PINOCYTOSIS AND PROTEIN DEGRADATION IN THE RAT VISCERAL YOLK SAC

by

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Post Script

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Abbreviations

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

E.I.	Endocytic Index
PTA	phosphotungstic acid
T.A.R.	tissue-associated radioactivity
TCA	trichloroacetic acid
y.s.	yolk sac
^{125}I-fdBSA	formaldehyde-denatured ^{125}I-labelled bovine serum albumin
^{125}I-PVP	^{125}I-labelled polyvinylpyrrolidone
^{125}I-ribonuclease	^{125}I-labelled ribonuclease
^{125}I-RNase	^{125}I-labelled ribonuclease

Abstract

The rat visceral yolk sac (17.5 days gestation) was incubated *in vitro* to try to elucidate some of the factors important in controlling pinocytosis and protein degradation. An investigation of four closely-related ribonuclease molecules revealed that a minor structural change in a protein (possibly of a type that occurs *in vivo*) greatly enhanced its rate of uptake by the yolk-sac tissue, supporting the suggestion that proteolytic "nicking" may be a signal for removal of a protein from the circulation. This study also showed there to be no mannose receptor on the surface of the yolk sac, and that the presence of a small oligosaccharide on the surface of the ribonuclease molecule neither hindered its entry into the tissue, nor affected its subsequent rate of digestion.

The maximum pinocytic rate was exhibited when yolk sacs were incubated in serum-free medium. Addition of serum (up to 50%, v/v) led to a progressive fall in both the pinocytic rate and the rate of degradation of internalized radiolabelled-protein. Addition of amino acids and formaldehyde-denatured BSA to the incubation medium also led to a fall in the rate of degradation of endogenous proteins (data provided by F.J.B. and S.E.K.). These findings support the suggestion that the control of pinocytosis and autophagy may be related by virtue of them both being membrane-related processes.

The effects of a variety of agents (insulin, cycloheximide, puromycin and bacitracin) on pinocytosis and on proteolysis of exogenous and endogenous proteins (the latter data compiled by F.J.B. and S.E.K.) were examined, and compared with the effects in other tissues, as reported in recent literature. Neither of two hypotheses (one based on membrane movement and one on response to intracellular amino acid

levels), postulated to relate the rate of pinocytosis to the rate of degradation of endogenous proteins, is supported by the data.

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

General Introduction

Every mammalian cell is bounded by its plasma membrane and, in order to survive, the cell needs to be able to ingest materials from its extracellular environment. Both low molecular weight and macromolecular materials can be ingested, and the cell has evolved a number of different mechanisms to achieve this. Certain low molecular-weight molecules (mainly non-polar) are able to diffuse across the lipid bilayer which surrounds the cell (e.g. O_2, CO_2 and H_2O) whereas the passage of small, polar compounds into the cell is achieved by active or passive transport mechanisms that involve membrane proteins (e.g. the transport of amino acids in the gut). The translocation of macromolecular material into the cell (important, for example, in the turnover of extracellular materials and in the defence of the organism) is realised by a different mechanism called endocytosis, which is the process by which such materials as serum proteins, bacteria and viruses, and antigen-antibody complexes are ingested by cells.

Endocytosis is a membrane-related phenomenon. The process often begins with a small depression in the plasma membrane which increases in size through a hemi-sphere to an almost complete sphere that communicates through a narrow neck with the extracellular space. Invagination is completed by the plasma membrane "pinching off" at the neck returning the cell surface to its former, smooth appearance and, at the same time, forming a vesicle located just beneath the surface of the plasma membrane. The cytoplasmic surface of the original plasma membrane becomes the cytoplasmic surface of this vesicle, and the extracellular surface of the plasma membrane becomes its internal surface. Any extracellular material in close proximity to the invaginating membrane can be captured by the cell and contained within the vesicle.

Endocytosis can be divided into two different forms. The first of these, phagocytosis, involves the ingestion of particulate material, and the size of vesicle formed is 1 μm, or larger, in diameter. Phagocytosis was first described by Metchnikoff in 1883, who followed the ingestion of particles by leucocytes under the light microscope. This process has a variety of important physiological roles such as providing a feeding mechanism for amoebae, and in enabling macrophages to engulf invading microorganisms as part of the organism's defence system. The second form of endocytosis is called pinocytosis, and is distinguishable from phagocytosis on two counts: firstly, pinocytosis involves the ingestion of extracellular fluid (not particles); and secondly, the vesicles formed are smaller than those formed in phagocytosis. Pinocytosis was first described by Lewis (1931) who observed the formation of small vesicles (0.2 to 1 μm in diameter), not containing particulate matter, from the plasma membrane of rat macrophages under the light microscope. Following the successful introduction of the electron microscope, Palade (1953) demonstrated that pinocytosis also occurred at the sub-light microscopic level in blood capillaries. To distinguish the electron microscopically-detectable pinocytic activity from that viewed in the light microscope, pinocytosis has been sub-divided into two forms, namely micropinocytosis and macropinocytosis respectively. Micropinocytosis has since been demonstrated, to different degrees, in virtually all mammalian cells; the vesicles formed are approximately 70 nm in diameter.

Conceptually, the process of pinocytosis is further sub-divided into two distinct types, fluid-phase and adsorptive (or surface) pinocytosis, but in the majority of cell types both occur concurrently. Fluid-phase pinocytosis is, as its name implies, simply the engulfing of extracellular fluid within the pinocytic vesicle as it forms.

Any solutes in the extracellular medium which have no affinity for the cell surface may be taken up into the cell by this route, and their rate of uptake will depend only upon their concentration in the extracellular fluid and upon the rate of vesicle formation by the cell (Williams et al., 1975a; Steinman et al., 1978). Adsorptive or surface pinocytosis, on the other hand, involves interaction of an extracellular solute with the plasma membrane, either by non-specific adsorption onto the surface of the cell, or by the binding of the component to a specific receptor molecule (Williams et al., 1975b; Brown et al., 1978). This effectively increases the concentration of the component within the forming pinosome, and therefore increases its rate of uptake by the cell. Mathematical models of these two forms of pinocytosis have been developed by Jaques (1969); these permit expression of the rate of uptake of extracellular components by these routes. The successful testing of these models has been performed by Williams et al. (1975a,b) and Roberts et al. (1977).

Once inside the cell, the pinosome is translocated through the cell with the contents becoming more concentrated, and perhaps fusion occurring with other incoming vesicles, until it comes into close proximity with lysosomes (membrane-bound vesicles containing a large range of acid hydrolases). Primary lysosomes are believed to be formed in a specialized portion of smooth endoplasmic reticulum, which is distinct from, but adjacent to, the concave face of the Golgi and is called the GERL (Golgi-associated Endoplasmic Reticulum from which Lysosomes arise). The incoming pinosome can fuse with a primary lysosome to form a secondary lysosome, and the contents of the pinosome become degraded by the hydrolytic enzymes from the lysosome; incoming pinosomes may also fuse with secondary lysosomes. Macromolecules within the secondary lysosome are degraded to the level of monomers and, since

the lysosomal membrane is permeable to components with a molecular weight of up to approximately 200 daltons, free amino acids produced by intralysosomal proteolysis are able to diffuse across the lysosomal membrane and escape from the cell. Following many fusion events, a secondary lysosome will eventually lose most of its hydrolytic capacity and be identified within the cell as a vesicle containing only indigestible remains, often appearing whorled under the electron microscope; such a vesicle is called a residual body. Continual removal of membrane from the cell surface during endocytosis quickly raises the problem of membrane replacement (Duncan & Pratten, 1977). Evidence is now accumulating that plasma membrane fragments are recycled back to the cell surface after transit through the lysosomal compartment (Schneider et al., 1978).

Other cell functions also depend on the vacuolar system of the cell but are not directly related to pinocytosis. Secretion is one such process where secretory vesicles, packed with secretory products from the Golgi, are translocated to the surface of the cell. Fusion of the secretory vesicle with the plasma membrane occurs by a process called exocytosis (the reverse of pinocytosis) resulting in the secretory components being expelled from the cell. If the cell is producing excess secretory products then lysosomes will fuse with the secretory vesicles before their arrival at the cell surface so that the products for export are catabolized; this process is called crinophagy. Pinocytosis following secretion may be envisaged as a mechanism for membrane recovery in such secretory cells. [Secretion has been extensively reviewed (Silverstein, 1978).] A process related to crinophagy is autophagy, and this appears to be present in all cell types. Its function is the turnover of intracellular materials, whether soluble intracellular proteins or organelles, and involves a

portion of the cytoplasm (often containing organelles) being surrounded by membrane (presumably originating from the endoplasmic reticulum) which eventually totally encapsulates the region. Fusion of such an autophagic vacuole with the lysosomal system will ensure the digestion of its contents. [This process has also been extensively reviewed (Rapoport & Schewe, 1978).]

In certain specialized cells, such as the endothelial cells lining the capillaries, pinosomes may be translocated right across the cell without any interaction with the lysosomal compartment; this is an exception to the usual rule of fusion between vesicles and lysosomes. On reaching the other side of the cell the vesicles fuse with the plasma membrane, extruding their contents into the extracellular medium, and this overall process is called diacytosis (Pitt, 1975). The function of diacytosis is to translocate extracellular components across the cell intact, and an example of this is the passage of immunoglobulins across cells (Wild, 1975).

The rate of uptake of a macromolecule by fluid-phase pinocytosis is dependent upon both the concentration of the extracellular material and the rate of vesicle formation by the tissue. The latter is greatly influenced by a number of other factors. The rate of pinocytosis is very dependent upon temperature, and a lowering of the temperature from 37^{o}C by only a few degrees can substantially reduce the pinocytic activity of cells (Duncan & Lloyd, 1978); pinocytosis is completely inhibited at 4^{o}C. The rate of pinocytosis is also known to be influenced by the presence of either metabolic inhibitors or microtubular inhibitors (Duncan & Lloyd, 1978; Pratten & Lloyd, 1979), implicating both metabolic energy and microtubular function as being important in the control of this membrane process.

Adsorptive pinocytosis, besides being dependent upon the factors

discussed above, is also dependent on additional factors. The number and type of binding sites or receptors on the surface of the cell at any one time is going to directly influence the rate of uptake of a particular ligand by the cell. Since it is inconceivable that every material has a specific receptor, the term binding site is used for an area of the plasma membrane where an affinity between a ligand and the cell surface exists and which is relatively non-specific in character, whereas the term receptor is reserved for a very specific interaction. The binding capacity of the plasma membrane for a ligand can in principle be determined by measuring the affinity of the ligand for the cell surface when pinocytosis is fully inhibited. Clearly the physical nature of a substrate is going to be of the utmost importance in determining its rate of uptake into cells, and many physical properties of the substrate may be important in this context: its general chemical structure (e.g. a protein, a glycoprotein, a glycolipid), the charge on the substrate (negative, positive or neutral), the size of the substrate (small or large, monomer or polymer), the conformation of a given substrate (whether in its native form or in some degree of denaturation). These considerations are more fully discussed in Section 3.1. Little is understood in general terms of the factors controlling the rate of formation of autophagic vacuoles in cells. The depletion of certain amino acids in the extracellular medium may be of some importance since autophagy has been shown to be stimulated under these conditions in a variety of cell types. Another possibly important controlling factor is the influence of hormones since glucagon has been shown to stimulate and insulin to depress this process in most cell types studied.

Methods used in the study of endocytosis usually involve following the fate of some tracer molecule within the cell, by either physical or chemical methods. These tracer molecules are usually labelled in

one of three ways: firstly, if the tracer molecule is an enzyme which is active, then its inherent "label" is its enzyme activity, secondly, tracer substrates may be labelled with radioactivity and thirdly, the tracer molecule may be chemically labelled. All of these methods are useful, but they all have their limitations which must be borne in mind during interpretation of results. Once an enzyme has been successfully pinocytosed by a cell, one would expect it to be degraded within the lysosomal system. If the level of tracer enzyme activity within the tissue falls, then one could attribute this to either loss of the tracer enzyme from the cell, its non-proteolytic inactivation or its proteolytic degradation. The problem is, of course, to determine the fate of the enzyme with confidence. If the tracer macromolecule is radiolabelled, then the problems experienced are different. Interference of the radiolabel with the normal functioning of the molecule may occur or, following degradation of the tracer, reincorporation of the label into other molecules may occur within the cell. The major problems associated with chemically-labelled substrates (e.g. macromolecules labelled with electron opaque or fluorescent moieties for detection by microscopy) are again that the addition of the tracer moiety may modify the behaviour of the molecule; also one can never be sure of the label remaining attached to the molecule so that the location of the labelling moiety does not necessarily indicate the location of the conjugate.

Both *in vivo* and *in vitro* methods have been used to measure rates of pinocytosis in tissues. *In vivo* experiments usually involve injection of the substrate under investigation into the circulation of the intact animal, and then following either its rate of clearance from the circulation or uptake by the different tissues. The limitations of this method are obvious if digestible substrates are used since

digestion within a tissue will be followed by release of digestion products into a common pool. Other practical considerations involve interanimal variations, the cost and convenience with regard to time-course experiments and the problem of low molecular weight markers being removed by the kidneys. A number of different in vitro systems have been used. Perfusion studies allow a substrate to come into contact with an organ and permit measurement of the amount of substrate retained by the tissue. Although this method allows the structural integrity of the tissue to be maintained, there are some drawbacks to this technique. It is not simple to set up, and again one will produce a common pool of digestion products, so again it is impossible to establish from which cell types digestion products arise. Another well-used in vitro method is that of cell culture, where cells isolated from a tissue are cultured in suspension or in monolayers, either as a mixed population of cells or after a preliminary separation step. The main advantages of these techniques are that they can be fairly simple, and also allow the study of different cells from a given tissue (e.g. Kupffer and parenchymal cells from liver). Unfortunately any vectorial feature of the cells is often, although not always, lost when the structural arrangement of the whole tissue is destroyed, and treatment with proteolytic enzymes to free the cells may damage protein components of the membrane surface; any residual proteolytic activity may also interfere in subsequent experiments. A valuable compromise between these two techniques is the culture of an organ or tissue excised from a freshly-killed animal and maintained in vitro until it begins to behave atypically; this method has the simplicity of the cell culture technique with the physiological advantages of the retention of the anatomical structure of the tissue. It is particularly suitable for tissues with a large surface area to volume ratio, and one such tissue

that has been extensively studied using this technique is the rat visceral yolk sac.

The rat visceral yolk sac, in the last third of gestation, surrounds both the fetus and the amnion and forms the outermost barrier between the fetus and the uterus. [Until 16 days of gestation another membrane, the parietal yolk sac, surrounds the visceral yolk sac. At 16 days this disappears, leaving the visceral yolk sac in contact with the uterine fluid (Beck & Lloyd, 1968).] It is the visceral yolk sac that is incubated *in vitro* in this well-documented method (Williams *et al.*, 1975a,b). The tissue is composed of three separate cellular layers: firstly the visceral endoderm, composed of columnar epithelial cells resting on a basement membrane; secondly the mesenchymal layer, composed of mesenchymal cells and blood capillaries resting on a thick, serosal basement membrane; thirdly a sparse layer of mesothelial cells (Seibel, 1974). It is the tightly packed layer of columnar epithelial cells in the first of the three layers that is pinocytically active; the apical faces of these epithelial cells are exposed to the uterine cavity (post 16 days gestation) and are microvillous in character.

Investigation of the visceral yolk sac is technically fairly easy. Yolk sacs are incubated with radiolabelled substrates in gassed (95% O_2/5% CO_2) medium 199, either in the presence or absence of calf serum (10%, v/v) for up to 6h. At the end of the incubation period, following their removal from the medium, the yolk sacs are washed in ice-cold saline and are assayed for both radioactivity and protein content; the residual medium is assayed for radioactivity. The yolk-sac tissue is fairly robust, and quantitative measurements of the pinocytic activity of the tissue have proved to be very reproducible. It therefore presents itself as an excellent system for studying factors affecting pinocytosis, and has already been used to investigate quite

a diverse range of factors that may control pinocytosis. The effects of temperature (Duncan & Lloyd, 1978), oxygen tension (Seymour, unpublished data), and removal of serum from the incubation medium (Ibbotson & Williams, 1979) have been investigated. The presence of metabolic inhibitors, microtubular inhibitors (Duncan & Lloyd, 1978) and proteolytic inhibitors (Livesey et al., 1980; Knowles et al., 1981) in the incubation medium and their effects on pinocytosis have been studied, and the properties of simple protein substrates (Livesey & Williams, 1981; Livesey & Williams, 1982), immunoglobulins (Weisbecker, 1981), denaturation of proteins and galactose moieties (Moore et al., 1977) on rates of protein uptake by the tissue have been examined. However, the yolk-sac incubation technique has left many questions unanswered. Very little has been found out about how subtle changes in protein conformation can affect protein uptake by the tissue, or about the presence of any receptors on the cell surface. Another area which is of great importance, but which has received very little attention, is the effect of the medium composition on the basal rate of pinocytosis in the yolk sac.

The aim of this project was to use the yolk-sac system to investigate two questions. The first question asked was: "Can subtle changes in the chemical nature of a simple protein substrate affect its rate of pinocytic uptake?"; the second: "Can changes in the composition of the incubation medium affect the pinocytic uptake of a substrate?". The first question was investigated by examining the rate of uptake of a family of closely-related ribonuclease molecules, to try to relate any differences in rates of pinocytosis to differences in structure, and to see if there were any differences in their subsequent degradation

by the yolk-sac tissue (Chapter 3). The second question was subdivided into three parts. The first was to investigate whether the basic composition of the incubation medium (presence of inorganic salts, vitamins, proteins and other components) could affect the rate of fluid-phase pinocytosis and the rate of adsorptive pinocytosis of a protein and its subsequent degradation within the yolk-sac tissue (Chapter 4). The second was to investigate the effect of hormonal influences upon these parameters (Chapter 5), and the third, to examine the effects of antibiotics upon these same parameters (Chapter 5). It would be interesting to establish whether factors controlling rates of pinocytosis also influence rates of degradation of intracellular proteins. This study was expanded by collaboration with F.J. Ballard and S.E. Knowles, (CSIRO, Division of Human Nutrition, Adelaide, S.A. 5000, Australia) who examined a number of factors that may affect the rate of endogenous protein degradation in the rat visceral yolk sac. It was envisaged that a comparison of the effects of these factors upon pinocytosis, and upon exogenous and endogenous protein degradation in the yolk sac, may provide evidence for some underlying control mechanism relating these phenomena.

CHAPTER 2

MATERIALS AND GENERAL METHODS

2.1 Materials

(i) Equipment

Centrifuge	Mistral 4L. M.S.E., U.K.
Gamma counter	5142 Selektronic Gamma Spectrometer. Packard Instruments Ltd., Caversham, Berks., U.K.
Spectrophotometer	CE 373 Linear Reading Grating Spectrophotometer. Cecil Instruments Ltd., Cambridge, U.K.
Water baths	Laboratory Thermal Equipment, A Searle Comp., Oldham, U.K.
LP3 tubes	Luckhams Ltd., Burgess Hill, Sussex, U.K.

(ii) Incubation Requirements

Medium 199	Powdered medium 199, with Earle's Salts, with L-glutamine; contained 100,000 units Penicillin-Streptomycin. Gibco Europe Ltd., Uxbridge, Middlesex, U.K.
Earle's B.S.S.	(IX) — Gibco Europe, Glasgow, Scotland, U.K.
M.E.M.	(Eagle) (IX) with Earle's Salts, without L-Glutamine — Gibco Europe, Glasgow, Scotland, U.K.
Saline (0.9%)	Sodium Chloride Injection B.P. 0.90% w/v for Intravenous Administration. Baxter Division, Travenol Laboratories Ltd., Thetford, Norfolk, U.K.
Calf Serum	Calf Serum 1, Inactivated. Wellcome Reagents Ltd., Beckenham, England, U.K.
Gas O_2 95%/CO_2 5%	British Oxygen Co. Ltd., Medical Gases Division, London, U.K.

(iii) Chemicals

$Na^{125}I$	Supplied as Sodium Iodide in Dilute NaOH Solution, pH 7-11, free from Reducing Agents (Code No. IMS.30) — Amersham International Ltd., Amersham, Bucks, England, U.K.
^{125}I-PVP	PVP (^{125}I) in Succinate Buffer Solution (Code No. IM.33P) — Amersham International Ltd., Amersham, Bucks, England, U.K.
Insulin	"Actrapid" MC Neutral Insulin Injection B.P. (mono component Porcine Insulin). Novo Industrai A/S, Denmark.

Bacitracin	(Code No. B 0125)	Sigma Chemical Co., London, U.K.
BSA	Cystallised and Lyophilized (Code No. A 4378)	
Cycloheximide	3 [2(3,5-Dimethyl-2-oxycyclohexyl)-2-hydroxyethyl] glutarimide (Code No. C 6255)	
2,4-DNP	Grade II (Code No. D 7004)	
Puromycin	Puromycin Dihydrochloride (Code No. P 7255)	
RNase A	Type X1-A, from Bovine Pancreas , Protease Free (Code No. R 5375)	
RNase B	Type XII-B, from Bovine Pancreas, Protease Free (Code No. R 5875)	
RNase S	Type XII-S, from Bovine Pancreas, Prepared from RNase XII-A (Code No. R 6000)	
RNase S protein	Type XII-PR, from Bovine Pancreas, Prepared from RNase XII-A (Code No. R 6250	

All other chemicals used were of Analytical Grade

2.2 Preparation of ^{125}I-labelled Proteins

2.2.1 Preparation of ^{125}I-labelled ribonucleases

The method used for radioiodination of ribonucleases was adapted from the method of Bocci (1969). Protein (10 mg) was dissolved in 4.25 ml phosphate buffer (0.05 M-Na_2HPO_4-KH_2PO_4, pH 8.0), and put on ice whilst being continuously stirred. Na^{125}I (1.0 mCi, usually in 10 µl solution) was added and the mixture stirred for 2 min. When 2.5 ml chloramine-T solution (2.0 mg in 2.5 ml water) had been added, the mixture was stirred for 8 min before the reaction was stopped by the addition of 2.5 ml sodium metabisulphite solution (2.0 mg in 2.5 ml water). After a further 2 min agitation, unreacted ^{125}I-iodide was diluted out by addition of excess KI (approximately 100 mg). The radiolabelled solution was transferred to Visking tubing (0.25" diam.) and dialysed at 4°C against 5 l NaCl (1%), the dialysis fluid being changed several times a day until its contained radioactivity gave a reading less than 5 times that of background. The ^{125}I-labelled protein solution was then removed from the tubing and stored at -20°C. The exact concentration of ^{125}I-labelled protein was measured spectrophotometrically via the A_{280} values.

Immediately after iodination the reaction vessel was washed out with medium 199 containing 10% (v/v) calf serum, and the total- and acid-soluble radioactivity levels were measured (see Section 2.4.2) in order to estimate both the labelling efficiency of the protein (usually 30 to 40%) and the quantity of unreacted ^{125}I-iodide discarded.

Before iodination began, the chloramine-T and sodium metabisulphite solutions were checked for reactivity. A few drops of the chloramine-T solution was added to a test-tube containing a little aqueous KI and some chloroform. This resulted in liberation of iodine, turning the dilute KI solution yellow and the chloroform pink. Addition of sodium

metabisulphite solution led to the iodine being reduced to KI, and both liquids becoming colourless again.

Labelling of ribonucleases with non-radioactive iodide was achieved by substituting $Na^{125}I$ with an equal weight of $Na^{127}I$, and then following the same procedure as above.

2.2.2 Preparation of formaldehyde-denatured ^{125}I-labelled BSA

The experimental procedure for the radioiodination of BSA was the same as is described in Section 2.2.1, up to the point when non-radioactive KI (100 mg) was added, with the following exceptions: twice the amount of protein (20 mg) was iodinated with 1.0 mCi of $Na^{125}I$; chloramine-T solution (4.0 ml) was added at a concentration of 1.0 mg/ml; and sodium metabisulphite solution (3.0 ml) was added at a concentration of 2.0 mg/ml. After addition of excess non-radioactive KI, 16.0 ml of formaldehyde solution (10%, w/v, in 0.5 M-$NaHCO_3$ buffer, pH 10.0) was added to the radiolabelled protein solution and kept at $4^{o}C$ for 24h. The solution was then transferred to Visking tubing (0.25" diam.) and dialysed at $4^{o}C$ against changes of 5 l NaCl (1%), after which it was handled as in Section 2.2.1. The labelling efficiency of formaldehyde-denatured ^{125}I-labelled BSA (^{125}I-fdBSA) was approximately 60%.

2.3 Rat Visceral Yolk-Sac Incubation Technique

The method used for the incubation of the rat visceral yolk sac was essentially as described by Williams et al. (1975a,b). Pairs of Wistar rats from an inbred colony were mated overnight in grid cages. If a sperm plug was detected underneath the grid the following morning, pregnancy was timed from midnight of the night of mating. After 17.5 days the rats were asphyxiated with carbon dioxide, and the uterus quickly removed and placed in a Petri dish containing incubation medium that had previously been warmed to 37°C. (The percentage calf serum present in the dissecting medium was the same as in the incubation medium for each separate experiment). The uterine wall was cut open longitudinally, and the conceptuses gently freed from the wall. The placental caps were removed, the cut edge of the yolk sacs enlarged by a single cut, and the fetuses dissected free. The yolk sacs were placed in fresh, warm incubation medium, and any remaining amniotic tissue was removed.

Each yolk sac was placed in a sterile 50 ml Erlenmeyer flask, containing 9.0 ml of incubation medium (either medium 199 alone or medium containing calf serum) that had previously been gassed with a mixture of 95% oxygen and 5% carbon dioxide, and sealed with a sterile silicone rubber bung. The flasks were placed in a water bath maintained at 37°C, with the shaker attachment set to a stroke of 3.4 cm at a frequency of 100 ± 5 strokes per min. After a preincubation period of 15 - 45 min, ^{125}I-labelled substrate was added to each flask as a solution in 1.0 ml of incubation medium. The flasks were then regassed and stoppered.

If the effect of some compound on the uptake of ^{125}I-labelled substrate was being investigated, the flask initially contained 8.0 ml of medium. After gassing and a preincubation of at least 15 min, the

compound was added as a solution in 1.0 ml of medium. After a further incubation period of 20 min, the ^{125}I-labelled substrate was, likewise, added in 1.0 ml of medium, and the flasks regassed and stoppered.

Yolk sacs were removed after different periods of incubation, washed three times for 2 min in approximately 30 ml ice-cold 1% NaCl, and stored in 5.0 ml volumetric flasks at -20°C until assayed. For non-degradable ^{125}I-labelled substrates (^{125}I-PVP), duplicate 1.0 ml samples of medium were pipetted into disposable 3 ml (LP3) tubes at the time of sampling, stoppered and stored at 4°C until assayed. For degradable ^{125}I-labelled substrates (^{125}I-labelled proteins), the flasks were returned to the water bath after removal of the yolk sac and kept there until the end of the experiment. This ensured that the effects of any proteolytic activity released into the medium, or of instability of the substrate, were the same in all flasks. Duplicate 1.0 ml samples were then pipetted into disposable 3 ml (LP3) tubes, stoppered and stored at -20°C until assayed. Two control flasks, containing both medium and ^{125}I-labelled substrate but no yolk sacs, were incorporated into each experiment with a degradable substrate. The medium in these flasks was sampled at the end of the experiment as above, and stored at -20°C until assayed.

2.4 Assay of Incubation Media and Yolk-Sac Tissue

2.4.1 Assay after incubation with ^{125}I-PVP

Samples of incubation media containing ^{125}I-PVP were assayed for radioactivity with a gamma spectrometer, using a constant geometrical relation of sample to detector.

NaOH (1.0 M) was added to each yolk sac in a 5.0 ml graduated flask and made up to the mark. The yolk sacs were then dissolved over a period of 1.0h at 37°C, the solutions being agitated at 0.5h and 1.0h. Duplicate 1.0 ml samples of the yolk-sac solution were pipetted into disposable 3 ml (LP3) tubes and assayed for radioactivity. The protein content of each yolk-sac solution was determined by the method of Lowry et al. (1951) with bovine serum albumin as reference protein. Duplicate 0.1 ml samples of each yolk-sac solution were made up to 1.0 ml by adding 0.4 ml 1 M-NaOH and 0.5 ml distilled water. Similarly, a calibration curve was constructed by assaying in triplicate a series of five solutions containing 0, 0.05, 0.10, 0.15 and 0.20 mg of BSA. To each solution was added 5 ml Folin A (100 ml 2% anhydrous sodium carbonate: 1 ml 1% copper sulphate: 1 ml 2% potassium sodium tartrate), and, after at least 20 min, 0.5 ml Folin B solution (Folin Ciocalteau's reagent freshly diluted with an equal volume of distilled water). After the Folin B solution was added the contents of each test tube were immediately vigorously mixed using a vortex-mixer. After a period of at least 45 min (the developed colour is stable up to 24h) the absorbance of each solution was read against a water blank at 750 nm. The number of milligrams of protein present in each sample was determined from the calibration curve, and this figure multiplied by fifty to give the total yolk-sac protein. Calibration curves were typically virtually linear between absorbances of 0.3 and 0.7.

2.4.2 Assay of incubation media containing ^{125}I-labelled proteins

Duplicate (1.0 ml) samples of incubation media were first assayed for their total content of radioactivity. Then, if the media contained no calf serum, 0.1 ml calf serum was added to each tube to act as a carrier protein in the precipitation of acid-insoluble radioactive material. (Media already containing calf serum required no such addition). If the ^{125}I-labelled protein under examination was ^{125}I-fdBSA, then precipitation of acid-insoluble material was achieved by addition of 0.5 ml 20% trichloroacetic acid (TCA) leaving TCA-soluble radioactivity in solution. If ^{125}I-RNases were being investigated, the precipitation was achieved by addition of 0.5 ml phosphotungstic acid solution (125 ml 1M-HCl, 10g sodium tungstate, 25.2g Na_2HPO_4, 9g NaCl, 1g phenol, made up to 1.0 litre) followed by 0.5 ml 20% TCA. After precipitation of protein in this manner, the tubes were centrifuged for 20 min at 1500g, the supernatant decanted and assayed for radioactivity; the pellets were discarded.

The radioactivity associated with the yolk-sac tissue was assayed by the same procedure as used when ^{125}I-PVP was the substrate (see Section 2.4.1).

2.5 Determination of Correction Factors for the Acid-Soluble Radioactivity Assay

Acid-soluble radioactivity was always assayed in a sample volume greater than 1.0 ml because of the addition of solutions of acidic reagents during the precipitation of acid-insoluble material (see Section 2.4.2). The observed radioactive counts had therefore to be corrected for two factors: first the loss of acid-soluble radioactivity occluded within the protein pellet after centrifugation; second, for the change in counting efficiency resulting from the increase in sample volume. Correction factors were established to permit calculation of the counts that would have been observed had all the acid-soluble radioactivity been contained within a 1.0 ml sample volume.

Williams et al. (1975,b) showed that after uptake of ^{125}I-fdBSA by the rat yolk sac, the major (95%) degradation product was ^{125}I-iodotyrosine; this compound was therefore used as the acid-soluble species in determinations of the correction factors.

^{125}I-labelled tyrosine was diluted in medium containing different amounts of calf serum to produce an acceptable number of counts. Six groups of ten tubes contained the following amounts of calf serum:-

Group A : 0% calf serum

Group B : 10% calf serum

Group C : 20% calf serum

Group D : 50% calf serum

Group E : 0% calf serum

Group F : 10% calf serum

Each 3 ml (LP3) tube contained 1.0 ml of the appropriate medium.

The tubes were counted for 30s on a gamma spectrometer. Calf serum (0.1 ml) was added to those tubes in groups A and E. Then protein

was precipitated by adding either 0.5 ml TCA (for tubes in groups A, B, C and D) or by adding 0.5 ml PTA followed by 0.5 ml TCA (for tubes in groups E and F). All tubes were then centrifuged for 20 min at 1500 g, (as in Section 2.4.2), and the supernatant decanted and counted for 30s.

The correction factors were determined from the following equation:-

$$\text{Correction Factor} = \frac{\text{Total counts (cpm, corrected for background)}}{\text{Observed acid-soluble counts (cpm, corrected for background)}}$$

The resulting correction factors are shown in Table 2.5a.

Table 2.5a Correction Factors Determined for Acid-Soluble Radioactivity Assays

Correction factors were determined as described in Section 2.5. Values reported are the means (± S.D.) of ten determinations.

Group	Initial Percentage Calf Serum	Method of Protein Precipitation	Correction Factor Mean ± S.D.
A	0	TCA	1.37 ± 0.07
B	10	TCA	1.29 ± 0.04
C	20	TCA	1.33 ± 0.06
D	50	TCA	1.47 ± 0.07
E	0	PTA + TCA	1.86 ± 0.04
F	10	PTA + TCA	1.80 ± 0.04

2.6 Quantitation of the Uptake of a Non-degradable Substrate

The only non-degradable substrate used in this study was ^{125}I-PVP. The uptake (and subsequent accumulation) of ^{125}I-PVP was expressed in a form in which the reproducibility of the results between experiments could be easily assessed, see Williams *et al.* (1975a), namely as the volume of culture medium whose contained substrate was captured by unit quantity of yolk-sac tissue (in microlitres per milligram yolk-sac protein). Uptake, determined after different periods of incubation, was expressed by the following equation:-

$$U = \frac{Y}{M.P}$$

where U = uptake (µl per mg yolk-sac protein)

Y = total radioactivity in whole yolk sac (cpm, corrected for background)

M = radioactivity per µl of incubation medium (cpm, corrected for background)

P = protein content of yolk sac (mg)

Since the rate of fall of the ^{125}I-PVP concentration in the incubation medium was equivalent to only approximately 0.1% per h (Williams *et al.*, 1975a), the concentration was effectively constant for the duration of the incubation, and the value of M above was therefore taken as the measured value of the radioactivity in the incubation medium at the end of an incubation period. Uptake values were routinely calculated by the simple program shown in Appendix I.

The rate of substrate uptake was the gradient of the plot of uptake against the period of incubation. This rate was termed the Endocytic Index (EI), and has the units microlitres/milligram yolk-sac protein per hour. After each plot had been visually inspected to confirm linearity, the gradient (EI), the intercept and the correlation coefficient for the best straight-line fit were calculated using a

standard statistical package on a computer. A correlation coefficient of well over 0.95 was usually obtained. However, linearity was always confirmed by visual inspection since the correlation coefficient is only a crude index of linearity; slight curvatures would not be detected by a fall in the correlation coefficient alone.

2.7 Quantitation of the Uptake of a Degradable Substrate

When using degradable substrates (^{125}I-labelled proteins), the bulk of the radiolabel taken up is not retained within yolk-sac tissue, but is released back into the medium as acid-soluble degradation products (see Williams et al, 1975b). These substrates are also taken up at a much higher rate than ^{125}I-PVP, resulting in a significant depletion of the amount of acid-insoluble radioactivity present in the medium during the incubation period. Both of these factors had to be taken into consideration when calculating the uptake of such substrates. Thus the expression suitable for describing the uptake of ^{125}I-PVP (see Section 2.6) has to be modified to:-

$$U = \frac{Y + 10(S-F)}{M'.P}$$

where U = uptake (µl per mg yolk-sac protein)

Y = total radioactivity retained in whole yolk sac (cpm, corrected for background)

S = acid-soluble radioactivity per ml of medium at end of incubation period (cpm, corrected for background)

F = acid-soluble radioactivity per ml of medium present at beginning of incubation, and that released by spontaneous degradation not associated with yolk sac during incubation (cpm, corrected for background)

P = protein content of yolk sac (mg)

M' = mean acid-insoluble radioactivity per µl medium over the particular incubation period (cpm, corrected for background)

The value of M' was calculated from M, the observed value of the acid-insoluble radioactivity per microlitre of medium, by adding one half of the acid-soluble radioactivity per microlitre of incubation medium generated by the yolk sac during the incubation period. The following expression was used:-

$$M' = M + \frac{(S-F)}{2 \times 10^3}$$

where M = Acid-insoluble radioactivity per µl of medium at end of incubation period (cpm, corrected for background)

The values of S and F were calculated using the correction factors evaluated to correct for loss of acid-soluble radioactivity occluded within the pellet after centrifugation following acid precipitation, and for the change in counting efficiency resulting from the volume increase (see Section 2.5). The value of S was calculated from:-

$$S = A.E$$

where S = acid-soluble radioactivity per ml of medium at end of incubation period (cpm, corrected for background)

A = acid-soluble radioactivity in whole medium sample at end of incubation period (cpm, corrected for background)

E = correction factor

The value of F was calculated from:-

$$F = B.E$$

where F = acid-soluble radioactivity per ml of medium present at beginning of incubation, and that released by spontaneous degradation not associated with yolk sac during incubation (cpm, corrected for background)

B = acid-soluble radioactivity in whole medium sample from control flask (cpm, corrected for background)

E = correction factor

Data were routinely processed by using the simple computer program shown in Appendix II. The rate of uptake of the substrate was determined as in Section 2.6, the resulting EI having the units microlitres/milligram yolk-sac protein per hour. A correlation coefficient of over 0.95 was usually obtained.

