The Mechanism of Transcellular Transport of Immunoglobulin G in Yolk-Sac Tissue.

by Leonard C. W. Seymour, BSc.

A thesis submitted to the University of Keele
in partial fulfillment of the requirements for
the degree of Doctor of Philosophy.

Biochemistry Research Unit,
University of Keele,
Staffordshire, U.K.

February 1985

I am indebted to a very great number of people who have helped, advised, assisted and threatened me over the last few years in the course of the work reported in this Thesis, and to thank them all individually would be to lengthen it considerably. In particular, though, I would like to thank my Supervisor, Dr. K.E.Williams, for his tireless assistance and endless patience, and my parents, Percy and Daisy Seymour, for their unnending support throughout the most difficult part of all, the writing up.

The work was supported by the S.E.R.C. in the form of a three-year postgraduate studentship.

ABSTRACT

The route of prenatal antibody transmission in both the rat and rabbit is via the yolk sac (Brambell, 1970), and a method for studying *in vitro* endocytosis in yolk sacs (Williams *et al*, 1975) was modified as a model for the process of transcellular immunoglobulin transport (Weisbecker, 1981). In this study, the validity of the model was substantiated and various investigations were performed.

The signal mediating specific protection *in vitro* was shown to reside in the F_c fragment of the IgG molecule, and carbohydrate moieties did not appear to be involved in the interaction.

Neuraminidase-treatment of IgG enhanced protection, while formaldehyde destroyed it. The inference was that the signal sequence was polypeptide in nature, contained a basic amino acid group, and was located close to one of the carbohydrate groups of the molecule, probably the asymmetric, hinge-linked one.

Various inhibitors were shown to inhibit equally uptake into the protective and degradative routes. The evidence suggested a common uptake stage in the uptake of IgG in both pathways.

Release of macromolecular material was found to be sensitive to metabolic inhibitors, but not so much to cytoskeletal ones, EGTA or bulk protein.

In contrast to the hypotheses of Brambell (1966) (where specific membrane-bound receptors are thought sterically to protect IgG from lysosomal degradation) and Wild (1975) (specific micropinocytic coated vesicles carry IgG from the plasmalemma to the basal or lateral membranes), it is proposed that IgG for protection enters common sorting vesicles along with other proteins (mainly destined for degradation) and fluid. It is sorted quickly (before the vesicle becomes too acid) and packaged into specific microvesicles that release their contents at the lateral or basal membranes. It is then free to make its way across the subcellular mesenchyme into the vitelline blood capillaries, and thence into the foetus intact.

CONTENTS

Chapter 1. General Introduction.

1.1 The cell membrane plays a vital role in regulating the behaviour of the eucaryotic cell. It has been implicated in controlling cell growth and division, in mediating cell-cell interaction and communication, in recognising hormones and regulating their responses within cells and in controlling the transport into and out of the cell of ions and small molecules (Knox, 1981). Its single most important function, however, is to provide a supportive but semi-permeable barrier separating cell organelles and the cytosol contents from the surrounding environment, thus allowing the maintenance of an isolated microenvironment to optimise cell processes, and to prevent the loss of certain metabolites and the entry of unwanted materials.

This leaves the cell with a substantial problem, however, with regard to the internalisation of desirable macromolecules that are too large to be transported across the membrane by specific protein carriers. It is essentially for this purpose that the process of endocytosis exists.

1.2 Endocytosis.

The term endocytosis describes uptake into the cell of extracellular material by any process that involves internalization of surface membrane to form vesicles. In many cases vesicles thus formed are involved in fusion with the lysosomal system, resulting in degradation of the endocytosed substrates. The degradation products may be used in cell metabolism (e.g. protein synthesis). However, some substrates taken up are directed toward different subcellular fates (e.g. the transport of intact proteins across the cell).

Endocytosis has traditionally been divided into two categories - phagocytosis ('eating by cells') and pinocytosis ('drinking by cells'). Phagocytosis was first observed using the light microscope, one hundred years ago in leucocytes (Metchnikoff, 1883) and was seen to involve the extension of folds of cell membrane around the prey, eventually surrounding it and fusing together, leaving it enveloped in a vesicle formed from plasmalemma-derived membrane. Pinocytosis, on the other hand, is probably a constitutive process in most mammalian cells, rather than a response to stimulation of the plasma membrane. Simple vesicle formation is sufficient to trap extracellular fluid, which is internalized together with any dissolved materials. Macromolecular material thus internalized

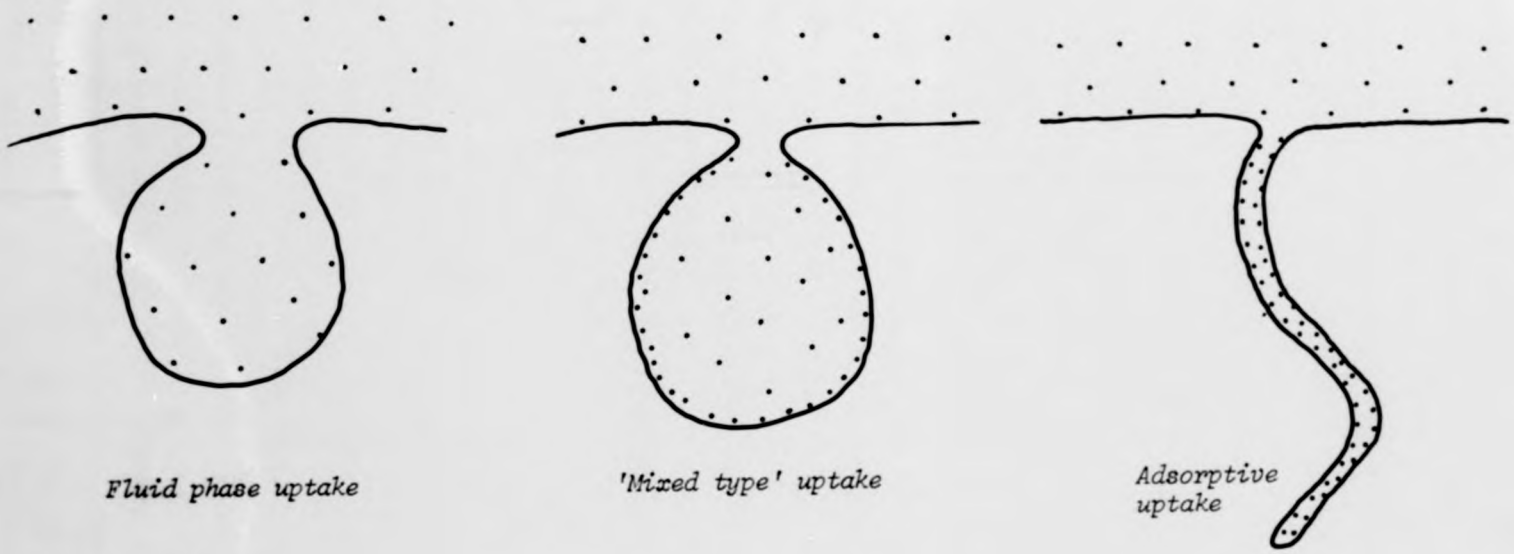
normally remains within the cell and becomes concentrated within vacuoles, while water and solutes of molecular weight less than approximately 250 can escape across the membrane of the vacuoles to the cytosol, and thence to the cell exterior. The captured prey, whether it be solid or solute is sent to its subcellular destination, usually the lysosomal system.

Despite the differences in definition between phagocytosis and pinocytosis, the two processes are thought to be mediated by essentially similar cellular mechanisms (Jacques, 1969). However, further observations of uptake have suggested that the process of endocytosis has more subdivisions than simply into solid or liquid substrate uptake. The first hint of the inadequacy of a division of uptake into simply phagocytosis or fluid-phase pinocytosis was communicated in the almost prophetic observations of Bennett (1956) in which he states : 'solid or soluble preys frequently adsorb at the surface of the phagocyte before being engulfed, whereas solutes and small particles can also be endocytosed merely owing to their presence in the extracellular fluid, when the latter is taken up in bulk'. The different mechanisms of substrate internalization will now be considered.

When a substrate interacts with the cell membrane prior to endocytosis, its rate of uptake is usually elevated relative to that of a substrate captured entirely by fluid-phase pinocytosis. Such interactions can be of a variety of types ranging from almost non-specific to highly specific. Most cell surfaces carry a net negative charge and hence positively charged substrates will adsorb to the plasma membrane and exhibit elevated rates of uptake (Kooistra et al, 1980). Similarly, hydrophobic substrates tend to be endocytosed faster than hydrophilic ones as a result of their interacting with hydrophobic regions of the plasma membrane. On the other hand, the interaction may be highly specific, for example the low-density-lipoprotein receptor on many cell types has a very high substrate specificity (Anderson et al, 1982). Between these two extremes lie a number of receptors possessed of a wider specificity, most notably the galactose receptor of hepatocytes (Pricer and Ashwell, 1971) that is responsible for the fast clearance from the bloodstream of a number of different asialoglycoproteins.

When adsorptive endocytosis is via cell-membrane receptors specific for certain substrates, then the process is renamed 'receptor-mediated

Figure 1.2.1 Models for different types of endocytosis.



Rate of uptake =

(a) Fp

(b) $Fp + \frac{SRp}{K + p}$

(c) $\frac{SRp}{K + p}$

R = adsorptive capacity S = rate of membrane engulfment K = efficiency of adsorption

F = fluid uptake rate p = substrate concentration

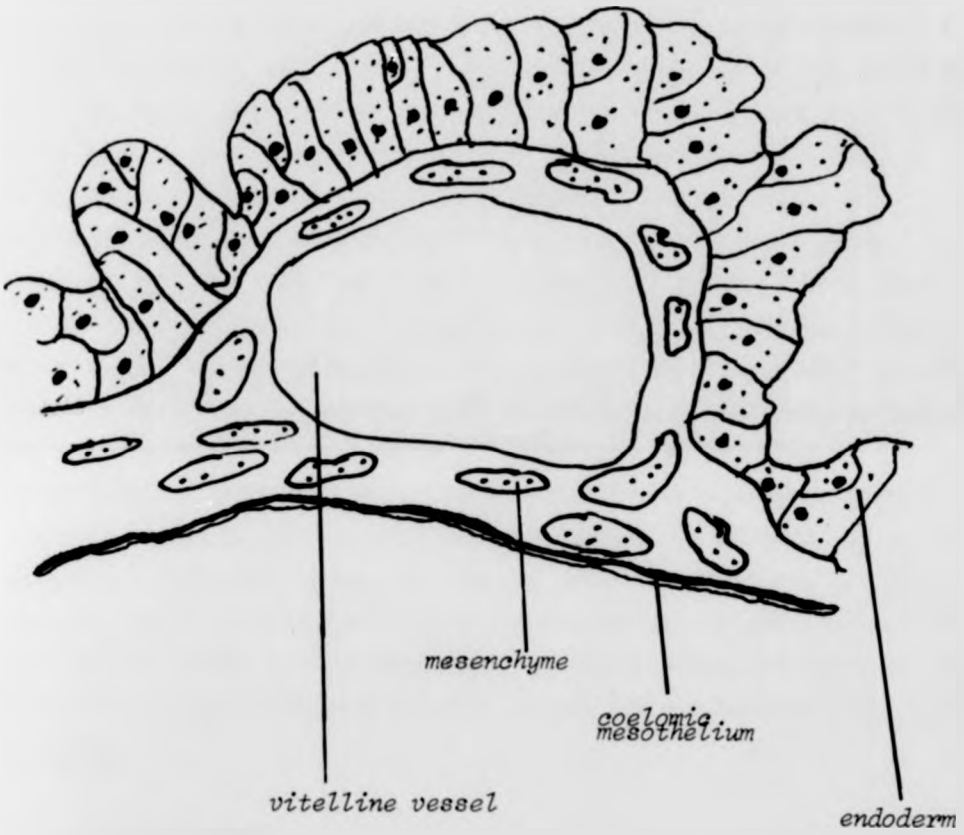
endocytosis'. This mode of uptake is generally observed for substrates intended for a specific fate rather than for degradation. For example, insulin activation of cells involves its receptor-mediated entry into the cell (Desbuquois et al, 1979), similarly the uptake of IgA into hepatocytes in its transcellular transport to the bile involves a specific receptor (Mullock and Hinton, 1981). (This latter is an example of the process of 'diacytosis', comprising endocytosis of the substrate, followed by transcellular transport and release at another surface of the cell).

The observed rates of endocytosis of substrates depends on a multitude of factors, including the molecular size and concentration of the substrate, together with other factors that may be hard to define. Moore et al (1977) observed a range of rates of uptake even for different preparations of the same protein (BSA), where the important discriminating factors were too subtle to be completely understood.

The opposite of adsorptive endocytosis, involving repulsion between substrate and cell membrane, leading to a rate of uptake lower than would be expected by simple pinocytosis, has been suggested. Such a system is referred to as 'repulsive endocytosis' (Jacques, 1969) but is not well documented. However, there is a substantial number of experimental observations that can possibly be explained in terms of this type of interaction. For example, Haxhe and Lesteus (1965) observed that the addition of heparin completely abolished the uptake into liver cells of denatured protein apparently without affecting pinocytosis of colloidal gold. It seems that repulsive endocytosis may be an important physiological process in terms of prolonging the lifetime of functional proteins, but it has been little studied in comparison with adsorptive endocytosis since in practice it is difficult to differentiate from effects that arise from competitive binding of a solute to the binding sites on the cell or from the formation of soluble complexes with different adsorptive properties.

Jacques (1969) produced a good review and a comprehensive, though simple, mathematical analysis of the various basic mechanisms of uptake. He was also one of the first to suggest the possibility that adsorptive or receptor-mediated endocytosis could proceed in the absence of fluid uptake (Fig. 1.2.1), although in theory to invaginate membrane without engulfing any fluid would seem extremely difficult.

Figure 1.3.1. Diagrammatic representation of section through rabbit yolk sac.



Representation of part of the yolk-sac splanchnopleur of a 24-day rabbit foetus, showing a large vitelline vessel in the mesenchyme, the endodermal epithelium of large cells on the outside next the uterine lumen and the thin squamous mesothelium on the inside next the exocoel.

Adapted from Brambell (1970).

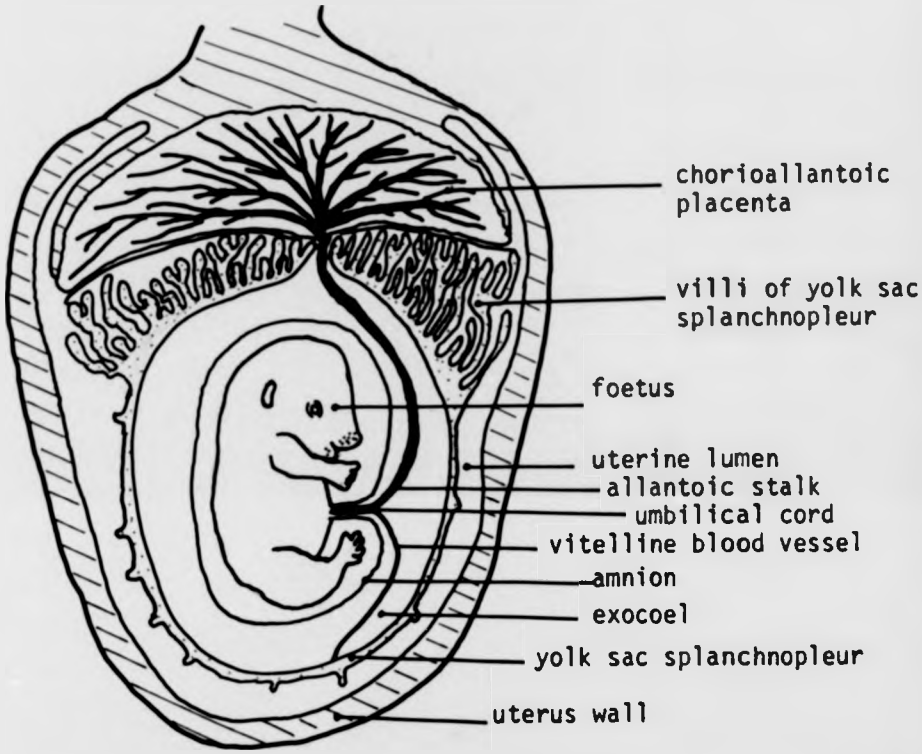
When Jacques published his review of endocytic mechanisms there was a dearth of hard, quantitative evidence available with which to check the relevance of his proposals. This was mainly because of the difficulties involved in *in vivo* experimentation in which substrates are invariably exposed to a variety of cell types and their concentration in the blood declines during the course of the experiment. The wide distribution of cells even of a single type relative to the circulatory systems is another source of complication, and, in practice, *in vitro* systems have been the major contributors to our understanding of endocytic processes. There have been two major types of *in vitro* investigation into endocytosis. The first, involving morphometric analysis of an electron-dense substrate or reaction product, requires complex interpretation of light or electron microscope pictures that show only one section of the cell at one time. The interpretation must take account of rates of release of material and the fact that the number of endocytic vesicles observed depends not only on the rate of endocytosis but also on the rate of fusion of vesicles. A rather more direct line of investigation is by the use of radioactively-labelled substrates, and this is the basis of the methodology developed by Williams *et al* (1975a and b) to study endocytosis in the rat yolk sac incubated *in vitro*. The yolk sac was the tissue of choice mainly because of its highly endocytic nature, its ready availability (each pregnant animal contains a large number of similar tissues) and its reproducible behaviour when incubated *in vitro*.

1.3 The Yolk Sac.

The word 'placenta' was redefined by Mossman, (1937) to mean 'an opposition or fusion of the foetal membranes to the uterine mucosa for the purpose of exchange', and by this criterion species such as the rabbit, guinea pig and rat are possessed of two effective placentae. In addition to the more familiar chorioallantoic placenta, which functions in essentially similar fashion to that in the human, the yolk-sac splanchnopleur (and possibly other foetal membranes too) fall into this category, being important routes of maternofoetal transmission of macromolecules and nutrients.

The outer surface of the rat yolk sac is composed of a layer of columnar epithelial cells whose microvillous membrane is bathed in uterine fluid (Fig. 1.3.1). The cells are joined together by tight

Figure 1.3.2 Gross arrangement of the foetal membranes of the 17.5 day rat.



Adapted from Brambell, 1970.

junctions that prevent the passage of material between them. They are attached to a fibrous basement membrane below which is a layer of mesenchyme containing collagen fibres, fibroblasts and macrophages as well as the vitelline blood capillaries, which are of foetal origin and connected to the foetus via the yolk-sac stalk (Brambell, 1970). Hence the foetus is possessed of three essentially different blood circulations, two of which are external and concerned with maternofoetal exchange.

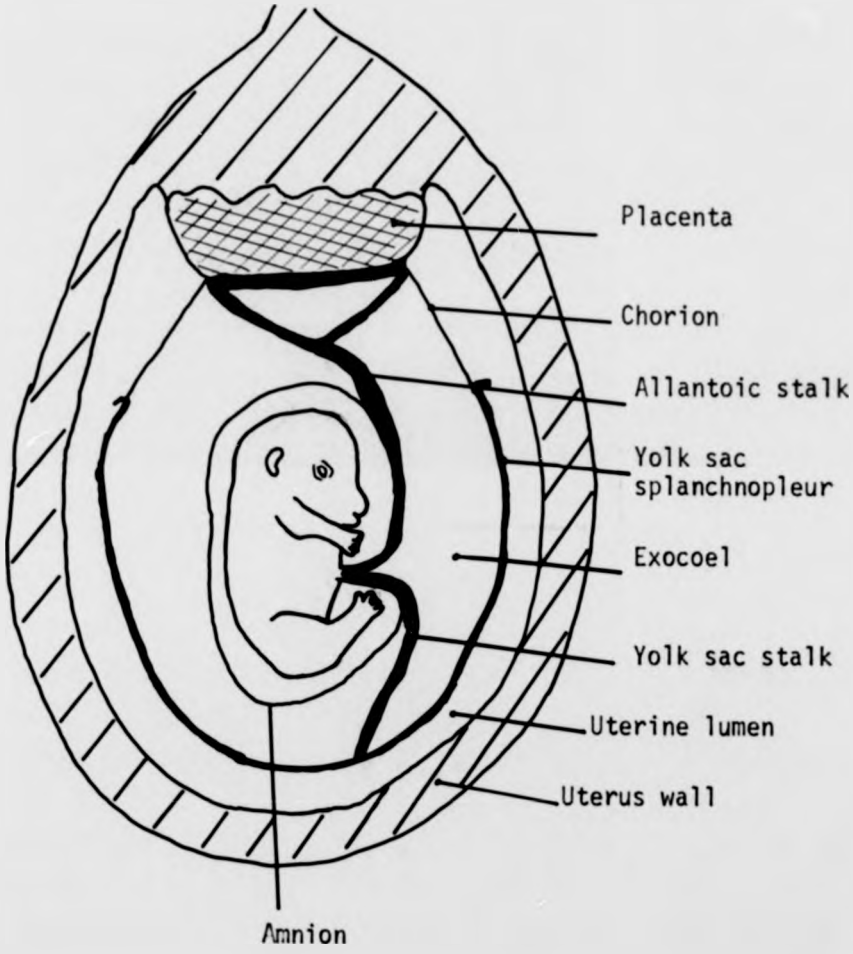
The rat yolk sac is composed of two main regions. The part adjacent to the chorioallantoic placenta (approximately one third of the total) is more highly villous than the remainder (Fig. 1.3.2) and is thought to be more active in endocytosis. This zonal feature differs in the rabbit, in which the highly villous region is absent, being replaced by the paraplacental chorion. Evidence (Wild, 1965) suggests that this region of the rabbit yolk sac is non-selectively permeable to material contained in the uterine fluid, and that this explains the difference between the rat and rabbit in the concentrations of materials, in particular antibodies, found in the exocoel and amniotic fluid (Wild, 1974). An alternative explanation for the apparent absence of antibodies from rat amniotic fluid is that the rat is able to absorb IgG via the foetal gut, an ability not shared by the rabbit. Hence any IgG reaching the amniotic fluid may be readily taken into the foetus, leaving the fluid depleted.

Surrounding the foetus itself, in both species, is a thinner membrane, the amnion, containing the amniotic fluid and believed to allow the passage of material by a system of molecular ultrafiltration that prevents the passage of very large molecules (Wild, 1974).

The pinocytic nature of the rat yolk sac, which may play a key role in its placental function, has allowed its exploitation for the study of endocytosis and protein catabolism by using it in the *in vitro* incubation system, developed by Williams *et al* (1975 a & b) and described more fully in Section 2.2.2.

1.4 Application of the in vitro incubation system to the study of maternofoetal immunoglobulin transmission.

Figure 1.3.3 Gross arrangement of the foetal membranes of the 24-day rabbit.



Adapted from Brambell, 1970.

It was first noted by Ibbotson (1977) in the rat system and then confirmed by Weisbecker (1981) in both rat and rabbit systems, that homologous IgG behaves in a special manner under in vitro incubation conditions. Unlike other proteins, homologous ^{125}I-IgG has the ability to be taken into these tissues by endocytosis and subsequently to be released in a macromolecular form, while all other proteins tested are invariably released only as degradation products. In view of the known route of maternofoetal transmission of intact antibodies (via the yolk sac) in both the rat and the rabbit (Brambell, 1970), while most other proteins are degraded within this tissue in vivo, the following question was posed. Does protection from degradation in the in vitro system reflect the process of maternofoetal transmission in vivo?

Preliminary experimentation by Weisbecker (1981) supported the suggestion that protection in vitro does mimic transmission in vivo. She reported that the rank order of protection in vitro of various heterologous IgGs was essentially the same as the rank order of transmission in vivo (Table 1.4.1) and that rabbit yolk sacs exhibit the capacity for more protection of IgG than do rat yolk sacs. These findings correlate well with the known importance of the route of transmission of antibodies in the two species. Antibody transmission in the rabbit occurs entirely via the yolk sac, while in the rat the majority of transmission occurs postnatally via the gut (Table 1.4.2). If in vitro protection reflects the in vivo transmission process then the in vitro system may prove useful for the investigation of the nature of the transmission mechanism. Studies with an in vitro system have many advantages over in vivo work, all essentially derived from the relative simplicity of the experimental design. The main disadvantages associated with an in vitor incubation system arise mainly from the absence of a functional blood circulation. This may cause an accumulation of material normally released into the vitelline circulation, which material may then be released in vitro by non-physiological mechanisms and routes. Therefore, interpretations of results, and in particular any observations on sites of release of material from cells, must be dealt with very cautiously.

1.5 Maternofoetal transmission of immunoglobulins.

The routes and times of transmission of passive immunity from mother to offspring vary between mammalian species (Table 1.4.2). By ligaturing

Table 1.4.1 Comparison of the rank orders of IgG transmission in vivo and of protection in vitro.

Rank order of maternofoetal transmission of various IgGs

Rabbit > Human > Guinea-pig > Canine > Bovine

(Batty et al, 1954)

Rank order of intact release from incubated yolk sacs of various IgGs.

Rabbit > Human > Rat > Bovine

(Weisbecker, 1981)

the yolk-sac stalk, Brambell elegantly demonstrated (1966) that the whole of such prenatal transmission in the rabbit occurs via the yolk sac, a similar route being important also in the rat (Brambell, 1970). Exactly how antibody molecules can pass from the uterine fluid into the vitelline circulation without being degraded in the lysosomal system has long been a matter of contention. It is evident and widely accepted that antibodies (in particular, IgG) must be possessed of some special feature that causes them to be specifically recognised by the tissue and treated in a special way. Using electron micrographic techniques, Wild (1970) has shown that the site of such selection is at the outer layer of epithelial cells, and it is also here that lysosomal degradation of nutrient proteins occurs. Chronologically, the first hypothesis of the mechanism for intact transcellular transport of immunoglobulins was proposed by Brambell (1966) and will hereafter be referred to as the 'Brambell Hypothesis'. According to this view (Fig. 1.5.1) immunoglobulins are internalised in the same pinosomes as other proteins destined for degradation. On the inside surface of the pinosomes are a number of receptors that are specific for immunoglobulins and these interact with some of the internalised antibodies, causing them to become closely associated with the vesicle wall. Upon fusion with the lysosomal system, the majority of contained substrate proteins are degraded, but the immunoglobulin bound to specific receptors is sterically protected from attack by lysosomal enzymes. According to the original hypothesis, following the completion of lysosomal degradation and diffusion out of the secondary lysosome of the degradation products, the material remaining inside the vesicle, including the protected immunoglobulin, is released at the lateral or basement membranes. The antibody then makes its way unhindered through the mesenchyme and vitelline epithelial cells into the foetal blood circulation.

A major alternative hypothesis proposed by Wild (1975) is supported by electron microscopic evidence. The 'Wild hypothesis' involves micropinocytic clathrin-coated vesicles (for discussion see the Introduction to Chapter 7) in the specific diacytosis of IgG (Figure 1.5.2). According to Wild, these coated vesicles form at the plasmalemmal surface of the epithelial cell, contain IgG only, and make their way across the cell eventually fusing with and releasing their contents at the lateral or basement cell membranes. There is no direct involvement with the lysosomal system at all, and Wild postulated that one of the functions of the protein coat might be physically to prevent fusion with

Table 1.4.2 General features of transfer of passive immunity in different mammalian species.

	Time of transfer		Route of transfer (where known)	Duration of period of transfer
	Prenatal	Postnatal		
Ox	0	+++	Gut	24 hours
Pig	0	+++	Gut	24-36 hours
Goat	0	+++	Gut	24 hours
Sheep	0	+++	Gut	24 hours
Horse	0	+++	Gut	24-36 hours
Wallaby	0	+++	Gut	180 days
Dog	+	++	Gut	1-2 days
Cat	+	++	Gut	1-2 days
Hedgehog	+	++	Gut	70 days
Mouse	+	++	Gut Yolk sac	16 days -
Rat	+	++	Gut Yolk sac	20 days -
Guinea Pig	+++	0	Yolk sac	-
Rabbit	+++	0	Yolk sac	-
Grey squirrel	+++	0	Unknown	-
Man	+++	0	Chorioallantoic placenta	-
Rhesus monkey	+++	0	Chorioallantoic placenta	-

Modified from Brambell, 1970.

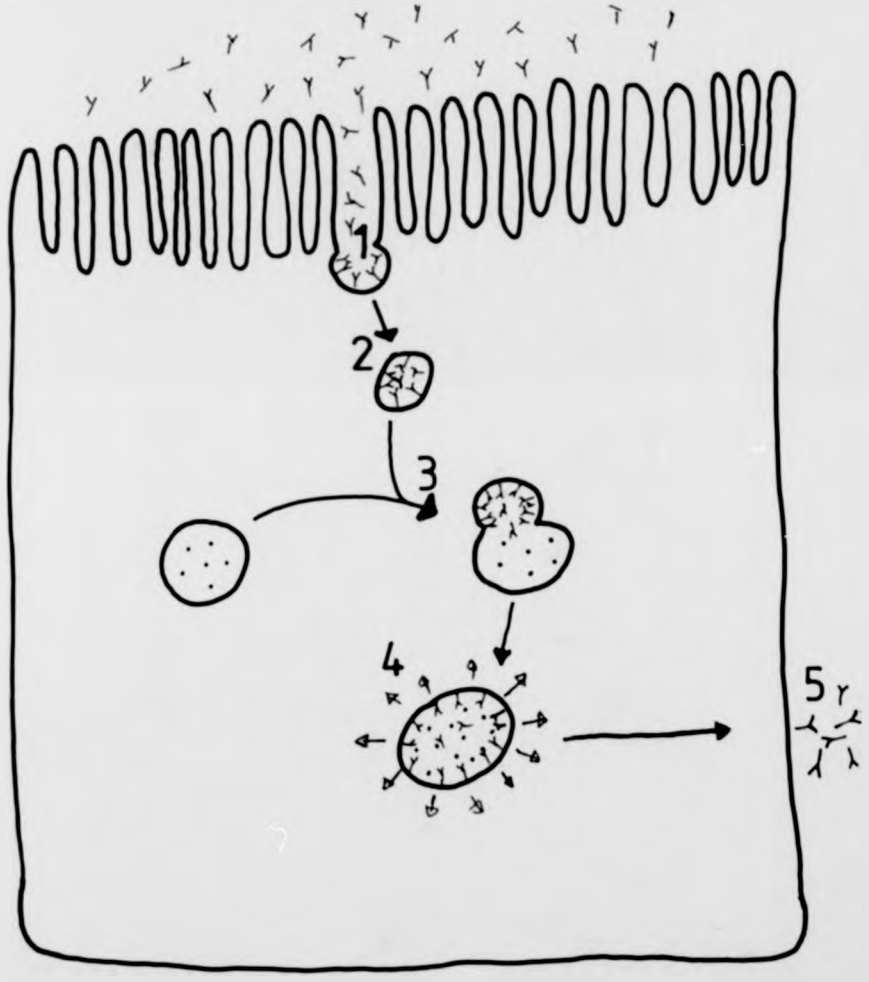
the lysosomes.

The original hypothesis of Brambell appears to be no longer tenable, however, following the demonstration (Weisbecker, 1981) that protection of material from lysosomal degradation (in this particular case, a non-degradable substrate , ^{125}I-PVP, was used) is not sufficient to cause its release from the tissue (an alternative experiment involved the use of ammonium ions to increase lysosomal pH, and thus inhibit proteolysis, but this also failed to increase the amount of degradable substrate released from the tissue in macromolecular form). The exit of ^{125}I-IgG from cells from cells appears to be a specific process, and a modofocation to the original Brambell hypothesis was proposed by Hemmings (1976) to allow for this. According to this hypothesis, molecules for degradation and also molecules of IgG in the diacytic pathway are taken into the cells in the same vesicles. Following rupture of these vesicles, IgG specifically is released intact at the lateral or basement membranes. The only evidence in support of this hypothesis to date is a series of electron micrographs showing ruptured vesicles inside the cell, and it fails to explain the mechanism of the specificity of diacytosis. In addition it must be questioned how the lysosomal degradation system can function efficiently when nutrient molecules are apparently dispersed in the cytoplasm. This is a relatively new hypothesis, however, and experimental support for it may be forthcoming in the future.

Another hypothesis has been recently proposed by King (1982a). This is supported by electron micrographic evidence and is essentially a derivative of the hypothesis of Wild. According to King, clathrin-coated vesicles specifically carry IgG to the lateral cell membrane. The vesicles may form from coated pits on the surface membrane, or may bud off from saccular invaginations that may still be attached to the surface or may have become internalised. Material remaining within these saccules is thought to be degraded within the lysosomal system. Essentially the main difference between this hypothesis and that of Wild (1975) is that the formation of microvesicles specific for IgG-transport is seen as perhaps being an intracellular event, rather than occurring solely on the cell surface.

Among these various hypotheses lie a multitude of possible hybrid alternatives. The purpose of this study was to attempt to find answers to certain discriminating questions in order to shed some light on the mechanism of transmission of passive immunity across the yolk sac.

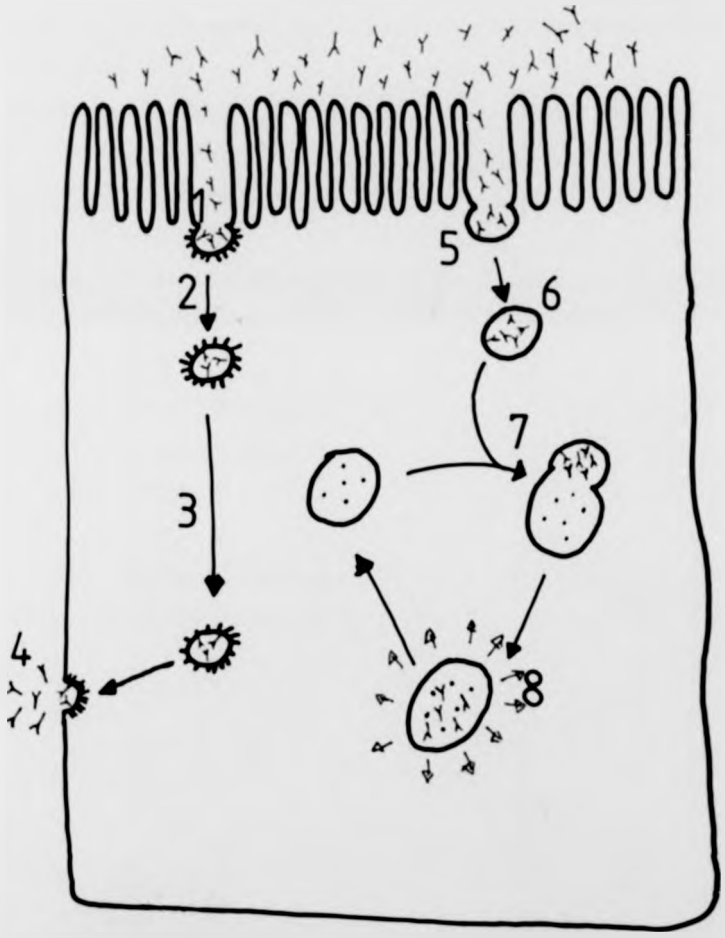
Figure 1.5.1 Brambell's hypothesis for the mechanism of diacytosis of IgG.



1. *IgG for transport adheres to specific receptors at the base of the microvilli.*
2. *Bound IgG is internalized in the same vesicles as other proteins, destined for degradation, and a certain amount of unbound IgG.*
3. *The endocytic vesicles fuse with lysosomes and all free proteins are degraded by lysosomal enzymes. Bound IgG is protected from degradation.*
4. *Degradation products diffuse through the lysosomal membrane into the cytosol.*
5. *Intact IgG is released at the* lateral cell membrane.

Figure 1.5.2 Wild's hypothesis for the mechanism of diacytosis of IgG.

1. *IgG for transport adheres to specific receptors at the base of microvilli.*
2. *IgG only is internalized in micropinocytic Clathrin-coated vesicles.*
3. *Coated vesicles traverse the cell without contact with the lysosomes.*
4. *Following fusion of the coated vesicles with the lateral or basal membranes, IgG is released intact.*



5. *Proteins for degradation are taken up in a separate class of vesicles that do not possess a Clathrin coat.*
7. *The vesicles fuse with lysosomes, and the contained proteins are degraded.*
8. *Degradation products are released into the cytosol.*

The different facets of the overall investigation are arranged in chapters in the following manner:

Chapter 2 decribes the standard materials and methods and gives a comparison of certain basic results with typical results from previous work.

Chapter 3 investigates the relevance of the *in vitro* protection process to *in vivo* transmission, as well as investigating the incubation system in greater detail.

Chapter 4 attempts to identify the precise part of the antibody molecule that mediates its protection *in vitro*.

Chapter 5 questions whether carbohydrate is involved in the specific recognition process.

Chapter 6 tries to discriminate between the degradative and protective routes of proteins through the cell by the application of various inhibitors.

Chapter 7 uses subcellular fractionation and related techniques to investigate preliminarily the nature of the IgG-protecting compartment of the cell.

The findings from each chapter are gathered together and collectively discussed in the General Discussion, Chapter 8.

Chapter 2. Materials and Methods.

INTRODUCTION

A simple, reliable and reproducible system for the quantitative study of pinocytosis was described by Williams et al (1975a & b). The tissue used was the 17.5-day rat yolk sac, its attributes being its highly endocytic activity, its accessibility by only a simple dissection procedure and the large number of similar tissues obtained from one animal. The system was developed because at the time there was a severe lack of quantitative data available for the investigation of endocytic processes. The same group has now gathered a large amount of data leading to a deeper understanding of the various mechanisms of protein uptake and degradation in the rat yolk sac (e.g. Livesey et al, 1980).

Most proteins are not released in macromolecular form from the incubated rat yolk sac after ingestion, and the rate of appearance of the metabolically-useless ^{125}I-tyrosine in the incubation medium can be readily determined. By measuring the rate of release from the tissue of ^{125}I-tyrosine, together with the level of radioactivity retained within the yolk sac, the rate of uptake of a protein substrate into the tissue can be calculated. The assumption is made that iodotyrosine is not used for the synthesis of new cell proteins, nor adheres to cell membranes or proteins. This assumption is discussed further in Section 2.2.5 and Chapter 3.

The observation (Williams & Ibbotson, 1979) that ^{125}I-labelled homologous gamma globulin had the ability to enter the rat yolk sac and be released again, apparently undegraded, into the medium, as well as undergoing normal degradation, suggested the possibility that the display of such a 'protective' mechanism by the in vitro yolk sac might be related to the process of transcellular transmission of IgG in vivo from mother to foetus.

U. Weisbecker (1981) extended the findings by the use of rabbit yolk sacs, which are more important sites of maternofoetal transmission of IgG than rat yolk sacs (Brambell, 1970), and found that they behaved well under the same conditions of incubation.

The following pages describe the details of the "traditional" system, and give some information on modifications made to the system for the purposes of this study together with various other techniques employed.

Some specialised procedures are presented in the appropriate chapters. The Results Section presents typical data obtained with the system and is a mixture of work performed both in the present study and previously, as indicated.

MATERIALS AND METHODS.

2.2.1 Radiolabelling

Various methods of radioiodination of tracer proteins were compared in terms of cost, efficiency and damage caused (See Chapter 3) and eventually a modified version of the chloramine-T method (Greenwood et al, 1963) was adopted for routine use. Sometimes the following procedure was scaled down, because of the small amount of the protein substrate available, but for most proteins 10mg was dissolved in 5ml of 0.05M phosphate buffer, pH 8.0, in a sterile bottle and stirred, magnetically, in an ice bath. A solution of ^{125}I-iodide (10 ul; 1 m Ci at reference date) was added using a Hamilton Syringe and the reaction mixture stirred for 2 mins before 1 ml of chloramine-T solution (1 mg/ml) was added. After 8 min, sodium metabisulphite (0.75 ml, 2mg/ml) was introduced to terminate the reaction. Stirring was continued for a further 2 min and then solid "cold" potassiom iodide (approx 100mg) was introduced to dilute and facilitate removal of the remaining free, ^{125}I-iodide. The mixture was then transferred into 0.25" dialysis tubing and dialysed at 4°C against frequent changes of 1% NaCl until virtually all of the free iodide was removed. The solution was then put in sterile 5ml bottles and frozen at minus 20°C. Protein concentration was determined either by the method of Lowry et al (1951) or by the extinction at 280 nm using a standard of albumin dissolved in saline (1% w/v).

Meanwhile, a small sample of the pre-dialysis mixture was diluted in a large volume of medium TC199 containing 10% calf serum and 1ml samples were counted in a gamma-counter. The samples were then treated with trichloroacetic acid (TCA), centrifuged (2000 g min) decanted into a clean disposable tube and the supernatant counted (See Section 2.2.5). Application of the relevant correction factor (dependent on the particular counter being used, exact counting geometry etc., Section 2.2.6) permitted simple estimation of the degree of incorporation of ^{125}I into protein i.e. the labelling efficiency.

2.2.2 Yolk-sac incubation method.

The method of Williams et al (1975) was used with slight modifications. Virgin female Wistar rats from an inbred colony were caged separately in grid cages and left overnight with a stud male. The

appearance of a sperm plug at the bottom of the cage next morning was taken as an indication of pregnancy. Mating was assumed to occur at midnight (0 days), and at 17.5 days the females were killed by either asphyxiation in CO_2 or by cervical dislocation.

Working as quickly as possible to prevent tissue death through anoxia, the uterus was exposed and removed. It was placed in a petri-dish containing medium 199 that had previously been gassed with a mixture of 95% O_2 : 5%CO_2 and warmed to 37°C. The uterus was then opened and the yolk sacs removed and freed from other foetal membranes - in particular the amnion - before being incubated separately at 37°C in sterile 50ml Ehrlemeyer flasks, each of which contained 9.0 ml of medium 199 under 95% O_2 : 5%CO_2 and were stoppered with sterile silicone rubber bungs. The flasks were incubated in a shaking water bath (thermostatted at 37°C) and at 100+4 strokes per minute, with a stroke length of 3.8cm.

The yolk-sacs were allowed to equilibrate for 15 min and then the radioactive substrate, dissolved in 1.0 ml of medium 199, was added. The flasks were regassed with 95%O_2 : 5%CO_2 and incubation was continued for up to 6.5h. The incubation of each individual flask was stopped at different times, the medium sampled and the yolk sacs removed, washed three times in ice-cold 1% saline for 2 min each, blotted dry and inserted into a 5ml volumetric flask for storage at -20°C.

2.2.3 Assay of radioactivity contained in yolk sacs.

Normally no distinction was made between different chemical forms in which radioiodide was present in the yolk sac after incubation, and simply the total contained radioactivity was determined. The yolk sacs were dissolved in a total of 5.0ml of 1M NaOH (37°C, 1h), and duplicate 1.0ml samples were taken and counted on a gamma counter in 3ml disposable plastic tubes (LP3, Luckham Ltd., Burgess Hill, Sussex).

In those cases in which information was required concerning the amounts of TCA-soluble and TCA-insoluble radioactivity, yolk sacs were homogenised in 2.5ml distilled water using a teflon-on-glass homogeniser. A portion of the homogenate (1.5ml) was taken and diluted to 3.0ml with 0.2% Triton-X100, before being counted as above and then precipitated with TCA and the supernatant counted (Section 2.2.9). A further 0.5ml of the homogenate was treated with 0.5ml 2M NaOH for 1h at 37°C, and then

used for protein estimation (Section 2.2.4).

2.2.4 Estimation of protein content of yolk sacs.

The protein content of the yolk sac was determined by the method of Lowry et al (1951). Each yolk sac was dissolved in 5.0ml 1M NaOH, and duplicate 0.1ml portions were diluted with NaOH (1M, 0.4ml) and distilled water (0.5ml), to a total volume of 1.0ml. These samples were treated with 5.0ml Folin A solution and, after standing for 20min, 0.5ml Folin-Ciocalteau reagent was added. Vortex mixing was thorough at each stage since the reaction is very dependent on pH, and poor mixing can give misleading results. Extinctions were read against a water blank at 750nm and compared with those from a calibration curve constructed by using BSA as a standard. The protein content of each yolk sac could thus be expressed as an equivalent number of mg of BSA.

2.2.5 **Assay for medium-contained radioactivity.**

At the end of the incubation, the medium present in each flask was sampled (2 x 1.0 ml) and counted in 3ml Luckhams tubes in a gamma-counter. By summing the amounts of radioactivity contained in the medium and the amount detected in the yolk sac, for each yolk sac on an individual basis, good estimates can be made of the total amount of radioactivity originally present in the incubation medium contained in each flask. This allows more accurate assay of the rate of fluid-phase pinocytosis than would be possible by assuming the same initial substrate concentration to be present in each flask, since that assumption allows small errors in substrate-pipetting to be reflected in the result.

If a ^{125}I-labelled substrate is degraded in the yolk-sac tissue, then some radioactivity may be present in the medium in the form of degradation products, in particular ^{125}I-L-tyrosine that has been produced by intracellular enzymatic degradation of the parent macromolecule. (Iodotyrosine is not incorporated into new proteins inside the cell (Hughes-Ryser, 1963), and diffuses out very quickly.) This degraded material must be taken into account when estimating the rate of uptake of the substrate by the tissue. A simple and quick way to estimate the quantity of medium-contained protein that has been degraded is to precipitate in 7% TCA. (Iodotyrosine is soluble in TCA, while most proteins and polypeptides are not.) To ensure complete precipitation of a

^{125}I-labelled tracer protein, it is essential that the medium contains sufficient non-radioactive protein to form a sizeable precipitate. Addition of serum to a final concentration (v/v) of 10% ensures this, thus in serum-free medium, 0.1 ml/ml of calf serum was routinely added before the addition of 0.5ml TCA (20%, w/v) solution.

After precipitation the samples, still in the 3ml Luckhams tubes, were centrifuged for 20min at 2000 rpm (MSE, 4L), and the supernatants decanted into fresh Luckhams tubes for recounting. The total volume of the supernatant will be greater than 1ml, however, and the observed count must be normalised to allow for this since the gamma-counter is more efficient when measuring small volumes. This correction factor will be dependent on at least three factors: (a) the geometrical features of the detector of the individual gamma-counter (b) the precise technique of decanting the supernatant and (c) whether the original medium contained 10% calf serum, or whether this was added just before the TCA (Section 2.2.6).

The TCA-soluble radioactivity that appears in the medium during the incubation period was assumed to have been released from the yolk sac, and was taken into account when estimating rates of uptake and degradation. It should be noted that if ^{125}I-iodotyrosine is able to associate with macromolecules or membranes, then the results obtained may be misleading since they would underestimate the amount of ^{125}I-iodotyrosine released from degraded protein. Such a phenomenon has been proposed by Udall *et al*, (1981), but to date there has been no firm indication that it is important in this system, and the frequently-observed linear rise in the amount of free ^{125}I-tyrosine detected with time suggests that the possibility is not crucial. It should, however, be borne in mind, and is discussed further in Chapter 3.

2.2.6 Estimation of correction factors.

As mentioned in the previous section, upon precipitation of 1.0ml samples with TCA and centrifugation, the supernatant volume of the sample increases to about 1.3ml. In order to permit meaningful comparison of observed cpm, a correction factor must be determined that will convert the count obtained from the larger sample to the count that would have been obtained if the same amount of radioactivity had been contained in 1.0ml of solution.

The correction factor is best determined by using a radioactive substrate that is completely soluble in 7% TCA, in this case ^{125}I-L-tyrosine. Six samples (1.0ml) of medium 199 containing ^{125}I-L-tyrosine (with and without 10% calf serum) were counted on the gamma counters used and precipitated, centrifuged and recounted in the usual way. It was assumed that no iodotyrosine was precipitated by the TCA, and that the volume of fluid entrapped in the pellet was constant.

Hence the correction factor required is the factor that will convert the count obtained from the second sample (after subtraction of background) to that obtained from the first, and this can be calculated by simple division.

Results were as follows:

Gamma counter	Medium contains calf serum (10%) throughout	Calf serum (0.1ml) added just prior to precipitation
Packard (Selectronik)	1.32	1.34
Packard (Modumatic 6) (newer)	1.22	1.26

These values were used as the appropriate correction factors throughout this thesis.

2.2.7 Incubation of rabbit yolk sacs.

The incubation system used for rabbit yolk sacs was essentially the same as that used for rat tissue (Section 2.2.2).

Californian White rabbits were mated and copulation was observed. On the 24th day of pregnancy the female was killed by cervical dislocation and the uterus removed as quickly as possible. Each yolk sac was dissected out and separated from the foetus, chorioallantoic placenta, paraplacental chorion and amnion in warm, gassed medium 199 (sometimes containing 10% calf serum, depending on the individual experiment).

Rabbit yolk sacs are substantially larger and rather more delicate than rat yolk sacs, and each was cut into 4-5 pieces of roughly equal size. Each piece was then treated in the same way as a complete rat yolk sac.

Weisbecker (1981) performed a detailed comparison of the viability of rabbit tissue and rat tissue in the incubation system. Both appeared to give reproducible and useful results.

2.2.8 Method of calculating the rate of uptake of ^{125}I-PVP.

In order to obtain an estimate of the rate of accumulation of a radiolabelled substrate that is not degraded by yolk sacs, a number of sacs were incubated for different lengths of time in medium 199, and the total amounts of radioactivity present in each yolk sac and the amount remaining in the medium were measured. To permit comparison of results, a unit of uptake was required that would enable uptake to be normalised for differences in size of yolk sacs. It is also desirable to avoid the need to correct the observed cpm values for a short-lived isotope like ^{125}I for decay of radioactivity. Williams *et al*, 1975(a) expressed uptake in units of 'the volume of medium that had been effectively cleared of substrate by unit weight of yolk-sac protein in unit time'.

$$A = \frac{B}{C \times D}$$

A = Uptake (ul/mg of tissue protein)

B = Total amount of radioactivity present in yolk sac (corrected cpm)

C = Total amount of substrate/ul medium (corrected cpm)

D = mg protein in the whole yolk sac

If values of uptake plotted against incubation time (h) result in a linear graph, then the slope of the line is termed the 'Endocytic Index' and it has the following units: ul/mg yolk-sac protein per h.

Throughout the incubation, as substrate accumulates in the yolk sac, the value of C, the substrate concentration in the medium, will fall. However, for ^{125}I-PVP, the only non-digestible substrate used, over a period of 6h less than 1% of the initial radioactivity in the medium is taken into the yolk sac. Hence no correction is made here for medium depletion of substrate since the error caused by this process is

negligible (<1%).

Finally, the concept of the Endocytic Index can be rather misleading. For materials that are engulfed entirely in the fluid phase, the rate of uptake (the Endocytic Index) is numerically equal to the rate of fluid uptake. However, for substrates taken into the cell by adsorptive pinocytosis, the value of the Endocytic Index will be much greater than the actual rate of uptake of fluid by the tissue. In such cases it could be argued that a less confusing unit of uptake would be ng substrate/mg tissue protein per h, but this unit is dependent on the precise concentration of substrate in the medium.

Throughout this thesis, each uptake rate is therefore reported in the form of an Endocytic Index. It is important to remember that it refers to 'that volume of medium whose contained substrate has been taken up by the yolk sac', and not directly to the volume of fluid ingested except for those substrates that are taken up entirely in the fluid phase. This is the case for ^{125}I-PVP (Ibbotson and Williams, 1979).

2.2.9 Calculation of the Endocytic Index for a degradable material.

If a radiolabelled substrate is degraded by lysosomal enzymes after entering the cell, then a substantial portion of the radioactivity originally taken up may be released again into the medium in the form of low molecular weight degradation products. In the case of radiolabelled proteins, the equation used for a non-degradable substrate:

$$A = \frac{B}{C \times D}$$

b

becomes modified to:

$$A = \frac{B + 10(E - F)}{C' \times D}$$

where E is the total acid-soluble radioactivity per ml of medium (cpm, corrected for background), and F is the acid-soluble radioactivity per ml of medium (corrected cpm) that arises from sources other than yolk sac-associated proteolysis. F is formed from two main components, acid-soluble radioactivity present in the stock solution due to incomplete dialysis or deterioration during storage, and non-yolk-sac-associated generation of acid-soluble radioactivity during

the incubation period e.g. by proteinases in the calf serum. The value of F was determined during each experiment by incubating a separate flask containing substrate but no yolk sac, and measuring the percentage of acid-solubles in the medium. C' represents the acid-insoluble radioactivity present in the medium, rather than the total radioactivity as was the case for non-degradable substrates.

If the uptake rate is high, medium depletion will potentially exert an important influence on the apparent rate of uptake. A correction is therefore necessary (Section 2.2.10).

2.2.10 Correction for medium depletion.

In the presence of 10% calf serum, ^{125}I-PVP is taken up with an Endocytic Index of approximately 2 ul/mg yolk-sac protein per h. Over a 6h period, under standard conditions, this leads to a negligible fall in the medium concentration of substrate. With most proteins, however, the rate of ingestion is very much greater than this. Thus a correction factor is usually required when the substrate is taken in by adsorptive endocytosis, and it is routinely calculated as follows:

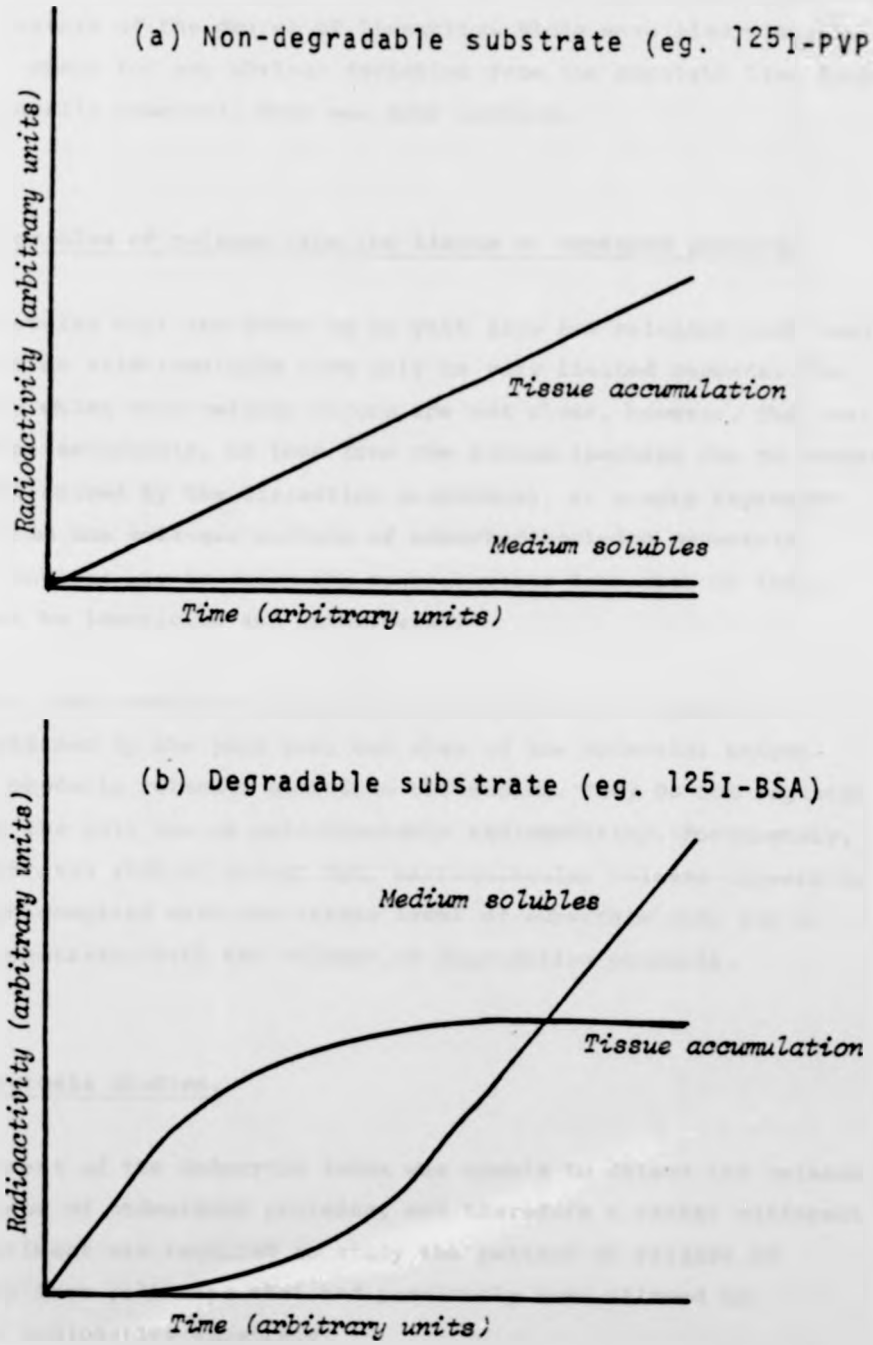
$$C' = C + \frac{1}{2}(E - F)$$

This adds to the observed TCA-insoluble radioactivity in the medium, half of the TCA-soluble radioactivity that appears in the medium during the whole incubation period. C' thus now estimates the mean quantity of TCA-insoluble substrate present in the medium during the incubation period, provided that the rate of substrate-depletion is linear with time. It ignores any radioactivity remaining within the yolk sac, but represents an important and useful correction.

2.2.11 Calculation of uptake values.

All data were processed using programs written in BASIC for an ICL 4080 (later updated to 4190) computer. Final versions are listed in Appendix 2.

Figure 2.3.1 Typical tissue accumulation and substrate degradation patterns for degradable and non-degradable substrates.



When a radiolabelled substrate is incubated in the presence of yolk-sac tissue, according to the usual technique (Section 2.2.2) it is taken into the tissue at a rate characteristic of the individual substrate. Upon reaching the lysosomes, the substrate may either be degraded - releasing iodotyrosine from the tissue- or, in the case of non-degradable substrates, stored. This latter behaviour gives rise to a linearly rising tissue level of radioactivity.

The plots of uptake against time were in general highly linear, in particular those data relating to uptake by yolk sacs incubated in the presence of 10% calf serum. Plots were analysed by a standard linear regression program, and correlation coefficients were calculated and used as a crude measure of the degree of linearity. Plots were also visually inspected to check for any obvious deviation from the straight line form that was generally observed. None was ever noticed.

2.2.12 The problem of release from the tissue of captured proteins.

Most proteins that are taken up by yolk sacs are released back into the medium in an acid-insoluble form only in very limited amounts. The mechanisms by which such release occurs are not clear, however. They may include either exocytosis, or loss from the tissue (perhaps due to damage or cell-death caused by the dissection procedure), or simply represent desorption from the yolk-sac surface of adsorbed/occluded materials. There is no obvious way in which the contributions from each of these processes can be identified and quantitated.

In short, the Endocytic Indices as calculated take account of substrate retained by the yolk sac, and also of low molecular weight degradation products released back into the medium. They do not register release from the yolk sac of acid-insoluble radioactivity. Fortunately, for all substrates studied except IgG, macromolecular release appears to be small when compared with the tissue level of substrate and, for a degradable substrate, with the release of degradation products.

2.2.13 Exocytosis studies.

Measurement of the Endocytic Index was unable to detect the release from the tissue of undegraded proteins, and therefore a rather different type of experiment was required to study the pattern of release of radioactivity from yolk sacs that had previously been allowed to accumulate a radioactive substrate.

The method was modified from that originally described by Williams _et al_ (1975b) in order to deal with degradable substrates. Rat and rabbit tissues were treated in essentially the same manner, and any differences

are mentioned below.

Three rat yolk sacs at 17.5 days of pregnancy, or three pieces of rabbit yolk sac at 24 days were incubated together in 10.0ml medium 199, in the same way as previously described (Section 2.2.2). The substrate was normally present at a concentration of 5ug/ml, and calf serum was usually omitted in order to maximise the rate of uptake. After 1.5h, all the yolk sacs were removed and washed quickly three times in warm, gassed medium, before being reincubated together in 10.0ml of fresh, gassed medium at 37°C in a 50ml Ehrlemeyer flask in the usual way. This fresh medium contained no radiolabelled substrate, although on some occasions it contained non-labelled substrate in order to attempt to displace any radiolabelled material that had simply adsorbed to the yolk-sac membrane. Duplicate 1.0ml samples were carefully removed at intervals up to 3h. They were replaced with 2.0ml fresh medium, and the flasks were regassed before being further incubated.

At the end of the reincubation the yolk sacs were removed, washed three times in ice-cold aq. NaCl (1%, w/v), and dissolved to a total volume of 5.0ml in aq. NaOH (1M). Samples were assayed for contained radioactivity, and the protein content was estimated using a slightly modified version of the Lowry technique (Section 2.2.4).

The medium samples were assayed in a gamma-spectrometer for total contained radioactivity, using standard counting geometry, and the total radioactivity released up to the time of taking of the n^{th} sample, T_n, was calculated from the expression:

$$T_n = 10C_n + 2\sum_{i=1}^{n-1} C_i$$

where C_i is the mean count obtained in the i^{th} sample (cpm, corrected for background).

In order to convert this value to 'ng of substrate release per mg of yolk-sac protein', T_n is divided first by D (mg of protein in the yolk sac), and then by G, where G is the cpm, obtained using standard counting geometry, of a 1ng/ml solution of substrate.

The samples were then precipitated with TCA and centrifuged in the normal way (Section 2.2.5). Measurement of the supernatant-associated

radioactivity allowed calculation of the amount of TCA-soluble radioactivity in the medium. If the soluble cpm in sample i is R_i, then the absolute (ng/mg) release of soluble radioactivity from the yolk sac up to the time of taking of sample n is given by S_n, where:

$$S_n = \frac{10R_n + 2\sum_{i=1}^{n-1} R_i}{D \times G} \qquad W_n = \frac{(10C_n + 2\sum_{i=1}^{n-1} C_i)}{D \times G} \quad \text{ng/mg}$$

The total amount of acid-insoluble radioactivity released by the n^{th} sample, I_n, is then equal to $(W_n - S_n)$, and it is measured in ng of substrate per mg yolk-sac protein.

2.2.14 Calculation of release parameters.

Release data were processed on a separate computer program (Appendix 2). The program was designed to list, for each sampling, the time of sampling, the percentage of the total yolk-sac associated radioactivity released by the time of sampling, the percentage of that radioactivity that was soluble in TCA, and to calculate weights of substrate (reported separately as both TCA-soluble and TCA-insoluble material) that had been released per mg yolk-sac protein by the time of sampling. In addition, on those occasions when the nature of the radioactivity remaining in the yolk sac at the end of the reincubation period was of interest (it was investigated by homogenising the yolk sac, rather than simply dissolving it in NaOH - Section 2.2.3), the program was able to calculate the amounts of acid-soluble and acid-insoluble radioactivity remaining in the yolk sac.

In general, the release of radioactivity from reincubated yolk sacs was far from linear (except in the case of ^{125}I-PVP, which maintains an approximately linear rate of release over the interval 1-6 hours, see Section 3.2.8). All points were routinely plotted to distinguish the nature of the release curves.

Release data were usually presented both as absolute weights and also as a percentage of the total tissue-associated radioactivity, since one without the other is open to misinterpretation.

2.2.15 Electrophoresis.

(a) Introduction

The main type of electrophoresis used in this work was SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE). After treatment with denaturing agents, proteins bind a quantity of the detergent sodium dodecyl sulphate in direct proportion to their molecular weight. Under electrophoresis conditions, SDS carries a net negative charge, hence the charge conferred on each substrate protein will be proportional to its molecular weight. By running well-characterized calibration proteins it is possible to estimate the molecular weights of unknown proteins. Mixtures of calibration proteins in the molecular weight range 14 000-76 000 daltons were obtained commercially for this purpose.

The major piece of apparatus used was an LKB 2117 Multiphor, together with power packs made by LKB (Model 2002) or BioRad (Model 1420). Methods used were essentially as described in LKB Application Note 306, with some modifications.

(b) Sample preparation

Protein solutions for use as electrophoresis samples must be initially almost free of salt, since a significant ionic content tends to upset the regular running of the gel, causing deviation of the bands that generally renders the results useless. Therefore the initial protein sample is dialysed, if necessary, against 0.01M phosphate buffer, pH 7.1, containing SDS (0.1%, w/v). 200ul of sample (1mg/ml approx.) is boiled with 100ul mercaptoethanol containing 6% (w/v) SDS for 5min, and allowed to cool. Bromophenol blue dye is added finally to mark the progress of the sample front.

(c) Casting the gel.

Slab gels were always used, and they were made using an LKB slot-former, rubber gasket and glass plates. In general the gel strength was 7.5% (w/v) acrylamide, and the composition was as follows:

H_2O	7.5 ml

phosphate buffer (0.2M, pH 7.1)	33.0 ml
acrylamide solution (22.2g acrylamide + 0.6g bis / 100ml)	22.0 ml
ammonium persulphate (150mg / 10ml)	3.2 ml
T.E.M.E.D.	0.1 ml

Ammonium persulphate and TEMED (NNN'N'-tetraethylene-diamine) are polymerisation catalysts, and they were added just before the gel was cast. Unpolymerised acrylamide is a potent neurotoxin, and the mixture was degassed and the gel cast in a fume cupboard. Care was taken not to introduce air bubbles into the gel since they would destroy the standard conductivity essential for meaningful electrophoresis.

After 40min, when the acrylamide had largely polymerised (the completion of the process can take up to 12h), the bulldog clips were removed and the apparatus was cooled in a fridge to facilitate removal of the slot-former and one of the glass plates. These were carefully peeled off and the gel was left in its final form, supported only by one thin glass plate which would remain in position throughout the electrophoresis. The gel was then stored overnight at room temperature in order to complete the polymerization and cross-linking reactions. (SDS-gels cannot be left for long at low temperatures since the detergent tends to crystallize out and the gels become useless, but they can be stored at room temperatures for three or four days without deteriorating). Normally the slot-former was used to give sample slots of 10ul capacity, since this volume of sample was found in general to give better results than 5ul.

(d) **SDS-PAGE operation.**

Unpolymerised and unwanted acrylamide was removed from the edges and base of the gel, and it was rested on the cooling plate of the multiphor (still supported on the thin glass plate). The contact between the gel plate and the cooling plate must be uniform (ie. no air bubbles) and the solution used for this purpose must be non-conducting (otherwise this will short-circuit the electrodes, and the gel will not function properly). A 0.1% (v/v) solution of the non-ionic detergent Triton X100

was used to make the contact.

Once the gel was in place, 1.1l of electrode buffer (phosphate buffer 0.1M, pH 7.1 containing 5g SDS/5l) was added to each of the buffer tanks in the multiphor, and a pad consisting of seven rectangles of Whatman number 1 filter paper soaked in buffer and applied as a wick to each side of the gel. The cooling-plate was maintained at 4°C and the pre-electrophoresis was carried out for 30min at 150mA current in order to stabilize the gel. Following this it was usually necessary to remove excess liquid from the edges and surface of the gel - and especially from the sample slots - with a piece of blotting paper. Sample solution (5 or 10ul) was then added to each of the slots using a Hamilton syringe. This operation, though delicate, was carried out as fast as possible once started, since Bromophenol blue marker begins to diffuse immediately into the gel.

The next stage in the procedure was a concentration step. A low (20mA) current was applied to the gel to ensure the proteins concentrate on the cathode side of the slots, but do not push into the gel. The effect was to give a slightly less diffuse spread of protein. After 10min, the current was increased to 200mA, and maintained for at least three hours. The proteins and bromophenol blue now migrated into the gel at their characteristic speeds. In order to maintain an even distribution of current in the gel, it was important not to let the sample slots dry out, and they were periodically topped-up with electrode buffer.

Immediately following electrophoresis, the distance travelled by the marker dye was measured in each lane, and the gel was submerged in fixer solution (in an LKB staining kit) to prevent further diffusion of the proteins.

(e) Staining the gel.

There are various methods for distinguishing the positions of proteins in polyacrylamide gels. Probably the currently most popular method is staining with Coomassie Blue, because of its simplicity and greater sensitivity relative to the traditional Amido Black method. However, there are now available a number of variations of a method of silver-staining that is supposed to be over 100x as sensitive as

Coomassie Blue, though rather more expensive and difficult to use. One particular variation of this method - that favoured by Oakley et al (1980) - has been repeatedly tried in this laboratory, but with no success. Since the method has failed in a variety of hands, it was abandoned.

One feasible alternative was to conjugate the various proteins in the sample to the fluorescent chemical 'Fluorescamine', which is marketed for that purpose (Sigma product no. F9878, Weigele et al, 1972). After conjugation the sample is treated in the usual way, and no modifications are made to the SDS-treatment and electrophoretic and fixation techniques. The gel is then scanned under u.v.light, and the proteins are seen as fluorescent spots, allowing their positions to be recorded. In principle this method sounds straightforward, but in practice a large amount of fluorescence was observed in the gel - and in the buffer solution - which made interpretation virtually impossible.

Finally, if the sample is radioactive, slicing the gel and counting the fragments in standard geometry in a gamma-spectrometer gives a simple and accurate determination of the positions of the constituent proteins, provided that they are all radioactive.

All the work presented in this thesis involving analysis of gels relied either on the radioactivity of the proteins to betray their positions or employed the Coomassie Blue staining technique. The latter technique is as follows:

The gel is fixed for 1h and then stained for 2h. The use of a high temperature staining solution was found to decrease the necessary staining time considerably, but was not found to be particularly beneficial in general. The gel is then destained against two or three changes of destaining solution (this is expensive, but can be simply regenerated by filtering through charcoal) and the protein bands appear. It is then soaked in preserving fluid for 1.0h and left to dry in a Cellophane preserving-sheet.

Details of the staining solutions are as follows:

Staining solution:	1.23g Coomassie brilliant blue R-250
	227 ml methanol
	227 ml water
	46 ml glacial acetic acid
Destainer:	1500 ml ethanol
	500ml acetic acid
	3000 ml water
Preserver:	300 ml ethanol
	100 ml acetic acid
	100 ml glycerol
	500 ml water

(f) **Molecular weight markers and calculation of results.**

The distance travelled by each protein is expressed as a fraction of the distance travelled by the marker dye front in that particular lane.

ie.relative mobility = distance moved by protein/distance moved by dye

In practice, a slight complication arises in that the dye position is measured before fixing, and the protein position after. If there is a change in the size of the gel during fixing, then this must be taken into account and a simple correction made.

A plot of the relative mobilities for different proteins against the log of their molecular weights should give a straight line. For calibration of the gel, BDH marker proteins (product number 442232U) were used. These consist of a series of oligomers formed by diethylpyrocarbonate cross-linking of the monomer protein.

In order of decreasing mobility they are:

	M.Wt.	Log_{10}
monomer	14 300	4.16
dimer	28 600	4.46
trimer	42 900	4.63
tetramer	57 200	4.76
pentamer	71 500	4.85

hexamer	85 800	4.93

These were either radioiodinated in the normal way before use, or stained together with the sample proteins after electrophoresis.

RESULTS

2.3 Results obtained using the conventional yolk-sac incubation system

The system of organ culture described in Sections 2.2.(1-15) has been used extensively in the laboratory to study the uptake into and subsequent release from yolk sacs of substrates, most of which are degradable within the lysosomal system of the yolk sac and a few of which are not (Williams et al, 1975a & b). In addition, the unusual behaviour of gamma globulin (70% of which is Immunoglobulin G) has been studied in some depth (Weisbecker et al, 1983, Williams and Ibbotson, 1979).

2.3.1 The uptake of ^{125}I-PVP into rat and rabbit yolk sacs.

The accumulation of ^{125}I-PVP by rat yolk-sacs incubated in vitro has previously been shown to be linear up to 18 hours (Williams, unpublished observation). The results expressed in Table 2.3.1 are in agreement with this, and the observed Endocytic Indices are shown to be dependent on the species of yolk sac and on the presence or absence of calf serum. These indices are assumed to be a measure of the rate of fluid phase pinocytosis, since ^{125}I-PVP has been shown (Roberts et al, 1977) not to adsorb to the plasma membrane of the rat yolk sac. Calf serum is often added to the incubation medium to a final concentration of 10% in order to increase the tissue survival time. One effect of this is to reduce the rate of accumulation of ^{125}I-PVP by the yolk sac.

Reincubation in fresh medium of yolk sacs that contain a large amount of ^{125}I-PVP shows release of only a small proportion of the contained radioactivity from the tissue. This is in agreement with the general view that PVP is not degradable in yolk-sac lysosomes and that no significant mechanism exists for its release intact from the tissue (Weisbecker, 1983). The observed low level of release of ^{125}I-PVP from the reincubated tissue is accounted for by incomplete washing or desorption from the plasmalemma and cell lysis, and is seen to rise slowly with time of reincubation. A possible alternative explanation, that ^{125}I-PVP is released from the reincubated tissue by fusion with the surface membrne of vesicles completing diacytosis, is rendered unlikely by the observation that this release continues at approximately the same rate for at least three hours (Figures 5.3.10 & Weisbecker (1981)), while diacytosis might be expected to be complete within a few minutes (see,

for example, the pattern of release of TCA-insoluble radioactivity derived from ^{125}I-IgG, Figure 3.3.2., which is almost complete within the first sixty minutes of reincubation.

The only differences observed between the behaviour of rat and rabbit yolk sacs under these conditions were that the rate of pinocytosis was substantially greater in rabbit tissue (Table 2.3.1) and that the inhibition of pinocytosis caused by the inclusion of 10% (v/v) calf serum in the incubation medium was greater for rabbit tissue (approx. 70%) than for rat (approx. 30%).

3.2.2 The behaviour of degradable substrates in incubated yolk sacs

^{125}I-labelled bovine serum albumin was used as a typical degradable protein substrate. It was found to be endocytosed at a constant rate that was decreased by the presence of calf serum and essentially the same behaviour was observed in both species - though uptake was much faster in the rat than in the rabbit (Table 2.3.2).

After a short exposure of the yolk-sac to ^{125}I-BSA (about fifteen min), TCA-soluble radioactivity begins to appear in the incubation medium. This activity has been shown (Livesey & Williams 1979) to be mainly in the form of ^{125}I-tyrosine which is presumed to result from lysosomal degradation of the ^{125}I-BSA.

Once a steady state of protein uptake has been attained, the level of medium ^{125}I-TCA-soluble material rises linearly and the tissue ^{125}I-level remains fairly constant (Figure 2.3.1). Hence the estimate of the rate of uptake of degradable ^{125}I-labelled-substrate is based both on the tissue level of radioactivity (cf. PVP) and on the level of TCA-soluble-radioactivity present in the medium (which must be corrected for the level of TCA-soluble radioactivity initially present).

When ^{125}I-labelled homologous gamma globulins are used in this system the results obtained are similar in every respect (Table 2.3.3).

If a yolk sac that has been incubated in medium 199 containing ^{125}I-BSA is washed and reincubated in fresh medium containing no radioactivity (Section 2.2.13), then the nature of the radioactivity released from the yolk-sac into the fresh medium can be monitored. As

shown in Figure 2.3.2b, over 90% of the released radioactivity is found to be soluble in TCA. This suggests that there is no important specific mechanism for the release from yolk sacs of intact ^{125}I-BSA. (The small amount of TCA-insoluble radioactivity that is released can be accounted for by desorption from the cell surface and/or by cell lysis).

If, however, ^{125}I-labelled homologous gamma globulins are studied in this way, significant differences are observed. It is found that a significant proportion of the radioactivity released from the yolk-sac into the fresh medium is TCA-insoluble (Figure 2.3.2a). This proportion varies depending on the exact incubation conditions, but tends to be greater in rabbit tissue than in rat.

The Release from Rat Yolk Sacs of Bovine Serum Albumin and Rat Gamma-Globulins. Figure 2.3.2.

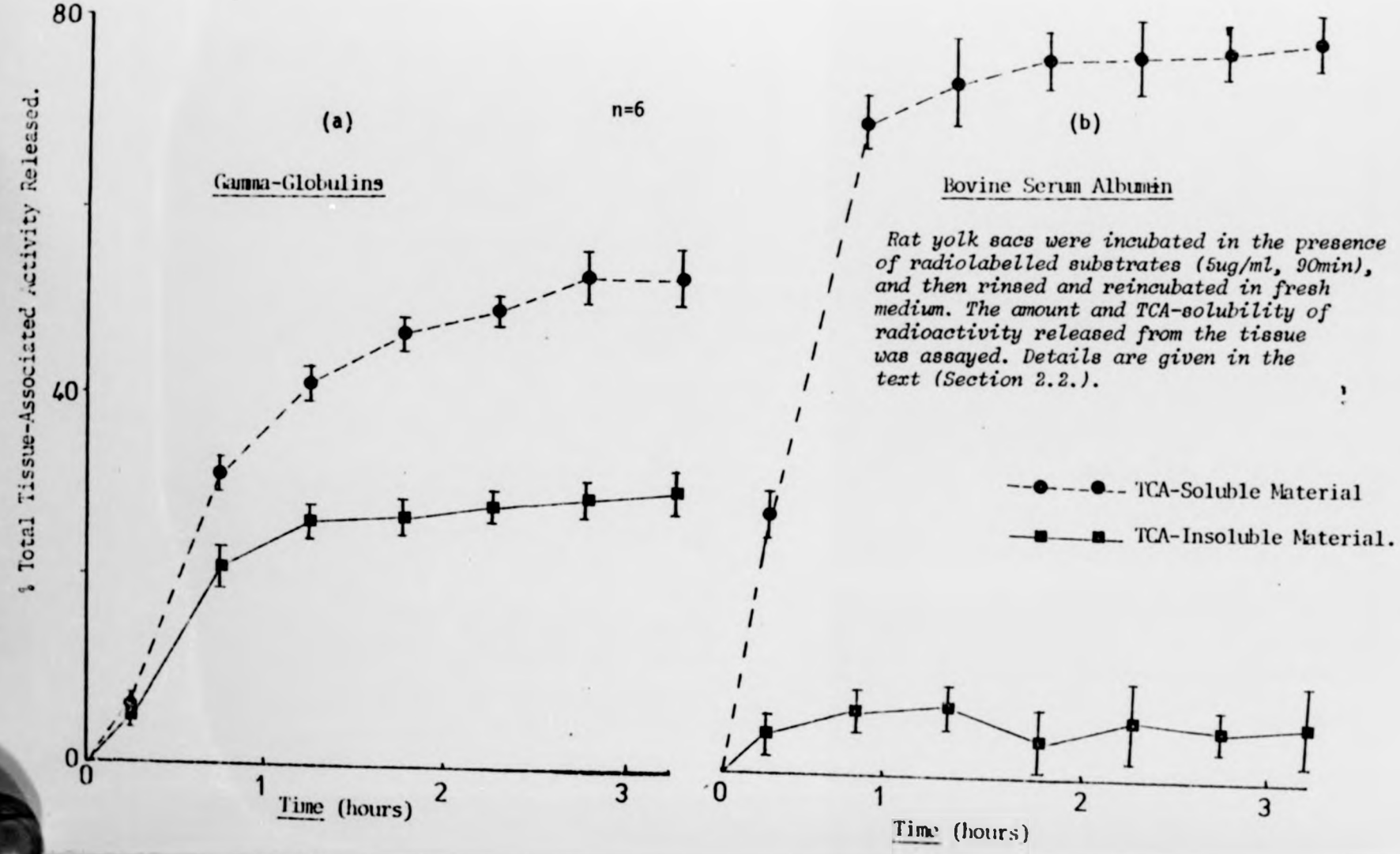


Table 2.3.1 **Uptake of PVP into rat and rabbit yolk sacs.**

a) **Rat tissue**

No calf serum			10% calf serum		
No. of yolk sacs	E.I.	R	No. of yolk sacs	E.I.	R
9	3.21	0.95	10	1.67	0.96
5	2.58	0.96	11	1.58	0.95
8	2.21	0.97	7	2.08	0.97
			11	1.81	0.99
			10	1.53	0.98
			10	1.74	0.99
			8	1.75	0.97
			5	2.12	0.99
			5	2.70	0.99
			6	2.02	0.93
			5	1.65	0.95
	2.67 ± 0.51			1.88 ± 0.34	

b) **rabbit tissue**

No calf serum			10% calf serum		
No of yolk sacs	E.I.	R	No. of yolk sacs	E.I.	R
10	12.6	0.89	10	3.48	0.80
10	16.3	0.93	5	2.35	0.92
5	10.0	0.97	5	2.50	0.92
5	7.2	0.95	8	1.48	0.88
5	9.2	0.98	8	1.20	0.80
8	9.0	0.70			
8	10.3	0.88			
8	5.7	0.92			
20	13.4	0.80			
10	9.1	0.77			
10	8.9	0.90			
8	9.1	0.68			
8	9.1	1.00			
5	8.0	0.96			
4	9.7	1.00			
10	6.7	0.85			
	9.64 ± 2.60			2.2 ± 0.9	

Table 2.3.2 **The uptake of BSA into rat and rabbit yolk sacs.**

Substrate (2ug/ml)	Rat tissue: No calf serum	Rat tissue: 10% calf serum	Rabbit tissue: no calf serum	Rabbit tissue: 10% calf serum
$_{nat}$BSA	N.D.	11.2±3.0	7.6±1.4*	N.D.
$_{fd}$BSA	381*	76.8±29.0*	11±1*	3.1*

Results marked * were obtained by Weisbecker, 1981.

Table 2.3.3 **The uptake of gamma globulins by rat and rabbit yolk sacs.**

Rat tissue				Rabbit tissue			
E.I.	R	n	% calf serum	E.I.	R	n	% calf serum
12.4	0.99	20	10	2.15*	0.81	8	10
15.2	0.97	10	10	3.57*	0.75	7	10
12.3	0.99	10	10	4.05	0.93	10	10
20.7	0.97	9	10				
68.7*	0.86	10	0	35.8*	0.95	9	0
				35.0*	0.85	8	0
				20.9*	0.92	9	0
				38.2*	0.94	9	0
				31.8*	0.82	9	0
				38.0	0.85	10	0

DISCUSSION

Rabbit and rat yolk sacs seem to behave in essentially the same ways under these *in vitro* incubation conditions, the major differences being in absolute rates of uptake of substrates.

The presence of 10% calf serum has been shown to increase tissue survival time (Ibbotson & Williams, 1979), but it has the effect of decreasing rates of uptake. It may do this in a variety of ways (see Chapter 3) but it exerts a greater inhibition on fluid phase (PVP) uptake in the rabbit (70%) than in the rat (30%), and this is one of the more obvious differences between the behaviour of yolk sacs from these two species.

Since calf serum contains a high level of BSA it might be expected to exert a greater inhibition on the uptake of ^{125}I-BSA than on ^{125}I-PVP because it will compete for binding sites as well as slowing the rate of fluid uptake. This is borne out by the observation of 80% inhibition of ^{125}I-BSA uptake by rat yolk sacs incubated in 10% calf serum. (The equivalent data for rabbit tissues are not available.)

Apart from the potential of the system in quantifying rates of protein catabolism in incubated yolk sacs the fundamentally useful observation is that homologous gamma globulin is treated in a special manner within the yolk sac. None of the other ^{125}I-proteins studied to date (Ibbotson, 1978) have exhibited such protection from degradation. This phenomenon of intracellular protection of IgG from degradation and subsequent macromolecular release from the tissue has been shown (Weisbecker (1981)) to be saturable, and therefore probably mediated by a receptor system. This, coupled with the observation (Weisbecker, 1981) that the rank order of *in vitro* protection of gamma globulins from various species is essentially the same as the rank order of transmission observed *in vivo* (Table 2.3.4) has led to the tentative suggestion (Weisbecker, 1981) that 'TCA-insoluble release' in the *in vitro* incubation system may mimic the process of transcellular (maternofoetal) transmission *in vivo*.

If this is indeed the case, then the far greater flexibility of an *in vitro* incubation system that is subject to relatively few of the restrictions inherent in dealing with live animals should have great

potential in exploring the nature of the transmission process.

As mentioned previously, the rabbit yolk sac is a more important site for maternofoetal immunoglobulin transmission than is the rat yolk sac, and *in vitro* it exhibits a greater adaptation for the protection of homologous IgG. Rabbit tissue was therefore the experimental tissue of choice, but rabbits were in short supply, and rat yolk sacs were used when rabbit tissue was not available.

Chapter 3. The in vitro Yolk-Sac Incubation Technique; an investigation into some unclear aspects.

INTRODUCTION

A large amount of information concerning endocytic mechanisms has now been obtained by the use of the yolk-sac incubation system described in Chapter 2. In order to verify the interpretations made of the data, most of the original assumptions have now been investigated and shown to be valid. For example, the assumption that substrate-degradation is an intracellular process and not mediated by plasmalemma-associated enzymes, which is of prime importance for the interpretation of the results in terms of endocytosis, has now (Weisbecker, 1981) been substantiated by the observation that degradation by intact tissue is halted by various inhibitors of endocytosis. Further, examination of proteolysis by tissue homogenates shows that it does not proceed at pH values greater than 5.5, suggesting that the proteolytic enzymes are located in acid compartments of the cell. Certain aspects of the experimental design and observed effects have not been properly clarified, however, and until such are done the interpretations made of the results obtained are open to criticism. The purpose of this chapter is to check out various aspects of the incubation system that are based on unsubstantiated assumptions, and hence to evaluate critically interpretations made of the results.

3.1.2 The use of pure IgG instead of whole gamma globulin.

The results presented and discussed in Chapter 2, like those from all the previous work performed in this system when investigating the behaviour of immunoglobulins, were obtained with a commercial preparation of gamma globulins as substrate. This preparation contains a mixture of the five different types of immunoglobulin: IgG, IgA, IgM, IgE and IgD, being 80% (w/w) IgG. These molecules have widely different structures and functions (see Chapter 4) and only IgG is thought to be able to pass from mother to foetus (there is some evidence for the transmission of a small amount of IgM in the rabbit (Hemmings, 1973)). Results obtained to date may therefore not accurately reflect the behaviour of IgG if the other immunoglobulins present in the substrate preparation behave differently in the incubation system. There is also the possibility that other immunoglobulins may be radioiodinated more efficiently than IgG and thus the relative amount of labelled IgG in the ^{125}I-labelled substrate preparation may be effectively less than 80%.

Comparison of the behaviour of ^{125}I-labelled gamma globulins with that of electrophoretically-purified (Sigma, commercial grade) ^{125}I-IgG in key experiments would indicate whether the data so far obtained reflect the behaviour of ^{125}I-IgG, and would show if any modification of the various interpretations made to date are necessary.

For example, a comparison of the observed Endocytic Index for ^{125}I-gamma globulins with that of the purified ^{125}I-IgG preparation in rat yolk sacs, together with the patterns for the release of radioactivity from reincubated yolk sacs would provide a measure of the validity of previous work.

3.1.3 The effects on immunoglobulin behaviour in the rat yolk-sac incubation system of radioiodination by different techniques.

A literature survey (see Appendix 1) revealed that opinions vary concerning the relative amounts of biological damage caused to proteins by different techniques of radioiodination.

In order to assess whether, under optimal conditions, the simple chloramine-T technique caused more damage than enzymic (and technically more complex) methods, a comparison was made between the behaviour *in vitro* of immunoglobulins labelled by chloramine T, lactoperoxidase and by an imido-ester conjugation technique. The last was included as a form of control, since iodide is not attached to tyrosine residues (conjugation is normally to the amine group of lysine residues via an amidine linkage) and there is no exposure of the protein to oxidising or reducing agents or to the possibly noxious ^{125}I-preparation.

3.1.4 The incorporation of ^{125}I-(L)-monoiodotyrosine into cell protein.

To equate the rate of release from the tissue of TCA-soluble radioactivity (mainly monoiodotyrosine) with the rate of intracellular degradation of the substrate necessitates two assumptions. First, the amount of free iodotyrosine retained within the tissue must be small, since this also results from intracellular substrate degradation and is not usually taken into account by the standard experimental regime - nor can the regime be simply modified to take account of it, since it is fairly hard to quantify - and secondly the amount of iodotyrosine incorporated into newly synthesised tissue protein must be small compared

with the amount produced by degradation of substrate.

The first of these points has been investigated previously (Duncan, personal communication), and the tissue level of ^{125}I-monoiodotyrosine shown to be low when ^{125}I-BSA was used as substrate. The second point has not been investigated in this system before, there being a widespread assumption, based on the work of Ryser-Hughes (1963) in Ehrlich Ascites tumour cells, that ^{125}I-iodotyrosine is not recognised by cellular tRNAs and therefore is not used at all for the synthesis of new proteins. There is no direct evidence available to confirm or refute the assumption that what is true for the tissue used by Ryser-Hughes is also true for the yolk sacs employed here. However, it is extremely important for the valid interpretation of the data that iodotyrosine is shown not to be reincorporated by the cell. This appears to be a simple matter to investigate since the yolk sac is highly permeable to iodotyrosine. Incubation of yolk sacs in ^{125}I-monoiodotyrosine for a few hours may result in a time-dependent cellular accumulation of radioactivity indicative of the incorporation of ^{125}I-tyrosine into new protein (or adsorption onto cell membranes or proteins). The rate of any such accumulation would indicate whether or not the standard interpretation of degradation data needs reappraisal.

3.1.5 The use of ^{125}I-PVP as a fluid phase pinocytic marker.

^{125}I-labelled poly(vinylpyrrolidone) has long been used as a non-digestible marker for pinocytosis and it has been shown apparently to internalise solely in the fluid phase in rat yolk sacs (Williams *et al*, 1975a). Its rate of uptake into rabbit yolk sacs, however, is very much greater (Table 2.3.1) and the possibility that it enters by adsorptive pinocytosis in this tissue must be considered. The observation that its rate of uptake is slowed 70% by the presence of 10% calf serum, while the rate of pinocytosis slows by only 30% under similar conditions in the rat, adds substance to this possibility. The widespread use of ^{125}I-PVP as as a marker of fluid phase pinocytosis in the rat yolk sac makes it of fundamental importance to the interpretation of equivalent data obtained from rabbit tissue to establish that there is no adsorptive component in its uptake.

The simplest way to investigate this is to measure Endocytic Indices for ^{125}I-PVP over a wide (non-toxic) range of substrate concentrations.

If there is an adsorptive component to uptake, a degree of concentration-dependence in the observed Indices would be expected. Fluid phase uptake alone would give rise to a constant value of the Endocytic Index that is independent of substrate concentration.

3.1.6 The effects of serum on endocytosis in rabbit yolk sacs.

The inclusion of mammalian serum in various culture media has been shown to increase tissue survival for a variety of cell types (Temin et al, 1972) but the exact mechanism of its action remains unclear. The effect of serum on pinocytosis seems to vary between tissues; swine serum (10% v/v) stimulates the rate of fluid pinocytosis in rat peritoneal macrophages (Kooistra et al, 1981), but calf serum (10%) has a depressive effect on the pinocytic activity of rat yolk-sac cells (Ibbotson & Williams (1979), Forster (1982)).

Calf serum (10%) is often added to the medium when rat yolk sacs are incubated in order to increase tissue survival. Its effect on pinocytosis could be mediated by one (or more) of three mechanisms: (i) 'hormonal' (possibly serum contains a hormone or metabolite that inhibits pinocytosis), (ii) the intracellular level of undegraded endocytosed protein may rise (in the presence of 10% calf serum) quickly and then exert a feedback effect on further substrate uptake, (iii) the presence of a large amount of protein non-specifically bound to the cell surface may cause the membrane to become less mobile. (Redistribution of receptors prior to endocytosis may be inhibited by cross-linking effects of the bulk protein and hence the rate of pinocytosis may be decreased.)

If (i) is correct, then (non-pregnant) rabbit serum might exert different effects on endocytosis in rabbit yolk sacs from those of calf serum. Further, pure preparations of individual proteins (e.g. BSA, casein) would be expected to have no effects of this nature.

If (ii) is true, then the tissue level of endocytosed protein when a steady state is reached would be expected to be constant, provided that the yolk sac is not endocytosing at maximal capacity. Further, the presence in the medium of similar concentrations (w/w) of amino acid mixtures or protein hydrolysates might exert similar effects if high intralysosomal levels of proteins signal their presence via an increased level of amino acids within the cells.

If (iii) is correct, then any protein at suitable concentration might be expected to exert the same effects, but amino acids and hydrolysates would be without effect. Also the tissue level of endocytosed protein would probably be lower in the presence of extremely high concentrations of protein than in only partially inhibited systems, since endocytosis would be halted almost as soon as the plasma membrane came into contact with the surrounding protein, and very little would be able to internalise.

Various experiments were carried out to distinguish between these possibilities, by testing the probable characteristics of each possibility, as outlined above.

3.1.7 The effects on rat yolk sac behaviour of lowering the oxygen tension, and characterisation of the TCA-insoluble ^{125}I-IgG radioactivity released from rat yolk sacs.

Under incubation conditions the yolk sac, which is a thin tissue, is in contact on both the outer and inner surfaces with medium 199. The ratio of O_2:CO_2 (95:5) may therefore be higher than is necessary to supply the cells with adequate oxygen. Further, such a high oxygen tension may have undesirable effects on the cells, in particular it may bring about cell lysis (D. New, personal communication).

The low rate of ^{125}I-PVP release from rat yolk-sac cells suggests, however, that the rate of epithelial cell-lysis is not sufficiently high under these conditions to cause seriously misleading results. On the other hand, it seems possible that the release of undegraded ^{125}I-IgG from reincubated yolk sacs may result not from specific protection from degradation of ^{125}I-IgG within the yolk sac and subsequent co-ordinated release, but from oxygen-induced lysis of some part of the yolk-sac to which ^{125}I-PVP does not penetrate (e.g.lysis of the endothelial cells of the stagnant vitelline circulatory system). The absence of intact protein released from reincubated yolk sacs previously exposed to ^{125}I-BSA could then be explained either by the inaccessibility to ^{125}I-BSA of this particular region of the tissue, or might be a result of the BSA molecule being degraded more rapidly than IgG in the lysosomes, thus giving rise to no detectable release of macromolecular ^{125}I-BSA from lysed cells. If it were conclusively shown, however, that the TCA-insoluble material released from yolk sacs previously exposed to ^{125}I-IgG was in fact

macromolecular IgG rather than large degradation products then this latter explanation loses credibility.

The behaviour of rat yolk sacs at lowered oxygen tensions was observed, and the TCA-insoluble ^{125}I-IgG-derived radioactivity released from reincubated yolk sacs was characterized by SDS-PAGE (rat tissue) and by gel chromatography (rabbit tissue).

3.1.8 The effects of cold gamma globulin on the ^{125}I-PVP release pattern from rat yolk sacs.

If IgG-specific vesicles internalise at the cell surface, then undergo diacytosis and release their contents at the lateral or basal membranes of the cell, the transcellular transport of a certain amount of fluid may be inevitable. Further, if the formation of IgG-specific vesicles is an inducible process then the addition of unlabelled rat gamma globulins to incubation medium containing a rat yolk sac, followed by a fluid-phase marker, would be expected to increase the amount of marker subsequently released from the reincubated tissue. The best fluid-phase marker readily available is ^{125}I-PVP (the molecular weight average of 40 000 is perhaps rather large, though small compared with IgG; ^{14}C-sucrose would have been a preferable marker).

In short, the aims of this chapter are to:

(i) Compare the behaviour in the *in vitro* system of ^{125}I-gamma globulins and commercial-grade ^{125}I-IgG in order to assess the value of work previously performed with the former preparation.

(ii) Examine the effects of radio-iodination by different techniques on the behaviour of gamma globulins in this system.

(iii) Establish whether monoiodotyrosine can be incorporated by rat yolk-sac cells during protein synthesis.

(iv) Check that ^{125}I-PVP enters rabbit yolk sac cells in the fluid phase only.

(v) Investigate the inhibitory effect of calf serum on rabbit yolk sac endocytosis.

(vi) Lower the oxygen tension in the system and observe any effects on the behaviour of rat tissues.

(vii) Characterize the ^{125}I-IgG derived radioactivity released from reincubated yolk sacs (both rabbit and rat).

(viii) Observe the effects on the pattern of release from rat yolk sacs of ^{125}I-PVP of the presence of unlabelled rat gamma globulins in the medium.

MATERIALS AND METHODS

3.2.1 SDS-polyacrylamide gel electrophoresis

Essentially the methods described in Section 2.2.15 were used but a few more details and slight modifications are mentioned here.

7.5% polyacrylamide gels were used throughout because these proved sufficiently robust for convenient handling and also allowed fairly fast migration of molecules under electrophoresis. Samples were always 10 ul volume and where gels were stained for protein, Coomassie Blue was always used. Gel slices (2mm wide), were cut by hand with a razor blade. For estimation of their relative content of radioactivity they were placed in the bottom of 3 ml plastic tubes (Luckham LP3) and counted on a gamma-scintillation counter in the usual way.

3.2.2 Gel chromatography

a) **Sephadex G-150.**

In order to minimise hydrophobic attachment of the protein to the gel particles, a fairly strong buffer was used throughout (0.2M sodium phosphate, pH 7.0, containing 0.02% sodium azide). Cold (unlabelled) BSA was routinely run through new columns in order to saturate any protein binding capacity which remained. (On reflection, cold gamma globulins would probably have been more efficient).

Samples of volume 1.0 ml were collected and the void volume was determined using blue dextran. Sephadex G-150 is reported (Pharmacia specifications, 1982) to exhibit good separation of molecules in the molecular weight range 5×10^3 to 3×10^5. In theory it should thus be useful for the separation of IgG and any major fragments thereof, but for larger immunoglobulins and aggregates a more porous gel would be expected to give better resolution.

