PROTEIN CATABOLISM IN CULTURED RAT YOLK SAC

by

Graham E. Ibbotson, G.R.I.C.

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Biochemistry Research Laboratory
Department of Biological Sciences
University of Keele

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ABSTRACT

An *in vitro* culture technique for the quantitation of pinocytosis in the rat visceral yolk sac was modified, by removing calf serum from the incubation medium, to permit determination of the rate of uptake of substrates in the absence of competing serum proteins. Study of the Endocytic Index of ^{125}I-labelled PVP in the absence of calf serum indicated that the substrate provided a suitable marker for determination of the rate of fluid ingestion in serum-free medium. From measurements of the ratesof ingestion of [U^{14}C] sucrose, ^{125}I-labelled dBSA and colloidal [^{198}Au] gold in the presence and absence of calf serum, it was concluded that the first substrate is ingested essentially in the fluid phase, while the other two are ingested mainly adsorbed to the plasma membrane. A detailed study of the uptake of ^{125}I-labelled dBSA in the presence of a non-radioactive analogue permitted a kinetic analysis of the affinity of this substrate for binding sites on the plasma membrane. The rate of ingestion of homologous ^{125}I-labelled IgG indicated that it is ingested mainly in the adsorbed phase but at a slower rate than ^{125}I-labelled dBSA. On re-incubation of yolk sacs 'loaded' *in vitro* with ^{125}I-labelled IgG a significant percentage of the released radioactivity was macromolecular and tentatively identified as ^{125}I-labelled IgG. This finding contrasted sharply with that for either ^{125}I-labelled PVP, with which little radioactivity was released, or ^{125}I-labelled dBSA, with which virtually all the radioactivity released was in a low molecular weight form. The observation that IgG can escape from the tissue intact is compatible with the suggestion that the rat yolk sac is instrumental in the transmission of passive immunity from mother to fetus prior to birth. A number of investigations were performed in an attempt to distinguish the mechanism of release. The findings suggest that release occurred by way of a separate transport vesicle as proposed by A. E. Wild.

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

Rat Visceral Yolk Sac

Throughout the work reported in this thesis the rat yolk sac has been employed as a model system for the investigation of the uptake of extracellular materials by pinocytosis. It is therefore pertinent to review the literature on the structure and function of this tissue in order that a detailed study of the mechanisms involved in the uptake of substrates may be related to the morphological and ultrastructural features of the tissue, enabling the observed pinocytic behaviour to be viewed in a broader context.

During the latter half of gestation, and following inversion of the embryo, the yolk sac of the rodent completes development by losing both Reichert's membrane and the parietal yolk sac so that the fetus is surrounded by only two membranes, the visceral yolk sac and the amnion (see Fig. 1a), both of which persist until term (Beck and Lloyd, 1968). The visceral yolk sac is composed of three cellular layers each separated by a fibrous membrane; Fig. 1b illustrates the arrangement of these layers. The external layer, which *in vivo* is in close contact with the uterine wall, consists of columnar vitelline epithelial cells attached to the fibrous visceral basement membrane. Below this membrane is a further layer consisting of mesenchymal or fibroblast-like cells (Wislocki and Padykula, 1953); within this layer the vitelline vessels, which extend around the yolk sac are found. The mesenchymal cells rest on another thick fibrous membrane known as the serosal basement membrane and below this layer, adjacent to the amnionic cavity is a final thin layer of mesothelial cells.

The fetus itself is contained in a second inner membrane, the amnion, which is entirely separate from the yolk sac.

The outer (uterine) surface of the yolk sac has two distinct regions. A highly folded or villous region extending from the chorioallantoic placenta and equivalent to approximately one third of the total

yolk sac area; the remainder of the surface is less villous but individual epithelial cells are identical at the ultrastructural level.

Gillman et al. (1948) and Everett (1933, 1935) showed that in vivo the yolk sac is highly active in the uptake of a number of dyes and further studies have shown that in vivo this tissue is able to ingest a variety of other substances. For example: ferritin (Lambson, 1966), ferritin and Trypan Blue (Krzyzowska-Gruca and Schiebler, 1967), horseradish peroxidase (Beck et al., 1967, 1969; Seibel, 1974), vitamin B_{12} (Padykula, 1966), thorotrast (Carpenter and Ferm, 1966) and gammaglobulin (Anderson, 1959; Halliday, 1957). Similarly, using an organ culture method ^{125}I-labelled PVP (Williams et al., 1975a), ^{125}I-labelled BSA (Williams et al., 1975b), [U^{14}C] sucrose and colloidal ^{198}Au (Roberts et al., 1977) have been shown to be accumulated by the tissue in vitro.

These studies indicate that the columnar epithelial cells are responsible for the ingestion of such materials, but the physiological significance of this intense pinocytic activity is not yet certain. One suggestion is that the yolk sac acts as a barrier to protect the developing fetus from foreign materials such as maternal antibodies and drugs which may be harmful (Wilson, 1959). Lloyd et al., (1974) showed that when a bisazo dye, Trypan Blue, was injected into the maternal system a considerable amount of dye could be detected in the epithelial layer at the yolk sac but none was found in the amniotic cavity or the fetus. A further function that has been proposed for the yolk sac is that the tissue plays a major role in embryotrophic nutrition (Beck and Lloyd, 1968). It was shown that after ingestion of horseradish peroxidase loss of enzymic activity occurred and it was suggested that hydrolysis of this exogenous protein occurred intralysosomally and that the amino acids so produced, are passed to the fetal tissues in vivo as a source of building materials (Beck and Lloyd, 1968; Beck et al., 1970). A further possible role is the transmission of passive immunity from mother to fetus. Brambell

(1970) was able to show the selective transport of immunoglobulins across the yolk sac of the rabbit and a similar process may well occur in the rat.

Whether or not the yolk sac can perform some of these functions *in vivo* is an open question but the demonstration of the full capacities of the epithelial cells of this tissue *in vitro* using culture techniques is likely to be informative. It is also of importance when considering properties of the yolk sac as an organ to consider its morphology. Its outermost layer is comprised of columnar epithelial cells whose nuclei are situated near the basal end of each cell. Furthermore, the apical surface of each cell has a prominent, microvillous brush-border which carries a glycocalyx that has been shown to be composed of mucopolysaccharide, by staining with ruthenium red and examination under the electron microscope (Jollie and Triche, 1971). It is further believed that this coating may extend across the whole surface, its role being to facilitate the binding of substrates prior to ingestion. Between the microvilli, caveoli extend into the cytoplasm of the cell and in some cases these have been shown to be several microns deep.

The caveoli too are found to be lined with the same glycocalyx that covers the base of the microvilli. The glycocalyx is also observed in vesicles close to the caveoli which appear to have formed from the latter. These vesicles appear to migrate further into the cytoplasm and fuse to form larger vesicles within the well-defined vacuolar system of these cells. The process of fusion has been suggested as playing an essential part in the process of membrane re-cycling (Duncan and Pratten, 1977). Furthermore, extensive membranous infoldings are observed in the form of an apical canalicular system which is an interconnected system of small tubules or canals sometimes observed to be connected to large vacuoles or small vesicles (Lambson, 1966). Presumably these vacuoles and vesicles are in the process of formation from the canalicular system.

Endocytosis

Endocytosis is a broad term used to describe general cellular uptake of substrates too large or polar to be taken up by diffusion, or for which no form of active transport exists. Presumably it is the major mode of entry of many small molecules into a number of tissues. The general process has been sub-divided into: 'Phagocytosis', 'Pinocytosis' and 'Micropinocytosis', although more recently these processes have become to be regarded as convenient classifications rather than processes each with a different mechanistic basis. Endocytosis in its most general form is the capture and ingestion of substrates by invagination of the plasma membrane adjacent to a particle or quantity of extracellular medium in which a soluble substrate is dissolved. Once the membrane completely surrounds a portion of medium the small 'droplet-like' body or vesicle detaches from the remainder of the apical membrane and migrates further into the cell where it fuses with other such bodies to form larger vesicles.

The essential distinguishing feature of the three sub-classes of the process is the size of the substrate ingested. Historically the first process to be described was 'Phagocytosis', a process first visualized at the light microscope level by Metchnikoff some hundred years ago. 'Phagocytosis' is now used to describe the ingestion of relatively large particulate material in the size-range from large colloidal material to particles such as bacteria or erythrocytes with dimensions in the order of 2.0 μm. Consequently such particles can be readily observed either under the light microscope or under only low magnification in the electron microscope. This type of uptake usually results in the phagocytic vesicle being completely filled with the ingested particle, there being little attendant ingestion of fluid.

'Pinocytosis' occurs by essentially the same process as 'Phagocytosis' but the term was coined by Lewis (1931) in order to describe the process of ingestion of fluid (containing finely divided colloidal or soluble

substrates, which cannot be visualized by light microscopy) rather than large particles. The size of the materials ingested by 'Pinocytosis' ranges from small molecules e.g. sucrose (just large enough not to diffuse across lysosomal membranes) up to colloidal material of approximately 20 nm dia., although this upper size limit should not be regarded as being rigidly defined. 'Pinocytosis' is also thought to be a continuous process in many mammalian cells but induced pinocytosis has been observed in the amoeba, an organism that undergoes a cycle of pinocytic activity lasting 30-60 min. in response to contact with the substrate (Chapman-Andresen, 1962).

In the rat yolk sac uptake of substrates is by pinocytosis or micropinocytosis (Williams et al., 1975a and b) rather than by phagocytosis. Therefore it is essential to review in more detail the processes involved in this type of uptake. The following steps outline the main features of pinocytosis; these are probably substantially the same in phagocytosis.

A substrate may enter a pinosome (pinocytic vesicle) either through being ingested in the bulk fluid or by attaching to binding sites on the cell surface prior to invagination of the plasma membrane. Jacques (1969) proposed a general model to describe the pinocytic uptake of solutes and derived a general equation that describes the uptake of solutes both in the fluid and adsorbed phases (see Chapter 3, equation 3.3). The initial surface adsorption of substrates has been investigated in a number of systems. The uptake of ^{131}I-labelled albumin by tumour cells (Sarcoma S 180) was followed by Ryser (1968). His data are compatible with a two stage process, namely a rapid phase of surface adsorption followed by a slower process of internalization of the radiotracer by pinocytosis. King (1968), using ^{131}I-labelled albumin, demonstrated binding to guinea pig lung-tissue slices and found treatment with strong urea solutions was required to reverse this process, while Gosselin (1967), in studying the uptake of colloidal [^{198}Au] gold by cultured

macrophages, was able to determine some physical parameters of the binding process. Similarly, through *in vivo* studies of the clearance of foreign particles from the blood stream of the rat by the liver, Normann (1974a and b) was able to demonstrate the importance of binding. Furthermore, by analysing the kinetics of the uptake process he derived constants for the receptor-substrate complex. Other studies by Soll *et al.* (1975), using an isolated brush-border membrane technique showed that the binding of insulin could be followed in detail, enabling a number of binding parameters to be determined; these included the substrate-receptor binding constants, the number of binding sites and the affinity of such sites for the substrate.

Whether or not binding occurs, the central event in pinocytosis is the internalization of a portion of the cell's membrane to form a vesicle. During this process a quantity of extracellular fluid (and possibly adsorbed substrate) is completely surrounded by a limiting membrane to form a pinosome which, once formed, fuses with other pinosomes to form larger vesicles that fuse with lysosomes on migrating deeper into the cytoplasm. This process was inferred from the findings of Straus (1962, 1963, 1964) who found that horseradish peroxidase was ingested by several mammalian tissues. By using histochemical techniques and either liver or kidney cells, he was able to show that peroxidase was initially confined to small vesicles and that with increasing time the peroxidase passed into the larger vesicles that were acid phosphatase positive. Mego and McQueen (1965) loaded rat liver *in vivo* with ^{131}I-labelled denatured albumin. On fractionating the tissue, at different time intervals after administering the radiolabelled protein, by density gradient centrifugation, the radioactivity was observed to move progressively from lighter to heavier particulate fractions and that proteolysis occurred in the latter. Likewise, Goetze *et al.* (1976) have recently shown that in subcellular fractions, prepared from

rat yolk sacs that have ingested ^{125}I-labelled human serum albumin *in vitro*, the ingested radioactivity moves from light to dense fractions as the time-interval between ingestion and homogenization of the tissue increases. Other workers, (Caulfield, 1963; Miller, 1960) have been able to visualise the passage of ferritin and haemoglobin into the vacuolar system of cells where the battery of lysosomal enzymes is able to hydrolyse such proteins. Obviously substrates which are not susceptible to hydrolysis are accumulated within these vesicles.

The primary lysosomes, with which the pinosomes fuse, originate from within the Golgi bodies. The lysosome consists of a limiting membrane containing a number of acid hydrolases which degrade biopolymers to their constituent monomers. The hydrolytic capacity of the lysosomal system was demonstrated *in vitro* by Mego and McQueen (1965) using rat liver heterolysosomes prepared from liver cells that were previously laden with ^{131}I-labelled denatured albumin. By working in osmotically protected media they were able to isolate an organelle fraction from this tissue which, on incubation at 37^{o}C, was found to progressively release the digestion product [^{131}I] iodo-tyrosine. Coffey and de Duve (1968) have also demonstrated the hydrolysis of radiolabelled substrates by isolated enzymic extracts from liver lysosomes. Further, the ability to hydrolyse ingested proteins has been demonstrated in a number of other intact cells. For example ^{131}I-labelled albumin by sarcoma cells (Gabathuler and Ryser, 1967), ^{131}I-labelled albumin and tritium-labelled haemoglobin by mouse macrophages (Ehrenreich and Cohn, 1967 and 1968), ^{125}I-labelled haemoglobin by trophoblastic cells in culture (Contractor and Krakauer, 1976) and ^{125}I-labelled asialo-fetuin by isolated hepatocytes in culture (Tolleshaug *et al.*, 1977).

Beck *et al.* (1967) were able to demonstrate, histochemically, the accumulation of horseradish peroxidase in the vacuolar system of the vitelline epithelial cells of the rat yolk sac and they further concluded

(Beck et al., 1969) that the slow hydrolysis of this exogenous enzyme was probably effected intralysosomally. Using an organ culture method Williams et al. (1975a, b) demonstrated the uptake and accumulation of ^{125}I-labelled PVP by the yolk sac and that in similar experiments with ^{125}I-labelled albumin, ^{125}I-labelled tyrosine was released back into the incubation medium. These findings suggested that the yolk sac ingests material by pinocytosis and passes it from pinosomes to secondary lysosomes where, in the case of biopolymers, digestion occurs. A general scheme for the uptake and digestion of substrates is shown in Fig. 1c. Williams et al. (1975a) and Roberts et al. (1977) showed non-digestible substrates to accumulate within the yolk sac tissue, and assumed that the sites of accumulation were pinosomes and secondary lysosomes. The rate of uptake of the non-digestible substrate, ^{125}I-labelled PVP, was calculated and expressed in the form of an 'Endocytic Index' which has the units µl/h/mg of yolk sac protein (Williams et al., 1975a). This form of expression permits the determination of the rate of uptake of a solute which accumulates within the tissue; any material released by the tissue following pinocytic capture makes no contribution to the rate of uptake so calculated. In studying the uptake of proteins (Williams et al., 1975b) the method of analysis was extended to allow for the effects of release from the tissue of hydrolysis products of labelled albumin in the form of trichloroacetic acid-soluble radioactivity (intact protein is insoluble in trichloroacetic acid). Again assuming the rate of exocytosis to be negligible, the total substrate uptake is then the sum of the 'tissue-associated radioactivity' and the 'TCA-soluble radioactivity' released back into the culture medium.

The following work extends that of Williams and Moore by investigating the effects on uptake of several substrates (including ^{125}I-labelled PVP and ^{125}I-labelled dBSA) of removing calf serum from

the culture medium. This has the effect of greatly simplifying the analysis of the kinetics and permits calculation of the associated kinetic constants.

In Chapter 5 the uptake of homologous ^{125}I-labelled IgG was followed in order to quantitate its rate of uptake, degradation and release. This study was extended in Chapter 6 in an attempt to probe the mechanism of transport of immunoglobulins across cellular barriers with attention focused on the hypotheses of Brambell (1958 and 1966) and Wild (1975). The suggestion by Brambell and Halliday (1956) that the yolk sac transports a higher fraction of the administered immunoglobulins as term approaches, prompted a study in Chapter 7 of the pinocytic capacity of the yolk sac tissue as a function of gestational age, using ^{125}I-labelled PVP and ^{125}I-labelled dBSA as substrates as well as ^{125}I-labelled IgG. In Chapter 8 preliminary studies of the effect of trypsinisation on the rates of uptake of ^{125}I-labelled PVP, ^{125}I-labelled dBSA and ^{125}I-labelled IgG by the yolk sac are reported together with the results of studies in which attempts were made to isolate the cell surface receptors.

Fig. 1a Diagrammatic Representation of the Arrangement of the Foetal Rat at 16 days of Gestation

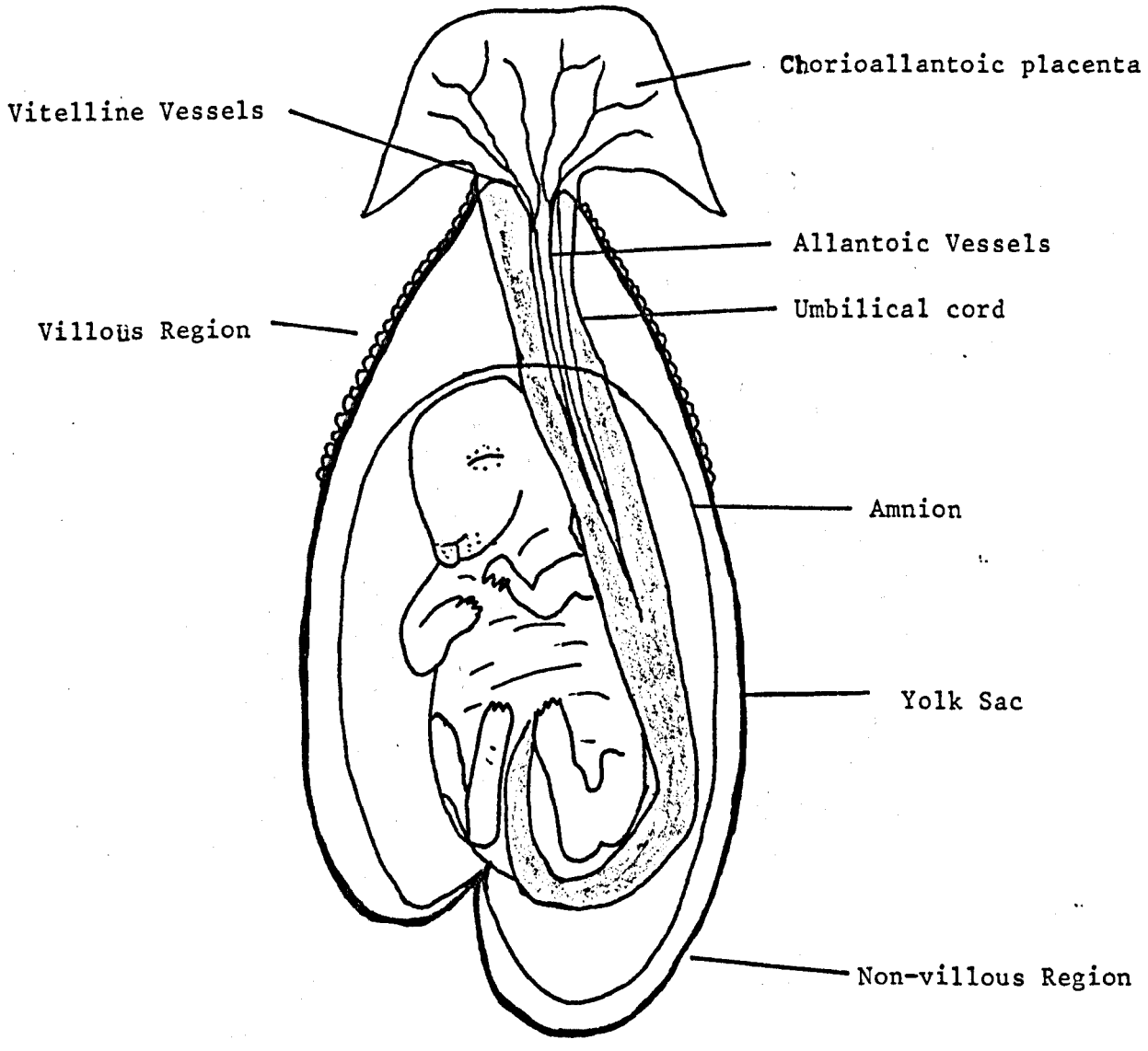


Fig. 1b Diagrammatic section through the rat yolk sac

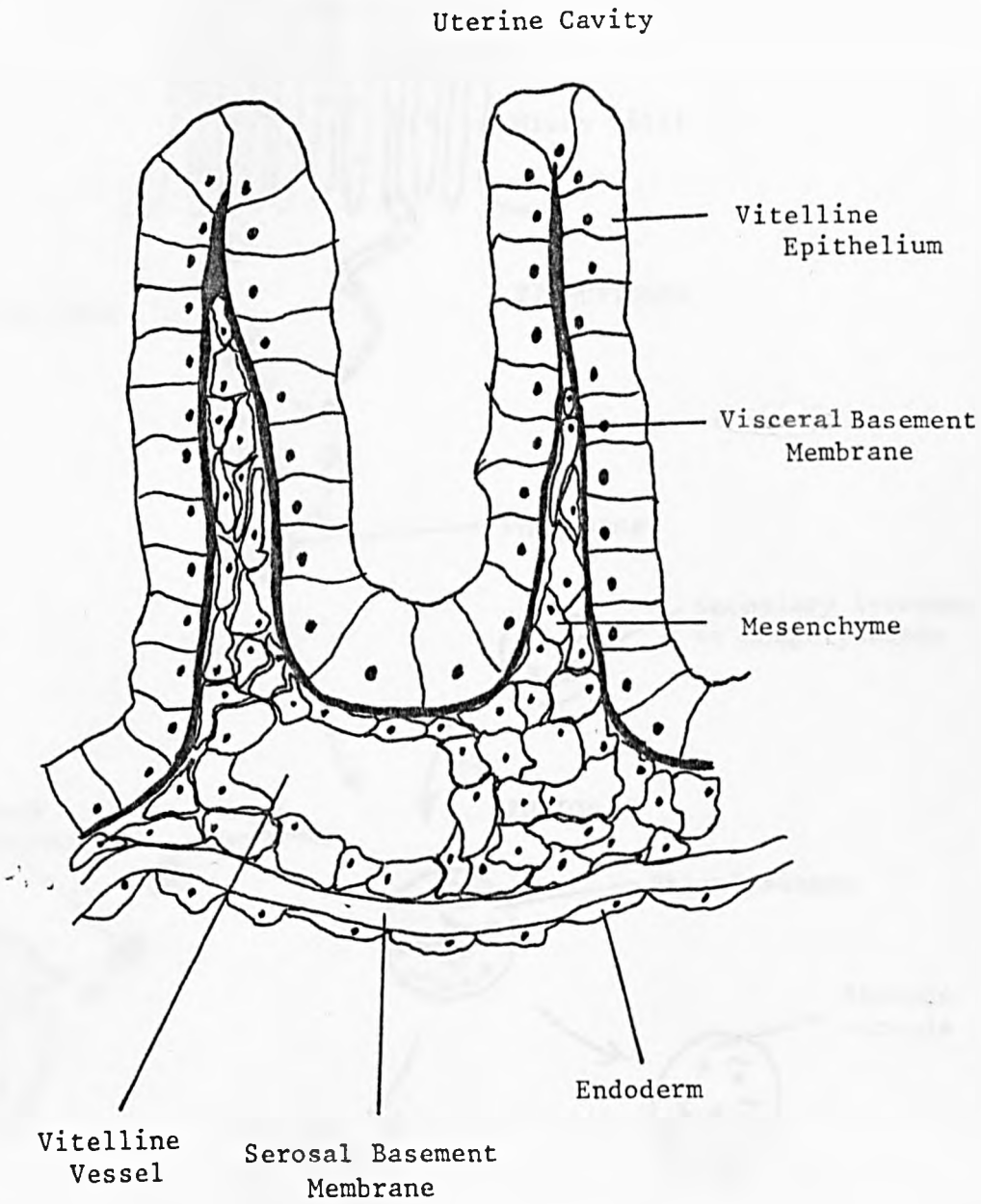
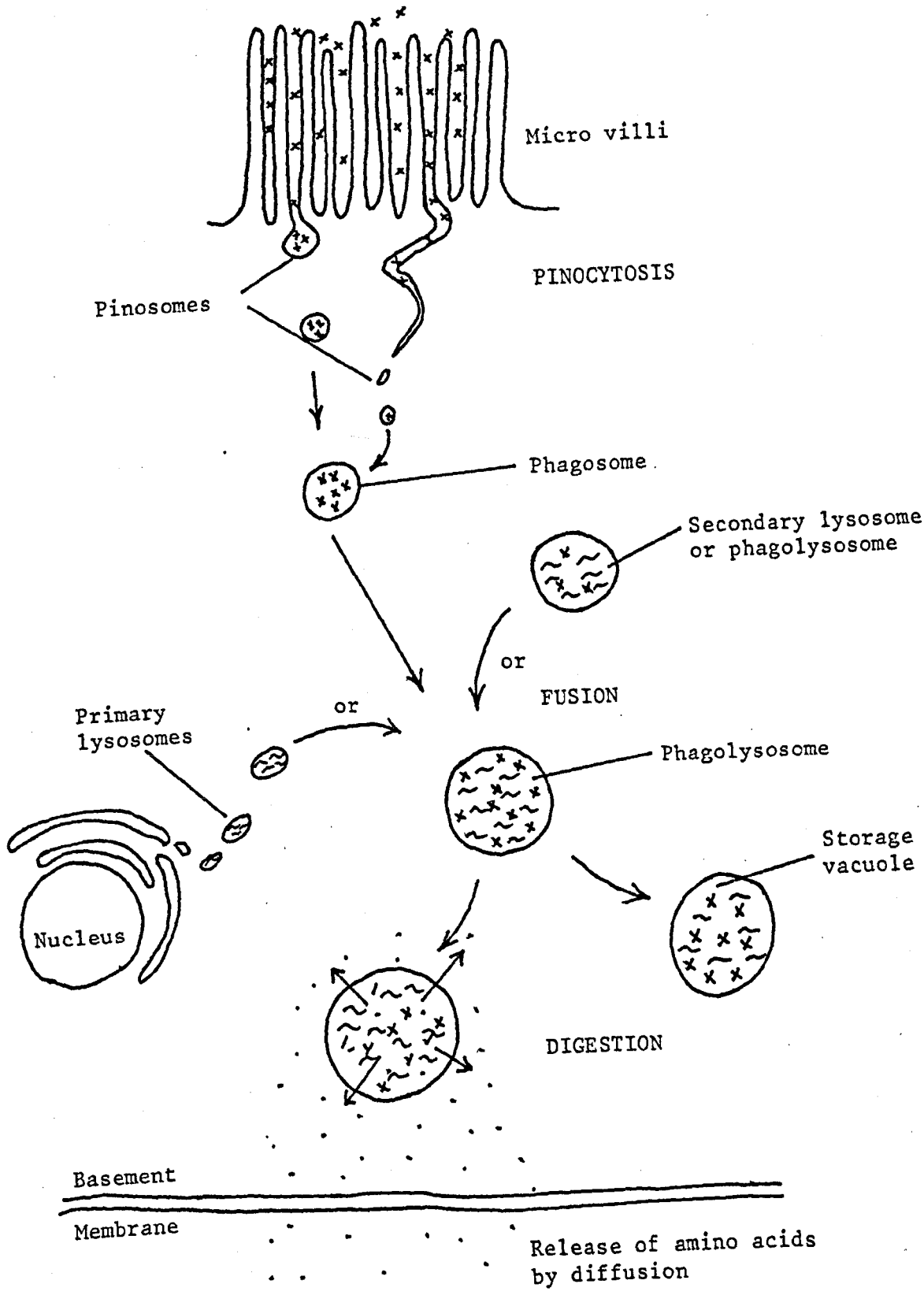


Fig. 1c General pathway for the uptake and digestion of solutes within the vitelline epithelium of the yolk sac



CHAPTER 2

MATERIALS AND METHODS

Chemicals

[^{125}I] iodide (preparation IMS.4, 5m Ci in 0.5 ml), ^{125}I-labelled poly(vinyl-pyrrolidone) [^{125}I-PVP: average molecular weight 30-40,000, preparation IM.33P], [U^{14}C] sucrose (preparation CFB.146, > 350 mCi/mM), and colloidal [^{198}Au] gold (preparation GCS. 1P, 4-12 mCi/mg Au) were purchased from the Radiochemical Centre, Amersham, Bucks., U.K. Bovine serum albumin (BSA, preparation 0142t), butyl PBD [5-(biphenyl-4-yl-)-2-(4-t-butylphenyl) 1,3,4-oxidiazole], 4-methylumbelliferyl 2-acetamido-2-deoxy-β-D-glucopyranoside and 4-methylumbelliferone were purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. Toluene (scintillation grade) was purchased from Hopkin and Williams, Chadwell Heath, Essex, U.K. Single strength tissue culture medium 199 (preparation TC 20, containing penicillin and streptomycin) and heat-inactivated calf serum (preparation CS07) were obtained from Wellcome Reagents Ltd., Beckenham, Kent. Glycyl-L-tyrosine, L-tyrosine, poly(vinyl-pyrrolidone) [average molecular weight 40,000], trypsin (preparation TO 134: type IX; crystallized from hog pancreas), glucagon (preparation G 4250), lactoperoxidase (preparation L-8503, from milk) and Nigrosin were purchased from Sigma Chemical Company Ltd., Surrey, U.K. Rat gamma globulins (IgG, fraction II) from Miles Laboratories Ltd., Slough, U.K. Oxygen/Carbon-dioxide (95:5) from British Oxygen Ltd., Manchester, U.K. All other chemicals were of analytical grade.

2.1. Preparation of labelled proteins

The method used was that of Williams et al. (1971). Bovine serum albumin (100 mg) was dissolved in phosphate buffer (45 ml of 0.05M Na_2HPO_4-KH_2PO_4, pH 8.0) and cooled in an ice-bath. Sodium [^{125}I] iodide (5 mCi) was added using a 1 ml disposable syringe and the ampoule rinsed with phosphate buffer (1.5 ml) and the washings added to the reaction vessel. After stirring for 2 min, chloramine-T solution (20 ml, 1.0 mg/ml) was added and the reaction allowed to proceed for 8 min before being stopped by the addition of sodium metabisulphite solution (15.0 ml, 6.0 mg/ml). Solid potassium iodide (500 mg) was then dissolved in the reaction mixture to aid displacement of unreacted [^{125}I] iodide during the subsequent dialysis. An equal volume of formalin (4% w/v formaldehyde in 0.5 MNa_2CO_3, pH 10) was added and the resulting solution allowed to stand for 72 h at 4°C (Moore et al., 1977). The solution was then freed from inorganic iodide and formaldehyde by dialysis (72 h at 4°C) in Visking tubing against at least four changes of aqueous sodium chloride (1%, w/v). The protein concentration of the dialysed solution was adjusted to 0.6 mg/ml using aq. sodium chloride and the resultant material dispensed into sterile 5 ml screw-top bottles. The whole batch (Batch V) of material was frozen and thawed several times, to ensure that any slight changes induced in the protein structure by this procedure were more likely to be complete before use. The whole batch was then finally stored at -20°C.

^{125}I-labelled IgG was prepared by the same method as ^{125}I-labelled dBSA but at a 1/5th scale, the formaldehyde treatment being omitted. The concentration was estimated from the extinction of 280 nm using a standard curve prepared in saline (1%, w/v).

2.2 Preparation of non-radioactive, formaldehyde-denatured ^{127}I-labelled albumin (^{127}I-labelled dBSA)

Bovine serum albumin was iodinated with ^{127}I-iodide, to the same

extent as the radioactive analogue, by substituting 0.38 µg of potassium iodide for every mCi of [^{125}I]iodide in the above method. All concentrations and volumes of reagents were increased to produce a final ^{127}I-labelled dBSA concentration of 1.7 mg/ml. The resulting solution was diluted with aq. NaCl solution (1%, w/v), to produce stock solutions of differing concentrations, then stored at -20°C.

Attempts to prepare samples of ^{127}I-labelled dBSA with a concentration in excess of 2.0 mg/ml resulted in precipitation of the protein during the dialysis procedure.

2.3 Technique for culturing yolk sacs in the presence of calf serum

The method of Williams et al. (1975a) was used with minor modifications. Wistar rats from an inbred colony were mated overnight, and pregnancy timed as from midnight if a vaginal plug was observed the following morning.

At 17.5 days of gestation the animals were killed by cervical dislocation. The yolk sacs were immediately removed and freed from other tissues by dissecting the uterus under a mixture of medium 199 and 10% (v/v) calf serum that had previously been gassed with oxygen/carbon dioxide (95:5) and warmed to 37°C (see Fig. 2a). Individual yolk sacs were each placed in a sterile 50 ml Erlemeyer flask, containing gassed medium (9.0 ml, consisting of 8.0 ml TC 199 and 1.0 ml calf serum). Each flask was sealed with a sterile silicon rubber bung and placed in a thermostatted reciprocating water-bath at a temperature of 37 ± 0.2°C and the shaking attachment set at 100 ± 4 strokes/min, with a stroke length of 3.8 cm. The yolk sacs were allowed to equilibrate under these conditions for about 20 min prior to addition of 1.0 ml of a stock solution of the radiolabelled substrate (in medium 199) using a 5 ml safety pipette. The final concentration of radioactive substrates in the incubation medium was usually in the range 2-5 µg/ml for ^{125}I-labelled PVP and 0.5-2 µg/ml for ^{125}I-labelled

dBSA.

After addition of the substrate the flasks were again gassed with oxygen/carbon dioxide (95:5), for a period of 10 sec each. Individual yolk sacs were removed at regular intervals, up to 6.5 h, washed three times for 2 min in changes of cold saline (20 ml, 1% w/v) then placed in a 5 ml volumetric flask and stored at -20°C until assayed (see below).

When ^{125}I-labelled dBSA was used as substrate the flasks containing the associated medium were returned to the water bath and incubated until the last yolk sac had been removed. This procedure ensured that the effects of any proteolytic activity in the culture medium on the labelled protein were uniform.

2.4 Assay of yolk sacs for their contained radioactivity

The yolk sacs were thawed and sodium hydroxide (1.0M) was added to a total volume of 5.0 ml. The volumetric flasks were then placed in a water-bath at 37°C for 1 h, prior to vigorous mixing using a 'Rotamixer'. The flasks were then returned to the water bath for a further 15 min to ensure complete solution of the tissue.

Duplicate samples of the yolk-sac solution (1.0 ml) were placed in 3 ml disposable plastic tubes (LP3, Luckham Ltd., Burgess Hill, Sussex) and the contained radioactivity assayed using a gamma spectrometer (Packard Instrument Ltd., Caversham, Berks.) with the tubes positioned in a standard geometrical relation to the crystal detector.

2.5 Determination of the protein content of yolk sacs

The protein content of each yolk sac was determined by the method of Lowry *et al.* (1951) using duplicate 0.1 ml portions of the yolk-sac solution as prepared above, and using bovine serum albumin (Sigma (London) Chemical Co. Ltd., London S.W.6; product no. A4378) as reference protein. The protein content of each yolk sac was expressed as an equivalent number of mg of bovine serum albumin.

2.6 Assay of culture medium from experiments using either ^{125}I-labelled PVP or colloidal [^{198}Au]gold as substrates

Duplicate 1.0 ml samples of medium from each culture flask were pipetted into 3 ml disposable plastic tubes and the radioactivity assayed using a Packard gamma spectrometer, usually with a counting period of 30 sec. The mean count (corrected for background) was termed the 'total radioactivity' in the culture medium.

2.7 Assay of culture medium from experiments using a ^{125}I-labelled protein as substrate

Since proteins are hydrolysed when incubated in the presence of yolk-sac tissue and digestion products are released back into the culture medium, any estimate of the rate of uptake of protein by the tissue must include not only the contribution from intact protein present in the tissue itself but also that from ingested protein that has been digested and released into the medium. The latter is conveniently measured as the increase of the trichloroacetic acid soluble radioactivity in the medium produced during the incubation of the yolk sac.

The 'total radioactivity' in the medium was determined by the same method (see 2.6) as used for non-digestible substrates, then trichloroacetic acid (20% w/v, 0.5 ml) was added to the duplicate samples to precipitate the contained protein. After centrifuging at 2000g for 20 min each supernatant (approx. 1.3 ml) was decanted completely into a new 3 ml plastic tube and assayed again for radioactivity with a counting period of 300 sec. This procedure yields a value for the amount of protein digested during the incubation period and the mean value (c.p.m., corrected for background) was termed the 'TCA-soluble radioactivity'. Since the volume of the decanted solution of TCA-solubles was 1.3 ml, the observed count was multiplied by an empirical correction factor (1.33) so as to give the count that would have been

observed if the same activity had been assayed using a counting volume of 1.0 ml. This normalization procedure removed any variation of the counting efficiency with the sample volume (see 2.11 for details of determination of the factor).

2.8 Assay of culture medium from experiments using [$U^{14}C$] sucrose

Incubation medium from experiments using [$U^{14}C$] sucrose as substrate was assayed by the method described by Roberts et al. (1977). Duplicate (1.0 ml) samples from each flask were placed in separate glass scintillation vials and scintillation 'cocktail' (10.0 ml of butyl-PBD; 6 g/l in toluene: Triton X-100, 2:1 v/v) was added. The vials were then assayed for their contained radioactivity on a Packard 2425 liquid scintillation spectrometer (Packard Instrument Ltd., Caversham, Berks.) with a sample-chamber temperature of 13°C. The presence of incubation medium in each sample vial provides a large number of substances which could 'quench' the observed count. It was therefore essential to correct the observed count for such 'quenching'. This was achieved by an internal standardization method using a small quantity or 'spike' of aq. [$U^{14}C$] sucrose solution (25 µl, containing approximately 10^5 d.p.m.) which was added to each vial from a stock solution. Four standard vials were prepared each containing the 'spike' of [$U^{14}C$] sucrose (25 µl), water (1.0 ml) and scintillation 'cocktail' (10.0 ml). The samples were then counted again and the relative counting efficiency calculated by the equation.

$$\frac{N_s - N_x}{N_o} = E \qquad 2.I$$

Where;

N_x = Observed sample count (c.p.m., corrected for background)

N_s = Observed count of sample after 'spiking' (c.p.m., corrected for background)

N_o = Observed count of 'spiked' standard (c.p.m., corrected for background)

E = Normalisation constant.

Each sample was then divided by its own normalisation constant, calculated as above, to give the count which would have been observed if the same amount of activity had been dissolved in water rather than in incubation medium. This individual correction factor was incorporated into the appropriate computer program for the calculation of uptake by the yolk sac using equation 2c (see Appendix for details).

2.9 Assay of radioactivity contained in yolk sacs from experiments with [U^{14}C] sucrose

Because of the possible effects of high pH or salt concentration on the stability of the scintillant emulsion, individual yolk sacs were dissolved in NaOH (0.25M, 2.0 ml) by sonication for 2 h at 37^{o}C. The yolk sac protein was then dispersed by vigorous mixing using a 'Rotamixer', neutralized with nitric acid (0.25 ml, 2.0 ml) and the total volume adjusted to 5.0 ml with water. Duplicate 1.0 ml samples were assayed for their contained radioactivity by the same method as used for assaying incubation medium containing [^{14}C] sucrose. In all cases the counting emulsion was found to be stable for at least five days.

2.10 Technique of culturing yolk sacs in the absence of calf serum

The method of Williams et al. (1975a) described previously (2.3) was used with several modifications.

Yolk sacs were removed from animals at 17.5 days of gestation and placed in gassed medium 199 containing antibiotics but no added calf serum. Yolk sacs were dissected as quickly as possible, (in less than 8 min) with the assistance of a second person, cultured in medium 199 alone and removed from the culture flasks at approximately 30 min intervals following addition of the radioactive substrate up to a maximum period of 5.0 h.

The methods of assay of both yolk sacs and the medium were the same as described above (see 2.4 - 2.7) except that the method of determination of the 'TCA-soluble radioactivity' in the medium was slightly changed as compared with 2.7. Before addition of trichloroacetic acid (20% w/v, 0.5 ml), 0.1 ml of calf serum was added to each tube to ensure complete precipitation of radioactive proteins. After decanting the supernatant (approx. 1.4 ml) the samples were counted (usually for 300 sec) and the resultant mean value (corrected for background) was multiplied by the appropriate empirical correction factor (1.35) to normalize the observed value of the 'TCA-soluble radioactivity' for altered counting geometry.

2.11 Determination of counting correction factor for change in sample volume

In sections 2.7 and 2.10 appropriate correction factors for different sample volumes were applied to the supernatant counts to enable the results to be rendered independent of the sample volume. The correction factors used were obtained in the following manner. Samples of ^{125}I-labelled iodotyrosine and glycyl[^{125}I]-iodotyrosine were prepared by essentially the same method as described in 2.1 then freed from [^{125}I] iodide by chromatography on Sephadex G-25. Calf serum was added to aqueous solutions of each compound to a concentration of 10% (v/v) to provide carrier protein in the TCA-precipitation. Six samples (1.0 ml) of each solution in 3 ml disposable tubes were counted under standard geometry on a Packard gamma spectrometer to give values of the total activity, T. Trichloroacetic acid (20% w/v, 0.5 ml) was then added and the sample centrifuged at 2000 g for 20 min. The supernatant was then decanted into fresh tubes and the TCA-soluble activity, S, assayed again on the same gamma spectrometer.
The correction factor C is then simply:

$$C = \frac{T}{S} \qquad (2.II)$$

Where T is the total activity in a 1.0 ml of sample (c.p.m., corrected for background) and S the activity in the decanted supernatant (c.p.m., corrected for background). This method not only corrects for volume change but also corrects for any loss of soluble activity through its being trapped in the precipitate.

To estimate the correction factor for media containing no calf serum, 0.1 ml of water was added to each sample tube prior to the addition of trichloroacetic acid (20% w/v, 0.5 ml). This volume of water compensates for the volume change owing to addition of calf serum as a carrier protein (see 2.10).

The values obtained were 1.33 $\pm$ 0.02 for cultures with calf serum and 1.35 $\pm$ 0.03 for cultures without calf serum. These two values were used as the appropriate correction factors throughout this thesis.

2.12 Calculation of accumulation by the yolk sac and Endocytic Index for non-digestible substrates

In order to correct for the variable size of individual yolk sacs and the change in substrate specific radioactivity through radioactive decay, Williams et al. (1975a) used the following expression for the accumulation of a non-digestible radio-labelled substrate by yolk-sac tissue.

$$A = \frac{Y}{M \times P} \qquad 2.III$$

Where A is the quantity of radioactive substrate accumulated in a yolk sac by a given time, Y is the total radioactivity in the whole yolk sac (c.p.m., corrected for background), M is the radioactivity per microlitre of medium (c.p.m., corrected for background) and P the protein content (mg) of the individual yolk sac.

This expresses the accumulation of substrate as the volume of culture medium whose contained substrate is internalised per mg yolk-sac

protein and has the units of µl culture medium per mg of tissue protein. This form of expression has the advantage not only of normalizing the results of each experiment for variation in tissue size but also renders the results of experiments, performed on different days, independent of the effect of radioactive decay in the substrate and from any drift in efficiency of the gamma counter. Plotting the accumulation of radio-labelled substrate, expressed in this form against incubation period (h) yielded a straight line. Williams et al. termed the gradient of this plot the "Endocytic Index". It has units of µl per milligram of yolk-sac protein per hour of incubation. Fig. 3a shows a typical plot.

The rates of accumulation of ^{125}I-labelled PVP, colloidal [^{198}Au] gold and [U^{14}C] sucrose were so low as to cause the concentrations of these substrates in the bulk medium to fall at the rate of only 0.1% per h. Hence even with the longest incubation period (7.0 h) the depletion of substrate in the bulk medium is less than 1% and a negligible error is introduced by using the substrate concentration of the medium at the end of the experiment in place of M in equation 2.III.

2.13 Calculation of an Endocytic Index for a digestible substrate

When ^{125}I-labelled proteins are used as substrates the yolk sac is found to degrade the ingested protein to the level of its constituent amino acids which, in the case of ^{125}I-labelled tyrosine at least, are released back into the culture medium. These digestion products appear as 'TCA-soluble radioactivity' which is estimated as described above (2.7). The expression for 'accumulation', A, the amount of substrate that would have accumulated in the tissue, had it not been subject to proteolysis, becomes:

$$A = \frac{Y + 10(S-F)}{M' \times P} \qquad 2.IV$$

As with non-digestible substrates, the numerator represents the total radioactivity 'processed' by the yolk sac. In the case of a

digestible protein this is the sum of two terms, Y, the total radioactivity retained in the whole yolk sac (c.p.m., corrected for background) and 10(S-F), the TCA-soluble radioactivity generated by the yolk sac and released into the culture medium (10 ml). S is the TCA-soluble radioactivity in the culture medium at the end of an incubation period (c.p.m. per ml, corrected for background). F is the amount of TCA-soluble radioactivity present that has been generated elsewhere than in the yolk sac; it is composed of two elements: TCA-soluble radioactivity present initially in the substrate preparation and any TCA-soluble radioactivity generated by proteolysis within the medium (e.g. by proteinase activity in the calf serum itself). The value of F is determined in each experiment by incubating a sample of medium, containing substrate but no yolk sac, for the duration of the experiment, then determining R, the fraction of the total radioactivity in the medium that is TCA-soluble. F, the TCA-soluble radioactivity of non yolk-sac origin is equal to M x R, where M is the total activity (c.p.m. per ml, corrected for background) in each individual incubation medium.

2.14 Modification of the calculation of the Endocytic Index to correct for depletion of substrate concentration

In equation 2.IV the total medium radioactivity M of equation 2.III is replaced by M'. This correction is necessary because in contrast to ^{125}I-labelled PVP (which is ingested with an Endocytic Index of 1.70 $\pm$ 0.30 µl/h/mg protein, see Section 3.5) protein ingestion occurs at a much greater rate (see Figs. 3.a and 3.b). This rapid ingestion leads to a significant fall in the concentration of the macromolecular form of the substrate in the medium during culture periods of 6 - 7 h (see Section 2.12). It was therefore necessary to correct this value to give the mean value of the 'TCA-insoluble radioactivity' in the medium (c.p.m. per ml, corrected for background) over

the individual incubation period for each yolk sac. The equation for calculation of the corrected medium radioactivity is:

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

This equation adds half the observed increase in the TCA soluble activity per ml of medium at the end of the culture period (c.p.m. per ml medium, corrected for background) to the value for the 'TCA-insoluble activity' as determined at the end of the same period (c.p.m. per ml medium, corrected for background), thus giving the mean quantity of TCA-insoluble substrate present in the medium in the incubation period of the yolk sac.

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

Equations 2.III and 2.IV enable uptake to be calculated, for non-digestible and digestible substrates, respectively, when the rates of exocytosis are negligible. When exocytosis cannot be ignored, these equations give only the net accumulation of substrate by the tissue. This quantity cannot be equated with the gross amount of substrate captured during the same period, since no allowance has been made for loss of the substrate from the tissue subsequent to capture. To calculate the gross rate of uptake, U, for a non-digestible substrate, equation 2.III has to be modified to correct for the loss by exocytosis and becomes:

$$U = \frac{Y}{M \times P} + R \qquad 2.VI$$

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

Likewise in calculating the gross rate of uptake of proteins, equation 2.IV becomes modified to:

$$U = \frac{Y + 10(S-F)}{M' \times P} + R' \qquad 2.VII$$

Where R' is the 'TCA-insoluble' radiotracer released from the yolk sac during its incubation period.

Thus, if R and R' can be determined accurately the true rates of uptake can be calculated. In practice, it is impossible to estimate either of these quantities accurately hence values of the Endocytic Index derived from the observed rates of uptake by the tissue will be lower than the true rates of uptake given in equations 2.VI and 2.VII, above. Providing the rates of release are negligible the 'true' and the 'observed' Endocytic Index will be indistinguishable, but, if a high rate of release of ingested substrate can be demonstrated, the 'observed' Endocytic Index calculated from either equation 2.III or 2.IV will be substantially lower than that derived by the above equations.

2.16 General treatment of uptake data

All uptake data were calculated using programs written for an ICL 4130 computer; these programs are given in the Appendix. The plots of uptake against time were all highly linear hence they were analysed by a standard linear regression program. Correlation coefficients greater than 0.95 were normally observed for all cultures containing calf serum; coefficients with values greater than 0.91 were normally observed for cultures without calf serum. In all cases experiments yielding correlation coefficients lower than above were rejected, as showing an abnormally high degree of scatter.

2.17 Exocytosis of radiotracer by the rat yolk sac

The rate of release of radio-labelled macromolecule associated with the yolk sac was studied by the method of Williams et al. (1975a) with certain modifications introduced to permit the study of digestible substrates. Three 17.5 day yolk sacs were incubated in a sterile 50 ml Erlenmeyer flask by essentially the same method as described in 2.10 but in the presence of ^{125}I-labelled PVP, ^{125}I-labelled dBSA or ^{125}I-labelled IgG, each at a higher than normal concentration

(5-10 μg/ml) in the culture medium. After addition of the substrate each flask was gassed again with oxygen/carbon dioxide (95:5) for 10 sec, sealed and re-incubated (for 3.0 h in the case of ^{125}I-labelled PVP and for 2.0 h for both ^{125}I-labelled dBSA and ^{125}I-labelled IgG). At the end of this incubation period the yolk sacs were removed from the flask, washed for 2 min in three changes of warmed,gassed medium 199 (30 ml) then placed in another flask containing fresh, gassed substrate-free medium 199 (10 ml), sealed and returned to the water bath.

Samples of culture medium (2 x 1.0 ml) were removed at regular intervals and placed in 3.0 ml disposable plastic tubes. The culture medium removed was replaced by 2.0 ml of fresh, warmed medium and the flask was gassed for 10 sec before being further incubated. The 'total radioactivity' present in each sample of medium was assayed on a gamma spectrometer using standard counting geometry.

The amount of 'TCA-soluble' radioactivity released when either ^{125}I-labelled dBSA or ^{125}I-labelled IgG was used as substrate was determined by adding first calf serum (0.1 ml) then trichloroacetic acid (20% w/v, 0.5 ml) to each tube to precipitate the protein. After centrifugation (2000 g for 20 min) the supernatants were decanted into new tubes and the 'TCA-soluble' radioactivity assayed. The observed count was normalised by multiplying by a factor of 1.35, the appropriate empirical correction factor.

At the end of the re-incubation the final medium samples were taken and the yolk sacs were removed, washed (3 x 2 min), in changes of cold (4°C) saline (20 ml, 1% w/v), then placed in a 5.0 ml volumetric flask and stored at -20°C until assayed for radioactivity as described in 2.4.

With ^{125}I-labelled PVP as substrate, the amount of substrate released over the re-incubation period was calculated using the formula:

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

Where T_n is the amount of 'total radioactivity' (c.p.m., corrected for background) released up to the time of the n^{th} sampling, and C_i the content of 'total radioactivity' (c.p.m. per ml medium, corrected for background) in the i^{th} sample of medium. The radioactivity released, T_n, was expressed in two ways. Firstly as the percentage of the radioactivity initially associated with the tissue (i.e. that remaining in the yolk-sac tissue after re-incubation plus the total released into the medium during the re-incubation period) and secondly as the amount (µg) of substrate released back into the medium from unit quantity of yolk-sac tissue. This second quantity was obtained by dividing T_n by the product of P, the protein content of the yolk sac and T, the activity associated with 1 µg substrate when assayed under standard counting geometry (c.p.m. corrected for background).

$$\frac{T_n}{T \times P} \qquad 2.IX$$

With either ^{125}I-labelled dBSA or ^{125}I-labelled IgG as substrate, two species of radioactivity (i.e. 'acid-soluble' and 'acid-insoluble') can be released into the medium during the re-incubation period rather than just a single species as in the case of ^{125}I-labelled PVP. It is therefore more informative to sum these species separately by applying the above calculations twice to the set of data, rather than to determine only the amount of undifferentiated activity released.

2.18 Release of β-N-acetylglucosaminidase by the rat yolk sac incubated in the presence and absence of homologous IgG.

The rate of release of the lysosomal enzyme N-acetylglucosaminidase was followed using the method described by Roberts et al. (1976) both in the presence and absence of homologous IgG at a concentration of 10 µg/ml.

Two yolk sacs were removed from a 17.5-day pregnant rat; one being placed in a flask containing medium 199 alone (15.0 ml) and the other in a flask containing medium 199 (15.0 ml) plus homologous IgG at a concentration of 10 µg/ml. The culture medium was maintained at 37°C under an atmosphere of O_2 + CO_2 (95:5) in a shaking water bath set at 100 strokes/min. Samples of the incubation medium (0.5 ml) were removed at intervals of 0, 15, 30, 60, 120, 180, 240 and 300 min the flasks being re-gassed for 10 sec with O_2 + CO_2 (95:5). Each sample was placed in a 3.0 ml disposable plastic tube and centrifuged at 1500 g for 15 min to remove detached and broken cells, decanted into fresh plastic tubes and stored -20°C until required for assay. At the end of the incubation period the yolk sac was removed from the flask and washed in 3 x 2 min changes of cold saline (1.0% at 4°C) and then homogenised using a Virtis homogenizer (Virtis Research Equipment, Gardiner, NY, USA.) at 4°C for 30 sec at speed setting 2 in 5 ml of 0.01M potassium phosphate buffer pH 7.4. The yolk-sac homogenates were then assayed for β-NAG activity.

2.19 Assay procedure for β-NAG

The assay procedure utilises the ability of β-NAG to hydrolyse the substrate 4-methylumbelliferyl 2-acetamido-2-deoxy-β-D-glucopyranosideto the fluorescent product 4-methylumbelliferone. The substrate was used at a concentration of 2.5mM dissolved in citrate/phosphate buffer, pH 4.3 (0.1M sodium citrate adjusted to pH 4.3 with 0.2M sodium phosphate). To 100 µl of substrate 50 µl of incubation medium was added and incubated at 37°C for 10 min when the reaction was stopped by addition of 2.8 ml of glycine-carbonate buffer (0.17M, pH 10.5). The yolk-sac homogenate was diluted 100-fold with 0.01M potassium phosphate buffer and duplicate 20 µl samples assayed for β-NAG activity in the same manner as the incubation medium. Standards containing 1.5nM-4-methylumbelliferone contained in glycine-carbonate

buffer (0.17M, pH 10.5) and suitable blanks were included with assay.

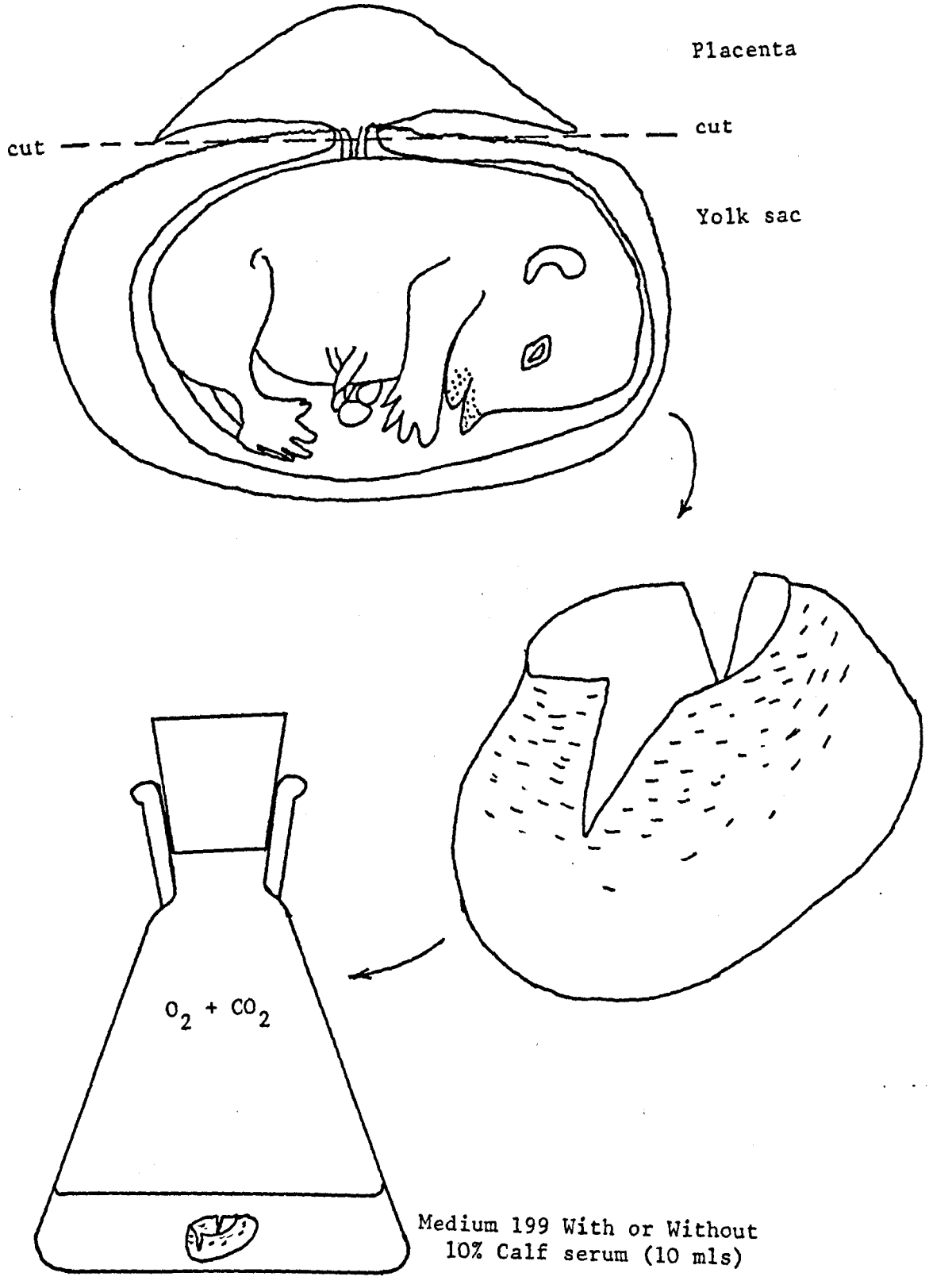
The activity of β-NAG enzyme was calculated in nmol. of substrate hydrolysed/h and the total activity (A_t) released for a given time interval was calculated using the equation

$$A_t = PV_t + \sum_{t=n}^{t=o} \frac{P}{2} \qquad 2.X$$

Where P is the activity of β-NAG in each ml of the re-incubation medium nmol./R, V_t is the volume of medium remaining at the sampling time t.

The released β-NAG activity was expressed as a percentage of the total β-NAG activity present in the yolk sac at the beginning of the incubation period. This value was obtained by adding the released β-NAG activity calculated for the final medium sample to the β-NAG activity determined for the yolk sac tissue at the end of the incubation period. The results calculated in this way are shown for a number of experiments in Chapter 6.