2.8 Effect of pH on the Degradation of ^{125}I-labelled Ribonucleases by Yolk-Sac Homogenate

The effect of pH on the degradation of ^{125}I-labelled ribonucleases (^{125}I-ribonucleases) by an homogenate of 17.5-day yolk sacs was examined over the pH range 3.0 to 9.0, at intervals of 0.5 pH unit. Sodium acetate buffer (0.1M, adjusted to the correct pH with glacial acetic acid) was used over the pH range 3.0 to 6.0. Tris [2-amino-2-(hydroxymethyl) propane-1,3-diol] (0.1 M, adjusted to the correct pH with HCl) was used over the pH range 6.5 to 9.0.

Rat yolk sacs were dissected as in Section 2.3, and washed in ice-cold saline (1%). The yolk sacs were blotted and homogenised in distilled water (1.0 ml/yolk sac) using a Potter-Elvehjem type Teflon-on-glass homogeniser (0.19 mm clearance). Four up-and-down strokes at a speed of 2,500 rpm were made within a 30s period. The homogenate was diluted with 2 volumes of distilled water, centrifuged at 150g for 10 min, the supernatant decanted and the pellet discarded. The supernatant (yolk-sac homogenate) was kept on ice until required, but used within 2h of preparation.

Incubations were performed in 3 ml (LP3) tubes initially containing 130 µl appropriate buffer and 20 µl substrate (0.5 mg/ml) and preincubated at 37°C, and the incubation started by addition of 50 µl yolk-sac homogenate (37°C) to each tube, followed by thorough mixing. Controls were performed by addition of 50 µl distilled water in place of substrate. Each incubation was allowed to proceed for 1.0 h before being terminated by addition of 0.5 ml ice-cold aqueous calf serum (10%), followed immediately by 0.5 ml PTA and 0.5 ml TCA.

The tubes were stoppered, the contents mixed and assayed for radioactivity. The tubes were then centrifuged at 1500 g for 20 min and the acid-soluble supernatant decanted and assayed for radioactivity;

the pellets were discarded. The percentage acid-soluble radioactivity present at each pH value was calculated, the observed acid-soluble radioactivity counts being corrected for the loss of acid-soluble radioactivity occluded within the pellet after centrifugation and for the change in counting efficiency resulting from the increase in sample volume. A suitable correction value was therefore evaluated by applying the method in Section 2.5 to the experimental conditions in this section; the correction value determined was 1.04.

The protein content of the yolk-sac homogenate was determined as described in Section 2.4.1.

2.9 Stability of ^{125}I-labelled Ribonucleases in "Conditioned Incubation Media"

Some "conditioned incubation media" were prepared by incubating normal media with or without yolk sacs; others were prepared by similarly incubating media to which 2,4-DNP had been added. A ^{125}I-labelled ribonuclease preparation was incubated in each of these media, and the amount of acid-soluble radioactivity produced during the incubation was measured.

Yolk sacs were dissected as in Section 2.3, and incubated individually in 50 ml Erlenmeyer flasks in a shaking water bath at 37°C for 3.0h in gassed serum-free or serum-containing (10%) medium, either in the absence or in the presence of 2,4-DNP. An identical set of flasks was incubated in the absence of any yolk sacs. At the end of the incubation period the yolk sacs (if present) were removed, washed for 3 x 2 min in changes of ice-cold saline (1%), and then placed in 5.0 ml volumetric flasks for protein estimations as in Section 2.4.1. The incubation medium from each flask was decanted into capped centrifuge tubes, centrifuged at 1500 g for 15 min, and 8.0 ml of the supernatant removed and placed in a fresh incubation flask. To each flask was added 1.0 ml fresh medium (either in the absence or presence of calf serum, 10% v/v, as appropriate) and 1.0 ml of the same medium containing ^{125}I-ribonuclease (to give a final ^{125}I-ribonuclease concentration of 1.0 µg/ml). These flasks were gassed and incubated for 3.0h, samples (2 x 1.0 ml) of the incubation medium being removed at 0, 1.0, 2.0 and 3.0h and assayed as in Sections 2.4.1 and 2.4.2.

2.10 Effect of Various Agents on the Release of a Non-Degradable Substrate from Yolk Sacs

Yolk sacs were dissected as in Section 2.3, and incubated in pairs in 50 ml Erlenmeyer flasks in shaking water baths at 37°C. Each flask contained 10.0 ml gassed medium 199 in which was dissolved the substrate (^{125}I-PVP, 2 µg/ml) and the agent of interest. After an incubation period of 2.5 to 3.0h the yolk sacs were removed, and 2 x 1.0 ml of this medium was sampled. The yolk sacs were washed for 3 x 2 min in warm (37°C) medium 199 containing both non-radioactive PVP (2 µg/ml) and agent, and then reincubated in the same pairs in fresh Erlenmeyer flasks containing 10.0 ml gassed medium 199 in which was dissolved non-radioactive PVP (2 µg/ml) and the agent. The yolk sacs were reincubated for a further 2.5 to 3.0h, 2 x 1.0 ml samples being taken at intervals of 0.25h for the first hour, and then at 0.5 or 1.0h intervals. After each sampling, 2 x 1.0 ml of medium containing non-radioactive PVP and agent were added back to the flask and regassed, to keep the volume in the flask constant. At the end of the incubation the yolk sacs were washed 3 x 2 min in ice-cold saline (1%) and assayed for protein as in Section 2.4.1. The samples were assayed for radioactivity as in Section 2.4.1.

In those experiments in which the effect of different media on the yolk-sac tissue was being investigated, medium 199 was simply replaced by the appropriate medium in all stages of the experiment, including the dissection.

2.11 Quantitation of Release of a Non-Degradable Substrate

The total activity released into the incubation medium over the reincubation period was calculated by the following formula:-

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

where T_n = radioactivity released up to the time of the nth sampling (cpm)

C_i = radioactivity per ml of incubation medium of the ith sample (cpm, corrected for background)

The sum of the total quantity of radioactivity released into the incubation medium over the reincubation period, and the quantity of radioactivity remaining associated with the tissue at the end of the reincubation period, is equal to the total amount of radioactivity associated with the yolk-sac tissue at the beginning of the reincubation period. Using these values it is possible to calculate the amount of radioactivity released during any part of the reincubation period as a percentage of the quantity of radioactivity associated with the yolk sac at the beginning of the reincubation. Likewise, knowing the amount of radioactivity equivalent to a given mass of labelled substrate, it is possible to calculate the amount of radioactivity released in ng per mg yolk-sac tissue. Data were routinely processed using the simple computer program shown in Appendix III.

2.12 Recovery of ^{125}I-PVP Uptake by Yolk Sacs after Exposure to Various Agents

Yolk sacs were dissected and incubated, as described in Section 2.3, in the presence of ^{125}I-PVP (2 µg/ml) and various agents. Yolk sacs were removed and washed for 3 x 2 min in ice-cold saline (1%), and 2 x 1.0 ml volumes of incubation medium were sampled at 0.5h intervals up to 2.5h. At 2.5h, all remaining unsampled yolk sacs were washed for 3 x 2 min in medium 199 (37°C), put into clean 50 ml Erlenmeyer flasks containing 10.0 ml medium with ^{125}I-PVP (2 µg/ml) only, gassed and then reincubated. These yolk sacs were sampled as above at 0.5h intervals for a further 2.5h. Yolk-sac tissue and samples of incubation medium were assayed as described in Section 2.4.1.

CHAPTER 3

PINOCYTOSIS AND DEGRADATION OF DIFFERENT RIBONUCLEASES IN THE RAT VISCERAL YOLK SAC

3.1 Introduction

Many factors affect the rate at which a substrate is pinocytosed by living cells. One group of factors, which may be sub-divided into more specific features is the physical characteristics of the ingested substance.

The size of a substance being pinocytosed by cells is probably important. Thus, Duncan *et al.* (1981) examined the effect of molecular size of ^{125}I-labelled poly(vinylpyrrolidone) (^{125}I-PVP) on its rate of pinocytosis by rat visceral yolk sacs and rat peritoneal macrophages *in vitro*. This compound has previously been shown to be a marker of fluid-phase pinocytosis in both of these systems (Williams *et al.*, 1975a; Pratten *et al.*, 1977). Varying the substrate concentration did not affect the rate of uptake of ^{125}I-PVP for a particular polymer size, but rat yolk sacs and macrophages each showed different preferences for different-sized fractions. (The molecular weights of the polymer fractions used were between 5×10^4 and 7×10^6 daltons). Macrophages preferentially captured the very high molecular weight fractions, whereas yolk sacs captured virtually none of these fractions, perhaps due to the limiting factor being the size of the pinosome. Yolk sacs captured the lowest molecular weight preparations most rapidly. Similarly, Nilsson *et al.* (1981) found that small chylomicrons produced *in vivo* were taken up more rapidly by rat hepatocyte monolayers *in vitro* than were large chylomicrons.

Another method of approaching the problem of the effect of size of a substrate on its rate of pinocytosis is by chemically creating aggregates from monomer proteins. Thus, Kooistra *et al.* (1979a) cross-linked ribonuclease with dimethyl suberimidate to form dimers and higher polymers in such a way that the charge of the protein was not altered. Monomer, dimer and higher polymer fractions were injected into

nephrectomized rats, and it was found that the polymer fraction was taken up by the liver faster than the dimer, which in turn was endocytosed more rapidly than the monomer; aggregated ribonuclease, prepared by freeze-drying the protein from 50% acetic acid, gave similar *in vivo* clearance results. They also formed dimers and polymers of hen egg-white lysozyme by cross-linking with dimethyl suberimidate (Kooistra *et al.*, 1980), and again found that the rate of clearance into the liver of the rat *in vivo* followed the same pattern as described above. In contrast to this finding, Livesey & Williams (1981) found that formaldehyde- and bicarbonate-induced aggregates of lysozyme were taken up more slowly by rat yolk sacs incubated *in vitro* than untreated monomer; formaldehyde-induced dimer had an uptake rate similar to that of the monomer. Another group that has examined bovine pancreatic ribonuclease A dimers is that of Bartholeyns & Baudhuin (1976). They found that hepatoma cells endocytosed the dimer much more avidly than the monomer form; the dimer also inhibited proliferation of the tumour cells. This work was extended to examine four different types of tumour cell and three normal cell lines (Bartholeyns & Zenebergh, 1979). These authors found that three of the four tumour cell types interiorized the dimer very rapidly and blocked proliferation; the normal cell lines did not take up the dimer to the same extent, and cell proliferation was unaffected. Ribonuclease A dimers were injected intramuscularly into rats in the vicinity of solid tumours, after which cellular proliferation was blocked. Also, development of tumours was inhibited if the dimer was injected prior to administration of hepatoma cells. Other evidence suggests that the dimer can inhibit fusion between phagosomes and lysosomes, and/or inhibit discharge of a protein (peroxidase) adsorbed to the phagosomal membrane after fusion (Bartholeyns *et al.*, (1979).

Another physical characteristic of molecules that may affect their rate of uptake by cells is their charge. Again there are two clear

avenues for the investigation of this property, examination of the effect of the inherent charge on a number of different substrates, and chemical modification of a single substrate so that its charge varies. Thus, Kooistra et al. (1979b) changed the positively-charged amino groups of the cross-linked ribonuclease molecules mentioned above to neutral or negatively-charged groups by acetylation or succinoylation respectively, and changed the positively-charged amino groups on lysozyme to neutral or negatively-charged groups by the same procedures (Kooistra et al., 1980). In both cases the rate of uptake of the positively-charged entity into the liver and spleen was greater than the rates for either the neutral or the negatively-charged derivative.

The work of the group of J.F. Dice and A.L. Goldberg has established a correlation between the rates of degradation of proteins in vivo and their isoelectric points. One such example of their work (Dice et al., 1979) is a report on soluble proteins from rat lung, heart and testes, and from human fibroblasts and mouse-embryo cells grown in culture. They found that the negatively-charged proteins tended to be degraded more rapidly than the neutral or basic ones; this finding is consistent with data they had previously reported relating to rat liver, skeletal muscle, kidney and brain, and mouse liver and skeletal muscle.

Proteins may be modified by different treatments to alter their conformation in a less specific manner than was achieved by Kooistra and co-workers in the work described above. For example, Moore et al. (1977) treated ^{125}I-labelled BSA with acetic acid in the pH range 2.5 to 3.5, with formaldehyde at pH 10.0 and with urea at pH 5.5. The rates of uptake of these treated forms of BSA by rat yolk sacs in vitro were much higher than that of the untreated form, and similar results were obtained for in vivo clearance of the substrates by rat tissues. In contrast, Livesey & Williams (1981) found that treatment of insulin, lysozyme and ribonuclease A with either acetic acid or urea had no

effect upon the rates of uptake of these proteins by rat yolk sacs *in vitro* compared to the rates of uptake of the corresponding untreated forms, and that treatment with formaldehyde led to a reduced rate of uptake. Livesey & Williams discuss these results and come to the conclusion that (unlike BSA) insulin, lysozyme and ribonuclease A suffer no permanent conformational change on exposure to either acetic acid or urea. Since lyosozyme and ribonuclease A are basic proteins, they may bind to negatively-charged groups on the yolk-sac membrane. The surface of the insulin molecule is hydrophobic in character, and may bind to hydrophobic patches on the yolk-sac plasma membrane. As the rate of uptake of BSA is lower than that of any of the smaller, simple proteins described above, and the albumin molecule is negatively-charged at physiological pH, Livesey & Williams suggest that this effect may be due to the inability of BSA to interact successfully with either negatively-charged or hydrophobic binding sites on the yolk-sac surface. They suggest that denaturation with, say, formaldehyde may cause hydrophobic groups normally buried within the albumin molecule to be brought to the surface, and therefore enhance uptake by binding to hydrophobic binding sites on the surface of the yolk sac.

The properties of charge and hydrophobicity of a protein molecule have been further studied by using the isoenzymes of lactate dehydrogenase. These proteins consist of four tetrahedrally-arranged subunits of equal size, and the two types of subunit have been called M and H. The attraction of working with these molecules is that subunit differences in surface charge and hydrophobicity can be studied without a change in molecular size. Sinke *et al.* (1979) studied two porcine lactate dehydrogenase isoenzymes, namely M4 and H4. They found that the isoenzyme M4 was cleared from the blood stream *in vivo* (into the liver and spleen) much more rapidly than was the H4 protein, and that it showed a half-life of about 30 min; the *in vivo* half-life of H4 was approximately

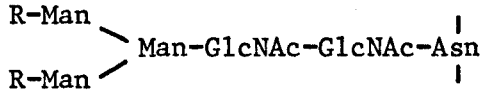
8h. Sinke et al. postulated that the differences in the amino acid residues on the surfaces of the isoenzymes were responsible for these differences, since at neutral pH the isoenzyme M4 carries a positive charge and H4 a negative one. This evidence is in agreement with data, discussed above, from which Kooistra et al. (1979b, 1980) found a more rapid in vivo clearance of positively-charged substrates.

Kooistra & Williams (1981) studied the in vitro uptake of lactate dehydrogenase by rat yolk sacs, using the same isoenzymes as Sinke et al. (1979). They found that the rates of uptake of the two proteins were very similar, the uptake of M4 being only slightly faster than that of H4. Competition studies led them to the conclusion that the binding of the H4 isoenzyme may be restricted to hydrophobic interactions with the yolk-sac surface, whereas the binding of the M4 isoenzyme probably involves both hydrophobic interactions and interactions between the positive charges on the surface of the isoenzyme and the yolk-sac plasma membrane.

Clearly, size and charge have a substantial effect upon the uptake of different molecules, both in clearance from the blood stream in vivo and in uptake experiments performed in vitro. Another outstanding feature from the data surveyed above is the diverse responses of the different tissues studied regarding their ability to endocytose molecules with certain physical characteristics, suggesting that differences between the surface characteristics of cells are as important as the differences between individual substrate molecules in determining the rates of endocytosis.

Another dominant feature of many proteins is the presence of carbohydrate moieties attached to the polypeptide chain. These glycoproteins have a wide range of diverse physical, chemical and biological properties dependent upon the type and amount of sugars present, and upon their distribution along the polypeptide chain. Marshall (1979) has reviewed some features of glycosylated proteins. The attachment of the carbohydrate moiety to the protein is almost always achieved by the

involvement of sugar tranferases, with the donor molecule being either a nucleotide monosaccharide or a dolichyl phosphate-linked oligosaccharide. One major class of glycoproteins contains a carbohydrate moiety linked at asparagine residues. These include glycoproteins found in serum and on the plasma membranes of cells. The number of glycosylated asparagine residues in any glycoprotein is usually small since the correct asparagine sequon (sequence of amino acids near to the asparagine residue) must be present. Two types of oligosaccharide have been found, the high-mannose type and the complex type; the biosynthetic pathways of these glycoproteins have been reviewed by Staneloni & Leloir (1979). Both types have a common pentasaccharide structure, but differ in the externally-located sugar residues:-



In the high-mannose type, the side chains have a variable number of mannose residues with different types of ramification, in addition to some acetylglucosamine residues; the complex type of side chain has galactose, N-acetylglucosamine and sialic acid, and occasionally three R-chains.

Another major class of glycoproteins contains O-glycosidically-linked carbohydrate moieties at the serine and threonine residues, and the protein backbone usually contains large amounts of proline. The number of carbohydrate moieties attached to a polypeptide chain may vary greatly; human apolipoprotein C-III contains only one carbohydrate moiety whereas in glycophorin A, 11 of the 15 carbohydrate chains occur within the first 25 amino acid residues from the N-terminus. This type of glycoprotein forms the mucous layer which protects epithelial layers from mechanical and osmotic shock.

Rauvala & Finne (1979) have reviewed the composition of the carbohydrate portions of both glycoproteins and glycolipids. They

point out that although the carbohydrate residues near to the protein or lipid part of the molecule can differ, there is a striking similarity between the terminal carbohydrate sequences. They suggest that this may indicate that terminal carbohydrate sequences may be synthesized by common glycosyl transferases for both types of molecule.

The effect of the extent of glycosylation of a protein upon its rate of clearance from the bloodstream *in vivo*, and upon its rate of uptake by various cell types *in vitro*, has been the subject of a large amount of recent literature. The experimental work consists mainly of removal of a terminal sugar residue; this often leads to an increased rate of clearance or uptake of the protein due to exposure of a particular carbohydrate residue. The importance of this exposed carbohydrate residue can be demonstrated by competition studies between the modified glycoprotein and various sugars. The general conclusions from such studies have been reviewed by Lloyd & Griffiths (1979). Different tissues recognise different terminal carbohydrate residues: hepatocytes recognise galactose residues; fibroblasts recognise phosphohexoses; N-acetylglucosamine and mannose are recognised by both Kupffer cells and macrophages. More recent literature (Burnside & Schneider, 1980 and Fischer *et al.*, 1980) have provided evidence for the presence of phosphomannosyl receptors on rat-liver lysosomal membranes which appear to be similar to the phosphohexose receptors determined for fibroblast plasma membranes.

Ribonucleases are a group of enzymes involved in the degradation of ribonucleic acid by splitting phosphodiester bonds, and in catalysing the hydrolysis of 2',3'-cyclophosphates of pyrimidine nucleotides to the corresponding 3'-nucleotides. Many different ribonucleases have been isolated from different tissues. The amino acid sequences and conformational

structures of many of these ribonucleases have been deduced, and it is now possible to synthesize ribonuclease A in the laboratory (Fujii & Yajima, 1981).

Baynes & Wold (1976) determined the half-lives of four bovine pancreatic ribonuclease isoenzymes when injected into the circulation of the rat. They used ribonucleases A, B, C and D which all have the same polypeptide chain backbone containing 124 amino acid residues, but all have different degrees of glycosylation at their asparagine-34 residues. Ribonuclease A contained no carbohydrate moiety; B had a simple oligosaccharide ($GlcNAc_2$ Man_{4-5}); C had a complex oligosaccharide ($GlcNAc_4$ Man_{2-3} Gal_2 Fuc $NeuAc_2$); and D had a different complex oligosaccharide ($GlcNAc_4$ Man_3 Gal_2 Fuc $NeuAc_4$). The *in vivo* clearance half-lives were 528-577 min, 15 min, 681-862 min and 839-941 min respectively, detected by measuring the ribonuclease activity present in the blood (the animals were nephrectomized to prevent rapid removal of these small proteins by the kidneys). When ribonuclease B was treated with α-mannosidase to remove 3 of the 4 mannosyl residues, this resulted in an elevation of the circulating half-life to 616-733 min. Baynes & Wold concluded that the presence of a complex oligosaccharide moiety had no apparent effect on the rate of clearance of these proteins from the circulation in the rat, but that the presence of the mannose residues in the simple oligosaccharide moiety resulted in rapid clearance; the recognition of mannose residues has since been attributed to Kupffer cells and macrophages (see above).

The presence of both glycosylated and non-glycosylated forms of a protein within an individual of a species may arise by three different ways: duplication of the gene specifying the protein chain; gene mutation; incomplete conversion within a cell of an apoprotein into the corresponding glycoprotein. The last-mentioned of these pathways is assumed to account for the different ribonuclease isoenzymes described above (Marshall, 1979).

Richards & Vithayathil (1959) modified bovine pancreatic ribonuclease A with subtilisin, resulting in a cleavage between amino acid residues

20 and 21. The eicosapeptide remained non-covalently associated with the rest of the protein, and this substance was called ribonuclease S. The enzymatic activity of ribonuclease S was negligibly changed from that of A, although various physical and chemical techniques indicated changes in the three-dimensional structure. Trichloroacetic-acid fractionation led to separation of the eicosapeptide from the main polypeptide chain, and these two separate substances were called S peptide and S protein, respectively.

The ribonuclease isoenzymes, native and modified, thus provide an excellent group of proteins in which to study the effect of the physical characteristics of a substrate upon its rate of uptake by cells. A comparison between ribonucleases A and B permits an examination of the effect of terminal mannose groups. Ribonucleases A and S allow a comparison between a native and a partially altered polypeptide chain, and also permit examination of the suggestion of Dice & Goldberg (1976) that proteolytic "nicking" outside of cells may be a signal for enhanced clearance of proteins from the circulation. Removal of the peptide fragment from ribonuclease S to form S protein will also provide an interesting variation on the influence of the composition of the polypeptide chain on uptake and degradation. Therefore the rates of uptake and subsequent degradation of these four ribonucleases (A, B, S and S protein) were studied in the rat yolk sac. (Unfortunately the C and D forms were not commercially available, hence could not be obtained for study.)

As an integral part of the comparative study of the rates of uptake of the different ribonuclease preparations mentioned above, it was necessary to investigate whether pinocytosis is a prerequisite of yolk-sac-mediated digestion of the ^{125}I-labelled forms of these proteins. If a significant fraction of the observed proteolysis occurs outside of the lysosomal system, there would be an over-estimate of the rate of pinocytic capture of these proteins. In the simplest case, extralysosomal

proteolysis could occur by proteinases released from the tissue into the incubation medium. In order to investigate this possibility, the activity of a cell-free yolk-sac homogenate towards the different ^{125}I-labelled ribonucleases was investigated over a wide pH range, to establish whether any activity could be exhibited in media of the same pH as the incubation medium. An alternative source of error in such uptake experiments would arise if the brush border of the yolk-sac epithelial cells carries proteinases that degrade a significant fraction of the ^{125}I-labelled protein to TCA-solubles; such activity would again lead to an over-estimate of the amount of proteolysis occurring within the lysosomal system subsequent to pinocytic capture. Inhibitors of yolk-sac pinocytosis (2,4-dinitrophenol and NH_4Cl) were therefore used to establish whether proteolysis of ^{125}I-labelled ribonuclease preparations was inhibited to the same degree as the uptake of ^{125}I-labelled PVP. Failure to abolish proteolysis when pinocytic capture has been stopped would, in the absence of any proteolysis by enzymes released into the medium, indicate that there is significant surface-bound proteinase activity present.

When investigating the rates of uptake of the different ^{125}I-labelled ribonuclease preparations, it was interesting to compare the relative effects of the presence, in the incubation medium, of calf serum (10% , v/v). Different degrees of competition between individual ribonuclease preparations and serum proteins may well help to indicate differences in the way(s) in which the different ribonucleases bind to sites on the surface of yolk-sac cells.

3.2 Materials and Methods

All the equipment and materials used in the experiments in this chapter were as described in Section 2.1. The medium used was 199, and calf serum (if present) was added at a concentration of 10% (v/v). ^{125}I-ribonucleases were radiolabelled as described in Section 2.2.1.

Almost all uptake experiments were performed as described in Section 2.3, and all assays as in Section 2.4. ^{125}I-PVP was always used at a concentration of 2 µg/ml, and ^{125}I-ribonucleases at a concentration of 1 µg/ml. The various agents were added 20 min prior to the addition of ^{125}I-labelled substrate, and the concentrations employed were as described in the text and in the legends to figures and tables. The only exception was when yolk sacs were preincubated in the presence of non-radioactive RNase S (see Table 3.3.2f). In this experiment some yolk sacs were preincubated with non-radioactively iodinated RNase S (^{127}I-RNase S) and control yolk sacs were not. Preincubation was performed in 50 ml Erlenmeyer flasks by placing ten yolk sacs in 20 ml gassed medium 199 containing ^{127}I-iodinated RNase S (10 µg/ml) and incubating them together in a shaking water bath at 37°C for 20 min. These yolk sacs were then removed from the flasks, washed for 3 x 2 min in gassed medium 199 (37°C), then each was put into a fresh 50 ml Erlenmeyer flask containing medium 199. Then ^{125}I-RNase S was added alone, or ^{125}I-RNase S plus 2,4-DNP were added together to make a final concentration of 1 µg/ml and 50 µg/ml, respectively, in a total volume of 10.0 ml medium 199. These flasks were gassed and then incubated for up to 3h, samples being removed at 0.5h intervals as described in Section 2.3, and incubation media and yolk-sac tissue were assayed as in Section 2.4. Control yolk sacs were preincubated as above in the absence of ^{127}I-iodinated RNase S (i.e. in medium 199 alone), washed, and incubated with ^{125}I-labelled RNase S either in the absence

or presence of 2,4-DNP, under the same incubation conditions as described above.

The experimental procedure to determine the effect of pH on the degradation of ^{125}I-labelled ribonucleases by yolk-sac homogenate was as described in Section 2.8, ^{125}I-ribonucleases being present at a concentration of 10 µg/200 µl incubation medium. The effect of "conditioned incubation media" on the stability of ^{125}I-ribonucleases was determined as described in Section 2.9, the concentration of ^{125}I-labelled substrate being 1 µg/ml.

3.3.1 The Rates of Uptake of Different ^{125}I-Ribonucleases by Yolk Sacs Incubated in the Presence and Absence of Serum

The uptake of the fluid-phase marker, ^{125}I-PVP, by yolk sacs in the presence and absence of calf serum is shown in Figure 3.3.1a. (In this series of experiments the concentration of calf serum, if present, was 10%, v/v). Uptake in the presence of serum was linear up to 6h, and the mean rate of uptake (or E.I.) was 1.92 µl/mg y.s. protein per h (see Table 3.3.1a) In the absence of serum the uptake of ^{125}I-PVP was higher, the mean E.I. being 3.39 µl/mg y.s. protein per h (see Table 3.3.1a), and the uptake plot was linear up to 3h (see Figure 3.3.1a). This indicates a higher rate of pinosome formation in the absence of serum, as was reported by Ibbotson & Williams (1979). Since the rates of uptake of all the ^{125}I-ribonuclease preparations were very high (particularly in the absence of serum, see below), leading to substantial depletion of their concentration in the incubation medium, incubations were routinely terminated at 3h; matched ^{125}I-PVP controls were also terminated at this time. (This point is more fully discussed later in this section.)

The time courses of uptake, in the presence of calf serum, of a number of different ^{125}I-ribonucleases, A, B, S and Spr were examined. The results are shown graphically in Figure 3.3.1b and in numerical detail in Table 3.3.1b. The plots of uptake against incubation time were linear for all the ribonucleases. The E.I's for ^{125}I-RNases S and Spr were very similar, 351 and 345 µl/mg y.s. protein per h respectively, and these values were higher than those of the other two ribonucleases; the E.I. of ^{125}I-RNase A was 187 µl/mg y.s. protein per h, and that of ^{125}I-RNase B was 139 µl/mg y.s. protein per h. The rate of uptake of ^{125}I-RNase A agrees well with the value of 147 µl/mg y.s. protein per h reported by Livesey & Williams (1981) for this protein in the presence of 10% serum.

Removal of serum from the medium resulted in an increase in the rates of uptake of all the ribonucleases, as shown in Figure 3.3.1c and Table 3.3.1c. Again the E.I's of both ^{125}I-RNases A and B were similar (515 and 538 µl/mg y.s. protein per h, respectively), but were lower than those of ^{125}I-RNases S and Spr (708 and 641 µl/mg y.s. protein per h, respectively). Livesey (1978) reported an E.I. of 638 µl/mg y.s. protein per h for ^{125}I-RNase A in serum-free medium.

Before further analysis of these data was initiated, the simplest hypothesis to account for the above results, namely that ^{125}I-ribonucleases stimulate the rate of fluid-phase pinocytosis, was examined. Yolk sacs were incubated with ^{125}I-PVP in serum-free medium in the presence of ribonucleases iodinated with non-radioactive iodide, ^{127}I-ribonucleases. The results of these experiments are given in Figure 3.3.1d and Table 3.3.1d. None of the four ^{127}I-iodinated ribonucleases altered the linearity or the rate of uptake of ^{125}I-PVP when present in the medium, indicating that iodinated ribonucleases do not affect fluid-phase pinocytosis in rat yolk sacs at the concentration used (1 µg/ml). Livesey & Williams (1981) found a similar result for the effect of ^{127}I-iodinated RNase A on ^{125}I-PVP uptake in the presence of serum.

The uptake of ^{125}I-ribonucleases by yolk sacs in the presence of serum, is accompanied by release of acid-soluble radioactivity into the incubation medium as shown in Figure 3.3.1e, indicating that degradation of these substrates occurs in the presence of the yolk-sac tissue. The release of acid-soluble radioactivity with incubation time was linear, and the rate of this release was virtually the same as the rate of uptake (E.I.) for each of the four ^{125}I-ribonucleases (compare mean E.I. values with rates of soluble release in Table 3.3.1b). The quantity of radioactivity associated with the yolk-sac tissue reached a constant level during the first hour of incubation, (see Figure

3.3.1f, part (ii)), and the mean value of this tissue-associated radioactivity (T.A.R.) was very similar for all the four ^{125}I-ribonucleases (see Table 3.3.1b), ranging from 99 to 119 µl/mg y.s. protein.

If all exogenous ^{125}I-labelled proteins were ingested at the same rate and into the same class of vesicle in the yolk-sac tissue, then any difference in the quantity of radioactivity associated with the tissue would be expected to reflect the speed at which the different proteins are degraded within the lysosomal system. Thus less readily degraded proteins would be expected to reach a higher steady-state concentration within lysosomes than those proteins that are more readily degraded, and hence the former would be expected to show a higher T.A.R. value than the latter. In practice, the T.A.R. values of many proteins have been observed to reach constant values within 1-2h of incubation indicating that steady-state levels are reached during the usual incubation period. However, the rates of uptake of individual proteins differ considerably, so that meaningful comparison of tissue levels is only possible if tissue levels are first normalized for differences in uptake rate. If, in the simplest case, tissue levels are constant beyond the first hour or so of incubation and the rate of uptake is also constant, the normalized tissue level is given as:

$$\frac{\text{T.A.R. (µl/mg yolk-sac protein)}}{\text{Endocytic Index (µl/mg yolk-sac protein per h)}}$$

This ratio has the units of time (h) and was termed by Livesey (1978) the 'Disposal Time' since he suggested that the magnitude of this ratio reflects the time taken for the protein to be fully degraded within the lysosomal system to fragments small enough to be released across the lysosomal membrane into the incubation medium. However, this interpretation assumes that both the quantity of undegraded protein within pinosomes that is in transit to the lysosomal system and the quantity of acid-soluble

material present in the tissue are negligible compared with the amount of protein undergoing degradation within lysosomes. An instantaneous value of the disposal time at any point during the incubation period may be calculated by normalizing the tissue-associated radioactivity for the amount of a substrate taken up during the period of incubation:

$$\frac{\text{T.A.R. (µl/mg y.s. protein) x period of incubation (h)}}{\text{Uptake over the incubation period (µl/mg y.s. protein)}}$$

The mean Disposal Times for the ^{125}I-ribonucleases in the presence of serum were calculated as [mean T.A.R. (µl/mg y.s. protein)/mean E.I. (µl/mg y.s. protein per h)], and these values are shown in Table 3.3.1b. The values for ^{125}I-RNases S and Spr were both approximately 0.3h, and for ^{125}I-RNases A and B were approximately 0.6h. The values of the Disposal Time following various periods of incubation were also calculated for the four ^{125}I-ribonucleases using the alternative equation (see Figure 3.3.1g). As expected, these Disposal Times did not change during the period of incubation; those of ^{125}I-RNases A and B were higher than those of ^{125}I-RNases S and Spr.

The duration of the experiments in the absence of serum was 3h only. This was because the rates of uptake of the ^{125}I-ribonucleases were so high that continuation of the incubations beyond 3h led to serious problems of substrate depletion, resulting in very scattered data with apparent falls in total uptake and tissue-associated radioactivity values (data not presented here.) This can easily be appreciated from a

simple calculation involving ^{125}I-RNase A. In the absence of serum the E.I. of this protein was 515 µl/mg y.s. protein per h. This is the volume of incubation medium whose contained substrate is cleared by each mg of yolk-sac protein during 1h. For a yolk sac containing 5 mg of protein, this represents the depletion of substrate from a volume of incubation medium of approximately 2,500 µl in 1h. If the incubations were allowed to proceed much over 3h, then well over half of the substrate would have been removed from the incubation medium during this time, leading to reductions in substrate concentrations and hence yielding data that would no longer be reliable.

