PROTEIN AND PEPTIDE CATABOLISM

WITHIN

THE RAT YOLK SAC

by

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ABSTRACT

Radio-labelled forms of ribonuclease, lysozyme and insulin were shown to be ingested by 17.5-day rat yolk sacs almost exclusively by adsorptive pinocytosis and were rapidly degraded by the tissue to the level of free amino acids. The rates of pinocytosis were shown to decrease markedly on treating these proteins with alkaline formaldehyde but not other denaturing reagents. Aggregates of radio-labelled lysozyme were ingested more slowly than the monomeric protein. Formaldehyde-denatured bovine serum albumin and ribonuclease were shown to adsorb to differing regions of the pinocytosing plasma membrane; such binding is discussed in terms of basic and hydrophobic regions on these protein molecules.

Radio-labelled bovine serum albumin was shown to be digested exclusively intralysosomally. The effects on this process of chloroquine, ammonium- and substituted ammonium ions were investigated; each was shown to inhibit both intralysosomal proteolysis and the rate of pinosome formation. In contrast a number of proteinase inhibitors (leupeptin, chymostatin, antipain, bestatin, elastatinal and pepstatin) were without effect on pinosome formation but several markedly inhibited intralysosomal digestion.

When radio-labelled forms of a number of proteins (formaldehyde--denatured albumin, rat immunoglobulin G, ribonuclease and lysozyme) and peptides (insulin, calcitonin, insulin B-chain and glucagon) were exposed to yolk-sac tissue, each showed a characteristic "lag-period" before digestion products appeared in the incubation medium. The durations of such lag-periods were shown to correlate well with the susceptibility of individual substrates to degradation by both disrupted rat-liver tritosomes and cell-free homogenates of yolk sacs. An attempt was made

to relate the effects of inhibitors of lysosomal proteolysis on exogenous protein breakdown to their effects on the breakdown of endogenous [3H]-leucine-labelled yolk-sac proteins to discover whether both groups of proteins are degraded intralysosomally. (This study was made in collaboration with S. Knowles and F.J. Ballard, CSIRO, Adelaide.)

CONTENTS

ABBREVIATIONS

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

EI	Endocytic Index.
CI	Catabolic Index.
Y.S.	yolk sac.
r.p.m.	revolutions per minute.
^{125}I-PVP	^{125}I-labelled poly(vinylpyrrolidone).
db-cAMP	dibutyryl cyclic AMP.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Preface

The ability of certain cells to ingest molecules, colloidal substances (0.2-20nm dia.) and particles of bacterial size (0.20μm dia.) that are unable to penetrate cytomembranes by diffusion or active transport is now well recognised and has been the subject of numerous reviews, most of which have appeared during the last decade (Mudd et al., 1934; Schechtman, 1956; Holter, 1959; Ryser, 1968; Jacques, 1969; Stockem & Wohlfarth-Botterman, 1969; North, 1970; Ryser 1970; Gordon, 1973; Chapman-Andresen, 1973; Simpson & Spicer, 1973; Allison & Davies. 1974; Stossel, 1974; Jacques, 1975; Stossel & Cohn, 1976; Chapman-Andresen, 1977; Stossel, 1977; Silverstein et al., 1977). The ingestion process (endocytosis) involves microscopic movements in the plasma membrane, the extracellular substrate being taken into the cell by either membrane invagination or evagination to encage the prey. In both cases membrane fusion occurs at the lips of the newly formed plasma membrane cavity to form free, membrane-limited vesicles within the cytoplasm of the cell. Since these vesicles usually fuse with lysosomes, a much reviewed organelle (see de Duve, 1959; Weissman, 1965; de Duve & Wattiaux, 1966; CIBA Foundation Symposium, 1963; Lysosomes in Biology and Pathology, 1969a,b, 1973, 1975, 1976; Holtzman, 1976; Dean & Barrett, 1976, see also Tissue Proteinases, 1977; Proteinases in Mammalian Cells and Tissue, 1977), digestible prey, e.g. serum proteins or peptides, can be catabolized following uptake via this route. Cell-proteins, however, are probably also digested by other routes (see Schimke & Doyle, 1970; Goldberg & Dice, 1974; Goldberg & St. John, 1976; Ballard, 1977 for reviews on this subject, see also Turk & Marks, 1977) but it is emerging that lysosomes might also be involved in the digestion of substrate captured by

autophagy. This thesis reports investigations of aspects of endocytosis in relation to the catabolism of proteins and peptides in the 17.5-day rat visceral yolk sac and (in collaboration with S. Knowles & F.J. Ballard, CSIRO, Division of Human Nutrition, Adelaide, Australia) the possible role of lysosomes in cell-protein catabolism. Before proceeding to these, however, it is appropriate to describe some of those studies which have contributed to the formulation of the current concepts of endocytosis and lysosomal proteolysis and to describe the structure and activities of the rat yolk sac that are relevant to its endocytic activity.

1.2 The concepts of endocytosis and intralysosomal proteolysis.

Uptake of erythrocytes, bacteria and non-living particles by leucocytes was observed with the light microscope by several workers including Metchnikoff (1883), who in 1892 used the term 'phagocytosis' to describe the process. The possibility of endocytosis at a sub-microscopic level was envisaged by Meltzer (1904) in his hypothesis of fluid uptake and passage through cells ("photocytosis") as an explanation of oedema in mammalian tissues; a phenomenon that was later demonstrated by electron microscopy (Palade, 1953). The physiological importance of the uptake of dissolved macromolecules was first appreciated by Lewis (1931) who suggested that: "By pinocytosis the cells are able to take in substances which cannot diffuse into them or be taken up by ordinary phagocytosis." However, uptake of soluble proteins by non-phagocytic cells in multicellular organisms was still questioned by Schechtman (1956): "... until we have definite evidence that such elements as hepatic cells, lymphocytes, heart myocardial cells, renal tubule epithelium, and ovarian eggs of birds carry on some form of pinocytosis, we can hardly

accord this process a role of general significance in macromolecular uptake". Nevertheless, the universality of pinocytosis became immediately apparent following electron microscopic observations made by Palade (1953, 1956) and Bennett (1956). In 1958, Holter and Holtzer, introduced the technique of following pinocytosis with fluorescein-labelled proteins and observed under the light microscope the ingestion of such proteins by cells in a wide variety of mammalian tissues. Straus (1961) made similar observations with the foreign enzyme horseradish peroxidase. Straus (1962), using histochemical methods, later observed that the peroxidase initially appeared in small phagosomes [a term coined by Straus (1959) to describe the vesicles which contained the endocytosed material] but later became concentrated in large phagosomes. Electron microscopic evidence using the electron opaque proteins ferritin (Caufield, 1963) and haemoglobin (Miller, 1960) gave similar results. Using histochemical methods, Straus (1964) elegantly demonstrated the fusion of peroxidase containing phagosomes with lysosomes [a particle discovered by de Duve (see de Duve, 1969) and now known to contain many hydrolytic enzymes]. Straus called the resultant particle a phagolysosome. In the meantime, work on leucocytes (Cohn & Hirsch, 1960a,b; Hirsch & Cohn, 1960; Zucker-Franklin & Hirsch, 1964) and later on macrophages (Cohn & Wiener, 1963a,b) identified the process whereby lysosomes merge with endocytic vacuoles and supply them with digestive enzymes. These observations gave a basis to the long inferred belief that endocytosed substrates are digested after being absorbed into the cell, an assumption that was made much earlier by Lewis (1931) to explain the disappearance of pinocytic vesicles that occurred after he had observed them travel toward the nucleus by time-lapse photography.

Straus (1964) announced his evidence for phagosome-lysosome

fusion, at about the same time that Mego & McQueen (1965a) demonstrated the digestion of ^{131}I-labelled albumin within particulate cell fractions isolated from the livers of adult mice that had been previously injected (i.v.) with the radiolabelled protein. Shortly afterwards (1965b) they indicated the digestive particles to be lysosomes, a point which Mego et al. (1967) established. The trichloroacetic acid-soluble digestion product released from the particulate fraction was found to be [^{131}]iodo-L-tyrosine (Mego et al., 1967). Radioiodinated L-tyrosine was also identified as the label appearing in the culture medium of mouse macrophages (Ehrenreich & Cohn, 1967, 1968; Unanue & Askonas, 1968) and sarcoma S180 cells (Gabathuler & Ryser, 1969) when radio-iodinated protein was digested after endocytosis. In vitro, lysosomal enzymes were shown to digest proteins to amino acids and oligopeptides, mainly dipeptides (Coffey & de Duve, 1968) and, in intact lysosomes, the lysosomal membrane was found to be permeable to amino acids and oligopeptides less than 200-240 molecular weight (Ehrenreich & Cohn, 1969; Lloyd, 1971).

1.3 The role of lysosomes in cell-protein catabolism.

The role of lysosomes in the catabolism of endocytosed proteins is now well established but the role of lysosomes in cell-protein turnover is not clear. Nevertheless several authors have postulated such a role (Gordon, 1973; Wibo & Poole, 1974; Dean, 1975a,b; Segal, 1976, Lloyd, 1976; Ballard, 1977) and Dean (1978) has even provided a hypothetical mechanism that would permit the selective catabolism of certain soluble cell-proteins by the invagination of the lysosomal membrane, a process that resembles pinocytosis. Such invaginations have been observed in mouse peritoneal macrophages treated with chloroquine (Fedorko et al., 1968a). Additional evidence for a lysosomal role in cell-protein breakdown [in addition to inhibition of lysosomal activity by weak

bases (see Chapter 5) and microbial proteinase inhibitors (see Chapter 6)] is as follows.

Stimulation of autophagy in rat liver can be achieved by the administration of glucagon. In such conditions electron micrographs of hepatocytes showed (de Duve & Wattiaux, 1966) autophagic vacuoles fusing with lysosomes. Neely et al. (1974) showed that in liver perfusion studies increased numbers of autophagosome-like bodies were found in hepatocytes and increased rates of protein breakdown were observed in isolated lysosomes. A demonstration of a rapid increase in the number of autophagosomes and of secondary lysosomes in cells exposed to an acute nutritional step-down (Mitchener et al., 1976) supports a lysosomal role in cell-proteolysis under conditions of accelerated catabolism. Amenta et al. (1977) showed that, when added to rat embryo fibroblasts in culture, microtubular poisons, which disorganise the vacuolar apparatus (Hoffstein et al., 1977) are inhibitors of the enhanced protein degradation induced by a nutritional step-down. Knowles & Ballard (1976) observed that the degradation of normal proteins in Reuber H35 hepatoma cells is between 28-44% inhibited by optimal concentrations of insulin, puromycin, cycloheximide, fetal calf serum, NH_4Cl, leupeptin, antipain and 7-amino-1-chloro-3-tosylamido-heptan-2-one. Combinations of the inhibitors present at optimal concentrations did not have additive effects also the inhibitors had no effect on the digestion of canavanine containing (i.e. short-lived) proteins. To explain their results it was proposed that the inhibitors completely inactivate a proteolytic pathway. The inactive pathway in cells incubated in the presence of insulin or serum became activated on nutritional step-down caused by the removal of these substances from the culture medium; suggesting that it may be an autophago-lysosomal pathway that is responsible for the digestion of long-lived proteins. An alternative

pathway, possibly not involving lysosomes, was suggested for the short lived proteins. Amenta et al. (1977) made similar proposals of a dual pathway in cell protein catabolism. More recently, Ward et al. (1977) provided evidence for the uptake of intracellular proteins by lysosomes during insulin and amino acid deprivation. Other factors involved in the regulation of cell-protein catabolism have been reviewed by Schimke & Doyle (1970), Goldberg & Dice (1974), Goldberg & St. John (1976) and Ballard (1977), only the last reviewer discusses a dual pathway of cell-protein catabolism.

For a further clarification of the role of lysosomes in cell--protein breakdown there is a need for specific inhibition of lysosomal function and an understanding of the inhibition process. Experiments aimed at answering questions in this area form the subject matter of the latter half of this thesis.

1.4 Morphology of endocytosis and the origin of intracellular vacuoles.

The simplest classification of endocytosis is a morphological one (see Allison & Davies, 1974) on the basis of the size of vesicle formed, its fate and its contents. Phagocytosis results in the entrapment of either solid or semi-solid particles within a vacuole formed by evaginations of the surface membrane that flow over the particle and fuse. The phagosome membrane adheres closely to the edocytosed particle so that the latter determines the size (usually about 1μm dia. or larger) and shape of the membrane-limited vacuole. Such vacuoles usually fuse with lysosomes.

Pinocytosis, by a process of membrane invagination and fusion results in the entrapment of fluid within a pinosome (pinocytic vesicle or phagosome). Micropinocytosis (observable by electronmicroscopy) and

macropinocytosis (observable by phase-contrast microscopy) have been described and result in the formation of micropinosomes (70-110nm dia.) and macropinosomes (0.2-1μm dia.). A specialized vesicle, called a coated micropinocytic vesicle has also been described, it is formed from dense caveolae (Fawcett, 1965) that further invaginate to develop into mature vesicles containing a condensed layer of glycocalyx. Roth & Porter (1964) suggested the glycocalyx may play an important role in the selective uptake of intact molecules. The detailed morphology of the coated micropinocytic vesicle is discussed by Ockleford (1976).

Micropinocytosis can take at least two main forms. One, called diacytosis, is classically seen in capillaries (Bruns & Palade, 1968). It is principally associated with the passage of fluid, in vesicles with a smooth inner surface, through cells by a process of endocytosis and exocytosis. The second form of pinocytosis is one in which the vesicles, often with an inner coat of mucopolysaccharide (Bennett, 1963), are delivered to and fuse with lysosomes where the endocytosed material is usually either digested or stored depending upon its susceptibility to attack by the lysosomal hydrolases. Coated micropinocytic vesicles can either release their contents at an opposing cell surface (Moxon et al., 1976) or empty their contents into lysosomes (Anderson et al., 1976, 1977). The contents of pinocytic vesicles have also been observed to be discharged into the cytoplasm by either vesicle rupture or dissolution (Wohlfarth-Bottermann, 1960; Casely-Smith, 1965; Florey, 1967; Bessis & Breton-Gorius, 1957, 1959). However, this process appears to be a rare event. In the absence of results that can be more confidently interpreted, it must be assumed not to be a process involved in normal cell physiology but to be the result of either some micropathological event or an artifact arising from the preparation of cells and tissues for ultrastructural

examination.

Not all cell vacuoles/vesicles arise from the plasma membrane. Primary lysosomes belong to a postulated GERL complex; Golgi-associated endoplasmic reticulum and lysosomes (Novikoff et al., 1971). Secretory vacuoles also arise from the Golgi apparatus. Fusion may occur between secretory vacuoles and primary lysosomes (Farquhar, 1969), a process called crinophagy. More frequently however, exocytic discharge of the exportable contents of the secretory vacuole occurs by its fusion with the plasma membrane; the membrane may later be retrieved by an endocytic mechanism. It is important to note that the contents of primary lysosomes, in some cell types, might also be discharged by exocytosis. This was proposed by Hickman & Neufeld (1972) as an explanation of the ability of normal fibroblasts to correct the lysosomal enzyme deficiency of mutant (I-cell) fibroblasts during co-cultivation. An alternative hypothesis (Lloyd, 1978) suggests that it is secondary lysosomes that discharge their contents extracellularly as a consequence of a postulated membrane recycling process. Neither hypothesis is supported by much experimental evidence. However, lysosomal enzymes are known to be released by several cell types under a variety of conditions (see Dean & Barrett, 1976) and often such release is associated with endocytic uptake of particulate materials (for a discussion of this subject see Ericsson et al., 1975).

Autophagic vacuoles may arise from the surrounding of cellular elements by a pair of smooth endoplasmic reticulum membranes, that fuse to form a double-membrane bounded vacuole. A second possible mechanism of formation is by invagination of the lysosomal membrane in a process that resembles pinocytosis; again a double membrane bounded vacuole is produced. Crinophagy is also a variant of autophagy but results in the formation of

a single membrane-bounded vacuole.

1.5 Mechanisms of endocytosis.

It is clear that endocytosis results in the internalization of membrane from the surface of the endocytosing cell. In the studies that follow, reference is often made to "the internalizing plasma-membrane" or "the plasma membrane from which pinosomes are formed", but it is not known, however, whether the membrane of the endocytic vesicle is a representative or a selected sample of the plasma membrane. Phagocytic membrane appears to be representative of the plasma membrane (see Silverstein et al., 1977) but the evidence that follows suggests pinocytic membrane is selected.

Anti-immunoglobulin binding-sites on B-lymphocytes are pinocytosed in the presence of anti-immunoglobulins but without perceptibly altering the binding of antibodies to other plasma-membrane components e.g. histocompatability antigens (Taylor et al., 1971). Similarly, Schneider et al. (1976) showed that specific anti-fibroblast plasma membrane antibodies were not interiorized but the same cells pinocytosed non-specific antibodies. After an examination of the lipid and protein components of isolated kidney brush border membranes and isolated pinocytic vesicles from the same tissue, Bode et al. (1976) discovered differences that led them to suggest that: "pinocytic vesicles are not derived from the brush border microvillous membrane but are independent entities that are newly synthesized during the pinocytic process".

The biochemical mechanisms underlying endocytic processes are poorly understood. Evidence is discussed by Allison & Davies (1974) for the proposal that formation of micropinocytic vesicles does not require metabolic energy and is unaffected by cytochalasin B, whereas the formation

of larger vacuoles in macropinocytosis and phagocytosis requires metabolic energy and is inhibited by cytochalasin B. Similar biochemical means were used to determine whether more than one endocytic mechanism underlies the formation of pinocytic vesicles (Gabathuler & Ryser, 1975). Cytochalasin B is a fungal product that is thought to inhibit both microfilament function (Wessels et al., 1971) and hexose trnasport in a number of phagocytes (Zigmond & Hirsch, 1972; Kletzien et al., 1972). However, its mode of action as an inhibitor of endocytosis appears to be the breakdown of microfilaments (Stossel, 1977). The observed inhibitions, by cytochalasin B, gave stimulus to the idea that contractile proteins (of the actin and myosin types) are involved in the formation of some endocytic vesicles. A hypothetical mechanism of how this might occur has been forwarded by Stossel (1977), who also reviews the evidence for the involvement of both contractile proteins and high energy compounds in endocytic uptake. Silverstein et al. (1977) also review these two aspects of endocytosis.

Whatever the mechanism underlying endocytic vesicle formation, pinocytosis is often an ongoing process that comes under some form of regulation e.g. in macrophages (Steinman & Cohn, 1972) and fibroblasts (Wenfeld et al., 1975). In certain cell types pinocytosis can be induced e.g. macrophages pinocytose several times more actively when obtained from inflammatory exudates (Edelson et al., 1975), cell-lines pinocytose 2-4 times faster on their becoming confluent (Steinman et al., 1974; Kaplan, 1976), synchronized hepatoma cells can interiorize horseradish peroxidase most actively in the G1 phase of the cell-cycle (Quintart et al., 1976) and partial hepatectomy induced an increased pinocytic activity in rat hepatocytes (Mori & Novikoff, 1977). The underlying regulatory mechanisms involved in these processes are, as yet, unknown.

Central to the problem of the control of protein turnover is the ability of some peptide hormones and proteins to modify the rate of pinosome formation, an ability which may well regulate a protein's own turnover. Thus stimululation of pinocytosis occurs in certain cell types: e.g. concanavalin A in mouse macrophages (Edelson & Cohn, 1974), maternal yolk-sac proteins in oocytes (Roth et al., 1976), a platelet--factor in arterial smooth-muscle cells (Davies & Ross, unpublished results), insulin in adipose cells (Barnette & Ball, 1959) and Hela cells (Paul & Person, cited in Becker & Ashwood-Smith, 1973) and thyroxin in epithelial cells of the thyroid (Wollman, 1969).

1.6 Adsorptive endocytosis.

A central feature of pinocytosis that similarly affects the rate of pinocytic ingestion in all cells, independently of the precise mechanism of pinosome formation or the way the process is controlled, is the extent to which a substrate is concentrated by binding to the surface of the membrane from which pinosomes are formed.

The adsorption of proteins (and other molecules) to the cell--surface prior to internalization was postulated by Bennett (1956) and confirmed in the amoeba by both morphological (Brandt, 1958) and biochemical (Schumaker, 1958) techniques. Chapman-Andresen & Holter (1955) had shown previously that serum albumin is ingested by the amoeba at a greater rate than glucose even when serum albumin was present with the latter. Since the rate of glucose uptake represents the maximum rate of fluid uptake, Holter (1959) concluded that the albumin must be concentrated by adsorption to the internalizing surface membrane and that: "surface adsorption is the dominant factor in pinocytosis, and that the uptake of fluid may, at least in certain cases, play a secondary role".

The method described above by Chapman-Andresen and Holter is still of great value in determining the extent to which proteins (and other substrates) bind to the membrane from which pinosomes are formed (see e.g. Williams et al., 1975b; Moore et al., 1977) and has been employed in the work reported in this thesis specifically for this purpose.

A theoretical treatment of the adsorption of substrate to membranes was left to Jacques (1975) who proposed that pinocytosis may take either one of three forms: uptake in the fluid phase only (substrate does not bind to the membrane), uptake in the adsorptive phase only (fluid being expelled from the forming pinocytic vesicle) and uptake in a combination of these two forms. In principle, negative adsorption is also possible where the substrate may be repelled from the external surface of the forming pinosome. An important feature of adsorptive pinocytosis is that, in theory at least, it can be selective. Those substrates that bind most extensively to the membrane that forms the pinosome will be ingested most rapidly. The occurrence of adsorption also permits competition between similar substrates for binding-sites. Fluid pinocytosis, however, can exhibit neither selection nor competitive uptake.

1.7 Morphology of the rat yolk-sac and its vacuolar system.

Prenatal transfer of passive immunity from a mother to her young is an important process in certain mammals but the placental tissues that immunoglobulins must penetrate to reach the embryo probably vary between species. In the rat it has been suggested that the visceral yolk sac may be involved in this process. This has helped to stimulate investigation of its morphological and ultrastructural features.

Development of the extra embryonic membranes of the rat occurs during the 23-day gestational period but is virtually complete by day 17 (Anderson, 1959; Sorkin & Padykula, 1964) the morphology then remains

essentially unchanged until term. A cross sectional drawing of placental tissue in an 18-day pregnant rat uterine horn, cut through a single conceptus, is shown in Fig. 1.1. The visceral yolk-sac membrane at its apex is smooth and lies closely adjacent to the epithelial cells of the uterus, but basally and laterally the yolk sac is thrown into folds or villi. Microscopic observations of the villous and non-villous regions (see Fig. 1.2) show it to include two basement membranes sandwiched between three cellular layers. Flattened mesothelial cells lie subjacent to a thick serosal basement membrane and face the amniotic cavity. On the opposite side of this membrane lie scattered mesenchymal cells and fetal, sinusoidal capillaries. Occasionally capillaries are found within villi where folding is extensive. Superior to these is the visceral basement membrane to which are attached the pinocytically active columnar epithelial cells that face the uterine cavity.

Histological and electron microscopic studies have shown the columnar epithelial cells of the yolk sac to be coated in a microvillous brush border and to be engaged in micropinocytosis (Sorkin & Padykula, 1964; Padykula et al., 1966; Lambson, 1966; Jollie & Triche, 1971; Seibel, 1974).

These cells have prominent basally-located nuclei. The apical plasma-membrane is evaginated into microvilli between which the membrane is often invaginated, forming caveolae. The latter seem to give rise to relatively electron-lucent micropinocytic vesicles. These apical structures each carry a fuzzy coat (glycocalyx) on their exoplasmic surface. Immediately inferior to the microvilli, in the same region as the electron--lucent vesicles, occur inherently electron-dense microvesicles and canaliculi; both seem not to be connected with the free, apical surface

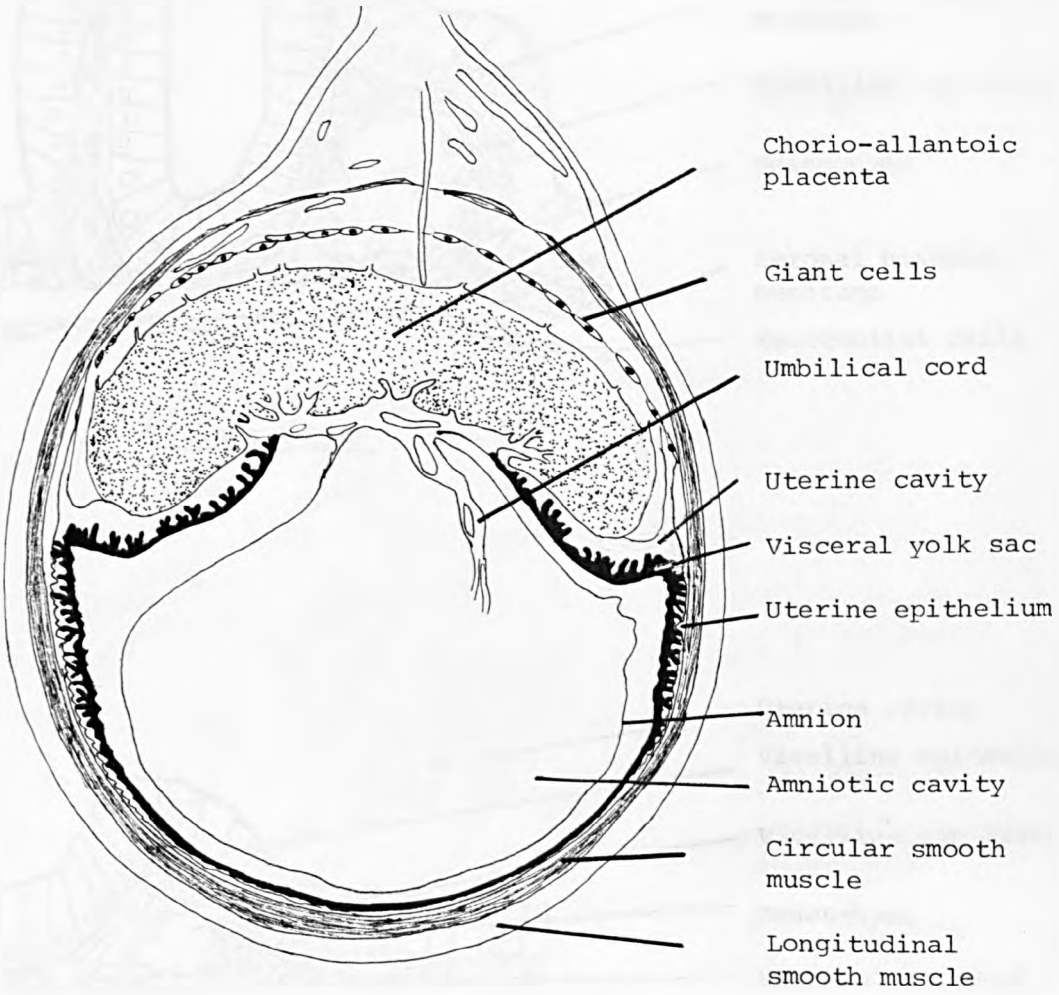
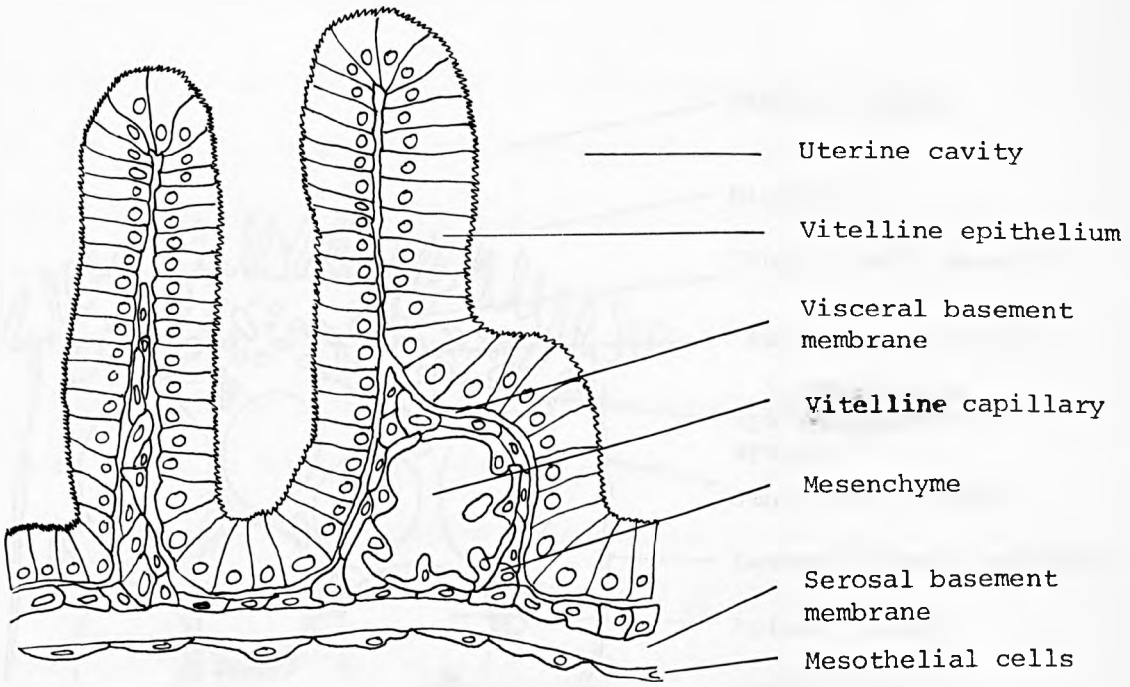


Figure 1.1 Cross-sectional drawing of the extraembryonic tissues in an 18-day pregnant rat uterine horn cut through a single conceptus.

(Richardson, 1963)



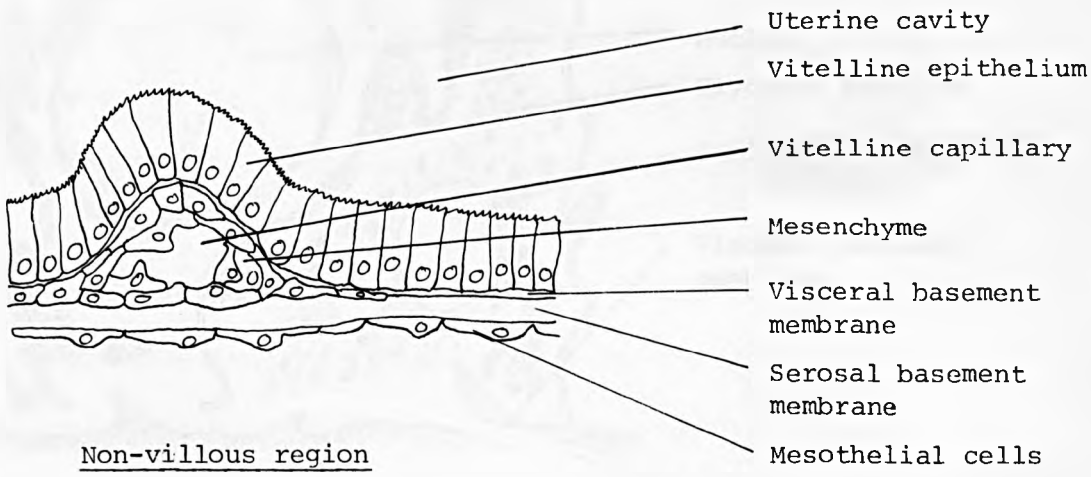


Figure 1.2 Drawing of sections through the villous and the non-villous regions of the rat visceral yolk-sac membrane.

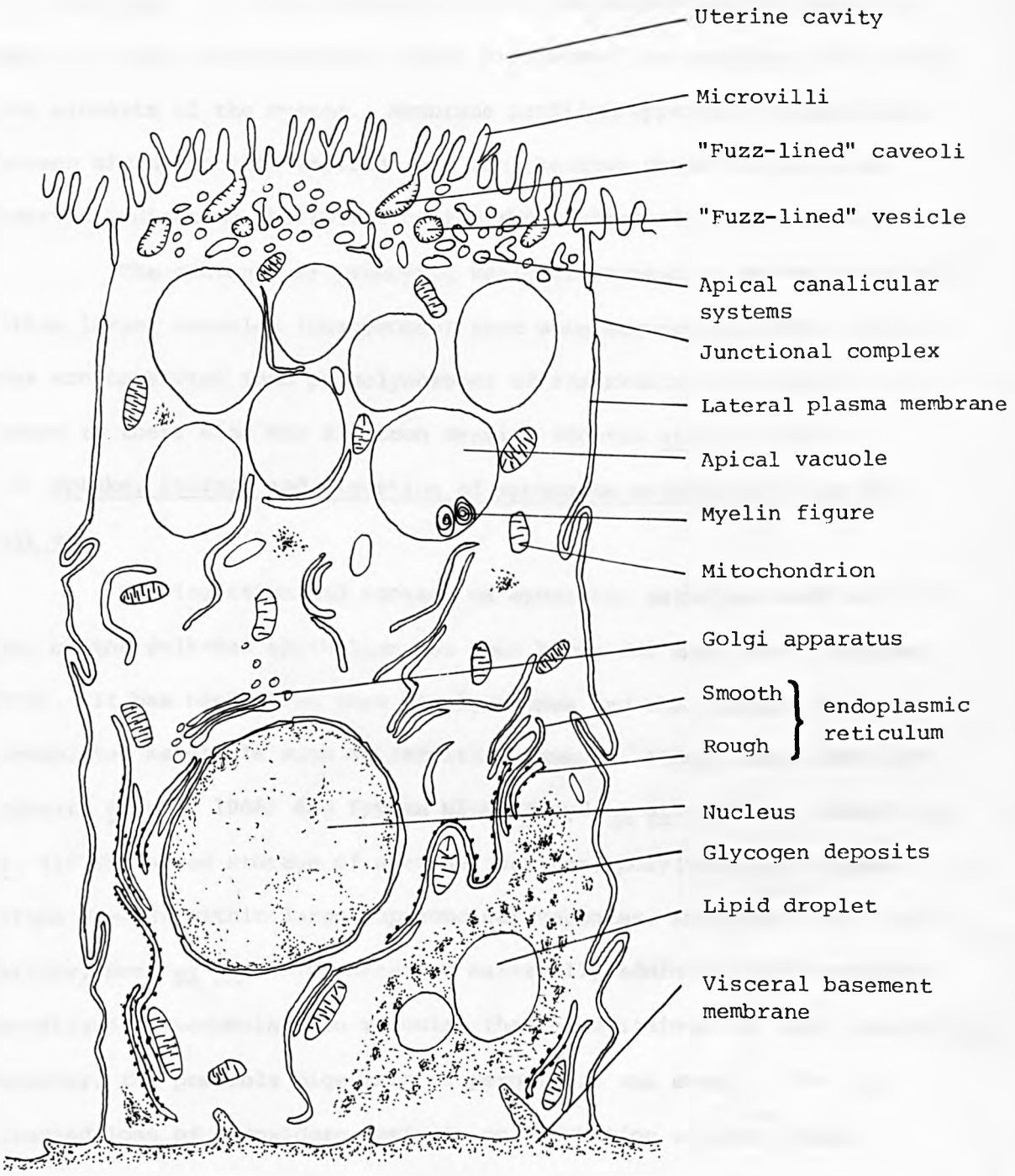


Figure 1.3 Drawing of a typical columnar vitelline epithelial cell from the rat visceral yolk-sac membrane.

of the cell. It is not certain whether the micropinocytic vesicles empty into the electron-dense system or whether they condense and reduce into elements of the system. Membrane profiles, apparently transitional between micropinocytic vesicles and the electron dense system, were observed indicating the latter of the above alternatives might occur.

The contents of pinocytic vesicles eventually become contained within larger vacuoles (phagosomes) that soon acquire lysosomal enzymes thus are converted into phagolysosomes of remarkable heterogeneity with regard to their size and electron density (Goetze et al., 1976).

1.8 Uptake, storage and digestion of exogenous materials by the rat yolk sac.

The ingestion and storage of exogenous materials such as vital dyes by the yolk-sac epithelium has been known for many years (Goldman, 1909). It has been shown that the lysosomes are the storage sites of accumulated materials such as ferritin (Lambson, 1966), Triton WR-1339 (Schultz et al., 1966) and Trypan Blue (Lloyd et al., 1968). Roberts et al. (1976) showed storage of sucrose, dextran, poly(vinylpyrrolidone), and Triton WR-1339 within large supranuclear vacuoles, inferred to be lysosomes. Earlier, Beck et al.(1970) observed maternally administered horseradish peroxidase to accumulate in vacuoles that also stained for acid phosphatase. Moreover, the possible digestion of peroxidase was suggested by the observed loss of peroxidase activity on incubation of such tissue without its recovery in the incubation medium. Conclusive evidence of the ability of yolk-sac lysosomes to digest pinocytosed protein (acid--denatured ^{125}I-labelled bovine serum albumin) was first provided by Williams et al. (1971). Kinetic evidence that the isolated 17.5-day rat yolk sac could accumulate (indigestible) ^{125}I-labelled poly(vinylpyrrolidone) and (digestible) acid-denatured ^{125}I-labelled bovine serum albumin was given by Lloyd et al. (1972) who used a technique that was developed to

enable quantitative kinetic experiments to be performed on endocytosis and lysosome function (see Williams *et al.*, 1975*a*,*b*). The kinetics of endocytosis in this system have since been examined by Roberts *et al.* (1977) using indigestible substrates ([^{14}C]sucrose, ^{125}I-labelled poly(vinylpyrrolidone) and colloidal [^{198}Au]gold) and by Moore *et al.* (1977) using digestible protein substrates (differently denatured preparations of ^{125}I-labelled bovine serum albumin and ^{125}I-labelled orosomucoid).

CHAPTER TWO

SELECTIVE PINOCYTOSIS OF ^{125}I-LABELLED PROTEINS BY RAT YOLK SACS

2.1 INTRODUCTION

Proteins are cleared from the mammalian circulation at widely differing rates and those factors that affect the rate of clearance have been reviewed by Bocci (1970). A continuing flow of more recent publications (see e.g. Ashwell & Morrel, 1974a,b; Rogers & Kornf eld, 1971; Kitani & Taplin, 1972. 1974; Buys et al., 1973, 1975; Normann, 1973, 1974a,b; Dice & Goldberg, 1976; Moore et al., 1974, 1977; Stahl et al., 1976a,b; Bose & Hickman, 1977; Furbish et al., 1978; Kooistra et al., 1978) indicates the area is still one of active research. Endocytosis is probably responsible for the in vivo clearance of proteins (Gordon, 1973) and this process has been studied more closely in suitable culture systems using either an intact tissue (see e.g. Williams et al., 1975a,b; Moore et al., 1974, 1977; Jirmanova et al., 1977) or isolated mammalian cells (see e.g. Kalderon & Wittner, 1971; Gabathuler & Ryser, 1975; Ryser, 1970; Wiesman, 1974; Hickman et al., 1974; Stein et al., 1976; Pratten et al., 1977; Nilsson & Berg, 1977; Tolleshaug et al., 1977; Munthe-Kaas, 1977; Ullrich et al., 1978; von Figura et al., 1978).

Of particular physiological interest, and possible pathological importance, are the demonstrations by Hickman et al. (1974) and Wiesman (1974) that lysosomal enzymes are selectively endocytosed by fibroblasts in culture but that lysosomal enzymes from patients with mucolipidoses types II and III are not (for review see Neufeld et al., 1977). Goldstein and co-workers (for reviews see Goldstein & Brown, 1976, 1977 and Brown & Goldstein, 1976) have shown that plasma low density lipoprotein is ingested by normal human fibroblasts in vitro by adsorptive endocytosis but that fibroblasts from homozygous hypercholesterolaemia patients appear to lack the necessary surface receptors, have a defective uptake of low density lipoprotein and consequently show impaired regulation of

cholesterol synthesis.

In general, the underlying mechanisms by which endocytic cells display sufficient specificity to account for the observed differences in uptake rate are poorly understood. A single exception exists as a result of detailed studies by Ashwell & Morell (for reviews see Ashwell & Morell, 1974a,b) who demonstrated conclusively that the rates of clearance of a number of human serum glycoproteins, from the circulation of the rat, are increased dramatically following removal of terminal sialic acid residues from the carbohydrate moiety of these glycoproteins. Removal of sialic acid exposes the penultimate galactose residues that participate in the selective binding of the desialylated glycoprotein to the receptor sites on the plasma membrane of hepatocytes. The mechanism of pinocytic recognition of certain lysosomal enzymes (Stahl et al., 1976a,b) is now also becoming clear and involves the participation of phosphohexosyl (seemingly phosphomannosyl) residues in the recognition site of the lysosomal enzyme (Kaplan et al., 1977a,b; Sando & Neufeld, 1977). Other pinocytic recognition systems, that are less well characterized, include the recognition of agalacto--glycoproteins in mammalian livers (Stockert et al., 1976) and avian livers (Lunney & Ashwell, 1976) and a system for the clearance of mannosyl-terminal glycoproteins (Winkel hake & Nicolson, 1976; Baynes & Wold, 1976). A rapid clearance of semi-synthetic D-glucosyl-protein has been reported and it seems to be mediated by binding to the same hepatic receptors that bind D-galactosyl-glycoproteins such as asialo-orosomucoid (Stowell et al., 1977). Dice & Goldberg (1976) have found that those rat--serum proteins with acidic isoelectric points are cleared from the circulation more rapidly than those with a basic isoelectric point and that those proteins cleared rapidly are more readily degraded by

both neutral and acid endoproteinases. Moreover, the half-lives of intracellular proteins also correlate with these two characteristics. The factors which determine the rate of catabolism of intracellular proteins are currently under active investigation (Dice et al., 1973; Dice & Goldberg, 1975a,b; Goldberg et al., 1976; Dean, 1975a; Schimke & Bradley, 1975; Momany & Larabee, 1976; Segal et al., 1974, 1976; Segal, 1976; Paskin & Meyer, 1977, 1978) and it has been suggested by Lloyd (1976) that the lysosomal system could be responsible for the selective degradation of cytoplasmic proteins if such proteins adsorb differentially to the cytoplasmic surface of the lysosomal membrane and subsequently enter lysosomes by budding of lysosomal membrane into the lysosomal interior, a process closely resembling pinocytosis.

Moore et al. (1974, 1977) have shown that treatment of ^{125}I-labelled bovine serum albumin with increasingly severe denaturing conditions, which induce a decrease in the α-helical content of the protein (as judged by optical rotatory dispersion), leads to a corresponding increase in the rate of uptake of this protein by the 17.5-day rat visceral yolk sac in culture. It was concluded that the elevated rate of clearance resulted from increased adsorption of these protein preparations to the plasma membrane. Exposure of chemical groupings, perhaps hydrophobic in nature, that are normally masked in the native conformation of the protein, is a possible explanation (Lloyd, 1976). However, for a number of reasons discussed below, it is not easy to identify those structural features of bovine serum albumin that are responsible for its increased rate of uptake.

Bovine serum albumin is a large single chain protein with microheterogenic forms. One cause of the heterogeneity is oxidation of the single, free sulphydryl group (Noel & Hunter, 1972). Oxidation

of bovine serum albumin has been shown to decrease its half-life in the circulation of the rat (Bocci, 1969). Pairing of disulphide bonds also accounts for heterogeneity (Sogami et al., 1969) and sulphydryl-disulphide exchange reactions, leading to a shuffling of disulphide bonds within the molecule, are enhanced under denaturing conditions (Foster, 1960).

Two distinct conformers of bovine serum albumin are known (Pederson & Foster, 1969; Foster, 1960) and transition from the compact (N) form to the expanded (F) form is associated with the exposure of hydrophobic surfaces. Buys (1973) has shown that denaturation of bovine serum albumin using alkaline formaldehyde results in a molecule with a larger Stokes radius (and a reduced half-life in the circulation of the rat) but it is not known whether this transition is of the type discussed by Pederson & Foster (1969) and Foster (1960). Wallevik & Mouridsen (1968) were able to separate human serum albumin into fractions (by ammonium sulphate precipitation) that show differences in the proportion of the N form to the F form at pH values between 4.5 and 3.7, but which show identical rates of clearance from the circulation of human subjects. However, it is not known if the albumin preparations have different proportions of the N form to the F form at physiological pH. In addition to the structural differences between individual albumin molecules within a single preparation, and which affect the physical behaviour of the preparation as a whole (Foster, 1960) albumin also binds many substances including: hormones, bile acids, vitamins, anions, neuro-transmitters, metal ions, dyes, drugs, antibiotics and, more importantly, fatty acids (Spector, 1975). Complete removal of fatty acids is very difficult, but Goodman (1957) has reduced fatty acid contamination to a vanishingly small level using 5% acetic acid in iso-octane (a treatment that is, however, also likely to denature the albumin) and

Verbruggen et al. (1968) have shown that removal of lipids from bovine serum albumin, by four consecutive extractions with carbon tetrachloride, changes the conformation of the albumin molecule.

Because of these factors, and because bovine serum albumin may not behave as a representative protein substrate in the rat visceral yolk-sac system, three less complex ^{125}I-labelled proteins: bovine insulin, hen egg-white lysozyme and bovine pancreatic ribonuclease A were studied in the same system. These proteins were chosen both because they are of lower molecular weight than albumin and because their structures and properties are well documented. The effects of treatment of these proteins with alkaline-formaldehyde (and other potential denaturing conditions) on their rates of endocytic capture were examined to discover whether, like albumin, their rates of uptake could be increased. It was hoped that a correlation would appear between the rates of ingestion of these protein molecules and some structural feature(s) thus permitting identification of the characteristic of a protein molecule that most strongly influences its ability to adsorb to the pinocytosing plasma membrane of the epithelial cells of 17.5-day rat visceral yolk sac.

2.2 METHODS

2.2.1 Methods for the culture of rat visceral yolk sac and the assay of yolk-sac tissue and culture medium.

(1) The culture technique. The method of Williams et al. (1975a,b) was used to determine the rate of pinocytic capture of substrates by the 17.5-day rat visceral yolk sac in culture. Wistar rats, from an inbred colony, were mated overnight; if a sperm plug was detected below the grid-cage the next morning, pregnancy was timed from midnight of the night of mating. After 17.5 days the rats were killed by cervical dislocation and the intact uterus immediately removed and placed in warm (37°C) culture medium [medium 199 (preparation TC20, containing penicillin, 200 units/ml and streptomycin, 100 µg/ml) supplemented with 10% (v/v) calf serum (preparation CS07); both products of Wellcome Reagents Limited, Beckenham, England]. The intact feto-placental units were removed from the uterus and each yolk sac dissected free from the placenta and fetus, then placed in fresh warm culture medium. The remaining amniotic tissue was removed and the yolk-sacs gently rinsed to remove any debris and blood.

Each yolk sac was placed in a separate sterile 50ml Erlenmeyer flask containing 9.0ml of gassed medium (8.0ml medium 199 and 1.0ml calf serum) under an atmosphere of 95% O_2 and 5% CO_2. This medium had been sealed in each flask with a sterile silicone-rubber bung and maintained at 37 ± 0.3°C for at least 15min prior to the introduction of the yolk sac. Each yolk sac was preincubated (15-20min) in a reciprocating water bath set with a stroke length of 3.4cm and stroke frequency of 100 ± 5 strokes per min, before 1.0ml of medium 199 containing dissolved substrate was added to each flask. The flasks were then re-gassed, re-stoppered and incubation continued for either 3h or 6-7h (according to the rate of substrate depletion).

Yolk sacs taken from the flasks at regular intervals over the 3h or 6-7h period were each washed three times to remove extracellular substrate, by agitating for 2min in 1% (w/v) aq. NaCl (approx. 30ml) at 4°C, then placed in a 5ml graduated flask and either assayed immediately for protein content and radioactivity (see below) or stored at -20°C until assayed. When protein substrates were used, incubation of the culture medium was continued after removal of the yolk sac. (This procedure was adopted to standardize any possible effects from de--iodination or hydrolysis of the substrate by enzymes present in the culture medium during the incubation period.) A control flask, containing both medium and labelled protein but no yolk sac, was also incubated in the same manner in each experiment and the amount of low molecular weight radioactive material present at the end of the incubation period determined. At the end of an experiment all media were either assayed for radioactivity immediately or deep frozen at -20°C until assayed.

Control experiments using ^{125}I-labelled poly(vinylpyrrolidone) [^{125}I-PVP] as substrate at a concentration of 2 or 5µg/ml of culture medium were performed at regular intervals throughout this study.

(2) The assay methods for yolk sacs and culture media when using the non-digestible ^{125}I-labelled poly(vinylpyrrolidone) as substrate. The yolk sacs, each contained in a 5.0ml graduated flask, were dissolved in 5.0ml of 1M-NaOH by warming at 37°C for 1h. Duplicate samples (1.0ml) of each yolk-sac solution were pipetted into 3ml disposable tubes (LP3, Luckhams Ltd., Burgess Hill, Sussex) and assayed for radioactivity in a 5142 Selektronic gamma spectrometer (Packard Instrument Ltd., Caversham, Berks.) using a standard counting geometry. The protein content of each yolk sac was determined by the method of Lowry et al. (1951), using bovine

serum albumin (Sigma, London) as the reference protein. Duplicate 0.1ml samples of yolk-sac solution were used for this purpose. (Yolk sacs from 17.5-day pregnant rats usually contained 4-7mg bovine serum albumin equivalents of protein.)

Duplicate samples (1.0ml) of medium were pipetted into 3ml disposable tubes, then assayed for radioactivity. The means of each pair of values (yolk-sac protein, radioactivity in the medium and in the yolk-sac solution) were used in subsequent calculations.

(3) The assay methods for yolk sacs and culture media when using digestible substrates (^{125}I-labelled proteins). Duplicate samples (1.0ml) of culture medium were first assayed, as in (2) above, for radioactivity ('total radioactivity') then the protein was precipitated with trichloroacetic acid and the radioactivity in the supernatant ('acid-soluble radioactivity') also assayed. For bovine serum albumin full precipitation was achieved by simply adding trichloroacetic acid (0.5ml, 20% w/v), but with bovine insulin, hen egg-white lysozyme and bovine pancreatic ribonuclease complete precipitation of protein required the addition of phosphotungstic acid solution* (0.5ml) before addition of the trichloroacetic acid as above. After thorough mixing, the tubes were immediately centrifuged at 2000g for 20min (MSE Mistral 4L) and the clear supernatant carefully decanted into another 3ml disposable tube and counted ('acid-soluble radioactivity'). The observed count was corrected for the change in counting geometry and for loss of acid-soluble radioactivity through occlusion within the precipitated protein, to give the count that would have been given if the entire acid-soluble

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*[Prepared by dissolving 10.0g sodium tungstate, 25.2g Na_2HPO_4, 9.0g NaCl and 1.0g phenol in 875ml of distilled water then adding 125ml of 1M-HCl.]

radioactivity had been assayed in a volume of 1.0ml. This was done by multiplying the observed count by an empirical correction factor, which differed according to the precipitation method used (see Appendix I for details).

The radioactivity and protein content of each yolk-sac solution was determined as in (2) above.

2.2.2 Calculation and expression of the pinocytic uptake data.

In quantitative studies of the pinocytic uptake of substrates it is desirable to express the rate of uptake in a form that permits the reproducibility of the results between experiments to be readily assessed. Even when uptake occurs at a constant rate, the factors whose effects must be eliminated are: the variable quantity of tissue in individual yolk sacs, the effect of isotopic decay on the specific radioactivity of the substrate and day to day variation in the sensitivity of the gamma spectrometer. For this reason the rate of uptake is expressed in the form described by Williams et al. (1975a) i.e. the volume of culture medium whose contained substrate has been captured by unit quantity of the yolk-sac tissue in unit time. This quantity, termed the Endocytic Index, and whose units are µl/h per mg of yolk-sac protein, is obtained from the gradient of the regression line fitted to a plot of uptake (in µl/mg yolk-sac protein) against incubation time (h).

The calculations of uptake (see Appendix II) and the linear regression analysis were made with an ICL 4130 computer. The associated Correlation Coefficient gives a crude index of the scatter of the points about the regression line, but is insensitive to slight curvature in a plot, hence each plot was inspected visually for linearity.

2.2.3 Preparation of protein substrates for pinocytosis experiments.

(1) Preparation of ^{125}I-labelled proteins. The method used for the radio-iodination of protein substrates was one adapted from the method of Bocci (1969), which itself is based on the original method of Hunter & Greenwood (1962). Usually 10mg quantities of protein were radio-iodinated, but when a different quantity was required, the quantities stated below were altered proportionately.

Bovine serum albumin (Koch-Light; preparation 0142t, purity 99%), bovine insulin (Sigma, London; approx. 25 IU per mg), bovine pancreatic ribonuclease A (Sigma preparations Type XA purchased as a solution and Type XIA purchased as a lyophilized powder) or hen egg-white lysozyme (Sigma, Grade I), were dissolved in 4.25ml of 0.05$\underline{\underline{M}}$-$Na_2HPO_4$-$KH_2PO_4$ buffer, pH 8.0, in a 25ml disposable glass bottle. The solution was stirred in an ice bath and $Na^{125}I$ (1mCi, approx. 0.25ml) added. After stirring for 2min, chloramine-$\underline{\underline{T}}$ (2mg in 2.5ml distilled water) was added and the solution stirred at 0°C for a further 8min. (Bocci has shown that for bovine serum albumin, the degree of labelling reached a maximum level at 8min and that further, unnecessary exposure of the protein to oxidising properties of chloramine-$\underline{\underline{T}}$ caused the half-life of the protein in the blood stream to decrease.) At 8min, the excess chloramine-$\underline{\underline{T}}$ was destroyed by the addition of sodium metabisulphite (2mg in 2.5ml of distilled water) and solid KI (approximately 20mg) was added to aid the displacement of unreacted ^{125}I in subsequent dialysis. A 50µl sample of the undialysed reaction mixture (total volume 9ml) was taken to determine its content of acid-soluble radioactivity, hence the labelling efficiency. The remainder of the final reaction mixture was dialysed at 4°C for 48-72h against 5 or 6 changes of 1% (w/v) aq. NaCl (5ℓ). The non-diffusible material was stored at -20°C.

Occasionally, when a large percentage of the radioactivity in the dialysis residue remained acid-soluble, the preparation was passed through a small column (10cm x 1.1cm) of Sephadex G-25, with 1% (w/v) aq. NaCl as elutant, to remove the low molecular weight radioactive material.

The labelling efficiencies of bovine serum albumin, insulin, ribonuclease (XA and XIA) and lysozyme were 60, 85, 5 and 5% repsectively. As 1mCi of radio-iodide was used for each 10mg of protein, the specific activities of the radio-labelled proteins were 60, 85, 5 and 5μCi per mg protein. It was calculated that the number of molecules of protein containing one atom of radio-iodide were: bovine serum albumin, 300 - 500; insulin, 2 800 - 4 400; ribonuclease, 20 000 - 31 000 and lysozyme 19 500 - 30 000 molecules.

(2) Treatment of ^{125}I-labelled proteins with acetic acid. A small volume (2 - 3ml) of the ^{125}I-labelled protein solution was titrated to pH 2.5 with glacial acetic acid using a glass microelectrode. The acidified solution was kept at 37°C for 2h before being dialysed as described in (1) above.

(3) Treatment of ^{125}I-labelled proteins with 4M-urea. A small volume (2 - 3ml) of the ^{125}I-labelled protein solution was mixed with an equal volume of aq. 8M-urea which had been adjusted to pH 5.0 with 1M-HCl. The resulting solution was kept at 22-25°C for 24h after which it was dialysed as described in (1) above.

(4) Treatment of ^{125}I-labelled proteins with formaldehyde. A solution of 10% (w/v) formaldehyde was made up in 0.5M-$NaHCO_3$ buffer, pH 10, and an equal volume added to a portion of the ^{125}I-labelled protein solution. The resultant 5% (w/v) formaldehyde solution was kept at 22-25°C for 72h before being dialysed as described in (1) above. A further portion of

the ^{125}I-labelled protein solution was treated with bicarbonate buffer alone.

After treatment with either formaldehyde (pH 10) or bicarbonate buffer (pH 10) alone, the lysozyme preparations were found to be heterogenous as judged by their elution patterns on Sephadex G-75. Each new species produced after treatment with either formaldehyde or alkali was isolated by Sephadex G-75 chromatography as described in Section 2.2.5 but using 1% (w/v) aq. NaCl as eluant. The ^{125}I-labelled protein contained in the pooled fractions of each peak was used separately as substrate.

(5) Assay of protein substrate concentration. In the above preparative procedures some of the protein substrate was incompletely transferred in certain manipulations (e.g. dialysis). It was therefore necessary to measure the protein content of each final preparation before its use in experiments. A spectrophotometric method was used as this caused minimal loss of material. (The possible effects of the various treatments on the optical properties of the proteins were ignored). The extinction, at 280nm, of each ^{125}I-labelled protein preparation was compared with extinction values measured using native protein solutions of known concentration in the 0 to 1mg range. The native proteins were dissolved in 1.0% (w/v) aq. NaCl that was adjusted to pH 5.6 with dilute HCl to give the same pH as observed in the dialysis of the ^{125}I-labelled protein preparations. [For native insulin and a portion of each ^{125}I-labelled insulin preparation, the solution was adjusted to pH 3.7 with a known quantity of 0.5M-HCl as insulin is not very soluble in the pH range 4 - 8].

2.2.4 Release of radioactivity on re-incubating yolk sacs that had previously ingested ^{125}I-labelled proteins in vitro.

The method used to calculate the Endocytic Index of a non-digestible substrate (see Section 2.2.2) assumed that the rate of release of substrate back into the culture medium from the yolk-sac tissue is negligible. Similarly, for digestible substrates, the method assumes that only acid--soluble digestion products are returned to the culture medium. Williams et al. (1975a) and Roberts et al.(1977) have shown that for ^{125}I-labelled poly(vinylpyrrolidone) the first of these assumptions is correct. To show that the second of these assumptions was correct for each protein, it was necessary to determine the rate of release of radioactivity from yolk sacs that had ingested the ^{125}I-labelled protein in vitro and to establish the nature of the products released into the medium.

(1) Method of determining the rate of release of radioactivity from yolk sacs containing ^{125}I-labelled proteins. Three 17.5-day yolk sacs were incubated together for 2h, essentially as described in Section 2.2.1 above, but in the presence of substrate(5μg/ml of medium). They were then washed three times for 2min in 10ml of fresh, gassed culture medium, initially containing no ^{125}I-labelled protein, at 37°C under the usual incubation conditions. (This washing procedure was found to be optimal, further washing resulted in excessive loss of tissue-associated radioactivity through substrate degradation, without significant desorption of substrate associated with extracellular surfaces.)

The washed yolk sacs were then incubated in 10.0ml of fresh, initially substrate-free culture medium for a further 2h period under the same conditions as described above. Samples (1.0ml) of medium were removed at 10min intervals throughout the 2h incubation period and replaced by an

equal volume of warm, substrate-free, gassed culture medium to maintain the volume at 10.0ml. The flasks were re-gassed after each sampling. At the end of the incubation period the yolk sacs were quickly rinsed in cold (4°C) aq. NaCl (1%, w/v) for 10s to remove culture medium, then dissolved in 20.0ml of aq. 1M-NaOH at 37°C and the contained protein and radioactivity assayed as described in Section 2.2.1. Each of the 1.0ml samples of culture medium taken throughout the re-incubation period, together with 3 x 1.0ml samples taken at the end of the initial incubation were assayed for total- and acid-soluble radioactivity as described in Section 2.2.1.

(2) Expression of the release of radioactivity from yolk sacs containing ^{125}I-labelled proteins. The quantities of total- and acid-soluble radioactivity released by the yolk sacs over the 2h re-incubation period were calculated according to the formula described by Williams et al. (1971):

$$T_n = 10C_{i(i=n)} + \Sigma_{i=0}^{i=(n-1)} C_i \qquad (2.1)$$

where T_n is the radioactivity (c.p.m.) released up to the time of the n^{th} sampling, and C_i the radioactivity (c.p.m., corrected for background) per ml of culture medium of the i^{th} sample.

To facilitate comparison between this data and that obtained in the pinocytosis experiments it was considered desirable to express the radioactivity released in the same units as those used for uptake (i.e. µl/mg yolk-sac protein). This can be achieved using the equation:

$$T_n' = T_n/P.M \qquad (2.2)$$

where T_n' is the radioactivity (µl/mg yolk-sac protein) released up to the time of the n^{th} sampling, T_n is as described for equation 2.1, P is

the total protein content of the yolk sacs (mg) and M is the acid--insoluble radioactivity (c.p.m., corrected for background and initial acid--soluble material) in 1µl of the culture medium containing the ^{125}I-labelled protein at a concentration of 5µg/ml of medium. The sum of the radioactivity remaining in the tissue at the end of the incubation period and the total radioactivity released by the yolk sacs at the end of the incubation period gave the radioactivity in the yolk-sac tissue at the time of transfer to the re-incubation medium. This value enables the total radioactivity released by the yolk sacs at the end of the incubation period to be expressed as a percentage of that associated with the yolk sac at the start of the re-incubation.

(3) Examination of the hydrolysis products derived from ^{125}I-labelled proteins. The procedure for the re-incubation of ^{125}I-labelled protein loaded yolk sacs, as described in (1) above, was used but no medium samples were taken during the 2h re-incubation period. Portions of the culture medium, taken from the end of the incubation and re-incubation period were centrifuged at 4°C (MSE Mistral 4L) at 500g for 10min to remove cellular debris. Each was then examined by gel-chromatography on Sephadex G-25 (Pharmacia, Uppsala, Sweden) and the pooled fractions containing low molecular weight digestion products were concentrated by lyophilization and further examined by eluting from a copper-complex of Sephadex G-25, which separates radioactive dipeptides containing [^{125}I]iodo-L-tyrosine from the [^{125}I]iodo-L-tyrosine (Fazakerley & Best, 1965).

Sephadex G-25 chromatography. Centrifuged culture medium (5.0ml) was applied to a Sephadex G-25 (fine) column (35cm x 1.7cm) that had been previously equilibrated with a solution suitable for the elution of a particular protein substrate. For ^{125}I-labelled insulin 0.05M-sodium acetate (pH 6.5) containing 0.1% (w/v) sodium azide was suitable but

this solution was unsuitable for ^{125}I-labelled lysozyme, which was found to adsorb strongly to the gel. This could be prevented by addition of 1% (w/v) NaCl (as described by Hampe, 1972). However, ^{125}I-labelled ribonuclease was found to bind to the gel when using the acetate solution even in the presence of 1% (w/v) NaCl, but almost 100% recoveries were achieved using 0.2M-NaH_2PO_4, pH 6.5, containing 0.1% (w/v) sodium azide.

After the 5.0ml sample had run into the gel it was further washed in using 2 x 1ml portions of the appropriate eluent before the column was eluted at 4°C at a flow rate of 24ml/h, collecting fractions (2ml) in glass test tubes. The fractions were decanted into 3ml disposable tubes and assayed for radioactivity as described in Section 2.2.1 above.

Chromatography on the copper-complex of Sephadex G-25. Sephadex G-25 (16g) was hydrated in distilled water and the 'fines' decanted off. The swollen gel suspension (125ml) was mixed with 0.16M-$CuSO_4$ (28ml) for 15min before 1M-NaOH (30ml) was added to precipitate the copper within the gel matrix. After a further 15min stirring, the gel was washed four times with 1 vol. of 0.5M-sodium borate, pH 11, and then packed into a column (30 cm x 1.7cm) which was equilibrated with the borax buffer at 4°C. The sample to be examined (2.0 - 3.0ml) was applied to the column, washed in with 2 x 1ml portions of borax buffer, then eluted with the same buffer at a flow rate of 24ml/h, and 2ml fractions collected. After 20 - 30 fractions had been collected, elution was continued with 0.4M-HCl, which solubilized the copper hydroxide in the gel matrix thus releasing the chelated [^{125}I]iodo-L-tyrosine. The fractions were assayed for radioactivity as described above.

Calculation of recovery values. The radioactivity recovered from a column after each chromatographic procedure was determined according to the

equation:

$$R = (\Sigma B_n . G/A)100 \qquad (2.3)$$

where R is the percentage recovery, G is an empirical correction factor (which corrects both for the loss of radioactivity on decanting the fractions from the glass collection tubes into the 3ml disposable plastic tubes and for the difference in counting geometry). G effectively converts the observed count in 1.6ml of solution to the number of counts that would have been observed if the entire fraction had been assayed in a volume of 1.0ml). B_n is the radioactivity contained in the n^{th} sample (c.p.m., corrected for background) and A is the total radioactivity applied to the column (c.p.m., corrected for background).

2.2.5 Analysis of ^{125}I-labelled protein substrates by Sephadex G-75 chromatography.

The possible presence of protein aggregates in both untreated ^{125}I-labelled protein preparations and those ^{125}I-labelled protein preparations which showed a modified rate of pinocytic uptake after treatments as described in Section 2.2.3 was investigated by chromatography on Sephadex G-75 (particle size 40-120μ).

The ^{125}I-labelled proteins (5-25μg) were analysed on a Sephadex G-75 column (80cm x 1.7cm) essentially as described for the gel-filtration studies in Section 2.1.4 (3). The column was eluted with an appropriate salt solution at 24ml/h and 4ml fractions collected. For ^{125}I-labelled insulin and ^{125}I-labelled ribonuclease, 0.2$\underline{M}$-NaH_2PO_4, pH 6.5, containing 0.1% (w/v) sodium azide was a satisfactory eluent and gave high recovery values but for ^{125}I-labelled lysozyme, 0.05$\underline{M}$-sodium acetate, (pH 6.5) containing 1% (w/v) NaCl and 0.1% (w/v) sodium azide was required to achieve high recoveries.

2.3 RESULTS

2.3.1 Uptake by the 17.5-day rat yolk sac in vitro.

Uptake data from a typical control experiment are shown graphically in Fig. 2.1 and the results of 29 such experiments are summarized in Table 2.1. The uptake of ^{125}I-labelled poly(vinylpyrrolidone) [^{125}I-PVP], a non-digestible substrate, is linear over the 6-7h incubation period (linearity has been shown to extend to 15h by Williams, K.E. - unpublished results, but 6-7h was used routinely for convenience). Little inter-experimental variation and no systematic drift in the individual values of Endocytic Indices (mean value 1.75 ± 0.33) were observed over the period of study (Table 2.1). This demonstrated the high degree of reproducibility of the pinocytic activity of the *in vitro* system.

A typical experiment in which yolk sacs were incubated in the presence of formaldehyde-denatured ^{125}I-labelled bovine serum albumin, a digestible substrate, gave the results shown in Fig. 2.2. Both the quantity of tissue-associated radioactivity and the quantity of acid-soluble radioactivity returned to the medium at different times up to 6h are shown. Overall uptake is calculated by summing these values at each time point. The quantities of tissue-associated radioactivity became constant within 2h so that the rate of release of acid-soluble radioactivity into the incubation medium became constant, and equal to the Endocytic Index in the period beyond 2h.

Results of equivalent experiments in which yolk sacs were incubated in the presence of ^{125}I-labelled insulin, lysozyme or ribonuclease (Type XA), eached stored at -20°C or further treated with either: acetic acid (pH2.5), 4M-urea, formaldehyde (pH 10) or bicarbonate

buffer (pH 10), can be seen in Tables 2.2a,b & c. The results of similar experiments with lyophilized ribonuclease (Type XIA) which had either been stored at -20°C or further treated with either: acetic acid (pH 2.5), 4M-urea or formaldehyde (pH 10) are shown in Tables 2.2d. The same Table also shows results of experiments with formaldehyde-denatured ^{125}I-labelled bovine serum albumin. The above results are summarized in Table 2.3. For comparison the results obtained by Moore et al. (1977) for differently treated ^{125}I-labelled bovine serum albumin preparations are also shown.

In the present investigation, as in those of Williams et al. (1975b) and Moore et al. (1974, 1977) the Endocytic Index of each ^{125}I-labelled protein substrate is markedly higher than that of ^{125}I-PVP. In contrast to ^{125}I-labelled bovine serum albumin the treatment of ^{125}I-labelled insulin, ^{125}I-labelled lysozyme and ^{125}I-labelled ribonuclease with either acetic acid (pH 2.5) or 4M-urea, results in little or no change in the Endocytic Index of these protein substrates. Where the students t-test of significance could be applied, no significant differences were found at the 0.05 level. Treatment with formaldehyde (pH 10) however, modified substantially the rate of uptake of each protein, but, unlike ^{125}I-labelled bovine serum albumin (where there is a 10-20 fold increase in the Endocytic Index after treatment with formaldehyde) these lower molecular weight proteins show a marked decrease in their rates of uptake. Except for ^{125}I-labelled lysozyme, treatment of the radiolabelled proteins with bicarbonate buffer (pH 10) alone produces little or no change in the observed rate of uptake, suggesting that the modified rate of uptake results from a reaction in the presence of formaldehyde rather than mere exposure to an alkaline solution alone. Unlike the ^{125}I-labelled forms of insulin and ribonuclease, ^{125}I-labelled lysozyme showed markedly reduced

rates of uptake on treatment with alkali. Table 2.2d shows that, after radio-iodination, a second Sigma preparation of ribonuclease was endocytosed at a lower rate than that shown in Table 2.2c. This second preparation differed from the first only in that it had been lyophilized prior to radio-iodination. Its rate of uptake was not modified by treatment with either acetic acid (pH 2.5), 4M-urea (pH 5.0) or formaldehyde (pH 10).

Because a 3h incubation period was used (except for the formaldehyde-denatured ^{125}I-labelled bovine serum albumin), it was not always possible to determine the steady-state quantity of radioactivity associated with the tissue as accurately as would have been possible in experiments of longer duration. However, Fig. 2.3 shows the levels of tissue-associated radioactivity over the 3h incubation period for each substrate. In general, the quantity of tissue-associated radioactivity increased rapidly during the first 30min, but then became virtually constant after 2-2.5h; this pattern is particularly pronounced for insulin.

Untreated, acetic acid- and urea-treated preparations of ^{125}I-labelled insulin all showed the quantity of tissue-associated radioactivity to be lower than that of the corresponding treated preparations of either ^{125}I-labelled ribonuclease or ^{125}I-labelled lysozyme (Fig. 2.3). Since, for all three proteins, the corresponding preparations have similar Endocytic Indices (see Table 2.3); this indicates that the yolk sac catabolises the ^{125}I-labelled insulin preparations more rapidly than those of the other two proteins.

The widely different Endocytic Indices observed between the non-digestible substrate, ^{125}I-PVP (1.75µl/h per mg yolk-sac protein) and the formaldehyde-denatured ^{125}I-labelled proteins (45-90µl/h per mg yolk-sac protein) and untreated, acetic acid- or urea-treated preparations

of ^{125}I-labelled insulin, lysozyme and ribonuclease (120-150µl/h per mg yolk-sac protein) may arise either from a modification of the rate of pinosome formation, in response to the presence of the different substrates, or from the substrates adsorbing differentially to binding sites on the pinocytosing plasma membrane. The results of experiments with ^{127}I-iodinated insulin, ^{127}I-iodinated lysozyme and ^{127}I-iodinated ribonuclease, as potential modifiers (Table 2.4a), and likewise the corresponding formaldehyde-treated ^{127}I-iodinated proteins (Table 2.4b), show that there is no significant modification of the rate of pinocytic capture of the ^{125}I-PVP. This indicates that the higher Endocytic Indices observed for the protein substrates result from their adsorbing to the pinocytosing plasma membrane. Moreover, the lowered rates of uptake of the formaldehyde-treated proteins arise from a lower degree of adsorption to the pinocytosing plasma membrane rather than from their inhibiting pinosome formation by cytotoxic or other effects.

2.3.2 Release of radioactivity from yolk-sac tissue and analysis of the hydrolysis products of ^{125}I-labelled protein substrates.

The patterns of release of total- and acid-soluble radioactivity from yolk sacs, that had previously ingested one of the ^{125}I-labelled protein preparations, into incubation medium containing no substrate are shown in Figs. 2.4a,b,c, & d. To facilitate comparison with pinocytosis experiments, release of radioactivity is expressed in the same units as uptake (i.e. µl/h per mg yolk-sac protein). The results for each substrate used (formaldehyde-denatured ^{125}I-labelled bovine serum albumin, ^{125}I--labelled insulin, ^{125}I-labelled lysozyme and ^{125}I-labelled ribonuclease) are similar. A large proportion of the tissue-associated radioactivity was released but only a small part was in the form of acid-insoluble

material. It is not clear from these experiments whether the released acid-insoluble radioactivity represents exocytosis of previously pinocytosed substrate or whether it arises from desorption of substrate from surface membranes of the tissue or leakage from dead or dying cells. Whatever the cause, the results indicate that following pinocytosis of any of the above ^{125}I-labelled protein, at least 90-95% of the radioactivity released is acid-soluble.

When yolk sacs, incubated for 2h in the presence of ^{125}I-labelled protein substrates, were subsequently washed at 4°C (as described for the pinocytosis experiments) rather than 37°C (as employed in the release experiments) the radioactivity remaining associated with the yolk-sac tissue was very much greater. The mean values for tissue-associated radioactivity, expressed as µl/h per mg yolk-sac protein following washing at 4°C (and 37°C) were: 60 (28), 52 (18), 106 (30), 86 (44) for the formaldehyde-denatured ^{125}I-labelled bovine serum albumin and the untreated ^{125}I-labelled proteins:insulin, lysozyme and ribonuclease respectively. Continued rapid hydrolysis of ingested protein substrate would be expected during the 37°C wash but not during the 4°C wash. Thus tissue washed at 4°C would be expected to retain virtually all its radioactivity. However, desorption would be expected to occur at both temperatures. The above observation therefore suggests the true proportion of acid-soluble radioactivity released from the yolk sacs is markedly greater than indicated by the data in Figs. 2.4 a,b,c, & d alone. In addition, the release of acid-insoluble radioactivity is complete within 20min for each substrate used, but acid-soluble radioactivity continues to be released after this time. This is more easily explained by the desorption of the acid-insoluble radioactivity from surface membranes and the release of acid-

-soluble radioactivity from an intracellular site, probably lysosomal.

The radioactive species present in the incubation media were examined by gel-filtration on Sephadex G-25. Figs. 2.5 a,b, & c show the elution patterns of intact substrates (^{125}I-labelled insulin, lysozyme and ribonuclease), incubation media in which yolk sacs had been incubated for 2h in the presence of a ^{125}I-labelled protein and incubation media in which yolk sacs, previously loaded with a ^{125}I-labelled protein, had been incubated for 2h in the absence of substrate. In each case incubation with a yolk sac caused a new peak of radioactivity to appear. This peak eluted after the free [^{125}I]iodide peak and in the same position as [^{125}I]iodo-L-tyrosine i.e. a little later than glycyl-[^{125}I]iodo-L-tyrosine. The pooled fractions of these peaks (A, B, C, D, E and F) were further examined using a copper-complex of Sephadex G-25, which resolved each into two peaks. Fig. 2.6 shows a typical elution profile using the low molecular weight material which appeared when yolk sacs were incubated with ^{125}I--labelled insulin (pooled fractions of peak A, Fig. 2.5a). Approximately 10% of the radioactivity is not retained by the copper-complex, and corresponds to oligopeptides containing [^{125}I]iodo-L-tyrosine. A summary of the results obtained for the pooled fractions of peaks A, B, C, D, E and F of Figs. 2.4 a, b & c is given in Table 2.5 together with results obtained with the markers [^{125}I]iodo-L-tyrosine and glycyl-[^{125}I]-iodo-L-tyrosine. Similar results, obtained by Williams *et al.* (1971) when using acid denatured ^{125}I-labelled bovine serum albumin are also presented for comparison. The close similarity in the proportions of radioactive oligopeptide and radioactive amino acid (in the low molecular weight material obtained when a ^{125}I-labelled protein substrate was incubated with yolk sacs and when yolk sacs previously loaded with

^{125}I-labelled protein substrate were incubated in the absence of substrate) suggests that, for all these substrates, the low molecular weight material obtained during the incubation of yolk sacs with ^{125}I-labelled protein results from intracellular digestion.

2.3.3 Analysis of ^{125}I-labelled protein substrates by Sephadex G-75 chromatography.

Each of the untreated ^{125}I-labelled proteins and each of those ^{125}I-labelled protein preparations that showed a modified rate of uptake by yolk sacs were analysed on Sephadex G-75 as described in Section 2.2.5.

Untreated and formaldehyde-treated ^{125}I-labelled insulin preparations each eluted as single components. Similar observations were made with untreated ^{125}I-labelled ribonuclease (Types XA & XIA) and formaldehyde-treated ^{125}I-labelled ribonuclease (Type XA). The resolving power of the Sephadex G-75 column was tested with a mixture of unlabelled ribonuclease aggregates, dimer and monomer prepared by lyophilization of ribonuclease from 50% acetic acid (Crestfield et al., 1962). Recoveries of the insulin and ribonuclease preparations were greater than 95%.

Unlike the insulin and ribonuclease elution profiles, those of untreated, formaldehyde-treated and alkali-treated ^{125}I-labelled lysozyme were complex, see Fig. 2.7. The untreated ^{125}I-labelled lysozyme preparation contained a major component, which co-eluted with unlabelled monomeric lysozyme, and a minor component of lysozyme aggregates a little later than bovine serum albumin (approx. molecular weight 60 000, possibly tetrameric lysozyme). Treatment of ^{125}I-labelled lysozyme with formaldehyde (pH 10) or bicarbonate (pH 10) increased the proportion of lysozyme aggregates. In addition, the formaldehyde-treatment produced a

new component which eluted in a similar position to α-chymotrypsin (molecular weight 24 000) which probably represents dimeric lysozyme rather than lysozyme in which disulphide bonds had been reduced, a species which elutes in a similar position from Bio-gels (Acharya & Taniuchi, 1976) but is severely retarded on Sephadex G-75 (see Fig 2.7) with poor recovery (16.5%). Following the formaldehyde-treatment of ^{125}I-labelled lysozyme no monomeric lysozyme remained.

The monomers, dimers and aggregates of ^{125}I-labelled lysozyme do not appear to be an equilibrium mixture as each gives a single peak of radioactivity in their respective positions on re-running on Sephadex G-75. Each species was found to be stable for at least one week when stored at -20°C also when incubated under the usual culture conditions (see Section 2.2.1).

