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ORIGIN OF GLYOXYSOMAL MEMBRANE PROTEINS

IN CASTOR BEAN ENDOSPERM

by

MICHAEL JOHN CONDER

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Department of Biological Sciences

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ABSTRACT

SUMMARY

Organelles isolated from castor bean (Ricinus communis L.) endosperm by sucrose density gradient centrifugation were assessed for purity by mixing isolated radiolabelled organelles with unlabelled homogenates and recentrifuging. The glyoxysomal fraction was found to be very pure but glyoxysomal membrane fragments contaminated the mitochondria and microsomes. Integral membrane proteins of the three major organelle fractions were isolated using the Triton X-114 method. SDS-PAGE analysis of Triton X-114 extracts revealed that the microsomes and glyoxysomes contained proteins of similar molecular weights. The slight glyoxysomal membrane contamination of the microsomes was dismissed as the cause of these similarities.

The e.r.-derived microsomal fraction was shown to possess lipid glucosyltransferase activity. The microsomal fraction was subfractionated on flotation gradients. Two major subfractions were obtained and appeared to represent microsomes of rough- and smooth-e.r. origin, the lighter subfraction being enriched in N-acetyl glucosaminyl and mannosyl transferases while the heavier subfraction was slightly enriched in protein fucosyltransferase.

Glycoproteins of the glyoxysomal membrane were shown by in vivo [<sup>3</sup>H] and [<sup>14</sup>C] sugar incorporation to possess oligosaccharide moieties containing glucosamine (GlcNAc), fucose and galactose residues. Mannose residues were detected by overlaying SDS-PAGE separated polypeptides with [<sup>125</sup>I] concanavalin A. The presence of GlcNAc and mannose indicated the presence of e.r. synthesized asparagine-linked oligosaccharide chains and this was confirmed by demonstrating (a) their susceptibility to endo-H and (b) inhibition of glycosylation by tunicamycin.

Glyoxysomal membrane protein synthesis was studied in vivo and in vitro. Structural similarities between shared microsomal and glyoxysomal membrane polypeptides were shown using antiserum to glyoxysomal Triton X-114 extracts. Castor bean mRNA translation in vitro demonstrated that the major glyoxysomal membrane proteins did not undergo co-translational insertion into canine microsomes and were possibly synthesized on free, cytosolic ribosomes.

The results are discussed with reference to recent models for microbody formation in other seeds and mammalian liver.

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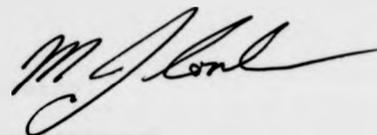
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DECLARATION

I hereby declare that the work presented here has not been used in any previous application for a degree. The work was carried out by myself at the Universities of Bradford, Warwick and the Technical University of Munich, Freising, FRG. The conclusions reached are my own, after many discussions with my supervisor Dr. J.M. Lord. All sources of information are acknowledged by reference.



M.J. CONDER

November 1984

ABBREVIATIONS

A6 <sub>3</sub> - Ag	-	antigen reacting with antibody secreted by cell-line A6 <sub>3</sub>
Anti GA <sub>S</sub>	-	antiserum to glyoxysomal integral membrane proteins
Asn	-	asparagine
ATP	-	adenosine 5'-triphosphate
Bis	-	N,N'-methylenebisacrylamide
BHT	-	butylated hydroxytoluene
BSA	-	bovine serum albumin
CDP	-	cytidine diphosphate
con A	-	concanavalin A
Da	-	dalton
DEAE	-	diethylaminoethyl
DMAE	-	dimethyl aminoethanol
DMSO	-	dimethyl sulphoxide
DNA	-	deoxyribonucleic acid
DOC	-	deoxycholic acid
dol	-	dolichol
dol-P	-	dolichol monophosphate
dol-PP	-	dolichol pyrophosphate
DTNB	-	5, 5' dithiobis- (2-nitrobenzoic) acid
DTT	-	dithiothreitol
EDTA	-	ethylenediaminetetra acetic acid
EGTA	-	ethyleneglycol-bis (2-aminoethyl ether) -N, N'-tetra acetic acid
ELISA	-	enzyme linked immunosorbant assay
endo-H	-	endo-N-acetylglucosaminidase H
e/m	-	region of sucrose gradient intermediate between e.r. and mitochondria
e.r./E.R.	-	endoplasmic reticulum
ERL - 4206	-	vinylcyclohexene dioxide

FITC	-	fluoresceine isothiocyanate
GAR	-	goat anti-rabbit IgG antiserum
GDP	-	guanosine diphosphate
GTP	-	guanosine triphosphate
GalNAc	-	N-acetyl galactosamine
Glc	-	glucose
GlcNAc	-	N-acetyl glucosamine
h	-	hours
Hepes	-	4- (2-hydroxyethyl)-1-piperazine ethanesulphonic acid
IAA	-	iodoacetamide
ICL	-	isocitrate lyase
IgG	-	immunoglobulin G
MDH	-	malate dehydrogenase (c, g & m denote cytosolic, glyoxysomal or mitochondrial forms respectively)
m/g	-	region of sucrose gradient intermediate between mitochondria and glyoxysomes
min	-	minutes
mRNA	-	messenger ribonucleic acid
NAD	-	nicotinamide adenine dinucleotide
NBA	-	nonenylsuccinic anhydride
N-P40	-	Nonidet P40
NS	-	null serum
oligo d(T) cellulose	-	oligo-deoxythymidylic acid cellulose
PAGE	-	polyacrylamide gel electrophoresis
PBS	-	phosphate-buffered saline
PLEP	-	phospholipid exchange protein
PMSF	-	phenylmethylsulphonyl fluoride
poly (A <sup>+</sup> )	-	polyadenylate
PPO	-	2, 5-diphenyloxazole
PT-114	-	precondensed Triton X-114

RNA	-	ribonucleic acid
r.p.m	-	revolutions per minute
SDS	-	sodium dodecyl sulphate
Ser	-	serine
TCA	-	trichloroacetic acid
TEMED	-	N,N,N',N'-tetramethylethylenediamine
Thr	-	threonine
Tricine	-	N-tris [hydroxymethyl] methyl glycine
Tris	-	2-amino-2 (hydroxymethyl) propane-1, 3-diol
tRNA	-	transfer ribonucleic acid
UDP	-	uridine diphosphate
UMP	-	uridine monophosphate
VSV	-	vesicular stomatitis virus
RuBPCase	-	ribulose bisphosphate carboxylase
Glyox	-	glyoxysomes
hER	-	heavy microsomal subfraction
lER	-	light microsomal subfraction
Mito	-	mitochondria

## INTRODUCTION

### I.1 - HISTORICAL AND DEFINITIONS

Eukaryotic cells, in contrast to prokaryotes, are characterised by extensive intracellular organisation. This organisation is, in part, represented by discrete compartments or organelles. One such organelle was first observed in mouse kidney cells in 1954 by Rhodin, a Swedish electron microscopist. Rhodin termed these particles, which were around  $0.5 \mu\text{m}$  in diameter, microbodies, although their function was at the time unknown. The subsequent development of cellular fractionation techniques led to the isolation and biochemical characterisation of animal microbodies. A leading figure in this research was the Belgian biochemist Christian de Duve, who has recently outlined these events in a Scientific American article (de Duve, 1983).

The metabolic roles of microbodies are many, their presence not being confined to the cells of kidney tissue. Indeed these organelles appear to be ubiquitously present in all eukaryotic cells. In a 1981 review Tolbert (1981) listed 40 enzymes which were associated with microbodies from only 5 different cell types.

It is now clear that the metabolic functions of microbodies vary depending upon the type of cell housing them. A feature of nearly all microbodies this far examined, however, is the presence of  $\alpha$  - hydroxy acid oxidase and catalase.

The presence of these marker enzymes, which respectively generate and degrade hydrogen peroxide, led to de Duve naming the organelles peroxisomes. This name has been widely adopted, although specialised forms of microbodies, such as glyoxysomes of fatty seeds, still retain the name originally ascribed to them. The major classes of microbody that have been studied and characterised are now briefly described.

#### I.1A - ANIMAL PEROXISOMES

These were the first peroxisomes to be isolated (de Duve et al., 1960). They are single membrane delimited organelles, 0.5 - 1.0  $\mu\text{m}$  in diameter, containing an electron dense protein matrix and, frequently, a crystalline inclusion or core. Most of the enzymes, e.g. catalase, are present in the matrix but at least one, uricase, is a known component of the crystalline core (Hruban and Swift, 1964). Although first observed in kidney cells, most of the biochemical and developmental characterisation data have been obtained from liver cell peroxisomes (de Duve, 1969; Lazarow et al., 1982). However, the precise role of these organelles still remains unclear. Initial suggestions were based on the protective advantages of isolating hydrogen peroxide - generating oxidases (which include amino acid oxidases, Baudhuin et al. 1964) with catalase, from the rest of the cytoplasm. More recently it has been found that these organelles also contain enzymes of the  $\beta$  - oxidation pathway (Lazarow, 1978).

A sub-population of animal peroxisomes was described by Novikoff and Novikoff (1972) in cells of mammalian small intestine. These "microperoxisomes", as they were called (being only approximately 0.1  $\mu\text{m}$  in diameter), were only visible when stained with alkaline 3,3' - diaminobenzidine (DAB), a stain specific for peroxidase enzymes. They have since been described in many mammalian tissues (Novikoff and Novikoff, 1982) though they have yet to be extensively characterised. The Novikoff's (1982) have tentatively assigned roles for microperoxisomes in "lipid metabolism", however, more specific data on their function(s) are lacking.

#### I.1B - LEAF PEROXISOMES

The oxygenase action of the chloroplast enzyme ribulose biphosphate carboxylase (RuBPCase) leads to the generation of phosphoglycolate. This compound is known to be the starting material for photorespiration, a process by which carbon dioxide, newly fixed by the plant cell during photosynthesis, is lost back into the atmosphere (Tolbert *et al.*, 1968; Tolbert, 1971; 1982). This carbon dioxide is released during the operation of a salvage pathway, the glycolate pathway, which converts two molecules of phosphoglycolate, via a series of intermediates, into one of phosphoglycerate. Most of the enzymic reactions responsible for this conversion are known to take place exclusively in leaf peroxisomes.

However, the close biochemical co-operation between leaf peroxisomes, chloroplasts and mitochondria in photorespiration is reflected by the close physical association of these organelles, as revealed by the electron microscope (e.g Newcomb, 1982). As with animal peroxisomes, hydrogen peroxide generating oxidases are functional in leaf peroxisomes, in this case during the conversion of glycolate into glyoxylate by peroxisomal glycolate oxidase and, once again, catalase is responsible for its detoxification.

#### I.1C - GLYOXYSOMES

A mechanism exists whereby acetyl-CoA can avoid the oxidative decarboxylation steps of the tricarboxylic acid (TCA) cycle. This alternative pathway is known as the glyoxylate cycle and is illustrated in fig. 1. By utilising the glyoxylate cycle, organisms are able to grow on acetyl-CoA or on substrates whose degradation yields acetyl-CoA.

The first illustration of this was provided by Hans Kornberg during the growth of E. coli on acetate (Kornberg and Krebs, 1957). Fatty seeds were also known to convert acetyl-CoA (derived during germination from stored fats via  $\beta$ -oxidation) into carbohydrate to nurture developing seedlings until they become photosynthetic. Kornberg and Beevers (1957) found that the glyoxylate cycle was responsible for this metabolism in the endosperm tissue of germinating castor bean.

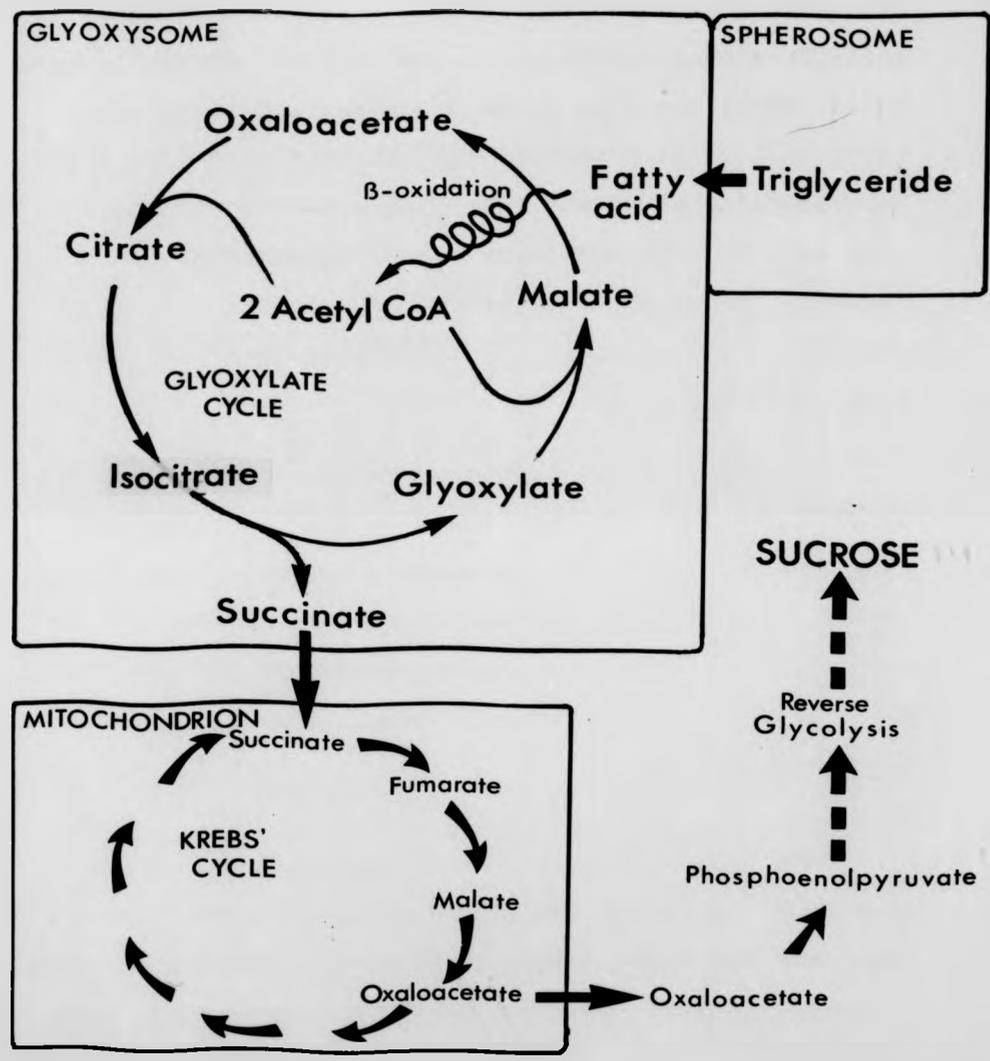


Fig. 1 The Glyoxylate Cycle as Operational in Fatty Seeds

This metabolic pathway, compartmented with the enzymes of fatty acid  $\beta$ -oxidation in glyoxysomes, utilizes acetyl-CoA derived from stored fat to synthesize succinate. This Krebs' cycle intermediate is metabolized in the mitochondrion to oxaloacetate which then leaves this organelle to be converted to sucrose by reverse glycolysis in the cytoplasm.

It was another decade, however, before the subcellular localisation of this pathway was discovered to be within a new kind of cytoplasmic organelle, the glyoxysome (Breidenbach and Beevers 1967). To date, castor bean glyoxysomes remain the best characterised form of microbody and have been the subject of several reviews (e.g Beevers, 1969; 1979; Lord and Roberts 1980, 1983).

Collaborative work between Müller, a then colleague of de Duve's, and Hogg on the ciliate, aerobic protozoan Tetrahymena pyriformis led to the demonstration that the peroxisomes of this organism housed the two key enzymes of the glyoxylate cycle, namely malate synthase (MS) and isocitrate lyase (ICL) (Müller et al 1968). This discovery prompted Beevers' group to look for the peroxisomal marker enzymes, catalase and hydrogen peroxide-generating oxidases, in their castor bean glyoxysomes with the result that glyoxysomes were indeed found to contain these enzymes (Breidenbach et al., 1968).

The glyoxysomes of castor bean endosperm and T. pyriformis differ in that MS and ICL are common to both organelles, whereas the other three glyoxylate-cycle enzymes (isozymes of which are to be found elsewhere in the cell) are found only in the glyoxysomes of the castor bean. de Duve argues that such differences warrant the abolition of the name "glyoxysome" and its replacement by the all-embracing term "peroxisome". I shall continue, however, to refer to the glyoxylate cycle - containing organelles of castor bean endosperm as glyoxysomes throughout this dissertation.

The glyoxylate cycle utilizes acetyl-CoA derived from the  $\beta$  - oxidation of fatty acids and so the discovery by Cooper and Beevers (1969 a,b) that the glyoxysomes of fat storing seeds contained all the  $\beta$  - oxidation enzymes showed that acetyl-CoA could be produced at the location of its utilisation (Beevers, 1969). Before this discovery the  $\beta$  - oxidation pathway was thought to be exclusively located in the mitochondria; it has since been demonstrated in liver peroxisomes and the microbodies of T. pyriformis (Lazarow, 1978; Blum, 1973). Leaf and kidney peroxisomes differ in this respect in that, to date, evidence for the  $\beta$  - oxidation enzymes has not been reported.

If a glyoxysome is defined as a microbody containing at least one of the two glyoxylate bypass enzymes, then the distribution of these organelles throughout eukaryotes becomes potentially very wide. I use the word "potentially" because in several organisms the presence of glyoxysomes is implied by the detection of MS and ICL activity in crude homogenates though the subcellular localization of these activities remains to be conclusively determined. This is particularly true in cases where one or both of these enzymes have been found in metazoan species, in phyla as distantly related as those containing insects (Carpenter and Jaworski, 1962) and mammals (Jones, 1980). McFadden and co-workers,

however, have provided strong evidence for glyoxysome-like particles in the nematode Caenorhabditis elegans (Patel and McFadden, 1977) where a glyoxylate cycle cycle functions in the larval stages of its life-cycle.

A more detailed description of glyoxysomes will be given in the following section, which summarises the state of our knowledge concerning the development of these organelles.

#### I.1D - MICROBODIES OF FUNGI AND YEASTS

These organelles are particularly interesting from a developmental point of view as their abundance varies not only with the stage of development but also with the type of nutrient on which the organism is growing. The fungus Neurospora crassa shows only low levels of the glyoxylate cycle enzymes when grown on sucrose-containing medium; if, however, the medium is changed to one containing acetate as the sole carbon source then, following a lag phase during which there is synthesis of glyoxysomal enzymes, growth resumes at the expense of acetate (Flavell and Fincham, 1968; Flavell and Woodward, 1971). Wanner and Theimer (1982) recently provided evidence for two types of microbody in a N. crassa mutant devoid of a cell wall (the "slime mutant"), one type containing the glyoxylate bypass enzymes and the other the catalase activity. Maxwell et al., (1977) have recently reviewed the microbodies present in plant pathogenic fungi.

Yeast microbodies were first detected in Saccharomyces cerevisiae by Avers' group (Avers and Federman, 1968; Avers, 1971), those of other species have since been shown to have biochemical functions unique to themselves when grown on media containing alkanes or methanol (Fukui and Tenaka, 1978). Candida tropicalis grown on alkanes contains catalase-containing particles which, in addition to MS and ICL, have a complete  $\beta$ -oxidation system and long-chain alcohol and aldehyde dehydrogenases. The mitochondria of these cells do not contain a  $\beta$ -oxidation pathway and so it is inferred that the microbodies play an important role in the synthesis of carbohydrate from long-chain fatty alcohols derived from the alkanes.

Another yeast, Hansenula polymorpha, can be grown on a medium where the sole carbon source is methanol. In such methanol-grown cells a very dramatic morphological change takes place coinciding with an equally dramatic biochemical adaptation (Fukui et al., 1975). These cells are seen to be filled with peroxisomes which are, in turn, filled with dense, crystalline inclusions composed of alcohol oxidase. The only other enzyme present to a large degree is catalase which seems to reduce the hydrogen peroxide, produced as a bi-product of the oxidation of methanol, to formaldehyde. The formaldehyde produced is then either oxidized to carbon dioxide with the co-reduction of two NAD molecules, or converted to dihydroxyacetone and from there to 3-carbon compounds and cell constituents (van Dijken et al., 1982).

Apart from the slime mutant microbodies, all of the microbodies discussed so far have been shown to contain catalase. It is the ubiquitous presence of catalase which most favours a common evolutionary origin to all of these microbody types. Other organelles, whose morphological characteristics indicate them to be related to microbodies, but which are devoid of catalase activity, have been described and I shall end this section with a brief discussion of these particles.

#### I.1E - "MISCELLANEOUS MICROBODIES"

The various oxidation reactions which occur in all of the above microbody-types may be thought of as a primitive respiratory pathway, differing from that which occurs in mitochondria in that the energy released is lost as heat rather than stored in phosphate bonds. Could peroxisomal respiration have been of importance before the evolution of eukaryotic mitochondrial respiration? An affirmative answer might be provided by an organism devoid of mitochondria but containing microbodies which were sites of usable energy production. At present no such organism is known, however, within the Trichomonads (flagellate protozoans responsible for certain sexually transmitted diseases in mammals) exist species which do not possess mitochondria but which are endowed with structures which can be classed, at least morphologically, as microbodies (Lindmark and Müller, 1973). The biochemical functions contained therein, however, are concerned with the metabolism of pyruvate, formed in glycolysis, to acetate via acetyl-CoA. Catalase, if present in these organisms, is cytosolic in location.

Thus it would seem that these organelles, which have been termed hydrogenosomes (because of an hydrogenase which transfers electrons from pyruvate to, ultimately, protons via ferridoxin with the release of molecular hydrogen) are not related, biochemically, to other microbodies. However, time and effort will, no doubt, tell whether structural and/or developmental similarities exist.

The other type of "miscellaneous microbody" of importance is the glycosome found in the protozoan family Trypanosomatidae. These organisms include the haemoflagellates responsible for nagana, in domestic cattle, and human sleeping sickness in Africa. Glycosomes are organelles 0.2 - 0.8 $\mu$ m in diameter which, like hydrogenosomes, resemble peroxisomes in appearance; however, their major enzymic complementation is unique in that it includes a large portion of the glycolytic pathway (Opperdoes and Borst, 1977). Glycolysis, the breakdown of glucose to pyruvate, occurs in the cytosol of most eukaryotic cells - why trypanosomes segregate this pathway is not at all clear, however, there is now evidence that the organelles which house it may be more closely related to peroxisomes and glyoxysomes than was once thought. Opperdoes, and colleagues, having extensively characterised the glycosomes of Trypanosoma brucei (Opperdoes et al., 1984) have found a common role for glycosomes and peroxisomes in the synthesis of alkylphospholipids (Opperdoes, 1984). There is also unpublished evidence for the presence of at least one  $\beta$ -oxidation enzyme (3 -  $\beta$  -hydroxyacyl-CoA dehydrogenase) in glycosomes.

This evidence is seen as sufficient for Opperdoes to conclude "that glycosomes and peroxisomes are related organelles".

Clearly, then, the roles of microbodies in general are great and varied, however, it is possible that this diversification stemmed from a single, ancient microbody population. Establishing the evolutionary origin of microbodies will be very difficult but progress will be made if a common method of biogenesis can be found to pertain to all such organelles. It is to this end that much work is being directed at the moment. The results presented in this thesis offer evidence for a possible mechanism of assembly of one type of microbody, the glyoxysome.

## I.2 - THE BIOGENESIS OF GLYOXYSOMES AND PEROXISOMES

### I.2A - INITIAL CONSIDERATIONS

Organisms expressing glyoxylate cycle enzymes usually do so for only relatively short periods of time, such as phases of rapid growth as in the case of germinating seeds. Fig. 2 shows the appearance and decline of one of the glyoxylate cycle enzymes, ICL, in two types of fat-storing seeds, castor bean and pumpkin. The lipid reserves are found in the endosperm tissue of the former and the cotyledons of the latter. The endosperm tissue of castor beans is senescing virtually from the onset of germination. After its formation in the developing seed, the endosperm undergoes no further cell division. After 6 - 7 days of germination, fusion of the protein body membranes has formed the vacuole which then occupies most of the cellular volume (Vigil, 1970). In cells adjacent to the expanding cotyledons, the cytoplasm occupies the region of the cell between the vacuole and the plasma membrane.

After 8 - 9 days the cotyledons of castor bean are green, photosynthetically competent structures and the endosperm is a thin layer of dead cells. In the case of pumpkin and other seeds which store their lipid reserves in the cotyledons, the glyoxysomal population initially present in the cotyledonary cells is replaced by one of leaf peroxisomes as the organ matures. A controversy surrounds this change in microbody population; are the leaf peroxisomes derived from the glyoxysomes by a gradual change in the enzymic complementation or are the leaf peroxisomes synthesized de novo? It seems reasonable to assume that leaf peroxisomes, like glyoxysomes, are assembled from basic

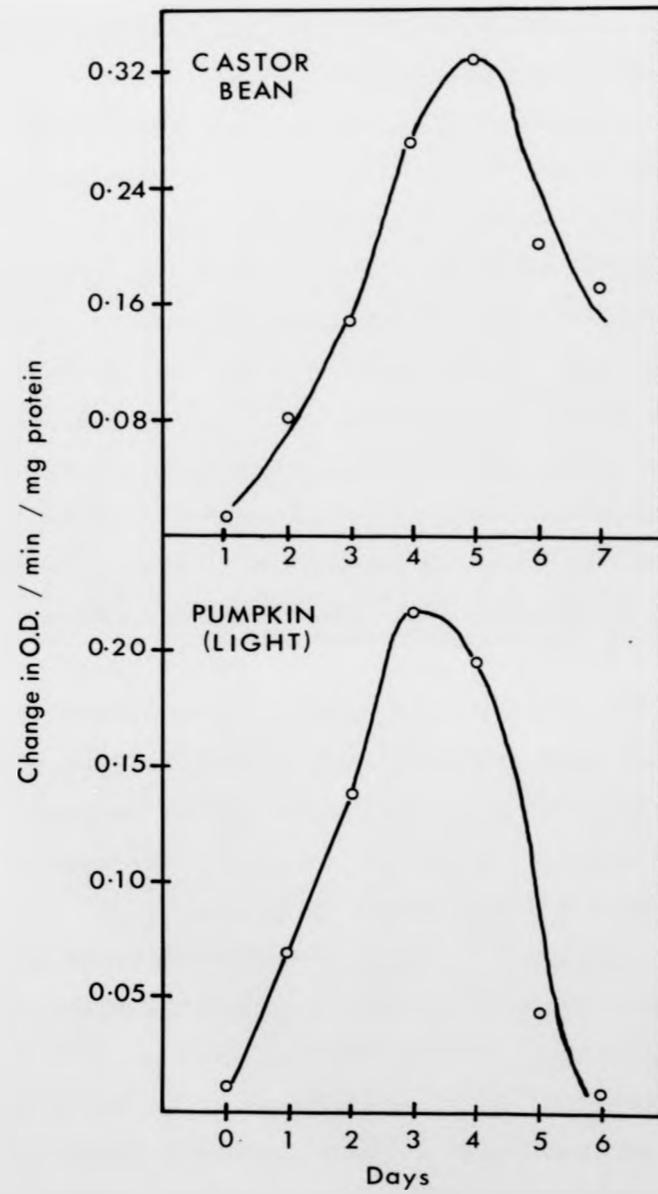


Fig. 2 Changes in Isocitrate Lyase Activity in  
Castor Bean and Pumpkin During Germination

(after Carpenter and Beevers, 1959)

components (Beevers, 1979). Another difference between seeds containing endosperm- and cotyledonary-stored lipid may lie in the time of the initial stages of glyoxysome biogenesis. Trelease's group initially presented evidence for glyoxylate cycle enzyme activity in the cotyledons of ripening cotton seeds (Choinski and Trelease, 1978). Köller later reported the presence of "incomplete glyoxysomes" in developing cucumber cotyledons (Köller et al., 1979b), "incomplete" in that ICL activity could not be detected. Doman and Trelease (1983), however, detected ICL by immunochemistry in mature cotton seeds. A possible role for glyoxysomes in the production of citrate, which accumulates in maturing cotton embryos, has been put forward by Miernyk et al. (1982). Whatever the role, a recent morphometric analysis by Kunc et al. (1984) indicates that these developing seed glyoxysomes proliferate during germination, by the post-translational incorporation of proteins from the cytosol and "transfer of membrane components probably from the endoplasmic reticulum". Such a situation is comparable to that found in watermelon in an ultrastructural investigation carried out by Wanner et al. (1982) where catalase-containing vesicles were observed in 2 day post germination cotyledons. These vesicles were found in close proximity, indeed possibly attached, to the lipid bodies. In addition, the vesicles were often observed with ribosome-bearing membrane fragments which these authors suggest to be of the e.r. origin, possibly remnants of the region of the e.r. involved in lipid-body ontogeny (Wanner et al., 1981) during seed maturation. Such findings have served to complicate the situation regarding glyoxysomal biogenesis even further,

however, in the case of castor beans, no evidence for glyoxysomes or glyoxysome-like structures in the developing, or mature, seed has been presented. Indeed, the complete absence of glyoxysomes in these seeds has been shown (Gerhardt and Beevers, 1970; Vigil, 1970). Thus glyoxysome assembly in castor bean endosperm will be assumed to be an event which occurs entirely post-imbibition.

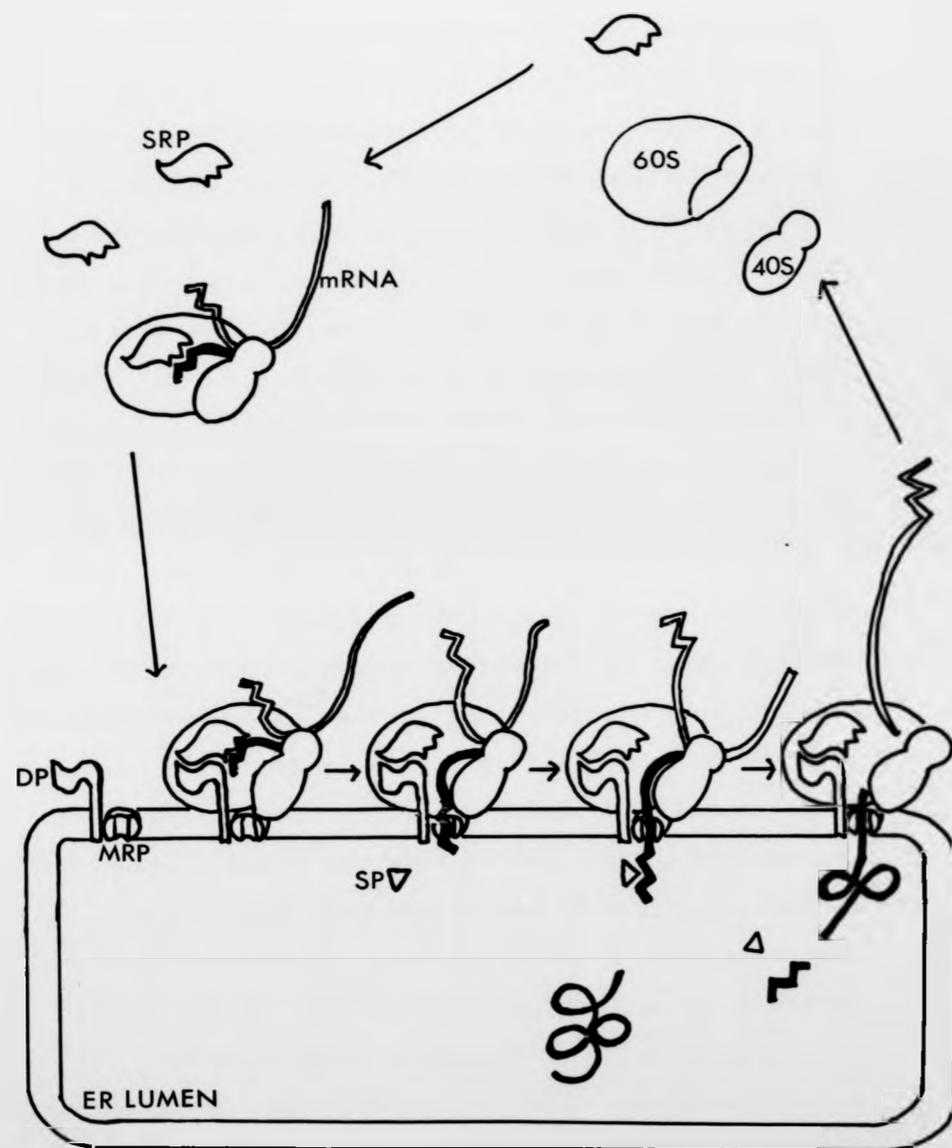
The assembly process itself has eluded biochemists and cell biologists for many years and is only now slowly beginning to be unravelled. With regard to the origin of the matrix components, a general mode of synthesis is now emerging.

#### I.2B - THE ORIGIN OF MICROBODY MATRIX PROTEINS

The first model for glyoxysome biogenesis was based on biochemical and morphological data. The biochemical evidence included the findings:- (i) that phosphatidyl choline is a major constituent of the glyoxysomal membrane (Donaldson et al., 1972) and (ii) that the site of synthesis of phosphatidyl choline, in castor bean endosperm, is the e.r. (Lord et al., 1973). The observations in electron micrographs of direct continuities between the e.r. and the glyoxysomal membrane (e.g., Vigil, 1970) supported a model which had glyoxysomal components synthesized at the e.r. and glyoxysomes arising by a process of vesiculation. The proteins of the glyoxysomal membrane and matrix were suggested to undergo co-translational insertion and sequestration in accordance with the, now widely

accepted, "signal hypothesis" of Blobel and Dobberstein (1975 a and b) (illustrated diagrammatically in fig. 3). This then was the basis of the so-called "classical model" of microbody assembly. However, it soon became apparent that the mode of translocation of the matrix proteins was not compatible with such a model. Preliminary evidence for a post-translational mechanism for microbody matrix protein sequestration came from Lazarow and de Duve (1973). These workers detected newly synthesized peroxisomal catalase in the cytosol of rat hepatocytes before its subsequent association with the peroxisomal fraction. More convincing data was provided in 1978 using cell-free protein synthesizing systems to translate the messenger RNA (mRNA) coding for peroxisomal catalase. Robbi and Lazarow (1978) showed that in vitro synthesized catalase monomer had a subunit molecular weight identical to that immunoprecipitated from peroxisomes in vivo. This implied that catalase was synthesized without a cleavable signal sequence. Goldman and Blobel (1978) took the story a stage further by translating mRNA associated with either free (cytosolic) or bound polysomes. The peroxisomal enzymes catalase and uricase were exclusively found in the polypeptide products encoded by free-polysome-associated mRNA. This was in contrast to the situation with mRNA encoding albumin, a known secretory protein, which was exclusively associated with bound polysomes, further, albumin was co-translationally inserted into canine pancreatic microsomes added to the in vitro system. Insertion of catalase and uricase into such microsomes could not be demonstrated. Thus in the case

Fig. 3 The Co-translation Sequestration of Proteins Across the E.R. Membrane (The Signal Hypothesis)



mRNA encoding a secretory or transmembrane protein associates with ribosomal subunits in the cytoplasm. Translation begins, the N-terminal "signal sequence" emerges from the ribosome and is recognised by the signal recognition protein (SRP) which binds and arrests polypeptide elongation until the ribosome/mRNA/SRP complex encounters the e.r. membrane. The SRP is recognised by a receptor or "docking" protein (DP) and the ribosome is anchored to the membrane by a membrane receptor protein (MRP) or ribophorin. Upon binding elongation recommences and the polypeptide is sequestered through the membrane co-translationally, possibly via a channel formed by the MRP. On the emergence of the signal sequence into the e.r. lumen it is removed by signal peptidase (SP). The remainder of the peptide chain is then either completely sequestered into the e.r. lumen (in the case of secreted and organelle matrix proteins) or in the case of integral membrane proteins translocation is terminated before translation has been completed. In this way the protein remains embedded in the e.r. membrane

of animal peroxisomes, the soluble enzyme components appeared to traverse the peroxisomal membrane post-translationally; but what of the situation regarding plant glyoxysomes? In the case of the castor bean organelles, elucidation of the situation was delayed by some confusing data concerning the glyoxylate cycle enzyme malate synthase (MS). In castor bean and other plant sources MS is peripherally associated with the internal surface of the glyoxysomal membrane (Huang and Beevers, 1973). A claim by Kindle and co-workers that cucumber and castor bean MS are integral glyoxysomal membrane proteins (Kindl *et al.*, 1980; Kindl, 1982) is no longer accepted. In addition to its membrane association, another feature of MS which distinguishes it from other microbody matrix proteins is the failure to detect activity in the cytosol fraction following castor bean endosperm fractionation on sucrose gradients (Gonzales and Beevers, 1976). Activity is found associated with the e.r. fraction however. The proportion of total MS activity which is e.r. associated varies from over 50%, in tissue homogenized early in germination, to less than 10% in older tissue. Lord and Bowden (1978) demonstrated that this activity could be chased into the glyoxysome fraction and Mellor *et al.* (1978) demonstrated, by periodic acid - Schiff (PAS) staining, castor bean MS to be a glycoprotein. The glycosylation of proteins will be discussed in more detail later, here it will suffice to state that one type, N-glycosylation, is known to occur exclusively in the endoplasmic reticulum and, furthermore, to be a co-translational event (Katz *et al.*, 1977). The detection of carbohydrate associated with castor bean MS thus

strengthened the support for a role for the e.r. in MS synthesis. Work on cucumber MS led to similar conclusions being drawn using  $^{125}\text{I}$ -labelled concanavalin A (con A) binding and gas chromatographic analysis of the sugars released from the purified enzyme (Reizman et al., 1980). Roberts has since demonstrated that the synthesis of castor bean MS in cell-free systems has features similar to those observed with the animal peroxisomal enzymes (Lord and Roberts, 1982). In vitro synthesized MS is apparently identical in size to in vivo synthesized enzyme and in translations supplemented with dog microsomes no MS is associated with these membranes. Taken together, these results imply that co-translational N-glycosylation does not occur. Thus an attempt must be made to explain the earlier observations. Kindl et al (1980) proposed that the e.r. - association of MS was due to a fortuitous co-sedimentation of aggregated forms of cytosolic MS. Gonzalez (1982) however showed this association to be a more complex one by subjecting castor bean microsomes to centrifugation for different times and at different rates. Some MS activity always remained associated with this fraction after these treatments suggesting that the association is real. The reported glycosylation observed by Mellor et al. (1978) can be explained by the unreliability of the PAS staining procedure. The findings of Reizman et al. are more difficult to explain unless their carbohydrate analysis was performed on a contaminant (such as a glycolipid) which had been carried through the MS purification procedure, as suggested by Lord and Roberts (1982). The Wisconsin group (Reizman et al.) have studied other glyoxysomal enzymes and their results

regarding each will now be discussed. Glyoxysomal catalase of cucumber has a  $M_r$  of 54,000 (Lamb *et al.*, 1978) (c.f. 60,000 in rat liver peroxisomes [Goldman and Blobel, 1978]). When a cell-free translation system prepared from wheat germ was used to translate cucumber cotyledonary mRNA, antibodies raised against purified catalase immunoprecipitated a protein of  $M_r$  55,000 (Reizman *et al.*, 1980). The same antibodies immunoprecipitated a protein, labelled *in vivo*, which showed the same mobility on SDS gels as purified catalase. The situation in cucumber glyoxysomes then is perhaps different to that in rat liver peroxisomes with regards catalase, the larger *in vitro* - synthesized cucumber form may represent a precursor with a signal sequence still attached. Further evidence to strengthen such a possibility and, indeed to show that this extension sequence is involved in the translocation of the protein across a membrane, has not been produced to date.

The study by Huang and Beevers (1973) showed MS and glyoxysomal citrate synthase to occupy similar locations within the cell; in the glyoxysome they are peripherally associated with the organelle membrane. Gonzalez (1982) has shown that they behave in an identical fashion during cell fractionation. To date there has been little or no work published concerning the synthesis of glyoxysomal citrate synthase and it will be interesting to compare its synthesis with its mitochondrial counterpart as has been done in the case of malate dehydrogenase (discussed later). Kindl (1982) has reported the successful *in vitro* post-translational

import of MS into isolated glyoxysomes. On incubating in vitro synthesized cucumber cotyledonary proteins with a crude glyoxysomal preparation, then isolating the glyoxysomes by density gradient centrifugation, it was found that the majority of immunoprecipitable MS was located inside the glyoxysomes (as shown by its protection from protease treatment). Such import experiments have been attempted by our group, using castor bean components, but without success.

In vitro import experiments have also been performed using Neurospora crassa glyoxysomes by Neupert's group (Desel et al., 1982). In these experiments translocation of in vitro synthesized ICL was investigated. Although protection from protease could be shown, the efficiency of import was very low (5 - 10% as estimated from an SDS gel) and could have been accounted for by the glyoxysomal vesicles (which Neupert admits to being very fragile - Desel et al., 1982 [discussion]) breaking and trapping some of the in vitro synthesized ICL upon resealing. In vitro synthesized Neurospora ICL was the same size as authentic, purified enzyme and similar results have been obtained by Roberts and Lord (1981 b) in the case of ICL from castor bean endosperm. The enzyme is synthesized on free polysomes and did not undergo co-translational insertion into canine pancreatic microsomes. Results with ICL from cotyledonary sources conflict with those obtained from castor bean and Neurospora. Firstly, it was reported by Frevert and Kindl (1978) that the cucumber enzyme was a glycoprotein though the converse situation was concluded to pertain to ICL from the same source by Reizman et al., 1980.

The Wisconsin group compared in vitro synthesized ICL with in vivo labelled enzyme. In both cases they found two polypeptides were immunoprecipitated using antiserum prepared against a purified ICL ( $M_r = 63,500$ ) (Lamb et al., 1978). In vivo the molecular weights of the bands were 63,000 Da and 61,500 Da and in vitro 61,500 Da and 60,000 Da. It is possible that the two forms in each case are the result of limited, specific proteolysis, ICL from several sources having been shown to be prone to such degradation (Theimer, 1976, Khan et al., 1979, Khan and McFadden, 1982). It is still difficult to explain the size difference between the in vivo and in vitro polypeptides, that synthesized in vivo being some 1,500 Da greater. There is no post-translational modification (apart from glycosylation) which could account for this size increase. It is possible that the 61,500 Da, in vitro polypeptide is a degradation product of the 63,000 Da primary translation product which is susceptible to a protease present in the in vitro translation system.

In the same study, results obtained with cucumber glyoxysomal malate dehydrogenase (gMDH) confirmed those of Walk and Hock (1978) using watermelon. The primary translation product from the mRNA coding for this enzyme is larger than the native, functional form. In the case of cucumber this size difference is some 5,000 Da compared with 8,000 Da in watermelon. Studies by Hock's group have shown there to be five isoenzymes of malate dehydrogenase; as well as gMDH there is mitochondrial (m) MDH and three cytosolic forms (cMDH I, II and III) (Hock, 1973). Both of the organelle forms have been

shown to be synthesized as higher molecular weight precursors (Gietl and Hock, 1982).

Protein translocation across biological membranes does not exclusively occur co-translationally. Other methods of protein translocation through membranes are known to occur where post-translocational membrane insertion follows synthesis on free, cytosolic ribosomes.

Wickner, in 1979, postulated the membrane-triggered folding hypothesis ("The Trigger Hypothesis", Wickner, 1979; Ito et al., 1979) as an alternative to the signal hypothesis. The trigger hypothesis explained how bacteriophage M.13 coat protein inserts itself into a lipid bilayer post-translationally. The essence of this model is that the protein is made in a soluble form, any hydrophobic stretches of non-polar amino acids being buried within its 3-D configuration. On encountering a membrane a conformational change is "triggered" so exposing the hydrophobic regions which embed themselves in the lipid bilayer of the membrane. As well as M.13 coat protein an Escherichia coli outer membrane protein (OmpA) has recently been shown to insert into the membrane post-translationally (Zimmerman and Wickner, 1983). A major difference between the signal and trigger hypotheses is in the involvement of other proteins. Whereas at least two polypeptide complexes are involved in co-translational insertion, namely the signal recognition protein (SRP) (Walter and Blobel, 1981) and the docking protein (Meyer et al., 1982; for review see Mayer, 1982) the post-translational

process does not seem to require other proteins. It is, however, possible that an existing membrane protein complex may be necessary to identify the membrane into which a post-translationally inserted membrane protein is to be incorporated (Hennig and Neupert, 1981; Zwizinski et al., 1983; Daum et al., 1982).

Another difference between the two methods of insertion lies in the energy source which powers the translocation process. In the case of the signal hypothesis it is thought that the process of polypeptide chain elongation drives the nascent chain through a channel in the membrane which forms as a result of ribosome binding (Blobel and Dobberstein, 1975 a). Chan et al. (1979) and von Heijne and Blomberg (1979), however, have independently questioned the formation of a hydrophilic pore and, as an alternative, suggest the "direct transfer" of the polypeptide through the membrane utilizing interactions between hydrophobic stretches of amino acids and the lipid bilayer. An alternative mechanism would be the adoption of a conformation, such as a helical hairpin (Engelman and Steitz, 1981) which allows the spontaneous insertion of a protein into the membrane. In the case of the trigger hypothesis, the energy source takes several forms including transmembrane electro-chemical potentials, as in the case of M.13 (Date et al., 1980) and ATP which is required for the import of some proteins by mitochondria (Zimmerman et al., 1981) and chloroplasts (Grossman et al., 1980). Initially the membrane trigger hypothesis

was presented as an alternative to the signal hypothesis but it is now widely accepted that both of these processes have a role in membrane and organelle biogenesis. Post-translational sequestration is a method used most frequently in prokaryotes and the semi-autonomous organelles, mitochondria and the plastids. However, there are examples of co-translational insertion of proteins into prokaryotic cell membranes (Michaelis and Beckwith, 1982), indeed most proteins of the inner bacterial membrane are co-translationally inserted (Wolfe and Wickner, 1984). There are also examples of post-translational incorporation of proteins into the e.r. of eukaryotes (Okada et al., 1982), the e.r. being the exclusive site of co-translational incorporation in such cells. It should be noted, however, that proteins inserted into the e.r. post-translationally (cytochrome  $b_5$  and NADH - cytochrome  $b_5$  reductase being the best characterized examples) are also present in mitochondria (Borgese et al., 1980). Although semi-autonomous, the great majority of mitochondrial and plastid protein components are encoded by the nuclear genome and enter the organelle after their synthesis on cytoplasmic ribosomes (Chua and Schmidt, 1979; Neupert and Schatz, 1981) In order to reach the site of their final location, some of these proteins must traverse up to three (in the case of chloroplast thylakoid membrane proteins) different membranes. A discussion of all the proteins studied with respect to import into these organelles is beyond the scope of this introduction, however, certain general characteristics should be mentioned. Firstly, many of

the proteins are synthesized with amino acid leader sequences which are subsequently cleaved following organelle uptake (e.g Dobberstein et al., 1977; Maccacchini et al., 1979). The peptidase responsible for this cleavage in chloroplast proteins has recently been characterized (Robinson and Ellis, 1984 a) and a similar activity is known to exist in the mitochondrial matrix (Gasser et al., 1982). The leader sequences of post-translationally sequestered proteins have been compared and have been found to be quite different in terms of hydrophobicity. The presence of hydrophobic regions are a ubiquitous property of signal sequences (Michaelis and Beckwith, 1982; von Heijne, 1983) whereas the extension sequences of post-translationally inserted proteins do not necessarily contain non-polar stretches of amino acids. Like the situation with M.13 coat protein, which also possessed a cleavable leader sequence (Konings et al., 1975; Sugimoto et al., 1977) a membrane potential or ATP is often required to drive the insertion processes (Daum et al., 1982). It is also becoming apparent that the presence of a cleavable sequence is not a prerequisite for membrane insertion or translocation (Wolf and Wickner, 1984; Schechter et al., 1979; Bonatti and Blobel, 1979; Rottier et al., 1984; Palmiter et al., 1978; Bar-Nun., et al., 1980; Smith et al., 1979).

Returning to the subject of microbody assembly there are only tentative data to indicate the post-translational incorporation of proteins into these organelles. As stated previously, MDH is the only glyoxysomal protein which is initially synthesized in

precursor form (Walk and Hock, 1978; Reizman *et al.*, 1980; Gietl and Hock, 1982). mMDH from rat liver is also synthesized as a higher molecular weight precursor with an amino terminal extension sequence of 1,000 - 2,000 Da (Chien and Freeman, 1984). The mitochondrial isoenzyme from watermelon is synthesized some 3,300 Da larger than the native enzyme. If the molecular weights of the *in vitro* synthesized precursors to both watermelon organelle forms are compared there is seen to be very little difference (41,000  $\pm$  200 Da for gMDH and 41,000  $\pm$  100 Da for mMDH) and the Freising group are now exploring the possibility that both the mitochondria and glyoxysomal forms share a common precursor (Gietl and Hock, personal communication). Lazarow's group have recently reported a peroxisomal protein, 3 - ketoacyl - CoA thiolase to be synthesized with a 6,500 Da cleavable extension sequence (Fujiki *et al.*, 1984) and this has been confirmed by Miura *et al.* (1984) though an extension sequence of only 3,000 Da is reported by this group. In the same paper these workers investigated the synthesis of two other peroxisomal proteins, acyl-CoA oxidase and a bifunctional protein containing enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities. mRNA's for both proteins were enriched in the free-polysome fraction and, in both cases, the primary translation product was the same size as the functional protein.

Thus the situation regarding the synthesis of microbody matrix proteins, and their mode of entry into the organelle, clearly does not comply with the original model for microbody biogenesis. Is a complete rejection of the classical model necessary however?

I.2C - THE ORIGIN OF THE MEMBRANE COMPONENTS

In this section I shall discuss what is known of the single membrane which delimits peroxisomes and glyoxysomes.

Beginning with the lipid components, Lord et al. (1972; 1973) showed the e.r. to be the exclusive site of synthesis of the major phospholipids constituting the glyoxysomal membrane i.e., phosphatidylcholine and phosphatidylethanolamine (Donaldson and Beevers; 1977). The transfer of phospholipids from the e.r. to the membranes of other subcellular organelles can occur by two mechanisms, phospholipid exchange and membrane flow. These processes have been reviewed by Lord and Roberts (1983), Wirtz (1974) and Morré (1979); briefly phospholipid exchange involves the transport of phospholipid, by a specific phospholipid exchange protein (PLEP), through the cytoplasm and its incorporation into the membrane of its final location. This would imply that a rudimentary acceptor-membrane must already exist (as in the case of mitochondria and chloroplasts) and therefore PLEP's could not be used in the assembly of an organelle de novo. Furthermore, the PLEP's operate only between cytoplasmically-adjacent surfaces of lipid bilayers, the transfer of lipid to the opposite leaflet of a bilayer (flip-flop) involves the passage of the polar head-group through the hydrophobic domain. This thermodynamically-unfavourable process occurs only at very low rates, probably too low to account for the rate at which the glyoxysomal membrane proliferates during germination. Lateral movement of phospholipids in a bilayer, unlike

the process of "flip-flop", can occur very easily and does so quite rapidly (Schlessinger *et al.*, 1977). This is the basis of membrane flow. Phospholipids are synthesized in the e.r. and because of the trans-membrane orientation of the choline and ethanolamine transferases the newly synthesized phospholipids can be inserted into both layers of the bilayer. They can then diffuse laterally thus expanding the e.r. membrane. At specific regions vesiculation may occur (in much the same way as membrane and secretory components are packaged for transport from the e.r. to the Golgi apparatus [Rothman, 1981]) to produce an organelle whose membrane composition is specific to its type with respect to proteins but which has a lipid composition very similar to that of the e.r. This membrane is then open to modifications independent of the e.r., such as lipid insertion via PLEP's and post-translational protein incorporation, to give a membrane unique to that organelle type. Depending on the degree of modification the membrane may still resemble its parent membrane, the e.r., as has been found in the case of the castor bean glyoxysomal membrane (Donaldson and Beevers, 1977; Bowden and Lord, 1976ab; Goldberg and Gonzalez, 1982).

The situation with regard glyoxysomal membrane lipid synthesis will remain speculative until a more detailed analysis of the various lipid components is made. The study by Donaldson and Beevers is open to questioning because the organelle membrane isolation procedure employed did not allow for cross-contamination of the various fractions. In the present study,

proteins of the glyoxysomal membrane have been isolated and characterised. Antiserum to these proteins was used to investigate their site(s) of synthesis in vivo and in vitro. The investigations have revealed that a single point of origin may not exist for these proteins and the e.r. would appear to play a role in the synthesis of several of them. This role was supported by results obtained using another approach where glyoxysomal membrane proteins were examined for a modification which could only have been acquired if the protein had passed through the e.r. The modification chosen was the possession of N-linked oligosacchoride chains, the e.r. being the exclusive site of N-glycosylation in castor bean endosperm (fig. 4) as well as other tissues.

#### I.2D - GLYCOSYLATION OF PROTEINS

Carbohydrate moieties may be linked to proteins in mainly two ways (Kornfeld and Kornfeld, 1976; 1980). The so-called O-glycosyl linkage has the carbohydrate attached via the hydroxy-group of serine, threonine, hydroxylysine or hydroxyproline. The other linkage, the N-glycosyl linkage, is between saccharide and the side-chain amino-group of asparagine. A major difference between O- and N-glycoproteins is in their mode and site of synthesis. In the case of O-linked glycoproteins it is thought that all the glycosyl-transferase reactions occur in the Golgi-apparatus (Hanover and Lennarz, 1981) although reports of co-translational (implying e.r. associated) O-glycosylation has been reported in yeasts (Haselbeck and Tanner, 1983; Larriba et al., 1976). N-glycosylation is a process which invariably begins in the e.r. with the addition of

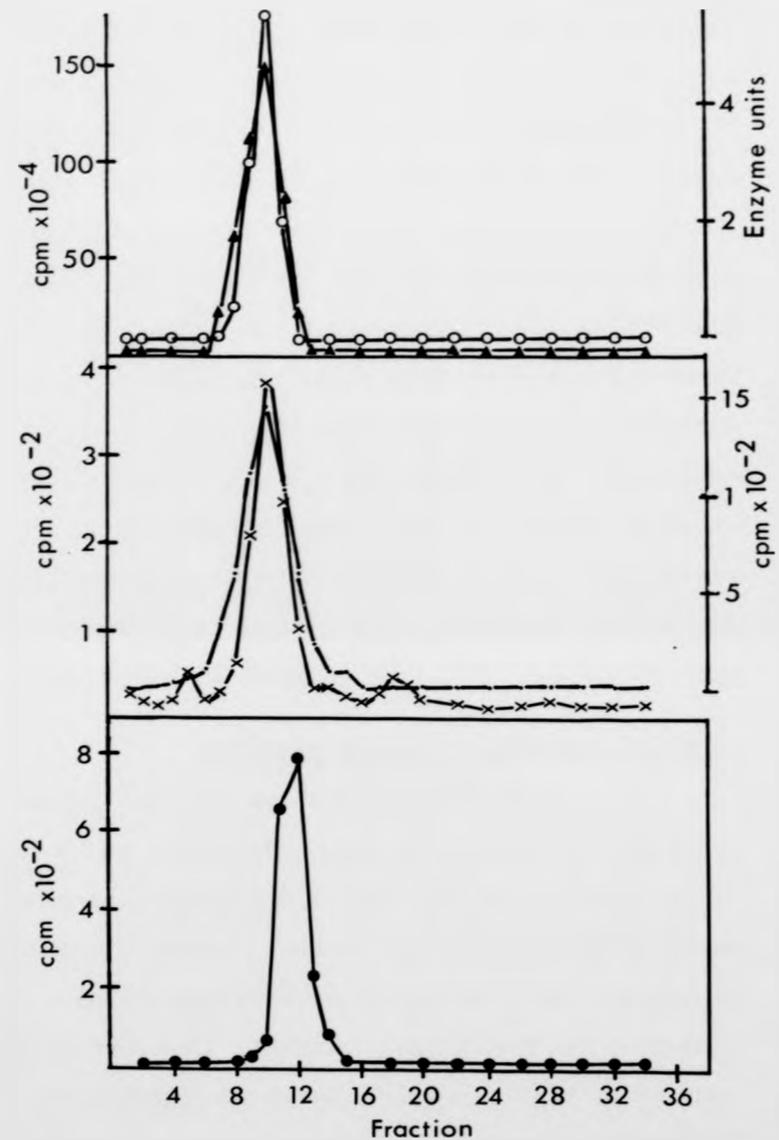


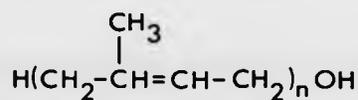
Fig. 4 The E.R. as the Site of Glycosylation in Germinating Castor Bean Endosperm Tissue

Assay of the e.r. marker enzymes NADH-cytochrome<sub>c</sub> reductase (o-o) and choline-phosphotransferase (▲-▲) identifies the microsomal fraction in a sucrose density gradient (top). The enzymes UDP-N-acetylglucosamine dolichol monophosphate GlcNAc transferase (x-x) and GDP-mannose dolichol monophosphate mannosyl transferase (----), involved in protein N-glycosylation, are similarly distributed (centre). Glycoprotein fucosyl transferase (●-●) is also associated with the e.r. in castor bean endosperm (bottom).

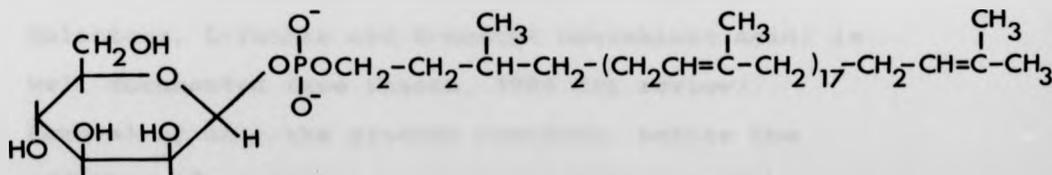
(Data from Lord, 1976; Mellor and Lord, 1979c; Mellor et al., 1980; Roberts et al., 1980).

an oligosaccharide core-structure to the growing polypeptide chain as it is sequestered through the e.r membrane. The process continues post-translationally with modifications to this core structure occurring in the e.r and Golgi apparatus.

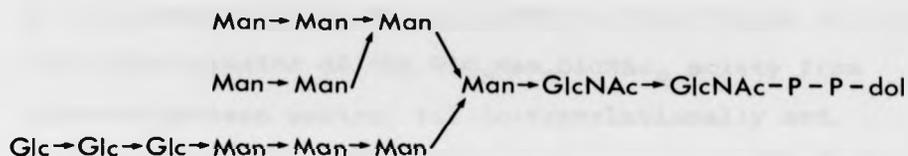
N-glycosylation requires the involvement of lipid-linked saccharide intermediates (Parodi and Leloir, 1979; Struck and Lennarz, 1980). Briefly, the process begins with the synthesis of monosaccharide-lipids in the lumen of the e.r. The lipid moiety is a monophosphoester of a polyisoprenol having the general structure:-



One family of polyisoprenols in particular (with  $n = 15-24$ ) has been shown to contain the lipids of choice in these reactions and these have been given the name dolichols. The monosaccharides which take part in these reactions are the nucleotide (activated) forms of N-acetylglucosamine (GlcNAc), mannose (Man) and glucose (Glc). The structure of a monosaccharide lipid (mannosylphosphoryldolichol, dol-P-Man) is shown below:-



In the case of GlcNAc, dolichol phosphate reacts with UDP-GlcNAc to produce dolichol phosphate-GlcNAc (dol-P-GlcNAc) and UDP or dolichol pyrophosphate - GlcNAc (dol-PP-GlcNAc) and UMP. The next step involves the formation of dol-PP-(GlcNAc)<sub>2</sub>, from dol-P-GlcNAc and dol-PP-GlcNAc, which then reacts with dol-P-Man to give dol-PP-(GlcNAc)<sub>2</sub>Man. Lipid-linked Man and GDP-Man donate their sugar moieties to the growing oligosaccharide chain then, finally, three Glc residues are transferred from dol-P-Glc to form the structure:-



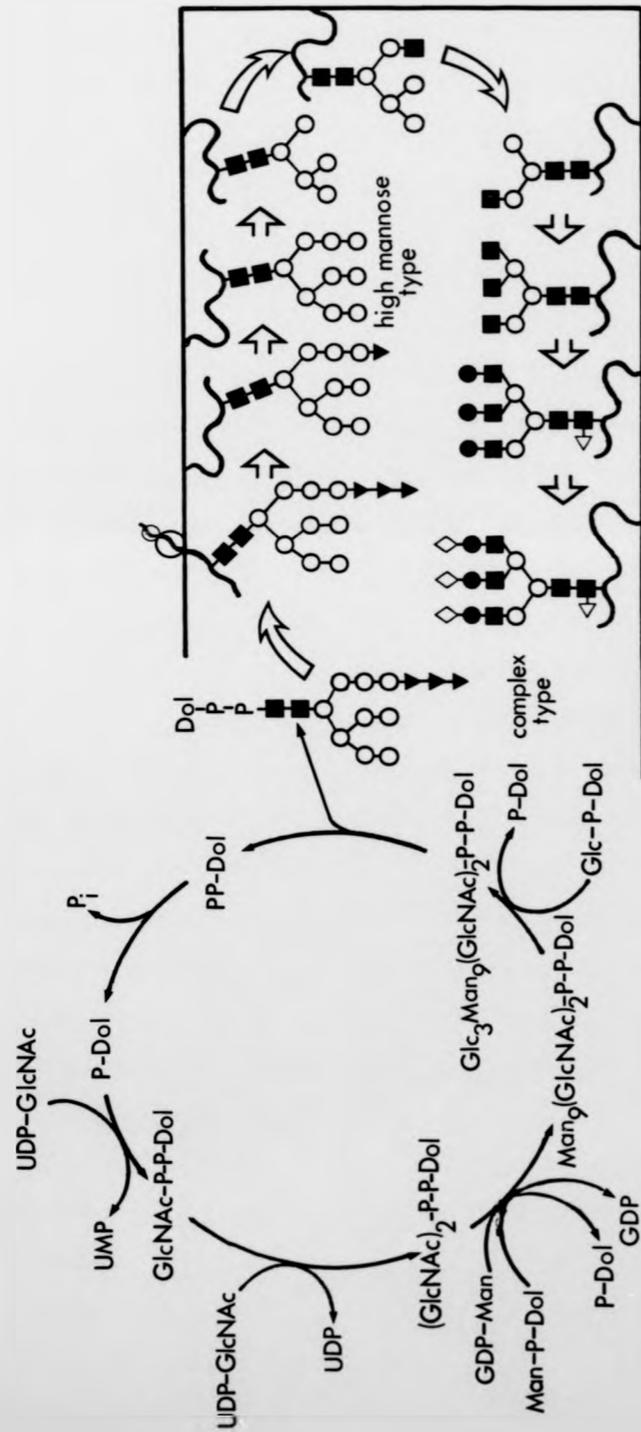
The oligosaccharide moiety of this molecule is then transferred, en bloc, to an asparagine residue of the glycopeptide. A general signal for glycosylation is the amino-acid sequence Asn-X-Ser/Thr (where X may be any amino acid except possibly aspartic acid), however, not all asparagines in such a sequence are glycosylated so a more complex recognition system is possibly involved (Struck and Lennarz, 1980).