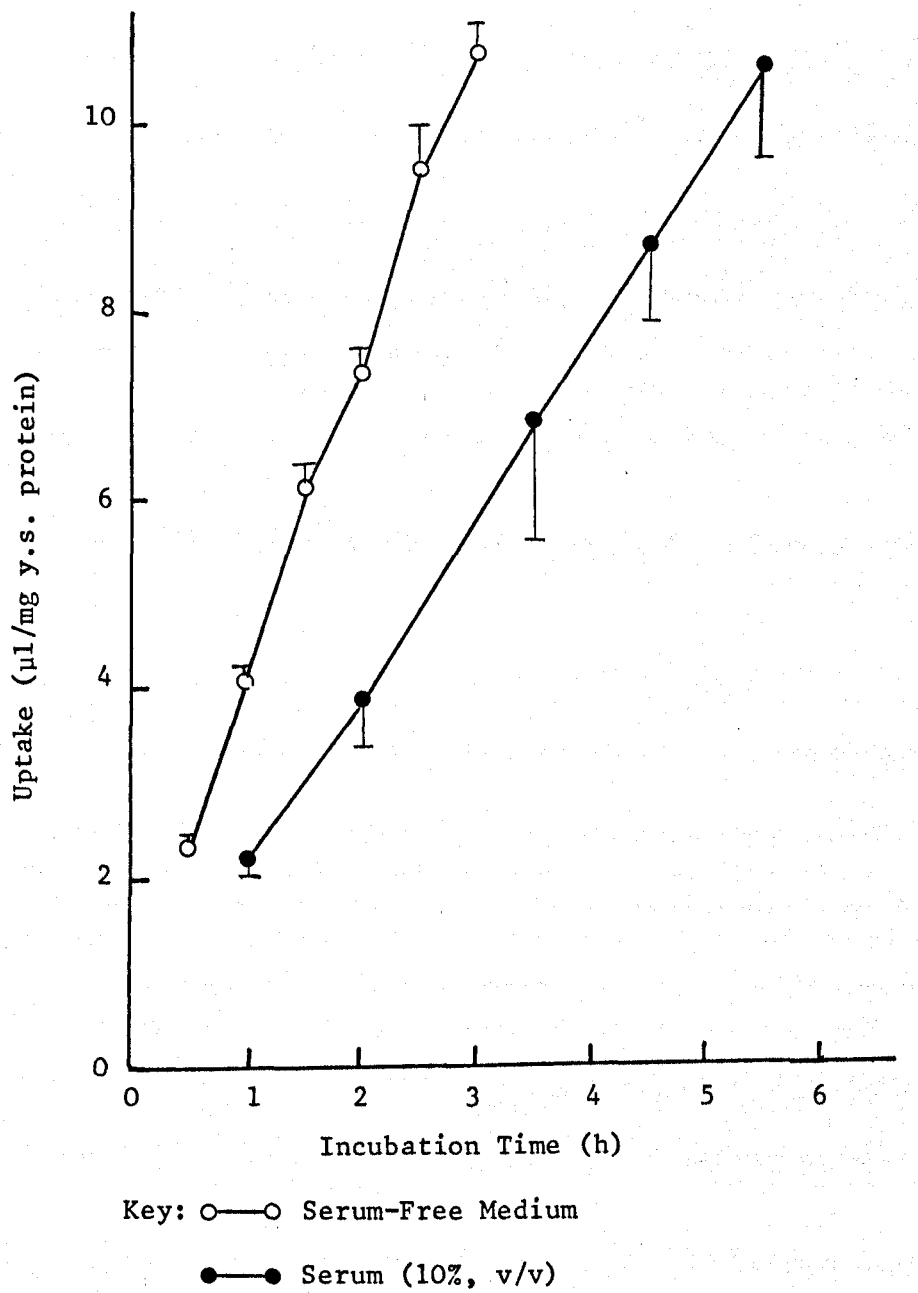
During uptake of ^{125}I-ribonucleases by yolk sacs incubated in serum-free medium, the amount of acid-soluble radioactivity released back into the incubation medium rose linearly with time (see Figure 3.3.1h). As in the experiments in the presence of serum, the rate of release of acid-soluble radioactivity was very similar to the rate of uptake for each of the substrates, and this is shown in Table 3.3.1c. The quantity of radioactivity associated with the tissue remained constant during the incubation period (see Figure 3.3.1f), and the mean T.A.R. values, as seen in Table 3.3.1c, were similar, ranging from 164 to 204 µl/mg y.s. protein.

The disposal times of the different ^{125}I-ribonucleases in the absence of serum are shown graphically in Figure 3.3.1g, part (i), and were constant during the period of incubation with very little difference between them. This is reinforced by the mean disposal times reported in Table 3.3.1c, which shows the values for ^{125}I-RNases A and B (0.32 and 0.33h, respectively) to be almost the same as those for ^{125}I-RNases S and Spr (0.29 and 0.26h, respectively). The disposal time for ^{125}I-RNase A is in agreement with the value of 0.36 h, as found by Livesey (1978). These findings contrast with those in the presence of serum, which show the disposal times for ^{125}I-RNases A and B to be higher than for ^{125}I-RNases S and Spr.

The release of acid-soluble radioactivity into serum-free medium during the first hour of incubation was carefully monitored, samples of medium being removed for analysis every 5 min during the first part of the incubation. Figure 3.3.1i shows these results. Each ^{125}I-ribonuclease had a lag-period of approximately 10 min before acid-soluble radioactivity release was detectable. Livesey (1978) also found a lag-period of 10 min for ^{125}I-RNase A in the absence of serum. This finding is in agreement with all four ^{125}I-ribonucleases having the same mean period of association with the tissue before release of acid-soluble fragments (the same disposal times) in serum-free medium.

In Table 3.3.1e the results for all the different substrates ingested in the presence of serum are expressed as percentages of the corresponding data in serum-free media. The close similarities between ^{125}I-RNases A and B, and between S and Spr are apparent from this table. From the effect on the rate of uptake of ^{125}I-PVP, the effect of serum on the rate of fluid-phase pinocytosis is to reduce it to 57% of the corresponding rate in serum-free medium. The effect on ^{125}I-RNases S and Spr is similar, being 50% and 54% respectively, but the relative effect of addition of serum on the rates of uptake of ^{125}I-RNases A and B is much greater, relative rates of uptake being 36% and 26%, respectively. These results are consistent with serum affecting the rates of uptake of ^{125}I-RNases S and Spr simply by decreasing the rate of fluid-phase pinocytosis, whereas the larger decreases in the rates of uptake of ^{125}I-RNases A and B suggest competition for plasma membrane binding sites between serum proteins and these two species of ribonuclease.

Figure 3.3.1a Uptake of ^{125}I-PVP by Yolk Sacs Incubated in the Absence and Presence of Calf Serum (10%, v/v)



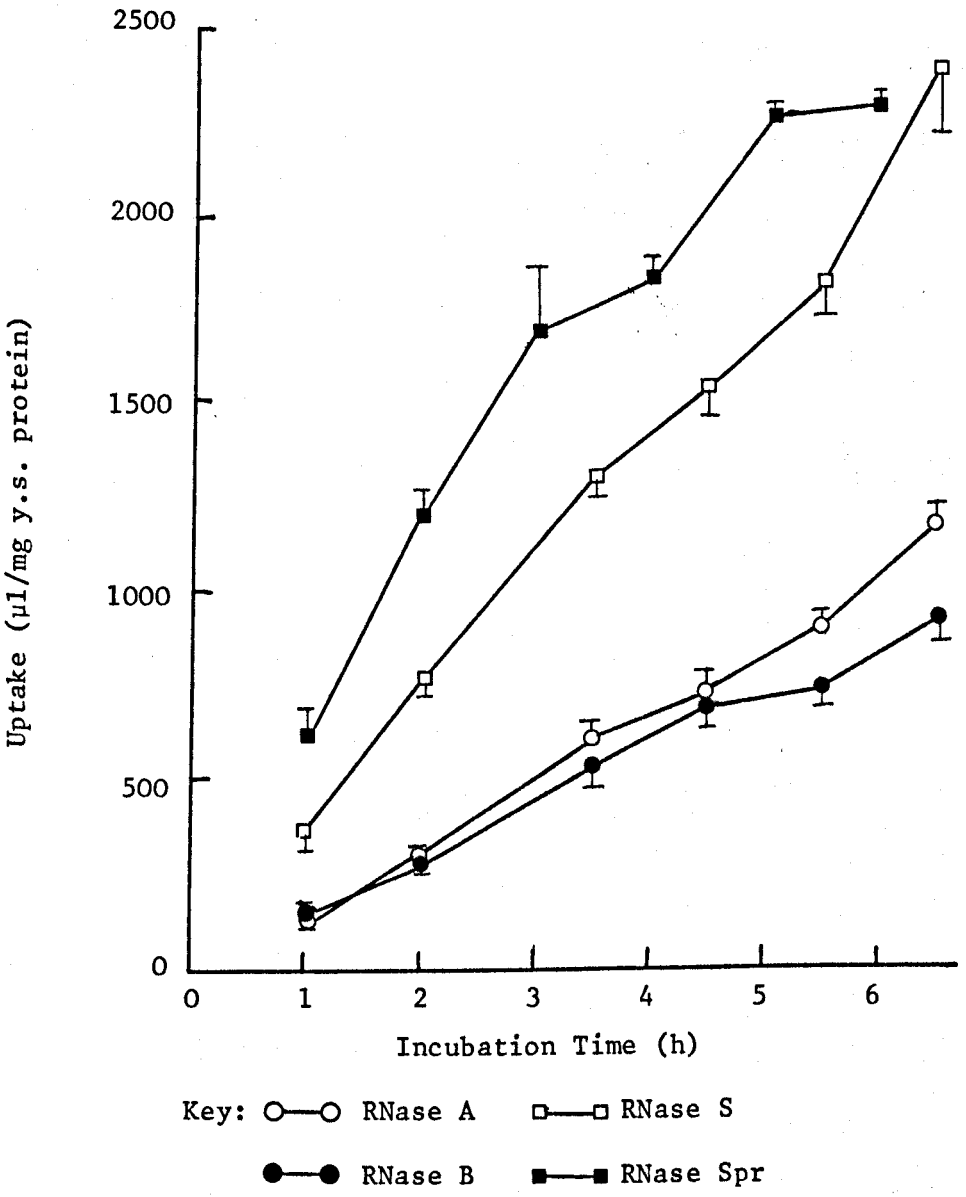
The method used was as described in Section 3.2. ^{125}I-PVP was present at a concentration of 2 µg/ml. Each uptake plot shows mean values (± S.E.M.) from 4 to 6 separate experiments. These results are summarized in Table 3.3.1a.

Table 3.3.1a Summary of Data for ^{125}I-PVP Uptake by Yolk Sacs Incubated in the Absence and Presence of Calf Serum (10%, v/v)

These data are from the experiments shown in Figure 3.3.1a. Rate of uptake is expressed as the Endocytic Index (E.I.), and is given with its correlation coefficient (Corr.Co.).

Serum Conc. (%)	E.I. (Corr.Co.) (µl/mg y.s. (protein per h)	Mean E.I. ± S.E.M. (µl/mg y.s. protein per h)
0	3.51 (0.972)	3.39 ± 0.10
	3.31 (0.984)	
	3.41 (0.946)	
	3.24 (0.992)	
	3.79 (0.984)	
	3.06 (0.977)	
10	2.51 (0.990)	1.92 ± 0.25
	2.17 (0.995)	
	1.48 (0.962)	
	1.52 (0.977)	

Figure 3.3.1b Uptake of Different ^{125}I-Ribonucleases by Yolk Sacs Incubated in the Presence of Calf Serum (10%, v/v)



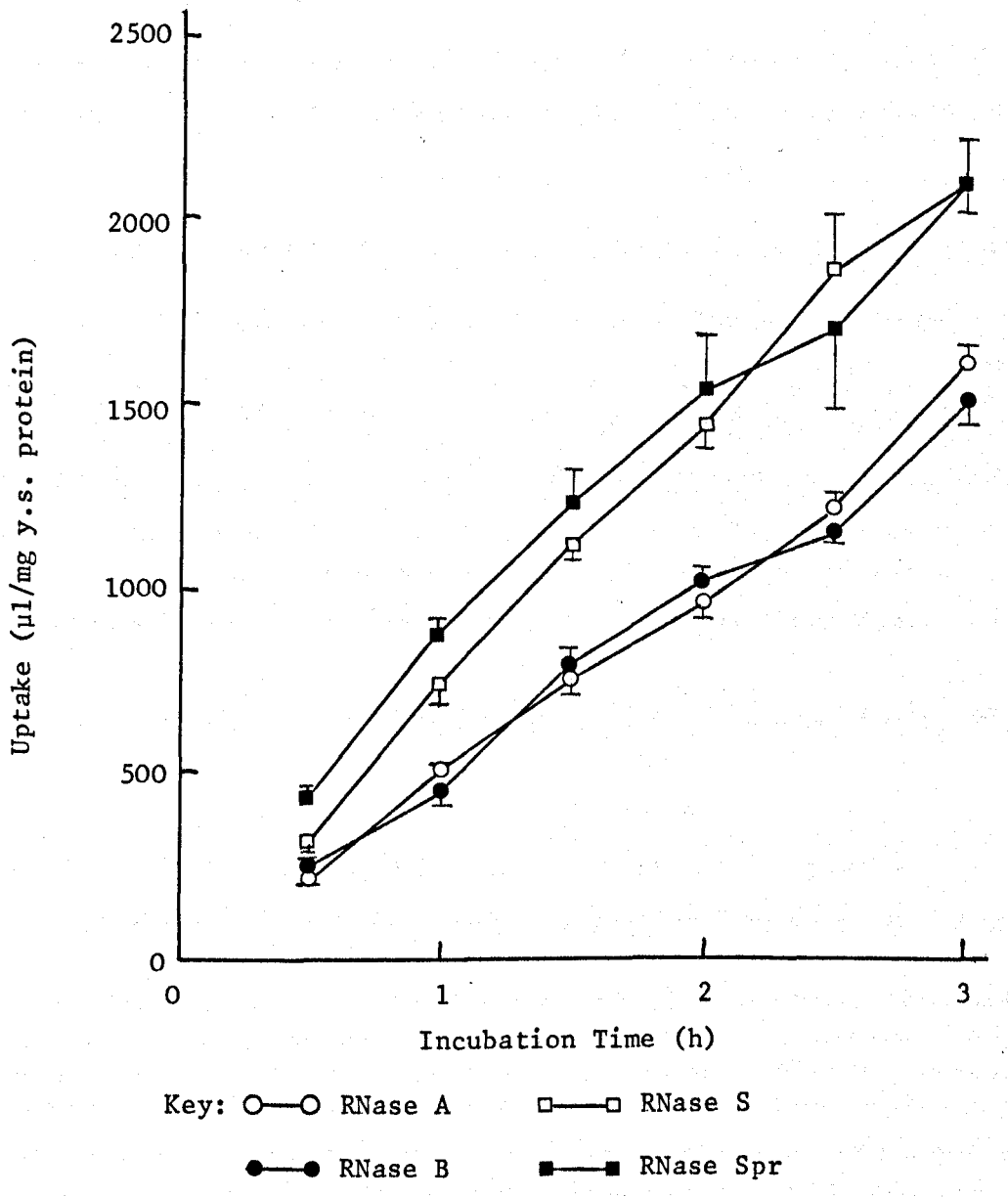
The method used was as described in Section 3.2. All ^{125}I-ribonucleases were present at a concentration of 1 µg/ml. Each uptake plot shows mean values (± S.E.M.) from 3 separate experiments. These results are summarized in Table 3.3.1b.

Table 3.3.1b Summary of Uptake Data for ^{125}I-RNases A, B, S and Spr by Yolk Sacs Incubated in the Presence of Calf Serum (10%, v/v)

The data reported below indicate the main features of the uptake and degradation of different ribonuclease preparations when incubated in medium containing 10% (v/v) calf serum. Rate of uptake is expressed as the Endocytic Index (E.I.), and is given with its correlation coefficient (Corr.Co.). Mean disposal time is calculated as (mean T.A.R./mean E.I.) and it represents the mean time that ^{125}I-ribonucleases are associated with the tissue before acid-soluble fragments are released. The rate of release of these acid solubles (Rate of Sol. Rel.) are also expressed. The data in all but the last column of this table are reported more fully elsewhere (Figures 3.3.1b, e and f).

RNase	E.I.(Corr.Co.) (µl/mg y.s. protein/h)	Mean E.I. (± S.E.M.) (µl/mg y.s. protein/h)	Rate of Sol. Rel. (µl/mg y.s. protein/h)	Mean T.A.R. (µl/mg y.s. protein)	Mean Disposal Time (h)
A	191 (0.988) 189 (0.958) 169 (0.988)	187 ± 7	175	105	0.56
B	123 (0.954) 153 (0.991) 141 (0.988)	139 ± 9	132	99	0.71
S	433 (0.974) 309 (0.977) 311 (0.971)	351 ± 41	344	119	0.34
Spr	364 (0.982) 345 (0.964) 326 (0.910)	345 ± 11	347	112	0.32

Figure 3.3.1c Uptake of Different ^{125}I-Ribonucleases by Yolk Sacs Incubated in Serum-Free Medium



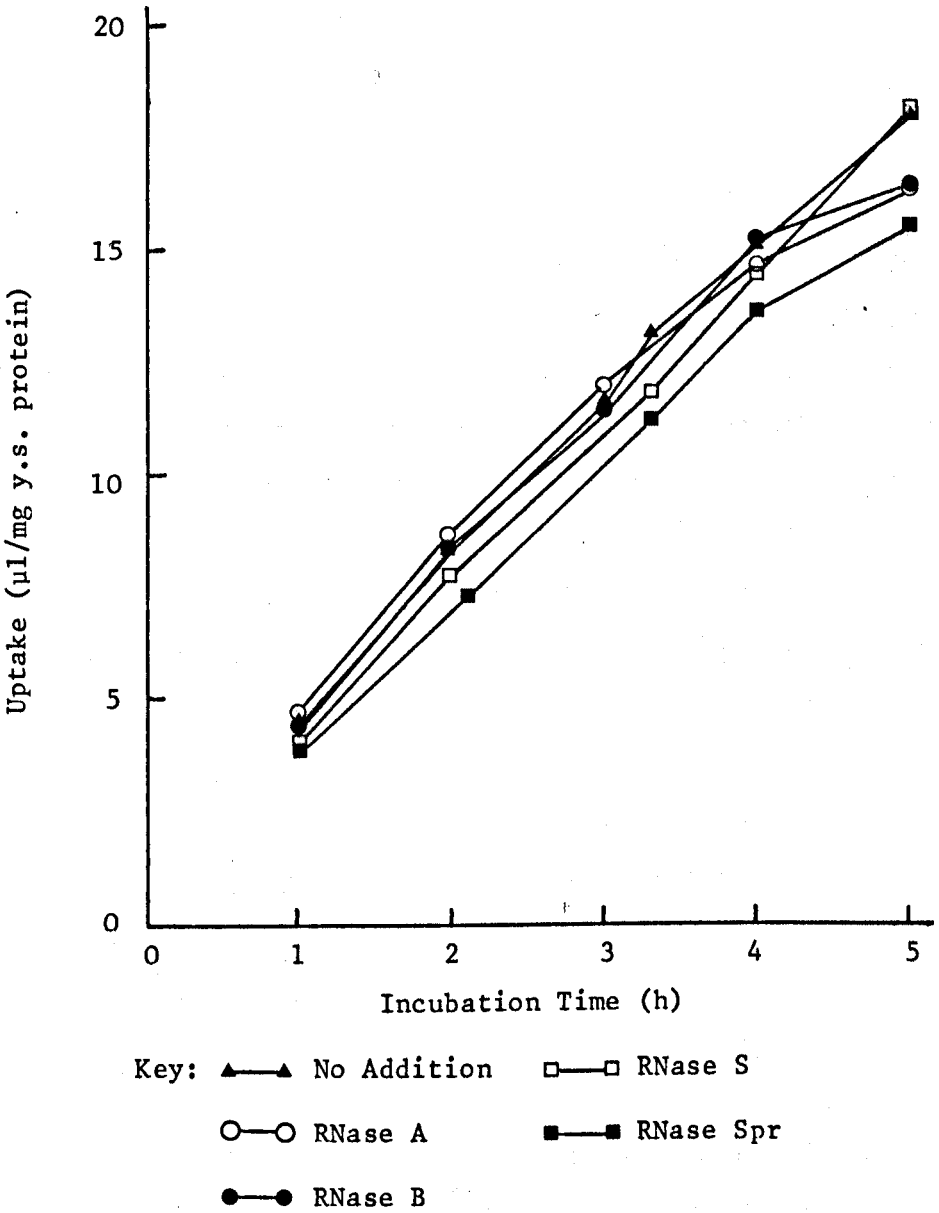
The method used was as described in Section 3.2. All ^{125}I-ribonucleases were present at a concentration of 1 µg/ml. Each uptake plot shows mean values (± S.E.M.) from 2 to 5 separate experiments. These results are summarized in Table 3.3.1c.

Table 3.3.1c Summary of Data for Uptake of Different ^{125}I-Ribonucleases by Yolk Sacs Incubated in Serum-Free Medium

These data are from the experiments shown in Figures 3.3.1c, f and h. The abbreviations used are as in Table 3.3.1b.

RNase	E.I.(Corr.Co.) (µl/mg y.s. protein/h)	Mean E.I. (± S.E.M.) (µl/mg y.s. protein/h)	Rate of Sol. Rel. (µl/mg y.s. protein/h)	Mean T.A.R. (µl/mg y.s. protein)	Mean Disposal Time (h)
A	518 (0.971) 535 (0.959) 525 (0.983) 553 (0.982) 446 (0.996)	515 ± 18	526	164	0.32
B	499 (0.972) 404 (0.980) 603 (0.971) 620 (0.969) 564 (0.991)	538 ± 39	484	179	0.33
S	720 (0.983) 600 (0.959) 772 (0.992) 740 (0.961)	708 ± 33	713	204	0.29
Spr	712 (0.985) 570 (0.975)	641	669	166	0.26

Figure 3.3.1d Effect of the Presence of Different ^{127}I-Ribonucleases on Uptake of ^{125}I-PVP by Yolk Sacs Incubated in Serum-Free Medium



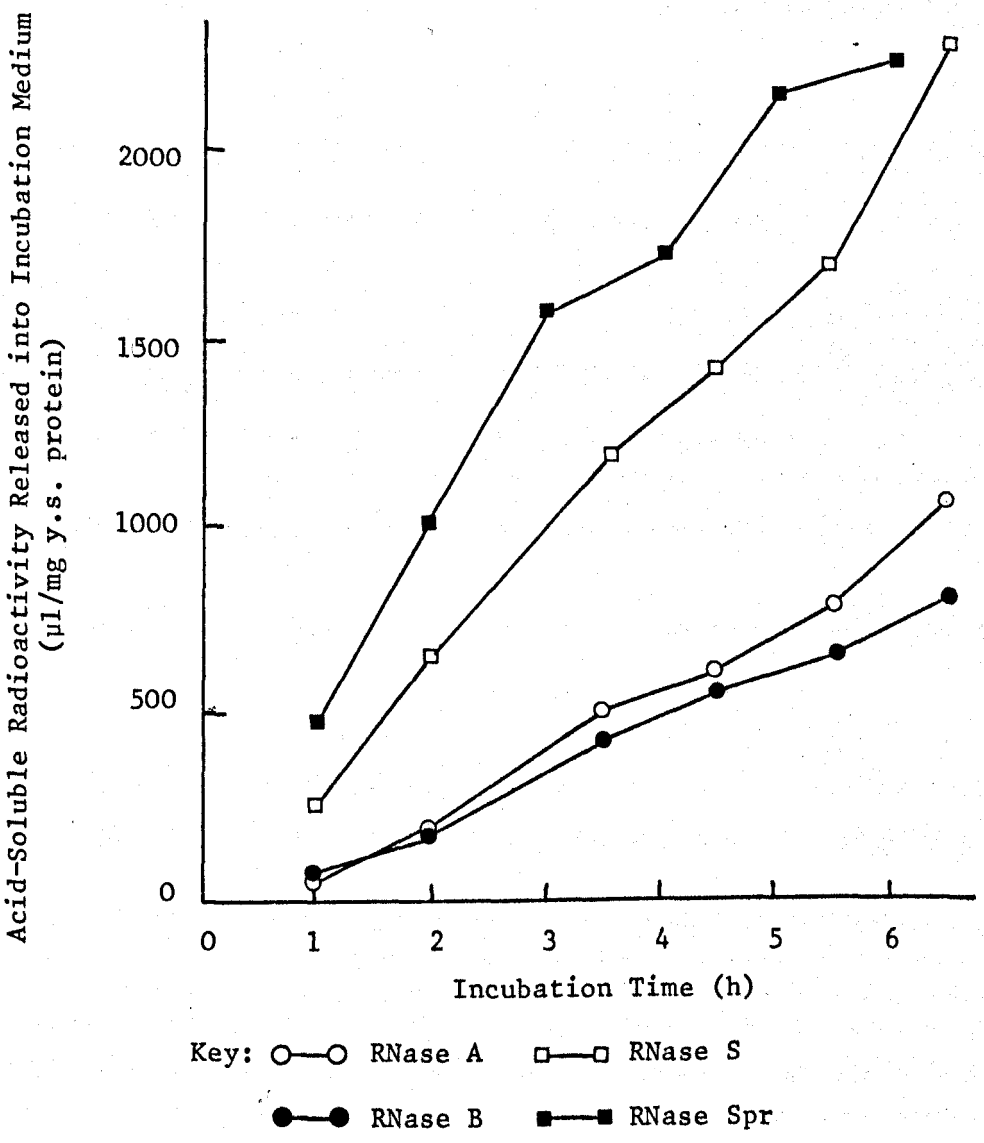
The method used was as described in Section 3.2. All ^{127}I-ribonucleases were present at a concentration of 1 µg/ml, and were added 20 min before addition of ^{125}I-PVP (2 µg/ml). Each uptake plot shows mean values from 2 to 6 separate experiments. Individual S.E.M. values of the means are not indicated but all means have a S.E.M. of less than 1.0. These results are summarized in Table 3.3.1d.

Table 3.3.1d Summary of the Effect of the Presence of Different ^{127}I-Ribonucleases on Uptake of ^{125}I-PVP by Yolk Sacs Incubated in Serum-Free Medium

These data are from the experiments shown in Figure 3.3.1d. Each E.I. value (rate of uptake) is given with its correlation coefficient (Corr.Co.).

RNase	E.I.(Corr.Co.) (µl/mg y.s. (protein per h)	Mean E.I. ± S.E.M. (µl/mg y.s. protein per h)	Mean E.I. as % of Control (%)
No Addition (Control)	3.01 (0.990)		
	3.28 (0.937)		
	4.22 (0.983)	3.38 ± 0.21	100.0 ± 6.2
	3.11 (0.974)		
	2.87 (0.985)		
	3.79 (0.996)		
	2.75 (0.973)		
A	3.10 (0.977)	3.06 ± 0.17	90.5 ± 5.0
	3.34 (0.976)		
	2.73 (0.959)		
B	2.83 (0.959)	3.06 ± 0.28	90.5 ± 8.3
	3.62 (0.976)		
	3.26 (0.989)		
S	3.16 (0.965)	3.21	95.0
	2.92 (0.983)		
Spr	2.67 (0.981)	3.02 ± 0.24	89.3 ± 7.1
	3.48 (0.959)		

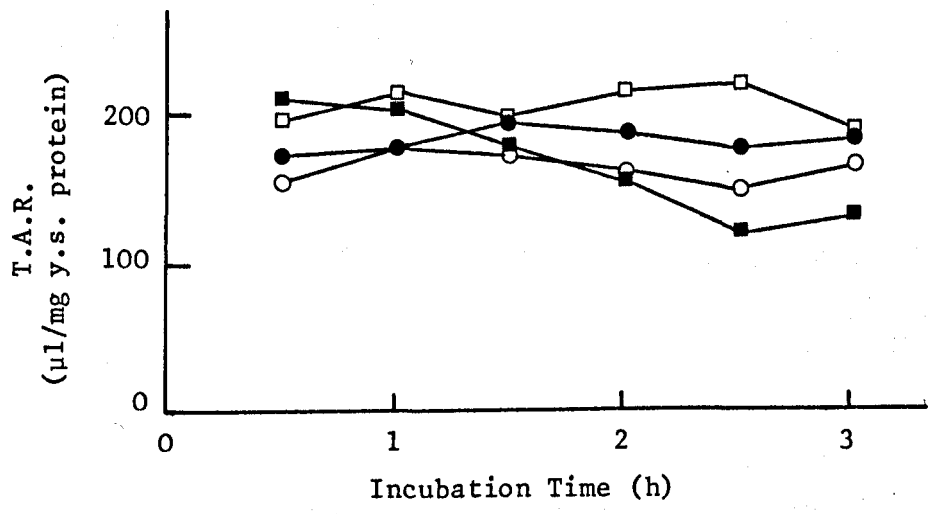
Figure 3.3.1e Release of Acid-Soluble Radioactivity into Incubation Medium by Yolk Sacs during Uptake of Different ^{125}I-Ribonucleases in the Presence of Calf Serum (10%, v/v)



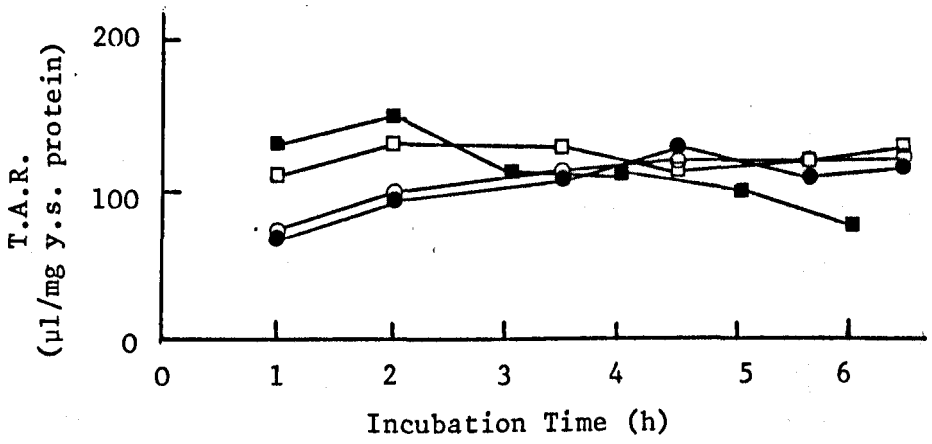
The acid-soluble radioactivity released into the incubation medium is expressed as mean values. These results are from the experiments shown in Figure 3.3.1b, and are summarized in Table 3.3.1b.

Figure 3.3.1f Quantity of Radioactivity that becomes Associated with Yolk-Sac Tissue during Uptake of Different ^{125}I-Ribonucleases when Incubated in the Absence and Presence of Calf Serum (10%, v/v)

(i) In absence of serum



(ii) In 10% serum



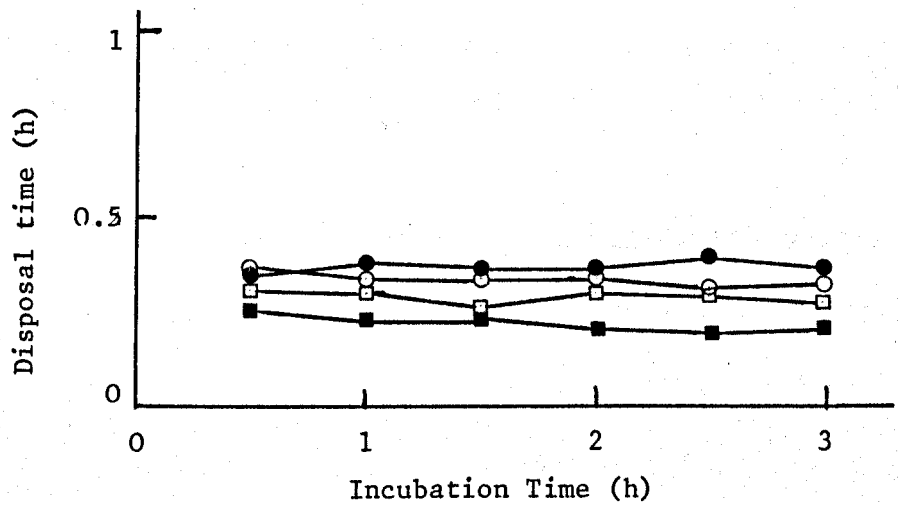
Key: ○—○ RNase A □—□ RNase S

●—● RNase B ■—■ RNase Spr

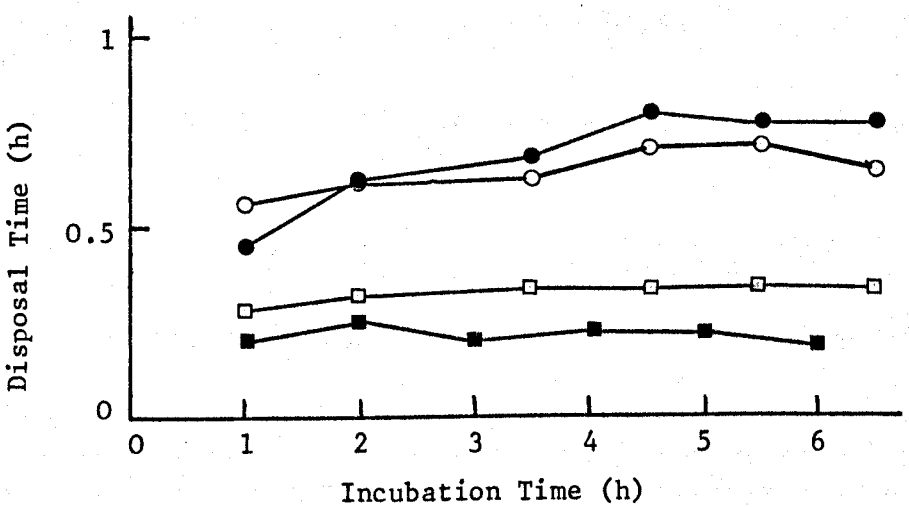
The quantity of radioactivity that becomes associated with yolk-sac tissue (tissue-associated radioactivity, T.A.R.) is expressed as mean values. Individual S.E.M. values of the means are not indicated, but all means have a S.E.M. of between 5 and 15. These results are from the experiments shown in Figures 3.3.1b and c, and are summarized in Tables 3.3.1b and c.