Fig.2a Diagrammatic representation of the dissection and incubation of the rat visceral yolk sac



CHAPTER 3

IN VITRO PINOCYTOSIS OF RADIOLABELLED SUBSTRATES BY THE RAT YOLK SAC

3.1 Introduction

Ryser (1968) described the process of protein uptake by mammalian cells as "An underdeveloped area" and put forward some criteria which a model culture system must fulfil in order to furnish meaningful results. The main criteria proposed were as follows. Firstly, the system used must permit distinction between adsorption and internalization. That is, the model system chosen and the methods of analysis used must enable the process of association with the surface of the cell/tissue without entry into the vacuolar system, to be distinguished clearly from the process of entry into the vacuolar system. He indicated that this is important since certain macromolecules are known to adsorb strongly to cell surfaces, this process being rapid and essentially independent of temperature. (Ryser further suggested that such temperature studies were a possible method of distinguishing between these two processes). Secondly, in any investigation of pinocytosis, the method must preserve integrity of the cells since cell lysis will obviously distort any uptake data gathered. Suitable techniques should allow optimum conditions to be maintained and cellular integrity to be estimated by the use of dye exclusion tests or some other method. Finally, Ryser considered the problem caused by the reincorporation of the hydrolysis products, arising from ingested proteins, into the tissue under investigation and proposed that this problem could be overcome by the use of the radio-iodinated proteins. The tyrosyl groups of proteins can be labelled with radioactive iodine (^{131}I or ^{125}I) and on hydrolysis within cells the iodiotyrosyl residues released are not re-utilized in protein synthesis but liberated from the cell, greatly simplifying the analysis of the experimental data.

Williams *et al.* (1975a) reported a tissue culture technique, based on the 17.5-day rat yolk sac incubated *in vitro*, and which fulfils Ryser's above criteria. The tissue was shown to survive well *in vitro*;

it accumulated the macromolecular substrate ^{125}I-labelled PVP linearly with time up to 18 h. Furthermore, a high degree of cellular integrity was observed both by ultrastructural studies and by measurement of the release of substrate from tissue that had previously been 'loaded' with ^{125}I-labelled PVP *in vitro*. When the protein ^{125}I-labelled BSA was used as a substrate, its ingestion was found to occur at a higher rate than that of ^{125}I-labelled PVP. The theoretical model proposed by Jacques (1969) was used to explain the mechanism of uptake. Further studies by Moore *et al*. (1977) using the same system showed adsorption of substrate to the cell surface prior to ingestion to play an important role in determining the rate of uptake of the substrate and that chemical modifications of ^{125}I-labelled BSA could radically increase the rate of ingestion of this protein.

Further work by Roberts *et al*. (1977) demonstrated the rat yolk sac's ability to ingest substrates of very different chemical properties. [U^{14}C] sucrose and colloidal [^{198}Au] gold were found to be ingested progressively over a 6.5 h incubation period and at a similar rate to ^{125}I-labelled PVP itself. Moreover, subsequent re-incubation in substrate-free medium showed only low rates of release, again demonstrating cellular integrity towards these substrates. Other proteins including ^{125}I-labelled insulin, ^{125}I-labelled glucagon and ^{125}I-labelled ribonuclease (Livesey, G., unpublished data) have also been investigated and shown to have high rates of uptake compared to ^{125}I-labelled PVP.

Histochemical studies by Beck *et al*. (1967) showed that following accumulation of horseradish peroxidase within the vitelline epithelium of the yolk sac, enzymic activity was lost and that the ingested material was almost entirely confined to the lysosomal system of these cells. *In vivo* studies on the uptake of ferritin (Lambson, 1966) demonstrated that this substrate was concentrated within the rat yolk sac and retained within it for long periods. The site of accumulation

was shown by electronmicroscopy to be the vacuolar system of the epithelial cells. Such observations provide strong circumstantial evidence that the observed hydrolysis of ^{125}I-labelled BSA and other proteins occurs within lysosomes. The possibility that hydrolysis of proteins may occur on the surface of cells by membrane-bound peptidases of the type described by Kerr and Kenny (1974) has recently been investigated by Livesey (unpublished data), but to date no evidence of such a process has been obtained. This re-enforces the belief that the lysosomal system is the major if not the only site of the observed protein catabolism.

All the in vitro studies reported above employed a tissue culture technique based on Eagle's medium containing 10% (v/v) calf serum (see Section 2.3 for method). This particular medium composition was selected because tissue incubated in it for several hours more closely resembled freshly excised tissue, when examined ultrastructurally, than did tissue incubated in medium 199 alone. Unfortunately, from the standpoint of analyzing the data on protein catabolism and pinocytosis this system has the complication that the vast excess of serum protein in the medium is able to compete with the tracer amounts of substrates offered to the tissue. This makes a rigorous analysis of the pinocytic process impossible when adsorption of a substrate occurs prior to ingestion. Hence the removal of these serum proteins becomes most desirable especially if the interaction of cell surface and substrate are to be investigated in detail. To this end Moore (1975) reported some preliminary findings on the uptake of ^{125}I-labelled PVP in the absence of calf serum and showed that under such conditions the tissue still ingested this substrate although the data were not very reproducible.

In this thesis the uptake experiments in the absence of calf serum were performed using a slightly modified version of the method of Moore

(see Section 2.8). The main differences were the dissection time was reduced to as short a period as possible to try and ensure better tissue survival and both the sampling intervals and overall incubation periods were reduced.

Using this technique, uptake of ^{125}I-labelled PVP was determined and compared to that in the presence of calf serum in order to establish a basal rate of pinocytosis in the absence of calf serum in the same manner as described by Williams et al. (1975a). Secondly, the method was employed to follow the effects of removing calf serum from the medium on the uptake of other substrates: namely [U^{14}C] sucrose, colloidal [^{198}Au] gold and ^{125}I-labelled dBSA which have previously been studied in the presence of calf serum (Roberts et al., 1977; Moore et al., 1977). Finally, the effects of increasing the overall PVP concentration were investigated in the absence of calf serum in order to determine whether ^{125}I-labelled PVP entered the cell solely in the fluid phase, as suggested by Williams et al. (1975a), or entered partly by adsorbing to the plasma membrane before ingestion.

METHODS

3.2 Uptake of ^{125}I-labelled PVP in the presence and absence of calf serum

17.5-day rat yolk sacs were incubated either in medium 199 plus 10% calf serum (see Section 2.3 for method) or in medium 199 alone (see Section 2.10 for method). ^{125}I-labelled PVP at a concentration of 2.0 - 5.0 µg per ml of medium, was used as substrate and incubations were terminated at regular intervals up to 6.5 h. The amounts of radioactivity in the yolk sac and medium were assayed as described in 2.4 and 2.6, respectively, and the protein content of each yolk sac determined (see 2.5). Uptake of substrate was plotted against time for each set of data and the related Endocytic Index calculated (see Section 2.12 for definition and units).

A typical uptake plot for ^{125}I-labelled PVP in medium 199 plus 10% calf serum is shown in Fig. 3a and a corresponding plot of uptake of the same substrate in medium 199 alone is shown in Fig. 3c. The results of a number of experiments of each type are given in Tables 3.Ia and 3.III. The results of Table 3.Ia are further summarized in Table 3.II.

3.3 Uptake of [U^{14}C]sucrose in the presence and absence of calf serum

Rat yolk sacs at 17.5 days of gestation, were incubated in medium 199 with 10% calf serum (see Section 2.3 for details) or in medium 199 alone (see Section 2.10 for details). The concentration of radiotracer employed was 0.1 µg/ml of culture medium and the incubations were terminated at regular intervals up to 6.5 h. The radioactivity was assayed as described in Sections 2.8 and 2.9 and the protein content of the yolk sacs was determined by the method of Lowry et al. (1951) described in Section 2.5.

The uptake of radiotracer was calculated using equation 2.III and the corresponding Endocytic Index calculated from the plot of uptake against time. Such results are shown in Table 3.Ib for experiments

in the presence and absence of calf serum, and summarized in Table 3.VII. Typical plots of uptake versus time are shown in Fig. 3a with calf serum and Fig. 3c in the absence of calf serum.

3.4 Uptake of colloidal [^{198}Au]gold in the presence and absence of calf serum

The methods used were identical to those for the uptake of ^{125}I-labelled PVP. The results for the uptake of colloidal [^{198}Au]gold in the presence and absence of calf serum are given in Table 3.Ic and are further summarized in Table 3.VII. Typical plots of uptake versus time are shown in Fig. 3a and Fig. 3d.

3.5 Uptake of ^{125}I-labelled dBSA in the presence and absence of calf serum

In similar experiments ^{125}I-labelled dBSA was used as substrate at concentrations of 0.6 - 1.0 µg/ml of medium. The 'total'- and 'soluble'-radioactivities in each sample of medium were assayed (see 2.6 and 2.7 for methods) together with the yolk sac radioactivity (see 2.4). From the results of each such experiment uptake of ^{125}I-labelled dBSA was plotted against time and the related Endocytic Index calculated as described in Section 2.13.

A typical uptake plot for ^{125}I-labelled dBSA in the presence of 10% calf serum is shown in Fig. 3b together with the levels of tissue-associated radioactivity from the same experiment. The amount of radioactivity associated with the tissue is expressed in the same units as uptake in order to facilitate comparison of the two quantities. Fig. 3e shows similar plots against time of both uptake and the quantity of tissue-associated radioactivity for a representative experiment in which ^{125}I-labelled dBSA is ingested in the absence of calf serum. The results of such experiments in both the presence and absence of calf serum, are shown in Tables 3.III and 3.VI and are summarized in Table 3.VII.

3.6 Uptake of ^{125}I-labelled PVP in the presence of unlabelled PVP (medium 199 alone)

In order to determine the effect of increasing the concentration of polyvinylpyrrolidone on the Endocytic Index of ^{125}I-labelled PVP, yolk sacs were incubated with ^{125}I-labelled PVP (2.0 µg/ml) in the presence of unlabelled polyvinylpyrrolidone of similar size (PVP-40) in the absence of calf serum. The Endocytic Index of ^{125}I-labelled PVP was determined at four different concentrations of PVP-40 (0, 10.0, 100.0 and 1000.0 µg/ml).

The results of each experiment are shown in Table 3.V and summarized in Table 3.VIII.

RESULTS

3.7 Endocytic Index of ^{125}I-labelled PVP, [U^{14}C]sucrose and colloidal [^{198}Au]gold in medium 199 plus 10% calf serum

Table 3.I shows the results obtained from the uptake of ^{125}I-labelled PVP from medium 199 containing 10% calf serum. The Endocytic Index (gradient) and correlation coefficient of the straight line, fitted by linear regression analysis, are shown for each plot together with the intercept on the ordinate axis. All plots with values of the Endocytic Index lower than 1.1 µl/h/mg protein or with correlation coefficients less than 0.95 were rejected as being atypical. (Plots which fell outside these limits showed either an unusually high degree of scatter or non-linearity but such plots constituted less than 10% of the total data.) In all experiments ^{125}I-labelled PVP was accumulated linearly in the yolk-sac tissue up to 7.0 h, the longest time period used in the current study. It should be noted that linear uptake has been observed for longer periods, up to 18 h by Williams (unpublished data). The average value for the Endocytic Index was found to be 1.70 $\pm$ 0.30 (S.D.) from a total of 55 experiments. This

figure is in close agreement with values obtained by Williams et al. (1975a), Moore et al. (1977) and Roberts et al. (1977) of 1.71; 1.5 ± 0.2 (S.D.); and 1.57 ± 0.35 (S.D.), respectively.

Table 3.II shows that there is little or no dependence of the Endocytic Index of ^{125}I-labelled PVP on the batch of material although Batches 142 BA to 149 BA showed a generally increased Endocytic Index compared to the other batches. This contrasts with the findings of Roberts et al. (1977) who observed a significant inter-batch variation in the Endocytic Index of colloidal [^{198}Au]gold in the yolk sac culture system. ^{125}I-labelled PVP therefore constitutes an ideal substrate with which to monitor a basal rate of pinocytosis in the tissue, and can be used both to check that no drift occurs in the performance of the culture system over a prolonged period of time and to monitor yolk sac function when modifications of the basic culture method are made (e.g. evaluating a new substrate for toxic or pharmacological effects that may alter the rate of pinosome formation, or on modifying the composition of the culture medium.)

The uptake of [U^{14}C]sucrose was, like that of ^{125}I-labelled PVP, highly linear in the presence of calf serum (see Fig. 3a) and the mean value of the Endocytic Index was found to be 3.69 ± 0.36 (S.D.) (Table 3.VII). This was somewhat higher than the value of 2.04 ± 0.21 (S.D.) obtained by Roberts et al. (1977) who also reported an Endocytic Index of 1.57 ± 0.35 (S.D.) for ^{125}I-labelled PVP. In the present study the mean value of Endocytic Index of ^{125}I-labelled PVP, in experiments performed in parallel with the sucrose experiments, was 2.05 ± 0.33 (S.D.).

Colloidal [^{198}Au]gold again showed highly linear uptake, (see Fig. 3a) with a mean Endocytic Index of 4.56 ± 0.62 (S.D.), more than twice that observed for ^{125}I-labelled PVP. Values of this magnitude have usually been found to be associated with Endocytic Indices obtained for proteins: for example ^{125}I-labelled BSA (unfrozen)

gave a value of 4.8 ± 1.5 (S.D.) (Moore et al., 1977) and ^{125}I-labelled IgG 6.27 ± 2.65 (S.D.) (see Chapter 5). Hence it would appear that colloidal [^{198}Au]gold may well be ingested in a manner more similar to that of proteins.

3.8 Endocytic Index of ^{125}I-labelled dBSA in medium 199 plus 10% calf serum

A typical plot of uptake against time for ^{125}I-labelled dBSA (Batch III) in the presence of 10% calf serum is shown in Fig. 3b. This plot shows highly linear uptake over a 6.0 h incubation period (Correlation Coefficient = 0.988). Also shown is the tissue level of ^{125}I-labelled dBSA i.e. the amount of radiotracer associated with the yolk-sac tissue at the time of sampling. It can be seen that after 2.0 h the tissue level reaches a constant value of about 80 µl/mg protein which is equivalent to approximately 0.08 µg of ^{125}I-labelled dBSA/mg of tissue protein. Table 3.IV shows the mean Endocytic Index for each batch of protein and also the mean quantity of radioactivity associated with the tissue (µl/mg protein) calculated, in each experiment, from the amount of radioactivity found associated with each yolk sac removed between 2.0 h of incubation and termination of the experiment. An overall mean was also calculated for each batch of ^{125}I-labelled dBSA from these same results (Table 3.VII).

No systematic variation in the overall mean value of the Tissue Radioactivity for the different batches of ^{125}I-labelled dBSA, ingested in the presence of calf serum, was observed (see Table 3.VII). Also there was no correlation between the value of the Endocytic Index and the Mean Tissue Radioactivity for a particular batch of ^{125}I-labelled dBSA.

Moore et al. (1977) found the Endocytic Index of formaldehyde-denatured bovine serum albumin to be 65.0 ± 11.0 (S.D.) µl/h/mg protein. This figure is in very close agreement with those values obtained in the current study: Batch II, 56.3 ± 10.3 (S.D.) Batch III, 64.8 ± 5.9

(S.D.); and Batch V, 69.5 ± 6.4 (S.D.) (see Tables 3.III and 3.VII). The relatively low Endocytic Index of 38.2 ± 2.7 (S.D.) observed for Batch I of this material can be attributed to its period of exposure to formaldehyde being only 3.0 h whereas all other batches were treated for 72 h (Table 2a).

3.9 Reproducibility of uptake experiments using medium 199 alone

Preliminary experiments using ^{125}I-labelled PVP as substrate, showed that in the absence of calf serum the reproducibility of the uptake data was reduced compared with that of similar experiments performed in the presence of 10% calf serum. Inspection of plots of ^{125}I-labelled PVP uptake in the absence of calf serum showed that the rate of uptake was substantially linear for the first few hours but an increased degree of scatter was observed over the remainder of the incubation period. A typical plot is shown in Fig. 3f and demonstrates the increased scatter that occurs at approximately 5 h in this case. This increase in scatter resulted in greatly reduced correlation coefficients for some incubations of more than 5 h duration hence for experiments in the absence of calf serum the maximum incubation period was reduced to 5 h and individual yolk-sac incubations were terminated either singly at 30 min intervals or in pairs at 60 min intervals.

A similar inspection of uptake plots for ^{125}I-labelled BSA in the absence of calf serum frequently showed a sharp reduction in the rate of uptake after 5 h of incubation as well as an increase in scatter. A typical plot of the uptake of ^{125}I-labelled BSA in the absence of calf serum is shown in Fig. 3g.

On inspecting several sets of such data with the aid of a visual display unit attached to the graph plotter of the ICL 4130 computer, it was observed that the majority of the plots showed a loss of linearity and increased scatter beyond 5 h of incubation. The overall incubation period was therefore limited to this time in subsequent

experiments of this type and sampling performed in the same manner as described for ^{125}I-labelled PVP.

Although the data collected for experiments where calf serum was not included in the culture medium showed slightly less reproducibility than for those cultures including calf serum, the sacrifice of a small degree of reproducibility is more than compensated for by the removal of a complex mixture of proteins. This allows a more detailed study of interactions between the yolk sac and substrate.

3.10 Endocytic Index of ^{125}I-labelled PVP in medium 199 alone

The results of uptake experiments in which yolk sacs were incubated with ^{125}I-labelled PVP in medium 199 alone are shown in Table 3.IV and a typical plot is shown in Fig. 3c. The mean Endocytic Index from 11 experiments (see Table 3.VII) was 2.47 $\pm$ 1.00 and is significantly higher than that from similar incubations in the presence of 10% calf serum (1.70 $\pm$ 0.30, S.D.). Removal of calf serum from the culture medium also leads to a fall in the reproducibility of such experiments as indicated by both a lowering of the correlation coefficient and an increase in the standard deviation of the mean Endocytic Index. The reduced reproducibility of such plots required correlation coefficients of 0.91 or greater to be accepted as being within the normal range as opposed to values in excess of 0.95 when calf serum was present.

Similarly, the removal of calf serum from the incubation medium resulted in an increase in the mean value of the Endocytic Index for [U^{14}C]sucrose although the standard deviations associated with these values were large (see Table 3.VII). For easy comparison a typical uptake versus time plot is shown in Fig. 3c along with a representative plot for ^{125}I-labelled PVP.

On the other hand, colloidal [^{198}Au]gold showed a dramatic increase in the Endocytic Index in the absence of calf serum,

(see Fig. 3d), rising from 4.56 ± 0.62 (S.D.) with calf serum to 55.62 ± 3.4 (S.D.) in the absence of calf serum; a 12-fold increase. This compares with similar large increases in Endocytic Index which were observed for ^{125}I-labelled dBSA (Table 3.VII) and ^{125}I-labelled IgG (Chapter 5) observed on removal of calf serum from the incubation medium.

3.11 Effect of increasing concentration of PVP-40 on the Endocytic Index of ^{125}I-labelled PVP in medium 199 alone

Tables 3.V and 3.VIII show the effect on Endocytic Index of ^{125}I-labelled PVP with increased concentration of PVP-40. These results clearly show that the Endocytic Index of ^{125}I-labelled PVP shows no significant change with increasing PVP-40 concentration over the range investigated.

3.12 Endocytic Index of ^{125}I-labelled dBSA in medium 199 alone

The rates of uptake of Batches II, III, IV and V of ^{125}I-labelled dBSA were determined using yolk sacs incubated in the absence of calf serum. Fig. 3e shows uptake with time for a typical experiment using ^{125}I-labelled dBSA (Batch V) in medium 199 alone. Again a highly linear plot is observed, similar to that for the same substrate (Batch III) incubated in medium 199 containing calf serum (Fig. 3b). Table 3.VI shows the results of a number of such experiments. The correlation coefficients of the plots of uptake against time are if anything slightly higher than those for ^{125}I-labelled PVP ingested in medium 199 alone, and the relative standard deviation in the mean Endocytic Index is generally found to be small. The most striking feature of these results is the large increase in Endocytic Index in the absence of calf serum relative to the Endocytic Index of the same substrate in the presence of 10% calf serum; Batches III and V show 3.7-5.5-fold increases in their rates of uptake, respectively.

The amount of radioactivity associated with the tissue in such

experiments shows that the absence of calf serum leads to a rise in tissue level to a maximum, at about 1.5 h, which subsequently falls with further incubation. This rise to a maximum value is not observed in plots where calf serum was present. The values of the Mean Tissue Radioactivity (μl/mg protein) are shown in Table 3.VI for each experiment and the value of overall Mean Tissue Radioactivity is shown in Table 3.VII. In the absence of calf serum both Batch III and V showed an increase in the value of the overall Mean Tissue Radioactivity of about 30 μl/mg protein relative to corresponding values from experiments in the presence of calf serum, but for Batch II no such increase was observed.

3.13 Comparison of results with ^{125}I-labelled PVP and ^{125}I-labelled dBSA as substrates

Table 3.VII summarizes the main findings with respect to the uptake of these two substrates by comparing the mean values of the Endocytic Indices determined from incubations performed both in the presence and absence of calf serum.

The uptake of ^{125}I-labelled PVP in 10% calf serum was observed to be highly linear up to 7.0 h and in such experiments the correlation coefficient of the uptake plot was usually in excess of 0.95. The Endocytic Index of ^{125}I-labelled PVP in the presence of 10% calf serum yields a valuable and reproducible reference value for pinocytic activity in cultured rat yolk sac (Table 3.Ia) and was used at regular intervals throughout the course of the work to confirm the reproducibility of the pinocytic activity of the incubated tissue.

Removal of calf serum from the culture medium gives rise to a slightly increased rate of uptake of ^{125}I-labelled PVP and somewhat greater variability between individual experiments so that the mean Endocytic Index is 2.47 $\pm$ 1.00 (S.D.) as opposed to 1.70 $\pm$ 0.30 (S.D.) for 10% calf serum incubations. Thus the presence of 10% calf serum

in the culture medium appears to be responsible for the depression of pinocytic uptake, equivalent to 31% when expressed relative to the Endocytic Index without addition of calf serum to the medium.

When ^{125}I-labelled dBSA was incubated with yolk sacs in the presence of calf serum, highly linear uptake was almost always observed up to 6.5 h with correlation coefficients in excess of 0.95. Again the absence of calf serum gave rise to slightly greater variability between individual experiments, as for ^{125}I-labelled PVP. The most striking effect of its removal from the medium was a large increase in Endocytic Index of ^{125}I-labelled dBSA relative to that of the same substrate in the presence of calf serum. Batches II, III and V showed an increase in Endocytic Index of between 4- and 6-fold.

In summary, the absence of calf serum from the culture medium results in a large increase in the Endocytic Index of ^{125}I-labelled dBSA but only a relatively small increase in that of ^{125}I-labelled PVP. Similarly, the Endocytic Index of [^{14}C]sucrose is little affected by the presence of calf serum whereas that of [^{198}Au]gold is greatly increased on removing calf serum from the incubation medium (see Tables 3.Ib and c).

3.14 Discussion

Jacques (1969) proposed that a macromolecular substrate entering a cell by pinocytosis has two potential mechanisms of ingestion. First, by fluid phase ingestion in which the substrate enters dissolved in a microscopic droplet of the extracellular liquid captured by movements of the cell membrane, and second, by prior adsorption of the substrate to that region of the plasma membrane that invaginates to form the limiting membrane of the pinosome. Both of these mechanisms are shown diagrammatically in Fig. 3 h.

If a substrate enters a cell in the fluid phase alone, the rate at which it enters is dependent on the product of the concentration of

the substrate in the extracellular liquid and the rate of fluid ingestion by pinocytosis, i.e.

$$Q = Fc \qquad 3.I$$

Where Q is the rate of uptake of the macromolecule (weight per unit time), F is the rate of fluid internalisation (volume per unit time) and c the substrate concentration in the extracellular liquid (weight per unit volume). Equation 3.I will fully describe the uptake of solutes that are not adsorbed to the plasma membrane. The rate of ingestion of such solutes will be dependent only on the concentration of the substrate in the extracellular liquid and the rate of pinosome formation. Furthermore, if two such substrates are offered simultaneously to the cell both should be ingested at rates equal to those for separate uptake of each substrate at the same concentration. Only if one of the substrates is present at a concentration which causes it to have a pharmacological effect on the process of pinosome formation, hence modify the value of F, will it influence the rate of ingestion of another substrate ingested in the same manner. Even when such an effect does occur, a pair of substrates undergoing simultaneous ingestion will be affected alike: i.e. both of them will show increased rates of ingestion or both will show decreased rates of ingestion.

At the opposite extreme, a pinocytic channel could be so flattened and narrow that extracellular fluid is effectively excluded from the nascent pinosome (although it may subsequently imbibe water intracellularly, to form a spherical pinosome, see Fig. 3h). In this mechanism, the only effective route of entry of the substrate is by adsorption to the plasma membrane. Jacques proposed the following equation to describe this mode of entry:

$$Q = \frac{SRc}{K+c} \qquad 3.II$$

Where K is the dissociation constant of the substrate-surface complex, R the maximum amount of substrate that can be adsorbed per unit area of cell surface (weight per unit area) and S the rate of internalisation of cell membrane (area per unit time). This formulation assumes that the process of adsorption of the substrate to the cell membrane is one in which equilibrium is rapidly established and, in its simplest form, involves a single class of adsorption sites with a relatively low degree of substrate specificity. The number of substrate binding sites is finite as implied by the presence of R in the above equation. Such a mechanism of uptake permits a pair of substrates to compete for receptors on the cell surface and thus reduce each other's rate of ingestion relative to that when ingested alone at the same concentration. This feature contrasts sharply with the model described above for uptake via the fluid phase alone, which precludes such competitive behaviour of substrates.

In a broader formulation of uptake the two mechanisms of ingestion can be envisaged to coexist, giving the general equation for uptake of:

$$Q = Fc + \frac{SRc}{K+c} \qquad 3.III$$

If no adsorption occurs, R will be zero and 3.III will simplify to 3.I whereas if adsorptive uptake is the major route of uptake fluid phase ingestion can effectively be ignored and 3.III will simplify to 3.II.

The Endocytic Index (E) as defined by Williams et al. (1975a) is given by 3.III divided by c, the concentration of the substrate, thus:

$$E = \frac{Q}{c} = F + \frac{SR}{K+c} \qquad 3.IV$$

For a non-adsorbed substrate the Endocytic Index is equal to F alone, hence is independent of substrate concentration and equivalent to the rate of capture of the extracellular liquid by the pinocytic cell.

It was proposed by Williams et al. (1975a) that pinocytosis was

entirely responsible for the progressive accumulation of ^{125}I-labelled PVP by the yolk sac. Furthermore, these authors tentatively equated the Endocytic Index of ^{125}I-labelled PVP, in 10% calf serum, with the rate of ingestion of fluid (Model I of Fig. 3h) on the assumption that ^{125}I-labelled PVP is not adsorbed to the plasma membrane prior to ingestion. This suggestion was supported by the observation that the Endocytic Index of ^{125}I-labelled PVP was independent of concentration over a substrate concentration range of 0.15 - 1000 µg/ml and is further supported by the observation that the Endocytic Index of ^{125}I-labelled PVP in the presence of ^{127}I-labelled BSA (Williams et al. 1975b) or ^{127}I-labelled dBSA (see Chapter 4 Section 4.) is not markedly depressed. From a parallel study (Roberts et al. 1977 and Table VII) it appears that [U^{14}C]sucrose is a second substrate that is ingested in the fluid phase alone, the Endocytic Index being close to that of ^{125}I-labelled PVP. Moreover, high concentrations of unlabelled sucrose (1000 µg/ml) did not cause a detectable fall in the Endocytic Index of [U^{14}C]sucrose (Roberts et al. 1977) when the latter was used as tracer in concentrations of only 0.1 µg/ml medium, suggesting that adsorption plays a negligible role in the ingestion of this solute. Again the removal of calf serum from the incubation medium resulted in very similar changes in the Endocytic Index for [U^{14}C]sucrose and ^{125}I-labelled PVP. These observations strongly suggest that both these substrates are ingested in the fluid phase.

A consequence of incubating yolk sacs in the presence of 10% calf serum is that the serum provides a high concentration of proteins that may well compete with the radiotracer for adsorption sites on the cell membrane, hence lower the Endocytic Index of the radiotracer. Williams et al. (1975b) modified the general equation for the Endocytic Index to allow for the effects of a second substrate, also ingested by adsorption to the same binding sites on the cell membrane, and

obtained the expression:

$$E = F + \frac{SRM}{KM + Ki + Mc} \qquad 3.V$$

Where M is the dissociation constant of the competitor-surface complex and i the concentration of competing substrate. This type of equation is appropriate when one additional substrate is present which competes for the same adsorption sites on the plasma membrane but should more than one additional species be present, as is probably the case with a complex mixture like serum, an even more complex expression becomes necessary. Thus to study quantitatively the competitive effects of substrates, the omission of calf serum from the medium becomes most desirable since equation 3.IV then fully describes the general uptake of a single substrate, providing it can be assumed that none of the low molecular weight components of medium 199 acts as an effective competitor for the same binding sites as used by proteins and other macromolecules. Likewise, if this same assumption can be made, equation 3.V will fully describe the competition that occurs between two substrates forthe same adsorption sites on a pinocytic cell.

In the case of ingestion of solute in the fluid phase only, the rate of uptake is given by equation 3.I. This equation divided by c, the concentration of substrate, gives an expression for the Endocytic Index (E)

i.e. $$E = \frac{Q}{c} = F \qquad 3.VI$$

This equation shows that for a non-adsorbing substrate the Endocytic Index is equal to the rate of fluid ingestion hence independent of substrate concentration provided the rate of pinosome formation is not altered by changes in the solute concentration.

Table 3.VIII shows the Endocytic Index of ^{125}I-labelled PVP to be independent of the concentration of PVP-40 up to a concentration of 1000 µg/ml of culture medium (PVP-40 was used as a substitute for the

radiotracer at high concentration because of the high cost of using ^{125}I-labelled PVP itself). However, since the ^{125}I-labelled PVP preparation routinely used was labelled to an extent of less than one atom of iodine per 100 molecules of polymer, it was thought justifiable to substitute unlabelled polyvinylpyrrolidone of a similar molecular weight (PVP-40) for the radiotracer.

The observation that the Endocytic Index of the ^{125}I-labelled PVP in the absence of calf serum is independent of the total PVP concentration substantiates the suggestion that ^{125}I-labelled PVP is ingested almost entirely via the fluid phase i.e. adsorption to the plasma membrane plays a negligible role in the uptake of this substrate. The experiments of Williams et al. (1975a) that showed the Endocytic Index of ^{125}I-labelled PVP to be independent of it s concentration were performed in 10% (v/v) calf serum; hence any component of calf serum that adsorbed on the binding sites more avidly than ^{125}I-labelled PVP could have totally displaced the ^{125}I-labelled PVP from the plasma membrane depressing the rate of substrate ingestion and rendering it independent of concentration. The observation of only a marginal increase in the Endocytic Index of ^{125}I-labelled PVP in the complete absence of calf serum indicates that this process can only be of limited quantitative significance, if any. Thus, the observed increase of 45% in the Endocytic Index of ^{125}I-labelled PVP in the absence of calf serum relative to that in the presence of 10% (v/v) calf serum can be attributed either to this effect or to the absence from the culture medium of some factor(s) present in calf serum that reduces the rate of vesicle formation.

In contrast, the absence of calf serum from the culture medium results in a large increase in the Endocytic Index of ^{125}I-labelled dBSA and colloidal [^{198}Au]gold. Of the batches of protein; Batch V shows the greatest effect giving a 5.5-fold increase in its Endocytic

Index whereas colloidal [^{198}Au]gold showed a 12-fold increase. This compares to only a 45% increase for ^{125}I-labelled PVP in the absence of calf serum. Clearly, the large increases in the rates of capture of these substrates cannot be accounted for solely by an increased rate of vesicle formation, since results with ^{125}I-labelled PVP as substrate indicate that no more than a 45% increase can be attributed to this origin. On the contrary, these results indicate that serum proteins compete with both ^{125}I-labelled dBSA and [^{198}Au]gold for adsorption sites on the plasma membrane. Moreover, an Endocytic Index of 2.47 µl/h/mg protein for ^{125}I-labelled PVP means that an average yolk sac of 5.0 mg protein ingests approximately 12.5 µl/h of the extracellular medium, assuming ^{125}I-labelled PVP is internalized in the fluid phase alone. If, on the other hand, ^{125}I-labelled dBSA were ingested entirely in the fluid phase then for Batch V (Endocytic Index 381.5 $\pm$ 24.8 µl/h/mg protein) an average yolk sac would ingest 1905 µl/h (nearly 2.0 ml per hour) of the extracellular medium. It would seem inconceivable that a yolk sac could turn over sufficient plasma membrane to maintain such a rate of internalization.

The uptake of radiotracer in the absence of calf serum is fully described by equation 3.IV. If, to a good approximation, the rate of fluid phase ingestion can be equated with the Endocytic Index of ^{125}I-labelled PVP in the absence of calf serum (i.e. F = 2.47 $\pm$ 1.0 µl/h/mg protein). Then for ^{125}I-labelled dBSA (Batch V) equation 3.IV becomes:

$$E = 381.1 = 2.47 + \frac{SR}{K+c}$$

$$\text{i.e. } \frac{SR}{K+c} = 381.1 - 2.47 = 278.63 \text{ µl/h/mg protein} \qquad 3.VII$$

Thus adsorption accounts for 278.6 µl/h/mg protein which is equivalent to 99.3% of the total ^{125}I-labelled dBSA ingested per hour.

The observed competition between ^{125}I-labelled dBSA and the serum

proteins demonstrates that the adsorption centres or loci present on the plasma membrane are essentially non-specific in nature, as would be expected for binding sites of this type, since it would be inconceivable that a highly specific receptor for ^{125}I-labelled dBSA should exist. However, it appears that the affinity of a protein for these binding sites is dependent on certain characteristics of the protein. Thus Moore et al. (1977) demonstrated that a number of preparations of bovine serum albumin that had been treated with a variety of chemical agents were ingested at substantially different rates even in the presence of 10% calf serum. In the current study, Table 3.VII shows the Endocytic Index of ^{125}I-labelled dBSA (Batch I) to be significantly lower than that for the other batches. This is probably a direct result of the shortened exposure of Batch I to the formaldehyde solution (3.0 h).

Moore found that formaldehyde-treatment resulted in a large increase in the rate of uptake of ^{125}I-labelled albumin and that such treatment was not accompanied by aggregation; hence the increased uptake must result from either chemical modification of certain groups or a change in the general conformation of the molecule. The most favoured reaction of formaldehyde is with the ε-NH_2 of lysine but reaction with the α-NH_2 and methylene bridge insertion into disulphide bonds also occurs. It is known that the reaction of formaldehyde with protein residues is complex and that reaction rates vary from very fast to very slow. This would appear to be consistent with pinocytosis results obtained for ^{125}I-labelled dBSA (Batch I) where a shortened exposure time (3.0 h) to formaldehyde results in a lower Endocytic Index than for batches exposed for longer periods (72 h). The dependence of the Endocytic Index of ^{125}I-labelled dBSA on the time of treatment with formaldehyde solution would seem to indicate incomplete interaction within a 3.0 h period. The longer treatment

would allow the slower reactions time to establish equilibrium which may modify the protein's subsequent interactions with the yolk-sac plasma membrane.

In a similar manner to ^{125}I-labelled dBSA, the increase in Endocytic Index of colloidal [^{198}Au]gold in the absence of calf serum indicates strong adsorption of this substrate to the cell surface prior to ingestion. It would seem that this substrate is treated essentially as a protein, at least during the ingestion phase. This is not so surprising, since the colloidal [^{198}Au]gold used was gelatin-stabilized thus the results are not for the uptake of particulate gold but for a gelatin-stabilized colloid. Moreover, Roberts et al. (1977) found the Endocytic Index of colloidal [^{198}Au]gold to fall progressively with increased concentration, demonstrating competition for binding sites on the plasma membrane; this behaviour is typical of a protein (see Chapter 4).

Table 3.VII shows that, for a particular batch of ^{125}I-labelled dBSA, the mean amount of tissue-associated radioactivity does not correlate with the Endocytic Index in the presence of calf serum. Removal of calf serum leads to a rise of approximately 31% (relative to the value of the tissue-associated radioactivity in the presence of 10% calf serum) for Batches III and V although no change was observed for Batch II. It should be stressed, however, that this increase is small compared with the increase in the Endocytic Index and indicates that a slightly higher steady state concentration of ^{125}I-labelled dBSA in the tissue accompanies the higher uptake of this labelled protein in the absence of competing serum proteins. If this increase were a consequence of saturating the digestive capacity of the yolk sac, then a progressive accumulation of radioactivity by the tissue would be expected.

On the contrary, inspection of Fig. 3e shows that in the absence

of calf serum the tissue radioactivity actually decreases with increased incubation time from a maximum at about 1.5 h. Analysis of several such plots showed that the fall in tissue radioactivity occurred for each batch of ^{125}I-labelled dBSA and was normally about 25% to 30% relative to the maximum and could be explained simply by depletion of radiotracer from the culture medium. For example, the rate of ingestion of a labelled albumin, with an Endocytic Index of 300 µl/mg protein, by an average sized yolk sac (containing 5.0 mg of tissue protein) would result in the substrate contained in 7500 µl of culture medium being ingested during a 5.0 h incubation. With a total volume of 10 ml of culture medium this is equivalent to a 75% depletion of available substrate, hence it seems likely that such a large fall in substrate concentration could adequately account for the fall in tissue radioactivity. (This effect is discussed further in Chapter 4, Section 4.7). In the presence of calf serum the Endocytic Index is considerably lower being about 60 µl/h/mg protein which results in capture of substrate from 1550 µl of medium. i.e. 15% depletion of radiotracer over 5.0 h. The much lower rate of radiotracer depletion in the presence of calf serum would account for the absence of a fall in the tissue radioactivity.

Table 3.Ia Endocytic Indices of ^{125}I-labelled PVP in medium 199 containing calf serum (10%, v/v)

Each row of data refers to individual experiments in which 17.5-day rat yolk sacs were incubated with ^{125}I-labelled PVP in the presence of calf serum.

Batch of ^{125}I-PVP	^{125}I-PVP Conc. (µg/ml)	No. of yolk sacs	Endocytic Index (µl/h/mg protein)	Corr. Coeff.	Intercept (µl/mg protein)
116A	5	10	1.85	0.953	+ 0.71
"	"	11	1.12	0.965	+ 0.63
"	"	7	1.72	0.983	+ 0.48
"	"	8	1.53	0.979	+ 0.26
"	"	10	1.68	0.988	+ 0.50
"	"	10	1.76	0.998	+ 0.19
117A	"	8	1.91	0.986	+ 0.50
"	"	6	1.81	0.961	+ 0.51
"	"	6	1.81	0.954	+ 0.82
119A	"	7	1.53	0.991	+ 1.56
"	"	6	1.51	0.993	+ 0.84
121A	"	7	1.44	0.991	+ 0.20
"	"	8	1.67	0.967	+ 0.26
"	"	6	1.12	0.950	+ 1.03
123A	"	10	1.92	0.971	+ 0.40
"	"	10	1.91	0.983	+ 0.11
"	"	10	1.40	0.958	+ 0.95
"	"	10	1.61	0.962	+ 0.26
"	"	10	1.41	0.979	+ 1.25
129BA	"	10	1.60	0.980	+ 1.70
"	"	8	1.58	0.973	+ 0.323
"	"	10	1.78	0.979	+ 0.66
"	"	10	1.47	0.961	+ 0.99
"	"	7	1.39	0.998	+ 0.87
130DA	"	10	1.33	0.952	+ 0.22
"	"	10	1.72	0.978	+ 0.97
"	"	10	1.72	0.965	+ 1.22

Table 3.Ia (continued) Endocytic Indices of ^{125}I-labelled PVP in medium 199 containing calf serum (10%, v/v)

Batch of ^{125}I-PVP	^{125}I-PVP Conc. (µg/ml)	No. of yolk sacs	Endocytic Index (µl/h/mg protein)	Corr. Coeff.	Intercept (µl/mg protein)
132DA	5	10	1.58	0.967	+ 0.98
"	2.0	10	2.08	0.994	+ 0.60
"	"	10	1.61	0.991	+ 0.39
"	"	10	1.42	0.968	+ 1.24
"	"	8	1.57	0.991	+ 1.07
"	"	10	1.50	0.986	+ 1.06
"	"	6	1.73	0.977	+ 0.15
136BA	"	9	1.34	0.965	+ 0.89
"	"	10	1.58	0.981	+ 1.41
"	"	10	1.27	0.985	+ 0.39
140 BA	"	10	1.20	0.991	1.02
"	"	10	1.48	0.974	1.50
"	"	10	1.78	0.985	I.10
142BA	"	10	2.06	0.986	0.20
145BA	"	10	1.48	0.982	0.79
"	"	10	2.15	0.975	- 0.22
"	"	7	2.20	0.977	0.80
"	"	9	2.00	0.966	0.70
"	"	10	2.10	0.979	0.41
146BA	"	10	2.17	0.991	0.32
"	"	10	1.97	0.975	0.97
"	"	10	1.88	0.987	0.71
"	"	10	1.89	0.986	0.68
149BA	"	10	2.52	0.994	0.13
"	"	10	2.17	0.968	0.86
"	"	10	2.00	0.989	1.15
"	"	10	1.95	0.962	0.17
"	"	10	1.61	0.963	1.40

Table 3.Ib Endocytic Indices of [$U^{14}C$]sucrose in medium 199 in the presence and absence of calf serum

Each row of data refers to individual experiments in which 17.5-day rat yolk sacs were incubated with [$U^{14}C$]sucrose either in the presence or absence of calf serum.

	[$U^{14}C$]sucrose Conc. (µg/ml)	No. of yolk sacs	Endocytic Index (µl/h/mg protein)	Corr. Coeff.	Intercept (µl/mg protein)
With calf serum (10%, v/v)	0.1	8	3.32	0.968	-0.18
	"	8	4.03	0.971	-0.24
	"	8	3.72	0.967	+2.09
Without calf serum	"	10	3.13	0.986	+0.63
	"	8	4.80	0.974	-0.41
	"	10	4.46	0.925	+0.81

Table 3.Ic Endocytic Indices of colloidal [^{198}Au]gold with 17.5-day yolk sacs incubated in medium 199 in the presence and absence of calf serum

Each row of data refers to individual experiments in which 17.5-day rat yolk sacs were incubated with colloidal [^{198}Au]gold either in the presence or absence of calf serum.

	[^{198}Au]gold Conc. (µg/ml)	No. of yolk sacs	Endocytic Index (µl/h/mg protein)	Corr. Coeff.	Intercept (µl/mg protein)
With calf serum (10%, v/v)	1.0	8	4.87	0.975	+ 1.63
	"	9	3.85	0.974	+ 4.29
	"	8	4.97	0.977	+ 2.55
Without calf serum	"	10	57.82	0.966	-21.11
	"	9	57.34	0.950	-23.12
	"	8	51.71	0.927	+30.76

Table 3.II Summary of Endocytic Indices for different batches of ^{125}I-labelled PVP (medium 199 plus 10% (v/v) calf serum)

Each row of data shows the mean Endocytic Index (± S.D.) calculated from a number of experiments with each batch of ^{125}I-labelled PVP (data summarised from Table 3.Ia).

Batch of ^{125}I-PVP (Radiochemical Centre Code No.)	^{125}I-PVP Concentration (µg/ml medium)	No. of Experiments	Mean Endocytic Index (± S.D.) (µl/h/mg protein)
116A	5	6	1.61 ± 0.26
117A	"	3	1.84 ± 0.06
119A	"	2	1.52 -
121A	"	3	1.41 ± 0.28
123A	"	5	1.65 ± 0.26
129BA	"	5	1.57 ± 0.15
130DA	"	3	1.59 ± 0.23
132DA	2	7	1.64 ± 0.22
136BA	"	3	1.40 ± 0.16
140BA	"	3	1.49 ± 0.29
142BA	"	1	2.06 -
145BA	"	5	1.99 ± 0.29
146BA	"	4	1.98 ± 0.14
149BA	"	5	2.05 ± 0.33

Table 3.III Pinocytosis results for ^{125}I-dBSA in medium 199 containing calf serum (10%, v/v)

Each row of data refers to uptake data obtained from 17.5-day rat yolk sacs incubated with ^{125}I-labelled dBSA as substrate in medium 199 containing 10% calf serum.

Batch of ^{125}I-dBSA	Conc. (µg/ml)	No. of yolk-sacs	Endocytic Index (µl/h/mg protein)	Corr. Coeff.	Intercept (µl/mg protein)	TCA-solubles in ^{125}I-dBSA (%)	Mean tissue radio-activity (µl/mg protein)
I	1.0	12	35.9	0.957	+28.6	1.69	70.6
"	1.0	6	41.2	0.970	-11.6	1.39	65.8
"	1.0	6	37.4	0.990	+16.9	1.73	55.8
II	1.0	9	72.5	0.984	-26.7	4.6	80.9
"	1.0	9	57.1	0.989	-30.9	3.5	69.2
"	1.0	10	54.7	0.971	-0.4	5.13	65.3
"	1.0	6	44.4	0.981	-17.7	5.13	58.8
"	1.0	7	52.7	0.955	-2.5	3.5	76.9
III	1.0	8	67.1	0.967	-20.2	1.16	73.4
"	1.0	8	64.8	0.988	-5.7	1.18	84.6
"	1.0	8	66.8	0.956	+51.5	0.92	102.0
"	1.0	8	70.4	0.986	+21.1	0.92	96.9
"	1.0	8	66.1	0.978	+ 3.4	2.18	119.5
"	1.0	8	53.3	0.971	+16.8	2.18	83.6
V	0.6	9	62.1	0.991	-12.7	1.05	46.5
"	0.6	9	63.8	0.981	+22.0	1.05	86.5
"	0.6	10	76.8	0.976	-35.5	2.9	80.4
"	0.6	10	78.9	0.936	-16.0	2.9	82.9
"	0.6	10	65.8	0.985	- 1.4	1.7	61.4
"	0.6	12	71.1	0.925	+100.5	0.02	61.2
"	0.6	12	68.1	0.970	+83.2	0.02	78.8
VI	1.0	10	87.8	0.985	+ 1.4	0.96	91.8
"	1.0	10	96.8	0.993	-10.0	1.02	93.1
"	1.0	10	77.3	0.990	10.4	0.93	83.8
"	1.0	10	65.2	0.985	10.1	0.96	71.5

Table 3.IV Pinocytosis of ^{125}I-labelled PVP in TC 199 alone

Each row of data refers to individual incubations performed with 17.5-day rat yolk sacs incubated with ^{125}I-labelled PVP in the absence of calf serum.

Batch of ^{125}I-PVP	^{125}I-PVP Concentration (µg/ml)	No. of Yolk-sacs	Endocytic Index (µl/h/mg protein)	Corr. Coeff.	Intercept (µl/mg protein)
116A	5.00	6	1.06	0.948	+3.3
"	"	10	2.95	0.939	+1.8
"	"	10	4.69	0.969	-0.8
"	"	10	3.46	0.979	+0.2
"	"	7	2.27	0.970	+2.4
117A	"	7	1.58	0.979	+3.8
"	"	6	1.5	0.924	+1.8
129BA	"	10	2.24	0.921	+2.1
130DA	"	10	2.35	0.989	+1.1
132DA	"	10	2.79	0.948	+3.1
"	"	10	2.26	0.966	+2.0

Table 3.V Endocytic Index of ^{125}I-PVP in the presence of unlabelled PVP in medium 199 alone

Each row of the table refers to uptake data for ^{125}I-labelled PVP by 17.5-day rat yolk sacs incubated in the presence of different concentrations of PVP-40 and a constant tracer concentration of ^{125}I-labelled PVP.

PVP-40 Conc. (µg/ml)	No. of yolk-sacs	Endocytic Index (µl/h/mg protein)	Correlation Coefficient	Intercept
10	10	2.06	0.986	+0.5
"	10	2.61	0.935	+2.1
"	8	4.10	0.973	+1.5
100	10	2.27	0.918	+2.0
"	10	2.20	0.938	+3.0
"	10	2.61	0.935	+2.1
1000	8	1.78	0.993	+0.8
"	9	1.97	0.983	+2.3
"	10	3.00	0.965	2.3
"	10	2.60	0.947	1.9

Table 3.VI Endocytic Indices of ^{125}I-labelled dBSA in medium 199 alone

Each row of the table refers to uptake data for 17.5-day rat yolk sacs incubated with ^{125}I-labelled dBSA in the absence of calf serum.

Batch of ^{125}I-dBSA	^{125}I-dBSA Conc. (µg/ml)	No. of yolk-sacs	Endocytic Index (µl/h/mg protein)	Corr. Coeff.	Intercept (µl/mg protein)	TCA-solubles in ^{125}I-dBSA (%)	Mean tissue radio-activity (µl/mg protein)
II	1.0	9	142.9	0.976	+115.7	4.4	73.2
"	1.0	8	208.9	0.987	- 20.7	4.6	77.4
"	1.0	6	124.0	0.986	+ 56.2	3.5	59.8
III	1.0	8	274.5	0.972	- 38.3	1.93	152.7
"	1.0	8	241.3	0.952	- 90.9	1.93	103.4
"	1.0	8	210.8	0.946	+120.3	0.90	117.8
IV	1.5	9	236.6	0.940	+ 95.3	1.4	86.3
"	1.5	10	228.6	0.948	+ 33.1	1.4	76.1
"	1.5	10	223.7	0.974	+ 15.8	3.1	86.8
"	1.5	9	230.3	0.954	+ 47.7	3.1	70.6
V	0.6	10	407.4	0.984	+ 0.6	2.98	101.3
"	0.6	10	378.9	0.981	+ 4.3	1.39	97.8
"	0.6	10	358.1	0.967	- 4.4	2.90	108.7

Table 3.VII Summary of the Endocytic Indices of ^{125}I-labelled PVP, ^{125}I-labelled dBSA, [U^{14}C]sucrose and colloidal [^{198}Au]gold in the presence and absence of calf serum

Summary of the uptake data obtained for 17.5-day rat yolk sacs incubated with ^{125}I-labelled PVP and ^{125}I-labelled dBSA in the presence and absence of calf serum.

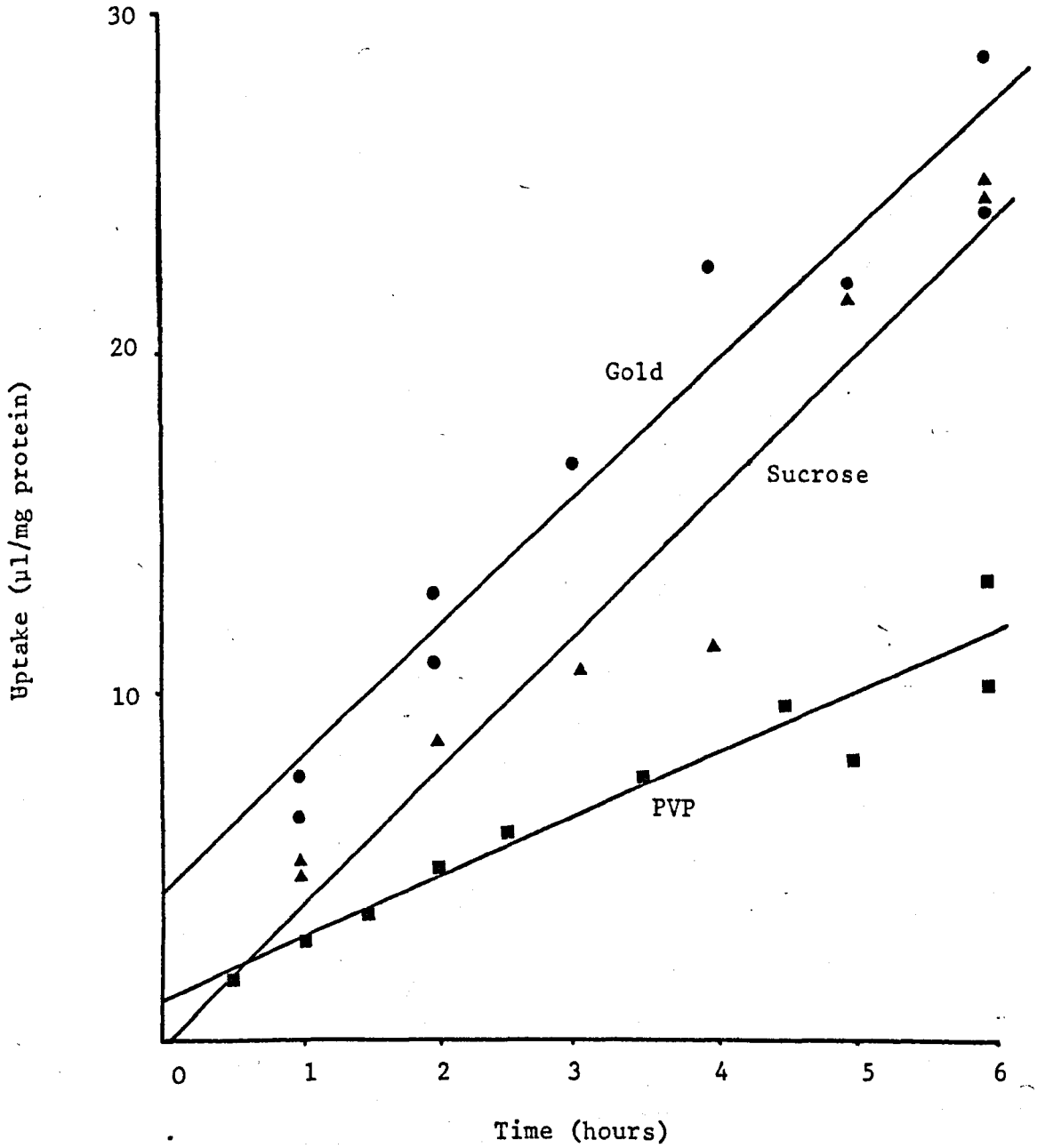
Substrate	Medium	No. of Experiments	Mean Endocytic Index, ± S.D. (µl/h/mg protein)	Overall mean tissue radioactivity (µl/mg protein
^{125}I-PVP	TC 199 + 10% calf serum	55	1.7 ± 0.30	-
^{125}I-PVP	TC 199 alone	11	2.47 ± 1.00	-
[U^{14}C]sucrose	TC199 + 10% calf serum	3	3.69 ± 0.36	-
[U^{14}C]sucrose	TC 199 alone	3	4.13 ± 0.88	-
[^{198}Au]gold	TC 199 + 10% calf serum	3	4.56 ± 0.62	-
[^{198}Au]gold	TC 199 alone	3	55.62 ± 3.40	-
^{125}I-dBSA (Batch I)	TC 199 + 10% calf serum	3	38.2 ± 22.7	64.1 ± 7.6
^{125}I-dBSA (Batch II)	TC 199 + 10% calf serum	5	56.3 ± 10.3	70.2 ± 8.7
^{125}I-dBSA (Batch III)	TC 199 + 10% calf serum	6	64.8 ± 5.9	93.3 ± 16.4
^{125}I-dBSA (Batch V)	TC 199 + 10% calf serum	7	69.5 ± 6.4	71.1 ± 14.8
^{125}I-dBSA (Batch VI)	TC 199 + 10% calf serum	4	81.8 ± 13.6	85.0 ± 9.9
^{125}I-dBSA (Batch II)	TC 199 alone	3	158.6 ± 44.6	70.1 ± 9.2
^{125}I-dBSA (Batch III)	TC 199 alone	3	242.2 ± 31.9	124.6 ± 25.4
^{125}I-dBSA (Batch IV)	TC 199 alone	4	229.8 ± 5.3	80.0 ± 8.0
^{125}I-dBSA (Batch V)	TC 199 alone	3	381.5 ± 24.8	102.6 ± 5.6

Table 3.VIII Summary of the Endocytic Index of ^{125}I-labelled PVP in the presence of PVP, medium 199 alone

Summary of data presented in Table 3.V

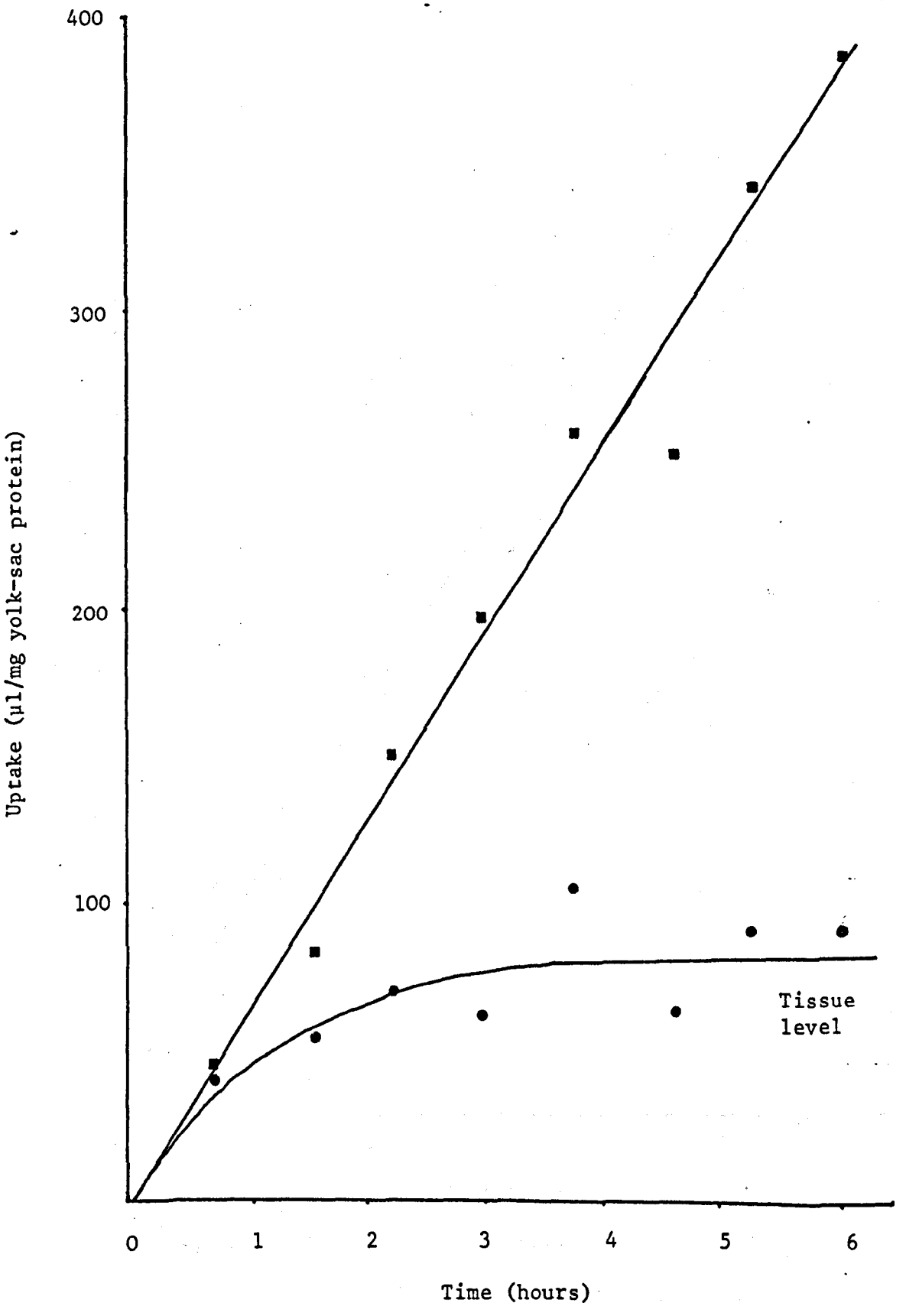
PVP-40 Concentration (µg/ml)	No. of Experiments	Mean (± S.D.) Endocytic Index (µg/h/mg protein)
0	11	2.47 ± 1.00
10	3	2.92 ± 1.06
100	3	2.14 ± 0.17
1000	4	2.34 ± 0.56