2.3.4 Uptake of the various ^{125}I-labelled lysozyme species isolated by Sephadex G-75 chromatography.

Five of the ^{125}I-labelled lysozyme species (see Fig. 2.7, peaks I-V) produced by treatment of a single ^{125}I-labelled lysozyme preparation with either formaldehyde (pH 10) or bicarbonate (pH 10) were separated on Sephadex G-75 as described in Section 2.2.3(4) but in the absence of NaN_3. Each lysozyme species was incubated with 17.5-day rat yolk sacs and its rate of uptake determined. The results are shown in Table 2.6. The Endocytic Index of the untreated ^{125}I-labelled lysozyme monomer (species I, 136.0 ± 6.8µl/h per mg yolk-sac protein) was similar to that reported earlier for the untreated ^{125}I-lysozyme preparation (129.9 ± 16.6, see Table 2.2b). The formaldehyde-induced dimer of ^{125}I-labelled lysozyme (species III) and the ^{125}I-labelled lysozyme monomer (species V) remaining after treatment with bicarbonate (pH 10) each have either lower

or unchanged rates of uptake. The aggregated species of ^{125}I-labelled lysozyme (II and IV) induced by either formaldehyde (pH 10) or bicarbonate (pH 10) have markedly lower rates of uptake compared to the other three species of ^{125}I-labelled lysozume (I, III and V). These results suggest that the decreased Endocytic Index observed for ^{125}I-labelled lysozyme following formaldehyde (pH 10) treatment is caused by the presence of ^{125}I-labelled lysozyme aggregates. The accumulation of the various ^{125}I-labelled lysozyme species by the yolk-sac tissue is shown in Fig. 2.3. In general, the features shown here are similar to those described for Fig. 2.3. Also, in general, the higher the degree of aggregation the lower is the observed level of tissue-associated radioactivity.

Table 2.1 Endocytosis of ^{125}I-labelled poly(vinylpyrrolidone) by 17.5-day rat yolk sacs incubated in medium 199 containing 10% (v/v) of calf serum.

Experiments were performed as in Section 2.2.1; yolk sacs were cultured with ^{125}I-PVP, at the concentrations indicated, for up to 6-7h. Values of Endocytic Indices more than 2 standard deviations away from the mean value were not included unless the associated correlation coefficient was greater than 0.95.

Expt. No.	^{125}I-PVP concentration (µg/ml)	Endocytic Index (µl/h per mg yolk-sac protein)	Correlation Coefficient	Intercept on ordinate (µl/mg yolk-sac protein)	No. of yolk sacs per experiment
1	5	1.604	0.977	+0.494	12
2	5	1.632	0.889	+0.570	12
3	5	2.149	0.983	+1.476	11
4	5	2.605	0.965	+0.388	12
5	5	1.848	0.934	+0.889	11
6	5	2.364	0.949	+0.883	12
7	5	2.278	0.998	+1.321	11
8	5	1.920	0.994	+1.227	12
9	5	1.696	0.988	+0.762	14
10	5	1.500	0.982	+0.700	13
11	2	2.220	0.987	-0.173	6
12	2	1.784	0.983	+0.624	7
13	2	2.042	0.985	+0.747	10
14	2	1.840	0.960	+0.595	11
15	2	1.078	0.977	+0.718	10
16	2	1.738	0.978	+1.988	9
17	2	1.678	0.978	+1.323	8
18	2	1.825	0.932	+0.293	10
19	2	1.629	0.971	+0.522	10
20	2	1.707	0.988	+0.006	10
21	2	1.462	0.985	+2.061	9
22	2	1.374	0.957	+1.346	10
23	2	1.322	0.958	+1.760	10
24	2	1.440	0.978	+1.972	10
25	2	1.535	0.991	+0.869	10
26	2	1.608	0.978	+0.614	10
27	2	1.532	0.983	+1.063	10
28	2	1.868	0.978	-0.088	10
29	2	1.586	0.974	+0.867	10
MEAN ± S.D. :-		1.75 ± 0.33			

Table 2.2a Endocytosis of ^{125}I-labelled insulin preparations by 17.5-day rat yolk sacs incubated in medium 199 containing 10% (v/v) of calf serum.

^{125}I-Labelled insulin was prepared as described in Section 2.2.3 and uptake experiments were performed as described in Section 2.2.1 using ^{125}I-labelled insulin at a concentration of 1 µg/ml; the duration of each experiment was 3 h. The results are also summarized in Table 2.3.

^{125}I-Insulin preparation	Expt. No.	% acid--solubles in preparation	Endocytic Index (µl/h per mg yolk sac protein)	Correlation Coefficient	No. of yolk sacs per experiment
Frozen (-20°C.)	1	2.1	120.8	0.918	10
	2	2.1	147.6	0.988	10
	3	2.1	130.1	0.944	9
	4	1.6	190.0	0.978	5
	5	1.7	157.7	0.857	8
	6	2.1	155.7	0.960	10
	MEAN ± S.D. :-		150.3 ± 24.3		
Acetic acid (pH 2.5)	1	1.3	154.2	0.940	10
	2	2.7	168.2	0.914	9
	3	1.7	152.4	0.873	10
	4	1.2	148.0	0.938	8
	MEAN ± S.D. :-		155.7 ± 8.7		
4M-Urea (pH 5)	1	1.5	172.0	0.929	8
	2	1.2	123.2	0.926	8
	3	1.6	148.3	0.962	10
	4	2.3	145.4	0.915	8
	5	1.7	142.8	0.928	8
	MEAN ± S.D. :-		147.3 ± 15.8		
Formaldehyde (pH 10)	1	0.6	62.9	0.985	10
	2	1.8	42.9	0.986	10
	3	3.1	30.6	0.874	10
	4	3.1	42.8	0.983	10
	MEAN ± S.D. :-		44.8 ± 13.4		
Bicarbonate buffer (pH 10)	1	0.4	139.5	0.985	10
	2	0.4	115.9	0.967	10
	3	0.4	116.5	0.960	8
	MEAN ± S.D. :-		129.3 ± 13.5		

Table 2.2b Endocytosis of ^{125}I-labelled lysozyme preparations by 17.5-day rat yolk sacs incubated in medium 199 containing 10% (v/v) of calf serum.

^{125}I-Labelled lysozyme was prepared as described in Section 2.2.3 and uptake experiments were performed as described in Section 2.2.1 using ^{125}I-labelled lysozyme at a concentration of 2.5 µg/ml; the duration of each experiment was 3 h. These results are also summarized in Table 2.3.

^{125}I-Lysozyme preparation	Expt. No.	% acid--solubles in preparation	Endocytic Index (µl/h per mg yolk sac protein)	Correlation Coefficient	No. of yolk sacs per experiment
Frozen (-20°C)	1	6.3	166.8	0.975	6
	2	6.3	121.9	0.966	6
	3	7.1	111.1	0.952	9
	4	8.1	146.6	0.978	10
	5	10.1	129.3	0.980	7
	6	8.2	104.0	0.974	10
	MEAN ± S.D. :-		129.9 ± 16.6		
Acetic acid (pH 2.5)	1	7.1	136.3	0.967	10
	2	10.0	136.3	0.978	7
	3	7.1	129.6	0.980	10
	MEAN ± S.D. :-		134.2 ± 3.9		
4M-Urea (pH 5)	1	4.8	130.0	0.995	8
	2	7.2	136.0	0.965	7
	MEAN :-		133.0		
Formaldehyde (pH 10)	1	2.2	103.6	0.993	9
	2	2.0	78.2	0.978	8
	3	2.5	80.0	0.957	9
	MEAN ± S.D. :-		87.3 ± 12.4		
Bicarbonate buffer (pH 10)	1	1.15	86.7	0.985	8
	2	9.2	60.3	0.955	10
	3	9.2	82.8	0.950	10
	MEAN ± S.D. :-		76.6 ± 14.3		

Table 2.2c Endocytosis of ^{125}I-labelled ribonuclease preparations by 17.5-day rat yolk sacs incubated in medium 199 containing 10% (v/v) of calf serum.

^{125}I-Labelled ribonuclease was prepared as described in Section 2.2.3 and uptake experiments performed as described in Section 2.2.1 using ^{125}I-labelled ribonuclease at a concentration of 1 µg/ml, the duration of each experiment was 3 h. These results are also summarized in Table 2.3.

^{125}I-Ribonuclease preparation	Expt. No.	% acid-solubles in preparation	Endocytic Index (µl/h per mg yolk-sac protein)	Correlation Coefficient	No. of yolk sacs per experiment
Frozen (-20°C)	1	27.0	120.1	0.970	10
	2	2.2	144.6	0.862	6
	3	2.2	135.3	0.954	6
	4	2.2	206.1	0.993	6
	5	2.2	201.7	0.963	6
	6	2.9	167.3	0.991	7
	7	3.0	100.7	0.948	10
	8	3.1	97.5	0.972	10
	MEAN ± S.D. :-		146.6 ± 42.0		
Acetic acid (pH 2.5)	1	11.1	140.5	0.994	9
	2	1.5	100.5	0.978	10
	MEAN :-		120.5		
4M-Urea (pH5)	1	1.1	139.3	0.996	7
Formaldehyde (pH 10)	1	7.8	60.8	0.988	10
	2	7.8	67.2	0.907	9
	3	9.6	53.3	0.939	10
	4	3.0	49.3	0.976	7
	MEAN ± S.D. :-		57.6 ± 7.8		
Bicarbonate buffer (pH 10)	1	9.1	117.3	0.981	10
	2	9.1	134.4	0.945	7
	3	9.4	112.3	0.980	8
	MEAN ± S.D. :-		121.1 ± 11.8		

Table 2.2d Endocytosis of lyophilized ^{125}I-labelled ribonuclease and formaldehyde-denatured ^{125}I-labelled bovine serum albumin preparations by 17.5-day rat yolk sacs incubated in medium 199 containing 10% (v/v) of calf serum.

Lyophilized ^{125}I-labelled ribonuclease and formaldehyde--denatured ^{125}I-labelled bovine serum albumin were prepared as described in Section 2.1.3 and the experiments were performed as described in Section 2.2.1. Ribonuclease preparations were cultured at 1 µg/ml, the albumin preparation at 0.6 µg/ml. The durations of the experiments were 3 h and 6 h for ribonuclease and albumin respectively. The results are also summarized in Table 2.3.

^{125}I-Labelled protein preparation	Expt. No.	% acid--solubles in preparation	Endocytic Index (µl/h per mg yolk-sac protein)	Correlation Coefficient	No. of yolk sacs per experiment
Lyophilized ribonuclease (Frozen, -20°C)	1	5.5	73.1	0.967	10
	2	5.5	69.4	0.965	8
	MEAN	:-	71.2		
Lyophilized ribonuclease. Acetic acid treated (pH 2.5)	1	2.8	58.8	0.947	10
	2	2.8	69.1	0.953	10
	MEAN	:-	63.4		
Lyophilized ribonuclease. 4M-Urea treated	1	2.7	56.9	0.984	9
	2	2.7	79.1	0.959	9
	MEAN	:-	68.0		
Lyophilized ribonuclease. Formaldehyde treated (pH 10)	1	0.8	56.5	0.992	10
	2	0.8	52.7	0.945	10
	MEAN	:-	54.5		
Bovine serum albumin. Formaldehyde treated (pH 10)	1	0.0	71.1	0.925	11
	2	0.0	68.1	0.970	11
	3	1.7	65.8	0.985	10
	4	1.5	80.3	0.986	9
	MEAN ± S.D.	:-	71.3 ± 6.4		

Table 2.3 Summary of Endocytic Indices for ^{125}I-labelled protein preparations incubated with 17.5-day rat yolk sacs.

^{125}I-Labelled protein preparations were treated as indicated (see Section 2.2.3) and incubated with 17.5-day rat yolk sacs as described in Section 2.2.1. The data show the mean Endocytic Index (µl/h per mg yolk-sac protein) for each preparation, and its standard deviation; the number of individual experiments is shown in parentheses. Results of individual experiments are shown in Table 2.2a,b,c & d. Also shown* are the results of similar experiments using bovine serum albumin preparations (from Moore et al., 1977).

	Treatment of ^{125}I-labelled protein preparations				
	Frozen (-20°C)	pH 2.5 Acetic Acid	4M-Urea	pH 10 Formaldehyde	pH 10 Bicarbonate
Insulin	150.3 ± 24.3 (6)	155.7 ± 8.7 (4)	147.3 ± 15.8 (5)	44.8 ± 13.4 (4)	123.9 ± 13.5 (3)
Lysozyme	129.9 ± 16.6 (6)	134.2 ± 3.9 (3)	133.0 (2)	87.3 ± 12.4 (3)	76.6 ± 14.3 (3)
Ribonuclease (Type XA)	146.6 ± 42.0 (8)	120.5 (2)	139.3 (1)	57.6 ± 7.8 (4)	121.1 ± 11.8 (3)
Lyophilized ribonuclease (Type X1A)	71.2 (2)	63.4 (2)	68.0 (2)	54.5 (2)	N.D.
Bovine serum albumin	8.9 ± 2.0 (5)*	14.5 ± 3.1 (4)*	73.3 ± 6.7 (2)*	65.0 ± 11.0 (4)* 71.3 ± 6.4 (4)	8.1* (1)

Table 2.4a Endocytosis of ^{125}I-labelled poly(vinylpyrrolidone) by 17.5-day rat yolk sacs incubated in the presence of ^{127}I-iodinated protein preparations in medium 199 containing 10% (v/v) of calf serum.

The uptake of ^{125}I-PVP at 2 µg/ml was determined as described in Section 2.2.1. The proteins were iodinated ostensibly by the same procedure as for radiolabelling with ^{125}I-iodide (see Section 2.2.3).

^{127}I-Protein preparation	Expt. No.	Endocytic Index (µl/h per mg yolk-sac protein)	Correlation Coefficient	Intercept on ordinate	No. of yolk sacs per experiment
(Matched controls)	1	1.609	0.977	+ 0.614	10
	2	1.532	0.983	+ 1.060	10
	3	1.868	0.978	- 0.090	10
	MEAN ± S.D. :- 1.670 ± 0.176				
^{127}I-Insulin (1 µg/ml)	1	1.237	0.974	+ 1.021	10
	2	1.691	0.960	+ 0.284	8
	3	1.936	0.960	+ 0.090	9
	MEAN ± S.D. :- 1.621 ± 0.355				
^{127}I-Lysozyme (2.5 µg/ml)	1	1.375	0.978	+ 0.583	9
	2	1.986	0.943	+ 0.229	10
	3	1.351	0.965	+ 0.370	10
	MEAN ± S.D. :- 1.580 ± 0.351				
^{127}I-Ribo-nuclease (1 µg/ml)	1	1.373	0.978	+ 0.422	10
	2	1.729	0.953	+ 0.057	10
	3	1.433	0.958	+ 0.332	10
	MEAN ± S.D. :- 1.511 ± 0.191				

Table 2.4b Endocytosis of ^{125}I-labelled poly(vinylpyrrolidone) by 17.5-day rat yolk sacs incubated in medium 199 containing 10% (v/v) of calf serum in the presence of formaldehyde treated ^{127}I-iodinated protein preparations.

The uptake of ^{125}I-PVP at 2 µg/ml was determined as described in Section 2.2.1. The proteins were iodinated ostensibly by the same procedure as for radiolabelling with ^{125}I-iodide and treated with formaldehyde as described for the ^{125}I-protein preparation in Section 2.2.3.

^{127}I-Protein preparation	Expt. No.	Endocytic Index (µl/h per mg yolk-sac protein)	Correlation Coefficient	Intercept on ordinate	No. of yolk sacs per experiment
(Matched controls)	1	1.322	0.953	+ 1.062	10
	2	1.586	0.963	+ 0.305	10
	3	1.148	0.963	+ 1.734	10
	MEAN ± S.D.:-	1.352 ± 0.220			
pH 10 Formaldehyde treated ^{127}I-insulin (1 µg/ml)	1	1.744	0.960	+ 0.671	10
	2	1.360	0.926	+ 0.984	10
	3	1.734	0.950	+ 0.661	9
	MEAN ± S.D.:-	1.613 ± 0.219			
pH 10 Formaldehyde treated ^{127}I-lysozyme (2.5 µg/ml)	1	1.882	0.918	+ 0.466	10
	2	1.648	0.994	+ 0.542	9
	3	2.404	0.887	+ 0.340	10
	MEAN ± S.D. :-	1.978 ± 0.387			
pH 10 Formaldehyde treated ^{127}I-ribonuclease (1 µg/ml)	1	1.475	0.974	+ 1.132	9
	2	1.376	0.928	+ 0.643	9
	3	1.649	0.968	+ 0.280	10
	MEAN ± S.D.:-	1.500 ± 0.138			

Table 2.5 Chromatography of the low molecular weight hydrolysis products of ^{125}I-labelled proteins on the copper complex of Sephadex G-25.

After concentration by freeze-drying, the pooled fractions (A,B,C,D,E and F) from Fig. 2.3 a,b & c, were further analysed on a copper--complex of Sephadex G-25 as described in Section 2.2.4(3). [^{125}I]Iodo--L-tyrosine and glycyl-[^{125}I]iodo-L-tyrosine were used as markers. Values obtained by Williams et al. (1971) for ^{125}I-labelled bovine serum albumin are also shown*. The distribution of the recovered radioactivity between the alkaline fractions and the acid fractions is given as a percentage. Total recovery of radioactivity is given as a percentage.

Low molecular weight hydrolysis products	Elution with borax buffer pH 11.0	Elution with 0.4M-HCl	Recovery of applied label
^{125}I-Iodo-L-tyrosine	2.5	97.5	102.0
^{125}I-Glycyliodo-L-tyrosine	88.4	11.6	88.0
^{125}I-Insulin peak A	11.6	88.4	93.0
^{125}I-Insulin peak B	11.4	88.6	92.0
^{125}I-Lysozyme peak C	8.6	91.4	98.6
^{125}I-Lysozyme peak D	14.4	85.6	98.2
^{125}I-Ribonuclease peak E	11.9	88.1	93.5
^{125}I-Ribonuclease peak F	11.6	88.4	107.0
^{125}I-Bovine serum albumin	14*	86.0*	98.4*

Table 2.6 Endocytosis of the various ^{125}I-labelled lysozyme species by 17.5-day rat yolk sacs incubated in medium 199 containing 10% (v/v) of calf serum.

Five species of ^{125}I-labelled lysozyme were prepared as described in Section 2.2.3(4) (see also Fig. 2.8) and uptake experiments were performed as described in Section 2.2.1. ^{125}I-Labelled lysozyme species were present at a concentration of 1 µg/ml; the duration of each experiment was 3 h.

^{125}I-Labelled lysozyme species	Expt. No.	% acid-soluble in preparation	Endocytic Index (µl/h per mg yolk-sac protein)	Correlation Coefficient	No. of yolk sacs per experiment
I Untreated monomer	1	7.1	140.3	0.989	8
	2	7.1	129.5	0.996	8
	3	7.0	130.9	0.989	9
	4	7.1	143.3	0.995	7
	MEAN ± S.D. :-		136.0 ± 6.8		
II Formaldehyde induced aggregates	1	0.9	63.3	0.994	9
	2	0.6	58.2	0.889	10
	3	0.7	53.5	0.943	10
	MEAN ± S.D. :-		58.3 ± 4.9		
III Formaldehyde induced dimer	1	0.5	109.3	0.939	10
	2	0.7	142.4	0.900	10
	3	0.6	123.1	0.953	10
	MEAN ± S.D. :-		124.9 ± 16.6		
IV Bicarbonate induced aggregates	1	6.0	34.7	0.953	10
	2	5.6	26.2	0.944	10
	3	5.7	33.4	0.978	6
	MEAN ± S.D. :-		32.3 ± 5.7		
V Bicarbonate treated monomer	1	3.9	114.5	0.989	10
	2	3.0	128.1	0.932	8
	3	3.1	113.0	0.922	10
	MEAN ± S.D. :-		118.5 ± 8.3		

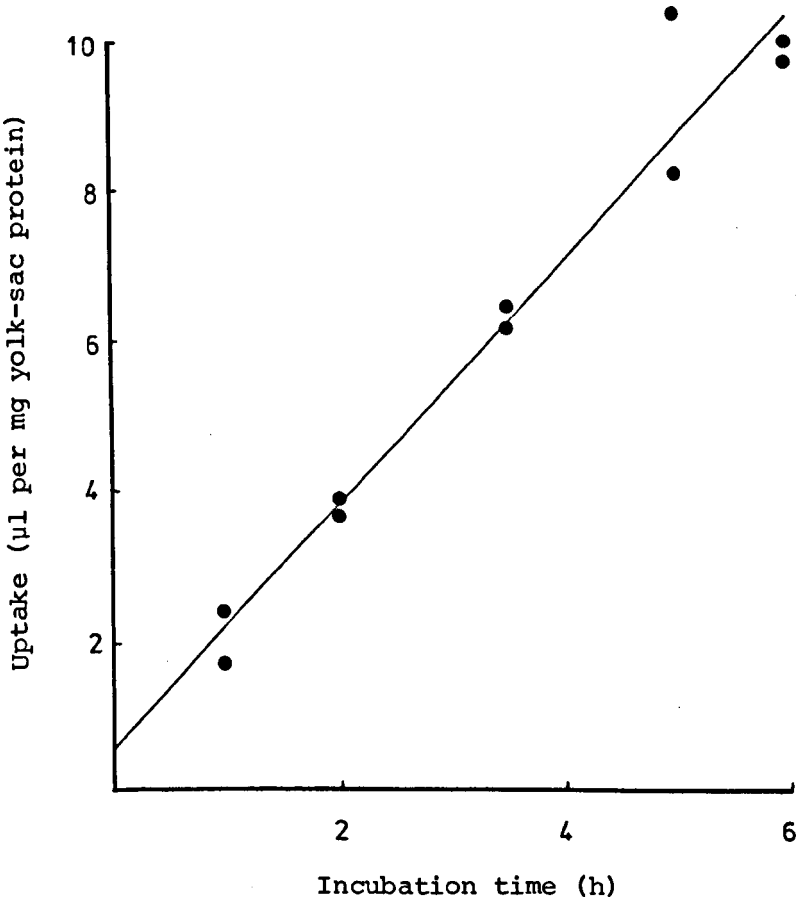


Figure 2.1 Uptake of ^{125}I -labelled poly(vinylpyrrolidone) by 17.5-day rat yolk sacs in culture.

Points are derived from data on single yolk sacs each taken from one animal and incubated separately in medium 199 + 10% (v/v) calf serum, as described in Section 2.2.1, with [^{125}I]-PVP (2 µg/ml of culture medium). Uptake is expressed as the number of microlitres of culture medium whose contained [^{125}I]-PVP has been ingested by unit quantity of tissue in a given time. The rate of uptake or "Endocytic Index" in the representative experiment shown above is 1.61 µl/h per mg yolk-sac protein and the Correlation Coefficient of the regression line is 0.978.

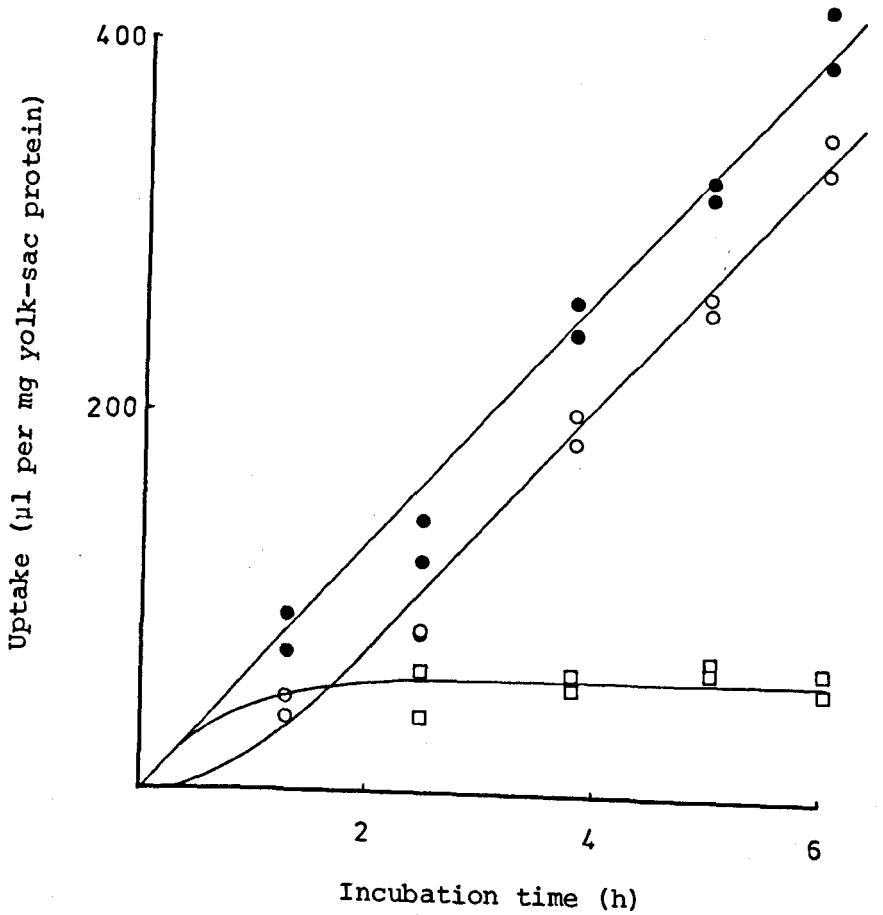


Figure 2.2 Uptake and digestion of formaldehyde-denatured ^{125}I-labelled bovine serum albumin by 17.5-day rat yolk sacs in culture.

Data at each time interval are derived from two yolk sacs, each incubated separately in medium 199 + 10% (v/v) calf serum as described in Section 2.2.1, with formaldehyde-denatured ^{125}I-labelled bovine serum albumin (0.6 µg/ml of culture medium). Uptake (●) is the sum of the tissue-associated radioactivity (□) and the acid-soluble radioactivity released into the culture medium (○). The rate of uptake or "Endocytic Index" is 65.8 µl/h per mg yolk-sac protein and the Correlation Coefficient of the regression line is 0.985.

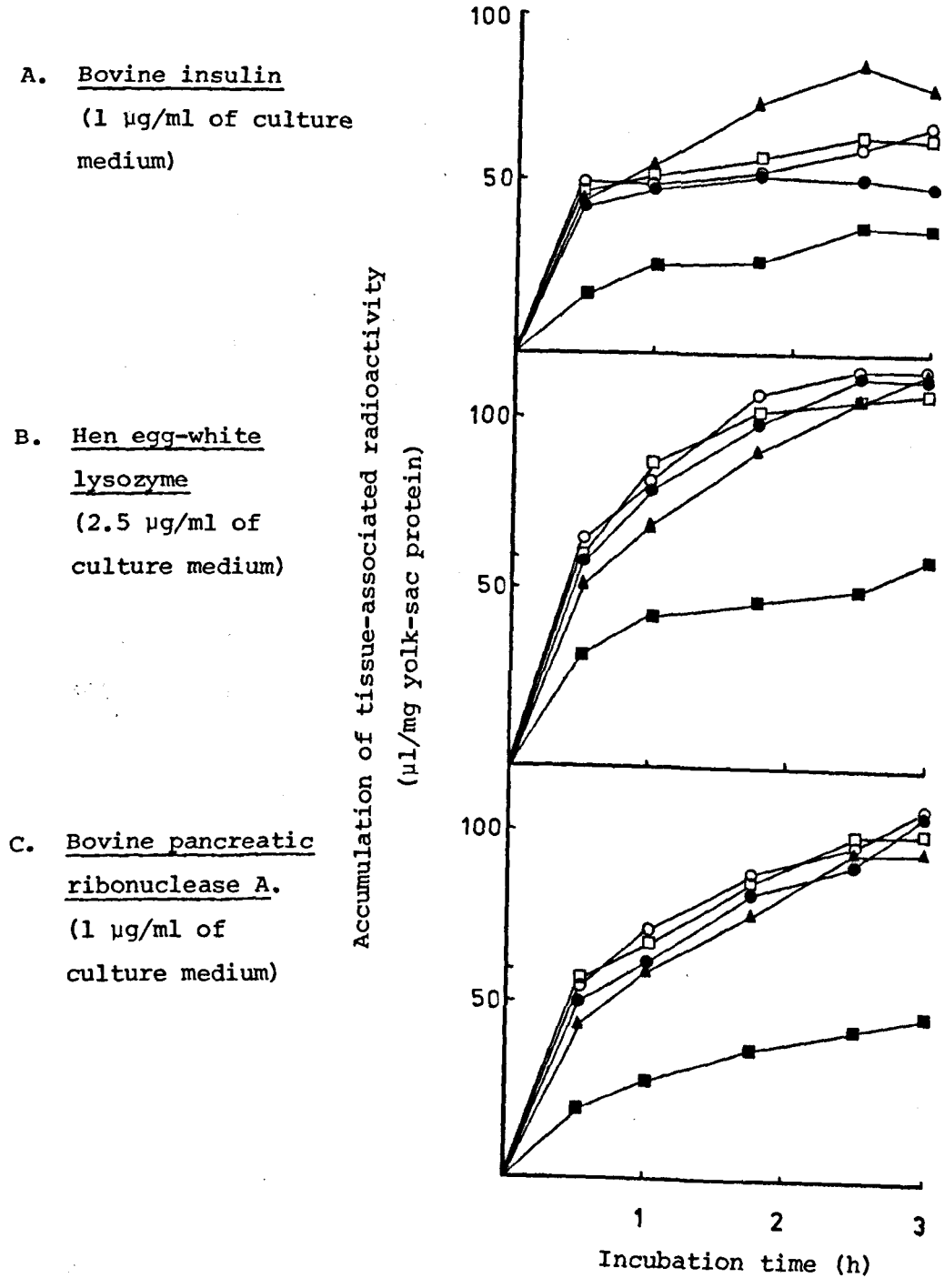


Figure 2.3 Tissue levels of the various preparations of ^{125}I-labelled proteins accumulated by 17.5-day rat yolk sacs in culture.

Each point represents the mean amount of tissue-associated radioactivity in 2-16 yolk sacs incubated with ^{125}I-labelled proteins in medium 199 + 10% (v/v) calf serum, as described in Section 2.2.1. (Individual values fell within ± 20% of the mean value).

Each preparation was either untreated (●), or treated with either: acetic acid, pH 2.5 (○); 4M-urea, pH 5.0 (□); formaldehyde, pH 10 (■); or bicarbonate, pH 10 (▲).

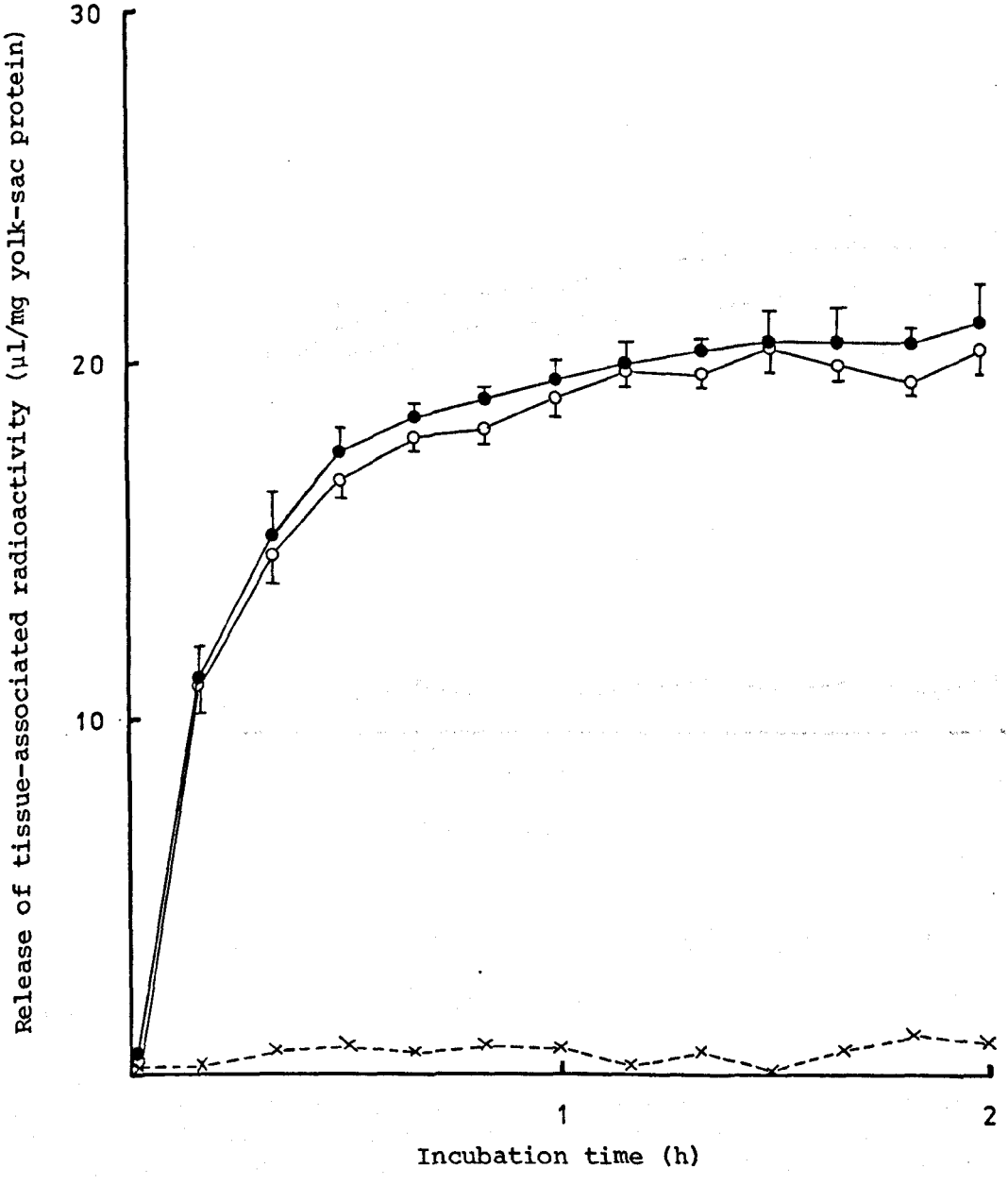


Figure 2.4a Release of tissue-associated radioactivity from yolk sacs previously loaded with formaldehyde-denatured ^{125}I-labelled bovine serum albumin.

Each point represents the mean quantity of radioactivity released by a given time in three separate experiments and the corresponding standard deviation (vertical bar). In each experiment three yolk sacs from one animal were incubated together in the presence of formaldehyde-denatured ^{125}I-labelled bovine serum albumin (5 µg/ml) and the release of total-(●) and acid soluble (o) radioactivity monitored after the transfer of the yolk sacs to fresh culture medium as described in Section 2.2.4. The difference between corresponding values of the total- and the acid-soluble radioactivity gives the acid-insoluble radioactivity released into the medium (×). At the end of the re-incubation period 76.8% of the radioactivity associated with the yolk sac at the start of re-incubation had been released.

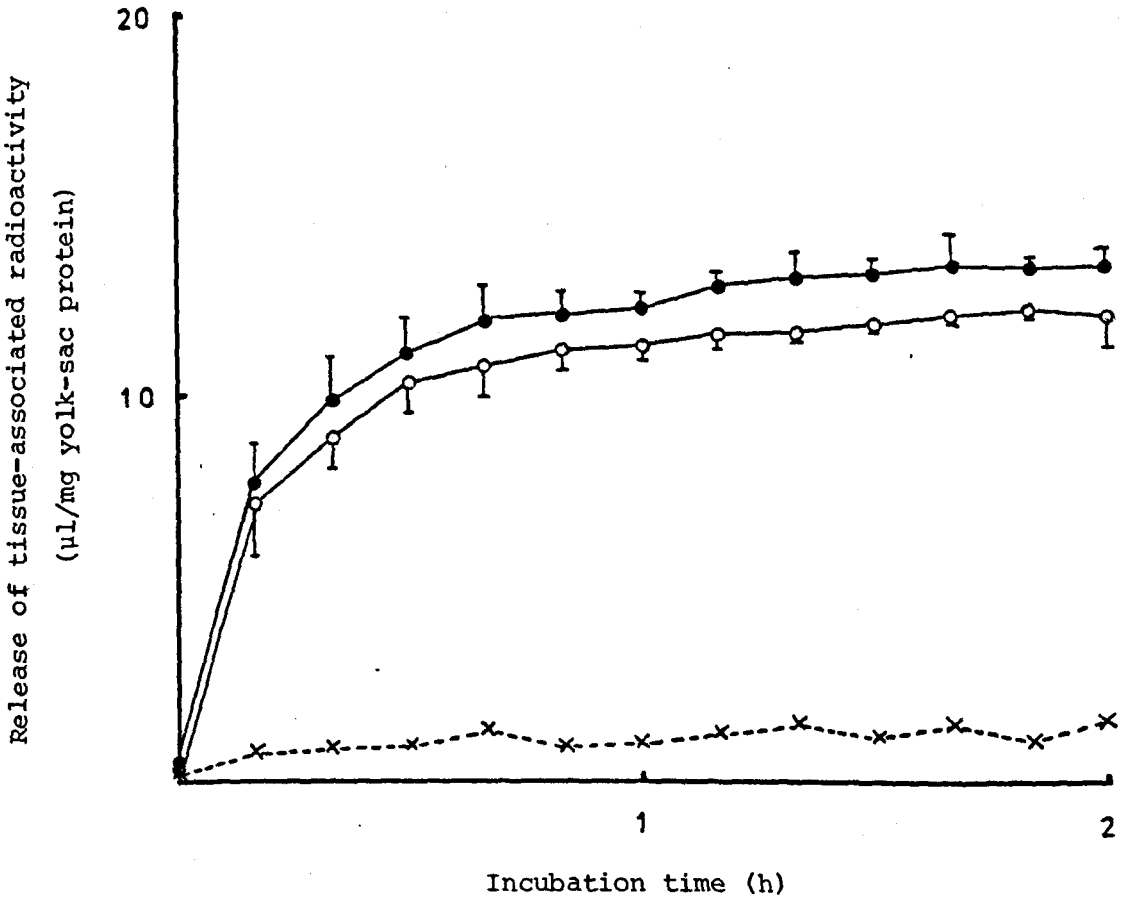


Figure 2.4b Release of tissue-associated radioactivity from yolk sacs previously loaded with ^{125}I-labelled insulin.

Each point represents the mean quantity of radioactivity released by a given time in three separate experiments and the corresponding standard deviation (vertical bar). In each experiment three yolk sacs from one animal were incubated together in the presence of ^{125}I-labelled insulin (5 µg/ml) and the release of total- (●) and acid-soluble (o) radioactivity monitored after the transfer of the yolk sacs to fresh culture medium as described in Section 2.2.4. The difference between corresponding values of the total- and the acid-soluble radioactivity gives the quantity of acid-insoluble radioactivity released into the medium (x). At the end of the incubation period 75.0% of the radioactivity associated with the yolk sac at the start of the re-incubation had been released.

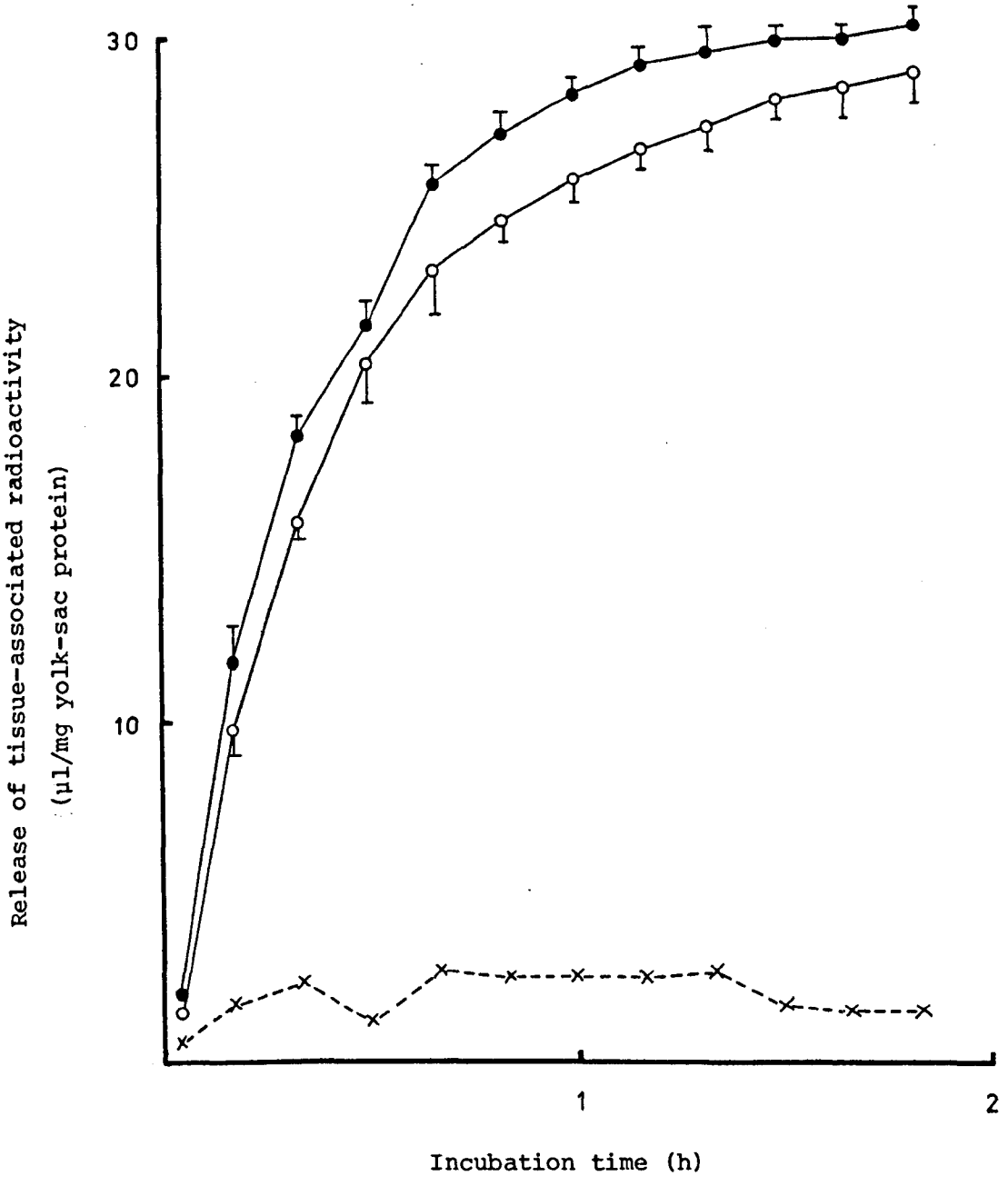


Figure 2.4c Release of tissue-associated radioactivity from yolk sacs previously loaded with ^{125}I-labelled lysozyme.

Each point represents the mean quantity of radioactivity released by a given time in three separate experiments and the corresponding standard deviation (vertical bar). In each experiment three yolk sacs from one animal were incubated together in the presence of ^{125}I-labelled lysozyme (5 µg/ml) and the release of total- (●) and acid-soluble (o) radioactivity monitored after the transfer of the yolk sacs to fresh culture medium as described in Section 2.2.4. The difference between corresponding values of the total- and the acid-soluble radioactivity gives the acid-insoluble radioactivity released into the medium (×). At the end of the incubation period 99.5% of the radioactivity associated with the yolk sac at the start of re-incubation had been released.

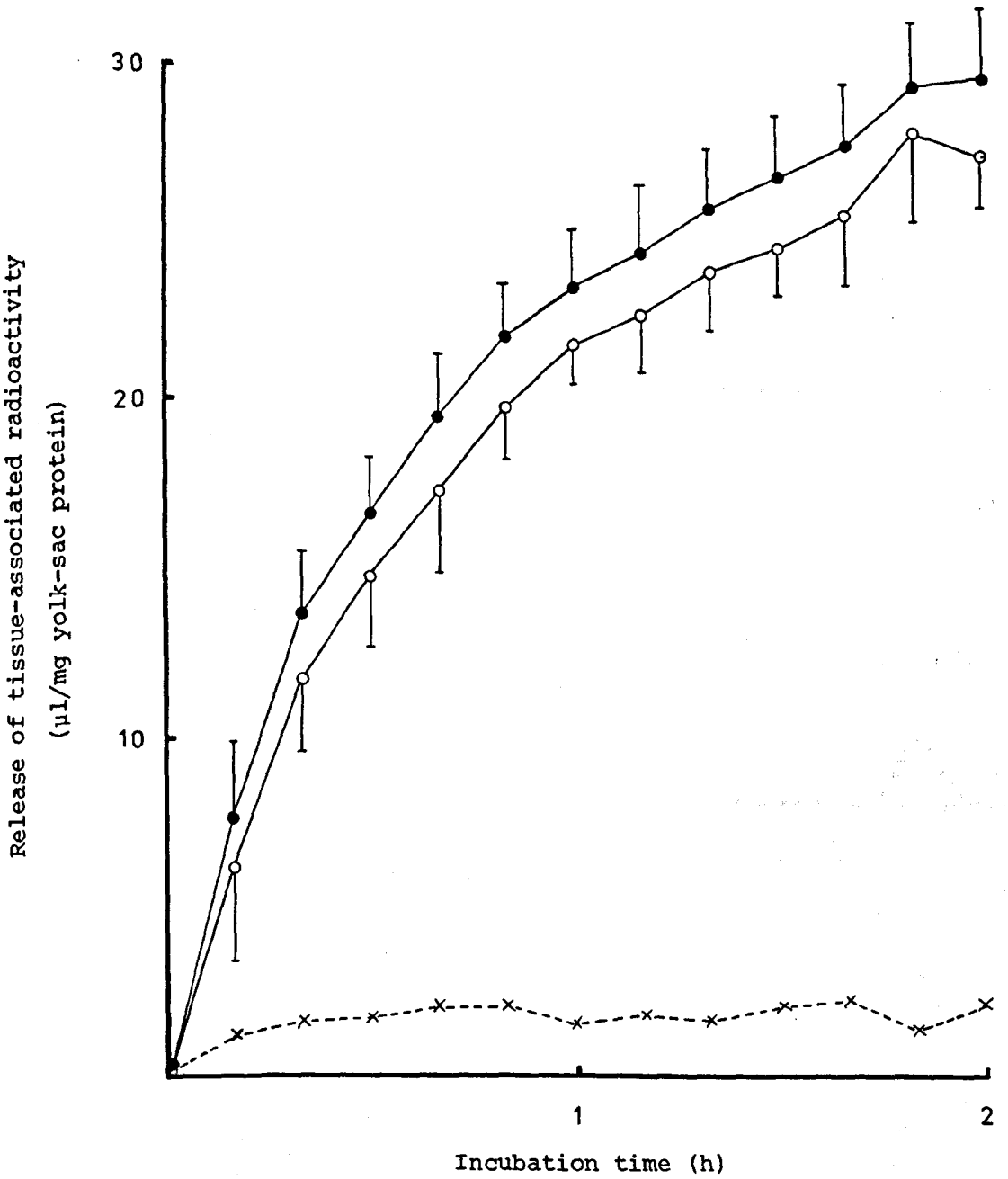


Figure 2.4d Release of tissue-associated radioactivity from yolk sacs previously loaded with ^{125}I-labelled ribonuclease A.

Each point represents the mean quantity of radioactivity released by a given time in three separate experiments and the corresponding standard deviation (vertical bar). In each experiment three yolk sacs from one animal were incubated together in the presence of ^{125}I-labelled ribonuclease (5 µg/ml) and the release of total- (●) and acid-soluble (o) radioactivity monitored after the transfer of the yolk sacs to fresh culture medium as described in the Section 2.2.4. The difference between corresponding values of the total- and the acid-soluble radioactivity gives the acid-insoluble radioactivity released into the medium (x). At the end of the incubation period 67.0% of the radioactivity associated with the yolk sac at the start of re-incubation had been released.

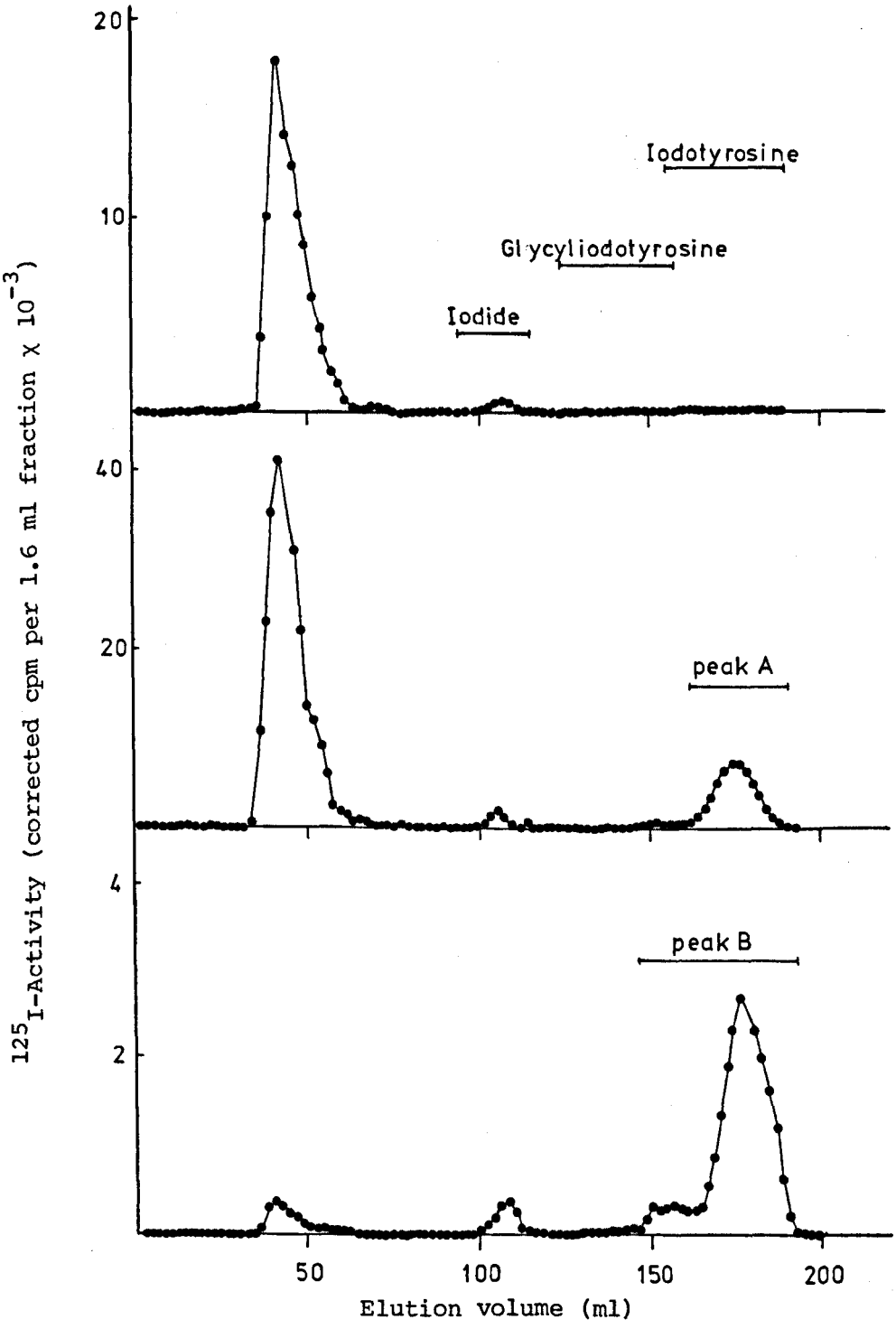


Figure 2.5a Sephadex G-25 chromatography of ^{125}I-labelled insulin hydrolysis products.

Upper, ^{125}I-labelled insulin alone; middle, ^{125}I-labelled insulin after incubation with yolk sacs; lower, radioactivity released on re-incubating yolk sacs previously loaded with ^{125}I-labelled insulin. Experiments were performed as described in Section 2.2.4. Recovery of radioactivity applied to the column was in each case 79%, 89%, 98%, respectively. The horizontal bars give the elution positions of [^{125}I]-iodide, glycyl-[^{125}I]iodo-L-tyrosine and [^{125}I]iodo-L-tyrosine. The pooled fractions of peaks A and B were further analysed on a copper-complex of Sephadex G-25.

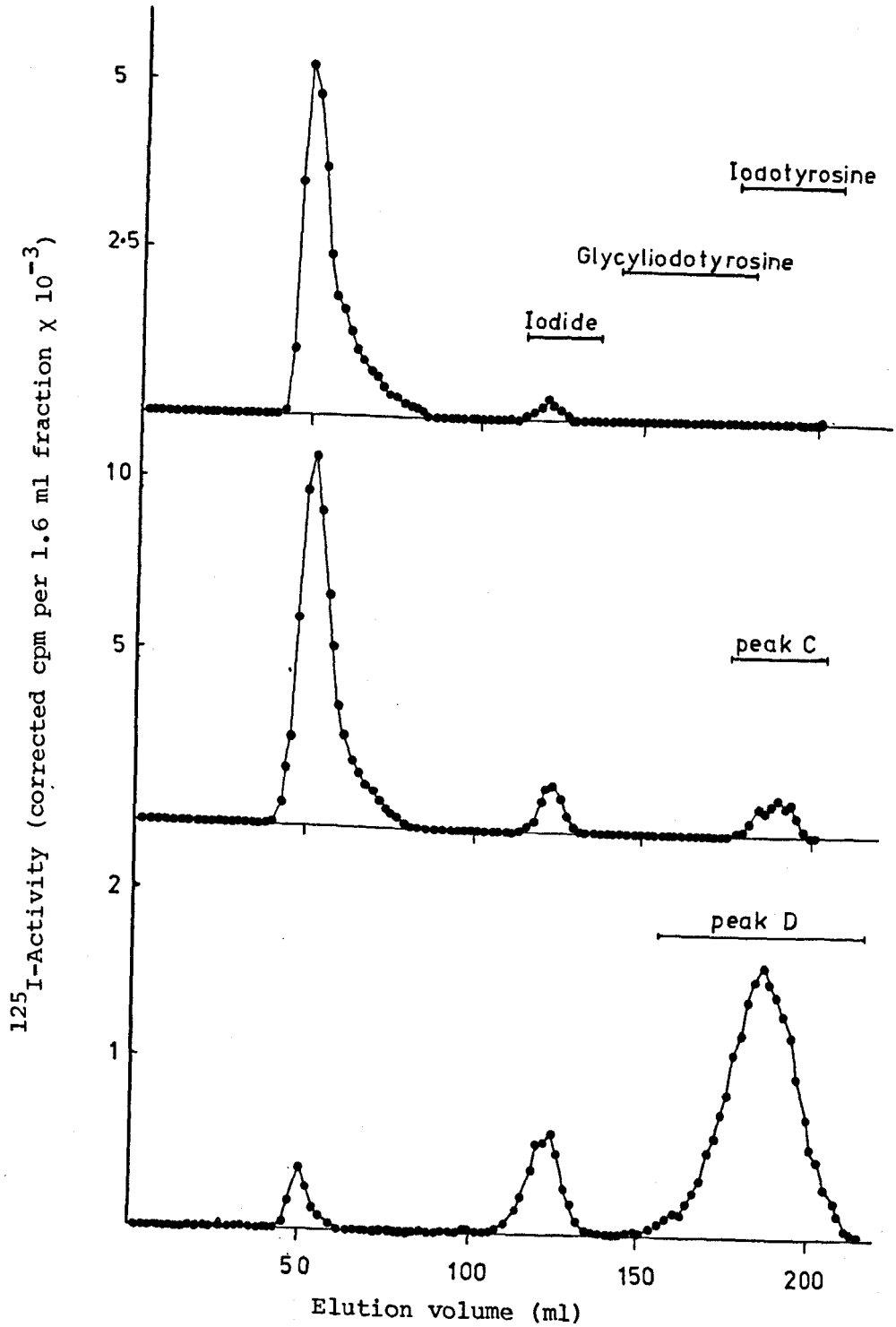


Figure 2.5b Sephadex G-25 chromatography of ^{125}I-labelled lysozyme hydrolysis products.

Upper, ^{125}I-labelled lysozyme alone; middle, ^{125}I-labelled lysozyme after incubation with yolk sacs; lower, radioactivity released on re-incubating yolk sacs previously loaded with ^{125}I-labelled lysozyme. Experiments were as described in Section 2.2.4. Recovery of radioactivity applied to the column was in each case 97%, 80%, 96%, respectively. The horizontal bars give the elution positions of [^{125}I]iodide, glycyl-[^{125}I]iodo-L-tyrosine and [^{125}I]iodo-L-tyrosine. The pooled fractions of peaks C and D were further analysed on a copper-complex of Sephadex G-25.

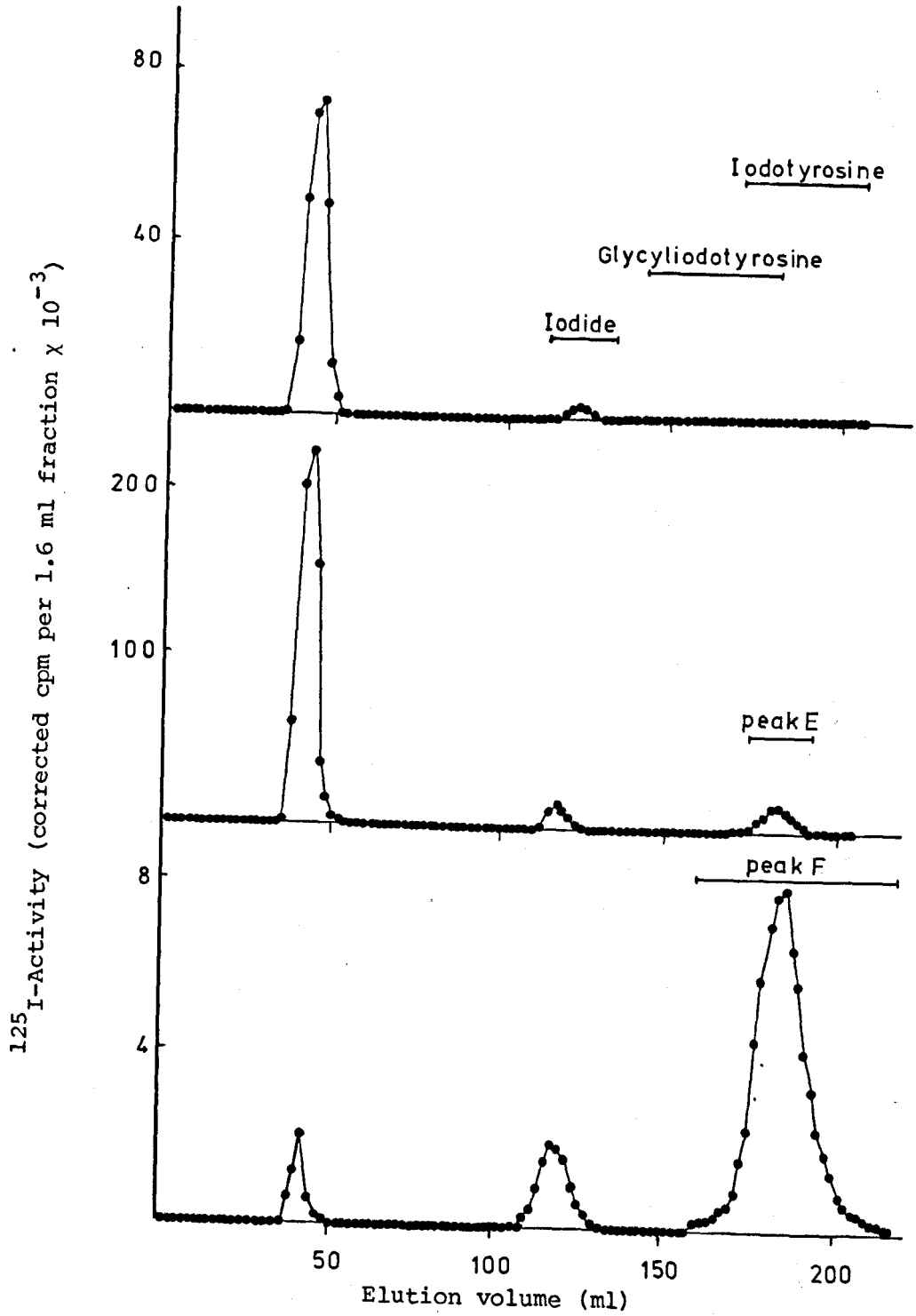


Figure 2.5c Sephadex G-25 chromatography of ^{125}I-ribonuclease hydrolysis products.

Upper, ^{125}I-labelled ribonuclease alone; middle, ^{125}I-labelled ribonuclease after incubation with yolk sacs; lower, radioactivity released from yolk sacs previously loaded with ^{125}I-labelled ribonuclease. Experiments were as described in Section 2.2.4. Recovery of radioactivity applied to the column was, in each case, 104, 108, 103, respectively. The horizontal bars give the elution positions of [^{125}I]iodide, glycyl-[^{125}I]iodo-L-tyrosine and [^{125}I]iodo-L-tyrosine. The pooled fractions of peaks E and F were further analysed on a copper-complex of Sephadex G-25.

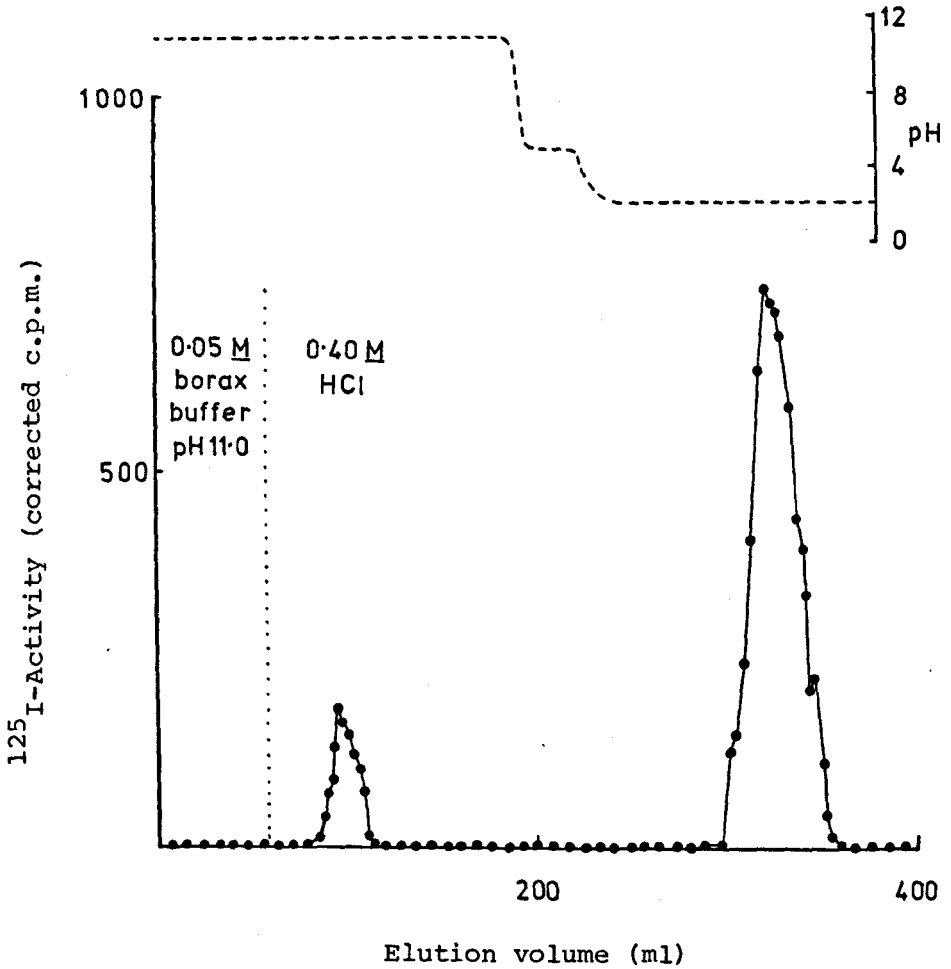


Figure 2.6 Chromatography of the low molecular weight ^{125}I-labelled insulin hydrolysis products on a copper-complex of Sephadex G-25.

The pooled and lyophilized fractions of peak A, Fig. 2.5a, were analysed on a copper-complex of Sephadex G-25 as described in Section 2.2.4(3). This procedure separates amino acids (acid peak) from dipeptide and oligopeptides (alkaline peak) as described by Fazakerley and Best (1965). The quantities of the recovered radioactivity present in the alkaline and acid peaks shown here is 11.6% to 88.4%. Recovery of the applied radioactivity was 93%. The pH of the effluent was measured using indicator paper. Results for similar experiments with the pooled and lyophilized fractions of peaks B, C, D, E and F of Figs 2.5a,b & c, are summarized in Table 2.5 together with those for the radioactive markers glycyl-[^{125}I]iodo-L-tyrosine and [^{125}I]iodo-L-tyrosine.

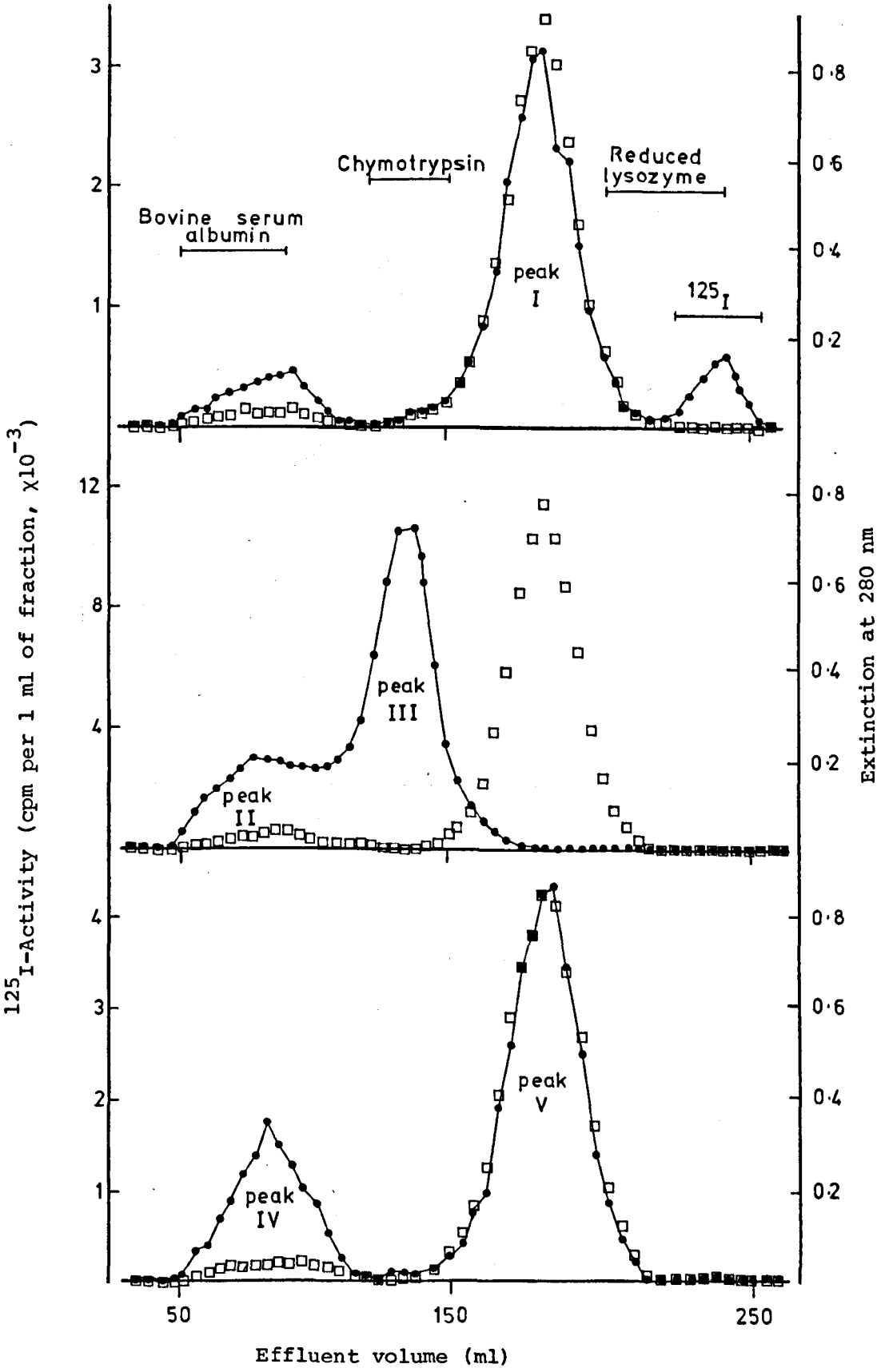
Figure 2.7 Sephadex G-75 chromatography of ^{125}I-labelled lysozyme preparations.

Upper untreated ^{125}I-labelled lysozyme

Middle formaldehyde (pH 10) treated ^{125}I-labelled lysozyme

Lower bicarbonate (pH 10) treated ^{125}I-labelled lysozyme.

In each case 5-25 µg of a ^{125}I-labelled lysozyme preparation in 10 mg unlabelled lysozyme (internal marker) was analysed as described in Section 2.2.5. Radioactivity (•) and extinction at 280 nm (□) of the eluate was monitored. The horizontal bars give the positions of marker substances. Reduced lysozyme was prepared as described by White (1967). Recovery of radioactivity was 98%, 84% and 92% for upper, middle and lower respectively. ^{125}I-labelled lysozyme species (peaks I-V) were prepared and their rates of uptake by the 17.5-day rat yolk sac determined (see Section 2.3.4).


Bovine serum albumin
Chymotrypsin
Reduced lysozyme
peak I
peak II
peak III
peak IV
peak V
125I
125I-Activity (cpm per 1 ml of fraction, χ10-3)
Extinction at 280 nm
Effluent volume (ml)
50
150
250

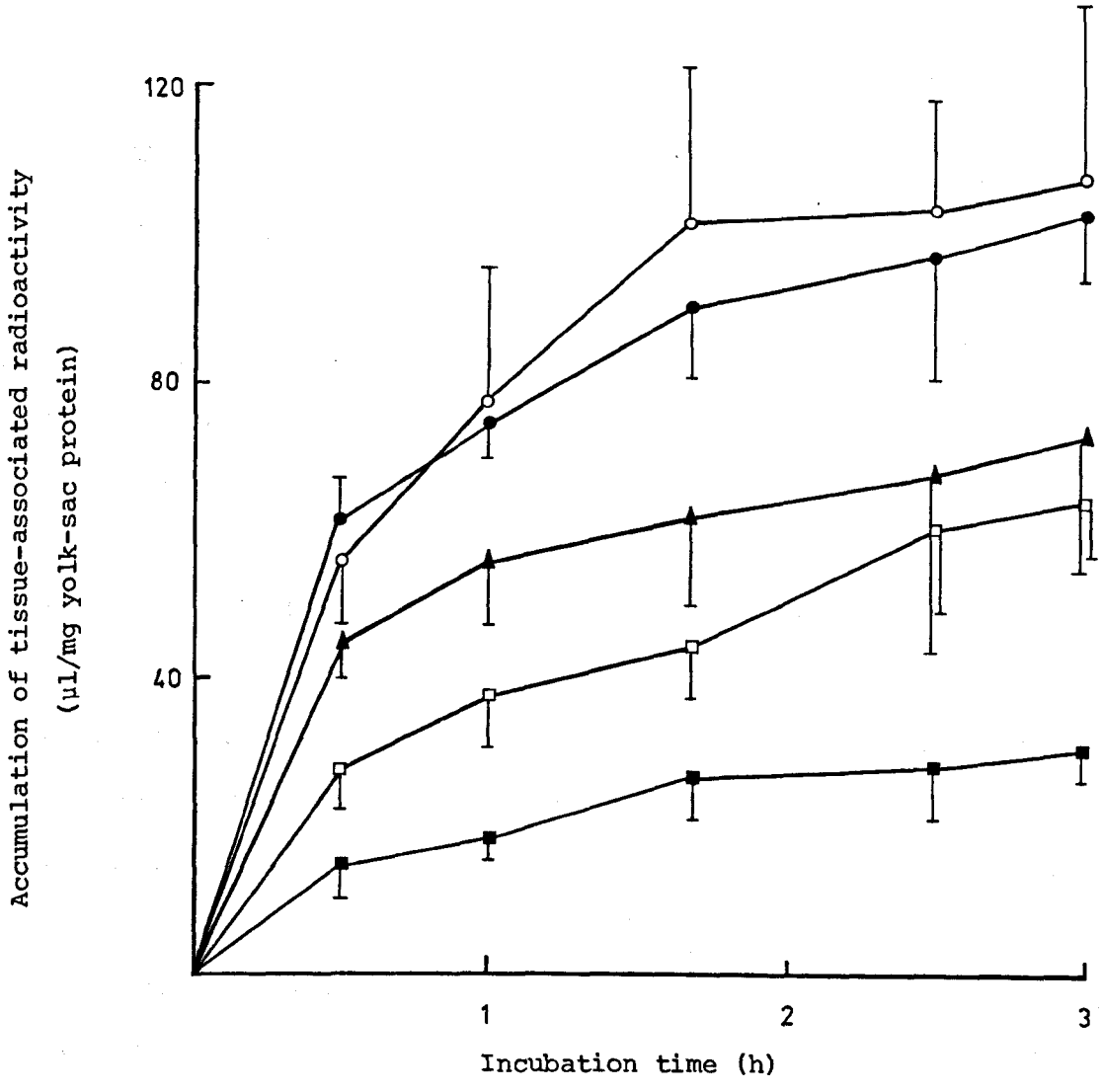


Figure 2.8 Tissue levels of the various preparations of ^{125}I-labelled lysozyme accumulated by 17.5-day rat yolk sacs in culture.

Each point represents the mean amount of tissue-associated radioactivity, and the vertical bar its standard deviation, for 4-8 yolk sacs incubated separately with a ^{125}I-labelled lysozyme preparation (1 µg/ml) in medium 199 + 10% (v/v) calf serum as described in Section 2.2.1.

The ^{125}I-labelled lysozyme preparations were: I, untreated-monomer (●); II, formaldehyde treated-aggregates (□); III formaldehyde treated-dimer (▲); IV, alkaline treated-aggregates (■); V, alkaline treated-monomer (○).

2.4 DISCUSSION

Many quantitative studies of the uptake of proteins by mammalian cells, either *in vivo* or *in vitro*, neglect the fact that, following endocytosis, proteins may be digested extremely rapidly and low molecular weight digestion products released back into the extracellular fluid. Consequently, the rate of uptake of different ^{125}I-labelled proteins, by the 17.5-day rat yolk sac incubated *in vitro*, was monitored by measuring both the amount of the protein label accumulated within the yolk-sac tissue and the amount of acid-soluble digestion products released back into the incubation medium over a given incubation period, then summing the two to give the amount of substrate that would have accumulated in the tissue if proteolysis had not occurred.

Calculation of the rate of uptake (Endocytic Index) of a protein substrate by the yolk-sac tissue in the above manner assumes: 1) that the observed proteolysis occurs exclusively intracellularly and 2) that once a protein is endocytosed it is not subsequently released back into the incubation medium, except as trichloroacetic acid-soluble (low molecular weight) digestion products. There is now conclusive evidence (see Chapters 4 & 7) that a number of protein substrates are digested solely at an intracellular site when they are incubated with yolk sacs, as suggested by Moore *et al.* (1977).

The elution profiles of both the ^{125}I-labelled protein digestion products found in the incubation medium and the radioactivity released on re-incubating yolk sacs previously loaded with the ^{125}I-labelled proteins each show (Figs. : 2.5a,b & c; 2.6 and Table 2.5) the bulk of the radioactive digestion products released by the yolk sacs to be [^{125}I]iodo-L-tyrosine with only a small amount (approx. 10%, see Table 2.5)

of oligopeptides containing an [^{125}I]iodo-L-tyrosyl residue. Williams et al. (1971, 1975b) obtained similar results with 17.5-day rat yolk sacs that had been previously loaded (either in vivo or in vitro) with denatured ^{125}I-labelled bovine serum albumin. These observations are consistent with the known permeability properties of lysosomal membranes (see Reijngoud & Tager, 1977) and suggest a lysosomal site of hydrolysis of each ^{125}I-labelled protein studied in this chapter.

Moreover, in addition to these findings, by studying the nature of the radioactivity released on reincubating yolk sacs previously loaded in vitro with one of the ^{125}I-labelled proteins (formaldehyde-denatured bovine serum albumin, insulin, lysozyme or ribonuclease) it was shown that essentially only acid-soluble radioactivity was returned to the medium (Figs. 2.4a,b,c & d). This indicates that the second of the above assumptions is substantially true for the yolk-sac system for all the above proteins, although Ibbotson (1978) found that this assumption does not hold for ^{125}I-labelled rat IgG preparations.

Steady-state tissue-levels were observed for ^{125}I-labelled albumin, insulin, lysozyme and ribonuclease (see Figs. 2.2 & 2.3). These occur when the tissue degrades the exogenous protein at a rate equal to that of its capture (Williams et al., 1975b). Pinocytic ingestion is thus the rate-limiting step and a possible control point in the overall process of ingestion and catabolism.

The rates of pinocytic ingestion of ^{125}I-labelled insulin, lysozyme and ribonuclease by the 17.5-day rat yolk sac are very rapid (Table 2.3) compared to that of ^{125}I-labelled poly(vinylpyrrolidone) [^{125}I-PVP]. The high Endocytic Indices observed for these proteins (130-150µl/h per mg yolk-sac protein) are approximately twice as large as the highest values previously reported for formaldehyde-treated

^{125}I-labelled bovine serum albumin by Moore et al. (1977; see also Table 2.3) and 14-17 times greater than that reported by the same authors for the equivalent, untreated ^{125}I-labelled bovine serum albumin. This demonstrates that the yolk-sac tissue has a very efficient system for the clearance of some proteins from its extracellular environment.

A number of explanations are possible for the widely different values of Endocytic Index observed for the various protein substrates (see Table 2.3). As described by Jacques (1975), the pinocytosis of a solute may occur in either of the adsorptive phase or the fluid phase or both (see Section 1.6). The upper limit to the rate of capture of fluid by the 17.5-day rat yolk sac [when incubated in medium 199 plus 10% (v/v) calf serum] can be no greater than the rate of uptake of ^{125}I-PVP (1.75 ± 0.33µl/h per mg yolk-sac protein, over this period of study), since this substrate probably enters the yolk-sac epithelial cells only in the fluid phase (Roberts et al., 1977; Ibbotson, 1978). Thus, a substrate with an Endocytic Index of 100µl/h per mg yolk-sac protein either enters 98% via the adsorptive route (due to its affinity for the pinocytosing plasma membrane) or enters via the fluid phase but pinocytosis is stimulated 50-fold,or both processes contribute. That no increase in the rate of accumulation of ^{125}I-PVP occurs when yolk sacs are incubated with either untreated- or formaldehyde-treated ^{127}I-iodinated analogues of each protein (Tables 2.4a & b) suggests that tracer quantities of these proteins do not modify the rate of pinosome formation. Thus the radiolabelled proteins studied here must enter almost entirely by the adsorptive route; the wide variation in the Endocytic Indices observed for the various proteins result from differential adsorption to the external surface of the internalizing plasma membrane. Modification of the adsorptive process should therefore regulate the rate of pinocytic capture of extracellular proteins and

their subsequent catabolism.