Figure 3.3.1g Disposal Time of Different ^{125}I-Ribonucleases after their Uptake by Yolk Sacs Incubated in the Absence and Presence of Calf Serum (10%, v/v)

(i) In absence of serum



(ii) In presence of serum

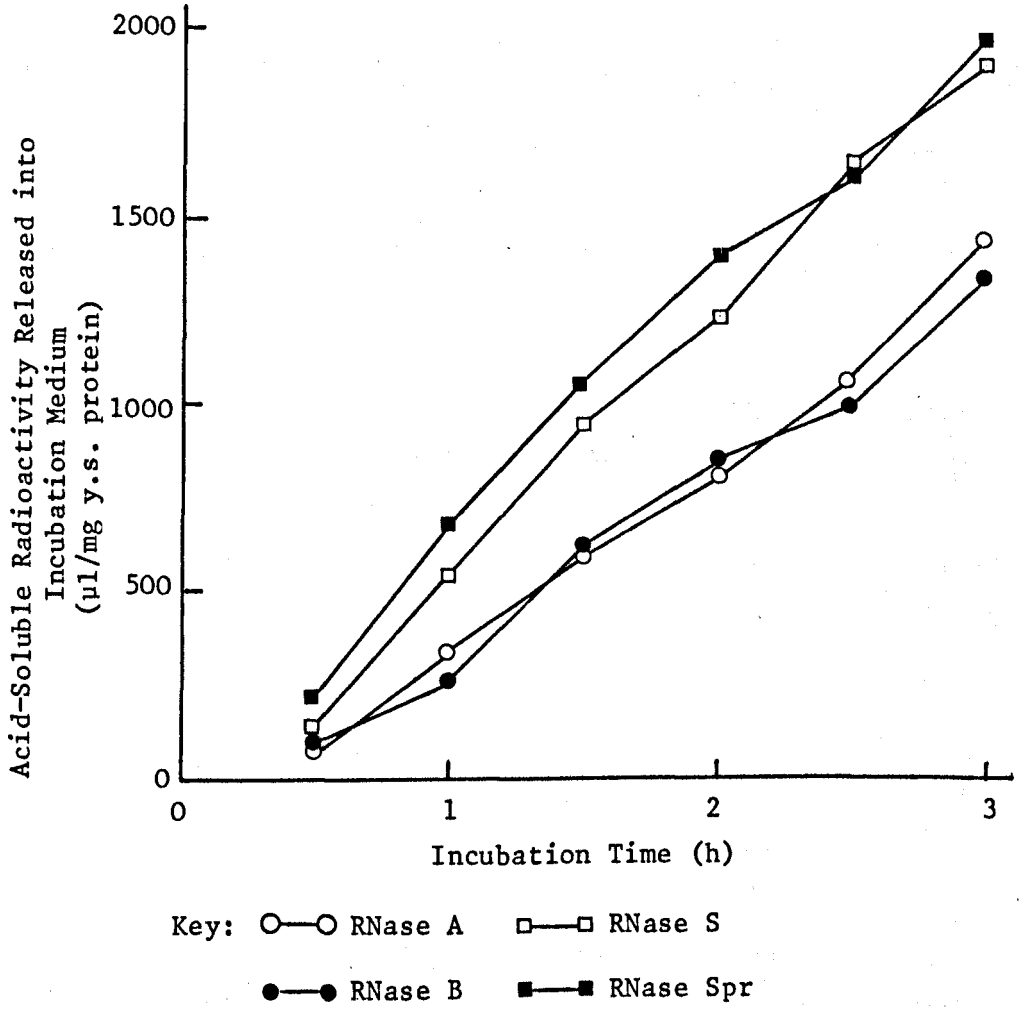


Key: ○—○ RNase A □—□ RNase S

●—● RNase B ■—■ RNase Spr

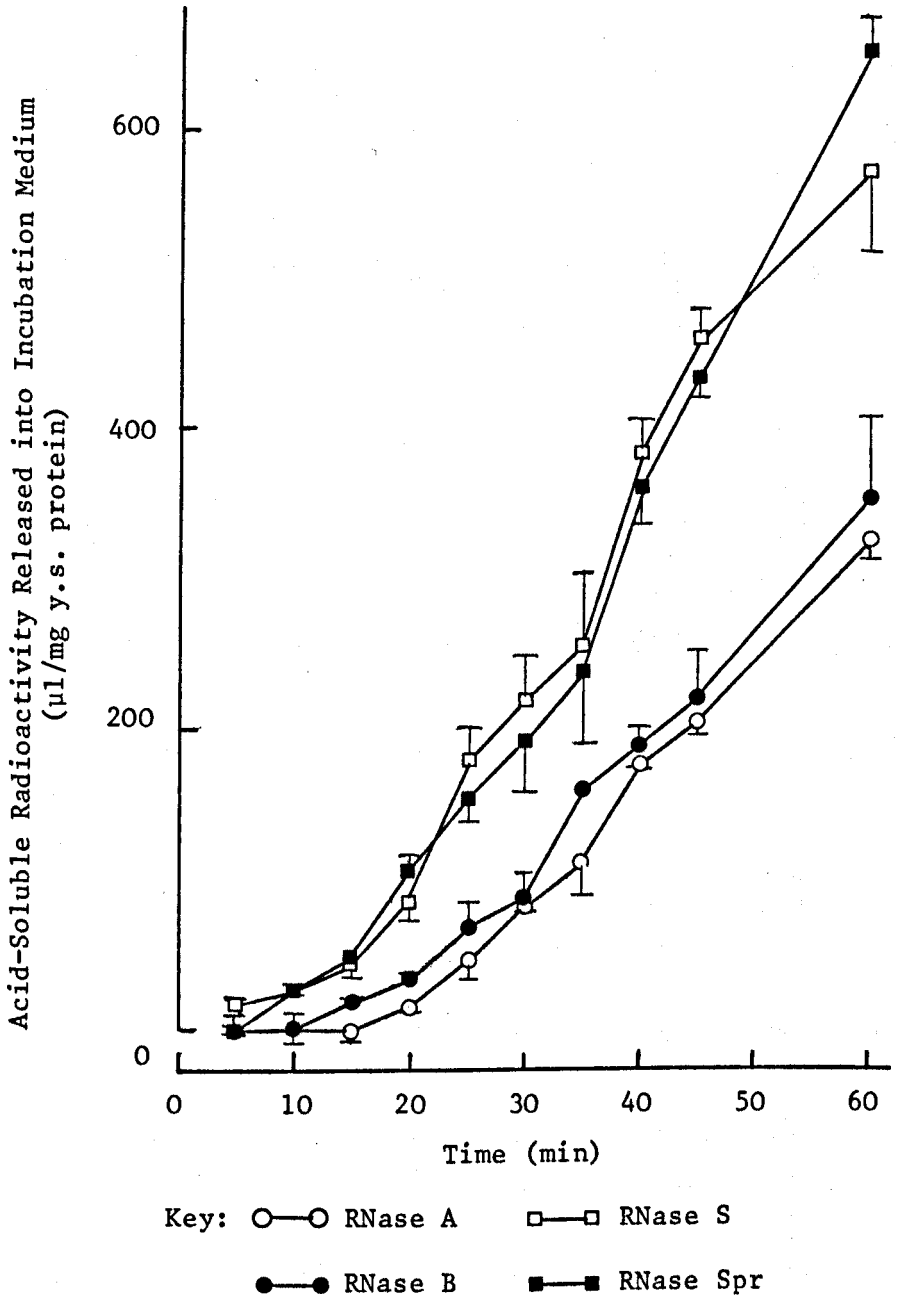
Disposal time is calculated as (T.A.R. x period of incubation (h)/ uptake), and it represents the mean time ^{125}I-ribonucleases are associated with the tissue before acid-soluble fragments are released. These results are from the experiments shown in Figures 3.3.1b and c.

Figure 3.3.1h Release of Acid-Soluble Radioactivity into Incubation Medium by Yolk Sacs during Uptake of Different ^{125}I-Ribonucleases in Serum-Free Medium



The acid-soluble radioactivity released into the incubation medium is expressed as mean values. These results are from the experiments shown in Figure 3.3.1c, and are summarized in Table 3.3.1c. A detailed study of the acid-soluble radioactivity released into the incubation medium during the first hour of incubation in serum-free medium is shown in Figure 3.3.1i.

Figure 3.3.1i Release of Acid-Soluble Radioactivity into Incubation Medium by Yolk Sacs during the First Hour of Uptake of Different ^{125}I-Ribonucleases in Serum-Free Medium



The acid-soluble radioactivity released into the incubation medium is expressed as mean values (± S.E.M.) from 3 separate experiments. The acid-soluble radioactivity released into the incubation medium during 3h of incubation in serum-free medium is shown in Figure 3.3.1h.

Table 3.3.1e Summary of the Relative Effects of Calf Serum (10%) on Uptake of ^{125}I-PVP and ^{125}I-Ribonucleases by Yolk Sacs Compared to their Uptake in Serum-Free Medium.

The data presented here are taken from Tables 3.3.1a, b and c, and the abbreviations and units used are as in Table 3.3.1b. All these data are expressed relatively by reporting the quantity in the presence of serum as a percentage of the equivalent quantity from experiments in the absence of serum.

Quantity Measured	Relative Effect (%) on a Given Substrate				
	PVP	RNase A	RNase B	RNase S	RNase Spr
Mean E.I.	57	36	26	50	54
Rate of Sol. Rel.	-	33	27	48	52
Mean T.A.R.	-	64	55	58	67
Mean Disposal Time	-	175	215	117	123

3.3.2 The Effects of NH_4Cl and 2,4-DNP on the Uptake of Different ^{125}I-Ribonucleases by Yolk Sacs.

The results in this section are illustrated graphically in figures, and presented in greater detail in corresponding tables. Since the variety of experimental conditions was large, the relative effects of the different agents used are summarized in tables at the end of the section (results being expressed as percentages of the results in the absence of any agent). The relative effects of the agents in the presence of serum are reported in Table 3.3.2g, and the relative effects in serum-free medium are reported in Table 3.3.2h.

Ammonium ions (in the form of 20mM NH_4Cl) were added to yolk sacs, and their effect on fluid-phase pinocytosis in serum-free medium is shown in Figure 3.3.2a. The uptake of the fluid-phase marker, ^{125}I-PVP, virtually ceased in the presence of the ammonium ions, the E.I. being 0.20 µl/mg y.s. protein per h (see Table 3.3.2a) which is similar to the value reported by Livesey et al. (1980) for experiments with 20mM NH_4Cl.

The effect of NH_4Cl on uptake of ^{125}I-ribonucleases from serum-free medium is shown in Figure 3.3.2b. Likewise, their uptake was virtually completely inhibited by 20mM NH_4Cl; the E.I. values are reported in Table 3.3.2a. The rate of release of acid-soluble radioactivity was similarly completely inhibited for each ^{125}I-ribonuclease, as would be expected from the uptake data. The relative effects of NH_4Cl (20mM) on uptake of ^{125}I-PVP and ^{125}I-ribonucleases in serum-free medium are summarized in Table 3.3.2h. The relative mean E.I's and rates of release of acid-soluble radioactivity are all 6%, or less, than control values (Livesey et al. (1980) found uptake of ^{125}I-PVP was 16% and uptake of formaldehyde-denatured ^{125}I-labelled BSA was 10% of control values).

The quantity of radioactivity associated with the yolk-sac tissue

(T.A.R. value) remained constant throughout the incubation period, although the mean values of this quantity for ^{125}I-RNases S and Spr were higher than those of ^{125}I-RNases A and B. The T.A.R. value for ^{125}I-RNases A and B in the presence of 20mM NH_4Cl is reduced to 10% of the control value, whereas the relative mean T.A.R. values for ^{125}I-RNases S and Spr are about 50% of the control values. This may be indicative of a higher non-specific binding of these last two ^{125}I-ribonucleases to yolk sacs over and above that of ^{125}I-RNases A and B.

Mean disposal times were not calculated from these data, since in the absence of any progressive uptake of radioactivity, the concept of the disposal time (the mean time for which radioactivity is associated with the yolk-sac tissue before its release as acid-soluble radioactivity) has no real meaning.

The rate of fluid-phase pinocytosis, as determined by the rate of uptake of ^{125}I-PVP, was measured in the presence of 2,4-DNP. The effect of 2,4-DNP at a concentration of 50 µg/ml in the presence of 10% calf serum is shown in Figure 3.3.2c, and the calculated E.I. values are reported in Table 3.3.2b. These data support the observation by Duncan & Lloyd (1978) that, under these conditions, the pinocytic uptake of ^{125}I-PVP was completely abolished. The uptake of ^{125}I-PVP in the presence of 2,4-DNP in serum-free medium was virtually completely inhibited at 50 µg/ml DNP, but inhibition was much less marked at a DNP concentration of 10 µg/ml (see Figure 3.3.2c and Table 3.3.2b). Similar findings were reported by Weisbecker (1981).

The effect of 2,4-DNP (50 µg/ml) on the uptake of ^{125}I-ribonucleases in the presence of serum is shown in Figure 3.3.2d. The uptake plots show that the rates of pinocytosis were greatly reduced under these conditions; these rates are reported in Table 3.3.2c. Again, as with inhibition of uptake by NH_4Cl, the rate of release of acid-soluble

radioactivity was very close to the observed E.I. The quantity of radioactivity associated with the yolk-sac remained constant during the incubation period, and was very low for ^{125}I-RNases A and B, but higher for ^{125}I-RNases S and Spr. The calculated mean disposal times were higher than in the absence of 2,4-DNP, ranging from 0.92 to 1.86 h.

The effect of 2,4-DNP (50 µl/ml) on the uptake of ^{125}I-labelled substrates from medium containing serum are summarized in Table 3.3.2g. The mean E.I. of ^{125}I-PVP was 4% of the control value. The relative uptake rate of ^{125}I-RNase A was very similar (3%), whereas the relative rates of uptake of the other ^{125}I-ribonucleases were slightly higher, ranging from 9% for ^{125}I-RNase B to 18% of the control value for ^{125}I-RNase S; these relative uptake rates reflect the relative rates of release of acid-soluble radioactivity. The relative mean disposal times were higher than control values, varying between 1.3- and 5.5-times the control values. [Calculation of a mean disposal time for ^{125}I-RNase A is questionable since uptake of this protein was completely abolished in the presence of 50 µg/ml 2,4-DNP (see above for discussion on this point), but it has been included for completeness.] These findings suggest that although 2,4-DNP (50 µg/ml) in the presence of serum can virtually fully inhibit fluid-phase pinocytosis, it is not as effective as NH_4Cl at inhibiting the apparent uptake and degradation of some of the ^{125}I-ribonucleases in serum-free medium.

The relative quantity of radioactivity associated with the tissue, when yolk-sacs are incubated in the presence of 50 µg/ml 2,4-DNP in medium containing serum, is 10% of the control values for ^{125}I-RNases A and B, which is similar to the effect for incubation in the presence of NH_4Cl in serum-free medium. Again the relative T.A.R. values are higher for ^{125}I-RNases S and Spr, being 100% and 50% of control values respectively; this gives further support to the suggestion that some

of the binding of ^{125}I-RNases S and Spr to rat yolk sacs may be non-specific compared with the binding of ^{125}I-RNases A and B.

The rates of uptake of ^{125}I-ribonucleases from serum-free medium were also examined in the presence of 2,4-DNP. Two different concentrations of 2,4-DNP (10 and 50 µg/ml) were used, as in the experiments with ^{125}I-PVP. The results for ^{125}I-RNases A and S are shown graphically in Figure 3.3.2e. The same trends were found with ^{125}I-RNases B and Spr, but the results for these were not included in this figure in order to help maintain clarity. Figure 3.3.2e shows that 2,4-DNP at a concentration of 50 µg/ml greatly inhibited uptake of ^{125}I-RNases A and S, and that at the lower concentration of 10 µg/ml the inhibition of uptake was not so great. These results are summarized in Tables 3.3.2d and e together with the equivalent results from experiments with ^{125}I-RNases B and Spr. Again, rates of release of acid-soluble radioactivity were very similar to mean E.I. values, and the mean T.A.R. values, which remained constant during the incubation period, were lower for ^{125}I-RNases A and B than for ^{125}I-RNases S and Spr. The lowering of uptake rates was accompanied by increases in mean disposal times.

The relative effects of 2,4-DNP in serum-free medium on uptake of ^{125}I-PVP and ^{125}I-ribonucleases are summarized in Table 3.3.2h. Fluid-phase pinocytosis virtually ceased in the presence of 50 µg/ml 2,4-DNP, the uptake rate falling to 5% of the control value. When the concentration of 2,4-DNP was decreased to 10 µg/ml, the relative E.I. rose to 26%. Again, the effect of 2,4-DNP on ^{125}I-RNase A was the same as on ^{125}I-PVP; the effect on ^{125}I-RNase B was also very similar. At a 2,4-DNP concentration of 50 µg/ml the relative rates of uptake of ^{125}I-RNases A and B were 5% and 10% respectively; at 10 µg/ml the corresponding values were 27% and 30%, respectively. At the highest 2,4-DNP concentration used in serum-free medium (50 µg/ml) the relative effects on the mean E.I. values of ^{125}I-RNases S and Spr were only

23% and 31% of the control values respectively, and at 10 µg/ml 2,4-DNP the uptake rate and the rate of release of acid-soluble radioactivity rose to 70% of the control value. The relative mean disposal times again showed no clear pattern, although at the higher 2,4-DNP concentration (50 µg/ml) the value for each ^{125}I-ribonuclease was greater than at 10 µg/ml 2,4-DNP. These values were in the same range as when the incubation medium contained 10% calf serum. (Again, calculation of a mean disposal time for ^{125}I-RNase A is questionable.)

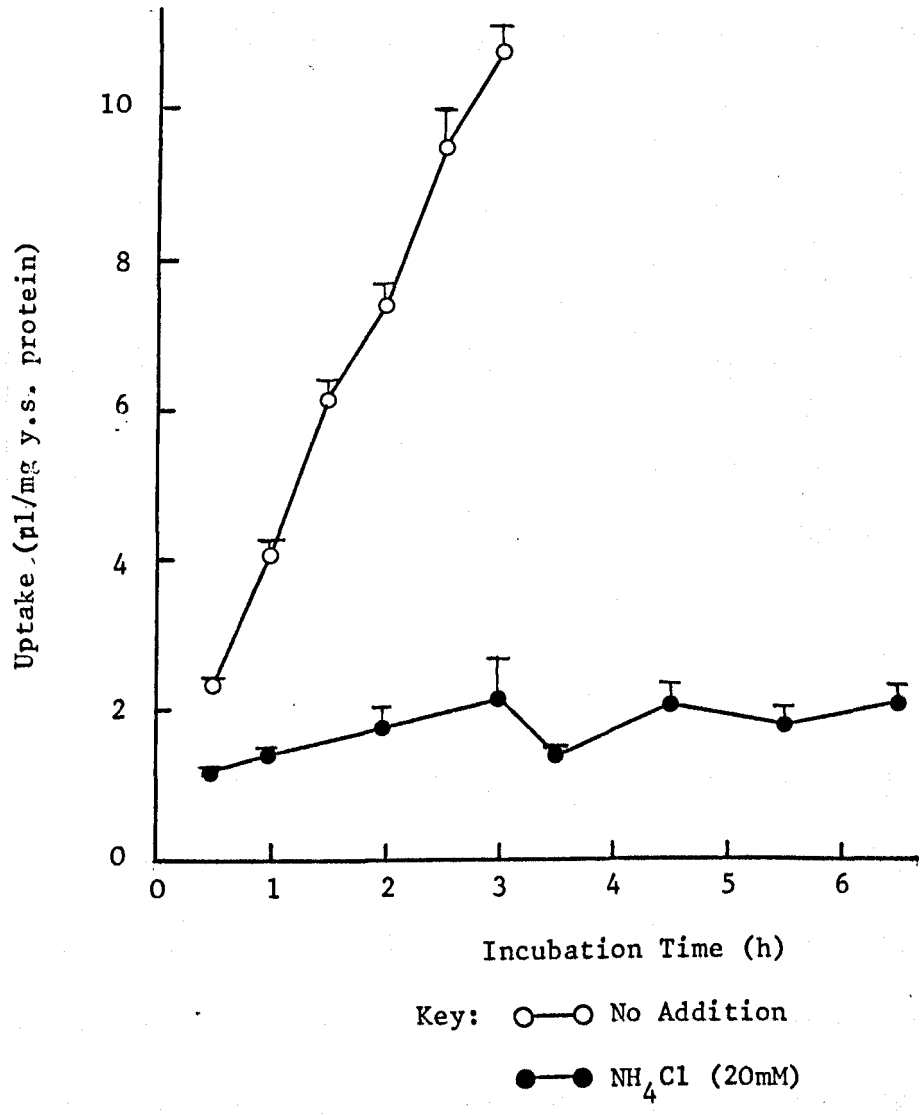
The quantity of radioactivity associated with yolk-sac tissue in the presence of 2,4-DNP in serum-free medium was again high. Mean T.A.R. values for ^{125}I-RNases A and B in the presence of 50 µg/ml were between 30% and 40% of control values, but at 10 µg/ml they were equal to the control values. The values for ^{125}I-RNases S and Spr were consistently greater than their control values. These high T.A.R. values may be indicative of strong non-specific binding of these ^{125}I-ribonucleases to the yolk-sac membrane.

Since the quantity of radioactivity associated with the yolk-sac tissue for ^{125}I-RNases S and Spr in the presence of 2,4-DNP was always very high, a single experiment was performed in which the yolk sacs were preincubated in serum-free medium with non-radioactive, ^{127}I-iodinated RNase S. This was followed by washing the tissue and reincubating it with ^{125}I-RNase S, both in the absence and presence of 2,4-DNP (50 µg/ml). The purpose of this experiment was to see if the saturation of any non-specific binding sites for ^{125}I-RNase S by the non-radioactive ^{127}I-iodinated RNase S could be achieved before addition of the radioactive analogue. This would be expected to reduce the mean T.A.R. values for ^{125}I-RNase S. This was not found (see Table 3.3.2f), and again the mean T.A.R. value was greater in the presence of 2,4-DNP. It is possible that non-radioactive ^{127}I-iodinated RNase S binds only very loosely to

the membrane, and that the washing procedure may remove a large proportion of it. But if this was the case the same would happen during the washing procedure at the end of conventional incubations. One may therefore conclude that the T.A.R. values measured following conventional incubations represent relatively strong binding (able to withstand the washing procedures), and that this binding is reversible (since an excess of ^{125}I-RNase S is able to displace any bound non-radioactive ^{127}I-iodinated RNase S).

The rate of release of acid-soluble radioactivity into the incubation medium from ^{125}I-RNases A and B in the presence of 2,4-DNP in serum-free medium paralleled the rate of uptake, and this inhibitor had the same relative effect on these substrates as on ^{125}I-PVP. One can conclude that these findings are due to the change in rate of pinocytosis in the rat yolk sac. However, more solubles are produced when yolk sacs are incubated with ^{125}I-RNases S and Spr than can be accounted for by the above reasoning. It is possible that the incubation medium has the capacity to liberate acid-solubles from ^{125}I-RNases S and Spr in the presence of 2,4-DNP, or some proteolytic activity may be present on the surface of the yolk sac and results in ^{125}I-RNases S and Spr being cleaved to form acid-soluble fragments in the presence of 2,4-DNP at physiological pH.

Figure 3.3.2a Effect of NH_4Cl on the Uptake of ^{125}I-PVP by Yolk Sacs Incubated in Serum-Free Medium



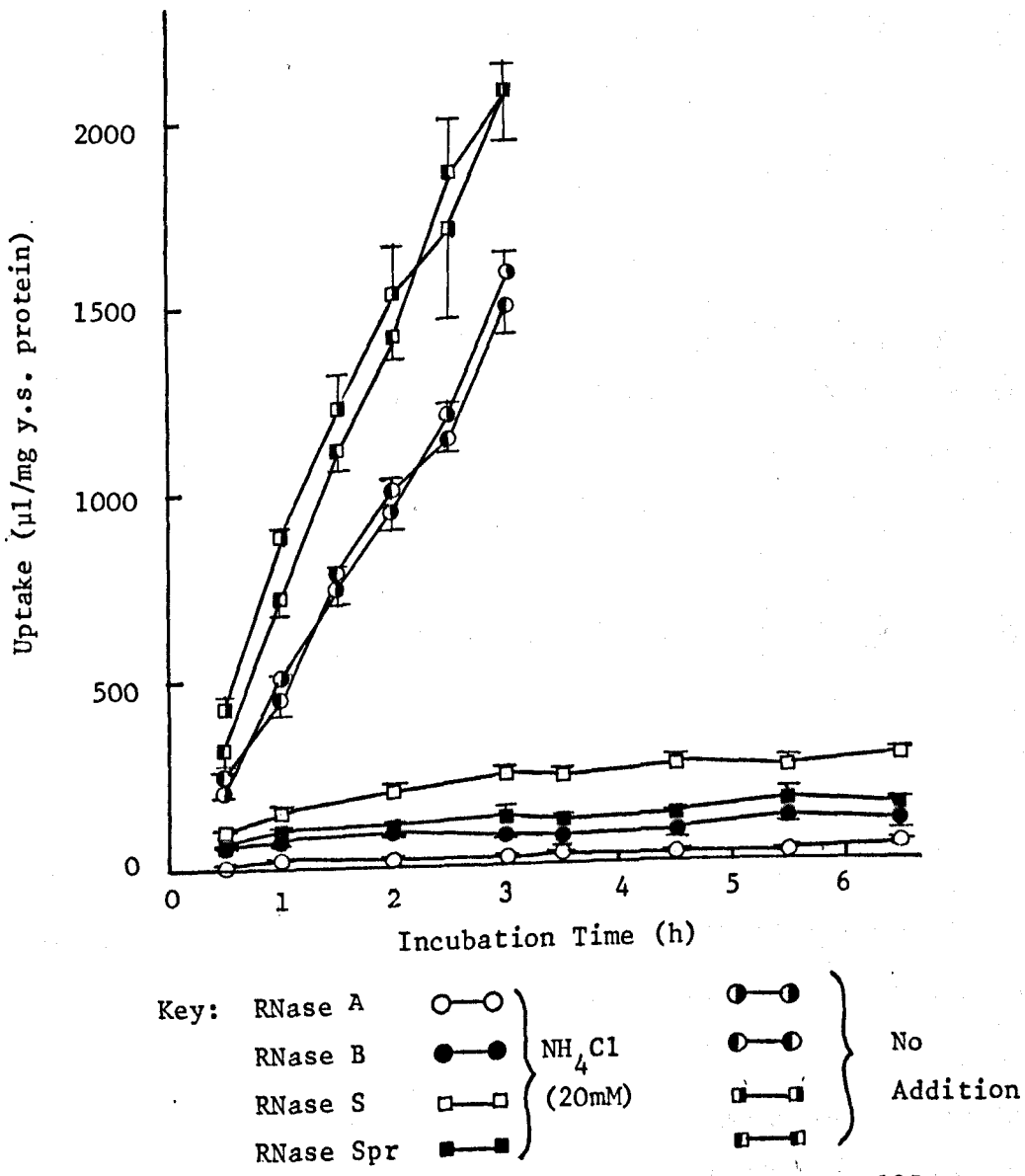
The method used was as described in Section 3.2. NH_4Cl (20mM) was added 20 min before addition of ^{125}I-PVP (2 µg/ml). Each uptake plot shows mean values (± S.E.M.) from 3 separate experiments. The results in the presence of NH_4Cl are summarized in Table 3.3.2a, and all these results are further summarized in Table 3.3.2h.

Table 3.3.2a Summary of the Effect of NH_4Cl on the Uptake of ^{125}I-PVP and of Different ^{125}I-Ribonucleases by Yolk Sacs Incubated in Serum-Free Medium.

These data are from the experiments shown in Figures 3.3.2a and b. The abbreviations used are as in Table 3.3.1b. These data are further summarized in Table 3.3.2h.

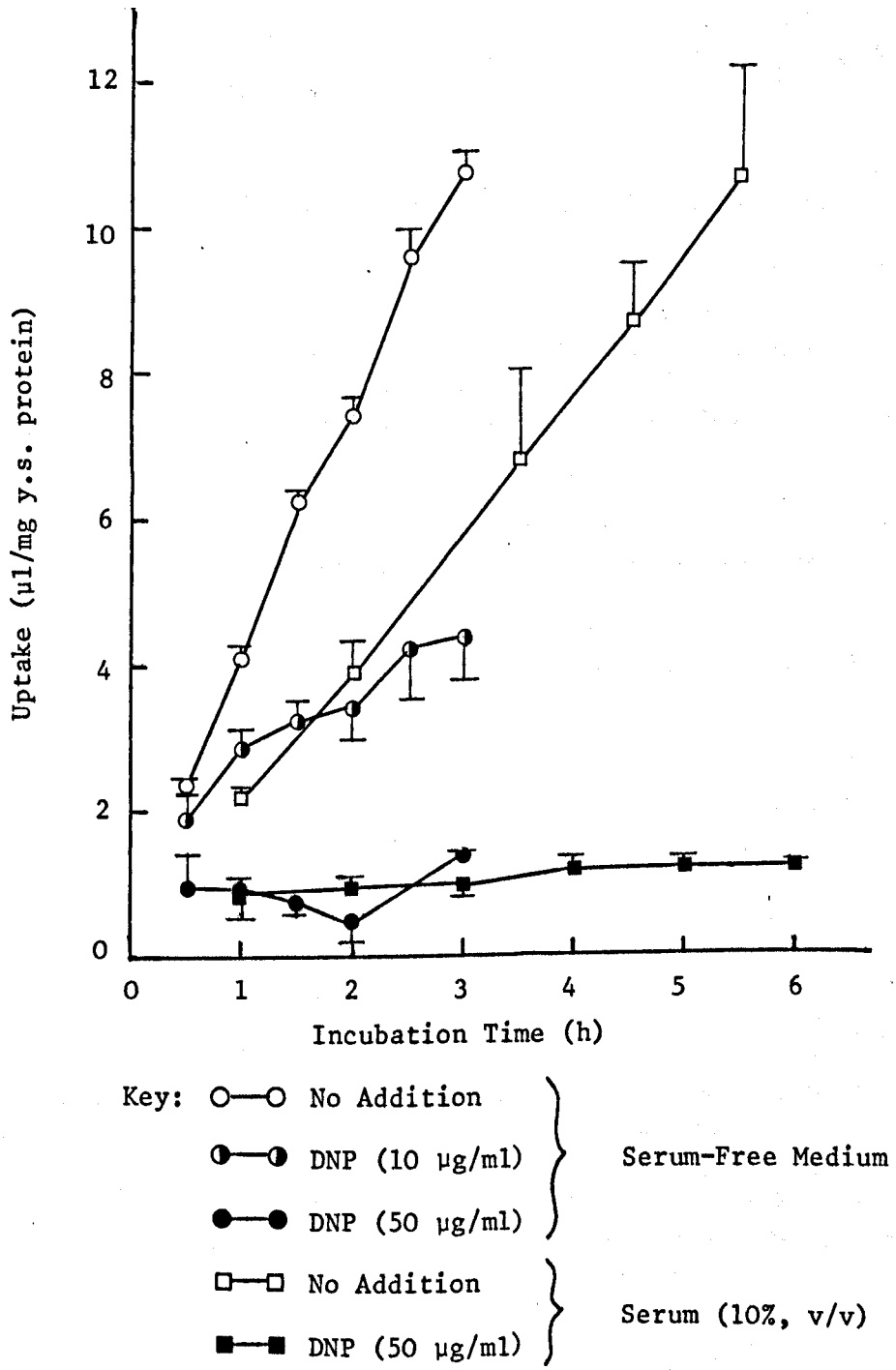
Substrate	E.I. (corr.co.) (µl/mg y.s. protein per h)	Mean E.I. ± S.E.M. (µl/mg y.s. protein per h)	Rate of Sol. Rel. (µl/mg y.s. protein per h)	Mean T.A.R. (µl/mg y.s. protein)
PVP	0.37 (0.795) 0.13 (0.514) 0.10 (0.633)	0.20 ± 0.09	-	-
RNase A	2.10 (0.3.77) 2.62 (0.537) 2.57 (0.681)	2 ± 2	2	17
RNase B	12.64 (0.837) 10.30 (0.617)	11	6	20
RNase S	24.00 (0.955) 15.66 (0.847)	20	16	122
RNase Spr	26.94 (0.821) 7.14 (0.803) 13.36 (0.834)	16 ± 6	11	81

Figure 3.3.2b Effect of NH_4Cl on Uptake of Different ^{125}I-Ribonucleases by Yolk Sacs Incubated in Serum-Free Medium



The method used was as described in Section 3.2. All ^{125}I-ribonucleases were present at a concentration of 1 µg/ml, and were added 20 min after addition of NH_4Cl (20mM). Each uptake plot shows mean values (± S.E.M.) from 2 to 3 separate experiments. The results in the presence of NH_4Cl are summarized in Table 3.3.2a, and all these results are further summarized in Table 3.3.2h.

Figure 3.3.2c Effect of 2,4-DNP on Uptake of ^{125}I-PVP by Yolk Sacs Incubated in Absence or Presence of Calf Serum (10%, v/v)



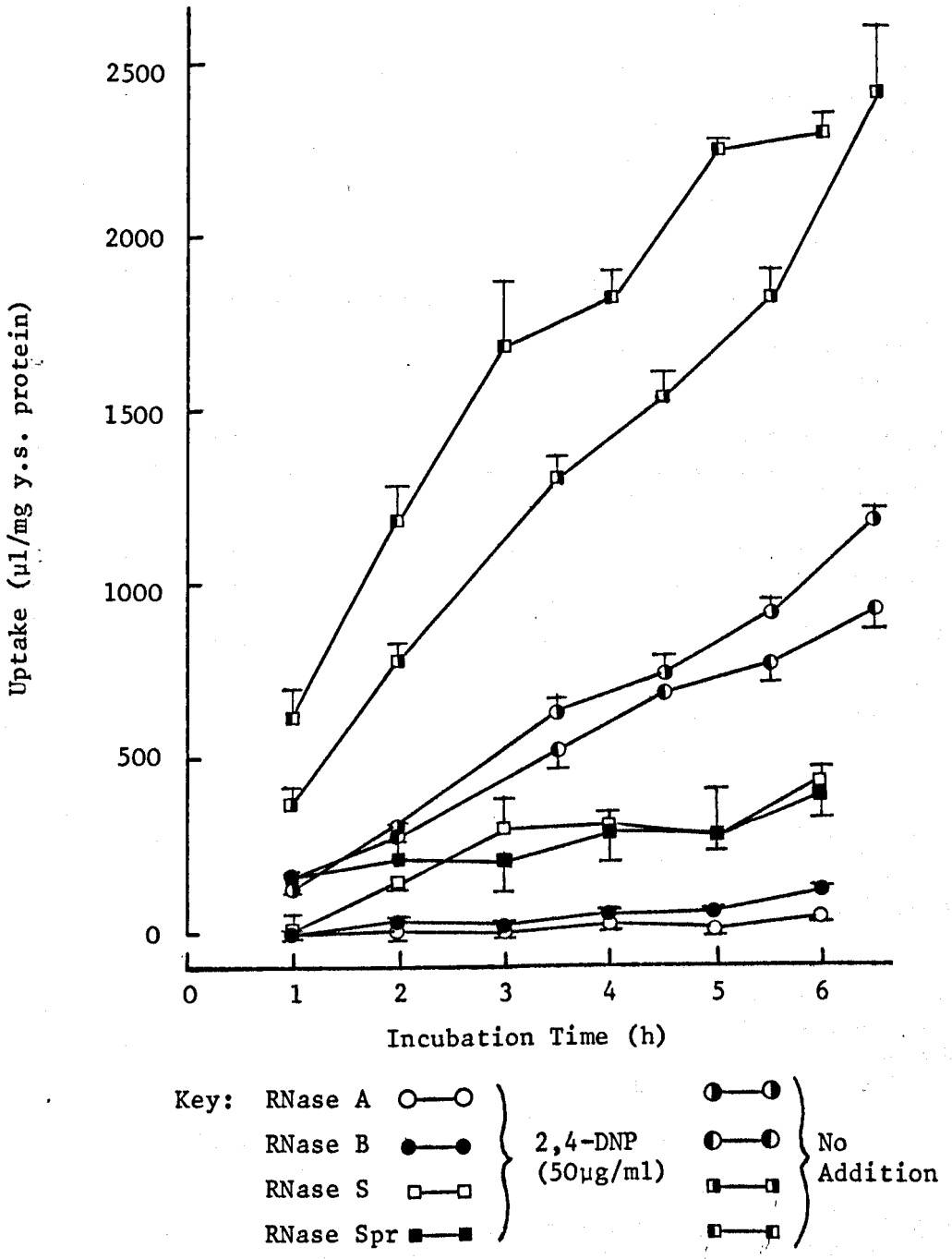
The method used was as described in Section 3.2. 2,4-DNP (10 or 50 µg/ml) was added 20 min before addition of ^{125}I-PVP (2 µg/ml). Each uptake plot shows mean values (± S.E.M.) from 4 to 6 separate experiments. The results in the presence of 2,4-DNP are summarized in Table 3.3.2b, and all these results are further summarized in Tables 3.3.2g and h.

Table 3.3.2b Summary of the Effect of 2,4-DNP on the Uptake of ^{125}I-PVP by Yolk Sacs Incubated in the Absence or Presence of Calf Serum (10%, v/v)

These data are from the experiments shown in Figure 3.3.2c, and are further summarized in Tables 3.3.2g and h. The abbreviations used are as in Table 3.3.1a.