Fig. 3a Plot of uptake against time for ^{125}I-labelled PVP, [U^{14}C] sucrose and colloidal [^{198}Au] gold in the presence of calf serum



[U^{14}C] sucrose	Endocytic Index	= 4.03 µl/h/mg protein
	(Correlation Coefficient	= 0.971)
[^{198}Au] gold	Endocytic Index	= 3.85 µl/h/mg protein
	(Correlation Coefficient	= 0.974)
^{125}I-labelled PVP	Endocytic Index	= 1.72 µl/h/mg protein
	(Correlation Coefficient	= 0.965)

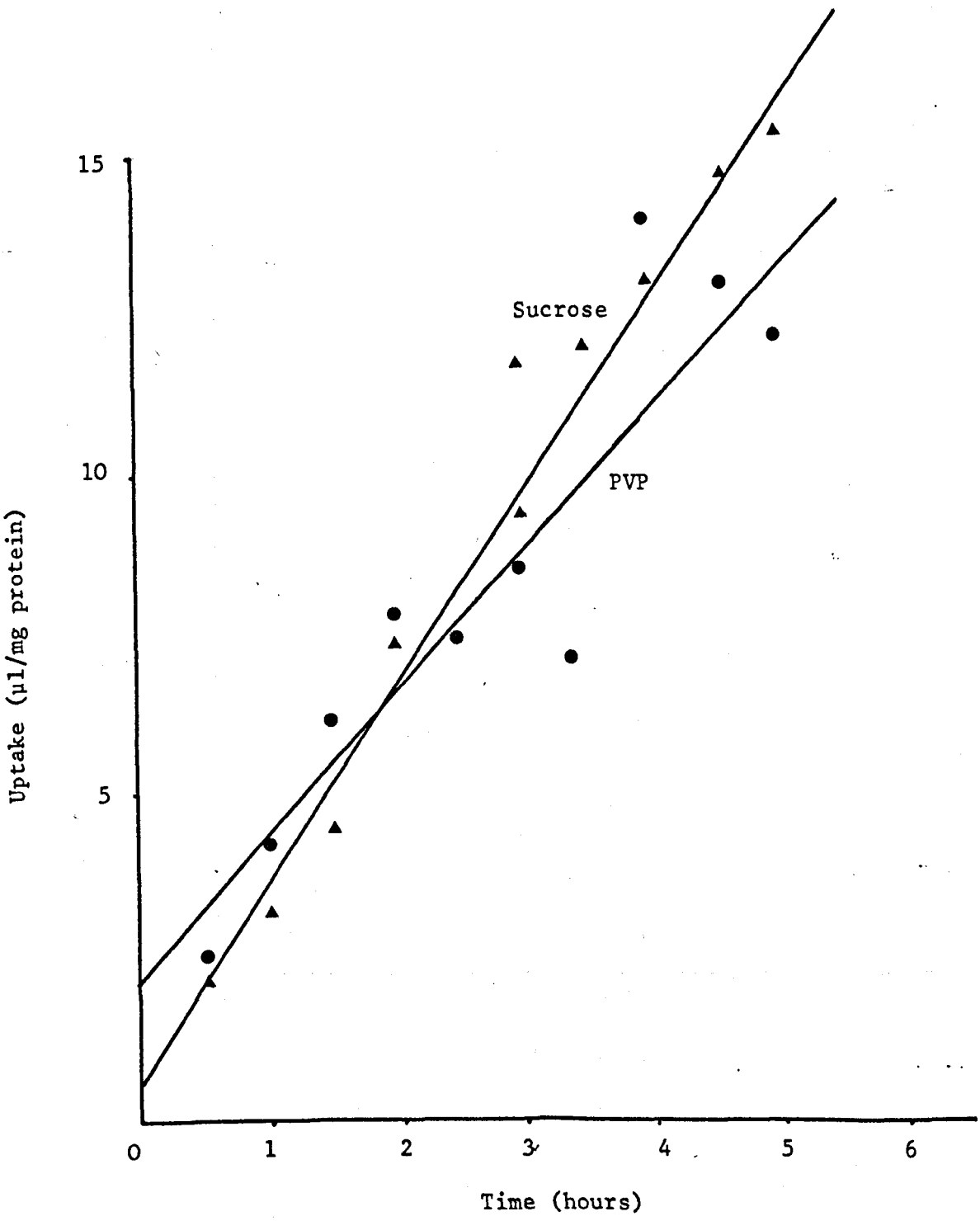
Fig. 3b Plots of uptake and tissue level against time for ^{125}I-labelled dBSA (Batch III) in the presence of calf serum



Endocytic Index = 64.8 µl/h/mg yolk-sac protein

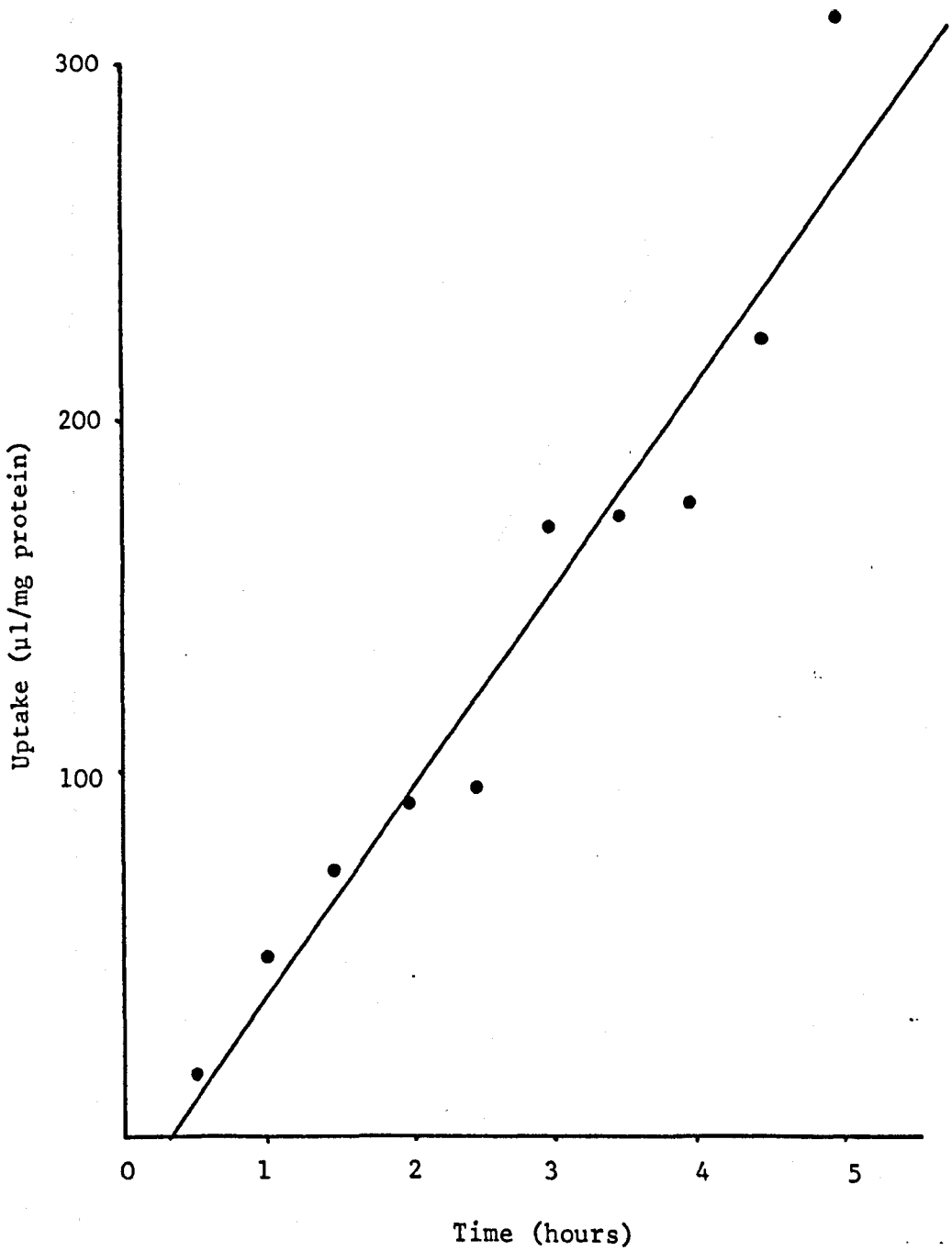
(Correlation Coefficient = 0.988

Fig. 3c Plot of uptake against time for ^{125}I-labelled PVP and [U^{14}C] sucrose in the absence of calf serum



^{125}I-labelled PVP	Endocytic Index	= 2.24 µl/h/mg protein
	(Correlation Coefficient = 0.921)	
[U^{14}C] sucrose	Endocytic Index	= 3.13 µl/h/mg protein
	(Correlation Coefficient = 0.986)	

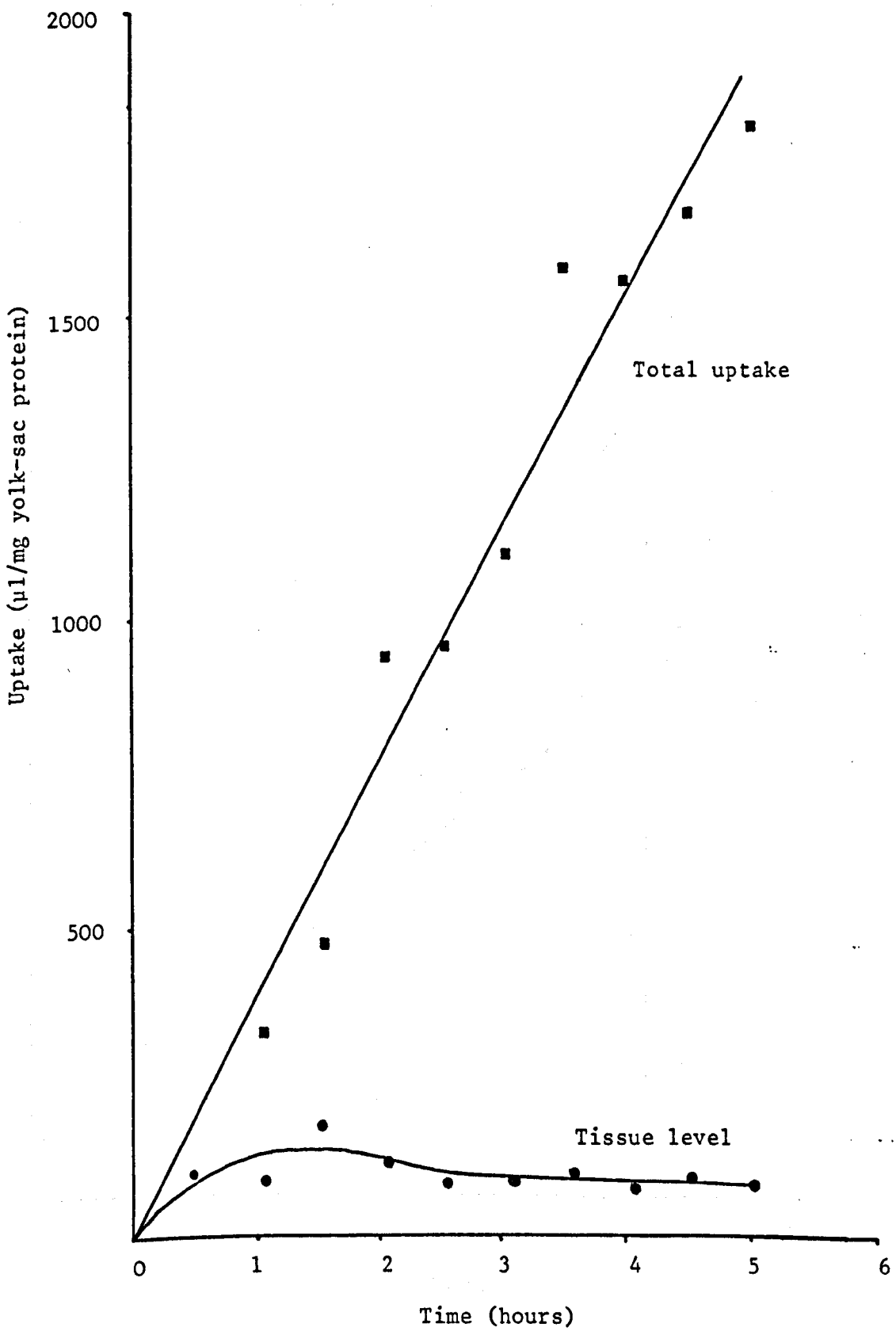
Fig.3d Plot of uptake against time for colloidal [^{198}Au] gold in the absence of calf serum



Endocytic Index = 57.82 µl/h/mg yolk-sac protein

(Correlation Coefficient = 0.966)

Fig.3e Plots of uptake against time for ^{125}I-labelled dBSA (Batch V) in the absence of calf serum

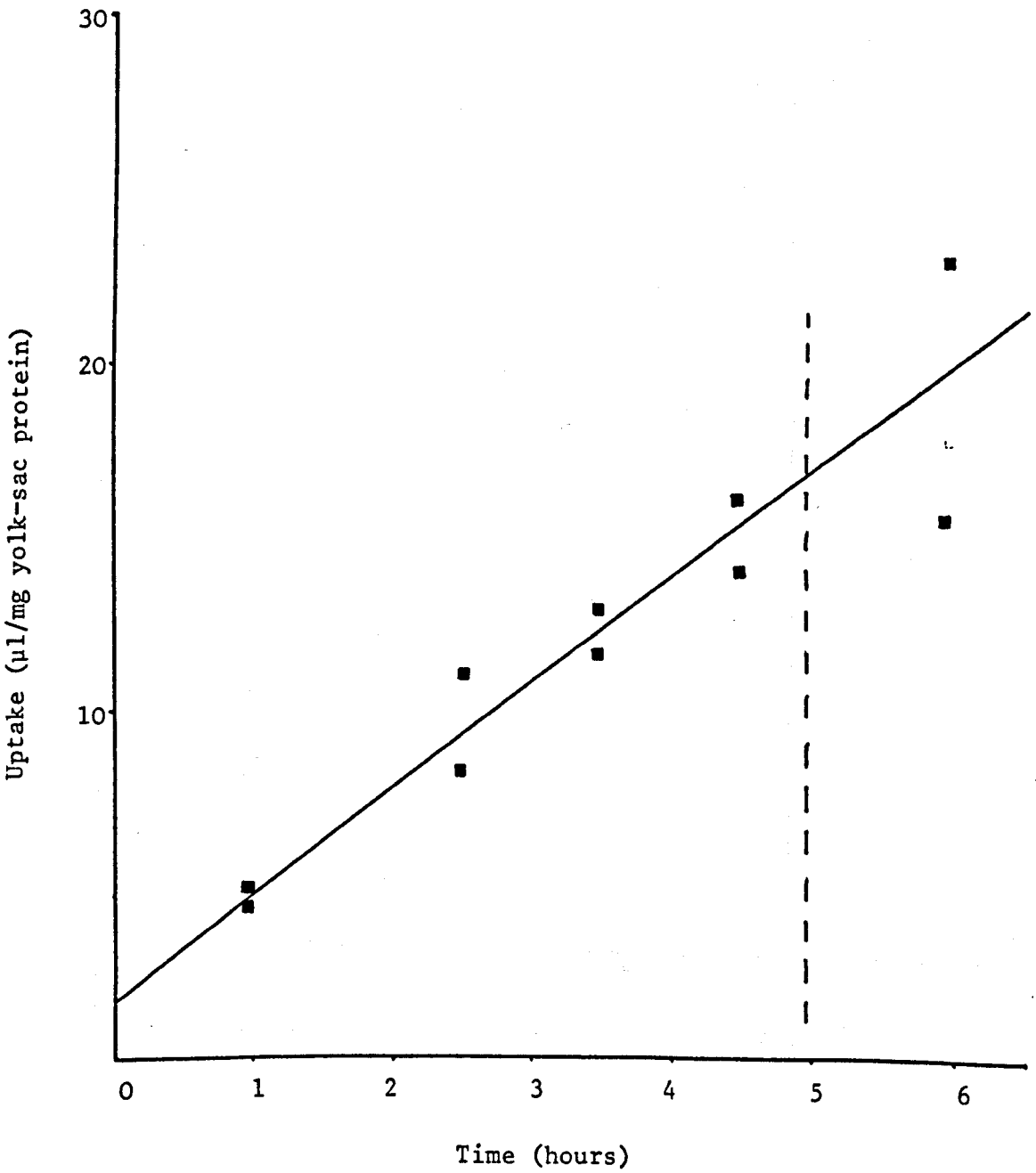


Endocytic Index = 378.9 µl/h/mg yolk-sac protein

(Correlation Coefficient = 0.981)

Fig. 3f Plot of uptake against time for ^{125}I-PVP in the absence of calf serum

Vertical bar indicates time beyond which loss of linearity is apparent.

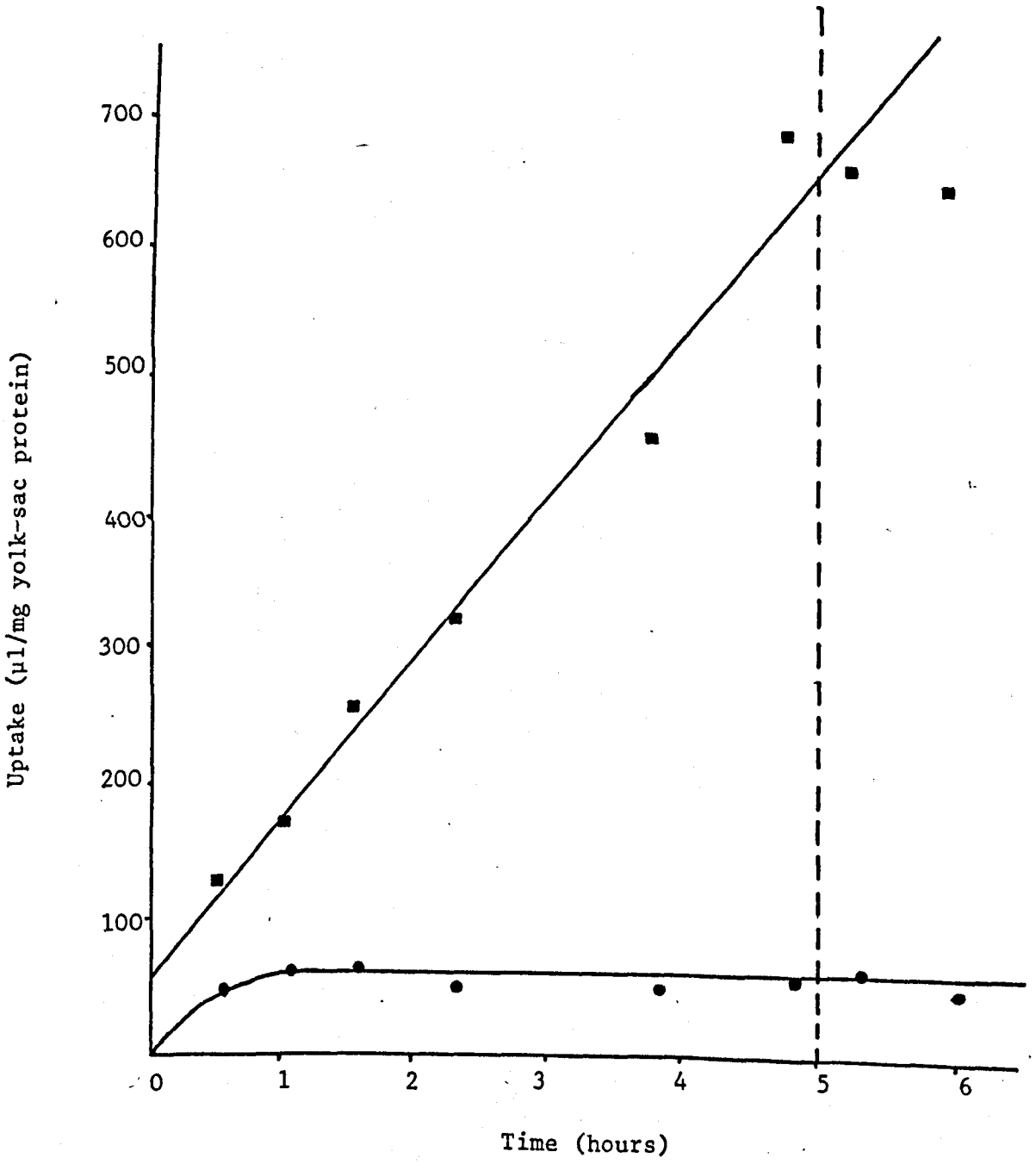


Endocytic Index = 2.96 µl/h/mg yolk-sac protein

(Correlation Coefficient = 0.939)

Fig. 3g Plot of uptake and tissue level against time for ^{125}I-dBSA (Batch II) in the absence of calf serum

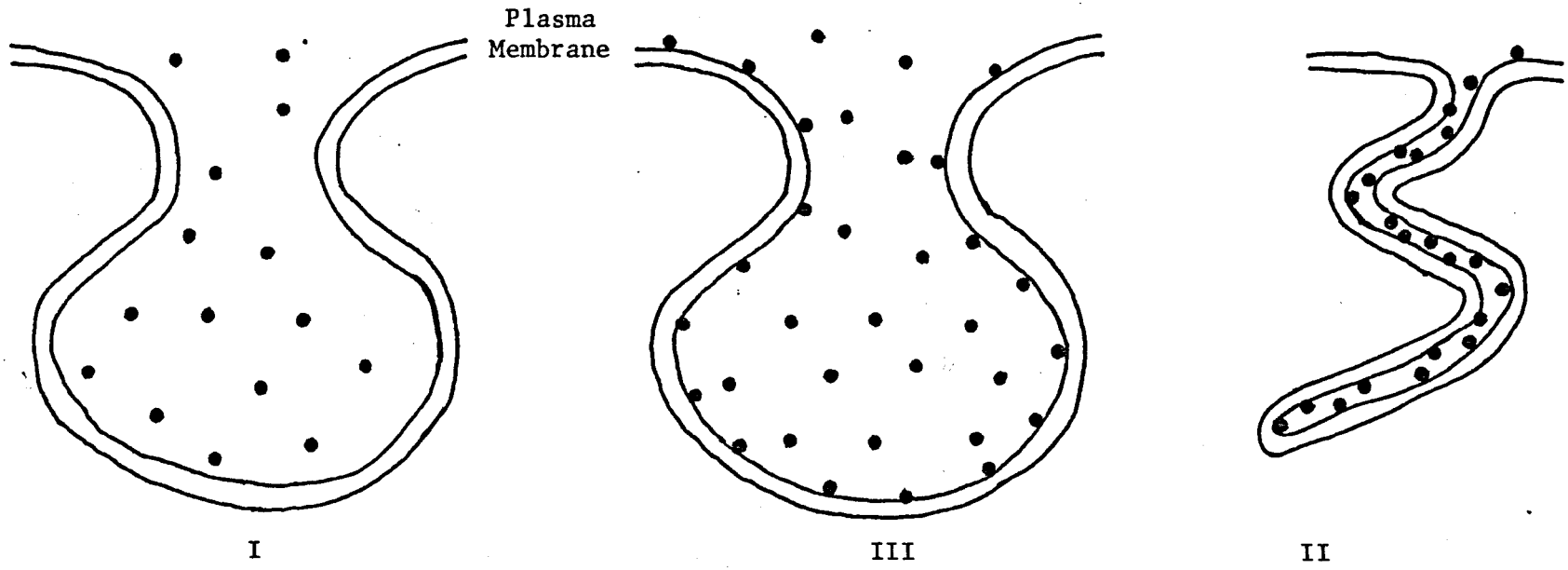
Vertical bar indicates time beyond which loss of linearity is apparent.



Endocytic Index = 124.0 µl/h/mg yolk-sac protein

(Correlation Coefficient = 0.986)

Fig. 3h Diagram of possible mechanisms of pinocytic ingestion of substrates (Jacques, P. J., 1969)



CHAPTER 4

ANALYSIS OF THE KINETICS OF INGESTION OF ^{125}I-LABELLED dBSA BY THE RAT YOLK SAC

4.1 Introduction

When the results reported in Chapter 3 are considered together with the findings reported by Williams et al. (1975b), Moore et al. (1977), and Roberts et al. (1977), they indicate that:

(a) the uptake of ^{125}I-labelled PVP occurs essentially in the fluid phase,

(b) that the uptake of ^{125}I-labelled albumin preparations occurs mainly by adsorption to the plasma membrane, and

(c) that calf serum competes with ^{125}I-labelled albumin for binding sites because adsorption plays a major role in determining the rate of internalisation of this protein substrate. In turn the affinity of the substrate for binding sites on the plasma membrane was shown by Moore et al. (1977) to be a function of the protein structure since chemical modification of bovine albumin greatly increases its ability to bind to the plasma membrane. Similarly Morrell et al. (1971) have demonstrated a relationship between the structure of a number of serum glycoproteins and their rates of uptake by the rat liver in vivo. These authors were able to show that removal of terminal sialic acid residues from these glycoproteins caused their half-lives in the circulation to fall dramatically. Furthermore, using isolated rat liver membranes, they were able to demonstrate that binding was the cause of the observed high rates of uptake and that desialylation of glycoproteins of the plasma membrane also resulted in a dramatic fall in the amount of binding (Lenten and Ashwell, 1971). This observation demonstrates that plasma membrane sialic acid residues also play an essential role in the binding process. The use of a quantitative assay technique enabled Lenten and Ashwell to demonstrate that the binding of substrate was a saturable process and these authors concluded that only a finite number of binding sites was available for the uptake of such substrates. Other workers (Rodbell, 1971) reached similar

conclusions for the binding of glucagon to liver plasma membrane. Cuatrecasas (1971) reported competitive binding of insulin to fat cells, while an analysis of insulin binding to isolated liver membranes (Soll et al., 1975) indicated the presence of two classes of receptor site.

In earlier studies, Gosselin (1956 and 1967) analysed the problem of surface binding of substrates in a study of the endocytic uptake of colloidal [^{198}Au]gold by macrophages in vitro. By applying a kinetic analysis, similar to that derived by Michaelis and Menten for the interaction of a substrate with an enzyme, he was able to derive a relationship between substrate concentration and the rate of endocytic uptake. Subsequently, by plotting the reciprocal of the rate of uptake against the reciprocal of the substrate concentration (Lineweaver-Burk plot) V_{max}, the maximal rate of substrate uptake, and K_s, the receptor-substrate binding constant, could be calculated. Cohen et al. (1968) analysed the kinetics of the disappearance of radiolabelled colloidal gold from the blood stream of the rat and in a later study Normann (1974a and b) analysed the kinetics of the clearance of foreign particles from the blood stream of this species. This latter author was able to calculate V_{max}, the "maximal phagocytic velocity", and K_c, the "Colloid-Membrane Constant", for several substrates including carbon, denatured BSA, lipids, colloidal gold, latex beads and formaldehyde-treated rabbit red cells. Moreover, he demonstrated that these substrates showed a degree of competitive inhibition when injected together indicating that their uptake was preceded by attachment to a limited number of essentially non-specific binding sites on the plasma membrane. In such studies the uptake of particles by macrophages and liver cells has been assumed to be entirely by prior adsorption to the cell membrane and uptake of substrate suspended or dissolved in the extracellular fluid has been ignored.

In this chapter the rate of uptake of ^{125}I-labelled dBSA by the

rat yolk sac was investigated in the presence of the non-radioactive competing substrate ^{127}I-labelled dBSA and also studied as a function of its own concentration. The application of a kinetic analysis analagous to that used in enzyme kinetics allows both the maximal uptake velocity, V_{max}, and the receptor-substrate dissociation constant, K, to be derived. Furthermore, by monitoring the effect of increased concentration of ^{127}I-labelled dBSA on the rate of uptake of ^{125}I-labelled PVP the rate of fluid ingestion could be monitored directly, avoiding any arbitrary assumptions as to its value.

METHODS

4.2 Uptake of ^{125}I-labelled PVP in the presence of ^{127}I-labelled dBSA

In order to determine whether iodinated, formaldehyde-denatured albumin modified the basal rate of pinocytosis when present in tracer concentrations, ^{125}I-labelled PVP was incubated with 17.5-day yolk sacs in the absence of calf serum (see Section 2.10 for method) but with addition of ^{127}I-labelled dBSA to the culture medium (see Section 2.2) for preparation of ^{127}I-labelled dBSA at three different concentrations: 4.25, 42.5 and 170.0 µg/ml of medium. The concentration of ^{125}I-labelled PVP was in the normal tracer concentration range (2.0 - 5.0 µg/ml of medium). Yolk sacs were removed at regular intervals up to 5.0 h and their contained radioactivity and protein content determined as described in 2.4 and 2.5, respectively. The medium radioactivity was determined as described in Section 2.6.

For each experiment, uptake, calculated by the method described in Section 2.12, was plotted against time (hours) and the Endocytic Index (µl/h/mg protein) was derived from the data by fitting a straight line to the points by linear regression analysis then determining its gradient. The individual values of the Endocytic Index of ^{125}I-labelled PVP in the presence of ^{127}I-labelled dBSA are shown in Table 4.I and summarized in Table 4.II, which also includes the value of the Endocytic Index of ^{125}I-labelled PVP at zero ^{127}I-labelled dBSA concentration in the absence of calf serum. (This value is taken from Table 3.V; see chapter 3 for details of these individual experiments).

The results given in Table 4.II are also presented graphically in Fig. 4a.

4.3 Uptake of ^{125}I-labelled dBSA in the presence of ^{127}I-labelled dBSA

The uptake of ^{125}I-labelled dBSA (Batch V) by 17.5-day yolk sac was studied in the absence of calf serum using a concentration of 0.6 µg/ml of ^{125}I-labelled dBSA and with ^{127}I-labelled dBSA present in

the culture medium at concentrations of 0-170 µg/ml. Tissue radioactivity and protein content were determined as described in 2.4 and 2.5. The medium total- and the TCA-soluble radioactivities were assayed by the methods given in section 2.6 and 2.7, respectively. Uptake of ^{125}I-labelled dBSA was calculated as described in section 2.13 and plotted against time (hours). The Endocytic Index was calculated in the usual manner using first the full set of data for 5.0 h incubations and secondly on restricting analysis of the same data to those obtained in the first 3.0 h of incubation. The corresponding results of each experiment are shown in full in Tables 4.IIIa (5.0 h period) and 4.IIIb (3.0 h period). Some of the sets of data that appear in Table 4.IIIb do not appear in Table 4.IIIa. This occurs because restricting the analysis of data to those gathered in the first 3.0 h of incubation often gives a linear regression line with an acceptably high correlation coefficient (i.e. in excess of 0.91) whereas the full set of data up to 5.0 h gives a correlation coefficient less than 0.91. Occasionally the converse is true, so that a set of data contributes a value to Table 4.IIIa but not Table 4.IIIb.

Table 4.IIIa also shows the mean amount of radioactivity associated with the tissue (µl/mg protein) in the period from 2.0 - 5.0 h. The mean value of the tissue associated radioactivity was not calculated for the shortened time period as there was an insufficient number of samples between 2.0 h and 3.0 h to give meaningful results.

The contents of Tables 4.IIIa and 4.IIIb are summarized both in Table 4.IV, which shows the mean values of the Endocytic Indices for both 3.0 h and 5.0 h sets of data together with the mean value of the tissue-associated radioactivity for the 5.0 h sets of data. Fig. 4b which shows the same data in graphical form.

4.4 Uptake of ^{125}I-labelled dBSA when present at different concentrations in medium 199 alone

A number of experiments were performed in which ^{125}I-labelled dBSA (Batch IV) was incubated with 17.5-day yolk sacs for 5.0 h. The experiments were performed in the same manner as those in 4.3 above but, instead of adding different concentrations of non-radioactive ^{127}I-labelled dBSA to the culture medium, the radiotracer alone was used at concentrations from 1.0 - 75 μg/ml of medium. All assay procedures were as in 4.2 above and the same methods of calculation and expression of results were used. The full sets of data relating to incubations up to 5.0 h were analysed.

The Endocytic Indices are shown in Table 4.V for each experiment together with the mean value of the tissue-associated radioactivity after the first 2.0 h of incubation. These same results are summarized in Table 4.VI and shown graphically in Fig. 4c.

RESULTS

4.5 Effect of increasing concentrations of ^{127}I-labelled dBSA on the Endocytic Index of ^{125}I-labelled PVP

Table 4.II and Fig. 4a show the effect on the Endocytic Index of ^{125}I-labelled PVP of different concentrations of ^{127}I-labelled dBSA. A slight fall in Endocytic Index over the initial concentration range of 0-50 μg/ml of ^{127}I-labelled dBSA is suggested but increasing the ^{127}I-labelled dBSA concentration from 50 to 170 μg/ml does not lead to a further fall. At no concentration was the Endocytic Index reduced by more than 20% as compared with that of ^{125}I-labelled PVP in medium 199 alone and, because of the large standard deviation associated with each mean value of the Endocytic Index, it is doubtful whether even the initial fall is significant. It is thus unlikely that concentrations of ^{127}I-labelled dBSA in the range 0-170 μg/ml have any effect on the rate of uptake of ^{125}I-labelled PVP.

4.6 Endocytic Index of ^{125}I-labelled dBSA (Batch V) in the presence of increasing concentrations of ^{127}I-labelled dBSA (in medium 199 alone)

The mean values of the Endocytic Index of ^{125}I-labelled dBSA in the presence of increasing concentrations of ^{127}I-labelled dBSA are shown in Table 4.IV and in Fig. 4b. The values of the Endocytic Index derived from analysis of both 3.0 h and 5.0 h sets of data differ little. Fig. 4b clearly shows a rapid fall in the Endocytic Index of ^{125}I-labelled dBSA with increasing concentration of ^{127}I-labelled dBSA. An increase in ^{127}I-labelled dBSA concentration from zero to 170.0 µg/ml of medium leads to a reduction in Endocytic Index, from 381.5 ± 24.8 to 96.8 ± 24.9 µl/h/mg protein i.e. approx, 75%. The magnitude of the reduction in Endocytic Index of ^{125}I-labelled dBSA (Batch V) caused by ^{127}I-labelled dBSA (170 µg/ml) is comparable to that caused by addition of 10% calf serum to the medium (see Table 3.IV) being 5.5- and 4-fold, respectively. A much higher concentration of protein is present, however, in 10% calf serum than in even the highest concentrations of ^{127}I-labelled dBSA used.

Fig. 4b also shows the mean tissue level of radioactivity (µl/mg protein) to fall as the concentration of ^{127}I-labelled dBSA (µg/ml) in the medium is raised. On increasing the ^{127}I-labelled dBSA concentration from 0 to 170 µg/ml medium a corresponding fall in tissue level activity from 102.5 ± 5.5 µl/mg to 23.7 ± 2.0 µl/mg is observed (i.e. approx, 77%). The steady fall in radioactivity associated with the tissue reflects the observed fall in Endocytic Index, and indicates that, at all ^{127}I-labelled dBSA concentrations investigated, the amount of radiotracer associated with the tissue remains constant and equal to approximately a quarter of the amount of protein ingested by the tissue every hour.

4.7 Endocytic Index of ^{125}I-labelled dBSA (Batch IV) at different concentrations (in medium 199 alone)

The results summarized in Table 4.VI and Fig. 4c show the dependence of the Endocytic Index of ^{125}I-labelled dBSA (Batch IV) on its concentration in the culture medium.

Inspection of Fig. 4c shows a rapid fall in Endocytic Index with increasing substrate concentration and this fall is comparable to that shown in Fig. 4b where the Endocytic Index for ^{125}I-labelled dBSA (Batch V) is plotted against concentration of ^{127}I-labelled dBSA. Although the Endocytic Index for ^{125}I-labelled dBSA (Batch IV) is lower than that of Batch V, at a particular substrate concentration both these plots show approximately a 60% fall in Endocytic Index between concentrations of 1.5 µg/ml and 75 µg/ml. Similarly, when the overall mean value of the tissue-associated radioactivity is plotted against tracer-protein concentration a progressive decrease with increasing substrate concentration is observed (Fig. 4c) and is comparable to that for ^{125}I-labelled dBSA (Batch V) shown in Fig. 4b.

DISCUSSION

4.8 Optimum incubation period when determining the Endocytic Index of a protein in the absence of calf serum

The Endocytic Index of formaldehyde-denatured bovine serum albumin, ingested by 17.5-day yolk sacs in the absence of calf serum, shows a very substantial increase when compared with that of the same preparation incubated in the presence of 10% calf serum (see Chapter 3 for determination of the latter).

This high Endocytic Index is accompanied by a rapid depletion of the available substrate in the culture medium. For example, an average sized yolk sac (containing 5.0 mg of protein) that is pinocytosing with an Endocytic Index of 300 µl/h/mg protein will capture, every hour, an amount of substrate equivalent to that contained in

1.5 ml of culture medium. Therefore, after 5.0 h of incubation, the substrate contained in more than half the original culture medium (10.0 ml total volume) will have been captured.

It should be noted, however, that the uptake of radiolabel is calculated using equation 2.IV (Chapter 2) and that the value for the amount of acid insoluble radioactivity in the medium, M', that is routinely used in these calculations of uptake is the mean amount of TCA-insoluble activity in the medium during an individual yolk sac incubation (obtained by adding, to the measured TCA-insoluble activity remaining in each culture medium at the end of its incubation period, one half of the quantity of TCA-soluble activity generated by the yolk sac in the same period). Even for a rapidly ingested protein, the value of M' so generated will be correct, provided that the substrate depletion proceeds in a linear manner over the incubation period. However, from Figs. 4b and 4c, it can be seen that the Endocytic Index rises as the substrate concentration falls, hence a linear decrease in substrate concentration may not necessarily occur.

In order to determine whether the effects of either substrate depletion or decreased tissue viability in the absence of calf serum affected the calculated values of the Endocytic Index for individual sets of data, despite the use of a mean value of M' calculated as described above, Endocytic Indices were calculated both for the data from the initial (3.0 h) incubation period and from the full sets of data relating to 5.0 h incubations. Either a fall in substrate concentration or an impairment of tissue function would be expected to be less over a 3.0 h incubation period than over a 5.0 h incubation period. Hence any substantial effect from either source would lead to deviation from a linear pattern of depletion and result in a significant difference between these pairs of values for the Endocytic Index in a given experiment.

The results shown in Tables 4.IIIa and 4.IIIb (and again graphically in Fig. 4b) show no significant difference between each pair of values for the Endocytic Index derived using first the full set of data relating to a 5.0 h incubation period, then only those data, from the same experiment, that had been gathered in the initial 3.0 h period. This finding implies that although the Endocytic Index might be expected to rise during the course of an experiment as a result of rapid substrate depletion, the net effect appears not to differ from that expected from a linear depletion with time. Hence the values derived from full, 5.0 h, sets of data were routinely analysed.

4.9 Kinetic Analysis

The general equation for the interaction of an enzyme, E, with its substrate, S, to give products, P, is:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E.S \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E + P \qquad 4.Ia$$

By restricting the analysis to the initial events only, the effect of the back reaction can be ignored and equation 4.Ia simplifies to:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E.S \overset{k_3}{\rightarrow} E + P \qquad 4.IIa$$

The initial rate of formation of products, V, is given by:

$$V = k_3[E.S] \qquad 4.IIIa$$

As the substrate concentration is increased saturation of the available enzyme occurs and 4.IIIa becomes:

$$V_{max} = k_3 [E'] \qquad 4.IVa$$

where E' is the total concentration of both free and bound enzyme. Using the above equations, Michaelis and Menten derived their well known relation between the observed reaction rate, V, at a particular substrate concentration, and the maximum rate of reaction, V_{max}, for a given amount of enzyme.

$$\frac{1}{V} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}} \qquad 4.Va$$

From this equation it can be seen that a plot of $\frac{1}{V}$ versus $\frac{1}{[S]}$ (Lineweaver Burk) gives a straight line with a gradient of $\frac{K_m}{V_{max}}$ and and an intercept of $\frac{1}{V_{max}}$ on the ordinate axis.

From a kinetic standpoint, the binding of a macromolecular substrate to a binding site on the plasma membrane prior to internalization may be regarded as being analogous to the binding of an enzyme to its substrate. Internalization of the binding site-substrate complex is equivalent to formation of product in enzyme kinetics. Thus, if uptake of a substrate occurs solely by its prior adsorption to the plasma membrane (i.e. no ingestion via the fluid phase), an equation similar to that describing the interaction of an enzyme and substrate to give products (4.Ia) may be stated as:

$$R + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} R.S \underset{k_4}{\overset{k_3}{\rightleftarrows}} R.P \qquad 4.Ib$$

Where R and S are cell surface receptor and substrate concentrations respectively, R.S the receptor substrate complex and R.P the receptor substrate complex that has been internalized by pinocytosis.

Restricting the study to systems where the rate of exocytosis of substrate is zero then the 'back reaction' can be ignored and 4.IIa becomes:

$$R + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} R.S \overset{k_3}{\rightarrow} R.P \qquad 4.IIb$$

From this equation the rate of internalization by adsorption to the membrane, q, is given as:

$$q = k_3.M\ [R.S] \qquad 4.IIIb$$

where M is the rate of membrane internalization.

The rate of substrate internalization, q, will reach a maximum

value, Q_{max}, when the substrate concentration increases to such a point that saturation of the cell surface binding sites occurs and the rate of uptake becomes independent of substrate concentration. Equation 4.IIIb then becomes:

$$Q_{max} = k_3 M[R'] \qquad 4.IVb$$

where R' is the total concentration of binding sites, both free and occupied. Assuming the rate of membrane internalization is not altered on increasing the substrate concentration then $k_3.M$ will be a constant (k_3') so that:

$$Q_{max} = k_3' [R'] \qquad 4.Vb$$

This equation is similar to equation 4.IVa. Likewise, an expression equivalent to 4.Vb is obtained which relates the observed rate of uptake, at a given substrate concentration, to the maximum rate of uptake (Q_{max}), i.e.:

$$\frac{1}{q} = \frac{K_c}{Q_{max}} \frac{1}{[S]} + \frac{1}{Q_{max}} \qquad 4.VIb$$

Hence, as in the case of enzyme kinetics, a plot of $\frac{1}{q}$ against $\frac{1}{[S]}$ should yield a straight line with an intercept on the ordinate axis of $\frac{1}{Q_{max}}$ and a gradient of $\frac{K_c}{Q_{max}}$.

Gosselin (1966) showed that the uptake of colloidal [^{198}Au]gold by the macrophage could be described by equation 4.VIb. Furthermore, in studying the clearance of foreign particles from the blood, (Normann, 1974a and b) obtained linear double reciprocal plots and also demonstrated competitive inhibition between different particles that had been administered together, intravenously. Both of these studies were concerned with the uptake of solutes by phagocytosis. It was assumed that the particles adsorbed to the cell surface prior to ingestion; fluid phase ingestion was considered not to be relevant.

Uptake of proteins by the yolk sac is known to be by pinocytosis

(Williams et al. 1975a, b) involving both fluid phase and adsorptive phase ingestion. This mode of uptake was previously discussed (Chapter 3 Section 3.12) in terms of the general formulation for Endocytosis proposed by Jacques (1969),

$$Q = Fc + \frac{SRc}{K+c} \qquad 4.Ic$$

(N.B. S in this equation does not represent substrate but the rate of ingestion of cell membrane)

A value for the rate of fluid ingestion, F, was derived from an investigation of the Endocytic Index of ^{125}I-labelled PVP (see Chapter 3) its mean value being 2.47 µl/h/mg protein in the absence of calf serum.

Rearranging equation 4.Ic and letting q equal the rate of ingestion by the adsorptive phase then:

$$q = (Q - Fc) = \frac{SRc}{K+c} \qquad 4.IIc$$

hence, taking reciprocals and separating terms:

$$\frac{1}{q} = \frac{K}{SR} \cdot \frac{1}{c} + \frac{1}{SR} \qquad 4.IIIc$$

Equation 4.IIIc is of the same form as equations 4.Va and 4.VIb and the Lineweaver-Burk plot should yield a straight line with an intercept of $\frac{1}{SR}$ (or $\frac{1}{Q_{max}}$) and slope of $\frac{K}{SR}$ (or $\frac{K}{Q_{max}}$).

The Endocytic Index of a substrate is equal to the rate of solute uptake, Q, divided by its concentration (Chapter 3, Equation 3.IV)

$$E = \frac{Q}{c} \qquad 4.IVc$$

Hence, e, the contribution to the Endocytic Index made by adsorptive phase uptake is given by:

$$e = E - F = \frac{SR}{K+c} \qquad 4.Vc$$

and substituting for E from 4.IVc and multiplying by c:

$$q = e.c. = Q - Fc \qquad 4.VIc$$

Using equations 4.Vc and 4.VIc the adsorptive phase rate of uptake, q (μg/h/mg protein), can be calculated.

Fig. 4a shows a plot of Endocytic Index of ^{125}I-labelled PVP versus various concentrations of ^{127}I-labelled dBSA (medium 199 alone). This plot clearly shows that there is no significant change in Endocytic Index of ^{125}I-labelled PVP on increasing the concentration of ^{127}I-labelled dBSA and supports the conclusion that the rate of fluid internalization is neither stimulated nor inhibited by the presence of formaldehyde-denatured albumins, hence the rate of fluid ingestion, F, can be assigned the constant value of 2.47 μl/h/mg protein in both the presence and absence of ^{127}I-labelled dBSA.

Using this value for the rate of fluid phase uptake (F) the Endocytic Index for adsorptive phase uptake, e (μl/h/mg protein), was calculated and hence q, the rate of uptake by adsorption. Table 4.VII shows the values of e and q obtained with ^{125}I-labelled dBSA Batches IV and V; the values for $\frac{1}{c}$ and $\frac{1}{q}$ are also shown in this table. These data are presented in the form of a Lineweaver-Burk plot in Fig. 4d. It should be noted, however, that the points derived from low substrate concentrations (1.5 μg/ml, Batch IV, 0.6 and 4.85 μg/ml, Batch V) were not included in either related plot. This was because of the difficulty in achieving precise measurement of low substrate concentrations. The error in measurement when expressed as a reciprocal number is large, whereas a similar error in a high concentration does not result in a significant error in the reciprocal. Furthermore, as a result of the high percentage change in the Endocytic Index with substrate concentration at low substrate concentrations (see above) the observed Endocytic Index may be difficult to determine precisely.

For these two reasons, points at low concentration were not included in the final plots. Fig. 4d shows the best straight line

fitted to each set of points by linear regression analysis. From the slopes and intercepts of these plots values for Q_{max} $(\frac{1}{SR})$ and K, respectively, were calculated (see Table VIII).

Table 4.I The Endocytic Index of ^{125}I-labelled PVP in the presence of ^{127}I-labelled dBSA (medium 199 alone)

Each row of table summarizes the uptake data from a single experiment in which a number of 17.5-day rat yolk sacs, taken from a single pregnant animal, were incubated separately at 37°C in medium 199 containing ^{125}I-labelled PVP (2.0-5.0 μg/ml) and removed from the medium at regular intervals up to 5.0 h.

^{125}I-PVP conc. (μg/ml)	^{127}I-dBSA conc. (μg/ml)	No. of yolk sacs	Endocytic Index (μl/h/mg protein)	Corr. Coeff.	Intercept (μl/mg protein)
5.0	4.25	10	2.10	0.954	+1.12
"	"	10	2.98	0.961	+3.20
"	"	9	2.24	0.925	+4.04
"	42.5	10	1.43	0.968	+1.26
"	"	9	2.33	0.975	+1.98
"	"	10	2.50	0.96	+1.99
"	170.0	10	2.18	0.964	+1.60
"	"	10	2.53	0.985	+1.74
"	"	10	1.67	0.972	+1.15

Table 4.II Summary of the Endocytic Index of ^{125}I-labelled PVP in the presence of ^{127}I-labelled dBSA (medium 199 alone)

Data presented in Table 4.I are summarized together with those from Table 3.V.

^{127}I-dBSA concentration (μg/ml)	No. of experiments	Mean Endocytic Index ± S.D. (μl/h/mg protein)
0	11	2.47 ± 1.00
4.25	3	2.44 ± 0.47
42.5	3	2.09 ± 0.58
170.0	3	2.13 ± 0.44

Table 4.IIIa Endocytic Index of ^{125}I-labelled dBSA (Batch V) (medium 199 alone) but in the presence of different concentrations of ^{127}I-labelled dBSA; data for full 5.0 h incubation period

Each row of the table summarizes the results of a separate experiment, as described in the caption to Table 4.I. In each experiment the concentration of ^{125}I-labelled dBSA was 0.6 µg/ml of medium.

^{127}I-dBSA conc. (µg/ml)	TCA solubles (%)	No. of yolk sacs	Endocytic Index (µl/h/mg protein)	Corr. Coeff.	Intercept (µl/mg protein)	Mean tissue activity (µl/mg protein)
0	2.98	10	407.4	0.984	+ 0.6	101.1
"	1.39	10	378.9	0.981	+ 4.3	97.81
"	2.90	10	358.1	0.967	- 4.4	108.6
4.25	2.98	9	218.7	0.972	+119.8	64.7
"	1.39	10	370.2	0.981	- 70.6	90.8
"	1.40	8	269.0	0.930	+ 8.4	76.1
"	2.90	10	255.5	0.973	+ 44.2	80.1
V 8.5	1.40	9	209.8	0.980	+ 61,5	52.1
"	1.40	9	190.8	0.960	+152,2	59.4
"	2.90	10	258.8	0.985	+ 68.5	73.3
17.0	1.40	10	200.5	0.922	+ 39.6	66.5
"	1.40	10	196.2	0.921	+139.8	62.6
"	2.90	10	226.6	0.957	- 70.8	57.7
"	2.98	10	167.6	0.935	- 62.7	35.9
42.5	2.90	10	122.3	0.951	+ 32.8	60.58
"	2.90	10	237.9	0.962	- 8.9	59.5
"	2.98	8	184.9	0.989	- 0.5	39.0
85.0	2.98	7	107.8	0.967	- 72.4	21.2
"	1.40	9	153.1	0.991	+ 26.0	36.6
"	2.90	10	123.7	0.948	+ 54.1	33.8
127.5	1.39	10	56.7	0.922	+ 18.0	16.5
"	1.14	9	82.9	0.977	+ 0.3	22.8
"	1.40	9	59.9	0.926	+ 78.9	16.1
"	2.90	10	115.0	0.916	- 10.9	27.4
170.0	1.39	8	72.1	0.946	+ 19.9	21.8
"	2.90	9	121.9	0.960	- 25.3	23.7
"	2.90	9	96.5	0.974	+ 26.7	25.7

Table 4.IIIb Endocytic Index of ^{125}I-labelled dBSA (Batch V) (medium 199 alone) but in the presence of different concentrations of ^{127}I-labelled dBSA; analysis of data restricted to those from the first 3.0 h of incubation only

Each row of the table summarizes the data from an experiment, as described in the caption to Table 4.I. In each experiment the concentration of ^{125}I-labelled dBSA was 0.6 µg/ml of medium.

^{127}I-dBSA conc. (µg/ml)	TCA solubles (%)	No. of yolk sacs	Endocytic Index (µl/h/mg protein)	Corr. Coeff.	Intercept (µl/mg protein)
0	2.98	6	420.9	0.995	- 38.7
"	1.39	6	403.4	0.963	- 70.0
"	2.90	6	388.2	0.956	- 54.0
4.25	2.98	6	231.1	0.939	+103.1
"	1.39	6	348.1	0.983	- 33.6
"	1.40	6	270.3	0.866	+ 54.6
"	2.90	6	335.5	0.996	- 81.9
8.5	1.40	6	205.8	0.967	+ 66.9
"	1.40	6	229.7	0.955	+102.3
"	2.90	6	274.5	0.983	+ 44.7
"	1.39	6	197.9	0.965	+ 43.5
17.0	1.40	6	298.5	0.990	- 18.0
"	1.40	6	287.5	0.978	+ 10.8
"	2.90	6	131.8	0.937	+ 72.2
"	2.98	6	116.3	0.987	30.9
42.5	1.40	5	140.3	0.994	- 6.0
"	2.90	6	147.7	0.891	- 6.6
"	2.90	6	252.6	0.953	- 34.9
85.0	2.98	6	102.8	0.946	- 62.7
"	1.40	6	172.7	0.991	+ 3.1
"	1.40	6	168.7	0.998	+ 34.2
"	2.90	6	170.4	0.990	- 18.5
127.5	1.39	6	59.8	0.972	+ 20.8
"	1.14	6	92.9	0.960	+ 74.6
"	1.40	6	86.7	0.946	+ 35.3
"	2.90	6	134.0	0.991	- 31.7
170.0	1.39	6	73.9	0.910	- 16.5
"	2.90	6	89.1	0.937	+ 23.3
"	2.90	6	118.9	0.979	- 13.7

Table 4.IV Summary of the Endocytic Indices of ^{125}I-labelled dBSA (Batch V) in medium 199 alone but in the presence of ^{127}I-labelled dBSA

Summary of data presented in Table 4.IIIa and 4.IIIb.

Analysed as 3.0 h incubations			Analysed as 5.0 h incubations		
^{127}I-dBSA concentration (µg/ml)	No. of experiments analysed	Mean (± S.D.) Endocytic Index (µl/h/mg protein)	No. of experiments analysed	Mean (± S.D.) Endocytic Index (µl/h/mg protein)	Overall mean (± S.D.) tissue radioactivity (µl/mg protein)
0	3	404.2 ± 16.4	3	381.5 ± 24.8	102.5 ± 5.5
4.25	3	304.9 ± 64.2	4	278.4 ± 64.8	77.9 ± 10.8
8.5	4	227.0 ± 34.5	4	214.7 ± 30.7	61.6 ± 10.8
17.0	4	208.3 ± 97.8	4	197.7 ± 24.2	55.7 ± 13.7
42.5	3	180.2 ± 62.8	4	171.3 ± 51.6	53.0 ± 12.2
85.0	4	153 ± 33.9	4	138.3 ± 27.6	30.5 ± 8.2
127.5	4	93.4 ± 30.6	4	78.6 ± 26.9	20.7 ± 5.4
170.0	3	94.0 ± 23.0	3	96.8 ± 24.9	23.7 ± 2.0

Table 4.V The effect of increasing the concentration of ^{125}I-labelled dBSA (Batch IV) in the medium on the Endocytic Index of ^{125}I-labelled dBSA (Batch IV)

Each row of the table summarizes the data relating to a separate experiment, as described in the caption to Table 4.I.

^{125}I-dBSA conc. (µg/ml)	No. of yolk sacs	Endocytic Index (µl/mg protein)	Corr. Coeff.	Intercept (µl/mg protein)	TCA solubles (%)	Mean tissue activity (µl/mg protein)
1.5	10	223.7	0.974	+15.8	3.1	86.4
"	10	228.6	0.948	+33.1	1.4	75.5
"	9	236.6	0.940	+95.3	1.4	85.9
"	9	230.3	0.954	+47.7	3.1	70.7
7.68	9	156.0	0.955	+58.7	0.85	64.5
"	9	158.8	0.938	+53.0	2.32	62.0
"	10	193.6	0.931	+45.5	1.4	73.0
"	10	193.5	0.956	-25.5	3.1	71.6
38.4	10	131.9	0.969	+ 3.5	2.32	41.7
"	8	128.7	0.993	+35.0	0.85	42.2
"	10	115.3	0.989	+20.0	3.1	34.8
"	10	162.1	0.961	+ 2.7	1.4	54.3
76.8	9	76.2	0.969	+45.9	3.1	26.7
"	10	121.5	0.967	- 9.3	3.1	42.3
"	8	116.9	0.994	-14.8	3.1	35.8

Table 4.VI Summary of the effect of increasing ^{125}I-labelled dBSA (Batch IV) concentration on the Endocytic Index of ^{125}I-labelled dBSA (Batch IV)

Summary of data presented in Table 4.V.

^{125}I-dBSA concentration (µg/ml)	No. of experiments	Mean ± S.D. Endocytic Index (µl/h/mg protein)	Overall mean tissue radioactivity (± S.D.) (µl/mg protein)
1.5	4	229.8 ± 5.3	79.6 ± 7.8
7.68	4	175.5 ± 20.9	67.8 ± 5.4
38.4	4	134.5 ± 19.8	43.3 ± 8.1
76.8	3	104.7 ± 24.9	34.9 ± 7.8

Table 4.VII Data for double reciprocal plot for ^{125}I-labelled dBSA (Batch IV)

The data in this Table were derived from Table 4.VI.

^{125}I-dBSA conc. (µg/ml)	$\frac{1}{c} \times 10^2$ (µg/ml)	Endocytic Index (µl/h/mg protein)	Rate of uptake $q \times 10^3$ (µg/h/mg protein)	$\frac{1}{q}$ (µg/h/mg protein)
1.5	66.7	229.8	341.0	2.933
7.68	13.0	175.5	1328.6	0.726
38.4	2.6	134.5	5068.8	0.197
76.8	1.3	104.7	7849.0	0.127

Table 4.VIII Data for double reciprocal plot for ^{125}I-labelled dBSA (Batch V)

The data in this table were derived from Table 4.IV.

Total dBSA conc. (µg/ml)	$\frac{1}{c} \times 10^2$ (µg/ml)	Endocytic Index (µl/h/mg protein)	Rate of uptake ($q \times 10^3$) (µg/h/mg protein)	$\frac{1}{q}$ (µg/h/mg protein)
0.6	166.7	381.5	227.4	4.40
4.85	20.62	278.4	1338.1	0.747
9.1	11.00	224.1	2016.7	0.496
17.6	5.68	197.7	3435.5	0.291
43.1	2.32	171.3	7275.3	0.138
85.6	1.12	138.3	11624.5	0.086
128.1	0.78	78.6	9748.4	0.103
170.6	0.59	96.8	16087.6	0.062

Table 4.IX Calculated values for Q_{max} (SR) and K for ^{125}I-labelled dBSA

Values calculated from the gradients and intercepts from Fig. 4d using a molecular weight of 65,000 for ^{125}I-labelled dBSA.