The subsequent processing of the Glc<sub>3</sub>-Man<sub>9</sub>-GlcNAc<sub>2</sub>-protein, which involves the removal of variable numbers of glucose and mannose residues and the addition of peripheral sugars (most frequently galactose, L-fucose and N-acetyl neuraminic acid) is well documented (see Sharon, 1984 for review). Removal of only the glucose residues, before the addition of peripheral sugars, results in "high mannose type" glycoproteins, whereas cleavage of mannose

residues, down to a  $\text{Man}_3\text{GlcNAc}_2$ -protein, and the subsequent addition of peripheral sugars gives rise to the so-called complex glycoproteins. The entire pathway to the synthesis of N-glycoproteins is summarised in fig. 5 and has been shown to be the same for glycoprotein synthesis in animal, plant and viral systems. Indeed the only life-form in which glycoprotein synthesis does not appear to occur is bacteria (Sharon, 1984).

Of central importance to the purpose of many of the experiments to be described in this thesis is the fact that transfer of the  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$  moiety from lipid to protein occurs, (i) co-translationally and, (ii) on the luminal side of the endoplasmic reticulum. It is therefore important to summarise the data which have established these points.

The asymmetrical insertion and co-translational glycosylation of an integral membrane protein was first convincingly demonstrated by Katz *et al* (1977) using an *in vitro* protein synthesizing system programmed with vesicular stomatitis virus (VSV) mRNA and supplemented with canine pancreatic microsomes to serve as receptor membranes. This pioneering work formed the basis of the methodology used to establish the general co-translational nature of the N-glycosylation of animal (e.g the four subunits of acetylcholine receptor in the electric eel [Anderson and Blobel, 1981]) and plant glycoproteins (such as the lectins of many plant seeds including castor bean [Roberts and Lord, 1981 a]) as well as other viral proteins (e.g the E1 glycoprotein of coronavirus mouse hepatitis virus A59 [Rottier *et al.* 1984]).



**Fig.5** Pathway of Protein Glycosylation

The oligosaccharide is assembled as detailed in the text. The tetradecasaccharide  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$  is then transferred to protein co-translationally in the e.r. lumen. Subsequent processing occurs in the e.r. and Golgi apparatus, the diagram illustrating only one of the many possible processing pathways (see Hubbard and Ivatt, 1981 for review). Processing need not go to completion as shown in the figure and both high-mannose and complex-type oligosaccharides may be end products. ■ = GlcNAc; O = Man; ► = Glc; ● = Gal; ◇ = Sialic acid; ▷ = Fucose. (After Schwarz and Datema, 1984).

Thus there is little doubt that N-glycosylation is a co-translational event but what of the sidedness, with respect to the e.r. membrane, of the carbohydrate attachment? Is the polypeptide chain's sequestration into the lumen of the e.r. a prerequisite for the addition of oligosaccharide? The answer to this question is yes, for the following two reasons:-

1. In the majority of co-translationally inserted transmembrane and secretory proteins the region of the polypeptide chain which interacts with the membrane is the hydrophobic stretches of the protein's signal sequence (Kriegl, 1981). These stretches are at, or very near, the amino-terminus of the polypeptide (an exception being chicken ovalbumin [Braell and Lodish, 1982; Meek *et al.*, 1982]) thus on emerging from the ribosome the signal sequence will enter the hydrophobic domain of the e.r. membrane almost immediately. The sequestration of the remainder of the polypeptide chain is thought to be driven initially by the chain elongation process (Davis and Tai, 1980) and so each emerging amino acid effectively pushes the previous one through the membrane. Were glycosylation to occur on the cytoplasmic side of the e.r. then the glycosylating apparatus, consisting of the glycosyl transferases and the  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2\text{-PP-dol}$  complex must fit between the ribosome and the e.r. membrane. Such a situation is difficult to envisage if, as is suggested by the data of Sabatini's group

(Kreibich *et al.*, 1978 a and b, Marcantonio *et al.*, 1982), the ribosome interacts with the membrane directly via the ribophorins, integral proteins of the rough e.r membrane. Further the "pore" through which the amino acids are channelled would have to increase quite substantially in size to allow the passage of a very hydrophilic core-glycosylated asparagine residue.

2. Reports by Eggens and Dallner (1982) and Hanover and Lennarz (1982) site the outer surface of microsomal vesicles as the location of the initial steps in dolichol mono- and pyrophosphate-mediated glycosylation, i.e the synthesis of oligosaccharide-lipid. The latter report also provides evidence for the presence of N,N'-diacetylchitobiosylpyrophosphoryldolichol (GlcNac<sub>2</sub>-PP-dol) on the luminal face of the rough e.r., implying that the GlcNac<sub>2</sub>-PP-dol is transported across the membrane in some way. Haselbeck and Tanner (1982) demonstrated the transfer of Man, from GDP-Man, across synthetic liposomal membranes composed of soybean lecithin, dolichol and a partially purified mannosyl transferase. The initial step was the transfer of Man to dolichol which, by some unknown means, effected its internalization as it was then able to be transferred to GDP present within the liposomes. It would appear then that most of the components of the core-oligosaccharide, i.e GlcNac<sub>2</sub>-PP-dol, Man-dol and GDP-Man, are located

on the luminal side of the e.r. membrane (a similar mechanism to the mannosyl transfer one possibly exists for glucose). Snider and Robbins (1982) showed  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2\text{-PP-dol}$  to be located on the luminal side of microsomal vesicles using the lectin concanavalin A as a non-penetrating probe. Taken together, these results indicate that the transfer of the core oligosaccharide from lipid intermediate to protein occurs in the rough e.r. lumen and therefore implies that any protein in possession of N-linked carbohydrate must have at some point penetrated the membrane. Evidence will be presented which strongly indicates the presence of N-linked glycoproteins in the membranes of glyoxysomes from germinating castor bean tissue so implying a role for the e.r. in glyoxysome formation. This is in agreement with the earlier findings of Bergner and Tanner (1981) who, in addition to showing glyoxysomal membranes to contain glycoproteins, demonstrated the glycosylation process responsible for them to be sensitive to the antibiotic tunicamycin, a specific inhibitor of N-glycosylation (Lehle and Tanner, 1976).

### I.3 - SUBCELLULAR FRACTIONATION

#### I.3A - GENERAL

Many of the experiments described below require a very pure glyoxysomal preparation. In this respect an advantage is offered by the relatively high density (i.e around 1.25 g/ml) to which microbodies in general migrate when centrifuged into sucrose gradients.

This high equilibrium density is due to their high protein content, their low membrane lipid content and the rapid rate at which they lose water to the sucrose gradient (Tolbert, 1981). As there are no other particles in a tissue homogenate of such a high density, relative to size, microbodies can be obtained which are essentially free from contamination by any other organelle. If we are to seek an e.r. origin to, at least, some microbody components then it is also essential that an e.r. fraction should be obtainable which is as free from contamination from other organelles, and organelle fragments, as possible. Castor bean endosperm offers several advantages over other tissues in this respect, for reasons to be discussed shortly.

In animal cells from which peroxisomes have been extensively characterized (e.g. mammalian hepatocytes and protozoans) there exists a wide degree of subcellular compartmentation by organelles and subcellular structures such as the e.r. and the Golgi. Homogenization of most of these structures causes disruption to varying degrees. For example, the e.r. retains very little of its in vivo morphology, forming a heterogeneous population of sealed vesicles which constitutes the microsomal fraction (Claude, 1943), whereas relatively intact mitochondria can be obtained from both plant (Douce et al., 1972) and animal (Beaufay et al., 1964) sources. The use of centrifugation procedures to isolate subcellular organelles is perhaps the simplest and quickest means of subcellular fractionation (speed being an important factor in the retention of in vivo function). Centrifugation,

however, relies on a large degree of uniformity in size, shape and density amongst the particles of a particular subcellular population and this uniformity, if it exists in vivo, cannot always be maintained on cellular disruption. For example, rough microsomes produce a very diffuse band on a linear sucrose gradient because of (i) non-uniformity of individual membrane vesicles and (ii) variation in the number of attached ribosomes. When mitochondria and rough microsomes are centrifuged into the same sucrose gradient there is severe cross-contamination due to the rough microsomes (whose equilibrium densities vary from 1.08 - 1.20 g/ml [Lord, 1983]) smearing through the mitochondrial band (at a density of approximately 1.18 g/ml [Quail, 1979]). Another problem is encountered in the separation of two or more sets of particles varying only slightly in size, shape and density, as in the case of smooth microsomes and small fragments of most membrane types. This is because (i) most membranes are of similar basic composition (i.e. protein and lipid) and (ii) several (e.g. plasma membrane and Golgi membrane) share a common origin in the e.r. As discussed previously, the e.r. is the site of synthesis of most membrane components, i.e. phospholipids and many integral proteins. Proteins destined for location in the plasma membrane may be present in the e.r., Golgi, secretory granules and the plasmalemma itself. Difficulties arise in distinguishing between proteins which are characteristic components of a particular membrane fraction and those of a contaminating fraction. In such situations more detailed biochemical analysis of the protein components must be performed, together with kinetic studies to

determine the significance of an association of a protein with a particular fraction. Such investigations have been performed using the e.r. fraction from germinating castor bean endosperm in attempts to find precursors to glyoxysomal components (Bowden and Lord, 1976 a and b; 1977) but have not provided unequivocal proof that glyoxysomes originate from the e.r. (Lord, 1980).

#### I.3B - CASTOR BEAN ENDOSPERM FRACTIONATION

Germinating castor bean endosperm, a senescing tissue, holds the following advantages which may be exploited in subcellular biochemical investigations:-

1. The cells are non-dividing and non-secreting, they do not therefore require a well-developed Golgi apparatus and this is reflected in both ultrastructural observations (Vigil, 1970) and the low levels of Golgi marker enzymes (Mellor and Lord, 1979 b).
2. In animal cells a significant degree of contamination of the peroxisomal fraction, from a sucrose gradient, is caused by lysosomes (de Duve, 1975). Lysosomes are absent from germinating castor bean endosperm cells (the lytic compartment of these cells is the tonoplast, or vacuole) and thus cannot contribute to glyoxysomal contamination.
3. The fact that the tissue is senescing increases its fragility and thus homogenization can be achieved with the minimum of mechanical force (in contrast to many plant tissues where the cell-wall structure must be subjected to quite large forces to achieve its disruption). This fragility allows the isolation of intact organelles in very high yield.

The fractionation of castor bean endosperm tissue into cytosolic, microsomal, mitochondrial and glyoxysomal fractions on sucrose gradients is a standard technique (Kagawa *et al.*, 1973) employed routinely in the experiments to be shortly described.

#### I.3C - SUBFRACTIONATION OF THE ENDOPLASMIC RETICULUM

The e.r. has been shown to be the site of N-glycosylation in castor bean endosperm (Mellor and Lord, 1979 a; Mellor *et al.*, 1980a). The reported absence of a Golgi, however, raises the question of where the addition of peripheral sugars takes place in this tissue. Galactose has been shown to be added in the "e.r." fraction (although the galactosyl transferase activity showed a similar distribution throughout the gradient to the Golgi marker-enzyme inosine diphosphatase) (Mellor and Lord, 1979 b) and Roberts *et al.* (1980) showed the e.r. to contain the largest complement of fucosyl transferase among cellular sub-fractions. It is nevertheless possible that the transferases responsible for the transfer of core-sugars (from nucleotide to lipid and lipid to protein) are located in different regions of the e.r. to the peripheral-sugar-transferases. Microsomal sub-populations may therefore exist which differ in glycosyl-transferase activity but which fail to separate on the standard sucrose gradient (Kagawa *et al.*, 1973). Microheterogeneities in e.r. subfractions have been reported in other tissues such as skeletal muscle (Salviati *et al.*, 1982). In the case of rat liver Beaufay *et al.* (1974a) initiated a series of detailed and elegant investigations into the subcellular membranes of this tissue. The microsomal fraction was

analysed with the aid of a completely novel rotor, the E-40 (Beaufay et al., 1974b), which allowed the further subfractionation of the e.r. into four microsomal subpopulations differing in marker enzyme complementation. In the fractionation of castor bean endosperm tissue the rough e.r. is stripped of attached ribosomes in the homogenization step and thus the e.r. fraction obtained, banding at a density of 1.12 g/ml (Kagawa et al., 1973), is a mixture of stripped rough- and smooth-microsomes.

Following the determination of the purity of the various subcellular fractions obtained from castor bean endosperm, the experimental section of this thesis investigates the role of the e.r. in transferring glucose (a "core-sugar" in most N-linked glycoproteins), galactosamine and arabinose (the latter two usually being added in the Golgi apparatus of other cells) to saccharide-linked lipids and polypeptides. This is followed by a more detailed investigation into the composition of the e.r. fraction. This investigation reveals heterogeneities with respect to glycosyl transferases and other, unspecified integral membrane proteins. Finally, having established that the e.r. is the site of N-glycosylation, its role in the synthesis of glyoxysomal membrane proteins is examined.

MATERIALS

Castor bean (Ricinus communis L.) seeds of mixed varieties were obtained from Croda Premier Oils, Hull, a generous gift from Dr. L.K. Evans.

Chemicals were obtained from the following sources:

Agar Aids (Stansted, Essex) -

Glutaraldehyde (E.M. grade)

Spurr's epoxy resin components: ERL - 4206  
DER - 736  
NBA  
DMAE, S-1

Amersham International p.l.c (Amersham, Bucks) -

All radioactive chemicals

Behringwerke AG (Marburg, FRG) -

Goat anti-rabbit-FITC conjugate

BDH Chemicals Ltd. (Atherstone, Warwickshire) -

Diethanolamine

2,5 - diphenyloxazole 'Scintran' (PPO)

EDTA

Folin and Ciocalteau's phenol reagent

$\beta$ -mercaptoethanol

Nonidet P-40

Osmium tetroxide

SDS

Boehringer-Mannheim

(BLC - The Boehringer Corporation Ltd. London) -

DTT

Micrococcal ribonuclease (EC 3.1.31.1)

Proteinase K (EC 3.4.21.14)

Tunicamycin

BRL (Cambridge) -

Oligo d(T) cellulose

Eastman Kodak Company (Rochester, NY, USA) -

N-N'-methylene bisacrylamide

Fisons Scientific Apparatus (Loughborough, Leics.) -

Acrylamide

DMSO

EDTA

Glycine

SDS

TCA

GIBCO (New York, USA) -

Freunds adjuvants

JMC Ltd. (Royston Herts.) -

Silver Nitrate

Pharmacia Fine Chemicals (Uppsala, Sweden) -

Protein A - Sepharose

Polaron Equipment Ltd. (Watford, Herts.) -

Sodium cacodylate

Uranyl acetate

Sigma Chemical Company (Poole, Dorset) -

Acetyl CoA

Amino acids

ATP

BSA

Butylated hydroxytoluene

Coomassie Brilliant Blue G

Creatine phosphate

Creatine phosphokinase (EC 2.7.3.2)

DEAE - cellulose

DTNB

EGTA

GTP

Hemin

Iodoacetamide

D-L Isocitrate

L-Malate

$\alpha$  -Methyl mannoside

NADH

Phenylhydrazine

Phosphatase (Alkaline EC 3.1.3.1) -

rabbit anti-mouse IgG conjugate

Phosphatase (104) substrate tablets

PMSF

Triton X-100

Triton X-114

Trizma base (Tris)

Tween 20

All other chemicals were of the highest grade and purity available.

Rabbits were purchased from Hylyne Rabbits Ltd., (Northwich, Cheshire) and nitrocellulose was obtained from Schleicher and Schull (Dassel, FRG)

METHODS

M.1 - TISSUE

Ricinus communis seeds were soaked overnight in running tap water and germinated in moist vermiculite in the dark at 30°C.

Developing seeds, used for mRNA extraction, were obtained from plants grown, from seed, in a greenhouse at 15°C under a 16h/8h light/dark cycle. Endosperm tissue was excised from the ripening seeds during testa formation.

M.2 - CELLULAR FRACTIONATION

M.2A GERMINATING TISSUE

Endosperm halves, from 2 - 3 day old seedlings and free from testa and cotyledons, were chopped in ice-cold grinding medium containing:-

150 mM tricine (pH 7.5)

1 mM EDTA (pH 7.5)

10 mM potassium chloride

1 mM magnesium chloride

100 mM lactose

12% (w/v) sucrose

Grinding medium was used at a rate of 4.5 ml per 15 endosperm halves and the chopping procedure utilised a single razor blade. After squeezing the finely chopped tissue through 4 layers of muslin, the resulting homogenates were centrifuged at 500 x g and 2°C for 10 minutes in an MSE Hi-spin centrifuge to pellet cellular debris, nuclei and the majority of plastids. The 500 x g supernatant was layered onto linear 60 - 30% (w/w) sucrose gradients overlaid with 20% (w/w) sucrose.

The two sizes of gradient used were:-

16 ml composed of	0.5 ml 60% sucrose
	10.0 ml 60 - 30% gradient
	3.5 ml 30% sucrose
	2.0 ml homogenate
and 38 ml composed of	2.0 ml 60% sucrose
	21.0 ml 60 - 30% gradient
	10.0 ml 20% sucrose
	5.0 ml homogenate

All sucrose solutions contained 1 mM EDTA (pH 7.6). The gradients were contained in cellulose nitrate tubes (Beckman) and organelle separation was achieved by centrifugation at 24,000 rpm (75,000 x g) and 2°C in an SW27 rotor (Beckman) on a Beckman L2, L5 or L8 ultracentrifuge for 3.5 h.

#### M.2B - DORMANT SEEDS

Protein bodies were isolated from dry seeds using the non-aqueous method described by Tully and Beevers (1976).

Endosperm, essentially free of testa, was homogenised in glycerol, using a mortar and pestle, at a rate of 2 halves per ml glycerol. The homogenate was filtered through 4 layers of muslin then centrifuged at 1,000 x g in an MSE Hi-spin centrifuge at 20°C for 5 min. The fat layer was discarded and the supernatant transferred to a clean tube and re-centrifuged at 40,000 x g for 20 minutes. The glycerol supernatant was discarded and the pellet resuspended in 5 - 10 times its volume of fresh glycerol. The protein bodies were repelleted at 40,000 x g for 20 minutes. This washing procedure was repeated once to yield a pure protein body preparation.

Protein body membranes were obtained by osmotically disrupting the protein bodies with ice-cold 5 mM tris-HCL (pH 8.5) then overlaying 5 ml portions onto sucrose gradients composed of:-

24 ml 68 - 30% (w/w) sucrose

4 ml 20% (w/w) sucrose

4 ml 10% (w/w) sucrose

These gradients were then subjected to centrifugation at 24,000 rpm (75,000 x g) and 2°C for 6 h.

#### M.2C - GRADIENT FRACTIONATION

Following centrifugation the gradients were fractionated by piercing the bottoms of the tubes and collecting 1 ml fractions in graduated plastic test tubes.

Alternatively, if only the major-organelle containing fractions were required then the bands composed of the relevant organelles, usually being clearly visible, were removed using a syringe or pasteur pipette.

#### M.3 ENDOPLASMIC RETICULUM SUB-FRACTIONATION

3 ml of an endoplasmic reticulum vesicle (microsomal) suspension, isolated as described above, was mixed with 2.5 ml 70% (w/w) sucrose in a 17.5 ml cellulose nitrate tube (Beckman) then overlaid consecutively with:-

3 ml 40% (w/w) sucrose

6.5 ml 30% (w/w) sucrose

2 ml 20% (w/w) sucrose

These, flotation, gradients were then centrifuged at 24,000 rpm (75,000 x g) and 2°C in a SW27 rotor.

Following centrifugation the gradients were collected as 1 ml fractions.

The sucrose concentration of the collected fractions from these, and the previous, gradients was determined refractometrically.

#### M.4 - ENZYME ASSAYS

##### M4A - ISOCITRATE LYASE (EC 4.1.3.1) -

Was assayed by the method of Dixon and Kornberg (1959). The reaction mixture, in a final volume of 1 ml, contained:-

100 mM sodium phosphate (pH 6.9)

10 mM magnesium chloride

5 mM dithiothreitol (DTT)

10 mM phenylhydrazine

10 mM D-L isocitrate

enzyme

The reaction was started by the addition of 100  $\mu$ l 100 mM D-L isocitrate and followed at 324 nm in a Gilford spectrophotometer. An increase of 17  $A_{324}$  units was taken to indicate the production of 1  $\mu$  mol glyoxylate phenylhydrazone.

##### M.4B - MALATE SYNTHASE (EC 4.1.3.2) -

Was assayed according to the method of Hock and Beevers (1966). The 1 ml reaction mixture contained:-

100 mM tris-HCl (pH 8.0)

5 mM magnesium chloride

100  $\mu$ M DTNB

33  $\mu$ M acetyl CoA

10 mM sodium glyoxylate

enzyme

The reaction was initiated by the addition of 100  $\mu$ l 100 mM sodium glyoxylate and followed in a Gilford spectrophotometer at 412 nm. A mM extinction coefficient of 13.6 was used to calculate enzyme activity.

M.4C - FUMARASE (EC 4.2.1.2) - Racker (1950) -

The reaction mixture contained, in a final volume of 1 ml:-

50 mM sodium phosphate buffer (pH 7.4)

50 mM L-malate (pH 7.4)

enzyme

The reaction was initiated by the addition of 100  $\mu$ l 500 mM l-malate (pH 7.4) and followed at 240 nm in a Gilford spectrophotometer. A decrease of 2.6 absorbance units indicated the conversion of 1  $\mu$ mol malate to fumarate.

M.4D NADH - CYTOCHROME C REDUCTASE (EC 1.6.2.4)

Lord et al (1973). The reaction mixture contained, in a final volume of 1 ml:-

20 mM potassium phosphate buffer (pH 7.2)

0.02 mM cytochrome c

0.2 mM NADH

10 mM potassium cyanide

The reaction was started by the addition of enzyme and followed at 550 nm. A mM extinction coefficient of 21 was used for the calculation of enzyme activity.

M.4E - CHOLINEPHOSPHOTRANSFERASE (EC 2.7.8.2) -

Lord et al. (1973)

This enzyme was assayed by incubating 1 ml samples (usually gradient fractions made 10 mM in magnesium chloride) with 0.02  $\mu$ Ci cytidinediphospho - [ $^{14}$ C] choline (CDP- $^{14}$ C] choline, S.A. 28  $\mu$ Ci/  $\mu$  mole, in 10  $\mu$ l ) at 25°C for 30 minutes then extracting phospholipid as follows:-

To each incubation mixture was added 2 ml absolute ethyl alcohol to precipitate proteins. The precipitate was removed by centrifugation in a bench top centrifuge and the pellet re-extracted with 1 ml ethyl alcohol. After a second centrifugation the ethanolic phases were pooled and mixed with 2 ml chloroform. The organic phase was washed twice with 5 ml portions of 2M potassium chloride and then twice with water to remove unreacted CDP - [<sup>14</sup>C] base. The residual chloroform phase, containing phosphatidyl - [<sup>14</sup>C] choline, was transferred to glass scintillation vials and evaporated to dryness. The radioactivity content was measured after the addition of 10 ml scintillation fluid (Beckman Ready - solv EP or "Brays's", Bray, 1960)

#### M.4F - GLYCOSYLTRANSFERASES

The transfer of radiolabelled sugars from activated (nucleotide) forms to glycoproteins and lipid-linked intermediates was assayed essentially as described by Forsee *et al.* (1976).

Assays were performed either on 1 ml gradient fractions or, for time course studies, a resuspended 20,000 x g pellet from a castor bean endosperm homogenate obtained as described in M.2A. The pellet was resuspended in:-

- 30 mM tris-HCl (pH 7.5)
- 10 mM magnesium chloride
- 20 mM  $\beta$  - mercaptoethanol
- 0.1% (v/v) Triton X-100

Where appropriate Triton X-100 stock, 1% (v/v), contained dolichol monophosphate to give a final dolichol content of 20  $\mu$ g/ml. A 2 ml assay contained the 20,000 x g pellet from 20 homogenised endosperm halves.

The reaction was started by the addition of 0.05  $\mu$ Ci UDP - [ $^{14}$ C] glucose (specific activity, S.A., 293 Ci/mol) then incubated at 30 $^{\circ}$ C. At various times 0.25 ml samples were removed and the incorporation of radioactivity into monosaccharide-lipid, oligosaccharide-lipid and TCA-insoluble residue determined as described below.

To determine glycosyl transferase activities in collected gradient fractions, each fraction was made 150 mM with respect to tris-HCL (pH 7.5), 10 mM in magnesium chloride and 20 mM in  $\beta$ -mercaptoethanol. To each was then added 0.1 ml 3% (v/v) Triton X-100 or 3% (v/v) Triton X- 100 containing 20  $\mu$ g dolichol monophosphate. Reactions were started by the addition of 0.01  $\mu$ Ci UDP- [ $^{14}$ C] glucose (S.A., 293 Ci/mol), 0.05  $\mu$ Ci GDP - [ $^{14}$ C] mannose (S.A., 199 Ci/mol), 0.05  $\mu$ Ci UDP-N-acetyl-D [ $^3$ H] glucosamine (S.A., 11 Ci/mmol) or 0.05  $\mu$ Ci GDP - L - [ $^{14}$ C] fucose (S.A., 24.5 Ci/mol) followed by incubation at 30 $^{\circ}$ C for 30 minutes. The reactions were terminated by the addition of 4 ml chloroform/methanol (2 : 1). After thorough mixing, the phases were separated by centrifugation (500 x g for 1 minute) and the lower organic phase, containing the monosaccharide lipid, was removed and saved. Following re-extraction of the aqueous phase and interface material with 2 ml chloroform, the second organic phase was combined with the first. Non-lipid material was removed from the organic phase by extracting with an equal volume of chloroform/methanol/water (3 : 48 : 47). The final organic phase was transferred to a scintillation vial, evaporated to dryness and the radioactivity determined after the addition of 10 ml scintillant.

To the aqueous phases and interface material from the initial extraction was added sufficient methanol to form a single phase. The insoluble residue was pelleted by centrifugation at 500 x g for 1 minute. The pellets were then washed twice with 50% methanol and the washings discarded. The washed pellets were re-suspended in 2 ml chloroform/methanol/water (1 : 1 : 0.3) and the suspensions were allowed to stand for 15 minutes at room temperature to extract oligosaccharide-lipid. After centrifugation, the supernatants were transferred to scintillation vials, evaporated to dryness and the radioactivity determined.

The residual pellets, containing the glycoprotein were resuspended in 8% (w/v) TCA and filtered through G/FA filter discs (Whatman). After washing the filters several times with 8% TCA they were immersed in 5 ml scintillant in plastic counting vials and their associated radioactivities determined.

In the case of the 17 ml flotation gradients, 400  $\mu$ l aliquots were used from each fraction to allow the effect of supplemented dolichol monophosphate to be determined on duplicate samples from the same fraction.

#### M.5 - PROTEIN DETERMINATION

Protein concentrations were determined by the method of Lowry *et al.* (1951).

To each 200  $\mu$ l sample, in duplicate, was added 1.5 ml of a solution comprised of:-

100 ml 2% (w/v) sodium carbonate in 0.1M NaOH

1 ml 1% (w/v) copper sulphate solution

1 ml 2% (w/v) potassium tartrate solution

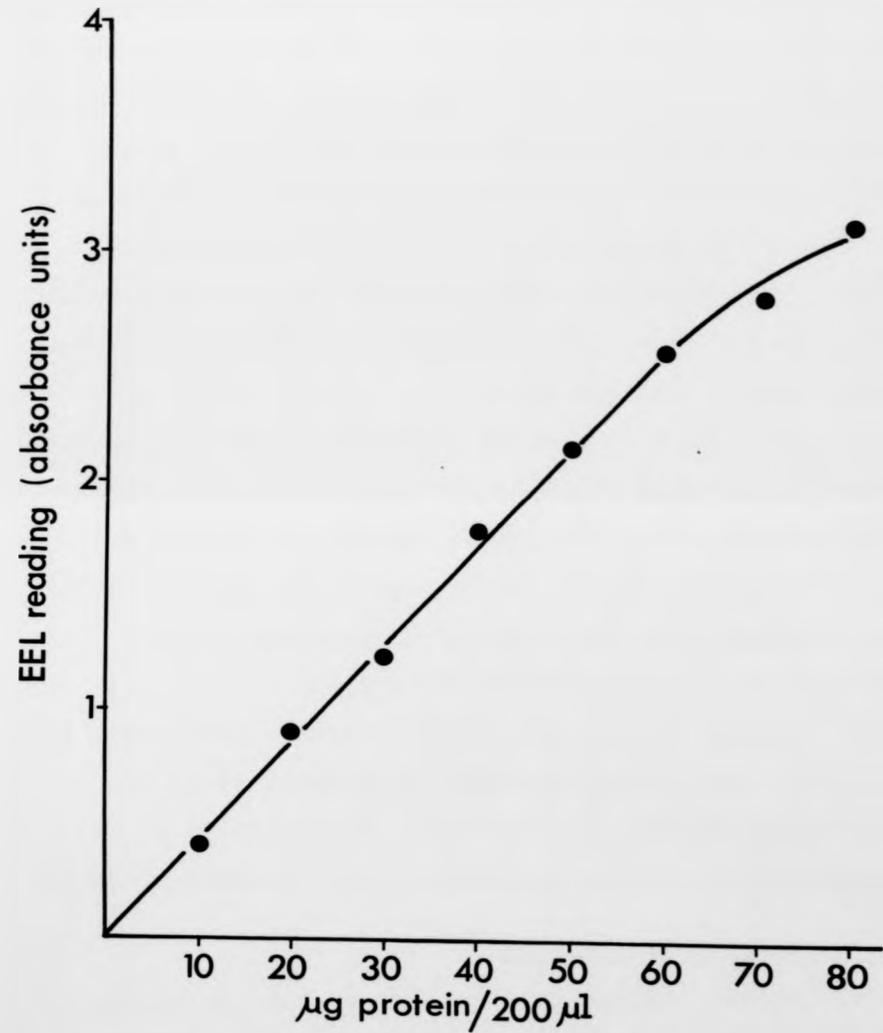


Fig. 6 Calibration Curve for the Determination of Protein

0.2 ml aliquots of standard solutions of BSA were treated as described in M.5. Optical density was read in an EEL colorimeter calibrated in arbitrary units and zeroed with a reagent blank.

After 15 minutes at room temperature a 1 : 1 dilution of Folin and Ciocalteu's phenol reagent was added. The samples were allowed to stand for a further 30 minutes before reading the colour intensity on an EEL colorimeter fitted with a red, O.R.1, filter. Standard solutions of bovine serum albumin (BSA, 0-400  $\mu\text{g/ml}$ ) were used to produce a calibration curve to which the sample readings could be related. An example of such a curve is shown in fig. 6.

#### M.6 - LABELLING OF INTACT TISSUE

The endosperm halves of beans 2 - 3 days post germination were excised from the testa and the cotyledons removed. The halves were placed in petri dishes with their abaxial surfaces in contact with moist filter paper. Depending on the required labelling, the adaxial surfaces were inoculated with:-

- 25  $\mu\text{Ci}$  [ $^{35}\text{S}$ ] - methionine  
(S.A., 750-1,000 Ci/mmol)
- 2  $\mu\text{Ci}$  [ $^{14}\text{C}$ ] - amino acid mixture  
(S.A. > 50 mCi/milliatom of carbon)
- 10  $\mu\text{Ci}$  D - [6 -  $^3\text{H}$ ] - glucose (S.A., 12.8 Ci/mmol)
- 10  $\mu\text{Ci}$  L - [1 -  $^3\text{H}$ ] - fucose (S.A., 5.4 Ci/mmol)
- 10  $\mu\text{Ci}$  N - acetyl - D - [1 -  $^3\text{H}$ ] glucosamine  
(S.A., 11 Ci/mmol)
- 10  $\mu\text{Ci}$  D - [1 -  $^3\text{H}$ ] - mannose (S.A., 5Ci/mmol)
- 10 or 100  $\mu\text{Ci}$  D - [6 -  $^3\text{H}$ ] glucosamine  
(S.A., 40 Ci/mmol)
- or 25 nCi CDP - [ $^{14}\text{C}$ ] - choline (S.A., 50 Ci/mol)

each in volumes of 10 - 25  $\mu\text{l}$ . The incubation times varied with the experiment. Except where stated,

this was either 2 hours at 30°C (when the subcellular site of initial incorporation was being investigated), or 1 h at 30°C followed by 16 h at 20°C (when samples were ultimately to be subjected to SDS - polyacrylamide gel electrophoresis and fluorography).

Following incubation the tissue was homogenised by the standard procedure (M.2) and loaded onto 16 ml gradients. Following centrifugation, the gradients were fractionated then the fractions, or 5 or 10  $\mu$ l aliquots thereof, were precipitated with cold 10% (final concentration) TCA. The TCA precipitates were filtered through Whatman GF/A discs then washed successively with 10% (w/v) TCA. chloroform/methanol (2 : 1) and chloroform/methanol/water (1 : 1 : 0.3) before immersing the filters in 5 ml scintillant and determining associated radioactivities.

#### M.7 - SODIUM DODECYL SULPHATE - POLYACRYLAMIDE

##### GEL ELECTROPHORESIS (SDS - PAGE)

Protein samples, as TCA or acetone precipitates or in solution, were prepared for SDS - PAGE by adding sample loading buffer:-

200 mM tris-HCl (pH 8.8)

0.5 M sucrose

5 mM EDTA

4% (w/v) SDS

1% (w/v) methionine

10 mM dithiothreitol (DTT)

0.01% (w/v) bromophenol blue

In the case of precipitated protein 30 - 50  $\mu$ l sample loading buffer was added directly to the protein, and in the case of protein solutions the sample was mixed with at least 5 times its volume of sample buffer.

A few crystals of solid tris base were usually required to restore the pH of a TCA precipitated sample to around 8.

On ensuring that the protein was completely dissolved in the loading buffer, the samples were heated to 95°C for 2 - 3 minutes. On cooling, iodoacetamide (IAA, freshly prepared) was added to a final concentration of 50 mM and the samples were then allowed to stand for 15 - 20 minutes before loading onto a polyacrylamide slab gel using a 100  $\mu$ l Hamilton syringe.

Slab gels were constructed as follows:- for a 30 x 20 cm resolving gel 80 ml of 10% acrylamide/Bis (30 : 0.8) in:-

0.4 M tris - HCl (pH 6.8)

0.1% SDS

0.05% TEMED

0.04% ammonium persulphate (fresh)

was poured and overlaid with a solution of water-saturated butan - 1 - ol. Following polymerization and extensive washing of the resolving gel, to remove the butanol, a 2 - 2.5 cm stacking gel was poured on top. This gel contained 5% acrylamide/bis (30 : 0.8) in:-

0.05 M tris - HCl (pH 8.8)

0.1% SDS

0.05% TEMED

0.04% ammonium persulphate.

Prior to polymerization of the stacking gel a 20 or 31 place spacer-comb was inserted into the gel to form sample wells. The polymerized gel was attached to a slab gel electrophoresis tank and the reservoirs filled with running buffer:

50 mM tris - 0.4 M glycine (pH 8.8)

0.1% (w/v) SDS

The samples were loaded and electrophoresis carried out, usually, for 16 h at 18 mA. Following electrophoresis the gels were fixed in either 10% (w/v) TCA (if the gel was to be processed for fluorography - M.8), 30% (v/v) methanol, 7% (v/v) acetic acid (if the gel was to be coomassie-blue stained) or 50% (v/v) methanol (in the case of gels to be silver stained). Coomassie staining was for 1 h in 0.2% (w/v) coomassie brilliant blue G, 30% (v/v) methanol and 10% (v/v) acetic acid, followed by destaining for several hours in 30% (v/v) methanol, 7% (v/v) acetic acid.

Silver staining was by the method of Wray et al (1981). Following fixation in 50% (v/v) methanol the gel was soaked in a further 2 changes of 50% methanol, the final change usually overnight to ensure the complete removal of tris/glycine.

The staining solution was prepared by adding, dropwise and with continuous vigorous stirring, 4 ml of 20% (w/v) silver nitrate to a solution comprised of 21 ml 0.36% NaOH and 1 ml 35% ammonium hydroxide. The volume of the staining solution was then increased to 100 ml. The gel was transferred to the stain solution (within 5 minutes of its preparation) and staining allowed to proceed for 15 minutes with gentle agitation. The gel was then washed in distilled water for a further 5 minutes during which time the developer solution (consisting of 500 ml 0.005% citric acid and 0.25 ml 38% formaldehyde) was prepared. The gel was then transferred to the developer and gently agitated until bands appeared. The background was cleared using a 1 in 10 dilution of Kodafix (Kodak), the action of which was stopped by transferring the gel to 50% methanol once more.

Gels were dried down at 60 or 80°C, under vacuum, following a 15 - 20 minute incubation in 5% (v/v) glycerol in either coomassie destain solution or 50% methanol.

Dried gels were photographed using Kodak Panatomic X film and, in the case of coomassie-stained gels, a yellow (Y2) filter.

#### M.8 - FLUOROGRAPHY

The processing of polyacrylamide gels for fluorography was essentially as described by Bonner and Laskey (1974). TCA fixed gels were soaked in 20 volumes of dimethyl sulphoxide (DMSO) for 30 minutes then transferred to fresh DMSO for a further 30 minutes. Impregnation with 2, 5 - diphenyloxazole (PPO) was achieved by immersing the gels in 4 gel volumes of 20% (w/w) PPO in DMSO for 3 h. The gels were then washed extensively with tap water until no crystals of PPO remained on the surfaces. Before drying, the gels were soaked in 5% glycerol for 15 - 20 minutes. After drying, the gels were mounted onto stiff card and placed in contact with Kodak X-Omat X-ray film and exposed at -80°C. The films were developed using Kodak DX -80 or Kodak LX -24 developer (under a Kodak orange, OA, safelight) and fixed in Kodak FX -40 fixer. Fluorographs were photographed using Kodak Panatomic -X film.

#### M.9 - GLYCOPROTEIN DETECTION USING [<sup>125</sup>I] CONCAVALIN A

##### M.9A - Con A IODINATION

The method was provided by Dr. D.J. Bowles (University of Leeds) To 100  $\mu$ l pre-reaction mix:

0.02 M sodium phosphate buffer (pH 7.4)

0.15 M sodium chloride

0.72 mM con A (subunit MW - 27,000 daltons)

0.72 mM potassium iodide

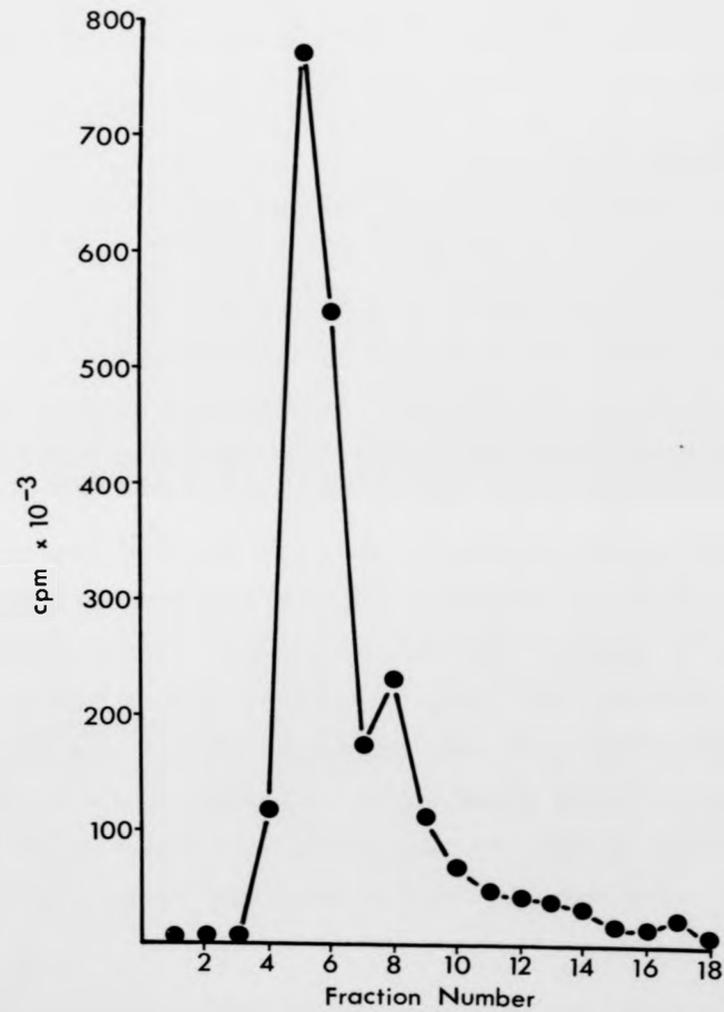


Fig. 7 Separation of [<sup>125</sup>I] Con A From Free [<sup>125</sup>I] By Sephadex G-50 Column Chromatography

4 mg (72 nmol) con A was iodinated with 400  $\mu$ Ci [<sup>125</sup>I] as detailed in M.9A. The [<sup>125</sup>I] con A was separated from the unreacted [<sup>125</sup>I] by gel filtration through Sephadex G-50 equilibrated in PBS. [<sup>125</sup>I] con A is represented by the peak associated with fractions 4 - 6.

0.20 M  $\alpha$ -methyl mannoside  
was added 400  $\mu$ Ci  $^{125}$ I, followed by 100  $\mu$ l 7.2 mM  
chloramine T (Sigma). The reaction mixture was  
incubated at room temperature for 5 minutes then the  
reaction stopped by the addition of 100  $\mu$ l sodium  
metabisulphite. The iodinated con A was separated from  
the free  $^{125}$ I, 10 minutes later, by passing the  
reaction mix down a 5 x 0.7 cm sephadex G-50 column  
equilibrated in phosphate buffered saline (PBS):

0.02M sodium phosphate buffer (pH 7.4)

0.15 M sodium chloride

0.3 ml fractions were collected and 1  $\mu$ l aliquots counted  
from the first 18 fractions. The distribution of  
radioactivity through these fractions is shown in fig. 7.  
Fractions 4 - 6 were taken to contain the iodinated con A,  
these were pooled to give 900  $\mu$ l containing  $2 \times 10^5$  cpm/ $\mu$ l  
which, if the entire 72 nmol (4 mg) con A is assumed  
to be contained in this volume, gives a specific  
activity in the order of  $4.5 \times 10^7$  cpm/mg protein. This  
is within the range ( $2 \times 10^7 - 10^9$  cpm/mg) quoted by  
Burridge (1978).

#### M.9B - GLYCOPROTEIN DETECTION AFTER SDS-PAGE

Following staining, with coomassie blue, the gel was  
transferred to PBS/azide:

0.02 M sodium phosphate buffer (pH 7.4)

0.15 M sodium chloride

0.02% (w/v) sodium azide

The gel was shaken in two changes of PBS/azide, each for  
2 h then transferred to overlay buffer:

0.02 M sodium phosphate (pH 7.4)

0.15 M sodium chloride

1% (w/v) BSA

0.02% sodium azide

After 3 changes of overlay buffer (two incubations of 2 h duration and a final, overnight incubation, all at 4°C on a rotary shaker) the gel was transferred to overlay buffer to which had been added (<sup>125</sup>I)-iodinated con A to a concentration of 10<sup>6</sup> cpm/ml.

The gel was incubated with the iodinated con A for 1 h at 4 C with rotation then washed extensively (i.e., for 24 h) with several changes of overlay buffer until the radioactivity associated with the washings was reduced to background levels.

Gels were then taken through control incubations which differed from the procedure described above in that all buffers i.e. PBS/azide and overlay buffer, contained 0.2 M  $\alpha$ -methyl mannoside.

Finally, gels were incubated in 5% glycerol before drying down as described previously (M.7) and exposing to X-ray film at room temperature for 3 - 5 days.

#### M.10 - EXTRACTION AND PURIFICATION OF POLYADENYLATED RNA

Total germinating castor bean RNA was extracted from 3 - 4 day-old tissue by the method of Keller and Taylor (1976) with modifications of Bowden-Bonnett and Lord (1979) and Roberts and Lord (1979). The tissue, free from testa and cotyledons, was frozen in liquid nitrogen and roughly ground using a mortar and pestle. The frozen granules were then added to a Waring Blender, together with 3 - 4 volumes of extraction buffer:

50 mM tris-HCl (pH 9.0)

150 mM sodium chloride

5 mM EDTA

5% (w/v) SDS

and homogenized at full speed for 2 minutes.

The resulting slurry was mixed with an equal volume of phenol/chloroform (1 : 1) and stirred for 5 - 10 minutes at room temperature. The mixture was centrifuged in batches in a bench-top centrifuge to separate the organic and aqueous phases. The latter were removed and pooled whilst the organic phases, together with the insoluble interface material, were re-extracted with a half volume of washing buffer:

20 mM tris-HCl (pH 9.0)

2 mM EDTA

Following phase separation, the aqueous phases were added to the aqueous phase pool from the first extraction and the organic phases discarded. The aqueous phase was then re-extracted twice with equal volumes of phenol/chloroform (1 : 1). The final aqueous phase was made 0.2 M in sodium chloride by the addition of an appropriate volume of 1.0 M NaCl. 2 volumes of absolute ethanol, which had been pre-cooled to  $-20^{\circ}\text{C}$ , were added and the nucleic acid allowed to precipitate overnight at  $-20^{\circ}\text{C}$ .

Harvesting of the nucleic acids was achieved by centrifugation at  $15,000 \times g$  and  $2^{\circ}\text{C}$  for 15 minutes in an MSE "18" centrifuge. The resulting pellets were resuspended in 3.0 M sodium acetate (pH 5.5) then recentrifuged at  $15,000 \times g$ . The process constituted one acetate wash. Following a further 3 acetate washes, which had been found necessary for the removal of polysaccharide, tRNA, 5s rRNA and DNA, the residual

pellet (by this time in a single, 30 ml siliconized "Corex" glass tube) was dissolved in the minimum volume of 0.3<sup>M</sup> NaCl and the RNA precipitated as before, with ethanol, at -20°C.

Following harvesting, the RNA was dissolved in buffer A:

20 mM tris-HCl (pH 7.6)

0.4 M sodium chloride

0.2% (w/v) SDS

ensuring that a concentration of 100 A<sub>260</sub> units/ml was not exceeded. Oligo (dT) cellulose, swollen in buffer A, was added at a ratio of 6 ml swollen cellulose per 1,500 A<sub>260</sub> units. 15 minutes, at room temperature, was then allowed for the polyadenylated (poly [A<sup>+</sup>]) RNA to bind to the cellulose; during which time the cellulose was kept in suspension by intermittent shaking. Following harvesting, in a bench-top centrifuge, the oligo (dT) cellulose was washed three times with buffer A then twice with buffer B:

20 mM tris-HCl (pH 7.6)

0.2 M sodium chloride

0.1% (w/v) SDS

The cellulose was again suspended in buffer B and transferred to a small glass column connected to an ISCO u.v monitor (model UA5 fitted with a type 6 optical unit) such that the absorbance of the column eluate could be monitored at 260 nm. The cellulose was washed with buffer B until the chart recorder indicated the eluate to be free of unbound RNA, poly (A<sup>+</sup>) RNA was eluted with 20 mM tris-HCl, pH 7.6 (buffer C) which had been pre-heated to 50°C, and precipitated, after

adjusting the NaCl concentration to 0.2 M, with 2.5 volumes of ethanol at  $-20^{\circ}\text{C}$  overnight. The precipitate was collected by centrifugation at  $12,000 \times g$  and  $2^{\circ}\text{C}$  for 30 minutes, washed three times with 70% (v/v) aqueous ethanol, dried under a stream of air then dissolved in 10 mM tris-HCl, pH 7.6 to a concentration of 1 mg/ml. 100  $\mu\text{l}$  aliquots were snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

M.11 - IN VITRO PROTEIN SYNTHESIS IN RABBIT RETICULOCYTE LYSATES

M.11A - LYSATE PREPARATION

Rabbit reticulocyte lysate was prepared by Dr.'s J.M. Lord and G. Valle by the method of Gilbert and Anderson (1971) and stored in 2 ml aliquots in liquid nitrogen until required.

M.11B - LYSATE PRE-TREATMENT FOR USE IN CELL FREE mRNA

TRANSLATION

A 2 ml aliquot of lysate was thawed and to it was added 80  $\mu\text{l}$  of 1 mM hemin in 90% ethylene glycol, pH 7.5 (the hemin solution was 20 mM with regard to tris and 5 mM in KCl; the hemin solid having originally been dissolved in 0.5 M KOH, which was diluted to a final concentration of 10 mM) and 20  $\mu\text{l}$  0.1% (w/v) creatine kinase in 50% (v/v) glycerol. The treated lysate was then quickly sub-divided into 100  $\mu\text{l}$  aliquots and snap-frozen in liquid nitrogen.

Prior to use, an aliquot was hand-thawed and to it was added 8  $\mu\text{l}$  of ribonuclease mix:

- 1 volume 100 mM calcium acetate
- 2 volumes 1.5 U/ml micrococcal ribonuclease
- 1 volume water

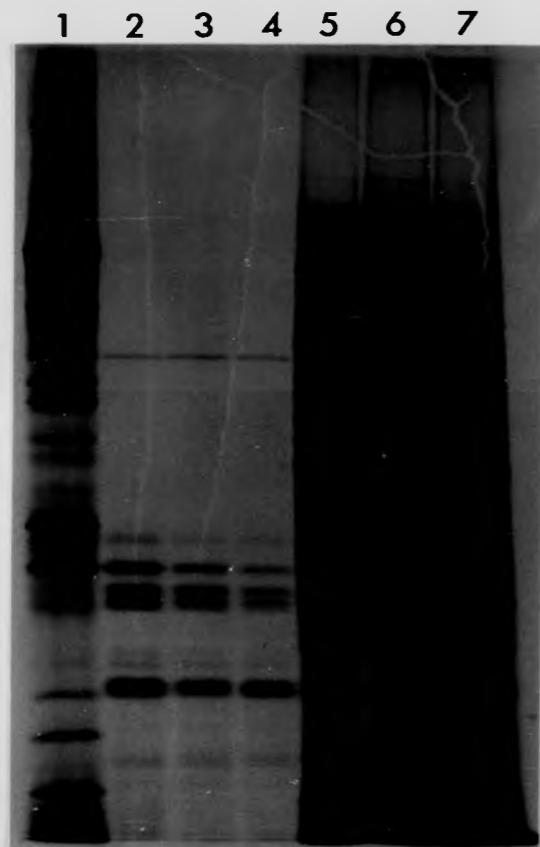


Fig. 8 Micrococcal Nuclease Treatment of Reticulocyte Lysates

Endogenous mRNA present in reticulocyte lysates was degraded with a  $\text{Ca}^{2+}$ -activated ribonuclease as detailed in M.11B. Track 1; polypeptide products of endogenous mRNA present in untreated lysate (see M.11C). Tracks 2 - 4; effect of nuclease treatment for 6, 8 and 10 minutes respectively on endogenous, mRNA translation products. Tracks 5 - 7; stimulation by exogenous germinating castor bean mRNA added to the lysates treated as in tracks 2 - 4.

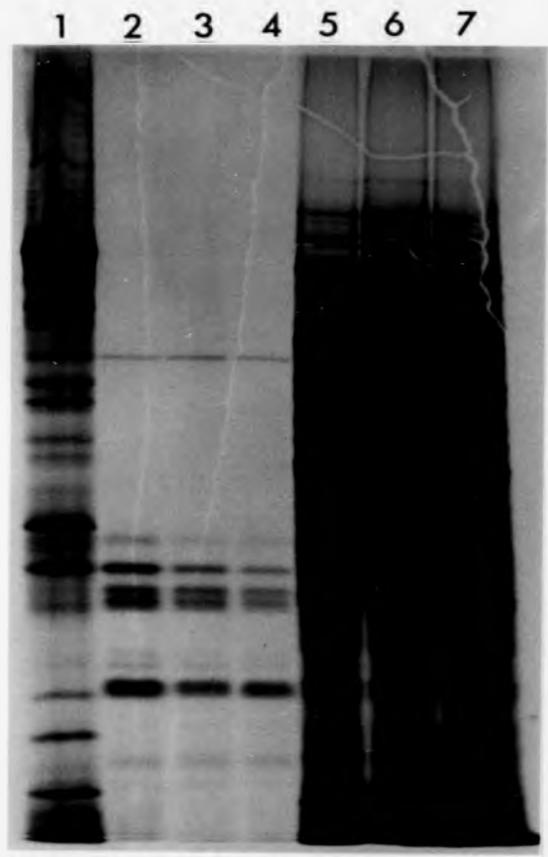


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Nuclease digestion was allowed to proceed for 8 minutes (as determined experimentally, see fig. 8) before its inactivation by the addition of 4  $\mu$ l 0.25 M EGTA. The lysate was then ready for use as a mRNA translation system. If it was not to be used straight away then the lysate was snap-frozen in liquid nitrogen immediately upon the addition of the EGTA.

M.11C - INCUBATION CONDITIONS FOR mRNA TRANSLATION IN VITRO

An energy mix was prepared containing:-

166 mM tris-HCl (pH 7.5)  
20 mM ATP  
4 mM GTP  
66 mg/ml creatine phosphate

and which was stored at  $-80^{\circ}\text{C}$  in 1ml aliquots

Into numbered, 600  $\mu$ l Eppendorf tubes was added 9  $\mu$ l reaction mix:

5  $\mu$ l 1.25 mM each of 19 unlabelled amino acids (excluding methionine)  
6.2  $\mu$ l energy mix (as described above)  
5.5  $\mu$ l 2 M potassium chloride  
1.8  $\mu$ l 40 mM magnesium chloride  
6.2  $\mu$ l [ $^{35}\text{S}$ ] methionine (approximately 100  $\mu$  Ci)  
3.2  $\mu$ l 100 mM tris-HCl (pH 7.4)  
28.8  $\mu$ l sterile water

for a final volume of 56.7  $\mu$ l (sufficient for 6 x 20  $\mu$ l translations). When appropriate the above volume of reaction mix was supplemented with 6  $\mu$ l of a preparation of rough microsomes from canine pancreatic tissue (a gift from Prof. V.R. Lingappa via Dr. A. Colman) with a corresponding adjustment in water volume.

Into the same tubes was then added 10  $\mu$ l nuclease-treated reticulocyte lysate. Protein synthesis was initiated by the addition of 1  $\mu$ l poly (A<sup>+</sup>) RNA and maintained by incubation at 37°C for, routinely 45 minutes, after which time there was little subsequent increase in [<sup>35</sup>S] methionine incorporation into protein. 2  $\mu$ l samples were removed and hot TCA insoluble radioactivity (an indication of the amount of [<sup>35</sup>S] methionine incorporated into protein) was determined essentially as described by Mans and Novelli (1961). The samples were spotted onto Whatman 3 M filter paper squares (approximately 1.5 x 1.5 cm) and allowed to dry before being plunged into cold, 10% TCA for 15 minutes. They were then taken through the following washing procedure:

Boiling 5% TCA	-	15 minutes
3 x cold 5% TCA	-	3 minutes each
Cold absolute ethanol	-	3 minutes
Ethanol/ether (1 : 1)	-	3 minutes

then dried either using an infra-red lamp or a stream of nitrogen. The filters were then immersed in 5 ml Beckman Read-Solv EP and the associated radioactivity determined.

#### M.12 - PROTEASE TREATMENT OF MEMBRANE VESICLES

In order to determine the topological location of proteins with respect to a membrane, samples were subjected to digestion by proteinase K in the presence or absence of sodium deoxycholate (DOC). Membranes treated were castor bean microsomes and glyoxysomes, from in vivo [<sup>35</sup>S] methionine-labelled tissue, and microsomal membranes from in vitro translation assays.

in vivo-labelled

The  $\lambda$  membrane suspensions typically contained the microsomal or glyoxysomal membranes from 7 - 10 endosperm halves in 200  $\mu$ l 50 mM tris-HCl (pH 7.5). For in vitro labelled samples 5 x 20  $\mu$ l translations were supplemented with pancreatic microsomes  $\lambda$  pooled to give 100  $\mu$ l to which was then added the microsomal fraction from approximately 5 endosperm halves in 100  $\mu$ l tris-HCl (pH 7.5). To these samples was added a half volume of 0.75 M sucrose in 50 mM tris-HCl or a similar sucrose solution containing 3% (w/v) DOC. After mixing and standing on ice for 10 minutes a 0.3% (w/v) proteinase K solution was added to give a final proteinase K concentration of 100  $\mu$ g/ml. Digestion was allowed to proceed for 90 minutes on ice before the addition of 10  $\mu$ l 10% (w/v) lysozyme or BSA and 10  $\mu$ l 1% (w/v) phenylmethylsulphonyl fluoride (PMSF) in ethanol.

Membranes which had been protease treated in the absence of DOC were removed from suspension by centrifuging at 100,000 x g and 4°C for 30 minutes in a Beckman Airfuge. Samples were then processed for SDS-PAGE directly or following Triton X-114 extraction as detailed below.

M.13 - TRITON X-114 EXTRACTION OF INTEGRAL MEMBRANE PROTEINS

M.13A - PRECONDENSATION OF TRITON X-114

This procedure was carried out essentially as described by Bordier (1981). To 20 g Triton X-114 was added 16 mg of butylated hydroxytoluene (BHT), an anti-oxidant. The Triton/BHT was added to 980 ml 10 mM tris-HCl (pH 7.4) containing 150 mM KCl. The Triton was dissolved by stirring at 0°C and then placed at 30°C overnight to allow separation into two phases.

The upper, aqueous, phase was discarded, after measuring its volume, and replaced by a similar volume of fresh 10 mM tris/150 mM KCl. The mixture was again stirred at 0°C to obtain a single phase then placed at 30°C overnight. This condensation procedure was repeated a further twice before storing the final detergent phase as 'precondensed Triton X-114' (PT-114). This solution was 15% in Triton X-114 as determined spectrophotometrically at 275 nm.

#### M.13B - MEMBRANE SAMPLE TREATMENT

All procedures, except where stated, were performed on ice. The starting membrane material was usually in the form of a membrane pellet containing 50 - 200  $\mu$ g protein. To the pellet was added 200  $\mu$ l 10 mM tris-HCl (pH 7.4), 0.4 M KCl then the pellet was roughly resuspended using a hypodermic syringe needle.

30  $\mu$ g PT-114 was added to the membrane suspension and the sample was then sonicated for 10 seconds using a Dawe - soniprobe, type 7530A, sonicator fitted with a microprobe. During sonication the fragmentation of large membrane aggregates could be observed. The sonicated sample was then allowed to stand for 30 minutes before centrifuging for 10 minutes at 4°C in an Eppendorf microfuge to pellet unsolubilized material. The supernatant from this step was then layered onto a sucrose cushion composed of:

10 mM tris-HCl (pH 7.4)

150 mM potassium chloride

6% (w/v) sucrose

~ 0.075% Triton X-114

The sucrose cushion was usually 300  $\mu$ l in volume, however, at this stage duplicate samples could be combined in which case two 200  $\mu$ l samples were layered onto a single 500  $\mu$ l cushion. An incubation period of 3 minutes at 30<sup>o</sup>C followed during which time clouding of the sample layer could be observed as the Triton solution separated into two phases. The Triton was centrifuged through the sucrose cushion, in a microfuge, for 10 seconds at room temperature. The sample layer was then removed and to it was added 20  $\mu$ l (per 200  $\mu$ l sample volume) PT-114. After mixing and cooling, to form one phase, the sample was again layered onto the original sucrose cushion from which it had been removed. After allowing phase separation at 30<sup>o</sup>C for 3 minutes the sample was again centrifuged for 10 seconds at room temperature, thus pooling the first and second Triton extracts, as an oily droplet, at the bottom of the sample tube.

The sample layer was again removed and given a final "Triton wash" by adding 50  $\mu$ l PT-114, cooling, mixing, placing at 30<sup>o</sup>C for 3 minutes and centrifuging for 1 minute at room temperature. The supernatant, above the Triton droplet, was then processed for SDS-PAGE after TCA precipitation (M.7).

The sucrose solution remaining above the Triton extract was aspirated and discarded. The Triton droplet was washed twice with 10 mM tris-HCl (pH 7.4), 0.4 M KCl and once with 10 mM tris-HCl only. A wash consisted of adding 600  $\mu$ l washing buffer to the Triton extract, mixing to disperse the droplet, then cooling to 0<sup>o</sup>C to form one phase. Following phase separation at 30<sup>o</sup>C for 3 minutes the Triton was harvested by centrifuging for 10 seconds.

The final Triton extract was then ready for use either as an antigen (see M.14) or in polypeptide analysis by SDS-PAGE. Samples to be analysed by SDS-PAGE were further processed by adding 1 ml of cold acetone and standing on ice for 30 minutes to precipitate the proteins. The proteins were pelleted by centrifugation in a microfuge at 4°C for 15 minutes and the supernatant, containing the Triton X-114, discarded. Residual acetone was evaporated at 56°C then the pellet taken up in SDS-PAGE sample buffer as described in M.7.

M.14 - PREPARATION OF ANTI-GLYOXYSOMAL MEMBRANE PROTEIN ANTISERUM

The glyoxysomal membranes from two sucrose gradients were Triton extracted as described above. The pooled Triton phases, containing approximately 50µg protein, were made up to 750µl with distilled water and mixed with an equal volume of complete Freund's Adjuvant. Emulsification was achieved by the passage of the adjuvant/antigen mixture between two 2 ml syringes. The emulsion was then injected intramuscularly into multiple sites on the hind legs of a lop-eared rabbit. A booster injection, containing a similar amount of protein but mixed with incomplete Freund's Adjuvant, was administered two weeks later. A final booster injection was given after a further two weeks and three days later the animal was bled from the ear.

The collected blood was allowed to clot overnight at 4°C then centrifuged at 2°C and 20,000 x g for 10 minutes in an MSE Hi-spin centrifuge.

The serum was removed using a Pasteur pipette and its volume measured. Keeping the serum on ice it was then made 50% saturated with ammonium sulphate to precipitate the immunoglobulins. The precipitated protein was harvested at 20,000 x g and 2°C for 15 minutes, then re-dissolved in a volume of 20 mM sodium phosphate buffer (pH 7.0) containing 0.15 M NaCl equal to half the original volume of serum. Ammonium sulphate was removed by extensive dialysis against 20 mM sodium phosphate, 0.15 M NaCl at 4°C.

The final antibody solution was divided into 1 ml aliquots and stored at -20°C until required.

