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THE SECRETION OF CHICK PROTEINS  
FROM XENOPUS OOCYTES  
AN INVESTIGATION INTO NON-PARALLEL SECRETION

Thesis submitted for the degree of  
Doctor of Philosophy  
of the University of Warwick

by  
Daniel F. Cutler

Department of Biological Sciences  
University of Warwick  
Coventry

October 1982

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### Summary

The oocyte of Xenopus laevis has been established as a system with which to study the export of many secretory proteins. When oocytes are micro-injected with hen oviduct mRNA, ovalbumin and lysozyme accumulate at different rates in the surrounding incubation medium. This thesis concerns an investigation of the molecular and cellular basis for this non-parallel secretion.

Kinetic studies confirm that the intrinsic rate of lysozyme secretion from oocytes is approximately twelve times that of ovalbumin and show that not all of the lysozyme is available for export. This slower rate of ovalbumin export is maintained following injection of a range of concentrations of oviduct mRNA or of purified ovalbumin messenger, the latter having been obtained by hybridisation to cloned ovalbumin complementary DNA. These results suggest that the differential rates of secretion of these two proteins observed in oocytes are not the consequence of competition for amphibian or avian factors and show that oviduct-specific proteins are not required for ovalbumin secretion.

To analyse the cellular basis for this non-parallel secretion, oocyte-fractionation protocols have been employed: if organelles of the secretory pathway could be separated, then it might be possible to distinguish between two possibilities. Firstly, that this non-parallel secretion reflects the relative retardation of ovalbumin during stages preceding the formation of the final exocytotic vesicles (containing both ovalbumin and lysozyme). Secondly, the two proteins might be segregated from one another and secreted by different tracks along the same pathway. Microsomal fractions containing oviduct proteins have been isolated from oocytes using sucrose gradients, but a demonstrable separation of Golgi- and ER-derived microsomal subfractions has not been obtained.

Analysis of pulse-labelled and pulse-chased oocytes on sucrose gradients show that non-secretory lysozyme slowly enters a novel high density oocyte compartment. Movement of lysozyme into this compartment may be prevented by the use of monensin.

### Acknowledgements

I am grateful to my supervisor, Alan Colman, for his continued interest, constructive criticism and support throughout this work. I would also like to thank members of the Department of Biological Sciences, particularly Giorgio Valle, Elizabeth Jones and Jenny Brown, for helpful and stimulating discussions. I wish to thank the Medical Research Council for financial assistance. Finally, I would like to thank my family for persistent encouragement and support throughout my education.

Declaration

Much of the work presented in Chapters 3 and 4 of this thesis has been published (Cutler, D., Lane, C. D. and Colman, A. (1981) J. Mol. Biol. 153, 917-931). All the work presented in this thesis is entirely that of the author.

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Abbreviations

BHK cells	: baby hamster kidney cells
Bisacrylamide	: N,N'-methylene bisacrylamide
BSA	: bovine serum albumin
cpm	: counts per minute
DTT	: dithiothreitol
EDTA	: ethylenediaminetetra-acetic acid
Endo H	: endo- $\beta$ -N-acetylglucosaminidase H
(r)ER	: (rough) endoplasmic reticulum
HEPES	: N-2-hydroxyethylpiperazine-N'-2-ethane-sulphonic acid
PBS	: phosphate buffered saline
PIPES	: piperazine-N,N'-bis (2-ethanesulphonic acid)
PMSF	: phenylmethylsulphonyl fluoride
POPOP	: 1,4-di(2(5-phenyl-oxazolyl))-benzene
PPO	: 2,5-phenyl oxazole
PVP	: polyvinylpyrrolidone
SDS	: sodium dodecyl sulphate
TCA	: trichloroacetic acid
TEMED	: N,N,N',N'-tetramethylethylenediamine
Trizma base	: 2-amino-2-hydroxymethyl propane-1,3-diol
VSV (-G)	: vesicular stomatitis virus (glycoprotein)
CHO cells	: Chinese hamster ovary cells

Chapter 1

Introduction

All cells produce proteins whose function requires insertion into, association with, or partition across one or more of the cell's membranes. This thesis concerns an investigation into aspects of the biogenesis of two proteins belonging to one particular class of localised proteins: those exported from the cell.

## 1. The Secretory Pathway

### a) Cellular Background

All eukaryotic cells have a number of organelles, including the endoplasmic reticulum (ER), Golgi apparatus, nucleus, mitochondria, lysosomes and a variety of other cell type-specific structures. Within cells specialised for secretion, two of these organelles are particularly strongly represented, the rough ER (rER) and the Golgi apparatus. For example, the cytoplasm of the pancreatic acinar cell is "packed with stacked ER cisternae studded with ribosomes" (Palade, 1975), i.e. with rER, and also has a great many structures derived from the Golgi apparatus present, such as condensing vacuoles and secretory or zymogen granules (see Caro and Palade, 1964). The relationship between these organelles and the secretion of proteins from cells, i.e. the route taken by secretory proteins on their way out of the cell, has been studied in some detail.

Initial studies of this problem used the application of autoradiography to electron microscopy (Caro and Palade, 1964). With in vivo labelling of whole guinea pigs, these authors showed that the majority of newly-synthesised protein in the pancreas moved from the rER to the zymogen granules (formed by a progressive concentration of secretory products within large Golgi-derived condensing vacuoles, and from whence discharge occurs) via the Golgi apparatus. These findings confirmed the observations of Siekevitz and Palade (1960)

who used subcellular fractionation of guinea pig pancreas to follow radiolabelled chymotrypsinogen from ribonucleoprotein particles via a microsomal fraction to the zymogen granules. Subsequent studies detailed passage through the Golgi apparatus (Jamieson and Palade, 1967a) and post-Golgi stages (Jamieson and Palade, 1967b) using subcellular fractionation to refine and confirm autoradiographic findings. A summary of this early work is shown in Fig. 1.1. Similar methods were then used to show that these observations could be extended to other secretory tissues, e.g. the parotid acinar cell of the rabbit (Castle et al., 1972); rat anterior pituitary (Hopkins and <sup>Farguhar</sup>  $\lambda$ , 1973) and frog pancreas (Slot et al., 1974). These studies have been thoroughly reviewed (Palade, 1975; Scheele, 1980).

#### b) Variations of the Secretory Process

As discussed by Palade (1975), there are variants to the secretory pathway. The commonest variation is that found in "non-regulatory" secretory cells (Tartakoff and Vassalli, 1978). These are cells lacking the short-term control of secretory rate found in the classic secretory cell such as the exocrine/endocrine cells used in the studies described above, where massive discharge of secretory protein occurs in response to some physiological signal. In these non-regulated cells (such as fibroblasts, chondrocytes and plasma cells) secretion granules (e.g. zymogen granules) of usual appearance are

Fig. 1.1 The Secretory Pathway

Diagram to show the route taken by secretory products in a generalised regulated secretory cell.

1. Synthesis and segregation into the rough endoplasmic reticulum.
2. Transport from the rough endoplasmic reticulum to the Golgi apparatus.
3. Transport through the Golgi apparatus (cis to trans).
4. Concentration within the trans-Golgi and condensing vacuoles, and packaging into secretory granules.
5. Exocytosis.

rER, rough endoplasmic reticulum.

TV, transporting vesicles.

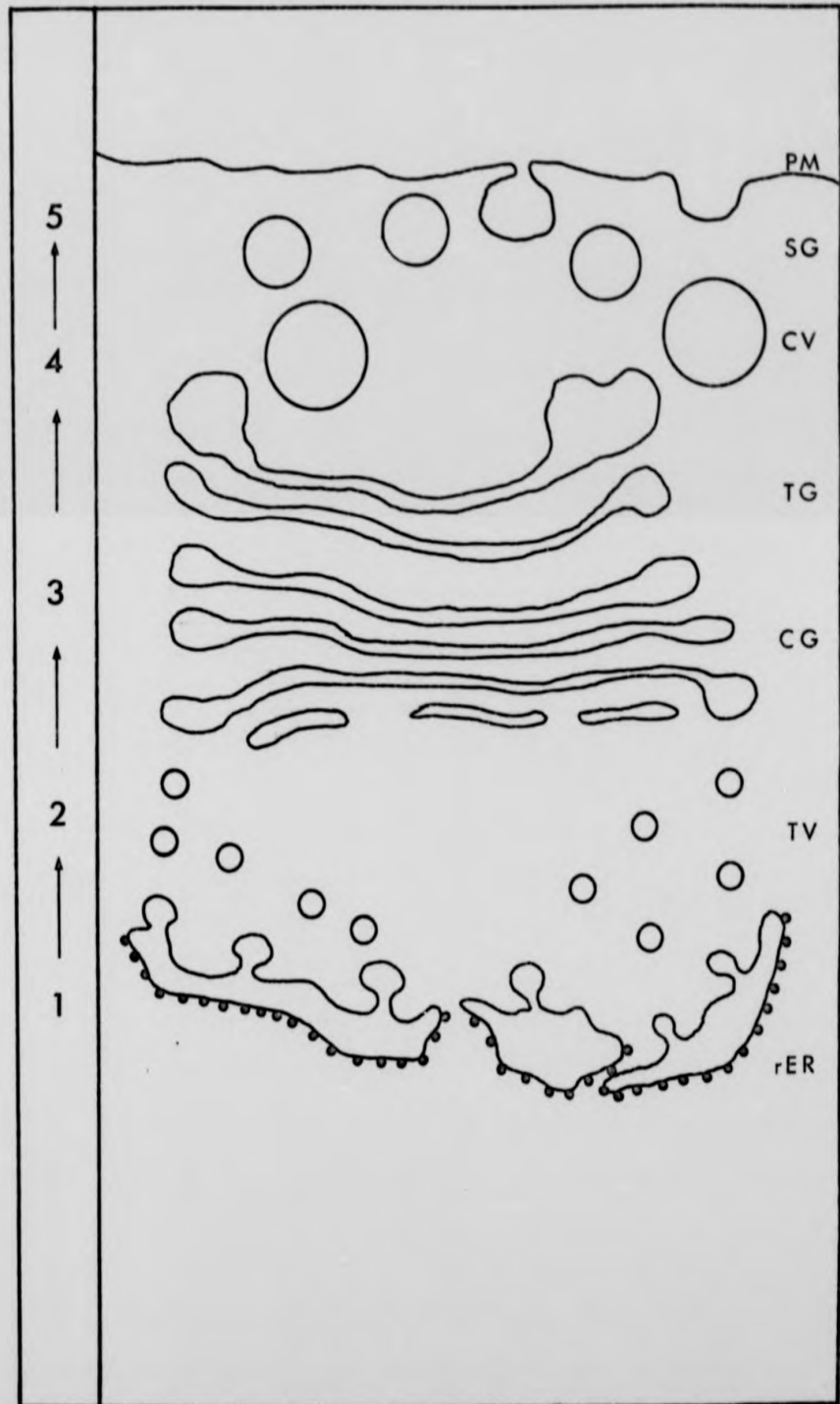
CG, cis-Golgi.

TG, trans-Golgi.

CV, condensing vacuole.

SG, secretory granule.

PM, plasma membrane.





absent since the concentration of secretory protein does not occur, intracellular storage is reduced in duration or eliminated and discharge takes place continuously; Golgi-derived vesicles are "presumed to pass to the cell surface where they fuse with the plasma membrane and release their contents by exocytosis" (Tartakoff and Vassalli, 1978). However, in this and other variants thus far studied (see Palade, 1975) all secretory proteins follow the same morphologically defined route until the Golgi apparatus is reached, cell function-related variation occurring in the late concentration/storage/discharge stages (Palade, 1975).

In a second type of variation, the secretory pathway (or portions of it) is used by a variety of proteins with destinies other than secretion. These alternative destinations include various cellular membrane-bound compartments and the plasma membrane. For reviews of various aspects of this problem see Blobel et al. (1979), Blobel (1980), Sabatini et al. (1982) and Strauss and Boime (1982). The only group of destination-variants that I will consider in detail are the plasma membrane proteins since they are among the most studied of all segregated proteins. This is because of the technical ease with which they can be studied and because their post-translational pathway is very similar to the secretory pathway.

The biogenesis of plasma membrane proteins has mainly been studied using viral membrane proteins as models, especially

the glycoprotein of Vesicular Stomatitis virus (VSV-G) and the membrane proteins of Semliki Forest and Sindbis viruses. These proteins behave as non-regulated secretory proteins, following the cellular pathway mapped out for secretory proteins - at least in the stages up to the Golgi apparatus (Green, Griffith et al., 1981; Bergmann et al., 1981; Wehland et al., 1982). However, instead of being transported along the secretory pathway free in the lumen of the various organelles involved, they are inserted into the membrane of the ER and are thought to cross the cell in this transmembrane configuration. Finally, on reaching the cell surface they are not released during exocytosis but remain inserted into the plasma membrane until viral budding releases them (Klenk and Rott, 1980; Simons et al., 1992; Simons and Garoff, 1980). Since these proteins remain inserted into membrane and do not even enter the lumen of the ER, it could be argued that their post-translational pathway diverges at a very early stage from the secretory pathway. However, they pass through all the same compartments and are subject to all the same post-translational events as secretory proteins up to exocytosis, and thus they diverge very late from the functionally defined secretory pathway.

c) Molecular aspects of Secretion

Having outlined the cellular background to the secretory process and introduced some of the variations encountered, I will now discuss what is known at a molecular level about secretion. The discussion will be split into sections that relate to the morphological background:

- i) Synthesis and Segregation into the Endoplasmic Reticulum.
- ii) Transport from the Endoplasmic Reticulum to the Golgi apparatus.
- iii) Processing inside the Golgi apparatus.
- iv) Post-Golgi events.

I shall be concentrating on what is common to all systems, ignoring post-translational segregatory phenomena such as movement into chloroplasts and mitochondria (reviewed in Sabatini et al., 1982) and discussing mainly work done with eukaryotes.

i) Synthesis and Segregation into the Endoplasmic Reticulum

The early stages in the secretory process, i.e. the synthesis and segregation of secretory proteins within the lumen of the ER, have been the subject of intense study. Due to the availability of suitable in vitro systems these events are well characterised at the molecular level.

In 1971, a hypothesis was presented to explain how secretory proteins become segregated within the ER. This hypothesis

(Blobel and Sabatini, 1971) suggested that all mRNAs encoding secretory proteins have a common 5' sequence which leads to binding of the nascent chain and polysome to the ER membrane. The nascent chain would then undergo vectorial co-translational translocation into the intracisternal space. There were two pieces of evidence for this hypothesis, the first being that secretory proteins are synthesised on membrane-bound polysomes. The first clear demonstration of this was that of Siekevitz and Palade (1960) who showed that after a short in vivo exposure to [ $^{14}$ C]-leucine, radioactive chymotrypsin is preferentially associated with membrane-attached polysomes isolated from the guinea pig pancreas. Subsequently a wide variety of secretory proteins were shown to be synthesised in this fashion (Redman, 1968; Redman, 1969; Hicks et al., 1969; Gaye and Denamur, 1970).

Secondly, studies by Redman and Sabatini (1966), Redman et al. (1966) and Sabatini and Blobel (1970) showed that protein synthesised on membrane-attached polysomes is released into the lumen of microsomal vesicles (equivalent to the lumen of the ER - Palade and Siekevitz, 1956), rather than into the cytosol. These experiments combined microsomal preparations with protein synthesis systems.

Subsequently, in 1972 Milstein et al. provided strong supporting evidence of, and further advancement of the Blobel-Sabatini hypothesis. They showed that a mouse myeloma

immunoglobulin light chain was synthesised in precursor form with an N-terminal extension in the RNA-dependent rabbit reticulocyte lysate system (Lockard and Lingrel, 1969) but as the mature protein in a system supplemented with a microsomal preparation. This maturation was shown to occur during and not after synthesis. The authors suggested that the N-terminal precursor sequence could be the signal for segregation. These findings were consolidated and advanced by the elegant studies of Blobel and Dobberstein (1975a, 1975b) who developed the cell-free protein translocation system that has become the basis for many further studies. They combined EDTA-treated microsomes derived from dog pancreas rER (which had thus been stripped of their ribosomes (Sabatini et al., 1966)) with the rabbit reticulocyte translation system. With this combination, using native small ribosomal subunits from reticulocytes, large ribosomal subunits from free polysomes of reticulocytes, stripped microsomes from dog pancreas and immunoglobulin light chain messenger RNA (mRNA) they showed that the immunoglobulin light chain is synthesised, processed and segregated, and that segregation and correct proteolytic processing occur during but not after completion of translation. These findings in such a heterologous system established that the information for segregation of a translation product is encoded in the mRNA itself and that translocation is a co-translational process.

It is now generally accepted that the N-terminal region of hydrophobic residues found on most secretory proteins is responsible

for triggering the segregatory step. Evidence giving rise to this belief is of three main types.

Firstly, support for the localisation of the signal sequence to the N-terminus of the nascent chain came from the work of Milstein et al. (1972) already described, and the subsequent finding that a wide variety of secretory proteins possess similar N-terminal extensions of between 15 and 30 amino acids (Scheele, 1980; Blobel et al., 1979). Secondly, studies using VSV-G by J. Rothman and Lodish (1977) in a synchronised protein synthesis system showed that the segregation of VSV-G was completely dependent on the time of addition of microsomal membranes to the system. The requirement for membranes during synthesis was only manifested over a few minutes when the putative 'signal sequence' had just appeared outside the ribosome, whereupon binding and translocation took place. Thirdly, bacterial studies using genetic and recombinant DNA techniques have shown that both prokaryotic (Emr et al., 1980) and eukaryotic (Talmadge et al., 1980) secretory and membrane proteins can be translocated from the cytoplasm to the periplasmic space of Escherichia coli by attaching amino-terminal sequences of appropriate secretory proteins. Since the translocation of bacterial proteins into the periplasmic space proceeds by a co-translational mechanism similar to that described for eukaryotes (Davis and Tai, 1980) it is argued that the periplasmic space is functionally equivalent to the lumen of the ER. These latter findings

constitute the best currently available, if indirect, support for this aspect of the signal hypothesis.

The current view of these events is diagrammed in Fig. 1.2.

Some co-translationally segregated proteins do not have proteolytically cleaved  $\text{NH}_2$ -signal sequences. The best characterised of these is hen ovalbumin, although others such as erythrocyte plasma membrane Band 3 protein (Sabban et al., 1981; Braell and Lodish, 1982) and other membrane-associated proteins such as cytochrome  $\text{P}_{450}$ , cytochrome  $\text{P}_{450}$  reductase and epoxide hydratase (Okada et al., 1982) have been identified as belonging to this group. When ovalbumin is synthesised in vitro in the absence of membranes, it has the same N-terminal sequence as the protein isolated from egg white and hence is not proteolytically processed during biogenesis (Palmiter et al., 1978). However, in the in vitro translocation system of Blobel and Dobberstein (1975b) it was found to compete with bovine prolactin for translocation into the lumen of the microsomal vesicles. This implied that ovalbumin contains the functional equivalent of a signal sequence (Lingappa et al., 1978). Subsequent controlled proteolysis showed that an internal fragment of the protein was responsible for this competition. (Doubt has now been cast on these in vitro competition experiments due to their requirements for very high levels of competing secretory proteins or peptides). Furthermore, in a synchronised

Fig. 1.2 Synthesis and Translocation of a Segregated Protein

This diagram shows the current model of how proteins become segregated, and incorporates recent evidence as to the rôle of various controlling proteins (see later this section). Initiation of translation occurs in the cytoplasm. Translation proceeds until the signal sequence emerges from the ribosome, whereupon it is blocked by Signal Recognition Protein. This arrest of translation persists until contact is made with Docking Protein. Translation then resumes, translocation proceeds and the signal sequence is cleaved from the nascent chain by signal peptidase as segregation is completed.

5' ——— 3', mRNA encoding segregated protein.

 , signal sequence on both mRNA and protein.

 , ribosome with nascent protein.

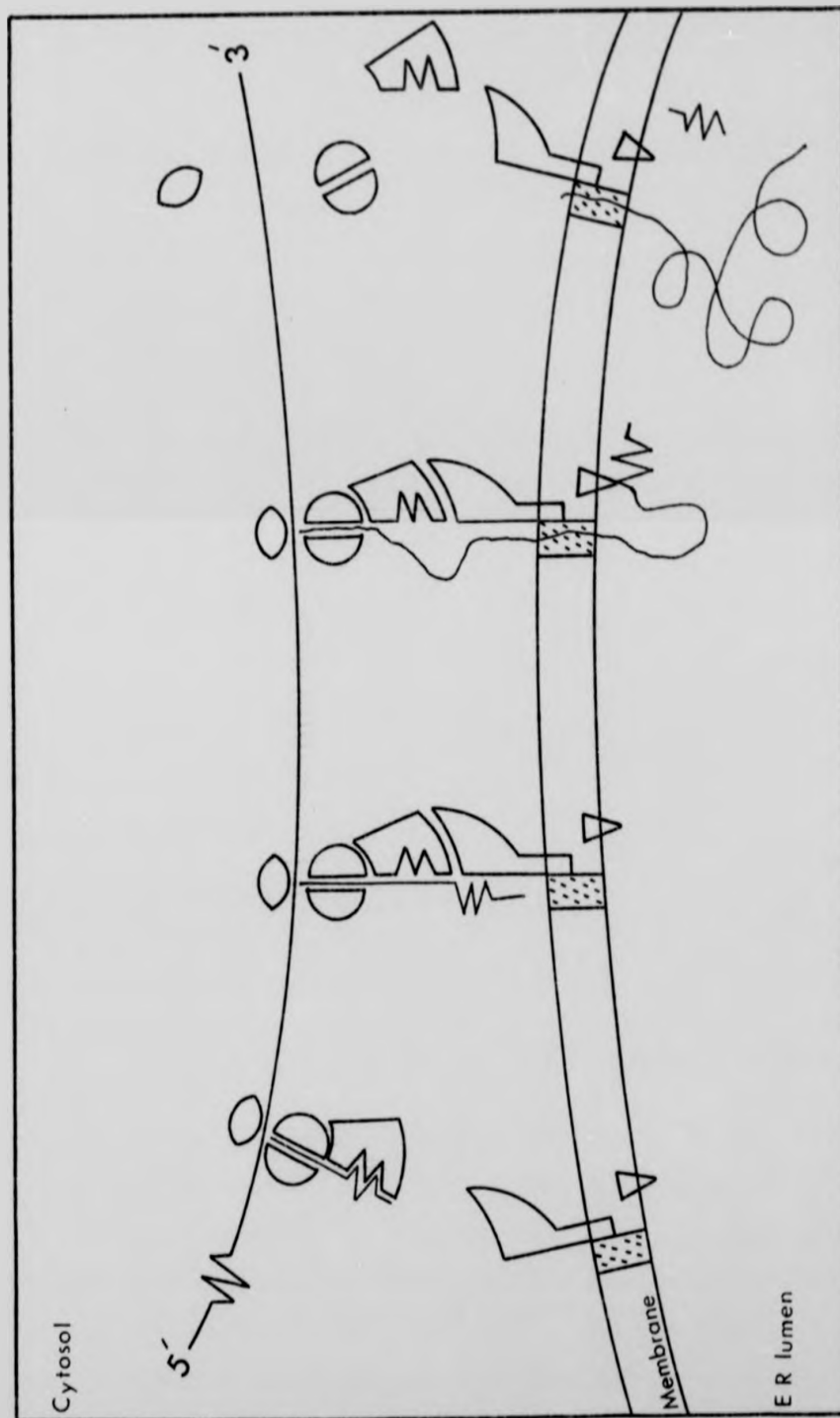
 , Signal Recognition Protein.

 , Docking Protein.

 , signal peptidase.

 , unknown component(s).





translation system, the critical chain length after which addition of microsomes did not lead to translocation was 250 (out of 385) amino acids. These two results (Lingappa et al., 1979) implied that an internal signal sequence was present in this protein. However, Meek et al. (1980) were unable to obtain the same results in a similar synchronous system. Their results imply an earlier membrane requirement for translocation, and Braell and Lodish (1982) have now shown that the ovalbumin signal sequence probably lies within the first 150 residues of this protein. Thus although it is agreed that ovalbumin contains a signal sequence, its precise location within the protein remains controversial.

Recently, work on this early portion of the secretory pathway has focused on the binding of the nascent chain to the membrane of the ER. Actual initiation of translation of secretory proteins probably occurs on free polysomes. Evidence for this is contained in the experiments of J. Rothman and Lodish (1977) using VSV-G. Biochemical dissection of the Blobel and Dobberstein (1975b) system has now shown that the binding of the nascent chain plus polysome to the membrane of the ER is mediated by several proteins. If the microsomes used are given a high salt wash (0.5 M), no translocation occurs (Warren and Dobberstein, 1978). The protein complex (Walter and Blobel, 1980) extracted in this way binds selectively to polysomes synthesising secretory proteins (Walter et al., 1981) and prevents synthesis beyond the signal sequence (Walter and Blobel, 1981b). This "Signal Recognition Protein" complex

mediates the binding of these polysomes to membranes (Walter and Blobel, 1981a). This binding can also be abolished by a salt-wash plus protease-digestion procedure developed by Meyer and Dobberstein (1980) which removes an integral membrane protein whose re-addition to the system specifically releases the translation block caused by Signal Recognition Protein (Meyer et al., 1982). The bound polysome may then interact with further proteins - possibly the ribophorins (see Sabatini et al., 1982). Thus the nascent protein is prevented from completing translation until it is bound to the membrane and in a position to be translocated (see Fig. 1.2).

Once inside the ER, many secretory proteins are glycosylated. The functional significance of glycosylation is, with one exception, unclear. The exception is the lysosomal hydrolases whose destination within the cell may depend on the presence of a specific oligosaccharide side chain attached to the protein (reviewed by Sabatini et al., 1982). No general conclusions about function have been drawn, mainly because the prevention of glycosylation may (Trowbridge et al., 1978; Hickman et al., 1977) or may not (Green, Meiss and Rodriguez-Boulan, 1981; Keller and Swank, 1978; Colman, Lane et al., 1981) affect the fate of the protein under study. It is within the ER that one of the two main classes of oligosaccharide-protein linkages is formed, N-type glycosylation. In the case of these N-glycosidic linkages there are two subtypes of chains, 'high mannose', e.g. those found on ovalbumin (Tai, et al., 1977) and 'complex' chains such as those found on

the heavy chain of human IgM immunoglobulins (Shimuzu et al., 1971). The assembly of N-linked oligosaccharide chains follows a pathway involving dolichol phosphate as the anchor for the sugars. Following completion of this chain (Fig. 1.3a) it is transferred en bloc from the lipid to an asparagine residue on a polypeptide chain. The asparagine residue glycosylated is invariably found within the sequence - Asn - X - Ser/Thr - (Hart et al., 1979). This transfer occurs in the ER. This conclusion is supported by two findings: firstly, that the enzymes involved in assembly of the intermediates plus those involved in transfer from lipid to protein are enriched in rER fractions (Czichi and Lennarz, 1977). Secondly, in the case of a number of glycoproteins including ovalbumin (Kiely et al., 1976) and VSV-G (Katz et al., 1977) it has been demonstrated that this glycosylation is a co-translational event. These various reactions are thought to take place on the luminal side of the ER (cf. Hanover and Lennarz, 1980; Hanover and Lennarz, 1981).

ii) Transport from the endoplasmic reticulum to the Golgi apparatus

From the ER, newly N-glycosylated, processed and segregated proteins are transported to the Golgi apparatus. This was first shown to be so in the pancreatic exocrine cells of the guinea pig where pulse-chase experiments showed that the

Fig. 1.3 Structures of Oligosaccharides

- a) Pyrophosphoryldolichol-linked oligosaccharide chain prior to transfer to suitable asparagine.
- b) Typical 'high mannose' oligosaccharide N-glycosidically linked to an asparagine.
- c) Typical 'complex' oligosaccharide N-glycosidically linked to an asparagine.
- d) Typical oligosaccharide o-glycosidically linked to a serine residue.

Abbreviations: Dol - PP - ; pyrophosphoryldolichol.

GlcNAc, Glc; N-acetylglucosamine, glucose.

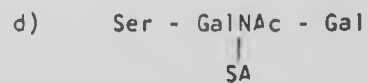
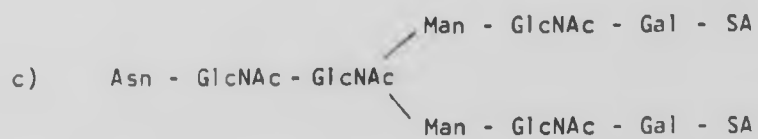
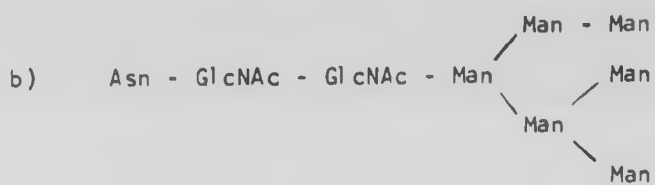
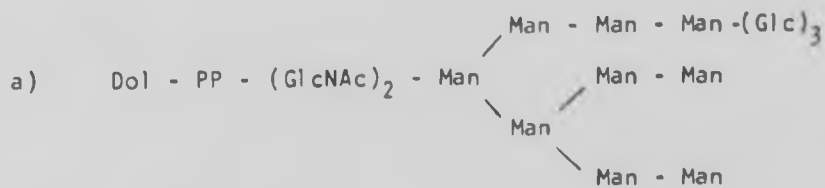
Man ; Mannose.

Asn, Ser ; Asparagine, serine.

SA ; Sialic acid

GalNAc, Gal; N-acetylgalactosamine,  
galactose.

Fig. 1.3 Structures of Oligosaccharides



pathway led from the rough ER to the transitional elements of this system then to the small peripheral vesicles on the cis side of the Golgi complex (Jamieson and Palade, 1967a). It is widely accepted that protein is ferried from the ER to the Golgi inside small vesicles (Palade, 1975). Molecular details of this process are almost unknown. Some electron microscopic investigations have suggested that the shuttling may take place inside clathrin-coated vesicles (Pearse, 1980) a view supported by the biochemical investigations of J. Rothman and co-workers using VSV-G protein.

J. Rothman and Fine (1980) have isolated clathrin-coated vesicles from pulse-chased CHO cells infected with VSV. Immediately following the pulse, G-protein isolated with these vesicles has the high-mannose type oligosaccharide characteristic of the ER attached to it. After a longer chase, isolated G-protein has a different oligosaccharide side-chain, of a type formed by the action of enzymes located in the Golgi complex (see later for detailed discussion). This change in carbohydrate can be assayed by its resistance to endo- $\alpha$ -N-acetylglucosaminidase H (endo H), an enzyme that can remove a substantial portion of the high mannose ER-characteristic oligosaccharide but not cleave the complex type of oligosaccharide characteristic of the Golgi apparatus. Thus the authors demonstrate using endo H that G-protein in a pre-Golgi state of glycosylation can be found in coated vesicles. This implies that it is being transported from the ER to the Golgi apparatus in these structures.

Another approach to understanding the relationship between the ER and Golgi apparatus is that recently taken by Palade and co-workers. They have been developing an immuno-affinity chromatography technique based on the use of antibodies raised against membrane-marker enzymes to pull out vesicles derived from ER or Golgi apparatus from a microsomal population. Preliminary results from this work (Farquhar and Palade, 1981) suggest that the distended rims of Golgi cisternae have ER-like membranes whereas the central part of the cisternae have Golgi-like membranes. The functional significance of these findings is not yet clear. However, it has been suggested (Farquhar and Palade, 1981) that they are consistent with the idea that transport between ER and Golgi is carried out in vesicles derived by pinching off from the ER and not in specialised structures. The ER-like character of the rims of Golgi cisternae would then be due to the fusion of ER-derived vesicles with the Golgi, concomitant with the transport of their content from one organelle to the other.

### iii) Processing inside the Golgi Apparatus

Once in the Golgi apparatus secretory proteins may be further post-translationally modified. Modifications include sulfation, phosphorylation, proteolysis or further glycosylation (Farquhar and Palade, 1981; Tartakoff, 1980). Since the state of glycosylation has been used as a marker of progress



along the secretory pathway, this aspect will be discussed further. N-linked oligosaccharide side-chains are secondarily processed in the Golgi<sup>body</sup>. In the case of the 'high mannose' type (Fig. 1.3b) processing merely involves the removal of terminal glucose residues and, at most, a few mannose residues (Hanover and Lennarz, 1981). In the case of complex chains (Fig. 1.3c), processing is more complicated and involves removal of all but three of the mannose residues followed by capping of the chain with N-acetyl glucosamine, galactose and sialic acid residues (Hanover and Lennarz, 1981). The enzymes involved in these later processing stages are located in the Golgi apparatus (Tulsiani et al., 1982; Dewald and Touster, 1973; Tabas and Kornfeld, 1979).

In the Golgi<sup>body</sup> the second main type of glycosylation occurs; O-glycosylation (reviewed in Hanover and Lennarz, 1981). O-glycosidically linked chains (Fig. 1.3d) are characterised by a linkage between an N-acetylgalactosamine and a serine or threonine residue in the polypeptide. This initial linkage is followed by the stepwise addition of single glycosyl units such as galactose, N-acetylglucosamine, sialic acid or fucose. The enzymes involved in these transfers are enriched in Golgi fractions (Hanover et al., 1980; Kim et al., 1971; Ko and Raghupathy, 1972). Kinetic evidence arising from studies of human chorionic gonadotropin, a protein with both O- and N-linked oligosaccharides, confirms the different subcellular sites proposed for N- and O-glycosylation (Hanover and

Lennarz, 1981). These various glycosylating reactions are, like those of the ER, thought to take place in the lumen of the organelle (Fleischer, 1981; Hanover and Lennarz, 1981), hence the restriction of glycosylation to membrane-translocated proteins.

After modification, secretory proteins free in the cisternal space (Tartakoff, 1980) may be transported from the cis to the trans side of the organelle (Farquhar and Palade, 1981), where concentration may take place, usually in the dilated rims of the transmost cisterns. However, in a few cell types (e.g. exocrine pancreatic cells) concentration takes place in specialised condensing vacuoles (Palade, 1975). Concentration takes place in many but not all cell types; for example, immunoglobulins in plasma cells are packaged in the Golgi complex and discharged by exocytosis in the usual manner, but the carrier consists of a fluid-filled vesicle instead of a dense granule (Farquhar and Palade, 1981). Where a concentration step is performed, the later discharge step is usually a regulated process occurring in response to some stimulus (Palade, 1975). In other secretory situations such as the plasma-cell immunoglobulin system, the process is 'non-regulatory', discharge taking place continuously.

#### iv) Post-Golgi events

The mode of transport from the Golgi apparatus to the cell surface where exocytosis takes place is dependent on both

the cell type and the protein being secreted. (For discussion of some variants see Palade, 1975 and also Gumbiner and Kelly, 1982). As with transport from the ER to the Golgi apparatus, very little molecular information is available about this stage of the secretory pathway.

In all cases, it is thought that the secretory proteins are ferried to their destination in membrane-bound vesicles or granules, and that the contents are discharged by a process whereby the membrane of the secretory vehicle fuses with the plasmalemma such that continuity is established between the granule compartment and the extracellular medium: exocytosis. The nature of the vesicles involved in these final stages of secretion is uncertain. Certain authors maintain that clathrin-coated vesicles are involved on the basis of biochemical evidence from the study of VSV-G, i.e. non-regulated intracellular transport (J. Rothman et al., 1980), or electron microscopic evidence, for example from a regulated secretory cell (Franke et al., 1976). Other authors maintain that clathrin-coated vesicles are only involved (transiently) in endocytosis (Pastan and Willingham, 1981; Wehland et al., 1982).

## 2. New systems with which to study secretion

It is clear from the above discussion of the secretory pathway that more detailed information has been obtained about the early stages of the pathway than about later stages. This partly reflects the lack of systems suitable for the analysis of these later stages. Some systems with which to study late stages are now being developed, some of which have already been discussed. One example is the work of J. Rothman and co-workers (Fries and J. Rothman, 1981; J. Rothman and Fries, 1981; J. Rothman, 1981) where the endo H-resistance of oligosaccharide side chains of VSV-G is used as a criterion for this protein's position along the secretory pathway, and the effects of various subcellular fractions and viral mutations on the movement of VSV-G analysed. Another Golgi-related system, with a novel technological basis, is the immuno-affinity chromatography of subfractions of microsomal vesicles being developed by Palade and co-workers (Farquhar and Palade, 1981). However, these in vitro systems have inevitable technical problems and are both at an early stage of development. Another kind of approach is to use the power of recombinant DNA technology plus whole-cell systems to try to analyse steps in the secretory pathway. The approach here is to attack the problem by altering the secretory protein itself rather than by fractionating the secretory apparatus. This approach is being exploited by several groups.