	Batch IV	Batch V
Q_{max} (SR)	16.05 µg/h/mg protein 0.247 n moles/h/mg protein	20.19 µg/h/mg protein 0.311 n moles/h/mg protein
K	81.97 µg/ml 1.26 µM	82.56 µg/ml 1.27 µM

Fig. 4a Plot of Endocytic Index of ^{125}I-labelled PVP versus concentration of ^{127}I-labelled dBSA in the absence of calf serum

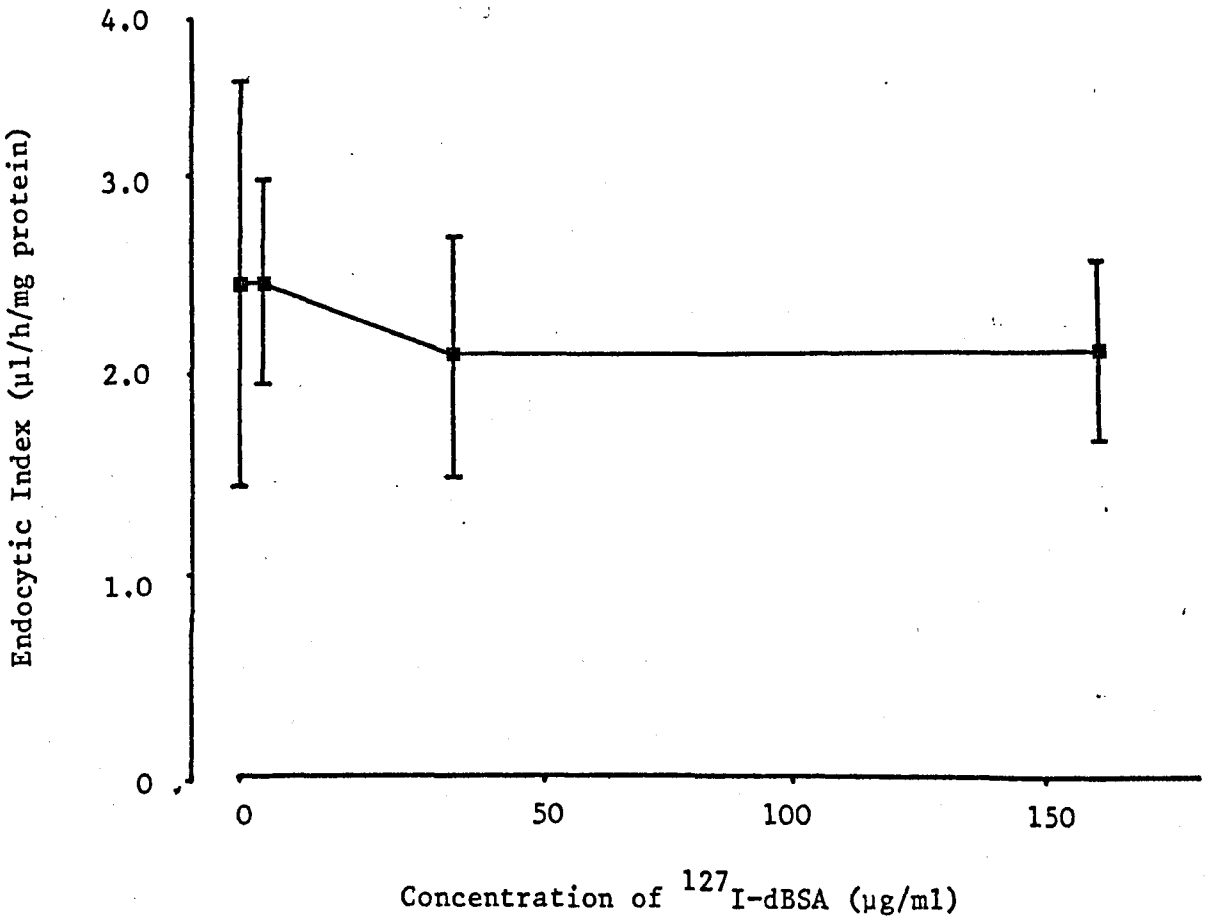


Fig. 4b Plot of Endocytic Index of ^{125}I-labelled dBSA (Batch V) versus concentration of ^{127}I-labelled dBSA in the absence of calf serum

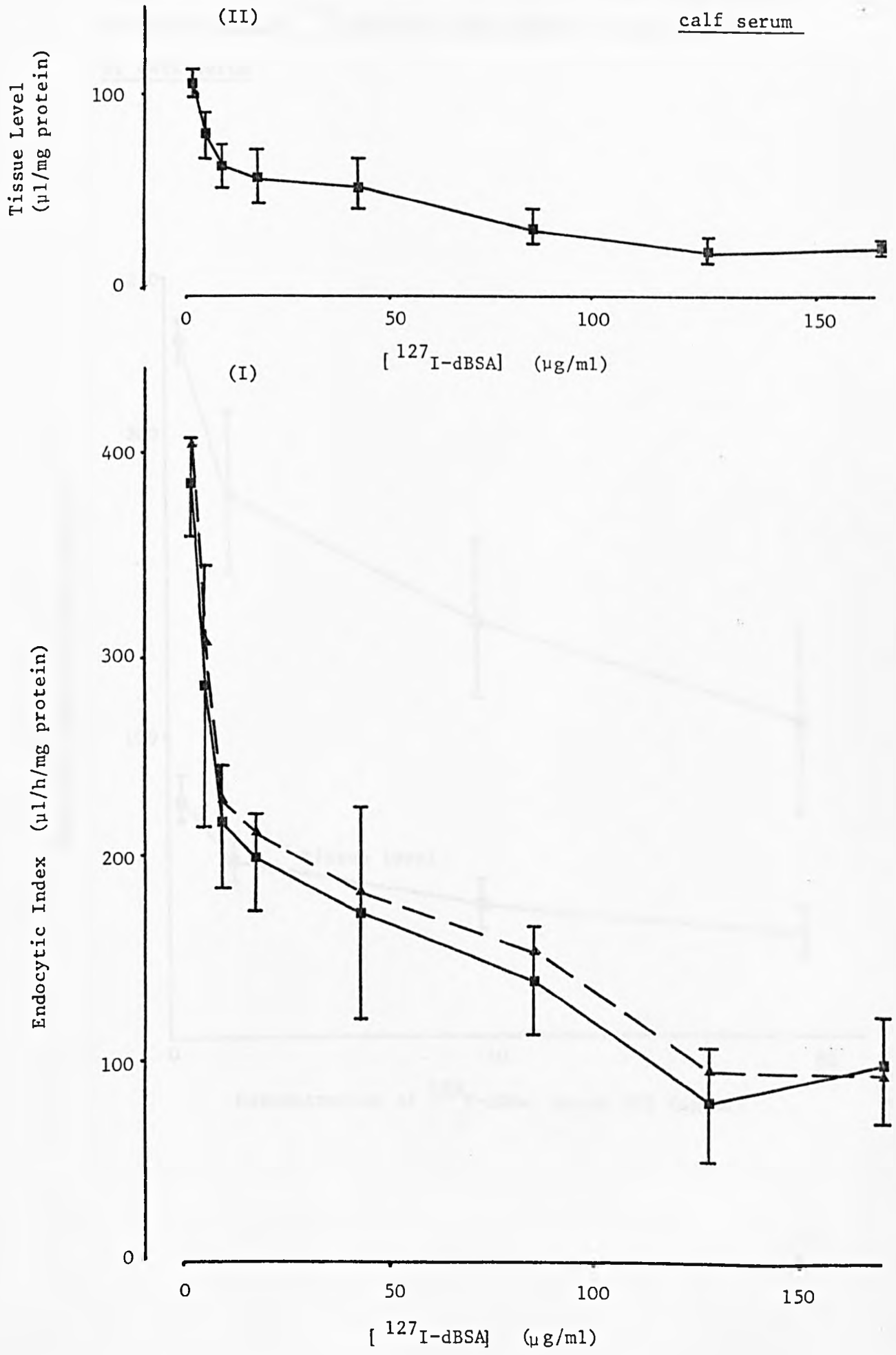


Fig. 4c Plot of Endocytic Index of ^{125}I-labelled dBSA (Batch IV) versus concentration of ^{125}I-labelled dBSA (Batch IV) in the absence of calf serum

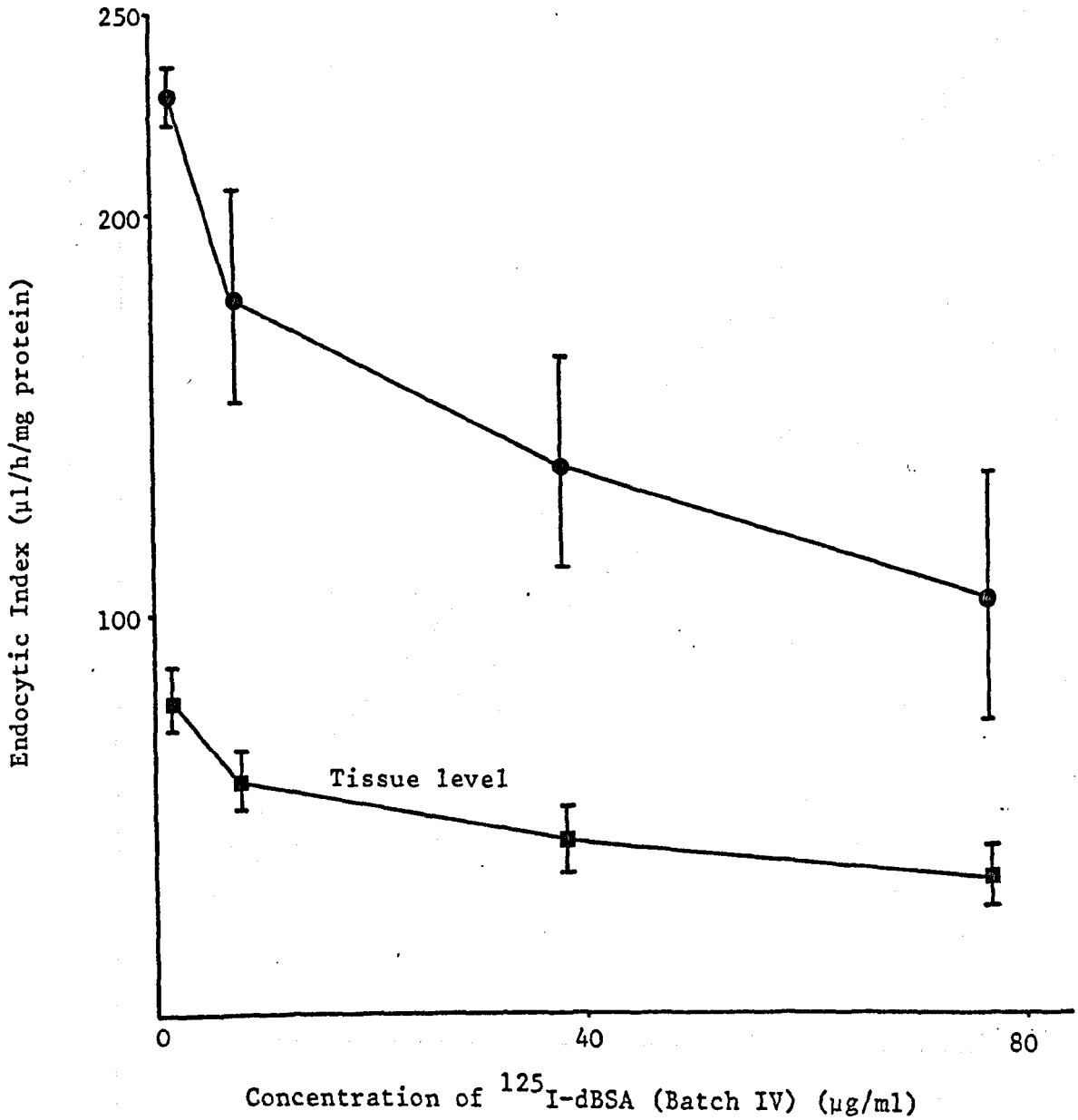
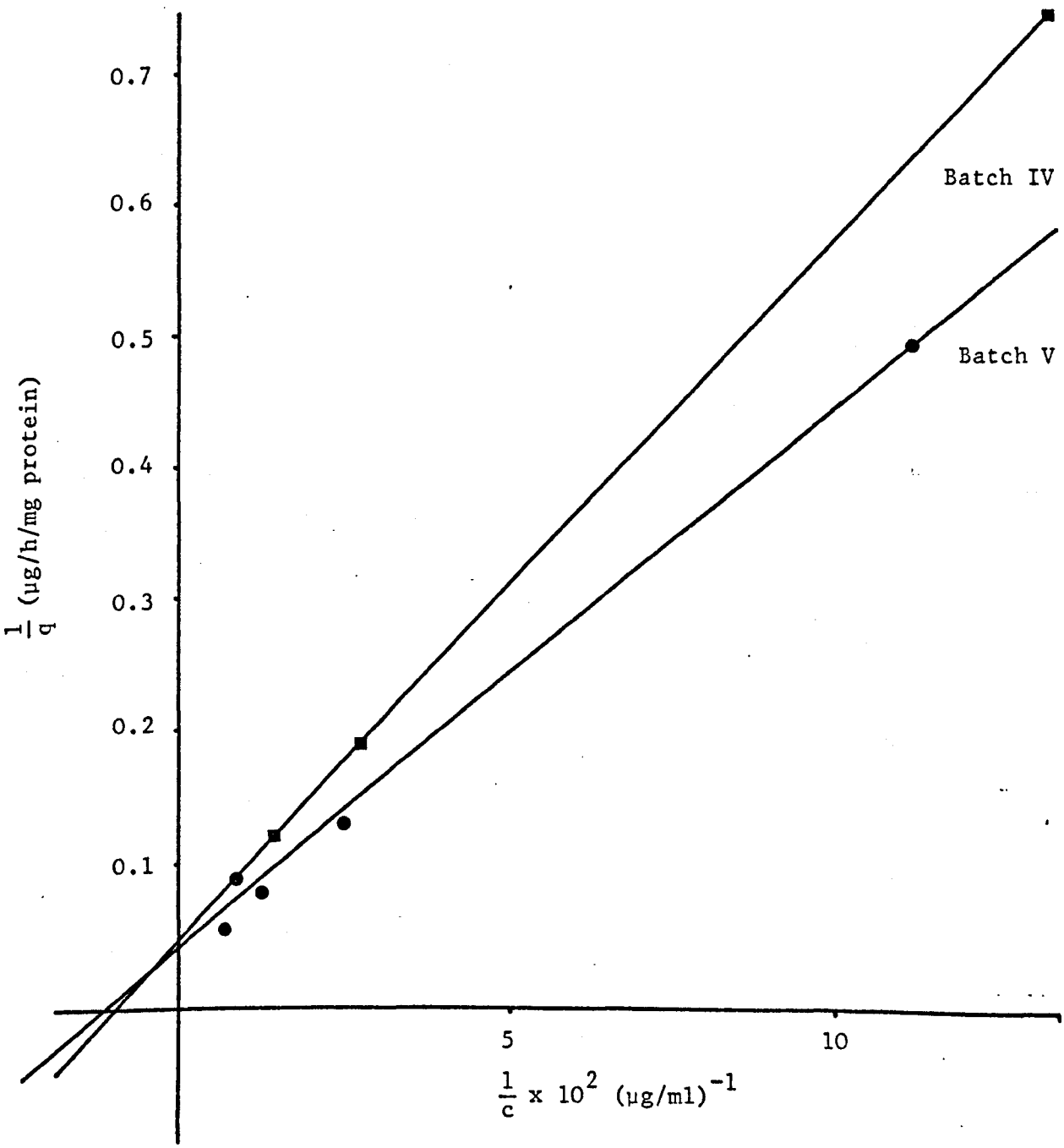


Fig. 4d Lineweaver Burk plot of $\frac{1}{q}$ versus $\frac{1}{c}$ for ^{125}I-labelled dBSA



CHAPTER 5

UPTAKE AND RELEASE OF RADIOLABELLED IMMUNOGLOBULINS BY THE RAT YOLK SAC IN VITRO

5.1 Introduction

In many mammalian species the fetus acquires passive immunity by transfer of IgG from the maternal to the fetal circulations; the route of transmission differs, however, from species to species. In man and the monkey, for example, transmission occurs before birth via the chorioallantoic placenta, whereas in the rabbit and guinea pig the yolk sac is instrumental in transmission (Wild, 1973). In other species such as the cow, pig, sheep, dog, rat and mouse, antibodies present in the colostrum or milk are adsorbed intact by the neonatal gut in the period immediately after birth (Halliday, 1955a, b). In the case of the rat, the major route of transfer has been shown to be post-natally via the gut, although pre-natal transmission has been shown by Brambell and Halliday (1956) and by Anderson (1959). These authors suggested that the pre-natal transport of IgG occurs by way of the visceral yolk sac, but this has been contested by Quinlivan (1964) who proposed that pre-natal transport in the rat occurs within the chorioallantoic placenta itself and not the yolk sac.

The precise mechanism of transcellular transport of IgG is not fully understood although two hypotheses have been proposed in relation to transport across the yolk sac of the rabbit. Brambell et al. (1966) suggested that selection of IgG for transmission occurs as an intracellular event; the IgG enters the vacuolar system but in some way is protected from degradation by lysosomal enzymes. Wild (1975), on the other hand, proposed that transfer of immunoglobulins in the rabbit occurs via a special transport vesicle which selectively ingests IgG then traverses the cell avoiding fusion with lysosomes (see Chapter 6, Section 6.1 for further detail).

Essentially all the investigations of the uptake and transport of IgG in the rabbit and rat have been performed in vivo. (Although Bamford (1966) studied the transmission of IgG across neonatal rat

gut sacs cultured *in vitro*). Thus, using *in vivo* techniques, Hemmings (1957) found that some 12% of an intrauterine dose of homologous IgG was transported across the yolk sac of the rabbit. Further studies by Batty *et al.* (1954) showed that in the rabbit most members of a series of heterologous IgG molecules were less readily transported than rabbit IgG itself in the order: rabbit > man > guinea pig > dog > horse > cow. Sonda and Schlomowitz (1972) also showed that the transmission of homologous IgG across the rabbit yolk sac *in vivo* to be independent of the concentration of rabbit serum albumin when both substrates were injected into the uterus together. These results demonstrate the selective nature of the transmission of immunoglobulins across cellular barriers with a marked preference for transport of the homologous immunoglobulin.

At the molecular level, the determinant for selection for transmission of IgG has been shown to reside in one specific region of the IgG molecule (Stanworth and Stewart, 1976). The IgG molecule (see Fig. 5n) consists of four polypeptide chains: two heavy chains (mol. wt. 50-75,000) each linked, by a number of disulphide bridges, to a light chain (mol. wt. 20-24,000); these chains can be separated by reducing the disulphide bonds. Digestion of the molecule with papain (Porter, 1959) results in cleavage across the heavy chains to give three fragments of similar size: an Fc region and two identical Fab fragments, while treatment with pepsin destroys the Fc region resulting in a divalent $F(ab')_2$ piece which retains antibody-binding activity.

The relative amounts of these fragments transported by the rabbit yolk sac *in vivo* were investigated by Brambell *et al.* (1959, 1960). Their findings showed the Fc region to be transmitted almost as readily as the intact molecule, whereas the Fab and $F(ab')_2$ fragments showed little or no transmission. Kaplan *et al.* (1965) by reducing

the disulphide bonds were able to show the heavy (H-chain) to retain the properties essential for transmission across the rabbit yolk sac but the Light (L-chain) showed little transmission. Furthermore, Morris (1963) found the Fc piece (administered to neonatal rat gut simultaneously with IgG) interfered with the uptake of IgG, whereas the Fab piece showed no interference at all when similarly administered. These findings are consistent with the existence of a specific recognition site on the IgG molecule within the Fc region which allows its transmission across the yolk sac.

In recent work Wild and Dawson (1977) have been able to demonstrate the presence of IgG specific receptors on the rabbit yolk sac. The technique was based on the method of Elson et al. (1975) in which a red blood-cell: antibody complex was presented to isolated rabbit yolk sac cells with the result that the complex binds to the yolk-sac epithelial cells, forming easily identified rosettes. Removal of the Fc region of the antibody resulted in loss of rosette formation. Other studies by Sonda and Schlamowitz (1972) also demonstrated the binding of ^{125}I-labelled IgG and ^{125}I-labelled rabbit serum albumin to formaldehyde-fixed rabbit yolk sac sections and that once membrane-bound, the IgG could not be displaced by rabbit serum albumin. These findings further support the conclusion that the sites to which IgG becomes bound are specific and it is proposed that, in the rabbit yolk sac at least, these binding sites are essential for transmission.

In the rat yolk sac, however, no similar evidence for the existence of IgG specific receptors has been obtained. In the previous chapter the pinocytic uptake of ^{125}I-labelled dBSA was investigated and a quantitative assessment of the fraction taken up by a route that involved plasma membrane binding was established. Further analysis of these data enabled the kinetic parameters relating to these albumin-binding sites to be calculated. The present chapter

reports a study of the rate of uptake of ^{125}I-labelled IgG by the rat yolk sac in the presence and absence of calf serum. Competition studies were also performed using non-radioactive, ^{127}I-iodinated dBSA in order to determine whether IgG-specific receptors were present on the rat yolk sac. Furthermore, a modified method of Williams et al. (1975a), for the study of the in vitro release of ingested substrates, was employed to follow any release of IgG (a phenomenon compatible with the existence of a route for the transmission of IgG across the rat yolk sac in vivo).

It was hoped that the use of these in vitro techniques with the rat yolk sac would enable more conclusive data to emerge on the question of the possible uptake and transmission of immunoglobulins across the visceral yolk sac of the rat in vivo.

METHODS

5.2 Uptake of homologous ^{125}I-labelled IgG by 17.5-day rat yolk sacs incubated in the presence and absence of calf serum

The rates of uptake of ^{125}I-labelled rat IgG (see Chapter 2 for preparation) by 17.5-day rat yolk sacs, cultured in either medium 199 alone or medium 199 containing calf serum (10%, v/v), were determined by the methods previously described (Sections 2.3 and 2.10). A concentration of 1.0 µg of radiotracer/ml of culture medium was employed in all experiments and yolk sacs were removed at regular intervals up to 6.5 h for incubations in the presence of calf serum and up to 5.0 h for incubations in the absence of calf serum. The protein content of each yolk sac was determined (see 2.5) and the quantity of 'total radioactivity' present in the yolk sac and the quantity of 'acid soluble radioactivity' in the medium were assayed as described in Section 2.4 and 2.6, respectively.

From each set of data, a plot of uptake of substrate (in µl/mg protein) against time enabled the related Endocytic Index to be calculated (see Section 2.13).

5.3 Effect of ^{127}I-iodinated dBSA on the uptake of ^{125}I-labelled IgG by 17.5-day yolk sacs incubated in medium 199 alone

In Chapter 4 it was demonstrated that competition occurred between ^{125}I-labelled dBSA (Batch V) and ^{127}I-iodinated dBSA when simultaneously ingested by 17.5-day yolk sacs incubated in the absence of calf serum (see Section 4.6). In order to determine whether a similar competitive effect could be observed when using ^{125}I-labelled IgG as substrate in place of ^{125}I-labelled dBSA, yolk sacs were incubated with ^{125}I-labelled IgG (Batch I, concentration of 1.0 µg/ml of medium) and with ^{127}I-iodinated dBSA (concentrations of 0, 42.5 and 170.0 µg/ml of medium). Again individual yolk sacs were removed at regular intervals up to 5.0 h and their radioactivity and protein

contents determined as described in Sections 2.4 and 2.5. The medium 'total radioactivity' and 'acid soluble radioactivity' were assayed by the methods described in Sections 2.6 and 2.7, respectively.

5.4 Release of ^{125}I-labelled IgG from 17.5-day rat yolk sacs re-incubated in the absence of calf serum

17.5-day yolk sacs were incubated in medium 199 alone (10.0 ml) containing ^{125}I-labelled IgG (Batch I) at a concentration of 5.0 μg/ml (see Section 2.17 for method). After 2 h incubation the yolk sacs were removed from their flasks and washed in warm (37°C), gassed medium 199 before being re-incubated for 3.0 h in fresh medium 199 (10.0 ml) containing no radiotracer. The quantities of 'total-' and 'acid soluble radioactivity' released by the re-incubated tissue up to a given time were calculated using equation 2.VIII (Chapter 2) then expressed as a percentage of the 'total radioactivity' associated with the yolk sac when transferred to the culture flask at the beginning of the re-incubation period. The percentage of 'acid insoluble radioactivity' released was obtained by subtracting the released 'acid soluble radioactivity' from the corresponding value for the released 'total radioactivity' at each time interval.

5.5 Release of ^{125}I-labelled IgG from 17.5-day yolk sacs re-incubated in the presence and absence of native IgG in the culture medium

In an attempt to determine whether the release of 'acid insoluble radioactivity' by a yolk sac on re-incubation was a result of desorption from the tissue surface rather than true exocytosis, the experiment described above (5.4) was repeated but with native IgG present in the re-incubation medium. Since a different batch of ^{125}I-labelled IgG was used, experiment 5.4 was also repeated as a necessary control.

Three yolk sacs, each taken from a different animal, were incubated in the same flask containing ^{125}I-labelled IgG (Batch III,

5.0 μg/ml) for 2.0 h then washed (see Section 2.17) and re-incubated at 37°C in separate flasks containing medium 199 alone. In a second series of experiments, a further 3 yolk sacs, taken from the same animals, were similarly incubated with ^{125}I-labelled IgG (Batch III, 5.0 μg/ml), washed as above but using medium 199 containing 10 μg/ml of unlabelled rat IgG, before re-incubation in medium 199 alone. Finally, another three yolk sacs from the same animals were incubated and washed as in the second series above, but on re-incubation the medium also contained unlabelled-IgG at a concentration of 5.0 μg/ml.

The release data were expressed in the same manner as described in detail in Section 2.17 of Chapter 2.

5.6 Effect of temperature on the rates of release of radioactivity from yolk sacs previously incubated with either ^{125}I-labelled IgG or ^{125}I-labelled dBSA

The effect of lowering the re-incubation temperature on the rate of release of ^{125}I-labelled IgG (Batch III) from a 17.5-day rat yolk sac, following its ingestion, was investigated using re-incubation temperatures of 21°C and 4.5°C. The experimental regime was the same as that described in Section 5.4 except that during the washing procedure the temperature of the medium was reduced to below 37°C and the yolk sacs re-incubated at the lower temperature.

The results shown in Tables 5.VIIId and e are for re-incubation temperatures of 21°C and 4.5°C, respectively. The quantities of the three species of released radioactivity, each expressed as a percentage of the total radioactivity associated with the tissue at the start of re-incubation, were plotted against time for each of the lower temperature experiments.

For comparative purposes, the rates of release of ^{125}I-labelled PVP and ^{125}I-labelled dBSA (Batch VI) at the same three temperatures were also investigated in equivalent experiments.

5.7 Chromatography of the radioactive species released from 17.5-day yolk sacs when re-incubated after ingesting ^{125}I-labelled IgG

Three 17.5-day rat yolk sacs, each from a different animal, were incubated together in a single flask for 2.0 h in medium 199 (10.0 ml) containing 5.0 µg/ml of ^{125}I-labelled IgG (Batch III). The yolk sacs were then removed, washed in warm medium 199 and re-incubated together in 10.0 ml of fresh medium. After incubation at 37°C for 3.0 h the yolk sacs were discarded, the remaining medium was centrifuged at 1000 g for 10 min to remove any detached cells and the supernatant was then deep-frozen until assayed.

The radioactivity released into the medium was analysed by gel filtration using both Sephadex G-25 and G-150 with 0.05M-phosphate buffered saline (pH 5.0) as elutant. A sample of the centrifuged medium (2.0 ml) was applied to a column (57 x 1.5 cm) of Sephadex G-25 and eluted at 4°C at a flow rate of 0.4 ml/min. Fractions (2.0 ml) were collected and assayed for their contained radioactivity. An identical 2.0 ml sample of the re-incubation medium was applied to a column (57 x 1.5 cm) of Sephadex G-150. Again 2.0 ml fractions were collected but using a slower elution rate (0.2 ml/min). To both columns samples of ^{125}I-labelled IgG (Batch III, [^{125}I]iodide, [^{125}I]iodo-L-tyrosine and glycyl [^{125}I]iodo-L-tyrosine were also applied to establish their relative elution positions. The void volume (V_o) of each column was estimated with Blue Dextran.

RESULTS

5.8 Endocytic Index of ^{125}I-labelled IgG in the absence and presence of calf serum

Figs. 5a and 5b show representative uptake plots for ^{125}I-labelled IgG (Batch III), in the presence and absence of calf serum respectively, together with the corresponding quantity of radioactivity associated with the tissue at each time interval. As with ^{125}I-labelled dBSA (Fig. 5c), the plots of total uptake (T+S) were substantially linear, enabling the rate of uptake to be expressed as an Endocytic Index.

The results of a number of experiments in which the rates of uptake of several different batches of ^{125}I-labelled IgG were determined in the absence and presence of calf serum are shown in Tables 5.I and 5.II respectively. These same results are summarized in Tables 5.III and 5.IV, respectively for ease of comparison. With each batch of ^{125}I-labelled IgG, the removal of calf serum from the culture medium resulted in a large increase in the observed Endocytic Index. Relative to corresponding experiments in the presence of calf serum Batches I - III showed increases of: 10.4-, 7.0- and 7.2-fold in the Endocytic Index of ^{125}I-labelled IgG on removing calf serum from the medium. The increases were higher than those observed with ^{125}I-labelled dBSA which, under the same conditions, showed corresponding increases of 3.7- and 5.5-fold in the Endocytic Index of Batches III and V, respectively (see Chapter 3).

It should be noted that in most experiments in which ^{125}I-labelled IgG was ingested in the presence of calf serum, a marked tendency for a progressive rise in the amount of radioactivity associated with the tissue was shown (Table 5.III) but no such accumulation was observed in corresponding incubations in which calf serum was absent from the culture medium (Table 5.IV). In both types of incubation the value of the mean quantity of tissue-associated radioactivity was

calculated by taking the mean of all values after the initial 2.0 h of incubation, whether or not accumulation of radiotracer occurred.

The results also indicate that, on removing calf serum from the medium, the quantity of tissue-associated radioactivity shows a marked increase relative to that in the presence of calf serum. This is a similar effect to that observed in corresponding experiments with ^{125}I-labelled dBSA (see Chapter 3). Tables 5.III and 5.IV show values of the overall mean value of the tissue-associated radioactivity for each batch of ^{125}I-labelled IgG in the presence and absence of calf serum, respectively. Taking Batch III as a typical example: a 4.55-fold increase in the amount of radioactivity associated with the tissue is observed on removing the calf serum from the culture medium, somewhat less than the corresponding increase in Endocytic Index (7.2-fold).

5.9 Endocytic Index of ^{125}I-labelled IgG in the presence and absence of ^{127}I-iodinated dBSA in medium 199 alone

In order to determine whether the binding sites on the plasma membrane, responsible for the high rate of ingestion of ^{125}I-labelled IgG, were specific or non-specific in character, the effect of ^{127}I-iodinated dBSA on the rate of uptake of ^{125}I-labelled IgG was studied in medium 199 alone, to avoid additional competitive effects from calf serum proteins.

Values of the Endocytic Index of ^{125}I-labelled IgG (Batch I) in the presence of increasing concentrations of ^{127}I-iodinated dBSA (0.0, 42.5, 170.0 µg/ml of culture medium) are shown in Table 5.V and summarized in Table 5.VI. Also shown in Table 5.V is the mean quantity of tissue-associated radioactivity (µl/mg protein) calculated for the period 2.0 h - 5.0 h for each set of data; these values are summarized in Table 5.VI under the heading of overall mean value of tissue-associated radioactivity.

The value quoted in Table 5.VI for the mean Endocytic Index of ^{125}I-labelled IgG (Batch I) at 0.0 μg/ml of ^{127}I-iodinated dBSA is the same as that reported in Table 5.IV,but has been stated again to aid comparison. The summarized data of Table 5.VI are shown graphically in Fig. 5d.

The presence of ^{127}I-iodinated dBSA at a concentration of 42.5 μg/ml in the culture medium resulted in a 38% fall in the Endocytic Index of ^{125}I-labelled IgG relative to the value when no competing proteins were present. Increasing the ^{127}I-iodinated dBSA concentration further to 170.0 μg/ml did not result in any further fall in the Endocytic Index.

Determination of the overall mean value of the tissue-associated radioactivity showed it to rise and fall in a similar manner to the corresponding value of the Endocytic Index, although the fall in the level of tissue-associated radioactivity caused by 42.5 μg/ml of ^{127}I-iodinated dBSA was approx. 30% less than the corresponding fall in Endocytic Index.

5.10 Release of ^{125}I-labelled IgG from 17.5-day yolk sacs re-incubated in the absence of calf serum

The release of radioactivity from 17.5-day yolk sacs after ingestion of ^{125}I-labelled IgG was investigated by the method described in Section 5.4 and the released radioactivity expressed as the percentage of the total radioactivity associated with the yolk sac when transferred to the re-incubation flask.

Results obtained using ^{125}I-labelled IgG (Batch I) as substrate and 3 different yolk sacs are shown in Table 5.VII. Plots of the mean percentage release for the three classes of radioactivity (i.e. 'total', 'acid soluble', and 'acid insoluble') are shown in Fig. 5e.

Inspection of Fig. 5e shows that after 3.0 h of re-incubation over 87% of the tissue-associated radioactivity was released into the

incubation medium, moreover, almost 50% of this was in the form of 'acid insoluble' species. This is in striking contrast to the results obtained using either ^{125}I-labelled PVP or ^{125}I-labelled dBSA (Batch VIII) in place of ^{125}I-labelled IgG (Batch I) (see Table 5.Xa and Fig. 5h for release of ^{125}I-labelled dBSA at 37°C). With ^{125}I-labelled dBSA as substrate only a small percentage (approx. 5.0%) of radiotracer was released as 'acid insoluble' species although the percentage of 'total radioactivity' released was similar.

5.11 Release of ^{125}I-labelled IgG from 17.5-day yolk sacs re-incubated in the presence of unlabelled IgG

The possibility that the above observation simply reflected de-adsorption of 'acid insoluble' radioactivity adhering to the tissue-surface, was investigated using the method described in Section 5.5. Tables 5.VIIIa, b and c show values of the mean percentage release which are also plotted together in Fig. 5f for ease of comparison. For purposes of clarity, the associated standard deviation for each point reported in these tables was not indicated in the plot.

For ^{125}I-labelled IgG (Batch III) the percentages of 'total radioactivity' released on re-incubation (Fig. 5f) show no significant differences between those experiments in which unlabelled-IgG was present in either the washing or re-incubation media and those in which yolk sacs were washed and re-incubated in medium 199 alone. The amount of radioactivity released by 3.0 h of re-incubation was approx. 87% of the total tissue-associated radioactivity. Similarly, the percentages of 'acid soluble' and 'acid insoluble radioactivity' released show no significant differences between each type of experiment.

5.12 Release of ^{125}I-labelled IgG, ^{125}I-labelled PVP and ^{125}I-labelled dBSA from 17.5-day yolk sacs re-incubated at lower temperatures

The effects of lowering the re-incubation temperature (from 37°C to either 21°C or 4.5°C) on the pattern of release of radio-labelled

species from ^{125}I-labelled IgG-laden yolk sacs are shown in Tables 5.IXa and b, respectively as well as in Fig. 5g. For purposes of comparison, Fig. 5h and Tables 5.Xa, b and c show the rates of release of radioactive species derived from ^{125}I-labelled dBSA in similar experiments at these same three temperatures.

In the case of ^{125}I-labelled IgG, lowering the temperature from 37°C to 21°C caused the amount of ingested radiotracer released over a 3.0 h period to fall from approx. 85% to less than 50%. Lowering the temperature further, to 4.5°C, resulted in an even slower release of 'total radioactivity', only about 21% over the same period. The amount of 'acid insoluble radioactivity' released is also shown in Fig. 5g. At 37°C a maximum of just over 40% of the 'total radioactivity' released was found to be 'acid insoluble' and lowering the temperature caused the 'acid insoluble radioactivity' released to drop in proportion to the observed fall in 'total radioactivity' released.

Fig. 5h shows the release of ^{125}I-labelled dBSA digestion products at three different temperatures, these experiments were performed in parallel with others in which the release of ^{125}I-labelled IgG digestion products was followed. At 37°C the amount of 'total radioactivity' released by 3.0 h was somewhat lower than in the corresponding experiments with ^{125}I-labelled IgG. For ^{125}I-labelled dBSA the maximum release was approx. 63% compared to over 85% for ^{125}I-labelled IgG. However, at 21°C and 4.5°C the amount of 'total radioactivity' released were found to be virtually identical with those for ^{125}I-labelled IgG.

With ^{125}I-labelled dBSA, the amount of 'acid insoluble radioactivity' released does not show any significant variation with temperature. It should be noted that after 50 min the release of 'acid insoluble radioactivity' is almost complete and amounts to only between 5 and 12% of the 'total radioactivity'.

The pattern of release of 'acid insoluble radioactivity' at 4.5°C after ingestion of ^{125}I-labelled IgG was virtually identical to that after ingestion of ^{125}I-labelled dBSA; at 50 min the amount released was again between 5 and 12% of the 'total radioactivity'. On the other hand higher temperatures would appear to facilitate the progressive release of 'acid insoluble radioactivity' derived from this substrate.

5.13 Chromatography of the digestion products of ^{125}I-labelled IgG and ^{125}I-labelled dBSA released from 17.5-day rat yolk sacs

The size of the radioactive molecules released by 17.5-day rat yolk sacs that had previously ingested either ^{125}I-labelled IgG or ^{125}I-labelled dBSA was investigated by gel chromatography on Sephadex G-25 and G-150 (see Section 5.7 for methods).

Figs. 5j and 5k show the elution patterns obtained on Sephadex G-25. The total recovery for ^{125}I-labelled IgG was calculated to be 88.5% of the applied radioactivity. Three main peaks were seen to emerge, Peak A (36.7% of recovered activity) was present in the void-volume, where ^{125}I-labelled IgG itself elutes, peak B (3.0%) was found in the same position as [^{125}I]iodide and the major peak, C (57.3%), eluted in a position corresponding to that where the markers [^{125}I]iodo-L-tyrosine and glycyl [^{125}I]-iodo-L-tyrosine both elute. The elution pattern for ^{125}I-labelled dBSA showed three major components A, B and C corresponding to 10, 8.8, and 80.5% of the applied activity, respectively. The recovery of activity applied to the column was about 99.0%. The three peaks eluted in positions corresponding to ^{125}I-labelled dBSA, ^{125}I[iodide] and that where both glycyl[^{125}I]iodo-L-tyrosine and [^{125}I]iodo-L-tyrosine elute.

The elution patterns obtained using Sephadex G-150 were similar to those obtained on G-25, see Figs. 5l and 5m.

DISCUSSION

The pattern of uptake of homologous ^{125}I-labelled IgG by the 17.5-day rat yolk sac when incubated in medium containing calf serum (10%, v/v) was similar to that observed with other substrates already investigated (^{125}I-labelled PVP and ^{125}I-labelled dBSA) in that it was linear with time up to at least 7.0 h, the correlation coefficients of the individual uptake plots being in excess of 0.95 (see Table 5.I). The associated Endocytic Indices varied little between different batches of this substrate and fell within the range 6-13 µl/h/mg protein (see Table 5.III). On comparing these values with the mean Endocytic Index observed for ^{125}I-labelled PVP, (Table 3.VI, Chapter 3) the overall rate of ingestion of ^{125}I-labelled IgG can be seen to be far greater but falls in the same range (8-150 µl/h/mg yolk-sac of/protein) as other ^{125}I-labelled proteins ingested in the presence of calf serum (see Moore *et al.*, 1977; Livesey, G., unpublished data). Therefore, unlike the uptake of either ^{125}I-labelled PVP or [^{14}C]sucrose (Chapter 3, Section 3.12), the uptake of ^{125}I-labelled rat immunoglobulins cannot occur solely by simple fluid phase ingestion into pinocytic vesicles and the major route must either involve prior attachment of the substrate to the plasma membrane before internalisation into such vesicles or occur largely by some additional, specific route available only to immunoglobulins.

On incubation with yolk sacs, a substantial portion of the ^{125}I-labelled IgG present in the medium was hydrolysed to give ^{125}I-labelled species that were soluble in trichloroacetic acid. Moreover, chromatography of these ^{125}I-labelled acid soluble species on Sephadex G-25 indicated that they consisted entirely of [^{125}I]iodo-L-tyrosine and small peptides containing [^{125}I]iodo-L-tyrosine. This pattern of hydrolysis is essentially the same as that observed with other ^{125}I-labelled proteins, including ^{125}I-labelled

dBSA, and has been attributed to full catabolism of such proteins within the lysosomal system of the epithelial cells of the yolk sac (Moore et al., 1977).

Williams et al. (1975b) showed the high rate of uptake of ^{125}I-labelled dBSA, relative to that of ^{125}I-labelled PVP, to arise from adsorption of this protein to the plasma membrane. If high values for the rate of uptake of ^{125}I-labelled IgG similarly result from the binding of ^{125}I-labelled IgG to the plasma membrane, the absence of any competing proteins would be expected to give an increase of the same type as observed with ^{125}I-labelled dBSA when calf serum is removed from the medium (see Chapter 3). Incubation of ^{125}I-labelled IgG with yolk sacs in the absence of calf serum indeed resulted in a 7- to 10-fold increase in Endocytic Index (Table 5.IV), as compared with a 3- to 6-fold increase observed with ^{125}I-labelled dBSA (Table 3.VI). The greater percentage increase observed with ^{125}I-labelled IgG is compatible with the suggestion that certain components of calf serum, possibly immunoglobulins, are able to compete more effectively with ^{125}I-labelled IgG than with ^{125}I-labelled dBSA for binding sites on the plasma membrane. This suggestion also gains support from the observation that ^{125}I-labelled IgG, ingested by yolk sacs incubated in serum-free medium 199 containing (non-radioactive) ^{127}I-iodinated dBSA at concentrations of up to 170 µg/ml of medium, showed a maximum reduction in Endocytic Index of only approx. 40% (Fig. 5d) as opposed to a reduction of approx. 75% in the Endocytic Index of ^{125}I-labelled dBSA in equivalent experiments (see Section 4.6).

Although the uptake of ^{125}I-labelled IgG by 17.5-day rat yolk sacs resembled the uptake of ^{125}I-labelled dBSA in the above respects, in two others there were marked differences between the two proteins. Firstly, ^{125}I-labelled IgG was seen to accumulate progressively in

the tissue at a significant rate when incubated for 6 h in medium containing 10% (v/v) calf serum (see Table 5.III). This is in sharp contrast to equivalent experiments with ^{125}I-labelled dBSA as substrate. Secondly, following *in vitro* accumulation of ^{125}I-labelled IgG by yolk sacs, re-incubation of the tissue in substrate-free medium led to a rapid release of radioactivity that was superficially similar to that observed for ^{125}I-labelled dBSA, but in contrast approximately half of the radioactivity released was found to be acid-insoluble.

Furthermore, the progressive rise in the level of the tissue-associated radioactivity (Fig. 5a) contrasts with the steady-state level observed with ^{125}I-labelled dBSA in either the presence or absence of calf serum. In the absence of calf serum, ^{125}I-labelled IgG probably does not accumulate in the tissue in the same progressive manner (Fig. 5b) but even in this case the mean level of the tissue-associated radioactivity was abnormally high compared with that anticipated from an analysis of the rate of production of acid-solubles (Livesey, G., unpublished data). By this empirical method, the slope of the plot of acid-soluble radioactivity against time (line S of Fig. 5c) is multiplied by x, the horizontal separation of the parallel portions of the plots S and (T + S) in hours (see Fig. 5c) to give the tissue content expected from the observed rate of production of acid-solubles. In the case of ^{125}I-labelled dBSA the agreement between the observed tissue level (97.5 µl/mg protein) and the calculated level (89.2 µl/mg protein) was close (9% underestimated). For a number of other protein substrates incubated with the 17.5-day yolk sacs a similar degree of agreement (i.e. within $\pm$ 10%) was invariably found (Livesey, unpublished data). For ^{125}I-labelled IgG, however, the calculated value of the tissue level (66.9 µl/mg protein) was some 25% less than the observed level, hence well outside

the normal range of error.

The observation that, following in vitro accumulation of ^{125}I-labelled IgG by the yolk sac, re-incubation of the tissue in substrate-free medium led to rapid release of radioactivity with approx. 50% of the radioactivity returned to the re-incubation medium at any instant being in the form of macromolecular IgG (Fig. 5e), contrasts sharply with the pattern observed in equivalent experiments with ^{125}I-labelled dBSA (Fig. 5h) which showed no progressive loss of equal amounts of acid-soluble and -insoluble activity. Thus, unlike any other ^{125}I-labelled protein investigated to date (Livesey, G., unpublished data), ^{125}I-labelled IgG alone shows release of a significant quantity of the tissue-associated protein back into the medium as acid-insoluble rather than acid-soluble species. On Sephadex G-150 the acid-insoluble material released eluted in the same position as ^{125}I-labelled IgG itself hence can tentatively be identified as such. This indicates that a portion of the ingested ^{125}I-labelled IgG somehow escapes hydrolysis within the lysosomal system and can pass back out of the epithelial cells into the medium.

A possible explanation of the observed release of macromolecular ^{125}I-labelled IgG from re-incubated yolk sacs is that ^{125}I-labelled IgG simply binds strongly to regions of the tissue that are not involved in pinocytic processes and desorbs only slowly, so does not become detached during the 3 x 2 min washing procedure prior to re-incubation in substrate-free medium. This explanation is rendered unlikely by the observation of identical patterns of release when tissue was either washed in the presence of unlabelled IgG before re-incubation or both washed and re-incubated in the presence of unlabelled IgG (Fig. 5f). Both procedures would be expected to favour the release of ^{125}I-labelled IgG adsorbed to the tissue surface and modify the rate of release of macromolecular ^{125}I-label during

the re-incubation period.

The rate of release of ^{125}I-labelled IgG at 37°C appeared to be rapid, there being little further release beyond the initial 60 min (Fig. 5g) by when some 35% of the tissue-associated radioactivity had been released in an acid-insoluble form. This contrasts with a release, under the same conditions, of only approximately 5% of the tissue-associated radioactivity in the case of ^{125}I-labelled PVP (Fig. 7a) and of a similar quantity of acid-insoluble radioactivity in the case of ^{125}I-labelled dBSA. This 5% release may represent either a basal level of exocytosis by the tissue or result from leakage from the cells following cell death or represent release of that residual activity, occluded between the microvilli of the tissue, that is not removed in the washing procedure.

Exocytosis experiments, using yolk sacs laden with ^{125}I-labelled IgG and re-incubation temperatures below 37°C, showed the release of both acid-soluble and acid-insoluble radioactivity to be equally temperature sensitive (Fig. 5g) whereas equivalent experiments with ^{125}I-labelled dBSA showed only the release of acid-soluble material to be temperature sensitive. These findings strongly suggest that the release of acid-insoluble ^{125}I-labelled IgG from the tissue cannot be attributed to release of protein occluded between the villi or from cell death hence the release almost certainly occurs by a process of physiological significance.

The observation of a high rate of exocytosis of ^{125}I-labelled IgG from the yolk-sac tissue implies that the true rate of capture will be considerably in excess of that indicated by the observed Endocytic Index, since that portion of the protein that enters the tissue and returns to the medium again in a macromolecular form will make no detectable contribution to the observed rate of uptake. Unfortunately, the net flux of macromolecular IgG out of tissue

cannot be calculated from the results of experiments of the type reported in Fig. 5e without a number of arbitrary assumptions being made (e.g. the amount of intact IgG lost by exocytosis during the 6 min wash period).

Although a high rate of exocytosis prevents any exact comparison of the Endocytic Index of ^{125}I-labelled IgG with those of other substrates, the striking qualitative differences observed with this protein are worthy of further discussion since they may well be of biological importance.

The rat is one of the species in which IgG is thought to be transmitted from the mother to the fetus partly across the yolk sac before birth but mainly via secretion in the milk and passage across the gut of the neonate during the first 21 days after birth, so that the pup gains passive immunity by two routes (Wild, 1973). From the experiments reported in this chapter, the fate of ^{125}I-labelled rat IgG within the 17.5-day rat visceral yolk sac incubated *in vitro* is atypical when compared with that of all other ^{125}I-labelled proteins studied in the same system. The observation that half of the ^{125}I-labelled IgG was completely hydrolysed to the level of amino acids, presumably upon entering the lysosomal system, while an equal quantity was released back into the culture medium without suffering any detectable hydrolysis, suggests that these two separate fates may reflect the existence of two distinct intracellular routes by which IgG is handled within the yolk-sac tissue. It is possible that the mechanism which enables the IgG to escape intact from the incubated tissue may be involved in the transfer of passive immunity across the yolk sac *in vivo*.

It is informative to consider the possible fates of IgG within yolk-sac tissue in relation to the ultrastructure of the tissue (Fig. 5i). At 37°C the rate of release of intact ^{125}I-labelled IgG

was far higher (approx. 10% per hour over the period 0.5-1.5 h) as opposed to a value of 1-2% over the same period for ^{125}I-labelled dBSA, ^{125}I-labelled PVP or colloidal [^{198}Au]gold (Roberts et al., 1977). It is conceivable that the observed high rate of release of intact IgG could arise from IgG simply entering the same pinocytic vesicles as used by other substrates. If the vesicles containing IgG remain in the apical region of the epithelial cells, rather than migrate rapidly towards the nuclear region to fuse with the lysosomal system, and if the membrane of the vesicles containing IgG becomes modified through the presence of IgG so that such vesicles are more prone to fuse again with the microvillous face of the tissue, then the higher rate of exocytosis of IgG over other substrates could be explained. If such a mechanism did indeed operate then the IgG would be released back into the medium from the same face of the tissue as involved in its uptake. (Unfortunately the design of the above experiments does not permit determination of the face of the tissue responsible for the observed release but the design of an incisive experiment should prove possible.) If, however, the IgG contained within pinocytic vesicles wasreleased by fusion of the vesicles with a region of the epithelial cell wall below the junction complex (Fig. 5i) it would then need to traverse the full thickness of the tissue (some 25 μ) to reach the opposite face. If, in vivo, the major fate of the non-degraded portion of the ingested ^{125}I-labelled IgG was release into the vitelline blood vessels followed by clearance from the vitelline circulation, rather than penetration of the serosal basement membrane to reach the exocoelom, then in vitro, where there is no circulation, it would be expected that the ^{125}I-labelled acid-insoluble activity would accumulate progressively in these stagnant vessels. The observed progressive accumulation of activity in the tissue when yolk sacs were incubated with ^{125}I-labelled IgG in the presence of 10%

(v/v) calf serum is compatible with this suggestion, but the failure of the tissue to accumulate activity at a significant rate in similar experiments performed in the absence of calf serum requires a separate explanation. Possibly the endothelial cells of the vitelline capillaries are sensitive to changes in the composition of the medium and become more permeable or loosely associated in the absence of serum, so that ^{125}I-labelled IgG entering the vitelline capillaries can diffuse out again readily.

Clearly, further experiments are needed to eliminate some of these possible explanations, but the above preliminary experiments indicate that the atypical fate of ^{125}I-labelled IgG in the *in vitro* system is worthy of further investigation, since it may well be related to the mechanism of transfer of passive immunity *in vivo*. The experiments, described in the following chapter, were designed to eliminate some of the possible explanations of the above results and to compare the indications from such experiments with established hypotheses for the transfer of passive immunity across tissue barriers.

Table 5.I Endocytic Index of ^{125}I-labelled IgG for 17.5-day rat yolk sacs cultured in vitro in medium 199 plus 10% (v/v) calf serum

Each row of data relate to a single experiment in which a number of yolk sacs taken from a 17.5-day pregnant animal were incubated separately at 37°C in medium 199 plus 10% (v/v) calf serum to which ^{125}I-labelled IgG was added at a concentration of 1.0 µg/ml of culture medium. Yolk sacs were removed at regular intervals up to 6.5 h.

			Total Uptake			Tissue Radioactivity		
Batch of ^{125}I-IgG	% TCA-solubles in ^{125}I-IgG preparation	No. of Yolk Sacs	Endocytic Index (µl/h/mg protein)	Correlation Coefficient	Intercept (µl/mg/protein)	Mean Tissue Radioactivity (µl/mg protein)	Tissue Accumulation Rate (µl/h/mg protein)	Correlation Coefficient
Batch I	1.54	10	5.62	0.968	+ 3.16	10.59	1.41	0.937
" "	1.54	9	4.00	0.983	+18.60	11.86	1.46	0.866
" "	1.54	9	9.18	0.975	+ 0.57	19.23	3.46	0.986
Batch II	3.5	9	7.10	0.953	+ 5.26	13.52	1.16	0.762
" "	3.5	10	5.74	0.964	+ 5.35	13.68	2.06	0.964
" "	3.6	10	14.53	0.975	+ 8.58	19.97	3.04	0.879
Batch III	1.79	10	15.83	0.955	- 7.24	21.71	4.38	0.892
" "	1.43	10	11.71	0.984	+ 4.90	16.46	2.05	0.898
" "	1.50	10	14.28	0.980	- 3.03	17.90	2.92	0.978
" "	2.60	10	11.67	0.951	+ 6.00	23.30	3.06	0.953

Table 5.II Endocytic Index of ^{125}I-labelled IgG for 17.5-day rat yolk sacs cultured in vitro in medium 199 alone

Each row of the table relate to single experiments as described in the caption to Table 5.I except calf serum was omitted from the culture medium, and yolk sacs were removed at regular intervals up to 5.0 h not 6.5 h.

Batch of ^{125}I-IgG	% TCA-solubles in ^{125}I-IgG preparation	No. of Yolk Sacs	Total Uptake			Tissue Radioactivity		
			Endocytic Index (µl/h/mg protein)	Correlation Coefficient	Intercept (µl/mg protein)	Mean Tissue Radioactivity (µl/mg protein)	Tissue Accumulation Rate (µl/h/mg protein)	Correlation Coefficient
Batch I	1.54	8	39.97	0.954	+ 82.20	78.36	4.08	0.483
" "	1.61	8	56.68	0.944	+ 84.60	72.68	0.51	0.096
" "	1.61	10	54.66	0.963	+ 89.30	89.90	5.25	0.457
" "	1.61	10	57.21	0.932	+ 53.21	70.48	2.89	0.290
" "	1.35	7	75.93	0.985	+ 48.46	75.90	12.00	0.886
Batch II	3.0	10	71.90	0.935	+144.68	99.51	0.98	0.085
" "	3.6	8	67.31	0.953	+ 95.64	107.10	16.47	0.840
" "	4.2	10	53.2	0.971	+ 34.42	84.60	5.50	0.678
Batch III	1.5	10	107.78	0.982	+ 26.22	112.38	15.27	0.816
" "	1.79	10	76.61	0.977	+ 15.67	96.98	10.42	0.805
" "	2.60	10	105.7	0.956	+ 42.89	83.62	1.17	0.117

Table 5.III Summarized values of the Endocytic Index of ^{125}I-labelled IgG for 17.5-day rat yolk sacs cultured in vitro in medium 199 plus 10% (v/v) calf serum

Summary of the data presented in Table 5.I

Batch of ^{125}I-IgG	No. of Experiments	Endocytic Index (µl/h/mg protein) Mean ± S.D.	Tissue Radioactivity (µl/mg protein) Overall mean ± S.D.	Tissue Accumulation Rate (µl/h/mg protein) Mean ± S.D.
Batch I	3	6.27 ± 2.65	13.89 ± 4.67	2.11 ± 1.17
Batch II	3	9.12 ± 4.73	15.72 ± 3.68	2.09 ± 0.94
Batch III	4	13.37 ± 2.04	19.84 ± 3.20	3.10 ± 0.96

Table 5.IV Summarized values of the Endocytic Index of ^{125}I-labelled IgG for 17.5-day rat yolk sacs cultured in vitro in medium 199 alone

Summary of data presented in Table 5.II

Batch of ^{125}I-IgG	No. of Experiments	Endocytic Index (µl/h/mg protein) Mean ± S.D.	Tissue Radioactivity (µl/mg protein) Overall mean ± S.D.	Tissue Accumulation Rate (µl/h/mg protein) Mean ± S.D.
Batch I	5	56.90 ± 12.78	77.46 ± 7.56	4.95 ± 4.32
Batch II	3	64.13 ± 9.74	97.07 ± 11.45	7.65 ± 7.97
Batch III	3	96.7 ± 17.44	97.66 ± 14.39	8.95 ± 7.16

Table 5.V Endocytic Index of ^{125}I-labelled IgG (Batch I) for 17.5-day yolk sacs incubated in the presence of different concentrations of ^{127}I-iodinated dBSA in medium 199 alone

Each row of data refers to a single experiment performed in the absence of calf serum with ^{127}I-labelled dBSA added (to the incubation medium) at different concentrations

^{127}I-dBSA Concentrations (µg/ml)	% TCA-solubles in ^{125}I-IgG preparation	No. of yolk sacs	Endocytic Index (µl/h/mg protein)	Correlation Coefficient	Intercept (µl/mg protein)	Mean Tissue Activity (µl/mg protein)
0	1.54	8	39.97	0.954	+ 82.20	78.36
"	1.61	8	56.68	0.944	+ 84.60	72.68
"	1.61	10	54.66	0.963	+ 89.30	89.90
"	1.61	10	57.21	0.932	+ 53.21	70.48
"	1.35	7	75.93	0.985	+ 48.46	75.90
42.5	1.61	6	32.1	0.939	+ 79.35	58.62
"	1.61	6	30.5	0.963	+ 62.3	46.00
"	1.61	6	43.18	0.957	+ 47.78	56.79
170.0	1.54	9	51.90	0.916	+102.65	79.19
"	1.61	9	33.55	0.927	+ 39.11	51.26
"	1.61	6	26.08	0.971	+ 40.18	38.63

Table 5.VI Summarized values of the Endocytic Index of ^{125}I-labelled IgG (Batch I) in the presence of different concentrations of ^{127}I-iodinated dBSA in medium 199 alone

Summary of data presented in Table 5.V

^{127}I-dBSA Concentration (µg/ml)	No. of Experiments	Endocytic Index (µl/h/mg protein) Mean ± S.D.	Tissue Radioactivity (µl/mg protein) Overall mean ± S.D.
0	5	56.90 ± 12.78	77.46 ± 7.56
42.5	3	35.26 ± 6.91	53.80 ± 6.82
170.0	3	37.18 ± 13.29	56.36 ± 20.76

In the following tables (5.VII - 5.Xc, inclusive) each of the columns of data represent mean values derived from three separate, identical experiments in each of which a single yolk sac, taken from a different animal, was used.