Blood from the same rabbit, before it had received the injection of antigen, was collected and fractionated in the same manner as described above, to give pre-immune serum (null serum, NS).

#### M.15 - PROTEIN BLOTTING ONTO NITROCELLULOSE PAPER AND ANTIBODY SCREENING

Following SDS-PAGE (during which the dye-front had entered the resolving gel to a maximum of 15 cm) the area of the gel containing the proteins of interest was excised and placed on a "Scotchbrite" pad soaking in transfer buffer:

25 mM tris-HCl, pH 8.3'

192 mM glycine

20% (v/v) methanol

A piece of nitrocellulose filter (BA 85, pore size 0.45 μm) was cut to the exact same size as the gel, soaked in transfer buffer then placed over the gel. A second layer of Scotchbrite completed the "sandwich" which was then inserted into a Biorad Trans-Blot Cell, previously filled with transfer buffer, with the filter nearer the anode.

The cell was connected to a Biorad 160/1.6 power supply and protein transfer accomplished by applying a potential of 50V for 3 h.

Following transfer, the filter was soaked for 1 h in:

100 mM sodium phosphate buffer, pH 7.2

1.5 M sodium chloride

8% (w/v) BSA

to block any remaining protein binding sites. 200  $\mu$ l anti-glyoxysomal membrane protein antiserum (anti-GMP) was added and the filter incubated at room temperature overnight with continuous, gentle agitation. The filter was then washed 3 - 4 times with PBS (above) then soaked again in PBS -8% BSA to which was added  $10^6$  cpm [ $^{125}$ I] - protein A (prepared by Dr. S. Hemmingsen, University of Warwick). Incubation with iodinated protein A was for 2 h at room temperature and with agitation. The filter was washed free of unbound [ $^{125}$ I] with PBS containing 1% Triton X-100 (until the background radioactivity was reduced to 10 - 20 cpm, as determined using a hand radiation monitor).

The filter was air-dried before placing it in contact with X-ray film in an X-ray cassette lined with intensifying screens. The film was exposed to the gel for 1 - 5 days at room temperature before developing.

#### M.16 - IMMUNOHISTOCHEMICAL LOCALIZATION

The tissue processing procedure was exactly as described by Sautter and Hock (1982) which, in turn, was based on those of Baumgartner *et al.* (1980) and Tokuyasu and Singer (1976). Using castor bean endosperm tissue 3 days after germination, 1mm thick sections were cut by

hand and fixed in 0.25% (w/v) formaldehyde in 0.5 M potassium phosphate buffer (pH 7.0). Fixation was for 1 h at 20°C and under slight evacuation. The samples were then carried through a series of increasing sucrose concentrations (0.25, 0.5 and 1.0 M in phosphate buffer) allowing 30 minutes in each solution. The sections were mounted on strips of aluminium foil and frozen in melting nitrogen (solid nitrogen was obtained by placing liquid nitrogen under vacuum for about 5 minutes). The frozen specimens were placed in liquid nitrogen until required for sectioning.

Sectioning was performed using a Leitz Grundschlittenmikrotom. 40 $\mu$ m thick sections were cut using a knife angle of 2° and at a temperature of -30°C. The sections were removed from the knife using a paint brush and collected on the surface of a large droplet consisting of 1% (w/v) gelatin and 0.3% (w/v) agarose in 10 mM potassium phosphate buffer (pH 7.0).

Staining was performed at 20°C, precisely as described by Tokuyasu and Singer (1976). A wire loop (approximately 5 mm in diameter) was used to lift a section from the gelatin/agarose droplet and into phosphate buffered saline (PBS, 10 mM potassium phosphate, pH 7.0, 0.8% NaCl) for a few seconds. The section was removed, again using the wire loop, and 5 $\mu$ l of undiluted antiserum applied. The antiserum was spread over the section using one eyelash mounted on the end of a syringe needle. The section was allowed to incubate with the antiserum for 10 minutes in a humid atmosphere. This period was followed by two washing steps,

each of 2 minutes duration, the first in PBS containing 10 mM glycine (the latter serving to block any non-specific protein-binding sites), and the second in PBS only. The section was counterstained using goat anti-rabbit antiserum linked to fluoresceine isothiocyanate (FITC) following the same procedure used in the primary antibody step. Finally, the section was mounted in phosphate buffer, labelled side up and covered with a coverslip.

Sections were examined under a Zeiss Photomicroscope II, equipped with epifluorescence (filter combination 450 - 490, FT 510, LP 520). Micrographs were taken on Ilford HP5 film.

#### M.17 - IMMUNOPRECIPITATION USING PROTEIN A-SEPHAROSE

Protein A-Sepharose was hydrated in 10 mM tris-HCl (pH 7.5) containing 1% (v/v) Nonidet P40 (N-P40) and 150 mM NaCl. The pH was adjusted to 7.5 then the Sepharose beads sedimented in a bench-top centrifuge. The supernatant was replaced with a volume of 10 mM tris-HCl (pH 7.5), 1% N-P40, 150 mM NaCl equal to the volume occupied by the Sepharose beads. This 1 : 1 slurry was stored at 4°C.

Immunoprecipitation from in vitro synthesized protein mixtures was performed as follows:

The cell free translation mixture (usually replicate 20 $\mu$ l translations were pooled at this stage) was mixed

in a 1.5 ml Eppendorf tube, with an equal volume of buffer consisting of:-

1% (v/v) NP-40

10 mM tris-HCl (pH 7.5)

150 mM NaCl

40  $\mu$ g /ml phenylmethylsulfonyl fluoride (PMSF)

30 minutes, at 20°C, was allowed for the solubilization of membranes, if present, before the addition of 1  $\mu$ l of pre-immune serum and 50  $\mu$ l protein A-Sepharose slurry. The mixture was incubated at room temperature, with occasional shaking, for 30 minutes, then the Sepharose sedimented by a brief, 10 second, centrifugation in an Eppendorf, model 5414, centrifuge. The supernatant was transferred to a clean tube and the Sepharose pellet discarded. 2  $\mu$ l of specific antiserum was added to the supernatant which was then incubated at room temperature for 1 h. The immunoglobulin, with specifically bound antigen, was harvested using 50  $\mu$ l protein A-Sepharose. Following a 30 minute incubation at room temperature, to allow the immunoglobulin to bind to the protein A, the Sepharose beads were collected by centrifugation and the supernatant discarded. The beads were then washed three times with:

20 mM tris (pH 7.4)

0.15 M sodium chloride

2 mM EDTA

0.2% NP-40

then twice with:

20 mM tris (pH 7.4)

0.5 M sodium chloride

2 mM EDTA

0.2% NP-40

and finally once with 10 mM tris (pH 7.4) only.

The beads were collected between each wash by a short centrifugation (10 seconds) with a longer centrifugation of 30 seconds following the final wash, after which 30 - 50  $\mu$ l PAGE sample buffer (M.7) was added directly to the Sepharose and the sample processed as described previously for gel electrophoresis.

Immunoprecipitation from in vivo labelled (with [<sup>35</sup>S] methionine) membrane samples was essentially the same as for the in vitro samples. Membranes were solubilized by the addition of an equal volume of 1% NP-40 buffer, containing PMSF, and incubated for 1 h at room temperature with occasional vortexing. Non-specific antibody-bound proteins were removed using 3 - 5  $\mu$ l pre-immune serum and 2 - 3  $\mu$ l specific antiserum was used for immunoprecipitation. All other procedures were as described for in vitro samples.

#### M.18 - ENZYME-LINKED IMMUNOSORBANT ASSAY (ELISA)

Monoclonal antibodies to Triton X-114 extracts of glyoxysomal membranes were raised in BALB/c mice by S. Varden (University of Bradford). Antibody-containing hybridoma supernatants from a cell-line, A6<sub>3</sub>, were used to probe for the presence of "A6<sub>3</sub> antigen" in 1 ml fractions from a normal 38 ml sucrose gradient. Each fraction was diluted with 4 ml 50 mM tris-HCl (pH 7.5) and centrifuged at 100,000 x g and 2°C for 1 h to pellet membranes. The pellets were re-suspended by brief (10 second) sonication in 1.2 ml ELISA coating buffer:

0.05 M sodium carbonate/bicarbonate (pH 9.6)

0.02% sodium azide

Following protein determinations, aliquots of each fraction were diluted to give a protein concentration of 10  $\mu$ g/ml and 100  $\mu$ l of each fraction were added, in duplicate,

to the wells of GIBCO-ELISA plates. After allowing 16 h at 4°C for the binding of protein to the plate wells, the wells were washed three times by flooding with PBS-Tween:

0.02 M sodium phosphate buffer (pH 7.4)

0.15 M sodium chloride

0.05% Tween-20

50  $\mu$ l of undiluted A6<sub>3</sub> supernatant was then added to each well and the plates incubated for 2 h at room temperature. The wells were again washed three times with PBS-Tween before the addition of 30  $\mu$ l alkaline phosphatase - rabbit anti-mouse Ig G conjugate (Sigma) which had been diluted 1 in 2,000 with PBS-Tween. Following an overnight (16 h) incubation at 4°C the wells were emptied and washed with PBS-Tween. Meanwhile the substrate solution was prepared by dissolving two 5 mg Sigma 104 phosphatase substrate tablets (containing p-nitrophenyl phosphate) in 10 ml diethanolamine buffer:

9.7% (v/v) diethanolamine-HCl (pH 9.8)

0.5 mM magnesium chloride

0.02 (w/v) sodium azide

100  $\mu$ l substrate solution was then added to each well and the reactions allowed to proceed for exactly 60 minutes before their termination by the addition of 50  $\mu$ l 3 M NaOH.

The intensity of the yellow reaction product, which is directly related to the amount of monoclonal antibody bound, was assessed by measuring the extinction at 410 nm using a Dynatech ELISA minireader, model MR590.

M.19 - ELECTRON MICROSCOPY

19A - ORGANELLE-CONTAINING GRADIENT FRACTIONS

Organelles were isolated from 80 germinating castor bean endosperm halves on six 38 ml sucrose gradients. Bands containing microsomes, mitochondria and glyoxysomes were collected and, in addition, 2 ml samples were removed from the regions of the gradients between the microsomal and mitochondrial bands (the e/m region) and between the mitochondrial and glyoxysomal bands (the m/g region). All fractions were diluted <sup>6-fold</sup> with 0.1 M sodium cacodylate buffer (pH 7.2) containing 5% (v/v) glutaraldehyde and sucrose, the concentration of which varied with the fraction being diluted i.e. 25% (w/w) sucrose in the case of the microsomal fraction, 45% sucrose in the cases of the e/m and mitochondrial fractions and 54% sucrose in the cases of the m/g and glyoxysomal fractions.

Glutaraldehyde fixation was for 4 h at 4°C (as recommended by Vigil, 1983) before diluting the fractions with 0.1 M cacodylate buffer (pH 7.2) to make the microsomal fraction 20% with respect to sucrose, the e/m and mitochondrial fractions 35% and the m/g and glyoxysomal fractions 40%. The suspensions were then centrifuged at 75,000 x g for 1 h to pellet organelles and membranes, Osmium fixation of the pellets was achieved by adding 1 - 2 ml of a 2% solution of osmium tetroxide in 0.05 M sodium cacodylate buffer containing 40% (w/w) sucrose and incubating for 2 h at 0°C in the dark. The pellets were washed three times (2 minutes each) with sucrose-free, 100 mM cacodylate buffer then incubated with a 0.5% (w/v) solution of uranyl acetate

in water for 20 minutes at room temperature, again in the dark. The pellets were fragmented by repeated pipetting with a Pasteur pipette then pieces less than  $1 \text{ mm}^3$  in size were dehydrated through 30, 50, 70, 80, 90 and 100% ethanol at room temperature, allowing 30 - 60 minutes in each solution. Final dehydration was achieved through 3 changes of 100% ethanol (30 minutes per change), then the sample fragments were transferred to Bijoux bottles containing a 1 : 1 mixture of ethanol/Spurr's epoxy resin. The Spurr's mixture comprised:

10 g ERL - 4206

4 g DER - 736

26 g NBA

0.4 g (exactly) DMAE, S-1

The samples were rotated continuously at 1.5 rpm for 1 h then the 1 : 1 mixture exchanged for a 1 : 2 mixture of ethanol/Spurr's resin. After a further hour, the 1 : 2 mixture was exchanged for 100% resin which was changed every 12 h for 3 days before transferring individual sample fragments to BEEM (type 00) embedding capsules. The capsules were filled with resin which was then hardened at  $80^\circ\text{C}$  for 3 days.

Silver sections (70 nm - 30 nm) were cut on a Reichert ultramicrotome using a diamond knife at a cutting angle of  $7^\circ$ . The sections were mounted onto 3.05 mm copper grids (type old 200) and viewed using a Jeol JEM - 100S electron microscope.

#### M.19B - INTACT ENDOSPERM TISSUE

3 - 4 day old castor bean endosperm tissue was excised and cut into cubes of  $1 \text{ mm}^3$  or less.

The cubes were fixed for 2.5 - 3 h at room temperature in a 2% solution of glutaraldehyde in 200 mM sodium phosphate buffer (pH 7.0). The tissue was then washed with 200 mM phosphate buffer for 1 minute and covered with a 2% solution of osmium tetroxide in 50 mM sodium cacodylate buffer (pH 7.2). Osmium fixation was performed overnight at 0 - 2°C in the dark.

Dehydration, infiltration with Spurr's mixture, embedding and sectioning were performed as with the organelle/membrane samples.

RESULTS I

R. 1 - SUBCELLULAR FRACTIONATION AND DETERMINATION OF FRACTION PURITY

R. 1A - CELLULAR FRACTIONATION ON SUCROSE DENSITY GRADIENTS

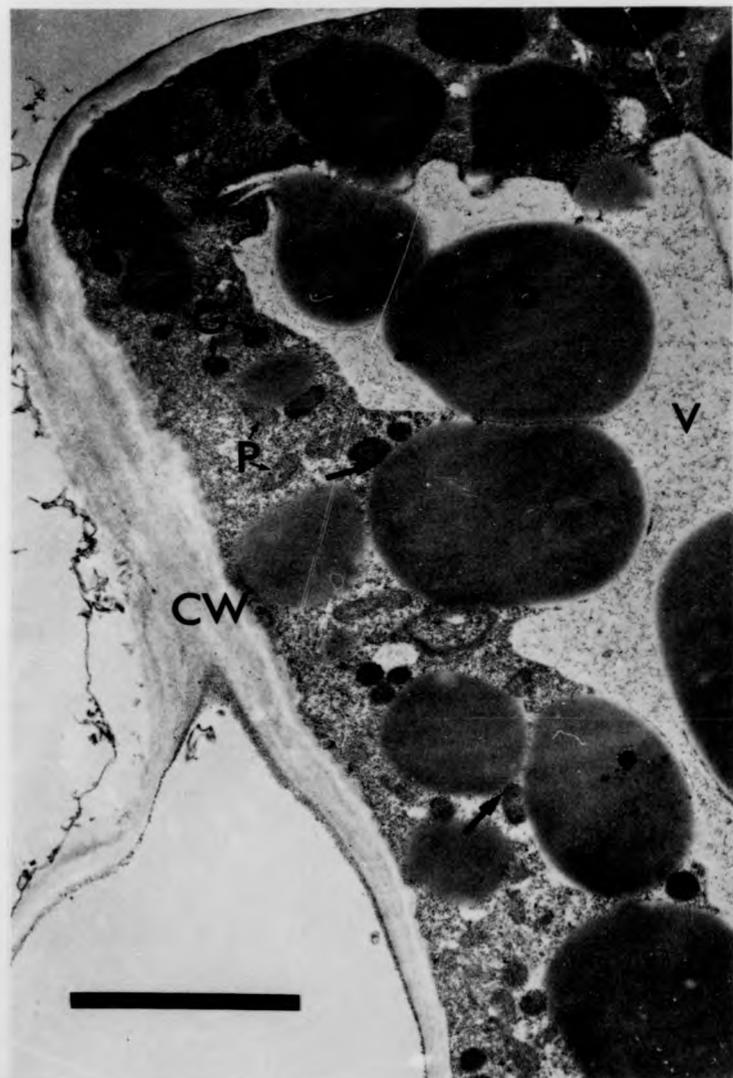
Fig. R1.1 shows the subcellular morphology of a typical castor bean endosperm cell 4 days post imbibition. The cell is characterized by a large central vacuole (V) occupying most of the cellular volume. The cytoplasm is restricted to the region of the cell 3 - 5  $\mu$ m from the plasma membrane/cell wall (CW) and is seen to contain various subcellular structures, the most prominent being the lipid-filled spherosomes (S). Often in close approximation to the spherosomes (fig. R1.1 arrows) can be seen the electron-dense glyoxysomes (G) with their crystalline-core structure visible in some cases. The granular appearance of the cytoplasmic matrix is due, in part, to an abundance of rough endoplasmic reticulum. The lighter-staining organelles of more variable size and shape are plastids (P). Mitochondria are not obviously distinguishable at this magnification (x 2000) and under these staining conditions. Tissue at this stage of germination was fractionated on sucrose gradients by the method of Kagawa *et al.* (1973) with slight modifications. These modifications consisted of the inclusion of 100 mM lactose in the tissue homogenization medium and an increase (from 5 ml to 10 ml) in the volume of 20% sucrose between the top of the sucrose gradient and the sample layer. A comparison of the appearance of the original and modified gradients together with the distribution of organelle marker-enzymes throughout the modified gradient is shown in fig. R1.2. The enzyme profiles indicate that

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E.M. of  
Fig R1.1  $\lambda$  Castor Bean Endosperm Cell Four Days  
Post-Imbibition

Tissue was prepared for electron microscopy as detailed in M.19B (p. 75). The larger arrows indicate tight junctions between glyoxysomes (G) and spherosomes (S). Other abbreviations used: CW, cell wall; P, plastids; V, vacuole. x 2000 (Bar = 5  $\mu$ m).

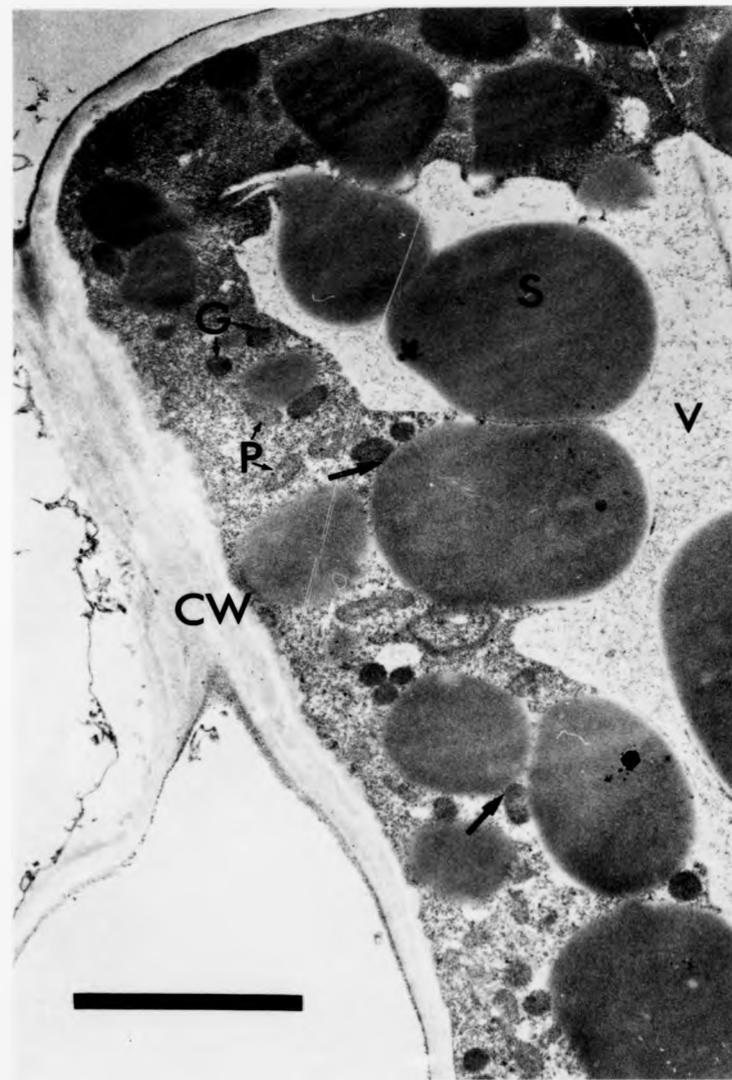


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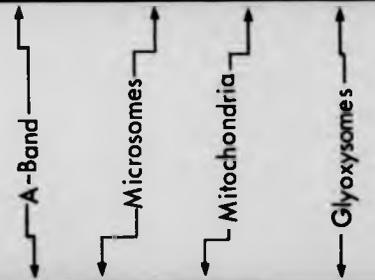
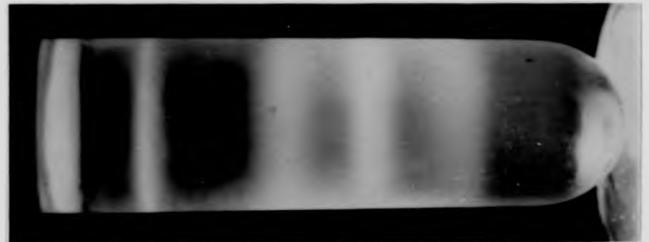
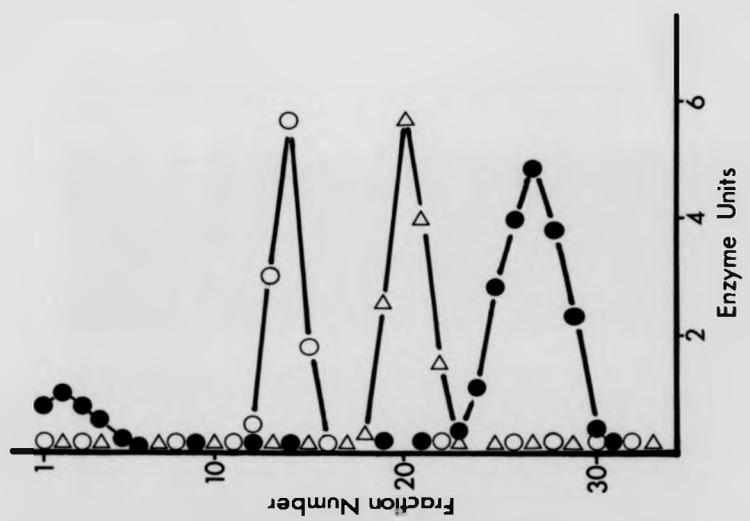


Fig. R1.2 Castor Bean Endosperm Tissue Fractionation on Sucrose Density Gradients

Gradients were loaded with the post 500 x g supernatant from 12 - 15 endosperm halves excised from 4-day-old seedlings. The gradient on the left was prepared as described by Lord *et al.* (1972) and that on the right had the 20% (w/w) sucrose step (see M2.A, p. 43) increased from 5 ml to 10 ml and included 100 mM lactose in the homogenization buffer. Organelle marker enzyme distributions throughout the modified gradient are shown, extreme right. Enzymes assayed were: cholinephosphotransferase (O), nmol/h. fraction x 20; fumarase (Δ), μmol/min. fraction x 10 and isocitrate lyase (●) μmol/min. fraction x 20.

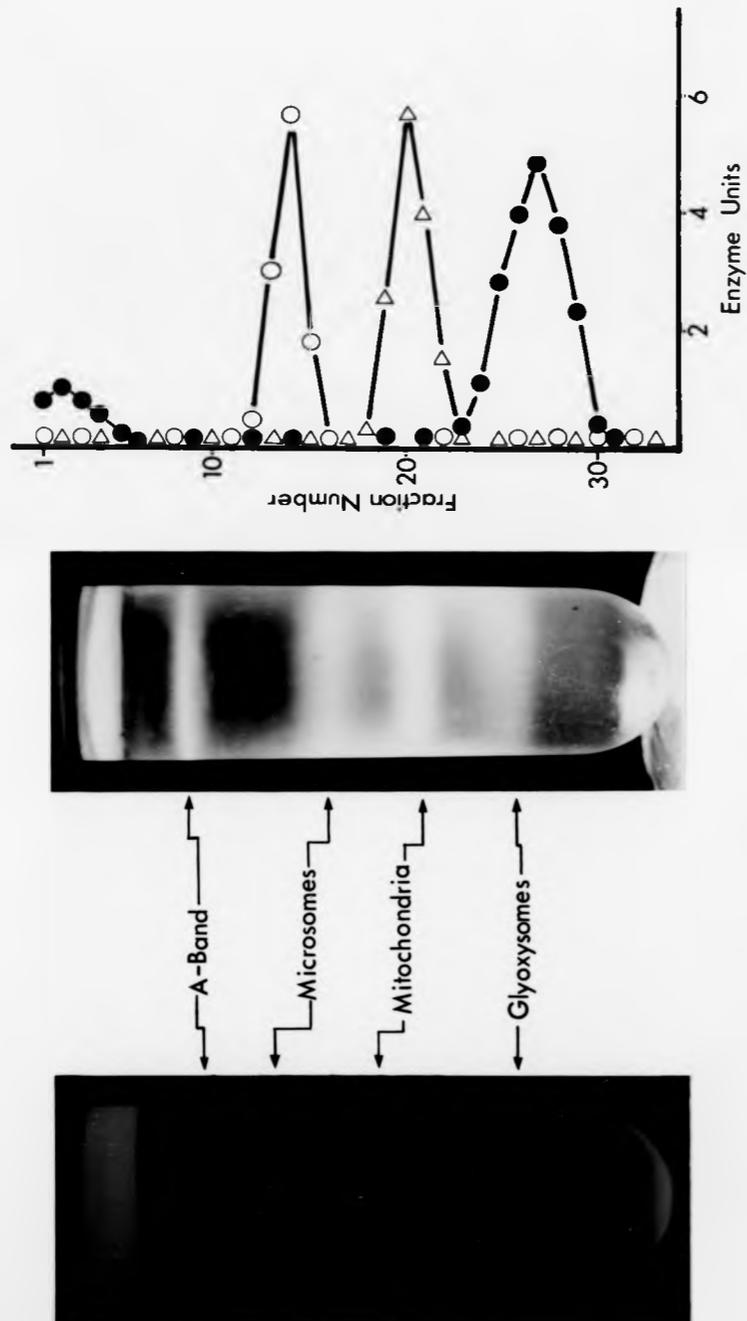


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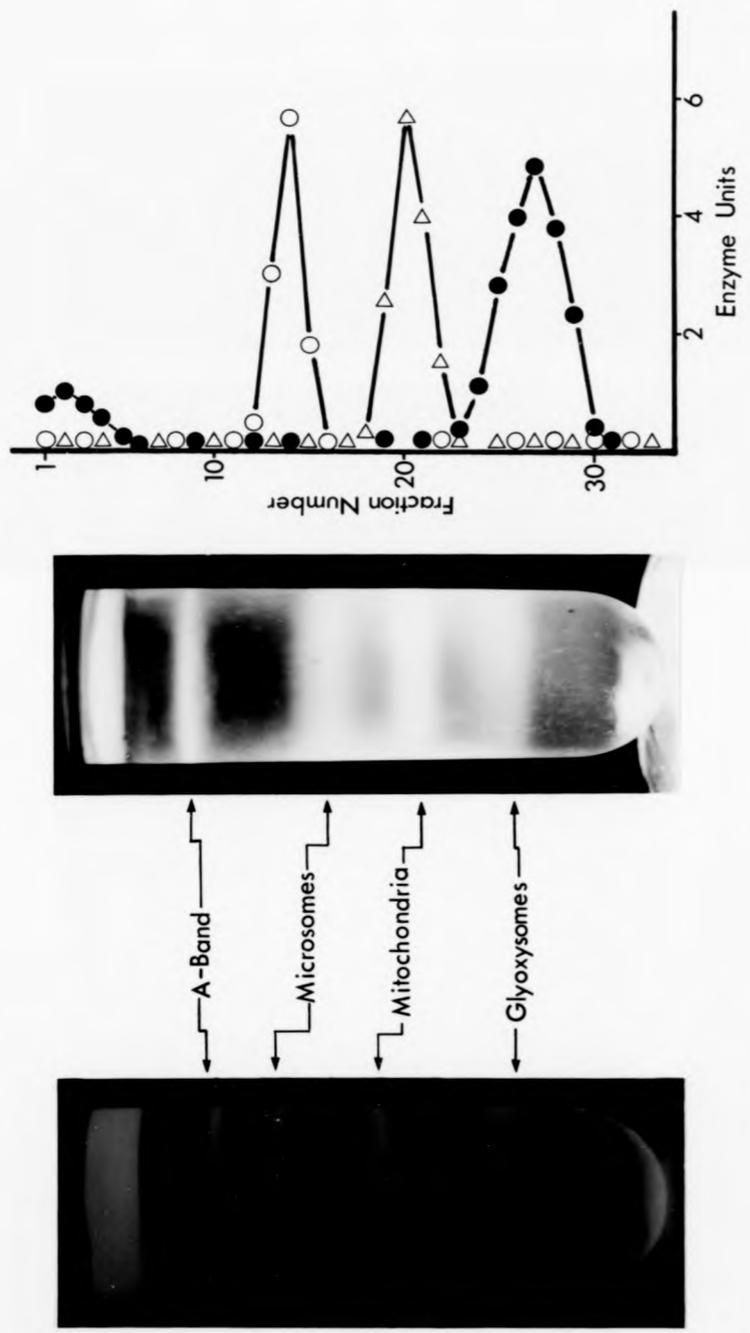


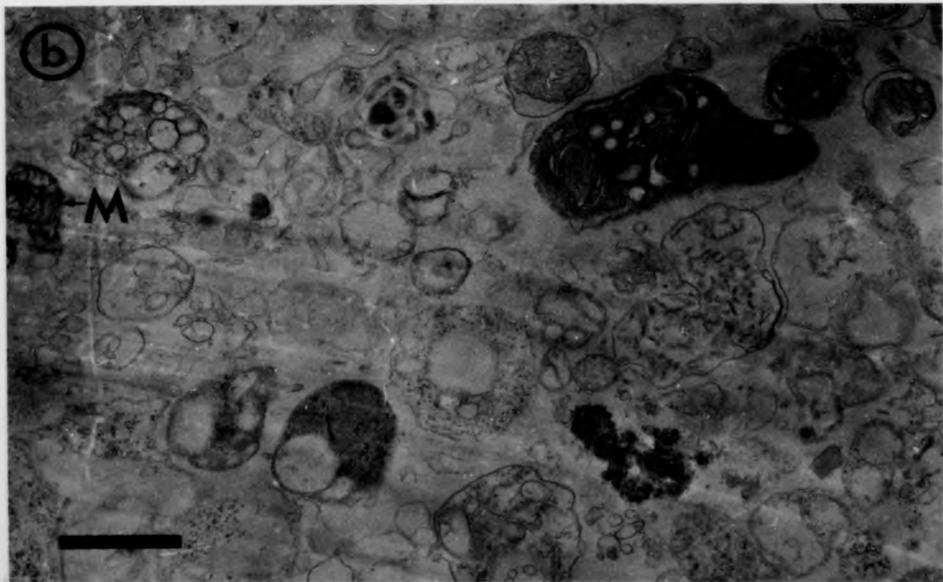
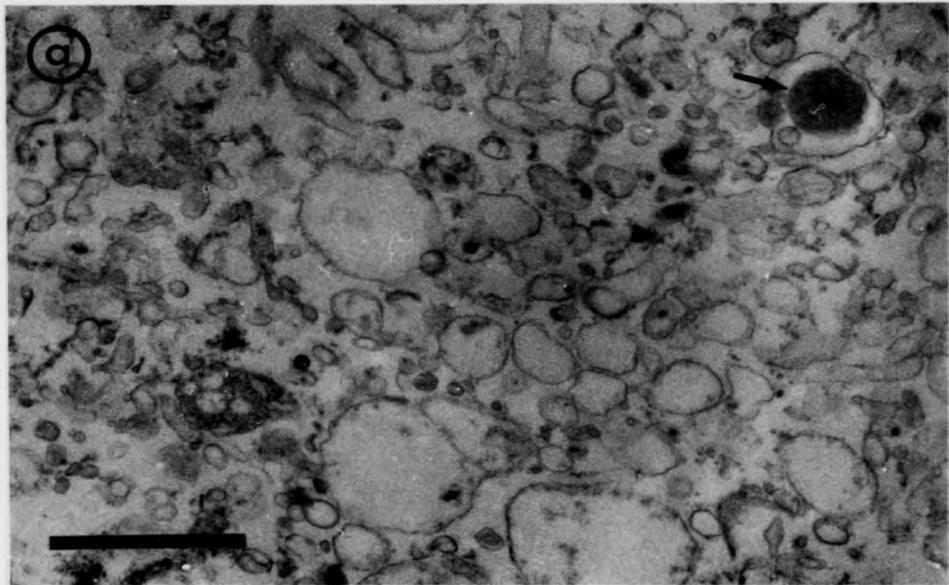
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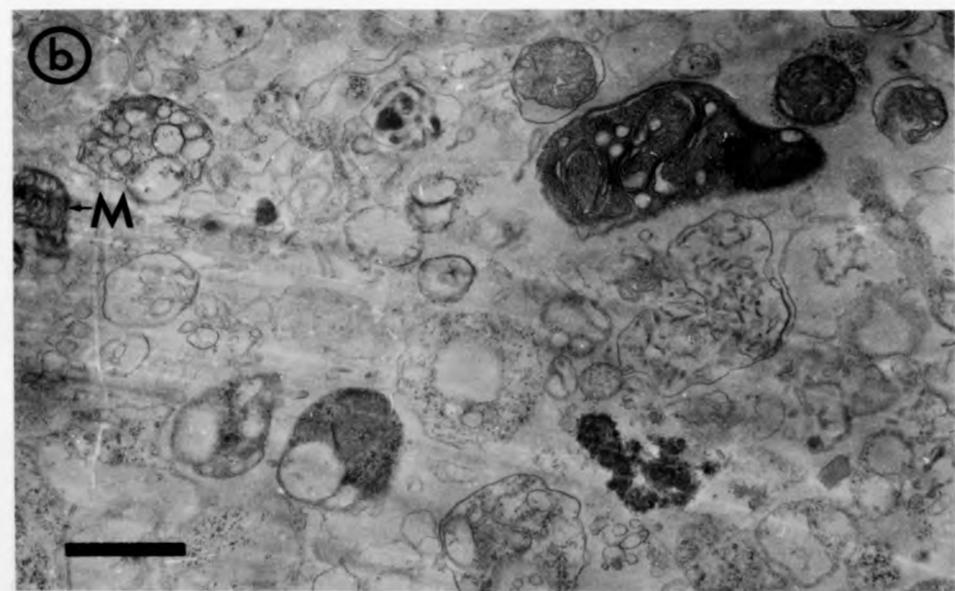
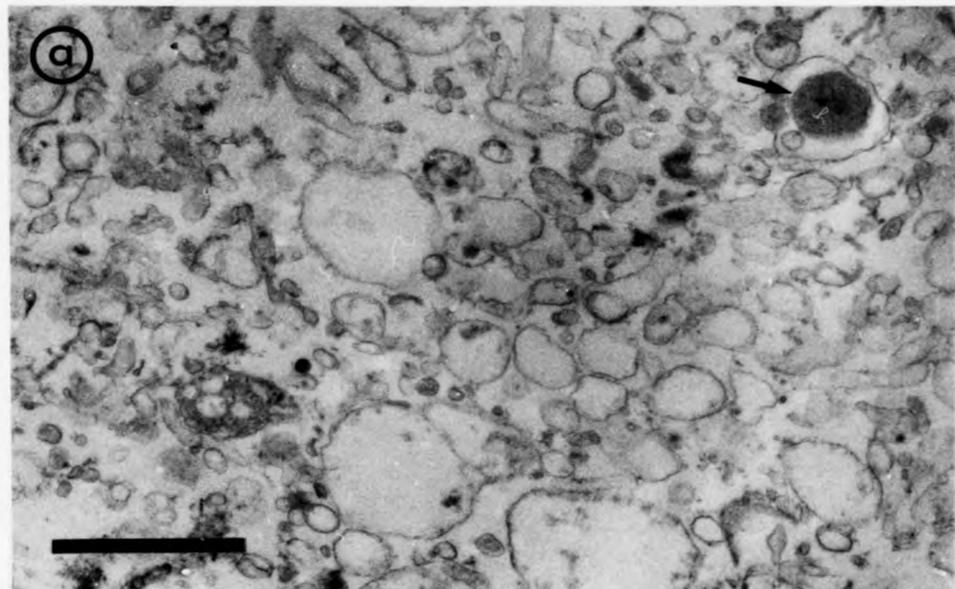
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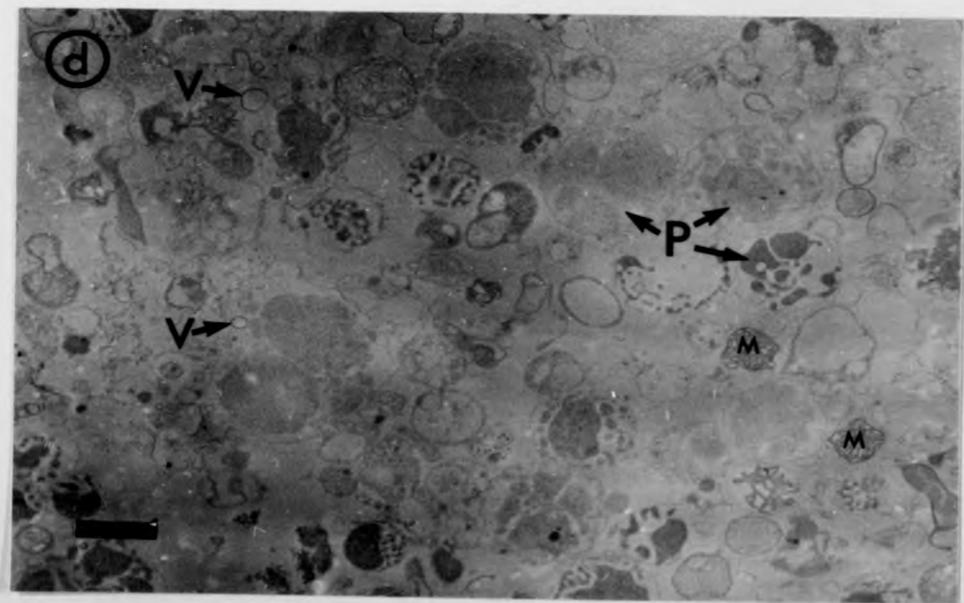
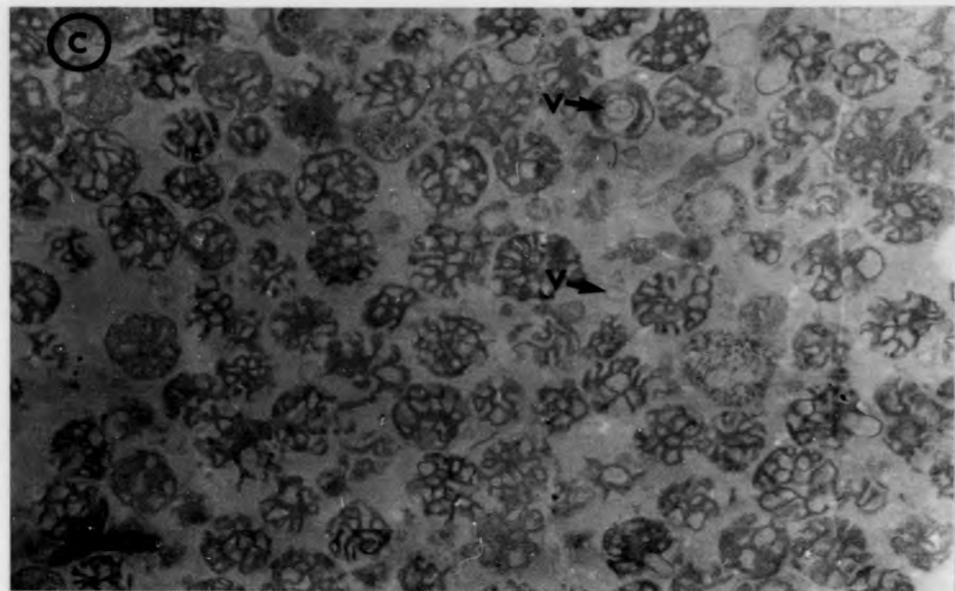
endoplasmic reticulum, intact mitochondria and intact glyoxysomes are well separated. Enzyme activity measurements at the top of the gradient indicate that although there appears to be negligible breakage of the mitochondria, approximately 20% of the total ICL activity has been released from the glyoxysomes (assuming endogenous cytosolic ICL levels to be negligible) presumably by breakage during the homogenization step.

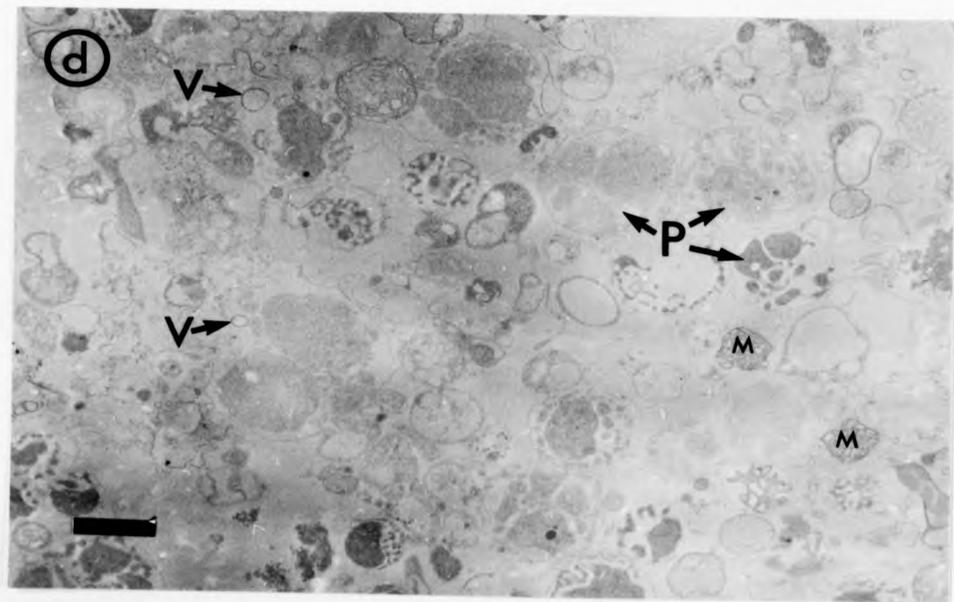
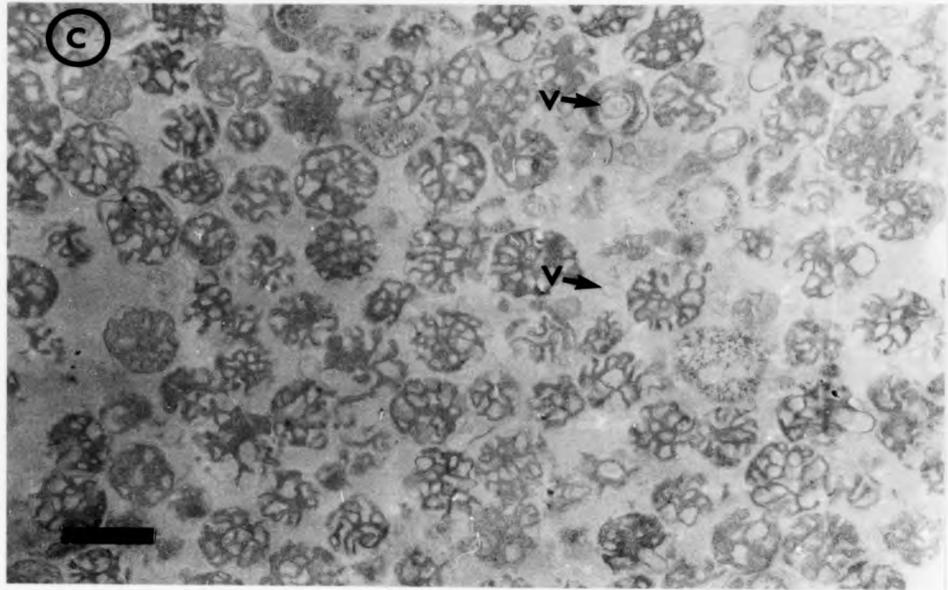
R.1B - ELECTRON MICROSCOPY OF ORGANELLE FRACTIONS

Samples from the three major organelle-containing regions of a sucrose gradient together with samples from the regions intermediate between the e.r. and mitochondria (the e/m region) and between the mitochondria and glyoxysomes (the m/g region) were processed for examination under the electron microscope. Fig. R1.3 (a - e) shows representative samples from these five fractions. The microsomal fraction (fig. R1.3a) is composed of membrane vesicles varying in size from less than  $0.1\mu\text{m}$  to  $1.0\mu\text{m}$  in diameter. A few electron-dense particles are visible, one (arrowed) is enclosed within a membrane vesicle. The origin of these particles is not known. The e/m region (fig. R1.3b) contains an assortment of membrane vesicles. In addition to vesicles resembling those found in the microsomal fraction there are mitochondria (M) and plastids (P), the latter distinguishable by the granular appearance of the matrix and the highly organized internal membrane structure. Fig. R1.3c shows the mitochondrial fraction to be very pure though again small (approximately  $0.1\mu\text{m}$ ) vesicles (arrowed) are present. The greatest variety of subcellular structures









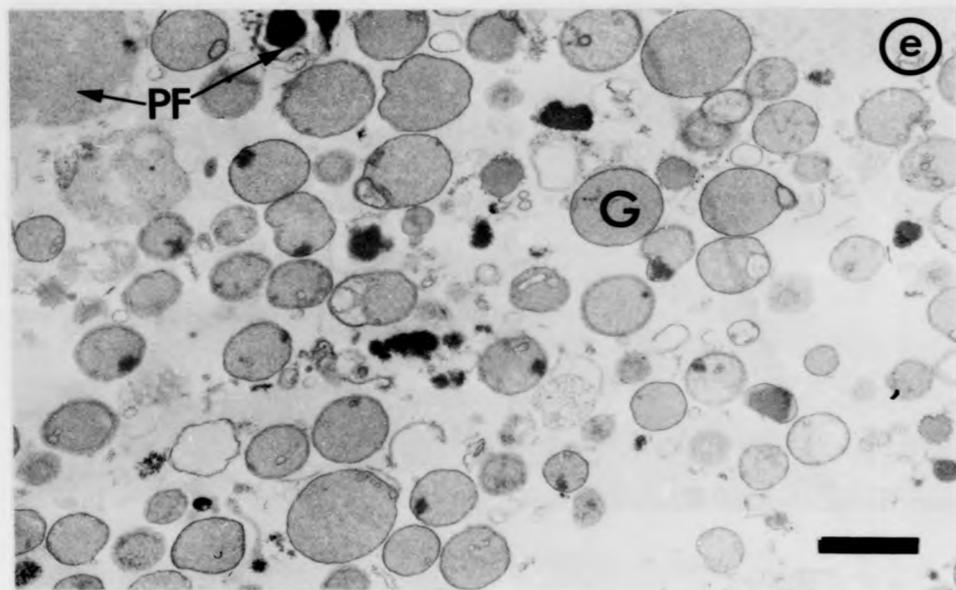


Fig. R1.3 Electron Microscopy of Organelle Fractions from Sucrose Gradients

Organelles were isolated on sucrose gradients and prepared for electron microscopy as detailed in M19.A (p. 74). Sections were post-stained with uranyl acetate: (a) microsomal fraction, arrow indicates membrane-bound electron dense particle (x 10,000); (b) gradient fractions intermediate between microsomes and mitochondria showing mitochondrion (M) and plastid (P) (x 8,000); (c) mitochondria with contaminating vesicles (V) (x 6,000); (d) gradient fractions intermediate between mitochondria and glyoxysomes. Plastids (P) and mitochondria (M) are seen together with vesicles (V) of unknown origin (x 6,000); (e) glyoxysomes (G), showing slight plastid (PF) contamination. (Bar, in all cases, = 1  $\mu$ m).

are seen in the m/g fraction (fig R1.3d). In addition to mitochondria there is an abundance of residual plastids which avoided sedimentation during the 500 x g centrifugation step. Small membrane vesicles of unknown, but possibly glyoxysomal (see later), origin are features of this fraction also. Finally, fig. R1.3e shows the composition of the glyoxysomal fraction to be highly pure glyoxysomes. The micrograph shown was chosen to illustrate the slight contamination by plastid fragments (PF). Mitochondria were found to be entirely absent from this fraction and smaller vesicles are in much lower abundance than in the m/g fraction. It should be noted that the glyoxysomes are virtually all intact with only a few examples of "ghosts", organelles which have completely lost their matrix contents.

#### R.1C - RECENTRIFUGATION OF CELLULAR SUBFRACTIONS

Following the labelling of intact tissue with either a [ $^{14}\text{C}$ ]-amino acid mixture or [ $^{14}\text{C}$ ]-choline and cellular fractionation, 1 ml gradient fractions were collected. The distribution of [ $^{14}\text{C}$ ] phosphatidyl choline throughout a gradient is shown in fig. R1.4. In keeping with the e.r. being the site of phospholipid synthesis (Lord *et al.*, 1973) approximately 80% of the phospholipid [ $^{14}\text{C}$ ]-choline is associated with this fraction. Much of the remainder is found in the mitochondrial fractions while the glyoxysomal region is labelled only to a very low extent.

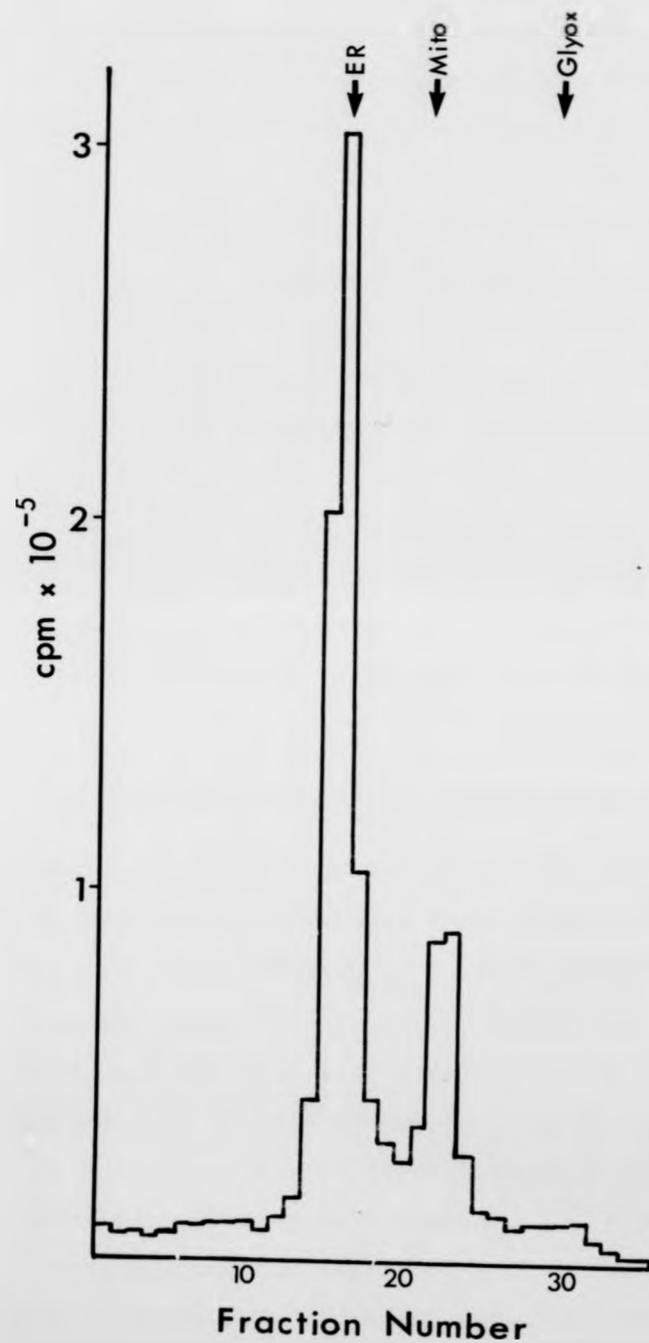
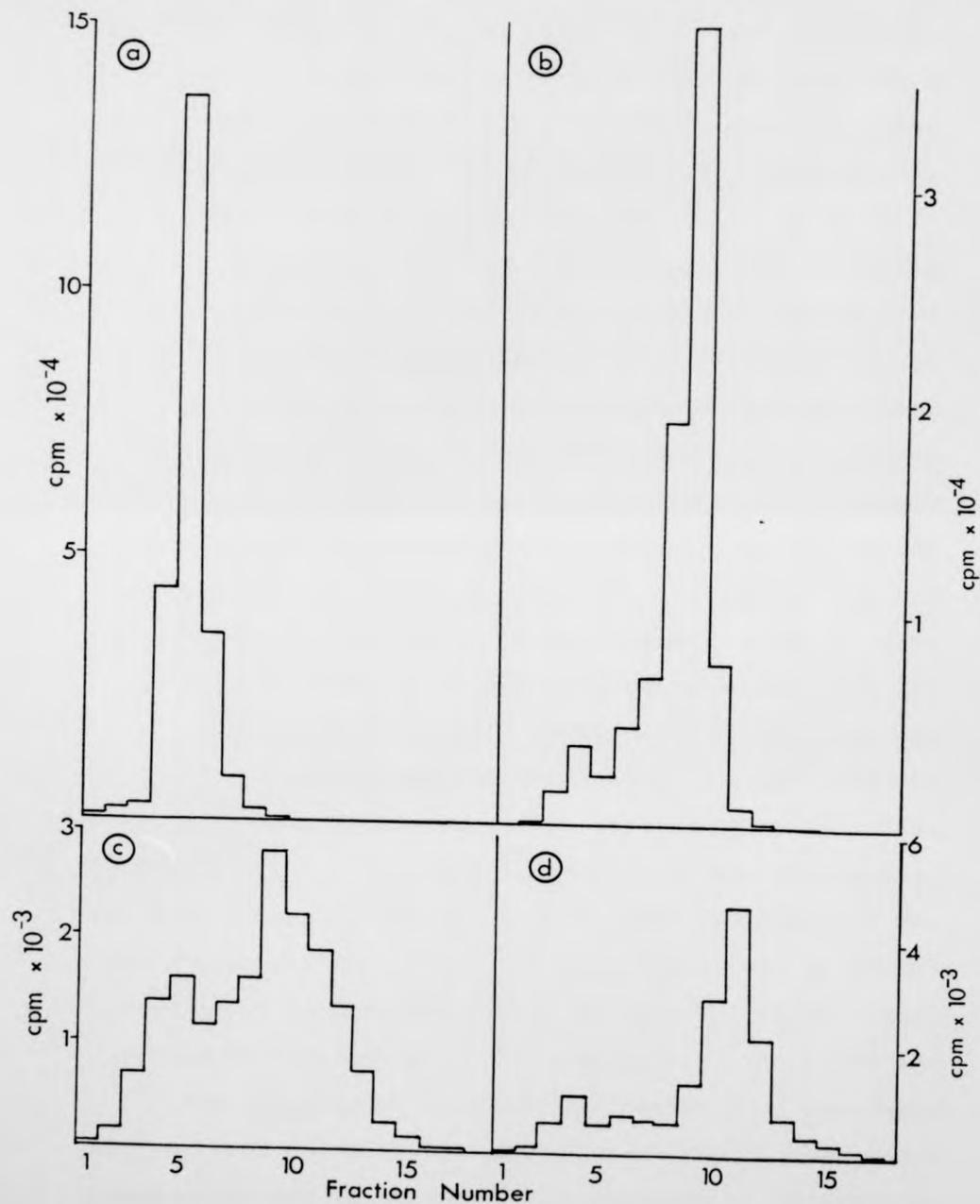


Fig. R1.4 Distribution of Newly Synthesized Phosphatidyl - choline Between Subcellular Fractions of Castor Bean Endosperm

Ten 3-day-old endosperm halves were each incubated with 10  $\mu$ Ci [ $^{14}$ C] choline for 18 h at 20°C before fractionating on a sucrose density gradient. Phospholipid was extracted from 100  $\mu$ l aliquots of each 1 ml fraction as detailed in the assay of cholinephosphotransferase (M.4F, P.47) and the associated radioactivity, as phosphatidyl- [ $^{14}$ C] choline, determined by liquid scintillation counting.

Individual fractions accounting for the microsomal, mitochondrial and glyoxysomal peaks were separately pooled, diluted with an equivalent volume of tris-buffer and the organelles pelleted by centrifugation at 100,000 x g. The pellets were resuspended in 2.5 ml tris-buffer and recentrifuged into 15 ml linear (20 - 60%) sucrose gradients. These gradients were fractionated and the radioactive phospholipid associated with each fraction extracted. The distribution of [ $^{14}\text{C}$ ]-phospholipid throughout gradients loaded with resuspended microsomes, mitochondria, glyoxysomes and the membranes of the m/g region of the original gradient is shown in fig. R1.5 (a - d). The microsomal fraction is again seen to band quite tightly in the 20 - 30% sucrose region of the gradient (fig. R1.5a). The mitochondria also retain essentially the same buoyancy characteristics as those applied to gradients immediately after tissue homogenization, although there is detectable radioactivity in fractions 4 and 5, the "microsomal fractions" (fig. R1.5b). Dilution of the mitochondrial fraction (in approximately 40% w/w sucrose) with an equal volume of buffer does not result in any organelle disruption. The m/g associated membranes re-equilibrate around fraction 11 (just below the mitochondria) though there is a significant amount recovered in fractions 10 and 4, mitochondria- and microsome-containing fractions respectively (fig. R1.5c). In contrast to mitochondria, dilution of the glyoxysomal fraction inevitably results in the osmotic disruption of the organelles and the loss of most of their matrix protein content. This disruption is reflected in fig. R1.5d which shows the glyoxysome-derived membranes





**Fig. R1.5** Recentrifugation of [<sup>3</sup>H] Choline-Labelled Membranes

Organelles were isolated from tissue labelled with [<sup>14</sup>C] choline (see fig. R1.4). The major organelle-containing peak fractions plus 2 ml from the region of the gradient intermediate between the mitochondria and glyoxysomes (m/g) were diluted with 50 mM Tris-HCl (pH 7.4) and the membranes harvested by centrifugation. The membrane-pellets were resuspended in 50 mM Tris-HCl and overlaid onto 15 ml linear 60 - 20% (w/w) sucrose gradients and centrifuged at 24,000 r.p.m and 2°C in a Beckman SW 27 rotor. The gradients were fractionated into 1 ml fractions and the phospholipids extracted as detailed in M.4E (p. 47). The distributions of radioactivity through the gradients loaded with phosphatidyl- [<sup>14</sup>C] choline-labelled microsomes (a); mitochondria (b); m/g-membranes (c); glyoxysomes (d) are shown.

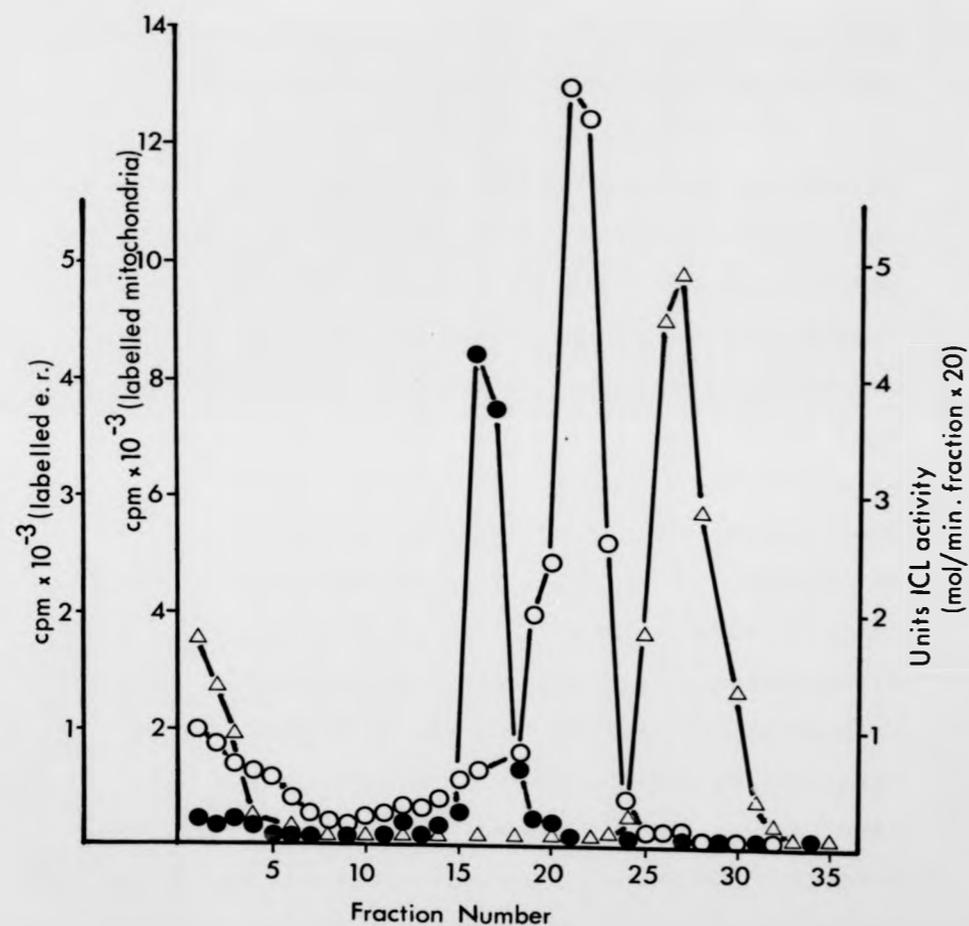
to smear throughout the second gradient but to peak in the microsomal (fractions 4 and 5) and mitochondrial (fractions 8 - 11) regions. Contamination of these two fractions with glyoxysomal membranes must therefore be assumed to occur, though probably to a lower degree when precautions are taken to minimise osmotic effects, as is the case during routine tissue fractionation.

The fractions containing [ $^{14}\text{C}$ ]-amino acid-labelled microsomes and mitochondria were gently diluted with 2.5x concentrated homogenization medium, from which sucrose had been omitted, and the diluted organelle suspensions used as homogenization buffer for two sets of 10 unlabelled endosperm halves. The resulting homogenates were centrifuged into normal sucrose gradients which were then fractionated. Each fraction was TCA precipitated and the associated radioactivity determined. The distribution of radioactivity down the two gradients is shown in fig. R1.6 together with the distribution of ICL throughout an identical gradient. A very slight cross-contamination of the microsomal fractions by mitochondria is revealed. Most importantly, the glyoxysomal region of the gradient (indicated by the ICL peak between fractions 26 and 30) is essentially free from contamination by the other two major organelle fractions.

