to mitochondrial membrane components.

Fig. 7.7a shows the binding pattern of [^{125}I]-Con A to mitochondrial membrane polypeptides. Detection of iodinated bands by autoradiography reveals 20 glycosylated components in the membrane mixture. In the inner membrane alone, 11 bands are visible. Similarly, the outer membrane shows up 10 Con A binding components. On comparison of the glycosylated components in the inner and outer, 8 bands appear common to both membranes. The binding of lectin to saccharide moieties is specific as the reaction is inhibited in the presence of 0.2M methyl-α-D-glucoside.

(b) Binding of WGA to mitochondrial membrane components.

The binding pattern of this lectin which is specific for GlcNAc containing saccharide sequences, although it is reported to bind to sialic acid (Bhavanandon and Katlic, 1979) is markedly different. Autoradiographic visualisation of the mitochondrial membrane polypeptides incubated with [^{125}I]-WGA, reveals only 6 bands. On comparison with the WGA immunoprecipitate, these correspond to those glycosylated components designated 1, 1a, 1b, 2a and 2b. The band below component 2a appears to be a new glycosylated component not

Fig. 7.7b.

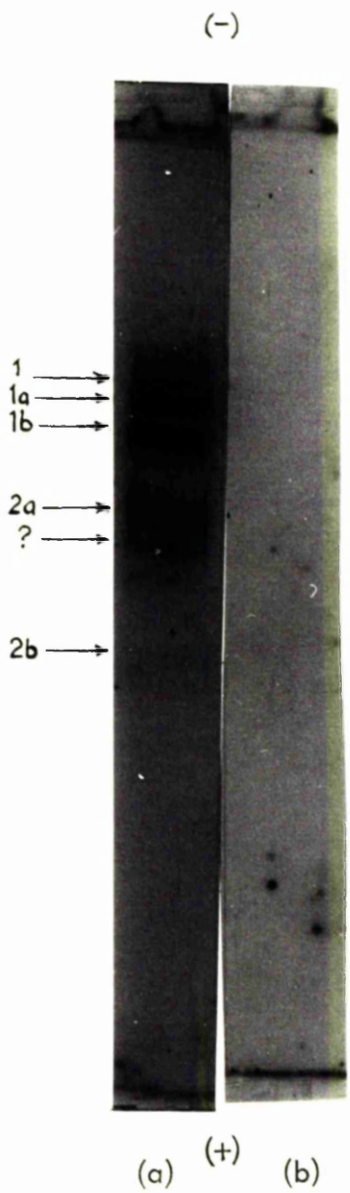
Binding of [^{125}I]-WGA to lectin binding receptors in rat liver mitochondrial membranes

Rat liver mitochondria are isolated (M 4.1) and sonicated for 2 x 30s. The membranes are pelleted, and further washed (x2) in 100mM-phosphate buffer, pH 7.5 at 100,000g for 90 min, before resuspending in 10mM-Tris, pH 7.2.

The remaining procedure is similar to that described in Legend 7.7a, except that [^{125}I]-WGA (1 x 10^6 cpm) is substituted for Con A.

(a) WGA receptors in an inner and outer membrane mixture

(b) control experiment performed in the presence of 0.2M-GlcNAc.


(−)
1
1a
1b
2a
?
2b
(a)
(+)
(b)

detected in the immunoprecipitation studies, probably due to lack of accessible tyrosine residues for radiolabelling. These represent specific lectin binding receptors, as the binding is inhibited in the presence of 0.2M-GlcNAc, Fig. 7.7b.

7.9 Discussion

The data in this chapter serves to confirm the fact that carbohydrate is still associated with the mitochondrial membranes and accessible to external ligands such as lectins, despite the removal of the loosely associated glycoprotein with low concentrations of digitonin or ionic strength.

No definitive evidence is available on the distribution of carbohydrate on the interior and exterior surfaces of the two membranes, although Glew et al. (1973) have suggested that 40% of the total Con A binding sites in purified liver mitochondria are exposed on the external surface of the organelle. This group did not check for the integrity of the outer membrane in their preparations, which is often badly disrupted during purification on sucrose density gradients. Similar problems arise in the interpretations of agglutination studies using Con A, WGA and SBA to probe for the presence of particular sugar residues on the mitochondrial surface, perhaps accounting for the major discrepancies reported in the data (Henning and Uhlenbruck, 1973; Nicolson et al., 1971). Our binding studies/agglutination studies are performed on the M_1 mitochondrial fraction in which the outer membrane is minimally damaged (3-5%).

Further, the mitochondria are washed in 0.12M-KCl buffer to remove the lossely associated carbohydrate material which may effect the data by allowing the mitochondria to bind adventitiously to the lectin.

Our binding studies have demonstrated that the number of binding sites for [^{125}I]-WGA available on the inner membrane is almost twice that on the outer surface. Lehninger (1964) has calculated the total surface area of a mitochondrion on the assumption that each organelle has 10 cristae. The total surface area of the inner membrane is $29\mu^2$ in comparison with outer membrane of surface area $13\mu^2$. On the basis of this calculation, the apparent surface area of the inner membrane is 2.2 times more than that of the outer membrane. Thus, it is justifiable to assume that the number of available lectin receptors on the inner membrane may also be larger. The lectin immunoprecipitation and lectin overlay data support this assumption; the majority of the WGA binding species appear to be inner membrane components (see Section 7.8).

The lectin binding studies confirm the presence of carbohydrate on the external surface of both inner and outer membranes. Although in this case the iodinated lectin could be specifically binding to mitochondrial contaminants in the M_1 fraction. However, the fact that mitochondria and mitoplasts can be directly agglutinated by WGA as monitored by the decrease in cytochrome c oxidase activity argues against the possibility.

In conclusion by these experiments we have established unequivocally the presence of carbohydrate on the external surfaces of the outer and inner mitochondrial membranes. This observation is significant in that it is an exception to the general rule that no carbohydrate is found on membrane surfaces directly in contact with the cytoplasm of the cell, e.g. plasma membrane and rough endoplasmic reticulum (Hirano et al., 1972).

Lectin immunoprecipitation has permitted us to assess that the majority of the proteins exposed on the outer surface of the organelle, namely bands 1, 3, 4, 5, 6 and 7 are glycoprotein in nature or are directly associated with either Con A or WGA receptors in the outer membrane. Only component 2 does not appear to interact with either of these lectins. Of the inner membrane species located on the external surface, components 1a, 1b, 1c, 2a, 2b, 4*, 5a, 6b and 7a again all appear to be glycosylated or interact with glycoproteins in mitochondria. The presence of at least 20 glycoproteins in mitochondria is confirmed by lectin overlay using [^{125}I]-Con A, although it has not been possible to correlate all these components with those observed in immunoprecipitation experiments to date. With WGA the data is not as expected, a number of low mol. wt. components are apparent in the immunoprecipitate, which are not detected by the lectin overlay method.

There is one tenable explanation for this. In the lectin staining method all the mitochondrial components are denatured and solubilised in SDS prior to gel electrophoresis

and detection of the lectin binding components by fluorography. In contrast, in lectin immunoprecipitation, the WGA binding receptors are solubilised in DOC which is shown to be most efficient in extracting membrane proteins and glycoproteins (Juliano and Li, 1978). However, as Con A is more easily denatured in DOC (Lotan et al., 1977) a 2-detergent buffer cocktail is made up which includes Nonidet P-40 and Triton X-100, as well as Ca^{2+} and Mn^{2+} to help maintain the biological activity. Thus, it is hoped that at the concentration of detergent employed in the immunoprecipitation studies which do not denature the lectin, the major lectin binding receptors are solubilised free of other molecular species. Hence, the bands visualised may represent lectin binding receptors as well as associated molecular species. For example, if one of the subunits of the cytochrome c oxidase is glycosylated, all 7 subunits could be immune-precipitated unless interactions between adjoining polypeptides are destroyed. Hence, it can be speculated that the 5 bands detected in the WGA lectin overlay, represent genuine GlcNAc containing lectin receptors. The remaining components detected in the immune -precipitate could be non-glycosylated polypeptides which are associated with the lectin receptors.

However, in the same light, lectin immunoprecipitation offers many advantages. It can be also used in conjunction with chemical cross-linking (Ji, 1976) to examine possible associations between surface glycoproteins and other non-glycosylated membrane components. This has been shown in

the case of lymphocytes, where a specific interaction between surface immunoglobulin and lectin has been demonstrated (Flanagan and Koch, 1978).

From our immunoprecipitation data there appears to be a great deal of similarity in the glycosylated components of the 2 membranes. Two reasons could account for this. If the contact points reported by Hackenbrock (1968a)do exist, it would be difficult to obtain mitoplasts completely free of outer membrane, consequently, the similarity could be attributed to contaminating membrane. Alternatively, it is possible to vary the protein/lectin ratios and allow subfractionation between the different glycoprotein populations. In our experiments this ratio is between 40/1 and 30/1. On a Scatchard plot (Juliano and Li, 1978) this corresponds to "high affinity" regions. On this basis it may be explained why, even though the outer membrane may only account for 10% contamination of mitoplasts, it may contain the "high affinity" binding population and consequently be preferentially immuno-precipitated.

There also appears to be a similarity in the lectin binding receptors for Con A and WGA on both surfaces, despite the fact both lectins have different sugar specificities. If mitochondria are further purified by sucrose density gradient centrifugation and then immunoprecipitated, no detectable differences in lectin binding receptors for either Con A or WGA are visible (data not shown). This suggests that the glycosylated species visible could not be attributed to a specific precipitation of serum glycoproteins or other smooth membrane contaminants. Further, it appears that the

glycoproteins are firmly associated with the membrane as they survive all the washing procedures, namely, after the iodination and immune-precipitation. The technique appears to be specific, in that the immune-precipitate which form in the presence of a hapten sugar have virtually no radioactivity.

The technique of lectin staining has many advantages in that a large number of samples can be rapidly analysed for saccharide containing moieties, at the same time as little as 40μg of membrane sample is sufficient. During the staining procedures, unlike Tanner and Anstee (1976) we have omitted the glutaraldehyde fixation of the gels, as we found that membrane proteins which have been fixed in acetic acid-isopropanol will remain immobilised in the gel during incubations and washes, thus obviating the need for a chemical cross-linking reagent. This finding is confirmed by Rostas et al. (1977). One disadvantage of this approach is that only isolated, denatured glycoprotein species can be examined. Hence, it is of little use in the study of the organisation of oligomeric enzyme complexes within the membrane, or possible linkages between trans-membrane glycoproteins and the cytosketal elements. However, in principle it should be possible to utilize this method for obtaining information regarding the nature and heterogeneity of the various lectin receptors in mitochondrial membranes on a quantitative basis.

CHAPTER 8

Isolation of "inside-out" vesicles and identification of the transmembrane proteins in the inner mitochondrial membrane.

8.1 Introduction

Our previous studies have demonstrated that the carbohydrate in mitochondria is present on the external surfaces of the inner and outer membranes. By analogy with the erythrocyte and lymphocyte cell membranes and including intracellular components, e.g. rough endoplasmic reticulum (Hirano et al., 1972) where the carbohydrate is asymmetrically distributed on the cisternal surface, it is feasible to propose that the carbohydrate in mitochondria has an asymmetric distribution too. The latter proposition is supported by the observation that neuraminidase treatment of a mixed mitochondrial inner membrane vesicle population causes a release of approx. 50% of sialic acid residues. This same effect is not apparent in vesicle populations which are unretarded on a Con A-Sepharose 4B column (G.K. Hunter and J.G. Lindsay, unpublished observations).