Serum Conc. (%)	DNP Conc. (µg/ml)	E.I.(Corr.Co.) (µl/mg y.s. protein per h)	Mean E.I. ± S.E.M. (µl/mg y.s. protein per h)
0	10	0.60 (0.686) 1.17 (0.853) 0.85 (0.896) 0.36 (0.656) 1.84 (0.926) 0.48 (0.447)	0.88 ± 0.22
0	50	0.15 (0.284) 0.04 (0.129) 0.13 (0.400) 0.27 (0.343) 0.26 (0.657)	0.17 ± 0.04
10	50	0.09 (0.630) 0.05 (0.198) 0.12 (0.713) 0.07 (0.614)	0.08 ± 0.01

Figure 3.3.2d Effect of 2,4-DNP on Uptake of Different ^{125}I-Ribonucleases by Yolk Sacs Incubated in Presence of Calf Serum (10%, v/v)



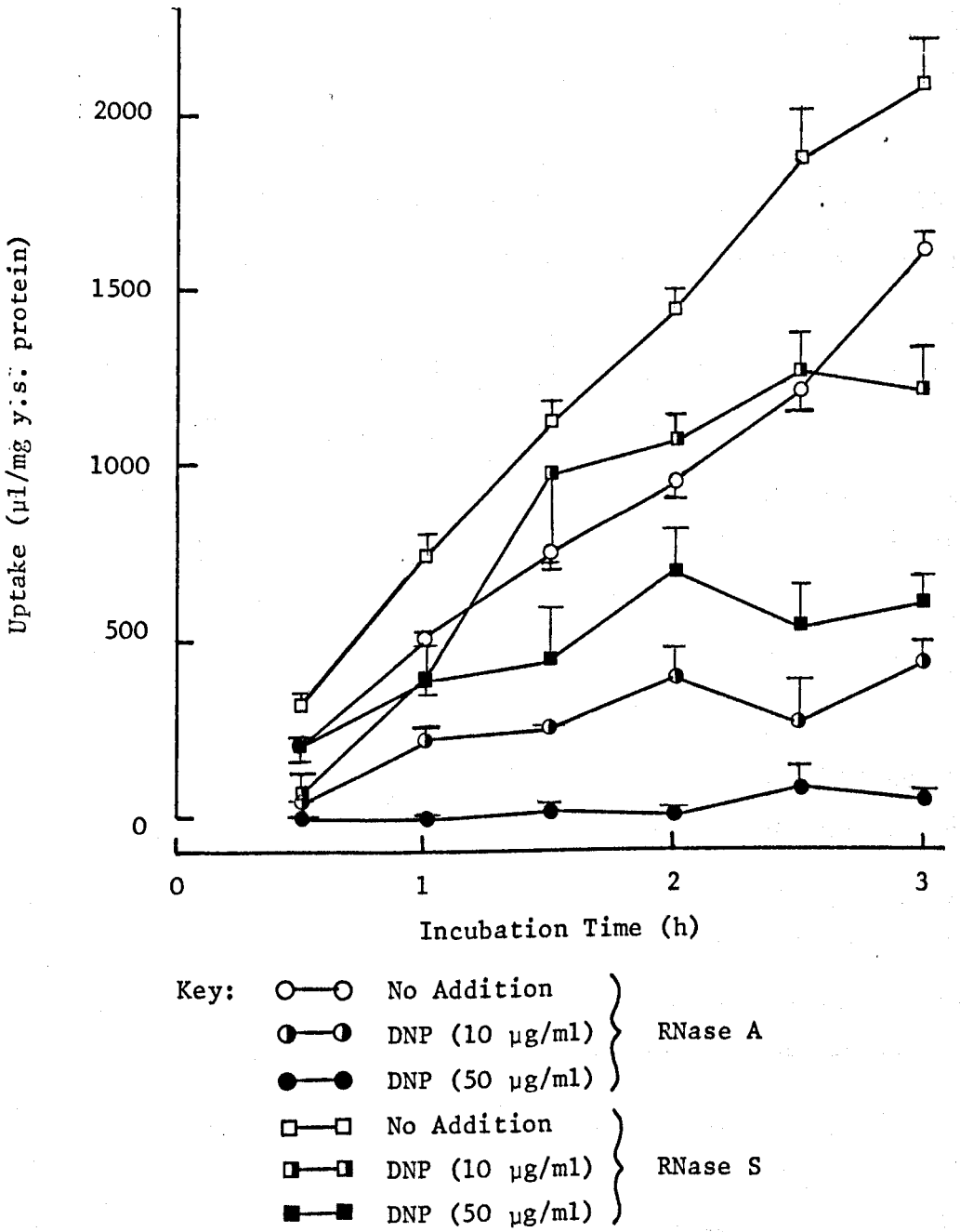
The method used was as described in Section 3.2. All ^{125}I-ribonucleases were present at a concentration of 1µg/ml, and were added 20 min after addition of 2,4-DNP (50µg/ml). Each uptake plot shows mean values (± S.E.M.) from 2 to 3 separate experiments. The results in the presence of 2,4-DNP are summarized in Table 3.3.2c, and all these results are further summarized in Table 3.3.2g.

Table 3.3.2c Summary of the Effect of 2,4-DNP on the Uptake of Different ^{125}I-Ribonucleases by Yolk Sacs Incubated in the Presence of Calf Serum (10%, v/v)

These data are from the experiments shown in Figure 3.3.2d. The abbreviations are as in Table 3.3.1b. Since the inhibition of uptake in the presence of DNP (50 µg/ml) is large, the errors in the mean E.I. and mean quantities of tissue-associated radioactivity (T.A.R. values) are larger than those of the controls. Therefore the calculated mean disposal times will also be subject to some error. These data are further summarized in Table 3.3.2g.

RNase	E.I.(Corr.Co.) (µl/mg y.s. protein/h)	Mean E.I. ± S.E.M. (µl/mg y.s. protein/h)	Rate of Sol.Rel. (µl/mg y.s. protein/h)	Mean T.A.R. (µl/mg y.s. protein)	Mean Disposal Time (h)
A	2 (0.215)	6	7	9	1.50
	11 (0.481)				
	15 (0.781)				
B	12 (0.575)	12 ± 2	17	11	0.92
	8 (0.540)				
	84 (0.692)				
S	63 (0.868)	64 ± 12	60	119	1.86
	44 (0.555)				
	30 (0.713)				
Spr	44 (0.915)	46 ± 10	43	56	1.22
	64 (0.956)				

Figure 3.3.2e Effect of 2,4-DNP on Uptake of Different ^{125}I-Ribonucleases by Yolk Sacs Incubated in Serum-Free Medium



The method used was as described in Section 3.2. All four ^{125}I-ribonucleases were used at a concentration of 1 µg/ml, and were added 20 min after addition of 2,4-DNP (10 or 50 µg/ml). Only the results for ^{125}I-RNases A and S are shown here; the results for ^{125}I-RNases B and Spr were similar to those of A and S respectively. Each uptake plot shows mean values (± S.E.M.) from 2 to 3 separate experiments. The above results in the presence of 2,4-DNP are summarized in Table 3.3.2d together with the results for ^{125}I-RNases B and Spr. These results are further summarized in Table 3.3.2h.

Table 3.3.2d Summary of the Effect of 2,4-DNP on the Uptake of Different ^{125}I-Ribonucleases by Yolk Sacs Incubated in Serum-Free Medium

These data are from the experiments shown in Figure 3.3.2e, and are for a DNP concentration of 10 µg/ml. The abbreviations are as in Table 3.3.1b. These data are further summarized in Table 3.3.2h.

RNase	E.I.(Corr.Co.) (µl/mg y.s. protein/h)	Mean E.I. (± S.E.M.) (µl/mg y.s. protein/h)	Rate of Sol. Rel. (µl/mg y.s. protein/h)	Mean T.A.R. (µl/mg y.s. protein)	Mean Disposal Time (h)
A	130 (0.799) 142 (0.676 152 (0.662)	141 ± 6	166	166	1.18
B	140 (0.722) 206 (0.887) 143 (0.836	164 ± 21	186	147	0.90
S	392 (0.796) 556 (0.963)	474	435	312	0.66
Spr	407 (0.875) 571 (0.972) 452 (0.918)	477 ± 49	471	268	0.56

Table 3.3.2e Summary of the Effect of 2,4-DNP on Uptake of Different ^{125}I-Ribonucleases by Yolk Sacs Incubated in Serum-Free Medium

These data are from the experiments shown in Figure 3.3.2e, and are for a DNP concentration of 50 µg/ml. The abbreviations are as in Table 3.3.1b. Since the inhibition of uptake in the presence of DNP (50 µg/ml) is large, the errors on the mean E.I. and mean T.A.R. values are larger than those of the controls. Therefore the calculated mean disposal times will also be subject to some error. These data are further summarized in Table 3.3.2h.

RNase	E.I.(Corr.Co.) (µl/mg y.s. protein/h)	Mean E.I. (± S.E.M.) (µl/mg y.s. protein/h)	Rate of Sol. Rel. (µl/mg y.s. protein/h)	Mean T.A.R. (µl/mg y.s. protein)	Mean Disposal Time (h)
	4 (0.134)				
A	46 (0.811)	24 (± 12)	33	53	2.21
	22 (0.238)				
	88 (0.973)				
B	71 (0.775)	56 (± 24)	61	67	1.20
	9 (0.265)				
	205 (0.771)				
S	59 (0.563)	160 (± 50)	156	248	1.55
	215 (0.754)				
	189 (0.901)				
Spr	264 (0.812)	198 (± 36)	207	226	1.14
	140 (0.731)				

Table 3.3.2f Effect of Preincubation with ^{127}I-RNase S on the Uptake of ^{125}I-RNase S by Yolk Sacs Incubated in Serum-Free Medium in Absence or Presence of 2,4-DNP

The details of this experiment are reported in Section 3.2. Yolk sacs were preincubated with ^{127}I-RNase S (10µg/ml), and washed in medium 199 (37°C) to remove any loosely-associated ^{127}I-RNase S. Then the substrate, ^{125}I-RNase S (1 µg/ml), was added at the same time as 2,4-DNP (50 µg/ml); usually 2,4-DNP was added 20 min before the substrate. This experiment was performed once only.

Preinc. with ^{127}I-RNase S (10µg/ml)	Inc. with 2,4-DNP (50µg/ml)	E.I.(Co.Coeff.) (µl/mg y.s. protein/h)		Mean T.A.R. (µl/mg y.s. protein)
0	0	894	(0.921)	152
+	0	828	(0.945)	171
0	+	576	(0.926)	337
+	+	629	(0.936)	404

Table 3.3.2g Overall Summary of the Effect of 2,4-DNP on Uptake of ^{125}I-PVP and of Different ^{125}I-Ribonucleases by Yolk Sacs when Incubated in the Presence of Calf Serum (10%,v/v)

These summary data are from the experiments shown in Figures 3.3.2c and d, and data from Tables 3.3.2b and c. The abbreviations and units are as in Table 3.3.1b. The relative effect of the agent, expressed as a percentage of the control value (no addition of agent), is in parentheses beside each experimental result:

Agent	PVP	RNase A	RNase B	RNase S	RNase Spr
(i) Mean E.I.	(µl/mg yolk-sac protein per h)				
No addition	1.92 (100)	187 (100)	139 (100)	351 (100)	345 (100)
DNP (50µg/ml)	0.08 (4)	6 (3)	12 (9)	64 (18)	46 (13)
(ii) Rate of Sol. Rel.	(µl/mg yolk-sac protein per h)				
No Addition	-	175 (100)	132 (100)	344 (100)	347 (100)
DNP (50µg/ml)	-	7 (4)	17 (13)	60 (17)	43 (12)
(iii) Mean T.A.R.	(µl/mg yolk-sac protein)				
No Addition	-	105 (100)	99 (100)	119 (100)	112 (100)
DNP (50µg/ml)	-	9 (9)	11 (11)	119 (100)	56 (50)
(iv) Mean Disposal Time	(h)				
No Addition	-	0.56 (100)	0.71 (100)	0.34 (100)	0.32 (100)
DNP (50µg/ml)	-	1.50 (270)	0.92 (130)	1.86 (550)	1.22 (380)

Table 3.3.2h Overall Summary of the Effects of NH_4Cl and 2,4-DNP on the Uptake of ^{125}I-PVP and of Different ^{125}I-Ribonucleases by Yolk Sacs when Incubated in Serum-Free Medium.

These summary data are from the experiments shown in Figures 3.3.2a,b,c and e, and data from Tables 3.3.2a,b,d and e. The abbreviations and units are as in Table 3.3.1b. The relative effect of each agent, expressed as a percentage of the control value (no addition of agent), is given in parentheses beside each experimental result.

Agent	PVP	RNase A	RNase B	RNase S	RNase Spr
(i) Mean E.I.	(µl/mg yolk-sac protein per h)				
No Addition	3.39 (100)	515 (100)	538 (100)	708 (100)	641 (100)
NH_4Cl (20mM)	0.20 (6)	2 (<1)	11 (2)	20 (3)	16 (2)
DNP (10µg/ml)	0.88 (26)	141 (27)	164 (30)	474 (67)	477 (74)
DNP (50µg/ml)	0.17 (5)	24 (5)	56 (10)	160 (23)	198 (31)
(ii) Rate of Sol. Rel.	(µl/mg yolk-sac protein per h)				
No Addition	-	526 (100)	484 (100)	713 (100)	669 (100)
NH_4Cl (20mM)	-	2 (<1)	6 (1)	16 (2)	11 (2)
DNP (10µg/ml)	-	166 (32)	186 (38)	435 (61)	471 (70)
DNP (50µg/ml)	-	33 (6)	61 (13)	156 (22)	207 (31)
(iii) Mean T.A.R.	(µl/mg yolk-sac protein)				
No Addition	-	164 (100)	179 (100)	204 (100)	166 (100)
NH_4Cl (20mM)	-	17 (10)	20 (11)	122 (60)	81 (49)
DNP (10µg/ml)	-	166 (101)	147 (82)	312 (153)	268 (161)
DNP (50µg/ml)	-	53 (32)	67 (37)	248 (122)	226 (136)
(iv) Mean Disposal Time (h)					
No Addition	-	0.32 (100)	0.33 (100)	0.29 (100)	0.26 (100)
DNP (10µg/ml)	-	1.18 (370)	0.90 (270)	0.66 (230)	0.56 (220)
DNP (50µg/ml)	-	2.21 (690)	1.20 (360)	1.55 (530)	1.14 (440)

3.3.3 The Influence of pH and the Presence of Yolk-Sac Tissue on the Stability of Different ^{125}I-Ribonucleases

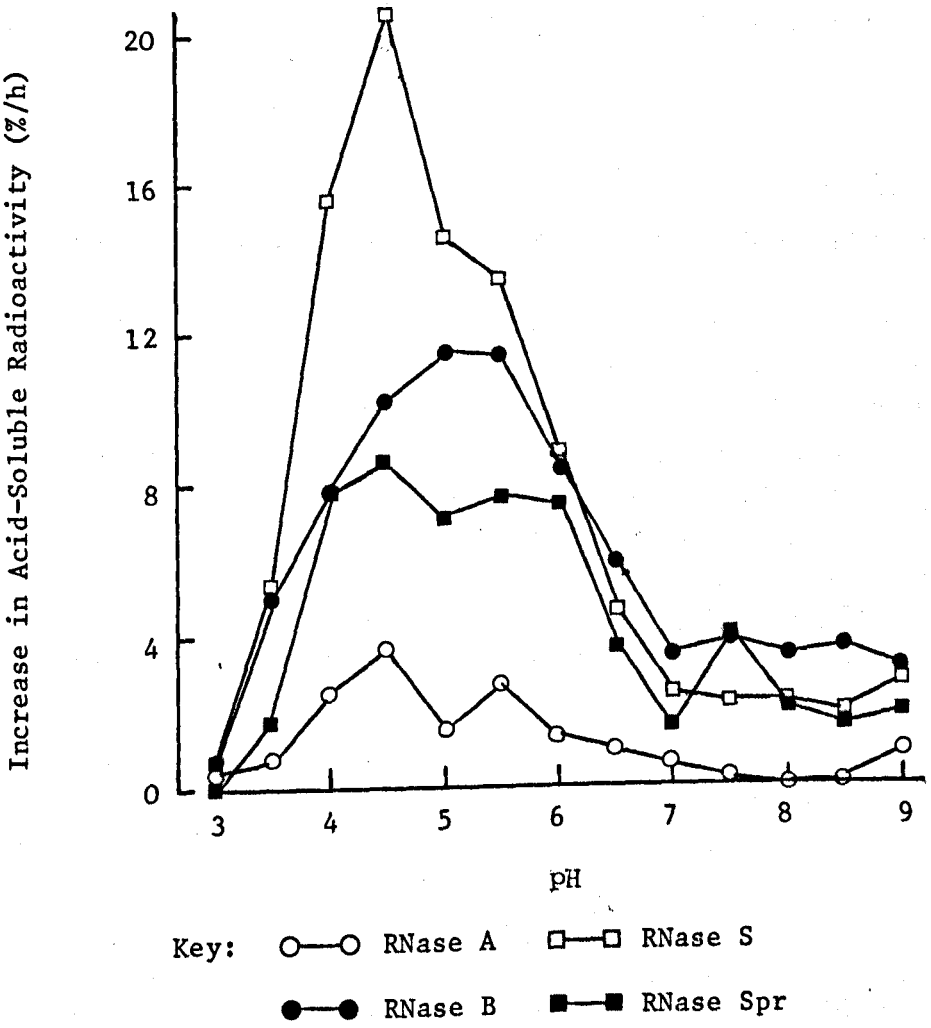
The effect of yolk-sac homogenate on the stability of different ^{125}I-labelled RNases over a range of pH values was examined. The stability of the substrates was determined by incubating them with yolk-sac homogenate and buffer, and determining the rate of production of acid-soluble radioactivity over a 1h incubation period; ^{125}I-ribonucleases were present at a concentration of 10 µg/200 µl, and each 50 µl aliquot of yolk-sac homogenate contained approx. 40 µg protein. The results of this investigation are shown in Figure 3.3.3a. Yolk-sac homogenate had the capacity to degrade all the ^{125}I-ribonucleases *in vitro*, the greatest rate of degradation being in the pH range from 4 to 5. In this pH range ^{125}I-RNase S had the highest rate of degradation with an increase in acid-soluble radioactivity of 20%/h; ^{125}I-RNases B and S_{pr} produced about 10% acid-soluble radioactivity per h, and ^{125}I-RNase A had the lowest rate, producing around 4% per h. At physiological pH (pH 7.4, the value at which ^{125}I-ribonucleases were routinely incubated with yolk sacs in medium 199), there was no production of acid-soluble radioactivity from ^{125}I-RNase A under the conditions used, but there was some acid-soluble radioactivity produced from ^{125}I-RNases B, S and Spr. Although this finding is compatible with the proposal that there may be some proteolytic activity associated with the yolk sac at neutral pH capable of degrading ^{125}I-ribonucleases, it provides no information as to where this proteolytic activity may be found, whether on the yolk-sac surface or at some intracellular site.

The stability of ^{125}I-ribonucleases to incubation media, or to media that had previously been exposed to yolk-sac tissue, was examined by measuring the production of acid-soluble radioactivity released from ^{125}I-ribonucleases during their incubation with "conditioned incubation

medium". "Yolk-sac conditioned medium" was prepared by incubating yolk sacs in either serum-free or serum-containing medium, and in either the presence or absence of 2,4-DNP. "Simple conditioned medium" was prepared as above but without any yolk sacs being present.

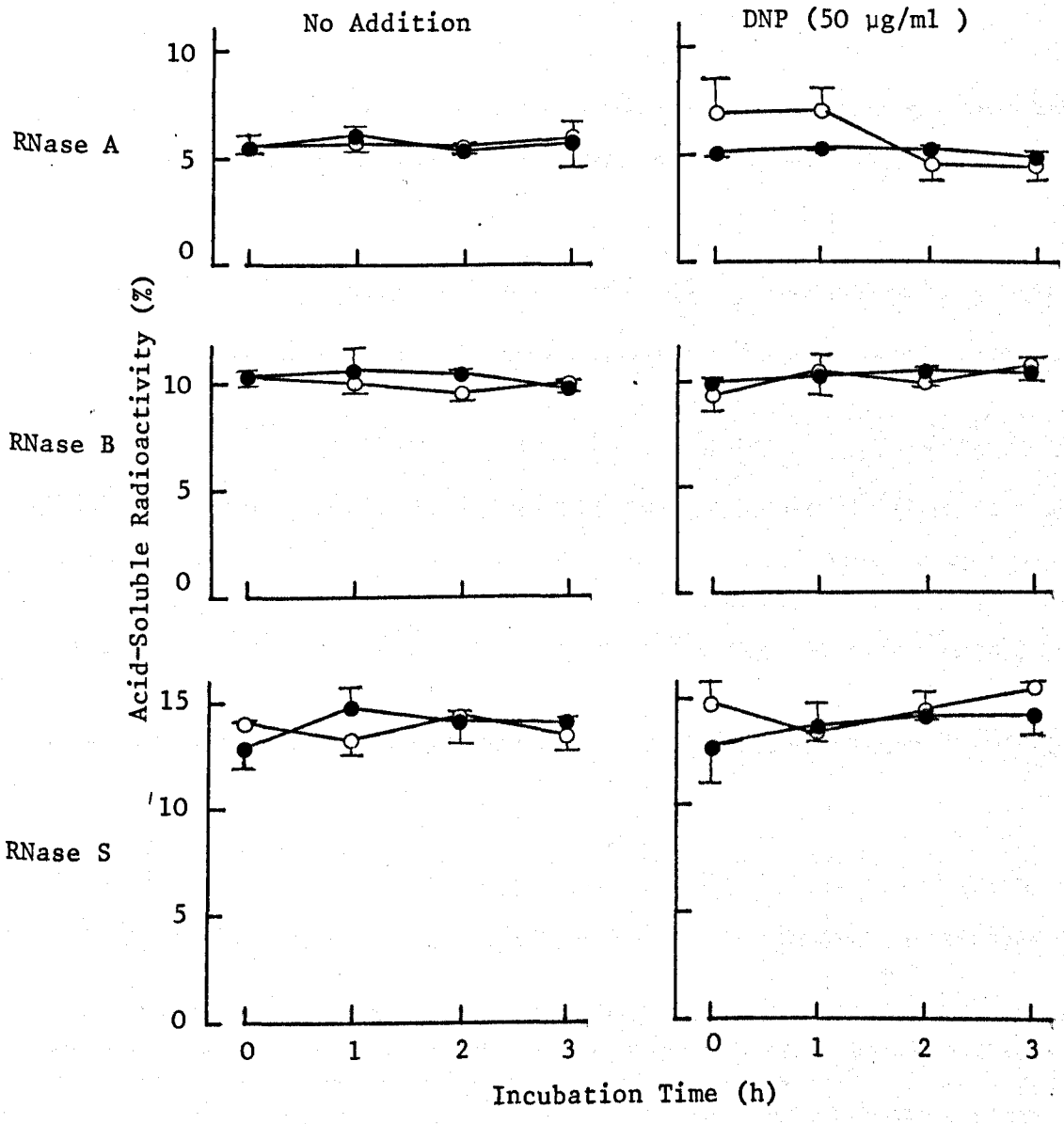
The results of these experiments are shown in Figure 3.3.3b in the presence of calf serum, and in Figure 3.3.3c in the absence of calf serum. The quantity of acid-soluble radioactivity released from any of the ^{125}I-ribonucleases studied did not alter with time. Neither the presence of calf serum nor the presence of 2,4-DNP appeared to make any difference to the percentage of acid-soluble radioactivity associated with each ^{125}I-ribonuclease. These data suggest that there is no enzymic activity associated with either the incubation medium or released from rat yolk sacs, that has the proteolytic capacity to degrade ^{125}I-ribonucleases at neutral pH.

Figure 3.3.3a Effect of pH on the Rate of Degradation of Different ^{125}I-Ribonucleases when Incubated with Yolk-Sac Homogenate



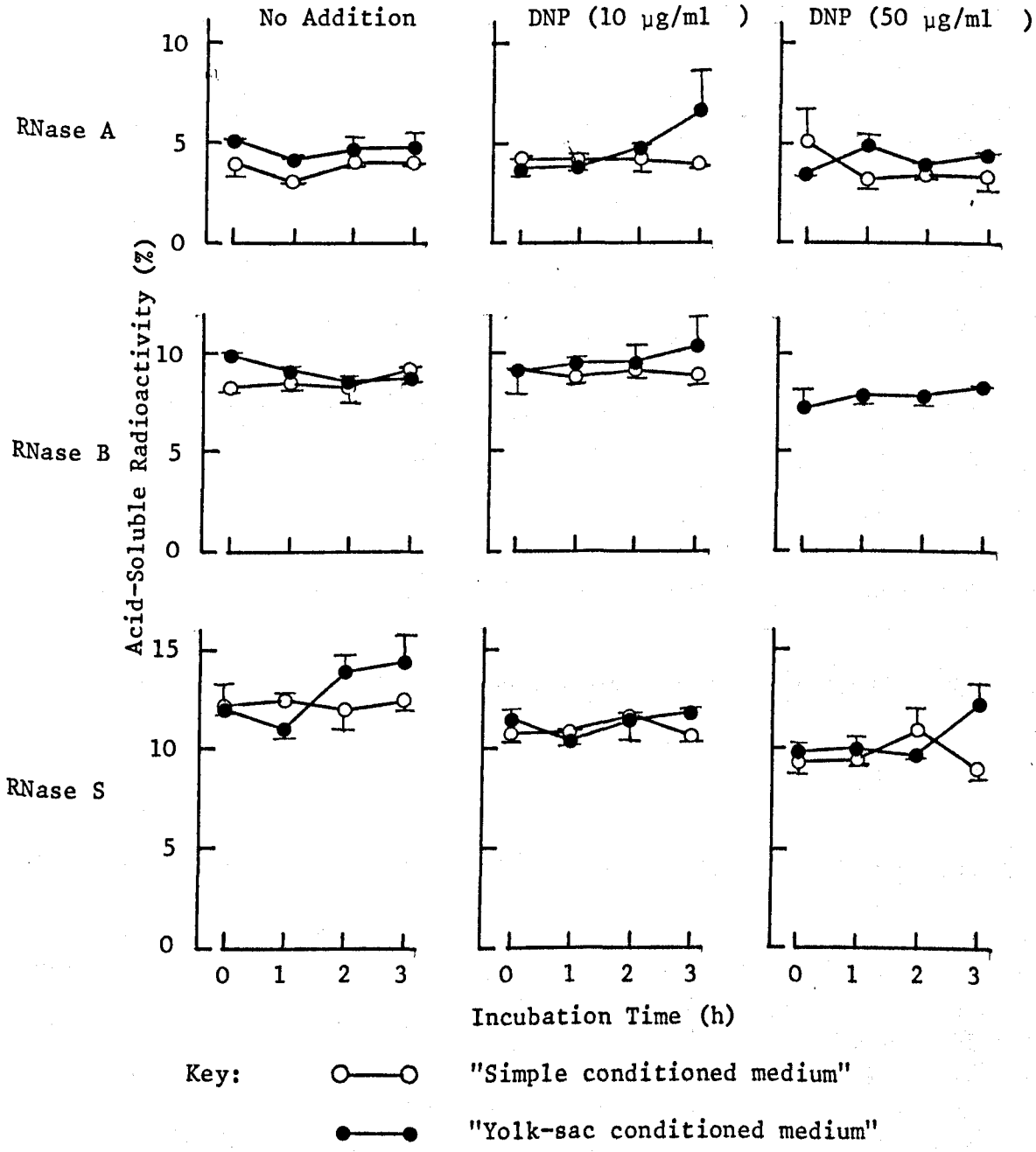
The method used was as described in Section 3.2. ^{125}I-ribonucleases were present at a concentration of 10 µg/200µl in an incubation mixture containing buffer and yolk-sac homogenate; control incubation mixtures contained buffer and distilled water. The duration of the incubation was 1.0h. The results are expressed as mean values of the increase in acid-soluble radioactivity [acid-soluble radioactivity of incubation mixture (%) - acid-soluble radioactivity of control incubation mixture (%)] from 2 separate experiments, each being performed in quadruplicate.

Figure 3.3.3b Effect of 2,4-DNP on the Degradation of Different ^{125}I-Ribonucleases when Incubated in Serum-Containing "Conditioned Incubation Medium"



The method used was as described in Section 3.2. "Conditioned incubation media" were prepared by incubating serum-containing medium with or without yolk sacs, either in the absence or presence of 2,4-DNP. ^{125}I-ribonucleases (1 µg/ml) were then incubated in the presence of these media, and the acid-soluble radioactivity produced was calculated. "Simple" and "Yolk-sac conditioned media" were in the absence (controls) and presence of yolk sacs respectively. The results are expressed as mean values (± S.E.M.) from 2 separate experiments, each being performed in duplicate.

Figure 3.3.3c Effect of 2,4-DNP on the Degradation of Different ^{125}I-Ribonucleases when Incubated with Serum-Free "Conditioned Incubation Medium"



The method used was as described in Section 3.2, and briefly outlined as in Figure 3.3.3b with the exception that serum was absent from the incubation media. The results are expressed as mean values (± S.E.M.) from 2 separate experiments, each being performed in duplicate.

3.4 Discussion

This study of the rates of uptake of a number of different ribonucleases by rat yolk sacs has produced one clearly dominant finding, namely the general similarity between ^{125}I-RNases A and B, and the similarity between ^{125}I-RNases S and Spr, regardless of the incubation conditions. The similarity of all the results obtained for ^{125}I-RNases A and B leads to the conclusion that there appears to be no preferential uptake by the rat yolk sac of a protein molecule proffering a terminal mannose residue, indicating that there is no mannose receptor on the rat yolk-sac surface. [This contrasts with the results obtained by Raynes & Wold (1976) who, as described in Section 3.1, found that the in vivo clearance of ribonuclease B was very much more rapid than that of ribonuclease A, due to the mannose residues on the surface of the B isoenzyme being recognised by the liver non-parenchymal cells and thence being removed from the circulation.] These same results in the yolk sac system also show that the introduction of the oligosaccharide moiety on the polypeptide backbone of ribonuclease A to form ribonuclease B does not appear to mask any determinant, based on amino acid residues, that is responsible for the rapid uptake of the simple "protein core".

The observation that the rates of capture of ^{125}I-ribonucleases A and B rise sharply in the absence of serum as compared with the rates of uptake in the presence of 10% calf serum (see Tables 3.3.2g & h), may in part be explained by the finding of Fuller & Maras (1979) that calf serum contains 70 µg of pancreatic ribonuclease *A* per ml, as measured by the degradation of yeast RNA. This would mean that, for experiments performed in the presence of 10% (v/v) calf serum, each molecule of ^{125}I-labelled ribonuclease would have been in competition with approx. 7 molecules of unlabelled ribonuclease. Recently, Livesey & Williams (1982) have shown that large amounts (100 µg/ml) of native ribonuclease A decreased the rate

of uptake of tracer amounts of ^{125}I-RNase A by approx. 90% when added to serum-free medium in which yolk sacs are incubated, a finding that gives substance to the above suggestion.

Baynes & Wold (1976) reported that the ribonuclease B that they obtained from Sigma was only 20% pure, the remaining 80% being contamination with ribonuclease A. More recently Kooistra et al. (unpublished data) have determined that the ribonuclease B preparations from Sigma have improved to 50% purity. The ribonuclease B preparation used in this study was also obtained from this source, but any such contamination with ribonuclease A (of the order of 50%) would have been unlikely to have prevented the detection of a mannose receptor on the rat yolk-sac surface in these experiments. Time allowing, the purity of the ribonuclease B preparation used here was to have been established.

The rates of degradation of ^{125}I-RNases A and B by the yolk-sac tissue, as indicated by the similarity in the disposal times, were the same. The influence of a carbohydrate moiety upon the rate of degradation of proteins is not readily predicted from the literature. For example, Kalish et al. (1979) found that among cytosol proteins from a variety of mammalian tissues, glycoproteins tend to be degraded more rapidly than non-glycoproteins. Conversely, Wang & Hirs (1977) found that enzymic removal of carbohydrate from porcine pancreatic ribonuclease resulted in an increase in sensitivity towards attack by subtilisin, indicating that heterosaccharides can exert a protective function against degradation. Brown et al. (1979) have studied the uptake and subsequent inactivation of native and deglycosylated yeast invertase in rat yolk sacs, by measuring the enzyme activity within the tissue with time. They found that the initial rate of uptake of the deglycosylated form was several-fold greater than that of the native molecule. They also found that, for the deglycosylated form, the accumulation of enzyme activity with time levelled off much more rapidly than that of the glycosylated form (indicating that, for the

deglycosylated form, a steady-state level within the tissue was more rapidly established). After preloading the yolk-sac tissue with the two different forms of invertase, the quantity of native invertase enzymic-activity associated with the tissue was constant up to 6h, whereas the activity of the deglycosylated form declined. These data indicate that the carbohydrate moiety protects the invertase molecule from intracellular protein inactivation; however, a similar conclusion could not be reached from the data presented in this chapter. The extent of glycosylation of the invertase molecule is very great in comparison to that of ribonuclease B, and this may be a key factor in the ability of the carbohydrate moiety to protect the molecule from attack in the case of invertase, but not in the case of ribonuclease B.

The rates of uptake of ^{125}I-RNases S and Spr by rat yolk sacs were always extremely similar. Comparison of the structures of these two ribonucleases is informative. (Ribonuclease S is formed by cleavage, with subtilisin, of the 124 amino acid chain of ribonuclease A between residues 20 and 21. However, the cleavage fragments remain held together by non-covalent forces in ribonuclease S; ribonuclease S protein is formed by removal of the eicosapeptide from ribonuclease S by acid precipitation.) This structural difference enables one to conclude that the removal of a small, non-covalently bound peptide does not appear to affect the ability of the yolk sac to pinocytose this protein. It would have been interesting to have examined the uptake and degradation of the isolated small peptide by rat yolk sacs. Unfortunately this peptide could not be labelled by the chloromine T method which was used to label the other ribonucleases, due to the absence of a tyrosine residue. As it was not desirable to label it via a different method the peptide was omitted from this study.

The rate of uptake of ^{125}I-RNase S was always much greater than that of ^{125}I-RNase A. The only difference between the structures of these two

molecules is that, in ribonuclease S, cleavage of a peptide bond has introduced into the protein a change in the conformation of the molecule. However, this change leaves the active site of the enzyme virtually unaltered. This rapid uptake of the partially denatured ribonuclease when compared to the native form is compatible with the suggestion of Dice & Goldberg (1976), that the rate-limiting step in the catabolism of serum proteins may be proteolytic "nicking", within the circulation, followed by selective pinocytosis of the partially-degraded protein.

The shortest measurable time between the addition of a radio-labelled protein to a yolk sac during incubation and the detection of acid-soluble radioactivity in the incubation medium (the lag-period) was the same for all four ribonucleases in serum-free medium. The calculated value of the mean disposal time (the mean time during which radioactivity is associated with yolk-sac tissue before its release as acid-soluble fragments) was also very similar for each of the four different ribonucleases. This suggests that, although the rates of uptake of the different ribonucleases by yolk sacs varied, once inside the tissue they are degraded with equal ease.

The influence of calf serum upon ^{125}I-RNases S and Spr was the same as on ^{125}I-PVP, the fluid-phase marker (see Table 3.3.1e). The effect of serum on ^{125}I-RNases A and B was always much greater, which suggests that these two ribonucleases are in greater competition with serum proteins for binding sites on the yolk-sac surface than the other two (vide supra). Since the ribonucleases S and Spr were produced by a proteolytic cleavage that induces a conformational change, it is possible that, during the conversion of ribonuclease A to S, some formerly buried domains within the polypeptide chain of ribonuclease A were uncovered and that these residues are responsible for the increased affinity of ^{125}I-RNases S and Spr for the yolk-sac surface. The finding of Moore *et al.* (1977) that bovine serum albumin is cleared more rapidly, both from the bloodstream *in vivo*

and from the incubation medium by yolk sacs *in vitro*, when the molecule has been exposed to urea, supports this suggestion that conformational changes within a simple protein can modify clearance rates.

The disposal times for ^{125}I-RNases A and B in the presence of 10% calf serum are higher than those of ^{125}I-RNases S and Spr (see Table 3.3.2g), which contrasts with the situation in serum-free medium (see Table 3.3.2h). This evidence suggests that for some reason, there is a lengthening of the time that the radioactivity remains associated with the tissue compared with ^{125}I-RNases S and Spr. For example, this may be indicative of a longer intralysosomal half-life of these proteins when serum is present. It would have been interesting to have measured the lag-phases of these four ribonucleases in the presence of serum.

Ammonium chloride has been shown to inhibit intralysosomal proteolysis in rat yolk sacs; at the same time it inhibits pinocytosis (Livesey *et al.*, 1980). In order to ensure that the ^{125}I-labelled ribonucleases were being digested only after pinocytic uptake into rat yolk sacs, a high concentration (20 mM) of ammonium chloride, sufficient to fully inhibit uptake of ^{125}I-labelled PVP, was added to the incubation medium to see whether production of acid-soluble radioactive fragments was completely abolished. Indeed, the uptake of all four ribonucleases was abolished by 20 mM ammonium chloride, as was the release of acid-soluble radioactivity into the incubation medium (see Table 3.3.2h). [The quantity of radioactivity associated with the tissue in the presence of 20 mM ammonium chloride was taken to represent the binding affinity of each ribonuclease for the yolk-sac surface; this was greater for ^{125}I-RNases S and Spr than for the other two.] The above findings were compatible with the assumption that all the ^{125}I-ribonucleases are pinocytosed by the tissue, and that acid-soluble radioactivity is generated mainly by intralysosomal proteolysis.

