THE TRANSCELLULAR TRANSPORT OF IMMUNOGLOBULIN G IN RAT AND RABBIT YOLK SACS

by

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ABBREVIATIONS

Abbreviations in this thesis comply with the policy of the Biochemical Journal (1978) but, in addition, the following are also used:

EI	Endocytic Index
TAR	Tissue Accumulation Rate
^{125}I-PVP	^{125}I-labelled poly(vinylpyrrolidone)
y.s.	yolk sac
fd-BSA	Formaldehyde-denatured bovine serum albumin
IgG	The term IgG is used throughout this thesis to refer both to experimental work and to literature on immunoglobulin G. In the former context, the term is used more loosely, for sake of brevity, to refer to a commercially available fraction of gamma globins (Cohn Fraction II, Miles Laboratories Ltd). This fraction is reported to contain at least 80% IgG.

ABSTRACT

An *in vitro* method, previously described for incubating rat yolk sacs for short periods, was adapted for the incubation of rabbit yolk sacs. It was shown that the endocytic properties of these two tissues are very similar as judged by their rates of uptake of ^{125}I-labelled PVP, albumin and homologous IgG. By using the *in vitro* methods for incubating rat and rabbit yolk sacs, it was possible to measure the rates of release of substrate (intact and degraded) from yolk sacs previously "loaded" *in vitro* with a marker protein. The latter variation of the method was used in an attempt to further investigate the cellular mechanism of prenatal transfer of passive immunity in the rat and rabbit. It was found that *in vitro* ^{125}I-labelled IgG internalized by the tissue can be released again into the incubation medium. When the fates of a number of ^{125}I-labelled homologous and heterologous IgGs were investigated, it was found that definite rank orders existed for the extent of release of intact IgG from the tissues. The rank orders were closely parallel to those reported for transplacental transfer by the same tissues *in vivo*.

Some assumptions underlying the Brambell, Wild and Hemmings' theories of IgG transfer in the rabbit yolk sac were investigated. Evidence for the presence of specific receptors or binding sites on the yolk-sac membrane was obtained both for molecules destined for transfer through the tissue and also for molecules destined for intracellular degradation. No exocytosis from heterolysosomes could be demonstrated, nor was an enhanced rate of release of ^{125}I-labelled homologous IgG from the rabbit or rat yolk sac *in vitro* observed when degradation of these substrates was inhibited by the addition of leupeptin to the incubation medium. These findings are more compatible with the operation of a two-vesicle system with separate vesicles for transcellular transport and degradation, as suggested by Wild.

CONTENTS

GENERAL INTRODUCTION

The observation that suckling animals can acquire immunity from their mothers was first made towards the end of the last century by Ehrlich (1892). The immune system of young animals is not very well developed at the time of birth. (Although Lewin & Altman (1976) report the ability of human foetuses to synthesize IgG, IgM, IgA and IgE themselves, the amounts are minute.) It is therefore vital that protection against infections should be gained from elsewhere for the first few weeks of life. Ehrlich's original observations were on suckling mice, but this process of transfer of passive immunity has turned out to be more universal, having now been investigated in a great number of mammals. Since this capacity is an obvious asset for survival, it has evolved in the various animal species in an analogous fashion (i.e. different organs or tissues have developed the same specialized functions). Thus, the timing and sites of antibody transmission are rather varied, as can be seen in the summary table (see page 1a). Attempts have been made to determine the basis of these species differences, but suggestions that the thickness and number of placental membranes determines whether transfer is pre- or postnatal are, on the whole, not very convincing.

A common feature in the transfer of passive immunity for all species investigated, is that some molecules are transferred preferentially. This selectivity in protein transfer operates for most species in favour of IgG. Although for cows, pigs and horses apparently no selectivity is evident during the uptake of proteins via the colostrum and the gut into the neonatal circulation, it should be pointed out that for these species a form of selectivity seems to operate at an earlier stage in the overall process, namely in the mother during the secretion of molecules into the colostrum by the mammary gland. In cows and horses IgG is preferentially secreted into the colostrum (Dixon et al., 1961;

General features of transfer of passive immunity in different mammalian species

	Time of transfer		Route of transfer (where known)	Duration of period of transfer	Representative literature
	Prenatal	Postnatal			
Ox	0	+++	Gut	24 hours	Balfour & Comline (1962)
Pig	0	+++	Gut	24-36 hours	Porter (1969); Payne & Marsh (1962)
Goat	0	+++	Gut	24 hours	Clarke & Hardy (1971)
Sheep	0	+++	Gut	24 hours	Halliday (1976)
Horse	0	+++	Gut	24-36 hours	Jeffcott (1972)
Wallaby (Setonix)	0	+++	Gut	180 days	
Dog	+	++	Gut	1-2 days	Schneider & Szathmary (1939)
Cat	+	++	Gut	1-2 days	Harding et al. (1961); Kulangara & Schechtman (1963)
Hedgehog	+	++	Gut	70 days	Morris (1961)
Mouse	+	++	Gut Yolk sac	16 days -	Koch et al. (1967); Gardner (1975)
Rat	+	++	Gut Yolk sac	20 days -	Clark (1959); Kraehenbuhl et al. (1969) Brambell & Halliday (1956)
Guinea Pig	+++	0	Yolk sac	-	Leisring & Anderson (1961); Dancis & Shafran (1958)
Rabbit	+++	0	Yolk sac	-	Brambell (1970)
Grey squirrel	+++	0	Unknown	-	Wild (1971)
Man	+++	0	Chorioallantoic placenta		Gitlin et al. (1964)
Rhesus Monkey	+++	0	Chorioallantoic placenta	-	Bangham (1960)

Jeffcott, 1972). Moreover, Brandon (1976) showed that in cows further selectivity can be demonstrated between IgG subclasses and favours IgG 1. Porter (1969) showed that in pigs IgG 2 and IgA are preferentially secreted into the colostrum. IgA is the major secretory product at a later stage (the molecule being a very powerful anti E. coli agent). The mechanism of selection operating in the neonatal gut (rats and mice) or in the placenta or yolk sac (rabbits, guinea pigs, humans and rats) poses some fascinating questions. Other proteins are also taken up into some of these tissues only to be degraded subsequent to capture. Homologous IgG and some heterologous IgG species, on the other hand, traverse the tissues without being broken down. The different fates of molecules are probably related to different physiological functions of the tissues in question. The placenta and yolk sac in particular are specialized to act as barriers between the foetus and mother. The foetus is in effect an intra-uterine allograft, being genetically dissimilar to the surrounding maternal uterine tissues. Thus, very important antigens, paternally-inherited histocompatibility antigens, have been demonstrated on the cell surface of the embryo at very early stages (Håkanson et al., 1975) and anti-HLA antibody responses can be demonstrated in some human females (Adinolfi & Billington, 1976). The foreign (to the mother) paternally-derived antigens should suffice to trigger off an immune reaction in the mother sufficient to destroy the embryo. The reasons given to account for the protection of the foetus against rejection by the mother are varied, but they include the presence of serum factors (specific or non-specific) in pregnant women that may suppress immunological responses (Gusdon, 1976) by, for example, binding to the cell surface in a non-harmful way and at the same time blocking either lymphocyte receptors or antigenic determinants of the target cell (Price & Robins, 1978). Other reasons for the protection of

embryos include the possible presence of T-lymphocyte suppressor cells in pregnant females (Chaonat et al., 1977) and, more important in the context of the present study, the presence of the trophoblast barrier. Even though there is some evidence pointing to the presence of HLA-antigens on even the mouse yolk sac (Jenkinson & Billington, 1974), the foetal membranes are, on the whole, regarded as effective barriers, acting in both directions. For an excellent review see Billington & Wild (1979). Thus, no foetally-derived material gets out and no maternally-derived material reaches the foetus intact. But, rather maternally-derived materials and also other potential harmful molecules are broken down to the level of small molecules (amino-acids, peptides) that can then be used for the embryonic nutrition (see Beck & Lloyd, 1968). The molecular breakdown is ensured by the large battery of enzymes contained in the lysosomal system of the placental and yolk-sac tissues. The ability of homologous IgG and some other proteins to escape degradation therefore constitutes an exception to the general rule. Likewise, in the neonatal gut of various animal species, all kinds of protein are degraded, whereas IgG is transported across the tissue intact. In all the tissues mentioned, large molecules are taken up by endocytosis and the pinosomes and phagosomes thus formed fuse with lysosomes to bring about degradation. Immunoglobulins thus seem to be treated exceptionally in this system. As the possibility of intercellular immunoglobulin transport has been excluded on the grounds of existing tight junctions between cells (Larsen, 1963), the molecules have to be taken up by cells but somehow escape degradation during their way through the cells.

For IgG transport across the rat gut in the postnatal period, it has been suggested that the different segments of the gut are specialized in either the transport or the catabolism of proteins (Rodewald, 1973).

Initially the distal part of the small intestine was suggested to be operative in antibody transfer (Clark, 1959; Kraehenbuhl et al., 1967; Kraehenbuhl & Campiche, 1969; Cornell & Padykula, 1969). However, considerably more evidence has been put forward for the involvement of the proximal half of the intestine (Rodewald, 1969, 1970, 1973, 1976a,b; Morris & Morris, 1974, 1976; MacKenzie, 1972; Jones, 1976a,b, 1979; Hemmings & Williams, 1977). The observation of protein transport at the distal end of the small intestine may be due to the failure in isolating the sites effectively, as Rodewald (1970) suggested. Alternatively, no clear distinction has been made by some workers between uptake and true transport. Uptake with subsequent degradation has been reported frequently for the ileum. Thus, Cornell & Padykula (1969) found great amounts of acid-phosphatase activity and other hydrolytic enzymes in supranuclear vacuoles from the distal part of the intestine. Rodewald (1970) showed that the proteolytic capacity of the distal half of the intestine exceeded that of the proximal half by 30-fold. Hemmings & Williams (1977), Noack et al. (1966) and Jones (1979) also showed that this region displays greater proteolytic capacity than the duodenum. Uptake into the ileum, on the other hand, has been reported by Rodewald (1973) for a variety of molecules (ferritin-conjugated IgG, free ferritin, ferritin-conjugated BSA) that were all taken up non-selectively.

From these findings it appears that along the gut of the neonatal rat, cells are specialized to perform different functions. Epithelial cells in the proximal part of the small intestine take up proteins destined for transmission to the circulation by receptor-mediated endocytosis (Rodewald, 1970; MacKenzie, 1972). The latter workers also showed that uptake is selective, e.g. sheep IgG2 is taken up preferentially to sheep IgG 1. Hemmings & Jones (1974) and Jones (1976) showed that for ^{125}I- or ^{131}I-labelled bovine IgG, the more basic fractions are

transmitted more readily as judged by isoelectric focussing analysis. Also Hammerberg et al. (1977) demonstrated the uptake of IgG1, IgG 2, IgM and, later in the development of the animal, IgA. When IgG2a and b were given orally, selective transport of the electrophoretically slower-moving subclass from gut to the serum was apparent. Concerning homologous and heterologous IgG species, Halliday (1955) established the following rank order for the transfer of IgG from different species: Rat > Mouse > Rabbit > Cow > Fowl. The distal part of the small intestine is also specialized, not for transfer, but for degradation of the internalized substrates. After a time span of 22 days following birth, the epithelial cells of the proximal intestine also lose their ability to transfer protein. But, during the period of transfer of passive immunity, it appears that the processes of substrate transfer and degradation are segregated and take place in entirely different regions of the gut. This is in stark contrast to the findings in the rabbit (and rat) yolk sac, where the selection takes place on the same cell surface and where both substrate transfer and breakdown happen in the same cell. This poses the fascinating question of how the cell can distinguish between molecules destined for transport and those destined for degradation by the lysosomal enzymes. In order to fully appreciate this problem it may be useful to have a short glance at the dynamics of the vacuolar system.

Uptake of exogenous substances into cells may be by a number of mechanisms. If the molecules are small enough (i.e. <220 Dalton), they can either diffuse across the plasma membrane or enter by active transport. For larger molecules, like IgG and particles, however, the cell membrane is not permeable in this way, but uptake may occur by endocytosis. Endocytosis describes a process of internalizing substrates into vesicles formed either by the plasma membrane invaginating or forming

pseudopodia engulfing the substrate. Depending on the size of the substrate taken up, endocytosis is further subdivided into phagocytosis and pinocytosis. Phagocytosis was first observed by Metchnikoff (1883) using the light microscope; pinocytosis was first defined and described by Lewis (1931). It has been suggested that phagocytosis and pinocytosis differ from each other not only with regard to substrate size but also with regard to energy requirements and kinetics (North, 1970); also pinocytosis has been divided into macro- and micropinocytosis on the basis of both vesicle size and energy requirements. Claims have been made that micropinocytosis requires no energy (Allison & Davies, 1974), but Duncan & Lloyd (1978) demonstrated that this distinction is not justified. Endocytosis has been demonstrated for a great number of cell types, both free cells and those within tissues, and a great variety of different substrates.

The process of endocytosis can be split up into a sequence of events. In phagocytosis, preceding membrane invagination or pseudopodium formation, contact of the substrate with the membrane and/or the presence of opsonins appears necessary to trigger membrane movement. Although pinocytosis may involve membrane/substrate interaction, vesicle formation has been shown to be a continuous and spontaneous process, not even demanding the presence of any substrate. The attachment phase in endocytosis is influenced by surface charge on particles (Stossel, 1973), the presence of hydrophobic groups on particles (Livesey, 1979), possibly the size of the ingested particle (Korn & Weisman, 1967) and the existence of specific receptors on the membrane (Steinman & Cohn, 1972). Internalisation may be by engulfment or invagination. With amoebae, pseudopodia can be seen to flow around the prey till it is totally enclosed (Christiansen & Marshall, 1965). Invagination may be mediated by the action of microfilaments (Goldman &

Representative sample of cells involved in endocytosis

Peritoneal macrophages	Davies et al. (1973); Ehrenreich & Cohn (1967) Lewis (1931); Robinovitch (1968), Caseley-Smith (1969)
Lung macrophages	Tsan & Berlin (1971); Stossel (1973, 1975)
Blastocysts	Parr & Parr (1978)
Neutrophils	Ward & Zwaiffler (1973)
Blood capillaries	Clementi & Palade (1969)
L-cells	Gordon & King (1960)
Renal proximal tubules	Maunsbach (1963)
Liver cells (Kupffer)	Mego et al. (1967); Munthe-Kaas (1976) Mori & Novikoff (1977)
Granulocytes	Stossel (1973)
Keratinocytes	Wolff & Konrad (1972)
Tumor cells	Ryser et al. (1962); Bruns & Palade (1968); Spivak (1973); Brunk et al. (1977)
Leucocytes	Sbarra et al. (1962); Robinovitch (1968)
Thyroid cells	Rodesch et al. (1970); Burke (1970); Zalin & Hoffenberg (1977)
Rat neurophysial axon	Theodoris et al. (1976)
Amoeba	Bowers & Olzewski (1972); Chapman-Andresen & Holter (1964); Christiansen & Marshall (1965); Bowers (1977)
Rat kidney cells	Strauss (1962, 1967)
Fibroblasts	Becker et al. (1973); Steinman et al. (1974); Warburton & Wynn (1976)
Glia cells	Brunk et al. (1977)

Representative sample of endocytosed materials

Paramecium aurelia	Christiansen & Marshall (1965)
Yeast cells	Nordenfelt (1970)
Pneumococci	Armstrong & D'Arcy Hart (1975)
Erythrocytes	Munthe-Kaas (1976); Spivak (1973)
Gold spherules	Komiyama & Spicer (1975)
Quarz	Mudd (1934)
Thorium dioxide	Caseley-Smith (1969)
Asbestos	Davies et al. (1974)
Silica, carbon	Fenn (1921); Roberts & Quastel (1963)
Glass	Gilfillan et al. (1970)
Polystyrene	Sbarra et al. (1962)
Latex beads	Wolff & Konrad (1972); Bowers (1977)
Coated oil droplets	Stossel (1973, 1975)
Starch	Sbarra et al. (1962)
Haemoglobulin	Contractor & Krakauer (1976)
Horseradish peroxidase	Mori & Novikoff (1977); Moxon et al. (1976); Steinman et al. (1974)
Glucose	Chapman-Andresen (1977)
Ferritin	Ryser et al. (1962); Bruns & Palade (1968)
Polyvinylpyrrolidone	Zalin & Hoffenberg (1977); Williams et al. (1975); Roberts et al. (1977)
^{131}I-Albumin	Ryser et al. (1962a); Mego et al. (1967)
Thyroglobulin, Fibrinogen	Zalin & Hoffenberg (1977)
^{125}I-Rat IgG	Williams & Ibbotson (1979)

Falett, 1969) or alternatively may be the result of a group of receptors moving together forming the limiting membrane that forms a vesicle (Goldstein et al., 1979; also see Ockleford, 1976, for the proposed mechanism for the formation of coated vesicles). The fates of vesicles are potentially three-fold. 1) Vesicles can move to the perinuclear region and fuse with lysosomes. This will lead to the degradation of the contained molecules. 2) Vesicles can exocytose their contents at the apical membrane. 3) Vesicles can traverse the cell and exocytose at the basal membrane (diacytosis). The last process is somewhat speculative, but Bruns & Palade (1968) have described the diacytosis of ferritin across endothelial cells .

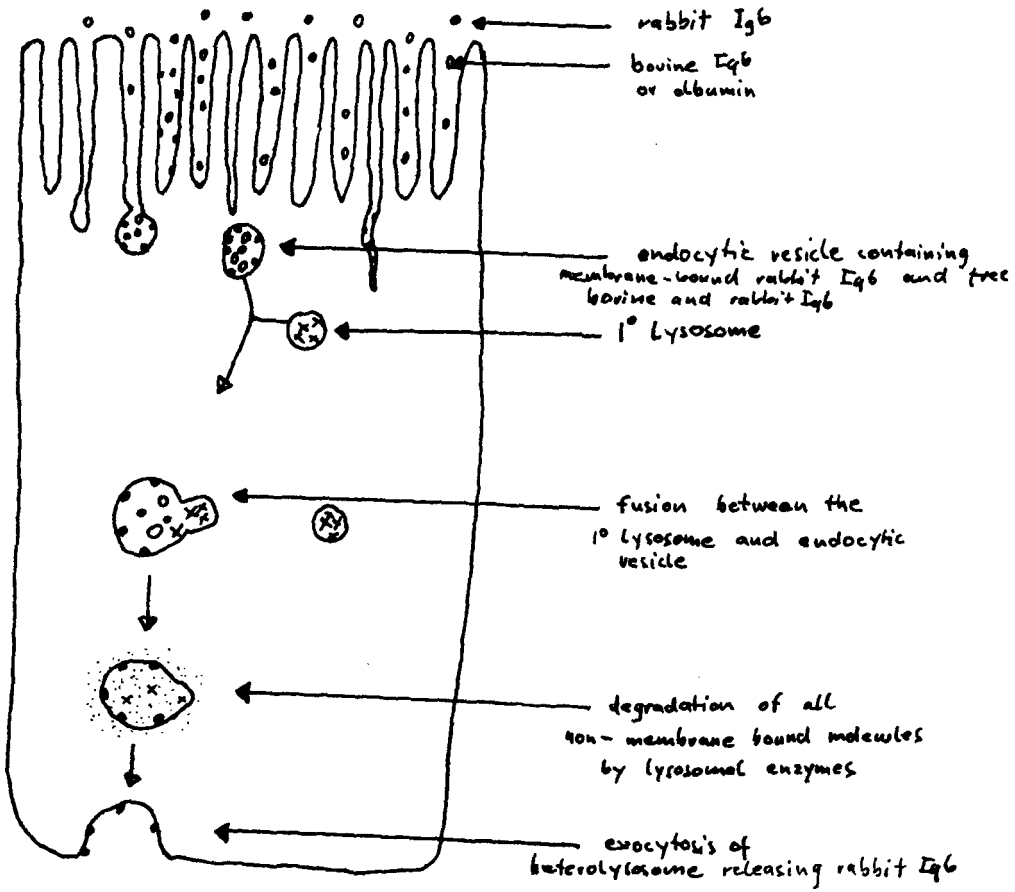
Endocytosis, being an energy-dependent process, can also be inhibited and stimulated by the addition of various effectors. The following are established as inhibitors in some systems: 2,4-dinitrophenol, iodoacetate (Chapman-Andresen, 1977; Casley-Smith; 1969); fluoride, sodium azide (Steinman et al., 1974) and certain weak bases including ammonium ions (Livesey et al., 1980). The following have been reported to act as stimulators: anionic compounds, dextran sulphate, albumin, fetuin (Cohn & Parks, 1967); strychnine (Teichberg et al., 1975).

A component of the vacuolar system that is vitally important to the discussion of the problem of transcellular IgG transfer is the lysosome. Lysosomes were discovered by de Duve (see de Duve, 1963; de Duve & Wattiaux, 1966 for reviews), and their function is the degradation of endogenous and exogenous materials in the cell. Biochemically, lysosomes can be described as unit-membrane-limited vesicles containing a battery of hydrolytic enzymes able to degrade biopolymers at acid pH. Primary lysosomes probably originate from the Golgi apparatus (GERL region). After fusing with either pinosomes, phagosomes or autophagosomes (vesicles containing endogenous materials) they are termed

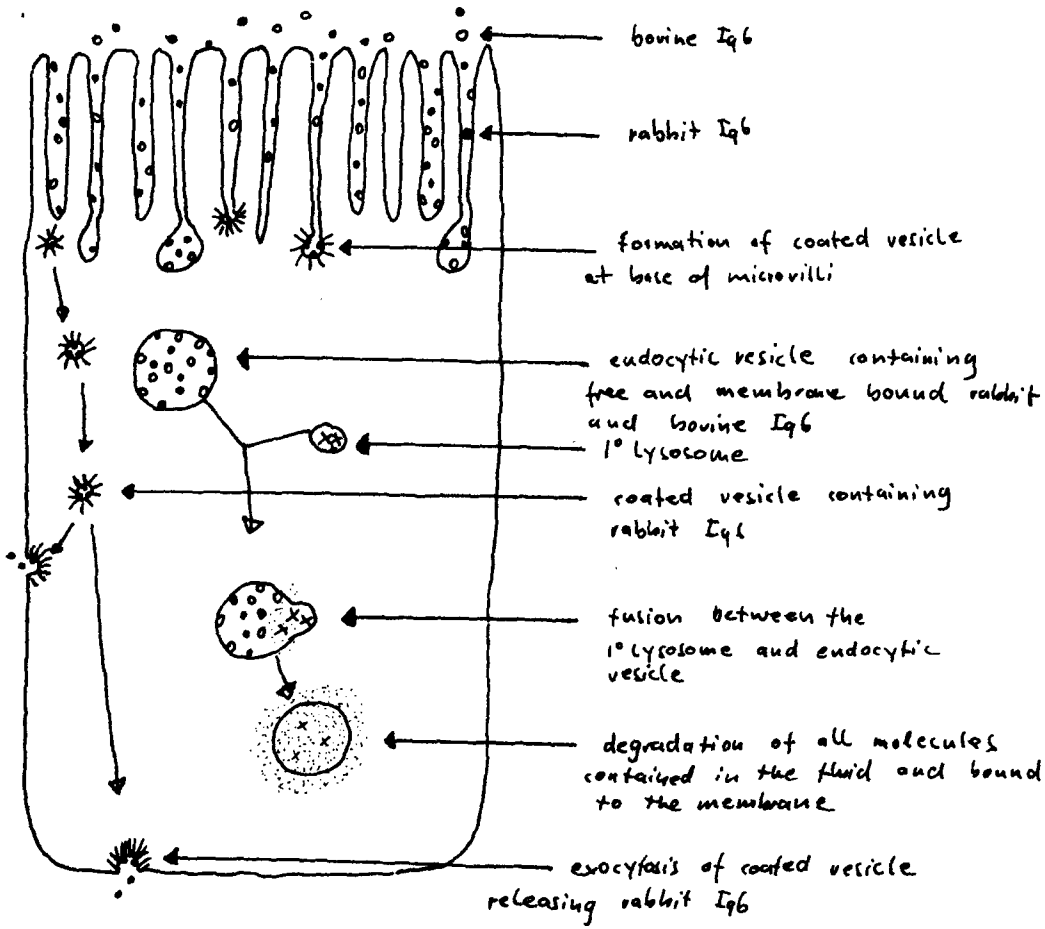
secondary lysosomes. The lysosomal membrane is permeable to molecules <220 Daltons so that small degradation products are released from the lysosome by diffusion whereas non-degradable substrates remain within the lysosome (then called a residual body).

Returning to the problem the yolk-sac or placental cell is faced with in the transport of IgG, it is clear that a very special mechanism must exist to effect selective degradation of proteins. Therefore, ever since Brambell et al. (1950) discovered this selection, ideas have been put forward to provide a mechanism for the protection of IgG. The first one chronologically, comes from Brambell (1958) and is shown diagrammatically on page 8a. Selective protection against degradation is supposed to occur within heterolysosomes by the binding of IgG molecules to specific receptors on the inner face of the heterolysosome; unbound proteins are degraded by lysosomal enzymes. The bound IgG,protected against enzymic attack in this manner,is released intact by exocytosis of the vesicle at the lateral or basal plasma membrane. An alternative model was proposed by Wild (1975, 1976), that involves coated micropinocytic vesicles, a separate class of vesicle (see diagram on p 8a). These vesicles have been shown to be involved in the transport of proteins in different cell types (see Pearse, 1980 and Goldstein et al.,1979 for reviews). According to Wild's theory, selection occurs on the outer face of the yolk sac by the binding of IgG to specific receptors prior to the formation of coated pinocytic vesicles. IgG, like other proteins, is also taken up into ordinary macropinocytic vesicles, but whereas the macropinocytic vesicles fuse with primary lysosomes leading to a degradation of their contents, coated micropinocytic vesicles do not fuse with lysosomes, but release their contents intact at the basal or lateral plasma membrane. This theory was raised in opposition to that of Brambell when a number of electron microscope findings failed to

BRAMBELL'S SCHEME



WILD'S SCHEME



support the Brambell model. Thus macropinocytic vesicles were not found in the basal region of cells and no ultrastructural evidence for exocytosis from heterolysosomes could be obtained. In contrast, coated micropinocytic vesicles containing HRP-conjugated human or rabbit IgG have been seen near the basal or lateral membranes and even in confluence with these membranes (Moxon et al., 1976).

Hemmings and Williams (1976) have forwarded yet another theory to account for the differential degradation of protein in the rabbit yolk sac. They proposed that all proteins are taken up indiscriminately by the yolk sac, but that subsequent to capture some vesicles burst for some undefined reason. (Broken vesicles have indeed been observed in a variety of cells fixed for electron-microscopy: Wild, 1970; Padykula et al., 1966 .) All proteins that are released into the cytoplasm in this manner escape lysosomal degradation, but all those proteins remaining within vesicles are subject to degradation. The release from the cell of those proteins that pass into the cytoplasm is supposed to be by differential diffusion across the basal plasma membrane.

Even though it has been formulated for another tissue, the theory of Rodewald (1973) accounting for IgG transfer across the rat gut should also be mentioned. The basic idea is that along the rat gut different cells are specialized to fulfil different functions. Antibody transport only takes place in the proximal part of the small intestine whose cells are morphologically different from those in the ileum or distal part. Only into the proximal cells are some antibodies selectively taken up (ferritin-conjugated bovine and rat IgG; Rodewald, 1973). Selection is supposed to happen during the uptake stage on the membrane at the base of tubular invaginations on the apical cell surface. Binding to receptors appears to be pH dependent. Both Rodewald (1976) and Wild & Richardson (1979) report that the initial pH optimum for the binding step is 6.0.

Comparison of the main features of the different mechanisms proposed for the selective transfer of IgG

Feature of the process considered	Predicted behaviour or feature in the model according to: Brambell	Wild	Hemmings & Williams
1. Mechanism responsible for selection of transferred IgG	Selective binding to membrane receptors	Selective binding to membrane receptors	Selective diffusion at basal membrane
2. Location of the specific receptors involved in IgG transmission	Inner face of pinosome or heterolysosome	Outer surface of plasma-membrane	No receptors postulated
3. Can the protective IgG route be saturated?	Yes	Yes	No
4. Location of IgG in the cell	Pinosome or heterolysosome	Pinosome, heterolysosome or coated vesicle	Pinosome, heterolysosome or cytosol
5. Location of protected IgG in the cell	Pinosome of heterolysosome	Coated vesicle	Cytosol
6. Location of protein destined for degradation	Pinosome or heterolysosome	Pinosome or heterolysosome	Pinosome or heterolysosome
7. Minimum number of vesicle-types proposed	1	2	1
8. Presence of lysosomal enzymes in transport vesicles is suggested?	Yes	No	Yes
9. Uptake of protein not destined for transport is by the following mechanism	Fluid-phase pinocytosis	Fluid-phase or adsorptive pinocytosis	Fluid-phase or adsorptive pinocytosis
10. Release of IgG from the cell is by the following mechanism	Exocytosis of pinosomes/ heterolysosomes at basal/ lateral membrane	Exocytosis of coated vesicles at basal/ lateral membrane	Differential diffusion at basal/lateral membrane
11. Is release of lysosomal enzymes or a non-degradable marker possible from transplant vesicles?	Yes	No	No

Dissociation of the IgG from the receptor at the basal membrane is supposed to be aided by the higher intracellular pH. During transport the tracer was observed in the tubular vesicles formed by invagination and later in coated vesicles pinching off from the surface of these tubular vesicles. Coated vesicles then transport the contained antibodies to the basal cell membrane, where they exocytose. Markers are subsequently also traced in the intercellular spaces. On closure (22 day after birth), the proximal cells undergo morphological changes and lose their ability to transfer proteins. Distal cells also endocytose all manner of proteins, but this uptake is followed by degradation. This theory is formulated specifically for the postnatal antibody transfer in the gut and will not be discussed further in the following work. For completeness' sake the final theory mentioned here, is that proposed by Kulangara & Schechtman (1962). They suggest that selection does not take place during transport across the yolk-sac splanchnopleur in the rabbit, but occurs in the foetal circulation by selective removal of transported proteins from the blood. As this theory assumes that transcellular transport without degradation can occur, but proposes no mechanism for the process, it will not be further discussed in this work.

Looking at the different theories it becomes apparent that,for each , different assumptions are made. The table on page 9a indicates the central assumptions of the theories of Brambell, Wild and Hemmings and their differences and in turn suggests how these theories can be examined critically. Three main categories of investigation have been used in the past: <u>in vivo</u> observations, <u>in vitro</u> binding to fixed tissues and ultrastructural investigations. (See Chapter 3 for an appraisal of respective merits of these methods .) This present work is concerned with applying a new method to the problem of selectivity, namely by using an <u>in vitro</u> incubation method to examine the behaviour of intact

tissues thought to be involved in selective transport of immunoglobulins (i.e. rat and rabbit yolk sacs). Encouraged by the findings of Williams & Ibbotson (1979) that rat yolk sacs incubated in vitro treated ^{125}I-labelled rat IgG in a significantly different manner to ^{125}I-labelled BSA, an attempt was made to modify the technique to permit investigation of rabbit yolk sacs in a similar manner. The use of rabbit yolk sacs was deemed extremely desirable, because the yolk sac has long been established as the site of transfer of passive immunity in the rabbit whereas in the rat the yolk sac is at best only a secondary site. Near term, when most transfer of passive immunity occurs, the arrangement of foetal membranes is very similar in rats and rabbits (see the diagrams on pages 11a and 11b). In both species the foetus is surrounded by the amnion and yolk sac, Reichert's membrane having disappeared . In the rabbit, the yolk sac is not joined to the placenta, but a strip of paraplacental chorion lies between those two tissues. Wild (1970) has demonstrated that this paraplacental chorion allows the non-selective diffusion of proteins into the exocoel and later into the foetal gut, where digestion occurs. In the rat this paraplacental chorion is not present, instead the endoderm of the yolk sac is highly villous near the placenta, and the placenta is permeated by the crypts of Duval. Selective protein transport to the foetal circulation, in both species, is thought to occur via the yolk sac, the ultrastructure of which is very similar in both tissues (for a diagrammatic scheme see page 11c). Immunoglobulins destined for transport must traverse a distance of approx. 24μm. Uptake is initially into the cells forming the low columnar epithelium. These cells are linked by desmosomes at the apical end. The IgG is then released into the mesenchyme of the epithelium where uptake has been observed into macrophages. To reach the foetus, IgG molecules have to pass through the cells lining the vitelline vessels and into the vitelline

DIAGRAM OF THE FOETAL MEMBRANES OF THE RAT

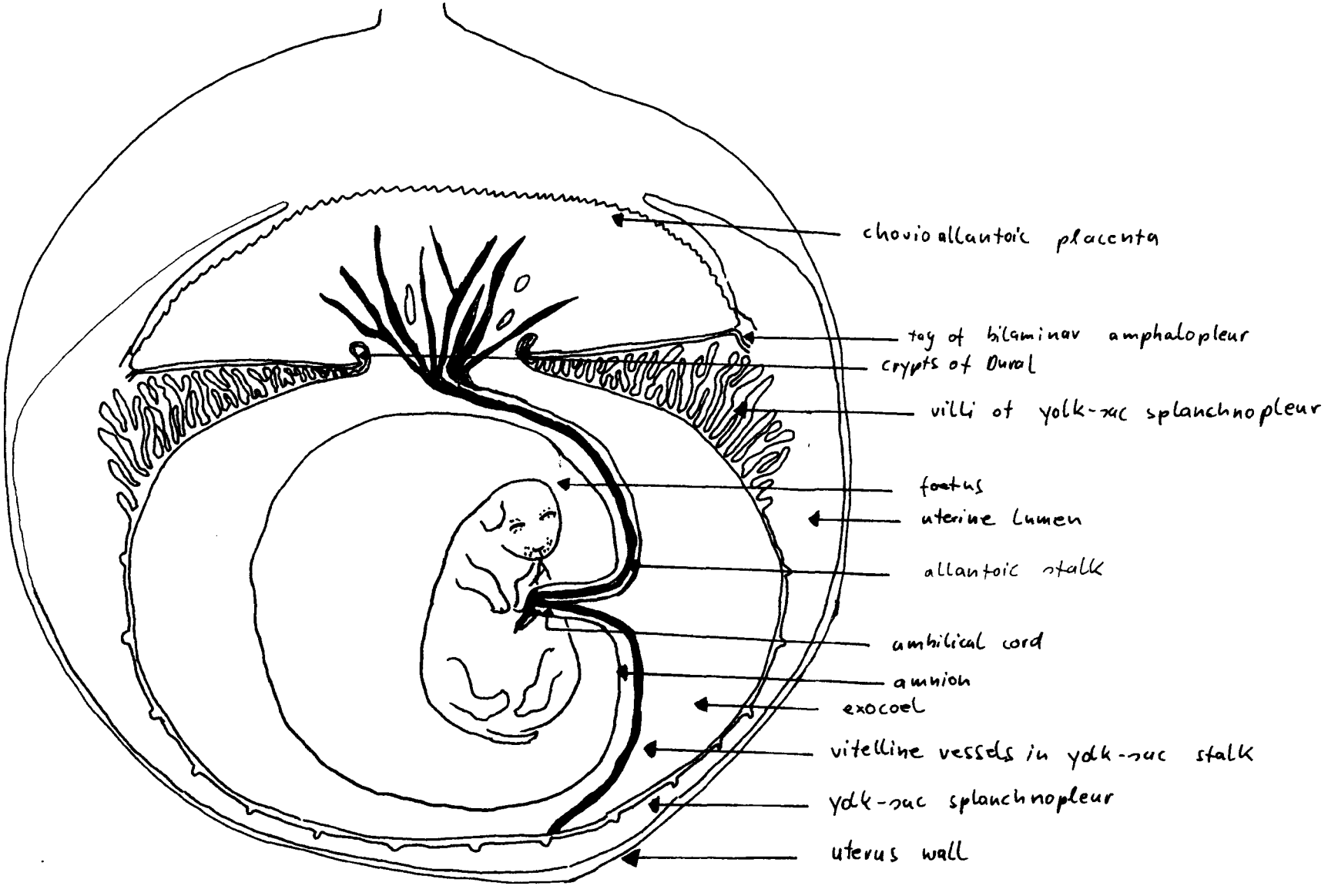
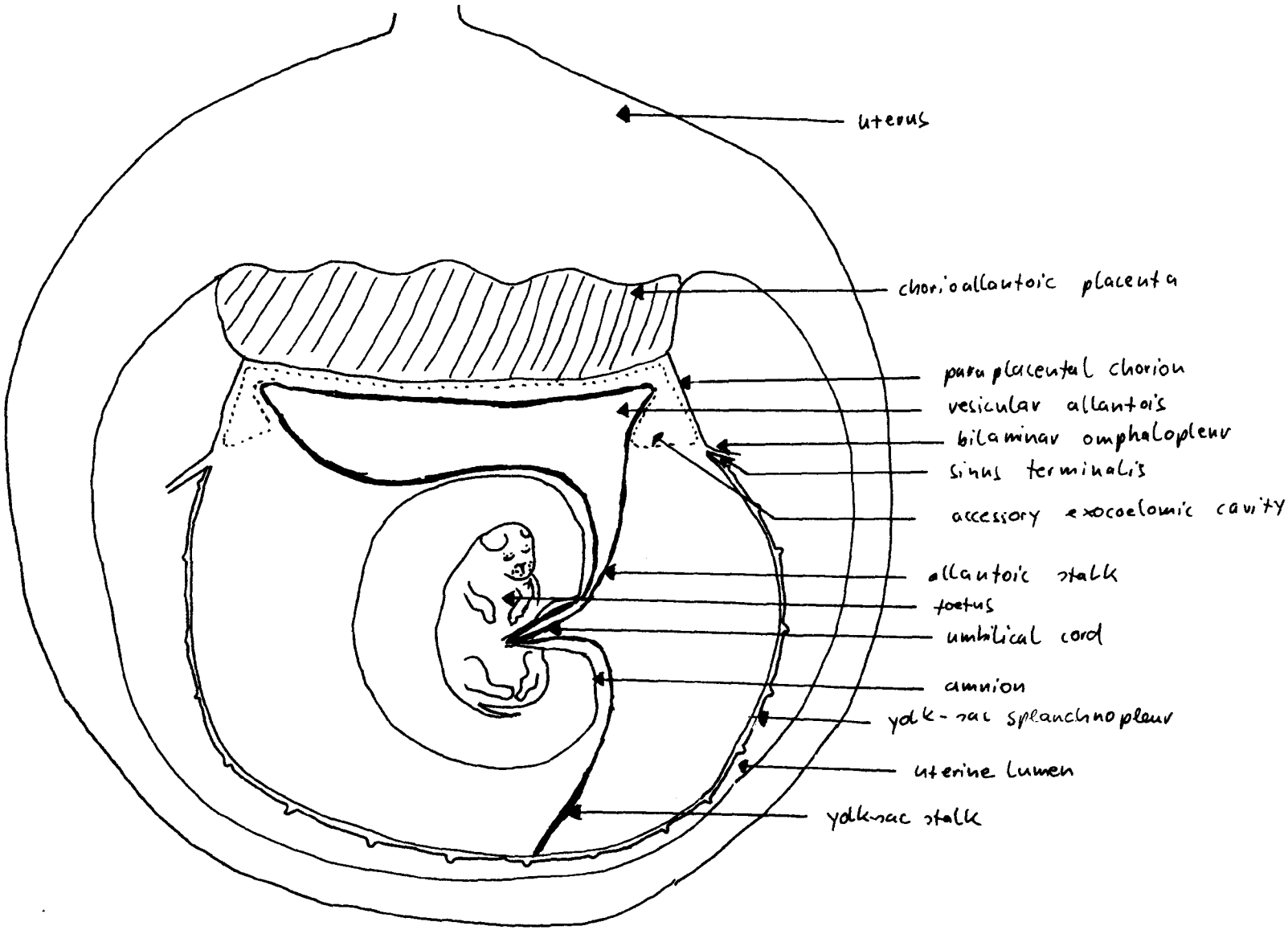
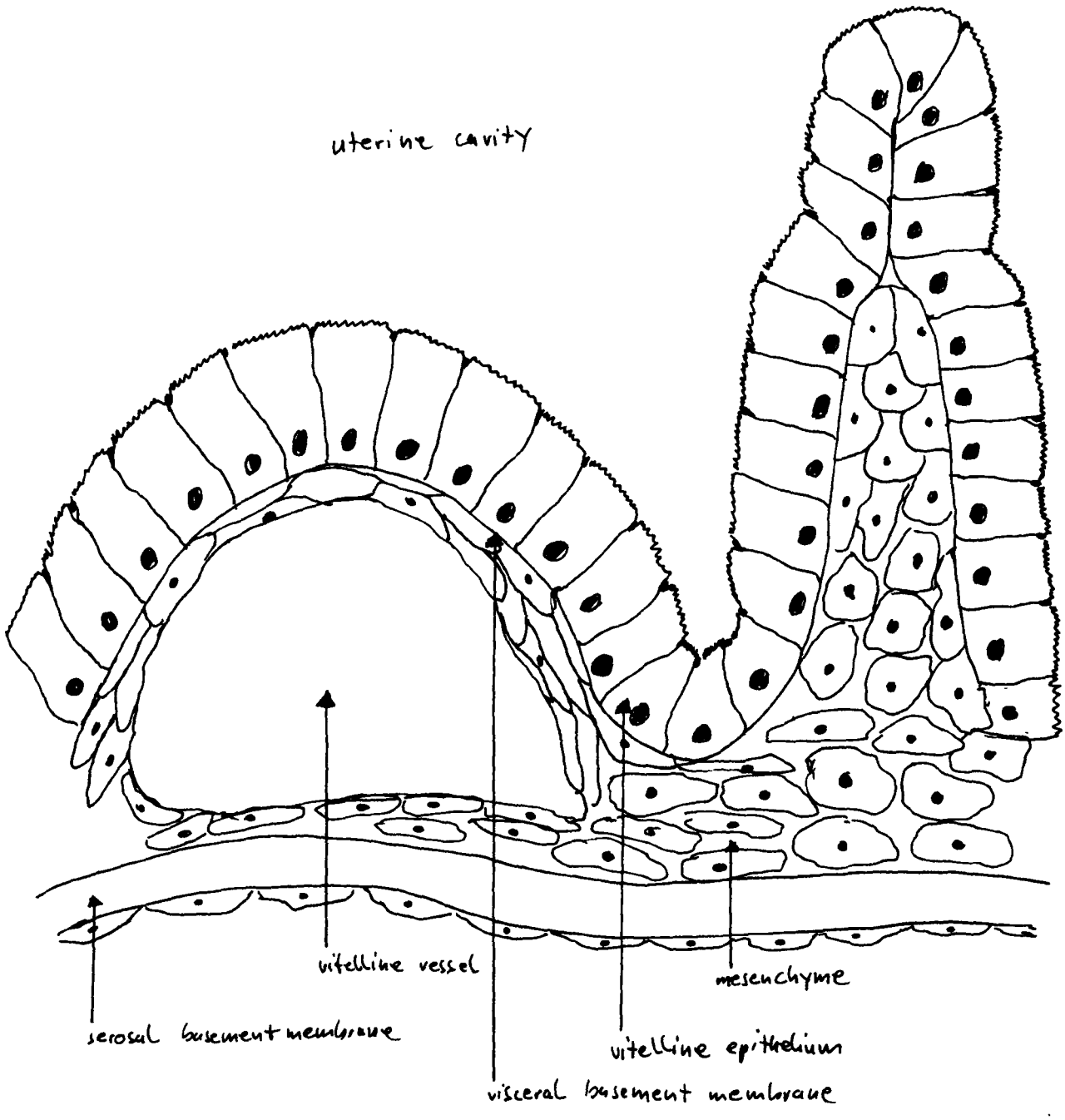


DIAGRAM OF THE FOETAL MEMBRANES OF THE RABBIT



DIAGRAMMATIC SECTION THROUGH THE YOLK SAC OF RAT OR RABBIT



circulation. Selection is thought to operate at the level of the epithelial cells, which in both species have a brush border of microvilli at the apical region facing the uterine lumen. In these cells invaginations can be seen between the bases of microvilli, often having a thickened filamentous lining which may present a special surface for protein attachment. The main functions of the rat yolk sac are probably to protect the foetus from harmful substances (Wilson et al., 1959) and in embryotropic nutrition (Beck & Lloyd, 1968). Structurally the rat and rabbit yolk sacs are quite similar and there is probably some overlap in the pathways by which IgG is acquired in vivo in the prenatal period.

The use of the in vitro incubation system for rat yolk sac has now been well documented both for studies of uptake (Williams et al., 1975a,b; Roberts et al., 1977; Moore et al., 1977, and for studies of "exocytosis" (Roberts et al., 1977; Williams & Ibbotson, 1979; Ibbotson & Williams, 1979). Therefore no detailed description will be given at this point. Briefly, in uptake studies yolk sacs are incubated for up to 7h in a suitable medium and radiolabelled proteins are allowed to be taken up by the tissue. The rates of uptake can then be determined, taking into account inter-experimental variables, e.g. radioactive decay, size of tissue, substrate concentration. The rate of uptake can be expressed as an Endocytic Index (i.e. the volume of culture medium, µl, whose contained substrate is ingested per mg yolk-sac protein per hour). In a variation on the basic method of yolk-sac incubation, a measure can be made of the amount of substrate released from the tissue intact. The tissue is first allowed to take up ^{125}I-labelled IgG and then during a reincubation period substrate release is monitored, and the amount of IgG escaping from the tissue intact can be measured. (More detailed descriptions of this method and its advantages are given in Chapter 3 .)

It is vitally important for the interpretation of any results obtained with this *in vitro* method that the tissue-associated IgG is fully internalized by the tissue and is not simply adsorbed to the outer yolk-sac membrane. (This question is investigated in detail in Chapter 4.) The remaining chapters are concerned with a critical examination of the assumptions and predictions of the theories of Hemmings, Wild and Brambell outlined in the Table on page 9a.

CHAPTER ONE

MATERIALS AND METHODS

1.1 Standard method for radioiodinating proteins

Proteins were labelled with [^{125}I]iodide by using the chloramine-T method described by Williams et al. (1971) with slight modifications. Proteins (10 mg) i.e. BSA (preparation 0142t; Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.), or rat gamma globulins (Fraction II; Code No. 82-504, Miles Laboratories Ltd., Slough, U.K.) or rabbit gamma globulins (Code No. 82-455) or bovine gamma globulins (Fraction II; Code No. 82-041) or human gamma globulins (Fraction II; Code No. 83-310) were dissolved in 0.05M-Na_2HPO_4-KH_2PO_4 buffer (10ml) at pH 8, in a sterile 50 ml beaker. After cooling the mixture in an ice-bath for 5 min, 1mCi of sodium [^{125}I]iodide (Preparation IMS.30, 5mCi in 0.5 ml, Radiochemical Centre, Amersham, Bucks., U.K.) was added using either a 1 ml disposable syringe or a Hamilton syringe. After stirring for 5 min, chloramine-T solution (2 ml, 200 µg/ml) was added. The reaction was allowed to proceed for 8 min, then stopped by adding sodium metabisulphite solution (2 ml, 200 µg/ml). To aid displacement of unreacted [^{125}I]iodide during the subsequent dialysis, solid NaI (50 mg) was added to the reaction mixture. The protein preparation was then dialysed for 48h at 4°C in Visking tubing (¼") against 2-3 changes (5 l) of aq. NaCl 1% (w/v), and finally dispensed into sterile 10 ml Bijou bottles and stored at -20°C until use.

1.2 Technique for incubating rat visceral yolk sacs in the presence of calf serum

The technique used was that of Williams et al. (1975a) with slight modifications. Virgin female Wistar rats from an inbred strain were put with a stud male animal over night in a grid-bottom cage. Mating was assumed to have occurred at midnight if a copulation plug was found below the grid next morning. At 17.5 days of gestation, rats were killed by cervical dislocation and the uterus was immediately

removed. The rest of the dissection was carried out in medium 199 (single strength, preparation TC 20 containing penicillin and streptomycin, Wellcome Reagents Ltd., Beckenham, Kent) containing 10% (v/v) calf serum (heat-inactivated, preparation CS07; Wellcome Reagents Ltd., Beckenham, Kent) that had previously been gassed with an O_2/CO_2 mixture (19/1). Each yolk sac was dissected out by removing the placental cap, conceptus and all amniotic tissue. The individual yolk sacs were placed in 50 ml Erlenmeyer flasks (previously sterilized by heating at 120°C in an air oven for 8-10 h), to which had been added 9.0 ml of gassed medium 199 containing 10% (v/v) calf serum. Flasks were sealed with sterile silicone rubber bungs and placed in a reciprocating water-bath at a temperature of 37°C (± 0.2°C) with the shaking attachment set at 100 ± 5 strokes/min and a stroke-length of 3.8 cm. The yolk sacs were allowed to pre-incubate for 5 min before adding 1.0 ml of a solution of either a ^{125}I-labelled protein or ^{125}I-labelled PVP (average molecular weight 30-40 000, preparation IM.33P, Radiochemical Centre, Amersham, Bucks., U.K.) dissolved in medium 199 containing 10% (v/v) calf serum, using either a Finnpipette or a 1 ml safety pipette. The final concentration of the radiolabelled substrate was 1-3 µg/ml for ^{125}I-labelled PVP and 0.7-2.0 µg/ml for ^{125}I-labelled proteins. After addition of substrate, each flask was regassed with O_2/CO_2 (19/1) for 10 sec before being returned to the water bath. Yolk sacs were removed at regular intervals up to 6.5 h and, unless stated otherwise, washed in 3 changes of 1% (w/v) ice-cold saline (approximately 30 ml) and stored in 5 ml volumetric flasks at -20°C until assayed.

1.3 Assay of yolk sacs for contained radioactivity

Yolk sacs were thawed and 1.0M-NaOH was added to give a final volume of 5.0 ml. The volumetric flasks were placed in a shaking

waterbath at 37°C for approximately 1h. After vigorous mixing with a Vortex mixer they were left in the waterbath for a further 30 min and then mixed again to ensure complete solution. Two aliquots of each yolk-sac solution were placed in 3 ml plastic tubes (LP3, Luckham Ltd., Burgess Hill, Sussex) and the associated radioactivity was assayed using a gamma spectrometer (Packard Instrument Ltd., Caversham, Berkshire.) Tubes were placed in the instrument in a standard geometrical relation to the crystal detector. An appropriate counting time was chosen to ensure a total count in excess of 10^3 in order to minimize associated counting errors.

1.4 Determination of the protein content of yolk sacs

The method employed to determine the protein content of yolk sacs was that of Lowry et al. (1951). BSA (Sigma (London) Chemical Co. Ltd, London S.W.6, Product No. A-4378) was used as reference protein. The protein content of each yolk sac or yolk-sac piece was determined by assaying 0.1 ml aliquots of the yolk-sac solution prepared above. The protein content of each yolk sac or yolk-sac piece was expressed as an equivalent number of mg of BSA.

1.5 Assay of radioactivity in the incubation medium from experiments with ^{125}I-labelled PVP as substrate

Duplicate samples (1.0 ml) of each incubation medium were pipetted into 3 ml disposable plastic tubes and their contained radioactivity was assayed, using a gamma spectrometer. The mean count, corrected for background, was termed the "total radioactivity" in the incubation medium.

1.6 Assay of radioactivity in the incubation medium from experiments with a ^{125}I-labelled protein as substrate

When a ^{125}I-labelled protein is used as substrate, hydrolysis within the yolk-sac tissue causes the release of radiolabelled degrada-

tion products back into the incubation medium (Williams et al., 1975b; Ibbotson & Williams, 1979; Livesey & Williams, 1979). Hence, to obtain a value for the net protein uptake into the tissue, these radioactive digestion products of the protein have to be taken into account as well as the tissue-associated radioactivity. The quantity of radiolabelled protein degradation products released into the medium was determined in the following way. First, the "total radioactivity" in the medium was measured in the manner outlined in Section 1.5, then aq. trichloroacetic acid (0.5 ml; 20% w/v) was added to each tube to precipitate the contained protein. After centrifugation (2000 g, 20 min) the supernatant was decanted into a new 3 ml plastic tube and recounted, using a counting time of suitable duration to minimize the associated error. This value, corrected for background, was termed the "TCA-soluble activity". A correction had to be applied to this quantity to allow for the change in sample volume caused by adding aq. trichloroacetic acid. This correction is necessary because the amount of radioactivity registered by the crystal detector falls as the sample volume increases. Hence the observed count, for a sample of volume other than 1.0 ml, was multiplied by an empirical correction factor to give that count that would have been observed if the contained radioactivity had been assayed in a sample of volume 1.0 ml. For "TCA-soluble activity" assayed in the above manner the appropriate factor was 1.3.

1.7 Technique for incubating rat visceral yolk sacs in serum-free medium 199

Yolk sacs were incubated by essentially the same procedure as described in Section 1.2, but incorporating minor variations described by Ibbotson & Williams (1979). The uterus was removed from rats at 17.5 days of gestation and put into serum-free medium 199. Care was

taken to dissect yolk sacs as quickly as possible since tissue survival seems to be rather poorer in the absence of calf serum (Ibbotson & Williams, 1979). Tissues incubated for 6.5h or longer frequently showed anomalous uptake data thus, in most experiments, the maximum incubation period was limited to only 5.5h. The methods for assaying medium "total radioactivity" and the radioactivity in yolk sacs and the protein content of yolk sacs were the same as described in Sections 1.3 to 1.5, but to obtain values for "TCA-soluble activity", 0.1 ml of calf serum had to be added to each tube before addition of trichloroacetic acid to ensure a complete precipitation of radioactive proteins. After centrifugation each supernatant (approximately 1.3 ml) was decanted into a fresh 3 ml tube and counted in the usual manner. The appropriate empirical correction factor for the TCA-soluble radioactivity was 1.33. Multiplying counts by this factor normalizes the observed values of this quantity for altered counting geometry.

1.8 Techniques for incubating rabbit yolk sacs in vitro

Rabbits of a Californian White strain were mated and copulation was observed. Animals were killed on the 24th day of gestation by cervical dislocation. The uterus was removed quickly and opened. Each yolk sac was cut away from the chorioallantic placenta, the conceptus and the amnion; the paraplacental chorion was then removed. The dissection was performed in warmed medium 199, that had previously been gassed with an O_2/CO_2 mixture (19/1) and containing either 10% (v/v) calf serum or no serum at all. Since rabbit yolk sacs are considerably larger than rat yolk sacs, each yolk sac was divided into 3-5 pieces of approximately equal size which were then incubated individually in 9.0 ml of either gassed medium 199 alone or medium 199 containing 10% (v/v) calf serum, depending on the experimental design. The remainder of the procedure was either the same as that described for rat yolk sacs (see Section 1.2 to

1.6) when rabbit yolk sacs were incubated in serum-containing medium 199, or as described in Section 1.7 for incubations in serum-free medium 199.

1.9 Calculation of the Endocytic Index and Tissue-Accumulation Rate for a non-digestible substrate

In order to obtain a meaningful plot of uptake against time, it is necessary to correct for the variable size of individual yolk sacs and the decay of the specific radioactivity of the substrate. This was achieved by using the following equation :

$$A = \frac{Y}{M \times P} \qquad 1.1$$

where A = quantity of radioactive substrate accumulated in the yolk sac at a given time (µl incubation medium/mg tissue protein)

Y = total radioactivity in the whole yolk sac (c.p.m., corrected for background)

M = radioactivity /µl of medium (c.p.m., corrected for background /µl)

P = protein content of individual yolk sac (mg)

Plotting A against time gives a straight line, the gradient of which has been termed "Endocytic Index" by Williams et al.(1975a) and which has the units: µl incubation medium per mg yolk-sac protein/h. It effectively reports the rate of uptake as the number of microlitres of medium whose contained substrate is captured by unit weight of tissue in unit time. For substrates with a very low Endocytic Index M is effectively constant so that any complications caused by medium depletion can be ignored (e.g. for ^{125}I-labelled PVP, the medium is depleted by less than 0.1% per h. Thus, if the substrate concentration in the medium at the end of the experiment is used for M in equation 1.1, this will

introduce only a negligible error.) The advantages of this form of expression are as follows :

a) The results for each experiment are normalized for variations in tissue size.

b) The results of experiments performed on different days are independent of radioactive decay in the substrate and of any drift in the efficiency of the gamma counter, provided media and yolk-sac counts are determined within a short period of each other.

For a non-degradable substrate the Endocytic Index and Tissue-Accumulation Rate are identical providing there is a negligible rate of release of the captured substrate by the tissue. This appears to be true for ^{125}I-labelled PVP captured by rat yolk sacs incubated in medium 199 containing 10% (v/v) calf serum (Williams et al., 1975a).

1.10 Calculation of the Endocytic Index and Tissue-Accumulation Rate for a ^{125}I-labelled protein

Using a ^{125}I-labelled protein as substrate complicates the calculation of the Endocytic Index in two ways:

1) If the protein is digested by the yolk sac and its degradation products (i.e. small peptides and amino acids) are released back into the incubation medium, this will affect the numerator of equation 1.1.

2) If the protein is rapidly captured, largely by adsorptive pinocytosis, its concentration in the medium may become noticeably depleted in the course of the 6-7h incubation period. This will mean that M in equation 1.1 can no longer be regarded as being effectively constant. Hence, to obtain a measure of A', the amount of substrate that would have been accumulated in the tissue, had it not been degraded, equation 1.1 has to be modified to:

$$A' = \frac{Y + 10\ (S-F)}{M' \times P} \qquad 1.2$$

The numerator represents the total radioactivity processed by the yolk sac. In addition to Y, the total radioactivity present in the whole yolk sac (c.p.m., corr. for background), another term, 10(S-F), has to be included to account for the substrate digested by the tissue and released back into the 10 ml of incubation medium. S is the TCA-soluble radioactivity in the incubation medium at the end of an incubation period (c.p.m./ml, corr. for background), and F is the amount of TCA-soluble radioactivity generated other than by proteolysis associated with the tissue (c.p.m./ml, corr. for background). F includes both the TCA-soluble radioactivity initially present in the substrate preparation and any TCA-soluble radioactivity generated by proteolysis within the medium. In each experiment F is determined by incubating 10 ml of medium, containing substrate but no yolk sac, for the duration of the experiment. Then R, the fraction of the total TCA-soluble radioactivity in this incubation medium is determined. F is then defined by the equation:

$$F = Q \times R \qquad 1.3$$

where Q is the total radioactivity in each individual incubation medium (c.p.m./ml, corr. for background).

Because the rates of pinocytic capture are generally higher for ^{125}I-labelled proteins (see Chapter 2), M, the concentration of the macromolecular form of the substrate in the medium falls with time. M must therefore be replaced by M', the mean value of TCA-insoluble radioactivity in the medium during the individual incubation period for each yolk sac (c.p.m./ml, corr. for background) which is defined as:

$$M' = M + \frac{(S-F)}{2} \qquad 1.4$$

The value of M' is obtained by adding to the observed value of M, the TCA-insoluble radioactivity at the end of an incubation period (c.p.m./ml, corr. for background), half the observed increase in the TCA-soluble activity/ml of medium at the end of the same incubation period (c.p.m./ml, corr. for background).

The gradient of the plot of A' (from equation 1.2) against time is the Endocytic Index, and the Tissue-Accumulation Rate is obtained from the gradient of the plot of A (from equation 1.1) against time.

1.11 Modification of the calculation of the Endocytic Index of a macromolecular substrate to allow for exocytosis

For most substrates, like ^{125}I-labelled PVP, colloidal [^{198}Au]gold and a great number of proteins it has been shown that exocytosis of the macromolecular form subsequent to capture occurs at such a low rate that it can be neglected. However, the same is not true for some IgG species, hence the general uptake equations 1.1 and 1.2 must be modified to allow for exocytosis of the macromolecular form of the substrate. The gross rate of uptake for a non-digestible substrate U, is then defined as:

$$U = \frac{Y}{M \times P} + R \qquad 1.5$$

where R is the amount of non-digestible substrate released from the tissue during the incubation of the individual yolk sac (µl/mg protein).

For a digestible substrate the corresponding expression for the gross rate of uptake, U', is :

$$U' = \frac{Y + 10\ (S-F)}{M' \times P} + R' \qquad 1.6$$

where R' is the TCA-insoluble radiotracer released from the yolk sac

during its incubation period (µl/mg protein).

In practice it is impossible to determine R and R' accurately and hence calculate the true rates of uptake. The values for the observed uptake by the tissue, obtained from equation 1.1 and 1.2, will necessarily be lower than those obtained by the above equations 1.5 and 1.6, which gives the true rates of uptake. Nevertheless, for most substrates the values of R and R' will be negligible, hence the true and the observed values of the Endocytic Index will be indistinguishable. But, when a high rate of exocytosis is found, the values calculated from the two sets of equations will differ significantly, so that equations 1.1 and 1.2 will only give net rates of uptake.

1.12 Exocytosis of radiotracer by the rat and rabbit yolk sac

In studying the release of radiolabelled macromolecules by the yolk sac, the original method of calculating release (Williams et al., 1975a) was modified as described by Ibbotson & Williams (1979) to include digestible substrates. The methods used for rat and rabbit yolk sacs were virtually identical, but any differences are mentioned below.

Three rat yolk sacs of 17.5 days gestational age or three pieces of rabbit yolk sac, removed at 24 days of gestation, were incubated in a sterile 50 ml Erlenmeyer flask by the method described in Section 1.7. Radiotracers (^{125}I-labelled PVP, ^{125}I-labelled BSA or ^{125}I-labelled IgG) were added at a concentration of 10 µg/ml, each flask was gassed for 10 sec. with an O_2/CO_2 mixture (19:1) and incubated with the tissue for 3h in the case of ^{125}I-labelled PVP and 2h in the case of proteins. At the end of the incubation period, the tissue was washed in 3 changes of warmed, gassed medium and then individually reincubated in a 50 ml Erlenmeyer flask containing 10 ml of fresh, gassed medium. Two aliquots (1.0 ml) were taken every 15 min up to 3h and placed in 3 ml disposable

plastic tubes. The medium removed was replaced by an equal volume of fresh medium. Flasks were re-gassed at each sampling interval. At the end of the re-incubation period yolk sacs were removed, washed in 3 changes of ice-cold saline and placed in 5 ml volumetric flasks and stored at $-20^{o}C$ until assayed by the procedures outlined in Sections 1.2 - 1.4. The medium samples were first assayed for their contained total radioactivity and then the amount of digested ^{125}I-labelled protein (TCA-soluble activity) was also determined by adding 0.1 ml of calf serum to each tube (to act as carrier protein) and precipitating the protein with 0.5 ml of TCA. After centrifuging the tubes (2000g, 20 min) the supernatants were each decanted into fresh tubes and their contained radioactivity determined on the gamma spectrometer. The observed counts were corrected for counting geometry by multiplying by a factor of 1.33 (see Section 1.7). For non-digestible substrates, the amounts of substrate released during the re-incubation period was calculated using the following equation:

$$T_n = 10\, C_{i\ (i=n)} + 2 \sum_{i=0}^{i=(n-1)} C_i \qquad 1.7$$

where T_n is the amount of total radioactivity (c.p.m., corr. for background) released up to the time of the n^{th} sampling, and C_i the content of total radioactivity (c.p.m./ml medium, corr. for background) in the i^{th} sample of medium. T_n, the radioactivity released, was expressed in 3 ways :

1) as the percentage of the radioactivity initially associated with the tissue,

2) as the amount (ng) of substrate released back into the medium from unit quantity of yolk-sac tissue. This value, S, is defined by the equation:

$$S = \frac{T_n}{T \times P} \qquad 1.8$$

where T is the activity associated with 1 µg substrate when assayed under standard counting geometry (c.p.m., corr. for background) and P is the protein content of the yolk sac;

3) as a percentage of the total radioactivity released at 180 min.

When a protein is used as substrate, at least two radioactive species are released: the digested and the undigested forms of the substrate. The amount released of these two species can be determined separately by applying the above calculations to each of the two sets of data.

1.13 General treatment of uptake data

Uptake data were calculated using programmes, initially written for an ICL 4120 computer and then modified to be run on an ICL 4082 computer (see Appendix for listing of programme). Plots of uptake (µl substrate/mg yolk-sac protein) against time were always found to be linear. This enabled them to be analysed by Multiple Regression Analysis (using a subroutine provided by the Computer Centre, Keele). This subroutine also permitted the calculation of correlation coefficients. These were used as a crude index of the degree of linearity of uptake plots before the plots were subjected to visual inspection to ensure that there was no under-lying curvature in the plots. For incubations in medium 199 containing 10% (v/v) calf serum, only those results were accepted that had a correlation coefficient higher than 0.95, for rat yolk sacs and 0.90 for rabbit yolk sacs. In the absence of calf serum these values were lowered to 0.90 and 0.85, respectively, since the degree of scatter appeared to be significantly greater in the absence of calf serum (as reported by Ibbotson & Williams, 1979).

2.1 INTRODUCTION

The yolk sacs of the rabbit and the rat are probably very specialized organs, and an outline of their possible physiological functions was given earlier (see General Introduction). The studies in this chapter are specifically concerned with the roles of these tissues in the transfer of passive immunity from mother to young.

For some time now it has been proposed that homologous IgG is transported intact across the yolk-sac membrane of the rabbit (Brambell et al., 1949, 1951; Brambell, 1954), but the precise mechanism of this process has not yet been firmly established. Since, in the yolk sac of the rat, proteins other than immunoglobulins are known to be degraded by lysosomal enzymes to the level of low molecular weight peptides and amino acids (Williams et al., 1971; Williams et al., 1975b; Livesey, Ph.D. Thesis, 1979; Moore et al., 1977), in any mechanism that is advanced it is necessary to explain why homologous IgG is in some way exceptional in being protected against the proteolytic action of lysosomal enzymes.

Published studies relating to the general fate of IgG in the rabbit yolk sac fall into 3 broad categories: in vivo studies, ultrastructural (electron microscopy) studies and in vitro binding studies. In vivo studies have prominence chronologically. Brambell & Mills (1947) first found evidence that in the rabbit the yolk-sac splanchnopleur was involved in antibody transfer. Brambell (1954, 1966 and 1970) expanded these findings by in vivo experiments involving ligaturing parts of the circulation and injection of antibody into selected maternal or foetal compartments; subsequent titrations of antibody in the foetuses gave information about the route of transfer of the antibodies. In other species too, in vivo investigations have established sites of transfer, thus cows transport passive immunity to newborn calves via

the colostrum (Balfour & Comline, 1962; Stanley et al., 1972; Brandon, 1976); the same route is used between sow and piglet (Payne & Marsh, 1962; Porter, 1969; Clarke & Hardy, 1971; Burton & Smith, 1977). Other species in which transfer has been investigated in vivo are: the horse (Jeffcott, 1972), sheep (Halliday, 1976), man (Gitlin et al., 1964), rhesus monkey (Bangham, 1960), guinea pig (Hartley, 1951; Dancis & Shafran, 1958; Leisring & Anderson, 1961), grey squirrel (Wild, 1971) and rat (Brambell & Halliday, 1956). Such studies have been excellently reviewed by Brambell (1970). Apart from initially stimulating research, in vivo studies also provide the ultimate test system for any theory of transfer, because only in vivo is the tissue undisturbed and in its natural condition. However, limitations of in vivo studies are also obvious. After injecting antibodies into a whole living animal it is very difficult to define the actual site of transfer, also the immunoglobulin molecules can be captured and modified by a variety of tissues other than the yolk sac. An attempt to overcome this problem has been made by ligaturing certain blood vessels (Brambell, 1954, 1966; Hemmings, 1973) but still the system is too complex to make any definite statement about the exact site of transfer of maternal antibodies. It is even more difficult for the same reason, to investigate the transfer mechanism at the cellular level. This has been tried slightly more successfully by combining in vivo methods with electron-microscopy. The fate of IgG can be followed by conjugating the immunoglobulin molecule to either a fluorescent or an electron-dense marker molecule (Wild, 1970; Slade, 1970; Hemmings, 1974; Wild et al., 1972; Slade & Wild, 1971). The conjugate is then injected into animals and after a time interval animals are sacrificed and the relevant tissue prepared for electron microscopy or autoradiography. By the use of this type of technique the presence of IgG conjugates in heterolysosomes and also in coated

micropinocytic vesicles has been demonstrated (Rodewald, 1973; Moxon et al., 1976). Some problems, however, arise from the use of conjugates. Principally the behaviour of the antibody molecule may be changed either by conjugating it to another molecule or by its exposure to the reagents used in the conjugation procedure. This may well alter the endocytic behaviour of the IgG molecule especially as a number of commonly used conjugating species are taken up as readily on their own as they are when conjugated to IgG molecules, for example, horseradish peroxidase and ferritin in the guinea-pig chorioallantoic placenta (King & Enders, 1970, 1971) and in the guinea pig parietal yolk sac (King & Enders, 1972) and ferritin in the rabbit yolk-sac splanchnopleur (Slade, 1970). Also, it is possible that, in such studies, the fate of the conjugated species is followed after the IgG, to which it was originally conjugated, has either been degraded or severely modified so that a false impression of the fate of IgG is obtained by following such conjugated species. Likewise, if the unconjugated species is itself transferred intact across membranes, as has been reported for ferritin and horseradish peroxidase in the suckling rat gut (Orlic & Lev, 1973; Hemmings & Williams, 1977), the method will give false impressions of the fate of IgG. Such problems can, to a certain extent, be overcome by the use of appropriate controls. This still leaves as the main limitation of electron microscopy in the context of these studies, the fact that it is essentially a static method whereas the cellular processes associated with IgG transport are by nature highly dynamic, so that even the application of morphometric methods to electron micrographs can give no information about rates of transfer.

In order to investigate certain facets of the cellular mechanisms of IgG transfer, especially the initial stage of binding of the substrate to the yolk-sac endodermal membrane and the presence of specific

receptors, *in vitro* binding studies offer certain advantages (Schlamowitz, 1976; Okafor *et al.*, 1974; Wild & Dawson, 1977; Linden & Roth, 1978). These studies include binding of IgG and its fragments to isolated and fixed membranes (Sonoda & Schlamowitz, 1972; Tsay & Schlamowitz, 1975; Schlamowitz, 1976), binding of substrate to vesicles consisting entirely of endodermal membranes (Schlamowitz *et al.*, 1975; Hemmings, 1975*a*,*b*; Schlamowitz, 1979), solubilization of receptors and investigating the extent of binding by precipitating a substrate-receptor complex (Schlamowitz, 1979) and binding of IgG to formalin-fixed yolk-sac discs (Hillman *et al.*, 1977). A related method is the rosetting antibody technique, which has been used to determine the presence of specific receptors. For example Elson *et al.* (1975) used this method to find Fc receptors on mouse placental and yolk sac cells and Wild & Dawson (1977) modified it to investigate the same receptor on rabbit yolk-sac membranes. Although these techniques are good for demonstrating the presence of specific receptors, they suffer from the same major limitation as electron microscopy studies involving the use of conjugates, namely they still give only a static picture of a highly dynamic process so can provide no information about the fluxes of receptors, membrane and substrate *in vivo*. Information is only given about the presence, specificity and distribution of receptors, but even here some doubts may be cast on the validity of some studies. In the isolation of receptors or membrane fractions, rather severe conditions and chemical agents are sometimes used (e.g. Schlamowitz, 1979, used formaldehyde-treated tissues), therefore the questions whether the receptors on such tissue fragments are still intact and whether their behaviour *in vitro* is representative of that of the tissue *in vivo* become important issues.

All these considerations make desirable a model system which involves living tissue in a viable form, but which avoids the complexity of the *in vivo* situation. Such a system would make it possible to study the

different fluxes associated with the transfer process. In addition, if the various fates of the molecules can be followed, it becomes possible to distinguish quantitatively between degradation and transport of antibodies (and other molecules). Even in the study of substrate : membrane binding, the use of living tissue affords the advantage of preserving the integrity of receptors.

An *in vitro* tissue incubation system was developed for the rat yolk sac by Williams *et al.* (1975a). Using this technique, Williams & Ibbotson (1979) found that the fate of homologous ^{125}I-labelled IgG in rat yolk sacs differed from that of all other proteins studied in the system (Williams *et al.*, 1975b; Moore *et al.*, 1977). They produced evidence that not all of the ^{125}I-labelled rat IgG that is endocytosed is digested but that some is released again from the rat yolk sac in a macromolecular form. In contrast, all other proteins tested in this system so far : bovine serum albumin, ribonuclease, calcitonin, insulin, orosomucoid and lactate dehydrogenase; (Moore *et al.*, 1977; Ibbotson & Williams, 1979; Livesey & Williams, 1981; Kooistra & Williams, 1981; Livesey, Ph.D. Thesis) are not released again from the tissue after pinocytic capture but are retained within the tissue until they undergo complete degradation within the lysosomal system. These observed differences in the fates of proteins are compatible with the postulated *in vivo* role of the rat visceral yolk sac in transfer of passive immunity, and hence the *in vitro* incubation system developed for the rat visceral yolk sac lends itself to adaptation for the further study of the mechanism of transfer of passive immunity across cells. But, although the visceral yolk sac is probably one of the sites where IgG transfer occurs prenatally in the rat, immunoglobulin transfer in this species occurs mainly postnatally via the neonatal gut (Halliday, 1959, 1955; Rodewald, 1970; Jones, 1972; Walker *et al.*, 1976). In the rabbit, on the other hand, all transfer of passive immunity occurs prenatally and the yolk-sac

splanchnopleur is the major, if not the only, site of transfer. For this reason, the rabbit yolk sac should be a better model system to work with, if a suitable technique for its in vitro incubation can be developed. Therefore, one of the main questions raised in the studies reported in this chapter was whether the same method as used for incubating rat yolk sacs can also be used for rabbit yolk sacs.

Whether or not an incubation method is successful can be judged by one or more of the following criteria:

1) Examining the ultrastructure of the tissue should determine whether integrity is retained in vitro. This can be evaluated by comparing electron micrographs of freshly excised tissues with those from tissues that have been subjected to the incubation procedure for differing periods of time (Williams et al., 1975a). However, a limitation of such studies is that only structural and not functional changes can be observed.

2) Biochemical measurement can give information on the rate of leakage of a cytosol enzyme (e.g. lactate dehydrogenase) or of a lysosomal enzyme (e.g. acid phosphatase) from the tissue (Livesey et al., 1980). In a healthy intact tissue, leakage should occur at only low rates and an increasing rate of leakage with increasing incubation time in vitro would suggest progressive deterioration of the tissue.

3) In addition, for an endocytic tissue like the yolk sac, information about the functional integrity of the tissue can be gained by looking at the pattern of uptake of a non-digestible compound (e.g. ^{125}I-labelled PVP or colloidal [^{198}Au] gold). The finding that the uptake of such molecules is linear as well as reproducible would be compatible with the survival and continued function of an endocytic tissue in vitro. Conversely, a levelling off of uptake with time or a lack of reproducibility in the uptake of a non digestible marker would suggest an impaired pinocytic behaviour of the tissue.

Williams et al. (1975a) used reproducibility and linearity of the uptake of ^{125}I-labelled PVP as criteria to test the structural and functional integrity of the rat yolk sac in vitro. In that system, linearity of uptake of the radioactive marker was demonstrated for at least 18h in rat yolk sac incubations in medium containing 10% (v/v) calf serum. Should initial investigations also establish linearity and reproducibility of uptake of ^{125}I-labelled PVP by the rabbit yolk sac too, then such incubations could also serve as a useful routine criterion of success of incubations in subsequent experiments.

Similarly, as well as investigating the endocytic capacity, the proteolytic capacity of the tissue can be checked by measuring the rates of uptake and of degradation of a representative non-transferred protein like ^{125}I-labelled BSA or formaldehyde treated ^{125}I-labelled BSA.

4) Another measure for the integrity of the tissue is the rate of leakage of a non-digestible compound (e.g. ^{125}I-PVP), accumulated in the vacuolar system. In studies of this type yolk sacs are allowed to take up a substrate and are then thoroughly washed to remove surface-associated activity before re-incubation in fresh substrate-free medium. ^{125}I-labelled PVP with a molecular weight of 30 000 - 40 000 should remain within the lysosomal system if the yolk-sac tissue remains intact in vitro.