Study of the topography of proteins within the bilayer is facilitated by comparative labelling of exposed proteins in homogeneous populations of RSO and ISO vesicles, as in the case of the erythrocyte plasma membrane (Reichstein and Blostein, 1975). To this end the mitochondrial inner membrane is particularly well suited for such studies because it can be obtained in either a normal orientation, as found in mitoplasts prepared by digitonin treatment, or in largely inverted orientation in sonically prepared SMPs.

Our evidence for the orientation of SMPs prepared from rat liver by marker enzymic criteria, such as cytochrome c oxidase activity which should be latent ISO vesicles, indicates that between 30-50% of the total cytochrome c binding sites are inaccessible in a normal preparation, indicating a hetergeneous vesicle population. However, this may not be the case for beef heart mitochondria which have a tremendously invaginated cristal membrane from which the SMP are derived (Racker et al., 1965).

This chapter describes the method we have evolved for isolation of homogeneous populations of ISO vesicles, and then subsequent lactoperoxidase-catalysed iodination of RSO and ISO vesicles to examine the organisation of proteins within the membrane.

8.2 Stimulation of cytochrome c oxidase activity in a sonicated inner membrane preparation on addition of DOC.

A valuable technique for assessing the purity of sealed membrane populations is the use of marker enzymic activities which are confined to either the interior or exterior face of the membrane. In the case of the mitochondrial inner membrane, cytochrome c oxidase activity provides us with a convenient marker since the binding site of cytochrome c is located on the outer surface of the inner membrane. Assessment of latent enzymic activity allows us to determine the homogeneity of SMPs prepared by sonication of rat liver mitochondria.

Cytochrome c oxidase activity is estimated in an aliquot of the SMP suspension as described in Fig. 8.1. On addition of 0.3% DOC (w/v), enzymic activity is stimulated routinely only 1.5-2.0 fold, indicating that only 30-50% of the total cytochrome c binding sites are inaccessible in a normal preparation. This result suggests that SMPs prepared from rat liver do not have a uniform "inside-out" orientation.

8.3 Selective immune-precipitation of "rightside-out" and non-sealed membrane fragments with WGA

It is commonly assumed that vesicles derived from the mitochondrial inner membrane have a reverse orientation to that of a mitochondrion (Racker et al., 1965). Our previous result is clearly in conflict with this idea. Hence, by employing different methodology based on the premise that the carbohydrate in mitochondria is asymmetrically distributed, we have attempted to purify ISO vesicles from a mixed population by selective immunoprecipitation of all membrane fragments and vesicles with accessible carbohydrate.

A mixed population of purified mitochondrial inner membrane vesicles are prepared by sonication of the inner membrane. The vesicles are treated with WGA and immune-precipitated using increasing quantities of anti-WGA as described in Fig. 8.2. The percentage of "inside-out" vesicles remaining in the supernatant fraction is assessed by measuring the latency of cytochrome c oxidase activity ± DOC (Wojtczak et al., 1972).

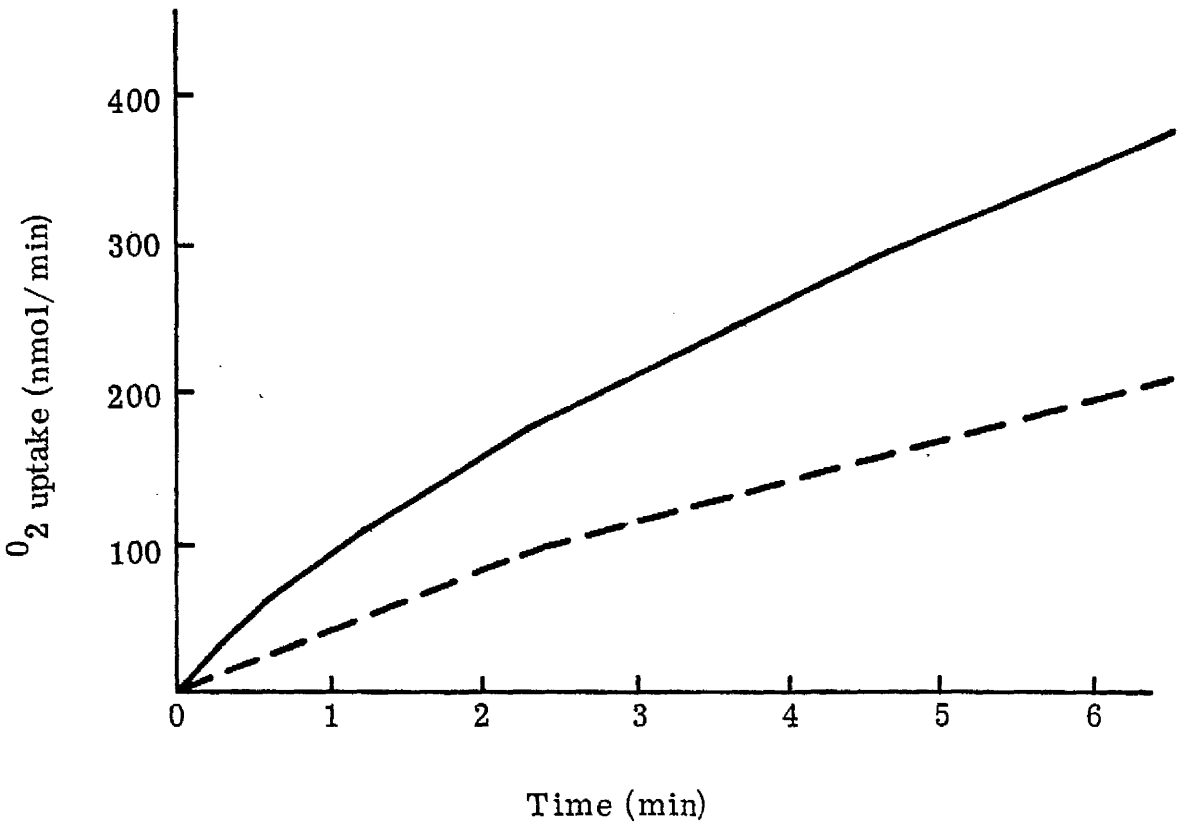


Fig. 8.1

Crypticity of cytochrome c oxidase activity in rat liver mitochondrial inner membrane vesicle populations.

Inner membrane vesicles (20mg) are prepared from isolated rat liver mitochondria (M 4.1) by sonication and separation of the membranes on a discontinuous sucrose gradient (M 4.3.1). The inner membrane subfraction is washed twice in 100mM-phosphate buffer, pH 7.5 at 100,000g for 40 min and the pellet is finally resuspended in the isolation medium (M 3.1.1).

An aliquot of the membrane suspension (50-100µg) is assessed for cytochrome c oxidase activity in the absence (----) and presence of 0.3% (___) (w/v) DOC (M 5.2(b)).

Fig. 8.2

Selective immunoprecipitation by WGA of mitochondrial inner membrane vesicles containing carbohydrate.

Inner membrane vesicles (20mg) are prepared as described in Legend 8.1. The final preparation is incubated at 25°C for 30 min with 5mg WGA before centrifugation and further washing (x3) in 100mM-phosphate, pH 7.5 containing 1mM-PMSF to remove excess and non-specifically bound lectin. Lectin-treated vesicles are subsequently incubated for 1h at 37°C and overnight at 4°C with increasing amounts of anti-WGA serum or normal serum (M 9.1). PMSF (1mM) is added to inhibit protease activity.

Immune-complexes are centrifuged at 1,000g for 10 min and residual cytochrome c oxidase activity in the supernatant fraction estimated as described in (M 5.2(b)).

(●) vesicles incubated with anti-WGA serum

(▲) vesicles incubated with anti-WGA serum treated with 0.3% (w/v) DOC before assay

(■) vesicles incubated with normal serum (rabbit)

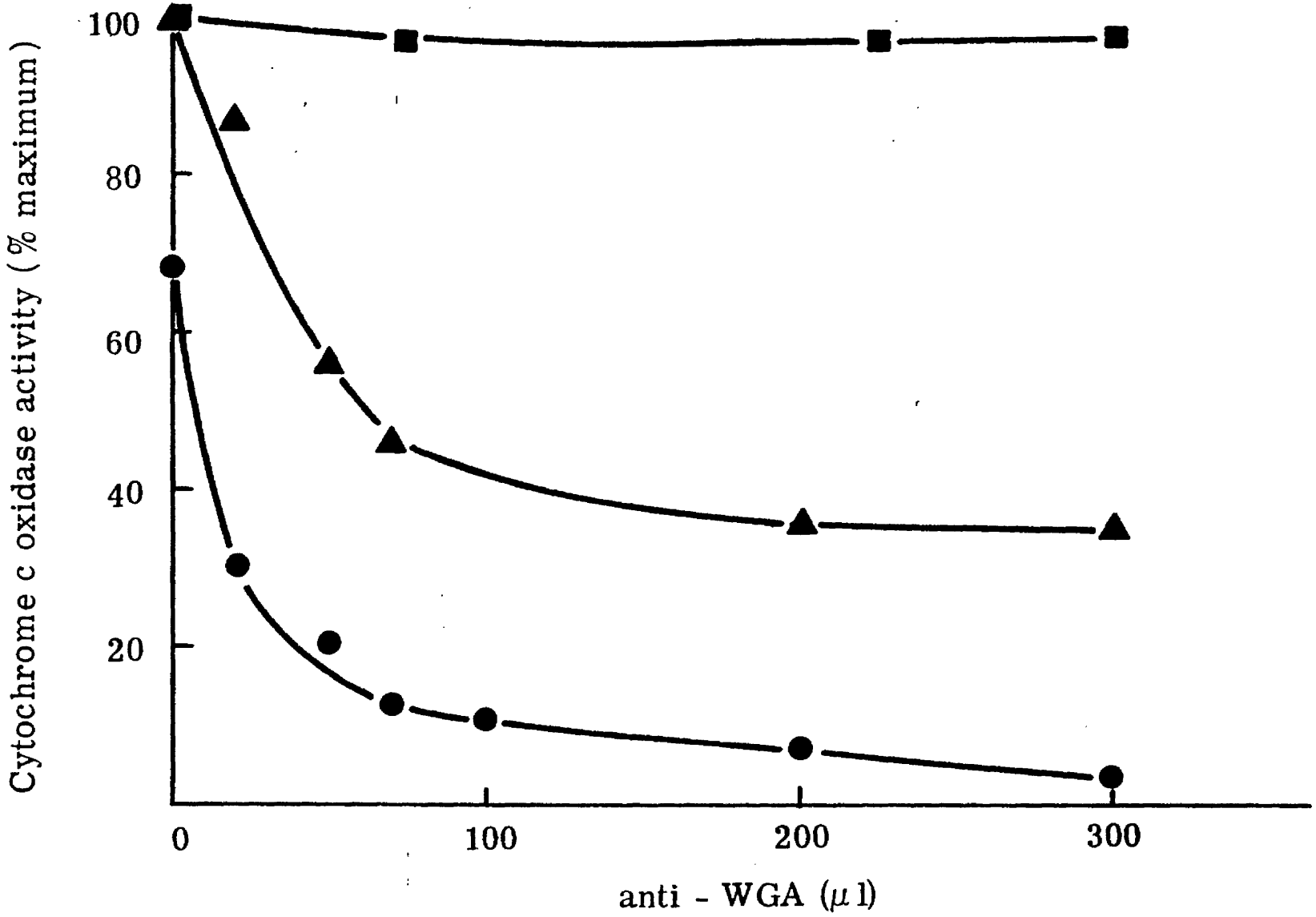

Cytochrome c oxidase activity (% maximum)
100
80
60
40
20
0
100
200
300
anti - WGA (μl)

Fig. 8.2 shows the percentage of cytochrome c oxidase activity remaining in the supernate when increasing quantities of anti-WGA IgG are added. Addition of 300µl of anti-WGA IgG causes total precipitation of all the lectin bound material. When 0.3% (w/v) DOC is added to the supernatant fraction it results in a 8-10 fold stimulation in cytochrome c oxidase activity, indicating that the "inside-out" population is between 90-95% pure. In comparison, enzymic activity in the original submitochondrial population is stimulated 1.5-2.0 fold with DOC, showing that only 30-50% of the total cytochrome c binding sites are inaccessible in a normal preparation (see Fig. 8.1).