Table 5.VII Release of radioactive species derived from ^{125}I-labelled IgG (Batch I) by 17.5-day rat yolk sacs re-incubated in the absence of calf serum

Time (min)	Mean Percentage radioactivity released ± S.D.		
	acid soluble	acid insoluble	total radioactivity
0	-1.0 ± 2.6	-0.6 ± 2.1	1.0 ± 0.1
15	12.6 4.9	27.7 5.6	40.3 5.2
30	25.0 7.7	34.2 9.4	59.2 4.3
45	30.0 9.9	40.5 11.6	70.5 4.1
60	32.7 10.2	42.8 11.2	75.5 4.8
75	34.1 11.0	45.4 12.0	79.5 3.9
90	35.4 12.2	46.1 12.5	81.6 3.9
105	36.5 12.7	47.0 13.4	83.5 3.9
120	36.5 13.0	47.8 14.0	84.3 4.0
150	37.5 13.8	49.0 14.7	86.5 3.5
180	38.4 14.5	49.7 15.1	88.1 3.5

Table 5.VIIIa Release of radioactive species derived from ^{125}I-labelled IgG (Batch III) by 17.5-day rat yolk sacs re-incubated at 37°C in the absence of calf serum

Time (min)	Mean Percentage radioactivity released ± S.D.		
	acid soluble	acid insoluble	total radioactivity
0	0.2 ± 0.3	0.5 ± 0.8	0.7 ± 1.1
5	7.5 2.8	10.1 2.5	17.7 5.2
15	17.0 4.5	18.6 2.1	35.3 5.7
30	26.8 6.9	27.2 3.5	54.0 8.6
45	32.6 8.4	34.0 3.5	66.4 11.6
60	35.3 8.9	35.0 4.8	70.3 11.2
75	39.5 10.5	37.5 4.4	75.4 11.2
90	39.7 9.0	38.3 4.6	78.0 10.9
120	42.2 9.2	39.8 4.4	82.2 10.2
150	44.9 8.1	40.2 6.1	84.9 10.4
180	44.9 8.7	42.4 5.4	87.3 10.1

Table 5.VIIIb Release of radioactive species derived from ^{125}I-labelled IgG (Batch III) by 17.5-day rat yolk sacs re-incubated at 37°C in the absence of calf serum, after washing in medium 199 containing unlabelled rat IgG (10.0 µg/ml)

Time (min)	Mean Percentage radioactivity released ± S.D.		
	acid soluble	acid insoluble	total radioactivity
0	0.4 ± 0.4	0.6 ± 0.6	1.0 ± 0.9
5	7.9 3.6	8.0 1.2	15.9 4.6
15	17.7 6.8	16.3 1.3	34.0 5.5
30	28.7 9.8	23.5 1.8	52.2 8.3
45	34.6 11.6	27.9 3.2	62.4 9.1
60	38.1 12.2	30.5 3.6	68.6 9.5
75	41.1 12.8	32.0 4.3	73.0 9.7
90	43.1 13.3	33.7 5.6	76.8 9.4
120	45.3 13.4	35.2 6.3	80.4 8.8
150	47.6 14.0	36.2 7.3	83.7 8.8
180	49.1 14.5	36.9 7.6	86.0 9.7

Table 5.VIIIc Release of radioactive species derived from ^{125}I-labelled IgG (Batch III) by 17.5-day rat yolk sacs re-incubated at 37°C in medium 199 containing unlabelled rat IgG (5 µg/ml) after washing in medium 199 containing unlabelled rat IgG (10.0 µg/ml)

Time (min)	Mean Percentage radioactiviy released ± S.D.		
	acid soluble	acid insoluble	total radioactivity
0	0.3 ± 0.4	0.4 ± 0.6	0.7 ± 1.1
5	7.9 3.6	7.4 1.8	15.3 5.0
15	18.1 7.0	14.7 0.2	32.8 6.9
30	29.4 11.2	22.8 1.8	52.2 9.5
45	35.1 12.8	27.0 2.5	62.2 10.5
60	38.8 13.5	30.8 4.1	69.6 11.7
75	42.1 14.8	31.9 5.0	74.0 11.5
90	44.2 15.1	32.5 6.2	76.8 10.5
120	46.8 15.1	34.3 7.4	81.1 8.8
150	49.3 14.8	35.3 7.5	84.7 8.7
180	51.5 16.5	34.9 10.1	86.4 9.0

Table 5.IXa Release of radioactive species, derived from ^{125}I-labelled IgG, by 17.5-day rat yolk sacs re-incubated at 21°C in the absence of calf serum

Time (min)	Mean Percentage radioactivity released ± S.D.		
	acid soluble	acid insoluble	total radioactivity
0	0.7 ± 1.3	1.7 ± 0.7	2.6 ± 0.8
5	2.4 0.9	4.7 2.7	7.1 2.0
15	5.0 1.0	8.4 3.0	13.4 2.4
30	8.4 0.8	11.2 2.7	19.6 2.2
45	12.1 0.5	13.5 2.6	25.6 3.0
60	13.2 1.3	16.5 3.9	29.7 3.8
75	15.0 2.1	19.2 3.9	34.2 3.1
90	17.5 3.2	18.9 2.6	36.3 2.8
120	18.4 2.6	25.3 6.1	43.6 4.4
150	19.7 3.4	26.8 7.6	46.5 5.2
180	22.0 4.4	27.5 6.4	49.5 3.7

Table 5.IXb Release of radioactive species, derived from ^{125}I-labelled IgG (Batch III), by 17.5-day rat yolk sacs re-incubated at 4.5°C in the absence of calf serum

Time (min)	Mean Percentage radioactivity released ± S.D.		
	acid soluble	acid insoluble	total radioactivity
0	0.7 ± 0.4	1.1 ± 0.6	1.7 ± 1.0
5	1.7 1.0	2.6 1.1	4.3 1.9
15	2.8 0.5	5.0 4.3	7.8 4.8
30	4.1 0.5	6.6 5.1	10.7 5.4
45	4.7 0.4	7.5 4.6	12.2 4.9
60	5.2 0.1	8.6 4.8	13.8 4.7
75	5.6 0.4	8.7 5.0	14.4 4.7
90	6.7 0.4	9.7 5.5	16.4 5.4
120	7.9 0.2	10.6 5.6	18.5 5.5
150	8.7 0.9	11.9 6.0	20.6 5.3
180	12.1 3.9	10.1 1.8	22.3 5.3

Table 5.Xa Release of radioactive species, derived from ^{125}I-labelled dBSA (Batch VI), by 17.5-day rat yolk sac re-incubated at 37°C in medium 199 alone

Time (min)	Mean Percentage radioactivitiy released ± S.D.		
	acid soluble	acid insoluble	total radioactivity
0	3.4 ± 3.6	-0.5 ± 3.8	3.2 ± 1.0
5	20.2 3.0	2.8 2.1	23.0 3.0
15	36.1 4.3	2.5 0.7	38.6 3.8
30	44.8 4.5	5.1 1.0	49.9 5.3
45	49.2 4.8	3.3 2.8	52.6 2.0
60	50.5 4.1	5.0 1.3	55.6 2.7
75	54.1 4.0	2.2 3.9	56.3 0.9
90	54.2 1.7	4.8 2.2	58.9 3.0
120	55.8 2.5	4.7 1.0	60.7 1.2
150	56.6 1.6	6.5 2.7	63.1 2.7
180	58.5 2.2	5.2 0.5	63.8 1.8

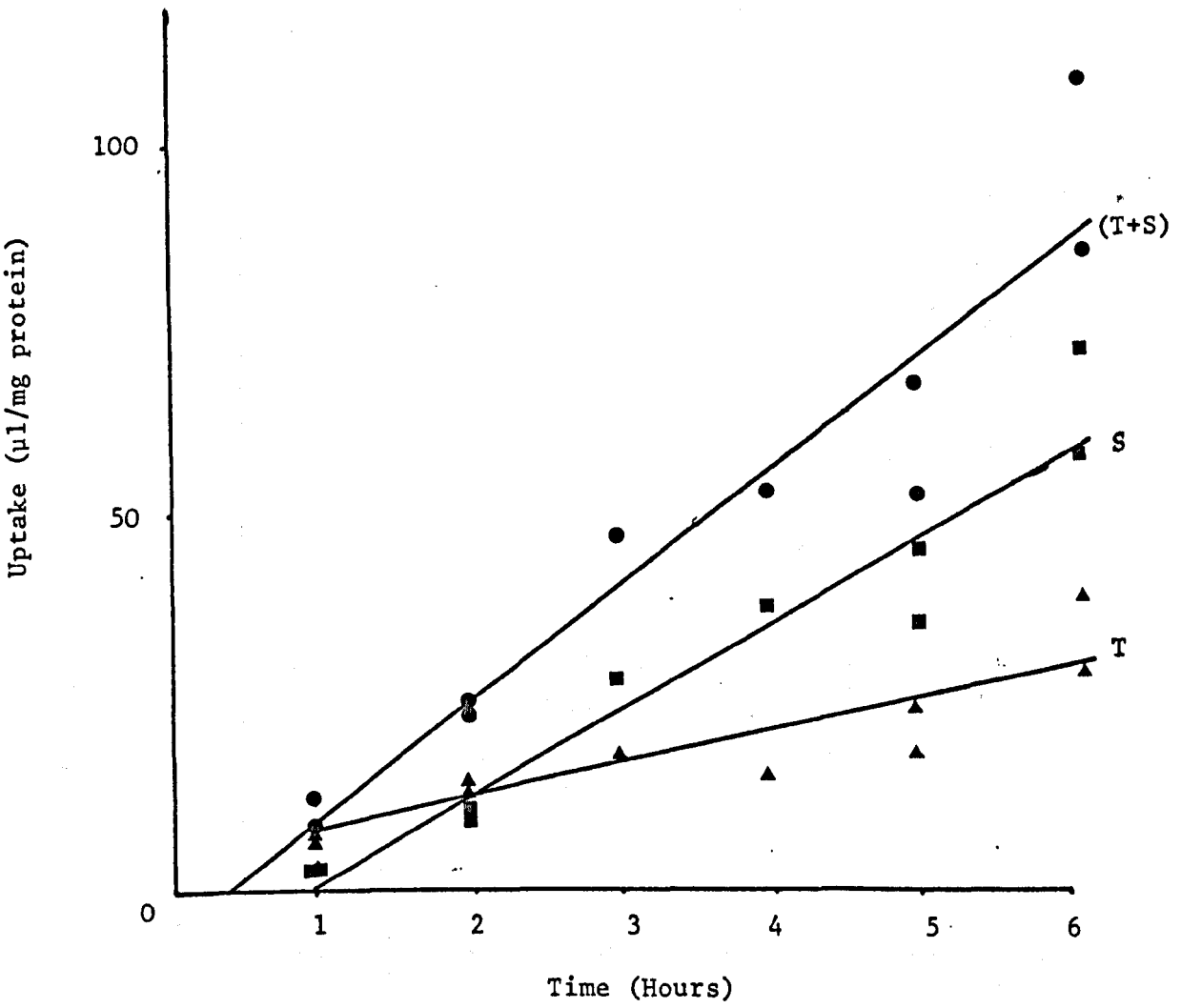
Table 5.Xb Release of radioactive species, derived from ^{125}I-labelled dBSA (Batch VI), by 17.5-day rat yolk sacs re-incubated at 21°C in medium alone

Time (min)	Mean Percentage radioactivity released ± S.D.		
	acid soluble	acid insoluble	total radioactivity
0	0.6 ± 1.8	- 1.2 ± 1.7	0.03 ± 0.4
5	6.0 2.5	1.3 2.0	4.7 1.6
15	13.1 3.7	1.5 0.7	14.6 4.4
30	18.3 4.4	2.1 3.7	20.4 2.1
45	24.4 5.2	3.5 3.2	27.9 4.9
60	27.8 5.2	4.5 3.1	32.3 2.6
75	31.0 8.2	4.7 2.8	35.5 5.9
90	33.7 5.8	4.3 3.2	38.0 5.3
120	36.6 8.1	10.6 8.5	47.2 7.0
150	37.5 5.5	9.6 3.7	47.1 2.0
180	39.3 8.4	11.9 8.2	51.3 1.3

Table 5.Xc Release of radioactive species, derived from ^{125}I-labelled ^{125}I-dBSA (Batch VI), by 17.5-day rat yolk sacs re-incubated at 4.5°C in medium alone

Time (min)	Mean Percentage radioactivity released ± S.D.		
	acid soluble	acid insoluble	total radioactivity
0	0.7 ± 0.6	-0.2 ± 1.0	0.5 ± 0.5
5	3.0 1.1	0.0 0.7	3.1 0.4
15	4.9 1.7	1.0 0.7	5.9 1.1
30	7.1 1.6	1.0 0.5	8.1 1.4
45	9.5 1.7	0.9 0.3	10.5 1.9
60	10.7 2.2	1.2 0.7	11.9 2.5
75	11.4 2.7	2.0 0.3	13.3 2.4
90	12.7 2.9	2.4 0.8	15.1 3.0
120	15.1 3.0	2.6 0.4	17.6 3.4
150	16.9 3.4	2.3 0.6	19.1 3.2
180	18.7 3.6	2.5 0.2	21.2 3.6

Fig. 5a Uptake plot versus time for ^{125}I-labelled IgG (Batch III) by 17.5-day rat yolk sac in the presence of calf serum (10% v/v)



Gradient of the line (T+S) gives value of;

Endocytic Index = 15.8 µl/h/mg protein)

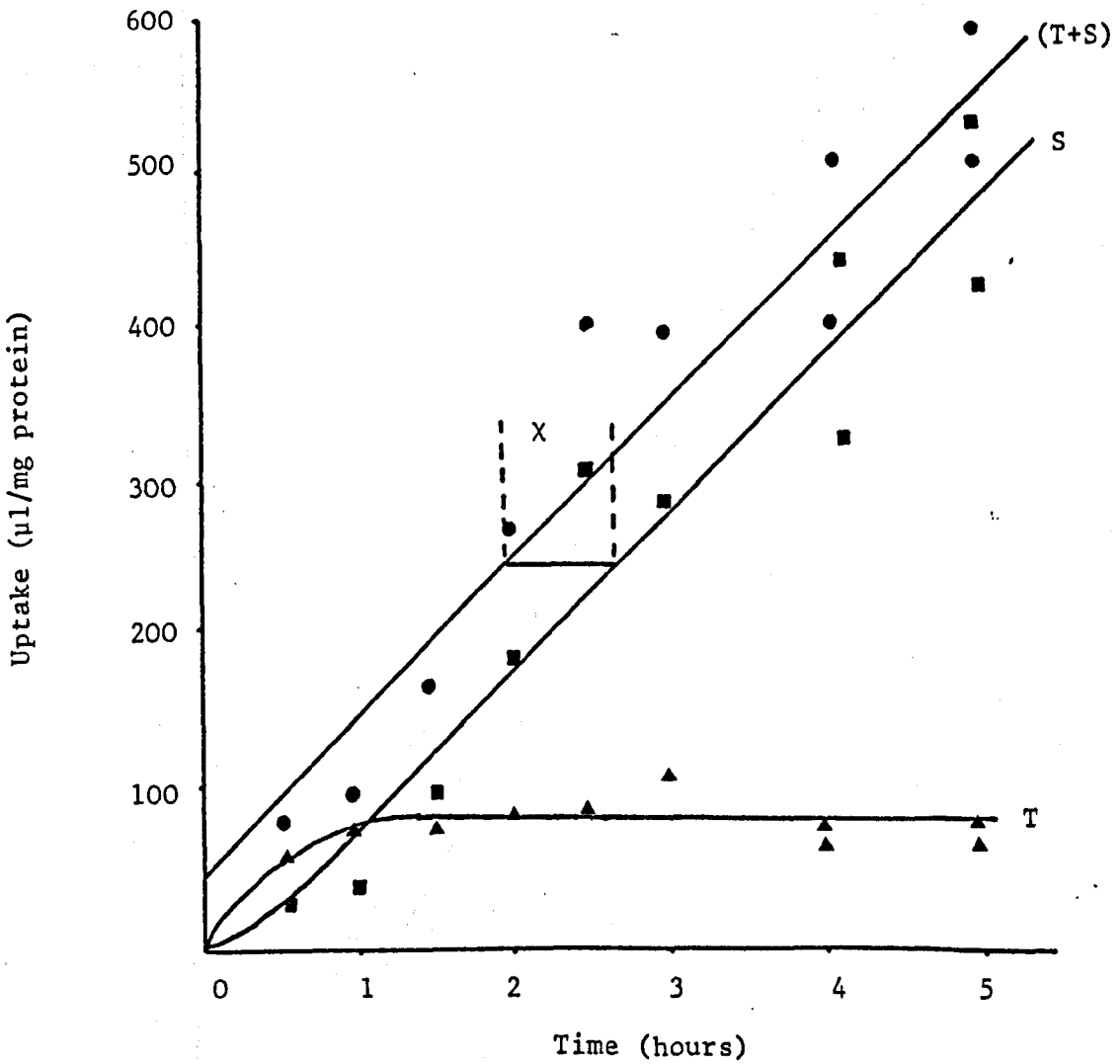
(Correlation Coefficient = 0.955)

Gradient of the line T (tissue associated radioactivity) gives value of:

Tissue Accumulation Rate = 4.38 µl/h/mg protein)

(Correlation Coefficient = 0.892)

Fig. 5b Plot of uptake against time for ^{125}I-labelled IgG (Batch III) by 17.5-day rat yolk sac in medium 199 alone



Gradient of the line (T+S) gives value of:

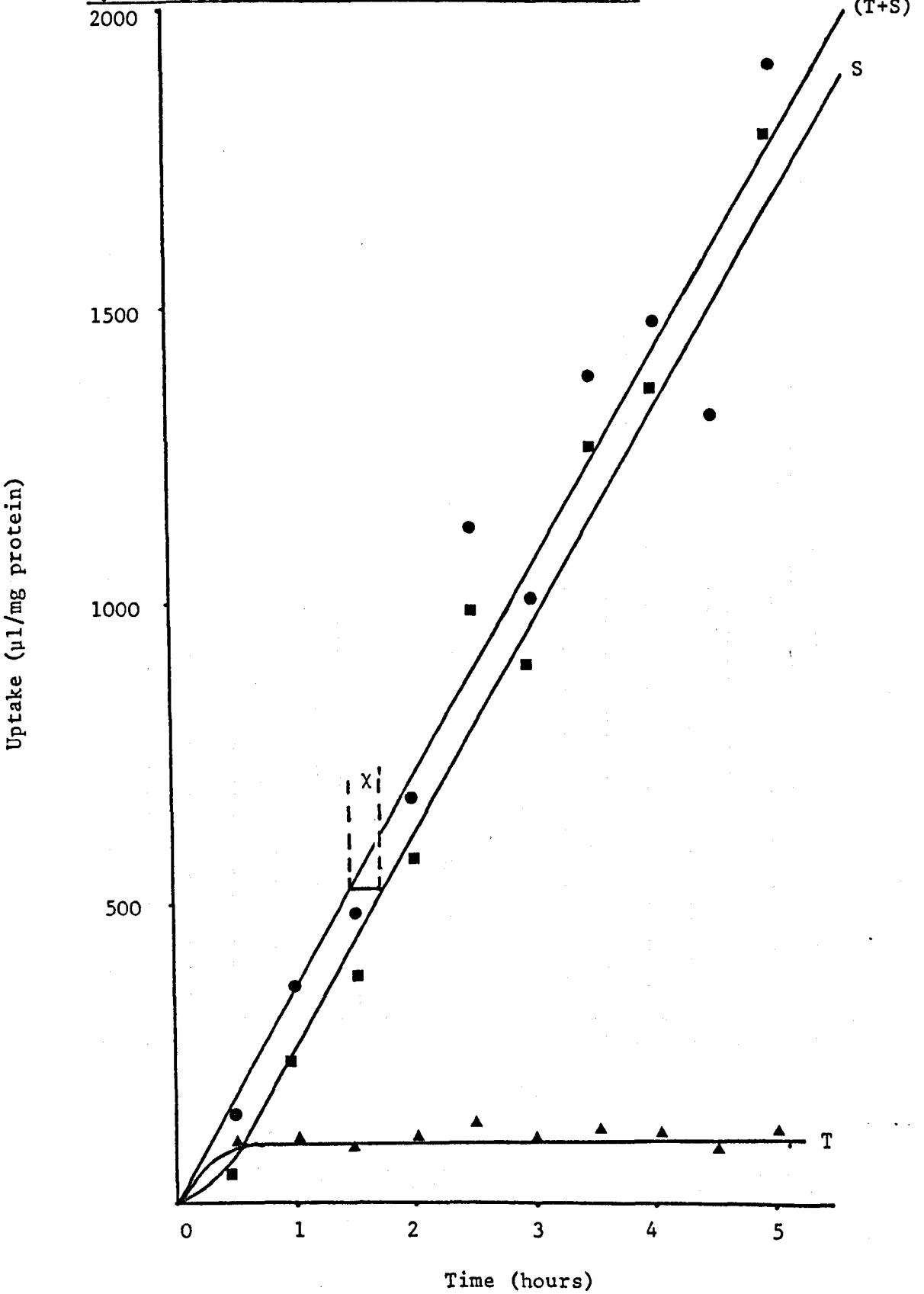
Endocytic Index = 105.7 µl/h/mg protein

(Correlation Coefficient = 0.956)

Horizontal displacement of plot S from line (T+S) gives value of:

Lag phase = χ hours

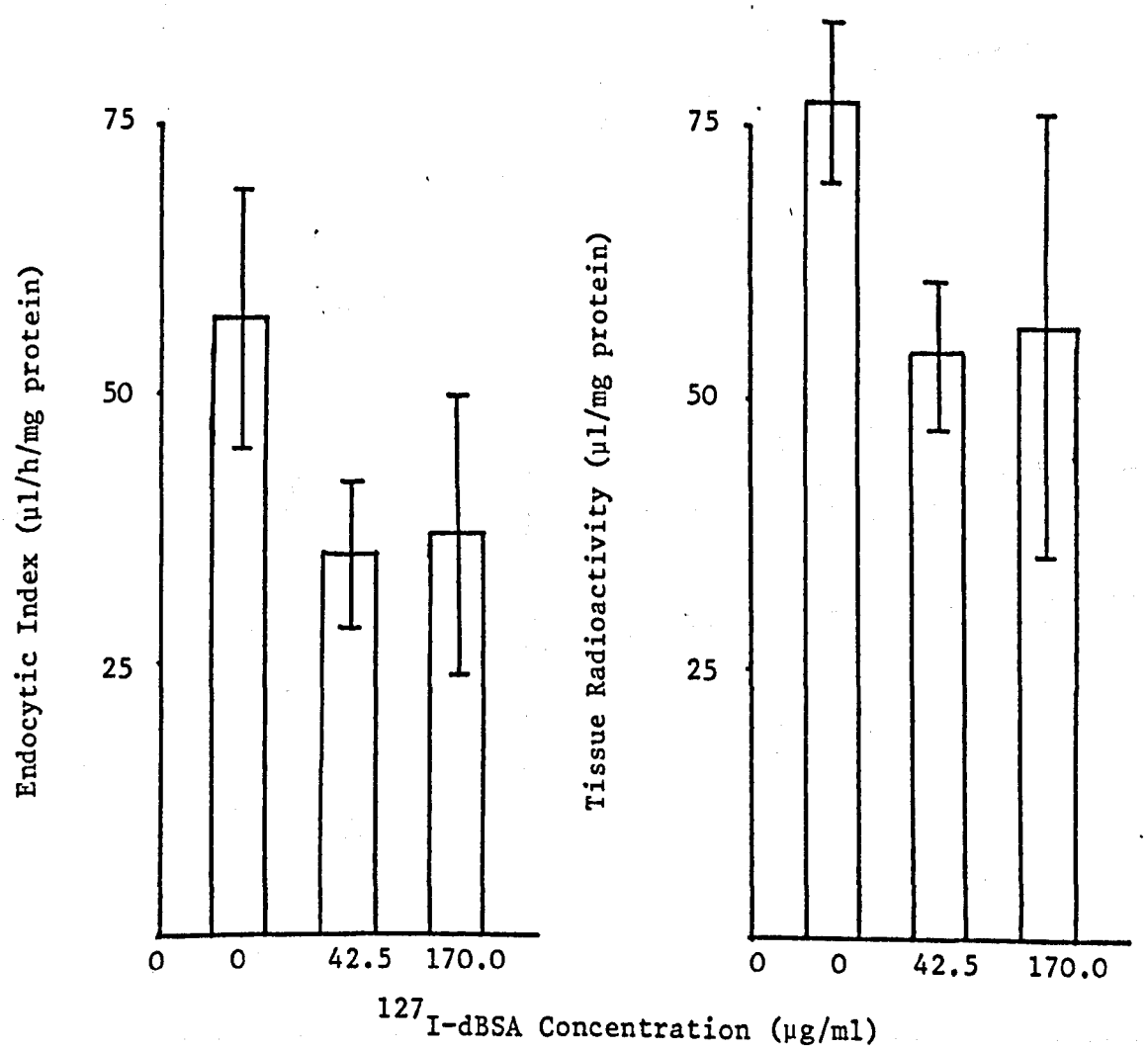
Fig. 5c Uptake plot versus time for ^{125}I-labelled dBSA (Batch VI) by 17.5-day rat yolk sac in medium 199 alone



Endocytic Index = 356.9 µl/h/mg protein

(Correlation Coefficient = 0.972)

Fig. 5d Effect of ^{127}I-iodinated dBSA on uptake of ^{125}I-labelled IgG by 17.5-day yolk sac



A. Endocytic Index of ^{125}I-IgG (Batch I) in the presence of ^{127}I-iodinated dBSA in medium 199 alone.

B. Overall mean tissue radioactivity in the presence of ^{127}I-iodinated dBSA in medium 199 alone.

Fig. 5e Release of ^{125}I-labelled IgG (Batch I) at 37°C from 17.5-day rat yolk sac re-incubated in medium 199 alone

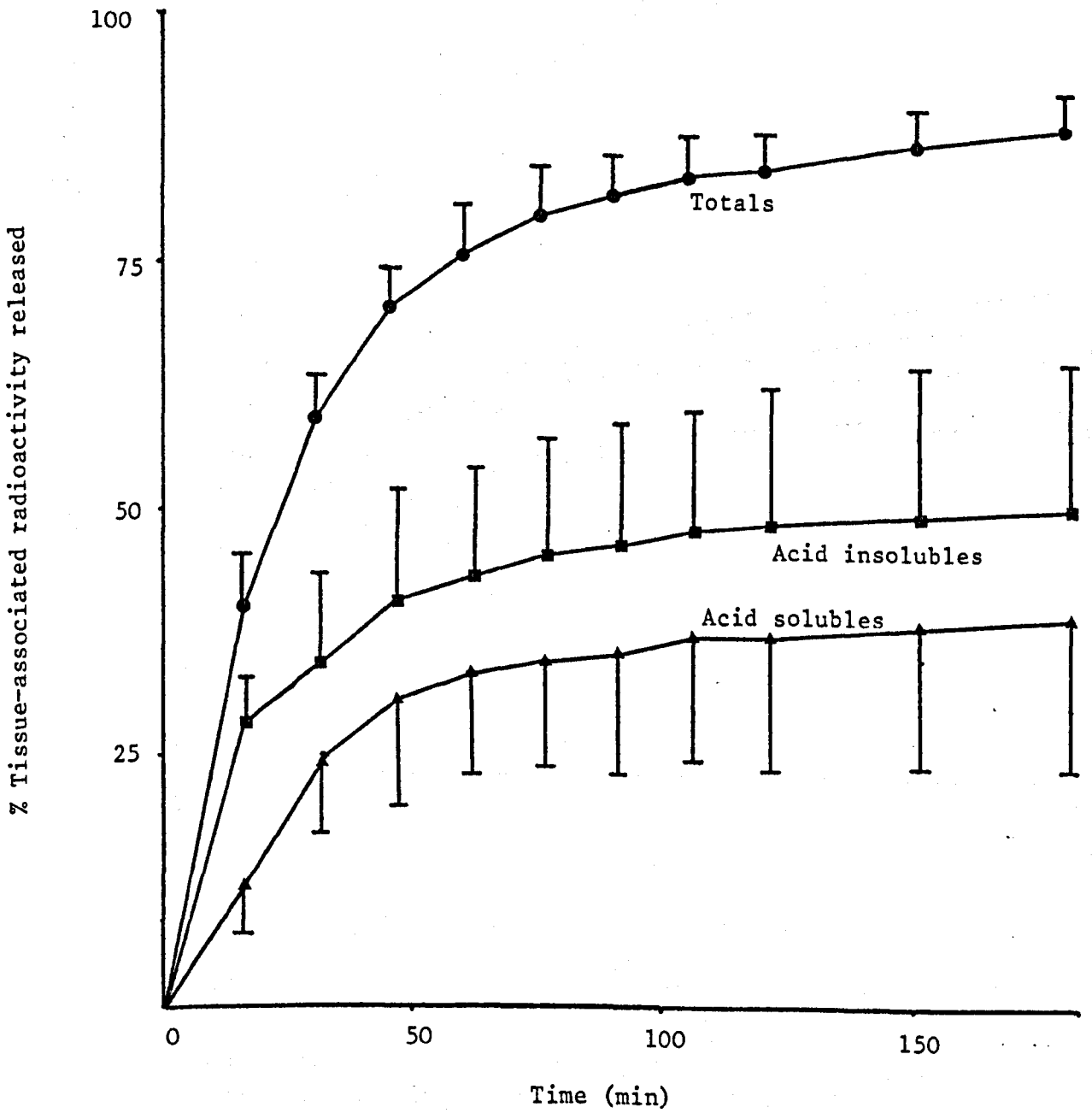
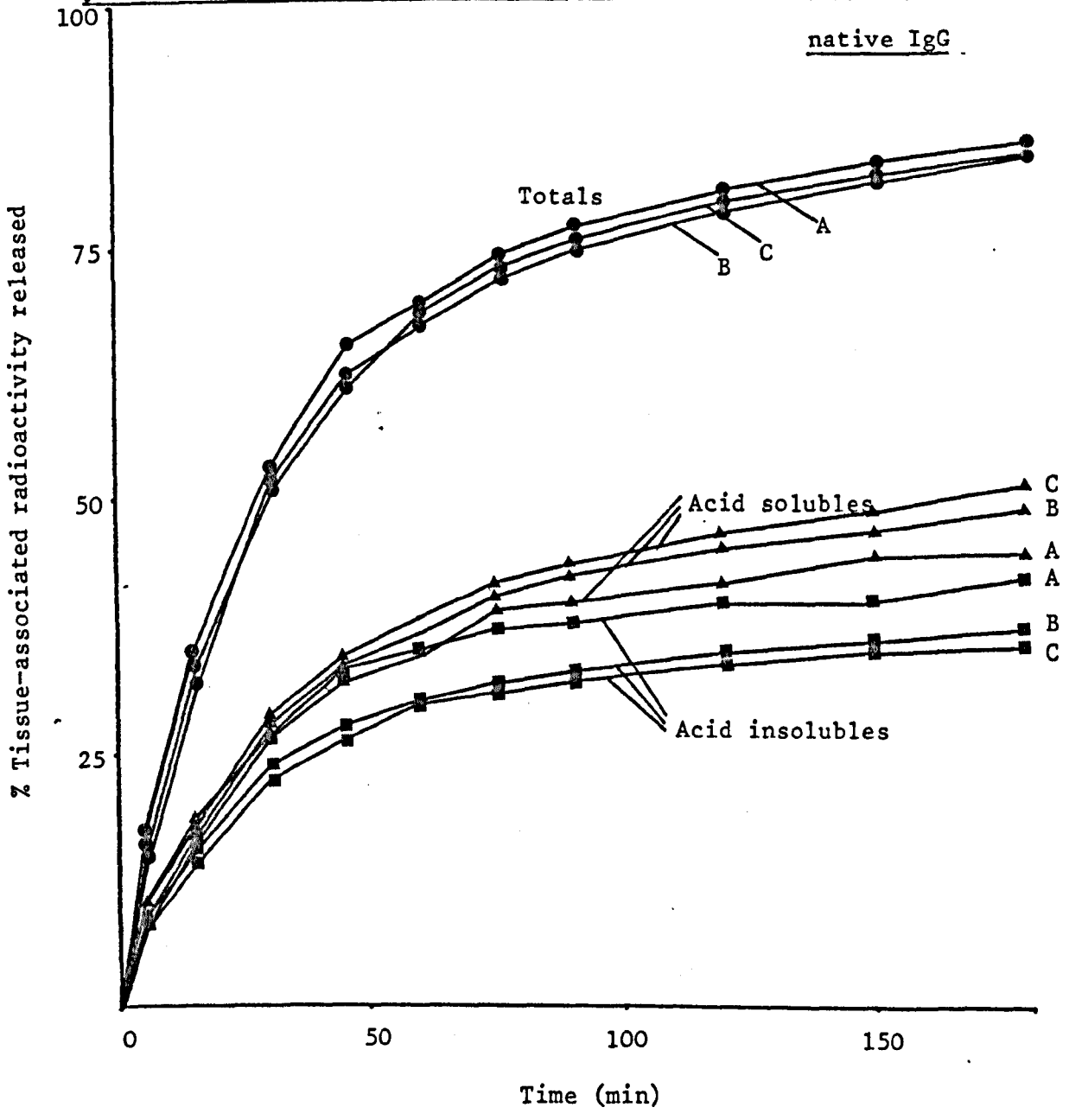


Fig. 5f Release of ^{125}I-labelled IgG (Batch III) from 17.5-day rat yolk sacs re-incubated at 37°C in the presence and absence of native IgG.



A = 17.5-day yolk sac re-incubated in medium 199 alone

B = 17.5-day yolk sac washed in medium 199 + IgG (10.0 µg/ml) and re-incubated in medium 199 alone

C = 17.5-day yolk sac washed in 199 + IgG (10.0 µg/ml) and re-incubated in medium 199 + IgG (5.0 µg/ml)

Fig. 5g Release of ^{125}I-labelled IgG (Batch III) from 17.5-day yolk sacs re-incubated at 37°C, 21°C and 4.5°C

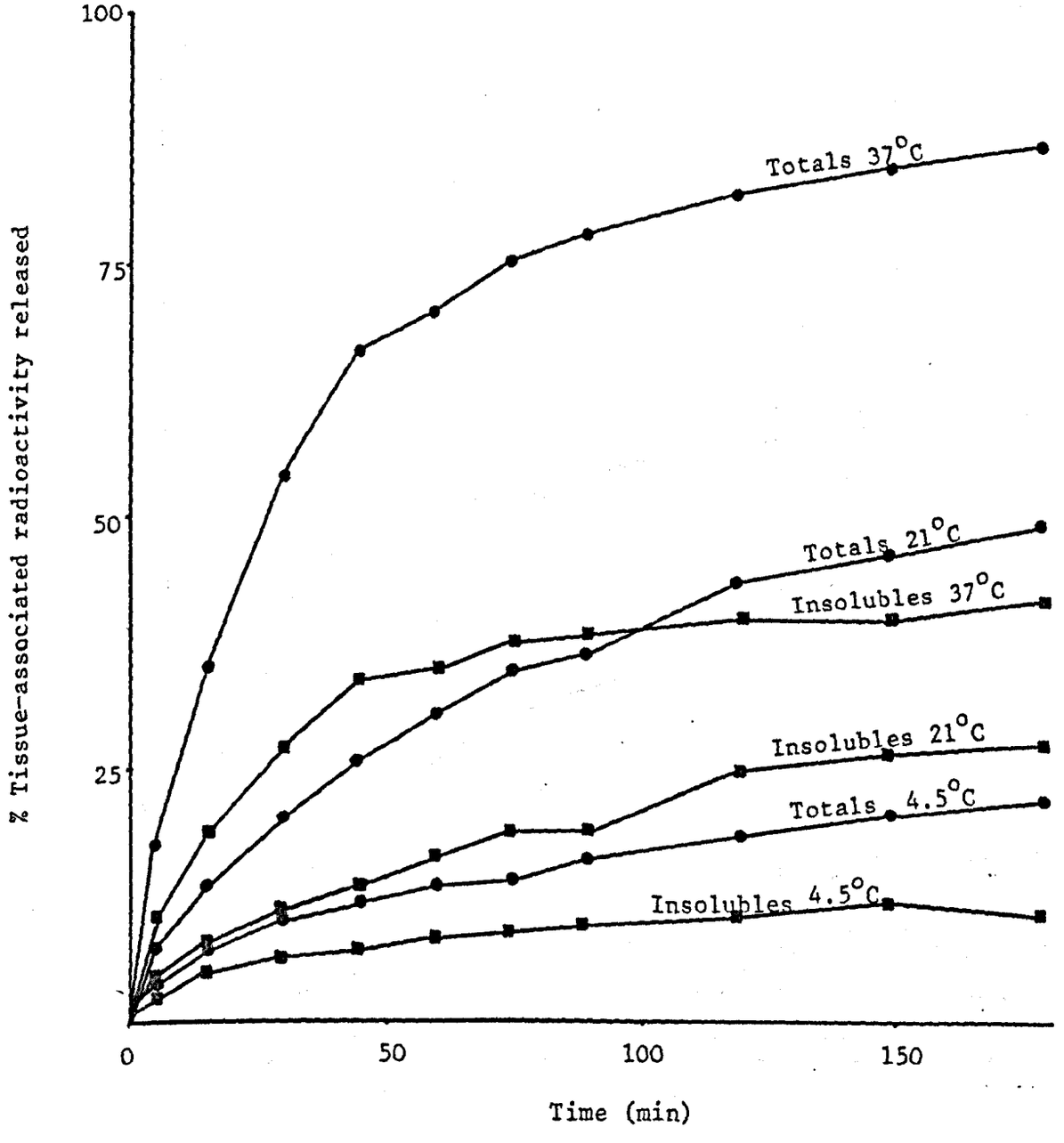


Fig. 5h Release of ^{125}I-labelled dBSA (Batch VI) from 17.5-day rat yolk sacs re-incubated at 37°C, 21°C and 4.5°C

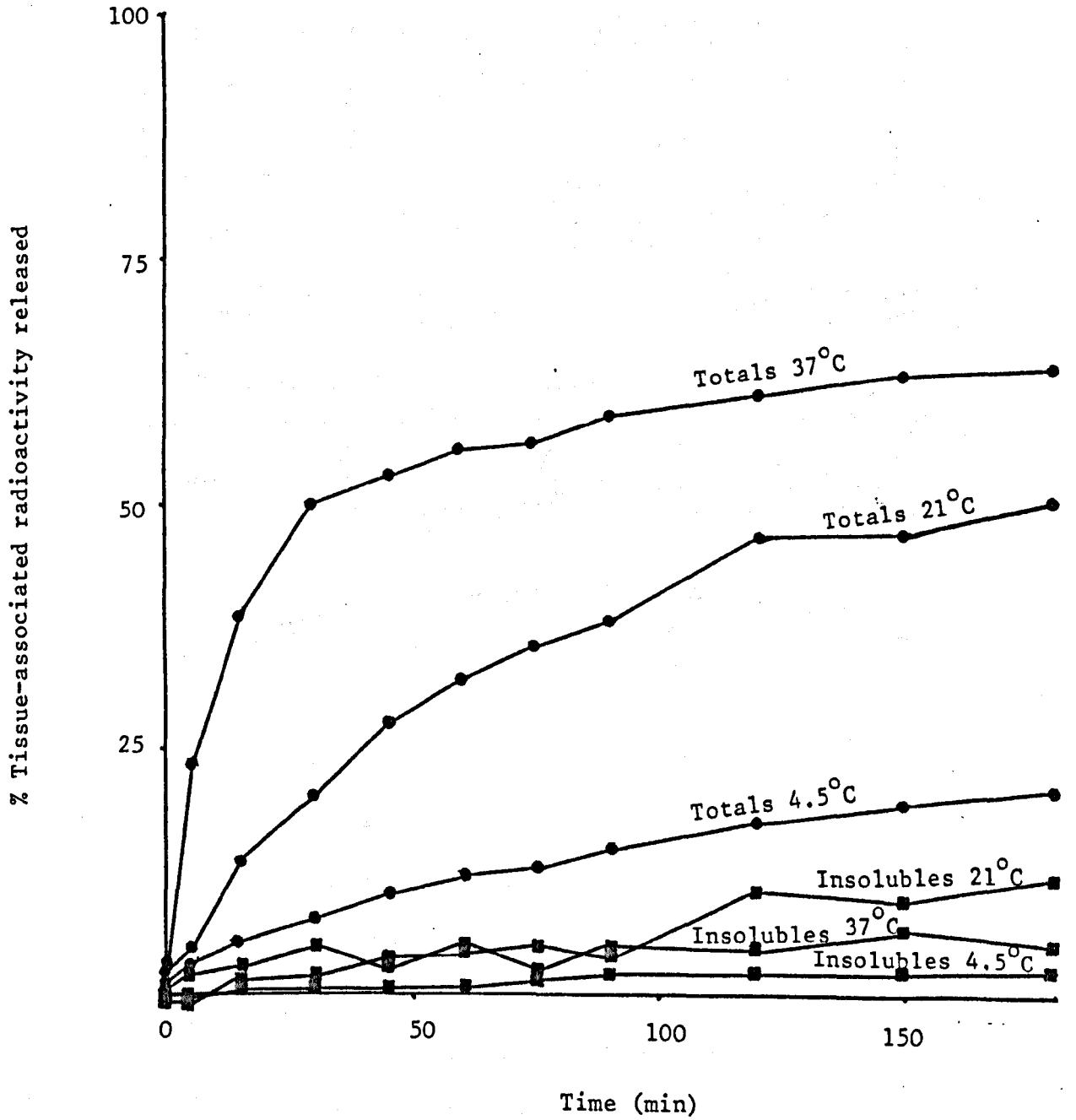
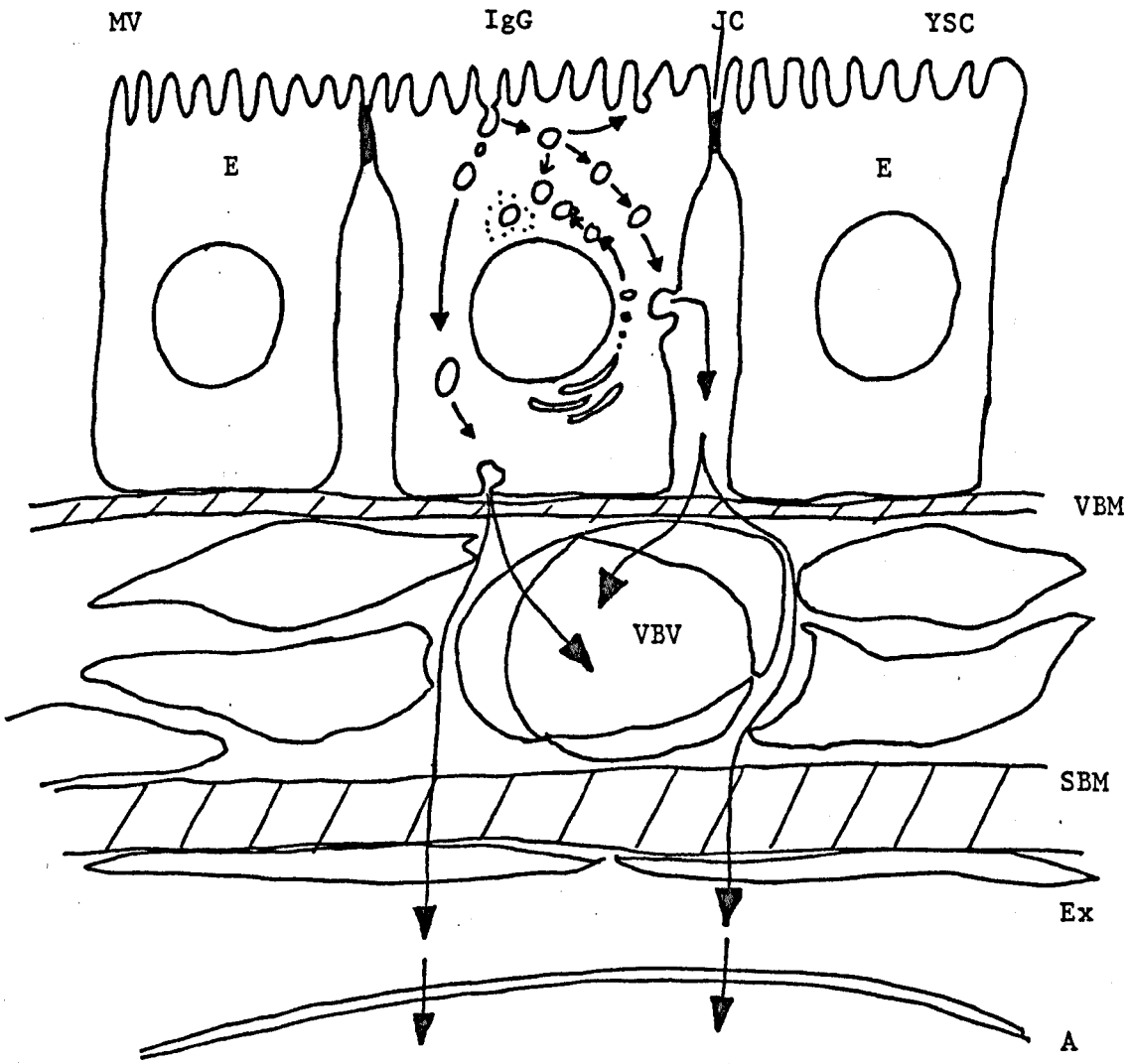


Fig. 5i Possible fates of ^{125}I-labelled IgG within 17.5-day rat yolk sac



A = Amnion

E = Epithelial cell

Ex = Exocoelom

JC = Junction complex

MV = Microvilli

SBM = Serosal basement membrane

VBM = Visceral basement membrane

VBV = Vitelline blood vessel

YSC = Yolk-sac cavity

Fig. 5j Chromatography on Sephadex G25 of the radioactive species released by 17.5-day rat yolk sacs 'loaded' with ^{125}I-labelled IgG and re-incubated in medium 199 alone

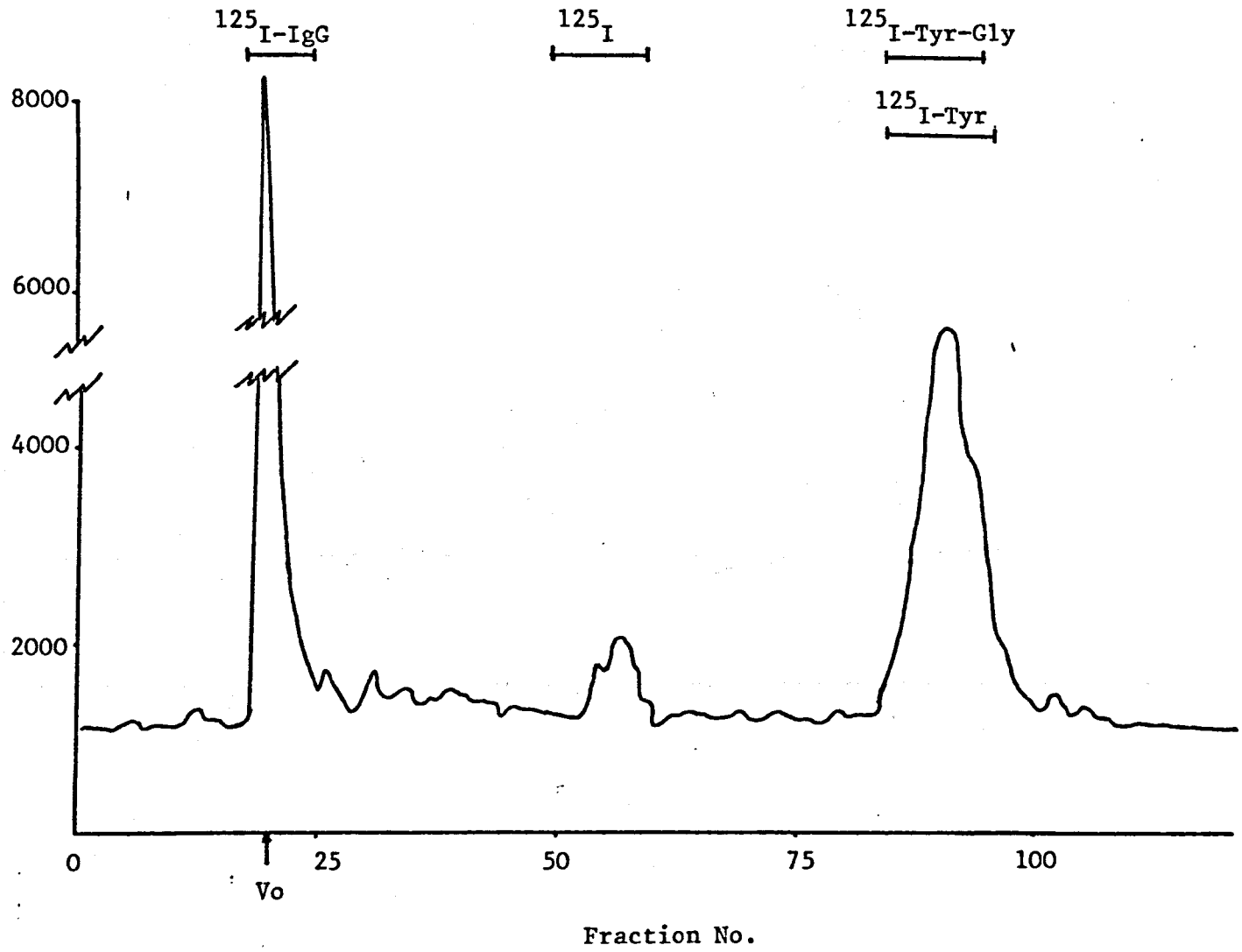


Fig. 5k Chromatography on 'Sephadex G25' of the radioactive species released by 17.5-day rat yolk sac 'loaded' with ^{125}I-labelled dBSA and re-incubated in medium 199 alone

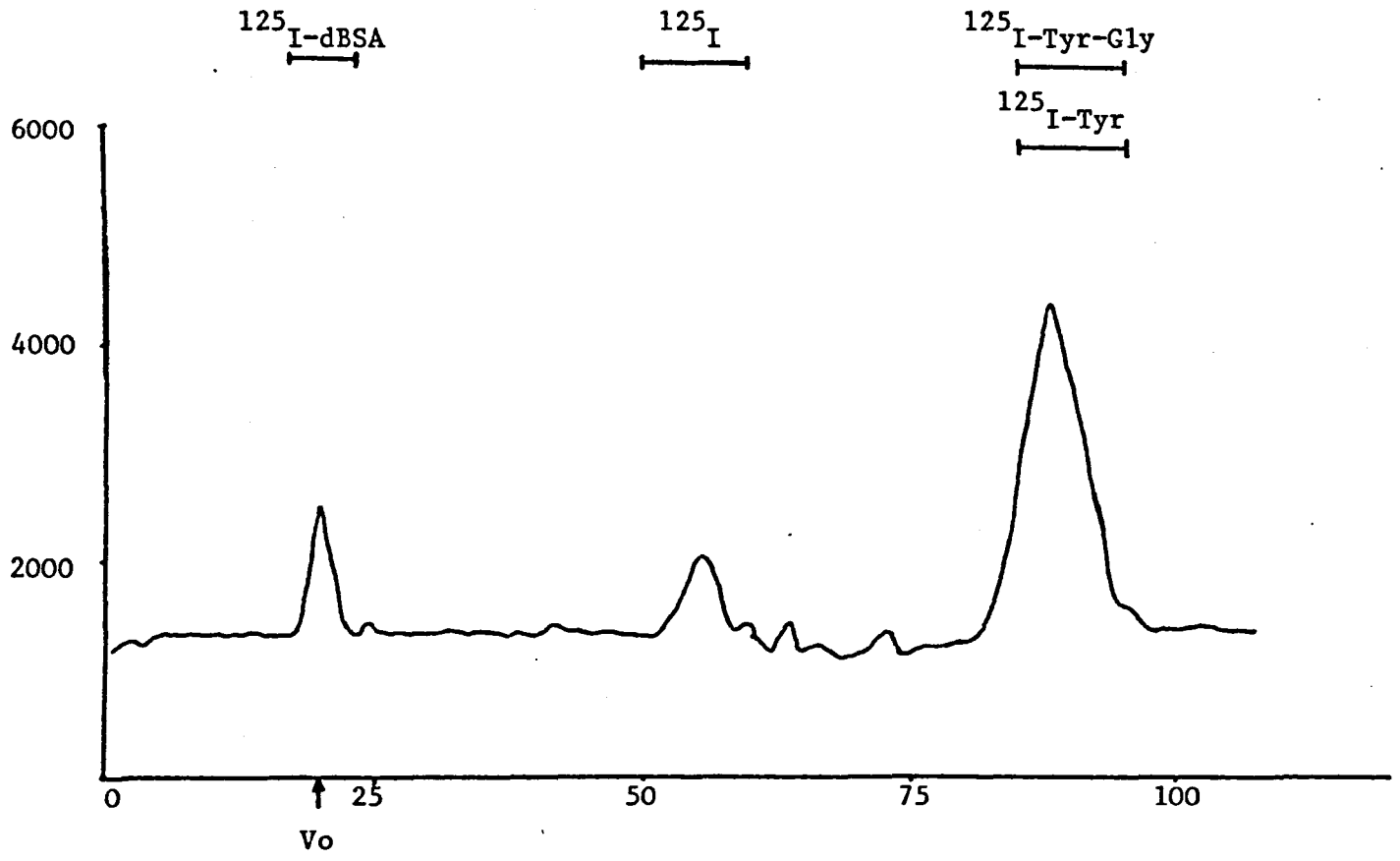


Fig. 51 Chromatography on 'Sephadex G150' of the radioactive species released by 17.5-day rat yolk sacs 'loaded' with ^{125}I-labelled IgG and re-incubated in medium 199 alone

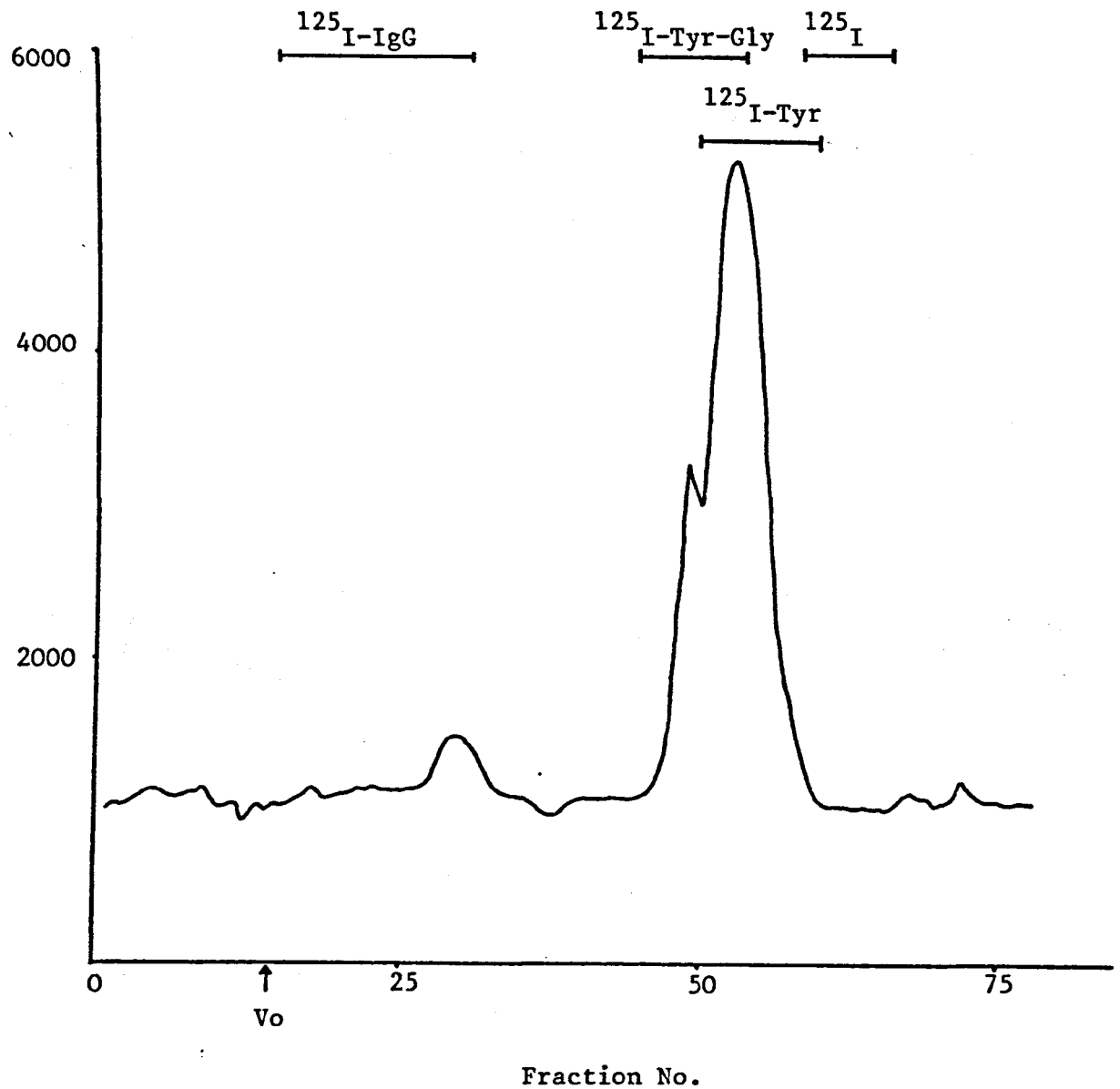


Fig. 5m *Chromatography on 'Sephadex G150' of the radioactive species released by 17.5-day rat yolk sacs 'loaded' with ^{125}I-labelled dBSA and re-incubated in medium 199 alone*

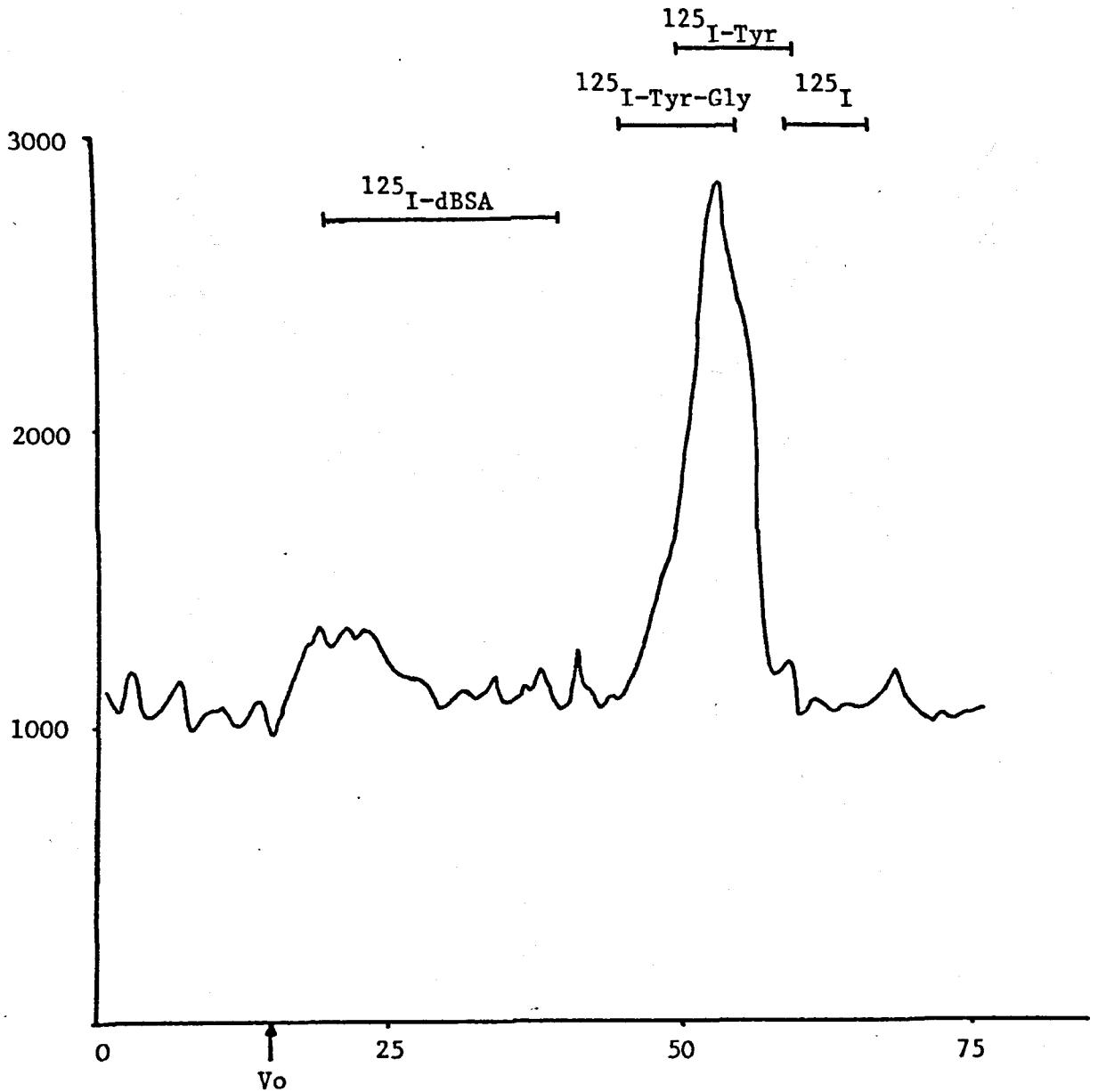
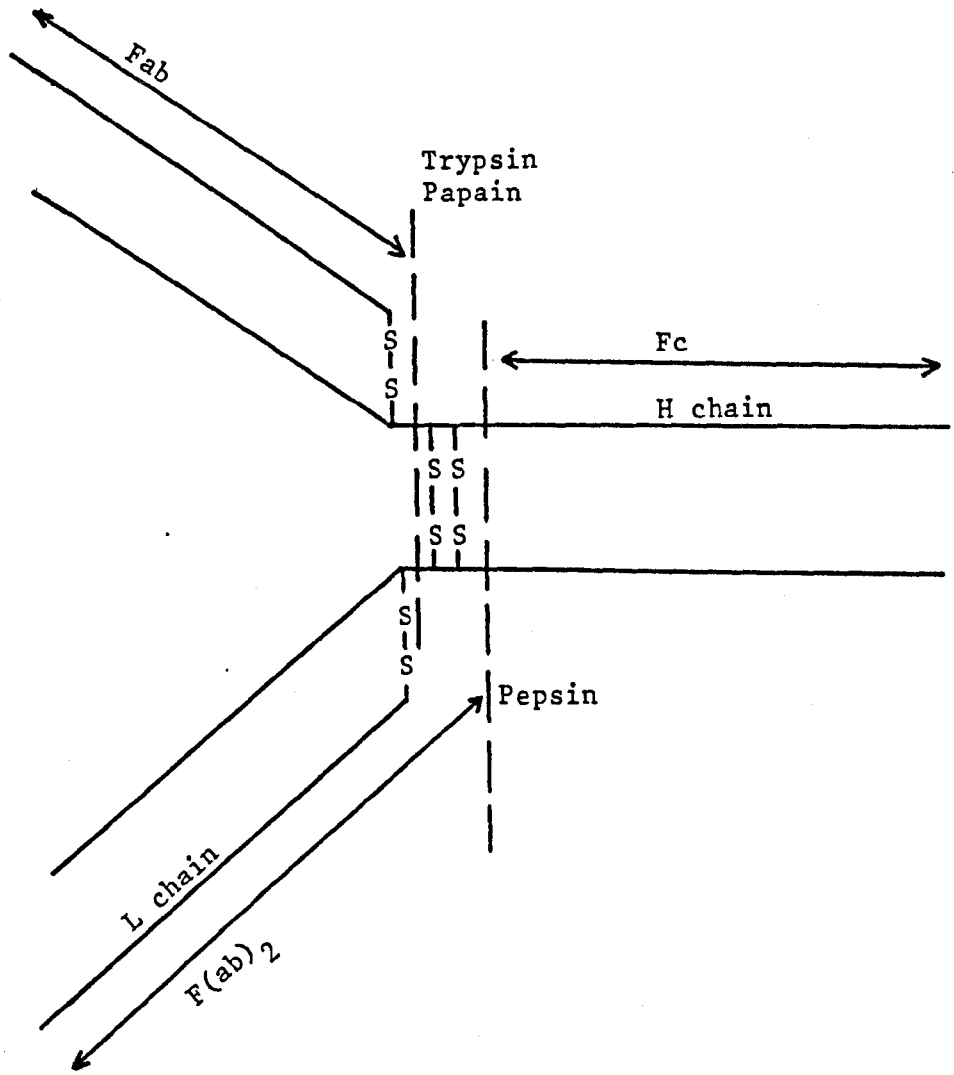


Fig. 5n Diagrammatic representation of the structure of IgG



CHAPTER 6

MECHANISM OF RELEASE OF IgG BY THE 17.5-DAY RAT YOLK SAC : AN IN VITRO STUDY

6.1 Introduction

The prenatal transmission of IgG in the rabbit has been investigated by a number of workers (Brambell,1949, 1966; Wild,1970, 1972, 1973; Hemmings,1957). Brambell *et al.* (1949) showed, by ligaturing the yolk sac stalk, that this membrane is the sole route by which passive immunity is transferred from mother to fetus in this species. In the rat, on the other hand, prenatal routes of transmission of immunoglobulins would appear less important since the greater part of the passive immunity is transferred after birth (during lactation) by way of the gut of the neonate (Halliday, 1955b; Bangham and Terry, 1957; Brambell *et al.*, 1961; Jones, 1972; Rodewald, 1970, 1973; Morris & Morris, 1974a, 1974b; Morris, 1974). It has been suggested that the passive immunity imparted to the rat fetus prior to birth is transmitted via the yolk sac (Brambell and Halliday, 1956) but this has been contested by Quinlivan (1964).