Using ^{125}I-labelled albumin instead of ^{125}I-PVP in such reincubation studies should add further information about the proteolytic capacity of the yolk sac. Homologous IgG, unlike albumin has been reported to be transported across the rabbit yolk sac intact (Brambell, 1954) and therefore an investigation of the release of this substrate from the rabbit yolk sac in vitro would be of greatest interest. The most important question is whether in in vitro uptake and release experiments

with the rabbit yolk sac, homologous ^{125}I-labelled IgG is treated atypically compared with other proteins, as is found to be the case in the rat system (Williams & Ibbotson, 1979).

Initially, *in vitro* incubation conditions always included 10% (v/v) calf serum in the medium, as in the original method of Williams *et al.* (1975a). However, if the presence of calf serum is not essential to tissue survival, its presence is a hindrance rather than a help when investigating in detail the pinocytic uptake of proteins (Ibbotson & Williams, 1979). For example, when investigating the uptake of ^{125}I-labelled IgG in the *in vitro* system, the unlabelled immunoglobulins present in the calf serum are likely to compete with the labelled substrate for possible binding sites and thus complicate the interpretation of data. Since Ibbotson & Williams, (1979) showed that the absence of calf serum did not abolish endocytic behaviour in the rat yolk sac, it was likewise important to establish whether the same held for the rabbit yolk sac. Accordingly, all uptake studies were conducted both in the presence and in the absence of calf serum.

In summary, the experiments reported in this chapter aimed to answer the following questions:

1) Can a viable *in vitro* incubation system be established for rabbit yolk sacs, comparable to that already established for rat yolk sacs? (This is tested by investigating the uptake of ^{125}I-labelled PVP.)

2) What is the fate of a representative, non-transportable protein (formaldehyde-treated ^{125}I-labelled BSA) after uptake by the rabbit yolk sac?

3) Is homologous ^{125}I-labelled IgG treated in the rabbit yolk sac in a manner different to that of other proteins, as is the case in the rat yolk sac?

4) Does the pattern of release of ^{125}I-labelled BSA differ from that of ^{125}I-labelled rabbit IgG, in rabbit yolk sacs? (In particular, do the quantities of acid-insoluble radioactivity released during reincubation experiments differ markedly?)

5) Is it possible to successfully incubate rabbit yolk sacs in serum-free medium as well as in serum-containing media?

CHAPTER TWO

Endocytic Behaviour of Rat and Rabbit Yolk Sacs Incubated In Vitro

2.2 METHODS

2.2.1 General comments

All the methods used are described in the general section on Materials and Methods (Chapter 1). However, some slight differences in the methods of incubating rat and rabbit yolk sacs are mentioned here. As the rabbit yolk sac is much larger than that of the rat, considerably more medium is needed for the dissection if the yolk sacs are to be completely immersed in medium at all stages. It is also an advantage to wash the yolk sacs thoroughly in medium before incubation, as very often large amounts of brownish, amorphous material are associated with the outer face of the yolk-sac membrane. The paraplacental chorion has to be cut away carefully, and it helps to do this under good illumination against a dark surface, as this tissue is more translucent than the yolk sac. Rabbit yolk sacs were always divided into 4-5 pieces of approximately equal size, each with a similar protein content to that of a typical whole rat yolk sac (i.e. approximately 5-7 mg equivalent of BSA).

2.2.2 Uptake of ^{125}I-labelled PVP in the presence and absence of calf serum in the rat and rabbit yolk sac

^{125}I-Labelled PVP was used as substrate at a concentration of 1-2μg/ml, and 17.5-day rat yolk sacs or 24-day rabbit yolk-sac pieces were incubated as described in Sections 1.2, 1.7 and 1.8. Tissue incubations were terminated at regular intervals up to 6.5h in the case of rat yolk sacs and up to 5.5h in the case of rabbit yolk sacs. Assays of the amount of radioactivity contained in the medium and in the yolk sacs were performed as previously described (see Sections 1.3 to 1.5 for details). Uptake of substrate was plotted against time for each set of data and the Endocytic Index derived (see Section 1.9

for details of calculations).

2.2.3 Uptake of ^{125}I-labelled BSA in the presence and absence of calf serum in the rat and rabbit yolk sac

17.5-Day rat yolk sacs or 24-day rabbit yolk-sac pieces were incubated in medium 199 alone or medium 199 plus 10% (v/v) calf serum, with ^{125}I-labelled BSA as substrate at a concentration of 2 µg/ml. Two different batches of ^{125}I-labelled BSA were used, one of which was treated with 10% (w/v) formaldehyde for 24h, instead of the usual 60-72h (Moore et al., 1977). Maximum times of incubation were 6.5h and 5.5h for rat and rabbit yolk sacs, respectively. The amount of radioactivity contained in the incubation medium, the amount of radioactivity associated with the yolk sac and the protein content of the yolk sac were each determined as previously described (see Sections 1.3, 1.4 and 1.6). Protein uptake was plotted against time and the Endocytic Index and Tissue-Accumulation Rate calculated (see Section 1.10 for details).

2.2.4 Uptake of homologous ^{125}I-labelled IgG in the presence and absence of calf serum in rat and rabbit yolk sacs

Homologous ^{125}I-labelled IgG was used as substrate following the same regime as above (2.2.3).

2.2.5 Exocytosis of ^{125}I-labelled BSA by rat and rabbit yolk sacs reincubated in serum free medium 199

The method is described in full in Section 1.12. Following a 2h incubation period in medium 199 containing ^{125}I-labelled BSA at a concentration of 10 µg/ml, 17.5-day rat yolk sacs or 24-day rabbit yolk-sac pieces were rinsed in warm, substrate-free medium 199 and then reincubated in fresh medium 199 (10.0 ml). Samples of medium were removed at 15 min intervals up to 3h and replaced by an equal

volume of fresh substrate-free medium. The total amount of radioactivity released and the TCA-soluble radioactivity released were determined. From these data, the amount of substrate released was calculated (see Section 1.12 for details of calculation).

2.2.6 Exocytosis of homologous ^{125}I-labelled IgG from rat and rabbit yolk sacs reincubated in serum-free medium 199.

Homologous ^{125}I-labelled IgG was used as substrate and the above method was followed (2.2.5). Some incubations were performed in the presence of calf serum.

2.3 RESULTS

2.3.1 Endocytic Indices of ^{125}I-labelled PVP for rat and rabbit yolk sacs incubated in the presence and in the absence of calf serum

As can be seen from Tables 2.1 and 2.2 and Figures 2.1 and 2.2, uptake of ^{125}I-labelled PVP is highly linear and reproducible in both the rat and the rabbit yolk sac. For rat yolk sacs incubated in medium 199 containing 10% (v/v) calf serum, the E.I. values are virtually the same as those previously reported (Williams et al., 1975a; Roberts et al., 1977) and the values of the E.I. for rat yolk sacs incubated in medium 199 alone are the same as reported by Ibbotson & Williams (1979). Furthermore, in the presence of 10% (v/v) calf serum, E.I. values for rat and rabbit yolk sacs are of similar magnitude. Incubating the yolk sacs in serum-free medium 199, however, makes the E.I. in the rat yolk sac increase 1.7-fold, as previously observed (Ibbotson & Williams, 1979), but in the rabbit yolk sac the increase is significantly greater (7-fold). It appears that in the first 3 incubations reported in Table 2.2, which also come first chronologically, the correlation coefficients as well as E.I. values are noticeably lower. This can be accounted for in terms of an initial failure either to dissect the yolk sacs out cleanly or quickly enough. Contamination of yolk sacs with paraplacental chorion and amnion will tend to lower the E.I., as can be seen from Table 2.2.c. The data in this table show that for incubations of amnion and paraplacental chorion uptake with time is very low and also not very linear. Similarly, prolonged dissection times will expose the tissue to stagnant medium and may cause the oxygen tension to fall to a level which leads to irreversible damage of the tissue. The adverse effects of prolonged dissection times have been observed in the dissection of rat yolk sacs (Williams, unpublished data).

2.3.2 Endocytic Indices and Tissue-Accumulation Rates of ^{125}I-labelled BSA and ^{125}I-labelled IgG ingested in medium 199 in the presence and in the absence of calf serum

Since the two batches of ^{125}I-labelled BSA used showed virtually the same endocytic behaviour in the rabbit tissue (Tables 2.4.b and 2.4.c) results gathered using these two batches of protein will be discussed together. The E.I. values of these preparations in the rat yolk-sac system are within the range of those previously quoted in the literature (Moore et al., 1977; Ibbotson & Williams, 1979). The E.I. values for ^{125}I-labelled albumin in the rat and rabbit yolk sacs are, however, very different, being much higher in the rat (Table 2.3) than in the rabbit (Table 2.4). Looking at the equivalent data obtained from rat and rabbit yolk sacs incubated in serum-free medium containing formaldehyde-treated ^{125}I-labelled BSA (Tables 2.3 and 2.4), a large (3-5 fold) rise in the E.I. can be observed for both tissues. Tissue Accumulation Rates were higher in the rat yolk sac and slightly lower in the rabbit yolk sac, compared with corresponding incubations in serum-containing medium. In the summary table (Table 2.7) tissue-accumulation is expressed as a ratio (T.A.R./E.I.) to give some information about the rate of accumulation of substrate relative to the rate of uptake. This table shows that in serum-free medium, tissue-accumulation is relatively lower than in serum-containing medium.

With homologous ^{125}I-labelled IgG as substrate, (Tables 2.5 and 2.6) the absence of calf serum makes the value of the E.I. increase in rat and rabbit tissues by 5-9 fold. The Tissue-Accumulation Rate (T.A.R.) increases to a comparable extent. The main difference between these two protein substrates lies in the values for the T.A.R. (which is defined as µl substrate accumulated within each mg yolk-sac tissue/h). Whereas the rate of tissue accumulation is relatively low for ^{125}I-

labelled BSA in all incubations, it is high for homologous ^{125}I-labelled IgG. However, for the homologous ^{125}I-labelled IgG there is a definite species difference, with tissue-accumulation accounting for a higher percentage of the total uptake in the rabbit compared with the rat. This is clearly seen on examining the data in the summary table (Table 2.7) which shows this effect by way of the ratio of T.A.R. to E.I. This ratio is generally higher for homologous ^{125}I-labelled IgG than for ^{125}I-labelled BSA. The exception is the rabbit yolk-sac incubation with ^{125}I-BSA in serum-containing medium 199; here the ratio T.A.R./E.I. is high, but this can be explained in terms of both T.A.R. and E.I. being very low in absolute terms.

2.3.3 Release patterns of ^{125}I-labelled PVP, formaldehyde-treated ^{125}I-labelled BSA and homologous ^{125}I-labelled IgG from rat and rabbit yolk sacs that had previously been incubated in the presence of these markers

In the rat and the rabbit yolk sac, ^{125}I-labelled PVP is hardly released from the tissue following incubation with this marker molecule. Expressed as a percentage, the extent of release (over the re-incubation period) of the total ^{125}I-labelled PVP-derived radioactivity associated with the yolk-sac tissue, was 7% for the rat yolk sac and 2.3% for the rabbit yolk sac (see Figures 2.8 and 2.9 for details).

The difference in the behaviour of ^{125}I-labelled albumin and ^{125}I-labelled homologous IgG, that was apparent from the different trends in the T.A.R.s in uptake experiments, becomes even more marked on examining the results of exocytosis experiments. In the rat tissue and with ^{125}I-labelled BSA as substrate, the majority (approx. 85%) of the radioactivity that becomes associated with the tissue is released as TCA-solubles (a finding that is compatible with this protein undergoing complete degradation in the lysosomal system after pinocytic capture).

In rabbit tissue and with ^{125}I-labelled BSA as substrate, the amount of the total radioactivity associated with the yolk-sac tissue that is released as TCA-solubles is slightly lower (approx. 65%). However, when looking at the total quantity of substrate-derived radioactivity that is released, release from the rabbit yolk sac (82.5 ng/mg tissue protein) amounts to only approximately 15% of that released from the rat yolk sac in equivalent experiments (522.5 ng/mg tissue protein) (Figures 2.3 and 2.4).

With ^{125}I-labelled homologous IgG as substrate, a far higher amount of the substrate is released from rat and rabbit yolk sacs in a TCA-insoluble from (Figures 2.5, 2.6 and 2.7). This value is higher for the rabbit yolk sac (58% released) than for the rat yolk sac (35% of the total released). A summary table, comparing the ratios of TCA-insoluble over total release is given for the two tissues and substrates used (see Table 2.8).

Table 2.1 Endocytic Indices of ^{125}I-labelled PVP ingested by 17.5-day rat yolk sacs incubated in medium 199 in either the presence or the absence of calf serum

Rat yolk sacs (17.5-day) were incubated for up to 6.5h with ^{125}I-labelled PVP (2 µg/ml of medium) either in medium 199 alone or in medium 199 containing 10% (v/v) calf serum, by using the method described in Section 1.5.

a) Experiments in medium 199 containing calf serum (10%, v/v)

No. of Yolk sacs	Endocytic Index (µl/mg protein per h)	Correlation Coefficient	Intercept (µl/mg protein)
12	1.55	0.966	1.36
12	1.96	0.998	-0.11
12	1.86	0.972	1.15
11	1.94	0.996	-0.27
8	1.38	0.970	1.85
10	1.86	0.982	-0.04
10	1.30	0.971	1.43
MEAN VALUES (± S.D.):	1.69 ± 0.28		0.84 ± 0.79

b) Experiments in serum-free medium 199

No. of Yolk sacs	Endocytic Index (µl/mg protein per h)	Correlation Coefficient	Intercept (µl/mg protein)
5	2.80	0.903	5.95
7	2.36	0.992	0.90
8	2.91	0.899	0.57
10	2.96	0.954	-0.67
MEAN VALUES (± S.D.):	2.76 ± 0.27		1.69 ± 2.92

Table 2.2 Endocytic Indices of ^{125}I-labelled PVP ingested by 24-day rabbit yolk-sac pieces incubated in medium 199 in either the presence or the absence of calf serum.

Rabbit yolk-sac pieces (24-day) were incubated for up to 5.5h in the presence of ^{125}I-labelled PVP (2 µg/ml of medium) either in medium 199 alone or in medium 199 containing 10% (v/v) calf serum by using the method described in Section 1.8.

a) Experiments in medium 199 containing calf serum (10%, v/v)

No. of Yolk-sac pieces	Endocytic Index (µl/mg protein per h)	Correlation Coefficient	Intercept (µl/mg protein)
9	0.82	0.810	1.63
10	0.93	0.958	1.16
10	0.72	0.819	2.57
10	1.91	0.956	-0.07
9	1.43	0.921	1.62
10	1.73	0.919	1.28
8	1.83	0.981	0.48
10	1.47	0.916	3.12
MEAN VALUES (± S.D.):	1.55 ± 0.36		1.27 ± 1.09

b) Experiments in serum-free medium 199

No. of Yolk-sac pieces	Endocytic Index (µl/mg protein per h)	Correlation Coefficient	Intercept (µl/mg protein)
9	13.18	0.971	-0.57
9	9.80	0.934	2.75
10	6.93	0.764	5.07
8	6.60	0.940	-1.19
9	13.24	0.871	-0.46
MEAN VALUES (± S.D.):	9.95 ± 3.22		1.12 ± 2.69

c) Experiments using amnion pieces in medium 199 containing calf serum (10%, v/v)

No. of amnion pieces	Endocytic Index (µl/mg protein per h)	Correlation Coefficient	Intercept (µl/mg protein)
9	0.38	0.31	8.17

Table 2.3 Endocytic Indices of formaldehyde-denatured ^{125}I-labelled BSA ingested by 17.5-day rat yolk sacs incubated in medium 199 in either the presence or the absence of calf serum (10%, v/v)

a) Experiments in medium 199 containing calf serum (10%, v/v)

Rat yolk sacs (17.5-day) were incubated with ^{125}I-labelled BSA (2 µg/ml of medium; Batch 1) as substrate. Samples were removed at intervals up to 6.5h. The values of both the Endocytic Index and the Tissue-Accumulation Rate were calculated (see Section 1.10).

TCA-solubles in the preparation	No. of yolk sacs	Endocytic Index (µl/mg protein per h)	Correlation Coefficient	Tissue-Accumulation Rate (µl/mg protein per h)	Correlation Coefficient
1.89	10	52.79	0.992	5.74	0.881
1.89	9	50.89	0.976	4.90	0.816
2.55	10	98.69	0.994	6.27	0.867
2.55	9	104.90	0.983	7.31	0.840
MEAN VALUES (± S.D.):		76.8 ± 29.0		6.1 ± 1.0	

b) Experiments in serum-free medium 199

The data quoted are taken from Ibbotson & Williams (1979, Figure 2) and represent the mean values (± S.D.) from 9 individual experiments with 4 batches of ^{125}I-labelled formaldehyde-denatured BSA. 17.5-Day rat yolk sacs were incubated in serum-free medium 199 with formaldehyde-denatured ^{125}I-labelled BSA (0.6 - 1.5 µg/ml medium) as substrate. Samples of medium were removed at intervals up to 5.5h.

Mean Endocytic Index (µl/mg protein per h)	Correlation Coefficient	Tissue-Accumulation Rate (µl/mg protein per h)
251 ± 84	0.940 - 0.987	approx. 12

In the following tables (Tables 2.4, 2.5 and 2.6) the full details of individual experiments are reported, but to conserve space the column headings have been abbreviated according to the scheme below:-

1	2	3
TCA-solubles in the ^{125}I-labelled protein preparation on the day of use (%)	No. of yolk-sac pieces used in the individual experiment	Endocytic Index (µl/mg of yolk-sac protein per h)

4	5	6
Correlation Coefficient of the plot uptake against time used to determine the Endocytic Index	Tissue-Accumulation Rate (µl/mg of yolk-sac protein per h)	Correlation Coefficient of the plot of tissue levels against time used to determine the Tissue-Accumulation Rate

The main findings reported in the above tables are summarized in Table 2.7 to permit ready comparison of data.

Table 2.4 Endocytic Indices of untreated and formaldehyde-treated ^{125}I-labelled albumin ingested by 24-day rabbit yolk-sac pieces incubated in the presence or absence of calf serum

a) Experiments in medium 199 containing calf serum (10%, v/v)

Rabbit yolk-sac pieces (24-day) were incubated with formaldehyde-treated ^{125}I-labelled albumin (2 µg/ml of medium; Batch 2) in medium 199 containing 10% (v/v) calf serum. Samples of medium were taken at intervals up to 5.5h and Endocytic Indices (column 3) and Tissue-Accumulation Rates (column 5) were calculated as outlined in Section 1.10.

1	2	3	4	5	6
1.37	7	3.62	0.711	1.51	0.888
1.37	8	2.51	0.894	1.29	0.909
MEAN VALUES:		3.07		1.40	

b) Experiments in serum-free medium

i) Rabbit yolk-sac pieces (24-day) were incubated with ^{125}I-labelled albumin (2 µg/ml of medium; Batch 3) as substrate in medium 199 alone, as described above (2.4.a)

1	2	3	4	5	6
1.26	8	9.32	0.778	0.44	0.437
1.26	8	5.95	0.784	0.06	0.054
3.61	9	7.31	0.875	1.05	0.807
3.61	9	7.77	0.885	0.07	0.058
MEAN VALUES (± S.D.):		7.59 ± 1.39		0.41 ± 0.46	

ii) Rabbit yolk-sac pieces (24-day) were incubated with formaldehyde-treated ^{125}I-labelled albumin (2 µg/ml of medium; Batch 2) as substrate in medium 199 alone, as described above (2.4.a)

1	2	3	4	5	6
0.81	9	11.12	0.856	0.11	0.092
1.22	9	9.99	0.869	0.76	0.426
1.22	9	12.99	0.943	1.83	0.711
1.22	9	10.73	0.955	0.89	0.712
MEAN VALUES (± S.D.):		11.21 ± 1.28		0.90 ± 0.71	

Table 2.5 Endocytic Indices and Tissue-Accumulation Rates of ^{125}I-labelled rat IgG ingested by 17.5-day rat yolk sacs incubated in medium 199 in the presence and absence of calf serum

a) Experiments in medium containing 10% (v/v) calf serum

Rat yolk sacs (17.5-day)were incubated with ^{125}I-labelled rat IgG (2 μg/ml of medium) in medium 199 containing 10% (v/v) calf serum. Samples of medium were removed at intervals up to 6.5h and Endocytic Indices (column 3) and Tissue-Accumulation Rates (column 5) were calculated as described in Section 1.10.

1	2	3	4	5	6
2.30	9	12.69	0.977	2.21	0.730
2.71	10	13.99	0.994	2.95	0.933
2.77	10	16.35	0.959	3.33	0.923
2.76	10	9.09	0.971	1.82	0.963
1.68	10	12.78	0.937	0.95	0.578
1.61	7	11.98	0.987	2.24	0.995
2.11	9	11.80	0.973	1.96	0.925
1.54	10	10.09	0.972	2.45	0.898
1.54	9	10.27	0.972	2.21	0.896
MEAN VALUES (± S.D):		12.12 ± 2.21		2.29 ± 0.68	

b) Experiments in serum-free medium

Rat yolk sacs (17.5-day) were incubated with ^{125}I-labelled rat IgG (2 μg/ml of medium) in serum-free medium 199. Samples were removed at intervals up to 5.5h and the Endocytic Indices (column 3) and Tissue-Accumulation Rates (column 5) were calculated as described in Section 1.10

1	2	3	4	5	6
2.32	10	76.15	0.910	3.35	0.322
2.11	7	76.00	0.910	11.49	0.776
2.11	8	80.76	0.939	14.68	0.954
4.76	8	66.63	0.956	11.51	0.761
3.68	9	84.74	0.953	12.79	0.875
3.07	10	52.01	0.938	8.04	0.874
4.41	8	53.91	0.901	11.24	0.784
3.67	9	98.77	0.950	14.05	0.909
3.15	9	45.21	0.893	6.84	0.676
2.30	10	43.45	0.933	6.81	0.708
MEAN VALUES (± S.D.):		66.86 ± 17.03		10.08 ± 3.66	

Table 2.6 Endocytic Indices and Tissue-Accumulation Rates of ^{125}I-labelled rabbit IgG ingested by 24-day rabbit yolk-sac pieces incubated in medium 199 in the presence and absence of calf serum

a) Experiments in medium 199 containing calf serum (10%, v/v)

Rabbit yolk-sac pieces (24-day) were incubated with ^{125}I-labelled rabbit IgG (2 µg/ml of medium) in medium 199 containing 10% (v/v) calf serum. Samples of medium were removed at intervals up to 5.5h and Endocytic Indices (column 3) and Tissue-Accumulation Rates (column 5) were calculated as described in Section 1.10.

1	2	3	4	5	6
2.16	8	2.15	0.809	1.68	0.806
2.09	7	3.57	0.745	1.48	0.854
2.70	10	4.05	0.933	1.51	0.693
2.52	9	4.92	0.478	2.42	0.441
MEAN VALUES (± S.D.):		3.67 ± 1.16		1.77 ± 0.44	

b) Experiments in serum-free medium

Rabbit yolk-sacpieces (24-day) were incubated with ^{125}I-labelled rabbit IgG (2 µg/ml of medium) in medium 199 alone. Samples were removed at intervals up to 5.5h and Endocytic Indices (column 3) and Tissue-Accumulation Rates (column 5) were calculated as described in Section 1.10.

1	2	3	4	5	6
2.60	9	35.79	0.947	22.60	0.851
2.72	8	34.97	0.852	13.01	0.717
2.34	9	20.90	0.916	10.27	0.706
3.29	9	38.19	0.943	17.22	0.859
3.29	9	31.83	0.823	14.89	0.638
3.15	10	37.98	0.847	15.89	0.741
3.15	9	44.42	0.725	13.72	0.415
MEAN VALUES (± S.D.):		34.87 ± 7.27		15.37 ± 4.25	

Table 2.7 Summary of Endocytic Indices and Tissue-Accumulation Rates of ^{125}I-labelled substrates ingested by rat and rabbit yolk-sac tissue

i) Rat yolk sacs:

Labelled Substrate	Calf Serum	Mean Endocytic Index ± S.D. (µl/mg protein per h)	Mean Tissue-Accumulation Rate ± S.D. (µl/mg protein)	$\frac{\text{T.A.R.}}{\text{E.I.}} \times 100$ (%)
PVP	+	1.69 ± 0.28	-	-
PVP	-	2.76 ± 0.27	-	-
fd-BSA (Batch 1)	+	76.82 ± 28.96	6.05 ± 1.01	7.88
fd-BSA (Batch Ibb4)	-	381.5	∿12	3
Rat IgG	+	12.12 ± 2.21	2.29 ± 0.68	18.89
Rat IgG	-	66.86 ± 17.03	10.08 ± 3.66	15.08

ii) Rabbit yolk-sac pieces:

Labelled Substrate	Calf Serum	Mean Endocytic Index ± S.D. (µl/mg protein per h)	Mean Tissue-Accumulation Rate ± S.D. (µl/mg protein)	$\frac{\text{T.A.R.}}{\text{E.I}} \times 100$ (%)
PVP	+	1.55 ± 0.36	-	-
PVP	-	9.95 ± 3.22	-	-
fd-BSA (Batch 2)	+	3.07	1.40	45.60
fd-BSA (Batch 2)	-	11.21 ± 1.28	0.90 ± 0.71	8.03
BSA (Batch 3)	-	7.59 ± 1.39	0.41 ± 0.46	5.27
Rabbit IgG	+	3.67 ± 1.16	1.77 ± 0.44	48.23
Rabbit IgG	-	34.87 ± 7.27	15.37 ± 4.25	44.08

^{125}I-labelled BSA (Batches 1, 2 and Ibb4) were formaldehyde-denatured and ^{125}I-labelled BSA (Batch 3) was untreated.

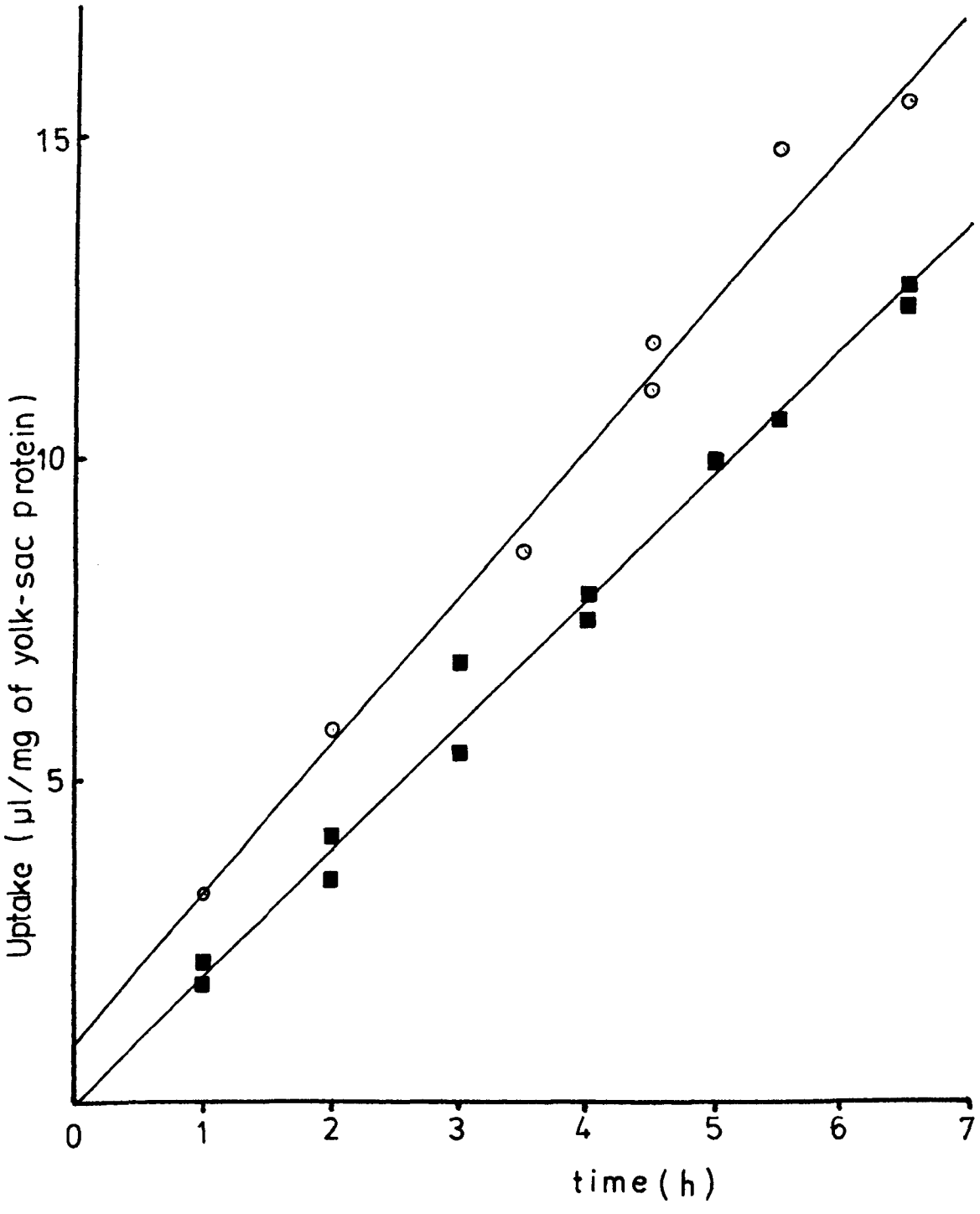


Figure 2.1 Representative uptake plots of ^{125}I-labelled PVP in rat yolk-sacs incubated in the presence and absence of calf serum

Uptake of data was plotted against time for 17.5-day rat yolk sacs incubated in serum-free medium 199 containing ^{125}I-labelled PVP (—o—) or incubated in medium 199 containing calf serum (10%, v/v) and ^{125}I-labelled PVP (—■—).

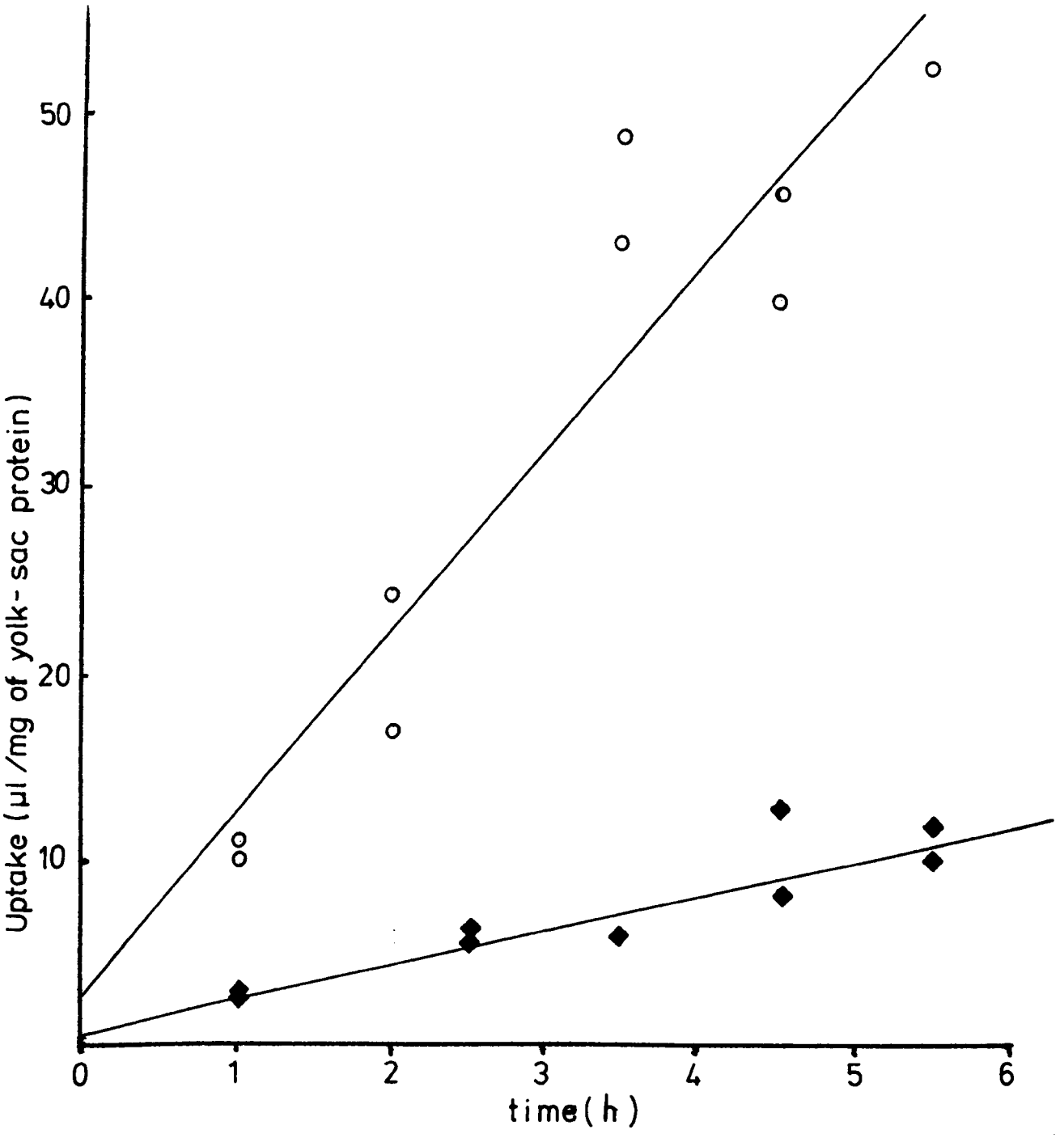


Figure 2.2 Representative uptake plots of ^{125}I-labelled PVP in rabbit yolk sacs incubated in the presence and absence of calf serum

Uptake of data was plotted against time for 24-day rabbit yolk sac tissue incubated in serum-free medium 199 containing ^{125}I-labelled PVP (—o—) or incubated in medium 199 containing calf serum (10%,v/v) and ^{125}I-labelled PVP (—◆—).

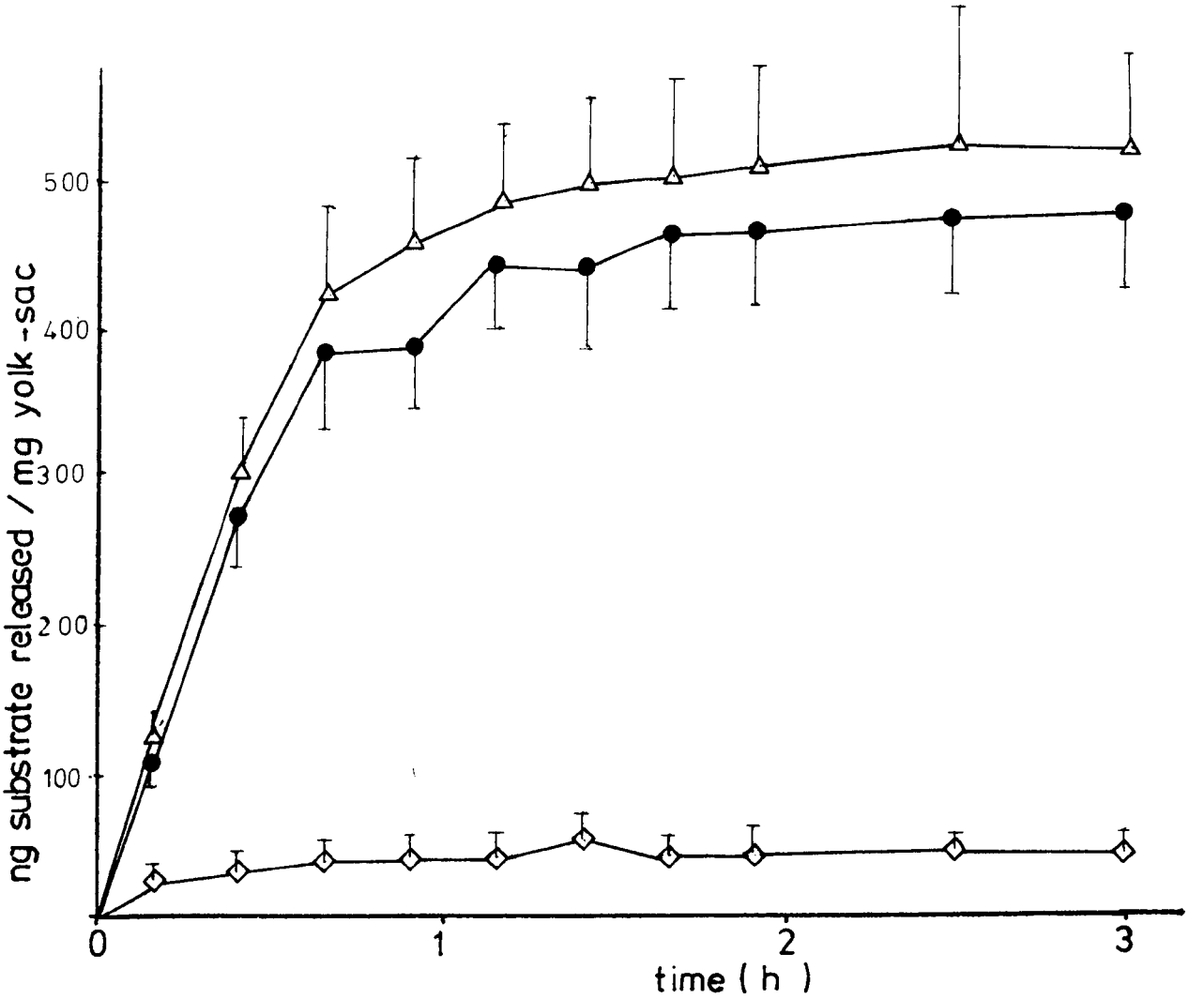


Figure 2.3 Time course of release of radioactive species derived from formaldehyde-treated ^{125}I-labelled BSA following incubation of rat yolk-sacs with substrate in serum-free medium 199

17.5-day rat yolk-sacs were first incubated for 2h in serum-free medium 199 in the presence of formaldehyde-treated ^{125}I-labelled BSA (10 µg/ml). Following washing they were reincubated in fresh medium and release of radioactivity was monitored up to 3h (see Section 1.12 for details). The total amount of substrate associated with the yolk-sac at the beginning of reincubation was 580 ng/mg yolk-sac tissue.
Total radioactivity released —Δ—;
TCA-soluble radioactivity released —●—;
TCA-insoluble radioactivity released —◇—.
The values shown are means (± S.D.) from 6 separate experiments performed with the same batch of formaldehyde-treated ^{125}I-labelled albumin used in the experiments reported in Table 2.3.a.

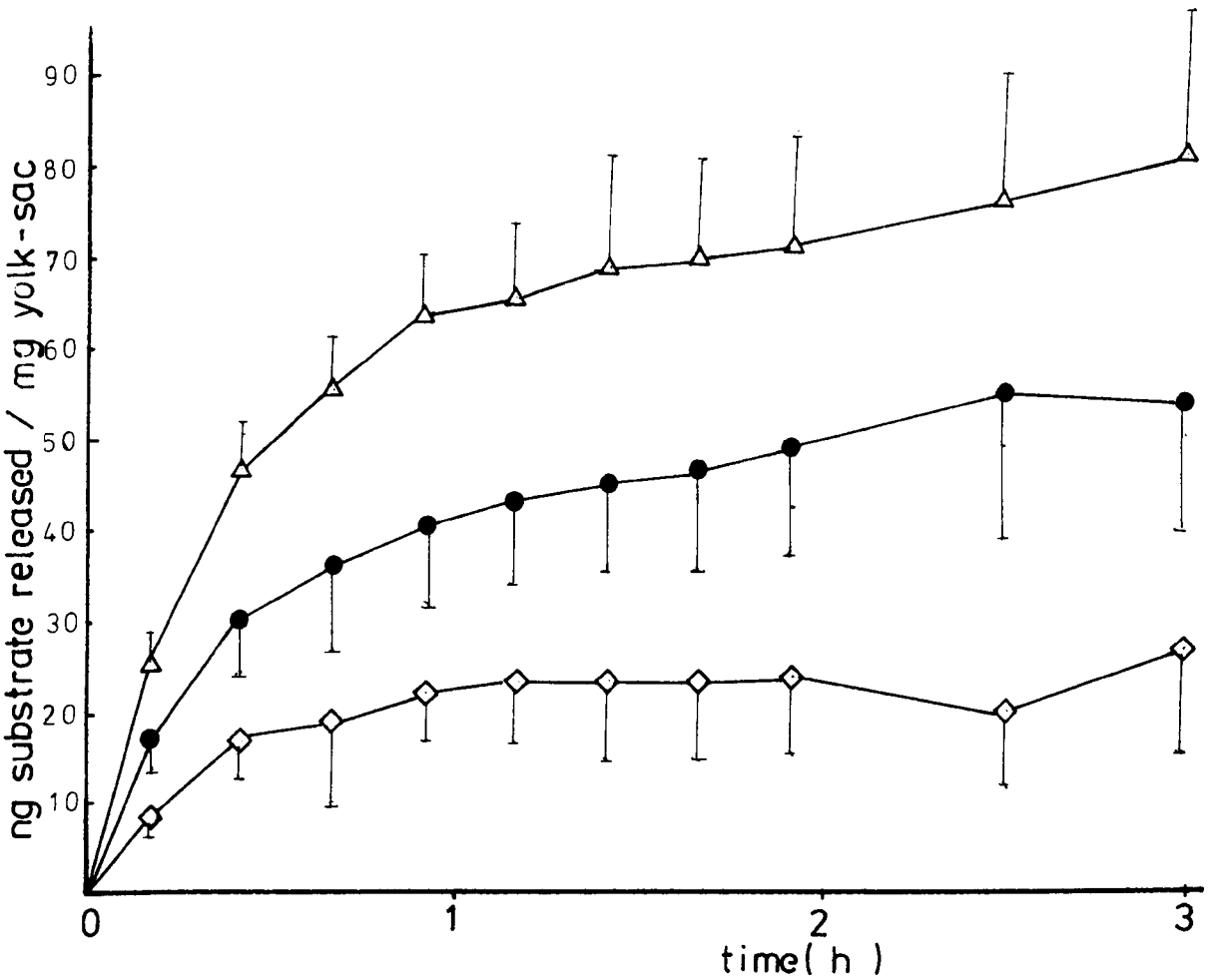


Figure 2.4 Time course of release of radioactive species derived from formaldehyde-treated ^{125}I-labelled BSA following incubation of rabbit yolk-sacs with substrate in serum-free medium 199

24-day rabbit yolk-sac pieces were first incubated for 2h in serum-free medium 199 in the presence of formaldehyde-treated ^{125}I-labelled BSA (10 µg/ml). Following washing they were reincubated in fresh medium, and release of radioactivity was monitored up to 3h (see Section 1.12 for details). The total amount of substrate associated with the yolk-sac at the beginning of reincubation was 102 ng/mg yolk-sac tissue.

Total radioactivity released —△—

TCA-soluble radioactivity released —●—

TCA-insoluble radioactivity released —◇—

The values shown are means (S.D.) from 5 separate experiments performed with the same batch of formaldehyde-treated ^{125}I-labelled BSA used in the experiments reported in Tables 2.4.a and 2.4.b.ii.

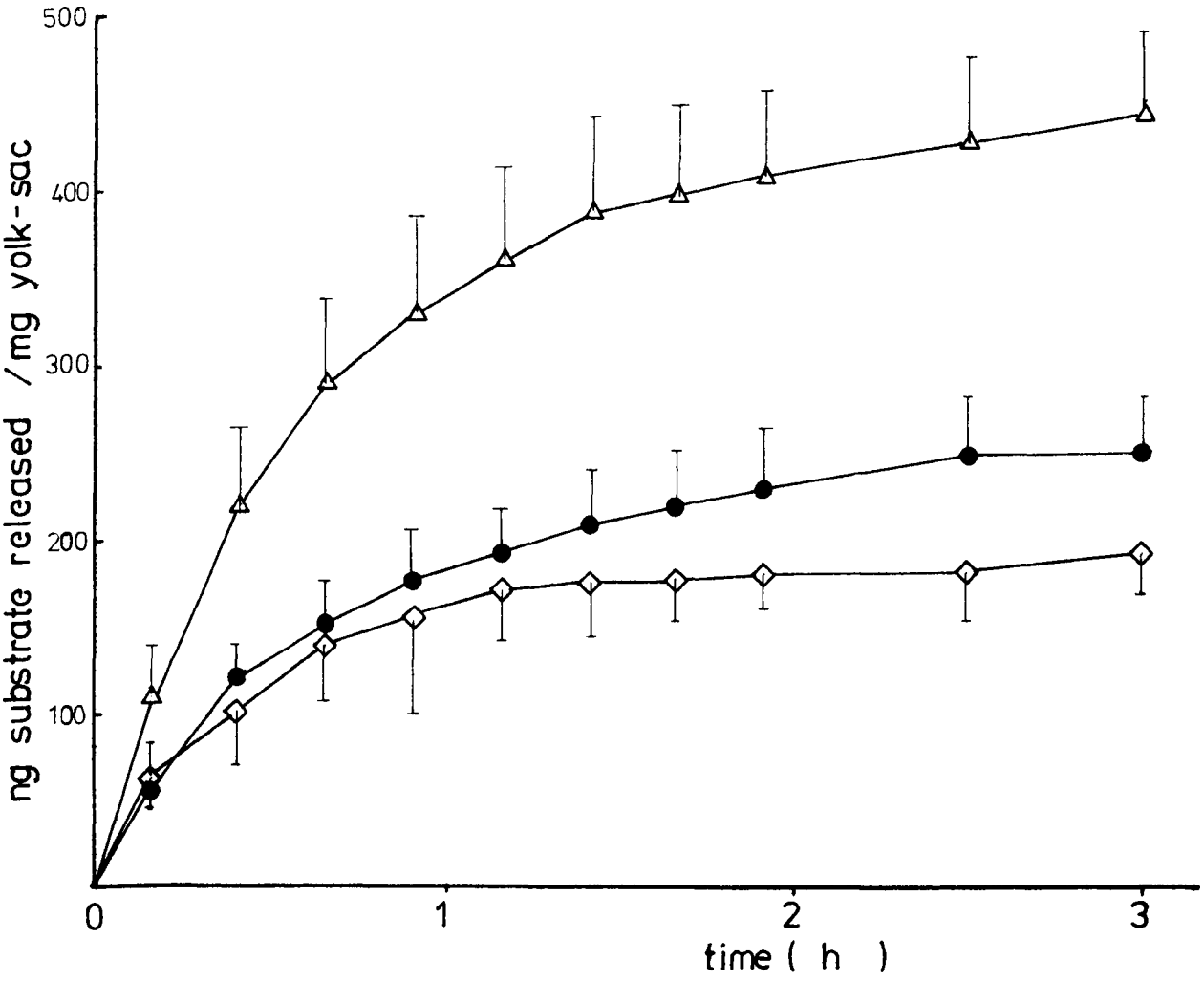


Figure 2.5 Time course of release of radioactive species derived from ^{125}I-labelled rat IgG following incubation of rat yolk-sacs with substrate in serum-free medium 199

17.5-day rat yolk-sacs were first incubated for 2h in serum-free medium 199 in the presence of ^{125}I-labelled rat IgG (10 µg/ml). Following washing they were reincubated in fresh medium, and release of radioactivity was monitored up to 3h (see Section 1.12 for details). The total amount associated with the yolk-sac at the beginning of reincubation was 528 ng/mg yolk-sac tissue.

Total radioactivity released —△—

TCA-soluble radioactivity released —●—

TCA-insoluble radioactivity released —◇—

The values shown are means (± S.D.) from 6 separate experiments.

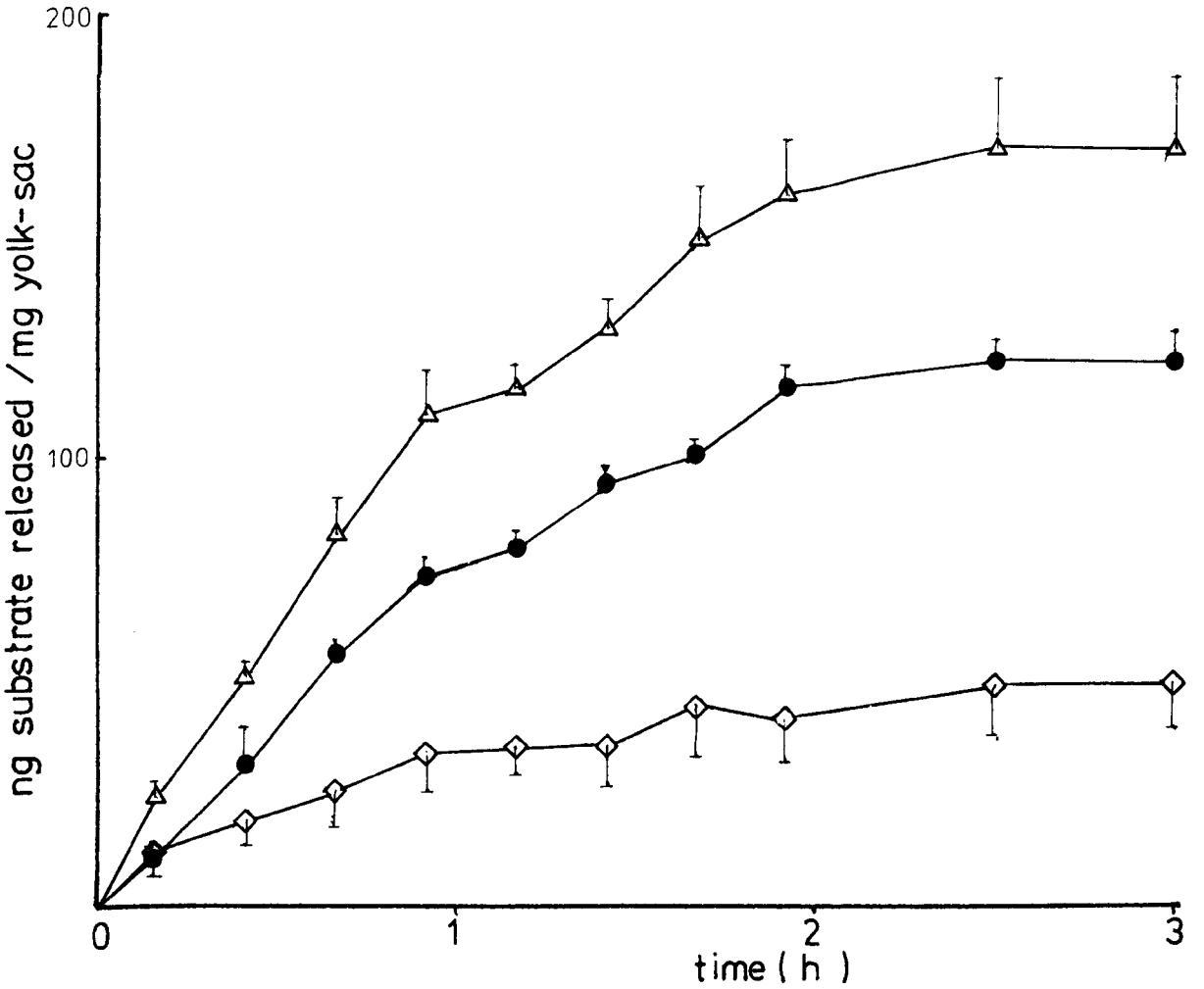


Figure 2.6 Time course of release of radioactive species derived from ^{125}I-labelled rat IgG following incubation of rat yolk-sacs with substrate in serum-containing medium 199

17.5-day rat yolk-sacs were first incubated for 2h in medium 199 containing 10% (v/v) calf serum in the presence of ^{125}I-labelled rat IgG (10 µg/ml). Following washing they were reincubated in fresh medium, and release of radioactivity was monitored up to 3h (see Section 1.12 for details). The total amount of substrate associated with the yolk-sac at the beginning of reincubation was 274 ng/mg yolk-sac tissue.

Total radioactivity released —Δ—

TCA-soluble radioactivity released —●—

TCA-insoluble radioactivity released —◇—

The values shown are means (S.D.) from 3 separate experiments

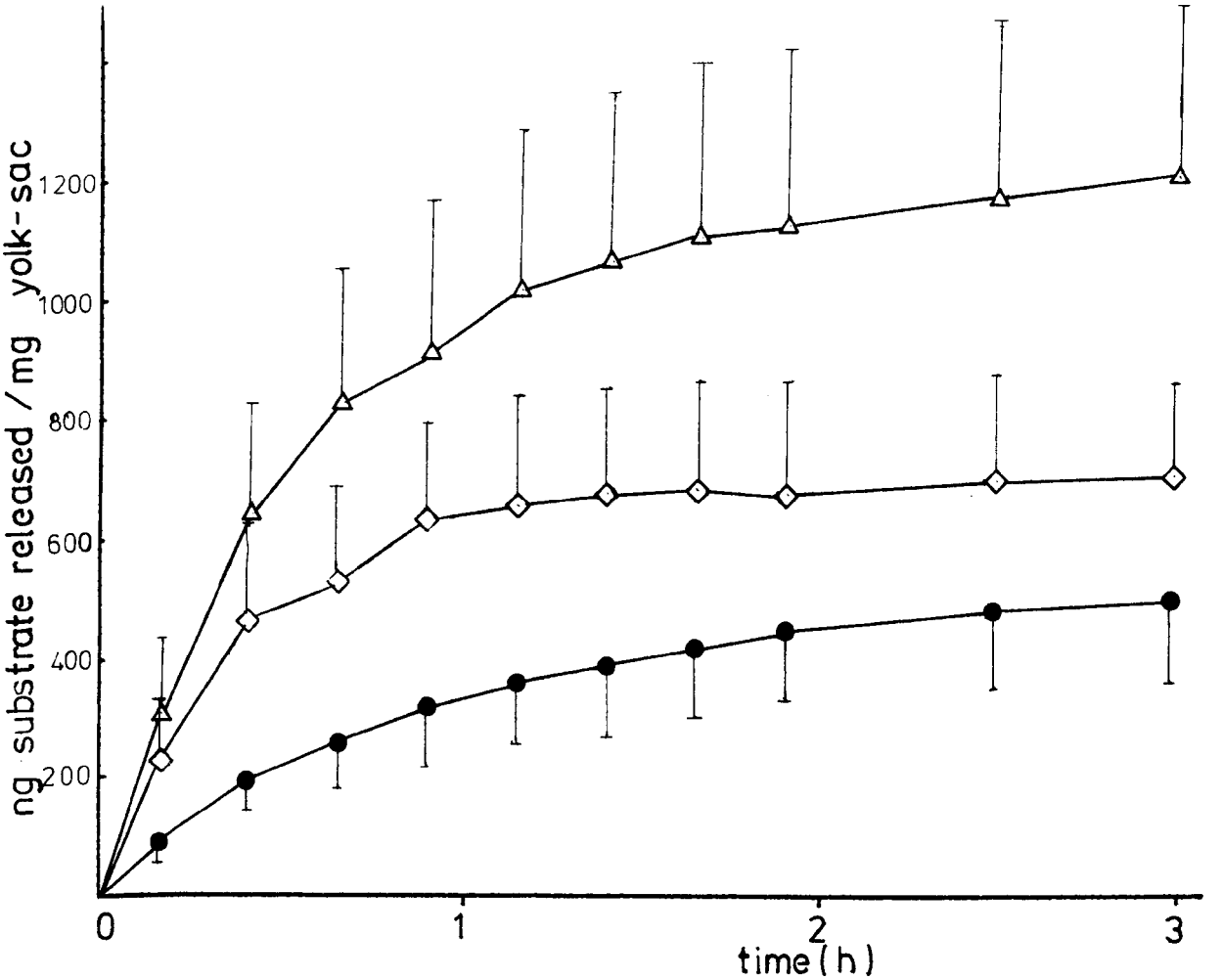


Figure 2.7 Time course of release of radioactive species derived from ^{125}I-labelled rabbit IgG following incubation of rabbit yolk-sacs with substrate in serum-free medium 199

24-day rabbit yolk sac pieces were first incubated for 2h in serum-free medium 199 in the presence of ^{125}I-labelled rabbit IgG (10 µg/ml). Following washing they were reincubated in fresh medium, and release of radioactivity was monitored up to 3h (see Section 1.12 for details). The total amount of substrate associated with the yolk-sac at the beginning of reincubation was 1510 ng/mg yolk-sac tissue.

Total radioactivity released —△—

TCA-soluble radioactivity released —●—

TCA-insoluble radioactivity released —◇—

The values shown are means (± S.D.) from 6 separate experiments.

Table 2.8 Summary of amounts of ^{125}I-labelled substrates released from rat and rabbit yolk-sac tissue during reincubation in fresh medium 199

In this table the findings displayed more fully in Figures 2.3 - 2.7 are summarized. The values quoted are the amounts of radioactivity released from rat and rabbit yolk-sac tissues after a 3h reincubation period. The last column gives a measure of the percentage of the total release of radioactivity that is acid-insoluble.

Labelled Substrate	Calf Serum	Radioactivity released (ng/mg yolk-sac protein)			$\frac{\text{TCA-insoluble}}{\text{Total}} \times 100$ (%)
		TCA-soluble	TCA-insoluble	Total	
i) Rat yolk sacs:					
BSA		472.8	48.2	522.5	9.23
Rat IgG	-	251.5	193.6	444.9	43.5
Rat IgG	+	123.9	50.9	174.1	29.3
ii) Rabbit yolk sacs:					
BSA		54.9	27.6	82.5	33.4
Rabbit IgG		504.1	712.4	1216.5	58.6

2.4 DISCUSSION

Uptake of ^{125}I-labelled PVP, which has been thoroughly investigated and quantitated in the rat yolk sac *in vitro* (Williams *et al.*, 1975a; Roberts *et al.*, 1977; Ibbotson & Williams 1979), was chosen to test the viability of rabbit yolk-sac tissue in the *in vitro* incubation system. When incubated with ^{125}I-labelled PVP under the same conditions as rat yolk sacs, rabbit yolk sac pieces continue to show endocytic behaviour over a period of at least several hours; this suggests that the rabbit yolk sacs remain viable in the *in vitro* system. In the presence of 10% (v/v) calf serum, uptake of this polymer by the rabbit yolk sac is highly linear and reproducible, and the rate of uptake is similar to that in the rat yolk sac. However, values of substrate uptake at 6.5h showed rather large standard deviations, therefore it was decided to follow the practice of Ibbotson & Williams (1979) and to incubate tissues for a maximum period of only 5.5h. ^{125}I-Labelled PVP has been shown to be captured exclusively via the fluid phase in the rat yolk-sac system (Roberts et al., 1977), and the observation that the Endocytic Indices of ^{125}I-labelled PVP ingested in the presence of calf serum are virtually identical in rat and rabbit yolk sacs lends support to the suggestion that fluid uptake proceeds at approximately the same rate in a variety of different tissues (see Pratten *et al.*, 1980 for further discussion). This is, however, challenged at once when the Endocytic Indices of ^{125}I-labelled PVP in serum-free medium 199 are compared for tissues from the two species. Whereas the rat yolk sac shows only a small increase (as compared with the value for equivalent incubations in the presence of calf serum), in the rabbit yolk sac the increase is considerably larger. Two explanations can be offered to account for this increase. First, ^{125}I-PVP may adsorb extensively to the rabbit yolk sac in the absence of any calf-serum proteins and thus

enter this tissue mainly by adsorptive pinocytosis in serum-free medium 199. Alternatively, the removal of serum proteins from the incubation medium may stimulate the rate of pinosome formation much more strongly in rabbit tissue than it does in rat tissues. Both these propositions can be tested, the former by increasing the concentration of PVP (unlabelled or ^{125}I-labelled) in the incubation medium. If the capture of ^{125}I-PVP in the absence of serum is mainly by adsorptive pinocytosis, the Endocytic Index would be expected to decrease on increasing the concentration of unlabelled PVP in the medium as binding sites become saturated with mainly non-radioactive forms of the polymer. The second possibility can be tested by incubating rabbit yolk sacs in serum-free medium 199 in the presence of some other established fluid-phase marker (e.g. ^{3}H-labelled inulin) which should show the same Endocytic Index as ^{125}I-labelled PVP if neither adsorbs to the membrane and the rate of pinosome formation is markedly elevated by the absence of serum from the incubation medium.

By following the fate of ^{125}I-labelled BSA as a representative non-transportable protein, it was hoped that some insight could be gained into the proteolytic capacity of the rabbit yolk sac. Tissue-Accumulation Rates were found to be very low in both tissues (in either the presence or the absence of calf serum) suggesting that most of the ^{125}I-labelled BSA captured by pinocytosis was subsequently degraded. As for the rat yolk sac, uptake in the rabbit yolk sac is linear with time and the Endocytic Index of ^{125}I-BSA is as reproducible in the rabbit yolk sac as in the rat yolk sac, although it is considerably smaller in magnitude compared to that for the rat. For rabbit yolk sacs the Endocytic Index of ^{125}I-labelled BSA when ingested either in the presence or in the absence of serum, is, surprisingly, almost identical to that observed for ^{125}I-PVP. Two possible ways of accounting for

these findings can be envisaged. First, both substrates (i.e. ^{125}I-BSA and ^{125}I-PVP) are pinocytosed almost entirely by way of the fluid phase in the rabbit yolk sac so that no membrane binding is involved. This would stand in strong contrast to the equivalent results in the rat yolk sac in which ^{125}I-PVP is thought to enter the cells only by fluid pinocytosis (Roberts et al., 1977), whereas ^{125}I-BSA enters mainly by adsorbing to the yolk-sac epithelial cell surface prior to internalisation (Williams et al., 1975b; Ibbotson & Williams, 1979). Second, both substrates adsorb to the membrane and do so to the same extent, either by using the same binding sites or by using different classes of binding site. (The existence of different classes of binding site for simple proteins has recently been shown in the rat yolk sac; Livesey, 1979).

However, in spite of these differences with formaldehyde-treated albumin, rat and rabbit yolk sacs each seem to treat homologous ^{125}I-labelled IgG in a similar manner. In both tissues, and in either the presence or absence of calf serum, a considerable amount of radioactivity becomes associated with the tissue (see also Williams & Ibbotson, 1979). By contrast, tissue-accumulation is much lower when using ^{125}I-labelled BSA as substrate. (This observation in the rat initially stimulated the in vitro work with IgG.) Homologous IgG is, according to in vivo observations, treated differently from other proteins, especially in the rabbit yolk sac. In vivo at least 12% of the total IgG present is transported across the yolk sac intact, following its administration into the uterus (Hemmings, 1956). Thus the observation of a lesser extent of degradation in vitro is in accordance with the in vivo observation.

Concerning the site at which substrate accumulates in the yolk sac during the incubation period, only speculations can be offered at this stage. Electron microscopy studies of rabbit yolk sacs, after in vivo exposure to homologous IgG coupled to horseradish peroxidase, have shown

the presence of IgG in the endodermal cells, in intercellular spaces, in the cells surrounding vitelline vessels and in mesenchymal macrophages (Moxon et al., 1976). These sites are likely to be the same in vitro, with the possible addition of the stagnant vitelline circulation (Ibbotson, Ph.D. thesis).

From reincubation studies, using ^{125}I-PVP as substrate, it is apparent that neither the rat nor the rabbit yolk sac releases this marker to any great extent over the 3h period of reincubation. This finding can be interpreted as evidence for an intact vacuolar system in yolk sacs incubated in vitro. From the equivalent reincubation studies using ^{125}I-BSA and ^{125}I-labelled homologous IgG as substrates, important additional information can be obtained, both on the extent of release of the intact protein and on the extent of degradation of the protein after internalization by the tissue. Differences between ^{125}I-labelled BSA and ^{125}I-labelled IgG are striking. For ^{125}I-labelled BSA, virtually all of the tissue-associated radioactivity is released in a TCA-soluble form, whereas for ^{125}I-labelled IgG a greater part is released in a TCA-insoluble form. In rabbit yolk sacs, the observation that not only Endocytic Indices are low for ^{125}I-BSA, but also that the total amounts of substrate released from the yolk sac during reincubation are low, suggests that this tissue has a rather low capacity for processing BSA within the lysosomal system. However, any ^{125}I-BSA that is actually taken up pinocytically by the tissue is mostly degraded.

The exact nature of the TCA-soluble radioactivity released into the incubation medium has been investigated by Ibbotson & Williams (1979) in the rat system using column chromatography (Sephadex G-25), and the major acid-soluble species present was found to be ^{125}I-labelled L-tyrosine. The released TCA-insoluble activity too has been investigated (Ibbotson et al., unpublished data) by both gel filtration studies

(Sephadex G-200) and by Ouchterlony diffusion analysis, and its behaviour is consistent with the material being intact, macromolecular IgG. In the rabbit yolk sac the percentage of the released ^{125}I-labelled IgG that remains undegraded (i.e. TCA-insoluble) is greater than in the rat yolk sac (58.6% c.f. 34.9%). This is compatible with the suggestion that the yolk sac is the major site for transfer of passive immunity in the rabbit but not in the rat.

In conclusion, these initial experiments have demonstrated the following points:

1) In the in vivo incubation system, the rabbit yolk sac is as viable and as likely to give physiologically relevant results as is the rat yolk sac.

2) In the absence of serum the rate of ingestion of ^{125}I-labelled PVP in the rabbit yolk sac differs markedly from that in the rat yolk sac, the Endocytic Index rising much more markedly in the rabbit tissue.

3) ^{125}I-labelled BSA is taken up more readily by the rat yolk sac than by the rabbit yolk sac.

4) The rabbit yolk sac does not seem to have as great a capacity for the uptake of ^{125}I-labelled BSA as it does for the uptake of homologous ^{125}I-labelled IgG.

5) Homologous ^{125}I-labelled IgG is treated differently from other proteins in the rabbit as well as in the rat yolk sac, a greater part of the protein being released in a TCA-insoluble (undegraded) form, during reincubation.

6) A greater proportion of homologous ^{125}I-IgG seems to be released undegraded after pinocytic capture in rabbit yolk sacs than in rat yolk sacs.

As a result of the above findings, in vitro studies using rabbit yolk-sac pieces were considered to be a valuable and worthwhile way of further investigating the cellular mechanism of IgG transport across living cells.

The results of experiments in which yolk sacs were incubated in either the presence or the absence of serum did not qualitatively differ from each other. General "patterns" did not change and tissue survival was good under both conditions. Therefore most of the experiments reported in the following chapters were performed in the absence of calf serum to simplify the system, by removing competing serum proteins.

CHAPTER THREE

The Effect of Chemical Treatments of IgG on the Behaviour of this Molecule in the Rat Yolk Sac System

3.1 INTRODUCTION

An earlier investigation of the fate of ^{125}I-labelled rat IgG in the rat yolk sac was made (Williams & Ibbotson, 1979) using the *in vitro* incubation technique of Williams *et al.* (1975a). In these experiments a number of different batches of ^{125}I-labelled rat IgG were prepared and used, and variations in the endocytic behaviour of the different preparations of this protein were noted (Ibbotson, Ph.D. thesis). Not only were significant differences in the Endocytic Indices of these preparations observed, but also different Tissue-Accumulation Rates and varying percentages of TCA-insoluble release during reincubation experiments.

Since the inter-batch variability seemed to exceed the reproducibility of uptake and release within each batch, it appeared that there was some variable that was not controlled in the preparation procedure, but which affected the properties of the individual preparations. Two principal possibilities can be envisaged. First, the commercial IgG preparation used as starting material may differ from batch to batch, either in content of protein contaminants (e.g. immunoglobulins other than IgG) or in the relative amounts of the different IgG sub-classes. Second, small variations in the labelling procedure may strongly affect the molecule and alter its endocytic properties. The first possibility cannot be held responsible for the observed variability since different preparations based on the same shipment of rat IgG also showed the same effect. Hence, in the current study, an attempt was made to establish whether any of the conditions in the iodination procedure itself had a decisive effect on the properties of the preparations of ^{125}I-labelled IgG in the *in vitro* system. By deliberately altering one experimental variable excessively, it was hoped to induce greater degrees of variation relative to those found in preparations that were ostensibly identical.

Identification of such a variable could have a profound effect on all the other quantitative data gathered from the yolk-sac system in subsequent experiments, hence this topic merited careful investigation at an early stage in the programme of investigations. In the labelling procedure a number of variables can be considered as potential candidates responsible for the observed differences in endocytic behaviour of ^{125}I-labelled IgG batches.

1) Reaction temperature. Sonoda & Schlamowitz (1970) using a chloramine-T method of labelling, found that the optimum reaction temperature was 2^{o}C. At higher temperatures they observed irreversible over-oxidation of the [^{125}I]iodide, whereas at low temperatures this problem was avoided.

2) Concentration of [^{125}I]iodide. Several workers found that high concentrations of [^{125}I]iodide, that lead to an incorporation of more than 1 atom into each protein molecule, caused progressive damage of proteins (Bocci, 1969; Schlamowitz, 1976). Also, Hemmings (1974) observed an increased rate of excretion of breakdown products when injecting over-iodinated IgG into suckling-rat or -mouse gut.

3) Protein concentration. This variable is related to the previous one. Sonoda & Schlamowitz (1970) showed a linear relationship between the extent of [^{125}I]iodide incorporation and the logarithm of protein concentration. To ensure maximal labelling efficiency, they used a concentration of 1 mg protein per ml of reaction medium, which is the same concentration used here.

4) pH of reaction. A neutral or slightly basic pH is quoted as the most favourable condition for activating [^{125}I]iodide and for the incorporation of the activated iodide into the protein during the iodination procedure. A pH of 7 has been suggested as optimal (Sonoda

& Schlamowitz, 1970), but Bocci (1969) preferred a pH of 8.

5) Ionic strength of buffer. Sonoda & Schlamowitz (1970) used 0.05M-$NaHSO_4$ as buffer in the radioiodination of IgG by a chloramine-T method. They found that increasing the buffer strength to 0.5M led to a decrease in labelling efficiency, accordingly in this study a buffer concentration of 0.05M was used during labelling.

If extremes of temperature, ionic strength and pH are avoided, variations in any of the above conditions will normally be expected to lead to only changes in labelling efficiency. Only changes in the degree of [^{125}I]iodide incorporation are expected to exert any significant effect on the biological activity of the labelled protein. Therefore, when keeping the degree of [^{125}I]iodide incorporation below the level of 1 atom per protein molecule, even small experimental variations of the other variables are not likely to lead to changes in general biological and endocytic properties of the labelled protein.

However, two more variables are considered below that are both concerned with the extent of exposure of the [^{125}I]iodide and the protein to the oxidising agent, chloramine-T.

6). Reaction time. (i.e. time of exposure of [^{125}I]iodide and protein to chloramine-T). Sonoda & Schlamowitz (1970) claim that activation of iodine takes a few seconds only, but the incorporation reaction is time-dependent. In their system, a plot of percentage iodide incorporation against reaction time shows that a plateau is reached at 30 minutes. From these results, by 8 min, the time normally used here (Section 1.1), the reaction should be almost completed.

7) Chloramine-T concentration. Sherman et al. (1974) found that the rate of clearance* of ^{125}I-labelled fibrinogen was dependent on the concentration of chloramine-T employed in the iodination procedure and

* from the bloodstream

was more rapid for protein preparations that were iodinated in the presence of higher concentrations of this reagent. Bocci (1969) noticed decreases in labelling efficiency with increasing chloramine-T concentrations. More importantly, he noticed that, whereas a protein: chloramine-T ratio of 200 does not modify the half life of ^{131}I-labelled IgG in the circulation of the rat (as determined by clearance studies by the rat liver), the use of larger amounts of oxidising agent significantly shortens the half-life of this protein. In this context it is helpful to consider the general role of chloramine-T during the labelling procedure and its effect on proteins. The radioiodination reaction is a biphasic process; first the generation of active iodide and second the incoporation of the iodide into the tyrosine residues of the protein molecule. Chloramine-T acts as an activator in the first stage (Sonoda & Schlamowitz, 1970). At the same time chloramine-T can have adverse effects on the protein at high concentrations; Sherman et al. (1974) reported polymerisation of the fibrinogen that was being labelled and this in turn led to more rapid clearance of such preparations when injected into the circulation of the rat. Bocci, (1969) found the same when using albumin, but with IgG as radioiodinated protein, the amount of polymerisation was negligible. Apart from this effect, other modifications of the proteins have also been observed. Sutcliffe et al. (1973) found that chloramine-T destroys essential methionyl residues in parathyroid hormone. For other proteins (e.g. calcitonin, Marx et al., 1973) no such effect has been reported. These considerations have led to controversies as to the usefulness of chloramine-T as a labelling agent for biologically active proteins.

In this chapter, the reaction conditions were altered in two ways: 1) by shortening the reaction time and hence the period of exposure to chloramine-T and 2) by deliberately increasing the chloramine-T

concentration to see whether this would induce any changes in the biological properties of the resultant preparations.

An earlier study (Moore et al., 1977) showed that very profound modifications of the uptake rate of ^{125}I-labelled BSA in the rat yolk sac in vitro could be induced by exposing different aliquots of a preparation of the ^{125}I-labelled protein to different chemicals. Increases in the Endocytic Indices of the ^{125}I-labelled BSA in the in vitro rat yolk-sac incubation system were observed on exposing the protein to dilute acetic acid. However, the most marked increase (ten-fold) in the Endocytic Index of ^{125}I-labelled BSA was induced by treating the labelled protein with either a 10% (w/v) formaldehyde solution or with urea. The increased uptake rate was attributed to a substantial change in 3^{o}structure leading to an unmasking of buried groups that bind to the plasma membrane, so increasing the rate of pinocytosis. Adsorptive pinocytosis is also responsible for the uptake of homologous ^{125}I-labelled IgG in the rat yolk sac (Williams & Ibbotson, 1979). It was therefore of interest to find out whether the endocytic behaviour of ^{125}I-labelled IgG in the rat yolk-sac incubation system can, likewise, be drastically altered on exposure to formaldehyde using the method employed to modify ^{125}I-labelled BSA. For this purpose a portion of ^{125}I-labelled IgG was treated with a 10% (w/v) formaldehyde solution subsequent to radioiodination and both uptake rates and release patterns were investigated. The effect of urea was similarly investigated.

In summary, the experiments reported in this chapter attempt to answer the following questions:

1) Does differential exposure of [^{125}I]iodide and homologous IgG to the oxidising reagent chloramine-T during radioiodination cause variations in the rate of endocytic capture of the ^{125}I-labelled IgG by the rat yolk sac in vitro?

2) Do differences in chloramine-T exposure during labelling of rat IgG exert any effect on the rate of breakdown of this protein in the rat yolk sac *in vitro*?

3) Is a radiolabelling method involving chloramine-T acceptable as a method for labelling IgG for *in vitro* studies?

4) Can greater variations of the Endocytic Index and the Tissue-Accumulation Rate of homologous ^{125}I-labelled IgG be induced by exposure to urea/formaldehyde?

5) Are release patterns, in rat yolk-sac reincubation experiments, modified after the ^{125}I-labelled IgG is exposed to formaldehyde or urea?

3.2 METHODS

3.2.1 Method of varying the exposure to chloramine-T during radioiodination

The radiolabelling method was essentially the same as outlined in Section 1.1, but in one preparation the chloramine-T and sodium metabisulphite concentrations during iodination were increased 10-fold, from 200 µg/ml to 2 mg/ml. The resulting preparation was termed "Strong" ^{125}I-labelled IgG. In another preparation the total exposure time of the protein and the [^{125}I]iodide to the chloramine-T was shortened from 8 min to 4 min, while leaving the rest of the procedure unchanged. This preparation was termed "Mild" ^{125}I-labelled IgG. Another batch of ^{125}I-labelled IgG was prepared, following the method outlined in Section 1.1 in all details. This preparation was termed "Normal" ^{125}I-labelled IgG.

3.2.2 Preparation of formaldehyde-treated ^{125}I-labelled rat IgG

A portion of stock solution of ^{125}I-labelled rat IgG (0.71 mg/ml) prepared as described in Section 1.1 was mixed with an equal volume of 10% (w/v) formaldehyde solution in 0.5 M-$NaHCO_3$ buffer, pH 10, and kept at room temperature for 72h. The solution was then dialysed exhaustively against a 1% NaCl solution. As a necessary control, a further aliquot of the protein stock solution was mixed with buffer only and then treated in the same way as the formaldehyde-containing solution.

3.2.3 Preparation of urea-treated ^{125}I-labelled rat IgG

A portion of stock solution of ^{125}I-labelled IgG (0.71 mg/ml), prepared as described in Section 1.1, was added to an equal volume of 8 M-urea, pH 5, and left at room temperature for 24h before dialysing against 2 changes (5 l) of 1% aq. NaCl (w/v).