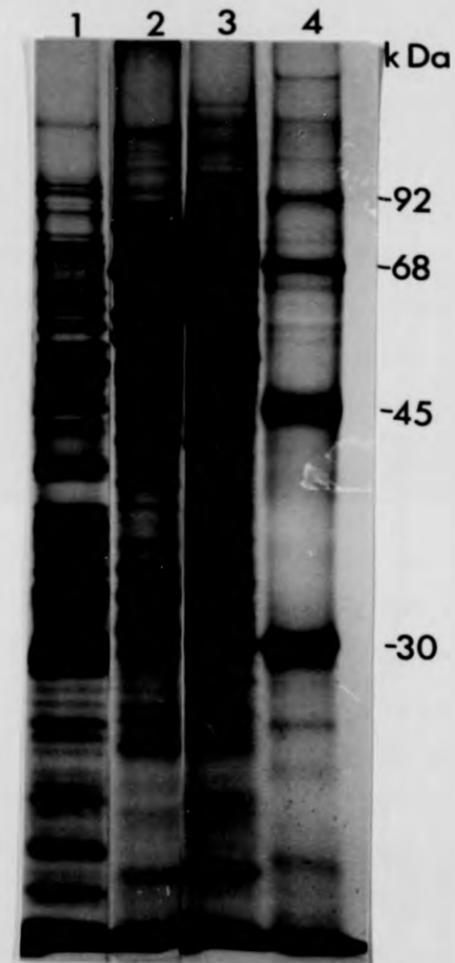
#### R.1D - POLYPEPTIDE PROFILES OF SUBCELLULAR FRACTIONS

The differences in polypeptide composition between 0.2 M KCl-washed [ $^{35}\text{S}$ ]-methionine labelled microsomal, mitochondrial and glyoxysomal membranes is shown in fig. R1.7. This illustration, however, represents only newly synthesized proteins and so does not show any of

Fig. R1.6 Recentrifugation of [<sup>14</sup>C] amino acid-labelled Microsomes and Mitochondria



Ten 3-day-old endosperm halves were incubated at 20°C for 18 h after adding 2 μCi [<sup>14</sup>C] amino acid mixture to each half. [<sup>14</sup>C]-labelled microsomes and mitochondria were isolated on a sucrose gradient as described in M2.A (p. 43). Diluted aliquots of the labelled organelle-suspensions were used as homogenisation media for batches of unlabelled endosperm tissue. The resulting 5 ml samples were recentrifuged into normal 38 ml gradients (M2.A). After centrifugation the gradients were fractionated into 1 ml fractions and the TCA-insoluble radioactivity associated with each fraction determined. The distribution of [<sup>14</sup>C] amino acid-labelled microsomes (●) and mitochondria (O) are shown together with the distribution of isocitrate lyase (Δ) throughout an identical gradient loaded with unlabelled endosperm homogenate.



**Fig. R1.7 Polypeptide Profiles of In Vivo [<sup>35</sup>S] methionine-Labelled KCl-washed Membrane Preparations**

[<sup>35</sup>S] methionine-labelled endosperm tissue (M.6, p. 51) was fractionated on sucrose gradients. The major organelle-containing fractions were removed from the gradients and diluted with 50 mM Tris-HCl, 400 mM KCl. The membranes were harvested by centrifugation then resuspended in 50 mM Tris-HCl (pH 7.4). Aliquots were TCA precipitated and the TCA-insoluble radioactivity associated with each membrane determined. Volumes containing approximately 100,000 cpm were centrifuged to pellet the membranes and the pellets solubilized in SDS-PAGE sample buffer. The proteins were separated by gel electrophoresis and visualized by fluorography. Lane 1, mitochondrial membranes; lane 2, glyoxysomal membranes; lane 3, microsomal membranes; lane 4, molecular weight markers.

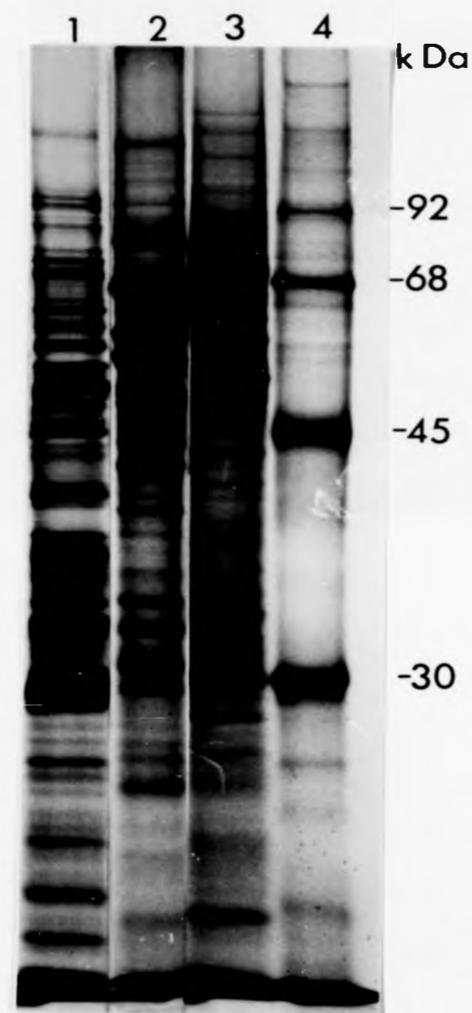
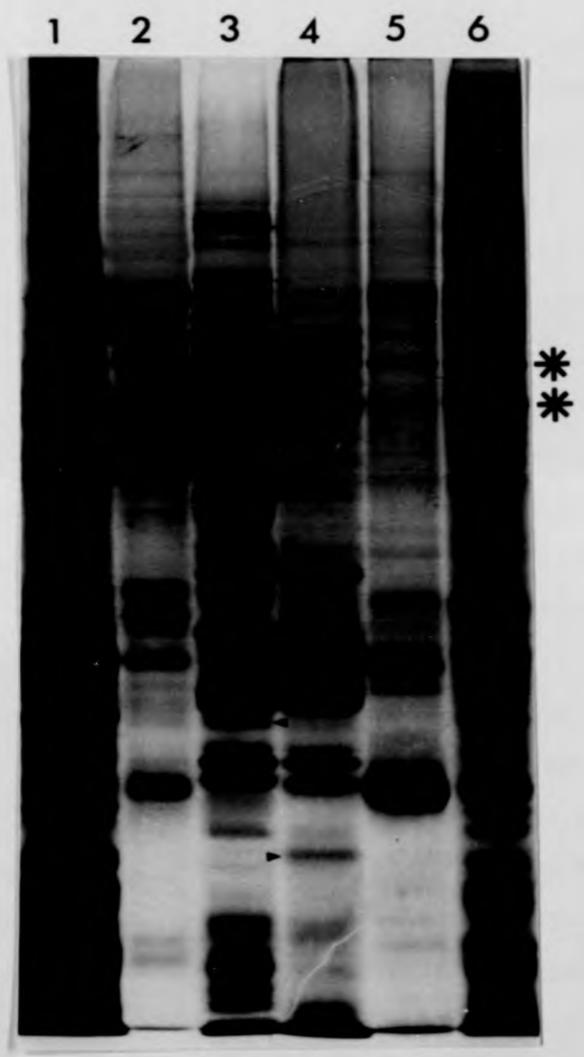


Fig. R1.7 Polypeptide Profiles of In Vivo [<sup>35</sup>S] methionine-Labelled KCl-washed Membrane Preparations

[<sup>35</sup>S] methionine-labelled endosperm tissue (M.6, p. 51) was fractionated on sucrose gradients. The major organelle-containing fractions were removed from the gradients and diluted with 50 mM Tris-HCl, 400 mM KCl. The membranes were harvested by centrifugation then resuspended in 50 mM Tris-HCl (pH 7.4). Aliquots were TCA precipitated and the TCA-insoluble radioactivity associated with each membrane determined. Volumes containing approximately 100,000 cpm were centrifuged to pellet the membranes and the pellets solubilized in SDS-PAGE sample buffer. The proteins were separated by gel electrophoresis and visualized by fluorography. Lane 1, mitochondrial membranes; lane 2, glyoxysomal membranes; lane 3, microsomal membranes; lane 4, molecular weight markers.

the polypeptides associated with unlabelled, pre-existing membranes (such as protein body membranes) which may be contaminating any one or all of the fractions. The polypeptide profiles of unlabelled organelle membranes were compared by SDS-PAGE following the specific extraction of integral membrane proteins using the detergent Triton X-114. This method of extracting hydrophobic proteins from biomembranes (based on the method of Bordier, 1981) utilizes the property of aqueous solutions of Triton X-114 of separating into a detergent phase and an aqueous phase at temperatures above 20°C. On solubilizing a membrane using a pre-condensed solution of Triton X-114 at 0 - 4°C then warming the solution to 20°C, the hydrophobic membrane components, i.e. lipids and amphiphilic proteins, associate with the detergent phase which can be separated from the aqueous phase by a simple centrifugation step. Fig. R1.8 shows the protein profiles of the detergent and aqueous phases after subjecting the three major organelle membrane types to Triton X-114 extraction. That a specific set of proteins are extracted by the detergent can be seen by comparing tracks 3 and 4, the mitochondrial hydrophilic and hydrophobic membrane protein profiles respectively. Both profiles contain proteins unique to themselves (arrowheads), however, there are also many examples of proteins of similar mobilities in both tracks, perhaps reflecting their incomplete partitioning into any one phase.

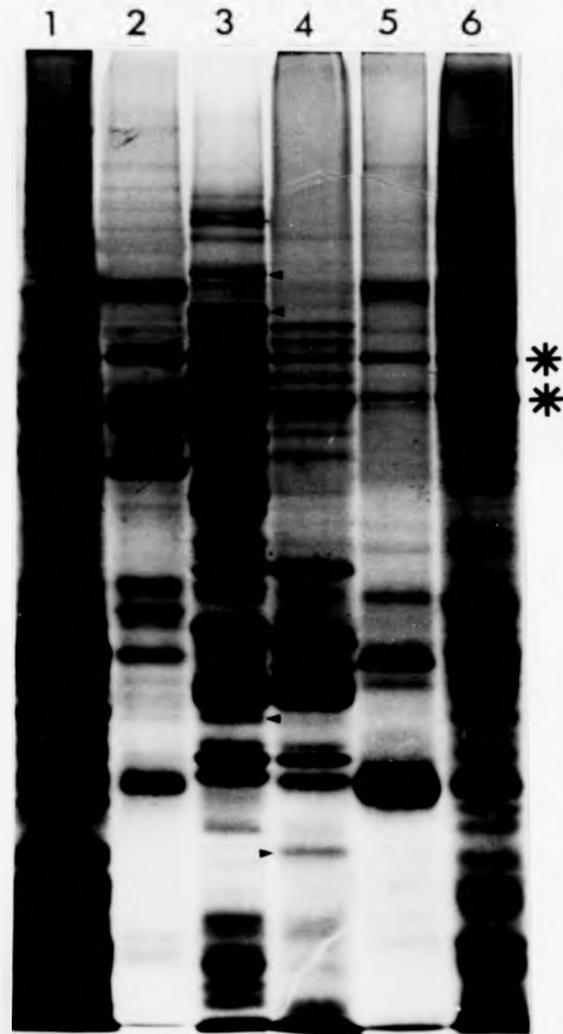
Comparing tracks 4, 5 and 6 in fig. R1.8, the hydrophobic protein profiles of the mitochondria, glyoxysomes and microsomes respectively, reveals an entirely unique polypeptide profile to



**Fig. R1.8** Dodecylsulphate-polyacrylamide Gel Electrophoresis of the Three Major Organelle-Fractions Following Triton X-114 Extraction

Endoplasmic reticulum, mitochondria and glyoxysomes were isolated from 4-day-old endosperm tissue by sucrose density gradient centrifugation. The collected organelle-suspensions were diluted with 50 mM Tris-HCl (pH 7.4) and the mitochondria disrupted by freezing, at -20°C, then thawing. Organelle membranes were harvested by centrifugation and the hydrophobic, integral membrane proteins extracted by phase-separation in Triton X-114 (M.13B p. 64). Detergent phase-associated and aqueous phase-associated proteins were separated by SDS-PAGE and visualized by silver-staining. lanes 1, 2 and 3, aqueous-phase-associated proteins from microsomes, glyoxysomes and mitochondria respectively; lanes 4, 5 and 6 detergent-soluble proteins from mitochondria, glyoxysomes and microsomes respectively.

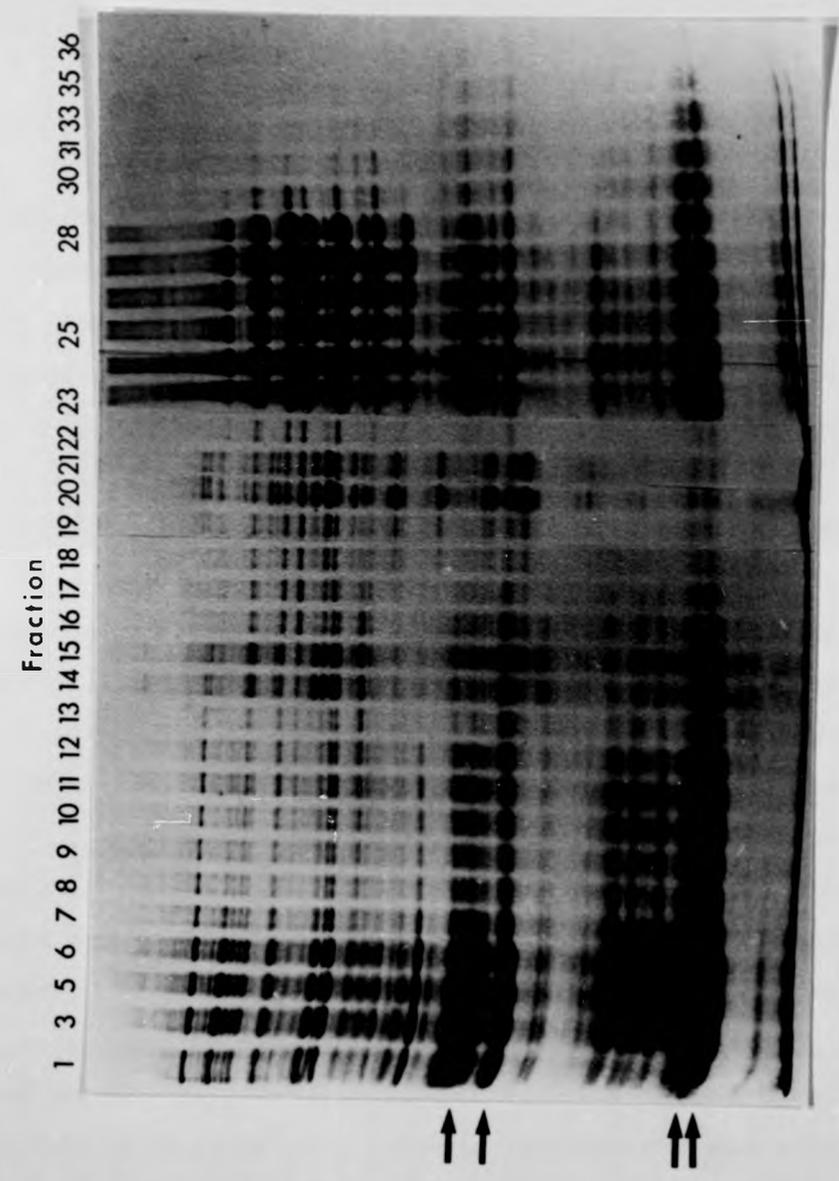
Fig. R1.8 Dodecylsulphate-polyacrylamide Gel  
Electrophoresis of the Three Major Organelle-  
Fractions Following Triton X-114 Extraction



Endoplasmic reticulum, mitochondria and glyoxysomes were isolated from 4-day-old endosperm tissue by sucrose density gradient centrifugation. The collected organelle-suspensions were diluted with 50 mM Tris-HCl (pH 7.4) and the mitochondria disrupted by freezing, at  $-20^{\circ}\text{C}$ , then thawing. Organelle membranes were harvested by centrifugation and the hydrophobic, integral membrane proteins extracted by phase-separation in Triton X-114 (M.13B p. 64). Detergent phase-associated and aqueous phase-associated proteins were separated by SDS-PAGE and visualized by silver-staining. lanes 1, 2 and 3, aqueous-phase-associated proteins from microsomes, glyoxysomes and mitochondria respectively; lanes 4, 5 and 6 detergent-soluble proteins from mitochondria, glyoxysomes and microsomes respectively.

pertain to each membrane type. However, within the microsomal profile can be seen counterparts, of similar mobility, to almost all of the proteins in the glyoxysomal track. That this is not due to glyoxysomal membranes contaminating the microsomal fraction is indicated by the differences in relative abundance of the proteins in the two profiles.

The possibility of organelle cross-contamination was further investigated by comparing the polypeptide profiles of each 1 ml fraction from a 38 ml sucrose gradient. Fig. R1.9 shows the total protein profiles of the gradient fractions following TCA precipitation of 10  $\mu$ l aliquots of fractions 1, 3, 5 - 12, 23 - 36 (omitting fractions 32 and 34) and 100  $\mu$ l aliquots of fractions 12 - 23. The different regions of the gradient are clearly distinguishable (soluble, fractions 1, 3, 5 and 6; e.r., fractions 14 and 15; mitochondria, fractions 20 and 21; glyoxysomes, fractions 23 - 28). The heavily-stained bands in fractions 1 - 6 (arrowed) are storage proteins, released from the protein bodies/tonoplasts on homogenization; they are seen to be present in most fractions but are very prominent in the area of the gradient occupied by the glyoxysomes. This would indicate either storage protein association with the glyoxysomes, possibly due to lectin-type binding to sugars in the glyoxysomal membrane, or the contamination of the glyoxysomal fraction with protein bodies. The latter possibility is unlikely in view of the observations made using the electron microscope which revealed no protein body-like structures in the glyoxysomal fraction. Selected fractions were then subjected to Triton X-114 extraction (after dilution and harvesting of any



**Fig. R1.9** Total-protein Profiles of Selected Fractions Throughout a 38 ml Sucrose Gradient Loaded with Castor Bean Endosperm Homogenate

3-day-old endosperm tissue was fractionated on sucrose density gradients and the gradients collected as 1 ml fractions. Aliquots from each fraction (see text) were TCA precipitated and the precipitate solubilized in SDS-PAGE sample buffer. The polypeptides were then separated by electrophoresis in a 10% polyacrylamide gel and the bands visualized by coomassie-blue staining. Arrows indicate the positions of storage protein subunits.

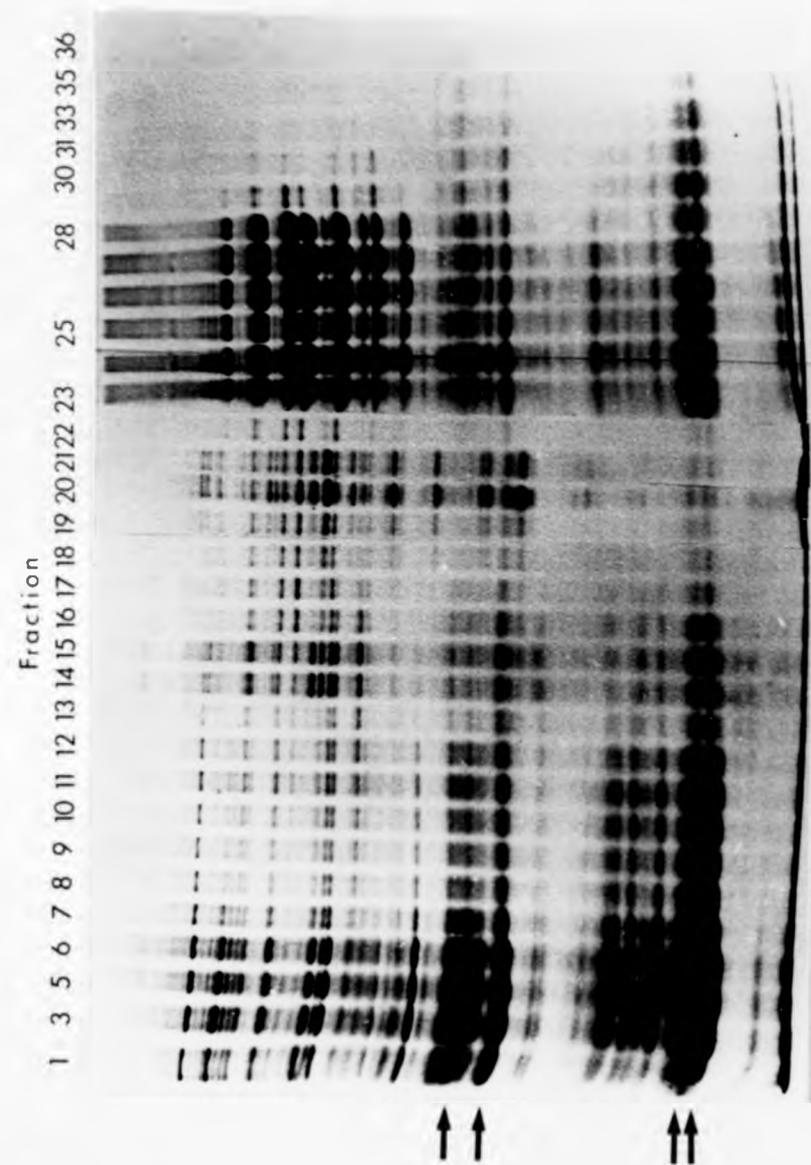


Fig. R1.9 Total-protein Profiles of Selected Fractions Throughout a 38 ml Sucrose Gradient Loaded with Castor Bean Endosperm Homogenate

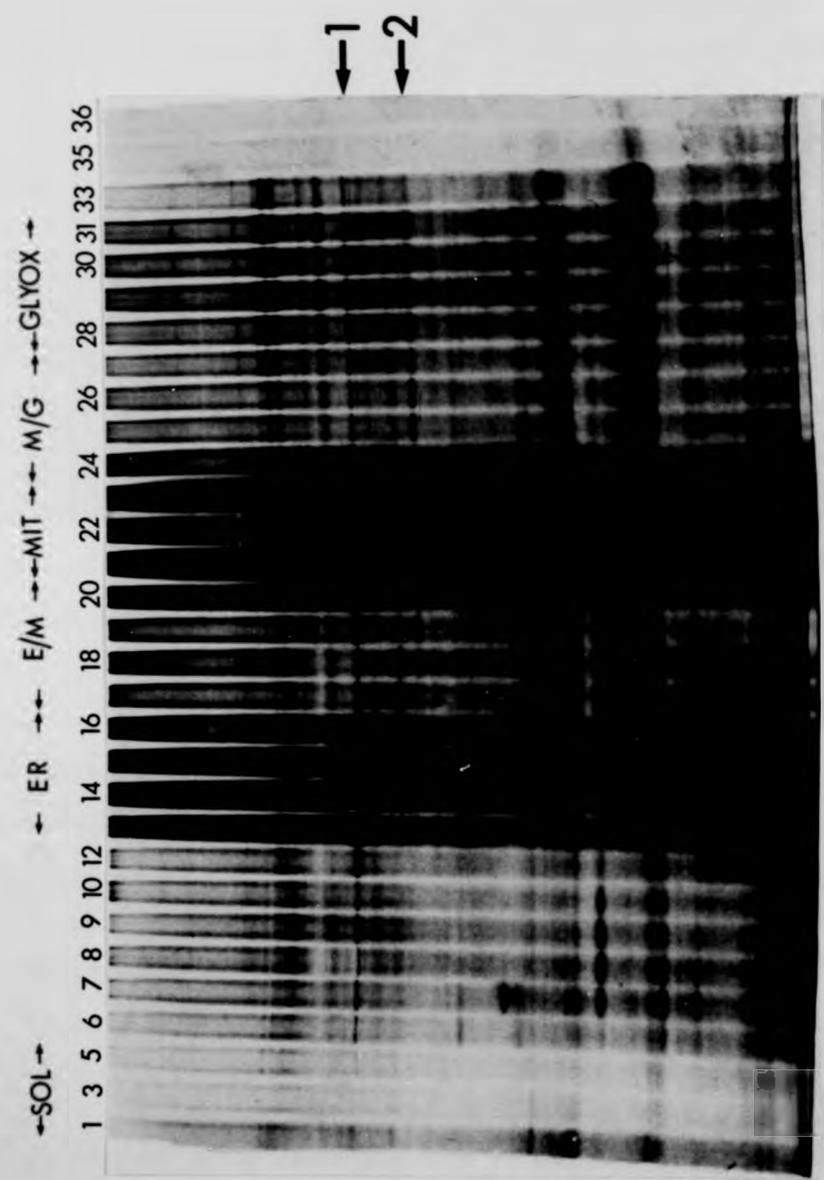
3-day-old endosperm tissue was fractionated on sucrose density gradients and the gradients collected as 1 ml fractions. Aliquots from each fraction (see text) were TCA precipitated and the precipitate solubilized in SDS-PAGE sample buffer. The polypeptides were then separated by electrophoresis in a 10% polyacrylamide gel and the bands visualized by coomassie-blue staining. Arrows indicate the positions of storage protein subunits.

particulate material by centrifugation). The coomassie-blue stained protein profiles are shown in fig. R1.10a. The most prominent tracks are 19 and 20 corresponding to the mitochondrial integral membrane proteins (this gradient being one fraction out of phase with that used to obtain fig. R1.9). The glyoxysomal membranes can again be seen to cover a broad area of the gradient, fractions 24 - 29 staining most heavily but similar profiles can be seen to extend to fractions of lower density than those containing the mitochondria. The position of the microsomes is not evident from fig. R1.10a, however, traces of Triton-extractable proteins can be seen in the gradient fractions from the top of the gradient. A similar gel was therefore stained using silver-stain in order to enhance the profiles of, in particular, the "soluble" fractions and those of the e/m and m/g regions of the gradient (fig. R1.10b). Silver-staining again reveals the three major-organelle-containing regions, however the intermediate fractions are also seen to stain quite heavily. It is interesting to note the similarities of the profiles of the m/g region with those of the glyoxysome-containing region and also the number of proteins associated with the glyoxysomes which have counterparts of similar mobility in the microsome-derived fractions.

R1.E - A6<sub>3</sub> ANTIGEN LOCALIZATION USING A MICRO ENZYME LINKED IMMUNOSORBANT ASSAY (ELISA)

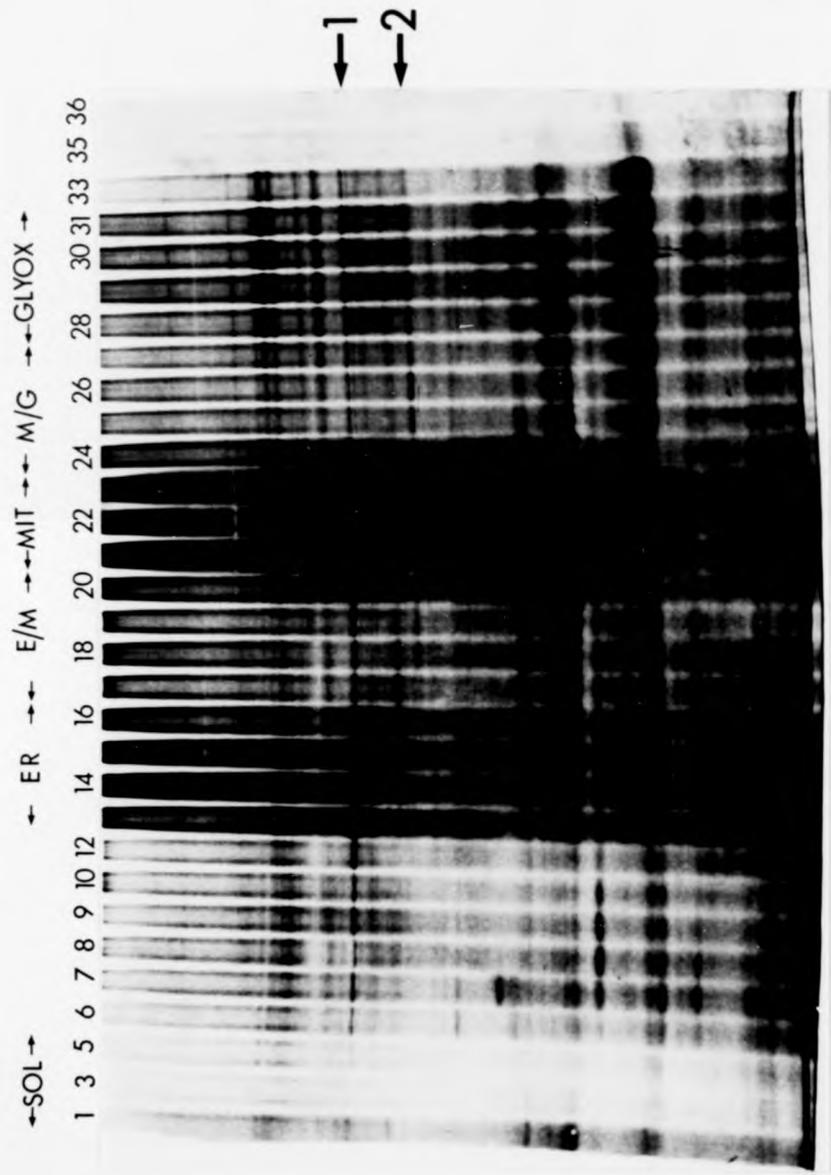
A hybridoma supernatant from a cell-line, A6<sub>3</sub> (which had been shown to react with an uncharacterized glyoxysomal membrane protein [S. Varden, unpublished data]) was used as a probe for the presence of "A6<sub>3</sub> antigen" throughout the subcellular fractions of germinating

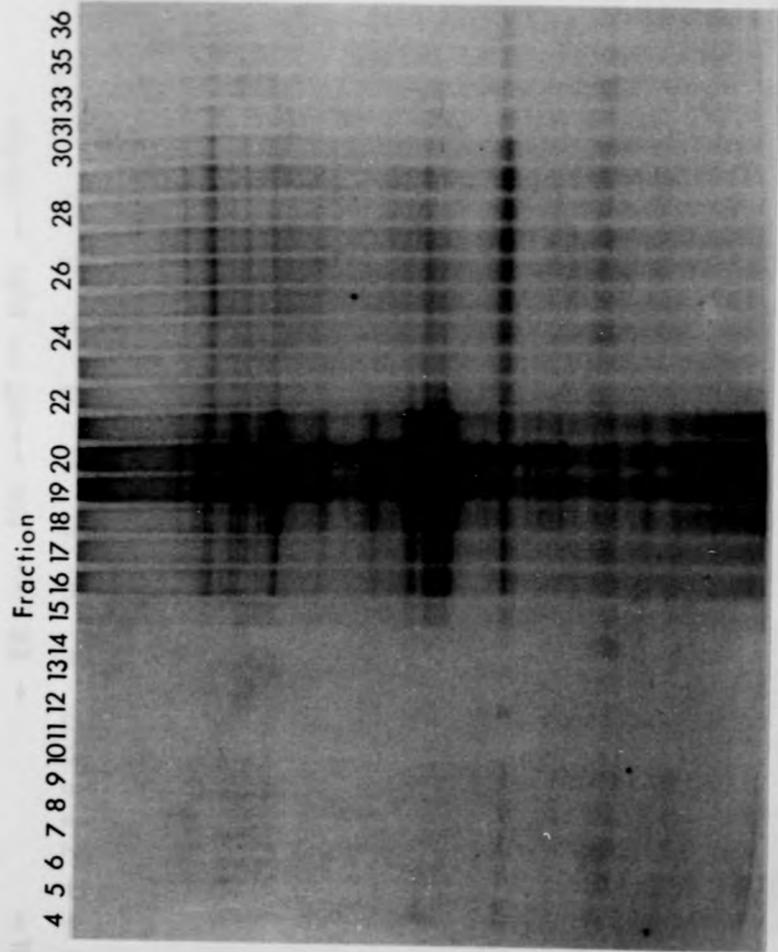
**b**



**c**

**b**

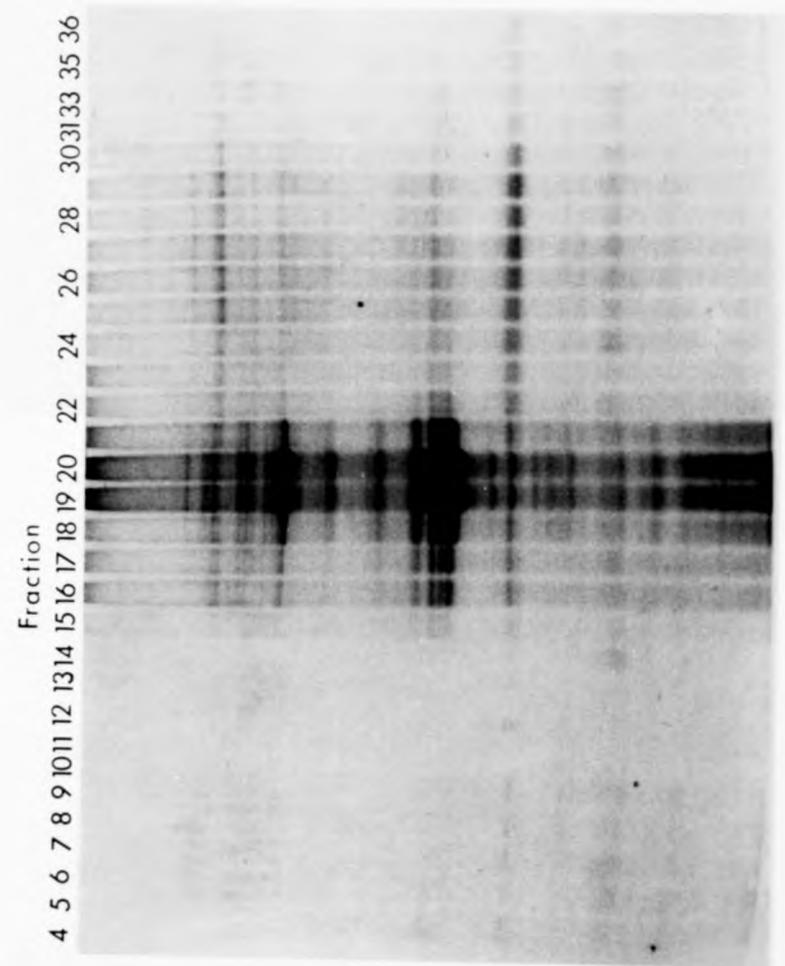




a

Fig. R1.10 Triton X-114-soluble Protein Profiles of Selected Fractions Throughout a 38 ml Sucrose Gradient Loaded with Castor Bean Endosperm Homogenate

Corresponding 1 ml fractions from duplicate sucrose gradients used to fractionate 3-day-old endosperm tissue were pooled then diluted with 50 mM Tris-HCl (pH 7.4). Membranes were pelleted by centrifugation for 1 h at 36,000 r.p.m and 2°C in a Beckman 40.3 fixed-angle rotor. The membranes were then Triton X-114 extracted and the detergent-soluble polypeptides separated by SDS-PAGE. Protein bands were visualized by coomassie-blue (a) or silver-staining (b).



a

Fig. R1.10 Triton X-114-soluble Protein Profiles of Selected Fractions Throughout a 38 ml Sucrose Gradient Loaded with Castor Bean Endosperm Homogenate

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castor bean endosperm cells. Following cellular fractionation by sucrose density gradient centrifugation, each 1 ml gradient fraction was subjected to Triton X-114 extraction. The distribution of extracted protein associated with each fraction is shown in fig. R1.11a. The mitochondria contain approximately 45% of the Triton-extractable protein, the e.r. 25% and the glyoxysomes 5% with the remaining 20% associated with the intermediate and "soluble" regions of the gradient. ELISA plates were coated with duplicate samples from each gradient fraction, 1 $\mu$ g protein being added to each well. The adsorbed protein was reacted with A6<sub>3</sub> supernatant and absorbance readings at 410 nm obtained for each fraction. The relative abundance of A6<sub>3</sub> antigen (per unit weight of total membrane protein) is shown in fig. R1.12a. A6<sub>3</sub> antigen is a glyoxysomal membrane protein, as indicated by its abundance in the glyoxysomal region (fractions 31 - 33) but it also constitutes large proportions of the Triton-extracted proteins derived from the cytosolic region at the top of the gradient and those regions intermediate between the e.r. and mitochondria and the mitochondria and glyoxysomes.

Electron microscopy showed the e/m and m/g regions of the gradient to contain small membrane vesicles. In order to determine whether these vesicles contained the A6<sub>3</sub> antigen, endosperm homogenates were subjected to an 8,000 x g centrifugation step and the resulting pellet

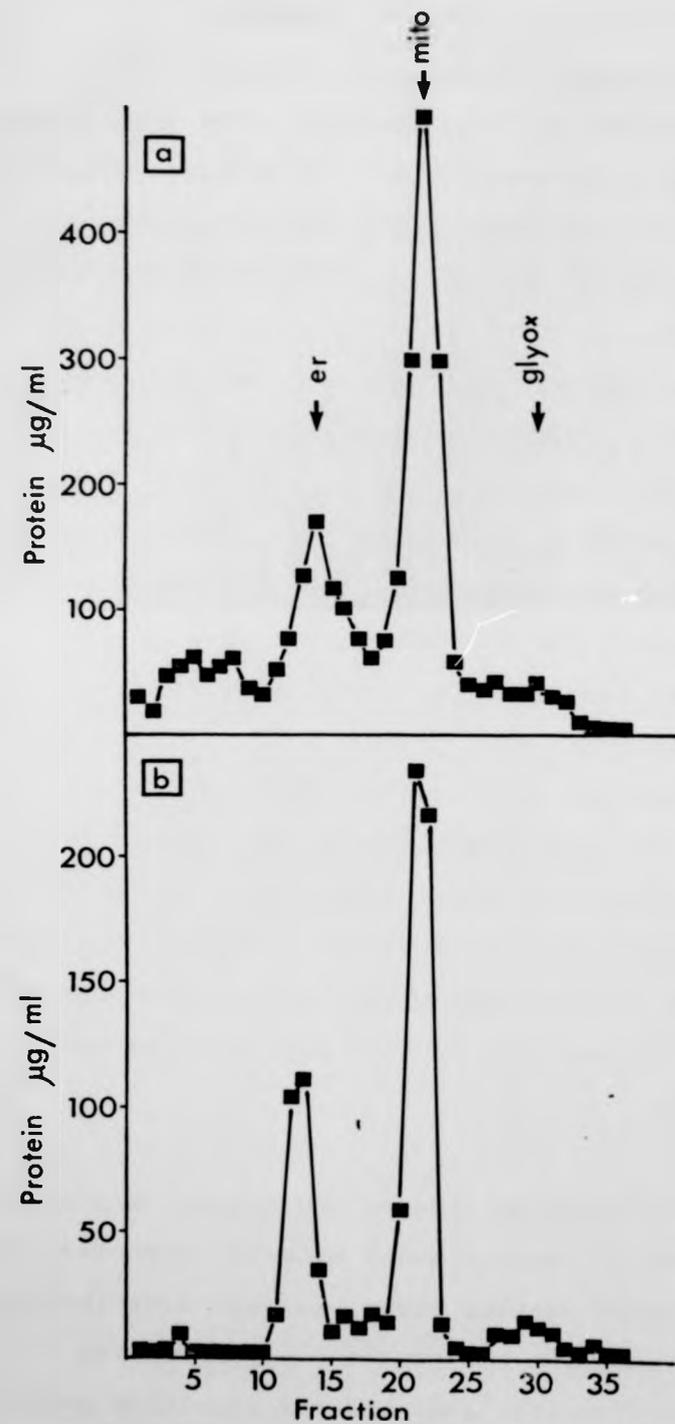


Fig. R1.11 Distribution of Triton X-114-soluble Membrane Proteins Throughout a Sucrose Gradient

(a) Twenty 4-day-old castor bean endosperm halves were fractionated on a 38 ml sucrose gradient. The collected 1 ml fractions were diluted with 50 mM Tris-HCl (pH 7.4) and subjected to centrifugation at 36,000 r.p.m and 2°C for 1 h in a Beckman 40.1 fixed-angle rotor. The membrane pellets were extracted with Triton X-114 and the detergent-soluble protein obtained from each fraction assayed as detailed in M.5 (p.50); (b) Eight 4-day-old endosperm halves were homogenized and the post 500 x g supernatant centrifuged for 10 minutes at 8,000 x g and 4°C. The resulting pellet was gently resuspended in homogenization buffer and centrifuged into a 38 ml sucrose density gradient. Collected 1 ml fractions were treated and assayed for protein as in (a).

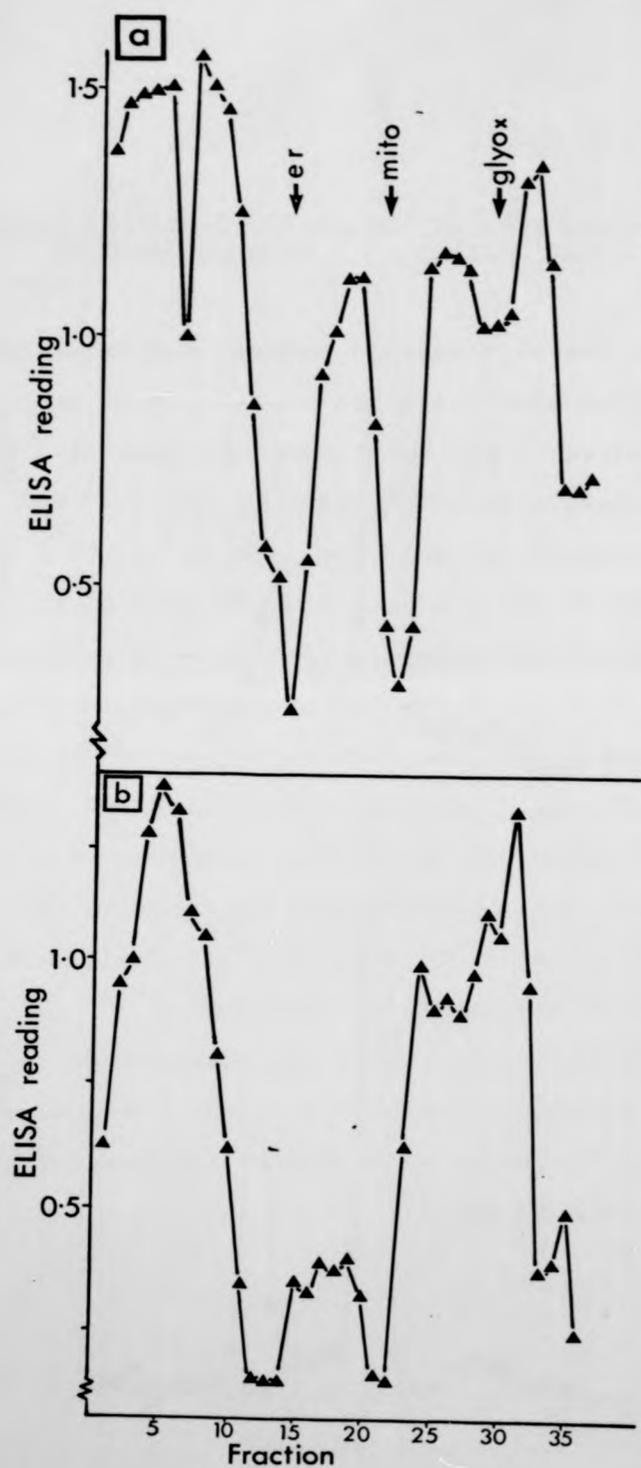


Fig. R1.12 Distribution of an Integral Glyoxysomal Membrane Protein Throughout Castor Bean Endosperm Tissue Fractionation Gradients

38 ml sucrose gradients were loaded with either a post 500 x g supernatant from an homogenate of 15 endosperm halves (a) or the resuspended 8,000 x g pellet (obtained as described in the legend to fig. R1.11b) from 8 endosperm halves (b). Following centrifugation, the gradients were collected as 1 ml fractions and each fraction assayed for antigen to a monoclonal antibody-secreting hybridoma cell-line, A6<sub>3</sub> (an integral glyoxysomal membrane protein) as detailed in M.18 (p. 72). The figure shows the distribution of "A6<sub>3</sub>-antigen" throughout the two gradients.

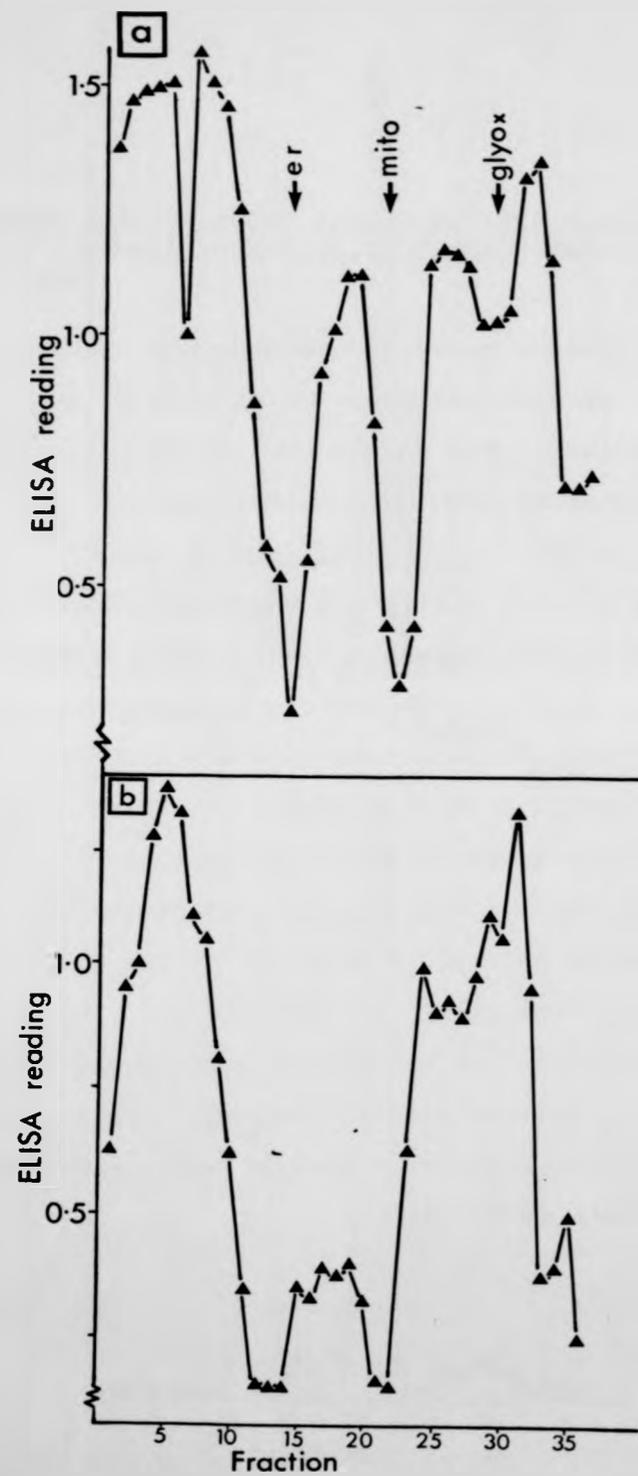


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fractionated on sucrose gradients following its gentle resuspension in homogenization medium. The protein distribution of Triton X-114 extracts of all 36 fractions from such a gradient is shown in fig. R1.11b. The effect of such a centrifugation step is to reduce the amount of Triton extractable (and, by inference, membrane associated) protein present in the fractions at the top of the gradient and also those of the e/m and m/g regions. ELISA's performed on these Triton extracts (fig. R1.12b) show that concomitant with the disappearance of the e/m and m/g membrane vesicles is a reduction in amount of A6<sub>3</sub> antigen. Surprisingly the Triton extractable protein remaining at the top of the gradient still reacted very strongly with A6<sub>3</sub> supernatant.

DISCUSSION I

The purity of the subcellular fractions from castor bean endosperm, obtained by sucrose gradient centrifugation, has been investigated in several ways. Marker enzyme activities (fig. R1.2) show the three major organelle fractions, i.e. microsomes, mitochondria and glyoxysomes to be well separated although the distribution of ICL indicates a 20% breakage of glyoxysomes during the fractionation procedure. The loss of matrix components following glyoxysomal breakage will alter the mean buoyant density of the residual membranes. Glyoxysomal membranes band at a density of 1.21 - 1.22 g/ml on sucrose gradients (Huang and Beevers, 1973) so membranes resulting from the 20% breakage during cell fractionation would be expected to be located between the mitochondria, at a density of 1.18 g/ml, and the glyoxysomes, at density of 1.25 g/ml. [<sup>14</sup>C] choline-labelled glyoxysomal membranes, when recentrifuged following glyoxysome disruption, were indeed found in the intermediate, m/g, region. There was a general smearing of glyoxysome-derived membranes throughout the gradient with peaks of radioactivity appearing in the microsomal and mitochondrial fractions of the gradient (fig. R1.5d) presumably as a result of "trapping" by the densely packed organelles in these regions. The association of glyoxysomal membranes with the microsomes and mitochondria was also indicated by the electron micrographs of these fractions (fig. R1.3a and c). Small membrane vesicles contribute to the morphology of both fractions but due to the vesicular appearance of

the microsomal fraction in general it is impossible to identify any of the vesicles associated with the microsomes as being of glyoxysomal origin. In contrast, the glyoxysomal fraction is not contaminated with either of the other two major organelle fractions. Neither microsomal- or mitochondrial-derived membrane lipid (fig. R1.5a and b) nor membrane protein (fig. R1.6) can be detected in the glyoxysomal region of the sucrose gradient.

The possibility of glyoxysomal contamination by other subcellular structures cannot, on this evidence alone, be eliminated; indeed the electron micrograph of the glyoxysomal fraction (fig. R1.3e) shows it to contain a few plastids. The contribution of these structures to the overall characteristics of the fraction which were investigated, i.e. the protein and glycoprotein composition of the glyoxysomal membrane, was thought to be negligible (a recent publication concludes that chloroplasts do not contain glycoproteins in significant amounts [Nagahashi and Beevers, 1978]). The high equilibrium density of the glyoxysomes (1.25 g/ml) eliminates the possibility of contamination by most other subcellular membranes and organelles (e.g. plasma membrane, spheroplasts and Golgi). There is, however, one other organelle found in castor bean endosperm cells, the protein body, which has a density as high, indeed higher (1.29 - 3.0 g/ml [Tully and Beevers, 1976]), than that of glyoxysomes, and thus could conceivably contribute to the glyoxysomal contamination. Protein bodies are the site of storage protein deposition in developing and dormant seeds. The stored protein is

broken down during germination to provide amino acids used for the synthesis of proteins expressed during germination. The high buoyant density of protein bodies is due to their high protein content, much of this protein being in the form of an insoluble crystalline structure, the crystalloid. The equilibrium density of protein bodies will decrease during germination as the protein reserves contained therein are depleted. Protein bodies of most oilseeds, including castor bean, disintegrate in the aqueous media used during tissue homogenization (Mikola *et al.*, 1975). The crystalloids released from the protein bodies can be sedimented by centrifuging for 5 minutes at 250 x g (Mettler and Beevers, 1979) and will therefore be removed from the homogenate by the 500 x g pre-centrifugation the homogenate receives prior to gradient fractionation. Thus it is unlikely that protein body contamination of the glyoxysomal fraction occurs to any large extent and this is further supported by the complete absence of any protein body-like structures in the glyoxysomal fraction when examined using the electron microscope (fig. R1.3e). The association of storage proteins with the glyoxysomal fractions, as revealed by SDS-PAGE of total TCA precipitates from collected gradient fractions (fig. R1.9a), is therefore likely to be the result of non-specific binding of these proteins to the glyoxysomes, possibly by lectin-like protein/sugar interactions, although the inclusion of 100 mM lactose in the homogenization medium was a precaution taken to minimise such interactions. Purified protein body membranes have a mean buoyant density of 1.21 g/ml (Mettler and Beevers, 1979) thus

one would expect these structures to be present in the m/g region of a standard fractionation gradient and not to contaminate the glyoxysomal fraction to any extent. The potential problem of protein body membranes contributing to glyoxysomal contamination and in particular being responsible for its glycoprotein complementation will be addressed in section 4.

Returning to the assessment of fraction purity in general, having concluded that the glyoxysomal fraction is highly pure, attention was turned to the microsomal fraction. The microsomes from germinating castor bean endosperm cells are collected at a 30/20% sucrose interface and therefore contain membrane vesicles which may vary in density between 1.08 and 1.12 g/ml. This wide density range allows the collection of all of the microsomal fraction, which is composed of vesicles of varying sizes (fig. R1.3a), as a tight band on the gradient. Glyoxysomal membranes, however, have been shown (fig. R1.5d) to be trapped within this fraction and it is likely that other membranes, such as plasma membrane, tonoplast and Golgi, are likewise present. Heterogeneity of the microsomal fraction with regards glycosyl-transferases has been established (Conder and Lord, 1983) and will be discussed in section 3. When glyoxysomes labelled with [<sup>14</sup>C] choline were purposely disrupted and recentrifuged, approximately 17.5% of the radioactivity was recovered in the microsomal region of the gradient (fig. R1.5d). This can be taken as the proportion on 100% glyoxysomal breakage. During normal homogenization procedures 20% glyoxysomal breakage has been shown to

occur, thus the proportion of the glyoxysomal fraction which can be expected to contaminate the microsomal fraction is approximately 3.5%. Fig. R1.8 compares the Triton X-114 extractable polypeptide profiles of the three major organelle fractions; if tracks 5 and 6 (corresponding to glyoxysomal and microsomal integral membrane proteins respectively) are compared, then it can be seen that counterparts to almost all of the glyoxysomal membrane proteins are to be found in the microsomal profile. If this were due solely to the glyoxysomal membranes which contaminate the microsomal fraction then the proteins common to both profiles would be expected to occur in similar proportions and clearly this is not the case (compare the intensities of the two asterisked protein bands in each track). Further evidence for the polypeptides common to both profiles being integrally associated with both membrane types comes from the comparison of the Triton X-114 extractable proteins through the entire gradient (fig. R1.10). Two proteins of the glyoxysomal membrane are arrowed (1 and 2). Protein 1 can be traced through the entire gradient and is particularly prominent in the microsomal fractions. Protein 2, on the other hand, is essentially absent from the microsomal region and indeed appears for the first time in fraction 18 in the e/m region which was seen in fig. R1.3b to be composed of a variety of membrane types. It is possible, though purely speculative, that the e/m region contains membranes undergoing transition from the e.r. to glyoxysomes, protein 2 being protein which is post-translationally inserted into an immature glyoxysomal

membrane of e.r. origin and protein 1 representing a glyoxysomal membrane protein which is inserted into the e.r. co-translationally. It is interesting to compare the results obtained with castor bean glyoxysomal membrane proteins with those obtained by Lazarow's group investigating the origin of rat liver peroxisomal membrane proteins (Fujiki *et al.*, 1982a). Using a sodium carbonate treatment (Fujiki *et al.*, 1982b) as opposed to Triton X-114, to strip the membranes of peripherally-bound proteins these workers found the polypeptide compositions of the e.r. and peroxisomal membranes to be completely different. The staining procedure following SDS-PAGE in this case, however, used coomassie blue which lacks the sensitivity of the silver stain used in the castor bean comparison. It is possible that polypeptides shared by rat liver e.r. and peroxisomes were not detected (fig. R3.8 shows a coomassie-stained comparison of e.r. and glyoxysomal membrane proteins and in this figure the similarities between the two profiles are much less apparent). The protein bands of similar mobilities present in both the microsomal and glyoxysomal fractions are assumed to represent identical polypeptides. It is possible, though improbable, that such proteins are structurally completely different. In order to demonstrate that the same protein is present in both the microsomal and glyoxysomal fractions a monoclonal antibody, raised against a glyoxysomal membrane total Triton X-114 extract, was used as a probe for glyoxysomal protein. Antigen to antibody secreted by cell-line A6<sub>3</sub> (A6<sub>3</sub>-A<sub>g</sub>) was indeed detected in the microsomal fractions (fig. R1.12). This protein is also present in other gradient

fractions, confirming that glyoxysomal membrane fragments are smeared throughout the gradient. The association of the A<sub>63</sub>-A<sub>g</sub> with the smaller membrane fragments, observed in the intermediate e/m and m/g fractions by electron microscopy, was shown by probing for A<sub>63</sub>-A<sub>g</sub> throughout a gradient loaded with a resuspended 8,000 x g pellet obtained from an endosperm homogenate. Such a centrifugation step serves to sediment the larger organelles and membrane fragments but leaves the smaller fragments in suspension. This procedure was also found to reduce the amount of A<sub>63</sub>-A<sub>g</sub> associated with the intermediate fractions. Although the identity of the A<sub>63</sub>-A<sub>g</sub> could not be established by conventional western-blotting techniques, it is clearly a glyoxysomal membrane component. Similar results were obtained with other hybridoma supernatants and polyclonal antibodies raised against Triton X-114 extracted integral proteins of purified glyoxysomal membranes.

In conclusion, the glyoxysomal preparation from fractionated castor bean endosperm tissue has been shown to be highly pure and to contain, in intact form, 80% of the extracted organelles. The membranes of the remaining 20%, spread throughout the rest of the gradient, contaminate other organelle fractions including the microsomes.

Polypeptide analysis of the various subcellular fractions reveals similarities between the microsomes and glyoxysomes which are greater than those which can be accounted for merely by cross-contamination. A role for the e.r. as the origin of some glyoxysomal polypeptides is therefore indicated.

Note added after examination

The data presented in Fig. R1.12, p 85b, shows the distribution of A6<sub>3</sub>-reactive polypeptide throughout a sucrose gradient fractionation of castor bean endosperm homogenate. However, since equal amounts of protein (10 μg) from each fraction were assayed by ELISA, the distribution shown does not reflect the relative abundance of antigen in the gradient fractions.

RESULTS II

R.2 - SUBCELLULAR LOCALIZATION OF GLYCOSYL-TRANSFERASES  
IN GERMINATING CASTOR BEAN ENDOSPERM

R.2A - GLUCOSYLTRANSFERASE

3 day-old castor bean endosperm tissue was fractionated on sucrose density gradients and 1 ml gradient fractions were assayed for the ability to transfer glucose from UDP - [<sup>14</sup>C] glucose to chloroform/methanol (2 : 1) soluble glucosyl-lipid. The distribution of this glucosyl-transferase throughout a normal 38 ml gradient is shown in fig. R2.1. The activity was greatest in the microsomal fraction (gradient fractions 8 - 12, the gradient in this case having only a 5 ml 20% (w/w) sucrose step between the 30% (w/w) sucrose and the sample). A second peak of activity was associated with the, largely uncharacterised, A-band which has previously been shown to possess phosphorylcholine-cytidine transferase activity (Lord et al., 1972). The mitochondria also showed some glucosyltransferase activity though none was associated with the glyoxysome-containing fractions. Glucose transfer to lipid is known to be an intermediate stage in the synthesis of N-linked glycoproteins in plants (Pont Lezica et al., 1978). The lipid involved in this process is a polyisoprenol of the dolichol-type. The addition of exogenous dolichol-monophosphate to glucosyl-transferase assay systems failed to stimulate incorporation of [<sup>14</sup>C] glucose into the chloroform/methanol (2 : 1) soluble product (fig. R2.2).

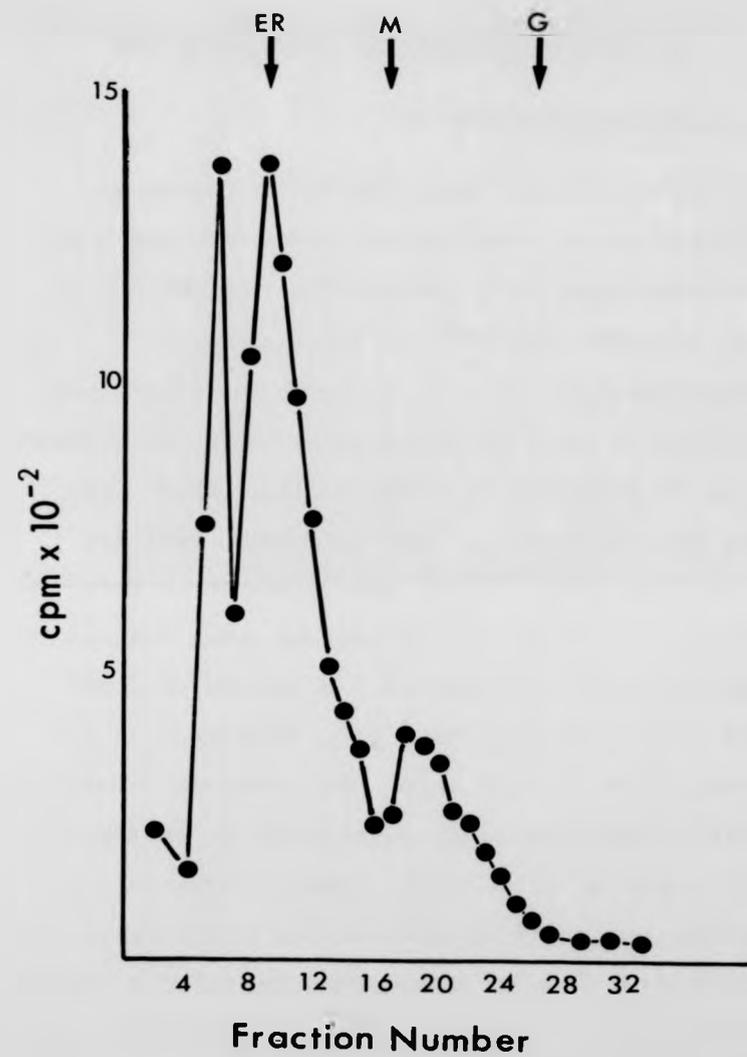
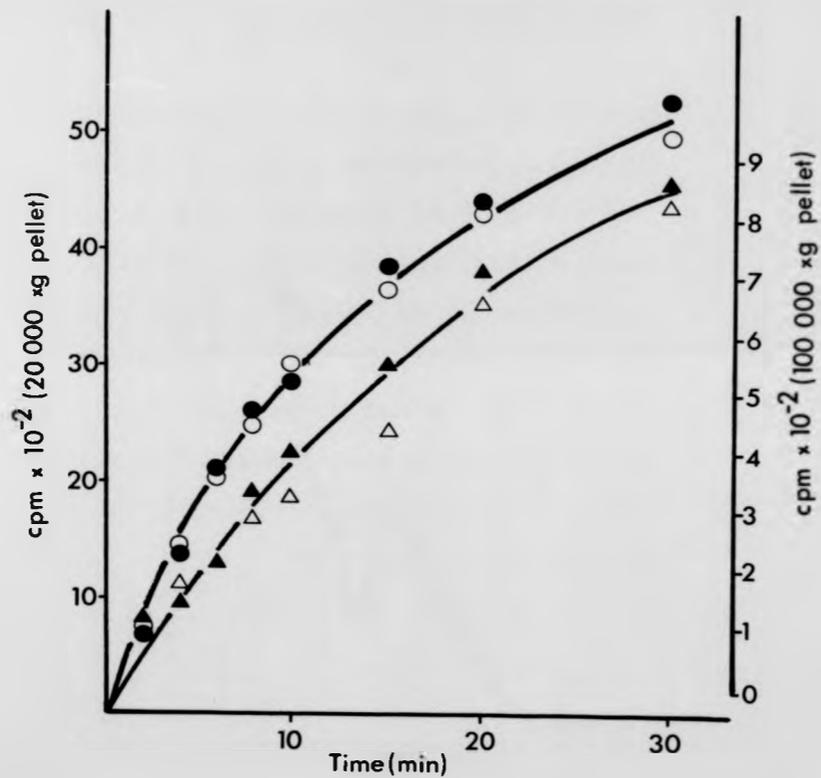


Fig. R2.1 Subcellular Localization of UDP-glucose Lipid Transferases in Castor Bean Endosperm

Following fractionation of castor bean endosperm tissue on sucrose density gradients, 1 ml fractions were collected and assayed for enzymes catalyzing the incorporation of UDP-[<sup>14</sup>C] glucose into chloroform/methanol (2 : 1) soluble glucosyl-lipid. E.R., M and G designate the peak activities of marker enzymes for the endoplasmic reticulum, mitochondria and glyoxysomes respectively.