It was desirable to reinforce this conclusion by the use of a different inhibitor of pinocytosis, 2,4-DNP (Duncan & Lloyd, 1978). This agent (50 µg/ml) causes a similar inhibition of the rate of uptake of ^{125}I-PVP as ammonium chloride (20 mM); the rate of uptake and rate of release of acid-soluble radioactivity from ^{125}I-RNases A and B were also similarly inhibited, but the inhibitions of these rates for ^{125}I-RNases S and Spr were less marked. (The quantity of radioactivity associated with the yolk-sac tissue for ^{125}I-RNases S and Spr was greater than that when the yolk sacs were incubated in the absence of any inhibitor.) However, when pinocytosis is noticeably inhibited, the apparent uptake which is calculated becomes greatly influenced by the quantity of radioactivity associated with the tissue. This latter value will be very strongly influenced by random experimental factors such as incomplete washing. (Compare Section 4.3.1, where one can follow experimentally an initial rapid release of ^{125}I-PVP from the surface of yolk sacs after putting them into substrate-free medium. Such release is presumably a result of the washing process being inefficient in removing surface-associated radioactivity). A lower concentration of 2,4-DNP (10 µg/ml) was therefore used to only partially inhibit pinocytosis. The effect of the inhibitor, at this concentration, on the rate of uptake and rate of release of acid-soluble radioactivity derived from ^{125}I-RNases A and B again paralleled the response of the tissue to the fluid-phase marker, ^{125}I-PVP (see Table 3.3.2h). The amounts of tissue-associated radioactivity for these two proteins were similar to the T.A.R. values in the absence of the inhibitors. The rate of uptake and the rate of release of acid-soluble radioactivity for ^{125}I-RNases S and Spr were again higher than for the other two proteins. Therefore the relative effect of the inhibitor upon ^{125}I-RNases S and Spr was less than the relative effect on the uptake of ^{125}I-PVP.

This large apparent uptake and release of acid-soluble radioactivity

from ^{125}I-RNases S and Spr by rat yolk sacs incubated in the presence of 2,4-DNP (10 µg/ml) was greater than would be expected from the pinocytic capacity of the yolk sac under these conditions. The experiments in Section 3.3.3 excluded the possibility of degradation in media alone, or by enzymes released from the yolk sac. The ability of a yolk-sac homogenate to degrade some of the ^{125}I-ribonucleases at physiological pH is consistent with, but not direct evidence for, the existence of some proteolytic activity on the surface of the yolk sac. Time permitting, the composition of the acid-soluble radioactivity would have been examined by column chromatography. It may be that some degradative enzyme activity, having the capacity to split ^{125}I-RNases S and Spr (but not ^{125}I-RNases A or B) into two or more large acid-soluble fractions, may be present on the yolk-sac surface. This, if true, would help to account for the high rate of production of acid-soluble radioactivity from ^{125}I-RNases S and Spr under conditions in which pinocytosis in the rat yolk sac is almost fully inhibited by 2,4-DNP.

CHAPTER 4

THE EFFECT OF INCUBATION MEDIUM COMPOSITION ON PINOCYTOSIS AND PROTEIN DEGRADATION IN THE RAT VISCERAL YOLK SAC

4.1 Introduction

The steady-state level of protein within a cell is a result of the balance between protein synthesis and degradation, the rates of which must be influenced by factors and processes both inside and outside the cell. Protein degradation is unlikely to be an entirely indiscriminate catabolic function since it can be very specific. For example, proteins such as proinsulin require the cleavage of specific peptide bonds to liberate the hormonally-active insulin molecule, and the selective removal of abnormal proteins, perhaps containing one or more incorrect amino acid residues, is probably essential to the efficient running of the cell (Knowles et al., 1975). Some of the functions of protein degradation are necessarily less specific, such as the digestion of exogenous proteins captured by endocytosis, and the digestion of endogenous proteins to provide amino acids as a fuel source in times of starvation.

It is clearly important to try to understand those factors that influence and control protein degradation, and to locate where proteolysis takes place within a cell. Any proposed theory of intracellular protein breakdown must be able to encompass the degradation of both exogenous and endogenous proteins, and specify their site(s) of degradation. The influence of extracellular factors, such as the general composition of the extracellular fluid including its hormone, vitamin, ion and protein content, must be accounted for. The influence of the extracellular medium on intracellular protein degradation, and also upon the rate of endocytosis of the cell, is likely to be important since the amino acid pool size within the cell may need expanding or contracting as metabolic conditions change. Much work has been done in the field of protein digestion, and amongst the suggestions that have been forwarded by different laboratories is a "dual-pathway" hypothesis of protein degradation within cells

(Ballard, 1975; Dean, 1978b). The "dual-pathway" hypothesis of intracellular protein degradation proposes that both a lysosomal and a non-lysosomal pathway are important in protein digestion. The lysosomal route is thought to be used by all exogenous proteins following their endocytic capture, and is also involved in the degradation of some endogenous proteins, for example by lysosomal reduction. [In lysosomal reduction it is envisaged that partially-denatured proteins can adsorb onto the cytoplasmic surface of lysosomes. Involution of the lysosomal membrane enables subsequent digestion of both the vesicle and its contents within the lysosome (Dean, 1978b).] Similarly, autophagy, which may proceed either at a basal rate or at an accelerated rate, provides a way by which endogenous proteins may be passed into the lysosomal system. The non-lysosomal route is proposed for the degradation of abnormal and short half-life proteins (Knowles et al., 1975).

Internalized exogenous proteins, taken into the cell by endocytosis, are degraded within the tissue, and this has generally been assumed to occur following fusion of the endocytic vesicles with lysosomes. Circumstantial evidence for this is not difficult to find. For example, evidence of exogenous material being degraded intracellularly within lysosomes is provided by Myagkaya et al. (1979) who performed an ultrastructural study on the breakdown of erythrocytes within lysosomes of trophoblastic epithelial cells from sheep placenta. They demonstrated acid phosphatase activity in the space between the erythrocyte membrane and the lysosomal membrane, but not inside the ingested erythrocytes. The erythrocyte plasmamembrane remained observable until the final stage of the breakdown process, and presumably formed a barrier preventing the penetration of lysosomal enzymes into the ingested erythrocytes during the earlier stages of degradation. But, conclusive evidence for the degradation of soluble proteins within the lysosomal system alone is much more difficult

to obtain. However, Livesey & Williams (1979) have provided substantial evidence that an exogenous protein, formaldehyde-denatured ^{125}I-labelled bovine serum albumin, is digested exclusively intralysosomally by rat yolk sacs *in vitro*. They found that more than 80% of the radiolabelled digestion products appearing in the incubation medium consisted of ^{125}I-labelled L-tyrosine, and that larger digestion products were only found in association with the yolk-sac tissue; association of trichloroacetic acid-insoluble radioactivity with the tissue always preceded detection of acid-solubles in the incubation medium. This degradation is believed to take place intralysosomally since the tissue-associated acid-insoluble radioactivity showed a lysosomal distribution on subcellular fractionation of tissue exposed to this marker protein, cell-free homogenates of yolk sacs degraded albumin only at acid pH values, and the rate at which acid-soluble radioactivity was formed was equal to the rate of uptake of acid-insoluble radioactivity by the tissue.

The rate of digestion of endogenous proteins has been the subject of a great deal of research. Kay (1978) has reviewed the main experimental approaches which have been used, and has discussed their merits and disadvantages. Many symposia have been held in this field [Schimke, R.T. & Katunuma, N. (1975); Rapoport, S. & Schewe, T. (1978); Evered, D. & Whelan, J. (1980)]. Many of the contributions in these works support and expand the suggestions made below, and are an excellent source of reference.

Evidence for lysosomes being involved in the breakdown of endogenous protein is also extensive. If one examines the rate of protein degradation of cultured cells *in vitro* under varying conditions of medium composition, one finds that the rate of proteolysis changes. Sommercorn & Swick (1981) showed that primary monolayer cultures of adult rat hepatocytes when incubated in a balanced salts medium degraded their endogenous protein

at a high rate, but, when incubated in a more complete medium, a lower rate of protein digestion was observed. Similarly, Frelin (1980) found that addition of serum strongly inhibited the rate of release of [^{3}H]leucine from the prelabelled, long-lived myocardial proteins of newborn rat heart cells in culture. The inhibiting serum factor was found to be heat stable. The possibility of this factor being albumin or fatty acids was ruled out.

Amenta & Brocher (1980b), culturing rat embryo fibroblasts in vitro, observed that the rates of protein degradation of cells in serum-free medium were greater that those for cells cultured in medium containing 10% serum. Addition of NH_4Cl (20mm) to the media resulted in inhibition of protein degradation and was accompanied by an accumulation of autophagic vacuoles (as observed by electron microscopy), particularly in the cells incubated in serum-free medium. When the cells were removed from media containing NH_4Cl and placed into fresh media, the accumulated material within the autophagic vacuoles was digested. From this they concluded that NH_4Cl inhibits rapidly and specifically the lysosomal proteolytic compartment and that this accounts for approximately 25% of protein turnover; proteolysis within this compartment can be stimulated to account for 50% of the protein degradation within the cell. They also stated that the major pathway for the degradation of fast-turnover proteins appeared to be separate from the lysosomal system.

The effect of "step-down" conditions (removal of serum from the incubation medium) on the rate of protein degradation in cultured cells was further investigated by Dean (1979). He concluded that the presence of 10% (v/v) serum in the incubation medium of cultured mouse macrophages established a basal rate of proteolysis in the cultures; this was assumed since insulin (1 pM-1 μM) and glucagon (10μM) had negligible effects on rates of protein degradation under these conditions (the effects of these hormones on protein degradation will be discussed more fully in Chapter 5).

Using the selective carboxyl proteinase inhibitor pepstatin, an inhibition of basal proteolysis of up to 40% was observed. If pepstatin can be assumed to enter the cells by pinocytosis, the inhibitor will probably remain within the lysosomal compartment following fusion between the incoming pinosomes and lysosomes. Cytochalasin B and the local anaesthetics lidocaine and procaine all had inhibitory effects on basal degradation, and since these agents all affect microfilaments, this inhibition of proteolysis may have resulted from a decrease in the rate of autophagy, thus implicating autophagy in basal proteolysis. Alternatively, lidocaine and procaine are weakly basic tertiary amines which may accumulate in lysosomes and thus inhibit proteolysis by altering the intralysosomal pH. Ward et al. (1979) found similar evidence in perfused rat liver and heart for the involvement of the lysosomal system in basal and deprivation-induced proteolysis.

Dean (1980), in an article in which he outlines some of the characteristics and inhibitory factors of endogenous protein degradation, presents further evidence for the involvement of lysosomes in basal proteolysis, by using an inhibitor of thiol proteinases, Z-Phe-Ala-CHN_2 (carbobenzoxycarbonyl-L-phenylalanyl-L-alanine-D-diazomethane). The inhibitor, like pepstatin, enters mouse peritoneal macrophages in culture quite slowly, probably by pinocytosis, and under basal conditions reaches a maximum of 30-40% inhibition of protein degradation.

The rate of endogenous protein degradation within cultured cells can readily be altered by using "step-up" or "step-down" conditions. This effect may be mediated by changes in levels of amino acids; this is possibly a physiologically relevant mechanism. Many examples of the absence of amino acids from incubation or perfusion media inducing increased rates in proteolysis are to be found in the literature. For example, three studies involving perfused liver [Neely et al. (1977),

Schworer et al. (1979) and Schworer & Mortimer (1979)] have shown that amino acid deprivation leads to increased rates of autophagy within the cells, and therefore enhanced rates of protein degradation. The reverse conditions elicit the reverse response. Kovács et al. (1981) inhibited lysosomal protein degradation (propylamine-sensitive degradation) by 70-75% in cultured isolated hepatocytes following addition of excess amino acids, and found that after incubation of the cells with the amino acid mixture for 1h, the formation of autophagic vacuoles was virtually completely inhibited. The amount of protein degradation that was attributed to lysosomes (75%) is much higher than in the examples quoted earlier in this section (25-40%). However, these earlier values were from cells of different tissues, and the high value quoted above is in agreement with those of Seglen & Gordon (1980). These authors examined a large range of different amine derivatives and found that all the lysosomotropic amines inhibited protein degradation to approximately 75% in isolated rat hepatocytes. It is perhaps useful at this point to make a general comment on experiments using combinations of inhibitors to try to estimate the percentage of the total proteolysis that involves lysosomes. Any residual activity after lysosomal inhibition may or may not provide a fair estimate of the contribution of a non-lysosomal degradative route (as outlined by Dean, 1980), since if one route is inhibited, another may undergo a compensatory acceleration.

More evidence for amino acids affecting lysosomal degradation was provided by Seglen et al. (1980) who, after looking at the inhibitory effect of seven different amino acids, determined that, in isolated rat hepatocytes, amino acids act exclusively on the lysosomal pathway of protein degradation. Sommercorn & Swick (1981) investigated the inhibition of endogenous protein degradation in isolated rat hepatocytes by amino acids in more detail, by trying to determine which amino acids

had most influence upon the rate of digestion. They found that in monolayer cultures of adult rat hepatocytes, the inhibition required at least two of the amino acids methionine, phenylalanine, tryptophan and, to a lesser extent, proline; phenylalanine and tryptophan were the most effective inhibitors. They suggested that the regulation of protein degradation by essential amino acids may be physiologically important in maintaining adequate intracellular amino acid pools when the exogenous supply of amino acids is diminished. It would be interesting to see if one can detect any lysosomal pools of amino acids. Harms et al. (1981) measured the amount of free (non-protein) amino acids in whole rat liver and in unmodified lysosomes prepared from rat liver by the technique of free-flow electrophoresis. They reported significant intralysosomal pools of threonine, serine, valine, cystine, isoleucine, leucine, methionine, tyrosine, phenylalaninine, lysine and arginine in rat liver lysosomes maintained at $0^{o}C$.

The literature reviewed so far has been involved in bulk proteolysis by the lysosomal system within cells. Before considering the evidence for a non-lysosomal route, it is important to ask the question whether the lysosomal route itself proffers any scope for selective degradation of proteins. Marzella et al. (1980) studied autophagic degradation in rat liver after vinblastine treatment. They found that vinblastine-induced autophagy enhanced degradation of both old and newly synthesized proteins, and that this was therefore a random process. But Hernandez-Yago et al. (1980) have provided some evidence that autophagy may be involved in selective protein degradation. Ferritin was trapped in negatively charged unilamellar liposomes and incubated with Hela cells for two hours when fusion of the liposome and plasma membrane occurred. They followed the fate of the ferritin within the Hela cells by electron microscopy and presented evidence that the free ferritin in the cytoplasm became

concentrated within lysosomes with time. The cytoplasmic ferritin formed clusters (not membrane bound) which were often observed in osmiophilic areas of the cytosol. They postulate that there could be a mechanism by which certain cytoplasmic areas are separated (by local lipid deposition isolating them from the rest of the cytoplasm) and act as a primary site for individual molecule selection. These electron opaque bodies may be incorporated into the vacuolar system by autophagy, and subsequently be degraded.

Another very indirect piece of evidence for selection in autophagy has been proposed by Dean (1978a). It has been observed that selective binding of cytosol and serum proteins can occur on hydrophobic supports and on membranes, and that the short half-life proteins tend to bind more avidly than proteins with longer half-lives. Since selective binding at the plasma membrane of the cell can lead to selective internalization and subsequent degradation of exogenous proteins, it is not unrealistic to propose that selective binding of proteins to cytoplasmic membranes performing autophagy may provide a mechanism of selective entry of proteins into the lysosomal degradative system. However, this still remains to be established.

The literature presented thus far indicates only indirectly the presence of a non-lysosomal proteolytic pathway within the cell by virtue of the fact that attempts to inhibit lysosomal degradation do not lead to complete inhibition of proteolysis. To date, there has been little direct evidence to characterise this alternative pathway of degradation. Kay (1980) has reviewed this topic in some detail, and has outlined several candidates as possible routes of extralysosomal degradation. Proteins that come into contact with the lysosomal system will experience a very acidic environment, and if this results in partial denaturation, attack by lysosomal proteinases may be readily initiated.

Extralysosomal degradation will not have this "initial advantage", and so it has been postulated that denaturating enzymes may exist to make proteins more susceptible to proteolytic activity. [Some evidence for intracellular membrane-bound enzymes with the capacity to inactivate cytoplasmic proteins is already available (Ballard & Hopgood (1976).] Proteins already in a partially-altered state would presumably be degraded faster than native proteins. Thus, Knowles et al. (1975) found that Reuber H35 hepatoma cells incorporate the arginine-analogue canavanine into cell protein when arginine is omitted from the incubation medium. They labelled arginine-containing proteins with [^{14}C]leucine, and canavanine-containing proteins with [^{3}H]leucine, and found that the canavanine-containing proteins were degraded at a rate several-fold greater than the normal proteins. Comparable results were found when 6-fluorotryptophan was used as an analogue for trytophan. From these results they concluded that the data supported the hypothesis that aberrant proteins are degraded more rapidly than native proteins. Data has also been found to show that metabolic energy is required for some non-lysosomal proteolysis [Seglen et al. (1979), Libby & Goldberg (1981)].

The effect of "step-up" and "step-down" conditions on endogenous protein degradation have been discussed (see above); "step-up" conditions lead to decreased protein degradation, and vice versa. It would be of great interest to see if there is a relationship between the rate of pinocytosis of a tissue and its rate of endogenous protein degradation under different media changes. Perhaps both are subject to some central homeostatic control mechanism. The rat yolk sac affords an excellent system for such a study since pinocytic and proteolytic rates are well quantitated as, to a lesser extent, are the rates of endogenous protein

turnover.

The rate of uptake of the fluid-phase marker ^{125}I-labelled polyvinylpyrrolidone (^{125}I-PVP) has been well established (Williams et al., 1975a). It was used by Ibbotson & Williams (1979) to investigate the effect of 10% (v/v) calf serum on the rate of fluid-phase pinocytosis in rat yolk sacs incubated in vitro. They found that the absence of serum resulted in an increase in the rate of fluid-phase pinocytosis of approximately 45%; experiments with [^{198}Au]gold and [U-^{14}C]sucrose gave similar results. Likewise, absence of serum led to an increase in uptake of formaldehyde-denatured ^{125}I-labelled BSA (^{125}I-fdBSA) up to 75%. Degradation of this protein has been well characterized in the rat yolk-sac system, and has been shown to be an intralysosomal event (Livesey & Williams, 1979). These studies provide a foundation for this proposed investigation.

Several collaborative studies have taken place between this laboratory and that of F.J. Ballard and S.E. Knowles of the CSIRO Division of Human Nutrition, Adelaide, South Australia. For example, the effects of weak bases on the degradation of endogenous and exogenous proteins by rat yolk sacs have been examined (Livesey et al., 1980). The data on exogenous protein (^{125}I-fdBSA) uptake and degradation were compiled in this laboratory, and the data on endogenous protein degradation were provided by F.J.B. and S.E.K. The conclusion drawn from this earlier study was that, since ^{125}I-fdBSA has been shown to be degraded intralysosomally within yolk sacs (see above), the results were compatible with the proteolysis of endogenous proteins being at two sites (lysosomal and non-lysosomal).

The purpose of the work reported in this chapter was to investigate more fully the effect of "step-up" and "step-down" conditions on the rate of fluid-phase pinocytosis (^{125}I-PVP) and on the rate of adsorptive pinocytosis of a protein (^{125}I-fdBSA) and its subsequent intralysosomal

degradation in rat yolk sacs. This study was performed in collaboration with F.J.B. and S.E.K., who provided data on the effect of "step-up" and "step-down" conditions on endogenous protein degradation in rat yolk sacs (these data will be presented and discussed in Section 4.4), to permit a comparison of the effect of different media changes on the rates of exogenous and endogenous protein degradation, and their relationship to the pinocytic rate in rat yolk-sac tissue.

4.2 Materials and Methods

All the equipment and materials used in the experiments reported in this chapter were as described in Section 2.1. Formaldehyde-denatured ^{125}I-labelled BSA (^{125}I-fdBSA) was prepared as described in Section 2.2.2. All the uptake experiments were performed as described in Section 2.3, and all assays as in Section 2.4.; ^{125}I-PVP was always used at a concentration of 2 μg/ml, and ^{125}I-fdBSA at a concentration of 1 μg/ml.

In Section 4.3.1, the serum-free media used were as described in the text and in the legends to figures and tables. The effects of the media on the release of ^{125}I-PVP from yolk sacs were measured as described in Section 2.10.

In Section 4.3.2, medium 199 was used throughout. Calf serum (if present) was used at the concentrations described in the text and in the legends to figures and tables.

The method of F.J. Ballard and S.E. Knowles for measuring the rate of endogenous protein degradation in the rat yolk sac (see Table 4.4a) was as described in detail by Livesey et al. (1980). Briefly, yolk sacs were incubated for 3h in the appropriate medium (see Table 4.4a) containing 2μCi of [^{3}H]leucine/ml and 10 μM-L-leucine, rinsed in radioisotope-free medium and incubated for a further 90 min in "degradation" medium [medium containing 2mM-L-leucine and 20mM-Tes; 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl] amino} ethanesulphonic acid, pH7.5] to degrade unstable proteins. The sacs were subsequently transferred to fresh "degradation" medium which, in some experiments, contained an agent (e.g. amino acids, bovine serum albumin), and were incubated, usually for 4h. Rates of degradation were expressed as the percentage of the ^{3}H-labelled protein that was degraded over this period. The concentrations of the various agents used were as described in Table 4.4a.

4.3.1 Effect of Different Media on Uptake of ^{125}I-PVP

The rates of uptake of ^{125}I-PVP were determined for rat yolk sacs incubated in three different serum-free media, namely medium 199, Minimum Essential Medium and Earle's Salts; physiological saline was also used. The compositions of these three media are outlined in Table 4.3.1a. The inorganic salt content is essentially the same in each case, as is the amount of glucose and phenol red present; Earle's Salts consists only of these compounds. In addition to these inorganic components, Minimal Essential Medium (M.E.M.) contains 13 amino acids and 8 vitamins. Medium 199 contains the entire composition of M.E.M. plus a further 8 amino acids and 9 vitamins, the antibiotics penicillin and streptomycin, and 14 "other components", including compounds such as adenosinetriphosphate (disodium salt), cholesterol and glutathione.

The effect of these three media on the rate of uptake of ^{125}I-PVP by yolk sacs is shown in Figure 4.3.1a. All the plots are linear up to 6h. Yolk sacs in the presence of 0.9% saline do not pinocytose any ^{125}I-PVP. The mean Endocytic Index (E.I.) of ^{125}I-PVP in the presence of medium 199 (control value) was 3.50 µl/mg yolk-sac protein per h (see Table 4.3.1b), and in Earle's Salts was 2.89 µl/mg yolk-sac protein per h. The mean E.I. of ^{125}I-PVP in Earle's Salts as a percentage of the control (in medium 199) was 83% ± 19%, indicating that the rates of uptake of ^{125}I-PVP in these two media are very similar. The rate of uptake of ^{125}I-PVP in M.E.M. was 2.64 µl/mg yolk-sac protein per h, or 75% ± 11% of the control value. This was slightly less than the rate of uptake in Earle's Salts. It therefore appears that the rates of fluid-phase pinocytosis of yolk sacs incubated in the three different media were very similar, although slightly lower in Earle's Salts and M.E.M. than in Medium 199.

The release of ^{125}I-PVP with time from yolk sacs that had previously been incubated in the presence of this radiolabelled substrate, washed

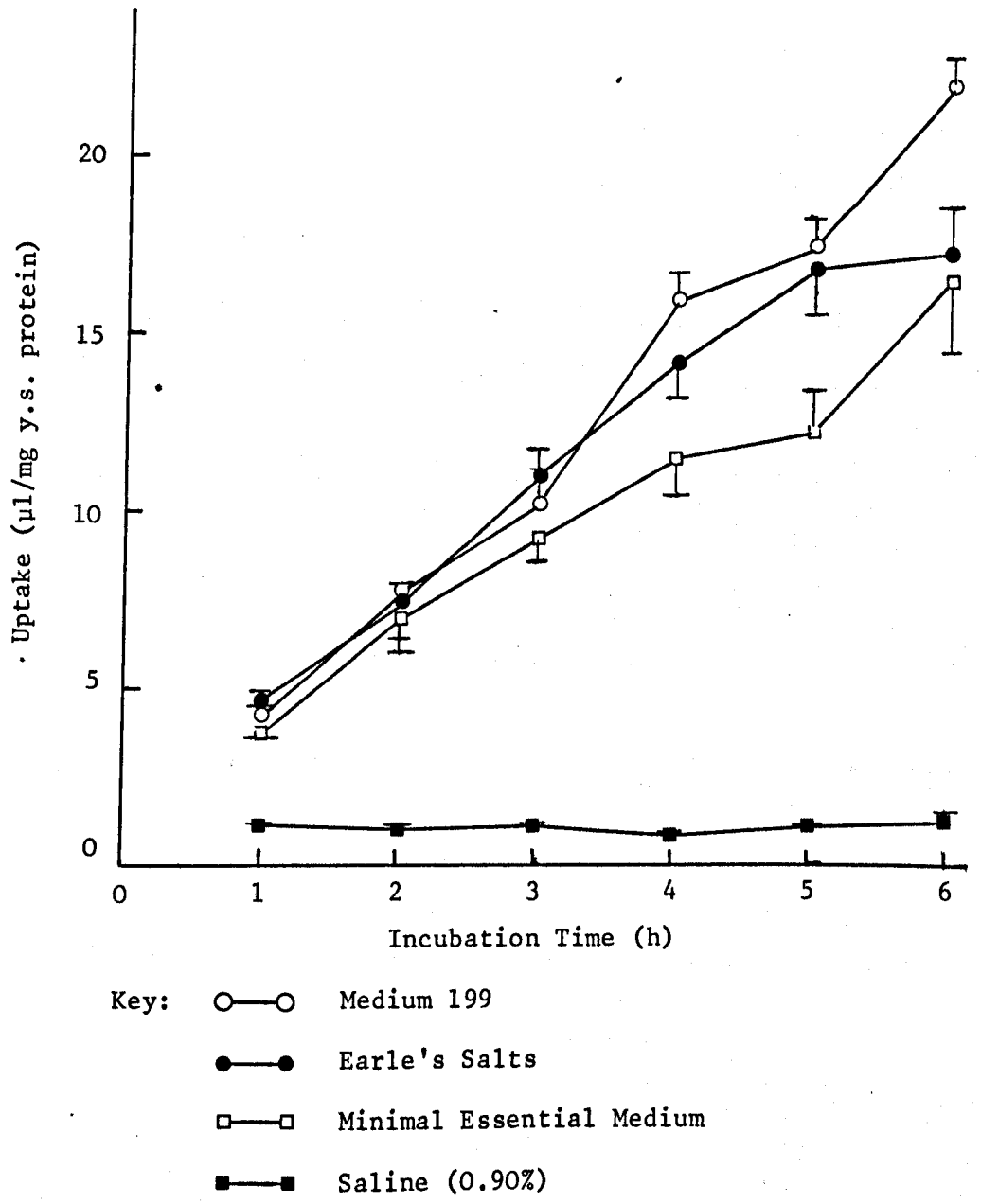
in substrate-free medium and then reincubated in substrate-free medium, is shown in Figure 4.3.1b, and was determined by reincubation in each of the three different media. In each case it can be seen that there was an initial rapid release of radioactivity from the yolk sacs. Presumably this is caused by incomplete removal of surface-associated radioactivity when the yolk sacs had been washed after their initial incubation with ^{125}I-PVP, before being reincubated in the absence of ^{125}I-PVP (the period being monitored). The quantity of radioactivity released with time was greatest in M.E.M. and least in medium 199, and the rates of release (after the initial rapid release) are shown in Table 4.3.1c. The rate of release of radioactivity from yolk sacs incubated in medium 199 was well under 1% per hour, and in Earle's Salts was approximately 1% per hour. These figures agree well with the value obtained by Williams *et al*. (1975,a), and indicate that the tissue is undamaged. Release of ^{125}I-PVP from yolk-sacs incubated in M.E.M. was only slightly faster than the control value, again indicating no appreciable loss of intregrity of the tissue.

Table 4.3.1a Composition of Medium 199, Minimal Essential Medium and Earle's Salts

The concentration of inorganic salts, glucose and phenol red are expressed as mg/l, and antibiotics as units/l. Numbers in parentheses represent the total number of different substances present. The amino acids and vitamins present in minimal essential medium are also present in medium 199, although not necessarily at the same concentrations.

Composition	Earle's Salts	Minimal Essential Medium	Medium 199
Inorganic Salts:			
$CaCl_2$	200	200	200
$Fe(NO_3)_3.9H_2O$	0	0	0.72
KCl	400	400	400
$MgSO_4$ (anhyd.)	0	0	97.67
$MgSO_4.7H_2O$	200	200	0
NaCl	6,800	6,800	6,800
$NaHCO_3$	2,200	2,200	2,200
$NaH_2PO_4.H_2O$	140	140	140
Other Components:			
Glucose	1,000	1,000	1,000
Phenol Red	10	10	20
Others	(0)	(0)	(14)
Amino Acids:	(0)	(13)	(21)
Vitamins	(0)	(8)	(17)
Antibiotics:			
Penicillin-Streptomycin	0	0	20,000

Figure 4.3.1a Uptake of ^{125}I-PVP by Yolk Sacs Incubated in Serum-Free Medium 199, Minimal Essential Medium, Earle's Salts and Saline



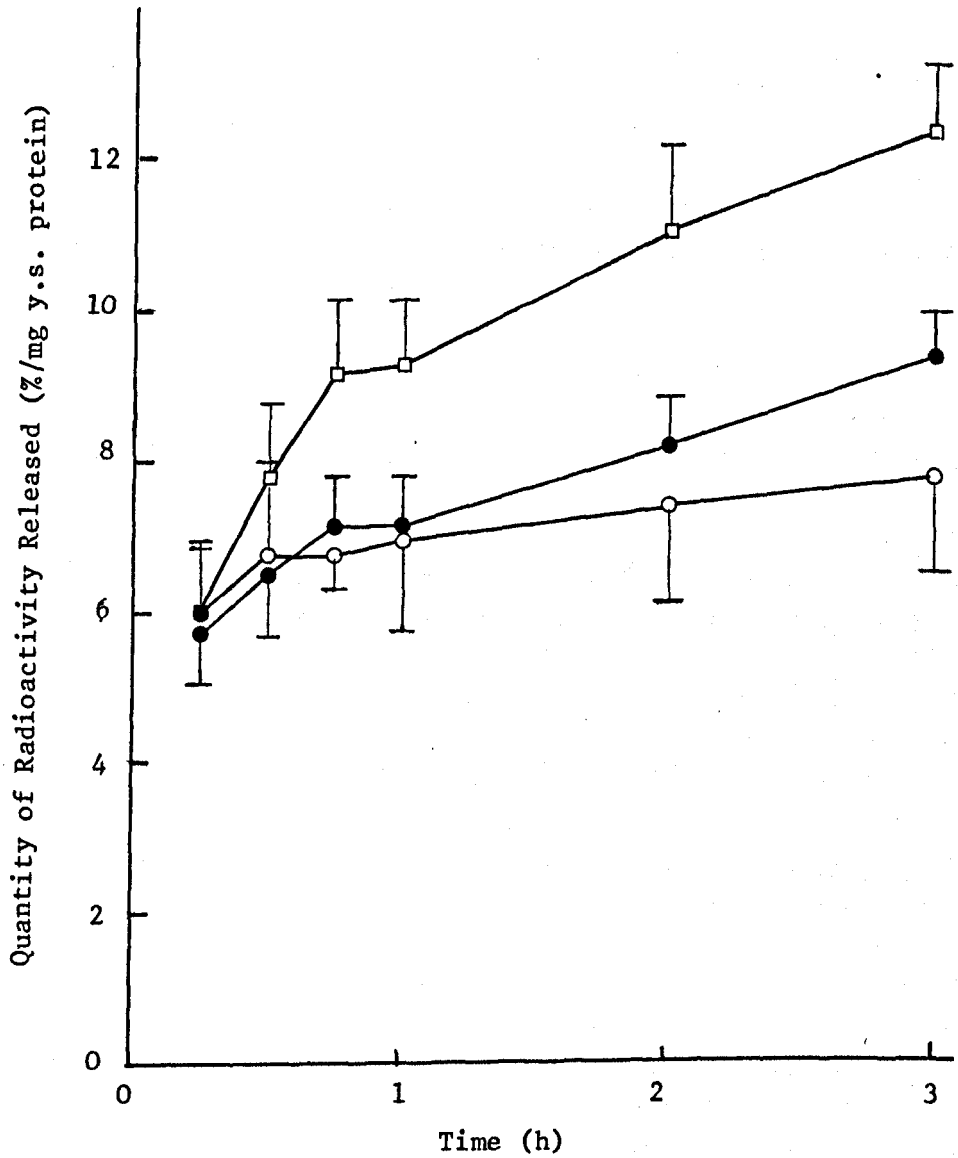
The method used was as described in Section 4.2. ^{125}I-PVP was present at a concentration of 2 µg/ml. Each uptake plot shows mean values (± S.E.M.) from 2 to 4 separate experiments. These results are summarized in Table 4.3.1b.

Table 4.3.1b Summary of Data for ^{125}I-PVP Uptake by Yolk Sacs Incubated in Different Serum-Free Media

These data are from the experiments shown in Figure 3.3.1a. Each rate of uptake is expressed as an Endocytic Index (E.I.), and is given with its correlation coefficient (Corr.Co.).

Medium	E.I.(Corr.Co.) (µl/mg y.s. protein per h)	Mean E.I ± S.E.M. (µl/mg y.s.) protein per h)	Mean E.I. as % of Control (%)
199 (Control)	3.57 (0.986) 3.91 (0.970) 3.03 (0.972)	3.50 ± 0.26	100.0 ± 7.4
Minimal Essential Medium	3.22 (0.951) 1.61 (0.935) 3.19 (0.917) 2.55 (0.964)	2.64 ± 0.38	75.4 ± 10.9
Earle's Salts	3.46 (0.945) 2.08 (0.946) 2.62 (0.946) 3.38 (0.980)	2.89 ± 0.66	82.6 ± 18.9
Saline (0.9%)	0.07 (0.376) 0.02 (0.106)	0.05	1.4

Figure 4.3.1b Release of ^{125}I-PVP from Yolk Sacs Incubated in Serum-Free Medium 199, Minimal Essential Medium and Earle's Salts



The method used was as described in Section 4.2. Each release plot represents the quantity of radioactivity released with time from yolk sacs that had previously been incubated in the presence of ^{125}I-PVP. Each experimental point is a mean value (± S.E.M.) from 5 separate experiments.

Table 4.3.1c Summary of the Data for Release of ^{125}I-PVP from Yolk Sacs Incubated in the Presence of Different Serum-Free Media

These data are from the experiments shown in Figure 4.3.1b. Each rate of release of radioactivity was calculated from the gradient of these plots between time points 0.75 and 3.0h.

Medium	Mean Total Uptake at Start of Reincubation Period (ng/mg y.s. protein)	Radioactivity Released (%/mg y.s. protein per h)
199 (Control)	30.76	0.43
Minimal Essential Medium	32.66	1.46
Earle's Salts	31.17	0.99

4.3.2 Effect of Serum on Uptake of ^{125}I-PVP and ^{125}I-fdBSA

Yolk sacs were incubated in medium 199 containing different amounts of serum, from 0% to 50% (v/v). The effect of the serum concentration upon the rate of fluid-phase pinocytosis was monitored using ^{125}I-PVP as substrate, and the results are shown graphically in Figure 4.3.2a. All the plots were linear up to 5h, and increasing the serum concentration decreased the rate of uptake. Table 4.3.2a shows the mean values of the Endocytic Index (E.I.) of ^{125}I-PVP for the different serum concentrations used. The addition of a small amount of serum (5%, v/v) resulted in a large fall in E.I. (to 70% of the control E.I. in the absence of serum). The addition of a further 5%, (to give a serum concentration of 10%, v/v) led to a relatively smaller fall to 64%. The 0% and 10% serum figures for the uptake of ^{125}I-PVP are in agreement with those determined by Ibbotson & Williams (1979). In the presence of 20% serum the E.I. fell to 50% of the control value, and at a concentration of 50% serum the E.I. was down to 29%.

Since the Endocytic Index of a substrate has units µl/mg yolk-sac protein per h, a potential factor contributing to the calculated fall in E.I. of ^{125}I-PVP, when incubated with increasing amounts of serum, could be the intracellular accumulation of large amounts of serum proteins before their degradation; this would increase the tissue-protein term in the denominator in the expression for calculation of uptake and hence cause the E.I. to fall. The possibility of this occurring was examined by plotting the mean protein content of yolk sacs against the time that the yolk sacs had been incubated. The protein content of yolk sacs from within a single mother can vary considerably (as much as ± 20%), and so it was desirable to utilize as many data as possible to average out such random variations. Many control experiments had been performed in the presence or absence of 10% (v/v) serum, and all the data from

November 1978 to November 1980 were pooled, and the results are shown in Figure 4.3.2b, part (1). From this graph it is clear that the presence of 10% serum in the medium in which yolk sacs were incubated made no difference to their mean protein content which did not vary with the duration of the incubation.