3.2.4 Uptake of ^{125}I-labelled IgG preparations, that had received differing exposures to chloramine-T during labelling

Rat yolk sacs (17.5-day) were incubated in medium 199 containing 10% (v/v) calf serum in the presence of ^{125}I-labelled IgG (2 µg/ml), that had been subjected to either the "Mild", "Normal" or "Strong" chloramine-T treatments outlined above (3.2.3). For each of the different IgG preparations a total of three uptake experiments were performed in medium 199 containing 10% (v/v) calf serum, and a single similar experiment was performed in medium 199 alone.

3.2.5 Uptake of formaldehyde- or urea-treated ^{125}I-labelled rat IgG in yolk sacs

Rat yolk sacs (17.5-day) were incubated as in the preceding section (3.2.4), but in the presence of formaldehyde-treated, urea-treated or buffer-treated ^{125}I-labelled IgG (2 µg/ml).

3.2.6 Exocytosis following incubation of rat yolk sacs in medium 199 in the presence of either "Mild", "Normal" or "Strong" chloramine-T treated ^{125}I-labelled IgG preparations

Rat yolk sacs (17.5-day) were incubated for 2h, in either medium 199 containing 10% (v/v) calf serum or in medium 199 alone and in the presence of either "Strong", "Normal" or "Mild" ^{125}I-labelled IgG (10 µg/ml). After washing, yolk sacs were reincubated in fresh medium 199 and 1 ml aliquots of medium were removed at 15 min intervals up to 3h. (For details of method see Section 1.12). For each ^{125}I-labelled IgG preparation 6 experiments were conducted.

3.2.7 Exocytosis following incubation of rat yolk sacs in serum-free medium 199 in the presence of formaldehyde-treated or urea-treated ^{125}I-labelled IgG

Rat yolk sacs (17.5-day) were incubated as in the preceding section

(3.2.6), but in the presence of formaldehyde-treated or urea-treated ^{125}I-labelled IgG. As controls, similar incubations were set up with ^{125}I-labelled IgG (10 µg/ml) that had either been 0.5M-$NaHCO_3$ buffer-treated or was untreated. For each ^{125}I-labelled IgG preparation 6 incubations were performed.

3.3 RESULTS

3.3.1 Uptake by the rat yolk sac in vitro of ^{125}I-labelled IgG that had received different exposures to chloramine-T during radioiodination

Uptake experiments conducted in serum-free medium 199 and with either the "Strong", "Normal" or "Mild" ^{125}I-labelled IgG preparation as substrate, resulted in Endocytic Indices that showed a tendency to decrease in the order "Strong" > "Normal" > "Mild" (Table 3.1). The results of equivalent experiments performed in the presence of serum (10%, v/v), gave lower Endocytic Indices but also showed the same trend (Table 3.1a). In the absence of calf serum from the incubation medium, the Endocytic Index of the "Mild" ^{125}I-labelled IgG preparation is only approx. 67% that of the other two preparations (Table 3.1b). However, since only one uptake experiment was performed with each preparation in the absence of serum, these findings are not conclusive and only suggest a possible trend towards a slightly increased rate of endocytosis with rising severity of chloramine-T exposure. Tissue-Accumulation Rates of "Strong", "Normal" and "Mild" ^{125}I-labelled IgG in the presence and absence of serum closely follow the pattern shown by the corresponding values of the Endocytic Index.

3.3.2 Release of radioactive species derived from different ^{125}I-labelled IgG preparations that had been exposed to different intensities of chloramine-T radioiodination

Following uptake of either "Strong", "Normal" or "Mild" ^{125}I-labelled IgG into rat yolk sacs *in vitro*, release of radioactive species derived from these substrates into fresh medium was monitored. The release patterns of "Strong", "Normal" and "Mild" ^{125}I-labelled IgG in medium 199 alone are shown in Figures 3.1, 3.2 and 3.3

respectively; they do not appear to be very different from each other. In Table 3.2 the same results are presented in the form of the amount of substrate (ng/mg yolk-sac protein) released into the fresh medium after 3h reincubation time, and also in the form of a percentage of the total radioactivity associated with the yolk-sac tissue at the beginning of the reincubation period. From this table it appears that the extent of release of "Normal" ^{125}I-labelled IgG in serum-free medium is rather lower than that of the other two preparations. This variation, however, can be said to be within the limits of experimental scatter since, on other days different amounts of substrate the same substrate preparation were released (e.g. 665 ng/mg yolk-sac protein, Chapter 2, Table 2.10).

When expressing substrate release as a percentage of the radioactivity initially associated with the yolk sac, it can be seen that greater amounts of the radioactivity are released in a TCA-insoluble form when using "Mild" ^{125}I-labelled IgG, than for the other two preparations (Table 3.2). On the whole, the release patterns of ^{125}I-labelled IgG preparations for incubations in serum-containing medium are similar to those in serum-free medium. The only difference lies in the total amount of substrate released, which is much lower in incubations in the presence of serum (see also Chapter 2, Table 2.5).

3.3.3 Uptake of formaldehyde- or urea-treated ^{125}I-labelled rat IgG by 17.5-day rat yolk sacs

Endocytic Indices and Tissue-Accumulation Rates of formaldehyde-, urea-, phosphate buffer-treated and untreated ^{125}I-labelled rat IgG in the rat yolk sac are quoted in Table 3.3 both for incubations performed in medium 199 alone and for those performed in medium 199 containing 10% (v/v) calf serum. For each substrate and each experimental condition only one incubation was performed, and subsequently the results can only

be used to indicate a general trend. In the presence of calf serum, both urea- and formaldehyde-treated ^{125}I-labelled IgG preparations appear to have enhanced E.I.s compared with the untreated controls. The T.A.R.s remain virtually unchanged (Table 3.3a).

In medium 199 alone (Table 3.3b) the values of the E.I. and the T.A.R. of the formaldehyde-treated ^{125}I-labelled IgG do not differ from those of either of the two controls. For urea-treated ^{125}I-labelled IgG in serum-free medium the T.A.R. is similar, but the E.I. is considerably lower than those of the other substrates. It may also be noted that there is a great difference between the tissue level of the urea-treated ^{125}I-labelled IgG and the other IgG preparations in the uptake in vitro incubations. [In the presence of 10% (v/v) calf serum the tissue-level of radioactivity is very much higher (Table 3.3a), whereas in the absence of serum the tissue-level appears somewhat lower than that of the other substrates (Table 3.3b).] The uptake of formaldehyde-treated IgG in the presence and absence of serum (Table 3.3) does not seem to differ from that of untreated ^{125}I-labelled IgG (see Table 2.5).

3.3.4 Release of radioactive species derived from different chemically-treated preparations of ^{125}I-labelled IgG

The results of the reincubation experiments using ^{125}I-labelled IgG, that had previously been treated with either urea or formaldehyde, are presented with the appropriate controls in Figures 3.4, 3.5 and 3.6 and Table 3.4. From the figures alone, which show the substrate-release patterns during reincubation of the "loaded" rat yolk sac in serum free medium 199, it appears that the release of TCA-soluble and TCA-insoluble radioactivity derived from urea-treated ^{125}I-labelled IgG follows the same pattern as the untreated "Normal" ^{125}I-labelled IgG (Figure 3.4 and Table 3.4). For formaldehyde-treated ^{125}I-labelled IgG, on the other hand, the release pattern

(Figure 3.5) is dramatically changed and resembles the pattern obtained with ^{125}I-labelled fd-BSA as substrate in the rat yolk sac (Chapter 2 , Figure 2.3). Of the total amount of radioactivity released subsequent to incubation of the rat yolk sac with formaldehyde-treated ^{125}I-labelled IgG, 95% is released in TCA-soluble form, compared with a value of 66% for the untreated ^{125}I-labelled IgG control and a similarly low value (67%), for the bicarbonate buffer-treated ^{125}I-labelled IgG control preparation. Another interesting feature concerned with the use of formaldehyde-treated ^{125}I-labelled IgG can be seen in the re-incubation studies in medium 199 containing 10% (v/v) calf serum. Unlike the other ^{125}I-labelled IgG preparations, the absolute values of the release from the yolk sac (in ng formaldehyde-treated ^{125}I-IgG) are not changed by the presence of serum in the incubation medium (Table 3.4b). Again, the greater percentage of substrate released is TCA-soluble. Urea-treated ^{125}I-labelled IgG does not differ from the "Normal" IgG control in its behaviour during release by the rat yolk sac.

Table 3.1 Endocytic Indices and Tissue-Accumulation Rates of three different batches of ^{125}I-labelled IgG each incubated with 17.5-day rat yolk sacs in medium 199 either in the presence or in the absence of calf serum

Rat IgG was radioiodinated with ^{125}I-iodide under conditions that were constant except that the exposure to chloramine-T during the labelling procedure was varied. The resulting ^{125}I-labelled IgG batches were termed "Strong", "Normal" and "Mild" ^{125}I-labelled IgG (see Section 3.2.1). Rat yolk-sacs (17.5-day) were incubated for up to 6.5h with either "Strong", "Normal" or "Mild" preparations of ^{125}I-labelled IgG (2 µg/ml of medium), either in medium 199 alone or in medium 199 containing 10% (v/v) calf serum. The methods are described in detail in Sections 1.7 and 1.8.

a) Experiments in medium 199 containing 10% (v/v) calf serum

Preparation of ^{125}I-IgG	1	2	3	4	5	6
Strong	1.25	9	21.32	0.967	4.46	0.926
Strong	1.22	10	15.25	0.991	3.77	0.928
Strong	1.38	10	17.21	0.997	4.31	0.961
MEAN VALUES (± S.D.):			19.93 ± 3.1		4.18 ± 0.36	
Normal	2.30	9	12.69	0.977	2.21	0.725
Normal	2.71	10	13.99	0.994	2.95	0.933
Normal	2.77	10	16.35	0.959	3.33	0.923
MEAN VALUES (± S.D.):			14.35 ± 1.88		2.83 ± 0.57	
Mild	4.02	10	17.27	0.968	2.44	0.738
Mild	4.06	10	12.19	0.966	2.31	0.851
Mild	4.19	10	12.15	0.833	2.36	0.645
MEAN VALUES (± S.D.):			13.87 ± 2.94		2.37 ± 0.06	

b) Experiments in medium 199 alone

Preparation of ^{125}I-IgG	1	2	3	4	5	6
Strong	1.33	9	77.19	0.946	4.51	0.579
Normal	2.32	10	76.15	0.910	3.35	0.322
Mild	5.07	10	51.62	0.955	1.93	0.579

Table 3.2 Release of radioactive species derived from "Strong", "Normal" and "Mild" ^{125}I-labelled rat IgG when 17.5-day rat yolk sacs were reincubated in medium 199 in either the presence or the absence of calf serum

Rat yolk-sacs (17.5-day) were first incubated for 2h in medium 199 containing 10% (v/v) calf serum or in medium 199 alone, in the presence of "Strong", "Normal" or "Mild" ^{125}I-labelled IgG (see Section 3.2.1 for details of preparation). Following washing they were reincubated in fresh medium, and the release of radioactivity was followed up to 3h (see Section 1.12 for details). In this table, only the amounts of substrate released at 3h of reincubation are reported. They are expressed as ng substrate released per unit weight of yolk-sac tissue (± S.D.) and as a percentage of the total amount of substrate associated with the yolk sac at the start of the reincubation period. The data reported in the Section b) of this table are also displayed graphically in Figures 3.1, 3.2, and 3.3.

a) Experiments in medium 199 containing 10% (v/v) calf serum

Preparation of ^{125}I-IgG	Substrate released (ng/mg yolk-sac)			Substrate released (%)		
	TCA-solubles	TCA-insolubles	Totals	TCA-solubles	TCA-insolubles	Totals
Strong	49.2 ± 14.4	41.0 ± 3.1	90.2 ± 10.5	43.0	35.8	78.8
Normal	46.1 ± 4.7	52.3 ± 13.1	98.4 ± 8.5	45.5	34.2	79.7
Mild	33.4 ± 1.1	40.5 ± 1.9	73.9 ± 3.0	33.5	40.6	74.2

b) Experiments in medium 199 alone

Preparation of ^{125}I-IgG	Substrate released (ng/mg yolk-sac)			Substrate released (%)		
	TCA-solubles	TCA-insolubles	Totals	TCA-solubles	TCA-insolubles	Totals
Strong	307.0 ± 64.4	156.8 ± 18.9	463.8 ± 64.6	56.3	29.2	86.4
Normal	228.7 ± 43.1	116.7 ± 22.6	345.4 ± 53.0	55.9	28.4	84.3
Mild	318.4 ±147.2	203.1 ± 65.3	521.6 ±209.6	52.4	33.4	85.8

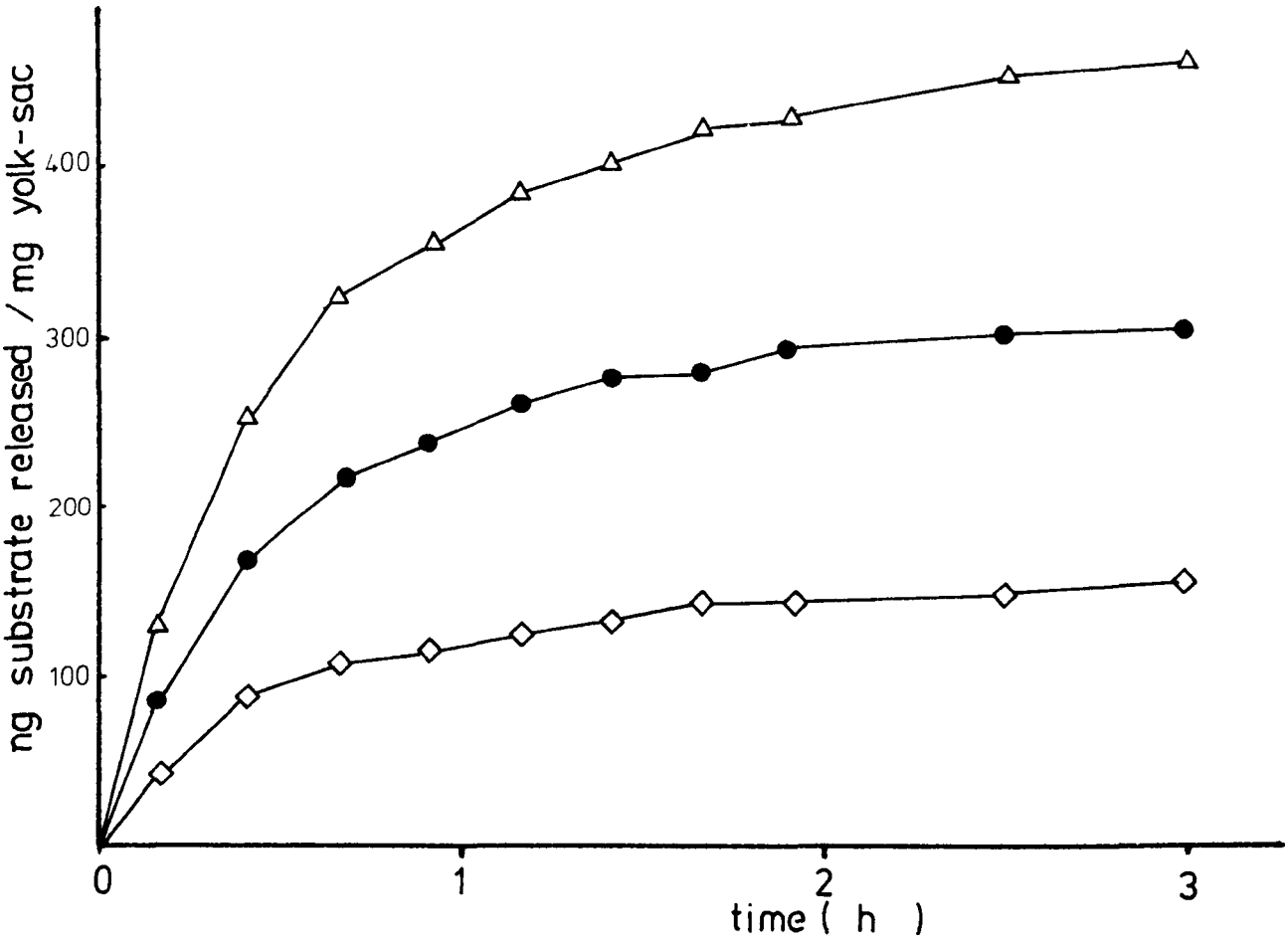


Figure 3.1 Time course of release of radioactive species derived from "strong" ^{125}I-labelled rat IgG following incubation of rat yolk-sacs with substrate in serum-free medium 199

17.5-day rat yolk sacs were first incubated for 2h in serum-free medium 199 in the presence of "strong" ^{125}I-labelled rat IgG (10 µg/ml). Following washing they were reincubated in fresh medium, and release of radioactivity was monitored up to 3h (see Section 1.12 for details). The total amount of substrate associated with the yolk-sac at the beginning of reincubation was 537 ng/ mg yolk-sac.
Total radioactivity released —△—
TCA-soluble radioactivity released —●—
TCA-insoluble radioactivity released —◇—
The values shown are means from 6 separate experiments performed with the same batch of "strong" ^{125}I-labelled rat IgG, used in the experiments reported in Table 3.1.

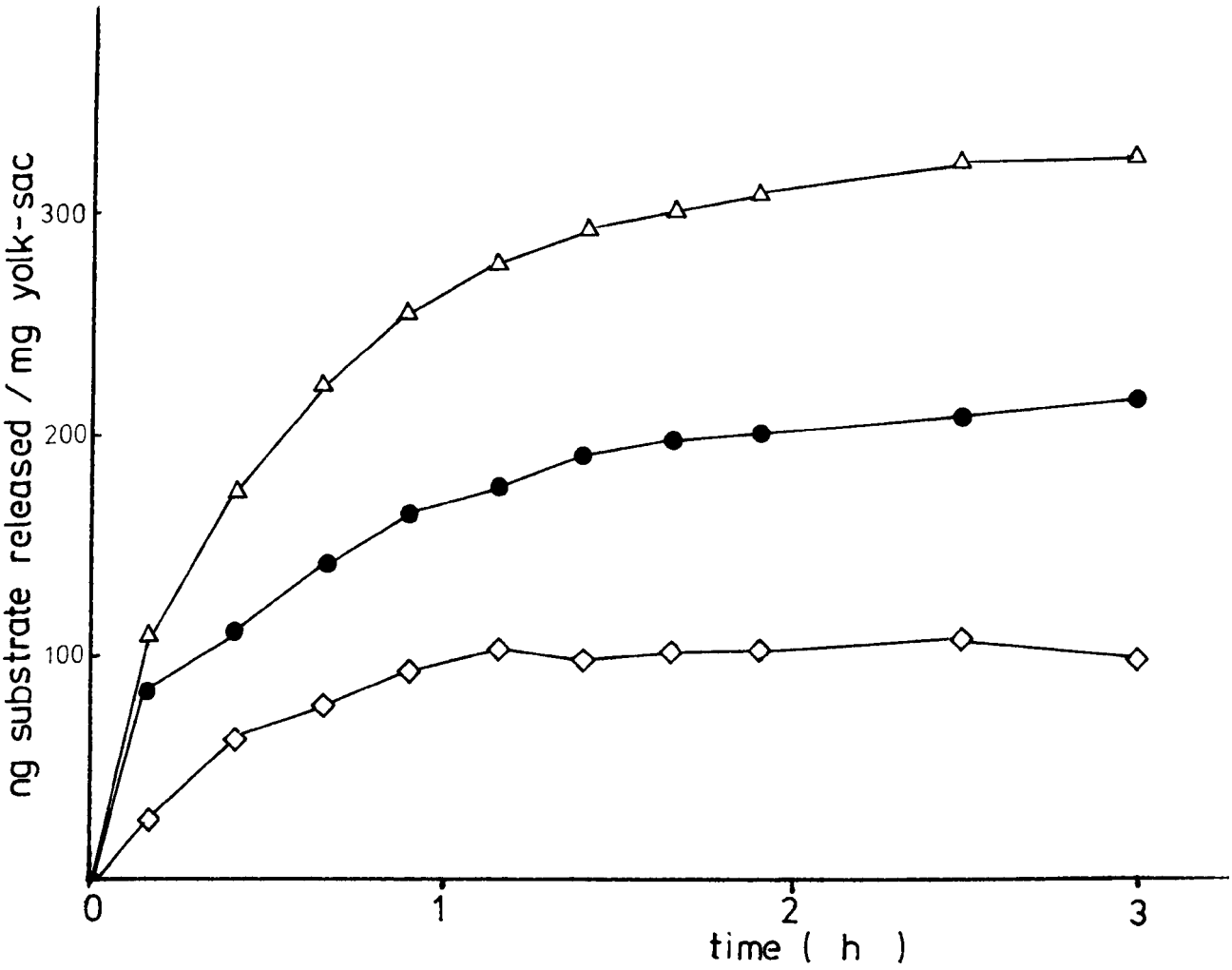


Figure 3.2 Time course of release of radioactive species derived from "normal" ^{125}I-labelled rat IgG following incubation of rat yolk-sacs with substrate in serum-free medium 199

17.5-day rat yolk-sacs were first incubated for 2h in serum-free medium 199 in the presence of "normal" ^{125}I-labelled rat IgG (10 µg/ml). Following washing they were reincubated in fresh medium, and release of radioactivity was monitored for up to 3h (see Section 1.12 for details). The total amount of substrate associated with the yolk-sac at the beginning of reincubation was 409 ng/mg yolk-sac protein.
Total radioactivity released —Δ—
TCA-soluble radioactivity released —●—
TCA-insoluble radioactivity released —◇—
The values shown are means from 6 separate experiments performed with the same batch of "normal" ^{125}I-labelled rat IgG used in the experiments reported in Table 3.1.

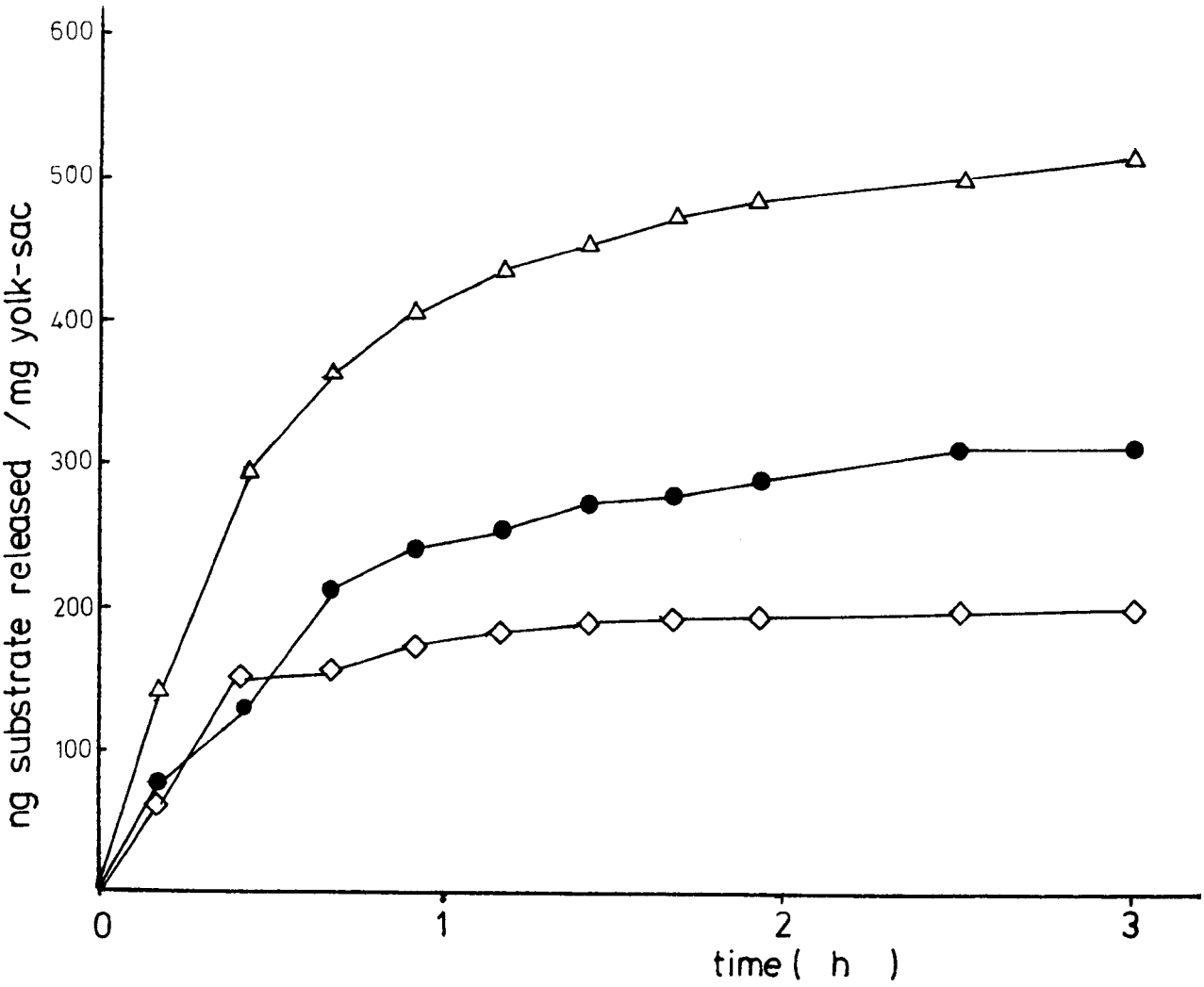


Figure 3.3 Time course of release of radioactive species derived from "mild" ^{125}I-labelled rat IgG following incubation to rat yolk-sacs with substrate in serum-free medium 199

17.5-day rat yolk-sacs were first incubated for 2h in serum-free medium 199 in the presence of "mild" ^{125}I-labelled rat IgG (10 µg/ml). Following washing, they were reincubated in fresh medium, and release of radioactivity was monitored up to 3h (see Section 1.12 for details). The total amount of substrate associated with the yolk-sac at the beginning of reincubation was 608 ng/mg yolk-sac.
Total radioactivity released —△—
TCA-soluble radioactivty released —●—
TCA-insoluble radioactivity released —◇—
The values shown are means from 6 separate experiments performed with the same batch of "mild" ^{125}I-labelled rat IgG used in the experiments reported in Table 3.1.

Table 3.3 Endocytic Indices and Tissue-Accumulation Rates of different preparations of ^{125}I-labelled rat IgG that had been subjected to differing chemical treatments, by rat yolk-sacs incubated in medium 199 in the presence and absence of calf serum

After radioiodination ^{125}I-labelled rat IgG was treated with either 8M-urea (see Section 3.2.3), a 10% (w/v) formaldehyde solution in a 0.5M-$NaHCO_3$ buffer (see Section 3.2.2) or 0.5M-$NaHCO_3$ buffer alone (see Section 3.2.2). Rat yolk sacs (17.5-day) were incubated for up to 6.5h in the presence of substrate (2 µg/ml), either in serum-free medium 199 or in medium 199 containing 10% (v/v) calf serum. The method is described in detail in Section 3.2.5.

a) Experiments in medium 199 containing 10% (v/v) calf serum

Chemical treatment of ^{125}I-IgG	1	2	3	4	5	6
Urea	2.7	10	18.85	0.907	2.49	0.563
Formaldehyde	1.8	10	14.98	0.989	1.96	0.905
Buffer	1.64	10	6.18	0.955	1.01	0.732
No treatment	2.76	10	9.09	0.971	1.82	0.963

b) Experiments in medium 199 alone

Chemical treatment of ^{125}I-IgG	1	2	3	4	5	6
Urea	3.08	10	36.71	0.939	2.58	0.768
Formaldehyde	3.16	10	74.99	0.965	3.41	0.703
Buffer	5.75	10	73.91	0.933	5.73	0.629
No treatment	2.32	10	76.15	0.910	3.35	0.322

Table 3.4 Release of radioactive species derived from urea- and formaldehyde-treated ^{125}I-labelled rat IgG when 17.5-day rat yolk sacs were incubated in medium 199 in either the presence of the absence of calf serum.

Rat yolk sacs (17.5 day) were first incubated for 2h in medium 199 alone or in medium 199 containing 10% (v/v) calf serum, in the presence of urea-treated (see Section 3.2.3), formaldehyde-treated (see Section 3.2.2), buffer-treated (see Section 3.2.2) or untreated ^{125}I-labelled rat IgG. After washing, yolk sacs were reincubated in fresh medium and release of radioactivity was followed for up to 3h (see Section 3.2.7 for details). In this table only the amounts of substrate released at 3h reincubation time are reported. They are expressed as ng substrate released per unit weight of yolk-sac tissue (± S.D.) and as a percentage release of the total amount of substrate associated with the yolk sac at the start of the reincubation period. The data reported in b) below are also displayed graphically in Figures 3.4, 3.5 and 3.6.

a) Experiments in medium 199 containing 10% (v/v) calf serum)

Chemical treatment of ^{125}I-IgG	Substrate released (ng/mg yolk sac)			Substrate released (%)		
	TCA-solubles	TCA-insolubles	Totals	TCA-solubles	TCA-insolubles	Totals
Urea	72.2 ± 18.8	52.9 ± 34.8	124.1 ± 20.0	47.1	35.5	82.6
Formaldehyde	219.9 ± 27.7	61.1 ± 9.4	281.1 ± 37.7	58.8	16.4	75.2
Buffer	60.2 ± 1.7	32.5 ± 4.6	92.8 ± 10.1	51.5	27.8	79.3
No treatment	46.1 ± 4.6	52.3 ± 13.1	98.4 ± 8.5	45.5	34.2	79.7

b) Experiments in medium 199 alone

Chemical treatment of ^{125}I-IgG	Substrate released (ng/mg yolk sac)			Substrate released (%)		
	TCA-solubles	TCA-insolubles	Totals	TCA-solubles	TCA-insolubles	Totals
Urea	296.5 ± 53.7	176.8 ± 38.8	473.3 ± 57.3	53.0	31.6	84.6
Formaldehyde	217.6 ± 53.4	20.1 ± 18.5	238.0 ± 33.3	70.9	6.6	77.5
Buffer	266.1 ± 68.9	134.7 ± 25.9	399.7 ± 84.9	56.6	28.7	85.0
No treatment	228.7 ± 43.1	105.6 ± 22.6	334.3 ± 53.0	56.9	25.8	82.7

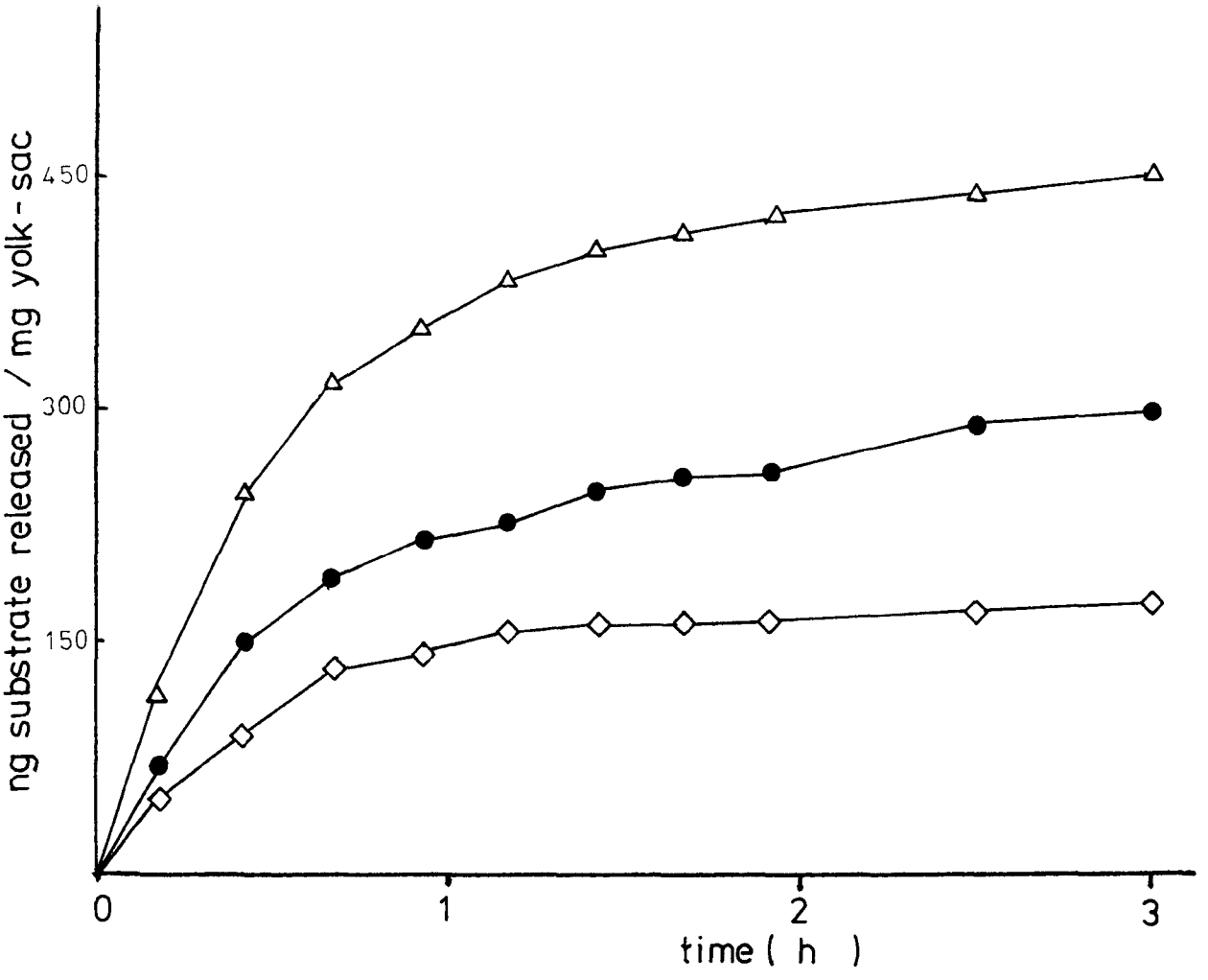


Figure 3.4 Time course of release of radioactive species derived from urea-treated ^{125}I-labelled rat IgG following incubation of rat yolk-sacs with substrate in serum-free medium 199

17.5-day rat yolk-sacs were first incubated for 2h in serum-free medium 199 in the presence of urea-treated ^{125}I-labelled rat IgG (10 µg/ml). Following washing, yolk-sacs were reincubated in fresh medium, and release of radioactivity was monitored up to 3h (see Section 3.2.7 for details). The total amount of substrate associated with the yolk-sac at the beginning of reincubation was 560 ng/mg yolk-sac.
Total radioactivity released —△—
TCA-soluble radioactivity released —●—
TCA-insoluble radioactivity released —◇—
The values shown are means from 6 separate experiments performed with the same batch of urea-treated ^{125}I-labelled rat IgG used in the experiments reported in Table 3.2.7 .

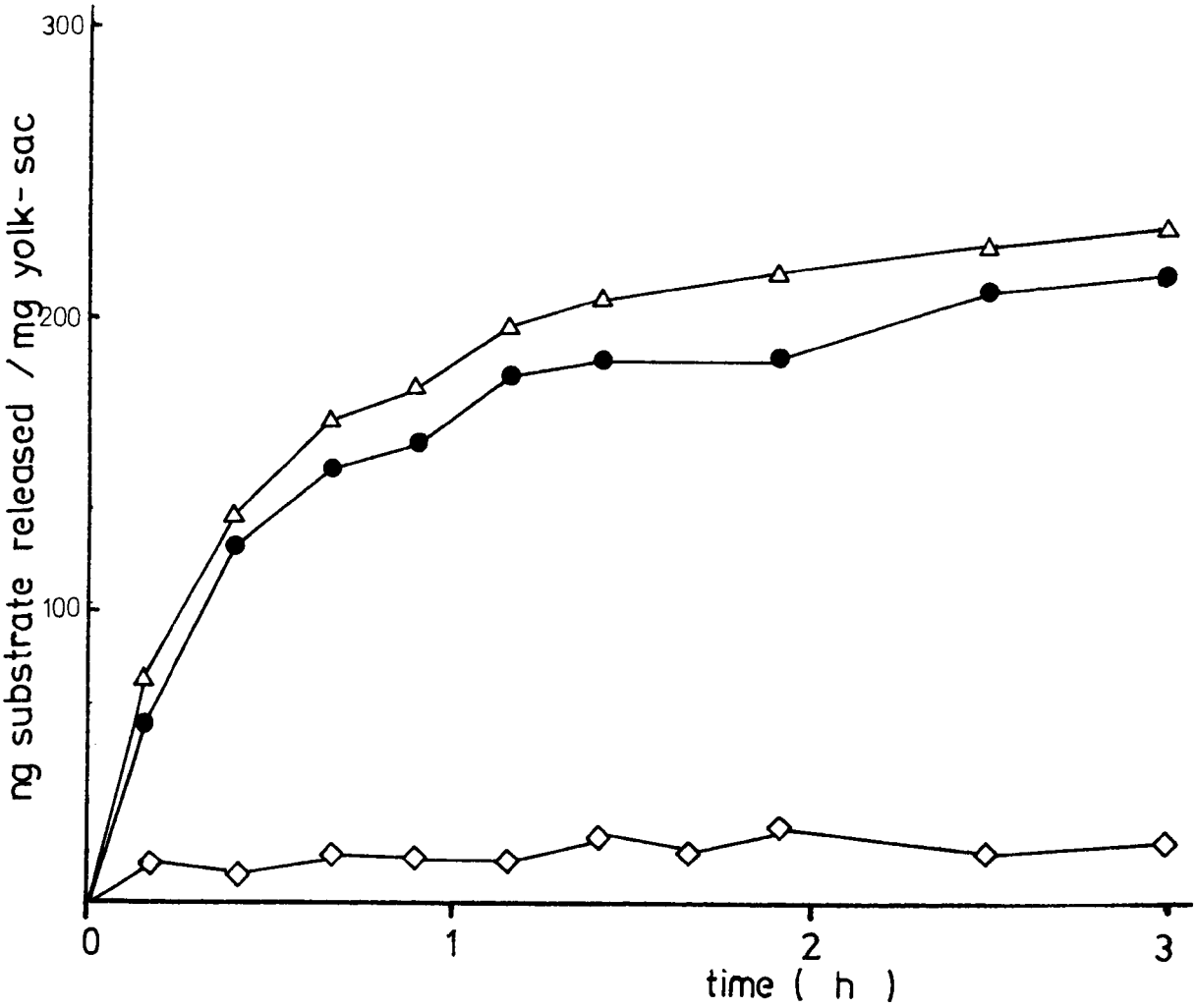


Figure 3.5 Time course of release of radioactive species derived from formaldehyde-treated ^{125}I-labelled rat IgG following incubation of rat yolk-sacs with substrate in serum-free medium 199

17.5-day rat yolk-sacs were first incubated for 2h in serum-free medium 199 in the presence of formaldehyde-treated ^{125}I-labelled rat IgG (10 µg/ml) . Following washing they were reincubated in fresh medium, and release of radioactivity was monitored up to 3h (see Section 3.2.7 for details). The total amount of substrate associated with the yolk-sac at the beginning of reincubation was 306 ng/mg yolk-sac.
Total radioactivity released —△—
TCA-soluble radioactivity released —●—
TCA-insoluble radioactivity released —◇—
The values shown are means from 6 separated experiments performed with the same batch of formaldehyde-treated ^{125}I-labelled rat IgG used in the experiments reported in Table 3.3 .

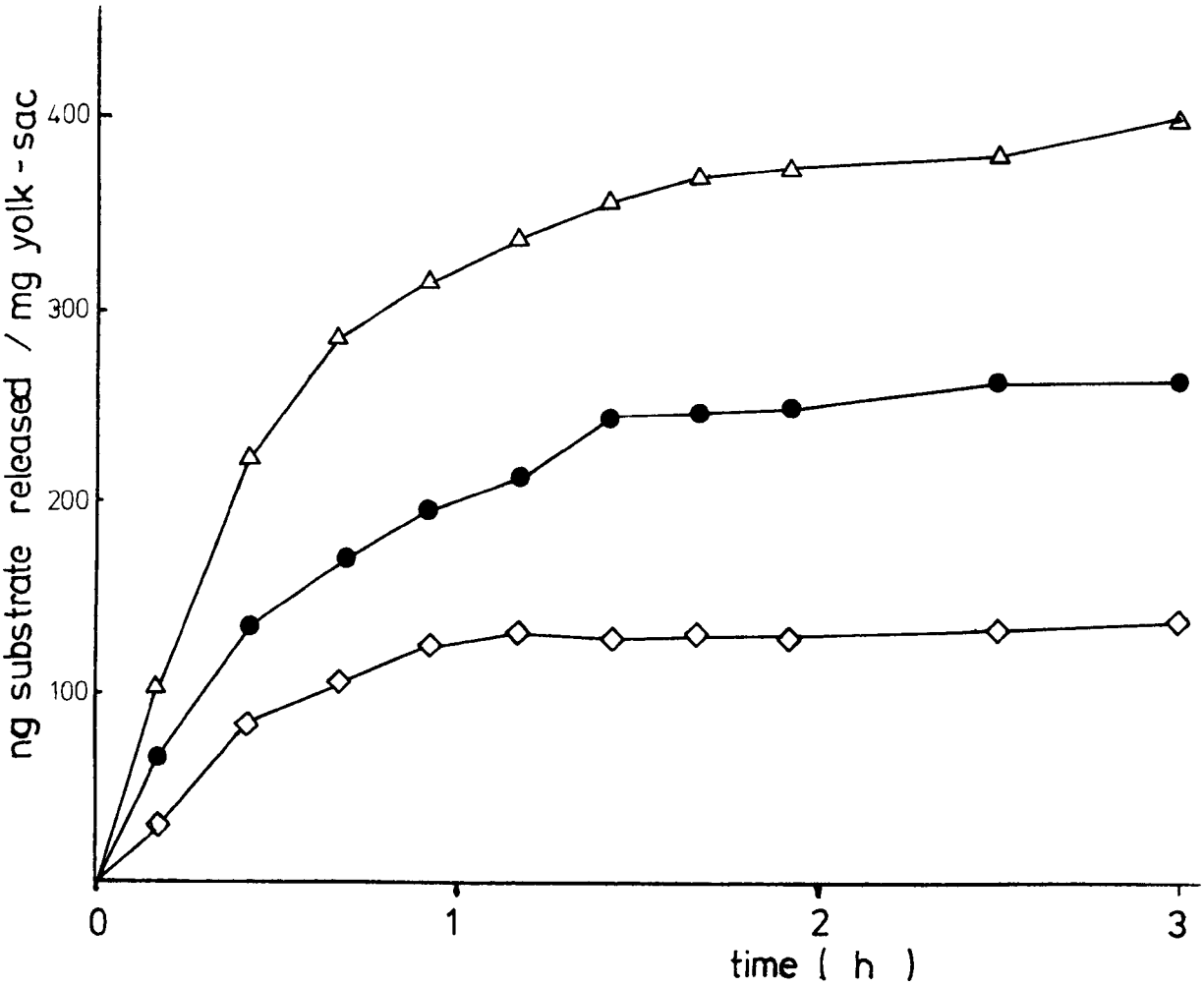


Figure 3.6 Time course of release of radioactive species derived from bicarbonate buffer-treated ^{125}I-labelled rat IgG following incubation of rat yolk-sacs with substrate in serum-free medium 199

17.5-day rat yolk-sacs were first incubated for 2h in serum-free medium 199 in the presence of 0.5M $NaHCO_3$ buffer-treated ^{125}I-labelled rat IgG (10 µg/ml). Following washing yolk-sacs were reincubated in fresh medium, and release of radioactivity was monitored up to 3h (see Section 3.2.7 for details). The total amount of substrate associated with the yolk-sac at the beginning of reincubation was 470 ng/mg yolk-sac.

Total radioactivity released ——△——

TCA-soluble radioactivity released —●—

TCA-insoluble radioactivity released —◇—

The values shown are means from 6 separate experiments performed with the same batch of buffer-treated ^{125}I-labelled rat IgG used in the experiments reported in Table 3.3 .

3.4 DISCUSSION

3.4.1 Effects of differential exposure to chloramine-T during labelling on the fate of ^{125}I-labelled rat IgG in the rat yolk sac in vitro

In an attempt to elucidate the cause of the previously reported inter-batch differences in the endocytic behaviour of different preparations of ^{125}I-labelled IgG by rat yolk sac (Ibbotson *et al.*, unpublished data), the radiolabelling procedure of the immunoglobulin was varied by changing the intesity of exposure of the [^{125}I]iodide and the protein to the oxidizing agent, chloramine-T. Neither an increased chloramine-T concentration nor halving the reaction time seemed to exert a very noticeable effect on the pinocytic uptake of the substrate by the rat yolk sac *in vitro*. Whereas Ibbotson *et al.** reported that different batches of ^{125}I-labelled IgG showed Endocytic Indices that ranged from 6.3 - 13.3 µl/mg protein per h (for incubations in medium 199 containing 10%, v/v, calf serum) and corresponding values of 57-97 µl/mg protein per h (for incubations in serum-free medium 199), the variations in the E.I.s quoted in this chapter are much slighter (see Table 3.1). The rate of uptake of ^{125}I-labelled IgG by rat yolk sacs *in vitro* is only slightly higher when a greater amount of chloramine-T was used in the labelling procedure. Therefore, it seems rather unlikely that small accidental variations in either reaction time or chloramine-T concentration during radioiodination can be responsible for the difference in E.I.s observed by Ibbotson *et al.** for a number of ^{125}I-labelled IgG batches. Equally, the degree of enzymic degradation within the rat yolk sac does not appear to differ between the "Strong", "Normal" and "Mild" ^{125}I-IgG preparations, as expressed in terms of either the Tissue-Accumulation Rate (i.e. that amount of substrate that is not degraded and released by the rat yolk sac *in vitro* subsequent to pinocytic capture, but remains in the tissue) or by the percentage of

*unpublished data

TCA-soluble radioactive species released during reincubation studies. Variations in TCA-soluble release and T.A.R. values are negligible, showing that the different ^{125}I-labelled IgG preparations are degraded at the same rates. These findings suggest no great influence of different chloramine-T exposure on the treatment by the rat yolk sac *in vitro*, of IgG that had been labelled by a method involving this reagent.

Possible harmful effects on the biological activities of a protein are suggested in the literature, but seem to depend on the protein in question. Heward *et al.* (1979a), iodinated alpha and beta melanotropin in the presence of chloramine-T according to the method of Greenwood *et al.* (1963) and reported a resulting inactivation of the molecules as measured by a frog-skin bioassay of a melanoma tyrosinase assay. In a later paper Heward *et al.* (1979b), correlated this inactivation of alpha and beta melanocyte-stimulating hormone to 4 changes in the molecule resulting from the labelling procedure: 1) oxidation of the methionine residue, 2) incorporation of iodine into the tyrosine residue, 3) modification of histidine and 4) modification of tryptophan. Other workers, using a variety of other proteins, claim no such effect of chloramine-T on the biological activity of their radiolabelled proteins; a few examples are quoted below. Hunter & Greenwood (1962) labelled human growth hormone with carrier-free [^{125}I]iodide by use of chloramine-T and found the iodinated molecule to be immunologically identical to unlabelled human growth hormone. Also, Lee & Ryan (1973) studied the interaction of human chlorionic gonadotropin with receptors in cellular fractions of rat ovaries and found full binding-activity, as determined by radio-receptor assay, when this gonadotropin had been labelled by a method based on that of Greenwood *et al.* (1963). Vihko et al. (1978) radiolabelled serum (prostate-specific)

acid phosphatase by a chloramine-T method and still observed a cross-reaction of this molecule with the antibody (prepared in sheep) to the native acid phosphatase. Similarly, Egorin et al., (1979) labelled pure isolectin by a chloramine-T method and noted subsequent binding of the isolectin molecule to human erythrocytes which have specific receptor sites for this protein. Also, Marx et al. (1973) showed that iodination of salmon calcitonin by chloramine-T had no effect on the biological activity of the hormone.

Faced with this ongoing general controversey concerning the use of chloramine-T as oxidizing agent in the iodination of biologically active molecules, it is important to ascertain whether the radioiodination method employed in this study is adequate for the production of labelled IgG species for use in the in vitro rat yolk-sac system. In this context, the recent contribution of Opresko et al. (1980) to the controversy merits special mention. These authors labelled proteins (vitellogenin, bovine serum albumin and X. laevis serum proteins lacking albumin) by a chloramine-T method modified from that of Greenwood et al. (1963). When incubating these ^{125}I-labelled proteins with Xenopus laevis oocytes they found that although the uptake rates of the proteins were not altered, the rate of protein degradation was abnormally high as compared with that of ^{3}H-labelled counterparts. In studies like the present one, that are concerned with the metabolic fate of labelled proteins, such an effect of chloramine-T on radio-labelled proteins would make any experimental findings unreliable. But, from the paper of Opresko et al. (1980), it is interesting to note that these workers used a very high chloramine-T concentration. Their final concentration of chloramine-T in the labelled protein solution was 390 µg/ml, which compared with 285 µg/ml for the "Strong" and 28.5 µg/ml for the "Normal" and "Mild" ^{125}I-labelled IgG preparations used here. It is possible that this very high level of chloramine-T

present in the radioiodination medium causes the observed effects. This suggestion is supported by the findings of McConahey & Dixon (1966). These authors state that the method of Greenwood et al. (1963), which was also used by Ospresko et al. (1980), involves too great a chloramine-T concentration, resulting in greater breakdown of molecules as judged by the in vivo half-lives of the iodinated proteins (40% of the bovine serum albumin injected, i.v., into rabbits was immediately eliminated). When a lower chloramine-T concentration (comparable to that used in this study) was used together with a longer reaction time, they still achieved the same labelling efficiency but did not observe detrimental effects on radioiodinated proteins. In the present study, the degree of molecular breakdown in rat yolk-sac cells was estimated in reincubation experiments, and the observation that the higher chloramine-T concentration does not cause the percentage of IgG molecules that are degraded to increase as compared to the "Mild" and "Normal" preparations, suggests that even this higher chloramine-T concentration is still below a level critical for molecular damage.

The particular concern of the current study is to ensure that the part of the molecule that ensures protection of the molecule from lysosomal attack, on association with the yolk-sac tissue, remains intact. Judging by the TCA-insoluble release during reincubation studies, a great percentage of even the "Strong" ^{125}I-labelled IgG is released in an undegraded form, having been protected from proteolytic attack. Sonoda & Schlamowitz (1972) present evidence that in the ^{125}I-labelled IgG molecule the main bulk of radioiodine is on the Fab region and thus the Fc region, which is believed to be responsible for specific binding during transport through the foetal tissue (Brambell, 1966; Morgan, 1964), is more likely to remain in its native state.

For the remainder of the work reported in this thesis, however,

the "Normal" labelling procedure was adopted, as it corresponds closely with the conditions regarded by other workers as "ideal". A worthwhile series of experiments to help resolve the controversy regarding chloramine-T methods of radiolabelling would have been an investigation of the effects of other different radioiodination methods on IgG behaviour in this *in vitro* rat yolk-sac system. An equivalent project has previously been carried out by Hemmings *et al.* (1974), using suckling-rat and mouse gut as the model system for physiological uptake. In the present work, however, such an investigation has been disregarded in favour of more physiologically relevant studies (Chapter 5).

3.4.2 Effects of formaldehyde- and urea-treatments subsequent to labelling, on the fate of ^{125}I-labelled rat IgG in the rat yolk sac in vitro

The interpretation of the results of uptake studies using formaldehyde- and urea-treated ^{125}I-labelled IgG has to be somewhat tentative because of the lack of full sets of experimental data. Nevertheless, they clearly stand in strong contrast to experimental data in which the E.I. of ^{125}I-labelled BSA was determined after the protein had been subjected to different chemical treatments. Treatment of ^{125}I-BSA with either dilute acid, urea or formaldehyde caused up to a 20-fold increase in the E.I. (Moore *et al.*, 1977), which was explained in terms of loss of helical content and a subsequent unmasking of moieties in the molecule that bind to the yolk-sac endodermal cell membrane. On the other hand a decrease in the rate of endocytic uptake of a molecule after, especially, formaldehyde-treatment, is in keeping with another effect that formaldehyde is known to exert, namely polymerisation of proteins (Meyers & Hardman, 1971; Galembeck *et al.*, 1977). Such formation of protein aggregates has been associated with alteration of the endocytic properties of the protein recognition sites involved in

adsorption of the protein to the plasma membrane; the recognition sites possibly becoming sheltered within the interfacial region between monomers (Livesey & Williams, unpublished data). However, using the in vitro yolk-sac incubation system with simple proteins as substrates, Livesey & Williams (1981) also made the observation that formaldehyde treatment of ^{125}I-labelled insulin, lysozyme and ribonuclease decreased the E.I. of these proteins, though for a reason other than polymerisation. These workers provided evidence of the presence of at least two classes of receptors on the rat yolk sac that bind hydrophobic and basic groups respectively (Livesey & Williams, 1981). In their native state ribonuclease and lysozyme are basic molecules and insulin is hydrophobic, and hence both classes of molecules have a great affinity for the respective yolk-sac membrane binding sites. Regions of the protein that are responsible for adsorption to these classes of binding site are likely to be destroyed by formaldehyde treatment, thereby decreasing the E.I. Serum albumin, on the other hand, carries a negative charge so has a low rate of endocytosis in its native state, but formaldehyde-treatment causes unmasking of recognition sites with a subsequent rise of the E.I. (Moore et al., 1977). ^{125}I-labelled IgG seems to fall between those two extremes of BSA and simple proteins so that neither urea nor formaldehyde have a significant net effect on the rate of uptake of the molecule .

The extent of enzymatic breakdown of chemically-treated ^{125}I-labelled IgG in the rat yolk sac was investigated in the reincubation studies. The results presented here can be discussed more conclusively than the uptake studies, since each experiment was replicated at least six times. Compared with controls, treating ^{125}I-IgG with urea seems to have no effect on the release patterns of this molecule from rat yolk sacs in vitro subsequent to capture. Formaldehyde-treatment, on the

other hand, alters the release pattern of ^{125}I-IgG considerably, both in the presence and absence of calf serum. Unlike the untreated control molecule, which shows at least 40% of the total radioactivity to be released in a TCA-insoluble form, urea- and formaldehyde-treated ^{125}I-labelled IgG are released mainly in the TCA-soluble form. [The bulk, 80%, of the released radioactivity from formaldehyde-treated ^{125}I-IgG is acid-soluble, a finding that resembles the fate of ^{125}I-labelled BSA in rat yolk sacs (see Chapter 2, Figure 2.3)]. It would appear that formaldehyde affects either the overall conformation of the molecule or affects that part of the IgG molecule responsible for ensuring its protection from degradation during transport through the yolk-sac endodermal cells. The notion of such a mode of action of formaldehyde in modifying important functional sites of a protein, can be supported in the literature. Thus Bizzini & Raynaud (1974), in a study of the mechanism of detoxification of protein toxins by formaldehyde, suggested that formaldehyde acts on the ε-amino groups of lysine residues and on other functional parts of the tetanus toxin (i.e. amide, guanidyl, imidazol, phenol and indol groups). Similarly, Feeney (1975) suggested that formaldehyde reacts with amino groups of amino acids. The reaction is considered to proceed to a dihydroxymethyl derivative by the addition of another molecule of formaldehyde to the hydroxymethyl already formed in the initial reaction step (Meens & Feeney, 1971). Although the action of formaldehyde on ^{125}I-IgG may be specific (e.g. affecting the CH1-CH2 domain of the IgG molecule) it is the Fc part of the molecule that has been suggested as the region that binds to specific yolk-sac membrane receptors and so prevents degradation in either the Brambell or the Wild mechanism (Schlamowitz, 1979). A general unfolding of the IgG molecule, as was observed for formaldehyde-treated albumin by Moore et al. (1977), could have the

effect of indirectly modifying the Fc region of IgG, rendering it unable to bind to specific membrane receptors. This suggestion, that the action of formaldehyde is not very specific is supported by another observation in the present study. When using untreated ^{125}I-IgG as substrate, the total amount of substrate released from the rat yolk sac subsequent to endocytic capture is much greater in medium 199 alone than in serum-containing medium. In contrast to this, the total amounts of radioactivity released, following uptake of formaldehyde-treated ^{125}I-IgG into rat yolk sacs are roughly equal in the presence and absence of serum (Table 3.4). This rather atypical behaviour, that has not been observed for any other protein (treated or untreated) suggests that the IgG molecule has been sufficiently denatured to not be "recognized" by any specific receptor at all. Accordingly, no competition should occur between the IgG contained in the serum and the radiolabelled formaldehyde-treated rat IgG.

3.4.3 Some aspects of future work

With formaldehyde and urea, a more general and non-specific denaturation of the IgG molecule has probably been achieved. It would be interesting to pursue the line of chemical treatment further, concentrating on agents that are known to have a more specific action on proteins and especially on different domains of the IgG molecule. Apart from Hemmings & Williams (1977) all workers concerned with the cellular aspect of IgG transport across cells, see the necessity of an initial step inhibiting selective binding to a specific membrane receptor for IgG to be protected against degradation (Brambell, 1966; Jones & Waldmann, 1972; Elson *et al.*, 1975; Rodewald, 1976). The part of the IgG molecule responsible for this binding has been shown to be the Fc region (Brambell, 1966; Morris, 1964). In this context it would be interesting to find a chemical agent that had a specific action on a part of this Fc region and to observe

its effect on endocytosis and release in the *in vitro* rat yolk-sac system. This could lead to a clearer definition of the moiety within the Fc region responsible for binding to yolk sac membrane receptors. In other studies (Stanworth & Turner, 1973; Ellerson *et al.*, 1976; Stanworth & Stewart, 1976) the Fc fragment has been broken down to smaller fragments so that it appears that some biological functions are regulated by either the CH2 or the CH3 domain independently (Yasmeen *et al.*, 1976; Hunneyball & Stanworth, 1976), while other biological processes demand the integrity of the Fc fragment. For example, Guyer *et al.* (1976) demonstrated that in the mouse intestine no subfragment of the Fc region showed any binding. More importantly, McNabb *et al.* (1976) found that, whereas the Fc fragment of human IgG is transported across the human placenta, its CH2 and CH3 subfragments are not. Here the integrity of the Fc fragment seems to be needed for recognition of the molecule by membrane receptors. But in all cases the precise nature of the receptor interaction is not known. Ghose (1972) presented evidence that the Fc region contains some carbohydrate (in the CH2 zone) and Deisendorfer *et al.* (1976) showed that this carbohydrate moiety is firmly attached to the CH2 portion, covering the C face of the molecule. Whether this carbohydrate moiety is involved in membrane binding could be tested in our system by treating the IgG molecule with some specific hydrolases prior to endocytosis. Also attached to some subclasses of IgG is an oligosaccharide moiety on the Fc part (Hinrichs & Smyth, 1970), the function of which could be tested by treatment of the molecule with periodate or another carbohydrate splitting agent.

The possibilities of chemical treatments are virtually unlimited as Argawal & Moore (1979) have demonstrated with albumin as the native substrate, and such an investigation could form an entire and separate project. In this present study, however, a different but related line

of investigation was pursued instead, which seemed to be of greater physiological significance: the comparison of homologous and heterologous IgG molecules with regard to their endocytic and catabolic properties (see Chapter 5). The structural differences between these molecules are probably much smaller and more specific than those that can be induced by chemical modifiers, but the differences in the degree of transport across cells or the degree of enzymic breakdown are startling according to in vivo studies (Koch et al., 1967; Brambell, 1966, 1970; Halliday, 1957).

In summary, the following conclusions have been reached in this chapter:

1) In the range of conditions studied, differential exposure of ^{125}I-iodide and homologous IgG to the oxidizing agent chloramine-T during radioiodination causes little or no variation in a) the rate of endocytic capture and b) the rate of breakdown subsequent to capture of the ^{125}I-labelled IgG in the rat yolk sac in vitro.

2) Chemical treatment of homologous ^{125}I-labelled IgG by either urea or formaldehyde does not induce great variations in either the Endocytic Index or the Tissue-Accumulation Rate.

3) Urea-treatment of homologous ^{125}I-labelled IgG has no effect on the degree of degradation of the substrate in the rat yolk sac in vitro.

4) Formaldehyde treatment of homologous ^{125}I-IgG causes the molecule to be almost entirely degraded by the rat yolk sac in the course of the in vitro incubation, rather than be released again intact from the yolk-sac endodermal cells.

5) Further studies investigating the use of other labelling regimes or other more specific chemical alterations of the IgG molecule were abandoned in favour of a more physiologically relevant line of pursuit.

CHAPTER FOUR

The Effects of Temperature and Metabolic Inhibitors on the Fate of Homologous IgG in Rat and Rabbit Yolk Sacs

4.1 INTRODUCTION

In the previous chapters it was shown that *in vitro* the rat and rabbit yolk sacs treat ^{125}I-labelled homologous IgG atypically as compared with other proteins, in that not all of the ^{125}I-labelled form of this protein is degraded after the protein becomes associated with the tissue. These observations are compatible with the *in vivo* findings that homologous IgG is transported intact across the rabbit yolk-sac splanchnopleur (Brambell *et al.*, 1951) and the rat visceral yolk sac (Mayersbach, 1958). It is thus desirable to try to further investigate the cellular mechanism(s) involved in IgG transport by using the *in vitro* systems developed in our laboratory. Before describing such studies, however, it is important to examine critically the limitations of the findings from the yolk-sac incubation systems.

It is possible to attribute the high release from yolk-sac tissue of TCA-insoluble radioactivity derived from ^{125}I-labelled IgG to the protein stimulating a general fusion of secondary lysosomes and/or phagosomes with the plasma membrane, resulting in the release of the molecules contained in these classes of vesicle. This explanation is rendered unlikely, however, by the observation of Williams & Ibbotson (1979) that tracer quantities of unlabelled IgG had no effect on the rate of release of ^{125}I-labelled PVP from rat yolk sacs previously loaded with this non-degradable macromolecular marker that accumulated in the lysosomal compartment. But, a more serious criticism of such observations is that neither uptake nor release studies give conclusive evidence that the substrate has actually been internalized by the yolk-sac endodermal cells before release from the tissue. It is possible that the ^{125}I-IgG simply adsorbs to the surface of the tissue and during reincubation slowly desorbs into the medium. A similar point, concerning the general mechanism of pinocytosis in mammalian cells was made earlier in a different

context by Ryser (1968) when he drew attention to the necessity of distinguishing between adsorption and internalisation. Thus, it is highly desirable to obtain more direct evidence to distinguish clearly between tissue adhesion and internalisation in the system used in this study, and to establish that nearly all of the tissue-associated ^{125}I-labelled IgG is truly internalized by the rat and rabbit yolk sac in the course of an incubation period.

Ryser (1968) claimed that one method to distinguish between the two possible processes is to modify the temperature of the incubation medium. He claimed that adsorption is expected to be essentially temperature independent, whereas endocytic internalization is not. An inhibition of pinocytosis at lower temperatures has been reported for a variety of cell types and substrates: (Steinman et al., 1974: horseradish peroxidase in mouse L-cells; Bowers & Olszewski, 1972: radioactively-labelled albumin, insulin, leucine and glucose in Acanthamoeba; Walker et al., 1972: horseradish peroxidase in gut-sacs of neonatal and adult rat; Munthe-Kaas, 1977: colloidal gold in Kupffer cells; Chapman-Andresen & Holter, 1964: glucose and albumin in Amoeba Chaos Chaos; Fridhandler & Zipper, 1964: [^{14}C]haemoglobin in in vitro rat yolk sacs. Also, Duncan & Lloyd (1978) found support for Ryser's suggestion by finding that in the rat yolk sac the accumulation of ^{125}I-labelled PVP is strongly temperature dependent.) Low temperature probably decreases the supply of metabolic energy necessary for membrane invagination during endocytosis and also decreases the mobility of membrane constituents, especially as the transition temperature of membrane lipids is approached. Neither effect would be expected to strongly affect adsorption of the substrate to the plasma membrane. Duncan & Lloyd (1978) found that 20°C was the temperature below which pinocytosis of ^{125}I-PVP in the 17.5-day rat yolk-sac system was almost completely inhibited. This observation was used in the current work as the basis of investigating the effect of

lowering the incubation temperature (to 10°C and 15°C) on the subsequent release of ^{125}I-labelled homologous IgG from rat and rabbit yolk sac. If adsorption of ^{125}I-labelled IgG to the yolk-sac surface rather than endocytic internalisation is to account entirely for the observed high release of substrate into the reincubation medium, the same extent of release and the same pattern of release would also be expected under conditions in which membrane invagination is inhibited. If, on the other hand, prior to release ^{125}I-labelled IgG is taken up by endocytosis into rat and rabbit yolk sacs in vitro, lowering the temperature of the incubation medium to below 20°C during the "loading" phase should severely inhibit release of radioactivity from yolk sacs during the reincubation phase. Yet another possibility that must be considered is that only a fraction of the tissue-associated ^{125}I-labelled IgG (i.e. that which is degraded by the yolk sac) is internalized, whereas the remainder of the molecules strongly adsorbs to the plasma membrane, but desorbs slowly during the reincubation. Such an effect would permit the majority of the observations of Williams & Ibbotson (1979) to be explained entirely in terms of the well-established catabolic capacity of rat yolk-sac tissue towards proteins and enable the transcellular transport interpretation of their observations to be completely discarded.

If the metabolic inhibitor, 2,4-dinitrophenol, rather than low temperature is used to inhibit pinosome formation in the rat and the rabbit yolk sac in vitro, similar results would be predicted. Moreover, the effect of low temperature on cells and tissues is not fully understood, but that of 2,4-dinitrophenol is more specific. This compound is known to decouple oxidative phosphorylation thus affecting the energy supply for cytoskeletal action and it has been used to inhibit pinocytosis and phagocytosis in a number of cell types. For example, Munthe-Kaas (1977) showed inhibition of uptake of colloidal [^{198}Au]gold into

Kupffer cells by 2,4-dinitrophenol, and Duncan & Lloyd (1978) demonstrated the inhibition of uptake of ^{125}I-labelled PVP into 17.5-day rat yolk sacs incubated *in vitro* in a medium containing calf serum (10%, v/v) and 2,4-dinitrophenol at a concentration of 50 μg/ml. Since this present work involves the use of serum-free medium, the effective concentration of 2,4-dinitrophenol had to be re-established for such a medium (since it may bind to serum proteins) before determining release patterns of yolk sacs "loaded" with ^{125}I-labelled IgG in the presence of this inhibitor. For this purpose, the general experimental practice of Duncan & Lloyd (1978) was followed and 17.5-day rat yolk sacs were incubated in serum-free medium in the presence of ^{125}I-PVP and 2,4-dinitrophenol at one of three different concentrations. Uptake data were reported in the form of Endocytic Indices.

The effect of another metabolic inhibitor, ammonium ions, was also investigated. The effects of ammonium ions on yolk sacs have been studied and are known to be two-fold; Livesey *et al*. (1980) showed that ammonium ions both strongly inhibit the endocytosis of ^{125}I-labelled PVP in rat yolk sacs *in vitro* in addition to the more widely reported effect of interference with the intralysosomal degradation of internalized proteins. The latter effect has been observed in a variety of cell types (Tolleshaug *et al*., 1977; Carpenter & Cohen, 1976; Hopgood *et al*., 1977; Seglen & Reith, 1976). The first effect makes it possible to use ammonium ions in much the same way as 2,4-dinitrophenol, that is to inhibit substrate internalisation in rat yolk sacs at 37^{o}C, without affecting membrane binding, and studying subsequent substrate release.

In summary, this chapter poses one central question. Is all the homologous ^{125}I-labelled IgG that becomes associated with rat and rabbit yolk sacs *in vitro* endocytically ingested, or does it merely adhere to the external surface of the plasma membrane thereby giving the misleading impression that IgG is protected against degradation after endocytic capture

by these tissues?

In an attempt to answer this important question, the effect on inhibition of endocytic uptake, while leaving substrate: membrane-binding unhindered, was investigated. Inhibition of uptake was induced by: a) low temperature, b) 2,4-dinitrophenol and c) ammonium ions.

4.2 METHODS

4.2.1 Exocytosis of homologous ^{125}I-labelled IgG by rat and rabbit yolk sacs that had previously been incubated with the substrate at different incubation temperatures in serum-free medium 199

The general method outlined in Section 1.12 was followed. Either 17.5-day rat yolk sacs or 24-day rabbit yolk-sac pieces were incubated for 2h in medium 199 containing homologous ^{125}I-labelled IgG (10 µg/ml). Incubation temperatures during the uptake phase were 15°C, 20°C, 25°C or 37°C for rat yolk sacs and 10°C, 20°C or 37°C for rabbit yolk sacs. Yolk-sac tissues were then rinsed in warm (37°C) substrate-free medium 199 and reincubated in fresh medium 199 at 37°C. Aliquots of medium were removed at 15 min intervals during the 3h reincubation period (for full method see Section 1.12). The total amount of radioactivity released and the amount of the TCA-soluble radioactivity released were each determined (see Section 1.6) and from these data the amount of acid-insoluble radioactivity released was calculated (see Section 1.12).

4.2.2 Uptake of ^{125}I-labelled PVP by rat yolk sacs, incubated in serum-free medium 199 or in medium 199 containing calf serum, in the presence of 2,4-dinitrophenol

^{125}I-labelled PVP was used as substrate at a concentration of 2µg/ml, and 17.5-day rat yolk-sac incubations were set up as described in full in Sections 1.2 and 1.7. The metabolic inhibitor 2,4-dinitrophenol was added to incubation flasks 1h before addition of the substrate at the concentration of 10, 20 or 40 µg/ml in serum-containing medium and at the concentrations of 5, 10, 20, 25, 40 or 50 µg/ml in serum-free medium 199. Tissue incubations were terminated at regular intervals up to 6.5h and assays of the amount of radioactivity contained in the medium

and in the yolk sacs were performed as previously described (Sections 1.4 and 1.5). Uptake of substrate was plotted against time for each set of data and the Endocytic Index derived (see Section 1.9 for details of calculation).

4.2.3 Exocytosis, from rat yolk sacs reincubated in serum-free medium 199 containing 2,4-dinitrophenol, of ^{125}I-labelled PVP accumulated by prior incubation in serum-free medium 199

The general method outlined in Section 1.12 was followed using ^{125}I-labelled PVP (10 µg/ml) as substrate. The initial incubation period of the 17.5-day rat yolk sacs in medium 199 in the presence of the substrate was 3h, and 2,4-dinitrophenol was added to the reincubation medium at a concentration of 10 µg/ml.

4.2.4 Exocytosis from rat and rabbit yolk-sac tissue of ^{125}I-labelled homologous IgG ingested from serum-free medium 199 containing 2,4-dinitrophenol

The general method outlined for rat and rabbit yolk sacs in Section 1.12 were followed. Using rat yolk sacs, 2,4-dinitrophenol (5, 10, 25, or 50 µg/ml) was added to the medium during the incubation stage 1h before the addition of the ^{125}I-labelled rat IgG (10 µg/ml). The total incubation period was 3h instead of 2h. Using rabbit yolk sacs, 2,4-dinitrophenol (10 or 25 µg/ml) was added at the same time as the substrate, ^{125}I-labelled rabbit IgG. The remainder of the procedure follows the general methods outlined earlier (see Section 1.12).

4.2.5 Uptake of ^{125}I-labelled rat IgG by rat yolk sacs incubated in serum-free medium 199 containing ammonium ions

Rat yolk sacs (17.5-day) were incubated in serum-free medium 199 as described in Section 1.2. Ammonium chloride (30mM or 5mM) was added to the incubation medium together with the substrate, ^{125}I-labelled rat IgG

(2 µg/ml). Yolk-sac incubations were then terminated at regular intervals up to 6.5h and assays of the amount of radioactivity contained in the medium and in the yolk sacs were performed (as outlined in Sections 1.4 and 1.6). Uptake of substrate was plotted against time for each set of data and the Endocytic Index derived (see Section 1.10 for details of calculation).

4.2.6 Exocytosis, from rat yolk sacs reincubated in serum-free medium 199 containing ammonium ions, of ^{125}I-labelled rat IgG accumulated by prior incubation in serum-free medium 199

The general method outlined in Section 1.12 was followed and 17.5-day rat yolk sacs were incubated in serum-free medium 199. Three different regimes were adopted for exposing the tissue to ammonium ions. 1) Ammonium chloride (30mM or 5mM) was added to the "loading"-phase medium at the same time as ^{125}I-labelled rat IgG (10 µg/ml). After washing, reincubation was in fresh medium 199 and samples were removed at regular intervals up to 3h. 2) The initial 2h incubation period of the rat yolk sacs was in serum-free medium 199 containing ^{125}I-labelled rat IgG (10 µg/ml). After washing, yolk sacs were reincubated in fresh medium 199 containing ammonium chloride (30mM or 5mM). Samples of medium were removed at regular intervals, taking care to replace the removed ammonium ions when replenishing the medium in order to maintain a constant concentration of ammonium ions. 3) Ammonium ions (30mM or 5mM) were present during both, the "loading" and the reincubation-phases.

4.3 RESULTS

4.3.1 The effect of low temperature on the subsequent release of homologous ^{125}I-labelled IgG from rat and rabbit yolk sacs

Duncan & Lloyd (1978) found that lowering the temperature of the medium to 20°C or below caused a marked decrease in the Endocytic Index of ^{125}I-PVP in 17.5-day rat yolk sacs. Uptake was reported to be inhibited to more than 80% at 20°C. This temperature gave a guideline to the temperature range (10-37°C) used in the current study. As might have been expected, the greatest effect on the substrate release patterns was observed when using the lowest temperature, 10°C in the rabbit and 15°C in the rat yolk sacs (see Figures 4.1 and 4.2). At these "loading" temperatures all release on subsequent incubation at 37°C is effectively abolished. The total amount of radioactivity released from yolk sacs does not appear to rise linearly with temperature. Figure 4.1, which represents the release of ^{125}I-labelled IgG from rat yolk sacs, shows a sharp rise of radioactive release between 20°C and 25°C. By the latter temperature, release resembles that of the control incubation at 37°C. For rabbit yolk sacs it is more difficult to come to any conclusion concerning linearity of inhibition with temperature because experiments were only performed at three different temperatures (Figure 4.2). The relative amounts of TCA-soluble and TCA-insoluble species derived from homologous ^{125}I-IgG stay the same throughout the temperature range tested (see Figures 4.1 and 4.2). In particular, no increase was observed in the relative amount of TCA-insoluble activity released on reincubating those tissues that were "loaded" at lower temperatures.

4.3.2 The effect of 2,4-dinitrophenol on the uptake and release of ^{125}I-labelled PVP in rat yolk sacs in vitro

In order to use 2,4-dinitrophenol as an inhibitor of substrate

internalisation during the uptake phase of a reincubation experiment, a concentration had first to be established at which this metabolic inhibitor would be effective in inhibiting endocytosis without being cytotoxic. Duncan & Lloyd (1978) found that in medium 199 containing 10% (v/v) calf serum, uptake of ^{125}I-PVP was completely inhibited at a concentration of 50 µg/ml. In serum-free medium 199 the concentration dependence had to be established. Figure 4.3 shows that in serum-containing medium the uptake of ^{125}I-labelled PVP is inhibited almost totally by a 2,4-dinitrophenol concentration of 20 µg/ml or more, supporting the findings of Duncan & Lloyd (1978). In serum-free medium 199, a lower concentration of 2,4-dinitrophenol (10 µg/ml) achieved the same effect, while 5 µg/ml lowered the E.I. of ^{125}I-PVP by 50%. In reincubation studies it was therefore decided to use 2,4-dinitrophenol concentrations of 10 µg/ml and higher. The possibility that 2,4-dinitrophenol may be cytotoxic to rat yolk sacs, was tested by determining its effect on the release, over a period of time, of ^{125}I-labelled PVP from tissues that had previously accumulated this compound. During this time 2,4-dinitrophenol was present at a constant concentration of 10 µg/ml. From Table 4.1 it appears that the presence of the metabolic inhibitor caused no difference in the release pattern between 15 and 180 min. Cell death would be expected to lead to enhanced release of this marker (see Livesey et al., 1980).