When Sephadex G-200 (Superfine) was used, technical difficulties were encountered in packing the column since the gel seemed to be able to pass through the LKB sealing filters. The inclusion of millipore filters at the ends of the column did not solve this problem and so no work carried out on Sephadex G-200 is presented here.

b) **Sephacryl S-200**

Sephacryl S200 is supplied in pre-swollen form, and it is therefore very simple to use. It is reported (Pharmacia specifications, 1982) to exhibit good separation of globular proteins over the molecular weght range $5x10^3$ to $2.5x10^5$. Columns were packed relatively quickly and void volumes determined using blue dextran, which was measured at 625mm in a spectrophotometer. The buffer used throughout was 4mM sodium acetate, pH 5.4, containing 0.02% sodium azide, and samples of volume 1.0ml were collected.

3.2.3 Methods of radioiodination of proteins

Chloramine-T

Chloramine-T was used to label proteins with iodide in the normal way, described in Section 2.2.1.

Lactoperoxidase

The method employed for iodination of proteins using lactoperoxidase was a modification of the original technique described by Marchalonis (1969).

Rat gamma globulins (7mg, Miles Laboratories Ltd., code 82-504) were dissolved in 7ml 0.05M phosphate buffered saline (PBS), pH 7.0, and stirred at room temperature. 1.0 mCi ^{125}I (NaI, aq) contained in 10 ul volume, dispensed using a Hamilton syringe, was added. Then 35 ug lactoperoxidase (Sigma, L 2005) contained in 1 ml PBS was added, followed by 0.12ml 30%(v/v) H_2O_2 solution. The mixture was stirred for 7min and another 0.12 cm^3 H_2O_2 solution was added. After a further 7min stirring, the mixture was placed in a dialysis bag and dialysed against six changes of 1% NaCl (aq) at 4^oC over a period of three days. It was then bottled and stored at -20^oC.

The efficiency of labelling was again calculated by taking a small sample of the final reaction mixture before dialysis and then analysing the contained radioactivity for solubility in TCA.

Conjugation technique

Methyl 3,5-di(^{125}I)iodohydroxybenzimidate (Radiochemical Centre, Amersham, code no. 1M.110) is a protein labelling reagent that preserves the charge on the protein, in contrast to many similar techniques that cause charge modification e.g. Bolton-Hunter reagent, see Appendix 1. At slightly alkaline pH the imido-ester derivative adds to protonated amine groups of proteins forming amidine linkages; full details are given in Appendix 1.

The protonated amidine formed is expected to have a pK in the range 11.5 to 12.5 (Albert et al, 1948) and is likely therefore to maintain the charge of the original amine group. This method of radiolabelling has the advantage of not exposing the protein to potentially noxious oxidising agents or polyiodides and also can be used to label proteins that do not contain tyrosine residues.

The regime of Wood et al (1975) was used to label rat gamma globulins: substrate (7mg) was dissolved in 4.5ml 50mM sodium borate buffer, pH 9.4, then 0.5 ml di^{125}I-MDHBIM was added and the mixture was incubated overnight at 37^{o}C. Dialysis was against six changes of 1% NaCl (aq) at 4^{o}C over a period of three days.

3.2.4 The incubation of rat yolk sacs in ^{125}I-L-tyrosine.

To study the possible incorporation into intracellular proteins of ^{125}I-L-tyrosine, rat yolk sacs were isolated in the normal way (Section 2.2.2) and incubated in medium 199 at 37^{o}C in a shaking incubator. A mixture of ^{125}I, ^{125}I-L-tyrosine and L-tyrosine (pure iodotyrosine would have been preferable, but was not immediately available) was added, to a final concentration of tyrosine of 1 ug/ml, and incubation was carried out for up to 20h. Yolk sacs were removed and washed in the normal way at various times, and their contained radioactivity was measured.

^{125}I has been previously shown (Duncan, unpublished results) not to accumulate progressively with time in rat yolk sacs and therefore it was assumed that any long-term increase in the amount of tissue associated radioactivity was due to the accumulation of ^{125}I-L-tyrosine.

3.2.5 The incubation of rabbit yolk sacs in high concentrations of PVP.

Rabbit yolk sacs were prepared as described in Section 2.2.7. In all incubations, a final concentration of ^{125}I-PVP of 2ug/ml of medium was used. When a higher concentration of PVP was required, unlabelled PVP-40 (Sigma PVP-40, which has similar molecular weight distribution to the labelled material) was added to increase the total PVP concentration.

3.2.6 Incubating rabbit yolk sacs in medium containing high concentrations of unlabelled proteins.

Rabbit yolk sacs were prepared as described in Section 2.2.7 and various concentrations of calf serum were added to the incubation medium as described in Section 2.2.2. When other proteins were used they were obtained as follows; BSA, and casein were obtained from Sigma Chemicals Ltd., (product no's A4378 and C0376 respectively), bovine gamma globulins from Miles Laboratories Ltd., (no. 82 041), and (D+)glycine from Sigma Chemicals Ltd., (G7126). Rabbit serum was prepared as follows;

A mature female Californian rabbit was loosely wrapped in a towel and placed on a bench top (it was found that restricting the movement of the animal in this way had a pacific effect on it). One ear was cleaned with hibitane and the vein was shaved with a large scalpel blade and wiped with xylol in order to make it stand out. Vaseline was then rubbed over the surface, to aid the free flow of blood across the surface of the ear, and a fairly deep incision was made along the vein with a scalpel blade. About 10ml blood was obtained in this way, before clotting became a severe problem, and then the operation was repeated using the other ear, whence another 10ml was taken. The blood was spun in a bench top centrifuge at 250 rpm x 3 min and the supernatant serum was collected. A total volume of about 11ml serum was obtained; its pink colour indicated that a certain degree of haemolysis had occurred.

3.2.7 The reversal of serum-induced inhibition of pinocytosis.

When the recovery of the pinocytic capacity of tissue following exposure to calf serum was investigated, two slightly different techniques were employed.

In all cases, following the preliminary incubation period in the presence of 10 or 20% calf serum, yolk sacs were harvested and washed three times in fresh, warm medium 199 which contained no calf serum. They were then reincubated in different flasks containing 10ml serum-free medium 199 and ^{125}I-PVP at a final concentration of 2 ug/ml.

The variation between the two techniques concerned the presence or absence in the preliminary incubation medium of radiolabelled substrate. The simpler experimental design is that in which radioactivity was not present in the preliminary uptake medium. In these cases the tissue-associated radioactivity at the time of sampling the yolk sac is a function only of the time of reincubation in fresh medium and of the Endocytic Index of ^{125}I-PVP in the reincubated tissue. When ^{125}I-PVP was present in the calf-serum-containing medium, a correction must be made for uptake which occurs during this incubation when estimating the Endocytic Index for ^{125}I-PVP during the reincubation.

3.2.8 Incubation of rat yolk sacs at lower oxygen tensions.

All gas mixtures used were obtained from BOC (Special Gases Division). Each gas contained 5% CO_2, the remainder being oxygen and nitrogen, except for the mixture referred to as '0% Oxygen', which was 100% Nitrogen.

Incubation flasks containing medium 199, and sometimes 10% (v/v) calf serum, were exhaustively gassed with the respective gas mixture prior to incubation. In order to allow random assortment of tissues from different animals in the various flasks, dissection was always carried out in medium gassed with 95% oxygen. As much medium as possible was removed from the yolk sacs, by blotting them, before they were inserted into the various incubation flasks. After addition of the substrate, which was also in medium gassed with 95% oxygen, the flasks were regassed with the appropriate gas mixture.

For incubations carried out at 0% oxygen, medium 199 was gassed with nitrogen for one hour, and dissection of yolk sacs and addition of substrate were carried out in this notionally '0%' oxygen medium. Hence there was no mixing of tissues from the same animals between control and test cultures for these experiments.

Release experiments were carried out as described in Section 2.2.12, except that, after sampling, 2ml medium gassed with the appropriate mixture was added.

3.2.9 Isolation of the macromolecular material released from yolk sacs previously exposed to ^{125}I-labelled immunoglobulins.

At the end of the reincubation period, when both yolk sacs and reincubation medium samples had been removed, the remaining medium was dialysed against six changes of distilled water at 4°C over a period of three days. The remaining material was freeze dried and dissolved in a small volume of water before being examined either on Sephacryl S200 (in the case of material released from rabbit yolk sacs and derived from homologous ^{125}I-IgG) or by SDS-PAGE (in the case of material released from rat yolk sacs and derived from ^{125}I-labelled homologous gamma globulins).

3.2.10 The inclusion of cold homologous gamma globulins in the uptake medium for ^{125}I-PVP into rat yolk sacs

When the effects of cold gamma globulins on the uptake behaviour of ^{125}I-PVP in rat yolk sacs was investigated, a final concentration of 5 ug/ml rat gamma globulins (Miles Laboratories Ltd., no 82-504) was included in the uptake medium before the yolk sac was added.

RESULTS

3.3.1. A comparison of the behaviour of homologous IgG and gamma globulins in rat yolk sacs incubated in vitro.

Electrophoretic analysis.

Examination of the SDS PAGE pattern of cold (non-radioactive) rat gamma-globulins (Figure 3.3.1) shows two major protein bands with associated molecular weights of approx. 25 000 and 50 000. (These results are expected since they correspond to the light and heavy chains that are obtained by denaturing a mixture of various gamma globulins). Presumably the 25 000 component is a mixture of kappa and lambda chains, and the 50 000 component must be a rather more heterogeneous mixture, being composed of alpha, delta, epsilon, gamma and mu chains, though mainly (approx.80%) gamma. The existence of a trace component of intermediate molecular weight (approx. 40 000) is rather more difficult to explain. This, contrary to the manufacturer's specifications, could be a contaminant, or could be formed by proteolysis of the heavy chain. The preparation is apparently not routinely screened for proteinases (Miles Laboratories, pesonal communication), and this latter explanation seems the most likely. There is, however, some evidence that it disappears after prolonged storage in aqueous solution at -20°C (see Section 3.3.2). This observation is difficult to interpret.

Pure, unlabelled rat IgG on SDS PAGE gives the two expected bands at 25 000 and 50 000, but no intermediate band. There is, however, some evidence for the presence of a heavier molecular weight component (approx. 70 000). The most likely origin of this material is incomplete denaturation of the original four-subunit molecule. (If one heavy and one light chain remain linked together by disulphide bonds, then the molecular weight associated with the unit would be approx.75 000). More extensive denaturation would probably split this unit. The presence of this band in the gels was therefore regarded as unimportant.

Hence SDS PAGE analysis of rat IgG and rat gamma globulins gives the same major features for both materials. Whether or not their behaviour in the *in vitro* yolk-sac system differed had to be established experimentally.

Release from reincubated yolk sacs.

The release pattern, from rat yolk sacs, of homologous ^{125}I-IgG was compared with the equivalent release pattern for ^{125}I-labelled rat gamma globulins. (In Figure 3.3.2, the radioactivity progressively lost from the tissue is expressed as a percentage of the total radioactivity associated with the tissue at the start of the reincubation). In general, a slightly greater percentage of the original activity taken up by the yolk sac remains associated with the tissue at the end of the release period for ^{125}I-IgG than for ^{125}I-labelled gamma globulins. Of the radioactivity released, a substantially greater percentage is in a form that is TCA-soluble (i.e. soluble in trichloroacetic acid) for ^{125}I-labelled gamma globulins than for ^{125}I-IgG.

The commercial preparation of rat gamma globulins is composed of only approx. 80% IgG, but the IgG preparation itself is over 99% pure. Hence the behavioural differences observed may either reflect a difference in supposedly similar molecules of IgG, or may be accounted for by the presence of 20% of non-IgG proteins in the gamma globulin preparation. These proteins may be more rapidly taken up and degraded in this system than IgG itself.

Gamma globulins were never used in the rabbit system in this study, but, for purposes of comparison, the release pattern of homologous ^{125}I-IgG from rabbit yolk sacs is shown in Figure 3.3.4 (two different experiments expressed in different units). The major difference between the rat and rabbit systems under these incubation conditions is that the release of TCA-insoluble material tends to dominate in the rabbit system, while the reverse is true in the rat.

3.3.2. The effects of labelling gamma globulins by different techniques on the analytical properties of the preparations and on their behaviour in the rat yolk sac system.

The results compare the behaviour of rat gamma globulins labelled by three different radio-iodination techniques; chloramine-T, lactoperoxidase and an imido-ester conjugation technique (see Section 3.2.3.). The different batches were compared on SDS PAGE, and their behaviour in the rat yolk-sac system was observed.

Analytical data: SDS gel electrophoresis.

Unlabelled gamma globulins gave two major bands (Figure 3.3.1) on SDS PAGE, (corresponding to molecular weights of 25 000 and 50 000) and one minor band at about 40 000. There is also evidence of other trace components. The origin of the 40 000 band and of the other traces is unclear, but the are perhaps formed by cleavage of the two main components.

Gamma globulins, freshly labelled by the lactoperoxidase or chloramine-T techniques, gave patterns that appeared identical with the unlabelled material when stained with Coomassie Blue. Without fail, however, older preparations that had been labelled by either method showed a different distribution on SDS gels. Invariably the bands at 50 000 and 40 000 were missing, and a new band appeared corresponding to a molecular weight of approx. 70 000. A similar band was observed in trace amounts with unlabelled IgG and was ascribed to incomplete denaturation of the molecule. The implication must be that prolonged storage of gamma globulins at -20^{o}C in water in some way stabilizes the molecule. Gamma globulins conjugated with the imido-ester also give a pattern similar to the one obtained with unlabelled material, but there is visual evidence on the gel for a large amount of material being left at the origin, presumably in an aggregated form.

Three preparations of labelled gamma globulins (two labelled by chloramine-T, one fresh and one six months old, and one freshly labelled by lactoperoxidase) were examined, and their distribution was assessed by measuring the radioactivity present in gel slices (Figure 3.3.5 (a)-(c)). Both of the fresh preparations showed the expected pair of peaks corresponding to molecular weights of 25 000 and 50 000, and the old chloramine-T-labelled preparation again exhibited a change in distribution with the replacement of the 50 000 band by a band corresponding to a molecular weight of approx.70 000. (The sensitivity of this technique is probably not sufficient to pick up the minor peak at 40 000 which is present on the stained gel). The most important observation to be made from the gel slices is the presence of a large amount of radioactivity at the origin for gamma globulins labelled by lactoperoxidase. This presumably represents aggregated material, and accounts for some 17% of the total radioactivity. Since the ratio of gamma globulins to lactoperoxidase (w/w) was 200:1, the lactoperoxidase

was unlikely to be easily detected on the gel.

When this freshly-prepared batch of lactoperoxidase-labelled gamma globulins was applied to a column of Sephadex G-150, in an attempt to remove the aggregated form, the pattern shown in Figure 3.3.6 was obtained. The aggregates elute slightly in advance of the major immunoglobulin peak, forming a distinct shoulder on its left hand side. (most of the non-IgG immunoglobulins in this preparation will also elute in this region, particularly IgM (M_r=900 000, 6% of total), and IgA (M_r=320 000, 13% of total). Hence a simple column procedure (which might be improved by the use of a more porous gel, e.g.Sephadex G200 or Sepharose 6B) is adequate to remove aggregates induced by the lactoperoxidase labelling procedure.

There is also evidence from this elution pattern of a lower molecular weight component, eluting at about fraction 85 (see Figure 3.3.6). This may correspond to the 40 000 component observed on SDS PAGE (see Section 3.3.1); its origin is unclear.

Behaviour of preparations in the <u>in vitro</u> incubation system.

When the batches of rat gamma globulins were examined in the <u>in vitro</u> system, the Endocytic Indices shown in Table 3.3.1 were obtained. Most of the gamma globulin preparations examined exhibited an uptake rate of about 15ul/mg tissue protein/h, except lactoperoxidase-labelled preparations, for which the uptake rates were slightly higher. This effect may perhaps be due to the presence of aggregated forms that had not been removed from the preparation.

The release patterns from rat yolk sacs for gamma globulins that had been labelled with either chloramine-T or lactoperoxidase were compared (Figures 3.3.7 and 3.3.8). The behaviour of the two preparations is essentially identical, with no evidence that either method of labelling significantly alters the capacity of the molecule to be protected from degradation and released from the yolk sac in a TCA-insoluble form.

3.3.3 The incorporation of ^{125}I-L-tyrosine into rat yolk sacs.

When rat yolk sacs were incubated in the presence of ^{125}I-L-tyrosine there was no clear evidence of significant progressive incorporation of radioactivity into newly-synthesised, tissue-associated proteins (Figure 3.3.9). The level of tissue-associated radioactivity, after the incubation has been stopped and the yolk sacs washed, remains apparently constant during the first few hours. (The radioactivity that is associated with the tissue at this stage is assumed to be free iodotyrosine that either did not have time to diffuse out of the tissue during the washing procedure or that is occluded at the cell surface and would be removed by longer washing.)

After 20h there is some evidence of increased tissue-associated radioactivity, but this could possibly be explained in terms of cell death. (At 20h a large proportion of the cells may well have lysed and the availability of intracellular membrane, onto which iodotyrosine can perhaps adsorb, could account for the rise in tissue levels of radioactivity.) An alternative explanation would be that the total protein content of the yolk sacs had fallen, thus causing an elevated amount of iodotyrosine per mg tissue protein. Results available to date are few in number, but they do appear to indicate a fall in the protein content of the yolk sac during long-term incubation (from 7.50 ± 0.36, n=8, to 7.07 ± 0.23, n=3). This small fall (approx. 5%) is not sufficient to account for the observed increase in tissue level of iodotyrosine, but further investigations may confirm it to be a contributory factor.

3.3.4 The uptake of polyvinylpyrrolidone by rabbit yolk sacs cultured in vitro.

The mean value of the Endocytic Index for the uptake of ^{125}I-PVP by rabbit yolk sacs (Figure 3.3.10 and Table 3.3.2) has a fairly high standard deviation, indicative of a high level of inter-rabbit variation. However, the data show no clear dependence of Endocytic Index on concentration of PVP. Tissue taken from a single animal invariably gave results similar to those shown in Figure 3.3.11. Here, for a limited range of substrate concentrations, there is no apparent dependence of the rate of uptake on concentration. This may be simply a result of the concentration range employed being related to the linear portion of the 'binding curve', however, and the use of a wider concentration range is

desireable to clarify this.

3.3.5 The effects of serum on endocytosis in rabbit yolk sacs.

Increasing concentrations of calf serum were shown to inhibit progressively the rate of ^{125}I-PVP accumulation by rabbit yolk sacs (Table 3.3.3). Rabbit serum, bovine serum albumin, casein and bovine gamma globulins were all found to inhibit pinocytosis by about the same amount at the same concentration (Figure 3.3.12). The inhibition of uptake caused by serum was shown to be immediately reversible by washing the tissue and reincubating in fresh medium (Figs.3.3.13 and 3.3.14).

If the inhibition is caused by a nutrient feedback, then high levels of exogenous amino-acids might be expected to have the same effect. If inhibition results from saturation of non-specific protein binding sites on the cell surface, however, simple amino acids and hydrolysed proteins might not have any effect if they are incapable of efficient binding.

In beginning to determine whether amino acids had any effect, the effects of casein hydrolysate and of glycine were investigated. Enzyme- and acid-hydrolysed casein were both used, but in all cases severe difficulty was experienced with solubility. Yolk sacs that were exposed to casein hydrolysates exhibited a higher than normal degree of tissue variability, and a distinct 'snowiness' of the incubation medium was noticed after a few hours. This may result from toxic effects of of the hydrolysate. Damage to the tissue could account for the observed inhibition of pinocytosis by the casein hydrolysate (Figure 3.3.12). However, similar concentrations of glycine caused no observable effects on pinocytosis, and hence high levels of this amino acid alone appear neither to be toxic nor to cause nutrient-feedback inhibition of pinocytosis.

Attempts were made to estimate the steady-state tissue levels of protein when high concentrations of cold protein were being used to inhibit endocytosis together with a low concentration of ^{125}I-labelled protein. External BSA concentrations of 0.4mg/ml and 0.8mg/ml, which inhibit rabbit yolk sac pinocytosis by 20-40%, allow the yolk sac tissue-level of ingested protein to reach steady-state after about 1h (Figure 3.3.15 (a)), but an external concentration of BSA of 7mg/ml (which inhibits pinocytosis by 80%) does not permit steady-state to be

attained in three hours, at which time the tissue level of ingested protein is still rising (Figure 3.3.15 (b)).

3.3.6. The effects of lower oxygen tension on the behaviour of rat yolk sacs in vitro.

When the oxygen tension was decreased from 95% to 5% there was no detectable effect on the rate of ^{125}I-PVP accumulation by rat yolk sacs in the presence or absence of 10% calf serum (Figure 3.3.16). Decreasing the oxygen tension as low as possible, by extensively gassing the incubation medium with nitrogen (see Section 3.2.8), also failed to disrupt the normal behaviour of the tissue.

In terms of the uptake rate of ^{125}I-labelled homologous gamma globulins , decreasing the oxygen concentration from 95% to 5% was seen (Figure 3.3.17) to have no observable effect.

Experiments were performed to investigate the effects of various oxygen tensions on the release from rat yolk sac tissue of homologous ^{125}I-labelled gamma globulins. Because this work was performed over a long period using different preparations of gamma globulins and incubation medium, a fairly wide spread of results was obtained. In all cases, however, matched controls were performed using 95% oxygen. In order to make the data strictly comparable they have been normalised relative to these matched internal controls. Decreasing the oxygen tension from 95% to 5% does not seem to modify either the uptake or release patterns for ^{125}I-gamma globulins (Figure 3.3.18).

3.3.7 The nature of the TCA-insoluble products released.

TCA-insoluble radioactivity that had been released from rat yolk sacs previously exposed to ^{125}I-labelled homologous gamma globulins was isolated as described (Section 3.2.9) and examined by SDS PAGE (Figure 3.3.19). Similarly, TCA-insoluble material released from rabbit yolk sacs that had been previously exposed to homologous ^{125}I-IgG was examined on Sephacryl S200 (Figure 3.3.21).

Essentially the same gel pattern is obtained for the activity released from rat yolk sacs as for the original preparation of gamma globulins (Figure 3.3.20). The expected two peaks are present, and there is no evidence for the presence of a substantial amount of partial

breakdown products, as compared with the original material.

The rabbit IgG-derived radioactivity gives the same elution pattern on Sephacryl S200 as the starting material (Figure 3.3.22), but there is a substantial tail to the peak, indicating the presence of fairly high molecular weight breakdown products, although the bulk of the material is intact.

3.3.8 The effects of cold gamma globulins on the release of ^{125}I-PVP from rat yolk sacs.

The inclusion of 5ug/ml cold rat gamma globulins in the uptake medium together with ^{125}I-PVP had no apparent effect on the subsequent release pattern of ^{125}I-PVP-derived radioactivity from the reincubated tissue (Figure 3.3.23).

In the following RESULTS section, as throughout the rest of this Thesis, the following key to symbols is observed, unless indicated otherwise in the legends.

TCA - soluble radioactivity is represented by open symbols - mainly squares or circles.

TCA - insoluble radioactivity is represented by closed symbols - mainly squares or circles.

$\pm$ Error bars represent $\pm$ Standard Deviations.

Figure 3.3.1 SDS PAGE pattern for various substrates.

band 20

band 1

Molecular weight markers were in bands 1, 10, 20

Chloramine T-labelled gamma globulins were: fresh, 14
old, 4
v. old, 15

Lactoperoxidase-labelled globulins were: fresh, 2, 19.
old, 17.

Imido-ester - labelled globulins were in 6.

Cold proteins were: globulins, 3, 18.
IgG, 5, 16.

Bands 7,8,9,11,12 and 13 were unrelated.

Figure 3.3.2 The release patterns from rat yolk sacs of radioactivity derived from ^{125}I-IgG and ^{125}I-gamma globulins.

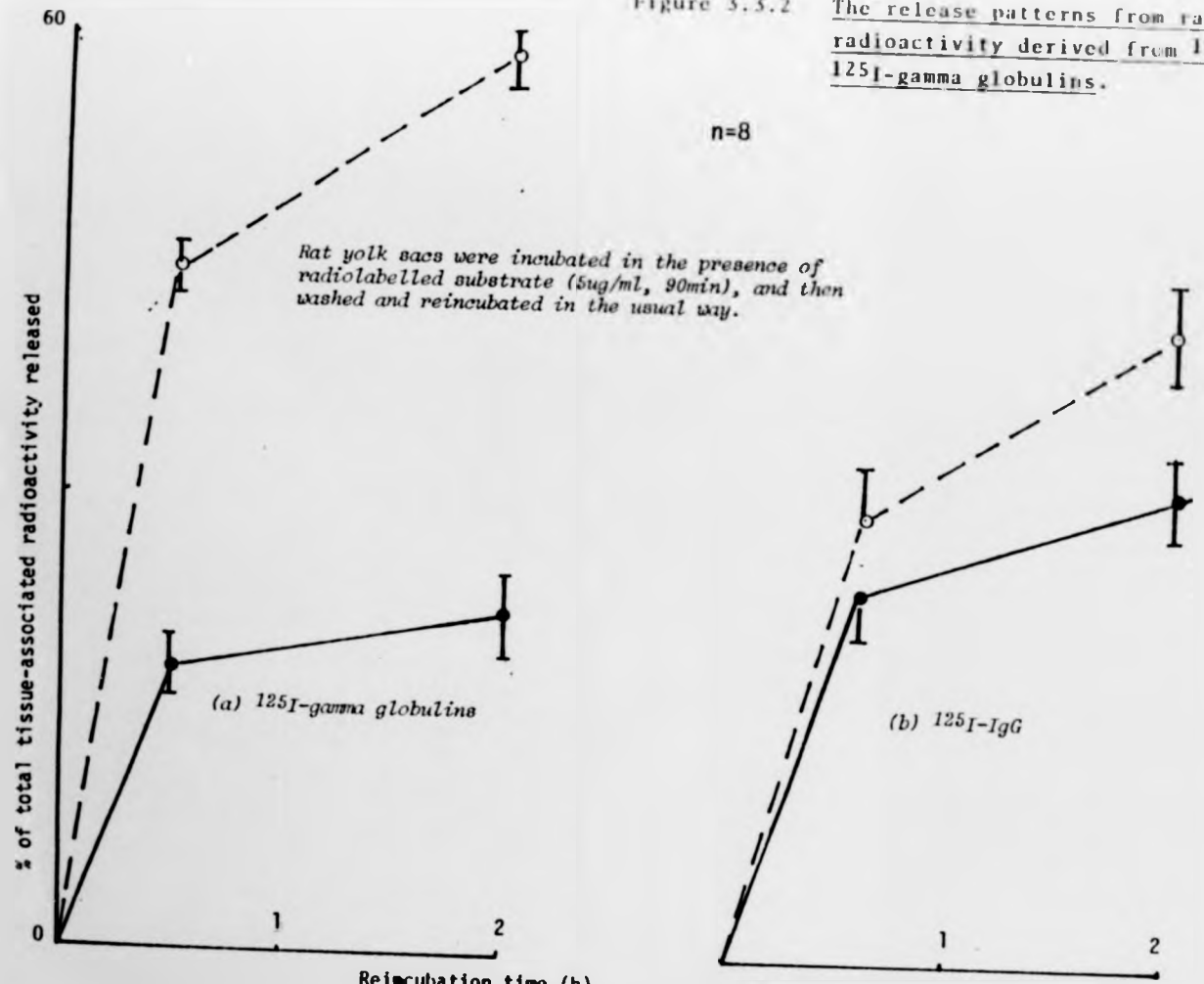


Figure 3.3.4 The release pattern from rabbit yolk sacs of rabbit ^{125}I-IgG-derived radioactivity.

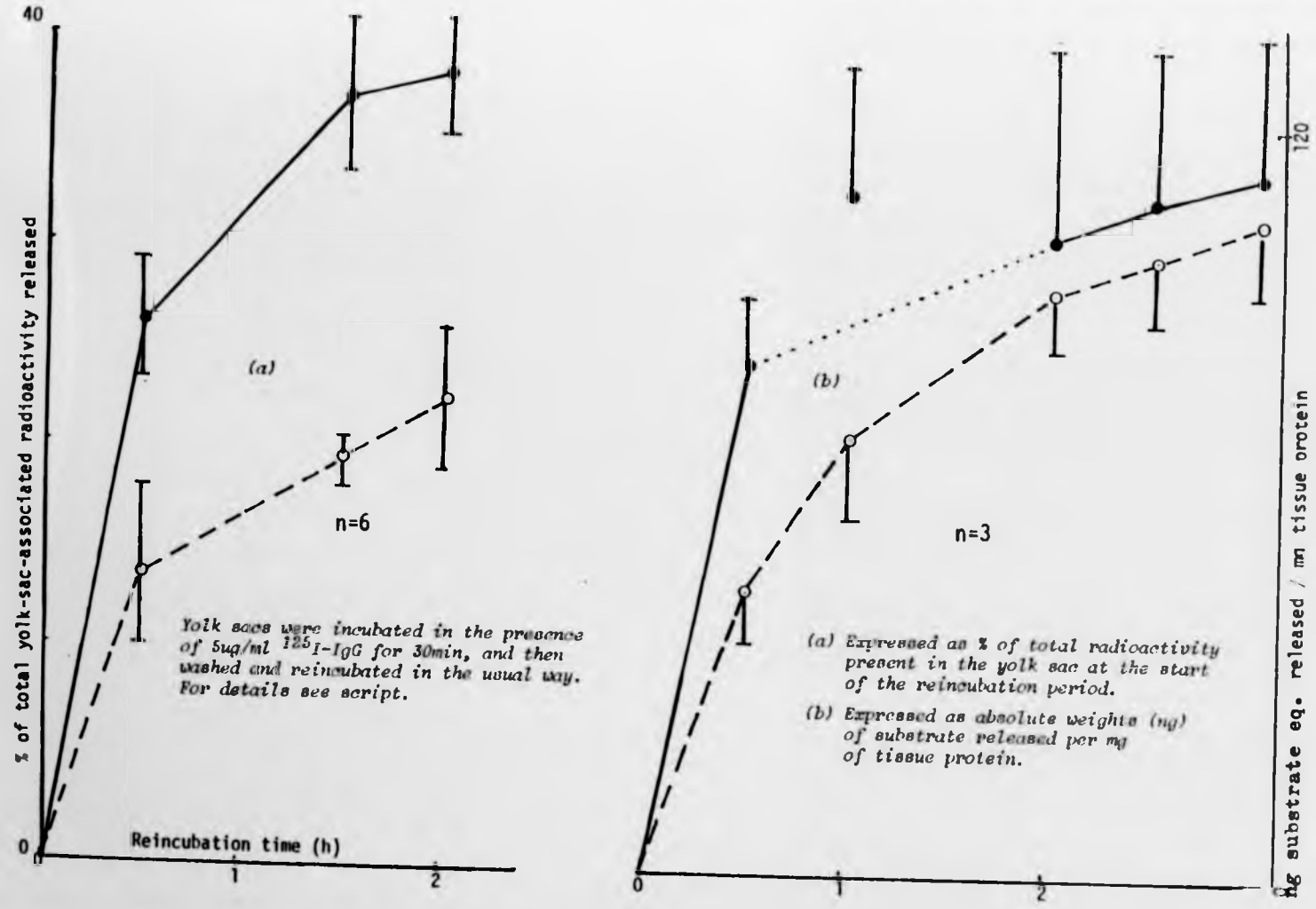


Figure 3.3.4 The release pattern from rabbit yolk sacs of rabbit ^{125}I-IgG-derived radioactivity.

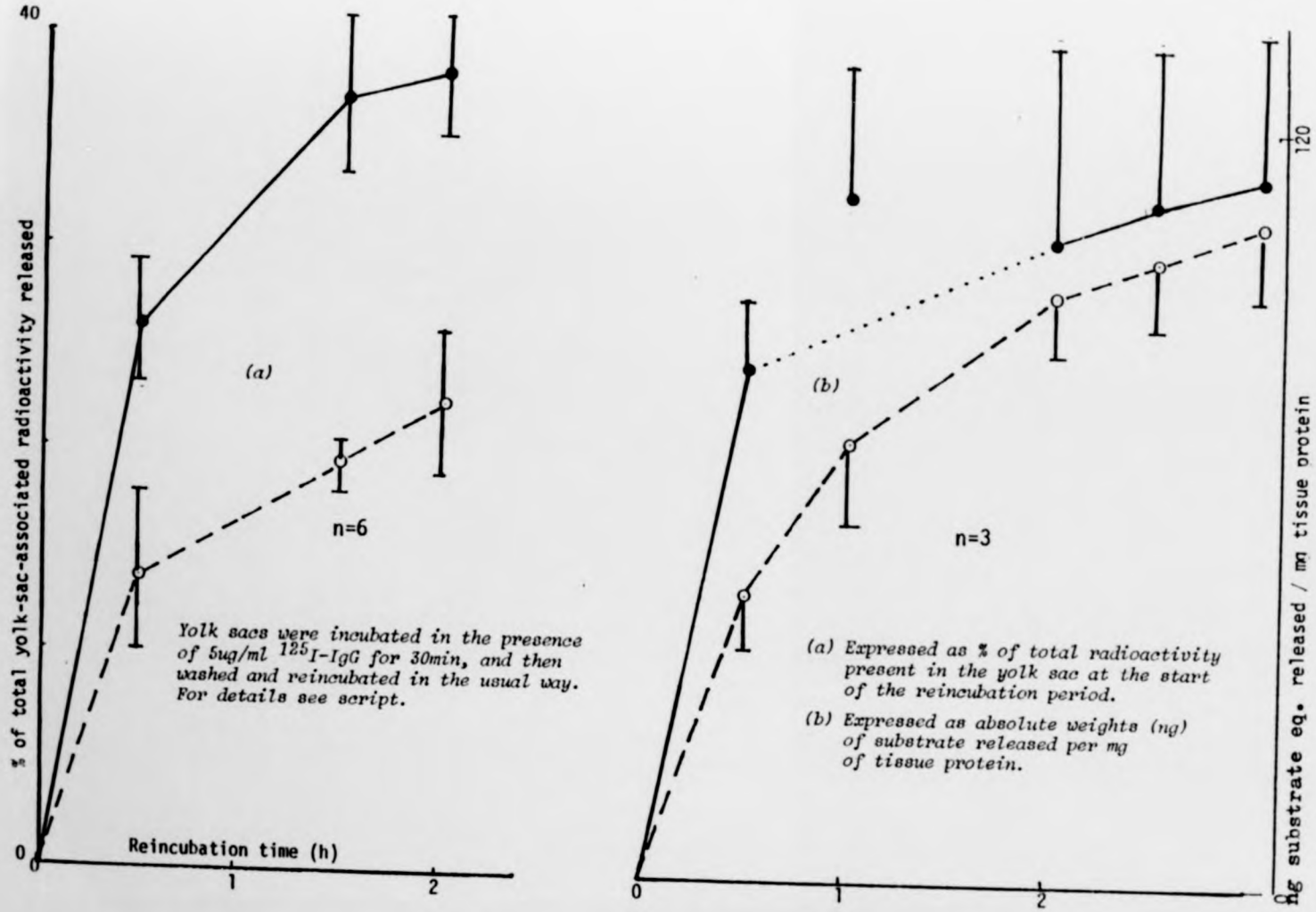
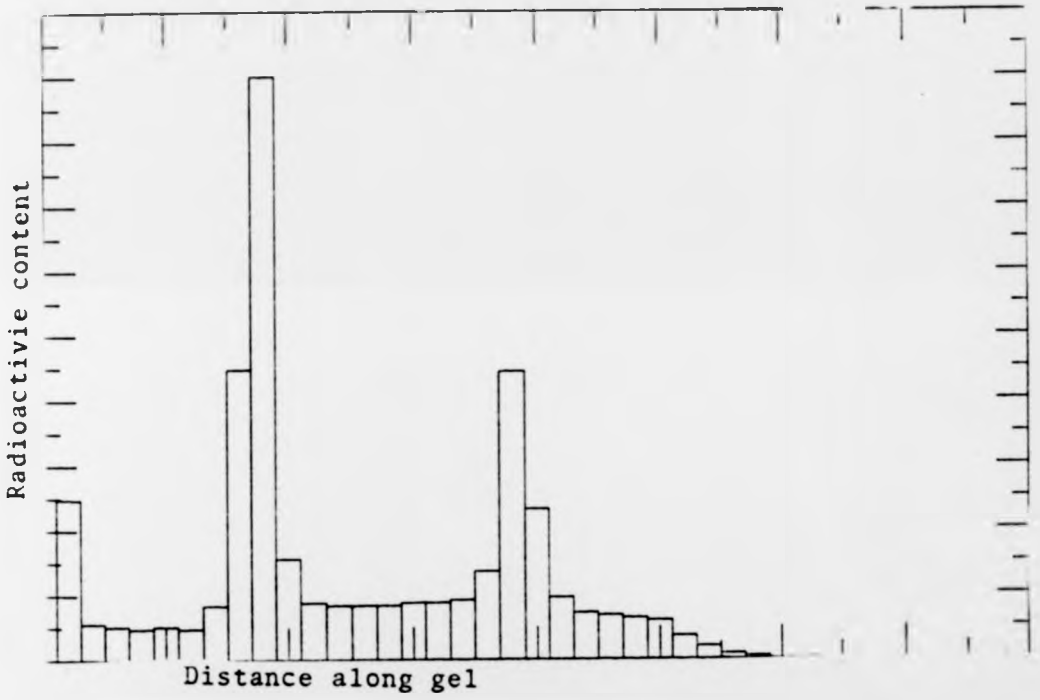


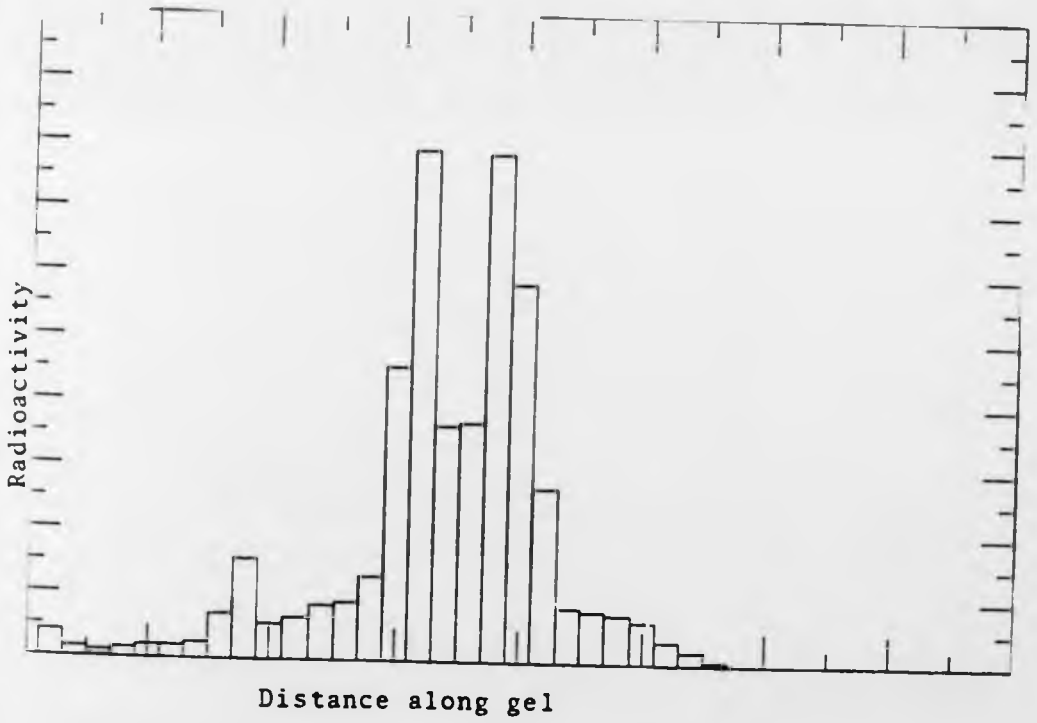
Figure 3.3.5a SDS-PAGE pattern for 'old' Chloramine T-labelled gamma globulins.



Gamma globulins (rat) were radioiodinated with Chloramine T in the usual way, and then stored for 6months at -20°C.

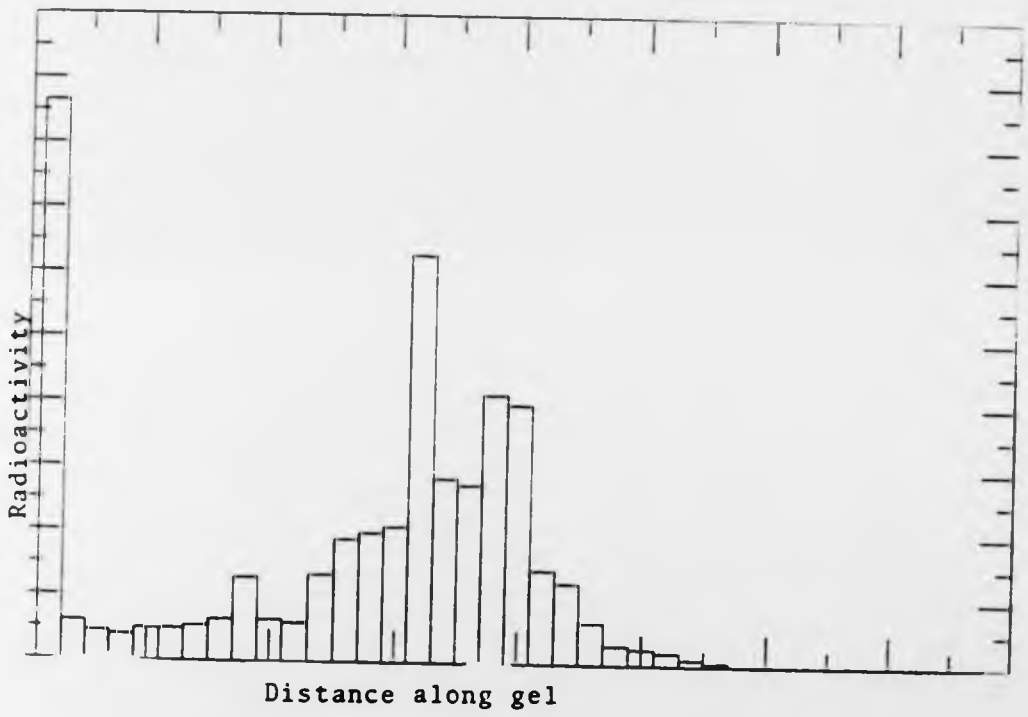
Full details are given in the text.

FIGURE 3.3.5b SDS-PAGE pattern for rat gamma globulins freshly labelled with Chloramine T.



Full details are given in the text

Figure 3.3.5c SDS-PAGE distribution for rat gamma globulins, freshly labelled using the lactoperoxidase method.



Full details are given in the text.

Figure 3.3.6 The elution pattern from Sephacryl S200 of gamma globulins labelled by the lactoperoxidase technique.

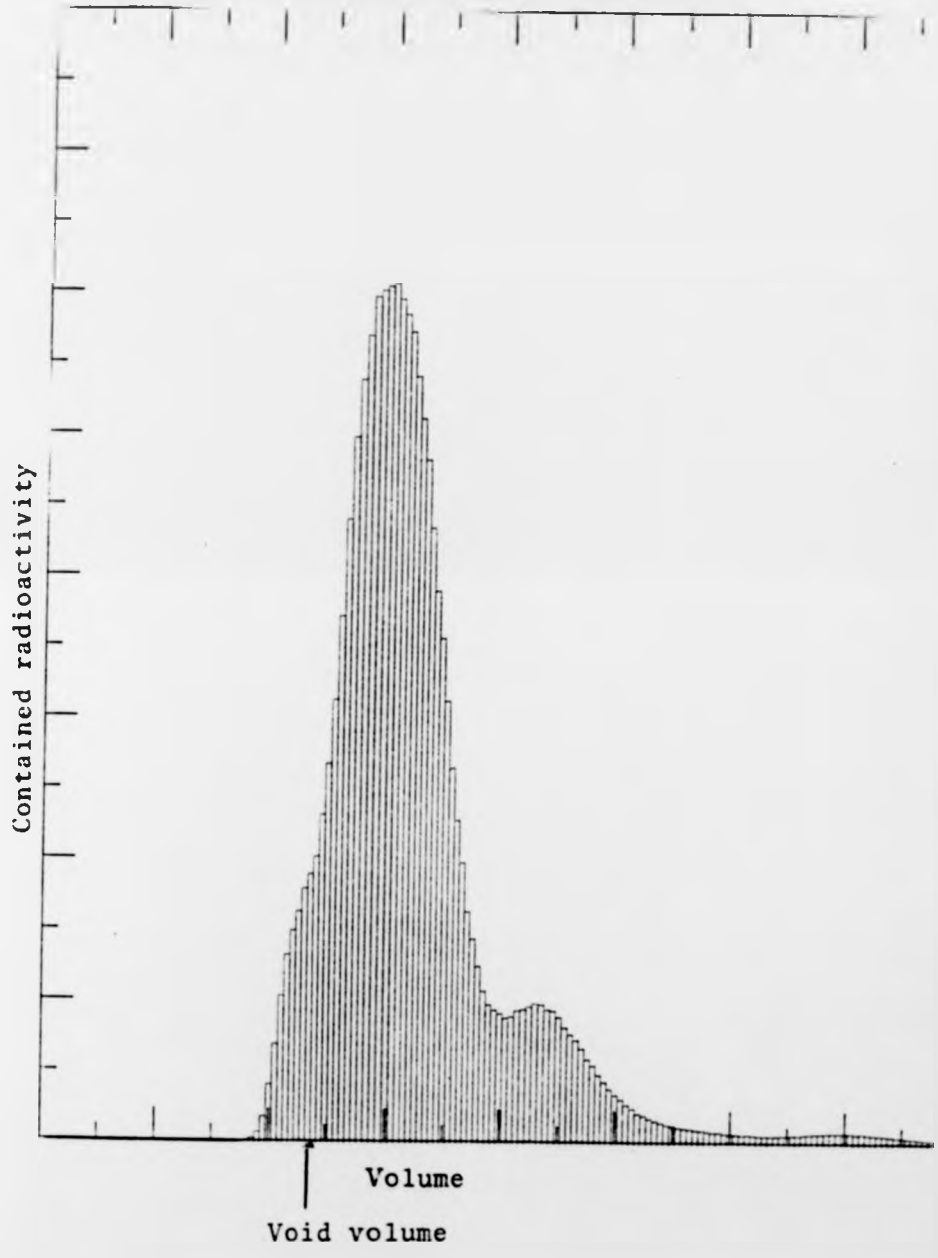


Figure 3.3.7 Release patterns from rat yolk sacs of radioactivity derived from homologous gamma globulins radiolabelled by the Chloramine-T and Lactoperoxidase methods.

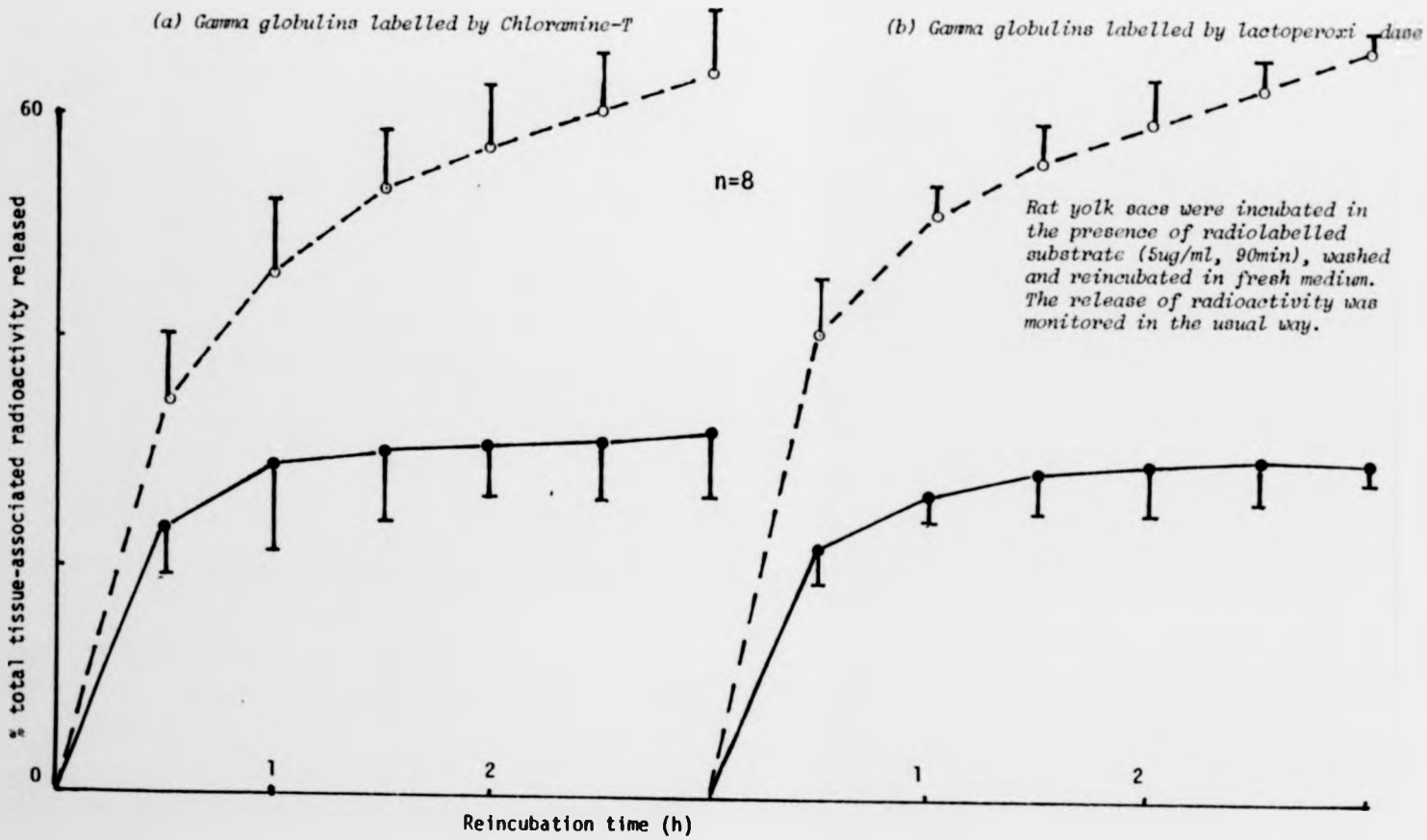


Figure 3.3.8 Release patterns from rat yolk sacs of radioactivity derived from homologous gamma globulins radiolabelled by the Chloramine-T and Lactoperoxidase techniques.

Rat yolk sacs were incubated in the presence of radiolabelled substrate (5ug/ml, 90min), washed and reincubated in fresh medium. The release of radioactivity was monitored in the usual way.

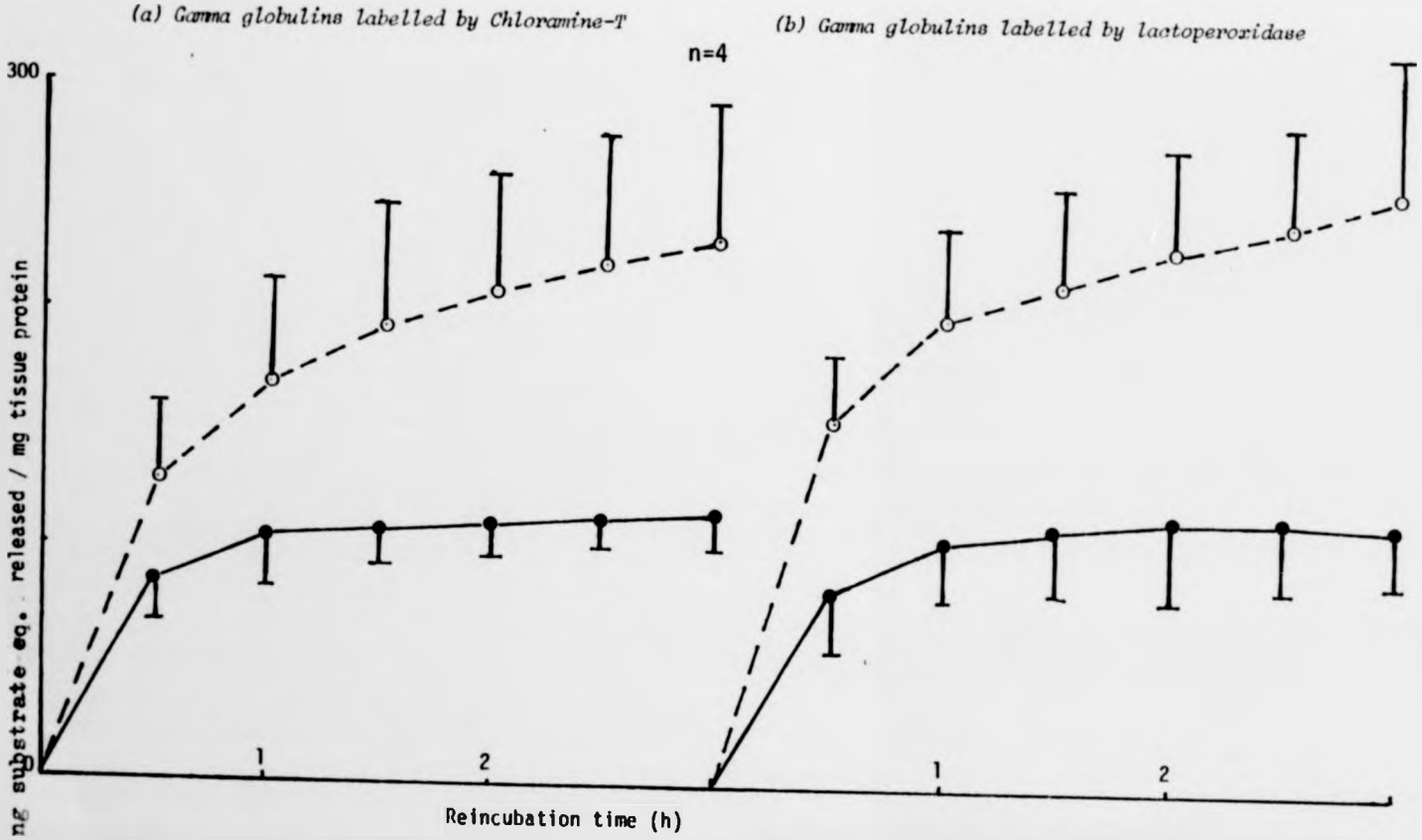


Figure 3.3.9 The incorporation of ^{125}I-(L)-tyrosine into rat yolk sacs.

Rat yolk sacs were incubated in the presence of 2ug/ml ^{125}I-L-tyrosine for a total of 21h, and the incorporation of radioactivity into the tissue was measured.

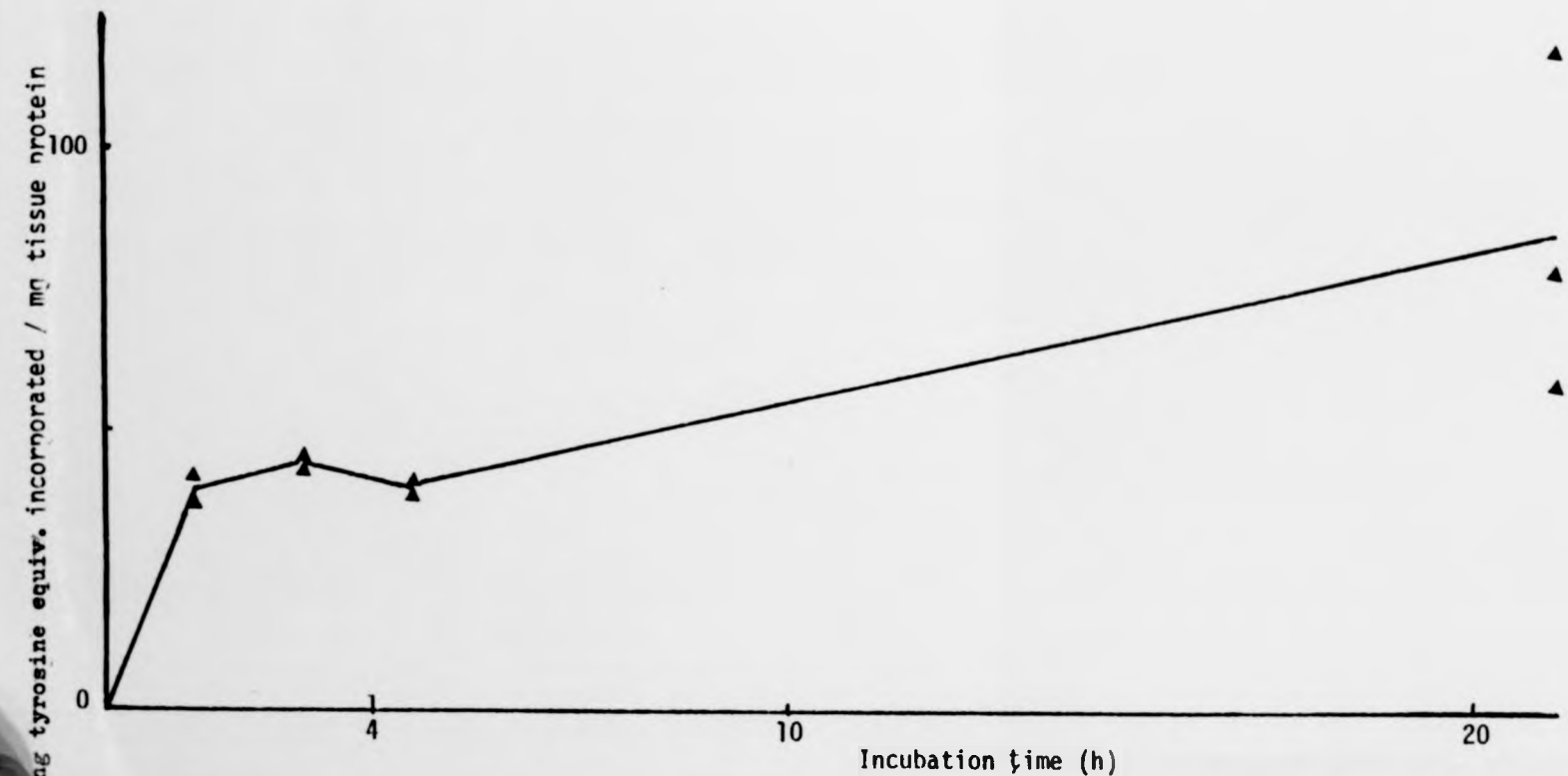
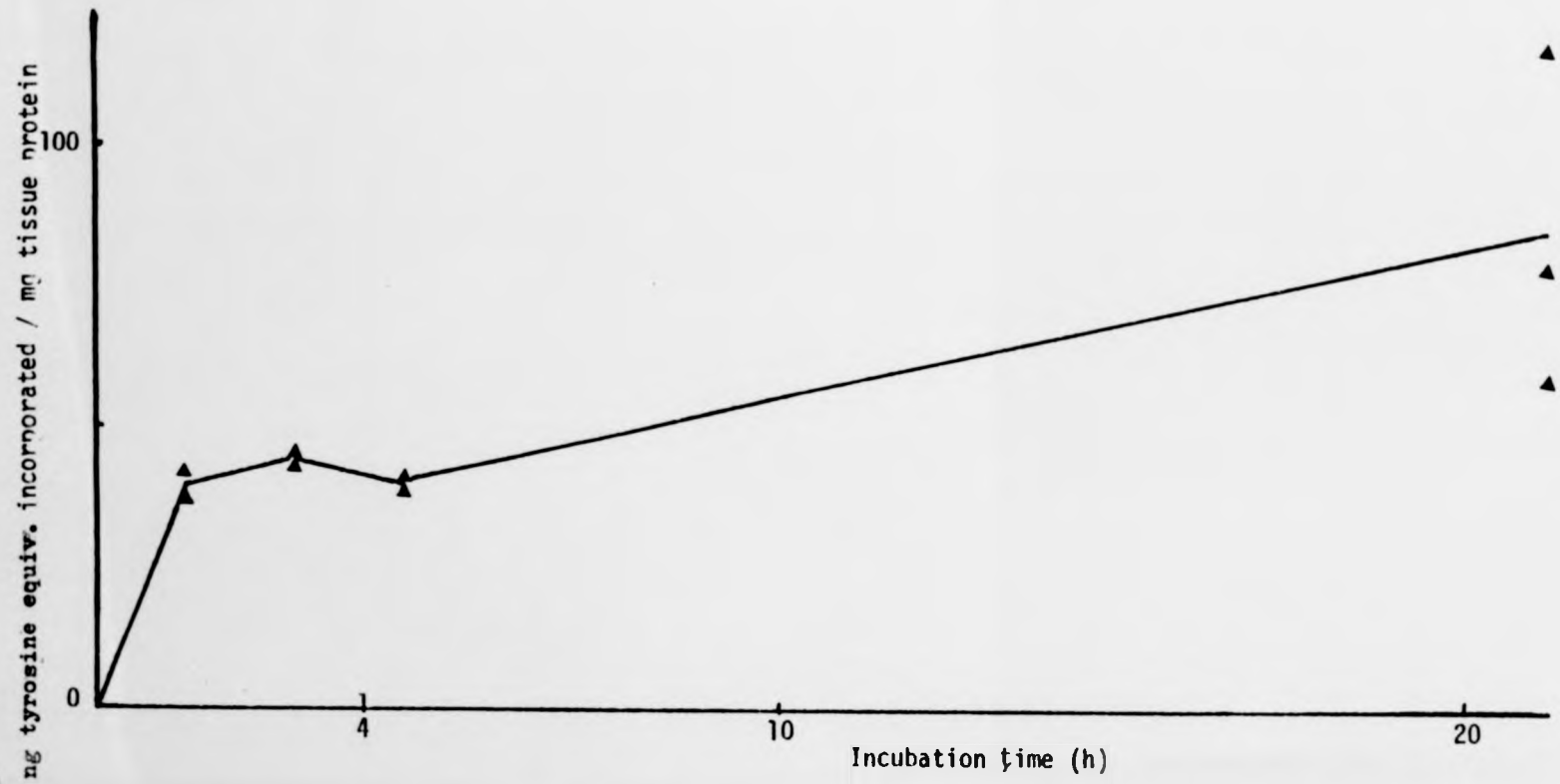


Figure 3.3.9 The incorporation of ^{125}I-(L)-tyrosine into rat yolk sacs.

Rat yolk sacs were incubated in the presence of 2ug/ml ^{125}I-L-tyrosine for a total of 21h, and the incorporation of radioactivity into the tissue was measured.



TIGHTLY
BOUND
COPY

Figure 3.3.10 The effect of substrate concentration on Endocytic Index for ^{125}I-PVP in rabbit yolk sacs.

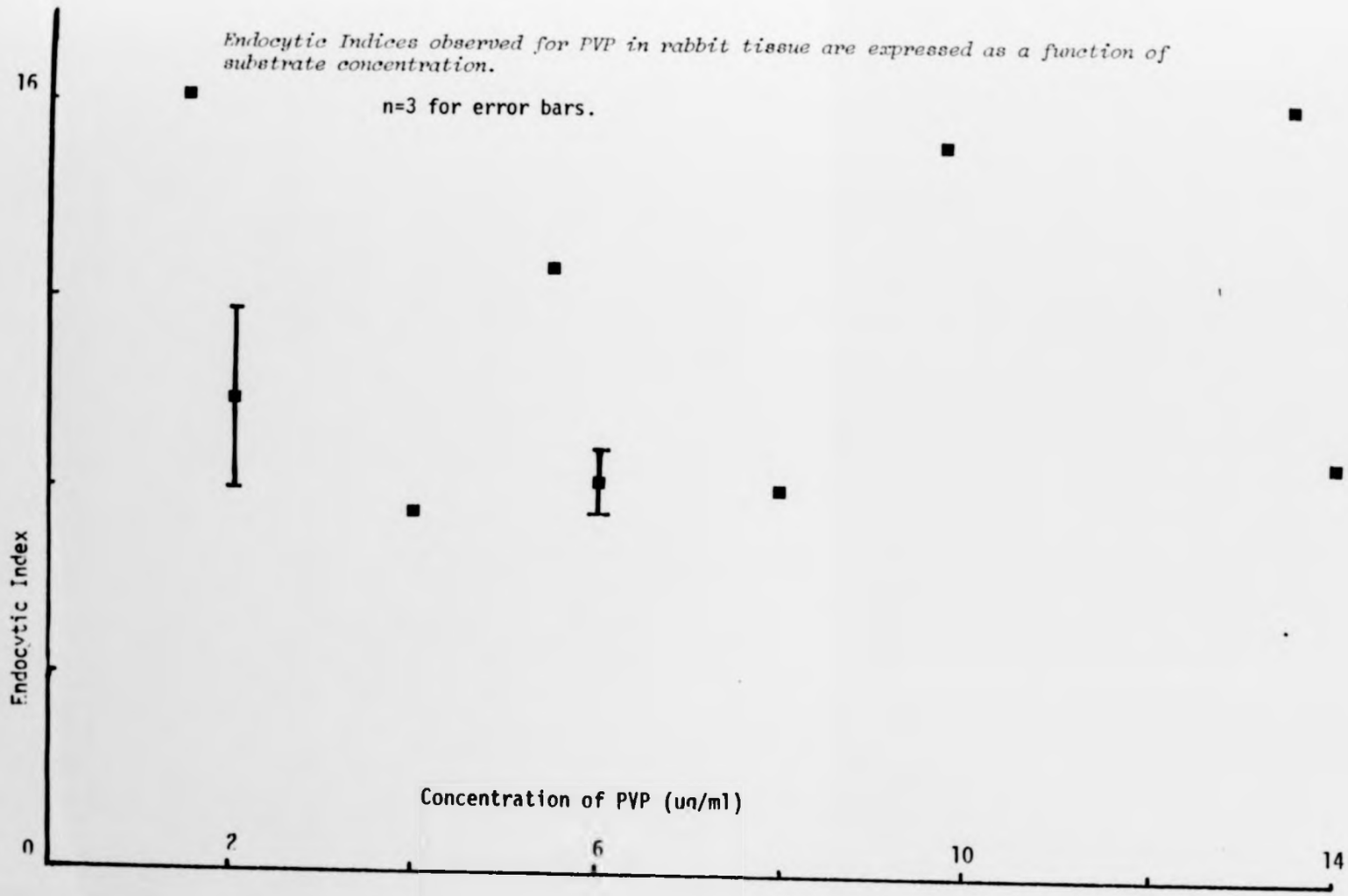
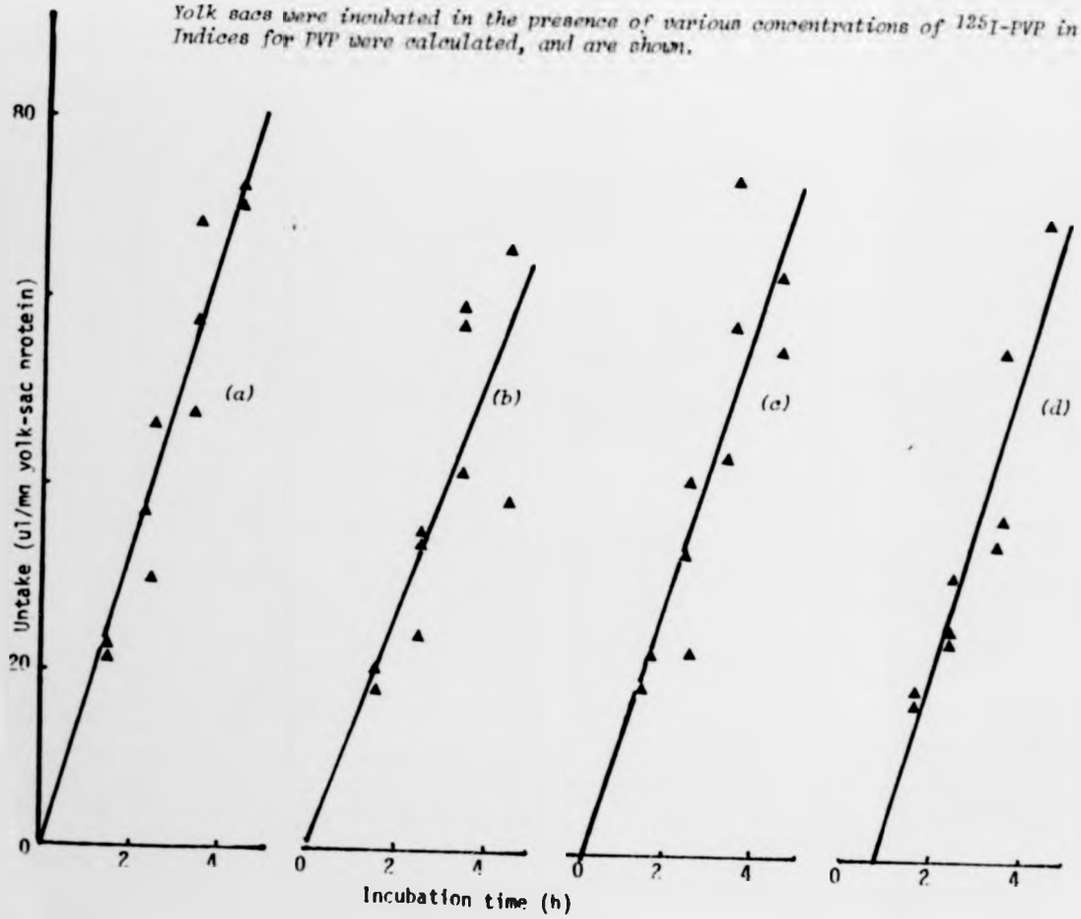


Figure 3.3.11 The influence of substrate concentration on Endocytic Index for ^{125}I-PVP in rabbit yolk sacs.

Yolk sacs were incubated in the presence of various concentrations of ^{125}I-PVP in the usual way. The Endocytic Indices for PVP were calculated, and are shown.



PVP concentration:
(a) 1.5 ug/ml
(b) 5.5 ug/ml
(c) 9.5 ug/ml
(d) 13.5 ug/ml

Endocytic Index:
(a) 16.3 ul/mg/h (0.93)
(b) 12.7 ul/mg/h (0.80)
(c) 15.3 ul/mg/h (0.84)
(d) 16.4 ul/mg/h (0.91)

Figure 3.3.12 The effects of serum and protein preparations on the uptake of ^{125}I-PVP by rabbit yolk sacs.

Rabbit yolk sacs were incubated in the presence of various sera and proteins, as shown, and the Endocytic Index for ^{125}I-PVP was measured in the usual way.

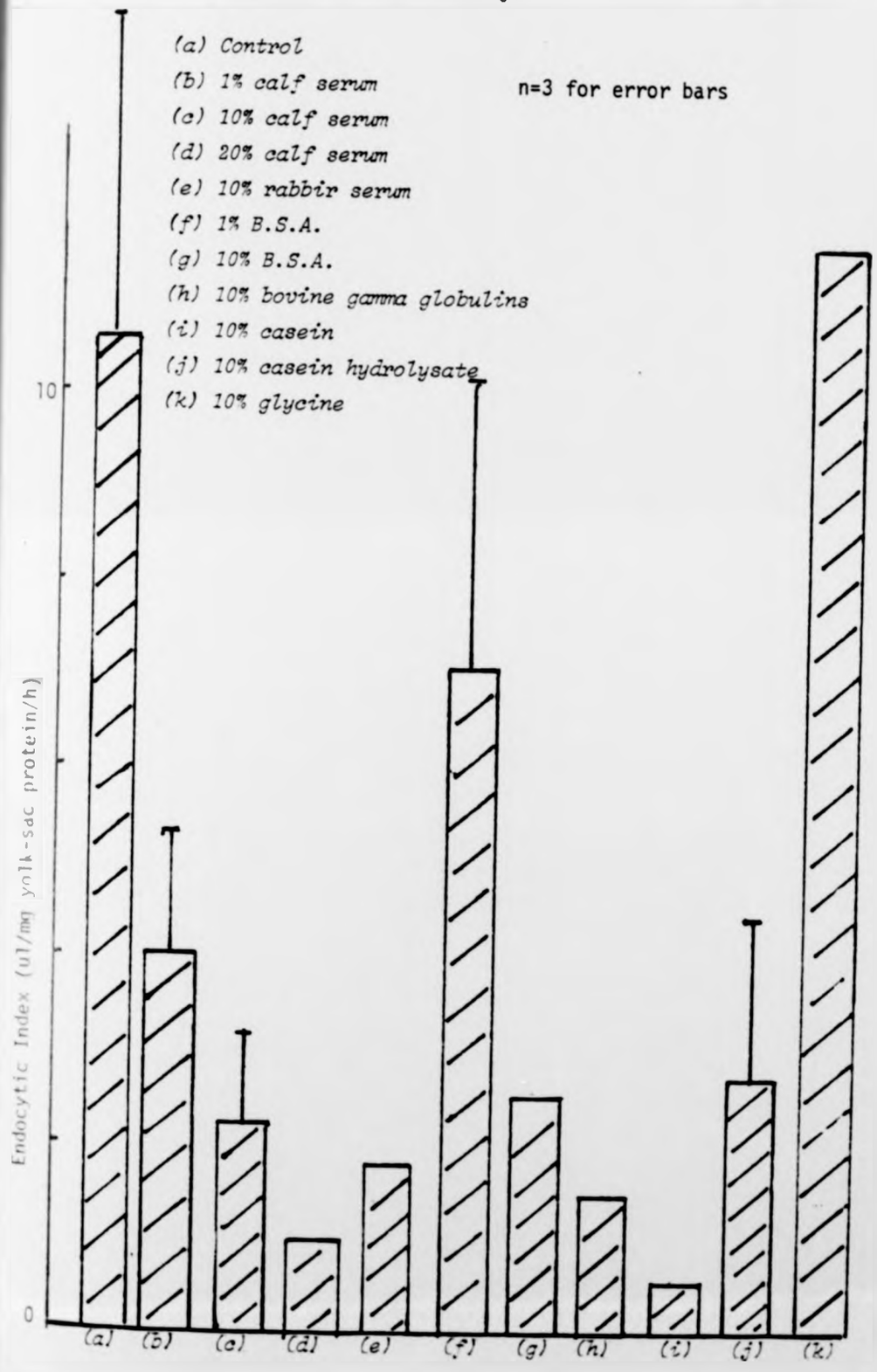


Figure 3.3.13 The reversibility of calf serum-induced inhibition of pinocytosis by rabbit volk sacs.

Control incubations were as usual. Test yolk sacs were incubated first in the presence of 10% calf serum, in the absence of substrate, for 2½h, then washed and reincubated in the absence of serum in substrate-containing medium (2ug/ml ^{125}I-PVP).

For simplicity, only points relating to experimental cases are shown. Control points are omitted.

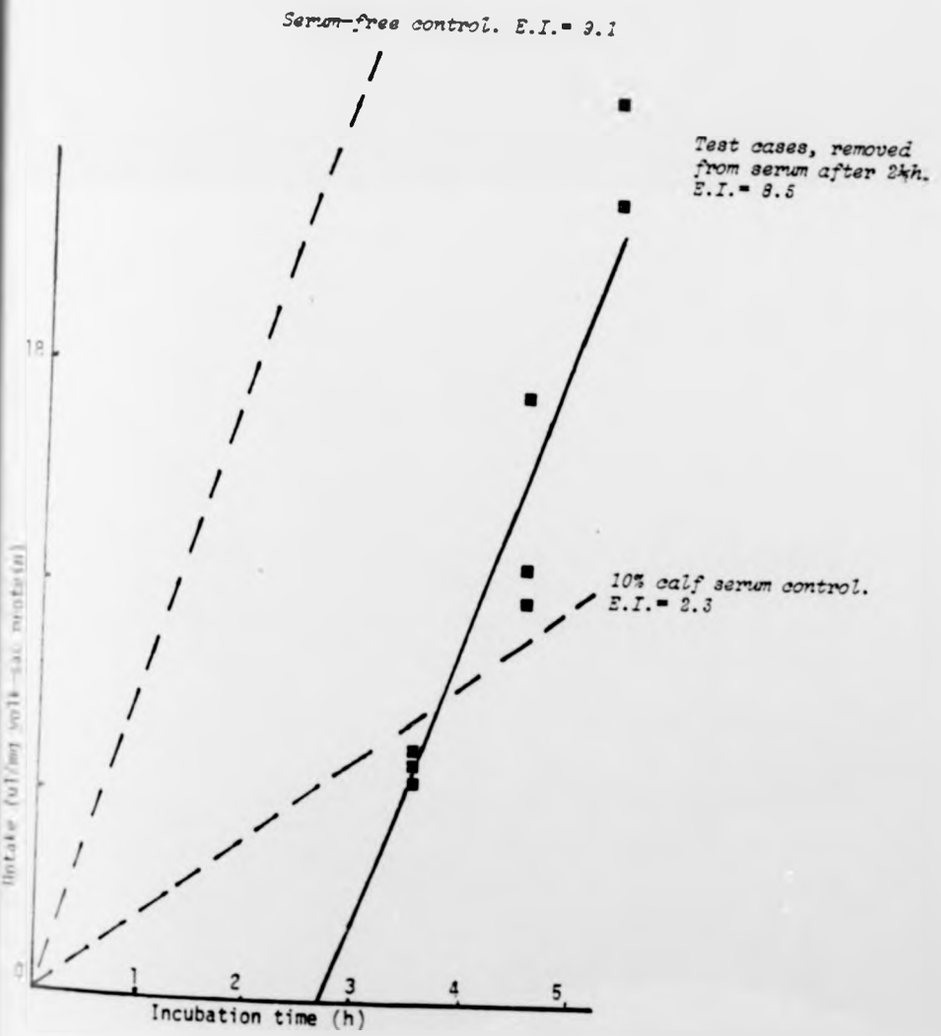


Figure 3.3.14 The reversibility of calf serum-induced inhibition of pinocytosis by rabbit volk sacs.

Rabbit yolk sacs were incubated with ^{125}I-PVP (2ug/ml) in either the presence or absence of 20% calf serum. In test cases, yolk sacs were removed after three hours from flasks containing calf serum, rinsed briefly, and then incubation continued in calf serum-free medium, still containing ^{125}I-PVP. Uptake of ^{125}I-PVP in both types of control and in test cases was monitored as usual.

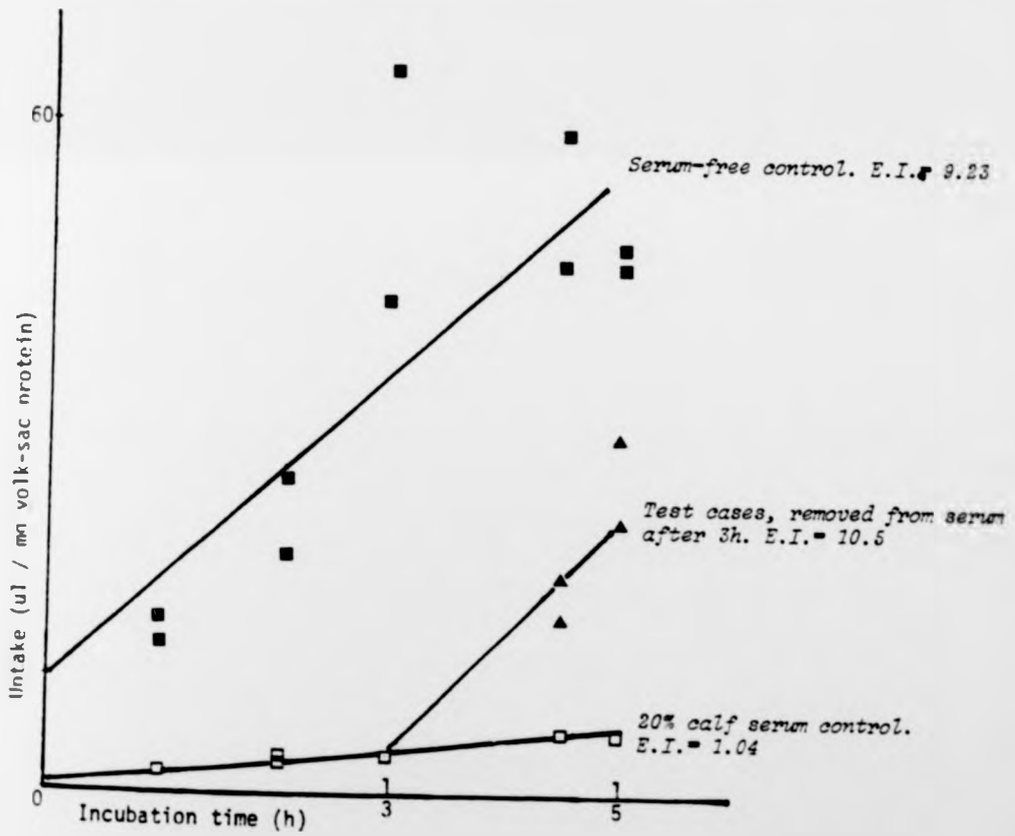


Figure 3.3.15 (a) Tissue level of ingested BSA in rabbit volk sacs at substrate concentrations of 0.4 and 0.8mg/ml.

Rabbit yolk sacs were incubated in the presence of BSA (0.4 or 0.8mg/ml) containing a 'spike' of ^{125}I-BSA. The tissue level was monitored, and hence the level of ingested protein was calculated.

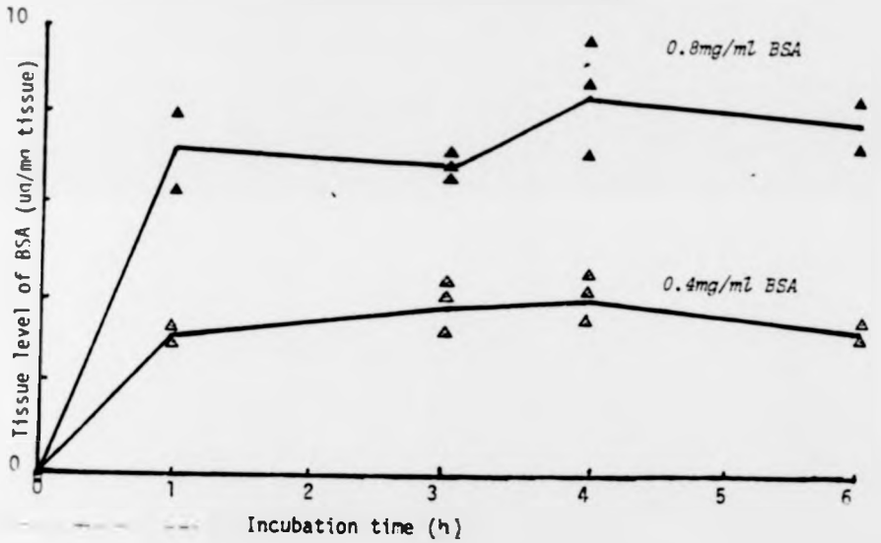


Figure 3.3.15(b) Tissue level of ingested BSA in rabbit yolk sacs at a substrate concentration of 7mg/ml.

Rabbit yolk sacs were incubated in the presence of 7mg/ml BSA, containing a 'spike' of ^{125}I-BSA. The tissue level of radioactivity was monitored, and hence the level of ingested BSA was calculated.

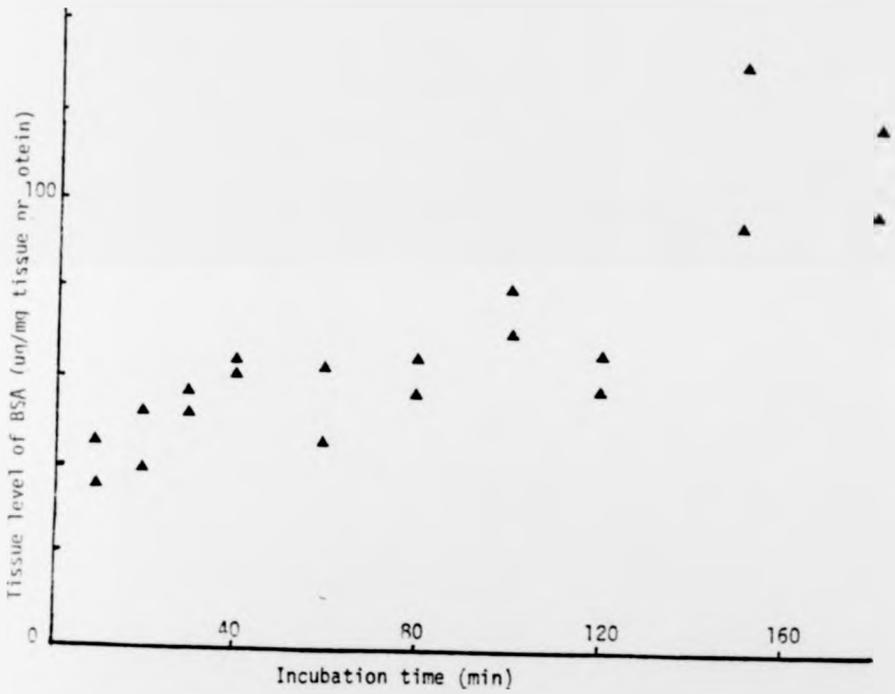
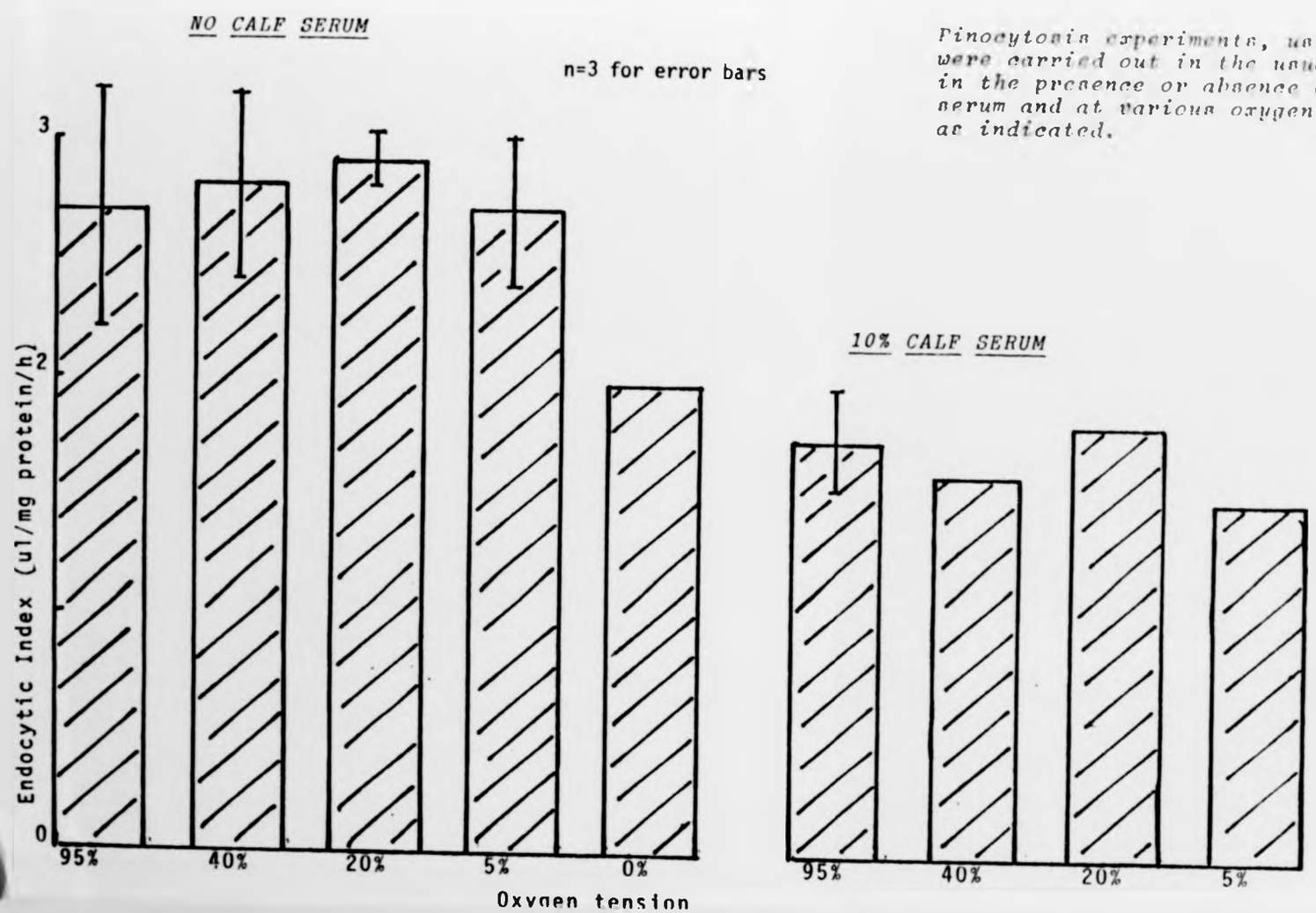


Figure 3.3.16 Endocytic Indices for the uptake of ^{125}I-PVP by rat yolk sacs at various oxygen tensions.



Pinocytosis experiments, using ^{125}I-PVP, were carried out in the usual way, but in the presence or absence of 10% calf serum and at various oxygen tensions, as indicated.

Figure 3.3.1 The effect of low oxygen tension on the uptake of ^{125}I-gamma globulins by rat yolk sacs.

Rat yolk sacs were incubated in medium 199 at 37°C in the presence of 2ug/ml ^{125}I-gamma globulins in the usual way, but at various oxygen tensions. The effect of dropping the oxygen tension from 95% to 5% is shown below.

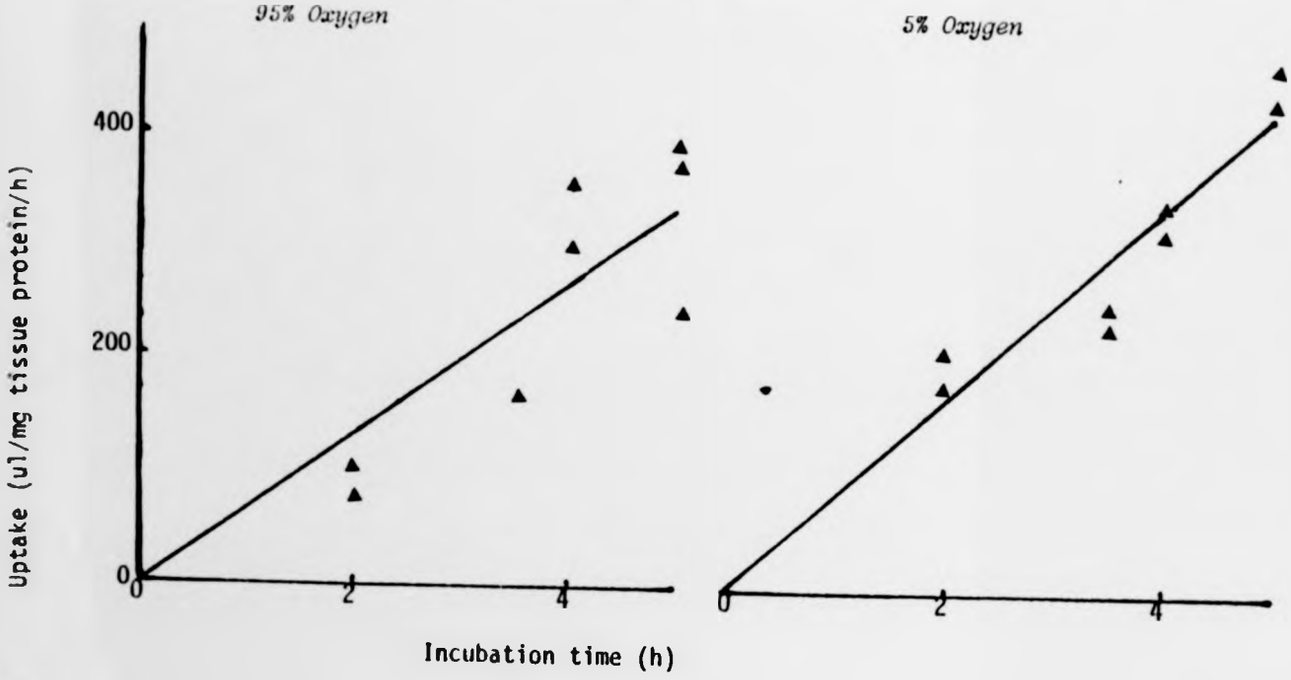


Figure 3.3.18 The effect of low oxygen tension on the release from rat yolk sacs of radioactivity derived from ^{125}I-gamma globulins.

Yolk sacs were incubated at various oxygen tensions in the presence of ^{125}I-gamma globulins, and then reincubated in fresh medium at the same oxygen tension. Exact experimental details varied, since the data were gathered over a long period of time, so the reults presented are normalised on matched controls (95% O_2), which were always carried out.

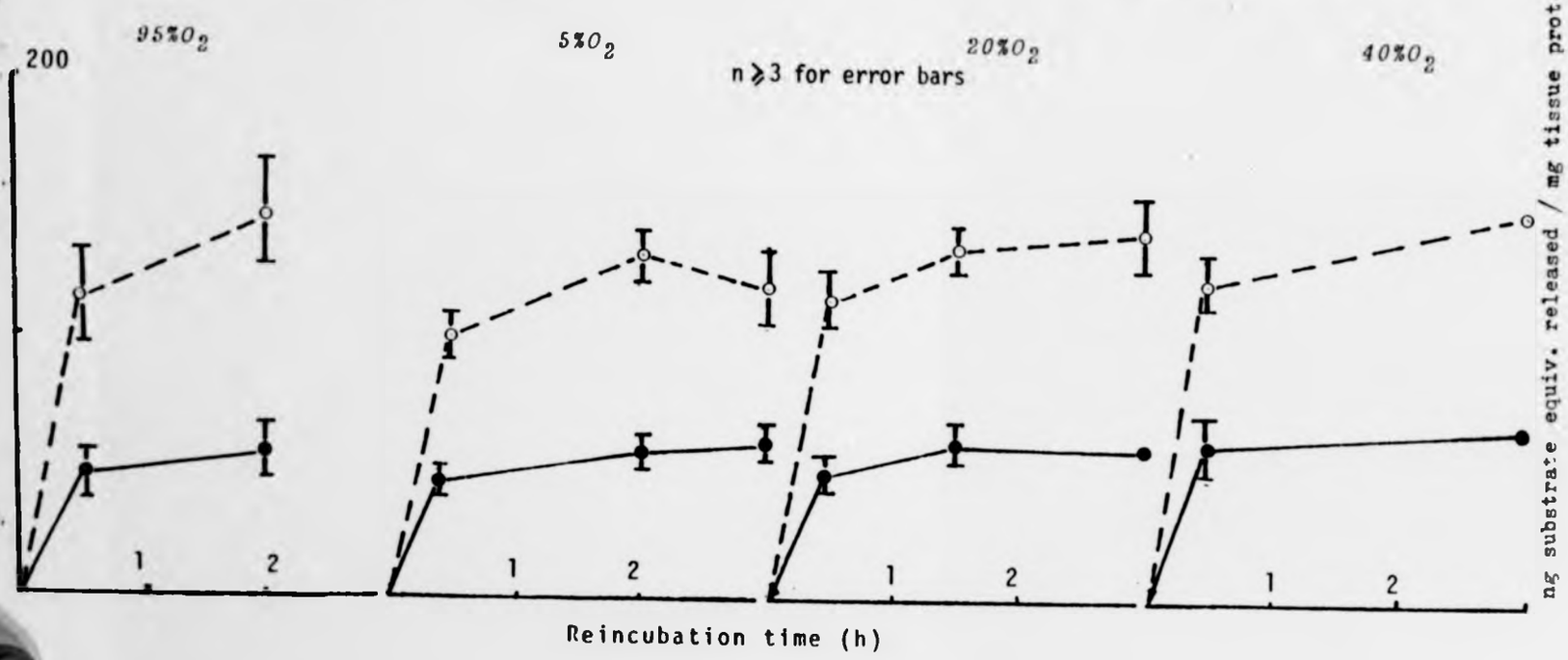


Figure 3.3.19 SDS-PAGE pattern for TCA-insoluble material released from rat volk sacs previouslv exposed to ^{125}I-gamma globulins.

Yolk sacs were incubated in medium 199 in the presence of 5ug/ml ^{125}I-gamma globulins. After 90min they were washed and reincubated in fresh, substrate-free medium. At the end of the 3h reincubation period, the medium was harvested, dialysed against 1% NaCl (aq.), and concentrated by freeze-drying. The protein content was examined by SDS-PAGE, in the usual way.