Influenza haemagglutinin, a viral membrane protein (Gething and Sambrook, 1981; Hartman et al., 1982) and human growth hormone (Pavlakis et al., 1981), a secretory protein, have been expressed in African Green Monkey Kidney cells using SV40 vehicles plus helper virus to infect these cells.

Thus far, expression and cell surface or extra-cellular location of the foreign proteins have been reported by these authors.

ro<sup>17</sup>  
A

The use of micro-injection of DNA constructs coding for viral membrane proteins into the nucleus of tissue culture cells has been pioneered by Henrik Garoff and co-workers. Here, Semliki Forest virus p62 protein and influenza haemagglutinin have both been expressed and found on the surface of Baby Hamster Kidney (BHK) cells by indirect immunofluorescence (H. Garoff, personal communication). Having achieved expression, these groups intend to manipulate the sequence of their chosen protein and look for changes in primary structure that affect that protein's passage through the cell.

There are drawbacks to these systems: firstly, using infection places limits on the cell type that may be used, an important point since, when using surrogate systems, it is useful to employ a range of cells in order to rule out cell-specific effects. Secondly, the maximum expression of the inserted sequence only shortly precedes the death of the cell. It is not clear whether the cytopathic results of

infection interfere in any way with the movement of protein. The micro-injection method of entry into the cell used by Garoff et al. avoids these problems but has the drawback of only producing relatively small amounts of expressed protein, since only small numbers of cells are involved in micro-injection.

Another related system, while not yet being used to investigate the interaction of primary sequence with transport machinery, has provided some insight into late stages of the secretory pathway while confirming in an elegant way what is known about the early steps. This is the oocyte of Xenopus laevis.

### 3. The Oocyte of Xenopus laevis: A system with which to study the whole secretory pathway.

Following Gurdon's demonstration that Xenopus oocytes could translate exogenous mRNAs (Gurdon et al., 1971), the oocyte has been used for the assay of a wide variety of mRNAs (Marbaix and Huez, 1980). In 1977 Zehavi-Willner and Lane showed that when mRNAs coding for secretory proteins were micro-injected into these giant cells (1.2 mm diameter) the protein products were segregated into a vesicle fraction. This segregation occurs during synthesis of the nascent polypeptide (Lane et al., 1979). Following this, Colman and Morser (1979) showed that human lymphoblastoid

interferon was secreted from oocytes injected with lymphoblastoid mRNA while interferon protein injected into the oocyte was rapidly degraded. A wide variety of mRNAs encoding secretory proteins derived from different sources were then micro-injected into oocytes and the results (Lane et al., 1980) showed that the secretory system of the oocyte is neither cell-type nor species-specific yet is completely selective, only exporting or segregating proteins that are similarly partitioned in their tissue of origin. Colman, Lane et al. (1981) followed this by showing that entry into the ER is necessary for secretion; ovalbumin in the cytosol of these cells is not secreted whereas molecularly identical protein present in the vesicle fraction of the cells is exported. This result is the clearest demonstration of the assertion by Palade (1975) that proteins destined for secretion are completely and continuously segregated in the membrane systems of the cell and that at no stage of the secretory pathway is there exchange between the cytosol and membraneous compartments of the cell as suggested by S. Rothman (1975). Thus work with the oocyte has been able to confirm and extend the observations made with cell-free systems to the ultimate event in the pathway, i.e. secretion.

Other studies have shown that the cytoskeleton has a rôle in secretion from oocytes (Colman, Morser et al., 1981), that in the uninjected oocyte the secretory system is active, secreting small amounts of endogenous proteins and that the surrounding

follicle cells are not necessary to the oocytes' secretory function (Mohun et al., 1981).

Other general features of the oocyte that render it suitable for the study of the secretory process are the ease with which cells this size can be manipulated (for example - manual enucleation is possible), and the ease and simplicity with which they can be cultured. Moreover, although studies thus far have utilised RNA, it is equally possible to inject DNA coding for the secretory protein under study into the oocyte nucleus (Wickens et al., 1980), therefore enabling the effects of recombinant DNA sequence manipulation of secretory proteins to be studied.

The fidelity of the oocyte's processing machinery has been of interest. It is clear that the oocyte will remove most signal sequences (Lane et al., 1980; Marbaix and Huez, 1980). The oocyte can also glycosylate, phosphorylate, N-acetylate and hydroxylate (Marbaix and Huez, 1980; Lane, 1981) as well as assembling multimeric proteins (Valle et al., 1981). However, reports of incomplete phosphorylation have appeared (Lane et al., 1979), and the oocyte can neither complete proteolytic processing of, nor secrete, Honeybee promelittin although initial processing and sequestration take place (Lane et al., 1981).

In addition, although the oocyte can glycosylate proteins it is not clear whether it is capable of both N and O



glycosylation, and Golgi-associated processing of N-linked core sugars may not occur (Mous et al., 1980; G. Valle unpublished data). These findings render certain kinds of detailed biochemical studies difficult. Other problems with the oocyte are difficulties with subcellular fractionation (see Chapter 5) and the fact that due to its size and pigmentation, immunofluorescent techniques cannot be used to study internal events in the whole cell.

#### 4. The problem of non-parallel secretion

One question that the oocyte has been particularly valuable in answering has been that of whether non-parallel secretion can occur. Originally, non-parallel secretion referred to the observations of S. Rothman and co-workers (reviewed S. Rothman, 1975) that the enzyme activities secreted by the acinar cell of the pancreas were present in different ratios to each other outside the cell from those present inside. This information was used to construct a model for the secretory pathway which proposed that secretory proteins were present in the cytosol of the cell at some stage during secretion (S. Rothman, 1975). Whilst this model accounts for his observations, it contradicted the model proposed by Palade (1975) stating that during secretion the secreted proteins were segregated away from the cytoplasm by membranes at all stages of the process. The data of S. Rothman were

attacked on technical grounds (Scheele and Palade, 1975) and a number of immuno-cytochemical studies showed that secretory proteins are never present in the cytoplasm (Geuze et al., 1979; Kraehenbuhl et al., 1977). Finally, the demonstration by Colman, Lane et al. (1981) that secretory protein present in the cytosol of oocytes was not secreted in contrast to identical protein present in the membranous compartments of the cell disproved the Rothman model.

Although the model remains discredited, its formulation stimulated research to see whether the phenomenon of non-parallel secretion did occur, albeit in a more orthodox form. The question now asked is whether, within the general scheme outlined by Palade there is micro-heterogeneity, with different proteins being transported in different compartments of the various organelles. Early attempts at answering this part of the question of parallelism were immunocytochemical or biochemical and were focused on the pancreatic secretory cell. Immunocytochemical studies of the bovine exocrine pancreatic cell show that RNAase (Painter et al., 1973) and trypsinogen (Kraehenbuhl and Jamieson, 1972) are found within all zymogen granules of any cell examined, and that these and other proteins (Kraehenbuhl et al., 1977) are found in qualitatively similar amounts in all compartments of the pathway.

Biochemical studies have been mainly concerned with comparing the contents of zymogen granules with the contents of other

cellular compartments or with secreted proteins from pancreatic acinar cells. Thus Greene et al. (1963) compared the enzymatic contents of zymogen granules from bovine pancreas with discharged proteins in the surrounding medium. Keller and Cohen (1961) used similar enzymatic methods to compare zymogen granule and microsomal fractions derived from bovine pancreas. In 1974 Tartakoff et al. compared zymogen granule and secreted proteins of the guinea pig pancreas using SDS-polyacrylamide gel electrophoresis and staining for protein. This was followed in 1975 by another study (Tartakoff et al.) of the same tissue comparing the contents of condensing vacuoles and zymogen granules using in vitro pulse-labelled and chased pancreas lobules followed by gel electrophoresis and autoradiography. All these studies came to the same conclusion; that secretory proteins moved from the zymogen granules to the external medium in parallel and that where analysed, earlier steps in the pathway gave the same picture.

These studies were mainly performed on cells stimulated to discharge by the action of secretagogues, although resting discharge has also been analyzed (Tartakoff et al., 1975). Finally, the enzyme assays, conditions of activation and discharge for seven proteins (three enzymes and four zymogens) were optimised and it was shown that each protein was discharged in parallel (i.e. in constant proportions) at various time points after stimulation with either carbamylcholine or caerulein. Furthermore, the proportions of the

enzymes and zymogens found in the tissue at the end of the experiment were identical to those discharged into the medium. The extreme care taken in these experiments (Scheele and Palade, 1975), and the immunocytochemical data discussed above seemed to establish finally that the various exported proteins of the pancreatic exocrine cell are discharged in parallel.

However, subsequent workers using different regulated tissues and a variety of secretagogues have demonstrated non-parallel secretion (reviewed in Scheele, 1980). The significance of these results, some of which are the result of long-term physiological changes in whole animals is not at all clear. Thus the clear demonstration by Colman and co-workers (Lane et al., 1980; Colman, Lane et al., 1981) that the ratio of synthesised to secreted protein varies following micro-injection into oocytes of RNAs coding for various secretory proteins was important. The work described in this thesis was aimed at a detailed analysis of the phenomenon of non-parallel secretion in the oocyte exemplified by the secretion of two proteins: chicken ovalbumin and lysozyme.

## Chapter 2

### Materials & Methods

## Materials

### 1. Chemicals, Biochemicals, Radiochemicals

All reagents not detailed below were bought as AnalaR grade (or highest grade available) from BDH Chemicals Ltd., Poole, Dorset.

Sigma London Chemical Co. Ltd., Poole, Dorset: Bovine serum albumin, L-methionine, heparin, hen ovalbumin (grade VI), hen lysozyme (grade III), Trizma base, HEPES, PIPES, TEMED, PMSF, DTT, iodoacetamide, PVP-360, Ficoll - type 400.

Pharmacia (GB) Ltd., London: CNBr-activated Sepharose 4B.

Whatman LabSales Ltd., Maidstone, Kent: Whatman 540 paper.

Boehringer Corporation (London) Ltd., Lewes, Sussex: Hind III, Proteinase K (from Tritirachium album).

Amersham International plc, Amersham, Bucks: [ $^{35}\text{S}$ ]-methionine (approximately 1200 Ci/mmole).

Agar Aids Ltd., Stansted, Essex: all materials used in electron microscopy.

Miles-Yeda Ltd., Rehovot, Israel: Rabbit anti-hen ovalbumin antiserum, goat <sup>and</sup> rabbit anti-mouse IgG antiserum, all approximately 2 mg/ml of antibody.

Uniscience Ltd., Cambridge: Oligo (dT)-cellulose, type T2 (Collaborative Research).

Eastman Kodak, Rochester, New York, USA: N,N'-methylene bisacrylamide.

Grand Island Biological Company, New York, USA: Freund's complete adjuvant, Freund's incomplete adjuvant.

Glaxo Laboratories Ltd., Greenford, Middlesex: Sodium benzylpenicillin (Crystapen), streptomycin sulphate (BP).

E. R. Squibb & Sons Ltd., Twickenham, Middlesex: Nystatin (Mycostatin).

Nicholas Laboratories Ltd., Slough: Gentamicin sulphate (Genticin).

May & Baker Ltd., Dagenham, Kent: sodium pentobarbitone (Euthatal).

## 2. Animals

Olac 1976 Ltd., Bicester, Oxon: Female New Zealand White Rabbits.

South African Snake Farm, Windhoek, Cape Province, South Africa: Female Xenopus Laevis.

Laying Rhode-Island Red hen a gift of Searle Scientific, High Wycombe, Bucks.

## Methods

### 1. Handling of oocytes

#### A. Culture and injection

Adult Xenopus laevis were kept at 19°C. Large X. laevis females were killed by the injection of 0.2-0.4 ml (depending on frog size) of sodium pentobarbitone (200 mg/ml). The ovary was removed, divided into small clumps of oocytes, and stored at 19°C in modified Barths saline: 88 mM NaCl, 1 mM KCl, 0.3 mM  $\text{Ca}(\text{NO}_3)_2$ , 0.41 mM  $\text{CaCl}_2$ , 0.82 mM  $\text{MgSO}_4$ , 2.4 mM  $\text{NaHCO}_3$ , 2.0 mM Tris HCl, pH 7.6, containing sodium penicillin (10 mg/ml), streptomycin sulphate (10 mg/ml), mycostatin (20 U/ml) and gentamycin (0.2 mg/ml).

Oocytes used for injection were first separated. Only undamaged oocytes were injected with approximately 30 nl of mRNA in distilled water. Injections were performed essentially as described by Gurdon (1974). After injection, oocytes were cultured at 19°C in modified Barths solution for 24 h (50 oocytes/ml), any damaged oocytes removed, and the remainder used for experiments.

#### B. Labelling regimes

Oocytes were labelled in small wells precoated with bovine serum albumin (BSA) by incubation with 0.5% BSA in water for



30 min at 37°C. Wells contained 30 µl of Barths solution plus [<sup>35</sup>S]-methionine (see figure legends for concentration) for various times at 19°C, with 5 oocytes per well. For those kinetic experiments where linear incorporation of [<sup>35</sup>S]-methionine was desirable, a small amount (0.1 mM) of cold L-methionine was added to the culture medium, in addition to [<sup>35</sup>S]-methionine. For other experiments, where high levels of incorporation were more important, carrier was left out. In pulse-chase experiments, oocytes were pulse-labelled for 24 h, removed, washed with Barths saline and re-incubated in small wells in Barths saline containing 10 mM unlabelled L-methionine for various times. Each well contained 100 µl incubation medium and 5 oocytes. Incubation medium was only analysed from those wells in which all the oocytes appeared morphologically healthy. At the end of the incubation period, oocytes and incubation media were separated, incubation media frozen at -20°C and oocytes either fractionated or frozen.

## 2. Preparation of Oviduct mRNA

The method used was adapted from Palacios et al. (1972) and Palmiter (1973). The oviduct from one laying Rhode Island Red hen was removed. This was divided into four portions (6 g each), each of which was minced with scissors and homogenised in 35 ml of a homogenisation buffer containing NaCl (25 mM),

MgCl<sub>2</sub> (5 mM), sodium deoxycholate (5%), Triton X-100 (5%), heparin (500 µg/ml) and Tris-HCl (25 mM), pH 7.6. Homogenisation was by a motorised Teflon/glass homogeniser, ten passes at the slowest speed. The homogenates were spun at 15,000 rpm (27,000 g maximum) in an angle rotor for 5 min. The supernatants were collected and 1 M MgCl<sub>2</sub> added, with mixing, to give a final concentration of 100 mM MgCl<sub>2</sub>. The mixture was stood in iced-water for 1 h. The precipitated polysomes were layered over 10 ml of homogenisation buffer minus detergents and made 17% sucrose (w/v). This was centrifuged for 10 min at 15,000 rpm in an angle rotor. The supernatant was removed with a vacuum line and the pellet suspended in 20 mM HEPES (pH 7.5) to a final volume of 15 ml. An equal volume of 0.2 M sodium acetate, pH 5, was added and then 10% SDS to a final concentration of 0.5% SDS. This mixture was extracted once with equal volumes of phenol/chloroform, extracted with chloroform twice and the aqueous phase finally made 0.2 M NaCl and precipitated with 2.5 volumes of ethanol at -20°C. RNA was pelleted, washed three times with ethanol and finally dissolved in 20 mM HEPES, pH 7.5. Poly(A) RNA was prepared using an oligo (dT) cellulose column.

### 3. Purification of Ovalbumin mRNA

Ovalbumin mRNA was purified by hybridisation to cloned ovalbumin cDNA (pOv230, McReynolds et al., 1977; a kind gift of M. Wickens) bound to diazobenzylloxymethyl (DBM) paper (Alwine et al., 1977) followed by elution with formamide buffer (Smith et al., 1979).

#### A. Preparation of DBM paper

One sheet of Whatman 540 paper (20 x 18 cm) was placed in a flat pan in a water bath at 60°C. To the paper was added 10 ml of 8% (w/v) nitrobenzylloxymethyl pyridinium chloride, 2.5% (w/v) sodium acetate. The solution was distributed evenly over the paper and allowed to dry. When dry, the paper was baked in a pre-heated oven at 130°C for 35 min, followed by washing with several changes of water at room temperature for 20 min. The wet paper was then washed for a further 20 min with several changes of acetone, air-dried and stored in this form (nitrobenzylloxymethyl (NBM) paper) at 4°C.

The conversion of NBM paper to DBM paper was performed immediately before use as the latter form is unstable. Three paper discs (1 cm diameter) were cut from the sheet of NBM paper. The discs were shaken at 60°C for 30 min in 20% (w/v) sodium dithionite (50 ml), then washed at room temperature with several changes of water for 20 min, followed by a further 20 min in 50 ml of 30% (v/v) acetic acid. The acetic acid

was washed away with several changes of water, leaving amino-benzyloxymethyl (ABM) paper.

ABM paper was converted to DBM paper in the cold room, with solutions at as close to 0°C as possible. The discs were incubated in 100 ml 12% (v/v) HCl, plus 2.7 ml 1% (w/v) NaNO<sub>2</sub> (freshly made) with continuous shaking. Diazotisation was allowed to proceed for 30 min then the paper was washed twice with water and DNA was bound.

#### B. Preparation of DNA-DBM paper discs

Thirty µg of pOv230 DNA was linearised by digestion with Hind III, ethanol-precipitated, and resuspended in 12 µl 2 mM NaPO<sub>4</sub> (pH 6.3), 48 µl dimethylsulphoxide (DMSO). Trace amounts of nick-translated plasmid pBR322 (gift of P. Turner) were added to monitor binding of DNA to filters. The DNA was melted at 80°C for 10 min, snap-cooled on ice and immediately added to the DBM paper discs in a plastic scintillation vial insert. The vial was capped and placed in the dark at room temperature overnight. The following morning the discs were washed twice with water and then treated with 0.4 M NaOH for 30 min at 37°C, before a further three washes in water and storage in 50% (v/v) formamide, 20 mM Tris-HCl, pH 6.3, at 4°C. Approximately 50% of the radioactive DNA had bound to the discs.

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C. Hybridisation of mRNA to DNA-DBM discs and elution of ovalbumin mRNA

RNA was ethanol-precipitated, washed, dried, and re-dissolved in 20  $\mu$ l 100 mM PIPES-NaOH (pH 6.4), 10  $\mu$ l 2% (w/v) SDS, 1  $\mu$ l EDTA (100 mM). When dissolved, de-ionised formamide (50  $\mu$ l) and NaCl (20  $\mu$ l, 4.5 M) were added. Thus the final hybridisation buffer was 20 mM PIPES-NaOH (pH 6.4), 0.2% SDS, 1 mM EDTA, 0.9 M NaCl and 50% de-ionised formamide. This RNA was added to the DNA disc (which had been pre-equilibrated in hybridisation buffer for 1 h at room temperature) and incubated in a shaking water bath at 37°C for 5 h. After hybridisation the DNA discs were removed and washed with five aliquots (5 ml) of a buffer (sodium citrate (16 mM), NaCl (40 mM), EDTA (2 mM), SDS (0.4%), de-ionised formamide (50%)) for 15 min each. The residual (unbound) mRNA was collected by ethanol precipitation, suspended in 25  $\mu$ l water, re-precipitated and finally suspended in 20  $\mu$ l of water.

RNA which was specifically hybridised to the immobilised DNA was eluted with 125  $\mu$ l of a buffer comprising PIPES-NaOH (10 mM, pH 6.4), EDTA (1 mM), SDS (0.5%) and de-ionised formamide (90%), by incubation with this buffer at 37°C, with shaking, for 30 min. The elution buffer was then collected, and the DNA discs washed with 300  $\mu$ l water which was added to the eluate. Ovalbumin mRNA was precipitated from this by the addition of 10  $\mu$ g carrier ribosomal RNA (*X. borealis*, gift of P. Turner), LiCl to 0.4 M, and 3 volumes of ethanol, followed

by incubation overnight at  $-20^{\circ}\text{C}$ . The bound RNA was re-precipitated twice and finally taken up in 7  $\mu\text{l}$  of water.

#### 4. Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was carried out using variations of the method described by Laemmli (1970). The gels were made using a series of stock solutions in various combinations.

Stock solutions:

- A. 40% (w/v) acrylamide, 0.2% (w/v) bisacrylamide.
- B. 30% (w/v) acrylamide, 0.825% (w/v) bisacrylamide.
- C. 30% (w/v) acrylamide, 0.45% (w/v) bisacrylamide.
- D. 3 M Tris-HCl, pH 8.8, 0.8% (w/v) SDS.
- E. 0.5 M Tris-HCl, pH 6.8, 0.8% (w/v) SDS.

##### i) 10-22% exponential gradient gels.

Two solutions were made:

	22% acrylamide	10% acrylamide
A	13.5 ml	-
B	-	24 ml
D	3 ml	9 ml

	22% acrylamide	10% acrylamide
2% polyacrylamide	2.4 ml	7.2 ml
water	5 ml	31.4 ml
TEMED	10 $\mu$ l	36 $\mu$ l
ammonium persulphate (10% w/v; APS)	125 $\mu$ l	384 $\mu$ l

The 22% acrylamide solution was placed in a 50 ml stoppered flask, with an outlet to the gel former and an inlet from the 10% acrylamide solution. Acrylamide was pumped through the sealed vessel (where mixing took place) into the gel former.

ii) 10-15% linear gradient gels.

Two solutions were made:

	10% acrylamide	15% acrylamide
B	15 ml	22.5 ml
D	6.3 ml	6.3 ml
water	24 ml	5 ml
60% (w/v) sucrose	-	11.3 ml
TEMED	22.5 $\mu$ l	22.5 $\mu$ l
APS	225 $\mu$ l	225 $\mu$ l

Gels were made using an MSE open-chamber gradient maker, in combination with a peristaltic pump.



iii) 12.5% gels.

12.5% gels contained the following:

B	12.5 ml
D	4.2 ml
water	13.3 ml
TEMED	15 $\mu$ l
APS	150 $\mu$ l

iv) 15% gels.

15% gels contained the following:

C	15 ml
D	4.2 ml
water	10.8 ml
TEMED	15 $\mu$ l
APS	100 $\mu$ l

v) Stacking gels.

All gels were made with stacking gels, which contained the following:

B (for i, ii and iii) or C (for iv)	4 ml
E	3 ml
water	16.8 ml
TEMED	15 $\mu$ l
APS	200 $\mu$ l

Gradient gels were 23 cm long, 16 cm wide and 0.15 cm deep.

Single percentage gels were 12 cm long, 14 cm wide and 0.12 cm

iii) 12.5% gels.

12.5% gels contained the following:

B	12.5 ml
D	4.2 ml
water	13.3 ml
TEMED	15 $\mu$ l
APS	150 $\mu$ l

iv) 15% gels.

15% gels contained the following:

C	15 ml
D	4.2 ml
water	10.8 ml
TEMED	15 $\mu$ l
APS	100 $\mu$ l

v) Stacking gels.

All gels were made with stacking gels, which contained the following:

B (for i, ii and iii) or C (for iv)	4 ml
E	3 ml
water	16.8 ml
TEMED	15 $\mu$ l
APS	200 $\mu$ l

Gradient gels were 23 cm long, 16 cm wide and 0.15 cm deep.

Single percentage gels were 12 cm long, 14 cm wide and 0.12 cm

deep. All solutions were degassed before use. The electrode buffer used for all these gels contained (per litre) SDS (1 g), Trizma base (6.04 g) and glycine (28.8 g).

After electrophoresis, gels were fixed (methanol (45%, v/v), acetic acid (10%, v/v)) for 1 h, dried down and autoradiographed or fixed for 20 min and fluorographed (Bonner and Laskey, 1974; Laskey and Mills, 1975).

## 5. Preparation of Samples for Gel Electrophoresis

### A. Oocyte samples

Oocytes were homogenised thoroughly in 0.1 ml Dounce homogenisers in 40  $\mu$ l/oocyte of homogenisation buffer: 0.1 M NaCl, 1% Nonidet P-40, 1 mM PMSF, 20 mM Tris-HCl (pH 7.6). The homogenate was centrifuged in an Eppendorf microfuge for 5 min and the middle region of soluble protein (below the lipid and above the yolk pellet) retained. Samples were either mixed with sample buffer (1:1) and electrophoresed directly ( $\frac{1}{8}$ th of an oocyte was loaded per track) or immunoprecipitated ( $\frac{1}{2}$  of an oocyte loaded per track).

#### B. Secreted Protein

Samples from pulse-labelling experiments were either diluted with sample buffer (1:1) and loaded directly (2 oocyte-equivalents per track) or immunoprecipitated (2 oocyte-equivalents per track loaded). Samples from pulse-chase experiments were immunoprecipitated (2 oocyte-equivalents per track finally loaded) or concentrated by precipitation for direct electrophoresis. Precipitation by TCA was performed as follows: the sample was made 10% TCA, left on ice for ten min, and spun in an Eppendorf microfuge for 2 min. The supernatant was removed with a vacuum line, the pellet washed with ice-cold acetone and then ice-cold ethanol, air-dried and taken up in sample buffer.

#### C. Samples from sucrose gradients

Except where detailed, the procedure was as follows: 50  $\mu$ l aliquots of samples were immunoprecipitated, resuspended in 50  $\mu$ l of sample buffer and 25  $\mu$ l loaded per track.

#### D. Sample buffers

Two different sample buffers were used. With gel types i and iii, the sample buffer used was: 20 mM Tris-HCl, pH 7.6, 20% (v/v) glycerol, 1% (v/v)  $\beta$ -mercaptoethanol, 2% (w/v) SDS, 1 mM PMSF, 0.01% bromophenol blue.

With gel types ii and iv, the sample buffer used was: 200 mM Tris-HCl (pH 8.8), 1.0 M sucrose, 5 mM EDTA, 2% (v/w) SDS, 10 mM dithiothreitol. All samples, once in sample buffer, were heated to 100°C for 2 min before loading.

#### E. Alkylation of samples

Samples electrophoresed on the gels shown in chapters 6, 7 and 8 (and immunoglobulin samples shown in chapter 5) were alkylated before loading. Alkylation was performed by incubating (15 min, at room temperature) samples that had been heated (100°C, 2 min) and cooled, with 5  $\mu$ l of 0.5 M iodoacetamide per 50  $\mu$ l of sample.

#### 6. Preparation of Rabbit anti-hen Lysozyme Antiserum

Large New Zealand White female rabbits were injected in each hind thigh muscle with 3 mg of lysozyme dissolved in 0.2 ml of phosphate buffered saline (PBS) and Freund's complete adjuvant. (PBS: NaCl (135 mM), KCl (3 mM), Na<sub>2</sub>HPO<sub>4</sub> (7 mM), KH<sub>2</sub>PO<sub>4</sub> (1.5 mM), pH 7.4). The animal was boosted twice, at 12 day intervals, with a further 6 mg of antigen per animal, but using incomplete adjuvant. Two weeks after the last injection, rabbits were exsanguinated. Antibody titre was

monitored by ear-bleeding, followed by an ouchterlony plate analysis. The final titre was approximately 0.02 mg antibody/ml of serum. Anti-lysozyme antibody was prepared from serum by an initial salt precipitation using  $\text{NaSO}_4$  (Kekwick, 1940) followed by an immunoaffinity column. The affinity column was prepared as follows: 2.5 g of CNBr-activated Sepharose 4B were washed on a glass filter with 500 ml of 1 mM HCl over a 20 min period. These pre-swollen beads were then shaken with 25 mg of lysozyme in 50 ml of 0.1 M borate, 0.3 M KCl, pH 8, for 2 h at room temperature. The beads were then washed with a further 500 ml of borate buffer, before shaking with 50 ml of 1 M ethanolamine (pH 8) for  $1\frac{1}{2}$  h at room temperature. The beads were then washed with 200 ml of 0.1 M sodium acetate, 0.3 M KCl (pH 4.0) alternating with 200 ml of borate buffer for three cycles. Finally, the beads were washed with 200 ml of PBS and poured into a column ready for use.

The affinity column was used as follows: 25 mg of serum protein, derived from the salt precipitations, was loaded in 1 ml of 0.15 M NaCl, 20 mM Tris HCl, pH 7.4 at room temperature. This was slowly run into the column, left for 5 min and then washed with phosphate buffered saline until the  $\text{OD}_{280}$  was negligible. The bound protein was then eluted with 4 M  $\text{MgCl}_2$ , 20 mM Tris HCl (pH 7.4) until the  $\text{OD}_{280}$  was again negligible. The eluted fractions were immediately dialysed overnight against PBS diluted with an equal volume of distilled water at  $4^\circ\text{C}$  and concentrated on an Amicon minicon concentrator to

5 mg/ml of protein. This antibody was tested by immunoprecipitation of samples from radiolabelled oocytes injected with oviduct mRNA.

#### 7. Immunoprecipitations

Immunoprecipitations were performed in a detergent buffer which comprised 100 mM KCl, 5 mM MgCl<sub>2</sub>, 1% Triton X-100, 0.5% SDS, 1% sodium deoxycholate and 100 mM Tris HCl, pH 8.2.

In a standard immunoprecipitation, 50 µl of sample was mixed with 450 µl of detergent buffer. Five µl of either rabbit anti-hen ovalbumin antiserum or affinity purified rabbit anti-hen lysozyme antiserum was added. (In the case of the latter, 5 µl 10 mg/ml ovalbumin was also added because the antiserum contained some anti-ovalbumin antibody). The mixture was stood on ice for 1 h before the addition of 50 µl of formalin-fixed Staphylococcus aureus envelopes (Kessler, 1975). After an overnight incubation the bacterial envelopes were pelleted, washed twice with 500 µl of detergent buffer and the bound protein eluted by heating (100°C for 2 min) in 50 µl sample buffer.

## 8. Quantitation of Ovalbumin and Lysozyme

### A. Quantitation of immunoprecipitates

Immunoprecipitates were quantitated in one of two ways: firstly, aliquots of immunoprecipitate were dissolved directly in a Triton/Toluene scintillation cocktail (PPO (0.4%), POPOP (0.02%), dissolved in toluene:Triton X-100 (2:1 (v/v)), and counted in a Packard Tri-Carb liquid scintillation counter.

Secondly, immunoprecipitates were electrophoresed, and autoradiographs or fluorographs prepared. The band of interest, located by using the autoradiograph or fluorograph, was excised from the dry gel and counted in a toluene scintillant (PPO (0.5%), POPOP (0.03%) dissolved in toluene).

### B. Quantitation of non-immunoprecipitates

Samples were either quantitated after gel electrophoresis (as above) or TCA-precipitated and filter counted in the toluene based scintillant described above.



#### 9. Microdensitometry

Microdensitometry was performed using a Joyce-Loebl double-beam scanning microdensitometer (model 3CS).

#### 10. Preparation of a Crude Vesicle Fraction from Oocytes

This method was modified from method 2 described in Colman and Morser (1979). Oocytes were homogenised (5 strokes, loose pestle, Dounce homogeniser) in 10% (w/v) sucrose dissolved in 100 mM KCl, 50 mM NaCl, 10 mM magnesium acetate, 20 mM Tris HCl (pH 7.6), at 30  $\mu$ l/oocyte. The homogenate was layered onto a step gradient consisting of 1 ml 20% (w/v) sucrose above 1 ml 50% (w/v) sucrose, both dissolved in 50 mM KCl, 10 mM magnesium acetate, 20 mM Tris-HCl (pH 7.6). The gradient was centrifuged in an MSE HS18 centrifuge using the 8x5 ml swing-out rotor at 12,000 rpm for 30 min at 4°C. The vesicle fraction was collected from the 20%/50% interface.

### Chapter 3

Ovalbumin and lysozyme are secreted  
at different rates from Xenopus oocytes

### Introduction

Because of the implications of non-parallel secretion for detailed models of the intracellular secretory pathway, the observations of Colman and co-workers (subsequently published in Lane et al., 1980; Colman, Lane et al., 1981) of different intracellular and extracellular ratios of ovalbumin and lysozyme synthesised in Xenopus oocytes were of interest. It was thought that if non-parallel kinetics of secretion could be unequivocally demonstrated, then it might be possible to investigate whether, within the general scheme outlined by Palade (1975), different proteins were taking different routes out of the cell in these circumstances. Accordingly, I set out to discover whether the differential accumulation of ovalbumin and lysozyme in the medium surrounding oviduct-mRNA-injected oocytes was a consequence of the two proteins' being exported at different speeds.

### Results

Oocytes were injected with oviduct mRNA, labelled for 24 h and groups of oocytes and the incubation medium surrounding them analysed by gel electrophoresis. A comparison of intracellular and secreted ovalbumin and lysozyme reveals (Fig. 3.1, tracks 1-4) that as much radioactive lysozyme as ovalbumin accumulates in the incubation medium although net ovalbumin synthesis is much greater<sup>†</sup>. The simplest explanation of this observation is that lysozyme is secreted faster than ovalbumin from Xenopus oocytes. However, alternative explanations of the data are possible.

Firstly, the observations could be the result of transitory changes in the rates of synthesis of individual proteins during the labelling period. The synthetic capacity of the oocyte and the synthesis and export of these two proteins during the 24 h labelling period were therefore studied. Under the experimental conditions employed, incorporation of <sup>35</sup>S-methionine into TCA-insoluble material during the 24 h period is fairly constant (Fig. 3.2). When the synthesis and secretion of ovalbumin and lysozyme are analysed at intervals during continuous labelling, it is clear that the disproportionality in extracellular accumulation is not a transitory phenomenon (Fig. 3.3).

<sup>†</sup> It should be noted that ovalbumin resolves to six spots on 2-dimensional gel electrophoretic systems, and to two or three bands on one-dimensional gels (see Colman, Lane et al., 1981).

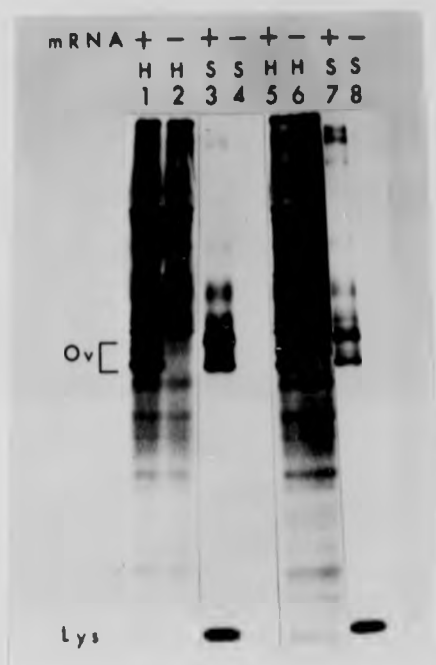
### Results

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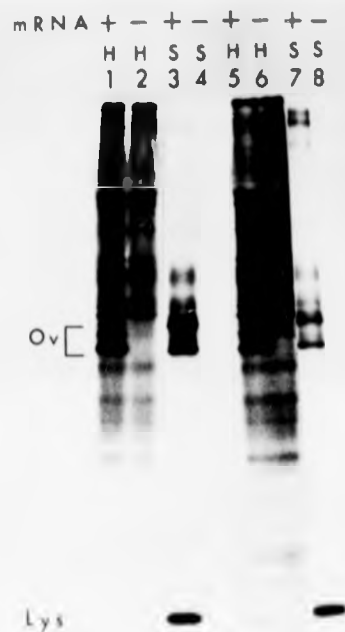
<sup>†</sup> It should be noted that ovalbumin resolves to six spots on 2-dimensional gel electrophoretic systems, and to two or three bands on one-dimensional gels (see Colman, Lane et al., 1981).