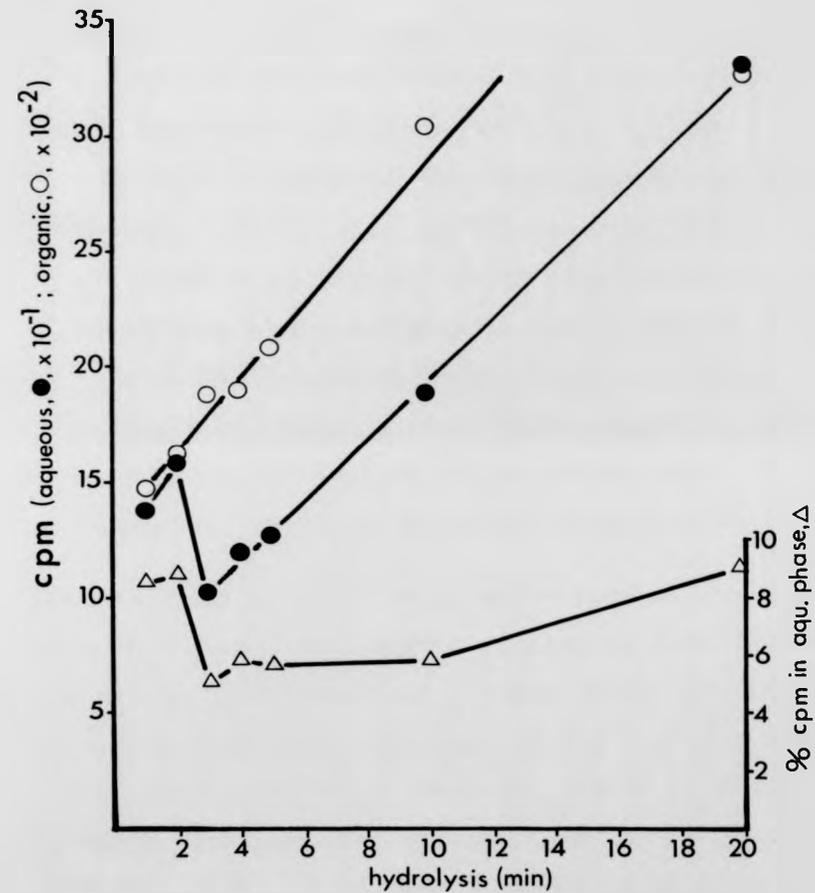
**Fig. R2.2** The Effect of Exogenous Dolichol on Glucose Incorporation Into Glucosyl-lipid

Twenty 3-day-old endosperm halves were homogenized and the homogenate subjected to consecutive centrifugation at 500 x g, 10 minutes; 20,000 x g, 45 minutes and 100,000 x g, 180 minutes. The pellets from the 500 x g step was discarded while those from the 20,000 x g (●,○) and 100,000 x g (▲,△) steps were each resuspended in 5 ml homogenization medium. 2 ml of each of the resuspended pellets were incubated with 0.05  $\mu$  Ci UDP-[<sup>14</sup>C] glucose in the absence (●,▲) or presence (○,△) of 50  $\mu$ g dolichol monophosphate. At various time points, 0.25 ml aliquots were assayed for [<sup>14</sup>C] glucose incorporation into chloroform/methanol (2 : 1) soluble glucosyl lipid as detailed in M.4F (p. 48).

In these assays the enzyme preparation was derived from either a 20,000 x g pellet from a post 500 x g endosperm homogenate supernatant or a 100,000 x g pellet from the 20,000 x g supernatant. The nature of the lipid with which the glucose became associated was therefore investigated. The chloroform/methanol (2 :1) soluble glycolipid was subjected to mild acid hydrolysis (fig. R2.3) but this failed to release the sugar from the lipid, suggesting that the lipid was not of the dolichol-type. This conclusion was further supported by the failure of the glucolipid to bind to a DEAE-cellulose column, unlike monosaccharide lipids known to contain dolichol. The elution profile of dolichol monophosphate mannose from such a column is shown in fig. R2.4. This behaviour is typical of a glycolipid of the dolichol-monophosphate-sugar type (Behrens *et al.*, 1971).

Glucose incorporation into TCA-insoluble material was investigated *in vivo* by incubating intact endosperm tissue with tritiated sugar. Following an incubation period of either 2.5 h or 16 h radiolabelled tissue was fractionated by sucrose gradient centrifugation. The distribution of TCA precipitable radioactivity after the two time periods is shown in fig. R2.5. After the 2.5 h incubation, TCA insoluble radioactivity was associated mainly with the A-band and microsome-containing fractions (fig. R2.5, fractions 5 and 9 - 12 respectively). There was a small peak associated with fraction 22 which did not correspond to either of the other major organelle-containing fractions but rather to the m/g region where residual plastids would be located (with a mean buoyant density of 1.23 g/ml [Breidenbach *et al.*, 1968]).

Fig. R2.3 The Resistance of the Glucosyl-lipid to Mild Acid Hydrolysis



Ten 3-day-old endosperm halves were homogenized in 2.5 ml 150 mM Tris-HCl (pH 7.5), 2 mM  $\beta$ -mercaptoethanol and 12% (w/v) sucrose. Following centrifugation at 500 x g the 500 x g supernatant was recentrifuged at 20,000 x g and 2°C for 15 minutes. The resulting pellet was resuspended in 3 ml of the homogenization medium described in M.4F (p. 48). 0.05  $\mu$  Ci UDP-[<sup>14</sup>C] glucose was added to 2 ml of the enzyme preparation and glucosyl-lipid synthesis allowed to proceed for 30 minutes at 30°C. The glucosyl-lipid was then extracted into chloroform/methanol (2 : 1) as described in M4.F. The final organic phase was evaporated to dryness and the residue redissolved in 3.6 ml 50% n-propanol. 0.4 ml 0.1 M HCl was added and the solution placed at 100°C. At various times 0.5 ml aliquots were removed, neutralized with 0.01 M NaOH and 1 ml chloroform/methanol (2 : 1) added. After separation of the organic and aqueous phases the radioactivity associated with each was determined.

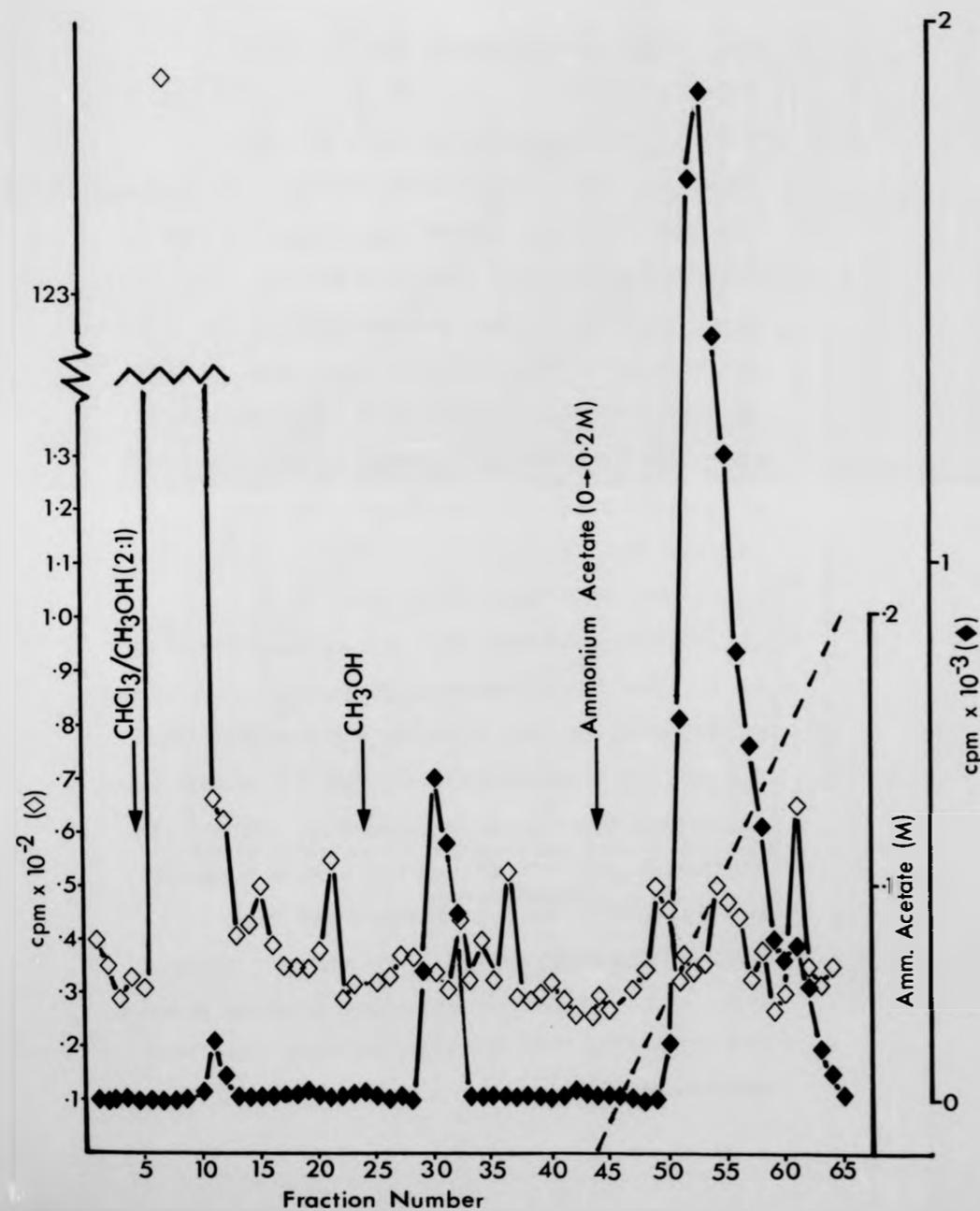


Fig. R2.4 DEAE Cellulose (Acetate) Column Chromatography of Mannosyl- and Glucosyl-Lipids

$^{14}\text{C}$  glucosyl lipid was synthesized and extracted into chloroform/methanol (2 : 1) as detailed in the legend to fig. R2.3, using the 20,000 x g pellet from 10 endosperm halves and 5  $\mu\text{Ci}$  UDP  $^{14}\text{C}$  glucose. 0.5 ml of the  $^{14}\text{C}$  glucosyl lipid was applied to a 7.5 x 120 mm DEAE cellulose (acetate) column which had been washed sequentially with 100 ml of glacial acetic acid, 100 ml of methanol and 100 ml of chloroform/methanol (2 : 1) before use. The column was eluted sequentially with 20 ml chloroform/methanol (2 : 1), 20 ml methanol and a 20 ml linear gradient of ammonium acetate (increasing from 0 to 0.2M). 1 ml fractions were collected, evaporated to dryness in scintillation vials and their radioactivity was determined ( $\diamond$ ). The elution profile of  $^{14}\text{C}$  mannosyl lipid, synthesized in a similar manner to the glucosyl lipid but substituting 0.6  $\mu\text{Ci}$  GDP  $^{14}\text{C}$  mannose for the radiolabelled glucose, is also shown ( $\blacklozenge$ ).

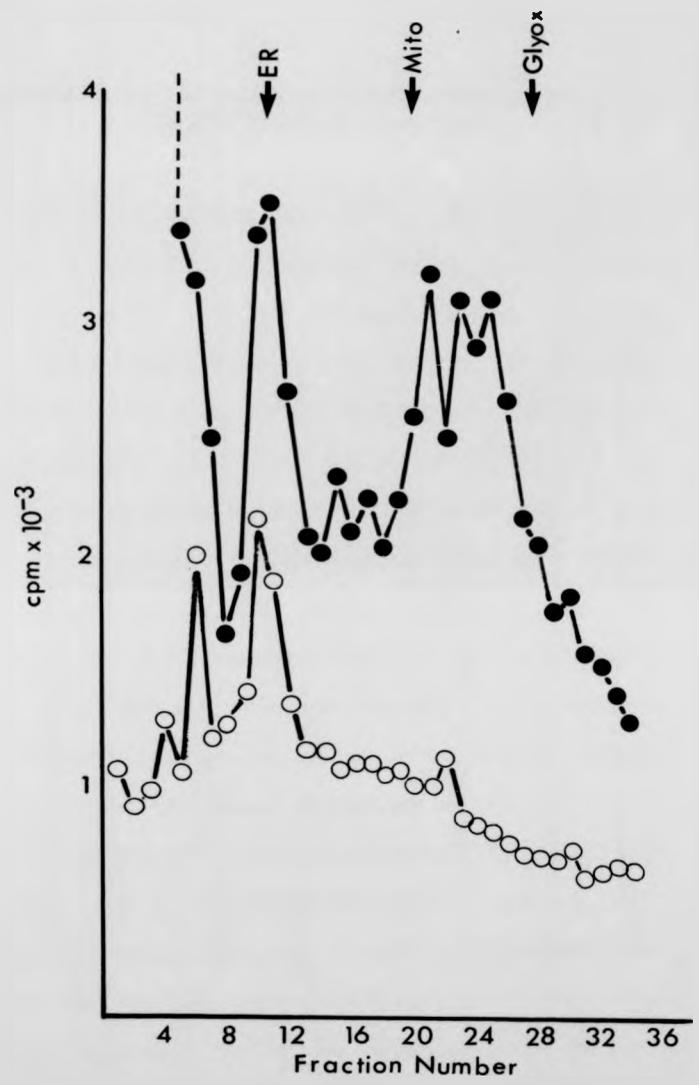


Fig. R2.5 Subcellular Distribution of TCA-insoluble [<sup>3</sup>H] glucose After In Vivo-labelling of Intact Tissue and Fractionation on Sucrose Density Gradients

Two batches of 20 3-day-old endosperm halves were incubated with [<sup>3</sup>H] glucose (2.5 μCi/half, as detailed in M.6, P.51) for 2.5 h (○) or 16 h (●). Following incubation the tissue was homogenized and applied to 38 ml sucrose density gradients which, following centrifugation, were collected as 1 ml fractions. The TCA-insoluble radioactivity associated with each fraction was determined as in M.6.

The association of radioactivity with the plastid region was more marked after a 16 h incubation of intact tissue with [<sup>3</sup>H] glucose. Also more pronounced was the microsomal peak, however by far the greatest amount of TCA insoluble radioactivity was located in the soluble region at the top of the gradient (a total of 72,500 cpm, representing approximately 50% of the total TCA precipitable radioactivity, being associated with the top 4 fractions). These results prompted an investigation into the glucose-containing TCA precipitate; in particular whether it was of a glycoprotein nature. The TCA-insoluble fraction from 30 endosperm halves labelled for 16 h with [<sup>3</sup>H] glucose was subjected to pronase digestion for approximately 90 h at 37°C. The amount of radioactivity solubilized by this treatment, compared with a control incubation, is shown in table 1 and indicates that the glucose-containing polymer was not of a proteinaceous nature. An alternative glucose-containing polymer which satisfies the criteria (a) of association with the plastid fraction and (b) of existing as chloroform/methanol (2 : 1) soluble intermediates is the polysaccharide, starch. In conclusion, it would appear that in castor bean endosperm the involvement of dolichol-linked glucose as an intermediate in glucose-containing glycoprotein synthesis may not be as prominent as in other eukaryotic cells.

R.2B - N-ACETYL GALACTOSAMINE INCORPORATION INTO TCA INSOLUBLE MATERIAL IN VIVO

N-acetyl-galactosamine (GalNAc) incorporation into subfractions of castor bean endosperm tissue was

TABLE 1

	+ Pronase	- Pronase
* Solubilized Radioactivity	5228	2810
* Insoluble Radioactivity Remaining	8750	10783
* Total	13978	13593
% Release	37.36	20.67

\* c.p.m

Table 1 Pronase Treatment of TCA-insoluble [<sup>3</sup>H] Glucose Incorporation Product

30 endosperm halves were incubated with 75  $\mu$ Ci [<sup>3</sup>H] glucose for 16 h at 20°C. The tissue was homogenized and centrifuged for 5 minutes at 500 x g and 2°C. The supernatant was increased to 12 ml with normal homogenization medium and 8 ml added to 50 ml chloroform/methanol (2 : 1). 17.5 ml water was then added. Following thorough mixing the phases were allowed to separate and the aqueous phase and interface material saved. After washing the aqueous phase with 20 ml chloroform it was made 8% in TCA. The precipitated material was washed with:

(a) 8% TCA; (b) 3 x 10 ml chloroform/methanol (2 : 1); (c) 3 x 10 ml chloroform/methanol/water (10 : 10 : 3). The final TCA precipitate was resuspended in 4 ml 0.2M NaCl, 0.1M sodium phosphate buffer (pH 7.4). 1 ml samples were placed in two tubes one of which contained 1 mg pronase. The pronase was dissolved and 1 drop of toluene layered onto both samples to prevent evaporation. The tubes were incubated at 37°C for 24 h then, after the addition of a further milligram of pronase to the treated sample, for for a further 65 h. The supernatants were then decanted into scintillation vials and the radioactivity determined in a lipid scintillation counter. The residue was filtered through Whatman G/FA filter discs and washed with 10% TCA before immersing the filters in scintillation fluid and measuring the associated radioactivity.

investigated by incubating intact tissue with [<sup>3</sup>H] GalNac for periods of 3 h and 16 h then fractionating the tissue by sucrose density centrifugation. The distribution of TCA insoluble [<sup>3</sup>H] GalNac is shown in fig R2.6. Radioactivity was associated with the cytosol (fractions 1 - 5) and the endoplasmic reticulum (fractions 11 and 12) after the 3 h incubation. After 16 h there was only a quantitative change in this distribution with a greater amount appearing in the microsomal fractions. There was a lower incorporation into fractions 16 - 25 (fig. R2.6) but the subcellular particles responsible for this incorporation were not specifically identified. An insignificant amount of [<sup>3</sup>H] GalNac was detected in the glyoxysomal fraction. GalNac being frequently present in O-glycosylated proteins, this result indicates a low abundance of this type of linkage in any glycoproteins associated with glyoxysomes.

R.2C - ARABINOSE INCORPORATION INTO TCA-INSOLUBLE MATERIAL IN VIVO

Fig. R2.7 shows the result of similar incubations to those in R.2B, using D-[<sup>3</sup>H] arabinose. Following a 3 h incubation period over 90% of the TCA-insoluble arabinose was associated with the cytoplasmic-derived fractions (1 - 8) with most of the remaining 10% located in the microsomal fractions. A possibly significant second peak was found in the fractions immediately below those containing the microsomes (fractions 16 and 17) and a small "smear" of elevated levels of radioactivity in the fractions intermediate between the mitochondria and glyoxysomes. Following an overnight (16 h)

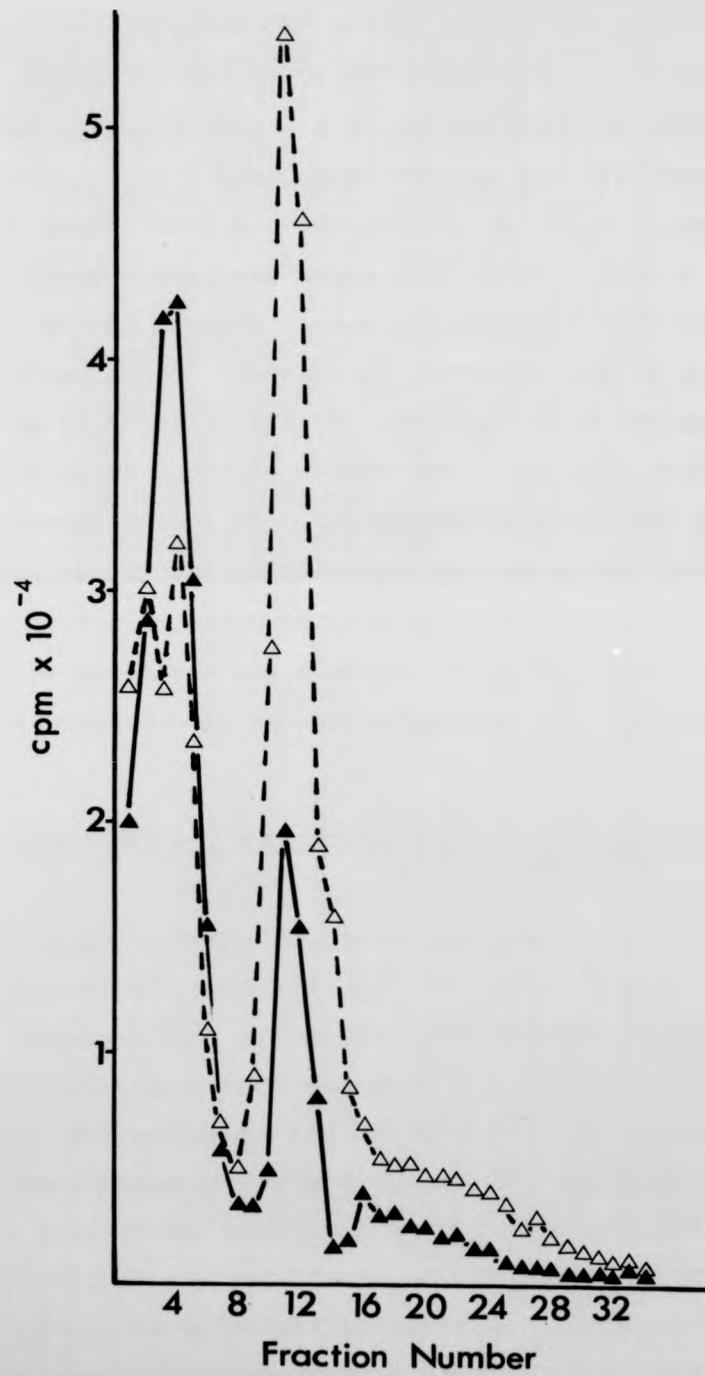


Fig. R2.6 Subcellular Distribution of TCA-insoluble N-acetyl-[<sup>3</sup>H] galactosamine After In Vivo-labelling of Intact Tissue and Fractionation on Sucrose Density Gradients

Tissue was labelled with N-acetyl-[<sup>3</sup>H] galactosamine (5  $\mu$ Ci/half) as detailed for [<sup>3</sup>H] glucose in the legend to fig. R2.5. Incubation with labelled sugar was for 3 h (▲) or 16 h (△) before tissue fractionation and the determination of TCA-insoluble radioactivity also as in fig. R2.5.

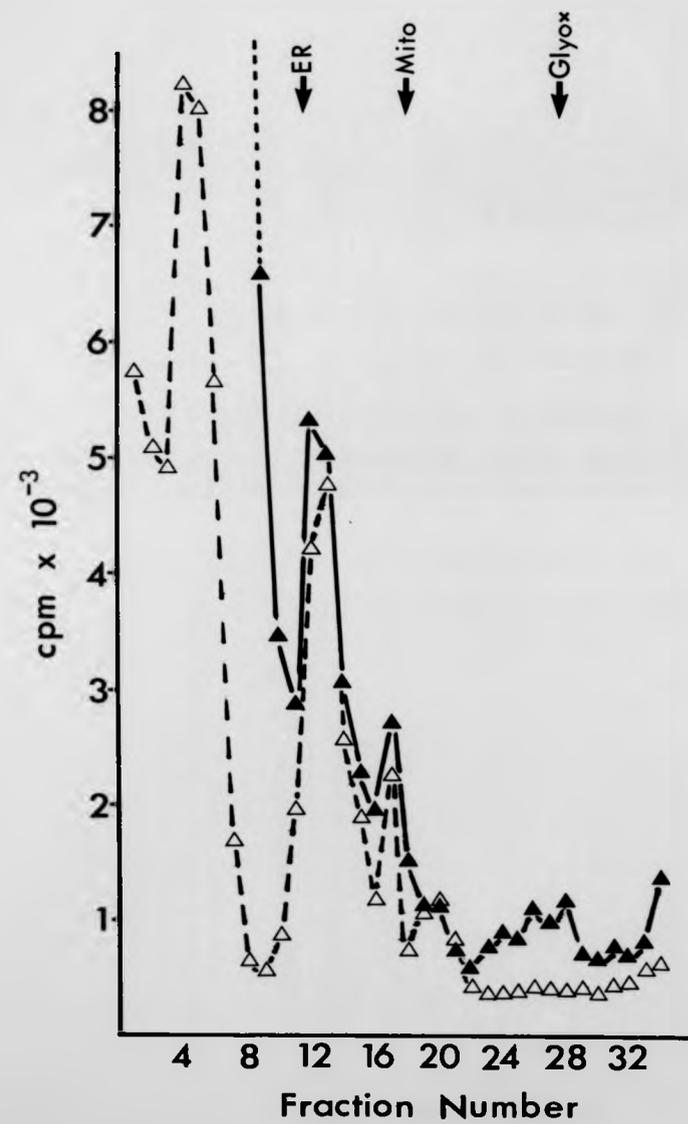


Fig. R2.7 Subcellular Distribution of TCA-insoluble [<sup>3</sup>H] arabinose After In Vivo-labelling of Intact Tissue and Fractionation on Sucrose Density Gradients

Tissue was incubated with [<sup>3</sup>H] arabinose (2.5 μCi/half) for 3 h (▲) and 16 h (Δ) periods before its fractionation on sucrose density gradients and the determination of TCA-insoluble radioactivity in collected 1 ml fractions as detailed in M.6 (p. 51) and fig. R2.5.

incubation there was a dramatic decrease in the number of counts incorporated in total, with the largest proportion disappearing from the cytosol. A peak of activity remained associated with the sample/20% sucrose interface. The radioactivity located in the microsomal and e/m fractions was much the same as that found after the 3 h incubation but there was a reduction in that associated with the m/g region. These results indicated a rapid metabolism of D-arabinose in this tissue

DISCUSSION II

D.2 - GLYCOSYL-TRANSFERASES IN GERMINATING CASTOR BEAN  
ENDOSPERM TISSUE

The involvement of lipid-linked intermediates in the synthesis of glycoprotein in castor bean endosperm has been extensively studied (Mellor and Lord, 1979 a, b and c; Mellor et al., 1979; 1980 a; Marriott and Tanner, 1979). In the cases of N-acetyl glucosamine and mannose (two of the components of the oligosaccharide core-structure in N-linked glycoproteins) involvement is consistent with the general mechanism of glycoprotein biosynthesis established for other systems (Parodi and Leloir, 1979; Struck and Lennarz, 1980).

The oligosaccharide moiety transferred en bloc to nascent glycopeptide chains is thought to have the general structure  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$  (Hubbard and Ivatt, 1981 for review) and dolichol-linked glucose has been implicated in the formation of this core-structure (Staneloni et al., 1980). No evidence for the synthesis of dolichylmonophosphate glucose could be found in the present study, although a glucosyl transferase was shown to incorporate glucose, from UDP-Glc into a chloroform/methanol (2 : 1) soluble compound. The lipid moiety of this compound was not of the dolichol type since (a) exogenously added dolichol failed to stimulate incorporation of [ $^{14}\text{C}$ ] glucose into the chloroform/methanol-soluble form (fig. R2.2), unlike castor bean endosperm mannosyl-lipid (Mellor and Lord, 1979 a and see Results III), (b) significant levels of radioactivity could not be detected in a chloroform/

methanol/water (10 : 10 : 3) soluble form which would have been indicative of incorporation into an oligosaccharide-lipid structure, (c) the glucosyl-lipid was not susceptible to mild acid hydrolysis (fig. R2.3) and (d) the glucosyl-lipid failed to bind to a DEAE-cellulose column equilibrated with chloroform/methanol (2 : 1) (fig. R2.4).

The involvement of UDP-glucose in the synthesis of macromolecules other than glycoproteins, namely the polysaccharides starch (Lavintman *et al.*, 1974) and cellulose (Hopp *et al.*, 1978), has been reported. In the latter case, synthesis was also shown to involve lipid-linked precursors though the lipid moieties functioning in this process also exhibited properties of polyisoprenols. Castor bean endosperm was not expected to be synthesizing cellulose to any great extent. Starch synthesis, on the other hand, has been shown to occur in plastids (Lavintman *et al.*, 1974) and most of the particulate-associated TCA-insoluble [<sup>3</sup>H] glucose is associated with sucrose gradient fractions known to contain these organelles (fig. R2.5). Mellor *et al.* (1980a) showed a glucosamine-containing TCA-insoluble product (obtained by incubating intact castor bean endosperm tissue with [<sup>3</sup>H] glucosamine) to be susceptible to digestion by pronase. Similar treatment of the TCA-insoluble [<sup>3</sup>H] glucose *in vivo* incorporation product failed to solubilize a significant amount of radioactivity, so providing further evidence for its non-proteinaceous nature. The further characterisation of the [<sup>3</sup>H] glucose-containing polymer and the [<sup>14</sup>C] glucose-containing glycolipid was not pursued. The

function of castor bean endosperm UDP-glucose glucosyl-lipid transferase therefore awaits further investigation. The association of the microsomal glucosyl-transferase with subfractions of the endoplasmic reticulum will be discussed in the following section.

The glucose residues in the  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$  oligosaccharide moiety are thought to be essential for its transfer from lipid to protein in calf thyroid tissue (Spiro et al., 1979 b). Yeast mutants, lacking the ability to glucosylate the  $\text{Man}_9\text{-GlcNAc}_2$  structure can, however, still transfer the non-glucosylated structure to protein (Runge et al., 1984). Further, the transfer of a similar unglucosylated core-structure is included in the normal phenotype of the protozoan species Trypanosoma cruzi (Parodi and Cazzulo, 1982). The function of the three glucose residues in the core-oligosaccharide is not known, however their association with the glycopeptide is only a transient one since they are removed from the glycosylated protein whilst the sequestered protein is still inside the lumen of the e.r. or shortly after it enters the Golgi apparatus for transport to its specific location within the cell (Spiro et al., 1979 a; Elting et al., 1980; Grinna and Robbins, 1980). Parodi et al. (1983) have suggested that the glucosylation/deglucosylation processes act as a recognition signal for an event such as assigning specific subcellular locations to different glycoproteins. Such a role, at the present time, remains highly speculative; whatever the role may be, however, its importance in glycoprotein synthesis is

seen to vary from one cell-type to another. The results presented have failed to provide evidence for the involvement of glucose and glucolipids in protein glycosylation in castor bean endosperm. These data are clearly preliminary, however, and a more detailed study would have to be undertaken before firm conclusions could be reached.

In many O-linked glycoproteins the monosaccharide residue directly involved with the attachment of the carbohydrate moiety to a serine or threonine in the polypeptide chain, is N-acetyl galactosamine (GalNAc) (Kornfeld and Kornfeld, 1980 for review). Castor bean endosperm would appear to be able to incorporate N-acetyl-[<sup>3</sup>H] galactosamine into a TCA insoluble form via an enzyme located in the e.r. and/or cytosol (fig. R2.6). The absence of any GalNAc in the glyoxysomal fraction, even after 16 h, confirms the result obtained by Mellor *et al.* (1980b) who, using gas liquid chromatography, were unable to detect GalNAc amongst the sugars released from glyoxysomal peptide components on their hydrolysis.

The pentose sugar D-arabinose has been found to occur in at least two plant glycoproteins, a glycopeptide obtained from potato lectin (Allen *et al.*, 1976) and the protein extensin, a component of plant cell walls (Lamport *et al.*, 1973). Castor bean endosperm was found to be capable of incorporating [<sup>3</sup>H] arabinose into a TCA-insoluble macromolecule (fig. R2.7) which was located primarily in the cell's cytoplasmic compartment. Once again, there was little association

with the mitochondria, plastids or glyoxysomes. Although by no means conclusive, the evidence provided by these latter two results would indicate that O-glycosylation does not feature prominently in the synthesis of organelle proteins, other than those of the e.r. and, possibly, Golgi in germinating castor bean endosperm tissue.

RESULTS AND DISCUSSION III

R/D.3 - THE HETEROGENEOUS DISTRIBUTION OF GLYCOSYL-  
TRANSFERASES IN THE ENDOPLASMIC RETICULUM  
OF CASTOR BEAN ENDOSPERM

Ribosome-denuded e.r. membranes were purified from castor bean endosperm homogenates by the standard sucrose gradient centrifugation procedure. The EDTA present in the homogenization medium and the sucrose solutions, used for gradient formation, served to disrupt ribosome attachment in the rough endoplasmic reticulum (which constitutes the bulk of the e.r. in the cells of this tissue [Vigil, 1970]). The membrane vesicles at a mean buoyant density of 1.12 g/ml therefore contained microsomes of both rough and smooth e.r. origin. Maintaining ribosome attachment (by omitting EDTA and increasing the magnesium ion concentration in the homogenization medium from 1 mM to 3 mM) results in the recovery of over 90% of the protein, phospholipid and marker enzyme activity present in the microsomal band (fractions 13 - 15 in fig R1.1) at higher densities (Lord et al., 1973).

The microsomal membranes were removed from the initial fractionation gradients and further subfractionated by flotation. The sucrose concentration of the microsomal suspension was increased to approximately 50% by the addition of a 70% (w/w) sucrose solution. A discontinuous sucrose gradient of 40%, 30% and 20% (w/w) sucrose was formed over the sample layer and the gradient centrifuged for 17 h, this prolonged period being necessary for the membranes to regain their

Fig. R3.1

Subfractionation of Isolated Endoplasmic Reticulum Vesicles By Centrifugation on a Flotation Gradient

Three ml of an endoplasmic reticulum vesicle suspension isolated from 3-day-old endosperm tissue by density gradient centrifugation (fractions 13 - 15, Fig. R1.2) were mixed with 2.5 ml of 70% (w/w) sucrose and placed in a centrifuge tube. This suspension was overlaid with 40%, 30% and 20% (w/w) sucrose and centrifuged at 24,000 r.p.m for 17 h at 2°C. After centrifugation two membrane bands were visible (arrows).

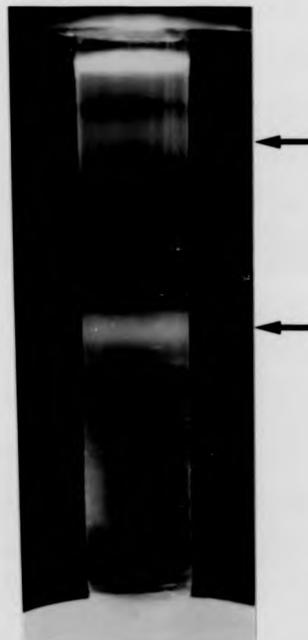
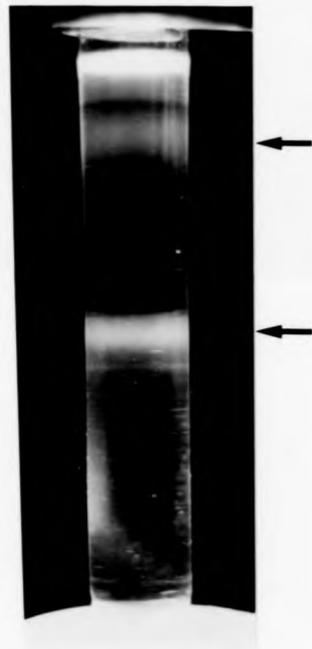


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Subfractionation of Isolated Endoplasmic Reticulum Vesicles By Centrifugation on a Flotation Gradient

Three ml of an endoplasmic reticulum vesicle suspension isolated from 3-day-old endosperm tissue by density gradient centrifugation (fractions 13 - 15, Fig. R1.2) were mixed with 2.5 ml of 70% (w/w) sucrose and placed in a centrifuge tube. This suspension was overlaid with 40%, 30% and 20% (w/w) sucrose and centrifuged at 24,000 r.p.m for 17 h at 2°C. After centrifugation two membrane bands were visible (arrows).



mean buoyant densities. The appearance of the gradient following centrifugation is shown in fig. R3.1. Two membrane bands are visible, at the 40 to 30% and the 30 to 20% sucrose interfaces. The uppermost band contained twice as much protein as the lower band and similar distributions of radioactive proteins and GlcNAC-containing glycoproteins were obtained following the fractionation of tissue labelled in vivo with either a [ $^{14}\text{C}$ ] amino acid mixture or [ $^3\text{H}$ ] GlcNAC (fig. R3.2a and b). The membrane subfractions are both apparently derived from the e.r. since they share the characteristic e.r. marker enzymes cholinephosphotransferase and NADH-cytochrome<sub>c</sub> reductase (fig. R3.3a). Both membrane subfractions accumulated [ $^{14}\text{C}$ ] phosphatidyl-choline synthesized in vivo by incubating intact tissue with CDP-[methyl  $^{14}\text{C}$ ] choline (fig. R3.3b).

Castor bean endosperm membrane glycoproteins contain N-glycosydically-linked oligosaccharide chains and the incorporation of [ $^{14}\text{C}$ ] glucosamine into TCA-insoluble membrane components is inhibited by prior treatment of the tissue with tunicamycin (Bergner and Tanner, 1981; section 4). The e.r. membrane has been shown to catalyze the synthesis of dolichol-linked intermediates containing N-acetyl glucosamine and mannose, their incorporation into mannose-rich oligosaccharide-lipid and the transfer of the oligosaccharide from its lipid carrier to asparagine residues in the acceptor proteins (Marriott and Tanner 1979; Mellor et al. 1979a; Nagahashi and Beevers, 1978). The e.r. subfractions obtained from flotation gradients were assayed for the presence of

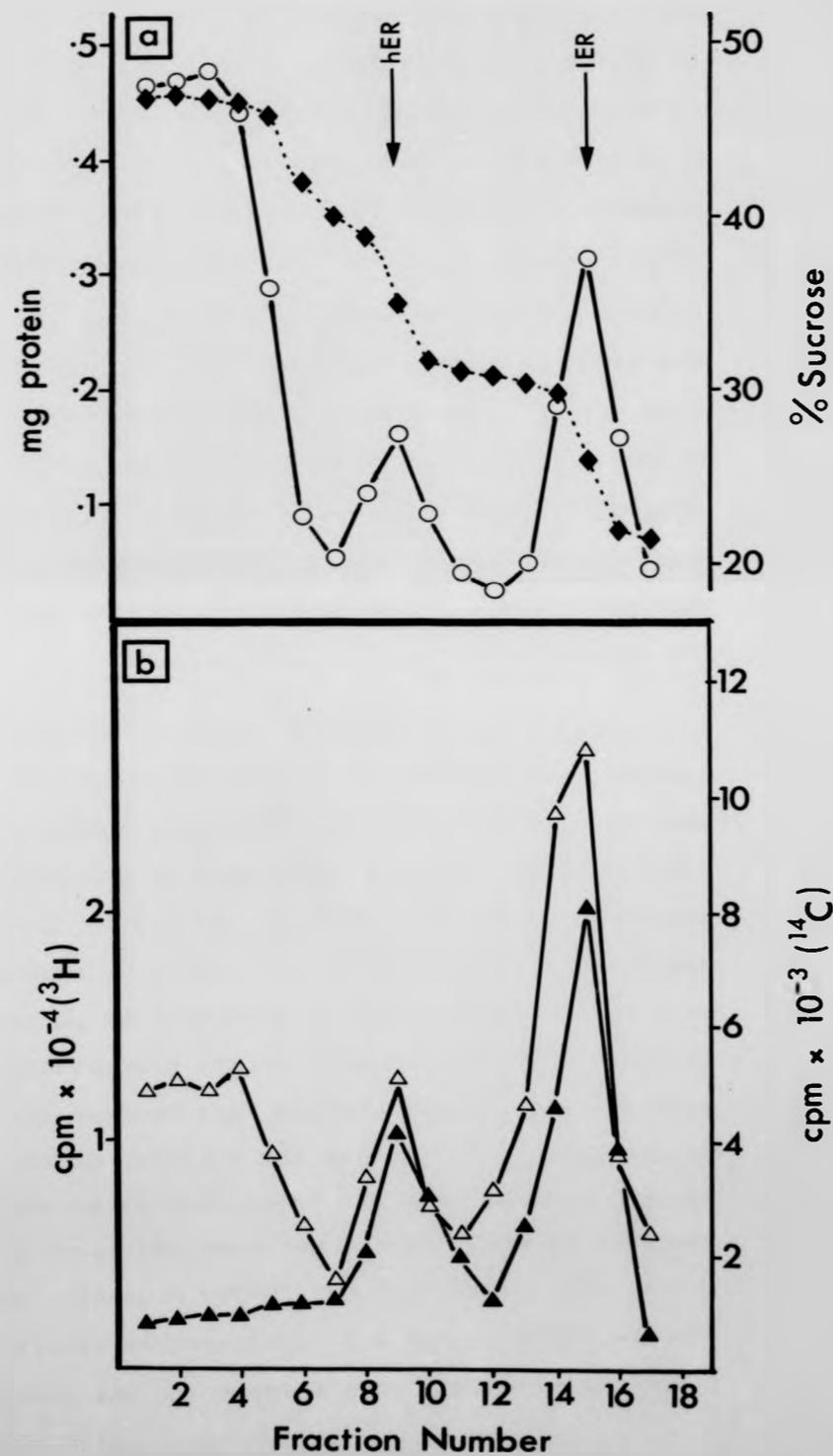
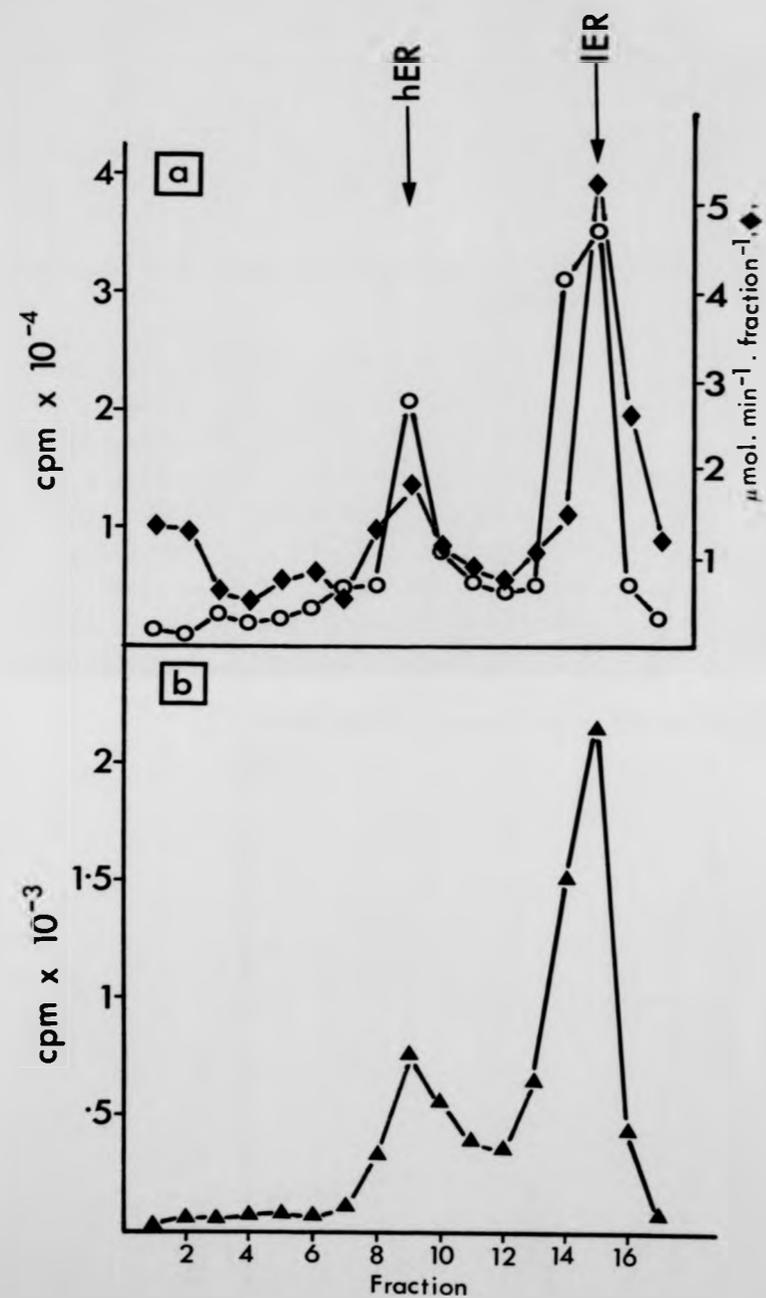


Fig. R3.2 Protein Distribution Between E. R. Vesicle Subfractions

After centrifugation, 1.0 ml fractions from the flotation gradient were assayed for protein (O) and sucrose (◆) content (a). (b), the distribution of TCA-insoluble radioactivity in fractions obtained using e.r. vesicles derived from [<sup>14</sup>C]-labelled amino acid (Δ, 2 μCi /endosperm half) or N-acetyl-[<sup>3</sup>H] glucosamine (▲, 10 μCi /half) labelled tissue. Incubation with label was for 2 h at 30°C.

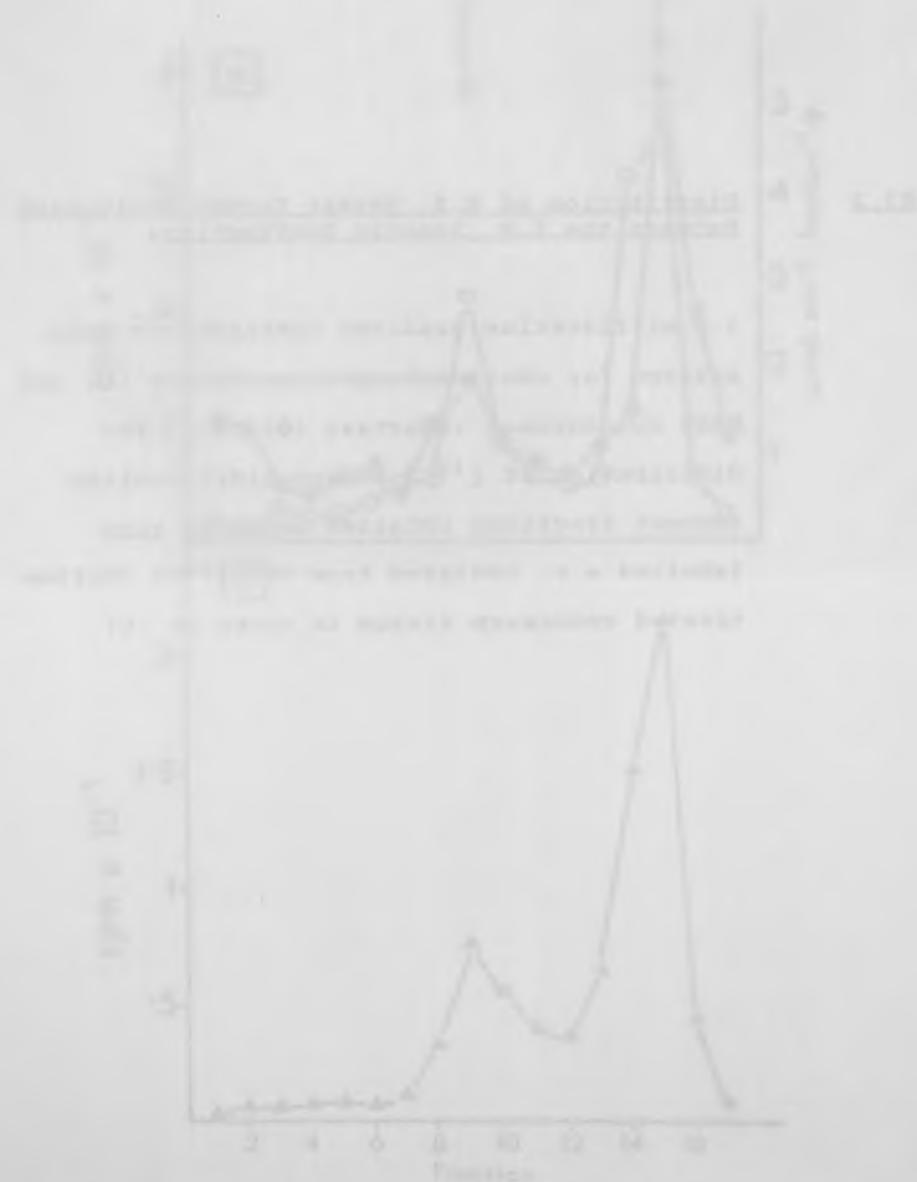


R3.3 Distribution of E.R. Marker Enzyme Activities Between the E.R. Vesicle Subfractions

1.0 ml flotation gradient subfractions were assayed for cholinephosphotransferase (○) and NADH cytochrome<sub>c</sub> reductase (◆) (a). The distribution of [<sup>14</sup>C] phosphatidyl-choline amongst fractions obtained using *in vivo* labelled e.r. isolated from CDP-[<sup>14</sup>C] choline treated endosperm tissue is shown in (b).

several glycosyl transferases catalyzing the transfer of sugar moieties from nucleotide diphosphate derivatives to a lipid acceptor and the assembly of oligosaccharide lipids from monosaccharide lipids. In the cases of GlcNAc and mannose the transferases catalyzing their incorporation into lipid-linked intermediates were located predominantly in the upper e.r. subfraction (figs. R3.4 and R3.5). The involvement of dolichol as the lipid carrier was shown by its stimulatory effect when added exogenously to the assay systems, thereby confirming the results of Mellor (Mellor and Lord, 1979a; Mellor *et al.*, 1980). In the case of monosaccharide-lipids, exogenous dolicholmonophosphate increased their synthesis 20- to 30-fold when added at a concentration of 30 $\mu$ g/ml (figs. R3.4a and R3.5a). The distribution of the enzymic activity catalyzing glucose transfer from UDP-glucose to chloroform/methanol-soluble glucolipid (section 2) between the two subfractions was also investigated (fig. R3.6) and was shown to be similar to the total protein distribution (table 2). The addition of exogenous dolicholmonophosphate had no stimulatory effect on glucolipid synthesis and glucose was not incorporated into oligosaccharide-lipid (fig. R3.6).

Glycoprotein fucosyl-transferase is another enzyme which has been localized in the e.r. of germinating castor bean (Roberts *et al.*, 1980). L-fucose is a peripheral sugar in complex-type oligosaccharide side-chains of N-linked glycoproteins. The location of the fucose molecule is usually via an  $\alpha 1 \rightarrow 6$  linkage to the GlcNAc residue involved in the attachment of the oligosaccharide chain to the receptor asparagine residue



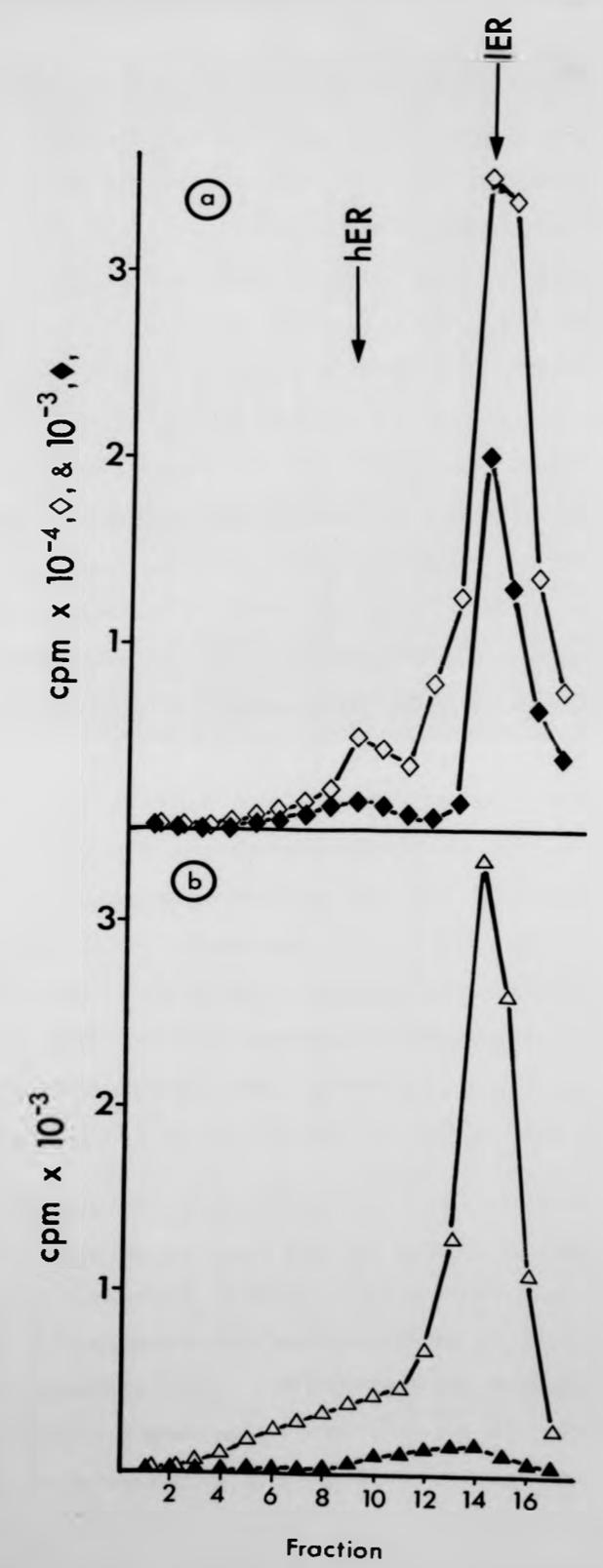


Fig. R3.4 Distribution of Glycolipid N-acetylglucosaminyltransferase Between E.R. Subfractions

Aliquots of collected flotation gradient fractions were incubated with UDP-N-acetyl [<sup>14</sup>C]-glucosamine at 30°C in the presence ( $\diamond$ ) or absence ( $\blacklozenge$ ) of exogenous dolichol-monophosphate. After 30 minutes, the incorporation of radioactivity into chloroform/methanol (2 : 1)-soluble monosaccharide lipid (a), or chloroform/methanol/water (10 : 10 : 3)-soluble oligosaccharide lipid (b) was determined.

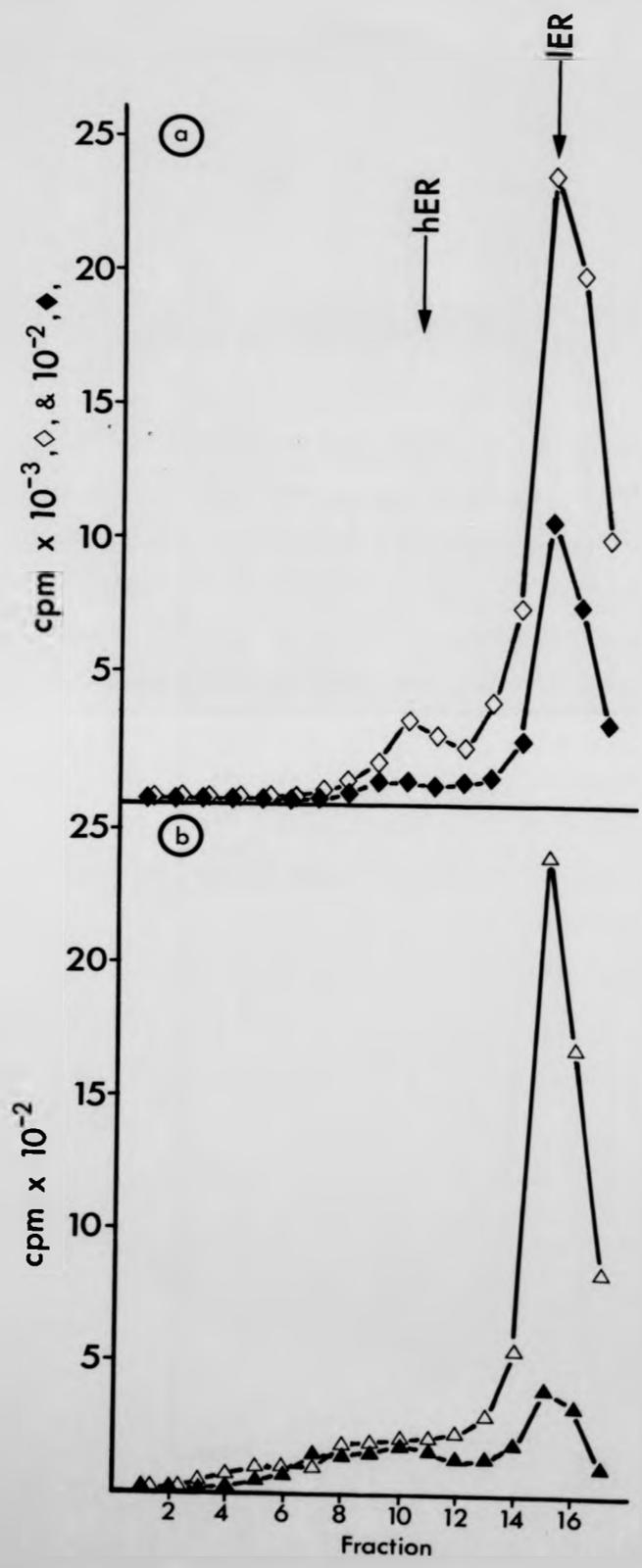


Fig. R3.5 Distribution of Glycolipid Mannosyltransferase Between E.R. Subfractions

Aliquots of collected flotation gradient fractions were incubated with GDP- $^{14}\text{C}$  mannose at  $30^\circ\text{C}$  in the presence ( $\triangle$ ) or absence ( $\blacktriangle$ ) of exogenous dolichol-monophosphate. After 30 minutes, the incorporation into chloroform/methanol (2 : 1)-soluble monosaccharide lipid (a), or chloroform/methanol/water (10 : 10 : 3)-soluble oligosaccharide lipid (b) was determined.

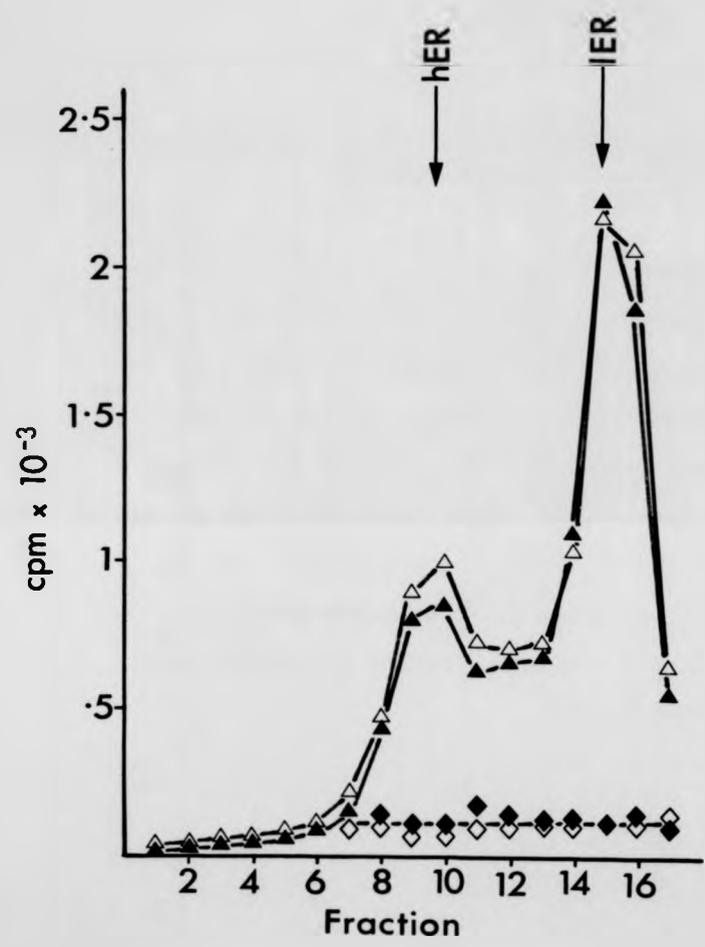
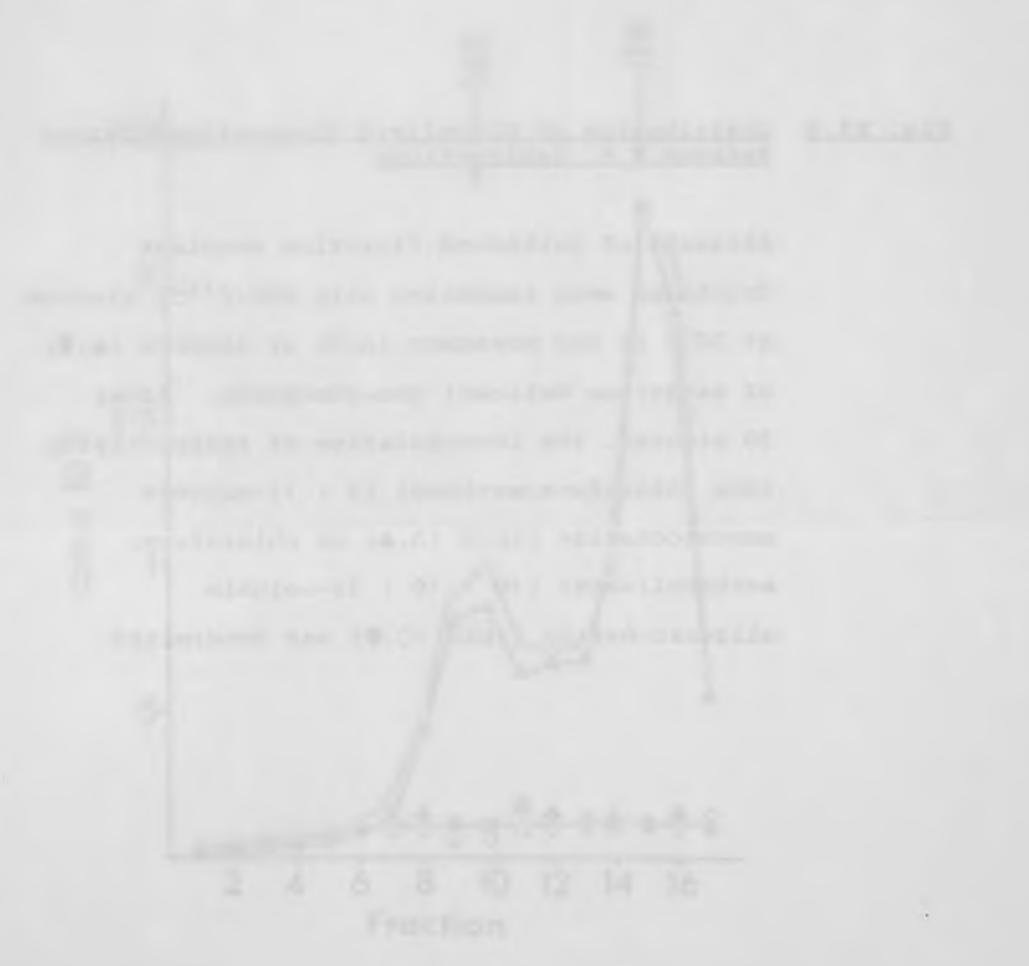


Fig. R3.6 Distribution of Glycolipid Glucosyltransferase Between E.R. Subfractions

Aliquots of collected flotation gradient fractions were incubated with UDP-[<sup>14</sup>C] glucose at 30°C in the presence (Δ,◇) or absence (▲,◆) of exogenous dolichol monophosphate. After 30 minutes, the incorporation of radioactivity into chloroform/methanol (2 : 1)-soluble monosaccharide lipid (Δ,▲) or chloroform/methanol/water (10 : 10 : 3)-soluble oligosaccharide lipid (◇,◆) was determined.