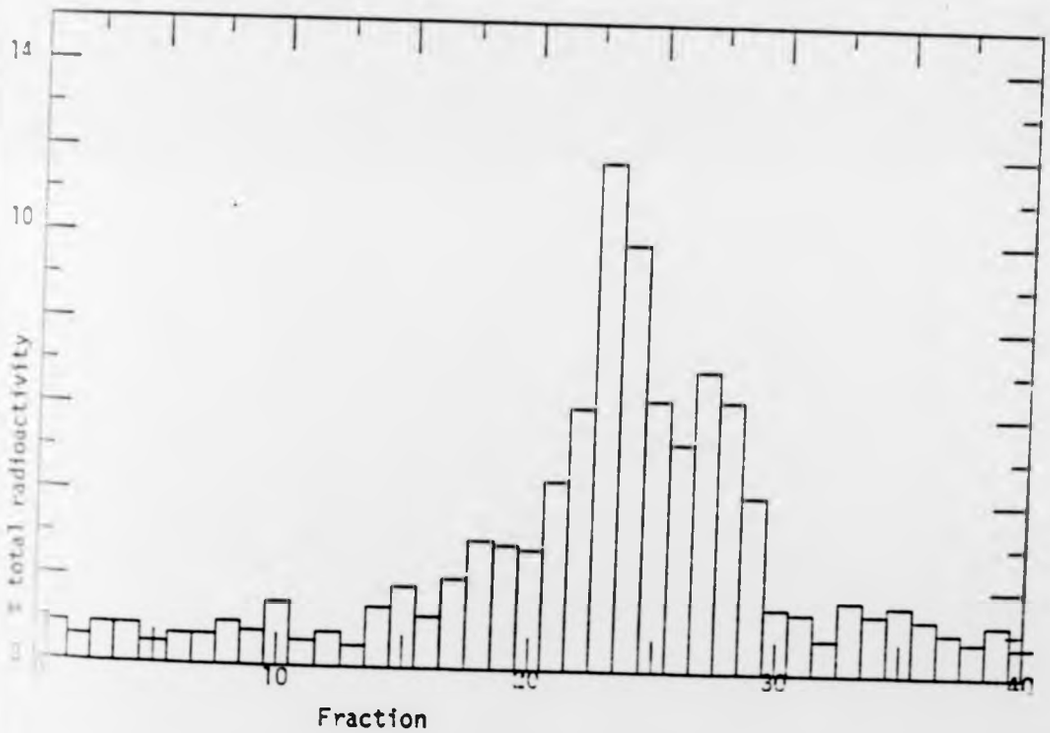


Figure 3.3.20 SDS-PAGE pattern for rat ^{125}I-gamma globulins.

Rat gamma globulins were examined by SDS-PAGE, as described in the text

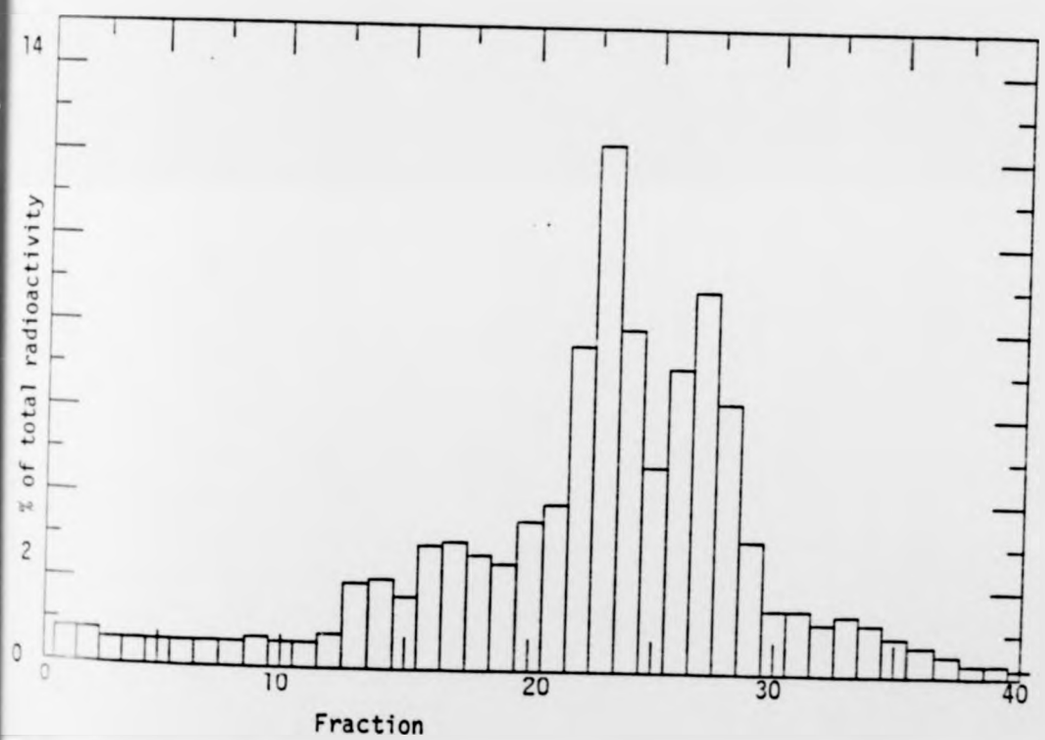


Figure 3.3.21

Elution Pattern on Sephacryl S200 of Products Released from Rabbit Yolk Sacs Previously Charged in Rabbit IgG (after dialysis).

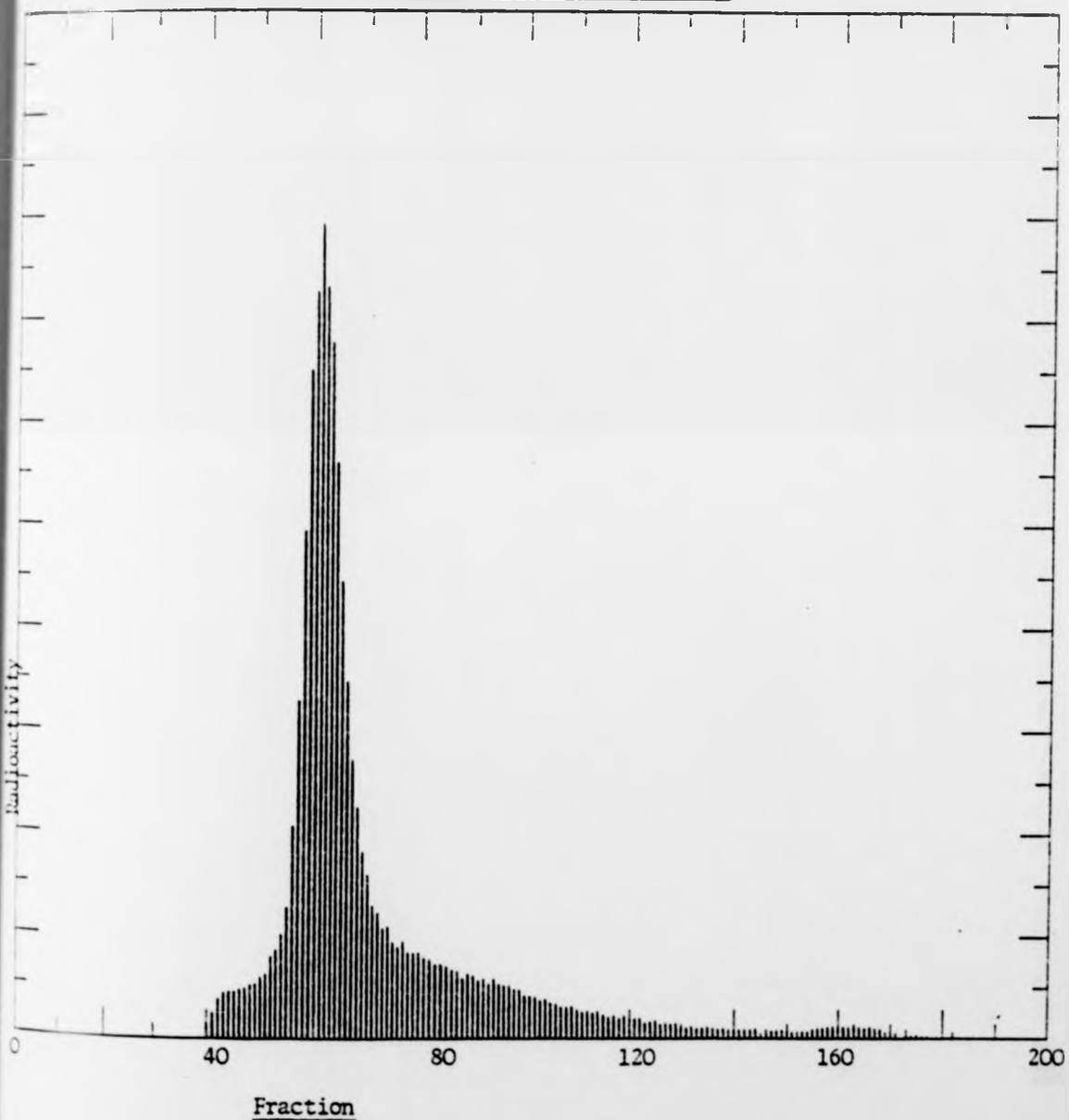


Figure 3.3.22

Elution Pattern of Untreated Rabbit IgG on Sephacryl S200.

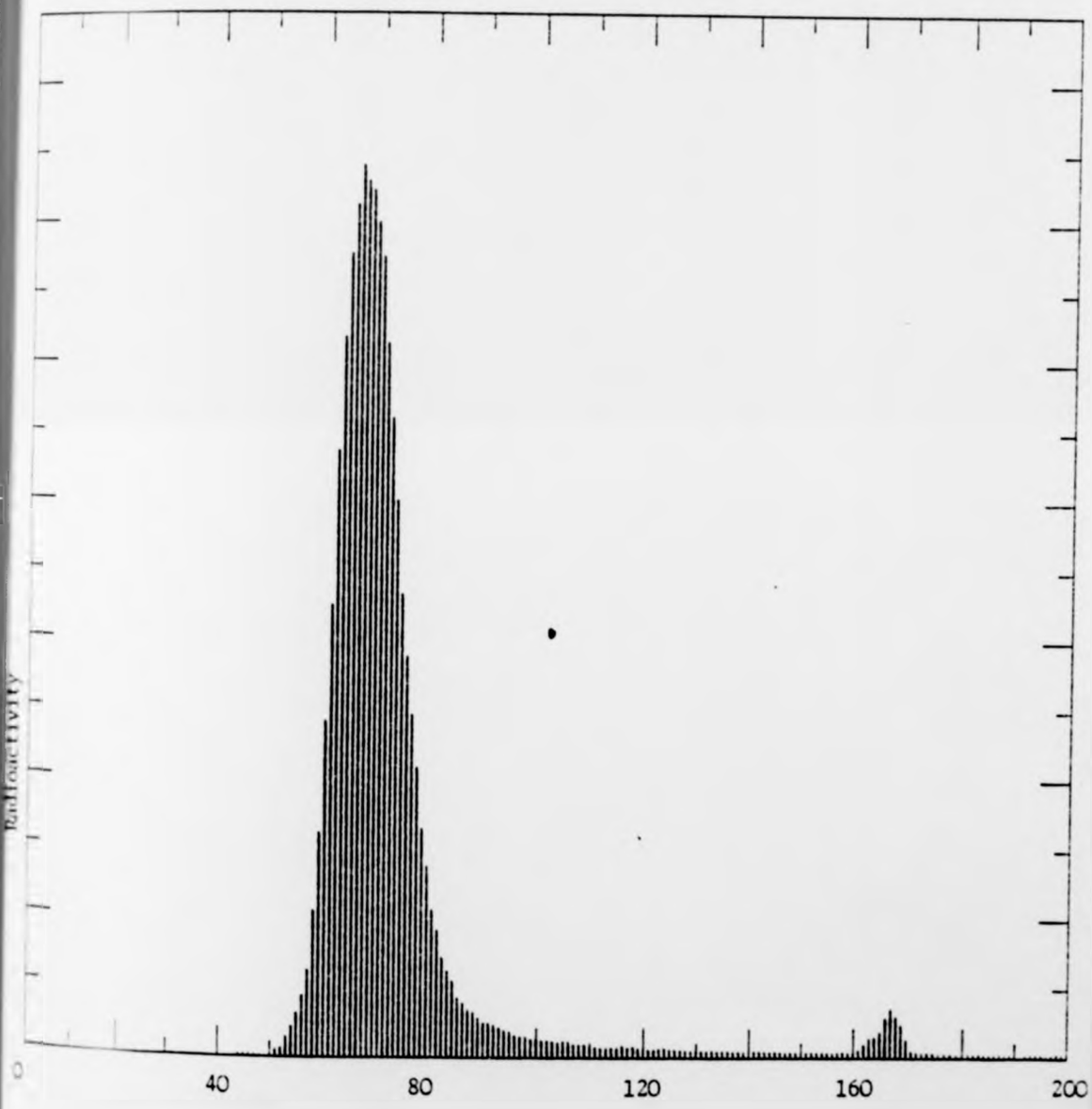
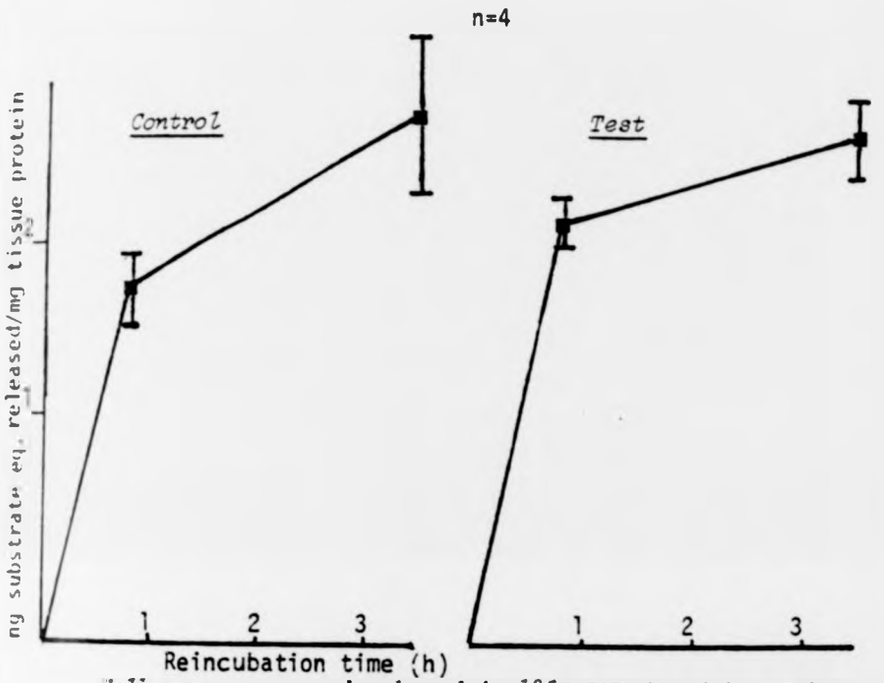


Figure 3.3.23 The effect of the inclusion of gamma globulins in the incubation medium on the pattern of uptake and release of ^{125}I-PVP.



Yolk sacs were preincubated in 125I-PVP (5ug/ml, 90min) and then washed and reincubated in fresh medium. The release of radioactivity was monitored. Test incubations contained 2ug/ml unlabelled gamma globulins throughout, controls did not.

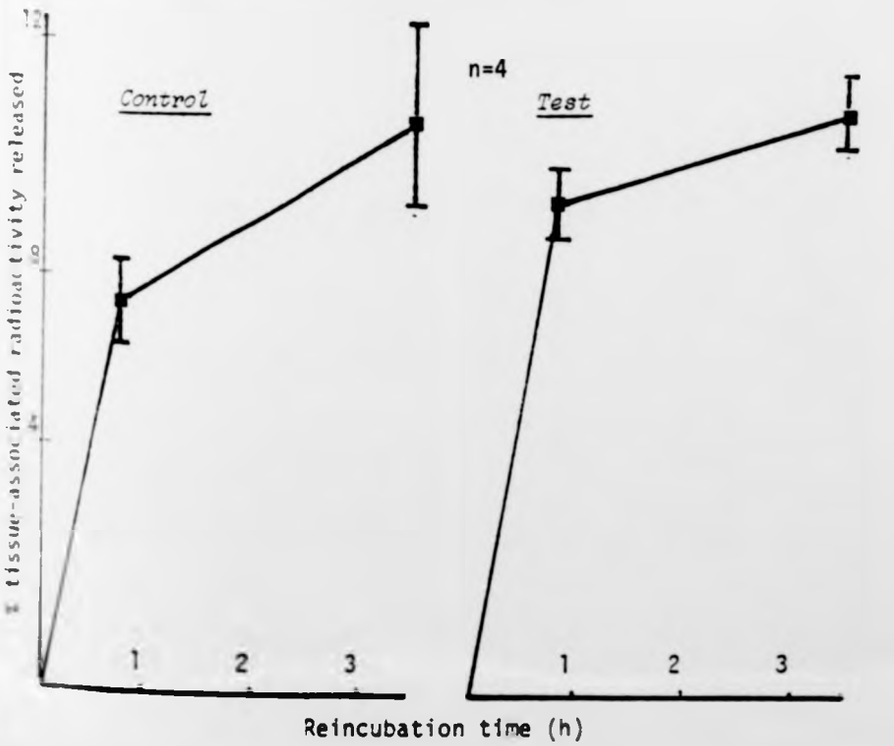


Table 3.3.1 **Endocytic Indices for rat ^{125}I-gamma globulins, labelled by different techniques.**

Labelling agent	No calf serum			10% calf serum		
	E.I.	R	n	E.I.	R	n
Chloramine-T	63.6	0.97	7	12.4	0.99	20
				15.2	0.97	10
(batch 1)				12.3	0.99	10
				20.7	0.97	9
Chloramine-T (batch 2)	68.7	0.86	10			
Chloramine-T (batch 3)				15.9	0.87	19
Lactoperoxidase				19.1	0.85	20
				16.0	0.89	10
(batch 1)				14.4	0.67	10
Lactoperoxidase				27.7	0.98	9
(batch 2)				34.7	0.99	5
Imido-ester				15.0	0.93	28

All experiments were performed in rat tissue at a final concentration of substrate of 2ug/ml.

Table 3.3.2 **The influence of substrate concentration on Endocytic Index for ^{125}I-PVP in rabbit yolk sacs.**

Concentration of PVP (ug/ml)	n	E.I. (ul/mg/h)	R	ng/mg/h
1.5	10	16.3	0.93	24.2
2.0	10	12.6	0.89	25.2
	10	10.0	0.97	20.0
	10	7.2	0.95	14.4
	10	9.2	0.80	18.5
	10	9.0	0.70	18.1
	10	10.3	0.88	20.6
	10	13.4	0.80	26.8
	10	9.1	0.77	18.2
	10	8.9	0.90	17.8
	10	9.1	0.68	18.3
	8	9.2	1.00	18.3
	10	7.9	0.96	15.8
	4	9.7	1.00	19.3
4.0	10	7.6	0.95	30.4
5.5	10	12.7	0.80	69.9
6.0	10	8.6	0.90	51.6
	10	7.9	0.99	47.4
8.0	10	8.2	0.97	65.5
9.5	10	15.3	0.84	145.4
13.5	10	16.4	0.91	217.8
14.0	10	8.6	0.99	120.4

Table 3.3.3 **The effects of calf serum on the uptake of ^{125}I-PVP by rabbit yolk sacs.**

% calf serum	E.I.	S.D.	n
0	10.6	3.4	21
1	4.0	-	2
10	2.3	0.8	11
20	0.9	-	2

DISCUSSION

3.4.1 Comparison of the analytical and behavioural features of IgG and gamma globulin.

The SDS-PAGE patterns obtained for rat IgG and gamma globulins (both unlabelled) were essentially identical, both showing the expected major molecular weight components of approx. 25 000 and 50 000. The first batch of gamma globulins appeared to have undergone a degree of protein cleavage and an intermediate band (M = 40 000) was detected that was not seen in subsequent batches of gamma globulins. There was some evidence of undenatured proteins: for example IgG displayed a trace of 70 000 component presumably consisting of one heavy and one light chain still linked together, although the method was not sufficiently sensitive to pick out the various molecular weight component molecules of the gamma globulins (e.g. the mu chain of IgM) preparation that are only present in small amounts. Essentially the purpose of this analysis was broadly to compare the composition of the IgG and gamma globulin preparation, and the results show that they behave very similarly on SDS-PAGE.

The behaviour of the preparations in the *in vitro* incubation system is rather different, however. Under the same experimental conditions a greater percentage of captured gamma globulin is released from reincubated rat yolk sacs in TCA-ssoluble form than for ^{125}I-IgG.

A likely explanation is that the non-IgG proteins present in the gamma globulin preparation are taken up and degraded rapidly in the yolk sac, and that this shifts the observed behaviour of the substrate away from the protective pathway towards the degradative one.

3.4.2 The effects of different radioiodination techniques on immunoglobulin behaviour

Freshly radioiodinated preparations of rat immunoglobulin were shown to behave on SDS gels in a manner indistinguishable from the unlabelled material. Older preparations however - whether labelled by Chloramine-T or lactoperoxidase - were found to be missing the 50 000 and 40 000 molecular weight components and to display a heavier unit of molecular weight approx. 80 000. One possible explanation is that this heavier unit is formed by linking together the two lighter ones. Since the 40 000

unit is thought to result from proteinase activity the exact nature of the units and of the time - dependent linkage process are not clear. Alternatively, storage of the labelled substrates may in some way stabilize the structure, perhaps allowing a unit composed of one heavy chain and one light chain to survive denaturation by mercaptoethanol and SDS. Further experiments are neede to resolve this question.

The visual evidence of aggregation (as detected by material remaining at the origin) induced by the imido-ester conjugation technique may at first sight be misleading. Although there is visibly more material left at the origin than for chloramine-T or lactoperoxidase-labelled preparations, there is also visibly more total protein on the gel for imido-ester than for the others. The percentage of the total protein applied to the gel that remains at the origin is approximately the same as for the other radioiodination methods and aggregation is probably not a problem peculiar to the imido-ester technique.

The distribution of radioactivity in SDS gel slices shows no modifications to the pattern induced by recent labelling of the protein with chloramine-T or lactoperoxidase, but the older chloramine-T-labelled preparation again shows the 80 000 molecular weight unit previously observed on the gel.

One important feature of the patterns of radioactivity observed in gel slices is the presence at the origin of over 15% of the total radioactivity for gamma globulins freshly labelled by lactoperoxidase. This was observed repeatedly and suggests that lactoperoxidase induces the formation of aggregates that are resistant to SDS-denaturing conditions. Application of the lactoperoxidase-labelled preparation to Sephadex G-150 allows simple removal of the aggregate, although a greater pore size (e.g. Sepharose 6B or Sephacryl S200) would be expected to allow a wider separation of the monomer from the aggregate.

The behaviour of the variously labelled preparations in the in vitro incubation system suggests that there is no significant difference in behaviour of molecules labelled by lactoperoxidase or by chloramine T. The slightly elevated Endocytic Index observed in lactoperoxidase-labelled preparation is assumed to result from the aggregate formed by the labelling procedure, and the best (and most simple) method of radioiodination is taken to be the Chloramine-T method,

with a small ratio of oxidising agent: protein, in order to minimise the possibility of oxidation damage.

Methods of radiolabelling involving conjugation of protein to a pre-labelled tag might be preferable - especially in terms of convenience (in general, there is no need for a dialysis step, since all the conjugation agent becomes attached to the protein). Unfortunately, they were found to be too expensive for regular use.

3.4.3 The incorporation of ^{125}I-(L)-tyrosine into yolk-sac tissue.

The preliminary experiments to investigate the possible incorporation of ^{125}I-iodotyrosine into newly synthesised protein, or sticking of it to cell membranes and proteins, did not completely dismiss the possible importance of these processes. However, the maximum incorporation rate compatible with these results was sufficiently low (about 2.4ng iodotyrosine/mg yolk-sac protein/h) to suggest that, under normal experimental conditions (where very much less iodotyrosine is present) the incorporation rate may be relatively small compared with the rate of substrate degradation. Observation of no progressive rise in tissue-associated level of radioactivity is another useful line of evidence suggesting that incorporation of iodotyrosine into new protein or sticking of it to cell membranes and proteins is unimportant in this experimental system. If such a process was important, the measured level of tissue-associated radioactivity would be seen to rise with time. That this does not occur to anything like the extent outlined above is demonstrated widely (eg Figures 4.3.4, 5.3.1), though a slow tissue-accumulation (referred to as the Tissue Accumulation Rate by Weisbecker (1981)) might be explained either in terms of such processes, or by gradual filling of unsaturated cell compartments. It would be wrong at this stage to dismiss the possible importance of iodotyrosine accumulation by the tissue, and the matter should be more fully investigated.

3.4.4 The uptake of ^{125}I-PVP by rabbit yolk sacs

The inter-animal variation observed for rates of pinocytosis in rabbit yolk sacs can have various origins. The exact time of pregnancy or the total number of foetuses may have an effect but more likely are genetic factors. The rat colony, which is heavily inbred, exhibits a high

degree of reproducibility, but the rabbits are of a more diverse background. Most of the females used were Californians, though of a variety of ages, and a few were Lops. The males were a more heterogeneous mixture however, the two major ones being Californian and the two minor ones Old English.

Despite inter animal variation, individual animals invariably demonstrated pinocytosis of ^{125}I-substrate at a rate independent of PVP concentration over the spread of concentrations examined. In retrospect, however, a wider concentration range should be employed in order to demonstrate the absence (or presence) of an adsorptive component to PVP uptake by rabbit yolk sacs, since the results obtained to date can perhaps be explained by the whole of the concentration range used being present on a linear portion of an adsorptive uptake mechanism. Alternatively, another notionally fluid-phase marker (eg ^{14}C-sucrose or ^{3}H-inulin) could be used to measure the rate of uptake. At this stage it has not been categorically established that PVP uptake into rabbit yolk sacs occurs solely in the fluid phase.

3.4.5 The effect of serum on rabbit yolk-sac endocytosis

The three major possible methods by which calf serum is able to inhibit endocytosis in rat and rabbit yolk sacs, outlined in the introduction were:

(i) hormone - like (ii) tissue level (of exogenous protein) feedback (iii) bulk-protein inactivation of the plasma membrane.

The ability of BSA, casein, bovine gamma globulins and (non-pregnant) rabbit serum to exert similar levels of inhibition suggested that (i) was an incorrect explanation, since it seems unlikely that all of these relatively pure protein preparations should contain even trace amounts of the relevant hormone or metabolite.

The almost-instantaneous reversal of the inhibition following removal of the inhibitor suggested that the effect was not caused by feedback from a high level of ingested substrate protein (ii), and this was further substantiated by the observation that the steady-state tissue level of BSA in yolk-sacs subject to inhibition was itself dependent on the external concentration of BSA. If nutrient feedback did cause the inhibition, then the tissue level of substrate would be expected to reach

a certain level before inhibition started, and thereafter that level would not rise greatly. That this is not so suggests no significant feedback inhibition.

Further, ingestion of high levels of protein-hydrolysates and preparation of amino acids might be expected to exert nutrient feedback inhibition in the same way as the ingestion of proteins. The inability of glycine at high concentrations to exert any effect does not support this mechanism, but further experiments, using a mixture of all amino acids, would be useful to clarify the situation.

The third possibility, inactivation of the membrane by bulk protein effects, seems therefore to be the best explanation to fit all the available evidence. Large proteins may bind to the membrane and effectively partially immobilise it. In vivo, the rabbit brush border membrane will be exposed, at this stage of gestation, to a total protein concentration in the uterine fluid of approx. 50 mg/ml (Wild, 1965), which is approximately the same total protein level as 80% calf serum in vitro, and therefore would be expected to cause very substantial inhibition of pinocytosis. Whether this inhibition occurs in vivo is an interesting question. The apposition of the yolk sac to the wall of the uterus may locally decrease the concentration of free protein to which the yolk sac surface is exposed, but the possibility nevertheless arises of an endocytosis-stimulating factor in the uterine fluid, and the effects of uterine fluid-proteins on the behaviour of rabbit yolk sacs in vitro might be extremely interesting.

3.4.6 The effects on tissue behaviour of lower O_2 tension

It was not possible to change the behaviour of rat yolk sacs incubated in vitro by decreasing the level of O_2 from 95% to 5%. Prolonged gassing of incubation medium with 100% N_2 failed to have any major effect on the rate of ^{125}I-PVP accumulation over 3h, suggesting that the amount of O_2 actually required by the yolk sac to maintain pinocytosis is very small.

No decrease in TCA-soluble ^{125}I-IgG release from reincubated tissue was observed, suggesting that this release is not caused by O_2-induced tissue damage.

In short, the yolk-sac incubation system would probably function as well in air as in 95% O_2, provided that the correct pH is maintained. The thinness of the tissue appears to allow fast diffusion of O_2 to the metabolising cells even at low oxygen tensions. Given that 95% O_2 appears to have no deleterious effects on the tissue, and in order to keep the number of experimental variables to a minimum, the remainder of the work in this study was nevertheless carried out in 95% O_2.

3.4.7 The nature of the TCA-insoluble released products.

The SDS-PAGE pattern for non-dialysable material released from reincubated rat yolk sacs previously exposed to ^{125}I-IgG is essentially identical to the pattern for fresh substrate, and the elution pattern on Sephacryl S200 for non-dialysable material released from reincubated rabbit yolk sacs previously exposed to ^{125}I-IgG is the same as that for unused ^{125}I-IgG. In neither case is there any strong evidence of non-dialysable material that is not apparently intact, and the conclusion is simply that ^{125}I-IgG-derived radioactivity released from reincubated yolk sacs is either dialysable or intact ^{125}I-IgG. Since dialysable material would be expected to be soluble in TCA, the implication is that 'TCA-insoluble material' can indeed be equated with 'intact material' when applied to released IgG. This is in agreement with the results of Williams & Ibbotson, (1979) who worked with rat yolk sacs and homologous gamma globulins.

3.4.8 The effect of unlabelled gamma globulins on ^{125}I-PVP transport in rat yolk sac tissue.

Since almost all of the contained ^{125}I-IgG is released from reincubated yolk sacs during the first hour of reincubation, while ^{125}I-PVP is released at a steady rate (apart from preliminary release of surface-adsorbed radioactivity) over at least 3h (Figure 5.3.7), this suggests that such release of ^{125}I-PVP is mainly not from the fluid phase of IgG-specific vesicles completing diacytosis.

On the other hand, it is hard to imagine how IgG-specific vesicles forming at the cell surface could exclude ^{125}I-PVP in the fluid phase, and the observation that the presence of cold gamma globulins does not stimulate ^{125}I-PVP transport' suggests that if IgG-specific vesicles do form at the cell surface prior to diacytosis then they do so

constitutively, and do not require induction by IgG.

The conclusions of this chapter are as follows:

(i) Rat gamma globulins behave similarly in the *in vitro* rat yolk-sac incubation system to rat IgG, except that under this experimental regime the gamma globulins exhibit a slight bias towards the degradative pathway. Electrophoretic analysis shows no important differences.

(ii) The behaviour of lactoperoxidase- and chloramine-T- radioiodinated preparations of gamma globulins is similar both in the rat yolk-sac incubation system *in vitro*, and under electrophoretic analysis. Evidence of some lactoperoxidase-induced aggregation means that chloramine-T - used with caution - is the method of choice for this study.

(iii) The incorporation of ^{125}I-(L)-tyrosine into newly synthesised tissue protein or onto cell membranes or fragments by the rat yolk sac incubated *in vitro* is probably not of sufficient magnitude to affect the valid interpretation of typical endocytosis results, but the possibility should be further investigated.

(iv) ^{125}I-PVP appears to be a good marker of fluid phase pinocytosis in rabbit yolk sacs incubated *in vitro* over the range of concentrations used, but a wider range must be used before a firm conclusion can be drawn. The use of another fluid-phase marker would be interesting.

(v) The inhibitory effects of serum on endocytosis in rabbit yolk-sac tissue appear more likely to be mediated by bulk protein-inactivation of the membrane than by hormonal or nutrient feedback mechanisms.

(vi) 95% O_2 is not essential for ongoing pinocytosis in the rat yolk sac *in vitro* since the rate of uptake of ^{125}I-PVP is undiminished in the presence of 5% O_2.

(vii) Few semi-degraded ^{125}I-IgG derived products are released from reincubated yolk sacs of both species. For ^{125}I-IgG, 'TCA-insoluble' can essentially be equated under these conditions with 'intact'.

(viii) Unlabelled rat gamma globulins in the incubation medium have no effects on 'PVP transport' in rat yolk sacs. This suggests either that

the cell-surface formation of IgG-specific vesicles is not inducible by IgG or that such formation somehow excludes fluid uptake very efficiently.

Chapter 4. The Use of Fragments of IgG to locate the Receptor Recognition Unit in vitro.

INTRODUCTION

4.1.1 Variety of immunoglobulins.

The roles of immunoglobulin molecules are many and diverse, and, in many cases, individual molecules are capable of performing functions that are essentially unrelated to each other. When Porter (1963) first elucidated the general structure of the immunoglobulin molecule, it was suggested that evolution had endowed the molecules with mixed functions by localising the effector sites of various functions to specific domains within the molecular structure. Hence different parts of the molecules might be capable, perhaps even in isolation, of performing different functions.

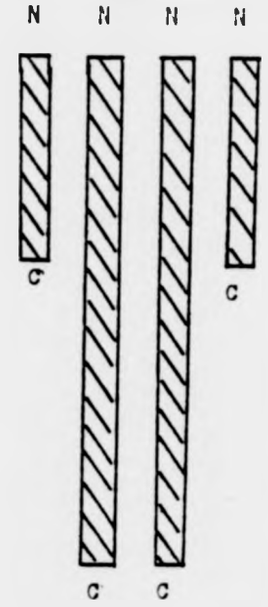
The five different classes of immunoglobulins - IgA, IgE, IgD, IgM and IgG - are all composed of similar polypeptide chains. There are two types of chains, commonly known as 'light' and 'heavy', both types being polypeptides with a small amount of carbohydrate attached. Light chains, of which there are only two types ('kappa' and 'lambda' chains) are common to the whole spectrum of immunoglobulins, though there is never a mixture of kappa and lambda chains in the same molecule, while heavy chains are class-specific. Hence there are five types of heavy chain, known as alpha, epsilon, delta, mu and gamma, corresponding to IgA, IgE, IgD, IgM and IgG respectively. Overproduction of heavy chains gives rise to 'H-chain disease', where incomplete and excess heavy chains are excreted in the urine; in cases of multiple myeloma excess light chains are excreted in the urine, where they are known as 'Bence-Jones proteins'.

The basic structural unit of all immunoglobulins is a monomer containing four chains: two identical heavy chains and two identical light chains (Figure 4.1.1) linked together by disulphide bonds. All four amino terminal ends of the polypeptides occur at the same end of the molecule.

IgM exists *in vivo* as a pentamer of this unit, containing twenty such chains in all, while all the other types of immunoglobulin occur mainly in the monomeric form (Weir, 1979). IgM is rapidly produced on invasion of the body by most antigens. Its specificity is fairly low and it is used as a quick first response to try to control an infection while

Figure 4.1.1

General immunoglobulin structure, showing N and C termini.



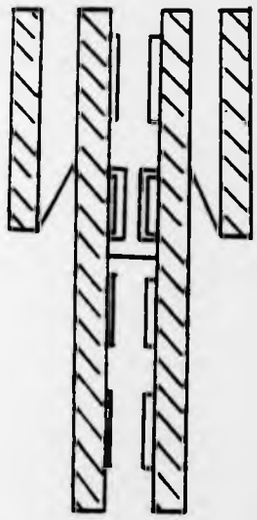
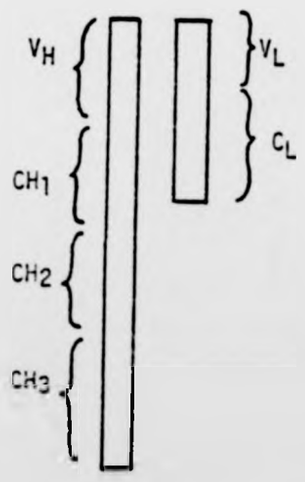


Figure 4.1.2

The arrangement of disulphide bonds in rabbit IgG.

The different regions of the molecule, and their abbreviations.



more specific antibodies are being produced. Its multivalency makes it a very efficient agglutinator of antigens, and it is extremely efficient at bringing about cell lysis by the classical activation of the complement pathway. It has a molecular weight of about 900 000.

IgA comprises about 15% of the total antibody content of most mammals, and it occurs in two distinct forms. Serum (or 'circulating')IgA exists primarily as a monomer and is thought to have a certain amount of antibody activity, particularly anti-toxin activity. The more important form of IgA is secretory IgA, which occurs as a dimer and in association with two other peptides which participate in the secretory process. The total molecular weight of the complex is about 380 000, and it is found in parotid saliva, tears, colostrum and various nasal, bronchial and intestinal secretions. Its function is to prevent the entry of micro-organisms into the body via the exposed mucous surfaces.

Both IgD and IgE have slightly longer heavy chains than the other immunoglobulins, with molecular weights of 184 000 and 195 000, respectively. They are present in most mammals only in trace amounts, and their functions are not yet clear. They are thought to be involved with the induction of immune tolerance in the developing immune system and with allergic reactions of various types, respectively.

IgG is easily the most abundant (approx.75% of total) and important class of immunoglobulins. When induced by a particular antigen, its specificity is high and it is able to fix complement by the classical pathway. With a molecular weight of approx. 150 000 it is the smallest of the antibody molecules, and since it is by far the most important for maternofoetal transmission (Stanworth and Stewart, 1976) its structure will be described in detail.

4.1.2 **The structure of IgG.**

The four chains that compose IgG (in this case, rabbit IgG) can be represented as shown in Figure 4.1.2. The N-terminal region of each chain consists of approximately 110 amino acid residues that exhibit a great deal of variation between molecules. Within these regions there are also some 'hot spots' of variation - the so-called 'hypervariable' regions. These exhibit very little sequence homology even between antibodies raised against very similar antigens. The remaining parts of both types

Figure 4.1.3

The site of papain cleavage of IgG

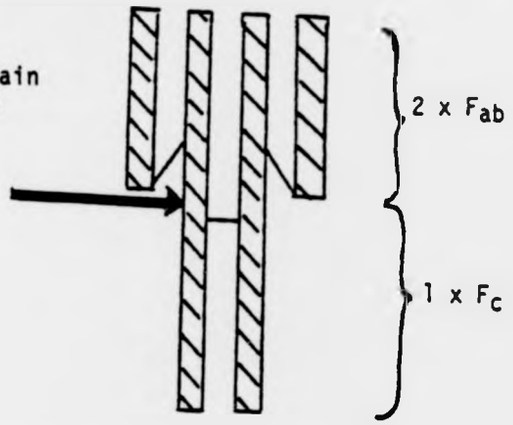
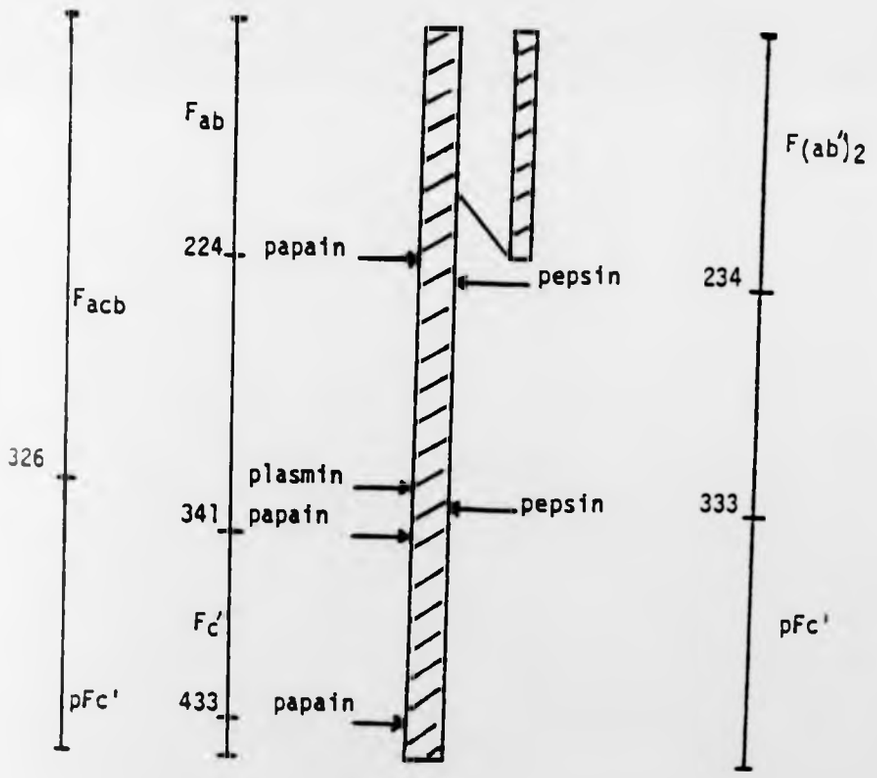


Figure 4.1.4

Some of the more useful fragments produced from IgG by enzymic cleavage.



of chain are relatively constant between molecules. The constant region of the light chain is thus the C-terminal half, which exists as a globular domain bonded via disulphide bonds to the heavy chain. The constant part of the heavy chain, which again is towards the C-terminal end, falls naturally into three regions, all of which are stabilized in a globular tertiary structure by intrachain disulphide bonds. For easy reference the various regions of the molecule are given specific names. Hence the use of 'H' to mean 'heavy chain', 'L' for 'light chain', 'V' for 'variable' and 'C' for 'constant' and a number to distinguish between the three 'CH' sites, allows all of the important regions to be simply denoted, as shown in Figure 4.1.2.

Rabbit IgG is unusual in that there is very little variation in the "constant" part of different molecules. Human IgG exhibits at least four distinct subclasses of IgG (types I, II, III and IV), while rabbit IgG has no important variations in the 'constant' polypeptide structure.

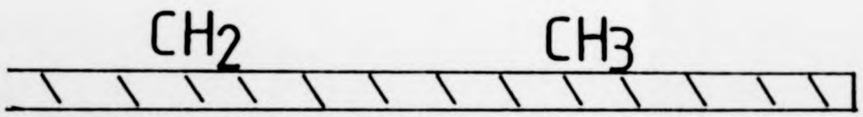
The functions of IgG are manyfold. They include:

antigen binding	reaction with protein A
complement binding	membrane transmission
macrophage binding	monocyte binding

Ever since Porter (1959) first cleaved IgG with the enzyme papain to produce the fragments shown in Figure 4.1.3, and the site of interaction with antigen was shown to be located in the F_{ab} fragment, the use of enzymes to attempt to isolate other functional sites has been intense. Edelman et al(1969), first proposed that the different functions associated with the IgG molecule might be mediated by discrete regions of the structure - in particular the globular CH_1, CH_2 and CH_3 domains of the heavy chain - and much effort has centred on attempting to cleave the molecule in the hinge regions which separate the constant domains. Some of the more useful fragments that have thus been isolated are shown in Figure 4.1.4.

The methods that have been used for preparing the different fragments are varied, some of them involving fairly harsh treatment of the protein. For example, that of Utsumi, (1969), included a reduction step using 14mM C_2H_6OS, and therefore it is not certain that fragments retained biological activity even though complete functional sites might

Figure 4.1.5 Suggested locations in the IgG molecule of various functional sites.



Function					
Classical complement fixation	1 6 2 7 3 10 4 5			3 17 4 18 14 19	
Passive cutaneous anaphylaxis		4 7 11	14	7 18 11 19 17 20	
Lymphocyte binding	8 9	5 12	12	8	
Macrophage binding	7	13	13 14 15 16	7 10 21	

(1) Kehoe & Fougereau, 1969
(2) Connell & Porter, 1971
(3) Ellerson et al, 1972
(4) Dorrington & Painter, 1974.
(5) MacLennan et al, 1974
(6) Colomb & Porter, 1975
(7) Ovary et al, 1976.
(8) Wisloff et al, 1974
(9) Michaelson et al, 1975.
(10) Yasmeen et al, 1976.
(11) Stewart et al, 1973.
(12) Ramasamy et al, 1975.
(13) Yasmeen et al, 1973.
(14) Minta & Painter, 1972.
(15) Okafor et al, 1974.
(16) Ciccimarra et al, 1975.
(17) Irimajiri et al, 1968.
(18) Utsumi, 1969.
(19) Frommel & Haig, 1970.
(20) Prahl, 1967.
(21) Abrams et al, 1970.

perhaps be contained within these regions under physiological conditions.

The results obtained to date are varied and sometimes contradictory. A summary of suggested locations for five of the functional sites is shown in Figure 4.1.5.

Few serious attempts have been made to localise within the molecule the site that mediates yolk-sac transmission, and those that have have usually met with difficulty. McNabb et al (1976) prepared and isolated CH_2 and CH_3 regions of human IgG by a procedure involving acid-pretreatment and tryptic cleavage of the parent molecule. They observed no binding of either fragment to placental membrane preparations, while the intact F_c region showed substantial interaction. Their conclusion was that the signal mediating placental transmission was a composite one, requiring both the CH_2 and CH_3 domains to be intact for it to be functional. Similar conclusions were reached by Guyer et al, (1976), who worked on in vivo transmission of fragments of IgG across the mouse gut.

Recently, Johanson et al, (1981), have demonstrated the binding to isolated, fixed rabbit yolk-sac membrane preparations of F_{acb} (160% of control intact molecule), but not of F_{ab} or pF_c'. Their conclusion was that the site mediating specific interaction with membrane-bound receptors lies in the CH_2 region of the molecule. This is in direct conflict with the work of Edelman and Gall (1969), who localised the site of IgG interaction with human placental receptors to the CH_3 portion of the molecule.

In order to attempt to clarify our understanding of the structural features responsible for the transmission of IgG, it was decided to observe the behaviour of different radiolabelled fragments of IgG in the rabbit yolk-sac incubation system. First the behaviour of F_{ab} and F_c fragments produced by pepsin cleavage was examined, as a test of the in vitro model as a reliable mimic of the in vivo transmission process, since the literature is agreed (eg. Kaplan, 1965) that F_c but not F_{ab} is transmitted in vivo.

To distinguish between CH_2-associated and CH_3-associated activities, the most useful degradation to carry out is with the enzyme plasmin, which is reported (Colomb and Porter, 1975) to cleave the molecule

between residues 326 and 327 under optimal conditions. This yields the pF_c' and F_{acb} fragments (Figure 4.1.4), the former containing apparently intact CH_3 domain, the latter the CH_2.

The possible existence within the IgG molecule of a site that is responsible for mediating the rate of catabolism of the molecule has been widely investigated (for review, see Waldmann and Strober,1969), but no such site has yet been pinpointed. It has been shown that human and rabbit F_{ab} and $F_{(ab')2}$ fragments are catabolised far more quickly than F_c fragments in a variety of species (Spiegelberg and Weigle, 1965 and Gitlin et al, 1964), exhibiting a half-life of only about 5h, compared with the F_c half-life of about a week. This rate of catabolism of F_c is about the same as that of the intact IgG molecule, and it has been shown (Fahey and Robinson, 1963) that the simultaneous injection of F_c fragment (but not F_{ab}) shortens the time of survival of intact IgG, and vice versa (Watkins et al, 1971). It seems that the F_c fragment contains a site that regulates the survival time of the molecule in vivo, and the observation (Watkins, Turner and Roberts, 1971) that human pF_c' fragment exhibits a short half-life in both man and the mouse suggests that this site is located nearer the N-terminal end of the F_c region of the molecule.

Exactly how such a site can mediate the rate of catabolism is unclear. Brambell et al, (1964), envisaged a saturable protection system involving regular uptake of IgG into pinocytic cells and its protection from breakdown by attachment to specific receptors followed by release back into the circulation. Hence the level of intact IgG in the circulation is directly related to the level of such specific receptors. Waldmann and Ghettie (1971), on the other hand, suggest that catabolism occurs only after a conformational change in the F_c region of the molecule exposes a catabolism-stimulating site. This does not explain the fast rate of degradation of the F_{ab} fragment, however, and such a mechanism is only really feasible if the conformational change occurs in the F_{ab} region, and may even be induced by the molecular cleavage needed to form the fragments. This seems unlikely though, since such a mechanism would not explain the competition, outlined above, between F_c and intact IgG for survival. Nor would it account for the short survival time of the pF_c' fragment.

It was hoped that various data obtained during this study might shed some light on this question.

In studies of cell-mediated proteolysis, the possible hydrolysis of substrates by membrane-bound proteinases on the outer face of the plasma membrane is a potential source of complication in the interpretation of data. Weisbecker (1981) was able to show, however, that degradation of rat IgG to TCA-soluble form by buffered preparations of homogenised rat yolk sac was not measurable at pH values greater than 4.5. This is in agreement with the results of other groups (eg. Ghetie and Motas, 1971; Fehr, LoSpalluto and Ziff, 1970) who estimate the optimal pH for degradation as about 3.5. This suggests there is little neutral proteinase activity associated with yolk sac tissue, and the possibility of substantial surface proteolysis seems unlikely.

Fragments of IgG smaller than the intact molecule might be expected, however, to be more susceptible to degradation by surface enzymes, since their smaller size might allow them to become more intimately associated with the membrane. F_c fragment has been shown (Ghetie and Motas, 1971) to be more susceptible to cathepsin degradation than F_{ab}, and it was considered important to check that neutral pH-degradation of fragments was not a significant factor in their breakdown during incubation.

The experimental animal used throughout this chapter was the rabbit.

In summary, the aims of this chapter were as follows:

(i) To obtain F_{ab} and F_c fragments of (human) IgG, and to observe their behaviour in the rabbit yolk-sac incubation system.

(ii) To attempt to prepare F_{acb} and pF_c' fragments of rabbit IgG by plasmin hydrolysis, and to observe their behaviour in the rabbit yolk-sac incubation system.

(iii) To monitor the degradation of fragments of IgG by rabbit yolk-sac homogenate preparations, and to investigate the effects of pH.

MATERIALS AND METHODS

4.2.1 SDS-PAGE analysis of fragments of human IgG.

Human IgG, together with its F_c and F_{ab} fragments, were a kind gift from Dr. D. Stanworth at Birmingham University.

SDS gels were prepared and used as described in Section 2.2.15. Instead of being stained for protein content, they were sliced by hand into 2mm thick slices and placed individually at the bottom of 3ml plastic tubes for assay of their contained radioactivity in a gamma scintillation counter.

When macromolecular material, released from reincubated yolk sacs that had been previously exposed to ^{125}I-labelled substrates, was examined, it was isolated by first dialysing the final incubation medium in Visking tubing against three changes of distilled water for 12h, and then freeze-drying the resulting solution to form a powder that could be redissolved in a small volume of water. Most of the components of the medium were lost during the dialysis procedure, and all that remained were released proteins of fairly high molecular weight.

4.2.2 The fragmentation of rabbit IgG by plasmin.

The method of Colomb and Porter (1975) was used, first with porcine plasmin and later with human plasmin. The method incorporates a low pH-pretreatment of the IgG that makes it more susceptible to the desired plasmin cleavage. The procedure is as follows:

Rabbit IgG (20 mg/ml) in 4mM sodium acetate buffer, pH 5.4, was adjusted to pH 2.5 by addition of 1M HCl with careful mixing. After 15 min of incubation at 30°C, the pH was rapidly raised to 7.0 with 1M NaOH, and plasmin added immediately to give 0.3 nkat/mg of IgG.

After incubation at 30°C for 10 min, the enzymic digestion was stopped by cooling the mixture to 0°C, then adding soya-bean trypsin inhibitor in 0.1M Tris-HCl buffer, pH 8.0, in twofold molar excess over plasmin. The products were then separated by gel chromatography.

This method has, according to the original authors, two main advantages over previous ones. First, the pretreatment at low pH increases the efficiency of the cleavage to about 95%, reducing contamination of the product with intact IgG, and secondly the addition of soya-bean trypsin inhibitor stops the subsequent non-specific degradation of the F_{acb} product by the plasmin.

Because of a low fragment yield, the method was eventually modified (referred to later as the 'strong' degradation) as follows. The preincubation time, at pH 2.5, was increased to 30 min, 0.6 nkat plasmin/mg IgG was used (and consequently double the amount of soya-bean trypsin inhibitor was added), and degradation was allowed to proceed at 37°C for 30 min.

All digests were examined on Sephacryl S200, which was prepared for use as described in Section 3.2.2.

4.2.3 pH-dependence of degradation of homologous ^{125}I-IgG and ^{125}I-F_c fragment by rabbit yolk-sac homogenate.

Four complete yolk sacs were homogenised in a total volume of 30 ml distilled water and centrifuged at 150g x 10 min (MSE 4L) to remove intact cells. Buffers (0.1M) were prepared so as to give every 0.5 unit pH increment between pH 3.5 and pH 9.0. (Acetate buffer was used up to pH 6.0, and Tris-HCl was used at pH 6.5 and above). Substrate solutions were made up at a concentration of 10 ug/ml in 1% NaCl(aq.).

Buffer (130ul) was warmed to 37°C in a shaking incubator and 20ul substrate solution added. The reaction was started by the addition of 50ul yolk-sac homogenate, and was allowed to proceed for 1h. The reaction was stopped by the addition of 0.8ml of 12.5% (v/v, aq.) calf serum, and the total contained radioactivity was assayed on a gamma scintillation counter. Aq. TCA (16.5% w/v, 0.6 ml) was added to precipitate proteins, and the estimation of TCA-soluble radioactivity was carried out in the usual way (Section 2.2.5).

Controls, containing water instead of yolk-sac homogenate, were used to estimate the quantities of TCA-soluble material in the substrate preparations. These values were subtracted from the quantities observed for both substrates at the various pH values.

RESULTS

4.3.1 SDS-PAGE analysis of human IgG fragments.

The F_c fragment, when examined by SDS-PAGE, forms one distinct band of radioactivity of molecular weight approx. 26 000 (Figure 4.3.1). This represents the carboxy-terminal portion of the H-chain which is formed by papain cleavage at the hinge region.

The F_{ab} fragment also shows one band, at a slightly lower molecular weight, approx. 24 000. This represents both the amino-terminal portion of the H-chain, formed by papain cleavage, and the intact light chain; both have similar molecular weights. The F_{ab} pattern, however, also shows the presence of a large molecular weight aggregate that remains at the origin (Figure 4.3.2). Since this aggregate accounts for 46% of the total F_{ab}-associated radioactivity, the behaviour of the F_{ab} preparation under incubation conditions was examined to see whether one form of the molecule was preferentially degraded by the yolk sac.

When non-dialysable radioactivity derived from ^{125}I-F_{ab} fragment, that had been incubated in medium 199 in the presence of a rabbit yolk sac, was isolated and examined by SDS-PAGE, to determine whether one of the bands observed in Figure 4.3.2. had been preferentially removed, the pattern shown in Figure 4.3.2a was obtained. The same band is observed at a molecular weight of approx. 24 000, but there is no evidence of an aggregated form.

4.3.2 The uptake of fragments of human IgG by rabbit yolk sacs.

Endocytic Indices in rabbit yolk sacs were determined by incubation in the absence of calf serum of intact human ^{125}I-IgG, ^{125}I-F_{ab} fragment and ^{125}I-F_c fragment (Figure 4.3.4). The net rate of uptake was slightly greater for ^{125}I-F_{ab} than for ^{125}I-IgG, although the maximum observed tissue level, when the tissue had reached equilibrium, was approximately the same for both. This is explained by the fact that the F_{ab} fragment was degraded and the resulting ^{125}I-iodotyrosine lost from the tissue faster than the parent IgG molecule.

^{125}I-F_c fragment has a substantially greater Endocytic Index, but its steady-state tissue level is also much higher (approx. 500% relative

to that of either ^{125}-IgG or ^{125}I-F_{ab} fragment). Since the tissue did not reach a steady state for at least 3h, the Endocytic Index for F_c fragment based on 4.5h uptake will be elevated because of an ongoing buildup in the release compartment (for further consideration of this point see the Discussion section of this chapter). As mentioned elsewhere, a buildup in the 'release compartment' is hard to distinguish experimentally from ongoing surface adsorption, and this latter process may also contribute to the high tissue level observed for the F_c fragment. Its actual rate of degradation as measured by release from the tissue of ^{125}I-tyrosine, was similar to or slightly less than the corresponding rates for the other two substrates.

4.3.3 The release of fragments of human IgG from rabbit yolk sacs.

Figure 4.3.5 shows a summary of all the release patterns obtained for human ^{125}I-IgG and its ^{125}I-F_{ab} and ^{125}I-F_c fragments from reincubated rabbit yolk sacs previously exposed to these substrates. In order to minimise the effects of inter-animal variation, the amounts of material released were expressed as percentages of the total radioactivity associated with the tissue at the start of the reincubation period, and plotted as a function of time.

The release pattern obtained for intact human ^{125}I-IgG is similar to the pattern for rabbit ^{125}I-IgG under the same incubation conditions (Figure 3.3.4), with approximately equal amounts of radioactivity being released in TCA-soluble and TCA-insoluble forms. When the substrate was ^{125}I-F_{ab}, only a very small portion of the tissue-associated radioactivity was released in TCA-insoluble form, nearly 70% of the total being released in degraded form. With ^{125}I-F_c as substrate, about 20% of the tissue-associated radioactivity was released in TCA-soluble form by 2.5h, but the greater part (approx. 55%) was released in TCA-insoluble form. In every case, approximately 25% of the original tissue-associated radioactivity remained associated with the tissue at the end of the reincubation.

4.3.4 Sephacryl-S200 examination of the products of plasmin digestion of IgG.

The void volume of the Sephacryl-S200 column was determined using Blue Dextran, and untreated rabbit ^{125}I-IgG applied to this column gave

the elution pattern shown in Figure 4.3.7. This shows only one peak, corresponding corresponds to the native IgG molecule.

When rabbit ^{125}I-IgG was treated with porcine plasmin and applied to this column, the pattern shown in Figure 4.3.8 was obtained. This shows a major peak in the same position as the untreated molecule, and there is very little evidence for the presence of degradation products.

More extensive treatment with human plasmin (Section 4.2.2) gave the pattern shown in Figure 4.3.9 (the protein distribution in this case was measured by E_{280} absorption, and here the presence of degradation products is much more evident).

4.3.5 pH-dependence of yolk-sac homogenate proteolytic activity.

The pH profile for the degradation of ^{125}I-IgG and ^{125}I-F_c fragment (Figure 4.3.3) shows much greater proteolytic activity at low values of pH than at higher ones. The greatest activity observed is at pH 3.5; above pH 4.5 there is relatively little proteolysis. Whether the activity observed at low pH is essentially enzymic or acid-mediated is unclear, but this is largely irrelevent in terms of the overall conclusion that there is no important proteolysis at neutral pH.

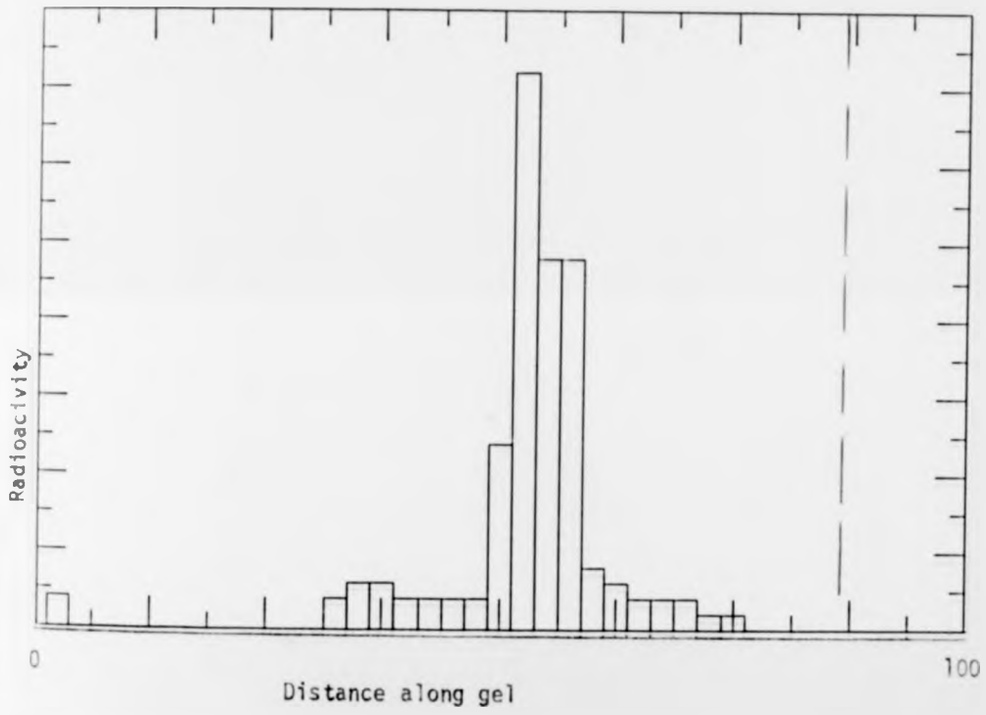
In the following RESULTS section, as throughout the rest of this Thesis, the following key to symbols is observed, unless indicated otherwise in the legends.

TCA - soluble radioactivity is represented by open symbols - mainly squares or circles.

TCA - insoluble radioactivity is represented by closed symbols - mainly squares or circles.

± Error bars represent ± Standard Deviations.

Figure 4.3.1 The SDS-PAGE pattern of human F_c fragment of IgG.



(Full experimental details are given in the text).

Figure 4.3.2 The distribution on SDS-PAGE of human F_{ab} fragment of IgG.

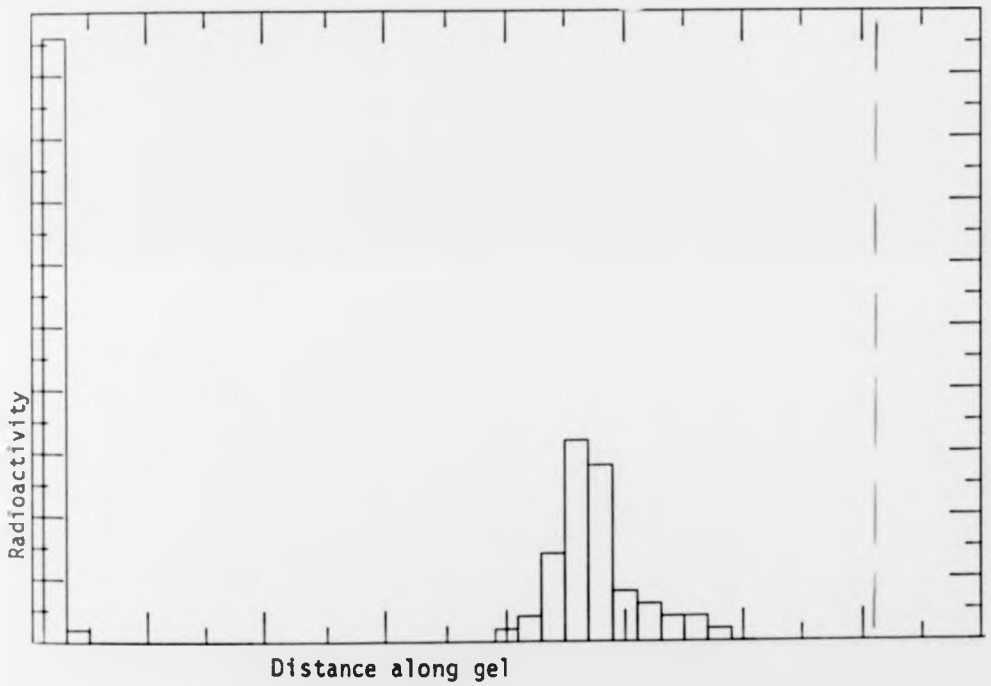
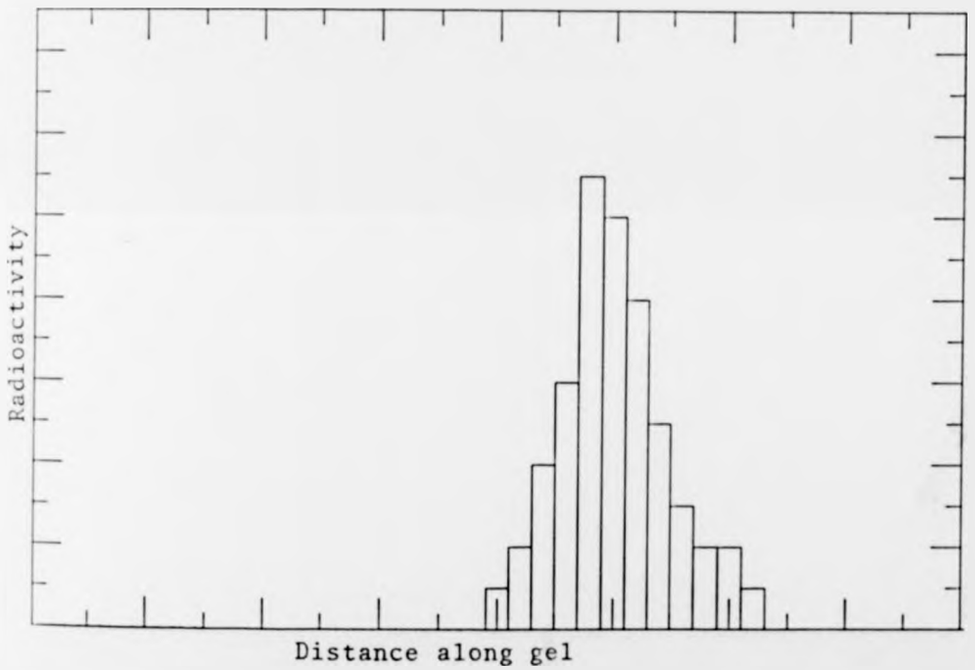


Figure 4.3.2a The SDS-PAGE pattern for F_{ab} fragment of human IgG, after reisolation from medium 199.



Full details are given in the text.

Figure 4.3.3 The degradation of human ^{125}I-IgG and ^{125}I-F_c fragment at varying pH, by rabbit yolk-sac homogenate.

The effect of changes in pH on the degradation of human IgG and F_c fragment by rabbit yolk-sac homogenate at 37°C was examined. Degradation was assessed by solubility of ^{125}I-tracer in TCA. The full method is described in Section 4.2.3.

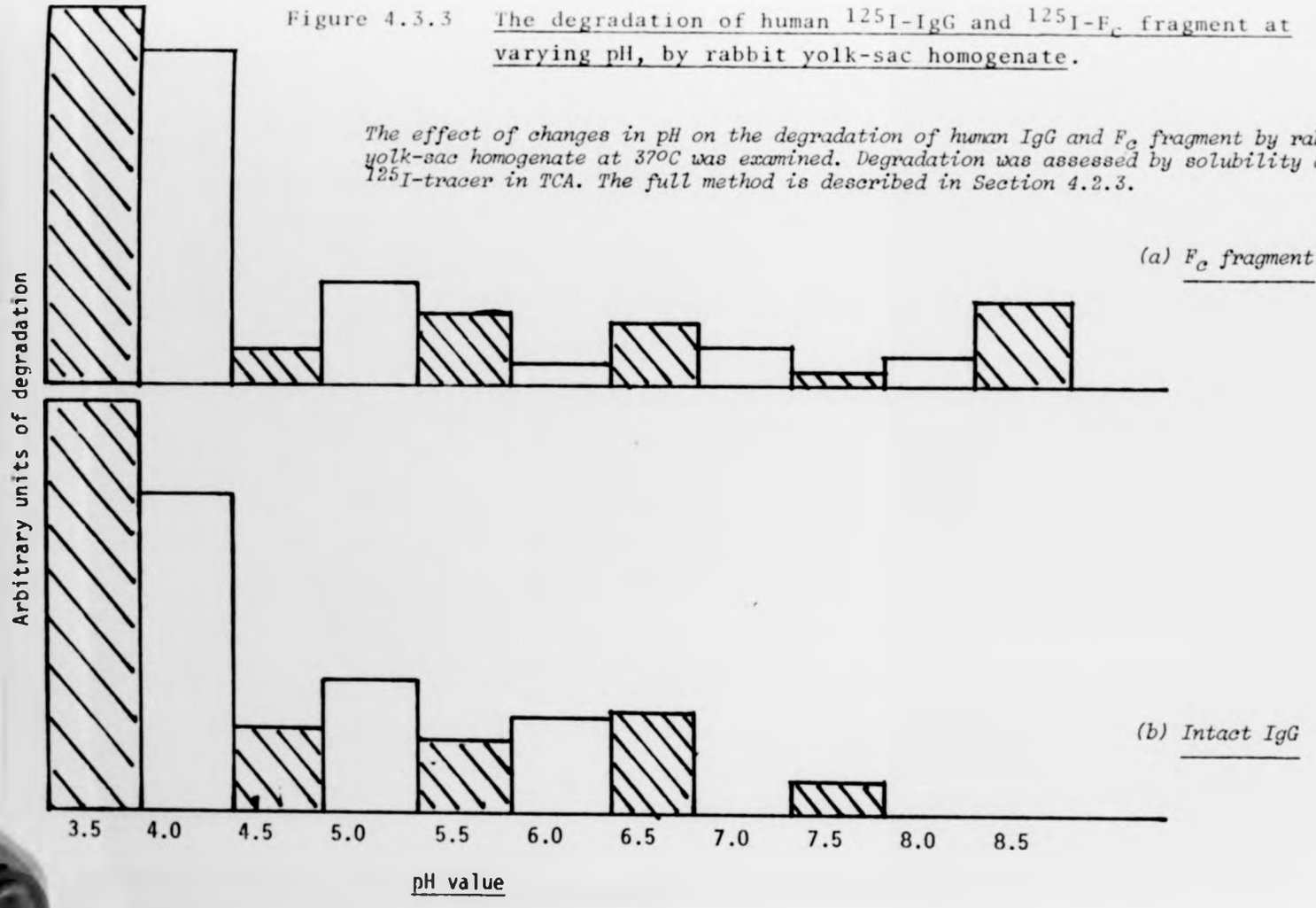


Figure 4.3.4 The uptake by rabbit yolk sacs of human ^{125}I-IgG and its ^{125}I-labelled F_{ab} and F_c fragments.

Yolk sacs were incubated in the presence of radiolabelled substrates, as indicated (2ug/ml), and uptake was measured in the usual way. A typical series of results is presented.

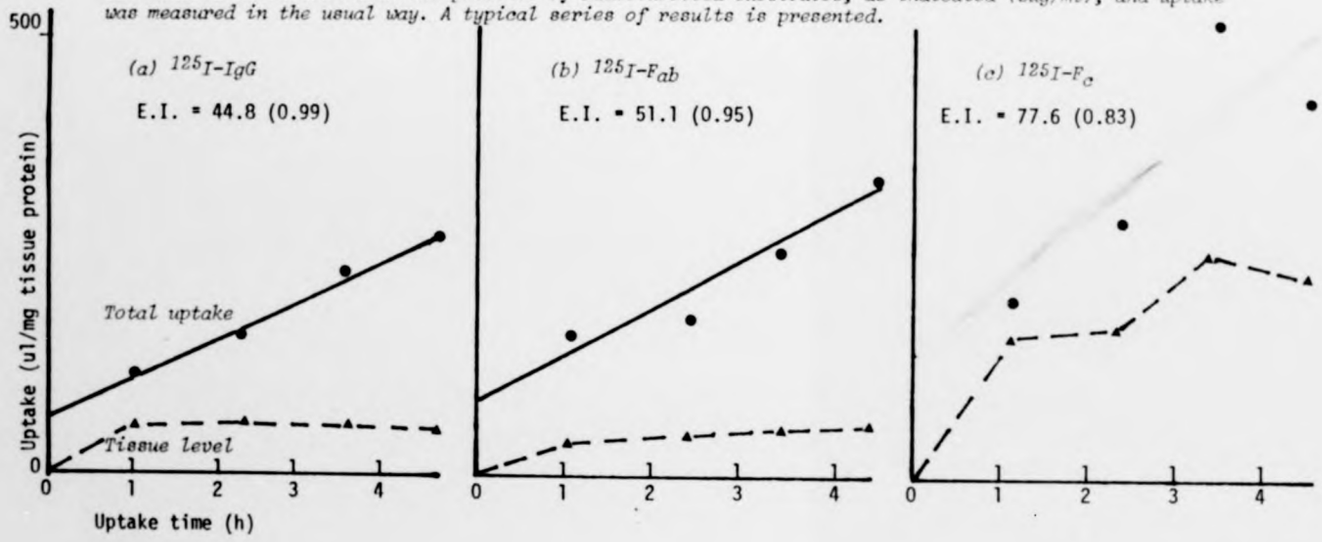


Figure 4.3.5 The release from rabbit yolk sacs of radioactivity derived from human ^{125}I-IgG and the ^{125}I-labelled F_{ab} and F_c fragments thereof.

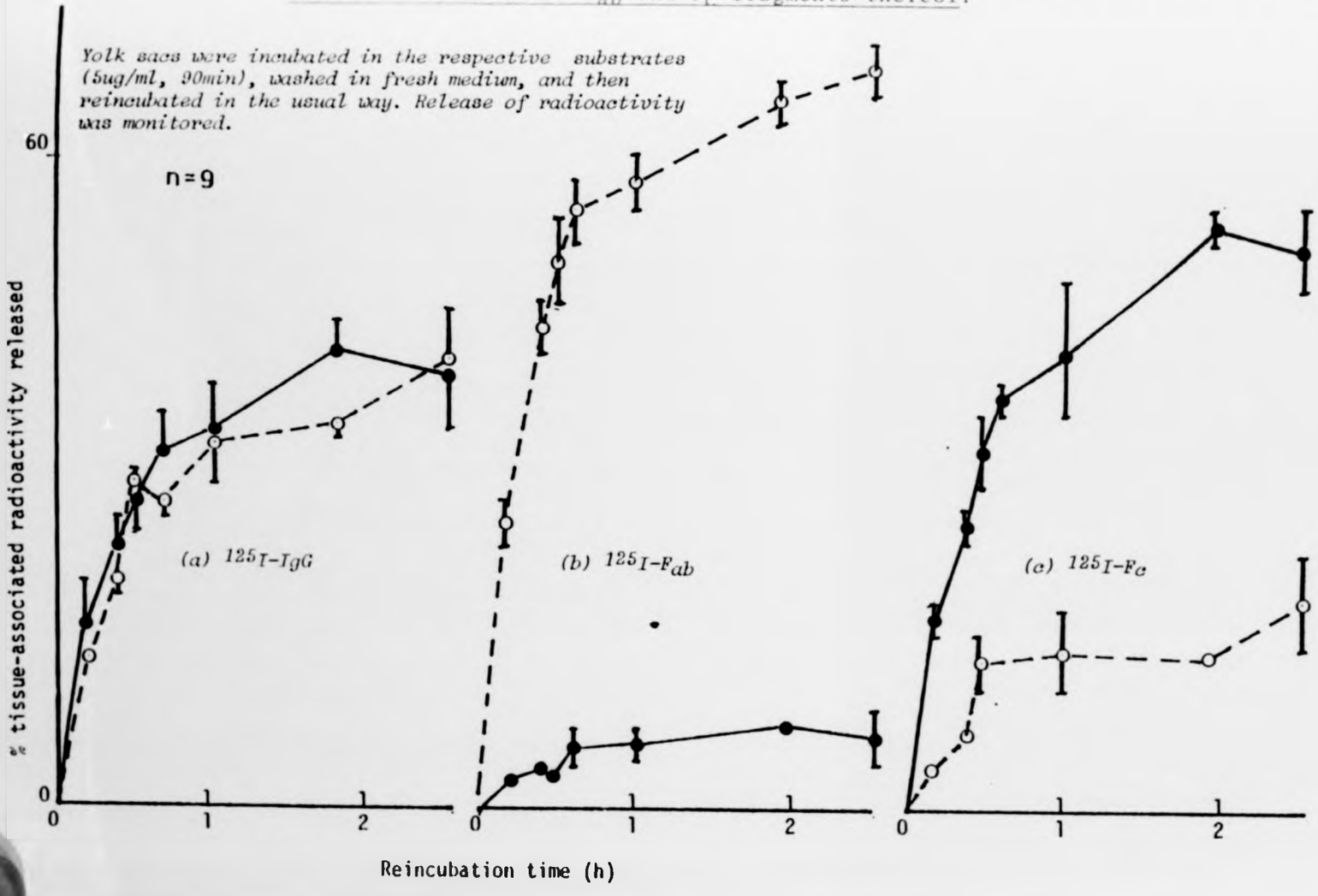


Figure 4.3.7 The elution pattern from Sephacryl S200 of untreated rabbit IgG.

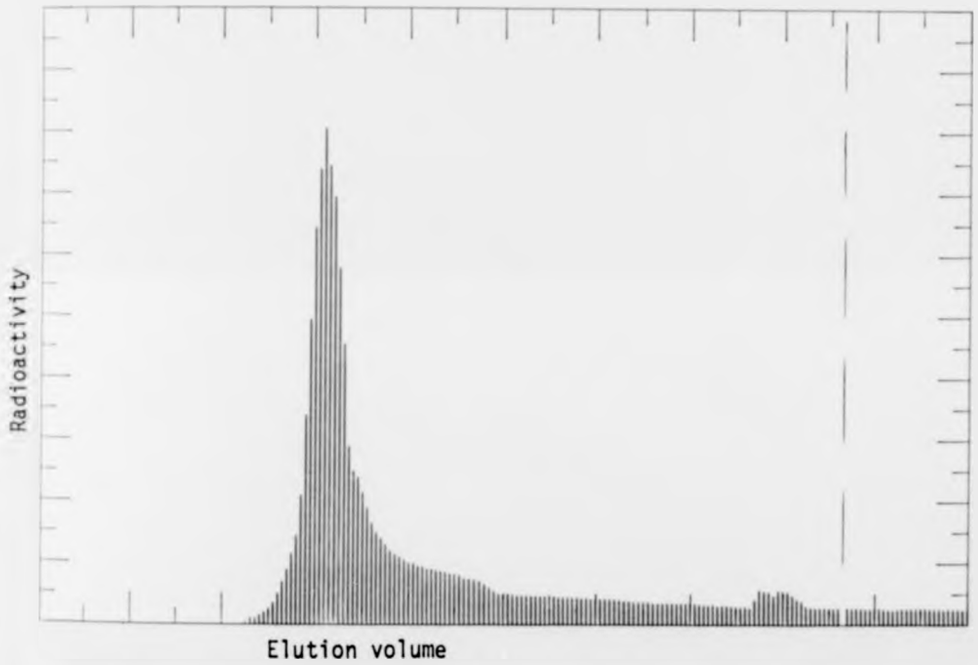


Figure 4.3.8 The elution pattern from Sephacryl S200 of rabbit IgG degraded by porcine plasmin.

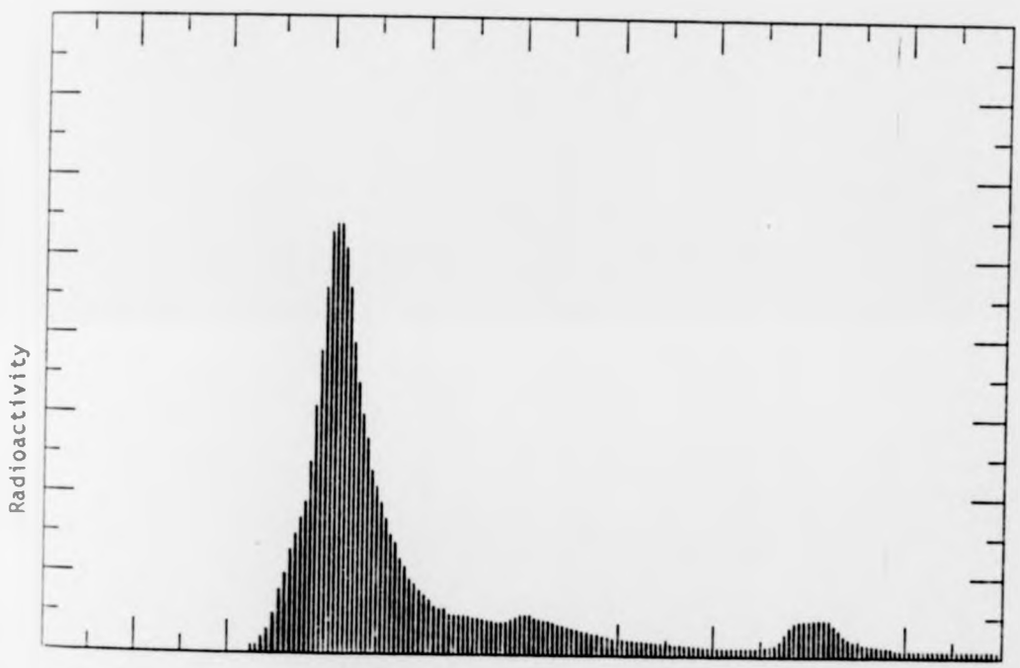
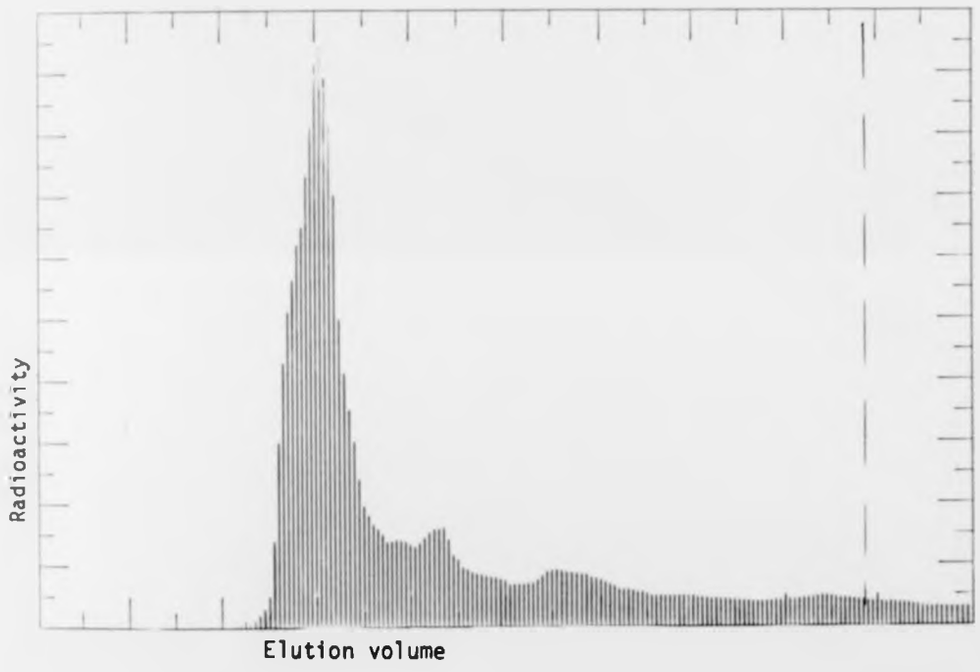


Figure 4.3.9 The elution pattern from Sephacryl S200 of rabbit IgG strongly degraded by human plasmin.



DISCUSSION

Cleavage of human IgG by papain between residues 224 and 225 would be expected to yield three fragments. Two should be identical, and capable of binding antigen (hence 'F_{ab}'), and the third should not bind antigen but should be easy to crystallise (hence F_c). Each F_{ab} contains two chains, linked by disulphide bonds, with a total molecular weight of about 49 000. Reduction of the disulphide bonds should yield two different fragments, each with a molecular weight of about 25 000. The F_c fragment will also be a two-chain structure, but in this case the chains are expected to be identical. Reductive cleavage of the fragment should therefore yield two fragments, each of molecular weight about 26 000.

Reduction of the F_{ab} and F_c fragments with C_2H_6OS and subsequent SDS-PAGE was shown to give results exactly in keeping with the expected results outlined above, and all indications were that the papain cleavage had proceeded as predicted. The demonstration of an aggregated form for F_{ab} but not for F_c is not easy to explain, but could perhaps result from the enzymic cleavage at the hinge region causing a rearrangement of the tertiary structure of the F_{ab} fragment involving the exposure of hydrophobic groups that are normally masked, and thus encouraging aggregation of the molecule. When the F_{ab} fragment was dissolved in Medium 199 and incubated at 37°in the presence of rabbit yolk sacs, the aggregated form quickly disappeared. The amount of degraded material produced was insufficient to suggest that the aggregate had been removed by preferential uptake and degradation by the yolk sacs, and the result is probably an effect of the salt content of the Medium 199 allowing the molecule to become more stable in solution. Similar effects have been observed with other proteins before in the laboratory (Livesey, unpublished results), and since there was no evidence for the persistence of the aggregated form when the substrate was added to incubation medium, the demonstration of its existence in the stock solution was assumed not to be crucial to the interpretation of these findings.

The Endocytic Index was originally designed as a measure of fluid phase pinocytosis, and when it is applied to substrates that are taken up solely in the fluid phase, its value (dimensions of volume per unit time) does represent the rate of fluid uptake. When it is used to describe the uptake of an adsorptive substrate, however, its value is meaningful only in so far as it represents the volume 'whose contained substrate is taken

up in unit time; the actual rate of fluid uptake being less than the Endocytic Index might appear to suggest.

Originally, using a non-degradable substrate that was not released from the tissue in any form after being taken up, the Endocytic Index was calculated simply by measuring the rate of association of radioactive substrate with the tissue. For degradable substrates it was necessary to measure also the rate of appearance in the medium of ^{125}I-iodotyrosine, and by assuming that this represented material released from the yolk sac after intralysosomal degradation of substrate, it was possible to calculate the true rate of uptake of substrate into the tissue. Release of degraded material was found not to occur until approximately twenty minutes after the first contact with substrate; during the first sixty minutes the amount of tissue-associated radioactivity was found to rise progressively with time. Eventually, though, the quantity of tissue-associated radioactivity reaches a steady-state, when the rate of degradation of substrate and release of TCA-soluble radioactivity into the medium equals the rate of uptake of substrate. Hence, for any substrate that is not released from the yolk sac in a macromolecular form, whether it is degradable or not, the value of the Endocytic Index will be the same regardless of the time period over which it is calculated, since the sum of the tissue-accumulation-rate and the rate of release of degraded products is a constant.

For a substrate that can be released in macromolecular form from the yolk sac, however, the situation is more complicated. Some material will be taken up and transported to the lysosomes, where it may be degraded in the usual way. Other material,though, is taken into the yolk sac and eventually released again intact. This second event has the effect of elevating the quantity of substrate associated with the tissue to a value higher than that concerned with degradation only, but once that higher level has been reached the net tissue-accumulation rate may fall to zero as substrate is released intact. There is no simple way routinely to measure the rate of release from the tissue of undegraded material, indeed such release may go undetected in the standard experimental procedure for estimating Endocytic Index. However, it would be expected that for a protected substrate, a consequence would be that the initial value of the Endocytic Index during the early part of the incubation period, when a steady-state level is being reached in the release compartment, will always be greater than its value later, when such a

steady-state has been reached. In practice, not only is it difficult to establish an accurate value of the Endocytic Index over the initial incubation period, but during this same period any rapid, non-pinocytic association of ^{125}I-labelled IgG with the tissue will be mistakenly ascribed to build-up in the release compartment. Thus, even if it were possible to determine accurately the initial value of the Endocytic Index, there would be serious reservations as to its reliability as a basis to calculate a rate of uptake into the release compartment.

Another set of results that must be treated with caution are the quantities of radioactivity released from the reincubated tissue previously exposed to ^{125}I-IgG or to the same concentration (w/v) of one of its ^{125}I-labelled fragments. In terms of release of undegraded substrate, the initial slope of the line representing the release from the tissue of TCA-insoluble radioactivity is an approximation to the maximum rate of release of intact ^{125}I-IgG in the steady state (although, in practice, this slope is very difficult to measure accurately because of the washing procedures necessary to remove adsorbed substrate). It appears that the F_c fragment is released more rapidly than the parent molecule, while the rate of release of the F_{ab} fragment is very low.

These observations give useful information concerning the site of the signal part of the immunoglobulin molecule that mediates protection from degradation and allows transcellular transport across the yolk sac. The F_{ab} fragment exhibits virtually no TCA-insoluble release from the tissue (similar behaviour to that observed for ^{125}I-BSA), while the F_c fragment has a greatly elevated potential for protection. It becomes evident that the signal mediating the special behaviour of IgG inside yolk-sac cells is located somewhere in the F_c fragment of the molecule. The greatly elevated amount of TCA-insoluble material released from the yolk sac when the F_c fragment is used as substrate (nearly three times as much as for the parent molecule) can be explained in various ways. It could be argued that the removal of the bulky F_{ab} portion of the molecule allows the F_c portion much more freedom of interaction with the receptor - indeed, the cleavage may allow more prominent orientation of the signal sequence. Probably more important is the fact that these experiments were conducted at the same concentration (wt/v) of the various substrates. Since the intact molecule has a molecular weight of 150 000, and the F_c fragment one of approximately 52 000, the F_c preparation contains nearly three times as many signal sequences per mg of substrate. This effective

tripling of the signal concentration would be expected to significantly elevate the amount of material specifically taken into the protective pathway, as is observed. A criticism of these data, however, is that they were obtained using different sized substrates at the same (w/v) concentrations. This makes interpretation rather difficult, and in retrospect a better experimental design would be to work at equimolar concentrations of the various radiolabelled substrates, and to express the results in corresponding units.

The lack of cleavage products obtained from rabbit IgG under the exact conditions outlined in the original method for plasmin degradation of IgG (Colomb and Porter, 1975) was disappointing. The same results were obtained with both porcine and human plasmin. When a 'strong' degradation (Section 4.2.2) was carried out using human plasmin, some degradation products were obtained, but the efficiency of the process was so low compared with the near-complete degradation claimed by the original authors that no attempt was made to isolate and characterise the products.

This was an unfortunate place to terminate these investigations since the system appears to be a very useful model of the *in vivo* transmission process, and the use of F_{acb} and pF_c' as substrates would shed more convincing light on the location of the signal than does the recent work of Schlamowitz (Johanson *et al*, 1983) which implicates the CH_2 region, but from data gathered using isolated and fixed yolk-sac membranes for binding studies. This contrasts with the methodology used in this chapter which employs intact, living cells, and hence is expected to be the more representative experimental system.

It is known that different classes of IgG heavy chains are transported to the rabbit foetus *in vivo* to different extents (eg. Hemmings, 1974, working with subclasses of human IgG). Hence another potentially useful application of the incubation system would be to examine the affinity of different subclasses of rat IgG for the protective internalization mechanism pathway of rat yolk sacs, with a view to further elucidation of the signal unit of the rat IgG molecule.

^{125}I-F_c fragment showed no significantly increased susceptibility to degradation by yolk-sac homogenates at neutral pH relative to the parent molecule. Very little breakdown was observed above pH 4.5, and therefore

the possibility of a significant proportion of the observed proteolysis occurring at extralysosomal locations seems remote, neither is there any evidence for important non-enzymic hydrolysis, or for hydrolysis involving lysosome-associated neutral proteases of the sort described by LoSpalluto et al, (1970).

In summary then, the conclusions of this chapter are as follows:

(i) The signal part of the IgG molecule which mediates protection on association with the yolk sac appears to be situated entirely within the F_c region of the molecule.

(ii) The existence of a catabolism-regulating site within the IgG molecule is uncertain. The F_{ab} fragment is taken up and degraded faster than the F_c fragment, and this may be an effect of a regulatory site in one of the fragments, or it may result from various less specific effects, including perhaps charge, size, shape, hydrophobicity or degree of denaturation.

(iii) The F_c fragment appears to be no more susceptible to degradation by membrane-bound neutral proteinases than is the intact parent molecule.

(iv) The potential of the further use of fragments in this system is considerable. It is unfortunate that none could be prepared.

Chapter 5. The involvement of Carbohydrate in the Protective Mechanism.

INTRODUCTION

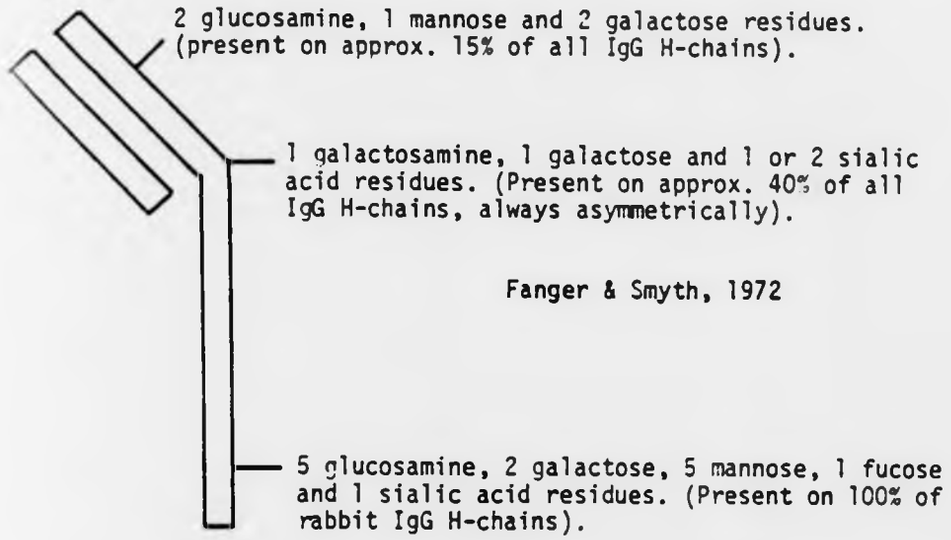
There are two possible ways in which carbohydrate may be involved in the specific interaction between IgG and the yolk-sac membrane. The oligosaccharide chains present within the IgG molecule itself may interact with the membrane to mediate specific uptake or, alternatively, the plasmalemma may carry glycoproteins whose oligosaccharide chains interact with the IgG molecule and thus bring about its transcellular transmission.

Consider first the carbohydrate present on the IgG molecule. There is relatively little carbohydrate on the light chain of rabbit IgG, and since transmission is a property of the F_c fragment, the light chain is probably not involved. The locations of the oligosaccharide chains on the heavy chain of rabbit IgG are shown in Figure 5.1.1 (Fanger and Smyth, 1972a). There are three known units, one being present in the F_d region of the molecule, one in the hinge, near the site of papain cleavage, and one in the CH_2 region, with the average compositions indicated in Figure 5.1.1. Again, since membrane transmission is a property of the F_c region, the F_d-linked carbohydrate is unlikely to be involved in the interaction.

The CH_2-linked carbohydrate is joined through the amide group of asparagine 297, and is present on all IgG heavy chains (Sutton and Phillips, 1983). It has a backbone structure composed of four glucosamine residues and three mannose residues. These may be supplemented by various other groups, and the complete structure (according to Rademacher *et al*, 1980) is shown in Figure 5.1.2. Analysis shows that 74% of the molecules are non-sialylated, 17% monosialylated and 9% disialylated.

The hinge-linked carbohydrate, which is bonded through the hydroxyl group of threonine 227, is usually terminated by either one or two sialic acid residues. This carbohydrate is only attached to about 40% of all rabbit IgG heavy chains and has been studied in depth by Fanger and Smyth (1972b). They found that the IgG molecule demonstrated asymmetric resistance to papain cleavage of the heavy chains, with one chain being substantially more susceptible than the other. This led to the isolation of the fragments shown in Figure 5.1.3 and to the suggestion that the hinge-linked carbohydrate was attached to only one of the heavy chains, affording it a certain amount of protection from papain degradation.

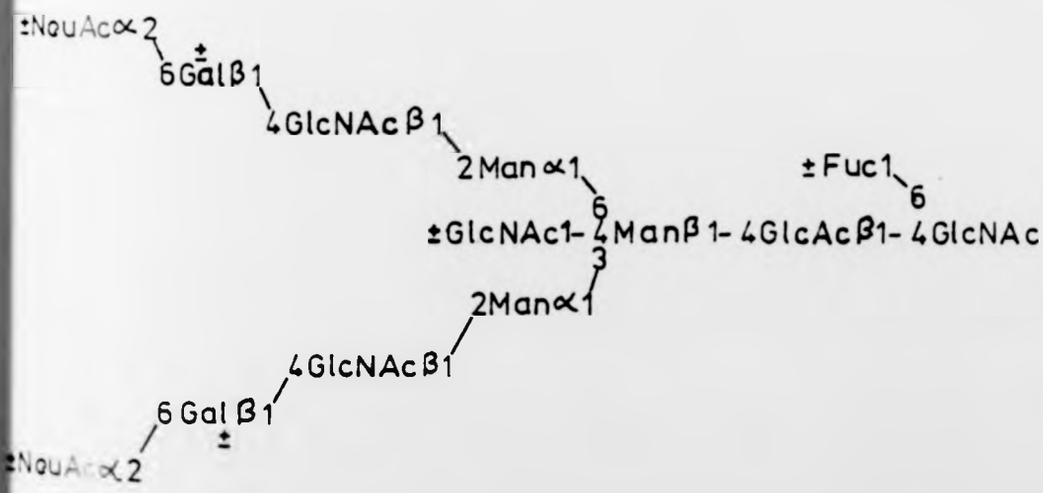
Figure 5.1.1 Carbohydrate groups on rabbit IgG.



Fanger & Smyth, 1972

Figure 5.1.2 The structure of the CH_2-linked carbohydrate.

(Rademacher, et al, 1983)



Further analysis of the fragment confirmed this suggestion; it appears that the hinge-linked carbohydrate group is never present on both heavy chains in the same molecule, and sometimes it is present on neither. The majority of rabbit IgG molecules contain just one hinge-linked carbohydrate moiety which is attached asymmetrically to one of the heavy chains. It is thus the only known asymmetric feature of the molecule, and is therefore of substantial biological interest.