Fig. 3.1 Secretion of Chick Proteins by Oocytes



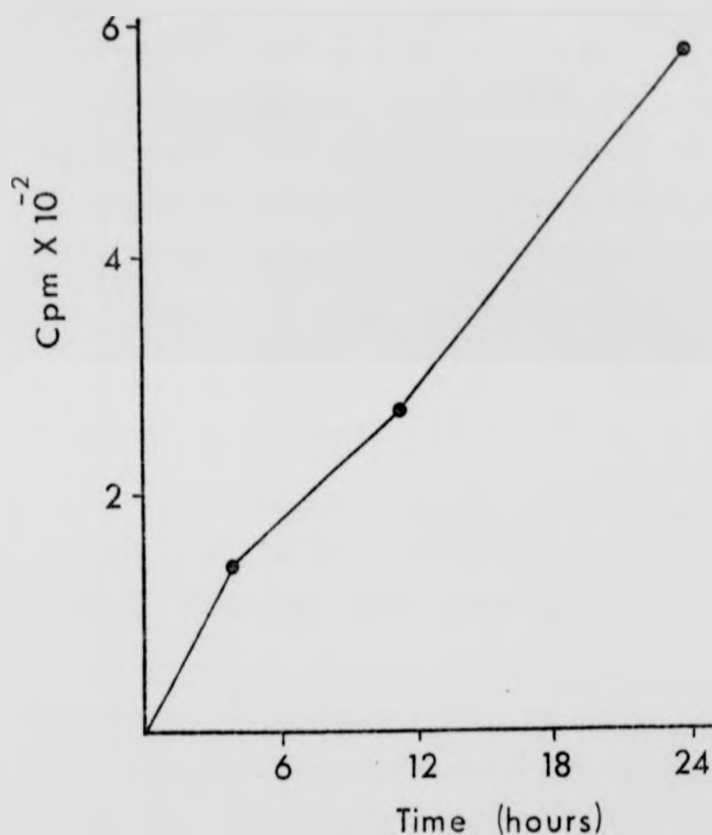
Oocytes were injected with oviduct mRNA (1 mg/ml) and cultured in modified Barth's saline for 24 h (tracks 1-4) or 72 h (tracks 5-8). Healthy oocytes were then labelled ( $[^{35}\text{S}]$ -methionine, 1 mCi/ml) for 24 h. Groups of 10 oocytes were homogenised and the homogenates (H) prepared for electrophoresis; incubation media (S) were prepared directly for electrophoresis. Samples were electrophoresed on a 10-22% exponential gradient SDS-polyacrylamide gel which was then fixed and fluorographed. Ov: denotes position of immunoprecipitable ovalbumin. Lys: denotes position of marker lysozyme and immunoprecipitable lysozyme. mRNA +/- describes injected or uninjected oocytes.

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Fig. 3.2 Incorporation of  $^{35}\text{S}$ -methionine into TCA-precipitable material



Oocytes were treated as in Fig. 3.1 tracks 2 and 4, except that labelling was performed in the presence of 0.1 mM L-methionine. At intervals during the labelling, groups of oocytes were removed, homogenised and aliquots (5  $\mu$ l) TCA-precipitated and filter counted. Each time point is the average of two aliquots.



Fig. 3.3 Kinetics of Chick Protein Secretion

A. Oocytes were injected with oviduct mRNA (1 mg/ml), cultured in unlabelled saline (24 h) followed by a further period of up to 24 h in saline containing [ $^{35}\text{S}$ ]-methionine (1 mCi/ml) and cold L-methionine (0.1 mM). Oocytes (tracks 1-8) and incubation media (tracks 9-16) removed at various times were immunoprecipitated and electrophoresed on a 12.5% SDS-polyacrylamide gel, which was fixed and fluorographed.

Tracks 1, 5, 9, 13 : 0-4 h      Tracks 2, 6, 10, 14: 0-12 h

Tracks 3, 7, 11, 15: 0-18 h      Tracks 4, 8, 12, 16: 0-24 h

Anti-ovalbumin immunoprecipitates: tracks 1-4, 9-12.

Anti-lysozyme immunoprecipitates: tracks 5-8, 13-16.

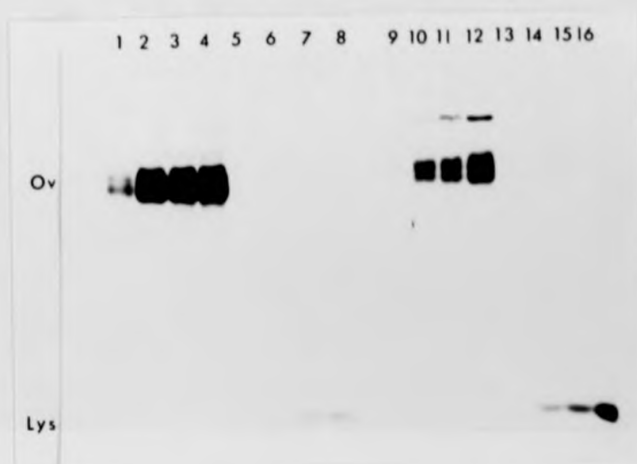
OV: position of ovalbumin.    lys: position of lysozyme.

B. Regions of the gel (A) corresponding to the positions of immunoprecipitated ovalbumin and lysozyme were excised and counted in a toluene-based scintillant. Data were corrected to show amount per oocyte.

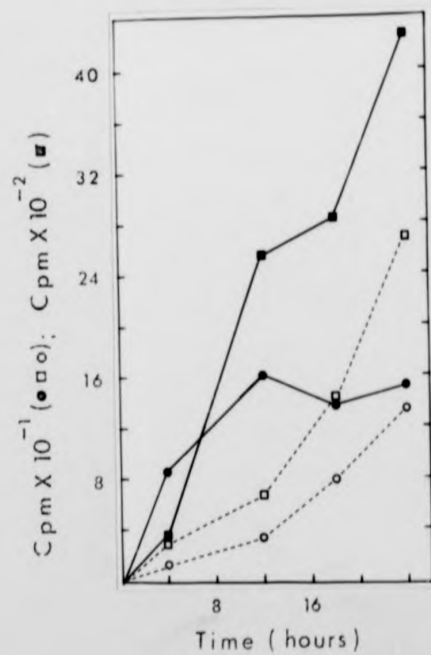
Ovalbumin: intracellular ; secreted 

lysozyme: intracellular ; secreted 

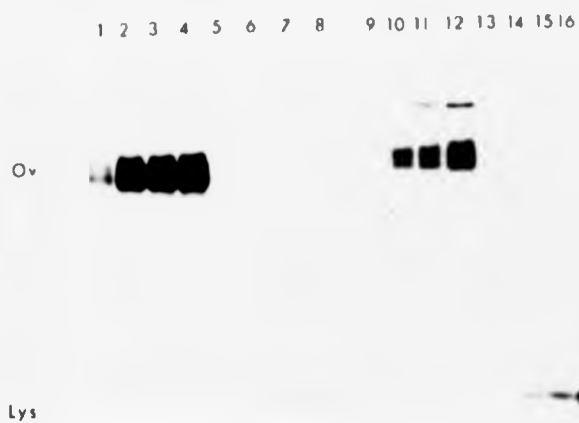
A



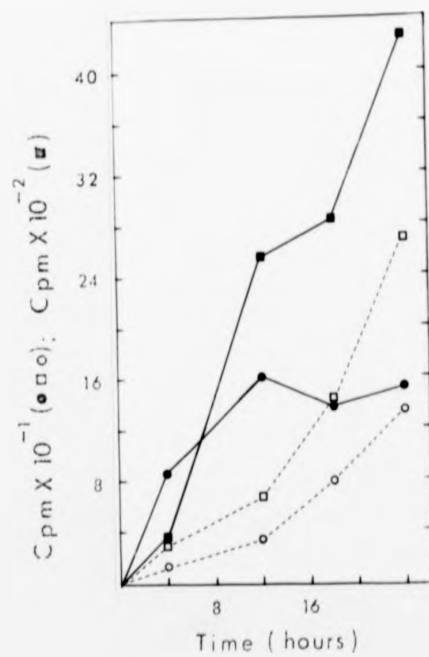
B



A



B



A second possible explanation is that lysozyme mRNA is preferentially degraded in the oocyte, thus diminishing lysozyme synthesis. To test this hypothesis a group of oocytes were micro-injected and split into two groups. One group was labelled from 24-48 h after injection and the other group was labelled from 72-96 h after injection. These two groups of oocytes and secreted proteins were then analysed (Fig. 3.1, compare tracks 1-4 with 5-8). It is clear that the mRNAs encoding both ovalbumin and lysozyme are stable for at least 96 h after injection into oocytes.

A third possibility is that not all of the ovalbumin synthesised by the oocyte is available for secretion. It has been demonstrated (Lane *et al.*, 1979; Colman, Lane *et al.*, 1981) that the bulk of oocyte-synthesised ovalbumin is present within these cells in a membranous fraction. However, most of this ovalbumin could be sequestered within some non-secretory compartment. Under these circumstances, the ratio between secreted and secretable protein could be similar for both ovalbumin and lysozyme. To test this possibility, pulse-chase experiments were employed. Injected oocytes were labelled for 24 h and then chased by incubation in medium supplemented with 10 mM L-methionine. It has been shown (Zehavi-Willner and Lane, 1977) that the presence of 10 mM L-methionine very rapidly dilutes the <sup>35</sup>S-methionine present inside the oocyte and effectively prevents any further incorporation of radioactive precursor into TCA-insoluble

material. In order to account for the differential accumulation of lysozyme and ovalbumin in the medium surrounding micro-injected oocytes, a large majority of the ovalbumin would have to be unavailable for secretion (assuming the intrinsic rate of secretion were similar for both proteins). By immunoprecipitation with an anti-ovalbumin antiserum it is possible to quantitate the amount of ovalbumin present inside the oocyte at the beginning and end of chase periods. Analyses of this kind show that about 40% of the ovalbumin present within the oocyte at the beginning of a chase is secreted within 72 h (Table 3.1). This result is incompatible with the hypothesis that only a small fraction of intracellular ovalbumin is potentially secretable. Moreover, longer chase periods show that ovalbumin continues to be secreted for up to two weeks after chase conditions are imposed (C. D. Lane, personal communication). After this length of time, cytopathic effects interfere with a quantitative analysis, but it is likely that most, if not all, of the ovalbumin synthesised within the oocyte is potentially secretable. A similar analysis performed using anti-lysozyme antiserum shows that about 60% of the lysozyme is secreted within 72 h (Table 3.1).

The complete recovery of radioactivity secreted from the oocyte into the extracellular medium (Table 3.1) indicates that neither protein is significantly degraded under the conditions used. This finding rules out a fourth possible explanation for the non-parallel extracellular accumulation of these two

Table 3.1 Secretion During Pulse-Chase Experiments

	Chase (h)	Oocyte		Medium	
		cpm $\times 10^{-3}$ /oocyte	Total loss cpm $\times 10^{-3}$ /oocyte	cpm $\times 10^{-3}$ /oocyte	Recovery <sup>*</sup> (%)
Ovalbumin	0	93.5	38.9	41.6	106
	72	54.6			
Lysozyme	0	6.1	3.5	3.3	94
	72	2.6			

Oocytes were injected with oviduct mRNA (1 mg/ml), cultured, labelled for 24 h ( $[^{35}\text{S}]\text{-methionine}$ , 1 mCi/ml) and chased for 72 hours. Samples were collected at 0 and 72 h and immunoprecipitated with anti-ovalbumin and anti-lysozyme antisera. Aliquots were directly counted.

\*The amount recovered in the medium is expressed as a percentage of the amount lost from the oocyte.

proteins; that extracellular ovalbumin is rapidly degraded.

An analysis of the appearance of these two proteins outside the oocyte during a chase clearly shows that lysozyme is secreted faster than ovalbumin (Fig. 3.4). However, after about 30 h, lysozyme secretion ceases while the secretion of ovalbumin continues at an unchanged rate (Fig. 3.4). Thus lysozyme secretion stops despite a substantial proportion (Table 3.1) of this protein remaining within the oocyte. Due to this phenomenon, the times taken for half the ovalbumin and lysozyme present inside the oocyte at the beginning of the chase to be secreted, cannot be compared. Instead, the time taken for half the exportable lysozyme to accumulate outside the oocyte (approximately 6 h; see Fig. 3.4) must be compared with the time taken for half the ovalbumin present at the beginning of a chase to be secreted (approximately 70 h; average of data shown in Table 3.1 and other experiments). Thus lysozyme is being exported approximately 12 times faster than ovalbumin from Xenopus oocytes.

Fig. 3.4 Pulse-chase of Oocytes making Chicken Ovalbumin and Lysozyme

A. Oocytes were injected with oviduct mRNA (1 mg/ml), cultured for 24 h and then labelled ( $[^{35}\text{S}]$ -methionine, 1 mCi/ml) for 24 h. The surrounding incubation media were then stored at  $-70^{\circ}\text{C}$ . The oocytes were then chased with saline containing 10 mM L-methionine. The medium surrounding chased oocytes was removed and replaced intermittently.

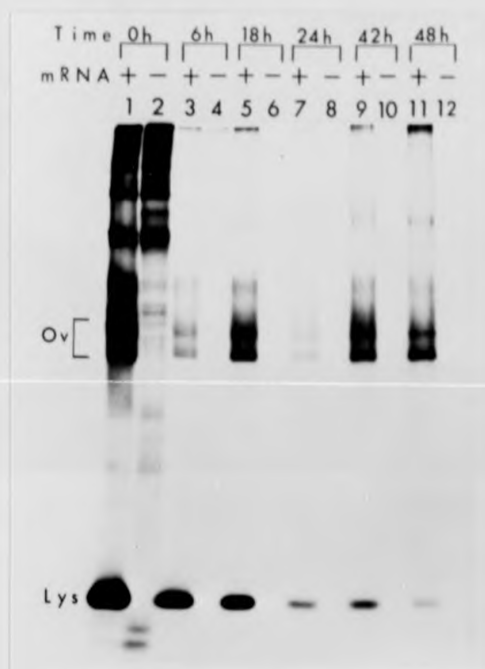
Track headings indicate the <sup>secreted</sup> protein accumulated before the beginning of the chase (0 h), between 0 and 6 h (6 h), 6-18 h (18 h), 18-24 h (24 h), 24-42 h (42 h), 42-48 h (48 h). Samples were electrophoresed on a 10-22% exponential gradient SDS polyacrylamide gel which was fixed and fluorographed. OV, ovalbumin. Lys, lysozyme.

B. Quantitation of the accumulation of extracellular ovalbumin and lysozyme was performed by excision and counting of regions of the gel (A) corresponding to the positions of ovalbumin and lysozyme. Data were corrected to show amount per oocyte.

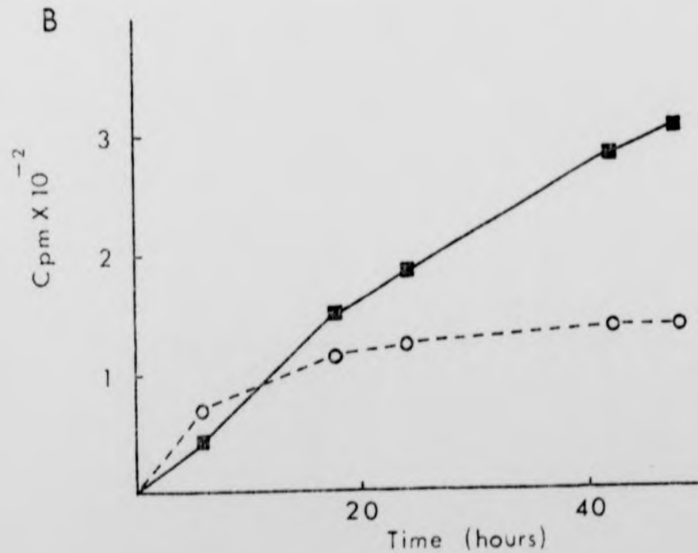
—■—■—, ovalbumin. o---o, lysozyme.



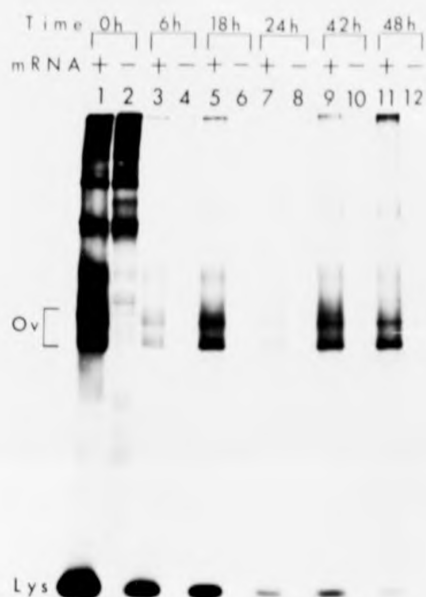
A



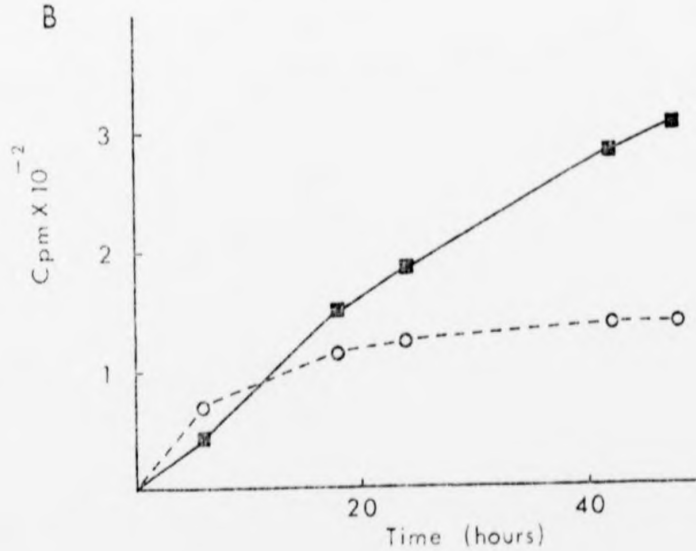
B



A



B



### Discussion

These data clearly demonstrate that ovalbumin and lysozyme are being secreted at very different rates from *Xenopus* oocytes. It seems unlikely that there is a trivial explanation for this phenomenon. For example, although ovalbumin is normally glycosylated whilst lysozyme is not (Warner, 1954), both glycosylated and non-glycosylated ovalbumin are secreted at the same rate from oocytes (Colman, Lane *et al.*, 1981). Moreover, guinea-pig  $\alpha$ -lactalbumin, a non-glycosylated protein that shows homology to chick lysozyme both in amino acid sequence and size (Brew, 1972) is secreted very slowly from oocytes (Lane *et al.*, 1980).

The retention within the cell of a substantial proportion of the lysozyme synthesised by the oocyte is of interest. This might reflect the fact that most oocyte secretory proteins are thought to be stored for later embryonic development (Zehavi-Willner and Lane, 1977); lysozyme may therefore be miscompartmentalised with these oocyte proteins.

## Chapter 4

The Rôle of extrinsic factors in the  
secretion of ovalbumin and lysozyme  
from Xenopus oocytes

### Introduction

Having clearly shown that ovalbumin and lysozyme are exported at very different rates from Xenopus oocytes, it became of interest to try to establish the cause of this differential extracellular accumulation. One question that arose was whether factors extrinsic to these two proteins are instrumental in causing this disparity in the rates of their secretion. More specifically, two hypotheses were formulated: firstly, that ovalbumin and lysozyme compete for some limiting oocyte factor necessary for export, that ovalbumin has a lower affinity than lysozyme for this factor, and is therefore retarded. A second hypothesis is that the secretion of ovalbumin is limited by or dependent on some avian protein whose encoding mRNA is co-injected into the oocyte along with the other oviduct mRNAs.

The in vitro competition experiments of Blobel and co-workers (Lingappa et al., 1978; Lingappa et al., 1979; reviewed Blobel et al., 1979) suggested to me that ovalbumin and lysozyme might be competing for some oocyte factor necessary for export. These experiments show that various secretory and other segregated proteins compete for entry into ER-derived vesicles in vitro. They concluded that this behaviour demonstrates that a single receptor operates to facilitate the translocation of segregated proteins across the membrane of the ER. However, these experiments have been criticised on the grounds that very high levels of competing proteins

were utilised, and it is not clear how far these in vitro results may be extrapolated to describe events occurring in whole cells. Thus it was of interest to see if it could be shown that ovalbumin and lysozyme compete for export from a living cell.

The second hypothesis - that ovalbumin export may be limited by, or dependent on some oviduct-specific protein - was suggested by both experimental data and theoretical considerations. The work of Paigen and others has shown that, in the mouse, the intracellular location of  $\beta$ -glucuronidase is affected by the presence or absence of another gene product, the protein egasyn (Ganschow and Paigen, 1967). Another example of this phenomenon is the requirement of histocompatibility antigens for  $\beta_2$  microglobulin in order to reach their normal cellular location (Ploegh et al., 1979; Krangel et al., 1979). Thus it is clear that in some cases not all the information necessary for the location of a protein is carried in that protein's primary structure. This has been discussed in theoretical terms by Blobel (1980), who postulated that proteins might reach their destinations by 'piggybacking' on other proteins. Blobel also proposed that 'carrier proteins' would be required to decode specific 'sorting sequences' on segregated proteins and thus direct them to correct destinations. All these considerations made it interesting to investigate the possibility that ovalbumin secretion was affected or effected by the presence of oviduct-specific accessory proteins.

## Results

### 1. Competition for Oocyte Factors

To test the hypothesis that the secretion of ovalbumin is retarded by the scarcity of some component of the oocytes' secretory apparatus, the effect of injecting a range of concentrations of oviduct messengers into the oocyte was investigated. It was reasoned that if ovalbumin and lysozyme compete for scarce oocyte factors, then reducing or increasing the amounts of ovalbumin or lysozyme being synthesised within the oocyte would affect this competition. This, in turn, would cause changes in the ratios of synthesised to secreted protein. However, there are limitations on the extent to which the concentration of mRNA injected into the oocyte can be usefully varied. At the top end of the scale, translational capacity becomes limiting, while quantitative detection of exogenous protein becomes impossible at low levels of injected mRNA. When the concentration of injected oviduct mRNA rises about 1 mg/ml (the standard concentration of mRNA used in oocyte-injection experiments), the oocytes translational capacity becomes limiting<sup>\*</sup>. For example, at 4 mg/ml, approximately 1.5 times as much ovalbumin is synthesised as at 1 mg/ml. The lower limit varies from experiment to experiment, depending on the particular batch of oocytes, but always being lower for ovalbumin than for lysozyme. It

<sup>\*</sup>There are problems with this interpretation. See final discussion, part 2.

is clear that when the oviduct mRNA is diluted from 1 mg/ml in three-fold steps before injection, the amount of synthesised and secreted ovalbumin falls in a corresponding fashion (Table 4.1). These data show that the ratio of synthesised to secreted ovalbumin remains unaltered over a twenty-seven fold range of mRNA concentration (1-0.037 mg/ml). Moreover, the linear fall in ovalbumin translation accompanying the mRNA dilution, implies that ovalbumin synthesis is not limited at 1 mg/ml. The ratio of synthesised to secreted ovalbumin and lysozyme remains similar (Fig. 4.1) over a range of mRNA concentrations from near to translational saturation (4 mg/ml) to the limits of quantitative lysozyme detection (0.2 mg/ml).

## 2. The Role of Avian Factors in the Secretion of Ovalbumin

The rôle of oviduct factors in the secretion of ovalbumin from oocytes was investigated by analysing the effect of injecting purified ovalbumin mRNA. The hypothesis was that if ovalbumin secretion is limited by, or dependent on, other oviduct proteins then its rate of secretion would be affected by their removal.

Ovalbumin encoding mRNA was purified by a hybrid-release protocol (for full details, see Methods). In outline, ovalbumin mRNA was hybridised to cloned ovalbumin complementary



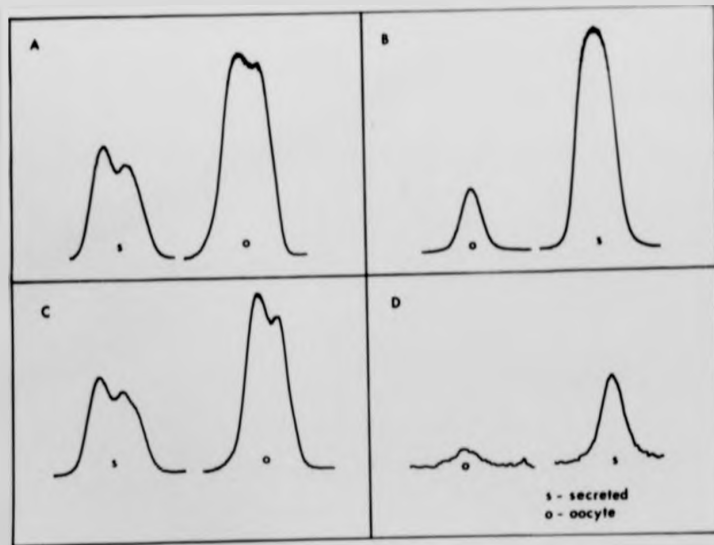
Table 4.1 Injection of diluted oviduct mRNA:  
Ovalbumin Synthesis and Secretion

Oviduct mRNA concentration (mg/ml)	cpm/oocyte in oocytes ( $\times 10^{-3}$ )	cpm/oocyte in media ( $\times 10^{-3}$ )
1	28.8	6.3
0.33	9.02	1.7
0.11	3.9	0.89
0.037	1.3	0.31
0.00	0.02	0.01

Oocytes were injected with a range of oviduct mRNA concentrations, cultured for 24 h and labelled ( $[^{35}\text{S}]\text{-methionine}$ , 1 mCi/ml) for 24 h. Samples of oocytes and incubation media were immunoprecipitated with anti-ovalbumin antiserum and electrophoresed. Regions from the gel corresponding to the position of ovalbumin were excised and counted.

Fig. 4.1 Injection of Different Concentrations of Oviduct mRNA

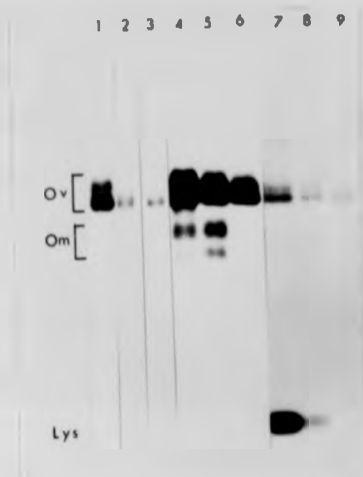
Oocytes were injected with oviduct mRNA <sup>at 4</sup> mg/ml or 0.2 mg/ml, cultured for 24 h and then labelled with [<sup>35</sup>S]-methionine, 1 mCi/ml) for 24 h. Incubation media (S) and oocyte (O) samples were then immunoprecipitated with anti-ovalbumin and anti-lysozyme antisera and electrophoresed on a 12.5% SDS-polyacrylamide gel. The gel was fixed, fluorographed and microdensitometry tracings prepared. Different exposure times and wedges were used in preparing the various panels which are therefore not directly comparable.



- A. mRNA at 4 mg/ml. Anti-ovalbumin immunoprecipitates.
- B. mRNA at 4 mg/ml. Anti-lysozyme immunoprecipitates.
- C. mRNA at 0.2 mg/ml. Anti-ovalbumin immunoprecipitates.
- D. mRNA at 0.2 mg/ml. Anti-lysozyme immunoprecipitates.

DNA (p0v 230, McReynolds et al., 1977) bound to diazobenzyl-oxymethyl (DBM) paper (Alwine et al., 1977) followed by elution with formamide buffer (Smith et al., 1979). The residual unbound oviduct mRNA was also recovered. The yields of bound and unbound mRNAs were low, possibly due to degradation during hybridisation. Purified mRNA, residual mRNA and the original oviduct mRNA were injected into oocytes which were then labelled and from which the synthesised and secreted proteins were then analysed (Fig. 4.2). The bound ovalbumin mRNA is shown to be pure by the absence of lysozyme and ovomucoid among the translation products of oocytes injected with this mRNA. Excess mRNA was used in the hybridisation and thus the unbound mRNA population contains ovalbumin mRNA. Similar amounts of ovalbumin were made in oocytes injected with bound or residual mRNA. More importantly, similar amounts of ovalbumin are secreted from both groups of oocytes. The unchanged rate of ovalbumin secretion in the absence of putative accessory factors implies that the rate of ovalbumin secretion is determined by the molecule itself.

Fig. 4.2 Injection of purified ovalbumin mRNA



Oocytes were injected with bound mRNA, residual mRNA or the original oviduct mRNA preparation (1 mg/ml). Following 24 h in culture, the oocytes were labelled ( $[^{35}\text{S}]$ -methionine, 1 mCi/ml) for 24 h. Oocytes and incubation media samples were then immunoprecipitated with anti-lysozyme or anti-ovalbumin antiserum, electrophoresed on a 12.5% gel, fixed and fluorographed. The anti-ovalbumin antiserum used in this experiment was a gift of M. Houghton and contains anti-ovomucoid activity. In order to visualise faint bands, exposures of 3 days (tracks 1-6) and 10 days (tracks 7-9) were used.

Secreted proteins; tracks 1-3. Intracellular protein; tracks 4-9.

Anti-ovalbumin immunoprecipitates; tracks 1-6.

Anti-lysozyme immunoprecipitates; tracks 7-9.

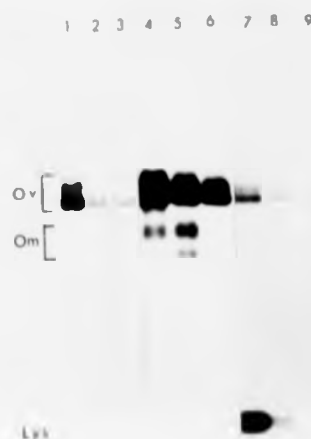
Untreated mRNA: tracks 1, 4 (0.15 vol loaded), 7.

Bound mRNA: tracks 3, 6, 9.

Residual mRNA: tracks 2, 5, 8.

Ov, ovalbumin; Om, ovomucoid; Lys, lysozyme.

Fig. 4.2 Injection of purified ovalbumin mRNA



Oocytes were injected with bound mRNA, residual mRNA or the original oviduct mRNA preparation (1 mg/ml). Following 24 h in culture, the oocytes were labelled ( $^{35}\text{S}$ -methionine, 1 mCi/ml) for 24 h. Oocytes and incubation media samples were then immunoprecipitated with anti-lysozyme or anti-ovalbumin antiserum, electrophoresed on a 12.5% gel, fixed and fluorographed. The anti-ovalbumin antiserum used in this experiment was a gift of M. Houghton and contains anti-ovomucoid activity. In order to visualise faint bands, exposures of 3 days (tracks 1-6) and 10 days (tracks 7-9) were used.

Secreted proteins; tracks 1-3. Intracellular protein; tracks 4-9.

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Anti-lysozyme immunoprecipitates; tracks 7-9.

Untreated mRNA: tracks 1, 4 (0.15 vol loaded), 7.

Bound mRNA: tracks 3, 6, 9.

Residual mRNA: tracks 2, 5, 8.

Ov, ovalbumin; Om, ovomucoid; Lys, lysozyme.

### Discussion

The general conclusion to be drawn from these experiments is that the different rates of ovalbumin and lysozyme secretion are the consequence of information contained within the two proteins themselves.

The results obtained by altering the concentration of oviduct mRNA injected must be interpreted with care. Unlike the in vitro experiments of Lingappa et al. mentioned earlier, these experiments are limited by the translational capacity of the oocyte. Thus the secretory pathway of the oocyte cannot first be saturated with ovalbumin and then be tested to see whether the introduction of other oviduct proteins reduces ovalbumin secretion (demonstrating competition) or leaves it unaffected (no competition). However, since alteration of the amounts of the chick proteins present within the oocyte does not affect their differential relative rates of secretion, it can be concluded that the oocyte has (for oviduct proteins) spare secretory capacity. This is not compatible with the hypothesis that ovalbumin export is being retarded by the scarcity of some oocyte factor necessary for the secretion of both ovalbumin and lysozyme.

The results obtained using purified ovalbumin mRNA demonstrate two things. Firstly, they rule out a rôle for any oviduct-specific protein in the secretion of this protein. Secondly, they imply that the post-translational modifications of

ovalbumin are performed in the oocyte by oocyte enzymes and not by enzymes encoded by oviduct mRNAs co-injected with ovalbumin mRNA; the ovalbumin made after injection of purified ovalbumin mRNA is indistinguishable (by SDS-polyacrylamide gel electrophoresis) from that synthesised when oviduct mRNA is injected.

Chapter 5

Subcellular fractionation of Xenopus  
oocytes on sucrose gradients



### Introduction

The demonstration that ovalbumin and lysozyme molecules are, on average, being exported at very different rates from Xenopus oocytes has implications for detailed models of the secretory pathway: if secretion in the oocyte proceeds in the same way as in other cells, and secretory proteins are transported from the Golgi apparatus to the extracellular space in discrete vesicles of mixed content, then this differential rate might reflect the relative retardation of ovalbumin during stages preceeding the formation of the final exocytotic vesicles. Alternatively, in the oocyte the two proteins might be segregated from one another and secreted by different tracks along the same pathway.

While the work presented in this thesis was in progress, Strous and Lodish (1980) showed that two proteins, VSV-G and transferrin (a hepatoma secretory protein) are translocated at different rates through rat hepatoma cells infected with VSV. Half of the transferrin present at the beginning of a 'chase' was secreted in 40 min whereas the time taken for half of the VSV-G to reach the plasma membrane was 23 min. They demonstrated that during these pulse-chase experiments, transferrin takes much longer to acquire resistance to endo H than does VSV-G. However, the time taken for both proteins, once endo H-resistant, to reach their final destinations is similar. Thus the authors argue that the difference between 23 and 40 min is due to a difference in rate of movement from

the ER to the trans-Golgi apparatus. If this result, comparing a membrane and a secretory protein, is relevant to the difference in rate of secretion of ovalbumin and lysozyme, then one might expect ovalbumin to be similarly retarded at an early stage along the secretory pathway. Unfortunately, this kind of endo H analysis cannot be carried out on these chick proteins in oocytes. Firstly, lysozyme is not glycosylated (Warner, 1954) and so a comparison with ovalbumin could not be made. Secondly, oligosaccharides present on ovalbumin extracted from egg white are of the high-mannose type, and are thus endo H-sensitive at all stages of biogenesis (Tai et al., 1975). Thirdly, the oocyte does not seem to synthesise endo H-resistant oligosaccharides. Immunoglobulins which do become endo H-resistant in myeloma cells remain sensitive when synthesised in oocytes (G. Valle, personal communication, and see Introduction).

However, one way to test the hypothesis that ovalbumin is being retarded relative to lysozyme between the ER and the trans-Golgi is to analyse microsomal fractions isolated from oocytes secreting oviduct proteins. If different microsomal subfractions (derived from the ER or the Golgi apparatus) contain markedly different ratios of ovalbumin to lysozyme, this would tend to confirm the hypothesis.

Published protocols for the subfractionation of oocytes give incomplete fractionation. There have been no successful attempts to separate the organelles involved in secretion

away from one another. This is primarily due to the nature of the cell; large amounts of sticky yolk protein makes separation difficult (Hurkman *et al.*, 1981), and compared with the specialised secretory cell (see Palade, 1975), the RER and other organelles are under-represented.

The method generally used to subfractionate the oocyte consists of gentle homogenisation followed by centrifugation through a suitably buffered linear 15-60% sucrose gradient. Several variations in methodology have been used. Opresko *et al.* (1980) added polyvinylpyrrolidone (2.5%) to increase the resolution and reproducibility of the method. Hurkman *et al.* (1981) used a high salt concentration (0.3 M NaCl) and EDTA to disaggregate and solubilise the yolk platelets. Zehavi-Willner and Lane (1977) added a second step gradient to the procedure in order to increase the purity of fractions. Interestingly, despite the differences in method, cellular fractions such as microsomes and mitochondria are found at remarkably similar densities in all three studies.

Analysis of the subcellular fractions obtained in these ways has mainly been done by electron microscopy. The three studies described above include electron micrographs of various cellular components such as lipid droplets, smooth vesicles, mitochondria and yolk platelets (listed in order from top to bottom of the gradient). Rough vesicles are not seen, probably because the ribosomes are removed during fractionation

or during the preparation of samples for electron microscopy (Zehavi-Willner and Lane, 1977). In addition to electron microscopy, attempts to characterise fractions using marker enzymes have been made. Both Zehavi-Willner and Lane (1977) and Hurkman et al. (1981) have been able to suggest the presence of elements of the ER in their smooth vesicle fraction using, respectively, membrane-associated NADH-ferricyanide reductase and NADH-cytochrome c reductase. However, these enzymes are not stringent markers (these enzyme activities are also found associated with other organelles; Zehavi-Willner and Lane (1977); Hurkman et al. (1981)). The position of mitochondria on these gradients was confirmed using cytochrome c oxidase. Stringent marker enzymes for ER, Golgi apparatus or plasma membrane, e.g. glucose-6-phosphatase, galactosyl transferase, 5'-nucleotidase, (Morre, 1971) have not been detected in oocytes. Thus vesicles derived from these membranes in oocytes have not yet been demonstrably separated. Because of this problem it was decided to use a novel strategy:

1. Isolate a population of microsomal vesicles containing oviduct secretory proteins from sucrose gradients.
2. Analyse the protein content of these vesicles to see if subfractions of different densities (hopefully containing different proportions of vesicles derived from the ER or the Golgi) possess different ratios of ovalbumin and lysozyme. Complete separation of ER and Golgi apparatus is unlikely since there will probably be vesicles with a range of densities

derived from each organelle.

3. Compare the results obtained with ovalbumin and lysozyme with those found when oocytes are micro-injected with mRNA encoding a mutant immunoglobulin with a non-secretory phenotype. The post-translational processing of this protein must diverge from that of secreted proteins and may therefore show a different pattern of distribution on the gradient from the oviduct proteins. If this is so, then the implication would be that a separation of functional components of the secretory pathway had been achieved.