(Kornfeld and Kornfeld, 1980 for review). The transferases involved in the addition of peripheral sugars in general, and fucose in particular, are located in the Golgi apparatus in mammalian tissues (Schacter *et al.*, 1970). The assay of e.r. subfractionation gradients for glycoprotein fucosyl-transferase revealed that this enzyme is distributed differently to the other glycosyltransferases with approximately 40% associated with the upper fraction, 30% with the lower e.r. band and the remaining 30% with the 50% sucrose-containing initial sample layer (fig. R3.7). The activity of the fucosyl-transferase, on a protein basis, is therefore greater in the lower fraction.

The heterogeneity in enzyme distribution between the two e.r. subfractions is summarised in table 2. The upper fraction contained approximately twice as much protein as the lower fraction. Likewise it contained 65 - 70% of the cholinephosphotransferase, NADH-cytochrome<sub>c</sub> reductase and glucosyltransferase which have similar activities in each subfraction. Over 90% of the total N-acetylglucosaminyl- and mannosyl-transferases were present in the uppermost subfraction at specific activities some 5-fold higher than those of the lower subfraction, whereas the specific activity of the fucosyl-transferase was some 1.5 times greater in the lower subfraction. The increased activity of the fucosyl-transferase in the lower fraction prevents attributing the reduced GlcNAc- and mannosyl-transferase levels to the destruction of nucleotide sugars by microsomal pyrophosphatase or glycosidase (Bergman and Dallner, 1978). Further, the total glycosyl-transferase



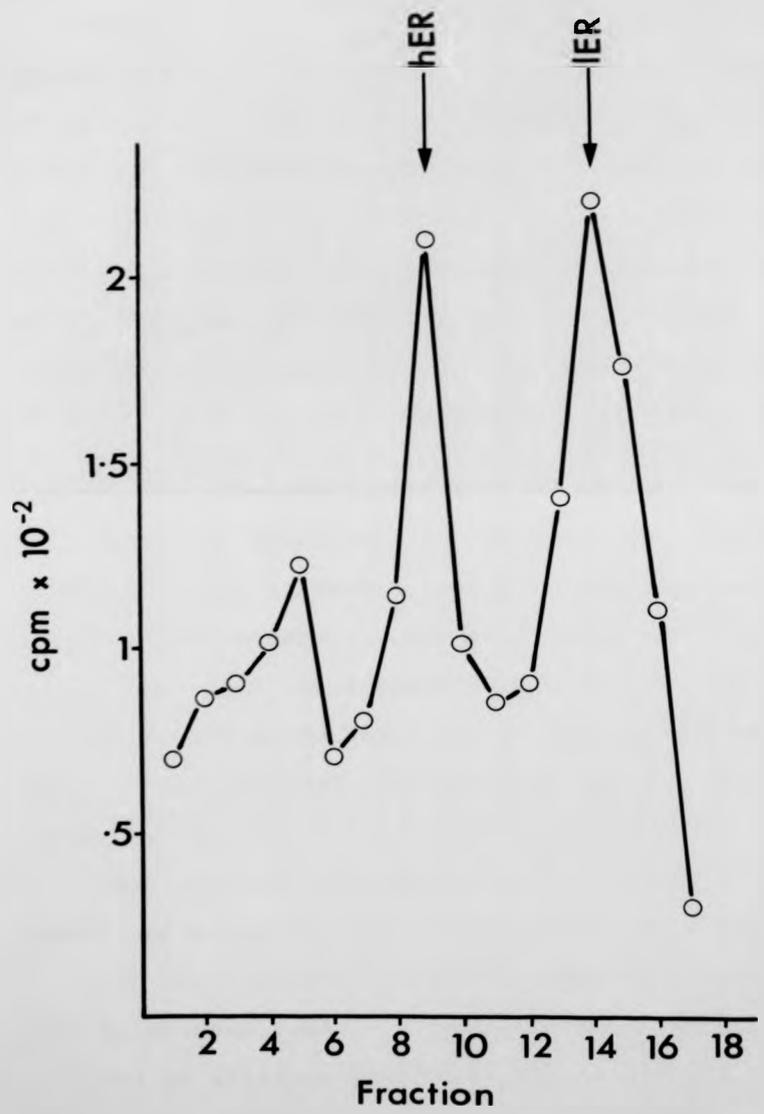


Fig. R3.7 Distribution of Glycoprotein Fucosyltransferase  
Between E.R. Subfractions

Isolated endoplasmic reticulum vesicles were subfractionated by flotation and collected gradient fractions were incubated with GDP-[<sup>14</sup>C] fucose at 30°C. After 30 minutes radioactivity present in trichloroacetic acid insoluble product was determined.

TABLE 2

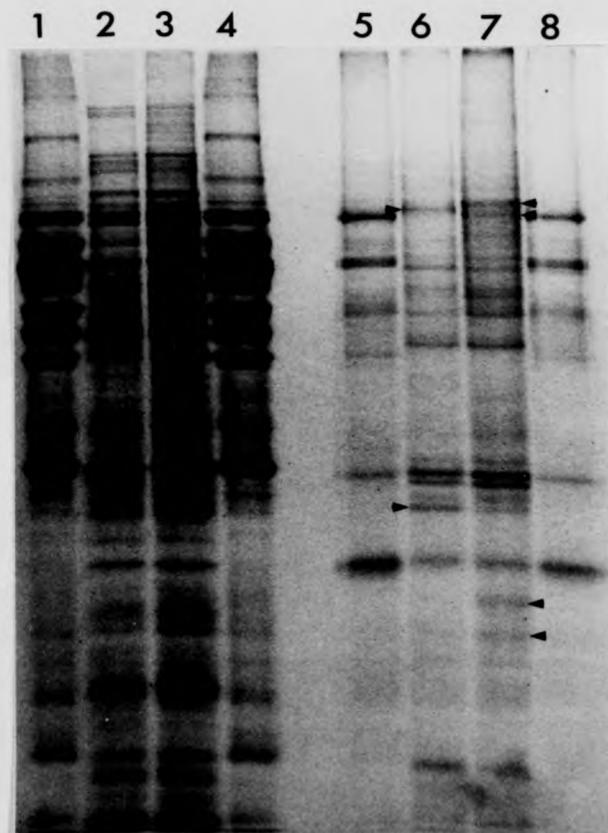
Constituent	LOWER E. R. BAND			UPPER E. R. BAND		
	Activity	Activity/mg Protein	% Total	Activity	Activity/mg Protein	% Total
Protein	380mg		34	730mg		66
Cholinephospho-transferase	30	78.9	30	70	95.9	70
NADH cytochrome c reductase	4.3	11.3	29	10.7	14.6	71
Fucosyl-transferase	9.3	27.2	22.4	14.1	19.89	34.1
Glucosyl-transferase	8.2	21.7	31	18.4	25.2	69
Mannosyl-transferase:-						
(a) monosaccharide lipid	68.2	179.5	10	600	821.9	90
(b) oligosaccharide lipid	5.4	14.2	9	54.8	75.1	91
N-acetylglucosaminyl-transferase:-						
(a) monosaccharide lipid	75.5	198.7	9	724.5	992.5	91
(b) oligosaccharide lipid	6.4	16.8	7	61.3	83.9	93

Table 2 Enzyme Distribution Between Endoplasmic Reticulum Subfractions

The distribution of enzymic activity between the lower gradient e.r. band and the upper gradient e.r. band is compared. With the exception of NADH cytochrome<sub>c</sub> reductase, for which activity is expressed as  $\mu\text{mol}/\text{min}$ , all other enzyme activities are given as  $\text{nmol}/\text{hr}$ .

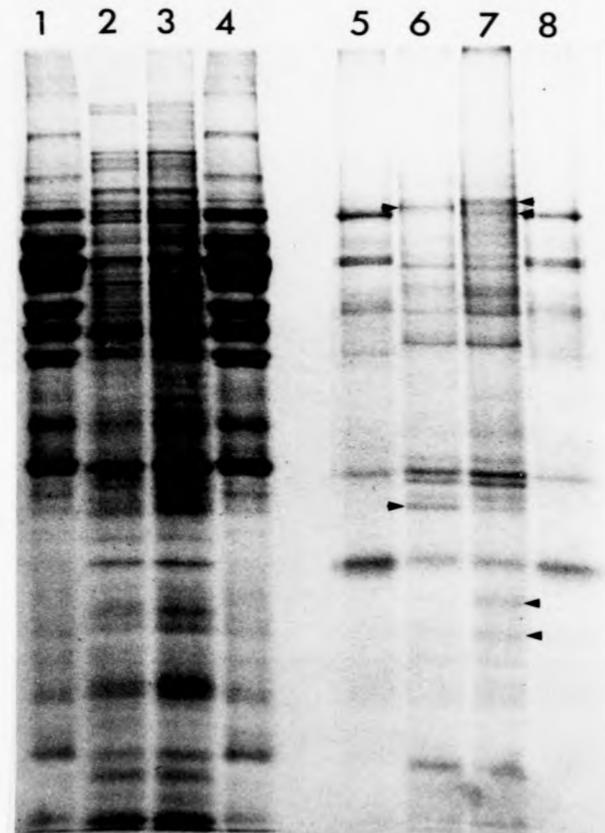


Fig. R3.8 Dodecylsulphate-Polyacrylamide Gel Electrophoresis of E.R. Membrane Proteins After Phase Separation In Triton X-114 Solution



E.R. and glyoxysomal membranes were isolated and the e.r. membranes were subfractionated by flotation. Membrane proteins were partitioned into water-soluble hydrophilic components and detergent-soluble amphiphilic integral membrane proteins by phase separation in Triton X-114 solution before being analysed electrophoretically. Lanes 1 and 4, water-soluble glyoxysomal membrane proteins: lane 2, water-soluble proteins from the lower e.r. subfraction: lane 3, water-soluble proteins from the upper e.r. subfraction: lanes 5 and 8, detergent-soluble glyoxysomal membrane proteins: lane 6, detergent-soluble proteins from the lower e.r. subfraction: lane 7, detergent-soluble proteins from the upper e.r. subfraction. Protein bands were visualised by coomassie blue staining.

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hydrophobic integral membrane protein profiles (fig. R3.8 lanes 6 and 7) reveals that the two e.r. subfractions share an almost identical set of integral polypeptides though some differences (fig. R3.8, arrowheads) were noted. Both the water-soluble and Triton-soluble polypeptide profiles from the e.r. subfractions were clearly different from those in corresponding fractions prepared from glyoxysomes (fig. R3.8, compare track 1 with 2, 3 with 4, 5 with 6 and 7 with 8), however, as in fig. R1.8, most of the glyoxysomal integral membrane proteins, as well as some of the water-soluble proteins, can be seen to have counterparts of similar mobility in the e.r. profiles.

Demonstrations of biochemical heterogeneity in microsomal vesicles has previously been restricted largely to results obtained using mammalian tissues (Beaufay *et al.*, 1974a; Salviati *et al.*, 1982). Numerous quantitative differences in enzymic distribution between rough and smooth e.r. and in subfractions derived therefrom have been reported (Beaufay *et al.*, 1974b; Dallman *et al.*, 1969; Svensson *et al.*, 1972). This heterogeneity suggests that there may be distinct regions of the e.r. specialised for particular functions, e.g. one such region may be enriched in dolichol-linked glycosyl-transferases involved in the assembly of the core-oligosaccharide structure transferred to proteins during glycosylation. Another may carry out the "trimming" of this core-structure and the addition of peripheral sugars such as L-fucose. It may be further speculated that the former

region, the upper e.r. fraction in the present study, is derived from the rough e.r. since (a) it is thought that the core-sugars are added to the nascent polypeptide chains before the termination of their synthesis (Katz *et al.*, 1977; Kiely *et al.*, 1976) and (b) rough e.r. has been identified as the intracellular location of dolichol-utilizing glycosyl-transferases (Czichi and Lennarz, 1977; Hannover and Lennarz, 1980; Nagahashi and Beavers, 1978).

RESULTS IV

R.4 - GLYCOPROTEINS OF THE GLYOXYSOMAL MEMBRANE

R.4A - LABELLING OF GLYOXYSOMAL MEMBRANE GLYCOPROTEINS

Intact endosperm tissue was pulse-labelled for 6 h with [<sup>3</sup>H] glucosamine then chased for various time intervals with a 2,000-fold excess of unlabelled glucosamine. The tissue was fractionated and the three major organelle bands removed. Equal proportions of each of the fractions were precipitated with cold TCA then the radioactivity associated with each precipitate determined. The membranes from the remainder of the fractions were Triton X-114 extracted and the aqueous and detergent phase-associated radioactivity determined after precipitation with cold TCA. The results are shown in fig. R4.1. Initially the glucosamine appears to become incorporated into the e.r. fraction, its association with other organelles, i.e. the mitochondria and glyoxysomes, was seen to increase after an initial lag phase of approximately 3 h and to reach a plateau after 12 h (fig. R4.1a). Analysing this incorporation more closely, i.e. into Triton-soluble and aqueous phase associated proteins, reveals that [<sup>3</sup>H] glucosamine is incorporated to a greater extent into the hydrophobic proteins of the membranes (fig. R4.1b and c). The amount of radioactivity incorporated into the glyoxysomal integral membrane proteins was consistently higher than that incorporated into those of the mitochondria (fig. R4.1b).

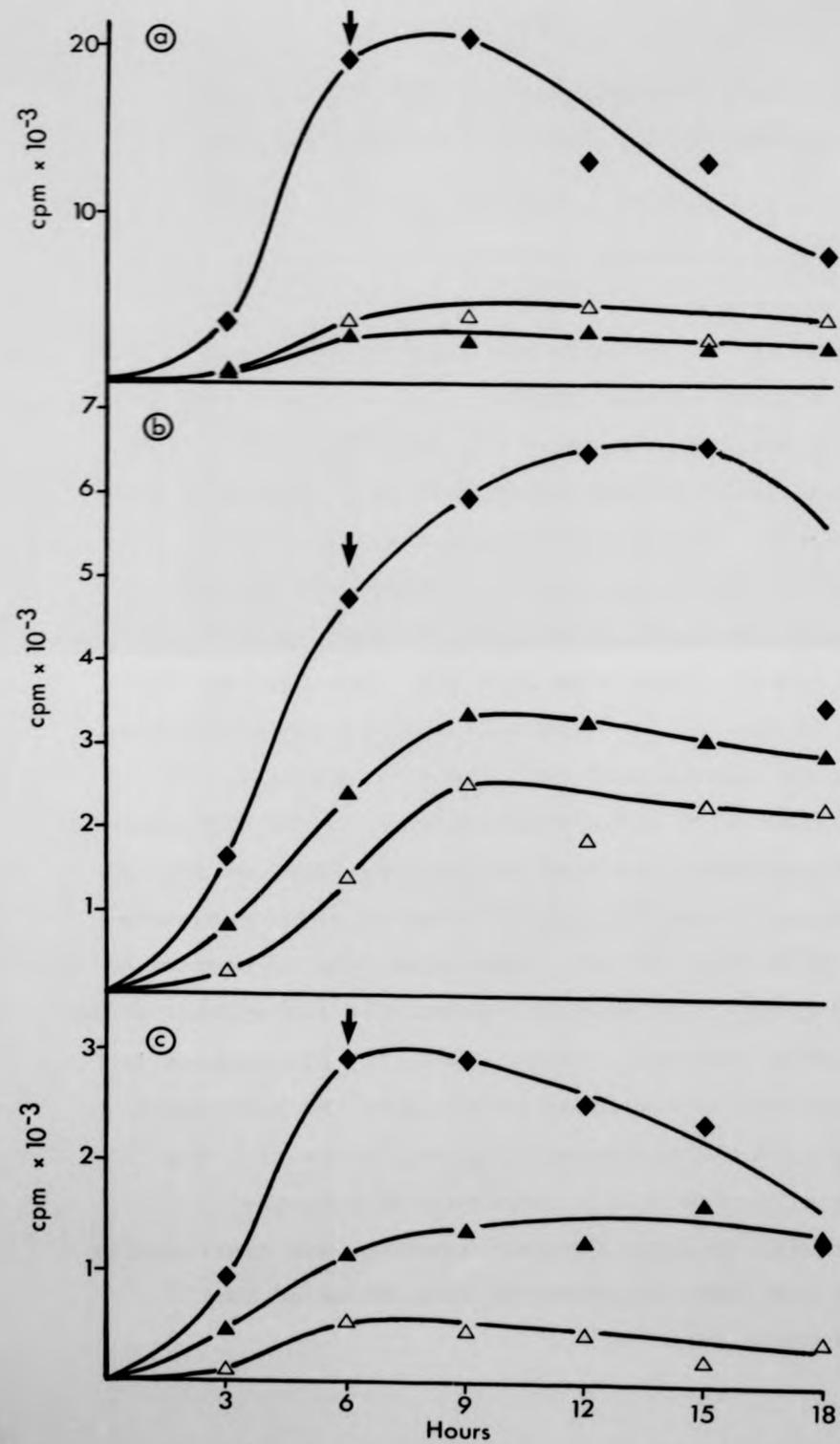


Fig. R4.1 Time Courses for  $[^3\text{H}]$  Glucosamine Incorporation Into E.R., Mitochondria and Glyoxysomes In Vivo

Batches of six 3-day-old endosperm halves were incubated at 20°C with either  $[^3\text{H}]$  glucosamine (5  $\mu\text{Ci}/\text{half}$ ) alone, for 3 or 6 h, or pulsed with  $[^3\text{H}]$  glucosamine, for 6 h, then chased with a 2,000-fold excess of unlabelled glucosamine for 3, 6, 9 and 12 h. The batches of tissue were then homogenized and fractionated on 17 ml sucrose gradients. The major organelle-containing bands were collected in standard volumes. Samples were removed from each for total TCA-insoluble radioactivity determination (a). The remainder of each sample was diluted with 50 mM Tris (pH 7.4), the membranes harvested by centrifugation and then subjected to Triton X-114 extraction. The TCA-insoluble radioactivity associated with the detergent and aqueous phases (b and c respectively) was then determined. C.p.m.'s associated with e.r., mitochondrial and glyoxysomal fractions represented by  $\blacklozenge$ ,  $\triangle$  and  $\blacktriangle$  respectively. Arrows indicate time chase applied.

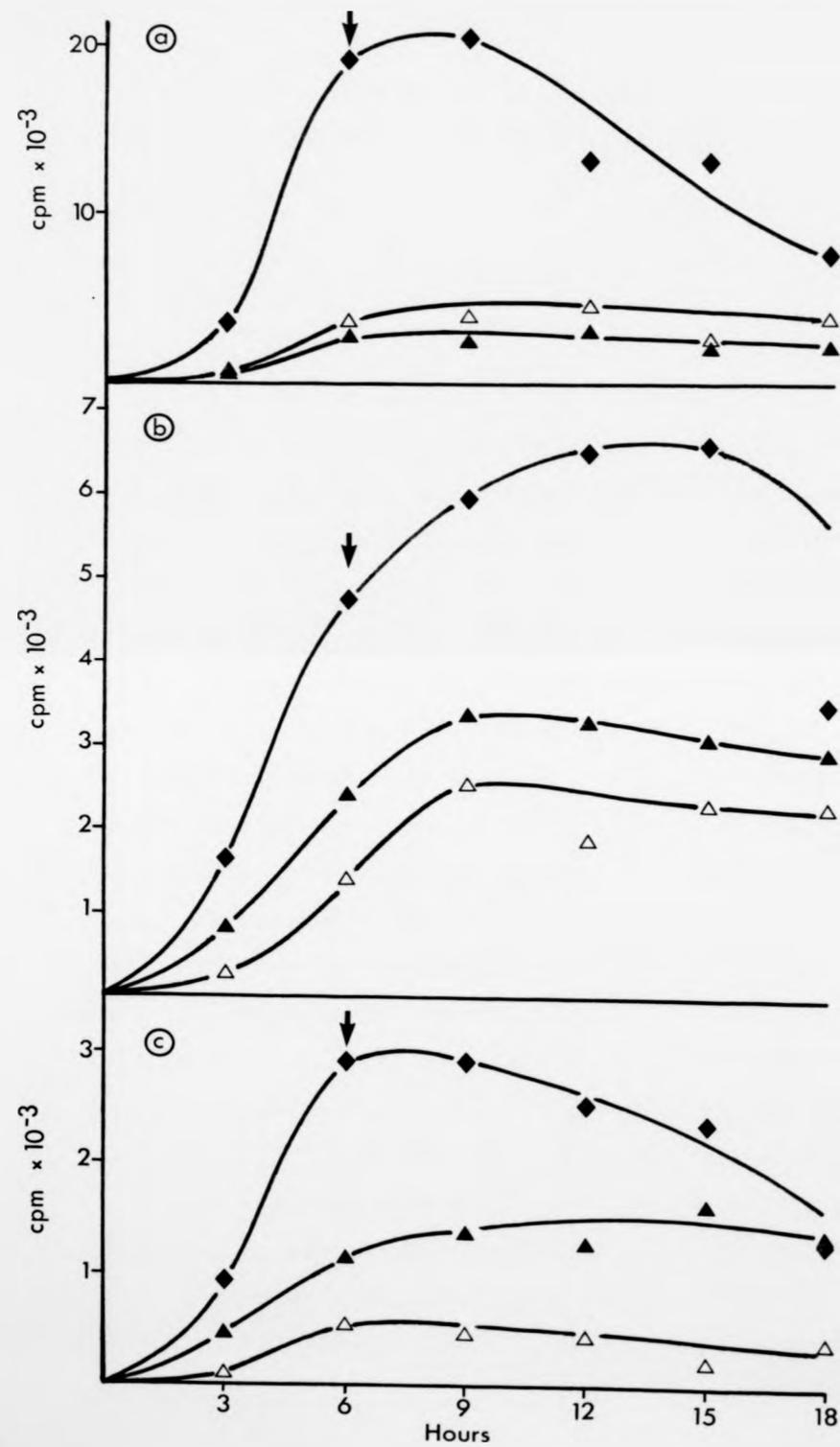


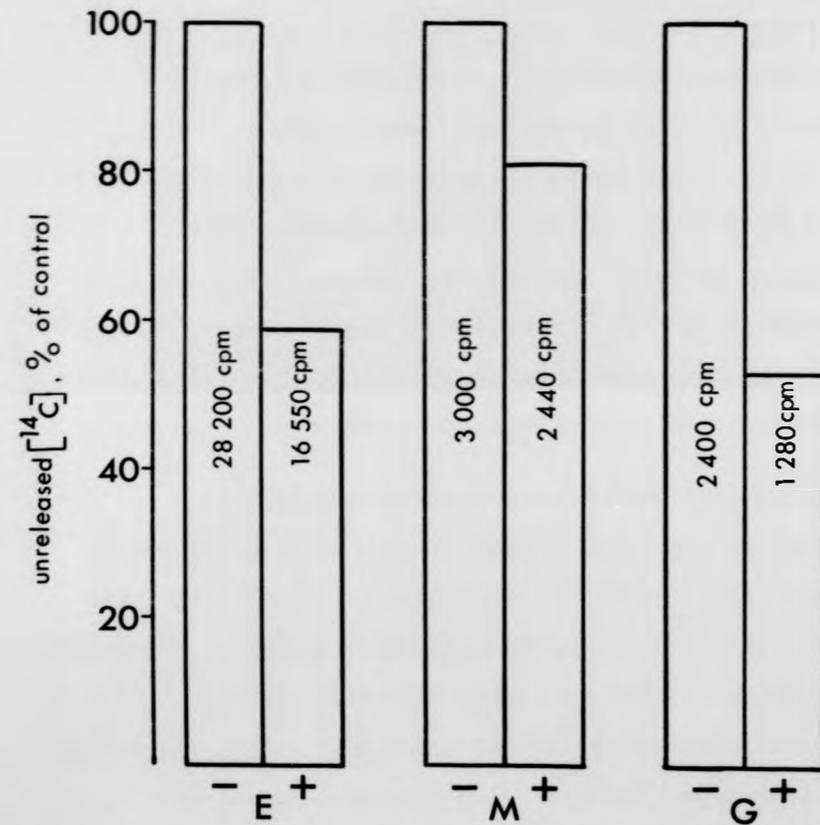
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In order to verify that the glucosamine had been incorporated into N-glycoproteins, [<sup>3</sup>H] glucosamine-labelled organelle membranes were subjected to digestion by endo-N-acetylglucosaminidase H (endo-H) for 24 h at 37°C in the presence of 0.2% NP-40. Following the 24 h incubation period the membrane suspension was TCA precipitated and the insoluble radioactivity compared to that associated with a TCA precipitate from a duplicate membrane sample which had not been exposed to endo H (fig. R4.2). The membrane-associated radioactivity was reduced by the endo-H in all three cases, the e.r. and glyoxysomes by some 40% and 50% respectively and the mitochondria by 20%. This latter figure may reflect the higher protein concentration in the incubation mixture containing mitochondria-derived membranes.

A further indication that glucosamine was incorporated into the organelle membrane proteins by N-glycosylation resulted from the inhibition of this incorporation by the antibiotic tunicamycin. Endosperm tissue was pre-incubated with tunicamycin for 30 minutes before inoculating with [<sup>3</sup>H] glucosamine and incubating for varying time periods. The incorporation of glucosamine into the microsomal, mitochondrial and glyoxysomal membranes were compared to that which occurred in the absence of tunicamycin treatment. The time-courses of [<sup>3</sup>H] glucosamine incorporation into the three organelle membrane-types are shown in fig. R4.3a-c. In all three cases incorporation was inhibited after tunicamycin pretreatment. In the case of the microsomes and mitochondria peak incorporation of radiolabel in the absence of tunicamycin, was reached after 6 h whereas

Fig. R4.2 Endo-H Treatment of [<sup>14</sup>C] glucosamine-labelled Organelle Membranes



Ten 3-day-old endosperm halves were incubated with [<sup>14</sup>C] glucosamine (1  $\mu$ Ci/half) for 18 h then the tissue was fractionated on sucrose density gradients. The major organelle-containing bands were collected and 250  $\mu$ l aliquots diluted with 100 mM citrate/phosphate buffer (pH 5.0) containing 0.4% (v/v) NP-40 and 80  $\mu$ g/ml PMSF (NP-40 buffer) or NP-40 buffer containing endo-H at 20 mU/ml. The mixtures were overlaid with toluene and incubated at 37°C for 24 h. The toluene layer was removed and the proteins precipitated with cold TCA. TCA-insoluble radioactivity was determined following filtration and washed with chloroform/methanol (2 : 1) and 10% TCA. Radioactivity (c.p.m) remaining after endo-H treatment (+) is expressed as a percentage of that associated with the untreated samples (-).

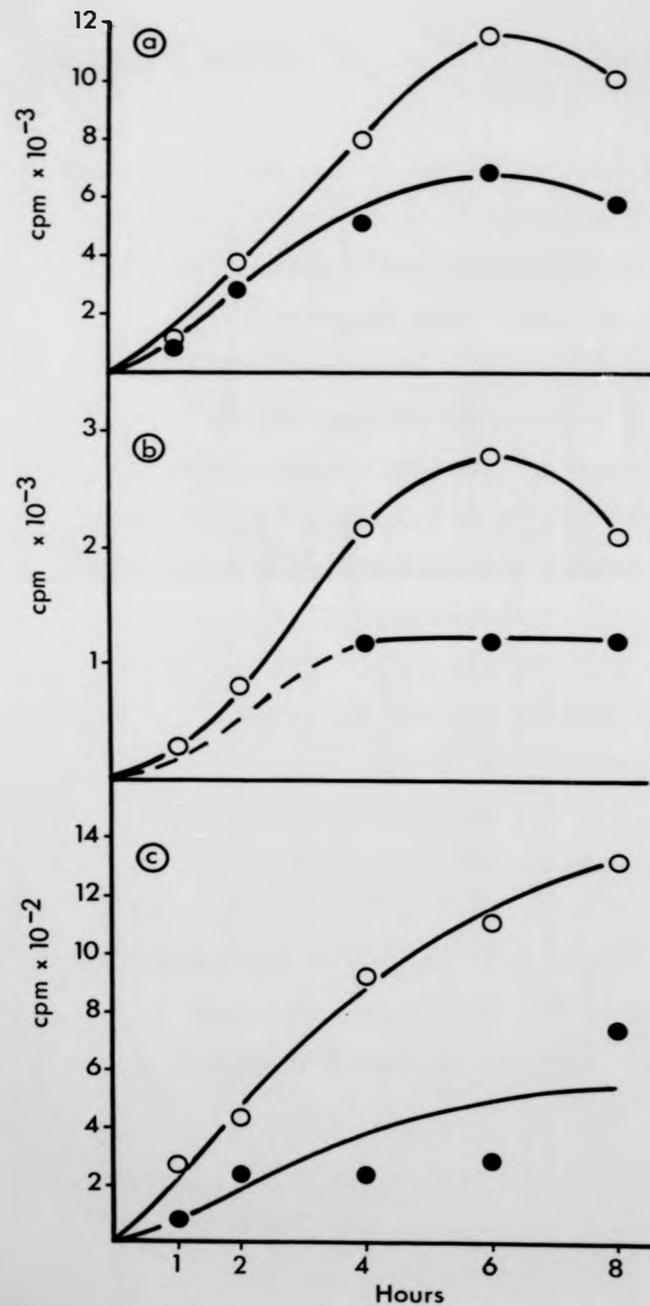


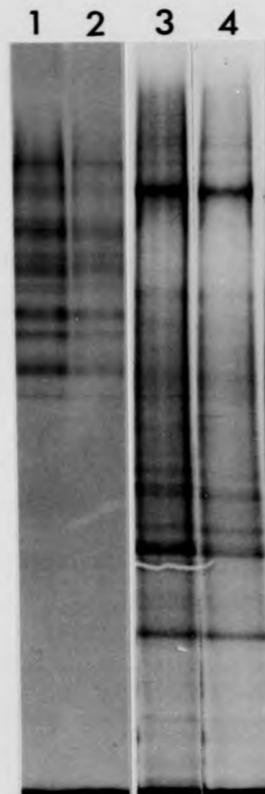
Fig. R4.3 The Effect of Tunicamycin on the Rate of Incorporation of [<sup>14</sup>C] glucosamine Into Organelle Fractions

Batches of 20 endosperm slices (two slices derived from one endosperm half) were pre-treated with tunicamycin, in 1 mM NaOH (20  $\mu$ g, in 20  $\mu$ l, per slice) or 1 mM NaOH only and incubated for 30 minutes at 30°C. The slices were then inoculated with [<sup>14</sup>C] glucosamine (0.05  $\mu$ Ci, in 10  $\mu$ l, per slice) and incubated for 1, 2, 4, 6 and 8 h periods before fractionating on sucrose gradients. The organelle-bands were collected and the TCA-insoluble radioactivity associated with each determined. Time courses for [<sup>14</sup>C] glucosamine incorporation into (a) e.r., (b) mitochondria and (c) glyoxysomes are shown both with (●) and without (○) tunicamycin pretreatment.

with the glyoxysomes [ $^3\text{H}$ ] glucosamine was incorporated at a linear rate for the entire 8 h experimental period. The glycopeptide profile of glyoxysomes labelled with [ $^3\text{H}$ ] GlcNAc with and without a preincubation with tunicamycin is shown in fig. R4.4 (lanes 1 and 2) and the reduced amount of incorporation in the presence of antibiotic is evident. This was in contrast to the situation with total protein labelling, with [ $^{35}\text{S}$ ] methionine, where no effect of tunicamycin was observed (fig. R4.4 lanes 3 and 4).

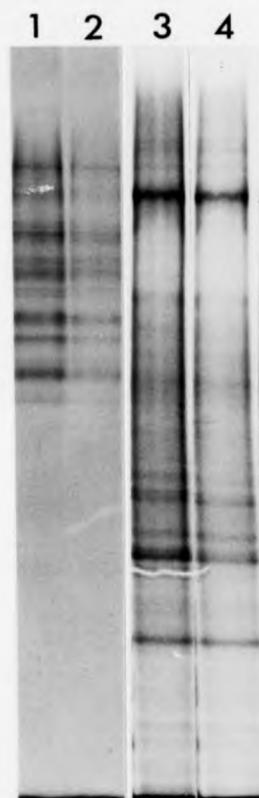
The [ $^3\text{H}$ ] glucosamine-containing glycopeptide profiles of the three major organelle membranes are shown in fig. R4.5 following the phase separation of integral and peripheral proteins by Triton X-114 extraction. The e.r., which is the site of [ $^3\text{H}$ ] glucosamine incorporation into glycoproteins, was the most heavily labelled fraction (fig. R4.5, lanes 3 and 5). Within the microsomal fraction it was the Triton-associated integral membrane proteins which incorporated the majority of the tritiated sugar. This is also true of the other membrane fractions though the most striking feature of the glyoxysomal and mitochondrial integral membrane glycoprotein profiles is their similarity (fig. R4.5, compare lanes 6 and 9). The aqueous phase-associated glycoprotein profiles of these latter membranes differ in intensity, the glyoxysomal profile (fig. R4.5, lane 7) indicating a much greater abundance of peripherally-associated glycoproteins in this fraction. The striking similarities between the detergent-associated profiles raises, once again, the question of cross-contamination between organelle fractions.

**Fig. R4.4** The Effect of Tunicamycin on the Polypeptide Profiles of the Glyoxysomal Membrane

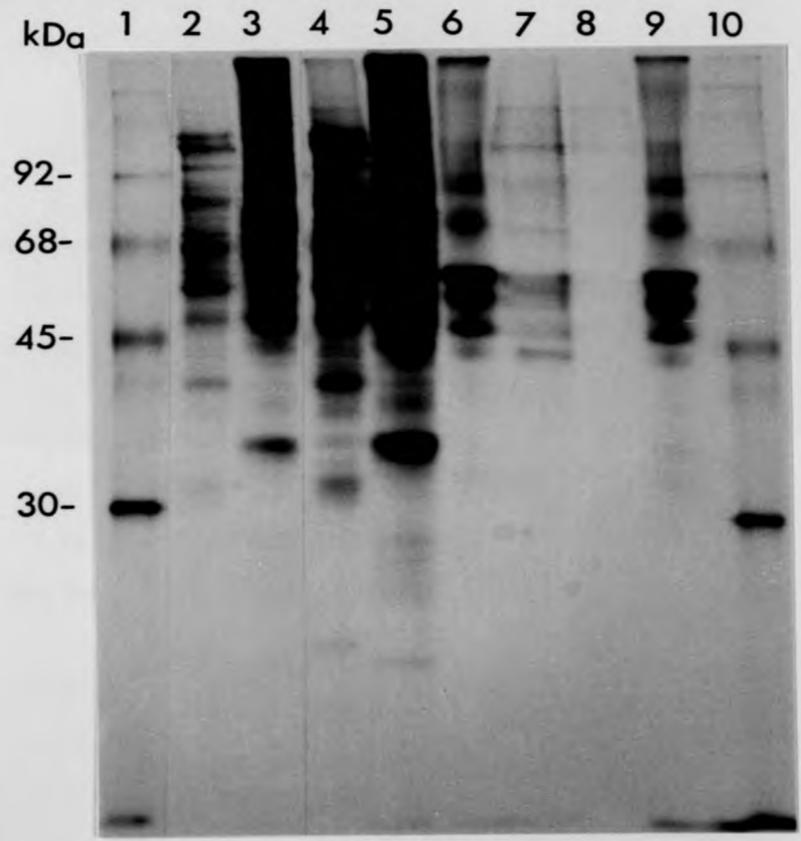


Batches of 10 endosperm slices were incubated with [<sup>35</sup>S] methionine (20  $\mu$ Ci/slice) or N-acetyl-[<sup>3</sup>H] glucosamine ([<sup>3</sup>H] GlcNAc, 10  $\mu$ Ci/slice) at 20°C with or without pre-treatment with tunicamycin (see fig. R4.3, legend). Incubation was for 18 h in the case of [<sup>3</sup>H] GlcNAc and 2 h in the case of [<sup>35</sup>S] methionine. The tissue was then fractionated on sucrose density gradients and the glyoxysomal bands removed. The glyoxysomal membranes were pelleted by centrifugation after diluting in 50 mM Tris-HCl (pH 7.4), 400 mM KCl and the [<sup>35</sup>S] methionine-labelled membranes subjected to Triton X-114 extraction before SDS-PAGE. The washed [<sup>3</sup>H] GlcNAc-labelled membranes were solubilized directly in SDS-PAGE sample buffer. Tracks 1 and 2, [<sup>3</sup>H] GlcNAc-labelled glyoxysomal membrane proteins without (-) and with (+) pre-treatment with tunicamycin. Tracks 3 and 4 [<sup>35</sup>S] methionine-labelled tissue without (-) and with tunicamycin pretreatment.

Fig. R4.4 The Effect of Tunicamycin on the Polypeptide Profiles of the Glyoxysomal Membrane



Batches of 10 endosperm slices were incubated with [ $^{35}\text{S}$ ] methionine (20  $\mu\text{Ci/slice}$ ) or N-acetyl- $^{3}\text{H}$  glucosamine ( $^{3}\text{H}$  GlcNAc, 10  $\mu\text{Ci/slice}$ ) at 20°C with or without pre-treatment with tunicamycin (see fig. R4.3, legend). Incubation was for 18 h in the case of  $^{3}\text{H}$  GlcNAc and 2 h in the case of [ $^{35}\text{S}$ ] methionine. The tissue was then fractionated on sucrose density gradients and the glyoxysomal bands removed. The glyoxysomal membranes were pelleted by centrifugation after diluting in 50 mM Tris-HCl (pH 7.4), 400 mM KCl and the [ $^{35}\text{S}$ ] methionine-labelled membranes subjected to Triton X-114 extraction before SDS-PAGE. The washed  $^{3}\text{H}$  GlcNAc-labelled membranes were solubilized directly in SDS-PAGE sample buffer. Tracks 1 and 2,  $^{3}\text{H}$  GlcNAc-labelled glyoxysomal membrane proteins without (-) and with (+) pre-treatment with tunicamycin. Tracks 3 and 4 [ $^{35}\text{S}$ ] methionine-labelled tissue without (-) and with tunicamycin pretreatment.



**Fig. R4.5** N-acetyl Glucosamine Incorporation into Castor Bean Endosperm Organelle Membrane Glycoproteins

Washed organelle membranes isolated from [<sup>14</sup>C] glucosamine-labelled castor bean endosperm tissue were fractionated into hydrophilic and hydrophobic protein components by phase separation in Triton X-114 solution. Lanes 1 and 10 molecular weight markers; lanes 4, 7 and 8 hydrophilic e.r., glyoxysomal and mitochondrial membrane glycoproteins, respectively; lanes 5, 6 and 9, hydrophobic e.r., glyoxysomal, and mitochondrial membrane glycoproteins, respectively. Lanes 2 and 3, as lanes 4 and 5 after exposure to the gel for a shorter period. Proteins were separated by SDS-PAGE and visualized by fluorography.

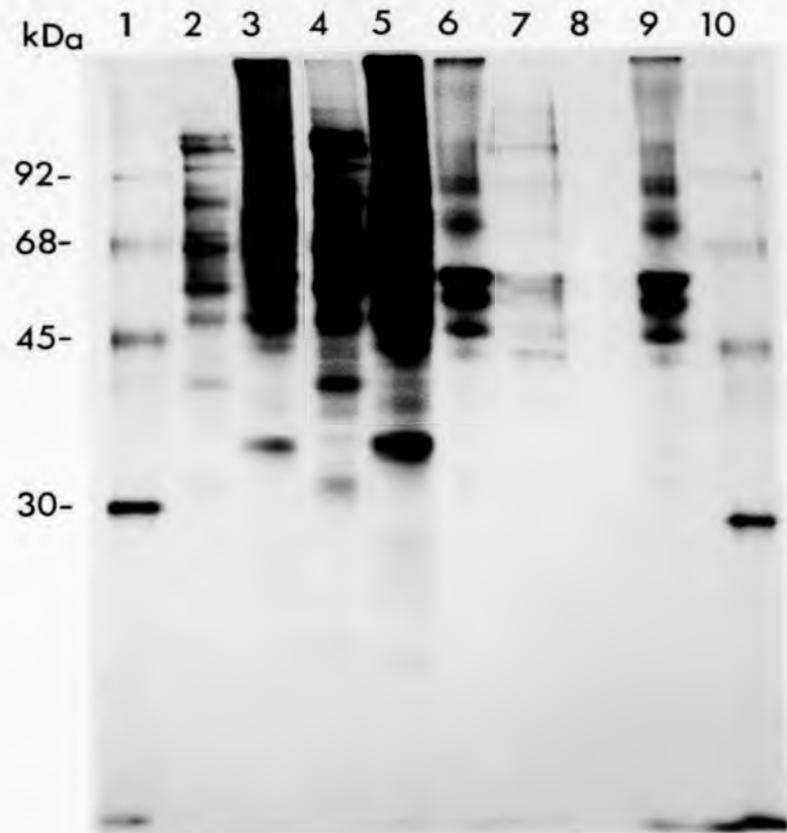


Fig. R4.5 N-acetyl Glucosamine Incorporation into Castor Bean Endosperm Organelle Membrane Glycoproteins

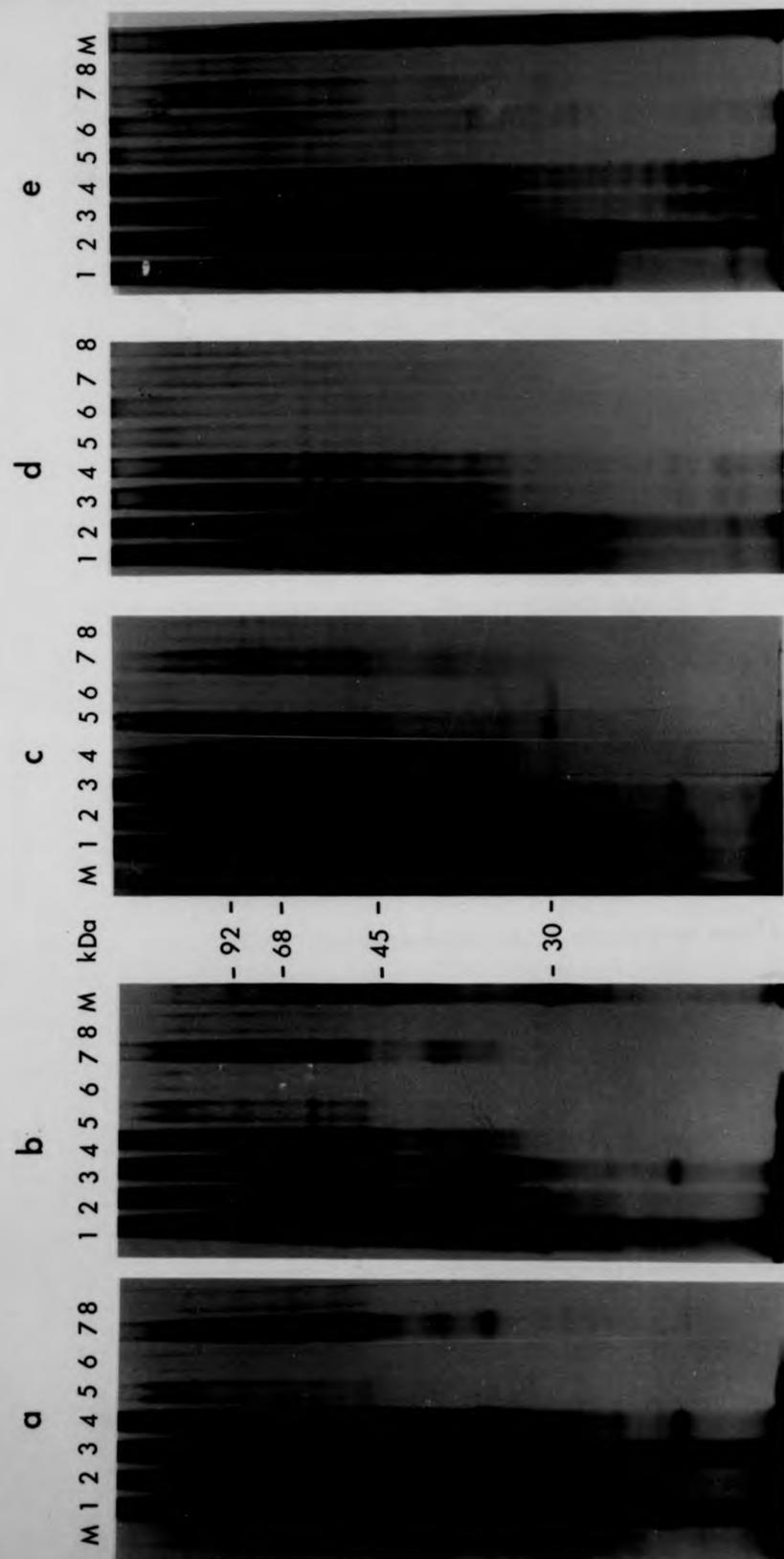
Washed organelle membranes isolated from [<sup>14</sup>C] glucosamine-labelled castor bean endosperm tissue were fractionated into hydrophilic and hydrophobic protein components by phase separation in Triton X-114 solution. Lanes 1 and 10 molecular weight markers; lanes 4, 7 and 8 hydrophilic e.r., glyoxysomal and mitochondrial membrane glycoproteins, respectively; lanes 5, 6 and 9, hydrophobic e.r., glyoxysomal, and mitochondrial membrane glycoproteins, respectively. Lanes 2 and 3, as lanes 4 and 5 after exposure to the gel for a shorter period. Proteins were separated by SDS-PAGE and visualized by fluorography.

Results obtained in section 1 indicated glyoxysomal-derived membrane contamination of the mitochondrial region of a sucrose fractionation gradient. Further, this contamination was not restricted to the mitochondrial fraction but occurred throughout the entire gradient. If the similarities between the glyoxysomal and mitochondrial glycopeptide profiles are the result of cross-contamination then it was thought most likely that this contamination was also caused by the presence of glyoxysomal membranes in the mitochondrial fractions rather than vice-versa. In order to confirm this, a similar strategy was applied as with the monoclonal antibody probe in section 1. Batches of endosperm halves were labelled with [<sup>35</sup>S] cysteine, [<sup>3</sup>H] glucosamine, [<sup>3</sup>H] galactose and [<sup>3</sup>H] fucose. Following homogenization, each homogenate was divided in half and one half from each treatment subjected to an 8,000 x g centrifugation step. The resulting pellet was resuspended in homogenization medium and applied to sucrose gradients. The uncentrifuged homogenates were also applied to sucrose gradients. Following centrifugation the microsomal, mitochondrial and glyoxysomal bands were collected in their entirety together with 2 ml samples from the regions intermediate between the microsomes and mitochondria (the e/m fraction) and the mitochondria and glyoxysomes (the m/g fraction). The membranes from each fraction were washed with 0.5 M KCl-containing tris-buffer and harvested by centrifugation. Without further treatment, to minimise protein losses, the membranes were solubilized in SDS-PAGE sample buffer and subjected to gel electrophoresis

and fluorography. The resulting profiles are shown in fig. R4.6a - e. All three sugars appear to be incorporated into polypeptides from all five fractions; the relative abundances differ for each sugar in the order glucosamine > fucose > galactose, though this order was maintained throughout the five different fractions. The [<sup>35</sup>S] cysteine<sup>e</sup>-labelled polypeptide profiles of the three major organelle fractions were very different. In the cases of the intermediate fractions however, the m/g fraction profile was identical to that of the glyoxysomal membrane whereas the e/m fraction profile shared similarities with both the microsomal and, to a lesser extent, the mitochondrial profiles. In contrast, the glycopeptide profiles of all fractions, except perhaps the [<sup>3</sup>H] glucosamine- and [<sup>3</sup>H] fucose-labelled microsomal proteins, are very similar. This would indicate the presence of a single glycosylated membrane-type throughout the gradient. As glyoxysomal membrane fragments have been shown to be smeared throughout most gradient fractions it is likely that the similarities in the glycopeptide profiles is due to the presence of glycosylated glyoxysomal membrane proteins.

R.4B - [<sup>125</sup>I] CONCAVALIN A BINDING TO ORGANELLE MEMBRANE PROTEINS

Organelle-associated glycoprotein detection was also achieved by overlaying polypeptides separated by SDS-PAGE with [<sup>125</sup>I] iodinated concanavalin A ([<sup>125</sup>I] con A). This binds to exposed mannose residues in glycoprotein side-chains. The result of overlaying microsomal and glyoxysomal membrane proteins, after Triton X-114 extraction, is shown in fig. R4.7. The



**Fig. R4.6** Radioactive Sugar-labelling of Organelle Membrane Glycoproteins - An Investigation Into Organelle Cross-contamination

Castor bean endosperm was labelled *in vivo* with [<sup>35</sup>S] cysteine, [<sup>3</sup>H] glucosamine, [<sup>3</sup>H] galactose or [<sup>3</sup>H] fucose. Following tissue homogenization and centrifugation at 500 x g the 500 x g supernatants were divided in half and one half from each treatment subjected to centrifugation 8,000 x g and 2°C for 10 minutes. The 8,000 x g pellets were resuspended in grinding medium and loaded, together with the remaining halves of the 500 x g supernatants, onto 17 ml sucrose gradients. Following centrifugation the three major organelle-containing bands from each gradient were collected together with standardized samples from the regions intermediate between these bands (the e/m and m/g fractions - see text). Washed membranes from each fraction were solubilized in SDS-PAGE sample buffer and the associated polypeptides separated by gel electrophoresis. (a - e) show the polypeptide profiles of the e.r., e/m, mitochondrial, m/g and glyoxysomal membranes respectively. Tracks 1 and 2 in each case are [<sup>35</sup>S] cysteine-labelled, tracks 3 and 4 [<sup>3</sup>H] glucosamine-labelled; tracks 5 and 6, galactose-labelled; tracks 7 and 8, [<sup>3</sup>H] fucose-labelled. Lanes 1, 3, 5 and 7, profiles from gradients loaded with post- 500 x g supernatants; lanes 2, 4, 6 and 8, profiles from gradients loaded with resuspended 8,000 x g pellets; lanes marked M, molecular weight markers.

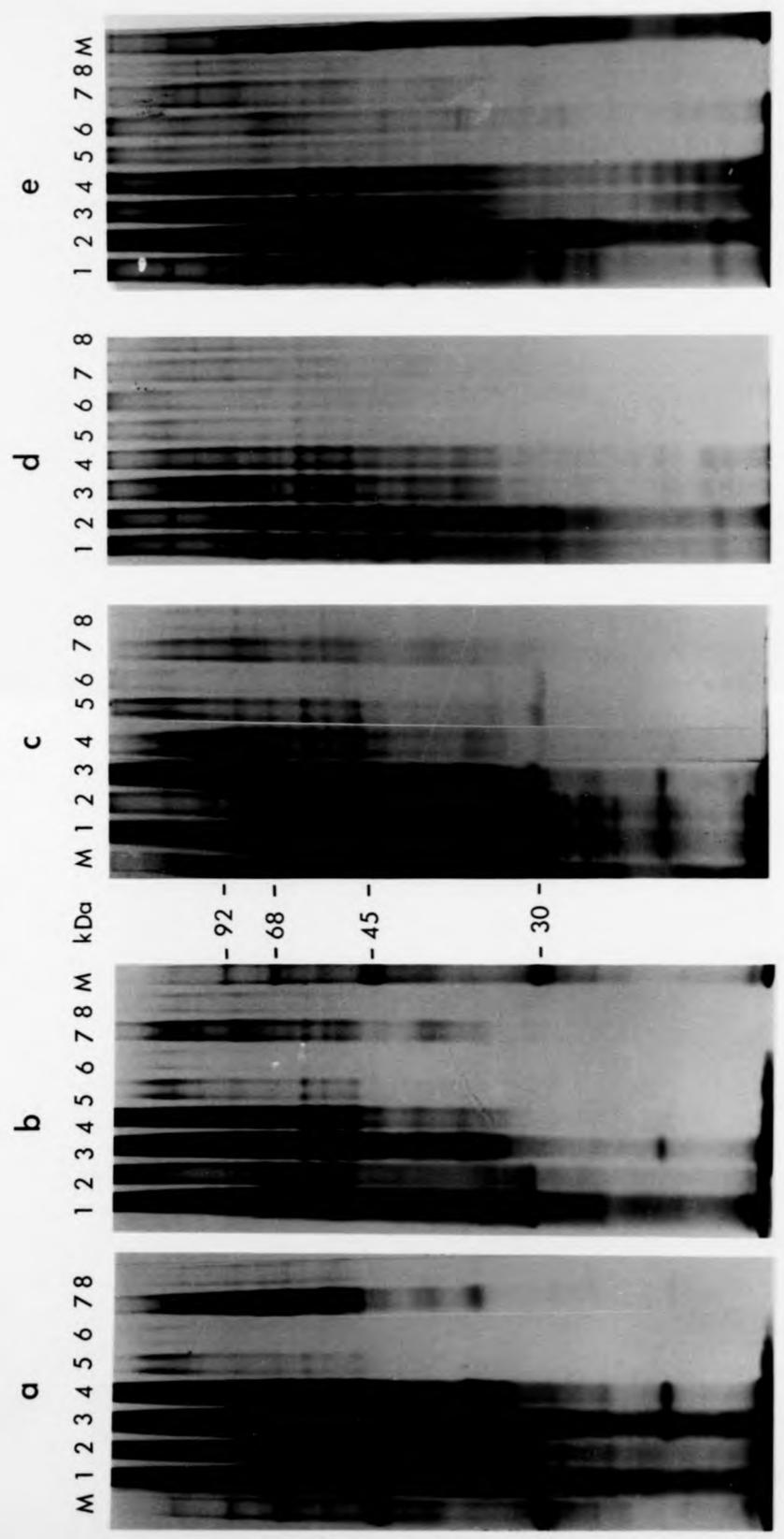


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Castor bean endosperm was labelled *in vivo* with [<sup>35</sup>S] cysteine, [<sup>3</sup>H] glucosamine, [<sup>3</sup>H] galactose or [<sup>3</sup>H] fucose. Following tissue homogenization and centrifugation at 500 x g the 500 x g supernatants were divided in half and one half from each treatment subjected to centrifugation 8,000 x g and 2°C for 10 minutes. The 8,000 x g pellets were resuspended in grinding medium and loaded, together with the remaining halves of the 500 x g supernatants, onto 17 ml sucrose gradients. Following centrifugation the three major organelle-containing bands from each gradient were collected together with standardized samples from the regions intermediate between these bands (the e/m and m/g fractions - see text). Washed membranes from each fraction were solubilized in SDS-PAGE sample buffer and the associated polypeptides separated by gel electrophoresis. (a - e) show the polypeptide profiles of the e.r., e/m, mitochondrial, m/g and glyoxysomal membranes respectively. Tracks 1 and 2 in each case are [<sup>35</sup>S] cysteine-labelled, tracks 3 and 4 [<sup>3</sup>H] glucosamine-labelled; tracks 5 and 6, galactose-labelled; tracks 7 and 8, [<sup>3</sup>H] fucose-labelled. Lanes 1, 3, 5 and 7, profiles from gradients loaded with post- 500 x g supernatants; lanes 2, 4, 6 and 8, profiles from gradients loaded with resuspended 8,000 x g pellets; lanes marked M, molecular weight markers.

coomassie blue stained gel (fig. R4.7a) shows that the polypeptide profiles of the Triton-soluble phases (lanes 2 and 3) were quite different as were the aqueous phase-associated proteins (lanes 1 and 4). The autoradiogram of the [ $^{125}\text{I}$ ] con A overlaid gel is shown in fig. R4.7b and the abundance of con A binding proteins is immediately noticeable (fig. R4.7c shows the result of overlaying an identical gel with [ $^{125}\text{I}$ ] con A in the presence of  $\alpha$ -methyl mannoside, which prevents con A lectin binding). Polypeptides of both phases and from both membrane types were seen to bind [ $^{125}\text{I}$ ] Con A, however, the microsomes appeared to have the greater glycoprotein complement (fig. R4.7b, lanes 1 and 2). The glycoprotein (con A-binding) bands of the glyoxysomal Triton-soluble profiles can all be aligned with proteins in the microsomal Triton-soluble track (compare fig. R4.7b, lanes 2 and 3). A noticeable feature of the glyoxysomal integral glycoproteins was their low abundance compared with the major coomassie-stained Triton-soluble proteins (compare fig. R4.7b, lane 3 with fig. R4.7a, lane 3). The major glyoxysomal con A binding proteins were seen in the aqueous phase track (fig. R4.7b, lane 4) with MW's of 31 kDa, 29 kDa and a diffuse smear between 20 and 22.5 kDa. These proteins did not align with coomassie-stained band and they were also present in the microsomal aqueous phase (fig. R4.7b, lane 1). No proteins of these MW's were labelled in vivo with [ $^3\text{H}$ ] glucosamine (fig. R4.5) and these bands, therefore, probably represent glycosylated lectins which bound to the e.r. and glyoxysomal membranes non-specifically. Support for this conclusion

**Fig. R4.7 Microsomal and Glyoxysomal Membrane Glycoproteins Binding [<sup>125</sup>I]-concanavalin A**

Microsomal and glyoxysomal membranes, obtained by tissue fractionation on sucrose density gradients, were subjected to Triton X-114 extraction and the hydrophobic and hydrophilic polypeptides separated by SDS-PAGE. (a) coomassie-stained gel: lane 1, glyoxysomal hydrophilic proteins; lane 2, glyoxysomal hydrophobic proteins; lane 3, microsomal hydrophobic proteins; lane 4, microsomal hydrophilic proteins. The gels were then overlaid with [<sup>125</sup>I] concanavalin A in the absence (b, tracks 1 - 4) or presence (b, tracks 5 - 8) of  $\alpha$ -methyl mannoside and, after washing free of unbound [<sup>125</sup>I] con A, exposed to X-ray film. (b), autoradiogram; lanes 1 and 2, microsomal aqueous- and detergent-phase associated con A-binding proteins respectively; lanes 3 and 4, glyoxysomal detergent- and aqueous-phase associated con A-binding proteins.