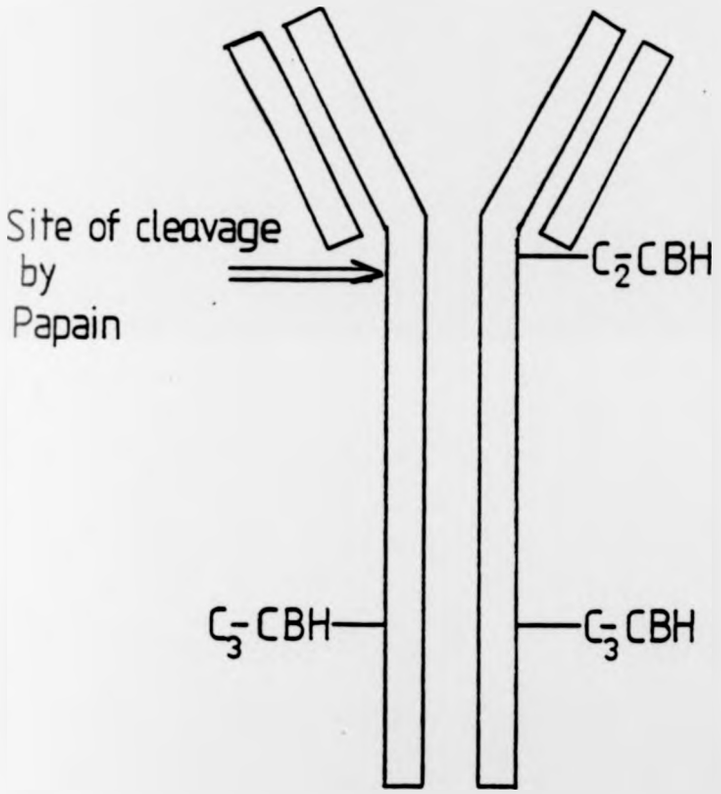
Periodate oxidation is a technique that has been used for many years in the analysis of carbohydrates. It causes cleavage of the bond between carbon atoms carrying either unsubstituted hydroxyl groups, or aldehyde or keto groups, and raises the oxidation state of each carbon by one level. Three adjacent carbon atoms bearing unsubstituted hydroxyl groups will thus give rise to one molecule of free HCO_2H, although this situation is not common in the sugar residues found in polysaccharides. Oxidation of the polysaccharide moiety of a glycoprotein normally breaks open every sugar ring but leaves the backbone of the oligosaccharide chain intact (see Figure 5.1.4). Hence any gross steric effects exhibited by the untreated molecule may be retained after oxidation, though any specific conformations dependent on the integrity of the carbohydrate will probably have been destroyed.

Many reports have been published concerning modification of cellular uptake of circulating glycoproteins following alteration of the terminal sugar residue of the carbohydrate part of the molecule. Morell et al (1971) have shown that rat hepatocytes are able specifically to recognise and remove from the circulation desialylated glycoproteins, and neuraminidase treatment of terminal sialic acid residues, to expose the penultimate galactose residues, substantially decreased the half-life of survival of such glycoproteins in the circulation (Van den Hamer, 1970).

More recently, Stockert et al(1976) demonstrated that sequential removal of sialic acid and galactose from alpha$_1$ acid-glycoprotein, to expose N-acetylglucosamine residues, results in rapid clearance of this molecule from the bloodstream via a pathway which, they postulate, is distinct from the one used for galactose-terminated oligosaccharides, although their evidence in support of this assertion is not presented. Other examples of desialylation causing rapid clearance from the circulation have been documented by Lunney and Ashwell (1976) and Tolleshaug et al(1977).

Figure 5.1.3 The asymmetric cleavage of IgG by papain.

from Fanger & Smyth, 1972b

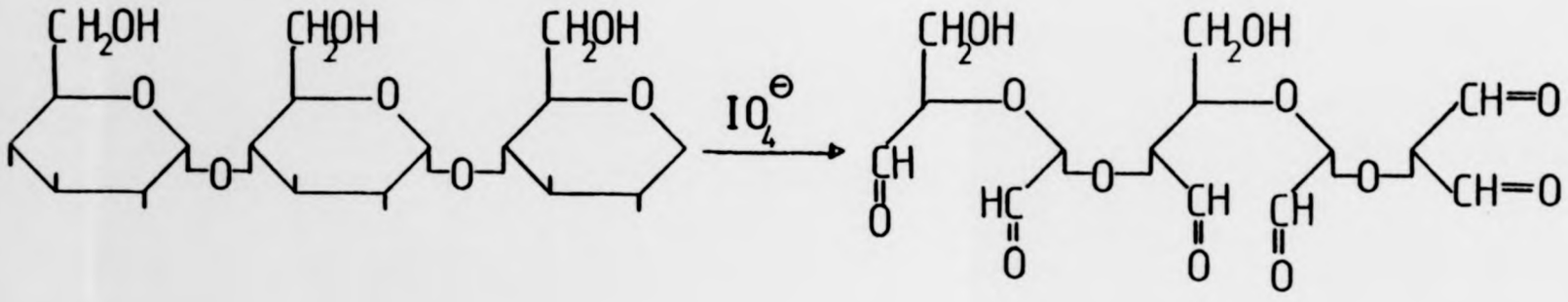


Hence the behaviour of neuraminidase-treated IgG in the incubation system would be of interest since the enzyme would be expected to remove the terminal sialic acid residues from the hinge-linked and perhaps from the CH_2-linked carbohydrates. Any decrease in the capacity of the molecule to be protected within the incubated yolk sac would implicate the involvement of sialic acid residues, in one or both of the F_c-linked carbohydrate groups, in the binding of the IgG molecule to the receptor in the tissue that is responsible for transcellular transport.

There are few reports in the literature of the involvement of membrane-linked carbohydrate groups in the specific substrate-membrane interactions in receptor-mediated uptake of substrates, though Pricer and Ashwell (1971) report the abolition of specific uptake of desialylated glycoproteins following treatment of the hepatocyte membrane with neuraminidase. Receptor activity could be restored to the membrane either by enzymic replacement of the sialic acid residues or by removing the next sugar (galactose) from the receptor carbohydrate. It was eventually established that the loss of activity of the receptor was caused by its ability to form a stable complex with its own exposed galactose residue. Perturbation of this complex, either by removal of the galactose residue with beta-galactosidase or by masking it with sialic acid, restored substrate-binding activity. (Stockert *et al*, 1977; Paulson *et al*, 1977).

There are many similarities between specific receptor-mediated uptake of molecules for transport or degradation and the stimulation of target cells by non-lipid-soluble hormones, a process that involves specific combination of the hormone with a membrane-bound receptor, and is followed by endocytosis of the complex (for review, see Cooke, 1982). There are well-documented examples of the involvement of sugar residues of receptors in the recognition process. For example, Haksar *et al* (1973) found that treatment of adrenocortical cells with neuraminidase abolished the induction of steroid production by ACTH, suggesting the involvement of terminal sialic-acid residues in the interaction. Similarly, treatment of adipose-tissue cells with neuraminidase followed by beta-galactosidase was shown (Cuatrecasas, 1974) to abolish insulin binding to the membrane, though neuraminidase alone did not have any effect. The implication is that the galactose residue adjacent to the terminal sialic-acid, but not the sialic-acid itself, is involved in binding to insulin.

Figure 5.1.4 Example of typical periodate oxidation of carbohydrate section.



On the other hand, there are many examples of hormone receptors that apparently contain no carbohydrate at all. Some findings are summarised in Table 5.1.1.

Most hypotheses of the mechanism of the maternofoetal transmission of antibodies now incorporate the existence of a membrane-bound, IgG-specific receptor that must interact with the 'signal' part of the antibody molecule and mediate its transmission to the foetus. By using a variety of techniques, the existence of such receptors has been demonstrated in a number of tissues (eg. rabbit yolk sac (Wild and Dawson, 1977), mouse placenta and yolk sac (Elson et al, 1975), chick yolk sac (Linden and Roth, 1978), rat yolk sac (Weisbecker, 1981), mouse intestine (Guyer et al, 1976), and rat intestine (Wild and Richardson, 1979; Rodewald, 1973). The work of Wild and Dawson (1977), on rabbit yolk-sac endoderm, involved the formation of rosettes of sheep red blood cells (SRBCs), coated with rabbit IgG, on the endodermal surface of rabbit yolk sacs. When the SRBCs were coated with F_{ab} or $F_{(ab')2}$ fragments, or IgM, no rosettes were formed. Rosetting of IgG-coated SRBCs could be inhibited by the prior exposure of the yolk sac to rabbit or human IgG, or rabbit F_c fragment. Bovine IgG had little inhibitory effect, and IgM, F_{ab} fragment and rabbit albumin were inactive. The conclusion was drawn that there was 'an association between the F_c receptor and selective immunoglobulin transport'.

A few attempts have been made to further characterise the IgG-receptor of rabbit yolk-sac membrane, but progress has been slow. Hillman et al (1977) confirmed by subcellular fractionation that the receptor is mainly associated with the brush border membrane, and concluded that it was a protein because its activity was destroyed by treatment of formalin-fixed yolk-sac membrane with trypsin and papain. The interaction with IgG was 60% inhibited by 0.4M NaCl, though similar concentrations of KI or KCl had no significant effect. Ca^{++} and Mg^{++} were not essential for the interaction, since pretreatment of the fixed tissue with EDTA did not noticeably influence binding.

Cobbs et al (1980) managed to solubilise and purify a component of the membrane that exhibited F_c-specific binding activity, and found it had a monomeric sedimentation coefficient of 7-8S. Phospholipase C was without effect on the receptor, which exhibited a buoyant density of 1.28g/ml. They concluded that the receptor was not a lipoprotein, and

Table 5.1.1 **Hormone receptors.**

Hormone	Tissue	Receptor	
L.H.	Ovary	protein/phospholipid	Dufau, 1974
	Testis	protein/phospholipid	Haour & Saxena, (1974)
F.S.H.	Testis	protein	Dufau, 1973
Prolactin	Liver	protein/phospholipid	Shiu & Friesen (1974)
G.H.	Liver	protein/phospholipid	Waters & Friesen, (1979)
Glucagon	Liver	protein/phospholipid	Giorgio et al, 1974
Insulin	Liver	glycoprotein	Cuatrecasas, 1972
	Placenta	glycoprotein	Petriozzelli, et al, 1982
Others:			
LDL	Adrenal cortex	glycoprotein	Schneider et al, 1982
Transferrin	HeLa cells	protein/phospholipid	Omary & Trowbridge, 1981
Asialoglyco-protein	Liver	protein/phospholipid	Harford, et al, 1982

that all of its known properties were compatible with it being a peripheral membrane protein.

There is no direct evidence to date of carbohydrate involvement in any cell-surface receptor, and the bulk of receptor-signal interactions characterised to date (for review see Neufeld and Ashwell, 1980) involve carbohydrate determinants present on the substrate rather than on the cell surface. Nevertheless, the effects of yolk-sac pretreatment with neuraminidase or beta-galactosidase would be a simple way to investigate any involvement of receptor carbohydrates terminated by sialic acid or galactose residues, respectively.

When a cell-substrate interaction is mediated by carbohydrate moieties the process can sometimes be partially inhibited by the presence of monomeric sugars and sugar phosphates (see e.g. Adams, 1982). It was therefore decided to investigate the effects, on the behaviour of rat IgG in rat yolk sacs incubated in vitro, of various sugars and sugar phosphates.

The type of experimental animal used was governed by the availability - or otherwise - of rabbit tissue. In fact, rat tissue was used for investigations involving periodate effects and the use of simple sugars as competitive inhibitors of uptake, and rabbit tissue was used for all other experiments - which mainly involved enzyme-treatment either of substrate or of cells.

In summary, the aims of this chapter were as follows:

(i) To investigate the effects of periodate treatment of rat IgG on its behaviour in the in vitro rat yolk-sac system.

(ii) To investigate any neuraminidase-induced modifications of the behaviour of rabbit IgG in the in vitro rabbit yolk-sac system.

(iii)To investigate any modifications caused to the behaviour of rabbit IgG incubated in vitro with rabbit yolk sacs by pretreatment of the yolk sacs with neuraminidase or beta-galactosidase.

(iv) To observe any competition for receptors between rat IgG and monomeric sugars and sugar phosphates present in the incubation medium at

fairly high concentrations.

MATERIALS AND METHODS

5.2.1 Periodate treatment of rat gamma globulins.

The method of periodate treatment described by Stahl et al (1976) was used with minor modifications. A reaction time of 3h was chosen since, according to these authors, this seemed to optimise carbohydrate modification (as measured by the rate of blood clearance) but maintained a high degree of binding to con-A-Sepharose for their particular substrate (beta-glucuronidase). The procedure was as follows:

Rat gamma globulins (1.5 mg/ml, Miles Laboratories) were dissolved in sodium phosphate buffer (20mM, pH 8.0) and adjusted with acetic acid to pH 6.0. Reaction was initiated by the addition of 1M sodium periodate (aq.) to a final concentration of 10mM. Incubation continued in the dark at 0°C for 3h, and the reaction was stopped by the addition of 1M ethylene glycol to a final concentration of 10% (v/v).

Radioiodination of the protein was carried out both before and after periodate treatment, in different experiments, in order to determine which was the more efficient. No modifications were made to the labelling procedure described in Section 2.2.1. Control gamma globulins were treated in exactly the same way, but without the addition of periodate.

5.2.2 Neuraminidase treatment of rabbit IgG.

Both neuraminidase-treated rabbit IgG and untreated IgG were a kind gift from Dr.D.Stanworth at Birmingham University. The small amount of neuraminidase used had not been removed from the preparation.

5.2.3 Neuraminidase treatment of rabbit yolk-sac tissue.

Two types of neuraminidase were used to modify the yolk-sac surface carbohydrates. Both were isolated from Clostridium perfringens and obtained through Sigma, but one preparation (Type X, N2133) was approximately 700x more active (manufacturer's specifications) than the other (Type V, N2876). Unless otherwise stated in the results section, neuraminidase treatment of yolk sacs was at a concentration of 2 units/10 ml for 30 min at 37°C in medium 199. Normally yolk sacs were then removed and reincubated in fresh medium containing radioactive substrate together

with a final concentration of 0.2 unit/10 ml neuraminidase.

For recovery experiments the tissue was removed from the pretreatment flask after 30 min and washed briefly in three changes of warm medium before being put into a fresh incubation flask containing 10ml medium 199, radioactive substrate (Section 2.2.12) but no neuraminidase. When samples of the reincubation medium were removed they were replaced with medium containing the same concentration of neuraminidase, and the yolk sacs were harvested at the end of the experiment in the normal way.

In order to minimise modification of the ^{125}I-IgG by the neuraminidase present in the uptake medium, rabbit IgG that had been pre-treated with neuraminidase was used as substrate throughout this series of investigations both for controls and for experimental incubations.

5.2.4 Beta-galactosidase treatment of rabbit yolk sacs.

Two types of beta-galactosidase, both isolated from E. Coli, were used to modify the yolk-sac cell surface carbohydrates. Sigma beta-galactosidase (Grade VIII, G5635) contains about 500 units/mg and Boehringer Mannheim (105 031) about 300 units/mg. The Sigma product contains low levels of Tris buffer and magnesium chloride which would not be expected to modify the behaviour of the yolk sac. The Boehringer Mannheim product, however, contains a high concentration (2.2M) of ammonium sulphate as a preservative (it is supplied in liquid form and stored at 4^{o}C). Ammonium ions at concentrations greater than 1mM cause modifications to the yolk sac behaviour (G. Livesey, 1977) and therefore the enzyme was dialysed overnight against cold 1% saline before use.

Pretreatment of yolk sacs, unless otherwise stated, was for 30 min at a concentration of beta-galactosidase of 1 unit/ml in medium 199, and uptake experiments were performed at an enzyme activity of 0.2 unit/ml in the usual way.

5.2.5 The use of sugars and sugar-phosphates as competitive inhibitors of uptake.

Adams (1982) used a wide variety of different sugars in his attempts to competitively inhibit endocytosis, but gives no explanation of the particular spread of concentrations employed. In the current study a uniform, fairly high (1mg/ml) concentration was used for each sugar, and rat yolk sacs were incubated in medium 199 containing the appropriate sugar for 1h before the addition of substrate. Uptake of rat ^{125}I-IgG proceeded for 1.1h at a substrate concentration of 5ug/ml and then yolk sacs were washed three times and reincubated in fresh medium 199 containing no added sugars. Samples were taken in the usual way.

The sugars and sugar phophates were all obtained from Sigma. They were; D(+)-galactose; D-mannose-6-phosphate (barium salt); D-fructose-1,6-diphosphate (trisodium salt); D-glucose-6-phosphate (monosodium salt); D-fructose-1-phosphate (barium salt) and D-galactose-6-phosphate (barium salt).

RESULTS

5.3.1 The behaviour of periodate-treated gamma globulins in rat yolk sacs.

During periodate-treatment of ^{125}I-labelled rat gamma globulins it was found that over 80% of the protein was lost during the final dialysis stage, due to its sticking to the dialysis tubing. When the protein was treated with periodate before being radiolabelled this was found not to be a significant problem. The efficiency of labelling was 52.6%, determined by the method described in Section 2.2.1, and this preparation was used for all the work reported here.

Periodate treatment increases the Endocytic Index observed in rat yolk sacs both in the presence and absence of calf serum (Figures 5.3.1 & 5.3.2). There is a slight increase in the tissue level of radioactivity, but this is not sufficient on its own to account for the increased Endocytic Index measured, which caused by an increased rate of uptake for degradation of periodate-treated rat gamma globulins.

Comparison of the release patterns of control and periodate-treated gamma globulins from rat yolk sacs both in the usual way (Figure 5.3.3) and also as a function of uptake time (Figure 5.3.4), shows clearly the elevated release of degraded material and also a slight fall in the amount of TCA-insoluble radioactivity released.

Periodate-treated gamma globulins, then, exhibit an increased Endocytic Index, a slightly increased tissue level, an elevated release of degraded material and a slightly decreased release of TCA-insoluble material.

5.3.2 The behaviour of neuraminidase-treated rabbit IgG in rabbit yolk sacs.

^{125}I-labelled neuraminidase-treated rabbit IgG exhibits a slightly decreased Endocytic Index in rabbit yolk sacs when compared with untreated ^{125}I-IgG. There is no obvious fall in the level of tissue-associated radioactivity (Figure 5.3.5).

The release pattern for neuraminidase-treated ^{125}I-IgG shows a slightly decreased release of degraded material from the yolk sac and a substantially elevated release of TCA-insoluble material (Figure 5.3.6). Neuraminidase treatment of rabbit IgG therefore decreases its extent of degradation in rabbit yolk sacs and increases its capacity for protection.

5.3.3 The effect of neuraminidase treatment of rabbit yolk sacs.

Treatment of rabbit yolk sacs with Sigma type V neuraminidase results in approximately equal falls in the release of TCA-soluble and TCA-insoluble material from rabbit yolk sacs previously exposed to ^{125}I-IgG (Figure 5.3.7). This was the result of a decreased rate of uptake of substrate into the yolk sacs, rather than inhibition of the mechanisms of release (uptake of ^{125}-PVP was inhibited approx.50% by neuraminidase-pretreatment of yolk sacs, Table 5.3.1). If the release graphs are expressed as '% of total tissue-associated radioactivity released' rather than in absolute weights of the substrate-equivalent of radioactivity released, the control and experimental results are virtually identical, there being no selective inhibition of just one pathway (Figure 5.3.7(a). In an attempt to discriminate between the two pathways, milder treatments were carried out on the yolk sacs (Figure 5.3.8), but again approximately equal falls were observed for the release of both forms of radioactivity.

Pretreatment of yolk sacs with type V neuraminidase resulted in treatment-dependent inhibition of pinocytosis, as measured by ^{125}I-PVP accumulation (Table 5.3.1). The inhibition of pinocytosis was found to be quickly reversed by removing the enzyme (Figure 5.3.9), and neuraminidase treatment did not increase the rate of cell lysis, as measured by the rate of loss of ^{125}I-PVP on reincubating tissue that had previously been allowed to accumulate this marker (Figure 5.3.10).

Sigma type X neuraminidase, used at the same activity, was shown to have no significant effects on ^{125}I-PVP accumulation (Figure 5.3.11) and did not modify the pattern of release from the tissue of ^{125}I-IgG (Figure 5.3.12).

5.3.4 The effect of beta-galactosidase treatment of rabbit yolk sacs.

Pretreatment of the tissue with beta-galactosidase failed to modify the release pattern of homologous ^{125}I-IgG from rabbit yolk sacs (Figures 5.3.13 & 5.3.14).

5.3.5 The effects of exogenous sugars and sugar-phosphates on the release pattern of homologous ^{125}I-IgG from rat yolk sacs.

In every case (Figure 5.3.15) the release of TCA-soluble material was unaffected by the presence of the sugar or sugar phosphate. D(+)glucose-6-phosphate and D fructose-1-phosphate seemed to cause a slightly elevated release of TCA-insoluble material, but no other effects were noted.

In the following RESULTS section, as throughout the rest of this Thesis, the following key to symbols is observed, unless indicated otherwise in the legends.

TCA - soluble radioactivity is represented by open symbols - mainly squares or circles.

TCA - insoluble radioactivity is represented by closed symbols - mainly squares or circles.

± Error bars represent ± Standard Deviations.

Figure 5.3.1 The uptake by rat yolk sacs of control and periodate-treated ^{125}I-gamma globulins in the presence of calf serum.

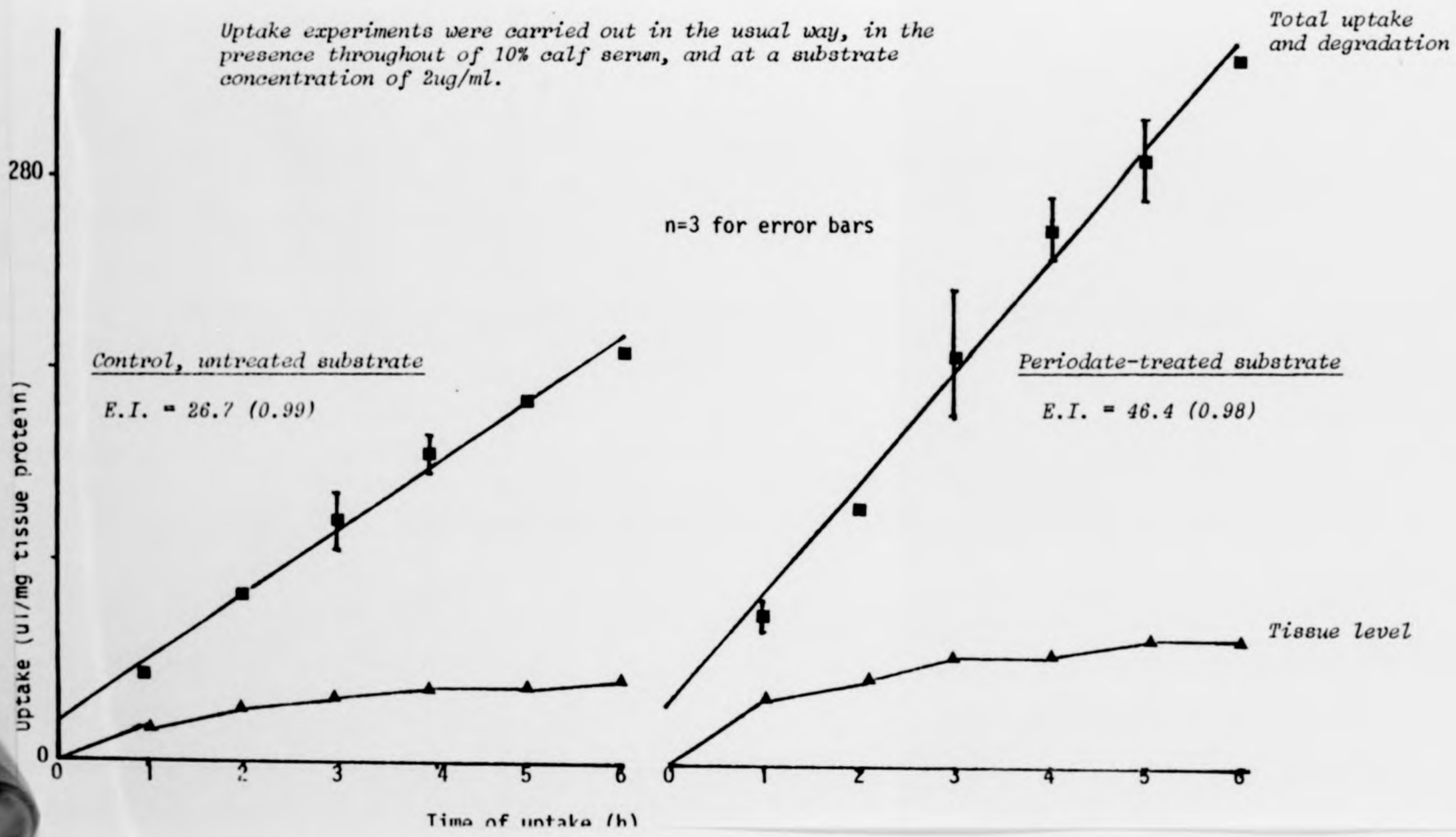


Figure 5.3.2 The uptake by rat yolk sacs of control and periodate-treated ^{125}I-gamma globulins, in the absence of calf serum.

Uptake experiments were carried out in the usual way, in the absence of calf serum, and with substrates present at a concentration of 2ug/ml.

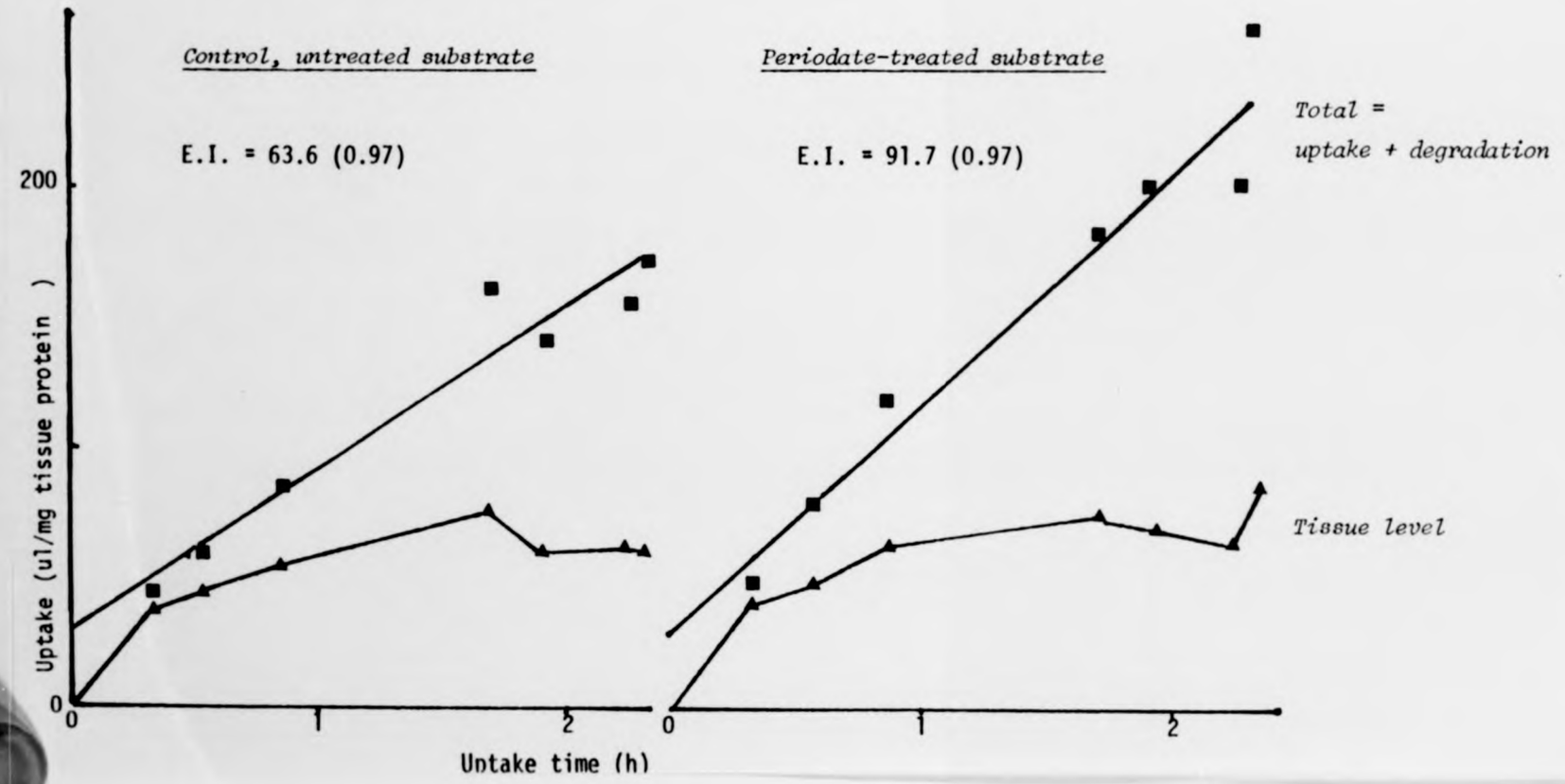


Figure 5.3.3 The effect of periodate-treatment of rat gamma globulin on its pattern of release from rat yolk sacs.

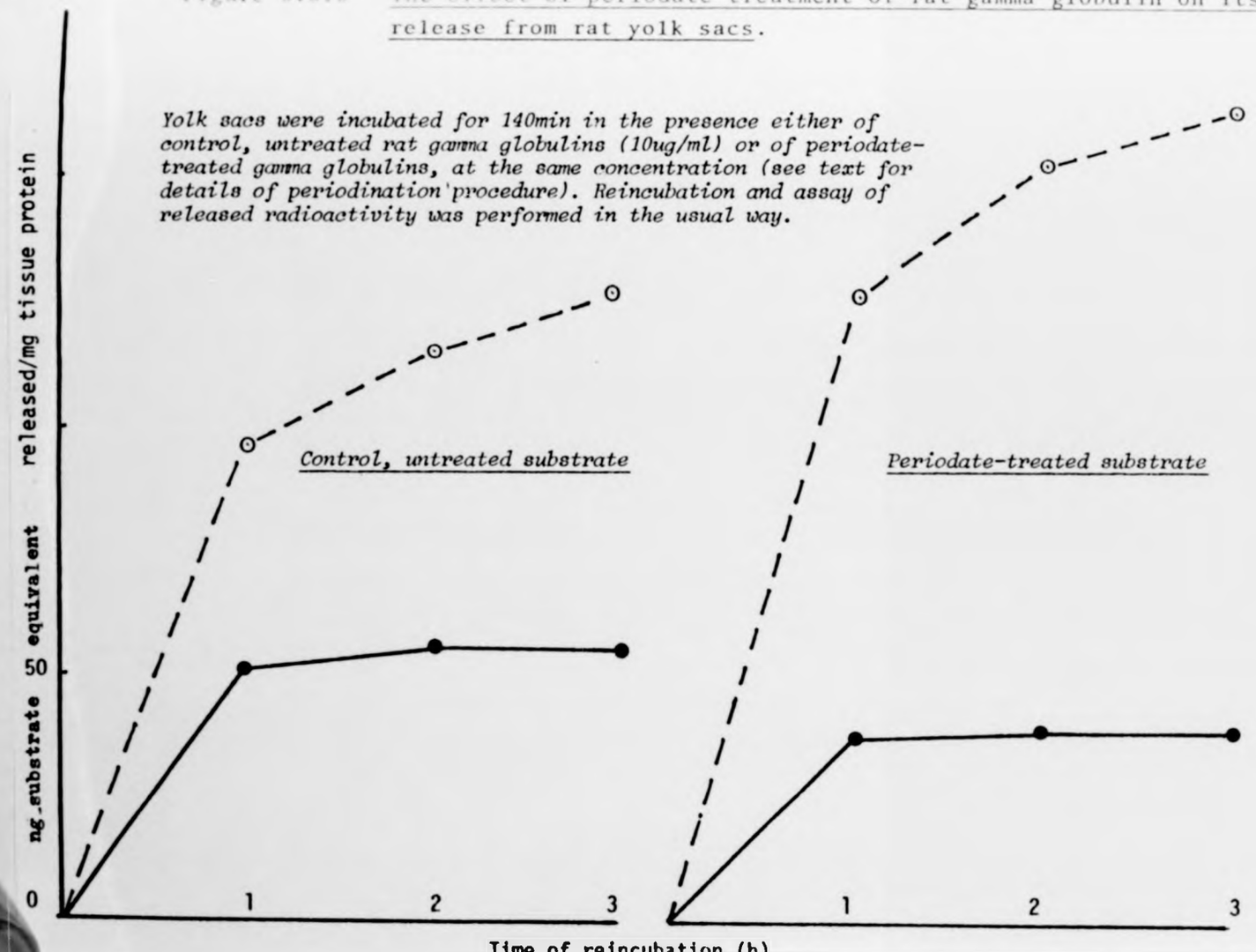
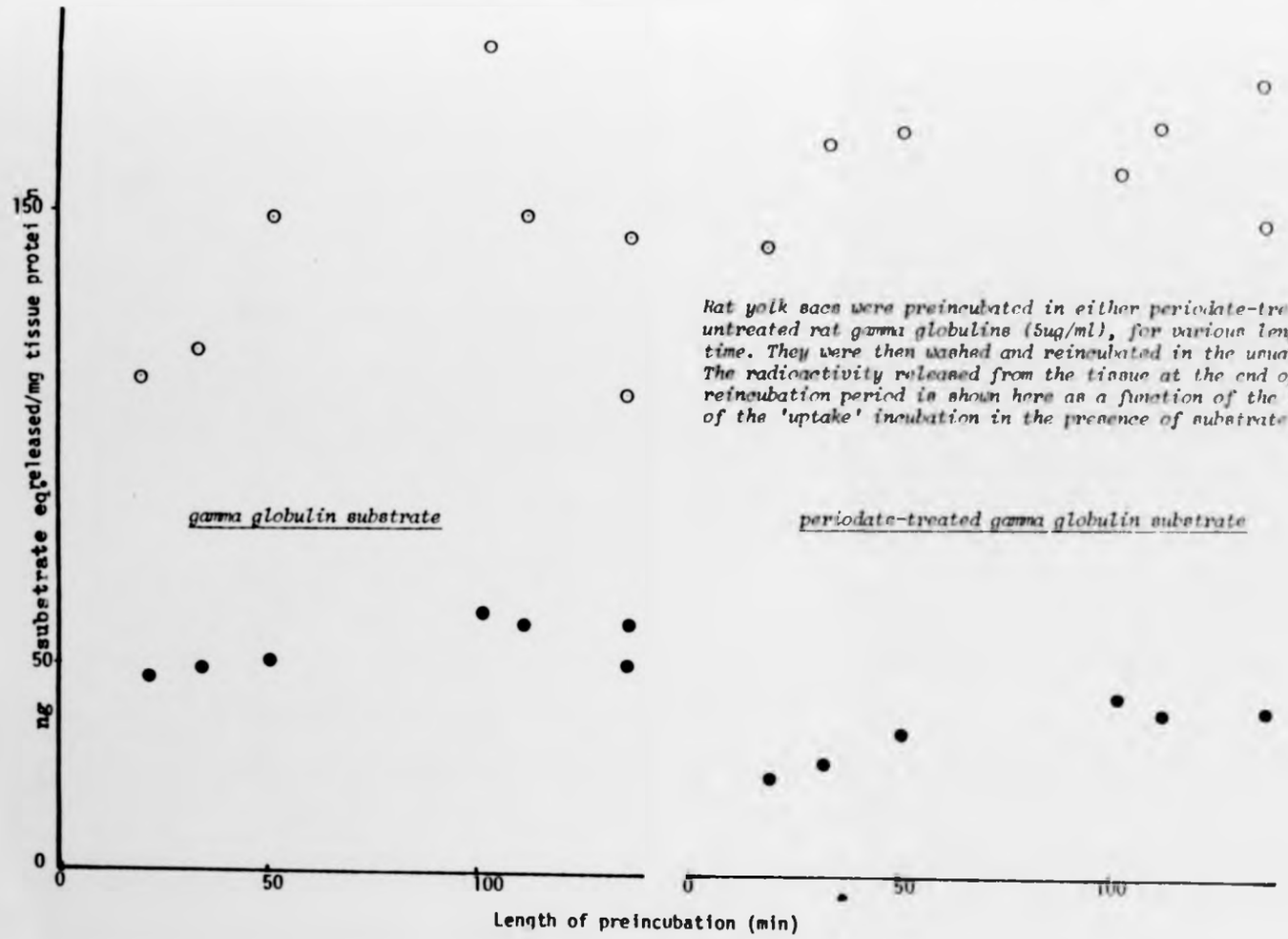


Figure 5.3.4 The release from rat yolk sacs of control and periodate-treated rat gamma globulin-derived radioactivity.



Rat yolk sacs were preincubated in either periodate-treated or untreated rat gamma globulins (5ug/ml), for various lengths of time. They were then washed and reincubated in the usual way. The radioactivity released from the tissue at the end of a 3h reincubation period is shown here as a function of the length of the 'uptake' incubation in the presence of substrate.

Figure 5.3.5 The uptake by rabbit yolk sacs of control and neuraminidase-treated homologous ^{125}I-IgG.

Uptake experiments were carried out as described in the text, with a substrate concentration of 2ug/ml.

n=3 for error bars

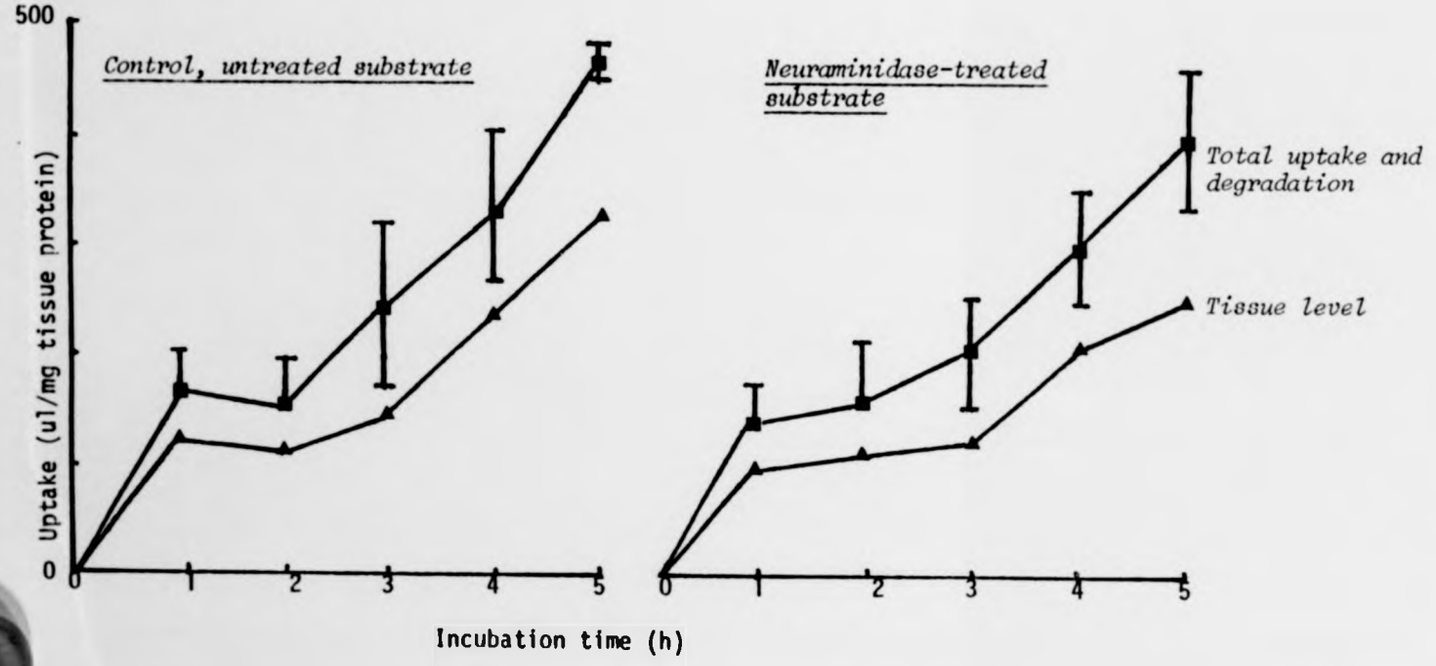


Figure 5.3.6 The release pattern from rabbit yolk sacs of control and neuraminidase-treated homologous ^{125}I-IgG.

Release experiments were carried out in the usual way, with a substrateoncentration of 5ug/ml and a preincubation time of 90min.

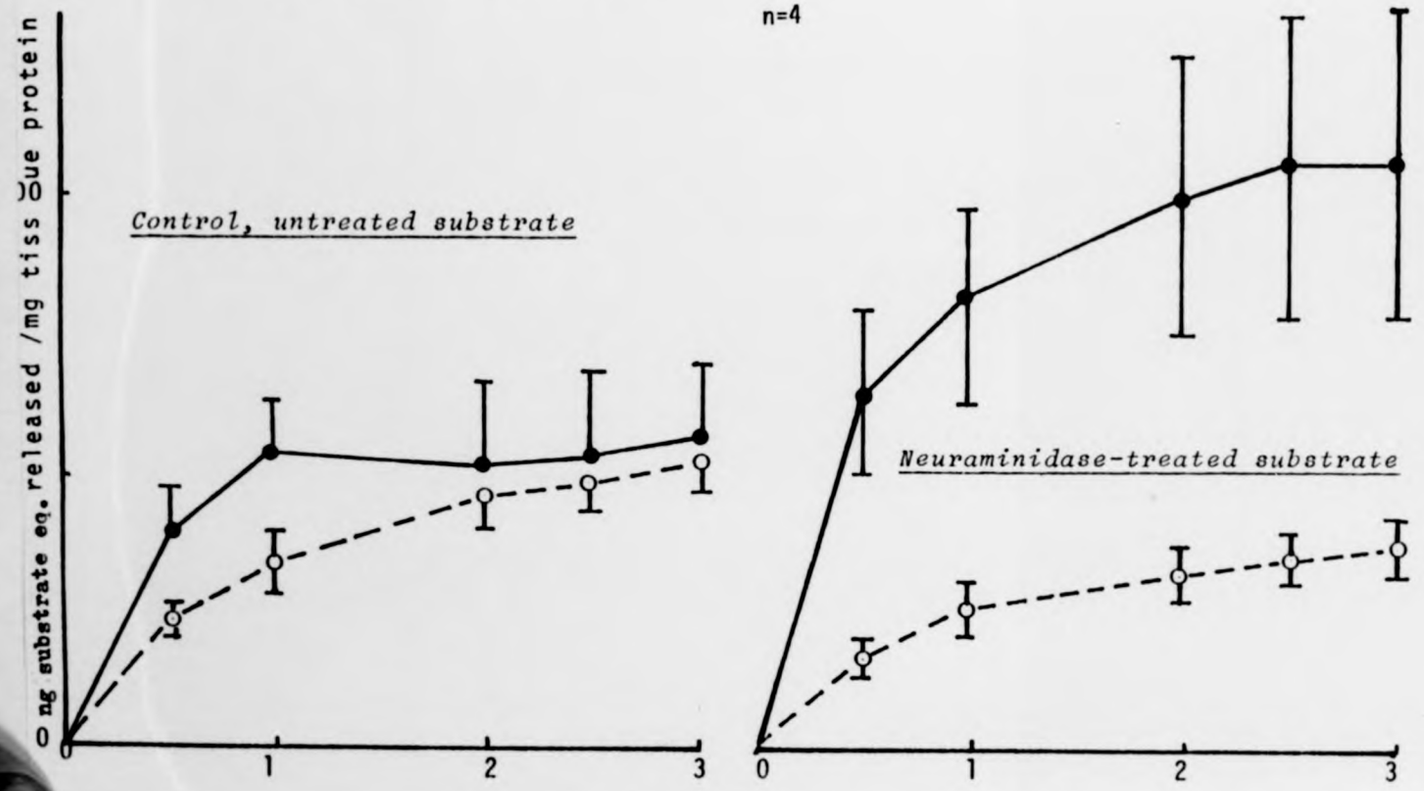


Figure 5.3.7 The effect of 'strong' type V neuraminidase pretreatment of rabbit yolk sacs on the release pattern of radioactivity derived from ^{125}I-IgG.

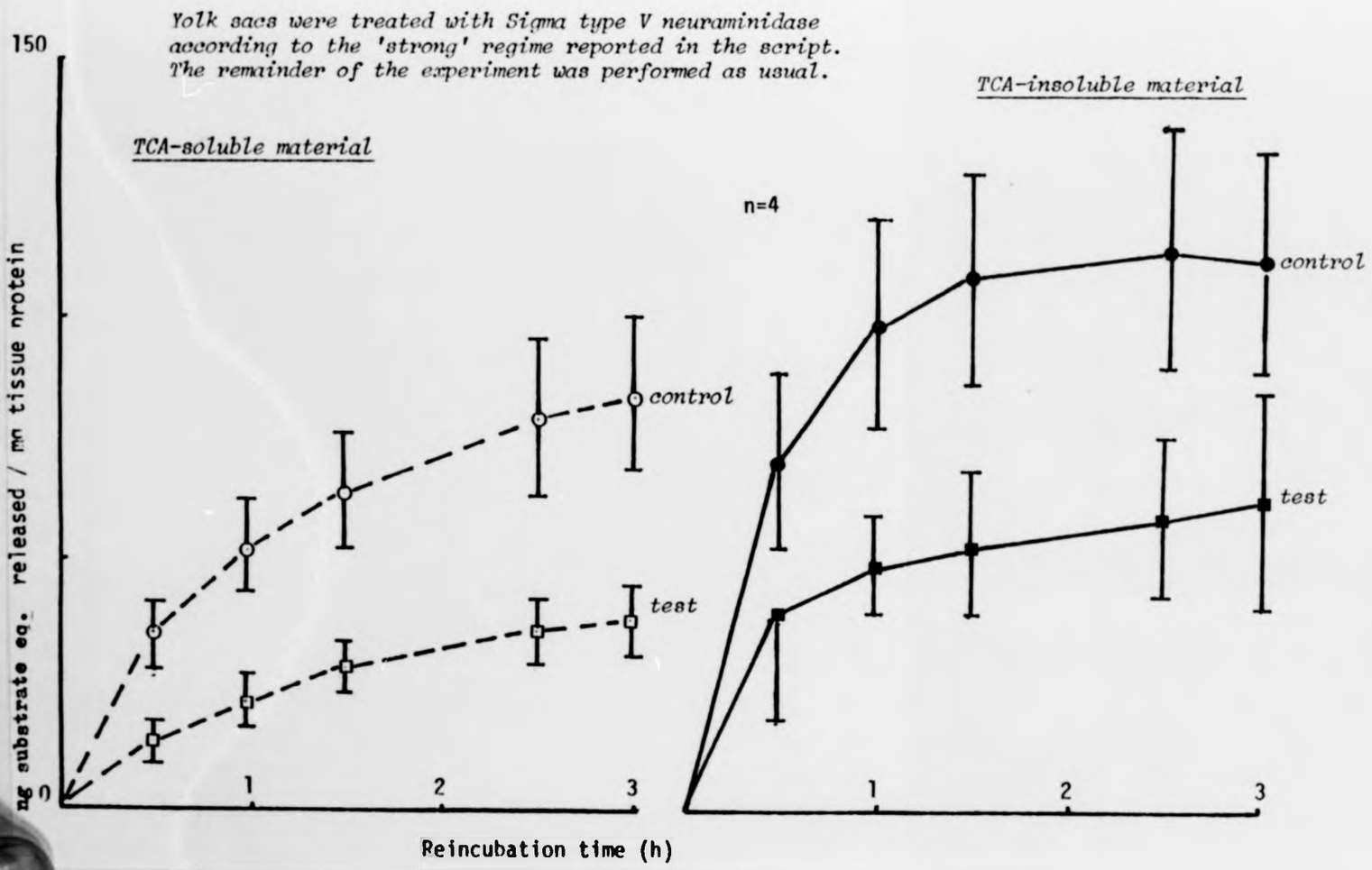


Figure 5.3.7a The effects of 'strong' type V neudaminidase pretreatment of rabbit yolk sacs on the release pattern of radioactivity derived from ^{125}I-IgG.

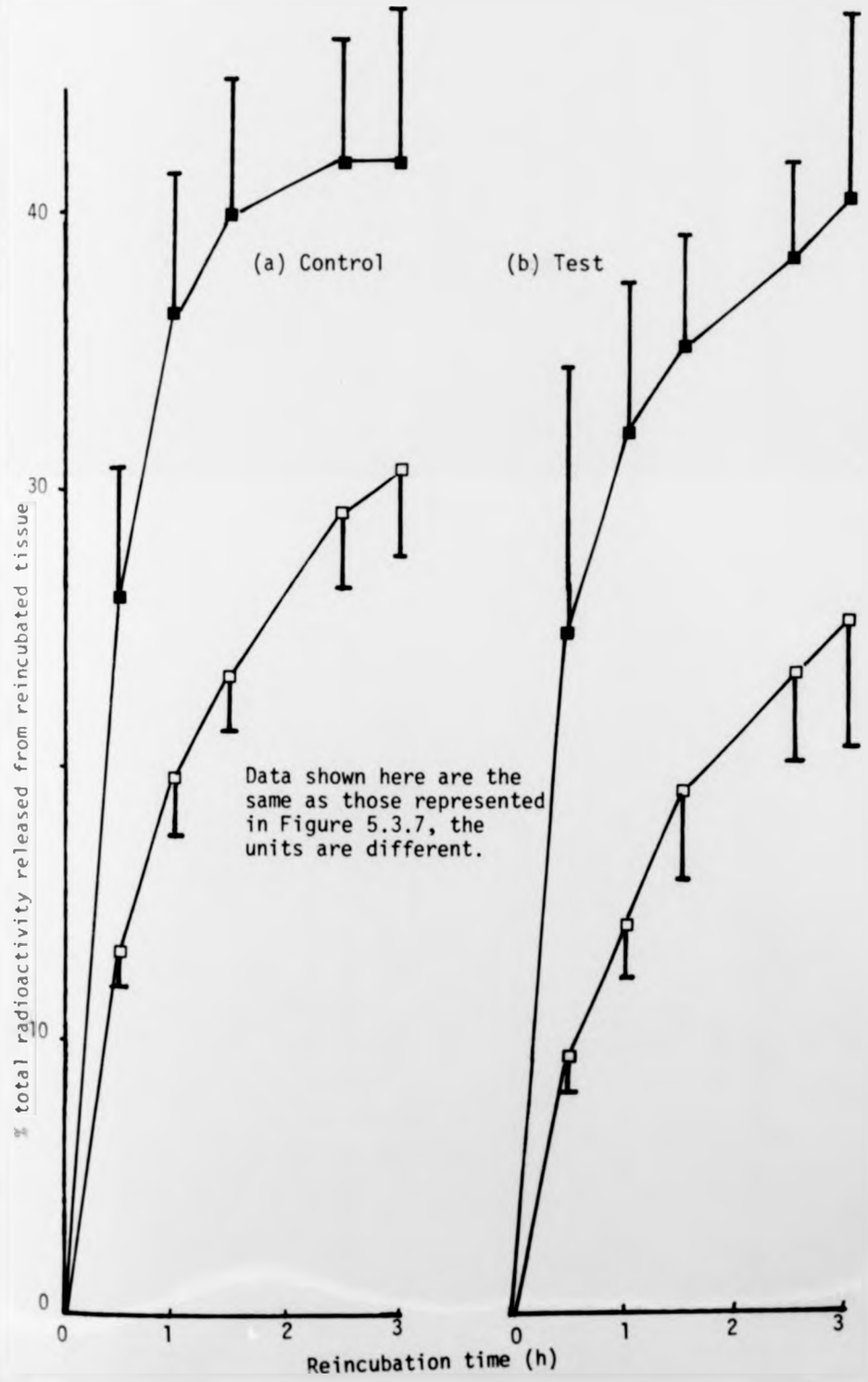


Figure 5.3.8 The effect of 'mild' type V neuraminidase pretreatment of rabbit yolk sacs on the release pattern of radioactivity derived from ^{125}I-IgG.

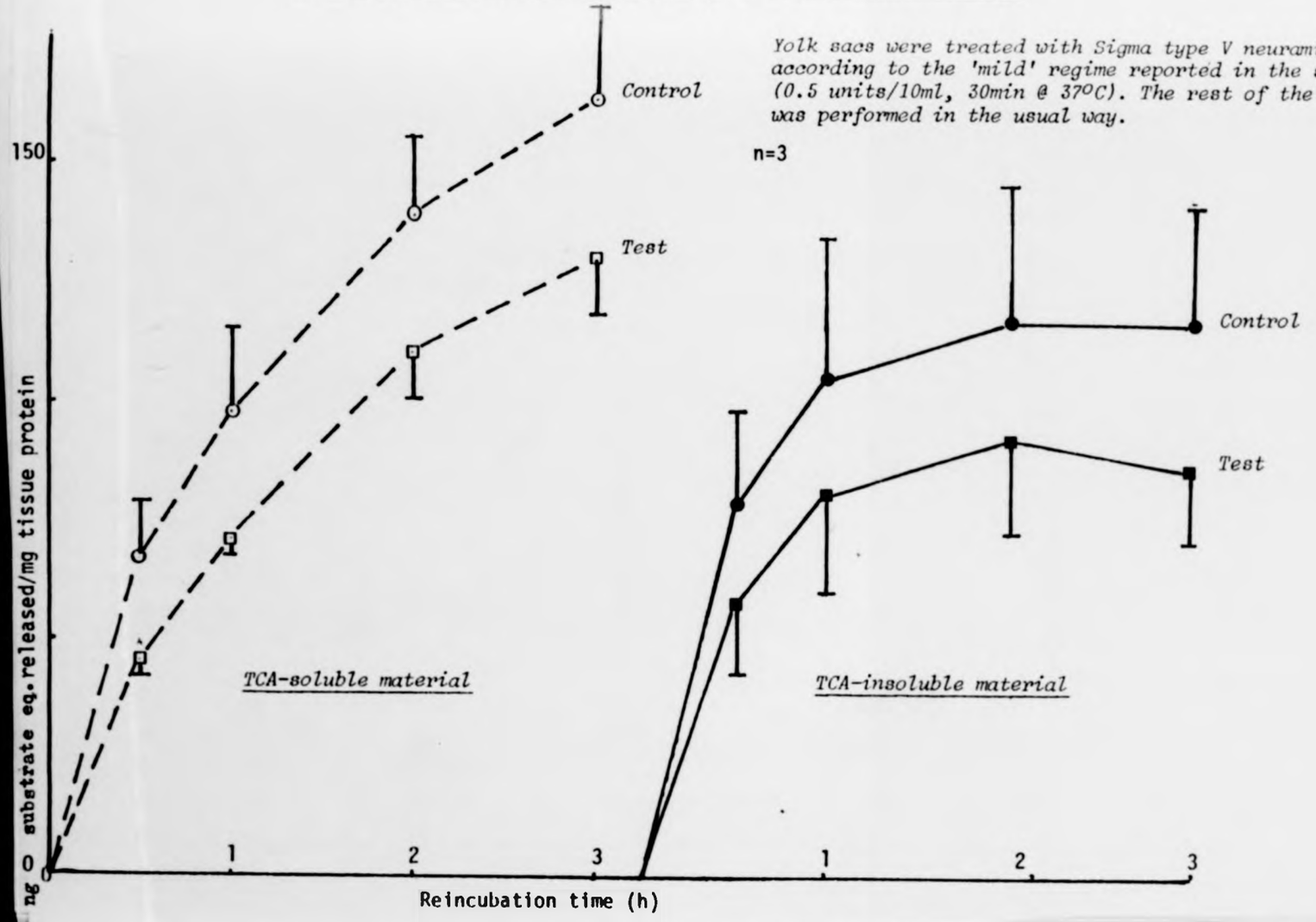


Figure 5.3.9 The reversibility of Sigma type V neuraminidase-induced inhibition of pinocytosis in rabbit yolk sacs.

Rabbit yolk sacs were pretreated with Sigma type V neuraminidase, according to the 'strong' regime, and were then exposed to ^{125}I-PVP for study of pinocytosis, in the usual way. Control yolk sacs had neuraminidase retained throughout the uptake phase, while 'test' cases were transferred to neuraminidase-free medium at the start of the uptake.

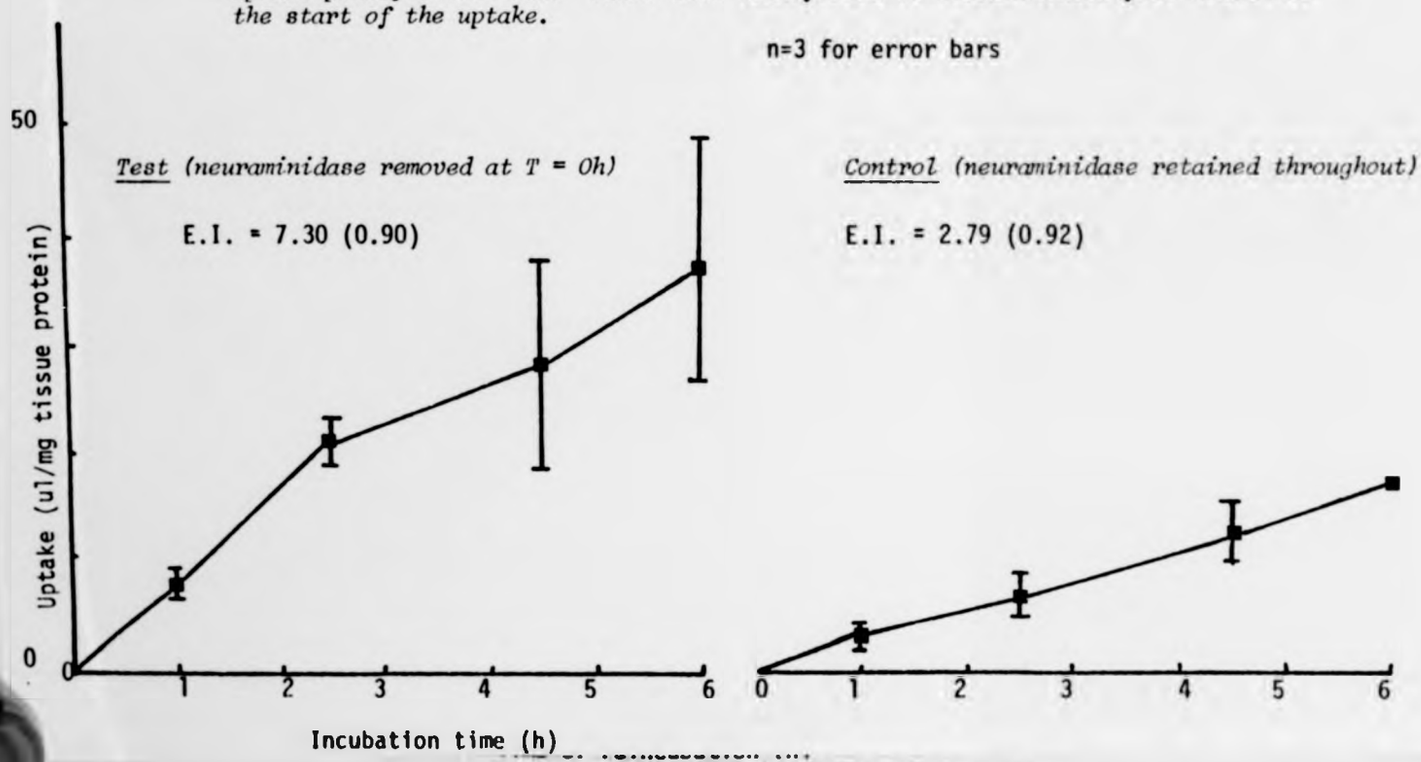


Figure 5.3.10 The effect of neuraminidase treatment on the rate of release of ^{125}I-PVP from rabbit yolk-sac tissue.

Rabbit yolk sacs were pretreated with Sigma type V neuraminidase before exposure to ^{125}I-PVP (3ug/ml, 3h). The tissues were then reincubated in fresh medium and the release of radioactivity was monitored.

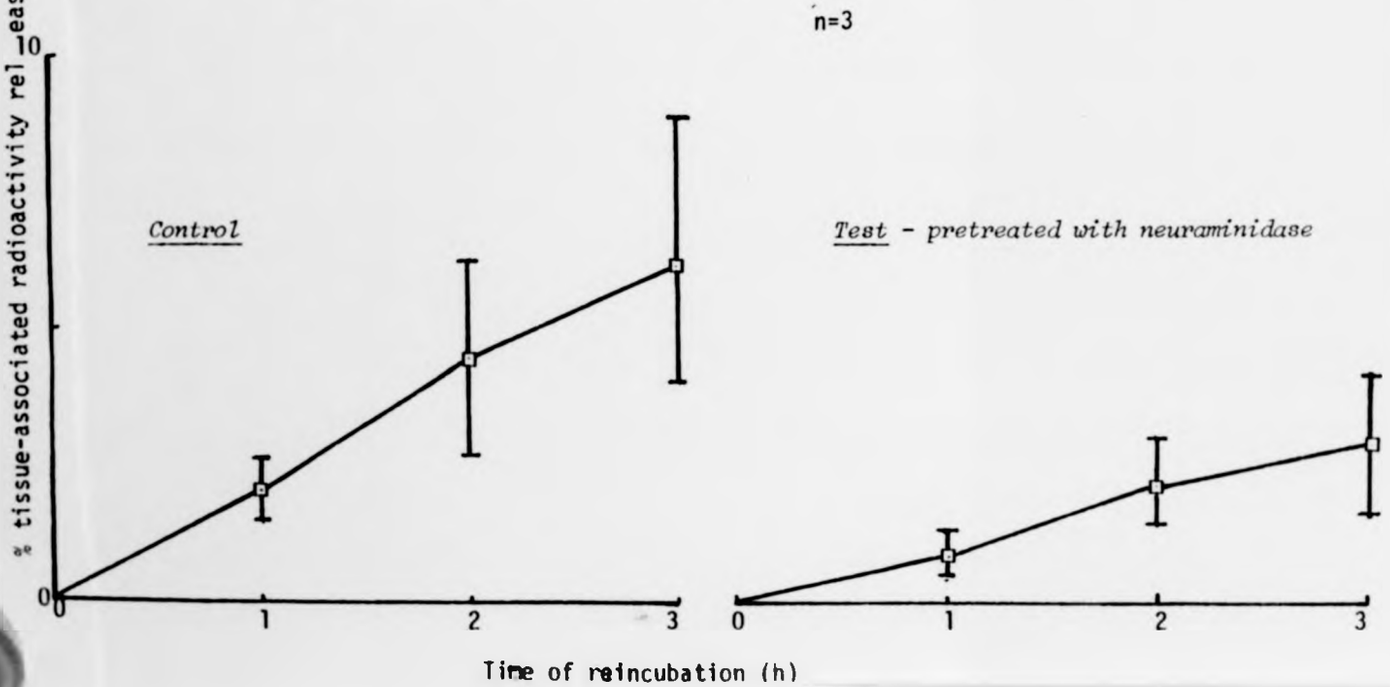


Figure 5.3.11 The effect of pretreatment of rabbit yolk sacs with Sigma type X neuraminidase on their subsequent uptake of ^{125}I-PVP.

Rabbit yolk sacs were treated with Sigma type X neuraminidase, as described in the text, before being exposed to ^{125}I-PVP for assay of pinocytic activity, in the usual way.

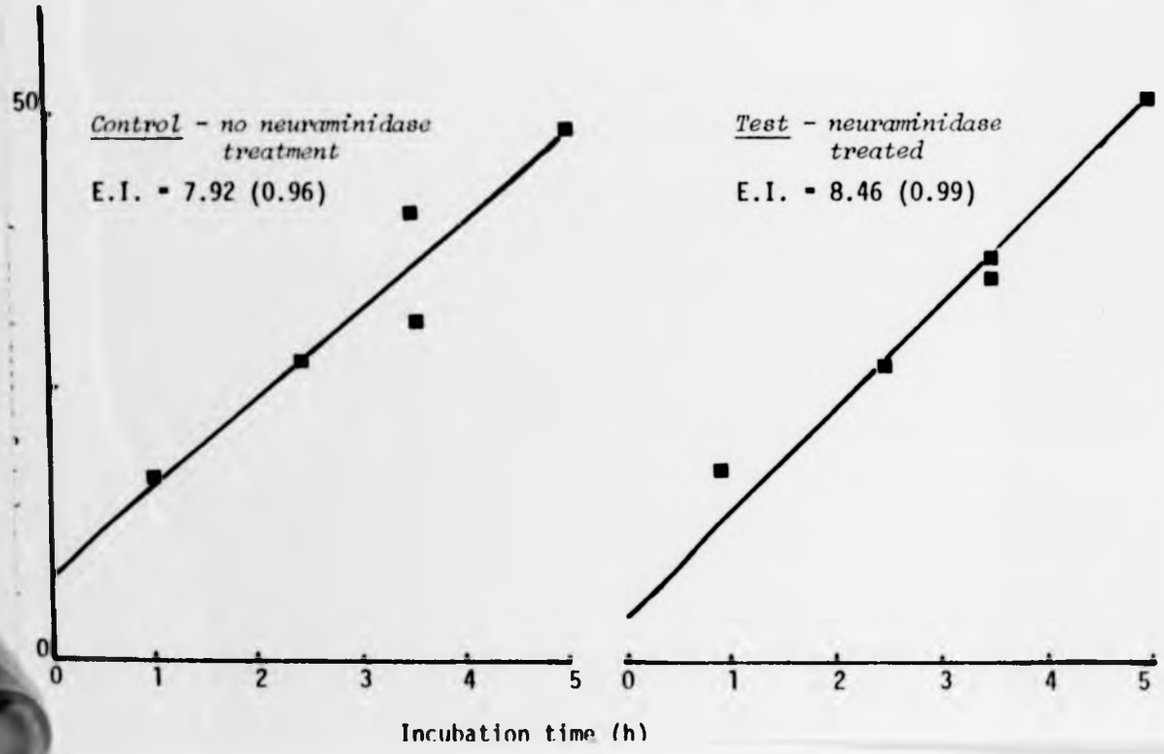


Figure 5.3.12 The effect of pretreatment of rabbit yolk sacs with Sigma type X neuraminidase on the pattern of release of ^{125}I-IgG-derived radioactivity.

Rabbit yolk sacs were treated with Sigma type X neuraminidase, according to the regime reported in the script, before being exposed to homologous ^{125}I-IgG (5ug/ml, 90min). The release of radioactivity on subsequent reincubation in fresh medium was monitored in the usual way.

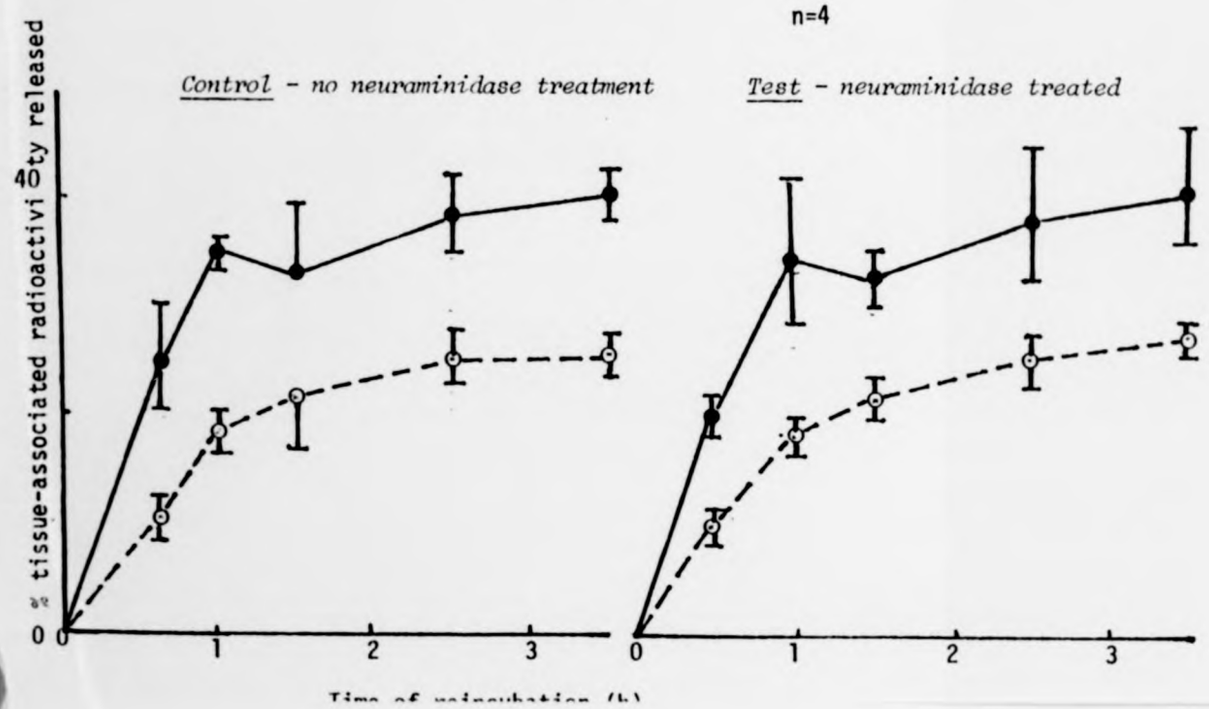


Figure 5.3.13 The effect of Boehringer-Mannheim β-galactosidase treatment of rabbit yolk sac on the release pattern for radioactivity derived from ^{125}I-IgG.

Rabbit yolk sacs were pretreated with Boehringer-Mannheim β-galactosidase, as described in the text, and then exposed to homologous ^{125}I-IgG (5ug/ml, 90min) before being reincubated in the usual way.

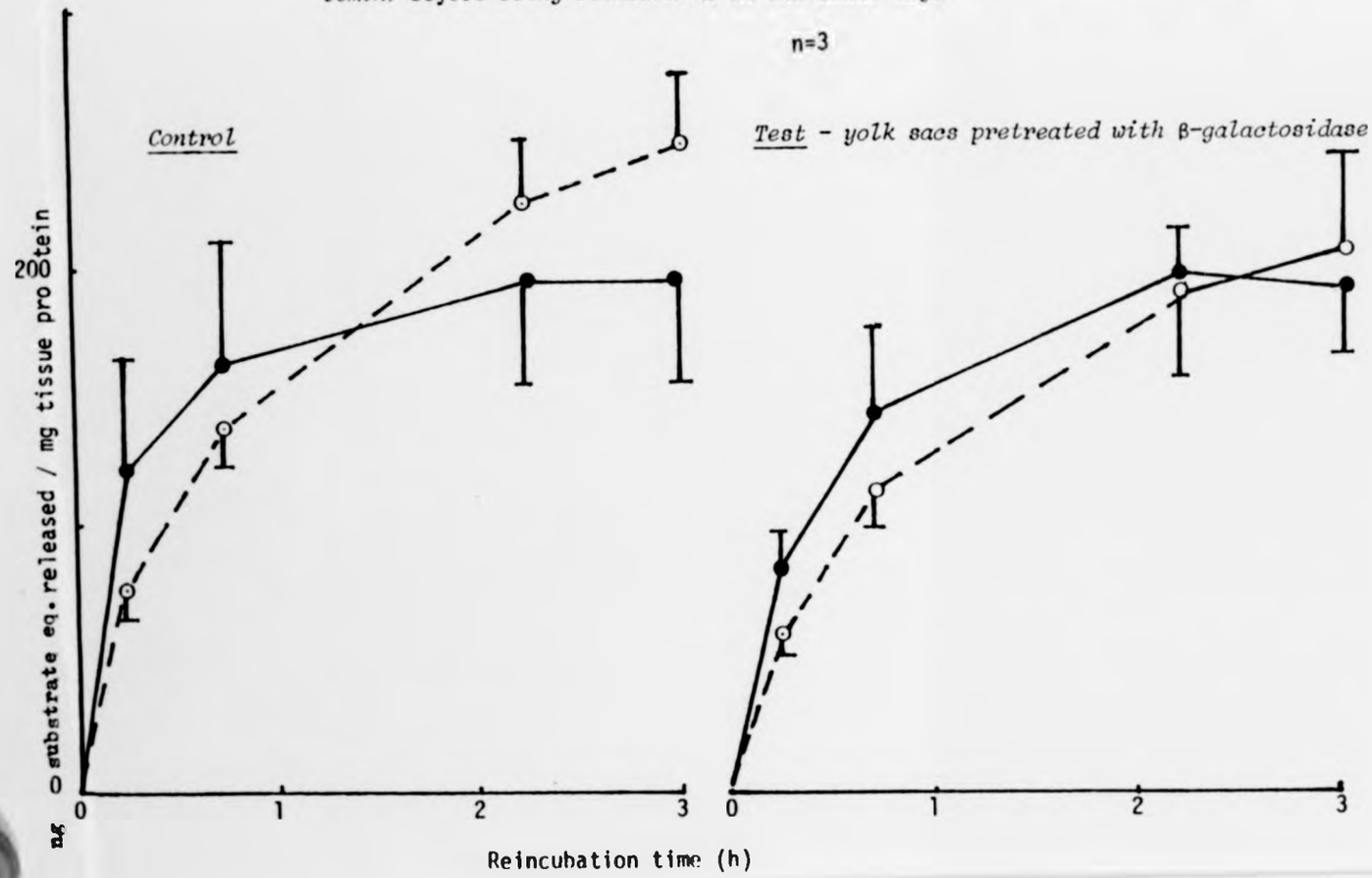


Figure 5.3.14 The effect of Sigma β-galactosidase-pretreatment of rabbit yolk sacs on the subsequent release pattern of radioactivity derived from ^{125}I-IgG.

Yolk sacs were treated with Sigma β-galactosidase before being exposed to homologous ^{125}I-IgG (5ug/ml, 90min). They were then reincubated in fresh medium, and the release of radioactivity was monitored as usual.

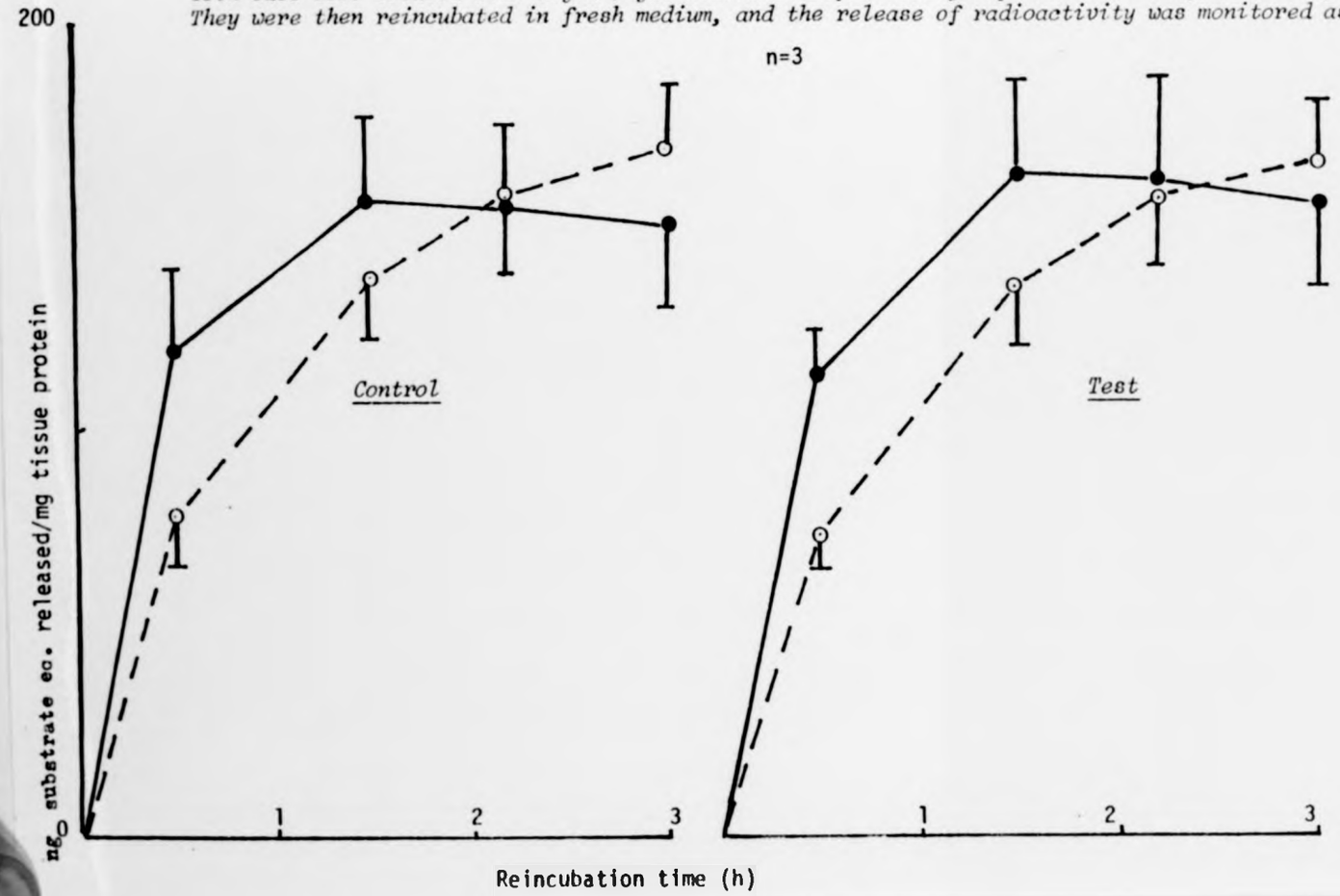


Figure 5.3.15 The effects of exogenously-added sugars and sugar phosphates on the release pattern from rat yolk sacs of radioactivity derived from ^{125}I-IgG.

Yolk sacs were preincubated in the presence of the various sugars and sugar phosphates, at a concentration of 1mg/ml, for 1h prior to the addition of ^{125}I-IgG (5ug/ml). After a further 1.1h, yolk sacs were washed and reincubated in fresh medium for assay of released radioactivity, in the usual way.

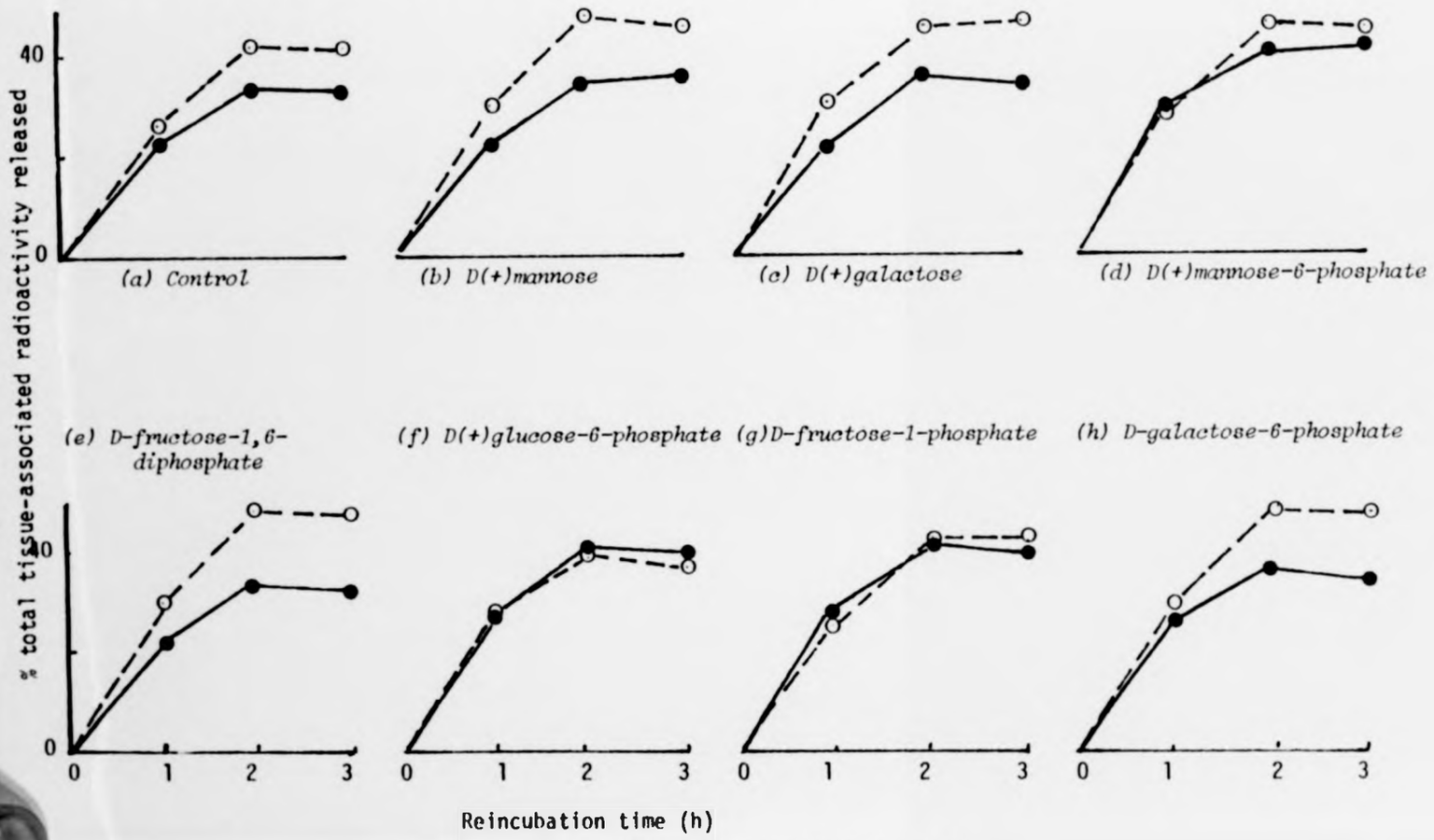


Table 5.3.2 The effect of Sigma Type V Neuraminidase-pretreatment of rabbit yolk sacs on the uptake of ^{125}I-PVP.

Units of neuraminidase in 10ml x 30min.	E.I.	R.	n
0	9.15	0.997	8
0.1	7.52	0.960	8
0.5	7.99	0.960	8
2.0	4.18	0.990	7

Rabbit yolk sacs were pretreated in the presence of Type V neuraminidase for 30min at the activity indicated, before the rate of uptake of ^{125}I-PVP was measured in the usual way. Full details are given in the text.

DISCUSSION

5.4.1 Periodate treatment of rat gamma globulins causes a slightly increased rate of uptake for catabolism into the rat yolk sac. This could arise as a result of carbohydrate groups on the native IgG molecule hindering a determinant that is involved in uptake by non-specific binding sites on the pinocytic cells. The effect of the periodate treatment may be to neutralise this masking and thus increase the observed rate of uptake and degradation. For example, periodate oxidation of the glycoprotein may cause the exposure of hydrophobic parts of the molecule that are normally hidden and thus increase its association with the cell membrane, accelerating uptake. It seems unlikely that the yolk sac is possessed of specific receptors for oxidised carbohydrate, and the increased rate of uptake must be mediated by some rather more general mechanism for the removal of damaged proteins, witness the increased rates of uptake and degradation caused by pretreatment of BSA with formaldehyde (Moore et al, 1977).

The pattern of release, from reincubated rat yolk sacs, of radioactivity derived from periodate-treated rat gamma-globulins differs slightly from that of controls. The amount of TCA-soluble material released is increased, in agreement with the observed elevation of the Endocytic Index outlined above, and the amount of TCA-insoluble material released is slightly decreased. The latter finding can be interpreted in various ways; it may suggest that the carbohydrate moieties are important in the specific 'signal' part of the molecule, and periodate oxidation causes them to function less efficiently, decreasing the rate of uptake for protection of the antibody molecule. On the other hand, periodate treatment of this intensity would be expected to exert a profound effect on any function of the molecule mediated by carbohydrate, and the observed slight decrease in the amount of material released from the protective pathway might be interpreted as indicative of no involvement of carbohydrate in the specific recognition 'signal'. The slight effect observed could be explained by masking of the signal sequence by the oxidised carbohydrate - either as a result of modified interaction between the carbohydrate and the signal sequence, or simply because of the changed conformation of the molecule as a whole.

A fuller measure of the effects of periodate-treatment of ^{125}I-IgG on its rate of uptake into rat yolk sacs is shown in Figure 5.3.4. Here,

each time point represents a different experiment, and the only variable between experiments is the length of the time of uptake. Hence the graph represents the amounts of radioactivity released after three hours from yolk sacs preincubated in hot substrates for various lengths of time. There is again some evidence of a slightly decreased rate of uptake into the protective pathway, and uptake for degradation is slightly stimulated.

A more specific modifier of carbohydrate was needed in order to clarify the possible alternatives, and, since terminal sugars tend to be the most important for recognition phenomena, neuraminidase was used to remove the terminal sialic-acid residues from the various oligosaccharide chains. The behaviour of neuraminidase-treated rabbit IgG in the rabbit yolk-sac system is rather difficult to interpret. The amount of TCA-soluble material released from reincubated yolk sacs, previously exposed to ^{125}I-labelled IgG, is slightly less when the IgG has been treated with neuraminidase than for control IgG. This suggests that removal from the CH_2 and hinge-linked carbohydrates of their terminal sialic-acid residues acts to decrease the rate of uptake for catabolism into the yolk sac. The only reasonable explanation is that neuraminidase treatment has slightly modified a site that mediates uptake into the degradative pathway present perhaps in the F_{ab} portion of the molecule. Although the effect was reproducible, the use of a different batch of neuraminidase-treated IgG is desirable to check its validity since it is in direct contradiction to most published findings (Stockert /et al/, 1977; Hinrichs and Smyth, 1969) which find terminal sialic-acid residues to be associated with protective functions.

On the other hand, neuraminidase-treated rabbit IgG invariably gave rise to a significantly elevated level of release of TCA-insoluble radioactivity from the reincubated rabbit yolk sac (Figure 5.3.6). This is quite the opposite of the result that might be expected if the F_c-linked carbohydrate groups are involved in the specific signal that mediates the characteristic behaviour of IgG both in the *in vitro* incubation system and *in vivo*. It suggests that the removal of terminal sialic-acid residues from the F_c-linked carbohydrates allows greater access to the signal part of the molecule that mediates protection from degradation, implying that the signal itself is located (at least in the physiological conformation of the molecule, if not in the amino acid backbone) very close to one of the carbohydrate groups. (It would have

been interesting to extend these findings to neuraminidase-treated F_c fragment of rabbit IgG, but unfortunately none was available for study).

Taken together, these two observations suggest that the signal mediating protection of IgG is a polypeptide sequence located close to either the CH_2 or hinge-linked carbohydrate groups.

Hinrichs and Smyth (1969) suggested that there might be a correlation between the ability of IgG (as compared with other immunoglobulins) to traverse the yolk sac or placenta, and its particularly low carbohydrate content (3%, compared with 9%, 12%, 12% and 12% for IgA, IgM, IgD and IgE respectively). They went on to investigate some possible biological roles for the (asymmetric) hinge-linked carbohydrate group. First they isolated rabbit IgG molecules containing the asymmetric group and molecules lacking it, and radiolabelled one form with ^{125}I, the other with ^{131}I. Upon injection of both forms into non-pregnant rabbits they found a significantly longer half-life for the carbohydrate containing molecules, and concluded that the asymmetric group confers on the molecule protection from uptake and degradation in the liver. They went on to measure the relative rates of maternofoetal transmission of the two types of IgG, and detected significantly greater transmission to the foetus of molecules lacking the asymmetric carbohydrate group. They took this as firm evidence that the hinge-linked carbohydrate actually mediates <u>against</u> <u>maternofoetal transmission</u>. This is in agreement with the results obtained in this study, and suggests that the important modification achieved by the neuraminidase is the removal of one or two sialic-acid residues from the hinge-linked carbohydrate, thus increasing physical access to the surrounding region.

In conjunction with the results available in the literature, then, these results suggest that the signal mediating maternofoetal transmission of IgG is located physically close to the hinge-linked carbohydrate.

Pretreatment of the rabbit yolk sac with two different concentrations of Sigma type V neuraminidase was shown to decrease the extent of release of radioactivity from the reincubated tissue without discriminating between TCA-soluble and TCA-insoluble material (Figures 5.3.7 and 5.3.8). This is probably a result of a general inhibition of

uptake mechanisms, as shown by the inhibition of the rate of accumulation of ^{125}I-PVP, rather than by inhibition of the various release mechanisms themselves, since there was no elevated tissue-level of radioactivity at the end of the reincubation period. The demonstration that the inhibition of pinocytosis (as measured by the accumulation of ^{125}I-PVP) is immediately reversed by reincubating the tissue in fresh medium containing no neuraminidase (Figure 5.3.9) suggests that the action of the preparation may not be an enzymic one, since the recovery of a full complement of sialic residues on the surface of the plasma membrane might be expected to take a longer time than the recovery period observed. This short recovery time is more compatible with the reversal of competitive inhibitory effects, mediated by non-toxic components of the enzyme preparation, than with the simple removal of sialic-acid residues from membrane proteins. These components did not appear to cause an elevated rate of cell death as judged by the loss from the tissue of intracellular ^{125}I-PVP.

The second of the above explanations was supported by the results of experiments in which a more highly purified preparation of neuraminidase was used at similar levels of enzyme activity. Yolk-sac treatment with the purer type X neuraminidase was found to exert no effect on pinocytosis (^{125}I-PVP) and not to modify significantly the pattern of release from the reincubated tissue of material of either form (degraded or intact). This suggests that the component of the Type V preparation that is responsible for the inhibition of pinocytosis of ^{125}I-PVP is not the enzyme itself.

If sialic-acid residues associated with the surface of yolk sac cells were involved in the specific recognition of ^{125}I-IgG for uptake and protection from degradation inside the yolk sac, then pretreatment of the yolk sac with neuraminidase would be expected to modify the uptake pattern and hence the release pattern of intact IgG. That this is not observed suggests very strongly that membrane-linked sialic-acid residues are not involved in the specific uptake for protection and release of intact rabbit ^{125}I-IgG. The only alternative explanation is that the turnover of the receptors is so rapid that they are replaced as fast as they are inactivated by the neuraminidase.

Another yolk sac-linked sugar residue that might be involved in the protective uptake of IgG is galactose. In the same way, pretreatment of

the yolk sac with beta-galactosidase might then be expected to modify the rate of uptake of ^{125}I-IgG into the protective pathway, and this would be reflected in the radioactivity-release pattern from the reincubated tissue. Two different preparations of beta-galactosidase were obtained and used, at the same levels of enzyme activity, to pretreat yolk sacs (Section 5.2.4). Neither type was shown to have any effect either on the subsequent accumulation of ^{125}I-PVP (pinocytosis) or on the release pattern from the reincubated tissue of either type of ^{125}I-IgG-derived radioactivity. This is a firm indication that membrane-associated terminal galactose residues are not important in the specific uptake of ^{125}I-IgG either for protection or for degradation.

On reflection, a more interesting series of experiments would have involved pretreatment of the yolk-sac membrane with neuraminidase and beta-galactosidase sequentially, since it seems likely that any galactose residues present on the surface may be masked by sialic acid ones.

The evidence gathered in this chapter is not indicative of any involvement of carbohydrate groups in the uptake of ^{125}I-IgG for protection or for degradation. It seems unlikely that carbohydrate groups present on the immunoglobulin molecule are involved in the process of specific recognition that leads to protection of IgG in the yolk sac, and the lack of involvement of membrane-linked terminal sialic-acid and galactose residues seems clear also. This is exactly in agreement with the work of Hillman *et al* (1977), who treated formaldehyde-fixed rabbit yolk-sac membranes with neuraminidase, beta-galactosidase and sodium periodate, and observed no modification to the amount of rabbit IgG that was able specifically to bind to the membrane. However, the present findings help to remove reservations about the possible effects of formaldehyde fixation in the experiments conducted by Hillman *et al*.

There are presently no indications of any involvement of carbohydrate groups present on the membrane or on the immunoglobulin in the process of specific recognition of IgG by yolk-sac endodermal cells.

A rather different method of investigation of the potential roles of carbohydrates in the various mechanisms of IgG uptake was the use of compounds that might compete for the site of carbohydrate interaction. Of the seven sugars and sugar phosphates used, none was found to bring about a fall in the amount of rat ^{125}I-IgG-derived radioactivity released in

TCA-insoluble form from reincubated rat yolk sacs. It seems likely, then, that none of these molecules was able effectively to compete with the immunoglobulin for binding sites on the membrane that lead to uptake.

All the evidence obtained suggests that carbohydrate is not specifically involved in the mechanism of uptake for protection of IgG in the yolk sac. It seems likely that the interaction is mediated by amino acid sequences of the IgG and receptor molecules. This is rather unusual as a mechanism for uptake, since many specific pinocytic interactions reported in the literature have been shown to involve carbohydrate. Although non-oligosaccharide interactions of substrates with specific membrane receptors are not common, they are known to operate for various hormone interactions. For example, both insulin and glucagon, being devoid of carbohydrate groups, must mediate their effects via specific interactions solely of their component amino acids. Likewise, there is plenty of evidence now available for adsorptive pinocytosis of simple proteins by rat yolk sacs (Moore et al, 1977; Livesey and Williams, 1979), suggesting that amino acid sequences alone can make up effective determinants for adsorptive uptake into yolk sac cells.

In summary, the conclusions drawn in this chapter are as follows;

(i) Periodate treatment of rat IgG only slightly damages the signal that mediates its uptake for protection in the rat yolk sac, though the mechanism of such uptake remains unclear.

(ii) Removal of any terminal sialic-acid residues from the oligosaccharide moieties of rabbit IgG increases the potential for its protection inside the yolk sac. This is possibly caused by decreased steric hindrance between the signal and receptor, and suggests that the signal part of the molecule lies spatially close to one of the F_c-linked carbohydrate groups. The literature supports this idea and nominates the hinge-linked carbohydrate.

(iii) Neuraminidase and beta-galactosidase modifications to the rabbit yolk-sac surface had no effect on the uptake of homologous ^{125}I-IgG for protection or for degradation.

(iv) At the concentrations used, simple sugars and sugar phosphates were not able to compete with ^{125}I-IgG for receptor interactions.

In short, the findings in this chapter gave no support to any ideas of specific carbohydrate involvement in the protection of IgG from lysosomal degradation *in vitro*, or, by inference, in the maternofoetal transmission of antibodies *in vivo*.

Chapter 6. The Use of Inhibitors to Try to Characterise the Mechanism of Uptake of IgG for Protection.

INTRODUCTION

At the two extremes of established hypotheses of the mechanism of transcellular transmission of IgG through yolk-sac endodermal cells lie the two mechanisms originally proposed by Brambell (1966) and by Wild (1975). The former postulates a close interrelation between the IgG-specific protective pathway and the pathway for degradation of proteins in general, while the latter claims no direct connections between the two. Many details that remain to be established concerning the mechanisms involved - for example, whether or not clathrin-coated vesicles play a part - are side issues as far as the fundamental mechanism is concerned, since such a feature could reasonably be incorporated into either model without contradiction. There are in fact only two central questions that serve to discriminate effectively between the two propositions:

(i) Does uptake of IgG into the cell occur in the same endocytic vesicles regardless of the ultimate fate of the molecule ?

(ii) Does IgG for transmission to the foetus ever enter into vesicles that contain active lysosomal enzymes ?

This chapter aims at answering the first of these questions, by making use of a variety of inhibitors of pinocytosis to attempt selectively to inhibit just one route of uptake.

If Brambell's hypothesis, which invokes the uptake of protein for transport and for degradation into the same endocytic vesicles, is correct, then every inhibitor of pinocytosis employed experimentally should cause exactly the same degree of inhibition of uptake of the IgG molecules destined for transport as of those destined for degradation. Inhibition of just one route of internalization would be extremely powerful evidence against a fundamental premise of the Brambell hypothesis. However, it would be difficult to discount the possibility that the interaction of the molecule with its receptor was the point of inhibition of uptake, rather than the process of pinocytosis itself. Inhibition of binding would seem to be more likely if the interaction between receptor and substrate was highly specific, rather than for more general uptake routes. Hence the uptake of IgG for transport might be expected to be more vulnerable to this sort of competitive inhibition than would the more general route of uptake for degradation. Hence, to

demonstrate selective inhibition of uptake into the degradative pathway whilst leaving uptake for transport unaffected would demolish Brambell's hypothesis in its original form, while the reverse would still constitute inconclusive evidence.

Since Wild (1975) proposed that uptake of IgG for degradation and for transport occurs via very different endocytic mechanisms, then, if his hypothesis is correct, it may be possible to inhibit specifically just one route of uptake. On the other hand, if both pathways respond to identical degrees to a wide variety of inhibitors of endocytosis used at various concentrations then this could be interpreted as indirect evidence against two separate internalization mechanisms. A limitation of this approach is that the only result that can be interpreted unambiguously is a sharp discrimination between the uptake of IgG for degradation and uptake for release, which result would support the hypothesis of Wild but be incompatible with that of Brambell. To categorically disprove Wild's hypothesis with these techniques would be impossible.

A wide variety of compounds have been reported to inhibit the uptake of substrates in many different endocytic tissues. Most fall into the categories of cytoskeletal inhibitors (microtubular and microfilament poisons) and metabolic inhibitors (eg.uncoupling agents), although there are a large number of additional compounds that interfere with other cellular processes.

Duncan and Lloyd (1978) examined the effects of various inhibitors on pinocytosis, as measured by the accumulation of ^{125}I-PVP, in the rat yolk sac. They used colchicine, a well-characterised microtubular poison that prevents the polymerisation of the tubulin subunits (Dustin, 1978). At low concentrations (1 ug/ml) it inhibits pinocytosis by 80% relative to controls. Increasing the concentration of colchicine to 10 ug/ml did not cause greater inhibition, and therefore they concluded that 20% of the cellular accumulation of ^{125}I-PVP was not dependent on microtubules. Starling *et al* (1983) found that colchicine (2.5 uM) or vinblastine (5 uM) were able completely to inhibit ^{125}I-PVP accumulation in the same system, but the effect was not observed until nearly three hours after the addition of the inhibitor. Colchicine was shown to enter the cell quickly, and hence it was concluded that the delay in action was

connected to the turnover time of the tubulin subunits which would not become inactivated until the microtubule was naturally broken down.