4.3.3. The effect of 2,4-dinitrophenol, present during the uptake phase, on the subsequent release of homologous ^{125}I-labelled IgG from rat and rabbit yolk-sac tissue

Having established the concentration of 2,4-dinitrophenol effective for inhibition of pinocytic uptake in rat yolk sacs when incubated in serum-free medium, the compound was added at this and higher concentration to the medium in which rat and rabbit yolk sac were incubated during the

uptake phase. From Figures 4.4 and 4.5 it appears that in both rat and rabbit tissues the subsequent release of homologous ^{125}I-labelled IgG was reduced considerably when the incubation medium contained 10 µg/ml or more of 2,4-dinitrophenol during the "loading" phase. For rat yolk sacs the decrease of substrate release is greater than for rabbit yolk sacs (at 10 µg/ml 2,4-dinitrophenol 89% and 72% of controls, respectively). This difference may arise from slight differences in the experimental regimes. In the rat yolk-sac incubations 2,4-dinitrophenol was added 1h before the substrate, whereas in the rabbit yolk-sac incubations both were added simultaneously. This difference resulted from a desire to keep the total incubation time of rabbit yolk sacs to a minimum because of the relatively lower period of survival of this tissue in vitro (see also Chapter 2). The relative percentage of TCA-soluble and -insoluble release remained constant for all 2,4-dinitrophenol concentrations tested in both rat and rabbit incubations (see Figures 4.4 and 4.5).

4.3.4 Effect of ammonium ions on the uptake of ^{125}I-labelled rat IgG in rat yolk sacs

Table 4.2 shows the Endocytic Indices and Tissue-Accumulation Rates of ^{125}I-labelled rat IgG in rat yolk sacs incubated in serum-free medium 199 in the presence of ammonium chloride at a concentration of either 5mM or 30mM. While both the Endocytic Index and the Tissue-Accumulation Rate are markedly reduced by the lower ammonium ion concentration, as compared with the control values, total inhibition of endocytosis can only be achieved by increasing the ammonium ion concentration to 30mM. It may be noted that, relative to control tissue, the lower ammonium-ion concentration (5mM) decreases the E.I. more strongly than it decreases the T.A.R. (67% vs. 39%; see Table 4.2).

4.3.5 The effect of ammonium ions, present during uptake of substrate, on the subsequent release of ^{125}I-labelled rat IgG from rat yolk sacs

In the exocytosis studies, the findings are rather complex. When ammonium chloride is present at a concentration of 30mM when yolk sacs are loaded with ^{125}I-labelled IgG uptake is inhibited and no appreciable amounts of radioactivity are released into the reincubation medium (Figure 4.6); these findings are expected from the data reported in Table 4.2. This effect is virtually the same as that observed when ammonium ions are present in the medium both during "loading" and during the reincubation period. However, when ammonium ions (30mM) are present in the reincubation medium only, and endocytosis is allowed to proceed unhindered during the "loading" phase, radioactivity is released in both TCA-soluble and TCA-insoluble forms, following a similar general pattern to the control, but with the amount of acid-solubles reduced by some 30%-40%; the total amount of radioactivity released is decreased to a similar extent.

When ammonium chloride (5mM) is present during only the uptake phase, the pattern of subsequent release of ^{125}I-labelled IgG does not differ noticeably from that of the control (Figure 4.7). Adding the inhibitor during the release phase as well, reduces total, TCA-soluble and TCA-insoluble release. Also, under these conditions only 59% of the total amount of radioactivity associated with the yolk sac at the beginning of the reincubation period is released, whereas for the control culture the value is 86%. The same effect, i.e. a lower total release of radioactivity, is also observed when ammonium ions (5mM) are present during only the exocytosis phase. At the same time the release of acid-solubles is decreased, below even the level of acid-insolubles. These effects are compatible with a decrease in the production of acid-solubles in the presence of this lower concentration of the base.

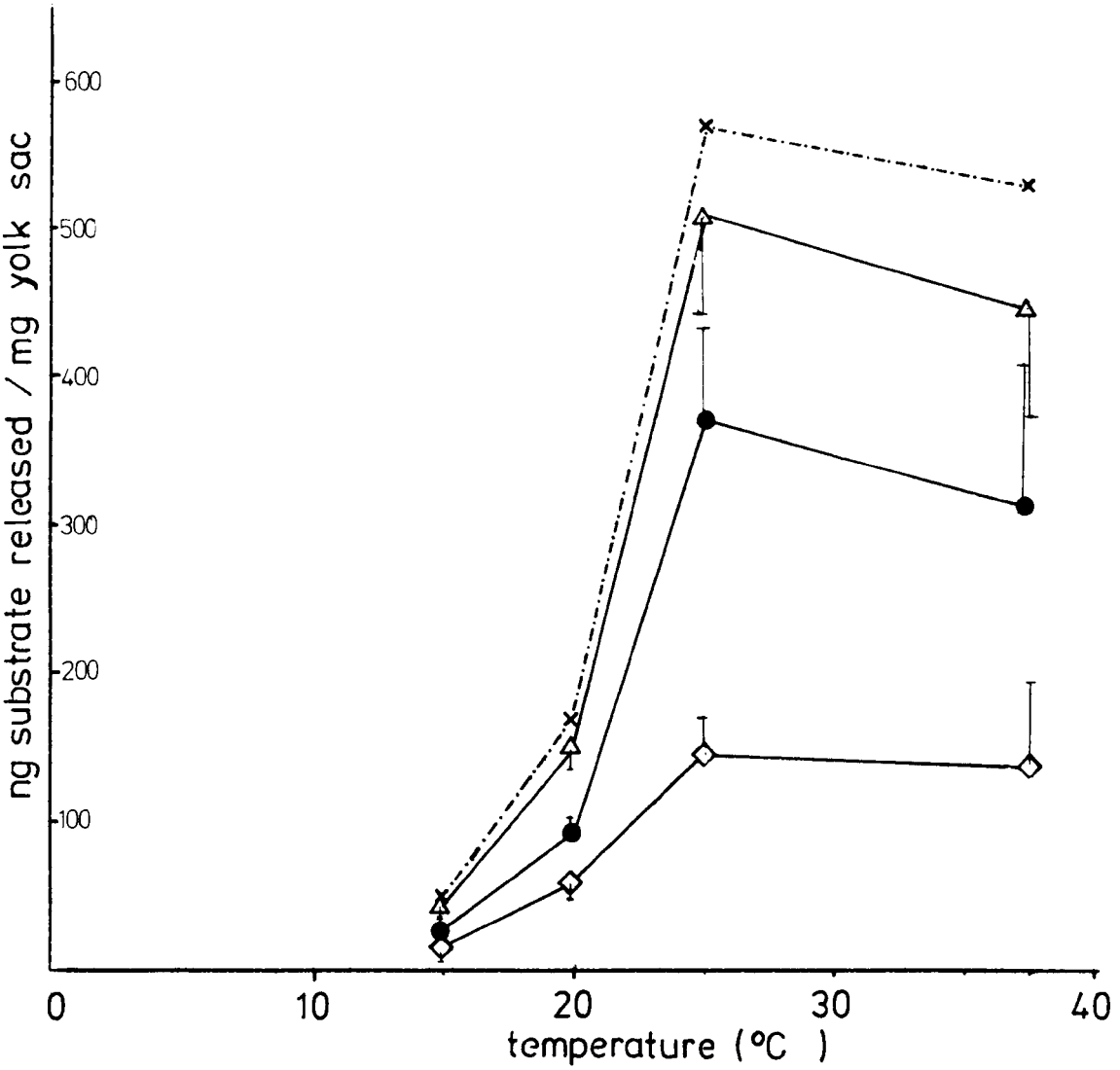


Figure 4.1 Effect of different incubation temperatures during the "loading" of rat yolk sacs with ^{125}I-labelled rat IgG on the subsequent release of this substrate at 37°C

17.5-day rat yolk sacs were first incubated for 2h in serum-free medium 199 in the presence of ^{125}I-labelled rat IgG (10 µg/ml). The temperature of the medium was 15°C, 20°C, 25°C or 37°C. Following washing, yolk sacs were reincubated in fresh medium 199 at 37°C, and the release of radioactivity was monitored up to 3h (see Section 4.2.1 for details).
Total radioactivity ——△——
TCA-soluble radioactivity ——●——
TCA-insoluble radioactivity ——◇—— released at 3h of reincubation time are shown. Also a measure is given of that amount of substrate associated with the yolk sac at the beginning of the reincubation period -·-·-·-×-·-·-·- . The values shown are means (± S.D.) of 6 (15°C), 9 (20°C), 12 (25°C) and 13 (37°C) separate experiments performed for each incubation temperature.

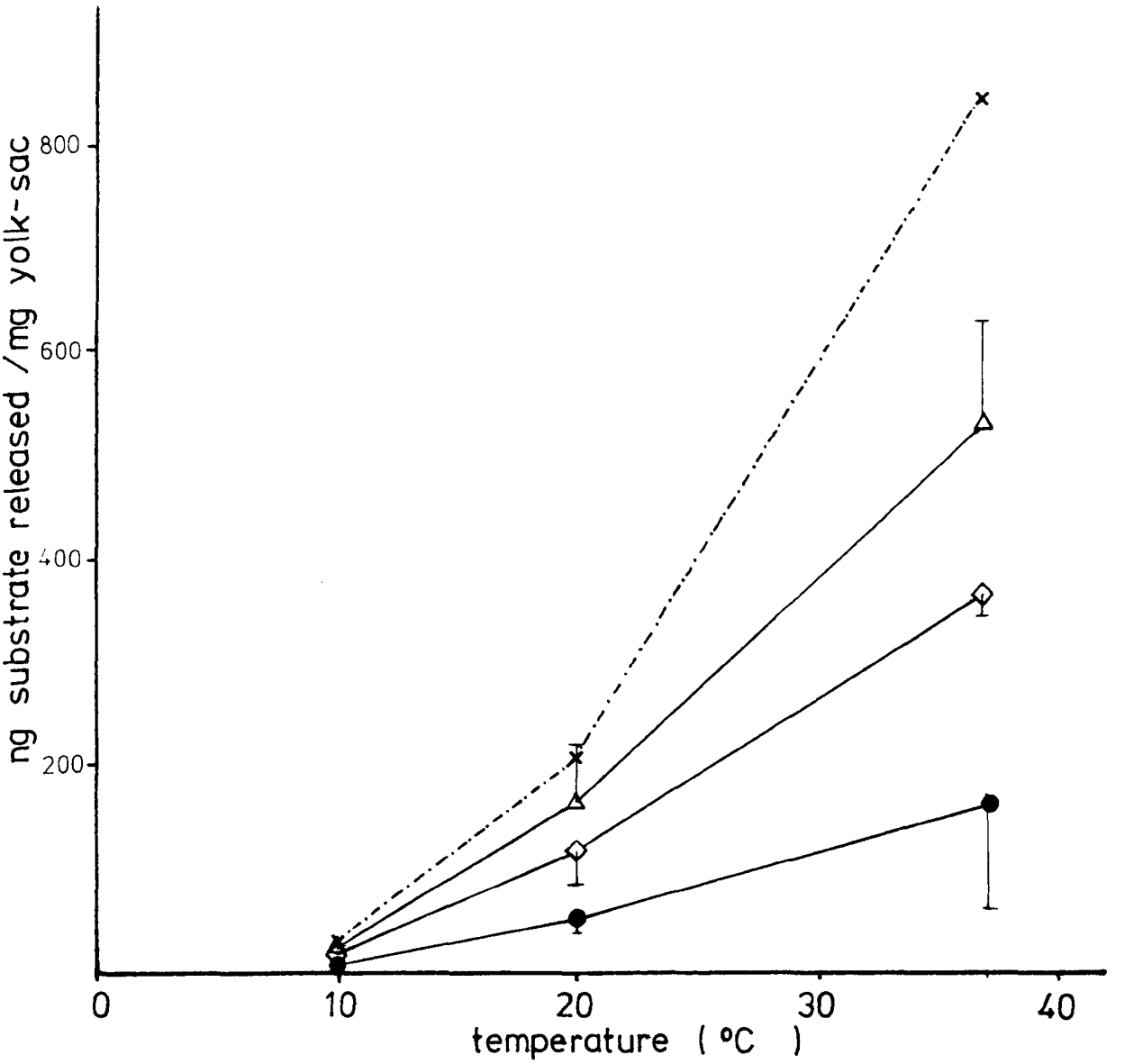


Figure 4.2 Effect of different incubation temperatures during the "loading" of rabbit yolk sacs with ^{125}I-labelled rabbit IgG on the subsequent release of this substrate at 37°C

24-day rabbit yolk-sac pieces were first incubated for 2h in serum-free medium 199 in the presence of ^{125}I-labelled rabbit IgG (10 µg/ml). The temperature of the medium was 10°C, 20°C or 37°C. Following washing, yolk-sac pieces were re-incubated in fresh medium 199 at 37°C, and the release of radioactivity was monitored up to 3h (see Section 4.2.1 for details). The amounts of total radioactivity ——△—— , TCA-soluble radioactivity ——●—— and TCA-insoluble radioactivity —◇— are shown. Also a measure is given of that amount of substrate associated with the yolk sac at the beginning of the reincubation period-·-·-×-·-·-. The values shown are means (± S.D.) of 6 separate experiments performed for each incubation temperature.

Table 4.1 Release of ^{125}I-labelled PVP from rat yolk sacs reincubated in the presence of 2,4-dinitrophenol

Rat yolk sacs (17.5-day) were first incubated for 2h in serum-free medium containing ^{125}I-labelled PVP (10 µg/ml). Following washing, they were reincubated in fresh medium 199 containing 2,4-dinitrophenol (10 µg/ml). The release of radioactivity from yolk sacs was monitored for 3h (see Section 1.12 for details). The equivalent control experiments, in which ^{125}I-labelled PVP release was in medium 199 alone for 3h, are reported Section b of this table.

a) Reincubation in the presence of 2,4-dinitrophenol

Values shown are means (± S.D.) of 5 separate experiments

Reincubation time (min)	Substrate release	
	ng/mg yolk-sac protein	percentage
15	8.47 ± 1.73	10.18 ± 0.52
30	8.52 ± 1.66	11.53 ± 1.66
45	9.04 ± 1.93	12.25 ± 2.18
60	9.92 ± 2.02	13.46 ± 2.48
90	10.07 ± 1.16	13.66 ± 0.95
120	10.42 ± 0.80	14.14 ± 0.29
150	10.61 ± 1.23	14.38 ± 0.13
180	11.01 ± 1.64	14.89 ± 1.26

b) Reincubation in medium 199 alone

Values shown are means (± S.D.) of 5 separate experiments

Reincubation time (min)	Substrate release	
	ng/mg yolk-sac protein	percentage
15	3.92 ± 1.67	3.01 ± 0.88
30	4.27 ± 1.62	3.42 ± 0.98
45	5.04 ± 1.67	3.91 ± 0.86
60	5.42 ± 2.14	3.84 ± 1.05
75	5.42 ± 2.09	4.16 ± 1.04
90	6.90 ± 1.60	5.55 ± 1.96
105	7.24 ± 1.38	5.82 ± 1.88
120	7.78 ± 1.48	6.23 ± 1.88
135	8.03 ± 1.31	6.44 ± 1.93
150	8.07 ± 1.43	6.46 ± 1.89
165	8.36 ± 1.26	6.73 ± 2.02
180	8.77 ± 1.08	7.00 ± 1.66

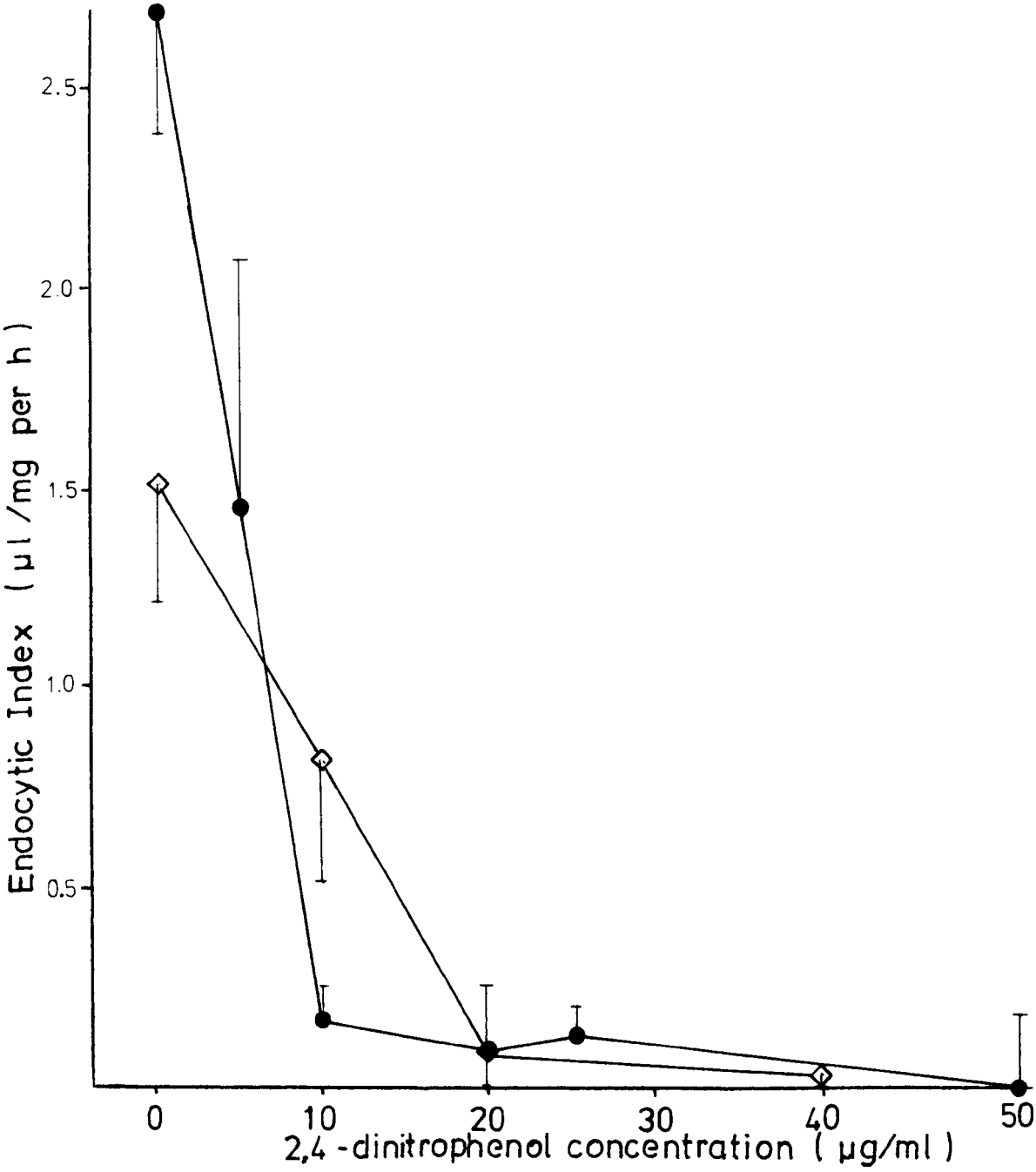


Figure 4.3 Effect of 2,4-dinitrophenol on the uptake of ^{125}I-labelled PVP in rat yolk sacs in vitro in the presence and absence of calf serum

17.5-day rat yolk sacs were preincubated for 1h in medium 199 containing 10%(v/v) calf serum or in serum-free medium 199 in the presence of 2,4-dinitrophenol at the concentrations indicated. ^{125}I-labelled PVP was then added and incubations were terminated at regular intervals up to 6.5h (see Section 4.2.2 for details). The graph represents mean Endocytic Indices (± S.D.) for serum-containing medium —◇— and for serum-free medium —●—. For each concentration and medium condition 3 separate experiments were performed.

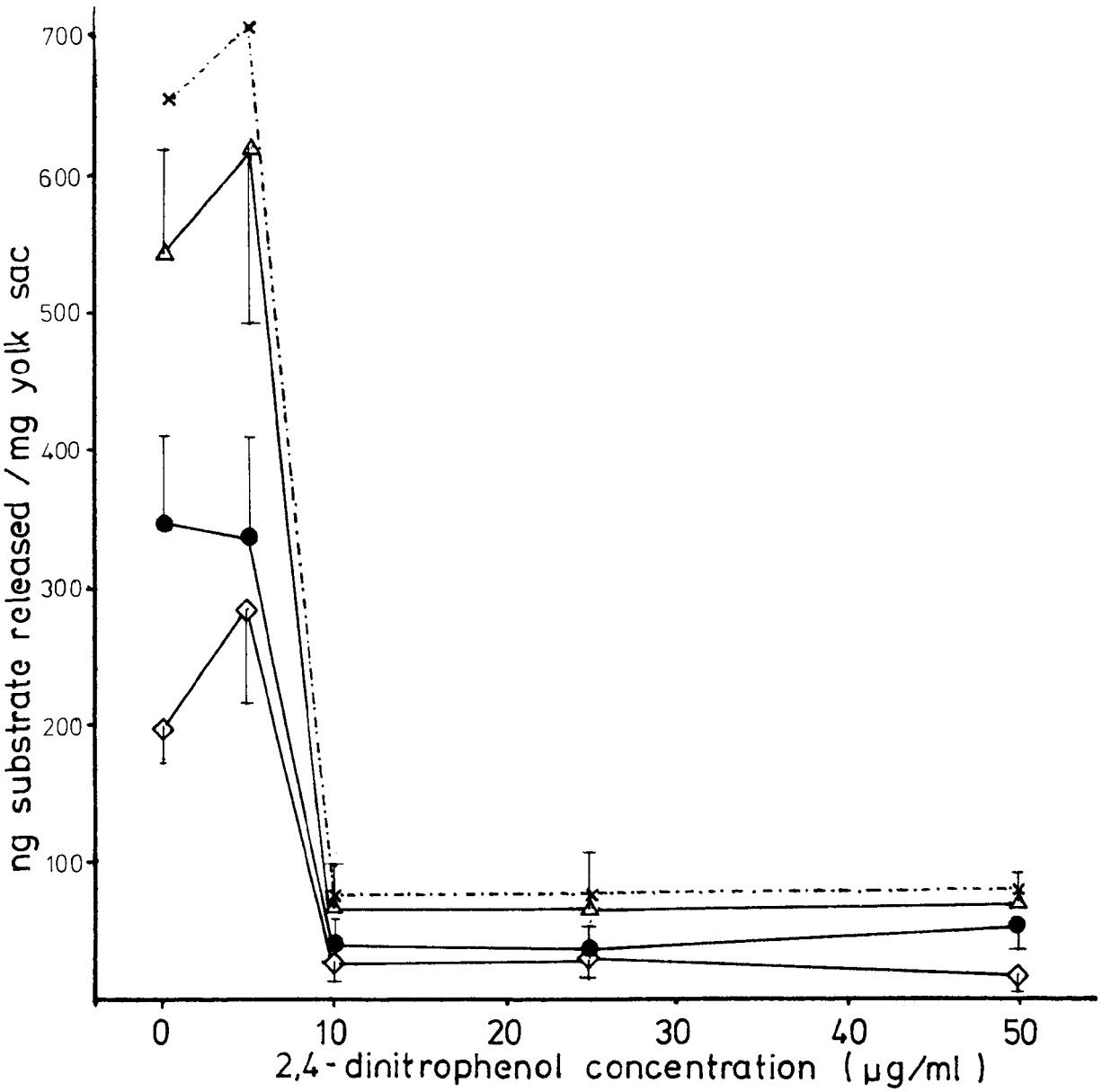


Figure 4.4 Effect of the presence of different concentrations of 2,4-dinitrophenol in serum-free incubation medium, during the "loading" of rat yolk sacs with ^{125}I-labelled rat IgG, on the subsequent release of this substrate

17.5-day rat yolk sacs were first incubated for 1h in serum-free medium 199 in the presence of 2,4-dinitrophenol (5, 10, 25 or 50 µg/ml) before addition of the substrate ^{125}I-rat IgG. After a further 2h of incubation yolk sacs were washed and reincubated in fresh medium 199 alone and the release of radioactivity was monitored up to 3h (see Section 4.3.2 for details). This graph represents the amounts of total radioactivity —Δ— , of TCA-soluble radioactivity —●— and of TCA-insoluble radioactivity —◇— released at 3h of reincubation time for each 2,4-dinitrophenol concentration. Also a measure is given of that amount of substrate associated with the yolk sac at the beginning of the reincubation time ---×---. The values shown are means (± S.D.) from 6 separate experiments performed for each 2,4-dinitrophenol concentration.

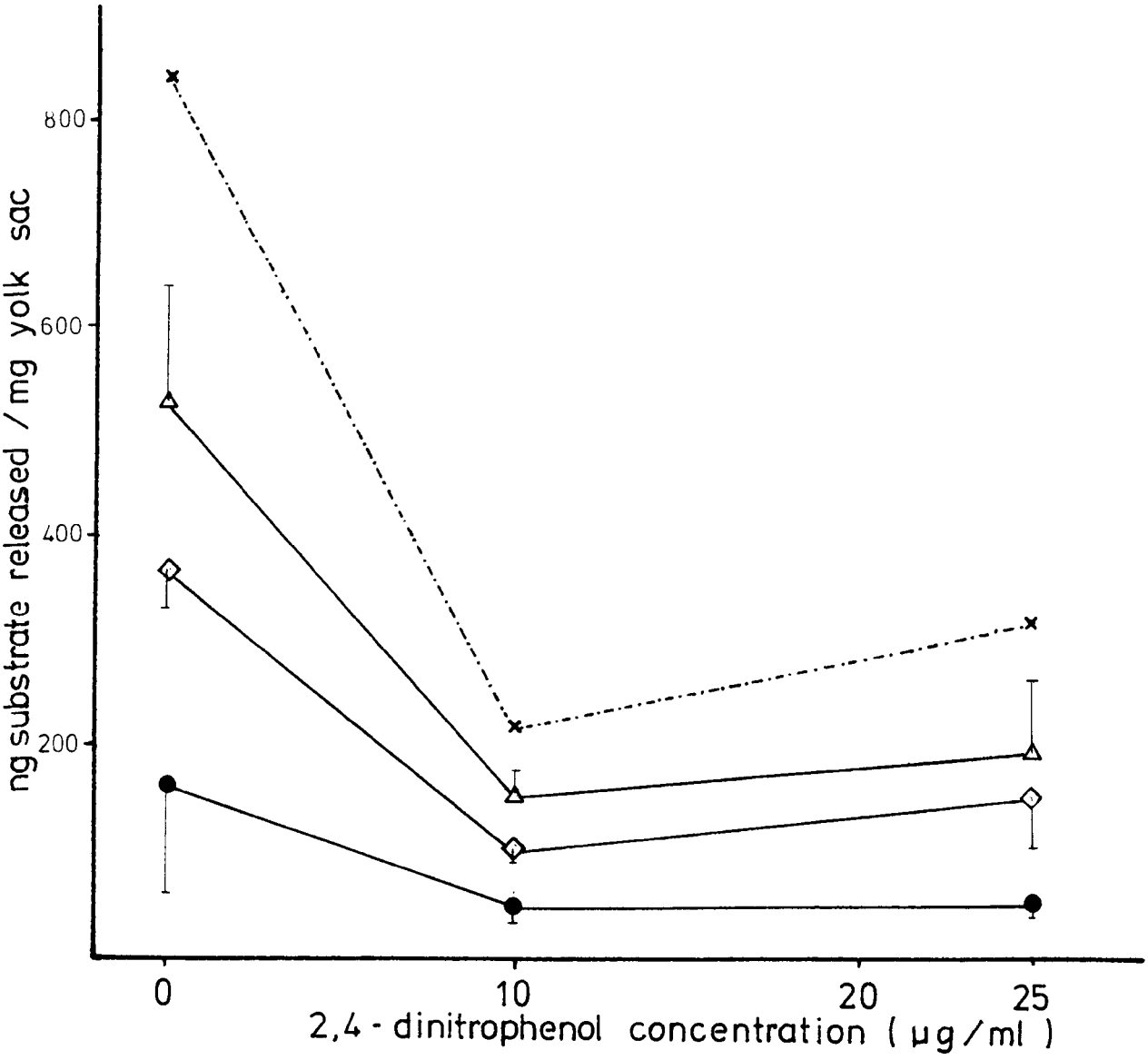


Figure 4.5 Effect of the presence of different concentrations of 2,4-dinitrophenol in the incubation medium during the "loading" of rabbit yolk sacs with ^{125}I-labelled rabbit IgG on the subsequent release of this substrate

24-day rabbit yolk-sac pieces were first incubated for 2h in serum-free medium 199 in the presence of ^{125}I-labelled rabbit IgG (10 µg/ml) and 2,4-dinitrophenol (10 or 25 µg/ml). Following washing yolk-sac pieces were reincubated in fresh medium 199 and the release of radioactivity was monitored for up to 3h (see Section 4.2.4 for details). The graph represents the amounts of total radioactivity ——△—— , of TCA-soluble radioacticity ——●—— and of TCA-insoluble radioactivity ——◇—— released at 3h of reincubation time. A measure is also given of that amount of substrate associated with the yolk sac at the beginning of the reincubation period -·-·-·-×-·-·-·-. The values shown are means (± S.D.) from 6 separate experiments performed at each 2,4-dinitrophenol concentration.

Table 4.2 Endocytic Indices and Tissue-Accumulation Rates of ^{125}I-labelled rat IgG in 17.5-day rat yolk sacs incubated in serum-free medium 199 in the presence of ammonium ions

Rat yolk sacs (17.5-day) were incubated for up to 6.5h with ^{125}I-labelled rat IgG (2 µg/ml medium) in serum-free medium 199 in the presence of ammonium chloride (30mM or 5mM), by using the method described in Section 4.2.5.

Concentration of NH_4Cl (mM)	TCA-solubles in preparation (%)	No. of yolk sacs	Endocytic Index (µl/mg protein per h)	Correlation Coefficient	Tissue-Accumulation Rate (µl/mg protein per h)	Correlation Coefficient
0	3.67	9	98.77	0.950	14.05	0.909
0	3.15	9	45.21	0.893	6.84	0.676
MEAN VALUES :			71.99		10.44	
5	3.51	10	12.23	0.885	5.21	0.831
5	3.51	10	19.69	0.932	5.50	0.604
5	3.51	10	23.93	0.960	8.29	0.892
MEAN VALUES (± S.D.):			18.61 ± 5.92		6.33 ± 1.70	
30	3.60	10	-1.57	-0.398	0.17	0.139
30	3.05	9	2.45	0.497	1.19	0.555
30	3.05	9	0.19	0.036	0.68	0.394
MEAN VALUES (± S.D.):			0.36 ± 2.01		0.68 ± 0.51	

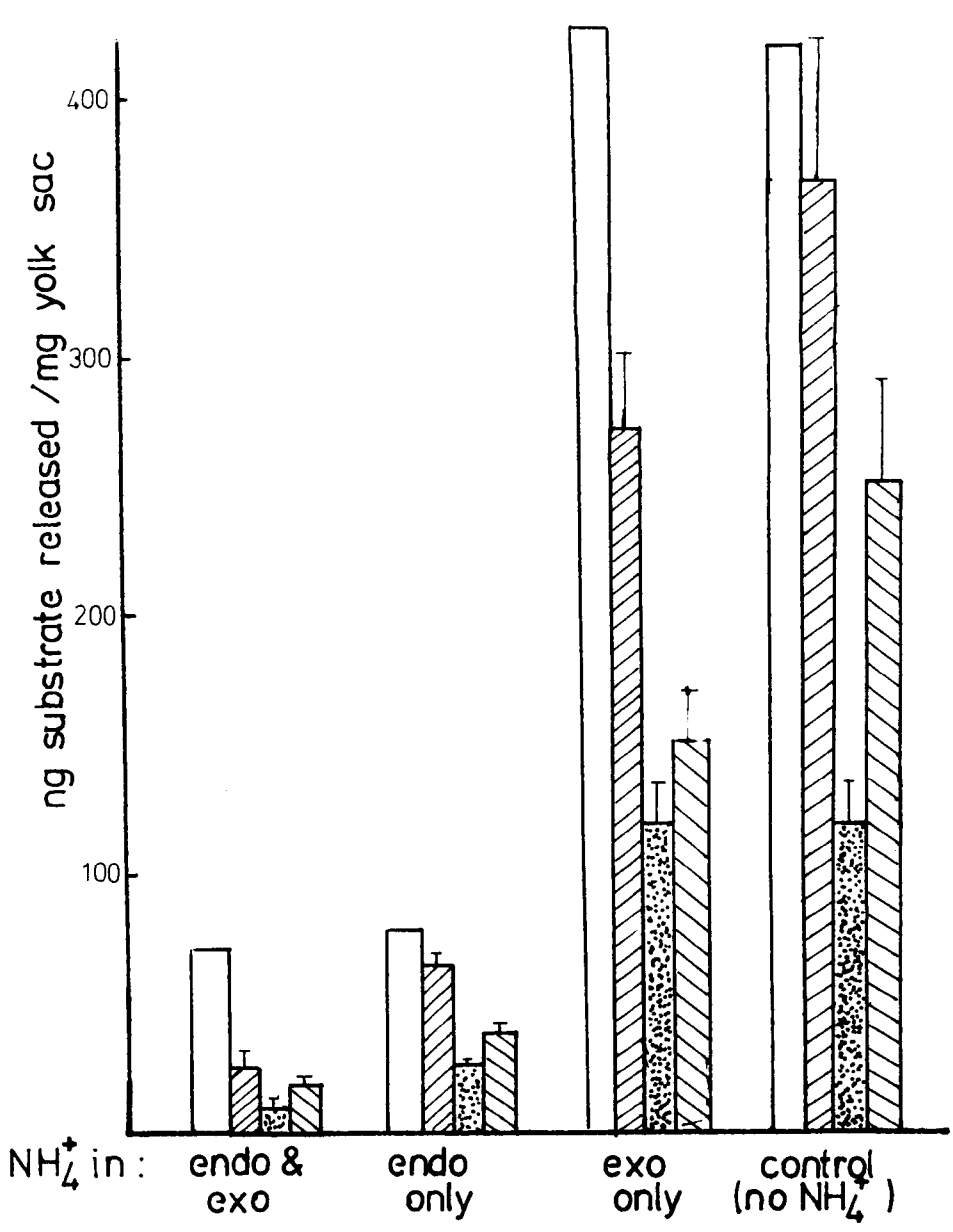


Figure 4.6 Effect of a high concentration of ammonium ions on the release of ^{125}I-labelled rat IgG from rat yolk sacs incubated in serum-free medium 199

17.5-day rat yolk sacs were incubated in serum-free medium 199 and ^{125}I-labelled rat IgG (10 µg/ml) then rinsed and reincubated in fresh medium. Ammonium chloride (30mM) was added in either the endocytosis phase (endo only) or the exocytosis phase (exo only) or both (endo & exo) as outlined in greater detail in Section 4.2.6 . Substrate release was monitored for 3h. The graph expresses the total radioactivity released ▨ , the TCA-soluble radioactivity released ▨ the TCA-insoluble radioactivity released ▩ at 3h of reincubation for each of the experimental regimes mentioned. A measure is also given of the total amount of substrate associated with 1 mg of yolk-sac protein at the beginning of reincubation □ Values shown are means (± S.D.) from 6 separate experiments for each condition.

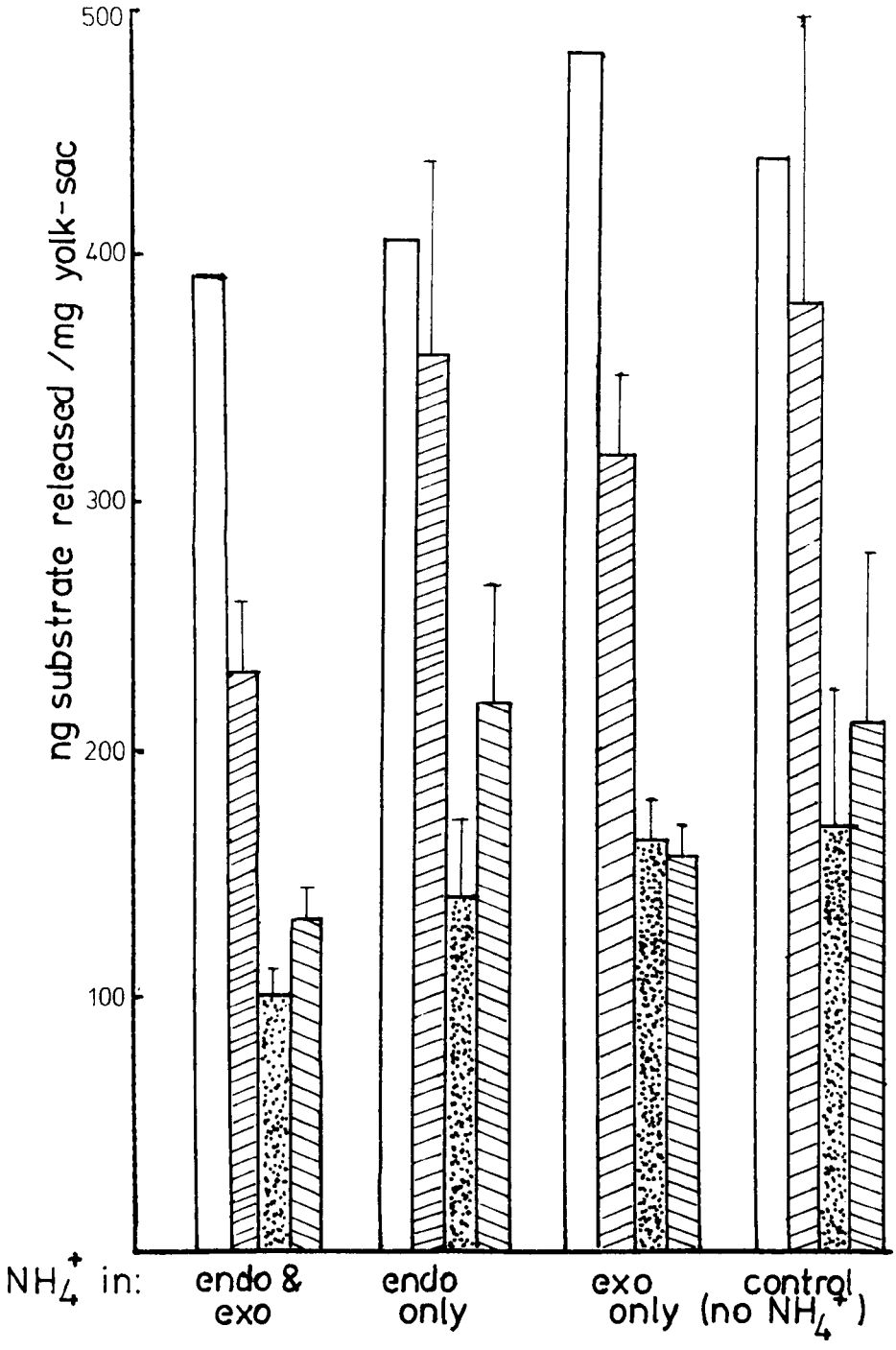


Figure 4.7 Effect of a low concentration of ammonium ions on the exocytosis of ^{125}I-labelled rat IgG from rat yolk-sacs incubated in serum-free medium 199.

17.5-day rat yolk-sacs were incubated in serum-free medium 199 and ^{125}I-rat IgG (10 µg/ml), then rinsed and reincubated in fresh medium. Ammonium chloride (5mM) was added in either the endocytosis phase (endo only) or the exocytosis phase (exo only) or both (endo & exo) as outlined in Section 4.2.6 . Substrate release was monitored for 3h. The graph expresses the total substrate associated with the yolk-sac at the beginning of reincubation ☐ , the total amount of substrate released ▨ , the TCA-soluble activity released ▧ and the TCA-insoluble activity released ▩ at 3h of reincubation for each of the experimental regimes mentioned. Values shown are means (± S.D.) from 6 individual experiments for each condition.

4.4 DISCUSSION

When Williams & Ibbotson (1979) first found that a significant fraction of the homologous ^{125}I-labelled IgG associated with rat yolk sacs was released again from the tissue in a macromolecular form, two explanations were put forward to account for this finding. First, ^{125}I-labelled rat IgG was transported across the rat yolk sac by a specific route with the molecule being protected against degradation by the mechanism proposed by Brambell following *in vivo* studies (Brambell, 1970). Second, ^{125}I-labelled rat IgG adsorbed strongly to the outer membrane of the rat yolk sac, without any internalisation taking place, and desorbed from the membrane slowly. The only experimental evidence that these authors forwarded against the second of these possible explanations was the observation that unlabelled IgG present in the reincubation medium (a condition that would be expected to enhance the displacement of reversible bound ^{125}I-labelled IgG from the tissue surface) was without effect. The experiments in this chapter were devised to test more rigorously the second of these proposals by inhibiting endocytic uptake of ^{125}I-labelled IgG by yolk sacs in a variety of ways. It was hoped that the experiments would give conclusive evidence concerning the mechanism by which IgG molecules associate with the yolk-sac tissue.

When using low temperature as an inhibitor of endocytosis subsequent release of radioactivity was virtually totally abolished at 10^{o}C for rabbit yolk sacs and at 15^{o}C for rat yolk sacs (Figures 4.1 and 4.2). Certainly no release was observed that was comparable to that in the corresponding control incubations, a finding that would have been compatible with extensive tissue adsorption. Neither was any elevation of TCA-insoluble release apparent after IgG exposure at the lower temperature. If it can be assumed that the yolk-sac membrane carries no extracellular proteases (an assumption which finds experimental support,

see Livesey & Williams, 1979), then the percentage of total radioactivity released that is in the form of TCA-insolubles would be expected to rise if reincubation at lower temperatures slows intracellular degradation down but does not inhibit desorption from the tissue surface. Thus the data presented here seem to be more compatible with the suggestion that, when membrane internalisation is inhibited, ^{125}I-labelled IgG does not become associated with the yolk-sac tissue to any extent and suggest that the only way such association takes place is by pinocytic uptake. It was claimed earlier (Chapter 2; Williams & Ibbotson, 1979) that ^{125}I-labelled IgG uptake by the rabbit and rat yolk sac proceed to a large extent by adsorptive pinocytosis rather than by fluid-phase pinocytosis only and it appears that substrate: membrane binding must be short-lived if not followed by internalisation, being fully reversible during the 6 min washing period (see Williams & Ibbotson, 1979). A closer look at the temperature profile from experiments with rat yolk sacs showed that in the plot of the release of radioactivity against temperature, the decrease in the quantity of radioactivity released is not linear with temperature; a sharp drop was observed between 20°C and 25°C. Duncan & Lloyd (1978), in a similar study, noted that the uptake of ^{125}I-labelled PVP by rat yolk sacs incubated in serum-containing medium dropped markedly between 30-37°C. The temperature difference may be related to the presence or absence of calf serum in the medium.

Moving on to the second attempt to solve the basic question of substrate internalisation by and adsorption to the rat and rabbit yolk-sac, experiments with 2,4-dinitrophenol as metabolic inhibitor yield results that are in keeping with the temperature studies. In serum-free medium, a concentration of 10 µg/ml of 2,4-dinitrophenol was sufficient to cause 90% inhibition of the uptake of ^{125}I-PVP, whereas for serum-containing medium the concentration of the inhibitor had to be higher, 20 µg/ml. This effect probably arises from binding of this

agent to serum proteins, so decreasing the concentration of the free inhibitor. Adding 10 µg/ml of 2,4-dinitrophenol to rat yolk sacs that had previously been "loaded" with ^{125}I-labelled PVP caused no drastic release of this marker (Table 4.1) during the period 15-180 min. [The variable rapid release in the first 15 min is probably the result of different degrees of removal of occluded medium during the washing stage; see Roberts *et al.* (1977) for fuller discussion.] An increased rate of release would have been expected if 2,4-dinitrophenol had been cytotoxic at this concentration, causing a disintegration of cells and a subsequent release of a marker that normally does not get released from cells. Such an effect was observed by (Livesey & Williams (1980) when yolk sacs were incubated in the presence of chloroquine. 2,4-dinitrophenol acts on tissues as a decoupler of oxidative phosphorylation, thereby inhibiting cytoskeletal action and would not be expected to affect the binding of molecules, in this case homologous ^{125}I-labelled IgG, to the cellular membranes. The finding that no significant amount of radioactive material (TCA-soluble or TCA-insoluble) derived from ^{125}I-labelled IgG is released from rat and rabbit yolk sacs upon inhibition of uptake of substrate by 2,4-dinitrophenol, suggests that IgG is normally internalized by the rabbit and the rat yolk sac *in vitro*.

Using ammonium ions as a metabolic inhibitor leads to rather more complex findings. Ammonium ions are reported to have a number of effects on an endocytosing system. *In vivo* they are toxic, causing severe neurological effects (Hindfelt *et al.*, 1977), but in the rat yolk sac *in vitro* they do not seem to be toxic (Livesey *et al.*, 1980). Ammonium ions are also claimed to be inhibitors of lysosome function (Seglen , 1975, 1977; Seglen & Reith, 1976; Hopgood *et al.*, 1977). For example, Tolleshaug *et al.* (1977) showed that digestion of asialofetuin, endocytosed by hepatocytes in culture is sensitive to ammonium ion inhibition, and Reijngoud *et al.* (1976) demonstrated the accumulation of

methylammonium ions in isolated rat liver lysosomes in vitro. These and other authors have suggested that proteolytic inhibition is due to a rise in intralysosomal pH, since lysosomal enzymes are less active at higher pH (Coff ey & De Duve, 1968). Another effect of ammonium ions is the inhibition of endocytic uptake. Livesey & Williams (1980) showed that at a concentration of 20mM the uptake of formaldehyde-denatured ^{125}I-labelled albumin is completely inhibited in rat yolk sacs in vitro. In this present study both effects can, to a certain extent, be demonstrated. When ammonium ions are present at a concentration of 30mM during uptake of ^{125}I-labelled rat IgG into rat yolk sacs, endocytosis is completely inhibited. Both, the E.I. and the T.A.R. are less than 1 µl/mg tissue-protein per hour (Table 4.2). When using ammonium ions at a lower concentration (5mM), although the total uptake is reduced by 67%, the decrease of the T.A.R. is only by 39%, i.e. tissue-accumulation is relatively increased (Table 4.2). In effect, a greater percentage of internalized substrate accumulates in the tissue, probably undegraded. This finding is compatible with the suggestion that ammonium ions inhibit intralysosomal digestion (Livesey & Williams, 1980). The results obtained in the release studies involving the presence of 5mM ammonium ions in the medium during the uptake and/or release phases can be interpreted in a similar way. Under all conditions tested, the total amount of ^{125}I-labelled rat IgG associated with the yolk-sac tissue at the beginning of the reincubation period is virtually the same (between 390 and 470 ng/mg yolk-sac tissue; Figure 4.7). Therefore no interference of ammonium ions (5mM) with uptake can be claimed here. This is even more strongly suggested by the similarity of the release patterns in controls and incubation in which 5mM ammonium ions were present in the uptake phase only. When ammonium ions (5mM) were present in the release phase, however, two effects can be noted. First, the total

release of substrate is markedly reduced and at the same time substrate accumulates in the tissue. Second, a relatively smaller release of TCA-soluble activity takes place. While the nature of the non-released material cannot be known for certain, a few tentative suggestions may be put forward. Assuming that release from yolk sacs is either by diffusion (Hemming & Williams, 1976), exocytosis from phagolysosomes (Brambell, 1970) or exocytosis of special micropinocytic vesicles (Moxon et al., 1976; Rodewald, 1973), ammonium ions may interfere with the release mechanism (especially exocytosis). More probable is a retention within lysosomes of substrate that remains at least partially undegraded due to the interference of ammonium ions with enzyme action. This would also explain the second effect of a decreased release of TCA-solubles.

Studies of the release of IgG in the presence of a higher concentration of ammonium ions (30mM) show a much greater effect on uptake (Figure 4.6), similar to the effect obtained with the metabolic inhibitor 2,4-dinitrophenol. When 30mM ammonium ions are present during the release phase only, the release patterns are similar to those observed at the lower concentrations, with a greater retention of substrate within the yolk sac and a slightly decreased TCA-soluble release. The explanation of this finding is therefore the same as put forward above for the lower inhibitor concentration. While the mere fact that yolk sacs can be induced to retain a greater percentage of ^{125}I-labelled rat IgG may serve as an indirect piece of evidence for the internalisation of substrate, what matters more to the aim of this chapter is the finding that, when substrate internalisation is inhibited, subsequent release of acid-insoluble radioactivity is likewise reduced.

In summary, the following conclusions can be drawn from the data presented in this chapter. When pinocytic uptake of homologous ^{125}I-labelled IgG into rat or rabbit yolk sacs is inhibited by either

low temperature or by the presence of 2,4-dinitrophenol or by ammonium ions, no appreciable release of acid-insoluble radioactivity can be observed on reincubation of substrate-loaded yolk sacs.

It could be argued, however, that the agents used to inhibit internalisation of substrate also decrease binding (e.g. low temperature may cause conformational changes of the membrane thereby decreasing the number of extracellular binding sites). Likewise, the chemical agents may form complexes with the substrate or the binding sites on the membrane thus lowering the degree of binding of the substrate to the yolk sac. This could possibly explain the failure to observe the release of acid-insoluble radioactivity on subsequent reincubation. However, since all three conditions (30mM ammonium chloride, 2,4-dinitrophenol and decreased temperature) produce extremely similar inhibitions of release, it is highly unlikely that either a decrease in the number of extracellular adsorption sites or the binding capacity of IgG can be held responsible for the observed decrease in release. This decrease is more readily compatible with the suggestion that *in vitro* rat and rabbit yolk sacs internalize homologous ^{125}I-labelled IgG, some of which is degraded subsequent to capture while the rest escapes degradation by a specific protection mechanism. Such considerations indicate that it is worthwhile to continue employing the *in vitro* yolk-sac incubation method to investigate the cellular mechanisms involved in IgG transport across membranes, since the original observation of release of acid-insoluble radioactivity from the yolk-sac tissue does not appear to be able to be explained in terms of release from extracellular adsorption sites.

CHAPTER FIVE

The Differential Fates of Homologous and Heterologous Species of IgG in the Rat and Rabbit Yolk Sacs

5.1 INTRODUCTION

In a previous chapter it was shown that when ^{125}I-labelled rat IgG was treated with formaldehyde the molecule lost its ability to remain undegraded in the in vitro rat yolk-sac system. In the work reported in the current chapter more subtle intermolecular differences are considered, namely those between the ^{125}I-labelled forms of homologous and heterologous species of IgG and the fates of these molecules in the rat and rabbit yolk sac in vitro.

Brambell et al. (1950) and later other workers, found that in vivo both transfer of proteins across the placental barrier in the rabbit, guinea pig, human and rhesus monkey (via either the chorioallantoic placenta or the yolk sac) and transfer across the neonatal rat and mouse gut are highly selective (Brambell, 1970; Hartley, 1951; Halliday, 1955; Brambell & Halliday, 1956; Bangham, 1960; Leisring & Anderson, 1961; Gitlin et al., 1964). Hemmings (1961) established that transfer of molecules across the rabbit yolk sac followed the order: ^{131}I-labelled IgG > ^{131}I-labelled BSA, whereas homologous alpha and beta globulins were hardly transported at all. Kulangara & Schechtman (1962) also reported a greater transfer of IgG than of human and bovine serum albumin across the same tissue. The latter findings were supported by Morgan (1964) who added transferrin to the list and found the order of transfer to be : IgG > albumin > transferrin. Slade & Wild (1971) reported a preference of uptake into rabbit yolk sacs of human IgG over that of ferritin or ferritin-conjugated IgG. Lambson (1966) observed the passage of ferritin through the same tissue. In other tissues too, similar selectivity could be demonstrated, for example Jollie (1968) investigated Reichert's membrane in the rat placenta and found that ferritin was taken up, whereas thorotrast was not. Also, Morgan (1964) found that, near term, the rat yolk sac transported

IgG in preference to either albumin or transferrin. Bangham & Terry (1957), while investigating the transfer of passive immunity in neonatal rats, noted that IgG was transported more readily across the gut than albumin. Bangham (1960) also investigated the process of selective transport in the rhesus monkey, a species in which the organ responsible is believed to be the placenta. In this species homologous IgG is transferred to the foetus twenty times more readily than albumin, while alpha and beta globulins are hardly transported at all.

These in vivo findings indicate a definite preference of transport of IgG over other proteins. In an early paper Halford (1930) suggested that molecular size was the basis of such selectivity, but Batty et al. (1954) disproved this by showing that some aggregated IgG molecules were transported across the rabbit placental membranes at a greater rate than other, non-aggregated molecules. After finding that bovine IgG was transmitted less readily than rabbit IgG to the rabbit foetus, at day-24 post coitum Brambell (1954) suggested that selectivity of transport of immunoglobulins depended on the species of origin of the molecule. In many subsequent studies, involving a variety of experimental animals and immunoglobulins obtained from different species, this concept of the preferential transfer of homologous IgG has been consolidated. Cohen (1950) noted a preferential transfer of rabbit IgG over bovine and human IgG to the foetal circulation in the rabbit. In a different species, the guinea pig, Hartley (1948) had previously found that guinea-pig diphtheria antitoxin was more readily transferred to the foetus than human diphtheria antitoxin. In the rat gut a selectivity of transmission in favour of the homologous IgG species was noted (Bamford, 1966). Moreover, for a group of heterologous IgG molecules an order of preference has been established, but this order differs from species to species and even from tissue to tissue within a given species. In

some cases selectivity may favour a heterologous IgG species rather than the homologous one! Below are mentioned but a few examples of *in vivo* experiments covering a variety of species:

Test animal	Order of transfer of IgG derived from different species	References
Rabbit	rabbit > human > guinea-pig > canine > equine > bovine	Batty *et al.* (1954)
Rat (prenatal)	guinea-pig > human > rat=mouse=bovine	Koch *et al.* (1967)
Rat (postnatal)	rat > rabbit=sheep	Bangham & Terry (1957)
	rat > mouse > rabbit > bovine > fowl	Halliday (1955)
Mouse (prenatal)	guinea-pig > human > rat > bovine=mouse	Koch *et al.* (1967)
Guinea-pig	guinea-pig > human > mouse	Koch *et al.* (1967)
Hedgehog	hedgehog > rabbit=guinea-pig > bovine	Halliday & Kekwick (1960) Morris & Baldwin (1962)

From such studies it is apparent that very definite patterns of selection of molecular transfer through cells exist. Since the lysosomal system is known to be very effective in the degradation of endogenous and exogenous materials within cells, the transfer of intact bipolymer molecules across cells is in itself very unusual and needs to be accounted for. In addition, a theory is needed to give a basis for selection. In the course of the years a number of theories have been put forward to explain this selection and protection of some molecules from degradation by enzymes within cells. The two main theories are briefly mentioned below.

According to Brambell, all protein molecules are taken up indiscriminately by rabbit yolk-sac cells into endocytic vesicles that subsequently fuse with lysosomes. Those molecules destined for transport bind firmly to specific receptors on the inner face of the vesicular membrane and thus are protected from the action of lysosomal enzymes; all non-bound molecules are degraded. Heterolysosomes then

fuse with the basal membrane to release the intact IgG molecules (Brambell, 1970). Wild, on the other hand, envisages that selection occurs at the cell surface itself, with a portion of the IgG binding to specific membrane receptors that are subsequently selectively taken up into coated micropinocytic vesicles, a class of vesicles distinct from general uptake vesicles and which does not fuse with lysosomes and can thus release their contents intact on fusion with either the basal or lateral plasmalemma (Wild, 1975, 1976, 1971). These two mechanisms cannot easily be distinguished by in vivo methods, as it is difficult, if not impossible, to observe transport on a cellular level in vivo (for further discussion see Chapter 2). An in vitro system like the one developed by Williams et al. (1975a) has the advantage that it deals, in isolation, with the tissue of interest and therefore it should be easier to investigate the question of molecular selection in some depth.

An important question to ask in the in vitro system is whether the in vitro findings show a pattern that reflects the selectivity observed in vivo in the rat and rabbit yolk sac, i.e. between homologous and heterologous ^{125}I-labelled IgG species. From previous chapters, a difference in the fate of ^{125}I-labelled albumin and ^{125}I-labelled homologous IgG has already been apparent (Chapter 2); likewise, the fate of formaldehyde-treated rat IgG differs from that of untreated rat IgG (Chapter 3). Both the ^{125}I-labelled albumin and formaldehyde-treated ^{125}I-labelled IgG are fully degraded in the rat yolk sac with little, if any, release of macromolecular material during reincubation, a finding that is compatible with failure of transport of the intact molecules across the tissue. It is therefore of interest to establish whether such selectivity extends further to homologous and heterologous IgG. Complete concordance of the rank orders of release in vitro and of transport in vivo would give strong support to the assumption that

the behaviour of the rat and rabbit yolk sacs in vitro reflect their in vivo functions. A further inherent shortcoming of in vivo experiments is that they use immunological methods to determine molecular transfer and therefore little can be deduced concerning the fate of those molecules that may be taken up and digested within the tissues rather than transferred intact across cells. This is regrettable since, in the Brambell mechanism in particular, degradation plays an integral part in the selection process. In vitro studies can thus serve to help elucidate the fate of immunoglobulins within the rat and rabbit yolk sac. Furthermore, in vivo studies are also largely qualitative and few workers (e.g. Brambell, 1966) have made attempts to quantitate transfer. In vitro studies offer the possibility of determining the rate of uptake of homologous and heterologous IgG and possibly also the rate of transfer of those molecules across the rat and rabbit yolk sac.

A further question to be investigated in this chapter is that of the actual site responsible for selection in the rat and rabbit. In the rabbit, the yolk sac has been firmly established as the tissue in which the selection of the immunoglobulins destined for transfer to the foetus occurs (Brambell, 1970; Wild, 1970), with the paraplacental chorion possibly serving as a non-selective permeable barrier to a number of molecules (Wild, 1970). The role of the rabbit yolk sac as the primary site of IgG transfer into the foetal circulation has only been challenged by Hemmings (1973), who found some evidence for selection in the rabbit placenta. In the rat, however, the situation is not as clearly defined and has not been investigated in the same depth as in the rabbit. Brambell & Halliday (1956), Anderson (1959) and Mayersbach (1958) proposed that the visceral yolk sac is the site where selection occurs, whereas Quinlivian (1964) gives evidence for the chorioallantoic placenta being the site of selection. An investigation of the differential treatment of homologous and heterologous IgG by the rat yolk sac in vitro

should provide evidence for or against the involvement of this tissue in the prenatal transfer of passive immunity from mother to young in this species. Such studies should prove to be particularly interesting if performed in parallel with equivalent experiments using rabbit yolk sacs, since any different trends in the properties of the two tissues should become apparent. If findings are similar to those obtained in the rabbit yolk sac, this could count as circumstantial evidence for the involvement of the yolk sac in selective protein transport in the rat.

In summary, this chapter poses the following questions:

1) Do rat and rabbit yolk sacs *in vitro* take up homologous and heterologous ^{125}I-labelled IgG at different rates, and can differential tissue-accumulation rates be observed?

2) Do homologous and heterologous ^{125}I-labelled IgG molecules differ in their degrees of degradation by rat and rabbit yolk sacs *in vitro*?

3) Can any correlation be found between the *in vitro* and the *in vivo* findings?

4) *In vitro*, do rat and rabbit yolk sacs show differences in their ability to ingest and degrade homologous and heterologous ^{125}I-labelled IgG?

5) Can any additional evidence be found to indicate that in either the rabbit or, especially in the rat, the yolk sac is the site at which selection takes place during transfer of passive immunity?

5.2 METHODS

5.2.1 Uptake of homologous and heterologous ^{125}I-labelled IgG in rat and rabbit yolk sacs in vitro

Rat yolk sacs (17.5-day) or 24-day rabbit yolk-sac pieces were incubated in medium 199 containing 10% calf serum or in serum-free medium 199. Substrate (^{125}I-labelled bovine-, human-, rabbit- or rat IgG) was present in the medium at a concentration of 2 µg/ml. The general methods outlined in Sections 1.2 (for rat yolk sacs incubated in serum-containing medium), 1.7 (for rat yolk sacs incubated in serum-free medium), 1.8 (for rabbit yolk sacs incubated in serum-containing and serum-free medium) were followed in all details. Endocytic Indices and Tissue-Accumulation Rates were calculated (see Sections 1.10 and 1.11 for details).

5.2.2 Release of homologous and heterologous ^{125}I-labelled IgG from rat and rabbit yolk sacs that had previously been loaded with these substrates in vitro

Rat yolk sacs (17.5-day) or 24-day rabbit yolk-sac pieces were incubated in serum-free medium 199, containing either ^{125}I-labelled bovine-, human-, rabbit- or rat IgG (10 µg/ml). Yolk sacs were then washed and reincubated in fresh medium 199 and release of radioactivity was monitored for 3h; substrate release patterns were plotted (see Section 1.12 for details).

5.3 RESULTS

5.3.1 Uptake of homologous and heterologous ^{125}I-labelled IgG by rat yolk sacs incubated in the presence or in the absence of calf serum

Tables 5.1 and 5.2 exemplify a point made earlier, namely that variations do occur in the behaviour of different preparations of ^{125}I-labelled IgG in the rat yolk-sac system. In these tables the Endocytic Indices and the Tissue-Accumulation Rates of ^{125}I-labelled bovine-, human-, rat- and rabbit IgG are shown in rat yolk sacs incubated in serum-containing medium. Endocytic Indices are all in the same range, between 6 and 13 µl/mg yolk-sac protein per h with the Endocytic Indices of the Batch 2 immunoglobulin preparation being slightly lower than those of the Batch 1 preparation. Interspecies differences are, however, maintained within both batches, with the Endocytic Indices of ^{125}I-labelled bovine- and rat IgG being higher than those of ^{125}I-labelled human- and rabbit IgG. A greater difference between the two batches can be observed in the Tissue-Accumulation Rates, which are higher for the Batch 2 material. Comparing the four ^{125}I-labelled IgG species when incubated in medium containing serum, the Tissue-Accumulation Rates tend to decrease in the order: rat > rabbit > human=bovine (Tables 5.2a & 5.6a).

On incubation of rat yolk-sacs in serum-free medium 199, results are very different from those obtained in serum-containing medium (Tables 5.3 & 5.6b). As in earlier experiments (Chapter 2), Endocytic Indices are increased by an order of magnitude. Endocytic Indices of ^{125}I-labelled bovine- and rat- IgG do not differ much from each other, but the Endocytic Index of ^{125}I-labelled human IgG is noticeably higher than that of the other two species. The main difference between homologous and heterologous IgG species can be observed in the Tissue-Accumulated Rates, whose ranking resembles that of the equivalent

incubations of rat yolk sacs in serum-containing medium, i.e.: rat > human > bovine (Tables 5.2, 5.3 and 5.6b). The difference between the Tissue-Accumulation Rates of ^{125}I-labelled rat and bovine IgG is much greater in the serum-free medium.

5.3.2 Uptake of homologous and heterologous ^{125}I-labelled IgG by rabbit yolk-sac pieces incubated in the presence or in the absence of calf serum

In the experiments performed with rabbit yolk sacs (Tables 5.4 and 5.6) Endocytic Indices are of the same order of magnitude as those found for equivalent rat yolk-sac incubations in the presence of serum (see Tables 5.1, 5.2 and 5.6). Interspecies differences are, however, more pronounced in the rabbit yolk-sac incubations. In incubations in serum-containing medium, Endocytic Indices decrease in the order: rat > human > rabbit (Table 5.6a) a pattern similar to that found in serum-free medium: human > rat > bovine=rabbit (Table 5.6b). In the absence of serum, Endocytic Indices rise by between 8- and 20-fold, a degree of elevation comparable to that observed in rat yolk sacs.

Tissue-Accumulation Rates for homologous and heterologous ^{125}I-labelled IgG species in serum-containing medium are slightly higher than the equivalent values in the rat yolk sac, but are of the same order of magnitude; their ranking order is: rat > bovine > human > rabbit. On removal of calf serum from the medium, Tissue-Accumulation Rates of all ^{125}I-labelled IgG species are increased, but to differing extents as outlined in Table 5.6, which expresses in percentages the rise of E.I's and T.A.R's on removing calf serum from the incubation medium. In serum-free medium, the ranking orders of T.A.R's of the homologous and heterologous ^{125}I-labelled IgG species are as follows: human > rabbit= rat > bovine (Table 5.6b).

5.3.3 Comparison of the ratios Tissue-Accumulation Rate to Endocytic Index for homologous and heterologous ^{125}I-labelled IgG ingested by rat and rabbit yolk sacs

In the above section (5.3.2) tissue accumulation of substrate was expressed in terms of Tissue-Accumulation Rates as defined in Section 1.10. However, in order to compare the relative extent of tissue accumulation of the various substrates between the two different tissues it is important to also keep in mind the net rate of uptake of these substrates. Thus, a substrate may have a very low Tissue-Accumulation Rate, but this may, nevertheless, be important if the net total uptake (Endocytic Index) is also low. For this reason, tissue accumulation was also expressed in terms of the ratio of the two rates, i.e. Tissue-Accumulation Rate over the net uptake rate (TAR/EI) (see Table 5.6). Expressed in this way, ranking orders of tissue accumulation for homologous and heterologous ^{125}I-labelled IgG species in rat and rabbit yolk sacs differ from the patterns quoted above (5.3.2). In the absence of serum, the ratio TAR/EI is the highest for the homologous IgG in both rat and rabbit yolk sacs (Table 5.6b); in the presence of serum the homologous IgGs fall second in the two rank orders (Table 5.6a).

5.3.4 Release, from rat and rabbit yolk sacs, of radioactivity derived from ^{125}I-labelled homologous and heterologous IgG previously ingested by the tissue in vitro

Release patterns of ^{125}I-labelled IgG from yolk sacs are presented graphically in Figures 5.1 - 5.7; Table 5.7 summarizes the same data by quoting the total amounts of substrates released by 3h reincubation together with the amounts of TCA-soluble and -insoluble ractivity released. In addition, release is expressed as a percentage of the total amount of substrate associated with the tissue at the beginning of the reincubation period. Looking at the total release of radioactivity expressed in this second manner, the amounts released are very similar

(79-91%)for all ^{125}I-labelled IgG species used as substrates in rat and rabbit yolk sacs. But, when release is expressed in absolute terms (ng substrate released/mg yolk-sac protein), total release decreases in the order human > rat > bovine for rat yolk sacs (Table 5.7a) and rabbit > rat > human > bovine for rat yolk sacs (Table 5.7b). For all ^{125}I-labelled IgG species, however, a greater release is reported from rabbit yolk sacs. Looking at the amount of substrate that is released from the yolk sac into the medium in an undegraded form (TCA-insoluble activity), a marked difference concerning the species of origin of the ^{125}I-labelled IgG is apparent. Whereas ^{125}I-labelled bovine IgG is released from both rat and rabbit yolk sacs mainly in a TCA-soluble form, i.e. degraded (Figures 5.1 and 5.4), human (Figures 5.2 and 5.5) and homologous (Figures 5.3 and 5.6) ^{125}I-labelled IgG remain undegraded to a larger extent in both yolk-sac systems. It may be of interest that, both in the rat and rabbit yolk sac ^{125}I-labelled rat IgG is degraded more readily than ^{125}I-labelled human IgG (Table 5.7).

One difference between rat and rabbit yolk-sac tissues that is apparent on examining these data is that homologous ^{125}I-IgG is released in an undegraded form to a greater extent (58%) from rabbit yolk sacs than from rat yolk sacs (36%).

In the following tables (Tables 5.1 - 5.5, inclusive) the full details of individual experiments are reported, but to conserve space the column headings have been abbreviated according to the scheme below:

1	2	3
TCA-solubles in the ^{125}I-labelled protein preparation on the day of use (%)	No. of yolk-sac pieces used in the individual experiment	Endocytic Index (µl/mg of yolk-sac protein per h)

4	5	6
Correlation Coefficient of the plot uptake against time used to determine the Endocytic Index	Tissue-Accumulation Rate (µl/mg of yolk-sac protein per h)	Correlation Coefficient of the plot of tissue levels against time used to determine the Tissue-Accumulation Rate

The main findings reported in the above tables are summarized in Tables 5.6a & 5.6b to permit ready comparison of data.

Table 5.1 Endocytic Indices and Tissue-Accumulation Rates of ^{125}I-labelled bovine-, human- and rat IgG for rat yolk sacs incubated in medium 199 containing calf serum

Rat yolk sacs (17.5-day) were incubated in medium 199 containing 10% (v/v) calf serum and ^{125}I-labelled bovine-, human- or rat IgG as substrate (2 µg/ml of medium). Yolk-sac incubations were terminated at intervals up to 6.5h. The values of both the Endocytic Indices and Tissue-Accumulation Rates were calculated (for details of methods see Section 1.10).

^{125}I-labelled IgG species	1	2	3	4	5	6
Bovine (Batch 1)	1.99	9	12.30	0.931	0.71	0.488
Bovine (Batch 1)	1.85	10	9.93	0.976	1.05	0.888
Bovine (Batch 1)	1.85	10	13.09	0.988	0.60	0.765
MEAN VALUES (± S.D.):			11.77 ± 1.64		0.79 ± 0.23	
Human (Batch 1)	2.35	10	12.58	0.953	1.22	0.390
Human (Batch 1)	2.19	9	7.38	0.979	1.18	0.846
Human (Batch 1)	2.14	9	8.08	0.925	0.45	0.262
MEAN VALUES (± S.D.):			9.35 ± 2.82		0.95 ± 0.43	
Rat (Batch 1)	1.68	10	12.78	0.937	0.95	0.578
Rat (Batch 1)	2.11	9	11.8	0.973	1.96	0.925
Rat (Batch 1)	1.61	7	11.98	0.987	2.24	0.995
MEAN VALUES (± S.D.):			12.18 ± 0.52		1.72 ± 0.68	

Table 5.2 Endocytic Indices and Tissue-Accumulation Rates of ^{125}I-labelled bovine-, human-, rabbit-, and rat IgG for rat yolk sacs incubated in medium 199 containing calf serum

Rate yolk sacs (17.5-day) were incubated in medium 199 containing 10% (v/v) calf serum and ^{125}I-labelled bovine-, human-, rabbit-, or rat IgG (2μg/ml of medium) as substrate. Yolk sac incubations were terminated at intervals up to 6.5h. The values of Endocytic Indices and Tissue-Accumulation Rates were calculated (for details of methods see Section 1.10).

^{125}I-IgG species	1	2	3	4	5	6
Bovine (Batch 2)	2.39	10	8.28	0.934	1.31	0.821
Bovine (Batch 2)	2.69	10	8.92	0.961	0.913	0.819
Bovine (Batch 2)	2.69	10	11.45	0.841	1.59	0.696
Bovine (Batch 2)	1.79	8	13.57	0.881	1.72	0.416
MEAN VALUES (± S.D.):			10.55 ± 2.43		1.38 ± 0.36	
Human (Batch 2)	2.01	10	6.11	0.912	1.30	0.949
Human (Batch 2)	2.58	8	5.26	0.949	1.39	0.729
Human (Batch 2)	2.58	9	7.78	0.954	1.54	0.624
MEAN VALUES (± S.D.):			6.38 ± 1.28		1.41 ± 0.11	
Rabbit (Batch 2)	2.76	10	6.02	0.937	2.19	0.949
Rabbit (Batch 2)	2.36	10	5.40	0.949	1.69	0.720
Rabbit (Batch 2)	2.67	9	8.01	0.954	2.85	0.624
MEAN VALUES (± S.D.):			6.48 ± 1.36		2.24 ± 0.58	
Rat (Batch 2)	1.54	10	10.09	0.972	2.45	0.898
Rat (Batch 2)	1.54	9	10.27	0.972	2.21	0.896
Rat (Batch 2)	1.98	8	8.46	0.743	3.32	0.898
MEAN VALUES (± S.D.):			9.61 ± 0.99		2.66 ± 0.58	

Table 5.3 Endocytic Indices and Tissue-Accumulation Rates of ^{125}I-labelled bovine-, human- and rat IgG for rat yolk sacs incubated in serum-free medium 199

Rat yolk sacs (17.5-day) were incubated in serum-free medium 199 containing ^{125}I-labelled bovine-, human- or rat IgG (2 µg/ml of medium). Yolk-sac incubations were terminated at intervals up to 5.5h. The values of Endocytic Indices and Tissue-Accumulation Rates were calculated (for details of methods see Section 1.10).

^{125}I-IgG species	1	2	3	4	5	6
Bovine (Batch 1)	1.85	9	98.45	0.955	3.11	0.445
Bovine (Batch 1)	1.85	8	21.93	0.962	-0.49	-0.064
Bovine (Batch 1)	1.85	9	74.00	0.985	0.56	0.206
MEAN VALUES (± S.D.):			65.03 ± 39.17		1.83 ± 1.80	
Human (Batch 1)	2.25	8	100.84	0.971	2.39	0.831
Human (Batch 1)	2.25	10	95.88	0.972	5.32	0.603
Human (Batch 1)	2.25	9	80.55	0.976	3.85	0.474
MEAN VALUES (± S.D.):			92.42 ± 10.58		3.85 ± 1.46	
Rat (Batch 1)	2.11	7	76.00	0.910	11.49	0.776
Rat (Batch 1)	2.11	8	80.76	0.939	14.64	0.954
Rat (Batch 1)	4.76	8	66.63	0.956	11.51	0.761
MEAN VALUES (± S.D.):			74.46 ± 7.19		12.56 ± 1.84	

Table 5.4 Endocytic Indices and Tissue-Accumulation Rates of ^{125}I-labelled bovine-, human-, rabbit- and rat IgG for rabbit yolk-sac tissue incubated in medium 199 containing calf serum

Rabbit yolk-sac pieces (24-day) were incubated in medium 199 containg 10% (v/v) calf serum and ^{125}I-labelled bovine-, human-, rabbit- or rat IgG (2 µg/ml of medium). The values of the Endocytic Indices and Tissue-Accumulation Rates were calculated (for details of methods see Section 1.10)

^{125}I-IgG species	1	2	3	4	5	6
Bovine (Batch 2)	2.05	10	8.19	0.819	4.36	0.768
Bovine (Batch 2)	1.90	10	3.66	0.845	3.37	0.913
Bovine (Batch 2)	2.01	8	5.51	0.871	2.24	0.770
Bovine (Batch 2)	2.57	8	5.79	0.822	3.75	0.747
MEAN VALUES (± S.D.):			5.79 ± 1.86		3.43 ± 0.89	
Human (Batch 2)	2.01	8	6.89	0.986	2.70	0.834
Human (Batch 2)	1.83	9	4.73	0.909	2.89	0.834
Human (Batch 2)	2.03	9	8.22	0.666	1.44	0.761
MEAN VALUES (± S.D.):			6.61 ± 1.76		2.34 ± 0.79	
Rabbit (Batch 2)	2.16	9	2.15	0.809	1.68	0.806
Rabbit (Batch 2)	2.09	7	3.57	0.745	1.48	0.845
Rabbit (Batch 2)	2.70	10	4.05	0.933	1.51	0.693
MEAN VALUES (± S.D.):			3.25 ± 0.99		1.56 ± 0.11	
Rat (Batch 2)	3.22	10	15.82	0.951	10.21	0.870
Rat (Batch 2)	1.76	9	11.05	0.923	7.53	0.867
Rat (Batch 2)	2.88	10	9.01	0.930	4.28	0.770
Rat (Batch 2)	1.64	10	10.54	0.920	5.05	0.871
MEAN VALUES (± S.D.):			11.60 ± 2.94		6.77 ± 2.68	

Table 5.5 Endocytic Indices and Tissue-Accumulation Rates of ^{125}I-labelled bovine-, human-, rabbit- and rat IgG in rabbit yolk-sac tissue incubated in serum-free medium 199

Rabbit yolk-sac pieces (24-day) were incubated in serum-free medium 199 containing ^{125}I-labelled bovine-, human-, rabbit- or rat IgG (2 µg/ml of medium). Yolk-sac incubations were terminated at intervals up to 5.5h. The values of Endocytic Indices and Tissue-Accumulation Rates were calculated (for details of methods see Section 1.10).