It has already been shown that denaturation of serum albumin increases this protein's rate of capture by rat liver *in vivo* (Kitani & Taplin, 1971, 1974; Buys *et al.*, 1973, 1975; Moore *et al.*, 1977) and by 17.5-day rat yolk sacs *in vitro* (Moore *et al.*, 1977). Buys and co-workers offered the explanation that formaldehyde-treatment of bovine serum albumin might result in either the 'unmasking' of recognition sites already existing within the protein molecule or the synthesis of new recognition sites, through chemical modification of functional groups at the surface of the molecule, to give a species that is more rapidly cleared from the rat blood stream. Lloyd (1976) preferred the first of these possibilities, and further suggested that the exposed recognition site is probably hydrophobic in character. Evidence that formaldehyde treatment does not always lead to an increase in the number of recognition sites on proteins (c.f. desialylation of glycoproteins) is shown here by the decreased rate of uptake by the rat yolk sac of ^{125}I-labelled insulin, lysozyme and ribonuclease following their formaldehyde treatment (Table 2.3). Here, the recognition sites, which already exist at the surface of these substrates, become modified so as to reduce their degree of adsorption to the pinocytosing plasma membrane.

We can still only guess at the factors responsible for the modified Endocytic Indices: conformational changes within the protein molecule, chemical modification of the recognition site(s) or the formation of protein aggregates, in which recognition sites become sheltered within the interfacial region between monomers, are all possible explanations. Clearly, the latter effect might explain the lower Endocytic Indices of lysozyme, following its treatment with either alkaline formaldehyde or alkali alone, since lysozyme aggregates are

formed in each of these processes (Fig. 2.7). [The properties of the lysozyme aggregates produced in the presence of alkaline formaldehyde must differ from those of aggregates produced in the presence of alkali alone, since Galenbeck et al. (1977) showed that, during treatment with alkaline-formaldehyde, [^{14}C]formaldehyde bound irreversibly to the lysozyme molecule. However, the chemical modifications that result from the binding of formaldehyde do not appear to appreciably modify the Endocytic Index of lysozyme aggregates (except possibly to enhance the rate of uptake rather than decrease it; see Table 2.6). Moreover, lysozyme dimers, which are presumed to bind formaldehyde (Galenbeck et al., 1977) show an Endocytic Index similar to that of untreated lysozyme monomers.]

The decreased values of the Endocytic Index of both ^{125}I-labelled insulin and ribonuclease, that accompany treatment with formaldehyde, could result from the formation of protein aggregates but this suggestion was excluded by gel-filtration studies. Chemical modifications or changes in the conformation of these macromolecules must therefore be considered. Formaldehyde reacts with proteins in many different ways (e.g. see Means & Feeney, 1968; Meyers & Hardman, 1971; Bizzini & Reynaud, 1974 & Feeney et al., 1975). Chemical modification of ribonuclease with formaldehyde has been reported by Galenbeck et al. (1977) but it is not known if insulin is similarly modified, although this is most likely (see Section 7.4). Likewise, the conformational states of both formaldehyde--treated ribonuclease and insulin have not been established. The inactivation of ribonuclease by formaldehyde has been reported by Zittle (1948), but this may result from chemical modification of the amino group essential for its enzymic activity (Means & Feeney, 1968). A strong indication that the conformation alone of the ribonuclease molecule might be important in determining the rate of clearance of this protein by the rat yolk sac

is given here by the markedly decreased rate of uptake of the lyophilized ribonuclease preparation compared to that of the ribonuclease preparation, isolated from bovine pancreas by the same method, but never lyophilized (Table 2.3). Both of these preparations were shown to be free of aggregates.

The observed decrease in the rate of uptake of the ^{125}I-labelled lysozyme aggregates compared to the ^{125}I-labelled lysozyme monomers contrasts with much of the literature on endocytosis which suggests that, in general, the effect of the formation of protein aggregates is to cause a more rapid rate of capture of a protein by endocytic cells. Thus Bocci, (1970) and Kitani & Taplin (1972, 1974) have shown that serum albumin aggregates are cleared from the rat blood stream more rapidly than albumin monomers. Sherman et al. (1974) suggested that the formation of fibrinogen aggregates following treatment of fibrinogen with high doses of chloramine-T is responsible for its more rapid clearance when radioiodinated using this method. Kooistra et al. (1978) indicated that the in vivo rates of uptake into rat-liver both of ^{125}I-labelled ribonuclease and of ^{125}I-labelled lysozyme were more rapid following chemical modification of these proteins with dimethylsuberidimate which produces protein dimers and higher polymers. Gabathuler & Ryser (1975) demonstrated that sarcoma S180 cells in culture endocytose ferritin more rapidly when it is presented to cells as ferritin aggregates. The actively phagocytic reticulo-endothelial system is responsible for the clearance of each of these protein aggregates from the blood stream and the enhanced endocytosis of ferritin aggregates is caused by an enhanced phagocytic uptake into the sarcoma S180 cells. The behaviour of endocytic cells in respect of their ability to ingest protein aggregates might depend on the relative contributions made to the endocytic process by both phagocytic and

pinocytic mechanisms of uptake (see Section 8.3). Morphological and biochemical evidence (Jollie & Triche, 1971; Goetze et al., 1976) show, however, that the rat yolk sac is not capable of phagocytosis, endocytic uptake being by micropinocytosis alone.

CHAPTER THREE

CHARACTERISTICS OF THE ADSORPTIVE PINOCYTOSIS OF FORMALDEHYDE--DENATURED BOVINE SERUM ALBUMIN AND RIBONUCLEASE BY RAT YOLK SACS

3.1 INTRODUCTION

A number of studies have been published on the rates of uptake of different proteins and, in some, attempts have been made at correlating the observed rates of uptake with certain characteristic properties of the proteins. Thus, Ryser (1970) found that, cultured sarcoma S180 cells endocytosed proteins with an isoelectric point above pH 7 (basic proteins) more rapidly than those with an isoelectric point below pH 7 (acidic proteins). However, Dice & Goldberg (1976) discovered that, *in vivo*, the more acidic of the rat-serum proteins turnover more rapidly than the neutral or basic ones. It is not clear, however, in these studies whether pinocytosis is the rate-limiting step in the turnover of the serum proteins, although this has previously been considered to be so (Mego & McQueen, 1965*a*, Kirsch *et al.*, 1972). Dice & Goldberg (1976) have suggested that limited proteolysis or the denaturation of serum proteins prior to pinocytosis may limit the rate of serum protein turnover. Alternatively, the more acidic serum proteins, which are known to be more resistant to degradation by endoproteinases (Dice & Goldberg, 1976) might resist lysosomal proteolysis and be released to re-enter the circulation (Gordon & Jacques, 1966; Jacques, 1974; Wild, 1973).

The relative rates of uptake of the ^{125}I-labelled basic proteins: lysozyme and ribonuclease (see Chapter 2 of this thesis) and those of the ^{125}I-labelled acidic proteins: bovine serum albumin and human orosomucoid (Moore *et al.*, 1977) in the 17.5-day rat yolk sac in culture are in keeping with the suggestion of Ryser (1970) that basic proteins are endocytosed more rapidly than acidic ones. However, the rapid endocytic uptake, by the 17.5-day rat yolk sac, of ^{125}I-labelled insulin and of formaldehyde-denatured ^{125}I-labelled bovine serum albumin (see Table 2.3) neither of which possess basic characteristics, suggests a factor

other than net charge might govern their rate of endocytosis. Lloyd (1976) has suggested that for the formaldehyde-denatured ^{125}I-labelled bovine serum albumin, hydrophobicity might be such a factor. This might also be true for the ^{125}I-labelled insulin since some 60% of the monomeric form of insulin is hydrophobic in character (Chothia & Janin, 1975).

Because their charge properties are different, ^{125}I-labelled ribonuclease and formaldehyde-denatured ^{125}I-labelled bovine serum albumin may bind to different parts of the plasma membrane that subsequently become pinocytically internalized. This possibility was examined in the section of work that follows.

3.2 METHODS

3.2.1 Method for the rapid determination of quantitative uptake data.

In order to more rapidly assess the effect of compounds on both the endocytosis and the degradation of ^{125}I-labelled proteins, the experimental procedure described in Section 2.2.1 was modified. The modified procedure also permits the use of smaller numbers of yolk sacs and smaller quantities of culture medium, substrate and effector compounds thus saving on animal and material costs.

(1) Rapid method to determine the rate of uptake and degradation of a ^{125}I-labelled protein by the 17.5-day rat yolk sac in serum-free medium 199.

Yolk sacs, dissected from 17.5-day pregnant rats were placed in 50ml Erlenmeyer flasks containing serum-free medium 199 then sealed with a silicone-rubber stopper and incubated at 37°C as described previously (see Section 2.2.1). Either 1 yolk sac was incubated in 19ml of medium 199 or 3 yolk sacs were incubated in 30ml. The experiments were either of 3-4h or less than 1h in duration, respectively. When experiments of 3-4h duration were performed, uptake of substrate commenced on addition of the substrate dissolved in medium 199 (1.0ml), but for experiments of less than 1h duration, substrate was contained within the 30ml of medium so that addition of the yolk sacs initiated uptake. At regular intervals aliquots of the medium (0.5ml, in the 3-4h experiments and 1.0ml in the shorter experiments) were removed, the flasks re-gassed and re-sealed and incubation continued. Incubation was terminated by the removal of yolk sacs from the incubation medium and a final sample of medium was then taken for analysis.

After removal from the incubation medium the yolk sacs were immediately washed in cold aq. NaCl (1% w/v, 4°C) to remove extracellular

substrate, and assayed for radioactivity and protein content as described in Section 2.2.1. Medium samples were assayed for both total- and acid-soluble radioactivity as described before [Section 2.2.1 (1)]. When 0.5ml samples were taken, 0.5ml of aqueous calf serum (20%, v/v) was added before assaying, both to standardize the counting volume to 1.0ml and to aid the precipitation of the ^{125}I-labelled protein substrate. With 1.0ml samples, this was achieved by the addition of 0.1ml of calf serum <u>after</u> assaying for total radioactivity. The acid-soluble radioactivity present in the incubation medium at the start of the uptake experiment was determined by assaying medium containing substrate but no yolk sac.

When effector compounds were studied the effector was added to the medium before introducing the yolk sacs.

(2) <u>Expression of data on uptake and degradation of ^{125}I-labelled proteins.</u>

Uptake (and degradation) data can be expressed conveniently in two ways. Firstly, as the volume of culture medium whose contained substrate has been captured by unit quantity of yolk-sac tissue (units: moles of substrate/mg yolk-sac protein or g of substrate/mg yolk-sac protein). The second expression is obtained from the first by multiplying the uptake value (µl/mg yolk-sac protein) by the mean quantity of substrate (in moles or g) contained in 1µl of culture medium over the interval of the experiment. Expressing the data in either of these ways has the merit of enabling a ready comparison to be made of the results from different experiments (for a list of the merits of the first form of expression see Section 2.2.2).

The volume, V, given by the first expression is equivalent to the Endocytic Index (EI) and can be calculated by the following equation:

$$V = EI = \frac{Y + T_t}{M' \times P \times t} \qquad (3.1)$$

where Y is the total radioactivity in the whole yolk-sac tissue (c.p.m., corrected for background), T_t is the total quantity of acid-soluble radioactivity released by the yolk-sac tissue throughout the incubation period of a given yolk sac (c.p.m., corrected for background), and M' is the mean concentration of radiolabelled substrate (c.p.m. per µl of culture medium, corrected for background) present in the medium over the particular incubation period, t, measured in hours. P is the protein content of the yolk-sac tissue (mg). T_t is calculated from equation 3.2, where T_t is simply the value of T_n at the end of the incubation period of a given yolk sac:

$$T_n = v_i \cdot C_{i(i=n)} + \Sigma_{i=o}^{i=(n-1)} C_i \qquad (3.2)$$

where T_n is the acid-soluble radioactivity (c.p.m.) released up to the time of the n^{th} sampling, v_i is the volume of the culture medium (ml) just prior to the removal of the i^{th} sample [v_i replaces the volume factor, 10, in equation 2.1, Section 2.2.4(2)] and accounts for the decrease in the volume of the culture medium over the period of incubation. C_i is the acid-soluble radioactivity per ml of culture medium in the i^{th} sample (c.p.m., corrected for background and initial acid-soluble radioactivity).

The mean concentration of radiolabelled substrate over a particular incubation period (M' in equation 3.1) is calculated according to equation 3.3.

$$M' = (M_o + M_i - C_i - 2F)\ 0.5 \times 10^{-3} \qquad (3.3)$$

where M_o is the total radioactivity (c.p.m., corrected for background) per ml of culture medium at the start of the incubation period, M_i is the total radioactivity per ml of culture medium of the i^{th} sample (c.p.m., corrected for background) and C_i is the same as in equation 3.2. F, a correction

factor, is the acid-soluble radioactivity per ml of culture medium at the beginning of the incubation period (c.p.m., corrected for background), it is calculated as described in equation A.3 in Appendix II.

Analysis of a plot of the acid-soluble radioactivity released by the yolk-sac tissue (according to equation 3.5) against time enables the proteolytic activity of the yolk sac to be measured. In some experiments the rate of degradation of radiolabelled substrate (µl/h per mg yolk-sac protein) was calculated from the slope of the linear part of the plot, using the expression:

$$T_n' = T_n/M'. P \qquad (3.5)$$

Here T_n' is the acid-soluble radioactivity released by the yolk sac up to the time of the n^{th} sampling (units: µl per mg yolk-sac protein). T_n, M' and P are as defined previously.

To express the data as the quantity of substrate captured or released by unit quantity of yolk-sac tissue, uptake and degradation data (expressed in µl/mg yolk-sac protein) were multipled by C, the quantity of substrate contained in 1µl of medium at the start of the experiment, according to equations 3.6 and 3.7.

$$Q = EI \times C \qquad (3.6)$$

$$Q_n = T_n' \times C \qquad (3.7)$$

where Q is the quantity of substrate captured by the yolk sac per hour of incubation per mg of yolk-sac protein, Q_n is the quantity of substrate degraded and released up to the time of the n^{th} sampling. For the purpose of calculating the substrate concentration in moles/µl, the molecular weights of the bovine serum albumin and ribonuclease were taken to be 68 000 and 13 700, respectively (Weber and Osborn, 1969).

3.1.2 Preparation of protein substrates and potential protein and peptide competitors.

Formaldehyde-denatured ^{125}I-labelled bovine serum albumin and ^{125}I-labelled bovine pancreatic ribonuclease A were prepared as described previously (Section 2.2.3, using Sigma ribonuclease A type XIA). Non-radioactive formaldehyde-denatured bovine serum albumin was prepared ostensibly by the same method as described for the radiolabelled analogue [see Section 2.2.3(4)]. Non-radioactive pancreatic ribonuclease A was Sigma preparation Type 1-A. Other potential competitor proteins and peptides were: tyrosyl-glycyl-glycine, bovine insulin and hen egg-white lysozyme (all from Sigma), bovine calcitonin (Calbiochem, San Diego, California) and bovine serum albumin (Koch-Light, preparation 0142t; purity 99%).

3.3 RESULTS

3.3.1 Endocytosis of formaldehyde-denatured ^{125}I-labelled bovine serum albumin and ^{125}I-labelled ribonuclease by the 17.5-day rat yolk sac incubated in serum-free medium 199.

(1) Effect of substrate concentration on the Endocytic Index. The values of the Endocytic Index for both formaldehyde-denatured ^{125}I-labelled bovine serum albumin and ^{125}I-labelled ribonuclease, in the presence of increasing concentrations of the non-radioactive substrate, were determined by the rapid method described in Section 3.2.1. The results of individual experiments are shown in Tables 3.1 and 3.2 and are summarised in Figs. 3.1 and 3.2. With both substrates a decrease in Endocytic Index was observed with increasing substrate concentration; the Index was most sensitive to changes of substrate concentration at the tracer concentrations normally used in pinocytosis experiments (viz. approx. 1µg/ml of medium). This effect was more pronounced for ribonuclease. Although the Endocytic Index of both protein substrates was substantially decreased in the presence of the corresponding unlabelled substrate (200µg/ml) it still remained far greater than that of ^{125}I-labelled poly(vinylpyrrolidone) a marker of the rate of fluid uptake.

(2) Kinetic analysis of the uptake data. A decrease in the Endocytic Index of a substrate with increasing concentration does not mean that less substrate is captured by the yolk sac, but merely that a smaller proportion of the substrate present in the extracellular fluid is captured. Indeed, expression of the uptake of substrate as p moles/h per mg yolk-sac protein shows that the yolk sac captures more substrate at higher substrate concentrations. This is shown in Tables 3.1 and 3.2 (see also the insert to Figs 3.3 and 3.4).

As stated by Jacques (1975), the rate at which a solute enters a cell by endocytosis is given by the sum of two terms, the first representing uptake in the liquid phase, the second uptake by adsorption on the pinocytosing plasma membrane. Thus, assuming an instantaneous and continuous adsorption equilibrium between substrate and binding sites:

$$Q = Fc + \frac{SRc}{K+c} \qquad (3.8)$$

where Q is the rate of uptake of solute, c its concentration in the external liquid, K the dissociation constant of the substrate-surface complex, and R the maximum amount of substrate than can be adsorbed per unit area of cell-surface. F and S are the rates of endocytic internalization of external liquid and of cell-surface, respectively. Given simple (Michaelis-Menten type) kinetic data, all the terms given in equation (3.8) are potentially variable. For any given substrate which adsorbs to a single class of receptor, R and K will be constant. For substrates with an Endocytic Index very much greater than the rate of fluid capture the term Fc in equation (3.8) becomes negligible so that the rate of uptake of a solute via the adsorptive phase, q, becomes:

$$q = \frac{SRc}{K+c} \qquad (3.9)$$

Taking reciprocals of these quantities an equation analogous to the Lineweaver-Burk equation, used in enzyme kinetics, is obtained:

$$\frac{1}{q} = \frac{K}{SRc} + \frac{1}{SR} \qquad (3.10)$$

Multiplication of each side by SRq gives equation 3.11 and re-arrangement and replacement of q/c by EI (Williams et al., 1975b) gives equation 3.12.

$$SR = \frac{q}{c} K + q \qquad (3.11)$$

$$q = -K.EI + SR \qquad (3.12)$$

Equation 3.11 (and 3.12) is equivalent to the Hoftsee equation used in enzyme kinetics. Like the Lineweaver-Burk equation, the Hoftsee equation gives a plot (here, q against EI) which, given simple kinetic data, is linear and the slope of the line is equal to -K and the intercept on the ordinate axis is equal to SR. The kinetic data obtained in this chapter are more suited to analysis by Hoftsee plot than by the Lineweaver-Burk plot, which would display a cramped distribution of points along its abscissa, enabling only poor interpretation of the data. Also, because the slope of the line in the Hoftsee plot is equal to -K (the dissociation constant of the substrate-cell-surface complex) one can see how K changes when complex kinetic data are plotted.

The effect of the substrate concentration on the rates of capture of formaldehyde-denatured bovine serum albumin and of ribonuclease are shown in Figs. 3.3 & 3.4 (inserts) respectively; hyperbolic plots were obtained for each substrate. The corresponding Hoftsee plots (Figs. 3.3 & 3.4) each gave non-linear plots, indicating that the kinetic data are not simple but complex. As the substrate concentration increases (Endocytic Index decreases) the gradient (negative) of the line increases so that K increases. The Hoftsee plots for both substrates therefore show negative co-operativity, which is defined by an increase in the value of K (i.e. a decrease of the "average affinity" of the substrate for the cell surface) with an increase of substrate concentration (Lewitzi & Koshland, 1970). In addition the data for both substrates show that K changes continuously over the range of substrate concentrations used; a feature,

which indicates that multiple forms of interactions occur between the substrates and the cell-surface.

(3) Effect of exogenous proteins and peptides on the Endocytic Indices of formaldehyde-denatured ^{125}I-labelled bovine serum albumin and ^{125}I-labelled ribonuclease. The decreasing Endocytic Indices observed above with increasing substrate concentration can be accounted for by the competition between each unlabelled protein substrate and its radioactive ^{125}I-labelled analogue both of which are captured adsorptively by the yolk-sac epithelial cells. The degree of specificity of each binding to the plasma membrane was tested by adding various proteins or peptides to the incubation medium before measuring uptake. The potential competitors were added at a concentration 100µg/ml of medium since additions of each non-radioactive substrate at this concentration had been shown to substantially inhibit uptake of its corresponding ^{125}I-labelled analogue.

As shown in Figs. 3.5 & 3.6 (see Tables 3.3 and 3.4 for results of individual experiments) a number of the potential competitors are able to inhibit the uptake of both formaldehyde-denatured ^{125}I-labelled bovine serum albumin and ^{125}I-labelled ribonuclease. This demonstrates that the binding process is not very specific. The tripeptide tyrosyl--glycyl-glycine had no measurable effect on the uptake of either of the protein substrates. Bovine serum albumin was similarly without measurable effect. Insulin, calcitonin and formaldehyde-denatured bovine serum albumin each inhibited the uptake of ^{125}I-labelled formaldehyde-denatured bovine serum albumin whereas the two basic proteins, ribonuclease and lysozyme, did not. With ^{125}I-labelled ribonuclease as substrate, substantial inhibition was observed with lysozyme, insulin, calcitonin and (non--radioactive) ribonuclease.

3.3.2 Effect of exogenous proteins and peptides on the fluid-phase uptake in 17.5-day rat yolk sacs incubated in serum-free medium 199.

The inhibition of ^{125}I-labelled protein uptake by exogenously added proteins can be readily explained by competition for binding-sites which are subsequently internalized during the process of pinosome formation. An alternative explanation is that exogenous proteins modify the rate of pinosome formation. The latter explanation was tested by measuring the Endocytic Index of ^{125}I-labelled poly(vinylpyrrolidone) in the presence of the various proteins and peptides used above (at a concentration of 100µg/ml of medium). Table 3.5 shows that only calcitonin and insulin substantially modified the rate of pinosome formation (approx. a 50% decrease). The lower rate of pinosome formation alone is sufficient to account for the inhibition of ^{125}I-labelled ribonuclease uptake by insulin and, similarly, the inhibition of formaldehyde-denatured ^{125}I-labelled bovine serum albumin uptake by calcitonin.

3.3.3 Effect of exogenous proteins and peptides on the proteolysis of ingested formaldehyde-denatured ^{125}I-labelled bovine serum albumin and ^{125}I-labelled ribonuclease by 17.5-day rat yolk sacs.

Lloyd (1976) concluded that the rate-determining step in the proteolysis of exogenous proteins by the rat yolk sac lies entirely in the uptake phase and that the proteolytic capacity of lysosomes apparently far exceeds demand. In agreement with this notion, a steady-state level of tissue-associated substrate has been observed for all protein substrates described in Chapter 2 (see Section 2.3.1). The precise value of the steady-state level is, however, dependent on the ease with which the proteolytic system can digest a particular protein and return the digestion products to the medium. As described in Section 2.3.1 the steady-state

tissue-level observed for the more easily degradable ^{125}I-insulin is less than that of ^{125}I-labelled ribonuclease, although both substrates have approximately the same Endocytic Index. An indication of the ease with which the yolk sac can digest a substrate and return its digestion products to the incubation medium following its capture by endocytosis is obtained from the ratio of tissue-associated radioactivity (during the steady-state period) to the Endocytic Index. The ratio is termed here the "Catabolic Index"; its units are hours.

Table 3.6 shows how the Catabolic Index differs for a number of different protein substrates (majority of data taken from Chapter 2; that on ^{125}I-labelled orosomucoid and bovine serum albumin from Moore *et al.*, 1977). Plots of tissue-associated radioactivity against Endocytic Index for both formaldehyde-denatured ^{125}I-labelled bovine serum albumin and ^{125}I-labelled ribonuclease, in the presence of either non-radioactive substrate at different concentrations or of a number of other exogenous proteins and peptides, are shown in Figs. 3.7 & 3.8. In each case all the data fall on a straight line which passes through the origin. The slope of each line was 0.477 and 0.425h for the albumin and ribonuclease substrates respectively. This suggests that, following pinocytosis, the digestion of both of the ^{125}I-labelled substrates is affected neither by the presence of increasing quantities of the substrate itself nor by the presence of substantial amounts of different exogenous proteins. This conclusion is supported by the finding that the observed lag-period (i.e. the period before radioactive digestion products are released from the yolk sac into the incubation medium) is also unchanged either on increasing the substrate concentration or by the presence of high concentrations of other exogenous proteins (see Tables 3.1, 3.2 and 3.4).

Table 3.1 Endocytosis of formaldehyde-denatured ^{125}I-labelled bovine serum albumin by 17.5-day rat yolk sacs incubated in serum-free medium : effect of increasing concentrations of non-radioactive formaldehyde-denatured bovine serum albumin.

The uptake of formaldehyde-denatured ^{125}I-labelled bovine serum albumin (1 µg/ml of medium) in the presence of increasing concentrations of non-radioactive formaldehyde-denatured bovine serum albumin was determined as described in Section 3.2.1. The total rate of uptake (µmoles/h per mg yolk-sac protein) was calculated by multiplying the Endocytic Index (µl/h per mg yolk-sac protein) by the total initial substrate concentration [pmoles of substrate (radioactive plus non-radioactive) per µl of culture medium].

Concentration of non-radioactive HCHO-denatured bovine serum albumin (µg/ml)	Expt. No.	Endocytic Index (µl/h per mg yolk-sac protein)	Tissue-associated radioactivity (µl/mg yolk-sac protein)	Total rate of uptake (pmoles/h per mg yolk-sac protein)	Duration of the lag period (min)
0	1	258.9	114.4	3.8	20.9
	2	337.5	152.3	5.0	18.3
	3	276.5	138.9	4.1	23.0
	4	262.1	132.5	3.9	27.0
	5	276.5	142.6	4.0	23.6
	MEAN ± S.D. :-	282.3 ± 31.8	136.2 ± 14.1	4.2 ± 0.5	22.7 ± 3.4
10	1	183.9	81.3	29.7	18.8
	2	199.1	81.9	32.2	19.3
	3	189.4	78.7	30.6	16.7
	MEAN ± S.D. :-	190.8 ± 7.7	80.6 ± 1.7	30.8 ± 1.26	18.2 ± 1.4
30	1	138.1	57.9	58.4	19.2
	2	173.3	73.3	79.0	22.9
	3	166.6	73.7	75.9	19.1
	MEAN ± S.D. :-	159.3 ± 18.7	68.3 ± 9.0	71.7 ± 11.1	20.4 ± 2.2
50	1	112.9	51.4	92.2	21.8
	2	117.2	54.8	87.8	24.2
	3	125.7	57.8	94.3	21.6
	MEAN ± S.D. :-	118.6 ± 6.5	54.7 ± 3.2	91.4 ± 3.3	22.5 ± 1.4
100	1	121.6	55.2	180.6	22.3
	2	136.7	64.2	195.3	20.6
	3	99.7	44.3	148.1	19.7
	MEAN ± S.D. :-	119.3 ± 18.6	54.7 ± 9.9	174.6 ± 24.1	20.9 ± 1.3
200	1	74.9	35.2	221.1	22.2
	2	90.3	43.9	266.9	19.5
	3	106.9	51.3	315.9	19.5
	MEAN ± S.D. :-	90.7 ± 14.5	43.5 ± 8.1	267.9 ± 47.4	20.4 ± 1.6

Table 3.2 Endocytosis of ^{125}I-labelled ribonuclease by 17.5-day rat yolk sacs incubated in serum-free medium : effect of increasing concentration of non-radioactive ribonuclease.

The uptake of ^{125}I-labelled ribonuclease (1 µg/ml of medium) in the presence of increasing concentrations of non-radioactive ribonuclease was determined as described in Section 3.2.1. The total rate of uptake was calculated as described in the legend to Table 3.1.

Concentration of non-radioactive ribonuclease (µg/ml)	Expt. No.	Endocytic Index (µl/h per mg yolk-sac protein)	Tissue-associated radioactivity (µl/mg yolk-sac protein)	Total rate of uptake (pmoles/h per mg yolk-sac protein)	Duration of the lag period (min)
0	1	721.3	312.4	50.8	19.5
	2	562.6	262.5	41.0	21.0
	3	606.1	266.3	44.1	21.9
	4	661.1	254.0	48.2	21.1
	MEAN ± S.D. :-	637.7 ± 68.7	273.8 ± 26.3	46.0 ± 4.3	20.9 ± 1.0
10	1	310.0	125.3	249.4	20.9
	2	288.5	100.6	231.5	20.2
	3	282.9	116.2	227.1	19.6
	MEAN ± S.D. :-	293.8 ± 14.3	116.7 ± 8.4	236.3 ± 11.5	20.2 ± 0.7
30	1	157.7	69.1	356.8	16.6
	2	164.0	66.1	371.3	21.3
	3	149.2	66.0	337.6	19.3
	MEAN ± S.D. :-	156.9 ± 7.4	67.0 ± 1.8	355.2 ± 16.9	19.1 ± 2.4
50	1	119.0	50.0	442.9	20.9
	2	112.8	45.1	420.0	18.8
	3	107.4	50.6	399.7	20.6
	MEAN ± S.D. :-	113.1 ± 5.8	48.6 ± 3.0	420.9 ± 54.2	20.1 ± 1.1
100	1	72.2	29.9	532.5	18.3
	2	74.9	30.0	552.4	21.3
	3	83.1	38.1	612.5	19.3
	MEAN ± S.D. :-	76.7 ± 5.7	32.6 ± 4.7	565.8 ± 41.6	19.6 ± 1.5
200	1	47.6	18.4	698.8	12.9
	2	56.4	25.2	828.8	16.2
	3	62.7	28.4	921.4	11.4
	MEAN ± S.D. :-	55.5 ± 7.5	24.0 ± 5.1	816.3 ± 111.8	13.5 ± 2.4

Table 3.3 Endocytosis of formaldehyde-denatured ^{125}I-labelled bovine serum albumin by the 17.5-day rat yolk sacs incubated in serum-free medium : effect of added (non-radioactive) exogenous proteins or peptides.

The uptake of formaldehyde-denatured ^{125}I-labelled bovine serum albumin (1 µg/ml of medium) in the presence of an added protein or peptide (100 µg/ml), was determined as described in Section 3.2.1. Controls (no addition of protein or peptide) were performed in parallel and are derived from Table 3.1.

Non-radioactive protein/peptide (Present at a concentration of 100 µg/ml)	Expt. No.	Endocytic Index (µl/h per mg yolk-sac protein)	Tissue-associated radioactivity (µl/mg yolk-sac protein)	Duration of the lag period (min)
None	MEAN ± S.D. :-	282.3 ± 31.8	136.2 ± 14.1	22.7 ± 3.4
Tyrosyl-glycyl-glycine	1	295.0	138.9	25.1
	2	230.2	140.4	23.4
	3	245.5	129.3	22.4
	MEAN ± S.D. :-	256.9 ± 33.8	136.2 ± 6.0	23.6 ± 1.4
Calcitonin	1	152.3	82.4	22.9
	2	219.3	100.2	20.6
	3	142.6	74.4	22.1
	MEAN ± S.D. :-	171.4 ± 41.8	85.7 ± 13.2	21.9 ± 1.2
Insulin	1	64.2	27.3	13.7
	2	85.9	27.9	25.0
	3	79.2	37.2	17.8
	MEAN ± S.D. :-	76.4 ± 11.1	30.8 ± 5.5	18.8 ± 5.7
Ribonuclease	1	365.9	161.0	27.2
	2	318.0	145.8	25.4
	3	317.3	141.2	22.1
	MEAN ± S.D. :-	333.7 ± 27.8	149.3 ± 10.4	24.9 ± 2.6
Lysozyme	1	310.8	124.1	21.5
	2	321.8	127.4	17.3
	3	301.4	127.8	20.6
	MEAN ± S.D. :-	311.3 ± 10.2	126.4 ± 2.0	19.8 ± 2.2
Bovine serum albumin	1	342.0	157.9	18.5
	2	274.7	147.9	20.0
	3	314.0	159.9	22.5
	MEAN ± S.D. :-	310.2 ± 33.8	155.2 ± 6.4	20.3 ± 2.0

Table 3.4 Endocytosis of ^{125}I-labelled ribonuclease by 17.5-day rat yolk sacs incubated in serum-free medium : effect of added (non-radioactive) exogenous proteins or peptides.

The uptake of ^{125}I-labelled ribonuclease (1 µg/ml of medium) in the presence of an added protein or peptide (30 or 100 µg/ml) was determined as described in Section 3.2.1. Controls (no addition of protein or peptide) were performed in parallel and are derived from Table 3.2.

Non-radioactive protein/peptide	Conc^{n}. (µg/ml)	Expt. No.	Endocytic Index (µl/h per mg yolk-sac protein)	Tissue-associated radioactivity (µl/mg yolk-sac protein)	Duration of the lag period (min)
No addition	MEAN ± S.D. :-		637.7 ± 68.7	273.8 ± 26.3	20.9 ± 1.0
Tyrosyl-glycyl-glycine	30	1	635.6	287.7	23.6
	100	1	690.6	293.2	20.0
		2	508.6	220.6	23.3
		3	676.4	313.6	22.7
	MEAN ± S.D. :-		625.2 ± 101.2	275.8 ± 48.9	22.0 ± 1.8
Calcitonin	30	1	214.3	103.4	24.1
		2	239.1	122.9	23.0
	100	1	134.5	57.7	17.3
		2	115.9	46.7	16.5
		3	138.3	61.6	22.9
	MEAN ± S.D. :-		129.6 ± 11.9	55.3 ± 7.7	18.9 ± 3.5
Insulin	30	1	454.5	210.3	17.5
	100	1	424.5	192.5	15.3
		2	346.4	172.4	25.0
		3	345.3	168.2	21.6
	MEAN ± S.D. :-		372.0 ± 45.4	177.7 ± 12.9	20.6 ± 4.9
Lysozyme	30	1	402.4	178.4	20.4
		2	461.8	228.3	21.7
	100	1	397.7	174.7	19.6
		2	398.5	185.5	20.0
		3	327.0	127.4	21.7
	MEAN ± S.D. :-		374.4 ± 41.0	162.5 ± 30.9	20.2 ± 0.7
Bovine serum albumin	30	1	570.1	265.4	18.7
		2	674.5	286.7	19.9
	100	1	603.5	256.2	18.0
		2	731.2	293.0	21.5
		3	543.4	245.9	21.3
	MEAN ± S.D. :-		626.0 ± 95.9	265.0 ± 24.7	20.3 ± 2.0
HCHO-denatured bovine serum albumin	100	1	510.7	221.7	23.6
		2	567.8	252.0	21.2
		3	602.2	254.8	20.0
	MEAN ± S.D. :-		560.2 ± 46.2	242.8 ± 18.3	21.6 ± 1.8

Table 3.5 Endocytosis of ^{125}I-labelled poly(vinylpyrrolidone) by 17.5-day rat yolk sacs incubated in serum-free medium : effect of added proteins or peptides.

The uptake of ^{125}I-PVP (2 µg/ml of medium) was determined as described in Section 2.2.1 but in the absence of calf serum.

Non-radioactive protein/peptide (present at a concentration of 100 µg/ml)	Expt. No.	Endocytic Index (µl/h per mg yolk-sac protein)	Correlation Coefficient	Intercept on ordinate (µl/mg yolk--sac protein)	No. of yolk sacs per experiment
Control (no addition)	1	2.226	0.941	+ 2.226	9
	2	3.421	0.975	+ 1.134	10
	3	4.580	0.964	+ 4.730	8
	4	4.165	0.974	+ 3.310	9
	5	4.949	0.932	+ 4.810	10
	6	3.956	0.956	+ 5.270	8
	7	3.985	0.940	+ 1.240	9
	8	2.965	0.969	+ 0.853	10
	MEAN ± S.D. :- 3.781 ± 0.881				
Tyrosyl-glycyl--glycine	1	3.679	0.956	+ 2.699	10
	2	3.552	0.961	+ 1.709	10
	3	3.383	0.974	+ 1.537	10
	MEAN ± S.D. :- 3.538 ± 0.148				
Calcitonin	1	2.192	0.925	+ 1.735	9
	2	1.624	0.968	+ 0.824	8
	3	2.005	0.967	+ 0.983	10
	MEAN ± S.D. :- 1.940 ± 0.289				
Insulin	1	1.640	0.934	+ 1.605	10
	2	2.303	0.957	+ 0.853	10
	3	1.661	0.940	+ 1.735	9
	4	1.917	0.953	+ 1.106	13
	MEAN ± S.D. :-1.880 ± 0.309				
Ribonuclease	1	3.128	0.975	+ 0.419	10
	2	2.517	0.955	+ 0.056	8
	3	2.966	0.988	+ 0.896	10
	MEAN ± S.D. :- 2.870 ± 0.316				
Lysozyme	1	2.682	0.981	+ 1.698	9
	2	3.555	0.930	+ 3.294	10
	3	4.302	0.951	+ 2.458	9
	MEAN ± S.D. :- 3.513 ± 0.811				
Bovine serum albumin	1	3.132	0.900	+ 1.941	11
	2	3.018	0.990	+ 0.435	12
	3	3.945	0.941	+ 1.311	9
	MEAN ± S.D. :- 3.365 ± 0.506				
Formaldehyde--denatured bovine serum albumin	1	3.406	0.972	+ 1.530	10
	2	2.165	0.976	+ 1.145	9
	3	2.682	0.958	+ 1.182	8
	MEAN ± S.D. :- 2.751 ± 0.623				

Table 3.6 Catabolic Indices of various ^{125}I-labelled protein substrates digested by 17.5-day rat yolk sacs that were incubated in medium 199 containing 10% (v/v) of calf serum.

The Catabolic Index, defined as the mean time taken by the yolk sac to digest and release a substrate once it has been captured by endocytosis, is the ratio of the tissue-associated radioactivity (in the steady state period) to the Endocytic Index. The Catabolic Index therefore has the units of hours. The Catabolic Indices were calculated from data presented in Chapter 2 and from data* published by Moore et al., (1977). In these experiments yolk sacs were cultured in medium containing 10% (v/v) calf serum.

Treatment (as defined in Section 2.2.3)	^{125}I-labelled substrate					
	*Oroso-mucoid	*Bovine serum albumin	Insulin	Lysozyme	Ribonuclease (Type XA)†	(Type XIA)†
Frozen	1.50	0.78	0.32	0.85	0.72	1.33
pH 2.5 Acetic Acid	-	0.86	0.42	0.86	0.78	1.31
Urea, pH 5.0	1.45	0.88	0.44	0.81	0.72	1.24
Formaldehyde	3.64	1.53	0.82	0.68	0.84	0.62
Bicarbonate	1.66	0.86	0.62	1.48	0.78	-

†See Section 2.2.3(1).

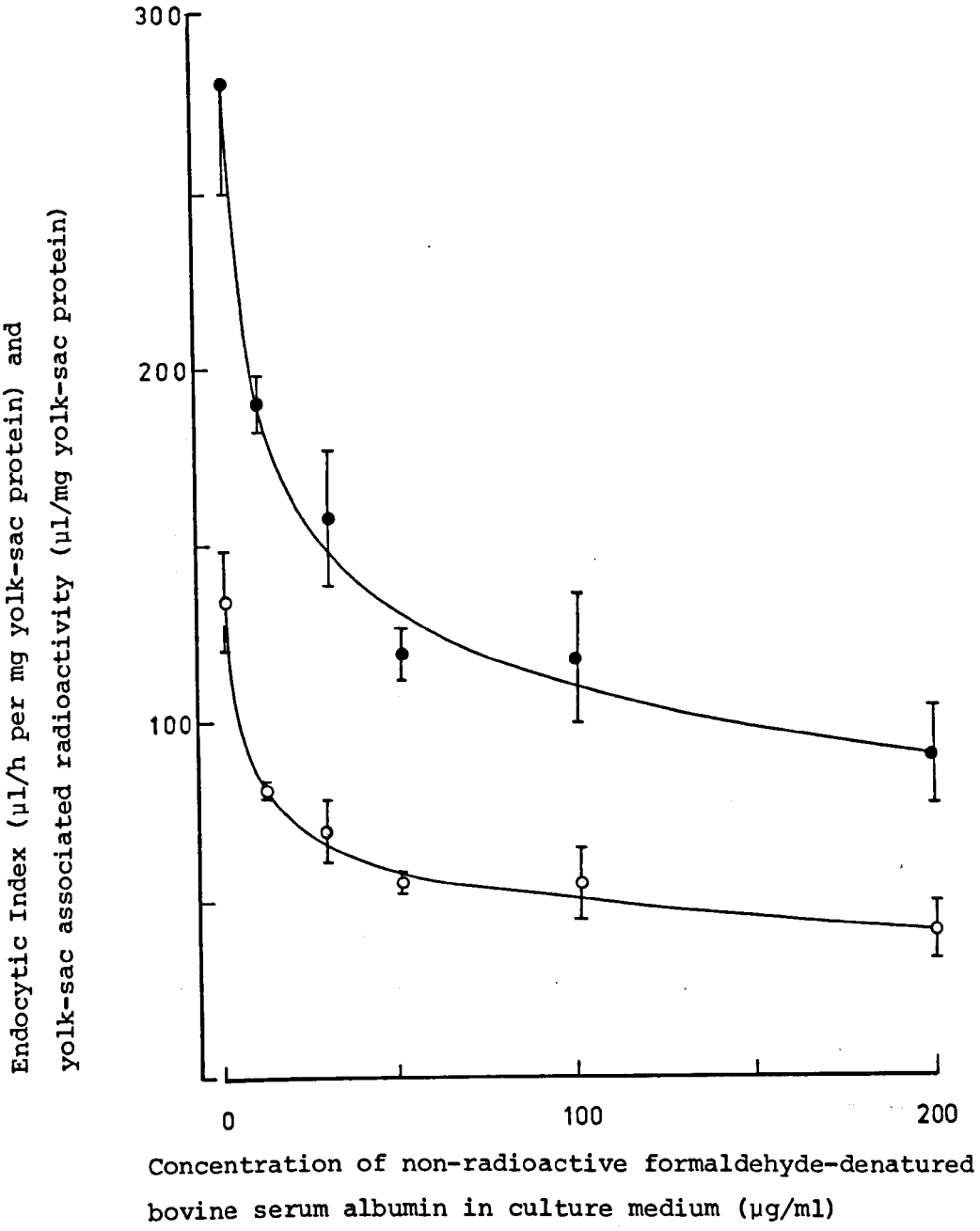


Figure 3.1 Endocytosis of formaldehyde-denatured ^{125}I-labelled bovine serum albumin by 17.5-day rat yolk sacs incubated in serum-free medium 199 in the presence of increasing concentrations of non-radioactive formaldehyde--denatured bovine serum albumin.

The Endocytic Index (●) and the yolk-sac associated radioactivity (o) were determined as described in Section 3.2.1. Each value shown is the mean (± S.D.) of results obtained using yolk sacs from three or five different animals (see Table 3.1).

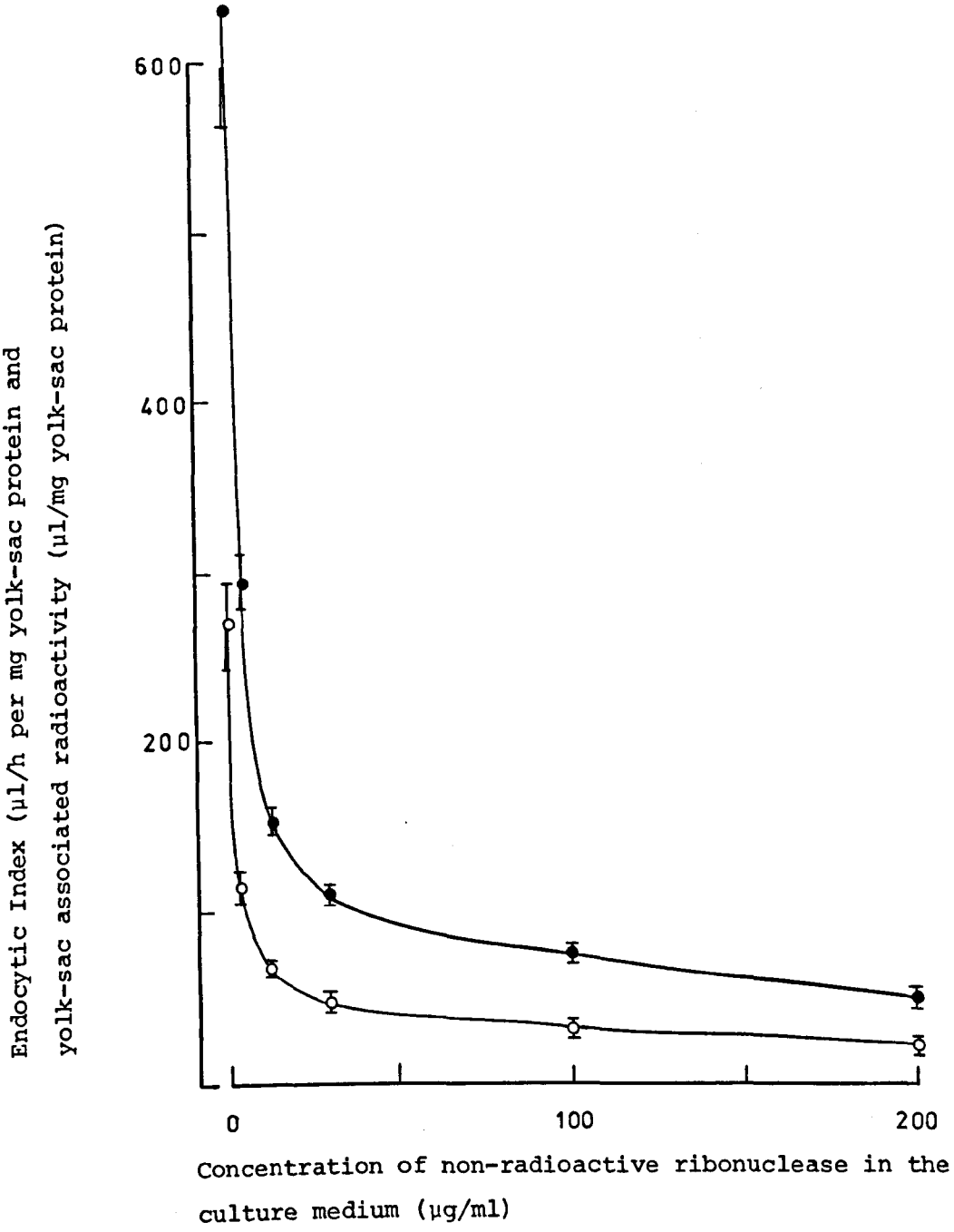


Figure 3.2 Endocytosis of ^{125}I-labelled ribonuclease by 17.5-day rat yolk sacs incubated in serum-free medium 199 in the presence of increasing concentrations of non-radioactive ribonuclease.

The Endocytic Index (●) and the yolk-sac associated radioactivity (o) were determined as described in Section 3.2.1. Each value shown is the mean (± S.D.) of results obtained using yolk sacs from three or four different animals (see Table 3.2).

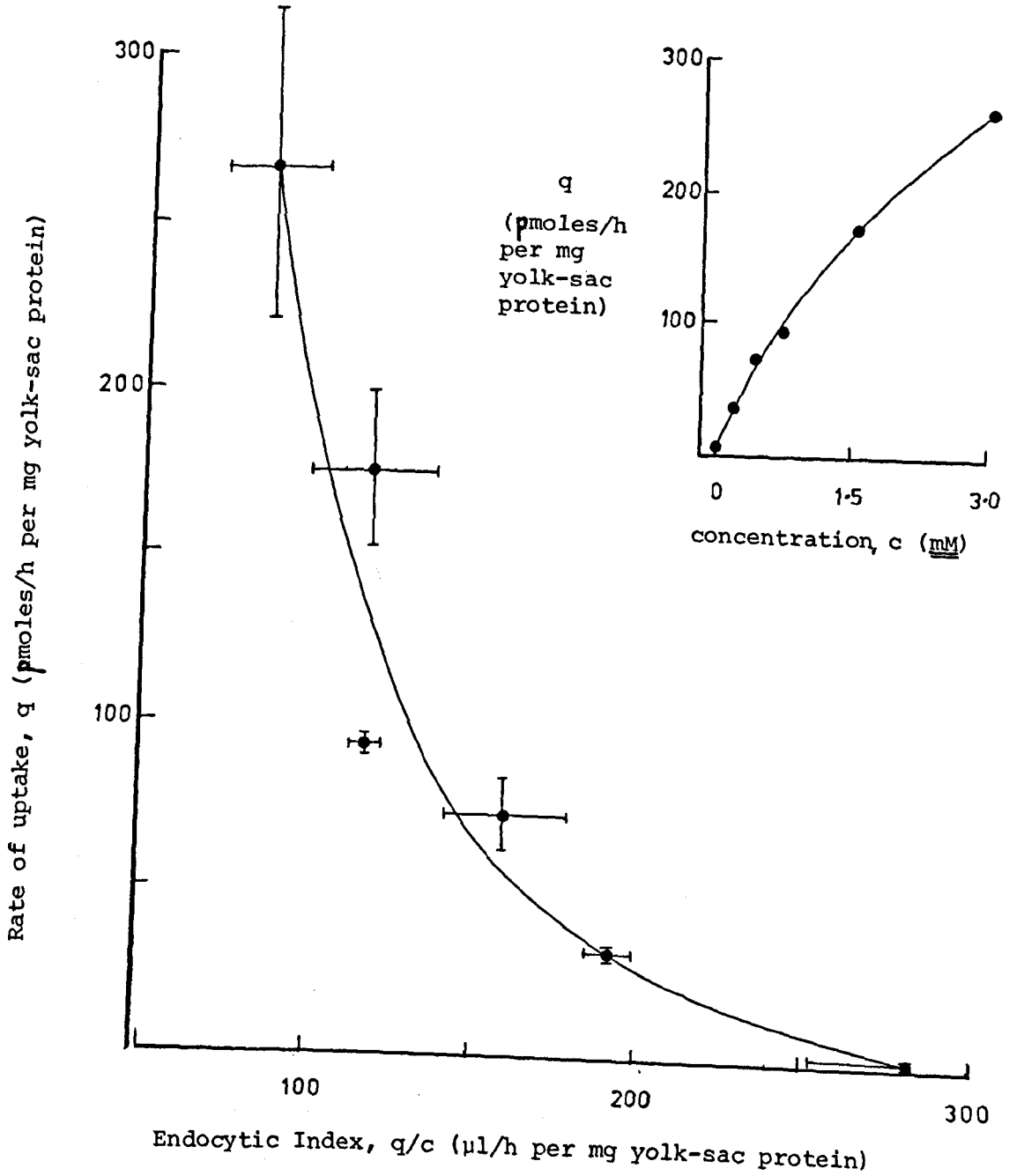


Figure 3.3 Hoftsee plot for the uptake of formaldehyde-denatured bovine serum albumin by 17.5-day rat yolk sacs incubated in serum-free medium 199.

The curve was plotted according to equation 3.12 ($q = -K.EI + SR$) using data from Fig. 3.1. To obtain both the mean value of q, and its standard deviation (vertical bar), the Endocytic Index, EI and its standard deviation (horizontal bar) were respectively multiplied by the associated initial substrate concentration (pmoles of radioactive plus non-radioactive substrate) in the culture medium.

The smaller inset figure shows how q increases with increasing substrate (radioactive plus non-radioactive) concentration.

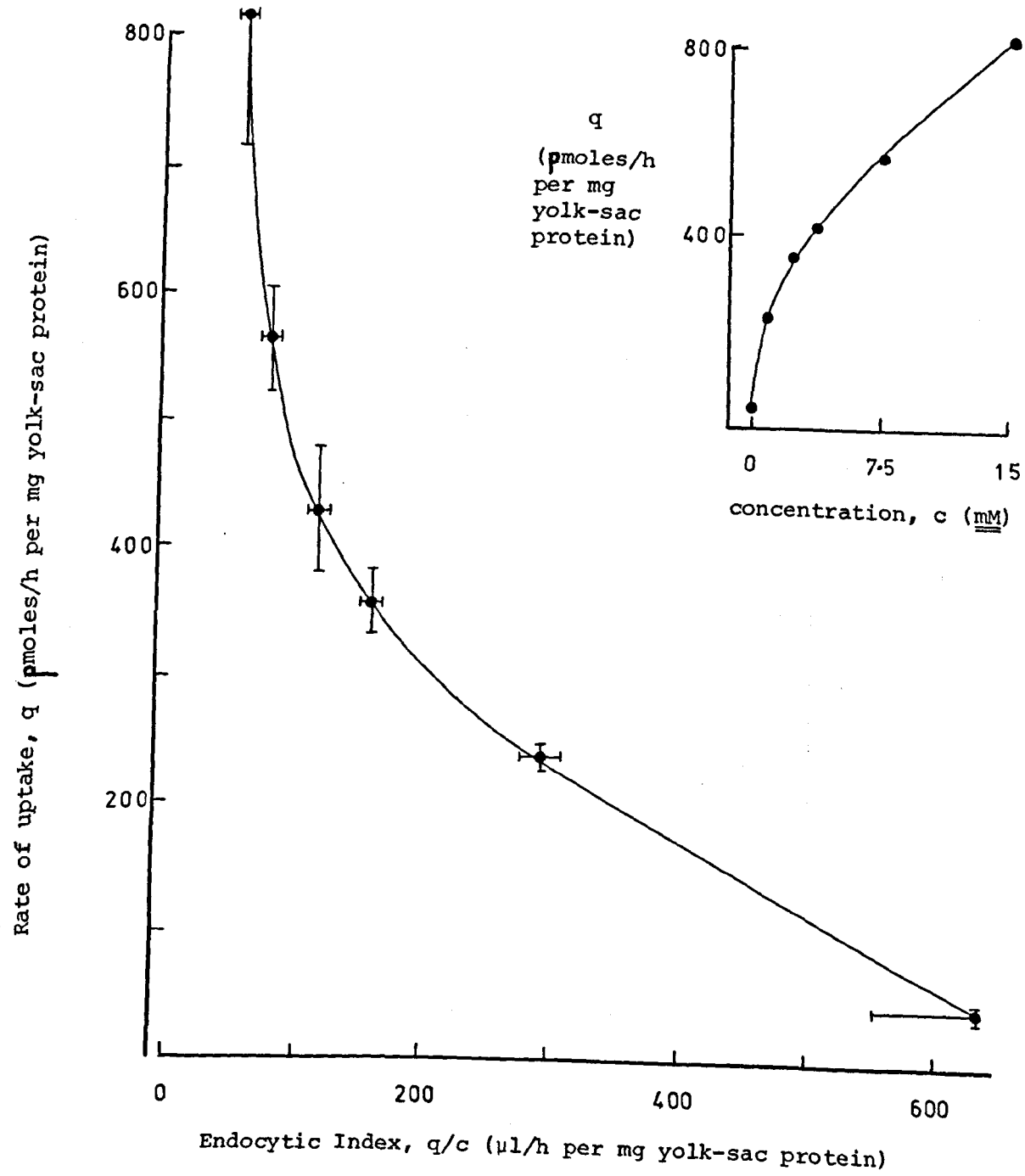


Figure 3.4 Hoftsee plot for the uptake of ribonuclease by the 17.5-day rat yolk sac incubated in serum-free medium 199.

The curve was plotted according to equation 3.12 ($q = -K.EI + SR$) using data from Fig. 3.2. To obtain both the mean value of q and its standard deviation (vertical bar), the Endocytic Index, EI and its standard deviation (horizontal bar) were respectively multiplied by the associated initial substrate concentration, c (pmoles of radioactive plus non-radioactive substrate) in the culture medium.

The smaller inset figure shows how q increases with increasing substrate (radioactive plus non-radioactive) concentration, c.

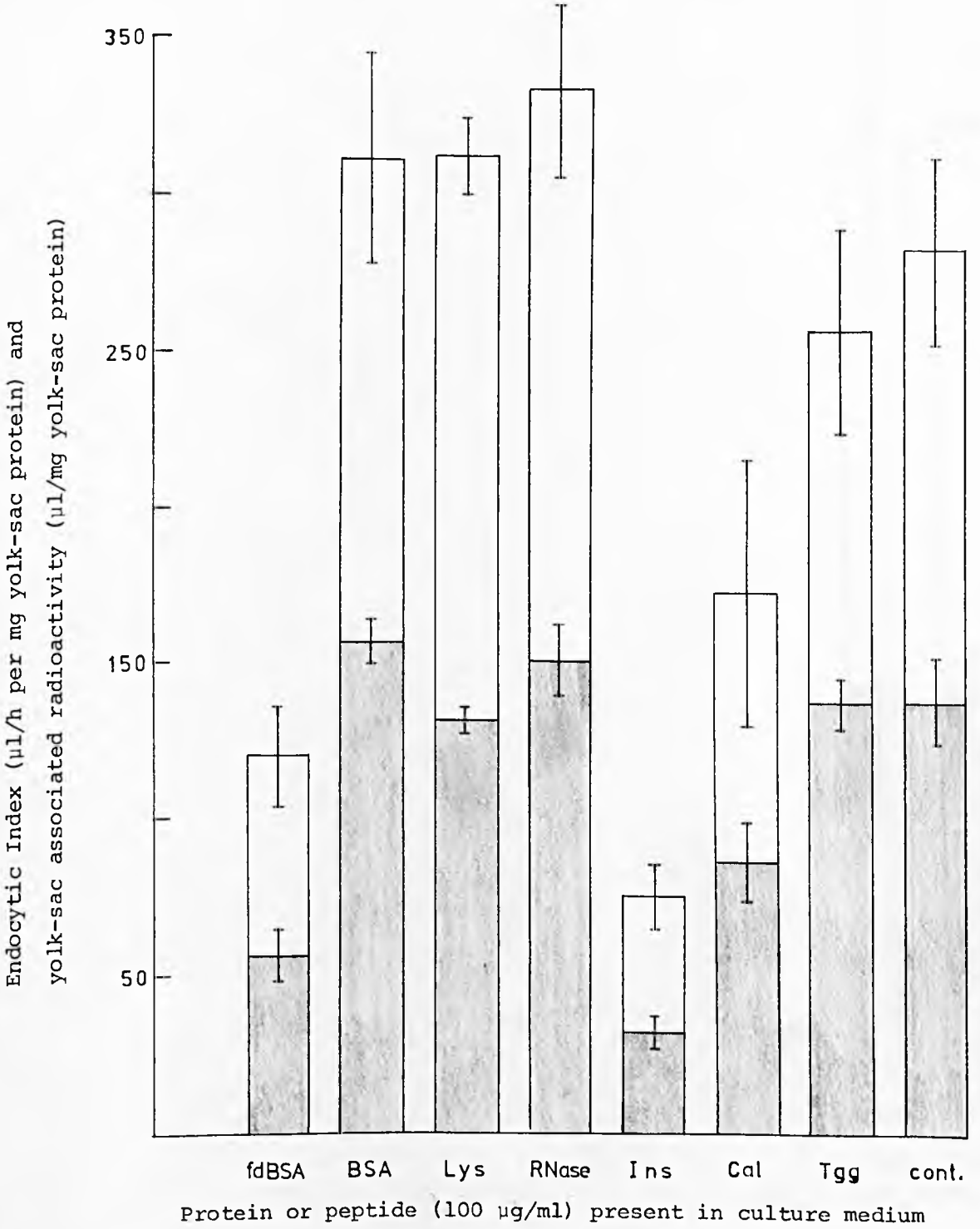


Figure 3.5 Endocytosis of formaldehyde-denatured ^{125}I-labelled bovine serum albumin by 17.5-day rat yolk sacs incubated in serum-free medium 199 in the presence of various proteins or peptides.

Values of the Endocytic Index (open bar) and the yolk-sac associated radioactivity (shaded bar) were determined as described in Section 3.2.1. Each value shown is the mean and standard deviation (vertical bar) of results obtained using yolk sacs from three or five different animals (see Table 3.3).

Abbreviations: fdBSA, formaldehyde-denatured bovine serum albumin; BSA, bovine serum albumin; Lys, lysozyme; RNase, ribonuclease; Ins, insulin; Cal, calcitonin; Tgg, tyrosyl-glycyl-glycine; cont., control (no additional protein/peptide).

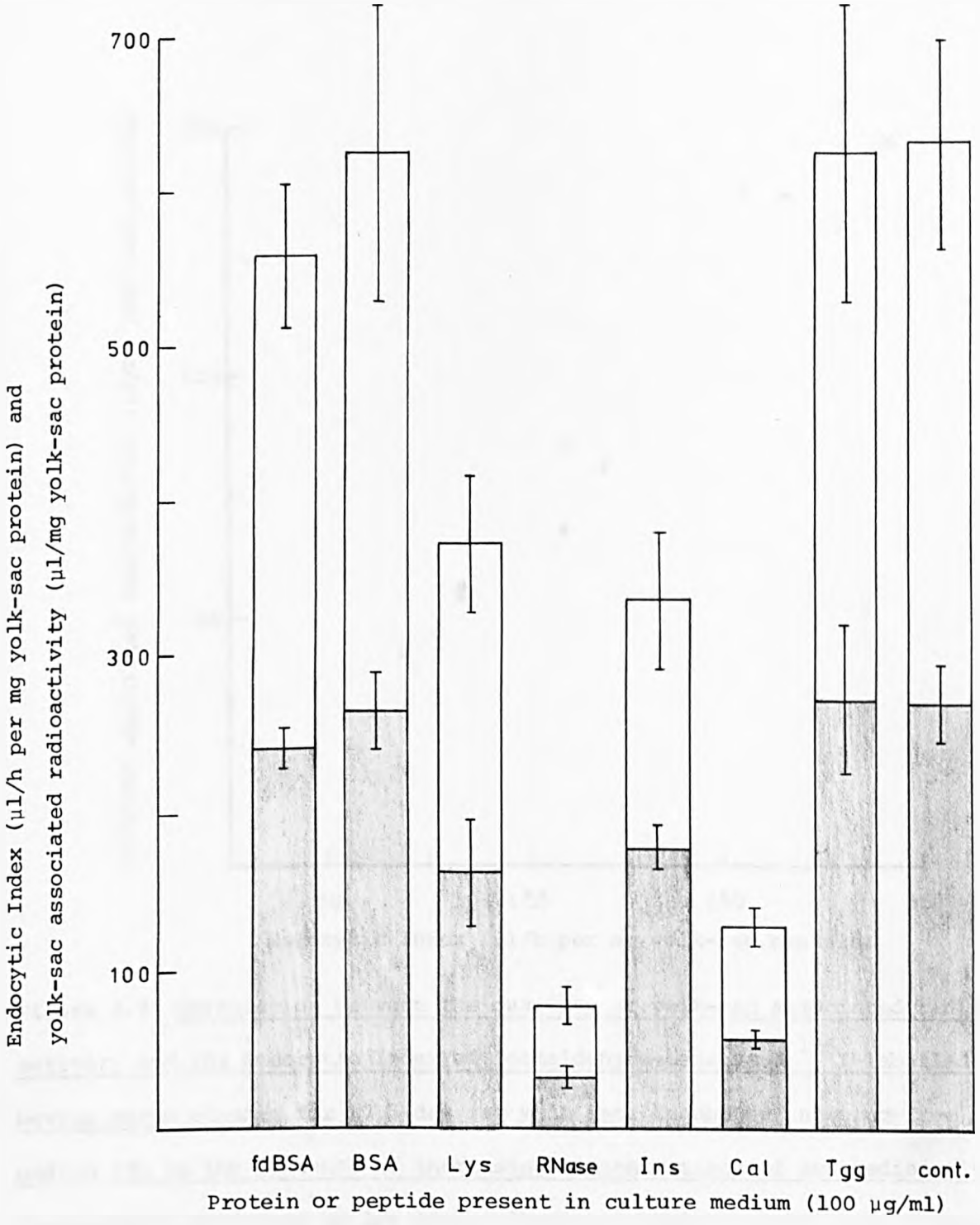


Figure 3.6 Endocytosis of ^{125}I-labelled ribonuclease by 17.5-day rat yolk sacs incubated in serum-free medium 199 in the presence of various proteins or peptides.

Values of the Endocytic Index (open bar) and the yolk sac associated radioactivity (shaded bar) were determined as described in Section 3.2.1. Each value shown is the mean and standard deviation (vertical bar) of results obtained using yolk sacs from three or four different animals (see Table 3.4).

Abbreviations: fdBSA, formaldehyde-denatured bovine serum albumin; BSA, bovine serum albumin; Lys, lysozyme; RNase, Ribonuclease; Ins, insulin; Tgg, tyrosyl-glycyl-glycine; cont., control (no additional protein/peptide).

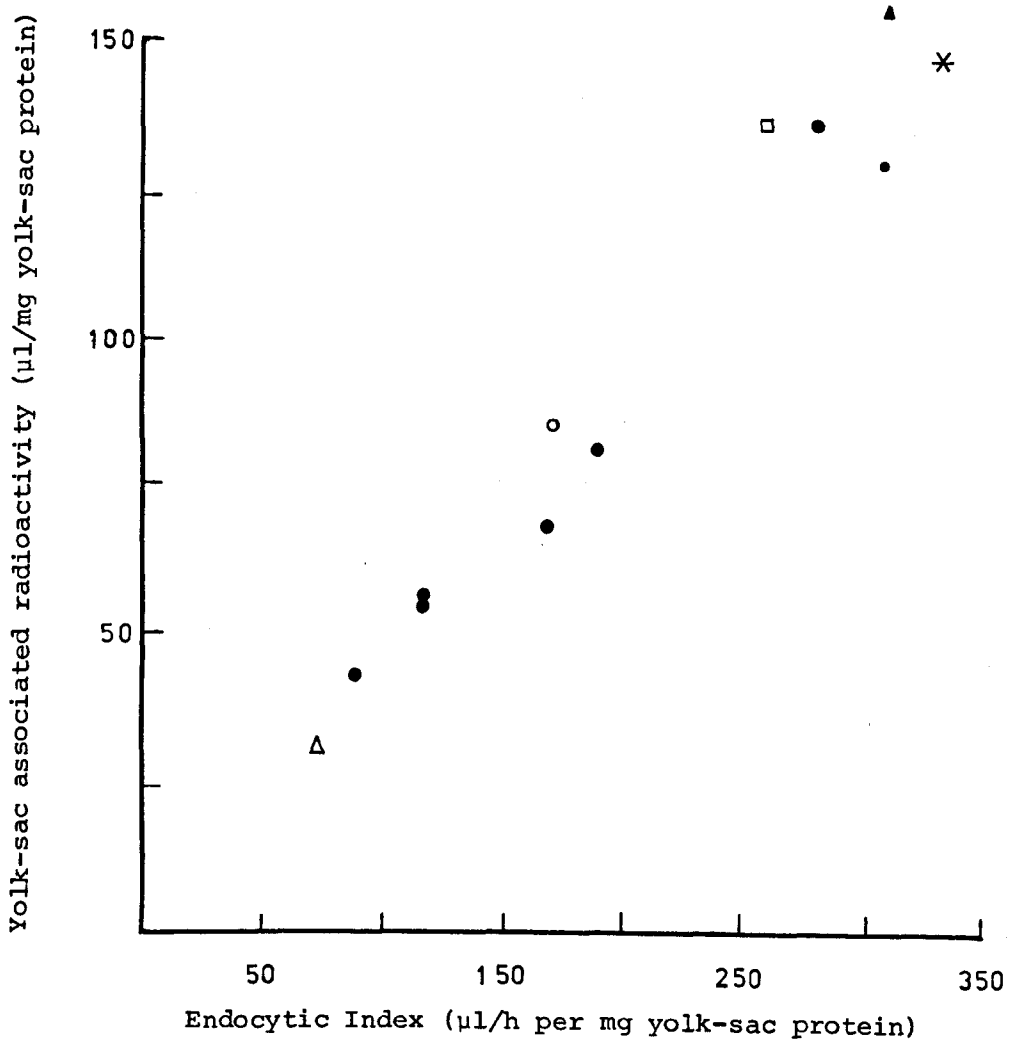


Figure 3.7 Correlation between the quantity of yolk-sac associated radioactivity and the Endocytic Index of formaldehyde-denatured ^{125}I-labelled bovine serum albumin for 17.5-day rat yolk sacs incubated in serum-free medium 199 in the presence of increasing concentrations of non-radioactive formaldehyde-denatured bovine serum albumin or various proteins or peptides.

Data are taken from Tables 3.1 & 3.3. Formaldehyde-denatured ^{125}I-labelled bovine serum albumin was present at an initial concentration of 1 µg/ml of culture medium.

(□)	Tyrosyl-glycyl-glycine	100 µg/ml
(o)	Calcitonin	100 µg/ml
(Δ)	Insulin	100 µg/ml
(✳)	Ribonuclease	100 µg/ml
(•)	Lysozyme	100 µg/ml
(▲)	Bovine serum albumin	100 µg/ml
(●)	Non-radioactive formaldehyde--denatured bovine serum albumin	0, 10, 30, 50, 100 and 200 µg/ml

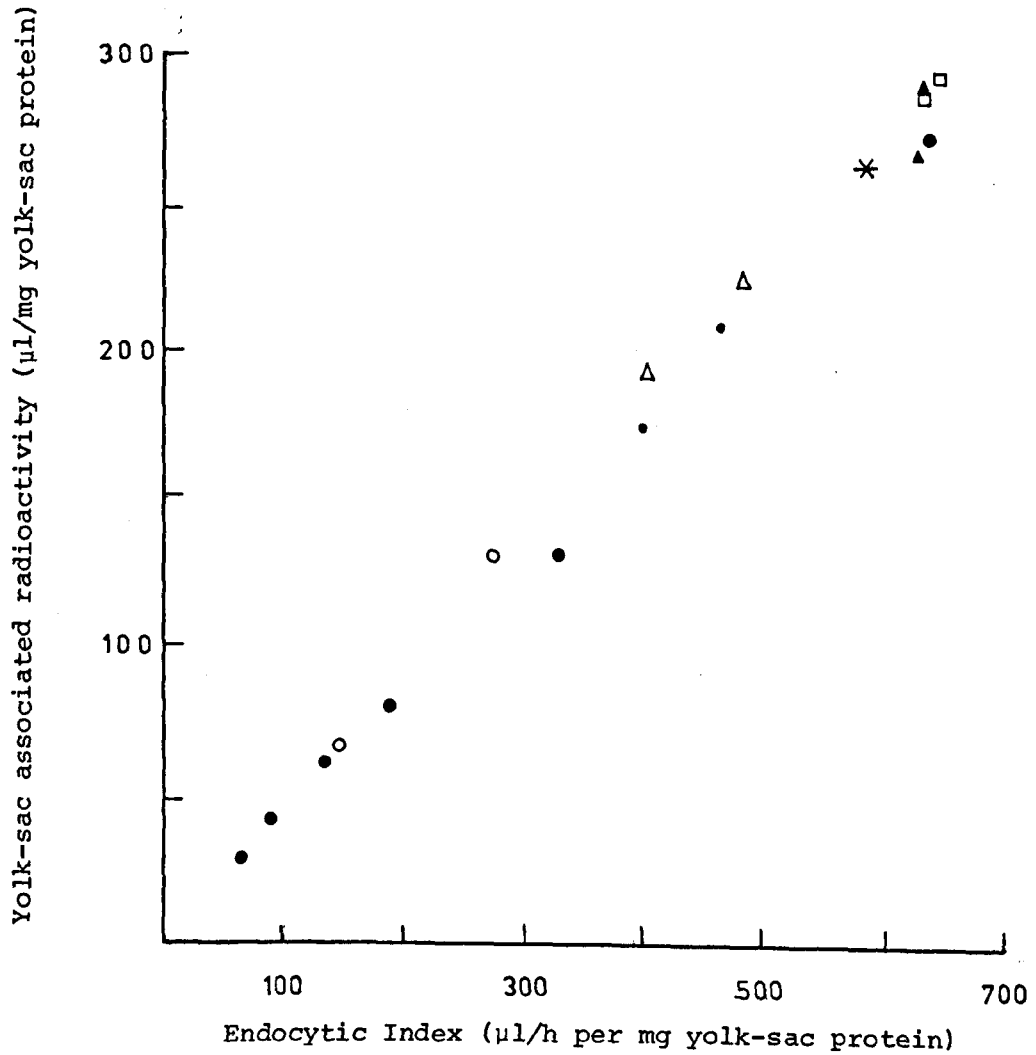


Figure 3.8 Correlation between the quantity of yolk-sac associated radioactivity and the Endocytic Index of ^{125}I-labelled ribonuclease for 17.5-day rat yolk sacs incubated in serum-free medium 199 in the presence of increasing concentrations of non-radioactive ribonuclease or various proteins or peptides.

Data are taken from Tables 3.2 & 3.4. ^{125}I-Labelled ribonuclease was present at an initial concentration of 1 µg/ml of culture medium.

(□) Tyrosyl-glycyl-glycine, 30 and 100 µg/ml
(o) Calcitonin, 30 and 100 µg/ml
(Δ) Insulin, 30 and 100 µg/ml
(●) Ribonuclease, 0, 10, 30, 50, 100 and 200 µg/ml
(•) Lysozyme, 30 and 100 µg/ml
(▲) Bovine serum albumin, 30 and 100 µg/ml
(✳) Formaldehyde-denatured bovine serum albumin, 100 µg/ml

3.4 DISCUSSION

Much of the foregoing discussion is based on the assumption that each of the ^{125}I-labelled protein substrates studied here interact with the surface of yolk-sac cells in the same manner as the corresponding unlabelled forms of the same protein. Sherman et al. (1974) have shown that radio-iodination of proteins by the chloramine-T method of Hunter & Greenwood (1962) can result in aggregation of monomeric protein molecules. But such observations were made only when high concentrations of chloramine-T were used. Sherman et al.(1974) found no evidence for the formation of macromolecular complexes when human serum albumin was iodinated using a chloramine-T to albumin concentration ratio of 2:1 (*w/w*). A much lower ratio (1:3) was used here. Moreover, gel-filtration studies of preparations of both formaldehyde-denatured ^{125}I-labelled bovine serum albumin (Moore et al., 1977) and of ^{125}I-labelled ribonuclease (see Chapter 2) exclude macromolecular-complex formation as a source of error. However, after iodination, some polypeptide hormones have been shown to differ in their behaviour compared to the native hormones (Stagg et al., 1970; Izzo et al., 1972; Marx et al., 1973; Sutcliffe et al., 1973; Desbusquois, 1975). Such differences include changes in the biological activity, membrane--binding properties, proteolytic susceptibility and rate of clearance from the bloodstream. The modification of the behaviour became more marked on increasing the number of atoms of iodine incorporated per molecule of polypeptide hormone (Izzo et al., 1972; Desbusquois, 1975). Usually only small differences in the behaviour between monoiodinated and native forms of a polypeptide hormone are reported. Thus Izzo et al. (1973) showed that after the iodination of insulin by the chloramine-T method, mono--iodoinsulin behaved like native insulin in two different biological assay systems. Terris & Steiner (1976) found close similarities in

the behaviour of various mixtures of native insulin and ^{125}I-labelled insulin containing 0.1-0.2 atoms of [^{125}I]iodine per molecule of insulin. Marx et al. (1973) showed that iodination of salmon calcitonin by the chloramine-T method, with an incorporation of 0.9-0.95 atoms of iodine per molecule of hormone, had no effect on the hormones biological activity. The incorporation of iodine into gastrin was without effect on its biological activity (Stagg et al., 1970) but required the presence of dimethylsulphoxide to protect the essential methionyl residue from the oxidative effects of chloramine-T. Chloramine-T has also been reported to destroy the essential methionyl residue in parathyroid hormone (Sutcliffe et al., 1973).

Both the formaldehyde-denatured ^{125}I-labelled bovine serum albumin and the ^{125}I-labelled ribonuclease preparations used here contain fewer than 1 atom of [^{125}I]iodine per 300 molecules of protein. It is therefore unlikely that the radioiodinated proteins studied differ substantially from the corresponding unlabelled proteins unless a methionyl residue is important in the adsorption of these proteins to internalizing binding sites on the surface of the yolk sac. Moreover, Davidson (1973) has shown that ^{125}I-labelled ribonuclease preparations, containing approximately 10 times more [^{125}I]iodine per mole of ribonuclease than the preparation used here, showed an undiminished ribonuclease activity. Furthermore, Lloyd (1976) reported that the conformation of native bovine serum albumin remains unchanged after its iodination with [^{127}I]iodine, as judged by optical rotatory dispersion studies. It seems reasonable, therefore, to assume a substantial similarity between the properties of radiolabelled proteins and the corresponding unlabelled proteins. However, since conclusive proof of this is lacking, the interpretation of some data may require caution.

Williams et al. (1975b) showed that the Endocytic Index of acid-denatured ^{125}I-labelled bovine serum albumin, in the rat yolk-sac system, was independent of the substrate concentration over the range 1-60 µg/ml of incubation medium, when the incubation medium contained 10% (v/v) calf serum. This finding did not seem compatible with their conclusion that the acid-denatured ^{125}I-labelled bovine serum albumin was ingested by adsorptive endocytosis, but was easily explained if proteins, present in the calf-serum, were also able to compete with acid-denatured ^{125}I-labelled bovine serum albumin for binding sites on the surface of the yolk sac. This conflict was resolved by Ibbotson (1978) who showed that calf-serum proteins did compete with denatured bovine serum albumin for binding sites on the surface of the yolk sac and that the Endocytic Index of formaldehyde-denatured ^{125}I-labelled bovine serum albumin in the yolk-sac system is substrate- concentration-dependent when yolk sacs are incubated in the absence of calf serum. In the experiments described in this chapter the yolk sacs were incubated in medium free of calf serum, the method avoids problems of the sort encountered by Williams et al. (1975b). The Endocytic Index of both formaldehyde-denatured ^{125}I-labelled bovine serum albumin (Ibbotson, 1978 and Fig. 3.1) and ^{125}I-labelled ribonuclease (Fig. 3.2), were observed to decrease with increasing concentration of the corresponding unlabelled proteins. If the radiolabelled and the corresponding unlabelled substrates behave alike, the shape of the curves in Figs. 3.1 and 3.2 also reflect the dependence of uptake on the concentration of the albumin and the ribonuclease. These observations confirm Ibbotson's findings with formaldehyde-denatured ^{125}I-labelled bovine serum albumin and further demonstrate that both substrates are ingested by adsorptive endocytosis.