Microfilaments are thought to be involved (Sundqvist and Ehrnst, 1976) in the redistribution of occupied receptors on the cell surface prior to endocytosis ('patching'). Cytochalasin B, a specific inhibitor of microfilaments, was found (Duncan and Lloyd, 1978) to inhibit the accumulation of ^{125}I-PVP in a dose-dependent manner; 10 ug/ml gave approximately 50% inhibition relative to controls, but required about three hours preincubation with the tissue to manifest any effects. The implication is that at least 50% of yolk-sac pinocytosis is directly or indirectly dependent on microfilaments. This is in agreement with the results of von Figura and Kresse (1974) who worked on the pinocytosis of lysosomal enzymes by cultured skin fibroblasts and found that 25 ug/ml cytochalasin B inhibited uptake by 50% after about 16 h.

Duncan and Lloyd (1978) also found that metabolic inhibitors (2,4-DNP, 50 ug/ml; monoiodoacetate, 10 ug/ml; low temperature, 15°C or EGTA, 5mM) were able to inhibit pinocytosis completely, in agreement with Bowers (1977) and Munthe-Kaas (1977). They further demonstrated that the EGTA effects were reversed by the addition of 5mM calcium chloride, suggesting that the inhibition was caused by a lack of Ca^{++} rather than some other toxic effect of EGTA, and found that Mg^{++} was unable to restore pinocytic activity.

Clustering of receptors and capping are now generally assumed (Pastan et al, 1981) to be a prerequisite for many types of receptor-mediated endocytosis. There is evidence that cross-linking of surface components is also involved in these processes, and transglutaminase has been proposed as an essential enzyme for internalisation to commence. Levitski et al (1980) report that capping for internalisation of alpha$_2$-macroglobulin in fibroblasts is inhibited by a wide range of transglutaminase-directed compounds, including dansylcadaverine and N-benzyloxy-carbonyl-5-diazo-4-oxonarvaline para-nitrophenyl ester, and at the same concentrations that are expected to cause inhibition of the enzyme. These findings are supported by Davies et al (1980, working on fibroblasts, and Haigler et al (1980), working on BALB 3T3 cells. In the absence of conflicting evidence it is now generally accepted that alpha-transglutaminase-mediated crosslinking of receptors is a necessary preliminary to specific receptor-mediated

endocytosis, at least in these cell types.

The effects of microtubular inhibitors (colchicine, vinblastine, nocodazole), microfilamentous inhibitors (cytochalasin B, dihydrocytochalasin B), metabolic inhibitors (2,4-DNP, rotenone, low temperature), inhibitors of transglutaminase (dansylcadaverine) and calcium chelating agents (EGTA) on the specific uptake of ^{125}I-IgG for degradation and for intact release were investigated in this study to establish whether the two types of uptake could be individually inhibited or if they are inseparable under these conditions.

MATERIALS AND METHODS

6.2.

Yolk sacs were incubated as described in Section 2.2, with a few modifications as outlined below.

Inhibitors were used at the various concentrations indicated in Section 6.3, and were added either dissolved in medium 199 or, if solubility was low, first dissolved in DMSO. In such cases the medium in the corresponding control experiments always contained the same amount of DMSO as the test incubations. Three basic regimes were used. Sometimes the yolk-sac tissue was preincubated in the presence of the inhibitor before the substrate was added, the preincubation period being varied between the different inhibitors, as indicated in the text. If the inhibitor was only to be present during the release phase, then it was only added to the reincubation medium, and not to the initial uptake medium. In the third type of experiment, the yolk sac was preincubated in the presence of a radiolabelled substrate before the inhibitor was added to the same flask. In these cases the inhibitor was added in a small volume of medium 199 (sometimes containing DMSO), the flask regassed with 95% O_2:5%CO_2 and the incubation continued. By allowing the yolk sacs to reach a steady-state in the presence of substrate before the effective application of the inhibitor, and by examining the new steady-state reached after the application of inhibitor, it was hoped that information could be gained concerning the susceptibilities to the inhibitor of the mechanisms of release of radioactivity from the cell, as well as the mechanisms of uptake.

When samples of medium containing inhibitor were removed from the reincubation flask, they were replaced with the same volume of fresh medium containing the same inhibitor at the appropriate concentration and temperature.

For low temperature incubations, all dissections were carried out in warm, gassed medium 199 as described in Section 2.2. Meanwhile, flasks containing medium 199 were equilibrated in shaking water baths at the appropriate temperatures before the yolk sacs were added. Working at different incubation temperatures with tissues from the same animal made it impossible always to dissect at the required temperature, and random assortment of dissected tissue was regarded as being more important than

the temperature of dissection.

Patterns of release of radioactive material from reincubated tissues can be usefully expressed either as absolute weights of substrate-equivalent released, or as percentages of the total tissue-associated radioactivity released in the two forms. The latter format has the advantage of including in the graph information concerning the percentage of the material that remains associated with the yolk sac at the end of the reincubation, while the former gives an indication of the total amount of material released from the tissue. The underlying assumption of this type of experiment is that provided there is no unusual tissue-accumulation of substrate, the amount of radioactivity released in TCA-soluble and TCA-insoluble form from reincubated yolk sacs reflects the amount of material taken into the degradative and protective, respectively. In this particular chapter, the experiments reported concern various degrees of inhibition of the mechanisms of uptake by applied inhibitors, and the overall endocytic activity of the yolk sac is of interest, as well as the distribution of the total substrate between the two pathways. For that reason, most of the release graphs are expressed in absolute weight-equivalents of substrate, and any unusual tissue accumulations of radioactivity are carefully monitored and mentioned in the text.

Rat and rabbit tissues were both studied, where possible, and the species of experimental animal used is indicated in the text.

RESULTS

6.3.1 Metabolic inhibitors.

(i) The effects of 2,4-dinitrophenol (DNP).

a The rat system.

Preincubation for 60 min in medium containing DNP (40ug/ml) caused the tissue level of radioactivity after subsequent exposure to ^{125}I-IgG (130 min, 5ug/ml) to fall by approximately 75% relative to controls. The release patterns (Figure 6.3.1) of radioactivity during reincubation show similar reductions (80% approx.) in the amounts of both TCA-soluble and TCA-insoluble material released into the medium over 2.5h.

When DNP (40ug/ml) was present only in the reincubation medium, no modification of the release pattern or of the level of radioactivity remaining in the tissue after 3h reincubation was observed (Figure 6.3.2).

When DNP (40 ug/ml) was added to the uptake medium after the yolk sac had been exposed to ^{125}I-IgG for a few minutes, and incubation continued for at least a further hour, a modification of the release pattern was observed. Invariably the level of TCA-soluble radioactivity released was substantially decreased compared with controls, but the amount of TCA-insoluble material released was decreased only slightly, if at all, compared with controls (Figure 6.3.3).

The inhibitory effect exerted by DNP on the release from tissue of radioactivity was shown to be substantially reversible (Figure 6.3.5). Yolk sac sacs that had been exposed first to ^{125}I-IgG for 10min and then to DNP (40ug/ml) released a greater percentage of their contained radioactivity on subsequent reincubation if there was no DNP present in the reincubation medium. Yolk sacs reincubated in the continuous presence of DNP released 38$\pm$6% of their total contained radioactivity over a reincubation period of 2.5h, while those reincubated in the absence of DNP released 74$\pm$3% of their total contained radioactivity during the same period, a figure comparable with control incubations.

b **The rabbit system.**

Preincubation of rabbit yolk sacs in medium containing 40ug/ml DNP for 80 min before exposure of the tissue to radiolabelled homologous IgG caused the amount of material released from the tissue on subsequent reincubation to be decreased by about 40%. No significant discrimination was observed between material released in TCA-insoluble and TCA-soluble forms, both forms being decreased by approximately 40% (Figure 6.3.4(b)).

When DNP (40ug/ml) was added to incubation media already containing radiolabelled IgG for the final 80 min of the uptake phase (Figure 6.3.4 (c)), the release pattern of radioactivity on subsequent reincubation was substantially modified. Release of TCA-soluble material was decresed by about 7 75%, while TCA-insoluble material only fell by about 35%. This effect is similar to, though less pronounced than, the effect of DNP in the rat system (Figure 6.3.3(b)).

(ii) The effects of rotenone.

a. **The rat system.**

When 10^{-5}M rotenone was present in the reincubation medium, no modification of the release pattern for ^{125}I-IgG-derived radioactivity was observed (Figure 6.3.6).

The addition of rotenone to the incubation medium after the yolk sac had been exposed to ^{125}I-IgG was seen to modify the release pattern (Figure 6.3.7) in a manner similar to DNP (Fig 6.3.4). In such experiments, release of TCA-insoluble material was largely unchanged, but the release of TCA-soluble material was decreased as a function of the time of exposure to rotenone. Rotenone achieved its maximum effect after about 90 min.

b. **The rabbit system.**

Preincubation of rabbit yolk sacs in medium containing rotenone (10^{-5}M, 80 min) before the addition of 5ug/ml ^{125}I-IgG was shown to modify the pattern of subsequent release of radioactivity from the tissue, but without discriminating between the two types (TCA-soluble and TCA-insoluble) of material released. Both types of release were decreased

(Figure 6.3.8) by about 77%.

The addition of rotenone (10^{-5}M) to the incubation medium after the yolk sac had been exposed to ^{125}I-IgG for 10min, and subsequent incubation of the yolk sac in the presence of both substrate and inhibitor for a further 80 min prior to reincubation, gave, relative to controls, a substantially modified pattern for the release of radioactivity from the tissue (Figure 6.3.8(c)). The quantity of TCA-soluble material released was low - approximately the same as that released by yolk sacs preincubated for 60 min in rotenone (Figure 6.3.8(b)) - but the quantity of TCA-insoluble radioactivity released from the tissue was only decreased by about 30%.

6.3.2 Inhibitors of microtubules.

(i) The effect of colchicine.

a. The rat system.

Rat yolk sacs were first exposed to ^{125}I-IgG (5ug/ml, 30 min) before the addition of colchicine (1uM final concentration, in medium). After a further 2h of incubation in this manner, the yolk sacs were transferred to fresh medium for reincubation in the usual way, and the release pattern of radioactivity was observed (Figure 6.3.9(b)). The release of both TCA-soluble and TCA-insoluble radioactivity was found to be decreased by approx.60%, though the evidence suggests slightly greater inhibition of TCA-solubles production.

b. The rabbit system.

Preincubation (150 min) of rabbit yolk sacs in medium containing colchicine at various concentrations before the addition of ^{125}I-IgG (for a further 2h) was shown to modify the subsequent release pattern in a dose-dependent manner (Figure 6.3.10). In every case, however, the release of TCA-soluble and TCA-insoluble material was inhibited to equal extents. No discrimination in inhibition was observed between the degradative pathway and the pathway giving rise to the release of macromolecules from the tissue. Colchicine had almost as pronounced an effect at 1uM as at 2uM, and the former concentration was held to be the optimum for this inhibitor in subsequent investigations.

The addition of colchicine (1uM) to incubation medium for the final 2h of the preincubation (Figure 6.3.11(c)) gave rise to a modified pattern for the release of radioactivity from the reincubated tissue. The release of TCA-insoluble material was reduced by about 85%, and the release of TCA-soluble radioactivity exhibited a high degree of variability, with a mean fall of 45% approx., when compared with controls (Figure 6.3.11(a)).

(ii) The effect of vinblastine.

a. The rat system.

The effects of the inclusion of vinblastine (10uM) in the reincubation medium only (Figure 6.3.12 (b)) indicate that it did not significantly modify the pattern of release of ^{125}I-gamma globulin-derived radioactivity from rat yolk sacs relative to matched controls (Figure 6.3.12a).

Preincubation of rat yolk sacs in medium containing vinblastine (10uM, 1h) before the addition of ^{125}I-gamma globulins, however, caused substantial reductions in the release of both TCA-soluble and TCA-insoluble radioactivity on subsequent reincubation (Figure 6.3.12 (c)). The presence or absence of vinblastine (10uM), in the reincubation medium was without effect on these results.

b. The rabbit system.

(No data available).

(iii) The effects of nocodazole.

a. The rat system.

(No data available).

b. The rabbit system.

Preincubation (150 min) of rabbit yolk sacs in medium containing various concentrations of nocodazole before exposure to ^{125}I-IgG (5ug/ml, 60min) caused inhibition of release in a dose-dependent manner (Figure 6.3.13) when the tissue was subsequently reincubated. The evidence

suggests a slightly greater fall in the release of TCA-insoluble material (decreased by approx. 60% at 1uM nocodazole) than degraded material (decreased by approx.20% at 1uM nocodazole).

If nocodazole (1uM) is added to the preincubation medium for the final 2h of uptake of ^{125}I-IgG, the subsequent release of radioactivity is decreased by about 50% in a non-specific manner. There is no evidence for selective inhibition of just one type of release (Figure 6.3.11 (d)).

6.3.3 Inhibitors of microfilaments.

(i) The effect of dihydrocytochalasin B.

a. The rat system.

Pre-incubation of rat yolk sacs in medium containing dihydrocytochalasin B (5ug/ml, 1h) before the addition of ^{125}I-IgG (5ug/ml) caused a non-selective inhibition of the release observed on subsequent reincubation (Figure 6.3.14). Release of both TCA-soluble and TCA-insoluble material was decreased by about 50%.

The addition of 10ug/ml dihydrocytochalasin B to the initial incubation medium for the final 2h of uptake, was found to decrease the release of radioactivity from the reincubated tissue by about 70% in a non-selective manner (Figure 6.3.9 (c)).

b. The rabbit system.

Preincubation of rabbit yolk sacs in the presence of dihydrocytochalasin B (10ug/ml, 80 min) before the addition of ^{125}I-IgG was found to non-selectively decrease the release of radioactivity from the reincubated tissue by about 20% (Figure 6.3.15).

When dihydrocytochalasin B (10ug/ml) was added to the initial incubation medium for the final period of exposure to ^{125}I-IgG, no significant modification of the subsequent release pattern was observed (Figs 6.3.11 (b) and 6.3.16 (c)).

(ii) The effects of cytochalasin B.

a. The rat system.

When cytochalasin B (10ug/ml) was added to the initial incubation medium for the final 80min of the exposure of the yolk sacs to ^{125}I-IgG, no modification of the subsequent release pattern was noted (Figure 6.3.16 (b)).

b. **The rabbit system.**

(No data available).

6.3.4 **The effects of dansylcadaverine.**

a. The rat system.

(No data available).

b. **The rabbit system.**

Preincubation of rabbit yolk sacs in the presence of dansylcadaverine for 1h before the addition of ^{125}I-IgG, decreased the subsequent release of radioactivity from the reincubated tissue in a dose-dependent manner (Figure 6.3.17).

The release of both TCA-soluble and TCA-insoluble radioactivity was decreased by approximately the same percentage, and no significant discrimination between the two types of release was observed.

6.3.5 **The effects of EGTA.**

a. The rat system.

Preincubation of rat yolk sacs in medium containing EGTA (30 min, 0.5mM) did not significantly modify the rates of uptake of ^{125}I-PVP or of ^{125}I-gamma globulins (Figure 6.3.18). However, preincubation for 30 min in the presence of 5mM EGTA caused a fall of 86% in the rate of pinocytosis, as measured by the accumulation of ^{125}I-PVP. This was

exactly paralleled by a drop of 86% in the observed Endocytic Index for homologous ^{125}I-gamma globulins (Figure 6.3.19). Under the same conditions the Endocytic Index for native ^{125}I-BSA was found to be too low to measure, reflecting an apparent fall due to EGTA of nearly 100%.

The inclusion of EGTA (5mM) in the reincubation medium only, caused no significant modifications to the observed pattern of release of radioactivity derived from ^{125}I-gamma globulins (Figure 6.3.20 (b)).

Preincubation in medium containing 5mM EGTA caused a large fall in the release of both TCA-soluble and TCA-insoluble radioactivity from the charged and reincubated tissue. No significant selective effect on either class of released radioactivity was noted (6.3.20 (c)).

b. **The rabbit system.**

Preincubation of rabbit yolk-sac tissue in EGTA (5mM, 30 min) prior to exposure to ^{125}I-PVP effectively abolished pinocytosis within the limits of experimental detection (Figure 6.3.21).

When yolk sacs were treated with EGTA (5mM, 30 min) before being exposed to ^{125}I-IgG, the radioactivity released from the reincubated tissue was decreased by about 90%, but there was no evidence of any selective effect on the release of either TCA-soluble or TCA-insoluble material (Figure 6.3.22).

6.3.6 **The effects of lower temperatures.**

a. **The rat system.**

When rat yolk sacs that had been exposed to homologous ^{125}I-IgG at 37°C were reincubated in fresh medium 199 at lower temperatures, the pattern of release of radioactivity from the tissue was modified. When release at 20°C was monitored (Figure 6.3.23b), the release from the tissue of TCA-insoluble radioactivity was not significantly different from controls (Figure 6.3.23 (a)). However, production of TCA-soluble material was substantially decreased. When the tissue was reincubated at 10°C a greater reduction in the release of both sorts of radioactivity was observed, but the production of TCA-soluble material was invariably slightly more inhibited than was the release of TCA-insoluble material

(Figure 6.3.23c).

When rat yolk sacs were preincubated at temperatures less than 37°C before the addition of ^{125}I-IgG, the uptake of the substrate and subsequent release pattern of radioactivity from the reincubated tissue (at the same low temperature) were substantially modified (Figure 6.3.24). At an incubation temperature of 20°C, the total quantity of radioactivity associated with the tissue at the end of the initial incubation was only 18% of the tissue level for the 37°C control. At an incubation temperature of 10°C, only 2% of the control level of radioactivity became associated with the tissue. Reincubation of such tissue showed approximately equal reductions in the release of TCA-soluble and TCA-insoluble radioactivity in each case, though slightly more TCA-insoluble radioactivity was invariably released.

b. **The rabbit system.**

When rabbit yolk sacs that had been exposed to ^{125}I-IgG at 37°C were reincubated at lower temperatures, interesting modifications to the release patterns were observed (Figure 6.3.25). At a reincubation temperature of 20°C the production of TCA-soluble material was decreased by about 85%; at 10°C by 95%. Surprisingly, the release of TCA-insoluble material was only decreased by 19% and 28% respectively.

When rabbit yolk sacs were preincubated at temperatures below 37°C in medium 199, before being exposed to homologous ^{125}I-IgG, exactly the same effects were observed as in the rat tissue. The total amount of radioactivity associated with the tissue at the end of the initial incubation period was decreased as a function of incubation temperature, and the pattern of release of radioactivity from the reincubated tissue showed approximately equal falls for both types of radioactivity, though slightly more TCA-insoluble material was invariably released (Figure 6.3.26).

The nature of the radioactivity still associated with the yolk sac at the end of the reincubation period in such experiments was also investigated (Figure 6.3.27). The differences between total amounts of residual radioactivity were always accounted for by TCA-insoluble material. The quantity of TCA-soluble radioactivity left in the yolk sac seemed to be essentially independent of reincubation temperature.

Attempts were made to reverse the temperature-induced inhibition of release of radioactivity from reincubated tissue (Figure 6.3.28). The evidence suggests that the inhibition is completely reversible by raising the temperature to 37°C. No difference was noted between the types and amounts of radioactivity finally released from yolk sacs that were initially reincubated at 10°C for 2h (before the temperature of the reincubation medium was raised to 37°C), compared with control yolk sacs that were reincubated at 37°C throughout.

In the following RESULTS section, as throughout the rest of this Thesis, the following key to symbols is observed, unless indicated otherwise in the legends.

TCA - soluble radioactivity is represented by open symbols - mainly squares or circles.

TCA - insoluble radioactivity is represented by closed symbols - mainly squares or circles.

± Error bars represent ± Standard Deviations.

Figure 6.3.1 The effects on the release pattern of ^{125}I-IgG from rat yolk sacs previously exposed to DNP.

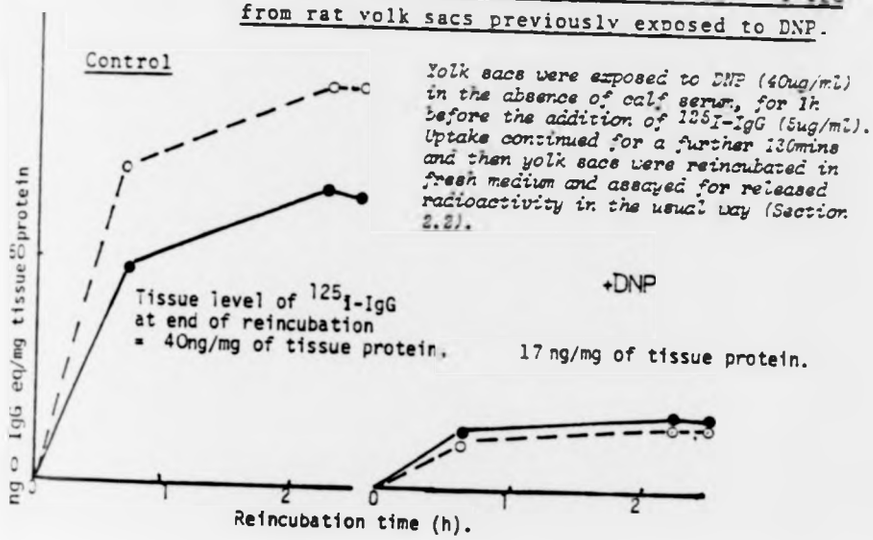


Figure 6.3.2 The effects of DNP in the release medium on the release pattern from rat yolk sacs of gamma-globulin-derived radioactivity.

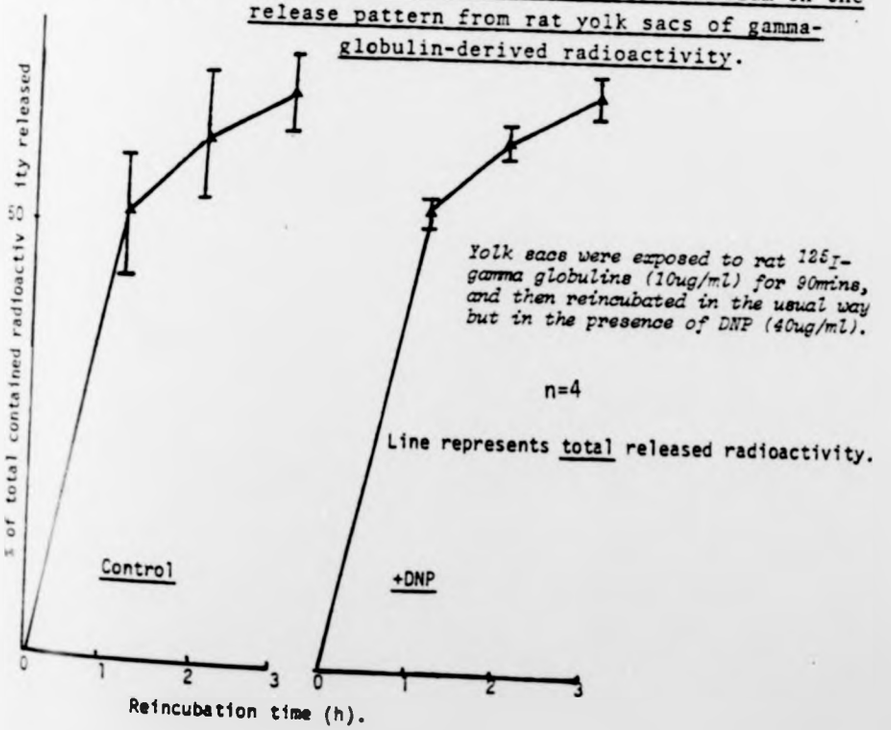


Figure 6.3.3 The effects of DNP, added after substrate, on the release from rat yolk sacs of radioactivity derived from ^{125}I-gamma globulins.

Rat yolk sacs were incubated for 10min in medium 199 in the presence of ^{125}I-gamma globulins (10ug/ml) before the addition of DNP (40ug/ml). Incubation continued for a further 130min before the yolk sacs were reincubated in the absence of DNP, and release of radioactivity monitored in the usual way. Controls encountered no DNP at all.

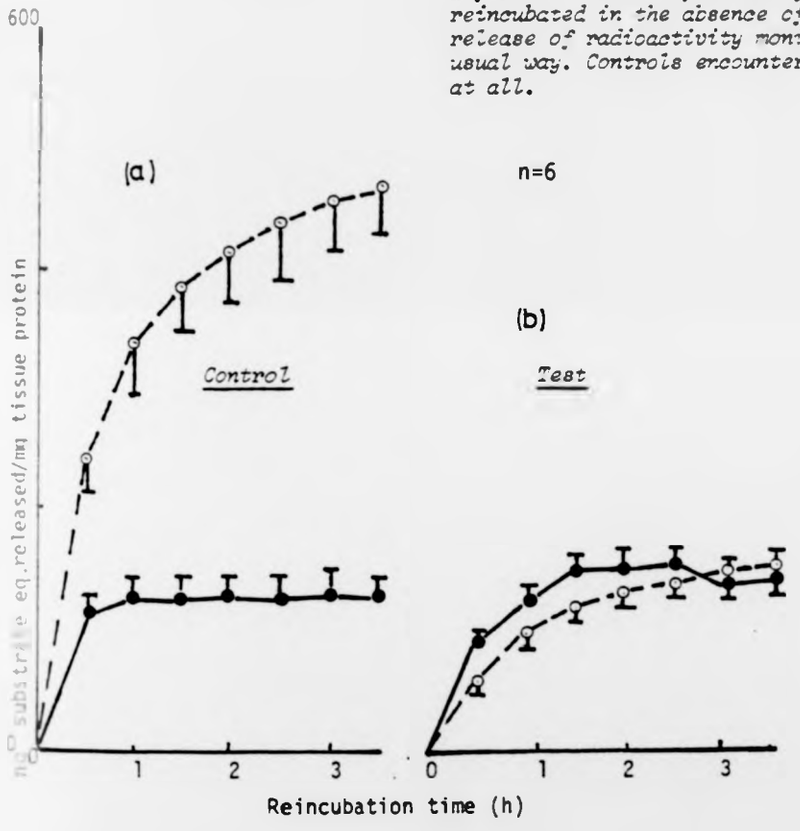


Figure 6.3.4 The effects on the release pattern of ^{125}I-IgG-derived radioactivity from rabbit yolk sacs of (b) preincubation in DNP and (c) the addition of DNP to the uptake medium for the final 80min.

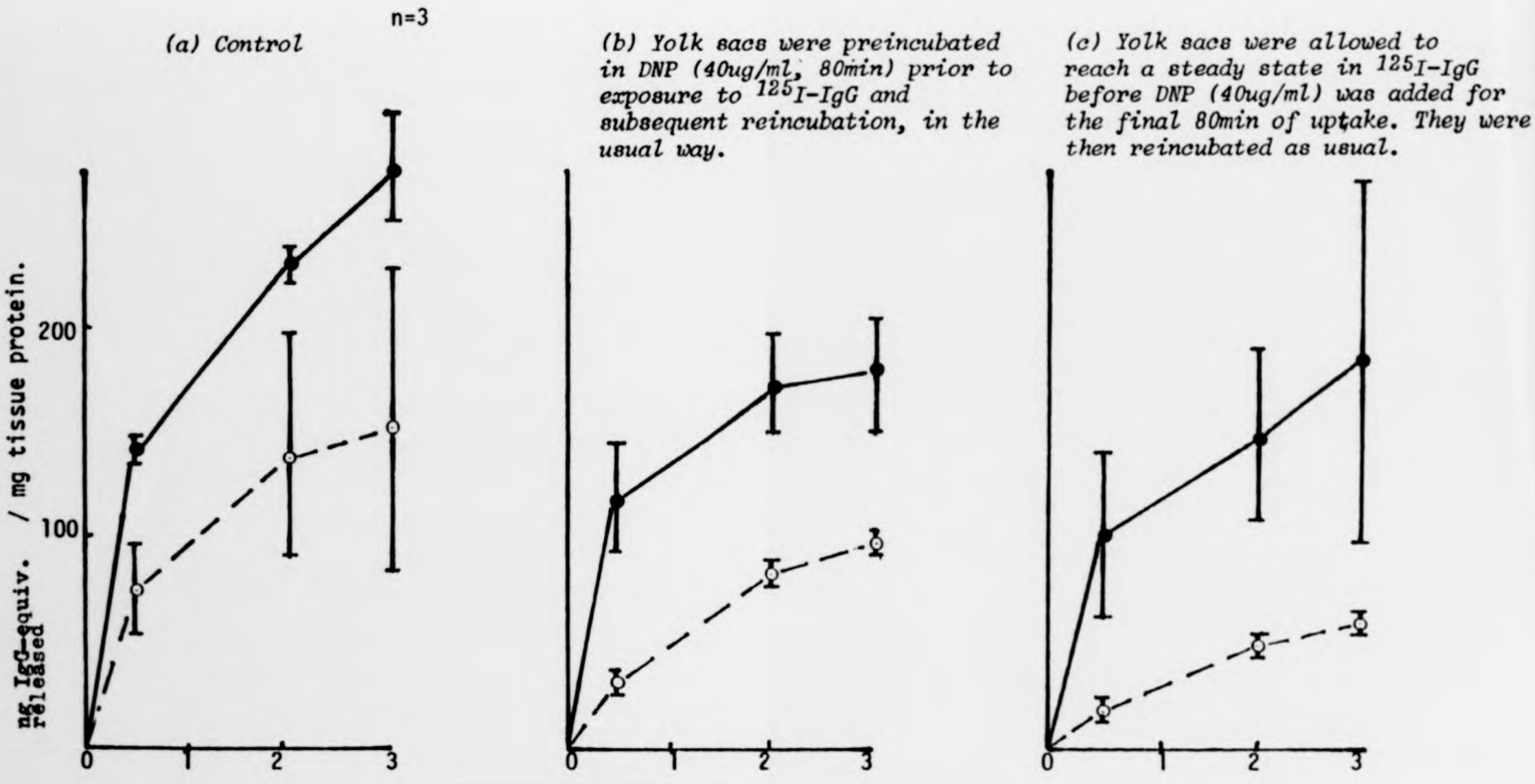
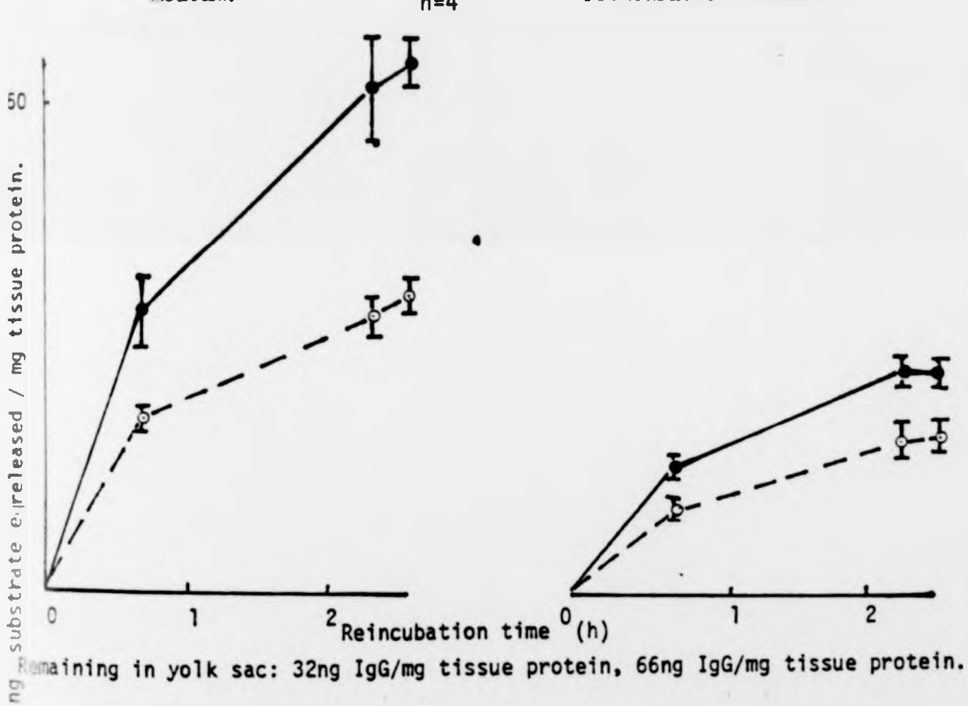


Figure 6.3.5 The reversibility of DNP-induced inhibition of ^{125}I-IgG release from rat yolk sacs.

DNP (40ug/ml) was added to the uptake medium for the final 80mins of uptake. In the subsequent reincubation, control yolk sacs were reincubated in DNP-containing medium, test ones in DNP-free medium.

Test: No DNP in reincubation medium. *Control: 40ug/ml DNP retained in the reincubation medium.*



Remaining in yolk sac: 32ng IgG/mg tissue protein, 66ng IgG/mg tissue protein.

Figure 6.3.6 The effect of rotenone in the release medium on the release from rat yolk sacs of ^{125}I-IgG-derived radioactivity.

Yolk sacs were preincubated in ^{125}I-IgG (5ug/ml, 90min.) and then reincubated in the presence of rotenone (10^{-5}M). The release of radioactivity was assayed in the usual way.

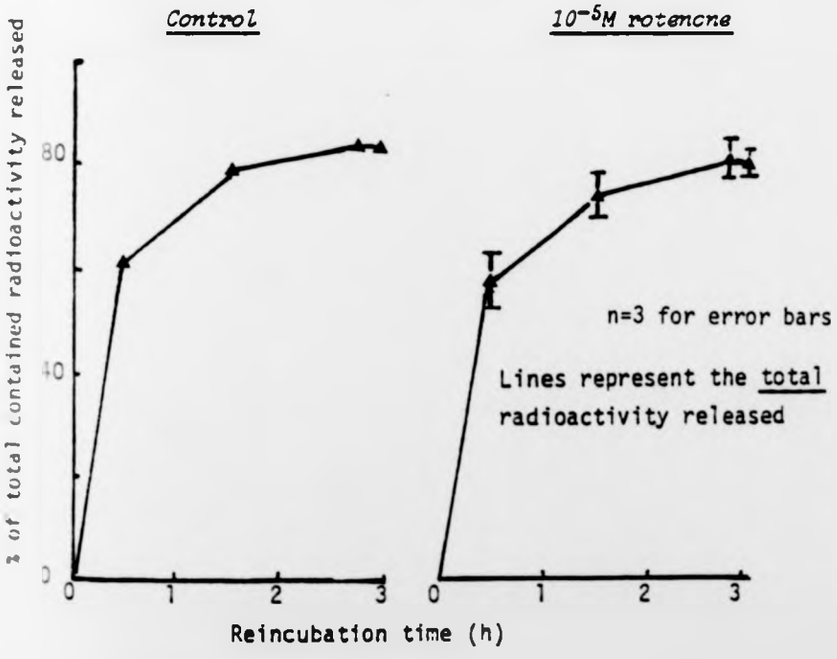


Figure 6.3.7 The effect of the addition of rotenone to the uptake medium for the final period of uptake on the subsequent pattern of release of ^{125}I-IgG-derived radioactivity.

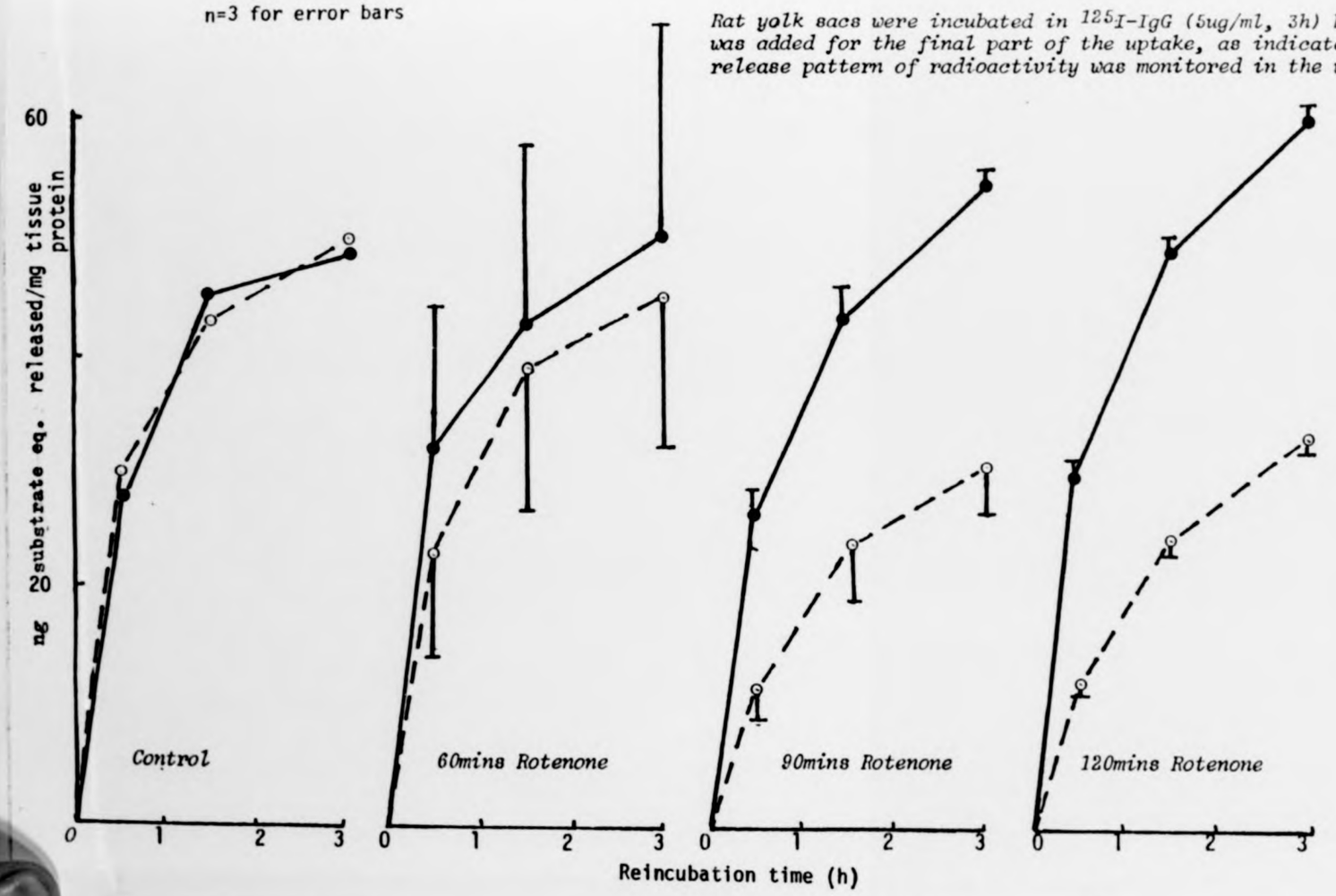


Figure 6.3.8 The effects of rotenone on the release from rabbit yolk sacs of ^{125}I-IgG-derived radioactivity

(a) Control Yolk sacs were exposed to ^{125}I-IgG prior to reincubation in the usual way. Exposure to ^{125}I-IgG was for 90mins.

(b) Yolk sacs were preincubated in rotenone (10^{-5}M) prior to the addition of ^{125}I-IgG.

(c) Rotenone (10^{-5}M) was added to the uptake medium for the final 80 min of uptake.

n=3 for error bars

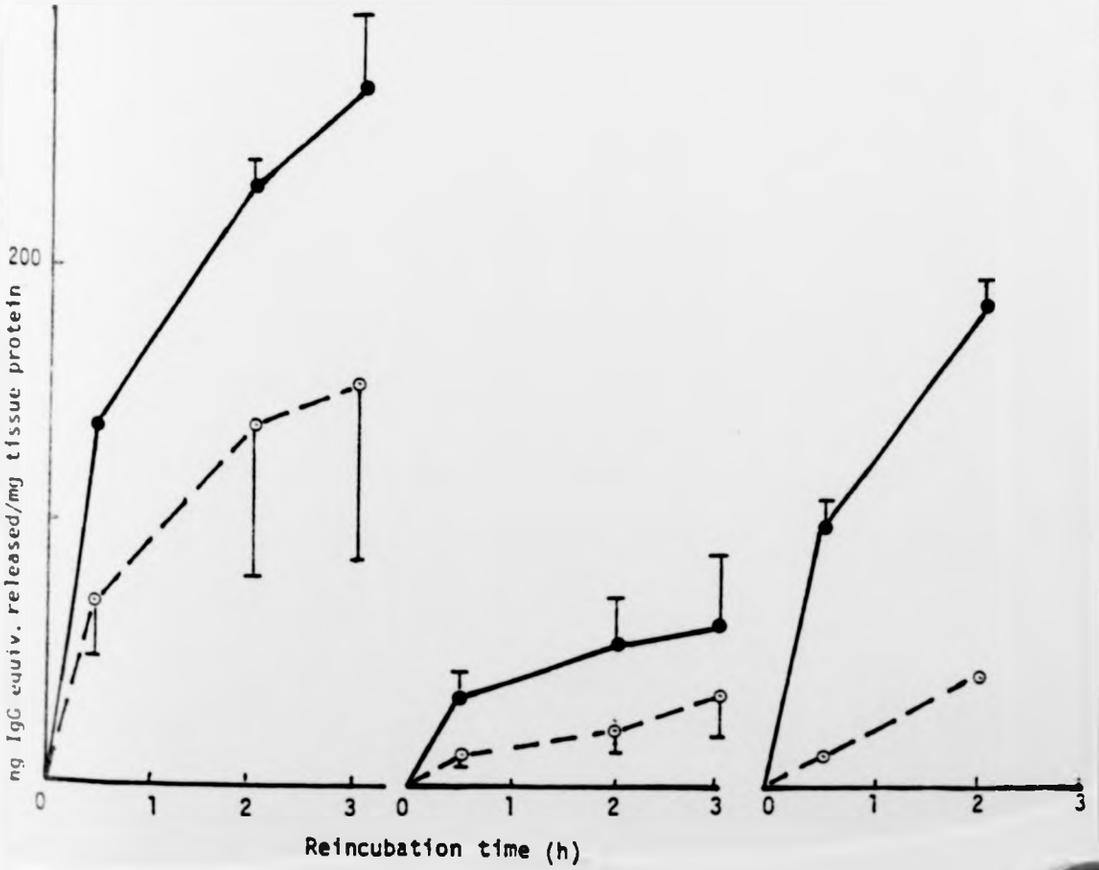


Figure 6.3.8 The effects of rotenone on the release from rabbit yolk sacs of ^{125}I-IgG-derived radioactivity

(a) Control Yolk sacs were exposed to ^{125}I-IgG prior to reincubation in the usual way. Exposure to ^{125}I-IgG was for 90mins.

(b) Yolk sacs were preincubated in rotenone (10^{-5}M) prior to the addition of ^{125}I-IgG.

(c) Rotenone (10^{-5}M) was added to the uptake medium for the final 80 min of uptake.

n=3 for error bars

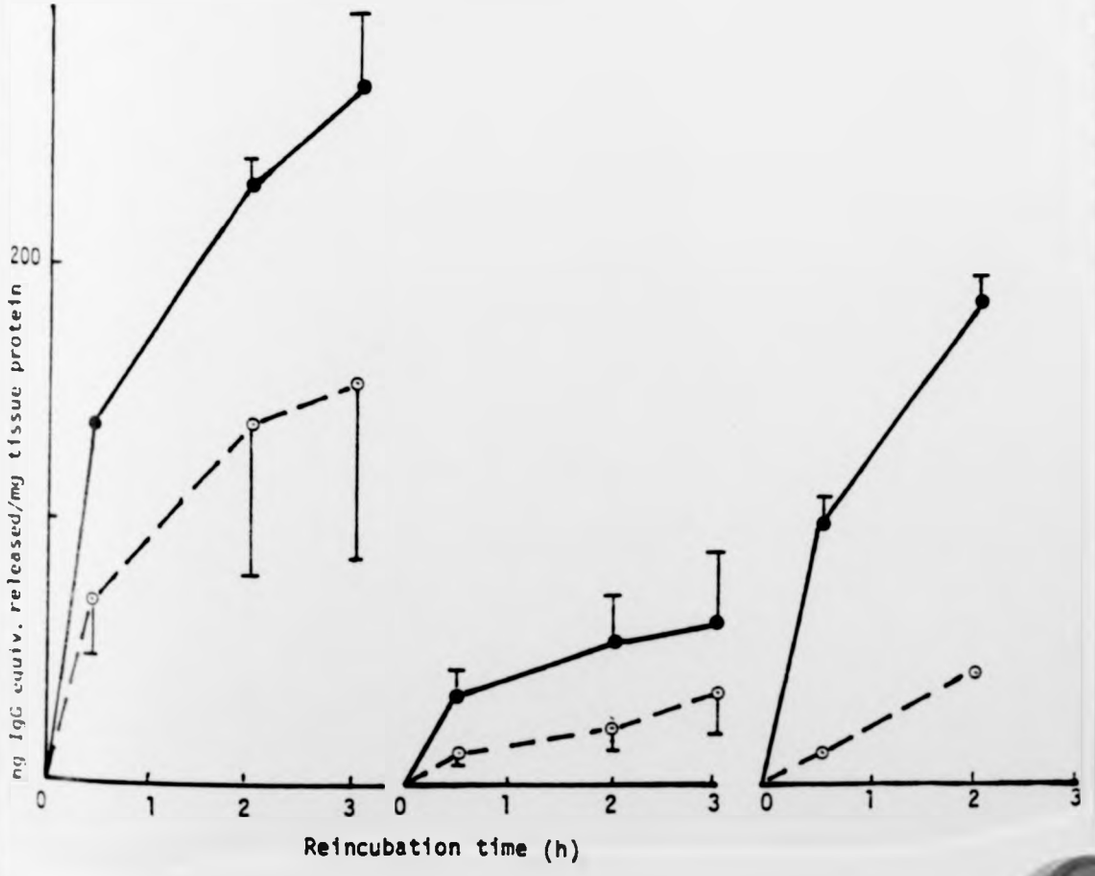


Figure 6.3.9 The effects of colchicine and dihydrocytochalasin B on the release from rat yolk sacs of ^{125}I-IgG-derived radioactivity.

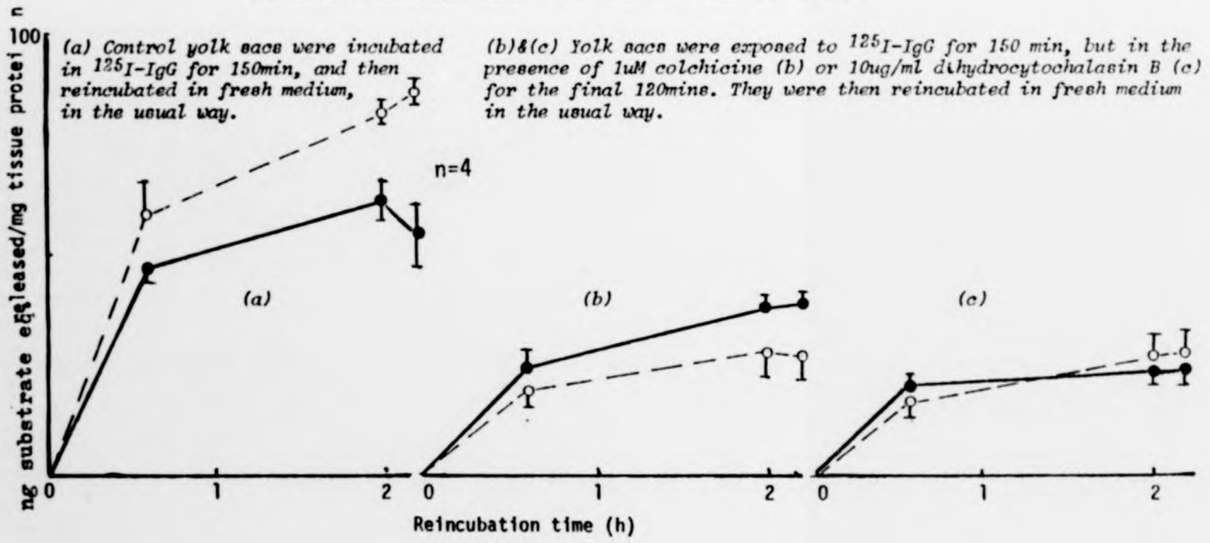


Figure 6.3.10 The Effects of Colchicine on the Pattern of Release of Rabbit IgG from Rabbit Yolk Sacs.

Rabbit yolk sacs were preincubated in colchicine at various concentrations before the addition to the incubation medium of ^{125}I-IgG. Uptake was for 2h, and the subsequent radioactivity release pattern was monitored in the usual way.

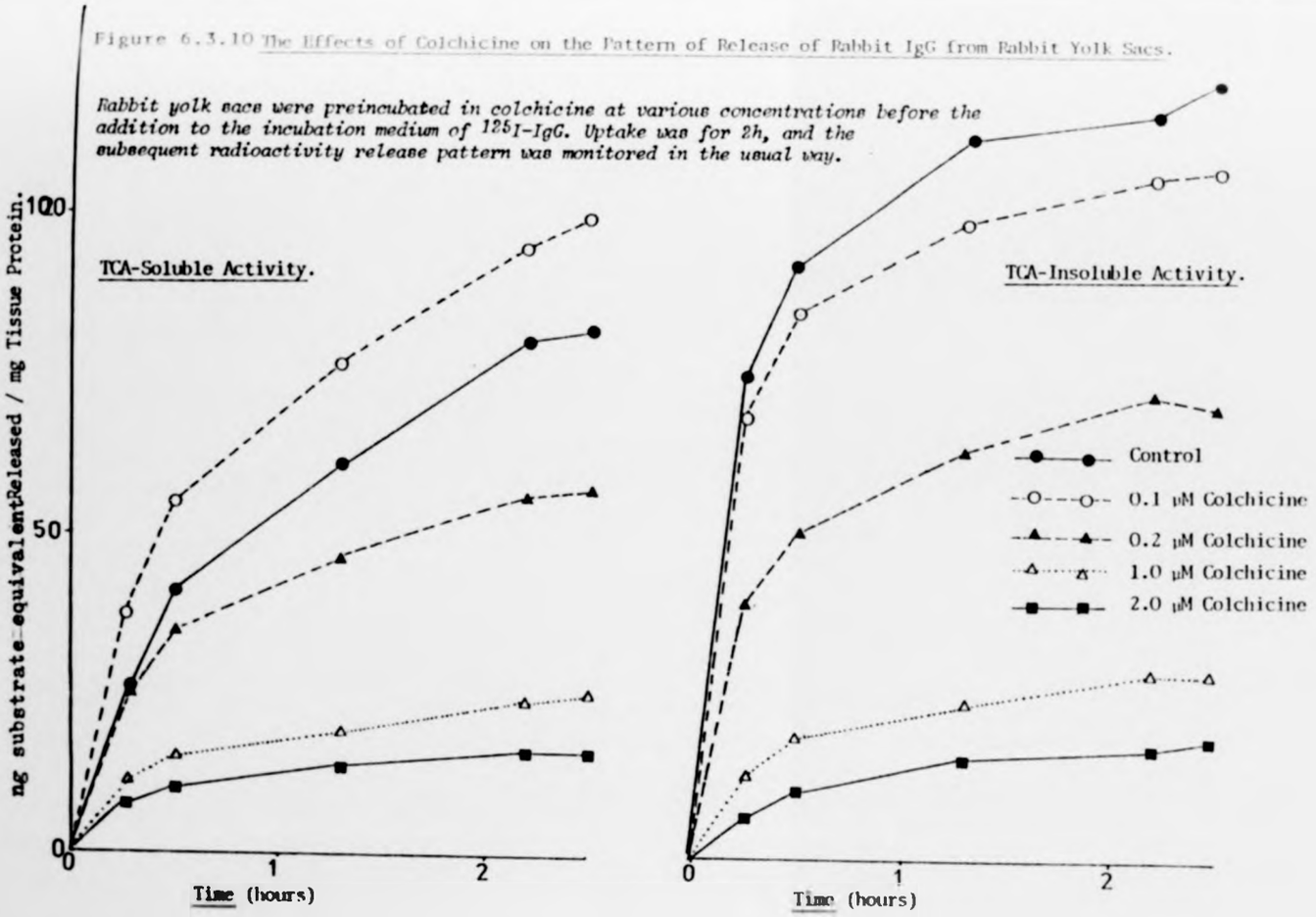


Figure 6.3.10 The Effects of Colchicine on the Pattern of Release of Rabbit IgG from Rabbit Yolk Sacs.

Rabbit yolk sacs were preincubated in colchicine at various concentrations before the addition to the incubation medium of ^{125}I-IgG. Uptake was for 2h, and the subsequent radioactivity release pattern was monitored in the usual way.

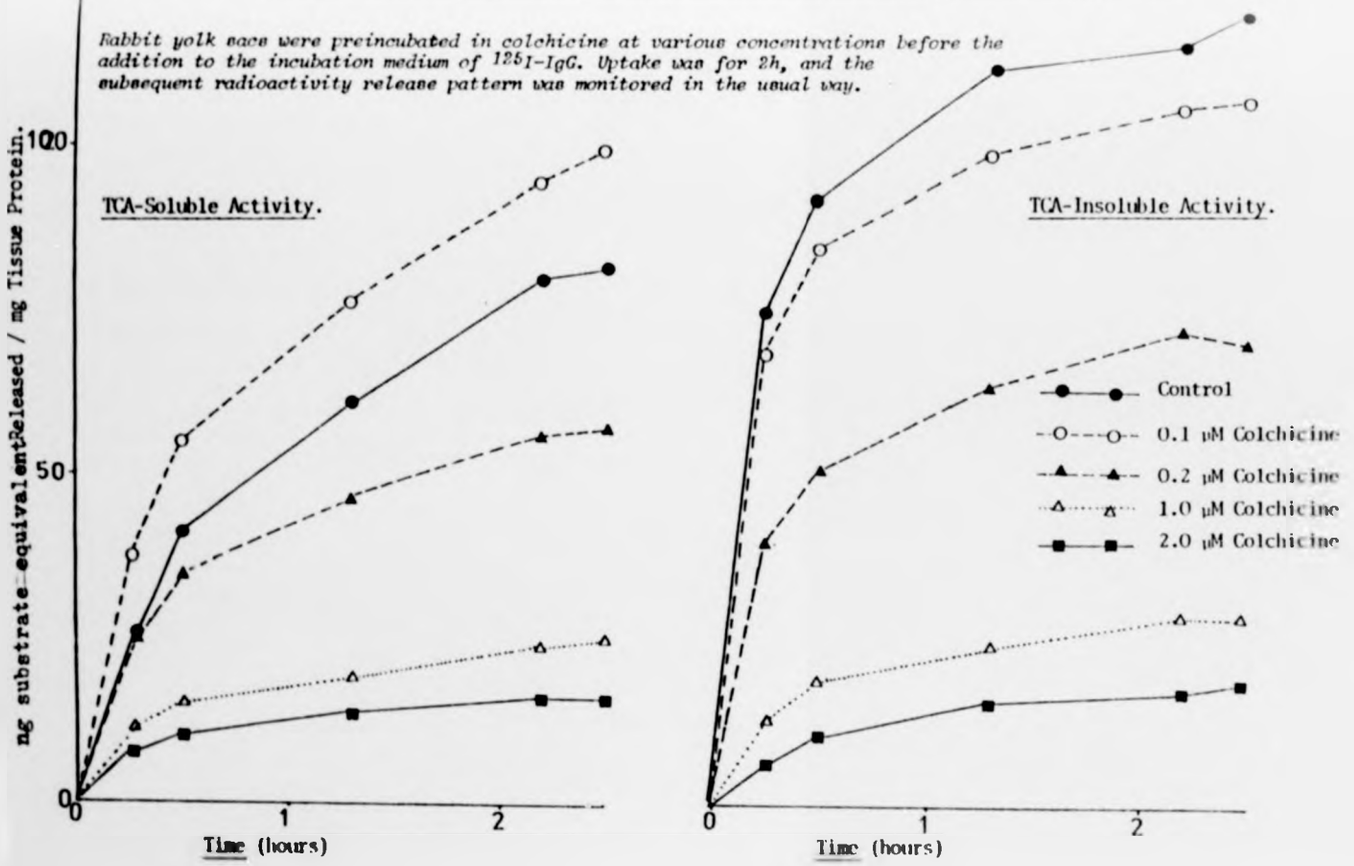
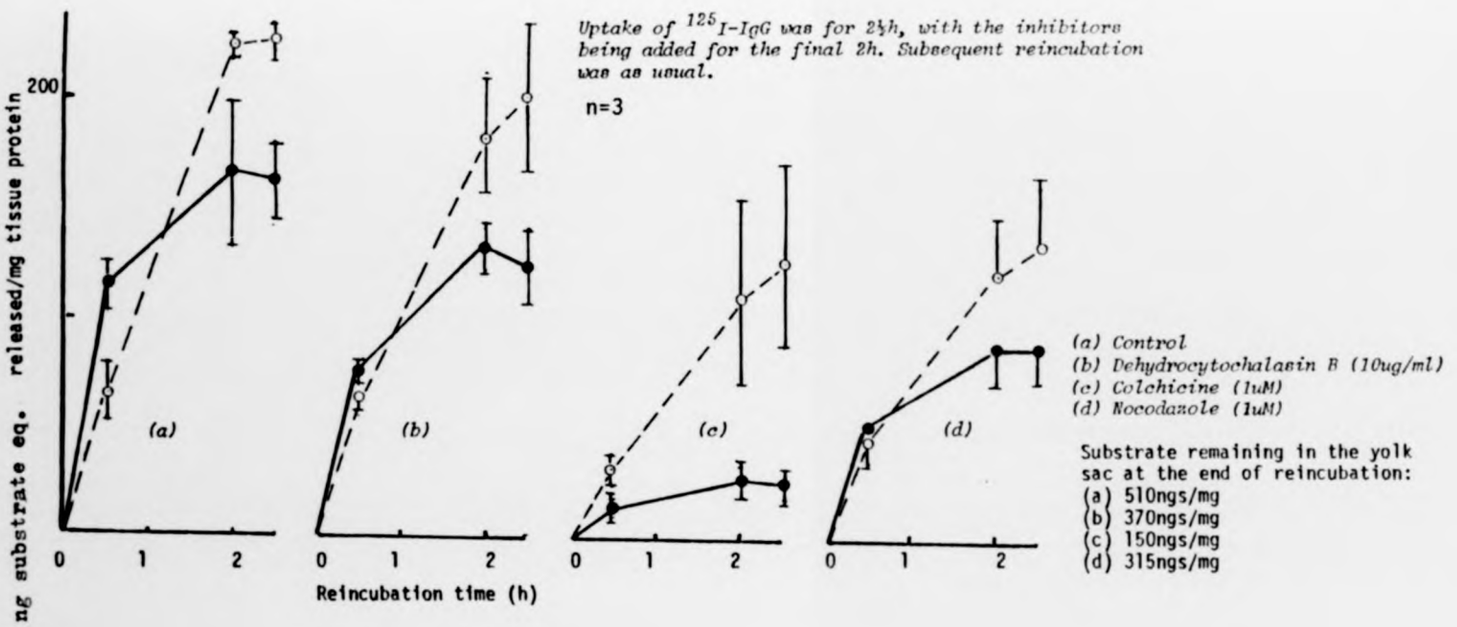


Figure 6.3.11 The effects of various inhibitors on the release from rabbit yolk sacs of radioactivity derived from ^{125}I-IgG.



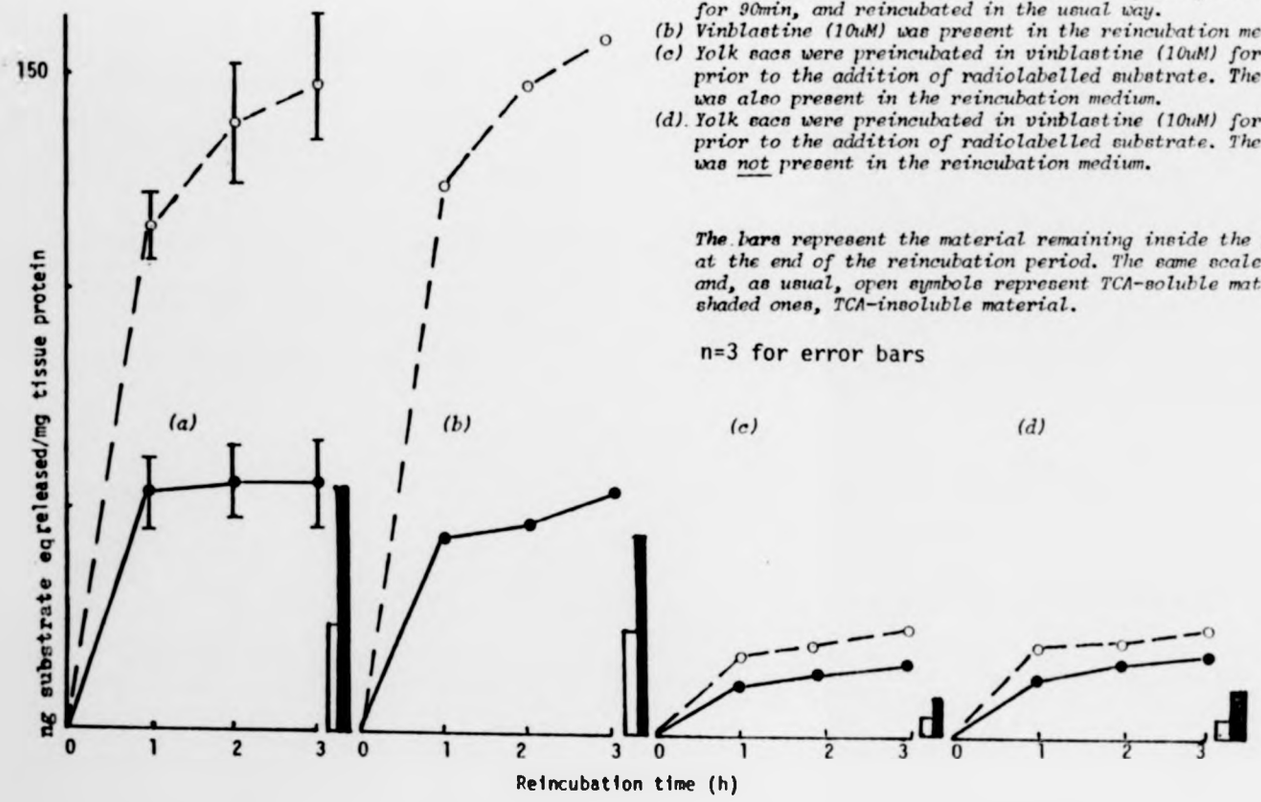
Uptake of ^{125}I-IgG was for 2½h, with the inhibitors being added for the final 2h. Subsequent reincubation was as usual.

n=3

(a) Control
(b) Dehydrocytochalasin B (10ug/ml)
(c) Colchicine (1uM)
(d) Nocodazole (1uM)

Substrate remaining in the yolk sac at the end of reincubation:
(a) 510ngs/mg
(b) 370ngs/mg
(c) 150ngs/mg
(d) 315ngs/mg

Figure 6.3.12 The effects of vinblastine on the release pattern from rat yolk sacs of radioactivity derived from ^{125}I-gamma globulins.



(a) Control yolk sacs were incubated in ^{125}I-gamma globulins for 90min, and reincubated in the usual way.
(b) Vinblastine (10uM) was present in the reincubation medium.
(c) Yolk sacs were preincubated in vinblastine (10uM) for 60min, prior to the addition of radiolabelled substrate. The inhibitor was also present in the reincubation medium.
(d) Yolk sacs were preincubated in vinblastine (10uM) for 60min, prior to the addition of radiolabelled substrate. The inhibitor was not present in the reincubation medium.

The bars represent the material remaining inside the yolk sac at the end of the reincubation period. The same scale is used and, as usual, open symbols represent TCA-soluble material, shaded ones, TCA-insoluble material.

n=3 for error bars

The Effects of Nocodazole on the Pattern of Release of Rabbit IgG from Rabbit Yolk Sacs. Figure 6.3.13

Yolk sacs were exposed to various concentrations of nocodazole for 2½h prior to exposure to ^{125}I-IgG.

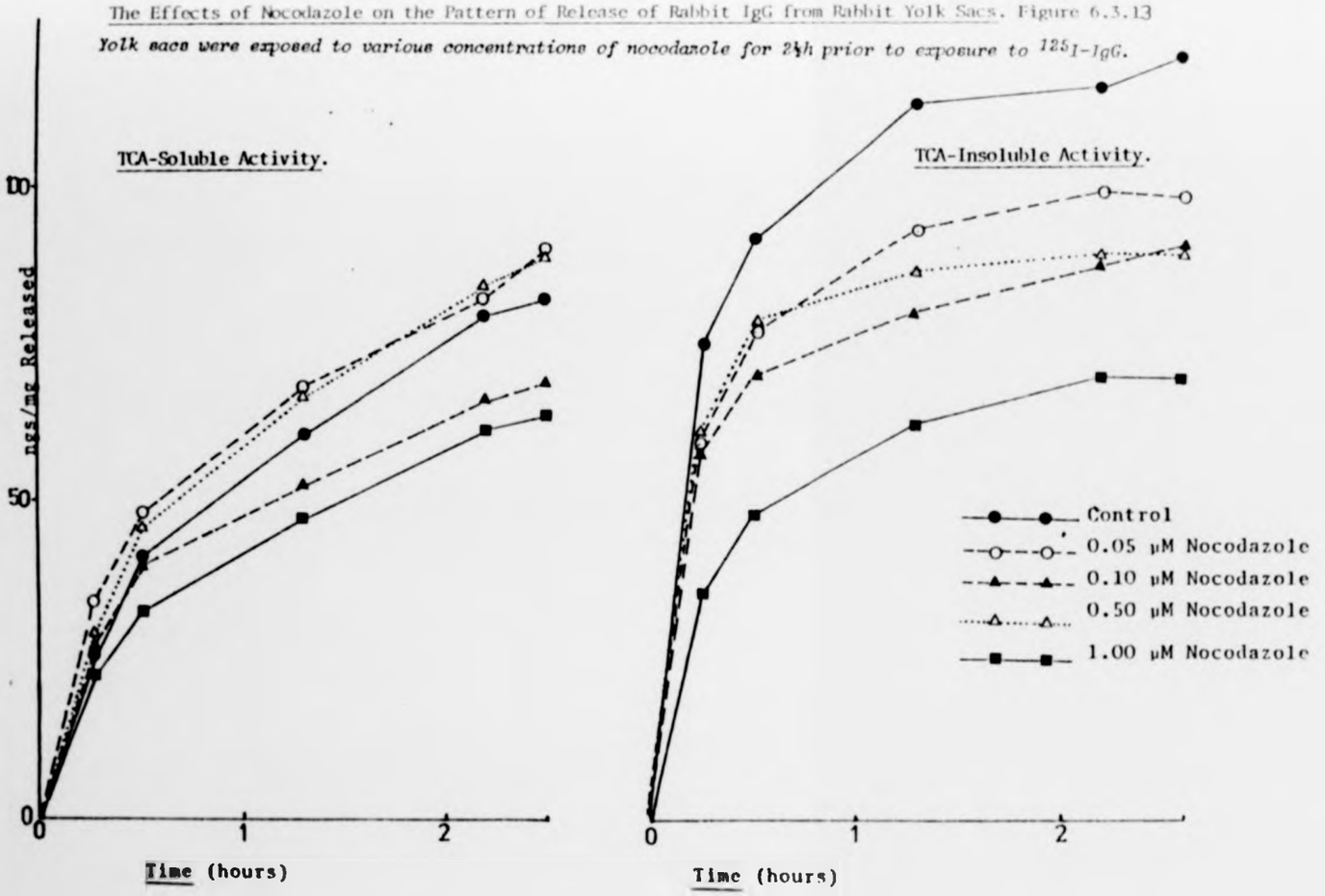


Figure 6.3.14 The effect of tissue-preincubation in dehydrocytochalasin B on the subsequent release pattern from rat yolk sacs of ^{125}I-IgG-derived radioactivity.

Rat yolk sacs were preincubated in dehydrocytochalasin B (5ug/ml, 1h) before the addition of ^{125}I-IgG (5ug/ml). Uptake was for a further 30min, and reincubation was carried out in the usual way.

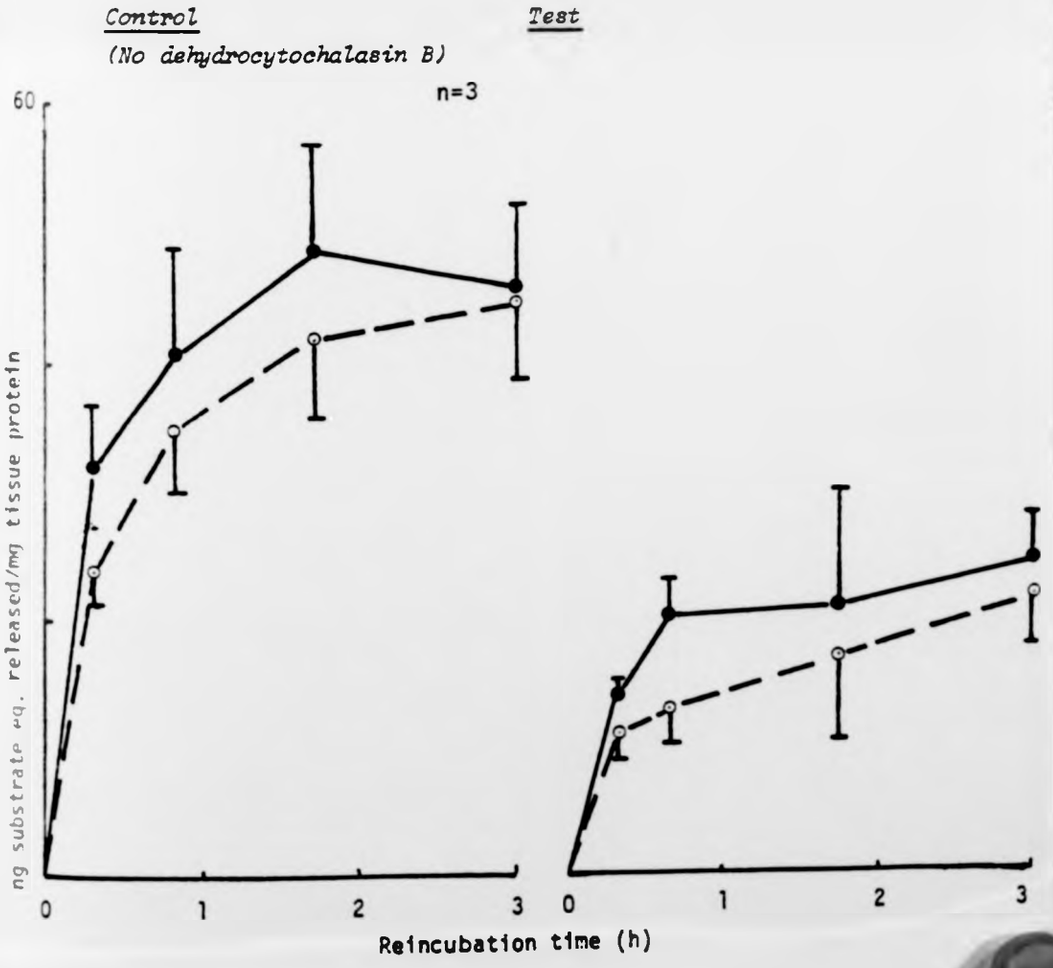


Figure 6.3.15 The effect of dihydrocytochalasin B on the release pattern from rabbit yolk sacs of ^{125}I-IgG-derived radioactivity.

Control: Yolk sacs were incubated in ^{125}I-IgG (5ug/ml) for 90min before being reincubated in the usual way.

Test: Dihydrocytochalasin B (10ug/ml) was added to the uptake medium for 30 min preincubation before the addition of ^{125}I-IgG (5ug/ml) and subsequent treatment in the usual manner.

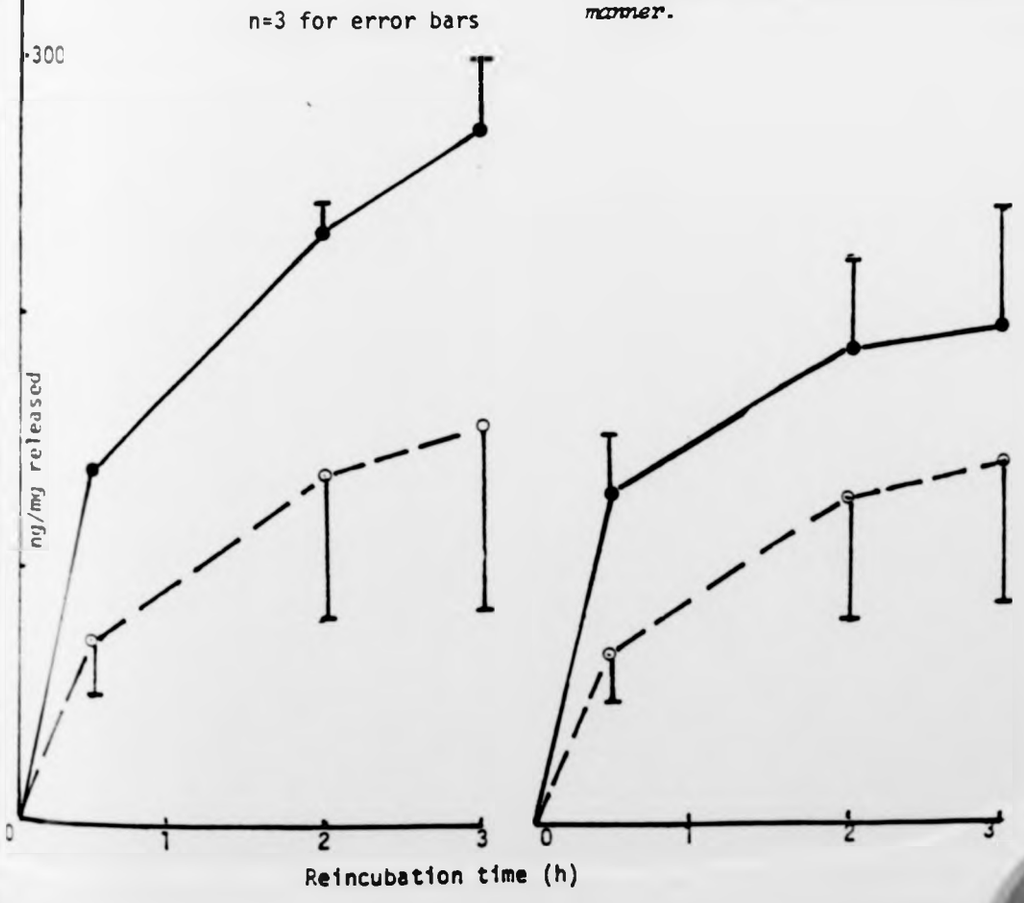


Figure 6.3.16 The effects of microfilament-inhibitors on the release from rabbit yolk sacs of ^{125}I-IgG-derived radioactivity.

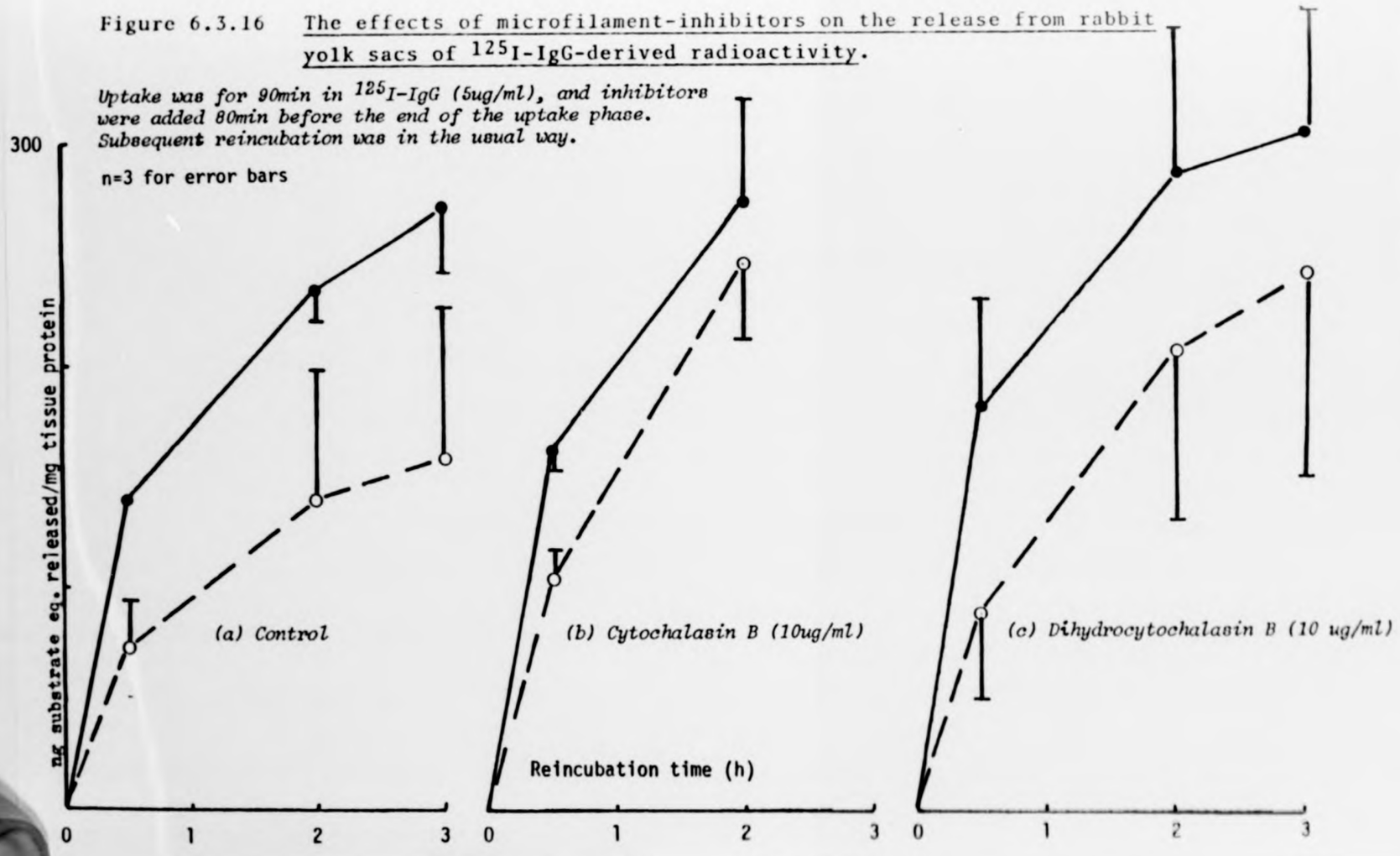


Figure 6.3.16 The effects of microfilament-inhibitors on the release from rabbit yolk sacs of ^{125}I-IgG-derived radioactivity.

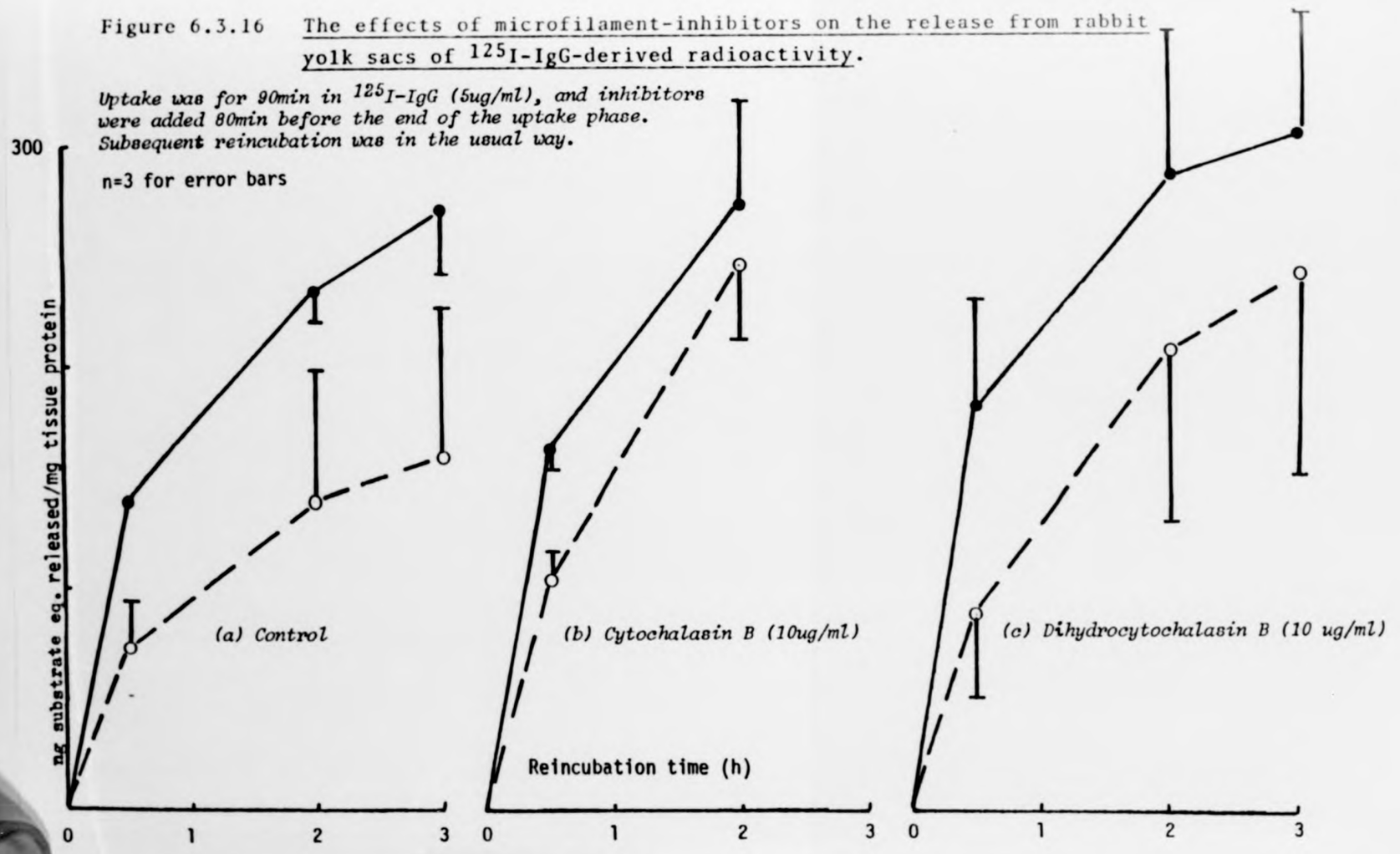


Figure 6.3.17 The effect of preincubation in dansylcadaverine on the release pattern from rabbit yolk sacs of ^{125}I-IgG-derived radioactivity.

Yolk sacs were preincubated for 1h in dansylcadaverine ((b), 250uM, (a), 500uM) before the addition of ^{125}I-IgG (5ug/ml). Uptake was for 90min, and reincubation was carried out in the usual way (in the absence of inhibitor).

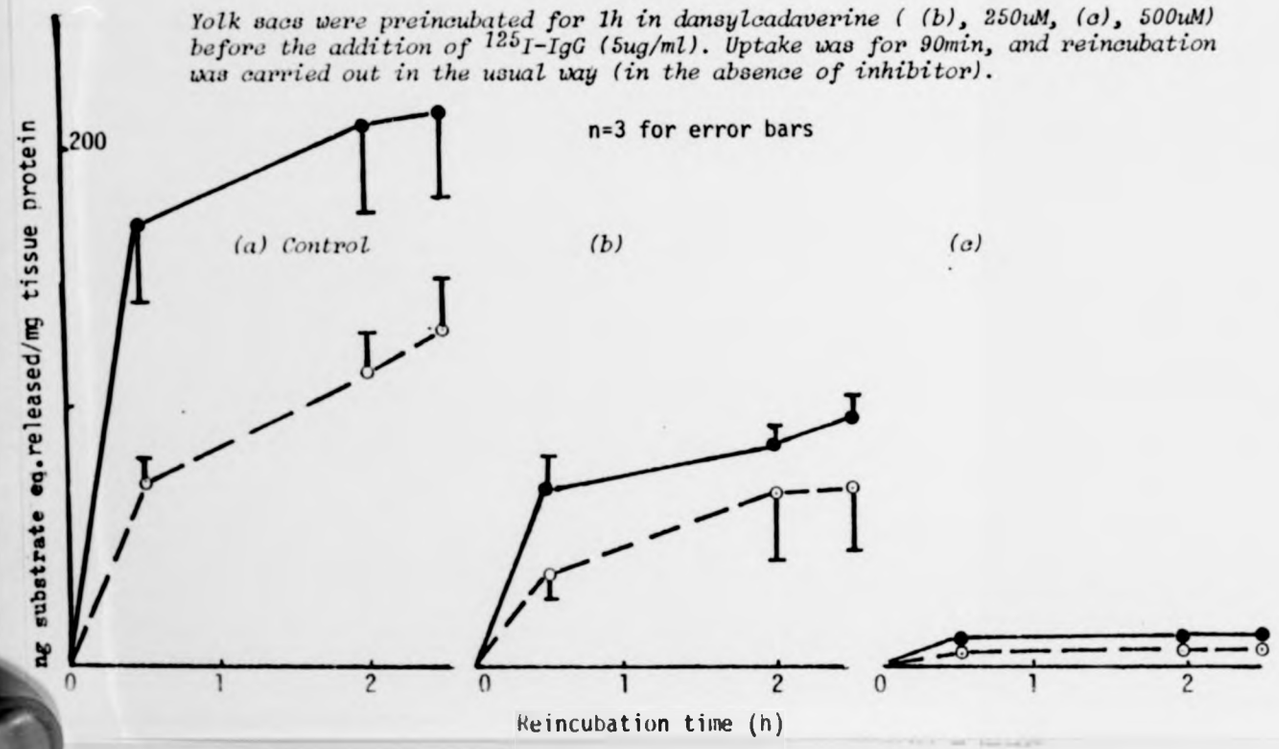
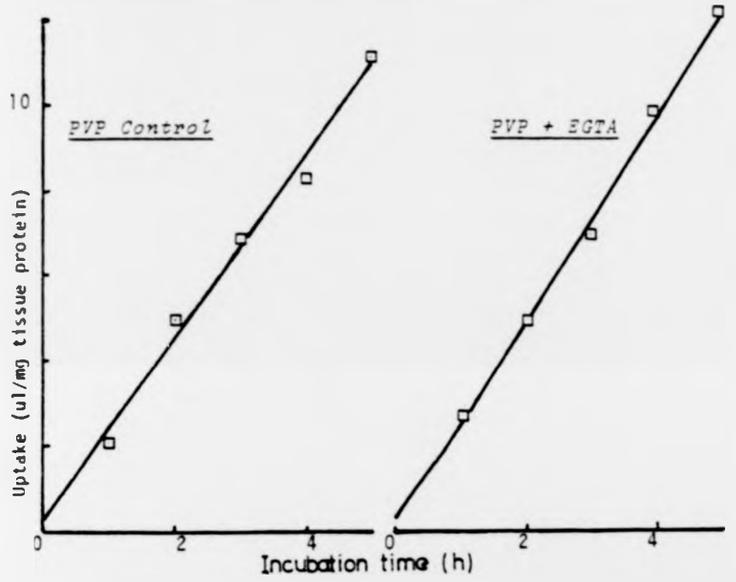


Figure 6.3.18 The effect of pretreatment in EGTA on the uptake into rat volk sacs of ^{125}I-PVP and ^{125}I-globulins.

Yolk sacs were preincubated for 1h in EGTA (0.5mM), before the addition of substrate. EGTA was retained in the uptake medium. No calf serum was used.



Endocytic Indices (Correlations)
(µl/mg tissue protein/h)

	PVP	Globulins
Control	2.12 (0.99)	26.7 (0.99)
+EGTA	2.36 (1.00)	25.2 (1.00)

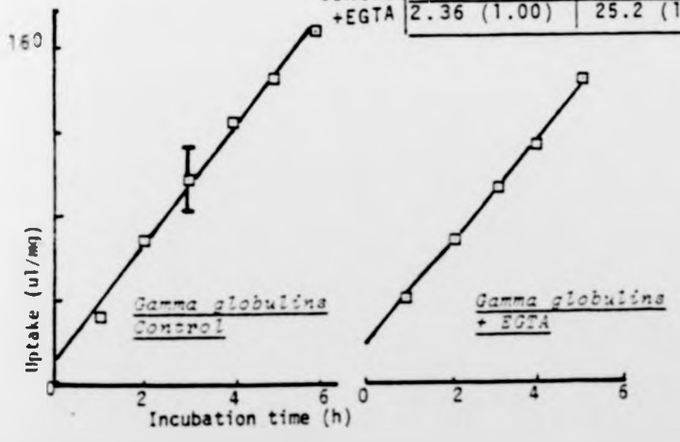
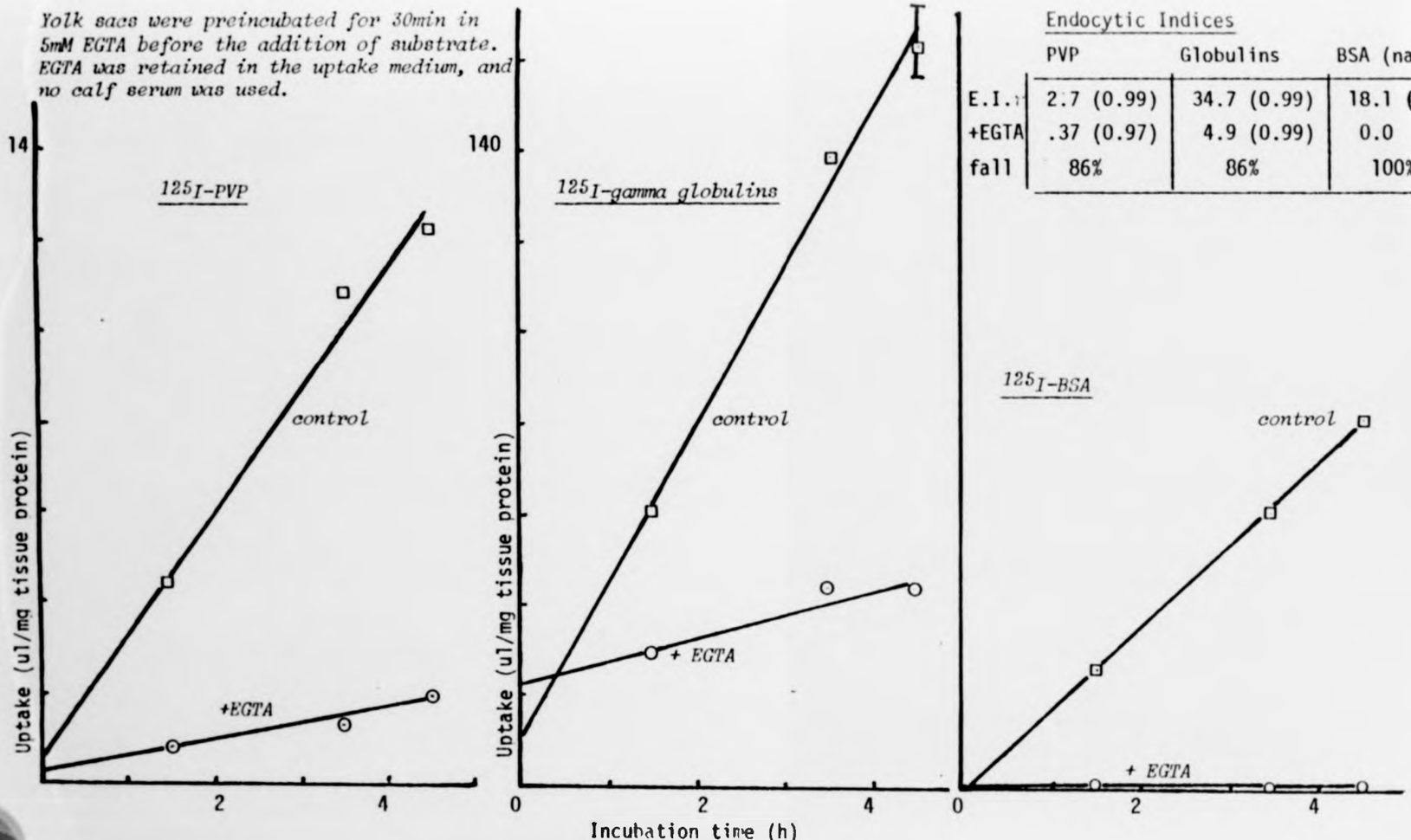


Figure 6.3.19 The effect of EGTA on the uptake of PVP, BSA and gamma globulins by rat yolk sacs.

Yolk sacs were preincubated for 30min in 5mM EGTA before the addition of substrate. EGTA was retained in the uptake medium, and no calf serum was used.



Endocytic Indices

	PVP	Globulins	BSA (nat)
E.I.	2.7 (0.99)	34.7 (0.99)	18.1 (1.00
+EGTA	.37 (0.97)	4.9 (0.99)	0.0
fall	86%	86%	100%

Figure 6.3.20 The effect of EGTA in the release medium on the pattern of release from rat yolk sacs of radioactivity derived from ^{125}I-gamma globulins.

(a) Control, (b) EGTA (5mM) was present only in the reincubation medium - all other procedures were as usual, (c) Yolk sacs were preincubated in EGTA (5mM) before the addition of substrate. The chelator was retained throughout the uptake and release incubations.

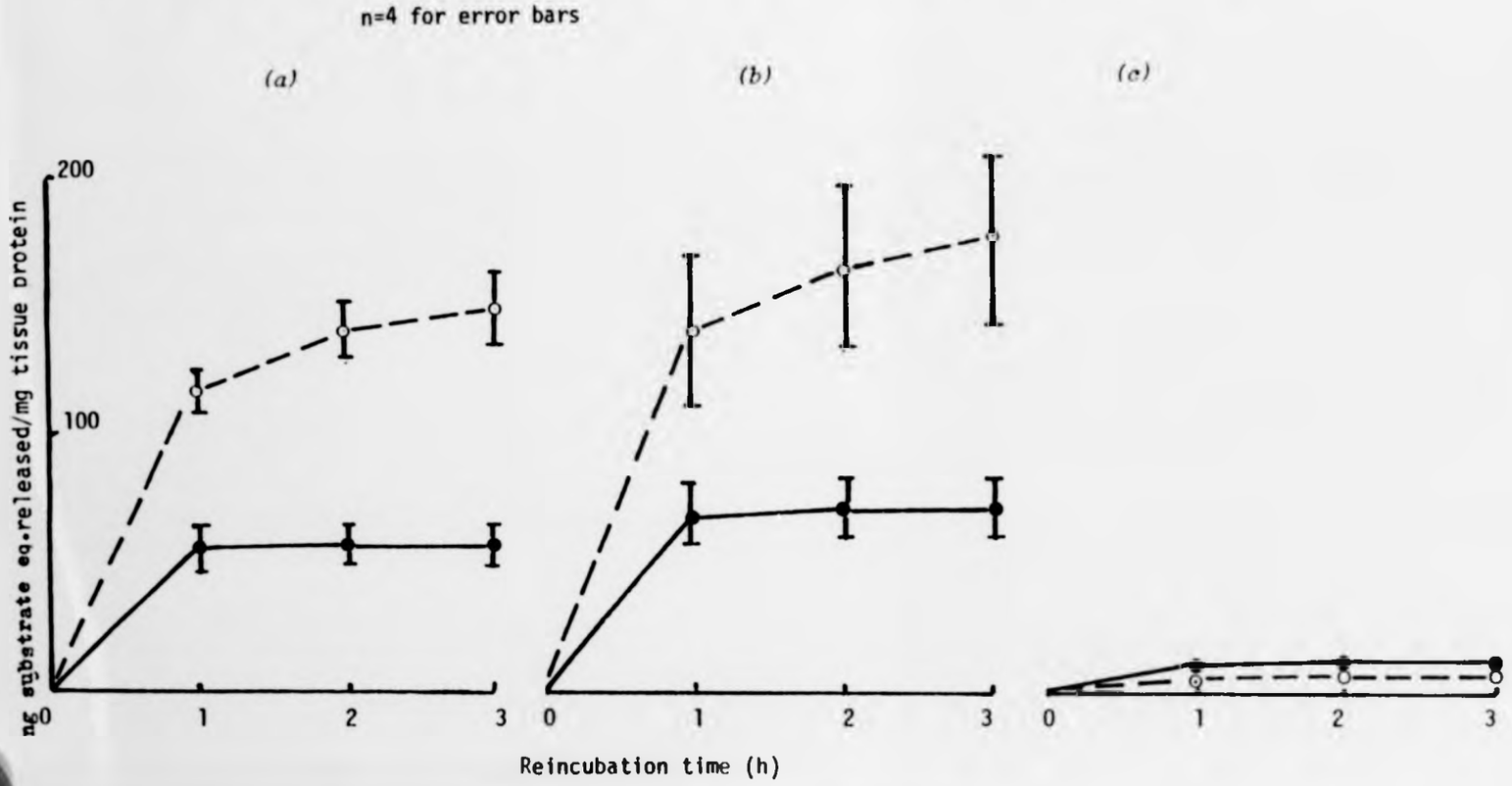


Figure 6.3.21 The effect of preincubation in EGTA on PVP accumulation by rabbit yolk-sac tissue.

Yolk sacs were incubated in serum-free medium in the presence of 5mM EGTA for 30min prior to the addition of ^{125}I-PVP. Controls were run without EGTA.