A number of *in vivo* studies have been performed in the rabbit and Hemmings (1957) has demonstrated that the transport mechanism for homologous immunoglobulins was selective although the total amount of IgG transmitted intact across the yolk sac was only about 12% of the total administered (Hemmings, 1956). Furthermore, the rabbit yolk sac was also shown to transmit IgM, but not IgA (Hemmings, Jones and Williams, 1973). Antitoxins, prepared in other species, were also found to be transported across the rabbit yolk sac (Batty *et al.*, 1954). Brambell (1966) using the same data, was able to calculate the relative rates of transmission. This investigation was extended (Brambell *et al.*, 1960) to include the rates of transmission of fragments of rabbit IgG, prepared by papain digestion (Porter, 1958, 1959), by the rabbit yolk sac.

In comparison with the rabbit, transmission across rat yolk sac has been little studied although Anderson (1959) showed,

autoradiographically, the presence of ^{131}I-labelled IgG within the yolk sac towards the end of pregnancy, confirming the ability of the tissue to ingest this substrate. In the rat the study of uptake and transmission of immunoglobulins has been largely restricted to transmission across the gut of young rats up to the age of 20 days, at which time Halliday (1955a) found that transport of IgG virtually ceased. Bangham and Terry (1957) calculated the proportion of the administered dose transmitted across the gut to be 7.5%. They also concluded that the transport was selective in nature, and Jordan and Morgan (1968) confirmed this by showing labelled IgG is transmitted but labelled albumin is not. In a later study, (Jones, 1972) obtained a value of 12% transmission of the administered dose. Morris and Morris (1976) demonstrated that the proximal intestine was responsible for transmission of some 40% of an administered dose of labelled IgG.

In vivo studies of the transmission of immunoglobulins, of the type described above, are accompanied by several difficulties which include: the possible existence of multiple sites of transmission, proteolysis of the administered substrate, dilution of transmitted antibodies within the rapidly growing offspring and uneven distribution of substrate at the transport sites. Such factors make quantitative assessment of the results difficult.

Potentially in vitro investigations could eliminate some of these problems and make more accurate assessment of the rates of uptake, hydrolysis and transmission by a given route possible. Bamford (1966), using a modified method of the culture technique developed by Wilson and Wiseman (1954a, b), was able to incubate inverted gut sacs, prepared from young rats, for several hours. Their viability was monitored by oxygen consumption and ability to transport L-histidine against a concentration gradient. Using this technique he was able

to show the transport of some IgG across the gut wall. Moreover, he found that IgG preparations from the rat were transmitted more readily than were similar fractions from the rabbit or cow. However, it has proved difficult to maintain cell viability in rat gut sacs incubated for long periods (Beck, personal communication), although more recently cell viability has been reported up to 3.0 h (Walker et al., 1976) and linear uptake of ^{125}I-labelled PVP has been observed over 3.0 h incubation periods with gut sacs from adult rats (Bridges, unpublished data).

Although in vitro study of the transport of immunoglobulins by rat yolk sac would avoid certain complications discussed earlier and clarify the role of this tissue in the transmission process, to date no attempts have been reported.

In Chapter 5 the uptake of ^{125}I-labelled IgG was studied by the in vitro culture techniques developed by Williams et al. (1975a and b) and the results compared with those of ^{125}I-labelled dBSA, a protein that is unlikely to be transmitted in vivo. With both substrates the results showed highly linear uptake over incubation periods of several hours, in the presence and absence of 10.0% calf serum in the culture medium. The release/exocytosis of ingested protein substrates was also investigated by a modified method Williams et al. (1975a). The results showed that while the non-specific protein ^{125}I-labelled dBSA underwent complete digestion, a considerable percentage of ingested ^{125}I-labelled IgG was released intact on re-incubation in fresh medium. The latter finding is compatible with the suggestion of Brambell and Halliday (1956) that the yolk sac is instrumental in transmitting immunoglobulins in the rat.

Currently there are two hypotheses in circulation to explain how, at the molecular level, IgG selectively crosses tissue barriers. That proposed by F. W. R. Brambell and co-workers, 1958, and later

revised (Brambell et al., 1966) states that the observed selective transfer of IgG is the result of intracellular events. The other, more recent, hypothesis proposed by Wild (1975) suggests that selective transfer of IgG is a result of extracellular events at the cell surface. According to the Brambell hypothesis transport across the rabbit yolk sac is envisaged to occur by IgG being ingested by pinocytosis, together with other proteins contained in the extracellular fluid present in the uterine lumen, and, following ingestion, a proportion of this IgG becomes bound to specific receptors on the internal face of the vesicles. As these pinocytic vesicles migrate further into the cell, they fuse with lysosomes, and bring the vesicle contents into contact with lysosomal enzymes thus permitting protein hydrolysis. It is during this digestion phase that selection of those molecules to be transported is envisaged to occur: those IgG molecules bound to specific receptors are thought in some way to be protected from digestion while any other proteins, and any IgG itself that is not bound in this manner, become completely hydrolysed (see Fig. 7a). After digestion the vesicle then proceeds to fuse with the basal membrane releasing its contents.

The Wild hypothesis differs from that of Brambell by proposing that IgG binds specifically to receptors on certain regions of the cell surface (possibly at the base of the micro-villi) prior to entering the cell. When the substrate has bound to these receptors this region of the plasma membrane invaginates to form small vesicles termed 'coated vesicles' (so called because of the presence of a 'fuzzy' coating which, under the electron microscope, can be seen on their cytoplasmic surface). It is proposed that these vesicles contain only IgG and that the physicochemical properties of the cytoplasmic surface differ from those of pinosomes arising from other regions of the plasma membrane. This allows them to

avoid fusion with the lysosomal compartment, hence traverse the cell and subsequently, on fusion with the basal membrane, release their contained IgG. Any IgG that does not bind to specific receptors would be taken up into normal pinosomes and be subject to intra-lysosomal hydrolysis.

The mechanism by which IgG is selectively transported across cells, according to the hypotheses proposed by Brambell and Wild, differ in certain details. In this chapter the component stages of the two mechanisms were first examined on a theoretical basis to establish the basic similarities and differences between them (see table below). This enabled a number of experiments to be designed in which significant differences of behaviour would be expected by the different hypothetical mechanisms.

Comparison of the Brambell and Wild models for selective transport of homologous IgG

Component of the overall process considered	Predicted behaviour of the model according to:-	
	Brambell	Wild
a. Molecular mechanism of the selection process for transported IgG.	Selective/preferential binding to membrane receptors.	Selective preferential binding to membrane receptors.
b. Location of the receptors involved.	Pinosome or lysosome (inner face).	Outer surface of plasma membrane (specific regions)
c. Saturation of the transport process with increasing IgG concentration possible?	Yes	Yes
d. Minimum number of discrete types of pinocytic vesicle proposed.	1	2
e. Presence of lysosomal enzymes in the transport vesicles is suggested?	Yes	No
f. Can proteolysis of excess IgG and other proteins occur?	Yes	Yes
g. Site of such proteolysis.	Transport vesicle (i.e. lysosomes)	Separate non-transport vesicle (i.e. lysosomes)

Component of the overall process considered	Predicted behaviour of the model according to:-	
	Brambell	Wild
In test situation:-		
h. Amount of IgG transported dependent on concentration of ingested substrate.	Yes	Yes
i. Could a non-digestible substrate be transported together with IgG?	Yes	No
j. Is release of lysosomal enzymes from the transport vesicles possible?	Yes	No
k. Uptake of proteins into the lysosomal system can be inhibited independently of IgG uptake (and vice versa).	No	Yes

In view of the factors considered in the above table, re-incubation experiments with rat yolk sacs appeared to offer a suitable in vitro system for further studies. The above table also outlines the design of three experiments aimed at assessing whether the observed release of ^{125}I-labelled IgG from yolk sacs in vitro is related to the phenomenon of transport across the tissue in vivo. Both hypotheses predict that increasing the concentration of extracellular labelled IgG will lead to a greater fraction of that ingested being degraded, rather than released again in macromolecular form, as the binding sites conferring protection on the ingested IgG become saturated and the excess is digested within the lysosomal system. Hence, if the in vitro release phenomenon is relevant to the in vivo transport route, this effect is anticipated by both of the above mechanisms. Secondly, according to the Brambell hypothesis, release of a non-degradable substrate that has been ingested into the same part of the vacuolar system as IgG would be expected to be released again on fusion of the secondary lysosome with the plasma membrane. The release of lysosomal enzymes would be expected at the same time.

The release of both ^{125}I-labelled PVP and the lysosomal enzyme β-N-acetylglucosaminidase were therefore studied during incubation of yolk sacs in medium containing homologous IgG.

METHODS

6.2 Release of ^{125}I-labelled IgG from 17.5-day rat yolk sacs 'loaded' at different substrate concentrations

Single yolk sacs removed from three different animals at 17.5 days of gestation were incubated in medium 199 alone (10.0 ml) containing ^{125}I-labelled IgG (Batch II) then re-incubated for 3.0 h in substrate-free medium. The experimental regime was the same as that described in Section 5.4 but differing concentrations of radiotracer (1.0, 5.0, 25.0, 75.0 and 100.0 µg/ml of medium were used in the initial [loading] stage). The radioactive species released were assayed by the method previously described (Chapter 2, Section 2.17).

The released radioactivities, when expressed as a percentage of the total radioactivity associated with the tissue at the start of the re-incubation period were observed to rise progressively with time, a pattern similar to that found with Batch I ^{125}I-labelled IgG (see Fig. 5e). The patterns again showed that by 120 min release was virtually complete, there being little or no further release in the remaining 60 min of incubation. In order to compare the amounts of ^{125}I-labelled IgG at each loading concentration, the released radioactivities were expressed in absolute units, rather than as a percentage of that contained in the tissue at the start of the re-incubation. The mean quantity of each of the different types of radioactivity released over the final 60 min of re-incubation was determined and each value expressed as µg ^{125}I-labelled IgG released per mg yolk-sac protein.

These mean values of the quantity of ^{125}I-labelled IgG released over the final 60 min of incubation, expressed in the above format are shown in Table 6.I; the individual time courses of release, from which these values were derived, are not reported. This table also shows the overall mean value of the released radioactivities for

each of the groups of three separate, identical experiments performed at a given 'loading' concentration. The same summary data are also expressed graphically in Fig. 6a.

Table 6.II shows the relative proportions of acid-soluble and acid-insoluble radioactivity in the released radioactivity. (N.B. These values are expressed as a percentage of the total released radioactivity rather than as percentage of the total tissue-associated radioactivity at the start of the re-incubation.) Fig. 6b shows the variation of these values at the different 'loading' concentrations of ^{125}I-labelled IgG.

6.3 Release of ^{125}I-labelled PVP from 17.5-day rat yolk sacs re-incubated in the presence and absence of unlabelled homologous IgG.

In order to determine whether IgG facilitated the release of a substrate contained within the lysosomal system, yolk sacs were loaded by incubating for 2.0 h in medium 199 containing homologous IgG (10.0 μg/ml) and ^{125}I-labelled PVP (5.0 μg/ml of medium) then re-incubated either in fresh medium 199 alone or in medium 199 containing homologous IgG (10.0 μg/ml). The experimental regime was that described in Section 2.17 and the released radioactivity was expressed as a percentage of the total tissue-associated radioactivity at the start of the re-incubation. These results are shown in Table 6.III and expressed graphically in Fig. 6c.

6.4 Release of the lysosomal enzyme β-N-acetylglucosaminidase (β-NAG) by 17.5-day rat yolk sacs incubated in the presence and absence of homologous IgG

The release of β-NAG by 17.5-day rat yolk sacs incubated in the presence and absence of homologous IgG (10.0 μg/ml) was measured to determine whether IgG influenced the rate of release of lysosomal enzymes. Six yolk sacs, taken as pairs from three different animals,

were incubated separately at 37°C, each in 15.0 ml of medium 199. Three of the incubation flasks contained medium 199 alone, the media in the other three flasks also contained homologous IgG (10.0 µg/ml). Samples (500 µl) of the culture medium were removed at regular intervals over a 5 h incubation period, centrifuged at 1500g for 15 mins to remove cell debris, then deep-forzen immediately until assayed for β-NAG. Full details of the experimental method the assay of β-NAG and calculation of the released enzyme activity can be found in Chapter 2, Section 2.18. The results obtained are shown in Table 6.IV and the calculated mean values of the amount of enzyme released in three separate experiments are expressed graphically in Fig. 6d.

6.5 Release of ^{125}I-labelled IgG from 17.5-day rat yolk sacs that had been 'loaded' with ^{125}I-labelled IgG in the presence and absence of glucagon

Yolk sacs, removed from three different animals at 17.5-days of gestation, were 'loaded' with ^{125}I-labelled IgG by being incubated either in medium 199 alone or in medium 199 containing glucagon at a concentration of 1.0 µg/ml; both incubation media contained ^{125}I-labelled IgG (Batch IV at a concentration of 5.0 µg/ml). On re-incubating the tissue, each species of released radioactivity was assayed as described in Chapter 2, Section 2.17. The progressive release was expressed in two ways, firstly as the percentage of the total radioactivity assocated with the yolk sac at the beginning of the re-incubation period. The values of total, acid-soluble and acid-insoluble radioactivities from experiments in the absence of glucagon, expressed in this form, can be found in Table 6.V and similar data from experiments in the presence of glucagon are shown in Table 6.VI. The mean value of each species of radioactivity from 3 separate, identical experiments are plotted against time in Figs. 6e and 6f.

Secondly, the amounts of substrate released in the three different forms were expressed as the percentage of the total radioactivity released by the yolk sac during the 3.0 h re-incubation period. Tables 6.VII and 6.VIII give the results of experiments in the presence and absence of glucagon, respectively, expressed in this format. Fig. 6g shows a plot of the mean values, from 3 separate experiments, of the total- and acid-insoluble radioactivities reported in Tables 6.VII and 6.VIII.

RESULTS

6.6 Release of ^{125}I-labelled IgG from rat yolk sacs 'loaded' at different substrate concentrations

The results of experiments in which the release of ingested radiotracer into fresh incubation medium by yolk sacs that had previously been exposed to ^{125}I-labelled IgG (Batch II) at a number of different substrate concentrations, are shown in detail in Table 6.I. The quantity of each species of radioactivity released during the first 2.0 h of re-incubation period in each experiment are shown together with the mean value for each group of three experiments performed at a given 'loading' concentration. The latter quantities are plotted in Fig. 6a which shows quite clearly that, at all substrate concentrations, the greater part of the total radioactivity released consists of acid soluble species but that the amounts of radioactivity released as acid-solubles rises progressively with increasing substrate concentration. Furthermore, this plot also shows the amount of acid insolubles released to increase from 24 to 890 µg/mg protein when the 'loading concentration' was raised from 1.0 to 100 µg/ml of medium. When expressed as a percentage of the total radioactivity released, the amount of acid-insolubles released was observed to fall from a maximum of 50%, at the lowest loading concentration (1.0 µg/ml), to approximately 20%, at a concentration of 25 µg/ml (Table 6.II). However, the percentage release remained virtually constant at 20% up to the highest loading concentration of 100 µg/ml. The acid soluble species showed, of course, the reverse pattern rising from 50% to 80% at the total radioactivity released when the 'loading concentration' was increased from 1 to 25 µg/ml of medium, then remained constant over the range 25-100 µg/ml of medium. Fig. 6b shows these results in graphical form.

6.7 Release of ^{125}I-labelled PVP by 17.5-day rat yolk sacs re-incubated in the presence and absence of homologous IgG

Table 6.III shows the activities released when homologous IgG is present and absent in the incubation medium, these values are also plotted in Fig. 6c. Linear regression lines were fitted to each set of data (excluding the samples taken at 0 min) and the corresponding rates of release (gradients) obtained. These were found to be 1.90%/h and 1.51%/h with and without added IgG, respectively. Inspection of Fig. 6c clearly shows the presence of homologous IgG at a concentration of 10 µg/ml in the re-incubation medium to have no significant effect on the rate of release of this substrate.

6.8 Release of β-NAG from 17.5-day rat yolk sacs incubated in the presence and absence of homologous IgG

As with ^{125}I-labelled PVP, the release of the lysosomal enzyme β-NAG was followed in the presence and absence of homologous IgG (Section 6.4). The results shown in Table 6.IV are expressed as a percentage of the total β-NAG activity which was present at the beginning of the incubation period. Plotting these values against time (Fig. 6d) showed the release of β-NAG to be slightly higher in the absence of IgG than when IgG was present in the incubation medium. Taking β-NAG to be a representative lysosomal enzyme, these results indicate that release of lysosomal enzymes is unaffected by the presence of IgG at a concentration of 10 µg/ml in the re-incubation medium.

6.9 Release of ^{125}I-labelled IgG in the presence and absence of glucagon

The release of radiotracer was followed after prior ingestion of ^{125}I-labelled IgG (Batch IV) in medium 199 alone (see Fig. 6e). Essentially the same release pattern was observed for Batches III and IV (see Chapter 5, Fig. 5 for Batch III). Close comparison of these

two plots shows that, although the overall release patterns are similar, there are several small differences which should be noted.

Firstly, the total radioactivity released in the case of Batch IV is some 10% greater than for Batch III (Section 5.11), the maximum released total-radioactivity being approximately 85% for Batch III, compared to some 95% for Batch IV. This difference would appear to indicate that Batch IV is more readily released by the yolk sac and may possibly result from a slightly more rapid rate of processing associated with this particular preparation. The quantity of acid soluble species released was also increased for this particular batch of IgG and reached a maximum value of approx. 65% compared with a value of nearer 45% for Batch III. Conversely, the amount acid insoluble species released was found to be somewhat less in the case of Batch IV, reaching a maximum at only 30% as opposed to a value nearer 40% for Batch III.

The presence of glucagon at a concentration of 1.0 μg/ml in the incubation medium caused a profound change in the relative amounts of acid soluble and acid insoluble species released, whereas the percentage of the total radioactivity released show no change. Re-incubation in the presence of glucagon caused the acid insoluble radioactivity released to rise to approximately 55% of the tissue-associated activity as opposed to a value of only 30% when glucagon was absent. (The acid soluble radioactivities showed a corresponding change but in the opposite direction, falling from a value of approx. 65% in the absence of glucagon to 40% when glucagon was present).

In order to compare the data more clearly the released radioactivities were re-calculated and expressed as a percentage of total radioactivity released by the yolk sac. Expressed in this format, each experiment showed the total released radioactivity to rise from virtually zero to 100% at the end of the incubation period (see Fig. 6g)

and no significant differences were observed between the two experiments. On the other hand, when plotting the acid insoluble radioactivity as a percentage of the total radioactivity released, a significant difference in the levels was evident. The inclusion of glucagon in the re-incubation medium resulted in a 22% increase in the radioactivity released in the form of acid insoluble species as compared with control experiments in which glucagon was not present. The acid soluble species showed a similar change but in the opposite direction (see Tables 6.VII and 6.VIII).

6.10 Discussion

Hemmings (1961) demonstrated that while homologous IgG was found to pass freely across the rabbit yolk sac a much smaller amount of homologous albumin was transmitted. Similarly, Brambell (1966) observed that heterologous immunoglobulins (from the dog, horse and ox) showed lower rates of transmission than homologous IgG or even homologous albumin. This evidence pointed to the presence of a highly selective transport mechanism in the rabbit yolk sac, specific to homologous immunoglobulins. It is conceivable that the rat yolk sac may also, but to a lesser degree, transport immunoglobulins selectively (Halliday, 1955b; Brambell and Halliday, 1956).

In vitro experiments (see Chapter 5) showed that 17.5-day rat yolk sacs released a considerable fraction of their ingested ^{125}I-labelled IgG in a virtually intact form, whereas ^{125}I-labelled dBSA was virtually completely degraded to the level of amino acids. Furthermore, it has been shown that other high molecular weight substrates such as ^{125}I-labelled PVP (Chapter 7) and colloidal [^{198}Au]gold (Roberts *et al.*, 1977), both of which are completely resistant to intralysosomal hydrolysis, were virtually completely retained within the rat yolk sacs and not released in the same manner as ^{125}I-labelled IgG. The striking difference between the release patterns with ^{125}I-labelled IgG as substrate and those with ^{125}I-labelled dBSA and other high mol.wt. substrates, in *in vitro* incubations, may reflect the existence of a process of physiological significance that occurs in the rat yolk sac *in vivo*, namely the transfer across it of passive immunity in the period prior to birth.

Clark and Hardy (1969) investigated the transport of polyvinylpyrrolidone (PVP 60; mol. wt. 60,000) across the ileal cells of the suckling rat. Although this substrate was not hydrolysed, relatively little transmission was observed. These findings would appear to

discount any suggestion that immunoglobulins are able to traverse a tissue simply as a result of their resistance to proteolysis.

In contrast to other proteins investigated in the present study, the uptake of ^{125}I-labelled IgG by the rat yolk sac *in vitro* was accompanied by a degree of accumulation within the tissue when incubated in the presence of 10% (v/v) calf serum (see Chapter 5). As already stated (Chapter 5), the progressive accumulation of a substrate would not appear to be a prerequisite for its subsequent transmission across the cell. There must then be some other reason for accumulation of this sort. If *in vivo* IgG passes through the epithelial cells of the yolk sac, enters the subjacent vitelline capillaries and is carried to the fetus by way of the bloodstream then *in vitro*, in the absence of any blood flow, the radiotracer would be expected to accumulate in the stagnant vitelline blood vessels of the yolk sac tissue. Unfortunately this explanation is not readily compatible with the fact that no tissue accumulation was observed when ^{125}I-labelled IgG was ingested in the absence of calf serum. Moreover, the suggestion that accumulation occurs in stagnant vitelline vessels is not compatible with the observation, in exocytosis experiments, of a rapid and ready release of the bulk of the tissue-associated acid insoluble radioactivity derived from ingested ^{125}I-labelled IgG, unless the integrity of the vitelline capillaries and retention of the acid insoluble species is in some way dependent on the presence of calf serum. Although this would seem an unlikely explanation, its validity could be tested by a study of the release of ^{125}I-labelled IgG in the presence of 10% (v/v) calf serum. An alternative explanation of the observed rise in the tissue levels of ^{125}I-labelled IgG in 10% calf serum incubation is that the protein enters the lysosomal system but the hydrolytic enzymes become saturated with proteins derived from the calf serum, leading to an accumulation of radiotracer within the epithelial cells

rather than within the vitelline vessels.

By Brambell's mechanism, the passage of immunoglobulins across cell barriers involves binding to specific receptors (present on the microvillous surface of the epithelial cells) prior to pinocytosis and requires the receptor-bound IgG molecules to be, in some way, protected from degradation by the lysosomal enzymes whereas neither unbound IgG nor other proteins are so protected hence are subject to hydrolysis (see Fig. 6h). When the vesicle finally traverses the cell, fusion with the lateral or basal region of the plasma membrane occurs and its contents are discharged. The amount of IgG so protected and transported is limited by the number of unoccupied receptors which will decrease with increasing concentration of immunoglobulins.

On loading yolk sacs at different concentrations of ^{125}I-labelled IgG then re-incubating them in substrate-free medium, the amount of intact radiotracer released was shown to be dependent on the loading concentration. Fig. 6b shows the amount of acid soluble and acid insoluble radioactivity as a percentage of the amount of radioactivity released by the yolk sac. The relationship between the amount of acid insoluble radioactivity released and the loading-concentration is compatible with either hypothesis but serves to demonstrate that the number of receptor sites is finite, as proposed by both hypotheses.

Release of IgG contained within secondary lysosomes would also lead to the release of any other molecules present in the same particles, including those lysosomal enzymes that are not bound to the lysosomal membrane. Similarly, if such secondary lysosomes contained a non-digestible substrate such as ^{125}I-labelled PVP, as well as unlabelled homologous IgG: then, during re-incubation, release of IgG would also be accompanied by the release of the ^{125}I-labelled PVP. Experiments where yolk sacs were loaded with ^{125}I-labelled PVP in the presence of homologous unlabelled IgG showed no change in the amount of the

radiotracer released during a subsequent re-incubation period (Fig. 6c). This finding is consistent with the finding (Wild, 1974) that the rate of transport of PVP to the fetal circulation of the rabbit was very low.

Wild (1975) also studied the release of a lysosomal enzyme, Cathepsin D, by using a fluorescent-antibody technique, but was unable to detect the release of this enzyme. In Fig. 6d the release of another lysosomal enzyme β-N-acetylglucosaminidase (β-NAG) was monitored both in the presence and absence of homologous IgG. Plots of released enzyme activity against time showed the release of β-NAG to be unaltered by the presence of homologous IgG.

In the rat yolk sac the binding of a substrate to the plasma membrane does not appear to automatically protect the substrate from degradation since ^{125}I-labelled dBSA (Moore *et al.*, 1977) was found to enter the cell by 98% of it being bound to the plasma membrane surface but hydrolysis of the substrate was virtually complete. Hence it would appear that transport of a protein substrate requires more than binding to the plasma membrane and that a very specific type of interaction must be proposed in order that IgG may be protected from hydrolysis if transport occurs by the mechanism proposed by Brambell.

On the contrary, by Wild's hypothesis binding of IgG occurs via specific receptors localized in areas of the plasma membrane from which coated micropinocytic vesicles form. Their small size and the presence of the glycocalyx may well prevent the inclusion of any other substrates present in the extracellular fluid. This would account for the fact that *in vivo* ^{125}I-labelled PVP is not transported in the presence of homologous IgG. Unfortunately the small size of micropinocytic vesicles makes it impossible to visualize, by immunofluorescence techniques, any proteins contained within them (Wild, 1975). If it is proposed that, once formed, a micropinocytic vesicles

containing only IgG migrate across the cell and fuses with the basement membrane where their contents are released, then to enable these vesicles to traverse the cell without fusion with the lysosomes they must have some surface property which either repels the lysosomes or in some other way prevents fusion. Possibly the specific receptor for IgG is a transmembrane protein and binding of IgG transmits some signal which causes a change in the properties of the outer surface of the vesicle, thus preventing fusion with the lysosomes. On the other hand, digestion of IgG occurs by its attachment to non-specific binding sites on the plasma membrane or its ingestion in the fluid phase, to give entry into 'normal' pinosomes that ultimately pass into the lysosomal system where hydrolysis of the contained IgG occurs.

The above theory suggests that the uptake and release of IgG and the uptake of other proteins takes place by two completely separate classes of vesicles: coated micropinocytic vesicles and pinocytic vesicles. The latter fuse with lysosomes and this results in digestion of their contained proteins.

If this model of Wild's is valid, and two distinct types of pinocytic vesicle do exist in yolk sac tissues, it is possible that exposure of rat yolk sac in vitro to certain exogenous agents may selectively inhibit the formation of one of the classes of vesicle, leaving the other unaffected. As a preliminary investigation of this possible difference, yolk sacs were incubated in the presence of glucagon an agent which has been shown (Livesey, unpublished data) to inhibit the uptake of ^{125}I-labelled dBSA.

Fig. 6g shows the release of ^{125}I-labelled IgG, expressed as a percentage of the total radioactivity released by the yolk sac, in the presence and absence of glucagon. Clearly there is a significant difference between the control experiment and that in which yolk sacs

were 'loaded' with IgG in the presence of glucagon, the latter showing an increase in the percentage of acid insoluble material released. It should be stressed, however, that if the quantity of 'acid solubles' is reduced because glucagon also inhibits intralysosomal proteolysis, then the corresponding quantity of acid insolubles released will automatically constitute a greater percentage of the total radioactivity released. Although the quantity of radioactive protein ingested and released by the yolk sacs exposed to glucagon was much lower, being only approx. 20% of that ingested in control experiments, Livesey (unpublished data) has found no evidence that glucagon interferes with intralysosomal proteolysis.

Although this study was only preliminary in nature, these data are best explained by proposing a two vesicle system in which the formation of transport vesicles is less readily inhibited than are those pinocytic vesicles destined to fuse with the lysosomal system. Thus this differential effect on the release of substrates is consistent with the mechanism proposed by Wild and cannot be explained by the mechanism proposed by Brambell where both the quantities, acid soluble and acid insoluble released would be expected to be affected to the same extent.

In summary, the rat yolk sac like that of the rabbit, appears to have the ability to treat homologous immunoglobulins in an atypical manner and, on the basis of the in vitro exocytosis experiments reported in this chapter, the circumstantial evidence favours a mechanism for the release of IgG from the rat yolk sac tissue, that is like that proposed by Wild. It is clearly desirable, however, that these preliminary indications of the existence of a separate class of pinocytic vesicle in rat yolk-sac tissue are substantiated by more direct evidence. Differential centrifugation techniques using density gradients similar to those recently reported by

Goetze et al. (1976), but using yolk sacs that have been loaded with IgG in vivo or in vitro appear to offer one obvious approach to investigating the existence of a separate type class of vesicle containing only ^{125}I-labelled IgG. Such studies seem worth exploring in future work.

Table 6.I Release of ^{125}I-labelled IgG (Batch II) by 17.5-day rat yolk sacs 'loaded' at different substrate concentrations in medium 199 alone

This table shows the results of 3 separate experiments in which the amounts (μg) of the different classes of radioactivity released on re-incubating single yolk sacs, that had been 'loaded' with ^{125}I-labelled IgG at different concentrations, were measured. The values reported refer to the final 60 min of the 3 h re-incubation period when release was virtually complete.

'Loading' concentration of ^{125}I-labelled IgG (μg/ml)	Mean quantity of radioactivity released by the final 60 min of re-incubation (μg/ml)			Activity released (μg/mg protein) Mean ± S.D.)
	Total radioactivity			
	Experiment A	Experiment B	Experiment C	
1.0	0.061	0.047	0.036	0.048 ± 0.013
5.0	0.213	0.220	0.290	0.241 0.043
25.0	1.192	0.991	1.155	1.113 0.107
75.0	1.838	2.573	2.408	2.273 0.386
100.0	4.146	3.788	6.743	4.892 1.613
	Acid soluble radioactivity			
1.0	0.023	0.029	0.019	0.024 ± 0.005
5.0	0.102	0.154	0.145	0.134 0.028
25.0	0.925	0.726	0.947	0.866 0.122
75.0	1.480	2.164	1.970	1.871 0.353
100.0	3.343	3.176	5.489	4.003 1.290
	Acid insoluble radioactivity			
1.0	0.039	0.018	0.017	0.024 ± 0.012
5.0	0.111	0.067	0.144	0.107 0.039
25.0	0.267	0.265	0.208	0.247 0.034
75.0	0.358	0.409	0.438	0.402 0.041
100.0	0.803	0.613	1.254	0.890 0.329

Table 6.II Variation in the relative quantities of acid soluble and acid insoluble species in the total radioactivity released from 17.5-day yolk sacs after 'loading' at different concentrations of ^{125}I-labelled IgG

The mean percentages of acid-soluble and acid-insoluble radioactivity released by the end of a 3.0 h re-incubation period (calculated from the data shown in Table 6.I) are shown as a function of the 'loading' concentration of ^{125}I-labelled IgG.

'Loading' concentration of ^{125}I-labelled IgG (µg/ml of medium)	Mean percentage of the radioactivity released in the form of:	
	Acid solubles	Acid insolubles
1.0	50.0	50.0
5.0	55.6	44.4
25.0	77.8	22.2
75.0	82.3	17.7
100.0	81.8	18.2

Table 6.III Release of ^{125}I-labelled PVP by 17.5-day rat yolk sacs re-incubated in the presence and absence of unlabelled homologous IgG

Yolk sacs were loaded with labelled substrate by incubating (2.0 h) in medium 199 containing ^{125}I-labelled PVP (5.0 µg/ml medium), then re-incubated either in fresh medium 199 alone or in fresh medium containing unlabelled homologous IgG (10.0 µg/ml of medium).

The quantity of radioactivity released is expressed as a percentage of that associated with the tissue at the start of the re-incubation period.

Re-incubation period (min)	Quantity of tissue-associated radioactivity released at a given time when tissue was re-incubated in medium 199 alone (% tissue-associated radioactivity)			Mean Released Activity ± S.D. (%)
0	Experiment A	Experiment B	Experiment C	
0	0.0	1.3	1.1	0.8 ± 0.7
15	4.5	5.6	5.4	5.2 ± 0.6
45	5.6	5.7	8.5	6.6 ± 1.7
90	5.9	7.9	8.1	7.3 ± 1.2
150	7.4	9.1	9.9	8.8 ± 1.3
210	9.4	10.3	11.9	10.5 ± 1.3
	Quantity of tissue-associated radioactivity released in the presence of IgG at a given time (% tissue-associated radioactivity)			
	Experiment A	Experiment B	Experiment C	
0	2.7	3.8	0.8	2.4 ± 1.5
15	4.6	7.2	3.2	5.0 ± 2.0
45	5.8	8.9	6.7	7.1 ± 1.6
90	5.2	9.9	7.2	7.4 ± 2.4
150	7.6	13.8	7.9	9.8 ± 3.5
210	9.8	14.5	10.9	11.7 ± 2.4

Table 6.IV Release of β-N-acetylglucosaminidase by 17.5-day rat yolk sacs incubated in medium 199 alone in the presence and absence of homologous IgG (10.0 μg/ml)

β-NAG activity was determined as described in Section 2.16. The enzyme activity released is expressed as a percentage of the total enzyme-activity associated with the tissue at the start of the incubation period.

Time (min)	β-NAG activity released in the absence of IgG (% tissue-associated activity)			Mean release, ± S.D. (%)
0	Experiment A	Experiment B	Experiment C	
0	0.0	0.2	0.4	0.2 ± 0.2
15	1.8	1.2	2.0	1.7 0.4
30	2.7	3.4	4.1	3.4 0.7
60	4.2	5.6	6.4	5.4 1.1
120	6.4	10.7	10.0	9.0 2.3
180	8.3	9.6	13.2	10.4 2.5
240	7.8	12.4	14.0	11.4 3.2
300	10.0	11.7	14.3	12.0 2.2
	β-NAG activity released in presence of IgG (% tissue-associated activity)			
	Experiment A	Experiment B	Experiment C	
0	0.5	0.3	0.2	0.3 ± 0.2
15	1.6	1.7	1.5	1.6 0.1
30	2.2	2.3	2.4	2.3 0.1
60	2.7	3.4	3.9	3.3 0.6
120	4.7	5.8	6.6	5.7 1.0
180	6.4	8.4	9.4	8.1 1.5
240	6.9	9.4	11.1	9.1 2.1
300	7.3	10.0	11.3	9.5 2.0

Table 6.V Release of ^{125}I-labelled IgG (Batch IV) by 17.5-day rat yolk sacs re-incubated in TC 199 alone

Each column of data gives the mean value from 3 experiments in each of which a single yolk sac, taken from a different animal, was used. The released radioactivities are expressed as a percentage of the total radioactivity associated with the tissue at the start of the re-incubation period.

Time (min)	Mean Percentage radioactivity released (± S.D.)		
	acid soluble	acid insoluble	total radioactivity
0	1.5 ± 0.8	0.5 ± 0.4	2.0 ± 0.4
5	13.0 1.5	6.6 1.5	19.6 2.2
15	27.3 3.0	13.8 3.3	41.2 5.4
30	39.6 2.8	21.6 4.6	61.3 5.0
45	48.7 4.3	24.2 3.5	72.9 3.7
60	51.1 3.4	26.6 1.9	77.8 3.4
75	58.3 2.3	26.5 1.1	84.8 1.4
90	59.6 3.5	28.6 2.7	88.3 1.4
120	63.7 3.5	30.0 2.6	93.7 1.8
150	65.3 2.5	30.2 1.9	95.5 1.6
180	67.3 2.4	28.8 2.5	96.2 1.7

Table 6.VI Release of ^{125}I-labelled IgG (Batch IV) by 17.5-day rat yolk sacs re-incubated in TC 199 plus glucagon (1.0 µg/ml)

Each column of data gives the mean value from 3 experiments in each of which a single yolk sac, taken from a different animal, was used. The released radioactivities are expressed as a percentage of the total radioactivity associated with the tissue at the start of the re-incubation period.

Time (min)	Mean Percentage radioactivity released (± S.D.)		
	acid soluble	acid insoluble	total radioactivity
0	0.8 ± 1.6	3.0 ± 2.1	3.7 ± 1.1
5	9.2 1.3	13.1 2.8	22.2 1.7
15	17.8 1.1	24.2 5.1	42.0 5.4
30	24.3 4.1	36.0 7.2	60.4 3.2
45	28.2 1.4	44.2 8.2	72.4 6.8
60	32.4 3.5	45.4 4.0	77.8 3.3
75	37.0 2.9	48.6 2.7	85.5 1.5
90	37.3 4.6	49.6 4.3	86.9 2.5
120	40.0 3.7	53.8 3.8	93.8 3.3
150	40.0 4.1	55.7 3.5	95.7 2.4
180	41.1 3.5	56.1 3.9	97.2 0.8

Table 6.VII Release of ^{125}I-labelled IgG (Batch IV) by 17.5-day yolk sacs re-incubated in medium 199 alone

Each column of data gives the mean value from 3 separate experiments in each of which the released radioactivities are expressed *as a percentage of the total radioactivity released by the yolk sac* during its 3.0 h re-incubation period.

Time (min)	Mean percentage radioactivity released (± S.D.)		
	acid soluble	acid insoluble	total radioactivity
0	1.6 ± 0.9	0.5 ± 0.4	2.0 ± 0.5
5	13.5 1.4	6.8 1.6	20.4 1.9
15	28.4 2.6	14.4 3.3	42.8 4.9
30	41.2 2.6	22.4 4.5	63.7 4.2
45	50.6 3.9	25.2 3.6	75.8 2.5
60	53.1 2.8	27.7 1.9	80.8 2.1
75	60.6 1.2	27.6 1.6	88.1 0.7
90	61.9 3.3	29.8 3.0	91.7 0.5
120	66.2 3.3	31.2 2.8	97.4 0.5
150	67.9 2.4	31.4 1.8	99.3 0.6
180	70.0 2.4	30.0 2.4	100.0 0.0

Table 6.VIII Release of ^{125}I-labelled IgG (Batch IV) by 17.5-day yolk sacs re-incubated in medium 199 plus glucagon (1.0 μg/ml)

Each column of data gives the mean value from 3 separate experiments in each of which the released radioactivities are expressed *as a percentage of the total radioactivity released by the yolk sac* during its 3.0 h re-incubation period.

Time (min)	Mean percentage radioactivity released (± S.D.)		
	acid soluble	acid insoluble	total radioactivity
0	0.8 ± 1.7	3.1 ± 2.2	3.9 ± 1.1
5	9.4 1.5	13.5 2.8	22.9 1.6
15	18.3 1.3	27.5 4.8	43.2 5.3
30	27.0 3.2	35.1 5.1	62.1 2.8
45	30.5 1.2	44.0 5.9	74.5 6.4
60	33.4 3.5	46.7 3.9	80.1 2.8
75	38.0 3.1	50.0 2.5	88.0 1.1
90	38.4 4.9	51.0 4.2	89.4 1.9
120	41.2 4.0	55.3 3.5	96.5 2.8
150	41.2 4.5	57.2 3.1	98.4 2.4
180	42.3 3.8	57.7 3.8	100.0 0.0

Fig. 6a Plot of radioactivity released by 17.5-day rat yolk sacs after ingestion of ^{125}I-labelled IgG (Batch II) at different concentrations

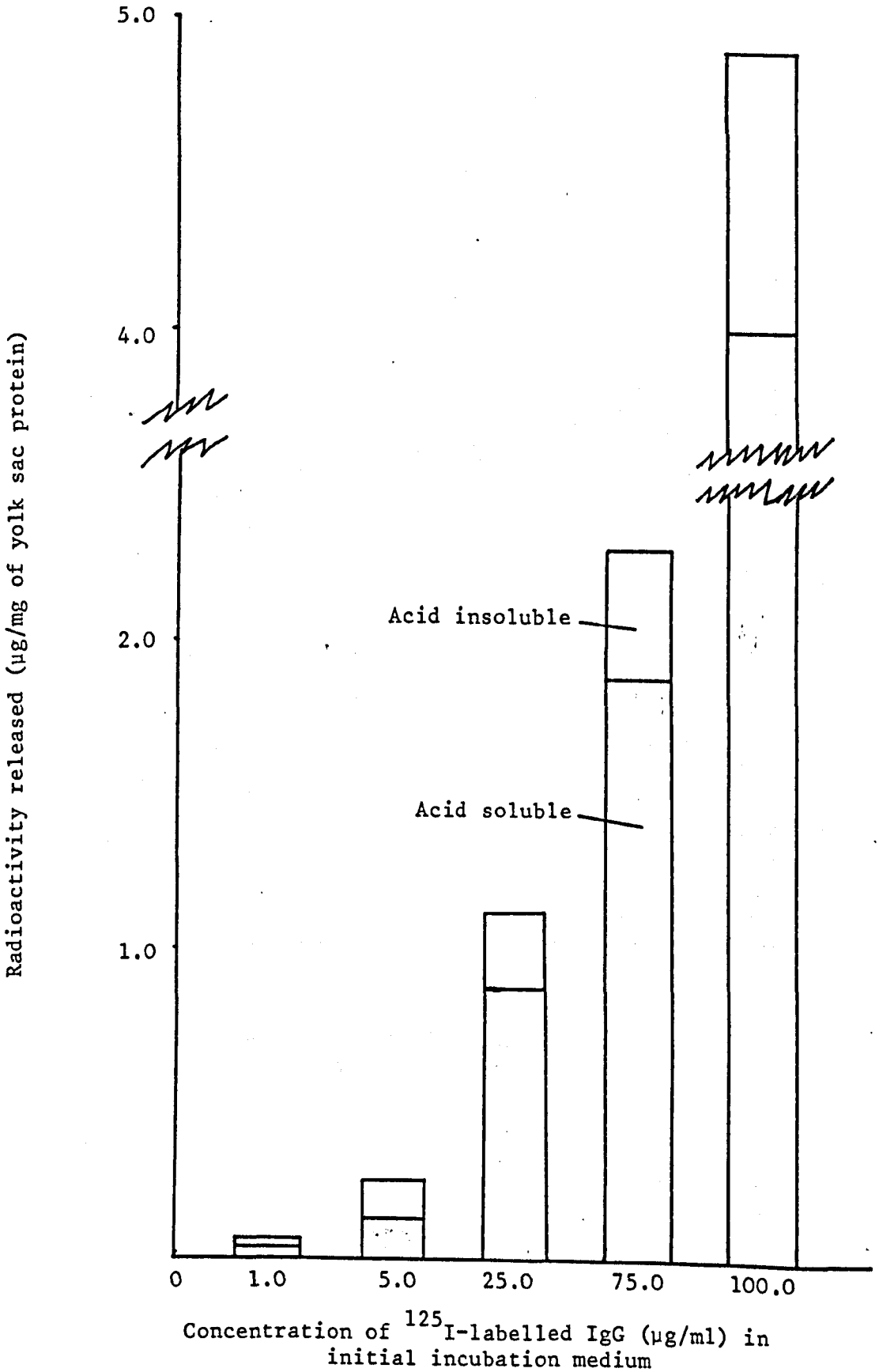


Fig. 6b Plot of acid soluble and acid insoluble radioactivity released by 17.5-day rat yolk sacs after ingestion of ^{125}I-labelled IgG (Batch II) at different concentrations

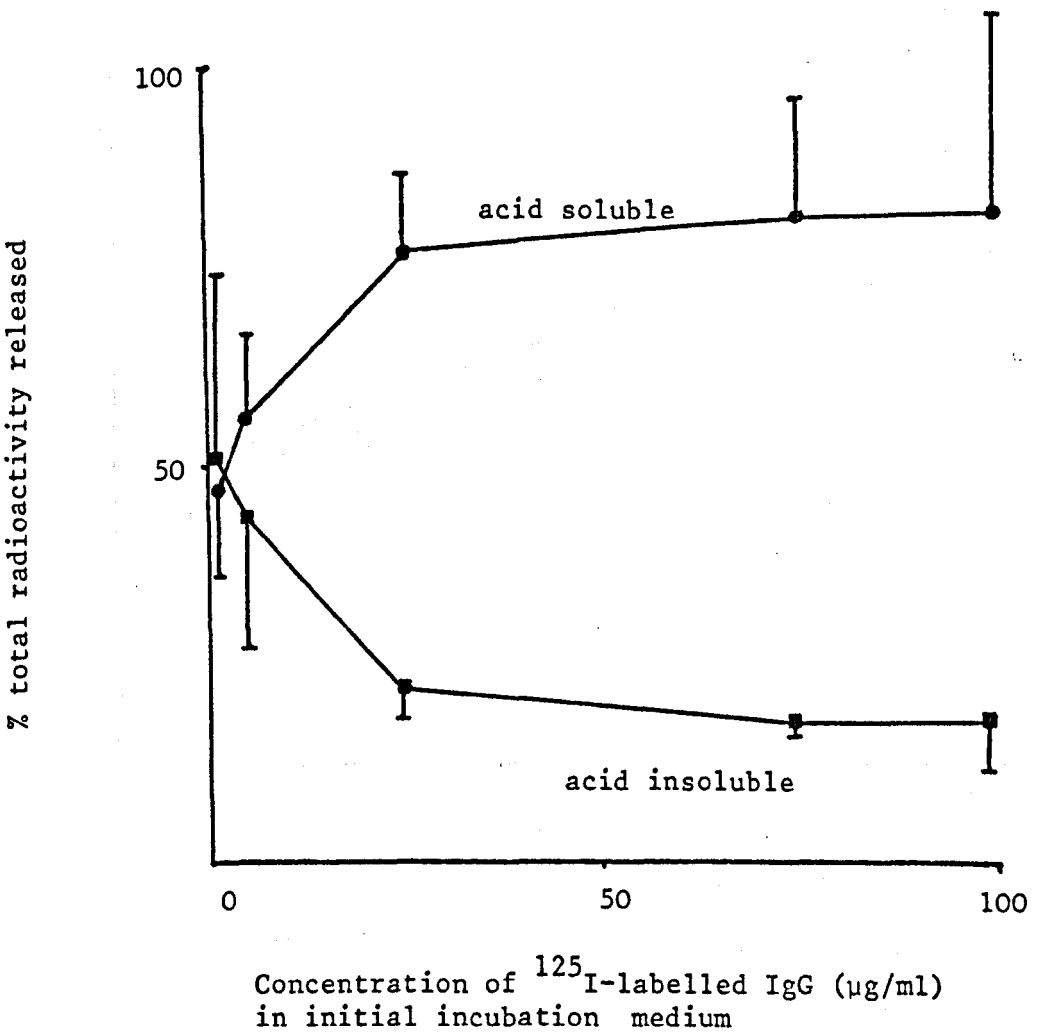
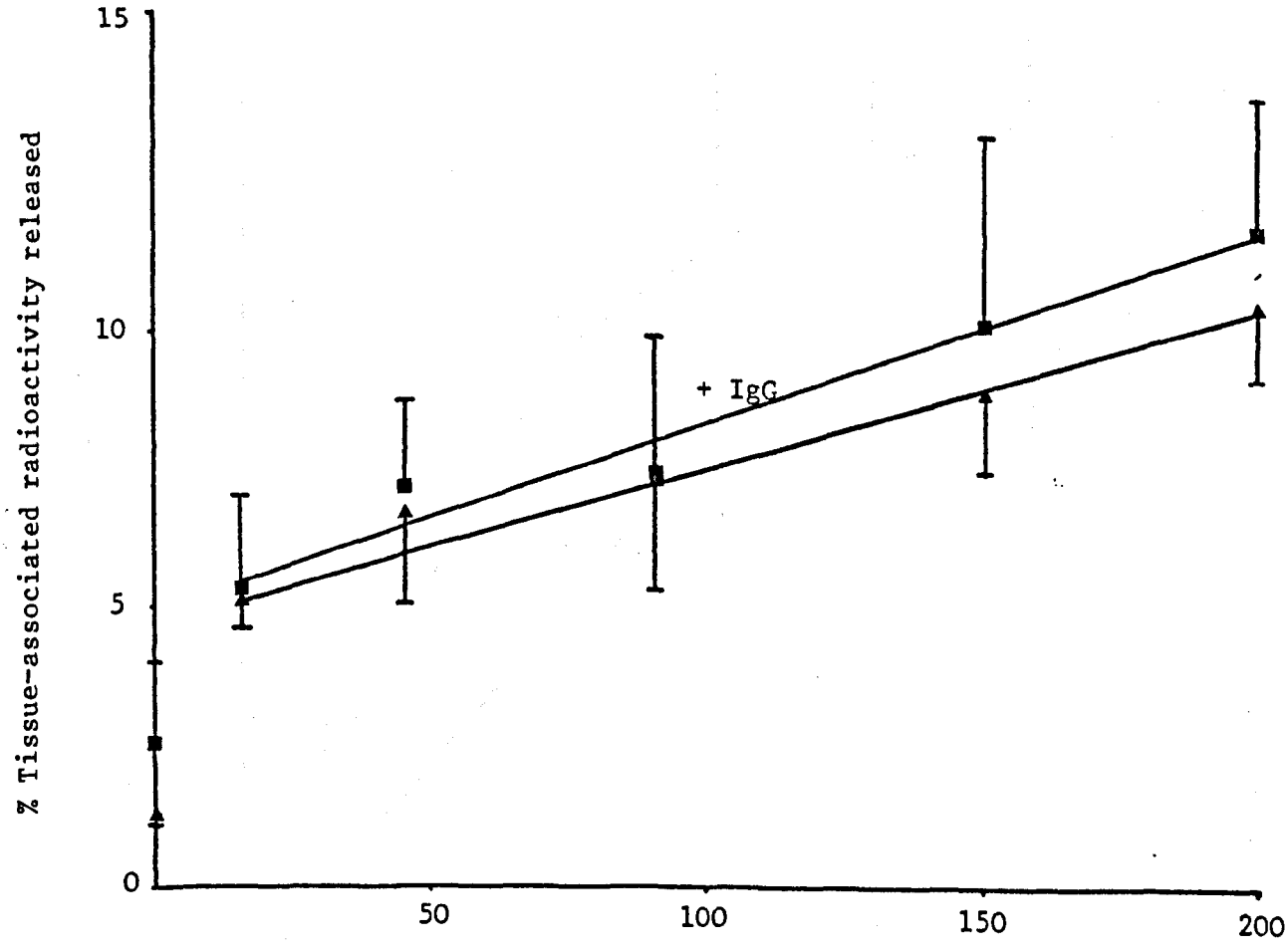


Fig. 6c Effect of homologous IgG (10 µg/ml) on the rate of release of ^{125}I-labelled PVP from 17.5-day rat yolk sacs incubated in medium 199 alone



Medium 199 plus IgG Gradient = 1.90 %/h

Medium 199 alone Gradient = 1.51 %/h

Fig. 6d Release of β-NAG by 17.5-day rat yolk sac re-incubated in the presence and absence of native IgG

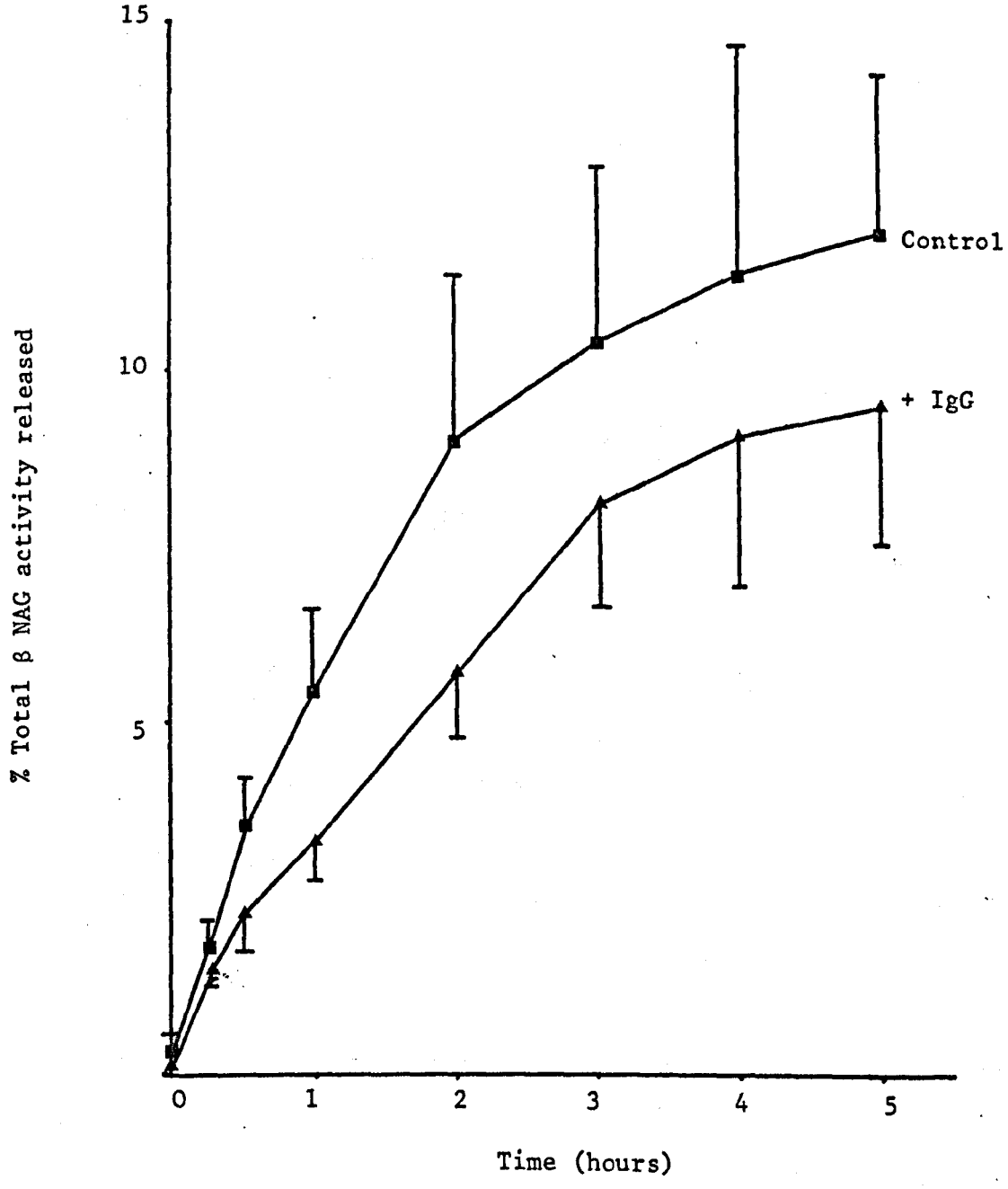


Fig. 6e Release of ^{125}I-labelled IgG (Batch IV) from 17.5-day rat yolk sac re-incubated in medium 199 alone