^{125}I-IgG species	1	2	3	4	5	6
Bovine (Batch 2)	3.05	9	95.08	0.862	15.97	0.621
Bovine (Batch 2)	4.57	9	53.63	0.901	3.63	0.368
Bovine (Batch 2)	2.95	9	69.53	0.958	7.06	0.639
MEAN VALUES (± S.D.):			72.7 ± 20.9		8.9 ± 6.4	
Human (Batch 2)	2.28	9	146.78	0.926	58.58	0.689
Human (Batch 2)	2.21	9	115.21	0.896	28.14	0.699
Human (Batch 2)	2.21	9	152.54	0.882	35.06	0.777
MEAN VALUES (± S.D.):			138.2 ± 20.1		40.6 ± 16.0	
Rabbit (Batch 2)	3.15	10	37.98	0.847	15.89	0.741
Rabbit (Batch 2)	3.51	9	44.42	0.725	13.72	0.415
Rabbit (Batch 2)	1.47	9	88.53	0.878	41.06	0.729
Rabbit (Batch 2)	1.47	8	94.97	0.837	27.60	0.847
MEAN VALUES (± S.D.):			66.5 ± 29.4		24.6 ± 15.4	
Rat (Batch 2)	5.96	9	88.70	0.989	22.80	0.904
Rat (Batch 2)	2.82	9	82.59	0.966	26.80	0.837
Rat (Batch 2)	3.47	9	65.14	0.545	14.94	0.345
Rat (Batch 2)	3.44	9	140.11	0.621	31.05	0.588
MEAN VALUES (± S.D.):			94.1 ± 32.2		23.9 ± 6.9	

Table 5.6a Comparison of Endocytic Indices and Tissue-Accumulation Rates of ^{125}I-labelled homologous and heterologous IgG species in rat and rabbit yolk sacs

This table serves as a summary of Tables 5.2-5.5, quoting only the Endocytic Indices and Tissue-Accumulation Rates of the various substrates that were incubated with rat and rabbit yolk sacs either in serum-free or in serum-containing medium 199. For the sake of comparison values of the ratio T.A.R. to E.I., are also quoted as a percentage.

Incubations in medium 199 containing calf serum (10% v/v)

^{125}I-IgG species	RABBIT			RAT		
	E.I.	T.A.R.	$\frac{\text{T.A.R.}}{\text{E.I.}}$	E.I.	T.A.R.	$\frac{\text{T.A.R.}}{\text{E.I.}}$
	(µl/mg protein per h)		(%)	(µl/mg protein per h)		(%)
Bovine (Batch 2)	5.79 ± 1.85	3.43 ± 0.89	59.2	10.6 ± 2.43	1.38 ± 0.36	13.1
Human (Batch 2)	6.61 ± 1.76	2.34 ± 0.79	35.4	6.38 ± 1.28	1.41 ± 0.11	21.8
Rabbit (Batch 2)	3.25 ± 0.99	1.56 ± 0.11	47.9	6.48 ± 1.36	2.24 ± 0.58	34.6
Rat (Batch 2)	11.6 ± 2.94	6.77 ± 2.68	58.4	9.61 ± 0.99	2.66 ± 0.58	27.7

Table 5.6b Comparison of Endocytic Indices and Tissue-Accumulation Rates of ^{125}I-labelled homologous and heterologous IgG species in rat and rabbit yolk sacs

This table serves as a summary of Tables 5.2-5.5, quoting only the Endocytic Indices and Tissue-Accumulation Rates of the various substrates that were incubated with rat and rabbit yolk sacs either in serum-free or in serum-containing medium 199. For the sake of comparison values of the ratio T.A.R. to E.I., are also quoted as a percentage.

Incubations in medium 199 alone

^{125}I-IgG species	RABBIT			RAT		
	E.I.	T.A.R.	$\frac{\text{T.A.R.}}{\text{E.I.}}$	E.I.	T.A.R.	$\frac{\text{T.A.R.}}{\text{E.I.}}$
	(µl/mg protein per h)		(%)	(µl/mg protein per h)		(%)
Bovine (Batch 2)	72.8 ± 20.9	8.89 ± 6.37	12.2	65.0* ± 39.2	1.83 ± 1.80	2.8
Human (Batch 2)	138.2 ± 20.1	40.6 ± 16.0	29.4	92.4* ± 10.6	3.85 ± 1.46	4.2
Rabbit (Batch 2)	66.5 ± 29.4	24.6 ± 15.4	37.0	- -	- -	-
Rat (Batch 2)	94.1 ± 32.2	23.9 ± 6.86	25.4	74.5 ± 7.19	12.6 ± 1.84	16.9

*Batch 1 results

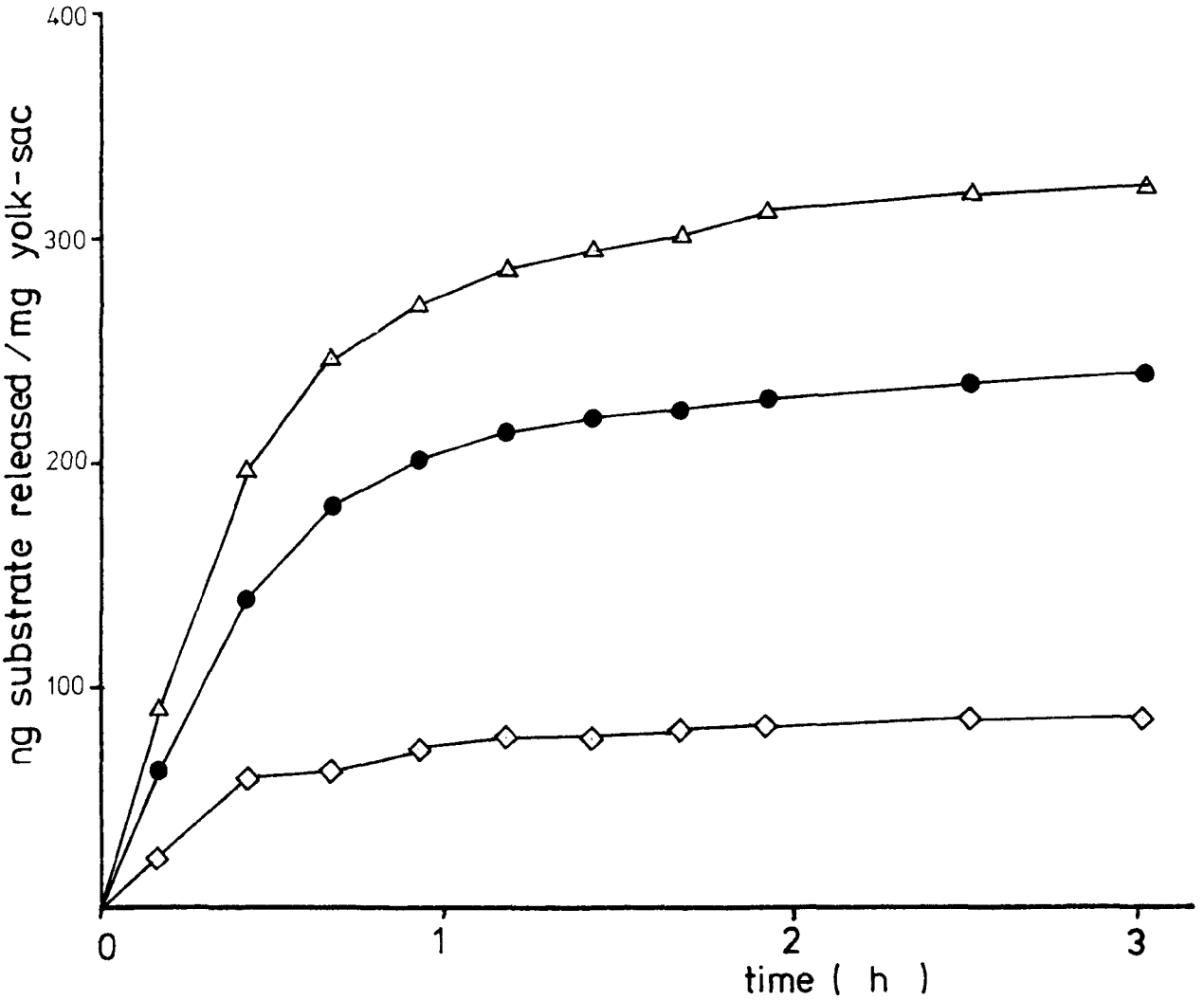


Figure 5.1 Time course of release of radioactive species derived from ^{125}I-labelled bovine IgG following incubation of rat yolk-sacs with substrate in serum-free medium 199

17.5-day rat yolk-sacs were first incubated for 2h in serum-free medium 199 in the presence of ^{125}I-labelled bovine IgG (10 µg/ml). Following washing, they were reincubated in fresh medium, and release of radioactivity was monitored up to 3h (see Section 5.2.2 for details). The total amount of substrate associated with the yolk-sac at the beginning of reincubation was 385 ng/mg yolk-sac.

Total radioactivity released —△—

TCA-soluble radioactivity released —●—

TCA-insoluble radioactivity released —◇—

The values shown are means from 6 separate experiments performed with the same batch (Batch 1) of ^{125}I-labelled bovine IgG used in the experiments quoted in Table 5.1.

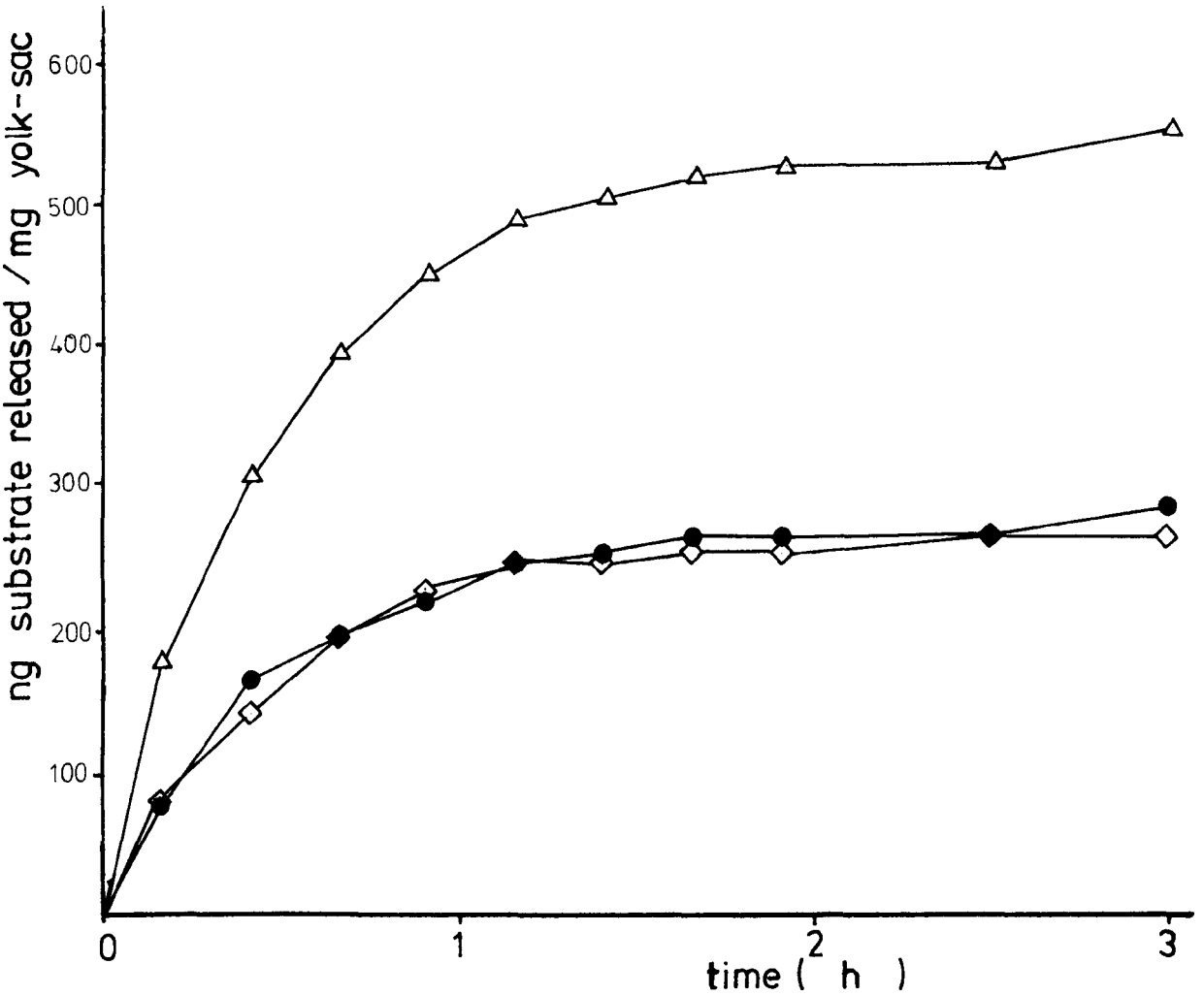


Figure 5.2 Time course of release of radioactive species derived from ^{125}I-labelled human IgG following incubation of rat yolk-sacs with substrate in serum-free medium 199

17.5-day rat yolk-sacs were first incubated for 2h in serum-free medium 199 in the presence of ^{125}I-labelled human IgG (10 µg/ml). Following washing, they were reincubated in fresh medium, and release of radioactivity was monitored up to 3h (see Section 5.2.2 for details). The total amount of substrate associated with the yolk-sac at the beginning of reincubation was 615 ng/mg yolk-sac.

Total radioactivity released —△—

TCA-soluble radioactivity released —●—

TCA-insoluble radioactivity released —◇—

The values shown are means from 6 separate experiments performed with the same batch (Batch 1) of ^{125}I-labelled human IgG used in the experiments quoted in Table 5.1 .

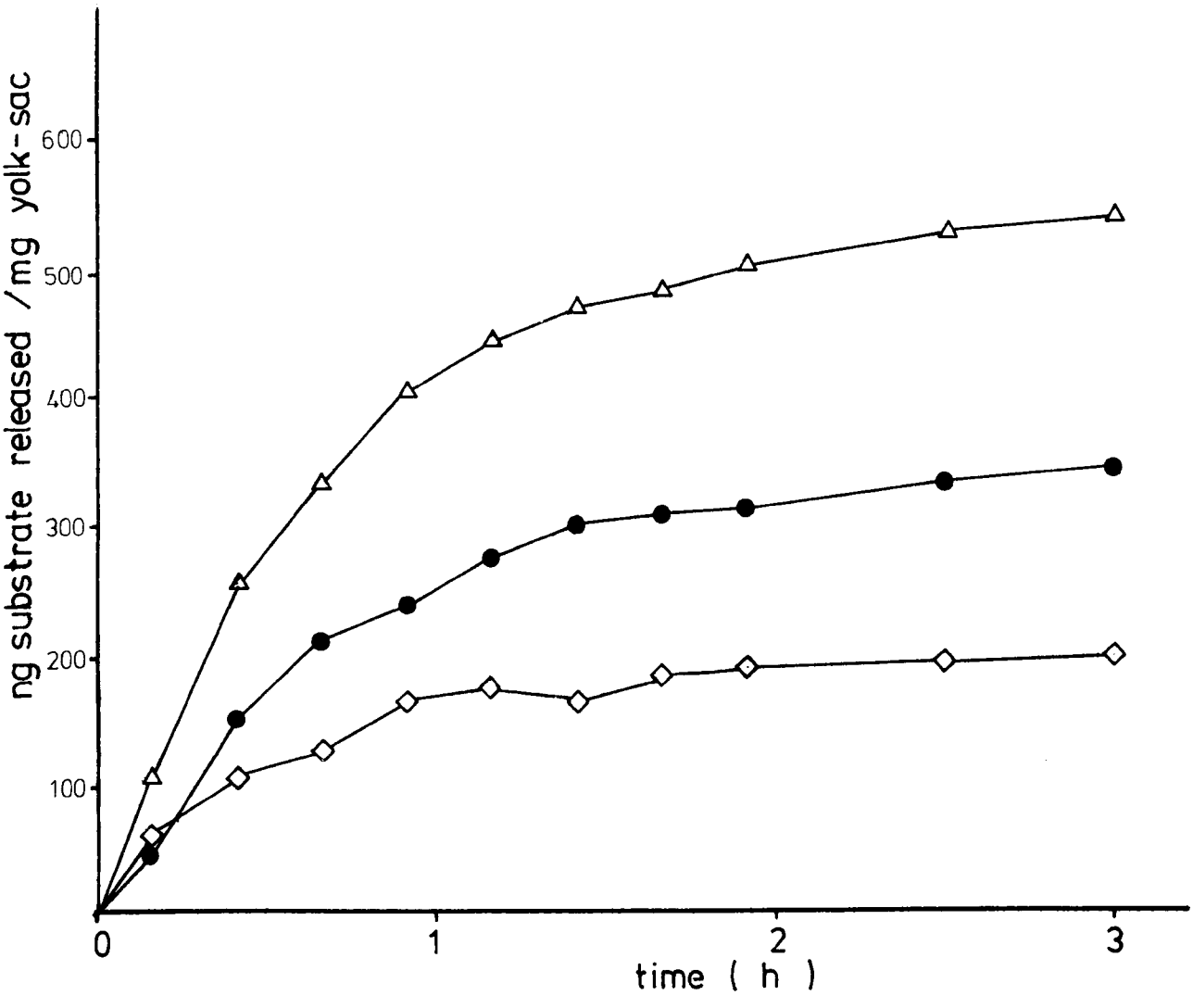


Figure 5.3 Time course of release of radioactive species derived from ^{125}I-labelled rat IgG following incubation of rat yolk-sacs with substrate in serum-free medium 199

17.5-day rat yolk-sacs were first incubated for 2h in serum-free medium 199 in the presence of ^{125}I-labelled rat IgG (10 µg/ml). Following washing, they were reincubated in fresh medium, and release of radioactivity was monitored up to 3h (see Section 5.2.2 for details). The total amount of substrate associated with the yolk-sac at the beginning of reincubation was 655 ng/mg yolk-sac.

Total radioactivity released ——△——

TCA-soluble activity released —— ● ——

TCA-insoluble radioactivity released —— ◇ ——

The values shown are means from 6 separate experiments performed with the same batch (Batch 1) of ^{125}I-labelled rat IgG used in the experiments quoted in Table 5.1 .

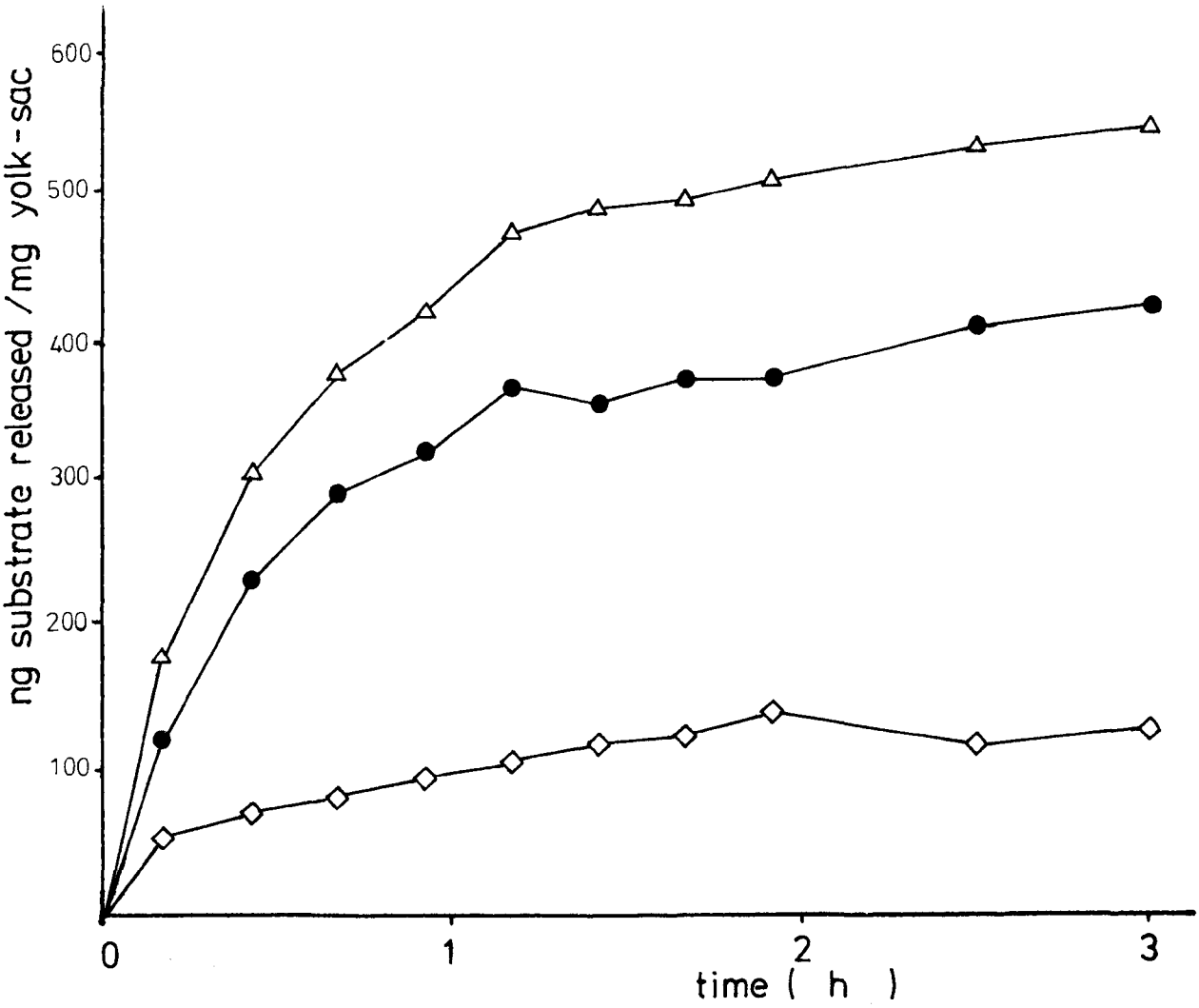


Figure 5.4 Time course of release of radioactive species derived from ^{125}I-labelled bovine IgG following incubation of rabbit yolk-sacs with substrate in serum-free medium 199

24-day rabbit yolk-sac pieces were first incubated for 2h in serum-free medium 199 in the presence of ^{125}I-labelled bovine IgG (10 µg/ml). Following washing, they were reincubated in fresh medium, and release of radioactivity was monitored up to 3h (see Section 5.2.2 for details). The total amount of substrate associated with the yolk-sac at the beginning of reincubation was 594 ng/mg yolk-sac.

Total radioactivity released ——△——

TCA-soluble radioactivity released ——●——

TCA-insoluble radioactivity released ——◇——

The values shown are means from 6 separate experiments performed with the same batch (Batch 2) of ^{125}I-labelled bovine IgG used in the experiments quoted in Tables 5.2 and 5.4 and 5.5 .

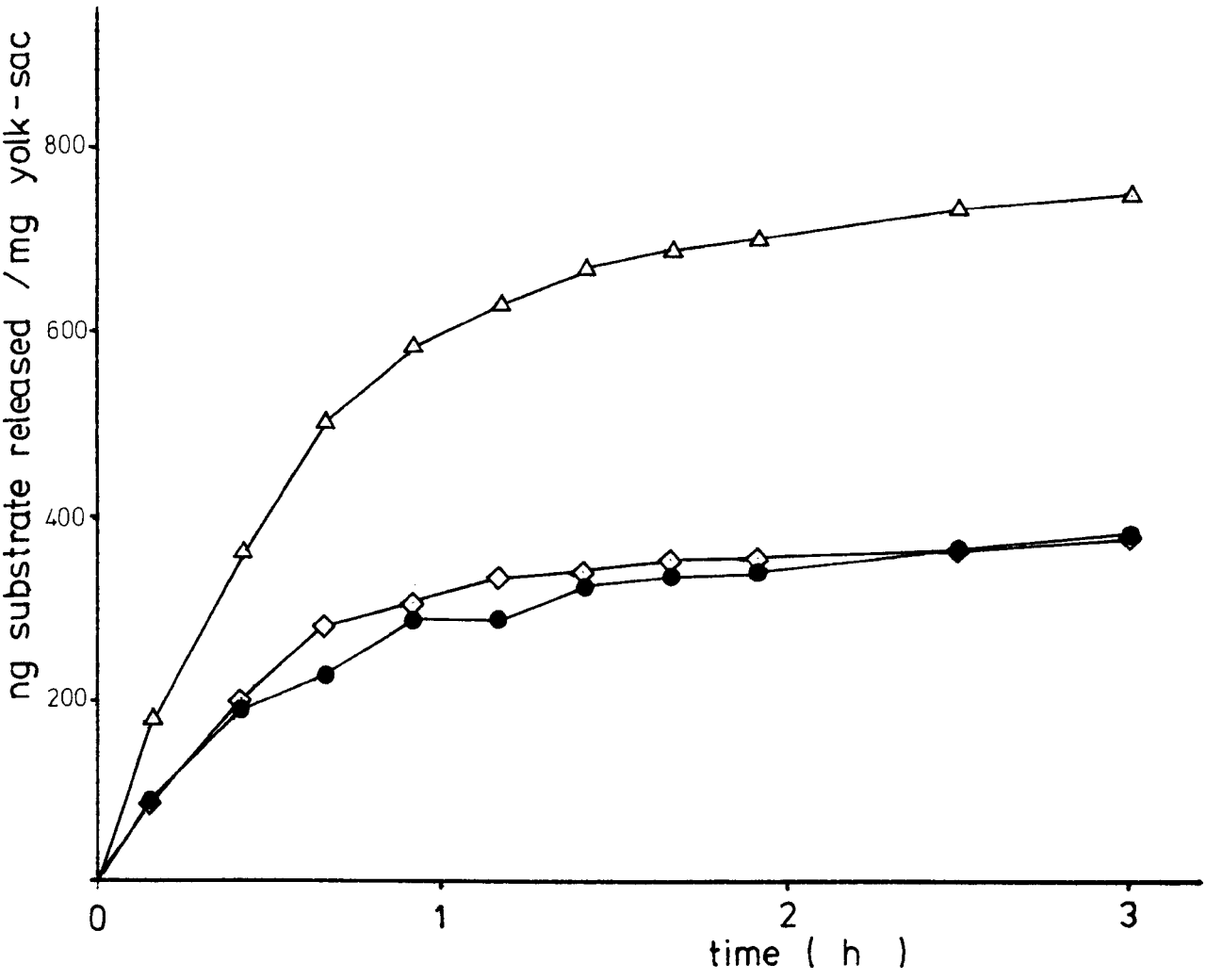


Figure 5.5 Time course of release of radioactive species derived from ^{125}I-labelled human IgG following incubation of rabbit yolk-sacs with substrate in serum-free medium 199

24-day rabbit yolk-sac pieces were first incubated for 2h in serum-free medium 199 in the presence of ^{125}I-labelled human IgG (10 µg/ml). Following washing, they were reincubated in fresh medium, and release of radioactivity was monitored up to 3h (see Section 5.2.2 for details). The total amount of substrate associated with the yolk-sac at the beginning of reincubation was 925 ng/mg yolk-sac.

Total radioactivity released —Δ—

TCA-soluble radioactivity released —●—

TCA-insoluble radioactivity released —◇—

The values shown are means from 6 separate experiments performed with the same batch (Batch 2) of ^{125}I-labelled human IgG used in the experiments quoted in Tables 5.2, 5.4 and 5.5 .

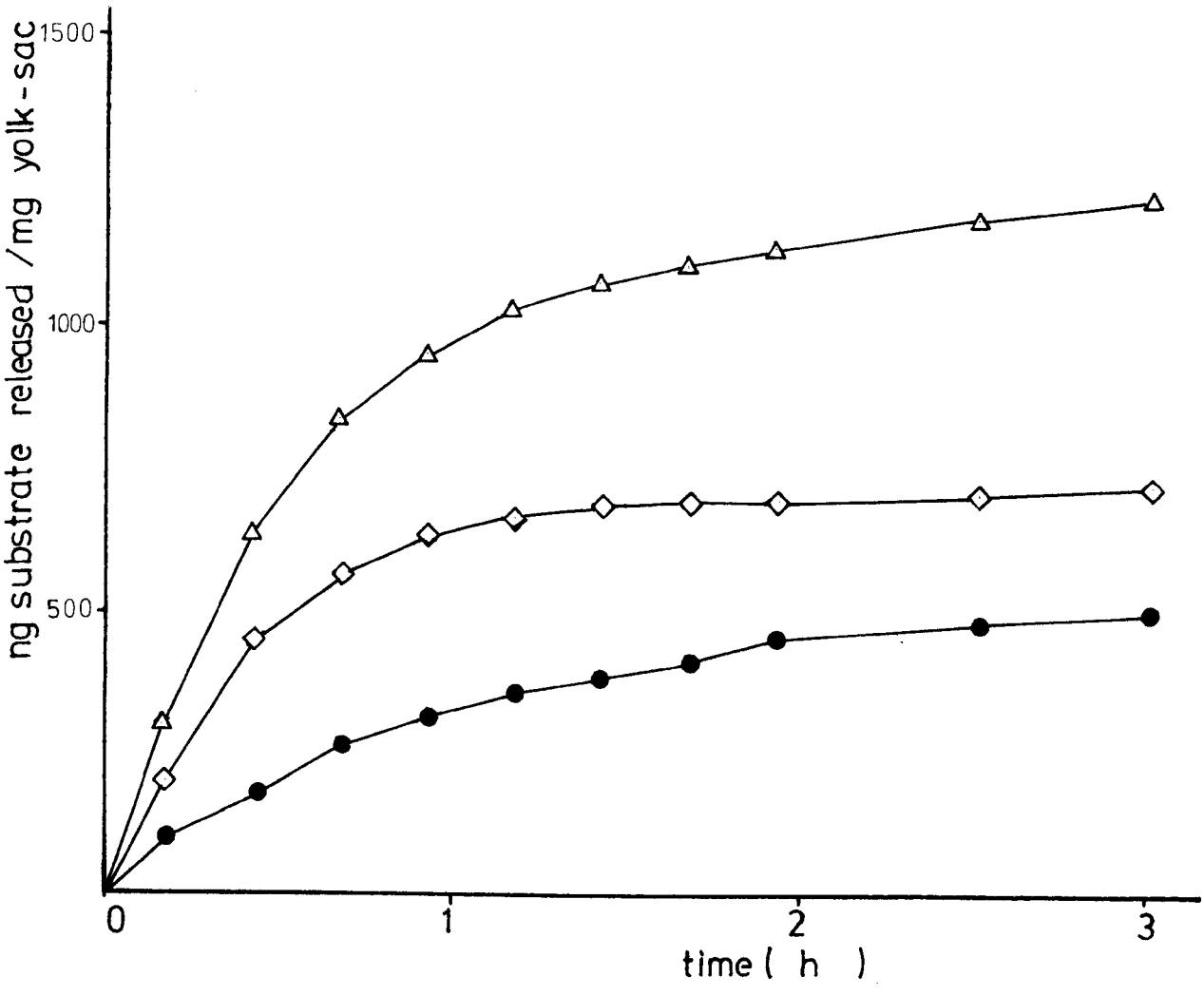


Figure 5.6 Time course of release of radioactive species derived from ^{125}I-labelled rabbit IgG following incubation of rabbit yolk-sacs with substrate in serum-free medium 199

24-day rabbit yolk-sac pieces were first incubated for 2h in serum-free medium 199 in the presence of ^{125}I-labelled rabbit IgG (10 µg/ml). Following washing, they were reincubated in fresh medium, and release of radioactivity was monitored up to 3h (see Section 5.2.2 for details). The total amount of substrate associated with the yolk-sac at the beginning of reincubation was 1509 ng/mg yolk-sac.

Total radioactivity released —Δ—

TCA-soluble radioactivity released —●—

TCA-insoluble radioactivity released — ◇ —

The values shown are means from 6 separate experiments performed with the same batch (Batch 2) of ^{125}I-labelled rabbit IgG used in the experiments quoted in Tables 5.2, 5.4 and 5.5.

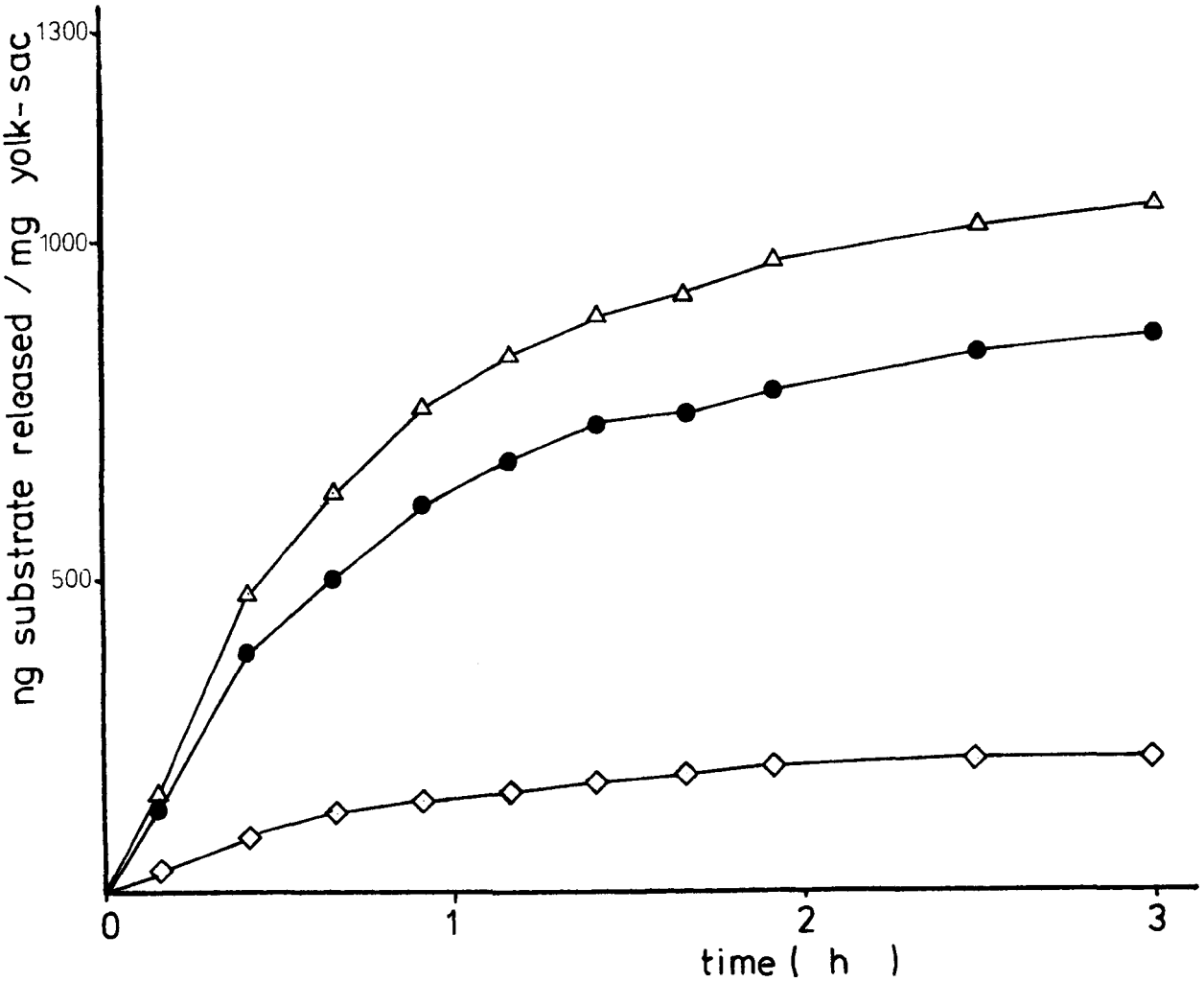


Figure 5.7 Time course of release of radioactive species derived from ^{125}I-labelled rat IgG following incubation of rabbit yolk-sacs with substrate in serum-free medium 199

24-day rabbit yolk-sac pieces were first incubated for 2h in serum-free medium 199 in the presence of ^{125}I-labelled rat IgG (10 µg/ml). Following washing, they were reincubated in fresh medium, and release of radioactivity was monitored up to 3h (see Section 5.2.2 for details). The total amount of substrate associated with the yolk-sac at the beginning of reincubation was 1335 ng/mg yolk-sac.

Total radioactivity released —△—

TCA-soluble radioactivity released —●—

TCA-insoluble radioactivity released —◇—

The values shown are means from 6 separate experiments performed with the same batch (Batch 2) of ^{125}I-labelled rat IgG used in the experiments quoted in Tables 5.2, 5.4 and 5.5 .

Table 5.7 Release from rat and rabbit yolk sacs of radioactivity derived from ^{125}I-labelled homologous and heterologous IgG species

This table represents a summary of the data presented in Figures 5.3-5.7. The data report the release of substrate-derived radioactivity by 3h of reincubation in fresh medium. In addition to the values of substrate release in absolute terms (ng/mg yolk-sac protein), release data are also expressed as a percentage of the total amount of substrate associated with the tissue at the beginning of the reincubation period. The S.D. for 3h values are reported in this table (c.f. Figures).

a) Rat yolk-sac incubations

^{125}I-IgG species	Quantity released by 3h			Percentage release		
	TCA-solubles	TCA-insolubles	Totals	TCA-solubles	TCA-insolubles	Totals
	(ng substrate released/mg yolk-sac tissue)					
Bovine (Batch 1)	142.1 ± 69.3	81.6 ± 22.1	325.2 ± 71.4	62.6	22.0	84.5
Human (Batch 1)	287.6 ± 29.4	265.5 ± 31.6	552.1 ± 30.7	46.7	43.2	89.7
Rat (Batch 1)	346.2 ± 65.3	197.6 ± 22.2	543.8 ± 78.9	52.6	30.4	83.0

b) Rabbit yolk-sac incubations

^{125}I-IgG species	Quantity released by 3h			Percentage release		
	TCA-solubles	TCA-insolubles	Totals	TCA-solubles	TCA-insolubles	Totals
	(ng substrate released/mg yolk-sac tissue)					
Bovine (Batch 2)	413.5 ±130.5	128.0 ± 59.2	540.5 ±108.7	69.6	21.6	91.2
Human (Batch 2)	374.1 ± 83.3	370.2 ±104.5	744.3 ±173.0	40.4	40.0	80.4
Rabbit (Batch 2)	504.1 ±137.3	712.4 ±175.5	1216.5 ± 291	33.4	47.2	80.6
Rat (Batch 2)	852.8 ±244.0	205.6 ± 96.3	1058 ±331	63.9	15.4	79.3

5.4 DISCUSSION

The transmission of passive immunity from mother to young has been shown by *in vivo* studies to be selective with regard to immunoglobulins from various species and also other protein molecules. The primary aim of this chapter was to see whether a similar kind of selectivity could be demonstrated in the rat and rabbit yolk sac *in vitro*, and then to attempt to elucidate possible interspecies differences between the two tissues with respect to selectivity. A brief glance at the main results (Tables 5.6 and 5.7) shows that the fates of homologous and heterologous ^{125}I-labelled IgG's do indeed differ from one another in both the rat and the rabbit yolk sac in the *in vitro* incubation system.

Looking first at the uptake data obtained from rat and rabbit yolk-sac incubations, it is apparent that homologous and heterologous ^{125}I-labelled IgG species differ quite strongly from each other in their fates (Table 5.6). This is manifest by different Endocytic Indices, Tissue-Accumulation Rates and relative degrees of tissue accumulation. At this point it is important to remember that Endocytic Indices measure only the net uptake of a substrate. The amount of substrate readily released back into the medium by the tissue after uptake is not taken into account in the expression of the Endocytic Index. As a result, Endocytic Indices cannot be taken as an index of transcellular transport. The Tissue-Accumulation Rate, on the other hand, should to some extent measure the quantity of material accumulated in a specific transport system. However, the Tissue-Accumulation Rate may also reflect the degree of intralysosomal accumulation of a poorly degraded protein, under conditions in which the rate of uptake exceeds the rate of degradation. Thus, T.A.R.s may either reflect differences in the amounts of the various IgG species being transported across the yolk sacs by a specific pathway, or differences in the structure of the IgG

molecules that affect their susceptibility to uptake and degradation within the lysosomal system. This latter possibility will be dealt with in more detail in Chapter 8. The implications of these considerations are that uptake data (that is E.I. and T.A.R.s) may not agree with data obtained from reincubation studies; of the in vitro findings, the latter are expected to give a more direct and reliable measure of transcellular transport.

Despite these reservations, a closer examination of the uptake data (Table 5.6) brings to light some rather interesting general findings. [For purposes of comparison, the uptake rates of the different ^{125}I-labelled IgG species and the rates of uptake of ^{125}I-labelled PVP under equivalent conditions (see Chapter 2) are all discussed here.] In the rat yolk-sac system, the rate of uptake of ^{125}I-labelled PVP has been established as the rate of fluid-phase uptake (Roberts et al., 1977). Even in the absence of definitive evidence, it should be fairly safe to assume that the same is the case in the rabbit yolk sac. Thus, it is obvious that in both the rat and rabbit yolk sac all ^{125}I-labelled IgG species have Endocytic Indices that are higher than that of ^{125}I-labelled PVP, even though the Endocytic Index of ^{125}I-labelled PVP does increase very sharply when serum is removed from the medium in which rabbit yolk sacs are incubated. In other words the greater proportion of all the species of IgG is taken up by adsorptive pinocytosis. In view of the theories put forward by Brambell and Wild for maternofoetal transport of IgG in the rabbit, this is not surprising since both workers postulate the presence of specific IgG receptors on the yolk sac membrane to ensure the protection of the bound molecules. Therefore by both theories it is expected that all IgG entering the "protective" IgG pathway is taken up by adsorptive pinocytosis. If, however, allowances are made for both fluid-phase uptake and tissue

accumulation, i.e. adding together these two rates for each substrate, the very surprising finding is that the actual Endocytic Indices exceed these values in all cases; the difference is particularly marked in serum-free medium. This means that more IgG binds to the cell-membrane surface than is transported across the tissue intact so that at least some of the adsorbed IgG must be degraded in the same manner as that taken up via the fluid phase. Hence, not all adsorption of IgG to the tissue leads to intralysosomal accumulation or transport. As this assumption is one of the major pillars supporting Brambell's theory, this hypothesis would have to be strongly modified to account for the existence of a receptor-mediated degradative IgG route. The above findings are true for all ^{125}I-labelled IgG species investigated in rat and rabbit yolk sacs. Interspecies differences between the ^{125}I-labelled IgG preparations will now be considered.

Looking first at the data obtained from rabbit yolk-sac incubations (Table 5.6), it is clear that the rabbit and bovine ^{125}I-labelled IgG differ strongly from each other in their fates. In the presence and absence of calf serum the homologous ^{125}I-IgG has the lowest Endocytic Index coupled with a relatively rather higher Tissue-Accumulation Rate (see Tables 5.4 and 5.6). A high T.A.R., as mentioned above, is simply an indication that not all the ^{125}I-labelled rabbit IgG taken up by the tissue is immediately released back into the incubation medium in a degraded form, but is accumulated in the tissue. These findings indicate that the greater part of the ^{125}I-labelled rabbit IgG taken up by the rabbit yolk sac is not readily degraded by the tissue. In contrast, in serum-free medium ^{125}I-labelled bovine IgG has a high Endocytic Index in the rabbit yolk sac and a very low Tissue-Accumulation Rate. As reincubation studies give a more reliable indication of the respective fates of IgG molecules in the yolk sac, it is interesting to note that up to 60% of the tissue-associated radioactivity derived from ^{125}I-labelled

rabbit IgG is released as TCA-insolubles, on reincubating rabbit yolk sacs that previously had accumulated this radioactive marker molecule (see Table 5.7 for details) whereas there is only a low release of TCA-insoluble activity from rabbit yolk sacs "preloaded" with bovine ^{125}I-labelled IgG (Table 5.7). These data suggest that, unlike rabbit IgG, most of the ^{125}I-labelled bovine IgG taken up by the rabbit yolk sac *in vitro* is degraded. (The values of the ratio TAR/EI for human and rat ^{125}I-labelled IgG fall between those for rabbit and bovine IgG, with the fate of ^{125}I-human IgG resembling more that of the ^{125}I-rabbit IgG, and ^{125}I-rat IgG being treated by the rabbit yolk sac *in vitro* in a manner more similar to ^{125}I-labelled bovine IgG.) Of the data quoted in the results section, the percentage release of TCA-soluble radioactivity (Table 5.7) gives the clearest indication of the degree of molecular degradation in the yolk sacs *in vitro*.

In the uptake studies discussed above, tissue accumulation, especially expressed as a percentage of total uptake, may possibly be a measure of protein accumulating undegraded in the yolk sac; the interpretation of the release of acid-insoluble radioactivity in re-incubation studies is less equivocal, hence more importance should be attached to these latter data. Quoting both T.A.R./E.I. ratios and percentage TCA-insolubles in rank order for the various ^{125}I-labelled IgG species gives the following patterns for rabbit yolk sacs:

TAR/EI (no serum in medium)	rabbit > human > rat > bovine (see Table 5.6)
Percentage release of TCA-insolubles	rabbit > human > bovine > rat (see Table 5.7)

The correlation between these two rank orders relating to "escape from being degraded" and the order of transfer of homologous and heterologous IgG species to the rabbit foetus *in vivo* (Cohen, 1950; Batty *et al.*, 1954) is striking see also Table in Introduction, 5.1).

The same selectivity between IgG species supports the contention that the mechanism that is operating *in vitro* is the same that is responsible for selective transfer *in vivo*.

Similarly, in the rat a parallel can be drawn between *in vitro* and *in vivo* findings. The ratio TAR/EI and the percentage release of TCA-insolubles for the homologous and heterologous ^{125}I-labelled IgG species fall in the following rank orders:

TAR/EI (no serum in medium)	rat > human > bovine	(see Table 5.6)
Percentage release of TCA-insolubles	human > rat > bovine	(see Table 5.7)

Koch *et al*. (1967) found the order of prenatal transmission of homologous and heterologous IgG in the rat *in vivo* to be: human > rat > bovine, which correlates with the rank order for *in vitro* release of TCA-insoluble radioactivity.

Having shown that, *in vitro*, the rabbit and the rat yolk sacs exhibit broadly the same pattern of selectivity towards homologous and heterologous species of IgG as exists in the respective tissues *in vivo*, the quantitation of uptake will now be considered in more detail. Experiments in which the rates of uptake of homologous and heterologous ^{125}I-labelled IgG were investigated were performed under two different conditions: in the presence and in the absence of calf serum. As noted before (Chapter 2), Endocytic Indices rose by an order of magnitude when calf serum was not included in the incubation medium in either the rat or the rabbit yolk-sac incubations. Such increases can be accounted for in terms of absence from the medium of serum proteins that compete with the ^{125}I-labelled IgG for membrane binding sites. Since the concentration of the serum proteins is much greater than that of the radioiodinated substrate, it is not surprising that such effects are marked.

Looking at the rates of uptake of homologous and heterologous ^{125}I-labelled IgG in detail (see Table 5.6), the rank order in the rabbit yolk sac is:

Endocytic Index (serum-containing medium)	rat > human > bovine > rabbit
Endocytic Index (serum-free medium)	human > rat > bovine > rabbit

Although those two patterns are similar, the uptake in serum-free medium is simpler to analyse because of the absence of any complications caused by the presence of serum proteins. The finding here is that the IgG species that is protected against degradation to the greatest extent (i.e. the homologous ^{125}I-labelled rabbit IgG) also has the lowest uptake rate. This may reflect the low affinity of rabbit IgG for nonspecific binding sites on rabbit yolk sac endodermal cells relative to other IgG molecules. The Endocytic Index of ^{125}I-labelled human IgG, which is equally well transferred *in vivo* in the rabbit and is protected against degradation *in vitro*, on the other hand, is very high, the non-transferred IgG species, rat and bovine, have intermediate uptake rates. However, in the rat yolk sac *in vitro* the range of variability in the rate of uptake is less than that in rabbit yolk sacs *in vitro*, so that attempts to put the Endocytic Indices of homologous and heterologous IgG's in rank order are not very meaningful, but show the following trend:

Endocytic Indices (serum-containing medium)	bovine > rat > rabbit=human
Endocytic Indices (serum-free medium)	human > rat > bovine

All the above findings are compatible with the functioning of two separate routes in the rabbit and also in the rat yolk sac *in vitro*; one of which is general and leads to the degradation of the ingested protein molecules, the other pathway is specific, transporting certain

protein molecules intact across the yolk sac. As was stated earlier, the "degradative" as well as the "protective" IgG route in yolk sacs seems to be mediated by selective adsorption (Chapter 2). The very different rates of uptake, especially in the rabbit yolk sac, could be accounted for by differences in the structures of the IgG molecules causing different degrees of adsorption to the binding sites on the plasma membrane. Looking in particular at the fates of ^{125}I-labelled human and rabbit IgG in the rabbit yolk sac, it is apparent that both of these molecules escape from being degraded in this tissue to a similar degree, as expressed by their high percentage of TCA-insolubles released from the yolk-sac tissue after previous "loading" with these substrates. That region in the IgG molecule responsible for "protective" binding may therefore be very similar in rabbit and human IgG. However, since these two substrates differ considerably in their Endocytic Indices it is very probable that the part of the IgG molecule responsible for binding to the "degradative" receptor differs in rabbit and human IgG. From these observations it appears that "protective" and "degradative" binding to the yolk-sac surface involves different regions of the IgG molecule.

Although a number of similarities have been shown between rat and rabbit yolk sacs in vitro, in a number of respects the two tissues differ in their treatment of homologous and heterologous ^{125}I-labelled IgG. The first of these differences is the rather low Endocytic Index for the homologous ^{125}I-labelled IgG in the rabbit coupled with the relatively high Tissue-Accumulation Rate. In the rat yolk sac, on the other hand, homologous ^{125}I-IgG has a higher E.I. and a lower T.A.R. This pattern is particularly noticeable in serum-free incubations. These findings suggest that more homologous IgG is degraded in a non-specific pathway in the rat than in the rabbit yolk sac in vitro. This is compatible with the yolk sac being the only site of transfer of passive

immunity in the rabbit, whereas in the rat, the neonatal gut is the major site, with the yolk sac providing only a secondary route (Halliday, 1955; Mayersbach, 1958; Williams & Ibbotson, 1979). This may also explain the second major difference between the rat and rabbit yolk sac *in vitro*. For all ^{125}I-labelled IgG species, the Tissue-Accumulation Rates in the rabbit are much higher than in the rat yolk sacs, especially when expressed as a percentage of the Endocytic Index (T.A.R./E.I.). This is compatible with the specific "protective" route involved in IgG transport being more developed in the rabbit than in the rat yolk sac. Finally, the rabbit yolk sac *in vitro* seems to have almost double capacity for "holding" IgG, this being determined as the total amount of substrate (ng per mg yolk-sac protein) released after previous"loading". A similarity between both tissues is that ^{125}I-labelled bovine IgG shows the lowest release of both total and acid-insoluble radioactivity (ng per mg yolk-sac protein), as well as the lowest Tissue-Accumulation Rate in serum-free medium. In all respects, this IgG molecule is treated more like ^{125}I-labelled BSA.

The fact, however, that both degradative and non-degradative pathways can be demonstrated in the rat as well as in the rabbit yolk sacs *in vitro* underlies the essential similarity in properties of the two tissues and in turn suggests that this tissue is involved in the transfer of passive immunity from mother to young in the rat as well as the rabbit. Further, and more conclusive, evidence is the finding that the *in vitro* selectivity follows the same pattern as *in vivo* selectivity in both species. Although such findings do not of course exclude the involvement of other tissues in prenatal transfer of immunity in rats and rabbits, they strongly suggest that yolk sacs play a similar role in both species, but with the selective route being more developed in the rabbit than in the rat.

In summary, the following conclusions are put forward:

1. The fates of homologous and heterologous ^{125}I-labelled IgG differ significantly in both rabbit and rat yolk sacs *in vitro*. All are taken up by adsorptive pinocytosis, but whereas some are almost totally degraded in the yolk sacs, others are either readily accumulated within the tissue or are readily released in an undegraded form on reincubating the tissue.

2. The rank order for release of TCA-insoluble radioactivity derived from different species of IgG from yolk-sac tissue follows closely the order of prenatal transfer from mother to foetus in rabbit and rat *in vivo*.

3. The similarity in the behaviour of both rat and rabbit yolk sacs *in vitro* towards homologous and heterologous ^{125}I-labelled IgG is compatible with the suggestion that in rats as well as in rabbits, the yolk sac is a site for selective transmission of immunoglobulins.

4. The observation that, *in vitro*, relative to the rat and the rabbit yolk sac has the better developed carrying capacity for IgG and a lower catabolic activity, is compatible with the yolk sac playing a greater role in the selective transfer of IgG *in vivo* in the rabbit than in the rat.

CHAPTER SIX

The Interference of Different Species of IgG with the Release of IgG from Rat and Rabbit Yolk Sacs

6.1 INTRODUCTION

In the previous chapter it was shown that the patterns of release of different ^{125}I-labelled IgG molecules, from rat and rabbit yolk sacs incubated *in vitro*, were entirely compatible with the published observations for the transfer of homologous and heterologous IgG from mother to foetus. But neither the published *in vivo* studies nor the *in vitro* studies reported in the earlier chapters give any information concerning the precise mechanism responsible for the protection of homologous IgG during its prenatal transport across foetal membranes.

In this chapter an attempt was made to investigate one of the features by which two of the current theories can be distinguished from the other, namely the suggested presence, on the outer face of the plasma membrane, of receptors that are specific for certain proteins. Both the models of Brambell and Wild require the presence, on the endodermal yolk-sac membrane of the rabbit, of receptors designed to bind to the Fc portion of the IgG molecule (Brambell, 1966, 1970; Wild, 1975, 1976). Similarly, Rodewald (1973, 1976) in a theory accounting for the selective transport of IgG across the neonatal rat gut, stipulates that specific receptors occur only in the proximal half of the rat gut. By contrast, Hemmings & Williams (1976) have proposed a theory according to which the first step in selective transport across the rabbit yolk sac involves non-selective uptake into vesicles with no specific receptors. Some vesicles are then purported to burst releasing their contents into the cytoplasm. According to this theory only those proteins released into the cytosol are candidates for transport, while molecules retained within intact vesicles are destined to undergo degradation by lysosomal enzymes. Selectivity is suggested to operate at the level of release from the basal and lateral membranes of the cell, but no detailed mechanism has been suggested so that it is

difficult to examine this theory experimentally.

Indirect information on the nature of the transport mechanism comes from *in vivo* studies of the kinetics of transfer, and rests on the assumption that models like those proposed by Brambell and Wild involve transport systems that are saturable and show zero or first order kinetics of transfer. In rabbit yolk sacs *in vivo*, Sonoda & Schlamowitz (1972) have indeed shown that maternofoetal transfer of rabbit IgG and of rabbit serum albumin proceeds according to a process that shows zero order kinetics.

Investigations, by a number of experimental techniques, have given more direct evidence for the presence of specific receptors on rabbit yolk sacs, rat gut, human chlorioallantoic placenta, etc. Thus, Morris, I.G. (1956) found that when sera of different species (man, rabbit, ox) were mixed with immune serum and administered by mouth to suckling rats or mice, they reduced the rate of entry of antibodies relative to a similar admixture with homologous non-immune serum. He termed this effect "interference" and Brambell *et al*. (1958) subsequently identified the serum component responsible as IgG, by achieving the same interference effect when replacing whole serum by unlabelled IgG. Like selection, "interference" displays a great degree of specificity. For example, Brambell *et al*. (1958) showed that the uptake of homologous IgG by the neonatal mouse gut *in vivo* could be interfered with by the addition of IgG molecules derived from various species and showed that their effectiveness followed the rank order : rabbit > guinea-pig > human > bovine > rat=hamster=sheep=mouse.

However, *in vivo* studies are not well suited to more detailed investigations of the nature of the IgG receptors because of: 1) the possible existence of multiple transmission sites in the animal; 2) uneven distribution of substrate at the transport sites and 3) the uncertainty of whether the molecule has been structurally or otherwise

modified in the animal prior to reaching the site of transport. Therefore attempts to characterize these proposed membrane receptors have been made using *in vitro* systems based on a variety of tissues. For example, Sonoda & Schlamowitz (1972); Tsay & Schlamowitz (1975); Schlamowitz *et al.* (1975) have demonstrated the presence of receptors on the rabbit yolk-sac membrane by using both formaldehyde-fixed tissue and also a special kind of vesicles ("Schlamowitz vesicles") claimed to consist of plasma membrane only. Similarly, using membrane suspensions isolated from human placenta, Balfour & Jones (1977) found a receptor specific for the light chain and the Fc regions of IgG. Likewise, by using antibody-coated red blood cells in a rosetting technique Elson *et al.* (1975) demonstrated Fc receptors on mouse placenta and yolk-sac cells and Wild & Dawson (1977), using the same technique, found Fc receptors on rabbit yolk-sac cells. In addition, Hillman *et al.* (1977) have characterized Fc receptors on rabbit yolk sacs by a different method, by binding IgG to brush-border membranes, tissue homogenate, plasma-membrane fractions and formalin-fixed discs of tissue. Experimental findings concerning selectivity of binding between homologous and heterologous IgGs are conflicting. Hemmings & Williams (1974) claim no selection in the extent of binding of rabbit and bovine ^{125}I-labelled IgG to membrane-containing cell-fractions of rabbit yolk sacs, whereas Tsay & Schlamowitz (1975) present evidence of such selectivity in the rabbit yolk sac.

Several aspects of the interpretation of data from such *in vitro* binding studies, as described above, are open to serious criticism. First, the behaviour of the living tissue *in situ* is very likely to differ from that of fragments of the same tissue isolated by various centrifugation techniques and in some cases even subjected to fixation in powerful reagents such as formalin, that are known to modify protein

structures. Second, apparent selectivity might be the result of general structural differences (size, shape and charge) of the entire IgG molecule and not of structural differences in the Fc region, as required in the receptor theory. Third, specific binding sites on cell membranes may be generated only during the formation of coated micropinocytic vesicles or normal pinosomes and might therefore not be apparent in non-living preparations. [For example, in the formation of coated micropinocytic vesicles a capping effect of ligand-receptor complexes may be essential. This has been postulated for IgG transport (Ockleford, 1976) and demonstrated in the interaction of immunoglobulins with the lymphocyte-surface receptors during the immune reaction (Taylor *et al.*, 1971).] Fourth, *in vitro* binding studies, like ultra-structural studies, could be very misleading, because in the molecular transfer across the membrane, a low capacity system could be operating but at very high speed. Using *in vitro* binding, this difference between the static and the dynamic carrying capacity of IgG by the tissue would be difficult, if not impossible to detect. However, an *in vitro* technique in which the intact, living tissue is maintained at all times under near physiological conditions is likely to differ less from the *in situ* tissue and hence should provide data that can be interpreted with few of the above reservations.

In the *in vitro* system one of the early findings (Moore *et al.*, 1977; Williams & Ibbotson, 1979) that is relevant to the issues raised above was the observation that adsorption to a plasma membrane alone does not ensure protection of IgG or other proteins from degradation. For example Moore *et al.* (1977) have shown that ^{125}I-labelled albumin is endocytosed by the rat yolk sac *in vitro* by adsorptive pinocytosis, as defined by Jacques (1969), but is entirely degraded within rat yolk-sac endodermal cells (Livesey & Williams, 1979). From the results of

Ibbotson & Williams (1979) and the reported findings (Chapter 5) it is apparent that *in vitro* ^{125}I-labelled IgG is also taken up by rat and rabbit yolk sacs mainly by adsorptive pinocytosis, the Endocytic Indices being higher (by at least an order of magnitude in serum-free medium) than the values accepted for fluid-phase pinocytosis (Pratten *et al.*, 1980). Thus it appears that the majority of all ^{125}I-labelled IgG, whether destined for transport or degradation, is taken up by the yolk sac by prior binding to the yolk-sac plasma membrane. This more general involvement of membrane binding in the rat yolk sac renders it important to distinguish at least two types of membrane binding, i.e. that associated with the "degradative" pathway and that associated with a "non-degradative" pathway.

It may be possible to block either the "protective" IgG receptor or the "degradative" IgG binding site to a given ^{125}I-labelled IgG species by exposing the yolk sac to sufficient quantity of an unlabelled species of homologous or heterologous IgG. The extent of such interference can be more easily quantified using an isolated intact tissue than from *in vivo* studies. Also, by attempting to block one pathway and by forcing substrate into the other pathway, information should be gained concerning the nature and possible saturability of receptors/binding sites involved in the separate routes, if there are indeed two different routes of protein transport into and through both the rat and rabbit yolk sacs.

In summary, this chapter is concerned with the following questions:

1) Can receptors, specific for homologous IgG be demonstrated on the intact rabbit and rat yolk sacs when incubated in the *in vitro* system?

2) Is it possible, in the *in vitro* system, to interfere with either : a) a specific (protective) IgG pathway, or b) a non-specific (degradative) IgG pathway for ^{125}I-labelled IgG molecules by using homologous and

heterologous unlabelled IgG species to block sites on the rat and rabbit yolk-sac plasma membrane?

3) Can "degradative" binding sites similar to those previously observed on the rat yolk sac, be demonstrated on the rabbit yolk sac in vitro?

4) Do rat and rabbit yolk sacs differ from each other with regard to interference in the uptake mechanism?

5) Can IgG be forced into the "degradative" pathway by effectively blocking the "specific" pathway by the addition of high concentrations of unlabelled IgG? Conversely, can blocking the "degradative" binding sites with unlabelled proteins leave only the "specific" pathway available to homologous IgG and so effectively abolish the degradation of tracer amounts of ^{125}I-labelled homologous IgG?

6.2 METHODS

6.2.1 Release of radioactive species, derived from ^{125}I-labelled bovine or rat IgG, from rat yolk sacs previously incubated with one of these substrates in the presence of either unlabelled homologous or heterologous IgG species

Rat yolk-sacs (17.5-day) were incubated for 2h in serum-free medium 199 containing both ^{125}I-labelled bovine (or rat) IgG (10 µg/ml) and native bovine (or human or rat) IgG at a higher concentration (100 µg/ml). After washing, yolk sacs were reincubated in fresh medium for up to 3h and aliquots of medium were removed at regular intervals. (The method is described in detail in Section 1.12). The amounts of total, TCA-insoluble and TCA-soluble radioactivity released were determined as outlined in Section 1.12.

6.2.2 Release, from rabbit yolk sacs, of radioactive species derived from ^{125}I-labelled bovine or rabbit IgG previously accumulated in the presence of unlabelled homologous or heterologous IgG species

Rabit yolk-sac pieces (24-day) were incubated for 2h in serum-free medium 199 containing radiolabelled substrate (^{125}I-labelled bovine or rabbit IgG at a concentration of 10 µg/ml). Also present in the incubation medium were unlabelled bovine, human, rabbit or rat IgG at a concentration of 100 µg/ml. The rest of the method followed closely the one outlined above (Section 6.2.1).

6.3 RESULTS

6.3.1 Presentation of results

Studies of the interference of unlabelled IgG with the uptake of ^{125}I-labelled homologous IgG, as determined by the subsequent release of radioactivity from yolk sacs took two forms. First, the effect of unlabelled homologous and heterologous IgGs present at a high concentration (100 μg/ml) on the total amount released of homologous ^{125}I-labelled IgG released was investigated. Second, attempts were made to interfere with the uptake and digestion of ^{125}I-labelled bovine IgG (an IgG species that is not transported from mother to foetus to any extent) by the addition to the uptake medium of homologous or heterologous IgG molecules at the same high concentration (100 μg/ml). Results are presented in the form of the time-course of substrate release (for some incubations only; Figure 6.5), histograms (Figures 6.1, 6.2, 6.3 and 6.4), and summary tables (Table 6.1 and 6.2). The format of histograms is the same as in Chapter 3. Histograms give an expression of interference of the unlabelled IgG with a) total tissue-accumulation, b) total release, c) TCA-soluble activity release (i.e. interference with the degradative pathway) and d) TCA-insoluble release (i.e. interference with the protective pathway) of the ^{125}I-labelled substrate. Summary tables (Tables 6.1 and 6.2) aid the establishment of trends in the data, so that relative effects can become obvious. In these tables the first of the four columns reports the fraction of the total radioactivity released that is acid-insoluble: i.e.

$$Y = \frac{\text{TCA-insoluble activity released}}{\text{Total activity released}}$$

The second column is an expression of the fractional release of acid-insolubles relative to controls, i.e.

$$Z = \frac{\left\{\begin{array}{l}\text{TCA-insoluble activity released by a yolk sac previously incubated in the presence of an unlabelled competing IgG species} \\ \hline \text{Total activity released by the same yolk sac}\end{array}\right\}}{\left\{\begin{array}{l}\text{TCA - insoluble activity released from control yolk sac (i.e. No competing IgG present)} \\ \hline \text{Total activity released from the control yolk sac}\end{array}\right\}}$$

(The third and fourth columns report the equivalent values for TCA-soluble activity.) The quantity will be referred to in the text as the "relative interference" with a pathway (protective or degradative) because it shows, relative to the control, how much IgG substrate is "pushed" into either the "degradative" or the "protective" pathway by the presence of unlabelled IgG in the incubation medium during the phase of substrate uptake. Thus, if this fractional release of acid-solubles (or acid-insolubles) expressed relative to controls is 100% in an experimental incubation with competing IgG species present, it means there is no difference between experimental and control tissues in the relative release of acid-solubles (or acid-insolubles). A percentage lower than 100%, however, indicates some degree of interference with the pathway investigated, whereas a percentage higher than 100% shows that some substrate has been "pushed" into this pathway, possibly by blocking the other, normally available pathway.

6.3.2 Interference in total tissue-accumulation and release, by rat and rabbit yolk sacs incubated in vitro, of ^{125}I-labelled homologous IgG by unlabelled bovine, human, rabbit or rat IgG

The possible effects of unlabelled immunoglobulins on the fate of ^{125}I-labelled homologous IgG within yolk-sac tissue are four-fold. First, interference with total tissue-accumulation; second, interference with the "protective" IgG route and third, interference with the

"degradative" IgG route. (These three possible effects are necessarily inter-related.) Finally, interference with the total amounts of protein released subsequent to uptake, without any attendant decrease in total tissue-accumulation.

Beginning with the rat yolk sac, these four possible effects will be investigated in turn for each species of "interfering" IgG (Figure 6.1). Bovine IgG causes a reduction in both total tissue-accumulation and release of ^{125}I-labelled rat IgG by rat yolk sacs; also release of TCA-soluble and -insoluble radioactivity are lower, with a relatively greater decrease in the release of the macromolecular material (Table 6.1). The presence of unlabelled human IgG during "loading" of yolk sacs with the homologous substrate causes no decrease in total tissue-accumulation, but TCA-insoluble release is markedly decreased with a simultaneous increase in the quantity of TCA-soluble material released. The presence of rat IgG as an interfering molecule gives rise to an intermediate pattern of results; total tissue-accumulation and release of substrate are decreased and the effects are almost entirely accounted for by the decrease in quantity of TCA-insoluble activity released (see Table 6.1). Thus, in the rat yolk sac, the "protective" IgG pathway is significantly interfered with only by human and rat IgG and the "degradative" route only by bovine IgG.

In the rabbit yolk sac (Figure 6.2) unlabelled bovine IgG does not noticeably affect the total tissue-accumulation of ^{125}I-labelled rabbit IgG or the relative release of TCA-insolubles and -solubles (Table 6.2). Unlabelled human IgG causes a decrease in the total tissue-accumulation and release of rabbit IgG, mainly at the expense of TCA-insolubles; TCA-solubles are only slightly decreased (Figure 6.2). A similar set of observations are made when unlabelled rabbit IgG is the competing species. Again, TCA-insoluble release is decreased, TCA-soluble release is little altered, but, whereas total

release of radioactivity is decreased, total tissue-accumulation is not (Figure 6.2). It is interesting to note that more than 50% of substrate remains in the tissue, even after 3h reincubation. This high tissue-retention of substrate can also be observed when rat IgG is the competing species. The other effect of the presence of rat IgG in the rabbit yolk-sac incubation medium during the "loading" phase is to decrease the total tissue-accumulation and release of ^{125}I-labelled rabbit IgG (Figure 6.2). The release of both TCA-soluble and TCA-insoluble activity are affected to the same extent (see Table 6.2 for relative values). Thus, in the rabbit yolk sac, the most noticeable interference effects of high concentrations of unlabelled homologous and heterologous IgG on ^{125}I-labelled rabbit IgG appear to be that human and rabbit IgG both cause a decrease in the amount of protein handled by the "protective" IgG pathway, bovine IgG has no effect on either pathway and rat IgG reduces both.

6.3.3 Interference with total tissue-accumulation and release of ^{125}I-labelled bovine IgG, by rat and rabbit yolk sacs when incubated in vitro, in the presence of unlabelled bovine, human, rabbit or rat IgG

In this series of experiments, the substrate was ^{125}I-labelled bovine IgG, a molecule that is not transported across the yolk sac *in vivo* (Brambell, 1970) and which has been shown to be almost entirely degraded in the rat and rabbit yolk sacs *in vitro* (see Chapter 5). Interfering with the total tissue-accumulation of this substrate in rat and rabbit yolk sacs *in vitro* by the addition of homologous and heterologous unlabelled IgG constituted an attempt to reduce the flux through the degradative IgG route in these tissues. At the same time the protective route would be expected to be blocked altogether, especially by the addition of rabbit IgG.

The data obtained for the rat yolk sac will be considered first (Figure 6.3). Here, none of the unlabelled IgG molecules caused a decrease in total tissue-accumulation and release of substrate. Rather there is possibly a slight increase in total tissue-accumulation and release in the presence of the competing proteins. The main effects observed are on the relative amounts of TCA-soluble and -insoluble radioactivity released. The presence of unlabelled bovine IgG during the "loading" phase causes little or no decrease in TCA-soluble release but there is a concommitant increase in TCA-insoluble release. Human IgG, on the other hand, has little effect on TCA-insoluble release, but increases TCA-soluble release. Rat IgG is like human IgG in effect. To summarize, there are no marked effects on either tissue-levels or release patterns (Figure 6.3). However, when looking at Table 6.1, it seems that bovine IgG interfers with the relative TCA-soluble release and human IgG interferes with the relative TCA-insoluble release of ^{125}I-labelled bovine IgG, but neither effect is very marked.

In the rabbit yolk sac, a greater complexity of findings is apparent (see Figure 6.4 and Table 6.2). Unlabelled bovine IgG has no detectable effect on either total tissue-accumulation or release of ^{125}I-labelled bovine IgG (Figure 6.4). The presence of human IgG in the uptake medium causes a marked decrease in total tissue-accumulation and release, but equally affects the release of acid-soluble and -insoluble breakdown products of bovine IgG (Table 6.2). Rabbit IgG as the interfering IgG species causes a greater decrease of radioactivity release than of total tissue-accumulation (as was also observed with ^{125}I-rabbit IgG as substrate). Looking at the relative TCA-soluble and -insoluble release (Table 6.2), the release of the macromolecular IgG species is decreased, whereas the relative release of the TCA-soluble radioactivity is, if anything, slightly increased. The addition of unlabelled rat IgG to the uptake medium causes the greatest effect on the total tissue-

accumulation and release of ^{125}I-labelled bovine IgG and also decreases markedly both the TCA-soluble and TCA-insoluble release from the tissue. Relatively, the protective IgG pathway is decreased to a greater extent (Table 6.2). In the rabbit yolk sac, therefore, an interference with both, protective and degradative pathways can be observed with human, rabbit and rat IgG exerting a strong absolute effect on the latter (Figure 6.4). Expressed in terms of "relative interference" (Table 6.2) the "protective" IgG route (TCA-insolubles) is decreased most by rat and rabbit IgG.

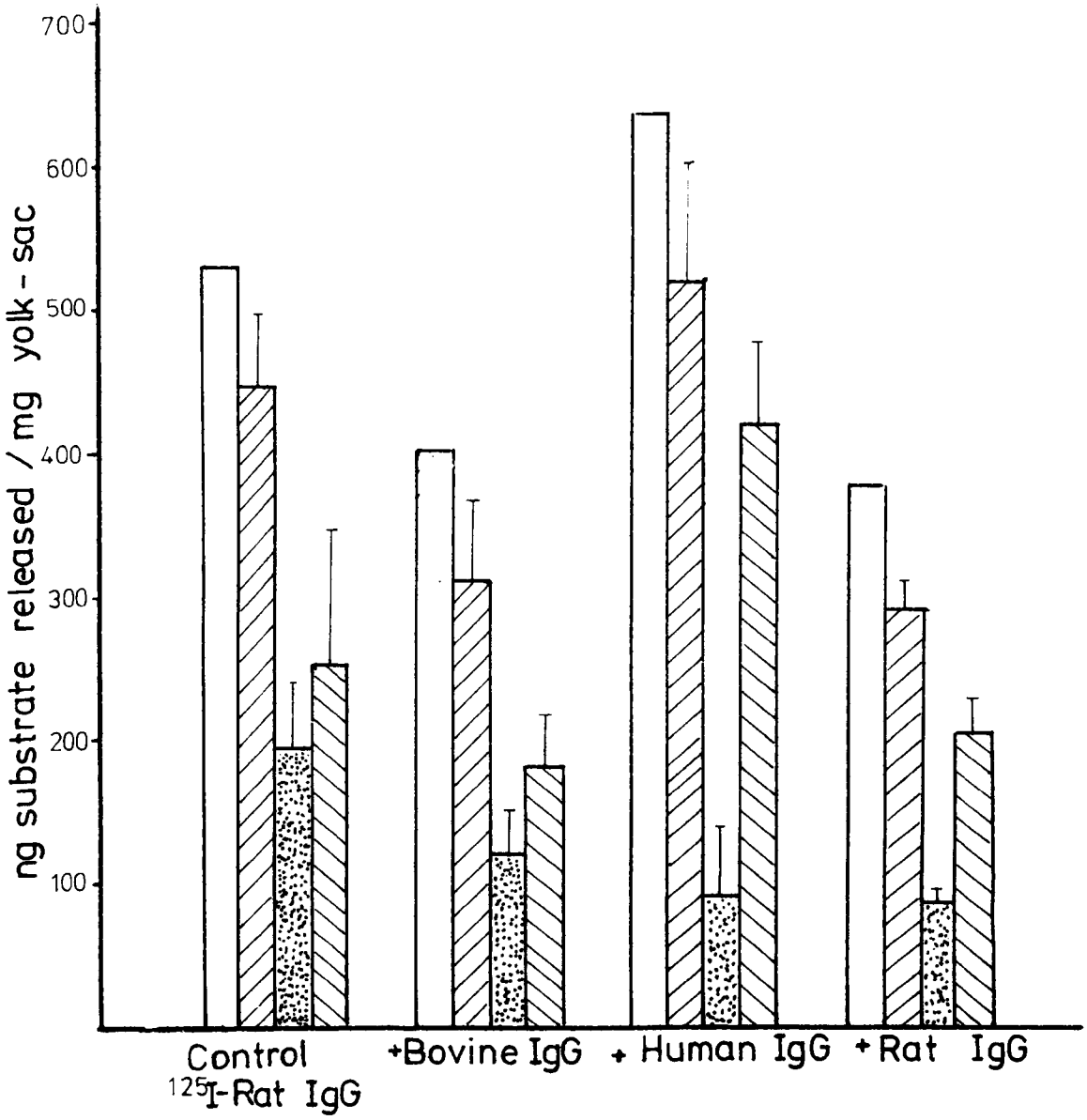


Figure 6.1 Release of ^{125}I-labelled species from rat yolk sacs on reincubating tissues previously exposed to ^{125}I-labelled rat IgG in the presence of various unlabelled IgG species

17.5-day rat yolk sacs were first incubated in serum-free medium 199 containing both ^{125}I-labelled rat IgG (10 µg/ml) and either bovine, human or rat IgG (100 µg/ml). After 2h, yolk sacs were washed and reincubated in fresh medium 199, up to a further 3h. Histograms show the amounts of total substrate associated with the tissue at the start of the reincubation period ☐,
total radioactivity release ▨,
TCA-soluble radioactivity release ▧
and TCA-insoluble radioactivity release ▩
after 3h of reincubation (see Section 6.2.2 for details). Values shown represent means (± S.D.) from 6 (control), 6(+bovine IgG), 6 (+ human IgG) and 5 (+rat IgG) separate experiments.