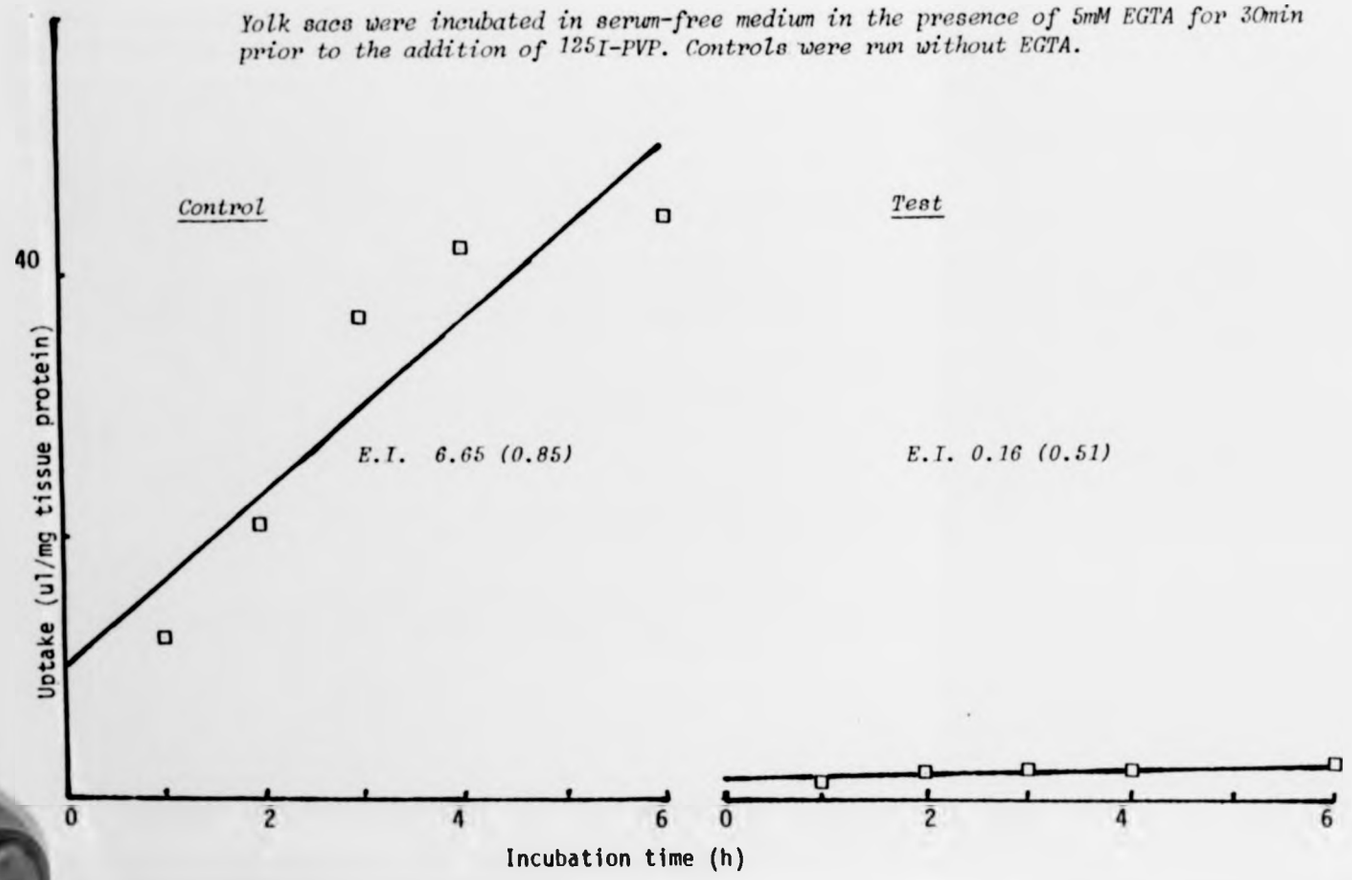


Figure 6.3.21 The effect of preincubation in EGTA on PVP accumulation by rabbit yolk-sac tissue.

Yolk sacs were incubated in serum-free medium in the presence of 5mM EGTA for 30min prior to the addition of ^{125}I-PVP. Controls were run without EGTA.

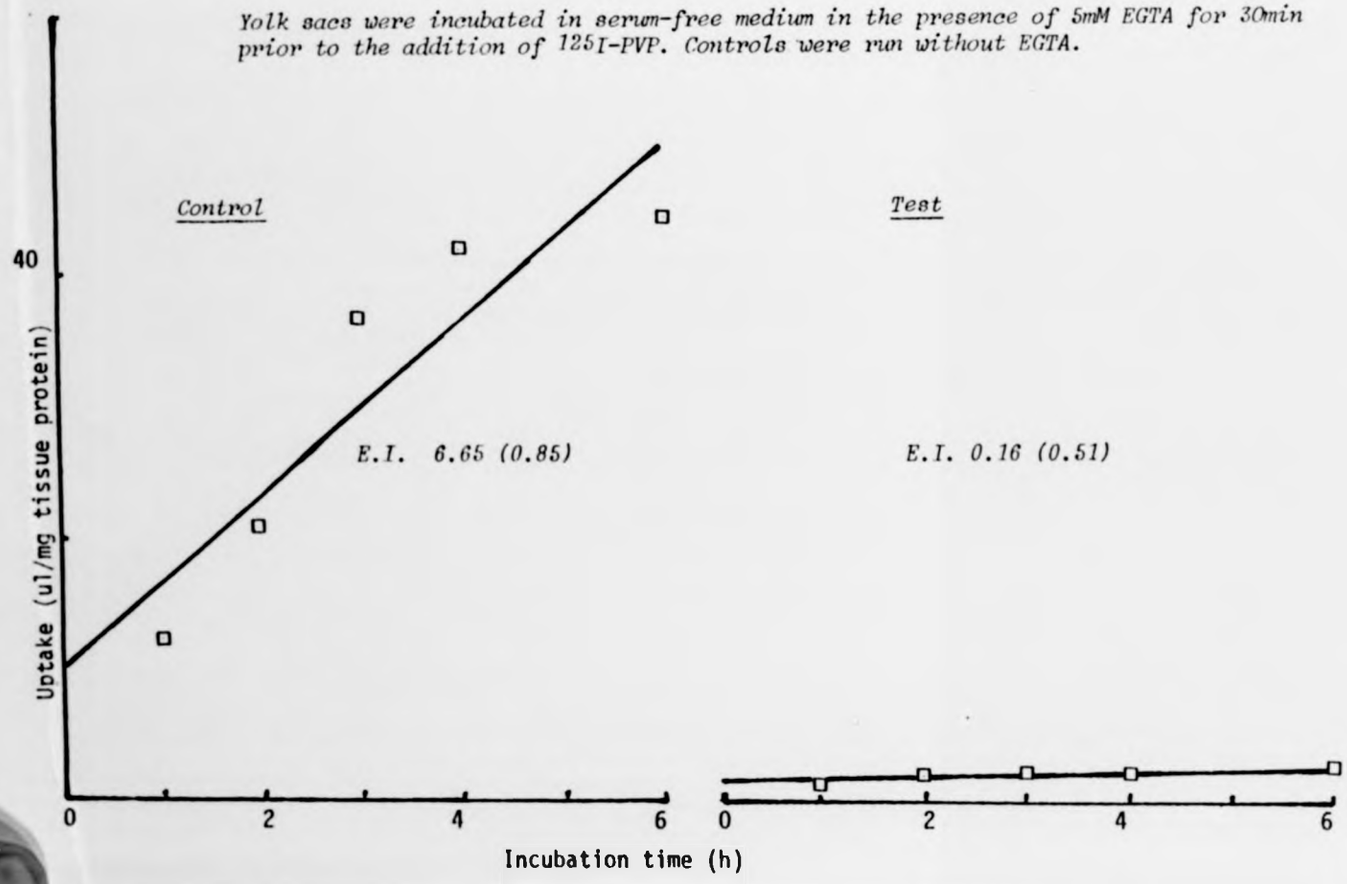


Figure 6.3.22 The effect of preincubation in EGTA on the release of ^{125}I-IgG-derived radioactivity from rabbit yolk sacs.

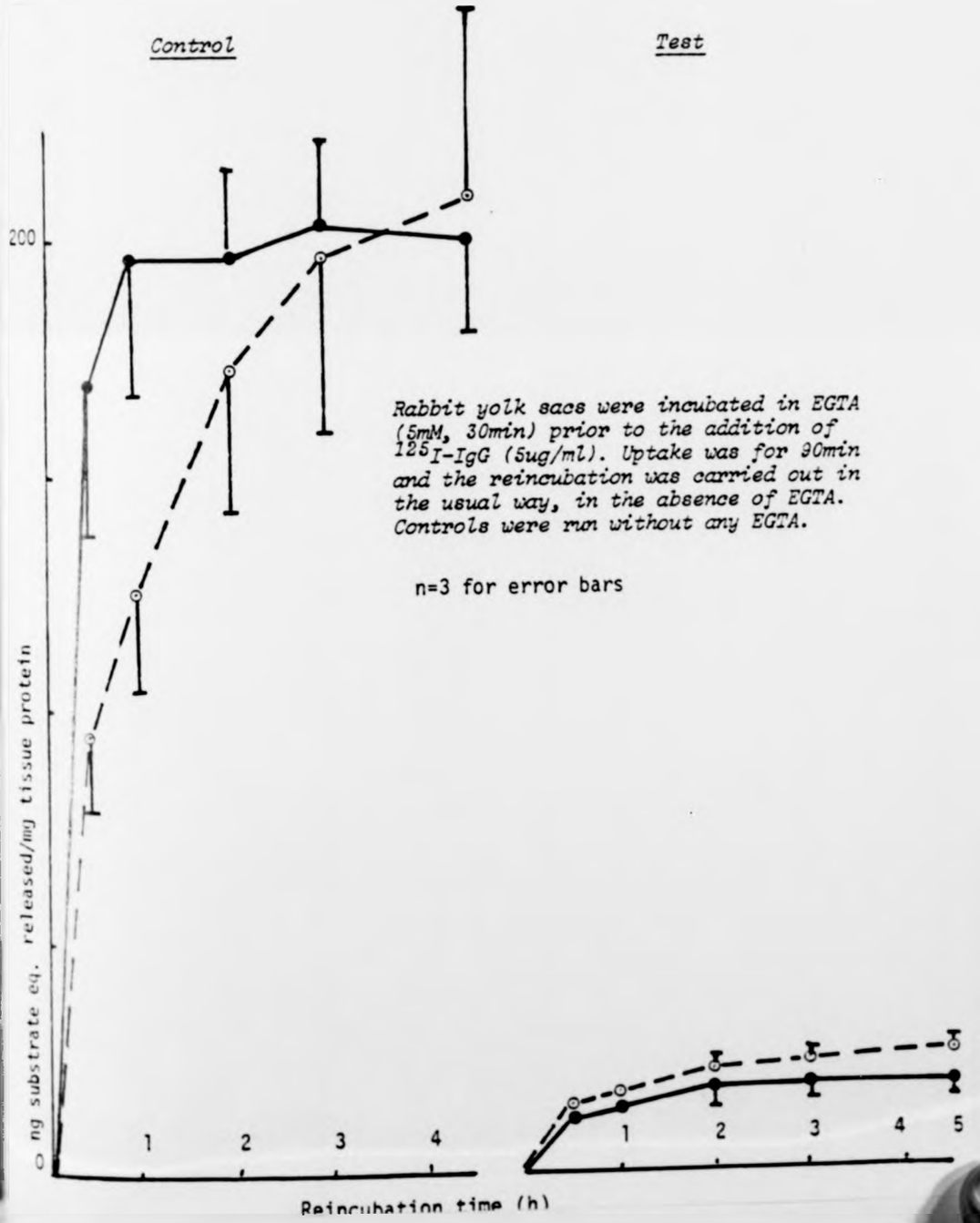
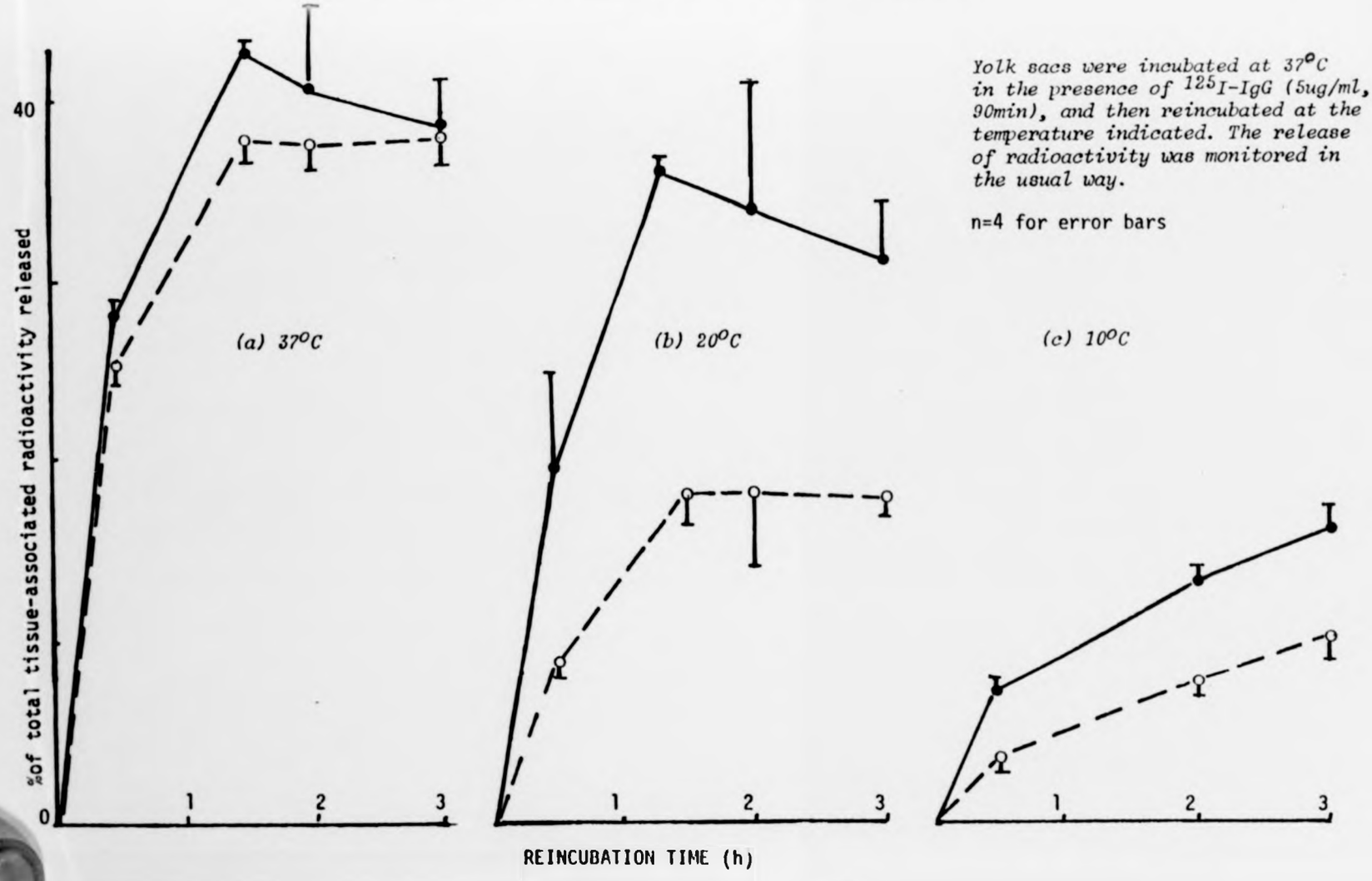


Figure 6.3.23 The effect of reincubation at lower temperatures on the pattern of release from rat yolk sacs of ^{125}I-IgG-derived radioactivity.



Yolk sacs were incubated at 37°C in the presence of ^{125}I-IgG (5ug/ml, 90min), and then reincubated at the temperature indicated. The release of radioactivity was monitored in the usual way.

n=4 for error bars

Figure 6.3.24 The effect of different incubation temperatures on the release pattern from rat yolk sacs of radioactivity derived from ^{125}I-IgG.

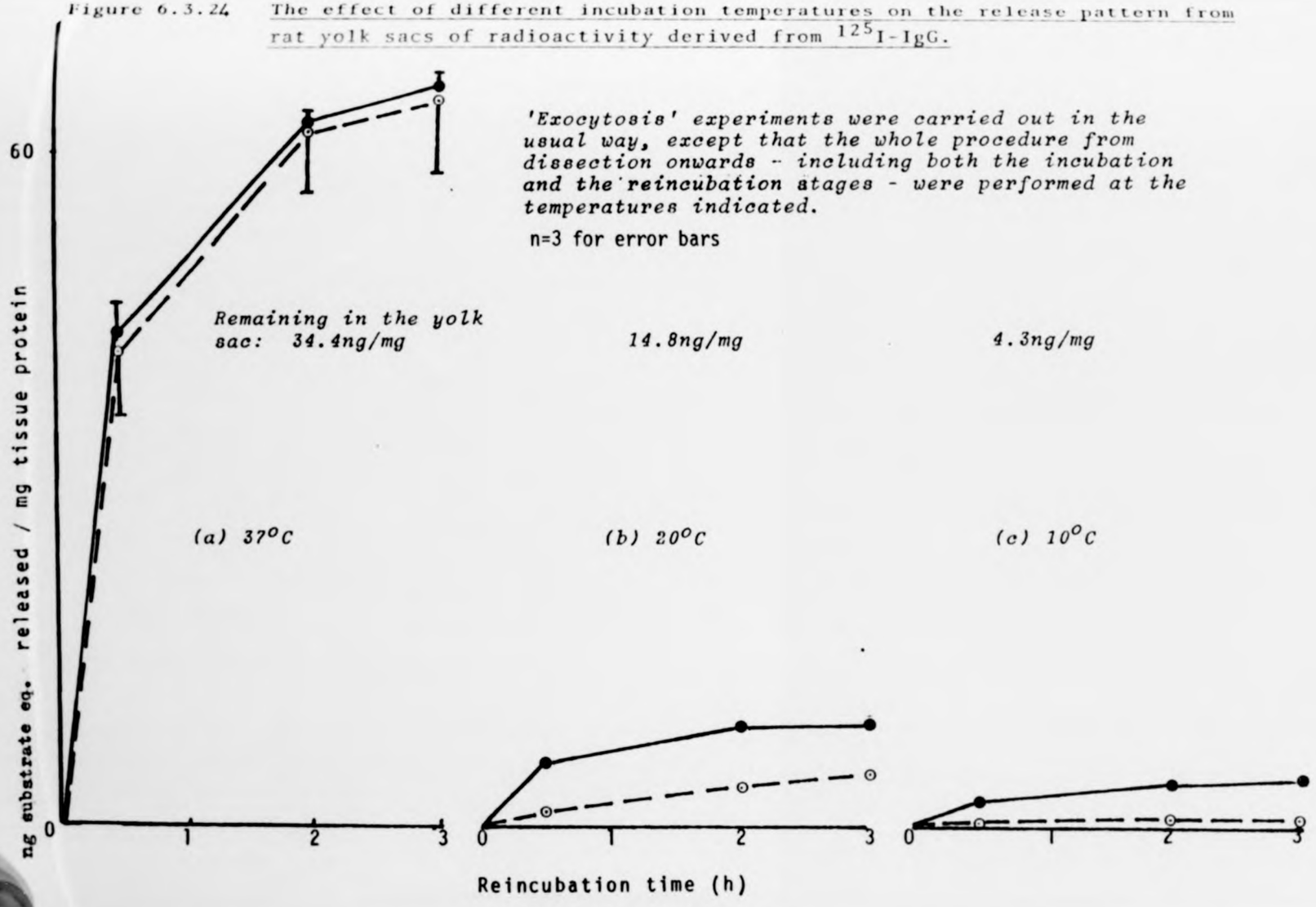


Figure 6.3.5 The effect of reincubation temperature on the release from rabbit yolk sacs of radioactivity derived from ^{125}I-IgG.

Rabbit yolk sacs were incubated in the presence of ^{125}I-IgG (90min, 5ug/ml), in the usual way, and then reincubated in fresh media at various temperatures, as indicated.

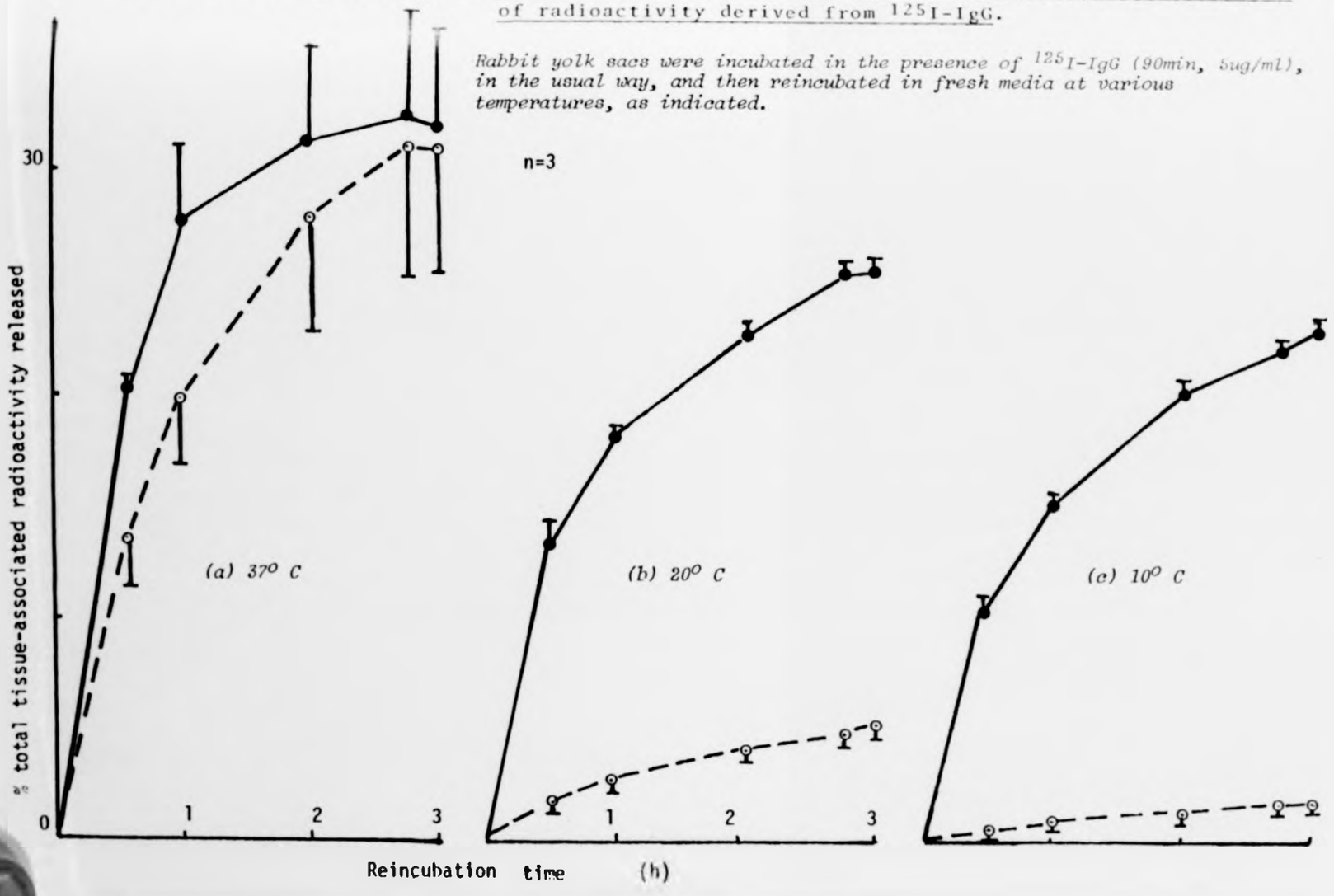


Figure 6.3.26 The effect of incubation at different temperatures on the pattern of release from rabbit yolk sacs of radioactivity derived from ^{125}I-IgG.

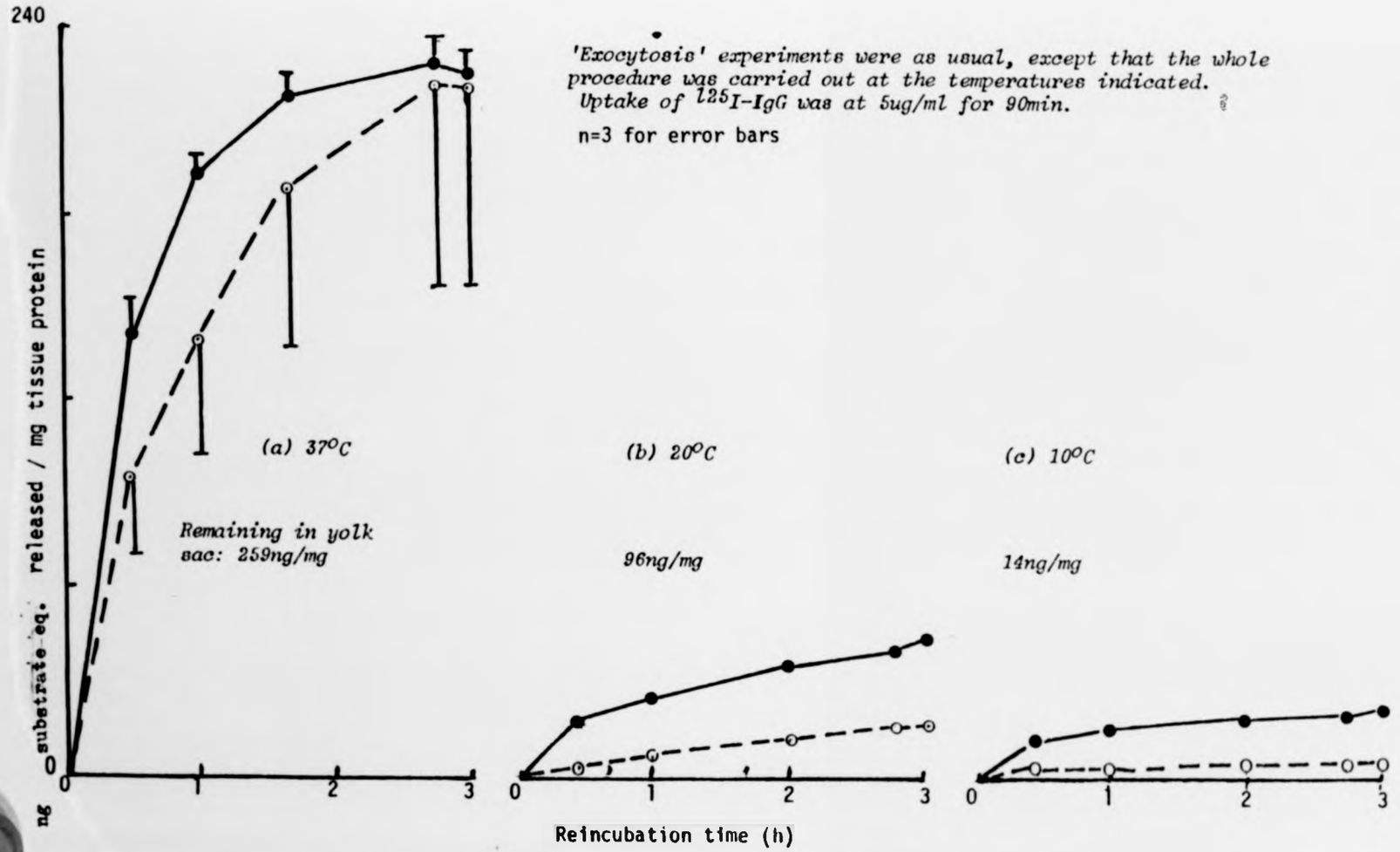
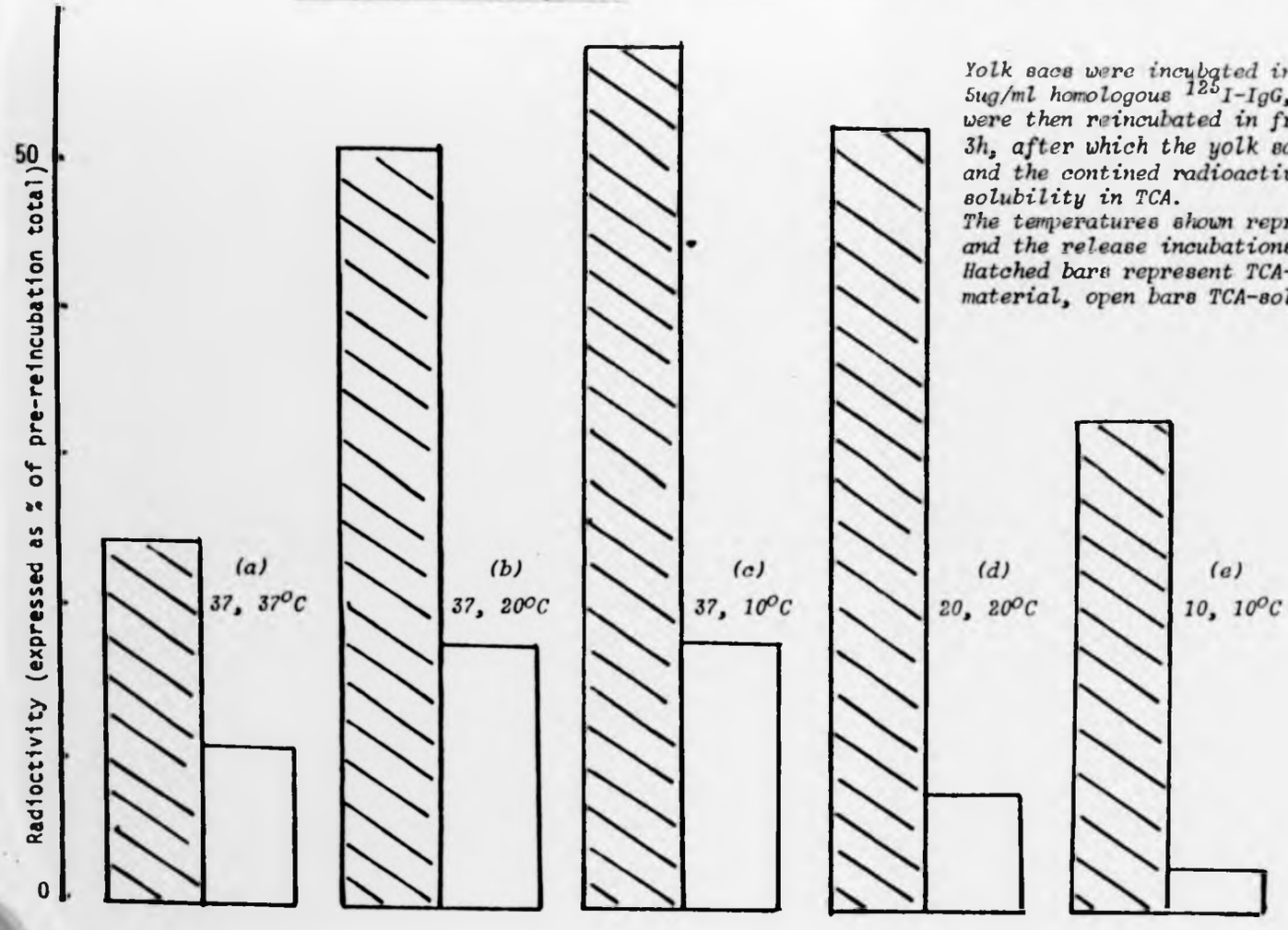
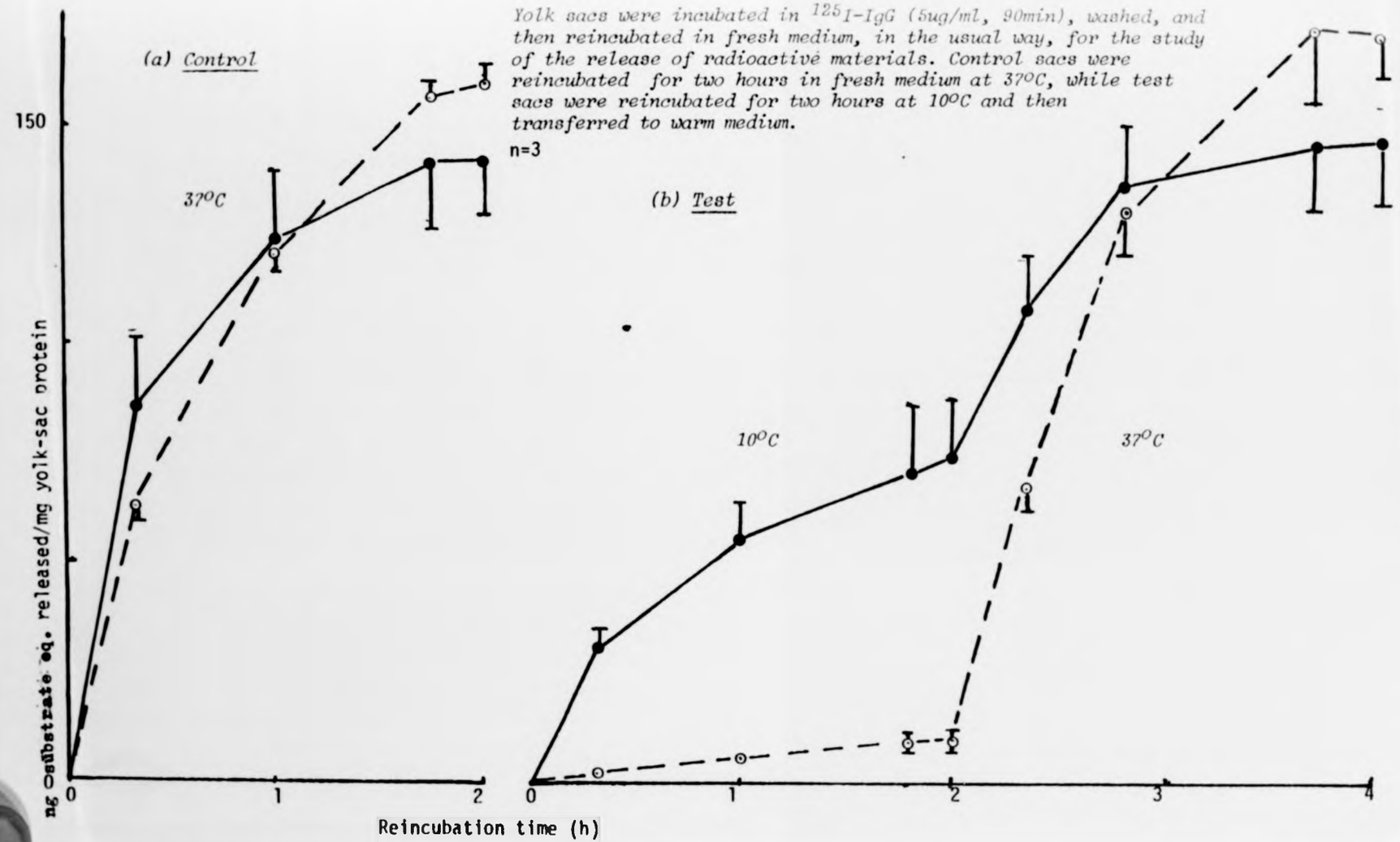


Figure 6.3.27 The nature of the radioactivity remaining in the rat yolk sac at the end of the reincubation period.



Yolk sacs were incubated in the presence of 5ug/ml homologous ^{125}I-IgG, for 90min. They were then reincubated in fresh medium for 3h, after which the yolk sacs were homogenised and the contined radioactivity assayed for solubility in TCA.
The temperatures shown represent the uptake and the release incubations respectively.
Hatched bars represent TCA-insoluble material, open bars TCA-soluble.

Figure 6.3.28 The reversibility of the inhibition by low temperature of the release from rabbit yolk sacs of radioactivity derived from ^{125}I-IgG.



Yolk sacs were incubated in ^{125}I-IgG (5ug/ml, 90min), washed, and then reincubated in fresh medium, in the usual way, for the study of the release of radioactive materials. Control sacs were reincubated for two hours in fresh medium at 37°C, while test sacs were reincubated for two hours at 10°C and then transferred to warm medium.

DISCUSSION

Casley-Smith (1969) observed pinocytic capture of various substrates by rabbit peritoneal macrophages in small vesicles, and found that the process was not inhibited by low temperature or by the presence of metabolic inhibitors. The idea was born that pinocytosis could be divided into two categories, macropinocytosis and micropinocytosis (Allison and Davies, 1974). The former process was defined as energy-requiring and was inhibited by cytoskeletal poisons. The latter required no metabolic energy and was thought to obtain sufficient energy from Brownian motion to fuel its requirements, nor was it affected by inhibitors of the cytoskeleton. The independence from energy-requirements of micropinocytosis has since been brought into question (Bowers, 1977). However, it is possible that two mechanisms of uptake, as different from each other as was originally proposed by Wild (1975), for the internalisation of IgG for degradation or for protection might show different responses to the presence of various inhibitors.

The major technique employed here, to monitor the effects of inhibitors on the two types of uptake, was to preincubate yolk sacs in the presence of the inhibitor, prior to the introduction of the radiolabelled substrate. The radioactivity released from the tissue when it was reincubated in fresh medium was monitored, and differentiated into two major categories - macromolecular, and degraded (on the basis of solubility in TCA). The amounts of radioactivity released in these two forms was taken to be indicative of the level of substrate taken into the protective and degradative pathways. A specific decrease in the amount of one particular form of radioactivity released from the reincubated tissue was assumed to result from specific inhibition of the pathway of uptake and release that gave rise to that form of released radioactivity. It is possible, however, that a specific fall in the amount of one particular form of radioactive species released may be caused by inhibition of processes other that the initial uptake. For example, inhibition of intralysosomal degradation might perhaps be caused by an inhibitor that caused the intralysosomal pH to rise, and this would probably result in a specific fall in the amount of TCA-soluble material released. It is therefore important to monitor the tissue level of radioactivity in order to detect any unexpected tissue accumulation of substrate.

The possibility that TCA-insoluble material released from reincubated yolk sacs was simply desorbing from the plasma membrane rather than being released from inside the cell was refuted by Weisbecker (1981) on the grounds that such release was prevented by inhibitors of endocytosis, suggesting that endocytic internalization was a prerequisite for this release to occur. The evidence accumulated in this study, using a much wider range of endocytosis inhibitors, supports and confirms that refutation. The process of uptake for intact release is evidently susceptible to EGTA and dansylcadaverine, as well as to metabolic and cytoskeletal inhibitors.

Preincubation of rat yolk sacs in DNP (details are given in the Methods section, results in Figure 6.3.1) caused equal falls in the amounts of both forms of radioactivity (^{125}I-IgG-derived) released from the reincubated tissue. This suggests that this regime of DNP-treatment does not specifically inhibit just one form of uptake of ^{125}I-IgG. Similar results were obtained when rabbit yolk sacs were pretreated with either DNP or rotenone (Figs 6.3.4(a) and 6.3.8(b), respectively), though rabbit tissue seemed less sensitive than rat tissue to DNP inhibition.

As well as membrane flexibility, low temperatures are expected to cause inhibition of the action of various enzymes and thus limit the supply of metabolic energy to cell activities as well as modify the rates of intralysosomal proteolysis. Tubulin polymerisation is also inhibited at low temperatures (Olmsted and Borisy, 1973), and the use of cold to investigate microtubular functions has been widespread - its action has been likened to that of colchicine (Dustin, 1978). Hence the effects of low temperature on yolk-sac treatment of IgG would be expected to reflect a complicated series of inhibitions. Preincubation with substrate of yolk sac at low temperatures followed by reincubation at the same low temperature appeared to cause non-specific falls in the amounts of both forms of ^{125}I-IgG-derived radioactivity released from rat (20°C, 10°C; Figs 6.3.23(b) and 6.3.23(c) respectively) and rabbit (20°C, 10°C; Figs 6.3.26(b) and (c) respectively) yolk sacs. It was concluded that these temperatures do not selectively inhibit one pathway of uptake of ^{125}I-IgG.

When tissue was preincubated in the presence of microtubular inhibitors before the addition of ^{125}I-IgG, the pattern of release of radioactivity from reincubated rat (vinblastine, Figure 6.3.12(b)) and

rabbit (colchicine, Figure 6.3.10; nocodazole, Figure 6.3.13) tissues gave no indication of any selective inhibition of either pathway of ^{125}I-IgG through the cell. The dose-dependent inhibition observed in rabbit tissue in the presence of colchicine (Figure 6.3.10) is not contradictory to the results of Duncan and Lloyd (1978) for rat tissue, since they found no increased inhibition of pinocytosis at colchicine concentrations over 1 ug/ml while the maximum concentration used in this study was 0.8 ug/ml (2 uM).

Preincubation of yolk sacs in microfilament-specific inhibitors (dihydrocytochalasin B, cytochalasin B) again gave no apparent indication of any discrimination of inhibition between the mechanisms of uptake of ^{125}I-IgG as judged by the patterns of radioactivity released from the reincubated yolk sacs (rat Figure 6.3.14, rabbit Figure 6.3.15).

Preincubation of tissue in 5mM EGTA (0.5 mM was shown to be without observable effect, Figure 6.3.18) was shown to inhibit pinocytosis of ^{125}I-PVP and the uptake of ^{125}I-IgG to similar extents (Figure 6.3.19) and to halt pinocytosis in rabbit yolk sacs (Figure 6.3.21). Uptake and release of ^{125}I-IgG in EGTA-pretreated tissue was shown to be virtually abolished (rat, Figure 6.3.20(b); rabbit, Figure 6.3.22). Although the effects of intermediate concentrations of EGTA on the ^{125}I-IgG release pattern would be interesting, there is no evidence to date of EGTA being able selectively to inhibit the uptake of ^{125}I-IgG into just one pathway.

The only other inhibitor investigated was dansylcadaverine, a specific and potent inhibitor of transglutaminases (Davies *et al*, 1980). Figure 6.3.17 shows the effects of preincubating rabbit yolk sacs in two concentrations of dansylcadaverine, before the addition of ^{125}I-IgG, on the pattern of radioactivity subsequently released from the reincubated tissue. Dansylcadaverine (250uM) caused a 50% fall in the amounts of both types of radioactivity released, and 500 uM abolished uptake and release entirely. The results suggest that transglutaminase activity is essential for both the degradative and the protective pathways to function properly.

The evidence gathered so far indicates that a wide variety of inhibitors at various concentrations are not able to cause any kind of selective inhibition of just one type of uptake of ^{125}I-IgG, leaving the other unaffected. The conclusion must be that the two processes are

extremely similar, if not identical. However, the two fates of IgG once associated with the cell are so different - one almost certainly involving specific receptors capable of mediating diacytosis, the other less specific binding sites that carry their substrates to the lysosomal system for degradation - that cell surface events leading to two separate types of endocytosis would not be expected to be identical. The most simple conclusion is that the two pathways share a common step, and that step is most likely to be the first stage of the pathway, the process of uptake. Endocytosis of ^{125}I-IgG destined for protection and for degradation may well occur into the same primary vesicles. This is in direct contradiction to one of the basic tenets invoked by Wild in his original hypothesis, but it does find a degree of support in the literature. The concept of common uptake of material intended for different fates in endocytic vesicles that fuse with larger vesicles near the cell surface, these larger vesicles being the primary site of sorting and directing of the various substrates to their different subcellular destinations - rather than the cell surface being the point of selection of materials for the different pathways - was originally proposed by Willingham and Pastan (1980), who coined the word 'receptosome' to describe the sorting-vesicle. Subsequently equivalent structures have been referred to as 'endosomes' (eg. Helenius, 1983), a rather less definitive label. The current thinking on this feature of pinocytosis is discussed more fully in Chapter 7.

Since is seemed impossible to discriminate between the two pathways at the uptake stage by the use of selective inhibitors, attempts were made to inhibit later stages of the pathways in order to gain information on the subcellular mechanisms involved.

Attempts to inhibit the release process by including inhibitors in the reincubation medium were frustrated by the length of time taken for the inhibitors to act compared with the relatively short length of time taken for the bulk of yolk-sac-associated radioactivity to be released from the tissue. Rotenone, DNP, EGTA and vinblastine were found to have no noticeable effects on the release of ^{125}I-IgG-derived radioactivity from the rat yolk sacs under these conditions (Figs 6.3.6, 6.3.2, 6.3.20(a) and 6.3.12(a) respectively), but the majority of release could already have occurred by the time any inhibitory effects became established. Thus it might be impossible to judge the effects of most inhibitors directly on the release process under these experimental

conditions. The only inhibitor used that acted sufficiently quickly to be effective on the release process was low temperature (Figs 6.3.24 - 6.3.28).

It was observed that if yolk sacs that had been preincubated at 37°C in the presence of ^{125}I-IgG were reincubated in fresh medium at lower temperatures, there were modifications to the pattern of release of radioactive materials from the tissue. Reincubation at 20°C caused a sharp fall in the amount of TCA-soluble radioactivity released, while the release of TCA-insoluble material was much less affected (Figs 6.3.23(b) and 6.3.25(b) for rat and rabbit tissues respectively). Reincubation at 10°C inhibited both types of release from rat yolk sacs to approximately equal extents, but seemed to cause total inhibition of release of TCA-soluble material from rabbit yolk sacs, with TCA-insoluble material largely unaffected (Figs 6.3.23(c) and 6.3.25(c) respectively). Evidently the production within rat yolk sacs of degraded material is very temperature-sensitive (this would be expected since degradation is essentially a lysosomal-enzyme-mediated process), but the release of TCA-insoluble material is less so. Compared with rabbit tissue, the release of TCA-insoluble material from rat yolk sacs seems to be more temperature dependent, and this suggests that the broad similarities that have been assumed between the two tissues (Weisbecker, 1981) may be an over simplification. In both tissues, however, the release of TCA-insoluble material appears to be less sensitive to low temperature than are the bulk of enzymic reactions, and thus the process has characteristics compatible with the hypothesis involving a separate pathway via microvesicles.

In order to investigate further the nature of the release process another type of experimental regime was developed. Yolk sacs were allowed to reach a steady state in the presence of radiolabelled substrate before the inhibitor was added to the uptake medium, allowing the yolk sac to reach a new steady state in the presence of the inhibitor.

When rat yolk sacs were incubated in the presence of ^{125}I-labelled rat gamma-globulins for 15 min before the addition of 40 ug/ml DNP, and then the incubation continued for a further 130 min before reincubation, the release pattern of radioactivity shown in Figure 6.3.3 was obtained. The pattern shows a specific fall in the amount of TCA-soluble material released, with the TCA-insoluble level being essentially unaffected. When

it was shown that DNP-inhibition was reversible (Figure 6.3.5) the following interpretation of the data (Figure 6.3.3) was made. The yolk sac reaches equilibrium in the presence of ^{125}I-IgG before the DNP begins to exert its effects. Uptake of ^{125}I-IgG into both pathways is then inhibited (see Figure 6.3.1), as is release of TCA-insoluble material. Degradation and release of TCA-soluble material continues, however, although a certain amount of ^{125}I-IgG destined for degradation is present in pre-lysosomal vesicles whose fusion with lysosomes may be prevented by the DNP, and the bulk of the ^{125}I-IgG destined for degradation is released back into the uptake medium in TCA-soluble form. The yolk sac thus becomes specifically depleted of ^{125}I-IgG destined for degradation whilst still in the uptake flask, and it contains a normal complement of ^{125}I-IgG for protection. Reincubation of the yolk sac in fresh medium allows the reversal of DNP-induced inhibition (Figure 6.3.5), and the amount of TCA-insoluble material released is similar to controls that contained no DNP. On the other hand, the level of TCA-soluble material released is low and results primarily from ^{125}I-IgG isolated in non-degradative vesicles by the effects of DNP finally being able to enter lysosomes for degradation. The release pattern obtained can only be explained by a step in the release pathway for TCA-insoluble material being equally susceptible to the effects of DNP as is the uptake process. If release is more susceptible to inhibition, an elevated level of yolk-sac TCA-insolubles would be observed; if less susceptible, an insolubles-depleted yolk sac would be observed.

This technique promises to give valuable information on the susceptibilities of the various release processes to different inhibitors.

Yolk sacs were exposed to various inhibitors according to the above regime. In the rat yolk sac neither mechanism of release appeared to be particularly sensitive to inhibition by the microtubular poison colchicine or the microfilament inhibitor dihydrocytochalasin B (Figure 6.3.9), though metabolic inhibitors were found to inhibit the release of TCA-insoluble material (DNP and rotenone, Figs 6.3.3 and 6.3.7 respectively) in a reversible manner. Microtubular inhibitors used with this technique in studies with rabbit yolk sacs had no selective effect on release, affecting both types slightly and suggesting that both mechanisms of release are equally susceptible to microtubular inhibition (colchicine, nocodazole; Figs 6.3.11(c) and 6.3.11(d) respectively). There was support for the previous conclusion relating to the data

reported in Figure 6.3.15, that uptake of ^{125}I-IgG by rabbit yolk sacs is not particularly sensitive to inhibitors of microfilaments, and the negligible modification to the release pattern caused by cytochalasin B and dihydrocytochalasin B (Figure 6.3.11 and 6.3.16) further suggests that the processes of release are no more sensitive to microfilament inhibitors than uptake.

The metabolic inhibitor rotenone, (Figure 6.3.8(c)), inhibited the release of TCA-insoluble material from rabbit yolk sacs (again, in a reversible manner), with less effect on the release of TCA-soluble material, and seemed to have a more profound effect at these concentrations than did DNP. In rat tissue DNP appeared to have a geater effect than rotenone, under similar conditions.

Despite some slight differences between the behaviours of rat and rabbit yolk sacs, the same general conclusions can be drawn for both tissues; all mechanisms of uptake of ^{125}I-IgG into yolk-sac endodermal cells are inhibited by metabolic inhibitors, cytoskeletal inhibitors, EGTA, low temperature and dansylcadaverine. Only low temperature has been shown to act sufficiently quickly to inhibit release of radioactivity when it is applied only to the reincubated tissue. Release of TCA-insoluble material does not appear to be highly sensitive to low temperatures, but lysosomal degradation of substrates and the subsequent release of TCA-soluble material is severely affected by falls in temperature.

The technique of adding the inhibitor to the uptake medium after the tissue has reached a steady state with regard to the radiolabelled substrate has shown the mechanism of release of TCA-insoluble material to be sensitive to metabolic inhibitors, but appently not to inhibitors of the cytoskeleton. Release of degradation fragments of ^{125}I-IgG from lysosomes was not affected by any of the inhibitors employed, and presumably occurs by simple diffusion. The implications for the mechanism of release of macromolecular ^{125}I-IgG are that the formation of the exocytic vehicle is an energy-requiring process, but that its movement is not directed by the cytoskeleton. It seems unlikely that the direction of movement is random however, since a large proportion of the protected ^{125}I-IgG would then never reach the lateral or basement membrane, returning instead to the plasmalemmal surface. On the other hand the mechanism of direction remains unclear - it could perhaps involve

cytoskeletal elements that are insensitive to microtubular and microfilamentous inhibitors.

In summary, the following conclusions were reached in this chapter;

(i) The susceptibility of the protection mechanism to inhibition by inhibitors of endocytosis suggests that TCA-insoluble ^{125}I-IgG released from the reincubated yolk sac does originate from inside the tissue and does not simply desorb from the plasma membrane. This conclusion is in support of that reached by Weisbecker (1981).

(ii) The difficulty experienced in trying to inhibit specifically either the mechanism of uptake of ^{125}I-IgG into yolk-sac cells for degradation or the process of uptake for diacytosis and intact release of ^{125}I-IgG, despite the use of a wide variety of inhibitors, suggests that the mechanisms of uptake leading to degradation and to protection of ^{125}I-IgG may share a common step. This step is most likely to be uptake, and the site of subcellular sorting may be vesicles that comprise the pre-lysosomal compartment.

(iii) Lysosomal degradation of ^{125}I-IgG and subsequent release of the degradation products is inhibited by low temperatures, but apparently not so much by metabolic or cytoskeletal inhibitors.

(iii) The release of TCA-insoluble radioactivity derived from ^{125}I-IgG from reincubated yolk sacs seems to be susceptible to inhibition by metabolic inhibitors but apparently not by cytoskeletal ones. The process appears to be less sensitive to low temperatures than is lysosomal degradation and the release of degraded products.

Chapter 7. A Preliminary Study into the Use of Subcellular Fractionation to Characterise the Vehicle(s) of Diacytosis of IgG.

INTRODUCTION

Immunoglobulin G has the ability to cross the endodermal layer of cells of the rat and rabbit yolk sacs without being degraded, and in doing so it provides the foetus with passive immunity derived from the mother's immune system. The route by which this transcellular passage occurs is not clearly understood, but the presence between the cells of tight junctions suggests that the pathway lies across the cells themselves, probably involving a system of endocytic vesicles. The aim of this chapter is to investigate what sort of information concerning the vehicles of transmission can be obtained by the use of techniques of tissue homogenisation and subcellular fractionation by centrifugation. Many of the experiments reported in this chapter are preliminary investigations, some having been performed on one occasion only. Others have been shown to be reproducible, and the degree of reliability of individual results is indicated in the text, and borne in mind during their interpretation.

In recent years, our understanding of mechanisms of endocytosis and subcellular routing of substrates has greatly developed. Although it is still assumed that mechanisms of endocytosis may vary significantly between different cell types and substrates, it is now widely believed that endocytic vesicles often fuse together inside the cell to form larger vesicles ('receptosomes' or 'endosomes') before fusion occurs with the lysosomal system (for review see Steinman et al, 1983). Hand in hand with this belief is the assumption that many different substrates (e.g. hormones, toxins, plasma proteins) may enter the cell initially via a common pathway, the receptosome being the important site of sorting substrates and directing them towards their various fates, including the separation of ligand from receptor, and the recycling of the latter back to the cell surface. Another feature that is now becoming widely accepted is the involvement of a protein coat (possibly acting as strength-giving scaffolding) in the process of endocytic vesicle formation for many sorts of endocytosis. In the absence of any viable alternative, current feeling is that specialised regions of the plasma membrane, visualised by electron microscopy (Heuser, 1980), are coated on their cytoplasmic surface with a coat composed mainly of the protein clathrin arranged in a tesselation of hexagons. It is in these regions that stimulated receptors (i.e. receptors that have bound their substrates) concentrate, possibly by some cross-linking mechanism involving transglutaminase (see

Chapter 6), prior to internalisation. An energy-dependent rearrangement, changing some of the hexagons to pentagons, is then thought to be involved, and this induces an increasing degree of curvature in the previously flat protein coat, since it is geometrically impossible for hexagons and pentagons to be mixed in a two dimensional lattice. Exactly why invagination rather than evagination occurs is unclear, but may reflect the possible involvement of microtubules or microfilaments in the vesicle-formation process.

It is at this stage of endocytosis that current opinions diverge. Traditional views involve the completion of vesicularization by the clathrin coat followed by movement of the coated vesicle away from the membrane and into the cytoplasm. Such vesicles could then exocytose their contents at the lateral or basal membranes (Wild, 1974) or fuse with other cell organelles. Since coated vesicles are traditionally assumed to be 'special', they were thought not to fuse with the lysosomal system (Pearse, 1980) but to proceed instead to other, more specialised, regions of the cell. Upon arriving at their destination the clathrin coat would be lost and would return to the surface membrane in the form of soluble clathrin monomers or trimers.

With the advent of the concept of a receptosome, this model became substantially modified. According to Helenius et al, (1983), coated vesicles are formed at the cell surface as outlined above, and move quickly into the cell. The protein coat is lost from the vesicle within the first minute and the clathrin recycled to the plasmalemma as before. Fusion of the endocytic vesicles with receptosomes allows sorting of the ligands and their individual redirection to different subcellular targets in uncoated vesicles, although some workers (e.g. Steinman et el, 1983) claim that even vesicles forming from receptosomes may carry a clathrin coat.

The other main current hypothesis is that proposed by Pastan and Willingham (1983). Despite the assertion of Helenius et al, (1983), that 'recent serial section analysis has unequivocally demonstrated the existence of endocytic coated vesicles as independent organelles', Pastan and Willingham (1983) believe that 'all coated structures which appeared morphologically in electron microscopic images to be coated vesicles located near the plasma membrane were functionally in communication with the cell exterior ... the explanation is that the necks connecting these

coated pits to the cell surface were out of the plane of section examined'.

Basing their argument esentially on their inability to detect inside fibroblasts any clathrin that is not associated with the surface membrane, and on the lack of effect on pinocytosis of micro-injection of anticlathrin antibodies, Pastan and Willingham deny the existence of free coated vesicles, instead preferring a mechanism of vesicle formation that involves retention of clathrin at the cell surface. They propose the existence of very deep clathrin coated pits in which occupied receptors congregate. Eventually - with no evidence presented or specifically quoted - they claim that the 'neck' of the coated pit constricts until it becomes so narrow that the coated part of the pit is still physically, but no longer functionally, in communication with the exterior. Basing their argument on one rather vague electron micrograph they propose the formation out of the semi-sealed coated pit of an uncoated vesicle. This vesicle contains the internalised substrates and carries them off to the receptosome for sorting.

In essence the hypothesis of Pastan and Willingham is an attempt to correlate the observation of apparently free coated vesicles with the absence of clathrin in the cytosol. The mechanism they propose, however, is open to severe criticism. Since the function of the coat is thought to be physically to support the formation of the endocytic vesicle, to propose the formation of an uncoated vesicle alongside the coated region (while the invagination is still in communication with the exterior), negates the whole assumed purpose of the coat unless two conditions are fulfilled. First, the neck of the coated pit must be sufficiently constricted to render unimportant the quantity of material that can pass through it during the uncoated vesicle formation step. Second, the coated region of the pit must be able to contract, in effect to 'blow up' the uncoated membrane into vesicular form. There is no known mechanism for coated pits to contract in a suitable manner, but unless both these criteria are met there appears to be no energetically feasible way that the uncoated vesicle could be formed.

Another possibility would be for the clathrin structure to be involved in vesicle formation but to spontaneously disassemble as vesicle formation is achieved, thus remaining associated with the cell surface. This would explain the inability to detect free clathrin in the

cytoplasm, but would not account for the coated-vesicle-like structures so often observed deep inside the cell by electron microscopy (e.g. King 1982b, Salisbury et al, 1980).

On the other hand, if the function of the clathrin coat is simply to provide a scaffold in order to allow vesicle formation, then, since any membrane reaching the receptosome from the cell surface must be recycled, and since it appears to be energetically more efficient to do so with the membrane in the form of vesicles rather than broken fragments, there may be a requirement for clathrin on the outside surface of the receptosome to allow formation of membrane vesicles for recycling. Since there has been no suggestion to date of a clathrin coat on the receptosome (Bequinot et al, 1983), this would necessitate plasma membrane-derived vesicles carrying their own coats with them in order to reform after fusion with the receptosome for membrane recycling. It seems unlikely that the methods of Pastan and Willingham would detect coated vesicles themselves, since vesicles are unlikely to be agglutinated by anti-clathrin antibodies, and even if they were observed by electron microscopy to interact specifically with anti-clathrin antibodies they would probably be interpreted by these workers as cross sections of deep coated pits.

If the assumption is made, however, that a supportive clathrin scaffold is always necessary for vesicle formation, then every vesicle formed must either carry a clathrin coat or be formed from an organelle that is itself coated (in the latter case the clathrin, after vesicle formation, might remain with the parent organelle). This would be necessarily true, by current concepts, of vesicle formation from receptosomes during subcellular direction of substrates following sorting. The morphological evidence available does not support either hypothesis, however, suggesting that the original assumption (the necessity of clathrin involvement in vesicularization) is invalid. It seems inescapable that some vesicles can form without the aid of a clathrin scaffold, and there are various feasible mechanisms for this. The most simple would be where the material to be encapsulated migrates into the base of a membranous tube whose walls then constrict and cause vesicularization. Reformation of the original tube could be accomplished by lateral growth of the membrane and hence the only novel requirement for such a method of vesicularization is the facility for the membranous tube to constrict locally.

Other mechanisms of non-coated vesicle formation might involve local expansion of the membrane to cause floppy regions that could be transformed into vesicles either by cytoskeletal involvement or perhaps by some form of local pressure effects. Such mechanisms are completely speculative, however, and a great deal remains to be understood of the precise mechanisms of endocytosis.

A principle that has recently become clear and met with widespread acceptance concerns the acidification of endocytic vesicles before they fuse with the lysosomal system. Until recently it has always been assumed that internalized substrates do not experience an acid environment until they reach the lysosomes, but various workers have now shown this to be incorrect. Helenius et al (1982) have shown that an acid environment is essential for Semliki Forest Virus infection since it catalyses the fusion of the viral envelope with cell membranes and thus releases the neucleocapsid into the cytosol. The presence of weak lysosomotropic bases inhibits such infection, presumably by raising the pH of the various vacuoles, but administration of bases 3-4 min after viral infection has no effect, the virus at that stage having already apparently reached acid compartments. Nucleocapsids are observed in the cytosol 10-15 min after infection but degradation of viral glycoproteins does not commence until at least 30 min after endocytosis. The inescapable conclusion is that the virus encounters a pre-lysosomal acid compartment, and this concept has received direct support from Galloway et al, (1983), who observed, in vitro, that purified endosomes decrease their internal pH by an ATP-dependent mechanism. The implicated H^+-ATPase is similar to that also detected in coated vesicles and it is thought that that could be derived from the plasmalemma.

The phenomenon of pre-lysosomal acidification adds new dimensions to our understanding of lysosome action, and it begs the question of primary lysosomal pH. It now seems feasible that the primary lysosome may have a relatively high pH, and hence the contained enzymes would be essentially inactive until fusion occurs with a substrate-containing vesicle (this perhaps explains why primary lysosomes do not seem to digest their own enzymes).

The purpose of pre-lysosomal acidification is thought to be concerned with recycling of internalized receptors since low pH often favours dissociation of substrates from their receptors (e.g.

phosphomannosyl glycoproteins (receptors for lysosomal enzymes), mannosyl glycoproteins and asialoglycoproteins. Low pH causes transferrin to dissociate from its iron cargo). Hence a fall in pH could facilitate recycling of receptors to the cell surface before they became exposed to degradative enzymes. This is a useful hypothesis, but begins to get extremely complicated when the pathway of substrates not intended for lysosomal degradation is considered. It seems likely that such materials will be directed towards their specific subcellular destinations before they dissociate from their specific receptors - otherwise the whole purpose of the specificity is lost - and therefore they must either never reach the receptosome or must leave it before it becomes appreciably acidic.

The original hypothesis of Brambell (1966) for the mechanism of transcellular passage of IgG specified the association of intact IgG first with macropinosomes, then with secondary lysosomes and later with an unspecified vehicle mediating exocytosis. One problem with this model is to suggest a feasible mechanism of release of intact IgG from secondary lysosomes into the extracellular compartment without loss of lysosomal enzymes and undegraded contents of lysosomes. The selective escape of the IgG from the lysosomes - either by leakage, lysosomal rupture or transmembrane transport - followed by diffusion of the antibody across the cell and its intact release at the lateral or basement membranes, comprises a possible model for transport. However, since the direction of diffusion would be random, such a mechanism would probably be extremely inefficient. Also, the observed sensitivity of the release mechanism to metabolic inhibitors (Chapter 6) suggests that the actual process is likely to be more complicated than this. Possibly IgG for release is packaged, after entry into lysosomes, into small vesicles that are formed by clustering of the immunoglobulin molecules on, and subsequent pinching off of, the lysosomal membrane. Alternatively, pieces of lysosomal membrane carrying IgG still attached to receptors may be directed towards and fuse with the lateral or basal plasma membranes, releasing the IgG into the subcellular mesenchyme. In each case the mechanism of direction of the IgG-containing vehicle towards the lateral or basement membrane is unclear, particularly in view of the apparent insensitivity of the process to cytoskeletal inhibitors (Chapter 6).

Wild's original hypothesis, on the other hand, suggested the involvement in the transport process of just one type of vehicle, the

micropinocytic clathrin-coated vesicle, formed at the brush-border plasma membrane, that travels to and fuses with the lateral or basal membranes.

Hence, in their original forms, the two hypotheses implicate two entirely different types of organelles in the transcellular transport of IgG. Wild invokes coated micropinocytic vesicles throughout, and Brambell suggests macropinocytic vesicles and either microvesicles or simple pieces of detached membrane. There are thus two important differences between these mechanisms that might be detected by the use of subcellular fractionation:

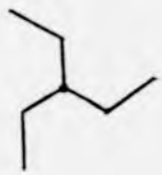
(i) Brambell's hypothesis claims at least transitory association of protected, intact IgG with secondary lysosomes.

(ii) In contrast, Wild's hypothesis invokes clathrin-coated micropinocytic vesicles that remain separate from the lysosomal system throughout.

When Wild (1975) first suggested the involvement of coated vesicles he envisaged the protein coat acting to prevent fusion of the vesicles with the lysosomal system and thus allowing them to cross the cell intact, finally to fuse with the basal plasma membrane. Other workers (eg. King, 1981; Huxhem and Beck, 1981) continue to support this view, but always with evidence based on electron microscopy.

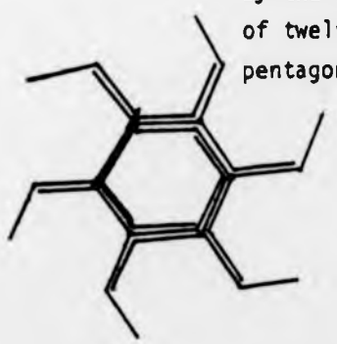
In addition to evidence from electron micrographs of cross-sections of cells that show small structures thought to be coated vesicles (eg Yu Fan, 1982), coated vesicles have been isolated as very pure preparations from cell homogenates (eg. Pearse, 1976), and their structures examined. In appearance a coated vesicle is a fairly regular polyhedron with a large number of hexagonal faces and twelve pentagonal ones. The major constituent of the coat is the protein clathrin (mol.wt.approx. 180 000) which comprises 40% of the total weight of the coat (Pearse, 1975), and the functional unit is the triskelion, which is a trimer of clathrin (Figure 7.1.1), in association with three lighter proteins. The lighter proteins are of two types, alpha and beta, with molecular weights of 36 000 and 33 000 respectively (Ungewickell and Branton, 1981). They are found in a ratio of 1:2, though whether or not each triskelion contains one alpha and two beta chains is not yet clear. The light chains seem to be located near the centre of the triskelion, and their purpose may be to

Figure 7.1.1



Clathrin trimer - the building block of the protein coat. Each trimer consists of three molecules ofClathrin arranged in a flat plane at 120°, and held rigid by various binding proteins. Full details are given in the text.

Overlapping the trimers as shown (or in a number of possible alternative structures) gives rise to a tesselation of regular hexagons. This would be a flat structure, and it is thought to be transformed to a vesicle by the gradual transformation of twelve of the hexagons to pentagons.



maintain it in its rigid state, with all three clathrin molecules in the same orientation. This has been suggested (Ungewickell and Branton, 1982) by elastase treatment of triskelions, which specifically attacks the light chains. Such treatment causes the triskelions to become less rigid and eventually to lose their 'handedness'(Schmid *et al*, 1982). The light chains are essential for polymerisation of the triskelions to form coated pits or vesicles.

Each of the three clathrin molecules of a triskelion extends a total of 44nm away from its centre, with a sharp kink at 16nm. If the centre of another triskelion is situated on each of these kinks, with one clathrin molecule pointing back towards the centre of the original triskelion, and so forth, a regular tessellation of hexagons is formed. Each side of the tessellation is formed of four strands - the proximal parts of the clathrins extending from the two nearest triskelions, and distal parts from the two triskelions one-step-removed (Crowther and Pearse, 1981). It is possible to form pentagons in a similar way by incorporating only a small amount of strain into the structure. Triskelions are thought to be attracted, by an unknown signal, to regions of the membrane where aggregation of specific receptors has occurred, and polymerisation occurs on the inside surface of the membrane to form a coat that gradually developes into a coated pit. It is presently unclear whether the coat itself has a role in causing the aggregation of receptors or whether its formation is indeed subsequent to the process of clustering. The coats of such coated pits are composed almost entirely of hexagons. It is proposed that at the time of endocytosis twelve hexagons change to pentagons and a coated vesicle is formed (Heuser, 1980) that is no longer part of the cell membrane. If the coat is now lost quickly it seems that its function is simply to serve as a scaffold for the construction of a vesicle out of a piece of flat membrane. It is conceivable that this is the only function of the coat, and that coated vesicles have a relatively short lifetime *in vivo*.

The release of clathrin from *in vitro* preparations of coated vesicles has been variously reported to be dependent on ATP and on an unidentified factor present in cytosol (Patzer *et al*, 1982), and to be induced by 0.5M Tris-HCl (Keen *et al*, 1979), 2M Urea, 0.25M $MgCl_2$ or pH 7.5 (Woodward and Roth, 1978). Optimal conditions for coat formation are reported (Crowther and Pearse, 1981) to be pH 6-6.5, in the presence of 20mM divalent cations. Crowther and Pearse (1982) also observed a

critical concentration of clathrin (50 ug/ml) below which coats disintegrated, though Jaarsfield et al (1981) found no evidence for this. In short, subtle control of these and other factors in the living cell may regulate the fate of the coat, and its stability in vitro may be a non-physiological result of the preparation technique employed. Hence, to demonstrate the absence of a protein coat on isolated ^{125}I-IgG-containing vesicles would not conclusively discriminate between the two original hypotheses, since a pre-existing coat may simply have been lost during the isolation procedure. On the other hand, the demonstration of the presence of a protein coat would shed very little light on the actual route of transmission, since the idea of a protein coat could simply be incorporated into a modified version of the Brambell Hypothesis. Nevertheless, demonstration of the involvement of coated vesicles in the process of transmission would represent an important step forward in our understanding of the mechanisms involved in this process of diacytosis and, experimentally, the coat-proteins would give a useful marker with which to identify a specific subpopulation of such vesicles.

Most of the evidence concerning the subcellular localisation of IgG has been obtained by electron microscopy of cells containing IgG conjugated to a marker molecule that can be detected by electron microscopic techniques (eg. IgG conjugated to ferritin). However, the presentation of such evidence can reflect the choices and interpretations of the individual experimenters. (Even the most honest workers are likely to present for publication those micrographs that most clearly illustrate and support their own hypotheses. Strictly, the interpretation of micrographic evidence should be based more on the large number of photographs that are never published, and the validity of the published conclusion is dependent on the ability of the author to give equal weight to all relevant micrographs of the same technical standard, and not to concentrate on the ones that show most clearly the features of interest). It was hoped that one of the strengths of the current study would be to obtain evidence based on techniques other than electron microscopic analysis in order to support or refute the suggestions made from ultrastructural studies.

Subcellular fractionation of homogenates of yolk sacs containing ^{125}I-IgG might yield information on the intracellular localisation of the antibody. A high level of intact ^{125}I-IgG in the lysosomal fraction might be viewed as strong pro-Brambell evidence, and a high level in the

microsomal fraction might allow characterisation of microvesicles carrying intact IgG and involved in the transmission process. For example, if the ^{125}I-IgG present in the cell is inside vesicles that sediment in the microsomal fraction, then preparation of the microsomal pellet under isotonic conditions might well preserve most of these vesicles intact. This in turn could lead to the bulk of the isolated ^{125}I-IgG being unavailable for interaction with applied anti-IgG antibodies. On the other hand, the ^{125}I-IgG present in the pellet might be bound to broken pieces of membrane. Some of this membrane may or may not have sealed to form vesicles with the IgG either on the outside or the inside surface. In the former case a substantial proportion of the IgG would be expected to be available for immunological interaction, and the application of a suitable anti-IgG antibody or an antigen to the contained IgG would yield useful information on the subcellular localisation of the immunoglobulin. If, on the other hand, it is shown that the IgG is unavailable for antibody-antigen interaction, and therefore probably contained within intact vesicles, then an investigation into the nature of the protective mechanism seems possible. If the IgG is contained inside a lipid membrane, then osmotic shock, detergent or freezing and thawing would be expected to expose the IgG for reaction. However, a complex membrane, perhaps including a protein coat, would possibly withstand such treatment and a more severe technique might be required to expose the IgG. Urea (2M) is reported (Woodward and Roth, 1978) to remove the protein coats from coated vesicles, and proteinases might be expected to have the same effect, perhaps leaving the IgG available for interaction with antibodies in the surrounding fluid.

Reincubation of ^{125}I-IgG-charged yolk sacs at low temperatures has been shown selectively to inhibit the release from the tissue of degraded material more than the release of macromolecular material (see Chapter 6).Such preincubation prior to homogenisation and centrifugation might yield useful information as to which subcellular compartments give rise to the release from the tissue of protected material. Alternatively, a comparison of the subcellular distribution pattern for native ^{125}I-IgG with that for formaldehyde-denatured ^{125}I-IgG (a treatment that has been shown to destroy its capacity for protection in the system, Ibbotson, 1978) also might elucidate which subcellular compartments are involved in diacytosis.

Another potentially useful line of investigation would be to incubate yolk sacs in medium containing ^{125}I-IgG for short times, and to observe the effects of the length of the pulse exposure on the subcellular distribution. Wild's hypothesis would predict a relatively high level of radioactivity in the microsomal fraction throughout, while Brambell's would predict an early concentration in the lysosomes, with a time-dependent shift to the microsomes. This latter situation might prove impossible to distinguish, however, from a mechanism involving intracellular sorting of substrates after uptake into a prelysosomal compartment, a possible mechanism that differs substantially from that proposed originally by Brambell, but which has certain features that would be difficult to differentiate experimentally from those of the Brambell model. The major discriminatory feature between the two is that substrate would be expected to reach an endosomal compartment within the first few minutes after internalization, while experimental observations (Livesey, 1978) suggest that material for degradation does not reach the lysosome of the rat yolk sac until approximately ten minutes after the commencement of internalization. Hence an early accumulation of substrate in 'lysosome-like' vesicles might represent material actually present inside endosomes, whle a later 'lysosome-like' accumulation would be compatible with material eventually passing from small endocytic vesicles into lysosomes.

The aim of this chapter was to begin to investigate the potential of subcellular fractionation studies as a means to probe the route of transmission of intact ^{125}I-IgG through the cell. In particular, preliminary experiments were aimed at:

(i) Developing a useful and reproducible scheme for the subcellular fractionation of yolk sacs.

(ii) Observing the organelles involved in transport of intact ^{125}I-IgG, and attempting to characterise them and the nature of their association with IgG.

(iii) Observing any time-dependent changes in the subcellular distribution of ^{125}I-IgG.

MATERIALS AND METHODS

7.2.1 Subcellular Fractionation Techniques.

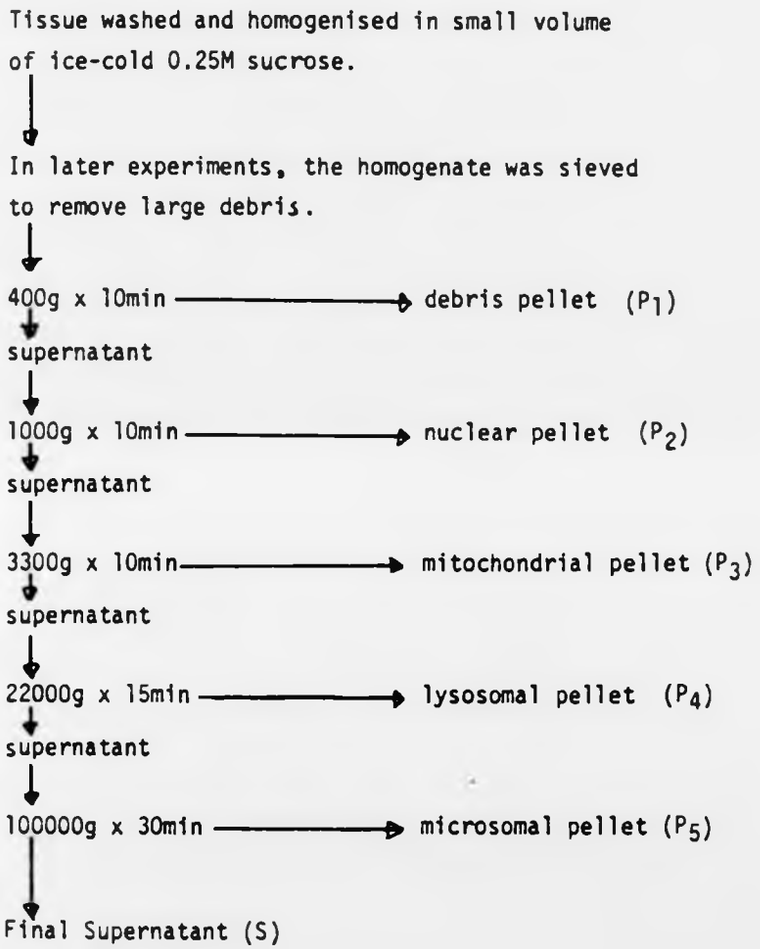
The basic techniques employed to fractionate the yolk-sac tissue were as described below. Minor variations to the technique are also described.

Homogenisation was usually performed in 0.25M sucrose, pH 6.0 (Goetze et al, 1976), but in certain experiments distilled water, 0.1% Triton X-100 or 2M urea were used instead. In the initial series of experiments, tissue was disrupted by using a motor-driven Tri-R homogeniser set to speed 4 for three double passes using a Teflon-on-glass homogeniser, but after a comparison of different methods (Section 7.3.3), hand homogenisation was routinely adopted. In later work the homogenate was strained through cotton gauze to remove pieces of undisrupted tissue. (The effect of this procedure is to reduce the size of the P_1 pellet, and probably to make it more representative of the nuclear material it is assumed to contain by removing unbroken cells and connective tissue that would spin down at very low speeds). Standard control experiments were carried out alongside every variation of technique to allow useful comparison of data gathered by slightly different experimental regimes.

The regime used for subcellular centrifugation was slightly modified from that of Williams et al (1971), and is outlined in Figure 7.2.1. Pellets were either dissolved in 1M NaOH to permit determination of their content of radioactivity and protein, or gently resuspended in various media (see Results) with a Teflon pestle that fitted snugly inside the centrifuge tube. This technique permitted fast, non-violent resuspension of even well-packed pellets.

Sometimes the first spin was omitted and the initial pellet was isolated at 10 000 gmin, and so contained nuclear material as well as any whole cells and debris that had not been removed from the homogenate by sieving. When the microsomal pellet was the only pellet of experimental interest, the first spin was often performed at $3x10^5$ gmin and the second at $3x10^6$ gmin. This gave just two pellets, one containing a mixture of cell organelles and debris, the other microsomes. This simple and rapid method for the isolation of the microsomal pellet was used regularly for

Figure 7.2.1 Differential centrifugation scheme used for the routine subcellular fractionation of rat and rabbit tissue homogenates.



the rat yolk-sac analysis reported in Section 7.3.1.

7.2.2 Marker enzyme assays.

Two marker enzymes were assayed in the yolk-sac fractions in order to clarify the distribution of organelles.

Arylsulphatase

Arylsulphatase is a lysosomal enzyme that hydrolyses various organic sulphates. The assay employed here measures the hydrolysis of nitrocatechol sulphate; the free nitrocatechol is highly coloured and easily measured by its extinction at 540 nm. In each estimation, known small amounts of resuspended pellet were added to 0.05M acetate buffer (pH 5.0) containing nitrocatechol sulphate and Triton X-100 (final concentrations of 5mM and 0.05% v/v, respectively), and the mixture incubated for 10 min at 37°C. Reaction was stopped by the addition of NaOH (final concentration 0.17M) and the amount of free nitrocatechol produced was estimated by measuring the absorbance at 540nm against water blanks and standards. This permitted calculation of the relative amounts of arylsulphatase present in the different fractions, and hence shows the distribution pattern in the homogenate of a typical lysosomal enzyme.

Glucose-6-phosphatase

Glucose-6-phosphatase has been used as a marker for liver microsomes. It was only detected in relatively small amounts in the yolk sac but has been used in this study on the assumption that it is characteristic of the microsomal fraction of the yolk sac. It hydrolyses glucose-6-phosphate to produce free glucose and inorganic phosphate, and the amount of free glucose is measured in the assay by the glucose oxidase method. Small (known) volumes of resuspended pellet are mixed with malate buffer (pH 6.0) containing glucose-6-phosphate (final concentrations 0.015M and 10mM, respectively) and incubated for 4h at 37°C. The reaction is stopped by heating the mixture briefly to 100°C, cooled, and then two volumes of TGO reagent (see below) are added. The tubes are incubated at 37° for 30 min, to allow the reaction to go to completion, and then an equal volume of 5M HCl is added. The extinction is measured at 525nm and corrected for blank readings (obtained by boiling the original homogenate before use). This corrects for the

presence of any free glucose, including any contaminating glucose present in the isotonic sucrose.

The TGO cocktail is a mixture of enzymes designed to detect glucose by quantitatively linking its oxidation to that of the chromogen dianisidine. The reagent is made as follows;

100g NaH_2PO_4, 72.6g Tris is adjusted to pH 7.0 with HCl and made up to 1l.

Glycerol (800ml) is added and the volume made up to 2l with water before 600mg glucose oxidase is stirred in, together with 60mg peroxidase. Finally, 200mg dianisidine, dissolved in a little warm water, is added, and the reagent is ready for use. It is best stored at low temperatures and used within 24h.

There must be reservations, however, concerning the interpretation of data obtained in this way since the assay will detect any glucose-6-phosphatase activity, including that mediated by non-specific phosphatases, and not just the activity of the pertinent enzyme.

7.2.3. Immunological techniques.

Due to technical problems with the commercial antibody preparations (they were not affinity-purified, and therefore not sufficiently specific to be used in the desired manner) no useful results were obtained using immunological techniques. Nevertheless, considerable time was spent in the design and developement of a potentially useful method, which is reported here as a possible basis for the design of more conclusive future studies.

Two regimes were used to demonstrate the availability - or otherwise - of immunoglobulins associated with the microsomal pellet for reaction with antibodies in the surrounding fluid. Each utilized the ability of the immunoglobulin to react with a radiolabelled species added after the pellet had been isolated and resuspended.

In the first regime (Figure 7.3.3), rat yolk sacs were preincubated in the presence of unlabelled rat IgG (5 ug/ml, 90 min, 37°C); matched controls were incubated in the absence of any such substrate. The washed tissue was homogenised in 0.1% Triton X100 and the microsomal pellet

isolated in the usual way. Pellets were resuspended in distilled water containing 5% (v/v) foetal calf serum (to combat nonspecific interaction of the IgG with the pellet), and a small amount of radiolabelled rabbit (anti-rat IgG)-IgG (final concentration of 5.6 ug/ml) was added. After incubation at 37°C for 15 min and measurement of the total contained radioactivity, the pellet was reisolated. The protein content of the pellet was estimated, in the usual way, and the contained radioactivity was assayed. Hence the binding of the ^{125}I-(anti-rat IgG)-IgG to the pellet was also estimated.

Significant differences in the amount of binding between experimental and control pellets might indicate specific binding of the ^{125}I-(anti-rat IgG)-IgG to the contained (unlabelled) rat IgG. The implication would then be that the rat IgG contained in the pellet is available for reaction, and therefore not present inside closed vesicles.

In the second regime (Figure 7.3.4), rabbit (anti-rat IgG)-IgG was used as the original (unlabelled) substrate. Experimental yolk sacs were incubated (5 ug/ml, 37°C, 90 min) in the presence of this substrate, control yolk sacs were incubated in the presence of nonspecific rabbit IgG, and homogenisation was in 0.25M sucrose, pH 6.0, the microsomal pellet being isolated in the usual way. After resuspension in distilled water containing 5% (v/v) foetal calf serum, the preparations were briefly frozen in liquid nitrogen before a small amount (final concentration 1.7 ug/ml) of ^{125}I-IgG (rat) was added. Following incubation for 15 min at 37°C and measurement of total contained radioactivity, the pellets were reisolated and their protein content and radioactivity measured as before. Any relative concentration of radioactivity in the experimental pellets, compared with the control pellets, was taken as evidence of the contained substrate being available for reaction and therefore not inside intact vesicles.

The design of the second regime is better in theory, since results obtained using the first might be misleading for the following reason. Exposure of substrate IgG to the second antibody (radiolabelled IgG anti-rat IgG) in test incubations implies that the corresponding empty receptors would be exposed to the second antibody in control incubations. That being so, the second antibody could bind to the test fractions in its capacity as (anti-rat IgG)-IgG (i.e. via its antigen binding sites), and it could bind to controls in its capacity as IgG molecule (i.e. via

its F_c region). Hence any result obtained using the first regime would be open to a variety of interpretations. Another advantage of the second regime is that the interaction makes use of the antigen-binding regions of the primary substrate, rather than its antigenic determinants (which lie primarily in the F_c region). Since the primary substrate is likely to be membrane-bound through its F_c regions, exploitation of its antigen binding capacity is more likely to give a successful interaction.

The functional integrity and specificity of the antibodies was checked by simple precipitation techniques. Solutions of equal concentration of antibody and antigen were placed in adjacent wells in agar gels (six wells were arranged in a circle around one in the middle. If the antibody was put into the middle well six antigens could be tested simultaneously). A line of precipitation in the gel was indicative of reaction.

Alternatively, careful decanting (without mixing) of equal-concentration solutions of antibody and antigen into a thin glass tube caused visible precipitation of complexes at the interface between the two solutions when reaction occurred.

Since the results obtained were inconclusive, perhaps because of the heterogeneity of the antibody preparations, no results are presented here.

7.2.4 **Presentation of results.**

The quantities of radioactivity and of enzyme reported in the Results section are expressed in two ways. The most simple is as ng of radioactive species-equivalent per mg yolk-sac protein (ng/mg). This form has only been used for radioactivity, usually to represent the content of substrate in subcellular fractions.

Activities were also expressed as 'relative specific activities' (sometimes referred to in the text as 'specific radioactivities') which are defined, for each component, as the percentage of the total enzyme activity present in that fraction divided by the corresponding percentage of the total tissue protein. This form has been used to normalise results from different experiments to facilitate comparison.

i.e. % total activity / % total protein = relative specific activity.

7.2.5 Formaldehyde treatment of rabbit IgG.

A modification of the method of formaldehyde treatment described by Moore et al (1977) was used to modify the structure of rabbit IgG and hence to alter its behaviour in tissues incubated in vitro.

A solution of rabbit IgG (1.0 mg/ml) was mixed with an equal volume of 10% formaldehyde in 0.5M $NaHCO_3$ buffer, pH 10, and reaction continued for 72h at room temperature. The mixture was then dialysed against three changes of 1% (w/v) saline at 4°C for a total of 48h, and stored at -20°C.

7.2.6 SDS-PAGE analysis of pellet-associated proteins.

To analyse the proteins contained in pellets obtained by subcellular fractionation of yolk sacs, the pellets were resuspended in a small volume (0.5 ml)of distilled water and freeze/thawed in liquid nitrogen before being denatured with mercaptoethanol and SDS in the usual way (Section 2.2.16 (b)).

Since electrophoresis of these pellets was expected to show the presence of a wide range of proteins, a good separation was important, and therefore it was decided to run the rectangular gel along its full length rather than across it in the usual way. Unfortunately no slot former was available that would form the slots at the end of the gel, and so blank gels were formed and the slots cut by hand in the required positions. A drop of polyacrylamide solution was then added to each slot to seal the bottom in order to prevent the sample from moving between the gel and the supporting glass plate by capillary action. Samples were applied in the usual way and no further modifications were made to the procedure outlined in Section 2.2.16, except that the running times were extended because of the increased path-length.

7.2.7 Experimental animals.

The work reported in this chapter was performed over a long period of time using rabbit tissue when it was available, and substituting with

rat tissue when rabbits were not available. The first series of experiments reported, using the simple centrifugation scheme, was performed with rat tissue only, while later work was performed variously on both rat and rabbit tissue. Hence the results must be viewed carefully with regard to the experimental species indicated in the text.

RESULTS

7.3.1 (a) Initial investigations into the subcellular distributions of various substrates in homogenates of rat yolk sacs.

In the initial series of exploratory experiments a simple centrifugation scheme was used to allow the fast routine isolation of a microsomal pellet from homogenates of rat yolk sacs. (Rat tissue only was used at this stage because of the preliminary nature of the experiments and the more pressing demand for rabbit tissue elsewhere in the studies. In this centifugation scheme only two spins were performed in order to isolate only the P_5 and supernatant fractions.

Three substrates were used, and the patterns obtained exhibited certain reproducible features of interest (Figure 7.3.1). ^{125}I-IgG invariably showed a high concentration in the P_5 pellet, while ^{125}I-PVP and ^{125}I-BSA were not detected in this pellet at anything like the concentration observed for ^{125}I-IgG. Indeed, for these other substrates, the microsomal pellet contained the lowest concentration of the three fractions isolated. More violent homogenisation led to both of these latter substrates becoming more concentrated in the supernatant fraction, suggesting the possibility of their initial containment in vesicles, perhaps small lysosomes. or pinosomes that could be ruptured, thus releasing their contents.

It is conceivable that the characteristic of the ^{125}-IgG responsible for its relatively high concentration in the P_5 pellet may also be concerned with its transcellular transport, a characteristic not shared by the other substrates. Therefore some preliminary investigations were made into the characteistics of its situation. These investigations were primarily comparisons of the behaviour of P_5-associated ^{125}I-IgG with that of other P_5-associated substrates in order to attempt to elucidate the special features responsible for its concentration in this pellet.

(b) The nature of the supernatant-associated radioactivity.

After homogenisation, more of the non-sedimentable radioactivity was found to be soluble in TCA when the substrate was ^{125}I-BSA (58.2% $\pm$ 4.3, n=6) than when it was ^{125}I-IgG 35.8% $\pm$ 1.8, n=6). The total amount of both substrates associated with the tissue was approximately equal, and

this difference may reflect the process of protection of IgG, with antibody being released during homogenisation from its protected site. Alternatively it may simply be caused by the IgG being less quickly degraded *in vitro* than BSA, and hence many TCA-insoluble fragments of ^{125}I-IgG might be released from the tissue during the homogenisation procedure due to the bursting of secondary lysosomes.

(c) The effects of resuspension in various media on the sedimentability of ^{125}I-PVP originally associated with the P_5 pellet.

When the P_5 pellet was resuspended in 0.1% Triton X100 prior to resedimenting of the pellet, virtually all (98.4 ± 1.7%) of the pellet associated ^{125}I-PVP was left in a non-sedimentable form. When the resuspension medium was distilled water 95.7 ± 3.2% of the ^{125}I-PVP was released into free solution, but when 0.25M sucrose was used, only about 70% (68.6 ± 1.8%) of the original ^{125}I-PVP remained in a non-sedimentable form.

This suggests that about 30% of ^{125}I-PVP present in the P_5 pellet under these conditions is held there by mechanisms that are susceptible to disruption. This is compatible with approx. one third of the ^{125}I-PVP being contained inside membrane-bound vesicles. The remainder of the ^{125}I-PVP is probably trapped in the pellet in the fluid phase or adsorbed to membranes. In any case it is released upon resuspension of the pellet in sucrose solution (0.25M). These results were reproducible.

(d) The effects on microsome-associated substrates of resuspension in Triton X100.

In order to assess whether the P_5 pellet-associated ^{125}I-IgG and ^{125}I-BSA were similarly released by membrane-disruption, the effect of resuspension of the pellet in detergent was examined.

Resuspension of the P_5 pellet in 0.1% Triton X100 was shown to liberate virtually all of the pellet-associated ^{125}I-PVP in a non-sedimentable form (Table 7.3.2). Similar treatment of native ^{125}I-BSA was found to release about two thirds of the total radioactivity into free solution, but when ^{125}I-IgG was used, less than 30% of the original pellet-associated radioactivity was found to be in a non-sedimentable form. (Freeze-thawing in liquid nitrogen had essentially similar effects

to the presence of Triton X100 (Table 7.3.2). The Triton X-100 results were reproducible, but the freeze-thawing experiment was performed on one occasion only with a number of replicates, as indicated.)

(e) **The effects of urea-treatment on the P_5 pellet-associated ^{125}I-IgG.**

The demonstration that not all of the P_5 pellet-associated ^{125}I-IgG was released by detergent action led to investigation into the effects on the sedimentibility of the substrate of treatment of the resuspended pellet with urea.

Resuspension in 2M (aq.) urea was found to decrease substantially the proportion of the original P_5-associated ^{125}I-IgG-derived radioactivity that remained in a sedimentable form . (Table 7.3.3 reports one typical experiment in detail, while Table 7.3.4 is a summary of a number of experiments.) Further investigation revealed, however, that a large proportion of the non-IgG proteins associated with the P_5 fraction were being solubilised by the urea, and that no obvious specific release from the pellet of ^{125}I-IgG was occurring (Tables 7.3.3 and 7.3.4).

Freeze/thawing of the resuspended pellet (in 2M urea) was found to increase the amount of ^{125}I-IgG released into solution, possibly by bursting residual lysosomes, but the effect was very small (Table 7.3.3). These results are preliminary, the experiment being performed on one occasion only. (A recurrent problem with results presented in these sections is that often minor improvements were made to experimental regimes between replicates. At the end of the day this has given a large amount of data relating to slightly different experimental regimes and are therefore not strictly cumulative. It is essentially in this sense that these particular results must be termed 'preliminary' - because the number of exact replicates of the reported experiments is small.)

(f) **The nature of the ^{125}I-IgG-derived material released by resuspension of the P_5 pellet in urea solution.**

When the P_5 pellet was resuspended in 2M (aq.) urea, and the radioactivity released into free solution analysed, it was found to be largely (92.9 $\pm$0.4%) insoluble in TCA; a high percentage of the radioactivity released by resuspension in water was found also to be TCA-insoluble (84.1 $\pm$ 1.3%). However, 2M (aq) urea caused the release of

about five times as much radioactivity (60.7 ± 0.14% of the total radioactivity, compared with only 12.5 ± 1.1% for resuspension in water).

These results were again preliminary (n=3), but are compatible with much of the radioactivity being released from a non-degradative compartment of the cell.

(g) **The subcellular distribution of ^{125}I-IgG added to the homogenate shortly before centrifugation.**

The results reported so far suggest that the association with the P_5 pellet of ^{125}I-IgG is rather stronger and more extensive than is the association with that pellet of ^{125}I-PVP or ^{125}I-BSA. It is possible that the association may be mediated by an IgG-specific receptor, and it would be of considerable interest to ascertain whether the putative receptor is available for reaction in the resuspended pellet, or whether it is contained inside intact or sealed vesicles. To that end, ^{125}I-IgG was added to the homogenate prior to centrifugation, and its distribution examined.

When homologous ^{125}I-IgG (2ug/ml) was added to an homogenate of rat yolk-sac tissue in 0.25M sucrose, the medium containing a large amount (350x as much as IgG, w/w) of unlabelled calf serum, added in order to minimise non-specific interaction of the ^{125}I-IgG with the pellet, the subcellular distribution of radioactivity described in Table 7.3.5 was obtained. The high concentration of ^{125}I-IgG in the P_5 pellet, so often observed when the substrate was added to the yolk sac during an incubation step, was again present.

When the concentration of ^{125}I-IgG was varied between 0.8 and 2.5 ug/ml of homogenate, the relative subcellular distribution remained almost constant (Figure 7.3.2), though at the higher concentrations a slightly greater percentage of the total radioactivity became associated with the supernatant at the expense of the pellets.

These results were reproducible in the sense that a number of slightly different experiments gave the same central conclusions, viz. the high concentration of ^{125}I-IgG, added after homogenisation and in the presence of excess unlabelled proteins, that was found associated with

the P_5 pellet. These results strongly suggest the existence in the P_5 pellet of an IgG-specific receptor. They also suggest that at least some of the receptor molecules are exposed and available for interaction - perhaps being held on broken pieces of membrane. The immunological investigation (outlined in Section 7.2.3, but sadly inconclusive due to technical problems) was aimed at further exploring the above suggestion.

(h) **The effect of the addition of competing unlabelled proteins to homogenates of rat yolk sacs that had been exposed to ^{125}I-IgG prior to homogenisation.**

An alternative way to investigate the resuspended P_5 pellet for available IgG-specific receptors was to homogenise rat yolk sacs (previously exposed to ^{125}I-IgG), to isolate the P_5 pellet and then to attempt to displace the ^{125}I-IgG with competing unlabelled proteins. Occupied specific receptors, if available for reaction, would be expected to release their bound ^{125}I-IgG in a competitive manner when challenged with cold homologous IgG.

When the P_5 pellet was resuspended in sucrose (0.25M, pH 6.0) neither the inclusion in the resuspension medium of 5ug/ml unlabelled rat gamma-globulins nor prolonged incubation of the resuspended material at 37°C caused any significant increase in the amount of ^{125}I-IgG-derived radioactivity released from the tissue. (The amount released was 16.5 $\pm$ 0.71% in control cases, 21.5 $\pm$ 6.4% with 5min reincubation in gamma globulins and 14.5 $\pm$ 0.71% with 60 mins reincubation).

The inclusion of a large amount of unlabelled IgG or BSA (Table 7.3.6 and 7.3.7) in the homogenisation medium and throughout the rest of the homogenisation procedure had no obvious effect on the subcellular distribution of radiolabelled IgG or BSA to which the yolk sac had been exposed prior to homogenisation. These results are preliminary in the sense that identical replicates of the experiment have not been performed, although the same overall conclusions have been drawn from a small number of experiments. In the first instance they suggest that there is no competition for binding, indicating that the interaction holding the ^{125}I-IgG to the pellet either is not a specific one, or the ^{125}I-IgG is not accessible under these conditions for competition. This is in apparent contradiction with the results of the previous section, and before too much weight is attached to these results further

replication is needed.