Analysis of the uptake data in Figs 3.1 and 3.2 showed, for both substrates (Figs. 3.3 & 3.4), a non-linear relationship between the rate of substrate uptake (in μmole/h per mg yolk-sac protein) and the Endocytic Index (in μl/h per mg yolk-sac protein). The upward concave shape of the curves in Figs. 3.3 and 3.4 indicates, for each substrate, that the apparent magnitude of the dissociation constant of the pinocytosing membrane-substrate complex (negative slope of the curve) increases with a decreasing Endocytic Index or increasing substrate concentrations. These data are, therefore, typical of substrate-receptor interactions that show negative co-operativity (Lewitzi & Koshland, 1960). The uptake of ^{125}I-labelled low density lipoproteins by human fibroblasts in culture also displayed negative co-operativity (Bachorik, 1976). Negative co-operativity has been found in both specific protein-receptor interactions (Kahn et al., 1974) and non-specific protein-receptor interactions (Jennissen, 1976), but not all such interactions show negative co-operativity and some (e.g. concanavalin A binding to isolated fat-cells in culture) show positive co-operativity (Ullrich & Wallack, 1976; Prujansky et al., 1978).

Ibbotson (1978) did not observe a co-operative interaction between formaldehyde-denatured ^{125}I-labelled bovine serum albumin and binding-sites on the surface of the rat yolk sac. It is not easy to explain this discrepancy. Negative co-operativity in substrate-receptor interactions is usually ascribed to the presence of either more than one receptor or more than one substrate species, or both. In the examples shown in Figs. 3.3 and 3.4 the curvature of each plot might be explained by differences in the behaviour of the ^{125}I-labelled substrates and the corresponding unlabelled substrates. However, if, as discussed above, both forms of the substrates behave alike in their interaction with the

yolk-sac cell-surface, the curvature might be explained by: 1) interaction of the yolk sac cell surface with a number of different recognition sites on each substrate molecule; 2) different binding sites existing on the surface of the yolk sac (each having different affinities for the substrate recognition site) and 3) a combination of both 1 & 2. It is also possible that an increasing concentration of substrate in the incubation medium results in an increasing rate of pinosome formation. If this is so, it would be necessary to postulate only a single type of interaction between each substrate and binding sites on the yolk-sac cell-surface. But this possibility can be excluded since no increases in the rate of ^{125}I-PVP uptake was observed with either formaldehyde-denatured bovine serum albumin or ribonuclease when either was present at a concentration of 100µg/ml of incubation medium.

The magnitude of the dissociation constant of the membrane--substrate complex obtained here for ribonuclease was 0.55 - 11.82 x 10^{-6} M over the substrate concentration range 1-200µg/ml of incubation medium, whereas for formaldehyde-denatured bovine serum albumin, over the same substrate concentration range, the dissociation was 0.29 - 4.72 x 10^{-6} M. Over a formaldehyde-denatured bovine serum albumin concentration range of 1-160µg/ml, Ibbotson (1978) obtained an average value, for the apparent dissociation constant, of 1.26 x 10^{-6} M. This value falls well within the range obtained here. The apparent affinity of the above substrates for the internalizing binding-sites is 2-4 orders of magnitude less than that for some polypeptide hormone receptor intereactions, but compares well with some other endocytic systems, and is two orders of magnitude greater than the non-specific interactions of phosphorylase b with methyl--Sepharose and myoglobin with phosphocellulose (see Table 3.7 for comparisons).

Table 3.7 Affinity constants of proteins for binding sites on the surface of cells, isolated membranes and synthetic materials.

Binding system	Substrate	Affinity constant (µg/ml)	Affinity constant (Molar conc.)	Reference
17.5-day rat yolk sac pinocytic membrane	Ribonuclease	8 - 173	$0.55\text{-}11.82\times10^{-6}$	Section 3.4
	Formaldehyde-denatured bovine serum albumin	20 - 320	$0.29\text{-}4.72\times10^{-6}$	Section 3.4
		86	1.26×10^{-6}	Ibbotson, 1978
Cultured Kupffer cell pinocytic membrane	Formaldehyde-denatured human serum albumin	4	0.06×10^{-6}	Nilsson & Berg, 1977
Cultured human fibroblasts	Low density lipoprotein	10 - 15	-	Goldstein & Brown, 1974 Brown & Goldstein, 1974
Cultured fat cell-surface	Concanavalin A	120	-	Cuatrecasas, 1973
	Wheatgerm agglutinin	8	-	
Cultured human fibroblasts	β-Glucuronidase	-	6×10^{-9}	Kaplan et al., 1977a
Isolated rat liver plasma membrane	Asialo-orosomucoid	-	6×10^{-9}	Van Lenten & Ashwell, 1972
Isolated liver cell plasma membranes (two receptors)	Insulin	-	2.0×10^{-9} 2.1×10^{-8}	Kahn et al., 1974
Isolated kidney brush border membranes	Calcitonin	-	1.1×10^{-10}	Marx et al., 1973
Cultured central smooth muscle cell-surface	Gastrin	-	2.0×10^{-9}	Baur & Bacon, 1976
Methyl-Sepharose	Phosphorylase b	-	$4 - 39\times10^{-4}$	Jennissen, 1976
Phosphocellulose	Myoglobin	-	$2 - 9\times10^{-4}$	

An easy comparison can be made between the kinetic constants of formaldehyde-denatured bovine serum albumin and ribonuclease in the yolk-sac system. The dissociation constant of the membrane-substrate complex (K) and the maximum rate of uptake (SR) were determined for each substrate, at a concentration of 10^{-7} M, from the slope of each curve (Figs. 3.3 and 3.4) and the intercept of each curve on the ordinate axis respectively. These values together with those of the associated Endocytic Index are shown below.

SUBSTRATE	ENDOCYTIC INDEX (µl/h per mg yolk-sac protein)	MEMBRANE--SUBSTRATE DISSOCIATION CONSTANT, K (µM)	MAX RATE OF UPTAKE, SR (pmoles/h per mg yolk--sac protein)
Formaldehyde--denatured bovine serum albumin	220.1	0.38	105
Ribonuclease	587.1	0.56	400

Although the formaldehyde-denatured bovine serum albumin, at a concentration of 10^{-7} M shows an apparent affinity for the internalizing binding sites on the yolk-sac surface that is almost one and a half times greater than that of ribonuclease at the same concentration, the Endocytic Index of ribonuclease is almost three times that of formaldehyde-denatured bovine serum albumin. This must result from a larger (approx. four times more) number of internalizing membrane binding sites available for the ribonuclease molecules than for the formaldehyde-denatured bovine serum albumin molecules. The large difference in the number of internalizing membrane binding sites available to these two substrates indicates that formaldehyde-denatured bovine serum albumin and ribonuclease adsorb, at least in part, to different binding-sites on

the plasma membrane from which pinosomes are formed.

The maximum rate of internalization of a substrate-binding-site complex is given in equation 3.12 ($q = -K.EI + SR$) by the term SR where S is the rate of internalization of unit area of membrane, and R is the number of receptors (binding sites) per unit area of membrane internalized. If the value of S is the same for both substrates (e.g. the substrates share common pinosomes) the number of binding-sites per unit area of plasma membrane internalized (R) must differ for each substrate (formaldehyde-denatured bovine serum albumin and ribonuclease). It is possible, however, that the value of R is similar for each of the two substrates. If this is so, the rate of internalization of unit area of the plasma membrane must differ for each substrate i.e. the two substrates are taken up into different pinosomes which are being formed at different rates. This was considered a possible explanation by Moore et al. (1977), for their observed differences between the rates of uptake of different ^{125}I-labelled bovine serum albumin preparations. There being no evidence to support this supposition it was dismissed by Moore and co-workers as being extremely improbable. Nevertheless, specific receptors for IgG are thought to exist on the surface of rabbit yolk sacs, and the IgG is considered to become internalized and transported within coated micropinocytic vesicles across the endothelial cell. (Moxon et al., 1976). Although coated micropinocytic vesicles have been seen in electron micrographs of the rat yolk sac (observations of A.J. Kenny and of C. Wilde, unpublished work), they probably do not contribute significantly to the uptake of either substrate studied here since electronmicroscopic evidence indicates that the number of such vesicles is very small in comparison with the abundant smooth micropinocytic vesicles. The different values of SR, for formaldehyde-denatured bovine serum albumin and ribonuclease, therefore

most likely reflect differences in R, the number of binding sites available per unit area of the internalizing plasma membrane.

The ability of a number of unlabelled proteins and peptides to compete with both formaldehyde-denatured ^{125}I-labelled bovine serum albumin and ^{125}I-labelled ribonuclease for binding sites on the surface of the rat yolk sac further distinguish the two types of binding sites (Fig. 3.5 & 3.6). The competitive studies were complicated by the ability of both calcitonin and insulin to inhibit significantly the rate of pinosome formation as judged by the inhibition of ^{125}I-PVP uptake (Table 3.5). Calcitonin and insulin are therefore not ideal competitive substrates in the rat yolk-sac system, but after correction for their inhibition of membrane internalization, an estimate is obtained for the inhibition of the adsorption of a ^{125}I-labelled protein substrate to binding sites at the yolk-sac cell-surface. Ribonuclease caused no detectable inhibition of formaldehyde-denatured ^{125}I-labelled bovine serum albumin uptake (Fig. 3.5). The converse was also true i.e. formaldehyde-denatured bovine serum albumin did not measurably modify the rate of uptake of ^{125}I-labelled ribonuclease (Fig. 3.6). It is clear that both formaldehyde-denatured bovine serum albumin and ribonuclease adsorb to different binding sites on the internalizing surface of the rat yolk sac. The question then arises as to what characteristic features of a protein molecule are recognised by each of the two different binding sites.

The basic protein lysozyme, was without effect on the adsorption of formaldehyde-denatured bovine serum albumin, but substantially inhibited ^{125}I-labelled ribonuclease adsorption. This supports the supposition that the basic characteristic of both ribonuclease (pI = approx. 9.5; Richards & Wyckoff, 1970) and lysozyme (pI = approx. 11.2; Imoto *et al.*, 1972) are responsible for their adsorption to the internalizing plasma membrane.

After correcting for the effects of non-competitive inhibition (on pinocytosis), insulin was found to competitively inhibit the uptake of formaldehyde-denatured ^{125}I-labelled bovine serum albumin, but was without measurable effect on the uptake of ^{125}I-labelled ribonuclease. This further supports the suggestion that both formaldehyde-denatured bovine serum albumin and insulin share a similar, if not common mechanism responsible for their adsorption to the plasma membrane. A hydrophobic interaction between these two proteins and the plasma membrane suggests itself. The hydrophobic interior of bovine serum albumin is almost certainly displaced to the surface of the protein molecule following its denaturation with alkaline formaldehyde (Lloyd, 1976) and approximately 60% of the surface of the insulin monomer contains hydrophobic residues (Chothia & Janin, 1975).

The rat yolk-sac plasma membrane is not unique in its ability to selectively bind protein substrates. If, using published data found in a literature survey, substrates are ranked according to the extent to which they adsorb to membranes (see Table 3.8) it can be seen that several membranes show a similar degree of specificity. In general, the ranking according to the extent of adsorption to plasma membranes is: insulin = ribonuclease > formaldehyde-denatured albumin > IgG. Albumin appears to adsorb to the membrane only to a small extent and poly(vinylpyrrolidone) either does not bind significantly to membranes (e.g. 17.5-day rat yolk sac and possibly rat liver cells) or shows only an extremely small extent of binding (human placental cell membranes). [A possible exception to these observations, however, is the finding of Zalin & Hoffenberg (1977) that ^{125}I-labelled poly(vinylpyrrolidone) appears to be accumulated by minced thyroid tissue more rapidly than [^{3}H]inulin; this implies that ^{125}I-PVP might be partly ingested by adsorption to pinocytic membrane.] Ashwell

Table 3.8 Selective adsorption of proteins to natural lipid membranes.

Key: Ins, Insulin; RNase, ribonuclease; fdBSA, formaldehyde-denatured bovine serum albumin; IgG^1, rat immunoglobulin G; IgG^2, human immunoglobulin G; BSA. bovine serum albumin; HSA, human serum albumin; PVP, poly(vinylpyrrolidone).

Binding Site		
17.5-Day rat yolk sac pinocytic membrane	Ins ≃ RNase > fdBSA > IgG^1 > BSA > PVP	Chapter 1, Moore et al. (1977) & Ibbotson (1978)
Kidney proximal tubule pinocytic membrane	Ins ≃ RNase, - , - > BSA, -	Cortney et al. (1970)
Liver Kupffer cell endocytic membrane	- , - , fdBSA, - > BSA > PVP	Moore et al. (1977) Millard, unpublished results
Human placental cell-membranes	Ins, - , - , > IgG^2 > HSA > PVP	Balfour & Jones (1977)
Human erythrocyte cell-membranes	- , - , - , IgG^2 > HSA, -	Balfour & Jones (1977)

& Morell (1974a,b) showed that the adsorption of asialo-glycoproteins to rat liver parenchymal cell membranes is mediated through a specific receptor protein. Polypeptide hormones are also recognised by specific receptor proteins in the plasma membranes of target cells (for reviews see Roth, 1973; Posner, 1975). However, it is not necessary to postulate the involvement of receptor proteins to account for the selective adsorption of proteins by yolk sac pinocytic membrane or plasma membranes from other tissues. Proteins are known to adsorb to lipid films and in particular lipid bilayers may selectively adsorb proteins (Tyrrell et al., 1976). Anionic lipids interacted with the basic proteins lysozyme and ribonuclease. Most phospholipids are negatively charged at physiological pH (Bangham, 1972) and those present in the membranes of yolk sac pinosomes might bind the basic proteins lysozyme and ribonuclease. Hydrophobic interactions might take place between the hydrophic lipid of the pinocytosing yolk-sac membrane and hydrophobic regions of exogenous proteins. These suggestions are not incompatible with the observed negative co-operativity in the interaction of formaldehyde-denatured bovine serum albumin and of ribonuclease with the pinocytosing plasma membranes of the 17.5-day rat yolk sac, but would suggest a tenable model for the negative co-operativity similar to the "sequential adsorption model" described by Jennissen (1976). However, further research is necessary to discover the nature of those regions of the pinocytosing plasma to which exogenous proteins bind.

CHAPTER FOUR

SITE OF DIGESTION OF FORMALDEHYDE--DENATURED ^{125}I-LABELLED BOVINE SERUM ALBUMIN BY RAT YOLK SACS IN VITRO

4.1 INTRODUCTION

When proteins are exposed to mammalian cells in culture, their digestion products are often observed to accumulate in the culture medium (Fridhandler & Zipper, 1964; Ehrenreich & Cohn, 1967, 1968, 1969; Gabathuler & Ryser, 1969; Kirsch et al., 1972; Edelson & Cohn, 1974; Williams et al., 1975b; Goldstein et al., 1975; Terris & Steiner, 1975; Stein et al., 1976; Tokes & Sorgente, 1976; Tokes & Cspike, 1977; Moore et al., 1977; Pratten et al., 1977, Nilsson & Berg, 1977; Tolleshaug et al., 1977). In the majority of these studies, protein digestion is assumed to occur by endocytic capture of the proteins by the cells and their subsequent proteolysis within the lysosomal system. While the lysosomes are undoubtedly a major site of digestion, a possible contribution from extracellular proteolysis cannot be excluded. Indeed, Tokes & Sorgente (1976) and Tokes & Cspike (1977) claim to have observed the extracellular digestion of casein (bound to plastic beads) in several cell types: T-lymphocytes, macrophages, bovine aorta endothelial cells, normal and transformed quail fibroblasts and normal and carcinogen-treated liver epithelial cells. Moreover, extracellular membrane-bound peptidases have been implicated in the digestion of several peptide hormones including insulin (Freychet et al., 1972) and glucagon (Pohl et al., 1972); Desbuquois & Cuatrecasas, 1972; Desbuquois et al., 1974) by liver plasma membranes, corticotrophin by crude preparations of adrenal-cortex membranes (Saez et al., 1975), calcitonin (Marx et al., 1972, 1973), glucagon and insulin B-chain by kidney plasma membranes (Kerr & Kenny, 1974).

It is not known whether the microvilli of the yolk sac, like those of the kidney proximal tubule (see Kenny, 1977), are endowed with neutral endopeptidases on their outer surface or whether, if present,

such endopeptidases can make a significant contribution to the overall catabolic activity of the rat yolk sac. Preliminary investigations of peptidase activity of membrane-rich fractions of the rat yolk sac showed very low specific activities (A.J. Kenny, unpublished work) in comparison with similar fractions from rabbit kidney proximal tubules (Kerr & Kenny, 1974). Nevertheless, even a small hydrolytic activity in the disrupted yolk-sac preparations does not exclude the possibility of a significant contribution of such enzymes to the hydrolytic capacity of the rat yolk sac in culture. Possibly the rat yolk sac membrane preparations examined by Kenny contained enzymes with very different specificities from those enzymes from rabbit kidney tubules so that only low activities were shown towards the substrate used in their assays.

When formaldehyde-denatured ^{125}I-labelled bovine serum albumin was incubated with 17.5-day rat yolk sacs, it was rapidly hydrolysed (Moore et al., 1974, 1977; see also Chapters 2 & 3). Moore et al. (1977) briefly discuss circumstantial evidence indicating that lysosomes are involved in the digestion process but there is, however, no direct evidence to suggest that formaldehyde-denatured ^{125}I-labelled bovine serum albumin is digested exclusively intracellularly. This protein has been employed by several workers (Moore et al., 1974, 1977; Roberts et al., 1977; Pratten et al., 1978; Ibbotson, 1978; Duncan & Lloyd, 1978; see also Chapter 2 and 3) in quantitative studies of pinocytosis in the rat yolk-sac culture system devised by Williams et al. (1975a,b). In each of the above studies, the quantity of albumin captured by pinocytosis at a given time was calculated by summing the quantity of substrate accumulated in the yolk-sac tissue and the quantity of substrate digestion products accumulated in the incubation medium during the same incubation period. The calculation of the rate of pinocytic capture of digestible proteins

in this manner requires the albumin to be digested exclusively intracellularly, but, to date, conclusive evidence of this has been lacking.

Williams et al, (1971) showed that when acid-denatured ^{125}I-labelled bovine serum albumin was injected into pregnant rats it accumulated within the lysosomal system of the visceral yolk sac. These observations led Williams et al. (1975b) to suggest that ^{125}I--labelled albumin was digested intracellularly, within the lysosomal system, when it was added to isolated yolk sacs in culture. These studies did not, however, exclude the possibility that in culture, extracellular proteolysis might also take place. Similarly, Moore et al. (1974, 1977) showed that formaldehyde-denatured ^{125}I-labelled bovine serum albumin incubated with 17.5-day rat yolk sacs was digested several times faster than acid-denatured ^{125}I-labelled bovine serum albumin (see Table 2.3). The higher rate of digestion of the formaldehyde-treated protein was attributed to its enhanced rate of uptake into the yolk-sac lysosomal system, but equally well could have been explained by an enhanced rate of extracellular hydrolysis. The latter explanation could not be excluded essentially because, as stated by Moore et al (1977), "a decisive experiment to differentiate extracellular from intracellular proteolysis has so far proved impossible to devise". Hydrolysis, due to the release of enzymes into the incubation medium after introduction of a yolk sac is readily identified but it is a more difficult task to distinguish between enzymes, active at an intracellular (lysosomal) site from enzymes of similar specificity that are active on the tissue--surface.

Pittman & Steinberg (1978) devised an ingenious method to show that some proteins added to human fibroblasts in culture are digested

exclusively intracellularly. The method requires that the protein to be studied is dual-labelled both with a marker which, after proteolysis, will diffuse from the lysosomes into the culture medium and with a marker which after proteolysis will not diffuse from the lysosomes. These authors used [^{125}I]iodide as the diffusible marker and succinylated [^{14}C]sucrose as the non-diffusible marker. When lipoproteins and albumin were dual-labelled by the above method and added to cells in culture the rate of accumulation of acid-soluble [^{125}I]-activity in the incubation medium was equal to the rate of accumulation of [^{14}C]-activity in the cells. The equality of the two rates demonstrated that the albumin molecule was digested entirely intracellularly. However, one minor criticism of the technique is that the labelling of the protein, with a marker large enough to be retained within lysosomes may modify either the rate of pinocytic uptake of the protein or of its rate of intralysosomal digestion, so that the results cannot be extrapolated to predict the behaviour of the unlabelled protein. Problems were also encountered in obtaining a homogeneously dual-labelled protein preparation. Nevertheless, an analagous principle: equating the rate of uptake of the lysosomally retained substrate, ^{125}I-labelled poly(vinylpyrrolidone), and the rate of appearance of acid-soluble radioactivity in the incubation medium, when formaldehyde-denatured ^{125}I-labelled bovine serum albumin is used as substrate, has been employed in the work reported in this chapter. Unfortunately ^{125}I-labelled poly(vinylpyrrolidone) is pinocytosed only in the fluid phase while formaldehyde-denatured ^{125}I-labelled bovine serum albumin is most probably ingested mainly by adsorptive pinocytosis (Moore et al., 1977) thus the absolute value for the rate of capture of the protein is far higher than that of ^{125}I-labelled poly(vinylpyrrolidone).

However, when changes are induced in the rate of pinosome formation in rat yolk sacs, the relative changes in the rate of uptake of ^{125}I-PVP and the rate of digestion of the ^{125}I-labelled albumin should be equal if digestion is exclusively an intralysosomal event. Duncan & Lloyd (1978) recently showed that decreasing the temperature at which yolk sacs are incubated lowers the rate of pinocytosis, they also showed an inhibition of pinocytosis by metabolic inhibitors. Similarly, Brown & Segal (1977) reported glucagon to be an inhibitor of pinocytosis in the rat yolk sac. Changes in temperature, the presence of either glucagon or the metabolic inhibitor, rotenone were therefore employed in the work reported in this chapter to induce changes in pinocytic activity. In addition, the kinetics of digestion of formaldehyde-denatured ^{125}I-labelled bovine serum albumin, immediately after its exposure to yolk-sac tissue were investigated and additional experiments were designed to discover whether lysosomes alone are responsible for the digestion of the ^{125}I-labelled albumin in this system.

4.2 METHODS

4.2.1 Assay of the uptake and degradation of ^{125}I-labelled substrates by 17.5-day rat yolk sac incubated in serum-free medium 199.

The uptake of ^{125}I-labelled poly(vinylpyrrolidone) was determined as described in Section 2.2.1. The rates of uptake and of digestion of formaldehyde-denatured ^{125}I-labelled bovine serum albumin (prepared as described previously; Section 2.2.3) were determined as described in Section 3.2.1 assuming an intracellular site of digestion. Yolk sacs were incubated singly in 20ml of serum-free culture medium. When yolk sacs were incubated in the presence of either rotenone or glucagon or dibutyryl-cyclic AMP (each from Sigma, London), radioactive substrate was added to the culture medium 30min after introduction of the yolk sacs to medium containing the inhibitor. Rotenone was initially dissolved in 95% (v/v) ethanol (1mg/ml); dilutions of the alcoholic rotenone solution to the required concentration were then made using medium 199. In the experiments where yolk sacs were incubated at different temperatures, the tissue was placed in media at the appropriate temperature and incubated for 15min before addition of the radioactive substrate.

Acid-soluble radioactivity in the alkaline yolk-sac solution was assayed essentially as described previously [see Section 2.2.1 (3), i.e. by the method used to assay acid-soluble radioactivity in the incubation medium using an empirical correction factor, see Appendix I, of 1.65] but 0.1ml of calf serum was added to 1.0ml of yolk-sac solution, followed by 1.0ml of 20% (w/v) trichloroacetic acid to achieve full precipitation. The formaldehyde-denatured ^{125}I-labelled bovine serum albumin was found to be slightly unstable in the aq. 1M-NaOH used to digest yolk sacs; at 37°C an increase in acid-soluble radioactivity of

<0.3%/h was observed, but at room temperature this fell to <0.1%/h. The instability of the formaldhyde-denatured ^{125}I-labelled bovine serum albumin in the 1M-NaOH will result in an overall error of only <2% in the values shown for the acid-insoluble and acid-soluble radioactivity present in yolk-sac tissues.

4.2.2 Centrifugal subcellular fractionation of 17.5-day rat yolk sacs that had accumulated formaldehyde-denatured ^{125}I-labelled bovine serum albumin *in vitro*.

Yolk sacs (12) were dissected from a 17.5-day pregnant rat and incubated at 37°C in serum-free medium 199 as previously described (Section 2.2.1). Formaldehyde-denatured ^{125}I-labelled bovine serum albumin was added to the culture medium (10µg/ml) and incubation continued for 1.5h. The yolk sacs were removed from the culture medium, washed (as described in Section 2.2.1) and homogenised in 2.5M-sucrose (10ml, adjusted to pH 7.4 with $NaHCO_3$) by using a Potter-Elvehjem type Teflon-on--glass homogeniser (with an 0.19mm clearance) at a speed of 2 500rpm. Four up-and-down strokes were made within a period of 30s. The homogenate was diluted with 20ml of cold 0.25 M-sucrose, pH 7.4, and fractionated by differential centrifugation. The fractionation scheme used (Lloyd & Beck, 1969) yields five particulate fractions, isolated with successively higher centrifugal forces, and a final supernatant (S). The five fractions are designated first nuclear (N_1, isolated at 150g x 10min), second nuclear (N_2, 1000g x 10min), mitochondrial (M, 3 300g x 10min), lysosomal (L, 16 000g x 20min) and microsomal (P, 100 000g x 30min). Fraction N_1 was obtained using an MSE Mistral 4L centrifuge, fractions N_2, M and L were obtained using an MSE High-Speed 18 centrifuge and fraction P was obtained using an MSE Superspeed 50 centrifuge. Each

pellet was resuspended in 0.25M-sucrose, pH 7.4 and all fractions were immediately assayed for total and acid-soluble radioactivity. After freezing (-20°C) each fraction was assayed for protein and acid-proteinase activity.

4.2.3 Assay of acid-proteinase activity.

Acid proteinase activity was assayed by the method of Anson (1937). An aqueous solution of bovine haemoglobin (Sigma, London) was dialysed against running tap-water for 3 days, diluted to 8% (w/v) and stored at -20°C until used. When required, the haemoglobin solution was thawed and diluted to 2% (w/v) with 0.4M-sodium acetate, pH 3.4 (final pH 3.6). To 1.0ml of 2% (w/v) haemoglobin was added 0.2ml of the cell fraction to be assayed. After incubation at 37°C for 1.5h, the protein was precipitated with 3.0ml of 5% (w/v) trichloroacetic acid and the incubation continued for a further 10min. When the mixture had cooled to room temperature it was centrifuged (500g x 20min, MSE Mistral 4L centrifuge). To 0.5ml of the supernatant, made alkaline with 2.0ml of aq. 1M-NaOH, was added 0.5ml of Folin Ciocalteu's Reagent. The extinction was measured at 750nm after 15-20min.

Blanks, incubated in parallel, contained 2% haemoglobin only but 0.2ml of a particular cell fraction was added just before the addition of the trichloroacetic acid. A standard curve was prepared using L-tyrosine.

4.2.4 Assay of the proteolytic activity (against formaldehyde-denatured ^{125}I-labelled bovine serum albumin) in a cell-free extract of 17.5-day rat yolk sacs.

Yolk sacs (10) were homogenised (as described in Section 4.2.2) in 10ml of distilled water. The homogenate was diluted with 20ml of

distilled water then centrifuged (150g x 10min) to remove intact cells.

The incubation mixture consisted of an appropriate buffer solution, 130µl; cell-free extract, 50µl (approx. 150µg protein) and 20µl of formaldehyde-denatured ^{125}I-labelled bovine serum albumin (10µg). After incubating the mixture at 37°C for 1h the reaction was stopped by adding 0.5ml of 20% (v/v) aq. calf serum immediately followed by 0.5ml of 20% (w/v) trichloroacetic acid. The precipitate was removed by centrifugation and the acid-soluble radioactivity was assayed (see Section 2.2.1). Blanks, containing appropriately buffered substrate were also incubated for 1h at 37°C.

Over a 3.0 to 6.5 pH range, 0.1M-sodium acetate was used as buffer but over the pH range 6.5 to 9.0 this was replaced by 0.1M-Tris [2-amino-2-(hydroxymethyl) propane-1,3-diol].

4.2.5 Analysis of the hydrolysis products derived from formaldehyde--denatured ^{125}I-labelled bovine serum albumin incubated with 17.5-day rat yolk sacs.

Analysis of the hydrolysis products derived from formaldehyde--denatured ^{125}I-labelled bovine serum albumin on incubation with 17.5-day rat yolk sacs in serum-free medium 199 was performed as described previously [Section 2.2.4(3)]. The gel columns were both equilibrated and eluted with 0.05 M-sodium acetate (pH 6.5) containing 0.1% (w/v) sodium azide.

When the radioactivity present in the yolk-sac tissue was analysed, the yolk sacs (3), loaded with ^{125}I-labelled albumin, were homogenised in 5ml of distilled water with a hand-held glass-on-glass Potter-Elvehjam type homogeniser. The radioactivity present in a 1.0ml sample of the homogenate was assayed (see Section 2.2.1), then the whole of the homogenate was applied to a Sephadex G-25 column. The rest of the analysis was performed as for the hydrolysis products present in the incubation medium [see Section 2.2.4(3)].

4.3 RESULTS

4.3.1 Association of formaldehyde-denatured ^{125}I-labelled bovine serum albumin with the yolk-sac tissue and the appearance of acid-soluble digestion products both in the yolk-sac tissue and in the serum-free incubation medium.

When yolk sacs were incubated in serum-free medium 199 containing formaldehyde-denatured ^{125}I-labelled bovine serum albumin, acid-soluble radioactive digestion products were found associated with the yolk-sac tissue and also in the incubation medium after a short period of time. If the digestion of the ^{125}I-labelled albumin occurs intracellularly, it is to be expected that acid-soluble radioactivity will appear inside the yolk-sac tissue before it appears in the incubation medium and that both these events will occur only after ingestion of the ^{125}I-labelled albumin by the yolk sacs.

This sequence of events was, indeed, observed (Fig. 4.1) in yolk sacs incubated in medium 199 containing formaldehyde-denatured ^{125}I-labelled bovine serum albumin (1µg/ml of incubation medium). The quantity of acid-insoluble radioactivity associated with the yolk-sac tissue increased steadily, from a measurable level at 3min to about 50ng/mg yolk-sac protein 25min after addition of the ^{125}I-labelled albumin, but then remained virtually constant. By 3min a measurable quantity of acid-soluble radioactivity could also be detected within the tissue but, although the quantity of acid-soluble radioactivity also increased steadily with time this rise occurred at a later time than that of the acid-insoluble radioactivity. In contrast, the appearance of acid-soluble radioactivity in the incubation medium was only observed after a lag-period of 12-15min. After 25min the rate of appearance of

the acid-soluble radioactivity in the incubation medium reached a maximum value (4.4ng/min per mg yolk-sac protein), that was virtually identical to the maximum observed rate of accumulation of acid-insoluble radioactivity by yolk-sac tissue in the initial 15min (4.3ng/min per mg yolk-sac protein).

These observations suggest a precursor-product relationship between the radioactive species monitored. Moreover, the close similarity between the maximum rate of accumulation of acid-insoluble radioactivity by the tissue and the maximum rate of release of acid-soluble radioactivity into the incubation medium indicates that the complete digestion of acid--insoluble radioactivity (i.e. the ^{125}I-labelled albumin) subsequent to its endocytosis can completely account for the observed rate of appearance of acid-soluble radioactivity in the incubation medium. Furthermore, the lag-period of 12-15min before the appearance of any acid-soluble radioactivity in the medium is consistent with an intracellular site of digestion of the ^{125}I-labelled albumin.

4.3.2 Subcellular distribution of the radioactivity accumulated within the 17.5-day rat yolk sac after incubation in serum-free medium 199 with formaldehyde-denatured ^{125}I-labelled bovine serum albumin and the digestion of the radiolabelled albumin by a cell-free extract of the yolk-sac tissue.

Fig. 4.2 shows the distribution of acid-proteinase activity, acid-soluble radioactivity and acid-insoluble radioactivity in subcellular fractions of yolk sacs that had previously ingested formaldehyde-denatured ^{125}I-labelled bovine serum albumin. The sum of the acid-soluble radioactivity present in all of the fractions was equivalent to 38% of the total radio-activity; this proportion compares with a value of 40% obtained in the yolk-sac homogenate assayed prior to centrifugation. The close similarity of these proportions indicates that no detectable digestion of the accumulated

formaldehyde-denatured ^{125}I-labelled bovine serum albumin occurred during the 2.5h period required to prepare the subcellular fractions. Fig. 4.2, which is presented in the manner described by de Duve et al. (1955), shows 60% of the acid-insoluble radioactivity to be particle-bound. The distribution of the acid-insoluble radioactivity closely parallels that of acid-proteinase activity and strongly suggests that the two are associated with the same organelle. The two patterns differ slightly in that the distribution of sedimentable acid-insoluble radioactivity is skewed toward the smaller particles. (A possible explanation of this trend is that some of the ^{125}I-labelled albumin is present in smaller pinocytic vesicles that have not fused with lysosomes.)

The sedimentable acid-soluble radioactivity showed a distribution essentially coincident with the sedimentable acid-proteinase activity. This suggests that hydrolysis of the ^{125}I-labelled albumin occurs in the organelle with which the acid proteinase activity is associated. But the largest proportion (74%) of the acid-soluble radioactivity was associated with the S-fraction. These observations are consistent with the entry of ^{125}I-labelled albumin into lysosomes, in which digestion takes place, and from which release of low molecular-weight digestion products occurs by diffusion into the cytosol throught the lysosomal membrane.

Cell-free extracts of yolk-sac tissue were shown to be capable of rapidly degrading the formaldehyde-denatured ^{125}I-labelled bovine serum albumin to acid-soluble material. The reaction showed a very sharp optimum at pH 4.0 with no measurable acid-soluble radioactivity produced above pH 6.0 (Fig. 4.3). This finding of an acid pH-optimum for the degradation of the ^{125}I-labelled albumin by a cell-free extract is consistent with the data in Fig. 4.2 which suggest that the observed degradation in intact cells (Fig. 4.1) occurs in lysosomes. Moreover, because yolk sacs

were incubated in a medium at pH 7.1, the lack of digestion of the ^{125}I-labelled albumin by the cell-free extract at this pH suggests that it is unlikely that extracellular tissue-associated enzymes could be involved in the observed digestion of the ^{125}I-labelled albumin by yolk sacs.

4.3.3 Analysis of the hydrolysis products derived from formaldehyde-denatured ^{125}I-labelled bovine serum albumin when incubated with 17.5-day rat yolk sacs in serum-free medium 199.

Fig. 4.4 shows Sephadex G-25 elution profiles of ^{125}I-labelled albumin that had been incubated either for 2h in medium 199 alone or for 1,2,3 or 6h in medium 199 containing yolk sacs (upper); yolk-sac homogenate prepared from tissue that had been incubated with ^{125}I-labelled albumin for 2h (middle); and radioactivity released into medium 199 during a 2h re-incubation of yolk sacs that had previously ingested the ^{125}I-labelled albumin (lower).

^{125}I-Labelled albumin incubated alone showed only a single peak that eluted at the void volume of the column. When yolk sacs were present, two new peaks of radioactivity (one minor and one major) appeared and the amount of radioactivity present in each peak progressively increased with time. The minor peak of radioactivity eluted at the same position as free [^{125}I]iodide and the major peak of radioactivity eluted at the same position as [^{125}I]iodo-L-tyrosine, and slightly later than the elution position of glycyl-[^{125}I]iodo-L-tyrosine. The amount of radioactivity contained in the minor peak was equivalent to 4.8, 4.4, 3.8 and 5.0% of the total radioactivity present in the combined minor and major peak at 1, 2, 3 and 6h respectively. No radioactivity was ever observed to elute between the ^{125}I-labelled albumin and the minor peak of radioactivity.

When yolk-sac associated radioactivity was examined by Sephadex G-25 chromatography (Fig. 4.4, middle) a major peak of radioactivity representing 29% of the total (recovered) radioactivity, eluted in the same position as [^{125}I]iodo-L-tyrosine. Again a minor peak of radioactivity eluted in the same position as free [^{125}I]iodide; the quantity of radioactivity in this peak represented 8.1% of that in the combined minor and major peaks. However, unlike the elution profiles of the incubation media, the elution profile of the yolk-sac associated radioactivity showed a small amount of radioactive material to elute between the ^{125}I-labelled albumin peak and the minor peak; this could represent intermediate digestion products.

When radioactivity, released on re-incubating washed yolk sacs that had previously accumulated formaldehyde-denatured ^{125}I-labelled bovine serum albumin, was examined, virtually none eluted in the position of ^{125}I-labelled albumin but the major and minor peaks of radioactivity eluted in the same positions as [^{125}I]iodo-L-tyrosine and free [^{125}I]-iodide respectively. The minor peak represented 7.4% of the combined radioactivity in both the minor and major peaks.

Examination of the radioactivity present in the major peaks [A (3h incubation), B and C] on a copper-complex of Sephadex G-25 showed that each contained approximately 75-90% of [^{125}I]iodo-L-tyrosine (Table 4.1).

4.3.4 Effect of temperature on the rate of uptake of ^{125}I-labelled poly(vinylpyrrolidone), the rate of digestion and putative rate of uptake of formaldehyde-denatured ^{125}I-labelled bovine serum albumin by the 17.5-day rat yolk sac incubated in serum-free medium.

Lowering the temperature at which yolk sacs are incubated in serum-free medium markedly inhibited the rate of accumulation of ^{125}I-labelled poly(vinylpyrrolidone) (Table 4.2), the rate of digestion

and the putative rate of uptake of formaldehyde-denatured ^{125}I-labelled bovine serum albumin (Table 4.3). A rapid decline in the rate of uptake of ^{125}I-PVP and in the rate of digestion of ^{125}I-labelled albumin occurred with only a small decrease in incubation temperature. At 15°C the two processes were fully inhibited. A closely parallel pattern of inhibition of the putative rate of uptake of the ^{125}I-labelled albumin by the yolk sac was also observed on lowering the temperature. When the data (Tables 4.2 and 4.3) are expressed as a percentage of the rates observed at 37°C, the parallel nature of these effects can be clearly seen (Table 4.4).

The quantity of radioactivity remaining associated with the yolk-sac tissue after 3h of incubation with ^{125}I-labelled albumin showed a biphasic effect on lowering of the incubation temperature; initially a small increase was observed, but, below 30°C, a rapid decline occurred. If the observed digestion of ^{125}I-labelled albumin is an intracellular event, the biphasic effect can be explained by the data in Fig. 4.5 which show the time-course of the appearance of acid-soluble radioactivity in the incubation medium. After an intitial lag-period, the amount of acid-soluble radioactivity appearing in the incubation medium increased linearly with time, at each incubation temperature above 15°C. With decreasing incubation temperature, the duration of the lag-period increased (values at 34, 30 and 25°C were 123, 230 and 423% of that observed at 37°C). The duration of the lag-period represents the time taken for the yolk sac to digest and release the ^{125}I-labelled albumin once captured by endocytosis. The longer this period the higher will be the quantity of radioactivity remaining associated with the tissue. Consequently, lowering the incubation temperature will elevate the level of the tissue-associated radioactivity until the decrease in rate of uptake of radioactivity (with

decreasing temperature) begins to have the predominant effect; the tissue level of radioactivity will then begin to decline.

The close parallels in the decline (on lowering the incubation temperature) in the rate of uptake of ^{125}I-PVP, the rate of digestion- and the putative rate of uptake of formaldehyde-denatured ^{125}I-labelled bovine serum albumin, strongly suggest that all three processes share a common rate-limiting step, namely pinocytosis. The increase in the duration of the lag-period and the attendant elevated quantities of tissue--associated radioactivity, observed with decreasing incubation temperature, are both consistent with an intracellular site of digestion of the formaldehyde-denatured ^{125}I-labelled bovine serum albumin by the yolk-sac tissue.

4.3.5 Effects of rotenone and glucagon on the uptake of ^{125}I-labelled poly(vinylpyrrolidone), the rate of digestion and the putative rate of uptake of formaldehyde-denatured ^{125}I-labelled bovine serum albumin by the 17.5-day rat yolk sac incubated in serum-free medium.

Table 4.5 shows that rotenone is a potent inhibitor of ^{125}I-PVP uptake and of the rate of digestion and putative rate of uptake of formaldehyde-denatured ^{125}I-labelled bovine serum albumin. The effects observed were dependent on the concentration of rotenone in the incubation medium. Little effect was observed with 10^{-11} M-rotenone, but above this concentration, increasing concentrations progressively inhibited all three processes. Fifty percent inhibition of ^{125}I-PVP uptake, of the rate of digestion- and of the putative rate of uptake of the ^{125}I-labelled albumin were observed at 1×10^{-8} M, 3×10^{-8} M and 5×10^{-8} M, respectively. Progressive inhibition of each of the three processes occurred up to 10^{-5} M-rotenone.

The quantity of radioactivity remaining associated with the yolk-sac tissue after the 3h incubation with ^{125}I-labelled albumin remained unchanged at 10^{-9} M-rotenone, a concentration which inhibited both ^{125}I-PVP uptake and ^{125}I-labelled albumin uptake (assuming that digestion occurs intracellularly). As with the temperature effects (see Section 4.3.4) this can be explained by the increase in the duration of the lag-period observed before acid-soluble radioactivity appears in the incubation medium (Fig. 4.6) when the yolk sacs are incubated in the presence of rotenone.

Fig. 4.7 shows that glucagon is also a potent inhibitor of ^{125}I-PVP uptake. Fifty percent inhibition of ^{125}I-PVP uptake by yolk sac was observed at a glucagon concentration of 2×10^{-8} M; at a concentration of 10^{-6} M maximum inhibition of ^{125}I-PVP uptake was observed but uptake was still linear with incubation time; the uptake of ^{125}I-PVP at 3h was equal to 30% of the control value. Table 4.6 shows that 10^{-6} M-glucagon inhibits both the rate of digestion and the putative rate of uptake of formaldehyde-denatured ^{125}I-labelled bovine serum albumin by about 60%. Glucagon is thought to act via the second messenger, cyclic 3'-5' AMP. Its analogue, dibutyl cyclic 3'-5' AMP was also shown (Table 4.6) to inhibit the digestion and putative uptake of the ^{125}I-labelled albumin.

The close similarity in the inhibitory effects, of both rotenone and glucagon, on the rates of uptake of ^{125}I-PVP and of both digestion and the putative uptake of formaldehyde-denatured ^{125}I-labelled bovine serum albumin is further evidence that the rates at which the three processes occur are determined by the same rate-limiting step.

Table 4.1 Chromatography of the low molecular weight hydrolysis products of formaldehyde-denatured ^{125}I-labelled bovine serum albumin on the copper complex of Sephadex G-25.

The pooled fractions A, B and C [see Fig. 4.4] were each concentrated by freeze-drying and further analysed on the copper-complex of Sephadex G-25, as described in Section 2.2.4(3). The distribution of the radioactivity between the fraction eluted by alkali and the fraction eluted with acid is reported as a percentage of the recovered radioactivity. The total recovered radioactivity is expressed as a percentage of the radioactivity applied to the column. The elution patterns of [^{125}]iodo-L-tyrosine and glycyl-[^{125}I]iodo-L-tyrosine are taken from Table 2.5.

Radioactivity applied to the column	Percentage of radioactivity eluting with borax buffer, pH 11	Percentage of radioactivity eluting with 0.4M-HCl	Percentage recovery of radioactivity from column
[^{125}I]iodo-L-tyrosine	2.5	97.5	102.0
Glycyl-[^{125}I]iodo-L-tyrosine	88.4	11.6	88.0
Peak A (Product produced from ^{125}I-labelled albumin incubated in the presence of a yolk sac)	10.7	89.2	79.8
Peak B (Yolk-sac associated radioactivity)	24.4	75.6	105.0
Peak C (Product released from a yolk sac loaded with ^{125}I-albumin)	17.8	82.2	100.4

Table 4.2 Effect of incubation-temperature on the rate of uptake of ^{125}I-labelled poly(vinylpyrrolidone) by 17.5-day rat yolk sacs incubated in serum-free medium 199.

The rate of ingestion of ^{125}I-PVP by yolk sacs was determined as described in Section 2.2.1. Yolk sacs were incubated for 15 min in serum-free medium 199 at the temperature indicated, before the addition of ^{125}I-PVP (2 µg/ml of medium), then further incubated for up to 3 h and yolk sacs removed at 0.5 h intervals.

Incubation temperature	Expt. No.	Endocytic Index (µl/h per mg yolk-sac protein)	Correlation Coefficient	Intercept on the ordinate (µl/mg yolk-sac protein)	No. of yolk sacs per experiment
37°C	1	+3.562	+0.959	+2.038	5
	2	+3.914	+0.988	+1.510	5
	3	+3.481	+0.996	+1.237	6
	4	+3.453	+0.960	+2.250	6
MEAN	S.D.:-	+3.602 ± 0.213			
34°C	1	+3.174	+0.989	+0.777	6
	2	+2.933	+0.989	+0.487	6
	3	+3.029	+0.997	+1.261	6
	4	+3.677	+0.993	+0.800	6
MEAN	S.D.:-	+3.203 ± 0.330			
30°C	1	+1.981	+0.899	+1.743	6
	2	+1.489	+0.796	+2.128	6
	3	+2.094	+0.981	+0.613	6
MEAN	S.D.:-	+1.854 ± 0.322			
25°C	1	+0.218	+0.293	+1.581	8
	2	+0.947	+0.656	+0.548	8
	3	+0.485	+0.666	+1.396	8
MEAN	S.D.:-	+0.564 ± 0.365			
15°C	1	+0.135	+0.250	+0.686	8
	2	+0.288	+0.453	+0.260	8
	3	-0.057	-0.124	+0.916	8
	4	-0.246	-0.267	+2.583	8
	5	+0.002	+0.003	+1.523	8
MEAN	S.D.:-	+0.024 ± 0.201			

[Results obtained at 25 and 15°C were provided by Mrs H. Cable].

Table 4.3 Effect of incubation temperature on the rate of digestion and on the putative rate of uptake of formaldehyde-denatured ^{125}I-labelled bovine serum albumin by 17.5-day rat yolk sacs incubated in serum-free medium.

The rate of digestion and the rate of uptake of formaldehyde-denatured ^{125}I-labelled bovine serum albumin (calculated assuming that digestion occurs intracellularly) were determined as described in Section 4.2.1. Yolk sacs were incubated, at the temperature indicated, for 15 min before addition of the ^{125}I-labelled albumin (1 µg/ml of culture medium) then incubation was continued for a further 3 h. The values reported in each individual experiment are the means of pairs of values obtained from two yolk sacs incubated separately (see Section 4.2.1).

Incubation temperature	Expt. No.	Rate of digestion (ng/h per mg yolk-sac protein)	Quantity of radioactivity associated with yolk-sac at 3 h (ng/mg yolk-sac protein)	Putative rate of uptake (ng/h per mg yolk-sac protein)
37°C	1	+387.9	124.3	391.6
	2	+274.1	142.5	299.6
	3	+352.8	130.2	341.1
	4	+366.9	206.3	379.7
	MEAN ± S.D. :-	+345.4 ± 49.7	150.8 ± 37.7	353.0 ± 41.6
34°C	1	+219.9	138.2	245.4
	2	+186.7	96.1	197.4
	3	+318.9	225.7	356.4
	4	+279.9	172.7	303.1
	MEAN ± S.D. :-	+251.3 ± 59.3	158.1 ± 54.8	275.5 ± 69.0
30°C	1	+131.0	155.3	159.2
	2	+126.8	163.6	154.8
	3	+118.3	131.6	137.1
	MEAN ± S.D. :-	+125.4 ± 6.4	150.2 ± 16.6	150.4 ± 11.7
25°C	1	+ 33.7	97.1	58.1
	2	+ 43.5	122.5	117.3
	3	+ 44.1	37.0	43.8
	MEAN ± S.D. :-	+ 40.5 ± 5.8	85.5 ± 43.9	73.1 ± 38.9
15°C	1	+ 1.0	31.4	10.8
	2	+ 0.1	11.4	4.6
	3	- 0.1	17.2	5.8
	4	- 0.6	32.8	11.3
	5	- 0.9	34.1	14.4
	MEAN ± S.D. :-	+ 0.1 ± 0.7	28.8 ± 7.8	10.6 ± 3.6

[Results obtained at 25 and 15°C were provided by Mrs H. Cable].

Table 4.4 Summary of the effects of incubation-temperature on the rate of accumulation of ^{125}I-labelled poly(vinylpyrrolidone), and on the rate of digestion and the putative rate of uptake of formaldehyde-denatured ^{125}I-labelled bovine serum albumin by 17.5-day rat yolk sacs incubated in serum-free medium.

From the data in Tables 4.2 and 4.3, the relative rates of uptake and digestion were calculated and expressed as a percentage (± standard deviation) of the mean value obtained at 37°C.

Incubation temperature (°C)	No. of expts.	Relative rate of accumulation of ^{125}I-labelled PVP (3.0 h period)	Relative rate of digestion of ^{125}I-labelled albumin (3.0 h period)	Relative putative rate of uptake of ^{125}I-labelled albumin (3.0 h period)
37	4	100.0 ± 5.9	100.0 ± 14.4	100.0 ± 11.8
34	4	88.9 ± 9.2	72.7 ± 17.2	78.0 ± 19.5
30	3	51.4 ± 8.9	36.3 ± 1.8	42.6 ± 3.3
25	3	15.6 ± 10.1	11.7 ± 1.7	20.7 ± 11.0
15	5	0.7 ± 5.8	0.1 ± 0.7	3.0 ± 1.0

Table 4.5 Effects of rotenone on the uptake of ^{125}I-labelled poly(vinyl-pyrrolidone) and on the rate of digestion and putative rate of uptake of ^{125}I-labelled formaldehyde-denatured bovine serum albumin by 17.5-day rat yolk sacs incubated in serum-free medium 199.

The quantity of ^{125}I-PVP accumulated during a 3.0 h period of incubation was determined as described in Section 2.2.1. The rate of digestion and the rate of uptake of formaldehyde-denatured ^{125}I-labelled bovine serum albumin (calculated by assuming that digestion occurs intracellularly) were determined as described in Section 4.2.1. In experiments with either substrate, yolk sacs were incubated in the presence of a given concentration of rotenone for 30 min before addition of substrate (^{125}I-PVP 2 µg/ml of culture medium; or ^{125}I-labelled albumin, 1 µg/ml of medium). The results shown for each experiment are derived from data on a single yolk-sac. Each experiment was performed with yolk sacs taken from a different animal.

Rotenone concentration (M)	Expt. No.	Uptake of ^{125}I-PVP at 3.0 h (µl/mg yolk-sac protein)	Rate of digestion of ^{125}I-labelled albumin (ng/h per mg yolk-sac protein)	Quantity of ^{125}I-labelled albumin accumulated in yolk-sac by 3.0 h (ng/mg yolk-sac protein)	Putative rate of uptake of ^{125}I-labelled albumin (ng/h per mg yolk-sac protein)
0 (control)	1	9.49	252.6	125.9	249.7
	2	10.08	283.9	150.4	286.7
	3	8.12	268.8	159.7	275.6
	4	9.92	333.5	171.6	344.0
MEAN ± S.D. :-		9.40 ± 0.88	284.7 ± 34.9	151.9 ± 19.4	289.0 ± 39.8
10^{-11}	1	10.24	234.7	145.6	236.3
	2	8.92	290.5	155.0	302.3
	3	9.52	280.0	134.4	285.4
	4	8.56	292.1	141.0	293.0
MEAN ± S.D. :-		9.31 ± 0.73	274.3 ± 26.9	144.0 ± 8.6	279.2 ± 29.4
10^{-9}	1	4.40	200.4	110.2	223.1
	2	8.31	224.8	134.2	228.9
	3	7.34	232.2	161.3	257.6
	4	4.83	286.0	194.0	280.3
MEAN ± S.D. :-		6.22 ± 1.90	235.9 ± 36.1	149.9 ± 36.0	247.5 ± 26.6
10^{-7}	1	2.69	107.4	94.2	110.2
	2	1.16	95.6	87.9	98.1
	3	3.50	142.6	127.0	166.8
	4	1.76	116.0	84.7	125.8
MEAN ± S.D. :-		2.39 ± 0.88	115.4 ± 19.9	98.4 ± 19.4	125.2 ± 29.9
10^{-5}	1	1.71	21.3	43.5	30.1
	2	0.96	32.4	54.7	45.3
	3	1.24	62.7	88.3	77.3
	4	1.02	32.1	48.4	39.5
MEAN ± S.D. :-		1.23 ± 0.34	37.1 ± 17.8	58.7 ± 20.2	48.1 ± 20.4

Table 4.6 Summary of the effects of rotenone on the uptake of ^{125}I-labelled poly(vinylpyrrolidone) and on both the rate of digestion and putative rate of uptake of formaldehyde-denatured ^{125}I-labelled bovine serum albumin by the 17.5-day rat yolk sacs incubated in serum-free medium 199.

From the data presented in Table 4.5 the relative rates of uptake and digestion were calculated and expressed as a percentage (± standard deviation) of the mean value obtained with control yolk-sacs.

Rotenone concentration (M)	No. of expts.	Relative uptake of ^{125}I-labelled PVP over a 3.0 h period	Relative rate of digestion of ^{125}I-labelled albumin (3.0 h period)	Relative putative rate of uptake of ^{125}I-labelled albumin (3.0 h period)
0 (control)	4	100.0 ± 9.4	100.0 ± 12.2	100.0 ± 13.8
10^{-11}	4	99.0 ± 7.7	96.3 ± 9.4	96.6 ± 10.1
10^{-9}	4	66.2 ± 2.0	82.8 ± 12.7	85.6 ± 9.1
10^{-7}	4	25.4 ± 9.4	40.5 ± 6.9	43.3 ± 10.3
10^{-5}	4	13.1 ± 3.6	13.0 ± 6.2	16.6 ± 7.0

Table 4.7 Effects of glucagon on the uptake of ^{125}I-labelled poly(vinylpyrrolidone) and on both the rate of digestion and putative rate of uptake of formaldehyde-denatured ^{125}I-labelled bovine serum albumin by 17.5-day rat yolk sacs incubated in serum-free medium 199.

The quantity of ^{125}I-PVP accumulated during a 3.0 h period of incubation was determined as described in Section 2.2.1. The rate of digestion and the rate of uptake of the ^{125}I-labelled albumin (calculated by assuming that digestion occurs intracellularly) were determined as described in Section 4.2.1. With either substrate, yolk sacs were incubated in the presence of the glucagon (or dibutyryl-cAMP) for 30 min before addition of substrate (^{125}I-PVP, 2µg/ml of culture medium; or ^{125}I-labelled albumin, 1 µg/ml of culture medium). The results shown for each experiment are derived from data on a single yolk-sac each taken from a different animal.

Effector compound	Expt. No.	Uptake of ^{125}I-PVP at 3.0 h (µl/mg yolk-sac protein)	Rate of digestion of ^{125}I-labelled albumin (ng/h/mg yolk-sac protein)	Quantity of ^{125}I-labelled albumin accumulated by the yolk sac at 3.0 h (µg/mg yolk-sac protein)	Putative rate of uptake of ^{125}I-labelled albumin (ng/h per mg yolk-sac protein.
(Control)	1	10.12	216.3	123.7	237.5
	2	8.45	196.1	153.3	227.7
	3	9.61	233.0	144.0	238.0
MEAN ± S.D. :-		9.39 ± 0.86	215.1 ± 18.4	140.3 ± 15.1	234.4 ± 5.8
(As % of Control:		100.0 ± 9.9	100.0 ± 8.5	100.0 ± 10.7	100.0 ± 2.4)
Glucagon 10^{-6}M	1	2.45	120.0	72.6	137.4
	2	3.56	47.1	25.7	42.0
	3	2.99	100.5	58.7	102.1
MEAN ± S.D. :-		3.00 ± 0.55	89.2 ± 37.7	52.3 ± 24.1	93.8 ± 48.2
(As % of Control:		31.9 ± 5.8	41.1 ± 17.5	37.2 ± 17.2	40.0 ± 20.5)
db-cAMP 5×10^{-4}M	1	-	170.3	133.8	187.6
	2	-	120.1	59.7	123.2
	3	-	130.0	52.1	131.8
MEAN ± S.D. :-		-	140.1 ± 26.6	81.8 ± 45.1	147.5 ± 34.9
(As % of Control :		-	65.1 ± 12.3	58.3 ± 32.1	62.9 ± 14.9)

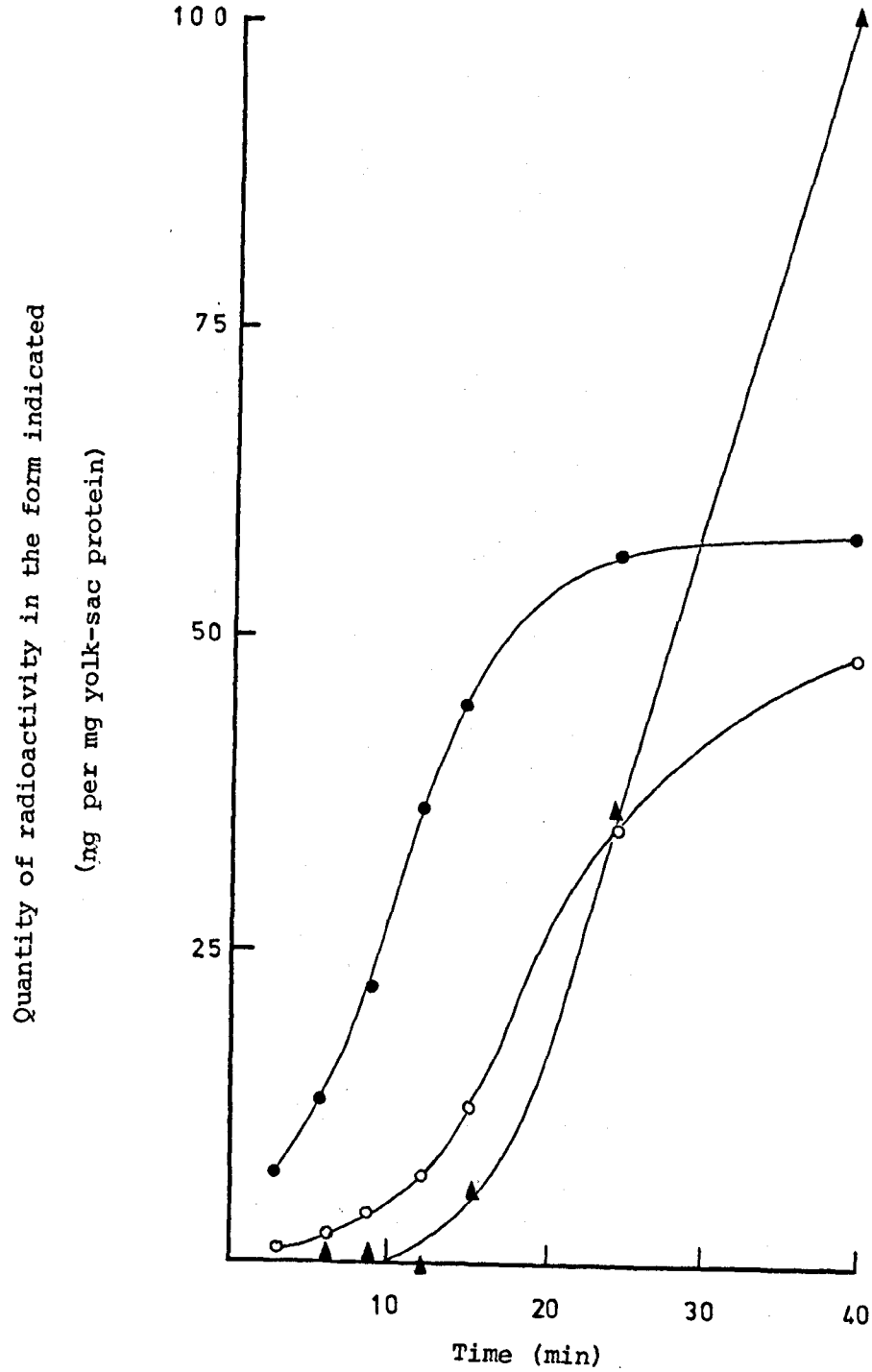


Figure 4.1 Time course of the appearance of acid-insoluble and -soluble radioactivity in the yolk-sac tissue and acid-soluble radioactivity in the culture medium when 17.5-day yolk sacs were incubated in the presence of formaldehyde-denatured ^{125}I-labelled bovine serum albumin in serum-free medium 199.

Yolk sacs were incubated in serum-free medium containing ^{125}I-labelled albumin (1 µg/ml of culture medium). At the indicated times, samples were taken to measure the quantities of tissue-associated acid-soluble (o) and -insoluble (●) radioactivity and the quantity of acid-soluble radioactivity (▲) in the culture medium. The points at each time interval were derived from single yolk sacs.

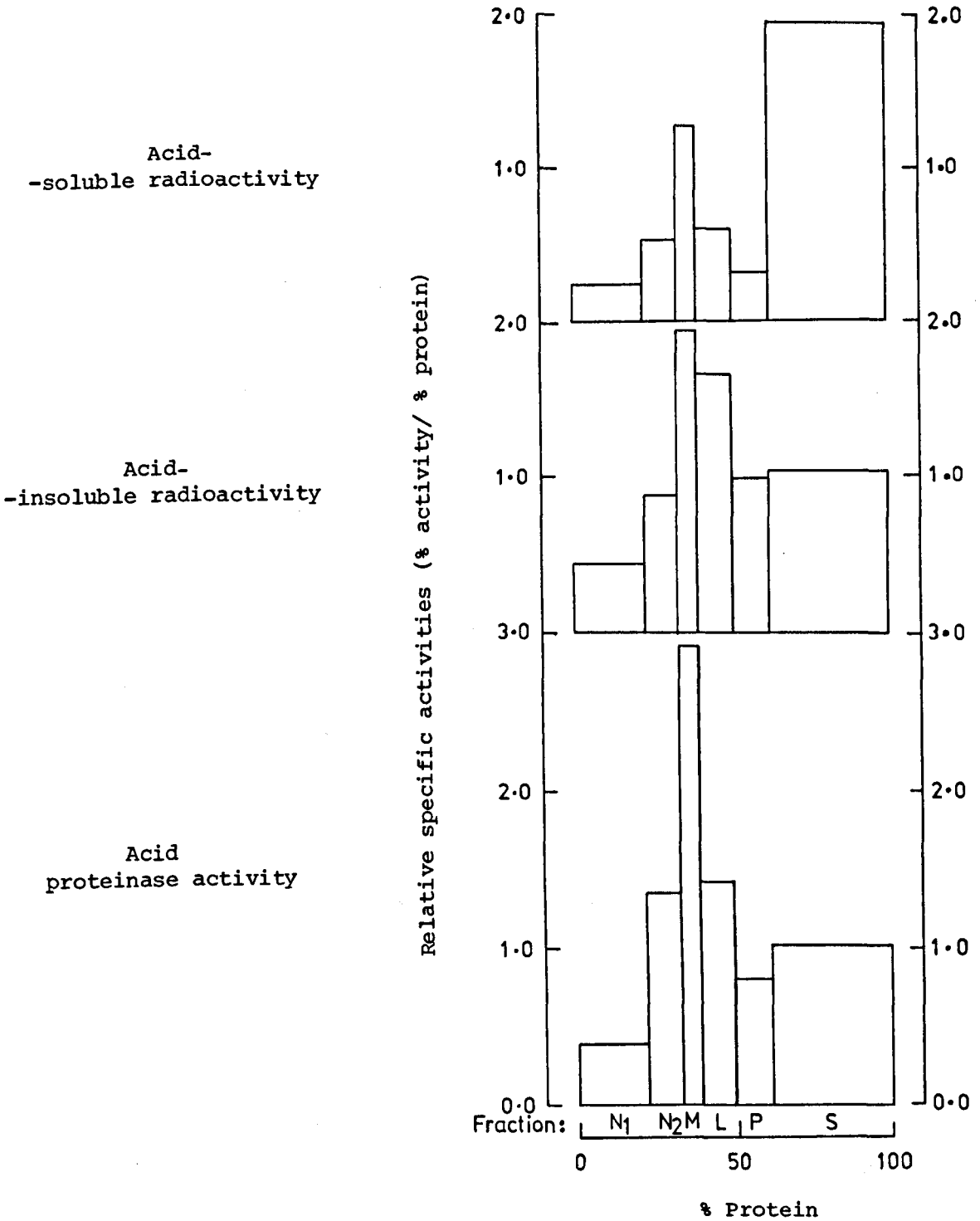


Figure 4.2 Distribution of acid-soluble radioactivity, acid-insoluble radioactivity and acid proteinase activity in subcellular fractions prepared from yolk sacs that had been previously incubated with formaldehyde-denatured ^{125}I-labelled bovine serum albumin in serum-free medium 199.

Graphical presentation as described by deDuve (1955). For experimental method see Section 4.2.2.

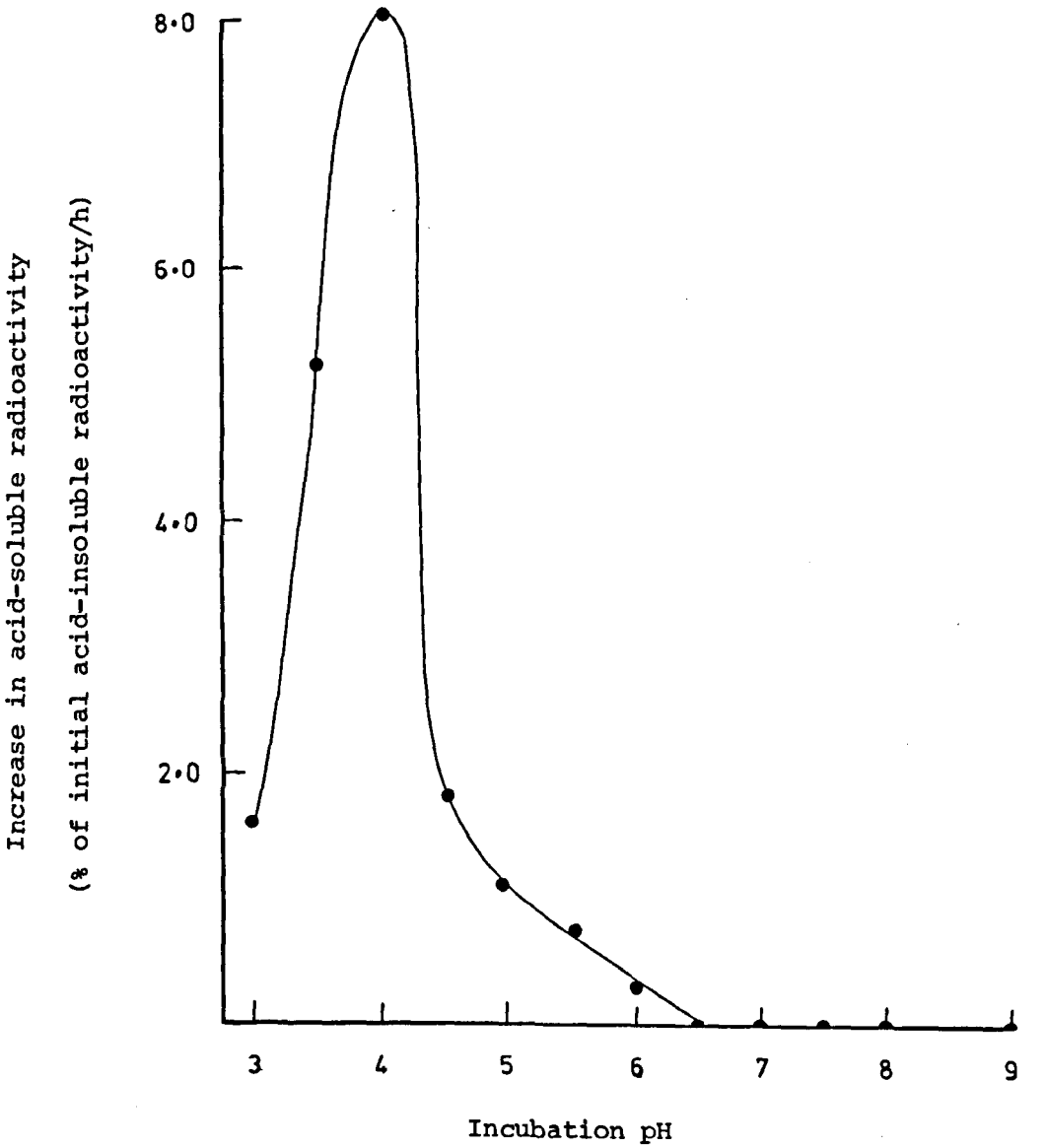


Figure 4.3 Effect of incubation pH on the release of acid-soluble radioactivity from formaldehyde-denatured ^{125}I-labelled bovine serum albumin by cell-free extracts of yolk sac.

The incubation medium contained cell-free extract (approx. 150 µg yolk-sac protein), formaldehyde-denatured ^{125}I-labelled bovine serum albumin (10 µg) and buffer solution (0.1M-Na acetate, pH 3.0 to 6.5 and 0.1M-TRIS pH 7.0 to 9.0). Each point is the mean of four individual determinations using a single preparation of cell-free extract. For methods see Section 4.2.4.

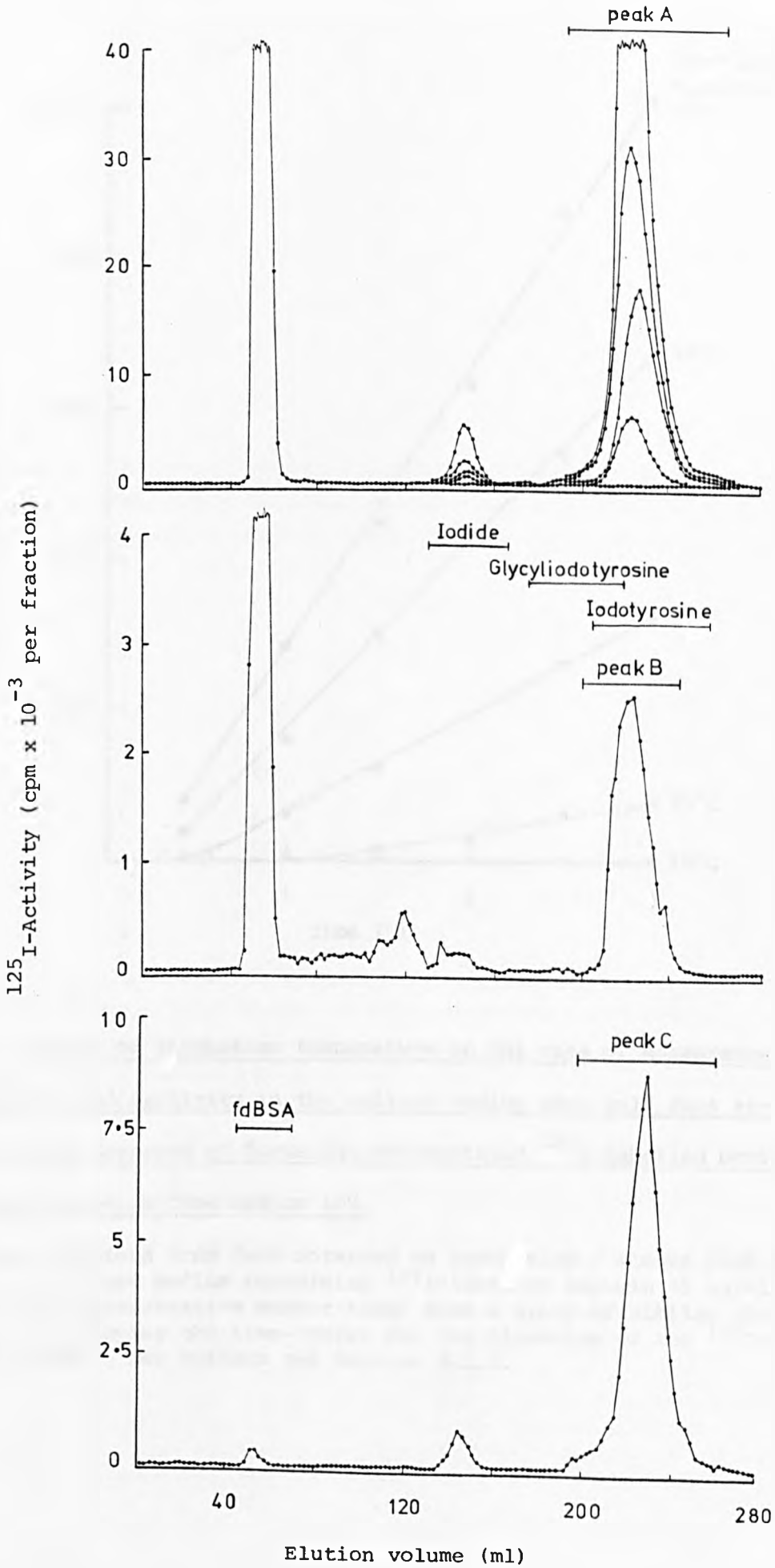
Figure 4.4 Sephadex G-25 chromatography of formaldehyde-denatured ^{125}I-labelled bovine serum albumin hydrolysis products.

Upper: ^{125}I-Labelled albumin incubated in the absence of yolk--sac tissue for 2h and in the presence of yolk-sac tissue for 1, 2, 3 & 6h. (The recovery values were: 107, 86, 96, 104 & 106% of the radioactivity applied respectively.)

Middle: ^{125}I-Activity associated with the yolk-sac tissue after incubation of the tissue with ^{125}I-labelled albumin for 2h. (The recovery value was 90% of the radioactivity applied.)

Lower: ^{125}I-Activity released during a 2h incubation period from the yolk-sac tissue previously loaded with ^{125}I-labelled albumin. (The recovery value was 109% of the radioactivity applied.)

For experimental details see Section 4.2.5. The elution positions of formaldehyde-denatured ^{125}I-labelled bovine serum albumin (fdBSA), [^{125}I]iodide, [^{125}I]iodo-L-tyrosine and glycyl-[^{125}I]iodo-L--tyrosine are indicated by the horizontal bars. The radioactivity contained in the fractions under the horizontal bars of peaks A (3h), B & C were separately pooled, lyophilized and further analysed on a copper-complex of Sephadex G-25.


peak A
40
30
20
10
0
Iodide
Glycyliodotyrosine
Iodotyrosine
peak B
4
3
2
1
0
peak C
fdBSA
10
7·5
5
2·5
0
40
120
200
280
125I-Activity (cpm x 10-3 per fraction)
Elution volume (ml)

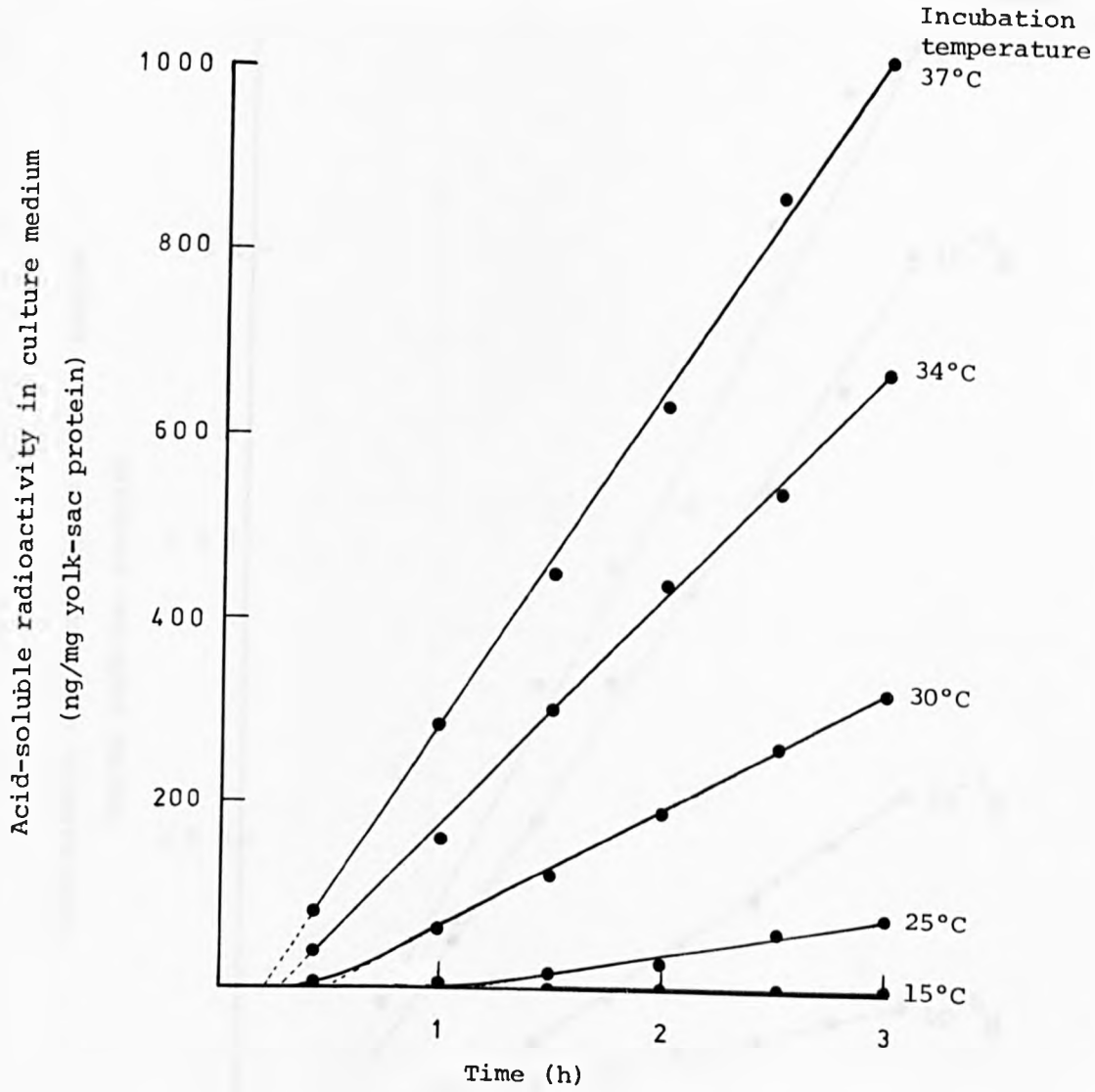


Figure 4.5 Effect of incubation temperature on the rate of appearance of acid-soluble radioactivity in the culture medium when yolk sacs are incubated in the presence of formaldehyde-denatured ^{125}I-labelled bovine serum albumin in serum-free medium 199.