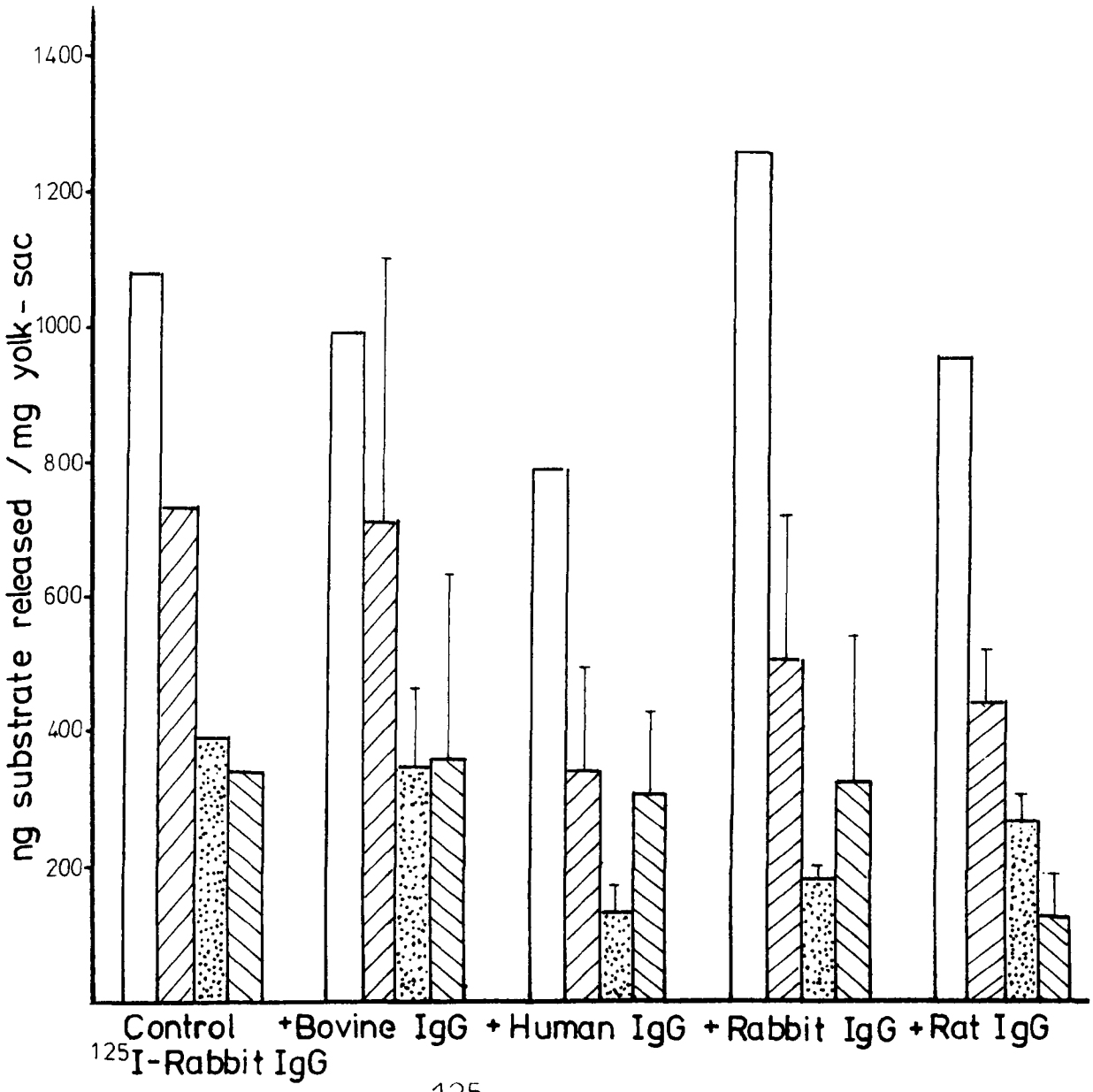


Figure 6.2 Release of ^{125}I-labelled species from rabbit yolk sacs on reincubating tissues previously exposed to ^{125}I-labelled rabbit IgG in the presence of various unlabelled IgG species

24-day rabbit yolk-sac pieces were first incubated in serum-free medium 199 containing both ^{125}I-labelled rabbit IgG (10µg/ml) and either bovine, human, rabbit or rat IgG (100 µg/ml). After 2h yolk-sacs were washed and reincubated in fresh medium 199, up to a further 3h. Histograms show the amounts of total substrate associated with the tissue at the start of the re-incubation period □ ,
total radioactivity release ▨ ,
TCA-soluble radioactivity release ▩
and TCA-insoluble radioactivity release ▩
after 3h of reincubation (see Section 6.2.2 for full method). Values shown represent means from 2 (control) and means (±S.D.) from 3 (all other incubation conditions) separate experiments each.

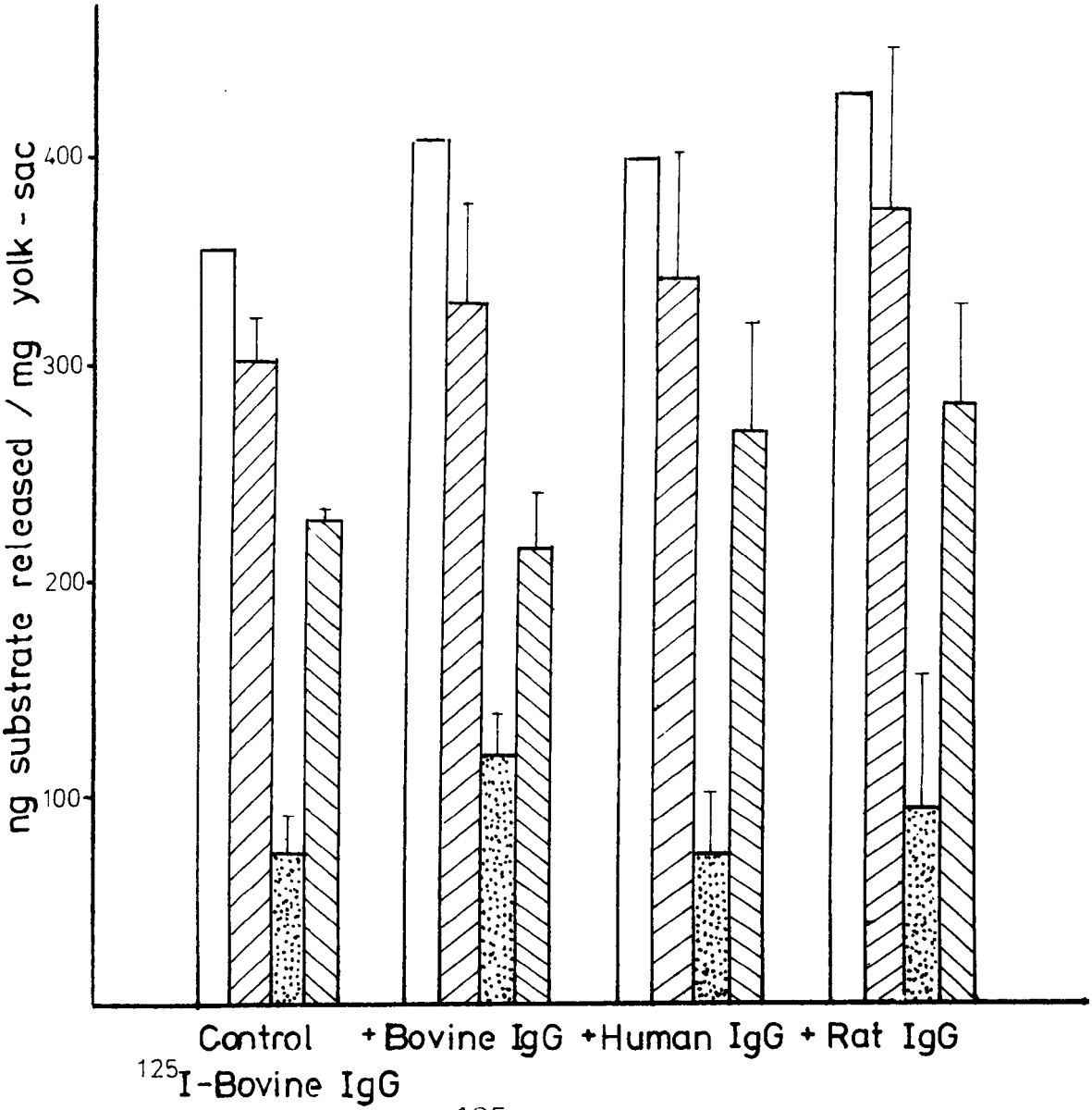


Figure 6.3 Release of ^{125}I-labelled species from rat yolk sacs on reincubating tissues previously exposed to ^{125}I-labelled bovine IgG in the presence of various unlabelled IgG species

17.5-day rat yolk sacs were first incubated in serum-free medium 199 containing both ^{125}I-labelled bovine IgG (10 µg/ml) and either bovine, human or rat IgG (100 µg/ml). After 2h, yolk sacs were washed and reincubated in fresh medium 199, up to a further 3h. Histograms show the amounts of total substrate associated with the tissue at the start of the reincubation period □, total radioactivity release ▨, TCA-soluble radioactivity release ▧ and TCA-insoluble radioactivity release ▩ after 3h of reincubation (see Section 6.2.2 for full method). Values shown represent means (± S.D.) from 3 (control), 5 (+ bovine IgG), 6 (+ human IgG) and 6 (+ rat IgG) separate experiments.

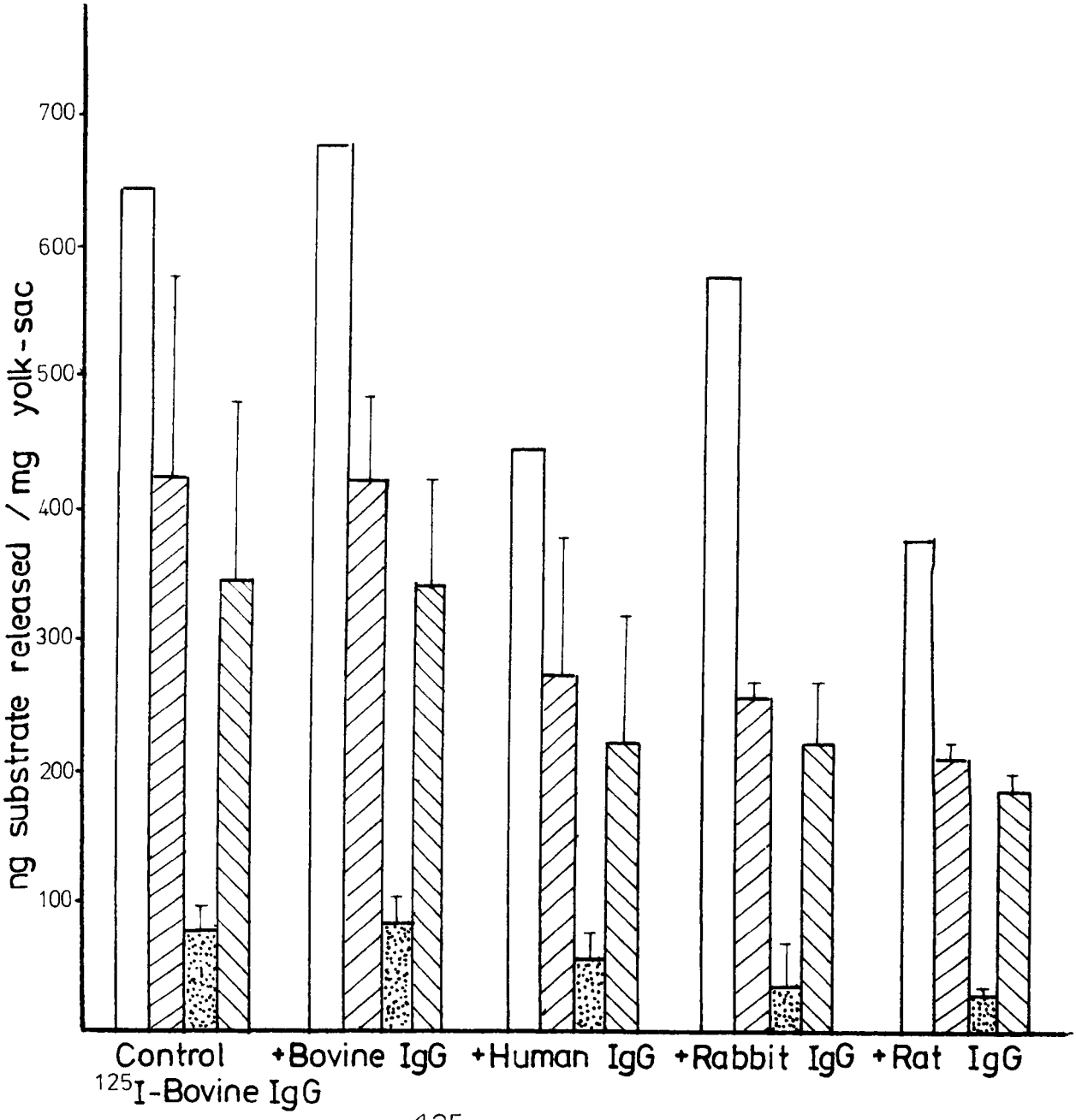


Figure 6.4 Release of ^{125}I-labelled species from rabbit yolk sacs on reincubating tissues previously exposed to ^{125}I-labelled bovine IgG in the presence of various unlabelled IgG species

24-day rabbit yolk-sac pieces were first incubated in serum-free medium 199 containing both ^{125}I-labelled bovine IgG (10 µg/ml) and either bovine, human, rabbit or rat IgG (100 µg/ml). After 2h, yolk sacs were washed and reincubated in fresh medium 199, up to a further 3h. Histograms show the amounts of total substrate associated with the tissue at the start of the reincubation period □,
total radioactivity release ▨,
TCA-soluble radioactivity release ▧
and TCA-insoluble radioactivity release ▩
after 3h reincubation. The method is described fully in Section 6.2.2 . Values shown represent means (± S.D.) from 3 separate experiments for each incubation condition.

a)

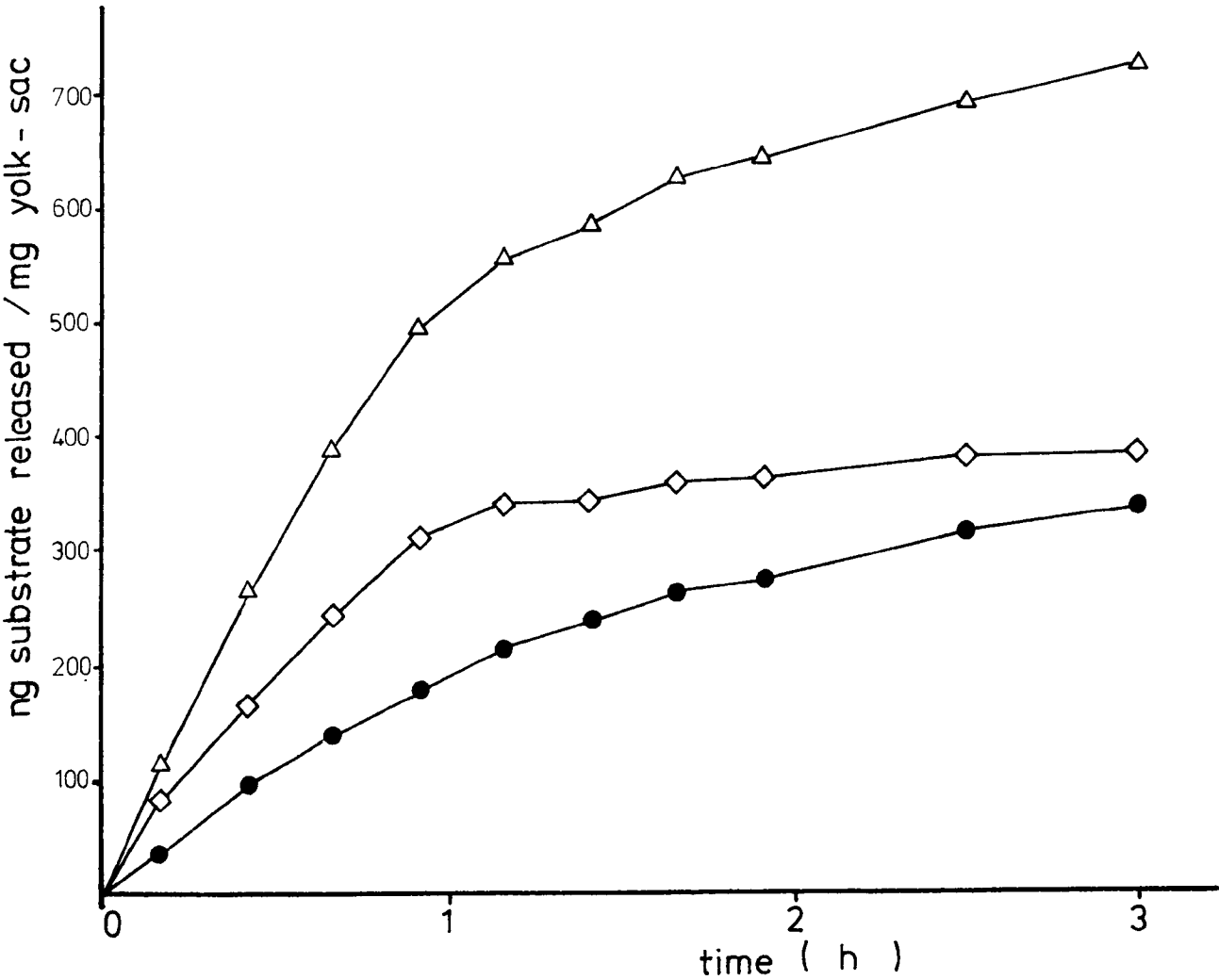


Figure 6.5 Time course of release of radioactivity derived from ^{125}I-labelled rabbit IgG after "loading" rabbit yolk sacs with this substrate in the presence of various unlabelled IgG species

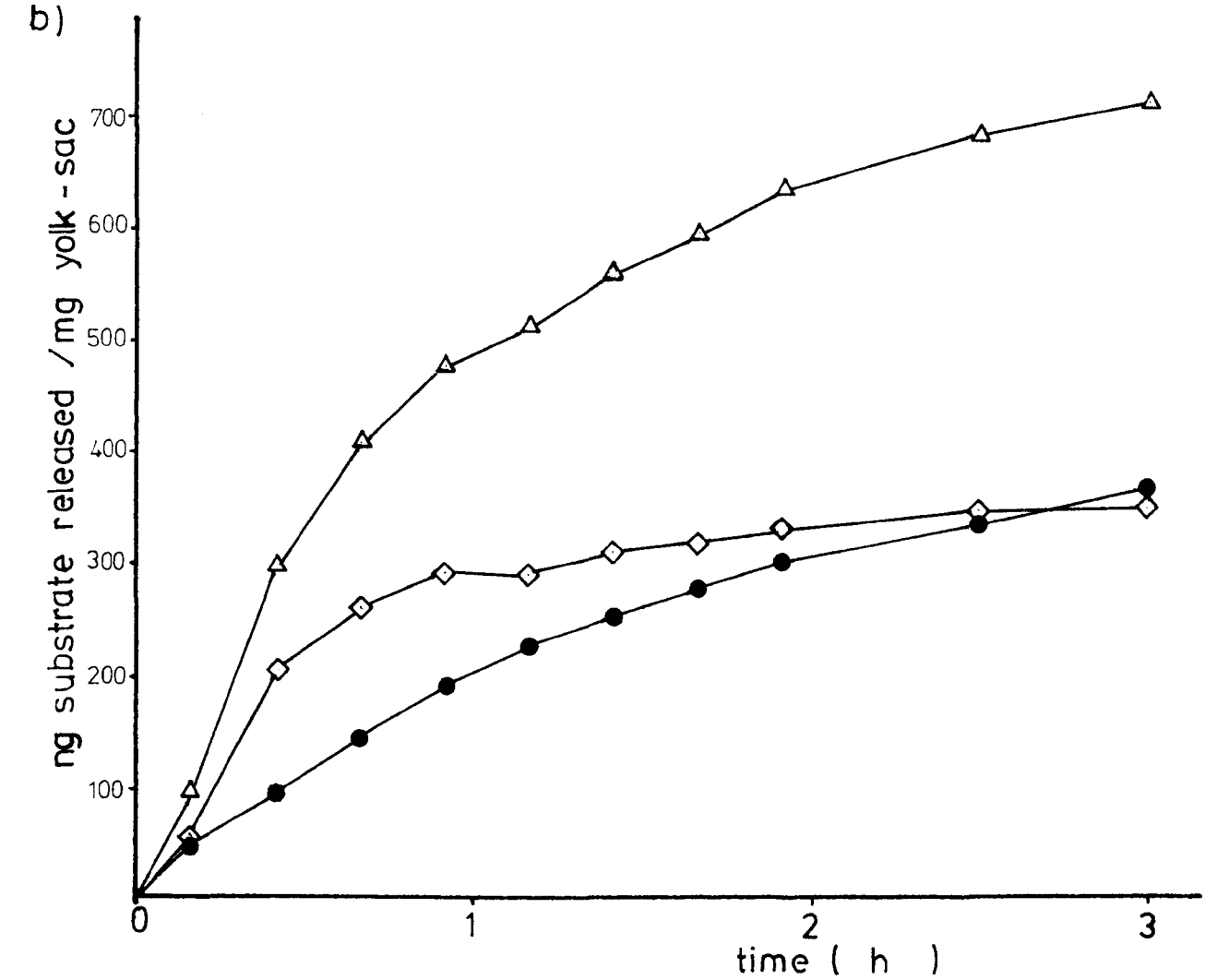
24-day rabbit yolk-sac pieces were incubated for 2h in serum-free medium 199 containing both ^{125}I-labelled rabbit IgG (10µg/ml) and 100 µg/ml of unlabelled bovine IgG (b), human IgG (c), rabbit IgG (d) or rat IgG (e). Figure 6.5.a is a control with no unlabelled IgG added. Following washing, yolk sacs were re-incubated in fresh medium 199 and medium samples were taken at regular intervals up to 3h. The method is outlined in Section 6.2.2 in detail.

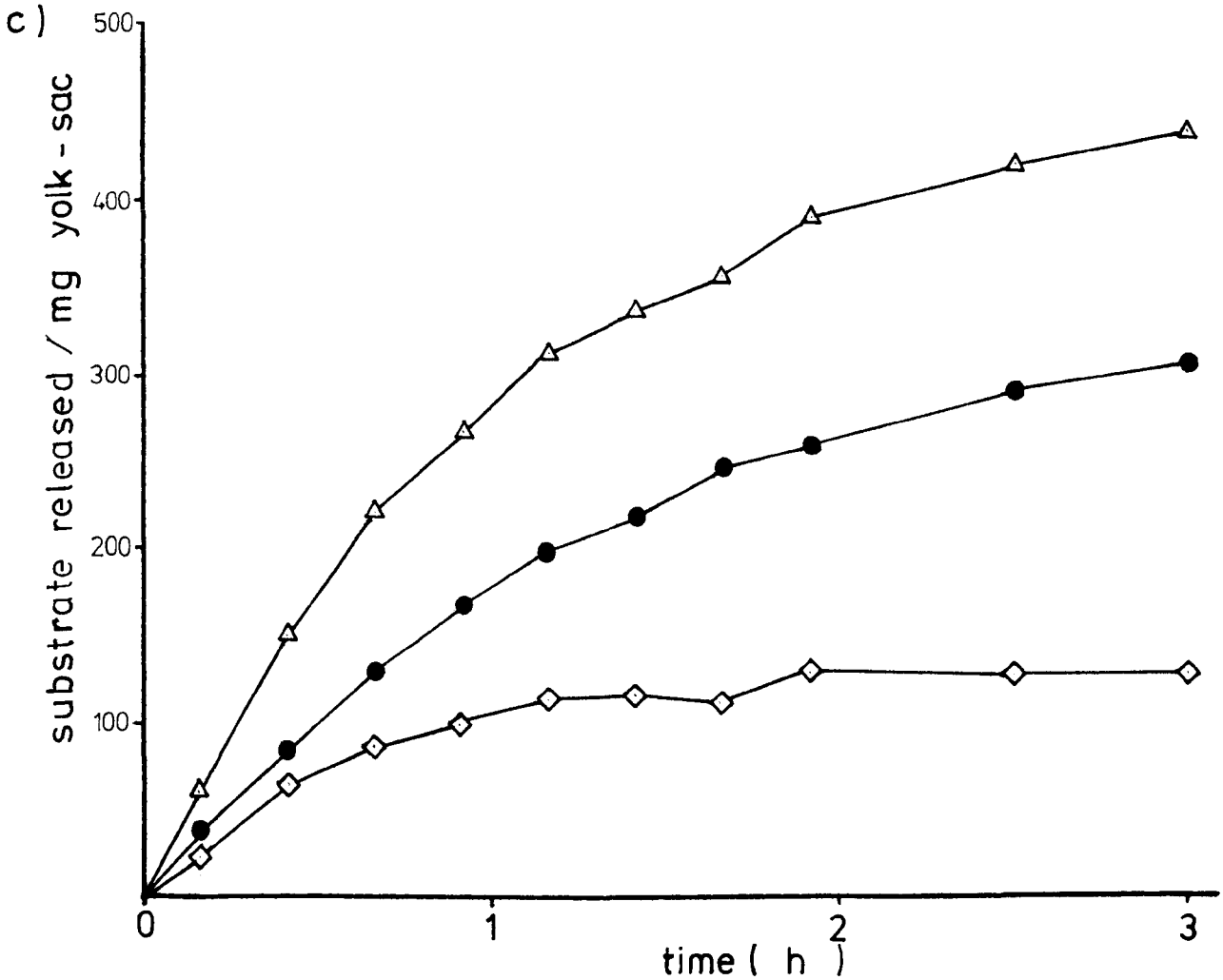
Total radioactivity released —— △ ——

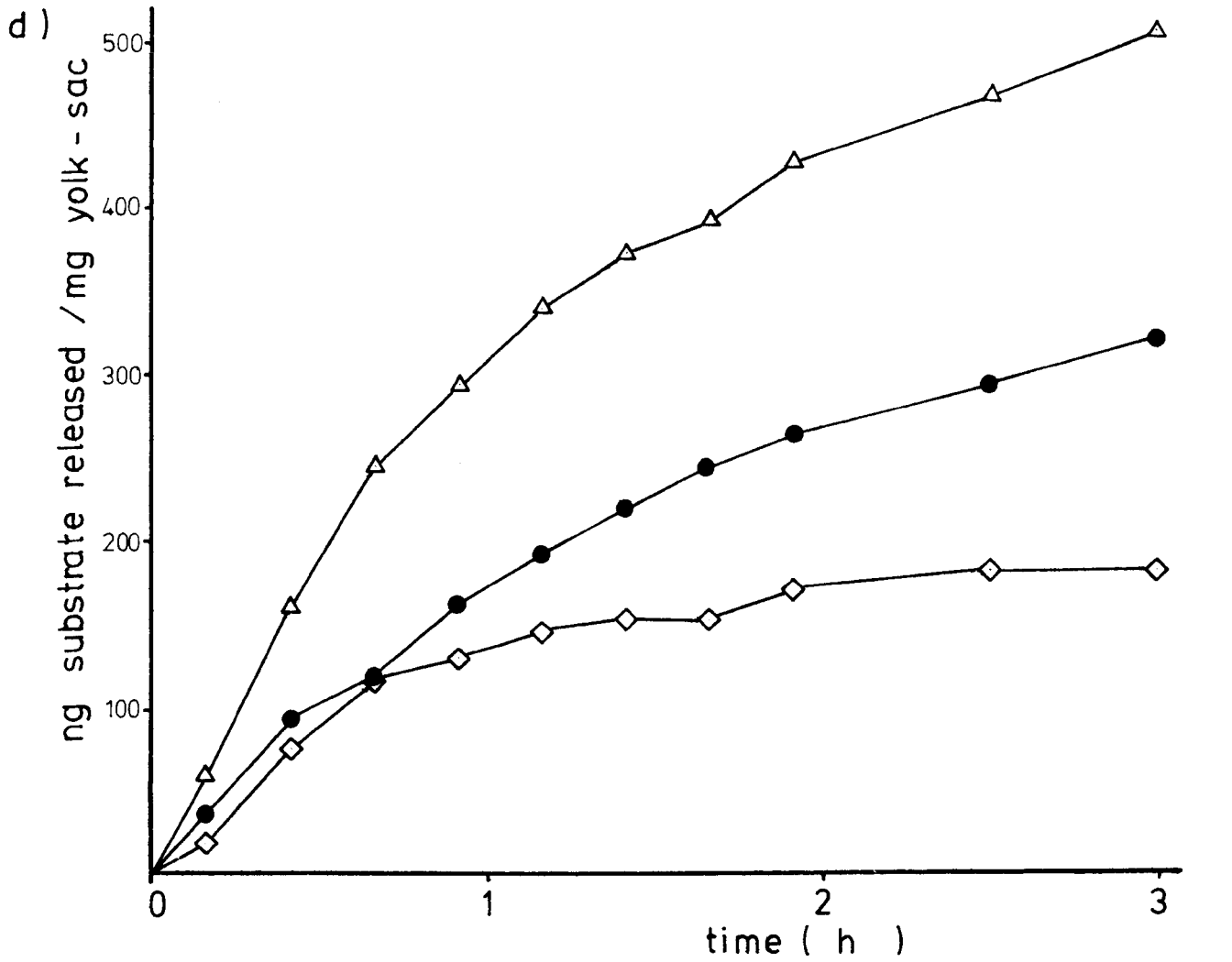
TCA-soluble radioactivity released — ● —

TCA-insoluble radioactivity released — ◇ ——

Values shown represent means from 2 (Figure 6.5.a) and 3 (Figures 6.5.b-e) separate experiments each. The data shown here is also summarized in Figure 6.2 .


b)
ng substrate released / mg yolk - sac
700
600
500
400
300
200
100
0
1
2
3
time (h)


c)
substrate released / mg yolk - sac
500
400
300
200
100
0
1
2
3
time (h)


d)
ng substrate released / mg yolk - sac
500
400
300
200
100
0
1
2
3
time (h)

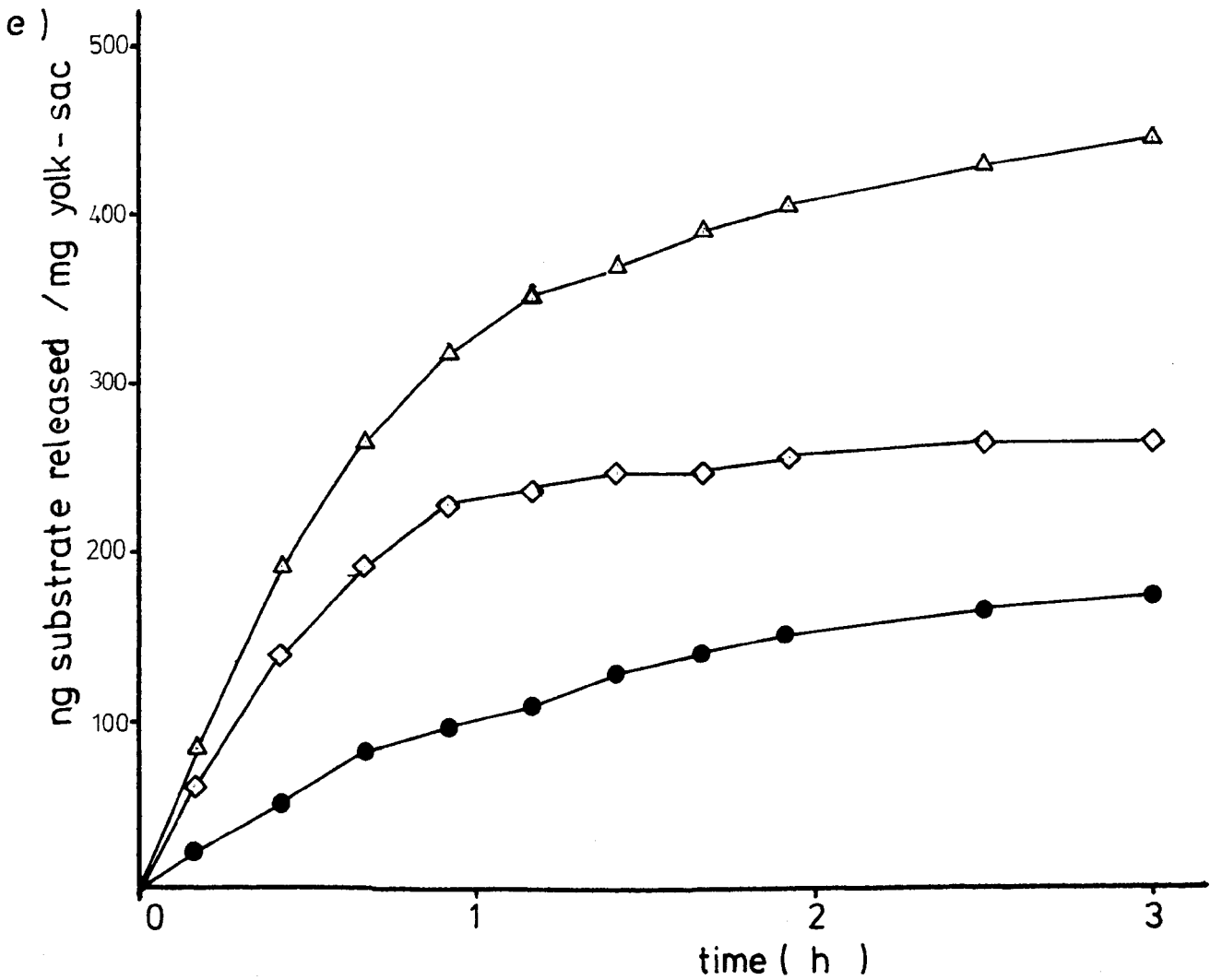

e)
ng substrate released / mg yolk - sac
500
400
300
200
100
0
1
2
3
time (h)

Table 6.1 Effect of the presence of different species of IgG on the pattern of release of ^{125}I-labelled products from rat yolk-sacs previously incubated with ^{125}I-labelled rat or bovine IgG in the presence of large quantities of another species of IgG (unlabelled).

This table summarizes the results shown in Figures 6.1 and 6.3, expressing interference in a relative way: a) as the ratio of TCA-solubles (and -insolubles) over total release for each incubation condition and b) as a relative effect by taking the values obtained with an interfering IgG species present and dividing each by the control value (see Section 6.2.1 for details).

^{125}I-labelled substrate (10 µg/ml)	Competing species of unlabelled protein (100 µg/ml)	Fraction of the total activity released that is acid-insoluble	Fractional release of acid-insolubles expressed relative to controls (%)	Fraction of the total activity released that is acid-soluble	Fractional release of acid-solubles expressed relative to controls (%)
Rat IgG	None (Control)	0.435	100.0	0.565	100.0
"	Bovine IgG	0.398	91.47	0.602	106.57
"	Human IgG	0.179	41.14	0.821	145.35
"	Rat IgG	0.295	67.87	0.705	124.75
Bovine IgG	None (Control	0.241	100.0	0.759	100.0
"	Bovine IgG	0.353	146.35	0.647	85.25
"	Human IgG	0.207	85.83	0.793	104.51
"	Rat IgG	0.250	103.48	0.750	98.89

Table 6.2 Effect of the presence of different species of IgG on the pattern of release of ^{125}I-labelled products from rabbit yolk sacs previously incubated with ^{125}I-labelled rabbit or bovine IgG in the presence of large quantities of another species of IgG (unlabelled)

This table summarizes the results shown in Figures 6.2 and 6.3, expressing interference in a relative way a) as the ratio of TCA-solubles (and -insolubles) over total release for each reincubation condition and b) as a relative effect by taking the values obtained with an interfering IgG species present and dividing each by the control value (see Section 6.2.1 for details).

^{125}I-labelled substrate (10 µg/ml)	Competing species of unlabelled protein (100 µg/ml)	Fraction of the total activity released that is acid-insoluble	Fractional release of acid-insolubles expressed relative to controls (%)	Fraction of the total activity released that is acid-soluble	Fractional release of acid-solubles expressed relative to controls (%)
Rabbit IgG	None (Control)	0.531	100.0	0.469	100.0
"	Bovine IgG	0.491	92.45	0.509	108.55
"	Human IgG	0.302	58.06	0.698	148.87
"	Rabbit IgG	0.358	67.47	0.642	136.83
"	Rat IgG	0.603	113.52	0.397	84.69
Bovine IgG	None (Control)	0.186	100.0	0.814	100.0
"	Bovine IgG	0.194	104.69	0.806	98.93
"	Human IgG	0.198	106.52	0.802	98.51
"	Rabbit IgG	0.139	74.68	0.862	105.77
"	Rat IgG	0.132	71.28	0.868	106.54

6.4 DISCUSSION

It was suggested earlier (Chapter 5) that both transport of IgG across the rat and rabbit yolk sac, as well as the degradative route, involve specific binding sites or receptors. One aim of the studies reported in this chapter was to provide further evidence for this assertion and to investigate the specificity of these receptors and binding sites more fully by interference studies using isolated tissues incubated *in vitro*. Whereas, in the neonatal rat-gut, transcellular transport of IgG and uptake of IgG with subsequent degradation happen at two different locations (the proximal and the distal part of the small intestine) in the rabbit and rat yolk sac both processes appear to take place in the same cell. It is fascinating then to ask how two distinct functional classes of receptor or binding site, specific for different molecules, can coexist on the surface of the same cell.

Some evidence for a specific protective IgG receptor on rabbit and rat (and mouse) yolk sacs has been obtained from *in vitro* binding studies (Tsay & Schlamowitz, 1975, 1978; Elson *et al.*, 1975; Schlamowitz, 1976, 1979; Hillman *et al.*, 1977; Wild & Dawson, 1977; Wild, 1979). For the degradative protein pathway, however, evidence of the nature of the binding site is more scanty. The finding in the previous chapter, that uptake of IgG into the degradative route cannot be accounted for simply in terms of fluid-phase uptake, suggests a general binding site/receptor-mediated uptake of IgG. Also, when re-examining Table 5.6 it appears that the rate of uptake (E.I.) of ^{125}I-labelled homologous and bovine IgG can be decreased to differing degrees by the addition of bovine calf serum to the incubation medium, suggesting that the degradative protein route can be interfered with. Interference in this way is indicative of binding sites that can be competed for. Before looking at the interference effects in detail, however, some

more general considerations need to be mentioned. The effects of interference have been expressed in terms of changes in the level of total tissue-accumulation of substrate, as well as in terms of changes in the amounts of substrate released. The level of tissue-accumulation provides, with reservations, a measure of total uptake. This is important, because mechanistically interference is, or should be, primarily taking place during the uptake of the substrate. A secondary effect of interference may be to cause intralysosomal accumulation of protein as a result of demand for degradative enzymes outstripping the available supply. Both Ibbotson & Williams (1979) and Livesey & Williams (m/s) have, however, shown that the level of radioactivity in the rat yolk-sac tissue is proportional to the Endocytic Index for ^{125}I-labelled albumin and ^{125}I-labelled ribonuclease. The big reservation, however, is the effect of possible differential rates of digestion of different protein substrates. Thus, a protein that is degraded by the tissue rapidly, while being taken up avidly (i.e. high Endocytic Index) will have a very low tissue-level of radioactivity. These considerations are also relevant to the interpretation of interference effects in terms of "protective" and the "degradative" IgG routes.

Obviously, the interpretation of interference effects in terms of the behaviour of the "degradative" IgG route are more subject to the limitations mentioned above. Therefore the major investigation in this chapter has been to block the "protective" transport route by observing the effect of homologous and heterologous unlabelled IgG on the "protective" route of ^{125}I-labelled IgG in rat and rabbit yolk sacs. Thus, in the rabbit yolk sac it is obvious (Figure 6.2) that the release of the macromolecular form of ^{125}I-labelled rabbit IgG is decreased considerably by the addition of either unlabelled human or rabbit IgG, but is not affected by bovine IgG. Interference with the protective IgG route is

also reflected in Table 6.2, which expresses the relative suppression of this specific route. To a certain extent ^{125}I-labelled rabbit IgG is also shown to be "pushed" into the degradative route by the action of the interfering molecules blocking the protective pathway. In the rat yolk sac similar observations were made (Figure 6.1). The addition of human or rat IgG decreased markedly the TCA-insoluble release as a result of interfering with the non-degradative route. As in the rabbit yolk sac, it can also be seen that a great amount of the radioactive homologous IgG has been "pushed" into a route leading to degradation (Table 6.1).

The interference of the uptake of a labelled molecule by a greater concentration of the same unlabelled molecules is taken to imply the presence of a finite number of specific receptors for this molecule on the membranes of the tissue investigated (Brambell et al., 1958). According to the findings here, in rat and rabbit yolk sacs such receptors have affinity not only for homologous IgG but also, to a greater or lesser extent, for IgG derived from other species. Expressing interference in "total" (Figures 6.1 and 6.2) and in "relative" (Tables 6.1 and 6.2) terms, the following rank orders can be drawn up for the extent of interference with the "protective"/transport receptor for ^{125}I-labelled homologous IgG by other IgG species:

Yolk Sacs	"Total" interference	"Relative" interference
Rabbit	human > rabbit > rat > bovine (Figure 6.2)	human > rabbit > bovine=rat (Table 6.2)
Rat	rat > human > bovine (Figure 6.1)	human > rat > bovine (Table 6.1)

These rank orders of interference are similar to those found in in vivo interference studies by Brambell et al. (1958), albeit in other tissues (the rat and mouse gut) in which uptake of homologous IgG could be interfered with by various IgG species in the following order:

rabbit > guinea-pig > human > bovine > rat=mouse=hampster=sheep. Also Gitlin & Gitlin (1974) found that unlabelled human IgG could interfere with the uptake of ^{125}I-labelled human and bovine IgG in the human placenta. In the same way, some *in vitro* binding studies have displayed a similar kind of selectivity in binding to homologous and heterologous IgG. Using an erythrocyte-antibody rosette technique Wild & Dawson (1977) found that whereas rabbit and human IgG bind to Fc receptors of the rabbit yolk-sac membrane, bovine IgG does not bind. Wild (1979) also stated that there is a good correlation between binding of human, rabbit and bovine IgG to rabbit yolk-sac membranes and the transfer of these molecules across the same tissue. Schlamowitz *et al.* (1975) found that "Schlamowitz" vesicles consisting of rabbit yolk-sac brush-border membrane and receptors bound FITC (fluorescein isothiocyanate conjugated) rabbit IgG but not FITC bovine IgG.

The rank orders of interference obtained here *in vitro* are also closely related to the order of materno-foetal transfer of IgG in the rat and rabbit *in vivo* (see Brambell, 1970) and *in vitro* (see Chapter 5). A slight difference, however, between transport and interference *in vitro* can be seen. Whereas in the rabbit yolk sac ^{125}I-labelled rabbit IgG escapes from being degraded best, human IgG is the best interfering IgG molecule. But differences between rabbit and human IgG in their extent of interference are not very great.

This demonstration of a receptor-mediated protective route of IgG transfer in the rabbit and rat yolk sacs renders Hemmings & Williams' (1976) theory of IgG transfer highly unlikely. According to this theory vesicles (pinosome or heterolysosomes) rupture releasing their contents into the cytoplasm, and IgG molecules can then move to the lateral and basal plasmalemma where they escape from the cell by selective diffusion. This random motion seems a far too slow process to account for the highly efficient process of IgG transport to the foetus. Moreover, on rupture of heterolysosomes it is expected that not only will immunoglobulins be

released into the cytoplasm but also lysosomal enzymes. The latter event would be expected to prove damaging to the yolk-sac cells. As the Hemmings' theory rests on the observation of free ferritin (and labelled homologous and heterologous IgG) in electron micrographs of rabbit yolk sacs, following uptake of these molecules, it is possible that this effect is due to bad fixation, as suggested by Slade (see Hemmings & Williams, 1976); or that the radioactive molecules detected in the cytoplasm are breakdown products of the injected radiotracers. A second finding of Hemmings (1974), that rabbit and bovine IgG bind indiscriminately to the rabbit yolk-sac membrane *in vitro*, is equally compatible with the occurrence of receptors for the specific (non-degradative) pathway being present alongside other binding sites involved in the degradative route of protein uptake.

As the protective IgG pathway has been shown to be receptor mediated, the degradative route will be considered next, looking first at those interference studies in which the substrate was homologous ^{125}I-labelled IgG. As mentioned above interpretations of results are more tentative. In the rabbit yolk sac, absolute TCA-soluble release is strongly decreased by only rat IgG (Figure 6.2). In the rat yolk sac, a significant decrease in the total amount of TCA-soluble radioactivity released can be noted with bovine IgG and to a lesser degree with rat IgG as competing species (Figure 6.1). [The "relative" figures differ little from the controls (Table 6.1); this reflects the fact that both degradative and protective routes are equally strongly interfered with by rat and bovine IgG.]

When ^{125}I-labelled bovine IgG is the substrate, interference effects are not as straightforward as with ^{125}I-labelled homologous IgG. In absolute terms, TCA-soluble release is decreased in the rabbit yolk sac by human, rabbit and rat IgG (Figure 6.4). Relatively, only human IgG suppresses the degradative pathway, though not greatly. In the rat

yolk sac (Figure 6.3) there is little or no effect to report. The conclusion drawn from these findings is that specific binding sites do exist for the degradative route in the rat and rabbit yolk sac. It is interesting to note that in the rat yolk sac the degradative IgG routes of both ^{125}I-labelled rat and bovine IgG are interfered with by bovine and rat IgG. In the rabbit yolk sac, on the other hand, the interfering IgG classes for the degradative binding sites for ^{125}I-labelled rabbit and bovine IgG differ. A short summary table may help to visualize these effects:

a) Rabbit yolk sacs

	^{125}I-rabbit IgG	^{125}I-bovine IgG
Substrate		
Total interference	rat IgG	human, rabbit, rat IgG
Relative interference	rat IgG	human, bovine IgG

b) Rat yolk sacs

	^{125}I-rat IgG	^{125}I-bovine IgG
Substrate		
Total interference	bovine, rat IgG	bovine IgG
Relative interference	———	bovine (rat) IgG

These findings point to a great complexity of binding sites for the degradative pathway on the rat and rabbit yolk sacs. The suggestion of the existence in IgG transport in the mouse of two receptors, both with a degree of specificity has also been made by Gitlin & Morphis (1969). They postulated two different transport systems for human, guinea-pig and rabbit IgG on the one hand and mouse and bovine IgG on the other hand in the mouse placenta.

The IgG transport receptor is probably specific for the Fc part of the IgG molecule (Kaplan *et al.*, 1965). Concerning the nature of the degradative binding site no suggestions have been made to date. Two possibilities can be envisaged. First the "degradative pathway"

binding sites also bind to the molecule via the Fc moiety of the IgG. Second, the binding sites show specificity on the basis of differences in charge, size or hydrophobicity etc. of the binding molecules. In support of the first suggestion are the findings that the Fc part of the IgG molecule is specialized to perform a great variety of different functions in a number of different cell types. (For example, Matre & Johnson (1977) found that in the human placenta multiple Fc receptors perform different functions. On the trophoblast, Fc receptors mediate maternofoetal transfer of IgG while on the placental endothelial cells, on the other hand, Fc receptors are involved in the protection of the foetus from immune complexes formed within the placenta, following transfer of maternal antibodies to paternally derived alloantigens of the foetus). Should the degradative as well as the protective receptor be Fc-specific, it may be difficult to account for selection of protein transport in a tissue like the rat yolk sac, where both receptors can be blocked by rat IgG.

The second possibility, that the binding site is specific for molecules of a particular charge, size or hydrophobicity, has some support from the findings of Livesey & Williams and Kooistra and Williams (unpublished data). In the rat yolk sac Livesey and Williams found evidence for two classes of binding sites involved in protein uptake, one specific for hydrophobic molecules and the other specific for positively charged molecules. Such a pattern of different binding sites could explain the very complex findings in the rabbit yolk sac although the equivalent experiments have yet to be performed with this tissue.

This brings to an end the most notable findings in this chapter. Nevertheless, some of the more minor observations also merit a mention. A very great tissue-accumulation is observed when unlabelled rabbit IgG is added to interfere with uptake of the substrate in the rabbit yolk sac

(Figures 6.2 & 6.3). A possible explanation of this finding is developed below. The release patterns in rabbit yolk sacs of ^{125}I-rabbit IgG, (Figure 6.5a-c) suggest that while TCA-insoluble release levels off after 70 min., TCA-soluble release continues to rise linearly with time. This finding may be accounted for by the suggestion that rabbit IgG is only slowly degraded in the rabbit yolk sac. Therefore when more unlabelled rabbit IgG is added to rabbit yolk sac incubations, an accumulation of the relatively poorly degraded substrate would be expected. Bovine IgG, on the other hand, seems to be more susceptible to degradation, and the presence of even large amounts of this substrate in the rabbit yolk sac should not lead to intracellular accumulation. This possibility of differential susceptibility of homologous and heterologous IgG to degradation by rabbit yolk sacs will be considered again in more detail in Chapter 8.

In conclusion:

1) The use of interfering species of IgG indicates that both rabbit and the rat yolk-sac cells appear to carry specific receptors that lead to the protection of the bound substrate against degradation.

2) In both tissues, the "protective" and "degradative" IgG routes have each been shown to be susceptible to interference by unlabelled IgG, suggesting the involvement of specific binding sites in both routes.

3) The binding sites on rat and rabbit yolk sacs differ in their specificity for IgG species.

4) Interfering IgG molecules can be shown to push the radiolabelled substrate into either the "protective" or the "degradative" IgG route, by effectively blocking the other route in either tissue.

CHAPTER SEVEN

Effects of Formaldehyde-treated Albumin and Rat IgG on the Uptake and Release of Rat IgG by the Rat Yolk Sac

7.1 INTRODUCTION

In the previous chapter some evidence was obtained for the existence, on the rat and rabbit yolk-sac membranes, of specific IgG receptors that are involved in the protection of homologous and some heterologous IgG species. Furthermore, evidence was advanced to suggest that uptake of those IgG molecules destined for degradation within either rat or rabbit yolk sac was also mediated by binding to the surface of the tissues.

Ideas were forwarded earlier (Chapter 6) concerning the nature of these degradative IgG receptors or binding sites. It has been suggested that the adsorption of IgG could involve either the Fc region of the molecule or could depend on some other more general feature of the molecule. Both suggestions find some support in the literature. Fc receptors are involved with the immune reaction and are found in a variety of cells. Bourgois et al. (1977) isolated Fc receptors from mouse B-cells, T-cells, macrophages, thymus cells and fibroblasts, and found that these receptors are so similar that there must either be only one Fc receptor molecule or a common precursor. Also Anderson & Grey (1977), to quote just one more group of workers from a large field, found Fc receptors on mouse macrophages, mastocytoma, lymphoma and Friend virus-induced leukaemias and remarked on the similarity of the receptor molecule. Despite this similarity, Fc receptors differ from each other not only in their display of selectivity, but they also appear to fall into two or more distinct classes, sometimes even on the same cell. Thus, Unkeless & Eisen (1975) found that Fc receptors on mouse macrophages show selectivity for IgG subclasses, with IgG_{2a} binding avidly, IgG_{2b} binding weakly and IgG1 not significantly. Unkeless (1977) expanded these findings and suggested that there are two classes of Fc receptors, one specific for IgG 2a and the other for IgG1. Also Matre & Johnson (1977) supplied some evidence for the existence of different classes of Fc receptor on the

human placenta. They found Fc receptors on the trophoblast that are thought to be involved in the transfer of IgG to the foetus and Fc receptors on the placental endothelium that may be involved in the protection of the foetus from immuno-complexes formed within the placenta following contact of maternal IgG antibodies with paternally derived alloantigens from the foetus.

When considering a variety of cell types, there is also some evidence for the binding sites responsible for uptake of that portion of IgG that is degraded being specific for some other molecular feature. Kaplan et al. (1975) found two types of binding sites on macrophages, one interacting with 1.1 μm latex beads and the other interacting with sensitized sheep red blood cells; both these receptors are distributed over the entire cell surface. Donelly & Bamford (1976) showed that IgG and amino acid transport are mediated through different membrane receptors in the neonatal rat intestine. The most relevant findings supporting non-Fc specific IgG binding sites on the rat yolk sac, however, come from recent work by Livesey & Williams (unpublished data). In the rat yolk sac they found evidence for two classes of binding site, one specific for hydrophobic molecules and the other specific for positively charged molecules; this conclusion has been supported by the results of subsequent studies (Kooistra & Williams, 1981).

In order to investigate these two possibilities concerning the nature of specificity of the binding sites involved in the degradative pathway for IgG, interference studies similar to those reported in Chapter 6 were carried out using rat yolk sacs. The substrate chosen was ^{125}I-labelled rat IgG, a molecule that enters the rat yolk sac by both the protective and the degradative route. The molecular species chosen in attempts to interfere with substrate uptake were: formaldehyde-treated rat IgG, buffer-treated rat IgG, formaldehyde-treated albumin and buffer-treated albumin. Bovine serum albumin is known to enter

the rat yolk sac by adsorptive pinocytosis and it is not transported to the foetus *in vivo*, neither does it evade being degraded in the rat yolk sac *in vitro* (Moore *et al.*, 1977). The same is also true for formaldehyde-treated albumin, a protein characterized by its much higher Endocytic Index that arises from an enhanced degree of binding of the substrate to the yolk-sac membrane. Both formaldehyde-treated and buffer-treated albumin are expected to interfere with the degradative IgG route if the receptor is not specific for the Fc part of the IgG molecule. Equally, formaldehyde-treated rat IgG has been shown earlier (Chapter 3) to be taken up by the rat yolk sac by the degradative route only, having lost all characteristics ensuring protection from enzymic digestion. The buffer-treated IgG would be expected to closely resembly native IgG hence it would be expected to interfere with the protective IgG receptor and, according to the findings in Chapter 6, also with the binding sites responsible for uptake into the degradative route, especially if the latter are Fc-specific.

In addition to testing the effect of interference during the "loading" stage on the subsequent release of substrate, the effect of interference on the Endocytic Index of ^{125}I-labelled rat IgG was also determined. As remarked earlier, the Endocytic Index is a true measure of net uptake into the degradative pathway, and interference with this route should result in a decrease in Endocytic Index.

In summary, the aim of this chapter is to throw light on the nature of the binding sites on the rat yolk-sac membrane that are involved in the uptake of IgG into the degradative pathway. The possibility that these binding sites show some specificity, not for the Fc part of the IgG molecule but for another molecular characteristic, was tested by trying to interfere with the uptake of ^{125}I-labelled rat IgG by the addition of a protein possessing either no Fc-determinant (BSA, foramldehyde-treated BSA) or a denatured Fc moiety (formaldehyde-treated IgG) or an intact Fc moiety (buffer-treated IgG).

7.2 METHODS

7.2.2 Effect of the presence of BSA, formaldehyde-treated BSA, rat IgG or formaldehyde-treated rat IgG in the incubation medium on the uptake of ^{125}I-labelled rat IgG by the rat yolk sac in vitro

Rat yolk sacs (17.5-day) were incubated for 5.5h in serum-free medium 199 containing ^{125}I-labelled rat IgG (2 µg/ml). Also present in the incubation medium was either BSA (1 mg/ml), or formaldehyde-treated BSA, buffer-treated BSA, formaldehyde-treated rat IgG or buffer-treated rat IgG (100 µg/ml). Incubations were terminated at regular intervals and Endocytic Indices and Tissue-Accumulation Rates were determined as described in Section 1.10.

7.2.3 Effect of the addition of BSA, formaldehyde-treated BSA, rat IgG or formaldehyde-treated rat IgG to the incubation medium during the loading phase of rat yolk sacs with ^{125}I-labelled rat IgG on the subsequent release of this substrate

Rat yolk sacs (17.5-day) were incubated for 2h in serum-free medium 199 in the presence of ^{125}I-labelled rat IgG (10 µg/ml). At the same time as the substrate was added, one of the following molecules was also added at a concentration of 100 µg/ml : buffer-treated BSA, formaldehyde-treated BSA, formaldehyde-treated rat IgG or buffer-treated rat IgG. The remainder of the method was as described in Section 1.12.

7.2.1 Preparation of unlabelled formaldehyde-treated BSA and rat IgG

Protein (rat IgG or BSA) was dissolved in 0.05M-Na_2HPO_4-KH_2PO_4 phosphate buffer, pH8, at a concentration of 1 mg/ml and then mixed with an equal volume of formaldehyde solution (10%, w/v, in an Na_2CO_3-bicarbonate buffer pH 10). The solution was left at room temperature for 72h and then dialyzed against 1% NaCl. As necessary controls, BSA and IgG were also treated with the buffer alone.

7.3 RESULTS

7.3.1 Effect of the addition of BSA, formaldehyde-treated BSA, rat IgG or formaldehyde-treated rat IgG on the rate of uptake of ^{125}I-labelled rat IgG

Addition of native BSA, formaldehyde- or buffer-treated BSA or formaldehyde- or buffer-treated IgG to the medium in which yolk sacs were incubated, caused the Endocytic Index of ^{125}I-labelled rat IgG to be decreased in some cases (Table 7.1 and Figure 7.1). The greatest decrease was observed on addition of a very high concentration of native BSA (1 mg/ml). This has the effect of decreasing the Endocytic Index by more than 50%. The effect of adding formaldehyde-treated and buffer-treated BSA at the lower concentration of 100 µg/ml was to produce no detectable decrease. The addition of formaldehyde-treated rat IgG to the incubation medium, on the other hand, caused a marked decrease of the E.I. of ^{125}I-labelled rat IgG. By contrast, and rather important to note, is the finding that the buffer-treated IgG produced, if anything, a slight elevation rather than a decrease in the E.I. of ^{125}I-labelled rat IgG. For the purpose of this study the Tissue-Accumulation Rates are not important and they are therefore reported in both Table 7.1 and Figure 7.1, but are not discussed at greater length.

7.3.2 Effect of the addition of either BSA, formaldehyde-treated BSA, rat IgG or formaldehyde-treated rat IgG to the incubation medium during the "loading" phase of rat yolk sacs with ^{125}I-labelled rat IgG on the subsequent release of this substrate

In the loading phase of reincubation studies of rat yolk sacs, a number of molecules were added to interfere with the uptake of ^{125}I-labelled rat IgG by the tissue. Formaldehyde-treated rat IgG and formaldehyde-treated and buffer-treated BSA were chosen because

they are taken up only by the general degradative protein route and should accordingly interfere only with that route. Buffer-treated rat IgG was chosen for a contrast, the native rat IgG having been shown to interfere with the protective pathway in the rat yolk sac (see Figure 6.1); it is also probably the only molecule of the four that has an intact Fc domain.

The effects of the four unlabelled protein preparations on the uptake and release of ^{125}I-labelled rat IgG from rat yolk sacs are varied (Figures 7.2 and Table 7.1). The format of histograms and the table are the same as in the previous chapter. It is apparent from Figure 7.2 that only formaldehyde-treated IgG interferes with the degradative pathway of IgG. Compared to the control, total tissue-accumulation and release are decreased, which can be mainly attributed to a decrease in TCA-insoluble release, accompanied by an increase in TCA-soluble release. The two BSA preparations do not interfere with uptake and release of ^{125}I-labelled rat IgG by the rat yolk sac. No evidence can be provided for a decrease of TCA-solubles, as would be indicative of interference of the BSA species with the "degradative" route.

Table 7.1 Endocytic Indices and Tissue-Accumulation Rates of ^{125}I-labelled rat IgG for rat yolk sacs incubated in serum-free medium 199 in the presence of both this substrate and various unlabelled proteins

Rat yolk sacs (17.5-day) were incubated in serum-free medium 199 containing ^{125}I-labelled rat IgG (2µg/ml) and one of the following unlabelled proteins: native albumin (1mg/ml), formaldehyde-treated albumin, buffer-treated albumin, formaldehyde-treated rat IgG, buffer-treated rat IgG (all 100 µg/ml). Incubations were terminated at regular intervals up to 5.5h and Endocytic Indices and Tissue-Accumulation Rates were determined (see Section 7.2.2 for details). The data are also summarized in Figure 7.1.

Competing protein (unlabelled)	1	2	3	4	5	6
Control (no protein added)	4.76	8	66.63	0.956	11.51	0.761
	3.68	9	84.74	0.953	12.79	0.875
	3.07	10	52.01	0.938	8.04	0.874
	3.91	8	52.35	0.755	13.22	0.697
	4.41	8	53.91	0.901	11.24	0.794
MEAN VALUES (± S.D.):			61.93 ± 14.11		11.36 ± 2.03	
Rat IgG_{HCHO} (100 µg/ml)	4.47	8	47.48	0.906	3.15	0.404
	4.47	8	35.64	0.898	0.53	0.055
	4.47	11	39.68	0.939	3.59	0.407
MEAN VALUES (± S.D.):			40.93 ± 6.02		2.09 ± 1.38	
Rat IgG_{Buffer} (100 µg/ml)	4.10	8	67.12	0.930	1.89	0.219
	4.10	8	63.89	0.919	1.78	0.360
	4.10	7	80.98	0.891	6.11	0.436
MEAN VALUES (± S.D.):			70.66 ± 9.08		3.26 ± 2.47	
BSA_{HCHO} (100 µg/ml)	4.65	10	83.73	0.933	10.50	0.748
	4.65	8	58.54	0.991	7.01	0.797
	4.65	10	79.40	0.994	7.50	0.660
	2.19	9	51.46	0.939	2.16	0.412
	3.82	8	40.45	0.990	4.94	0.784
MEAN VALUES (± S.D.):			57.59 ±20.77		5.73 ±3.25	
BSA_{Buffer} (100 µg/ml)	5.01	9	65.95	0.928	5.51	0.736
	3.39	10	75.11	0.908	12.59	0.848
	3.39	8	41.99	0.960	4.15	0.573
	3.39	10	37.03	0.932	4.95	0.610
MEAN VALUES (± S.D.):			55.02 ± 18.41		6.80 ± 3.90	
BSA (1 mg/ml)	2.97	10	14.95	0.832	0.280	0.062
	2.97	10	28.54	0.971	4.96	0.891
	2.97	10	26.14	0.968	3.79	0.803
MEAN VALUES (± S.D.):			23.22 ± 7.23		3.01 ± 2.43	

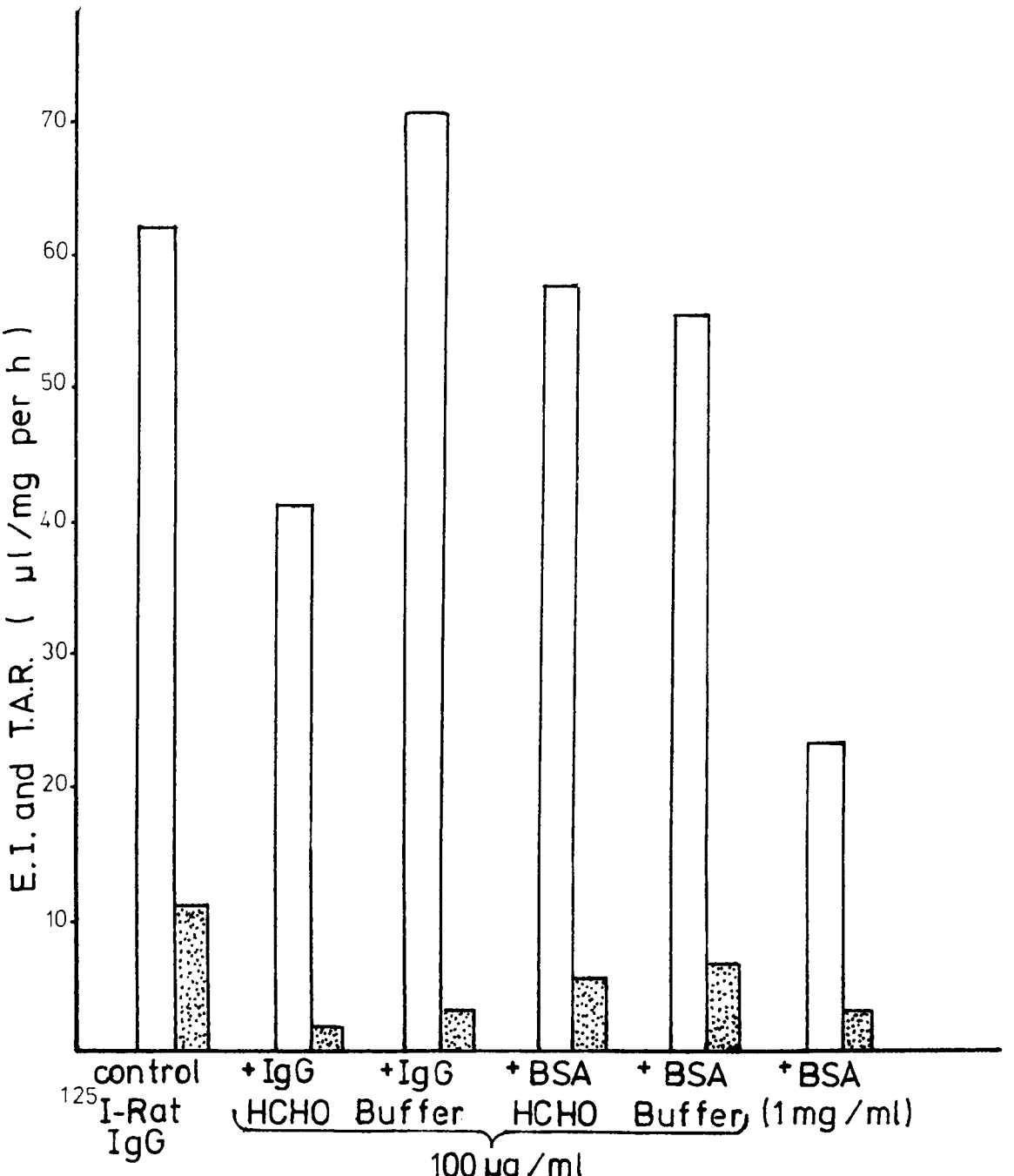


Figure 7.1 Endocytic Indices and Tissue Accumulation Rates of ^{125}I-labelled rat IgG in rat yolk-sacs incubated in serum-free medium 199 in the presence of substrate and various unlabelled proteins

17.5-day rat yolk-sacs were incubated in serum-free medium 199 containing ^{125}I-labelled rat IgG (2 µg/ml) and one of the following unlabelled proteins: native albumin (1mg/ml), formaldehyde-treated albumin, buffer-treated albumin, formaldehyde-treated rat IgG, buffer-treated rat IgG (all 100 µg/ml). Incubations were terminated at regular intervals up to 5.5h and Endocytic Indices □ and Tissue-Accumulation Rates ▨ were determined (see Section 7.2.2 for details). The above data are also presented fully in Table 7.1 and represent the mean Endocytic Indices and Tissue Accumulation Rates from between 3 and 5 separate experiments for each incubation condition.

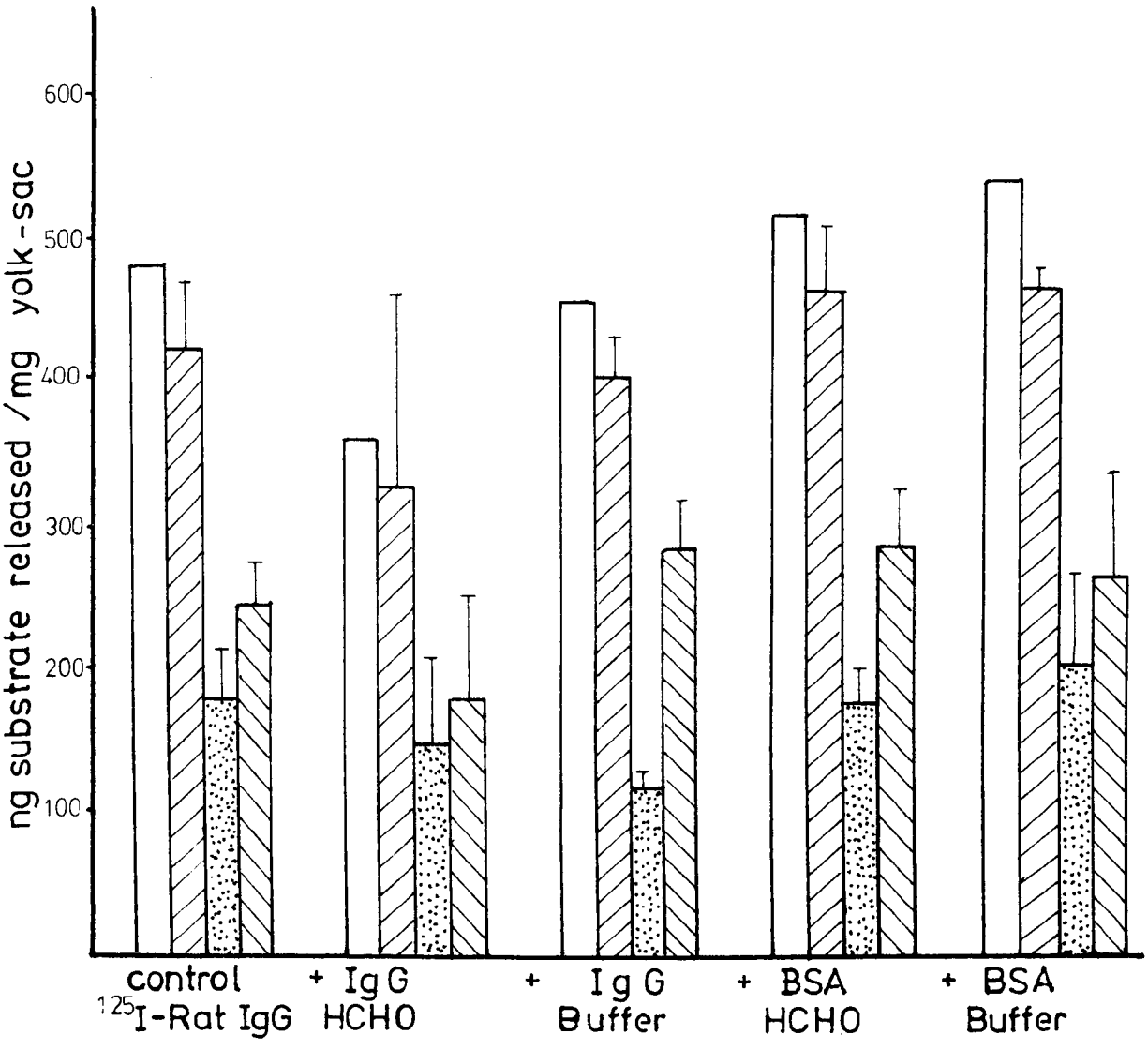


Figure 7.2 Release of ^{125}I-labelled species from rat yolk-sacs on reincubating tissues previously exposed to ^{125}I-labelled rat IgG in the presence of various unlabelled protein species

17.5-day rat yolk-sacs were first incubated in serum-free medium 199 containing both ^{125}I-labelled rat IgG (10 µg/ml) and either formaldehyde-treated rat IgG, buffer-treated rat IgG, formaldehyde-treated albumin or buffer-treated albumin (all 100 µg/ml) or native albumin (1mg/ml). After 2h yolk-sacs were washed and reincubated in fresh medium 199, up to a further 3h. Histograms show the amounts of total substrate associated with the tissue at the beginning of the reincubation period □ total radioactivity release ▨, TCA-soluble radioactivity release ▧, and TCA-insoluble radioactivity release ▩ after 3h reincubation(see Section 7.2.3 for details). Values shown represent means (± S.D.) from 6 separate experiments for each incubation condition.

Table 7.2 Effect of the presence of different species of protein on the pattern of release of ^{125}I-labelled products from rat yolk sacs previously incubated with ^{125}I-labelled rat IgG in the presence of large quantities of another species of protein

This table summarizes Figure 7.2, expressing interference in a relative way: a) as the ratio of TCA-solubles (or -insolubles) to total release, for each incubation condition, and b) as the ratio obtained by taking these values, when an interfering protein species is present, and dividing each value by the control value (see Section 6.2.1 for details).

Radiolabelled substrate (10 µg/ml)	Competing species of protein (100 µg/ml)	Fraction of the total activity that is acid-insoluble	Fractional release of acid-insolubles expressed relative to controls (%)	Fraction of the total activity released that is acid-soluble	Fractional release of acid-solubles expressed relative to controls (%)
^{125}I-Rat IgG	None (Control)	0.423	100.0	0.577	100.0
	IgG_{HCHO}	0.452	106.88	0.548	94.94
	IgG_{Buffer}	0.294	69.42	0.706	122.43
	BSA_{HCHO}	0.381	90.12	0.619	107.25
	BSA_{Buffer}	0.434	102.59	0.566	98.09

7.4 DISCUSSION

The aim of this chapter was to expand the findings of Chapter 6 concerning the nature of the binding sites involved in the uptake into the degradative route of the rat yolk sac. In this tissue, bovine and rat IgG were shown to interfere with the uptake of ^{125}I-labelled rat and bovine IgG into the degradative pathway, whereas human IgG showed no such effect (see table in Discussion of Chapter 6). No conclusive evidence could be produced to establish the basis of selectivity between these IgG species, although two possibilities were put forward. The binding site could either involve the Fc moiety of the IgG molecule or could be dependent on some other physical or chemical characteristic. The proteins tested for interference in Chapter 6 were all IgG molecules, which left both possibilities open. Therefore, in this chapter other molecules were investigated for their effects in interfering with uptake into the degradative route of ^{125}I-labelled rat IgG by the rat yolk sac. Native, buffer-treated and formaldehyde-treated BSA species were chosen because they have no Fc region and because formaldehyde-treated BSA, in particular, is known to be taken up by the rat yolk sac at a very high rate due to extensive tissue adsorption (Moore et al., 1977). Formaldehyde-treated rat IgG is a molecule in which the Fc region has probably been modified, so it was interesting to compare the interference effect of this modified IgG molecule with that of the native rat IgG molecule.

The effect of the BSA molecules on the Endocytic Indices and release patterns of ^{125}I-labelled IgG will be considered first. When present in the incubation medium at a concentration of 100 μg/ml, buffer-treated and formaldehyde-treated preparations of BSA caused a barely detectable decrease in the E.I. of ^{125}I-labelled rat IgG. Likewise, buffer-treated albumin was without effect, but a strong effect was

observed when native albumin at a concentration of 1 mg/ml was used (see Figure 7.1). In reincubation studies the effects of only the formaldehyde-treated and buffer-treated albumin preparations, at lower concentration, were tested; in both cases release patterns of ^{125}I-labelled rat IgG were the same as in the control incubations. It therefore seems likely that although BSA does compete with rat IgG for degradative binding sites, it does so only at very high concentrations. This could be accounted for in at least three ways: either the number of such binding sites is so large that it takes a lot of molecules to saturate them, or the binding site is fairly specific and BSA fits the requirements for binding only imperfectly, or such high concentrations of albumin modify the rate of pinosome formation. The finding with formaldehyde-treated albumin, however, is more positive. Since this compound has little or no effect on the uptake of ^{125}I-labelled rat IgG into the degradative pathway (see Table 7.1) and since Livesey and Williams (unpublished data) suggest that this compound is captured by adsorption to hydrophobic sites on the rat yolk sac, the lack of competition suggests that entry of rat IgG into the degradative pathway involves adsorption to sites other than those that bind hydrophobic proteins.

Rather surprizingly, the presence of formaldehyde-treated rat IgG (100 µg/ml) in the incubation medium decreases the Endocytic Index of ^{125}I-rat IgG considerably (by about 30%), whereas the buffer-treated rat IgG at the same concentration has no effect on the Endocytic Index. This difference in interference also shows up in the reincubation studies (see Figure 7.2) which show that, of all the molecules tested for interference, only formaldehyde-treated rat IgG affects the release of TCA-soluble activity derived from ^{125}I-labelled rat IgG, decreasing it by 25%. In contrast, the buffer-treated IgG interferes with only the

protective IgG route in a similar way to that observed in Chapter 6. These findings give some evidence to support the suggestion that the binding sites responsible for the uptake of protein into the degradative pathway are extensive in number and are not specific for the Fc region but rely on some other characteristic of the molecule.

In future experiments it would be very interesting to extend these findings and make them more conclusive by testing the Fc, Fab and other fragments of the IgG molecule for interference with the protective and the degradative routes in the rat and rabbit yolk sacs. Also, following on from the findings of Livesey & Williams, it would also be of interest to investigate further the characteristics of the binding site(s) involved in the uptake into the degradative pathway by interference studies using differentially charged and hydrophilic and hydrophobic molecules.