(i) **The effects on its subcellular distribution of increasing the incubation concentration of ^{125}I-BSA.**

One possible source of the high level of P_5-associated ^{125}I-IgG in homogenates of rat yolk sacs may be vesicularization of the plasma membrane during homogenisation. If the vesicles thus formed are small, and if the vesicularized membrane carries with it an appreciable amount of substrate, either previously attached to its surface or perhaps occluded between the microvilli and encapsulated during vesicularization, such a process may result in a high concentration of substrate in the P_5 pellet. One way to examine this possibility would be to expose yolk sacs to different concentrations of substrate and to observe the changes in apparent subcellular distribution, since increasing the amount of surface-associated substrate would be expected to increase (approximately linearly) the amount of material taken into the P_5 pellet by the process just outlined.

Doubling the incubation concentration of ^{125}I-BSA from 5 ug/ml to 10ug/ml caused its concentration in each subcellular fraction to increase as follows (Figure 7.3.5);

P_1 increased to 208%
P_2 increased to 188%
P_3 increased to 230%
P_4 increased to 187%
P_5 increased to 138%
Supernatant increased to 216%

Hence the P_3 pellet saw the greatest relative increase in its w/w content of BSA/mg total protein, and the P_5 pellet the smallest relative increase.

The observation that the amount of substrate associated with the P_1 pellet can more than double, while that associated with the P_5 pellet increases by only about 40% could be taken as preliminary evidence against the importance of plasmalemma-derived vesicles in the substrate complement of the P_5 pellet, since the P_1 fraction is expected to contain a large amount of surface membrane and related structures (largely as

unbroken cells) and would therefore seem likely to reflect a high concentration of substrate on the cell surface. Again, however, the results are preliminary and require further verification, perhaps using ^{125}I-IgG as substrate also.

(j) **The effect of exposing rat yolk sacs to cold rat IgG before and after incubation in ^{125}I-IgG on the subsequent subcellular distribution of ^{125}I-IgG-derived radioactivity.**

Incubation of yolk sacs in cold IgG prior to ^{125}I-IgG would be expected to saturate any permanent membrane-IgG binding sites, whereas post ^{125}I-IgG exposure of yolk sacs to cold rat IgG should substantially displace any temporarily bound ^{125}I-IgG on the cell surface. Hence yolk-sacs given this combined treatment should be essentially free from surface membrane-bound ^{125}I-IgG. There was no obvious effect of this combined treatment on the subsequent subcellular distribution pattern for ^{125}I-IgG-derived radioactivity (Table 7.3.8). The implication from these results, which were obtained in duplicate but which would benefit from further verification, is that the high level of P_5 pellet-associated ^{125}I-IgG may not result from vesicularization of substrate-bearing plasmalemma.

(k) **The effects of time of exposure to ^{125}I-IgG on the subcellular distribution of ^{125}I-IgG-derived radioactivity.**

A preliminary experiment showed that the subcellular distributions of ^{125}I-IgG-derived radioactivity in rat yolk sac homogenates show some slight changes as a function of the time of exposure of the yolk sacs *in vitro* to ^{125}I-IgG immediately prior to homogenization and subcellular fractionation (Figure 7.3.7). (Fractionation was by the more complex regime, outlined in Section 7.2.1).

The quantity of radioactivity associated with the tissue appears to reach a maximum after 30 min, and the typical distribution pattern seems to be established after only about 5 min exposure to substrate (Figure 7.3.6 (a)).

If the distribution is expressed as a relative specific activity, however, it shows some minor changes during the first hour of exposure (Figure 7.3.6 (b)). After 5 minutes exposure to ^{125}I-IgG the most

concentrated radioactivity is found in the P_5 pellet, with a strong concentration also in the P_4 pellet and a relatively low level detected in the supernatant.

By 20min the amount in the P_4 pellet has fallen slightly relative to the others, and that in the P_5 pellet is the most concentrated. The relative activity present in the supernatant has risen slightly, and continues to rise until 30 min, by which time that in the P_5 pellet has fallen slightly. After 60min, the relative specific activity present in both the P_4 and P_5 pellets has fallen, with the P_5 pellet remaining the most concentrated site of radioactivity, in terms of absolute weights, and the relative specific activity of the supernatant fraction has again risen. By this time, equilibrium is assumed to have been attained.

The changes with time in the distribution of radioactivity (expressed as relative specific activity) though slight, are indicative of a large proportion of ingested material being contained, in the first few minutes, in fairly large vesicles. After 20 min the microsomes have become more important locations of radioactivity, and from then on, whilst the other fractions remain approximately constant, the level of radioactivity present in the supernatant (both relative and absolute) continues to rise until equilibrium is reached.

Some of the ^{125}I-IgG contained in these cells is being degraded, whilst the rest is specifically protected from degradation. Since the results outlined above reflect material present in both pathways, they are open to various interpretations (see Discussion for details).

It must be emphasised, however, that these results are preliminary, being gleaned only from three replicates of the same experiment performed on the same day on tissues from the same rats. Further replication is essential for meaningful interpretation.

(1) **SDS-polyacrylamide gel electrophoresis of proteins present in the P_5 pellet.**

When denatured and solubilised preparations of the P_5 pellet were applied to SDS polyacrylamide gels and electrophoresis and protein staining of the gel carried out as usual, no distinct protein bands were seen. Increasing the sample strength up to 0.25 mg vesicle protein/0.1 ml

sample volume did not improve the results. Presumably there are so many different proteins present in this pellet that each individual one is insufficiently concentrated to form a distinct band.

Further purification and greater quantities of the starting material may give worthwhile results, but there appear to be no particular problems inherent in the modified methodology employed (Section 7.2.6).

7.3.2 Subcellular distributions in rat and rabbit yolk sacs of various ^{125}I-substrates.

Following the exploratory experiments described in the previous section, a more complex fractionation scheme (outlined in Section 7.2.1) was used to compare the subcellular distribution patterns of ^{125}I-PVP, ^{125}I-BSA and ^{125}I-IgG in rat and rabbit tissues.

Rat tissue fractions.

The three substrates used gave very different subcellular distribution patterns. ^{125}I-PVP was invariably associated mainly with the heavier fractions (P_1-P_4 pellets) and the supernatant, with the P_5 pellet invariably containing a relatively small amount of radioactivity (Figures 7.3.7, 7.3.8 & 7.3.9). Preliminary investigations showed native ^{125}I-BSA to exhibit its greatest concentration in the supernatant fraction, its lowest level being in the P_5 pellet (Figure 7.3.9). ^{125}I-IgG was reproducibly found to show its greatest concentration in the microsomal (P_5) pellet, with a fairly high level in the P_4 pellet also, the amounts present in the other fractions were lower and approximately equal. to each other.

Experiments tended to vary slightly in their design, and the spread of results reflects this, but the reproducible points that emerge are as follows: radioactivity derived from ^{125}I-PVP and, provisionally, ^{125}I-BSA is usually associated with the supernatant and also the heavier fractions; ^{125}I-IgG was found invariably to concentrate in the P_5 pellet.

Rabbit tissue fractions.

In rabbit tissue (Figure 7.3.10) the corresponding distribution patterns are substantially different. (The large amount of protein

present in the P_1 pellet was due to a weakness in the experimental design; the experiments were performed at an early stage, before routine sieving of the homogenate was adopted, and the P_1 pellet contains a large amount of whole cells and debris).

These preliminary experiments showed that ^{125}I-PVP again concentrates in the heavier fractions - in particular the P_2 ('mitochondrial') pellet, and there is less in the supernatant than there was with rat tissue. There is a very low level of radioactivity derived from ^{125}I-PVP associated with the ('microsomal') P_5 pellet. In contrast, ^{125}I-BSA is found once more at its most concentrated in the supernatant fraction, while the level associated with the P_5 pellet is so low as to nearly defy accurate measurement.

In rabbit tissue, the amount of radioactivity detected in the P_5 pellet for ^{125}I-IgG is a substantially greater percentage of the whole tissue-associated radioactivity than for either ^{125}I-BSA or ^{125}I-PVP (Figure 7.3.10), suggesting a relative concentration of ^{125}I-IgG in the microsomal pellet, but the effect is not so marked as for rat tissue (Figure 7.3.9). Otherwise the ^{125}I-IgG seems to concentrate in the heavier (P_1 and P_2) fractions, with relatively little in the supernatant. Again these results are not conclusive, but the same general indications were apparent from a number of experiments of slightly different design, of which only one is shown.

7.3.3 **The subcellular distribution of marker enzymes in yolk-sac tissue.**

In order to assess the distribution of different organelles in the fractionation, and in particular to assess the problem of cross-fraction contamination, assay of a number of 'marker' enzymes would be of interest. Preliminary experiments were performed using two such enzymes, arylsulphatase and glucose-6-phosphatase.

Arylsulphatase was assumed to be a typical non-membrane-linked lysosomal enzyme, and its distribution was taken as indicative of the positions of lysosomes and free lysosomal enzymes in the fractionated tissue. The rat yolk-sac homogenate, prepared with a motorized homogenizer, displayed an apparent concentration of arylsulphatase in the P_3 pellet (Figure 7.3.11), indicating that lysosomes were being isolated

most abundantly in the 'second nuclear' fraction. Initial separations using rabbit tissue showed a smaller concentration of arylsulphatase in the P_3 fraction, with much more being detected in the heavy (P_1 and P_2) fractions (Figure 7.3.11).

Glucose-6-phosphatase was used as a marker enzyme for microsomes, although in retrospect the method used would measure any activity capable of hydrolysing glucose-6-phosphate, and not just the specific enzyme. In both rat and rabbit tissues the greatest concentrations of activity were detected in the P_5 pellet (Figure 7.3.11), though the relative concentration in this fraction was greater for the rat than for the rabbit. In rabbit tissue, a large proportion of the activity was detected in the supernatant, this being the second most concentrated fraction isolated.

These results are very tentative, having been obtained once only, but preliminary interpretations suggest that in both tissues many arylsulphatase-containing vesicles are not being ruptured in the process of tissue homogenisation, and are found associated mainly with the heavier (P_1, P_2 & P_3) fractions. There is preliminary evidence for the presence of a relatively high concentration of arylsulphatase activity in the P_3 pellet from rat yolk sac, but not from rabbit.

Initial indications are that glucose-6-phosphatase activity is most concentrated in both tissues in the P_5 pellet, though more so for rat than for rabbit tissue. Both species exhibit a substantial amount of activity associated with the supernatant fraction, though more so with rabbit tissue than with rat. This raises the possibility that very small vesicles are not being sedimented in the P_5 pellet, but are remaining associated with the supernatant, particularly in rabbit tissue. More vigorous centrifugation, or other analysis of the supernatant-associated activity would be of interest to establish whether the activity is in free solution or not.

7.3.4 The effects of different homogenisation procedures on the subcellular distribution pattern of ^{125}I-PVP in rat yolk sacs.

When rat yolk sacs were subjected to motor-driven homogenisation procedures of different severities before subcellular fractionation, the distributions shown in Figure 7.3.8 were obtained. In these experiments

the greatest concentration of ^{125}I-PVP was found in the P_2 pellet, and the level of radioactivity detected in the supernatant was assumed to be a rough measure of lysosomal rupture. There seemed to be little difference between the three distributions examined, though this preliminary evidence suggests that higher speeds of pestle rotation may cause more damage to the lysosomes than multiple passes at lower speeds. Essentially the differences between the three distributions were small.

When the effects of motor-driven homogenisation of rat yolk sacs was compared with homogenisation by hand, the distributions shown in Figure 7.3.7 were obtained. In this experiment the ^{125}I-PVP was found to concentrate in two particular fractions - the P_4 pellet and the supernatant. With motor-driven homogenisation, however, the concentration of ^{125}I-PVP in the supernatant fraction was greater than in the P_4 pellet, perhaps indicating a substantial degree of lysosomal rupture, while hand-homogenisation always preserved the greatest concentration of ^{125}I-PVP in the P_4 pellet. Hand-homogenisation also gave slightly higher concentrations of radioactivity in the heavier fractions. The concentration of radioactivity in the P_5 pellet remained low, regardless of the method of homogenisation. While further experiments need to be carried out, preliminary indications are that hand-homogenisation better preserves lysosomal integrity in this tissue than does motor-driven homogenisation.

7.3.5 The effects of reincubation of yolk sacs at different temperatures on the subcellular distribution of ^{125}I-IgG in yolk-sac cells.

Rat tissue

Rat yolk sacs, exposed to homologous ^{125}I-IgG prior to homogenisation, invariably show the greatest concentration of radioactivity in the P_5 (microsomal) pellet. A preliminary investigation into the effects of reincubation for 2h at 37°C before homogenisation, during which time a large proportion of the contained radioactivity is released, showed the subcellular distribution to remain essentially the same, though much less total radioactivity is present. The most concentrated radioactivity is still detected in the P_5 pellet (Figs 7.3.12 and 7.3.13).

Reincubation at 20°C has been shown (Figures 6.3.23 and 7.3.12) to decrease the release of TCA-soluble material from the rat yolk sac more than the release of TCA-insoluble material. The subcellular distribution pattern of ^{125}I-IgG radioactivity after reincubation of the yolk sac at 20°C shows a slightly modified pattern (Figs 7.3.12 and 7.3.13). The most concentrated fraction is now the P_4 pellet, the greatest release of radioactivity (w/w) having been from the P_5 pellet. Whether or not these preliminary findings do indicate a reproducible effect is unclear. The results were reproducible in the sense that duplicate treatments of tissue from the same set of animals on the same day gave similar results, but a more extensive investigation is needed. The preliminary observation is in agreement with that reported in the following sections, namely that much of the radioactivity released from reincubated rat yolk sacs in TCA-insoluble form is shown to be located in the undischarged yolk sac, in the microsomal and supernatant fractions.

Rabbit tissue

This section reports the findings of one experiment, performed in duplicate on one occasion only, using tissue from only one animal. While the results obtained were similar for both duplicates, and no experimental problems were identified, the experiment should be repeated on tissue from different animals.

Rabbit yolk sacs that had been exposed to ^{125}I-IgG immediately before homogenisation and subcellular fractionation exhibited the subcellular distribution of radioactivity shown in Figure 7.3.14.(a). The greatest total concentration of radioactivity was found in the P_3 pellet, and the greatest concentration of TCA-insoluble radioactivity was in the P_4 pellet, as shown. A relatively low level of radioactivity was detected in the supernatant.

A comparison was made of the subcellular distribution patterns for rabbit yolk sacs that had been exposed to ^{125}I-labelled homologous IgG and then reincubated at either 37°C or 10°C in fresh medium (the corresponding release patterns are shown in Figure 6.3.25) prior to homogenisation and subcellular fractionation (Figure 7.3.14). The results obtained show essentially the same relative distribution, one being approximately twice the absolute magnitude of the other. The only exception is the supernatant radioactivity, which is approximately the

same. in either case.

More enlightening are the difference patterns (obtained by subtracting the distributions of radioactivity that remains in the yolk sac after reincubation at 37°C or 10°C from the distribution in the undischarged yolk sac). These patterns (Figure 7.3.15) represent the radioactivity that has been released from the yolk sac during the reincubation period prior to homogenisation. Figure 7.3.15 (a) represents the radioactivity that is lost from the tissue during reincubation at 37°C, while Figure 7.3.15 (b) represents material released at 10°C, and is thus regarded as 'uninhibited' by low temperature in its release from rabbit yolk sacs. Subtracting (b) from (a) gives Figure 7.3.15 (c). This represents material that is normally lost from the yolk sac during reincubation at 37°C but is not released during reincubation at 10°C. Its release is therefore 'inhibited' by low temperature. It should, perhaps, be emphasised again that the data being treated in this rather exploratory manner derive from one experiment only, and have not been shown to be reproducible.

It was shown in Figure 6.3.25 that material released from rabbit yolk sacs during reincubation at 10°C (ie. uninhibited) is almost entirely insoluble in TCA (also Figure 7.3.16). The bulk of this material (Figure 7.3.15 (b)) evidently originates, at least in this particular experiment, from the supernatant fraction, though the greatest relative loss is from the P_3 and P_4 fractions.

Material whose release is inhibited by low temperatures emanates in this case almost completely from the sedimentable fractions (Figure 7.3.15 (c)). The only supernatant-associated radioactivity whose release is inhibited by low temperatures appears to be destined for release in TCA-soluble form.

Supernatant-associated radioactivity could be derived from material that was originally present free in the cytosol of the intact cell, alternatively it might have been released by rupture of lysosomes or other vesicles during the homogenisation procedure. If lysosomal rupture is an important source of cytosol-associated material, then the cytosol would be expected to contain also a substantial amount of free lysosomal enzymes. The distribution of arylsulphatase (Figure 7.3.11) seems to suggest that lysosomal rupture is not sufficient to account for the high

level of radioactivity detected in the supernatant, and hence the preliminary implication is that, in this case, most of the radioactivity concerned was present free in the cytosol before the tissue was disrupted by homogenisation.

A simple calculation (Table 7.3.1) demonstrates that the total TCA-soluble radioactivity associated with the yolk-sac tissue at the start of the reincubation at 10°C is apparently sufficient to account for all the TCA-soluble radioactivity detected in the system at the end of the reincubation. There is, to date, no evidence of any protein degradation at 10°C, and this observation complements the observation that most of the radioactivity lost from the supernatant fraction at 10°C is released in macromolecular form.

7.3.6 The rate of uptake and subcellular localisation of formaldehyde-treated ^{125}I-IgG in rabbit yolk sacs.

Figure 7.3.17 shows that the observed Endocytic Index for formaldehyde-treated rabbit ^{125}I-IgG was approximately four times greater than the Endocytic Index for the untreated molecule. In these preliminary experiments the observed tissue level of substrate was found also to be elevated (approx. 860 ng/mg fraction protein) for formaldehyde-treated substrate compared with control (approx. 270 ng/mg fraction protein).

Formaldehyde-treatment also appeared to destroy the potential of the molecule for protection from degradation inside the rabbit yolk sac (Figure 7.3.18). Although the molecule was avidly taken up by the yolk sac, no measurable amount of TCA-insoluble radioactivity was released from the tissue on subsequent reincubation.

The observed subcellular distributions of native and formaldehyde-treated rabbit ^{125}I-IgG are shown in Figure 7.3.19. Native IgG was found to exhibit no concentration within a particular subcellular fraction, while formaldehyde-treated material was most prominent in the heavier fractions, relatively little being detected in the P_5 pellet and supernatant. Since none of the formaldehyde-treated IgG is apparently inside the protective pathway, while a substantial proportion of the control substrate might be expected so to be, it might be useful to compare the two fractionation patterns with a view to identifying

differences between them that might be caused by material contained inside compartments of the protective pathway.

A cursory glance at Figure 7.3.19 might suggest the simple message that since formaldehyde-denatured substrate is found predominantly in the heavier fractions, while native substrate appears to be spread more evenly across all the fractions isolated, material in the protective pathway may be located substantially in the lighter fractions, particularly the P_5 pellet and supernatant. It is not possible, however, with the available data, to make a solid analysis of the differences between the distributions, for two reasons. The first drawback is that approximately 40% of the control substrate-derived radioactivity present inside the yolk sac is in an unknown form (that is the % that is found not to be released from the yolk sac during 3h reincubation), and hence although it may be correct to assume that the fractionation pattern derived from the denatured substrate arises only from material present in the degradative pathway, it would still not be clear what proportion of material present inside control yolk sacs is present in that pathway, and how much is inside the protective pathway. Hence no clear pattern of the distribution of protected substrate could be established.

There is another complicating factor, arising out of the elevated rate of uptake of formaldehyde-denatured IgG into the degradative pathway. If the rate of substrate uptake into the cell is too high, then saturation of the proteolytic compartments of the cell may occur as substrate accumulates. Hence the shape of the subcellular fractionation profile given by denatured ^{125}I-IgG under these conditions, may not be of the same general shape as the component of the 'control pattern' that is due to substrate in the degradative pathway. The two probably would be similar in circumstances where the proteolytic capacity of the cells was not being taxed under experimental conditions, but it is important to ascertain that there is no long-term time-dependent tissue-accumulation of radioactivity using formaldehye-denatured substrate. If there was, the distribution pattern observed might be misleadingly biased towards the saturated components of the cell.

In summary, this approach appears promising, but many more experiments need to be performed before the results can be precisely interpreted.

In the following RESULTS section, as throughout the rest of this Thesis, the following key to symbols is observed, unless indicated otherwise in the legends.

TCA - soluble radioactivity is represented by open symbols - mainly squares or circles.

TCA - insoluble radioactivity is represented by closed symbols - mainly squares or circles.

$\pm$ Error bars represent $\pm$ Standard Deviations.

Figure 7.3.1 The subcellular distribution of various substrates in rat yolk sacs.

Yolk sacs were incubated in the presence of the various substrates (2h, 5ug/ml) and then washed, homogenised and fractionated by the rapid scheme designed for the quick isolation of the 'microsomal' fraction. This gives three fractions, loosely described as (in reverse order) supernatant, microsomal and heavier material, the latter being assumed to contain a certain amount of material that should ideally be found in the other two fractions. Results are expressed as (a) specific radioactivities, and (b) ng substrate/mg tissue protein.

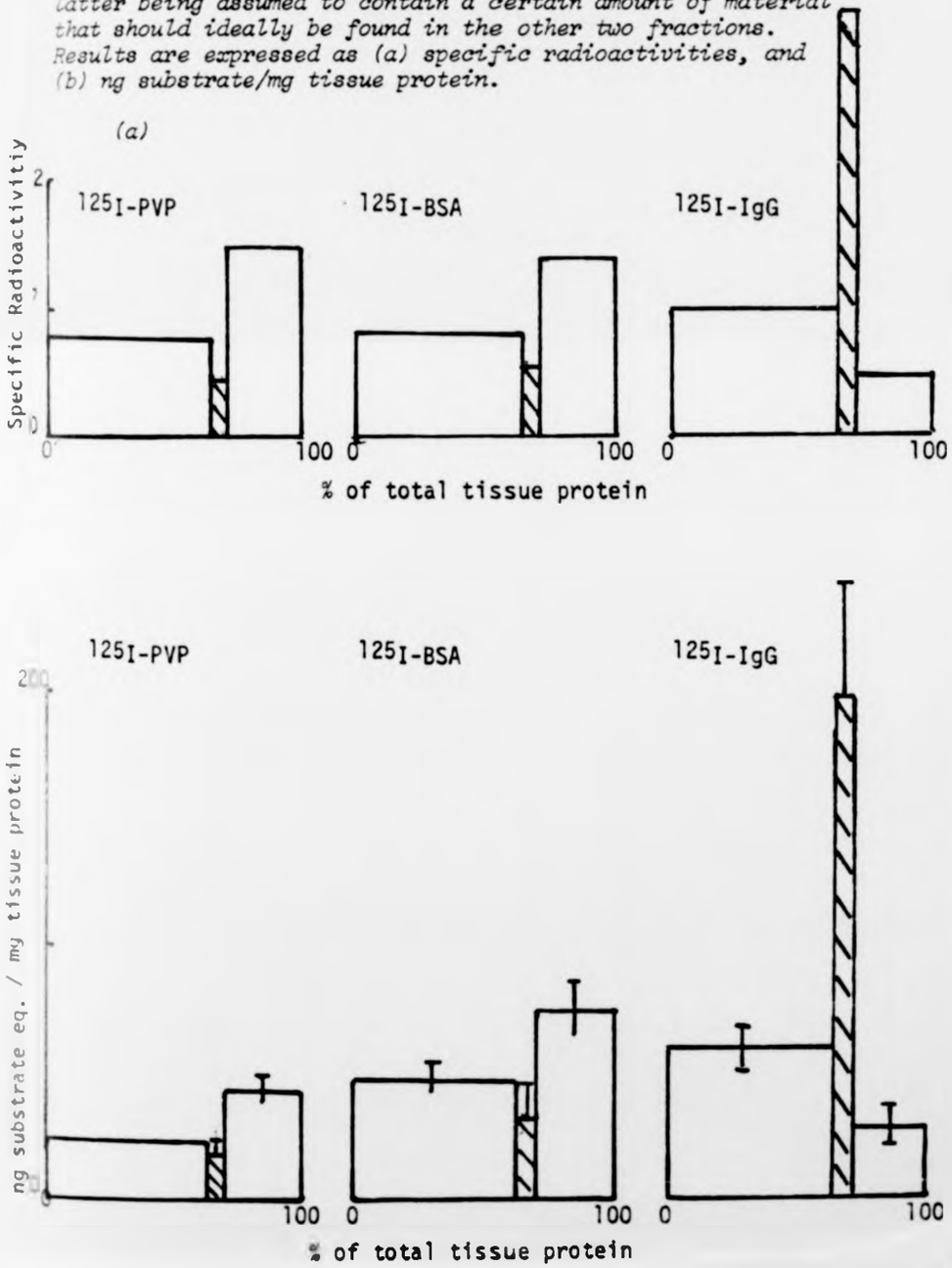
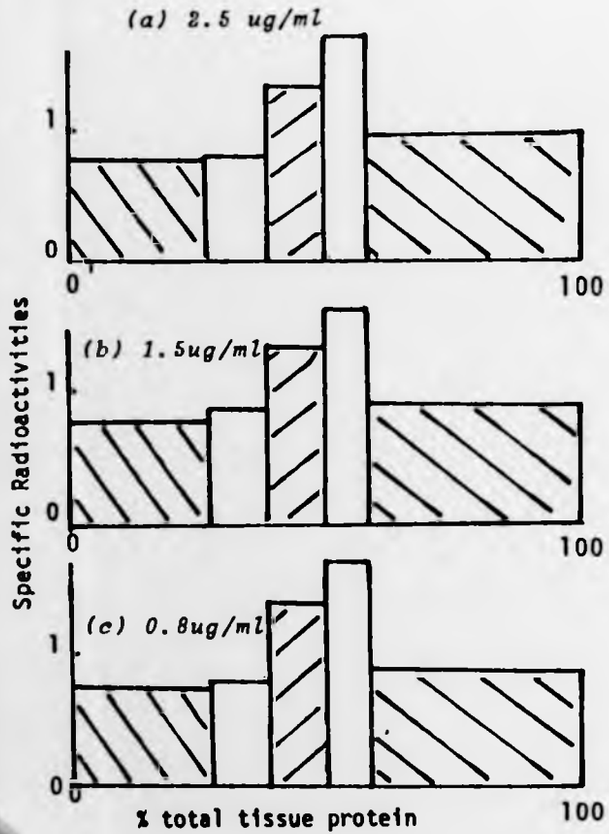


Figure 7.3.1 The distribution pattern of ^{125}I-IgG added to rat yolk sac homogenates shortly before subcellular fractionation.



Rat yolk sacs were incubated briefly in medium 199 (15min) and then homogenised with a motor-driven Teflon-on-glass homogeniser. ^{125}I-IgG was then added, to the final concentrations indicated, and the homogenate was centrifuged according to the usual regime for the isolation of five fractions.

Figure 7.3.3 **Immunological technique 1. Using rat IgG as primary substrate and radiolabelled rabbit anti-rat-IgG IgG as second antibody.**

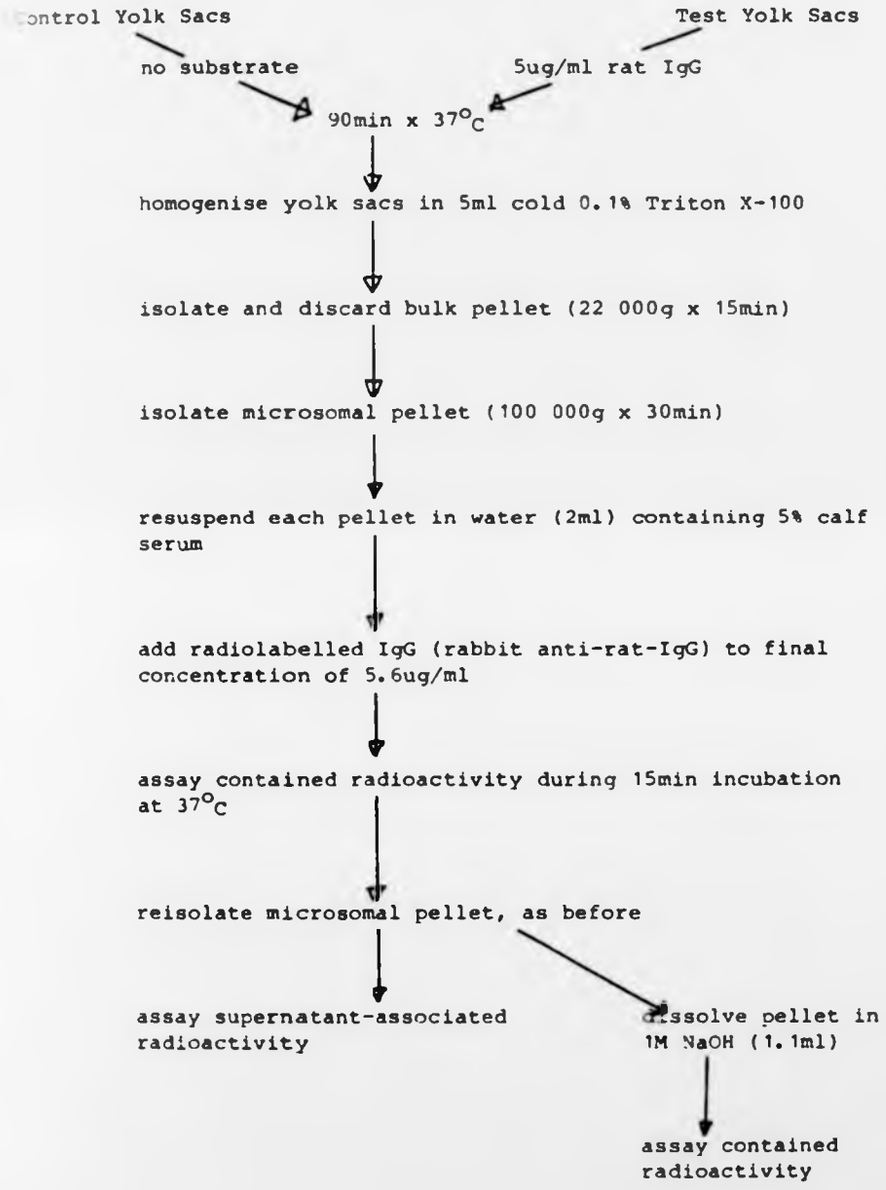


Figure 7.3.4. **Immunological technique 2. Using rabbit anti-rat-IgG as substrate and radiolabelled rat IgG as second antibody.**

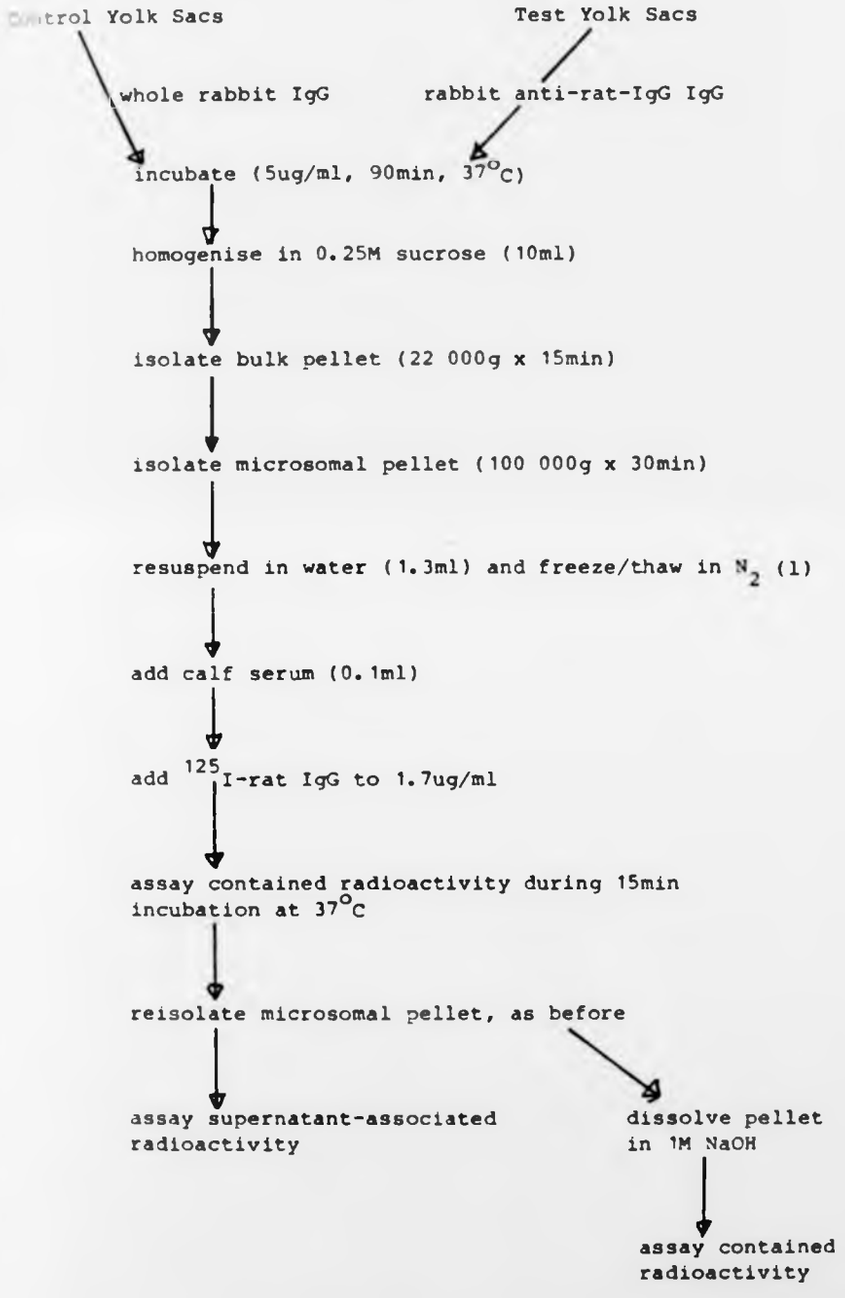


Figure 7.3.5 The effect of incubation concentration on the subcellular distribution of ^{125}I-BSA in rat yolk sacs.

Rat yolk sacs were incubated in medium 199 (90min) containing ^{125}I-BSA at various concentrations. The yolk sacs were then washed, homogenised and fractionated by the usual regime for the isolation of five fractions. The radioactivity content of each fraction was assayed and is expressed here both as ng BSA/mg tissue protein and also as specific radioactivities.

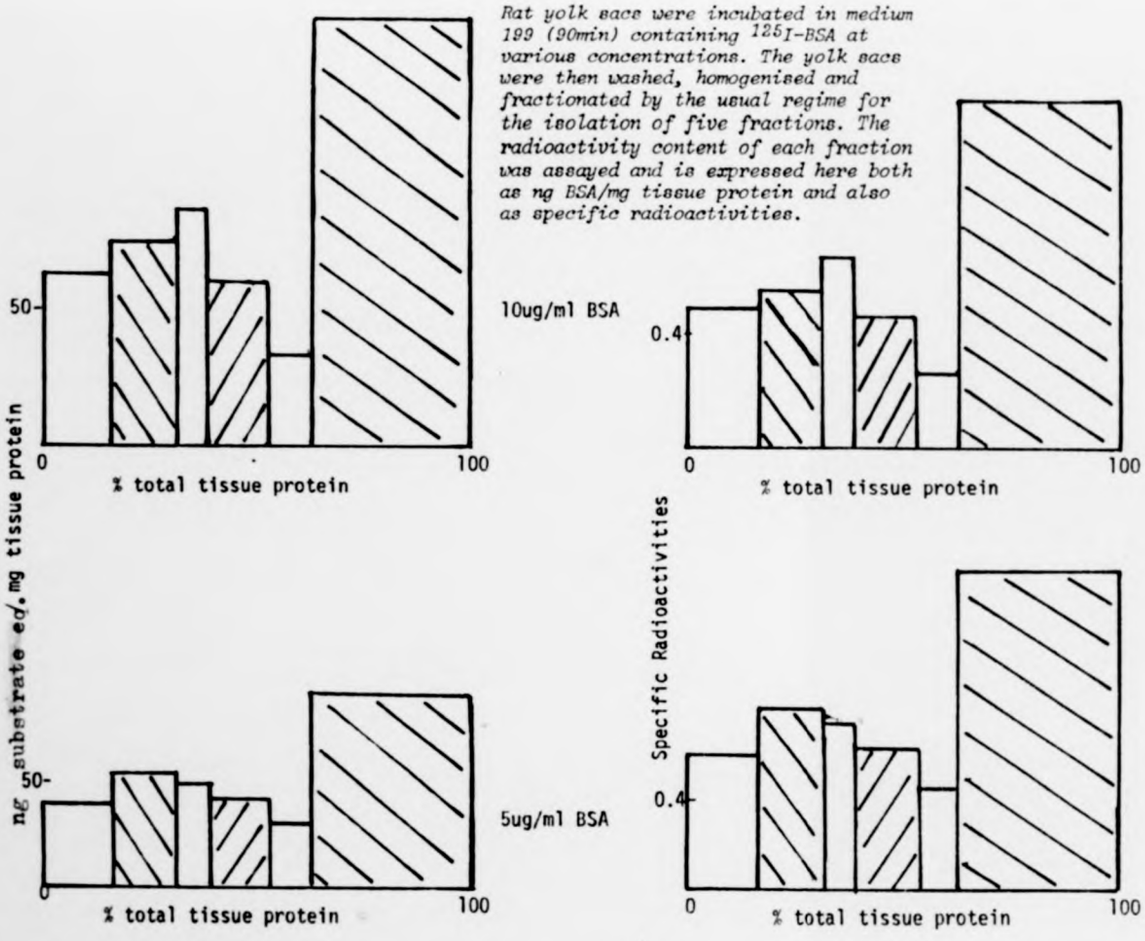


Figure 7.3.6 The effect of incubation time on the subcellular distribution of ^{125}I-IgG-derived radioactivity in rat yolk sacs.

Rat yolk sacs were incubated in medium 199 containing ^{125}I-IgG (5ug/ml) for different lengths of time (as shown). The yolk sacs were then homogenised and fractionated in the usual way, and five fractions were isolated. The radioactivity content of each fraction was measured, and the results are expressed here as (a) ng substrate/mg tissue protein, and (b) specific radioactivities.

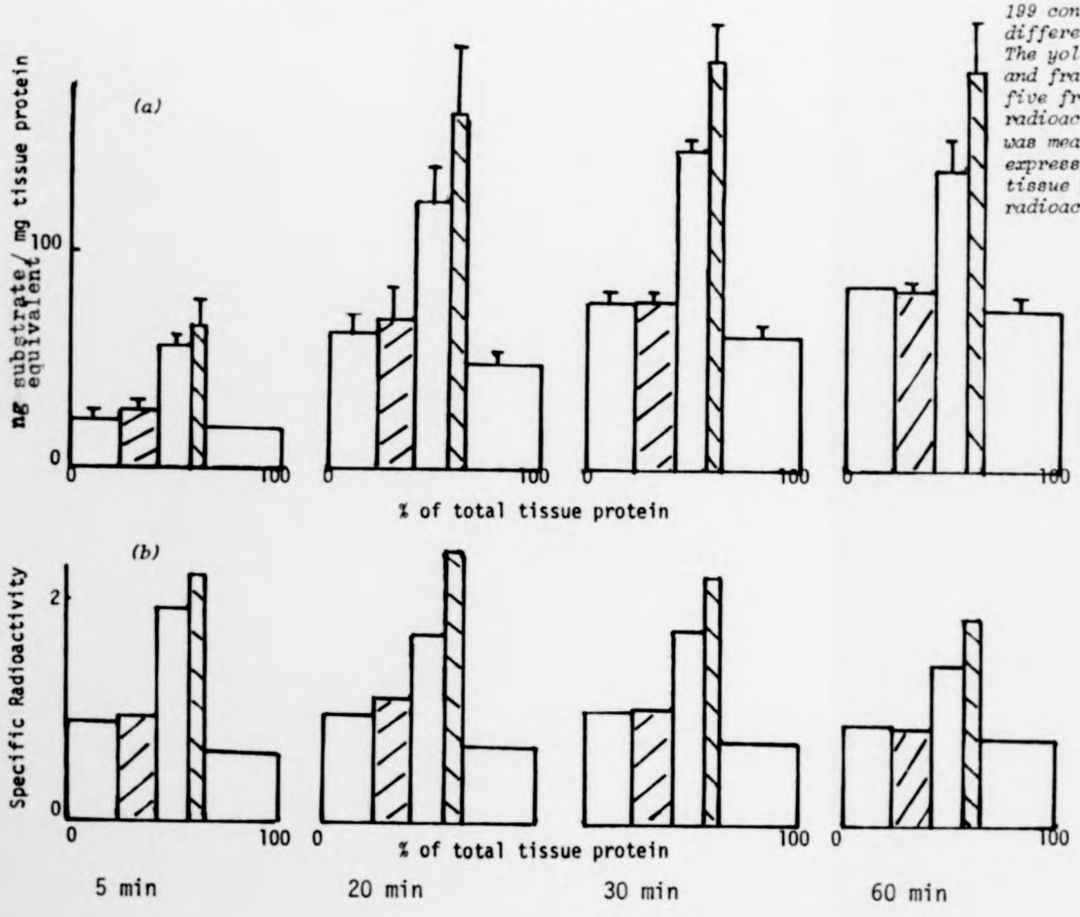
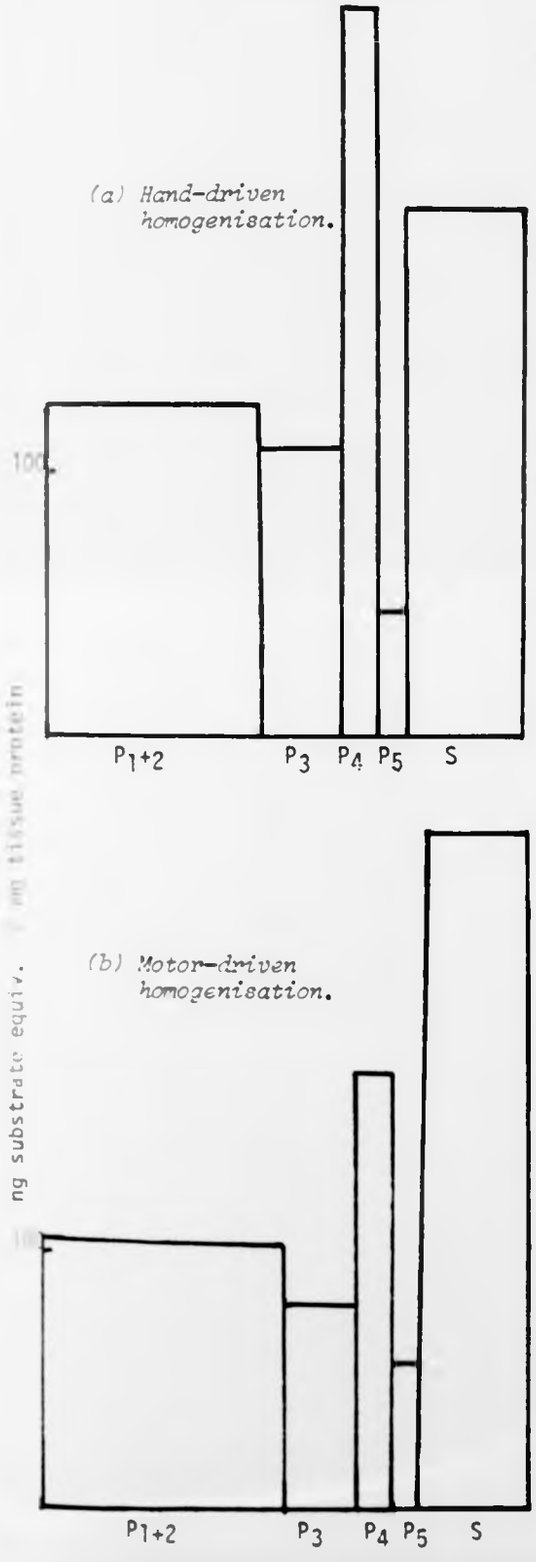


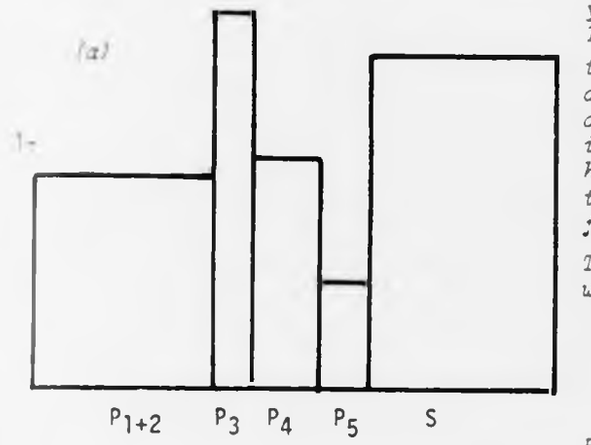
Figure 7.3.7 A comparison of the effects of hand- and motor-driven Teflon-on-glass homogenisation on the subcellular distribution of ^{125}I-PVP in rat yolk sacs.

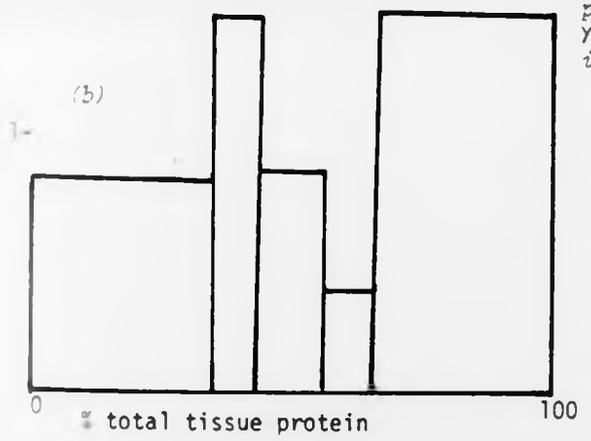


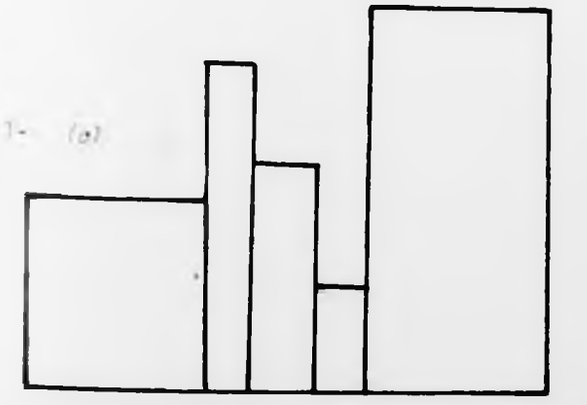
Rat yolk sacs were incubated in medium 199 containing ^{125}I-PVP (3h, 2ug/ml) and then washed and homogenised in 0.25M sucrose. Hand-homogenisation comprised six double passes of the Teflon pestle, at which stage most of the tissue appeared to be disrupted. Motor-homogenisation involved three double passes of the motor-driven Teflon pestle, set to Mark 3 on the Tri-R motor.
Subcellular fractionation was as described in the script for the isolation of five fractions. The homogenate was not sieved prior to the isolation of fraction 1.

The x-axis represents the mg of total tissue-protein contained in that fraction, the y-axis the concentration of radioactivity, expressed as ng PVP/mg fraction *protein.*

Figure 7.3.8 The effects of different regimes of motorized homogenisation on the subcellular distribution of ^{125}I-PVP in rat yolk sacs.





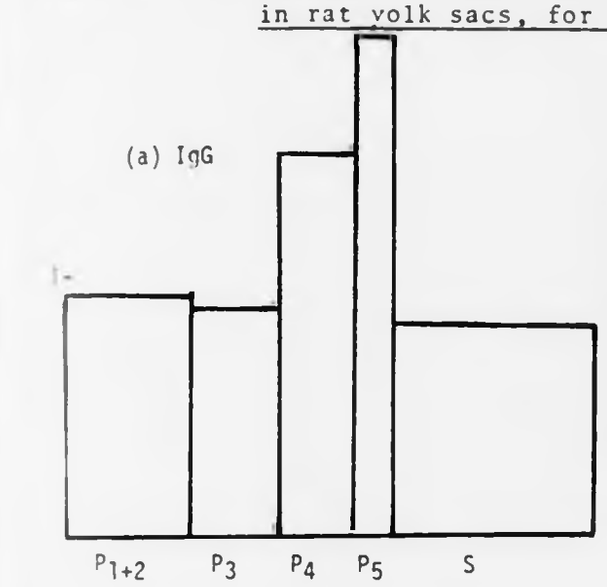


Yolk sacs wereincubated in ^{125}I-PVP, (3h, 2ug/ml), prior to washing, homogenisation and subcellular fractionation, as described in the text for the isolation of five pellets. The homogenate was not sieved prior to the isolation of the first fraction.

The regimes of homogenisation were as follows:
(a) 1 double pass, speed 2.
(b) 1 double pass, speed 4.
(c) 3 double passes, speed 3.

Results are expressed as specific activities - the x-axis represents the % total protein present in the fractions, the Y-axis the specific activity ie. the $\frac{\%\text{ total radioactivity}}{\%\text{ total protein}}$.

Figure 7.3.9 Subcellular distribution of radioactivity in rat yolk sacs, for three different substrates.



Rat yolk sacs were incubated in the presence of substrate (2h, approx.), then washed, homogenised by motor-driven Teflon pestle, and fractionated as described in the text. Protein and radioactivity content was assayed for each fraction and results are expressed as specific radioactivities. Minor differences in experimental design (eg. different number of pellets) arose because the various experiments were executed at widely different times.

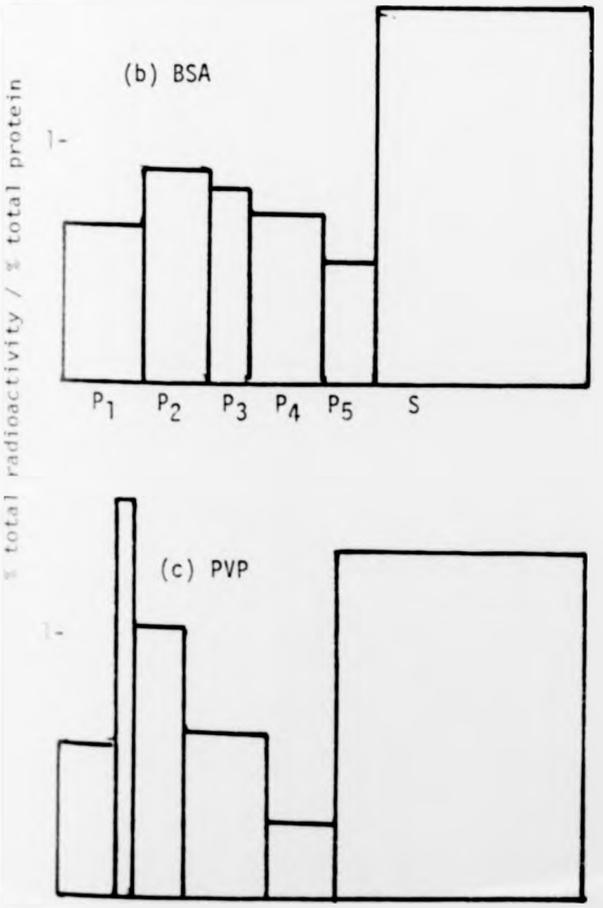
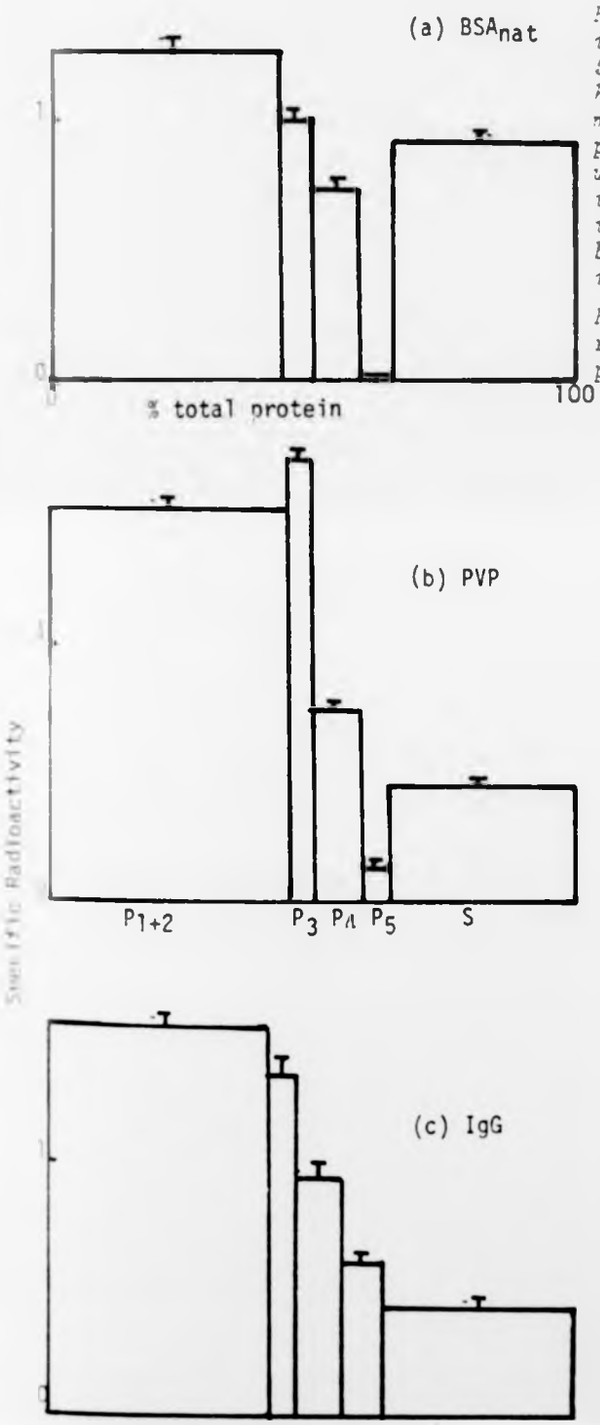


Figure 7.3.10 The subcellular distribution in rabbit volk sacs of radioactivity derived from three substrates.



Rabbit yolk sacs were incubated in the presence of substrate (3h, 5ug/ml) before being washed and homogenised in 0.25M sucrose, by motor-driven Teflon-on glass pestle. Subcellular fractionation was as desribed in the text for the isolation of five pellets, but the homogenate was not sieved before the first fraction was isolated.

Results are expresses as specific radioactivities against % total protein.

Figure 7.3.11 The distribution of marker enzymes in homogenates of rat and rabbit yolk sacs.

Glucose-6-phosphatase and arylsulphatase, conventional microsomal and lysosomal marker enzymes, respectively, were assayed in homogenates of rat and rabbit yolk sacs, as described in the text.

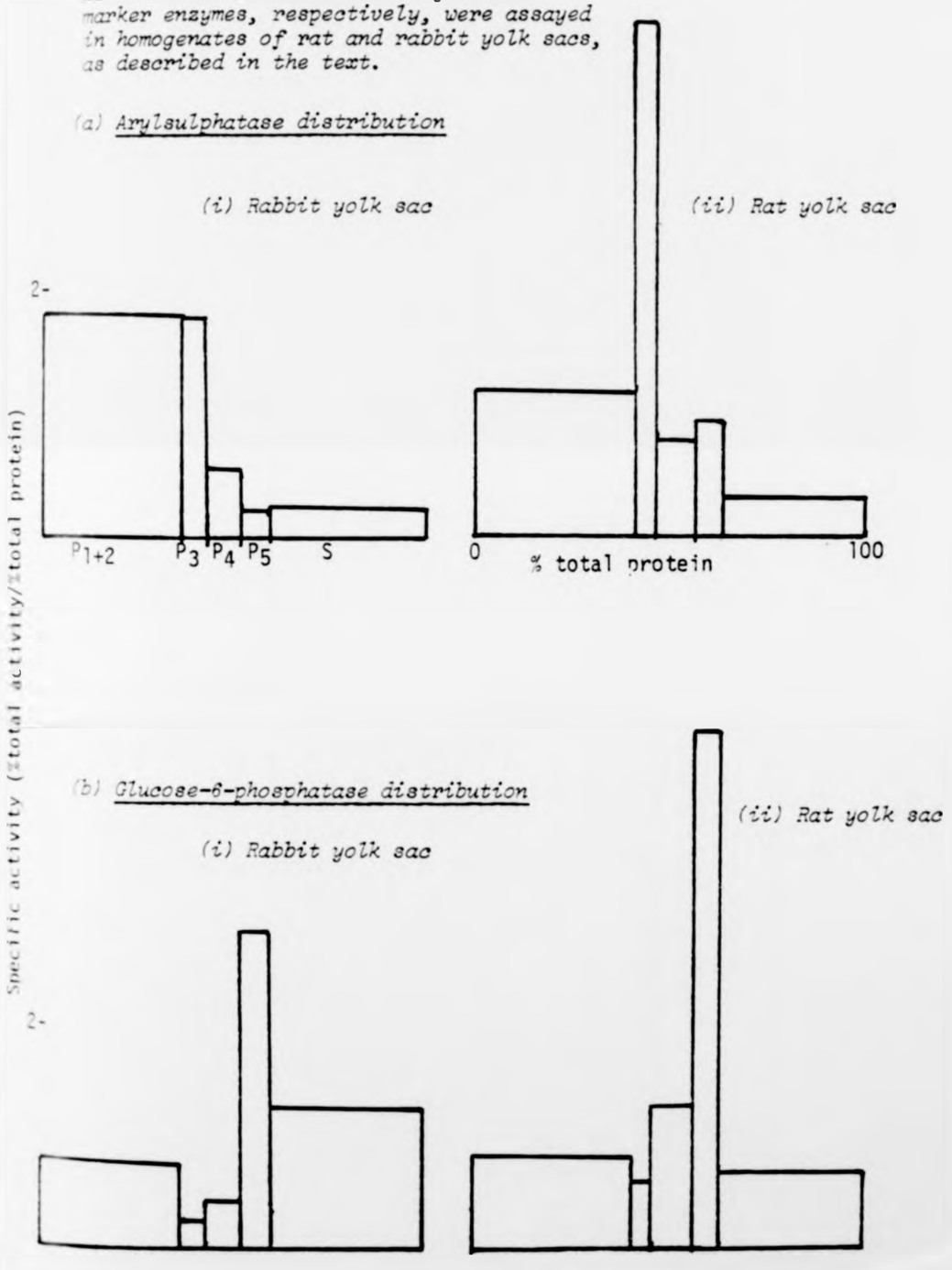


Figure 7.3.12 The subcellular distribution of radioactivity derived from ^{125}I-IgG in rat yolk sacs following reincubation at various temperatures.

Rat yolk sacs were preincubated in medium-containing ^{125}I-IgG (5ug/ml, 90min, 37°C) and then subjected to various treatments before being homogenised and fractionated in the usual way.

(a) Reincubated (2h) @ 37°C *(b) Reincubated (2h) @ 10°C* *(c) Not reincubated.*

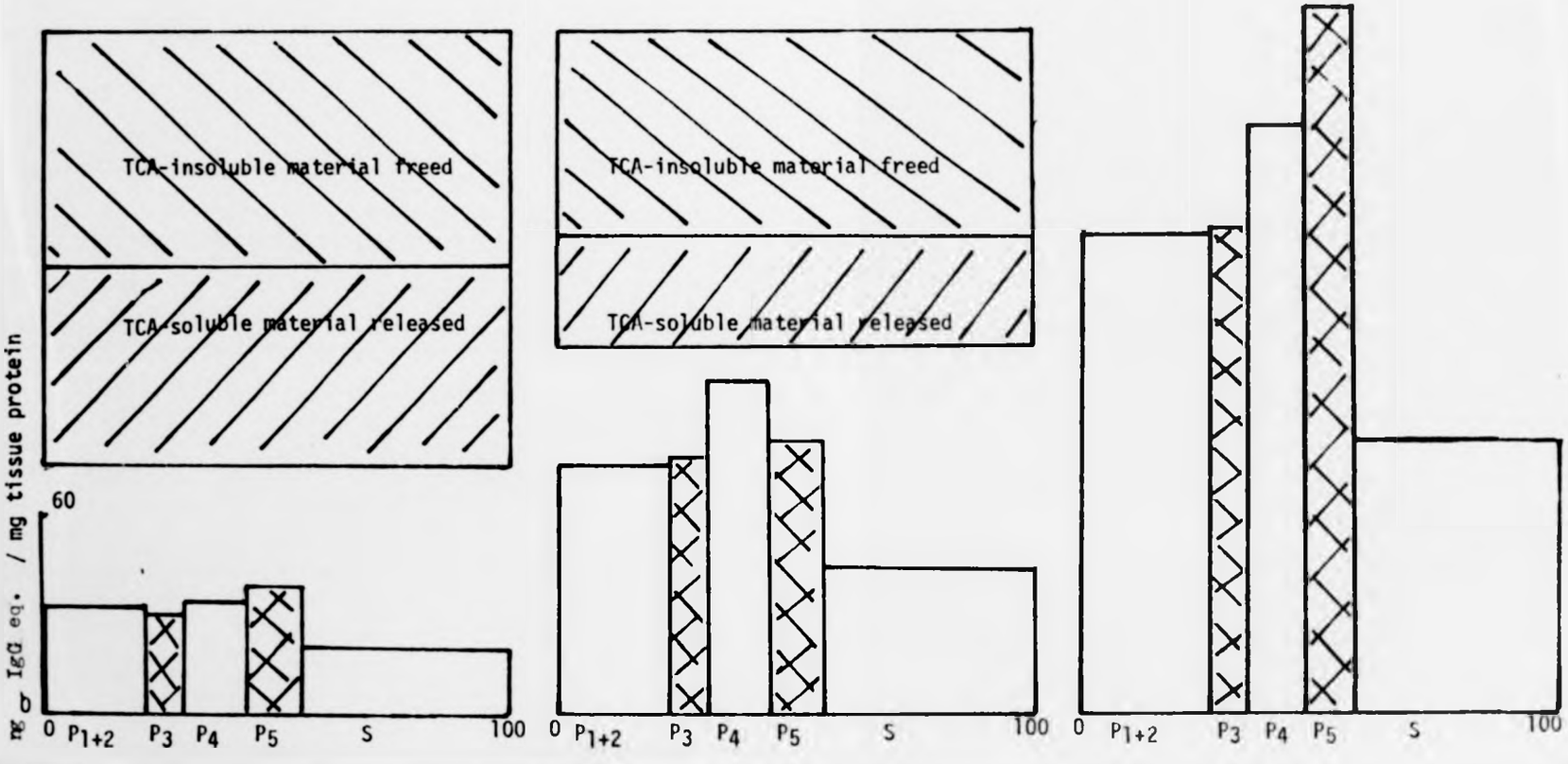


Figure 7.3.13 The subcellular distribution of ^{125}I-IgG-derived radioactivity in rat yolk sacs.

Yolk sacs were incubated in the presence of ^{125}I-IgG (5ug/ml, 90min) and then either homogenised, as described in Section 7.2.1, or reincubated and then homogenised, as detailed below. The radioactive and protein content of each of the subcellular fractions was measured, and the results are presented here as 'specific activities' - the % of the total tissue-associated radioactivity that is present in each fraction divided by the % of the total tissue-protein also present in that fraction.

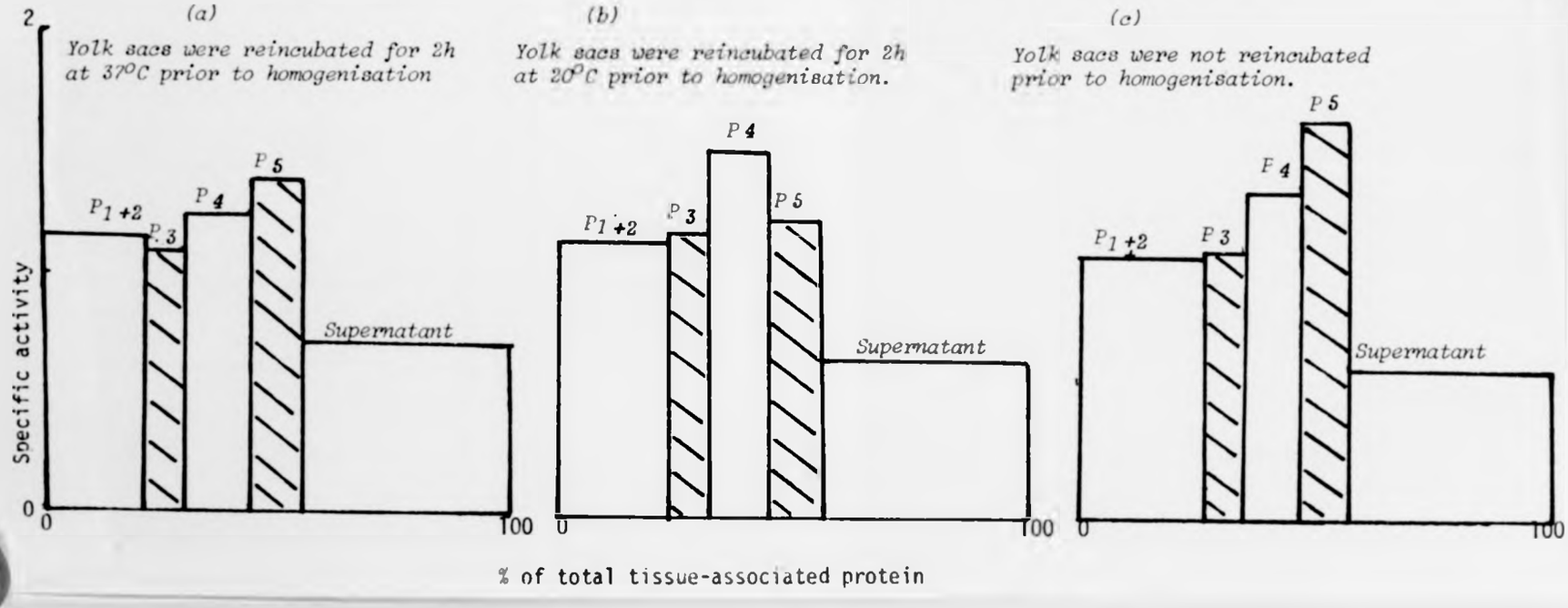
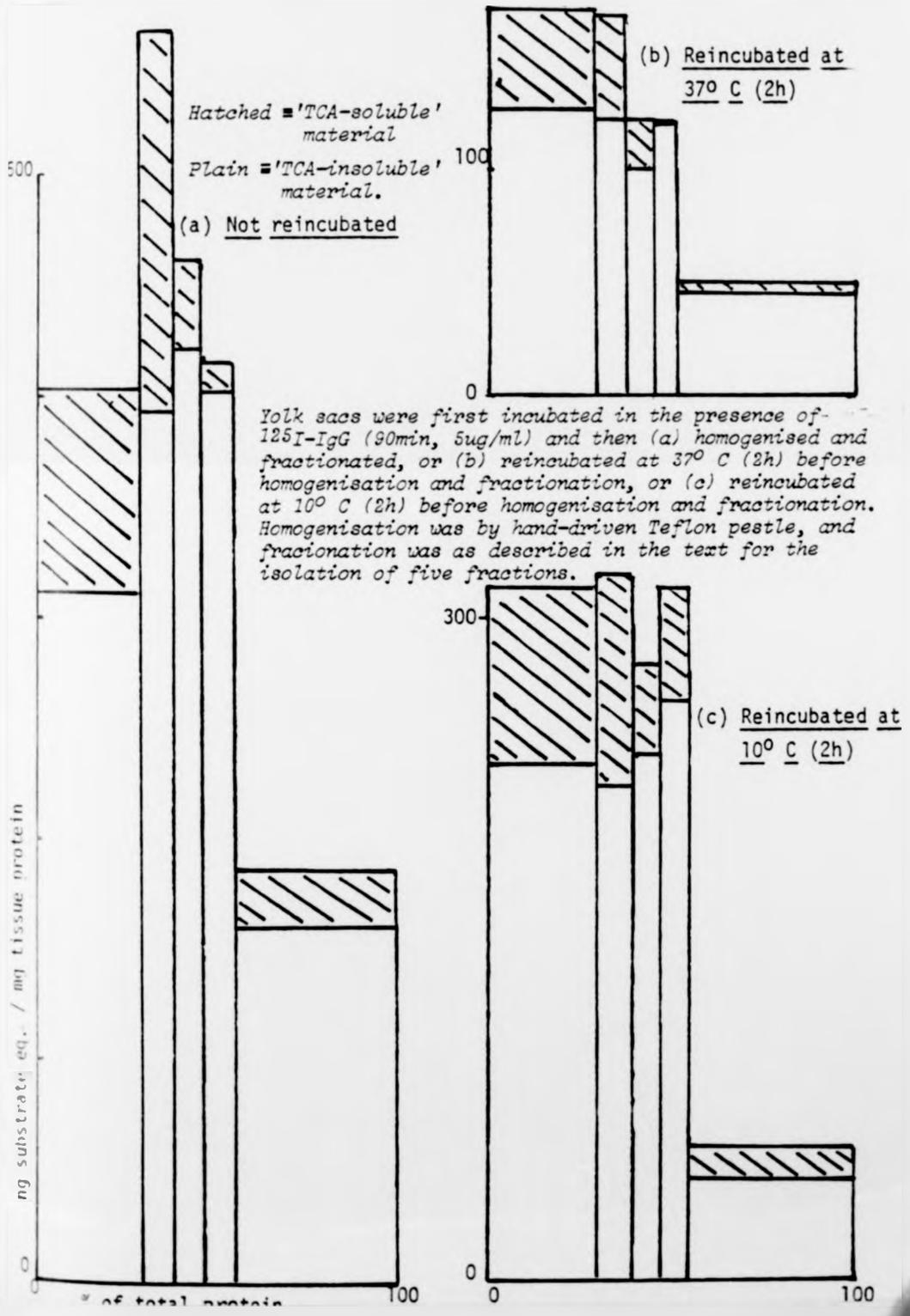


Figure 7.3.14 The subcellular distribution of radioactivity derived from ^{125}I-IgG in rabbit volk sacs following reincubation of the volk sacs in substrate-free media, at various temperatures.



Yolk sacs were first incubated in the presence of ^{125}I-IgG (90min, 5ug/ml) and then (a) homogenised and fractionated, or (b) reincubated at 37° C (2h) before homogenisation and fractionation, or (c) reincubated at 10° C (2h) before homogenisation and fractionation. Homogenisation was by hand-driven Teflon pestle, and fracionation was as described in the text for the isolation of five fractions.

Figure 7.3.15 Subcellular sites of ^{125}I-IgG-derived radioactivity in rabbit volk sacs.

These data are derived from Figure 7.3.6, as follows:

(i) 6(a) minus 6(b) ≡ radioactivity that is lost from the yolk sac during 2h reincubation @ 37° C ≡ 'TOTAL LOSEABLE RADIOACTIVITY'. Represented in (a).

(ii) 6(a) minus 6(c) ≡ radioactivity that is lost from the yolk sac during 2h reincubation @ 10° C ≡ 'UNINHIBITIBLE RELEASE'. Represented in (b).

(iii) 7(a) minus 7(b) ≡ radioactivity released during 2h reincubation at 37° C, but not at 10° C ≡ 'INHIBITIBLE RELEASE'. Represented in (c).

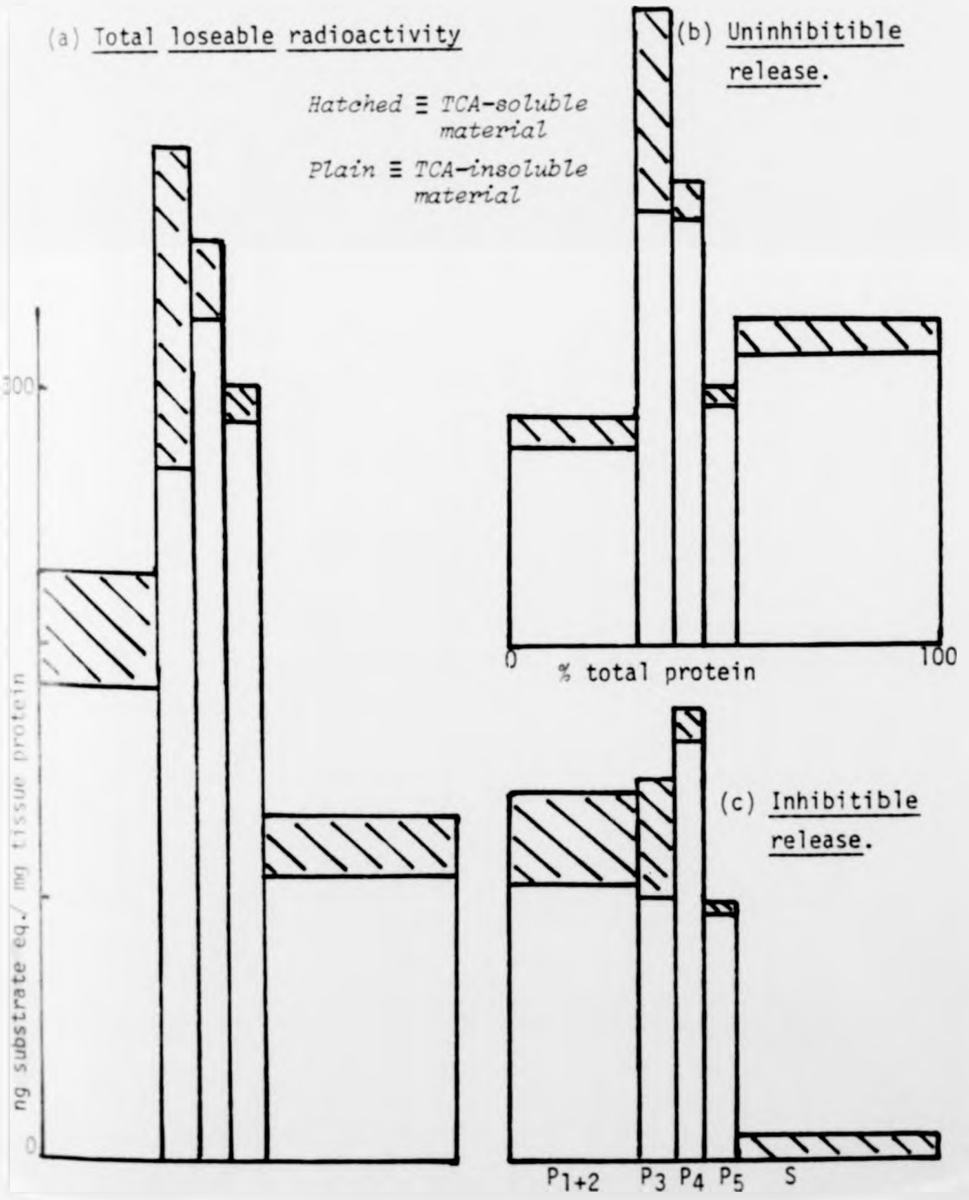


Figure 7.3.16 The composition of the ^{125}I-IgG-derived radioactivity released from rabbit yolk sacs reincubated at different temperatures.

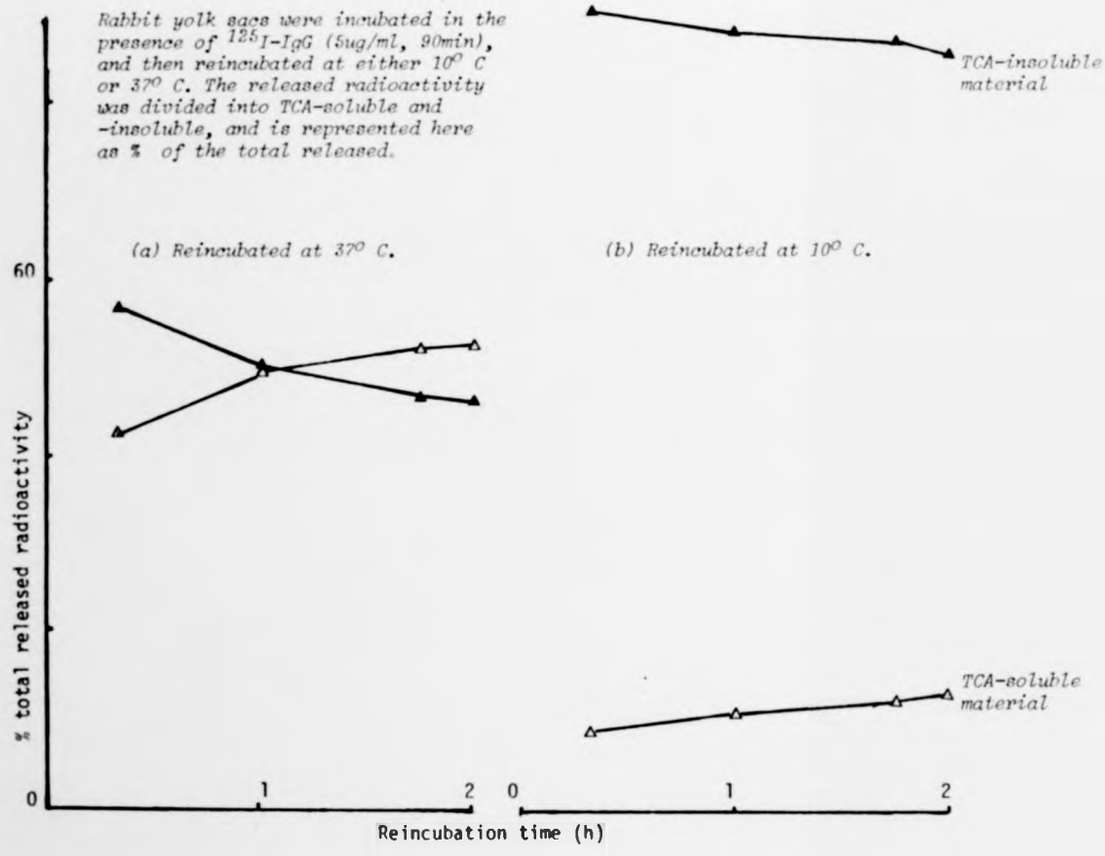


Figure 4.37 The uptake by rabbit yolk sacs of native and formaldehyde-treated rabbit IgG.

Rabbit yolk sacs were incubated in substrate (2ug/ml), and uptake was monitored in the usual way.

n=3 for error bars

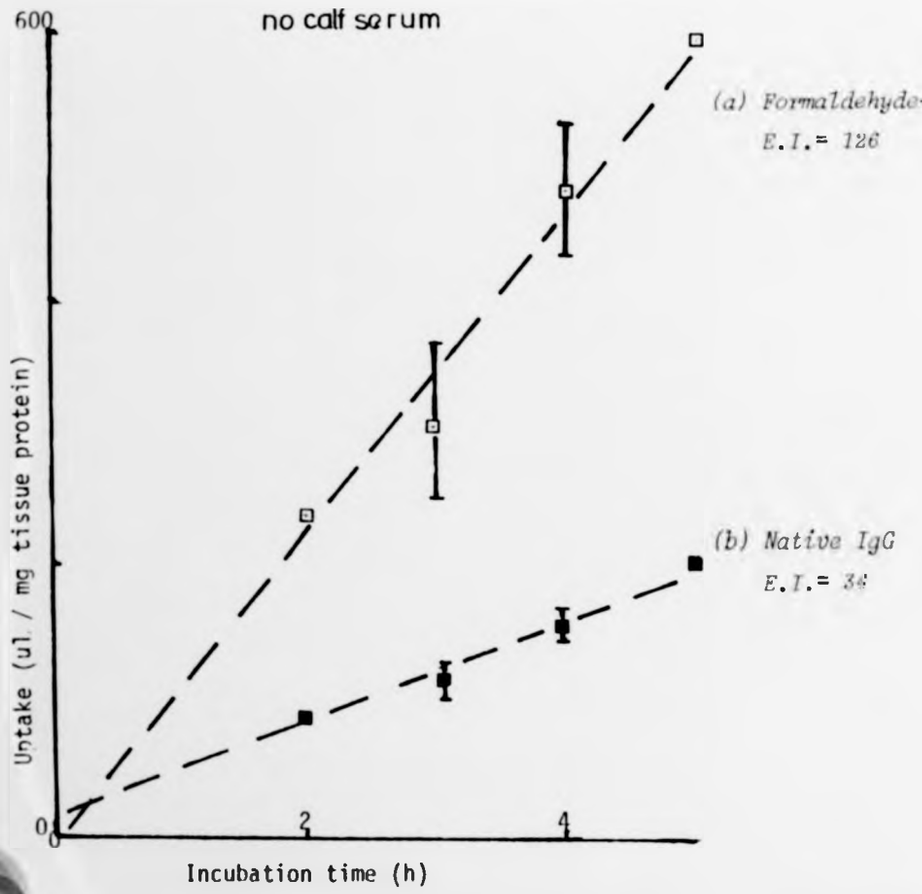


Figure 7.3.18 The release pattern from rabbit yolk sacs of radioactivity derived from native and formaldehyde-treated ^{125}I-IgG.

Yolk sacs were incubated in the presence of substrate (5ug/ml, 30min) and then washed and reincubated in fresh medium in the usual way. The release of radioactivity was monitored. No release of insoluble radioactivity derived from formaldehyde-treated IgG was detected.

n=3 for error bars

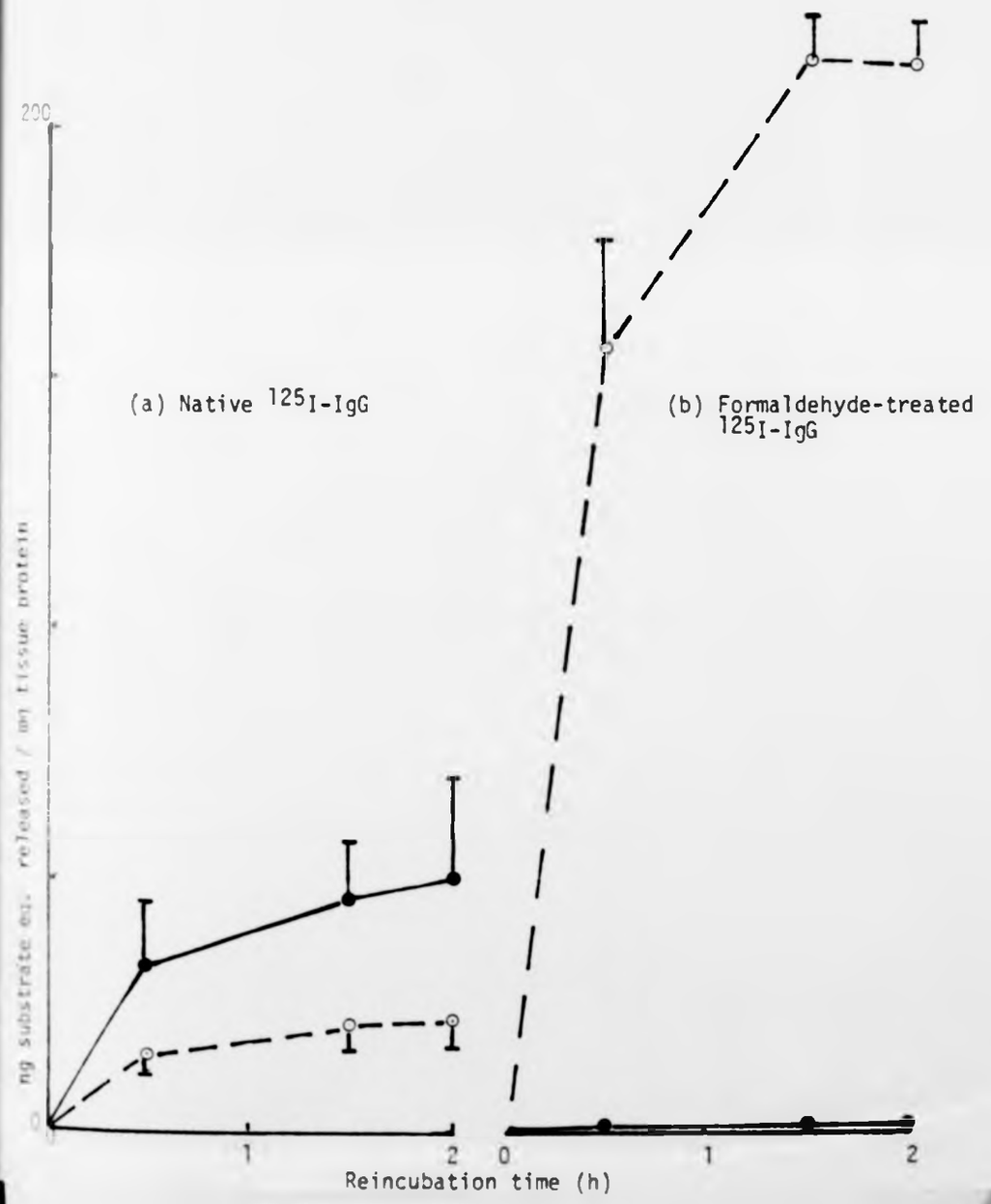


Figure 7.3.19 Subcellular distribution patterns in rabbit yolk sacs for radioactivity derived from native and formaldehyde-treated rabbit ^{125}I-IgG.

Rabbit yolk sacs were incubated in substrate (5ug/ml, 30min), then washed and homogenised (by hand) in 0.25M sucrose. Subcellular fractionation was as described in the text for the isolation of five fractions, the homogenate being initially sieved to remove large debris.

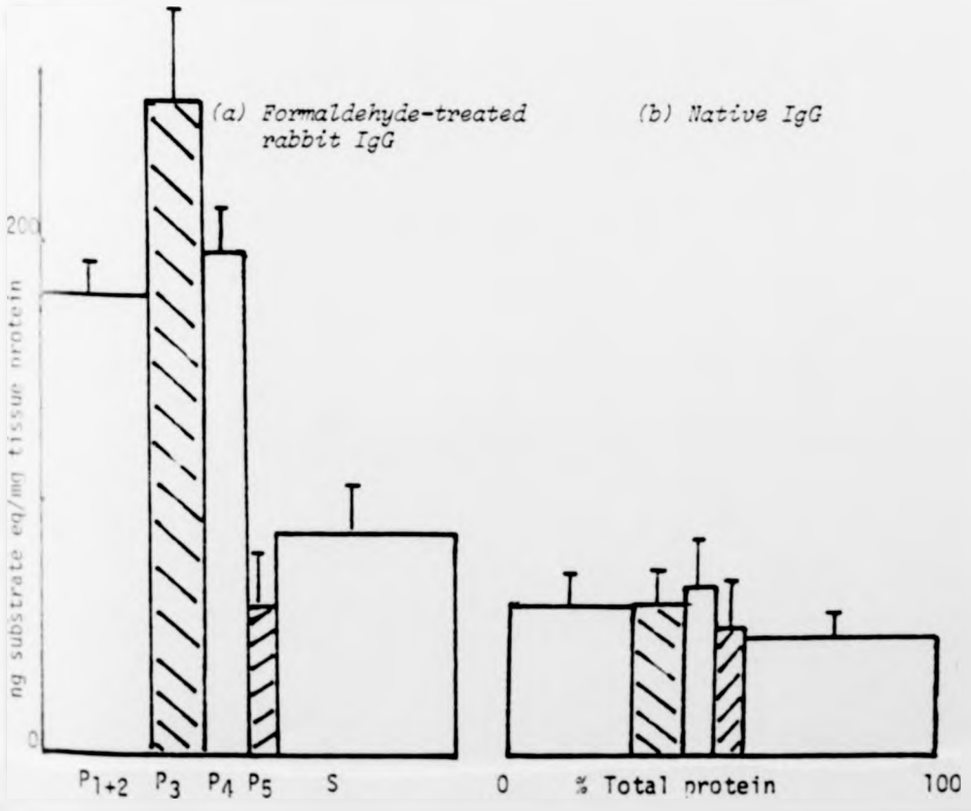


Table 7.3.1 **An investigation into the rate of degradation of ^{125}I-IgG in rabbit yolk sacs at 10°C.**

Fraction	ng sols/mg protein at start of reinc.	ng sols/mg protein at end of reinc.	ng sols/mg protein released	total mgs protein	total ngs of sols freed
Nuclear	92.5±5	80±4	12	5.7	68
Mitoch ondrial	169	93±20	76	1.65	125
Lysosomal	41±3	22±6	19	1.35	26
Microsomal	13±1	6±1	7	1.45	10
Supernat.	28±1	15±1	13	8.3	108

Total = 337ngs sols

Observed total TCA soluble material released = 253ng.

Rabbit yolk sacs were incubated in medium containing ^{125}I-IgG at 37°C for 90min. Different batches were homogenised and fractionated before or after a 2h cold-chase at 10°C, and the radioactivity contained in the different fractions was assayed. The total amount of TCA-soluble radioactivity found in yolk sacs that had not been reincubated was found to be of the same order of magnitude as the amount found in yolk sacs that had been reincubated added to the amount released into the medium during the reincubation procedure. Therefore, there is no evidence for intracellular protein breakdown at 10°C.

Table 7.3.2 **The effects of resuspension in Triton-X100 and freezing on the sedimentability of microsome pellet-associated substrates.**

Substrate	% released by resuspension in 0.1% Triton-X100	% released by freeze-thawing in N_2(l)
^{125}I-PVP	98.4 ± 1.7 (n=6)	74.7 ± 2.5 (n=4)
^{125}I-nat.BSA	67.4 ± 22.3 (n=6)	69.3 ± 1.0 (n=4)
^{125}I-IgG	28.4 ± 11.3 (n=16)	29.4 ± 1.9 (n=6)

Rat yolk sacs were incubated in the presence of the indicated substrates and then homogenised and the microsomal pellet isolated, in the usual way. The pellet was resuspended in a small volume of either 0.1% Triton-X100 or distilled water, and its contained radioactivity was assayed. Pellets resuspended in water were frozen rapidly in liquid nitrogen and then allowed to thaw. The pellet was reisolated in the same way, and the contained radioactivity again assayed, together with that liberated into the medium.

Table 7.3.4 **The effects on microsome-associated ^{125}I-IgG of resuspension in 2M urea.**

% IgG retained in pellet	ng IgG retained per mg pellet protein	ng IgG released per mg released protein
34.7	56	72
36.9	65	70
42.9	70	62
42.7	69	60
44.2	58	51
40.0	60	52
40.2 ± 3.8	63.0 ± 5.9	61.2 ± 8.8

Rat yolk sacs were incubated in the presence of ^{125}I-IgG, homogenised, and the microsomal pellet isolated in the usual way. Resuspension of the pellet in 2M urea, followed by assay of its contained radioactivity and resiolation was found to have no selective effect on the release from the pellet of IgG compared with other proteins.

Table 7.3.3 **The effect on microsomal pellet-associated ^{125}I-IgG of freezing and of treating with urea.**

The microsomal fraction of a batch of yolk sacs preincubated in ^{125}I-IgG was isolated in the usual way, and then subjected to various treatments in the resuspension stage. When the pellet was reisolated, the effects of the resuspension on the sedimentability of the contained ^{125}I-IgG was assessed., and protein content was measured (Lowry et al, 1951).

This table reports the results of one particular experiment.

Preliminary distribution in the fractionated homogenate:

Pellet	Contained protein (mg)	Amount of associated IgG (ng/mg)	% total IgG	% total protein	specific radioactivity
P_1	304	29	68.9	72.6	0.95
P_2	12.6	125	12.4	3.1	4.00
S	101.8	23	18.7	24.3	0.77

Treatment of the microsomal pellet:

A - Resuspend in 2M urea and freeze/thaw in N_2(l)

B - Resuspend in 2M urea

C - Resuspend in 0.25M sucrose

Treatment	IgG retained in pellet (% original)	protein retained in pellet (% original)	ng IgG/mg protein in pellet	ng IgG/mg protein in supernatant
A	35.8 ± 1.6	34.1 ± 0.7	78	71 ± 1.4
B	42.8 ± 0.1	35.4 ± 0.3	84 ± 4	61 ± 1.4
C	73.8 ± 0.7	53.9 ± 0.9	91	33 ± 3

Table 7.3.5 **The distribution of ^{125}I-IgG added to the homogenate before fractionation.**

Fraction	ng ^{125}I-IgG per mg pellet protein	specific radioactivity
P_{1-4}	229 ±20	0.96
P_5	723 ± 60	3.02
S	177 ± 19	0.74

Rat yolk sacs were homogenised in 0.25M sucrose (approx. 90mg protein in 8ml) and ^{125}I-IgG was added (final concentration 2ug/ml). The homogenate was then fractionated in the usual way, and the distribution of radioactivity observed. 350x excess (wt/wt) of calf serum over rat IgG was used throughout in order to minimise non-specific binding.

Table 7.3.6 **Effects of the inclusion of cold proteins throughout the homogenisation on the retention of substrates in the microsomal pellet.**

Cold protein	amount of substrate that was reisolated after resuspension in Triton X100. (ng/mg protein)		% freed by Triton BSA	IgG
Control	3.1	220	93.5	29.2
5ug/ml BSA	9.0	N.D.	77.0	-
50ug/ml BSA	3.5	181	89.0	27.4
5ug/ml IgG	N.D.	134	-	26.7
Control	N.D.	142	-	27.2

When cold proteins were included throughout the homogenisation procedure and fractionation, the amount of substrate that was released from the microsomal pellet by resuspension in Triton-X100 was not different from controls. The difference between the controls must arise from inter-animal variation.

Table 7.3.7 **The effects of the inclusion of cold rat IgG throughout the homogenisation procedure.**

Fraction	Concentration of IgG (ng ^{125}I-IgG per mg pellet protein)	
	Control	5ug/ml cold IgG
P_{1-4}	64 (1.03)	58 (0.97)
P_2	195 (3.15)	187 (3.11)
S	31 (0.50)	39 (0.65)

Specific radioactivities are shown in brackets.

Rat yolk sacs were incubated in the presence of ^{125}I-IgG in the usual way, and then homogenised in 0.25M sucrose. Tests included 5ug/ml cold rat IgG in the homogenisation medium in order to observe its effects on the distribution pattern.

Table 7.3.8 **The effect of pre- and post-chase in cold rat IgG on the subcellular distribution of ^{125}I-IgG.**

Fraction	Control		Test	
	% total ^{125}I-IgG	specific radioactivity	% total ^{125}I-IgG	specific radioactivity
P_{1-4}	69.2 54.5	1.01 0.96	70.0 52.0	1.06 0.95
P_5	9.3 7.4	2.24 1.80	10.0 8.9	2.19 2.09
S	21.4 38.1	0.78 0.97	20.0 39.1	0.67 0.95

Test rat yolk sacs were incubated first in the presence of cold rat IgG (10min, 5ug/ml), then ^{125}I-IgG (90min, 5ug/ml) and finally cold IgG (10min, 5ug/ml) again, prior to homogenisation and subcellular fractionation in the usual way.
Control yolk sacs were not exposed to cold IgG.

DISCUSSION

A rapid method, involving just two centrifugal spins, was developed for the isolation of the P_5 fraction from rat yolk-sac homogenates. It was observed that ^{125}I-IgG characteristically exhibited a high concentration in the P_5 pellet while ^{125}I-PVP and, provisionally, ^{125}I-BSA were least concentrated in this fraction (Figure 7.3.1). This led to the suggestion that the microsomal pellet might represent a relevant compartment in the process of intracellular protection, and some investigations were carried out on the precise location of the ^{125}I-IgG within the P_5 fraction.

Although the concentration of ^{125}I-PVP in the P_5 pellet was shown to be relatively low, the observation that nearly half of the P_5 fraction-associated ^{125}I-PVP could be resedimented after resuspension in 0.25 M sucrose (Section 7.3.1 (c)) suggested that much of it might be present inside vesicles rather than in free solution. The method used to resuspend the pellet (Teflon-on-glass hand-homogenisation of the pellet) would be expected to have some disruptive effects, and hence this measure of the amount of ^{125}I-PVP present inside vesicles may be an underestimate. On the other hand, resuspension of these pellets in water or 0.1% Triton X-100 liberated essentially all of the ^{125}I-PVP in a non-sedimentable form, supporting the view that the encapsulating vesicles are of lipid composition, and readily burst by osmotic shock or detergent action. An alternative view would be that the ^{125}I-PVP had simply adsorbed to the outside surface of a population of microsomes during the homogenisation procedure and was released by the resuspension process. However, the observation that resuspension in 0.25M sucrose preserves, in sedimentable form, more of the ^{125}I-PVP than does resuspension in water, tends to indicate against this possibility.

When ^{125}I-IgG was used as substrate, the bulk (70%) of the P_5 pellet-associated radioactivity was found not to be released by resuspension in Triton X100 or distilled water, but to remain in a sedimentable form (Section 7.3.1 (c)). The behaviour of ^{125}I-BSA (Table 7.3.2) appeared to be intermediate between ^{125}I-PVP and ^{125}I-IgG, about 70% of P_5-associated radioactivity being released by resuspension in 0.1% Triton X100.