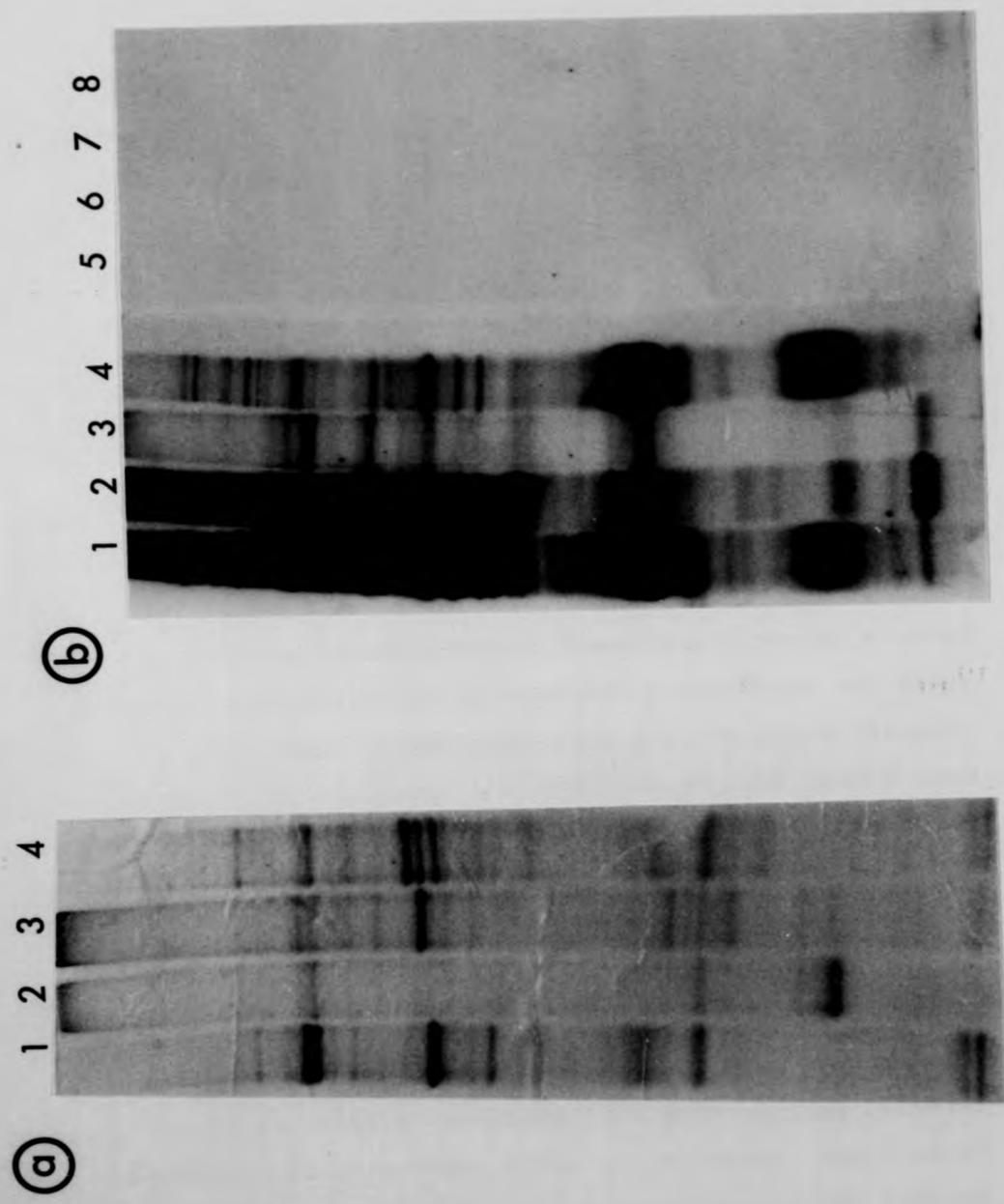
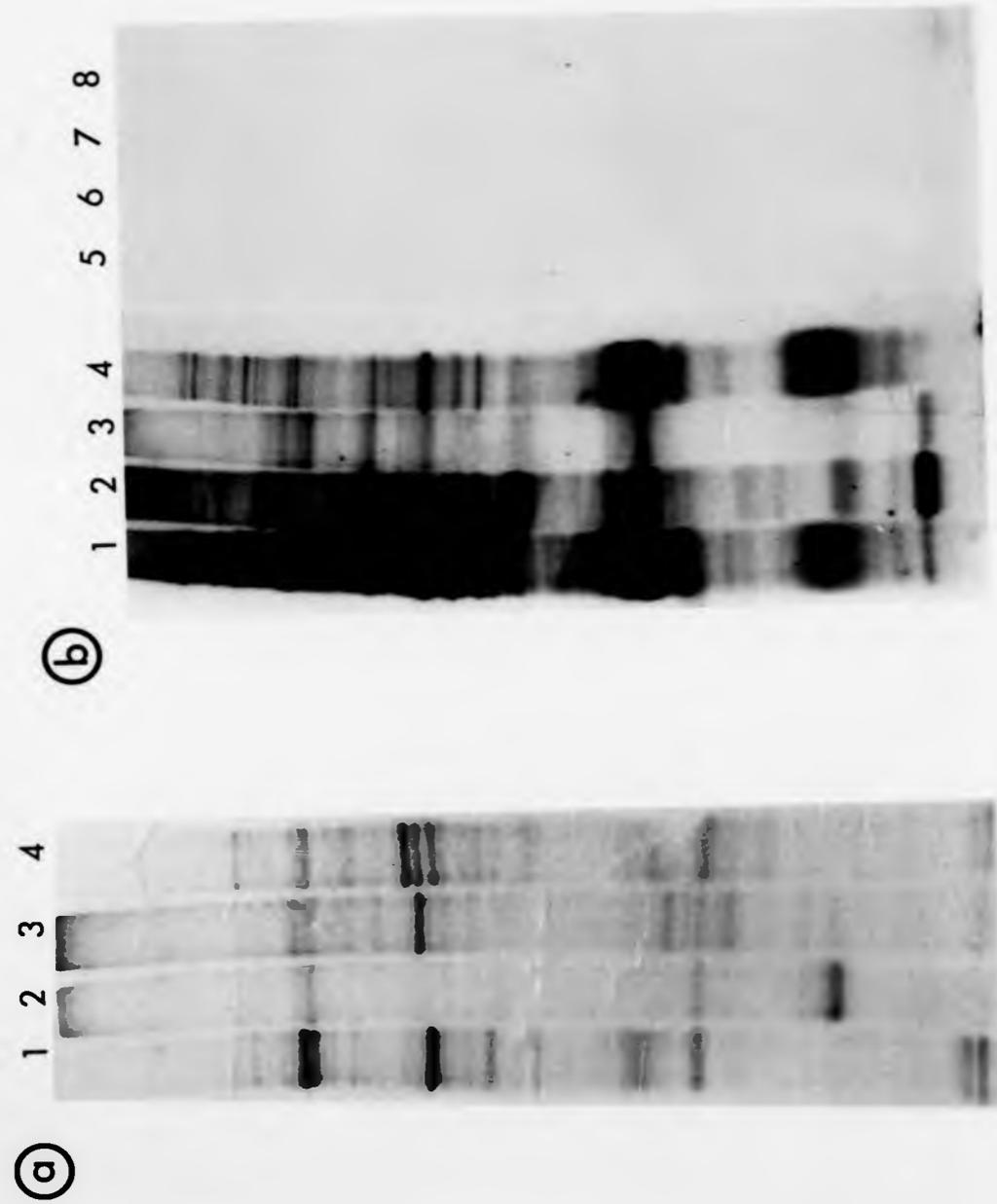


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was obtained when total protein profiles from each 1 ml fraction down an entire gradient were overlaid with [ $^{125}$ I] con A (fig. R4.8). Here again the microsomal fractions (lanes 12 - 15) were seen to contain the greatest number of different glycoproteins. However, [ $^{125}$ I] con A binding was also seen in the fractions known to contain the glyoxysomes (lanes 21 - 24). All fractions contained the 29 - 31 kDa proteins and most fractions the 20 - 22.5 kDa smear which had been seen associated with the microsomes and glyoxysomes in fig. R4.7b.

The fractions containing the mitochondria showed very low levels of [ $^{125}$ I] con A binding (fig. R4.8, lanes 17 - 20). These profiles, however, represent mainly the mitochondrial matrix proteins. In order to investigate whether the mitochondrial membranes contained glycoproteins, mitochondria were isolated by sucrose gradient fractionation, Triton X-114 extracted and the Triton-soluble protein profile overlaid with [ $^{125}$ I] con A after SDS-PAGE. Fig. R4.9a shows the comparison between coomassie-stained mitochondrial and glyoxysomal membrane proteins and fig. R4.9b an autoradiogram of the same gel after [ $^{125}$ I] con A overlaying. Con A binding to mitochondrial integral membrane proteins was indeed observed. The contamination of this fraction by glyoxysomal membranes was not responsible for this binding<sup>as</sup> could be seen by comparing the mitochondrial integral glycoproteins (fig. R4.9b, lane 2) with the glyoxysomal integral proteins (fig. R4.9b, lane 3). The two profiles were completely different. That the mitochondrial [ $^{125}$ I] con A binding profile was indeed due to the presence of

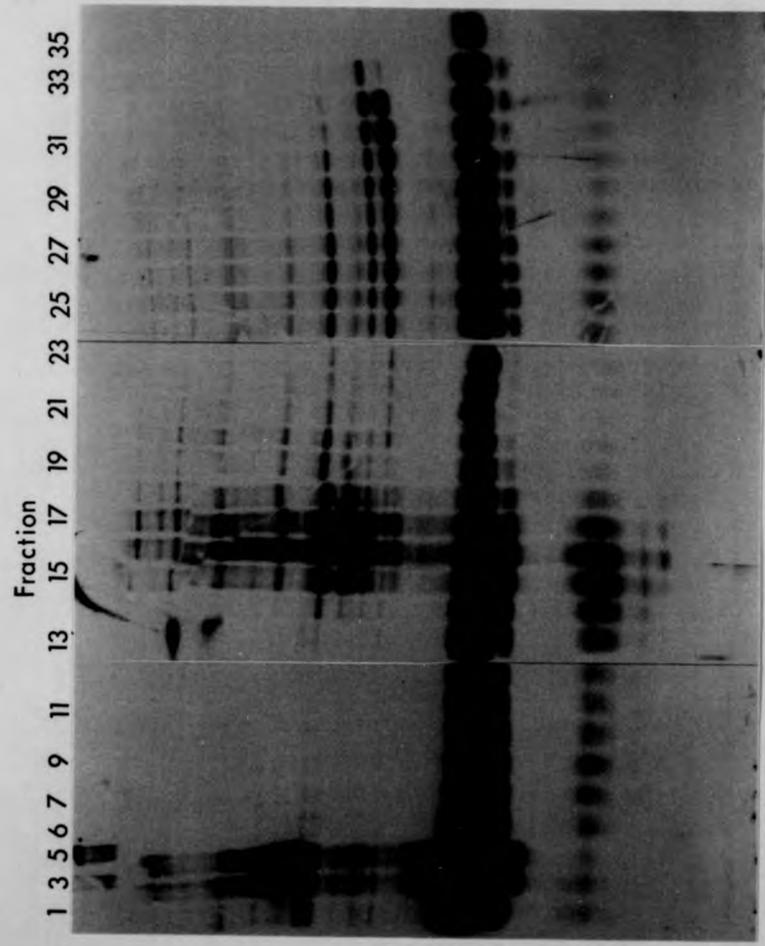


Fig. R4.8 The Distribution of Con A Binding Proteins In Sucrose Gradient Fractions Following Castor Bean Endosperm Tissue Fractionation

Endosperm tissue was fractionated on a 38 ml sucrose gradient which was collected as 1 ml fractions. Aliquots of each fraction were TCA precipitated and the TCA-insoluble polypeptides subjected to SDS-PAGE as detailed in results 1 (see fig. R1.9). The gel was overlaid with [<sup>125</sup>I] con A and exposed to X-ray film. Numbers above lanes on autoradiogram refer to fraction number, beginning at the top of the gradient.

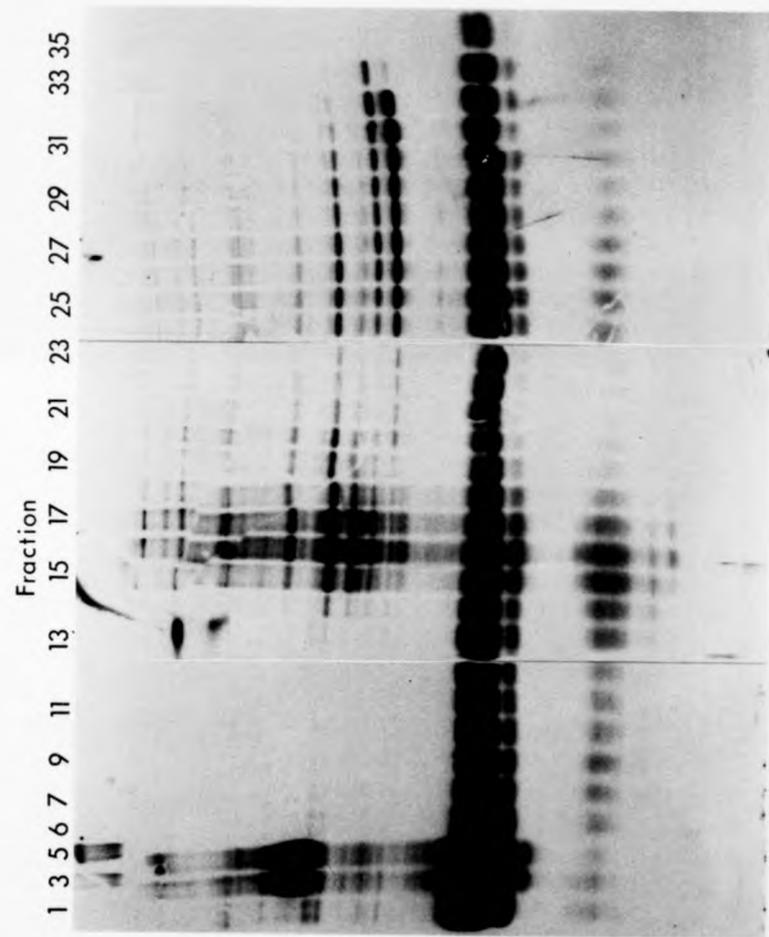
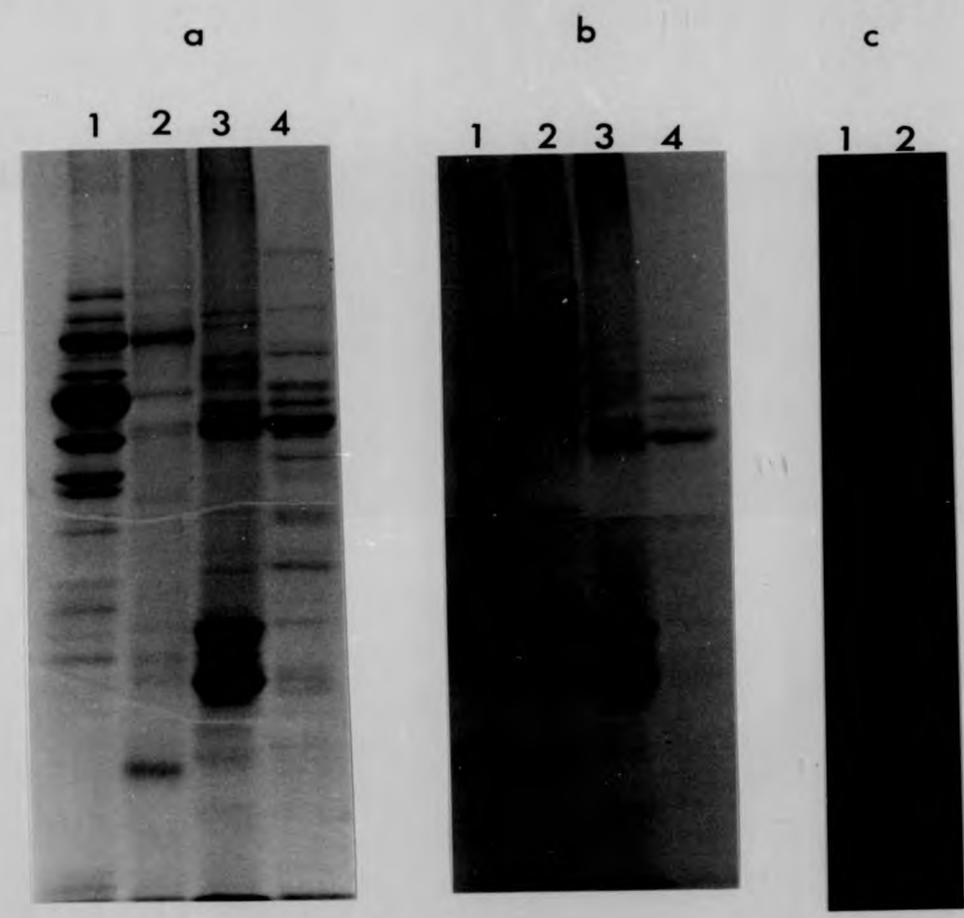


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**Fig. R4.9 Comparison of Mitochondrial and Glyoxysomal Mannose-containing Glycoproteins**

Mitochondrial and glyoxysomal membranes were subjected to Triton X-114 extraction and the hydrophilic and hydrophobic proteins compared as described for microsomes and glyoxysomes in fig. R4.7. (a), coomassie-stained gel: lanes 1 and 4, hydrophilic proteins of glyoxysomal and mitochondrial membranes respectively; lanes 2 and 3, hydrophobic proteins of glyoxysomal and mitochondrial membranes respectively. The gel was overlaid with [<sup>125</sup>I] con A and the resulting autoradiogram is shown in (b): lanes numbered as in (a). Mitochondrial membranes from endosperm tissue labelled with [<sup>3</sup>H] mannose in vivo in the absence (lane 1) or presence (lane 2) of tunicamycin were also subjected to SDS-PAGE (c) as detailed for fig. R4.4.

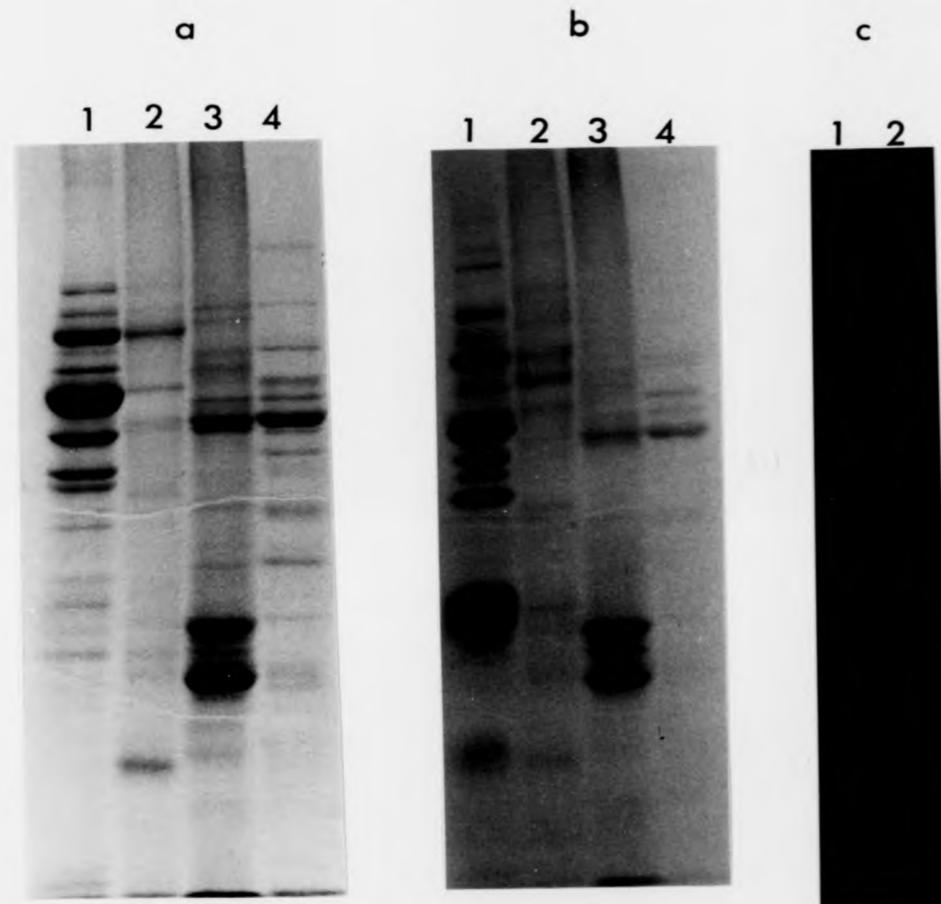
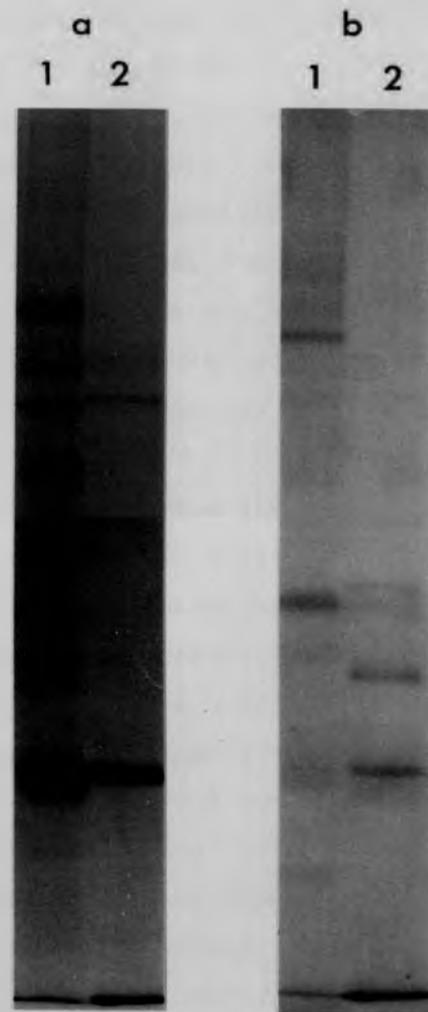


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glycoproteins and not, for instance, due to non-specific hydrophobic interactions (Dean and Homer, 1973), was shown by labelling intact tissue with [ $^3\text{H}$ ] mannose, isolating the mitochondria and Triton extracting the integral membrane proteins. The mitochondrial [ $^3\text{H}$ ] mannose-containing glycoprotein profile is shown in fig. R4.9c, and is seen to be very similar to that obtained with the [ $^{125}\text{I}$ ] con A overlay profile. The [ $^3\text{H}$ ] mannose labelling was also carried out after tissue pre-incubation with tunicamycin and the effect of this treatment, also shown in fig. R4.9c. was to reduce [ $^3\text{H}$ ]-mannose incorporation into the mitochondrial membrane proteins.

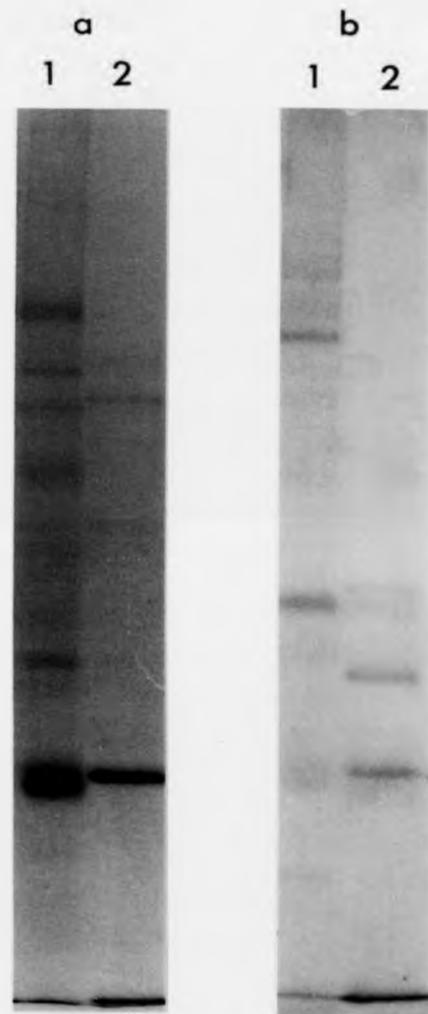
The presence of protein body-derived proteins in the glyoxysome-containing fractions indicated possible protein body contamination of the isolated glyoxysomes. Protein body membrane glycoproteins were therefore compared with those from glyoxysomes for [ $^{125}\text{I}$ ] con A binding. Protein bodies were isolated from dry castor beans and the membranes subjected to Triton X-114 extraction before the separation of the integral membrane polypeptides by SDS-PAGE. A comparison of the coomassie stained protein profiles of protein body and glyoxysomal integral membrane proteins is shown in fig. R4.10a and the autoradiogram of the same gel after [ $^{125}\text{I}$ ] con A overlaying is shown in fig. R4.10b. The differences in the profiles of the con A binding proteins is quite obvious. In addition to this evidence, it should be remembered that protein body membrane synthesis is an event which occurs during the development of the seed, thus radioactive sugar incorporation, *in vivo*, would not be expected to occur during germination.



**Fig. R4.10 Comparison of the Mannose-containing Glycoproteins of Glyoxysomal and Protein Body Membranes**

Glyoxysomal membranes were isolated from 4-day-old endosperm tissue and the integral membrane proteins extracted using Triton X-114. Protein bodies were prepared from dry seeds and the membranes isolated on a sucrose density gradient as described in M2.B (p. 45). The protein body membrane bands were removed from the 10/20% and 20/30% (w/w) sucrose interfaces and, following dilution and pelleting, also subjected to Triton X-114 extraction. The detergent-soluble proteins were separated by SDS-PAGE and coomassie-stained: (a), lane 1, glyoxysomal membrane proteins; lane 2, protein body membrane proteins. The gel was overlaid with [<sup>125</sup>I] con A and the resulting autoradiogram is shown in (b), lane 1, glyoxysomal membrane [<sup>125</sup>I] con A binding protein profile; lane 2, protein body membrane [<sup>125</sup>I] con A binding protein profile.

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DISCUSSION IV

The presence of glycoproteins in the glyoxysomal membranes of germinating castorbean endosperm has been demonstrated by two methods, (a) the direct, in vivo labelling of membrane proteins with radioactive sugars and (b) the detection of exposed mannose residues using [<sup>125</sup>I] labelled concanavalin A. The results presented here are in agreement with the findings of Bergner and Tanner (1981) who concluded that the glycoproteins of the glyoxysomal membrane are of the N-linked type. Evidence that N-glycosylation was responsible for the incorporation of sugars into glyoxysomal proteins came from (a) the <sup>partial</sup> susceptibility of the oligosaccharide moiety to digestion by endo-H and (b) the partial inhibition of the glycosylation process by the antibiotic tunicamycin whose mode of action is to inhibit the formation of GlcNAc-P-P-dolichol. Although [<sup>3</sup>H] glucosamine was used in the incorporation study, its conversion, following tissue penetration, to N-acetyl glucosamine has been demonstrated in other tissues (Roberts and Pollard, 1975; Nagahashi et al., 1980) and can therefore be assumed to occur in castor bean endosperm. In demonstrating the presence of N-linked glycoproteins in glyoxysomal membranes, a role for the e.r. in glyoxysomal biogenesis is strongly implicated as this compartment has been shown to be the exclusive site of N-glycosylation in castor bean endosperm tissue (Mellor and Lord, 1979c; Roberts et al., 1980). The structures of the glyoxysome-associated glycoproteins were not determined in any detail, however, the in vivo labelling data indicated the presence of GlcNAc, fucose

and galactose. This latter sugar was recently shown to be the one of greatest abundance in glycopeptides derived from washed glyoxysomal membranes (Mellor *et al.*, 1980). Con A binding indicated the presence of mannose though direct labelling with [<sup>3</sup>H] mannose proved difficult, only low rates of incorporation being observed (data not shown). These low rates may have been due to poor tissue impregnation though it is unlikely in light of the fact that label as [<sup>3</sup>H] mannose was incorporated into the mitochondria (fig. R4.9c). The mitochondria have been reported to be the site of mannose incorporation into glycoproteins (Morelis *et al.*, 1974) and lipid-linked intermediates have been implicated in this glycosylation process (Gateau *et al.*, 1978). The mitochondria of castor bean endosperm were also observed to possess a low level of mannosyl-transferase activity (Mellor and Lord, 1979c) though these mitochondrial glycoproteins have not been studied in any great detail.

Galactose and fucose are generally regarded as peripheral sugars in the oligosaccharide moieties of asparagine-linked glycoproteins. Their addition to the Glc<sub>3</sub>Man<sub>9</sub>GlcNAc<sub>2</sub>-core structure occurs in the Golgi apparatus (Schacter *et al.*, 1970) after the removal of the glucose residues in the e.r. (Spiro *et al.* 1979a) and the trimming of the residual structure by mannosidases also present in the Golgi (Tabas and Kornfeld, 1979; Dunphy and Rothman, 1983). In castor bean endosperm however, some galactose transfer to glycoproteins has been shown to proceed via a dolichol-linked intermediate and the UDP-galactose dolichylmonophosphate transferase

has been localized in the e.r. (Mellor and Lord, 1979c). GDP-fucose glycoprotein fucosyltransferase has also been shown to be an e.r. associated enzyme, though perhaps in regions of the e.r. distinct from those containing the GlcNAc and mannosyl-transferases (Conder and Lord, 1983., section 3). The synthesis of glyoxysome-associated glycoproteins containing GlcNAc, mannose, galactose and fucose is therefore feasible without the involvement of the Golgi, an organelle which is not implicated in any model for microbody biogenesis and indeed which is poorly developed in germinating castor bean endosperm cells.

Peripheral sugar addition to mannosidase-trimmed oligosaccharide moieties can result in the formation of complex oligosaccharides and also occurs as the glycoprotein passes through the Golgi apparatus (Dunphy and Rothman, 1983; Hubbard and Ivatt, 1981 for review). The non-involvement of the Golgi in glyoxysomal glycoprotein synthesis implies that their oligosaccharide moieties are of the high mannose type. This is further indicated by their susceptibility to endo-H digestion. The presence of fucose,  $\alpha 1 \rightarrow 6$  linked to the proximal core GlcNAc in complex-type oligosaccharide blocks the action of endo H (Tarentino and Maley, 1975). The apparent resistance of some of the glycoproteins to endo-H treatment (fig. R4.2) may have been due to some of the oligosaccharides being of the complex-type or merely due to an inaccessibility of the remaining oligosaccharide chains to the endo H because of the proteins conformation. This could be tested by denaturing the proteins to expose their

oligosaccharide chains, as recently described for the "inaccessible" oligosaccharides of ovalbumin (Trimble and Maley, 1984).

The role played by these glycoproteins in the membrane of glyoxysomes is unknown, thus their importance in glyoxysome biogenesis or function cannot be assessed. The peroxisomes of rat liver have been analysed for glycoprotein components using immobilized lectin columns. Protein from both the peroxisomal matrix and the membrane failed to bind to such columns and it was concluded that glycoproteins play no major role in these organelles (Volk and Lazarow, 1982). The oligosaccharide chains of asparagine-linked glycoproteins have been shown to function in protein recognition and clearance from the circulation by the mammalian reticuloendothelial system (Ashwell and Harford, 1982) and in the targeting of proteins to subcellular locations such as the lysosomes (Neufeld and Ashwell, 1980). Any role I might suggest for the glycoproteins of the glyoxysomal membrane is purely speculative however. Bearing in mind that they seem to constitute a relatively minor portion of the membrane protein complement, a possible function could be in the initial identification of a region of the endoplasmic reticulum where glyoxysome vesiculation may begin.

RESULTS V

R.5 - THE FURTHER CHARACTERIZATION OF THE POLYPEPTIDES ASSOCIATED WITH THE GLYOXYSOMAL MEMBRANE

R.5A - ANTISERUM TO GLYOXYSOMAL MEMBRANE PROTEINS AND ITS USE IN IMMUNOHISTOCHEMICAL LOCALIZATION STUDIES

Total Triton X-114 extracts of glyoxysomal membrane proteins were used to raise antisera in two rabbits. The antisera (A and B) were tested for specificity to integral glyoxysomal membrane proteins by reacting them with glyoxysomal polypeptides which had been transferred to nitrocellulose paper by western-blotting. Autoradiograms of the blots, after binding of anti-glyoxysomal antiserum (anti-GA<sub>S</sub>) and [<sup>125</sup>I] iodinated rabbit anti-mouse 1gG, are shown in fig. R5.1. Both anti-GA<sub>S</sub>-A and anti-GA<sub>S</sub>-B were seen to react with specific detergent phase-associated proteins (fig. R5.1, lanes 5 and 7). Particularly prominent were bands at molecular weights of 21 kDa and 25 kDa. There were major differences between the specificities of the antisera towards higher molecular weight proteins however, anti-GA<sub>S</sub>-B reacting with at least 7 discrete polypeptides of molecular weights (MW) between 35 kDa and 85 kDa, whereas anti-GA<sub>S</sub>-A reacted with only 4 or 5 polypeptides in the range of 60 kDa to 85 kDa. Cross-reactivity with proteins associated with the aqueous phase after Triton X-114 extraction was apparent with both batches of antisera (fig. R5.1, lanes 6 and 8). A protein with a MW of 58 kDa, probably malate synthase, reacted very strongly with anti-GA<sub>S</sub>-B (fig R5.1, lane 8). Cross-reactivity with Triton X-114 extracts of microsomal membranes was investigated using anti-GA<sub>S</sub>-B. Fig. R5.2 shows

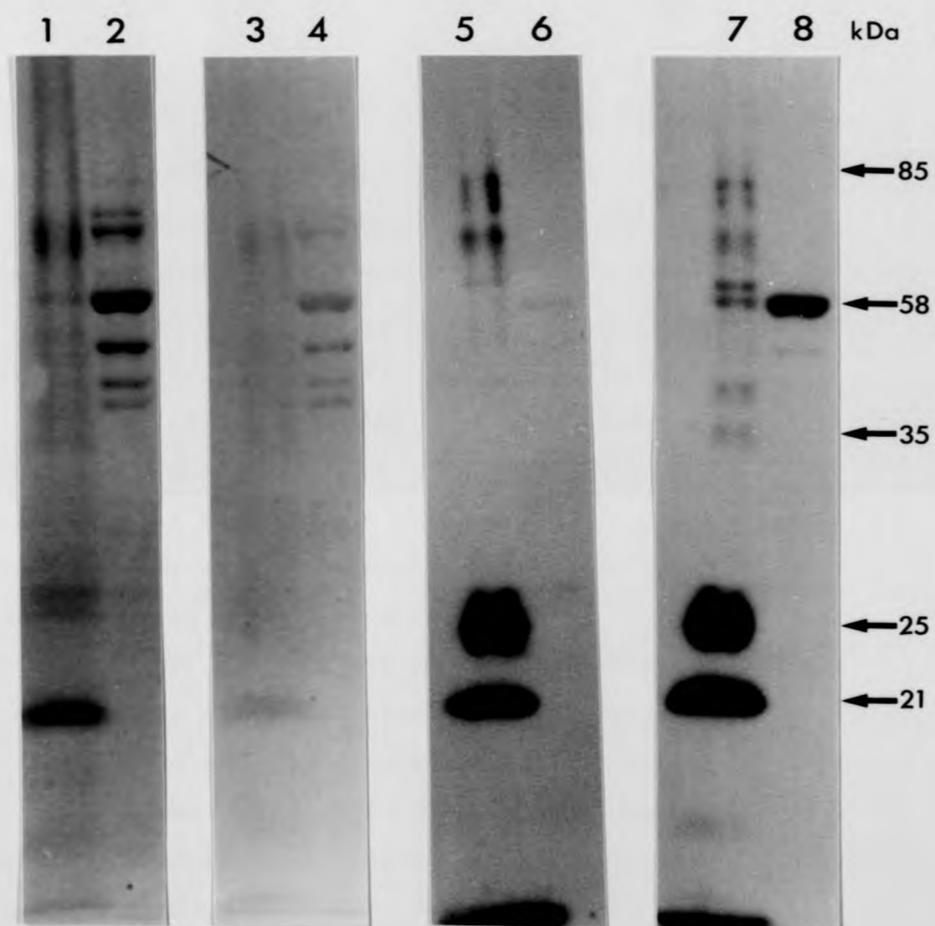


Fig. R5.1 Western Blot Analysis of Polypeptides Reacting With Anti-glyoxysomal Membrane Protein Antisera

Glyoxysomal membranes were Triton X-114 extracted and the hydrophobic and hydrophilic proteins separated by SDS-PAGE. The polypeptides were then electrophoretically transferred to nitrocellulose and probed with anti-glyoxysomal membrane antisera (anti-GA<sub>S</sub>) A and B. Reacting polypeptide bands were visualized by autoradiography after secondary binding of [<sup>125</sup>I]-protein A as detailed in M.15 (p. 67): lanes 1 and 2, hydrophobic and hydrophilic proteins respectively before transfer from gel; lanes 3 and 4, corresponding polypeptide profiles after transfer to nitrocellulose (visualized using imido black staining); lanes 5 and 6, hydrophobic and hydrophilic proteins, respectively, reacting with anti-GA<sub>S</sub>-A; lanes 7 and 8 as lanes 5 and 6 but probed with anti-GA<sub>S</sub>-B.

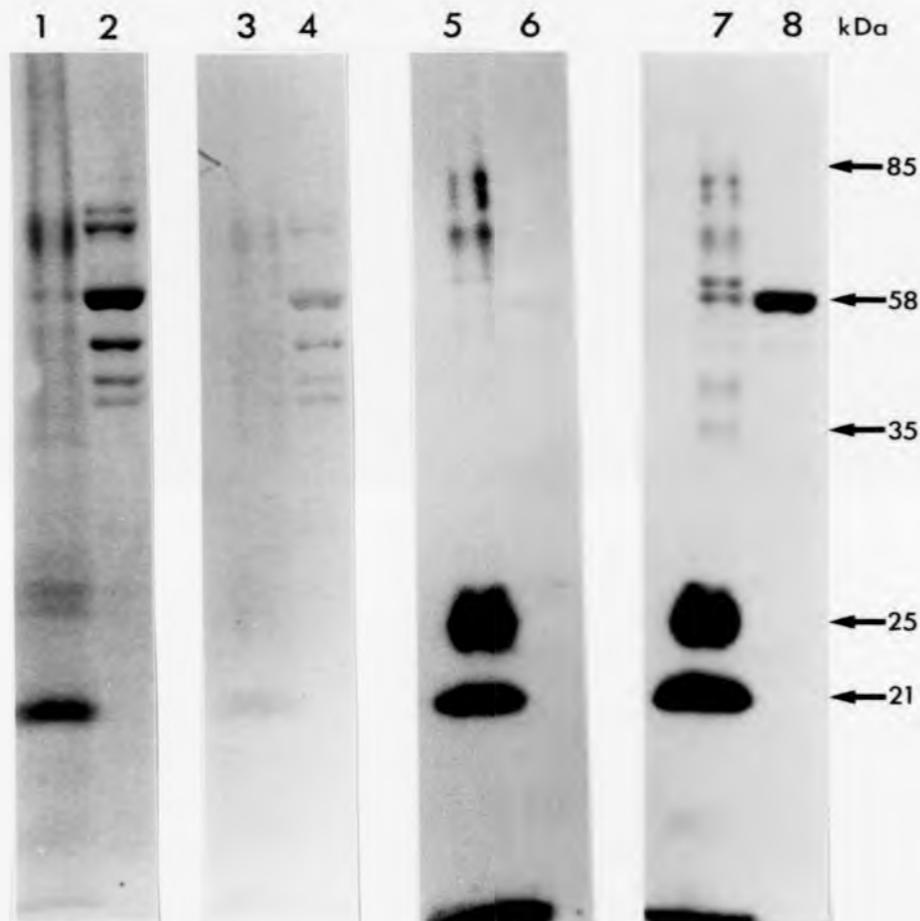


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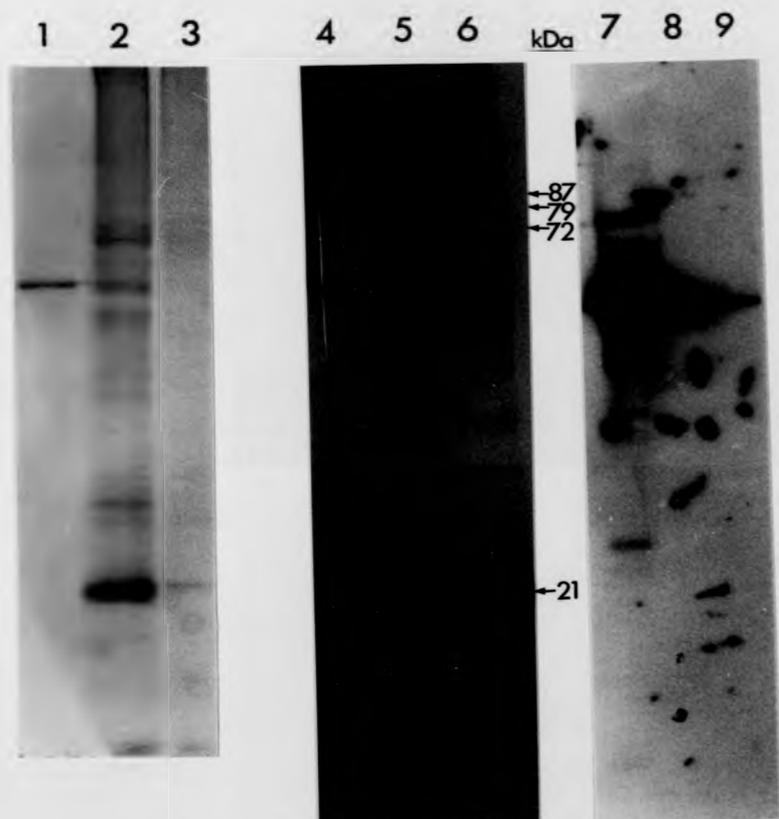


Fig. R5.2 Cross-reactivity of Anti-glyoxysomal Integral Membrane Protein Antiserum with Integral E.R. Polypeptides

Microsomal and glyoxysomal membranes were Triton X-114 extracted and the detergent-soluble polypeptides separated by SDS-PAGE. The proteins were electrophoretically transferred to nitrocellulose and probed with anti-GA<sub>S</sub>-B followed by [<sup>125</sup>I]-protein A. As a control purified ribulose bis phosphate (RuBP) carboxylase large subunit binding protein (BP) was subjected to SDS-PAGE alongside the membrane polypeptides and anti-BP antiserum used to probe for non-specific binding of integral membrane proteins: Lanes 1, 2 and 3, purified BP, microsomal and glyoxysomal membrane polypeptides, respectively, before transfer from the gel; lanes 4, 5 and 6, as lanes 1, 2 and 3 following transfer and probed with anti-GA<sub>S</sub>-B; lanes 7, 8 and 9, as lanes 1, 2 and 3 following transfer and probed with anti-BP antiserum.

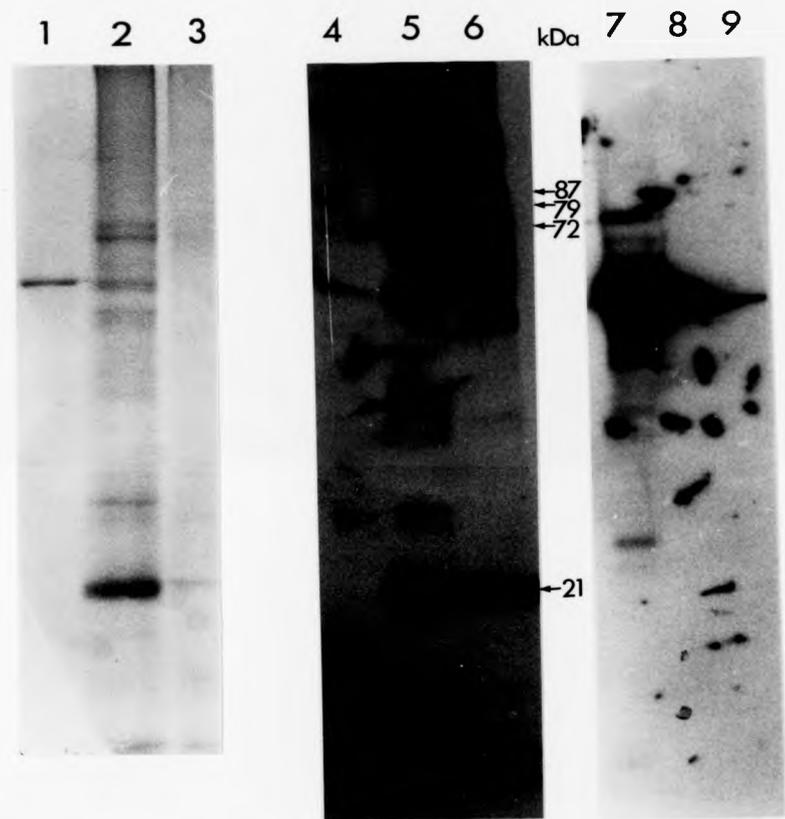


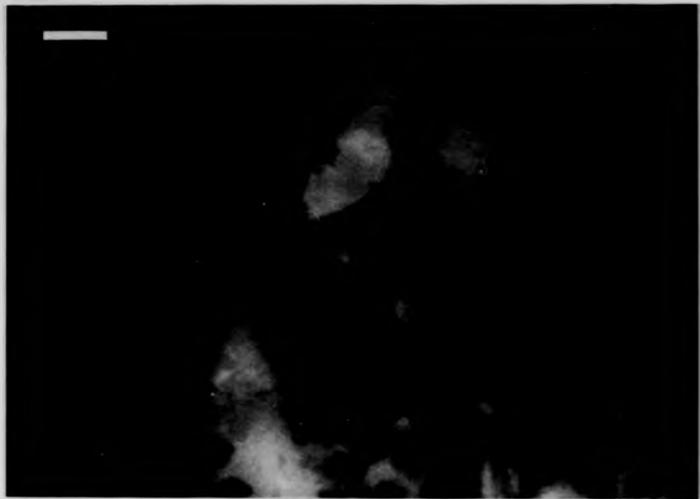
Fig. R5.2 Cross-reactivity of Anti-glyoxysomal Integral Membrane Protein Antiserum with Integral E.R Polypeptides

Microsomal and glyoxysomal membranes were Triton X-114 extracted and the detergent-soluble polypeptides separated by SDS-PAGE. The proteins were electrophoretically transferred to nitrocellulose and probed with anti-GA<sub>5</sub>-B followed by [<sup>125</sup>I]-protein A. As a control purified ribulose bis phosphate (RuBP) carboxylase large subunit binding protein (BP) was subjected to SDS-PAGE alongside the membrane polypeptides and anti-BP antiserum used to probe for non-specific binding of integral membrane proteins: Lanes 1, 2 and 3, purified BP, microsomal and glyoxysomal membrane polypeptides, respectively, before transfer from the gel; lanes 4, 5 and 6, as lanes 1, 2 and 3 following transfer and probed with anti-GA<sub>5</sub>-B; lanes 7, 8 and 9, as lanes 1, 2 and 3 following transfer and probed with anti-BP antiserum.

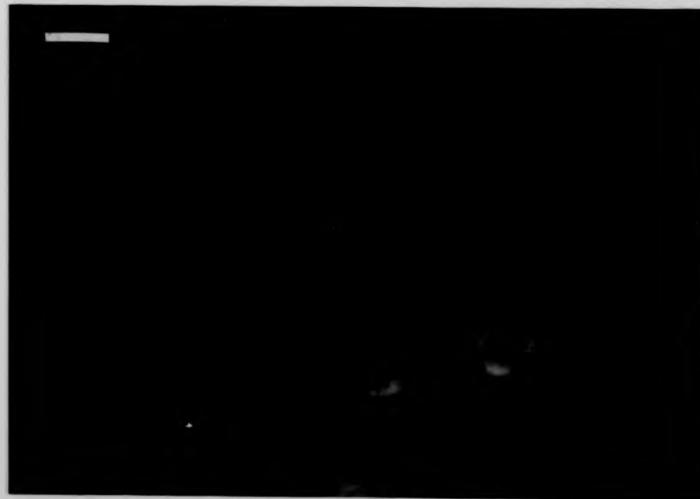
that antibody binding to e.r. integral membrane proteins did occur, in particular to the 21 kDa protein present in both the e.r. and glyoxysomal profiles (fig. R5.2, lanes 5 and 6). That this was not due to cross-contamination of the e.r. by glyoxysomes is indicated by the differences in antibody-binding to proteins of higher molecular weight, especially in the 80 kDa region. Two proteins, with MW 87 kDa and 79 kDa, were more prominent in the e.r. than the glyoxysomal membrane, whereas a protein of MW 72 kDa is more prominent in the glyoxysomes. Non-specific binding was checked by reacting microsomal and glyoxysomal integral membrane proteins with an antiserum raised to a protein entirely foreign to castor bean glyoxysomes, i.e. pea chloroplast RuBP carboxylase large subunit binding protein (prepared by Dr. S. Hemmingsen, University of Warwick). No binding was obtained using this antiserum (fig. R5.2, lanes 8 and 9).

Sections of castor bean endosperm tissue were cut at a temperature of  $-30^{\circ}\text{C}$  and incubated with anti-GA<sub>s</sub>-B. The sections were counterstained with goat anti-rabbit IgG (GAR) conjugated to fluoresceine isothiocyanate (FITC) to identify regions of antibody binding when the sections were viewed under epifluorescence. Fig. R5.3a shows the appearance of the castor bean cells under bright field illumination, the only discernible subcellular structures were the protein bodies (PB). When the sections were incubated with anti-GA<sub>s</sub>-B/GAR-FITC and viewed under epifluorescence small (0.5 - 1.0  $\mu\text{m}$ ) subcellular particles, presumably glyoxysomes, were visible (fig. R5.3b). There was also faint staining of a subcellular network (arrowed) which

c



d



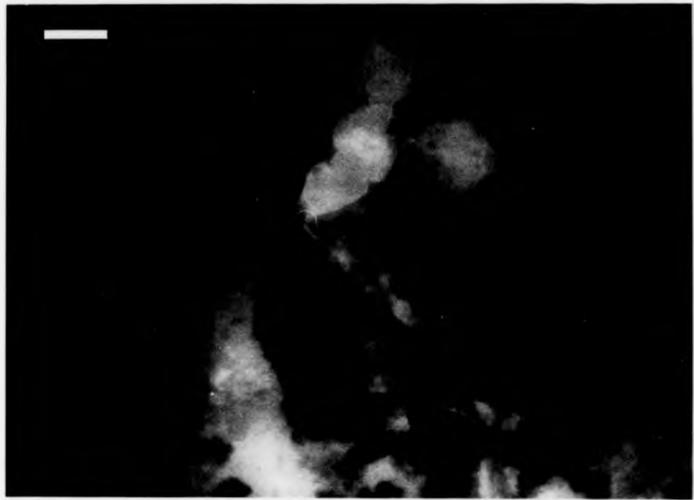
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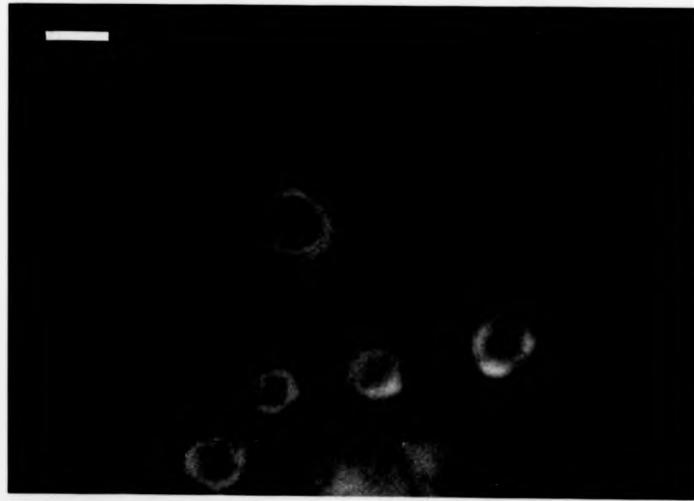
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13

c



d



a



b

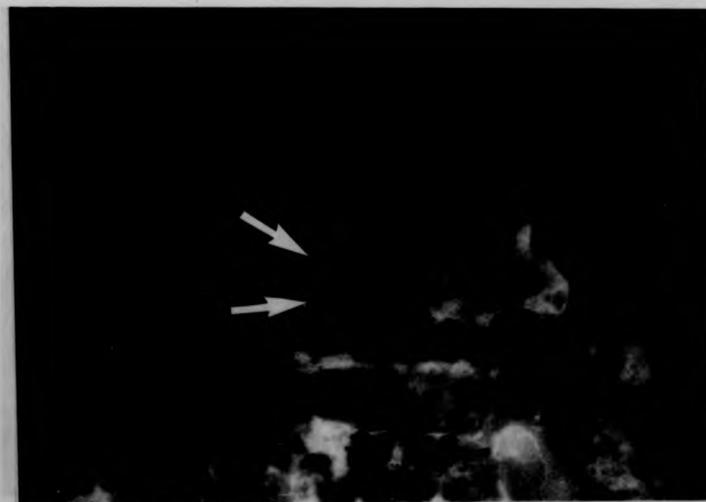
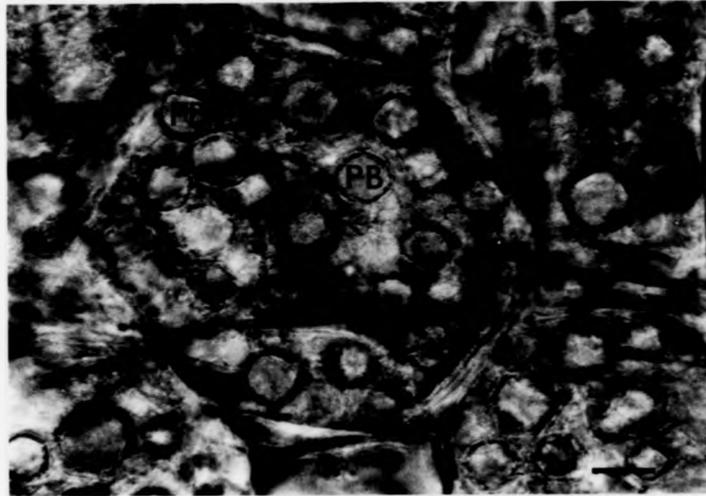


Fig. R5.3 Immunofluorescent Labelling of Glyoxysomes by Rabbit anti-Glyoxysomal Membrane Protein Antiserum

Frozen sections of 4-day-old endosperm tissue were cut and "stained" using antibodies as detailed in M.16, p. 68: (a) brightfield micrograph of endosperm cell (PB, protein bodies); (b) section treated with anti-GA<sub>s</sub>-B followed by FITC-labelled antibodies (arrows indicate possible e.r. localization); (c) section treated with pre-immune serum followed by FITC-labelled secondary antibodies; (d) section treated with secondary, FITC-labelled, antibodies only. Bars, 10  $\mu$  m.

a



b

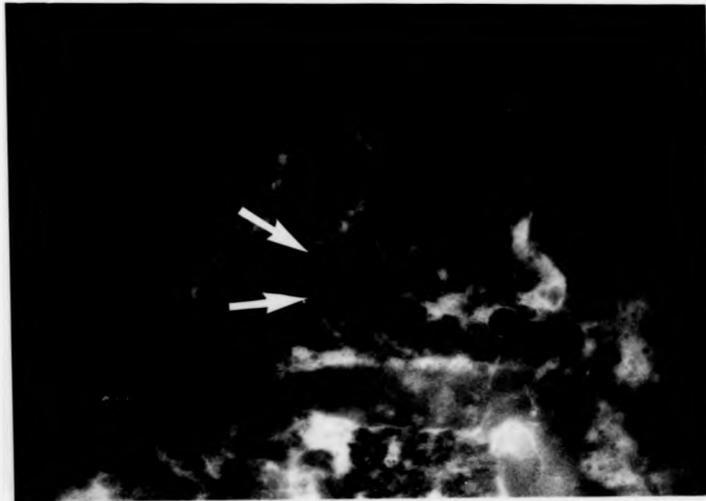


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**Fig. R5.4** Newly-synthesized Integral Membrane Protein Profiles of Microsomes, Mitochondria, the M/G-Fraction and Glyoxysomes

Endosperm tissue was incubated with [<sup>35</sup>S] methionine for 18 h then fractionated on sucrose density gradients. Organelle-membranes were subjected to Triton X-114 extraction and the detergent-soluble polypeptides separated by SDS-PAGE: lane 1, microsomal membrane proteins; lane 2, mitochondrial membrane proteins; lane 3, proteins from membranes from the m/g region of the gradient; lane 4, glyoxysomal membrane proteins.



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could indicate the presence of glyoxysomal membrane proteins in the membrane of the endoplasmic reticulum. The most prominent areas of fluorescence, however, were in association with the protein bodies. The specificity of the antiserum to the protein bodies was therefore investigated by (a) "staining" the sections with pre-immune serum prior to incubation with GAR-FITC and (b) applying GAR-FITC without a primary antibody treatment. The results of these treatments are shown in fig. R5.3c and d respectively. It can be seen that the glyoxysomes and the subcellular network were not stained in these instances, however, the protein body-associated fluorescence was at least as prominent in these sections as in those treated with anti-GA<sub>S</sub>-B. This non-specific binding to protein body components could not be reduced either by pretreatment of sections with galactose (the hapten sugar of castor bean agglutinin) or porcine gastric mucin, a highly glycosylated protein (data not shown). The cause of this non-specific binding remains unidentified.

R5.B - STUDIES ON THE SYNTHESIS OF GLYOXYSOMAL MEMBRANE PROTEINS

(i) Integral Membrane Protein Synthesis In Vivo

Following the labelling of intact tissue with [<sup>35</sup>S] methionine and fractionation by sucrose gradient centrifugation, the major organelle bands were removed together with a sample from the intermediate region between the mitochondria and glyoxysomes. The membranes were harvested by dilution and centrifugation then subjected to Triton X-114 extraction. The polypeptide profiles of the four membrane types are shown in fig. 5.4.

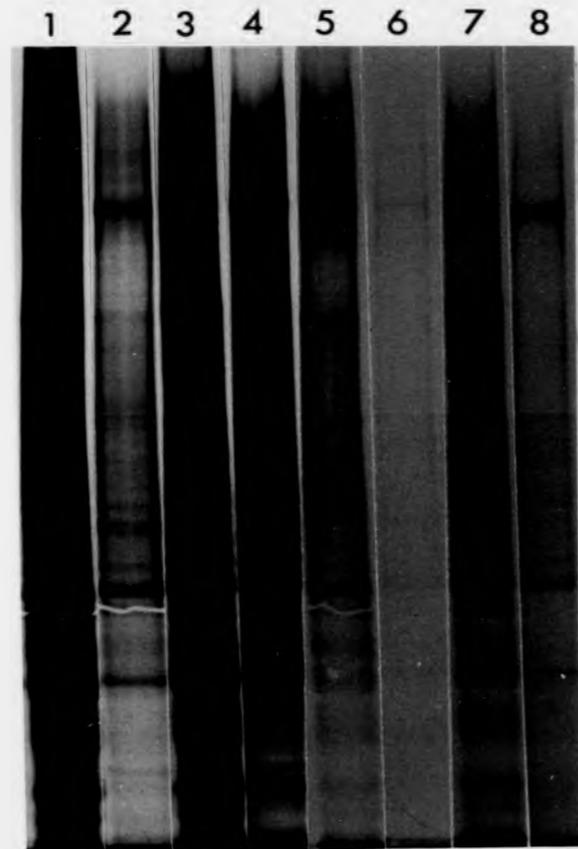
Similarities between the m/g and glyoxysomal fractions were again obvious (fig. R5.4, lanes 3 and 4) as were the differences between the three major organelle-derived membrane protein profiles. Once again however, polypeptides of similar mobilities to those in the glyoxysomal profile were present in the microsomal fraction. Evidence that these similarities were not due to glyoxysomal membrane contamination of the e.r. fraction (in addition to that provided in section 1) was obtained by incubating intact tissue with [<sup>35</sup>S] methionine for 2 h and 18 h time periods before the tissue was fractionated on sucrose gradients. The microsomal and glyoxysomal fractions were Triton X-114 extracted and the detergent phase-associated proteins separated by SDS-PAGE (fig. R5.5). Once again, counterparts to glyoxysomal membrane proteins could be seen in the microsomal polypeptide profile. The presence of these microsomal proteins could not be accounted for by contamination with glyoxysomal membranes since it had been shown in section 1 that a maximum of only 3.5% of the glyoxysomal membrane contaminated the microsomes and the intensities of the shared bands in fig. R5.5 were in fact greater in the microsomal profile. Structural similarities between the proteins common to both membrane types was shown by probing both sets of membranes with anti-GA<sub>s</sub>-B antiserum. Fig R5.6 (lane 2) shows the total Triton X-114 associated proteins from glyoxysomal membranes after in vivo labelling with [<sup>35</sup>S] methionine. Lane 3 shows nine polypeptides which were immunoprecipitated by the antiserium. Eight of these proteins were also immunoprecipitated from [<sup>35</sup>S] methionine-labelled microsomal membranes by anti-GA<sub>s</sub>-B

Fig. R5.5 SDS-PAGE Analysis of Microsomal and Glyoxysomal Integral Membrane Proteins After Short or Long Pulse In Vivo Labelling

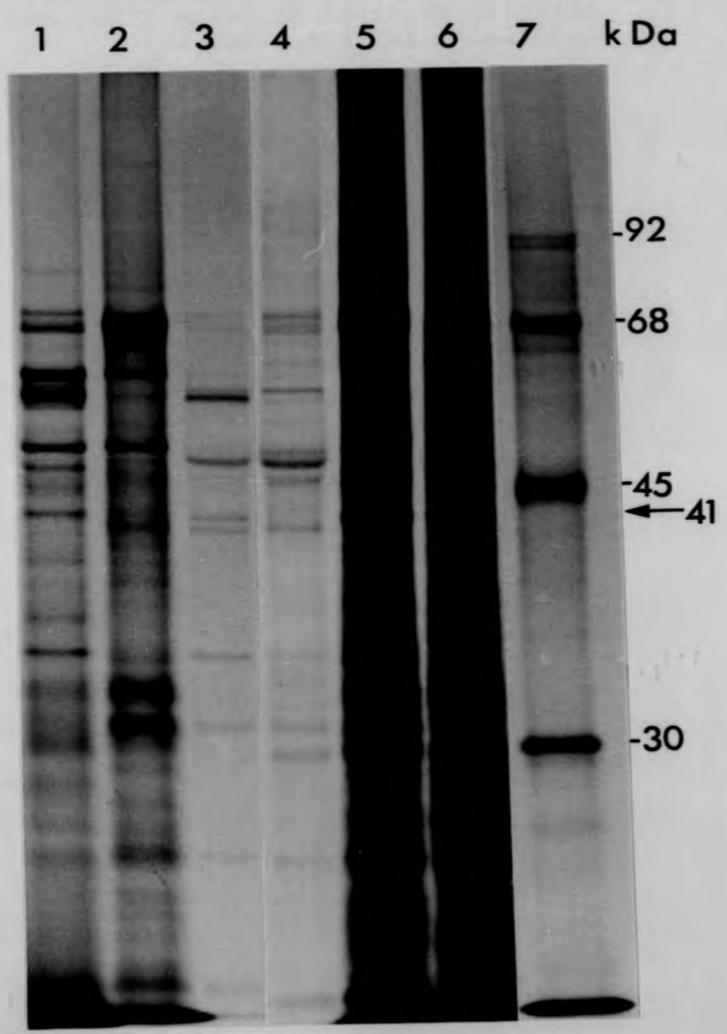


Batches of ten 3-day-old endosperm slices were incubated with [<sup>35</sup>S] methionine (20 μCi/slice) for 2 h or 18 h before fractionating the tissue on two 17 ml sucrose gradients. The microsomal and glyoxysomal bands were collected from each gradient and the associated membranes subjected to Triton X-114 extraction. The detergent-soluble proteins were separated by SDS-PAGE and visualized by fluorography: Lanes 1 and 2, microsomal and glyoxysomal integral membrane proteins, respectively, after 2 h pulse; lanes 3 and 4, microsomal and glyoxysomal integral membrane proteins after 18 h pulse; lanes 5 - 8, as lanes 1 - 4 after shorter exposure to the gel.

Fig. R5.5 SDS-PAGE Analysis of Microsomal and Glyoxysomal Integral Membrane Proteins After Short or Long Pulse In Vivo Labelling



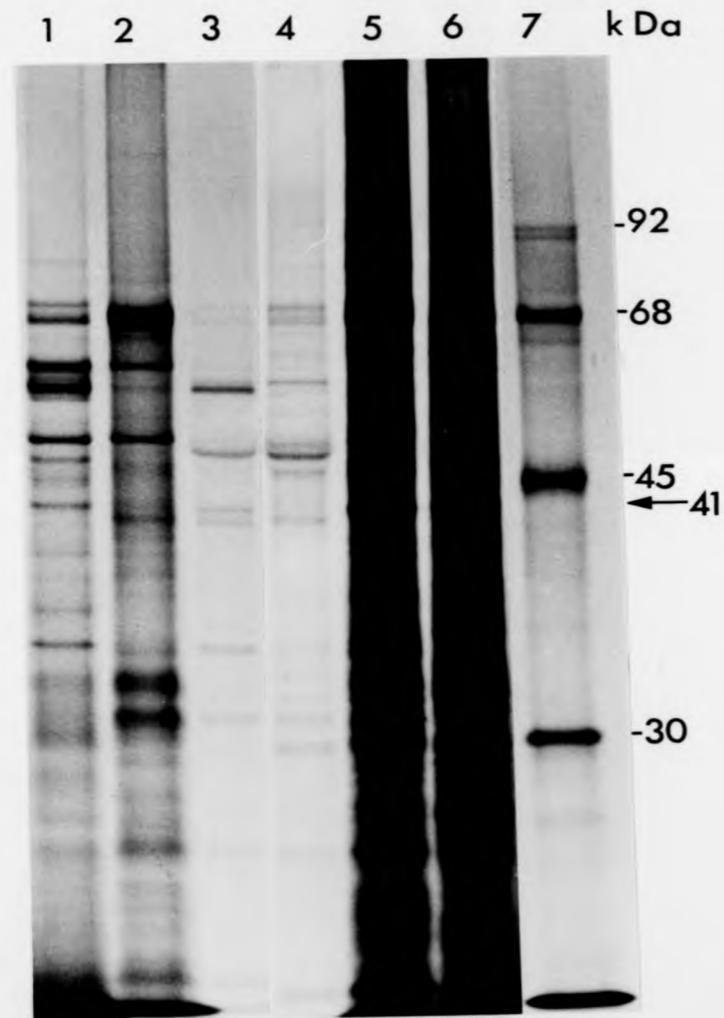
Batches of ten 3-day-old endosperm slices were incubated with [<sup>35</sup>S] methionine (20  $\mu$ Ci/slice) for 2 h or 18 h before fractionating the tissue on two 17 ml sucrose gradients. The microsomal and glyoxysomal bands were collected from each gradient and the associated membranes subjected to Triton X-114 extraction. The detergent-soluble proteins were separated by SDS-PAGE and visualized by fluorography: Lanes 1 and 2, microsomal and glyoxysomal integral membrane proteins, respectively, after 2 h pulse; lanes 3 and 4, microsomal and glyoxysomal integral membrane proteins after 18 h pulse; lanes 5 - 8, as lanes 1 - 4 after shorter exposure to the gel.