From this experiment we have concluded that SMP have accessible carbohydrate and can be selectively precipitated with WGA and anti-WGA IgG. The SMPs remaining in the supernate contain no accessible carbohydrate and the cytochrome c binding site must also be inaccessible. As it is normally on the outer surface of the inner membrane, such vesicles must be "inside-out". Finally, IgG from normal serum does not cause precipitation of WGA bound material indicating the validity of our method.

8.4 Identification of transmembrane proteins in the inner mitochondrial membrane

Identification of proteins which traverse the lipid bilayer is facilitated by comparative labelling of sealed "right side-out" and "inside-out" vesicles.

Treatment of mitochondria with digitonin (digitonin/protein ratio: 0.2) yields mitoplasts which are reported to be sealed "right side-out" particles (Schnaitman et al., 1967). The orientation of these particles could be further assessed by the extent of labelling of carbamyl phosphate synthetase (Clarke, 1976a). The source of ISO vesicles is described in Fig. 8.2.

The result in Fig. 8.3 shows representative patterns of the iodinated polypeptides in RSO (mitoplasts) and ISO vesicles as revealed by polyacrylamide gel electrophoresis in SDS. Two new high mol. wt. components as well as one in the 10,000 mol. wt. range are visible in ISO vesicles and assigned to the non-cytoplasmic surface of the inner membrane. Other visible differences are; component 1d is 4.4 times more labelled in ISO vesicles suggesting that the bulk or possibly all the polypeptide is located on the inside of the inner membrane. In the latter case, the faint labelling of band 1d in mitoplasts could be owing to a population of disrupted vesicles as previously observed in Chapter 6. Also, component 2b is 2.2 times more heavily labelled in mitoplasts as compared by a densitometric scan.

Polypeptide components 1, 2b, 5, 5a, and 6a appear to traverse the inner membrane by virtue of the fact they are labelled in both populations of vesicles. Track (c) represents the polypeptides that are labelled in the original SMP population. All the bands correspond to protein species in either RSO or ISO vesicles. In addition, two other components appear; one between bands 2b and 5, the other

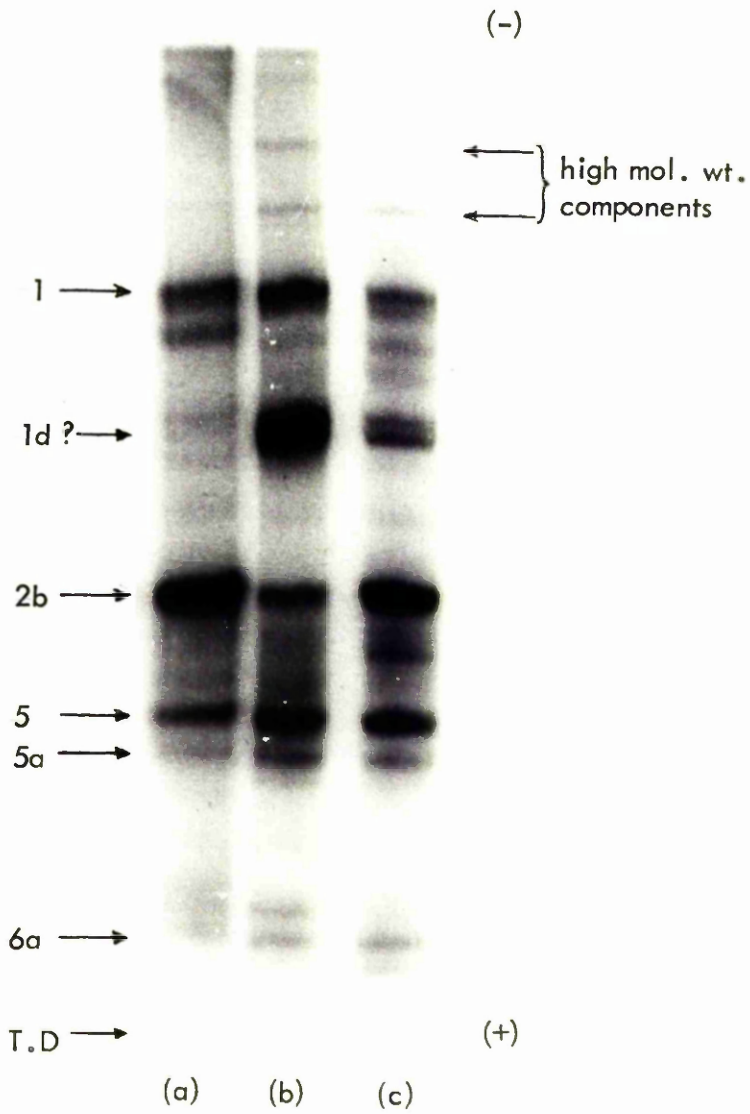
Fig. 8.3

Identification of transmembrane proteins in the inner membrane of rat liver mitochondria by SDS-polyacrylamide gel electrophoresis

Mixed vesicle populations are prepared by sonicating the purified mitochondrial inner membrane (M 4.3.1). The vesicle mixture (5mg) is washed twice in 100mM-phosphate, pH 7.5 and pelleted at 10,000g after 90 min. The vesicles are then incubated with 2mg of WGA and washed as described in Legend 8.1. The final preparation (0.3ml) is incubated with 0.6ml of purified anti-WGA IgG (M 9.3) as described in the legend to Fig. 8.2. The supernatant fraction containing vesicles with no accessible carbohydrate is then centrifuged at 100,000g for 40 min to pellet the ISO vesicles and separate the excess anti-WGA IgG.

Purified vesicles (200µg) (b), mitoplasts (200µg) (a) and the inner membrane mixture (200µg) (c) are simultaneously iodinated by the lactoperoxidase method (M 7.1). Samples are denatured in-SDS (M 8.4.1) and aliquots containing equal radioactivity (300,000cpm) electrophoresed on 10% (w/v) slab gels (M 8.2). Gels are processed for fluorography (M 8.5.2) and iodinated polypeptides visualised as described in (M 8.5.4), after a 2 day exposure.

The bands arrowed in the Fig. represent transmembrane proteins.


(-)
high mol. wt.
components
1
1d ?
2b
5
5a
6a
T.D
(+)
(a)
(b)
(c)

in the low mol. wt. region. The possible origins of these bands will be considered later.

8.5 Discussion.

In order to study the topography of proteins within the lipid bilayer it is convenient to have sealed membrane vesicles of defined polarity, in particular, a sealed inside-out population. Analysis of proteins exposed on the exterior surface can be readily performed on intact mitochondria (outer membrane) or mitoplasts (inner membrane).

It is commonly assumed that vesicles derived from mitochondrial inner membrane are ISO both from a structural (electron microscopic, antibody studies Racker, 1970) and functional (H^+ translocation) standpoint. However, disruption of cells or organelles generally leads to the formation of mixed populations containing ISO, RSO, and non-sealed fragments. If sonic oscillation of the inner membrane does result in a mixed populations of vesicles rather than the uniform reversed orientation, the classical experiments of Racker (1970) (reverse proton translocation and antibody studies) would still remain undisputed. It could however be argued that cytochrome c which is easily extracted, (Jacobs and Sanadi, 1960) accounts for the loss of functional proton translocation. The argument also holds for the lack of activity observed when antibody is directed against cytochrome c. Therefore, although it is well established that SMP represent ISO vesicles, we feel that the assumption ought to be reviewed in the light of

specific marker enzymic activities which are associated with a particular face of the membrane.

By analogy with the distribution of glycoprotein in the plasma membrane of other cell types especially erythrocytes (Steck, 1974), it seems likely that the carbohydrate moieties are located on the external surface of the membrane (Hirano et al., 1972; Nicolson and Singer, 1974). Using lectins (WGA) as an external ligand for exposed carbohydrate residues, we have selectively purified homogeneous populations of ISO vesicles by an immuno-precipitation technique.

The conclusions from this experiment have indicated that a proportion of SMP have exposed carbohydrate residues and can be specifically immune-precipitated with WGA and the respective anti-serum. The remaining population in the supernate has low cytochrome c oxidase activity unless disrupted with DOC.

Thus, with access to vesicles of defined orientation, we have performed surface labelling studies which provide information on the topography of proteins within the bilayer, and in particular identify transmembrane proteins. The significance of these iodination studies depends on vectorial labelling by lactoperoxidase. Lack of $[^{125}I]$-label in carbamyl phosphate synthetase (Clarke, 1976a) indicates that the mitoplasts are not leaky to lactoperoxidase and by analogy in ISO vesicles. The difference in the labelled polypeptides of mitoplasts and ISO vesicles shows that we have indeed obtained vesicles of defined orientation and suggests that 5 of the polypeptides are transmembrane including bands

designated 1, 2b, 5, 5a and 6a.

In our initial surface labelling studies of intact mitochondria and mitoplasts, bands 1 and 5 have been shown to traverse the outer membrane. In this experiment bands 1 and 5 appear to traverse the inner membrane too. We thus propose in a novel fashion that from our studies band 1 and 5 span both mitochondrial membranes. The hypothesis is feasible particularly in the light of contact points that have been reported between the 2 membranes (Hackenbrock, 1968a). These points could represent regions which facilitate the orientation of polypeptides across the two membranes. The appearance in the iodinated mixture of a component of approx. mol. wt. 25,000 together with one of lower mol. wt. which do not correspond to any of the bands visualised in mitoplasts or ISO vesicles, could actually represent inner membrane proteins that get extracted during the vesiculation process. A similar situation is evident in the erythrocyte membrane on manipulation of the ionic gradient to form vesicles. In this case band 6 (extrinsic protein) is nearly completely released from ghosts but retained in intact erythrocytes in the presence of 0.15M-NaCl-5mM phosphate, pH 8.0 (Fairbanks et al., 1971).

In evaluating these results we have made one important assumption. The absence of a particular polypeptide on a membrane face is attributed to its asymmetric orientation across the lipid bilayer rather than the lack of accessible tyrosine or histidine residues: a limitation of the lacto-peroxidase labelling method. In a similar vein, when a

component e.g. 2b is labelled to different extents on both sides of the membrane, we have interpreted the result by assuming the polypeptide is asymmetrically orientated across the bilayer, the bulk appearing on the cytoplasmic surface of th membrane. This situation is reminiscent to the orientation of PAS 1 in the erythrocyte plasma membrane which is reported to have only one tyrosine residue near the carboxy terminus of the polypeptide chain, but is better accessible to lactoperoxidase at the NH_2-terminus. (Segrest et al., 1973).

This aspect of the project is still in its infancy and we have not yet been able to equate any of the surface components with iodinated polypeptides. However, component 1d which we suggest is located on the inside of the inner membrane, closely corresponds in mol. wt. to the α and β subunits of the F_1-ATPase (58,000 and 54,000 respectively) (Schatz, 1979).