4. If positive results are obtained, then further functional characterisation of the microsomal subfractions could be performed. By monitoring the protein content of vesicles under a variety of experimental conditions, it might be possible to confirm the organelle of origin of subsets of vesicles; for example, monensin - a drug known to block exit of immunoglobulin from the Golgi apparatus of plasma cells (Tartakoff and Vassalli, 1977) - might cause a rise in the proportion of secretory protein found in one subset of vesicles. Another approach would be to pulse-label injected oocytes and then to subject them to long periods of chase conditions which should cause a rise in the ratio of intracellular secretory protein found in the Golgi apparatus to that found in the ER (Tartakoff and Vassalli, 1979). The effects of this treatment on the distribution of ovalbumin and lysozyme on gradients would be monitored. These approaches might give information as to the whereabouts of vesicles

derived from these two organelles on sucrose gradients.

This strategy avoids the problem of a lack of the enzyme markers which would be required if a conventional approach to the problem were used. Thus, I set out to isolate intact microsomal vesicles from oocytes. Several methods were tried - the results obtained with each will be described and their relative merits discussed.

### Methods

All procedures referred to in this chapter but not detailed below were carried out as described in Chapter 2.

#### Linear Sucrose Gradients

Linear 11 ml gradients of 19-50% (w/v) sucrose in HEPES buffer (5 mM HEPES, pH 7.5) were poured. Gradients were made in an MSE gradient maker. Thorough mixing of these viscous solutions was made possible by the simultaneous use of both a magnetic follower and a vibrating corkscrew. 0.8 ml Paraffin oil (Boots) was layered over the 19% sucrose solution to prevent backflow from the reservoir containing 50% sucrose. This gradient was pumped by a Gilson minipuls 2 peristaltic pump onto a 1 ml cushion of 60% sucrose (w/w) in HEPES buffer in a Beckman 14 ml cellulose nitrate centrifuge tube. The gradient was pumped (at the slowest speed consistent with maintaining a continuous flow) down the side of the centrifuge tube which was held at a slight angle from the vertical. These gradients were stored at 4°C for up to 18 h before use. Oocytes for fractionation were washed in modified Barth's solution and homogenised in 1 ml of 15% sucrose (w/w) in HEPES buffer also containing 1 mM PMSF. Forty oocytes were used per gradient; this included a variable number of injected and labelled oocytes (see figure legends) plus uninjected cold oocytes added as carrier. Homogenisation was in a 7 ml Dounce homogeniser (Wheaton Scientific, USA) using 15 strokes of the loose-fitting pestle. The homogenate was layered onto

the gradient and centrifuged in a Beckman model L ultra-centrifuge at 40,000 rpm for 12 h at 4°C in an SW40 rotor. All operations involving homogenised oocytes were carried out as rapidly as possible at 4°C.

Three variants of this basic gradient were used in experiments. These variants (consisting of salt and/or polymer additions to the sucrose solutions) will be described in the relevant results sections of this chapter.

Fractions from sucrose gradients were collected in one of two ways:

1. Gradients were pumped out of the top of the centrifuge tube using a saturated  $\text{CsSO}_4$  solution (with added bromophenol blue) at 1 ml/min in an Instrumentation Specialities Co. (ISCO) density gradient fractionator model 640. 0.6 ml Fractions were collected. The optical density ( $\text{OD}_{254}$  or  $\text{OD}_{280}$ ) was monitored in a 1 cm cell and recorded on an ISCO absorbance monitor (Model UA5).
2. The gradient was removed from the top of the tube downwards by a Gilson densiflow moving-probe fractionator. The  $\text{OD}_{280}$  was followed using an LKB Uvicord S (model 2210) dual channel chart recorder. Fractions were collected in an LKB redirac fraction collector.

The linearity of gradients was checked with an Abbé refractometer.



### Electron Microscopy

Samples were collected from high-salt (100 mM KCl, 50 mM NaCl) 19-50% linear sucrose gradients, pooled, diluted six-fold in 5 mM HEPES (pH 7.5), 100 mM KCl, 50 mM NaCl, and centrifuged at 4°C for 1 h at 39,000 rpm in the SW40 rotor in a Beckman L8 ultracentrifuge. The supernatant was discarded and the pellet fixed overnight in 2% glutaraldehyde in 0.2 M cacodylate (pH 7.5). The fixed pellet was rinsed twice in 0.2 M cacodylate (pH 7.5) and incubated in 1% OsO<sub>4</sub>, 0.1 M cacodylate for 1 h at room temperature. The pellet was thoroughly rinsed with 0.1M cacodylate (pH 7.5) and stained with 2% uranyl acetate in 10% acetone for 10 min. The stained pellet was rinsed in 30% acetone and transferred to a Beem capsule for dehydration in a series of acetone rinses. The pellet was left in 30%, 70% and 95% acetone for two periods of 5 min at each stage. Finally it was incubated in 100% acetone for three periods of 7 min each. One volume of Spurr's resin (firm mix, Spurr, 1969) was added to the final acetone rinse, mixed, left for 30 min and another volume of resin mixed in. This procedure was repeated three times, the resin replaced with fresh 100% resin, left for 30 min and heated to 70°C for at least 8 h. Gold sections were cut with a Reichert (Om U2) ultramicrotome from blocks produced in this way, post-stained with uranyl acetate and lead citrate (Hayat, 1970) and viewed with a Jeol JEM 100S microscope.

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## Results

### 1. Fractionation of Oocytes on Sucrose Gradients containing Polyvinylpyrrolidone and EDTA

#### A. Distribution of UV-absorbing material on polyvinylpyrrolidone (PVP)-EDTA gradients

The first method of oocyte fractionation tried was that of Opresko et al. (1980). These authors emphasise the reproducibility of their method and this was seen as necessary to the proposed experimental strategy. Thus sucrose gradients as described in the methods section (this chapter) but also containing the additional components PVP (2.5% w/v) and EDTA (1 mM) were made and used. These gradients were linear and reproducible with respect to their refractive index profile (Fig. 5.1) and reproducible with respect to their OD profile (Fig. 5.2a). In all gradient experiments, fraction number 1 is at the top of the gradient. Three different oocyte fractions were visible on these gradients (Fig. 5.2b); lipid at the top of the gradients, an opalescent fraction in the middle of the gradients and yolk at the bottom of the gradients. These results are similar to those obtained by Opresko et al. (1980).

#### B. Distribution of oviduct proteins on PVP-EDTA gradients

The distribution of newly synthesised ovalbumin and lysozyme on these gradients was then examined. Oocytes injected with

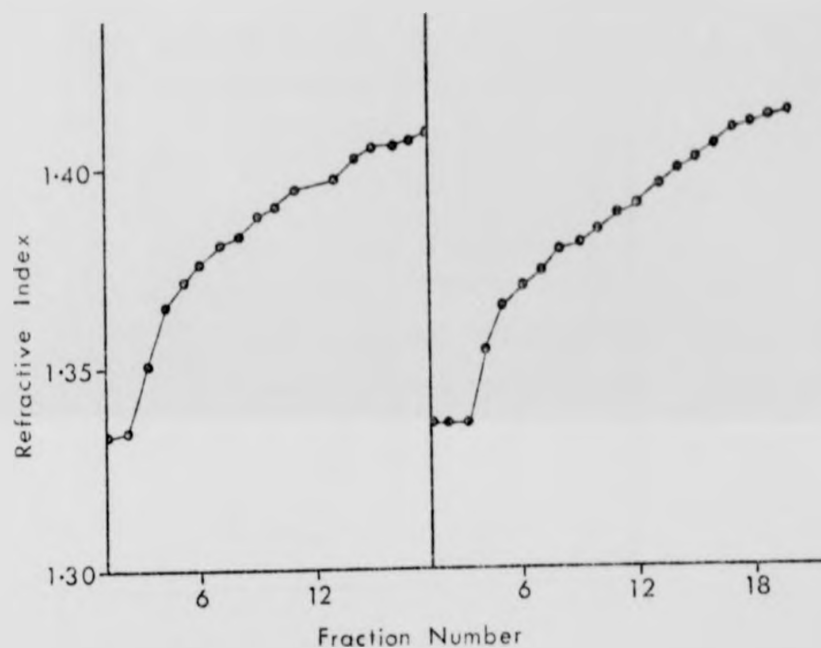


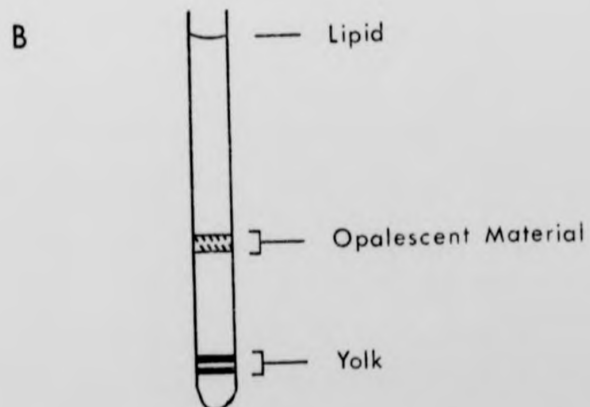
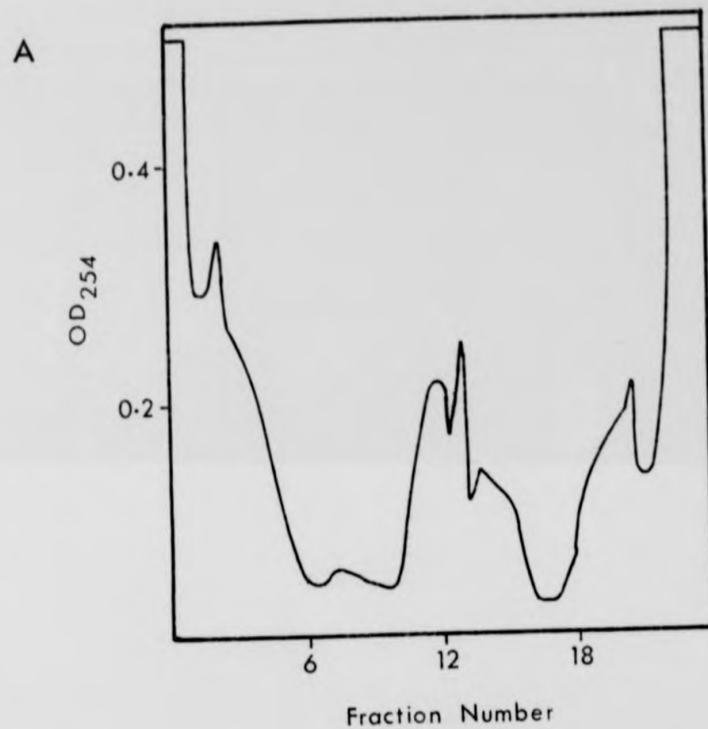
Fig. 5.1 Refractive Index Profile of PVP-EDTA Gradients

PVP-EDTA gradients were made as described. Successive samples from gradients after centrifugation were analysed with an Abbé refractometer. Gradients from separate experiments are shown.

Fig. 5.2 Visual and OD<sub>254</sub> Profiles of PVP-EDTA Gradients

A. OD<sub>254</sub> profile of typical PVP-EDTA gradient after centrifugation. Gradient monitored and sampled with the LKB/Gilson equipment as described.

B. Visual profile of PVP-EDTA gradient. Scale drawing ( $\frac{1}{2}$  size) of gradient after centrifugation. Density of shading indicates relative density of band.



oviduct mRNA and incubated in [ $^{35}$ S]-methionine for 24 h were fractionated. Uninjected radiolabelled oocytes were processed in parallel. Trichloroacetic acid (TCA)-insoluble counts present in injected and uninjected oocytes show a different distribution down these gradients (Fig. 5.3). In both cases the bulk of the counts are at the top of the gradient where the cytoplasm is expected to be (cf. Zehavi-Willner and Lane, 1977). However, in the injected oocytes there is a secondary peak centred around fraction number 13. The distributions of ovalbumin and lysozyme on a similar gradient, as determined by immunoprecipitation and gel electrophoresis, show a peak approximately coincident with this secondary peak (Fig. 5.4). The portion of the gradient around fraction number 13 corresponds in density to the region described by Opresko *et al.* (1980), Hurkman *et al.* (1981) and Jared *et al.* (1973) as containing ER-derived smooth vesicles. Quantitation of the distribution of immunoprecipitable material on gradients from this and one other experiment is shown in Fig. 5.5. In these and all subsequent experiments, the ISCO fractionation equipment was used. These results (Fig. 5.5) show that lysozyme and ovalbumin are similarly distributed over PVP-EDTA gradients, three regions containing exogenous secretory protein being clearly distinguished.

I believe that the peak at the top of the gradient (density  $\sim 1.08$  g/ml) represents ovalbumin and lysozyme leaked from microsomal vesicles during homogenisation. The reasons for this are as follows: the density of this region corresponds

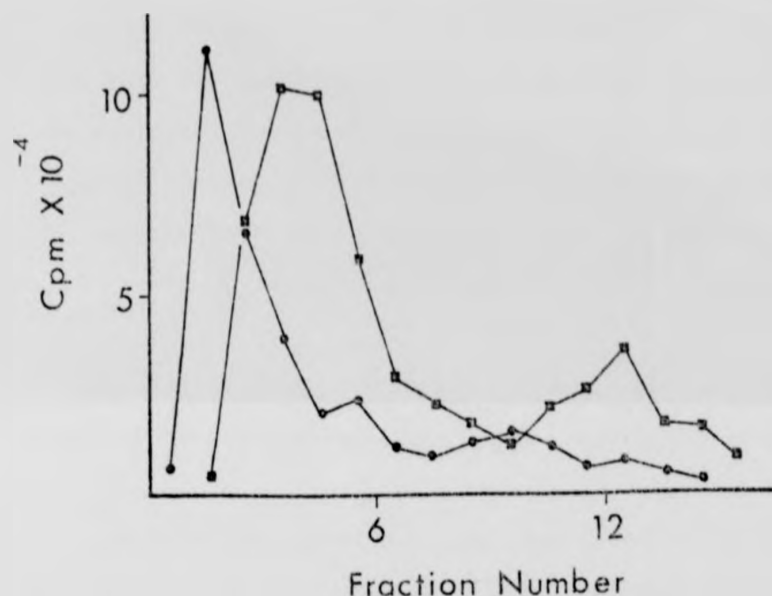


Fig. 5.3 Distribution on PVP-EDTA Gradients of TCA-insoluble Counts from Injected and Uninjected Oocytes

Oocytes were injected with oviduct mRNA (1 mg/ml) and labelled ( $^{35}\text{S}$ -methionine, 1 mCi/ml) for 24 h in parallel with uninjected oocytes. Both groups of oocytes were fractionated on PVP-EDTA gradients, samples collected and duplicate aliquots (2  $\mu\text{l}$ ) TCA-precipitated and counted.

- , uninjected oocytes. 40 labelled oocytes, no carrier.
- , injected oocytes. 40 injected oocytes, no carrier.



Fig. 5.4 Distribution of Ovalbumin and Lysozyme on  
PVP-EDTA Gradients

Oocytes were injected (oviduct mRNA, 1 mg/ml), labelled ( $r^{35}\text{S}$ -methionine, 4 mCi/ml) for 24 h, and fractionated on PVP-EDTA gradients. Samples were collected, immunoprecipitated and electrophoresed on 10-15% SDS-polyacrylamide gels.

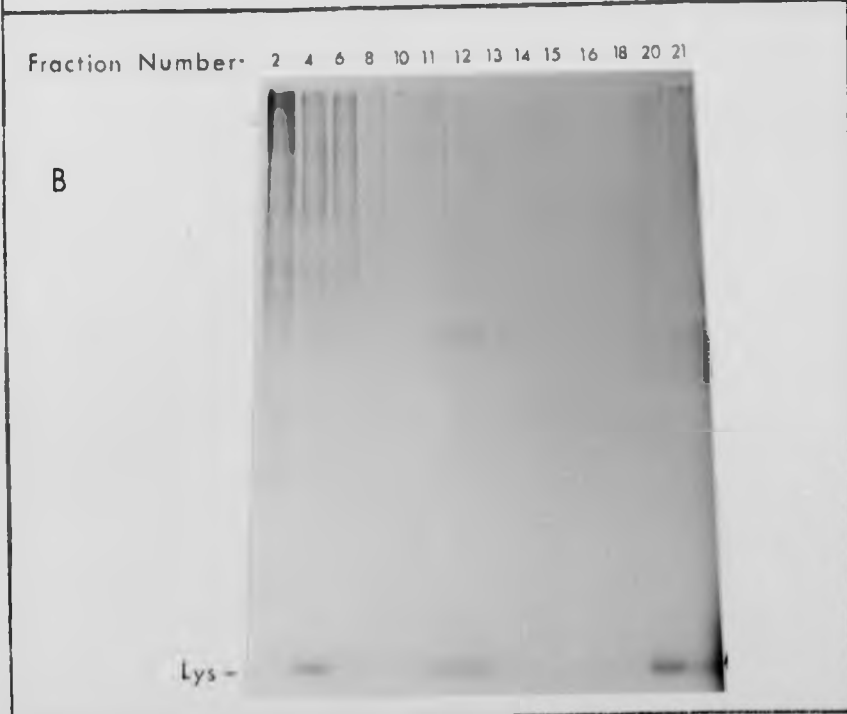
A. Fluorograph of anti-ovalbumin immunoprecipitates.

OV = ovalbumin;  $\text{OV}_M$  = miscompartmentalised ovalbumin.

B. Fluorograph of anti-lysozyme immunoprecipitates.

Lys = lysozyme.

This gradient contained 9 injected/labelled, and 31 carrier oocytes.



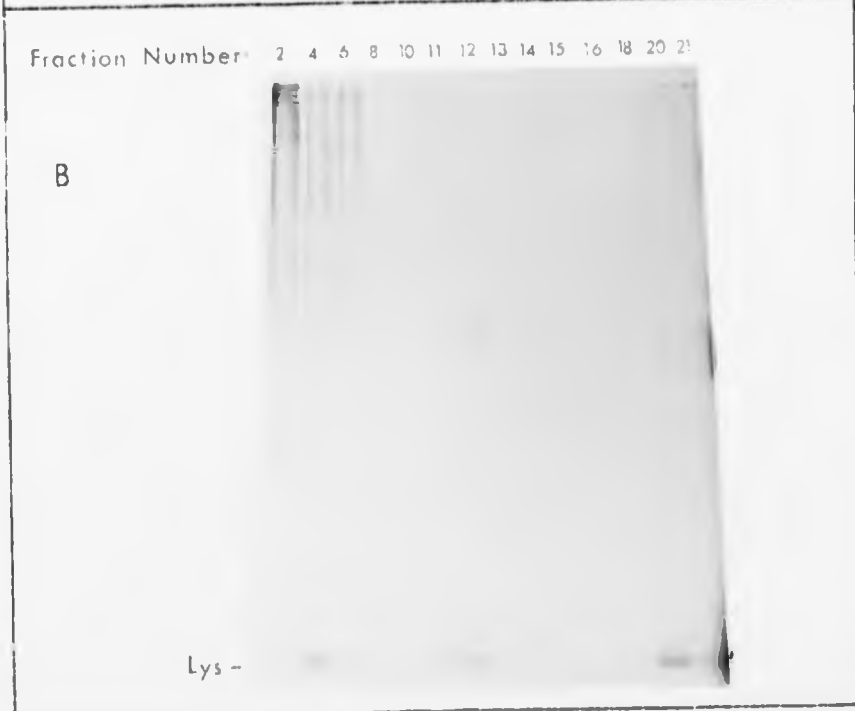
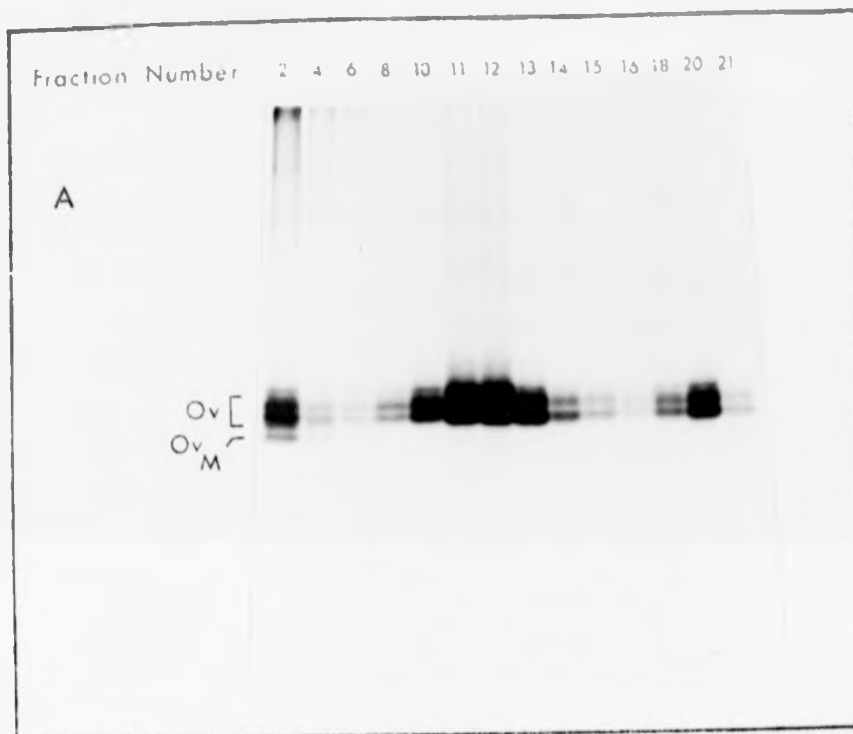
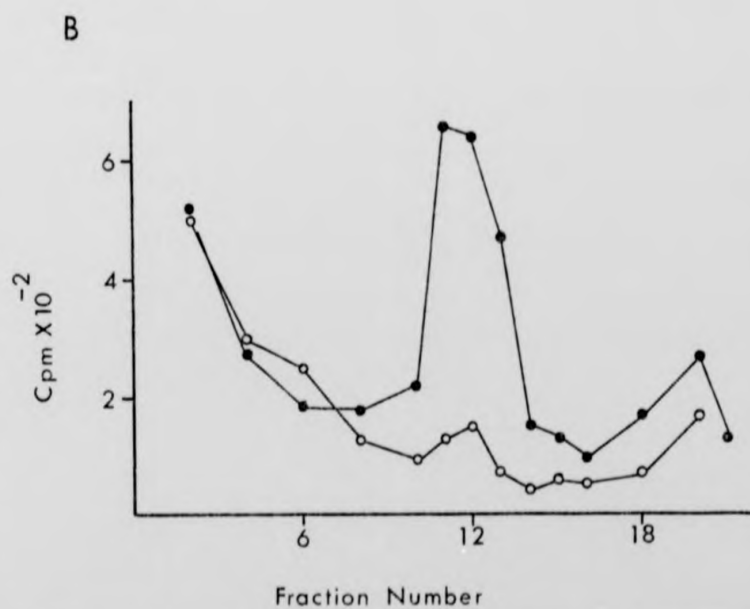
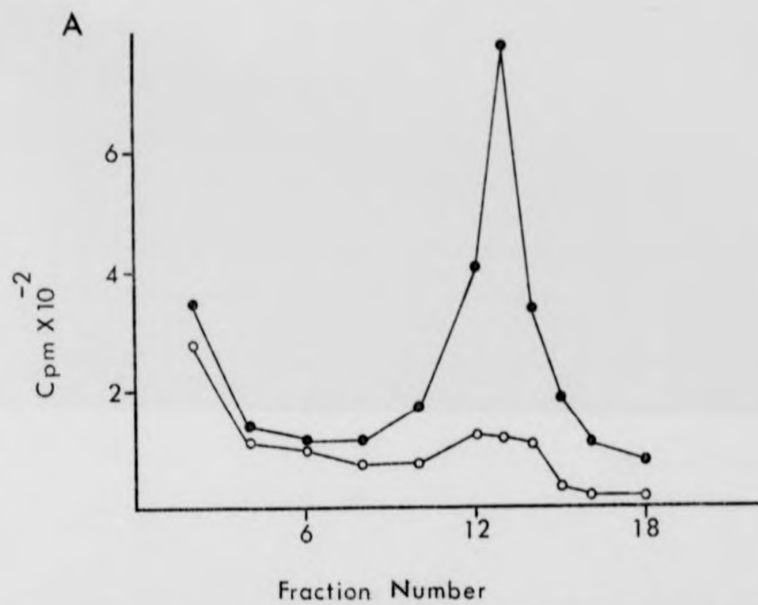


Fig. 5.5 Quantitative Distribution of Ovalbumin and Lysozyme on PVP-EDTA Gradients

Oocytes were injected (oviduct mRNA, 1 mg/ml) and labelled ( $[^{35}\text{S}]$ -methionine, 4 mCi/ml) for 24 h. Fractionation on PVP-EDTA gradients was followed by immunoprecipitation with anti-ovalbumin and anti-lysozyme antisera. Duplicate samples of immunoprecipitates were directly counted. Data from two separate experiments are shown. The samples shown in B are those shown in Fig. 5.4.

●—●, ovalbumin; o—o, lysozyme

- A. Gradient containing 10 injected/labelled, and 30 carrier oocytes.
- B. Gradient containing 9 injected/labelled, and 31 carrier oocytes.



to that at which Zehavi-Willner and Lane (1977) found cytoplasmic proteins, and this is where the non-glycosylated, cytoplasmic form of ovalbumin ( $Ov_m$ , Fig. 5.4a) present due to miscompartmentalisation during synthesis (Colman, Lane et al., 1981) is found. However, the other, glycosylated forms of ovalbumin are also found in this region of the gradient. Since glycosylation is known to occur exclusively in the ER, and since the ER has been shown to be present in the middle portion of these gradients (see the oocyte fractionation studies referred to above), the presence of glycosylated ovalbumin at the top of the gradient must reflect leakage from broken and damaged vesicles.

The peak of ovalbumin and lysozyme seen in the middle of the gradient probably represents secretory protein within microsomal vesicles since this peak corresponds in density to the microsomal fractions shown in all other published studies. Additionally, I will show in the following chapter that the secretory protein in this region is relatively resistant to protease. The bottom peak probably represents aggregated material (see Hurkman et al., 1981).

It is apparent from these experiments that the fraction of lysozyme present in the part of the gradient normally occupied by microsomes (see confirmatory electron microscopic study, section 3, this chapter) is smaller than the fraction of ovalbumin similarly confined. If the above interpretation of

the gradient-distribution of secretory protein is correct, then this represents differential leakage during homogenisation. The greater leakage of lysozyme might reflect its lower molecular weight and therefore greater rate of diffusion than ovalbumin.

C. Distribution of non-secretory mouse immunoglobulin on PVP-EDTA gradients

In accordance with the strategy outlined in the introduction to this chapter, mRNA encoding the non-secretory light chain immunoglobulin NS1 (Valle *et al.*, 1981) (mRNA a kind gift of G. Valle) was injected into oocytes and the distribution of this protein within oocytes determined by fractionation on PVP-EDTA gradients. The result (Fig. 5.6) shows that NS1 has a similar distribution to that of ovalbumin and lysozyme on these gradients.

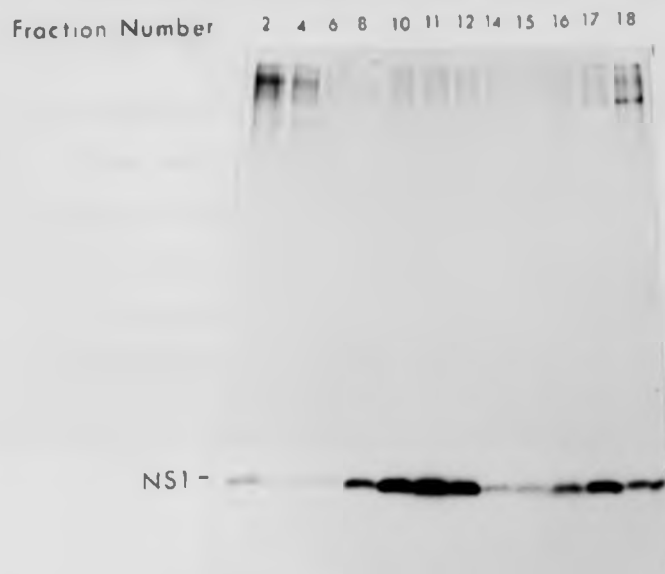


Fig. 5.6    Distribution of NSI Light Chain Immunoglobulin  
on PVP-EDTA Gradients

Oocytes were injected with NSI mRNA (0.5 mg/ml), labelled ( $r^{35}\text{S}$ ]-methionine, 4 mCi/ml) for 24 h and fractionated on a PVP-EDTA gradient. Immunoprecipitation of samples with rabbit anti-mouse IgG antiserum was followed by electrophoresis on a 10-15% SDS-polyacrylamide gel. The gel was fluorographed. NSI = position of marker NSI. This gradient contained 17 injected/labelled and 23 carrier oocytes.



Fraction Number    2   4   6   8   10 11 12 14 15 16 17 18

NSI -



Fig. 5.6    Distribution of NSI Light Chain Immunoglobulin  
on PVP-EDTA Gradients

Oocytes were injected with NSI mRNA (0.5 mg/ml), labelled ( $^{35}\text{S}$ ]-methionine, 4 mCi/ml) for 24 h and fractionated on a PVP-EDTA gradient. Immunoprecipitation of samples with rabbit anti-mouse IgG antiserum was followed by electrophoresis on a 10-15% SDS-polyacrylamide gel. The gel was fluorographed. NSI = position of marker NSI. This gradient contained 17 injected/labelled and 23 carrier oocytes.

## 2. Fractionation of Oocytes on Sucrose Gradients containing Ficoll and EDTA

The second method tried used sucrose gradients containing Ficoll. There were two reasons for trying Ficoll. Firstly, it might give greater resolution of the microsomal fraction, and secondly, even in the absence of improved resolution, Ficoll-EDTA gradients have a lower viscosity than PVP-EDTA gradients and would thus be easier to handle.

### A. Distribution of UV absorbing material on Ficoll-EDTA gradients

Gradients were prepared as before except that Ficoll at three concentrations (0, 1.5%, 2.5%, w/v) was added in place of PVP. Results obtained using these gradients are shown in Fig. 5.7. The visual profile and OD<sub>280</sub> tracings are similar to those obtained with PVP (in this experiment the visual profiles were particularly clear). Clearly 2.5% Ficoll gives a better separation of subfractions than 0, or 1.5% Ficoll as judged by these means.

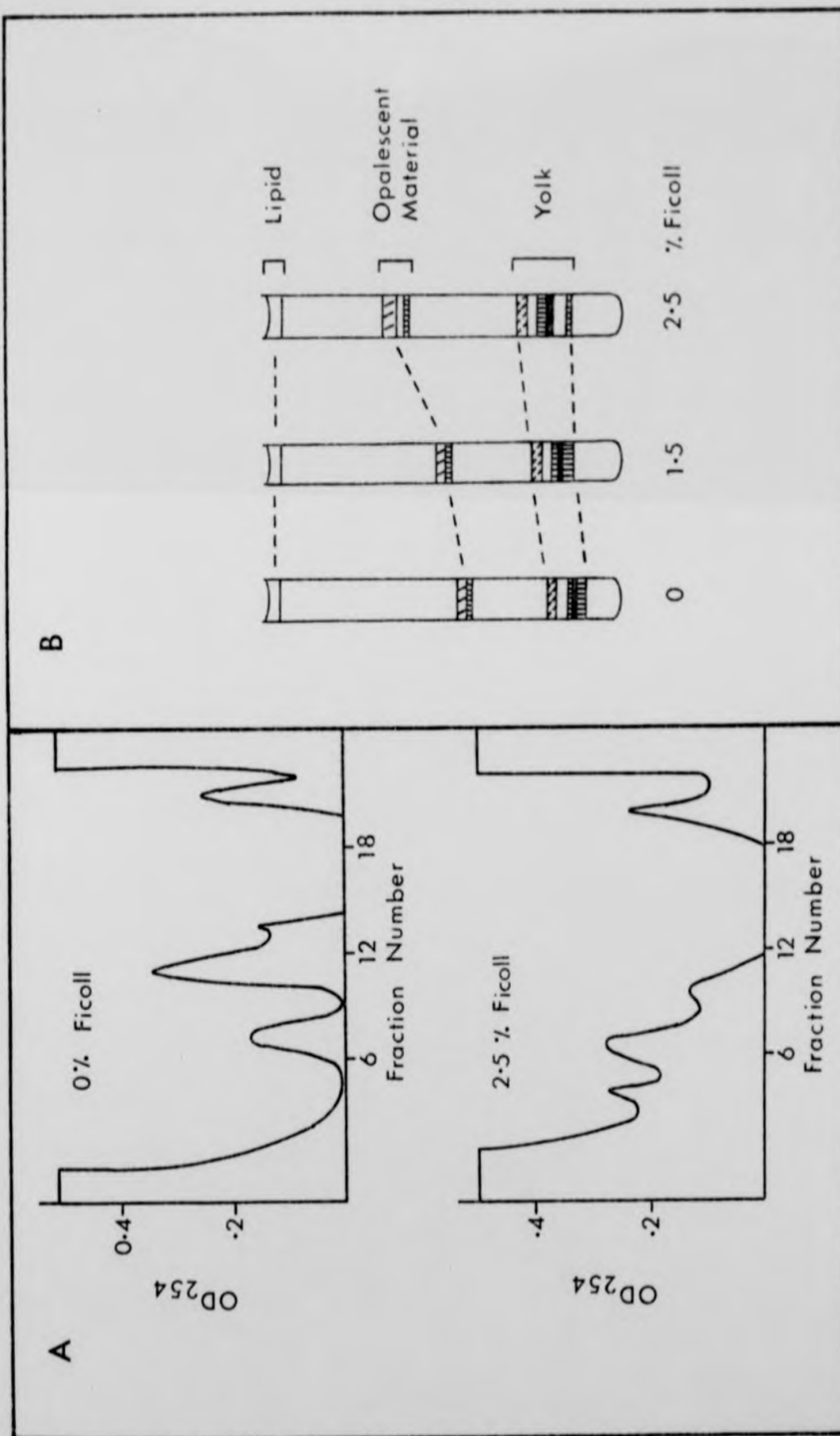
### B. Distribution of oviduct protein on Ficoll-EDTA gradients

Oocytes injected with oviduct mRNA were labelled and fractionated on 2.5% Ficoll-EDTA gradients. The distribution of ovalbumin and lysozyme on these gradients was then analysed by anti-ovalbumin and anti-lysozyme immunoprecipitation followed by gel electrophoresis (Fig. 5.8). The results show

Fig. 5.7 Visual and OD<sub>254</sub> Profiles of Ficoll-EDTA  
Gradients

Oocytes were fractionated on sucrose-EDTA gradients containing varying amounts of Ficoll. After centrifugation, visual and OD<sub>254</sub> profiles of the gradient were obtained.

- A. OD<sub>254</sub> profiles of gradients with 0% and 2.5% Ficoll.
- B. Visual profiles of gradients with 0%, 1.5% and 2.5% Ficoll.



Fraction Number 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

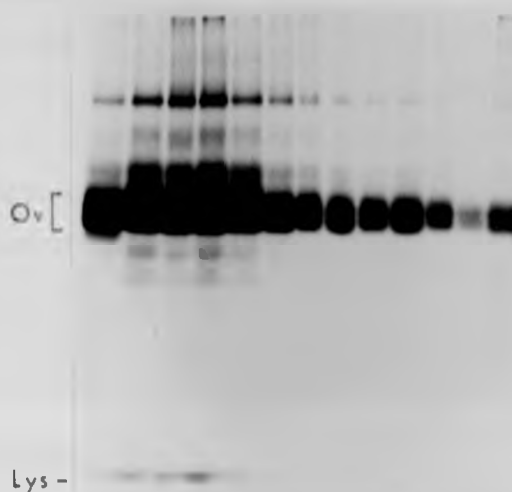


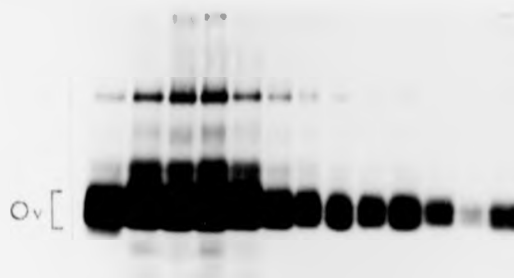
Fig. 5.8 Distribution of Ovalbumin and Lysozyme on a Ficoll-EDTA Gradient

Oocytes were injected (oviduct mRNA, 1 mg/ml) and labelled ( $[^{35}\text{S}]\text{-methionine}$ , 1 mCi/ml) for 24 h. Fractionation on a 2.5% Ficoll-EDTA gradient was followed by immunoprecipitation of samples with anti-ovalbumin and anti-lysozyme antisera. The immunoprecipitates of each sample were mixed and co-electrophoresed on a 10-15% SDS-polyacrylamide gel, which was fluorographed.