**Fig. R5.6 Immunoprecipitation of In Vivo-labelled Glyoxysomal and Microsomal Integral Membrane Proteins**

Ten 3-day-old endosperm halves were incubated with [<sup>35</sup>S] methionine (20 μCi/half) for 16 h at 20°C. Labelled microsomes and glyoxysomes were isolated by sucrose density gradient centrifugation. One half of each organelle suspension was used for immunoprecipitation of proteins reacting with anti-GA<sub>s</sub>-A antiserum as detailed in M.17, p.70. The remaining organelle suspensions were divided into two equal volumes one of which was treated with proteinase K (as described in M.12, p. 62) in each case. The treated and untreated organelles were harvested by centrifugation and the integral membrane proteins extracted with Triton X-114: Lanes 2 and 5, total glyoxysomal and microsomal Triton-soluble proteins respectively; lanes 3 and 4, polypeptides immunoprecipitated from glyoxysomal and microsomal membranes respectively; lanes 1 and 6, Triton-soluble polypeptide profiles of glyoxysomes and microsomes after treatment with proteinase K; lane 7, molecular weight markers.

Fig. R5.6 Immunoprecipitation of In Vivo-labelled Glyoxysomal and Microsomal Integral Membrane Proteins



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(the exception being a protein with an MW of 41 kDa). However, thirteen additional protein bands also appeared in the microsomal fraction immunoprecipitation profile (lane 4).

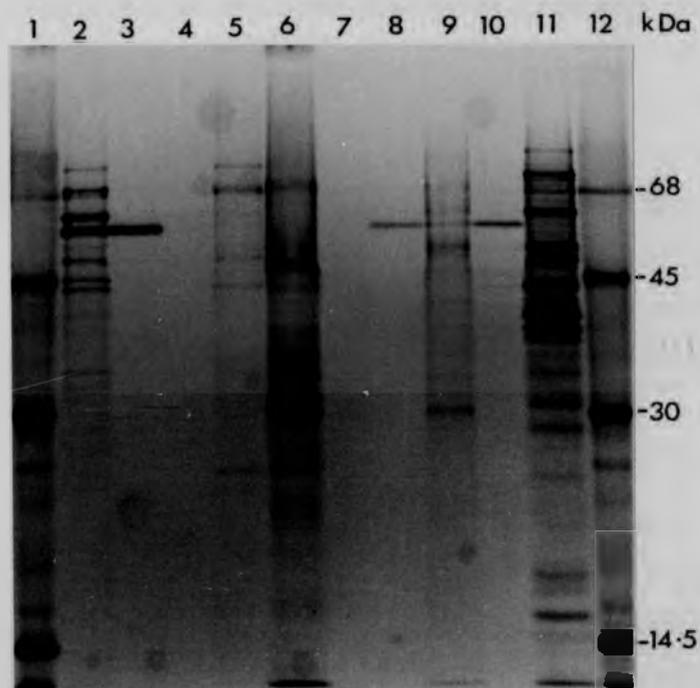
Microsomal and glyoxysomal membranes were subjected to proteinase K digestion in order to show that some protection against proteolytic attack was afforded to proteins embedded in them. Fig. R5.6, lanes 1 and 6, show the effect of proteinase K on the mobilities of Triton X-114-soluble proteins from the glyoxysomes and microsomes respectively. Most proteins with MW's above 70 kDa were degraded by this treatment. At lower MW's however, proteins of both membranes were seen to be susceptible to proteolysis to varying extents and this may reflect the different topological positions occupied by the proteins in relation to the membrane.

(ii) Malate Synthase, A Peripheral Membrane Protein

Huang and Beevers (1973) and Bieglmayer *et al.* (1973) independently concluded that the glyoxylate cycle enzyme malate synthase (MS) is a peripheral glyoxysomal membrane protein in castor bean endosperm. Further evidence for MS's non-integral association with the glyoxysomal membrane was provided by immunoprecipitating [<sup>35</sup>S] methionine-labelled MS from the detergent and aqueous (KCl extracted) phases following Triton X-114 extraction of glyoxysomal membranes. Endosperm tissue was labelled with [<sup>35</sup>S] methionine for 16 h prior to fractionation on a sucrose gradient. The microsomes and glyoxysomes were collected and the membranes extracted with Triton X-114. To the Triton phase, in a volume of

50  $\mu$ l, was added 200  $\mu$ l 10 mM Tris (pH 7.4) followed by 250  $\mu$ l NP-40 buffer (normally used for membrane solubilization, see Methods M.17). To the aqueous phase, in a volume of 200  $\mu$ l, was added 50  $\mu$ l precondensed Triton X-114 and the volume again doubled with NP-40 buffer. Immunoprecipitation was carried out as normal using rabbit anti-MS antiserum raised against MS purified by Dr. L.M. Roberts (University of Warwick). 250  $\mu$ l from the top of the gradient, representing a cytosolic-derived sample, was also taken for immunoprecipitation. The result is shown in fig. R5.7. The total protein profiles of the aqueous and Triton phases from the glyoxysomal membranes is shown in fig. R5.7, lanes 2 and 5 respectively. Immunoprecipitation of MS from each of these fractions reveals MS to be exclusively located in the aqueous phase (fig. R5.7, lane 3).

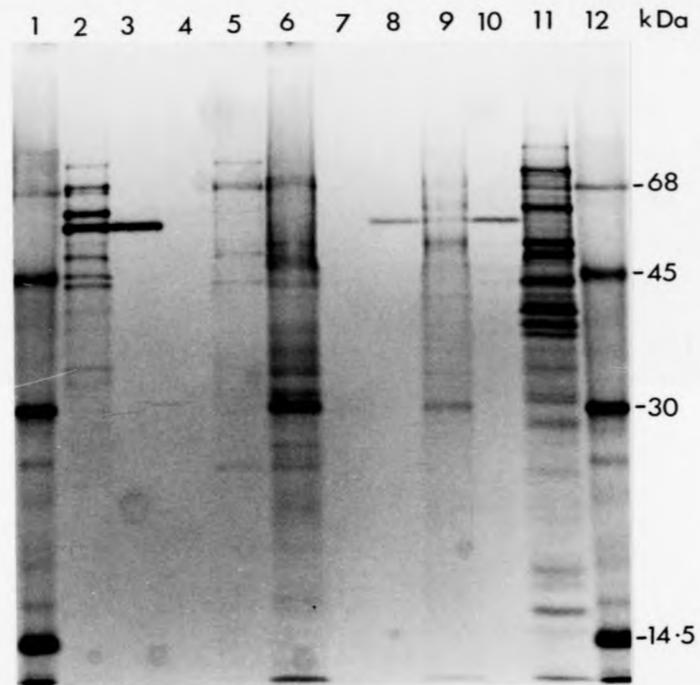
In castor bean endosperm cells, malate synthase activity is additionally associated with the endoplasmic reticulum membrane fraction (Gonzalez and Beevers, 1976; Gonzalez, 1982). Fractionation of [<sup>35</sup>S] methionine labelled e.r. membranes into aqueous hydrophilic and Triton X-114 soluble hydrophobic proteins followed by immunoprecipitation confirmed the hydrophilic, peripheral nature of membrane-associated malate synthase (fig. R5.7, lanes 7 and 8). MS was also present in the soluble fraction obtained at the top of the gradient after centrifugation (fig. R5.7, lanes 10 and 11); this soluble MS presumably represents newly synthesized protein which had not been translocated across a membrane at the time of tissue homogenization (Kindle *et al.*, 1980).



**Fig. R5.7** The Identification of Malate Synthase as a Peripheral Glyoxysomal Membrane Protein

Endosperm tissue was labelled with [<sup>35</sup>S]-methionine and fractionated by sucrose density gradient centrifugation. Glyoxysomal and e.r. membranes were obtained and their constituent proteins were separated into hydrophilic or hydrophobic components using Triton X-114. Lanes 1 and 12, molecular weight markers; lane 2, hydrophilic glyoxysomal membrane proteins; lane 3, malate synthase immunoprecipitated from the proteins in lane 2; lane 5, hydrophobic glyoxysomal membrane proteins; lane 4, malate synthase immunoprecipitated from proteins in lane 5; lane 6, hydrophobic e.r. membrane proteins; lane 7, malate synthase immunoprecipitated from proteins in lane 6; lane 9, hydrophilic e.r. membrane proteins; lane 8, malate synthase immunoprecipitated from proteins in lane 9; lane 11, total soluble proteins; lane 10, malate synthase immunoprecipitated from proteins in lane 11.

Fig. R5.7 The Identification of Malate Synthase as a Peripheral Glyoxysomal Membrane Protein

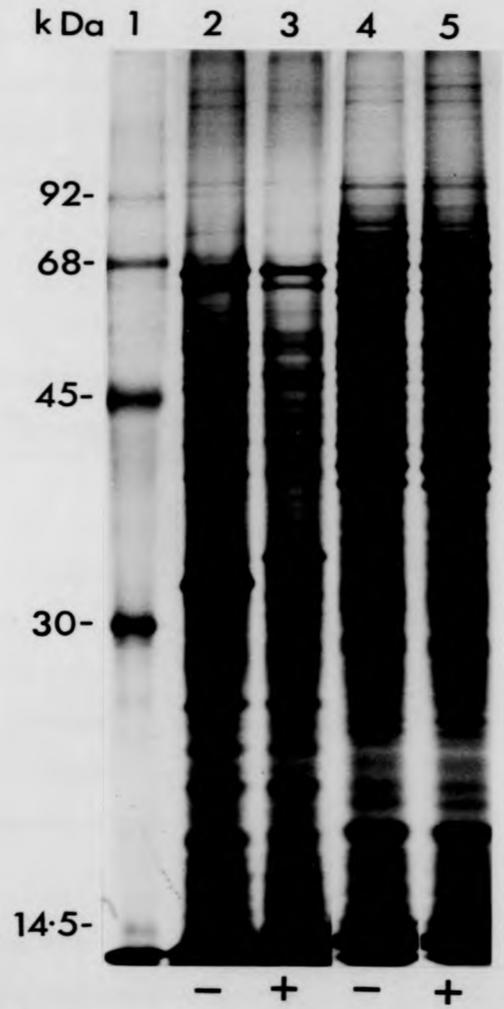


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(iii) Integral Membrane Protein Synthesis In Vitro

Messenger RNA (mRNA) from germinating castor bean endosperm was translated in a rabbit reticulocyte-derived cell-free translation system. Proteins were synthesized in the absence and presence of canine pancreatic microsomes in order to determine if the co-translational translocation of any proteins could be demonstrated. In agreement with Roberts and Lord (1981b), fig. R5.8 (lanes 4 and 5) shows that no obvious differences in the total polypeptide products synthesized in the presence or absence of the dog membranes could be seen. This is in contrast to translations programmed with polyadenylated RNA from developing castor beans (fig. R5.8, lanes 2 and 3) where the processing of several polypeptides was observed. Most obvious was the molecular weight shift exhibited by a protein synthesized, in the absence of dog microsomes, with an MW of 32.5 kDa. When canine microsomes were present during translation this polypeptide was cleaved to a MW of 31 kDa. Such processing is a characteristic of this protein, which is thought to be the precursor to the 2-S albumins located in the protein bodies of castor bean endosperm (Butterworth and Lord, 1983).

The polypeptides of the microsomal and glyoxysomal membranes are probably only minor components of the total polypeptide products of cell-free mRNA translation. In order to enrich for hydrophobic proteins, microsomes, which had been present during in vitro protein synthesis, were isolated, washed by centrifugation through a 0.2 M sucrose cushion, and then subjected to Triton X-114 extraction. The microsomes from a second



**Fig. R5.8** Translation of Ripening and Germinating Castor Bean Endosperm mRNA In Vitro

Total poly (A<sup>+</sup>) RNA from ripening seed endosperm (tracks 2 and 3) and germinating seed endosperm (tracks 4 and 5) were translated in rabbit reticulocyte lysate cell-free systems (as detailed in M.11C, P.61) in the presence (+) or absence (-) of canine pancreatic microsomes. Track 1, molecular weight markers.

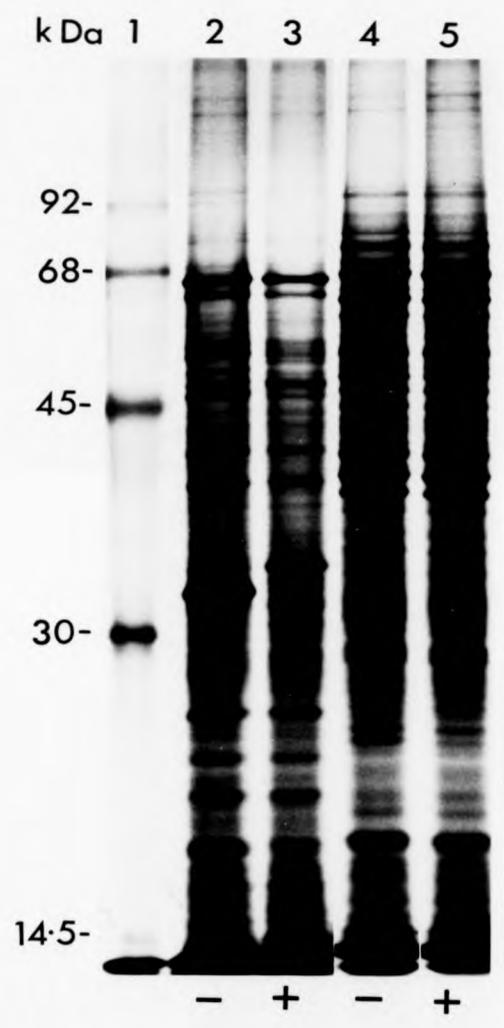
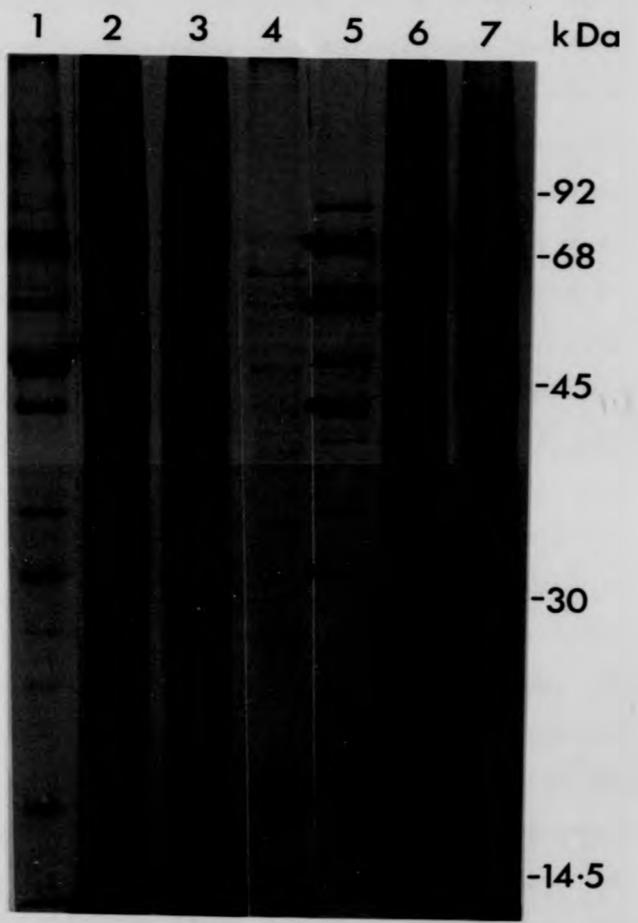


Fig. R5.8 Translation of Ripening and Germinating Castor Bean Endosperm mRNA In Vitro

Total poly (A<sup>+</sup>) RNA from ripening seed endosperm (tracks 2 and 3) and germinating seed endosperm (tracks 4 and 5) were translated in rabbit reticulocyte lysate cell-free systems (as detailed in M.11C, P.61) in the presence (+) or absence (-) of canine pancreatic microsomes. Track 1, molecular weight markers.

series of translations were washed then solubilized in NP-40 buffer and immunoprecipitated using anti-GA<sub>S</sub>A. The resulting polypeptide profiles are shown in fig. R5.9 together with Triton X-114 extracts and immunoprecipitates (again using anti-GA<sub>S</sub>A) from castor bean microsomes and glyoxysomes labelled *in vivo*. Several bands in the canine microsomal polypeptide profile (fig. R5.9, lane 3) can be aligned with Triton X-114-extractable proteins from castor bean microsomes (lane 2) the most prominent being a protein with a MW of 51 kDa. A protein of this mobility is also seen in the Triton extract from glyoxysomal membranes (lane 6), however the antiserum failed to precipitate this polypeptide (lane 5). Studying the profiles of polypeptides immunoprecipitated by anti-GA<sub>S</sub>A more closely reveals similarities between those of castor bean microsomes and glyoxysomes (fig. R5.9, compare lanes 1 and 5 corresponding to microsomal and glyoxysomal immunoprecipitates respectively); however, only five bands in the canine microsomal immunoprecipitate profile can be aligned with bands immunoprecipitated from labelled glyoxysomal membranes (fig. R4.9, asterisks between lanes 4 and 5). Such a situation is perhaps to be expected since proteins destined for the glyoxysomal membrane may undergo post-translational modifications en route to the glyoxysomal membrane from their point of synthesis in the e.r.

The association of anti-GA<sub>S</sub> immunoprecipitated proteins with the canine microsomes was investigated further by removing the microsomes from the translation mixtures after *in vitro* protein synthesis and



**Fig. R5.9 Immunoprecipitation of Integral Glyoxysomal Membrane Proteins Synthesized In Vivo and In Vitro**

Castor bean microsomes and glyoxysomes were labelled with [<sup>35</sup>S] methionine in vivo. Total Triton X-114-soluble proteins from microsomal and glyoxysomal membranes are shown in lanes 2 and 6 respectively. Germinating castor bean mRNA was translated in vitro in reticulocyte lysates in the presence of canine pancreatic microsomes. The microsomes were harvested and also subjected to Triton X-114 extraction. Detergent-soluble polypeptides are shown in lane 3. Glyoxysomal integral membrane proteins were immunoprecipitated from in vivo-labelled castor bean microsomes and glyoxysomes and canine microsomes from in vitro mRNA translations: Lanes 1, 4 and 5 are the immunoprecipitates from castor bean microsomes, canine microsomes and castor bean glyoxysomes respectively. Lane 7, molecular weight markers.

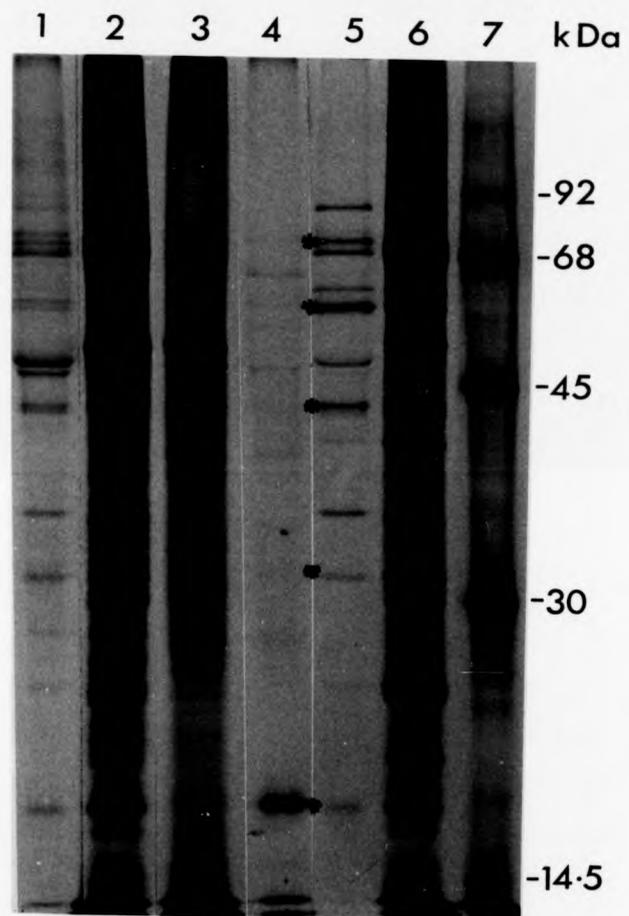


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immunoprecipitating glyoxysomal membrane proteins from the solubilized membrane pellet and the membrane-free lysate supernatant. A similar procedure was followed using a "pellet" and supernatant from pooled translations carried out in the absence of dog membranes. The results are shown in fig. R5.10. Once again there was no difference in the total products synthesized in the absence and presence of canine microsomes (fig. R5.10, lanes 2 and 7). Further, the majority of immunoprecipitable proteins were associated with the lysate supernatant and not the "pellet" regardless of whether canine microsomes were present during translation. Two exceptions to this general observation were polypeptides with MW's of 60 kDa and 22 kDa. In both cases they were more prominent in the microsomal pellet (fig. R5.10, lane 6), however there was no indication of processing or sequestration by the canine membranes. Further evidence to indicate a possible inability of dog microsomes to process castor bean membrane polypeptides was then sought. Following germinating seed mRNA translation in the presence of dog membranes the membranes were removed from suspension and the pellet and supernatant once again subjected to immunoprecipitation by anti GA<sub>5</sub> antiserum. Fig. R5.11 shows that once again the antiserum precipitated more proteins from the lysate supernatant than the microsomal pellet (fig. R5.11, lanes 4 and 3 respectively) but the 22 kDa polypeptide was again predominantly associated with the membranes. Lanes 5 and 6 represent the immunoprecipitates from *in vivo* labelled e.r. and glyoxysomes respectively. The 22 kDa protein is seen to

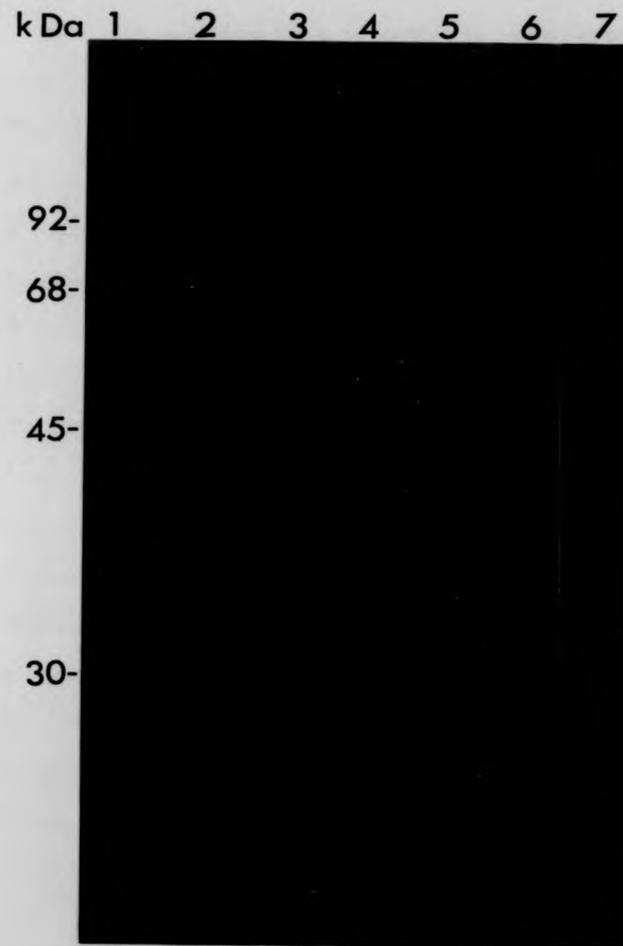


Fig. R5.10 Analysis of Membrane Association of In Vitro Synthesized Glyoxysomal Membrane Proteins

Germinating castor bean mRNA was translated *in vitro* in reticulocyte lysates in the absence and presence of dog microsomes. Following protein synthesis the lysates were centrifuged at 100,000 x g for 30 minutes in a Beckman Airfuge. Glyoxysomal integral membrane proteins were immunoprecipitated from the pellets and supernatants so obtained: Lane 1, molecular weight markers; lanes 2 and 7, total polypeptides synthesized in the absence and presence of canine microsomes; lane 6, glyoxysomal membrane proteins immunoprecipitated from the 100,000 x g pellet from translation mixes containing canine microsomes; lane 5, glyoxysomal membrane proteins immunoprecipitated from the microsome-containing, 100,000 x g supernatant; lanes 3 and 4, as lanes 6 and 5 but from translations carried out in the absence of canine microsomes.

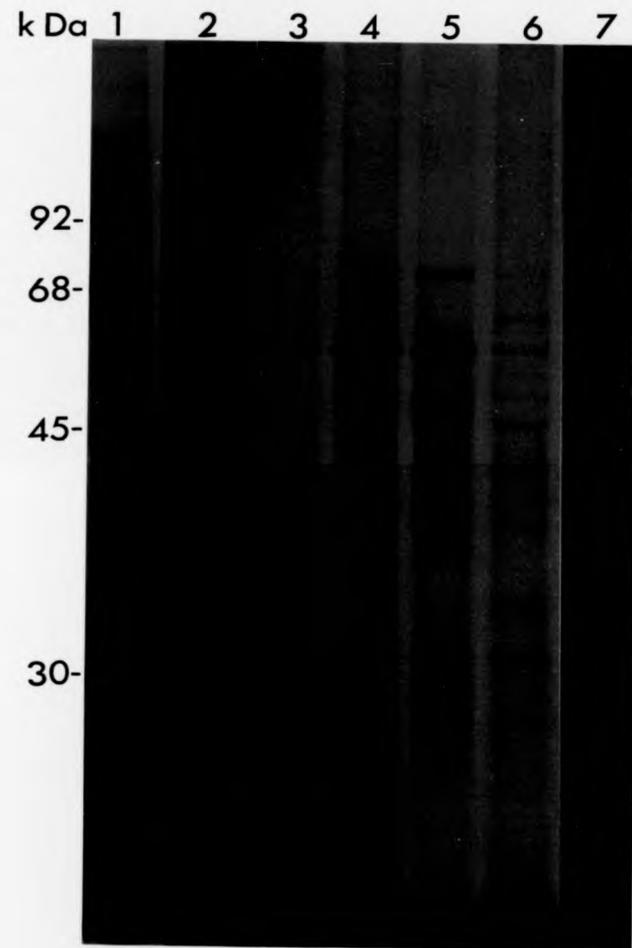
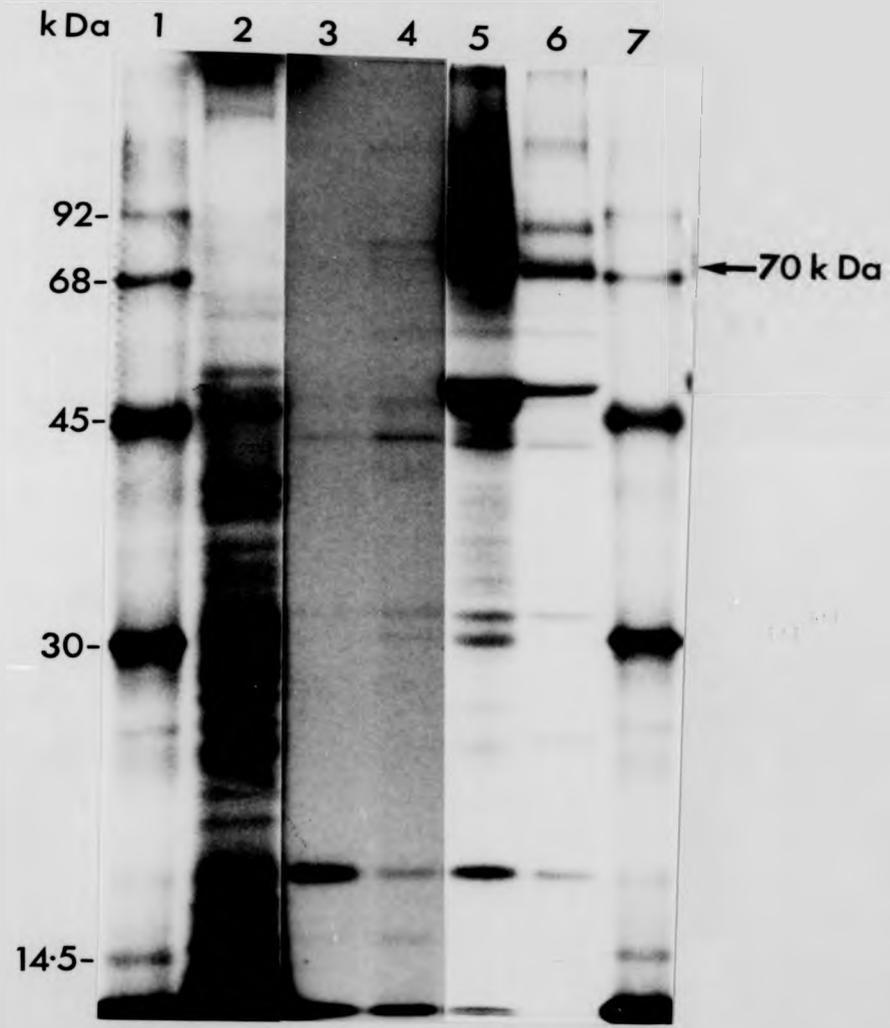


Fig. R5.10 Analysis of Membrane Association of In Vitro Synthesized Glyoxysomal Membrane Proteins

Germinating castor bean mRNA was translated in vitro in reticulocyte lysates in the absence and presence of dog microsomes. Following protein synthesis the lysates were centrifuged at 100,000 x g for 30 minutes in a Beckman Airfuge. Glyoxysomal integral membrane proteins were immunoprecipitated from the pellets and supernatants so obtained: Lane 1, molecular weight markers; lanes 2 and 7, total polypeptides synthesized in the absence and presence of canine microsomes; lane 6, glyoxysomal membrane proteins immunoprecipitated from the 100,000 x g pellet from translation mixes containing canine microsomes; lane 5, glyoxysomal membrane proteins immunoprecipitated from the microsome-containing, 100,000 x g supernatant; lanes 3 and 4, as lanes 6 and 5 but from translations carried out in the absence of canine microsomes.



**Fig. R5.11 Comparison of Integral Glyoxysomal Membrane Proteins Synthesized In Vitro and In Vivo**

[<sup>35</sup>S] methionine-labelled microsomes and glyoxysomes were isolated from in vivo-labelled tissue and glyoxysomal integral membrane proteins immunoprecipitated from each (lanes 5 and 6, immunoprecipitates from microsomal and glyoxysomal membranes respectively). Glyoxysomal integral membrane proteins were also immunoprecipitated from a post translational 100,000 x g supernatant (lane 4) and a canine microsomal pellet (lane 3) following castor bean mRNA translation in vitro. The total polypeptide products from the cell-free translation are shown in lane 2. Lanes 1 and 7, molecular weight markers.

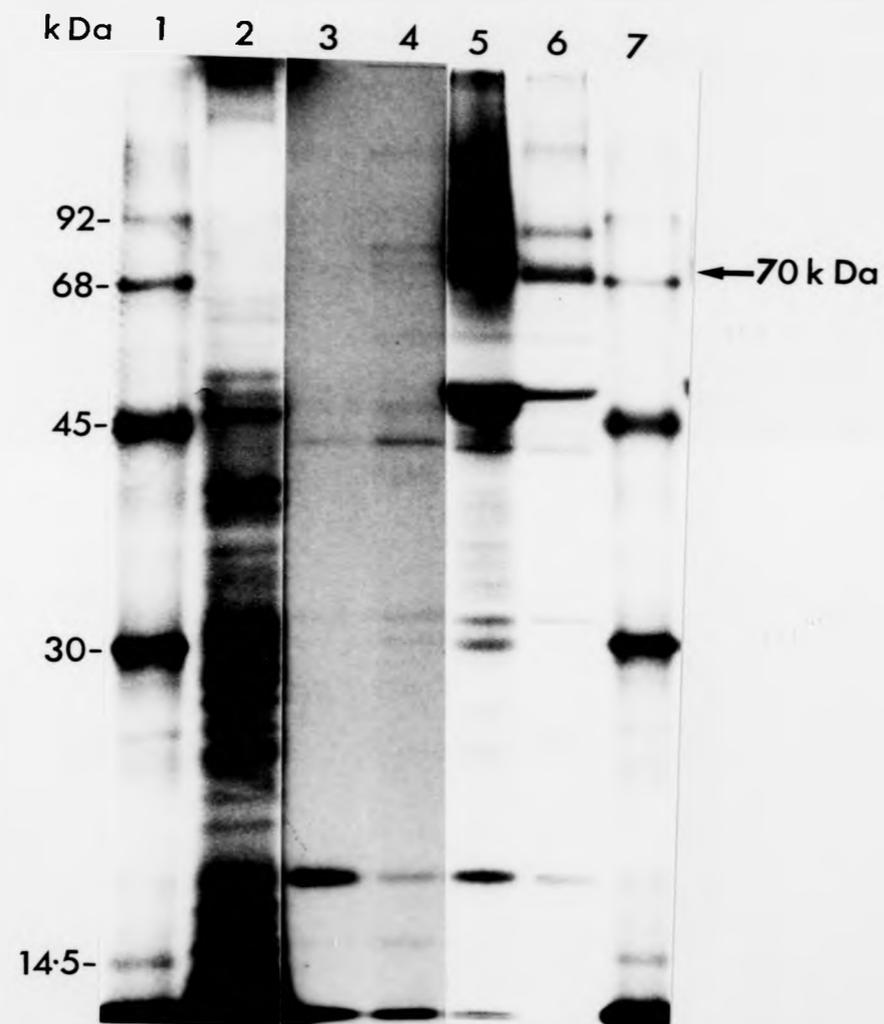


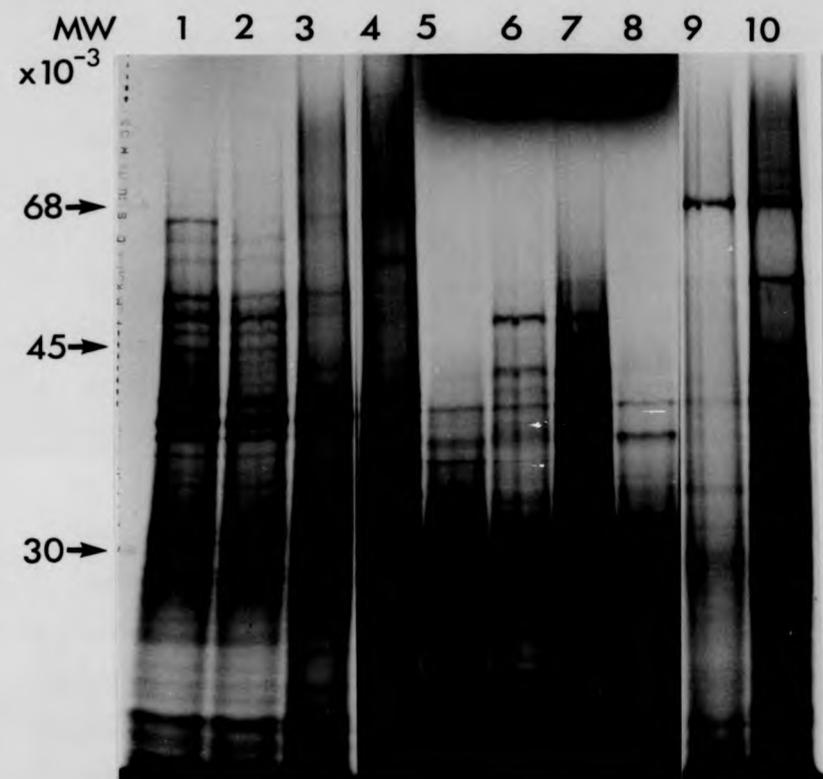
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be present in both membranes. If this protein is synthesized on membrane-bound ribosomes and co-translationally sequestered into the e.r. then transported to the glyoxysomal membrane it would appear that no processing of the protein occurs en route. A different situation may exist with other membrane proteins. For example, a polypeptide doublet, with MW's of 74 and 70.5 kDa, was seen in the castor bean microsomal membrane profile (fig. R5.11, lane 5). In the glyoxysomal membrane a doublet was also evident, however the MW's of these proteins was estimated at 71.5 and 69.5 kDa. If the glyoxysomal bands are derived from those in the microsomes then the change in mobilities indicated some kind of processing to have occurred. Whether this processing was due to proteolysis and/or carbohydrate trimming cannot be deduced from these data. In vitro however, proteins constituting a similar doublet to that observed in the castor bean microsomes were synthesized with MW's of 75 kDa and 71.5 kDa (fig. R5.11, lane 4). If these proteins were indeed counterparts to those of the castor bean microsomes then it would appear that they undergo some kind of processing, such as signal sequence cleavage, in vivo which cannot be demonstrated in the in vitro system. The reason for the lack of processing of proteins translated from germinating castor bean mRNA is not known.

Polypeptides synthesized in the presence of dog microsomes were tested for protection against proteinase K to obtain evidence for insertion into the microsomal membranes. Translations carried out in the absence and

presence of dog microsomes were incubated with proteinase K in the absence and presence of the detergent deoxycholate (DOC). The effects of such treatments on the polypeptide profiles is shown in fig. R5.12. Lanes 4 - 8 are from a fluorogram which had been exposed to the gel for approximately five times longer than the remaining tracks. Comparison of the total Triton X-114 extractable proteins from translations in the absence and presence of dog microsomes (fig. R5.12, lanes 3 and 10) reveals negligible differences. Following proteinase K treatment and Triton X-114 extraction there appeared to be a selective degradation of some of the proteins synthesized in the presence of dog microsomes (compare lanes 9 and 10), however this was probably not due to the protection of regions of these polypeptides by the microsomal membranes since similar proteins, synthesized in the absence of dog membranes underwent proteolysis to a similar extent (the polypeptide profile of fig. R5.12, lane 4 showed bands of similar intensities of those in lane 9 after equal exposure to the gel). If DOC was included during the protease treatment then the effect on the proteins synthesized in the absence and presence of dog microsomes was as shown in fig. R5.12, lanes 5 and 8 respectively (note - DOC prevented the separation of Triton X-114 solutions into two phases thus Triton X-114 extraction could not be performed on DOC-containing samples). Again similar profiles were obtained indicating (a) the DOC was not increasing the accessibility of membrane proteins to protease digestion and (b) the period of incubation of the protein mixtures



with proteinase K was of 15 minutes to allow the complete degradation of any the polypeptides present. The DOC did, however, appear to allow access of the proteinase K to polypeptide regions which were inaccessible in the absence of DOC. Data being for

Fig. R5.12 Protease Treatment of Integral Membrane Proteins Synthesized In Vitro

Lanes 1 and 2, total polypeptide profiles from cell-free translation of castor bean mRNA in the absence and presence of canine pancreatic microsomes respectively. Lanes 3 and 10, total detergent-soluble proteins from translations in the absence and presence of dog microsomes after Triton X-114 extraction; lanes 4 and 9, as lanes 3 and 10 but following the treatment of the lysates with proteinase K; lanes 6 and 7, as lanes 4 and 9 but without subjecting the proteins to Triton X-114 extraction following proteinase K treatment; lanes 5 and 8, as lanes 6 and 7 but with the inclusion of 1% DOC during incubation with proteinase K.

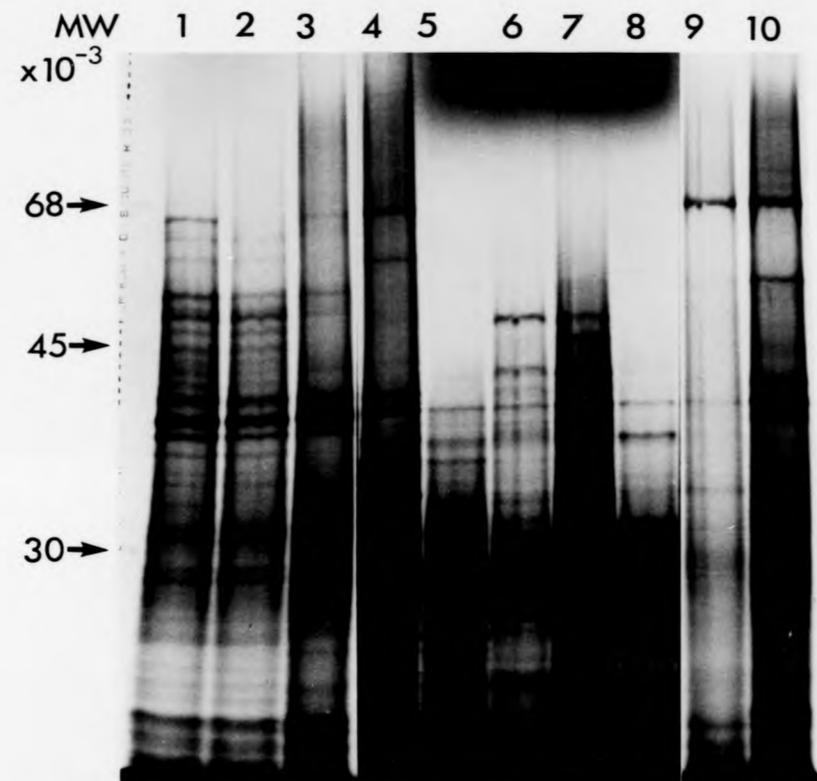


Fig. R5.12 Protease Treatment of Integral Membrane Proteins Synthesized In Vitro

Lanes 1 and 2, total polypeptide profiles from cell-free translation of castor bean mRNA in the absence and presence of canine pancreatic microsomes respectively. Lanes 3 and 10, total detergent-soluble proteins from translations in the absence and presence of dog microsomes after Triton X-114 extraction; lanes 4 and 9, as lanes 3 and 10 but following the treatment of the lysates with proteinase K; lanes 6 and 7, as lanes 4 and 9 but without subjecting the proteins to Triton X-114 extraction following proteinase K treatment; lanes 5 and 8, as lanes 6 and 7 but with the inclusion of 1% DOC during incubation with proteinase K.

with proteinase K was of insufficient length to allow the complete degradation of all the polypeptides present. The DOC did, however, appear to allow access of the proteinase K to polypeptide regions which were inaccessible in the absence of DOC; there being no discrete polypeptide bands in the mixtures treated in the presence of DOC (fig. R5.12, lanes 5 and 8) with MW's above approximately 40 kDa. This effect is more directly comparable to proteins treated with protease in the absence of DOC but which had not been subjected to Triton X-114 extraction (fig. R5.12, lanes 6 and 7). In this case polypeptides with MW's above 40 kDa could be seen and which were resistant to further proteolytic degradation by proteinase K.

DISCUSSION V

The search for the subcellular origin of microbodies has tended to concentrate on the soluble proteins of the microbody matrix (e.g. Goldman and Blobel, 1978; Walk and Hock, 1978; Roberts and Lord, 1981b) or proteins peripherally associated with the internal surface of the surrounding membrane, e.g. malate synthase (Kindl, 1982; Lord and Roberts, 1982). In all these cases a general post-translational mode of membrane translocation has emerged.

Only recently have efforts been focused on the elucidation of the origin of the proteins integrally associated with the microbody membrane (Fujiki *et al.*, 1982b; 1983). A comparison of the integral membrane proteins of rat liver peroxisomes with those of e.r. from the same cells showed, on a molecular weight basis, that the polypeptide composition of the two membranes was completely different (Fujiki *et al.*, 1982b) and this was taken as additional evidence for a non e.r. origin to the peroxisomal membrane. A more recent report by Fujiki *et al.* (1983 [Abs]) claimed that an integral polypeptide of the peroxisomal membrane is synthesized on free, cytosolic polysomes hence implying a post-translational mechanism for its insertion into the membrane. Results from the studies with castor bean glyoxysomal membrane proteins, presented here, conflict with those of Fujiki *et al.* (1982) in that the polypeptide profile of the glyoxysomal membrane shares many features with that of the e.r., further, these features cannot be explained by cross-contamination of

subcellular organelle fractions for reasons illustrated in fig. R5.5 and other sections, i.e. 1 and 4.

The characterization of castor bean glyoxysomal membrane components has been confined largely to the membrane lipids (Kagawa et al., 1973; Lord, 1976; Donaldson and Beevers, 1977) where a role for the e.r. in glyoxysome ontogeny has been convincingly demonstrated (reviewed by Lord and Roberts, 1983). The protein complement of castor bean glyoxysomal membranes however, has only been studied to a limited degree (Bieglmayer and Ruis, 1974). Earlier developmental and serological investigations demonstrated similarities to exist between the polypeptides of these membranes and those of the e.r (Bowden and Lord, 1977; 1978; Brown et al., 1976). These latter studies were performed on membrane proteins which had been subjected to a less rigorous selection procedure than the Triton X-114 method of integral membrane protein extraction used in this study. The extraction of integral membrane polypeptides using Triton X-114, originally described by Bordier (1981), has been used to selectively extract the E1 glycoprotein of coronavirus mouse hepatitis virus A59 (Rottier et al., 1984) where its specificity for hydrophobic integral membrane proteins was demonstrated. Triton X-114 extracts of glyoxysomal membranes were used to raise antibodies in rabbits. Immunofluorescence histochemistry was used to show that the antisera was indeed directed against proteins of glyoxysomal origin and the staining of an intracellular network hinted that common antigens were present in the e.r. also (there being no evidence, to date, for a glyoxysomal equivalent

to the "peroxisomal reticulum" postulated by Lazarow et al. [1982]).

None of the proteins extracted by Triton X-114 was specifically identified. Indeed only a few enzymes are known to be integrally associated with the glyoxysomal membrane, namely an alkaline lipase (Muto and Beevers, 1974) and possibly the constituents of a primitive electron transport chain (Donaldson et al., 1981; Hicks and Donaldson, 1982). The only specific protein investigated in the present study was the peripheral glyoxysomal membrane protein, malate synthase. Although this protein has been described as an integral membrane protein in glyoxysomes of cucumber cotyledons (Kindl, 1982) it is clearly peripherally associated in castor bean. In addition to confirming the hydrophilic nature of this protein in castor beans (fig. R5.7), the immunoprecipitation of MS served as a control illustrating the lack of non-specific precipitation of hydrophobic proteins by non-membrane protein-directed antiserum. Thus the polypeptides immunoprecipitated by the anti GA<sub>S</sub> were almost certainly the result of specific antibody/antigen interactions.

The anti GA<sub>S</sub> reacted with approximately 10 proteins with molecular weights varying from 20 kDa to 85 kDa. From this preliminary study it seemed possible that different glyoxysomal membrane polypeptides arrived at their location in the membrane by following one of several routes, not all of which necessitated e.r. involvement. The route followed most commonly appeared to begin with protein synthesis on free ribosomes so

implying their post-translational insertion into the glyoxysomal membrane. This was supported by the results of in vitro germinating castor bean mRNA translation studies carried out in the absence and presence of canine pancreatic microsomes. Following the separation of the microsomes from the reticulocyte lysate by centrifugation, and immunoprecipitation of glyoxysomal membrane proteins from the solubilized membranes and the lysate supernatant, the majority of immunoprecipitated proteins were found associated with the supernatant (fig. R5.10). There were two exceptions to this, a minor protein with a MW of 60 kDa and another protein of MW 21 -22 kDa. This latter polypeptide was synthesized in large amounts and was the most abundant protein associated with the polypeptide profile of the immunoprecipitate from the dog microsomes. A protein of the same mobility was the major immunoprecipitated protein from the lysate "supernatant" following mRNA translation in the absence of dog microsomes. Referring back to the profile of Triton X-114 extracted proteins from the glyoxysomal membrane (fig. R1.8 and R3.8), the major polypeptide of the glyoxysomal membrane is also of 21 kDa MW. This protein is perhaps analogous to the 21.7 kDa protein of rat liver peroxisomes which has been reported to be synthesized on free polysomes (Fujiki et al., 1983). Further work is required before it can be stated with certainty that the 21 - 22 kDa glyoxysomal membrane protein is co-translationally inserted. Protection experiments (figs. R5.6 and R5.12), which involved subjecting glyoxysomal and microsomal vesicles (the latter from both castor bean and canine pancreas)

to protease treatment following protein synthesis in vivo and in vitro, provided inconclusive results. Interpretation of the data was difficult for several reasons. Unlike the situation with secretory proteins (e.g. Blobel and Dobberstein, 1975b) and organelle matrix proteins (e.g. Lord and Roberts, 1981a) where the entire polypeptide chain traverses a membrane and thus becomes resistant to protease digestion, stretches of membrane proteins may remain exposed to the vesicles external environment and thus to the protease. Evidence for membrane protein insertion would therefore be a partial protection of the inserted protein from protease, indicated by a molecular weight shift on SDS-PAGE (Katz et al., 1977). The mixed antisera used in the present study precipitated several proteins thus making it impossible to identify polypeptide fragments which appeared following protease treatment.

Had the 21 kDa polypeptide shown signs of some kind of processing, e.g. signal sequence cleavage or glycosylation (there is no evidence of a glycosylated protein of this MW in the glyoxysomal membrane glycoprotein profiles [section 4]) then this would have been strong evidence for co-translational insertion into the dog microsomes. The MW's of the protein synthesized in the absence and presence of dog microsomes appears to be exactly the same (the protein migrating to a region of a 10% polyacrylamide gel where a difference of only a few hundred daltons would alter the proteins mobility quite markedly). The absence of cleaved signal sequences cannot be taken as evidence for non-cotranslational membrane insertion, several proteins having been

identified which contain "internal" signal sequences (Meek et al., 1982 Wolfe and Wickner, 1984). Indeed there is emerging evidence for the major protein of castor bean protein body membranes being synthesized in this way (C. Halpin, University of Warwick, personal communication). This protein, with a MW of 24.5 kDa, is synthesized in vitro but appears in abundance only when canine pancreatic microsomes are included in the translation mixture. In this respect the synthesis of the protein body membrane protein serves as an additional control to illustrate that castor bean mRNA is capable of directing the synthesis of a membrane protein which is recognised as such by the canine microsomes. A recent study of the synthesis of a 25 kDa integral protein of the protein body membrane of Phaseolus vulgaris has shown it to be initially inserted into the e.r. membrane. This protein was not glycosylated and it was not processed to any major extent during its transport from the e.r. to the protein body membrane (Mader and Chrispeels, 1984). The possibility of the 21 kDa castor bean proteins' association with the dog microsomal pellet being due to some post-translational hydrophobic interaction remains to be tested. This could be done by adding canine pancreatic microsomes to an in vitro translation system after the termination of protein synthesis. Proteins which post-translationally integrate into microsomal membranes are rare (the cytochrome  $b_5$ 's being the best known examples [Borgese et al., 1980; Okada et al., 1982]). However, proteins such as apocytochrome<sub>c</sub> have been shown to be capable of spontaneous insertion into membranes (Dumont and Richards, 1984).

Stronger evidence for e.r. involvement in glyoxysomal membrane protein synthesis comes from the comparison of in vivo-labelled glyoxysomal membrane Triton X-114 extracts with those from dog microsomes after the latter's inclusion in the cell-free translation of germinating castor bean mRNA. Of the proteins immunoprecipitated by anti-GA<sub>s</sub>, five were associated with both the dog microsomes and the glyoxysomal membrane though, with the exception of the 21 kDa protein, none of these proteins appeared as major components of the membrane. It is clear that future studies should concentrate on a single glyoxysomal membrane protein. For convenience one which is present in large abundance could be chosen, however, it is possible that, as in the case of the rat liver peroxisomal membrane protein, the major glyoxysomal membrane proteins are synthesized without the involvement of the e.r. The proteins in the MW range 69 - 72 kDa would appear to be of considerable interest since (a) in vivo proteins of this MW are present in both the e.r. and the glyoxysomes (figs. R5.9, R5.11), (b) the protein profile of glycosylated proteins (fig. R4.5) shows the presence of glycosylated proteins in this MW range and (c) in vitro-synthesized polypeptides of appreciably larger size than those synthesized in vivo were immunoprecipitated and this could imply the presence of cleavable signal sequences. However, should this prove to be the case it will be of interest to determine why these proteins, when synthesized in vitro in the presence of dog pancreatic microsomes, failed to be co-translationally incorporated into the membranes.

To summarise then, the results presented in this section are from preliminary studies and are hence somewhat confusing. The synthesis of the glyoxysomal membrane is clearly more complicated than was once imagined; greater importance must be placed on a post-translational mechanism of protein incorporation into the membrane following the synthesis on free ribosomes. This would appear to be the route adopted by the major protein components. A co-translational route, via the e.r. may also exist however, though possibly only for minor polypeptides. The most compelling evidence for this is the presence of oligosaccharide moieties attached to some of the proteins via N-glycosidic linkages (see section 4).

FINAL DISCUSSION AND CONCLUDING REMARKS

The recent accumulation of evidence for the synthesis of microbody proteins on ribosomes not bound to the e.r. has necessitated the revision of the importance of this organelle in microbody biogenesis. Investigations carried out with rat liver have led some workers to conclude that the e.r. plays no role whatsoever in peroxisome synthesis (Lazarow et al., 1982). In the case of plant microbodies, glyoxysomes in particular, recent revisions of the "classical" e.r.-vesiculation model (Hruban and Recheigl, 1969) still retain a role for the e.r. in supplying components for glyoxysome construction. The first of these revised models (Wanner et al., 1982) attempts to account for glyoxysome formation in watermelon seedlings. Rather than glyoxysomes "budding" from the e.r., this model has rudimentary "glyoxysomes" (identified by catalase staining) appearing as rough-e.r. cisternae which fuse with the lipid bodies. They then proceed to grow around the lipid body which diminishes in size as the lipid reserves are depleted. The proponents of this model state that glyoxysomal proliferation occurs throughout the period of germination and is initiated from the rough-e.r. cisternae. Glyoxysome growth, however, would seem to be completely independent of e.r. involvement and thus post-translational synthesis of both matrix and membrane proteins is implied. The second alternative-model (Kunce et al., 1984), based on morphometric data, concerned glyoxysome biogenesis in cotton seeds. In this case developing seeds, as opposed to germinating seedlings, were studied since by the the onset of

germination de novo glyoxysome synthesis had been completed. Glyoxysome proliferation throughout germination occurred purely by the growth of preformed immature organelles. The e.r. in this case was proposed as the site of synthesis of membrane components which were then transferred post-translationally to the immature glyoxysome "targets". The synthesis of "incomplete glyoxysomes" during seed maturation has also been described in cucumber (Koller et al., 1979a and b) although biochemical evidence for or against a role for the e.r. in the formation of these progenitor organelles has not been presented. The mature glyoxysomes of watermelon, cotton and cucumber are highly pleomorphic structures (Wanner et al., 1982; Kuncze et al., 1982) and thus differ from the uniform glyoxysomes of castor bean with which the present study has been concerned. There is no evidence for significant glyoxysomal synthesis in developing castor beans. If immature glyoxysomes are present in dry castor beans they are considerably less abundant than in cotton seeds where they can be seen using the light microscope. This would imply that glyoxysome biogenesis occurs in castor beans throughout germination and the prime aim of the work presented in this thesis was to assess if any role was played by the e.r. in this process. As it became more apparent that the matrix proteins of glyoxysomes and indeed, microbodies in general, are translocated across the "glyoxysomal" membrane post-translationally, it was decided that attention should be turned to the integral polypeptides of the glyoxysomal membrane.

The de novo synthesis of the glyoxysomal membrane without the involvement of the e.r. is difficult to envisage. A "chicken and egg" situation exists in that a glyoxysomal membrane is necessary for a glyoxysomal membrane protein to insert itself into, yet a glyoxysomal membrane without protein components already being present, i.e. a liposome, would not be expected to possess characteristics which allowed the selective insertion of specific proteins. In other cases where membrane proteins are synthesized on free ribosomes, e.g. mitochondrial proteins (Zimmermann et al., 1981; Gasser et al., 1982; Mihara et al., 1982) the membranes into which the proteins insert are preformed. Whereas preformed microbody membranes may exist in some cases (e.g. rat liver [Lazarow et al., 1982]) in castor bean endosperm they almost certainly do not.

The involvement of the e.r. in glyoxysomal membrane biogenesis was investigated using two approaches. The first involved showing that glyoxysomal membrane proteins underwent a modification which could only occur during insertion into the e.r. membrane. The modification chosen was N-glycosylation which has been shown to be a co-translational event in other systems (Katz et al., 1977; Anderson and Blobel, 1981; Roberts and Lord, 1981a). Prior to showing that proteins present in the glyoxysomal membrane were indeed glycosylated it was necessary to establish that glyoxysomal preparations were free from contamination by other subcellular fractions. Though free from contamination themselves glyoxysomal membrane fragments

were shown to be present in the other two major organelle fractions, i.e the mitochondria and the e.r.-derived microsomes, obtained by sucrose gradient centrifugation of tissue homogenates. It was then necessary to verify that the similarities which existed between the polypeptide profiles of the microsomal and glyoxysomal bands were not merely due to the presence of glyoxysomal membranes in the microsomal fraction. It was unlikely that the similarities in glycopeptides was the result of such contamination since the glyoxysomes had been shown to be devoid of protein glycosyltransferase activities (Mellor and Lord, 1979c; Mellor *et al.*, 1980). The endoplasmic reticulum was therefore investigated as the unique site of glycoprotein synthesis in order to confirm, and extend, the results of Mellor. In addition to showing that the e.r. was responsible for the biosynthesis of mono- and oligosaccharide lipids (involved in the assembly of the core-oligosaccharide transferred co-translationally to nascent glycopeptides) this activity was localized to a subfraction of the e.r. tentatively assigned to be of rough-e.r. origin. This subfraction was distinct from a denser subfraction, more highly enriched in fucosyl- and possibly glucosyl-transferases, though the role of the glucosyltransferase in protein glycosylation was left open to further investigation.

The presence of glycoproteins in the glyoxysomal membrane was established using radioactive sugars to label tissue *in vivo*. Glycoproteins had already been detected by other workers (Bergner and Tanner, 1981) and Mellor *et al.* (1980) had undertaken an analysis of the

sugars present in the membrane proteins from all three organelle fractions. The study by Mellor, however, failed to acknowledge the possible contamination of the mitochondrial fraction by glyoxysomal membranes. The sugar analysis of the mitochondrial membrane may therefore be somewhat inaccurate. Comparing the glycoprotein profiles of mitochondria and glyoxysomes after overlaying with [ $^{125}$ I] con A indicated that the mitochondrial glycoprotein content was not due solely to glyoxysomal membrane protein contamination. The mitochondrial profile was very different to that of the glyoxysomal membranes but it was very similar to the profile obtained following the labelling of mitochondria in vivo with [ $^3$ H] mannose. GDP-mannosyl dolichylmonophosphate transferase activity is associated with the mitochondrial fraction of castor bean (Mellor and Lord, 1979c) and has been shown in other tissues (Morelis et al., 1974). No glycosyltransferase activity has ever been found associated with the glyoxysomes however. The glycoproteins present in glyoxysomal membranes is the best evidence to date for the direct involvement of the e.r. in glyoxysomal biogenesis.

I began preliminary investigations into the synthesis of glyoxysomal proteins in vitro in an attempt to gain evidence for a co-translational mechanism for their insertion into the e.r. membrane. For this purpose canine pancreatic microsomes were used which had been shown to possess translocational activity in cell-free protein synthesizing systems programmed with ripening castor bean mRNA. The data obtained only tentatively suggested any co-translational insertion to be occurring

and it appeared that, like the matrix proteins, the majority of glyoxysomal membrane proteins are synthesized on free-ribosomes. A similar conclusion was recently reported in the case of the major rat liver peroxisomal membrane protein (Fujiki *et al.*, 1983). However, it is evident that some proteins of the glyoxysomal membrane, and indeed the e.r. membrane, are glycosylated, thus it may be that the mRNA's encoding these proteins are in very low abundance. The results of the study were complicated by the use of a mixed antiserum (raised against a glyoxysomal membrane Triton X-114 extract) which immunoprecipitated a wide range of proteins. Clearly an effort should now be made to concentrate on a specific, preferably glycosylated, protein of the glyoxysomal membrane, purify it to homogeneity and raise antiserum to it. The mRNA encoding the protein should be enriched (initially, possibly, by mRNA fractionation on sucrose gradients) and the protein's synthesis on membrane-bound polyribosomes confirmed. The antiserum could also be used for immunohistochemical localization studies at both the light and, more importantly, electron microscope levels (e.g. as used by Bendayan and Shore, 1982 for mitochondrial protein localization). These studies would (a) confirm that the protein was of glyoxysomal membrane origin, (b) perhaps reveal the protein to be present in the e.r., presumably en route to the glyoxysomes and (c) possibly give clues to the protein's function if, for example, the protein was seen to have a particular distribution in the glyoxysomal membrane.

A final word should be said concerning the assigning of a general mode of synthesis to all forms of microbody, whatever the origin. The presence of microbodies in organisms as diverse as man, Tetrahymena, Neurospora and castor beans suggests they originated before the divergence of the plant and animal kingdoms (between 0.5 and 1 billion years ago). Subsequent evolution may have generated differences in the mode of microbody biogenesis in rat liver and castor bean and may also have emphasised any differences between glyoxysomal biogenesis in castor bean and, e.g. watermelon and cucumber. It should therefore not be so surprising that "no glycoproteins were detected in rat liver peroxisomes" (Volk and Lazarow, 1982). Trelease (1984) ends the most recent review on glyoxysome biogenesis by stating, essentially, that variations on the e.r-vesiculation model may hold for some systems but others, e.g. rat liver peroxisomes, may rely on a completely different mode of development. The next major goal in the field of microbody biology, following the elucidation of the biosynthesis of the individual species types, may therefore be the pinpointing of the divergence points of these different types on the evolutionary scale.

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