Experiments in which the serum concentration was higher than 10% were also examined, but, since fewer such experiments were performed compared with those with 0% and 10% serum, only 0% and 10% serum data produced during the period of the high serum concentration experiments (November 1980 to January 1981) were compared with these high serum data for variations in the yolk sac protein content; these data are shown in Figure 4.3.2b, part (2). Although the data from the more substantial series of incubations in the presence of 0% and 10% serum indicate that, in these data collected over a period of 2 years, there is no difference in the mean protein content of the yolk sacs [Figure 4.3.2b (1)], for the more limited series of such incubations the results are less clear cut. There is even an apparent difference in the 0% and 10% serum results for this limited series of incubations that served as matched controls to the incubations in higher levels of serum [Figure 4.3.2b (2)]. At first glance it may appear that the mean yolk-sac protein content was slightly greater for yolk-sacs incubated in the presence of higher serum concentrations (>10%), but this most probably represents scatter caused by so few measurements. Even if this were a true effect, it would not be sufficient to cause any marked difference to the mean calculated values of the E.I.

Formaldehyde-denatured ^{125}I-bovine serum albumin (^{125}I-fdBSA) has been extensively studied in the rat yolk-sac system (Moore *et al.*, 1977), both in the presence and absence of 10% calf serum (Ibbotson & Williams, 1979), and has been shown to be degraded exclusively intracellularly,

within lysosomes (Livesey & Williams, 1979) after being captured by adsorptive endocytosis. The effect of calf serum concentration (up to 50%, v/v) on the rate of uptake of this protein by rat yolk sacs incubated in medium 199 was investigated. The results are shown in Figure 4.3.2c, the uptake being linear up to 5h of culture, with increasing serum concentrations resulting in decreasing rates of uptake. The mean E.I. of ^{125}I-fdBSA in serum-free medium was 355 µl/mg yolk-sac protein per h, and in the presence of 10% calf serum the E.I. fell to 92 µl/mg yolk-sac protein per h, which was only 26% of the control value (in serum-free medium). Increasing the calf serum concentration to 50% led to virtually complete inhibition of uptake of ^{125}I-fdBSA, the mean E.I. being only 5% of the control values. However, the correlation coefficients of these plots are greater than 0.95, indicating that the calculated uptake, despite being so low, increases linearly with time. The contrast in the relative effect of serum on the rate of fluid-phase pinocytosis (as measured by uptake of ^{125}I-PVP) and on the adsorptive uptake of ^{125}I-fdBSA, is shown in Figure 4.3.2d. Serum has a much greater relative effect on the uptake of ^{125}I-fdBSA, reflecting competition between this substrate and serum proteins for binding sites on the yolk-sac membrane.

The effect of serum on the release of acid-soluble radioactivity into the incubation medium from yolk sacs incubated in the presence of ^{125}I-fdBSA is shown in Figure 4.3.2e. This release was virtually linear beyond the first hour of incubation for serum concentrations up to 20% (v/v), and the rate of the release, shown in Table 4.3.2b, was the same as the rate of uptake (mean E.I.) for each serum concentration up to 20%. In the presence of 50% serum, however, there was no detectable release of acid-soluble radioactivity until about 3h.

The quantity of radioactivity that became associated with the yolk-sac tissue during incubation with ^{125}I-fdBSA (T.A.R.) is shown in Figure 4.3.2f. In the absence or presence of 10% (v/v) calf serum, the T.A.R.

values remained approximately constant beyond the first 2h of incubation, reflecting a steady-state relation between the rate of uptake of radio-labelled substrate and the rate of release of acid-soluble radioactive fragments following intralysosomal degradation. The absolute T.A.R. values in the absence of serum were greater than in the presence of serum, due to the higher rate of uptake in serum-free medium.

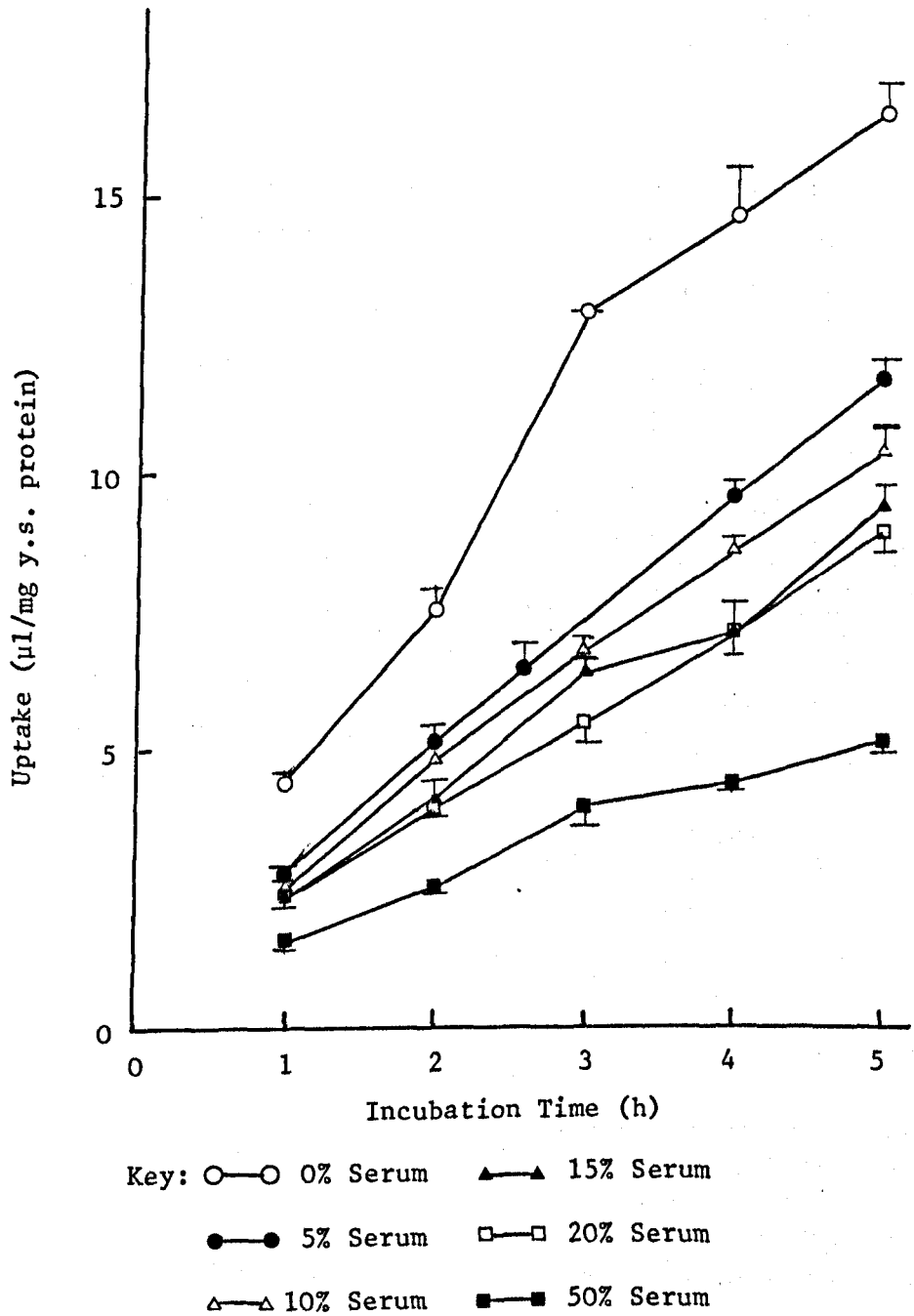
At high serum concentrations (20% and 50%) the T.A.R. values at 1h were lower than described above because of the lower rate of uptake of ^{125}I-fdBSA under these conditions. During the incubation the T.A.R. values did not remain constant as above, but instead rose progressively. This cannot be due to changes in pinocytic rates within the tissue since uptake of both ^{125}I-PVP and ^{125}I-fdBSA were linear with time at these serum concentrations. It is unlikely that the time taken for the release of radioactive fragments from the lysosomes to appear in the incubation medium had increased since these radioactive fragments, ^{125}I-tyrosine, move by diffusion. The most likely explanation for this finding is that there has been an inhibition of the capacity of the lysosomal enzymes to degrade the large amounts of serum proteins presenting themselves to the lysosomes.

This explanation was further supported by examination of the calculated disposal times of ^{125}I-fdBSA (the mean time that ^{125}I-fdBSA is associated with the tissue before acid-soluble fragments are released, see Figure 4.3.2g). In the absence or presence of 10% (v/v) serum, the disposal time remained constant during the period of the incubation, the disposal time in serum-free medium being the smaller. At higher serum concentrations the disposal time increased during the incubation period. The effect is only slight at a serum concentration of 20% (v/v), but is marked when the concentration is increased to 50%.

The effect of calf serum on the uptake and degradation of ^{125}I-fdBSA

by yolk sacs during the first hour of incubation is shown in Figures 4.3.2h and i. The first of these two figures shows that uptake began immediately under all the conditions studied. The release of acid-soluble radioactivity (see Figure 4.3.2i) was detectable at 6 minutes in serum-free medium, and at 15 min in the presence of 10% (v/v) serum. At a concentration of 20% serum the release of acid-soluble radioactivity was not detectable until after 0.5h, and in the presence of 50% serum there was no acid-soluble radioactivity released during the first hour of incubation (as was expected from Figure 4.3.2e).

Figure 4.3.2a Effect of Calf Serum Concentration on the Uptake of ^{125}I-PVP by Yolk Sacs



The method used was as described in Section 4.2. ^{125}I-PVP was present at a concentration of 2 µg/ml. Each uptake plot shows mean values (± S.E.M.) from 3 to 4 separate experiments. These results are summarized in Table 4.3.2a.

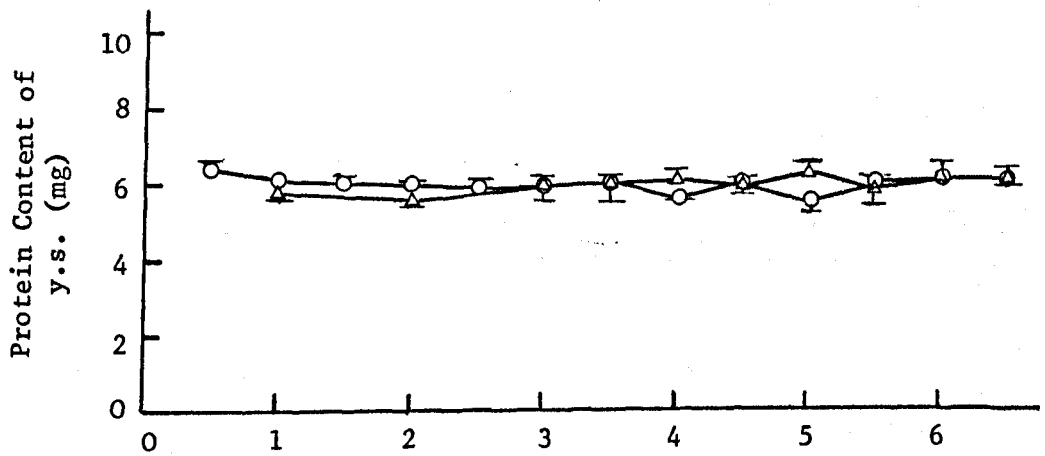
Table 4.3.2a Summary of the Effect of Calf Serum Concentration on the Uptake of ^{125}I-PVP by Yolk Sacs

These data are from the experiments shown in Figure 4.3.2a. The rate of uptake is expressed as the Endocytic Index (E.I.), and is given with its correlation coefficient (Corr. Co.).

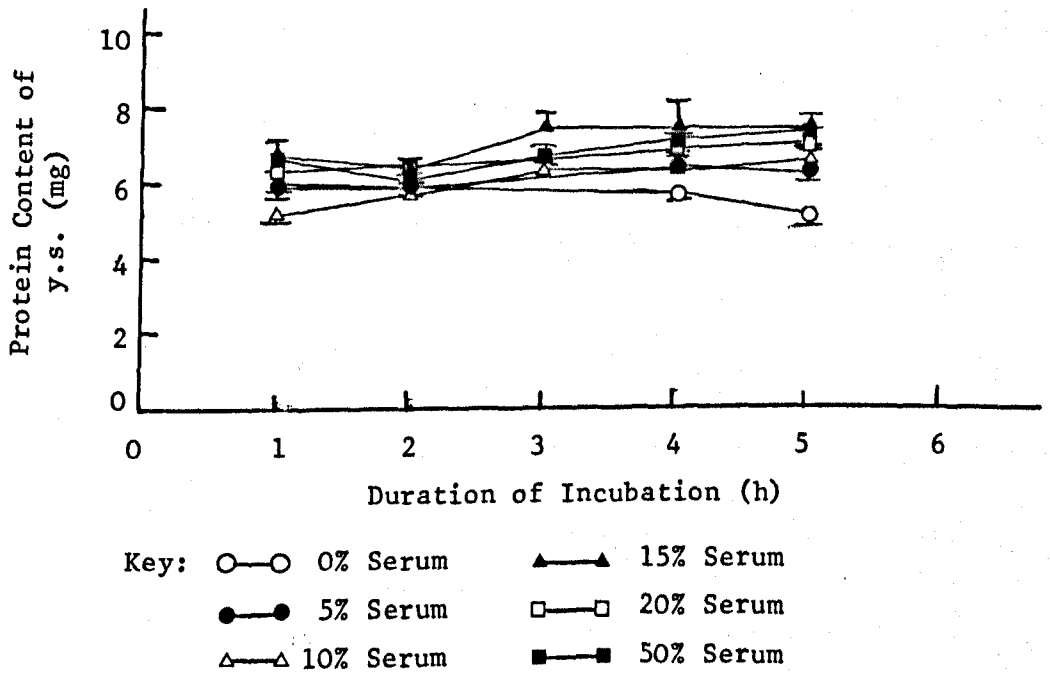
Serum Conc. (%)	E.I.(Corr. Co.) (µl/mg y.s. protein per h)	Mean E.I. ± S.E.M. (µl/mg y.s. protein per h)	Mean E.I. as % of Control (%)
0 (Control)	3.10 (0.944) 2.89 (0.984) 3.35 (0.991)	3.11 ± 0.13	100 ± 4.2
5	2.31 (0.988) 2.06 (0.983) 2.31 (0.977)	2.23 ± 0.08	71.7 ± 2.6
10	2.09 (0.973) 1.93 (0.985) 1.92 (0.990)	1.98 ± 0.06	63.7 ± 1.9
15	1.81 (0.975) 1.68 (0.995) 1.38 (0.988) 1.89 (0.949)	1.69 ± 0.11	54.3 ± 3.5
20	1.81 (0.983) 1.59 (0.994) 1.24 (0.956) 1.54 (0.989)	1.55 ± 0.12	49.8 ± 3.9
50	0.84 (0.980) 0.95 (0.959) 0.87 (0.976)	0.89 ± 0.03	28.6 ± 1.0

Figure 4.3.2b Effect of Calf Serum Concentration on the Protein Content of Yolk Sacs during Incubation with ^{125}I-PVP

(1) Experiments from November 1978 to November 1980

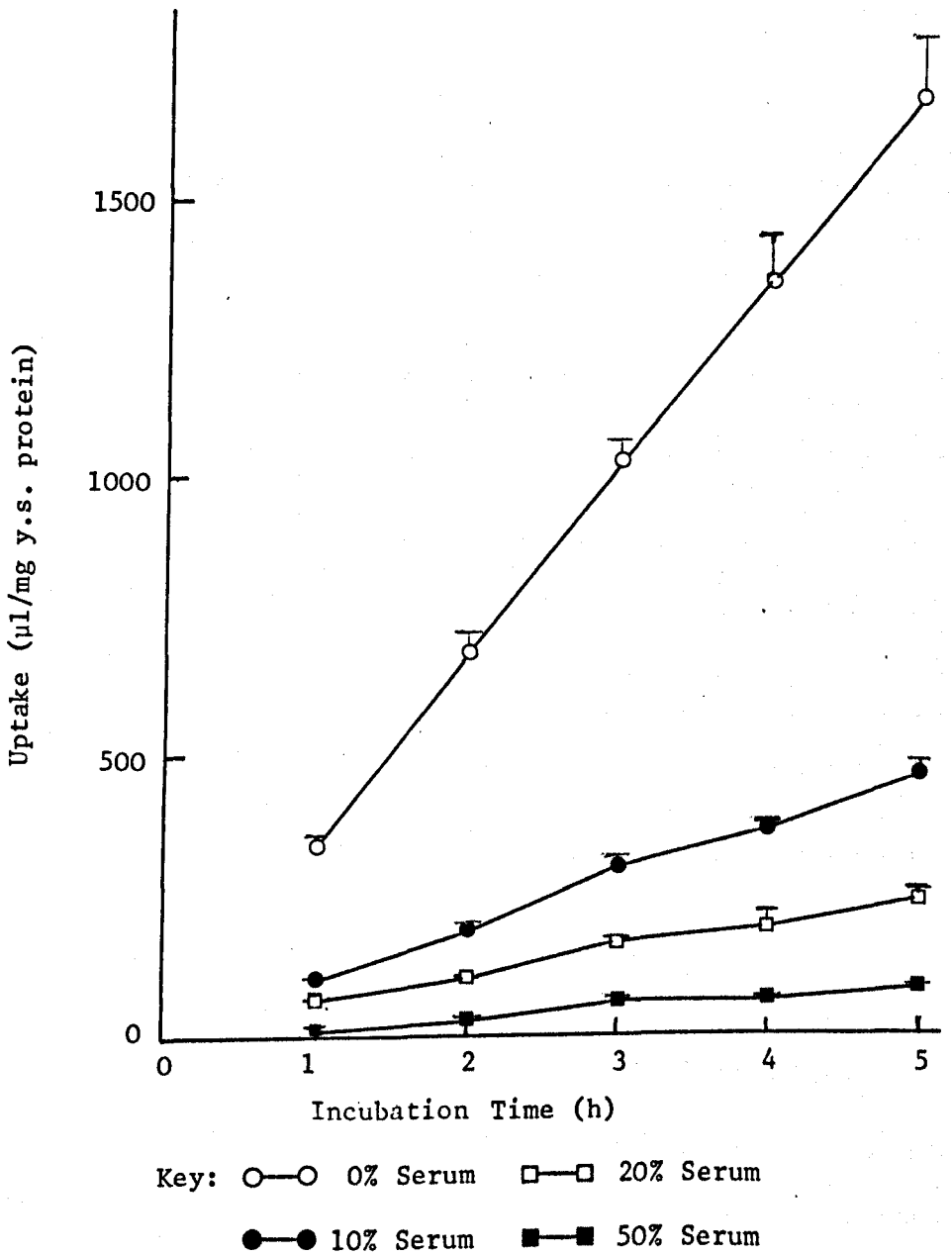


(2) Experiments from November 1980 to January 1981



The method used was as described in Section 4.2. ^{125}I-PVP was present at a concentration of 2 µg/ml. The protein content of the yolk sacs is expressed as mg equivalent of BSA (mean ± S.E.M.) from between 6 and 24 individual determinations in the presence of serum, and between 12 and 83 individual determinations in serum-free medium in part (1). In part (2), the mean protein content of the yolk sacs represents between 4 and 10 individual determinations.

Figure 4.3.2c Effect of Calf Serum Concentration on the Uptake of ^{125}I-fdBSA by Yolk Sacs



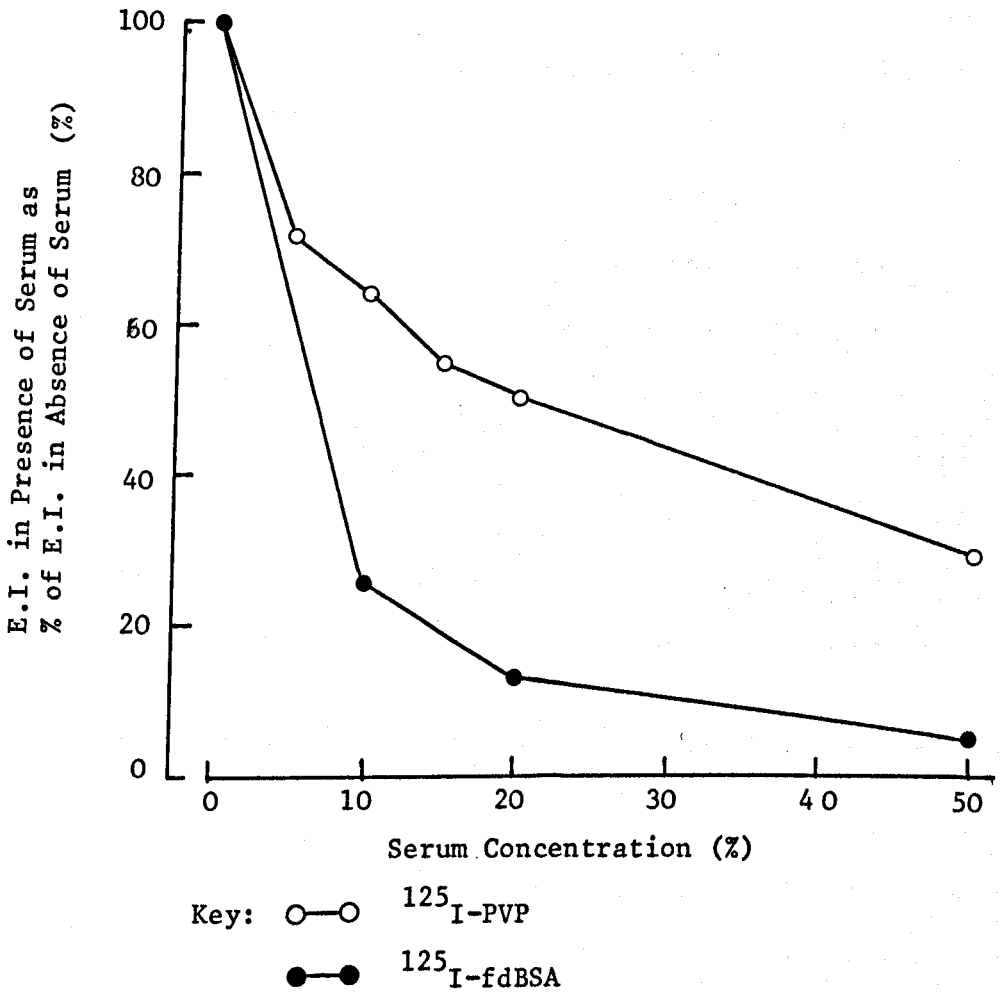
The method used was as described in Section 4.2. ^{125}I-fdBSA was present at a concentration of 1 µg/ml. Each uptake plot shows mean values (± S.E.M.) from 3 to 5 separate experiments. These results are summarized in Table 4.3.2b. A detailed study of the uptake during the first hour of incubation is shown in Figure 4.3.2h.

Table 4.3.2b Summary of the Effect of Calf Serum Concentration on the Uptake of ^{125}I-fdBSA by Yolk Sacs

These data are from the experiments shown in Figures 4.3.2c and e. Rate of uptake is expressed as the Endocytic Index (E.I.), and is given with its correlation coefficient (Corr. Co.). The rate of release of acid-soluble radioactivity from the tissue is denoted by "Rate of Sol. Rel.".

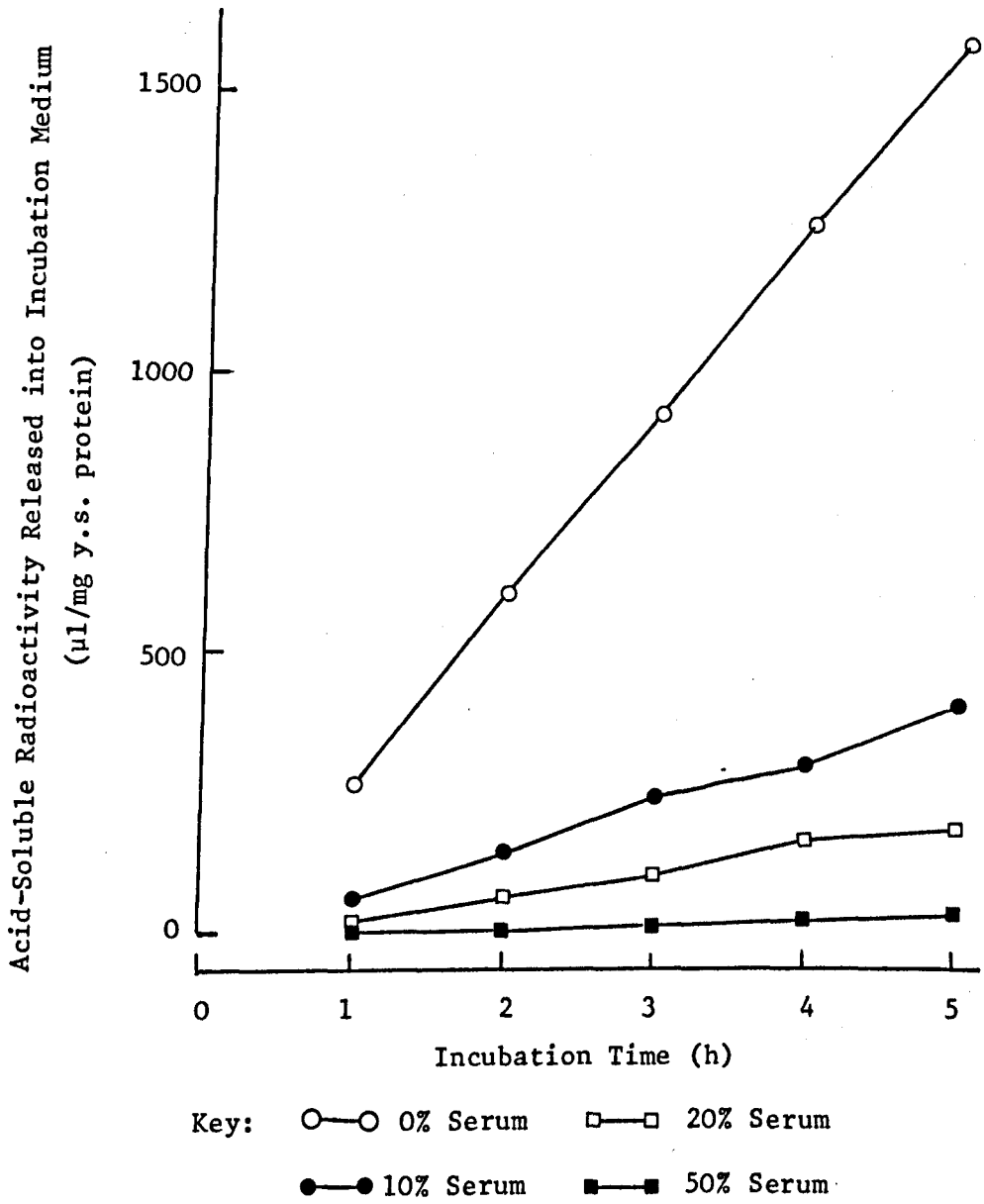
Serum Conc.(%)	E.I.(Corr.Co.) (µl/mg y.s. protein per h)	Mean E.I. ± S.E.M. (µl/mg y.s. protein per h)	Relative E.I. as % of Control (%)	Rate of Sol. Rel. (µl/mg y.s. protein per h)
0 (Control)	377.47 (0.916) 430.58 (0.991) 290.27 (0.982) 385.66 (0.980) 289.67 (0.908)	354.73 ± 27.94	100.0 ± 7.9	329.44
10	113.17 (0.940) 92.40 (0.988) 69.72 (0.975) 90.98 (0.983)	91.57 ± 8.87	25.8 ± 2.5	84.04
20	41.63 (0.986) 38.10 (0.985) 37.68 (0.989) 60.22 (0.989)	45.53 ± 5.27	12.8 ± 1.5	41.30
50	17.24 (0.987) 16.87 (0.992) 17.00 (0.957)	17.04 ± 0.11	4.8 ±<0.1	9.82

Figure 4.3.2d Effect of Calf Serum Concentrations on the Relative Endocytic Indices of ^{125}I-PVP and ^{125}I-fdBSA by Yolk Sacs



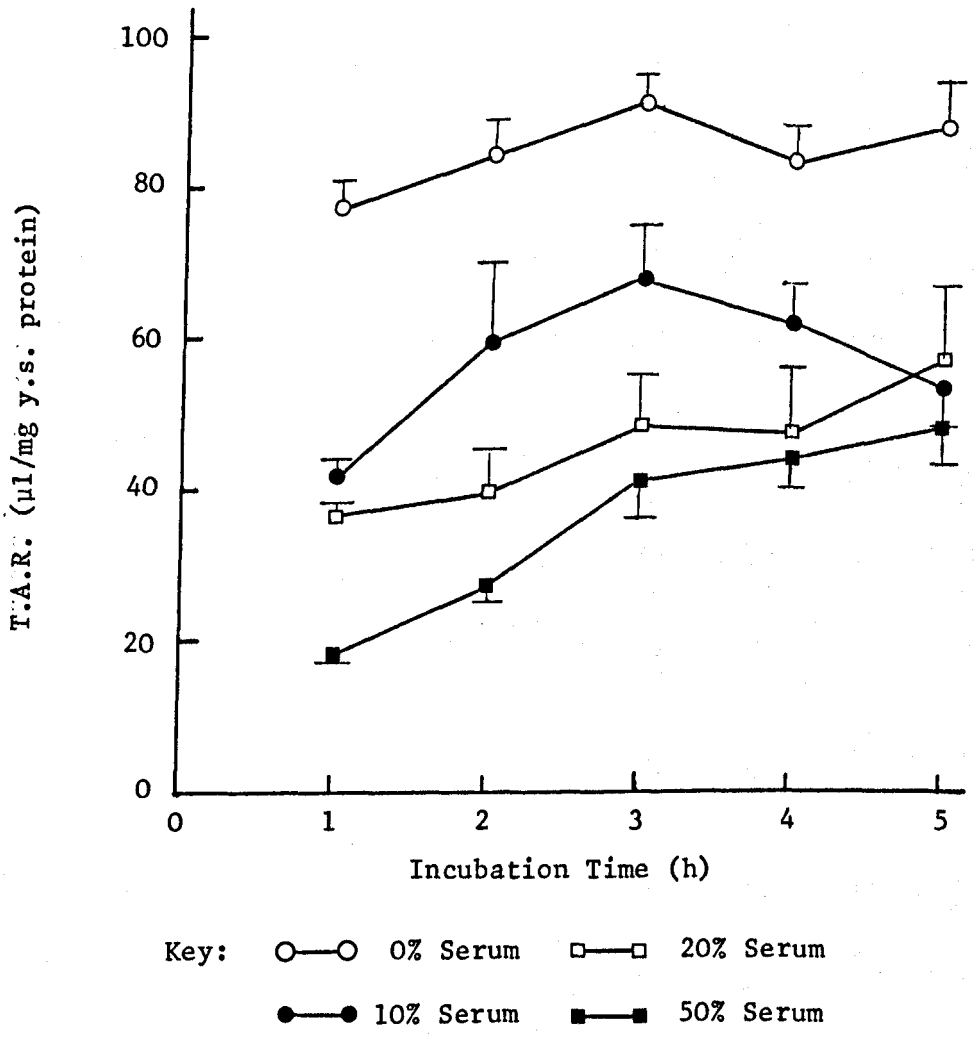
Each point represents the mean E.I. of the substrate at a particular serum concentration expressed as a percentage of the mean control E.I. in the absence of serum. These results are from the data summarized in Tables 4.3.2a and b.

Figure 4.3.2e Effect of Calf Serum Concentration on the Release of Acid-Soluble Radioactivity into the Incubation Medium by Yolk Sacs during Uptake of ^{125}I-fdBSA.



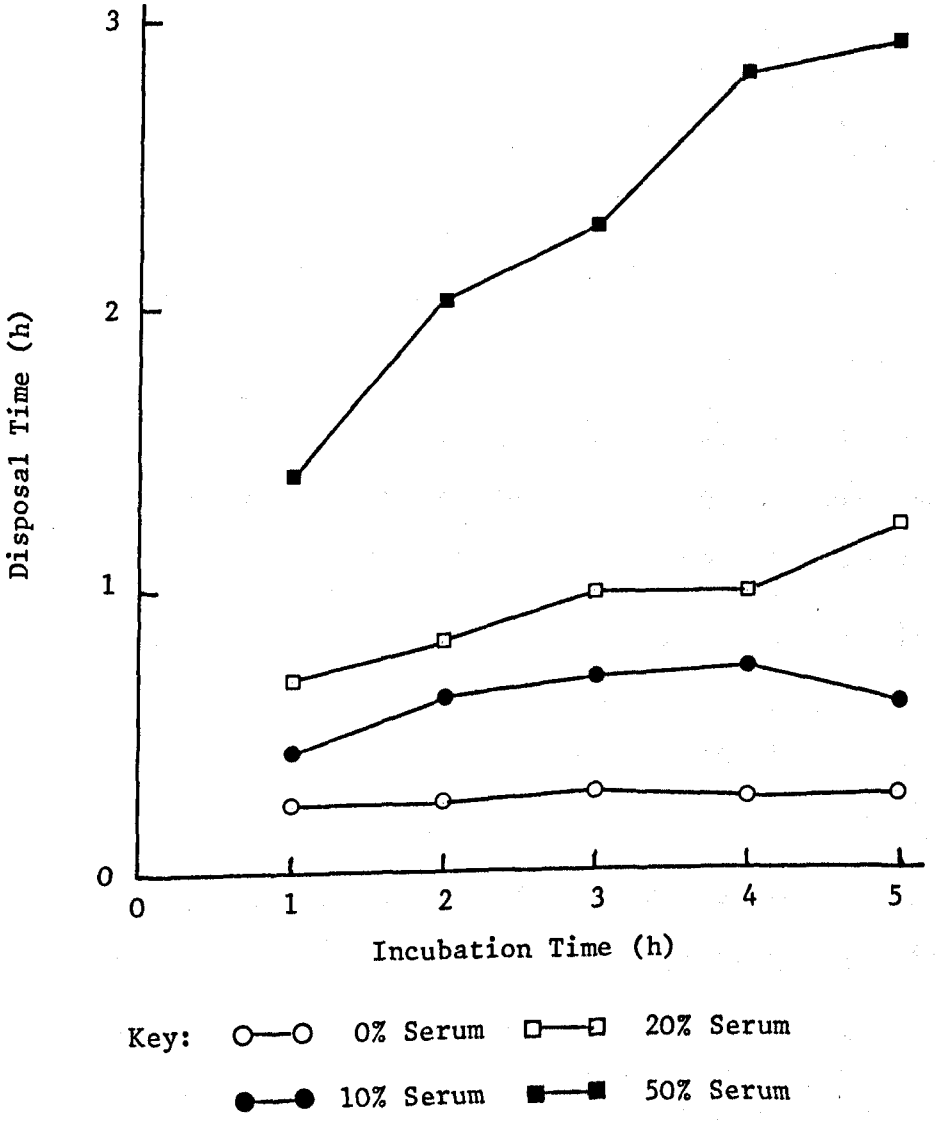
The acid-soluble radioactivity released into the incubation medium is expressed as mean values. These results are from the experiments shown in Figure 4.3.2c, and are summarized in Table 4.3.2b. A detailed study of the acid-soluble radioactivity released into the incubation medium during the first hour of incubation is shown in Figure 4.3.2i.