Ov = ovalbumin; Lys = lysozyme.

This gradient contained 37 injected/labelled, and 3 carrier oocytes.

Fraction Number 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17



Lys -

Fig. 5.8 Distribution of Ovalbumin and Lysozyme on a Ficoll-EDTA Gradient

Oocytes were injected (oviduct mRNA, 1 mg/ml) and labelled ( $^{35}\text{S}$ -methionine, 1 mCi/ml) for 24 h. Fractionation on a 2.5% Ficoll-EDTA gradient was followed by immunoprecipitation of samples with anti-ovalbumin and anti-lysozyme antisera. The immunoprecipitates of each sample were mixed and co-electrophoresed on a 10-15% SDS-polyacrylamide gel, which was fluorographed.

Ov = ovalbumin; Lys = lysozyme.

This gradient contained 37 injected/labelled, and 3 carrier oocytes.

Fraction Number 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

Ov[

Lys -

Fig. 5.8 Distribution of Ovalbumin and Lysozyme on a Ficol1-EDTA Gradient

Oocytes were injected (oviduct mRNA, 1 mg/ml) and labelled ( $[^{35}\text{S}]\text{-methionine}$ , 1 mCi/ml) for 24 h. Fractionation on a 2.5% Ficol1-EDTA gradient was followed by immunoprecipitation of samples with anti-ovalbumin and anti-lysozyme antisera. The immunoprecipitates of each sample were mixed and co-electrophoresed on a 10-15% SDS-polyacrylamide gel, which was fluorographed.

Ov = ovalbumin; Lys = lysozyme.

This gradient contained 37 injected/labelled, and 3 carrier oocytes.

that Ficoll does not stabilise microsomal vesicles under the conditions used here, since all the secretory protein is at the top of the gradient, where it would be if complete leakage during homogenisation had occurred. If this interpretation is correct, then this result also shows that the  $OD_{280}$  profile of a gradient gives no information as to the integrity of the microsomal vesicles.

### 3. Fractionation of Oocytes on Sucrose Gradients containing Salt

#### A. Gradient composition

The third approach to isolating intact ER and Golgi apparatus-derived vesicles was based on the methods of Zehavi-Willner and Lane (1977). They describe the isolation of such vesicles using sucrose gradients supplemented with salt to stabilise the membranes.

Four gradients were made as described. The salt conditions used were:

- A. 10 mM magnesium acetate, 50 mM NaCl
- B. 10 mM magnesium acetate, 50 mM NaCl, 100 mM KCl
- C. 50 mM NaCl, 100 mM KCl (2 gradients).

The conditions described in A are those of Zehavi-Willner and Lane (1977). The conditions described in B are a modification



of A suggested by the observation of C. D. Lane (personal communication) that this higher salt concentration may further stabilise oocyte microsomal vesicles and that NaCl and KCl have similar effects on oocyte vesicles. Moreover, Hurkman et al. (1981) state that high salt concentrations tend to solubilise the yolk and increase the resolution of oocyte subfractions on sucrose gradients. The conditions described in C were suggested by observations on the effect of various ions on microsomal vesicles reviewed by Dallner and Ernster (1968). They suggest that divalent ions can interact with smooth ER-derived vesicles to cause irreversible aggregation, which may be a problem with oocyte vesicles (see Results 1). EDTA was left out of these gradients, since a separation of smooth and rough vesicles (Golgi-derived and rough ER-derived) is desirable and EDTA is known to strip ribosomes from the ER (Sabatini et al., 1966). The effects of reducing the extent of homogenisation (reducing the number of strokes of the pestle from 15 to 5) were also monitored in this experiment.

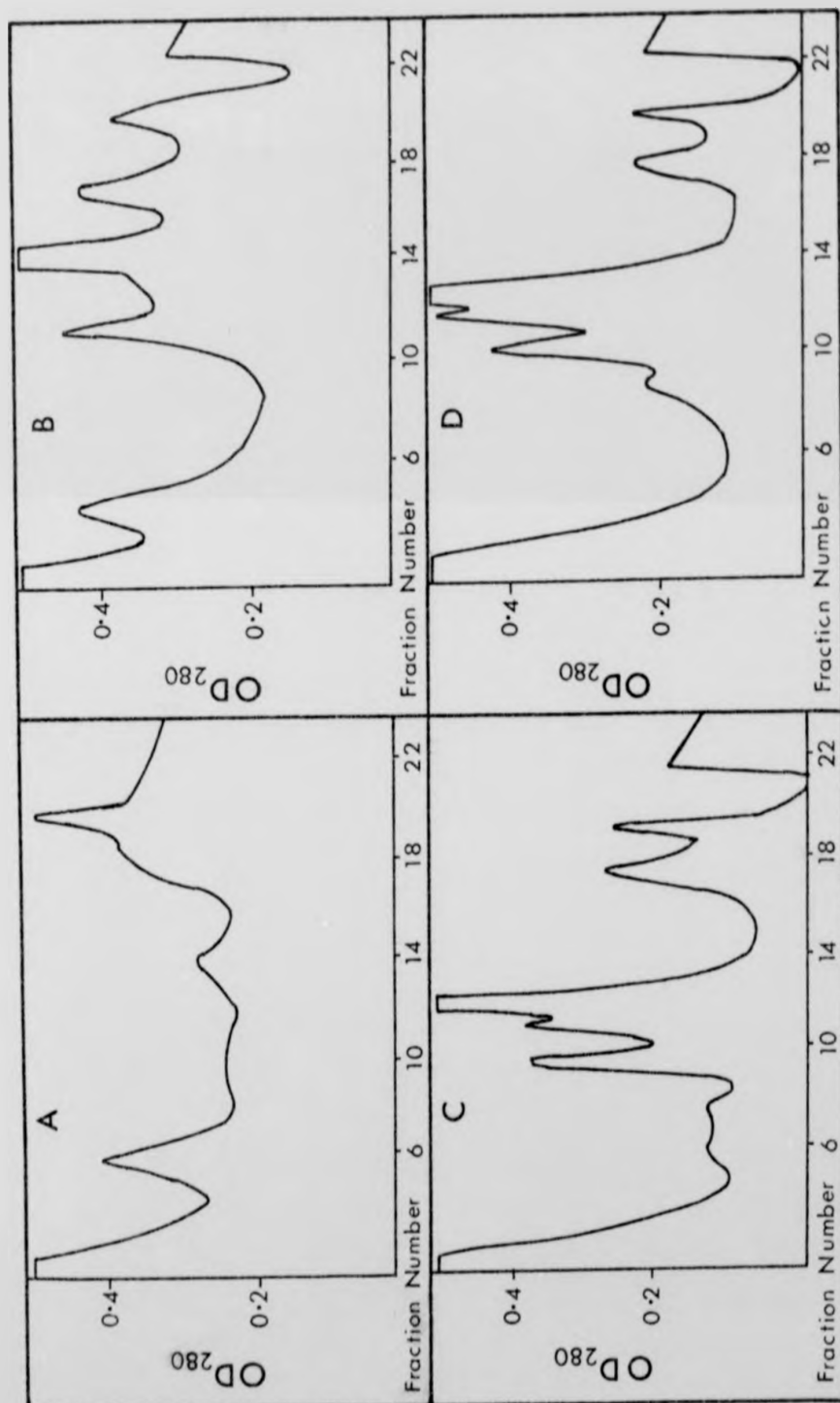
#### B. Distribution of UV absorbing material on sucrose-salt gradients

The effects of the various salt conditions on gradient OD profiles are shown in Fig. 5.9. The visual profiles are similar to those obtained with PVP-EDTA gradients. However, the higher salt concentrations used here (B and C) resulted in the band of yolk seen at the bottom of the PVP-EDTA

Fig. 5.9 OD<sub>280</sub> Profiles of Sucrose-Salt Gradients

- A. sucrose, 10 mM magnesium acetate, 50 mM NaCl.
- B. sucrose, 10 mM magnesium acetate, 50 mM NaCl,  
100 mM KCl.
- C. sucrose, 50 mM NaCl, 100 mM KCl.
- D. sucrose, 50 mM NaCl, 100 mM KCl.

All gradients contain 40 oocytes. Homogenisation of oocytes for A, B, C was by 5 strokes of the loose-fitting pestle in a Dounce homogeniser. Homogenisation of oocytes for D was by 15 strokes of the pestle.



gradients disappearing - presumably solubilised (see Hurkman et al., 1981). The OD profiles shown (Fig. 5.9) indicate that changing the salt conditions has a marked effect on the distribution of cellular components on these gradients. It is clear that the OD profile is unchanged by altering the extent of homogenisation (compare 5.9c and 5.9d) and that the conditions described in C give the best resolution in the area of interest - the middle of the gradient.

#### C. The distribution of oviduct proteins on sucrose-salt gradients

The distribution of ovalbumin was assayed here as before (see Fig. 5.5) by immunoprecipitation followed by counting of immunoprecipitates in a Triton X-100/toluene based scintillant (Fig. 5.10). However, unlike previous experiments where there was an extremely good correlation between results obtained by this method and by gel electrophoresis of immunoprecipitates (compare Fig. 5.4 with 5.5b), results in this and subsequent experiments obtained by direct counting of immunoprecipitates were less satisfactory. Thus this method, while quicker and more convenient than gel analysis, could only be used to monitor ovalbumin and not lysozyme distribution since the background contributed by non-specific immunoprecipitation is higher in lysozyme immunoprecipitations and the distribution of lysozyme can be obscured (see Fig. 7.1). Even with ovalbumin immunoprecipitates counted in this way, background in samples taken from the top and the bottom of

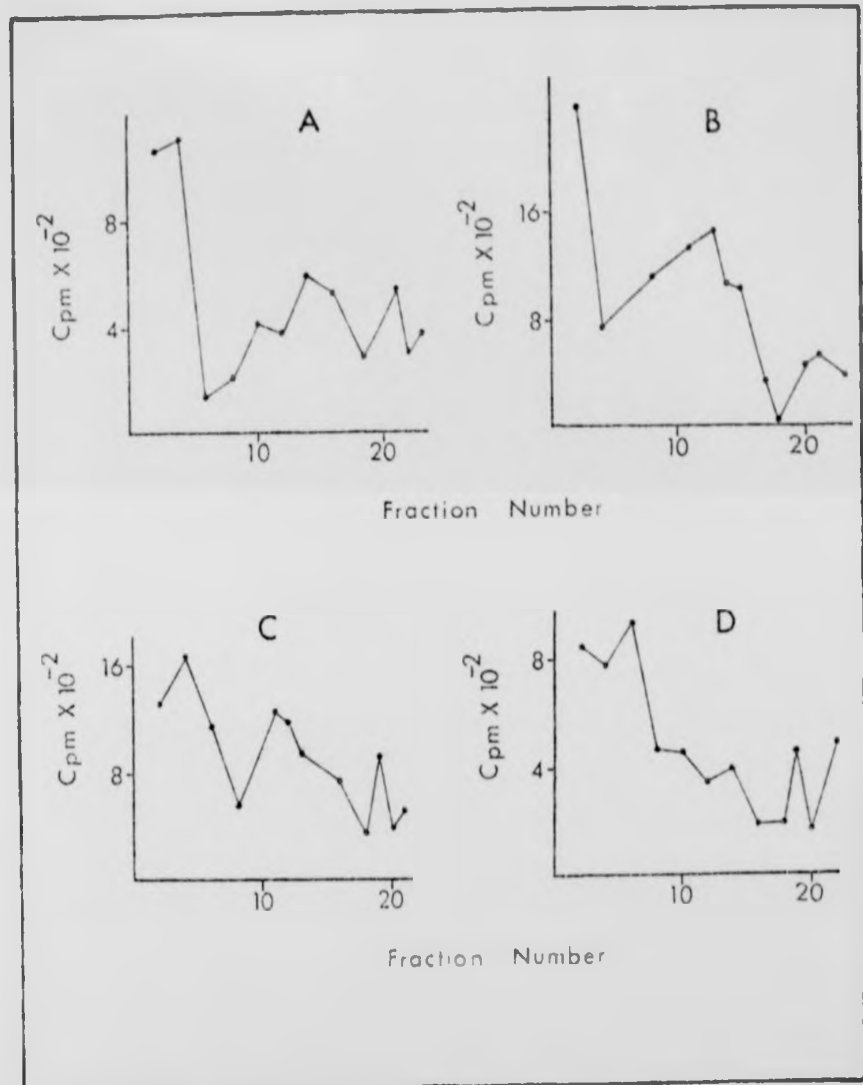


Fig. 5.10 Distribution of Ovalbumin on Sucrose-Salt Gradients

Oocytes were injected (oviduct mRNA, 1 mg/ml), labelled ( $[^{35}\text{S}]$ -methionine, 1 mCi/ml) for 24 h and fractionated on various sucrose-salt gradients. Samples were immunoprecipitated with anti-ovalbumin antiserum and immunoprecipitates directly counted. Results are not quantitatively comparable, B and C were counted on a different machine to A and D. A, B, C and D are as described (Fig. 5.9, legend). All gradients contained 7 injected/labelled, and 33 carrier oocytes.

gradients is often high (see Fig. 7.1) and results must therefore be treated with caution. Since the methodology was not changed from that employed earlier, this rise in background may be due to changes in the oocytes. When the distribution of ovalbumin on these gradients (Fig. 5.10) is considered, four conclusions may be drawn:

1. The methods of Zehavi-Willner and Lane (1977) give rise to an ovalbumin distribution similar to that obtained with PVP-EDTA gradients, but with a broader 'microsomal' peak.
2. The addition of 100 mM KCl does not appear to stabilise the vesicles, but does reduce the bottom 'aggregate' peak.
3. Leaving out the magnesium acetate may stabilise the vesicles and may also increase resolution in the middle of the gradient as judged by the OD<sub>280</sub> profile and the ovalbumin distribution.
4. Reducing the number of pestle-strokes from 15 to 5 increases the fraction of ovalbumin present in the microsome-containing portion of the gradient (compare Fig. 5.10 c and d). Since the OD<sub>280</sub> profiles of these two gradients were very similar (compare Fig. 5.9 c and d), this would imply that 5 strokes of the pestle are sufficient for fractionation, and that further homogenisation just breaks up the vesicles. It was decided to continue experiments with salt conditions C and to use 5 pestle strokes in homogenisation.

The distribution of ovalbumin and lysozyme on high-salt (salt conditions C will be referred to as 'high salt') gradients was analysed by gel electrophoresis and quantitation from these gels (Fig. 5.11). These data show that the distribution of ovalbumin is much broader over high-salt gradients than over the previously used PVP-EDTA gradients. Lysozyme, although equally widely distributed, is concentrated further up the gradient. The pattern of distribution of ovalbumin and lysozyme on high-salt gradients may reflect increased leakage of secretory protein from microsomal vesicles, lysozyme leaking faster than ovalbumin - as seen with PVP-EDTA gradients.

#### D. Distribution of non-secretory mouse immunoglobulin on high-salt gradients

The distribution of a non-secretory immunoglobulin on high-salt gradients was analysed. Oocytes were injected with mRNA encoding mouse MOPC 43 lamda light chain (Valle *et al.*, in press; mRNA a kind gift of G. Valle) and labelled. MOPC 43 has a secretory phenotype similar to that of NS1 (NS1 mRNA was not available for this experiment). An analysis of the result of fractionation of these oocytes (Fig. 5.11) on high-salt gradients shows that MOPC 43 lamda light chain has the same distribution as ovalbumin on these gradients.

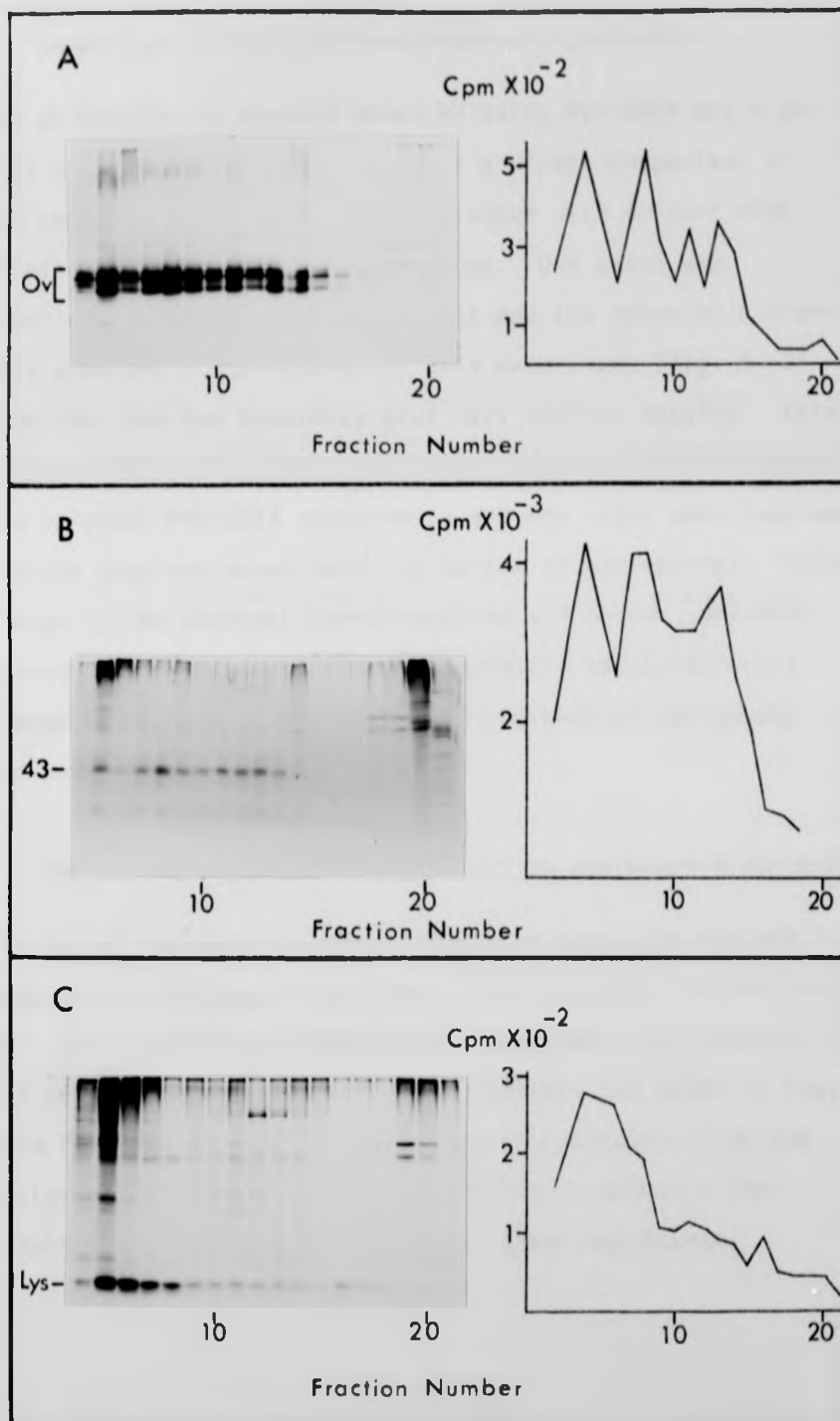
Fig. 5.11 Distribution of Ovalbumin, Lysozyme and  
MOPC 43 on a High-Salt Gradient

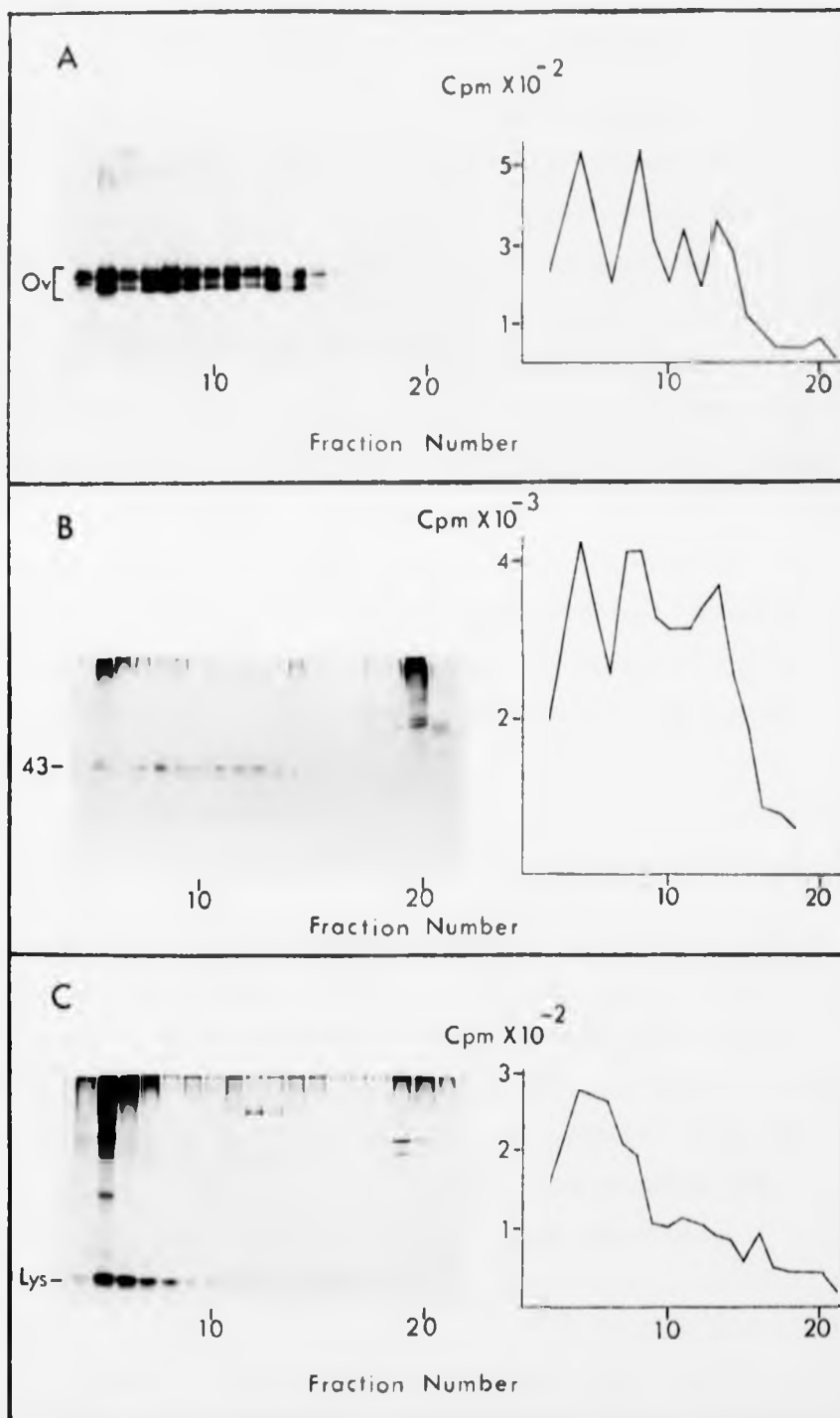
Oocytes were injected with oviduct mRNA (1 mg/ml) or MOPC 43 mRNA (0.5 mg/ml), and labelled ( $r^{35}\text{S}$ -methionine, 4 mCi/ml) for 24 h. Both groups of oocytes were fractionated on one high-salt gradient (50 mM NaCl, 100 mM KCl). Samples were collected, immunoprecipitated with anti-ovalbumin, anti-lysozyme or anti-mouse IgG antiserum, followed by electrophoresis on 15% SDS-polyacrylamide gels.

- A. Fluorograph and quantitation by excision from gel of anti-ovalbumin immunoprecipitates. Ov = ovalbumin.
- B. Fluorograph and quantitation by excision from gel of anti IgG immunoprecipitates. 43 = MOPC 43 light chain.
- C. Fluorograph and quantitation by excision from gel of anti-lysozyme immunoprecipitates. Lys = lysozyme.

The gradient contained 8 oviduct mRNA injected, 10 MOPC 43 mRNA injected and 22 uninjected oocytes.







#### E. Comparison of PVP-EDTA and high-salt gradients

The difference in results obtained using PVP-EDTA and high-salt gradients was investigated by a direct comparison of the two methods. Oocytes were injected with oviduct mRNA, labelled and divided into two groups. One group was fractionated on a PVP-EDTA gradient and the other on a high-salt gradient. The results of this experiment (Fig. 5.12) show that the two gradients give very similar results. This implies that the oocytes had changed during the time between the original PVP-EDTA experiments and the later salt-supplemented sucrose gradient experiments (a matter of six months). This change may be seasonal (Lane, Woodland and Colman, personal communications). The simplest hypothesis to explain this change is that the intracellular membranes of the oocyte had become more fragile.

#### F. The effect of a PVP-salt gradient on ovalbumin distribution

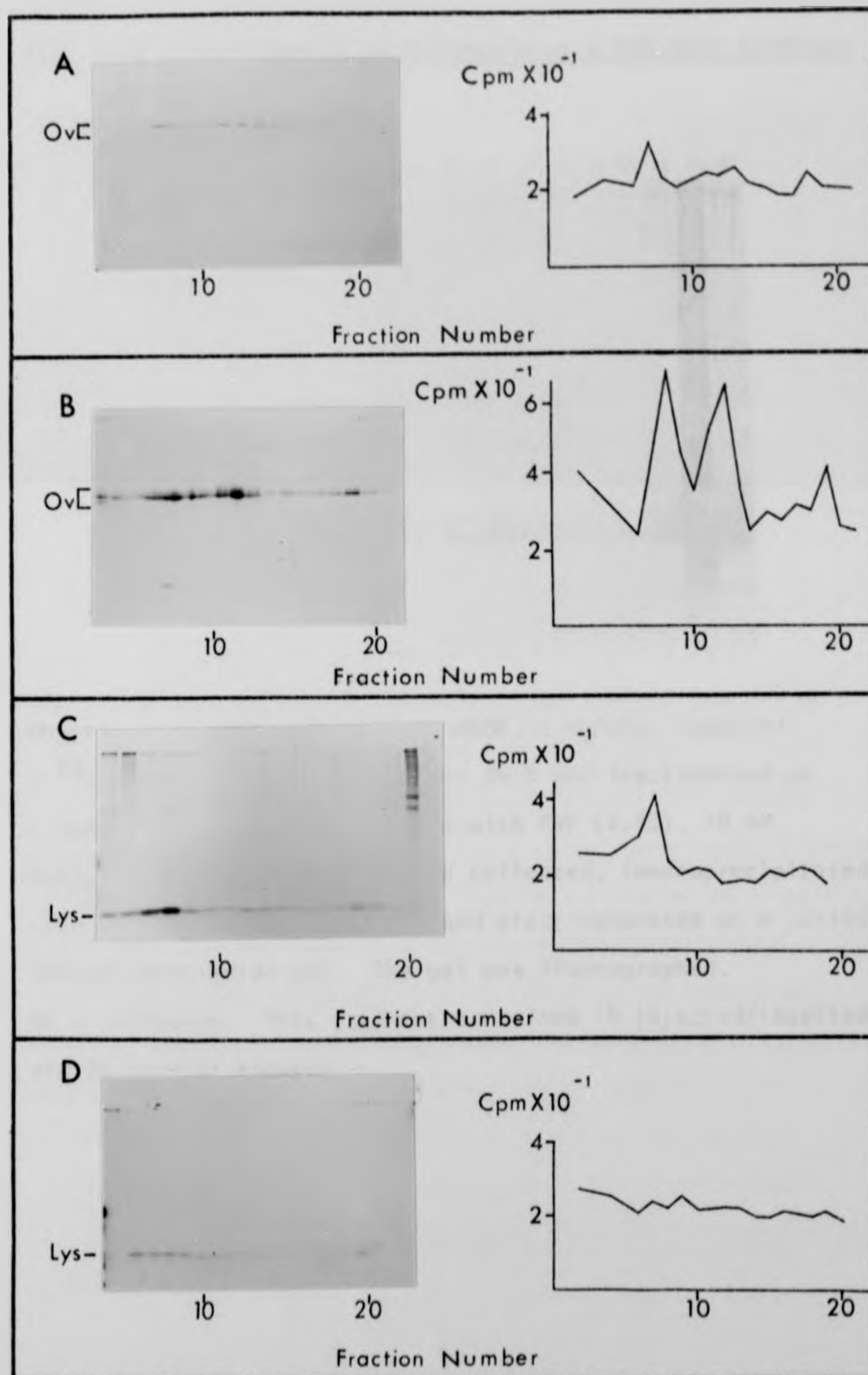
In view of the partial effectiveness of both salt and PVP in stabilising microsomal vesicles, the combination of PVP plus salt was tried in an attempt to stop leakage from vesicles. The results obtained using these gradients are shown in Fig. 5.13. This result indicates that the bulk of ovalbumin is at the bottom of the gradient. This pelleting is probably the result of vesicle aggregation under these conditions.

Fig. 5.12 Comparison of Ovalbumin and Lysozyme  
distribution on High-Salt and PVP-EDTA  
Gradients

Oocytes were injected (oviduct mRNA, 1 mg/ml), labelled ( $r^{35}\text{S}$ -methionine, 4 mCi/ml) for 24 h, and fractionated on a PVP-EDTA gradient or a high-salt gradient. Samples were collected, immunoprecipitated with anti-ovalbumin or anti-lysozyme antiserum and electrophoresed on 15% SDS-polyacrylamide gels.

- A. Autoradiograph and quantitation by excision from gel of anti-ovalbumin immunoprecipitates from high-salt gradient.
- B. Autoradiograph and quantitation by excision from gel of anti-ovalbumin immunoprecipitates from PVP-EDTA gradient.
- C. Autoradiograph and quantitation by excision from gel of anti-lysozyme immunoprecipitates from high-salt gradient.
- D. Autoradiograph and quantitation by excision from gel of anti-lysozyme immunoprecipitates from PVP-EDTA gradients.

Ov = ovalbumin; Lys = lysozyme. Both gradients contained 8 injected/labelled oocytes and 32 carrier oocytes.



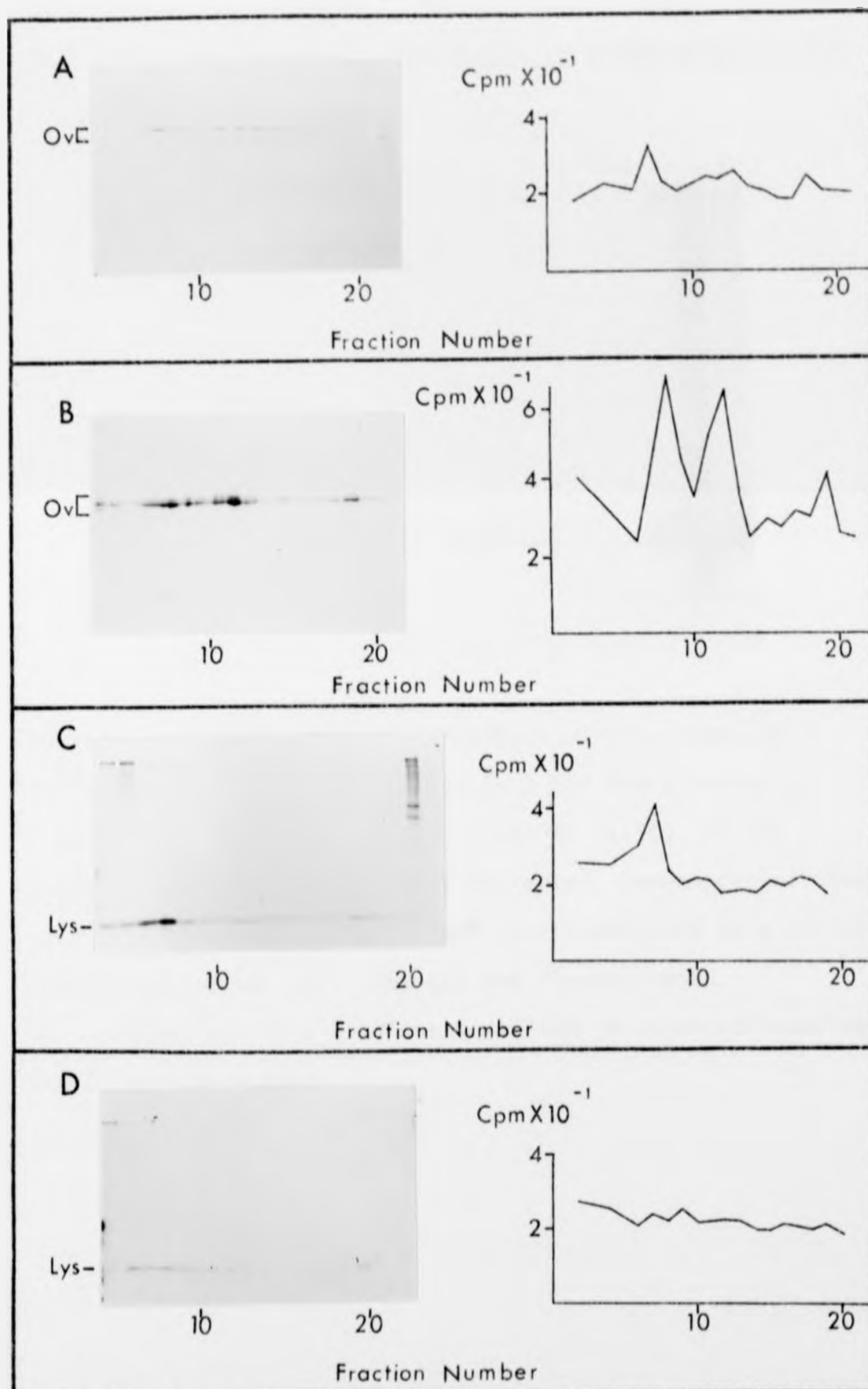
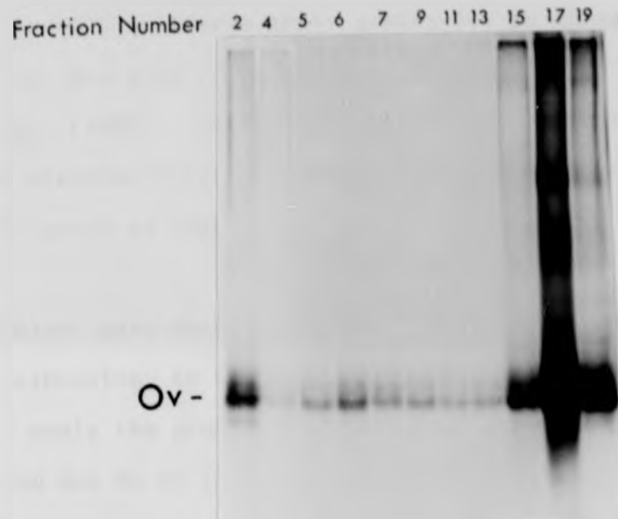
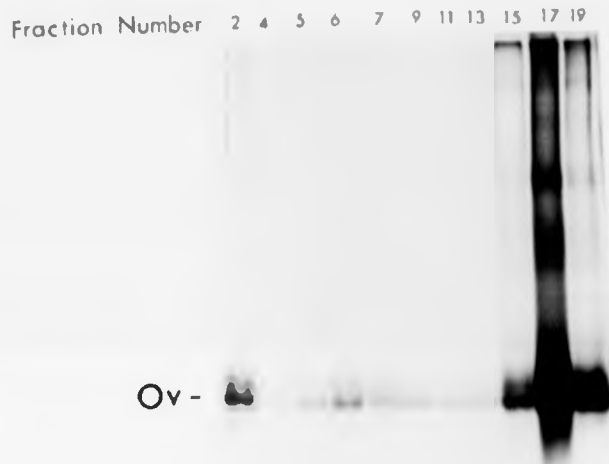


Fig. 5.13 Distribution of Ovalbumin on a PVP-Salt Gradient



Oocytes were injected (oviduct mRNA, 1 mg/ml), labelled ( $[^{35}\text{S}]$ -methionine, 4 mCi/ml) for 24 h and fractionated on a sucrose gradient supplemented with PVP (2.5%), 10 mM MgAc, 50 mM NaCl. Samples were collected, immunoprecipitated with anti-ovalbumin antiserum and electrophoresed on a 10-15% SDS-polyacrylamide gel. The gel was fluorographed. Ov = ovalbumin. This gradient contained 14 injected/labelled, and 26 carrier oocytes.

Fig. 5.13 Distribution of Ovalbumin on a PVP-Salt Gradient



Oocytes were injected (oviduct mRNA, 1 mg/ml), labelled ( $^{35}\text{S}$ -methionine, 4 mCi/ml) for 24 h and fractionated on a sucrose gradient supplemented with PVP (2.5%), 10 mM MgAc, 50 mM NaCl. Samples were collected, immunoprecipitated with anti-ovalbumin antiserum and electrophoresed on a 10-15% SDS-polyacrylamide gel. The gel was fluorographed. Ov = ovalbumin. This gradient contained 14 injected/labelled, and 26 carrier oocytes.