Further work on the identification of protein components in terms of molecular functions is anticipated by preparing antiserum to defined surface components and then analysing the respective immune-precipitates by SDS-polyacrylamide gel electrophoresis.

The technique of peptide mapping as described by Bretscher (1971) will be very useful for study of the orientation membrane proteins and glycoproteins. After labelling and separation of mitochondrial proteins in RSO and ISO vesicles by use of a membrane impermeable reagent, it should be possible to elute specific protein bands and subject them to detailed peptide mapping subsequent to

enzymic hydrolysis. This type of data will be useful for distinguishing between polypeptides which span the membrane, or those that are symmetrically distributed in both halves of the lipid bilayer.

CHAPTER 9

Possible functional roles for glycoproteins in rat liver mitochondria.

9.1 Introduction

It has been suggested by Guidotti (1976) that all transport processes carried out across eukaryotic membranes should be catalysed by oligomeric glycoproteins which span the membrane. Of the 2 major energy linked functions taking place in mitochondria : ATP synthesis and Ca^{2+} transport, the latter is reported to be mediated via glycoprotein (Sottocasa *et al.*, 1972). Preliminary evidence has also been presented of a role for carbohydrate in the ATP synthetase complex (Andreu *et al.*, 1977). However, the detailed function of the 10-12 glycoproteins (see Chapter 7) associated with the inner and outer mitochondrial membranes remains largely unresolved.

One method for probing specific alterations in functions of mitochondrial glycoproteins is to specifically "block" these macromolecules. This is achieved by incubation with lectins or specific antibodies after mild hypotonic shock or digitonin treatment to render the outer membrane permeable to these high mol. wt. proteins. In this manner, using antibodies against Ca^{2+} binding glycoprotein Panfili *et al.* (1976) have specifically inhibited Ca^{2+} transport in mitochondria and mitoplasts.

By employing lectins as specific "blocking agents", we have monitored the effects on two of the best characterised energy-linked protein functions in mitochondria and mitoplasts.

Any inhibition by lectin treatment will implicate membrane glycoprotein for an active role in that particular function.

9.2 Determination of P:O ratios in rat liver mitochondria

The yield of oxidative phosphorylation is generally expressed by the P:O ratio, that is the ratio of nmol ADP phosphorylated (to ATP) to the number of ng-atoms of O_2 consumed. Frequently the degree of "respiratory control" is of interest. This is defined as the respiratory rate in the presence of ADP to the rate obtained following its (ADP) consumption. This assay is based on the phosphorylation of exogenous added ADP and Pi, which is directly coupled to FAD-linked reduction of succinate.

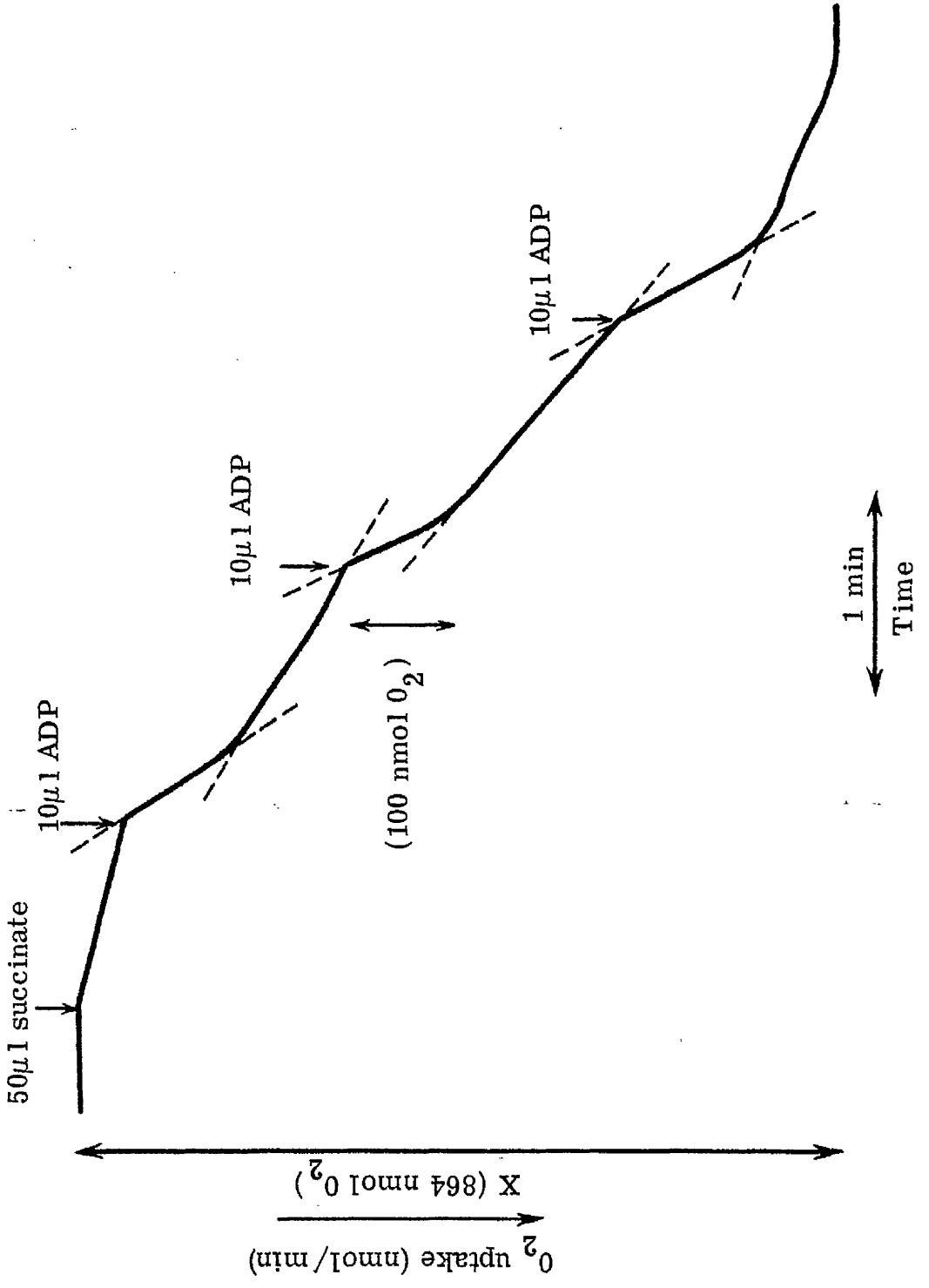
The experiment shows the routine method by which we have measured P:O ratios. Energy-linked phosphorylation in mitochondria is measured by the oxygen electrode method (Estabrook, 1967). Into the cuvette of the O_2 electrode containing the assay buffer is added an aliquot of isolated mitochondria and respiration initiated by addition of substrate succinate. As illustrated in Fig. 9.1 after the linear rate of respiration is established in the presence of substrate, addition of ADP causes an immediate increase in the rate of O_2 consumption. The amount of O_2 utilised is proportional to the amount of ADP phosphorylated to ATP.

The P:O ratio is calculated by determining the number of n mol of O_2 consumed for the quantity of ADP added. In the example illustrated in Fig. 9.1 the medium contains 240.0 n mol O_2/ml (Handbook of Chemistry and Physics, 33rd Ed., 1951-1952. p.1481); X = 80.0 recorder divisions; Y = 9.5 divisions

Fig. 9.1.

Determination of ADP:O ratios in freshly isolated rat liver mitochondria.

Rat liver mitochondria are isolated (M 4.1) and finally resuspended in MS-MOPS-Pi, pH 7.2 (M 3.8.1). Phosphorylation of ADP is monitored by oxygen uptake in an assay medium containing 3.4ml MS-MOPS-Pi buffer, pH 7.2, 100µl mitochondrial protein (approx. 3mg), 0.1ml BSA (1mg/ml), succinate (50µmol) and ADP 390 nmol) are added as indicated. The calculation of ADP:O is described in the text.


O_2 uptake (nmol/ min)
X (864 nmol O_2)
50µl succinate
10µl ADP
10µl ADP
10µl ADP
(100 nmol O_2)
1 min
Time

(average of 2 values); therefore 200.0/n mol of O_2 is utilised during active (state 3) respiration. A 10µl aliquot of 39mM-ADP is added equivalent to 390n mol. Correspondingly, the calculated P:O ratio is 1.95. A value of 2.0 is expected for FAD-linked substrates.

9.3 Measurement of energy-linked Ca^{2+} transport in rat liver mitochondria.

Mitochondria can accumulate certain ions from the suspending medium in a process linked to electron transport. When accumulation of divalent cations occurs e.g. Ca^{2+}, Sr^{2+}, it replaces oxidative phosphorylation. These processes are therefore alternate and each is stochiometric with electron transport. This experiment measures the energy-linked uptake of Ca^{2+} by monitoring the O_2 consumption.

A known amount of Ca^{2+} is added to mitochondria respiring in the presence of substrate, and the amount of extra oxygen uptake is recorded. Fig. 9.2. shows the stimulation of respiration obtained on addition of Ca^{2+} to respiring mitochondria (state 4). It is calculated that 49.3 n mol O_2/mg of protein is consumed on addition of 2500 n mol of Ca^{2+}. On addition of an aliquot of La^{3+}, there is almost complete inhibition of Ca^{2+} uptake, as shown by lack of respiration. The n mol of O_2 consumed being reduced to 8.5 n mol/mg protein.

9.4 Effect of lectins on Ca^{2+} transport in rat liver mitochondria and mitoplasts.

By using lectins to specifically "block" mitochondrial glycoprotein functions, we have investigated their effect on

Fig. 9.2.

Investigation of energy-linked Ca^{2+} uptake in freshly isolated rat liver mitochondria.

Rat liver mitochondria are isolated (M 4.1) and finally resuspended in MS-MOPS, pH 7.2 (M 3.8.1). Energy linked Ca^{2+} uptake is monitored by its effect on oxygen consumption in an assay medium containing 3.4ml MS-MOPS buffer, pH 7.2, 100µl mitochondrial protein (2.5mg), 0.1ml BSA (1mg/ml), succinate (50µmol) and Ca^{2+} (2.5µmol) are added as indicated.

In a separate experiment, indicated by the broken line La^{3+} (10µmol) is added prior to addition of Ca^{2+} (2.5µmol).