Evidently at least some of the ^{125}I-IgG isolated in the P_5 fraction is protected from being released by osmotic shock or detergent action. The mechanism of such protection is open to question, but the involvement of protein seems likely - either as a particularly resilient receptor maintaining the IgG bound to the wall of the burst vesicle or a plasma membrane fragment, or in the form of a protein coat, giving the vesicle added strength and keeping it intact even in the presence of Triton X100. Urea (2M) has been reported (Woodward & Roth 1978) to remove the protein coats from clathrin-coated vesicles, and therefore the effects of 2M urea on the sedimentability of P_5 pellet-associated ^{125}I-IgG were of interest. Since 2M urea was reported to remove effectively all the coats from the vesicles investigated, if a protein coat was the only special factor conferring protection on the P_5-associated-^{125}I-IgG, then the application of 2M urea in the presence of detergent or freeze-thawing might be expected to release virtually all of the contained ^{125}I-IgG. In fact, such treatment (Table 7.3.3, 7.3.4) releases only about 40% of the contained ^{125}I-IgG (an increase of approx. 10% over simple resuspension in Triton X-100) and many other proteins are released also (Table 7.3.4). Interpretation of these data must again be carefully considered since non-specific release of protein from the pellet could be a misleading phenomenon. There is no evidence for specific release from the pellet of ^{125}I-IgG, and hence it seems unlikely that a simple, urea-susceptible protein coat is the only IgG-protecting feature of this fraction.

Preliminary analysis (Section 7.3.1(f)) of the ^{125}I-IgG released from the resuspended P_5 pellet by 2M urea showed it to be virtually all TCA-insoluble. It seems this fraction may contain either pre-lysosomal material or material intended for protection from degradation.

The next feature of the P_5 fraction-associated ^{125}I-IgG-derived radioactivity to be investigated was whether it was available for interaction with the surrounding solution (before intentional disruption of any vesicles present), or whether it was located inside intact vesicles. A number of approaches to the problem were considered;

(i) The addition to the (rat yolk-sac) homogenate of a small amount of ^{125}I-IgG followed by differential centrifugation in order to indicate whether the IgG-specific receptors present in the homogenate were available for interaction with the added ^{125}I-IgG. (Cold bulk protein was routinely added to help to minimise non-specific binding of ^{125}I-IgG to membranes.) The reproducible high concentration in the (microsomal) P_5

pellet (Table 7.3.5) of ^{125}I-IgG indicates that the receptors are available for binding after resuspension of the pellet in 0.25M sucrose and they bind ^{125}I-IgG as well as, if not better than, the microsomal fraction isolated from yolk sacs that had been incubated. Increasing the concentration of exogenously added ^{125}I-IgG shifted the bulk of the material towards the supernatant fraction, but an initial indication was that the IgG-specific receptors were not fully saturated when exposed to the amount of ^{125}I-IgG that they normally associate with under incubation conditions.

The major indication of these (reproducible) results is that many of the IgG specific receptors are present either on the outside surface of vesicles or on broken pieces of membrane when the P_5 pellet is isolated from rat yolk sacs homogenised in 0.25M sucrose.

(ii) Preliminary investigations revealed that the inclusion throughout the rat yolk-sac homogenisation procedure of a large excess of unlabelled rat IgG (Table 7.3.7) or BSA (Table 7.3.6) was without apparent effect on the pattern of subcellular distribution of radioactivity derived from the ^{125}I-IgG present in the medium in which yolk sacs had been preincubated. Neither did resuspension of the P_5 pellet in a solution of unlabelled IgG cause any measureable displacement of contained radioactivity (7.3.1 (h)). There is therefore no evidence to date of any competition for receptors between radiolabelled immunoglobulin prebound to receptor and unlabelled molecules in solution. This is not conclusive evidence concerning the subcellular location of the ^{125}I-IgG, since the lack of competition could result from spatial inaccessibility of the receptor to the unlabelled immunoglobulin, or it could be a characteristic of this particular interaction that binding under these conditions is essentially irreversible. However, it is generally accepted (Wild, personal communication) that specific interactions between substrate and receptor are usually susceptible to competition from other substrate molecules, and that an interaction that is not reversible is usually of a fairly low specificity.

The indications from these two sections are in apparent contradiction - the former firmly demonstrates the availibility of receptors in the resuspended P_5 pellet for specific interaction with IgG, the latter implying (though only in preliminary terms) that this is not so. This contradiction could perhaps be resolved by further

experimentation, but presently the bulk of the evidence suggests that at least some IgG-specific receptors are exposed and available for interaction in the resuspended P_5 pellet.

(iii) The time course for the release of radioactivity from the resuspended P_5 pellet after the addition of a mixture of exopeptidases is an approach that has some potential in answering the question of whether the substrate in the microsomal pellet is inside protein-coated vesicles. If the immunoglobulin is immediately available for reaction then the release course might be approximately linear, but if a protective protein coat has to be digested before the enzymes can attack the immunoglobulin, then a lag phase might be observed. Preliminary experiments were executed without conclusive results, and none are presented in this report.

In summary, although the body of evidence needs to be enlarged and much of the work needs to be repeated, (especially the immunological investigations), the preliminary indication is that much of the ^{125}I-IgG isolated in the P_5 pellet from homogenised rat yolk-sacs previously exposed to this substrate is available for reaction when the pellet is resuspended in a suitable medium. This suggests that the receptors are either present on pieces of broken membrane or on the outside of microvesicles that remain intact under homogenisation conditions.

The formation of small vesicles from plasma membrane during homogenisation is well documented, and such a phenomenon could perhaps explain the existence of microvesicles carrying IgG-receptors on their outside surface. If this is an important source of the P_5-associated substrate then it could seriously effect interpretation of results and observations. The possibility was investigated in two ways;

If microvesicles bearing ^{125}I-IgG (on their inside or on their outside surfaces) are formed from the plasmalemma during homogenisation, then incubation of the intact tissue in unlabelled IgG prior to homogenisation might be expected to displace any ^{125}I-IgG specifically bound to the plasma membrane surface, and hence to decrease significantly the level of radioactivity detected in association with the microsomes. Such treatment (Table 7.3.8) was observed not to have an marked effect, suggesting that plasmalemma-derived vesicles isolated in the P_5 fraction may not be of fundamental importance in the interpretation of these results. The P_5 pellet still exhibits a very great concentration of

radioactivity even under conditions expected to minimize the contribution from plasmalemma-binding.

Alternatively, if the P_5-associated radioctivity is indeed derived mainly from the radioactive material originally present on the cell surface during homogenisation, then doubling the incubation concentration of substrate (in this case, ^{125}I-BSA) might be expected to cause a large increase in the level of P_5 pellet-associated radioactivity. Preliminary indications are that this is not observed (Figure 7.3.5), suggesting that plasmalemma-derived vesicles carrying radiolabelled substrates may not be of crucial importance in the substrate-complement of the P_5 pellet. The evidence available to date preliminarily suggests that the P_5-associated ^{125}I-IgG-vehicle may be already inside the cell prior to homogenisation, and not formed solely as an artefact of homogenisation. Further experiments are required to elucidate this matter, but it appears at this stage that the characteristics of the P_5-associated ^{125}I-IgG observed, and outined above, may result from its association with an important vehicle involved in the transcellular transport process.

The results discussed so far were all obtained using rat tissue and a motor-driven Teflon-on-glass homogenisation technique that proved both simple and convenient. When a comparison of the effects on the subcellular fractions was made between homogenates prepared with the homogeniser driven by its motor and with those prepared by using the same homogeniser driven by hand, however, important differences were noted. Hand homogenisation of rat yolk sacs containing ^{125}I-PVP, though slower and more difficult to perform, seems to lead, in the experiments performed to date, to a greater amount of the ^{125}I-PVP being associated with sedimentable organelles during subcellular fractionation than for motor-driven homogenisation (Figure 7.3.1). The shear forces experienced by tissue components passing a continuously rotating pestle appear to be greater than those caused by the same pestle driven by hand, and hence more organelles survive hand-homogenisation intact. Varying the speed of pestle rotation and the number of passes with motor homogenisation did not modify significantly the ^{125}I-PVP subcellular distribution observed (Figure 7.3.8), suggesting that uneccessary damage was being caused to the tissue even at the slowest possible speed of motorized homogenisation, and therefore a change of technique was made partway through this work, from motorized to hand-driven, and the method of homogenisation (indicated in the text) may have some bearing on the

interpretation of the various results.

Using motor homogenisation of rat yolk sacs and a more comprehensive scheme of fractionation (Figure 7.2.1), ^{125}I-PVP was found to concentrate mainly in the P_2 (second nuclear) and supernatant fractions (Figure 7.3.9), perhaps reflecting its presence in intact secondary lysosomes and its release from broken ones, respectively. The experimental technique employed (one fewer fraction was isolated for ^{125}I-IgG) picked up (in initial experiments) an equivalent P_2 pellet-concentration for ^{125}I-BSA, while the distribution pattern obtained for ^{125}I-IgG was reproducible and seemed to be characteristic only of that substrate. By far the greatest concentration of ^{125}I-IgG in the fractionation profile is found associated with the P_5 (microsomal) pellet; this was not observed for any other protein substrate investigated, reinforcing the earlier suggestion that microsome-associated ^{125}I-IgG might represent an important compartment of the diacytic route.

Complementary experiments with rabbit yolk sacs (homogenised by hand) gave a high concentration of ^{125}I-PVP in the P_1-P_3 region, with relatively little in the supernatant fraction (Figure 7.3.10). ^{125}I-BSA was provisionally detected in approximately equal amounts (measured in terms of the relative specific activity) in all fractions except the P_5, which, in these experiments, contained no detectable radioactivity at all. ^{125}I-IgG concentrated in the heavier (P_1-P_3) fractions in the same manner as ^{125}I-BSA and ^{125}I-PVP, but some concentrated in an atypical manner relative to the other two substrates in the P_5 (microsomal) pellet. (A possibly important difference between the behaviours of rat and rabbit tissue became obvious with the absence of such a marked concentration of ^{125}I-IgG in the P_5 pellet from rabbit yolk sac homogenate, although compared with both ^{125}I-PVP and ^{125}I-BSA there is a relative concentration of ^{125}I-IgG in the P_5 fraction). Perhaps the application of the same homogenisation technique to the two tissues causes a lower degree of disruption of the rabbit tissue since, for all three substrates, much more radioactivity was detected in the P_1 and P_2 pellets than in equivalent rat tissue homogenates. This suggestion found some support from the observed distribution of the marker enzyme arylsulphatase (specific for lysosomes), but the distribution of glucose-6-phosphatase activity suggests that cells are being broken. The results therefore indicate that lysosomes from both tissues are being isolated in rather heavier fractions than would normally be expected

(Goetze et al, 1976), particularly in the case of rabbit tissue. The microsomes appear invariably most abundant in the P_5 pellet, as would be expected, though rabbit microsomes seem perhaps to be rather more difficult to sediment. This is in broad agreement with the observed high level of glucose-6-phosphatase activity detected in the supernatant fraction from rabbit yolk-sac homogenates, but the specificity of this activity as a microsomal marker has not been established in this system. The possibility that rabbit-microsomes are remaining unsedimented under these conditions would be an interesting feature to investigate more fully, either by more vigorous centrifugation of the homogenate or by analysing the form of the supernatant-associated radioactivity by gel chromatography. The possibility that the glucose-6-phosphatase activity detected may not be mediated by that enzyme but by less specific phosphatases that may not be restricted to microsomal compartments of the cell could perhaps be investigated by the inclusion in the assay mixture either of specific inhibitors of these other enzymes, or of an excess of alternative phosphates that are not suitable substrates for glucose-6-phosphatase but would probably occupy the less specific enzymes. Assay of the production of glucose - rather than of phosphate - would then give a better indication of the activity of the microsomal marker enzyme.

The observation that ^{125}I-PVP concentrated in the P_2 fraction (Figures 7.3.9 and 7.3.10) is not at odds with the assumption that its accumulation is lysosomal, and the surprisingly low concentration of ^{125}I-IgG in the rabbit P_5 pellet (Figure 7.3.10) can perhaps be correlated with the possible presence of a relatively high level of unsedimented microsomes in homogenates of that tissue.

The location of 'protected' ^{125}I-IgG in rat yolk sacs.

The preliminary subcellular radioactivity distribution patterns for rat yolk sacs preincubated with ^{125}I-IgG and then reincubated at 37°C and 20°C in the absence of substrate, before homogenisation and subcellular fractionation, were compared with those for undischarged yolk sacs (Figures 7.3.12, 7.3.13). In these experiments, yolk-sacs reincubated at 37°C had lost approximately equal amounts of TCA-soluble and TCA-insoluble radioactivity, while those reincubated at 20°C had lost relatively more TCA-insoluble material. Hence the differences between the subcellular radioactivity distributions for the reincubated tissues

might give a preliminary indication of the subcellular locations of protected ^{125}I-IgG in rat yolk sacs.

In Figure 7.3.13 the distribution patterns are expressed as relative specific activities, and it can be seen that (a) (tissue reincubated at 37°C) and (c) (not reincubated) have almost exactly the same distribution, with a peak in the P_5 fraction. On the other hand, (b), the pattern for tissue reincubated at 20°C, and which had lost proportionately more TCA-insoluble material than TCA-soluble material, shows a shift in the highest specific concentration of radioactivity from fraction P_5 to P_4. This suggests that ^{125}I-IgG destined for release intact from the tissue may be concentrated in the P_5 fraction, in accordance with the proposal that some of the microsomes isolated in the P_5 pellet are specific ^{125}I-IgG-carrying vehicles. These data should be interpreted with caution, however, in view of the fairly large amount of material lost from the reincubated yolk sacs (Figure 7.3.12) compared with the amount remaining. To derive firm conclusions concerning the nature of the released material from the distribution of that remaining may be misleading, and further experiments would be useful to establish the reproducibility of the results.

The location of protected ^{125}I-IgG in rabbit yolk-sacs.

When the preliminary results desribing the subcellular distribution of ^{125}I-IgG-derived radioactivity in rabbit yolk sacs preincubated in medium containing this substrate before reincubation in fresh, substrate-free, medium at 37°C or at 10°C were compared, it was shown (Figs 7.3.16, 6.3.25) that tissue reincubated at 37°C had lost approximately equal amounts of TCA-soluble and TCA-insoluble radioactivity while tissue reincubated at 10°C had lost essentially only TCA-insoluble material. Hence subtraction of the amounts of radioactivity detected in each subcellular fraction of the material that had been discharged at 37°C prior to homogenisation from the corresponding values for 'undischarged' tissue gives a pattern for 'releasable' radioactivity, while similar comparison of tissue discharged at 10°C with undischarged tissue gives a pattern for 'uninhibitible' radioactivity. It should again be emphasised that this discussion is based on duplicate results obtained using experimental tissue from one animal on one occasion only. The experiment must be repeated before the degree of reproducibility of the results can be established.

To facilitate the following discussion, three related terms are coined. It may prove useful to define them rigorously:

'Releasable radioactivity' - the total radioactivity that is found to be released from the yolk sac under optimal conditions ie. reincubation in fresh medium at 37°C for 2h.

This can be differentiated into:

'Inhibitible releasable' - that part of the releasable radioactivity that is not released when reincubation is at 10°C.

and

'Uninhibitible releasable' - that part of the releasable radioactivity that is still released when reincubation is at 10°C.

The difference between the subcellular distributions of 'uninhibitible' releasable radioactivity and 'releasable' radioactivity gives a distribution of 'inhibitible' releasable radioactivity. The patterns obtained from rabbit tissue, in this particular experiment, for 'releasable', 'uninhibitible releasable' and 'inhibitible releasable' radioactivity derived from ^{125}I-IgG are shown in Figure 7.3.15 (a), (b) and (c) respectively.

From these initial investigations, the most clear-cut observation is that virtually all of the supernatant-associated releasable radioactivity is apparently uninhibitible. The small amount that is inhibitible is found to be almost completely TCA-soluble.

Since 'uninhibitible releasable' radioactivity is probably mainly TCA-insoluble (Figure 6.3.25) it seems that the bulk of insoluble material may be released (Figure 7.3.15 (b)) from the supernatant fraction, but with the greatest concentration (in terms of ng of substrate per mg total pellet protein) being lost from the P_3 and P_4 fractions.

It was indicated in the results section (7.3.5) that the material apparently released from the supernatant fraction at 10°C may have been originally present in the cytoplasm, and not released from lysosomes that burst during the homogenisation procedure. (The possibility that the radioactivity is released from fragile vesicles that do not contain arylsulphatase must be borne in mind as a possible alternative explanation). This appears to form the bulk of the TCA-insoluble material released, and therefore this material may have been lost from microvesicles (non-sedimented at 3×10^6 gmin) or from free solution in the cytoplasm. There is some evidence (Figure 7.3.11(c)) of a fairly large quantity of microvesicles in rabbit yolk-sac homogenate supernatant, and easy ways to resolve this problem would be either to pass the supernatant fraction through a suitable gel-chromatography column and observe the elution profile for the radiolabelled IgG contained, or to centrifuge with greater force (eg. 60mins x 100 000g). Unfortunately, there was insufficient time to complete this investigation.

'Inhibitible releasable' (i.e. mainly TCA-soluble) radioactivity seems to originate (Figure 7.3.15(c)) from the P_1-P_5 fractions, with virtually none being lost from the supernatant. This suggests that material for degradation may be present in association with large organelles, and perhaps never free in the cytoplasm or in microvesicles that do not sediment at 3×10^6 gmin. The small amount of material lost from the supernatant fraction was originally present in TCA-soluble form and presumably in the process of diffusing out of the cells when homogenisation occurred.

In summary of this section of the overall investigation:

Preliminary observations suggest that:

(i) There is no detectable degradation of ^{125}I-IgG in rabbit yolk sacs at 10°C (Table 7.3.1).

(ii) Most ^{125}I-IgG for degradation may be present in association with sedimentable organelles, not in free solution.

(iii) The bulk of ^{125}I-IgG-derived radioactivity released in TCA-insoluble form from the cell may not be in association with large organelles that survive homogenisation. Therefore, in the intact cell, it may be present either free in the cytoplasm, contained in small vesicles that behave as unsedimented microvesicles or present in a non-lysosomal compartment that bursts easily during homogenisation.

The use of formaldehyde-denatured ^{125}I-IgG in rabbit yolk sacs.

Formaldehyde-treatment of ^{125}I-IgG causes its Endocytic Index in rabbit tissue to increase by a factor of approximately four (Figure 7.3.17), together with an increase in the steady-state tissue level of substrate of approximately 250%. Examination of the subsequent pattern of release of radioactivity from the reincubated tissue (Figure 7.3.18) showed that, in agreement with previous work (Weisbecker, 1981), formaldehyde-treatment effectively abolished the potential for protection of the molecule inside the yolk *in vitro*. Simple comparison of the subcellular fractionation patterns obtained for control ^{125}I-IgG and formaldehyde-denatured ^{125}I-IgG (Figure 7.3.19) suggests that the latter substrate is present more predominantly in heavier cell compartments. This may imply that the vehicles of the protective pathway are lighter but, as discussed more fully in the Results section (7.3.6), there are various sources of complication in a direct comparison of the patterns given by these two substrates, and more experimental work needs to be performed prior to precise analsis of the data.

Time course studies on rat yolk sacs.

A preliminary investigation into the route of ^{125}I-IgG through the cell was carried out by pulse-exposing rat yolk sacs to ^{125}I-IgG *in vitro* for various lengths of time and examining the effects on the subcellular distribution patterns for radioactivity obtained after (motor driven) tissue homogenisation. The results (Figure 7.3.6) are compatible with a gradual, time-dependent, shift of relative amounts of radioactivity associated with tissue from heavier (P_4 and P_5 fraction-associated) organelles to the nonsedimentable (supernatant) fraction, and this may arise by material present in the lysosomes being released after degradation and diffusing across the cytoplasm and thus out of the cell. The P_5 pellet-associated radioactivity appears to reach its maximum level very quickly (after about 20 min), and it seems to be independent of the level of radioactivity present in the supernatant fraction (which is still apparently rising after 30 min). If ^{125}I-IgG associated with the P_5 fraction is specifically destined for release in intact form from the cell, then the implication from these preliminary results is that protected material reaches the microsomes in significant amounts during the first five minutes. It is impossible to conclude whether this is indicative of micro-endocytosis or the rapid formation of IgG-containing

microsomes from receptosomes of some description, but the evidence available suggests that after 5 min the P_5 fraction does not contain such a relatively high concentration of radioactivity compared with other pellets as it does after 20 min or more. This, though flimsy evidence, is compatible with the possibility that endocytosis is not directly into microvesicles but into another, larger compartment, possibly a receptosome. Microvesicles may then form quickly from the receptosome and the protective pathway apparently reaches a steady state within 30 min of initial exposure to ^{125}I-IgG.

There appears to be substantial potential in the use of this investigative technique to follow the distribution of ^{125}IgG in yolk sac cells during the first few minutes - or even seconds - of endocytosis.

In summary of the various investigations performed in this chapter;

i) Many different lines of evidence have suggested that in both rat and rabbit yolk sac homogenate preparations, the microsomal pellet isolated contains an important component of the cellular protection process of IgG. The evidence available suggests that the corresponding rabbit-organelle may be more difficult to sediment than that of rat, although otherwise both seem very similar.

ii) Preliminary studies on rat tissue can be interpreted to indicate a short lag phase in the entry of ^{125}I-IgG into the P_5-compartment, being perhaps indicative of the prior involvement of a larger vesicle, suggestive of a receptosome or lysosome.

iii) The bulk of the evidence accumulated to date suggests that many of the IgG-receptors in the P_5 pellet isolated under these conditions from rat tissue are available for reaction, and it seems likely that they are attached either to flimsy vesicles that are easily burst, or to the cytoplasmic surface of vesicles that may remain intact. Alternatively, they may be in association with pieces of membrane, subject to observation iv), that the membrane pieces are probably not derived from the plasmalemma.

iv) Preliminary results indicate that the process of vesicularization of the plasma membrane during homogenisation is probably not an important source of the high level of microsomal-pellet-associated ^{125}I-IgG

detected in rat yolk sacs.

Chapter 8. General Discussion.

DISCUSSION

The purpose of this final discussion is to bring together observations and conclusions from the various experimental chapters, and to attempt to integrate them into a coherent reappraisal of the possible mechanisms for the transcellular transport of immunoglobulins from mother to foetus across the yolk sac.

First a summary of the conclusions reached in earlier chapters;

(Chapter 3) (a) Experimental results obtained previously with gamma globulins are essentially similar to those obtained using pure IgG in the rat yolk-sac incubation system. Interpretations made of previous data therefore appear to be valid.

(b) Various methods of radio-iodination, the oxygen tension in the incubation system and the incorporation of iodotyrosine into or onto cell membranes or newly-synthesised protein do not in practice appear significantly to affect the results obtained with the rat yolk-sac incubation system.

(c) Bulk protein appears to saturate and partially immobilise the cell surface of the rat yolk sac, thus slowing down the rate of endocytosis.

(d) In rat tissue, no detectable amount of fluid is transported across the cell in inducible IgG-specific vesicles.

(Chapter 4) (e) The signal sequence mediating protection inside the rabbit yolk sac is sited in the Fc region of IgG.

(f) Rabbit yolk-sac homogenates exhibit very little proteolytic activity towards IgG at pH values greater than pH 5. Surface-bound proteinases therefore appear unimportant in the catabolism of this protein.

(Chapter 5) (g) Neuraminidase and beta-galactosidase treatment of the rabbit yolk-sac surface suggest no carbohydrate involvement in the specific recognition of IgG.

(h) Rabbit IgG-linked carbohydrate seems to mediate against specific protection. The simplest explanation is that the receptor recognition unit is a polypeptide sequence located spatially close to the hinge-linked carbohydrate.

(i) There was no observed competitive inhibition of the

interaction between the signal and receptor mediating *in vitro* protection of IgG, by a variety of simple sugars and sugar phosphates, in rat tissue.

(Chapter 6) (j) The characteristics of endocytic uptake of IgG for protection and for lysosomal degradation seem to be incapable of differentiation by a variety of inhibitors, both in rabbit and in rat tissue.

(k) Release from yolk sacs of both species, of undegraded material appears to be inhibited by metabolic inhibitors, but not so much by cytoskeletal poisons, EDTA or 10% (v/v) calf serum.

(l) The release of the bulk of the macromolecular material is less sensitive to low temperatures than is the formation and release of TCA-soluble material, from tissues of both species.

(Chapter 7) (m) IgG-containing organelles have essentially similar subcellular distributions in both rat and rabbit tissue homogenates. There is a lysosomal concentration and also a concentration in a lighter fraction that behaved as a microvesicle in rat tissue preparations but was thought to be largely unsedimented in rabbit tissue homogenates.

(n) Short-term pulsing of rat yolk sacs with substrate gave preliminary indications in keeping with the possibility that protected IgG passes from the (heavy) lysosomal fraction towards the (light) microsomal one in the first few minutes after internalization.

(o) Many IgG-specific receptors appear to be exposed to the surrounding fluid when rat microsomes are isolated under these experimental conditions.

(p) The IgG-containing microsomes appear not to be substantially surface membrane-derived during homogenation of rat yolk sacs.

The investigations carried out in Chapter 3 seem to have covered all the obvious potential sources of error in the incubation system and did not detect any important faults. Previous work, and in particular the thesis of U. Weisbecker therefore stands on a solid premise, and there is no need for substantial modification of her interpretation of the data. Essentially, the evidence available to date suggests, without contradiction, that IgG is treated in a special way within yolk sacs incubated *in vitro*. It is endocytosed in an energy-dependent manner, some of it is degraded in the lysosomes and some is released again by an

energy dependent process into the surrounding medium in an intact form. Protection from degradation is shown to be a characteristic of the Fc part of the molecule, and the property is shown to be dependent on the species of origin of the IgG and also of the yolk sac. Taken together these observations suggest that the yolk sac is performing a process *in vitro* very similar to its normal *in vivo* role of maternofoetal IgG transmission.

Essentially the only feature of the *in vitro* process that still requires fundamental clarification is the mechanism and route of release of the undegraded material. *In vivo* the intact IgG is released at the lateral or basement membrane, and makes its way through the subcellular mesenchyme into the vitelline blood circulation and thence directly to the foetus. It is possible that the exit route from the tissue of intact IgG *in vitro* is also via the vitelline circulation into the incubation medium, but it seems likely that this circulation will be effectively stagnant under incubation conditions. However, if stagnation of the vitelline circulation causes the IgG to be released from the tissue by non-physiological mechanism, then *in vitro* investigation into the nature of release processes may give misleading results. Preliminary attempts to study the site of release of intact material from the tissue are outlined in Appendix 3.

Despite a lack of clear understanding of whether the *in vitro* release of intact IgG was vectorial, there is no evidence to suggest that the phenomenon of IgG-protection *in vitro* is an artifact induced by the experimental conditions. The similarities observed between *in vitro* protection and *in vivo* transmission are sufficient to justify the provisional assumption that the *in vitro* model is a good mimic of the physiological process of the maternofoetal transfer of passive immunity.

Stanworth *et al* (1976) postulated that the CH_3 region of the immunoglobulin molecule contains most of the binding sites employed by the molecule in situations in which the activity of the Fab region was still important e.g. binding sites for macrophages, mast cells and cytotoxic lymphocytes, while the CH_2 region contained most of the effector sites e.g. histamine releasing, phagocytosis stimulating, complement activating and the auto-antigenic sites. Since this broad generalisation still appears to be essentially correct, the site of the IgG molecule that mediates maternofoetal transmission would be expected,

by these guidelines, to be present in the CH_2 region of the molecule.

The observation in chapter 5, that cleavage but not disruption of the immunoglobulin-linked carbohydrate groups causes an increase in the degree of protection in the yolk sac, suggests that the site of the specific signal sequence is polypeptide in nature and situated spatially close to either the hinge-linked or the CH_2-linked carbohydrate. In conjunction with the literature (Hinrichs & Smyth 1976), the implication was that the group in question was the hinge-linked carbohydrate, and therefore that the polypeptide sequence mediating protection (transport) was situated near to Threonine 227. This again is in agreement with Stanworth's generalization (above) since during transport the molecule is not intended to be biologically active in terms of interacting with antigens, and therefore there is no reason for the signal sequence to be present in the CH_3 region. If the signal is in fact a simple polypeptide sequence, rather than a complex three-dimensional structure formed by the tertiary folding of the molecule, then preparation of the various amino acid sequences that might form the signal would permit identification of it.

The observation that formaldehyde treatment of IgG completely destroys its potential for protection inside the yolk sac (Weisbecker, 1981, and Chapter 7) is supportive of the idea that the signal is polypeptide in nature. Formaldehyde readily combines with unprotonated amine residues to give methylol derivatives;

$$R_2CHNH_2 + 2HCHO \quad R_2CHN(CH_2OH)_2$$

and this strongly suggests the involvement of an unprotonated basic amino acid residue (Lys, Arg or His) in the signal sequence.

The local sequence of amino acids, adjacent to the CH_2-carbohydrate-linked Threonine residue is

N CBH C

Ser-Lys-Pro-Thr-Cys-Pro-Pro-Pro-Glu-Leu

the only basic residue being lysine.

An area of investigation that would be interesting to pursue in the future would be to study the behaviour in the <u>in vitro</u> system of both

whole IgG and fragments of IgG that have been modified by reaction with chemical reagents that are more selective in their modification of specific amino acid residues than is formaldehyde. Of particular interest would be the effects on IgG behaviour of radiolabelling the molecule with a lysine-conjugating agent (eg. Bolton-Hunter reagent, or the imido-ester technique outlined in Appendix 1). By attaching to lysine residues, these reagents might be observed to cause a specific inhibition of the interaction of the IgG molecule with its receptor, thus impairing its capacity for protection whilst perhaps having no other deleterious effects at all.

Alternatively, synthesis of a peptide (for example a decapeptide) starting from proline and extending towards the N-terminal, and its subsequent testing either in vivo or in vitro (such a small molecule would be best labelled either with ^{14}C or ^{3}H or used as a competitive inhibitor of transport of the intact whole molecule), might give interesting information on the site and nature of the signal unit. An alternative approach to this type of enquiry would be to study the degree of protection in the in vitro rat yolk-sac incubation system afforded to the different subclasses of rat IgG of known structure. Comparison of their observed behaviour with their carbohydrate and amino acid structures might provide useful information concerning the signal unit. This latter type of investigation is likely to become very complex, however, particularly if the unit is composed by cooperative action of non-adjacent parts of the molecule, and high technology computer software would probably be required to investigate the question properly.

The other main line of investigation in this study was concerned with the cellular mechanism of diacytosis of homologous IgG in rat and rabbit yolk sacs. No firm evidence was found to indicate a profound difference between the mechanisms in the two species and the assumption is made that they are essentially similar, although there was some evidence that the microsomal carrier vesicles were less easily sedimented on centrifugation of homogenates of rabbit tissue than were those in homogenates of rat yolk sacs.

The apparent difficulty (Chapter 6) of differentiating between the uptakes of ^{125}I-IgG and of ^{125}I-PVP by the use of various inhibitors suggests that they may be taken up in the same vesicles, and there is a large amount of morphological evidence for association between IgG and

whole IgG and fragments of IgG that have been modified by reaction with chemical reagents that are more selective in their modification of specific amino acid residues than is formaldehyde. Of particular interest would be the effects on IgG behaviour of radiolabelling the molecule with a lysine-conjugating agent (eg. Bolton-Hunter reagent, or the imido-ester technique outlined in Appendix 1). By attaching to lysine residues, these reagents might be observed to cause a specific inhibition of the interaction of the IgG molecule with its receptor, thus impairing its capacity for protection whilst perhaps having no other deleterious effects at all.

Alternatively, synthesis of a peptide (for example a decapeptide) starting from proline and extending towards the N-terminal, and its subsequent testing either in vivo or in vitro (such a small molecule would be best labelled either with ^{14}C or ^{3}H or used as a competitive inhibitor of transport of the intact whole molecule), might give interesting information on the site and nature of the signal unit. An alternative approach to this type of enquiry would be to study the degree of protection in the in vitro rat yolk-sac incubation system afforded to the different subclasses of rat IgG of known structure. Comparison of their observed behaviour with their carbohydrate and amino acid structures might provide useful information concerning the signal unit. This latter type of investigation is likely to become very complex, however, particularly if the unit is composed by cooperative action of non-adjacent parts of the molecule, and high technology computer software would probably be required to investigate the question properly.

The other main line of investigation in this study was concerned with the cellular mechanism of diacytosis of homologous IgG in rat and rabbit yolk sacs. No firm evidence was found to indicate a profound difference between the mechanisms in the two species and the assumption is made that they are essentially similar, although there was some evidence that the microsomal carrier vesicles were less easily sedimented on centrifugation of homogenates of rabbit tissue than were those in homogenates of rat yolk sacs.

The apparent difficulty (Chapter 6) of differentiating between the uptakes of ^{125}I-IgG and of ^{125}I-PVP by the use of various inhibitors suggests that they may be taken up in the same vesicles, and there is a large amount of morphological evidence for association between IgG and

coated pits on the cell surface (e.g. Moxon et al, 1976). This suggests that the first stage of diacytosis of IgG is via clathrin coated pit-mediated uptake, and the first destination may be the receptosome for sorting from other substrates. The pH-dependence of interaction between the IgG and its specific receptor has not been properly documented (an unreported attempt in this study was abandoned because such small amounts of IgG bound to yolk sacs at 4°C even at physiological pH that it was not reliably measureable), but binding is thought to be greater at pH 6-6.5 than at pH 7-7.5 (Balfour & Jones, 1977). This has often been correlated with the increase in pH experienced in passing from the uterine lumen to the vitelline circulation, this change being assumed to cause release into the circulation of IgG that was firmly bound to its receptor on the maternal side of the cell. Such resistance to acid of the IgG-receptor interaction suggests that it may persist in a slightly acid receptosome, although it would seem unlikely that the interaction persists at lysosomal pHs (this has never been shown, but would be a very simple experiment). Even if the interaction were stable at lysosomal pHs, the concept of protection from lysosomal degradation by binding to specific membranous receptors seems both physically unlikely and biologically inefficient.

Since specific exit of IgG from the secondary lysosome must occur eventually it would seem to be far more simple to segregate the IgG into a specific vesicle well before fusion occurs with the lysosomal system. The preliminary evidence obtained in Chapter 7 suggests that this specific vesicle is very small, perhaps without a protein coat and appears to be either fairly easily ruptured or displays the IgG receptors on its cytoplasmic surface. Its fusion with the lateral or basal cell membrane is shown to be dependent on metabolic energy, but apparently not mediated by cytoskeletal elements, and if the existence of the carrier vehicle is induced by the presence of IgG, it does not appear to carry any measurable amount of fluid across the cell as measured by release of ^{125}I-PVP. Of the published formal hypotheses of the diacytic mechanism, these observations are in best agreement with that proposed by King (1982a), who envisaged the formation of specific transport vesicles to be largely an intracellular event.

In summary, the interpretation of the evidence obtained in this study concerning the mechanism of maternofoetal transmission of antibodies is as follows:

(i) The determinant on the IgG that mediates maternofoetal transmission is probably polypeptide in nature, located near the hinge-linked carbohydrate of IgG, and may well depend on a basic group (e.g. Lys) for its action.

(ii) The specific membrane-bound receptor does not appear to involve carbohydrate in its uptake of IgG.

(iii) IgG may be internalised in the same vesicles as fluid phase markers and is probably quickly sorted in receptosomes. Specific microvesicles, containing IgG but very little fluid, may form before fusion occurs with the lysosomes, and they carry the IgG to the lateral or basement cell membranes. Fusion with these membranes allows low pH-induced desorption of IgG from its receptor and it makes its way through the subcellular mesenchyme and vitelline epithelium into the vitelline circulation intact.

(iv) Although clathrin coats are undoubtedly important scaffolds facilitating the formation of many types of vesicles, exactly which subcellular organelles possess them and whether any sorts of vesicles carry their own around with them remains unresolved. No evidence was obtained in this study to suggest that the microvesicles concerned with specific release of intact IgG are possessed of clathrin coats.

APPENDICES.

APPENDIX 1

A critical comparison of various radiolabelling techniques.

Radiolabelling of substrates has been widely used for many years to facilitate monitoring of their behaviour in biological systems. Careful careful use of the available techniques can efficiently label most molecules without significantly modifying their biological activities.

In this particular study, the majority of radioactive tracing involved using various immunglobulin preparations, the molecule of greatest interest being IgG. The first decision to be made was with which particular isotope to radiolabel the proteins of interest, widely used possibilities being ^{3}H, ^{14}C, ^{125}I and ^{131}I. The disintegration rate for ^{125}I is 600 x as great as that of ^{3}H, and 52000 x that of ^{14}C, and it was apparent that the small amount of substrate metabolised by yolk-sac cells would necessitate labelling of substrate to high specific activity to give useful results. A major problem with iodine is that its large size (approx = benzene ring) may cause some modifications to molecular behavioru whereas ^{14}C and ^{3}H can be incorporated into the normal structure of the substrate itself, and therefore remain biologically indetectable. It was felt that provided suitable controls were incorporated to establish that the behaviour of the labelled molecule was not significantly different from that of the unlabelled species, iodine was the element of choice.

The next decision was whether to use ^{125}I or ^{131}I. Table A.1 shows that ^{131} has a far greater specific activity but a very much shorter half life than ^{125}I. Since substrates, once labelled, would be used over a period of weeks in order to carry out sufficient replications, ^{125}I was selected as the only suitable isotope.

Direct ^{125}I-labelling of proteins usually occurs by iodination of the benzene ring of tyrosine residues, some tyrosine residues labelling more readily than others. Provided that these iodinated residues are not involved in the active sites of the substrate, associated biological activities may be unaffected.

A major source of difficulties experience with radioiodinated substrates is damage caused to the proteins during the various iodination procedures themselves, usually by oxidizing or reducing agents. The changes, induced in this way, may modify the subsequent biological behaviour quite independently of the introduction of an iodine atom into the molecule.

The ideal method of direct radioiodination, then, is one that causes no alteration to the protein other than that induced by the actual physical presence of iodine.

For most purposes, investigators use proteins iodinated to an extent of less than one iodine atom per molecule of protein, hence effectively tracing only a subpopulation of molecules that may not be representative of the rest. This is because an iodination ratio of greater than one atom per molecule is more likely to induce unnecessary damage in the protein structure, either during iodination or during the storage of the product (self-irradiation damage).

In most iodination reactions, the attacking species is I^+, which reacts with ionized tyrosyl residues and substitutes an I atom for the H atom on the carbon adjacent to the oxygen-carrying carbon. (Figure A.1 shows the fundamental reaction).

Early methods of radioiodination caused molecular iodine to disproportionate (induced by oxidising agents such as persulphate, iodate or nitrite) and hence generated the attacking species. In theory this method can only be 50% efficient, and in practice the levels of oxidising agent used tended to cause severe damage to the substrates. In chronological order of their proposal, the main methods of direct iodination of proteins are as follows.

The iodine monochloride technique

This method was originally proposed by McFarlane (1958) and relies on the exchange of I^+ between $^{125}I_2$ and ^{127}ICl to yield in effect a low concentration of free $^{125}I^+$. Addition of protein substrate will allow iodination to proceed relatively slowly, but as I^+ is removed from solution, so more is released from ICl and the reaction continues. The

reaction is only useful at neutral pH, and there will always be some incorporation of $^{127}I^{+}$ but this technique has the advantage of not exposing the protein to any damaging oxidising agents.

Chloramine T technique

This method was originally employed by Greenwood and Hunter (1963) and it makes use of a fairly mild oxidising agent, Chloramine T (which is the sodium salt of the N-mono-chloro derivative of p-toluene sulphonamide), to produce the reactive $^{125}I^{+}$ from $Na^{125}I$. The effects of pH, time of reaction, amounts of Chloramine T and protein on the reaction are shown in Figure A.2 (taken from V. Bocci, 1969). Maximum efficiency is seen to be achieved with a Chloramine T/protein ratio of approx 1/200 (Greenwood & Hunter used one of 20 i.e. 4000 x excess) and it is widely accepted (Freeman, 1959) that too much chloramine T is a major source of damage to proteins being radiolabelled, possibly by causing the formation of IO_3^{-} and IO_4^{-}.

Electrolytic iodination

The method originally proposed by Rosa *et al* (1963) was claimed to label proteins to a high specific activity, to cause no vapourization of ^{125}I and give a 'favourable turnover' time in humans compared with ^{35}S-labelled proteins, though this latter point was not supported with data. Its biggest advantage was that the protein was never exposed to oxidising agents and were therefore not unnecessarily damaged. In practice, however, it involves a long exposure to iodide solution (and there are suggestions in the literature (Hunter, 1971) of the presence of noxious contaminants (e.g. polyiodides that may cause protein damage) in the commercial preparations. Also, the large scale of the preparative procedure is rather awkward for small amounts of protein and there is some evidence that iodine ends to become dissociated from the protein over a period of days (Beahon, unpublished observations).

Enzymatic methods

The first enzyme to be used as a protein iodinating agent was lactoperoxidase (Marchalonis, 1969). Other enzymes have been tried since, but lactoperoxidase remains the most widely used. Peroxide is needed to

activate the enzyme to carry out the iodination, which can occur over a wide range of pH (pH 3-8). Hence the optimum pH for the individual protein can be utilized, and the efficiencies of labelling can simultaneously be very high (Thorell & Johansson, 1971; see Table A.2), although this can vary from protein to protein. The main disadvantage of the technique is that it is difficult to remove the enzyme from the preparation after labelling, unless it is pre-fixed to beads (Karonen et al, 1975), although it is itself poorly labelled and for most purposes therefore unimportant. The peroxide may also have deleterious effects on the protein and is usually added in small amounts during the reaction rather than all at the start.

These four are the main methods of direct protein radioiodination. Many comparisons have been made between them in the literature, of which a few will be mentioned here. In selecting a suitable method of radio-iodination there are two main criteria to consider: (i) The protein must not suffer damage that significantly modifies its behaviour, and (ii) the level of iodination achieved must be adequate for experimental purposes.

For most protein substrates, the second of these requirements can usually be fulfilled using any of the techniques described. The question of damage, however, is much more difficult to define, and therein may lie many of the apparent contradictions that are widely encountered in literature comparisons. For example, a radioiodinated protein may, at the same time, retain its immunoreactivity with a specific antiserum but have lost its biological activity completely, meaning that various workers assessing the same effects by measuring different parameters may reach contradictory conclusions.

Another important source of contradiction in comparisons of the effects of different techniques of protein iodination is misuse of the techniques themselves. For example, the comparison of methods of albumin iodination by Karonen et al (1975) assesses damage to protein by electrophoretic mobility changes and is very critical on these grounds of Chloramine T as a radioidination reagent (Table A.3). Their use of a Chloramine T/protein ratio (w/w) of 8/1 (ie. 1600 x too much, according to Bocci, 1969) would be expected to cause an unnecessarily large amount of protein damage, and hence any criticism based on these particular

results should not be taken as valid criticism of the Chloramine T technique itself. Similarly, Holohan et al (1973) used a Chloramine T/protein ratio of 4/1 (800 x excess) and observed a high level of damage caused to polypeptide hormones by Chloramine T compared with lactoperoxidase, as judged by immunoprecipitation.

Krohn et al (1972) compared different techniques of radioiodinating fibrinogen and used Sepharose 4B chromatography to assess damage (Table A.4). For biological work they recommended the iodine monochloride technique and found that Chloramine T induced a degree of aggregation.

Hemmings & Redshaw (1974) labelled IgG by various methods - including a modification of the original Chloramine T technique (Sonoda & Schlamowitz, 1970) - and recommended lactoperoxidase for biological work, although they did not try iodine monochloride..

Krohn et al (1976) carried out an in-depth investigation of various effects of labelling fibrinogen by four different techniques (Table A.6) and eventually concluded that none of the four was obviously preferable.

One problem common to all techniques of direct iodination of proteins is that tyrosine residues are usually the points of attachment of the iodine atom. This can be inconvenient when either the substrate has tyrosine in its active site or alternatively contains no tyrosine (e.g. secretin). The idea of conjugating the protein to an iodine-containing acylating agent was first formulated by Bolton and Hunter (1973) who used iodinated 3-(4-hydroxyphenyl)propionic acid N-hydroxysuccinimide ester to form amide bonds to free amine groups of the protein. Hormones labelled thus exhibited superior immunoreactivity over Chloramine-T-iodinated preparations, but the main advantages of the technique are the absence of exposure of the protein to oxidising or reducing agents or to possibly noxious contaminants of the $Na^{125}I$ solution. Further, the possibility of labelling groups other than tyrosyl residues is a distinct advantage for certain proteins with 'active' tyrosine residues (Figure A.3).

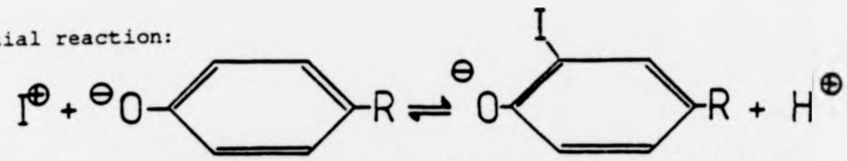
The main disadvantage in the use of the 'Bolton-Hunter' reagent is that it causes a significant alteration in the charge of the protein, which may have deleterious effects on the biological activity. This has

now been overcome by the introduction (Wood *et al*, 1975) of an imido-ester conjugation technique using Methyl 3, 5-di(^{125}I)iodohydroxybenzimidate (Figure A.4) that preserves the charge on the amine group of the protein via amidine linkage.

In summary, whatever the criteria used to assess its value, the best technique for iodination may vary between different proteins, and it is important that any method, in the form stated in the literature, is not applied to a particular problem without critical evaluation of the final labelled product.

Figure A.1 **Methods of radio-iodinating proteins.**

Essential reaction:



.....

Original methods (a) $^{125}I_2 \rightarrow {}^{125}I^+ + {}^{125}I^-$

(b) reaction assisted by persulphate, iodate or nitrite.

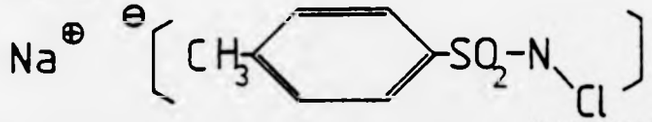
.....

Iodine Monochloride (MacFarlane, 1958) (a) $^{125}ICl \rightarrow {}^{125}I^+ + Cl^-$

(b) $ICl + {}^{125}I_2$ - equilibrium

.....

Chloramine-T (Greenwood et al, 1962) A mild oxidising agent:



.....

Electrolytic (Rosa et al, 1963) "Controlling current controls degree and rate of iodination."

.....

Enzymatic (Marchalonis, 1969). Lactoperoxidase most popular. HRP less efficient. Beware of azides, cysteine, mercaptoethanol etc. Can usually be immobilised if necessary.

Figure A.2 Factors affecting Chloramine-T iodination of rabbit serum proteins.

Taken from Bocci, 1969.

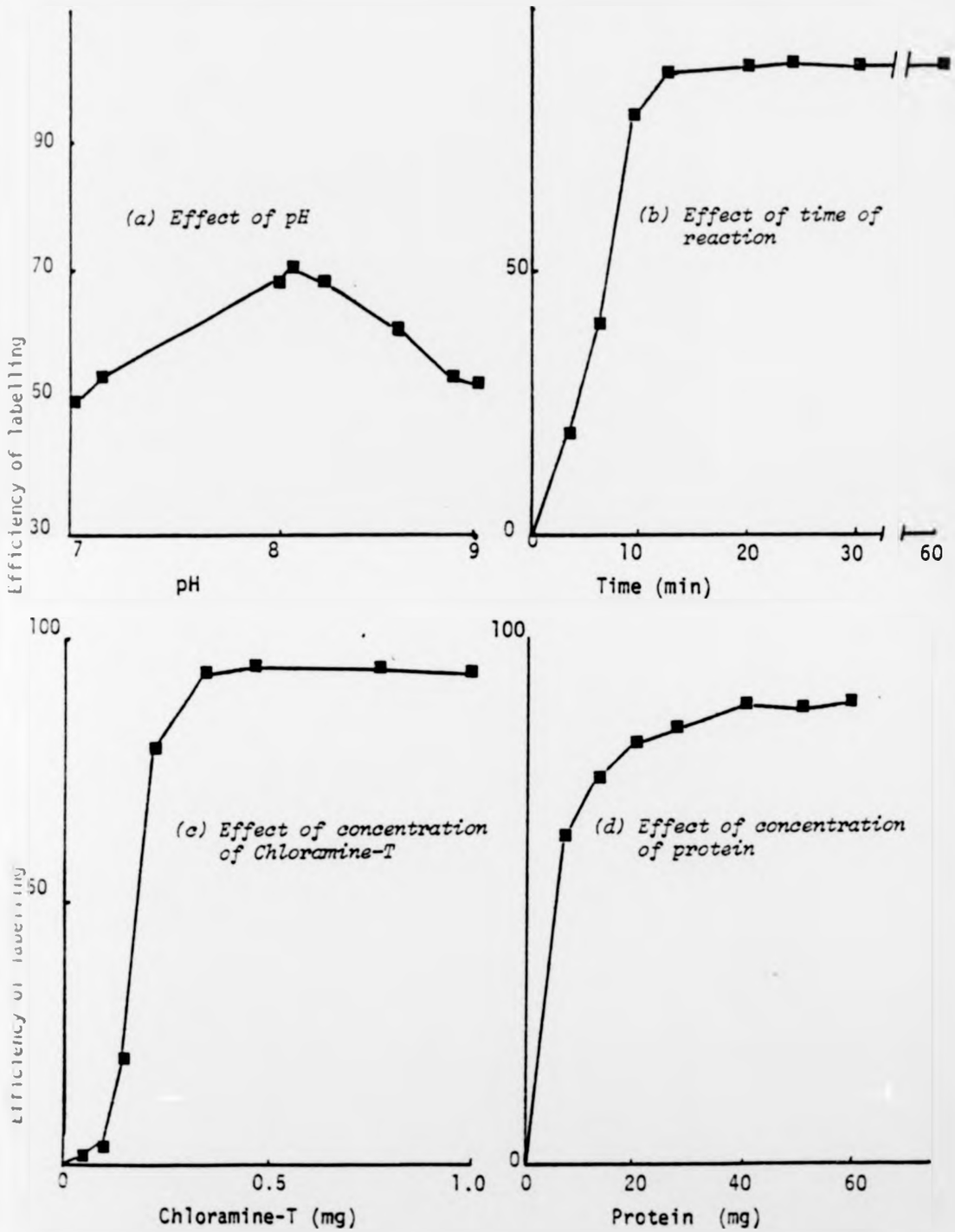
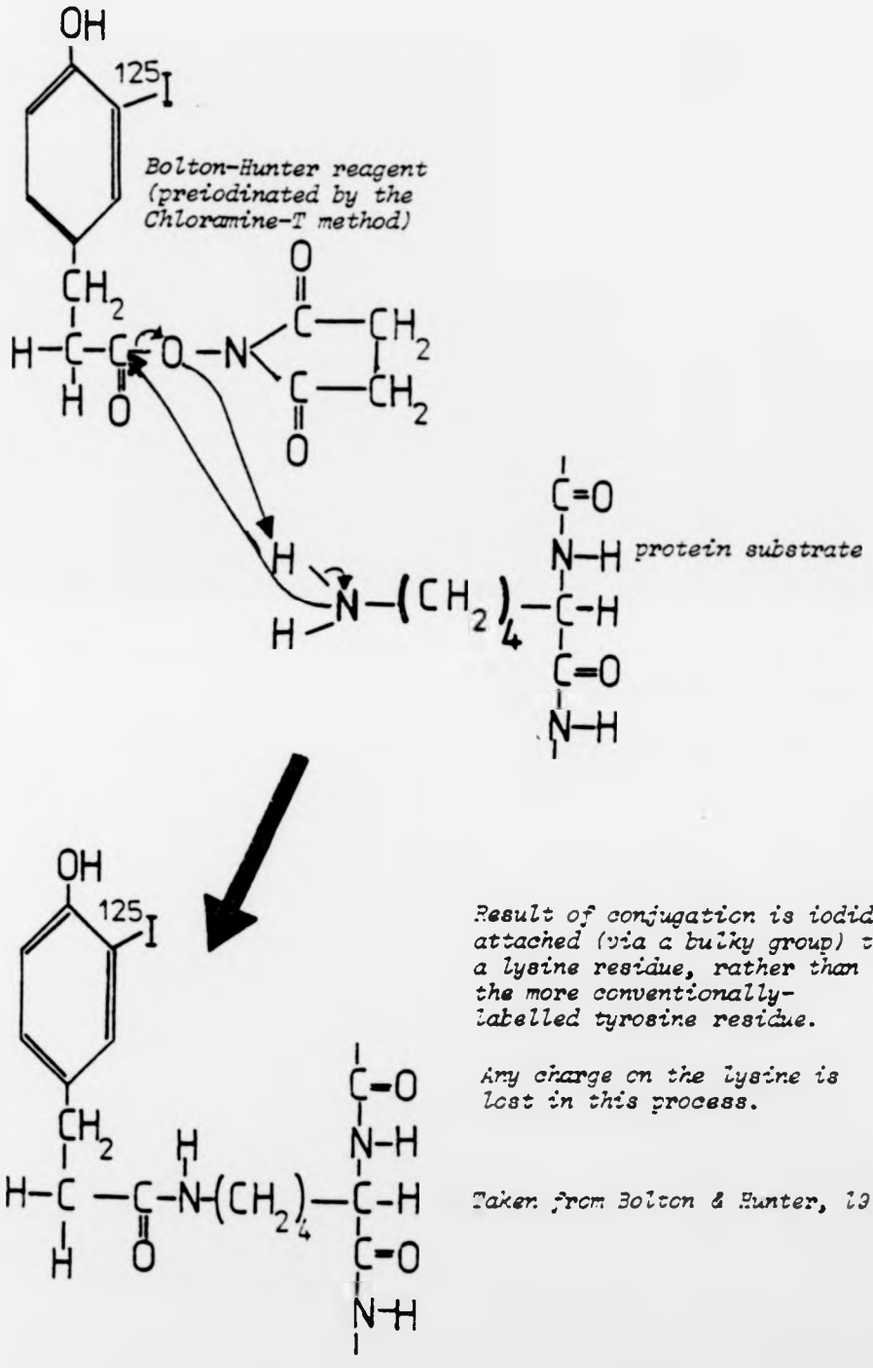


Figure A.3 Conjugation labelling 1. The Bolton - Hunter reagent.

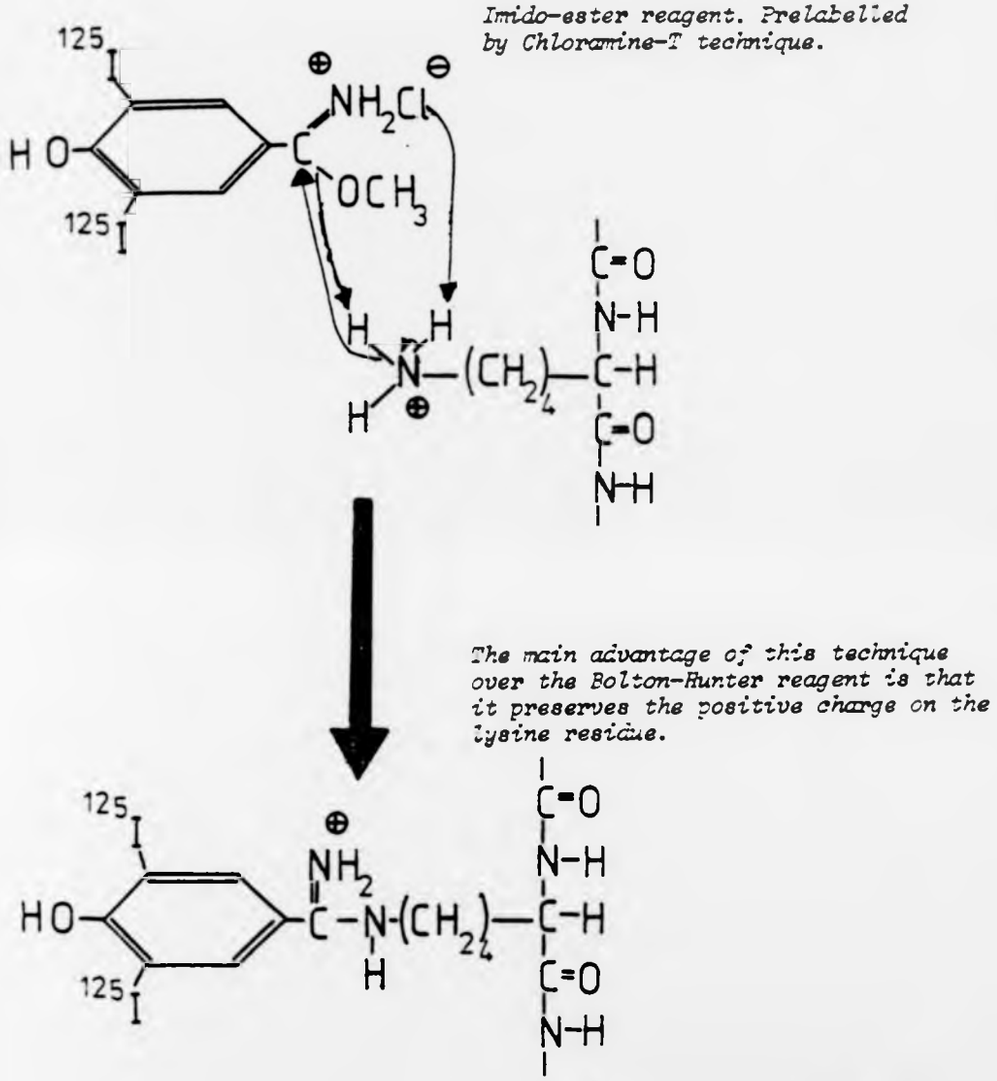


Result of conjugation is iodide attached (via a bulky group) to a lysine residue, rather than the more conventionally-labelled tyrosine residue.

Any charge on the lysine is lost in this process.

Taken from Bolton & Hunter, 1973.

Figure A.4 Conjugation labelling 2. Imidoester reagent.



Taken from Wood et al, 1975.

Table A.1 **The properties of ^{125}I and ^{131}I.**

	^{125}I	^{131}I
Specific activity for 100% isotopic abundance (mCi/ug, theoretical)	18	125
Isotopic abundance available in carrier-free preparations*	80 - 95%	15 - 20%
Counting efficiency in NaI well-type gamma counter	80%	40%
Half life (days)	60	8

* Carrier-free ie. in the absence of added carrier. In practise, especially with ^{131}I, cold iodide (^{127}I) is always present.

Table A.2 **Iodination by lactoperoxidase (Thorell & Johansson, 1971).**

Protein	Incubation time (s)	mCi ^{125}I	Yield (%)	Adsorption to Whatman 3MC	Binding to antibody
Insulin	2	1.0	88	95	93
Insulin	60	1.8	96	94	?
G.H.	2	1.0	72	80	83
G.H.	60	1.8	92	80	?
Glucagon	60	0.5	94	89	?
Glucagon	60	1.8	88	94	?
L.H.	60	1.0	82	?	85
F.S.H.	60	1.0	89	?	82
Thyrotropin	60	1.0	86	?	88
IgG	60	1.2	95	?	?
Albumin	2	1.8	82	?	?
Lactoperoxidase	20	1.0	10	?	?

In each case 5ug substrate and 4ug lactoperoxidase was used, except for 250ug IgG.

These authors took binding to Whatman 3MC in buffer flow chromatography as being indicative of undamaged protein.

Note the inefficient self-labelling of lactoperoxidase.

Table A.3 **The iodination of albumin (Karonen et al, 1975).**

Method	% Efficiency of labelling	Specific activity	Damage caused
Chloramine-T	49	42	26
HRP (aq. phase)	11	10	?
HRP (sol. phase)	3	3	?
Lactoperoxidase (aq.)	47	40	¨5
Lactoperoxidase (sol.)	39	34	¨5

Damage was assessed by electrophoretic mobility.

N.B. The conditions used for Chloramine-T labelling were not optimal (see text).

Table A.4 **Labelling of fibrinogen by different techniques (Krohn et al, 1972).**

Method	% staying at top of Sepharose 4B column.	% fibrinogen	% I^-	Other
a) Chloramine-T				
CT/protein ratio (w/w)				
10, +reducing agent	79	10	6	5
1.3, +reducing agent	70	9	21	
0.2, no reducing agent	15.6	51.6	32.3	
0.05, +reducing agent	6	85	9	
0.002, no reducing agent	0.005	0.5	99.5	
b) Electrolytic				
70min, 250uA	17	21	0	62 IO_3^-
250min, 70uA.	29	31	40	
120min, 300uA	15.6	16.4	68	
c) Iodine monochloride				
MacFarlane (1963) buffer. pH 7.9, 0.2I/fibrinogen	"1	71	28	slight high m.wt. tail
Helmkamp (1967) buffer pH 8.0,, 0.5I/fibrinogen	"1	57	42	

In vivo studies (not reported) "raise doubts as to the validity of the use of the electrolytic or Chloramine-T preparations for clinical studies". NB. The ratio of protein to Choramine-T is not optimal (see text).

Table A.5 **Radio-iodination of IgG (Hemmings and Redshaw, 1974).**

Method of Iodination	According to	% yield	Specific activity (Ci/g)	C.Q.	Standard error
Chloramine-T	Hunter & Greenwood (1962)	35	2.05	0.008	0.0005
Chloramine-T	Sonoda & Schlamowitz (1970)	67	0.067	0.255	0.032
Hypochlorite	Redshaw & Lynch (1974)	76	6.7	0.201	0.027
Electrolytic	Rosa et al (1964)	55	0.027	0.280	0.028
Lactoperoxidase	David (1972)	56	0.056	0.372	0.040

Table A.6 **Enzymatically hydrolysed iodinated fibrinogen Krohn et al, 1976.**

Product	ICl	Lact'ase	Chloramine-T CT/Prot ratio			Electrolytic	
			1/9	1/1.4	1/0.6	25I/mol	5I/mol
I^-	6.6	8.1	5.4	19.2	24.3	16.7	32.9
IO_3^-	1.6	0.5	0.8	1.7	4.1		
Monoiodohist.	1.5	1.8	1.6	1.7	3.4	2.5	1.6
Diiodohist.	0.7	-	0.7	0.9	4.2	-	-
3-iodotyr.	78.3	84.3	82.3	62.9	21.4	57.8	56.4
Oxidised iodotyr.	-	-	0.9	1.2	5.2		
Iodophe.	-	-	-	0.3	3.5	-	-
Diiodotyr.	5.2	-	1.5	8.8	12.1	21.0	7.3

APPENDIX 2

Programme used to calculate the uptake of non-degradable substrates.

```
0010  DIM C£30
0020  REM KEW 125-IPVP PROGRAM,MODIFIED NOV.,1974
0027 REM FURTHER MODIFIED DECEMBER 1980 BY LEN
0030  DIM H(15),I(15),K(15),L(15),R(15)
0060  PRINT "NON-DIGESTIBLE PROG. ENTERED"
0070 % "EXPERIMENT CODE =";
0080 INPUT Z£
0090  PRINT "BACKGROUND IN CPM=";
0100  INPUT A
0110  PRINT "COUNTING TIME FOR EACH ML OF MEDIUM, SECS=";
0120  INPUT C
0130  PRINT "COUNTING TIME FOR EACH ML OF YS SOLUTION, SECS=";
0140  INPUT D
0150  PRINT "NO. OF POINTS IN PLOT=";
0160  INPUT G
0165  PRINT "NAME OF DATA FILE:";
0167  INPUT F1£
0170  PRINT "NAME OF REGRESSION FILE:";
0174  INPUT F2£
0176  FILES ,F1£,,F2£
0180  PRINT "NAME OF RESULTS FILE:";
0184  INPUT F3£
0186  FILES ,F3£
0190  FOR X=1 TO G
0200  INPUT , H(X),I(X),K(X),L(X),
0210  NEXT X
0220  FOR X=1 TO G
0230  LET M=(I(X)*60/C)-A
0240  LET Q=((K(X)*60/D)-A)*5
0250  LET N=M+Q/20
0260  LET R(X)=(Q*1000)/(N*L(X))
0265
0266
0270 % ,H(X),",",R(X)
0280  NEXT X
0310  PRINT , " "
```

```
0315 % ,"THIS IS EXPT CODED ";Z£
0320  PRINT , " "
0330 % ,"INCUBATION TIME";"   PROTEIN IN Y.S. ";"   UPTAKE "
0332 % ,"(HOURS)"
0340  PRINT ,
0350  FOR X=1 TO G
0360 % ,H(X),L(X),R(X)
0370  NEXT X
0380  STOP
```

Program used for the calculation of uptake of degradable substrates.

```
0020  REM PROTEIN PROGRAM ,REVISED OUTPUT ,NOV. 1974
0030  DIM E(12),H(12),I(12),J(12),K(12),L(12),S(12)
0040  DIM C£30
0045 DIM V£30
0060  PRINT "PROTEIN PROGRAM ENTERED"
0070 PRINT "EXPERIMENT CODE =";
0080 INPUT V£
0090  PRINT  "BACKGROUND IN CPM=";
0100  INPUT  A
0110  PRINT  "PERCENT SOLUBLES IN PREP=";
0120  INPUT  B
0130  PRINT  "COUNTING TIME MEDIUM TOTALS,SECS=";
0140  INPUT  C
0150  PRINT  "COUNTING TIME MEDIUM SOLUBLES,SECS=";
0160  INPUT  Z
0170  PRINT  "YS COUNTING TIME,SECS=";
0180  INPUT  D
0190  PRINT  "CORRECTION FACTOR FOR MEDIUM TOTALS=";
0200  INPUT  Y
0210  PRINT  "CORRECTION FACTOR FOR MEDIUM SOLUBLES=";
0220  INPUT  R
0230  PRINT  "NO. OF POINTS IN PLOT=";
0240  INPUT  G
0264  PRINT "NAME OF DATA FILE:";
0265  INPUT F1£
0266  PRINT "NAME OF REGRESSION FILE:";
0267  INPUT F2£
0269  FILES ,F1£,,F2£
0270  PRINT "NAME OF RESULTS FILE:";
0272  INPUT F3£
0275  FILES ,F3£
0277  FOR X=1 TO G
0280   INPUT , H(X),I(X),J(X),K(X),L(X),
0290  NEXT X
0300  FOR X=1 TO G
0310  LET M=(I(X)*60/C-A)*Y
0320  LET N=(J(X)*60/Z-A)*R
0330  LET O=N-(M*B/100)
```

```
0340  LET Q=((K(X)*60/D)-A)*5
0350  LET P=(M-N)+O/2
0360  LET F=((10*O)+Q)*1000
0370  LET E(X)=(Q*1000)/(L(X)*P)
0380  LET S(X)=F/(L(X)*P)
0385
0387 % ,
0390 % ,H(X),",",S(X)
0400  NEXT X
0405 % ,
0430  PRINT , " "
0435
0440  PRINT , " "
0445 % ,
0450  PRINT , " INCUBATION ","PROTEIN","MICROLITRES","     UPTAKE"
0460  PRINT , "TIME (HOURS)"," IN YS "," PER MG YS "
0465 % ,
0470  FOR X=1 TO G
0480 % ,H(X),L(X),E(X),S(X)
0490  NEXT X
0500  STOP
```

Programme used to study release of substrates from reincubated tissue.

```
0010 REM EXOCYTOSIS PROGRAMME,DECEMBER 1980
0020 DIM P(20),Q(20),R(20),S(20),T(20),U(20),W(20)
0030 DIM A(20),B(20),C(20)
0040% "EXOCYTOSIS PROGRAMME ENTERED"
0050 % "EXPERIMENT NUMBER = ";
0060 INPUT V1£
0070 % "BACKGROUND IN CPM =";
0080 INPUT D
0090 % "TOTAL VOLUME OF UPTAKE MEDIUM,ML =";
0100 INPUT B1
0104 % "TOTAL VOLUME OF RELEASE MEDIUM,ML =";
0106 INPUT B2
0110 % "NUMBER OF SAMPLES AT EACH TIME =";
0120 INPUT K
0130 % "NUMBER OF TIMES SAMPLES WERE TAKEN =";
0140 INPUT E
0150 % "MG SUBSTRATE/ML UPTAKE MEDIUM =";
0160 INPUT Q1
0170 % "MEAN CPM PER ML UPTAKE MEDIUM =";
0180 INPUT L2
0190 % "CORRECT CPM IN 5ML Y.S. SOLUTION =";
0200 INPUT A
0210 % "MG OF Y.S. PROTEIN =";
0220 INPUT Y
0230 FOR I=1 TO 2
0240 % "DATA FOR ";
0250 IF I=2 THEN 280
0260 % " TOTALS"
0270 GOTO 290
0280 % " SOLUBLES"
0290 % "COUNTING TIME,SECS =";
0300 INPUT C(I)
0310 % "CORRECTION FACTOR =";
0320 INPUT M(I)
0330 NEXT I
0340 % "NAME OF DATA FILE =";
0350 INPUT P1£
0360 % "NAME OF RESULTS FILE =";
```

```
0370 INPUT F2£
0380 FILES ,F1£,,F2£
0390 J=0
0400 L=0
0410 FOR X=1 TO E
0420 INPUT ,P(X),Q(X),R(X),S(X)
0430 NEXT X
0440 FOR X=1 TO E
0450 F=((R(X)*60.0/C(1))-D)*M(1)
0460 J=J+F*K
0470 REM J IS COUNTS SO FAR REMOVED
0480 T(X)=J+((B2-K)*F)
0490 REM T(X) IS TOTAL COUNTS SO FAR EXOCYTOSED
0500 G=((S(X)*60.0/C(2))-D)*M(2)
0510 L=L+G*K
0520 REM L IS SOLUBLES SO FAR REMOVED
0530 U(X)=L+((B2-K)*G)
0540 REM U(X) IS TOTAL SOLUBLES SO FAR EXOCYTOSED
0550 NEXT X
0560 V=T(E)+A
0570 REM V IS TOTAL ACTIVITY TAKEN UP
0580 FOR X=1 TO E
0582 A(X)=1000*T(X)*B1*Q1/((L2*B1+V)*Y)
0584 B(X)=1000*U(X)*B1*Q1/((L2*B1+V)*Y)
0586 C(X)=A(X)-B(X)
0590 T(X)=100*T(X)/V
0600 U(X)=100*U(X)/V
0610 REM CONVERTED TO PERCENTAGES
0620 W(X)=100*U(X)/T(X)
0625 M4=100*V/(B1*L2+V)
0627
0628
0630 NEXT X
0631 GOTO 930
0632 % ,
0634 % ,
0635 % ,"THIS IS EXOCYTOSIS EXPT CODED ";V1£
0640
0643 M5=Q1*B1*V/((B1*L2+V)*Y)
0645 % ,"EFFICIENCY OF UPTAKE =";M4;"%"
```

```
0646 % ,"TOTAL UPTAKE PER MG PROTEIN (MG) =";M5
0647
0648
0650 % ,
0660 % ,
0670 % ,"SERIAL NO.","TIME","% ACTIVITY ","% SOLUBLES"
0680 % ,"OF SAMPLE","(HOURS)","EXOCYTOSED","SO FAR"
0690 % ,
0700 % ,
0710 FOR X=1 TO E
0720 % ,P(X),Q(X),T(X),W(X)
0730 NEXT X
0740 % ,
0750 % ,
0760 % ,
0765 % ,"MICROGRAMS","MICROGRAMS","MICROGRAMS"
0770 % ," PROTEIN"," SOLUBLES","INSOLUBLES"
0780 % ,"EXOCYTOSED ","EXOCYTOSED ","EXOCYTOSED "
0790 % ,"PER MG YS ","PER MG YS ","PER MG YS "
0800 % ,
0805 FOR X=1 TO E
0810 % ,A(X),B(X),C(X)
0820 NEXT X
0825 IF P3=2 THEN 900
0826 % ,
0828 % ,
0830 % ,"YS INSOLUBLES =";Z1;"MICROGRAMS/MG"
0835 % ,"YS SOLUBLES =";Y9;"MICROGRAMS/MG"
0840 Z2=Y9+B(E)
0845 Z3=Z1+C(E)
0850 Z4=100*Z2/(Z2+Z3)
0855 % ,
0860 % ,"TOTAL SOLS PRODUCTION = ";Z2;" MICROGRAMS PER MG"
0865 % ,"TOTAL INSOLS PRODUCTION = ";Z3;" MICROGRAMS PER MG"
0870 % ,"THIS IS ";Z4 ;"% SOLUBLES"
0900 STOP
0930 % "DO YOU HAVE DATA FOR YS SOLS(1 FOR Y,2 FOR N)"
0935 INPUT P3
0940 IF P3=2 THEN 632
0945 %"INPUT CORRECT SOLS CPM IN WHOLE YS "
```

```
0950 INPUT Y4
0955 Y9=1000*Y4*B1*Q1/(Y*(B1*L2+V))
0960 Z1=(1000*A*B1*Q1/(Y*(B1*L2+V)))-Y9
0965 GOTO 632
9300
```

APPENDIX 3

Some preliminary attempts (not reported in detail) were made to determine the direction of macromolecular release of ^{125}I-IgG from reincubated rat yolk sacs.

Previous work employed Perspex blocks containing hollows filled with medium over which yolk sacs were placed and secured by the application of rubber O-rings that were screwed down onto the block (Figure A6). Incubation of the apparatus in medium 199 at 37°C containing radiolabelled substrate was intended to establish whether trans-tissue passage of substrate occurred by sampling the medium in the recess. No useful results were obtained, however, because the apparatus tended to damage the tissue, allowing non-specific passage of substrates like ^{125}I-PVP through the membrane.

A modified version of essentially the same apparatus in which the yolk sac was placed over the ball of a 'Quickfit' ball and socket joint was constructed. Careful insertion into the socket caused the tissue to be held in position while sustaining minimal damage. The apparatus used is represented in Figure A7, but various difficulties were experienced with its use. It is important to have exactly the same amount of liquid in both flasks at any time in order to avoid subjecting the yolk sac to hydrostatic pressures, and it is important also to have efficient circulation and oxygenation of the medium on either side of the tissue, otherwise the yolk sac may be subject to an non-uniform local environment, (anoxic, or gradients of metabolites established) and the results misleading. Again no useful results were obtained, this time because of the small size of the functional aperture. No transfer of radioactivity was detectable and simple calculation shows that even at maximal transport rates it would not be expected to be detectable. Since increased incubation time or larger aperture size were essential the best design would involve a larger aperture but with some form of mesh-support for the yolk sac. This support would need, however, to be on both sides of the yolk sac and the inherent technical difficulties made the construction of such an apparatus impractical in this study.

In this apparatus, the yolk sac was placed over the well, and the top secured in place.

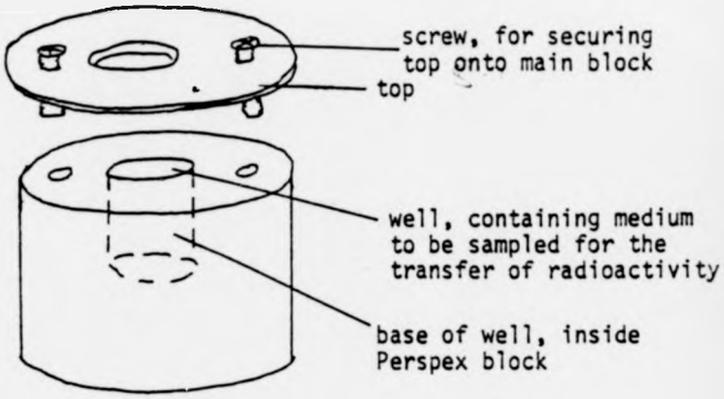
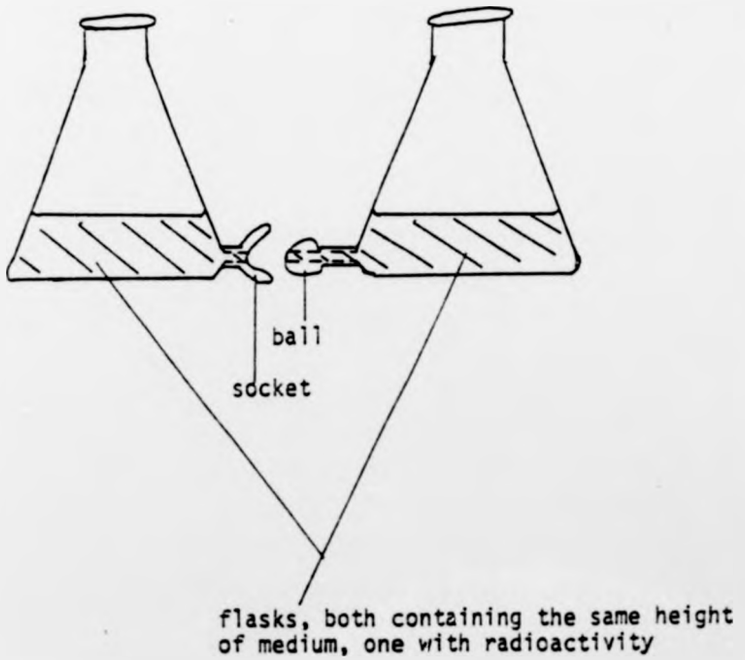


Figure A5 Previous 'Ussing-apparatus'.

Figure A6 Ball and socket type 'Ussing apparatus.'

In this apparatus, the yolk sac was placed over the ball, and the socket secured in place.



References

Abramson N., E.W.Gelford, J.H.Jandl & F.W.Rosen, (1970). J. Exp. Med. 132, 1207

Adams C.J., (1982). J. Cell Biol., 93.

Albert et al, (1948). J. Chem. Soc. Lond., 2240.

Allison, A.C. & Davies, P, (1974). Symp. Soc. Exp. Biol. et al, et al,VIII, 419.

Anderson, R.G.W., J.L.Goldstein & M.S.Brown. (1982). J. Cell Biol., 93, 523.

Balfour, A.H.& Jones, E.A., (1977). Clin. Sci. Mol. Med., 52, 383.

Batty, I., Brambell, F.W.R., Hemmings, W.A. & Oakley, C.L., (1954). Proc. Roy. Soc. B., 142, 452.

Bennett, (1956). J. Biophys. Biochem. Cytol., 2, suppl. 99.

Bequinot et al, (1983). J. Cell Biol., 94, abstract 394.

Bocci, V., (1969), Ital. J. Biochem., 18, 346.

Bolton, A.E.& Hunter, W.M., (1973). Biochem. J., 133, 529.

Bowers, B., (1977), Exp. Cell Res., 110, 409.

Brambell, F.W.R., W.A.Hemmings & I.G.Morris, (1964). Nature (Lond), 203, 1352.

Brambell, F.W.R., (1966), Lancet, 19/11/66, 1087.

Brambell, F.W.R., (1970). The transmission of passive immunity from mother to young, Frontiers of Biology vol. 18, North Holland, Lond.

Casley-Smith, J.R., (1969). J. Microscop., 90, 15.

Ciccimarra, F., F.S.Rosen & E.Merler, (1975). P.N.A.S., 72, 2081.

Cobbs, C.S., A.R.Shaw, K.Hillman & M.Schlamowitz, (1980). J. Immunology, 124 (4), 1648.

Colomb, M., & Porter, R.R.,, (1975), Biochem. J., 145, 177.

Connell, G.E. & Porter, R.R., (1971), Biochem. J., 124, 53P.

Cooke, (1982). p327 in Biological Membranes, vol. 4 (D.Chapman, Ed.).

Crowther, R.A. & Pearse, B.M.F., (1982). J. Cell Biol., 91, 790.

Cuatrecasas, P., (1972). J. Biol. Chem., 247, 1980.

Cuatrecasas, P., (1974). Ann. Rev. Biochem., 43, 169.

David, G.S., (1972). Biochem. Biophys. Res. Commun., 48, 434.

Davies D.R., A.Levitski, P.Maxfield, P.Milhand, M.C.Willingham & I.H.Pastan,(1980). Nature (Lond.), 283, 162.

Desbuquois, B., J.Willeput & A.Huet de Frobeville, (1979). Febs Let.s, 106 (2), 338.

Dorrington, K.J. & Painter, R.H., (1974). Progress in Immunology II, vol.1.

Dufau, M.L., E.H.Charreau + K.J.Catt, (1973). J. Biol. Chem., 248, 6973.

Dufau, M.L., E.H.Charreau, D.Ryan + K.J.Catt, (1974). Febs Lett.s, 39, 149.

Dufau, M.L., A.J. Bankel, D.Ryan & K.J.Catt, (1977). Mol. Cell Endocr., 6, 253.

Duncan, R. & Lloyd, J.B.L., (1978). Biochem. Biophys. Acta., 544, 674.

Dustin, P, (1978). Microtubules, Springer Verlag, N.Y.

Edelman G.M., (1969). P.N.A.S., 63, 78.

Edelman G.M. & Gall W.E., (1969). Ann. Rev. Biochem., 38, 415.

Ellerson, J.R., D.Yasmeen, R.H.Painter & K.J.Dorrington, (1972). Febs Lett.s, 24, 318.

Elson, J., E.D.Jenkinson & W.D.Billington, (1975). Nature, 255, 412.

Fahey, J.L. & Robinson, A.G., (1963). J.Exp. Med., 118, 845.

Fanger, M.W.& Smyth, D.G., (1972a). Biochem. J., 127, 757.

Fanger, M.W. & Smyth, D.G., (1972b). Biochem. J., 127, 767.

Fehr, K., J.LoSpalluto & M.Ziff, (1970). J. Immunology, 105 (4), 973.

Forster, S., (1982). PhD Thesis, University of Keele, U.K.

Freeman, (1959). Clin. Chim. Acta, 4, 788.

Frommel, D. & Hong, R., (1970). Biochem. Biophys. Acta., 200, 113.

Galloway, C.J., G.E.Dean, M.Marsh, G.Rudnick & I.Mellman, (1983). P.N.A.S. (USA)

Ghetie, V. & Motas, C., (1971). Immunochemistry, 8, 89.

Giorgio, N.A., C.B.Johnson & M.Blecher, (1974). J. Biol. Chem., 249, 428.

Gitlin, D., J.Kumate, J.Urrusti & C.Morales, (1964). J. Clin. Invest., 43, 1938.

Goetze, T., H.Franke, W.Huhn, R.Tonjes, B.Schlag & E.Goetze, (1976). Cytobiologie, 12, 274.

Greenwood, F.C. & Hunter, W.M., (1963). Biochem. J., 89, 114.

Guyer, R.L., M.E.Koshland & P.M.Knopf, (1976). J. Immunology, 117 (2), 587.

Haigler, H.T., M.C.Willingham & I.Pastan, (1980). J. Biol. Chem., 255 (4), 1239.

Haksar, P., S.Baniuki & F.G.Peron, (1973). Biochem. Biophys. Reps, 52, 959..

Haour, F. & Saxena, B.B., (1974). J. Biol. Chem., 249, 2195.

Harford, J., M.Lowe, H.Tsunoo & G.Ashwell, (1982). J. Biol. Chem., 257, 12685.

Haxhe, J.J. & Kestens, P.J., (1965). In Radioaktive isotope in klinik und Forschung, 4, 450. Fellinger & Hofer, eds.

Helenius, A., M.Marsh & J.White, (1982). J. Gen. Virol., 58, 47.

Helenius, A., I.Mellman, D.Wall & A.Hubbard, (1983). Trends in Biochemical Sciences, p245.

Helmkamp, R.W., M.A.Contrera & M.J.Izzo, (1967). Int. J. Appl. Radiat. Isotop., 18, 747.

Hemmings, W., (1973). Immunology, 25, 165.

Hemmings, W., (1974) Immunology 27, 693

Hemmings, W. & Redshaw, M.R., (1974). Int. J. Appl. Rad., 26, 429.

Hemmings, W., (1976). in Maternofoetal Transmission of Immunoglobulins, W.Hemmings, ed. C.U.P.

Heuser, J., (1980). J. Cell Biol., 84, 560.

Hillman, K., M. Schlamowitz & A.R.Shaw, (1977). J. Immunology, 118 (3), 782.

Hinrichs, W.A. & Smyth, D.G., (1970), Immunology, 18, 759.

Holohan, K.N., R.F.Murphy, K.D.Buchanan & D.T.Elmore, (1973). Clin. Chim. Acta., 45, 153.

Hugues-J.P.-Ryser, (1963). Lab. Invest., 12 (10), 1009.

Hunter, W.M., (1971). in Radioimmunoassay Methods; European Workshop, Kirkham & Hunter (eds), Churchill-Livingstone, Ed. and Lond.

Hunter, W.M. & Greenwood, F.C., (1962). Nature (Lond.), 194, 495.

Huxham, M. & Beck, F., (1981). Cell Biol. Int. Reps., 5 (12), 1073.

Ibbotson, G., (1978). PhD Thesis, University of Keele, UK.

Ibbotson, G. & Williams, K.E.W., (1979). Biochem. J., 178, 785.

Irimajiri, S., E.C.Franklin & B. Frangione (1968). Immunochemistry, 5, 383.

Jaarsfield, P.P.V., P.K.Nandi, R.E.Lippoldt, H.Saroff & H.Edelhoch, (1981). Biochemistry, 20, 4129.

Jacques, P.J., (1969). in Lysosomes in Biology and Pathology, vol. 2, Dingle & Fell (Eds), North Holland Publishers, Amsterdam.

Johanson, R.A., A.R.Shaw & M.Sclamowitz, (1981). J. Immunology, 126 (1), 194.

Kaplan, K.C., E.A.Catsoulis & E.C.Franklin, (1965). J. Immunology, 8, 354.

Karonen, S.L., P.Morsky, M.Siren & U.Siederli, (1975). Analyt. Biochem., 67, 1.

Keen, J.H., M.C.Willingham & I.Pastan, (1979). Cell, 16, 303.

Kehoe, J.M. & Fougereau, M., (1969). Nature, 224, 1212.

King, B.F., (1982a). J. Ultrastruct. Res., 79, 273.

King, B.F., (1982b). Anatomical Record, 202, 221.

Knox, P., (1981). Biochemistry of Cellular Regulation. Vol. 4, The Cell

Surface. CRC Press, Fl, USA.

Kooistra, T., A.M.Duursma, J.M.W.Bouma & M.Gruber, (1980). Biochem. Biophys. Acta., 631, 439.

Kooistra, T., M.K.Pratten & J.B.Lloyd, (1981). Bioscience Reports, 1, 587.

Krohn, K.A., M.A.Sharman & M.Welch, (1972). Biochem. Biophys. Acta., 285, 404.

Krohn, K.A., L.C.Knight, J.F.Harwig & M.J.Welch, (1976). Biochem. Biophys. Acta., 490, 497.

Lehninger, A.L., (1975). Biochemistry. Worth Publisher, N.Y.

Levitski, A., M.C.Willingham & I.Pastan, (1980). P.N.A.S. (USA), 77 (5), 2706.

Lewis, W.H., (1937). Am. J. Cancer, 29, 666.

Linden, C.D. & Roth, T.F., (1978). J. Cell Sci., 33, 317.

Livesey, G., (1978). PhD Thesis, University of Keele, U.K.

Livesey, G. & Williams, K.E., (1979). Biochem. J., 184, 519.

Livesey, G., K.E.Williams, S.E.Knowles & F.J.Ballard, (1980). Biochem. J., 188, 895.

LoSpalluto, J., K.Fehr & M.Ziff, (1970). J.Immunol. 105(4), 886.

Lowry, O.H., N.J.Rosebrough, A.L.Farr & R.J.Randell, (1951). J. Biol. Chem., 193, 265.

Lunney, J. & Ashwell, G., (1976). P.N.A.S.(USA), 73, 341.

McFarlane, A.S., (1958). Nature, 182, 53.

McFarlane, A.S., (1963). J. Clin. Invest., 42, 346.

McNabb, T., T.Y.Koh, K.J.Dorrington & R.H.Painter, (1976). J. Immunology, 117 (3), 882.

MacLennon, I.C.M., G.E.Connell & F.M.Gotch, (1974). Immunology, 26, 303.

Marchalonis, J.J., E.H.M.Ealey & E.Diener, (1969). Biochem. J., 113, 299.

Metschnikoff, E., (1883). Biologisches Zentralblatt, 3, 560.

Michaelson, T.E., F.Wisloff & J.B.Natvig, (1975). Scand. J. Immunol., 4, 71.

Minta, J.O. & Painter, R.H., (1972). Immunochemistry, 9, 1041.

Moore, A.T., K.E.Williams & J.B.Lloyd, (1977). Biochem. J., 164, 607.

Morell, A.G., G.Gregoria, I.H.Schienbe, J.Hickman & G.Ashwell, (1971). J. Biol. Chem., 246, 1461.

Mossman, (1937). Contr. Embryol., 479, 129.

Moxon, L.A., A.E.Wild & B.S.Slade, (1976). Cell & Tissue Research, 171.

Mullock, B. & Hinton, R., (1981). Trends in Biochemical Sciences, p188.

Muntheka, A.C., T.Berg & R.Seljelid, (1977). Exp. Cell Res., 107, 55.

Neufeld, E. & Ashwell, G., (1980). in Biochemistry of Glycoproteins and

Proteoglycans, Lennarz (Ed.), Plenum Press.

Oakley, B.R., D.R.Kirsch & N.R.Morris (1981). Analyt. Biochem. 119, 164.

Okafor, G.O., M.W.Turner & F.C.Hay, (1974). Nature, 248, 228.

Olmsted, J.B. & Borisy, G.G., (1973). Biochemistry, 12, 4282.

Omary, M.B. & Trowbridge, I.S., (1981). J. Biol. Chem., 256, 12888.

Ovary, Z., P.H.Saluk, L.Quizada & M.E.Lamm, (1976). J. Immunology, 116 (5), 1265.

Pastan, I., H.Haigler, R.Dickson, S.Cheng & M.C.Willingham, (1981). Miami Winter Symposia, vol. 18, p137.

Pastan, I. & Willingham, M.C., (1983). Trends in Biochemical Sciences, p250.

Patzer, E.J., D.M.Schlossman,& J.E.Rothman, (1982). J. Cell Biol., 93, 230.

Paulson, J.C., R.L.Hill, T.Tanabe & G.Ashwell, (1977). J. Biol. Chem., 252, 8624.

Pearse, B.M.F., (1975). J. Mol. Biol., 97, 93.

Pearse, B.M.F., (1976). J. Mol. Biol., 103, 785.

Pearse, B.M.F., (1980). Trends in Biochemical Sciences, p131.

Pearse, B.M.F. & Bretscher, M.S., (1981). Ann. Rev. Biochem., 50, 85.

Petruzelli, L.M., S.Ganguly, C.J.Smith, M.H.Cobbs, C.S.Rubin & O.M.Rosen, (1982). P.N.A.S.(USA), 79, 6792.

Porter, R.R., (1959). Biochem. J., 73, 119.

Porter, R.R., (1963). Brit. Med. Bull., 19 (3), 197.

Prahl, J.W., (1967). Biochem. J., 104, 647.

Pricer, W.E. & Aswell, G., (1971). J. Biol. Chem., 246, 4825.

Rademacher, T.W., S.W.Homans, D.L.Fernandes, R.A.Dweck, T.Mizuochi, T.Tanginuchi & A.Kobata, (1983). Biochem. Soc. Trans., 11, 132.

Ramasamy, R., D.S.Secher & K.Adetugbo, (1975). Nature, 253, 656.

Redshaw, M.R. & Lynch, S.S., (1974). J. Endocrinol., 527.

Roberts, A.V.S., K.E.Williams & J.B.Lloyd, (1977). Biochem. J., 168, 239.

Rodewald, R., (1973). J. Cell Biol., 58, 189.

Rosa, U., G.A.Scassellati, F.Pennisi, N.Riccioni, P.Giagnoni & R.Giordani, (1964). Biochem. Biophys. Acta., 86, 519.

Salisbury, J.L., J.S.Condeelis & P.Satir, (1980). J. Cell Biol., 87, 132.

Schmid, S.L., A.K.Matsumoto & J.E.Rothman, (1982). P.N.A.S.(USA), 79, 91.

Schneider, W.J., U.Beisiegel, J.L.Goldstein & M.S.Brown, (1982). J. Biol. Chem., 257, 2664.

Shiu, R.P.C. & Friesen, H.G., (1974). J. Biol. Chem., 249, 7902

Sonoda, S. & Schlamowitz, M., (1970). Immunochemistry, 7, 885.

Spiegelburg, H.L. & Wiegle, W.O., (1965). J. Immunol., 95, 1034.

Stahl, P., H.Six, J.Rodman, P.Schlesinger, D.R.P.Tulsiani & O.Touser, (1976). P.N.A.S.(USA), 73 (11), 4045.

Stanworth, D.R. & Stewart, G.A., (1976). In Maternofoetal Transmission of Immunoglobulins, Hemmings (Ed), C.U.P.

Stanworth, D.R. & Turner, (1979). In Handbook of Exp. Immunol., vol. 1, Weir (Ed), Blackwell.

Starling, D., R.Duncan & J.B.Lloyd, (1983). Cell Biol. Int. Reps., 7 (8).

Steinman, R.M., I.S.Mellman, W.A.Muller & Z.A.Cohn, (1983). J. Cell Biol., 96, 1.

Stewart, G.A., A.K.Smith & D.R.Stanworth, (1973). Immunochemistry, 10, 755.

Stockert, R.J., A.G.Morell & I.H.Schienberg, (1976). Biochem. Biophys. Res. Commun., 68 (3), 988.

Stockert, R.J., A.G.Morell & I.H.Schienberg, (1977). Science, 197, 66.

Strober, W. & Waldmann, T.A., (1969). Unpublished, cited by Weir, 1979.

Sundqvis, K.G. & Ehrnst, A., (1976). Nature, 264, 226.

Sutton, B.J. & Phillips, D.C., (1983). Biochem. Soc. Trans., 11, 130.

Temin, H.M., R.W.Pierson & N.C.Dulak, (1972). In Growth, Nutrition & Metabolism of Cells in Culture, Rothblatt & Cristofallo (Eds), vol. 1, Acad. Press.

Thorell, J.I. & Johansson, B., (1971). Biochem. Biophys. Acta., 251, 363.

Tolleshaug, H., T.Berg, M. Nilsson, & K.R.Norum, (1977). Biochem. Biophys. Acta., 499, 73.

Udall, J.N., K.J.Bloch, L.Fritze & W.A.Walker (1981). Immunology, 42, 251.

Ungewickell, E. & Branton, D., (1981). Nature, 289, 420.

Ungewickell, E. & Branton, D., (1982). T.I.B.S., p358.

Utsumi, S., (1969). Biochem. J., 112, 343.

Van Den Hamer, C.J., A.G.Morell, I.H.Schienbe, J.Hickman & G.Ashwell, (1970). J. Biol. Chem., 245, 4397.

Von Figura, K. & Kresse, H., (1974). Eur. J. Biochem., 48, 357.

Waldmann, T.A. & Strober, W., (1969). In Progr. Allergy (Karger), Basel and N.Y., 13, 1.

Waldmann, T.A. & Ghettie, V., (1971). In Workshop 10 in Progress in Immunology, Amos (Ed), London & N.Y., Academic Press.

Waters, M.J. & Friesen, H.G., (1979). Proc. Austr. Biochem. Soc., 11, 101.

Watkins et al, (1971). in Protides of the Biol. Fluids (Peeters, Ed), 19, 461.

Weir, D.M., (1979). Handbook of Exp. Immunol., vol 1, Blackwell, Lond.

Weisbecker, U., (1981). PhD Thesis, University of Keele, U.K.

Weisbecker, U., G.Ibbotson, G.Livesey & K.E.Williams, (1983). Biochem. J., 214, 815.

Wild, A.E., (1965). Proc. Roy. Soc. Biol., 163, 90.

Wild, A.E., (1970). J.Embryol.Exp.Morph., 24, 313.

Wild, A.E., (1974). in Transport at the Cellular Level, Sleigh & Jennings (Eds), Soc. Exp. Biol. Symp., 28, C.U.P.

Wild, A.E., (1975). Phil. Trans. R. Soc. Lond. B., 271, 395.

Wild, A.E. & Dawson, P., (1977). Nature, 268, 443.

Wild, A.E. & Richardson, L.J., (1979). Separatum Experientia, 35, 838.

Williams, K.E., E.M.Kidston, F.Beck & J.B.Lloyd, (1975a). J. Cell Biol., 64, 113.

Williams, K.E., E.M.Kidston, F.Beck & J.B.Lloyd, (1975b). J. Cell Biol., 64, 123.

Williams, K.E. & Ibbotson, G., (1979). in Protein Transmission through Living Membranes, Hemmings (Ed), Elsevier North-Holland Biomedical Press.

Willingham, M.C., (1983). Trends in Biochemical Science. p250.

Willingham, M.C., (1984). Trends in Biochemical Science. p93.

Willingham, M.C. & Pastan, I., (1980). Cell, 21, 67.

Willingham, M.C., (1981). Exp. Cell Res., 132, 329.

Wisloff, F., T.E.Michaelson & S.S. Froland, (1974). Scand. J. Immunol., 3, 29.

Wood, F.T., M.W.Wu & J.C.Gerhart, (1975). Analytical Biochem., 69, 339.

Woodward, M.P. & Roth, T.F., (1978). P.N.A.S.(USA), 75 (9), 4394.

Yasmeen, D., J.R.Ellerson, K.J.Dorrington & R.H.Painter, (1973). J. Immunol., 110, 1706.

Yasmeen, D., J.R.Ellerson, K.J.Dorrington & R.H.Painter, (1976). J. Immunol., 116, 518.

Yu Pan, J., J.L.Carpentier, P.Gorden, E.V.Obberghen, N.M.Blackett, C.Grunfeld & L.Occi, (1982). P.N.A.S.(USA), 79, 7788.