#### G. Electron microscopy

Electron microscopy was used to confirm the content of high-salt gradients and to assess their similarity to those of Zehavi-Willner and Lane (1977), Hurkman et al. (1981) and Opresko et al. (1980). The method used to prepare gradient samples for electron microscopy was a modification (after repeated failures) of that described in Opresko et al. (op cit).

Several problems were encountered in attempting to apply published methodology to these gradient samples. The first attempts to apply the protocol described by Opresko et al. (1980) failed due to an inability to obtain a firm pellet of material from the sample. These authors pellet gradient fractions in the presence of glutaraldehyde and this gives rise to an extremely friable pellet, which gradually breaks down during all subsequent manipulations until it becomes unmanageable. To counteract this, attempts were made to embed the fixed, pelleted sample into agarose, in order to preserve the pellets' integrity. When blocks of agarose containing the fixed sample were processed for microscopy, the sample was found to be fragmentary and diluted, making analysis impracticable. The fragmentary nature of samples prepared in this way may be due to the need to use molten agarose (45°C) during the embedding of samples. The final method, (see Methods, this chapter) involving fixation after pelleting, avoided these problems.

The results obtained using electron microscopy are shown in Fig. 5.14. These results are similar to those presented by other investigators (Zehavi-Willner and Lane, 1977; Hurkman et al., 1981; Opresko et al., 1980). However, like Zehavi-Willner and Lane, and unlike Opresko et al. and Hurkman et al., the microsomal and mitochondrial fractions have not been separated on high-salt gradients. Thus membranous material (presumably derived primarily from the ER and Golgi apparatus) is found in a broad peak in the middle of the gradient, along with mitochondria, and yolk platelets are found at the bottom of the gradient. In addition, some membranous material is found at the same density as yolk. This may represent aggregated vesicles. The wide distribution shown by exogenous secretory proteins on high-salt gradients does not appear to correspond to the distribution of the microsomal fraction.

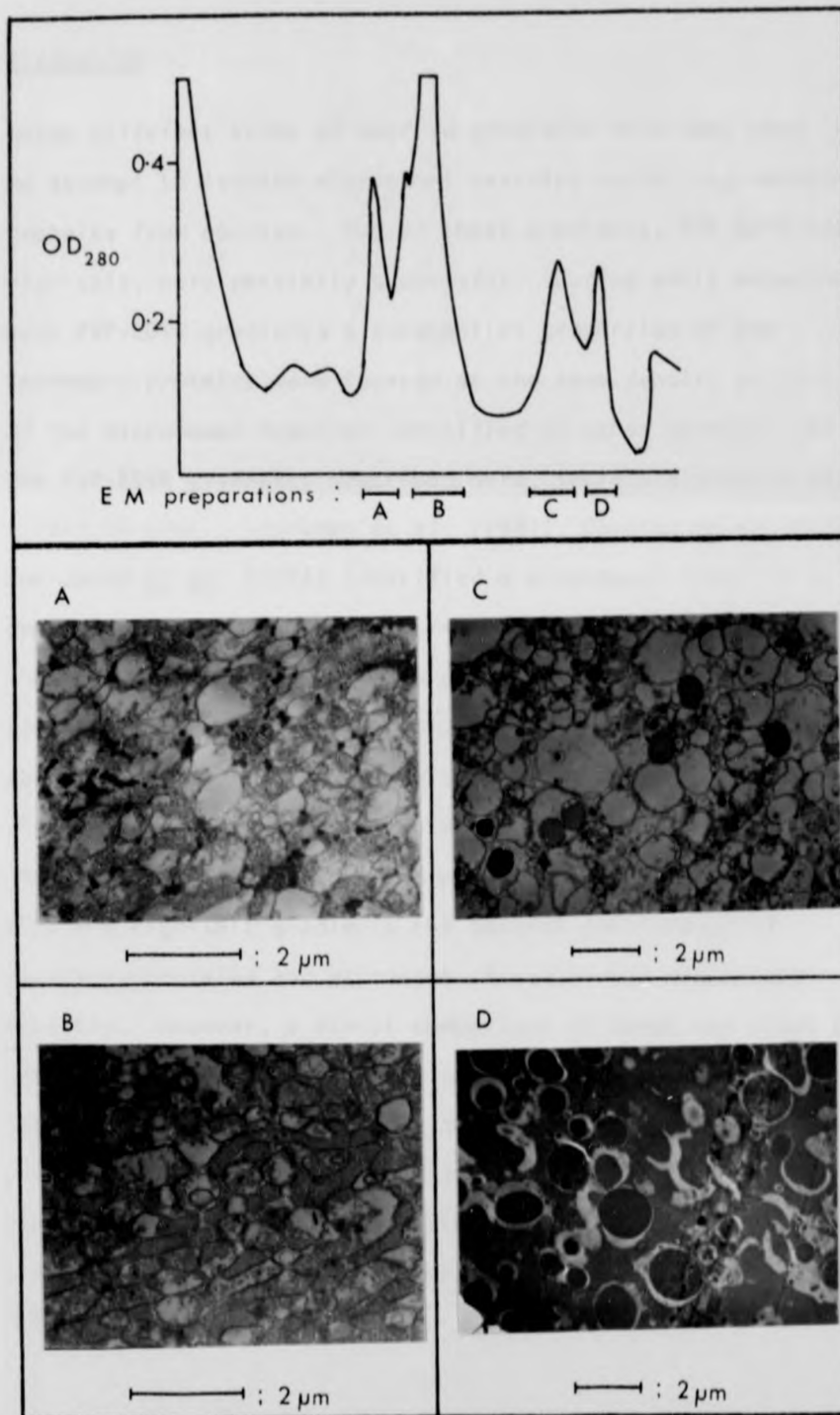
Fig. 5.14 Electron Microscopy of Samples from High-Salt Gradients

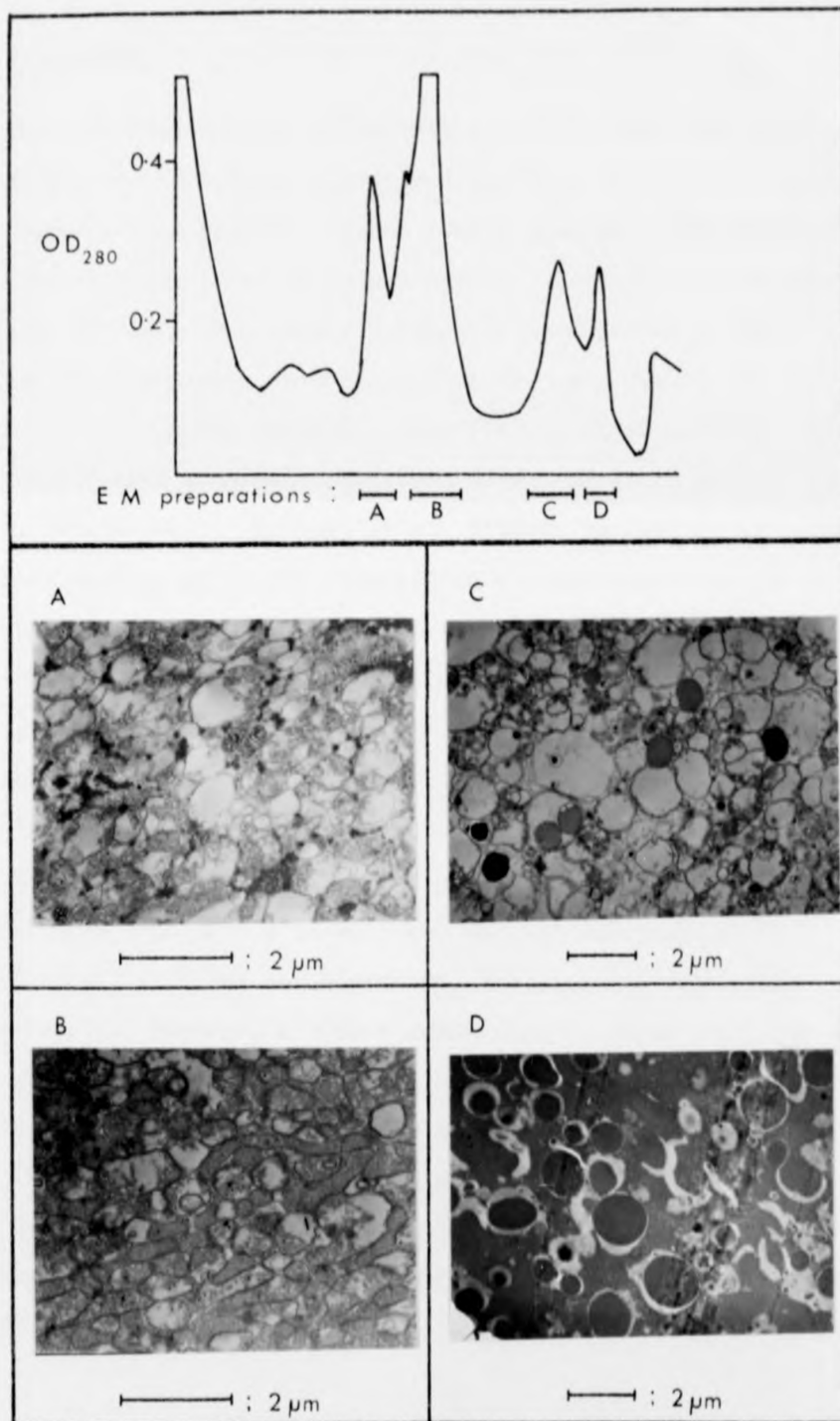
Oocytes were fractionated on four high-salt gradients (40 oocytes/gradient ), and samples from four regions (A, B, C, D) pooled and prepared for electron microscopy (Methods, this chapter).

The figure shows the OD profile of one of these gradients with the regions from which A, B, C and D were collected, and photographs of samples of each region.

- A. Photograph of sample prepared from region A showing membranous vesicles and mitochondria.
- B. Photograph of sample prepared from region B showing mitochondria and membranous vesicles.
- C. Photograph of sample prepared from region C showing membranous material and yolk platelets.
- D. Photograph of sample prepared from region D showing yolk platelets and proteinaceous background.

Samples prepared from regions above A or below D show amorphous material only.





### Discussion

Three different kinds of sucrose gradients have been used in an attempt to isolate microsomal vesicles containing secretory proteins from oocytes. Two of these gradients, PVP-EDTA and high-salt, were partially successful. During early experiments with PVP-EDTA gradients a substantial proportion of the secretory proteins were located at the same density as that of the microsomal fraction identified by other workers. On the PVP-EDTA gradients described here, secretory protein bands at 1.12-1.14 g/ml. Hurkman et al. (1981), Opresko et al. (1980) and Jared et al. (1973) identified a microsomal fraction at densities of 1.13 g/ml, 1.12 g/ml and 1.125 g/ml respectively. The similarity in distribution of oocyte subfractions on the various sucrose gradients described in the studies referred to above, indicates that the results obtained by electron microscopy from high-salt gradients are probably similar to those that would be obtained from PVP-EDTA or Ficoll gradients. With the high-salt gradients the partial coincidence of secretory proteins and microsomal fraction was determined directly. However, a direct comparison of these two kinds of gradients performed six months after the initial PVP-EDTA experiments revealed a similar distribution of secretory protein, and therefore the analysis of these gradients was continued using the more convenient low viscosity high-salt gradients. The pattern of distribution of secretory proteins will be discussed in chapter 6.

## Chapter 6

Further analysis of the distribution of  
ovalbumin and lysozyme on sucrose gradients

### Introduction

In the previous chapter, data have been presented showing the distribution of ovalbumin, lysozyme and non-secretory immunoglobulin on various sucrose gradients. Using electron microscopy, the distribution of cellular components on high-salt gradients was also determined. The two sets of results do not match up: the distribution of ovalbumin, lysozyme and immunoglobulin does not correspond with the distribution of microsomal vesicles on these gradients.

This anomaly raises several questions: for example, does the finding of secretory protein in the microsomal portion of the gradient reflect adventitious adsorption to this subcellular fraction? The fact that the miscompartmentalised form of ovalbumin is confined to the top of PVP-EDTA gradients (see Fig. 5.4a) would argue against this. In this chapter an experiment to investigate the adsorptive properties of lysozyme is described.

A second question is whether the secretory protein that is not coincident with the microsomal fraction is present inside a small group of vesicles (so rare as to be missed by electron microscopic analysis) with a large range of densities, or whether the distribution of this protein reflects leakage from, or breakdown of, vesicles. These latter possibilities, referred to jointly as 'leakage', have been advocated in the previous chapter, and since it is extremely difficult to



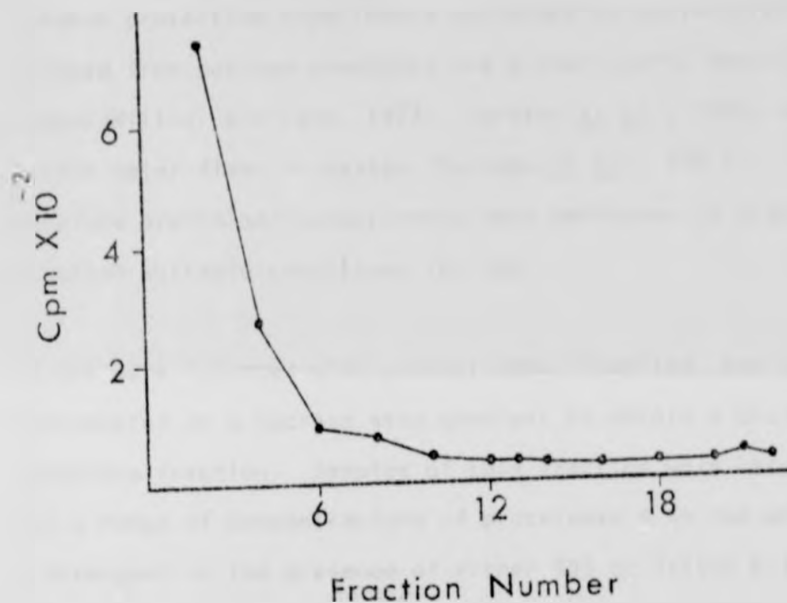
distinguish between them, this term will continue to be used to describe both. The extent of leakage can be assessed by employing a protease protection assay to detect extra-vesicular secretory protein.

### Results

To discover whether lysozyme leaked during homogenisation could be carried into the microsomal region of the gradient by adsorption, radiolabelled, secreted oviduct proteins were collected and homogenised in the presence of 40 unlabelled oocytes. This mixture was left for 5 min at 4°C to facilitate any adsorption that might be occurring and then fractionated on a standard high-salt gradient. The distribution of lysozyme over the gradient was analysed by direct counting of immunoprecipitates - accurate in this case (cf. chapter 5, section 3.C) because of the lack of any radiolabelled oocyte proteins. The result (Fig. 6.1) shows that the lysozyme is confined to the top of the gradient and that any ovalbumin or lysozyme found further down the gradient must have reached its position inside a microsomal vesicle.

Having cleared up the problem of adsorption, a direct test of the extent of leakage on high-salt gradients was set up. Protease protection experiments were employed. These involve incubating gradient samples with a protease in the presence or absence of a detergent. If the secretory proteins are digested only in the presence of detergent, then it is assumed that the detergent has broken down the microsomal membranes and allowed access of protease to secretory proteins. This result (100% protection) would imply that there was no leaked secretory protein present in that sample (Sabatini and Blobel, 1970; Blobel and Dobberstein, 1975b). This assay has been

Fig. 6.1 The Distribution of Secreted Lysozyme on a High-Salt Gradient



Oocytes were injected (oviduct mRNA, 1 mg/ml) and labelled ( $[^{35}\text{S}]$ -methionine, 4 mCi/ml) for 24 h. Incubation medium surrounding 10 healthy oocytes (60  $\mu\text{l}$ ) was collected. This sample was made 5 mM HEPES (pH 7.5), 50 mM NaCl, 100 mM KCl (final volume 100  $\mu\text{l}$ ) and added to 900  $\mu\text{l}$  high-salt homogenisation buffer. Forty oocytes were homogenised into this mixture, which was left at 4°C for 5 min before fractionation on a high-salt gradient. Samples were collected, immunoprecipitated with anti-lysozyme antiserum and directly counted. ●—● = lysozyme.

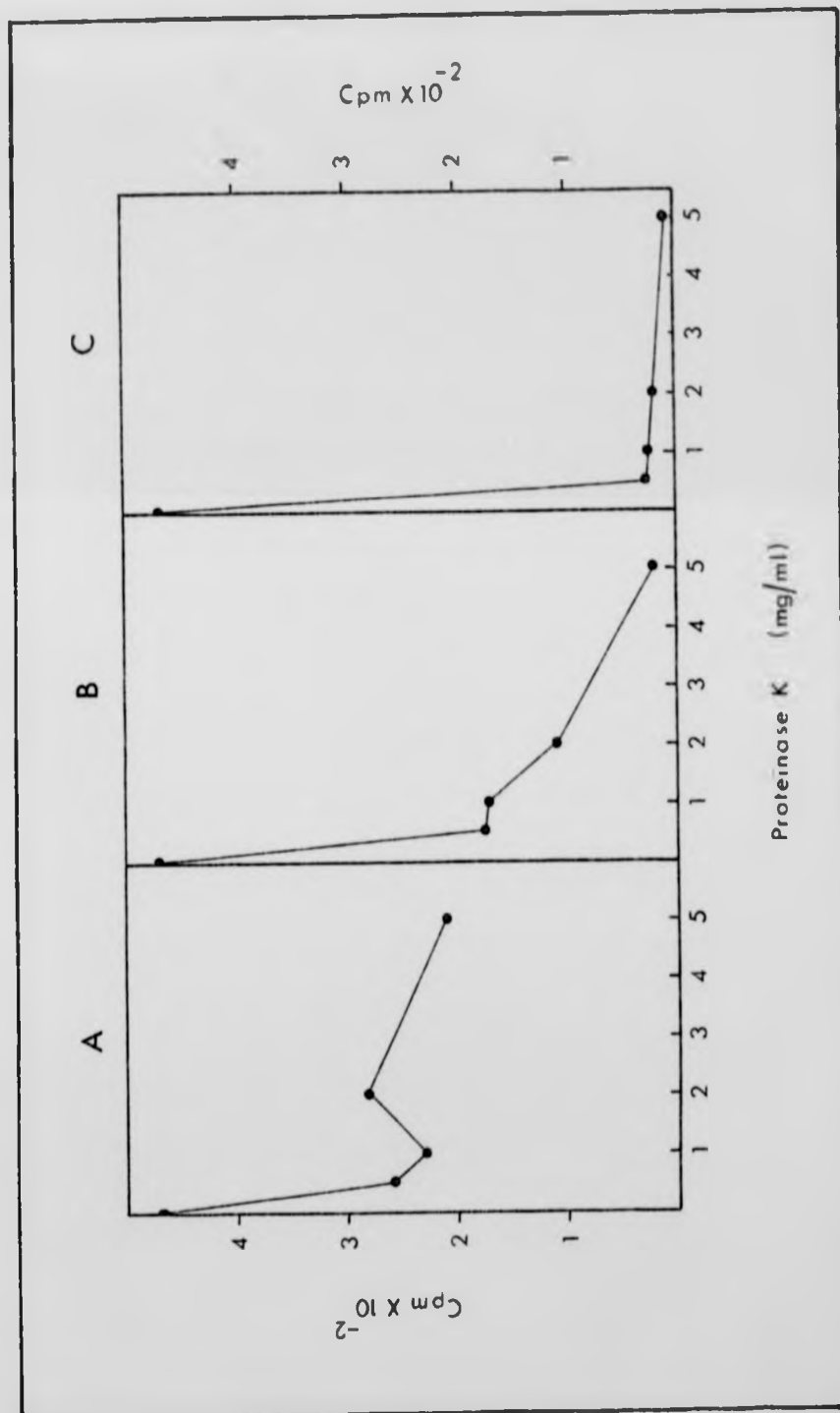
an integral part of in vitro translocation systems (see Blobel and Doberstein, 1975b; Braell and Lodish, 1982). Protease protection experiments performed on oocyte microsomes isolated from sucrose gradients are either poorly described (Zehavi-Willner and Lane, 1977; Opresko et al., 1980) or contain major flaws in design (Hurkman et al., 1981). Therefore preliminary experiments were performed in order to establish suitable conditions for use.

Oocytes were injected with oviduct mRNA, labelled, and then fractionated on a sucrose step gradient to obtain a crude membranous fraction. Samples of this fraction were incubated with a range of concentrations of proteinase K in the absence of detergent or the presence of either SDS or Triton X-100. Concentrations of proteinase K used are higher than those published (see Braell and Lodish, 1982), but the enzyme available was known to be two years old and to have been repeatedly frozen and thawed. The effects of these incubations on ovalbumin present in this vesicle fraction is shown in Fig. 6.2. In the absence of detergent, approximately 50% of the ovalbumin was protected from proteinase K. In the presence of 0.1% Triton X-100, complete digestion of ovalbumin was obtained only at the highest concentration of protease. In the presence of 0.1% SDS, complete digestion was obtained at all protease concentrations. These results imply that 50% of the ovalbumin present in these reactions was sequestered inside microsomal vesicles and that 0.1% SDS breaks down the

Fig. 6.2 Protease Digestion of Microsomal Ovalbumin

Ten oocytes were injected (oviduct mRNA, 1 mg/ml), labelled ( $[^{35}\text{S}]$ -methionine, 1 mCi/ml) and fractionated on a sucrose step gradient (Methods, chapter 2). The microsomal fraction (350  $\mu\text{l}$ ) was collected and diluted with 3 volumes of 20 mM Tris-HCl (pH 7.5), 100 mM KCl, 50 mM NaCl, 10% sucrose (w/v). Samples (100  $\mu\text{l}$ ) were incubated with various concentrations of proteinase K in the absence of detergent or the presence of either 0.1% SDS or 0.1% Triton X-100 at 0°C for 90 min. Reactions were terminated by the addition of PMSF to 3 mM and freezing. Samples were immunoprecipitated with anti-ovalbumin antiserum and electrophoresed on a 15% SDS-polyacrylamide gel. Regions corresponding to the position of ovalbumin were cut from the gel, counted and results plotted against protease concentration.

- A. No Detergent.
- B. 0.1% Triton X-100.
- C. 0.1% SDS.



vesicles much more effectively than does 0.1% Triton X-100.

On the basis of these results, and the knowledge that varying concentrations of sucrose and membranes would be present in gradient samples, 0.1% SDS and 1 mg/ml proteinase K were chosen as conditions with which to test gradient samples. Oocytes were injected, labelled and fractionated on a high-salt gradient. This gradient was standard except that PMSF was omitted to avoid inhibition of proteinase K. The results (Fig. 6.3) show that compared to undigested controls, ovalbumin is not completely digested by proteinase K in the absence of SDS in any part of the gradient. In the presence of SDS, complete digestion occurred in each sample (data not shown). This result implies that vesicles containing ovalbumin are scattered over the entire gradient. However, one further control was performed:  $^{35}\text{S}$ -methionine-labelled secreted oviduct proteins (30  $\mu\text{l}$ ) were mixed with 60  $\mu\text{l}$  of HEPES-buffered sucrose (18% w/v), made 100 mM KCl, 50 mM NaCl and then incubated with proteinase K (1 mg/ml) at  $0^\circ\text{C}$  for 90 min. This digestion was designed to check that ovalbumin was fully susceptible to proteinase K in the absence of detergent or membranes. However, under these conditions, digestion was not completed; 22% of the ovalbumin remained intact after the incubation. This result indicates that ovalbumin is more susceptible to proteinase K in the presence of SDS than in its absence. This may be due to denaturation of ovalbumin by this detergent. Moreover, because non-sequestered ovalbumin

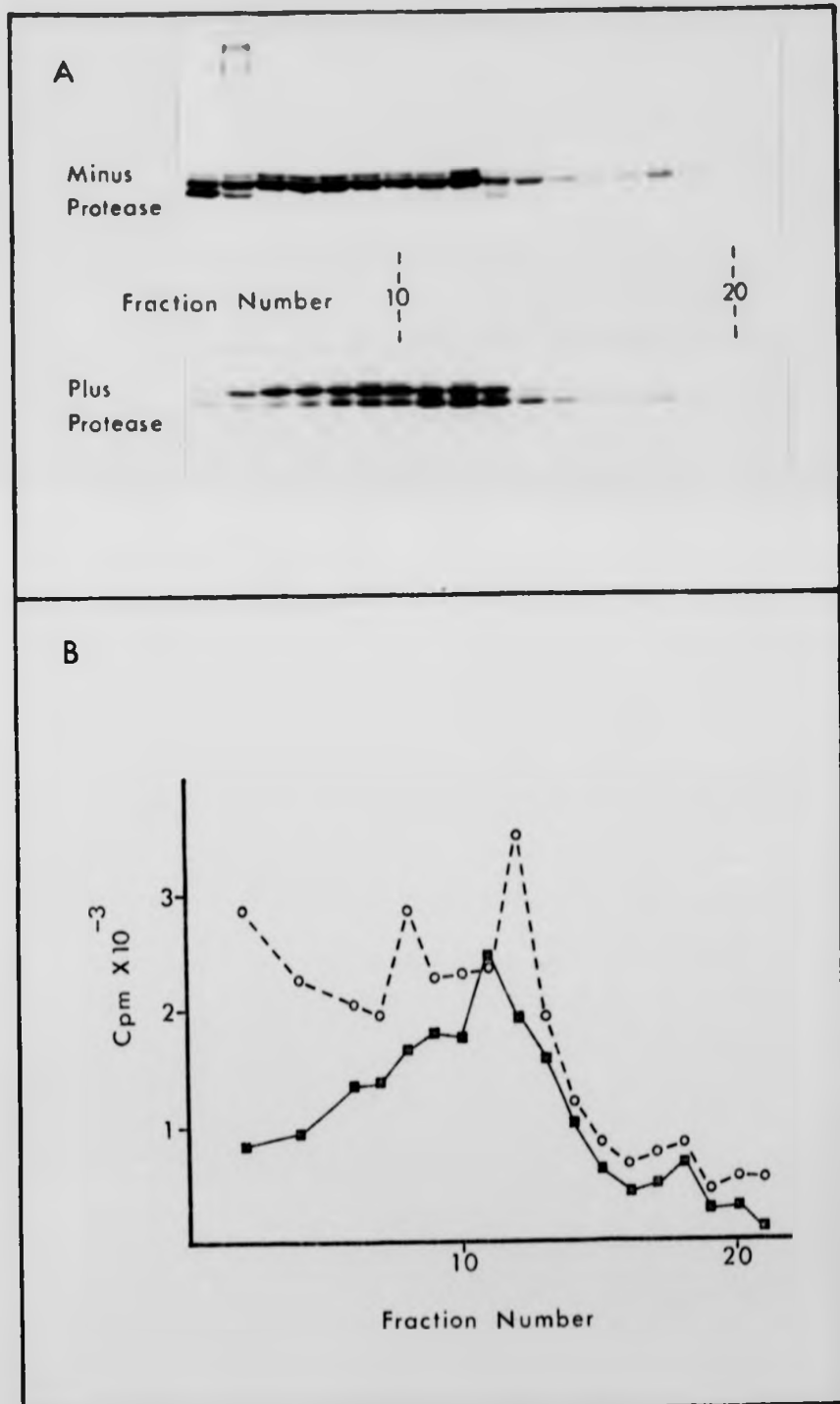
Fig. 6.3 Distribution of Protease-Susceptible Ovalbumin on High-Salt Gradients

Forty injected (oviduct mRNA, 1 mg/ml) oocytes were labelled ( $[^{35}\text{S}]$ -methionine, 4 mCi/ml) for 24 h and fractionated on a high-salt gradient. Samples (30  $\mu\text{l}$ ) were incubated with 60  $\mu\text{l}$  of a buffered sucrose-salt solution (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 50 mM NaCl, 7.5% sucrose) and 10  $\mu\text{l}$  of proteinase K (10 mg/ml) for 90 min at  $0^\circ\text{C}$  in the presence or absence of 0.1% SDS. Reactions were terminated by the addition of PMSF to 3 mM. Samples were immunoprecipitated with anti-ovalbumin anti-serum and electrophoresed on 15% SDS-polyacrylamide gels.

- A. Fluorographs showing anti-ovalbumin immunoprecipitates from proteinase digestions plus and minus protease.
- B. Relevant regions of the gels shown in A were excised and counted.

o---o, minus protease; ■—■ plus protease.





A

Minus  
Protease

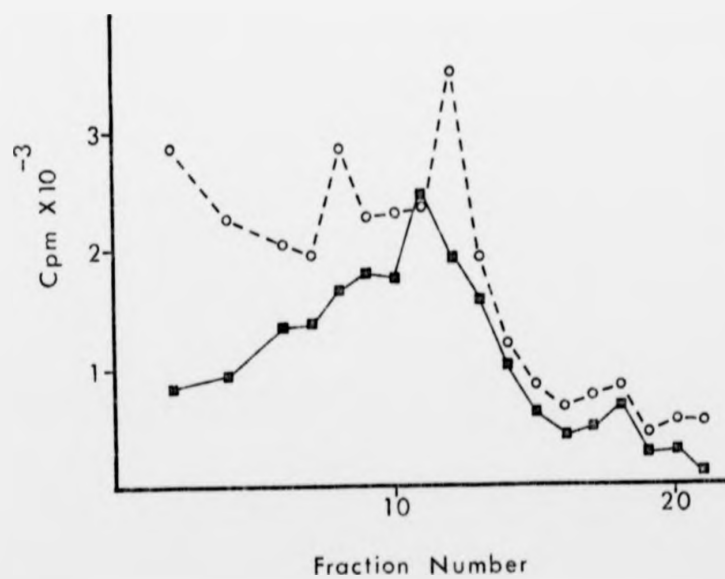
Fraction Number

10

20

Plus  
Protease

B



is incompletely degraded under the conditions used, then the results shown in Fig. 6.3 must be re-interpreted. The interpretation that best fits all the data is that there is a single peak of ovalbumin-containing vesicles centred around fraction number 11. Above this, most (if not all) of the ovalbumin is present as the result of leakage during homogenisation or during centrifugation. Below this, even less protease action is apparent and this is consistent with the hypothesis that secretory proteins present in the lower portion of the gradient are present in aggregates of vesicles. If this interpretation is correct then the distribution of lysozyme (Fig 5.11) is likely to be the result of almost complete leakage from microsomal vesicles during homogenisation and fractionation.

Similar experiments to those described above, performed to assess the status of lysozyme on high-salt gradients, were inconclusive. Under the conditions described in Fig. 6.3 (legend) lysozyme was completely unaffected by proteinase K. Moreover, when the susceptibility of lysozyme to proteinase K in the absence of membranes was investigated, only 15% of the lysozyme was digested, in conditions where only 22% of the ovalbumin remained intact (data not shown).

### Discussion

The first conclusion arising from the studies presented in this and the previous chapter, is that a microsomal fraction containing secretory protein can be isolated from oocytes. The second conclusion is that ovalbumin, lysozyme and selected immunoglobulins all show similar - though not identical - patterns of gradient distribution under a variety of conditions. There are three possible reasons for this coincident distribution:

1. Ovalbumin, lysozyme and immunoglobulin have a coincident distribution among the organelles involved in secretion.
2. Ovalbumin, lysozyme and immunoglobulin are differentially distributed between secretory organelles, but these gradients are incapable of resolving the microsomal vesicles into subsets derived from different organelles.
3. There is both sufficient resolution of organelles on these gradients and differential distribution of secretory proteins, but this is masked by the leakage of microsomal content into the surrounding medium and subsequent diffusion.

It is not possible, on the basis of current data, to distinguish between these three hypotheses. The major reason for this impasse is the problem of leakage, compounded by the failure of the protease-protection experiments, which meant that

leaked protein could not be clearly distinguished from sequestered protein on these gradients. This was further complicated by the apparently different rates of leakage shown by different secretory proteins. Due to this differential and extensive leakage, and subsequent diffusion, of secretory protein from microsomal vesicles, the original amount of ovalbumin, lysozyme or immunoglobulin present within each subfraction could not be determined.

It may be that if effective assays for the relevant stringent marker enzymes could be developed, then a clear ER-Golgi separation could be obtained by using combinations of discontinuous gradients and pelleting procedures as developed for other tissues. However, the degree of leakage - especially of lysozyme - found under the very gentle conditions described in these two chapters implies that any microsomal fractions isolated in this way from oocytes would consist of empty vesicles. Because of this microsomal fragility, the methods of, for example, Fries and J. Rothman (Fries and J. Rothman, 1981; Dunphy *et al.*, 1981) may only be effective when a membrane-bound protein such as VSV-G is used, and are unlikely to provide satisfactory approach to the isolation of oocyte vesicles containing secretory proteins. If this kind of approach is ruled out, and if no biochemical markers of progress along the secretory pathway (such as endo H-sensitivity) are actually present on the secreted protein itself, there does not seem to be any fractionation method currently available

which could be used to solve the problem of the cellular basis for non-parallel secretion in the oocyte.

which could be used to solve the problem of the cellular basis for non-parallel secretion in the oocyte.

## Chapter 7

Identification of a high-density oocyte  
fraction containing non-secretory lysozyme



### Introduction

The rationale underlying the fractionation approach developed in the previous chapter was that the movement of a protein along the secretory pathway must result in that proteins' sequential association with elements of different densities; this movement could therefore be monitored by density gradient analysis. As discussed in the previous chapter the approach failed. Although the reasons for this failure remain unclear, it is possible that all the secretory elements have densities so similar as to prevent their resolution. This problem might not apply to storage compartments or to other elements not normally associated with secretion. Hurkman *et al.* (1981) have injected oocytes with mRNA encoding maize storage proteins and have been able to demonstrate that vesicles containing these storage proteins (zeins) are clearly separated on sucrose gradients from the microsomal fraction that Zehavi-Willner and Lane (1977) showed was associated with secreted proteins.

In chapter 3 it has been shown that a substantial proportion of the lysozyme synthesised in oocytes is not secreted, possibly because it has been miscompartmentalised into a storage compartment. The experiments described in this chapter were designed to investigate the possibility that the sucrose gradients developed in chapter 5 would be capable of resolving non-secretory lysozyme from secretory lysozyme and ovalbumin. It has been shown (chapter 3) that under chase

conditions, the oocyte rapidly secretes all the potentially exportable lysozyme. Therefore, after a 24 h chase almost all the intracellular lysozyme should be present within the putative storage compartment. Thus to compare the subcellular distribution of secretory ovalbumin and lysozyme with non-secretory lysozyme, the distribution on sucrose gradients of oviduct proteins from pulse-labelled and pulse-chased oocytes was investigated.

### Results

The protocol used in these experiments was designed to ensure that both pulse-labelled and pulse-chased oocytes were processed after identical times in culture following micro-injection and that both sets of oocytes were fractionated in parallel. This design should rule out any possible differences in results between the two groups caused by the effects of micro-injection or length of time in culture on the oocytes. Accordingly, oocytes were micro-injected and split into two groups (day one). The first group were left overnight, healthy oocytes selected and then labelled on day two for 24 h. On day three these oocytes were placed in medium supplemented with 10 mM L-methionine and chased for 24 h. The second group of oocytes were cultured in normal incubation medium until day three when they were labelled for 24 h. On day four both groups of oocytes were homogenised, fractionated overnight and then samples collected and frozen down on day five. Immuno-analysis of both pulse-labelled and pulse-chased oocyte gradients was then performed. The results show (Fig. 7.1) that although the distribution of ovalbumin on sucrose gradients is similar for both pulse-labelled and pulse-chased oocytes, that of lysozyme changes dramatically. This experiment has been repeated four times and in each case the same result was obtained. The only difference in result between experiments was in whether or not a small percentage of the lysozyme was present in a high density portion of the gradient on which pulse-labelled

1. The first part of the report is a general introduction to the subject of the study. It discusses the importance of the study and the objectives of the research.

2. The second part of the report is a detailed description of the methodology used in the study. It includes information about the sample size, the data collection methods, and the statistical analysis techniques.

3. The third part of the report is a presentation of the results of the study. It includes tables and graphs showing the data and the findings of the research.

4. The fourth part of the report is a discussion of the results and their implications. It discusses the strengths and limitations of the study and provides recommendations for future research.

5. The fifth part of the report is a conclusion and a summary of the findings. It provides a final statement on the results of the study and the overall conclusions.

The results of the study show that there is a significant difference between the two groups. The first group showed a higher level of performance than the second group. This difference was statistically significant at the 0.05 level.