In summary, the data presented are not very conclusive yet, but the available evidence points to the involvement, in the degradative pathway for IgG in the rat yolk sac, of a binding site that is specific for neither the Fc part of the IgG molecule nor for a hydrophobic region of the molecule, but is dependent on some other as yet uncharacterized feature of the molecule.

CHAPTER EIGHT

The pH Profile for the Degradation of Various IgG Species and BSA by Yolk Sac Homogenates and Effect of "Loading" Concentrations on Release from Yolk Sacs

8.1 INTRODUCTION

In previous chapters IgG transport into both the protective and the degradative pathway in the rat and rabbit yolk sacs was shown to be mediated by the interaction of IgG with the plasma membrane. Uptake into the protective pathway most likely involves a receptor specific for the Fc domain of the IgG molecule (Brambell, 1966), whereas uptake into the degradative route occurs by way of less specific sites on the plasma membrane that probably bind molecules with a common physical characteristic (e.g. hydrophobicity or positive charge). According to both the Brambell and Wild theories the protective receptors should be readily saturable, since only a limited amount of IgG is transferred to the foetus intact. The observation that the protective IgG route can be interfered with more effectively by the addition of substrate molecules than can the degradative route, is compatible with the suggestion that protective uptake involves a limited number of high affinity receptors. It was shown in Chapter 7, however, that the degradative route could also be interfered with by the addition of formaldehyde-treated IgG, suggesting that the more general binding sites may likewise become saturated. Determination of the saturation characteristics of both types of binding site was one of the aims of this short chapter.

A second aim was to investigate an alternative way of interpreting the interference effect in the degradative pathway. The interpretation of the data presented in Chapters 6 and 7 had been by regarding interference in terms of competition of two or more substrates for the same class of membrane receptor or binding site. An alternative possibility is that uptake of the radioactive marker is unhindered by the addition of non-radioactive molecules, at even high concentrations, and that the real interference effect takes place at the level of degradation.

In support of this suggestion Tolleshaug et al. (1977) found that in the uptake of ^{125}I-asialofetuin by isolated rat hepatocytes, the rate of uptake exceeded the rate of degradation hence degradation was the rate-limiting step in the process. If, for example, the added non-radioactive protein is more susceptible to degradation or simply has a greater affinity for the degradative enzymes than the marker, the degradative enzymes may bind to the unlabelled protein rather than to the labelled marker protein and thus effectively less of the degradative enzymes will be available to degrade the marker. Against such an interpretation of interference at the level of degradative enzymes is the finding of Lloyd et al. (1976) that in the process of endocytosis of ^{125}I-labelled albumin by the in vitro rat yolk sac, membrane adsorption of the substrate, not subsequent degradation of the substrate, was the rate-limiting step. This may not necessarily be the case for the rabbit yolk sac, since there is only a low total percentage release of substrate after "loading" this tissue with ^{125}I-labelled rabbit IgG. The observed effect could possibly arise from slow intralysosomal degradation of this substrate by the tissue. However, it may be remembered that ^{125}I-labelled human IgG, a molecule that is protected in a similar way to the homologous IgG by the rabbit yolk sac, does not give rise to such a high tissue retention. (Incidentally, the Endocytic Indices of both substrates are also very different, low for the ^{125}I-labelled rabbit IgG and high for ^{125}I-labelled human IgG.) Up to now, these findings have been explained in terms of a difference in the specificity of the binding sites involved in the degradative pathway. An alternative explanation could be differential rates of degradation of the two substrates by the rabbit yolk sac. This possibility will be considered here by determining the difference in ease of degradation of homologous and heterologous IgG by enzymes derived from the rat and

rabbit yolk sacs.

In summary, the questions raised in this chapter are:

1. Can the degradative and non-degradative pathways of IgG transport in the rabbit yolk-sac be saturated?

2. Do differences exist between the proteolytic susceptibility of homologous and heterologous IgG species by the rat and rabbit yolk-sac enzymes?

3. In rat and rabbit yolk sacs, is there any relation between the proteolytic susceptibility of homologous and heterologous IgG species and their release from the tissue?

8.2 METHODS

8.2.1 Release patterns of ^{125}I-labelled rabbit IgG from rabbit yolk sacs following "loading" with this substrate at various different concentrations

The method in Section 1.12 was followed; ^{125}I-labelled rabbit IgG was present at one of the following concentrations: 1, 5, 10, 25, 50, 75, 100, 150, or 200 µg/ml.

8.2.2 Assay of the proteolytic activity (against homologous and heterologous ^{125}I-labelled IgG species and ^{125}I-labelled albumin) of cell-free extracts of 17.5-day rat yolk sacs and 24-day rabbit yolk sacs

The method used was essentially the same as described by Livesey and Williams (1979) for rat yolk sacs. Rat yolk-sacs (10) or rabbit yolk sacs (3) were washed in distilled water and homogenized in 10 ml of distilled water by using a Potter-Elvehjem type Teflon-on-glass homogenizer, with an 0.19 mm clearance, rotating at a speed of 2500 rpm. After diluting the homogenate with 20 ml of distilled water it was centrifuged for 10 minutes at 150 g to remove intact cells. 50 µl aliquots of this cell-free extract were incubated with 130 µl of a buffer solution (pH 3.0 - pH 6.0, 0.1M-acetic acid / sodium acetate buffer; pH 6.5 - pH 9.0, 0.1M-HCl/Tris) and 20 µg of substrate (^{125}I-labelled bovine, human, rabbit, rat IgG or ^{125}I-labelled albumin). The mixture was incubated for 1h at 37^{o}C then the reaction was stopped by first adding 0.5 ml ice-cold aq. calf serum, 20% (v/v), followed by 0.5 ml of 20% (w/v) trichloroacetic acid. Tubes were counted for contained total radioactivity and afterwards the precipitate was recovered by centrifuging the 3 ml Luckhams tubes, used for the reaction, for 20 min at 2000 g. Supernatants were decanted into fresh 3 ml Luckhams tubes and the contained TCA-soluble radioactivity counted. Blanks, containing

no cell-free extract but just appropriately buffered substrate, were also incubated under the same conditions as experimental tubes.

8.3 RESULTS

8.3.1 Release patterns of ^{125}I-labelled rabbit IgG from rabbit yolk sacs following "loading" with this substrate at different concentrations

Rabbit yolk sacs were first incubated with one of an increasing series of concentrations of ^{125}I-labelled rabbit IgG, then the release patterns were determined on reincubating the tissue. Figure 8 and Table 8.1 show how the following quantities vary with substrate concentration: total radioactivity associated with the yolk sac at the beginning of the reincubation period and the 3h values for the total substrate released, the TCA-solubles released and the TCA-insolubles released. It can be seen (Figure 8.2) that with increasing loading concentration the percentage total release decreases, i.e. the retention of substrate in the tissue effectively increases and that, for all concentrations up to 150 µg/ml, the quantity of TCA-insoluble radioactivity released is higher than the amount of TCA-solubles released. This, however, is reversed at the concentration of 200 µg/ml of ^{125}I-labelled rabbit IgG. Figure 8.1 shows that whereas the plot of TCA-insolubles against substrate concentration levels off (i.e. reaches saturation point) the plot of TCA-solubles against substrate concentration continues to rise progressively. This effect is also reflected in Figure 8.2 in which the percentages of total, TCA-soluble and TCA-insoluble radioactivity released are plotted against the loading concentration of ^{125}I-labelled rabbit IgG. Although the percentage TCA-insoluble release is higher than the percentage TCA-soluble release up to a substrate concentration of 150 µg/ml, it falls progressively while the percentage TCA-soluble release rises. For purposes of comparison, equivalent findings in the rat yolk sac (Ibbotson, Ph.D. thesis) are quoted in Figure 8.3. For all concentrations of ^{125}I-labelled rat IgG investigated, TCA-soluble

release by the rat yolk sac is considerably higher than TCA-insoluble release, although both rise with increasing substrate concentration. The main difference between the rat and rabbit yolk sacs lies in the amounts (both relative and absolute) of radioactivity released in TCA-soluble and -insoluble forms. In the rabbit yolk sac, more radioactivity is released in the TCA-insoluble form, except at the highest substrate concentration. In Table 8.2, TCA-soluble and TCA-insoluble release of ^{125}I-labelled rabbit IgG from rabbit yolk sacs are expressed in the form of the gradient of the plot of release against time over the last 80 min of the reincubation period. The value for TCA-insoluble release gives information about the rate of release of undegraded substrate from the yolk sac, and the value for TCA-soluble release is a measure of the rate of degradation of the substrate. Such rates are expected to reflect any saturability of a particular protein route (degradative or protective). The gradients of release of TCA-solubles are lower than those for TCA-insolubles with ^{125}I-labelled rabbit IgG at concentrations of 1 and 5 μg/ml, but are higher at all other concentrations.

8.3.2 Assay of the proteolytic activity (against homologous and heterologous ^{125}I-labelled IgG species and albumin) of a cell-free extract of 17.5-day rat yolk sacs and 24-day rabbit yolk sacs

Figures 8.4 and 8.5 show the pH-profiles of the proteolytic activity of the enzymes derived from the rabbit and rat yolk sacs, respectively, for a number of ^{125}I-labelled protein substrates. The percentage of substrate that has become degraded in 1.0h (measured as TCA-soluble radioactivity, corrected for the TCA-soluble activity contained in the substrate preparation itself or generated spontaneously during incubation without enzymes present) is plotted against the pH of the

reaction medium. Both rat and rabbit yolk-sac enzymes show maximal proteolytic activity against the added substrates (^{125}I-labelled albumin and bovine, human, rabbit and rat IgG) at pH 3.5 to 4.5. As the protein-content of the cell-free extracts derived from the two tissues are the same, it is possible to compare the relative proteolytic capacities of the two tissues (expressed as percentage degradation of substrate) and it is apparent that all substrates are degraded to a greater (x5) extent when the cell-free extract from rabbit yolk sacs is added rather than that from rat yolk sacs. (Ideally an incubation period of 5h would have been used in the determination of the degradation with the rat yolk-sac extract to reduce the degree of scatter in these data.) From the two figures it can be seen that individual substrates differ in their degree of susceptibility to degradation. With the rabbit yolk-sac extract the rank order is : bovine IgG > human IgG > BSA > rat IgG > rabbit IgG; with the rat yolk-sac extract the ranking order is more tentative, but suggests the following order : bovine IgG > BSA > rabbit IgG > human IgG > rat IgG. An interesting finding is that both patterns are similar, in that the homologous IgG is the least susceptible to breakdown by the yolk-sac degradative enzymes and bovine IgG is the most susceptible. Albumin is highly susceptible to degradation by the cell-free extract of both tissues.

Table 8.1 The effect of different substrate concentrations on the release of ^{125}I-labelled rabbit IgG from rabbit yolk sacs.

^{125}I-labelled rabbit IgG at one of the following concentrations (1, 5, 10, 25, 50, 75, 100, 150 or 200 µg/ml). Yolk sacs were then washed and reincubated for 3h in fresh, serum-free medium 199. At regular intervals up to 3h, samples were taken and the released TCA-soluble, TCA-insoluble and total radioactivity determined. The data, from 3h reincubations, presented here are also shown graphically in Figure 8.1 but without standard deviations.

Number of individual incubations	Substrate concentrations (µg/ml)	Mean quantity of substrate released (±S.D.)			Total activity associated with the yolk sac (ng/mg protein)
		TCA-soluble activity	TCA-insoluble activity (ng/mg yolk-sac protein)	Total radioactivity	
6	1	27.69 ± 8.55	53.11 ± 17.68	80.98 ± 15.12	111
6	5	154.61 ± 43.23	350.70 ± 108.07	488.64 ± 40.43	663
6	10	504.10 ± 137.40	712.38 ± 175.52	1226.47 ± 291.80	1510
6	25	434.13 ± 92.80	1248.06 ± 198.75	1679.19 ± 286.40	2140
6	50	914.28 ± 294.61	3122.10 ± 925.91	4036.38 ± 948.29	5557
6	75	1296.38 ± 213.95	2506.93 ±1028.78	3770.04 ± 852.83	4957
3	75	1488.73 ± 586.43	3194.64 ± 464.50	4650.01 ± 282.45	8221
6	100	1180.49 ± 799.08	3346.13 ±2173.79	4518.77 ±2969.25	6959
3	100	2196.10 ± 789.32	1972.38 ± 25.26	4168.38 ± 809.53	7186
3	150	1840.90 ± 368.17	2455.77 ± 461.29	4296.66 ± 436.51	8441
3	200	3981.07 ± 126.86	3013.71 ± 274.85	6994.81 ± 178.54	14283

Table 8.2 The effect of different substrate concentrations (^{125}I-labelled rabbit IgG) during the loading phase on the rate of release of substrate from reincubated rabbit yolk sacs

From plots of the individual time courses of release of ^{125}I-labelled rabbit IgG from rabbit yolk sacs, the gradient was determined over the last 80 mins of release. This should indicate whether, during this period, substrate release was almost complete or still ongoing.

Substrate concentration (μg/ml)	Gradient (ng per h) of the plot of release against "loading" concentration last 80 min.		Total release (%)
	TCA-soluble activity released	TCA-insoluble activity released	
1	0.29	0.36	72
5	1.51	2.91	73
10	4.98	1.95	80
25	4.05	2.89	78
50	10.30	9.00	72
75	10.55	8.74	76
75	14.20	15.41	56
100	9.60	6.99	64
100	19.28	8.73	58
150	15.43	11.32	50
200	36.60	19.16	48

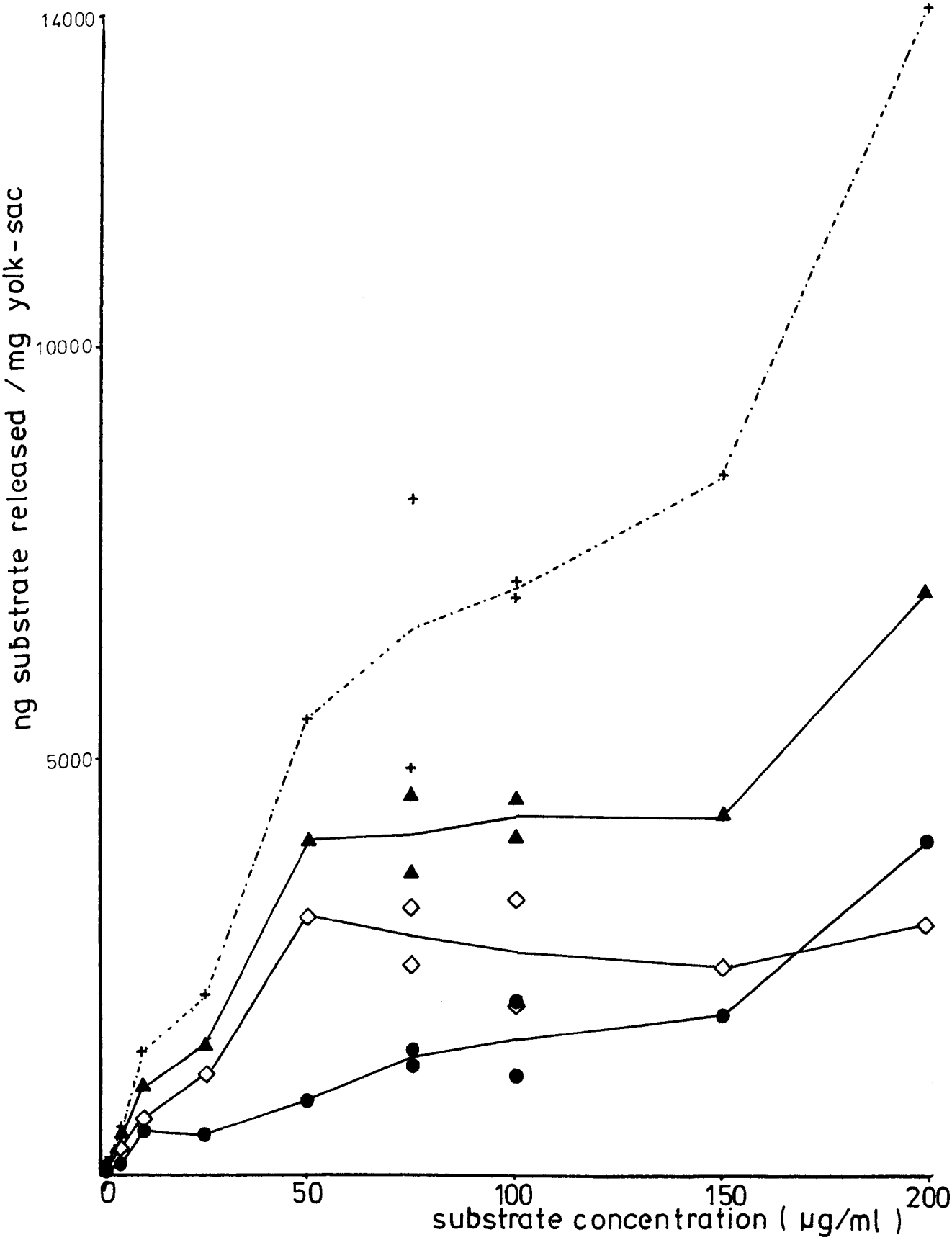


Figure 8.1 The effect of different concentrations of ^{125}I-labelled rabbit IgG on the subsequent release of radioactivity from rabbit yolk sacs

The data shown are those reported in Table 8.1. Total amount of substrate associated with the yolk sac at the beginning of reincubation (+-·-·-+), total radioactivity released (▲——▲), TCA-solubles released (●——●), TCA-insolubles released at 3h of reincubation (◇——◇).

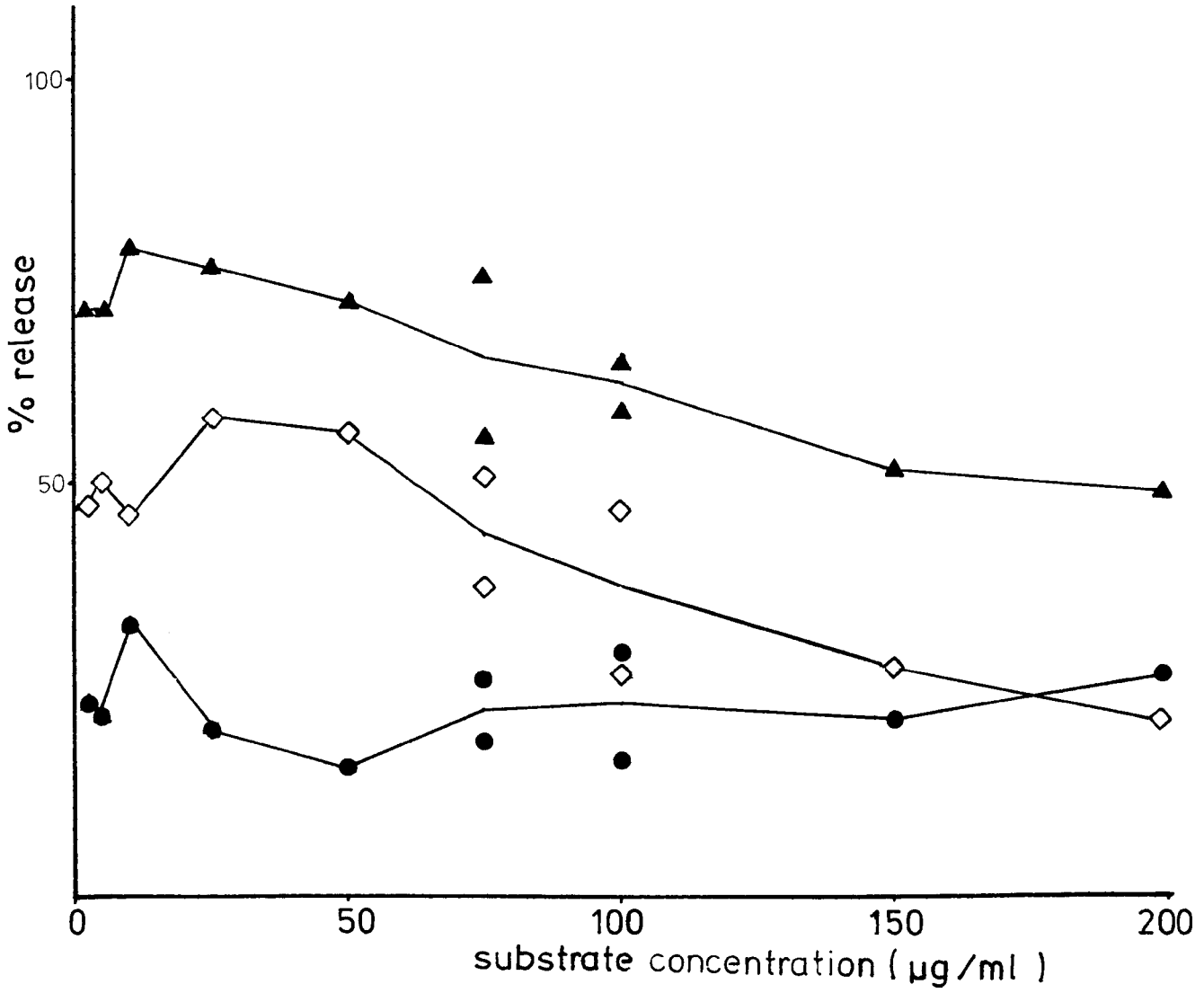


Figure 8.2 The effect of different substrate concentration on the percentage release of ^{125}I-labelled rabbit IgG from rabbit yolk sacs during the incubation period

In these plots the data from Table 8.1 are presented in a different way. Total radioactivity (▲———▲), TCA-soluble radioactivity (●———●) and TCA-insoluble radioactivity (◇———◇) released at 3h of reincubation are shown as a percentage of the total radioactivity associated with the yolk-sac at the beginning of reincubation.

Figure 8.3 The effect of different concentrations of ^{125}I-labelled rat IgG on the subsequent release of radioacti from rat yolk sacs

The data presented here are taken from Ibbotson (Ph D Thesis) and represent the results from experiments in which 17.5-day rat yolk sacs were first incubated for 2h in serum-free medium 199 containing ^{125}I-labelled rat IgG at a different concentration (1, 25, 75, or 100 µg/ml). Following washing, yolk sacs were reincubated and samples were taken at regular intervals for up to 3h.

Total radioactivity released: ▲——▲——▲——▲,

TCA-soluble radioactivity released: ●——●——●,

TCA-insoluble radioactivity released at 3h of reincubation ◇——◇——◇——◇.

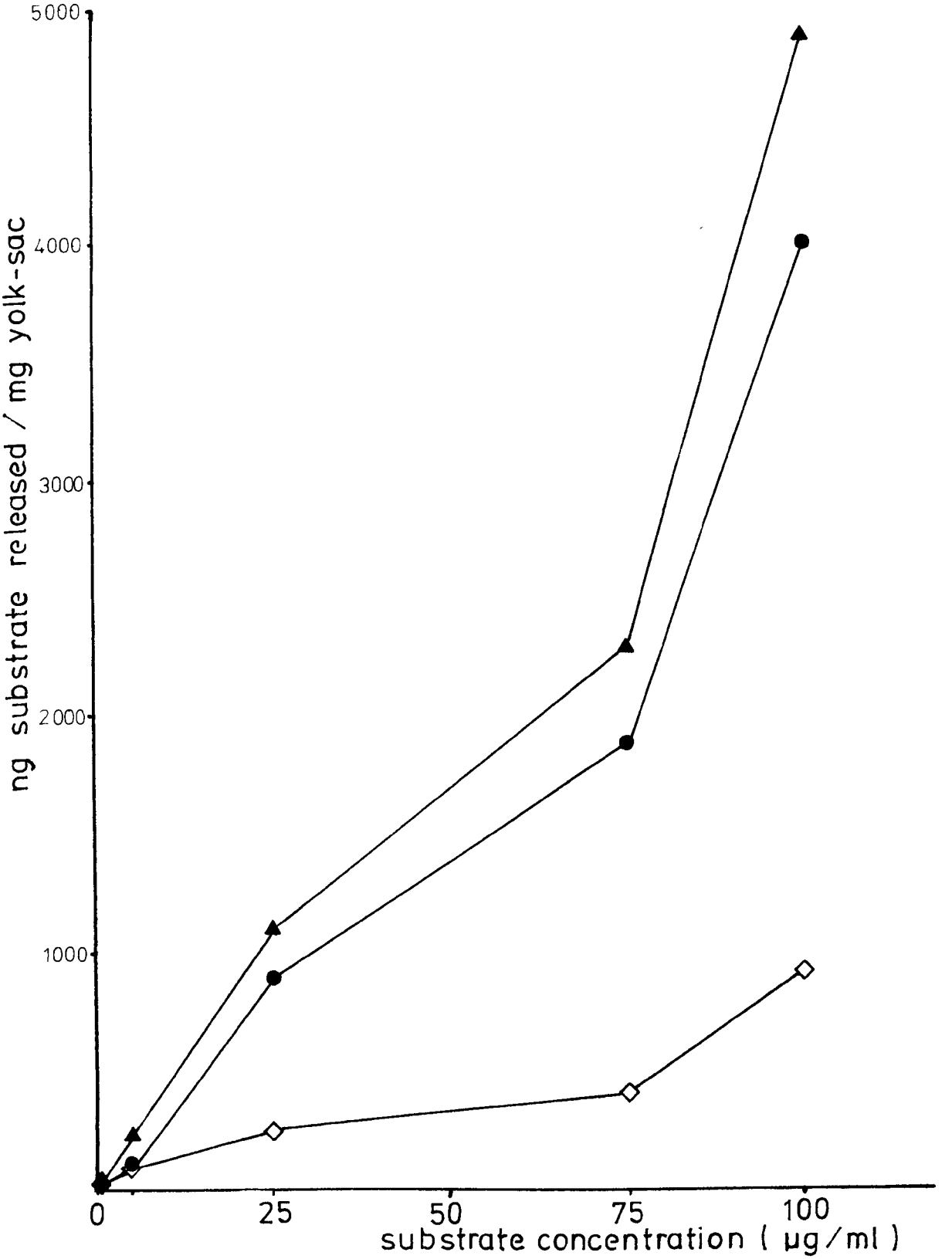

5000
4000
3000
2000
1000
ng substrate released / mg yolk-sac
0
25
50
75
100
substrate concentration (µg/ml)

FIGURE 8.3

Figure 8.4 pH profile for the degradation of ^{125}I-labelled albumin, bovine,human, rabbit and rat IgG by enzymes derived from cell-free extracts of rabbit yolk sacs

A cell-free extract of rabbit yolk sacs was prepared and incubated together with substrate (^{125}I-labelled albumin, bovine,human, rabbit,rat IgG) in either a 0.1M-sodium acetate/acetic acid buffer (pH 3.0-6.0) or a 0.1-Tris/HCl buffer (pH 6.5-9.0). Incubation was for 1h and the degree of substrate degradation was determined. (For detailed method see Section 8.2.2) This graph is a plot of the percentage of the total substrate that is degraded against the pH of the reaction mixture. Data are means from 4 experiments for each pH and substrate.
Bovine serum albumin, ●——● ; Bovine IgG, ◇——◇ ; Human IgG, +——+ ; Rabbit IgG, ○——○ ; Rat IgG, ▲——▲

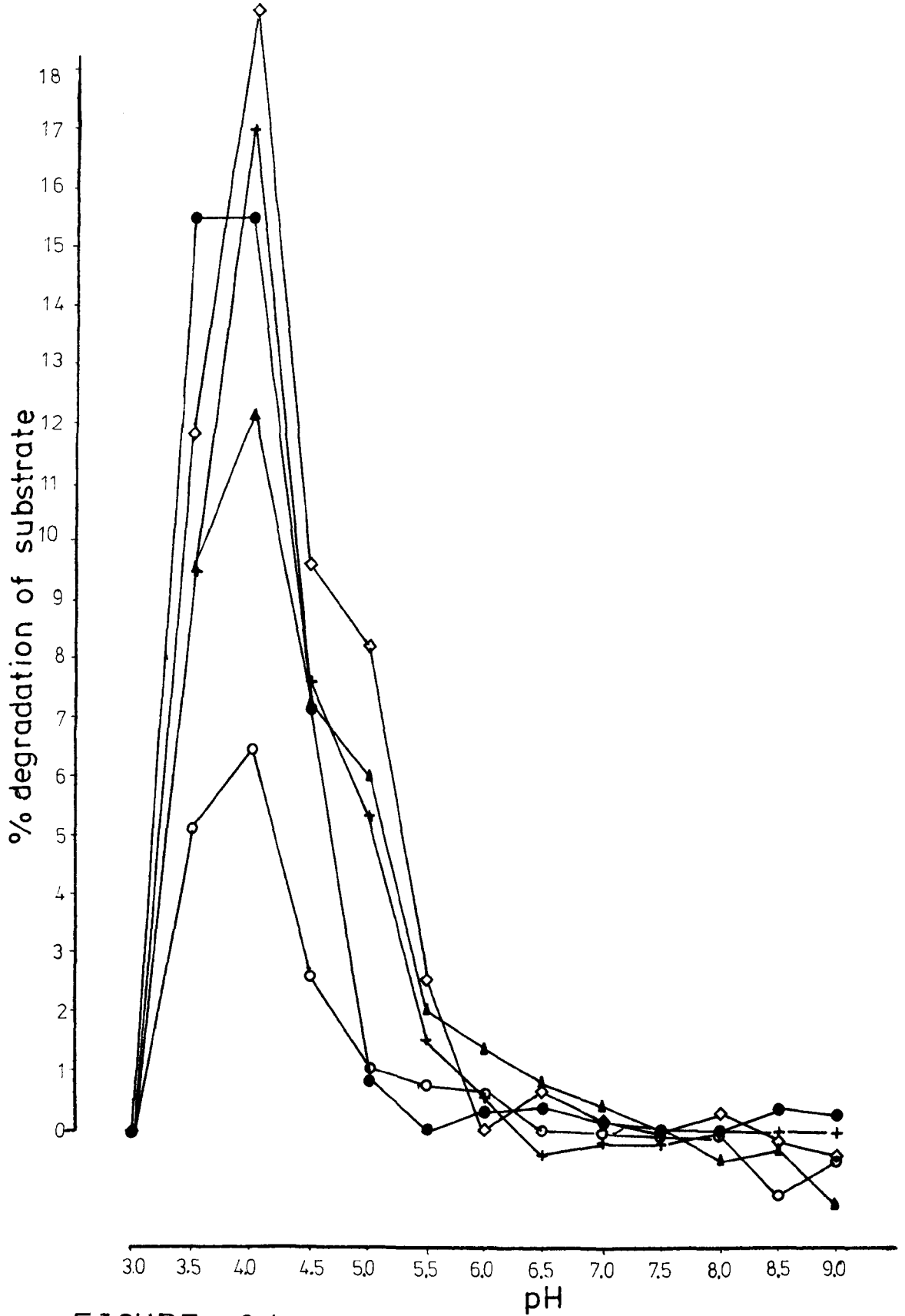

% degradation of substrate
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
pH

FIGURE 8.4

Figure 8.5 pH profile for the degradation of ^{125}I-labelled albumin, bovine,human, rabbit and rat IgG by enzymes derived from cell-free extracts of rat yolk sacs

A cell-free extract of rat yolk sacs was prepared and incubated together with substrate (^{125}I-labelled albumin, bovine,human, rabbit,rat IgG) in either a 0.1M-sodium acetate/acetic acid buffer (pH 3.0-6.0) or a 0.1-Tris/HCl buffer (pH 6.5-9.0). Incubation was for 1h and the degree of substrate degradation was determined. (For detailed method see Section 8.2.2). This graph is a plot of the percentage of the total substrate that is degraded against the pH of the reaction mixture. Data are means from 4 experiments for each pH and substrate.

Bovine serum albumin, ●——● ; Bovine IgG, ◇——◇ ; Human IgG, +——+ ; Rabbit IgG, ○——○ ; Rat IgG, ▲——

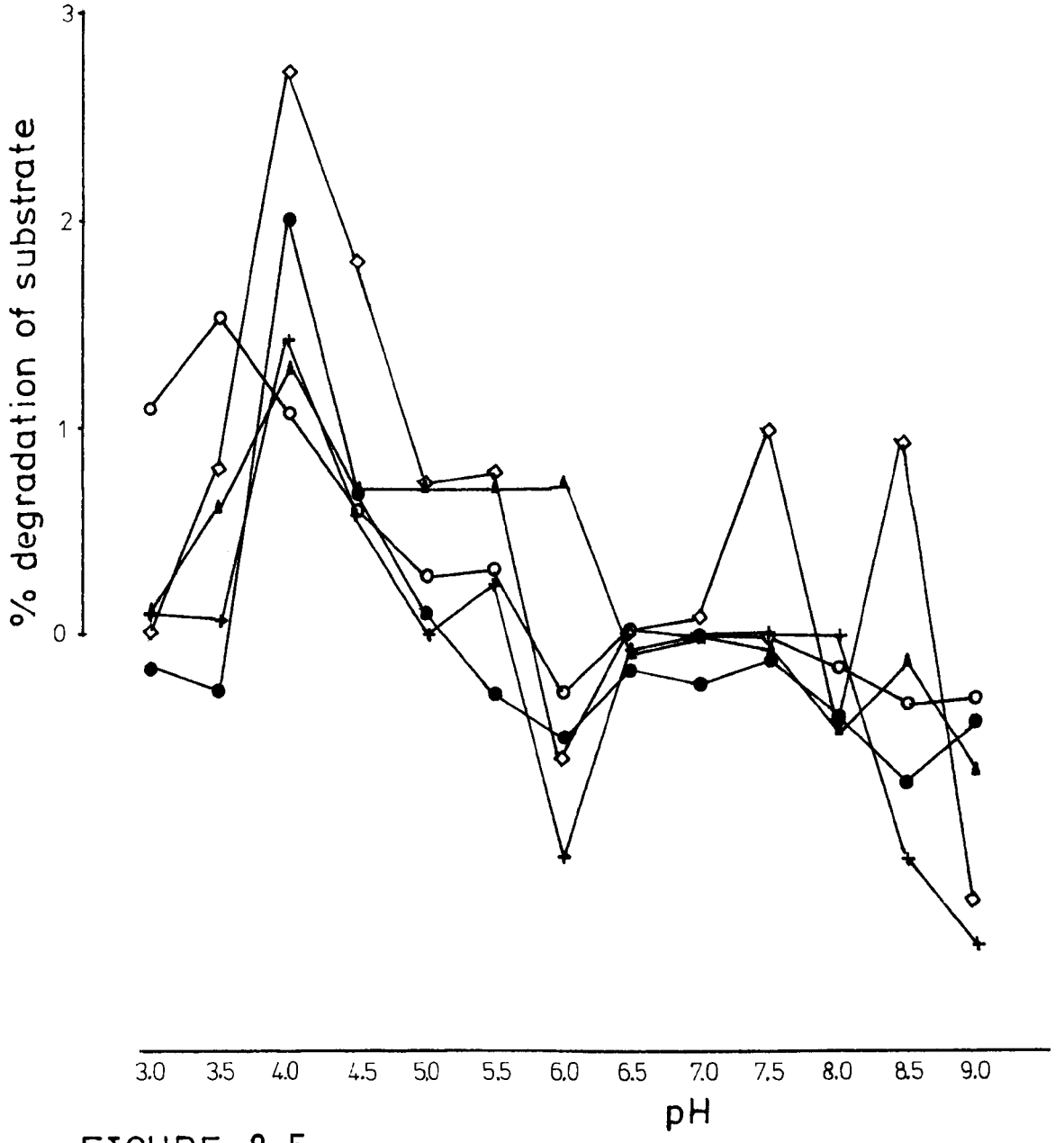

% degradation of substrate
3
2
1
0
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
pH

FIGURE 8.5

8.4 DISCUSSION

After demonstrating specific binding sites on the rat and rabbit yolk-sac membranes for the protective and degradative pathways, more information was sought about the properties of these two routes, especially the carrying capacity and ease of saturation. Williams & Ibbotson (1979) showed, in an earlier study on the rat yolk sac (data quoted in Figure 8.3 for comparison), that increasing the "loading" concentration of ^{125}I-labelled rat IgG led to an increased release of radioactivity in the form of TCA-solubles. Thus, when the loading concentration of ^{125}I-labelled homologous IgG was 5.0 µg/ml, 55.6% of the radioactivity released was acid-soluble, but with a loading concentration of 100 µg/ml 81.8% was acid-soluble. Likewise, the data reported in this chapter show that in the rabbit yolk sac an increase in the release of acid-solubles is observed with increasing loading concentration of ^{125}I-labelled rabbit IgG. In contrast, the TCA-insoluble release is considerably higher than the acid-soluble release up to a loading concentration of 150 µg/ml. Loading with 200 µg/ml of substrate, however, leads to a rise in TCA-soluble release, with the TCA-insolubles remaining constant. From Figure 8.1, a plot of substrate release (in ng) against loading concentration, it is evident that the release of radioactivity in the TCA-insoluble form does not exceed approx. 3000 ng/mg yolk-sac tissue. This figure is almost reached by a loading concentration of 50 µg/ml. The TCA-soluble release, on the other hand, seems to rise progressively with the loading concentration. This pattern suggests a protective route of IgG transport that is both saturable and chosen preferentially by the ^{125}I-labelled rabbit IgG. At low concentrations of substrate during the endocytosis phase, most of the substrate appears to be taken up into the transport pathway and so is subsequently released intact. A smaller amount of substrate

enters the degradative pathway. This could reflect differences in either the relative number or the affinity of "protective" and "degradative" binding sites present on the rabbit yolk-sac membrane. With higher concentrations of substrate, the protective pathway becomes saturated and more rabbit IgG is taken up into the degradative route where it is broken down and released. There is some evidence to indicate that ^{125}I-labelled rabbit IgG is degraded more slowly than other proteins that have been studied. Thus, in Table 8.2 an impression is given of the time-course of release, quoting the gradient of the plot of substrate release (acid-solubles and acid-insolubles) against time over the last 80 minutes of the 3h reincubation period. A low gradient indicates that release is almost complete after the first 80 min. A low gradient is evident for TCA-insoluble release at most concentrations, but a higher gradient is found for the release of TCA-solubles, especially at the higher loading concentrations. These findings, together with the great amount of substrate accumulated in the rabbit yolk-sac tissue following loading with high substrate concentrations, indicate that the release of radioactivity is almost complete after the first 80 min of reincubation. No saturation level of the degradative pathway is apparent in contrast to the findings with the protective IgG pathway. In Figure 8.2 where percentage release at 3h reincubation is plotted against the loading concentration, a gradual decrease in the total percentage release can be observed, reaching a level of less than 50% at a substrate concentration of 200 μg/ml. Expressed differently, more than 50% of the substrate taken up remains within the tissue. This great accumulation of ^{125}I-labelled rabbit IgG by the rabbit yolk sac (also observed in Chapter 6) could be indicative of either an overloading, by high quantities of ^{125}I-labelled rabbit IgG, of the lysosomal enzyme in the rabbit yolk sac, making breakdown the

rate-limiting process, or alternatively, of an accumulation of rabbit IgG in the protective pathway, either in coated vesicles (Wild,1975) or adsorbed to protective sites on the walls of ordinary pinosomes or heterolysosomes.

The first possibility is more likely, since support for this suggestion comes from the experiments investigating the proteolytic capacity of enzymes derived from rabbit yolk sacs against a number of proteins (^{125}I-labelled albumin and ^{125}I-labelled bovine, human, rabbit and rat IgG) at different pH values. ^{125}I-labelled rabbit IgG is by far the least susceptible to breakdown by the proteolytic enzymes derived from the rabbit yolk sac (Fig. 8.4). (Incidentally, in the rat yolk sac too the homologous IgG species may be the one that is least readily degraded.) These findings have very interesting implications in the ongoing Brambell/ Wild controversy about the mechanism of transfer of passive immunity. Unlike the Wild hypothesis, the Brambell hypothesis advocates differential degradation of proteins within the same vesicle followed by exocytosis of substrate at the basal or lateral plasmalemma. Brambell's suggested cause of this selective degradation was the presence of protective receptors on the inner membrane of pinocytic vesicles. If, in addition to such receptors, the homologous IgG is also especially resistant to the degradative enzymes contained in the heterolysosomes, the result would be a greater release of the intact homologous IgG. Such an increased resistance of the homologous IgG to degradation would add a new dimension to the Brambell theory without however, fundamentally modifying it. In the terms of the Wild hypothesis, an increased resistance of homologous IgG to degradation by the yolk sac would be entirely incidental and not advantageous. Resistance to degradation would not lead to an enhanced transport across the cell, but rather to an accumulation of the undegraded species in the heterolysosomes, since the

latter are not expected to exocytose. Thus, if it is possible to experimentally render the homologous IgG harder to degrade, the predictions that arise concerning the fate of the homologous IgG in the rat and rabbit yolk sac, using Brambell's theory, differ from those that would arise according to Wild's theory. This idea will be further developed and discussed in Chapter 9, with the hope that the evidence would point to one or other of the prevailing theories of IgG transfer.

The finding that the rank order of susceptibility to degradation of the various proteins is the reverse of the order observed for transfer across the tissue, is thus rather interesting, since it suggests that those protein species that are least susceptible to degradation are most readily transported intact across the yolk sac. It would even be possible to postulate that selectivity in IgG transmission is due merely to the differential susceptibility of the proteins to lysosomal enzymes. This is an extremely attractive suggestion to bear in mind if one tries to correlate the observation that IgG subclasses differ in their susceptibility to degradation with their ease of transfer *in vivo*. (Rousseau *et al.* (1980), using IgG subclasses derived from rat immunocytomas, found interesting differences in both their degradation by trypsin, pepsin, plasmin and papain and in their extents of transfer.) The one protein, however, that does not fit this scheme in the rabbit yolk sac is ^{125}I-human IgG. Although degradation of this substrate by the cell-free rabbit yolk-sac extract is rapid, transfer *in vivo* and release of TCA-insolubles *in vitro* following "loading" of the yolk sac with this substrate are both high. These findings do not support the suggestion that susceptibility to degradation alone can determine the degree of transport of this substrate.

The finding that cell-free extracts from both rat and rabbit yolk

sacs degrade all substrates maximally within the pH range of 3.5 - 4.5, that is, in the region of the pH optimum of lysosomal enzymes, parallels the findings of Ghetie & Motas (1971) on the degradation of rabbit IgG by cathepsin-rich subcellular fractions derived from rat hepatocytes, where the pH optimum was also 3.5. In the present work no significant peaks were found elsewhere in the pH range tested (3.0 - 9.0), which is indicative of an exclusive degradation of these IgG species and albumin within the lysosomes. As the amounts of cell-free rat and rabbit yolk sac extract used were equal (i.e. same protein content) the percentage degradation could be compared meaningfully, and it seems that the catabolic potential of the rabbit yolk sac exceeds that of the rat yolk sac for the protein species tested. Thus it is difficult to explain the elevated transport capacity of the rabbit yolk sac, relative to that of the rat, in terms of a corresponding decrease in its catabolic capacity. There appears to be plenty of opportunity for endocytic vesicles to fuse with lysosomes in rabbit yolk sacs so that it would appear that any vesicle-mediated transcellular transport must somehow avoid direct confrontation with this system.

In summary, the most important findings in this chapter are:

1. The protective IgG pathway in the rabbit yolk sac is saturable, but only at high concentrations of homologous IgG. In contrast, the degradative pathway does not seem to be readily saturable.
2. A preference of ^{125}I-labelled homologous IgG for the non-degradative IgG route in the rabbit yolk sac is apparent.
3. In their basic behaviour rat and rabbit yolk sacs do not differ, but the capacity for IgG transport is greater in the rabbit than in the rat yolk sac.
4. ^{125}I-labelled albumin and homologous and heterologous IgG species are degraded by enzymes derived from both rat and rabbit yolk sacs

at pH 3.5 - 4.5, i,e, in the pH region considered optimum for lysosomal enzymes.

5. Differences between the proteolytic susceptibility of homologous and heterologous IgG species to degradation by rat and rabbit yolk sac enzymes can be demonstrated. Homologous IgG is the least susceptible and bovine IgG the most susceptible to degradation by yolk-sac extracts from both species.

6. The proteolytic capacity of rabbit yolk sac seems to exceed that of rat yolk sac hence it is not possible to explain the greater transport capacity of rabbit yolk sacs in terms of a decreased or absent proteolytic enzyme capacity.

CHAPTER NINE

An Investigation of the Effects of Leupeptin on the Release of Homologous IgG from Rat and Rabbit Yolk Sacs

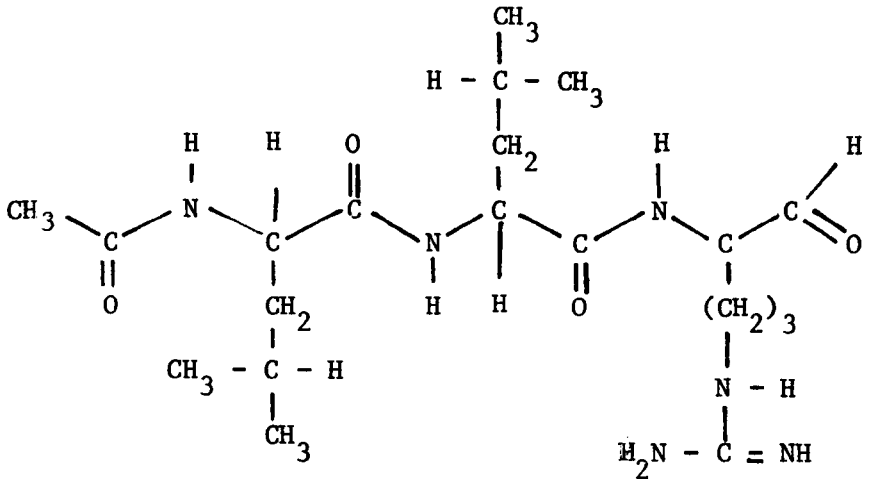
9.1 INTRODUCTION

Of the three theories advanced to account for the selective transfer of proteins across the yolk sac of the rat and rabbit, that put forward by Hemmings & Williams (1976) was shown to be highly unlikely (see Chapters 6 and 7). So far, however, no conclusive evidence has been found either for or against the Brambell or the Wild theories. In this chapter an attempt will be made to investigate some factors that may help to distinguish between these two theories.

According to Brambell, the IgG to be transferred is taken up into ordinary endocytic vesicles that later fuse with lysosomes to form heterolysosomes. It is suggested that protection of IgG molecules against the degradative action of lysosomal enzymes occurs by adsorption to specific receptors on the inner face of the vesicle membrane. In order to transfer the protected proteins to the foetal circulation, these heterolysosomes are postulated to release their contents at the lateral or basal plasmalemma by exocytosis. During this process of exocytosis, however, it would be expected that other contents of the heterolysosomes would also be released. Hence, both lysosomal enzymes and any non-digestible marker molecules would be expected to be released in the same manner as adsorptively protected IgG. Therefore, "loading" the rat and rabbit yolk sacs with ^{125}I-labelled PVP should result in a release of this polymer during reincubation in fresh medium if Brambell's theory is correct. If, however, Wild's theory is valid, no great amount of PVP would be expected to be released because, according to this hypothesis, only coated micropinocytic vesicles exocytose their contents (homologous and some heterologous IgG) at the basal and lateral plasmalemma (Wild, 1975).

In a study using rat yolk sacs incubated in vitro, Williams & Ibbotson (1979) could find no evidence for the release of PVP from

preloaded yolk sacs; likewise, no attendant release of lysosomal enzymes could be detected (Ibbotson, thesis). It remains to be established whether the same holds for the rabbit yolk sac. Thus, a study of the release of a non-degradable marker molecule from the rabbit yolk sac (that is, an attempt to demonstrate exocytosis from heterolysosomes) should be informative in deciding whether the Brambell theory is correct. Moreover, it would be expected, according to Brambell, that a greater release of IgG would result if non-adsorbed IgG, free within the lumen of the heterolysosomes, could somehow be prevented from being degraded by lysosomal enzymes. The finding in Chapter 8, that rabbit IgG is very poorly degradable at acid pH by a cell-free extract of rabbit yolk sac, gives some support to Brambell's theory, expanding it somewhat by providing a second mechanism responsible for protection. If degradation could be further prevented, for example by the addition of a specific inhibitor of lysosomal proteinases, then according to the Brambell mechanism a greater amount of IgG would be expected to be transported across yolk sac *in vivo* or released in the *in vitro* system. According to Wild's theory, no such enhanced release of intact immunoglobulin should result on inhibiting intralysosomal proteolysis, because heterolysosomes are not expected to exocytose their contents. In Chapter 4 ammonium ions were shown to be inhibitors of lysosomal enzymes, but they also strongly inhibit pinocytosis (Livesey *et al.*, 1980). These workers found leupeptin to be a more specific, hence a more suitable, inhibitor of lysosomal proteolysis (Knowles *et al.*, 1981). Leupeptin is the name given to a group of compounds, comprising methyl-, acetyl- or propionyl-L-leucyl-L-leucyl-L-arginal and their analogues in which a leucine may be replaced by either isoleucine or valine (Kondo *et al.*, 1969; Kawamura *et al.*, 1969). The structure of the molecule used in this study is as follows :



Leupeptin is of microbial origin and is reported to inhibit competitively plasmin, trypsin, kallikrein, thrombokinase, papain and cathepsin B (Aoyagi et al., 1969; Aoyagi & Umezawa, 1975; Fritz et al., 1973). It also inhibits cathepsin L, but not cathepsin D (Kirschke et al., 1977). The advantages of using leupeptin to inhibit the action of lysosomal enzymes are as follows : 1) it has been shown to be non-toxic at even quite high concentrations. Umezaywa & Aoyagi (1977) showed that the LD_{50} (i.e. the quantity of leupeptin needed to kill half the test population) was 118 mg/kg body weight in mice, when the inhibitor was injected intraveneously. 2) Leupeptin has been shown not to affect pinocytic uptake of ^{125}I-labelled formaldehyde-treated BSA into the rat yolk sac (Knowles et al., 1981), while inhibiting the degradation of this molecule by more than 50%. 3) Leupeptin has a molecular weight of 300, and this implies that it is taken up into the tissue by pinocytosis only.

In summary, the questions posed in this chapter are:

1) Does a non-degradable marker, like ^{125}I-labelled PVP get released from rabbit yolk sacs that have been loaded with this substrate in vitro, or is it retained in the same manner as observed in

rat yolk sacs?

2) Is it possible to induce a greater amount of homologous ^{125}I-labelled IgG to be released intact from rat and rabbit yolk sacs *in vitro*, by inhibiting lysosomal enzymes with leupeptin?

9.2 METHODS

9.2.1 Exocytosis, from rat and rabbit yolk sacs reincubated in serum-free medium 199, of ^{125}I-labelled PVP accumulated by prior incubation in serum-free medium 199

The general method outlined in Section 1.12 was followed using ^{125}I-labelled PVP as substrate. The initial incubation period in the presence of ^{125}I-PVP (10 µg/ml) was 2h for 24-day rabbit yolk sacs and 3h for 17.5-day rat yolk sacs.

9.2.2 Uptake of homologous ^{125}I-labelled IgG by rat and rabbit yolk sacs incubated in serum-free medium 199 containing leupeptin

Rat yolk sacs (17.5-day) or rabbit yolk-sac pieces (24-day) were incubated in serum-free medium 199 as described in Section 1.7. Leupeptin (30 µg/ml) was added to the incubation medium together with the substrate, homologous ^{125}I-labelled IgG (2 µg/ml). Yolk-sac incubations were then terminated at regular intervals up to 5.5h, and assays of the amount of radioactivity contained in the medium and in the yolk sacs were performed as outlined in Section 1.4 and 1.6. Uptake of substrate was plotted against time for each set of data and the Endocytic Index and the Tissue-Accumulation Rate derived (see Section 1.10 for details of calculations).

9.2.3 Exocytosis from rabbit and rat yolk sacs of homologous ^{125}I-labelled IgG accumulated by the tissue during a prior incubation in medium 199 containing leupeptin

The general method outlined in Section 1.12 was followed and either 17.5-day rat yolk sacs or 24-day rabbit yolk-sac pieces were incubated in serum-free medium 199. Three different regimes were adopted for exposing the tissues to leupeptin.

1. Leupeptin (30 μg/ml) was added to the "loading" phase medium at the same time as ^{125}I-labelled homologous IgG (10 μg/ml). After washing, the tissue was reincubated in fresh serum-free medium 199, containing neither ^{125}I-labelled IgG nor leupeptin, and samples were removed at regular intervals up to 3h.

2. The initial 2h incubation period of the yolk sacs was in serum-free medium 199 containing only homologous ^{125}I-labelled IgG (10 μg/ml) but, following washing, yolk sacs were reincubated in fresh medium 199 containing leupeptin (30 μg/ml). Samples were removed at regular intervals, taking care to replace the removed leupeptin when replenishing the medium in order to maintain a constant concentration of leupeptin.

3. Leupeptin (30 μg/ml) was present during both, the "loading" and the reincubation phases.

9.3 RESULTS

9.3.1 Release of ^{125}I-labelled PVP from rat and rabbit yolk-sac tissue that had previously been exposed to this marker macromolecule in vitro

The time-course of release of ^{125}I-labelled PVP from rat yolk sacs is presented in Figure 9.1 and for rabbit yolk sacs in Figure 9.2. Over the 3h reincubation period only a very small amount of the marker accumulated in either tissue was released into the reincubation medium. Expressed as a percentage of the total, rat yolk sacs released 6.7% and rabbit yolk sacs released 2.3% of the ^{125}I-labelled PVP. In contrast, when ^{125}I-labelled homologous IgG replaced the ^{125}I-labelled PVP, 83.0% (rat) and 80.6% (rabbit) of the radioactivity contained in the yolk sacs was released (544 ng/mg and 1216 ng/mg; see Table 5.7). It should be noted that the absolute amount (ng) of ^{125}I-labelled PVP released is considerably smaller than the quantity TCA-insolubles of homologous ^{125}I-labelled IgG released.

9.3.2 Uptake of ^{125}I-labelled homologous IgG by rat and rabbit yolk sacs incubated in serum-free medium 199 containing leupeptin

The Endocytic Indices of ^{125}I-labelled homologous IgG, obtained in rat and rabbit yolk sacs in serum-free medium, differ considerably (Tables 9.1 and 9.2). (This point has been discussed previously, see Chapter 2). The same differences between tissues are observed for Tissue-Accumulation Rates. The addition of leupeptin to the incubation medium caused no change in the E.I.s for either substrate. The T.A.R.s, however, rose sharply for ^{125}I-labelled homologous IgG in rat and rabbit yolk sacs incubated in serum-free medium in the presence of 30 µg/ml of leupeptin. Expressed in the form of the ratio T.A.R./E.I., the rise was from 47.2% to 71.8% in the rabbit yolk sac and from 9.5% to 55.8% in the rat yolk sac.

9.3.3 Release from rabbit and rat yolk sacs of ^{125}I-labelled homologous IgG accumulated by the tissue during a prior incubation in medium 199 containing leupeptin

In considering the effects of the presence of leupeptin in the incubation medium on the release of ^{125}I-labelled homologous IgG from rat and rabbit yolk sacs, data obtained with rat tissue will be considered first (Figure 9.3). In the histograms, the total amounts of substrate-derived radioactivity released by 3h under the various conditions of reincubation are reported. A value is also given for the total amount of radioactivity associated with the tissue before reincubation. Without leupeptin present, in the rat yolk-sac incubations most (80%) of the ^{125}I-labelled rat IgG associated with the tissue is released. The presence of leupeptin, however, either during the loading or the reincubation phase, or both, caused a considerable amount of IgG to accumulate in the yolk sac, but not to be released. The greatest percentage tissue-accumulation or tissue-retention of substrate was observed when leupeptin was present in both loading and reincubation phases (47%), followed by leupeptin in only the loading phase (39%), and the least when leupeptin was present during only the reincubation phase (31%). It may also be noted that under these conditions the total amount of substrate accumulated in the rat yolk-sac tissue was markedly higher than for the controls. Again the highest values were recorded for rat yolk-sac incubations with leupeptin present in the loading phase or during the entire length of loading and reincubation, but even with leupeptin present during only reincubation phase, the elevation in the total tissue-accumulation level is marked. The total amounts of radioactivity released were very similar under all conditions. When leupeptin was present in either the loading or reincubation phases or both, the extent of release of TCA-solubles was slightly higher than for

the controls.

With rabbit yolk sacs, even in the control series the total percentage release of substrate from tissue loaded with ^{125}I-labelled rabbit IgG was not as high as in corresponding incubations with rat yolk sacs. This tended to mask the effect of any tissue-retention induced by the presence of leupeptin (Figure 9.4). Nevertheless, the accumulation of substrate by the yolk sac appeared to be slightly higher when leupeptin was present either during loading alone or during both loading and reincubation. When leupeptin was present during the reincubation phase only, the retention of substrate in the tissue appeared to be even lower than in the control incubation. TCA-soluble release remained the same whether leupeptin was present or not in the incubation medium, and more importantly, no increase of TCA-insoluble activity release was recorded. Rather, when leupeptin was present in the reincubation phase only, TCA-insoluble release was, if anything, slightly decreased.

Summarizing the most important of the above findings, the presence of leupeptin in the medium at any phase of incubation did not significantly affect the release of the TCA-insolubles. That is, release of the macromolecular form of the homologous ^{125}I-labelled IgG molecule does not appear to be enhanced by the presence of leupeptin in either the "loading" or the reincubation media.

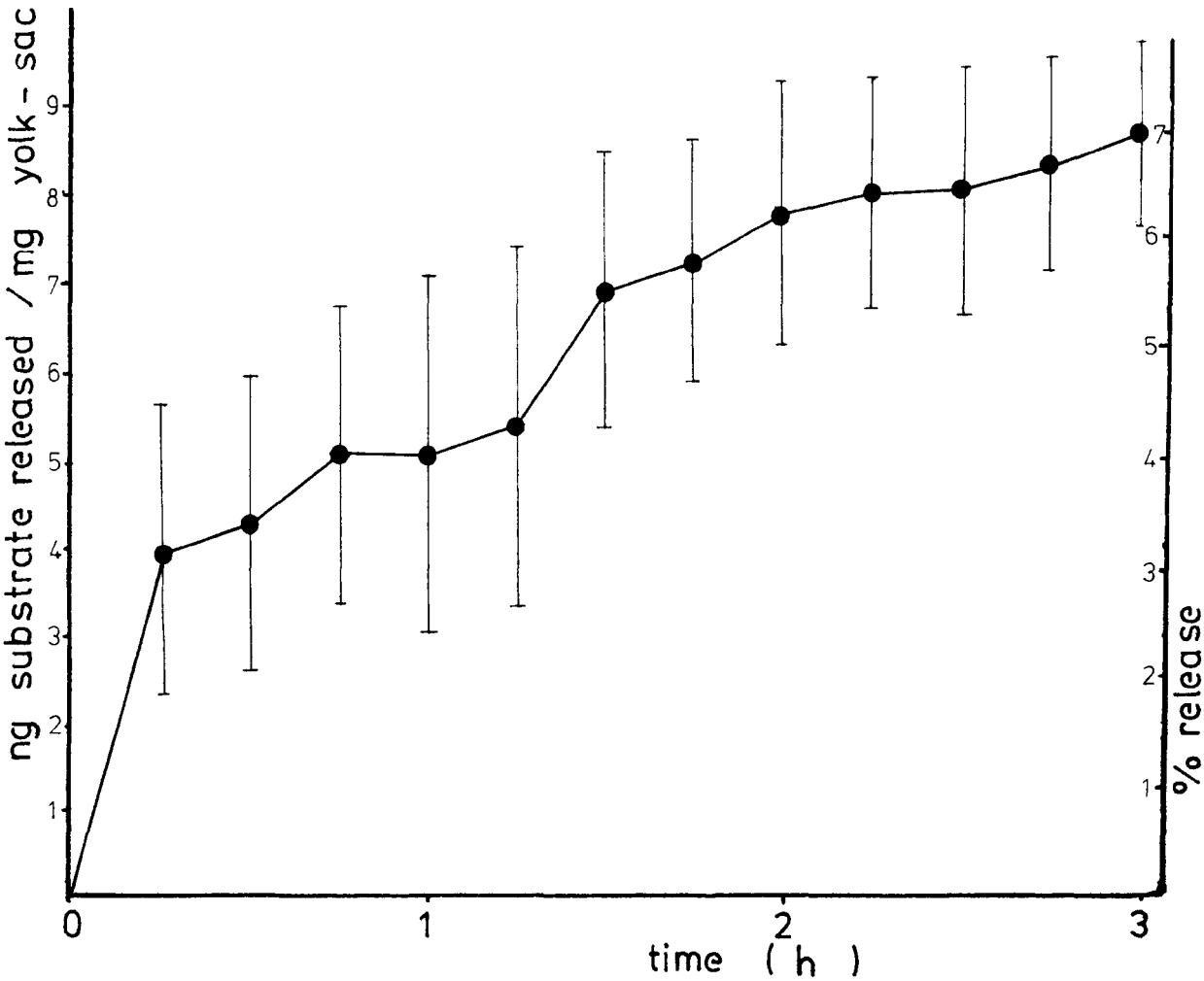


Figure 9.1 Time course of release of ^{125}I-labelled PVP from rat yolk-sacs previously exposed to this marker macromolecule in vitro

17.5-day rat yolk-sacs were first incubated in serum-free medium 199 containing ^{125}I-labelled PVP (10 µg/ml). After 3h yolk-sacs were washed and reincubated in fresh medium 199, up to a further 3h. Values shown are means (± S.D.) from 6 separate experiments.

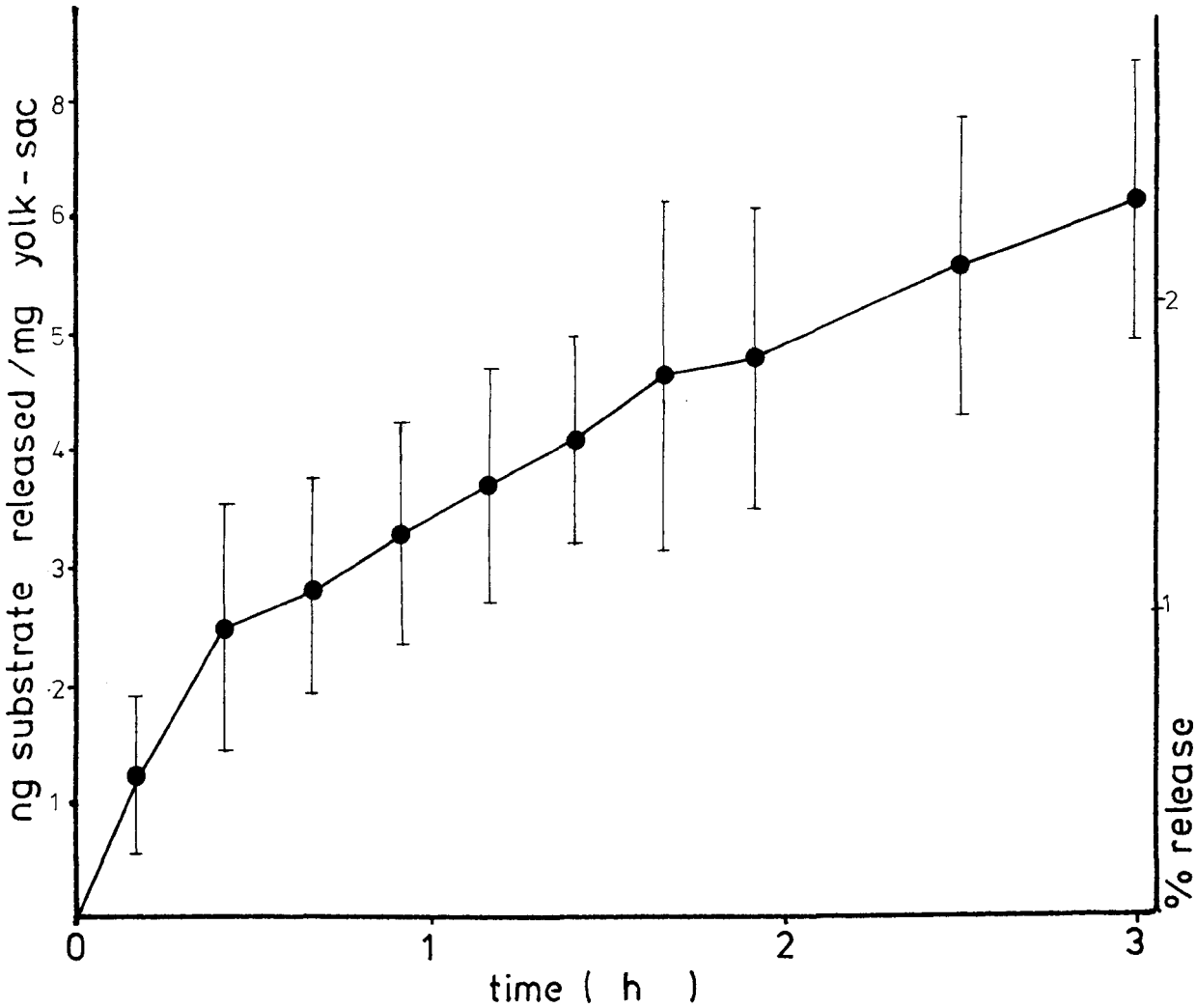


Figure 9.2 Time course of relase of ^{125}I-labelled PVP from rabbit yolk-sac previously exposed to this marker macromolecule in vitro

24-day rabbit yolk-sac pieces were first incubated in serum-free medium 199 containing ^{125}I-labelled PVP (10 µg/ml). After 2h yolk-sacs were washed and reincubated in fresh medium 199, up to a further 3h. Values shown are means (± S.D.) from 6 separate experiments.

Table 9.1 The effect of the presence of leupeptin in the incubation medium on the rates of uptake, proteolysis and tissue-accumulation of ^{125}I-labelled rat IgG in rat yolk sacs

Rat yolk sacs (17.5-day) were incubated in serum-free medium 199 containing ^{125}I-labelled rat IgG (2 µg/ml) and leupeptin (30 µg/ml). Incubations were terminated at regular intervals up to 5.5h. (For details of this method see Section 9.2.2.) From the plots of TCA-solubles released and total tissue uptake against time, the rates of proteolysis, the tissue-accumulation rate and the Endocytic Index could be calculated. The correlation coefficients of the plots are shown in parentheses.

Concentration of leupeptin (µg/ml)	No. of yolk sacs	Endocytic Index (µl/mg per h)	Tissue Accumulation Rate (µl/mg per h)	Rate of Proteolysis (µl/mg per h)
0 (control)	10	64.15 (0.793)	6.13 (0.680)	57.20 (0.904)
30	10	52.92 (0.955)	29.29 (0.904)	24.47 (0.938)
30	10	71.11 (0.887)	43.91 (0.881)	27.79 (0.894)
30	10	70.45 (0.921)	35.23 (0.878)	35.23 (0.999)
	MEAN VALUES (±S.D)	64.83 ± 10.51	36.14 ± 7.35	29.16 ± 5.51

Table 9.2 The effect of the presence of leupeptin in the incubation medium on the rates of uptake, proteolysis and tissue-accumulation of ^{125}I-labelled rabbit IgG in rabbit yolk sacs

Rabbit yolk-sac pieces (24-day) were incubated in serum-free medium 199 containing ^{125}I-labelled rabbit IgG (2 µg/ml) and leupeptin (30 µg/ml). Incubations were terminated at regular intervals up to 5.5h. (For detailed method see Section 9.2.2.) Other details as in the legend to Table 9.1

Concentration of leupeptin in incubation medium (µg/ml)	No. of yolk sacs	Endocytic Index (µl/mg per h)	Tissue Accumulation Rate (µl/mg per h)	Rate of Proteolysis (µg/mg per h)
0	8	34.97 (0.852)	13.01 (0.717)	23.23 (0.935)
0	9	39.79 (0.947)	22.60 (0.851)	17.09 (0.937)
0	9	20.19 (0.916)	10.27 (0.706)	11.56 (0.902)
0	9	38.19 (0.943)	17.22 (0.857)	20.76 (0.869)
0	9	31.83 (0.823)	14.89 (0.638)	18.42 (0.919)
	MEAN VALUES (± S.D.)	32.99 ± 7.78	15.59 ± 4.67	18.21 ± 4.39
30	10	21.46 (0.898)	11.95 (0.828)	10.11 (0.897)
30	9	20.53 (0.968)	15.39 (0.943)	5.61 (0.911)
30	9	33.80 (0.920)	26.47 (0.877)	7.37 (0.857)
30	9	37.90 (0.963)	27.88 (0.932)	9.57 (0.892)
	MEAN VALUES (± S.D.)	28.42 ± 8.75	20.42 ± 7.94	8.16 ± 2.07

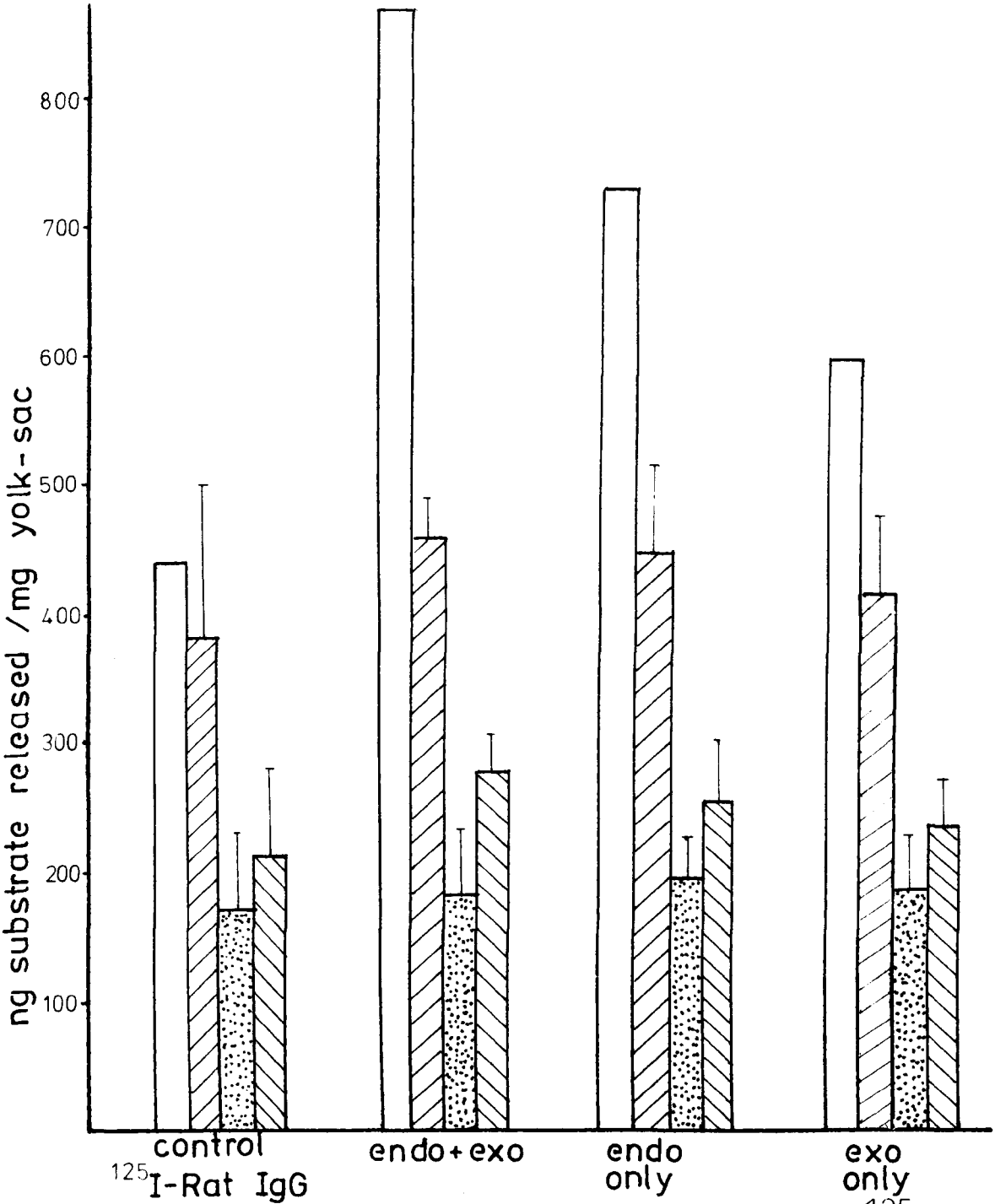


Figure 9.3 Effect of leupeptin on the release of ^{125}I-labelled rat IgG from rat yolk-sacs incubated in serum-free medium 199

17.5-day rat yolk-sacs were incubated in serum-free medium 199 and ^{125}I-labelled rat IgG (10 µg/ml), then rinsed and reincubated in fresh medium. Leupeptin (30 µg/ml) was added in either the endocytosis phase (endo only) or the exocytosis phase (exo only) or both (endo + exo) as outlined in Section 9.2.3. Substrate release was monitored for up to 3h. The graph expressed the total amount of substrate associated with the yolk-sac at the beginning of reincubation □ , the total amount of substrate released ▨ , the TCA-soluble activity released ▧ and the TCA-insoluble activity released ▩ at 3h of reincubation for each of the experimental regimes mentioned. Values shown are means (± S.D.) from 6 separate experiments for each condition.

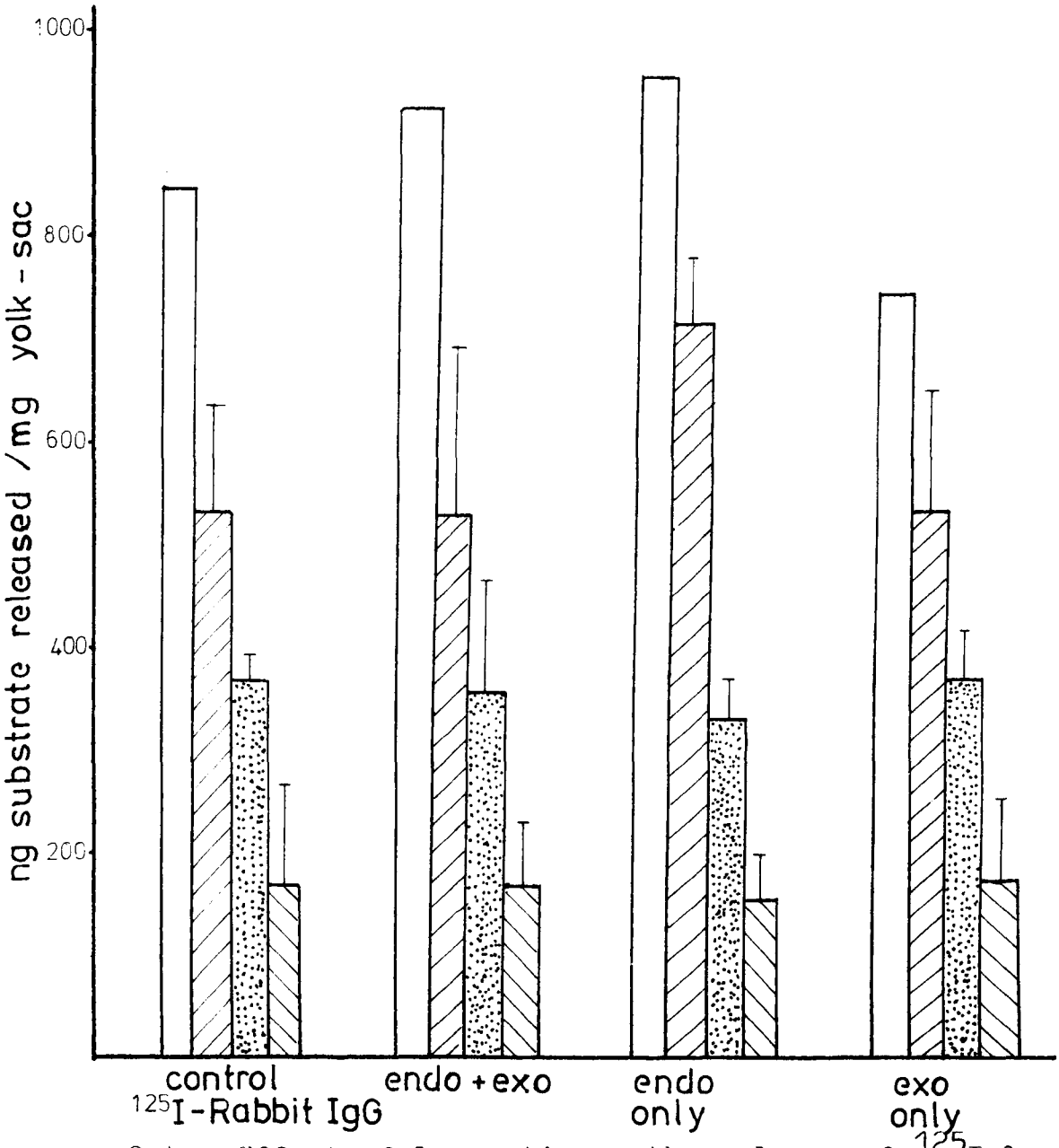


Figure 9.4 Effect of leupeptin on the release of ^{125}I-labelled rabbit IgG from rabbit yolk-sacs incubated in serum-free medium 199

24-day rabbit yolk-sac pieces were incubated in serum-free medium 199 and ^{125}I-labelled rabbit IgG (10 µg/ml), then rinsed and reincubated in fresh medium 199. Leupeptin (30 µg/ml) was added in either the endocytosis phase (endo only) or the exocytosis phase (exo only) or both (endo + exo) as outlined in greater detail in Section 9.2.3. Substrate release was monitored for up to 3h. The graph expresses the total amount of substrate associated with the yolk-sac at the beginning of reincubation, the total amount of substrate released, the TCA-soluble activity released and the TCA-insoluble activity released at 3h of reincubation for each of the experimental regimes mentioned. Values shown are means (± S.D.) from 3 separate experiments for each condition.