Each plot was obtained from data obtained on incubating a single yolk-sac in serum-free culture medium containing ^{125}I-labelled albumin (1 µg/ml). Each plot is a representative member taken from a group of similar plots (see Table 4.3) showing the time-course for the digestion of the ^{125}I--labelled albumin. For methods see Section 4.2.1.

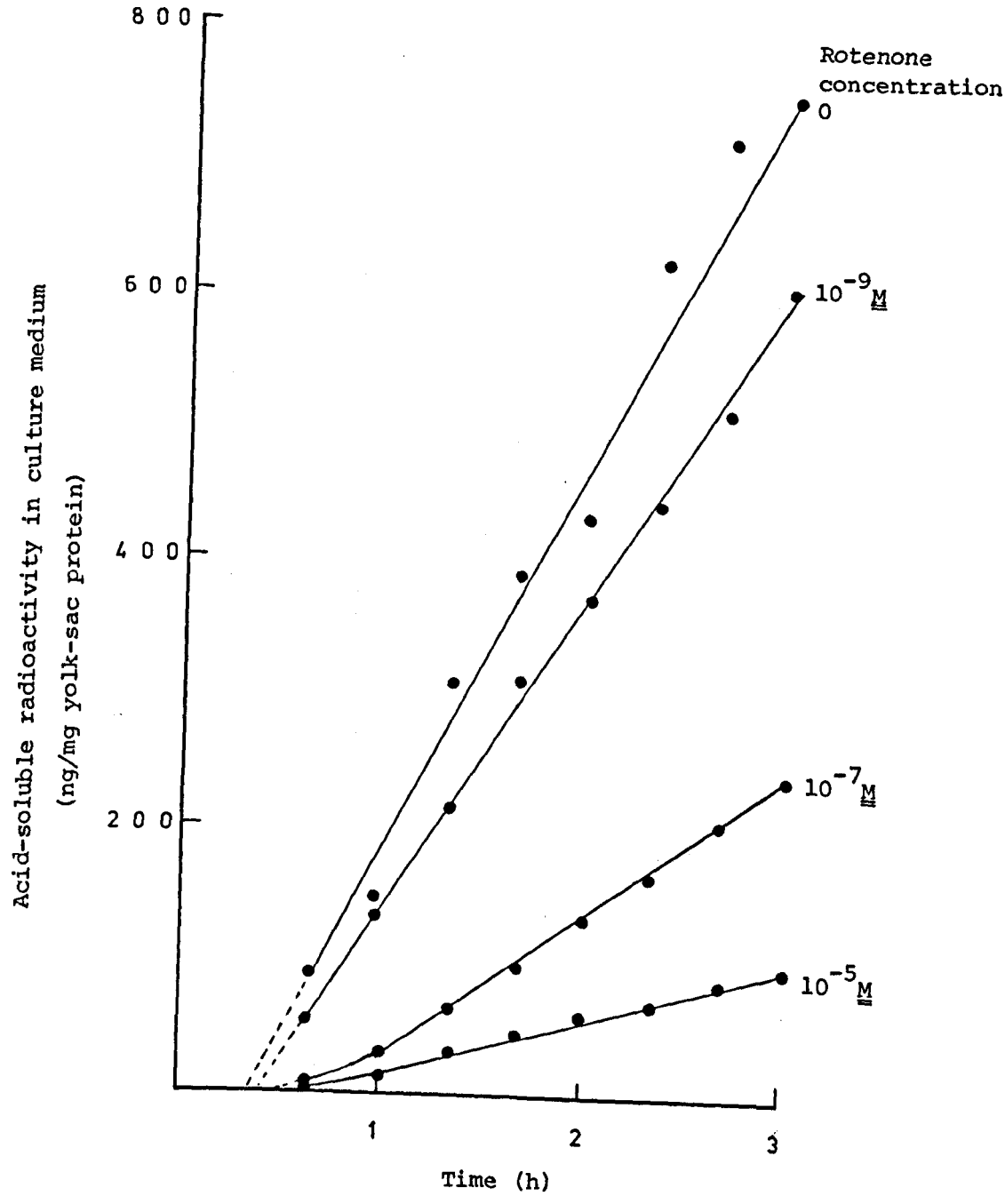


Figure 4.6 Effect of rotenone on the rate of appearance of acid-soluble radioactivity in the culture medium when yolk sacs are incubated in the presence of formaldehyde-denatured ^{125}I-labelled bovine serum albumin in serum-free medium 199.

Each plot was obtained on the incubation of a single yolk sac in serum-free culture medium containing ^{125}I-labelled albumin (1 µg/ml of culture medium). Each plot is a representative member taken from a group of similar plots (see Table 4.4), showing the time course for the digestion of the ^{125}I--labelled albumin. Yolk sacs were incubated in the presence of rotenone for 30 min before the substrate was added. For methods see Section 4.2.1.

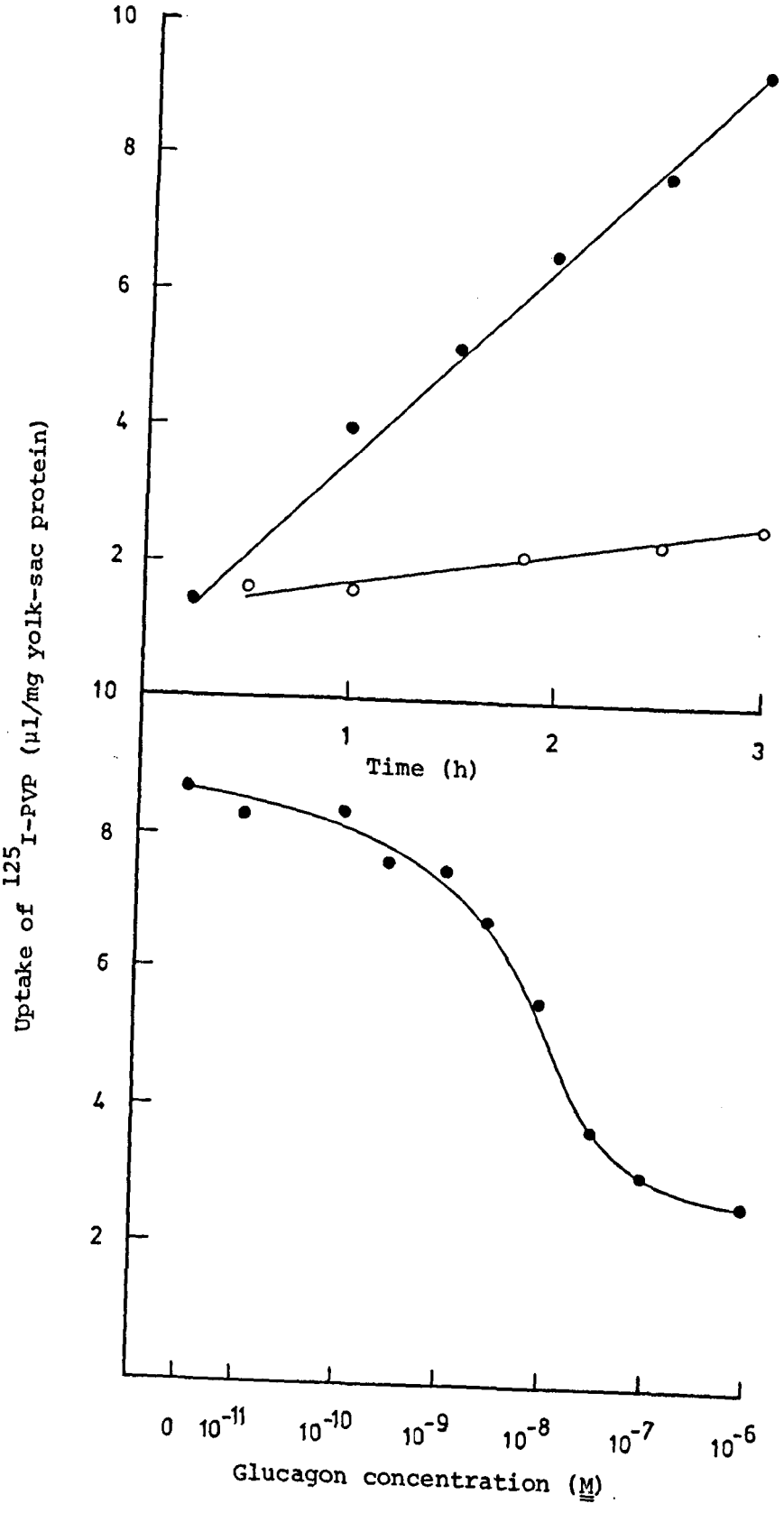


Figure 4.7 Effect of glucagon on the uptake of ^{125}I-labelled poly(vinyl-pyrrolidone) by 17.5-day rat yolk sacs incubated in serum-free medium 199.

Uptake of ^{125}I-PVP (2 µg/ml) was determined as described in Section 2.2.1.

Upper: control, (●); 10^{-6}M-glucagon (○).

Lower: uptake (3 h) at differing glucagon concentrations.

4.4 DISCUSSION

In previous quantitative studies of pinocytosis, using the yolk-sac culture system, several authors (Moore et al., 1974, 1977; Roberts et al., 1976; Pratten et al., 1978; Duncan & Lloyd, 1978; Ibbotson, 1978 and Chapters 2 & 3 of this thesis) have assumed that formaldehyde-denatured ^{125}I-labelled bovine serum albumin, added to the incubation medium, is digested entirely intracellularly by the yolk-sac tissue. Except for Moore et al. (1977) who briefly discussed some evidence (unpublished work, mainly derived from this thesis), none of the above authors gave evidence for the site of the albumin digestion being intracellular. Several lines of evidence presented here show that, when incubated with rat yolk-sacs, formaldehyde-denatured ^{125}I-labelled bovine serum albumin is, indeed, digested entirely intracellularly within lysosomes.

From an analysis of the distribution of radioactive species during the course of an incubation experiment with formaldehyde-denatured ^{125}I-labelled albumin, the maximum rate of accumulation of acid-insoluble radioactivity (representing macromolecular albumin) in the yolk-sac tissue, in the initial stages of the incubation was found to be equal to the rate of appearance of acid-soluble radioactivity in the incubation medium at a later stage, i.e. when the tissue-associated radioactivity had reached a steady-state level (Fig. 4.1). Provided that the rate of pinosome formation does not decrease with increasing incubation time (there is no evidence to suggest that it does), the rate of appearance of acid-soluble radioactivity in the incubation medium can be fully accounted for by the complete digestion of that albumin that has been captured by pinocytosis. The sequence of events shown in Fig. 4.1 is of the form expected if a precursor-product relationship exists between the acid-

-insoluble- and acid-soluble radioactive-species in the tissue, and between the acid-soluble radioactivity in the tissue and that in the incubation medium. These events are entirely consistent with the formaldehyde--denatured ^{125}I-labelled bovine serum albumin being ingested by pinocytosis, its accumulation in the vacuolar system prior to digestion within lysosomes, then diffusion of the digestion products from the lysosomes, through the cytoplasm to the incubation medium, where they accumulate.

Digestion of formaldehyde-denatured ^{125}I-labelled bovine serum albumin at an extracellular site could also lead to an initial lag-period, before products of digestion small enough to be acid-soluble appear in the incubation medium, but, if this explanation is valid, it would be expected that detectable amounts of intermediate-sized digestion products would be present in the medium. Alternatively, the observed lag-period could represent a delay in secretion of neutral proteinase activity into the incubation medium. This latter possibility can be dismissed since no hydrolysis of the formaldehyde-denatured ^{125}I-labelled bovine serum albumin occurred on its addition to incubation medium in which yolk-sacs had previously been incubated for 3h. The lag-period observed here (15-20min) is of the same magnitude as other lag-periods recently reported in the literature: approx. 20min for the digestion of formaldehyde-denatured ^{125}I-labelled human serum albumin by non-parenchymal cells isolated from rat liver (Nilsson & Berg, 1977), 30min for the digestion of ^{125}I-labelled plasma low density lipoprotein by normal human fibroblasts (Stein et al., 1976) and 7-8min for the intracellular digestion of ^{125}I-labelled insulin by cultured hepatocytes (Terris & Steiner, 1975). Terris & Steiner also showed that increasing concentrations of insulin do not shorten the lag-period and suggested that this is consistent with an obligatory

translocation of the insulin from a binding-site to an intramembranous or intracellular site of degradation. Increasing the concentration of formaldehyde-denatured bovine serum albumin (0-200µg/ml, Table 3.1) did not shorten the lag-period in the rat yolk-sac system. Nilsson & Berg (1977) suggested that the lag-period is indicative of the time required for fusion of phagosomes with lysosomes, but evidence presented in Chapter 7 of this thesis indicates that it is at least in part dependent on the susceptibility of the endocytosed substrate to digestion by the lysosomal enzymes subsequent to phagosome-lysosome fusion.

A strong piece of evidence for the site of hydrolysis being intracellular comes from the demonstration that the calculated concentration of acid-soluble radioactivity in the yolk-sac tissue is several orders of magnitude greater than that in the incubation medium. Yolk sacs were found to contain approx. 110mg protein (BSA equivalents) per g wet-weight of tissue. Assuming the tissue to have an approximate specific gravity of 1.0, the volume of the tissue was calculated to be 8.85µl/mg yolk-sac protein. After 40min of incubation with formaldehyde-denatured ^{125}I--labelled bovine serum albumin (1µg/ml of incubation medium), the quantity of acid-soluble radioactivity associated with the yolk-sac tissue was 43ng/mg yolk-sac protein (Fig. 4.1); this is equivalent to a concentration in the yolk-sac tissue of 43/8.85 or 4.85ng/µl. At the same time (40min) the concentration of acid-soluble radioactivity in the incubation medium, determined from Fig. 4.1, was [101ng/mg x 5mg yolk-sac protein/20 000µl of incubation medium] or 0.025ng/µl assuming the incubation had been performed with an averaged-sized yolk sac containing 5mg of protein. These results therefore indicate that the concentration of acid-soluble radioactivity in the yolk-sac tissue is 4.85/0.25 or 194 times greater than the concentration in the medium after 40 min of incubation. [This value must be regarded as a conservative estimate of the actual ratio of the

concentration, since not all the space within the yolk-sac tissue will be available to the acid-soluble radioactivity.] The much higher tissue concentration of the radioactive digestion products compared to the concentration in the incubation medium suggests that the direction of the flow of the digestion products is from within the tissue, out into the incubation medium.

The presence of measurable levels of intermediate products of digestion in the incubation medium (Fig. 4.4) would be expected if extracellular digestion of the albumin substrate occurred. The Sephadex G-25 profiles of the digestion products that appear in the medium on incubating yolk sacs with formaldehyde-denatured ^{125}I-labelled bovine serum albumin shows only peaks corresponding to [^{125}I]iodo-L-tyrosine and oligopeptides containing this amino acid residue. Similar profiles are also obtained on analysing the radioactivity released from yolk sacs previously loaded with the ^{125}I-labelled albumin substrate. This indicates that the products of digestion can be accounted for by the radioactivity released from the yolk-sac tissue. Moreover, the presence of only small amounts of intermediate products of digestion in the yolk-sac homogenate indicates that degradation, once begun, proceeds rapidly. This is concordant with the "all or none" hypothesis of Huisman et al. (1974) in which they suggest that the initial proteolytic cleavage is the rate-limiting step in the digestion of a protein intralysosomally. The appearance, in the incubation medium, of only end-products of digestion, is in full agreement with a lysosomal site of digestion in which the size of the digestion products released into the incubation medium is determined by the permeability preperties of the lysosomal membrane (see Reijngoud & Tager, 1977, for a discussion of this subject).

Further evidence for a lysosomal site of digestion was obtained

on examining the distribution of ^{125}I-radioactivity among subcellular fractions, obtained by differential centrifugation (see Section 4.3.2), from homogenates of yolk sacs that had been loaded with formaldehyde--denatured ^{125}I-labelled bovine serum albumin. The distribution of acid-insoluble ^{125}I-activity in the various centrifugal fractions closely paralleled that of acid-proteinase activity. Similar observations were reported by Williams et al. (1971) using acid-denatured ^{125}I-labelled bovine serum albumin and by Goetze et al. (1976) using formaldehyde--denatured ^{125}I-labelled human serum albumin. Moreover, the digestion of formaldehyde-denatured ^{125}I-labelled bovine serum albumin by cell-free homogenates of 17.5-day rat yolk sacs showed an acid pH optimum, a finding that is consistent with digestion involving lysosomal cathepsins (Coffey & de Duve, 1968). No measurable digestion occurred with the cell-free extract at neutral pH. This result indicates that the tissue does not contain, intracellularly or extracellularly, neutral proteinases, with either sufficient activity or with the appropriate specificity to digest the formaldehyde-denatured ^{125}I-labelled bovine serum albumin, and that such enzymes cannot be involved in the digestion of this protein when incubated with yolk sacs in medium 199 (pH 7.1).

The digestion of formaldehyde-denatured ^{125}I-labelled bovine serum albumin by yolk sacs was inhibited by decreasing the temperature at which yolk sacs were incubated, by the presence of the metabolic inhibitor rotenone, and by the presence of the peptide hormone glucagon. These conditions were also shown to inhibit the pinocytic uptake of ^{125}I--poly(vinylpyrrolidone) [^{125}I-PVP]. It is difficult to envisage how each of the three conditions might also inhibit a possible extracellular proteolytic enzyme activity. The pattern of the inhibition of ^{125}I-PVP uptake by the yolk sacs on incubation at lower temperatures in serum-free

medium closely parallels that obtained by Duncan & Lloyd (1978) for yolk sacs incubated in the presence of 10% calf serum. The inhibition of pinosome formation, in the rat yolk sac, by rotenone has not been previously reported. Here glucagon, present at a concentration of 10^{-6} M, maximally inhibited the uptake of ^{125}I-PVP by approximately 70%; half-maximum effect of glucagon was obtained with a concentration of 10^{-8} M-glucagon. The observations made here with yolk sacs incubated in serum-free medium compare well with those of Brown & Segal (1977) who used medium containing 10% calf serum. These authors observed a 75% inhibition of the uptake of yeast invertase by 10^{-6} M-glucagon; half-maximum effect being at 3×10^{-8} M--glucagon.

The change in the rate of digestion of formaldehyde-denatured ^{125}I-labelled bovine serum albumin paralleled the change in the rate of uptake of ^{125}I-PVP when either the incubation temperature was progressively decreased (Table 4.4) or the rotenone concentration progressively increased (Table 4.6) or 10^{-6} M-glucagon was present (Table 4.7). Such parallelism strongly indicates that the rate of digestion of the formaldehyde-denatured ^{125}I-labelled bovine serum albumin is limited by the rate of pinosome formation and that no detectable extracellular digestion of the ^{125}I-labelled albumin occurs.

The data reported in this chapter validate the previous assumption that formaldehyde-denatured ^{125}I-labelled bovine serum albumin is digested exclusively intracellularly and within lysosomes. Consequently, the putative rate of uptake of the ^{125}I-labelled albumin reported here represents the actual rate of uptake of this protein into epithelial cells of the rat yolk sac. Thus formaldehyde-denatured ^{125}I-labelled bovine serum albumin is a suitable substrate for use in the yolk-sac culture system to investigate aspects of pinocytosis and lysosome function.

CHAPTER FIVE

EFFECTS OF WEAK BASES ON PROTEIN CATABOLISM WITHIN RAT YOLK SACS

5.1 INTRODUCTION

The weak base chloroquine, an anti-malarial and anti-inflammatory drug, has been shown to inhibit the digestion of several substances that gain entry into the lysosomes of intact cells via either autophagy or heterophagy. Wibo & Poole (1974) used chloroquine to partially inhibit the digestion of cellular-proteins within rat fibroblasts in culture. Lie & Schofield (1973) showed that human fibroblasts incorporate [^{35}S]-sulphate into mucopolysaccharides and that, in the presence of chloroquine, these cellular ^{35}S-labelled mucopolysaccharides accumulate within lysosomes, so that normal cells exposed to chloroquine were indistinguishable from cells with a genetic defect in a lysosomal enzyme responsible for the digestion of mucopolysaccharide. When macrophages were allowed to engulf [^{3}H]leucine-labelled bacteria (de Duve *et al.*, 1974), complete inhibition of the release of acid-soluble radioactivity was achieved by chloroquine present in the incubation medium.

The lysosomal digestion of lipoproteins, both *in vivo* and by cells in culture, is also sensitive to inhibition by chloroquine. Stein *et al.* (1977) showed that both human- and rat-plasma low density lipoproteins are cleared from the circulation of the rat by the liver and that recovery of the lipoproteins from the liver was increased by pretreating the animals with chloroquine, a result compatible with a decreased rate of hydrolysis of the lipoproteins by the liver. Goldstein *et al.* (1975) discovered that chloroquine inhibits the lysosomal proteolysis of low density lipoproteins that had been endocytosed by human fibroblasts in culture. The lysosomal hydrolysis of the cholesteryl ester associated with lipoproteins was also inhibited (Goldstein *et al.*, 1975; Brown *et al.*, 1975). Moreover, Floren & Nilsson (1977) showed an inhibition, by chloroquine, of cholesteryl ester degradation, subsequent

to the endocytosis of chylomicron remnant particles by rat hepatocyte monolayers.

The ability of chloroquine to inhibit the lysosomal breakdown of a variety of substrates does not seem to be a property peculiar to this drug, but has led to it being regarded as a specific inhibitor of lysosome function. Ammonium ions (and methylammonium- and ethylammonium ions) are also inhibitors of lysosome function, but have been used less extensively for this purpose than has chloroquine. Seglen (1975, 1977) and Seglen & Reith (1976) observed that ammonium ions inhibited the autophagic digestion of cellular proteins in hepatocytes in culture. This observation was confirmed by Hopgood et al. (1977), who also showed that methylammonium- and ethylammonium ions had a similar but more potent inhibitory effect. The greater potency was, however, considered by these authors to be due to the inability of the hepatocytes to detoxify the substituted ammonium ions via the urea cycle. Tolleshaug et al. (1977) also showed that the digestion of asialo-fetuin, endocytosed by hepatocytes in culture, is sensitive to ammonium ion inhibition (and to inhibition by chloroquine). Moreover, Carpenter & Cohen (1976) discovered that ammonium ions are potent inhibitors of the degradation of endocytosed epidermal growth factor by human fibroblasts in culture. Glimelius et al. (1977) discovered that human glial cells incorporate [^{35}S]sulphate into glycosoaminoglycans and that these labelled macromolecules accumulate in the cells when incubated in medium containing ammonium ions; no such accumulation was observed when these cells were incubated in the absence of ammonium ions.

It is not entirely clear how chloroquine or ammonium ions (and other weak bases) exert an inhibitory effect on lysosomal digestion, but it is apparent that these substances gain access to the lysosomal interior.

Several basic (and neutral) substances have been shown to accumulate in the lysosomes of mammalian cells (Allison & Young, 1964). Chloroquine is taken up by HeLa cells (Polet, 1970), rat fibroblasts (Wibo & Poole, 1974), human fibroblasts (Lie & Schofield, 1973; Donato et al., 1976), macrophages (Fedorko et al., 1968a,b) and L-cells (Fedorko et al., 1968b) and has been shown to accumulate in lysosomes (Fedorko et al., 1968b; Allison & Young, 1964; Reijngoud & Tager, 1976). Morphological and biochemical evidence suggest that ammonium ions and other weak bases accumulate in lysosomes of isolated rat hepatocytes (Seglen & Reith, 1976) and that methylammonium ions can accumulate in isolated rat-liver lysosomes in vitro (Reijngoud & Tager, 1973 & 1977; Reijngoud et al., 1976; Henning, 1975; Goldman & Rottenberg, 1973). Morphological studies indicate that vacuolation (i.e. an increase in both the size and the number of vacuoles found in the cytoplasm) accompanies the uptake of both chloroquine (Fedorko et al., 1968a,b) and ammonium ions (Seglen & Reith, 1976) so that morphologically, the cells resemble those from individuals with a genetically induced lysosomal storage disease (Hers & Van Hoof, 1973).

Two different routes of entry of the weak bases into the lysosomes of intact cells have been suggested. Polet (1970) argued that for chloroquine an endocytic route could be involved. He observed that the cytoplasm of chloroquine-treated HeLa cells contained chloroquine-rich vacuoles, which he suggested resembled pinocytic vacuoles. But de Duve et al. (1974) suggested that the rate of entry of chloroquine into cells by this route would be too slow to explain the observations on chloroquine induced lysosomal dysfunction but also suggested that the alternative route (by permeation of both plasma and lysosomal membranes) could be slow, since, at neutral pH, chloroquine is almost entirely

in the protonated form. Homerwood et al. (1972), partially resolved this problem when they explained that, at physiological pH, chloroquine, with ionization constants of pKa_1 = 10.2 and pKa_2 = 8.1 (Irvin & Irvin, 1947), is 18% in the monoprotonated form and only a negligible amount is in the non-protonated form. The monoprotonated form, Homerwood and co-workers suggest, is soluble in lipid, consequently this form is capable of passing through cell membranes (Albert, 1968; see below) hence could readily enter lysosomes and then be retained within the more acidic lysosomal environment. This hypothesis was corroborated by the observation that [^{14}C]chloroquine is accumulated by isolated rat liver lysosomes (Reijngoud & Tager, 1976) a finding which refutes the suggestion that the only route of entry into the lysosomes is one involving endocytosis. Reijngoud & Tager (1976), however, assumed that chloroquine enters isolated lysosomes in the unprotonated form. But Albert (1968) writes "... a non-penetrating ion can often be made penetrating by the addition of a lipophilic group; the chloro- and other lipophilic-groups of the antimalarials mepacrine ('Atebrin') and chloroquine may assist thus in the necessary penetration [of the membranes] of red blood cells."

A non-endocytic route of entry into lysosomes for ammonium ions (also methylammonium- and ethylammonium ions) also seems probable since isolated rat-liver lysosomes accumulate [^{14}C]methylamine (for review see Reijngoud & Tager, 1977), a process that can be inhibited by ammonium ions in the incubation medium (Reijngoud et al., 1976). Hence, both ammonium- and methylammonium ions are able to enter lysosomes by permeation of membrane elements, but, unlike chloroquine, which might penetrate cell membranes in the monoprotonated form, it seems likely that ammonium ions (and probably the substituted ammonium ions) pass through the cell membranes in the unprotonated form (Verity & Brown, 1973).

There is little doubt that, once inside a lysosome, weak bases become trapped on protonation (de Duve et al., 1974; Reijngoud & Tager, 1977). It is not known, however, to what extent the protonated weak bases become bound to large anions within lysosomes. Glycoproteins in lysosomes have, in general, acidic-isoelectric points (Tappel, 1969), and Henning et al. (1973) showed the interior side of rat-liver lysosomal membranes to contain acidic-glycolipids. Dingle & Barrett (1969) extracted from kidney lysosomes an acidic substance, of apparent glycolipid nature, with a strong affinity for acridine orange and other cations [Acridine orange is a basic dye, that accumulates in lysosomes (Robbins & Marcus, 1963) and fluoresces orange at acid pH; it is now commonly used as a histological stain for this organelle.] Such substances might also contribute to the lysosome's ability to trap and accumulate weak bases. Henning et al. (1973) and Reijngoud & Tager (1977) suggested that such anionic substances are responsible for the generation of the acidic interior of the lysosome. Binding of protonated weak bases [and other cations which can permeate the lysosomal membrane, (Henning et al., 1975)] could therefore interfere with the ability of such anionic substances to maintain an acid environment within lysosomes.

The accumulation of weak bases by lysosomes is associated with a rise in the pH of the lysosome interior; this has been observed to occur both with methylamine (Reijngoud & Tager, 1973, 1977; Reijngoud et al., 1976; Henning, 1975) and with chloroquine (Reijngoud & Tager, 1976). In some of these studies a concomitant decrease in the rate of digestion of endocytosed formaldehyde-denatured ^{125}I-labelled bovine serum albumin was also observed. The proteolytic inhibition was considered to result, in part, from an elevation of the intralysosomal pH toward a value at which the lysosomal enzymes were less active (Coffey & de Duve, 1968).

Choroquine also directly inhibits cathepsin B (Wibo & Poole, 1974) aryl-sulphatase A (Smith et al., 1976) and the activities of several other poorly-defined hydrolytic enzymes (Cowey & Whithouse, 1966).

The above studies illustrate that both chloroquine and ammonium ions (and the substituted ammonium ions) can be usefully employed to demonstrate, in several cell types, the involvement of lysosomes in the digestion of several classes of substrate. However, a literature survey indicates that no quantitative studies of the effects of the weak bases on lysosome function and on endocytosis have been conducted. Many of the investigations of the effects of weak bases on the digestion of substrates by intact cells have been unsuitable for the quantitative estimation of lysosome function.

The contribution of non-lysosomal digestion to the breakdown of cellular proteins is not known so that an investigation of the effects of weak bases on the inhibition of total cell-protein breakdown would not permit an estimate to be made of the degree of inhibition of lysosomal activity. If, however, the degree of lysosomal inactivation were known precisely, such studies would permit an estimate to be made of the contribution of non-lysosomal digestive processes to the total cell-protein breakdown. This has been part of the aim of the investigations reported in this chapter.

Neufeld and co-workers (Fratantoni et al., 1968, 1969 ; Neufeld & Fratantoni, 1970) described a technique to measure the normal catabolic function of lysosomes in cultured fibroblasts. The technique takes advantage of the fact that when mammalian cells are cultured in the presence of inorganic [^{35}S]sulphate, the only macromolecules to become labelled are the mucopolysaccharides. The majority of the newly synthesized

mucopolysaccharide is rapidly secreted from the cell as proteoglycans, but a minor portion is transferred to the lysosomes where it must be digested to small molecules before passing out of the cell. The rate of disappearance of the ^{35}S-labelled mucopolysaccharide in "chased" cells was used as an index of lysosome function. This rate, however, must also depend on the rate of transfer of the mucopolysaccharide to the lysosomes, a quantity that was not measured. Consequently, Neufeld's index of lysosome function might not be reliable quantitatively.

Measurement of the inhibition, by weak bases, of the digestion of endocytosed exogenous substrates should permit a quantitative estimate of the degree of inhibition of lysosomal activity providing any concomitant effects of the inhibitors on the uptake of the substrate into the lysosomes are taken into account, and that the substrate used for the investigation is digested exclusively within the lysosomal system. (Evidence of this is not always given in many published studies.)

It has been already established (see Chapter 4) that the digestion of endocytosed formaldehyde-denatured ^{125}I-labelled bovine serum albumin by the 17.5-day rat yolk sac occurs exclusively within lysosomes. In the work reported below the effects of weak bases on the endocytosis and lysosomal digestion of the ^{125}I-labelled albumin has been studied quantitatively. This in turn permits comparison of the effects of the same concentrations of a given weak base on lysosomal inactivation and the degree of inhibition (by similar concentrations of weak bases) of the rate of degradation of endogenous yolk-sac proteins labelled with [^{3}H]leucine. The latter study was conducted concurrently by Knowles and Ballard (CSIRO, Division of Human Nutrition, Adelaide, Australia).

5.2 METHODS

5.2.1 Assay of the uptake and degradation of ^{125}I-labelled substrates by 17.5-day rat yolk sacs in serum-free medium 199.

The uptake of ^{125}I-labelled poly(vinylpyrrolidone) was determined as described in Section 2.2.1. Two different methods were used to assay the uptake and degradation of formaldehyde-denatured ^{125}I-labelled bovine serum albumin. Each has been described previously (Sections 2.2.1 and 3.2.1). When the rapid method was used (Section 3.2.1) yolk sacs were incubated singly and when the method described in Section 2.2.1 was used, yolk sacs were again incubated singly but in 20ml of serum-free medium 199. Substrates were prepared as described previously (Sections 2.2.1 and 2.2.3). When yolk sacs were incubated with inhibitors [ammonium chloride, methylamine hydrochloride and chloroquine diphosphate, (each from Sigma, London) and ethylamine hydrochloride (Cambrian Chemicals, Beddington Farm Road, Croydon, U.K.)] the radioactive substrate was added to the medium 30min after the introduction of the yolk sacs to medium containing the inhibitor.

5.2.2 Expression of the uptake and degradation data for the ^{125}I-labelled substrate incubated with rat yolk sacs: a quantitative estimation of lysosomal activity.

In Chapters 2 & 3 both uptake and degradation data have been expressed as the volume (µl) of incubation medium whose contained substrate has been captured by unit quantity of yolk-sac tissue (mg yolk-sac protein). This form of expression (µl/mg yolk-sac protein) is appropriate for substrates (e.g. ^{125}I-PVP) which are ingested only by fluid endocytosis but, for a substrate like formaldehyde-denatured

^{125}I-labelled bovine serum albumin that is ingested mainly by adsorptive endocytosis, the rate of uptake is better expressed as the quantity (e.g. ng) of substrate captured by unit quantity of yolk-sac tissue (mg yolk-sac protein). This avoids the possible inference that a high rate of substrate uptake is associated with an increased rate of fluid capture.

When an effector compound substantially modifies the rate of uptake of a digestible substrate, the overall rate of digestion of the substrate will also be modified; this can mask any possible effects of such compounds on the digestion of a substrate once it has been endocytosed. To determine the precise action of an effector compound on the digestive process it is necessary to correct the overall rate of substrate digestion for the modified rate of substrate uptake. This can be achieved simply by expressing the overall rate of substrate digestion (ng/h per mg yolk-sac protein) as a percentage of the rate of uptake (ng/h per mg yolk-sac protein) to give a dimensionless quantity. When the overall rate of substrate digestion is limited by the rate of endocytic uptake (see Section 2.4) a value of 100% should be obtained, but when the digestion step is rate limiting, a value <100% should be obtained. However, it follows that any effector compound which enhances the rate of digestion by stimulating the digestive process will not affect this quantity (the rate of digestion cannot exceed the rate of substrate uptake).

A similar analysis can be made of the quantity of substrate accumulated by the yolk-sac tissue. But, to obtain a dimensionless quantity, the <u>average</u> rate of substrate accumulation by the yolk sac (ng/h per mg yolk-sac protein) must be expressed as a percentage of the rate at which the substrate is endocytosed (ng/h per mg yolk-sac protein). This has been done irrespective of whether the time-course of the substrate

accumulation within the tissue is linear or non-linear. When the digestive process is completely inhibited, so that no digestion products are released back into the medium following the endocytosis of a digestible substrate, the value of the average rate of tissue accumulation when expressed as a percentage of the rate of uptake should be 100%. In the absence of an effector compound this value should be <100% (say x%); the precise value will depend on the ease with which an individual substrate can be digested within the yolk sac. An effector compound that inhibits the digestive process should give a value >x%, whereas an effector compound that stimulates the digestion of a substrate once it has been captured (so that the digestion products are released back into the culture medium more rapidly than would occur under control conditions) should give a value <x%.

5.2.3 Release of both radioactivity and enzymes from yolk sacs that have ingested ^{125}I-labelled substrates in vitro

A decrease in the quantity of a non-digestible ^{125}I-labelled substrate accumulated within the rat yolk sac, due to the presence of an effector compound, can result from either an inhibition of pinocytosis or an accelerated rate of release of the pinocytosed substrate from the yolk sac. When digestible substrates are being investigated an inhibition of the digestion process could leave more intact radiolabelled substrate within the tissue to be subsequently exocytosed. It was therefore important to investigate the potential effects of the effector compounds on the rates of release of the endocytosed substrates.

1) Assay of the release of radioactivity from yolk sacs that have ingested formaldehyde-denatured ^{125}I-labelled bovine serum albumin in vitro. This assay was performed essentially as described in Section 2.2.4(1) except that yolk sacs were allowed to ingest substrate over an incubation period of 3h in serum-free medium 199. When the effect of a weak base on the release of substrate from a yolk sac was being investigated, the weak base was present in the culture medium during the ingestion, washing, and re-incubation phases of the experiment. The release of both acid-insoluble and acid-soluble radioactivity were monitored as described previously [Section 2.2.4(1)].

(2) Assay of the release of radioactivity from yolk sacs that have ingested ^{125}I-labelled poly(vinylpyrrolidone) in vitro. This assay was performed essentially as described in (1) above, but using ^{125}I--labelled poly(vinylpyrrolidone) at a concentration of 10µg/ml of medium 199, instead of the radiolabelled protein substrate; a 3h ingestion period was again used.

The yolk sacs were washed for slightly longer periods (3 x 5min). Some yolk sacs were allowed to ingest ^{125}I-labelled poly(vinylpyrrolidone) for 2h, in the absence of a weak base, washed in medium 199 alone then placed into medium containing a particular weak base and the incubation continued. The release of radioactivity was monitored as described previously [Section 2.2.4(1)].

(3) Assay of the release of lactate dehydrogenase activity and β-N-acetylglucosaminidase activity from yolk sacs that have ingested ^{125}I-labelled poly(vinylpyrrolidone) in vitro. The release of both lactate dehydrogenase activity and β-N-acetyl-

-glucosaminidase activity from yolk sacs that had previously ingested ^{125}I-labelled poly(vinylpyrrolidone) were monitored together with the release of radioactivity. Yolk sacs were allowed to ingest the radiotracer for 2h, in the absence of a weak base, washed in medium 199 alone then re-incubated in the presence of a weak base at concentrations of 0-200μM as described in (2) above. The release of enzymic activity and radioactivity were monitored by taking 1.0ml samples of culture medium at 0.5h and 3.0h after introducing the washed yolk sacs into the re-incubation medium. Incubations were terminated at 3h and the medium samples were centrifuged at 1500g for 15min to remove cell debris and the supernatant assayed for radioactivity as described previously [Section 2.2.1(3)] then stored at -20°C before assaying lactate dehydrogenase and β-N-acetyl-glucosaminidase activities. The radioactivity and enzymic activities of each sample were used to calculate the total radioactivity and total enzymic activities released into the medium over the period 0.5h to 3.0h of incubation. At the end of the incubation, the yolk sacs were homogenized at 4°C for 30s in 5ml of 0.01M-K_2HPO_4 buffer, pH 7.4, by using a Virtis homogenizer (Virtis Research Equipment, Gardiner, N.Y., U.S.A.) set at speed 2. The homogenates were assayed immediately for lactate dehydrogenase activity, β-N-acetylglucosaminidase activity and radioactivity.

(4) Calculation and expression of the quantities of radio- and enzymic activities released from yolk sacs that have ingested ^{125}I-labelled substrate. The amounts of radioactivity and enzymic activities released from the yolk sacs were calculated by the method described in Section 2.2.4(2). The activities released were then expressed as a percentage of that activity associated with the yolk sac at the time of transfer to the re-incubation medium. As described before [Section 2.2.4(4)], the total

amounts of radioactivity and enzymic activities present in the yolk sac at the time of transfer to the re-incubation medium were calculated by adding the total radioactivity or enzymic activity in the yolk-sac homogenate to the total radioactivity or enzymic activity in the culture medium at the end of the re-incubation period.

5.2.4 Recovery of pinocytic and digestive activities of rat yolk sacs previously exposed to ammonium chloride.

Three yolk sacs from a single 17.5-day pregnant rat were incubated together for 1h at 37.0 ± 0.3°C under 95% O_2: 5% CO_2 in 20ml of medium 199 containing ammonium chloride at a concentration of either 6.32mM or 20.0mM. The yolk sacs were then washed three times for 2min in 20ml of warm, gassed medium 199 containing no ammonium chloride and placed into 19ml of warm, gassed medium 199, containing no ammonium chloride, and incubation continued. The uptake and degradation of formaldehyde--denatured ^{125}I-labelled bovine serum albumin by each yolk sac was assayed by the rapid method as described in Section 4.2.1 by adding the radioactive substrate (dissolved in 1ml of medium 199) to a flask containing a washed yolk sac either at 8, 60 or 120min after the transfer of the yolk sacs from the medium containing ammonium chloride to the fresh medium 199.

Control experiments were performed using three yolk sacs from the same animal as used above but excluding ammonium chloride during the 1h incubation prior to washing the yolk sacs. The incubation media containing the ammonium chloride to which the yolk sacs had been exposed was reutilized to determine the effect of continuous exposure to ammonium chloride on the uptake and degradation of formaldehyde-denatured ^{125}I-labelled bovine serum albumin. This was done by introducing into this medium a freshly dissected yolk sac, taken from a second 17.5-day pregnant

rat. After 30min the uptake and degradation of formaldehyde-denatured ^{125}I-labelled bovine serum albumin was assayed by the rapid method as described in Section 4.2.1 by adding radioactive substrate dissolved in 50µl of aq. NaCl (1% w/v).

5.2.5 Assay of lactate dehydrogenase activity.

Lactate dehydrogenase activity was measured by the method of Lowry et al. (1951). A mixture was prepared containing: sodium pyruvate (5.5mg in 0.5ml distilled water), reduced nicotinamide adenine dinucleotide (35.5mg in 0.5ml distilled water) and 0.1M-Na_2HPO_4, pH 7.5 (4.0ml). To 20µl of this mixture was added 10µl of culture medium or appropriately diluted yolk-sac homogenate [Section 4.2.3(3)] and the mixture was incubated at 37°C for 15min. The reaction was terminated and the remaining reduced nicotinamide dinucleotide destroyed by adding 0.4M-HCl (4.0µl). After at least 30s, 10M-NaOH (120µl) was added and incubation continued at 37°C for 30min. To the reaction mixture was added 2.0ml of distilled water and the fluorescence was determined in a Perkin-Elmer 1000 fluorimeter, using an excitation wavelength of 365nm and an emission wavelength of 470nm. Standards containing up to 2ng of nicotinamide adenine dinucleotide were incubated and assayed.

5.2.6 Assay of β-N-Acetylglucosaminidase activity.

β-N-Acetylglucosaminidase was assayed by using 2.5mM-4-methylumbelliferyl 2-acetamido-2-deoxy β-D-glucopyranoside (Koch-Light Ltd., Colnbrook, Bucks, U.K.) as substrate in 0.1ml of citrate/phosphate buffer pH 4.3 (0.1M-citric acid adjusted to pH 4.3 with 0.2M-Na_2HPO_4). To this was added 10µl of incubation medium (or appropriately diluted yolk-sac homogenate [Section 4.2.3(3)] and the mixture was incubated at 37°C for 15min. The reaction was terminated by addition of 2.5ml of 0.17 M-glycine/-

carbonate buffer, pH 10.5. The fluorescence was determined in a Perkin-Elmer 1000 fluorimeter, using an excitation wavelength of 365nm and an emission wavelength of 450nm. Standards containing up to 1nmole of 4-methylumbelliferone in glycine/carbonate buffer, pH 10.5, were also assayed.

5.3 RESULTS

5.3.1 Uptake and digestion of formaldehyde-denatured ^{125}I-labelled bovine serum albumin by 17.5-day rat yolk sacs incubated in serum-free medium 199 containing 20mM-ammonium chloride.

A preliminary study showed the presence of ammonium chloride (20mM) in the incubation medium to completely inhibit the production of trichloroacetic acid-soluble radioactivity from formaldehyde-denatured ^{125}I-labelled bovine serum albumin by 17.5-day rat yolk sacs; a marked inhibitory effect on the uptake of this substrate was also noted. The time course of these effects, after a 30min pre-incubation of yolk sacs in 20mM-ammonium chloride, is shown in Fig. 5.1 together with the corresponding results for a control experiment. In the control experiment the level of tissue-associated radioactivity rose during the first hour but after this period it remained constant, so that the rates of digestion and of uptake of the ^{125}I-labelled albumin became equal. By contrast, yolk sacs incubated in the presence of ammonium chloride (20mM) continued to accumulate radioactivity at a constant rate throughout the 3h incubation but no acid-soluble digestion products were released into the incubation medium, so that the rate of tissue accumulation of radioactivity was equal to the rate of substrate ingestion. These rates were, however, markedly decreased in the presence of ammonium chloride and were equal to approximately 10% of the control rate of uptake.

Ammonium chloride therefore appears to have two major effects: firstly, inhibition of the endocytic process and secondly, an inhibition of the proteolytic process responsible for the digestion of the ingested ^{125}I-labelled albumin.

5.3.2 Effects of ammonium-, methylammonium- and ethylammonium chloride on the uptake and digestion of formaldehyde-denatured ^{125}I-labelled bovine serum albumin by 17.5-day rat yolk sacs incubated in serum-free medium 199.

To determine whether inhibition of both substrate uptake and proteolysis occurred in the presence of other weak bases, and to determine whether proteolysis could be inhibited without any concomitant effect on uptake, the effects of methylammonium- and ethylammonium ions on both ingestion and digestion were also examined.

Table 1 shows that, at 20mM concentrations, ammonium-, methylammonium- and ethylammonium chlorides each inhibited the rate of ingestion to a similar extent and also almost fully inhibited the proteolysis of the ^{125}I-labelled albumin. Only a small proportion of that radiolabelled protein ingested by the yolk sac was hydrolysed, and the average rate of tissue accumulation of radioactivity became almost equal to the rate of uptake. Each weak base at a concentration of 5mM inhibited both uptake and digestion, but to lesser extents than at a concentration of 20mM. The amount of acid-soluble radioactivity released into the incubation media containing a weak base at a concentration of 5mM increased linearly with time after an initial lag-period. No inhibition of proteolysis was observed without an attendant effect on ingestion also being observed. These effects did not result from changes in the pH of the incubation media since all media, at 37°C under an atmosphere of 95% O_2: 5% CO_2, showed a pH of 7.1 whether they contained ammonium-, methylammonium- or ethylammonium chloride (20mM) or no weak base at all.

5.3.3 Effect of different concentrations of ammonium chloride on the uptake and digestion of formaldehyde-denatured ^{125}I-labelled bovine

serum albumin by 17.5-day rat yolk sacs incubated in serum-free medium 199.

The inhibitory effects of ammonium chloride on both uptake and digestion are clearly concentration dependent phenomena (Table 5.1). Fig. 5.2 shows the extent of the same effects over a range of ammonium chloride concentrations. The rate of uptake of ^{125}I-labelled albumin decreased on increasing the ammonium chloride concentration from approx. 0.2mM to 20mM. Similarly, the overall rate of appearance of acid-soluble radioactivity progressively decreased over the same ammonium chloride concentration range. The effect on digestion was more pronounced than the inhibitory effect on ingestion and reached approx. 100% inhibition in 20mM-ammonium chloride; at all other ammonium chloride concentrations the rate of appearance of acid-soluble radioactivity was measurable and was constant over the remainder of the 3h incubation period beyond the initial lag-period. A biphasic effect on the accumulation of radiolabelled substrate by the yolk-sac tissue was observed. No measurable effect on the tissue-accumulated radioactivity occurred below an ammonium chloride concentration of 2mM. At concentrations above 2mM-ammonium chloride the amount of tissue-associated radioactivity increased to reach a maximum equal to approx. 210% of control (at 6.32mM) then decreased again, but, even with 20mM-ammonium chloride, the quantity of tissue-associated radioactivity was not measurably lower than that observed for yolk sacs incubated in the absence of ammonium chloride. On increasing the ammonium chloride concentration, the acid-insoluble radioactivity accumulated by the tissue constituted a greater proportion of the total yolk-sac associated radioactivity and was found to reach a maximum value, equivalent to approx. 310% of the level in the control, at an ammonium chloride concentration of 6.32mM. At 20mM this value was approx. 165% of the control.

Because ammonium chloride inhibits the uptake of the ^{125}I-labelled albumin, it is not easy to distinguish the extent of the effect of ammonium chloride on the proteolytic process when the data are presented in the format used in Fig. 5.2. However, if the rate of digestion and the average rate of tissue accumulation of ^{125}I-labelled albumin are each corrected for the modified rate of uptake, as described in Section 5.2.2 it becomes clear that one effect of ammonium chloride is to inhibit the lysosomal proteolytic process (Fig. 5.3). As the corrected rate of digestion progressively decreases, with increasing concentrations of ammonium chloride, a corresponding rise in the corrected rate of tissue accumulation of radioactivity occurs. Fig. 5.3 (lower) shows the proportion of the total tissue-accumulated radioactivity that is acid--soluble at each ammonium chloride concentration; this is observed to decrease with increasing ammonium chloride concentration.

5.3.4 Effects of chloroquine on the uptake and digestion of formaldehyde--denatured ^{125}I-labelled bovine serum albumin by 17.5-day rat yolk sacs incubated in serum-free medium 199.

Chloroquine, like ammonium-, methylammonium- and ethylammonium chlorides is a weak base and reports in the literature have indicated that in some cell types it can inhibit both endocytosis and lysosomal digestion of proteins (see de Duve et al., 1974). It seemed that chloroquine might have similar properties to ammonium chloride and therefore decrease both the rate of uptake and the rate of digestion of proteins by the rat yolk sac. In order to investigate this possibility, the uptake and digestion of the ^{125}I-labelled albumin by the rat yolk sac was measured in the presence of chloroquine phosphate at concentrations which did not modify the pH of the incubation medium. Fig. 5.4 shows that chloroquine does

indeed have effects which are qualitatively similar to those already described above for ammonium chloride. As with the other weak bases, the rate of appearance of acid-soluble radioactivity in each separate experiment, at a given chloroquine concentration, was constant after an initial lag-period. As the chloroquine concentration was increased a progressive decrease occurred both in the rate of uptake and in the rate of digestion of the radiolabelled protein. A biphasic effect on the accumulated tissue radioactivity was also observed when such results, from a number of experiments at different chloroquine concentrations, were compared.

After correcting both the overall rate of digestion and the average rate of tissue accumulation of radioactivity for the chloroquine--induced decrease in the rate of uptake of the radiolabelled substrate, the effects of chloroquine on the lysosomal proteolysis of the radiolabelled substrate can be clearly seen (Fig. 5.5). As with ammonium chloride (Fig. 5.3), an increase in chloroquine concentration caused a marked decrease in the rate of proteolysis and a corresponding rise in the corrected rate of accumulation of tissue-radioactivity. The proportion of the tissue-accumulated radioactivity found to be acid-soluble also decreased with increasing chloroquine concentration.

The results showed that chloroquine was a more potent inhibitor of both the uptake and of the proteolytic processes than was ammonium chloride. Fifty percent inhibition of uptake occurred at a concentration of approx. 100$\underline{\underline{\mu M}}$-chloroquine, but the same result was only achieved with approx. 10$\underline{\underline{mM}}$-ammonium chloride (i.e. 100 times greater than for chloroquine). Similarly, 50% inhibition of the proteolytic process occurred at a chloroquine concentration of approx. 75$\underline{\underline{\mu M}}$, while the concentration of ammonium chloride that gave the same result was approximately 9$\underline{\underline{mM}}$ (i.e.

120 times greater than for chloroquine).

5.3.5 Effect of different concentrations of ammonium chloride and of chloroquine on the uptake of ^{125}I-labelled poly(vinylpyrrolidone) by 17.5-day rat yolk sacs incubated in serum-free medium.

To determine whether ammonium chloride or chloroquine caused a decreased rate of pinosome formation or whether the inhibition of formaldehyde-denatured ^{125}I-labelled bovine serum albumin uptake arose from an interference with the adsorption of the macromolecule to pinocytosing plasma membrane, it was necessary to determine the effects of these compounds on the rate of uptake of fluid, as measured by ^{125}I-labelled poly(vinylpyrrolidone) [^{125}I-PVP] ingestion. Both ammonium chloride (Fig. 5.6) and chloroquine (Fig. 5.7) inhibited the uptake of ^{125}I-PVP, indicating that the observed inhibition of ^{125}I-labelled albumin uptake, in the presence of these inhibitors, is a result of a decreased rate of pinosome formation. Brown & Segal (1977) reported that chloroquine was without effect on the uptake of invertase by 17.5-day rat yolk sacs. However, these authors incubated yolk sacs in the presence of calf serum. It therefore seemed possible that calf serum might quench the effect seen here with chloroquine. But, when calf serum (10% v/v) was added to the incubation media chloroquine still inhibited ^{125}I-PVP uptake (Fig. 5.7).

5.3.6 Release of radioactivity from yolk sacs that had previously accumulated either ^{125}I-labelled poly(vinylpyrrolidone) or formaldehyde--denatured ^{125}I-labelled bovine serum albumin.

The effects of ammonium-, methylammonium- and ethylammonium chlorides and chloroquine on the rates of release of radioactivity from yolk sacs that had previously accumulated ^{125}I-labelled substrates were determined to establish whether the lower rates of accumulation of substrate

by the yolk sac in the presence of these weak bases could arise from more rapid rates of release of captured substrate.

When yolk sacs were allowed to accumulate ^{125}I-PVP in the absence of a weak base then transferred to media containing ammonium-, methylammonium- or ethylammonium chlorides (20mM), the rate of release of radioactive substrate remain essentially the same as that observed in control yolk sacs re-incubated in media containing no weak base (Table 5.2). In contrast, chloroquine at 200μM caused a rapid rate of release of radioactivity (approx. 13%/h; c.f. control rate of 1.1%/h), but even this enhanced rate of release was not sufficient to completely account for the decreased rate of accumulation.

Similar experiments, in which yolk sacs were allowed to accumulate ^{125}I-PVP in the presence of either ammonium chloride (20mM) or chloroquine (200μM) before monitoring the release of radioactivity on re-incubation in the presence of the same weak base, gave similar results (Table 5.2). When the latter experiment was repeated with yolk sacs that had accumulated the ^{125}I-labelled albumin, chloroquine caused a rapid rate of release of acid-insoluble radioactivity but ammonium chloride did not (Fig. 5.8). An inhibitory effect of these weak bases on the proteolysis of the radiolabelled protein was also observed. In control experiments acid-soluble radioactivity was rapidly released from the yolk sacs, whereas the rates of release of acid-soluble radioactivity in the presence of either ammonium chloride (20mM) or chloroquine (200μM) were negligible.

5.3.7 Release of radioactivity, lactate dehydrogenase activity and β-N-acetylglucosaminidase activity from yolk sacs that had previously accumulated ^{125}I-labelled poly(vinylpyrrolidone).

To determine whether chloroquine (200μM) was toxic to the yolk-sac

cells, or whether it specifically elevated the rate of exocytic regurgitation of either lysosome or pinosome contents, the rate of release of ^{125}I-PVP, lactate dehydrogenase activity and β-N-acetyl-glucosaminidase activity from yolk sacs was measured with different concentrations of chloroquine in the re-incubation medium.

Fig. 5.9 shows that only small amounts of ^{125}I-PVP, lactate dehydrogenase activity and β-N-acetylglucosaminidase activity are released in the presence of 0, 10 and 100$\mu\underline{\underline{M}}$ chloroquine, but above 100$\mu\underline{\underline{M}}$ all three markers are rapidly released, indicating that cytolysis occurs at the higher concentrations of chloroquine.

5.3.8 Recovery of the endocytic and proteolytic activities of 17.5-day rat yolk sacs following exposure to ammonium chloride.

It seemed possible that the ammonium chloride induced inhibitions of both pinocytosis and protein digestion by the rat yolk sac could arise from general irreversible cytotoxic effects. Results of recovery experiments (Table 5.3) indicate that this is not so. After a 1h exposure to 6.32$\underline{\underline{mM}}$ ammonium chloride [sufficient to cause approx. 25% inhibition of pinocytic activity and an approx. 50% inhibition of the proteolytic process when continuously present] yolk sacs immediately showed a complete recovery of both activities. Following exposure to 20$\underline{\underline{mM}}$-ammonium chloride, a complete recovery of pinocytic activity was not achieved but it did rapidly return to 85-90% of that observed for control yolk sacs. However, a complete recovery of the proteolytic capacity was achieved, even after exposure to ammonium chloride at a concentration sufficient to inhibit this process by 100%.

Table 5.1 Effects of ammonium, methylammonium and ethylammonium ions on the rates of uptake and digestion of formaldehyde-denatured ^{125}I-labelled bovine serum albumin by 17.5-day rat yolk sacs incubated in serum-free medium 199.

The rates of uptake and digestion of formaldehyde-denatured ^{125}I-labelled bovine serum albumin were determined by the method described in Section 3.2.1. The ^{125}I-labelled albumin (1 μg/ml of medium) was added 30 min after placing the yolk sacs in serum-free medium 199 containing the weak bases at the concentrations indicated. Each value reported represents the mean (± S.D.) of 3 - 4 separate determinations each over a 3 h uptake period.

Inhibitor	Inhibitor concentration (mM)	Rate of uptake (% of control)	Rate of digestion (% rate of uptake)	Average rate of tissue accumulation over the 3.0 h period (% rate of uptake)	Proportion of the radioactivity in the yolk sac (at 3 h) that is acid-soluble (%)
Control	0	100.0 ± 8.9	95.1 ± 5.6	16.3 ± 2.7	39.2 ± 5.3
NH_4^+	5	55.2 ± 9.8	68.8 ± 4.9	48.6 ± 3.6	18.1 ± 1.7
$MeNH_3^+$	5	57.4 ± 19.8	67.0 ± 13.5	48.6 ± 8.0	17.4 ± 3.4
$EtNH_3^+$	5	65.5 ± 12.6	59.0 ± 13.8	46.0 ± 7.3	14.8 ± 2.3
NH_4^+	20	10.8 ± 2.0	5.3 ± 7.2	92.0 ± 9.7	7.5 ± 1.9
$MeNH_3^+$	20	9.2 ± 5.2	-2.8 ± 9.2	102.0 ± 10.0	6.8 ± 0.6
$EtNH_3^+$	20	9.8 ± 2.8	4.5 ± 1.4	97.7 ± 5.3	6.8 ± 1.7

Table 5.2 Release of radioactivity from 17.5-day rat yolk sacs, that had accumulated ^{125}I-labelled poly(vinylpyrrolidone) in vitro, on re-incubating the tissue in the presence of ammonium, methylammonium, or ethylammonium ions or chloroquine.

Yolk sacs (3) each from a different animal were loaded in vitro with ^{125}I-PVP in the presence or absence of a weak base for 2 h, washed to remove extracellular substrate as described in Section 5.2.3(2). After washing, each yolk sac was re-incubated separately in fresh tracer-free medium 199, with or without (control) a weak base, and the release of ^{125}I-PVP was monitored as described in Section 5.2.3(2). Each value of the rate of release is the mean (± S.D.) value, over the period 1 - 3 h, from 3 separate determinations.

Weak base	Loaded in the presence (+) or absence (-) of the weak base	Concentration of weak base in loading and re-incubation media	Rate of ^{125}I-PVP release (% tissue-associated radio-activity released per h.)
Control	-	0	1.16 ± 0.17
	-	0	1.17 ± 0.14
HNH_3^+	-	20 mM	1.23 ± 0.12
	+	20 mM	0.79 ± 1.54
$MeNH_3^+$	-	20 mM	2.14 ± 1.15
$EtNH_3^+$	-	20 mM	2.26 ± 1.42
Chloroquine	-	200 µM	13.07 ± 1.90
	+	200 µM	11.84 ± 1.22

Table 5.3 Recovery of the endocytic and proteolytic activities of 17.5-day rat yolk sacs that had previously been exposed to ammonium chloride.

Following a 1.0 h exposure of yolk sacs to ammonium chloride, at the concentrations indicated, the tissues were washed as described in Section 5.2.4, then placed in fresh, serum-free medium 199 containing no ammonium chloride. Uptake and digestion (determined as described in Section 3.2.1) of formaldehyde-denatured ^{125}I-labelled bovine serum albumin commenced on addition of substrate (1 µg/ml of medium) to the flasks containing the washed yolk sacs. Such additions were made at 8, 60 or 120 min after removing the yolk sacs from medium containing the ammonium chloride.

The inhibitory effects, on endocytic and proteolytic activity, of the continuous presence of ammonium ions were also investigated. Freshly dissected yolk sacs were placed in media that had previously been used to expose yolk sacs to ammonium chloride and, after a 30 min period, the uptake and degradation of ^{125}I-labelled serum albumin studied on adding this substrate.

	Continuous presence of NH_4^+-ions in the culture medium		Recovery after 1.0 h exposure to NH_4^+-ions		
Concentration of NH_4^+-ions in medium	Rate of uptake (% of control)	Rate of digestion of ingested protein (as a percentage of the rate of uptake)	Period before addition of substrate	Rate of uptake (% of control)	Rate of digestion of ingested protein (as a percentage of the rate of uptake)
6.32 mM	76.1 ± 2.5	54.4 ± 8.8	8 min	119.4 ± 13.3	99.2 ± 1.2
			60 min	91.2 ± 13.6	100.2 ± 4.0
			120 min	98.5 ± 14.7	96.4 ± 2.5
20.0 mM	20.0 ± 8.5	-0.04 ± 1.18 (100% inhibition)	8 min	89.6 ± 17.8	102.9 ± 2.3
			60 min	84.2 ± 8.5	98.9 ± 2.4
			120 min	86.4 ± 14.9	94.9 ± 2.5

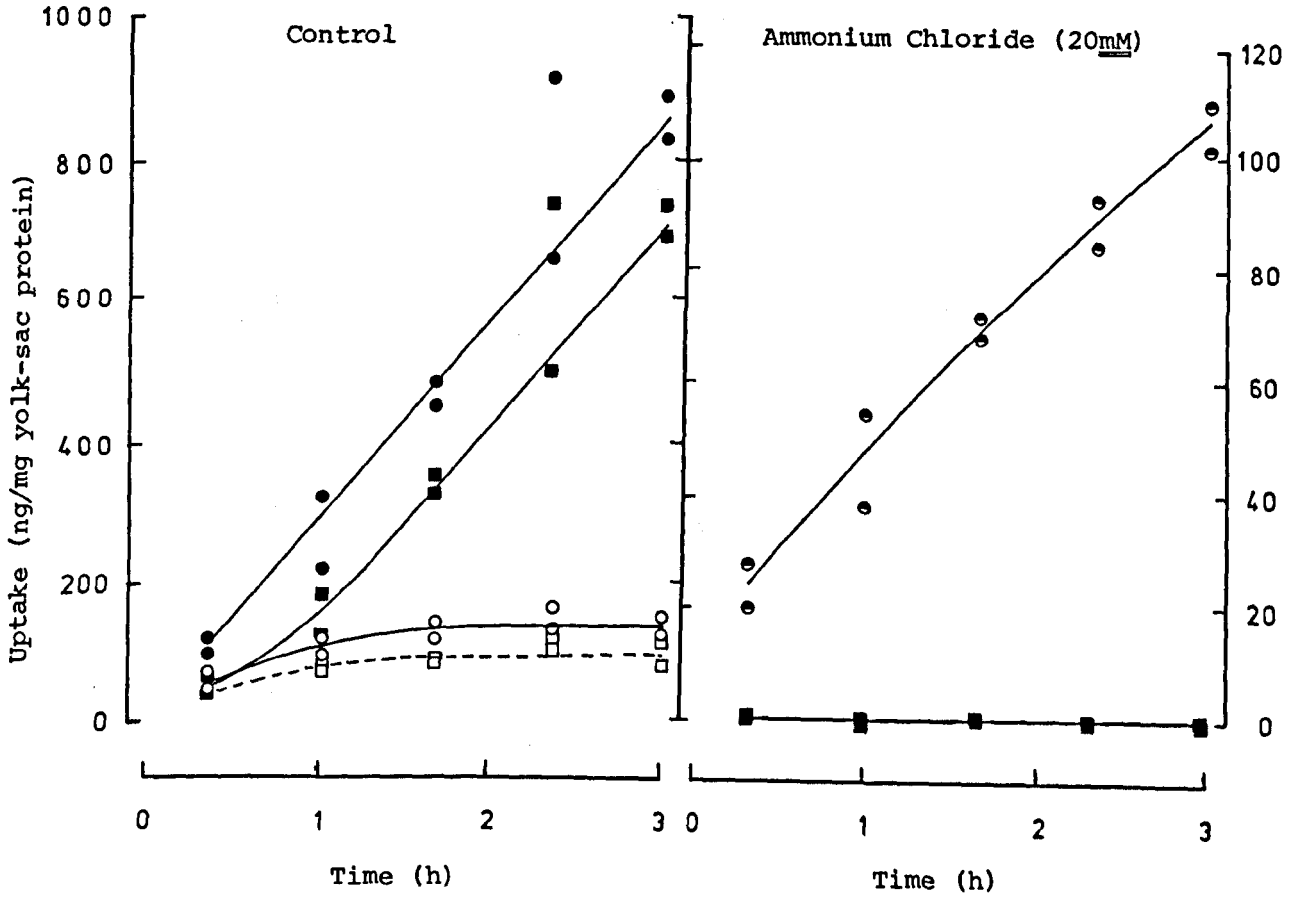


Figure 5.1 Effects of ammonium chloride (20 mM) on the uptake and degradation of formaldehyde-denatured ^{125}I-labelled bovine serum albumin by 17.5-day rat yolk sacs incubated in serum-free medium 199.

The uptake and degradation of formaldehyde-denatured ^{125}I-labelled bovine serum albumin were determined as described in Section 2.2.1. Yolk sacs were incubated with ammonium chloride for 30 min before uptake commenced on adding substrate (1 μg/ml of culture medium). Each experiment was performed using yolk sacs from a single animal.

Values of the tissue-associated radioactivity, (○); acid-soluble radioactivity released into the culture medium, (■); sum of tissue-associated radioactivity and acid-soluble radioactivity released into the culture medium (●); acid-insoluble radioactivity in yolk-sac tissue, (□) are shown at each time point for two yolk sacs that have each been incubated separately.

In the presence of 20 mM-ammonium chloride the tissue-associated acid-insoluble radioactivity at each time point was equivalent to 94.4 - 96.5% of the total tissue-associated radioactivity.

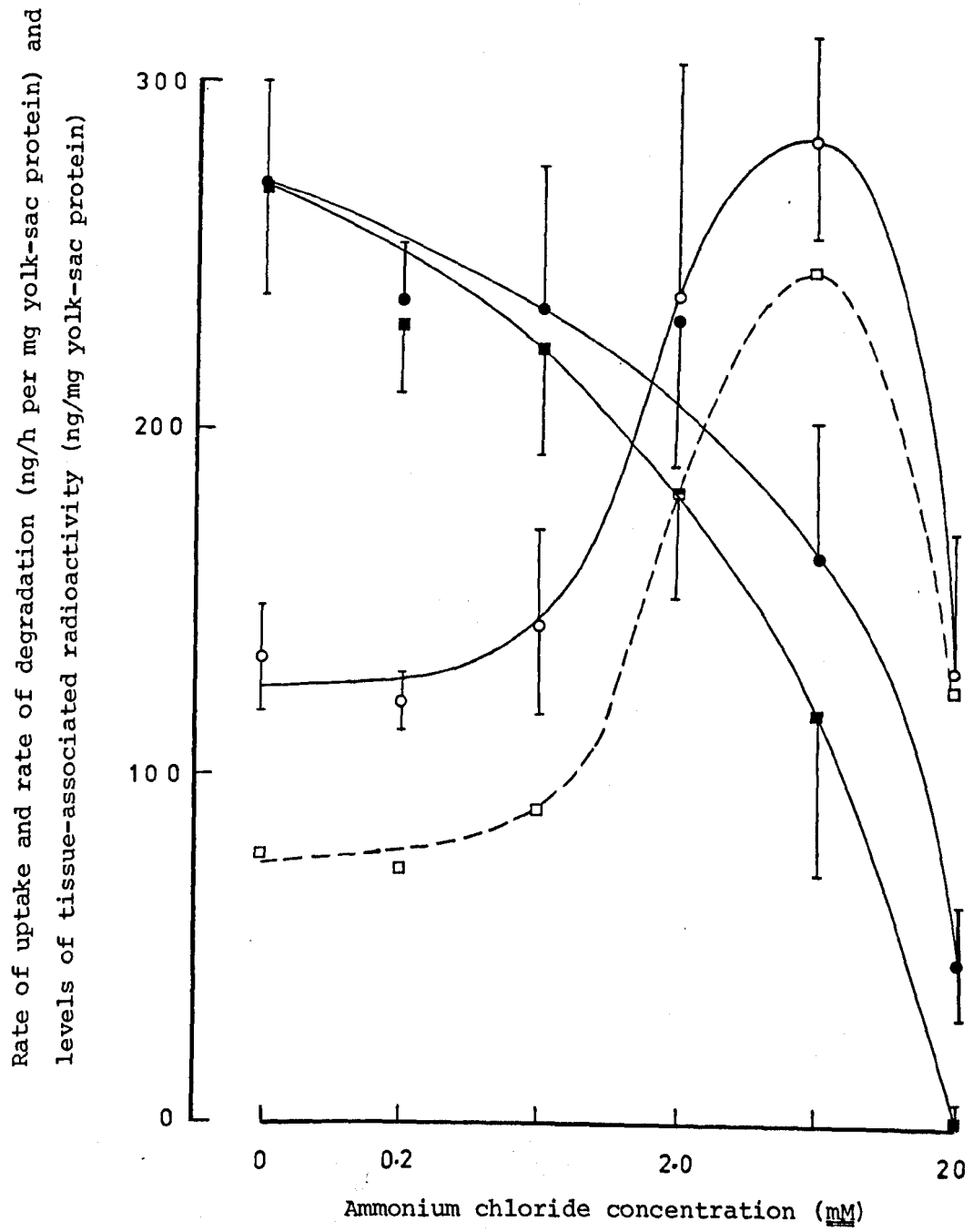


Figure 5.2 Effects of different concentrations of ammonium chloride on the rates of uptake and digestion of formaldehyde-denatured ^{125}I-labelled bovine serum albumin by 17.5-day rat yolk sacs incubated in serum-free medium 199.

The rates of uptake and rates of degradation of formaldehyde-denatured ^{125}I-labelled bovine serum albumin were determined as described in Section 3.2.1. Yolk sacs were incubated for 30 min in medium containing ammonium chloride, at the concentrations indicated, before uptake was started by the addition of substrate (1 µg/ml of culture medium) Each value is the mean (± S.D.) from 4 separate determinations each using a single yolk sac from a different animal.

Rate of uptake, (●); rate of degradation, (■); total tissue-associated radioactivity, (○); tissue-associated acid-insoluble radioactivity, (□).

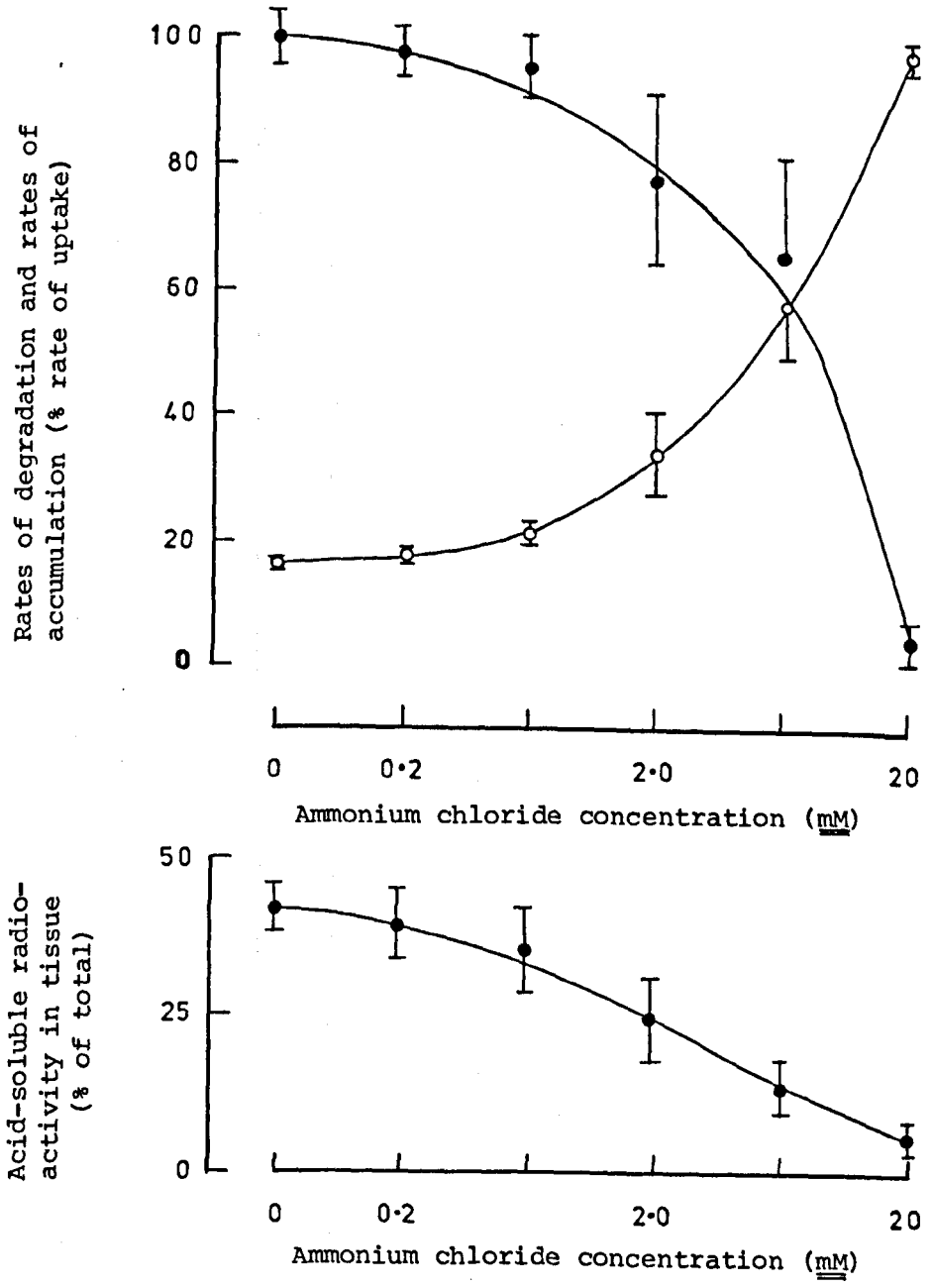


Figure 5.3 Effects of different concentrations of ammonium chloride on the proteolysis of pinocytosed formaldehyde-denatured ^{125}I-labelled bovine serum albumin by 17.5-day rat yolk sacs incubated in serum-free medium 199.

Upper — These data (means ± S.D.) are taken from Fig. 5.2 but differ in that the rates of degradation and rates of tissue--accumulation have been corrected for changes in the rate of uptake. Rate of degradation, (●); rate of accumulation, (o).

Lower — Shows the proportion (means ± S.D.) of the tissue-associated radioactivity that is acid-soluble.

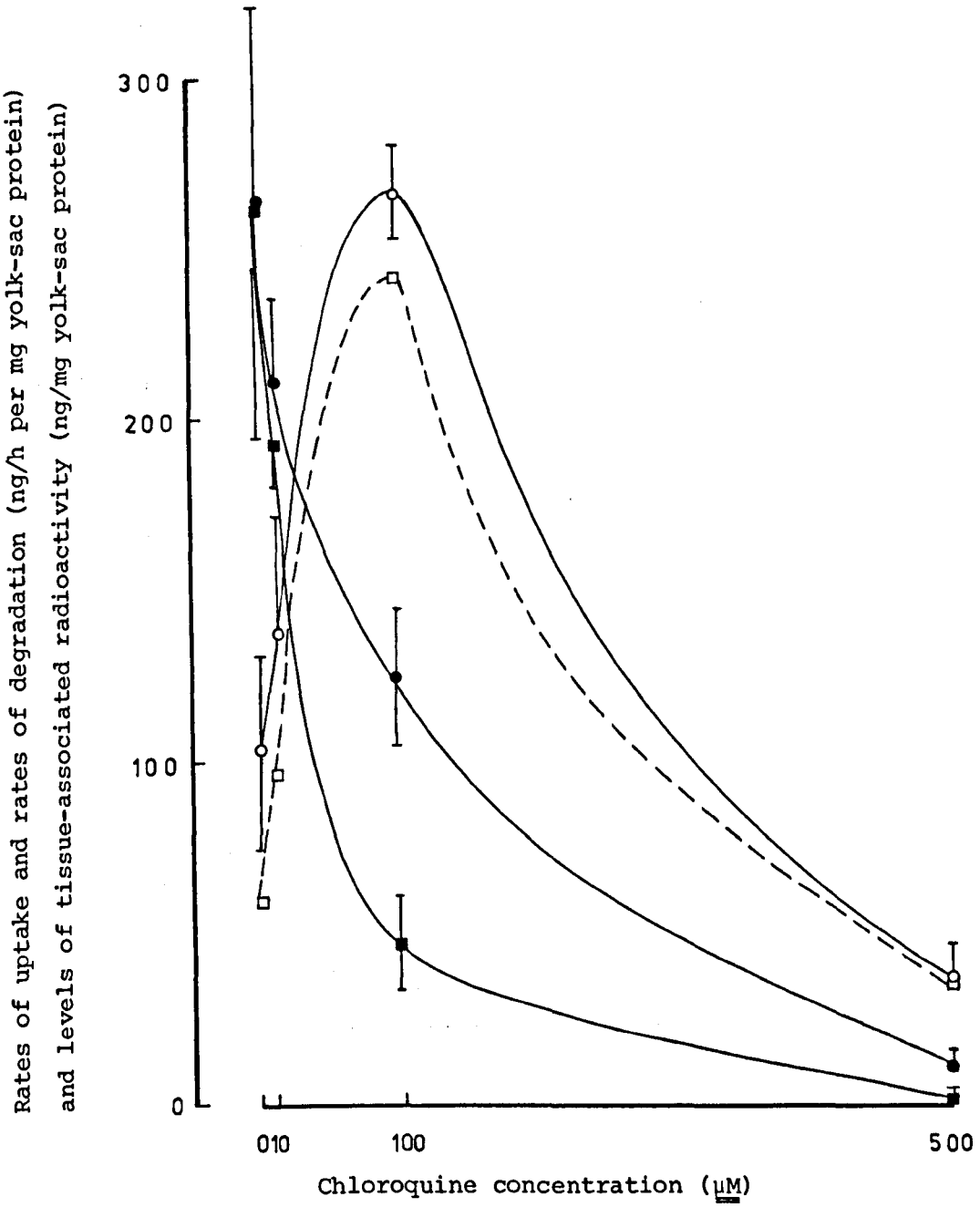


Figure 5.4 Effects of different concentrations of chloroquine on the rates of uptake and degradation of formaldehyde-denatured ^{125}I-labelled bovine serum albumin by 17.5-day rat yolk sacs incubated in serum-free medium 199.