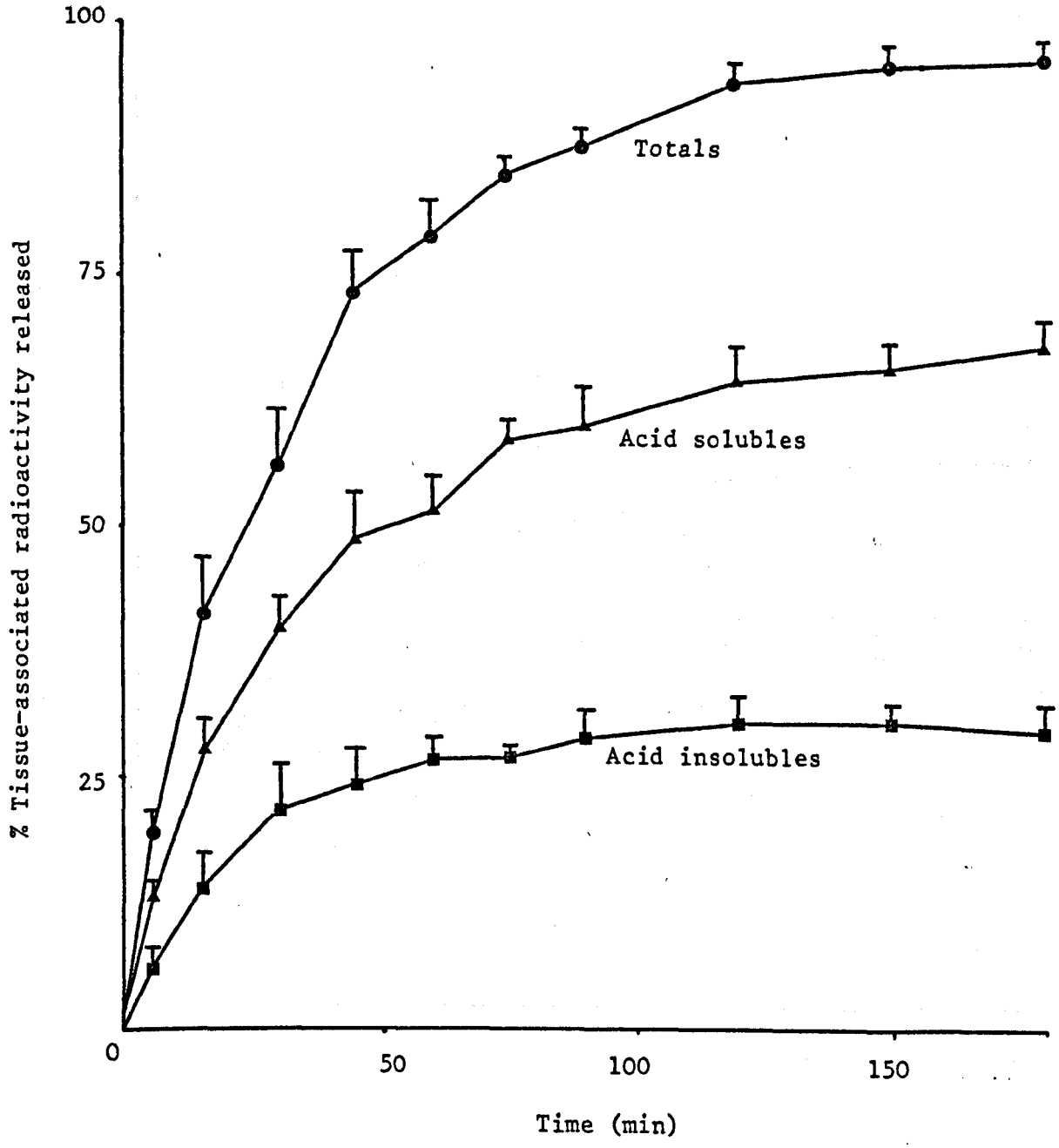


Fig. 6f Release of ^{125}I-labelled IgG (Batch IV) from 17.5-day rat yolk sac re-incubated in medium 199 plus glucagon (1.0 µg/ml)

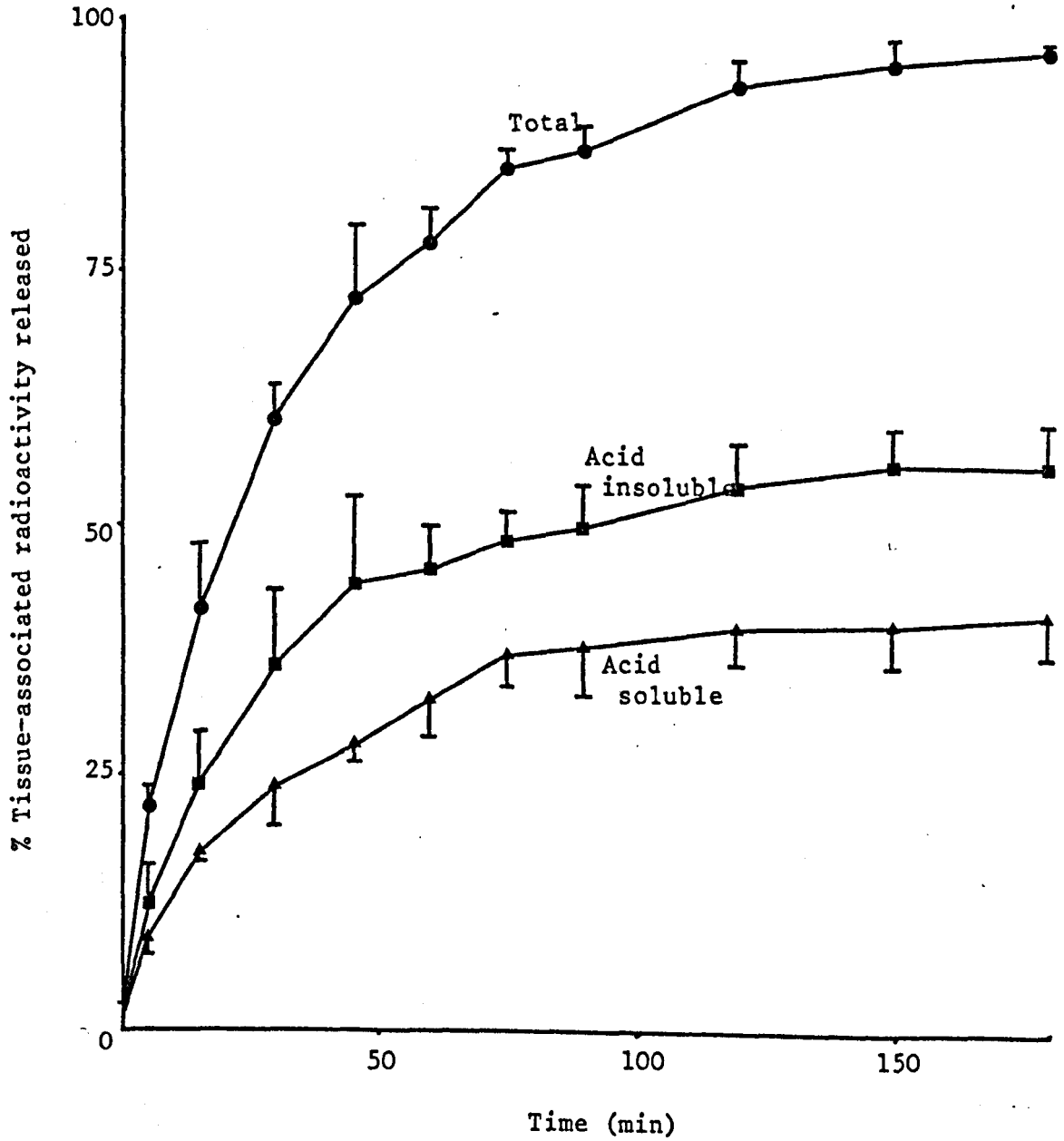


Fig. 6g Release of ^{125}I-labelled IgG from 17.5-day rat yolk sacs expressed as a percentage of the Total Radioactivity released by the tissue

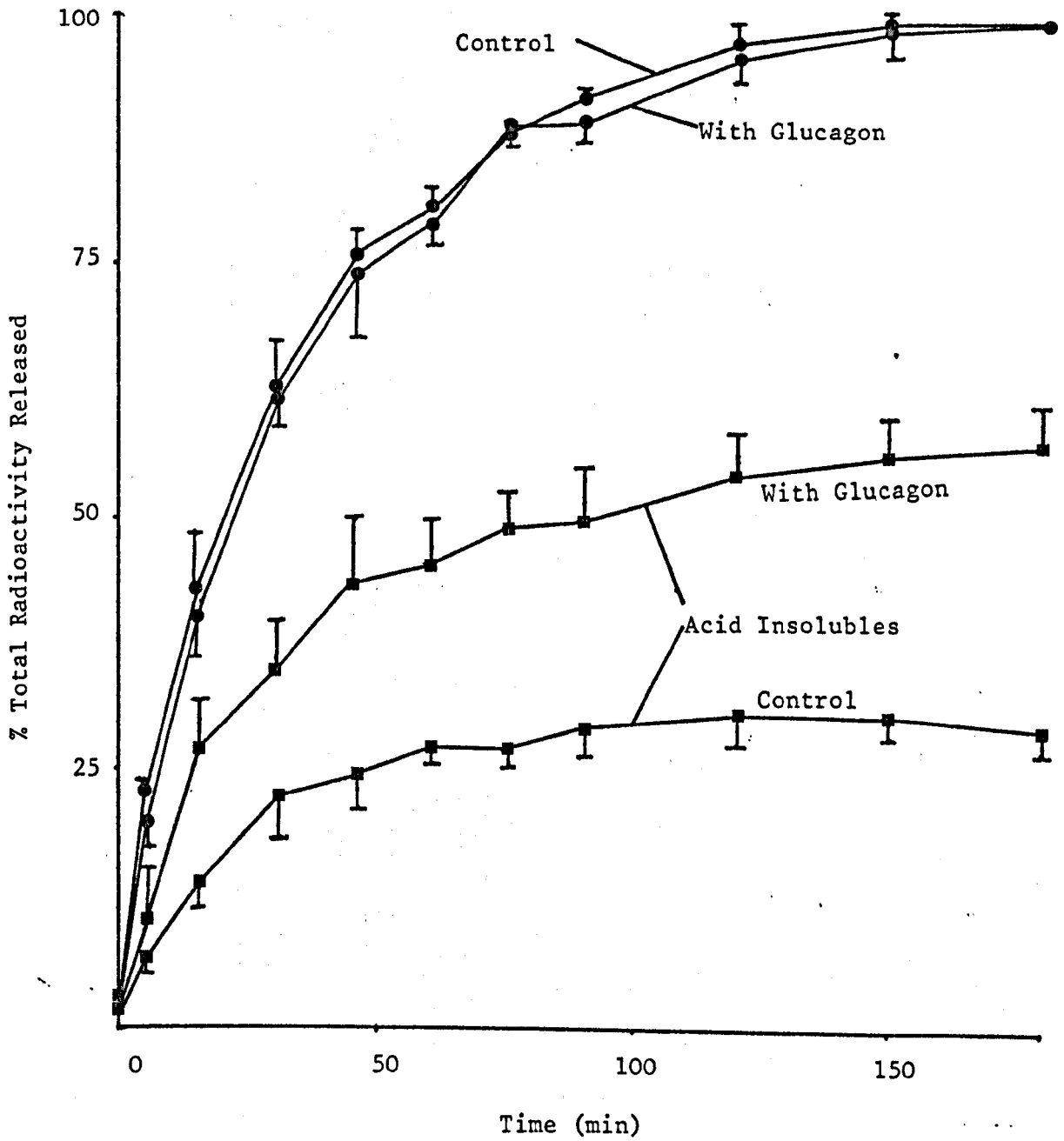
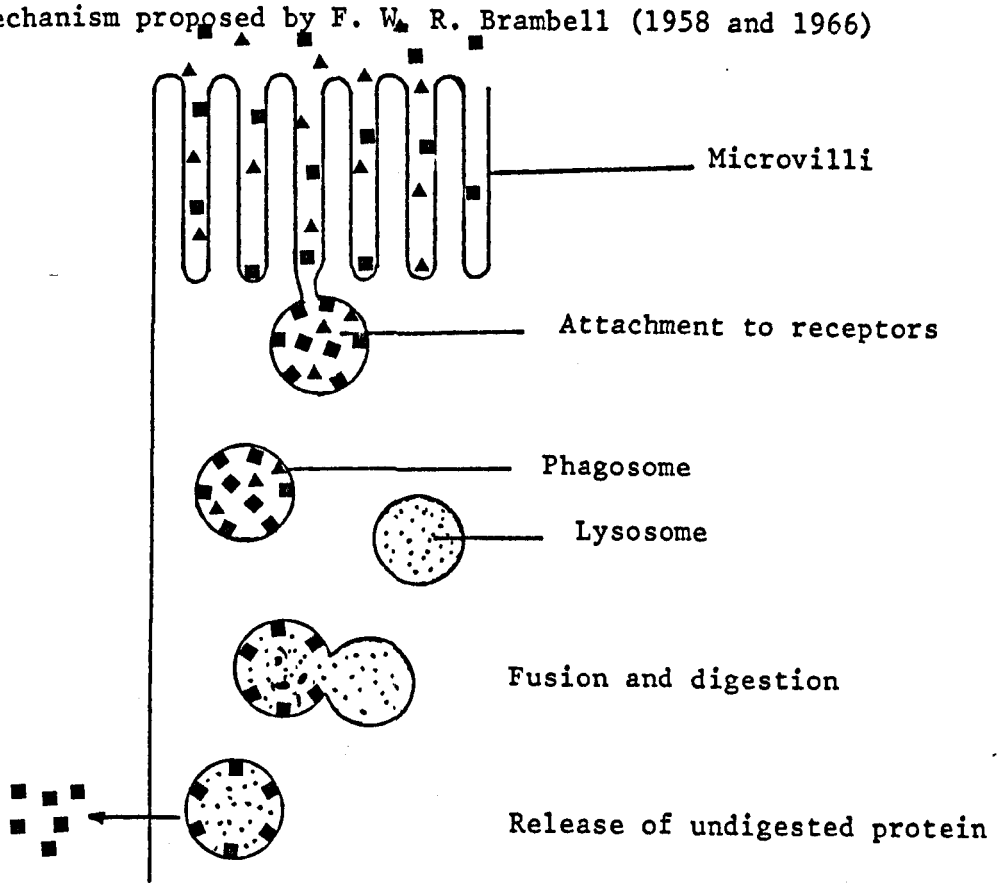
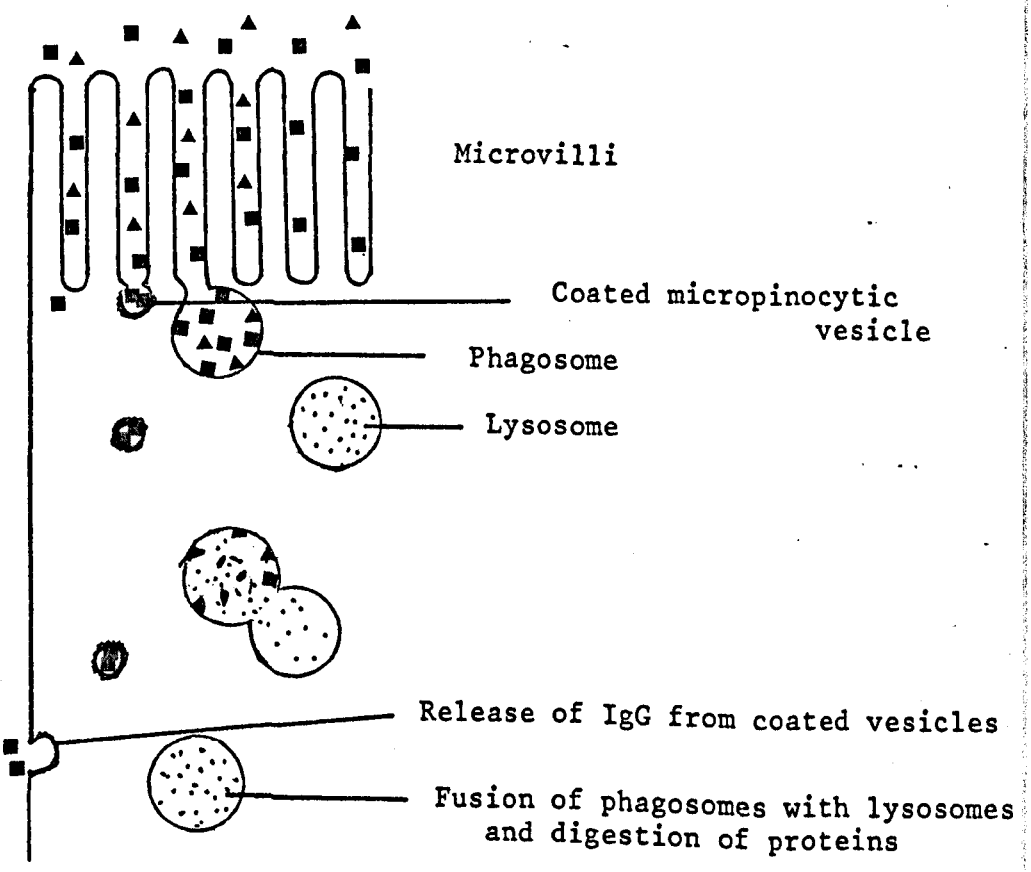


Fig. 6h Diagrammatic representation of the proposed mechanisms for the transmission of IgG across cellular barriers

a) Mechanism proposed by F. W. R. Brambell (1958 and 1966)



b) Mechanism proposed by A. E. Wild (1975)



CHAPTER 7

UPTAKE, DIGESTION AND RELEASE OF MACROMOLECULAR SUBSTRATES BY THE RAT YOLK SAC AT DIFFERENT GESTATIONAL AGES

7.1 Introduction

It has been suggested (Brambell and Halliday, 1956) that prenatal transmission of immunoglobulins in the rat occurs by way of the yolk sac. In the previous chapter the possible mechanisms of transmission were discussed and a number of *in vitro* experiments using yolk sacs removed from rats at 17.5 days of gestation were described. At this stage of gestation the ability of the tissue to remain viable in culture is now well established and the experiments described in Chapters 5 and 6 of this thesis establish the capacity of the 17.5-day tissue to release homologous IgG that has associated with it. Although Williams *et al.* (1976) have made a preliminary report of the rate of ingestion of ^{125}I-labelled PVP *in vitro* at slightly earlier stages of gestation, the ability of the tissue to ingest and release homologous IgG has only been investigated at 17.5 days of gestation.

The passage of immunoglobulins from the mother to the fetal rat was the subject of an *in vivo* study by Halliday (1955b). In this communication the amount of antibody in the fetal blood was expressed as a concentration quotient (C.Q.) with respect to the maternal serum (Batty *et al.*,1954). Halliday found the C.Q. to rise from $^{1}/_{128}$ at 17 days to $^{1}/_{32}$ at 20 days of gestation: indicating the progressive transmission of antibodies prior to birth. Unfortunately, these data do not demonstrate that the capacity of the transporting tissue actually increases with age but only that the net amount of antibody in the fetal blood compared to the maternal serum increases towards the end of gestation.

In order to investigate whether the cells of the yolk sac change in their ability to ingest and transport materials as a function of gestational age, experiments were designed with the objectives of answering the following questions:

1. Does the rate of pinosome formation change with age?

2. Do the rates of ingestion of either a non-specific protein or homologous immunoglobulins alter with age?

3. Are the rates of release of ingested substrates, especially homologous IgG, affected by the gestational age of the tissue?

4. Does the proteolytic capacity of yolk-sac tissue change significantly towards the end of gestation?

The rates of uptake of ^{125}I-labelled PVP, ^{125}I-labelled dBSA and ^{125}I-labelled IgG were therefore determined using yolk sacs taken at 17.5, 19.5 and 20.5 days of gestation. In other experiments, the amount and nature of the radioactivity released on re-incubating tissues that had ingested one of these substrates were also investigated in an attempt to answer the third of these questions. Finally, the proteolytic capacity of the yolk sac towards ^{125}I-labelled dBSA and ^{125}I-labelled IgG was compared at different stages of gestation.

METHODS

7.2 Uptake of ^{125}I-labelled PVP, ^{125}I-labelled dBSA and homologous ^{125}I-labelled IgG by rat yolk sacs removed at 17.5, 19.5 and 20.5 days of gestation

The rates of uptake of the substrates ^{125}I-labelled PVP, ^{125}I-labelled dBSA (Batch VI) and homologous ^{125}I-labelled IgG (Batch III) were determined in the presence of calf serum (10%, v/v) using yolk sacs taken from 17.5-, 19.5- and 20.5-day pregnant animals (see Section 2.3 for details of method). The quantities of total radioactivity present in the yolk sacs and in the media were assayed as described in Section 2.4 and 2.6, respectively and the protein content of each yolk sac was determined by the method of Lowry (see Section 2.5). The concentration of radiotracer employed was 1.0 µg/ml for both ^{125}I-labelled dBSA and ^{125}I-labelled IgG and 2.0 µg/ml for ^{125}I-labelled PVP.

Uptake, expressed as µl/mg yolk-sac protein, was plotted against time and the related Endocytic Index calculated as described in Sections 2.12 and 2.13. The results, for each substrate at the different days of gestation, are shown in Tables 7.I to 7.VI.

7.3 Release of ^{125}I-labelled PVP, ^{125}I-labelled dBSA and ^{125}I-labelled IgG on re-incubating 17.5-, 19.5- and 20.5-day rat yolk sacs that had accumulated these substrates in vitro

Single yolk sacs, taken from three different animals on one of the above days of gestation, were incubated in medium 199 alone (10.0 ml) containing the radiotracer at a concentration of 5.0 µg/ml (see Section 2.17 for method). In the case of ^{125}I-labelled IgG, 15.5-day yolk sacs were also studied in equivalent experiments. After 2 h of incubation the yolk sacs were removed from each flask, washed (3 x 2 min) in changes of warm (37°C) gassed medium then re-incubated for 3.0 h in fresh substrate-free medium.

The radioactivity released was assayed by the methods described in Chapter 5, Section 5.4. For ^{125}I-labelled dBSA and ^{125}I-labelled IgG the quantities of total, acid soluble, and acid insoluble radioactivity released by 17.5-, 19.5- and 20.5-day yolk sacs are each shown in Tables 7.VIIa and b, 7.VIIIa and b. [The rates of release of these substrates from 17.5-day yolk sacs are taken from Chapter 5; Tables 5.IXa (for ^{125}I-labelled dBSA) and Table 5.VIII (for ^{125}I-labelled IgG)]. The percentage of tissue-associated radioactivity released from 17.5-, 19.5- and 20.5-day yolk sacs that had ingested ^{125}I-labelled PVP are shown in Table 7.IX.

The amounts of ^{125}I-labelled PVP released by 17.5-, 19.5- and 20.5-day yolk sacs are plotted together against time in Fig. 7a; corresponding results for the release of ^{125}I-labelled dBSA (Batch VI) are shown separately in Figs. 7b-7d. Similarly, Figs. 7e-7g show the results of equivalent experiments using ^{125}I-labelled IgG (Batch III) and 17.5-, 19.5 and 20.5-day yolk sacs, respectively.

Since insufficient Batch III IgG was available to include 15.5-day tissues in the same series of experiments, the release of ^{125}I-labelled IgG by 15.5-day yolk sacs had to be determined with a fresh batch of ^{125}I-labelled IgG (Batch IV). A further set of data on 17.5-day tissues was also collected for this material to provide a necessary control. These data are shown in Figs. 7h and 7i, respectively.

RESULTS

7.4 Endocytic Indices of ^{125}I-labelled PVP, ^{125}I-labelled dBSA and ^{125}I-labelled IgG for yolk sacs at 17.5, 19.5 and 20.5 days of gestation

The results of uptake experiments, using ^{125}I-labelled PVP as substrate are presented in Tables 7.I and 7.II. Those data relating to 17.5-day yolk sacs are derived from Table 3.Ia (Chapter 3) but are quoted again for purposes of comparison. It can clearly be seen from Table 7.II that the Endocytic Index of ^{125}I-labelled PVP shows no significant change with increasing age of the yolk sac. Likewise, with ^{125}I-labelled dBSA as substrate, no significant change in Endocytic Index was evident with increased age (see Tables 7.III and 7.IV). In addition, with ^{125}I-labelled dBSA as substrate, the overall mean value of the tissue-associated radioactivity did not differ significantly between 17.5- and 19.5-day yolk sacs but an increased level was observed at 20.5 days although this latter value was subject to a large standard deviation.

Unlike the Endocytic Index of either ^{125}I-labelled PVP or ^{125}I-labelled dBSA, the observed Endocytic Index of ^{125}I-labelled IgG (Batch III) given in Tables 7.V and 7.VI shows a slight decrease with increased gestational age, falling from 13.4 µl/h/mg protein at 17.5 days to 9.3 µl/h/mg protein at 20.5 days of gestation. Unfortunately only two satisfactory sets of data relating to the uptake of ^{125}I-labelled IgG by 20.5-day yolk sacs were obtained. [Although several other sets were obtained, the resultant uptake plots were non-linear or showed a high degree of scatter and consequently were rejected (see Chapter 2, Section 2.15). As a result of this difficulty, this particular batch of substrate became exhausted before any further acceptable plot could be obtained.] Furthermore, with ^{125}I-labelled IgG as substrate, the overall mean value of the tissue-associated radioactivity was higher

for 17.5-day yolk sacs than for either 19.5- or 20.5-days yolk sacs, the latter two showing little difference in values (Table 7.VI). As with the value of the tissue-associated radioactivity, the mean rates of accumulation by the tissue also showed no significant difference between 19.5 and 20.5 days, but the values on both of these days were slightly lower than that at 17.5 days.

7.5 Release of ^{125}I-labelled PVP, ^{125}I-labelled dBSA and ^{125}I-labelled IgG from 17.5-, 19.5- and 20.5-day yolk sacs on re-incubation in substrate-free medium

The amounts of the non-digestible substrate, ^{125}I-labelled PVP, released up to a given time on re-incubating yolk sacs taken at 17.5, 19.5 and 20.5 days of gestation are plotted in Fig. 7a. For 17.5- and 19.5-day yolk sacs the amount of substrate released was only a small percentage of that ingested by the tissue, this being no more than 6% over the 3.0 h re-incubation period. Fig. 7a also clearly shows the release patterns at these two times to be virtually identical, each showing a slight gradient, equivalent to a rate of release of approx. 1% per h, beyond the first 30 min of re-incubation.

In contrast, equivalent plots for 20.5-day yolk sacs showed the amount of ^{125}I-labelled PVP released to reach a value of approx. 10% after 150 min of re-incubation. Beyond this time a rapid and atypical increase in the amount of released radiotracer occurred, the amount of total radioactivity released rising to more than 20% over the next 30 min. Calculated over the period 60 to 150 min, the rate of release was found to be approx. 2.0% per h; this is almost twice that for 17.5 or 19.5-day yolk sacs over the equivalent time interval.

Equivalent experiments using the readily degraded protein ^{125}I-labelled dBSA as radiotracer showed a very different pattern of results compared with those for ^{125}I-labelled PVP. Figs. 7b, 7c and 7d show the release of radioactivity with time for 17.5-, 19.5- and 20.5-day

yolk sacs, respectively. The total radioactivity released rose rapidly to a maximum level of 60 to 75% of the radioactivity associated with the tissue at the beginning of the re-incubation period. The differences between each age group were only marginal. In each case the acid soluble radioactivity released closely followed the total radioactivity while the difference between each corresponding pair of these values (the acid insoluble radioactivity) was equivalent to only approx. 5-10% after 3.0 h re-incubation. The quantities of acid soluble and acid insoluble radioactivity released at 3.0 h of re-incubation showed no significant dependence on the day of gestation.

Figs. 7h and 7i show the release of radioactivity from 17.5-day and 15.5-day yolk sacs after ingestion of a different batch of ^{125}I-labelled IgG (Batch IV). The amount of total radioactivity released by the yolk sacs appears to be somewhat lower at 15.5-days than at 17.5-days of gestation (i.e. 20% lower after 3.0 h re-incubation). On the other hand the amount of acid soluble radioactivity released appears to be approximately the same on both days so that a significantly lower amount of acid insoluble radioactivity is released in the case of 15.5-day yolk sacs. When expressed as a percentage of the amount of tissue-associated radioactivity, the acid insoluble radioactivity released in the case of the 15.5-day yolk sac is much less than for 17.5-day yolk sacs. Thus these results suggest that at the earlier age the yolk sac is restricted in its ability to release ^{125}I-labelled IgG intact.

7.6 Discussion

In the previous chapter the mechanism by which ^{125}I-labelled IgG is ingested by and released from the yolk sac *in vitro* was investigated in relation to the hypotheses advanced by Brambell (1966) and Wild (1975). While the bulk of the work reported in this thesis relates to yolk sacs taken at 17.5 days of gestation, the investigations in

this chapter were designed to determine whether the rate of pinocytic uptake, the proteolytic capacity of the tissue or the rate of release of any of the three different substrates from the tissue depended significantly on the gestational age of the yolk sacs.

The non-digestible substrate, ^{125}I-labelled PVP, was shown in Chapter 3 to be ingested and progressively accumulated by the 17.5-day yolk sac and to enter essentially in the fluid phase. From the Endocytic Indices shown in Table 7.II it is apparent that the rate of pinocytosis remains unaltered in yolk sacs removed at 17.5 - 20.5 days of gestation. Williams et al. (1976) found similar results with yolk sacs taken at 15.5 and 17.5 days of gestation. Taken together, these findings indicate that the yolk-sac tissue ingests the surrounding medium at a rate of approximately 2 µl/h/mg protein and that this capacity remains substantially the same over the period 15.5 - 20.5 days of gestation. Moreover, the rate of release of this substrate was very low, being about 1.0% per h for 17.5- or 19.5-day yolk sacs and slightly higher (2.0% per h) for 20.5-day yolk sacs. This release of substrate may well result from one of several causes. Firstly, the lysis of cells resulting in liberation of their contained substrate; secondly, exocytosis of substrate from the vacuolar system and, thirdly, slow release of extracellular substrate during the re-incubation period due to its slow de-adsorption from the cell surface or diffusion out of pockets of bulk medium occluded between the microvilli that cover the uterine face of the tissue. Nevertheless, such small differences in the rates of release at different stages of gestation would not affect significantly the calculated values for the rate of fluid ingestion.

The rate of uptake of the digestible substrate ^{125}I-labelled dBSA was also observed to remain independent of the gestational age of the tissue. Since the basal rate of uptake of the non-digestible substrate ^{125}I-labelled PVP was also constant, the finding that the

rate of uptake of the digestible substrate, ^{125}I-labelled dBSA (a substrate whose major route of uptake has been shown to be by adsorption to the tissue surface), is also independent of the gestational age, implies that the number of available binding sites for this protein also remains unchanged. Furthermore, these results indicate that the capacity of the yolk sac to ingest and degrade proteins to the level of their constituent amino acids remains a prominent characteristic of the tissue and shows no signs of diminished activity as the end of gestation approaches.

It is interesting that the Endocytic Index of ^{125}I-labelled IgG was observed to fall with increased age of the yolk sac. This does not necessarily indicate reduced uptake of this substrate by the tissue since, in the two previous chapters, it was observed that on re-incubating yolk sacs that had previously ingested ^{125}I-labelled IgG, a substantial proportion of the ingested substrate was released in a macromolecular form. The observed fall in Endocytic Index with age may well result from a modification of the percentage of ingested IgG so released rather than the amount ingested (since the method of deriving values of the Endocytic Index would neglect the contribution to the total uptake made by substrate released in a macromolecular form).

Analysis of the release patterns for ^{125}I-labelled IgG indicates that no change occurs between 17.5 and 19.5 days but, at 20.5-days, the percentage of the tissue-associated radioactivity released over a 3.0 h re-incubation period was reduced and,of that portion released, a greater fraction was in the form of acid insoluble species. This observation is compatible with the above suggestion that the fall in the Endocytic Index on day 20.5 may well be a reflection of the increased proportion of acid insoluble radioactivity released from the tissue rather than a reduction in the pinocytic capacity of the tissue towards ^{125}I-labelled IgG.

The results obtained for 15.5-day yolk sacs would appear to support this finding, in that less ^{125}I-labelled IgG was released by yolk sacs of this age compared to yolk sacs taken at 17.5-days of gestation. From these data it would appear that during the time interval 15.5 to 20.5 days of gestation the yolk sacs' ability to release immunoglobulins intact actually increases.

A final point worthy of note is that during the latter days of gestation the protein content of the yolk sac increases rapidly from about 5-6 mg at 17.5 days to some 10-12 mg at the age of 20.5 days, as a result of rapid growth of the tissue. Furthermore, it was also noted that the diameter of the yolk sac increases rapidly, more than doubling in the same time period. Such an expansion in diameter requires an attendant 4-fold increase in surface area (assuming the yolk sac to be approximately spherical in shape). During this time the amount of tissue protein only doubles hence a certain amount of stretching or unfolding at the yolk sac must occur. The rapid expansion may well be the cause of the frailty which has been generally observed to be associated with rat yolk sacs towards the end of gestation especially those at 20.5-days (Roberts, G.; personal communication). The more delicate state of the yolk sac at 20.5 days probably explains why such tissues failed to show reproducible uptake of ^{125}I-labelled IgG, showed rapid release of ^{125}I-labelled PVP after 150 min of re-incubation and showed decreased reproducibility in the release plots. Possibly, weakening of the yolk sac immediately prior to birth would be advantageous to the fetus in that its escape through this cellular barrier would be made considerably easier, hence decreasing the neonatal mortality rate for the species.

The above findings may be relevant to the biological function of the tissue. If the yolk sac is involved in the transmission of IgG to the rat fetus in the last few days of gestation, as suggested by

Brambell and Halliday (1956), then, as term approached, if a relatively greater percentage of ingested ^{125}I-labelled IgG was released from the yolk sac rather than digested within its lysosomal system, then *in vivo* a greater amount could be transmitted to the fetus to provide it with passive immunity immediately before birth.

Table 7.I Uptake of ^{125}I-labelled PVP by 17.5-, 19.5- and 20.5-day rat yolk sacs from medium 199 containing calf serum (10%, v/v)

Each line of the table shows the data from a separate experiment in which yolk sacs, taken from a single animal at the day of gestation indicated, were incubated separately in medium 199 containing 10% calf serum and ^{125}I-labelled PVP (2.0 µg/ml of medium).

Age of yolk sacs (days)	No. of yolk sacs	Endocytic Index (µl/h/mg protein)	Correlation Coefficient	Intercept (µl/mg protein)
17.5	10	2.10	0.979	+0.41
"	10	2.17	0.991	+0.32
"	10	2.52	0.994	+0.13
"	10	1.61	0.963	+1.40
19.5	10	1.88	0.987	+0.71
"	10	2.17	0.968	+0.86
"	10	1.89	0.986	+0.68
20.5	10	1.97	0.975	+0.97
"	10	2.00	0.989	+1.15
"	10	1.95	0.962	+0.17

Table 7.II Summary of the Endocytic Indices of ^{125}I-PVP for 17.5-, 19.5- and 20.5-day rat yolk sacs

This table summarizes the data of Table 7.I.

Age of yolk sac (days)	No. of experiments	Mean Endocytic Index (S.D.) (µl/h/mg protein)
17.5	4	2.10 ± 0.38
19.5	3	1.98 ± 0.17
20.5	3	1.97 ± 0.03

Table 7.III Uptake of ^{125}I-labelled dBSA (Batch VI) by 17.5-, 19.5- and 20.5-day rat yolk sacs in medium 199 containing calf serum (10%. v/v)

Age of yolk sacs (days)	No. of yolk sacs	Endocytic Index (µl/h/mg protein)	Correlation Coefficient	Intercept (µl/mg protein)	% TCA Solubles in ^{125}I-dBSA	Mean Tissue Radioactivity (µl/mg protein)
17.5	(See Chapter 3 Table 3.II for individual results)					
19.5	10	103.6	0.981	-18.3	0.60	74.9
"	10	78.6	0.980	+13.3	0.60	61.3
"	10	92.6	0.970	-20.5	0.93	82.3
20.5	10	66.9	0.972	- 7.9	0.63	50.7
"	10	86.0	0.984	- 3.7	0.96	120.3
"	10	104.0	0.984	-26.3	1.04	146.9

Table 7.IV Summary of the Endocytic Indices of ^{125}I-labelled dBSA (Batch VI) for 17.5-, 19.5- and 20.5-day rat yolk sacs

Age of yolk sacs (days)	No. of Experiments	Mean ± S.D. Endocytic Index (µl/h/mg protein)	Overall mean tissue radioactivity; ± S.D. (µl/mg protein)
17.5	4	81.8 ± 13.6	85.0 ± 9.9
19.5	3	91.6 ± 12.5	72.8 ± 10.7
20.5	3	85.6 ± 18.5	106.0 ± 49.7

Table 7.V Endocytic Indices of ^{125}I-labelled IgG (Batch III) for 19.5- and 20.5-day rat yolk sacs in the presence of calf serum (10%, v/v)

Age of yolk sac (days)	No. of yolk sacs	Endocytic Index (µl/h/mg protein)	Correlation Coefficient	Intercept (µl/mg protein)	% TCA Solubles in ^{125}I-IgG	Mean Tissue radioactivity (µl/mg protein)	Rate of accumulation of tissue radioactivity (µl/h/mg protein)	Correlation Coefficient
17.5	See Chapter 5 Table 5.I for individual results							
19.5	10	8.82	0.972	+2.79	2.01	13.19	1.15	0.885
"	9	10.45	0.965	+0.35	2.60	13.16	1.21	0.652
"	10	10.95	0.993	-3.00	2.01	14.51	1.99	0.918
20.5	10	10.36	0.980	+1.9	1.79	16.00	2.13	0.891
"	10	8.16	0.952	+6.45	2.39	12.55	0.85	0.695

Table 7.VI Summary of the Endocytic Indices of ^{125}I-labelled IgG (Batch III) for 17.5-, 19.5- and 20.5-day rat yolk sacs

Age of yolk sacs (days)	No. of Experiments	Mean Endocytic Index; ± S.D. (µl/h/mg protein)	Overall mean tissue radioactivity, ± S.D. (µl/mg protein)	Mean rate of accumulation of tissue radioactivity (µl/h/mg protein)
17.5	4	13.4 ± 2.0	19.8 ± 3.2	3.1 ± 0.96
19.5	3	10.07 ± 1.11	13.62 ± 0.77	1.45 ± 0.47
20.5	2	9.26 -	14.28 -	1.49 -

Tables 7.VIIa to 7.X

Each set of results relates to data from three separate, identical experiments in each of which a single yolk sac, taken from a different animal, was used.

Table 7.VIIa Release of ^{125}I-labelled IgG (Batch III) by 19.5-day rat yolk sacs re-incubated at 37°C in medium 199 alone

Time (min)	Mean Percentage radioactivity released ± S.D.		
	acid soluble	acid insoluble	total radioactivity
0	1.5 ± 0.4	2.3 ± 0.5	3.8 ± 0.7
5	6.8 ± 1.2	8.9 ± 1.2	15.7 ± 1.5
15	15.3 ± 1.5	17.1 ± 2.4	36.9 ± 3.8
30	22.4 ± 3.7	28.3 ± 4.4	50.6 ± 4.2
45	25.6 ± 4.5	33.7 ± 4.9	59.3 ± 4.8
60	27.4 ± 4.0	37.9 ± 6.0	65.3 ± 3.7
75	31.7 ± 5.0	39.8 ± 6.9	71.5 ± 2.0
90	32.4 ± 5.4	41.9 ± 6.6	74.3 ± 2.5
120	35.3 ± 5.4	44.1 ± 7.5	79.3 ± 2.2
150	36.5 ± 5.7	46.5 ± 7.1	83.0 ± 3.4
180	37.7 ± 5.0	47.1 ± 7.1	84.8 ± 2.6

Table 7.VIIb Release of ^{125}I-labelled IgG (Batch III) by 20.5-day rat yolk sacs re-incubated at 37°C in medium 199 alone

Time (min)	Mean Percentage radioactivity released ± S.D.		
	acid soluble	acid insoluble	total radioactivity
0	-0.6 ± 0.3	0.2 ± 0.7	0.6 ± 0.6
5	4.3 ± 1.3	5.3 ± 2.2	9.6 ± 0.9
15	9.5 ± 2.4	15.1 ± 3.8	24.7 ± 5.7
30	15.4 ± 3.9	19.4 ± 2.6	34.9 ± 5.7
45	18.7 ± 4.8	23.5 ± 2.5	42.2 ± 7.0
60	20.0 ± 4.8	27.9 ± 1.4	48.0 ± 5.7
75	20.8 ± 4.1	28.9 ± 2.7	49.7 ± 6.7
90	22.1 ± 4.3	30.5 ± 2.5	52.5 ± 6.8
120	22.5 ± 4.6	32.0 ± 1.5	55.5 ± 6.1
150	22.9 ± 4.1	34.5 ± 1.0	57.4 ± 4.9
180	22.3 ± 4.1	36.4 ± 1.3	58.7 ± 5.2

Table 7.VIIIa Release of ^{125}I-labelled dBSA (Batch VIII) by 19.5-day rat yolk sacs re-incubated at 37°C in medium 199 alone

Time (min)	Mean Percentage radioactivity released ± S.D.		
	acid soluble	acid insoluble	total radioactivity
0	2.9 ± 0.2	0.8 ± 0.8	3.6 ± 0.7
5	17.7 2.0	0.2 3.4	17.8 3.0
15	32.0 3.8	3.2 1.6	35.1 4.5
30	42.8 4.1	3.2 2.1	47.0 4.3
45	47.1 2.7	5.5 2.9	53.5 4.2
60	50.6 2.3	3.8 2.3	55.8 3.6
75	56.9 0.9	5.9 0.7	63.2 0.8
90	58.6 0.3	5.4 1.6	64.9 0.9
120	61.7 0.4	6.4 1.2	68.1 1.0
150	61.6 1.4	7.1 0.9	68.7 0.8
180	64.6 0.9	7.2 0.8	71.9 1.3

Table 7.VIIIb Release of ^{125}I-labelled dBSA (Batch VI) by 20.5-day rat yolk sacs re-incubated at 37°C in medium 199 alone

Time (min)	Mean Percentage radioactivity released ± S.D.		
	acid soluble	acid insoluble	total radioactivity
0	1.0 ± 0.2	-0.1 ± 0.3	0.9 ± 0.2
5	12.3 1.5	1.7 0.7	14.0 1.3
15	28.2 4.4	1.9 0.6	30.0 4.4
30	40.9 7.3	3.6 1.3	44.5 8.5
45	46.5 8.5	3.2 1.0	49.6 7.4
60	49.2 8.0	4.2 0.2	53.2 8.1
75	52.5 9.3	4.8 0.6	57.3 9.4
90	55.1 10.1	6.9 2.3	62.0 11.7
120	59.0 10.6	9.1 4.4	68.2 14.3
150	61.1 9.5	10.3 5.0	71.3 13.5
180	64.7 4.4	11.1 5.7	73.1 13.6

Table 7.IX Release of ^{125}I-labelled PVP by 17.5-, 19.5- and 20.5-day rat yolk sacs re-incubated at 37°C in medium 199 alone

Time (min)	Mean Percentage radioactivity released ± S.D.		
	19.5-day	20.5-day	17.5-day
0	0.3 ± 0.3	0.6 ± 0.8	0.3 ± 0.4
5	1.2 0.2	4.0 1.6	2.0 0.5
15	1.8 0.1	5.1 1.7	2.9 0.3
30	4.0 1.7	6.5 1.9	2.9 0.6
45	3.6 0.7	7.0 1.4	3.2 0.5
60	3.7 1.0	7.4 1.7	3.1 0.5
75	3.9 0.9	8.1 1.5	3.4 0.4
90	4.3 0.9	8.6 1.7	4.0 0.7
120	4.7 1.0	9.6 1.7	4.0 0.6
150	5.4 0.9	10.3 2.0	4.4 0.2
180	6.5 0.8	20.6 7.7	5.1 0.6

Table 7.X Release of ^{125}I-labelled IgG (Batch IV) by 15.5-day rat yolk sacs re-incubated at 37°C in medium 199 alone

Time (min)	Mean Percentage radioactivity released ± S.D.		
	acid soluble	acid insoluble	total radioactivity
0	1.3 ± 0.6	0.2 ± 0.5	1.5 ± 0.2
5	11.8 2.2	3.9 1.2	15.8 2.8
15	27.9 5.3	7.5 1.6	35.4 5.7
30	37.6 5.3	10.9 3.4	48.5 8.5
45	44.7 7.7	11.5 2.0	56.2 9.6
60	49.5 7.4	11.6 3.3	61.1 9.3
75	49.7 6.9	14.0 4.6	63.7 9.8
90	54.1 6.3	11.8 2.9	65.9 8.8
120	57.4 7.8	13.1 2.3	70.6 9.7
150	59.4 7.2	13.5 2.9	72.8 9.3
180	60.9 7.6	13.8 3.3	74.7 9.4

Fig. 7a Release of ^{125}I-labelled PVP from 17.5, 19.5 and 20.5-day rat yolk sacs re-incubated in medium 199 alone

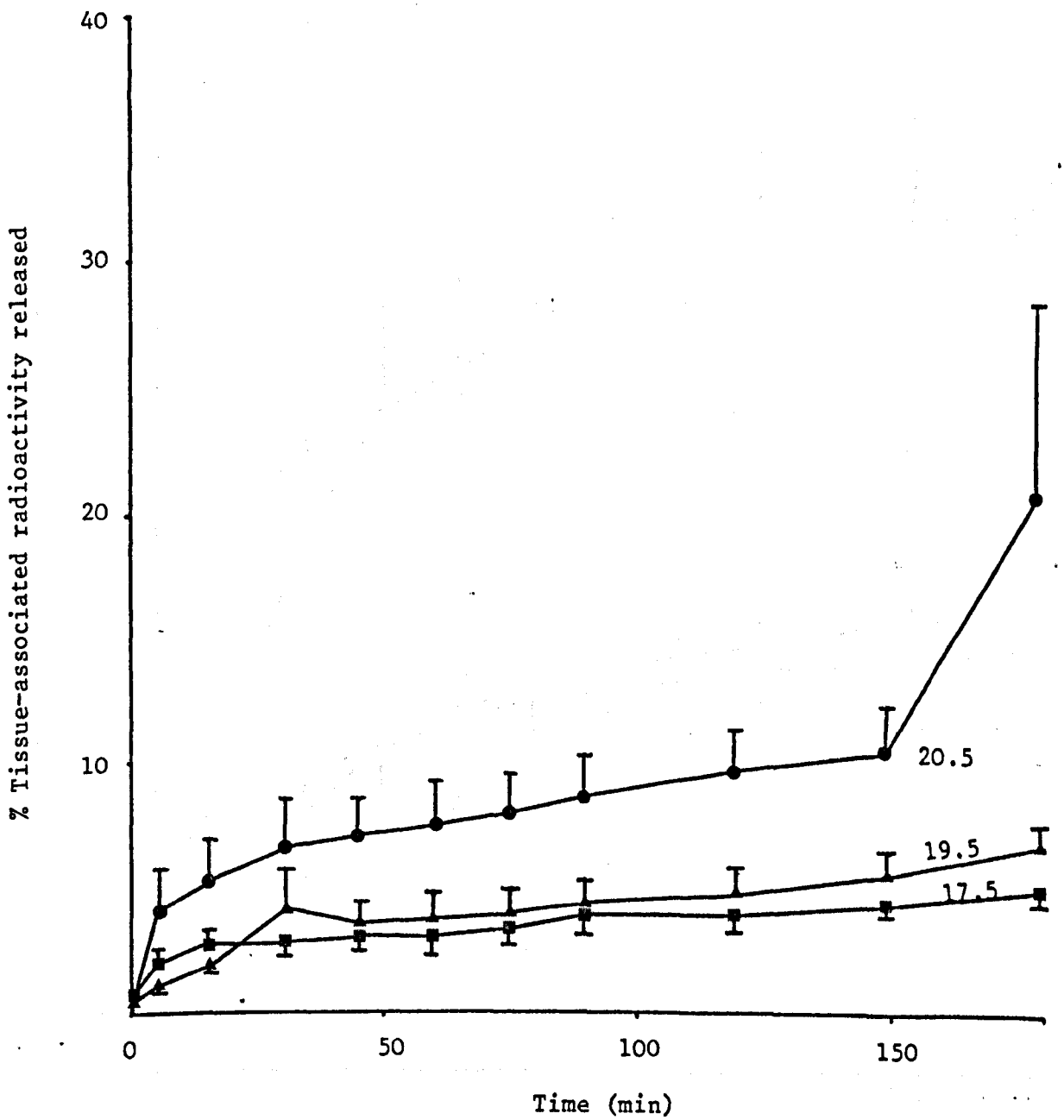


Fig. 7b Release of ^{125}I-labelled IgG (Batch III) by 17.5-day rat yolk sacs re-incubated in medium 199 alone

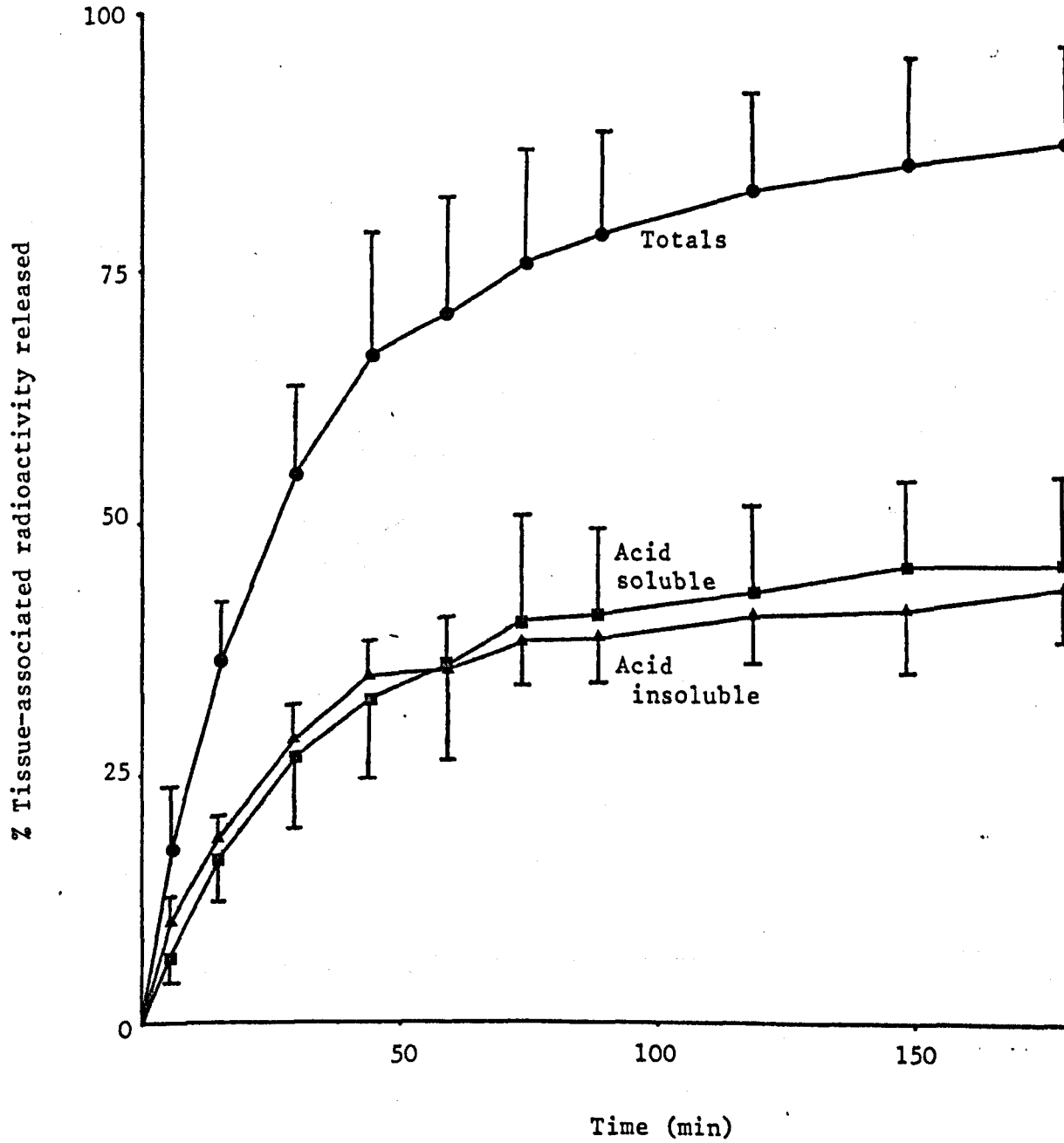


Fig. 7c Release of ^{125}I-labelled dBSA (Batch VI) from 19.5-day rat yolk sacs re-incubated in medium 199 alone

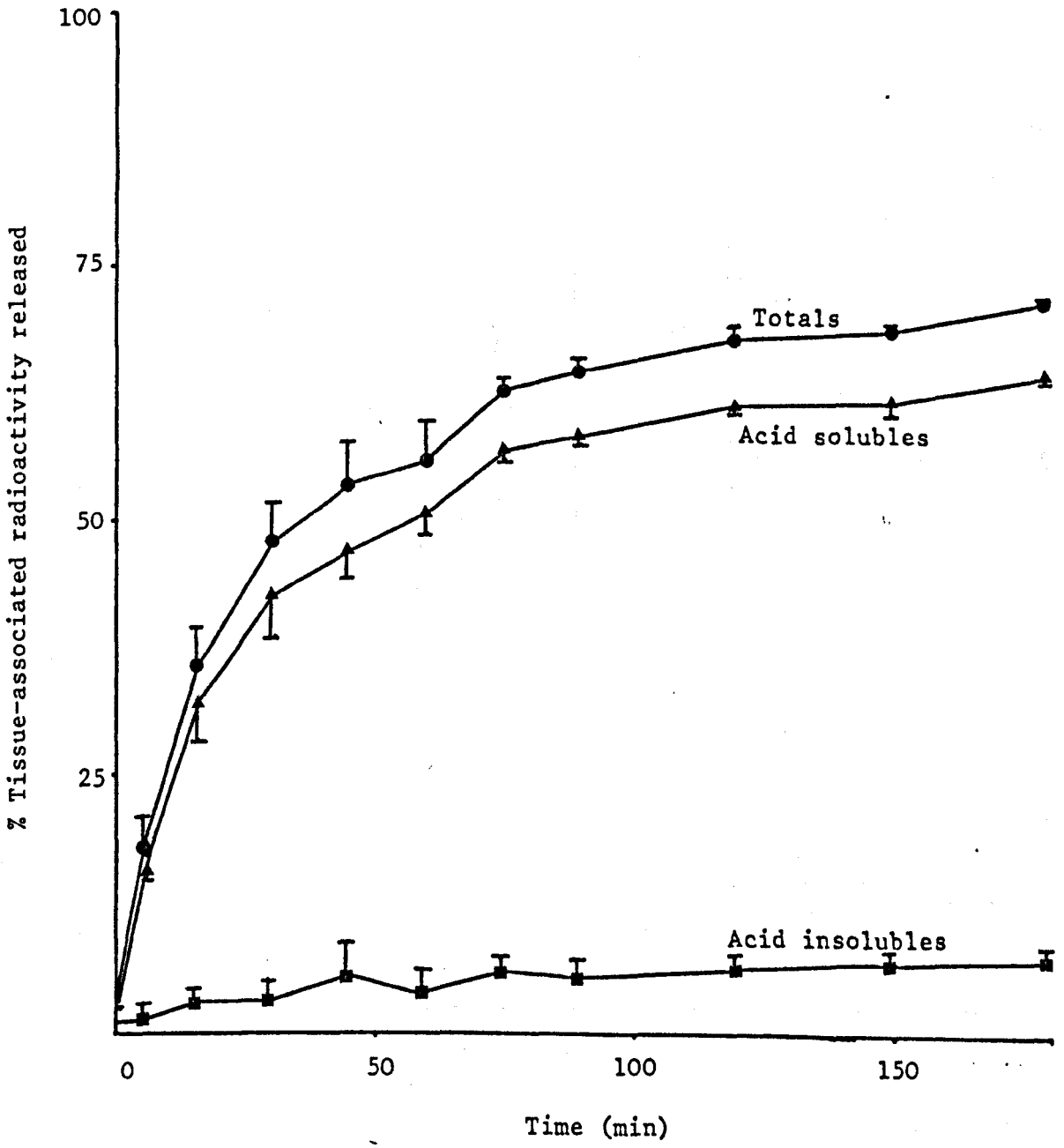


Fig. 7d Release of ^{125}I-labelled dBSA (Batch VI) from 20.5-day rat yolk sacs re-incubated in medium 199 alone

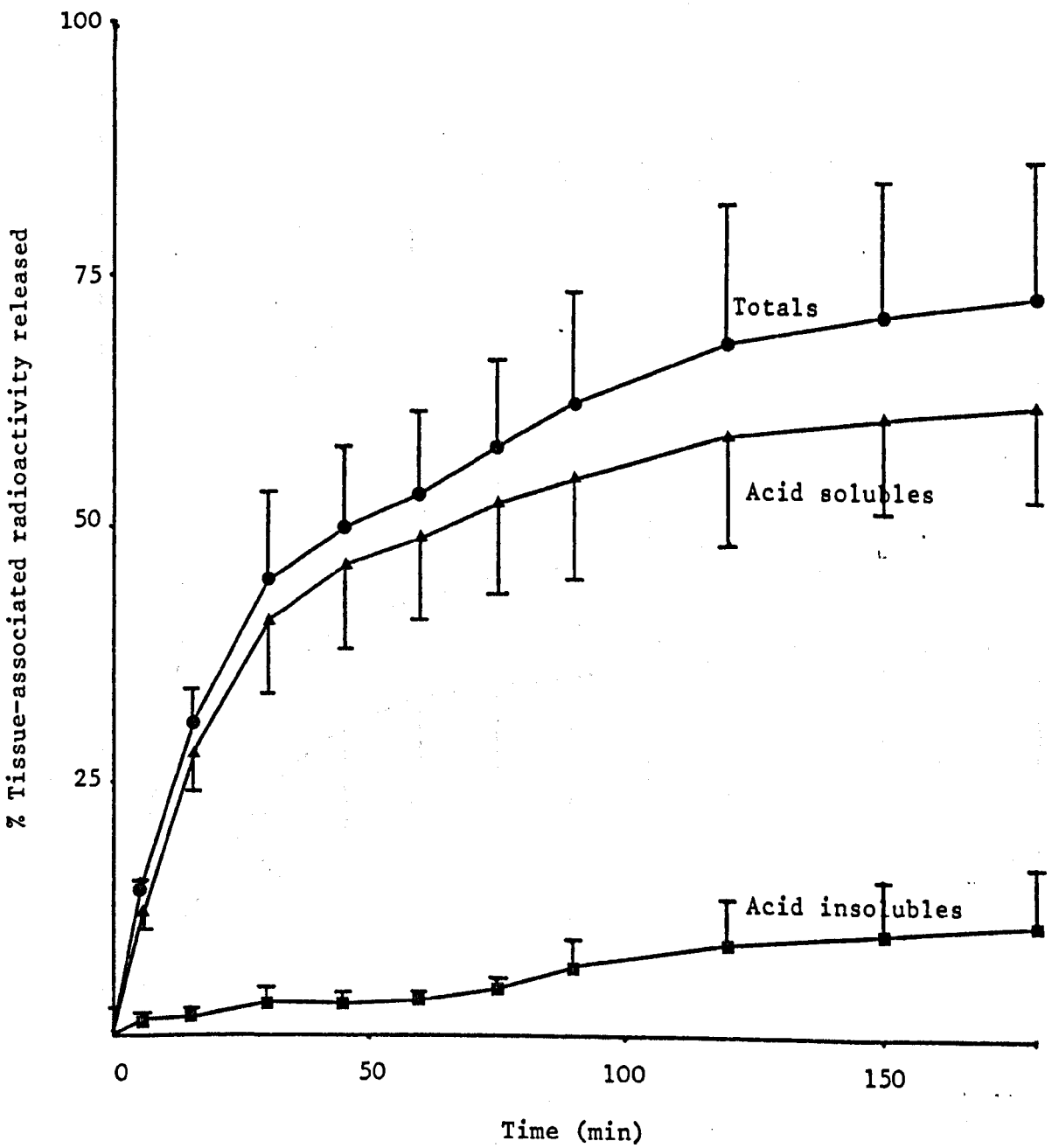


Fig. 7e Release of ^{125}I-labelled IgG (Batch III) from 17.5-day yolk sacs re-incubated in medium 199 alone