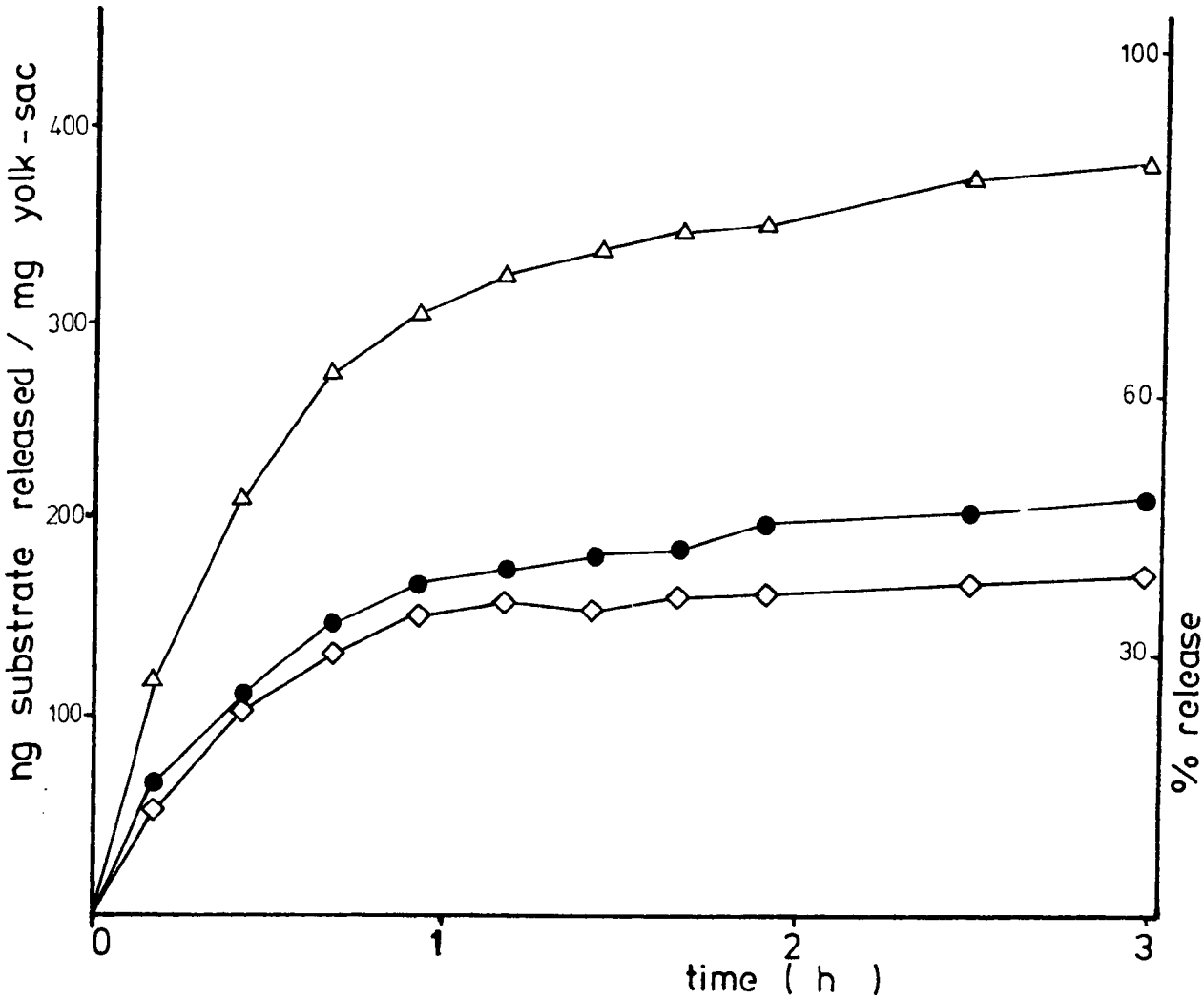


Figure 9.5 Time course of release of ^{125}I-labelled species from rat yolk-sacs on reincubating tissues previously exposed to ^{125}I-labelled rat IgG

17.5-day rat yolk-sacs were first incubated in serum-free medium 199 containing ^{125}I-labelled rat IgG (10 µg/ml). After 2h yolk-sacs were washed and reincubated in fresh medium 199, up to a further 3h (see Section 9.2.3 for details). Values shown represent means from 6 separate experiments. Figure 9.5 serves as control for Figures 9.6, 9.7 and 9.8 , where data is presented from rat yolk-sac incubations with leupeptin,as well as substrate, present.

Total radioactivity released —— △ ——

TCA-soluble radioactivity released — ● —

TCA-insoluble radioactivity released —— ◇ ——

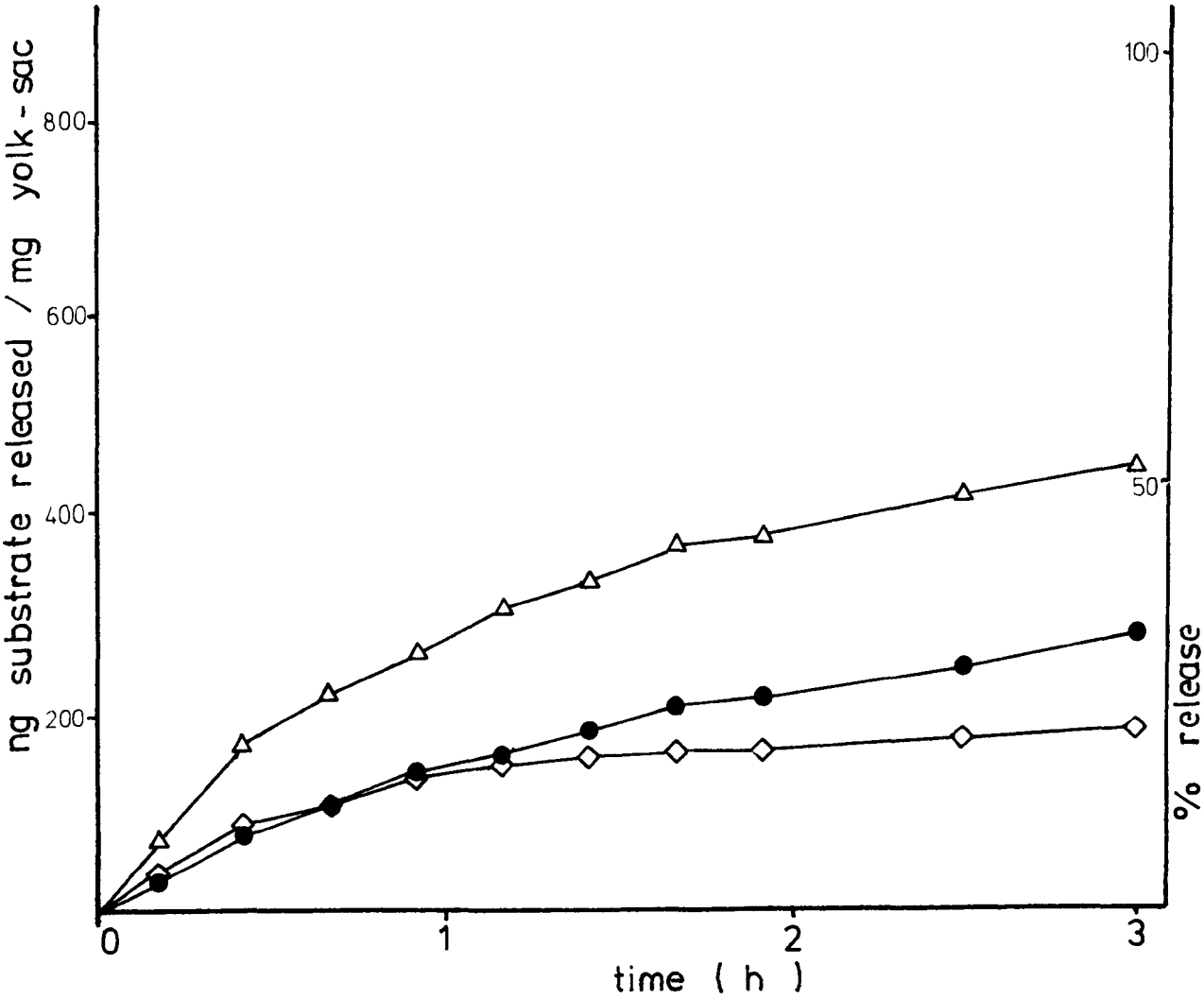


Figure 9.6 Time course of release of ^{125}I-labelled species from rat yolk-sacs on reincubating in leupeptin-containing medium 199 tissues previously exposed to ^{125}I-labelled rat IgG and leupeptin

17.5-day rat yolk-sacs were first incubated in serum-free medium 199 containing both ^{125}I-labelled rat IgG (10 µg/ml) and leupeptin (30 µg/ml). After 2h yolk-sacs were washed and reincubated in fresh medium 199 containing leupeptin (30 µg/ml), up to a further 3h (see Section 9.2.3 for details). Values shown represent means from 6 separate experiments.

Total radioactivity released ——△——

TCA-soluble radioactivity released — ● —

TCA-insoluble radioactivity released —— ◇ ——

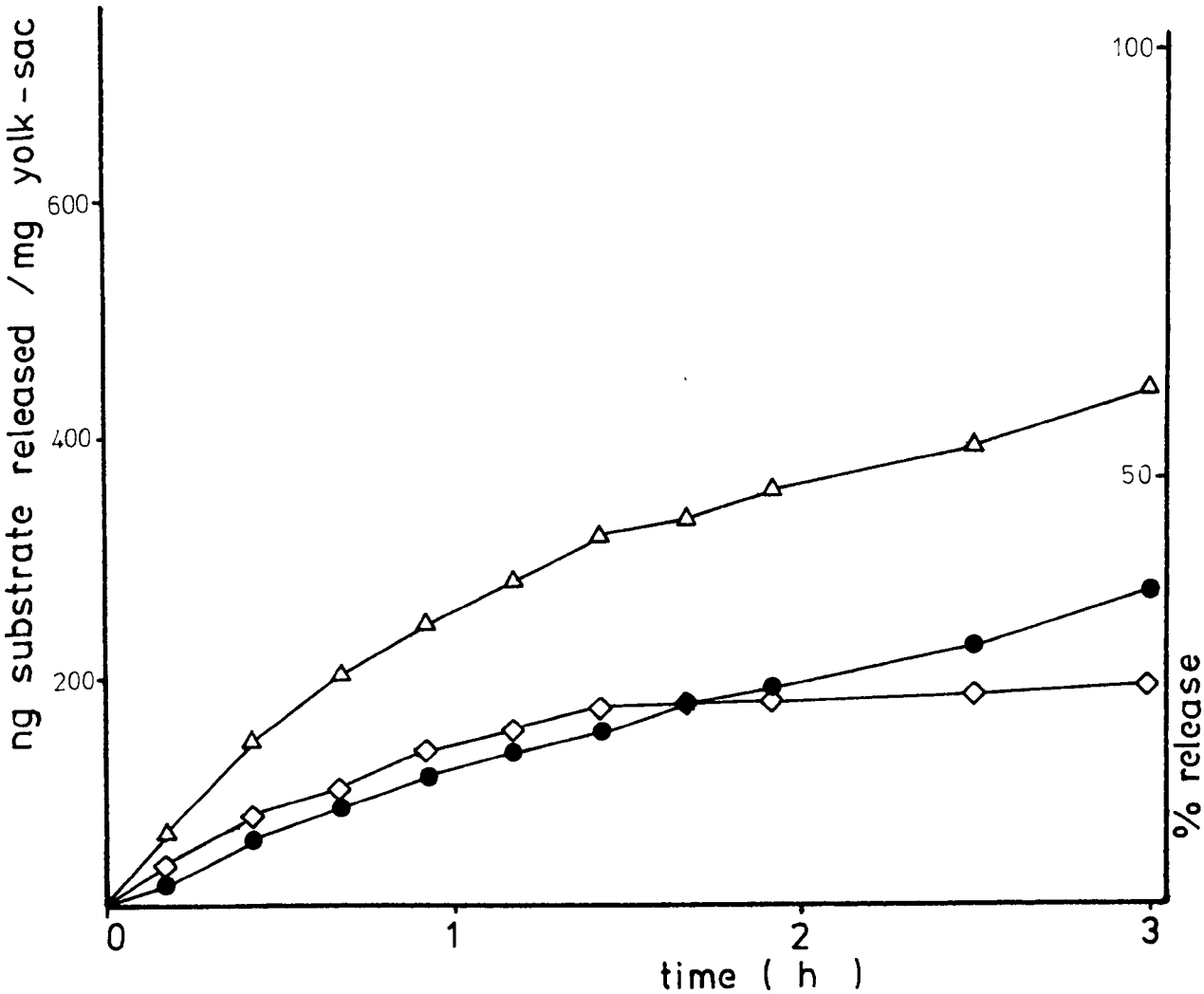


Figure 9.7 Time course of release of ^{125}I-labelled species on reincubating tissues previously exposed to ^{125}I-labelled rat IgG in the presence of leupeptin

17.5-day rat yolk-sacs were first incubated in serum-free medium 199 containing both ^{125}I-labelled rat IgG (10 µg/ml) and leupeptin (30 µg/ml). After 2h yolk-sacs were washed and reincubated in fresh medium 199, up to a further 3h (see Section 9.2.3 for details). Values shown represent means from 6 separate experiments.

Total radioactivity released —— △ ——

TCA-soluble radioactivity released — ● ——

TCA-insoluble radioactivity released — ◇ ——

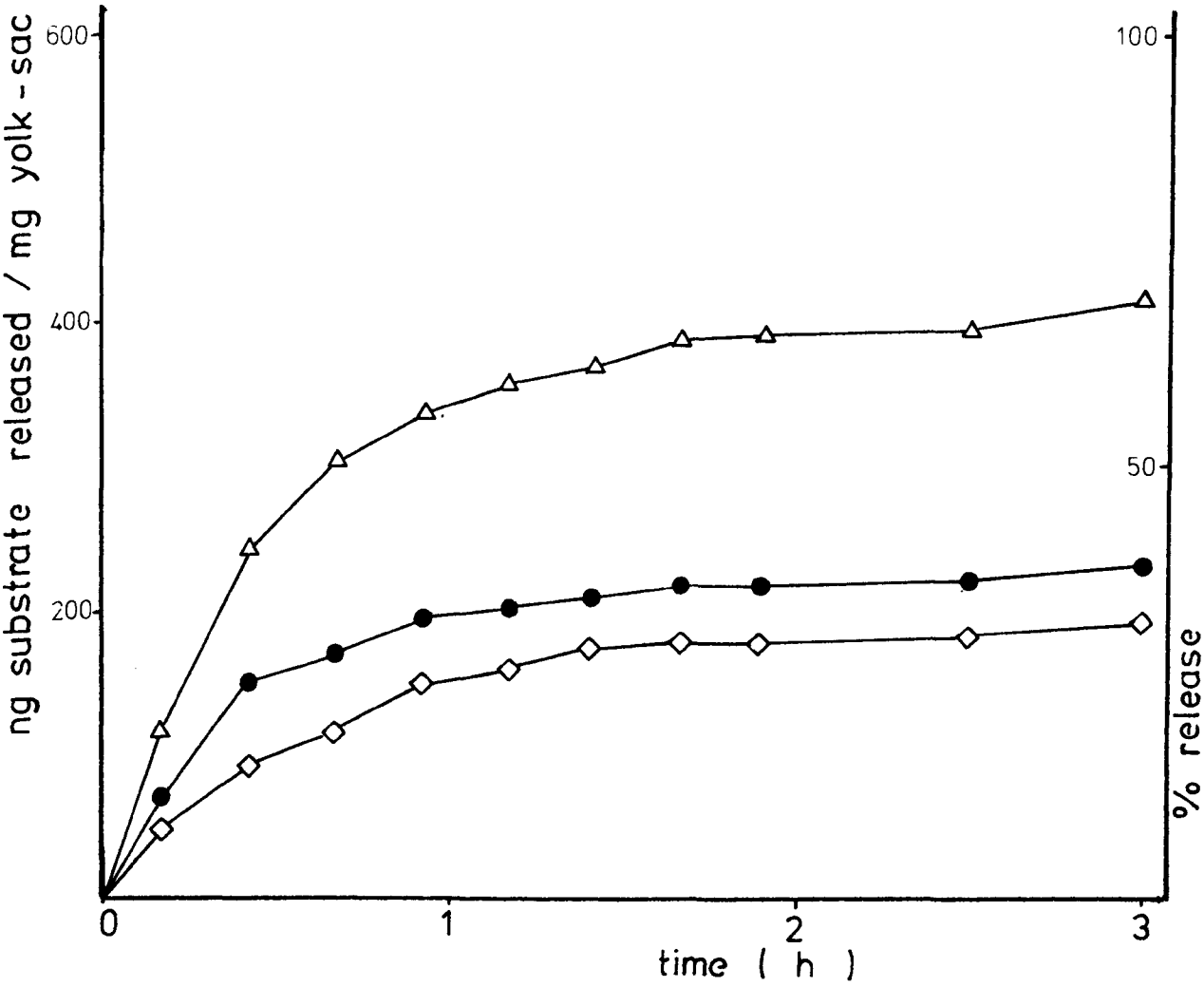


Figure 9.8 Time course of release of ^{125}I-labelled species from rat yolk-sacs on reincubating in leupeptin-containing medium 199 tissues previously exposed to ^{125}I-labelled rat IgG

17.5-day rat yolk-sacs were first incubated in serum-free medium 199 containing ^{125}I-labelled rat IgG (10 µg/ml). After 2h yolk-sacs were washed and reincubated in fresh medium 199 containing leupeptin (30 µg/ml), up to a further 3h (see Section 9.2.3 for details). Values shown represent means from 6 separate experiments.

Total radioactivity released —— △ ——

TCA-soluble radioactivity released —— ● ——

TCA-insoluble radioactivity released —— ◇ ——

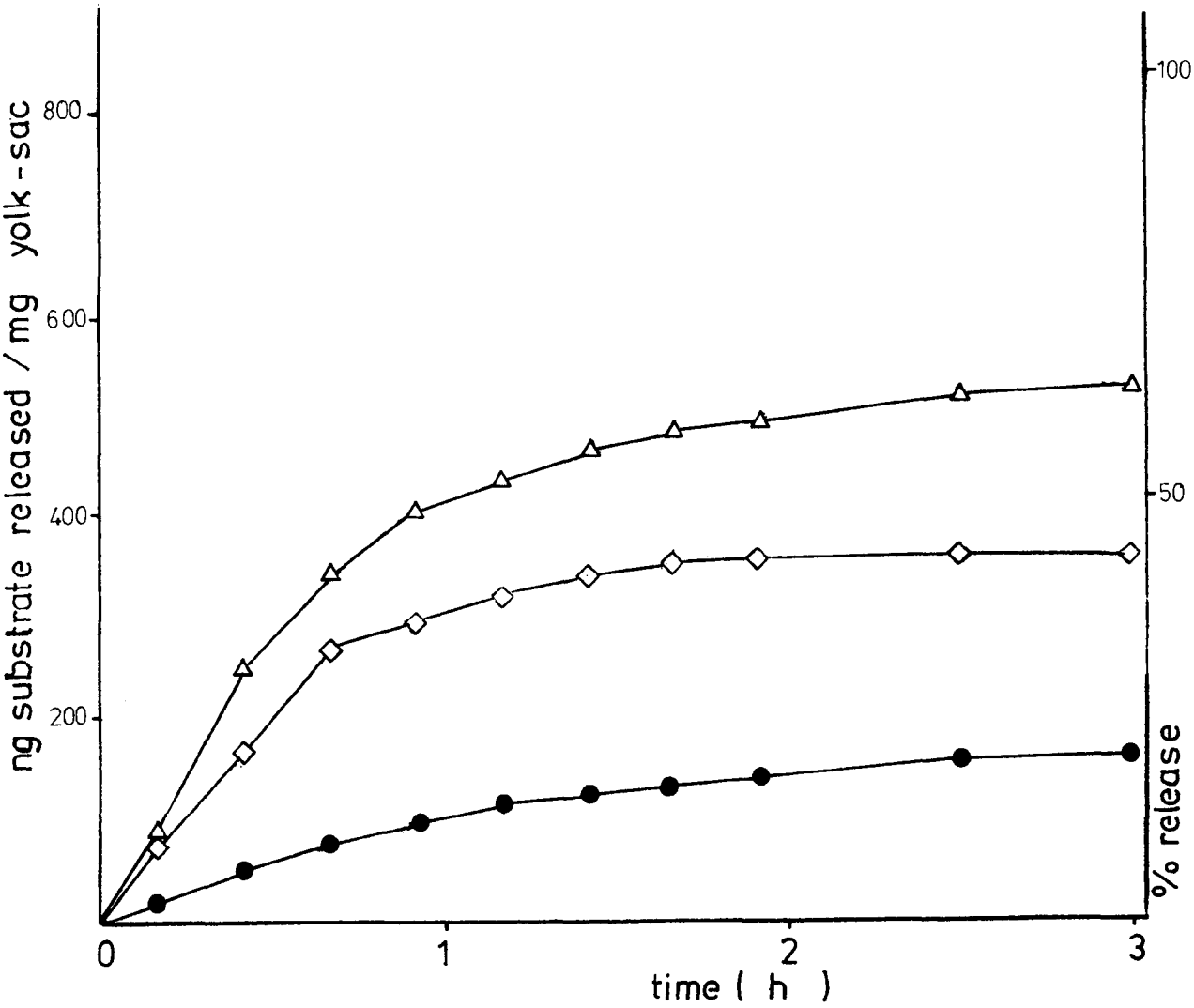


Figure 9.9 Time course of release of ^{125}I- labelled species from rabbit yolk-sac on reincubating tissues previously exposed to ^{125}I-labelled rabbit IgG

24-day rabbit yolk-sac pieces were first incubated in serum-free medium 199 containing ^{125}I-labelled rabbit IgG (10 µg/ml). After 2h yolk-sacs were washed and reincubated in fresh medium 199, up to a further 3h (see Section 9.2.3 for details). Values shown represent means from 3 separate experiments. Figure 9.9 serves as control for Figures 9.10, 9.11 and 9.12, where data is presented from rabbit yolk-sac incubations with leupeptin, as well as substrate, present.

Total radioactivity released —— Δ ——

TCA-soluble radioactivity released —— ● ——

TCA-insoluble radioactivity released —— ◇ ——

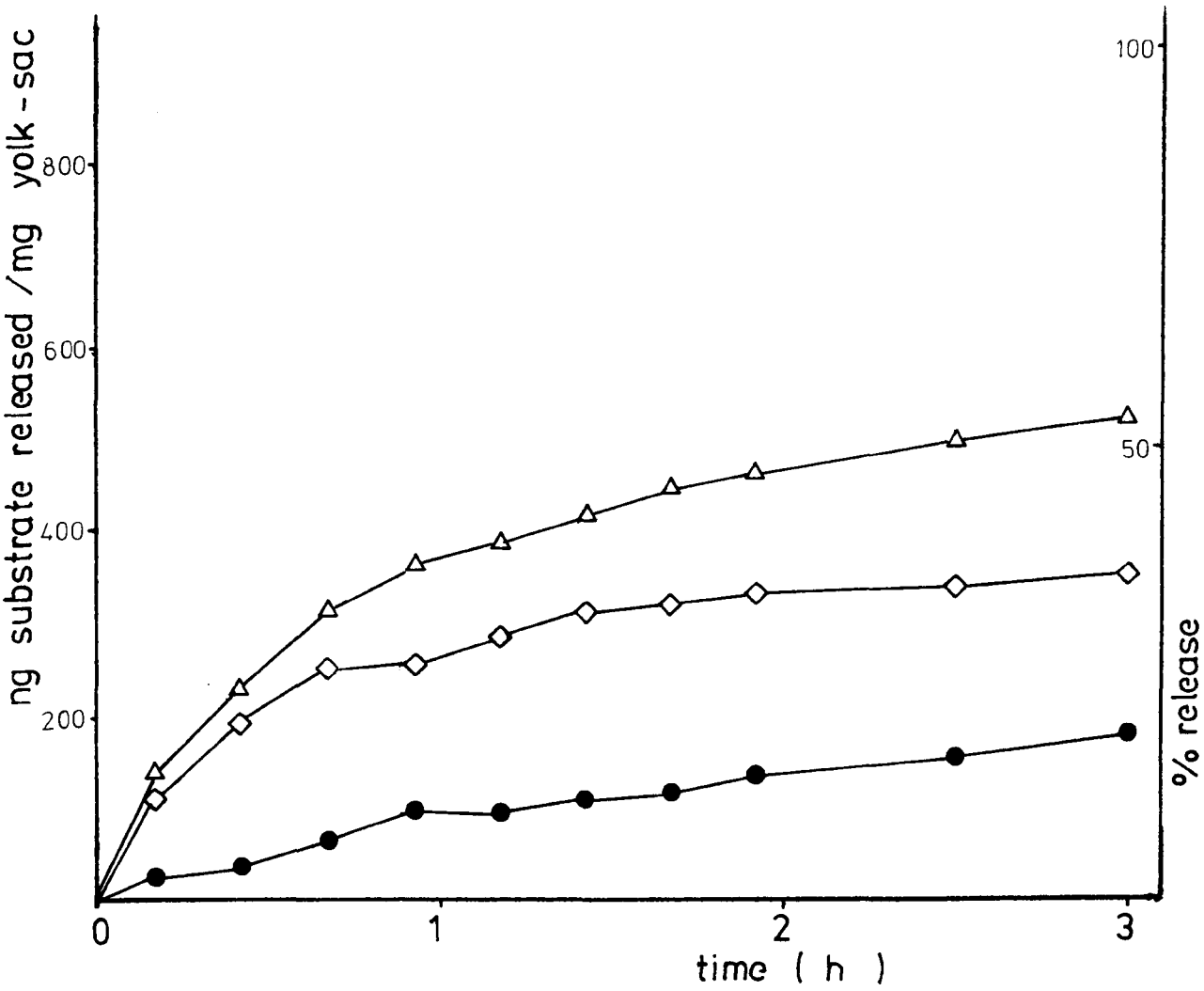


Figure 9.10 Time course of release of ^{125}I-labelled species from rabbit yolk-sacs on reincubating in leupeptin-containing medium 199 tissues previously exposed to ^{125}I-labelled rabbit IgG and leupeptin

24-day rabbit yolk-sac pieces were first incubated in serum-free medium 199 containing both ^{125}I-labelled rabbit IgG (10 µg/ml) and leupeptin (30 µg/ml). After 2h yolk-sacs were washed and reincubated in fresh medium 199 containing leupeptin (30 µg/ml), up to a further 3h (see Section 9.2.3 for details). Values shown represent means from 3 separate experiments.

Total radioactivity released ——△——

TCA-soluble radioactivity released —— ● ——

TCA-insoluble radioactivity released —— ◇ ——

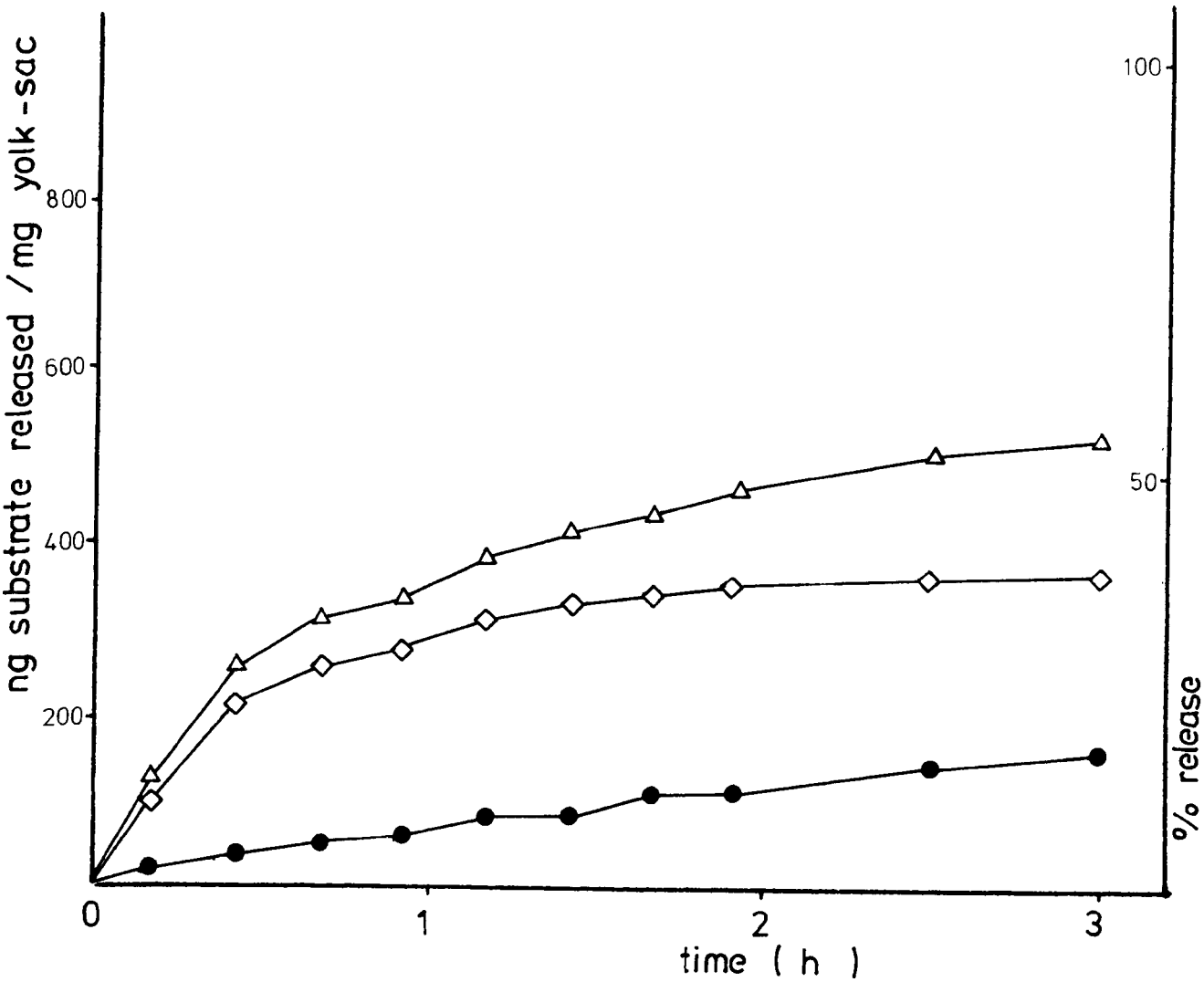


Figure 9.11 Time course of release of ^{125}I-labelled species on reincubating tissues previously exposed to ^{125}I-labelled rabbit IgG in the presence of leupeptin

24-day rabbit yolk-sac pieces were first incubated in serum-free medium 199 containing both ^{125}I-labelled rabbit IgG (10 µg/ml) and leupeptin (30 µg/ml). After 2h yolk-sacs were washed and reincubated in fresh medium 199, up to a further 3h (see Section 9.2.3 for details). Values shown represent means from 3 separate experiments.

Total radioacitvity released —Δ—

TCA-soluble radioactivity released — ● —

TCA-insoluble radioactivity released — ◇ —

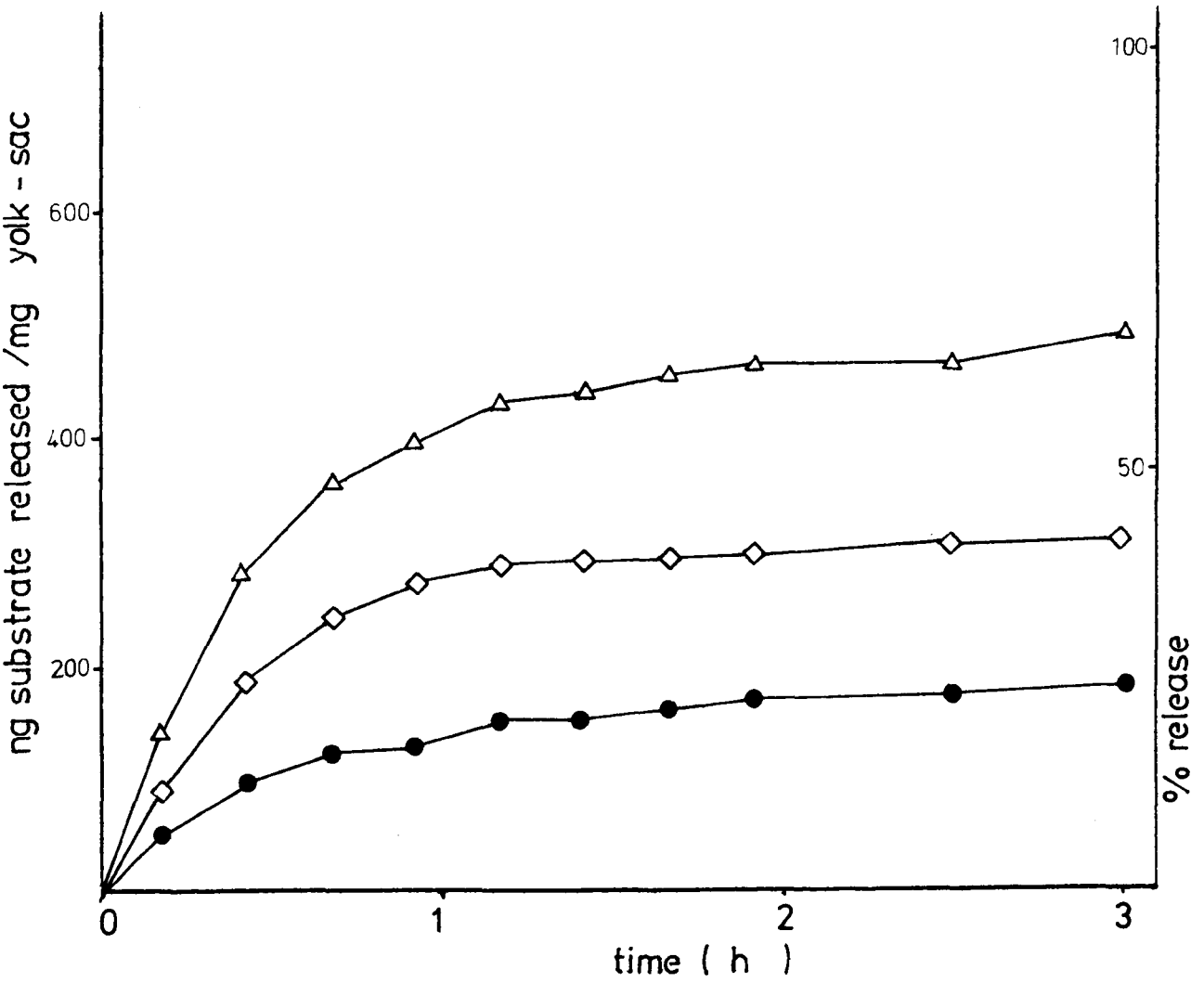


Figure 9.12 Time course of release of ^{125}I-labelled species from rabbit yolk-sacs on reincubating in leupeptin-containing medium 199 tissues previously exposed to ^{125}I-labelled rabbit IgG.

24-day rabbit yolk-sac pieces were first incubated in serum-free medium 199 containing ^{125}I-labelled rabbit IgG (10 µg/ml). After 2h yolk-sacs were washed and reincubated in fresh medium 199 containing leupeptin (30 µg/ml), up to a further 3h (see Section 9.2.3 for details). Values shown represent means from 3 separate experiments.

Total radioactivity released — △ —
TCA-soluble radioactivity released — ● —
TCA-insoluble radioactivity released — ◇ —

9.4 DISCUSSION

One of the main differences between the Brambell and the Wild theories for selective transport of IgG across rabbit foetal membranes lies in the mechanism of release of the intact IgG from the tissue. In the Brambell mechanism, heterolysosomes exocytose their contents at the lateral and basal plasmalemma. In the Wild mechanism, only micropinocytic coated vesicles release IgG at these sites by exocytosis; heterolysosomes do not fuse with the plasma membrane. (In support of the latter point, Moxon et al., 1976 in an ultrastructural study on rabbit yolk sacs, never observed macropinocytic vesicles near the basal and lateral plasma membrane.) The assumption in the Brambell theory, that heterolysosomes can take part in exocytosis, provides a means of testing this theory experimentally in an in vitro system in which living cells undergo pinocytosis and exocytosis. Not only should IgG be exocytosed, but also other macromolecular materials that have been captured by pinocytosis but which are resistant to hydrolysis by lysosomal enzymes. It should therefore be easy to find experimental support for this theory if it is valid. From Figures 9.1 and 9.2, however, it is apparent that only a very low release of the non-digestible marker ^{125}I-labelled PVP occurs on reincubating rat and rabbit yolk sacs loaded with this substrate. As the release of marker from yolk sacs is reported in absolute terms (ng/mg tissue), it is possible to compare the amounts of ^{125}I-labelled PVP released with the amounts of ^{125}I-labelled homologous IgG released from the same tissues (see Table 5.7), where the loading concentration of the substrate was the same. Not only is the total release of homologous ^{125}I-labelled IgG from rat and rabbit yolk sacs considerably higher than ^{125}I-PVP release, but with labelled IgG the TCA-insoluble activity released exceeds ^{125}I-PVP release 22-fold for the rat and 116-fold for the rabbit. In other words,

more homologous IgG seems to be transported across the rat and rabbit yolk sacs intact compared with the amount of ^{125}I-labelled PVP being released from the tissue. The implications of this observation are that heterolysosomes do not exocytose their contents into the intercellular spaces or, if exocytosis does occur, it is not sufficient to account for the great amount of IgG getting released intact from the rat and rabbit yolk sacs *in vitro*.

The second strand of the investigation pursued in this chapter was to examine another of Brambell's assumptions: namely that within rabbit yolk-sac heterolysosomes the IgG that is free in the lumen becomes degraded by lysosomal enzymes and so is no longer intact when exocytic release of the contents occurs. An attempt was made to increase the amount of unbound but intact IgG within heterlysosomes by the use of leupeptin, an inhibitor of lysosomal enzymes. If the Brambell theory is correct, the amount of IgG released intact from heterolysosomes containing this inhibitor should rise sharply. Table 9.1 and 9.2 show the action of leupeptin on uptake of homologous ^{125}I-labelled IgG by rat and rabbit yolk sacs. In agreement with the findings of Knowles *et al.* (1981) who used formaldehyde-treated BSA as a substrate in the rat yolk sac, the Endocytic Indices of ^{125}I-labelled homologous IgG was not changed in either the rat or the rabbit yolk sac when leupeptin was present in the incubation medium. The Tissue-Accumulation Rates, on the other hand, increased in both tissues, especially in the rat. Hopgood *et al.* (1977) showed that in isolated rat hepatocytes leupeptin inhibited endogeneous protein breakdown; Knowles & Ballard (1976) similarly demonstrated leupeptin to be an effective inhibitor of protein breakdown in Reuber H35 hepatoma cells. Also Knowles *et al.* (1981) by incubating rat yolk sacs in medium containing ^{125}I-labelled formaldehyde-treated albumin and leupeptin found digestion of the substrate very much

impaired, as expressed by a very high accumulation of the albumin in the tissue. The high Tissue-Accumulation Rates for homologous ^{125}I-labelled IgG in the presence of leupeptin can therefore be explained in terms of intralysosomal accumulation of undegraded or poorly degraded material in the rat and rabbit yolk sacs. It was important to establish such tissue-accumulation of ^{125}I-labelled IgG in the rat and rabbit yolk sacs and to be sure that the concentration of leupeptin used was effective, before going on to reincubation studies. By making use of leupeptin in reincubation studies, the Brambell versus Wild argument could be investigated more thoroughly.

The greatest effect of leupeptin on intralysosomal degradation is expected when the inhibitor is present during loading as well as during the reincubation phases. Looking at incubation of rat and rabbit yolk sacs under different conditions (Figures 9.3 and 9.4), it is indeed apparent that the inhibition of degradation is strongest when leupeptin is present both during uptake and during reincubation. In both tissues the total percentage release of ^{125}I-labelled IgG is lower than for the controls and for the other incubation conditions. In this context it is appropriate to comment on one apparently unusual finding, in the rat yolk sac. In this tissue, leupeptin seems to exert an effect, even when present during only the reincubation phase, not only on the tissue retention of substrate, but also on the total tissue-accumulation level, by increasing both. While the first effect is not surprizing (leupeptin should decrease the breakdown of substrate during the reincubation phase and hence lead to an accumulation in the tissue), the second finding is more unexpected. But, as the tissue level is determined during the uptake phase, it is obvious that this elevation cannot be due to any action of leupeptin; instead, it must be accounted for by a general variability in uptake and total tissue-accumulation of substrate by yolk sacs. This suggestion finds support in the data reported in Figures 2.5, 3.2, 4.1,

4.6 and 5.7 for other incubations of rat yolk sacs with ^{125}I-labelled rat IgG followed by release in fresh medium. In these experiments, a variability in the amounts of total tissue-accumulation is also apparent (409 ng - 655 ng/mg tissue).

Returning to the main findings, when degradation of ^{125}I-labelled IgG is inhibited this substrate accumulates in the rat and rabbit yolk sacs. But, according to the hypothesis for selectivity of IgG transport forwarded by Brambell, undegraded IgG would be expected to be released in the undegraded form, rather than be accumulated. However, looking at the release of TCA-insolubles (Figures 9.3 and 9.4), no such elevated release can be observed in either the rat or the rabbit yolk-sac tissues, thus giving little evidence to support the Brambell hypothesis. The high retention of substrate in the tissue is more compatible with the suggestion that non-degraded IgG, being of too high a molecular weight to diffuse across the heterolysosomal membrane, is not released from the tissue by exocytosis but rather stays within those vesicles. This behaviour would in fact be expected if the Wild hypothesis were correct. Unless leupeptin interferes with the formation of coated vesicles, TCA-insoluble release should remain unaffected by the addition of leupeptin to the incubation medium during loading, reincubation or both, because protective IgG transport is supposed to take place in only coated vesicles, hence there is no necessity for heterolysosomes to engage in exocytosis.

Looking at the figures depicting the detailed time-course of release Figs 9.5-9.12, it is very interesting to note that the release of TCA-solubles is very much affected by the presence of leupeptin in the various stages of incubation. When the inhibitor is present during both the loading and the reincubation periods in both tissues, release of TCA-solubles (i.e. degraded ^{125}I-labelled IgG) is very low, but continues to rise almost linearly with reincubation time. When leupeptin was present

during only the 'loading" phase, the pattern of release of TCA-solubles was very similar, but perhaps with a slightly more pronounced rise of release towards the end of the reincubation period, suggesting that the action of leupeptin on lysosomal enzymes in the rat and rabbit yolk sacs may be a short-term effect. When leupeptin was present in the reincubation period only, it had no detectable effect on the release of TCA-insoluble activity relative to controls. As in control incubations, TCA-soluble release reached almost its final level by 70 min. Such results indicate that leupeptin needs to be endocytosed continuously by the tissue to be effective as lysosomal enzyme inhibitor. Exposure of the tissue to leupeptin after it had accumulated ^{125}I-labelled IgG had little effect, because leupeptin being taken up by the tissue endocytically needed some time to reach a sufficiently high concentration in the tissue to be effective in inhibiting enzyme action.

The most important finding, however, related to the release of TCA-insolubles. Both the total amounts and the time-courses of TCA-insolubles release remained virtually unaffected by the presence of leupeptin at any stage of incubation of rat and rabbit yolk sacs. This observation is most convincingly explained in terms of a specialized IgG transport system independent of the lysosomal system and therefore unaffected by leupeptin. Coated micropinocytic vesicles as suggested by Moxon *et al*. (1976) fit the criteria for such a special transport system.

In summary, the findings in this chapter are:

1) As in the rat yolk sac, the non-digestible macromolecular marker ^{125}I-labelled PVP is not released when rabbit yolk sacs are reincubated after loading with this substrate *in vitro*.

2) Leupeptin decreases the rate of intralysosomal degradation of homologous ^{125}I-labelled IgG while leaving endocytosis of these

proteins unaffected.

3) The presence of leupeptin in the incubation medium either during loading or reincubation or both did not cause the rate of release of intact IgG (TCA-insoluble material) to increase.

The main implications of the findings reported in this chapter are that they lend more support to the Wild than to the Brambell theory, having undermined two of the main assumptions of the latter.

GENERAL DISCUSSION

GENERAL DISCUSSION

The principal aim of the work reported in this thesis has been to investigate the controversy surrounding the mechanism of transfer of IgG across the rabbit (and rat) yolk sac by employing a different experimental technique. It was hoped that a novel approach, in which it was possible to study the isolated tissue in vitro, would give a new insight into the dynamics of transcellular transfer of IgG.

The first and vital step was to determine whether the method developed for incubating rat yolk sacs in vitro was also adaptable for incubating rabbit yolk sacs, this tissue having been established as the principal site of IgG transfer in the rabbit. The preliminary experiments reported in Chapter 2 indicate that in vitro the rabbit yolk sac, like the rat yolk sac, is an endocytically active tissue. In fact, both rat and rabbit yolk sacs show very similar endocytic properties with respect to most substrates investigated; also the catabolic capacities towards homologous and heterologous species of IgG were similar. However, in the rabbit yolk sac a slightly higher catabolic capacity was demonstrated.

At this point it is pertinent to comment on the various IgG preparations used in this study. The homologous and heterologous IgG fractions used were commercially purchased and not further purified (for sources see General Materials and Methods Section, Chapter 1). The use of highly purified IgG fractions or, even better, monoclonal antibody subclasses would have been an advantage in this study, as any specific differences in endocytic or transport properties of homologous and heterologous species of IgG could thus be more readily explained in terms of structural features of the molecules. As it is, possible impurities in the preparations may be partly responsible for some of the observed effects. The use of crude IgG preparations can,

however, be justified on as least two grounds. First, a less refined commercial preparation of homologous and heterologous IgG will contain a more respresentative cross section of gamma globulins so will more closely resemble the population of molecules that come into contact with the yolk sac *in vivo* than will some arbitrarily chosen, purified IgG fraction the behaviour of which may not be representative of the other IgG molecules. The second and more obvious reason for using the commercial IgG preparation is a financial one. Highly purified preparations of IgG are much more expensive (whether purchased or prepared). In view of the rather large quantities of IgG used in "Interference" studies (Chapter 6) and "Loading concentration" studies (Chapter 8), the use of highly purified IgG could not be justified in an initial round of investigations. Since the findings of these initial experiments were quite interesting, it does suggest that certain key experiments should be repeated with purer fractions of IgG to check that the trends established in the work reported in this thesis hold good.

As *in vitro* techniques can often be criticized on the grounds that they do not truly represent *in vivo* conditions, the next chapters were devoted to establishing that the behaviour of rat and rabbit yolk sacs *in vitro* was compatible with that of the same tissues *in vivo*. Thus it was shown (Chapter 4) that substrates (especially homologous IgG) were indeed internalized endocytically by the tissues. This was a very important point to establish, as the yolk-sac reincubation technique used relied on the internalisation of the substrate to yield interpretable results.

In vivo the rabbit yolk sac and also the rat yolk sac have been shown to be very selective in the transfer of homologous and heterologous IgG species (Brambell, 1970). This same selectivity has been shown (Chapter 5) to be a characteristic of the tissues *in vitro*, with the

rank orders being extremely similar. Such similarities could possibly be fortuitous, but are compatible with the *in vitro* behaviour of the tissue reflecting its *in vivo* function. After these preliminary investigations, the further work dealt more directly with the question of the cellular mechanism of IgG transfer across the rat and rabbit yolk sac. It may be remembered that in the General Introduction a table was compiled giving the assumptions and predicted behaviour of the yolk sac according to the Brambell, Wild, Hemmings theories; this table is shown again overleaf. These predictions will now be discussed in detail in the light of both the findings presented in this work and the published literature.

The first point concerns the nature of the mechanisms proposed for protein selection in the process of IgG transfer. On this issue Brambell and Wild both propose the presence of specific receptors to which IgG binds selectively. In this respect these authors are similar and differ from Hemmings who is less specific about the mechanism of selection, but, when pressed, envisages the site of selection to be entirely different, namely the basal membrane which is thought to operate some vague diffusion-based selection process in favour of some IgG species. But diffusion across plasma membranes has to date not been demonstrated for any molecules in the molecular weight region of IgG or even anywhere near it, as pointed out before by Lloyd (1976) in response to the first proposal of the Hemmings theory. The presence of receptors, specific for homologous and some heterologous IgG species, on the other hand, has not only been proposed, but has also been experimentally demonstrated (Wild & Dawson, 1977; Hillman *et al.*, 1977; Sonoda & Schlamowitz, 1972; Tsay & Schlamowitz, 1975; Schlamowitz *et al.*, 1975). This puts the greater weight of experimental evidence behind the Brambell and Wild theories. In Chapter 6 the presence of specific protective IgG receptors has also been demonstrated in the

Comparison of the main features of the different mechanisms proposed for the selective transfer of IgG

	Feature of the process considered	Predicted behaviour or feature in the model according to: Brambell	Wild	Hemmings & Williams
1.	Mechanism responsible for selection of transferred IgG	Selective binding to membrane receptors	Selective binding to membrane receptors	Selective diffusion at basal membrane
2.	Location of the specific receptors involved in IgG transmission	Inner face of pinosome or heterolysosome	Outer surface of plasma-membrane	No receptors postulated
3.	Can the protective IgG route be saturated?	Yes	Yes	No
4.	Location of IgG in the cell	Pinosome or heterolysosome	Pinosome, heterolysosome or coated vesicle	Pinosome, heterolysosome or cytosol
5.	Location of protected IgG in the cell	Pinosome or heterolysosome	Coated vesicle	Cytosol
6.	Location of protein destined for degradation	Pinosome or heterolysosome	Pinosome or heterolysosome	Pinosome or heterolysosome
7.	Minimum number of vesicle-types proposed	1	2	1
8.	Presence of lysosomal enzymes in transport vesicles is suggested?	Yes	No	Yes
9.	Uptake of protein not destined for transport is by the following mechanism	Fluid-phase pinocytosis	Fluid-phase or adsorptive pinocytosis	Fluid-phase or adsorptive pinocytosis
10.	Release of IgG from the cell is by the following mechanism	Exocytosis of pinosomes/ heterolysosomes at basal/ lateral membrane	Exocytosis of coated vesicles at basal/ lateral membrane	Differential diffusion at basal/lateral Membrane
11.	Is release of lysosomal enzymes or a non-degradable marker possible from transplant vesicles?	Yes	No	No

dynamic yolk-sac system (rat and rabbit) by interference studies. The degree of specificity displayed by those receptors closely parallelled the rank orders for IgG transport *in vivo* in these tissues.

The second point concerns the location of these protective receptors on the yolk sac. In the Wild mechanism, active receptors have to be located on the outer face of the plasma membrane, whereas for Brambell it is important that receptors are functional on the inner face of the limiting membrane of the endocytic vesicle. Considered in isolation, the interference experiments conducted in Chapters 6 may not give conclusive and satisfying evidence for the location of the receptors on the outer plasma membrane. It is just possible to envisage interference taking place subsequent to internalisation rather than during or before the invagination process. However, when considered together with the results of specific binding of IgG to non-living membrane preparations, that show the rank order of binding to be the same as those from experiments with the living tissue, it is possible to postulate that the receptors are localized on the outer plasma membrane. This in itself does not invalidate the Brambell theory, because it can be envisaged that IgG receptors are distributed over the entire outer yolk-sac plasma-membrane facing the uterus. Binding of IgG occurs at these sites followed by vesicle formation, and protection of the homologous IgG against proteolytic enzymes is ensured as postulated. However, to have demonstrated specific receptors on the outer yolk-sac cell membrane clearly goes against the findings of Hemmings and Williams (1976) but, by itself, is insufficient evidence to disprove the Hemmings' theory. If selection does happen at the cell surface, it is hard to imagine why an additional mechanism of selection should be necessary at the basal membrane. In addition, the third point in the table picks out another characteristic that distinguishes the Wild and Brambell theories from that of Hemmings, namely the

saturability of the protective route. A receptor-mediated transport process must of necessity be saturable if only a limited number of receptors is present. A process, on the other hand, without receptors and relying on diffusion or a similar mechanism should not be limited in such a way. In Chapter 8 rather interesting findings are presented which show that in both rat and rabbit yolk sacs the protective route can be saturated, albeit over different concentration ranges. In keeping with the proposal that the rabbit yolk sac is specialized for the transport of IgG, the transport capacity of the rabbit yolk sac is greater than that of the rat yolk sac. Thus, the experimental findings are again more compatible with the Brambell and Wild theories, rather than the Hemmings' theory.

The next three points (4,5 and 6) will be considered together, as they are related. All 3 theories agree that pinosome/heterolysosome are one possible location for internalized IgG, especially that IgG destined for degradation. But, whereas for Brambell this is the only intracellular location, Wild proposed coated micropinocytic vesicles as an additional site whereas Hemmings' mechanism predicts that free IgG will also be found in the cytosol. In support of Wild's model, Moxon, Wild & Slade (1976) showed the presence of HRP-conjugated human, rabbit and bovine IgG in coated micropinocytic vesicles; rabbit anti-HRP antibodies, free HRP and human IgG also became localized within coated vesicles. All these proteins plus bovine anti-HRP antibodies, IgG-ferritin conjugates and free ferritin were also found in smooth macropinocytic vesicles and dense bodies. (A possible argument against these findings is that some markers like HRP have a very "fuzzy" appearance under the electron microscope. This may well cause difficulties in deciding whether a vesicle is coated or not, but Rodewald (1973) in the suckling rat gut also produces evidence for the

presence of ferritin-conjugated IgG within coated vesicles.)

Hemmings & Williams (1976) found, in a study of IgG transport across the rabbit yolk-sac, that a lot of ferritin and ^{125}I-labelled bovine and human IgG were located in the cytoplasm together with the presence of broken pinocytic vesicles, after yolk sacs had been exposed to these markers in vitro. These findings, together with those from a previous investigation, suggested that no selective binding occurred on the membrane between bovine and rabbit IgG. These findings caused Hemmings and Williams (1976) to formulate their alternative theory. When the theory was put forward at the 1976 Brambell Symposium (see Hemmings, 1976) in the discussion following the paper Slade suggested that free substrate in the cytoplasm together with the presence of broken vesicles may well occur as an artefact of fixation. The occurrence of broken vesicles has been more universally supported by observations (Wild, 1970; Padykula et al., 1966). These workers, however, could not discern any purpose for their presence in IgG transfer and it could be argued that broken vesicles too, may simply be artefacts.

The seventh point, concerning the minimal number of distinct vesicles postulated by the respective theories, can be passed over quickly. The presence, in yolk-sac cells, of a variety of distinct vesicles (primary lysosomes, heterolysosomes, coated vesicles, pinosomes, autosomes, etc.) has been demonstrated by electron microscopy studies. Whether any of these vesicles are specialized to perform the function of exclusively transporting homologous and some heterologous IgG species to the foetal circulation is an open question.

At this point is may be concluded that the overall evidence accumulated renders the Hemmings' theory highly unlikely. Therefore the further discussion will concentrate on the remaining two theories.

Turning to point 8 in the table, it can be seen that the presence of lysosomal enzymes in transport vesicles is suggested by the Brambell theory but not by the Wild theory. This is an important point, since a consequence of this factor in the Brambell mechanism is that the exocytosis of lysosomal enzymes together with the transferred IgG at the basal or lateral plasma membrane is predicted. Now, the exocytosis of lysosomal enzymes has indeed been frequently observed from cultured cells. Ibbotson (Ph.D. thesis) tried to demonstrate the same for rat yolk sacs in vitro. He incubated 17.5-day rat yolk sacs in the presence of ^{125}I-labelled rat IgG, washed yolk sacs after 2h and reincubated them in fresh medium (same method as outlined in Section 1.12). The medium was then assayed for intact and degraded ^{125}I-labelled IgG and also for β-NAG, a lysosomal enzyme. Plenty of intact ^{125}I-labelled rat IgG was found in the medium, but little or no β-NAG, leading to the claim that the presence of lysosomal enzymes in the transport vesicles is highly unlikely. Also Wild (1975) could not demonstrate the presence of cathepsin D outside rabbit yolk-sac endodermal cells, which should release this enzyme if secondary lysosomes are undergoing exocytic discharge. The presence of IgG in vesicles containing lysosomal enzymes has not yet been shown. However, to demonstrate IgG in heterolysosomes would still be compatible with Wild's hypothesis because he postulated that surplus IgG (i.e. not transported IgG) is treated in the same manner as any other protein and is degraded in heterolysosomes. If the presence of lysosomal enzymes and substrate in coated vesicles (as has been suggested by Friend & Farquhar, 1967 in the rat vas deferens) could be demonstrated, this would constitute evidence against the Wild hypothesis, but no such evidence has yet been forwarded.

Point 9 concerns the uptake of those proteins not destined for transport, i.e. the normal mode of protein uptake. For Brambell, the

mechanism of uptake of protected IgG and that of other proteins destined for degradation differ in that the former process, involves specific receptors; the remainder of protein ingested was proposed to be captured by fluid-phase pinocytosis. The distinction between those two types of uptake was made by Jacques (1969). Later it was experimentally shown that the rates of these two types of uptake process differed considerably, being higher when receptors were involved (see Pratten et al., 1980 for a review). Lloyd (1976) then argued that since the uptake of ^{125}I-labelled BSA into the rat yolk sac was by adsorptive pinocytosis (as shown by the very much higher Endocytic Indices than are accepted for fluid-phase uptake), the mere fact that a protein adsorbs to the plasma membrane prior to invagination cannot ensure its protection within the cell. In Chapter 6 of this thesis it was also shown that both homologous and heterologous IgG species enter the rat and rabbit yolk-sac cells by adsorptive endocytosis and the rates of uptake in the absence of calf serum are considerably higher than the sum of the rates of fluid-phase and "protective" uptake. Consequently, it was suggested that the uptake of homologous IgG and heterologous IgG destined for degradation was by adsorptive pinocytosis. From the additional findings that in the rabbit yolk sac ^{125}I-labelled rabbit and human IgG are released intact to almost equal degrees upon reincubation (Chapter 5), but have very different Endocytic Indices, it was deduced that the receptors or binding sites mediating the two protein routes must differ in their specific requirements for binding. Also connected with this point and a consequence of the observed differences between human and rabbit IgG is the following conclusion. Different parts of the IgG molecule must be responsible for binding to the two types of receptors/binding sites. The existence on the plasma membrane of two or more types of binding site seems perfectly compatible with the Wild theory, especially

if, as Anderson et al. (1977) proposed, the coated vesicle receptors all cluster in a specific region that invaginates to internalize any protein bound to this area. On the other hand, the unoccupied receptors may be distributed randomly over the plasma membrane in which case "capping" would have to occur before invagination.

Up to this point the experimental findings presented in this work and also in the literature are more compatible with the Wild theory than with the original Brambell theory. The latter theory could, however, be modified to fit the experimental evidence. For this purpose it would be necessary to propose that both the specific (transport) receptors and the non-specific (degradative) binding sites for homologous IgG are located on the outer face of the plasma membrane and that binding takes place there, instead of after internalization. If uptake of IgG into the degradative route occurs by adsorption to the non-specific binding sites of limited specificity, it is probable that the differential effect on degradation in secondary lysosomes could result from binding to protective IgG receptors being either firmer or causing a steric hindrance to the lysosomal enzymes. The binding of IgG to "degradative route binding sites", on the other hand,must be without impairment of the lysosomal enzymes.

In the following aspect of IgG transport considered (Point 10) a very clear distinction is made between the two theories considered. The point in question is the mechanism of release of transported IgG from the cell. According to Brambell, transport takes place in heterolysosomes, and the proposed release is by exocytosis from these vesicles. Wild, on the other hand envisages transport as occurring in coated vesicles and it is therefore only these vesicles that must exocytose. Indeed, Moxon et al. (1976) claims to have observed such exocytosis from coated vesicles i.e. coated invaginated areas on the basal and lateral membrane and coated vesicles in confluence with

the plasma membrane at these sites. Wild & Richardson (1979) showed by SRBC (Sheep red blood cell) binding that F_c receptors are located not only on the apical cell membrane but also at the basal cell face, an observation that they ascribed to receptors being returned to the plasma membrane by exocytosis before being possibly recycled.

The electron micrographs, although they seem convincing, suffer from the same shortcoming that was pointed out earlier, namely, that the behaviour of a dynamic system is being interpreted from a static snapshot. Expressed most simply, it is impossible to decide the direction of movement, i.e. whether endocytosis or exocytosis is being observed. Still, in such coated vesicles the presence of HRP-conjugated homologous and human IgG, rat anti-HRP antibody and human IgG are demonstrated very frequenctly, a finding predicted by Wild's theory.

The lack of experimental support for exocytosis from heterolysosomes was instrumental in triggering off research to find a possible alternative to the Brambell theory. Wild (1975, 1976) and Moxon *et al.* (1976) claim that heterolysosomes are not even to be found near the basal membranes. But not only is supporting evidence absent, it is also possible to test this particular aspect of the IgG transfer mechanism experimentally using the *in vitro* system, because of the predictions that can be made if it is valid. Namely, if heterolysosomes exocytose IgG, other molecules contained in the vesicle must be released as well. It has already been shown that lysosomal enzymes are not released (Wild, 1975; Ibbotson, 1979). Small degradation products are difficult to trace, but it is easy to demonstrate the release of a non-digestible macromolecule following its uptake by the tissue. Using ^{125}I-labelled PVP as a test macromolecule it was demonstrated (Williams & Ibbotson (1979) for the rat yolk sac, and in Chapter 9 of this work for the rabbit yolk sac) that no significant amount of this

marker (at least not comparable to IgG release) is released from the cells. Instead, the marker was shown to accumulate steadily in the tissue.

Another more subtle and even more convincing experiment makes use of the homologous IgG itself as the non-degradable substrate. In Chapter 8 it was shown that the homologous IgG species is the most resistant of the proteins to digestion by enzymes derived from rat and rabbit yolk sacs. This finding alone would give a boost to the Brambell theory by also providing (in addition to specific and protective membrane binding) a different protective mechanism for the homologous IgG. Considering this finding and the proposed exocytosis of heterolysosomes it would follow that more homologous IgG could be transferred through the cells intact if it was rendered more resistant to degradation. Furthermore, if the action of lysosomal enzymes could be inhibited by the addition of a very potent and specific agent (e.g. leupeptin), more IgG would be expected to be released from the cells intact according to Brambell's theory. In Chapter 9 data were collected from such experiments with rat and rabbit yolk sacs. The finding in all cases was that, although degradation of IgG was clearly inhibited, no increased release of homologus IgG could be demonstrated. These findings are, most emphatically not compatible with the Brambell theory and can be taken as strong evidence against this theory.

Considering the leupeptin experiments and the predictions of Wild's hypothesis, no increased release of homologous IgG is expected when the degradation of this substrate is inhibited, because the degradative and protective routes are entirely separate. The surplus IgG would merely accumulate in heterolysosomes when leupeptin is present. Such accumulation has in fact been demonstrated in reincubation studies, where a very great tissue retention of substrate was observed especially

when leupeptin was present at all times of incubation of rabbit yolk sacs (see Chapter 9, Figure 9.4).

To conclude this section on the assumptions and predictions put forward in the table it is quite clear that the experimental findings presented in this work support the Wild rather than the Brambell (or the Hemmings) theories. In the following section, therefore, a critical appraisal of the Wild theory will be attempted.

At first sight it appears like an easy way out of a dilemma to propose the presence of a different class of vesicle to carry IgG when difficulties seem to have arisen with an existing theory based on transport within heterolysosomes. However, coated micropinocytic vesicles have been demonstrated in a great number of cells and now constitute a distinct subclass of vesicle. The difficulties connected with the Brambell theory are so numerous and grave that apparently no modification of the original theory can satisfactorily account for the body of conflicting experimental evidence that can now be assembled. A possible reservation concerning the Wild theory comes from another quarter and is connected with the nature and properties of coated vesicles themselves. Evidence for the role of coated vesicles in the transfer of passive immunity comes from Wild and his group for the rabbit yolk sac (Moxon et al., 1976) and also from Rodewald (1973) for the rat gut. Also in the rat yolk sac coated vesicles could be documented (Lambson, 1966; Jollie & Triche, 1971). But coated vesicles have been more universally found, for example in the oocyte of mosquito aedes aegypti L. (Roth & Porter, 1964), toad spinal ganglia (Rosenbluth & Wissig, 1963), rat cerebellum (Palay, 1963), rat intestine (Palay, 1963; Cardell et al., 1967), pericardial cell of aphids (Bowers, 1964), erythroblasts of guinea pig bone marrow (Fawcett, 1964), rat vas deferens (Friend & Farquhar, 1967), rat ganglion nodosum neurons (Holtzman et al., 1967), nerve endings of guinea pig brain (Kanaseki &

Kadota, 1969), guinea pig yolk sac (King & Enders, 1970), endodermal chick yolk-sac cells (Lambson, 1966), guinea pig chorioallantoic placenta (King & Enders, 1971) frog neuromuscular junction (Heuser & Reese, 1973), rat eosinophilic leucocytes (Komiyama & Spicer, 1975), implanting mouse blastocyts (Prelmann, 1975), rat sinusoidal endothelial cells of bone marrow (de Bruyns et al., 1975), lactating rat mammary epithelial cells (Franke et al., 1976), human placenta (Ockleford, 1976, Ockleford et al., 1977) porcine brain, adrenal medulla, lymphoma cell line (Pearse, 1975, 1976), human cultured fibroblasts (Anderson et al., 1977; Goldstein et al., 1979), porcine brain, chicken oocytes (Woods et al., 1978) and arachnoid of optic nerve (de la Motte, 1979).

The functions that coated vesicles perform in the above systems are by no means uniform but fall into two major categories: 1) membrane recycling and 2) protein transport, endocytosis or secretion. Membrane recycling as a function has been reported for synapses after neurotransmitter release (Heuser & Reese, 1973), but Franke & Herth (1974) also gives some evidence for that role of coated vesicles in exponentially growing plant cells. Protein transport can follow uptake of exogenous materials, mainly proteins, by the cell into coated vesicles (e.g. uptake of yolk protein in the oocyte of mosquito aedes aegypti L. (Roth & Porter, 1964), uptake of ferritin by toad spinal ganglia after intraperitoneal injection of this marker (Rosenbluth & Wissig, 1963), uptake of gold in rat eosinophilic leucocytes (Komiyama & Spicer, 1975), ferritin uptake in rat yolk sacs (Lambson, 1966) ferritin and HRP uptake in the guinea pig chorioallantoic placenta (King & Enders, 1971). Such coated vesicles are thought to form at the outer cell membrane. Another type of coated vesicle apparently forms, not at the plasma membrane, but within the cell. Thus, Rodewald (1973) envisages a scheme in the formation of coated vesicles at the ends of tubular vesicles (see also Wild, 1979). Geuze & Kramer (1974) found that in stimulated

exocrine pancreas cells of rats some coated vesicles originated by pinching off from mature Golgi cisternae and condensing vacuoles and they propose that the possible function of coated membrane is the concentration of exportable protein within forming secretory granules. Friend & Farquhar (1967) also say that some coated vesicles originate from the Golgi region and they claim that such small vesicles show acid phosphatase activity so are primary or secondary lysosomes. The same has been proposed by Holtzman et al. (1967) and de Bruyns et al. (1975). It is obvious that, as far as IgG transport is concerned, these findings present a potential problem. Another series of observations consolidates this problem, namely that at least some coated vesicles apparently fuse with other vesicles and primary lysosomes (Roth & Porter 1964; King & Enders, 1970, 1971; Anderson et al., 1977; de Bruyns et al., 1975). To highlight the difficulty posed to the Wild theory it must be remembered that one of the aspects of this theory was that the vesicle coat has the function of preventing fusion of the vesicle with lysosomes (Wild, 1975). If such fusion is generally reported for coated vesicles, either the Wild hypothesis has to be reconsidered or alternatively the claimed fusion of coated vesicles with other vesicles examined more closely. It is apparent that, in all cells investigated to date, preceding fusion coated vesicles shed their coats (Roth & Porter, 1964; King & Enders, 1970; de Bruyns et al., 1975). This could support the point made by Wild (1976) that the vesicle coat has a protective function and prevents fusion with other vesicles. But an interesting question raised at the same time concerns the mechanism of the coat shedding. Formulated simply, what determines whether the vesicle coat is retained or shed? An answer to this question is urgently needed.

A second argument in favour of the Wild hypothesis is that in many cell types at least two distinct classes of coated vesicles have been traced (Prelmann, 1975; de Bruyns et al., 1975; Worthington & Graney, 1973;

Bowers, 1964; Friend & Farquhar, 1967) differing from each other in size. Some workers also claim that they differ in function, the larger class of coated vesicles (> 100 nm) being involved mainly with uptake of exogenous protein and found mainly in the apical region. The smaller coated vesicles (< 70 nm) are located in the Golgi region and are either involved in the transport of enzymes to vesicles or in membrane recycling (Friend & Farquhar, 1967; Holtzman et al., 1967). If coated vesicles can be subdivided into distinct classes, then it would be easy to envisage that a subpopulation was specialized to transport antibodies and to avoid fusion with other vesicles in the process in a Wild type mechanism. A point against this is that Ockleford et al. (1977), by contrast, cannot see size groups of coated vesicles corresponding to different functions. In this he has support from the group of workers investigating the structures of coated vesicles who all comment on a great similarity of coated vesicle structure independent of origin (Pearse, 1976, 1980; Ockleford, 1976; Crowther et al., 1976; Woods et al., 1978). They found that coated vesicles are made up from varying numbers of identical subunits organized in pentagons and hexagons (Crowther et al., 1976) consisting mainly of the protein termed clathrin (Pearse, 1976). A certain uniformity in structure has been shown for coated vesicles derived from the adrenal medulla and a lymphocyte cell line (Pearse, 1976), pig brain and chicken oocytes (Woods et al., 1978) and the human placenta (Ockleford, 1976). In this context, however, it is interesting that Woods et al. (1978) found in chicken oocytes the presence of two other major proteins apart from clathrin, in the coat of coated vesicles, and they suggest that one of these may be a building block of a specific receptor protein. If it could be shown that there is indeed a marked interspecies heterogeneity caused by specific receptors in the coat, it would not be difficult to claim different special functions for coated vesicles depending on the cell of origin.

One experimental step towards resolving this problem of a coated vesicle specialized for IgG transport in the rabbit yolk sac can be proposed by making use of the in vitro yolk-sac incubation method employed in this thesis. If rabbit yolk sacs were "loaded" in vitro with ^{125}I-labelled homologous IgG, then coated vesicles isolated from such tissues (the method could be modified from Pearse, 1975, 1976), it should be possible to assay the coated vesicles for their contained immunoglobulin and lysosomal enzyme activity.

In conclusion of this section, even though coated vesicle have been shown to sometimes contain lysosomal enzymes and also to fuse with other vesicles, thus presenting problems to the Wild theory, the Wild theory is still tenable. Assuming then that the coated vesicle theory put forward by Wild (1975, 1976) is correct, it is interesting to speculate about the mechanism of receptor segregation in the formation of these vesicles. It has been shown in Chapters 6 and 7 that receptors or binding sites are involved in the specific, protective uptake as well as in the uptake of proteins in the degradative system of the yolk sac cell (see also Kaplan et al., 1975 and Unkeless, 1977 for similar findings in the mouse macrophage). With two receptor-mediated uptake processes taking place on the plasma membrane of the same cell at the same time, there must be some control mechanism operating. Two mechanisms can be suggested for the organisation of receptors. First there may be localized concentrations of receptors distributed over the cell surface, similar to the coated regions observed by Anderson et al., 1977a, for low density lipoprotein uptake in human fibroblasts. Such coated regions seem to exist on the cell surface without the necessity of the presence of LDL. Also Ockleford & Menon (1977) showed that in the human placenta distinct regions of the plasma membranes apparently specialized for iron uptake could be demonstrated. These workers go as far as to raise these regions to the status of separate organelles.

If the plasma membrane can be differentiated, in this manner, into a number of distinct regions, each with a different function, the mechanism of segregating different proteins into those destined for transport or degradation arises simply by different specialized vesicles arising from different regions of the cell surface.

The second possible mechanism for the organisation of "protective" and "degradative" receptors on the rat and rabbit yolk sac is slightly more complex and is based on the observation by a number of workers that receptors are, at least initially, freely distributed over the entire cell surface and that subsequently clustering or capping of coat/receptor complexes occurs (Kaplan et al., 1975; Taylor et al., 1971; Ockleford & Whyte, 1977; Anderson et al., 1977b; Goldstein et al., 1979). Regarding the mechanism of capping, these workers fall into two groups. Anderson et al. (1977b) and Goldstein et al. (1979) think that clustering of receptors is independent of the presence of any bound proteins or ligand and happens more or less spontaneously. This suggestion is also supported by their earlier findings (Anderson et al. (1977a) that ready-formed coated regions were even found on formaldehyde-fixed tissues and that a "receptor mislocation mutation" of the fibroblasts prevented the clustering process. The other group of workers, by contrast, suggests the involvement of effectors in cap formation. Thus Taylor et al. (1971) say that lymphocyte surface immunoglobulins are possibly induced by anti-immunoglobulin antibodies to gather over one pole of the cell. As cap formation was shown to be an energy-dependent process by these workers, the interaction and activation of microfilaments by the bound proteins was postulated. This involvement of the microfilaments in receptor clustering is also proposed by Ockleford & Whyte (1977), who elaborate this point by saying that receptors probably repel each other and need ligands to bind them together and cause capping at the base of microvilli. Divalent cations

have been forwarded for the role as ligands. For example Maxfield *et al.* (1978) showed that extracellular Ca^{++} was required for the aggregation of ligand/receptor complexes over coated pits. Davies *et al.* (1980) recently suggested that the intracellular enzyme transglutaminase (which is known to covalently cross-link proteins and requires Ca^{++} for its activity) may have a major role to play in receptor clustering in coated pits. When these findings are applied to the protein segregation of the yolk-sac cell surface the following, speculative, picture emerged. At least two distinct types of receptors/binding sites are distributed randomly over the outer cell membrane surface (having been synthesized within the cell or recycled). Receptors of the one type may cluster spontaneously (Anderson *et al.*, 1977*a*,*b*) taking up proteins and other substrates. Receptors of the other type need the previous binding of ligands (immunoglobulins) to trigger off clustering preceding coated vesicle formation with the protection of the contained immunoglobulins. These schemes are, of course, speculative, but they serve to highlight that protein segregation on the membrane is theoretically possible. The possible differential involvement of cations in vesicle formation could be investigated by use of the *in vitro* incubation technique of yolk sacs. Adding, for example Ca^{++} to the incubation medium (during "loading") may well lead to a decrease in the transport of intact IgG while leaving the "degradative" protein route unaffected.

In summary, the main body of evidence obtained by incubating rat and rabbit yolk sacs *in vitro* supports the Wild hypothesis of IgG transport to the foetus by a separate class of vesicle.

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