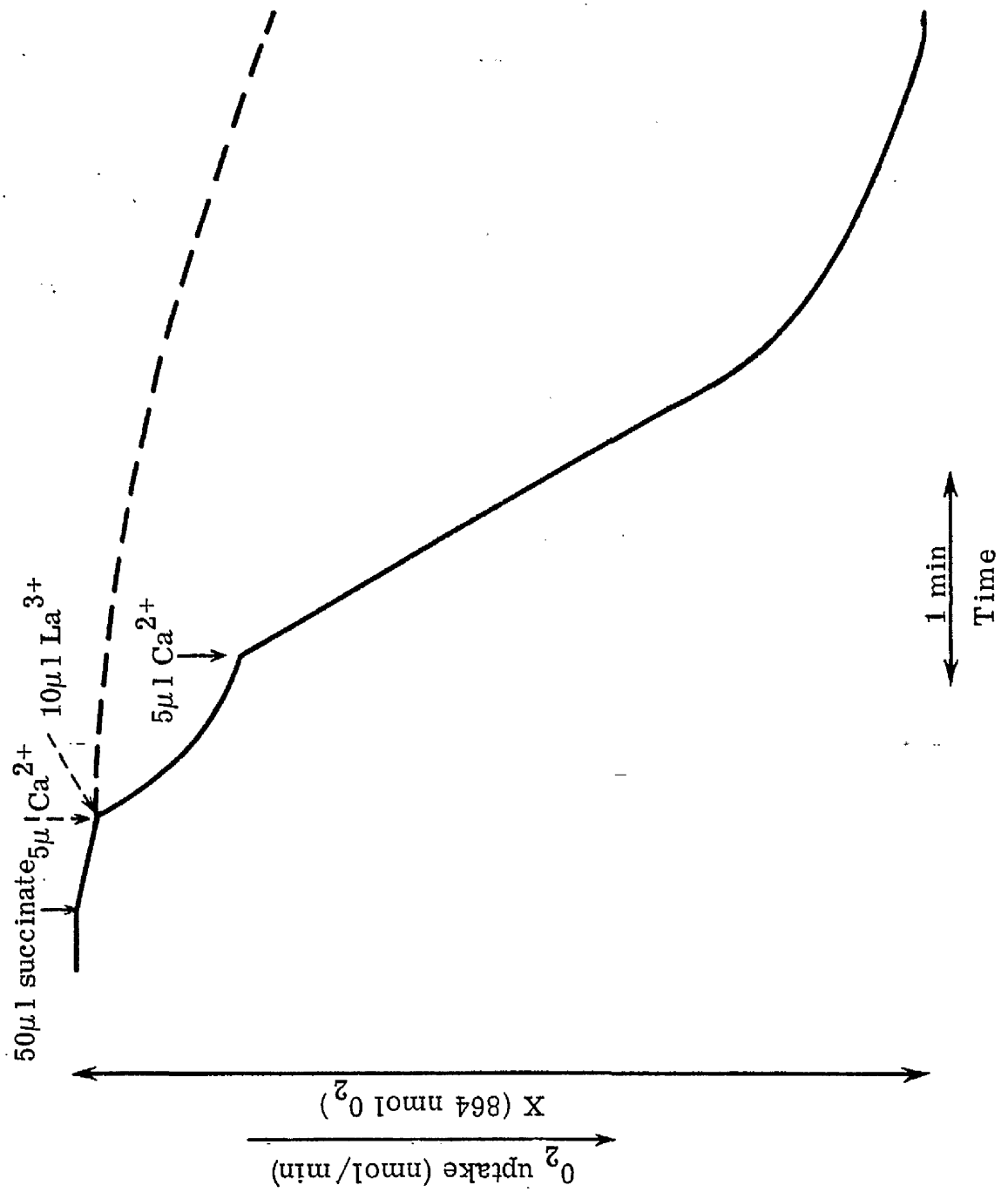

50µl succinate
5µl Ca2+
10µl La3+
5µl Ca2+
1 min
Time
O2 uptake (nmol/min)
X (864 nmol O2)

Table 9.1.

Investigation of energy-linked Ca^{2+} uptake into rat liver mitochondria and mitoplasts pretreated with lectins.

Rat liver mitochondria are freshly isolated (M 4.1) and resuspended in MS-MOPS, pH 7.2 (M 3.8.1). An aliquot of the mitochondrial protein (20mg) is treated with digitonin (0.11mg of digitonin/mg protein) as described in (M 4.3.2), except that the swelling and shrinking stage is omitted.

Mitochondria (10mg) and mitoplasts (10mg) are treated with SBA, WGA and Con A (2mg) by incubation for 1h at $4^{o}C$. The lectin-treated mitochondria and mitoplasts are washed twice in MS-MOPS, pH 7.2 to remove unbound lectin and finally resuspended in 1ml of the same buffer.

Energy-linked Ca^{2+} uptake is monitored in an oxygen electrode by addition of 3.5ml MS-MOPS buffer, 0.1ml BSA (1mg/ml and 100µl mitochondrial suspension. Respiration is initiated by addition c succinate (50µmol), and Ca^{2+} (2.5µmol) is added once a steady stat of respiration is achieved. Ca^{2+} uptake is monitored by stimulation in the rate of respiration.

Table 9.1

Effect of lectins on Ca^{2+} transport

Sample	Lectin Treatment	Other Additions	n mol O_2/mg protein/min
Mitochondria	-	-	12.7
Mitochondria	-	Ca^{2+}	98.6
Mitochondria	-	Ca^{2+}, La^{3+}	15.9
Mitochondria	WGA	Ca^{2+}	104.2
Mitochondria	Con A	Ca^{2+}	105.4
Mitochondria	SBA	Ca^{2+}	58.6
Mitoplasts	-	-	11.1
Mitoplasts	-	Ca^{2+}	94.4
Mitoplasts	WGA	Ca^{2+}	49.4
Mitoplasts	Con A	Ca^{2+}	98.4
Mitoplasts	SBA	Ca^{2+}	52.8

transport of the cation.

Isolated mitochondria and mitoplasts are pre-incubated with different lectinsas described in Table 9.1. Preliminary data indicates that SBA specifically affects Ca^{2+} transport in intact mitochondria and mitoplasts, whereas with WGA Ca^{2+} transport is effected only in mitoplasts. No observable effect of Con A on Ca^{2+} transport could be detected. This could be attributed to the presence of mannitol and sucrose in the assay buffer which may act as competitive hapten inhibitors for the lectin binding sites.

9.5 Effect of lectins on P:O ratios in rat liver mitochondria and mitoplasts.

In order to investigate if glycoproteinsare involved in energy-linked phosphorylation, the effect of lectins on P:O ratios have been monitored.

The P:O ratios for control and lectin treated mitochondria using succinate as substrate is tabulated in Table 9.2. No respiratory control is observed in SBA treated mitochondria, indicating that the lectin interferes with the phosphorylation. The other lectin treated samples demonstrate usual P:O ratios. In mitoplasts, a much lower P:O ratio is observed, possibly due to extraction of extrinsic respiratory chain components e.g. cytochrome c during the digitonin fractionation. No respiratory control is observed in mitoplasts pre-treated with lectins (WGA, Con A and SBA), although there is a stimulation of respiration on addition of ATP.

Table 9.2

Measurement of P:O ratios in rat liver mitochondria and mitoplasts pretreated with lectins.

Rat liver mitochondria and mitoplasts are prepared and treated with lectin as described in Table 9.1. P:O ratios are estimated as described in Fig. 9.1 in an oxygen electrode by addition of 3.5ml MS-MOPS-Pi, pH 7.2, 0.1ml BSA (1mg/ml) and 100μl of mitochondrial protein (3mg). Respiration is initiated by addition of succinate (50μmol), and ADP (390nmol) is added at intervals once a steady state of respiration is achieved.

Table 9.2

Effects of lectins on P:O ratios

SAMPLE	Amount of O_2 consumed (nmol)	n mol ADP	P:O ratio
Control Mitochondria	200.0	390	1.95
Mitochondria - WGA	226.0	390	1.73
Mitochondria - Con A	200.0	390	1.95
Mitochondria - SBA	No respiratory control	390	-
Control Mitoplasts	248.4	390	1.57
Mitoplasts - WGA		390	-
Mitoplasts - Con A	No respiratory	390	-
Mitoplasts - SBA	control	390	-

9.6 Discussion

Mitochondrial glycoproteins can be specifically modified by incubation with purified lectins which act as "blocking agents". In our studies, the marked inhibitory effects observed on Ca^{2+} transport in mitochondria which are treated with either WGA or SBA, underline the value of this approach in determining the individual roles of mitochondrial glycoproteins.

The only mitochondrial glycoprotein in which a transport function is implicated involves active Ca^{2+} transport (Sottocasa et al., 1972). The isolated glycoprotein does not interact with Con A (Hughes, 1976) in agreement with our data, thus suggesting that α-mannosides and glucosides are not present in the glycoslyated portion of the protein.

The Ca^{2+} binding glycoprotein is reported in the literature as having a mol. wt. ranging from 40,000 (Sottocasa et al., 1972) to 31-33,000 (Carafoli, 1976). This apparent discrepancy could be attributed to the anomalous behaviour of glycoproteins during polyacrylamide gel electrophoresis. Also, the fact that the protein is partly located in the inter-membrane space could result in different quantities of the species being extracted during the various experiments. In relation to the immunoprecipitation studies and lectin overlay data which demonstrates proteins that are specifically bound by WGA, component 2a (mol. wt. 43,000) appears to be similar to the Ca^{2+} binding glycoprotein. However, further confirmation of this fact by use of specific antisera against the glycoprotein is anticipated.

We have found that pre-incubation for 1h is necessary to obtain complete combination between lectin and glycoprotein as no effect on Ca^{2+} uptake is observed if this step is omitted (data not shown). It is difficult to predict the mechanism by which the lectins effect Ca^{2+} transport, that is by specifically impeding a translocase process, or modulating membrane interactions with the microfilament system which impairs the transport system. The latter suggestion is also put forward by Li and Kornfeld (1977) for the cytotoxic effect of WGA on several tissue culture lines which inhibit transport of non-utilizable amino acids.

Finally, the fact that SBA affect P:O ratios only in intact mitochondria, indicates that it may be blocking uptake of ADP via non-specific "pores" present in the outer membrane. It still remains to be documented whether the inhibitory effect of this lectin is owing to binding to the respiratory chain components or interference at the energy coupling stage. The latter effect would be of particular interest as the presence of carbohydrate is suggested in the F_1 ATPase (Andreu et al., 1977).

CHAPTER 10

General Discussion

10.1 Conclusions on the topography and organisation of the mitochondrial membrane proteins and glycoproteins

In this project we have established that carbohydrate is present on the external surface of both inner and outer mitochondrial membranes, largely associated with glycoprotein. We have confirmed that approx. 70% of this material is readily solubilised on disruption of the organelle. A large portion of this glycoprotein fraction (50%) is loosely-bound to the outer surface of the organelle and released under controlled conditions which do not disrupt the integrity of the outer membrane.

Specific surface-labelling of intact rat liver mitochondria (outer membrane), and mitoplasts (inner membrane) have allowed us to establish the major polypeptides species accessible at the outer surface of these membranes. This approach has been extended using immunoprecipitation techniques to demonstrate the glycoprotein nature of the majority of these components.

Lectin affinity procedures have been developed to fractionate heterogeneous populations of inner membrane vesicles. Criteria for the evaluation of the polarity of isolated vesicles has been developed based on the sidedness of known enzymes, e.g. the binding site of cytochrome c, the substrate for the transmembrane cytochrome c oxidase complex,

is normally located on the outer phase of the inner membrane. Thus in sealed ISO populations this activity is latent unless disrupted by detergents. By employing vesicles of defined orientation in surface labelling studies, we have been able to examine the topography of the inner membrane and in particular identify transmembrane proteins.

Using lectins as specific "blocking agents", we have assessed the role of glycoproteins in the 2 major energy-linked functions in mitochondria: ATP synthesis and Ca^{2+} transport. We have shown that SBA specifically diminishes Ca^{2+} transport in intact mitochondria. Both SBA and WGA have a similar effect on Ca^{2+} transport if the outer membrane is partially removed. Conversely, SBA effects P:O ratios only in intact mitochondria.

The model in Fig.10.1 depicts our current prejudices on the way we visualise the topography, organisation and function of the major proteins/glycoproteins associated with mitochondrial membranes.

Our surface-labelling studies on intact mitochondria and mitoplasts have clearly established the major polypeptides present on the surface of inner and outer mitochondrial membranes. We have designated the polypeptides into one of four categories. The first category represents proteins that are exclusively present on the outer membrane: bands 1, 2, 3, 4, 5, 6 and 7. In category 2 are proteins which appear when the outer membrane is partially disrupted, and disappear on complete removal of the membrane suggesting an inter-membrane location for these components; 1c, 2a and 6c.