The rates of uptake and the rates of degradation of formaldehyde-denatured ^{125}I-labelled bovine serum albumin were determined as described in Section 3.2.1. Yolk sacs were incubated for 30 min in medium containing chloroquine, at the concentrations indicated, before uptake was started by the addition of substrate (1 μg/ml of culture medium). Each value is the mean (± S.D.) from 4 separate determinations each using a single yolk sac from a different animal.

Rate of uptake, (●); rate of degradation, (■); total tissue-associated radioactivity, (○); tissue-associated acid-insoluble radioactivity, (□).

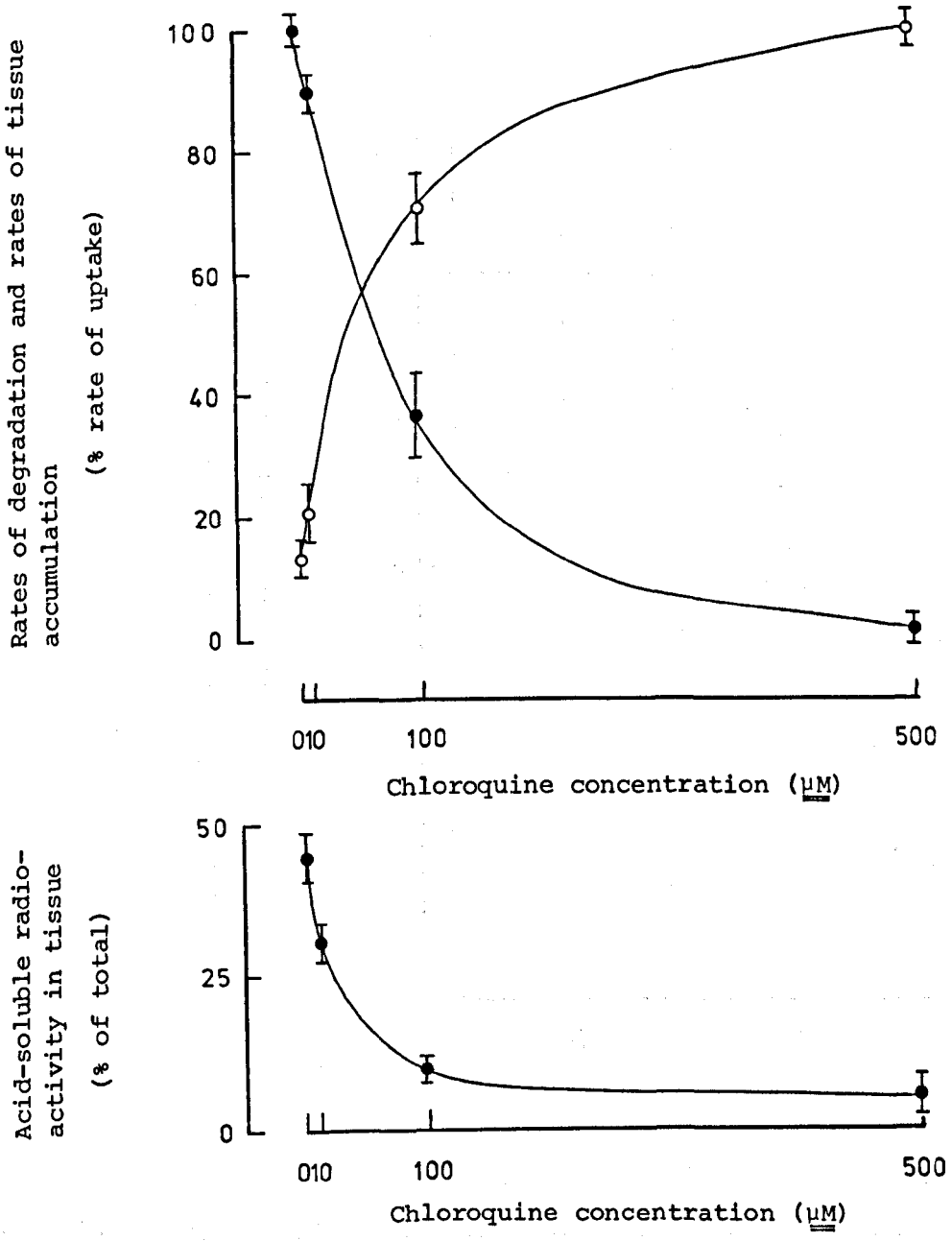


Figure 5.5 Effects of different concentrations of chloroquine on the proteolysis of pinocytosed formaldehyde-denatured ^{125}I-labelled bovine serum albumin by 17.5-day rat yolk sacs incubated in serum-free medium 199.

Upper — These data (means ± S.D.) are taken from Fig. 5.4 but differ in that the rates of degradation and rates of tissue-accumulation have been corrected for changes in the rate of uptake. Rate of degradation, (●); rate of accumulation, (○).

Lower — Shows the proportion (means ± S.D.) of the tissue-associated radioactivity that is acid-soluble.

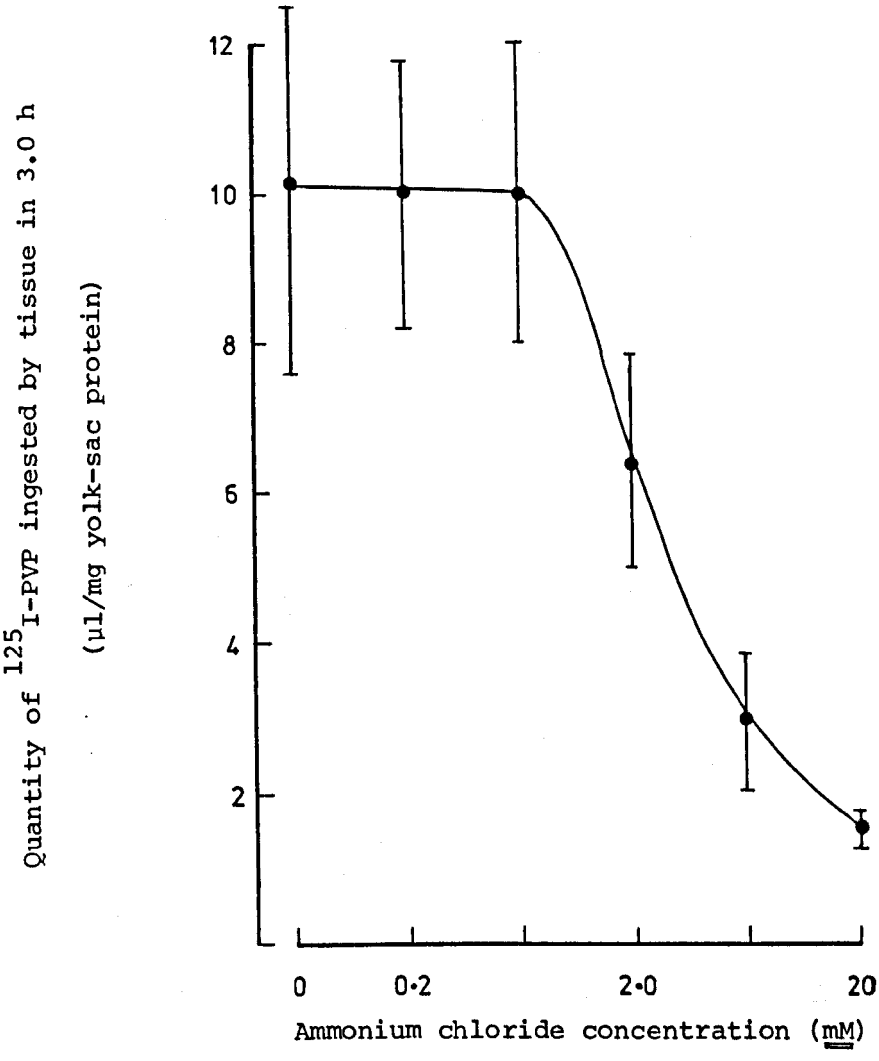


Figure 5.6 Effect of different concentrations of ammonium chloride on the ingestion of ^{125}I-labelled poly(vinylpyrrolidone) by 17.5-day rat yolk sacs incubated in serum free medium 199.

The quantity of ^{125}I-PVP ingested by yolk sacs was determined as described in Section 2.2.1. Yolk sacs were incubated in medium containing ammonium chloride for 30 min before uptake was started by the addition of ^{125}I-PVP (2 µg/ml of culture medium) and incubation continued for a further 3 h. Each reported value for uptake is the mean (± S.D.) of 4-5 determinations each with a single yolk sac taken from a different animal. Uptake is expressed as the volume of medium (µl) whose contained substrate has been captured over a 3.0 h period.

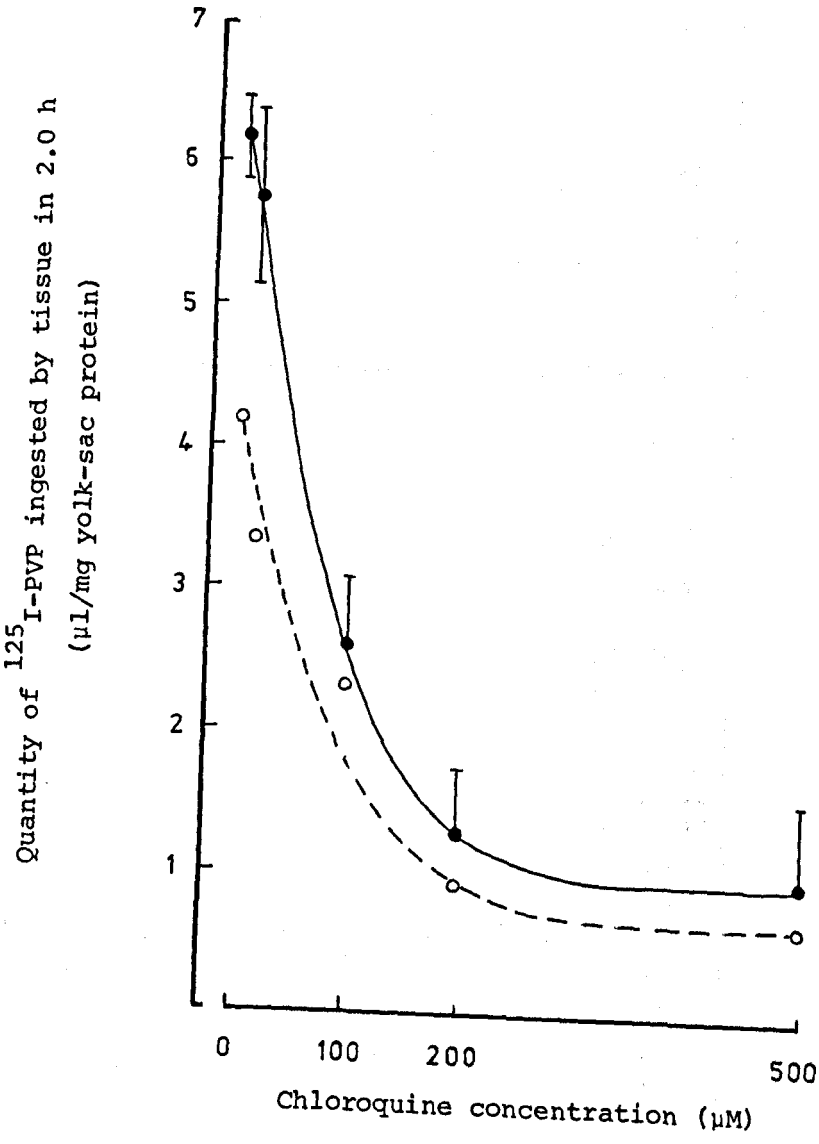


Figure 5.7 Effect of different concentrations of chloroquine on the ingestion of ^{125}I-poly(vinylpyrrolidone) by 17.5-day rat yolk sacs incubated in serum-free medium 199.

The quantity of ^{125}I-PVP ingested by yolk sacs was determined as described in Section 2.2.1. Yolk sacs were incubated for 30 min in medium containing chloroquine, at the concentration indicated, before uptake was started by the addition of ^{125}I-PVP (2 µg/ml of culture medium) and incubation continued for a further 2 h. Each reported value (●) is the mean (± S.D.) of 3 determinations each with a single yolk sac taken from a separate animal. (Values (o) relate to similar experiments in which the culture medium contained 10% (v/v) calf serum.)

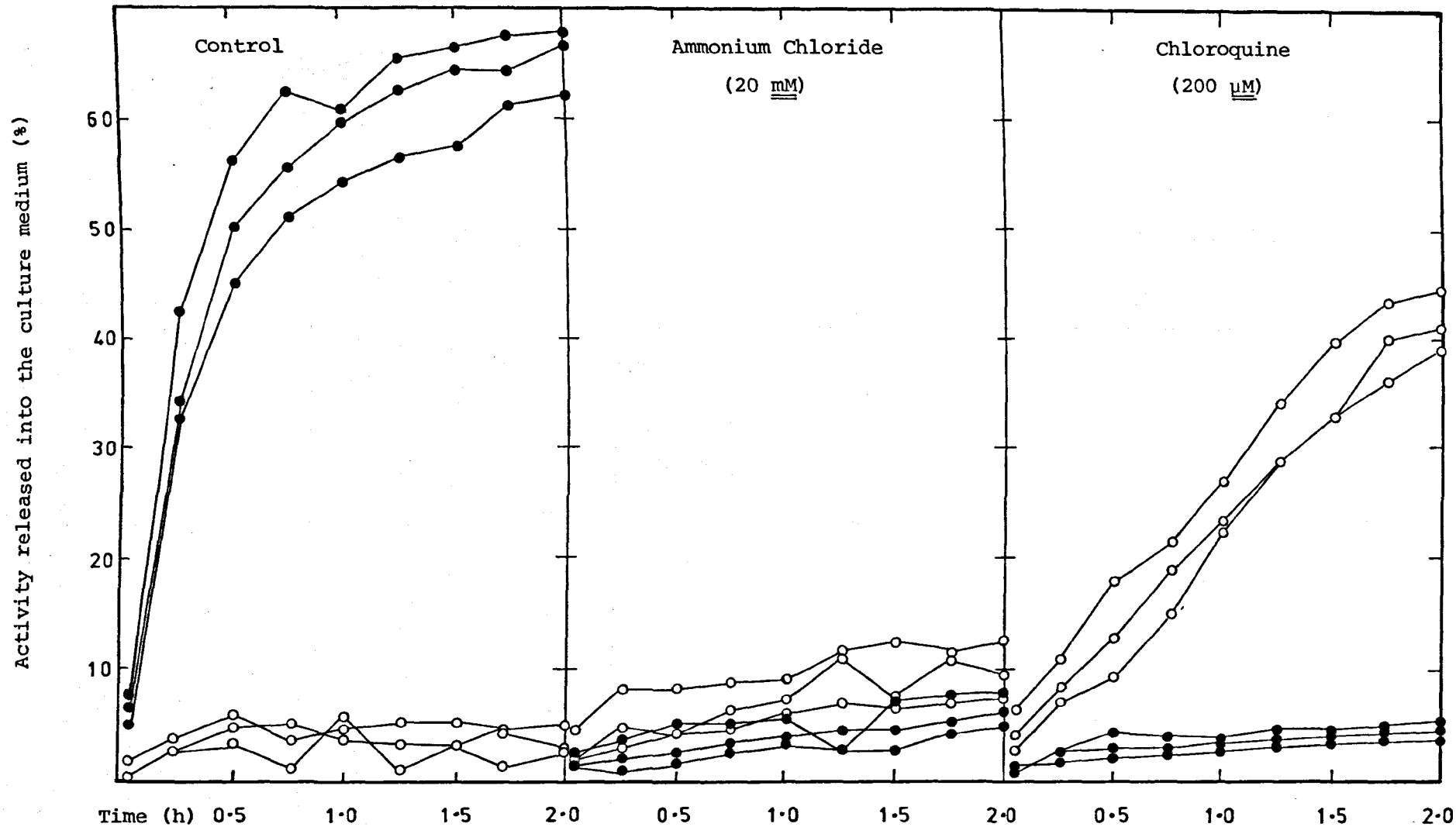


Figure 5.8 Effects of ammonium chloride or chloroquine on the release of formaldehyde-denatured ^{125}I-labelled bovine serum albumin from 17.5-day rat yolk sacs.

For experimental details see Section 5.2.3. The released acid-soluble radioactivities (●) and acid-insoluble radioactivities (○) are expressed as a percentage of that radioactivity initially associated with the yolk-sac tissue.

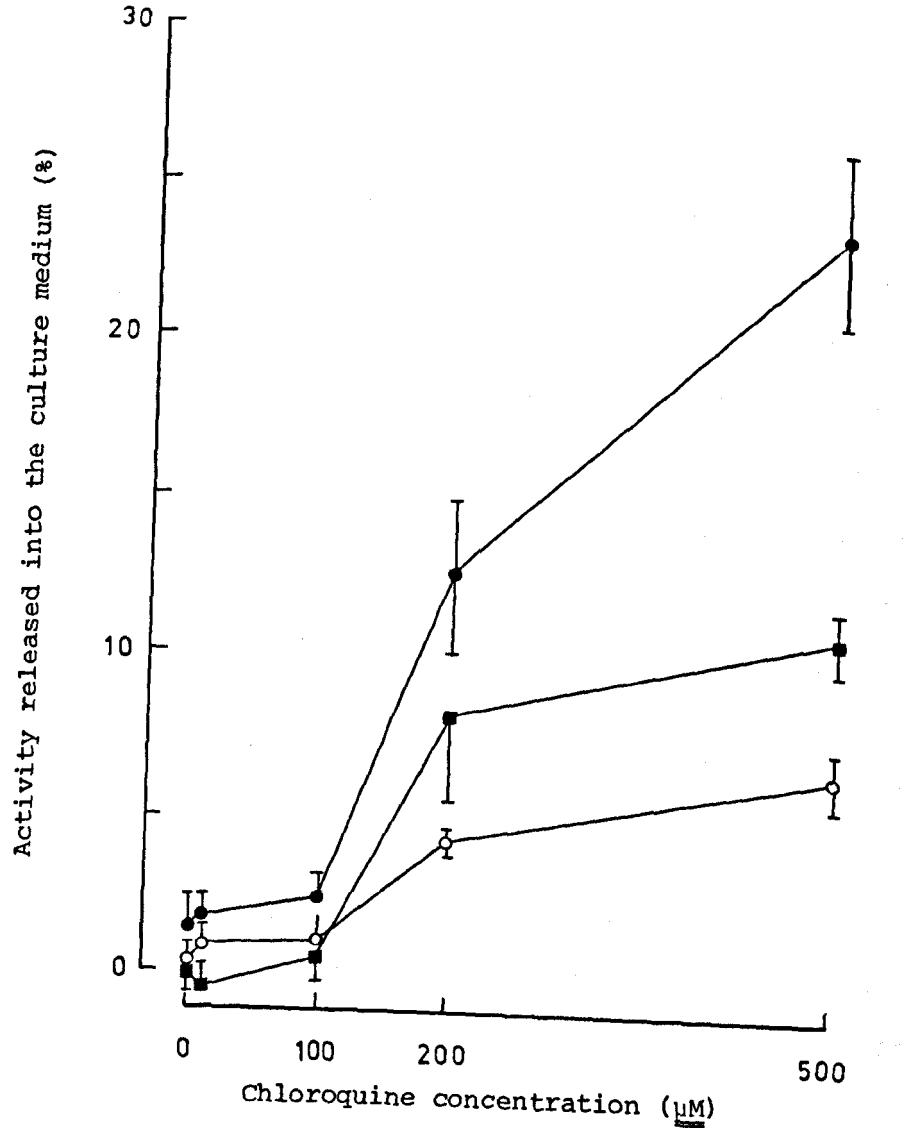


Figure 5.9 Release of ^{125}I-labelled poly(vinylpyrrolidone), lactate dehydrogenase and β-N-acetylglucosaminidase activities on re-incubating 17.5-day rat yolk sacs in the presence of different concentrations of chloroquine after the tissue had accumulated ^{125}I-labelled poly(vinyl-pyrrolidone) in vitro.

Yolk sacs were loaded in vitro with ^{125}I-PVP for 2 h as described in Section 5.2.3. After washing, each yolk sac was re-incubated separately in fresh culture medium containing chloroquine, at one of the concentrations indicated, but initially no ^{125}I-PVP. The quantities of ^{125}I-PVP, lactate dehydrogenase activity and β-N-acetylglucosaminidase activity released into the culture media between 0.5 h and 3.0 h were determined as described in Section 5.2.3. The values shown are the means (± S.D.) of results from 3 yolk sacs, each taken from a separate animal. The results are expressed as the proportion of the initial yolk-sac activities released between 0.5 h and 3.0 h of reincubation.

^{125}I-PVP, (●); lactate dehydrogenase (■); β-N-acetyl-glucosaminidase (○).

5.4 DISCUSSION

The marked inhibitions by ammonium-, methylammonium- and ethylammonium ions and chloroquine of the digestion of formaldehyde--denatured ^{125}I-labelled bovine serum albumin subsequent to its ingestion by yolk sacs, and the concomitant accumulation of the ^{125}I-labelled albumin in the yolk-sac tissue are in full agreement with the conclusion, in Chapter 4, that formaldehyde-denatured ^{125}I-labelled bovine serum albumin is digested by the yolk sacs intracellularly and within lysosomes. The highest concentration of each weak base used completely inhibited the observed lysosomal digestive activity (Table 5.1 & Figs 5.3 and 5.5). De Duve *et al.* (1974) showed the digestion of [^{3}H]leucine-labelled bacteria engulfed by macrophages in culture to be completely inhibited by 100$\mu\underline{\underline{M}}$ chloroquine and Floren & Nilsson (1977) showed that 50$\mu\underline{\underline{M}}$ chloroquine virtually completely inhibited the digestion, by hepatocytes in culture, of cholesterylester associated with chylomicron remnant particles (formed by the action of a lipoprotein lipase on chylomicrons).

The results of a literature survey indicate that a complete inhibition of a lysosomal activity by ammonium- and substituted ammonium ions has not been previously reported. Reijngoud *et al.* (1976) measured the inhibitory effects of ammonium- and methylammonium ions on the digestion of endocytosed formaldehyde-denatured ^{125}I-labelled bovine serum albumin contained in isolated rat-liver tritosomes; both weak bases were equally inhibitory. Current results (Table 5.1) also show ammonium- and methylammonium ions to be equally inhibitory of lysosomal digestive activity in the intact cell. This is in full agreement with the findings of Reijngoud and co-workers in their cell-free system. However, in the cell--free system only 40-50% inhibition of proteolysis occurred at a 20$\underline{\underline{m}}$M

concentration of each weak base whereas approximately 100% inhibition was observed in yolk-sac tissue when 20mM concentrations of the same weak bases were used. Maximum inhibitory effects (60% inhibition) in the cell-free system were observed only when the tritosomes were incubated at pH 7.5 in the presence of a 50mM concentration of either weak base (Reijngoud et al., 1976). The large discrepancy between the extent of the weak-base inhibition in the yolk-sac system and that in isolated tritosomes must be a result of inherent differences in the experimental models used or the different source of lysosomes or the presence of Triton WR-1339 within the liver lysosomes. Reijngoud and co-workers measured the rate of production of acid-soluble radioactivity over the first 10min of incubation. A short delay (approx. 5min) between uptake of a weak base and the inhibition of digestion in the tritosomes would explain the discrepancy, but there is however, no evidence to suggest this. [^{14}C]-Methylammonium ions reach a steady-state level in isolated tritosomes within 45s (Reijngoud et al., 1976), therefore incomplete uptake of the weak bases by isolated tritosomes would not explain the discrepancy unless an additional mechanism, involved in the uptake of the weak bases by the lysosomes present in the intact cell, enhances the weak-base inhibition of lysosomal proteolysis. Such a mechanism might be energy dependent. Polet (1970) observed that chloroquine uptake by Hek cells in culture occurred by both energy-dependent and independent mechanisms. An alternative explanation of the discrepancy (that might be related to Polet's findings) can be forwarded; it assumes that the interior of isolated lysosomes is less acidic than the interior of the lysosomes within the cytoplasm of the intact cell. If this is the case, the control rate of proteolysis measured by Reijngoud et al. (1976) in isolated tritosomes would underestimate lysosomal activity in the intact cell, also the amount of weak base accumu-

lated would be less than that which would accumulate in lysosomes within the intact cell. The net result of both effects would be that the inhibition by each weak base of the proteolytic activity of isolated lysosomes would be less than the inhibition of the proteolytic activity of lysosomes present in the intact cell. The idea that the pH of the interior of isolated lysosomes is greater than that in lysosomes present in the intact cell seems feasible if, as discussed by Casey et al. (1978), an energy-dependent mechanism is involved in the maintenance of an acidic intralysosomal pH in the intact cell. Such a mechanism could operate in addition to the Donnan equilibrium (energy independent) mechanism discussed by Reijngoud & Tager (1977).

From the experiments performed in this chapter it is not possible to establish the mechanism of the lysosomal inhibition by the weak bases. It is possible that an inhibition of heterolysosome formation could explain the observations made here, but evidence in Chapter 7 indicates that such an inhibition does not occur. It is probable that the weak bases directly inhibit the lysosomal digestion process due to an elevation of the intralysosomal pH (de Duve et al., 1974; Reijngoud & Tager, 1977; see also Section 5.1). But it is possible that the weak bases are also able to selectively inhibit certain lysosomal proteinases. Chloroquine is an inhibitor of cathepsin B (Wibo & Poole, 1974). Should cathepsin B contribute to the observed digestion of the formaldehyde-denatured ^{125}I-labelled bovine serum albumin (see Chapter 6) it is possible that the chloroquine would interfere with the lysosomal digestion of the ^{125}I--labelled albumin independently of and in addition to any elevation of the intralysosomal pH. However, it does not seem likely that ammonium- and substituted ammonium ions could act other than by elevating the intralysosomal pH since neither ammonium- nor methylammonium ions inhibited the

digestion of formaldehyde-denatured ^{125}I-labelled bovine serum albumin by broken lysosomes (Reijngoud et al., 1976).

Knowles & Ballard (unpublished work), again working with the 17.5-day rat yolk sac, discovered that 20mM concentrations of ammonium-, methylammonium- and ethylammonium chloride each inhibited the normal breakdown of endogenous [^{3}H]leucine-labelled proteins by 47.1% ± 5.3%, 44.6 ± 7.6 and 45.6 ± 3.4 (mean ± S.D.) of the control rates respectively. The extents of these inhibitions of endogenous protein degradation by each weak base are approximately equal, as were the weak-base inhibitions of the lysosomal proteolysis of the ^{125}I-labelled albumin in the rat yolk sac (Table 5.1) and in isolated lysosomes (Reijngoud et al., 1976). Knowles & Ballard also showed that chloroquine (500μM) inhibited the breakdown of the endogenous proteins of the 17.5-day rat yolk sac by 36.1 ± 9.8% (mean ± S.D.).

These values for the inhibition of endogenous protein breakdown obtained by Knowles & Ballard contrast with the almost complete inhibition of the lysosomal digestion of formaldehyde-denatured ^{125}I-labelled bovine serum albumin (Table 5.1 and Figs. 5.3 & 5.5). One explanation of these observations is that more than 50% of the breakdown of the endogenous proteins is not intralysosomal, and that the breakdown of cellular proteins is evenly divided between a lysosomal and a non-lysosomal site. An alternative explanation is that all cellular proteins are digested in lysosomes but that weak bases selectively inhibit the lysosomal digestion of some proteins more than others so that only half of them are susceptible to the weak-base inhibition. This possibility is examined in Chapter 7 of this thesis where the results indicate that not all lysosomal enzymic activity is inhibited by the weak bases and that selective inhibition of proteolysis does indeed occur.

The inhibitory effects of the weak bases on the rate of pinosome formation (as judged by an inhibition of both ^{125}I-PVP and ^{125}I-labelled albumin uptake and by the observation that, except for chloroquine, the weak bases do not enhance the release of the endocytosed substrates) are almost as marked as the observed inhibition of lysosomal proteolysis of the ^{125}I-labelled albumin. Both inhibition of pinocytic uptake and inhibition of lysosomal digestion of the ^{125}I-labelled albumin occur over the same concentration range with a given weak base. Tulkens et al. (1970) showed that when lysosomal function, in cultured fibroblasts, was inhibited by anti-lysosomal antibodies endocytosis was also impaired. These results might indicate that the two processes are linked in some way. It is possible that an inhibition of lysosome function could result in a feedback inhibition of pinosome formation. This would have advantages for the cell since it would prevent the engorgement of the vacuolar system with extracellular material.

Both ammonium ions and chloroquine are vacuolating agents (Seglen & Reith, 1976, and Fedorko et al., 1968a,b, respectively) and Ballard (personal communication) has recently reported very marked vacuolation in yolk-sac tissue exposed to 20mM-NH_4^+ ions. However, Roberts et al. (1977) showed that after vacuolation was induced (using either Triton WR-1339 or sucrose or PVP or Dextran) in rat yolk sacs in vivo neither atypical pinocytic activity nor atypical proteolytic activity against formaldehyde-denatured ^{125}I-labelled bovine serum albumin occurred when the vacuolated yolk-sac tissue was investigated in vitro. The observations of Roberts and co-workers using the non-ionic vacuolating agents are in marked contrast to the observations made here using the weak bases. The observed inhibitions of pinocytosis and lysosomal digestion by the weak bases cannot be attributed to vacuolation, unless

tissue undergoing vacuolation behaves differently to tissue already vacuolated at the start of the experiment (as used by Roberts et al., 1977). A criticism of the design of experiments of the type reported by Roberts et al. is that any inhibition dependent on the inhibitor being present in nascent secondary lysosomes could be missed. Also, if the tissue rapidly recovers from the effects of a small diffusible vacuolating agent, like the ammonium-and substituted ammonium ions, again any effect would be missed.

Feedback-inhibition of pinosome formation might operate through stopping membrane re-cycling at a stage after phagolysosome formation. Alternatively, plasma membrane replenishment might result from a secretory mechnism whereby membrane synthesized in the Golgi region is formed into vesicles which fuse with the plasma membrane. Ammonium ions at a similar concentration to those used here have been shown to inhibit secretion of proteins by hepatocytes (Seglen & Reith, 1977) and might also inhibit membrane replenishment by the above mechanism should it operate in the rat yolk sac. A more readily tested explanation of the inhibition of pinocytosis is, however, an inhibition by the weak bases of energy producing processes. Knowles & Ballard (unpublished work) showed (see below) that the levels of ATP and of the ATP/ADP ratio in yolk-sac tissue were decreased when the tissue was exposed to either ammonium- or methylammonium- or ethylammonium ions.

Weak Base	ATP content (nmole/mg yolk-sac protein)	ATP/ADP
Control	14.19 ± 0.45 (12)	5.00 ± 0.30 (12)
5mM-ammonium	12.75 ± 0.56 (6)	4.69 ± 0.24 (6)
20mM "	10.84 ± 0.32 (13)	3.19 ± 0.15 (13)
5mM-methylammonium	12.09 ± 0.39 (6)	4.28 ± 0.15 (6)
20mM "	9.80 ± 0.31 (13)	2.76 ± 0.12 (13)
5mM-ethylammonium	13.74 ± 0.09 (3)	5.41 ± 0.33 (7)
20mM "	11.68 ± 0.64 (3)	3.65 ± 0.24 (3)

Yolk sacs, from 17.5-day pregnant rats, were incubated in the presence of a weak base for 2h before assaying for the adenine nucleotide levels. The data show the mean values (± S.E.M.) with the number of individual determinations shown in parentheses (Knowles & Ballard, unpublished data).

The metabolic inhibitors rotenone (Chapter 4), dinitrophenol and iodoacetate (Duncan & Lloyd, 1978) are also known to inhibit pinocytosis of substrates by the 17.5-day rat yolk sac. The effect of ammonium ions on intermediary metabolism and energy production has been studied in the liver (Katunuma et al., 1966) kidney (Vinay, 1976) and brain (Hindfelt, 1977).

An ammonium-ion load on kidney was shown by Vinay (1976) to have very little effect on the energy status of the kidney cells of fasted rats. In contrast, ammonium ions markedly decreased the energy status of both liver and brain tissues. In the brain, 10min after injection of ammonium ions into rats (that had been made chronically

hyperammonaemic by portal-systemic shunting 8 weeks previously), creatine phosphate levels were found to decrease whereas the level of the hydrolysis products, creatine and phosphate, increased. By 60min this effect was more pronounced and ATP levels were also diminished as was the ATP/ADP ratio. Measurements of the pyridine nucleotide reduction-potential in the cytoplasm and mitochondria indicated an increased NAD+/NADH ratio in the mitochondria and a decreased NAD+/NADH ratio in the cytoplasm. Hindfelt et al. (1977) suggested that the decreased level of ATP resulted from inhibition of respiration which occurred secondary to changes in the pyridine nucleotide levels. In the brain ammonia-loading decreases the tissue-level of asparate [considered by Hindfelt et al., (1977) to be the result of a net transamination between asparate and pyruvate giving rise to alanine and oxaloacetate]. Glutamate levels are also decreased as a result of the detoxification of ammonium ions in the brain, a process brought about by the synthesis, from glutamate and ammonium ions, of glutamine. The fall of both glutamate and aspartate levels, Hindfelt and co-workers suggested, inhibits the "malate-aspartate shuttle" which normally functions to transfer reduced equivalents of NADH from the cytoplasm to the mitochondria. A deficiency of intramitochondrial NADH was suggested to inhibit oxidative energy coupling and ultimately lead to a fall in ATP levels.

A similar event might also occur in the liver, but in addition, Katunuma et al. (1966) discovered that, following ammonia intoxication, the total level of mitochondrial pyridine nucleotides markedly decreased. Katanuma and co-workers discovered that ammonia activated a new pathway of pyridine nucleotide catabolism. In this pathway NADH and NADPH were enzymically converted by a pyrophosphatase enzyme to NMNH that, after

oxidation, was further converted to nicotinamide. The consequent decrease in the levels of mitochondrial pyridine nucleotide would necessarily bring about an inhibition of respiration.

The effects of ammonium, methylammonium- and ethylammonium ions on intermediary metabolism and the levels of pyridine nucleotides in the yolk sac are not known, but it is possible that the observed decreases in ATP levels and ATP/ADP ratios could be the result of changes similar to those described by Hindfelt et al. (1977) and Katunuma et al. (1966).

It is also possible that the inhibition of pinocytosis in the 17.5-day rat yolk sac by chloroquine is due to an interference with the energy status of the yolk-sac epithelial cells, but data on this is not available. Chloroquine, however, has been reported to decrease the cellular level of ATP. (see Goldstein & St. John, 1976, unpublished results).

Morphological studies indicate that chloroquine also inhibits pinosome formation in macrophages (Fedorko et al. 1968b). Chloroquine at a concentration of 5μM completely inhibited the uptake of arylsulphatase A by human fibroblasts whereas the uptake of [^{14}C]dextran was only inhibited by about 5-10% (see Fig. 5 of Weismann, 1974). This suggested to Weisman that chloroquine might mask the cell-surface binding-site for the lysosomal enzyme. [It is interesting to note however, that chloroquine inhibits arylsulphatase enzymic activity (Smith et al., 1976) which also suggests that chloroquine binds to the lysosomal enzyme, possibly masking the recognition site on the enzyme surface.] Brown & Segal (1977) reported that chloroquine had no effect on the pinocytic activity of the 17.5-day rat yolk sac. This is a marked contradiction of the observations made here showing chloroquine to inhibit the uptake of [^{125}I]-labelled substrates in both serum-free and serum-containing incubation medium. However, because

Brown & Segal (1977) do not present any data on the uptake of substrates by the 17.5-day rat yolk sac in the presence of chloroquine and because they do not state the concentration of chloroquine used it remains impossible to explain this discrepancy. Nevertheless, a lack of effect of chloroquine would be surprising since chloroquine is a toxic substance. Chloroquine toxicity has been described both in vivo and in vitro. The injection of chloroquine into rats at doses higher than 75mg/kg body-weight is usually lethal (Stein et al., 1977). Deleterious effects of chloroquine, at doses higher than 50$\mu\underline{\underline{M}}$, were observed on human fibroblasts in culture (Lie & Schofield, 1973) and 55µg/ml (approx. 100$\mu\underline{\underline{M}}$) concentrations of chloroquine in the culture medium produced severe toxic effects with macrophages, most of the cells being dead or detached by 2h. Moreover, Goldstein & St. John (1976) report that their unpublished data show chloroquine to cause the release of endogenous proteins from within cells. Concentrations of chloroquine in excess of 100$\mu\underline{\underline{M}}$ were found to be severely toxic to yolk sacs, and resulted in the release of ingested substrates, endogenous β-N-acetyl-β-D-glucosaminidase and endogenous lactate dehydrogenase.

In contrast to the observed toxicity of chloroquine to intact cells, the drug has been shown to stabilize lysosomal membranes (Weismann, 1965, 1966, 1967; Ignarro & Colombo, 1972). However, a biphasic effect of chloroquine on the stability of erythrocyte membranes has been reported by Inglot & Wolna (1968). These authors reported that low concentrations (approx. 100$\mu\underline{\underline{M}}$) of chloroquine stabilized the membranes but higher concentrations (approx. 1$\underline{\underline{mM}}$) labilized them. Cells in culture can accumulate chloroquine to produce an intracellular concentration many times that in the extracellular medium (Polet, 1970; Wibo & Poole, 1974) and might

therefore produce intracellular concentrations of chloroquine which are sufficiently high to labilize the cytomembranes even though low concentrations of chloroquine are present in the incubation medium.

Ammonia is known to be toxic *in vivo* causing severe neurological side effects (Wergedal *et al.*, 1964, Hindfelt *et al.*, 1977) but *in vitro* ammonia appears to be less toxic. Here ammonium- methylammonium- and ethylammonium ions at effective inhibitory levels did not cause the same degree of release of endocytosed substrates as did chloroquine (Table 5.2). Moreover, the inhibitory effects of ammonium ions on both endocytic and proteolytic activities were found to be reversible (Table 5.3); complete recovery was observed except after exposure to 20mM-ammonium ions for 1h when the endocytic activity of the exposed rat yolk sacs remained 10-15% lower than in control yolk sacs. Seglen (1975) obtained a complete recovery in the autophagic digestive activity of hepatocytes in culture after exposure to 5mM-ammonium ions and rat hepatocyte monolayers incubated with 10mM--ammonium ions for several days showed an undiminished viability (Seglen, 1977). Knowles & Ballard (unpublished data) showed that after exposure of 17.5-day rat yolk sacs to 20mM-ammonium ions for 2h, the yolk sacs, after re-incubation in medium free of ammonium ions, fully regained their ability to degrade endogenous proteins. Indeed, a higher rate of such digestion was observed during re-incubation, an effect which might be explained by the accumulation of cellular material within lysosomes during the exposure to ammonium ions so that on removal of the ammonium ions, more cellular protein was immediately available for digestion. Knowles & Ballard also showed a recovery in both the ATP levels and the ATP/ADP ratios after removal of ammonium ions:

		Recovery period in serum-free medium		
		0h	1h	2h
ATP (nmoles/mg yolk-sac protein)	control	14.19±0.45 (12)	13.61±0.49 (6)	14.12±0.73 (5)
	20mM-NH_4^+ ions	10.84±0.32 (13)	11.37±0.41 (6)	11.54±0.28 (4)
ATP/ADP	control	5.00±0.30 (12)	5.11±0.23 (6)	5.73±0.40 (5)
	20mM-NH_4^+ ions	3.19±0.15 (12)	4.92±0.31 (6)	5.10±0.30 (4)

Adenine nucleotide levels in control tissue and rat yolk sac after exposure to 20mM-NH_4^+ ions for 2h followed by 0, 1 and 2h recovery in medium free of NH_4^+-ion. The mean values (± S.E.M.) of the ATP levels (nmole/mg yolk-sac protein) and ATP/ADP ratios are shown together with the number of individual determinations shown in parentheses. (Knowles & Ballard, unpublished data).

The ATP/ADP ratio clearly returns to almost normal within 1h but the recovery of ATP levels is slow and incomplete by 2h. The rapid recovery of pinocytic activity (Table 5.3) and the ATP/ADP ratios, but not ATP levels, in yolk sacs after exposure to 20mM-ammonium ions suggests that it is the ATP/ADP ratio that probably regulates the pinocytic activity of the rat yolk sac. ATP levels might, however, limit the pinocytic activity of the yolk--sac and the incomplete recovery of pinocytic activity after exposure to ammonium ions might be the result of a slower recovery of ATP levels.

The inhibition of pinocytic activity of the rat yolk sacs observed in the presence of ammonium-, methylammonium- and ethylammonium ions is a new finding (as indicated by the results of a literature survey) and adds to the many effects that ammonium ions are reported to have. Ammonium ions inhibited the toxic action of diphtheria toxin on mammalian cells in culture (Ivins et al., 1975) but the ammonium ions at the protective

levels did not alter the uptake of the toxin (Bonventre, 1975). Lectin--induced lymphoblastogenesis was reported to be inhibited in a tissue culture medium, and the inhibitor was identified as ammonia, generated by deamination of glutamine (Baechtel et al. 1976). Ammonium chloride buffers were accidentally discovered to markedly reduce the ability of lymphoid cell preparations to function as attacking cells in an antibody-dependent cell-mediated cytotoxicity test, but the cells retained their ability to adhere to antibody-coated target cells (Yust et al., 1976). Seglen & Reith (1977) discovered that ammonium ions inhibited protein secretion in isolated rat hepatocytes. All these effects might be the result of the interference of ammonium ions on energy production. Stimulatory effects of ammonium ions have, however, also been reported: increased iodide uptake by thyroid cells (Burk & Kowalski, 1971), enhanced Ca^{++}-dependent catacholamine secretion from adrenal glands (Sorimachi, 1968), enhanced aldosterone production by rat adrenal sections (Muller, 1965) and increased gluconeogenesis in isolated rat hepatocytes (Zahlten et al., 1974).

CHAPTER SIX

EFFECTS OF MICROBIAL PROTEINASE INHIBITORS ON PROTEIN CATABOLISM WITHIN RAT YOLK SACS

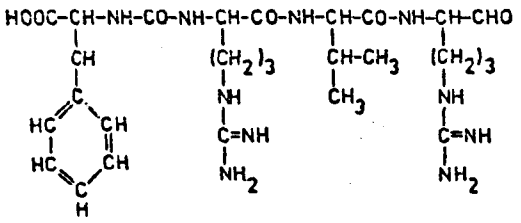
6.1 INTRODUCTION

In the previous chapter evidence was forwarded that the digestion of endogenous yolk-sac proteins occurs at both a lysosomal and a non-lysosomal site. The evidence was gained through the use of weak bases that were shown to inhibit both lysosomal proteolysis and the breakdown of endogenous proteins. The inhibition of proteolysis by the weak bases was not, however, a specific effect; concomitant decreases were observed in the tissue-level of ATP, in the ATP/ADP ratio and in the rate of pinosome formation. Simpson (1953) as well as Steinberg & Vaughan (1956), showed agents that inhibited the generation of ATP to also inhibit protein degradation in liver slices. It has now been observed, in many cell types, that protein degradation is inhibited by compounds that interfere with energy production (Goldberg & St. John, 1976). Consequently, the observed inhibition, by weak bases, of endogenous yolk-sac protein breakdown may, in addition to any inhibition of intra-lysosomal proteolysis, be caused by an inhibition of some non-lysosomal energy-requiring process essential to protein breakdown. For example, autophagosome formation, which appears to contribute to the breakdown of endogenous proteins in other cell types(Dean & Barrett, 1976; Ballard, 1977) might also be inhibited by weak bases in a manner analogous to the inhibition of heterophagosome formation observed in the previous chapter.

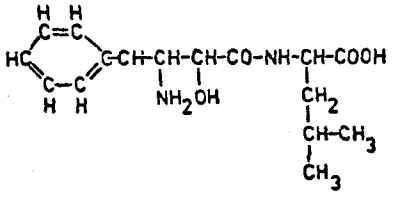
To further investigate the contribution of lysosomal and non-lysosomal enzymes to the breakdown of endogenous yolk-sac proteins and to further investigate the hydrolysis of endocytosed protein substrates, it was desirable to discover lysosomal enzyme inhibitors free of any attendant complications of the type met in studies made with weak bases, and which preferably act solely on lysosomal enzymes. It was the aim of the work reported in this chapter to discover such inhibitors.

A new group of peptide proteinase inhibitors isolated from Streptomycese culture filtrates (for reviews see Aoyagi & Umezawa, 1975 and Umezawa & Aoyagi, 1977) have been usefully employed to specifically inhibit certain groups of proteolytic enzymes. Six such microbial compounds have been utilized in the studies that follow. Pepstatin is a N-acyl-pentapeptide of which several distinct chemical forms have been isolated (Aoyagi & Umezawa, 1975) The structure of pepstatin A is shown in Diagram 6.1; it is thought to be a transition state, pseudo-irreversible, competitive inhibitor of acid proteinases (Marcinszin et al., 1976). Pepstatin specifically inhibits the acid-proteinases pepsin and renin (Aoyagi et al., 1971, 1972) and Cathepsins D and E (Barrett & Dingle, 1972; Woessner, 1972). Dean (1975b) showed that pepstatin, entrapped in liposomes, was taken up by rat liver, where it inhibited endogenous protein digestion.

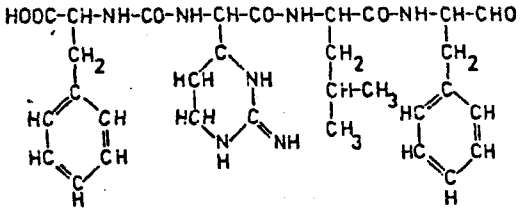
Hopgood et al. (1977) showed that, in addition to pepstatin, the microbial proteinase inhibitors antipain, bestatin and leupeptin could also be used to inhibit endogenous protein breakdown in isolated rat hepatocytes. Antipain and leupeptin were also effective inhibitors of protein breakdown in Reuber H35 hepatoma cells (Knowles & Ballard, 1976). Antipain (Diagrams 6.1) is an inhibitor of papain, trypsin, thrombokinase, plasmin, cathepsin A and cathepsin B (Suda et al., 1972; Ikezawa et al., 1972). Bestatin (Diagrams 6.1) inhibits the enzymes aminopeptidase B and leucinaminopeptidase but not aminopeptidase A or endopeptidases (Umezawa & Aoyagi, 1977). Leupeptin is the name given to a group of compounds comprising acetyl-(or proprionyl-)-L-leucyl-L-leucyl-L-arginal and their analogues in which leucine is replaced by either isoleucine or valine (Kondo et al., 1969; Kawamura et al., 1969). The structure of the leupeptin, used in the studies that follow is in Diagram 6.1. Leupeptin inhibits (competitively): plasmin, trypsin, kallikrein, thrombokinase,



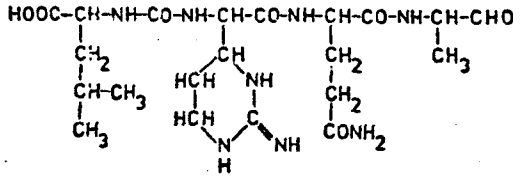
Antipain



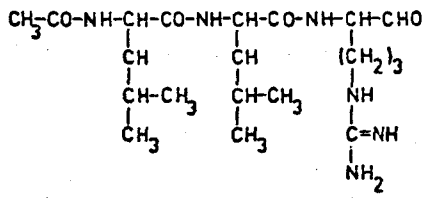
Bestatin



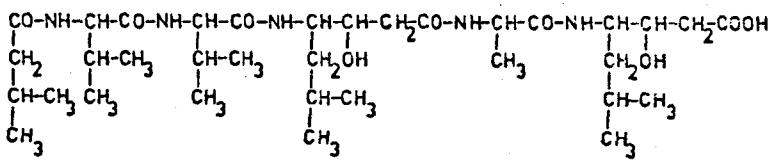
Chymostatin



Elastatinal



Leupeptin



Pepstatin

Diagram 6.1 Structures of six peptide proteinase inhibitors of microbial origin.

Antipain: [(S)-1-carboxyl-2-phenylethyl]-carbamoyl-L-arginyl-L-valylargininal (Umezawa et al., 1972). Bestatin: (2S,3R)--3-amino-2-hydroxy -4-phenylbutanoyl-L-leucine (Umezawa & Aoyagi, 1977). Chymostatin: N-[(S)-1-carboxy-2-phenylethyl]--carbamoyl-α-N[2-iminohexahydro-4(S)-pyromidyl]-(S)-glycyl-L-leucyl-phenylalaninal (Tatsuta et al., 1973). Elastatinal: N-[(S)-1-carboxy-isopentyl]-carbamoyl-α-[2-iminohexahydro -4(S)-pyrimidyl]-(S)-glycyl-(S)-glutaminyl-(S)-alaninal (Okura et al., 1975). Leupeptin: acetyl-L-leucyl-L-leucyl-L-argininal (Kondo et al., 1969). Pepstatin: isovaleryl-L--valyl-L-valyl-AHMHA-L-alanyl-AHMHA, where AHMHA is 4-amino-3-hydroxy-6-methylhepatonoic acid (Morishima et al., 1970).

papain and cathepsin B (Aoyagi et al., 1969; Aoyagi & Umezawa, 1975; Umezawa, 1975; Fritz et al., 1973) and cathepsin L (Kirschke et al., 1977) but not cathepsin D (see Kirschke et al., 1977). In addition to the above microbial inhibitors, chymostatin, an inhibitor of chymotrypsin (Umezawa et al., 1970) and elastatinal, a specific inhibitor of elastase (Umezawa et al., 1973) might also inhibit lysosomal proteolysis in the yolk-sac culture system. Chymostatin is a good inhibitor of cathepsin B but poorly inhibits cathepsins A and D (Ikezawa et al., 1971). Riemann & Hanson (1978) have recently reported that chymostatin is also an inhibitor of cathepsin L.

Two characteristics of the above microbial proteinase inhibitors make them suitable for use as potential specific inhibitors of lysosomal proteolytic activity in the yolk-sac system. Firstly, they have been reported to be non-toxic. Umezawa & Aoyagi (1977) indicate that an intravenous injection of antipain, 250mg/kg body-weight, and intraperitoneal injection of bestatin, 300mg/kg body-weight, caused no death in mice. No toxicity in mice was observed after an intravenous dose of 250mg elastatinal per kg body-weight. The quantity of leupeptin (given intravenously) and pepstatin (given intraperitoneally) that killed half the number of mice was reported as 118mg/kg body-weight (125mg/kg in rats) and 1 090mg/kg body-weight, respectively. Secondly, cellular membranes, will not normally permit molecules of molecular weight >150 to permeate through them (Albert, 1968) and lysosomal membranes are not permeable to peptides with a molecular weight exceeding 200-250 (for review see Reijngoud & Tager, 1977). It is, therefore, most unlikely that the above microbial peptide proteinase inhibitors, each with a molecular weight that exceeds 300, will reach intracellular proteinases other than those within

the lysosomal system. Also this implies that for inhibition of lysosomal proteolysis to occur, the inhibitors must be taken up by endocytosis.

Substances, other than microbial proteinase inhibitors, known to inhibit enzymes of the type that occur in lysosomes include: heavy metal ions (Huisman et al., 1973; Mego, 1976), iodoacetate (Huisman et al., 1973, 1974), diazonium compounds (e.g. diazoacetylnorleucine methylester) in the presence of Cu^{2+} (Sodek & Hofmann, 1968), Tosyl derivatives (Barrett, 1977), anti-proteinase antibodies (Dingle, 1971; Tulkens et al., 1970) and protein proteinase inhibitors isolated from either plants and animals (Kassell, 1970; Loskowski & Sealock, 1971) or microbes (Kakinuma et al., 1978). The possibility of using protein proteinase inhibitors was considered to be of little interest here since it is likely they would be rapidly digested once inside lysosomes. Antilysosomal-enzyme antibodies were shown to impair pinocytosis in fibroblasts (Tulkens et al., 1970) and iodoacetate a powerful inhibitor of lysosomal catheptic activity (Huisman et al., 1973) was shown to inhibit pinocytosis in the rat yolk-sac system (Duncan & Lloyd, 1978). The diazonium compounds are slow acting and require the presence of Cu^{2+}, which like the heavy metal ions, might be expected to be generally toxic; like metal ions and iodoacetate, Tosyl derivatives might not be expected to limit their antiproteolytic activities to the lysosomes. Also, Tosyl derivatives have been shown to inhibit protein synthesis at extremely low concentrations (Chou, et al., 1974).

The microbial proteinase inhibitors seem to offer the best prospect of a specific inhibition of lysosomal proteolytic activity provided that adequate quantities can be taken into the lysosomes by endocytosis. Leupeptin is a potent inhibitor of proteolysis in broken lysosome preparations (Huisman et al., 1974) and an inhibitor of endogenous protein catabolism in intact cells (Hopgood et al., 1977; Neff et al.,

1977; Libby & Goldberg, 1977). In this chapter the effects of leupeptin on endocytosis and lysosomal proteolysis in the 17.5-day rat yolk sac were studied in detail; possible effects of antipain, bestatin, chymostatin, elastatinal and pepstatin on this system were also investigated. A parallel study of the effects of these inhibitors on endogenous protein breakdown in the 17.5-day rat yolk sac is, at the time of writing, being conducted by Knowles & Ballard.

6.2 METHODS

All methods used in this chapter were essentially the same as described in previous chapters. Two different methods (see Section 5.2.1) were used to assay the uptake and degradation of formaldehyde--denatured ^{125}I-labelled bovine serum albumin. [Two different batches of formaldehyde-denatured ^{125}I-labelled bovine serum albumin were used in the experiments reported in this chapter and are designated batch I and batch II. Each was prepared ostensibly by the same method (see Section 2.2.3).] When yolk sacs were incubated with inhibitors, the radiolabelled substrate was added to the incubation medium 30min after the introduction of the yolk sacs to medium already containing the inhibitor. Antipain [(1-carboxy-2-phenyl-ethyl) carbamoyl-L-arginyl-L-valyl-L--arginine] and bestatin [3-amino-2-hydroxy-4-phenylbutanoyl-L-leucine] were kind gifts from Dr. H. Umezawa, Microbial Chemistry Research Foundation, 14-23 Kamiosaki, 3-Chome, Shingana-Ku, Tokyo, Japan. Chymostatin [N-(1-carboxy-2-phenylethyl)-carbamoyl)-α-(2-iminohexahydro--4-pyrimidyl)-L-glycyl-L-leucyl-phenylalaninal] and Elastatinal [N-(1--carboxyisopentyl) carbamoyl-α-(2-imino-hexohydro-4-pyrimidyl)-glycyl-glutaminyl-alininal] were kind gifts from Dr. W. Troll, Department of Environmental Medicine, Obstetrics and Gynaecology, New York University Medical Centre, 550 First Avenue, New York 1006, U.S.A. Leupeptin [acetyl-L-leucyl-L-leucyl-L-arginal] and pepstatin [isovaleryl-L-valyl-L--valyl-AHMHA-L-alanyl-AHMHA; where AHMHA is 4-amino-3-hydroxy-6-methyl--hepatonoic acid] were purchased from the Peptide Institute Inc., 476 Ina, Minoh-shi, Osaka 562, Japan.

The method used to determine the recovery of the yolk-sac proteolytic activity after exposure to leupeptin was the same as that

described for recovery from ammonium chloride exposure (see Section 5.2.4). The effects of proteinase inhibitors on the release of endocytosed formaldehyde-denatured ^{125}I-labelled bovine serum albumin were determined as described for the effect of the weak bases (see Section 5.2.3(1)) except that a 2h incubation period was used to load the yolk sacs with ^{125}I-labelled albumin.

The proteolytic activity (against formaldehyde-denatured ^{125}I-labelled bovine serum albumin) of cell-free extracts of 17.5-day rat yolk sacs was assayed essentially as described in Section 4.2.4. The incubation mixtures contained the following: buffer solution [250µl, 0.1M-sodium acetate, either pH 4.0 or 5.5]; Triton X-100 [150µl, 0.75% (v/v) dissolved in the appropriate buffer solution]; cell-free extract [100µl, containing 100µg yolk-sac protein]; inhibitor [11µg in 50µl of distilled water]; formaldehyde-denatured ^{125}I-labelled bovine serum albumin [10µg in 50µl of 1% (w/v) aq. NaCl]. When assays were performed in the absence of Triton X-100, 150µl of Triton-free buffer solution was added in place of the Triton solution.

6.3 RESULTS

6.3.1 Uptake and digestion of formaldehyde-denatured ^{125}I-labelled bovine serum albumin by 17.5-day rat yolk sacs incubated in serum-free medium 199 containing leupeptin (20µg/ml).

A preliminary study showed that the digestion of formaldehyde-denatured ^{125}I-labelled bovine serum albumin, when incubated with 17.5-day rat yolk sacs, was markedly inhibited by leupeptin at a concentration of 20µg/ml of serum-free medium 199. No effect on the rate of uptake of the substrate was observed. The time course of the effect is shown in Fig. 6.1 together with the corresponding control. In the control (as described previously, Section 5.3.1) the tissue-associated radioactivity rose during the first hour but then remained constant, so that the rates of digestion and of uptake of the ^{125}I-labelled albumin became equal. In contrast, yolk sacs incubated in the presence of 20µg/ml leupeptin continued to accumulate radioactivity throughout the 3h incubation period; also the rate of substrate digestion was markedly decreased (by approx. 50%) but the rate of uptake of the ^{125}I-labelled albumin was not altered. Moreover, the overall quantity of ^{125}I-labelled albumin endocytosed by the yolk sac, the quantity of acid-soluble radioactivity released into the incubation medium and the quantity of radioactivity accumulated by the yolk-sac tissue each increased linearly with time. Such linearities indicate that, if a progressive accumulation of the leupeptin within lysosomes occurs during the course of incubation it does not result in a progressively greater inhibition of the lysosomal digestive process.

6.3.2 Effects of different concentrations of leupeptin on the uptake and digestion of formaldehyde-denatured ^{125}I-labelled bovine serum albumin by 17.5-day rat yolk sac incubated in serum-free medium 199.

The inhibitory effect of leupeptin on the digestion of formaldehyde-denatured ^{125}I-labelled bovine serum albumin by yolk sacs was shown to be dependent on its concentration in the incubation medium. Fig. 6.2 shows the time-course of the appearance of acid-soluble radioactivity in the incubation medium when yolk sacs were incubated with the ^{125}I-labelled albumin in the presence of different concentrations of leupeptin (0-100µg/ml of incubation medium). At each leupeptin concentration the acid-soluble radioactivity appearing in the medium, after an initial lag-period, increased essentially linearly with time over the remainder of the 3h incubation period. The rate of production of acid-soluble radioactivity did not show any indication that it was progressively decreasing throughout the incubation period as might be expected if the leupeptin progressively accumulated within the lysosomes of the yolk sacs.

Fig. 6.3 shows the effects of the different concentrations of leupeptin on the rates of uptake and of digestion of the ^{125}I-labelled albumin and on its accumulation by yolk sacs. A small (18%) inhibition of uptake was observed in the presence of leupeptin at a concentration of 100µg/ml, but this was not statistically significant ($P < 0.10$). As the leupeptin concentration was increased, the rate of digestion of the ^{125}I-labelled albumin progressively decreased, reaching about 20% of the control rate of digestion in the presence of 100µg/ml of leupeptin. The decreased rate of digestion resulted in an observed concomitant increase in the 3h-level of tissue-associated radioactivity, which, at 40µg/ml of leupeptin, reached a maximum equivalent to 413% of that in control yolk sacs. The corresponding increase in the acid-insoluble radioactivity associated with the yolk sacs was significantly higher, 593%. The quantity of acid-soluble radioactivity in the yolk-sac tissue remained approximately constant (34-50ng/mg yolk-sac protein) at each leupeptin

concentration so that the proportion of tissue-associated radioactivity that was acid-soluble decreased with increasing leupeptin concentration (Fig. 6.4, lower). The difference was found to be statistically significant ($P < 0.02$) even at a leupeptin concentration of 1µg/ml of medium. Fig. 6.4 (upper) shows the effects of different concentrations of leupeptin on the rate of digestion of the ^{125}I-labelled albumin and the average rate of accumulation of radioactivity by the yolk sac, but after corrections for the observed fluctuations in the rates of uptake. These data show more accurately the inhibitory effects of leupeptin on the digestion of the ^{125}I-labelled albumin within the lysosomal system of yolk-sac tissue.

6.3.3 Recovery of the proteolytic activity of 17.5-day rat yolk sacs after exposure to leupeptin.

The lack of a progressive decrease in the rate of digestion of formaldehyde-denatured ^{125}I-labelled bovine serum albumin, with increasing duration of incubation of yolk sacs in the presence of leupeptin (Fig. 6.1 and 6.2), suggests either that the endocytosed leupeptin is continually inactivated or that newly-formed pinosomes containing the ^{125}I-labelled albumin do not fuse with heterolysosomes containing previously endocytosed leupeptin. In either case a recovery of the proteolytic activity of the yolk sacs following exposure of the tissue to leupeptin would be expected. Fig. 6.5 shows that yolk sacs, exposed to concentrations of 40µg/ml of leupeptin for 1h, showed an initial rapid recovery of a large proportion of their proteolytic activity on being washed and re-incubated in inhibitor-free medium 199 containing the ^{125}I-labelled albumin (1µg/ml of medium). A slow, progressive recovery of the remainder of the proteolytic activity was observed over an 8-120min washing period,

but complete recovery was never observed even after 2h of incubation in leupeptin-free medium before re-determining the proteolytic activity of the tissue. (It is possible, however, that a complete recovery might have been achieved if a much longer recovery period had been used.) The results obtained with the control yolk sacs (Fig. 6.5) show them to maintain a constant proteolytic capacity throughout the duration of the experiment; at each time interval the ^{125}I-labelled albumin endocytosed was completely digested so that the level of tissue-associated radioactivity remained constant.

6.3.4 Effects of chymostatin and leupeptin on the release of formaldehyde--denatured ^{125}I-labelled bovine serum albumin by 17.5-day rat yolk sacs.

Yolk sacs, incubated in serum-free medium 199, were permitted to accumulate formaldehyde-denatured ^{125}I-labelled bovine serum albumin in the presence of either chymostatin (an inhibitor of the digestion of ^{125}I-labelled albumin by yolk sacs, see Table 6.1) or leupeptin. After washing the yolk sacs (in medium 199 containing the appropriate inhibitor, see Section 6.2), they were re-incubated in medium containing the appropriate inhibitor but no ^{125}I-labelled albumin. Fig. 6.6 shows that, compared with corresponding control experiments, both chymostatin and leupeptin inhibited the release of acid-soluble radioactivity into the incubation medium; leupeptin was more effective than chymostatin. However, such inhibition of the digestion of the ^{125}I-labelled albumin did not enhance the proportion of the tissue-associated radioactivity that was released into the incubation medium as acid-insoluble radioactivity when the tissue was re-incubated. Indeed the proportion (<0.2%/h in the presence and absence of the inhibitors) was, if different, less when the inhibitors were present.

6.3.5 Effects of six different microbial proteinase inhibitors on the rates of uptake and of digestion of formaldehyde-denatured ^{125}I-labelled bovine serum albumin by 17.5-day rat yolk sacs incubated in serum-free medium 199.

The above experiments show that leupeptin is a powerful inhibitor of the lysosomal digestion of formaldehyde-denatured ^{125}I-labelled bovine serum albumin. Five other microbial proteinases (antipain, bestatin, chymostatin, elastatinal and pepstatin) were tested to discover whether they too inhibited proteolysis in the yolk-sac system. Results of such experiments are shown in Table 6.1. (The inhibitory effects of leupeptin were again measured so that a more direct comparison of the inhibitory effects of the various inhibitors could be made. This was necessary as the batch of formaldehyde-denatured ^{125}I-labelled bovine serum albumin used in the following experiments differed from that used previously.) In control experiments, the rate of uptake of the ^{125}I-labelled albumin used here (batch II; 208.8 ± 23.8ng/h per mg yolk-sac protein, see Table 6.1) was less than that observed in the previous control experiments (batch I; 275.6 ± 32.9ng/h per mg yolk-sac protein, see Fig. 6.3).

None of the microbial proteinase inhibitors, present at either 10 or 30μg/ml of incubation medium, modified the rate of uptake of the ^{125}I-labelled albumin (Table 6.1, columns 1 and 2). Leupeptin was found to be the most potent inhibitor of digestion, showing a rate of digestion equal to 52.4 ± 11.2% (mean ± S.D.) of the rate of uptake (Table 6.1, column 4). Antipain and chymostatin also showed substantial inhibitory powers; chymostatin appeared to be slightly more effective but this difference was not statistically significant ($P < 0.10$). Bestatin and elastatinal showed a small inhibitory effect when present at 30μg/ml of

incubation medium, but this was only statistically significant in the case of bestatin ($P < 0.02$). No inhibition of ^{125}I-labelled albumin digestion was observed in the presence of either 10 or 30µg/ml concentrations of pepstatin.

Whenever an inhibition of the digestion of ^{125}I-labelled albumin was observed with any of the microbial inhibitors, a corresponding increase in the tissue-associated radioactivity was seen. Thus the quantity of tissue-associated radioactivity increased along the series: control = pepstatin = elastatinal = bestatin << antipain = chymostatin << leupeptin (Table 6.1, columns 5 and 6). The proportion of the tissue-associated radioactivity that was acid-soluble decreased in the same order (Table 6.1 column 7).

6.3.6 Effects of the six microbial proteinase inhibitors on the digestion of formaldehyde-denatured ^{125}I-labelled bovine serum albumin by a cell-free extract of 17.5-day rat yolk sacs.

The lack of inhibition of the digestion of formaldehyde--denatured ^{125}I-labelled bovine serum albumin by yolk sacs incubated in the presence of some of the above microbial proteinase inhibitors (Section 6.3.4) might be explained in a number of different ways. Either some of the microbial compounds have intrinsically low inhibitory powers against cathepsins in general or they are active against cathepsins other than those involved in the rate-limiting steps of the overall digestion of proteins, or their intralysosomal concentration never reaches an effective level (either because they are poorly endocytosed or because they are rapidly inactivated within lysosomes). A further way in which the microbial compounds might inhibit intralysosomal proteolysis of endocytosed protein substrate is by preventing lysosome-pinosome fusion. The last of these

possibilities was tested by determining the effects of the inhibitors on the digestion of the ^{125}I-labelled albumin by a cell-free extract of 17.5-day rat yolk sacs. In this system the membrane barriers that exist in the yolk sac are disrupted so that the added inhibitors have free access to the cathepsins. However, the intralysosomal pH within intact tissue is not known precisely hence cell-free extracts were incubated with ^{125}I-labelled albumin at two pH's: 4.0 and 5.5 (i.e. an optimal- and suboptimal pH, see Fig. 4.3). Also, because a possible binding of inhibitors to disrupted membranes might decrease their effective concentrations (especially pepstatin which is very hydrophobic) the determinations at both pH's were also made in the presence and absence of 0.2% (v/v) Triton X-100. Results of such experiments are shown in Table 6.2.

In the presence of Triton X-100 at pH 4.0 the order of the effectiveness of the inhibitors in preventing the hydrolysis of the ^{125}I-labelled albumin by the cell-free extract of yolk sacs was: leupeptin > chymostatin = antipain > bestatin = elastatinal = pepstatin > control. In the presence of Triton X-100 at pH 5.5 the potency of each inhibitor was, in general, decreased, except for pepstatin, which (unexpectedly) was more inhibitory at pH 5.5 than at pH 4.0. This effect was reproducible. A similar decrease in the extent of inhibition by pepstatin occurred on increasing the pH of the Triton-free assay medium from 4.0 to 5.5. As in the presence of Triton X-100, all the other proteinase inhibitors were less effective at the higher pH. These results, in general, indicated that leupeptin is the most potent inhibitor, antipain and chymostatin were good inhibitors at pH 4.0 whereas pepstatin was a good inhibitor at pH 5.5. Elastatinal and bestatin were only poor inhibitors. The presence of Triton X-100 did not affect the general patterns of inhibition. These results suggest that the differential

inhibitory effects of the microbial compounds does not reside in a differential inhibition of lysosome-pinosome fusion but resides in a direct inhibition of those cathepsins involved in the rate-limiting step of the overall proteolysis of the ^{125}I-labelled albumin after reaching an intralysosomal site.

Table 6.1 Effects of microbial proteinase inhibitors on the rates of uptake and of digestion of formaldehyde-denatured ^{125}I-labelled bovine serum albumin by 17.5-day rat yolk sacs incubated in serum-free medium 199.

The rates of uptake and of digestion of formaldehyde-denatured ^{125}I-labelled bovine serum albumin were determined by the method described in Section 3.2.1. ^{125}I-labelled albumin (Batch II 1µg/ml of incubation medium) was added 30min after placing the yolk sacs in incubation medium containing the inhibitor at the concentrations indicated. Each value reported represents the mean (± S.D.) of 3-8 separate determinations each over a 3h uptake period.

Inhibitor	Conc. µg/ml	Rate of uptake		Rate of digestion		Tissue-associated radioactivity (ng/mg) at 3h	Average rate of tissue accumulation over 3h period (% rate of uptake)	Proportion of the radioactivity in the yolk sac (at 3h) that is acid-soluble	No. of determinations
		ng/h/mg yolk-sac protein	(% of control)	ng/h/mg yolk-sac protein	(% of rate of uptake)				
Control	-	208.8 ± 23.8	(100.0)	204.9 ± 25.8	(97.9 ± 1.9)	99.1 ± 17.0	15.8 ± 2.4	36.4 ± 3.2	8
Antipain	(10)	227.2 ± 16.0	(108.8)	201.8 ± 11.7	(88.9 ± 3.7)	149.8 ± 13.7	22.0 ± 2.1	26.3 ± 3.1	4
	(30)	199.8 ± 23.2	(95.7)	151.6 ± 34.9	(75.2 ± 10.2)	218.9 ± 34.2	37.1 ± 8.5	15.7 ± 3.2	4
Bestatin	(10)	216.2 ± 26.1	(103.5)	214.2 ± 21.5	(99.2 ± 2.5)	107.7 ± 16.4	16.6 ± 1.3	38.4 ± 5.5	3
	(30)	196.9 ± 25.6	(94.5)	185.5 ± 22.2	(94.3 ± 1.2)	107.0 ± 12.9	18.1 ± 0.2	34.7 ± 2.9	3
Chymostatin	(10)	215.1 ± 20.6	(103.0)	180.6 ± 18.3	(84.0 ± 3.4)	181.1 ± 26.5	28.1 ± 3.3	19.6 ± 3.9	4
	(30)	208.5 ± 28.6	(99.8)	173.7 ± 16.2	(71.8 ± 8.8)	241.3 ± 26.2	39.1 ± 6.9	14.8 ± 3.6	4
Elastatinal	(10)	200.0 ± 7.8	(95.7)	193.6 ± 2.7	(97.0 ± 4.9)	90.6 ± 12.3	15.0 ± 1.6	36.7 ± 4.4	3
	(30)	196.4 ± 29.2	(94.0)	182.1 ± 26.1	(92.9 ± 6.9)	101.4 ± 31.2	16.9 ± 3.3	30.8 ± 4.9	3
Leupeptin	(10)	212.2 ± 59.5	(101.6)	167.3 ± 45.4	(79.0 ± 5.6)	207.5 ± 56.9	32.8 ± 3.1	17.7 ± 3.6	4
	(30)	214.2 ± 33.4	(102.4)	114.8 ± 40.2	(52.4 ± 11.2)	314.2 ± 54.6	54.8 ± 8.9	9.6 ± 2.3	3
Pepstatin	(10)	211.0 ± 23.5	(101.0)	207.3 ± 23.0	(98.3 ± 2.3)	98.1 ± 14.4	15.4 ± 0.9	36.6 ± 3.2	3
	(30)	195.7 ± 21.3	(93.7)	196.4 ± 16.9	(100.5 ± 3.3)	100.5 ± 18.5	16.9 ± 1.8	35.2 ± 3.0	3

Table 6.2 Effects of microbial proteinase inhibitors on the digestion of formaldehyde-denatured ^{125}I-labelled bovine serum albumin by a cell-free extract of the 17.5-day rat yolk sac.

The effects of the six microbial proteinase inhibitors on the rate of digestion of formaldehyde-denatured ^{125}I-labelled bovine serum albumin (Batch II) was determined as described in Section 6.2.1. The data show the mean (± S.D.) of four replicate determinations. In each case the maximum amount of substrate digested did not exceed 3% of that initially present. Each of the inhibitors was present at a concentration of 20 µg/ml of incubation mixture.

	Relative rates of digestion of ^{125}I-labelled albumin (Control = 100%)			
	Presence of Triton X-100		Absence of Triton X-100	
	pH 4.0	pH 5.5	pH 4.0	pH 5.5
Control	100.0 ± 5.7	100.0 ± 14.3	100.0 ± 7.5	100.0 ± 9.9
Antipain	14.6 ± 4.3	84.5 ± 5.3	18.8 ± 7.3	48.9 ± 9.3
Bestatin	75.0 ± 10.9	98.3 ± 1.8	89.6 ± 8.5	88.4 ± 16.1
Chymostatin	11.9 ± 6.4	84.7 ± 15.7	23.6 ± 4.4	69.0 ± 11.0
Elastatinal	71.8 ± 12.9	83.5 ± 4.0	57.4 ± 5.2	71.1 ± 9.0
Leupeptin	5.3 ± 5.5	49.3 ± 6.5	10.0 ± 2.0	25.1 ± 10.7
Pepstatin	77.4 ± 4.6	63.3 ± 14.5	50.8 ± 5.9	19.4 ± 6.3

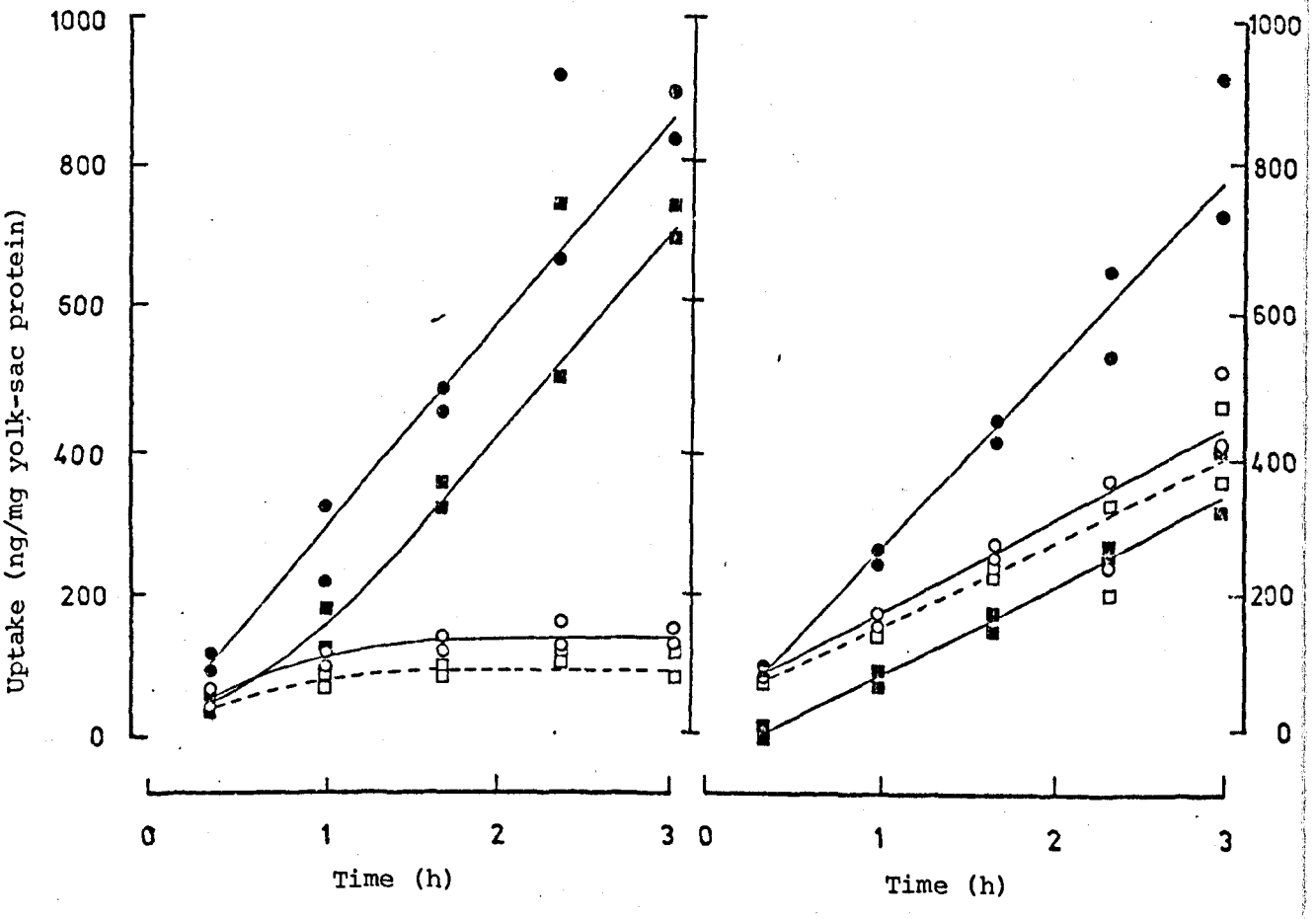


Figure 6.1 Effect of leupeptin (20 µg/ml) on the uptake and digestion of formaldehyde-denatured ^{125}I-labelled bovine serum albumin by 17.5-day rat yolk sacs incubated in serum-free medium 199.