The findings of the study suggest that the first group had a higher level of skill and knowledge than the second group. This could be due to a variety of factors, including differences in training, experience, and motivation.

The study has several limitations. First, the sample size was relatively small, which may have affected the results. Second, the study was conducted in a laboratory setting, which may not reflect real-world conditions.

Despite these limitations, the study provides valuable information about the differences between the two groups. It suggests that the first group has a higher level of performance and that this difference is statistically significant.

Further research is needed to explore the reasons for these differences and to determine the best way to improve performance. This could involve larger studies, more realistic settings, and more detailed analysis of the factors involved.

Fig. 7.1 Gradient Analysis of Pulse-Labelled and Pulse-Chased Oocytes

Oocytes were split into two groups for processing as follows:

Group 1 (pulse-labelled)	Group 2 (pulse-chased)	
injection	injection	Day 1
culture	labelling (24 h)	Day 2
labelling (24 h)	chase (24 h)	Day 3
fractionation	fractionation	Day 4
sample collection and immuno-analysis	sample collection and immuno-analysis	Day 5

Injection was of oviduct mRNA (1 mg/ml). Labelling was with [ $^{35}\text{S}$ ]-methionine (4 mCi/ml). Fractionation was on high-salt gradients. Samples were immunoprecipitated with anti-ovalbumin and anti-lysozyme antisera and electrophoresed on 15% SDS-polyacrylamide gels. After exposure, gels were cut up for quantitation. Results of two experiments are shown. Data from gradient (I) have been shown before (Fig. 5.11).

A. Anti-lysozyme immuno-analysis

- I. pulse-labelled oocytes, fluorograph and quantitation.
- II. pulse-chased oocytes, fluorograph and quantitation.
- III. pulse-labelled oocytes, fluorograph and quantitation.
- IV. pulse-chased oocytes, fluorograph and quantitation.

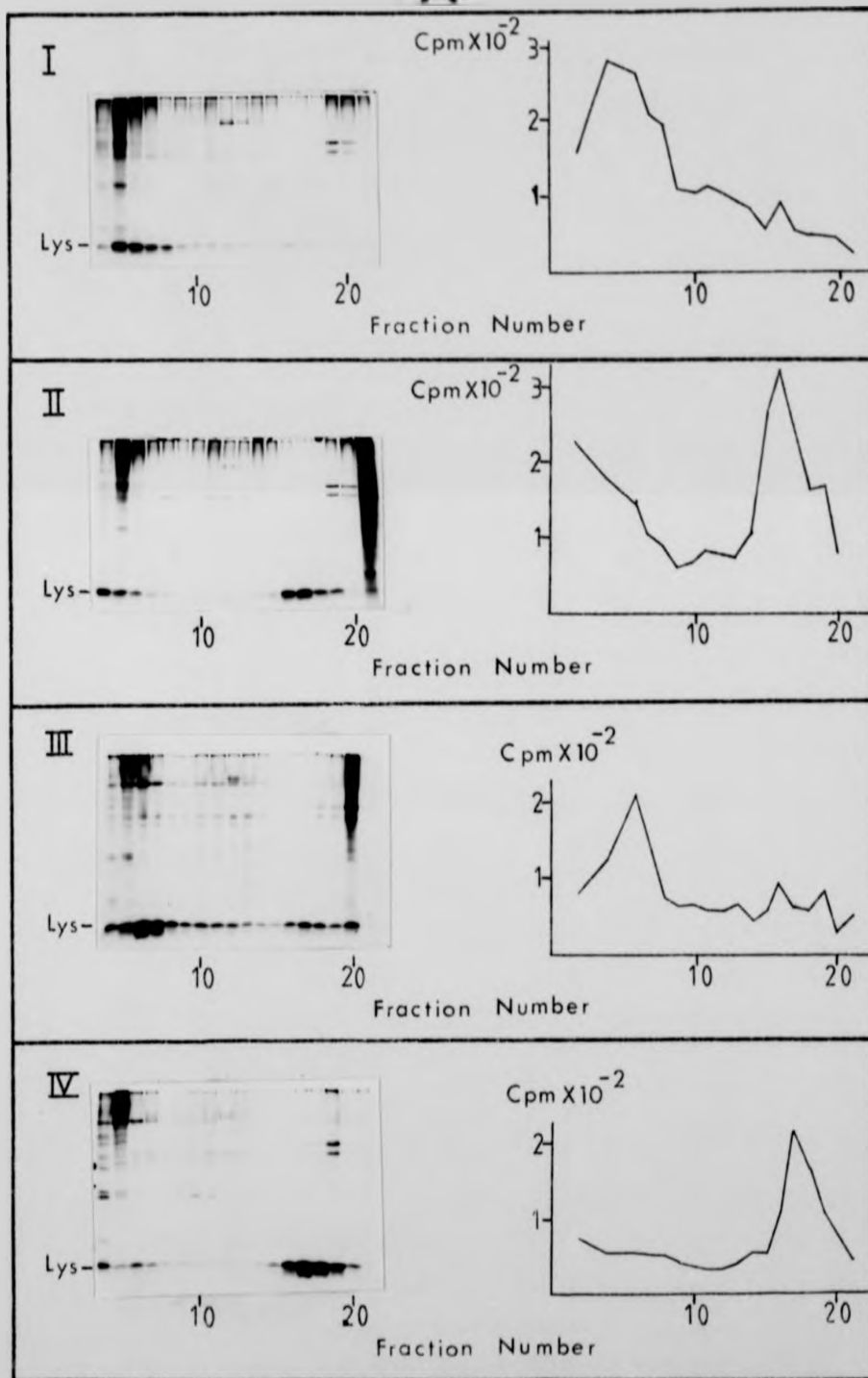
I and III are from one experiment, II and IV from another.

B. Anti-ovalbumin immuno-analysis

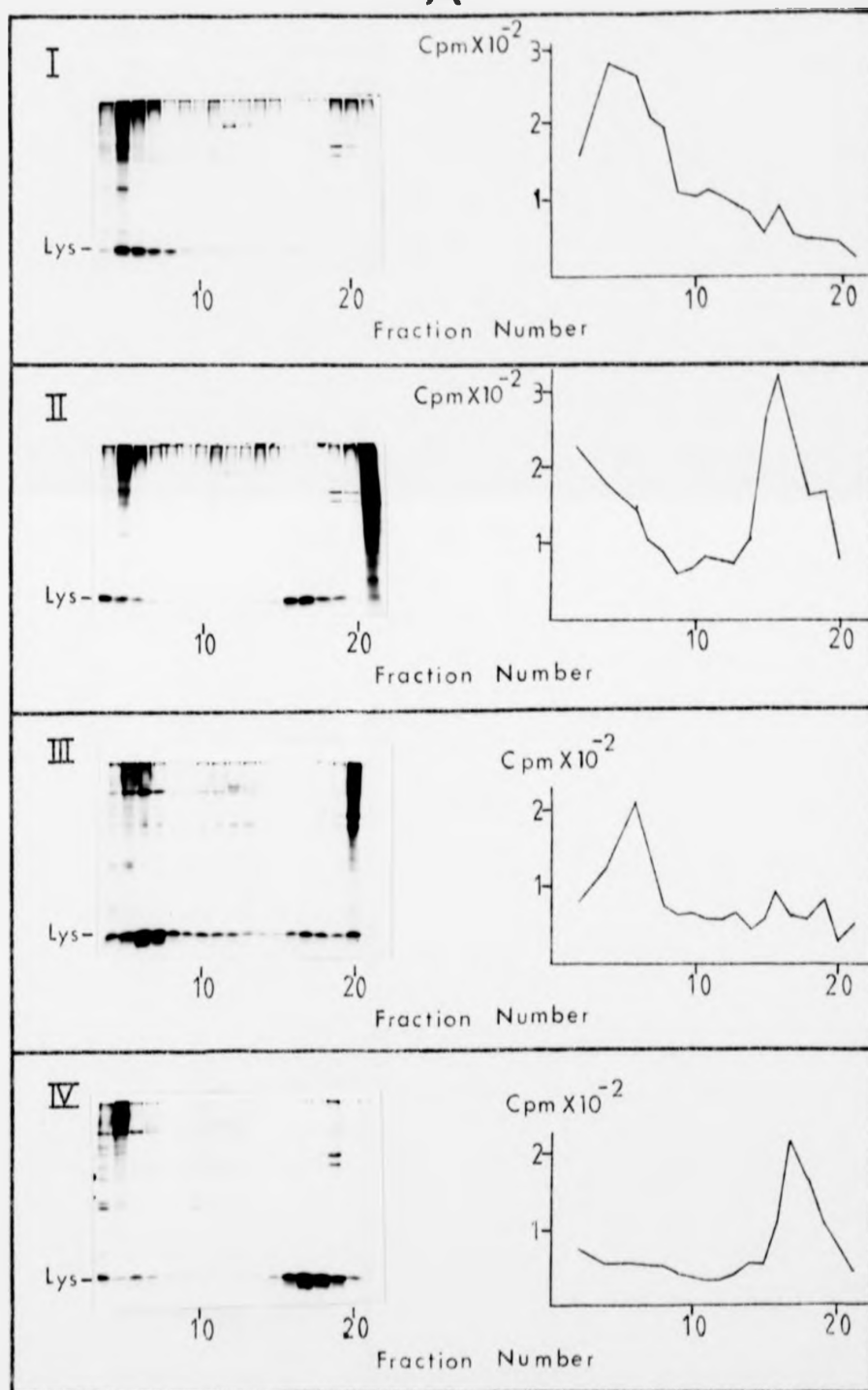
I, II, III, IV correspond to the same gradients as in A.

Gradient I contained 8 injected/labelled, and 32 carrier oocytes  
II contained 15 injected/labelled, and 25 carrier oocytes  
III contained 7 injected/labelled, and 33 carrier oocytes  
IV contained 10 injected/labelled, and 30 carrier oocytes

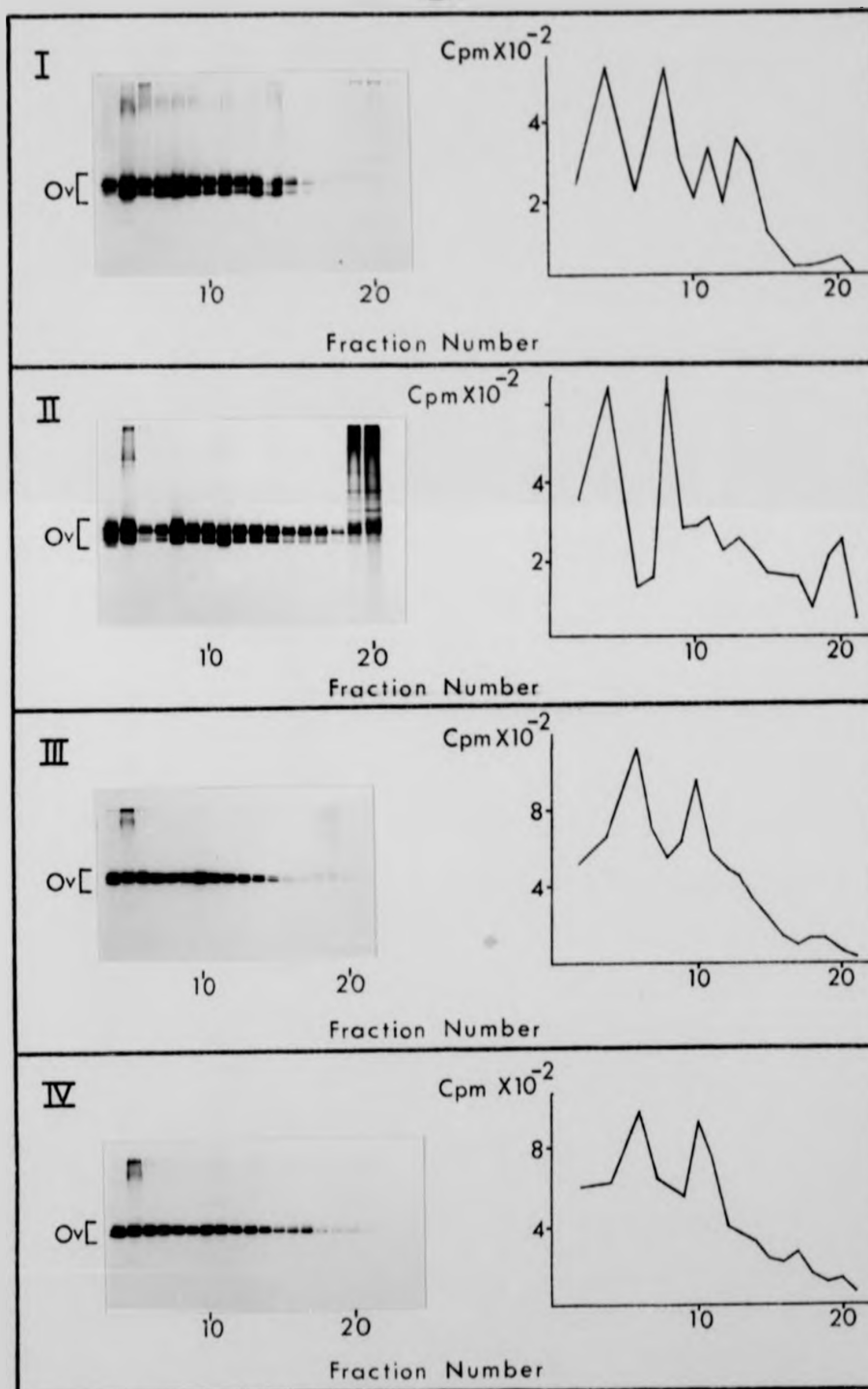
A



A

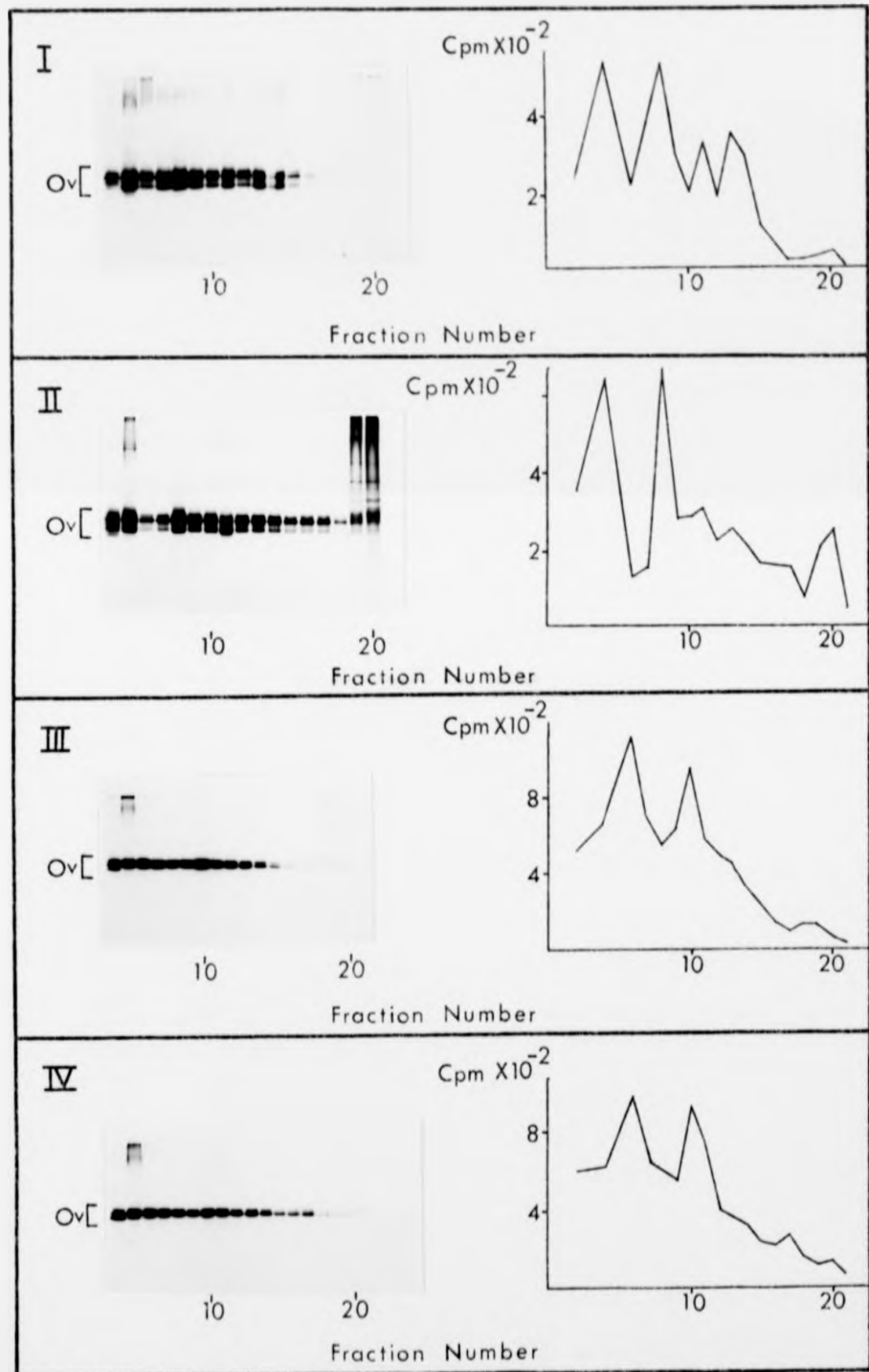


# B





# B



oocytes were fractionated. Both kinds of result are shown.

One possible interpretation of these results is that non-secretory lysozyme is moving slowly into a high-density oocyte fraction. The presence or absence of high-density lysozyme on the 'pulse' gradient would then reflect variation in the rate at which this miscompartmentalisation occurs in different batches of oocytes. If lysozyme is moving slowly into a high density oocyte fraction then lengthening the 'chase' should cause the ratio of lysozyme at the top of the gradient (presumably mainly leaked secretory protein) to that at the bottom of the gradient to fall. To test this prediction, injected oocytes were split into three groups; the first group was pulse-labelled, the second group was labelled and then chased for 24 h and the third group was labelled and then chased for 48 h. The three groups of oocytes were fractionated in parallel and samples analysed to show the effects of the three labelling regimes on the gradient-distribution of lysozyme (Fig. 7.2). These results show that the ratio of lysozyme at the top of the gradient to that in a high density fraction does fall with time. Thus lysozyme seems to be slowly moving into a high density fraction within the oocyte.

To rule out the possibility that the movement of lysozyme into a high density fraction is an artefact caused by the salt conditions used, oocytes were labelled in parallel with groups

Fig. 7.2 Movement of Lysozyme into a High-Density Fraction

Oocytes were split into 3 groups and processed as follows:

Group C	Group B	Group A	Day
injected	injected	injected	1
labelled (24 h)	cultured	cultured	2
chased (24 h)	labelled (24 h)	cultured	3
chased (24 h)	chased (24 h)	labelled (24 h)	4
fractioned	fractioned	fractioned	5
samples collected	samples collected	samples collected	6

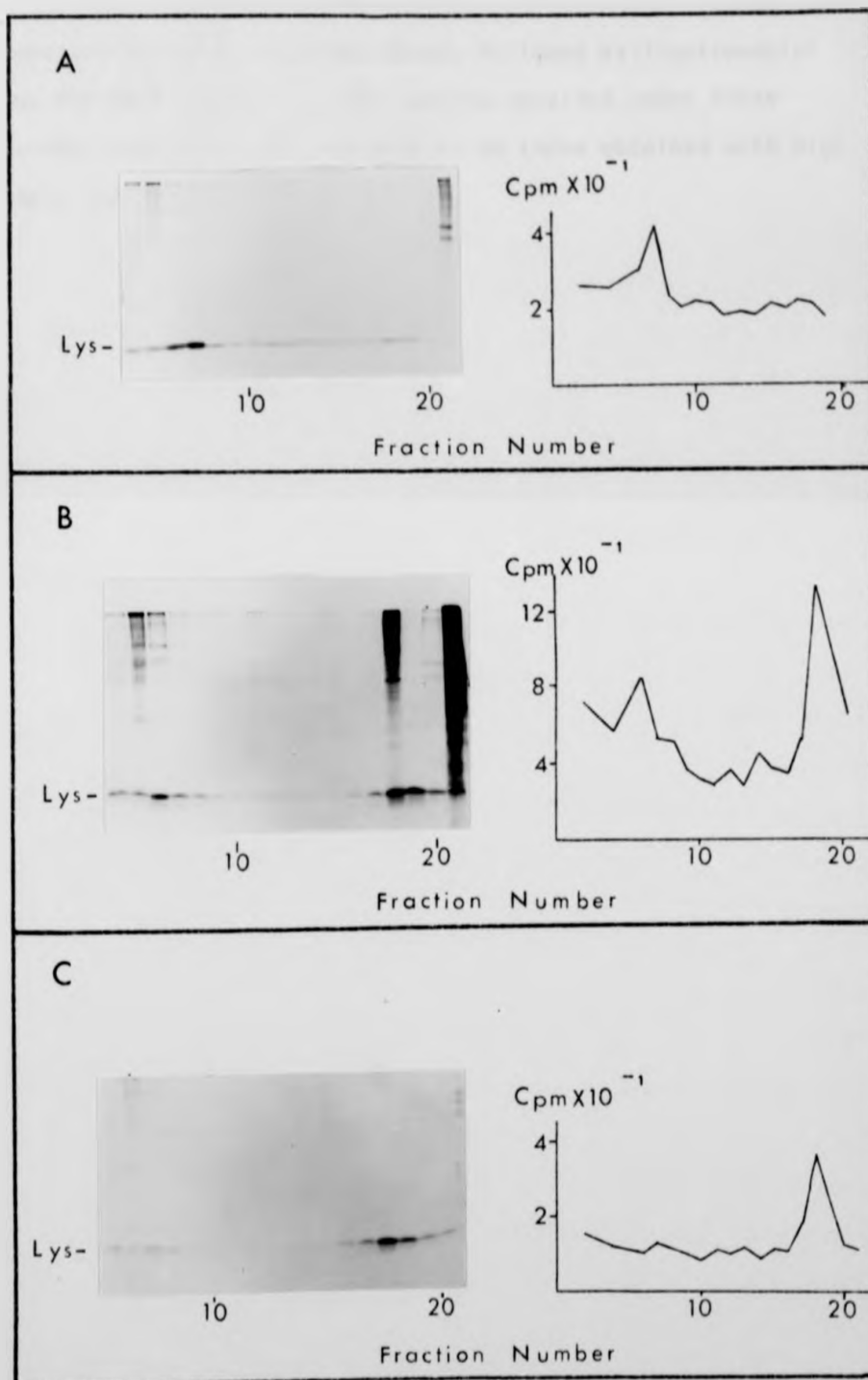
Injection was of oviduct mRNA (1 mg/ml). Labelling was with [ $^{35}\text{S}$ ]-methionine (4 mCi/ml). Fractionation was on high-salt gradients. Samples were precipitated with anti-lysozyme anti-serum and electrophoresed on 15% SDS-polyacrylamide gels. After exposure, gels were cut up and regions corresponding to the position of lysozyme counted. Data in A have been shown before (Fig. 5.12).

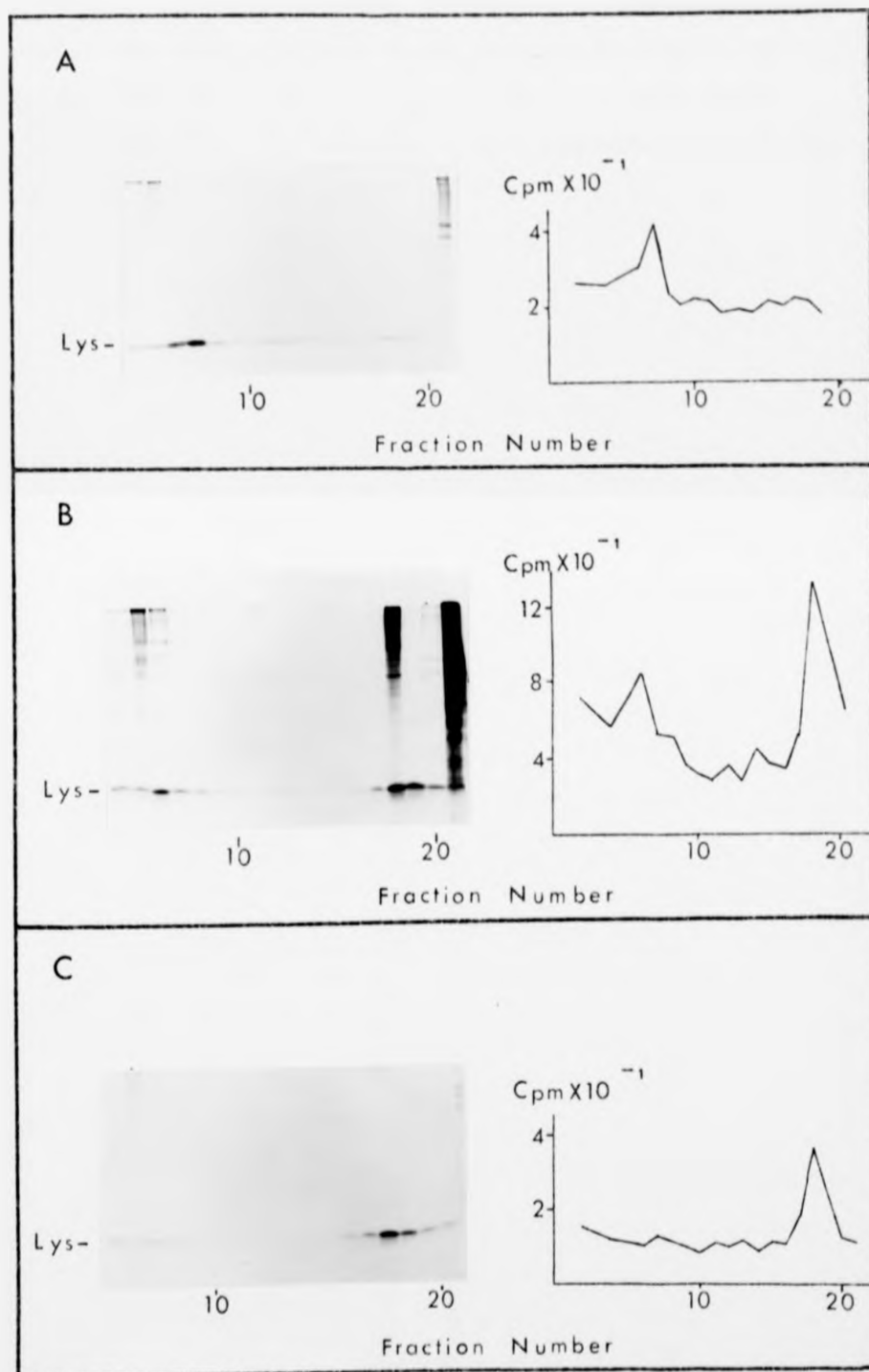
- A. Autoradiograph and quantitation of samples from group A (above)
- B. Fluorograph and quantitation of samples from group B (above)
- C. Fluorograph and quantitation of samples from group C (above)

Gradient in A contained 8 injected/labelled, and 32 carrier oocytes

Gradient in B contained 16 injected/labelled, and 24 carrier oocytes

Gradient in C contained 10 injected/labelled, and 30 carrier oocytes



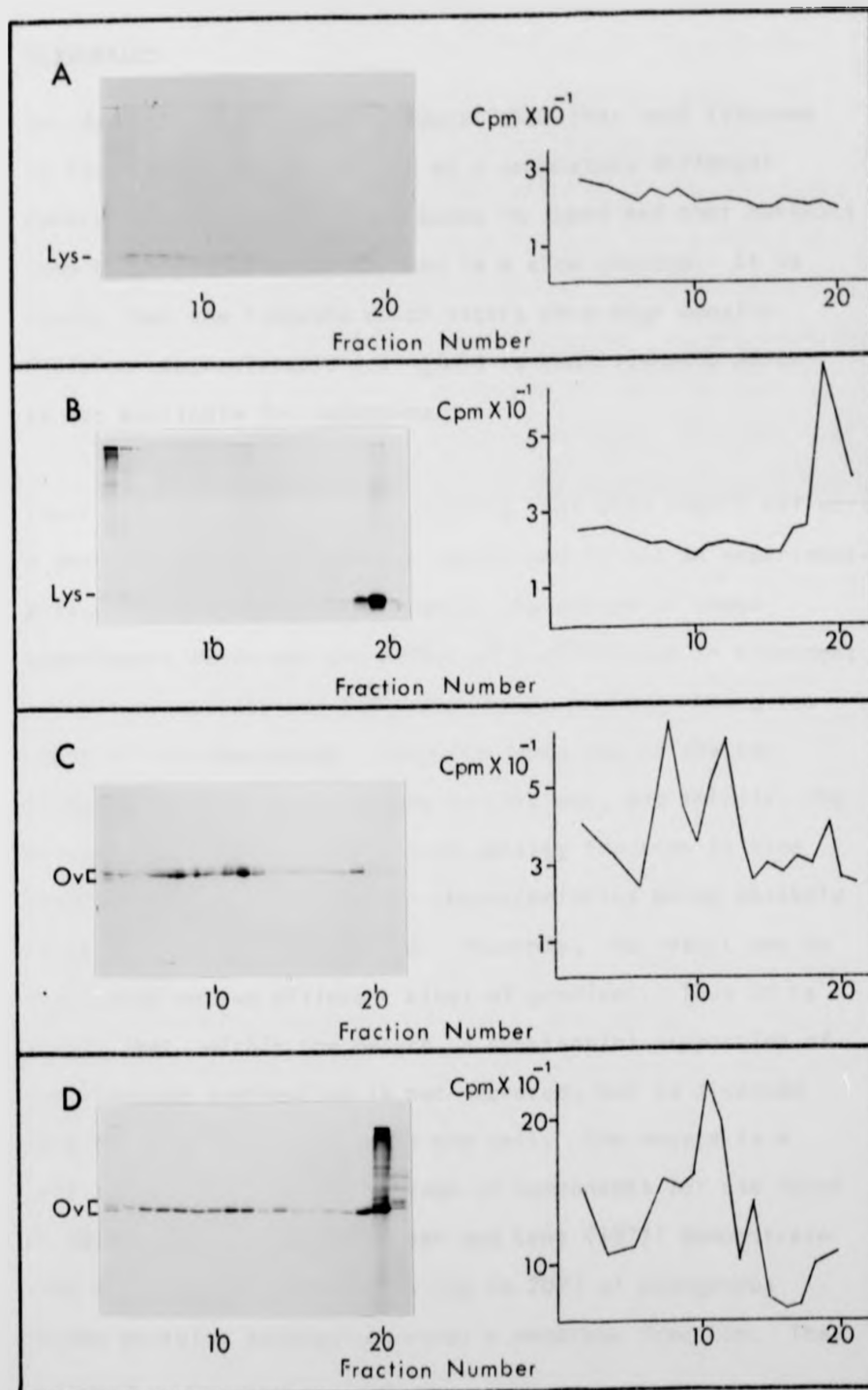


one and three as described above, followed by fractionation on PVP-EDTA gradients. The results obtained under these conditions (Fig. 7.3) are similar to those obtained with high salt gradients.

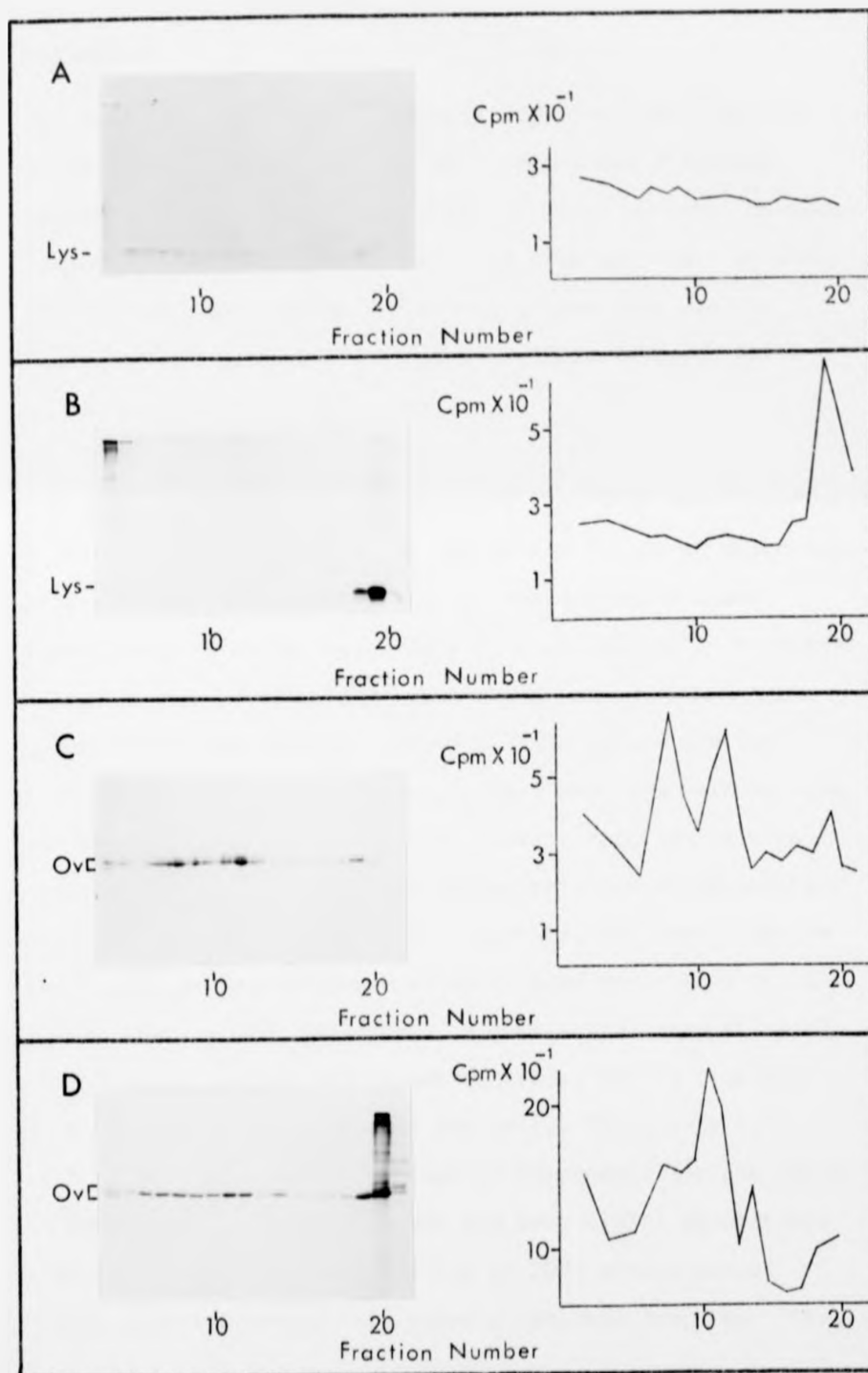
Fig. 7.3 Fractionation of Pulse-Labelled and Pulse-Chased Oocytes on PVP-EDTA Gradients

Oocytes were processed as in groups A and C of Fig. 7.2. Fractionation was performed on PVP-EDTA gradients. Samples were collected, immunoprecipitated with anti-ovalbumin and anti-lysozyme antisera and electrophoresed on 15% SDS-polyacrylamide gels. After exposure, gels were cut up and regions containing ovalbumin and lysozyme counted. Data in A and C shown before (Fig. 5.12). Ov = ovalbumin; Lys = lysozyme.

- A. Autoradiograph and quantitation of lysozyme from pulse-labelled oocytes.
- B. Autoradiograph and quantitation of lysozyme from pulse-chased oocytes.
- C. Autoradiograph and quantitation of ovalbumin from pulse-labelled oocytes.
- D. Autoradiograph and quantitation of ovalbumin from pulse-chased oocytes.







### Discussion

The data presented in this chapter show that some lysozyme is found on sucrose gradients at a completely different density to that at which ovalbumin is found and that movement into this high density fraction is a slow process. It is likely that the lysozyme which enters this high density fraction (approximately 1.21 g/ml) is that lysozyme which is not available for secretion.