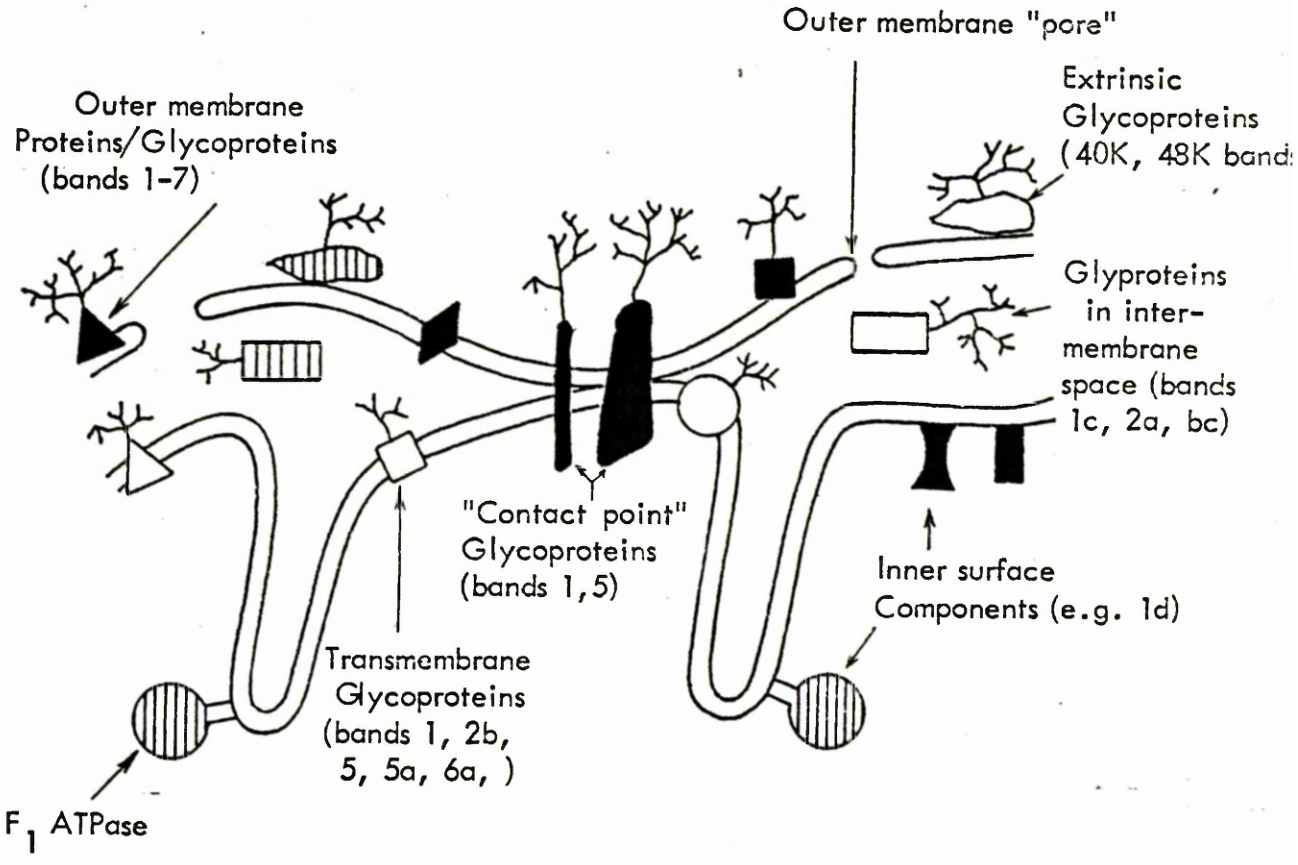


Fig. 10.1

Diagrammatic representation of the proteins and glycoproteins identified in rat liver mitochondrial membranes.

Category 3 includes proteins that appear to be inner membrane components: bands 1b, 2b, 5a and 6b. Finally, into category 4 are assigned polypeptides which appear to traverse both membranes: bands 1 and 5. Several of these major polypeptide components are glycosylated or as shown by immunoprecipitation studies and include bands 1, 1a, 1b, 1c, 2a, 2b, 4, 5, 5a, 6, 6b. 7 and 7a.

10.2 Criteria for homogeneity of "inside-out"vesicles

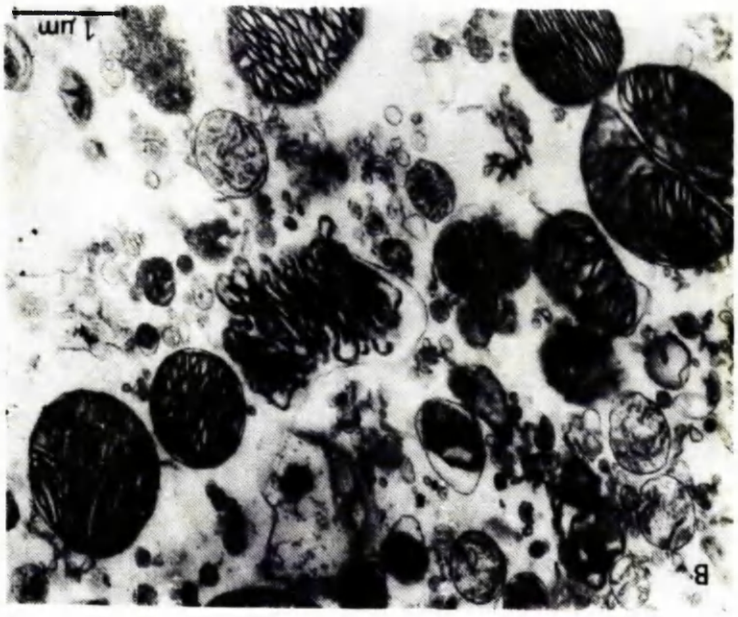
In order to determine the orientation of proteins within the lipid bilayer it is convenient to have access to defined populations of ISO vesicles. Comparative labelling studies on vesicles of defined polarity will ascertain the topography of proteins within the membrane; in particular identify trans-membrane proteins. From a historical standpoint, it has been documented that sonication causes a fragmentation and "pinching off" of the cristae, giving rise to vesicles whose outer surface corresponds to the original inner surface of the cristae. This is visualised by the position of the repeating ATPase units (F_1) on electron micrographs and other functional criteria, such as reverse proton uptake which indicates that vesicles appear to have an opposite polarity to intact mitochondria (Lee and Ernster, 1966).

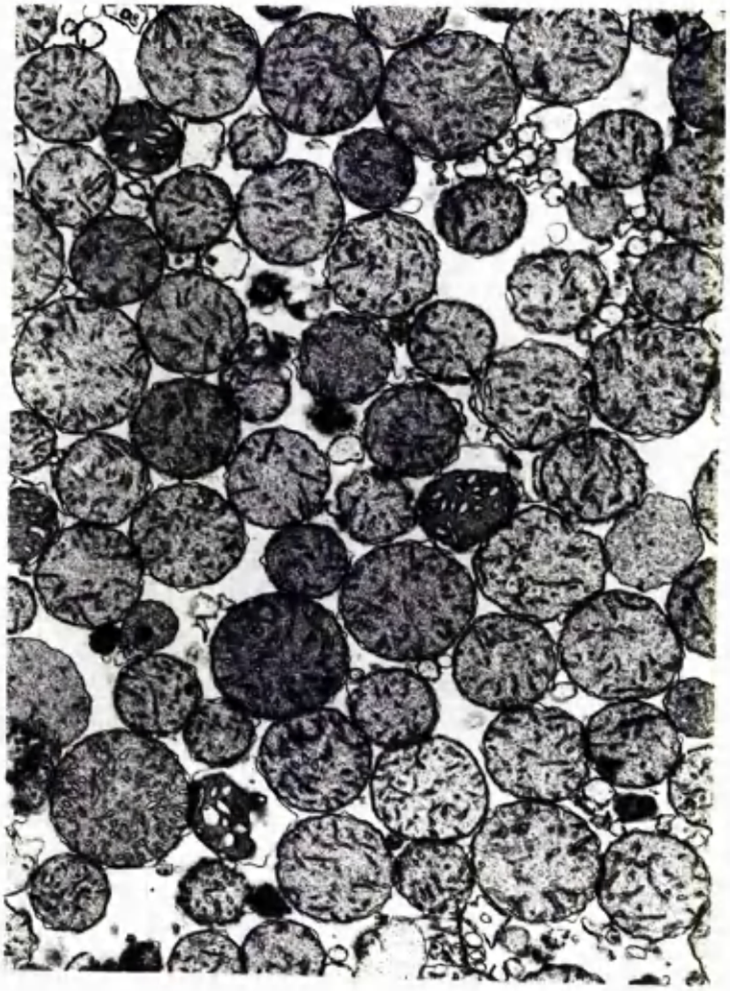
In the case of beef heart mitochondria which has a very convoluted inner membrane comprising of 50-60% (w/w) of the total mitochondrial protein (as compared to 15-25% (w/w) in rat liver), it is possible that sonication gives rise to a homogeneous population of ISO vesicles. However, in inner membranes

Fig. 10.2.

Comparison of the cristal structures in mitochondria isolated from rat heart and rat liver.

(reproduced from Acta Biochim. Polonica. 19, L. Wojtczak, H. Zaluska, A. Wroniszewska and A.B. Wojtczak (1972) and J. Cell Biol. 37, C.R. Hackenbrock (1968b).


1 µm
B



prepared from rat liver mitochondria which do not have such a convoluted cristal structure, it seems doubtful that SMPs represent a sealed homogeneous population of ISO vesicles. Disruption of the organelle in general leads to the formation of mixed populations containing ISO, RSO and non-sealed fragments with some "scrambled" membrane induced by the fusion of vesicles of opposite sidedness. Fig. 10.2.

In the inner mitochondrial membrane where good criteria exist for assessing "sidedness", we have developed a procedure for isolation of purified ISO vesicles from mixed membrane fractions. This technique is based on the ability of lectins to interact with specific monosaccharides in the carbohydrate chains of membrane glycoproteins.

An important corollary from this experiment is the asymmetric distribution of carbohydrate on the external surface of the inner membrane, as ISO vesicles are neither absorbed nor precipitated by lectins. Although Andreu et al. (1978) have reported the presence of carbohydrate in the α and β - subunits of the F_1 ATPase from beef heart mitochondria, there still appears to be much controversy about the result. Nalin et al. (1979) have reported a complete lack of carbohydrate in the F_1 ATPase as detected by identical means (PAS stain). We tend to favour the latter result as it supports our observations on the asymmetric distribution of carbohydrate on the external surface of the membrane. Alternatively, if the ATPase is glycosylated, we can unequivocally conclude that it possesses no WGA binding sites.

This technique has enormous potential for use in any membrane system where the carbohydrate is asymmetrically distributed, particularly if marker enzymes are available to assess vesicle sidedness. A similar principle has been utilised by Walsh et al., (1976) to isolate ISO fragments from lymphocyte plasma membranes.

10.3 Speculations on the role of the mitochondrial outer membrane.

The presence of carbohydrate on the outer mitochondrial membrane is of considerable significance. The glycoproteins of cell membranes are largely intrinsic proteins, anchored by hydrophobic interactions in the lipid bilayer with the carbohydrate groups facing towards the outside, as in band 3 and glycophorin of the red blood cell membrane (Steck, 1974). The sugar groups from complex arrays with considerable variation from protein to protein. The polypeptide portions may be anchored in the lipid bilayer by a sequence of hydrophobic amino acid residues in an α-helical form that may traverse the bilayer, but in some cases several polypeptides may collectively form a complex that traverses the membrane. The importance from a functional point of view is that protein continua span the bilayer so that they are in contact with both the external and cytoplasmic environments.