The uptake and digestion of formaldehyde-denatured ^{125}I-labelled bovine serum albumin were determined as described in Section 2.2.1. Yolk sacs were incubated (in 20 ml medium 199) with leupeptin for 30 min before uptake commenced on adding substrate (Batch I, 1 µg/ml of incubation medium). Each experiment was performed using yolk sacs from a single animal. The control experiment was performed in parallel and is the same as that shown in Fig. 5.1. Values at each time point are from two yolk sacs incubated separately. Tissue-associated radioactivity, (○); acid-soluble radioactivity released into the incubation medium, (■); sum of tissue-associated radioactivity and acid-soluble radioactivity released into the incubation medium (●); acid-insoluble radioactivity in the yolk-sac tissue, (□).

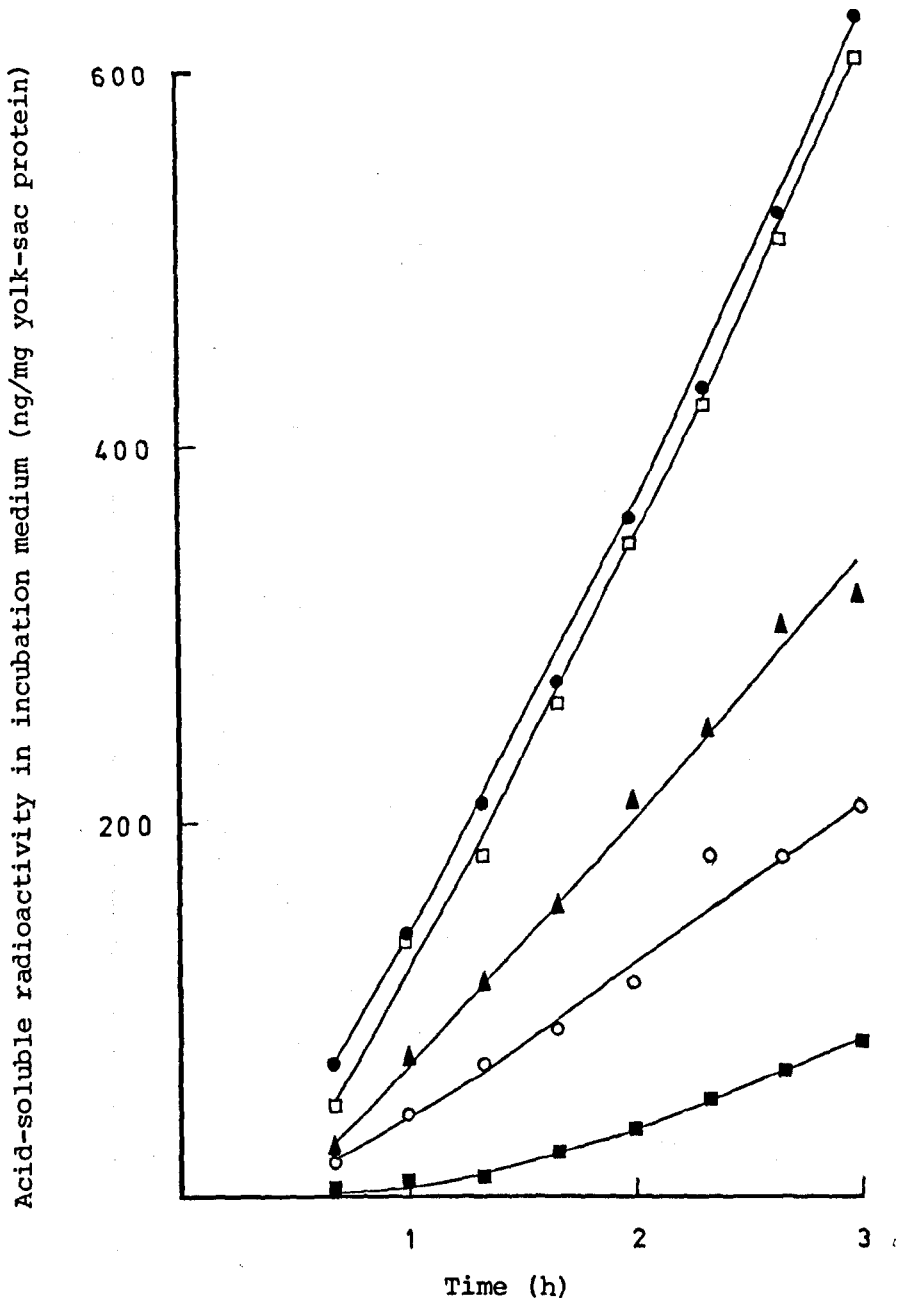


Figure 6.2 Effects of different concentrations of leupeptin on the rate of appearance of acid-soluble radioactivity in serum-free incubation medium when yolk sacs were incubated in the presence of formaldehyde--denatured ^{125}I-labelled bovine serum albumin.

Each curve was obtained on incubating a single yolk sac in serum-free medium 199 containing ^{125}I-labelled albumin (Batch I, 1 µg/ml) (for method see Section 6.2). Yolk sacs were incubated in serum-free medium 199 containing leupeptin for 30 min before addition of the ^{125}I-labelled albumin. Each curve was selected, from a group of curves, to show a typical rate of digestion of the ^{125}I-labelled albumin. The leupeptin concentrations (µg/ml) were: (●), 0; (□), 1; (▲), 20; (○). 40; (■), 100.

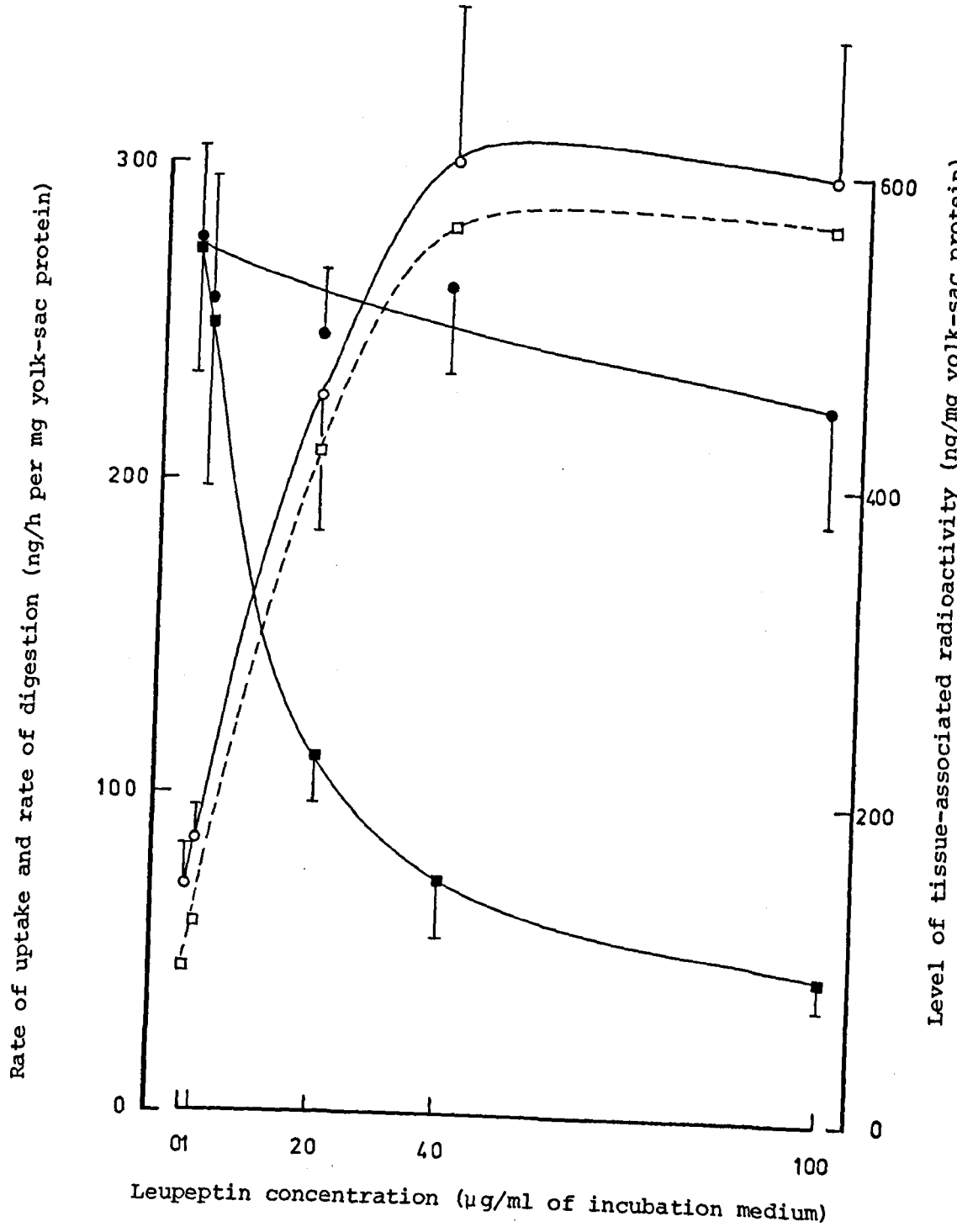


Figure 6.3 Effects of different concentrations of leupeptin on the rates of uptake and digestion of formaldehyde-denatured ^{125}I-labelled bovine serum albumin by 17.5-day rat yolk sacs incubated in serum-free medium 199.

The rates of uptake and digestion of formaldehyde-denatured ^{125}I-labelled bovine serum albumin were determined as described in Section 6.2. Yolk sacs were incubated for 30 min in medium containing leupeptin, at the concentrations indicated, before uptake was started by the addition of substrate (Batch I, 1 µg/ml of incubation medium). Each value is the mean (± S.D.) from 4 separate determinations each using a single yolk sac from a different animal.

Reading from left-hand scale: rate of uptake, (●) and rate of digestion (■). Reading from right-hand scale: total tissue-associated radioactivity, (○) and tissue-associated acid-insoluble radioactivity, (□)

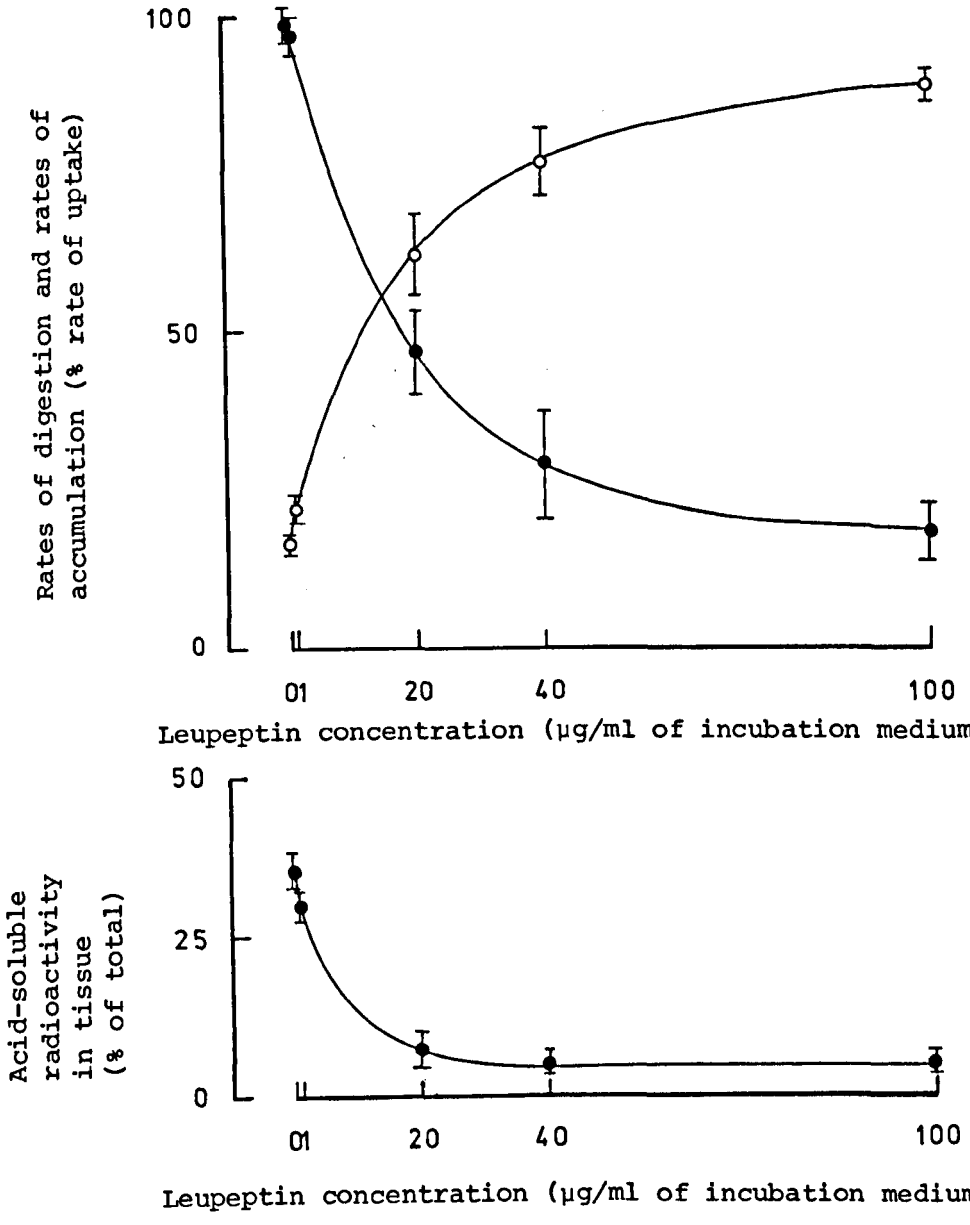


Figure 6.4 Effects of different concentrations of leupeptin on the rates of digestion and of accumulation of pinocytosed formaldehyde-denatured ^{125}I-labelled bovine serum albumin by 17.5-day rat yolk sacs incubated in serum-free medium 199.

Upper These data (mean ± S.D.) are taken from Fig. 6.2 but differ in that the rates of digestion and rates of tissue-accumulation have been normalized for experimental variations in the rate of uptake. Rate of digestion, (●); rate of accumulation, (o).

Lower Shows the proportion (mean ± S.D.) of the tissue-associated radioactivity that is acid-soluble.

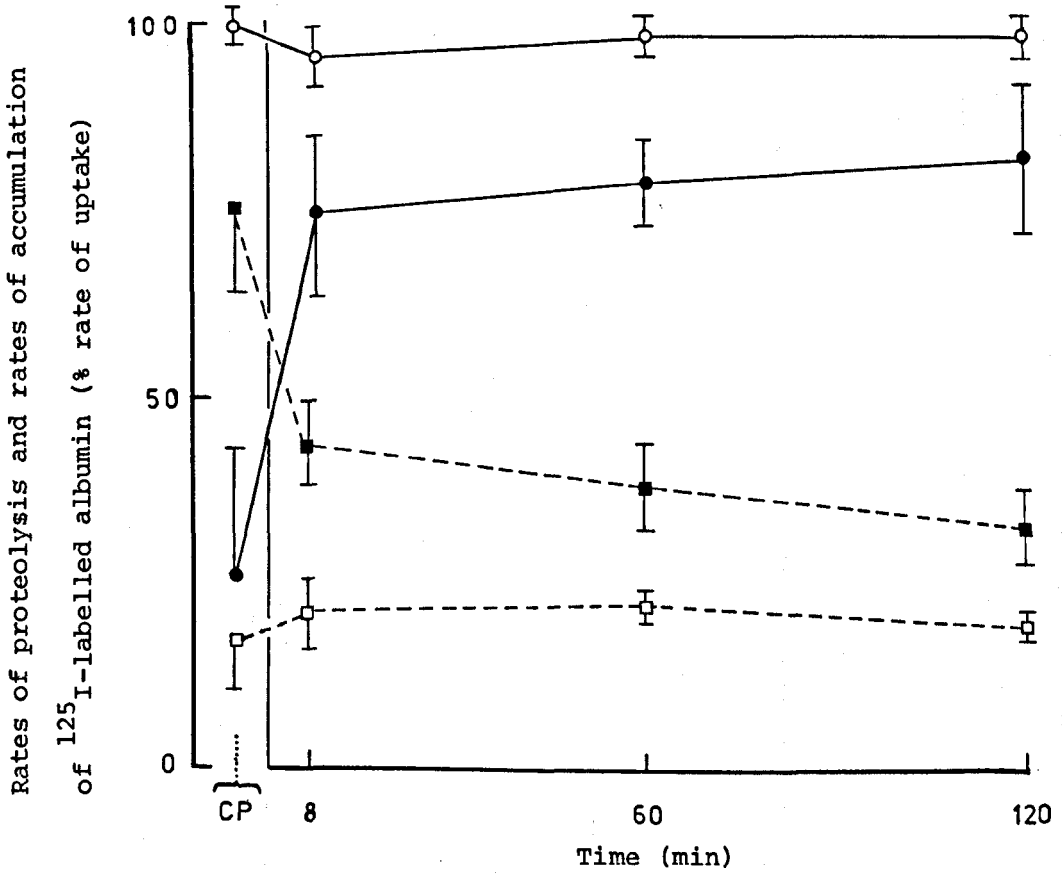


Figure 6.5 Recovery of proteolytic activity by 17.5-day rat yolk sacs following their exposure to leupeptin.

Following a 1.0 h exposure of yolk sacs to leupeptin (40 µg/ml of incubation medium), the tissues were washed as described in Section 5.2.4, then placed in fresh incubation medium containing no leupeptin. Uptake and proteolysis (determined as described in Section 6.2) of formaldehyde-denatured ^{125}I-labelled bovine serum albumin commenced on addition of substrate (Batch I, 1 µg/ml of medium) to the flasks containing the washed yolk sacs. Such additions were made at 8, 60 or 120 min after removing the yolk sacs from medium containing the leupeptin.

To determine the degree of inhibition of proteolysis caused by the continuous presence of leupeptin, media, that had been previously used to expose yolk sacs to leupeptin, were re-utilized. Freshly dissected yolk sacs were placed in such media and, after a 30 min period, the uptake and proteolysis of ^{125}I-labelled albumin monitored on adding this substrate.

Each value is the mean (± S.D.) of four separate determinations using yolk sacs taken from different animals. Data indicated by "CP" on the abscissia are those obtained from experiments in the continuous presence of leupeptin and of the corresponding controls.

Control yolk sacs that had not been exposed to leupeptin were incubated in parallel. Rate of digestion: (○) control; (●) leupeptin (40 µg/ml). Rate of tissue accumulation: (□) control; (■) leupeptin (40 µg/ml).

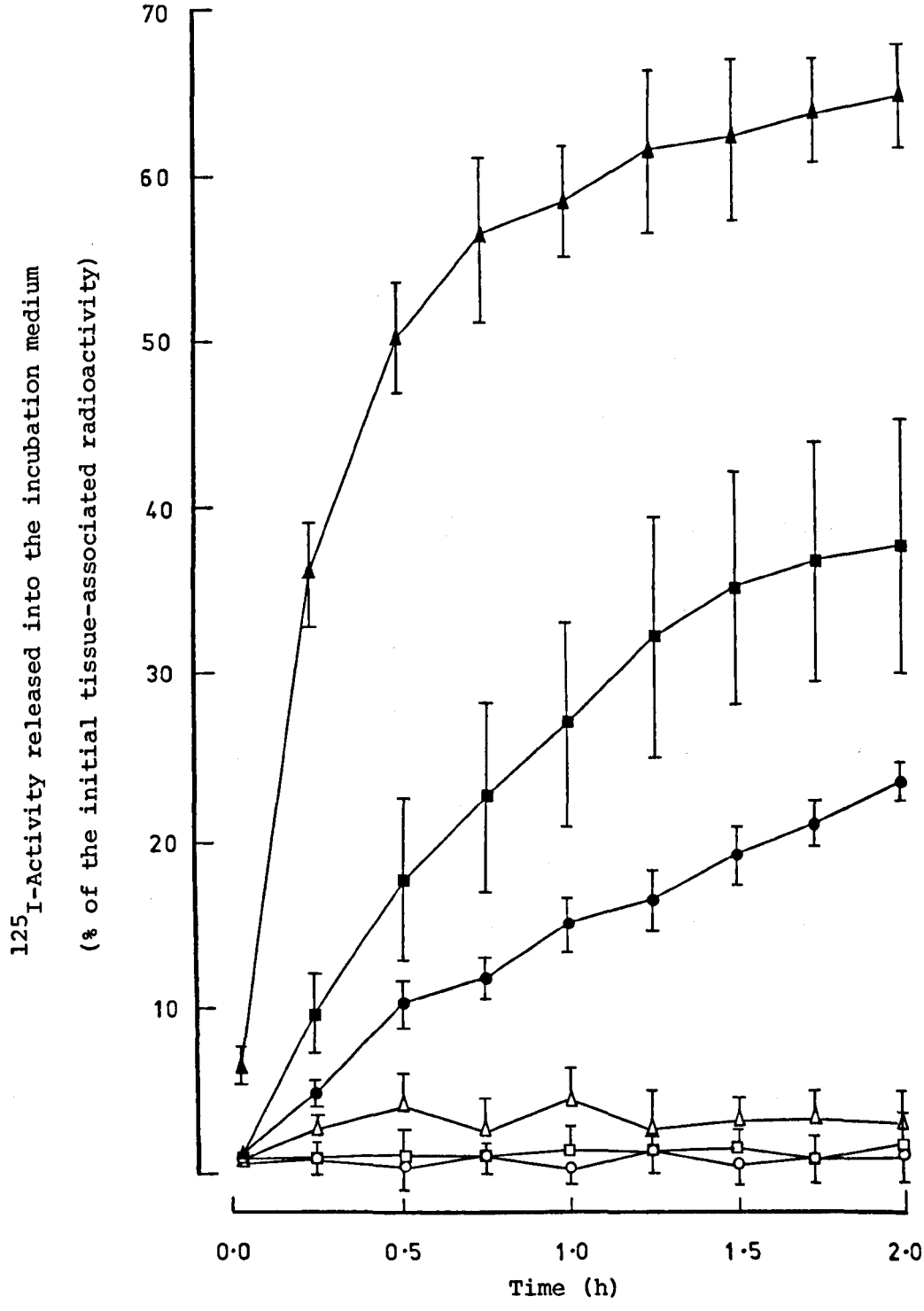


Figure 6.6 Effects of chymostatin and leupeptin on the release of formaldehyde-denatured ^{125}I-labelled bovine serum albumin from 17.5-day rat yolk sacs.

Three yolk sacs, each from a different animal, were loaded with formaldehyde--denatured ^{125}I-labelled bovine serum albumin (Batch II, 5 µg/ml) in the presence or absence (control) of either chymostatin (40 µg/ml) or leupeptin (40 µg/ml). After washing, each yolk sac was re-incubated separately in fresh serum-free medium 199 with or without (control) inhibitor but initially containing no formaldehyde-denatured ^{125}I-labelled bovine serum albumin. The quantities of released acid-soluble- and acid-insoluble radioactivity were monitored as described in Section 2.2.4(1) and expressed as a percentage of that radioactivity associated with the washed yolk sac when transferred to the fresh incubation medium. Values shown are the mean (± S.D.) of three determinations. Acid-soluble radioactivities: control, (▲); chymostatin, (■); leupeptin, (●). Acid-insoluble radioactivities: control, (Δ); chymostatin, (□); leupeptin, (○).

6.4 DISCUSSION

An inhibitor of proteolytic enzymes will only inhibit protein breakdown at an intracellular site within an intact cell if the inhibitor can penetrate the cell to the site of proteolysis and the inhibited proteinase is essential to the digestion process (Ballard, 1977). Because the microbial peptide proteinase inhibitors used here (see Fig. 6.1) are too large to penetrate cell membranes, they are not expected to inhibit intracellular proteinases except those confined to the vacuolar system (see Section 4.1), namely, the lysosomal cathepsins; their only envisaged route of access to the lysosomes is by endocytosis. The endothelial cells of the rat yolk sac are actively engaged in pinocytosis (see Chapter 1) and might therefore concentrate larger quantities of these inhibitors than some of the other cell types used to study endogenous protein breakdown (see Goldberg & St. John, 1976). This makes the study of the inhibition of the breakdown of endogenous proteins by yolk-sac cells more amenable to investigation by the use of microbial peptide proteinase inhibitors. [Such a study (Knowles & Ballard, unpublished work) has been conducted in parallel with the current investigation.]

None of the six microbial proteinase inhibitors had measurable effects on the rate of uptake of formaldehyde-denatured ^{125}I-labelled bovine serum albumin by 17.5-day rat yolk sacs. This observation is in marked contrast to the observed inhibitions of pinocytosis by the weak bases used in Chapter 5; hence the microbial compounds should prove more useful as specific inhibitors of lysosomal proteolysis than the weak bases. Moreover, an inhibition of intralysosomal proteolysis (see Table 6.1) does not result in any feedback-inhibition of pinocytosis of the type discussed in Section 5.4. Nagai *et al.* (1978) showed chymostatin to be an inhibitor of phagocytosis by guinea pig macrophages, thus demonstrating the participation of

a chymotrypsin-like enzyme in the phagocytic uptake process. This observation corroborates the observations of Perlman et al. (1969) and Mussen & Becker (1977) that serine proteinase inhibitors are inhibitors of phagocytosis in both human and rabbit neutrophils. As none of the six microbial compounds modified the rate of pinocytosis of the ^{125}I-labelled albumins, no proteinases either to which the microbial compounds might gain access or of the type described by Nagai et al. (1975) are necessary for continued micropinocytosis in 17.5-day rat yolk sacs.

Although much circumstantial evidence is available that suggests antipain, leupeptin and chymostatin inhibit intralysosomal proteolysis when added to cells in culture (see Knowles & Ballard, 1976; Hopgood et al., 1977; Neff et al., 1977; Libby & Goldberg, 1977; Riemann & Hanson, 1978) the observations made here (Table 6.1), to my knowledge, give the first direct evidence. No inhibition of intralysosomal proteolysis was observed when yolk sacs were incubated in the presence of pepstatin. A lack of inhibition by pepstatin in the yolk-sac system was also observed by Moore (1975) who used both formaldehyde-denatured ^{125}I-labelled bovine serum albumin and ^{125}I-labelled fibrinogen as substrates. In marked contrast, pepstatin was shown here (Table 6.2) to inhibit the digestion of formaldehyde--denatured ^{125}I-labelled bovine serum albumin by a cell-free extract of rat yolk sacs. The reason for this discrepancy is not known. A possible explanation is that cathepsins D and E, which are specifically inhibited by pepstatin (Barrett & Dingle, 1972; Woessner, 1972), might not catalyse the rate-limiting step in the intralysosomal proteolysis of the ^{125}I-labelled albumin. Indeed, cathepsin D might not necessarily contribute to the hydrolysis of ^{125}I-labelled albumin in intact lysosomes. An alternative and equally feasible explanation is that yolk-sac epithelial cells are unable to transport pepstatin into their lysosomes. Concordant with this

suggestion is a report in Turk & Marks (1977), [see Goldberg & St. John, 1976] that pepstatin does not readily enter cells. Also, Dean (1975b) reported that pepstatin does not seem to permeate macrophage plasma membranes (work of A.J. Barrett & C.G. Knight). Evidence that peptstatin is capable of inhibiting intralysosomal proteolysis was provided by Dean (1975b) who found that liposomally entrapped pepstatin was endocytosed by rat liver and caused up to 50% inhibition of the breakdown of endogenous proteins.

The lack of inhibition of formaldehyde-denatured ^{125}I-labelled bovine serum albumin digestion by bestatin and by elastatinal, in the yolk--sac system, correlates with their poor inhibition of the digestion of the same substrate in the cell-free system. So, even if these inhibitors were endocytosed, no marked inhibition of intralysosomal digestion would be expected.

Leupeptin was discovered to be the most potent inhibitor of lysosomal proteolytic activity in both the intact yolk-sac tissue (up to 80% inhibition, see Table 6.1) and the cell-free system (up to 95% inhibition, see Table 6.2). Leupeptin is a powerful inhibitor of cathepsin B (Huisman et al., 1974) and cathepsin L (Kirsche et al., 1977). These are the only lysosomal cathepsins known to be inhibited by leupeptin. Moreover, both enzymes (especially cathepsin L) readily hydrolyse protein substrates. Hence, either one, or both together are expected to play a major role in the digestion of formaldehyde-denatured ^{125}I-labelled bovine serum albumin within yolk-sac lysosomes.

Antipain and chymostatin (Ikezawa et al., 1972 and 1971, respectively) are also inhibitors of cathepsin B but are less potent inhibitors of this enzyme than is leupeptin (Aoyagi & Umezawa, 1975). Chymostatin is also an inhibitor of cathepsin L (Riemann & Hanson, 1978).

In the current study, both antipain and chymostatin were found to be less active than leupeptin as inhibitors of the ^{125}I-labelled albumin digestion in both the yolk-sac system and the cell-free system. [It is important to note that the comparison of the inhibitory activities of these compounds is made here on an equal weight basis, and that Aoyagi & Umezawa (1975) also compared their activities on the same basis.] It seems probable, therefore, that the inhibitions of digestion observed with antipain and chymostatin are a consequence of the inhibition of cathepsin B (or cathepsin L or both). Antipain is also an inhibitor of cathepsin A (Suda et al., 1972; Ikezawa et al., 1972) and chymostatin is a weak inhibitor of both cathepsins A and D (Aoyagi & Umezawa, 1975). An inhibition of the digestion of the ^{125}I-labelled albumin as a result of an interference with cathepsins A and D cannot therefore be ruled out.

Tappel (1969) postulated that during the degradation of denatured proteins by lysosomal extracts, the first bonds to be split were those hydrolysed by cathepsin D, while the large peptide fragments formed were broken down by other proteases and peptidases. Coffey & de Duve (1968) also suggested an important role for cathepsin D. However, Huisman et al., (1973, 1974) demonstrated that thiol enzymes were important in the digestion of several protein substrates and concluded (1974) that, for serum albumin, the first peptide bonds to be broken are those cleaved by either cathepsin B or another leupeptin-sensitive thiol enzyme. Riemann & Hanson (1978) indicate that such thiol-dependent proteinases include cathepsin L. The results reported here, especially those obtained using the cell-free system (see Table 6.2), also indicate this may be true for the digestion of formaldehyde-denatured ^{125}I-labelled albumin by the yolk-sac lysosomal cathepsins.

It is an implicit assumption that, to exert an inhibitory action

on intralysosomal proteolysis, antipain, chymostatin and leupeptin, are accumulated within the yolk-sac lysosomes. Evidence that the inhibitions of intracellular proteolysis of the ^{125}I-labelled albumin is the result of a direct inhibition of lysosomal cathepsins, rather than an inhibition of lysosome-pinosome fusion, was gained by showing that, except for pepstatin, the pattern of inhibitions by the microbial compounds could be reproduced in the cell-free digestive system. If the inhibitors continued to accumulate in an active form throughout the 3h incubation period employed in these studies, it might be expected that the rate of digestion of the formaldehyde-denatured ^{125}I-labelled bovine serum albumin would decrease with increasing time of incubation. There is, however, no evidence of this; the rate of digestion of the ^{125}I-labelled albumin was constant (after an initial lag--period) throughout the 3h incubation in the absence and presence of all the inhibitors at each of the inhibitor concentrations used. It seems likely, therefore, that the intralysosomal concentrations of these three microbial-peptide proteinase inhibitors must reach a steady-state level so that the yolk-sac lysosomes continue to digest incoming ^{125}I-labelled albumin at a constant but lowered rate. This would occur either if the microbial peptide proteinase inhibitors were inactivated within the lysosomes or if newly-formed pinosomes fuse only with primary lysosomes. The latter explanation would imply an extremely rapid turnover of lysosomes and does not, therefore, seem feasible since Wang & Touster (1975) estimated the average half-life of lysosomal constituents to be four days. The rapid recovery of proteolytic activity following the exposure of yolk sacs to leupeptin (Fig. 6.5) is consistent with the contention that the microbial proteinases are inactivated within the lysosomes. Neff et al. (1977) also observed a recovery of proteolytic activity in hepatocytes 30min after exposure to leupeptin.

Knowles & Ballard (unpublished work) showed leupeptin, chymostatin and antipain (at concentrations of 50µg/ml of medium) to be inhibitors of endogenous yolk-sac protein breakdown; inhibitions of 15-20%, 15-20%, and 15% of total yolk-sac protein breakdown were shown, respectively. They also showed pepstatin, bestatin and elastatinal (and phosphoramidone) to have no measurable inhibiting effects on endogenous yolk-sac protein breakdown. The inhibitions demonstrate a lysosomal site of digestion of endogenous yolk-sac proteins (since these inhibitors will be restricted to the lysosomal compartment). However, these inhibitions are not as marked as those for an exogenous protein (^{125}I-labelled albumin). A possible interpretation of these findings is that endogenous yolk-sac proteins are also digested at an extra-lysosomal site. But such a conclusion would be based on the supposition that the exogenous protein (^{125}I-labelled albumin) has a susceptibility to hydrolysis by lysosomal enzymes that is representative of the bulk of endogenous proteins, so that the microbial compounds will cause a similar degree of intralysosomal inhibition. This could only be expected to arise from a very fortuitous choice of exogenous proteins.

When the six microbial inhibitors are ranked according to their inhibitory powers against both endogenous protein digestion and exogenous protein digestion the ranking orders are seen to be the same. This indicates that for those endogenous yolk-sac proteins that are digested intralysosomally, the rate-limiting step of their intralysosomal catabolism is catalysed by similar enzymes to those that catalyse the rate-limiting step of the intralysosomal digestion of formaldehyde-denatured ^{125}I-labelled bovine serum albumin.

CHAPTER SEVEN

PINOCYTOSIS OF ^{125}I-LABELLED PROTEINS AND PEPTIDES AND THEIR SITES OF DIGESTION BY RAT YOLK SACS IN VITRO

7.1 INTRODUCTION

The ability of several types of mammalian cells in culture to endocytose and digest various exogenous proteins is well known (see Chapters 1, 2 & 4) but little is known about the fate of polypeptides in such systems. Ryser (1970) suggested that polypeptides with a molecular weight less than 1000 are probably not endocytosed and that large polypeptides and proteins are endocytosed more rapidly than small ones. The observations made in Chapter 2, however, are not concordant with Ryser's suggestion; ^{125}I-labelled preparations of lysozyme, ribonuclease and insulin were shown to be pinocytosed more rapidly by the rat yolk sac *in vitro* than were ^{125}I-labelled preparations of the larger proteins orosomucoid and bovine serum albumin (results of Moore *et al*, 1977). [These observations are more readily explained in terms of the surface charge and hydrophobicity of the protein preparations, see Section 3.4.] There may, however, be a "cut-off" point in the size of peptide molecule below which it can carry no determinant for selective endocytic uptake. It was therefore of interest to determine whether ^{125}I-labelled polypeptides smaller than ^{125}I-labelled insulin (molecular weight 5 700, the smallest polypeptide investigated in the yolk-sac system, see Chapter 2) are endocytosed by the rat yolk sac and, if so, at what rates they are ingested. The possible uptake of ^{125}I-labelled preparations of calcitonin (molecular weight 4 500), insulin B-chain (3 500) and glucagon (3 650) were therefore investigated.

The above substrates were chosen for investigation since they are precipitatable with trichloroacetic acid, but, of more physiological importance, they also enabled a concomitant study to be made of any extracellular digestion by peptidases associated with the cell surface of the 17.5-day rat yolk sac. Extracellular digestion of the peptides:

insulin, calcitonin, insulin B-chain and glucagon has been suggested to occur in tissues other than the rat yolk sac (for review see Kenny, 1977; see also Section 4.1). For insulin, however, lysosomes are suggested as a possible site of digestion (Bohley et al., 1971; Terris & Steiner, 1975; Grisolia & Wallace, 1976; see also Chapter 2).

Direct evidence that formaldehyde-denatured ^{125}I-labelled bovine serum albumin is digested exclusively intracellularly is given in Chapter 4. Firm circumstantial evidence for an intralysosomal site of digestion is given in Chapter 2 for the ^{125}I-labelled substrates: lysozyme, ribonuclease and insulin, and by Ibbotson (1978) for rat immunoglobulin G. These digestible substrates were therefore re-examined here in order to compare results with those for the ^{125}I-labelled peptides.

The susceptibility of proteins to digestion by proteinases is of current major interest since this might regulate the rate at which cell proteins turnover in vivo (Goldberg & Dice, 1976; Ballard, 1977). Bohley et al. (1971) discovered that, in general, polypeptide substrates were more rapidly degraded by enzymes contained in lysosomal extracts than were larger protein substrates. Also, Marco et al. (1978) observed a similar increasing resistance to proteinases with increasing size of the protein substrate. These observations are contrary to those of Dice et al. (1973) and Dice & Goldberg (1975); both publications report the converse relationship.

If the peptide substrates investigated here are endocytosed by the rat yolk sac and their rates of uptake can be accurately determined, it becomes possible to discover whether the observations made by Bohley and Marco and their co-workers also hold for digestion within intact yolk-sac lysosomes in situ. The susceptibility to proteolysis of the proteins

and peptides was judged by their Catabolic Indices (see Section 3.3.2).

The principle used here to determine the contribution of intracellular digestion to the overall digestion observed, when ^{125}I-labelled proteins (rat immunoglobulin G, formaldehyde-denatured bovine serum albumin, lysozyme and ribonuclease) and ^{125}I-labelled peptides (insulin, calcitonin, insulin B-chain and glucagon) were added to the 17.5-day rat yolk sac in culture, is the same as that described in Section 4.1. Here inhibition of pinocytosis was achieved using decreased incubation temperature, the metabolic inhibitor rotenone and the weak base ammonium chloride.

The effect of ammonium chloride, also an inhibitor of lysosomal proteolysis (see Chapter 5), on the intracellular digestion of each protein and peptide substrate is of particular interest. Complete inhibition of the lysosomal digestion of formaldehyde-denatured ^{125}I-labelled bovine serum albumin by yolk sacs can be achieved by including a 20mM concentration of ammonium chloride in the incubation medium (see Chapter 5). But it is not known whether this effect is the result of a complete inhibition of all lysosomal proteolytic activity or whether the result with formaldehyde--denatured ^{125}I-labelled bovine serum albumin is atypical. Weak bases are regarded as specific inhibitors of lysosome function (see Section 5.1). It is generally assumed that the digestion of all proteins within lysosomes will be inhibited by weak bases but this remains unproven.

Finally, the early events in the digestion of the ^{125}I-labelled proteins and peptides have been examined here. It was shown in Chapter 4 (see Fig. 4.1) that degradation products of formaldehyde-denatured ^{125}I-labelled bovine serum albumin, added to 17.5-day rat yolk sacs in culture, appeared in the incubation medium only after an initial lag--period. Since the lag-period with ^{125}I-labelled albumin was observed to

increase with decreased incubation temperature, the possibility that similar observations would be made with other substrates was investigated.

7.2 METHODS

7.2.1 Preparation of ^{125}I-labelled protein and peptide substrates.

The ^{125}I-labelled proteins formaldehyde-denatured bovine serum albumin, bovine pancreatic ribonuclease A (type XIA), hen egg-white lysozyme and bovine insulin were prepared as before [see Section 2.2.1(3)]. Rat immunoglobulin G (Pentex Fraction II; Miles Laboratories, Stoke Poges, Bucks.) bovine calcitonin (Calbiochem, San Diego, California) bovine/porcine glucagon (Sigma, London) and carboxymethylated insulin B-chain (a kind gift from Dr. A.J. Kenny, Department of Biochemistry, University of Leeds) were each iodinated with [^{125}I]iodide as described in Section 2.2.3(1). When glucagon (10mg) was added to the phosphate buffer (pH 8.0, 4.25ml) it did not completely dissolve but remained in suspension in which form it was iodinated. (Since the ^{125}I-labelled glucagon remained insoluble it could be dialysed [Section 2.2.3(1)] to remove [^{125}I]iodide). After dialysis, the ^{125}I-labelled glucagon preparation was titrated to pH 3.5 with dilute acetic acid to solubilize it. Insulin B-chain was soluble in aqueous solution but separation of the ^{125}I-labelled insulin B-chain from free [^{125}I]iodide and other salts was achieved on a Sephadex G-25 column (30cm x 1.7cm), equilibrated and eluted with distilled water by the method described in Section 2.2.4(3). Virtually all of the radioactivity applied to the column was eluted; 95% being associated with the insulin B-chain. The iodination efficiency for this peptide was therefore 95%. Determination, by the acid-precipitation method [see Section 2.2.3(1)] of the labelling efficiencies of the immunoglobulin G, calcitonin and glucagon gave values of 60, 35 and 75% respectively. The specific activities of the ^{125}I-labelled substrates at the time of preparation were 60, 35, 75 and 95μCi/mg for immunoglobulin G, calcitonin, glucagon and

insulin B-chain; the corresponding numbers of protein or peptide molecules containing one atom of [^{125}I]iodine were 700 - 1 200; 8 000 - 13 000; 3 500 - 6 000 and 2 500 - 4 000, respectively.

7.2.2 Handling of the yolk-sac tissues in experiments with ^{125}I-labelled peptides.

Yolk sacs, dissected from 17.5-day pregnant rats by the method described in Section 2.2.1, and placed in serum-free medium 199 were found to digest ^{125}I-labelled insulin B-chain at a very rapid rate (approximately 20%/h for a single yolk sac in 20ml of medium 199 containing 20µg of ^{125}I-labelled insulin B-chain). Only half of the digestive activity was eliminated on removing the yolk sac from the incubation medium, indicating that degradative enzymes were released into the incubation medium from the incubated tissue.

A more thorough washing of the freshly dissected yolk sacs, with medium 199, decreased the quantity of degradative enzymes released. Deliberately cutting such yolk sacs with scissors or gripping them between a pair of forceps resulted in the release of large quantities of the degradative enzymes. Also, a single well-washed amnion (previously dissected free of the yolk-sac tissue) digested ^{125}I-labelled insulin B-chain (20µg) at a rate of 1.15%/h when both were incubated together in 20ml of medium 199. Approximately one third of the digestive activity remained associated with the incubation medium on removal of the amnion after 3h of incubation.

Because of the extreme ease of release of degradative enzymes from the yolk-sac tissue, special care was required when handling it to reduce such release to a minimum. Additional procedures were therefore adopted when preparing yolk sacs for experiments. Care was taken to ensure that all the amnion was dissected away from the yolk-sac tissue (by increasing the

contrast between the tissues by dissecting over a black background) also yolk-sac tissue was handled as little as possible. As an additional precaution before their use in digestion studies with peptides, yolk sacs completely free of amniotic tissue were washed (3 yolk sacs per 20ml of medium 199) for 10min under the usual incubation conditions; the washing was repeated twice more before introducing the tissue into the experimental incubation medium. Care was required when transferring the yolk sacs from one medium to another. To transfer the yolk sacs, the wash medium and yolk sacs were decanted from the incubation flask into a clean Petri dish. Each yolk sac was scooped out of the wash medium, on the top of a clean pair of curved forceps, and the yolk sac allowed to float off the tip of the forceps after submerging both in fresh medium.

7.2.3 Assay of the uptake and digestion of ^{125}I-labelled proteins and peptides by 17.5-day rat yolk sacs incubated in serum-free medium 199.

The rates of uptake and digestion of ^{125}I-labelled proteins and peptides by 17.5-day rat yolk sacs were each assayed by the rapid method described in Section 3.2.1. When the acid-soluble radioactivity released into the incubation medium was monitored over a 0-50min period, either 1 or 3 yolk sacs were incubated in 20.0 or 30.0ml of serum-free medium respectively. For the ^{125}I-labelled substrates immunoglobulin G, formaldehyde-denatured bovine serum albumin, ribonuclease, lysozyme, glucagon and insulin, three yolk sacs were incubated in 30.0ml of serum--free medium. With ^{125}I-labelled calcitonin and ^{125}I-labelled insulin B-chain, yolk sacs were incubated singly in 20.0ml of serum-free medium. [The reason for the two different methods employed is historical; initially it was considered that to obtain measurable quantities of radioactive digestion products during the first few minutes of incubation,

the larger quantity of yolk-sac tissue would be required, but this was not generally the case.] All the yolk sacs were incubated at an appropriate temperature for 15min before addition of the ^{125}I-labelled substrate at an intial concentration of 5µg/ml of medium. Medium samples (1.0ml) were taken at frequent periods and immediately mixed with 0.1ml of calf serum quickly followed by the addition of 20% (w/v) trichloroacetic acid before the acid-soluble radioactivity was assayed as described in Section 2.2.1(3).

When an incubation period of 3h duration was employed, the yolk sacs were incubated singly in 20.0ml of serum-free medium 199. This procedure was adopted for all the ^{125}I-labelled protein and peptide substrates used. Substrates, initially present at 1µg/ml of incubation medium, were always added to the incubation medium at least 30min after the introduction of the yolk sac. Duplicate samples (0.5ml) of medium were taken at frequent time intervals. Each sample was immediately mixed with 0.5ml of 20% (v/v) aq. calf serum, quickly followed by the addition of 20% (w/v) trichloroacetic acid (0.5ml) to only the second of the duplicate samples. The second sample was used to assay acid-soluble radioactivity, the first to assay the total radioactivity. [The immediate addition of trichloroacetic acid to the second sample was essential to prevent the continued digestion of the ^{125}I-labelled peptides, subsequent to taking the sample out of the incubation medium, but was not necessary for the sample used to determine the total radioactivity present at each time interval.] When the quantity of acid-soluble radioactivity in medium containing either ^{125}I-labelled ribonuclease or ^{125}I-labelled lysozyme was measured, 0.5ml of phosphotungstic acid [prepared as described in Section 2.2.1(3)] was added to the sample prior to the addition of the trichloroacetic acid.

When the ^{125}I-labelled substrates, insulin, calcitonin, insulin B-chain and glucagon were being investigated the incubation medium was incubated for a further 3h after removal of the yolk sac. When either ammonium chloride or rotenone was added to the incubation media, the addition was made 30min prior to the addition of the substrate (see Section 4.2.1 & 5.2.1).

7.2.4 Calculation and expression of the pinocytic uptake and digestion data.

The quantities of the ^{125}I-labelled proteins (immunoglobulin G, formaldehyde-denatured bovine serum albumin, ribonuclease and lysozyme) ingested and digested were each calculated as described in Section 3.2.1(2). For the ^{125}I-labelled peptide substrates that became digested by degradative enzymes released into the incubation medium, additional calculations were required to obtain data on that fraction of the total digestion that arose from enzymes associated with the yolk-sac tissue.

Fig. 7.1 shows the time-course of digestion of ^{125}I-labelled insulin B-chain by yolk sacs incubated singly in either the presence or the absence of rotenone (an inhibitor of pinocytosis). In both cases the rate of digestion decreases after removal of the yolk-sac tissue from the incubation medium. For the purpose of calculating the rate of tissue--associated digestion it was assumed that the rate of extracellular digestion over the period 3-6h (after removal of the yolk sac) was identical to that in the presence of the yolk sac over the period 0-3h. This assumes that the release of the enzymes from the yolk sac is complete before the ^{125}I-labelled substrate is added to the incubation medium. The assumption was not made without experimental support; the quantities of enzyme present in incubation medium in which washed yolk sacs had been

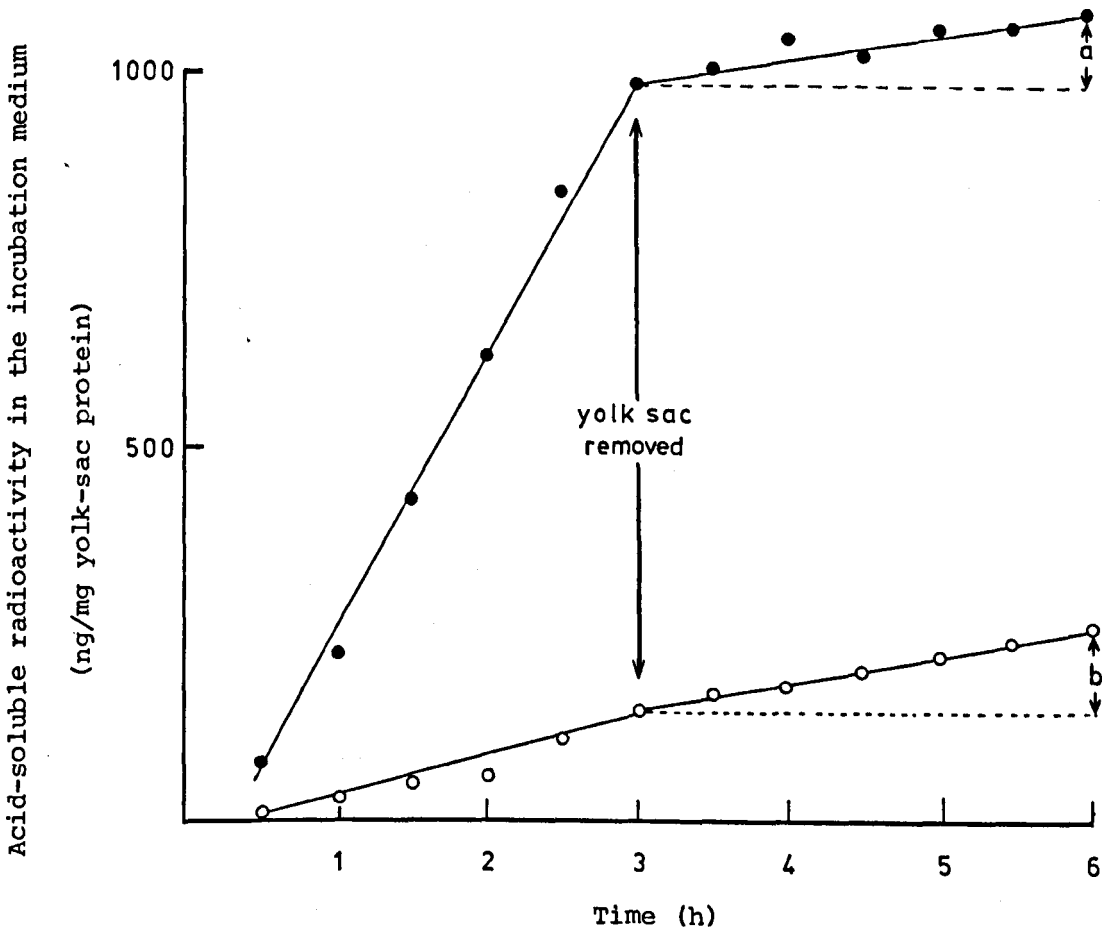


Figure 7.1 Digestion of ^{125}I-labelled insulin B-chain by 17.5-day rat yolk sacs incubated in serum-free medium 199.

The data show two typical experiments in which a single yolk sac was incubated in 20 ml of serum-free medium 199 containing ^{125}I-labelled insulin B-chain (1 µg/ml of medium) in the presence (o) or absence (●) 10^{-5}M-rotenone, an inhibitor of pinocytosis. For experimental details see Section 7.2.3. The rate of yolk-sac associated digestion is calculated by subtracting the rate of digestion at 3-6h from the rate of digestion at 0-3h and assumes that the rate of digestion in the medium itself is constant over the period 0-6h. The quantity of acid-soluble radioactivity generated by yolk-sac associated digestion during the 0-3h period of incubation is calculated by subtracting the quantity of acid-soluble radioactivity generated over 3-6h (a or b) from the corresponding quantity of acid-soluble radioactivity present at 3h.

previously incubated for either 0.5, 1.0, 1.5 or 2.0h were found to be the same when assayed using ^{125}I-labelled insulin B-chain as substrate. Furthermore, in the absence of yolk sacs the rate of degradation of ^{125}I-labelled insulin B-chain over a 6h period, by the degradative enzymes released from yolk sac, was constant. These observations indicate that the above assumption is valid.

The data as presented in the form shown in Fig. 7.1 were derived using equations 3.1 & 3.6 in Section 3.2.1(2). [For the purpose of calculating the amount of digestion that occurs after removal of the yolk-sac tissue (3-6h), P in equation 3.1 was made equal to the quantity of yolk sac protein.] The quantity of acid-soluble radioactivity produced by the tissue-associated degradation process throughout the 0-3h incubation period (T_t) is calculated according to the equation:

$$T_t = T_3 - (T_6 - T_3) = 2T_3 - T_6 \qquad (7.1)$$

where T_t is as defined above [see also Section 3.2.1(2)], T_3 is the total amount of acid-soluble radioactivity generated in the 0-3h period of incubation by both intracellular and extracellular digestive processes and T_6 is the total amount of acid-soluble radioactivity generated in the period 0-6h by both the tissue-associated degradation process and the degradative enzymes present in the culture medium. [$T_6 - T_3$ is the same as the quantity indicated by 'a' or 'b' in Fig. 7.1.] T_3 and T_6 are calculated according to equations 3.2 in Section 3.2.1(2):

[$T_n = V_i \cdot C_{i(i=n)} + \Sigma_{i=o}^{i=(n-i)} C_i$] where T_n is equal to T_3 or T_6 when n is the serial number of the sample taken at 3.0h or 6.0h respectively. The rate of pinocytic uptake of a ^{125}I-labelled peptide was calculated (assuming that the tissue-associated digestion process is intracellular) according to

equations 3.1 & 3.6 in Section 3.2.1(2) using values of T_t claculated as described above.

The rate of digestion of a ^{125}I-labelled peptide by the tissue--associated digestion process is calculated according to the equation:

$$R_1 = R_2 - R_3 \qquad (7.2)$$

where R_1 is the rate of digestion of the ^{125}I-labelled peptide by the tissue-associated digestion process, R_2 is the rate of production of acid-soluble radioactivity in the presence of the yolk sac (0-3h), and R_3 is the rate of production of acid-soluble radioactivity in the absence of the yolk sac (3-6h).

All uptake and digestion data are expressed as the quantity (ng) of substrate ingested or digested per unit quantity of yolk-sac tissue (mg yolk-sac protein).

7.2.5 Preparation of rat-liver tritosomes.

Rat-liver tritosomes were isolated by the method of Trouet (1974). Two male Wistar rats (each weighing approx. 250g) were injected intra-peritonealy with 2.5ml of Triton WR-1339 solution [20% (v/v) in 0.9% (w/v) aq. NaCl]. Four days later, they were killed after overnight fasting, the livers quickly removed then washed in ice-cold 0.25M-sucrose. The washed livers were pressed through a wire gauze (1mm mesh-size) and the liver pulp homogenized, in ice-cold 0.25M-sucrose (5ml per g of liver), using a Potter-Elvehjem type Teflon-on-glass homogenizer (with an 0.19mm clearance) at a speed of 2 500r.p.m. Four up-and-down strokes were made within a period of 30s.

The homogenate was centrifuged at 2 200r.p.m. for 10min (6 500 g-min) in a refrigerated MSE High-Speed 18 centrifuge. After removing the supernatant, the sediment was again homogenized in 0.25M-sucrose (3ml per g of liver) and

the suspension centrifuged at 2 000r.p.m. for 10min (5 000g-min). The pooled supernatants were made up to a volume of 10ml per g of liver by addition of 0.25M-sucrose then centrifuged at 17 000r.p.m. for 10min (340 000g-min). The supernatant and the pink fluffy layer which covered the light brown pellet were removed, the pellet suspended in 0.25M-sucrose (5ml per g of liver) and again centrifuged at 17 000r.p.m. for 10min. The supernatant and fluffy layer were removed from above the pellet. The pellet was carefully suspended in 45% (w/v) sucrose (1ml per g of liver) by one up-and-down stroke in the homogeniser. A volume (5-7ml) of suspension was placed in a 25ml centrifuge tube and layered successively with 10ml of 34.5% (w/v) sucrose and 5ml of 14.3% (w/v) sucrose, by gently pipetting one solution on top of the previous one. The sucrose gradient was then centrifuged at 17 500r.p.m. for 2h (34 000 g-av.) in a refrigerated MSE Superspeed 50 centrifuge. The tritosomes, which congregated at the interface between the 34.5% and the 14.3% sucrose layers, were removed and kept at -20°C until required. The tritosome preparation and a sample of the liver homogenate were each assayed for acid phosphatase activity [method of Torriani (1960) and preincubating the fraction for 10min to inactivate microsomal phosphatases as described by Neil & Horner (1964)] and for protein content (method of Lowry et al., 1951). The relative specific activity of the acid phosphatase in the tritosome preparation was 33.5; this compares with a value of 32 ± 3.5 (n=22) reported by Trouet (1974).

7.2.6 Assay of the digestive activity of disrupted rat-liver tritosomes using various ^{125}I-labelled proteins and peptides as substrates.

The method used to assay the digestive activity of disrupted tritosomes was essentially the same for each substrate

investigated, but, when using a substrate for which the tritosomes have a low specific activity, it was necessary to increase both the quantity of tritosomes utilized and the duration of the incubation period in order to obtain measurable quantities of acid-soluble digestion products. The digestive activity was also measured at several pH values (4.0, 5.0 & 6.5) using 0.1M-sodium acetate and at pH 8.0 using 0.1M-Tris [2-amino-2-(hydroxymethyl) propane - 1,3-diol].

The incubation mixtures contained buffered 0.32% (v/v) Triton X-100 (350µl), tritosomes (5-200µl, containing 0.5µg protein per µl), sufficient distilled water to bring the volume to 550µl and (after a 10min preincubation at 37°C) ^{125}I-labelled protein or peptide substrate (10µg in 20µl of 1.0% (w/v) aq. NaCl). The incubations were performed in 3ml disposable tubes (LP3, Luckhams Ltd., Burgess Hill, Sussex). After incubation for an appropriate interval, the reaction was stopped by the addition of 20% (v/v) aq. calf serum (0.5ml) immediately followed by 20% (w/v) trichloroacetic acid (0.5ml). When ^{125}I-labelled lysozyme was used as substrate, phosphotungstic acid [0.5ml, prepared as described in Section 2.2.1(3)] was added prior to the trichloroacetic acid. Substrate blanks, that contained distilled water in place of the tritosomes, were incubated in parallel. To determine the initial acid-insoluble radioactivity, total radioactivity was assayed in un-incubated substrate blanks prior to the addition of aq. calf serum, precipitation and assay of acid-insoluble radioactivity.

Both total- and acid-soluble radioactivity were assayed using a well-type gamma counter. Empirical correction factors, required to correct the observed acid-soluble counts for counting geometry (see Appendix I), were determined for a sample volume of 0.570ml (the volume

of the reaction mixture) using ^{125}I-labelled-L-tyrosine as the acid--soluble radiotracer. The values were 1.67 and, when phosphotungstic acid was used to aid precipitation of the protein substrate, 2.17.

7.3 RESULTS

It is advantageous, for the sake of clarity, to discuss some of the results from this chapter at the time of their presentation. A more general discussion is given in Section 7.4.

7.3.1 Stability of ^{125}I-labelled proteins and peptides under various incubation conditions in serum-free medium 199.

Table 7.1 shows the rate of appearance of acid-soluble radioactivity derived from various ^{125}I-labelled proteins and peptides incubated under various conditions. The rates of appearance of the acid-soluble radioactivity are each expressed as the percentage of the initial acid-soluble radioactivity rendered acid-soluble per h of incubation. Values below 0.1%/h are considered to be negligible [0.1%/h corresponds to a value of 4ng/h per mg yolk-sac protein if the incubation medium (20ml) contained yolk-sac tissue equivalent to 5mg yolk-sac protein.]

Only ^{125}I-labelled insulin B-chain and ^{125}I-labelled glucagon showed significant rates of production of acid-soluble radioactivity when present in fresh serum-free medium 199 alone. This indicates that the majority of the ^{125}I-labelled substrates employed are stable under the usual incubation conditions. When yolk sacs were added to the incubation medium, significant quantities of each of the ^{125}I-labelled substrates were digested. Removal of the yolk sacs from the medium completely halted the digestion of the ^{125}I-labelled protein substrates (immunoglobulin G, formaldehyde-denatured bovine serum albumin, ribonuclease and lysozyme) showing that the digestive activity toward these substrates is entirely tissue-associated. However, the ^{125}I-labelled peptide substrates (insulin, calcitonin, insulin B-chain and glucagon) continued to be digested in the incubation medium in which yolk sacs had been previously

incubated. The quantities of ^{125}I-labelled peptide digested in such conditioned medium were always greater than the corresponding amount digested in (unconditioned) medium 199 alone. This suggests that incubating yolk sacs in medium results in the release of significant amounts of degradative enzymes. Nevertheless, the amount of ^{125}I-labelled peptide digested in the conditioned medium was always significantly less than that in the presence of a yolk sac. The ^{125}I-labelled peptide substrates therefore appear to be digested mainly by a tissue-associated process and, to a much lesser extent, by degradative enzymes present in the incubation medium. Homogenization of the yolk-sac tissue in medium 199 [1 yolk sac was homogenized in 5ml of medium (pH 7.1), using a Potter-Elvehjem type glass-on-glass homogenizer (0.19mm clearance), then centrifuged at 300g x 5min to remove intact cells] fully abolished their digestive activity toward the ^{125}I-labelled protein substrates. In marked contrast, the digestive activity of the yolk-sac tissue toward the ^{125}I-labelled peptide substrates was dramatically increased following its homogenization. The lack of digestion of the ^{125}I-labelled protein substrates by the yolk-sac homogenate shows that intact cells are required to maintain protein digestion and, unless they are rapidly inactivated by homogenization, the yolk sac contains no extracellular, cell-surface associated enzymes that could be responsible for the observed digestion in the presence of the intact yolk-sac tissue. The increased ability of the yolk-sac tissue to digest the ^{125}I-labelled peptide substrates on homogenization suggests that a barrier must separate the bulk of the degradative enzymes from the ^{125}I-labelled peptide substrates in the intact yolk-sac tissue. It is probable that the bulk of the degradative enzymes is located within some intracellular compartment and is not freely accessible to the incubation medium. (The alternative explanation,

that homogenization activates extracellular, cell-surface associated enzymes seems implausible.)

The ranking of the relative rates of digestion of the ^{125}I-labelled peptides by the yolk-sac homogenate is the same as the ranking of the relative rates of digestion in the conditioned medium. In each case the most rapidly digested ^{125}I-labelled substrate is glucagon, followed by insulin B-chain, calcitonin and lastly insulin. This provides strong circumstantial evidence to suggest that the digestive activity present in the conditioned medium is the result of the leakage or transport of intracellular enzymes into the medium. The proportion of the yolk--sac ^{125}I-labelled peptide-degradating activity that must be released into the incubation medium to account for the digestion of ^{125}I-labelled calcitonin, ^{125}I-labelled insulin B-chain and ^{125}I-labelled glucagon in the conditioned medium was calculated to be 0.16, 0.15 and 0.18% respectively for a typical yolk sac containing 5mg protein. (The value for ^{125}I-labelled insulin was 0.06%, but the rates of digestion in the conditioned and unconditioned medium were too small to make this determination accurate.)

The location within the intact yolk sac of the enzymes that digest the ^{125}I-labelled peptides is not clear. The digestive activity of an unfractionated, cell-free, yolk-sac homogenate measured at several pH values between 3.0 and 9.0 (essentially by the method described in Section 4.2.4) showed the highest activity against ^{125}I-labelled insulin B-chain at pH 3.5 to 5.0. The digestive activity of the homogenate at the acid pH values was largely inhibited by a mixture of pepstatin and leupeptin (each at 10µg/ml). The distribution of the ^{125}I-labelled peptide-degradating enzymes (measured at neutral pH with ^{125}I-labelled insulin B-chain as substrate) among subcellular fractions obtained by

centrifugation (for method see Section 4.2.2) suggested a lysosomal location, except that a large fraction (52%) of the digestive activity was associated with the cytosol fraction whereas the activities of the lysosomal markers: acid-proteinase (assayed as described in Section 4.2.3), β-N-acetyl-glucosaminidase (assayed as described in Section 5.3.6) and acid phosphatase (assayed as indicated in Section 7.2.5) in the cytosol fraction were 39.5, 10.5 and 24.8% of the total, respectively. Nevertheless, the digestive activity of the cytosol fraction at several pH values (from 3.0 to 9.0) against ^{125}I-labelled insulin B-chain, paralleled that in the lysosomal fraction; each peaked at pH 3.5-5.0, this suggested that the peptide-degrading enzymes found in the cytosol fraction were derived from disrupted lysosomes. Although this data suggests that the digestive activity associated with the medium after introduction of a yolk sac arises at least in part from a lysosomal location it does not exclude a possible contribution from enzymes normally located in the cytosol or other organelles.

7.3.2 Effects of ammonium chloride and rotenone on the uptake and digestion of ^{125}I-labelled proteins and peptides by 17.5-day rat yolk sacs incubated in serum-free medium 199.

It was shown above that each of the ^{125}I-labelled proteins and peptides was digested mainly, if not entirely by a tissue-associated mechanism. Should the site of digestion be lysosomal, inhibitors of the endocytic process should also inhibit the observed tissue-associated digestive activities, an effect that would not be expected if the underlying digestive process was associated with enzymes present on the extracellular cell-surfaces of the yolk-sac tissue. Ammonium ions and rotenone were each shown (Section 4.3 & 5.3) to be inhibitors of pinocytosis in 17.5-day rat yolk sacs.

The effects of 5mM and 20mM concentrations of ammonium ions and of 10^{-5}M-rotenone on the putative rate of uptake, the rate of digestion and on the quantity of substrate that becomes tissue-associated radioactivity (at 3h) for each of eight ^{125}I-labelled proteins and peptides are shown in Tables 7.2 a,b,c & d. Tables 7.2 c & d also show the rates of digestion of the ^{125}I-labelled peptides in the presence (0-3h) and absence (3-6h) of the yolk-sac tissue. A linear increase with time in the quantity of acid-soluble radioactivity in the incubation medium was observed in all incubations, both in the presence and absence of the yolk-sac tissue and in the presence and absence of each of the inhibitors (e.g. see Fig. 7.1).

Table 7.3 summarizes the data on the effects of the inhibitors on the rates of uptake and digestion of the ^{125}I-labelled substrates and includes data on the inhibition of the uptake of ^{125}I-labelled poly(vinyl-pyrrolidone) (data derived from Fig. 5.6 and Table 4.6). Both the rate of tissue-associated digestion and the putative rate of uptake, for each of the ^{125}I-labelled proteins and peptides, were decreased by increasing concentrations of ammonium chloride and by 10^{-5}M-rotenone. Except for ^{125}I-labelled glucagon, the decreased rates of tissue-associated digestion and the putative rates of uptake of each ^{125}I-labelled substrate parallel the decreased rate of uptake of ^{125}I-labelled poly(vinylpyrrolidone) [^{125}I-PVP]. A possible exception (in addition to ^{125}I-labelled glucagon) is ^{125}I-labelled calcitonin, which, other than at a 20mM-ammonium ion concentration, shows a rate of tissue-associated digestion and a putative rate of uptake that are consistently higher than the rate of uptake of ^{125}I-PVP. The differences are not, however, statistically significant. The observed correspondence of parallelisms strongly suggest that, except

for glucagon, the tissue-associated digestion of each of the ^{125}I-labelled proteins and peptides occurs mainly, if not exclusively, intracellularly. The data on the inhibition of ^{125}I-labelled glucagon uptake and digestion by ammonium ions is difficult to interpret; glucagon is itself an inhibitor of pinocytosis in 17.5-day rat yolk sacs (see Section 4.3) and the combined effects of glucagon and ammonium ions on the uptake and digestion processes are not known. In general, the rate of tissue-associated digestion could not exceed, and indeed would be expected to be less than, the putative rate of uptake when the yolk sacs are incubated in the presence of the inhibitors because each inhibitor is also an inhibitor of intracellular proteolysis. This effect was more pronounced for the ^{125}I-labelled proteins than for the peptides (see Section 7.3.4).

7.3.3 Time-course of the appearance of acid-soluble digestion products derived from ^{125}I-labelled proteins and peptides when incubated with 17.5-day rat yolk sacs in serum-free medium 199.

It is expected that, following addition to the incubation medium of a substrate that is digested intracellularly by the yolk sac, there will be a lag in the appearance of acid-soluble digestion products in the medium. The duration of the lag-period will depend on the time taken for the tissue to internalize, digest and release the radioactive hydrolysis products of the substrate. Such a lag was previously shown for the substrate formaldehyde-denatured ^{125}I-labelled bovine serum albumin (see Fig. 4.1). Except for glucagon, Fig. 7.2 shows such a lag to occur for each ^{125}I-labelled protein and peptide investigated. Moreover, the duration of the lag-period was shown to have characteristically different value for each of the ^{125}I-labelled substrates examined.

The effects of decreasing the incubation temperature on the length of the lag-period for each ^{125}I-labelled peptide (i.e. glucagon,

insulin B-chain, calcitonin and insulin) are shown in Figs 7.3a & b. Decreasing the temperature at which the yolk sacs were incubated increased the duration of the lag-period; at temperatures of 34°C, and less, even ^{125}I-labelled glucagon showed a lag-period. With ^{125}I-labelled calcitonin, an unusual effect was observed at each incubation temperature. After an initial lag-period, the quantity of acid-soluble radioactivity in the incubation medium increased linearly with time but, after several minutes, the rate of appearance of the acid-soluble radioactivity suddenly increased. This suggests either that the ^{125}I-labelled calcitonin preparation is heterogenous and contains at least two radioactive species, each with a significantly different lag-period, or that a single species of substrate molecule exists but the ^{125}[I]iodo-L-tyrosyl-residues are attacked at very different rates by the enzymes of the yolk-sac tissue.

On decreasing the temperature at which the yolk sacs are incubated with each ^{125}I-labelled peptide, the rate of appearance of acid-soluble radioactivity in the incubation medium, after the initial lag-period, was decreased. Fig. 7.4 shows that, for each ^{125}I-labelled peptide, the rate decreases essentially in parallel with the decrease in the rate of uptake of ^{125}I-PVP (data derived from Table 4.4) and also parallels the decrease in the rate of digestion of formaldehyde-denatured ^{125}I-labelled bovine serum albumin (data derived from Table 4.4). The high degree of scatter in the results is possibly the result of the variation in the small contribution of digestive activity associated with the incubation medium.

These results further indicate that the ^{125}I-labelled peptides are digested intracellularly and also suggest an intracellular digestion site for ^{125}I-labelled glucagon.

7.3.4 Effects of ammonium chloride and of rotenone on the intracellular proteolysis of ^{125}I-labelled proteins and peptides.

Table 7.4 shows a summary of the data on the effects of ammonium ions and of rotenone on the intracellular proteolysis of ^{125}I-labelled protein and peptide substrates. The data assume, as suggested by the earlier observations, that the tissue-associated digestion of each substrate is intracellular. For each substrate, most of the ingested radioactivity is subsequently released back into the incubation medium in an acid-soluble form. In control experiments the rates of digestion of ^{125}I-labelled immunoglobulin G, ^{125}I-labelled lysozyme, ^{125}I-labelled calcitonin and ^{125}I-labelled glucagon each fell slightly below the corresponding value of the rate of uptake, indicating that some of the yolk-sac associated substrate is either not readily made available for intracellular digestion or that some digestion products accumulate within the tissue. Rotenone (10^{-5} M) inhibited the intracellular proteolysis of each endocytosed ^{125}I-labelled substrate investigated (^{125}I-labelled glucagon was not investigated). With ammonium ion (20 mM) inhibition of intracellular proteolysis was not invariably observed, but where ammonium ion inhibition occurred, it was concentration dependent.

The ^{125}I-labelled proteins and peptides can be grouped according to their susceptibility to inhibition of proteolysis by ammonium ions (20 mM): ^{125}I-labelled immunoglobulin G and formaldehyde-denatured ^{125}I-labelled bovine serum albumin each showed almost complete inhibition, ^{125}I-labelled ribonuclease and ^{125}I-labelled lysozyme each showed more than 50% inhibition, ^{125}I-labelled insulin and ^{125}I-labelled calcitonin each showed less than 50% inhibition and ^{125}I-labelled insulin B-chain and ^{125}I-labelled glucagon each showed no inhibition. Where inhibition of intracellular proteolysis occurred, the relative rate of accumulation of the ^{125}I-labelled substrate

label in the tissue was increased (Table 7.2 a,b,c & d).

7.3.5 Digestion of ^{125}I-labelled proteins and peptides by disrupted rat-liver tritosomes.

If the inhibition of intracellular proteolysis by ammonium ions results from an elevation of the intralysosomal pH (see Sections 5.1 & 5.4), it seemed possible that the enzymes that are rate-limiting for the digestion of the ^{125}I-labelled proteins might be more sensitive to increases in pH than those that are rate-limiting for the digestion of ^{125}I-labelled peptides. (Thus explaining the selective inhibition of the intracellular proteolysis of ^{125}I-labelled proteins by the ammonium ions.) This possibility was tested using disrupted rat-liver tritosomes incubated at pH values between 4.0 and 8.0 and measuring their digestive activity against the various ^{125}I-labelled proteins and peptides. [Rat liver tritosomes were used in preference to rat yolk-sac tritosomes because the purity of the latter after isolation is not yet known and because the former can be isolated in larger quantities.]

The effects of increasing the pH of incubation on the rate of digestion of ^{125}I-labelled protein and peptide substrates by rat-liver tritosomes are shown in Table 7.5. Increasing pH caused similar decreases in the relative hydrolytic activity of the tritosomes towards protein and peptide substrates. However, the specific activities of the tritosome preparation, at each pH, were several orders of magnitude higher for the ^{125}I-labelled peptide substrates than for the ^{125}I-labelled protein substrates.

7.3.6 Calculation of the Catabolic Index for each ^{125}I-labelled protein and peptide digested by 17.5-day rat yolk sacs incubated in serum-free medium 199.

The Catabolic Index of a digestible substrate is defined as the

mean time taken for the substrate to be digested and released from a cell once it has been captured by endocytosis. [It is the ratio of the steady-state level of the tissue-associated substrate label to the rate of uptake of the substrate by the endocytic cell, and has the dimension of time (see Section 3.2.2).] Table 7.6 shows the rate of uptake of each ^{125}I-labelled protein and peptide investigated in this chapter, and the corresponding quantity of substrate associated with the tissue at 3h. (The rate of uptake and the level of tissue-associated substrate label at 3h for ^{125}I-labelled glucagon are also shown. These values have been corrected to give those values that would have been observed if the glucagon was not an inhibitor of pinocytosis.)

In general, Table 7.6 shows the ^{125}I-labelled peptides are ingested more rapidly than the ^{125}I-labelled proteins (^{125}I-labelled ribonuclease, however, contradicts this general trend) but generally the levels of the tissue-associated radioactivity for the ^{125}I-labelled proteins are greater than the corresponding levels for the ^{125}I-labelled peptides. This would not be expected if each ^{125}I-labelled protein and peptide was digested in the yolk-sac tissue with the same efficiency before the acid-soluble digestion products were returned to the incubation medium. The Catabolic Indices for the eight digestible substrates investigated are shown in Table 7.6. In general, the smaller the size of the substrate the smaller the Catabolic Index. This suggests that the smaller substrates are digested more efficiently once inside the yolk-sac epithelial cells.

Table 7.1 Stability of ^{125}I-labelled proteins and peptides under various incubation conditions in serum-free medium 199.

The rates of appearance of acid-soluble radioactivity in medium 199 containing ^{125}I-labelled protein or peptide (1µg/ml of medium) were measured over a 3h incubation period under the following culture conditions: (A) medium 199 [20ml] alone; (B) medium 199 [20ml] containing a single 17.5-day rat yolk sac; (C) uncentrifuged- and (D) centrifuged (300g x 5min) medium 199 [20ml] in which a single 17.5-day rat yolk sac had been previously incubated for 3h; (E) medium 199 [20ml] containing a cell-free, yolk-sac homogenate [approx. 1mg yolk-sac protein].

Each value shown is the mean rate [± standard deviation) of 3 separate determinations and is expressed as the % of the initial acid-soluble radioactivity hydrolysed per h. Data marked † are initial rates measured over the period 0-20min.

^{125}I-Labelled protein/peptide	(A) 199 alone (%/h)	(B) 199 + yolk sac (%/h)	(C) Conditioned 199 uncentrifuged (%/h)	(D) Conditioned 199 centrifuged (%/h)	(E) 199 + yolk-sac homogenate (%/h)
Rat IgG	-0.08 ± 0.05	+1.44 ± 0.02	–	+0.08 ± 0.08	+0.01 ± 0.01
Formaldehyde--denatured BSA	-0.05 ± 0.05	+7.05 ± 0.79	+0.05 ± 0.16	+0.06 ± 0.02	-0.02 ± 0.04
Ribonuclease	+0.08 ± 0.35	+16.79 ± 2.62	-0.06 ± 0.25	+0.09 ± 0.03	+0.09 ± 0.01
Lysozyme	+0.04 ± 0.12	+6.78 ± 0.29	+0.07 ± 0.19	+0.15 ± 0.06	+0.08 ± 0.03
Insulin	+0.05 ± 0.02	+10.61 ± 0.47	+0.12 ± 0.08	+0.09 ± 0.02	†25.6 ± 3.3
Calcitonin	+0.00 ± 0.10	+13.10 ± 0.61	+0.34 ± 0.01	-	†42.1 ± 1.5
Insulin B–Chain	+0.28 ± 0.34	+10.50 ± 0.62	+0.97 ± 0.41	-	†90.2 ± 4.2
Glucagon	+0.42 ± 0.49	+4.26 ± 0.51	+1.48 ± 0.53	-	†116.3 ± 2.5

Table 7.2a Effects of ammonium ions and of rotenone on the uptake and digestion of ^{125}I-labelled rat immunoglobulin G and of formaldehyde-denatured ^{125}I-labelled bovine serum albumin by 17.5-day rat yolk sacs incubated in serum-free medium 199.

For experimental details see Section 7.2.3. Each value reported represents the mean (± S.D.) of 3-4 separate determinations with yolk sacs from different animals. The data for the albumin are summarized from Tables 4.5 & 5.1. Values marked † show data for which the rate of uptake was too small to calculate accurate values.