Same data as shown in Fig. 5e

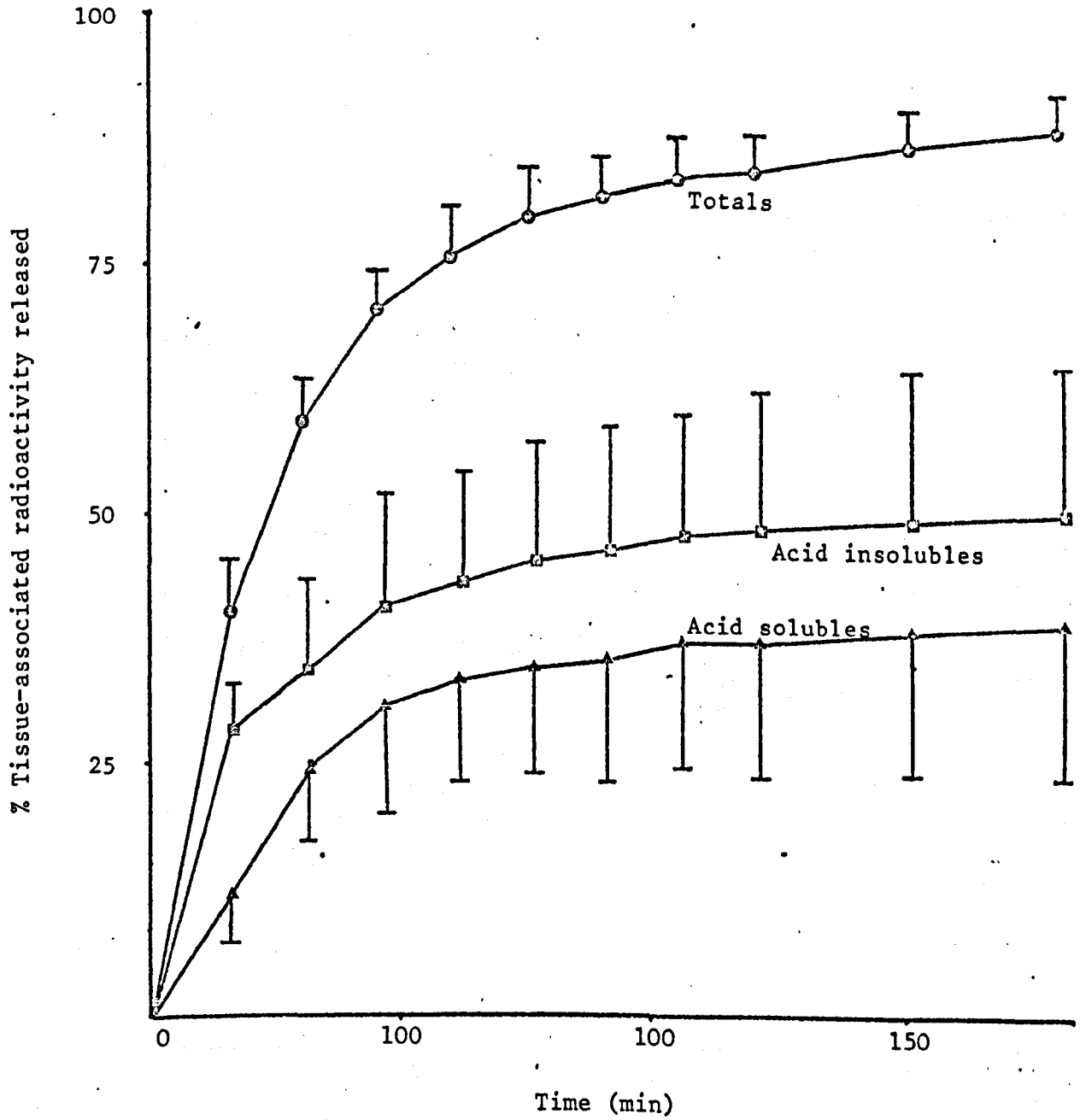


Fig. 7f Release of ^{125}I-labelled IgG (Batch III) from 19.5-day rat yolk sacs re-incubated in medium 199 alone

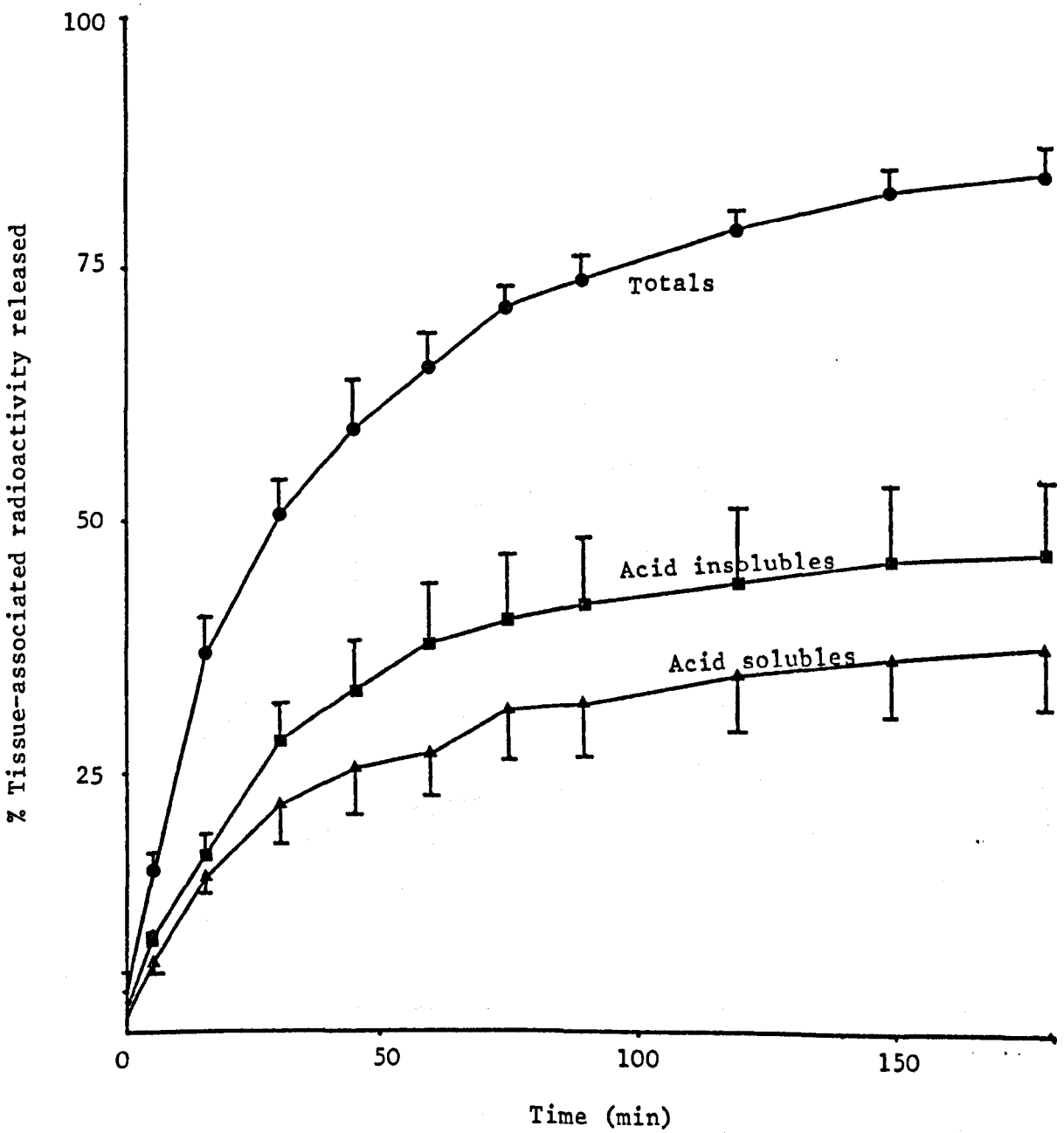


Fig. 7g Release of ^{125}I-labelled IgG (Batch III) from 20.5-day rat yolk sacs re-incubated in medium 199 alone

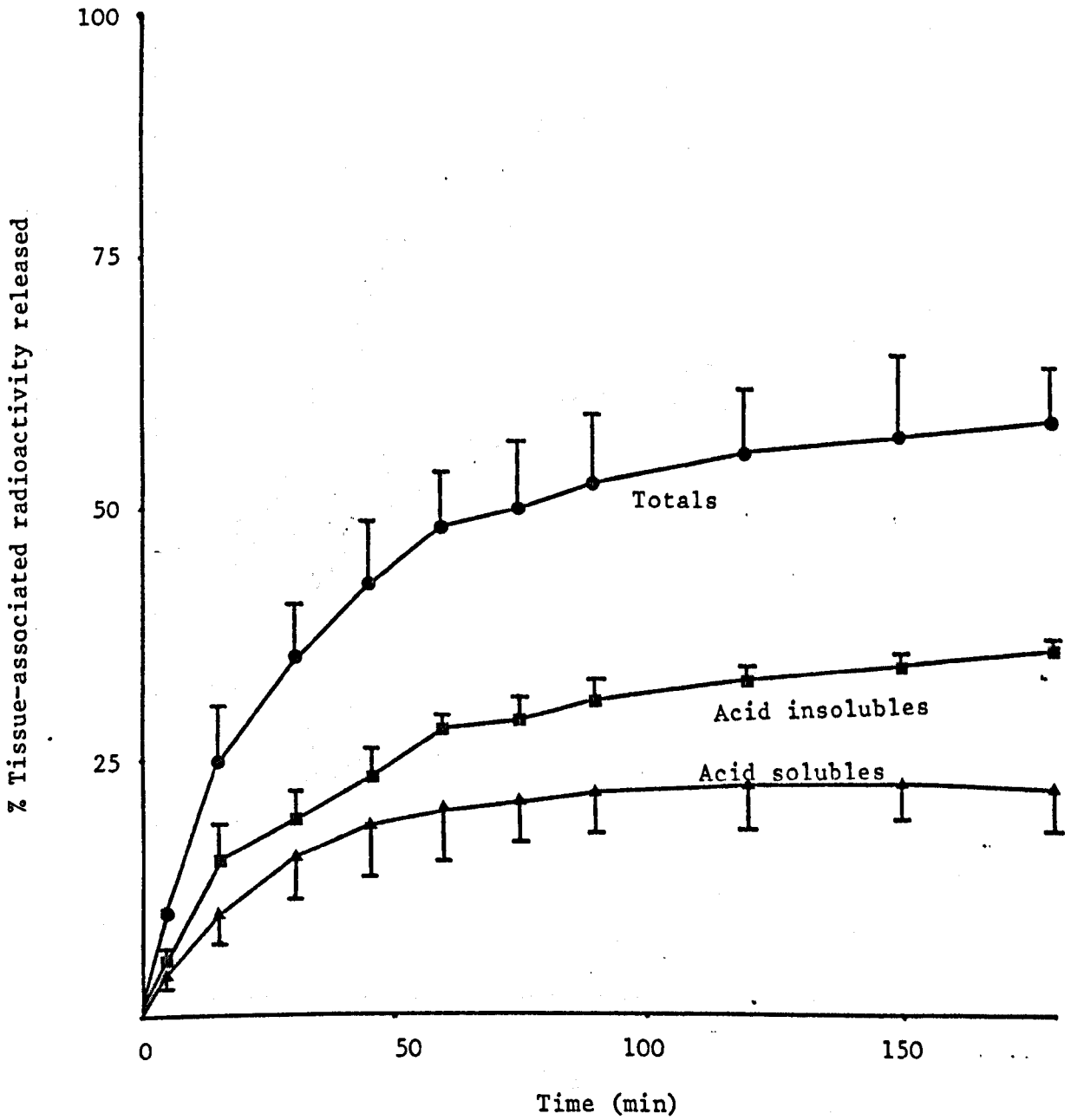


Fig. 7h Release of ^{125}I-labelled IgG (Batch IV) from 17.5-day yolk sacs re-incubated in TC 199 alone

(Same data as presented in Fig. 6e)

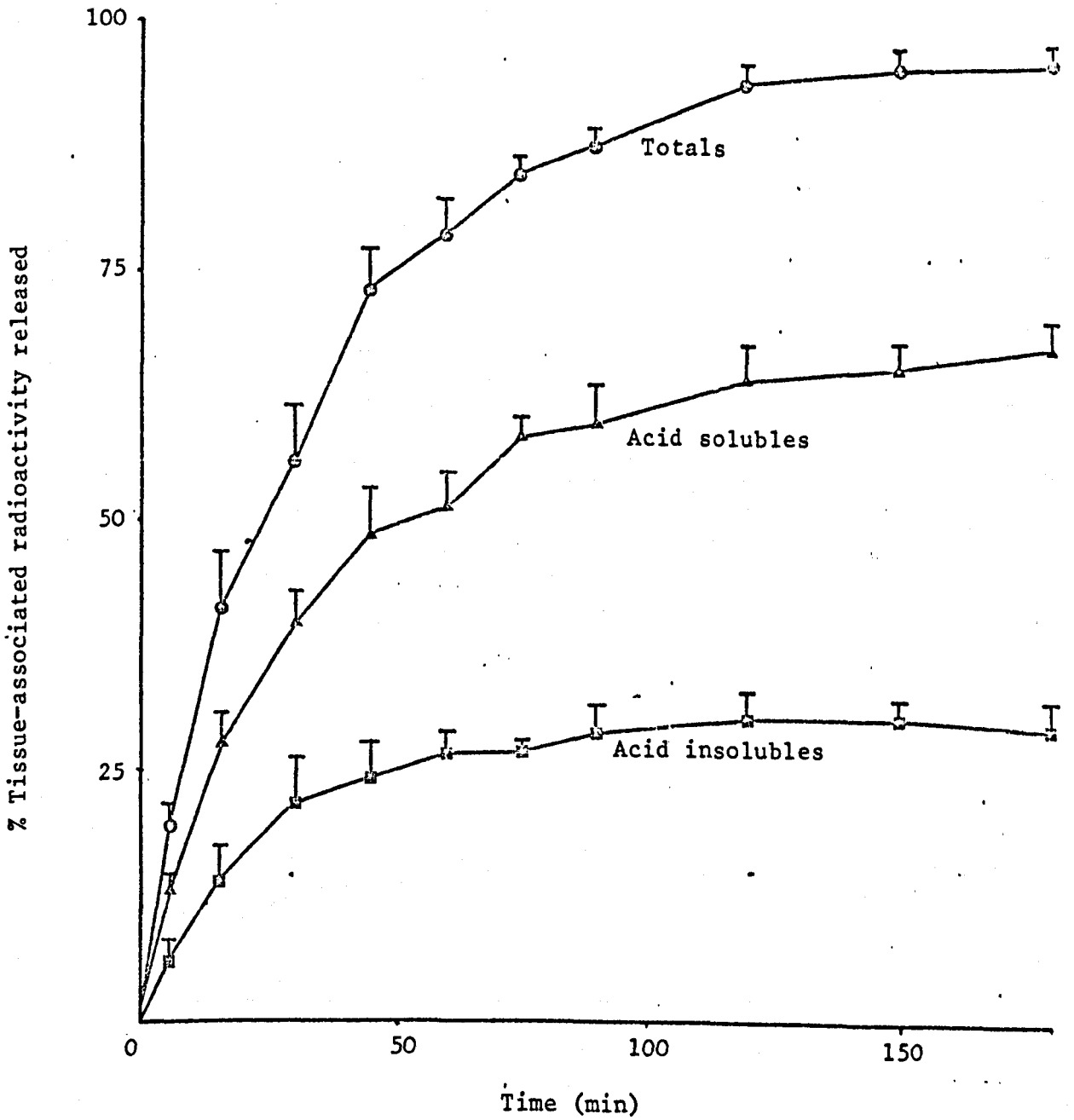
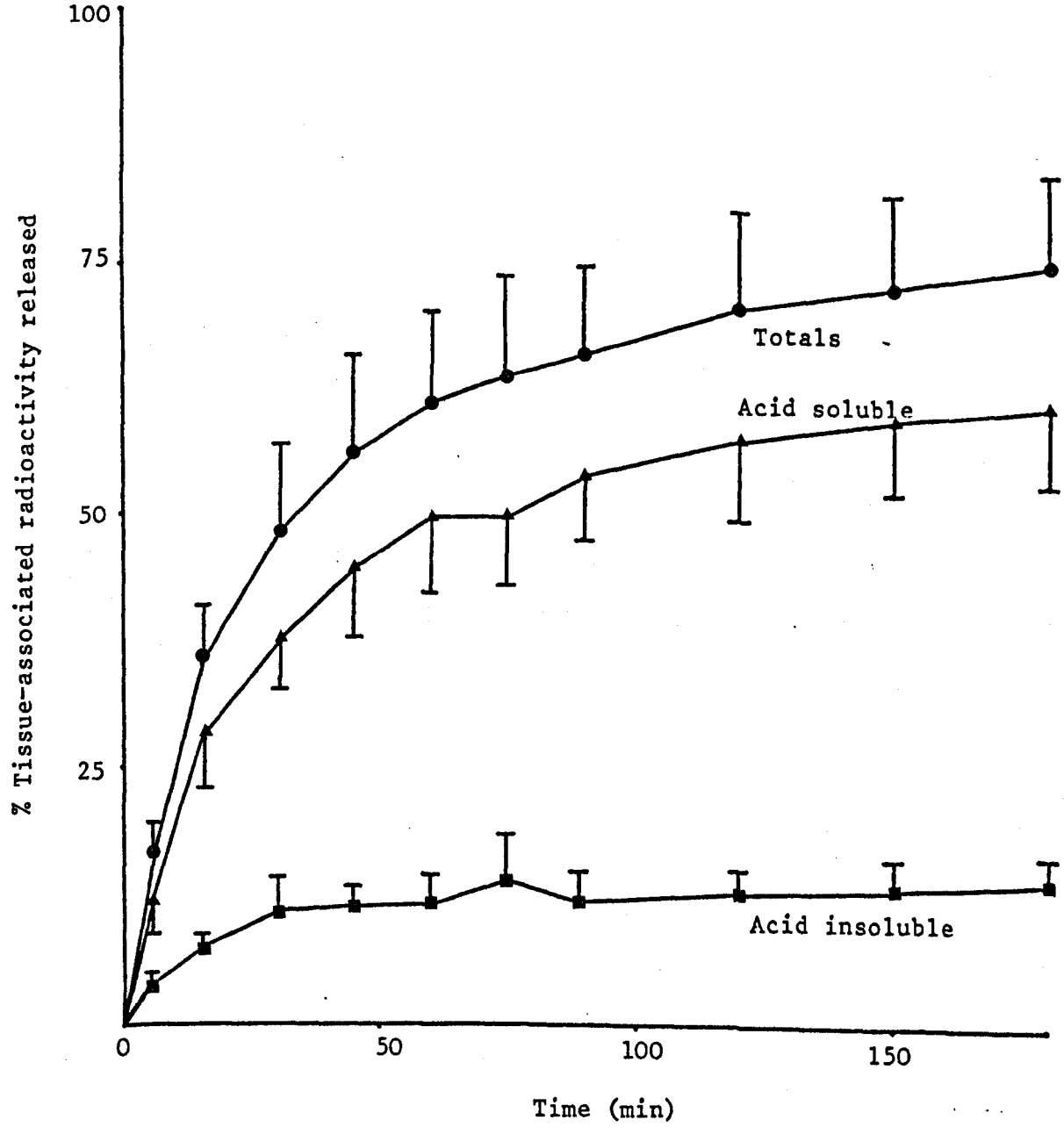


Fig. 7i Release of ^{125}I-labelled IgG (Batch IV) from 15.5-day rat yolk sacs re-incubated in medium 199 alone



CHAPTER 8

STUDY OF THE ROLE OF MEMBRANE PROTEINS OF THE RAT YOLK SAC

8.1 Introduction

The existence of protein molecules within a 'fluid' lipid bilayer (Singer, 1972) is now a generally accepted feature of current models for the structure of cellular membranes. The purpose of many of these proteins is to act as specific recognition or receptor sites for the binding of exogenous compounds. Subsequent to such binding one of a number of different events may occur. The proteins at such sites may be enzymic in nature and be responsible for effecting some change in the bound species, e.g. degradation. A second consequence is that the binding of the species (in this case a hormone) results in the transmission of a 'signal' across the membrane causing changes in the metabolic activity of the cell. One of the most well known examples of this type of effect is that shown by insulin. Alternatively, binding to the receptor site may result in phagocytosis or pinocytosis leading to:

(a) Degradation of the internalized substrate,

(b) Accumulation as in the case of non-digestible substrates,

or (c) Transmission across the cell, as in the case of IgG.

In previous chapters the binding of radiolabelled substrate to the plasma membrane (prior to pinocytosis) by the rat yolk sac maintained in culture has been one area of investigation. Further to these studies the experiments described in this chapter, although preliminary in nature, have been conducted in order to ascertain the role of membrane proteins in the uptake of substrates by pinocytosis. Also, the isolation of receptor proteins has been attempted by gel chromatography techniques.

A number of studies both in intact erythrocyte and isolated membranes (Winzler *et al.*, 1967; Tripplett and Carraway, 1972; Steck *et al.*, 1971) and similar studies on Sarcoma 180 tumour cells (Hunt and Brown, 1974; Huggins *et al.*, 1975) have also been conducted. In all these studies attention was focussed on the digestion of receptor sites with reference

to the released digestion products.

In this chapter mild proteolysis by trypsin was employed to study the role of receptors during pinocytosis. This investigation utilized ^{125}I-labelled PVP as a substrate which was shown in previous studies (Williams et al., 1975a and Chapter 3) to have an Endocytic Index which directly reflects the rate of pinocytic capture of fluid. Furthermore, the role of the receptor(s) as binding sites for proteins was followed by the rate of uptake of ^{125}I-labelled dBSA and ^{125}I-labelled IgG. A study of the digestion products released by trypsinisation was not undertaken. Although such information would be valuable in assessing the structural features of the receptor proteins in relation to the the processes of pinocytosis and substrate binding, it was first necessary to investigate the gross effect of trypsin on pinocytic events.

The second line of investigation involved the labelling of membrane proteins with ^{125}I[iodide] using lactoperoxidase and normal yolk sacs. The isolation of such proteins from the homogenized yolk sac by gel chromatography was attempted. Demonstration of the binding of the labelled receptors to protein substrates was also investigated by incubation of ^{125}I-labelled cell homogenate with BSA and IgG before chromatography and observing shifts in the elution patterns.

METHODS

8.2 Uptake of ^{125}I-labelled PVP, ^{125}I-labelled dBSA (Batch VI), and ^{125}I-labelled IgG by trypsinized 17.5-day rat yolk sacs

A total of 20 yolk sacs were removed from two 17.5-day pregnant rats and pooled before placing 10 in a 250 ml Erlenmeyer flask containing a solution (100 ml) of hog pancreas trypsin (0.5 lg/ml; Type IX, Sigma London) in medium 199. The remaining 10 yolk sacs were placed in a similar flask containing medium 199 alone. Both flasks were gassed with oxygen/carbon dioxide (95:5) for 30 sec and placed in a shaking water-bath at 100 strokes/min at 20°C. After 10 min the yolk sacs were removed from the water-bath and each transferred to a single flask containing medium 199 (8.0 ml) and calf serum (1.0 ml) gassed with O_2/CO_2 (95:5) for 10 secs and maintained at 37°C in a shaking water-bath at 100 strokes/min. Substrate was added after 20 min incubation in medium 199 (1.0 ml) to give final concentrations of 2.0 µg/ml for ^{125}I-labelled PVP and 1.0 µg/ml for both ^{125}I-labelled dBSA and ^{125}I-labelled IgG. Yolk sacs were removed at regular intervals up to 3.0h and washed three times (2 min each) in changes of cold saline (1.0% w/v, 4°C) and stored at -20°C until assayed.

Incubation medium and yolk sacs were assayed as described in Sections 2.6 and 2.4 respectively for incubations in which ^{125}I-labelled PVP was used as substrate, by the methods described in Section 2.4 and 2.7 when either ^{125}I-labelled dBSA or and ^{125}I-labelled IgG was used as substrate. The results were processed as described in Section 2.12 and 2.13 for non-digestible and digestible substrates, respectively.

In order to determine whether treatment with trypsin resulted in loss of cells by the yolk sac upon re-incubation, 1.0 ml samples of the incubation medium were removed from each flask, pooled and centrifuged at 1,000 g for 10 min to precipitate any detached cells. The supernatant was removed and the cells washed with phosphate-buffered

saline (0.1 M phosphate in 1.0% w/v saline; 5.0 ml) re-centrifuged at 1,000 g for 10 min and the supernatant removed. Finally the cells were re-suspended in phosphate-buffered saline and the volume adjusted to 0.5 ml. A small sample of the suspension was placed on a haemocytometer slide with improved Neubauer rulings (Weber and Sons, Lancing, Sussex) and whole cell count performed by the usual platelet counting method, under a phase contrast microscope. Cell integrity was judged by Nigrosin exclusion. From the remaining suspension two 200 µl samples were removed and assayed for protein content by the method of Lowry et al. (1951).

The rates of uptake of ^{125}I-labelled PVP, ^{125}I-labelled dBSA and ^{125}I-labelled IgG are shown in Tables 8.I, 8.II and 8.III, respectively, and further summarized in Table 8.IV.

8.3 Chromatography of ^{125}I-labelled membrane proteins of the rat yolk sac

The surface proteins of the epithelial cells on the uterine surface of the visceral yolk sac were labelled with [^{125}I]iodide using the method of Hunt and Brown (1975). A single intact conceptus was removed from a 20.5-day pregnant rat and ligatured around the yolk sac stalk, just under the placental cone, and suspended by the thread in 2.0 ml of phosphate-buffered saline (0.05 M in 1.0% (w/v) saline). The intact conceptus was suspended in the solution in order to minimize the contact between the solution and the placental cone and maximize contact between the visceral yolk sac and solution. The solution was stirred at room temperature and sodium [^{125}I]iodide (200 µCi) and lactoperoxidase (200 µg) were added to the solution. To the reaction mixture the oxidizing agent hydrogen peroxide (20 µl of 0.06%, v/v) was added at 2 min intervals until some 10 such aliquots had been added. The mixture was stirred for a further 10 min prior to the addition of KI (500 µg) to displace ^{125}I-iodide which was not tightly bound. The

conceptus was then removed from the flask and washed in phosphate-buffered saline containing KI (1.0 mg/ml). The yolk sac was then dissected free and homogenized in 2.0 ml of Tris/SDS buffer (Tris (hydroxymethyl) aminomethane, 0.05 M, in sodium dodecyl sulphate, 0.1% w/v, adjusted to pH 7.0 with 0.5 M H_2SO_4) using a Virtis homogenizer at speed setting 6 for 2 min.

The homogenate was centrifuged and an aliquot (0.5 ml) was applied to a column of Sephadex G-200 (57 x 1.7, cm) and eluted with Tris/SDS buffer at flow rate of 0.2 ml/min. The eluted radioactivity was assayed on a gamma scintillation counter and plotted against fraction number. Two 0.5 ml samples of the homogenate were incubated; one with homologous IgG (10 µg/ml) and the other with BSA (10.0 µg/ml) at 4°C in a shaking water-bath set at 100 strokes/min for 6.0 h. The samples were then applied separately to a column of Sephadex G-200 and eluted as described above. The three elution patterns are shown in Fig. 8a.

RESULTS

8.4 Uptake of substrates by trypsin treated yolk sacs

Table 8.IV shows a summary of the Endocytic Indices for ^{125}I-labelled PVP, ^{125}I-labelled dBSA and ^{125}I-labelled IgG obtained with yolk sacs which received prior treatment with trypsin (Section 8.2). In the case of each of the substrates the results show a fall in the Endocytic Index upon treatment with 0.5 µg/ml of trypsin prior to incubation. The data for ^{125}I-labelled PVP show the Endocytic Index to drop to 61.6% of that for non-treated yolk sacs. Similarly, with ^{125}I-labelled dBSA and ^{125}I-labelled IgG the Endocytic Indices dropped to 66.2% and 58.9% of the corresponding control values, respectively.

It is clear that trypsin treatment depresses the rate of fluid ingestion as reflected by the uptake of ^{125}I-labelled PVP. It is also evident that the rate of uptake of substrate ingested in the adsorbed phase is also lowered but this would seem to be as a result of an overall fall in the rate of pinosome formation rather than by specific alterations to the receptor sites.

For almost every experiment the number of detached cells and the amount of protein loss per yolk sac were both estimated. Generally trypsin treatment resulted in neither a greater detachment of cells nor a greater protein loss upon re-incubation compared to the non-trypsinised tissues.

8.5 Gel Chromatography of membrane proteins

Fig. 8a(i) shows the elution pattern on a Sephadex G-200 column of a centrifuged homogenate prepared from a ^{125}I-labelled 20.5-day rat yolk sac. Clearly evident are two major protein peaks followed by a third smaller peak. (This in turn is followed by a very large peak of radioactivity (fractions 55-75) corresponding to that of free [^{125}I] iodide.) Incubation of the yolk sac homogenate with native IgG

and BSA followed by chromatography resulted in the elution patterns shown in Fig. 8a(ii) and Fig. 8a(iii). The observed patterns are essentially the same as that for homogenate alone, except for a slight change in the size of the third peak is observed in the case of IgG. This peak would appear to be reduced in size compared to the other two elution patterns.

This observation is consistent with the binding of IgG to this membrane protein causing a fraction of it to elute at some earlier position between the void volume and the original position i.e. at a position corresponding to a complex of IgG and the membrane protein.

8.6 DISCUSSION

It should be noted that the studies presented in this chapter are of only a preliminary nature and further work must be undertaken in order to confirm and extend these findings. Nevertheless, the results collected showed a number of features worthy of discussion.

Trypsinization of yolk sacs was performed at 20°C, a temperature at which pinocytosis has virtually ceased (Duncan, unpublished data) in order to prevent internalization of the enzyme and the possible consequences of internal cellular damage. The results show that when ^{125}I-labelled PVP was used as a substrate, in order to follow any changes in the rate of fluid ingestion, trypsin treatment caused a fall in the Endocytic Index compared to the matched (non-trypsinized) control experiments. Control experiments demonstrated good recovery of the tissue when transferred from medium 199 at 20°C to freshmedium in incubation flasks at 37°C. Furthermore, experiments, in which the rate of uptake of ^{125}I-labelled PVP was measured at 37°C, showed a mean Endocytic Index of 1.7 $\pm$ 0.3 (S.D.) compared to 1.77 $\pm$ 0.23 (S.D.) for the trypsinization control experiments. These findings demonstrate that the mechanical and culture manipulations associated with the trypsinization process do not interfere with the pinocytic capacity of the tissue and that such changes which were observed are solely the result of the presence of trypsin during the enzyme treatment stage.

The Endocytic Index of ^{125}I-labelled PVP for trypsinized yolk sacs was reduced by approximately a third, reflecting a fall in the pinocytic activity of the tissue to this extent. Whether the reduced pinocytic activity was the result of digestion of the cell membrane proteins or because trypsin itself irreversibly inhibits pinocytosis cannot be judged from these results.

Similar experiments using ^{125}I-labelled dBSA and ^{125}I-labelled IgG showed equivalent percentage falls in the Endocytic Indices compared

to the appropriate control experiments. Considering these data together, it would seem that these changes in the Endocytic Index are due to an alteration in pinocytic activity of the tissue and that the binding process for these two proteins at least is unaffected. As a result of this action, trypsinization of this tissue may well be able to yield valuable data relating to the mechanisms involved in the pinocytic process itself rather than that of protein binding.

Obviously more work is required to confirm these findings and extension of the study to investigate the site of action of trypsin must be undertaken. Furthermore, analysis of the membrane protein fraction could yield valuable data on the effects of enzyme treatment of the yolk sac tissue. With this type of investigation in mind a method is described in this chapter using gel chromatography of the homogenized tissue. Gel electrophoresis methods similar to those described by Fairbanks et al., 1971; Triplett and Carraway, 1972; Phillips and Morrison, 1970; Hunt and Brown, 1974, 1975 were investigated as alternatives to gel filtration.

Further studies using other enzymic treatments would seem a logical extension of this work. Two other enzymes of potential interest are papain and neurominidase but unfortunately time did not allow work planned with the latter to be carried out. The digestion of yolk sacs with neurominidase may well result in interesting findings when the uptake and release of ^{125}I-labelled IgG are investigated by the methods already described in Chapter 5. It has been suggested (Sonda et al., 1973; Wild, 1975) that the glycocalyx may be involved in the binding of immunoglobulins prior to transport across the cell. Digestion of the glycocalyx with neurominidase which would result in the removal of the terminal sialic acids may well have profound effect on binding processes and transport. Studies of a similar nature (Cuatrecasas and Illiono, 1971) have demonstrated the role of sialic acid in the action of insulin

on adipose tissue, and similar studies (Pricer and Ashwell, 1971) using membranes from hepatocytes were able to show a relationship between de-sialation and protein binding.

Again it should be stressed that the studies reported are only preliminary and the lack of time prevented a more exhaustive investigation. I should therefore like to suggest a continuation of this investigation should involve attention to the following points.

1) Confirmation of existing data.

2) Monitoring of trypsin released digestion products.

3) Analysis of membrane proteins before and after trypsinization.

and 4) Use of other enzymes; for example neurominidase and papain.

In conjunction with the above studies a method of isolating the receptor sites by chromatography was used, similar to that employed by Dufau et al. (1973). The elution patterns from a Sephadex G-200 column are shown in Fig. 8a. It is evident that there are three major bands of activity probably corresponding to membrane proteins, although these bands could well represent exogenous proteins associated with the yolk sac which became iodinated during the labelling procedure. One such protein may well be lactoperoxidase itself. It is therefore essential that the elution pattern of this enzyme be established in further investigations.

Incubation of the yolk sac homogenate with BSA resulted in no change in the elution pattern but incubation with rat IgG resulted in a slight reduction in size of the smallest peak. This shift in peak size may be a result of binding between a receptor protein and IgG.

These preliminary studies would appear to demonstrate the presence of proteins in the yolk sac membranes and may well offer a method of studying substrate-receptor interactions especially those changes induced by enzymic treatments of the yolk sac. Unfortunately an alternative method employing polyacrylamide gel electrophoresis was unsuccessful

in isolating bands of radioactivity because of the small amount of sample (20 µl) applied to the gel. In order to make this method usable a much higher specific radioactivity would have to be achieved during the iodination of the tissue.

Table 8.I Endocytic Index of ^{125}I-labelled PVP in the presence and absence of trypsin treatment

Each row of data is for a single experiment, several of which included treatment of the yolk sacs with Trypsin 0.5 mg/ml for 10 mins at 20°C prior to incubation with the substrate. A number of experiments were also performed in the same manner except Trypsin was omitted from the medium (see Section 8.2 for method).

	Endocytic Index (µl/h/mg protein)	Correlation Coefficient	Total Cell Count	Percentage Protein loss
Without Trypsin	1.47	0.929	0.97×10^6	1.04
	2.00	0.960	0.93×10^6	1.00
	1.60	0.990	-	2.20
	1.80	0.977	0.98×10^6	1.46
With Trypsin	1.02	0.957	0.76×10^6	1.60
	0.93	0.968	0.74×10^6	0.10
	1.07	0.931	0.60×10^6	0.50
	1.00	0.980	- -	1.50
	1.26	0.991	0.70×10^6	1.94

Table 8.II Endocytic Index of ^{125}I-labelled dBSA (Batch VI) by trypsinized and non-trypsinized yolk sacs

Each row of data is for a single experiment, several of which included treatment of the yolk sacs with Trypsin 0.5 mg/mg for 10 mins at 20°C prior to incubation with the substrate. A number of experiments were also performed in the same manner except Trypsin was omitted from the medium (see Section 8.2 for method).

	Endocytic Index (µl/h/mg protein)	Correlation Coefficient	Total Cell Count	Percentage Protein loss
Without Trypsin	89.4	0.959	1.1×10^6	0.8
	42.0	0.969	0.5×10^6	0.9
	63.0	0.924	1.7×10^6	0.2
With Trypsin	41.7	0.957	1.44×10^6	0.9
	42.1	0.995	1.10×10^6	1.7
	45.0	0.952	0.80×10^6	0.1

Table 8.III. Endocytic Index of ^{125}I-labelled IgG (Batch III) by trypsinized and non-trypsinized yolk sacs

Each row of data is for a single experiment, several of which included treatment of the yolk sacs with Trypsin 0.5 mg/ml for 10 mins at 20°C prior to incubation with the substrates. A number of experiments were also performed in the same manner except Trypsin was omitted from the medium (see Section 8.2 for method).

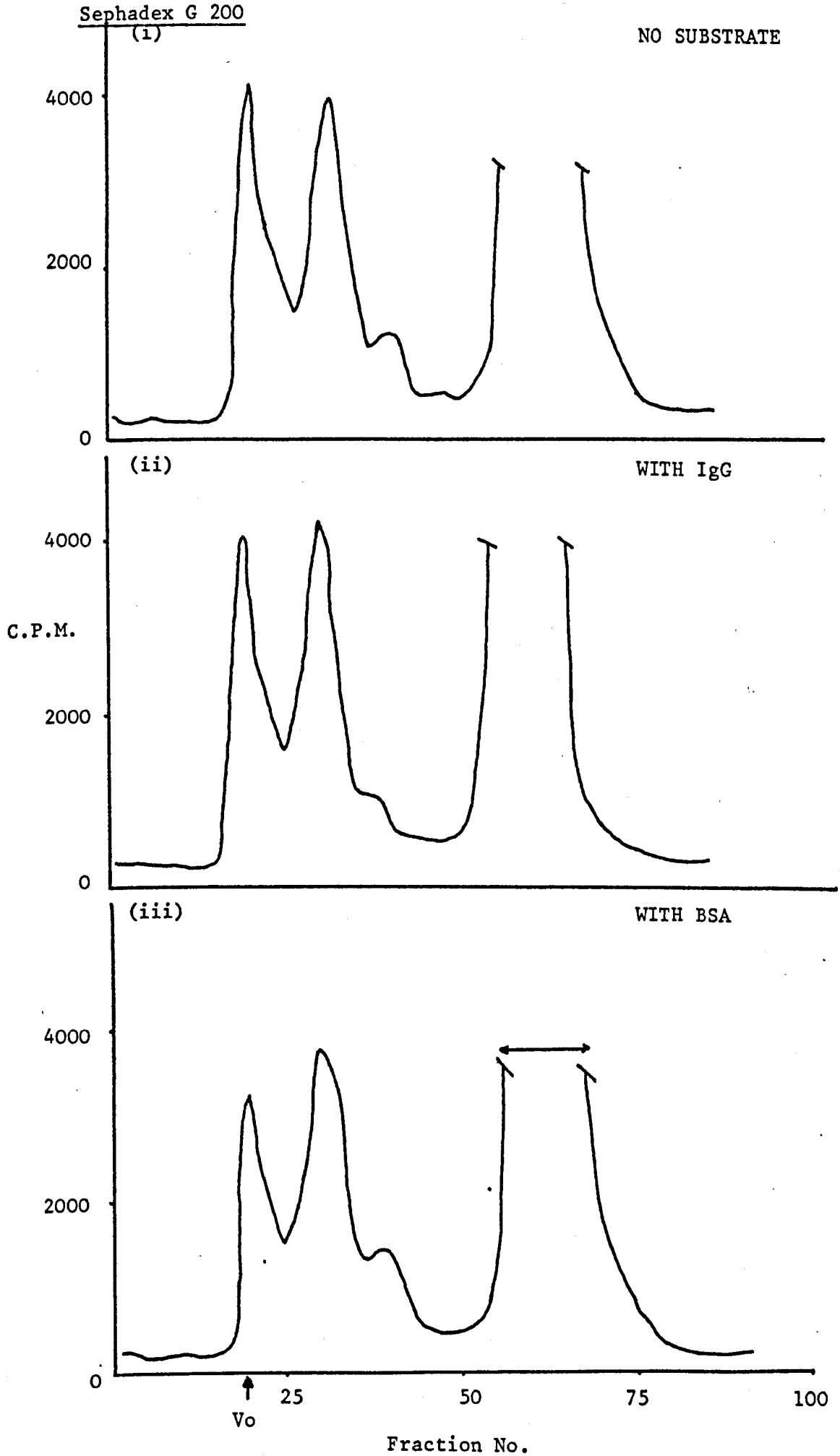
	Endocytic Index (µl/h/mg protein)	Correlation Coefficient	Total Cell Count	Percentage Protein loss
Without Trypsin	7.2	0.913	1.42×10^6	0.7
	5.6	0.887	- -	2.8
	9.0	0.990	0.8×10^6	1.0
With Trypsin	4.3	0.703	0.67×10^6	0.25
	2.2	0.406	- -	1.20
	6.5	0.476	1.02×10^6	1.55

Table 8.IV Summary of the Endocytic Index for ^{125}I-labelled PVP, ^{125}I-labelled dBSA and ^{125}I-labelled IgG for trypsinized and non-trypsinized yolk sacs

Summary of data from Tables 8.I, 8.II, and 8.III

Substrate	Mean ± S.D. Endocytic Index µl/h/mg protein	Number of Experiments	Trypsin Concentration (µg/ml)
^{125}I-labelled PVP	1.72 ± 0.23	4	0.0
	1.06 ± 0.13	5	0.5
^{125}I-labelled dBSA	64.8 ± 23.8	3	0.0
	42.9 ± 1.8	3	0.5
^{125}I-labelled IgG	7.3 ± 1.7	3	0.0
	4.3 ± 2.2	3	0.5

Fig. 8a Chromatography of homogenized ^{125}I-labelled rat yolk sac on Sephadex G 200



CHAPTER 9

GENERAL DISCUSSION

GENERAL DISCUSSION

The work reported in this thesis is based on the experimental technique for the *in vitro* culture of rat yolk sac, in medium 199 containing calf serum, developed by Williams *et al.* (1975a, b). This technique was modified (see Chapter 3) in order to study the ability of this tissue to ingest substrates in the absence of calf serum and thus simplify the analysis of the kinetics. These findings, in the absence of 10% (v/v) calf serum, indicated a fluid phase rate of ingestion of about 2.5 l/h/mg of protein as determined by the rate of ingestion of ^{125}I-labelled PVP. Williams *et al.* (1975b) and Moore (1977) showed the major route of uptake of ^{125}I-labelled bovine albumin preparations to involve adsorption to the plasma membrane; a finding which was confirmed in Chapter 3 for ^{125}I-labelled formaldehyde-denatured BSA. Moreover, some 99% of this substrate was found to enter the cell bound to the plasma membrane when yolk sacs were incubated in medium 199 alone. These findings are consistent with the mechanism proposed by Jacques (1969) for the uptake of substrates by pinocytosis. Furthermore, the presence of calf serum in the incubation medium lowered the value of the Endocytic Index of ^{125}I-labelled dBSA relative to that in the absence of calf serum; this observation is consistent with the existence of non-specific binding sites on the plasma membrane.

In Chapter 4 the value of the Endocytic Index of ^{125}I-labelled dBSA was studied as a function of the overall albumin concentration and was shown to fall with increased concentration indicating saturation of binding sites. Subsequent analysis of the kinetics enabled the parameters K, the substrate: binding-site dissociation constant, and SR, the maximal rate of ingestion, to be estimated. Unlike previous work by Gosselin (1966) and Normann (1974) the uptake of substrate by the adsorptive route was differentiated from fluid-phase ingestion, hence allowing the calculation of the constants for the adsorption

phase only without any arbitrary assumptions being made about the contribution from fluid phase ingestion.

Much work has been reported on the uptake of immunoglobulins by various mammalian tissues and in some cases the transport of intact IgG across such cellular barriers. Moreover, the transport of IgG has been postulated to depend on its binding to specific IgG receptors present on the cell surface (Brambell, 1966 and Wild, 1975). An attempt to investigate the existence of receptor sites for IgG by using competitive experiments, similar to those used for ^{125}I-labelled dBSA, resulted in only limited competition being observed. The failure of this pair of substrates to compete effectively probably resulted from two causes. Firstly, IgG-receptor specificity and secondly the release of macromolecular IgG from the yolk sac, which makes quantitative assessment of uptake via analysis of Endocytic Indices impossible.

Investigation of specific IgG-binding may well be approached by studying the amounts of ^{125}I-labelled IgG bound to the yolk sac at 4^{o}C, at which temperature pinocytosis is almost 100% inhibited (Duncan, unpublished data). Investigations of the interaction of IgG with tissues have been performed by Sonda and Schlamowitz (1972) who were able to demonstrate specific binding of IgG to rabbit yolk sac sections. Unfortunately this technique involved formaldehyde-fixation of the tissue which probably leads to damage of the receptor sites, making calculated parameters such as Km the substrate/receptor binding constant rather arbitrary and physiologically meaningless.

An interesting feature of the <u>in vitro</u> uptake of ^{125}I-labelled IgG, in the current study, was the accumulation of this substrate within the yolk-sac tissue when incubations were performed in the presence of calf serum. However, this effect was not observed in the absence of calf serum. In Chapter 5 it was suggested that the site of accumulation may be within the vitelline capillaries and that the absence of calf serum results in a loss of structural integrity of

these vessels, permitting the ^{125}I-labelled IgG to leak freely into the re-incubation medium. An alternative explanation (Chapter 6) was that the presence of substantial quantities of calf serum in the medium results in the simultaneous ingestion of large quantities of serum proteins along with the radiotracer. This may cause saturation of the lysosomal enzymes with serum proteins and cause a slower rate of degradation of the radiotracer hence lead to its accumulation. Unfortunately, the results reported in this thesis do not permit these two possible explanations to be differentiated, but it should prove possible to design experiments to decide conclusively whether the intracellular site of accumulation of ^{125}I-labelled IgG is the lysosomal system of epithelial cells or the vitelline capillaries. Isolation of a lysosome rich fraction, from yolk-sac tissue that has incubated with ^{125}I-labelled IgG in the presence or absence of 10% calf serum, by a differential centrifugation technique is one method by which intralysosomal accumulation may be investigated. By the use of autoradiographic techniques and sections of these same tissues, it should be possible to obtain evidence of accumulation within the vitelline vessels.

The observation that ^{125}I-IgG was released, apparently intact, from the yolk sac in the *in vitro* system is compatible with the *in vivo* findings of Brambell and Halliday (1956) who were able to demonstrate the pre-natal transmission of immunoglobulins in the rat. Although not ideal in design, the *in vitro* method of studying the release of ingested substrates provided an opportunity to probe certain facets of the mechanisms proposed for the transport of immunoglobulins (Brambell, 1966; Wild, 1975) and to establish the atypical character of the behaviour of homologous IgG in the system compared with all other proteins investigated to date. A number of test situations proposed in Chapter 6 were investigated in order to attempt to define the process

by which release of IgG occurs. Unfortunately the evidence was not wholly conclusive, but strongly supported the hypothesis advanced by Wild. A potential extension of this investigation is to attempt the isolation of vesicles of the class involved in the transport of immunoglobulins. A promising technique for this task is density gradient centrifugation. By this technique Mego and co-workers (1965, 1967) were able to isolate liver lysosomes and, in a later study, Goetze et al. (1976) using a similar technique isolated vesicles of various sizes from homogenized rat yolk sacs. Applications of a variation of this technique to yolk sacs which have previously ingested radiolabelled immunoglobulins may well produce conclusive evidence for or against the existence of two classes of vesicle within the rat yolk sac.

Studies of the physicochemical characteristics of the IgG receptor is a field open to further investigations. To this end preliminary studies were undertaken in Chapter 8 using trypsin in attempts to modify the binding sites responsible for the adsorptive uptake of proteins. Extending this study by the use of other enzymes may well be informative. Neuraminidase has been used as a tool to elucidate the role of certain carbohydrate moieties , especially sialic acid, in membrane binding phenomena and is an obvious choice in further studies of the binding of proteins to yolk sac membranes.

Finally, further work on the radiolabelling of the surface receptor proteins and subsequent gel chromatography of SDS solublized fractions is required in order to obtain conclusive results. The data so far gathered seem promising and the isolation of a particular receptor type, specific for immunoglobulins is not now beyond the realms of possibility.

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A P P E N D I X

The following computer programs were used to calculate the majority of the results presented in this thesis.

Program A Uptake of non digestible substrates by the rat yolk sac

Program B Uptake of digestible substrates by the rat yolk sac

Program C Uptake of [$U^{14}C$] sucrose by the rat yolk sac

Program D Release of non digestible radiotracers from the rat yolk sac

Program E Release of digestible radiotracers from the rat yolk sac

Program A

```
5 REM KEW 125-IPVP PROGRAM,MODIFIED NOV.,1974
10 DIM H(15),I(15),K(15),L(15),R(15)
12 IF W=1 THEN 84
13 LET W=1
16 PRINT "NON-DIGESTIBLE PROG. ENTERED"
17 PRINT "EXPT. NO. (DIGITS ONLY)=";
18 INPUT Z
19 PRINT "BACKGROUND IN CPM=";
20 INPUT A
35 PRINT "COUNTING TIME FOR EACH ML OF MEDIUM,SECS=";
40 INPUT C
45 PRINT "COUNTING TIME FOR EACH ML OF YS SOLUTION,SECS=";
50 INPUT D
75 PRINT "NO. OF POINTS IN PLOT=";
80 INPUT G
82 STOP
84 LET W=0
95 FOR X=1 TO G
98 INPUT H(X),I(X),K(X),L(X)
99 NEXT X
100 FOR X=1 TO G
101 LET M=(I(X)*60/C)-A
135 LET Q=((K(X)*60/D)-A)*5
136 LET N=M+Q/20
140 LET R(X)=(Q*1000)/(N*L(X))
145 PRINT FRE(5);H(X),FRE(6);R(X)
150 NEXT X
152 PRINT FRE(5);-1,FRE(6);Z
155 ORSPEC TO OWN
157 PRINT " "
158 PRINT " "
160 PRINT "INCUBATION TIME(HOURS)",
          "PROTEIN IN YS","    UPTAKE"
165 PRINT
190 FOR X=1 TO G
195 PRINT "      ",FRE(5);H(X),FRE(6);L(X),FRE(6);R(X)
200 NEXT X
205 STOP
```

Program B

```
1 REM PROTEIN PROGRAM ,REVISED OUTPUT ,NOV. 1974
10 DIM E(12),H(12),I(12),J(12),K(12),L(12),S(12)
12 IF W=1 THEN 84
13 LET W=1
14 PRINT "PROTEIN PROGRAM ENTERED"
15 PRINT "EXPT. NO. (DIGITS ONLY)=";
16 INPUT V
19 PRINT "BACKGROUND IN CPM=";
20 INPUT A
25 PRINT "PERCENT SOLUBLES IN PREP=";
30 INPUT B
35 PRINT "COUNTING TIME MEDIUM TOTALS,SECS=";
40 INPUT C
41 PRINT "COUNTING TIME MEDIUM SOLUBLES,SECS=";
42 INPUT Z
45 PRINT "YS COUNTING TIME,SECS=";
50 INPUT D
60 PRINT "CORRECTION FACTOR FOR MEDIUM TOTALS=";
65 INPUT Y
67 PRINT "CORRECTION FACTOR FOR MEDIUM SOLUBLES=";
68 INPUT R
75 PRINT "NO. OF POINTS IN PLOT=";
80 INPUT G
82 STOP
84 LET W=0
95 FOR X=1 TO G
98 INPUT H(X),I(X),J(X),K(X),L(X)
99 NEXT X
100 FOR X=1 TO G
101 LET M=(I(X)*60/C-A)*Y
102 LET N=(J(X)*60/Z-A)*R
105 LET O=N-(M*B/100)
135 LET Q=((K(X)*60/D)-A)*5
136 LET P=(M-N)+O/2
137 LET F=((10*O)+Q)*1000
138 LET E(X)=(Q*1000)/(L(X)*P)
139 LET S(X)=F/(L(X)*P)
140 PRINT FRE (5);H(X),FRE(6);S(X)
150 NEXT X
151 PRINT FRE(5);-1,FRE(6);V
152 DRSPEC TO OWN
153 PRINT " "
```

```
154 PRINT " "
155 PRINT " INCUBATION ","PROTEIN",
          "MICROLITRES","   UPTAKE"
160 PRINT "TIME (HOURS)"," IN YS "," PER MG YS "
190 FOR X=1 TO G
195 PRINT FRE(5);H(X),FRE(6);
196 PRINT L(X),FRE(6);E(X),FRE(6);S(X)
200 NEXT X
205 STOP
```

Program C

```
5 REM 14-C PROGRAM REVISED FORMAT,FEB 1977
10 DIM J(20),H(20),I(20),K(20),L(20),R(20),M(20)
11 IF W=1 THEN 84
12 LET W=1
13 PRINT "KEN'S SUCROSE PROGRAM ENTERED"
14 PRINT " N.B.-ALL COUNTS ENTERED IN
           IN PROGRAM ARE ASSUMED TO"
15 PRINT "BE C.P.M. (UNCORRD. FOR B.G. )
          PER ML SOLUTION"
16 PRINT "     "
17 PRINT "EXPT. NO. (DIGITS ONLY)=";
18 INPUT V
19 PRINT "BACKGROUND IN CPM=";
20 INPUT A
65 PRINT "MEAN COUNT OF SPIKED 1.0
          ML WATER BLANK,C.P.M.=";
70 INPUT F
75 PRINT " NO. OF POINTS IN PLOT=";
80 INPUT G
81 PRINT " "
82 STOP
84 LET W=0
```

```
95 FOR X=1 TO G
98 INPUT J(X),H(X),I(X),K(X),L(X),R(X)
99 NEXT X
100 FOR X=1 TO G
101 LET N=(H(X)-A)
105 LET O=(I(X)-A)
110 LET P=(O-N)
115 LET Q=N*F/P
120 LET S=(K(X)-A)
125 LET T=(L(X)-A)
130 LET Y=(T-S)
135 LET Z =S*F/Y
140 LET U=Q+(5*Z/20)
142 LET M(X)=5*Z*1000/(U*R(X))
145 PRINT FRE (5);J(X),FRE(6);M(X)
150 NEXT X
153 PRINT FRE(5);-1,FRE(6);V
155 DRSPEC TO OWN
156 PRINT " "
157 PRINT " "
160 PRINT "INCUBATION TIME'(HOURS)",
          "PROTEIN IN YS","    UPTAKE"
190 FOR X=1 TO G
195 PRINT "     ",J(X),R(X),M(X)
200 NEXT X
205 STOP
```

Program D

```
 5 REM EXOCYTOSIS PROGRAM , NOV. 1976
 10 DIM P(30),Q(30),R(30),T(30),U(30)
 11 DIM V(30)
 12 IF W=1 THEN 56
 14 LET W=1
 16 PRINT "EXOCYTOSIS PROGRAM ENTERED"
 18 PRINT "(PROGRAM ASSUMES 1.0 ML SAMPLES"
 19 PRINT "TAKEN AT EACH TIME INTERVAL )"
 20 PRINT "EXPT. NO. (DIGITS ONLY)=";
 22 INPUT A
 24 PRINT "TOTAL VOLUME OF MEDIUM,ML=";
 26 INPUT B
 28 PRINT "NO. OF SAMPLES TAKEN AT EACH TIME INTERVAL=";
```

```
30 INPUT K
32 PRINT "COUNTING TIME,SEC=";
34 INPUT C
36 PRINT "BACKGROUND ON COUNTER , C.P.M. =";
38 INPUT D
40 PRINT "DENOMINATOR (CPM PER ML,CORRD.BG)=";
42 INPUT L
44 PRINT "CORRECTION FACTOR=";
46 INPUT M
47 PRINT "NO. OF TIMES SAMPLES WERE TAKEN=";
48 INPUT E
49 PRINT "CORR CPM. IN 5ML YS. SOLN=";
50 INPUT C1
52 PRINT "  "
53 PRINT
54 LET S=0
55 STOP
56 PRINT "SERIAL NO","  TIME OF ",
         "ACTIVITY ","ACTIVITY     ",
         "% TOTAL"
57 PRINT "OF SAMPLE","SAMPLING ",
         "RELEASED  ","RELEASED     ",
         "ACTIVITY"
65 LET W=0
66 FOR X=1 TO E
68 INPUT P(X),Q(X),R(X)
70 NEXT X
72 FOR X=1 TO E
74 LET F=((R(X)*60/C)-D)*M
76 LET S=S+K*F
78 LET T(X)=S+((B-K)*F)
80 LET U(X)=(T(X)/L)*1000
86 NEXT X
88 LET S1 = T(E) + C1
90 FOR X=1 TO E
95 LET V(X) = T(X) / S1 * 100.0
100 NEXT X
105 FOR X=1 TO E
110 PRINT FRE(5);P(X),FRE(5);
111 PRINT Q(X),FRE(6);T(X),FRE(5);
115 PRINT U(X),FRE(5);V(X)
120 NEXT X
125 STOP
```

Program E

```
100 REM EXOCYTOSIS PROGRAM ,DEC. 1976
200 DIM C(2),M(2)
300 DIM P(20),Q(20),R(20,2),T(20),U(20)
400 DIM V(20)
500 IF W=1 THEN 3450
600 LET W=1
700 PRINT "EXOCYTOSIS PROGRAM ENTERED"
800 PRINT "TOTAL VOLUME OF MEDIUM,ML=";
900 INPUT B
1000 PRINT "NO. OF SAMPLES IN EACH TIME INTERVAL=";
1100 INPUT K
1200 PRINT "BACKGROUND=";
1300 INPUT D
1400 PRINT "DENOMINATOR=";
1500 INPUT L
1600 PRINT "NO. OF TIMES SAMPLES WERE TAKEN=";
1700 INPUT E
1800 PRINT "CORR CPM. IN 5ML YS. SOLN=";
1900 INPUT C1
2000 FOR I=1 TO 2
2100 PRINT "DATA FOR ";
2200 IF I=2 THEN 2600
2300 PRINT "TOTALS";
2400 GOTO 2700
2600 PRINT "SOLUBLES";
2700 PRINT
2800 PRINT "COUNTING TIME,SECS=";
2900 INPUT C(I)
3000 PRINT "CORRECTION FACTOR=";
3100 INPUT M(I)
3200 NEXT I
3300 LET S=0
3400 STOP
3450 PRINT
3460 PRINT
3500 PRINT "SERIAL NO"," TIME OF ",
           "ACTIVITY ","ACTIVITY    ",
           "% TOTAL"
3600 PRINT "OF SAMPLE","SAMPLING ",
           "RELEASED  ","RELEASED     ",
           "ACTIVITY"
```

```
3700 PRINT
3800 LET W=0
4000 FOR X=1 TO E
4100 INPUT P(X),Q(X),R(X,1),R(X,2)
4200 NEXT X
4300 FOR I=1 TO 2
4350 LET S=0
4400 FOR X=1 TO E
4500 LET F=((R(X,I)*60.0/C(I))-D)*M(I)
4600 LET S=S+K*F
4700 LET T(X)=S+((B-K)*F)
4800 LET U(X)=(T(X)/L)*1000
4900 NEXT X
5000 IF I<>1 THEN 5200
5100 LET S1 = T(E) + C1
5200 FOR X=1 TO E
5300 LET V(X) = T(X) / S1 * 100.0
5400 NEXT X
5900 FOR X=1 TO E
6000 PRINT FRE(5);P(X),FRE(5);
6050 PRINT Q(X),FRE(6);T(X),FRE(5);
6100 PRINT U(X),FRE(5);V(X)
6200 NEXT X
6300 PRINT
6400 PRINT
6500 NEXT I
6600 STOP
```