The set of functions in which glycoproteins seem to play an important and specific role, is the response of cells to environmental substances, a function particularly applicable to bands 1 and 5. The response involves 3 types of interactions associated with 3 different parts of the glycoprotein

molecule. The first is recognition (specific binding) of an extracellular molecular e.g. antibody or lectin or soluble glycoprotein. The second involves a triggering reaction (or form of communication) across the membrane-spanning segment. The third involves interactions at the inner face of the membrane between the membrane glycoproteins and other proteins. This interaction may involve responses such as release of cAMP, phosphorylation of protein, release of Ca^{2+}, or modulation of the microfilament system.

The fact that mitochondria are able to respond to external hypo- or hyper-tonic media by swelling and contraction of the inner membrane (Hackenbrock, 1968a) indicates the existence of a microfilament system in the organelle. This is supported by the observation that cytochalasin B at concentrations of around 10^{-4} inhibits contraction of mitochondria induced by ATP and Mg^{2+} (Lin et al., 1973). The alkaloid has a similar effect on ATP synthesis, but to a lesser extent.

No direct role can presently be given for glycoproteins in membrane movements or flow, or in the maintenance of special forms of membrane architecture, although all these processes may be modulated by external factors through the mediation of surface glycoproteins.

Another probable role for the outer membrane could be to provide a "frame-work" into which the hydrophobic portions of amphipathic proteins are anchored. This would allow the hydrophilic, catalytically active portion of the molecule access to the membrane interface and its catalytic effectors in the cytoplasm. An example of this situation has been presented

for the two microsomal enzymes, cytochrome b_5 and cytochrome b_5 reductase. Both enzymes contain distinct hydrophobic segments which provide firm attachment of the proteins to the endoplasmic reticulum. The hydrophobic segments containing the catalytic sites are oriented towards the cytoplasm, permitting interactions of the polar catalytic heme with either reductase, cytochrome c or the desaturase of the electron transfer system (Spatz and Strittmatter, 1971; Strittmatter et al., 1972).

Finally, it is proposed that the outer membrane plays a special structural role in preventing osmotic swelling and lysis of the organelle, particulary during the morphological changes observed in respiration (Lemaster, 1978).

10.4 Mitochondrial-cytoplasmic interactions

Components which traverse both membrane are of particular biochemical significance in the case of mitochondria, for it is well established that the majority of mitochondrial proteins are synthesised on cytoplasmic ribosomes and then transported into the organelle (Schatz and Mason, 1974). Maccecchini et al. (1979) have recently documented evidence for the existence of larger precursor forms of the three larger subunits of the soluble F_1 ATPase. Also subunit V of the mitochondrial bc_1 complex is synthesised as a larger polypeptide in vitro (Côté et al., 1979). Transport of these proteins into the mitochondrion is accompanied by cleavage of a signal sequence which converts the protein into a mature form in the organelle. Thus it appears that translation and import of proteins are two

independent processes (Maccecchini et al., 1979; Côté et al., 1979). A similar import mechanism appears to operate during the transport of polypeptides into chloroplasts (Highfield and Ellis, 1978), and perhaps also during the transport of bacterial toxins across the plasma membrane of prokaryotes and eukaryotes (Neville and Chang, 1978).

Schatz (1979) has suggested that the mitochondrial polypeptide precursors migrate to contact points in the membrane. Interactions with the precursor opens up a pore across both membranes which allows the polypeptide to equilibrate across the membrane barrier. Unindirectional transport is achieved by irreversible proteolytic processing of the precursor in the matrix. The process of protein import is energy dependent and does not occur if ADP-ATP transport is inhibited or oxidative phosphorylation uncoupled (Schatz, 1979).

In this regard the saccharide moieties of bands 1 and 5 (traverse both membranes) could serve as receptors for the precursor protein, triggering of interactions with the cytoskeletal system, which may modulate formation of a protein lined pore through which nascent polypeptides are inserted. Other facts which support this mode of protein import into mitochondria are the observed contact points between inner and outer membranes (Hackenbrock, 1968a) which could facilitate the formation of a transmembrane polar channel. Also, examination of double membrane fracture faces of mitochondria from yeast (Packer et al., 1973) and mammalian cells (Melnick and Packer, 1971) reveals the existence of double membrane

fracture faces showing large regions where the outer and inner membranes are fused together.

From a theoretical point of view this is feasible. The membrane thickness is approx. 8nm, thus at the reported contact points (Hackenbrock, 1968a)the total membrane width expected is 16nm. Based on this figure, it is possible to calculate that in a α-helix configuration a protein of approx. mol. wt. 10,000 spans the contact points.

The porous nature of the outer membrane has been well documented (Pfaff et al., 1968). The membrane is permeable to solutes of mol. wt. up to 4,000 suggesting "holes" with an approx. diameter of 2nm. This is supported by the fact that cytochrome c which has an effective spherical diameter of 34°A (Dickerson, 1969) does not penetrate the outer membrane (Wojtczak and Zaluska, 1969). The physiological significance of these "holes" other than for the passage of small solutes is unknown. Whether bands 1 and 5 are actually integrated into the lipid bilayer of the outer membrane, or are simply exposed on the cytoplasmic surface by penetrating these "holes" is a question which requires further investigation.

The synthesis of membrane proteins probably begins on free ribosomes. The N-terminal sequence of the new peptide then causes attachment of the ribosome to the endoplasmic reticulum. Evidence that all glycoproteins, including membrane glycoproteins are assembled on membrane bound ribosomes has been presented (Morrison and Lodish, 1975).

Recently, the presence of a signal sequence of approx. 16 amino acids of a transmembrane glycoprotein of vesicular

stomatitis virus has been demonstrated (Lingappa et al., 1978). The ribosomes become attached to the microsomal membrane via a ribosome binding protein, which is postulated to assemble into a hydrophilic channel for the passage of nascent polypeptides through the membrane. After translation is complete, the channel closes leaving the C-terminal exposed on the cytoplasmic surface and the ribosomes become "unbound". The signal sequence is cleaved off and carbohydrate incorporation begins within the lumen of the endoplasmic reticulum.

In view of the hypothesis put forward by Schatz (1979) for import of cytoplasmically synthesised proteins into mitochondria, it appears likely that these species are synthesised on free polysomes and therefore remain unglycosylated. It is possible that glycosylation of nascent polypeptide chains occurs in mitochondria by a mechanism remote from the endoplasmic reticulum and Golgi apparatus. It seems improbable, however, that glycosylated proteins are imported into mitochondria as the membrane shuttle hypothesis, (migration of vesicles containing newly synthesised glycoproteins which fuse into the existing plasma membrane, Steiner et al., 1974) would result in the reverse orientation of glycoproteins to that reported in this study.

In the light of this data, we would favour a mechanism by which glycosylation of nascent mitochondrial polypeptide chains occurs within the organelle, independent of the endoplasmic reticulum and Golgi apparatus. Support for this suggestion comes from the demonstration that radioactively labelled sugar nucleotides are incorporated into acid

precipitable glycoproteins in intact mitochondria (Bosmann, 1971).

10.5 Functional correlations with glycoproteins.

Future work.

The central problem of membrane structure and its correlation with physiological and biochemical functions is to define the organisation of constituent molecules. This is a necessary pre-requisite before a function can be allocated to the components in molecular terms. Two important processes in mitochondria which have been correlated with specific protein transporters are : Ca^{2+} and ADP/ATP transport.

Ca^{2+} transport thought to be mediated via a specific glycoprotein located within the inner membrane and inter-membrane space. (Sottocasa et al., 1972). This component closely corresponds to band 2a identified in our system. However, confirmation of this fact is anticipated by use of specific antisera against a purified glycoprotein implicated in Ca^{2+} binding and transport.

In our system component 2b appears to strongly correlate with the adenine nucleotide translocase. Riccio et al. (1975) have isolated this protein on a column of hydroxyapatite from mitochondria that are pretreated with radio-labelled carboxyatractyloside. Later, Boxer et al. (1977) have identified the CAT binding protein as the major component that is labelled on the outer surface of the inner membrane in ox heart mitochondria. In rat liver mitochondria, we have shown

the first time that component 2b is glycosylated and
verses the inner membrane supporting its function of
leotide translocation. Correlation of component 2b
n the adenine nucleotide translocase is anticipated by
lating the protein from rat liver mitochondria and
sequent identification by gel electrophoresis.

In view of research on red blood cells and other plasma
oranes, glycoproteins frequently if not always span the
id bilayer and are important in recognition and transport
ctions. Preliminary investigation into the functions of
identified glycoproteins by the use of lectins on observed
transport and ATP synthesis underline the value of this
roach in determining the individual roles of mitochondrial
coproteins. In this context, we hope to extend our
ctional studies to cover the full range of mitochondrial
and substrate transport systems to monitor the effects
specific lectins and antibodies.

APPENDIX

Development of 2-dimensional electrophoresis for separation of rat liver mitochondrial membrane proteins.

A.1. Introduction

During the course of this investigation, we felt it would be useful to have a technique by which we could obtain detailed peptide maps of mitochondrial membrane proteins. Minor components, or even relatively abundant proteins cannot be distinguished if their mol. wt. is the same as that of one of the major components; thus resolution obtained by one-dimensional dodecyl sulphate gel electrophoresis is not adequate in many cases.

In this appendix we describe a two-dimensional separation of membrane proteins which offers a much greater power of resolution (O'Farrell, 1975; Ames and Nikaido, 1976). The method involves isoelectric focusing (IEF) of proteins in the presence of non-ionic detergents and urea in the first dimension, followed by SDS-polyacrylamide gel electrophoresis which separates proteins on the basis of mol. wt. in the second dimension.

The object of these experiments is to characterise using IEF, the banding pattern of mitochondrial membrane proteins. We have also attempted to optimise the procedure for further separation in the second dimension. Although, we were unable to continue this technique on a routine basis owing to the technical difficulties experienced in obtaining reproducible results, further work is envisaged, particularly as regards detailed peptide mapping of the five inner membrane

complexes in terms of their topographical distribution and possible functional roles.

A.2. Isoelectric focusing of mitochondrial membrane proteins.

In developing this method of membrane protein analysis we have aimed at obtaining effective solubilisation of membrane proteins in SDS, followed by IEF in the first dimension. An important reason for separating proteins by IEF, is that large amounts of material (200µg) can be applied to the gel without running into overloading problems; as compared to a dodecyl sulphate gel. This is particularly advantageous as the problem of low specific activity of radio-labelled proteins and difficulty in detecting minor species can be circumvented.

Isolated mitochondrial inner, outer and membrane mixture are solubilised by using SDS as denaturing agent as described in Materials and Methods (M 8.4.2). The prepared samples are then polymerised into the gel mixture containing urea and Nonidet P-40 and electrophoresed in a cylindrical gel apparatus as described in (M 8.3.1).