^{125}I-labelled protein	Inhibitor	Rate of Uptake		Rate of Digestion		Quantity of radioactivity associated with tissue at 3h (ng/mg y.s.protein)	Mean rate of tissue accumulation over 3h period (% rate of uptake)
		ng/h per mg yolk sac protein	% of control	ng/h per mg yolk-sac protein	% of rate of uptake		
Rat immunoglobulin G	Control	66.0 ± 2.9	100.0 ± 4.4	57.6 ± 0.9	87.4 ± 2.4	74.8 ± 1.8	37.7 ± 1.2
	NH_4^+, 5mM	18.2 ± 8.4	27.8 ± 13.7	10.7 ± 6.6	57.7 ± 18.7	35.5 ± 10.4	68.1 ± 11.4
	NH_4^+, 20mM	3.2 ± 0.9	4.9 ± 1.3	-0.8 ± 0.7	approx.0†	12.7 ± 3.2	approx.100†
	Rotenone, 10^{-5}M	8.8 ± 1.7	13.2 ± 2.8	4.3 ± 1.2	48.3 ± 5.9	16.5 ± 2.6	62.6 ± 3.6
Formaldehyde denatured bovine serum albumin	Control	257.3 ± 23.1	100.0 ± 8.9	244.2 ± 16.0	95.1 ± 5.6	131.7 ± 28.4	16.5 ± 2.8
	NH_4^+, 5mM	141.6 ± 24.2	55.2 ± 9.8	98.2 ± 23.2	68.8 ± 4.9	206.1 ± 40.2	48.5 ± 3.8
	NH_4^+, 20mM	32.7 ± 6.7	10.8 ± 2.0	1.6 ± 1.9	5.3 ± 7.2	75.8 ± 8.5	91.9 ± 9.7
	Control	289.0 ± 39.8	100.0 ± 13.8	284.7 ± 34.9	98.6 ± 1.9	151.9 ± 19.4	17.5 ± 1.2
	Rotenone, 10^{-5}M	48.1 ± 20.4	13.2 ± 6.9	37.1 ± 17.8	76.1 ± 5.8	58.7 ± 20.2	41.8 ± 4.4

Table 7.2b Effects of ammonium ions and of rotenone on the uptake and digestion of ^{125}I-labelled ribonuclease and of ^{125}I-labelled lysozyme by 17.5-day rat yolk sacs incubated in serum-free medium 199.

For experimental details see Section 7.2.3. Each value reported represents the mean (± S.D.) of 3 separate determinations with yolk sacs from different animals. The rates of uptake were calculated assuming that the observed digestion occurred intracellularly.

^{125}I-labelled protein	Inhibitor	Rate of Uptake		Rate of digestion		Quantity of radioactivity associated with tissue at 3h (ng/mg y.s.protein)	Mean rate of tissue accumulation over 3h period (% rate of uptake)
		ng/h per mg yolk-sac protein	% of control	ng/h per mg yolk-sac protein	% of rate of uptake		
Ribonuclease	Control	682.2 ± 88.7	100.0 ± 13.0	671.6 ± 105.7	98.3 ± 2.9	245.5 ± 10.3	12.1 ± 1.6
	NH_4^+, 5mM	195.1 ± 44.2	29.7 ± 10.3	147.7 ± 38.3	75.3 ± 3.3	185.2 ± 36.8	31.8 ± 1.8
	NH_4^+, 20mM	26.5 ± 8.5	3.8 ± 7.5	6.6 ± 3.2	27.0 ± 14.0	62.9 ± 28.5	77.4 ± 11.6
	Rotenone, 10^{-5}M	78.4 ± 17.4	11.5 ± 1.7	59.2 ± 11.8	75.0 ± 5.2	88.2 ± 20.6	36.9 ± 0.5
Lysozyme	Control	302.8 ± 5.7	100.0 ± 1.2	271.2 ± 11.8	89.5 ± 2.9	249.9 ± 11.1	27.4 ± 0.8
	NH_4^+, 5mM	140.5 ± 21.6	46.3 ± 6.2	86.3 ± 20.6	61.0 ± 9.5	179.1 ± 23.7	42.6 ± 2.8
	NH_4^+, 20mM	43.4 ± 4.6	14.3 ± 1.3	20.8 ± 5.9	47.5 ± 9.7	77.9 ± 6.6	61.8 ± 10.7
	Rotenone, 10^{-5}M	72.4 ± 5.2	23.9 ± 1.8	37.7 ± 2.8	51.9 ± 0.2	113.6 ± 8.4	52.6 ± 0.7

Table 7.2c Effects of ammonium ions and of rotenone on the uptake and digestion of ^{125}I-labelled insulin and of ^{125}I-labelled calcitonin by 17.5-day rat yolk sacs incubated in serum-free medium 199.

For experimental details see Section 7.2.3. Each value reported represents the mean (± S.D.) of 3 separate determinations with yolk sacs from different animals. The rates of uptake were calculated assuming that the yolk-sac associated digestion occurred intracellularly.

^{125}I-Labelled peptide	Inhibitor	Rate of uptake		Rates of digestion				Quantity of radioactivity associated with tissue at 3h (ng/mg y.s.protein)	Mean rate of tissue accumulation over 3h period (% rate of uptake)
		ng/h per mg yolk-sac protein	% of control	with yolk sac	without yolk sac	yolk-sac associated	(% of rate of uptake)		
				(ng/h per mg yolk-sac protein)					
Insulin	Control	411.4±18.4	100.0 ± 4.5	424.6±18.9	8.9±3.9	415.6±15.7	(102.1 ± 0.5)	113.1±15.6	9.1 ± 0.8
	NH_4^+, 5mM	88.8 ±8.7	21.5 ± 1.5	82.2 ± 9.6	4.8±1.0	77.3 ± 8.6	(87.0 ± 1.8)	88.4 ± 3.7	20.4 ± 1.0
	NH_4^+, 20mM	11.3 ±1.3	2.7 ± 0.4	10.8 ± 1.9	3.7±1.3	7.4 ± 1.3	(66.9±18.2)	18.8 ± 0.4	56.0 ± 5.5
	Rotenone 10^{-5}M	43.4±15.6	10.6 ± 4.2	39.9±16.9	1.9±1.5	38.0±18.2	(77.2 ± 5.2)	38.8 ± 3.8	31.9 ± 9.3
Calcitonin	Control	450.4±16.6	100.0 ± 3.7	438.4±20.2	11.6±0.4	426.8±19.2	(94.6 ± 1.6)	177.9±16.9	13.1 ± 1.4
	NH_4^+, 5mM	197.2±42.1	43.9±10.7	181.8±51.9	10.7±4.8	171.1±49.6	(85.6 ± 7.6)	121.0±15.7	21.4 ± 7.0
	NH_4^+, 20mM	68.7 ± 3.6	15.2 ± 1.0	40.0 ± 5.3	1.5±1.6	38.6 ± 4.5	(56.4 ± 9.0)	100.0±21.3	50.1 ± 7.8
	Rotenone, 10^{-5}M	129.6±20.2	27.0± 5.3	93.4±24.8	12.9±2.4	80.4±27.0	(64.1±11.4)	140.3±27.2	38.9±13.7

Table 7.2d Effects of ammonium ions and of rotenone on the uptake and digestion of ^{125}I-labelled insulin B-chain and of ^{125}I-labelled glucagon by 17.5-day rat yolk sacs incubated in serum-free medium 199.

For experimental details see Section 7.2.3. Each value reported represents the mean (± S.D.) of 3 separate determinations with yolk sacs from different animals. The rates of uptake were calculated assuming that the yolk-sac associated digestion occurred intracellularly.

^{125}I-Labelled peptide	Inhibitor	Rate of uptake		Rates of digestion				Quantity of radioactivity associated with tissue at 3h (ng/mg y.s.protein)	Mean rate of tissue accumulation over 3h period (% rate of uptake)
		ng/h per mg yolk-sac protein	% of control	with yolk sac	without yolk sac	yolk-sac associated	(% of rate of uptake)		
				(ng/h per mg yolk-sac protein)					
Insulin B-chain	Control	374.1±37.4	100.0±10.0	420.2±24.5	44.4±26.0	375.8±36.4	(100.4±0.9)	61.0±15.4	8.2±3.9
	NH_4^+, 5mM	120.4±20.4	32.0± 2.5	168.7±17.4	46.1±4.2	122.6±20.5	(101.7±0.2)	16.5± 3.3	4.5±0.1
	NH_4^+, 20mM	40.2± 5.3	10.7± 1.6	65.8± 7.7	25.8± 2.6	40.1± 6.1	(99.6±1.8)	7.8± 1.1	6.6±1.9
	Rotenone, 10^{-5} M	33.7± 7.3	9.0± 2.0	66.8±14.4	36.5±12.8	30.2± 7.6	(88.9±3.9)	16.2± 0.9	16.5±3.6
Glucagon	Control	170.2±20.3	100.0±11.9	240.6±79.2	77.1±65.6	163.5±20.9	(94.3±1.3)	38.2± 5.0	7.5±1.5
	NH_4^+, 5mM	132.2±39.4	75.1±15.9	193.4±42.6	67.3± 7.0	129.1±35.6	(95.8±1.9)	33.1± 2.7	9.1±3.7
	NH_4^+, 20mM	86.3±23.9	50.5±15.4	151.7±44.6	69.2±21.5	82.4±23.9	(95.1±1.4)	15.9± 1.6	6.3±1.5

Table 7.3 Summary of the effects of ammonium ions and of rotenone on the rates of uptake and digestion of ^{125}I-labelled substrates by 17.5-day rat yolk sacs incubated in serum-free medium 199.

Data are summarized from Tables 7.2a,b,c & d [protein substrates] and Table 4.6 and Fig. 5.6 [poly-(vinylpyrrolidone)]. The rates of uptake were calculated assuming that the yolk-sac associated digestion occurred intracellularly. N.D.: not determined.

^{125}I-Labelled substrate	Rates of uptake (% of control)			Rates of digestion (% of control)		
	NH_4^+, 5mM	NH_4^+, 20mM	Rotenone, 10^{-5}M	NH_4^+, 5mM	NH_4^+, 20mM	Rotenone, 10^{-5}M
PVP	30.4 ± 10.8	15.9 ± 2.9	13.1 ± 3.6	-	-	-
Rat IgG	27.8 ± 13.7	4.9 ± 1.3	13.2 ± 2.8	18.5 ± 11.4	-1.4 ± 2.0	7.4 ± 2.1
Formaldehyde--denatured albumin	55.2 ± 9.8	10.8 ± 2.0	13.2 ± 6.9	40.2 ± 9.5	0.6 ± 2.1	13.0 ± 6.2
Ribonuclease	29.7 ± 10.3	3.8 ± 7.5	11.5 ± 1.7	21.9 ± 5.7	0.9 ± 0.5	8.8 ± 1.7
Lysozyme	46.3 ± 6.2	14.3 ± 1.3	23.9 ± 1.8	31.8 ± 7.5	7.6 ± 2.1	13.7 ± 1.0
Insulin	21.5 ± 1.5	2.7 ± 0.4	10.6 ± 4.2	18.6 ± 2.0	1.8 ± 0.3	9.1 ± 4.3
Calcitonin	43.9 ± 10.7	15.2 ± 1.0	27.0 ± 5.3	40.0 ± 11.6	9.0 ± 1.0	18.8 ± 6.3
Insulin B-chain	32.0 ± 2.5	10.7 ± 1.6	9.0 ± 2.0	43.5 ± 5.4	10.6 ± 1.6	8.0 ± 2.0
Glucagon	75.1 ± 15.9	50.5 ± 15.4	N.D.	78.9 ± 21.8	50.4 ± 14.6	N.D.

Table 7.4 Summary of the effects of ammonium ions and of rotenone on the intracellular proteolysis of ^{125}I-labelled proteins and peptides ingested by 17.5-day rat yolk sacs incubated in serum-free medium 199.

The data are summarized from Tables 7.2a,b,c & d. The data were calculated assuming that the yolk--sac associated digestion occurred intracellularly. N.D.: not determined.

^{125}I-labelled substrate	Rates of digestion (% rate of uptake)			
	Control	NH_4^+, 5mM	NH_4^+, 20mM	Rotenone, 10^{-5} M
Rat IgG	87.4 ± 2.4	57.7 ± 18.7	approx. 0	48.3 ± 5.9
Formaldehyde--denatured albumin	95.1 ± 5.6 (Batch I) 98.6 ± 1.9 (Batch II)	68.8 ± 4.9 -	5.3 ± 7.2 -	- 76.1 ± 5.8
Ribonuclease	98.3 ± 2.9	75.5 ± 3.3	27.0 ± 5.2	75.0 ± 5.2
Lysozyme	89.5 ± 2.2	61.0 ± 9.5	47.5 ± 9.7	51.9 ± 0.2
Insulin	102.2 ± 0.5	87.0 ± 1.8	66.9 ±18.2	77.2 ± 5.2
Calcitonin	94.6 ± 1.6	85.6 ± 7.6	56.4 ± 9.0	64.1 ± 11.4
Insulin B-chain	100.4 ± 0.9	101.7 ± 0.2	99.6 ± 1.8	88.9 ± 3.9
Glucagon	94.3 ± 1.3	95.8 ± 1.9	95.1 ± 1.4	N.D.

Table 7.5 Rates of digestion of ^{125}I-labelled proteins and peptides by disrupted rat-liver tritosomes and the effects of incubation pH on the relative rates of digestion.

The increase in acid-soluble radioactivity, on incubating each of the ^{125}I-labelled proteins or peptides (10µg in buffered 0.2% (v/v) Triton X-100) with rat-liver tritosomes (2.5-100µg tritosome protein) for the duration and at the pH shown, was determined as described in Section 7.2.6. The data show, for each substrate, the mean specific activity of the tritosome preparation (when incubated at pH 4.0) and the mean value (± standard deviation, from 3 or 4 separate determinations; using a single tritosome preparation) of the increase in acid-soluble radioactivity at pH 4-8, expressed as a percentage of that observed at pH 4.0. Incubation conditions were such that the maximum increase in acid-soluble radioactivity did not exceed 25% of the initial acid-insoluble radioactivity. The specific activities are expressed as the quantity of protein or peptide (pg) hydrolysed per µg of tritosome protein per h of incubation.

^{125}I-labelled substrate	Quantity of tritosomes added (µg protein)	Duration of incubation at 37°C	Specific activity at pH 4.0	Incubation pH			
				4.0	5.0	6.5	8.0
Rat IgG	100.0	24h	9.3×10^2	100.0 ± 4.8	62.5 ± 6.5	0.9 ± 0.9	-0.3 ± 1.7
Formaldehyde--denatured albumin	100.0	24h	5.3×10^2	100.0 ± 10.4	102.3 ± 9.8	3.3 ± 0.8	-0.8 ± 0.3
Lysozyme	100.0	24h	4.9×10^2	100.0 ± 12.6	119.3 ± 3.2	0.2 ± 4.8	-2.7 ± 2.5
Insulin	10.0	1h	1.2×10^5	100.0 ± 5.9	62.1 ± 6.0	0.9 ± 0.5	0.3 ± 0.5
Calcitonin	10.0	15min	6.7×10^5	100.0 ± 8.2	82.1 ± 8.4	20.6 ± 2.7	1.0 ± 1.1
Insulin B-chain	2.5	10min	5.5×10^6	100.0 ± 14.1	72.5 ± 8.5	1.3 ± 5.1	0.6 ± 0.1
Glucagon	5.0	15min	1.2×10^6	100.0 ± 12.6	70.3 ± 2.6	1.7 ± 4.5	1.1 ± 2.7

Table 7.6 Summary of the rates of endocytic uptake of ^{125}I-labelled proteins and peptides by 17.5-day rat yolk sacs incubated in serum--free medium 199 and their associated Catabolic Indices.

The rates of endocytic uptake are derived from Tables 7.2a,b,c & d. For formaldehyde-denatured ^{125}I-labelled bovine serum albumin (denatured BSA) data are included from Tables 3.1, 4.3, 4.5, 4.7, 6.1 & 5.1 and Figs. 5.1, 5.2, 5.4 & 6.3. Two groups of data are presented for glucagon; the second group is corrected to give the rate of uptake that would have been observed if the glucagon was not an inhibitor of endocytosis in the rat yolk sac (10^{-6}M glucagon inhibits ^{125}I-labelled poly(vinylpyrrolidone) uptake by 68.1%, see Table 4.7). The Catabolic Index (the mean time taken to digest and release a substrate once it has been endocytosed) is calculated from the ratio of the yolk-sac associated radioactivity (at its steady-state level) to the rate of uptake. The rates of uptake shown are numerically equivalent to the Endocytic Index (µl/h per mg yolk-sac protein).

^{125}I-labelled protein/peptide	No. of expts	Rate of uptake (ng/h per mg yolk-sac protein)	Yolk-sac associated radioactivity (ng/mg yolk--sac protein)	Catabolic Index (h)
Rat IgG	3	66.0 ± 2.9	74.8 ± 1.8	1.132 ± 0.035
Denatured BSA	37	272.8 ± 45.5	133.7 ± 25.6	0.493 ± 0.076
Ribonuclease	3	682.0 ± 88.7	247.5 ± 10.3	0.364 ± 0.049
Lysozyme	3	302.8 ± 5.7	249.9 ± 11.1	0.822 ± 0.024
Insulin	3	411.4 ± 18.4	113.1 ± 15.6	0.273 ± 0.025
Calcitonin	3	450.4 ± 16.6	177.9 ± 16.9	0.393 ± 0.042
Insulin B-chain	3	374.1 ± 37.4	61.0 ± 15.4	0.246 ± 0.117
Glucagon	3	170.2 ± 20.3	38.2 ± 5.0	0.223 ± 0.046
Glucagon corrected		533.5 ± 63.6	119.7 ± 15.7	-

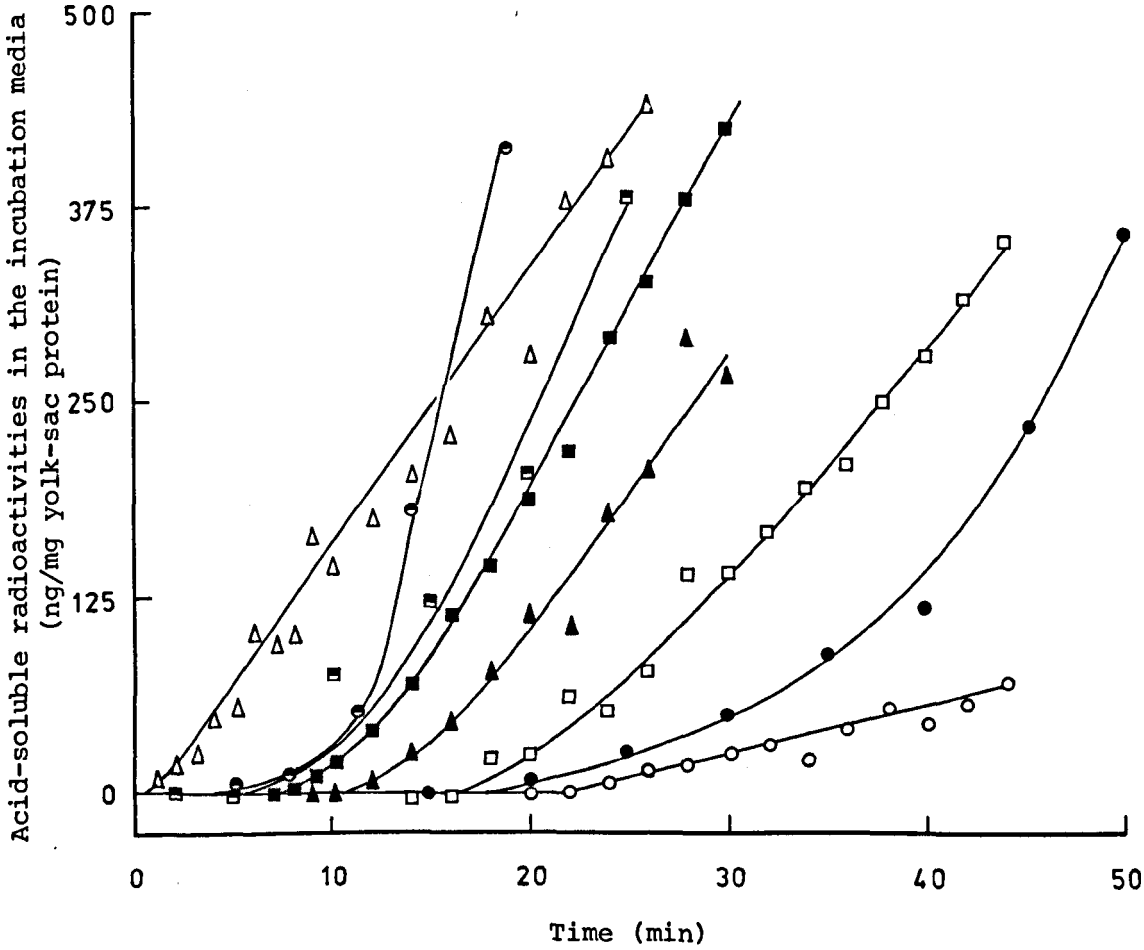


Figure 7.2 Appearance of acid-soluble radioactivity in the incubation medium when ^{125}I-labelled proteins and peptides were incubated with 17.5-day rat yolk sacs in serum-free medium 199.

Each ^{125}I-labelled substrate was present at 5 µg/ml of incubation medium. For experimental details see Section 7.2.3. The ^{125}I-labelled substrates were: glucagon, (Δ); insulin B-chain, (●); calcitonin, (▪); insulin, (■); ribonuclease, (▲); formaldehyde-denatured bovine serum albumin, (□); lysozyme, (●); rat immunoglobulin G (○).

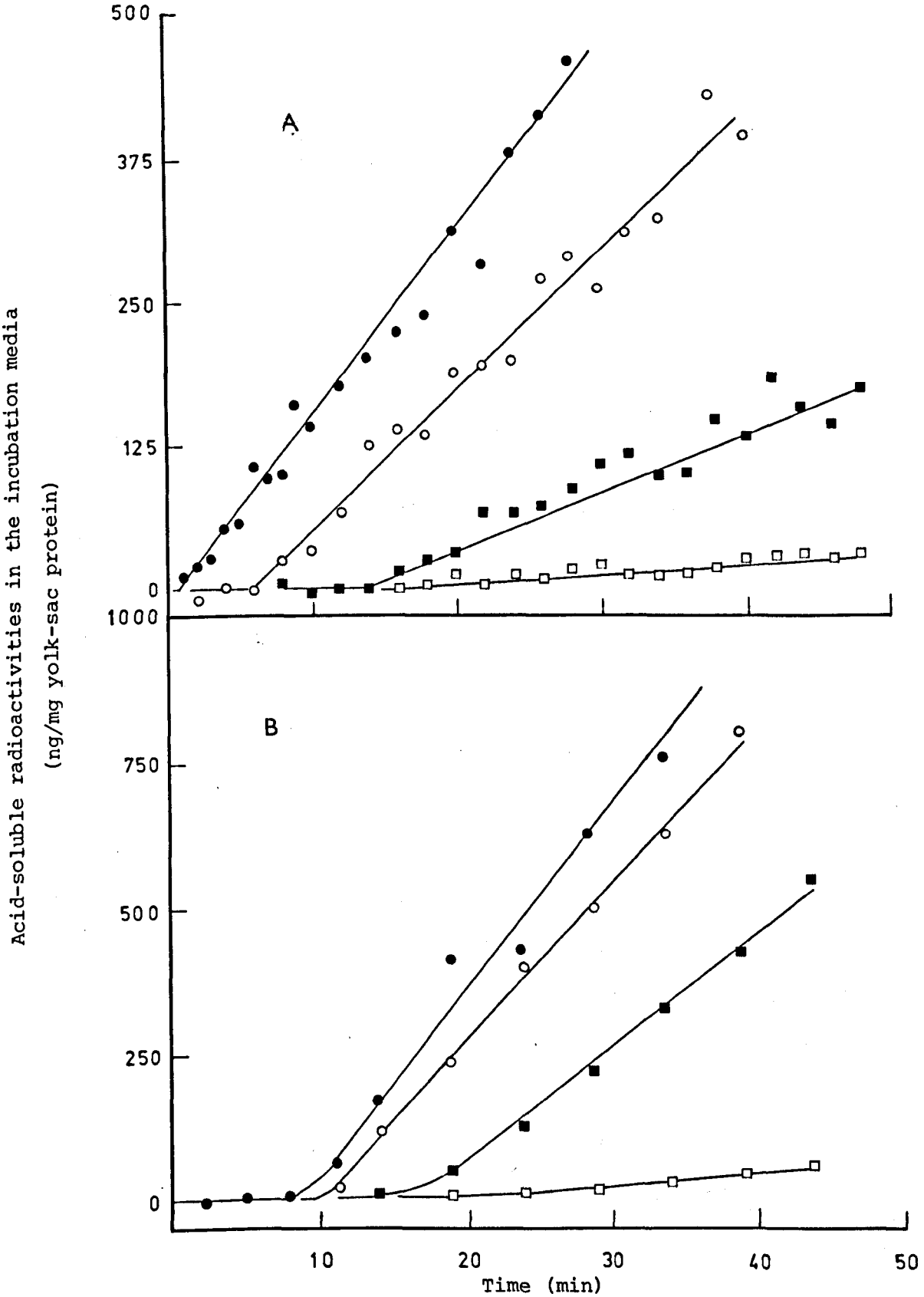


Figure 7.3a Effect of incubation temperature on the time of first appearance of acid-soluble radioactivity in the incubation medium when either ^{125}I--labelled glucagon (A) or ^{125}I-labelled insulin B-chain (B) were incubated with 17.5-day rat yolk sacs in serum-free medium 199.

For experimental details see Section 7.2.3. The incubation temperatures (°C) were: 37, (●); 34, (○); 30, (■); 20, (□).

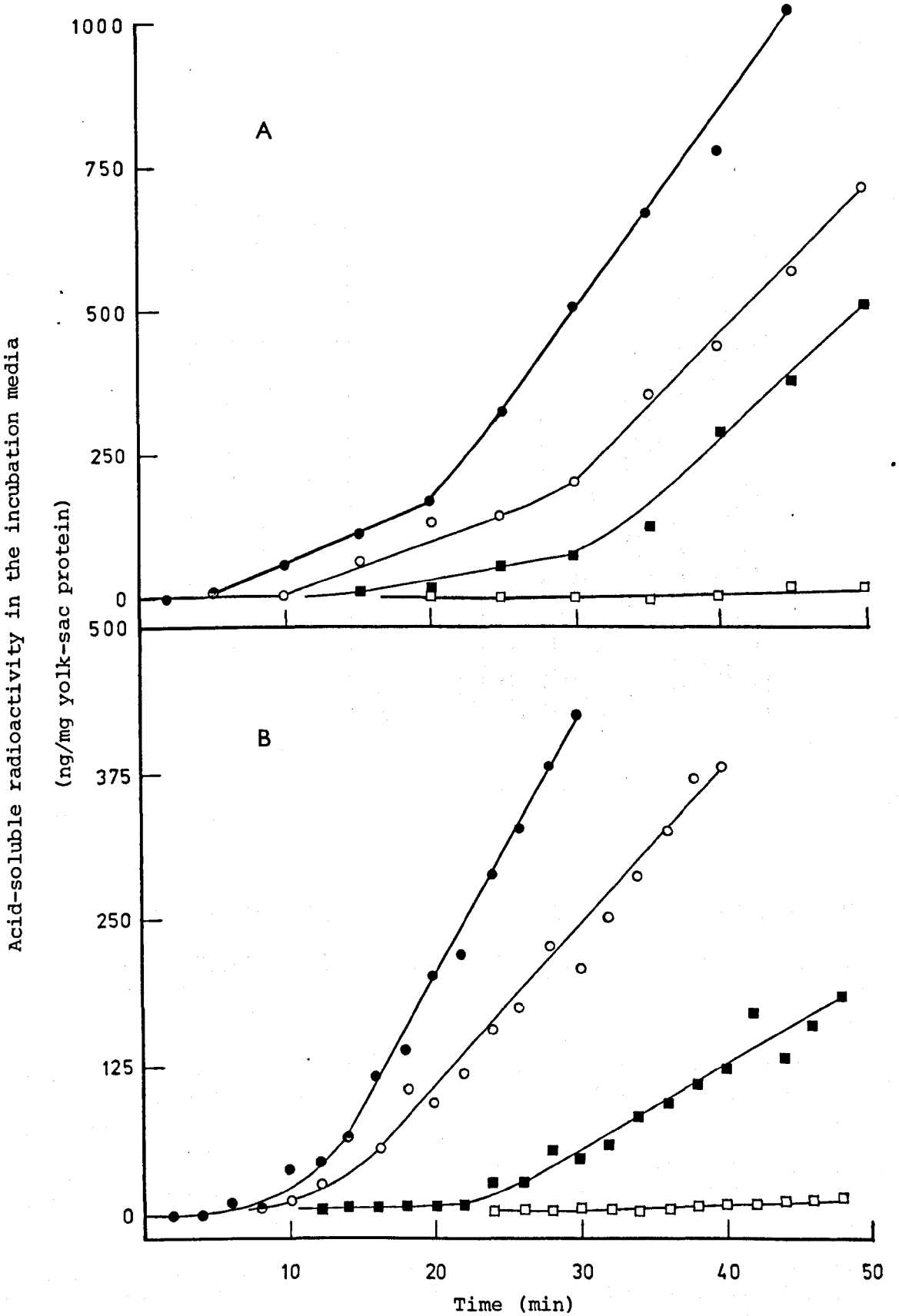


Figure 7.3b Effect of incubation temperature on the time of first appearance of acid-soluble radioactivity in the incubation medium when either ^{125}I-labelled calcitonin (A) or ^{125}I-labelled insulin (B) was incubated with 17.5-day rat yolk sacs in serum-free medium 199.

For experimental details see Section 7.2.3. The incubation temperatures (°C) were: 37, (●); 34, (○); 30, (■); 20, (□).

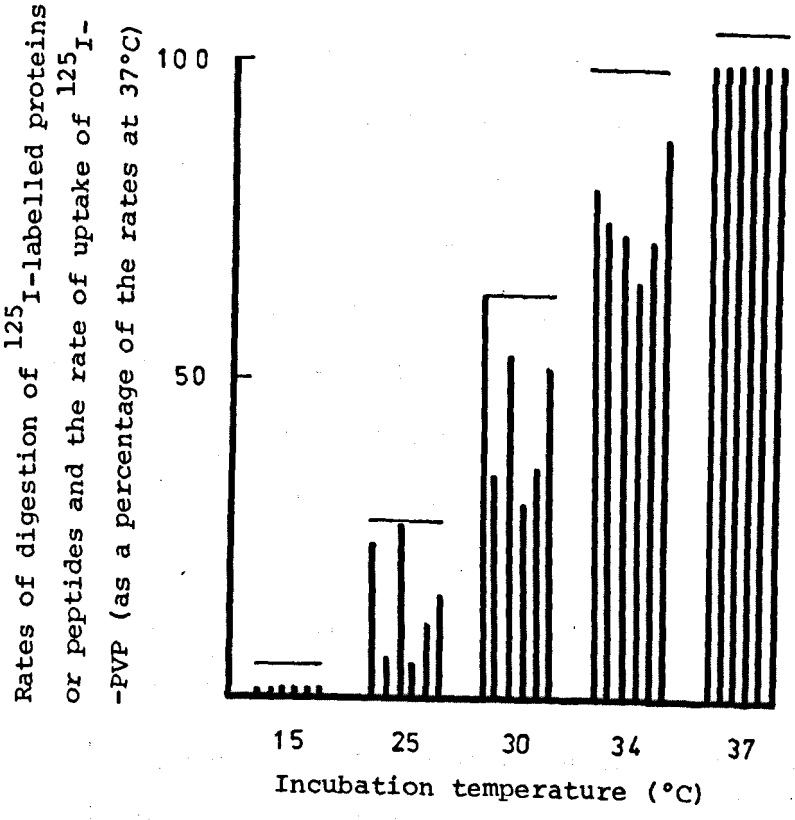


Figure 7.4 Effects of incubation temperature on the rates of digestion of ^{125}I-labelled proteins and peptides by the 17.5-day rat yolk sacs incubated in serum-free medium 199.

For experimental details see Section 7.2.3. The data for formaldehyde--denatured ^{125}I-labelled bovine serum albumin and ^{125}I-labelled poly-(vinylpyrrolidone) are derived from Table 4.4.

The vertical bars represent from left to right the ^{125}I-labelled substrates: insulin B-chain, glucagon, calcitonin, insulin, formaldehyde-denatured bovine serum albumin, and PVP.

An indication of the size of the standard deviation in each set of data, relating to a given incubation temperature, is given by a horizontal bar representing the value of the mean rate of uptake of PVP plus its standard deviation (mean + S.D.).

7.4 DISCUSSION

For each of the ^{125}I-labelled proteins and peptides investigated, the above results on the inhibition of pinocytosis by ammonium ions, rotenone and decreased temperature (as judged by the decreased putative rates of uptake and decreased levels of tissue-associated substrates), are in agreement with the findings in Chapters 4 & 5. The rates of yolk--sac associated digestion of each ^{125}I-labelled protein and peptide (immunoglobulin G, formaldehyde-denatured bovine serum albumin, ribonuclease, lysozyme, insulin, calcitonin, insulin B-chain and glucagon) were also decreased by the inhibitors of pinocytosis. The inhibitions (up to 90%), except for ^{125}I-labelled glucagon, paralleled the inhibition of uptake of ^{125}I-labelled poly(vinylpyrrolidone) and formaldehyde-denatured ^{125}I--labelled bovine serum albumin, a substrate known to be digested exclusively intracellularly by the 17.5-day rat yolk sac (see Chapter 4). These results would not be expected if a significant proportion of the protein or peptide substrates was digested by extracellular, plasma-membrane associated enzymes.

The lack of extracellular, yolk-sac associated proteolytic activity against the ^{125}I-labelled proteins was not unexpected. Firm circumstantial evidence (see Chapter 2) indicated an intracellular site of hydrolysis of the ^{125}I-labelled proteins: ribonuclease, lysozyme and insulin. Also, Ibbotson (1978) indicated that rat immunoglobulin G was digested intracellularly by the 17.5-day rat yolk sac. The results presented in this chapter, however, give direct evidence of an intracellular site of digestion of these substrates, unless the unlikely explanation is advanced that extracellular yolk-sac associated enzymes are inhibited to precisely the same extent as pinocytosis.

The lack of evidence for an extracellular site of yolk-sac associated digestion of the ^{125}I-labelled peptide substrates is compatible with the results of preliminary studies by A.J. Kenny (unpublished results), who investigated the peptidase activity in plasma membrane-rich fractions of rat yolk-sac and discovered only very low specific activities in comparison with similar fractions from the rabbit kidney proximal tubule. Such findings, however, do not exclude the possibility that extracellular, yolk-sac associated peptidases exist, but, either they make very little contribution to the overall catabolic activity of the rat yolk sac (too little to be detected by the experiments performed here), or only have specificities for substrates other than those used in this study. (In the latter case, the putative enzymes must have an exceptionally narrow range of specificity.)

It is not possible to explain adequately the fate of ^{125}I--labelled glucagon added to rat yolk sacs in culture, but evidence suggests it is digested, at least in part, intracellularly. Possibly ^{125}I-labelled glucagon is digested entirely intracellularly, as are other ^{125}I-labelled proteins and peptides, but further evidence is necessary to support such a conclusion. The inhibitory effects of ammonium ions on ^{125}I-labelled glucagon digestion are difficult to interpret. Both ammonium ions and glucagon are inhibitors of pinocytosis in the rat yolk sac (see Chapters 4 & 5). An understanding of the combined inhibitory effects of these pinocytosis inhibitors would help interpretation of the inhibitory effects of ammonium ions on ^{125}I-labelled glucagon digestion. However, in support of the notion that ^{125}I-labelled glucagon is digested by yolk sacs mostly, if not entirely, at an intracellular site, is the observed complete inhibition of ^{125}I-labelled glucagon digestion at 15-20°C, temperatures at which pinocytosis is also completely inhibited in the rat

yolk sac in the absence of calf serum (see Fig. 7.4). Moreover, the increase in the lag-period (Fig. 7.3a) on decreasing the incubation temperature suggests an intracellular site of digestion. Furthermore, an examination of the digestion products of ^{125}I-labelled glucagon after its incubation (2h) with well-washed yolk sacs (results not shown) showed that all the radioactivity rendered acid-soluble eluted coincidentally with the marker substances [^{125}I]iodo-L-tyrosine and glycyl-[^{125}I]iodo--L-tyrosine, an observation that is concordant with hydrolysis occurring at a lysosomal site in which the permeability properties of the lysosomal membrane limit the size of the digestion products released into the incubation medium (Reijngoud & Tager, 1977).

The duration of the lag-period observed at 37°C is a characteristic of the particular ^{125}I-labelled protein or peptide incubated with the yolk sacs. In general, the larger the size of the substrate the longer is the duration of the lag-period. The duration of the lag-periods for the ^{125}I-labelled peptides (see Fig. 7.2) correlate well with the initial rate of digestion of the substrates by cell-free extracts of the rat yolk sac (see Table 7.1, column E). Thus, ^{125}I-labelled glucagon has the shortest lag-period, followed by insulin B-chain and ^{125}I-labelled calcitonin then ^{125}I-labelled insulin; this is also the order of susceptibility to digestion by disrupted rat-liver tritosomes (see Table 7.5). Fig. 7.3b shows that ^{125}I-labelled calcitonin has two lag-periods: an initial lag-period in which no acid-soluble radioactive digestion products are released into the incubation medium, followed by a second period in which the rate of appearance of the acid-soluble radioactivity is slower than the final rate of appearance. This was observed at each temperature investigated (except at 20°C, at which temperature all

digestion had ceased). This probably relates to the occurrence of two sites at which the radioiodide attaches to calcitonin the label at one site being more easily released than that at the other. In support of this notion, the digestion of ^{125}I-labelled calcitonin (but not ^{125}I-labelled insulin, insulin B-chain and glucagon) by the cell-free extracts of the rat yolk sac was also biphasic. More than 90% digestion of ^{125}I-labelled peptides was generally achieved with the cell-free extract during a 3h incubation, whereas the digestion of ^{125}I-labelled calcitonin was initially rapid but after 18% of the substrate had been digested, very little further digestion occurred. The idea that the duration of the lag-period relates to the susceptibility of the substrate label to proteolytic release from the substrate is also supported by the observations that those substrates with a short lag-period are more readily digested by disrupted rat-liver tritosomes (Table 7.5). The correlation is not perfect in so much that ^{125}I-labelled insulin B-chain was found to be a better substrate for the tritosomal enzymes than was ^{125}I-labelled glucagon. [Bohley (1971), using a lysosomal fraction of rat liver found the reverse to be the case, however, this author used oxidized insulin B-chain whereas the reduced form was used here]. The discrepancy in these results may arise from differences in the complement of enzymes found in liver lysosomes and yolk-sac lysosomes, since, for the peptide substrates, a better correlation between lag-period and susceptibility to proteolysis was obtained when a cell-free extract of yolk sacs was used in place of tritosomes as a source of proteolytic enzymes (Table 7.1, column E).

It is conceivable that factors other than proteolytic susceptibility might also contribute to the observed differences in lag-periods. The duration of the lag-period must be the sum of the time

taken for each of the following processes to occur:

a) adsorption of the substrate to the internalizing plasma membrane,

b) pinosome formation,

c) intracellular movement of the newly formed pinosomes to the lysosomes (including pinosome-pinosome fusion),

d) fusion of pinosomes with lysosomes,

e) proteolytic cleavage of the substrate-label from the substrate, (including desorption of the substrate from the interior side of the heterolysosomal membranes if the latter is necessary for digestion),

f) diffusion of the digestion products from the lysosomes into the cytoplasm and subsequently into the incubation medium.

It was suggested above that factor e) is an important determinant of the duration of the lag-period for a particular substrate but it is not known whether the time taken for the substrate to desorb from the interior side of heterolysosomes would make a significant contribution to the lag-period of any of the substrates. It has generally been regarded that a) is an instantaneous event; this is not without experimental support. Fig. 4.1 shows the uptake of formaldehyde-denatured ^{125}I-labelled bovine serum albumin into the yolk sac tissue to occur, in effect, immediately the substrate is added to the yolk sac. The time taken for f) to occur must be similar for each of the ^{125}I-labelled substrates (IgG, lysozyme, ribonuclease, insulin, formaldehyde-denatured bovine serum albumin and probably glucagon) since, in each case, the radioactive digestion product is essentially the same (approx. 90% [^{125}I]iodo-L-tyrosine, see Chapter 2,4 & 7 and Ibbotson, 1978). Thus the lag-period most likely represents the time taken to translocate substrate to the lysosomes [i.e. b), c) and d) above] plus the time

taken to digest the substrate intralysosomally [e) above].

Since more than 98% of uptake of each substrate appears to take place by adsorptive endocytosis, it is possible that such adsorption of substrate modifies the properties of the pinosome membrane, hence modifies factors b), c) and d), above. However, experimental evidence suggests that this is not the case. The durations of the lag-periods for both formaldehyde-denatured ^{125}I-labelled bovine serum albumin and ^{125}I-labelled ribonuclease were each independent of the concentrations of the corresponding non-labelled substrate (Tables 3.1 & 3.2) and of the presence of different concentrations of (non-labelled) proteins or peptides in the incubation medium (Tables 3.3 & 3.4). These findings suggest that factors b), c) and d) are not affected by substrate adsorption hence these factors probably make a constant contribution to the lag-periods at 37°C. However, the time taken for these three processes to occur must be less than the shortest total lag-period observed. Thus, if ^{125}I-labelled glucagon is, as suggested earlier, digested mostly intracellularly, this time must be less than 3min, and, unless each of the eight substrates is taken up into a different class of vesicle, the major contribution made to the observed lag-periods of the other substrates must be the time taken to digest the substrate within lysosomes. The above considerations, and the observed correlation between the duration of the lag-period (Fig. 7.2) and the susceptibility of these substrates to digestion by cell-free extracts of the yolk-sac (Table 7.1, column E) or disrupted tritosomes (Table 7.5), indicate that the duration of the lag-period observed for a particular substrate when the yolk sacs are incubated at 37°C is dependent mostly on the time taken to digest the substrate once it is within the lysosomes.

Determination of the lag-period for a particular substrate is

therefore one way in which one can assess the susceptibility of a substrate to digestion by lysosomal enzymes within the intact cell. An alternative method, which was suggested in Chapter 3, is the calculation of the Catabolic Index, that is, the ratio of the tissue-associated substrate label, at a steady-state level, to the Endocytic Index. The Catabolic Index for each of the eight digestible substrates is shown in Table 7.6. The values obtained are all larger than the corresponding value for the initial lag-period as shown in Fig. 7.2. This presumably is because the lag-period represents the most rapid time in which a substrate can be digested and returned to the incubation medium, whereas the Catabolic Index represents the mean time taken for this process to occur. As expected, however, the ranking of substrates according to Catabolic Index is the same as that according to the lag-period provided that, when ranking the lag-periods, ^{125}I-labelled calcitonin takes the position dictated by the end of its second lag-period (see Fig. 7.3b). Table 3.6 shows the Catabolic Index (CI) of several ^{125}I-labelled protein substrates when incubated with 17.5-day rat yolk sacs in the presence of 10% calf serum. The native substrate least susceptible to digestion is ^{125}I-labelled orosomucoid (CI = 1.5h), the value for native ^{125}I-labelled bovine serum albumin is 0.78h. and the value for the most susceptible substrate, ^{125}I-labelled insulin is 0.32h. Treatment of these substrates with formaldehyde (pH 10), a procedure that generally makes proteins more resistant to proteolytic attack through their chemical modification (Feeney et al., 1975) also made the above substrates less susceptible to proteolysis by enzymes within yolk-sac lysosomes. The Catabolic Indices for the above substrates were doubled giving values of 3.64, 1.53 and 0.82h respectively. This effect was not observed, however, for ^{125}I-

-labelled lysozyme and ^{125}I-labelled ribonuclease. It will be noticed that the Catabolic Index for a protein substrate digested by the 17.5-day rat yolk sac in the presence of 10% calf serum (see Table 3.1) is always greater than the corresponding value for the same protein digested by yolk sacs incubated in serum-free medium (see Table 7.6). This suggests that 10% calf serum, in addition to inhibiting pinocytosis by up to 50% [Ibbotson, 1978; also compare value for the rate of uptake of ^{125}I-poly(vinylpyrrolidone) in Tables 2.1 and 3.5] also inhibits the proteolysis of a substrate once it has been endocytosed. The underlying mechanisms for these inhibitions are not known. However, any explanation must also take into account the fact that the presence of 10% calf serum does not modify the initial lag-period for formaldehyde-denatured ^{125}I-labelled bovine serum albumin (results not shown). The times taken for the yolk sac to dispose of the ^{125}I-labelled substrates discussed above compare well with those observed by other research workers, who, more generally have calculated the half-lives of endocytosed proteins. Davidson et al, (1971) reported a half-life of 14min for the disposal of ^{125}I-labelled ribonuclease A by mouse-kidney lysosomes. Mego & McQueen (1965) showed the half-life of denatured serum albumin digested by liver cells to be approx. 0.75h. Cohen (1975) graphically illustrated the disappearance of endocytosed proteins digested by mouse macrophages in culture; for serum albumin, haemoglobin, horseradish peroxidase and lysozyme. The half-lives were 5, 20, 30 and 35h, respectively. In comparison to the 17.5-day rat yolk sac, macrophages seem to clear endocytosed proteins at a very slow rate. For cells in culture, lag--periods of 7-8, 20 and 30min have been reported for ^{125}I-labelled insulin, formaldehyde-denatured ^{125}I-labelled human serum albumin and

and ^{125}I-labelled plasma low-density lipoproteins (see Section 4.4).

The pattern of ammonium ion inhibition of the digestion of endocytosed ^{125}I-labelled proteins and peptides also correlates with the substrate's susceptibility to attack by lysosomal enzymes (see Table 7.5). The mechanism of the lysosomal inhibition by ammonium ions is uncertain, but an elevation of the intralysosomal pH is inferred, thus inhibiting the action of all lysosomal enzymes with an acid pH optimum rather than the inhibition of particular enzymes (see Chapter 5). The following scheme offers a possible explanation of how in the yolk--sac system ammonium ions might inhibit the lysosomal digestion of the protein substrates but not the peptide substrates. If, for the protein substrates, the digestive capacity of the lysosomal enzymes is only a little greater than the endocytic capacity of the yolk-sac epithelial cells, normally all the endocytosed proteins will be digested. But if lysosomal enzymes are inhibited by ammonium ions the endocytosed proteins will accumulate in the lysosomes in an undigested form, because the digestive capacity will now be substantially less than the endocytic capacity. However, if the peptide substrates are endocytosed at a rate similar to that of protein substrates but are much more susceptible to attack by lysosomal enzymes, the lysosomal capacity for digestion even after strong inhibition by ammonium ions, will still remain far greater than the endocytic capacity; consequently, there will be no apparent inhibition of the digestion of the endocytosed peptides.

It was suggested in Chapter 5, that a possible explanation for the complete inhibition, by 20mM-ammonium ions, of formaldehyde-denatured ^{125}I-labelled bovine serum albumin digestion by cultured rat yolk sacs might be an inhibition of pinosome-lysosome fusion. Clearly the evidence here rules out this possibility. Such an inhibition would result in a

decreased digestion of the protein and peptide substrate alike.

Finally, the possibility that the adsorptive pinocytosis of the ^{125}I-labelled peptides by the rat yolk sac might occur at a low rate, or not at all, as suggested by the findings of Ryser (1970) was not the case. Table 7.6 shows that, in general (with ^{125}I-labelled ribonuclease being a remarkable exception), the smaller the substrate the greater is its rate of uptake by adsorptive pinocytosis.

CHAPTER EIGHT

GENERAL DISCUSSION

GENERAL DISCUSSION.

After a general introduction to the concepts of endocytosis, lysosomal protein digestion and the vacuolar system of animal cells and a description of some morphological and biochemical studies of endocytosis in the rat visceral yolk sac, reports have been presented of investigations of various aspects of endocytosis, lysosomal protein catabolism and (in collaboration with S. Knowles & F.J. Ballard, CSIRO, Division of Human Nutrition, Adelaide, Australia) the role of lysosomes in endogenous yolk-sac protein breakdown. Individual chapters of this thesis generally contain substantial introductions, and the investigations reported have been discussed at length at the end of each chapter. Therefore, in order to avoid overlap with the material presented previously, only particular aspects of the work that merit further discussion in a broader context will be discussed below along with areas of work that need further research.

8.1 Selective pinocytic uptake of proteins and peptides by 17.5-day rat yolk sacs.

Adsorptive pinocytosis is concluded to be the dominant mechanism of uptake by 17.5-day rat yolk-sacs of all the proteins and peptides investigated. The ^{125}I-labelled forms of insulin, ribonuclease and lysozyme (Chapter 2) are captured virtually entirely by an adsorptive mechanism; these results extend the findings of Moore et al. (1974, 1977) and Ibbotson (1978) that ^{125}I-labelled rat immunoglobulin G and several denatured forms of ^{125}I-labelled bovine serum albumin are captured by the 17.5-day rat yolk sac by adsorptive endocytosis. It can also be reasoned that the ^{125}I -labelled peptides: calcitonin, insulin B-chain and glucagon are captured almost entirely in the same manner. Although direct experiments

were not performed to determine whether these peptides, at the concentrations used in pinocytosis experiments, stimulate pinocytosis, results (in Chapters 3 & 4 respectively) show that calcitonin (100µg/ml) and glucagon (several concentrations) are in fact inhibitors, and not stimulators of rate of pinosome formation. The effect of insulin B-chain is unknown, but there is no reason to suppose that it stimulates pinosome formation. [Glucagon might have an important role to play in controlling yolk-sac pinocytic activity, thus regulating some, as yet unidentified but important, process necessary to the normal reproductive physiology of the rat (and possibly other animals, e.g. rabbits). A clue to the possible importance of glucagon in reproductive physiology is the observed rise in serum glucagon in rats during late pregnancy (Girard et al., 1974; Metzger et al., 1974; Seudek, 1975).]

The widely differing rates of uptake of the various ^{125}I-labelled proteins and peptides can thus be explained solely by the differential extents of their adsorption to pinocytosing plasma membrane (Section 2.4). The specificity of the selective adsorption process was examined in Chapters 2, 3 & 7. Hydrophobic and basic regions (Chapter 3) of substrates seem to be recognised (c.f. formaldehyde-denatured bovine serum albumin and ribonuclease see Section 3.4) and seem to adsorb to different regions of the pinocytosing plasma membrane. Chapman-Andresen (1977) commented on the nature of the pinocytosing plasma membrane of the 17.5-day rat yolk sac. She inferred that the adsorptive uptake of denatured ^{125}I-labelled albumin (that is expected to be negatively charged at neutral pH) "indicates a net positive charge on the surface of the (yolk-sac) tissue". Clearly for this inference to be correct it denies that more than one type of interaction (namely electrostatic) can occur between a substrate protein and pinocytosing plasma membrane. If (as indicated in Chapter 3)

hydrophobic and basic regions of substrate are both recognised by the pinocytosing plasma membrane, using the same argument presented by Chapman-Andresen (1977), it can be inferred that the extracellular surface of this membrane is both hydrophobic and negatively charged (and not positively charged). The possibility that yolk-sac pinocytic-membrane recognition of regions of molecules depends neither on hydrophobic nor basic characteristics has yet to be investigated. Trifluoroacetylated bovine serum albumin is rapidly endocytosed by the rat yolk sac (P. Agarwal & A.T. Moore, unpublished results). This molecule is highly polar and negatively charged, but, it is not yet fully characterised so that it may either have regions that are hydrophobic, or the highly polar trifluoroacetyl groups may bind cations thus acquiring an overall net positive charge at the surface of the molecule. In Section 2.1 examples of several glycoprotein-substrate receptor recognition systems were described. The well-characterized asialoglycoprotein recognition system that binds these proteins to mammalian liver parenchymal cells (Ashwell & Morell, 1974a,b) was in fact shown not to be present in the yolk-sac tissue (Moore *et al.*, 1977). This system is, indeed, peculiar to the liver parenchymal cells. Nevertheless, it is not known whether the rat yolk sac can selectively endocytose lipoproteins, lysosomal enzymes and certain types of naturally occurring or semi-synthetic glycoproteins of the types indicated in Section 2.1.

There appears to be, perhaps superimposed upon the recognition of hydrophobic, basic and possibly other characters, a preferential uptake of peptide substrates over the larger proteins (Chapter 7). In agreement with this generalization, is the demonstrated lower rate of uptake of lysozyme aggregates compared with lysozyme monomers (Chapter 2).

Possible reasons for this latter phenomenon have been discussed in Section 2.4. In addition, it is possible that preferential uptake of smaller macromolecules occurs because they present a larger surface area per unit weight of substrate than do the larger macromolecules. Alternatively, the latter may be sterically hindered from interacting with the surface of the pinocytosing plasma membrane. Such steric hindrance of large macromolecules might occur if they are unable to filter through the dense regions of the cell-surface glycocalyx that are observed to line the cavities from which pinosomes are formed (Jollie & Triche, 1971).

The above observations contrast with those of Ryser (1970) and Gabathuler & Ryser (1975) who investigated the endocytosis of peptides and proteins in Sarcoma S180 cells in culture. These authors presented evidence indicating that large macromolecules are taken up more rapidly than small ones. Also ferritin aggregates are endocytosed more rapidly than ferritin monomers. The latter phenomenon was attributed to enhanced phagocytic activity (Gabathuler & Ryser, 1975) and is in agreement with the higher rates of uptake of protein aggregates, compared with the monomeric forms of the corresponding proteins, by the phagocytic Kupffer cells, examples of which are cited in Section 2.4. The earlier observation of Ryser (1970) might also be attributed to enhanced uptake of the larger substrates by phagocytosis. Morphological (Jollie & Triche, 1971; see also Section 1.7) and biochemical evidence (Goetze et al., 1976) indicate that endocytic uptake in the rat visceral yolk sac is by micropinocytosis alone. The above differences between selective uptake in a phagocytic system and the selective uptake in the micropinocytically active rat yolk sac suggest an hypothesis that is readily amenable to investigation. That is, micropinocytosis selectively captures soluble substrates and shows a preference for macromolecules of a small size

whereas phagocytosis selectively captures substrates with a preference for materials of larger size. A mechanistic basis for such a difference has not been established but, all else being equal, adsorption to endocytic membranes may show a preference for small macromolecules, but, in micropinocytosis, no induction of uptake is required whereas in phagocytosis uptake needs to be induced (Stossel, 1973; Silverstein et al., 1977) and this is only achieved by the larger macromolecules.

The hypothesis purposely does not include phenomena that might be associated with macropinocytosis. It is envisaged that the mechanism underlying macropinosome formation in some instances may resemble phagocytosis and in other micropinocytosis.

8.2 Proteolytic activities of the 17.5-day rat yolk sac.

Knowledge of the proteolytic enzymology of the rat yolk sac is poor. It is now clear, however, from the studies described herein and those of others (Williams et al., 1971; Goetze et al., 1976) that the rat yolk sac has a highly active proteolytic system, namely the lysosomal system. As with many other cells and tissues the existence of particular cathepsins within lysosomes of yolk sacs can be only inferred. The inhibitions in intact yolk-sac tissue of the digestion of formaldehyde-denatured ^{125}I-labelled bovine serum albumin caused by leupeptin, chymostatin and antipain (see Chapter 6) and the inhibitions caused by these inhibitors and pepstatin of the digestion of this substrate by a cell-free extract of yolk sacs, strongly suggest the presence of enzymes resembling cathepsin D or cathepsin L, or both, and of cathepsin D or cathepsin E, or both. Before any particular enzymes can be held responsible for the digestion of the endocytosed ^{125}I-labelled albumin further investigations are required.

The presence of proteolytic enzymes associated with the extra-

cellular cell-surface of the rat yolk sac is possible [c.f. those present on the surface of kidney brush border membranes (Kenny, 1977)]. But if such enzymes do exist, they have negligible digestive activity toward several ^{125}I-labelled substrates: rat immunoglobulin G, formaldehyde-denatured bovine serum albumin, lysozyme, ribonuclease, insulin, calcitonin and insulin B-chain. Each of these substrates is rapidly digested by cultured rat yolk sacs but at an intracellular site (see Chapters 2,4 & 7). The only mechanism envisaged for the translocation of each of the above substrates from the extracellular medium to an intracellular site is pinocytosis; fusion of the pinocytic vesicles with lysosomes resulting in the observed proteolytic activity. A lysosomal site of digestion was confirmed for formaldehyde-denatured ^{125}I-labelled bovine serum albumin digestion (Chapter 4). The intracellular site of the digestion of the remainder of the above protein and peptide substrates can safely be considered to be lysosomal as discussed in Chapters 2 & 7.

It has been suggested that in hepatocytes, and other insulin target cells, the peptide hormone insulin might gain access to the cytoplasm and there be degraded by either an insulin specific proteinase (Brush, 1971) or a glutathione-insulin transhydrogenase (Thomas, 1973), or both. It is not known whether similar enzymes exist in the rat yolk sac, but if they do, it is difficult to conceive of a mechanism that might translocate insulin into the cytoplasm of cells either within the yolk-sac tissue or the liver thus permitting such enzymes to play an active role in insulin catabolism. Grisolia & Wallace (1976), however, have postulated a model for a role of lysosomes in peptide hormone degradation, and suggest that insulin can be partially degraded in hepatic lysosomes by a lysosomal glutathione-insulin transhydrogenase. The occurrence of such an enzyme in yolk-sac lysosomes is unknown but

its existence might explain how the disulphide linkages of insulin (and possibly other proteins) are cleaved.

It is possible that a lysosomal site of digestion of insulin within hepatocytes could be demonstrated using the microbial proteinase inhibitors that were shown in Chapter 6 to be inhibitors of intralysosomal proteolysis. A possible complication, however, is that insulin is easily and rapidly digested by lysosomal enzymes in comparison to some other proteins (see Chapter 7) so that substantial inhibition of lysosomal catheptic activity might be necessary to prevent intralysosomal insulin digestion. This is apparent from the observations in Chapter 7 that the intralysosomal digestion of insulin is very much less sensitive to weak--base inhibition than is the intralysosomal digestion of protein substrates.

The digestion of ^{125}I-labelled peptides (insulin, calcitonin, insulin B-chain and glucagon) by disrupted rat-liver lysosomes, isolated by the method of Trouet (1974), is several orders of magnitude more rapid than the digestion of ^{125}I-labelled proteins (rat immunoglobulin G, denatured bovine serum albumin, and lysozyme). This observation (Chapter 7) is in full agreement with the "all-or-none" hypothesis of Huisman (1974); that was forwarded to explain the apparent absence of intermediate sized digestion products derived from proteins hydrolysed intralysosomally. It is envisaged that after initial proteolytic attacks by the cathepsins on proteins to yield peptide fragments, the fragments are digested extremely rapidly and completely so that only very small quantities of intermediate sized digestion products are present in the lysosomes at any one time. However, the observed differences in the lag-periods (shown in Chapter 7) for the various protein and peptide substrates during their digestion by cultured rat yolk sacs, indicates that for some proteins (e.g. formaldehyde--denatured bovine serum albumin), once they have reached an intralysosomal

site, a considerable period of time elapses before the protein is completely digested. Consequently, this period must represent the time taken for the initial rate-limiting cleavages to occur which result in the formation of the rapidly degradable peptide fragments. The difference in the duration of the lag-periods between the peptide and protein substrates suggest that more than one, and probably several, initial degradative attacks must occur before the rapidly digestible fragments are produced. It is possible that the initial intralysosomal degradative attacks on the above protein substrates are not peptide-bond cleavages but disulphide bond reductions. If this is the case, the durations of the lag-period for different sized proteins that contain no disulphide bonds should be similar and this should be short in comparison to lag-periods for proteins containing disulphide bonds. Further research is required to discover whether disulphide reductions constitute an initial, rate-limiting attack on disulphide containing proteins or whether the initial attacks are indeed a series of peptide-bond cleavages that result in the formation of proteins that remain relatively intact before the "last" of such initial attacks result in the formation of the rapidly digestible peptide fragments.

If the latter of the above occurs, it is possible that substrates that have undergone limited proteolysis can be isolated. Analysis of these fragments to determine the site of cleavage would give valuable information that could be compared with the specificity of the known lysosomal cathepsins, thus enabling the identification of those cathepsins that are responsible for the initial rate-limiting attacks on proteins.

The lysosomal enzymes β-N-acetylglucosaminidase and β-galactosidase are released from 17.5-day rat yolk sacs incubated in medium 199 containing 10% calf serum (Roberts et al., 1977). The rate of release was found to be approx. 1% of the tissue-associated enzyme activity per h.

If the lysosomal cathepsins are also released at this rate then substantial extracellular digestion of peptide substrates would be expected to occur when they are added to yolk sacs incubated in medium containing 10% calf serum. The value of 1%/h is approx. 15 times greater than would be predicted from results presented in Chapter 7 (on the digestion of peptides by enzymes present in the incubation medium). After incubating yolk sacs in serum-free medium 199 for 3h, less than 0.2% of the catheptic activity of the tissue was present in the incubation medium. From the above observations it can be inferred either that cathepsins are not released at the same high rate as the glycosidases (thus possibly explaining the apparent lack of lysosomal storage disease associated with protein catabolism c.f. mucolipidoses, Neufeld et al., 1977) or that the presence of calf-serum promotes the exocytic release of lysosomal hydrolases. Further research is necessary to determine whether either of these inferences is correct. It was shown (see Fig. 5.9) however, that β-N-acetylglucosaminidase is released at a rate of 0.3%/h when incubated in serum-free medium thus suggesting the latter explanation might be incorrect. This, however, is not consistent with the hypothesis of Lloyd (1978) that lysosomal enzymes are exocytosed into the extracellular medium consequent on recycling of pinocytic membrane since the extent of the latter would be expected to decrease secondarily to the approximately two-fold decrease in rate of pinosome formation in yolk sacs incubated in the presence of 10% calf serum.

It is interesting to note here, that whereas glucagon can inhibit exogenous protein catabolism through inhibition of pinocytosis, once a protein is captured within pinosomes its lysosomal catabolism progresses at the normal rate. This is also true when dibutyryl cyclic AMP is used in place of glucagon. Both these compounds had no effect on the

rate of digestion of formaldehyde-denatured ^{125}I-labelled bovine serum albumin when this was expressed as a proportion of rate of uptake of the substrate (values taken from Table 4.7, were: control, 97.5% ± 12.8%; 10^{-6} M-glucagon, 100.5 ± 13.1; 5 x 10^{-4} M-db-cAMP, 100.9 ± 4.7. Similar values for the average rates of tissue accumulation, expressed as a percentage of the rate of substrate uptake were: control, 21.9% ± 5.7%; 10^{-6} M-glucagon, 19.5 ± 1.8; 5 x 10^{-4} M-db-cAMP, 22.6 ± 4.7). These results indicate that the glucagon and the db-cAMP (present at concentrations that are sufficient to inhibit the rate of pinosome formation by 58.9% ± 17.5% and 34.1 ± 12.3 respectively) controls neither pinosome fusion with lysosomes nor intralysosomal proteolysis. Moreover, although glucagon is a stimulator of endogenous protein breakdown in perfused liver (Woodside et al., 1974) and in isolated hepatocytes (Hopgood et al., 1977) it is without effect on the rate of digestion of endogenous yolk-sac proteins (Knowles & Ballard, unpublished work). Nutritional step-down , achieved by removing calf serum from medium in which yolk sacs are incubated, enhanced the rate of pinosome formation, but no effect is observed on the rate of endogenous yolk-sac protein breakdown (Knowles & Ballard, unpublished work). Furthermore, insulin, an inhibitor of hepatic protein breakdown (Mortimore & Mondon, 1970; Knowles & Ballard, 1976), is without effect on yolk-sac protein breakdown (Knowles & Ballard, unpublished work). Clearly endogenous yolk-sac protein catabolism is under very different controls to that found in the liver. [This is probably a marked reflection on the very different metabolic roles of the two tissues.] Also, whereas exogenous protein catabolism in the rat yolk sac may be under external controls that of endogenous protein catabolism does not seem to be.

8.3 Role of lysosomes in the catabolism of endogenous yolk-sac proteins.

An attempt has been made to identify inhibitors of intralysosomal catheptic activity. The digestion, by yolk sacs, of formaldehyde-denatured ^{125}I-labelled bovine serum albumin was employed to monitor this activity since it was shown (Chapter 4) that it is hydrolysed exclusively within lysosomes. The weak bases (Chapter 5): ammonium-, methylammonium- and ethylammonium chlorides and chloroquine and the microbial proteinase inhibitors (Chapter 6): leupeptin, chymostatin and antipain (but not: pepstatin, elastatinal and bestatin) were shown to inhibit intralysosomal catheptic activity within intact yolk-sac tissues. Knowles & Ballard (unpublished work) showed that each of the above weak bases and each of the above inhibitory microbial compounds also inhibited the breakdown of endogenous yolk-sac proteins previously labelled *in vitro* with [^{3}H]leucine.

Wibo & Poole (1974) and Hopgood *et al.* (1977) suggested that results of the types discussed above for weak bases and microbial proteinase inhibitors, indicate that lysosomes are involved in endogenous protein digestion. However, the weak bases were shown to inhibit energy production (Knowles & Ballard, unpublished results and Goldberg & St. John, 1976 report of unpublished results; see Section 5.4). Since endogenous cell-protein breakdown is energy dependent (Ballard, 1977) and the nature of the energy requirement is unknown, it is not possible to conclude with confidence that the inhibition by weak bases of endogenous cell--protein breakdown in intact cells is a result of lysosomal inactivation. Thus, results of the type presented by Wibo & Poole (1974) and in Chapter 5 of this thesis can not be argued to be evidence for a lysosomal role in endogenous cell-protein turnover without also having shown either one or a combination of: (a) accumulation of cell-protein within lysosomes, (b) a lack of inhibition of cell-protein breakdown under

conditions in which energy production is inhibited or (c) that the energy requirement of endogenous cell-protein breakdown is solely necessary for either autophago-lysosome formation or the maintenance of a normal lysosomal hydrolytic capacity (e.g. maintenance of an acid intralysosomal pH). None of these conditions were met either by Wibo & Poole (1974) or in Chapter 5 of this thesis.

It is concluded, however, that since antipain, chymostatin and leupeptin are inhibitors of endogenous, yolk-sac protein breakdown (up to 20% when present at 50μg/ml of incubation medium) lysosomes are indeed involved in endogenous yolk-sac protein breakdown. It is possible that lysosmes are involved in very much more than 20% of such breakdown since further inhibition of lysosomal proteolysis of the exogenous protein is obtained with higher concentrations of leupeptin (Chapter 6). Moreover, endogenous yolk-sac protein hydrolysis in lysosomes may well be less susceptible to inhibition by the microbial compounds than is formaldehyde-denatured ^{125}I-labelled bovine serum albumin.

It was hoped that monitoring the digestion of formaldehyde--denatured ^{125}I-labelled bovine serum albumin by yolk sacs would enable a quantitative estimation to be made of lysosomal activity in the presence of the various proteolytic inhibitors. It was further hoped that a comparison of the extent of lysosomal inactivation, measured by the observed impaired digestion of the exogenous protein could be compared with the extent of the inhibition of endogenous cell-protein breakdown under identical culture conditions. It was shown (Chapter 7) however, that the extent of the observed lysosomal inactivation by the weak base ammonium chloride (20mM) was dependent on the exogenous substrate used to monitor lysosomal digestive activity. Values were obtained indicating

that the lysosomal inactivation by 20mM-ammonium chloride ranged from 0% to 100%! Although lysomal inactivation does indeed occur in the presence of the 20mM-ammonium chloride, the lysosomal capacity for the digestion of some substrates (the peptide substrates) remained far greater than the endocytic capacity of the yolk-sac tissue (see Section 7.4). The results clearly showed that a comparison of the extent of inhibition by the weak bases, between exogenous substrate digestion and endogenous substrate digestion yields no meaningful information from which the extent of lysosomal participation in cell protein breakdown can be estimated. It remains to be investigated whether a selective inhibition of the intralysosomal digestion of various exogenous protein and peptide substrates occurs when the microbial compounds (Chapter 6) are used in place of the weak bases. If similar extents of inhibitions are achieved it is then possible to quantitate the lysosomal catheptic activity and thus obtain some estimates of the extent to which lysosomal and non-lysosomal digestive activities are involved in endogenous cell-protein breakdown. If a pattern of inhibition occurs similar to that obtained with 20mM-ammonium ions, then only when the exogenous substrate and the endogenous substrate are the same can the results of such studies be interpreted with confidence.

APPENDIX I

Determination of empirical correction factors for the acid-soluble radioactivity assays.

When measuring the acid-soluble radioactivity present in culture medium as described in Section 2.2.1(3), the supernatant obtained following the trichloroacetic acid precipitation and centrifugation procedure contains only a part of that present in the original 1.0ml of culture medium (due to some of the acid-soluble radioactivity becoming occluded within the pellet of precipitated protein). The acid-soluble radioactivity present in the supernatant is also contained in a larger volume of solution (due to the addition of acid precipitant). Both the loss of acid-soluble radioactivity and the increase in the volume of the solution counted in the Packard Selektronic gamma spectrometer will decrease the observed count for the acid-soluble radioactivity. In order to obtain accurate values for the quantity of acid-soluble radioactivity and to standardize the counting geometry to 1.0ml it was necessary to correct the observed count to that which would have been observed if all the acid-soluble radioactivity had been present in 1.0ml of culture medium. This was achieved by multiplying the observed count for the supernatant (corrected for background) by an empirical correction factor, E.

Empirical correction factors were obtained using acid-soluble radiotracers. Either sodium [^{125}I]iodide, [^{125}I]iodo-L-tyrosine or glycyl-[^{125}I]iodo-L-tyrosine was added to culture medium 199 containing 10% (v/v) calf serum to give 50-150 x 10^3 c.p.m. per millilitre. When [^{125}I]iodide was used, solid KI was added to 1% (w/v) to prevent excessive binding of the [^{125}I]iodide to the assay tubes. Portions (1.0ml) of the

culture medium were pipetted into 3ml disposable polystyrene tubes and the radioactivity counted (total acid-soluble radioactivity). The protein present in each 1.0ml of culture medium was then precipitated using either trichloroacetic acid (0.5ml) or phosphotungstic acid (0.5ml) followed by trichloroacetic acid (0.5ml) as described in Section 2.1.1(3). The supernatant obtained after the centrifugation procedure was decanted into a new 3ml polystyrene tube and the radioactivity counted as before (observed acid-soluble radioactivity). The empirical correction factor, E, can be simply calculated by dividing the total acid-soluble radioactivity, A (c.p.m. in 1.0ml of culture medium, corrected for background) by the observed acid-soluble radioactivity, B (c.p.m. in the supernatant, corrected for background) according to the equation: $E = B/A$.

When the acid-soluble radioactivity was present in culture medium deficient in calf serum, 0.1ml of calf serum was added to the 1.0ml of culture medium just before the addition of acid. This procedure resulted in a further dilution of the acid-soluble radioactivity, consequently a different set of empirical correction factors needed to be calculated.

Table A.1 shows the empirical correction factors determined for both the trichloroacetic acid and the phosphotungstic acid-trichloroacetic acid protein precipitation methods, using the three different acid-soluble radiotracers present in the culture medium, both with and without added calf serum. In general, [^{125}I]iodo-L-tyrosine and glycyl-[^{125}I]iodo-L--tyrosine gave similar empirical correction factors, whereas [^{125}I]iodide consistently gave smaller values. Because, [^{125}I]iodo-L-tyrosine was always found to be the major hydrolysis product when using ^{125}I-labelled protein substrates in the yolk-sac culture system, the empirical correction factors obtained for this acid-soluble radiotracer were used in experiments.

Table A.1 Empirical correction factors determined for the acid-soluble radioactivity assay.

The empirical correction factors were determined as described in the text. Values are given as the mean ± standard deviations of six determinations.

Percentage of calf serum present in the original culture medium	Acid-soluble radiotracer	Precipitation method	
		Trichloroacetic acid (TCA)	Phosphotungstic acid + TCA
10%	[^{125}I]iodide	1.259 ± 0.025	1.480 ± 0.095
	[^{125}I]iodo-L-tyrosine	1.274 ± 0.011	1.514 ± 0.14
	glycyl-[^{125}I]iodo-L-tyrosine	1.291 ± 0.014	1.559 ± 0.034
0%	[^{125}I]iodide	1.294 ± 0.016	1.546 ± 0.017
	[^{125}I]iodo-L-tyrosine	1.330 ± 0.022	1.624 ± 0.021
	glycyl-[^{125}I]iodo-L-tyrosine	1.328 ± 0.056	1.617 ± 0.025

APPENDIX II

Expression of pinocytic uptake data for experiments described in Section 2.2.1

As described before (see Section 2.2.2), the method used to calculate and express pinocytic uptake data was that of Williams et al. 1975a,b). For reasons discussed in Section 2.2.2, pinocytic uptake is expressed as the volume of incubation medium whose contained substrate is captured by unit quantity of the yolk-sac tissue. This volume (whose units are microlitres of incubation medium per mg of yolk-sac protein) is given by the expression

$$V = Y/(M.P) \qquad (A.1)$$

where Y is the total radioactivity (in c.p.m., corrected for background) in the whole yolk sac, M is the radioactivity (in c.p.m., corrected for background) per microlitres of incubation medium and P is the protein content (in milligrams) of the yolk-sac tissue. Thus a value of uptake is obtained for each yolk sac at each time interval in the experiment. The rate of uptake [^{125}I]labelled poly(vinylpyrrolidone) [^{125}I-PVP] is derived from a plot of uptake against the duration of the respective incubation, (see e.g. Fig. 2.1). The slope of the plot gives the rate of uptake, and is called the "Endocytic Index" or "EI"; its units are microlitres per milligram of yolk-sac protein per hour of incubation.

In all the experiments reported in this thesis, uptake of ^{125}I-PVP by the yolk sac resulted in a detectable fall in the ^{125}I-PVP concentration in the incubation medium but the rate of fall was never greater than approximately 0.1%/h in medium plus 10% calf serum or 0.2%/h in serum-free medium. Thus the concentration of ^{125}I-PVP in the incubation medium is effectively constant for the duration of the incubation,

therefore the measured value of the radioactivity in the medium at the end of an incubation period can be used for M in equation A.1.

In experiments where ^{125}I-labelled protein substrates were used, a large proportion of the ingested radio-label was not retained within the yolk-sac tissue, but was released back into the medium as "acid-soluble" digestion products. It is appropriate therefore to modify the numerator in the uptake equation A.1, to give:

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

where 10(S-F) is the total quantity of "acid-soluble" radioactivity released into the incubation medium (10.0ml) during the incubation period. Y is the total radioactivity in the whole yolk-sac (c.p.m., corrected for background), S is the acid-soluble radioactivity in the incubation medium at the end of an incubation period (c.p.m. per ml of incubation medium, corrected for background). F, a correction term, is the sum of the acid-soluble radioactivity present at the start of an incubation and that radioactivity which may be lost from the protein during the incubation period by processes not associated with the yolk sac. F was determined by assaying the percentage acid-soluble radioactivity in the incubation medium in the control flask at the end of each experiment (i.e. the incubated flask which contained medium and dissolved substrate but no yolk sac). The correction term F in equation A.2 can be derived from equation A.3:

$$F = M'' \times Q \qquad (A.3)$$

where M" is the total radioactivity present in 1.0ml of incubation medium from the control flask (c.p.m., corrected for background). Q is

the fraction of radioactivity in the control medium that is acid-soluble. [$Q = E.C_1/C_2$, where C_1 is the mean acid-soluble radioactivity (c.p.m. per ml, corrected for background) and C_2 is the mean total radioactivity of the incubation medium (c.p.m. per ml, corrected for background). E is an empirical correction factor (see Appendix I) that corrects for both the loss of acid-soluble radioactivity and a change in counting geometry when assaying the incubation medium for acid-soluble radioactivity.]

In all the experiments with a labelled protein as substrate, it was found that the rate of uptake was much greater than for ^{125}I-PVP. Hence there was significant depletion of the substrate in the incubation medium over the period of incubation [almost 10% per hour when culturing a yolk sac, containing 7mg protein, in the presence of a substrate with an Endocytic Index of 150µl/h per mg yolk-sac protein]. It was therefore no longer accurate to use the total medium radioactivity M, as in equation A.1 but to use M', the mean concentration of radiolabelled substrate over the particular incubation period (c.p.m. per µl of incubation medium, corrected for background). M' is calculated from equation A.4:

$$M' = M - [(S-F)/2 \times 10^3] \qquad (A.4)$$

where S and F are as defined in equation A.2.

The uptake, V, in equation A.2, again has the units of microlitres of incubation medium per milligram yolk-sac protein. A plot of the values of uptake against time (see e.g. Fig. 2.2) should be linear over the incubation period used. The slope of the plot gives the value of the Endocytic Index.

[The term F in equation A.2, A.3 and A.4, in the experiments described in this thesis, represents, almost exclusively, the acid-soluble radioactivity present in the incubation medium at the start of the incubation.

Evidence that this is so comes from the observations that: firstly, no increase in acid-soluble radioactivity was observed in control experiments where radiolabelled substrate was incubated without yolk sac, and second, incubation medium, removed at intervals (up to 6h) from flasks containing yolk sacs and incubation medium without added substrate, contained no significant hydrolytic activity when re-incubated under the usual incubation conditions with added substrate.]

Calculation of pinocytic uptake data.

Computer programs (BASIC) were written for the calculation of data for both the uptake of the non-digestible substrate ^{125}I-labelled poly(vinylpyrrolidone) and the digestible ^{125}I-labelled protein substrates, and are shown below. Since uptake was obviously linear with time, a best straight line was fitted to each set of points for uptake against time by linear regression analysis. Values of the slope and the intercept of these lines were obtained from a standard computer program along with the value of the association Correlation Coefficients. The calculations and the linear regression analysis were made with an ICL 4130 computer.

(1) Program for uptake of an indigestible substrate [e.g. ^{125}I-labelled poly(vinylpyrrolidone)]

"Input" data are as defined in the program but the assay data were collected as follows:

H(X) Duration of the yolk sac incubation period (h).

I(X) Mean observed radioactivity count for 1.0ml of incubation medium.

K(X) Mean observed radioactivity count for 1.0ml of alkaline yolk-sac solution.

L(X) Total protein content of the incubated yolk sac (mg).

```
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  IMPUT 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 DRSPEC 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
```

(2) Program for uptake of a digestible substrate [e.g. ^{125}I-labelled proteins]

"Input" data are as defined in the computer program but the assay data were collected as follows:

H(X) Duration of the yolk sac incubation period (h).

I(X) Mean observed radioactivity count for 1.0ml of incubation medium.

J(X) Mean observed "acid-soluble" radioactivity count for 1.0ml of incubation medium after the precipitation and centrifugation procedure.

K(X) Mean radioactivity count for 1.0ml of the alkaline yolk-sac solution.

L(X) Total protein content of the incubated yolk sac (mg).

```
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*Q)+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);L(X),FRE(6);E(X),FRE(6);S(X)
200 NEXT X
205 STOP
```

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