There are four reasons for supposing that this result reflects a phenomenon occurring in the oocyte and is not an experimentally-generated artefact. Firstly, the design of these experiments rules out any effect of a difference in treatment between pulse-labelled and pulse-chased oocytes' being the cause of the phenomenon. Secondly, only one of the two proteins being studied behaves in this way, and thirdly, the movement of lysozyme into a high density fraction is time dependent, both of the latter characteristics being unlikely to be artefactually generated. Fourthly, the result can be duplicated on two different kinds of gradient. Thus it is likely that, within the oocyte, a substantial proportion of the lysozyme synthesised is not secreted, but is diverted to a novel compartment within the cell. The oocyte is a cell specialised for the storage of components for use later in development. Zehavi-Willner and Lane (1977) demonstrate that a significant proportion (up to 20%) of endogenous oocyte proteins synthesised enter a membrane fraction. They

speculate that this fraction represents an oocyte storage system. Thus the idea of a membranous storage compartment within oocytes is not new. More recently, Hurkman *et al.* (1981) injected mRNA encoding maize storage proteins (zeins) into oocytes. They find that these proteins are present in a high density compartment (approximately 1.22 g/ml) within the cell following synthesis. The authors argue that because, during endosperm development, zeins are synthesised and deposited as dense, insoluble protein bodies within the rough ER, the same process is occurring in the oocyte; i.e. protein bodies forming inside ER vesicles. The implication of this argument is that the high density fraction within which zeins are recovered from oocytes is not a natural oocyte compartment, but a novel compartment generated by the unusual nature of these ethanol-soluble proteins. However, the results presented in this chapter show that a soluble, secretory protein may be entering a high density oocyte compartment (approximately 1.21 g/ml). Unfortunately, there are no criteria other than density by which the zein-occupied and lysozyme-occupied compartments may be compared. It is possible that there may be a high density compartment normally present within oocytes and that exogenous storage proteins may be capable of entering this novel compartment.

## Chapter 8

The effect of monensin on lysozyme  
distribution on sucrose gradients

### Introduction

In the previous chapter it was shown that non-secretory lysozyme has a different distribution on sucrose gradients from secretory ovalbumin and lysozyme. It was also demonstrated that the differential distribution results from the slow movement of lysozyme into a high density fraction. It was argued that this differential distribution reflects a phenomenon occurring in the oocyte and that this high density fraction may represent some storage compartment within these cells. One question in particular about this phenomenon remains unanswered: at what stage in its intracellular movement is lysozyme diverted from secretion to storage? This question relates to a point of major interest. It is generally argued (see Farquhar and Palade, 1981; Tartakoff, 1980) that all intracellular traffic passes through the Golgi apparatus. If this is so, then one would expect all lysozyme within the oocyte to pass into this organelle and then be sorted into two subpopulations, that destined for secretion and that destined for the high density fraction. If this model is correct, and lysozyme destined for the high density fraction passes through the Golgi, then blocking secretion from the oocyte with a drug that disrupts the Golgi apparatus should also block movement of lysozyme into the high density fraction. Monensin, a carboxylic ionophore (Pressman, 1976), has been shown to affect the intracellular transport and secretion of a variety of proteins (see Tartakoff, 1980; Tartakoff, 1982), blocking intracellular transport in the

proximal (or cis) Golgi. If oocytes are treated with monensin and secretion is blocked, and if movement of lysozyme into the high density fraction should prove also to be blocked, this would be evidence that lysozyme destined for this alternative fate reaches the Golgi apparatus before diverging from secretory lysozyme. Thus an opportunity to assess the rôle of the Golgi in a novel sorting operation is available.

## Results

The use of monensin on oocytes has not been reported. Two preliminary experiments aimed at establishing the concentration of drug that would block secretion but not kill the oocytes were therefore performed. The first experiment was designed to find the highest concentration of drug that could be used without damaging these cells. The toxicity was monitored by the effect on protein synthesis. Groups of oocytes were incubated with various levels of monensin in the presence of  $^{35}\text{S}$ -methionine. The incorporation of label into protein at several time points was measured by TCA-precipitation of oocyte samples. The results (Fig. 8.1) show that protein synthesis is unaffected up to 20  $\mu\text{M}$  monensin, but that at 50  $\mu\text{M}$  monensin, protein synthesis in the oocyte is severely depressed.

The second preliminary experiment was designed to check that monensin would block secretion from oocytes at concentrations below 50  $\mu\text{M}$ . Two concentrations of monensin were tested. 15  $\mu\text{M}$  was chosen as being well below the toxic level, and 30  $\mu\text{M}$  was chosen as being close to the concentration of monensin that causes oocyte death. Injected oocytes were labelled in the presence of 0, 15 or 30  $\mu\text{M}$  monensin. After incubation the oocytes and surrounding medium were collected and analysed. The results of this analysis (Fig. 8.2) show that monensin does have a small effect on protein synthesis even at 15  $\mu\text{M}$  (compare tracks 1 and 4) but that increasing

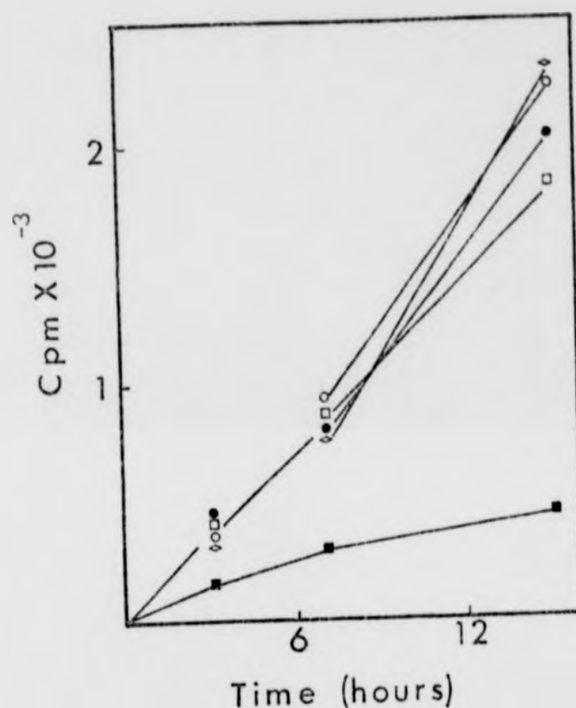


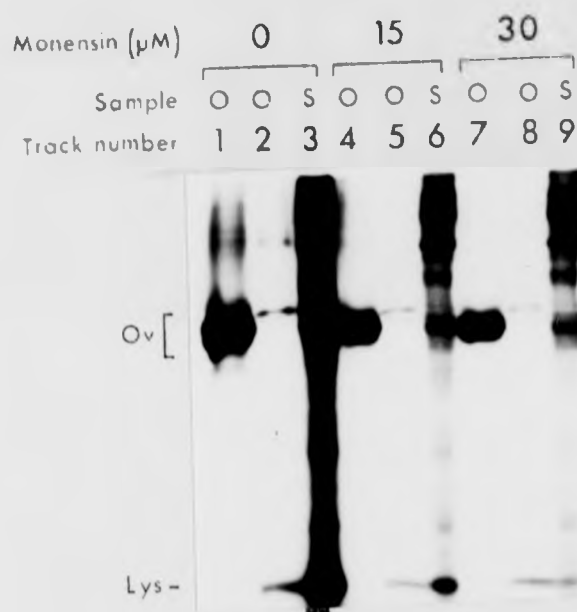
Fig. 8.1 The Effect of Monensin on Protein Synthesis in Oocytes

Oocytes were labelled ( $[^{35}\text{S}]$ -methionine, 0.5 mCi/ml) in medium supplemented with monensin (0, 1, 10, 20, 50  $\mu\text{M}$ ). At intervals, groups of oocytes were removed and frozen. Samples were homogenised and duplicate aliquots (5  $\mu\text{l}$ ) were TCA-precipitated and counted.

○—○ = 0  $\mu\text{M}$ ; ◇—◇ = 1  $\mu\text{M}$ ; □—□ = 10  $\mu\text{M}$ ; ●—● = 20  $\mu\text{M}$ ;  
■—■ = 50  $\mu\text{M}$ .



Fig. 8.2 The Effect of Monensin on the Secretion of Ovalbumin and Lysozyme from Oocytes



Oocytes were injected (oviduct mRNA, 1 mg/ml) and labelled ( $r^{35}\text{S}$ -methionine, 1 mCi/ml) for 20 h in the presence of 0, 15 or 30  $\mu$ M monensin. Healthy oocytes and the medium surrounding them were collected and frozen for analysis. Samples were electrophoresed on a 12.5% SDS-polyacrylamide gel, which was fluorographed. Oocyte samples (O) are immunoprecipitates. Secretable protein (S) was prepared directly for electrophoresis.

Tracks 1, 4, 7; anti-ovalbumin immunoprecipitates.

Tracks 2, 5, 8; anti-lysozyme immunoprecipitates.

Ov = ovalbumin; Lys = lysozyme.

Fig. 8.2 The Effect of Monensin on the Secretion of Ovalbumin and Lysozyme from Oocytes



Oocytes were injected (oviduct mRNA, 1 mg/ml) and labelled ( $^{35}$ S-methionine, 1 mCi/ml) for 20 h in the presence of 0, 15 or 30  $\mu$ M monensin. Healthy oocytes and the medium surrounding them were collected and frozen for analysis. Samples were electrophoresed on a 12.5% SDS-polyacrylamide gel, which was fluorographed. Oocyte samples (O) are immunoprecipitates. Secretable protein (S) was prepared directly for electrophoresis.

Tracks 1, 4, 7; anti-ovalbumin immunoprecipitates.

Tracks 2, 5, 8; anti-lysozyme immunoprecipitates.

Ov = ovalbumin; Lys = lysozyme.

the concentration to 30  $\mu$ M does not reduce protein synthesis further (compare tracks 4 and 7). However, the addition of monensin dramatically reduces the amounts of ovalbumin and lysozyme secreted by these oocytes. This is most clearly seen in the changing ratio of intracellular to secreted lysozyme. At 30  $\mu$ M monensin the amount of ovalbumin and lysozyme being exported is a very small fraction of that normally secreted.

From these results it was concluded that a concentration of 30  $\mu$ M should be used to assess the affect of monensin on the movement of lysozyme into the high-density fraction.

Oocytes were injected and labelled before division into two groups. After a 24 h chase in the presence or absence of 30  $\mu$ M monensin, both groups of oocytes were then fractionated on high-salt gradients.

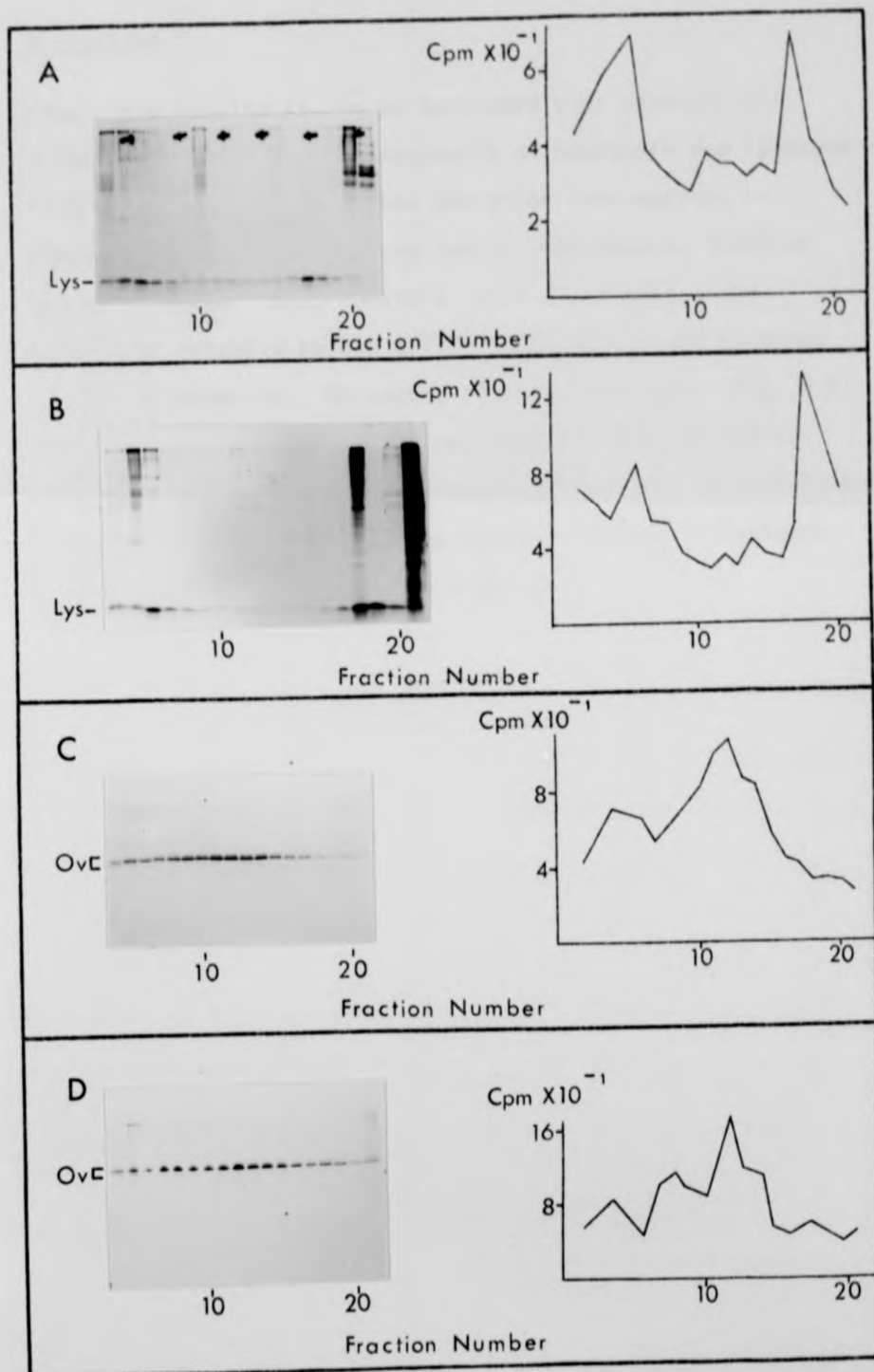
Immuno-analysis of the distribution of lysozyme on these gradients (Fig. 8.3) shows a result suggestive of an impaired movement into the high-density fraction when monensin is present. However, no firm conclusions can be drawn because the experiment was not repeated. The distribution of ovalbumin on these gradients remains unchanged, within the natural variability generated by this system. In this experiment, the secretion of ovalbumin and lysozyme from monensin-treated oocytes was 9% and 30 % (respectively) of that from untreated oocytes.

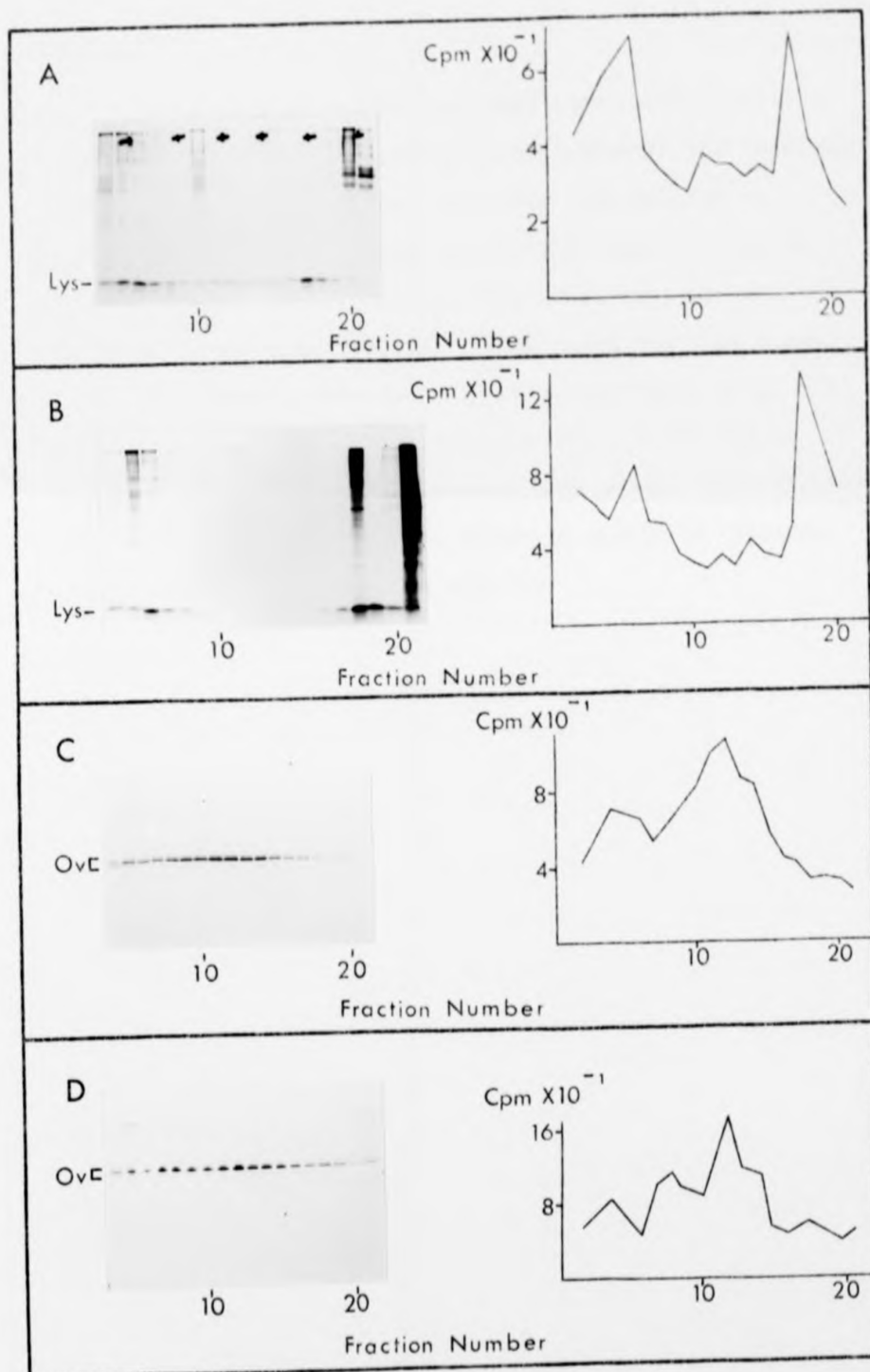
Fig. 8.3 The Effect of Monensin on the Gradient-  
Distribution of Ovalbumin and Lysozyme from  
Pulse-Chased Oocytes

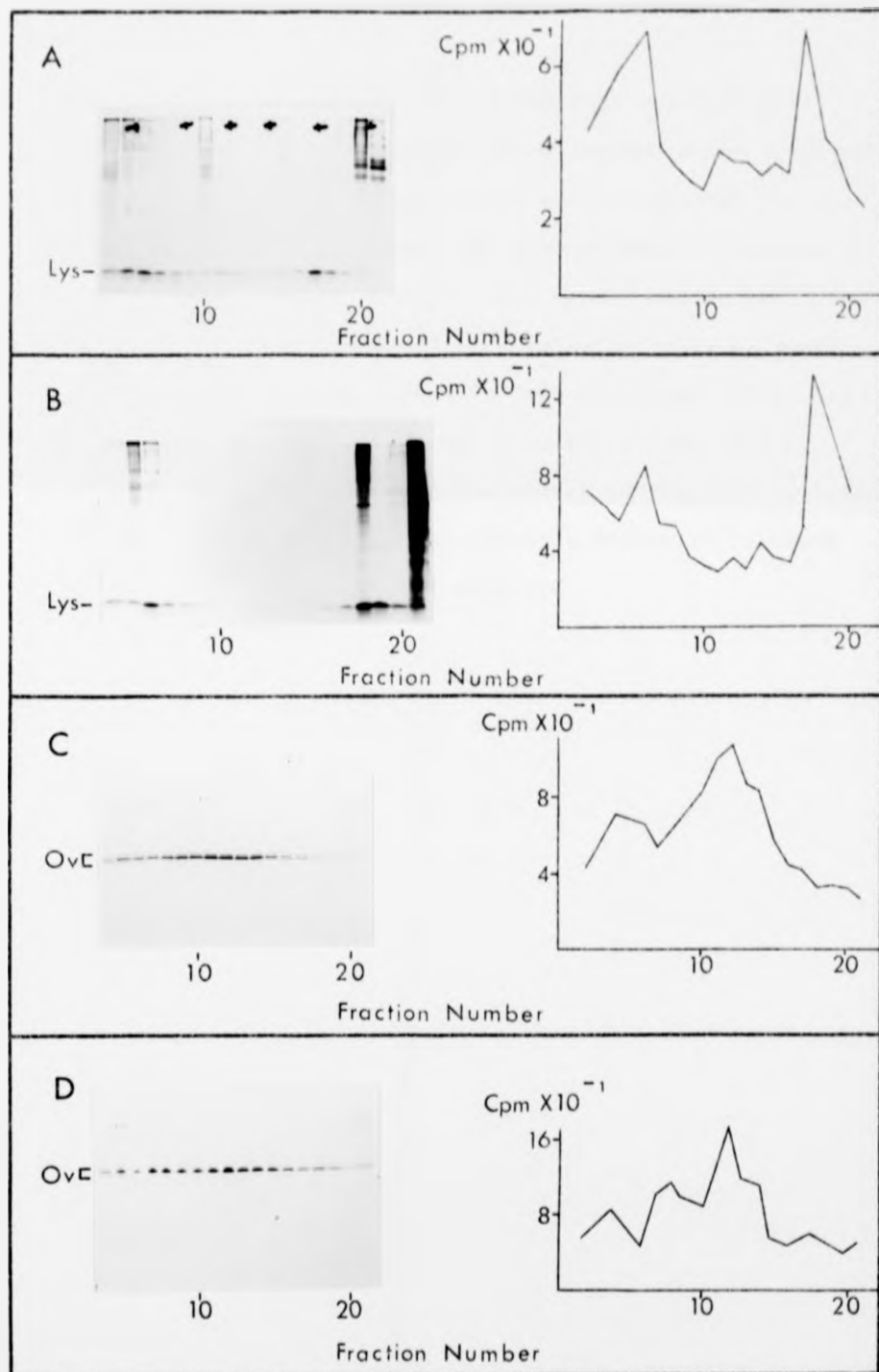
Oocytes were injected (oviduct mRNA, 1 mg/ml) labelled ( $r^{35}\text{S}$ -methionine, 4 mCi/ml) for 24 h, and chased in the presence or absence of 30  $\mu\text{M}$  monensin for a further 24 h. Fractionation on high-salt gradients was followed by collection of samples, immunoprecipitation with anti-ovalbumin and anti-lysozyme antisera and electrophoresis on 15% SDS polyacrylamide gels. Gels were fluorographed, exposed, and regions containing ovalbumin and lysozyme excised and counted. Data in B has already been shown (Fig. 7.2). Ov = ovalbumin; Lys = lysozyme.

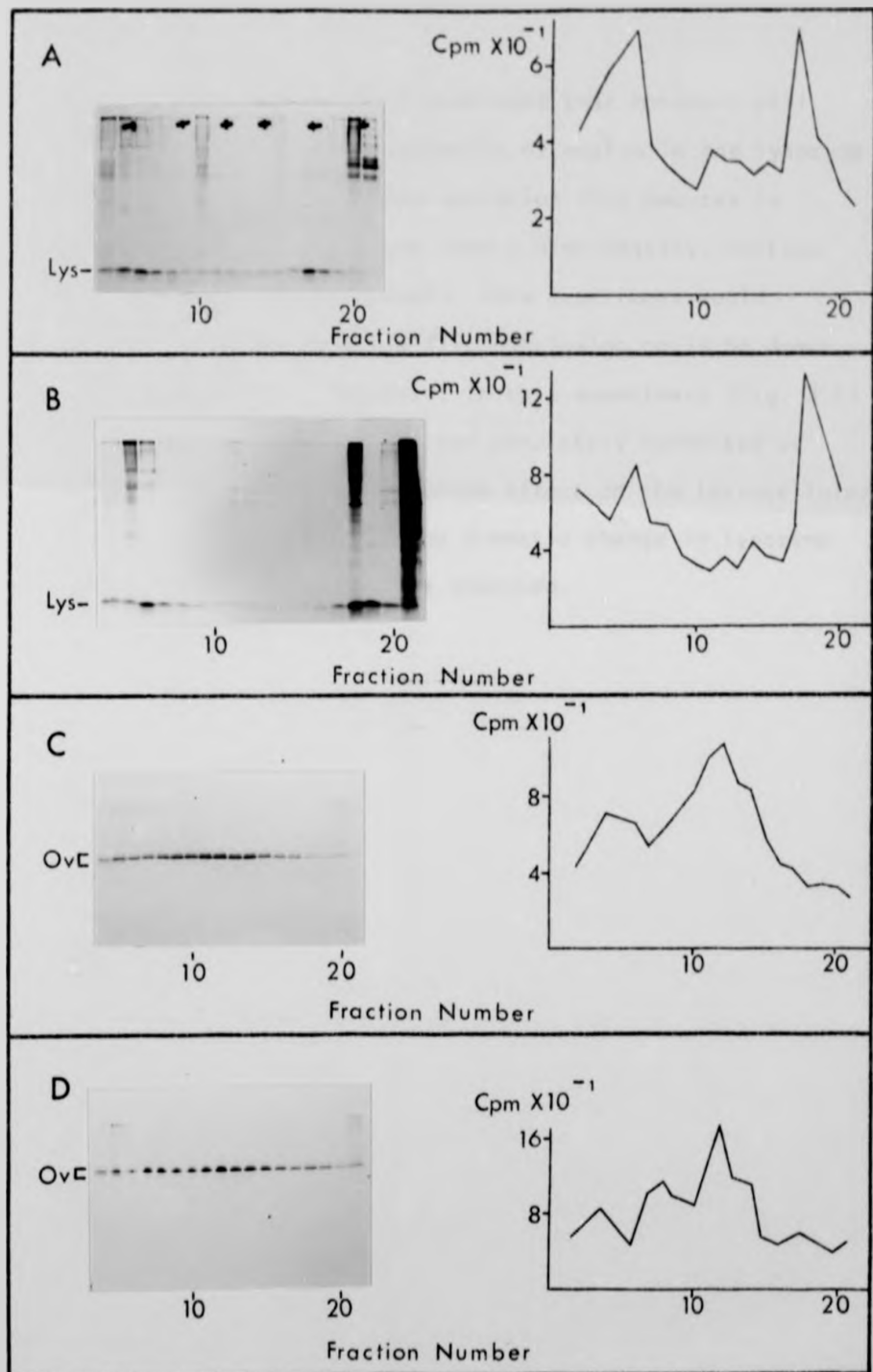
- A. Fluorograph and quantitation of anti-lysozyme immunoprecipitates. Plus monensin.
- B. Fluorograph and quantitation of anti-lysozyme immunoprecipitates. Minus monensin.
- C. Fluorograph and quantitation of anti-ovalbumin immunoprecipitates. Plus monensin.
- D. Fluorograph and quantitation of anti-ovalbumin immunoprecipitates. Minus monensin.

Both gradients contained 16 injected/labelled and 24 carrier oocytes.











### Discussion

From these results it can be concluded that monensin will substantially inhibit the secretion of ovalbumin and lysozyme from oocytes. Moreover, when secretion from oocytes is blocked, movement of lysozyme into a high-density fraction may be affected. Unfortunately, this experiment would need to be repeated before a firm conclusion could be drawn on this observation. Moreover, in this experiment (Fig. 8.3) the secretion of lysozyme was not completely inhibited by monensin; thus only an incomplete effect on the intracellular movement of this protein and no dramatic change in lysozyme gradient-distribution could be expected.

## Chapter 9

### General Discussion

In this final discussion, I shall expand on some of the points raised during this thesis, discuss the relationship of this work to that of others, and discuss the rôle of the oocyte system in future studies of secretion.

### 1. Non-parallel Kinetics of Secretion from Oocytes

The experimental work presented in chapter 3 shows that secretable lysozyme is exported approximately twelve times faster than ovalbumin from Xenopus oocytes. The observation that two proteins with similar fates can be secreted at different rates has also been made by other workers. Giannattasio et al. (1980) showed that growth hormone and prolactin are secreted with different kinetics from bovine pituitary slices. However, leakage from tissue slices is always a problem in secretory studies (Palade, 1975). Moreover, each slice comprises more than one cell type. A more satisfactory study is that of Strous and Lodish (1980) who demonstrated that albumin is secreted twice as fast as transferrin from rat hepatoma cells in culture. These examples are from non-regulated secretion (as are the oocyte data) and it may be that different kinetics of export for different proteins is a characteristic of non-regulated secretion. The early studies showing parallel kinetics of secretion (see Introduction) were all performed on regulated systems. Most of these studies concentrated on comparing the ratio of secretory proteins in the zymogen granules of

the pancreatic acinar cell with the ratio of those proteins found in incubating media after stimulation of discharge. The conclusion drawn from these studies was that discharge of the various proteins occurred in parallel. Only one study (Keller and Cohen, 1961) compared the enzymatic composition of a bovine pancreatic microsomal fraction with zymogen granules and pancreatic juice and concluded that they were all similar. However, protein leakage in some of these early experiments was very high, and was protein- and fraction-specific in the extent to which it occurred (Scheele *et al.*, 1978). Moreover, due to the concentration of secretory product in zymogen granules, any contamination of a microsomal fraction with this cellular fraction would have a substantial effect on the observed ratio of secretory protein. Thus these data may not be reliable. In summary, the extensive studies of the pancreatic secretory process have only really established kinetic parallelism for the final stage of the process, from zymogen granule to discharge, and it would in fact be surprising if the proteins found outside the cell did not reflect the contents of the zymogen granules.

The immunocytochemical studies thus far discussed were qualitative. Recently, Bendayan *et al.* (1980) performed a quantitative immunocytochemical analysis of nine different secretory proteins in rat acinar cells. They showed that the ratio of secretory proteins present in the different organelles involved in secretion varies from organelle to organelle. In the absence

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of pulse-chase studies, they ascribe these differences to differential concentration of the various proteins. These data and all the previous studies could be interpreted as showing that the various proteins are transported at different rates through the cell until they reach the condensing vacuole/zymogen granule stage whereupon accumulation occurs. These large concentrations of secretory protein are then discharged in parallel by exocytosis. Thus I would argue that all secretory proteins may be exported with non-parallel kinetics and that parallel discharge may only be a consequence of the special circumstances of regulated secretion.

In the context of this argument, a recent paper by Gumbiner and Kelly (1982) is of interest. They show that both regulated and non-regulated secretion (of adrenocorticotrophic hormone and the envelope protein of murine leukaemia virus respectively) can occur from a pituitary cell line. This kind of system might allow a thorough investigation of the relationship between these two processes. In addition, it would be instructive to analyse the kinetics of secretion of pancreatic secretory proteins from Xenopus oocytes.

## 2. Amphibian Factors in the Secretion of Exogenous Proteins from Oocytes

The data presented in chapter 4 lead to the conclusion that the differential rates of secretion observed in oocytes are not the consequence of competition for amphibian factors.

Since these experiments were performed, evidence showing that segregated proteins (zeins and ovalbumin) compete with one another for translation in oocytes (Richter and Smith, 1981) has been published. These authors show that mRNAs encoding zeins saturate the oocyte's translational machinery at much lower levels ( $< 10$  ng/oocyte) than cytosolic proteins ( $> 100$  ng/oocyte), and they show that this saturation may be partially relieved by the micro-injection of dog pancreas microsomes. This result, and those of Blobel, Dobberstein and their co-workers on the initiation of translocation (see introduction), implies that the translational saturation may be due in part to translocational limitations. Thus, since the authors show competition for translation between mRNAs encoding zeins and ovalbumin, it could be argued that they are in fact demonstrating competition for some component of the oocyte's translocatory apparatus. This is in apparent contrast to the data presented in chapter 4 which show that the non-parallel secretion of ovalbumin and lysozyme does not reflect competition for some component of the oocyte's secretory apparatus. This apparent contradiction cannot be resolved, since experiments designed to investigate translation/

translocation cannot easily be compared with experiments designed to investigate secretion. Nevertheless, certain differences between the two sets of data need to be explained. For example, the level of mRNA encoding zeins which Richter and Smith report as saturating the oocyte's translation/translocation machinery is much lower than the level of oviduct mRNA which we find to be saturating ( $> 120$  ng/oocyte, see chapter 4). Furthermore, the stability of mRNA in oocytes reported by these authors is much lower than the stability of oviduct and other mRNAs in our oocytes (see chapter 3). (However, the amounts of injected mRNAs coding for specific proteins cannot be directly compared because the precise degree of activity of the messengers used has not been established in either study). The choice of zeins by Richter and Smith with which to perform these studies further complicates the issue. Zeins are unusual proteins, forming dense, insoluble deposits within rER membranes (Hurkman *et al.*, 1981), and this may affect the secretion of other proteins.

In principle, the two sets of results need not necessarily be contradictory; proteins could compete for translation/translocation at levels which do not generate competition for subsequent components of the secretory pathway. However, the experiments described in chapter 4 were not designed to investigate this question and further experiments would be required to resolve the issue.



### 3. Oocyte-Specific Phenomena

One major concern when using a heterologous system of the type exploited for these studies is that any data obtained may only reflect the system used, and have no general relevance. Thus both the non-parallel secretion of ovalbumin and lysozyme and their different distributions on sucrose gradients during pulse-chase experiments might be oocyte-specific phenomena.

Non-parallel secretion of ovalbumin and lysozyme does not seem to occur in the oviduct. The proportion of ovalbumin and lysozyme in the egg (Warner, 1954) reflects their steady-state concentration in the tubular gland cell (the secretory cell) in the oviduct (Palmiter, 1972). However, when the oviduct mRNA used in these experiments is micro-injected into heterologous tissue culture cells (BHK cells) and ovalbumin and lysozyme localised within these cells by indirect immunofluorescence, the two proteins are differentially located (Garoff and Colman, personal communication). This result implies that these non-oviduct cells may also translocate oviduct proteins with non-parallel kinetics. This difference between oviduct and other cells may be explained by the nature of secretion from gland cells of the oviduct. Secretion appears to occur in response to the mechanical effect of an object passing through the oviduct magnum, since not only an ovum, but also a ping-pong ball (or a cockroach!) will stimulate secretion of egg albumen (Nalbandov, 1976). Thus, if a kind

of regulated secretion is occurring in the tubular gland cell, then by analogy with the pancreatic acinar cell the proteins should be exported in parallel. That they are exported in a non-parallel fashion from other cells supports the idea that parallel secretion merely reflects the final, regulating effect of short-term physiological control on the secretory process.

At present there is no evidence to show whether the retention of a substantial proportion of lysozyme within a high-density fraction of the oocyte is a cell-type-specific phenomenon or not. However, the oocyte does stockpile materials (such as ribosomes, mRNAs, mitochondria) for use in embryogenesis (see Lane, 1981), and most segregated proteins within the oocyte may be thus stored (Zehavi-Willner and Lane, 1977). Lysozyme may be stored, in error, with endogenous proteins (although it should be noted that the density of the storage compartment proposed by Zehavi-Willner and Lane (1977) does not correspond to that of the compartment in which lysozyme accumulates). In this context, it is of interest that lysozyme is a member of a family of widely distributed (Dixon and Webb, 1979) and highly conserved proteins (see Matthews *et al.*, 1981). Thus hen lysozyme might resemble an as yet unidentified lysozyme-like endogenous protein and be miscompartmentalised, in error, with this protein. It would be of interest to see whether guinea-pig  $\alpha$ -lactalbumin, a protein which shows homology to hen lysozyme (Brew, 1972) in amino acid sequence and is secreted slowly from oocytes (Lane *et al.*, 1980) is similarly accumulated by *Xenopus* oocytes.

#### 4. The Oocyte and Secretion: Future Prospects

It is clear, both from the introductory survey, and the work presented in this thesis, that the oocyte can be used to gain valuable information about the secretory process. However, now that technology enabling other cell types to be micro-injected with nucleic acids and proteins has been developed, does the oocyte have a future rôle? There are clearly problems with the oocyte; difficulties with cell fractionation, problems with glycosylation (for example, preventing the use of endo H), and the fact that internal events cannot be monitored by immunofluorescence on whole cells. These are balanced against the advantages of using such a large cell; the ease of culture and manipulation, and the fact that large amounts of exogenous protein can be produced by micro-injection, with much greater ease with the oocyte, than with tissue culture cells. However, the use of a viral system to introduce material into tissue culture cells (see Gething and Sambrook, 1981), although much less flexible, can overcome the problem of producing efficient material for biochemical analysis.

Despite the increasing number of alternative surrogate systems coming into use, I believe that there are reasons why the oocyte will continue to be of use. Firstly, it is always an advantage to employ more than one surrogate system, in order to be able to rule out cell-type-specific effects; also, the ease and speed with which results can be produced using the oocyte should favour its use as a supplementary system or as

a testing-ground for new investigations. Secondly, the oocyte's large size enables large objects such as organelles to be introduced. The injection of microsomal vesicles by Richter and Smith (1981) shows that this approach can be extremely productive. Finally, the robustness of the oocyte system enables different materials to be introduced into the same cell at different times using multiple injections (Colman et al., 1982). Thus I believe that although the oocyte is no longer the only system where the interaction of materials from different sources can be observed in a living cell, for some studies it will still be the system of choice.

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