Fig. 1 shows the polypeptide components of mitochondrial membranes as visualised by Coomassie Blue staining (Vesterburg et al., 1977). The pH gradient is measured by use of an antimony microelectrode system (Beeley et al., 1972). The gradient is linear ranging from 3.5 to 9.5, comparable with that obtained by Beeley et al., (1972) for the ampholine range pH 3.5 to 10.0 (see Fig. 11). There are fewer proteins

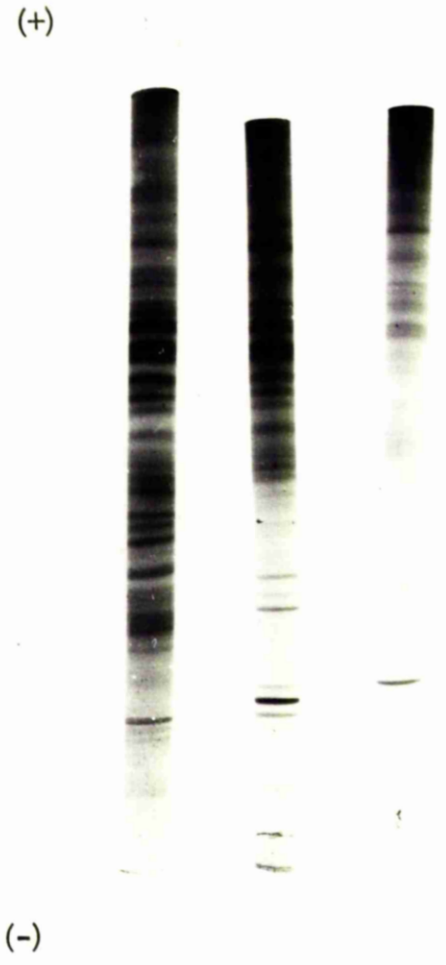
Fig. I.

Isoelectric focusing of the proteins of inner and outer rat liver mitochondrial membranes.

Rat liver mitochondria are isolated (M 4.1) and purified on a discontinuous sucrose density gradient as described in (M 4.3.1). The membranes are pelleted by centrifugation at 100,000g for 40 min and an aliquot (3mg) solubilised as described in (M 8.4.2).

IEF gels are prepared and the sample polymerised into the gel mixture prior to electrophoresis as described in (M 8.3.1). Extruded gels are fixed overnight in TCA (10% w/v) and proteins visualised by Coomassie Blue staining (M 3.5.3).

(a) membrane mixture
(b) inner membrane
(c) outer membrane


(+)
(-)
(a)
(b)
(c)

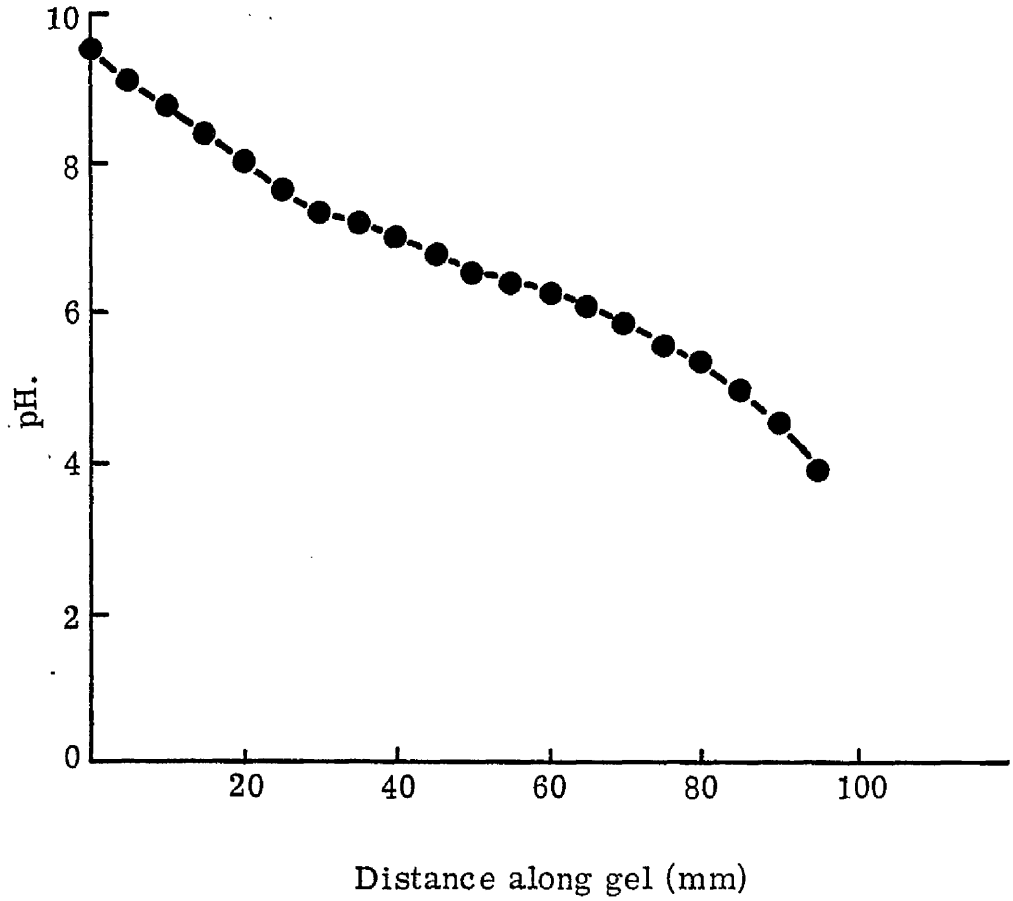


Fig. II.

Measurement of the pH gradient of an IEF gel

A sample containing a mixture of inner and outer membrane (200µg) is focused as described in Fig. I. The extruded gel is washed twice in distilled water and the pH along the gel measured at intervals (0.5cm) using an antimony microelectrode, pre-calibrated with standard solutions of known pH (M 8.3.3).

present at the basic end of the gel than the acidic, this resolution could be increased by incorporating a acidic ampholine range into the gel. In all cases however, over 30 bands could be resolved using ampholine range of pH 3.5 to 10.0.

A.3. Sodium dodecyl sulphate gel electrophoresis

After IEF of the mitochondrial membrane mixture, the extruded gels are equilibrated in SDS sample buffer (M 3.4.3.9) and fixed horizontally to a 10% (w/v) acrylamide slab gel with an agarose solution as described in (M 8.3.2). Electrophoresis in the second dimension resolves 70-80 spots as detected by Coomassie Blue staining (see Fig. III).

From the photograph, it can be seen that the resolving power of this method is much higher than any single dimension separation of proteins. Increased sensitivity could be achieved by radio-labelling the membrane, e.g. with $[^{125}I]$ and detecting the spots by autoradiography or fluorography.

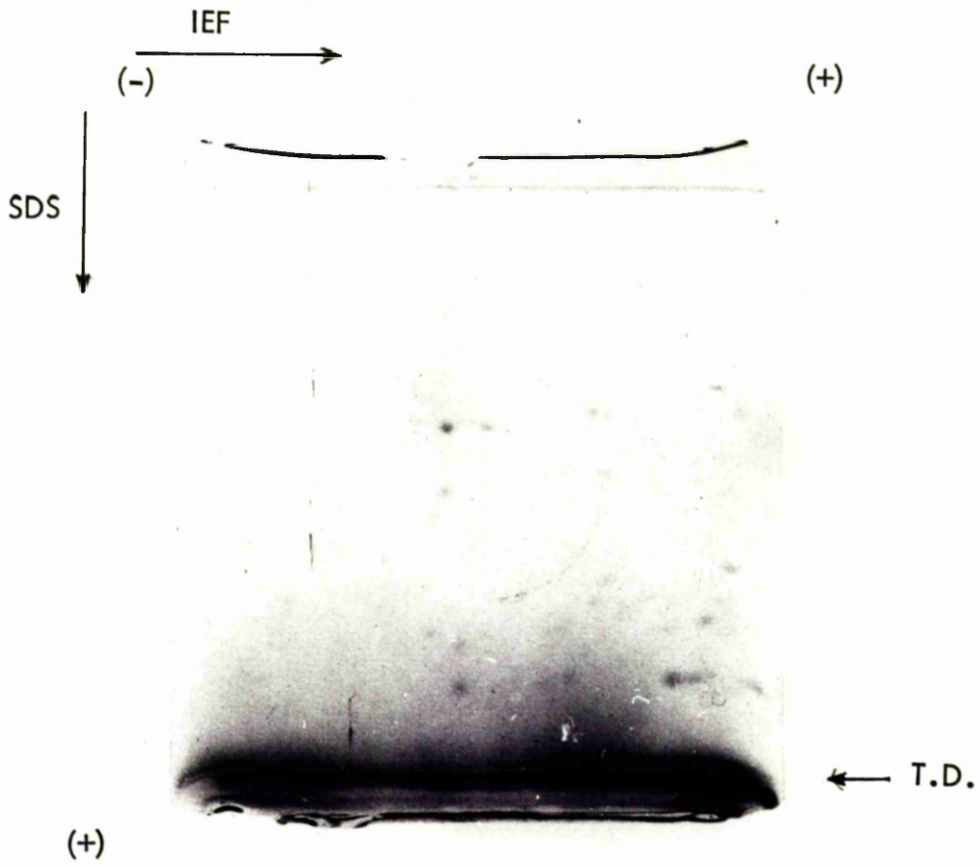
On some occasions, there appears to be a small amount of streaking at the acidic end of the gel. This could be due to a variety of reasons including poor solubility of proteins in that region or the presence of nucleic acids as discussed by O'Farrell (1975). Although SDS binds to protein with a high binding constant (Ray et al., 1966), it comes off the protein and forms micelles with Nonidet P-40. These migrate to the acidic end of the gel and could be responsible for streaking. Sometimes streaking is also observed at the dye front. This could be due to the

Fig. III.

Analysis of rat liver mitochondrial membrane proteins by 2-dimensional separation on polyacrylamide slab gels.

Rat liver mitochondrial are isolated (M 4.1) and sonicated for 2 x 30s. The membranes are pelleted by centrifugation at 100,000g for 40 min. An aliquot of the mitochondrial membranes (3mg) is solubilised as described in (M 8.4.2). The sample is polymerised into the IEF gel mixture (200µg/gel) and electrophoresis conducted as described in (M 8.3.1).

After equilibration of the extruded gels in SDS-buffer (M 3.4.3.9), gels are electrophoresed in the second dimension as described in (M 8.3.2). Protein spots are then visualised by staining with Coomassie Blue staining (M 3.5.1).


IEF
(-)
(+)
SDS
T.D.
(+)

ampholines in the gel which behave like very small proteins; they bind SDS and the vast majority migrate to the dye front. In the presence of SDS, the ampholines are acid precipitable and remain in the gel, observable as a dense line at the dye front after Coomassie Blue staining. This could mar the appearance of small proteins migrating near the dye front.

A.4. Conclusions and future use of the technique.

In conclusion, this method offers much potential for analysis of membrane proteins because of its capability to solubilise and then resolve denatured proteins by two different parameters: IEF, which separates proteins by virtue of their net intrinsic charge allows application of large quantities of solubilised sample. This should be useful in cases where it is not possible to prepare labelled membrane of high specific activity. The polypeptides can then be separated in the second dimension on the basis of size and visualised either by Coomassie Blue staining or autoradiographic/fluorographic procedures.

Using this technique in conjunction with autoradiography/fluorography it should be possible to detect e.g. [^{125}I]-labelled spots (accessible proteins at membrane surface), [^{3}H]-glucosamine-labelled spots (membrane glycoproteins) or [^{35}S]-methionine-labelled spots after in vivo incorporation of the radio-labelled amino acid (total membrane protein). A longer term objective is to attempt detailed mapping and characterisation of the inner membrane proteins. The availability of specific mitochondrial complexes e.g. succinate-cytochrome c reductase,

cytochrome c oxidase, will be useful in permitting preliminary assignment of the majority of inner membrane proteins to particular complexes on 2-dimensional analysis. Other individual proteins can be identified by their characteristic properties e.g. the Ca^{2+} binding glycoprotein (murexide staining) or the adenine nucleotide translocase (^{14}C-carboxyatractyloside tagging).

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