Figure 4.3.2f Effect of Calf Serum Concentration on the Quantity of Radioactivity that becomes Associated with Yolk-Sac Tissue during Uptake of ^{125}I-fdBSA



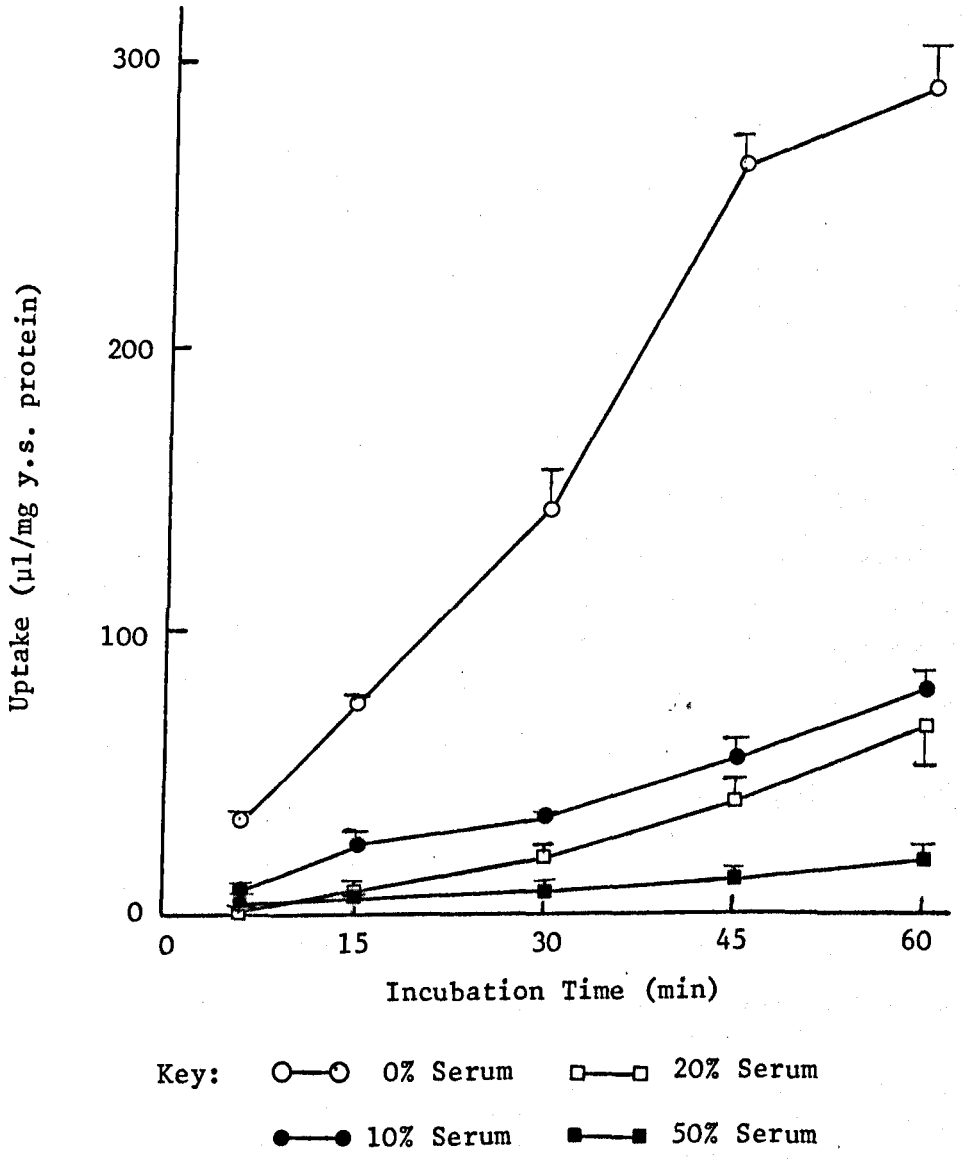
The quantity of radioactivity that becomes associated with yolk-sac tissue (tissue-associated radioactivity, T.A.R.) is expressed as mean values (± S.E.M.). These results are from the experiments shown in Figure 4.3.2c.

Figure 4.3.2g Effect of Calf Serum Concentration on the Disposal Time of ^{125}I-fdBSA after Uptake by Yolk Sacs



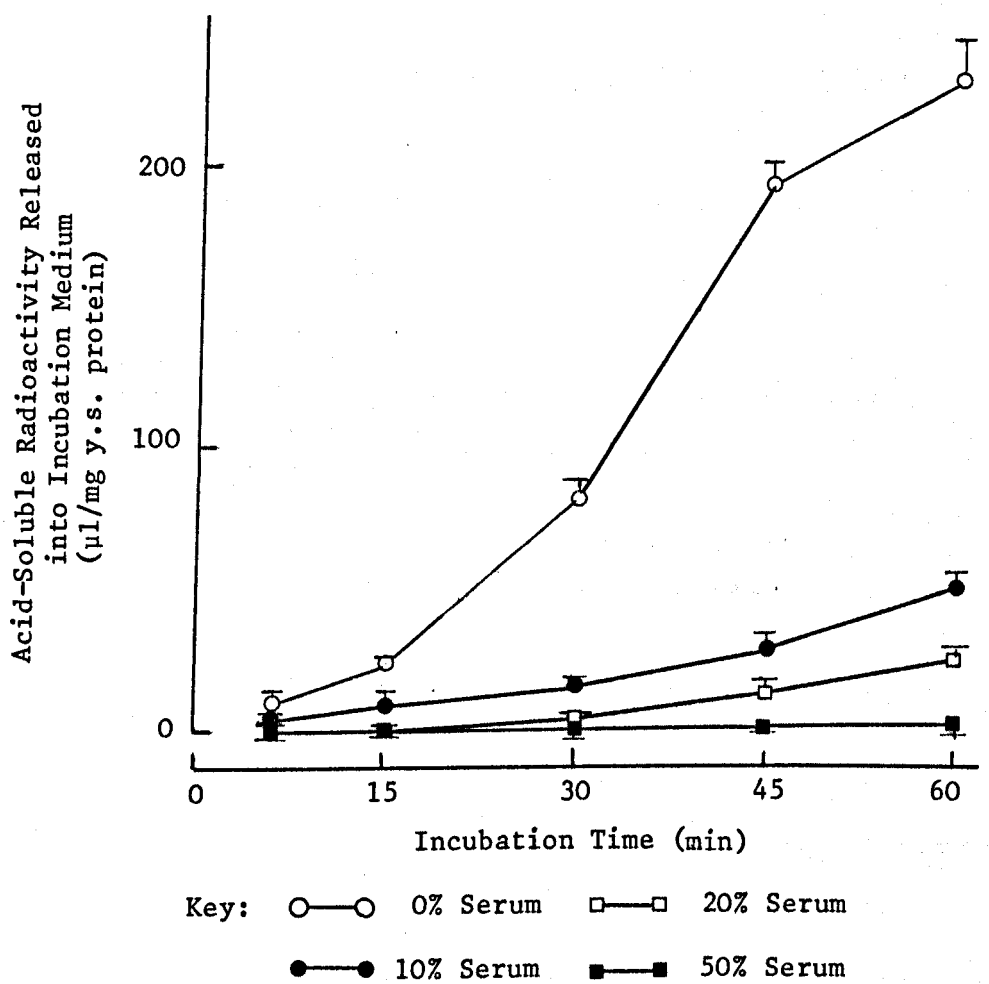
Disposal time is calculated as (T.A.R. x period of incubation (h)/ uptake), and it represents the mean time ^{125}I-fdBSA is associated with the tissue before acid-soluble fragments are released. These results are from the experiments shown in Figure 4.3.2c.

Figure 4.3.2h Effect of Calf Serum Concentration on the Uptake of ^{125}I-fdBSA by Yolk Sacs during the First Hour of Incubation



The method used was as described in Section 4.2. ^{125}I-fdBSA was present at a concentration of 1 µg/ml. Each uptake plot shows mean values (± S.E.M.) from 3 to 4 separate experiments. The uptake during 5h of incubation is shown in Figure 4.3.2c.

Figure 4.3.2i Effect of Calf Serum Concentration on the Release of Acid-Soluble Radioactivity into the Incubation Medium by Yolk Sacs during the First Hour of Uptake of ^{125}I-fdBSA.



The acid-soluble radioactivity released into the incubation medium is expressed as mean values (± S.E.M.). These results are from the experiments shown in Figure 4.3.2h. The acid-soluble radioactivity released into the incubation medium during 5h of incubation is shown in Figure 4.3.2e.

4.4 Discussion

The effect of altering the composition of the incubation medium on pinocytosis in the yolk sac was investigated both by changing the non-macromolecular components of the medium and by altering the amount of serum present. First, the effect of changing the composition of the non-macromolecular components of the medium in which yolk sacs were incubated on the rate of fluid-phase pinocytosis was examined. The uptake data for ^{125}I-PVP were more scattered when yolk sacs were incubated in either Minimal Essential Medium or Earle's Salts rather than in Medium 199, indicating that incubations in the more complex medium may yield more reproducible results. However, substituting either Earle's Salts or Minimal Essential Medium for Medium 199 had very little effect on the rate of fluid-phase pinocytosis in yolk sacs, despite the yolk sacs experiencing "step-down" conditions. Perhaps the yolk sac contains sufficient reserves of most major nutritional compounds to allow it to maintain its usual pinocytic rate over a 6 hour incubation period. However, yolk sacs incubated in physiological saline showed no evidence of pinocytosis. Evidently tissues require the presence of certain components in the medium for pinocytosis to occur. Extracellular calcium ions, for example, have been shown to be required for pinocytosis in both the rat yolk sac (Duncan & Lloyd, 1978) and the rat peritoneal macrophage (Pratten & Lloyd, 1979). By adding various compounds to a basal fluid such as physiological saline in which yolk sacs are incubating, it may be possible to identify some of the components that are essential for ongoing pinocytic activity.

Secondly, the effect of "step-up" conditions on the rate of fluid-phase pinocytosis in the yolk sac was investigated by increasing the concentration of serum in the incubation medium. It was found that increasing the proportion of serum in the incubation medium led to a

fall in the rate of uptake of ^{125}I-PVP by the yolk sac. These data extend the observation of Ibbotson & Williams (1979) that addition of serum (10%, v/v) to Medium 199 induces a fall in the Endocytic Index of the tissue. However, addition of serum to medium 199 will dilute the concentrations of some of the components of the medium, and at a dilution of 50% [i.e. in the presence of 50% (v/v) serum] this may be important. One could postulate that the fall in the pinocytic rate may be due to a decrease in the concentration of some of these components, rather than an increase in the amount of serum. It seems unlikely that the dilution of some of the more complex components of the medium will affect the rate of pinocytosis of the tissue, since incubation of the yolk sacs in Earle's Salts or Minimal Essential Medium had no detectable effect on the rate of fluid-phase pinocytosis. But it is possible that a dilution of some of the low-molecular weight compounds may have occurred (despite the presence of many organic and inorganic constituents provided by the serum), and these may influence the rate of uptake of ^{125}I-PVP. [This could easily be investigated by using a more concentrated stock solution of Medium 199 so that dilution with serum will always result in a concentration of each of its components that is at least equal to that in single-strength Medium 199.]

Finally, the effect of "step-up" conditions on the rate of adsorptive pinocytosis in the yolk sac was examined by measuring the effect of serum on the rate of uptake of ^{125}I-fdBSA. It was found that increasing the serum concentration led to a decrease in the E.I. of ^{125}I-fdBSA. However, the fall in the rate of uptake of the ^{125}I-labelled protein was proportionately much greater than the fall in the rate of uptake of ^{125}I-PVP, so that the effect could not be explained entirely in terms of a decrease in the rate of fluid uptake,

and suggests that there is competition between ^{125}I-fdBSA and the serum proteins for binding sites on the surface of the plasma membrane.

When the serum concentration was very high, not only did the rate of pinocytic uptake fall, but the quantity of radioactivity associated with the tissue (the T.A.R. value) increased with time, as did the disposal time. The last two of these changes are indicative of large amounts of protein reducing the capacity of the tissue to degrade the radiolabelled protein, and several alternative suggestions can be proposed to explain these findings. First, the presence of large amounts of protein may inhibit the fusion of pinosomes with lysosomes. Second, the total amount of protein (labelled and unlabelled) accumulated within the lysosomes may be too great for the lysosomal enzymes to cope with, so that the degradative step, rather than pinocytosis, becomes rate-limiting in the overall process of uptake and digestion. Third, the lysosomal enzymes may degrade the unlabelled serum proteins in preference to the radiolabelled protein. Finally, the degradation of large amounts of protein may increase the intracellular levels of amino acids, thereby reducing the need for the pinocytic capture of extracellular proteins. One could therefore envisage that elevated pools of intracellular amino acids constitute a negative feedback signal to inhibit pinocytosis. It is therefore interesting to extend the findings by investigating the effects of varying the incubation medium composition on the rate of degradation of endogenous proteins in the yolk sac. If a fall in the rate of pinocytosis results in a loss of nutritional components (e.g. amino acids) entering the cell, the cell may respond by increasing the rate of degradation of endogenous proteins as a short-term solution to a loss of an essential nutrient. Alternatively, since pinocytosis and autophagy (possibly an important route of degradation of endogenous proteins in some cells) are both membrane-related phenomena, then a stimulus

that causes a fall in the pinocytic rate may also cause a fall in the rate of degradation of endogenous proteins. (Some homeostatic mechanism containing elements of both of these extremes may also be envisaged.)

The data on the effect of different media composition on the rate of degradation of endogenous protein in the rat yolk sac were compiled by F.J. Ballard and S.E. Knowles in a collaborative study, and their data are shown in Table 4.4a. A comparison of the control data for each set of results shows that the mean rate of protein degradation is greater with yolk sacs incubated in Earle's Salts than in Minimal Essential Medium. This finding is in agreement with findings with other cell types (see Section 4.1) which show that degradation of endogenous proteins increases under "step-down" conditions. The addition of increasing amounts of fetal calf serum led to a decrease in the rate of degradation of endogenous protein. Adding formaldehyde-denatured BSA also led to a decrease in the rate of degradation of endogenous protein, although the extent of the inhibition was approximately the same on addition of 0.5 and 3.0 mg/ml, presumably because the effect was maximal at 0.5 mg/ml; addition of native BSA had no inhibitory effect upon the rate of degradation of endogenous protein. Addition of amino acids decreased the rate of degradation of endogenous protein and the effect of amino acids at twice the normal concentration was the same as that at five-times the normal concentration. Neely *et al.* (1977) found that addition of amino acids to perfusedrat livers caused a reduction in total proteolysis, and at both four-and ten-times the physiological concentration, the extent of inhibition was the same; this inhibition was to fifty per cent of the control value. The effects of these components on the rate of degradation of endogenous protein in rat yolk sacs generally follow the predicted results for "step-up"

conditions. These effects are very small, but are none-the-less consistent. It is also important to bear in mind that if the proportion of the degradation of endogenous protein which is sensitive to changes in the extracellular medium is lysosomal and is of the order of 20-30% of the total (as discussed in Section 4.1), then this represents a substantial inhibition of this lysosomal component.

In summary, the maximal rate of fluid-phase pinocytosis was observed in serum-free medium 199: the effect of "step-up" conditions (i.e. increased serum concentrations) on the rat yolk sac was to reduce the rate of both fluid-phase and adsorptive pinocytosis. The reduction in the rate of pinocytosis was accompanied by a fall in the rate of degradation of exogenous proteins following their accumulation by the tissue, implying an inhibition of the intralysosomal digestive capacity of the yolk sac. If the fall in the rate of degradation of endogenous proteins within the tissue arises from a reduction in the rate of intralysosomal digestion (effectively resulting from a fall in the rate of autophagy), then the stimulus that induced a fall in the rate of pinocytosis (possibly the intracellular levels of amino acids) may also have induced a fall in the rate of autophagy by virtue of them both being membrane-related processes. Such a stimulus, however, appears to elicit a differential level of response in these two membrane processes.

Table 4.4a Effect of Fetal Calf Serum, Amino Acids and BSA on Intracellular Protein Degradation by Yolk Sacs Incubated in Different Serum-Free Media (F.J. Ballard & S.E. Knowles: Unpublished Data)

The method used was as described in Section 4.2. Yolk sacs were incubated in Minimal Essential Medium (M.E.M.) or Earle's Salts (E.S.), and the percentage degradation of intracellular proteins is expressed as the mean over a 4h period of 3 to 4 separate determinations. BSA was added either in its native form, or after being denatured with formaldehyde (fdBSA).

Medium	Addition	Mean Protein Degradation (%)	Mean Protein Degradation as % of Control (%)
M.E.M.	None (Control)	15.67	100.0
	Fetal Calf Serum (1%)	15.41	98.3
	Fetal Calf Serum (10%)	14.66	93.6
M.E.M.	None (Control)	13.55	100.0
	fdBSA (0.5 mg/ml)	12.76	94.1
	fdBSA (3.0 mg/ml)	12.70	93.7
	Native BSA (3.0 mg/ml)	13.30	98.2
E.S.	None (Control)	14.74	100.0
	fdBSA (0.5 mg/ml)	13.56	92.0
	fdBSA (3.0 mg/ml)	13.96	94.7
	Native BSA (3.0 mg/ml)	14.96	101.5
M.E.M.	None (Control)	15.78	100.0
	Amino Acids (x2)	14.67	93.0
	Amino Acids (x5)	14.65	92.8
E.S.	None (Control)	16.27	100.0
	Amino Acids (x2)	14.52	89.2
	Amino Acids (x5)	14.40	88.5

CHAPTER 5

THE EFFECTS OF INSULIN AND ANTIBIOTICS ON PINOCYTOSIS AND PROTEIN DEGRADATION IN THE RAT VISCERAL YOLK SAC

5.1 Introduction

Both *in vivo* and *in vitro*, cells are greatly influenced by the composition of their extracellular environment. For example, in the yolk-sac system the presence or absence of a variety of nutrients has been found to be of great importance in influencing both the rate of pinocytosis of exogenous substrates and the subsequent rate of digestion of ^{125}I-labelled exogenous proteins; the rate of degradation of [^{3}H]leucine-labelled endogenous protein is likewise affected (see Chapter 4). Hormones have been found to influence the rate of degradation of endogenous protein, for example a lot of work has been done on the effect of glucagon on the rate of digestion of endogenous protein by *in vitro* cell systems. Using electron microscopy Shelburne *et al.* (1973) demonstrated that intraperitoneal injection of either cyclic AMP or dibutyryl cyclic AMP induced a wave of autophagy in rat liver which closely resembled that produced by glucagon (5'-AMP failed to produce a wave of autophagy), and they presented this as preliminary evidence that glucagon-stimulated autophagy is mediated, at least in part, by cyclic AMP. Hopgood *et al.* (1980) augmented these findings by measuring the effect of glucagon and dibutyryl cyclic AMP on the rate of proteolysis in rat hepatocyte monolayer cultures; proteolysis was stimulated by both of these agents, and, since their effects were not additive, it was proposed that glucagon may act via cyclic AMP to stimulate cellular autophagy and thus increase proteolysis. The effect of glucagon on autophagy in perfused rat livers has been further investigated by Schworer *et al.* (1979) who found that glucagon was capable of affecting the distribution of lysosomal markers by enhancing lysosomal density. Schworer & Mortimore (1979) investigated the effect of glucagon on autophagy and proteolysis in perfused rat livers, and found that the ability of glucagon to increase autophagy and protein degradation was only elicited at certain amino acid concentrations; their results indicated that this effect was mediated by an hormonally-

induced depletion of the amino acids glycine, alanine, glutamate and glutamine. They have therefore expanded the hypothesis proposed above to suggest that glucagon acts on gluconeogenesis, amino acid transport and metabolic interconversions, leading to an intracellular amino acid depletion which induces autophagy, and results in an increase in endogenous protein digestion.

Another hormone that has been extensively studied for its effect on the rate of endogenous proteolysis *in vitro* is insulin. Ballard and co-workers (Ballard *et al.*, 1980; Gunn *et al.*, 1980) have found a consistent and marked inhibition of endogenous protein breakdown by insulin in a variety of normal and transformed cell lines in culture. Libby & Goldberg (1981) found the same pattern in cultured chick-embryo muscle cells, Pfeifer (1977) presented electron microscopic evidence for inhibition of autophagic vacuole formation by insulin in rat liver *in vivo* (following an intraperitoneal injection of the hormone) and both Amenta & Brocher (1980a) and Marzella *et al.* (1980), working on rat embryo fibroblasts and rat liver respectively, found that insulin blocked induced proteolysis, but had little or no effect on basal and non-inducible proteolysis. Amenta & Brocher also found no "catch up" proteolysis when cultures in "step-down" medium containing insulin were transferred to fresh insulin-free medium, and there was no accumulation of autophagic vacuoles in insulin-treated cells. This is further evidence that insulin inhibits endogenous proteolysis by inhibiting the formation of autophagic vacuoles. Hopgood *et al.* (1980) demonstrated that insulin decreased the rate of proteolysis in cultured hepatocyte monolayers *in vitro*. They found that insulin decreased glucagon-stimulated proteolysis, but not the glucagon-elevated intracellular cyclic AMP concentration. Since glucagon-induced inhibition of autophagy is believed to be mediated via cyclic AMP, it was concluded that the

inhibition of proteolysis by insulin was independent of cyclic AMP. (They also found that the continuous presence of either insulin or glucagon was necessary for full expression of the effects.) However, such studies do not always show a strong insulin effect since Woodside & Massaro (1979) found no effect of insulin on the rate of degradation of either short or long half-life proteins in rabbit pulmonary macrophages.

Other growth factors have also been shown to influence endogenous protein degradation in vitro. Gunn et al. (1980) demonstrated a suppression of endogenous proteolysis in normal and transformed monolayer cell cultures with epidermal growth factor (EGF) and fibroblast growth factor (FGF). This study was expanded to include insulin-like growth factor-I (IGF-I) and insulin-like growth factor-II (IGF-II) [Ballard et al., 1980].

Another group of substances, some of which have been shown to have an effect upon endogenous protein degradation are the antibiotics. Schwarze & Seglen (1981) showed that streptomycin, penicillin and Garamycin (a mixture of gentamycins and antioxidants) all inhibited protein synthesis in primary rat hepatocyte cultures at concentrations above 0.1 mM. However, the extent of inhibition of proteolysis differed for each antibiotic. Penicillin was found to be effective at inhibiting protein degradation at concentrations above 0.1 mM, whereas streptomycin and Garamycin showed only slight inhibition at concentrations greater than 1 mM. It was not possible to comment on the mechanism of proteolytic inhibition since no attempt was made to distinguish lysosomal from non-lysosomal degradation.

Another antibiotic that has been studied to a greater extent is cycloheximide. This well-known inhibitor of protein synthesis has been examined for its effect upon endogenous protein degradation, and there are now many reports of this antibiotic inhibiting proteolysis.

For example, Hopgood et al. (1980) have shown that cycloheximide can inhibit protein breakdown in hepatocyte monolayers. The decreased proteolytic capacity found in the presence of the antibiotic was still responsive to glucagon but not to insulin; this gave support to the suggestion that cycloheximide inhibits autophagocytosis. Amenta et al. (1978) demonstrated that, in cultured rat embryo fibroblasts, cycloheximide depressed protein synthesis by up to eighty per cent and proteolysis by up to seventy per cent. The inhibition of basal proteolysis was not immediate but gradually increased over twenty-four hours, whereas the inhibition of induced protein degradation (thought to be effected by increased activity of the autophagic-lysosomal system) was almost immediate. They also measured the level of activity of three different lysosomal enzymes during the course of these experiments, and found them to vary independently of each other. From their data they postulated that the cell proteinases are inactivated by a mechanism that is not sensitive to cycloheximide, and that under such conditions the new steady-state levels of the enzymes remain exactly proportional to the level of protein synthesis within the cells; they state that the proposed mechanism relates protein synthesis to protein breakdown.

The addition of hormones or antibiotics to the extracellular medium in vitro poses several questions. For example, how do they gain entry into the cell, and what effect do they have on cellular processes (other than protein synthesis and degradation) such as endocytosis? Some of the antibiotics are fairly large molecules and are probably too large to enter the cell by diffusion so may well be taken up partly or even completely by endocytosis (fluid-phase or adsorptive). Insulin is known to enter cells via receptor-mediated pinocytosis although its fate, once inside the cell, is under dispute. Carpentier et al. (1979) produced evidence that ^{125}I-labelled insulin

was localized on the plasma membrane of isolated rat hepatocytes, but was subsequently internalized and became preferentially associated with lysosomes. This finding was supported by Marshall & Olefsky (1979, 1980) who examined the effects of two lysosomotropic agents (chloroquine and NH_4Cl) on the interaction between ^{125}I-labelled insulin and rat adipocytes. They presented data which indicated that the ^{125}I-labelled insulin was taken into the tissue by an energy-dependent mechanism, possibly pinocytosis, and,when lysosomal function was inhibited, the radiolabelled substrate accumulated intracellularly. (In the absence of inhibitors, degradation products were normally detected within five minutes of endocytosis occurring). They suggest their data indicate that insulin is degraded by a lysosomal pathway. However, other workers in this field have obtained evidence to suggest that internalized ^{125}I-labelled insulin does not become associated with lysosomes in the cell. Three such studies have involved intravenous injection of ^{125}I-labelled insulin into rats, followed by rapid excision of the liver and examination of the sub-cellular distribution of the ^{125}I-labelled hormone (Desbuquois et al., 1979a,b; Papachristodoulou et al., 1979). Each group found that the radiolabelled substrate became associated with the microsomal fraction and that little or no radioactivity became associated with the mitochondrial-lysosomal fraction; however radioactivity was released into the cytosol as ^{125}I-labelled monoiodotyrosine. Bergeron et al. (1979) also studied capture of ^{125}I-labelled insulin by rat liver following its injection, and followed its fate by electron microscope autoradiography. After 3 min the insulin was found bound to the hepatocyte plasmamembrane, and then later associated with the Golgi. At ten minutes both secretory elements of the Golgi and lysosome-like vacuoles were labelled, although the latter did not stain for acid phosphatase and were presumably not lysosomal. These three studies, in contrast to the ones presented above, do not suggest a

prominent role for lysosomes in the degradation of ^{125}I-labelled insulin.

The effects of hormones and antibiotics on the endocytic activity of tissues has been studied to a limited extent. Brown & Segal (1977) and Livesey (1978) examined the effect of glucagon (10^{-11}M to 10^{-6}M) on the rate of pinocytosis in the rat yolk sac. Uptake of both ^{125}I-PVP (Livesey, 1978) and yeast invertase (Brown & Segal, 1977) were inhibited strongly by glucagon; rates of uptake were 30% of those of controls in the presence of 10^{-6}M glucagon; the uptake of formaldehyde-denatured ^{125}I-labelled BSA was similarly decreased. Haigler et al. (1979) studied the effect of epidermal growth factor (EGF) on the rate of fluid-phase pinocytosis (as measured by uptake of horseradish peroxidase) in human carcinoma cells. They reported that addition of low concentrations of EGF produced a ten-fold increase in the rate of pinocytosis within thirty seconds, but that this effect was transient, the pinocytic rate returning to its control value within fifteen minutes.

The effect of tunicamycin (an antibiotic that specifically inhibits the formation of N-acetylglucosaminylpyrophosphorylpolyisoprenol, a precursor of oligosaccharide side chains N-glycosidically-linked to a protein core) on the transport of lysosomal enzymes in cultured skin fibroblasts has been investigated (von Figura et al,,1979). It was found that tunicamycin did not affect receptor-mediated endocytosis in a general manner, but instead inhibited uptake of β-N-acetylglucosaminidase that had been formed in the presence of tunicamycin. From this they deduced that tunicamycin interfered with receptor-mediated pinocytosis by influencing the expression of phosphomannose receptors on the cell surface of fibroblasts and the synthesis of phosphomannosyl residues on lysosomal enzymes.

Another antibiotic that has been studied for its effect upon

pinocytosis is bacitracin (Maxfield et al., 1979; Davies et al., 1980; Dickson et al., 1981). They found that bacitracin (0.7 mM) inhibited clustering of rhodamine-labelled epidermal growth factor or α_2-macroglobulin in chinese hamster ovary (CHO) cells and BALB/c3T3 cells. At a concentration of 2 mg/ml, bacitracin was found to be an inhibitor of α_2-macroglobulin clustering in rat kidney fibroblasts, CHO and 3T3 cells. They looked for a correlation between inhibition of transglutaminase activity and inhibition of clustering and subsequent internalization of α_2-macroglobulin; their observations indicated that a transglutaminase is required for receptor-mediated endocytosis of certain ligands. This model was expanded to accommodate the observation that bacitracin directly blocks binding to high-affinity sites in plasmamembranes of cultured fibroblasts, and it is suggested that a transglutaminase-like activity is required for conversion of low affinity receptors to high affinity receptors in the membrane (the data did not support a model in which bacitracin affects only receptor recycling).

It is conceivable that endocytically-active cells normally gain appreciable quantities of amino acids either by ingesting free amino acids by fluid-phase pinocytosis or by degradation within lysosomes of proteins captured by adsorptive pinocytosis. Inhibition of endocytosis could therefore produce a similar effect to a "step-down" change in the incubation medium. Both manipulations may result in a fall in the intracellular levels of amino acids in some common pool. A fall in the levels of certain amino acids could well act as a signal to trigger either an increase in autophagy or a general increase in the degradation of endogenous proteins to restore the amino acid levels to normal. There may thus be reason to anticipate an inverse relation between inhibition

of pinocytosis and the degradation of endogenous proteins if this "nutritional hypothesis" is valid. Conversely, as lysosome reduction and autophagy are membrane-related events, then agents that interfere with the mechanism of pinocytosis may also have a similar effect on these processes. Thus agents that interfere with endocytosis would also be expected to reduce the rate of turnover of endogenous proteins. Observation of a parallel decrease in the degradation of endogenous protein and the ingestion of exogenous protein would be compatible with this "membrane-mediated hypothesis".

Both hormones and antibiotics are important constituents of the cellular environment _in vivo_ and _in vitro_ respectively, and thus serve as obvious agents to investigate for their effects on endocytosis and intracellular proteolysis and to test the above hypotheses. The rat yolk-sac system is an excellent system for quantitating pinocytosis and has also been used to determine the rates of degradation of endogenous proteins (Chapter 4). Therefore a collaborative investigation was instigated with F.J. Ballard and S.E. Knowles (see Chapter 4) to see if there was any effect of these agents on rates of fluid-phase pinocytosis and intracellular protein degradation in rat yolk sacs.

Insulin was chosen for investigation of its effects upon pinocytosis and proteolysis of exogenous proteins since glucagon, the agonist of insulin, had already been investigated in this system (see above). F.J.B. and S.E.K. measured the effects of both of these hormones on the rate of endogenous proteolysis in rat yolk sacs. It is also of great importance to examine the effects of antibiotics because they are common constituents of virtually all of the commonly-used cell culture media, and it is desirable to know whether, at the concentrations at which they are normally used, they greatly affect cellular processes so that _in vitro_ cell culture results may be interpreted with confidence.

Cycloheximide was one of the compounds chosen for study since it has already been shown to be an inhibitor of endogenous protein degradation in a variety of cell types (see above). Another antibiotic (which is well-known because of its mechanism of inhibition of protein synthesis), puromycin, was studied to see if its effects are similar to those of cycloheximide. Again, studies of the rates of fluid-phase pinocytosis in the rat yolk sac were complemented by measurements of the rates of endogenous protein degradation within yolk sacs, made by F.J.B. and S.E.K. (Data obtained by F.J.B. and S.E.K. will be presented in Section 5.4.) The final antibiotic chosen for study was bacitracin. This cyclic dodecapeptide antibiotic was selected since it is reported to have an effect on receptor-mediated pinocytosis by inhibiting receptor clustering for certain ligands (see above), and it is also known to inhibit various proteolytic enzymes (Makinen, 1972). Using the rat yolk-sac system the effects of bacitracin both on the rate of fluid-phase pinocytosis (as determined by the rate of uptake of ^{125}I-PVP) and on the rate of adsorptive pinocytosis of formaldehyde-denatured ^{125}I-labelled BSA were established, to see if bacitracin interfered either with uptake or with the intralysosomal degradation of this protein.

5.2 Materials and Methods

All the equipment and materials used in the experiments in this chapter were as described in Section 2.1. Formaldehyde-denatured ^{125}I-labelled BSA (^{125}I-fdBSA) was prepared as described in Section 2.2.2. All the uptake experiments were performed as described in Section 2.3, and all assays as in Section 2.4. Medium 199 was used throughout, and all experiments were performed in the absence of serum. ^{125}I-PVP was always used at a concentration of 2 µg/ml and ^{125}I-fdBSA at a concentration of 1 µg/ml. The various agents were added 20 min prior to the addition of ^{125}I-labelled substrate, and the concentrations employed were as described in the text and in the legends to figures and tables.

In Sections 5.3.2 and 5.3.3, the measurement of the recovery of ^{125}I-PVP uptake by yolk sacs after exposure to various agents was as described in Section 2.12. In Section 5.3.3, the effect of bacitracin on the release of ^{125}I-PVP from yolk sacs was measured as described in Section 2.10.

The method of F.J. Ballard and S.E. Knowles for measuring the rate of endogenous protein degradation in the rat yolk sac (see Tables 5.4a and b) was as described in detail by Livesey *et al.* (1980) and as outlined in Section 4.2. The concentrations of the various agents used, and the period of preincubation (if appropriate) were as described in Tables 5.4a and b.

5.3.1 Effect of Insulin on the Uptake of ^{125}I-PVP and ^{125}I-fdBSA by Yolk Sacs

The effect of the hormone insulin on the rate of fluid-phase pinocytosis in the rat yolk sac was investigated by measuring the rate of uptake of ^{125}I-labelled PVP (^{125}I-PVP). The uptake of ^{125}I-PVP with time in the presence of a range of concentrations of insulin (from 10^{-13} to 10^{-6}M) in serum-free medium 199 is shown in Figure 5.3.1a. The uptake plots were linear with time under all the conditions tested, and in the presence of the higher insulin concentrations, there appeared to be a slight inhibitory effect upon fluid-phase pinocytosis. (Error bars are not shown on this graph; their presence would introduce unnecessary confusion since most of them would overlap.) The values of the rates of uptake (Endocytic Indices) of ^{125}I-PVP in the presence of different concentrations of insulin are shown in Table 5.3.1a. The mean E.I. in the absence of any insulin was 3.15 ± 0.19 µl/mg yolk-sac protein per h, which compared well with values found previously (see Chapters 3 and 4). Addition of insulin to the incubation medium up to a concentration of 10^{-11}M caused virtually no change in the rate of uptake of ^{125}I-PVP; at a concentration of 10^{-11}M the mean E.I. was 89 ± 8% of the control value in the absence of insulin. Further increasing the insulin concentration up to 10^{-6}M led to a fall in the mean E.I. to 72 ± 5% of the control value.

The effect of insulin on the rate of adsorptive pinocytosis in rat yolk sacs was determined by following its effect on the rate of uptake of formaldehyde-denatured ^{125}I-labelled BSA (^{125}I-fdBSA). This protein was chosen since its fate in the rat yolk-sac incubation system has been well documented, and it is known to be degraded intralysosomally; this has been discussed in Chapter 4. Results from Chapter 4 led to the conclusion that not only was the rate of uptake of

^{125}I-fdBSA affected by the presence of calf serum in the medium, but the rate of degradation within the lysosomal system was also affected. Therefore, in order to remove this complication from these experiments, all the incubations performed in this chapter (including Sections 5.3.2. and 5.3.3) were in serum-free medium 199. The effect of different insulin concentrations (from 10^{-11} to 10^{-8}M) on the uptake of ^{125}I-fdBSA in serum-free medium is shown in Figure 5.3.1b. As with ^{125}I-PVP, all the plots were linear, and the lower concentrations of insulin appear to have very little effect upon the rate of uptake of ^{125}I-fdBSA; in the presence of 10^{-6}M insulin there is some indication of a slight inhibitory effect. The rates of uptake (the gradients of these plots) are summarized in Table 5.3.1b. In the absence of insulin (the control) the mean E.I. was 77 ± 2 µl/mg yolk-sac protein per h. The addition of 10^{-11}M insulin to the medium resulted in no detectable change in this value. At the higher concentrations of insulin, the mean E.I. was 109 ± 8% of the control value in the presence of 10^{-8}M insulin, and 88 ± 3% in the presence of 10^{-6}M; this very slight inhibition at 10^{-6}M insulin was less than was found with ^{125}I-PVP. This finding (that insulin at a concentration of 10^{-6}M appeared to have a slightly greater relative inhibitory effect upon the rate of uptake of ^{125}I-PVP compared to that of ^{125}I-fdBSA) is illustrated in Figure 5.3.1c. The observation that the uptake of ^{125}I-PVP is more affected by increasing insulin concentrations than is the uptake of ^{125}I-fdBSA is not easy to explain. Competition between insulin and ^{125}I-fdBSA for binding sites is thought to occur (Livesey & Williams, 1982) but would result in the pattern being the opposite of that observed. A possible explanation of this effect is that insulin decreases the radius of the nascent pinosomes (without affecting the number formed in unit time). Since volume is proportional to r^3 whereas area is proportional

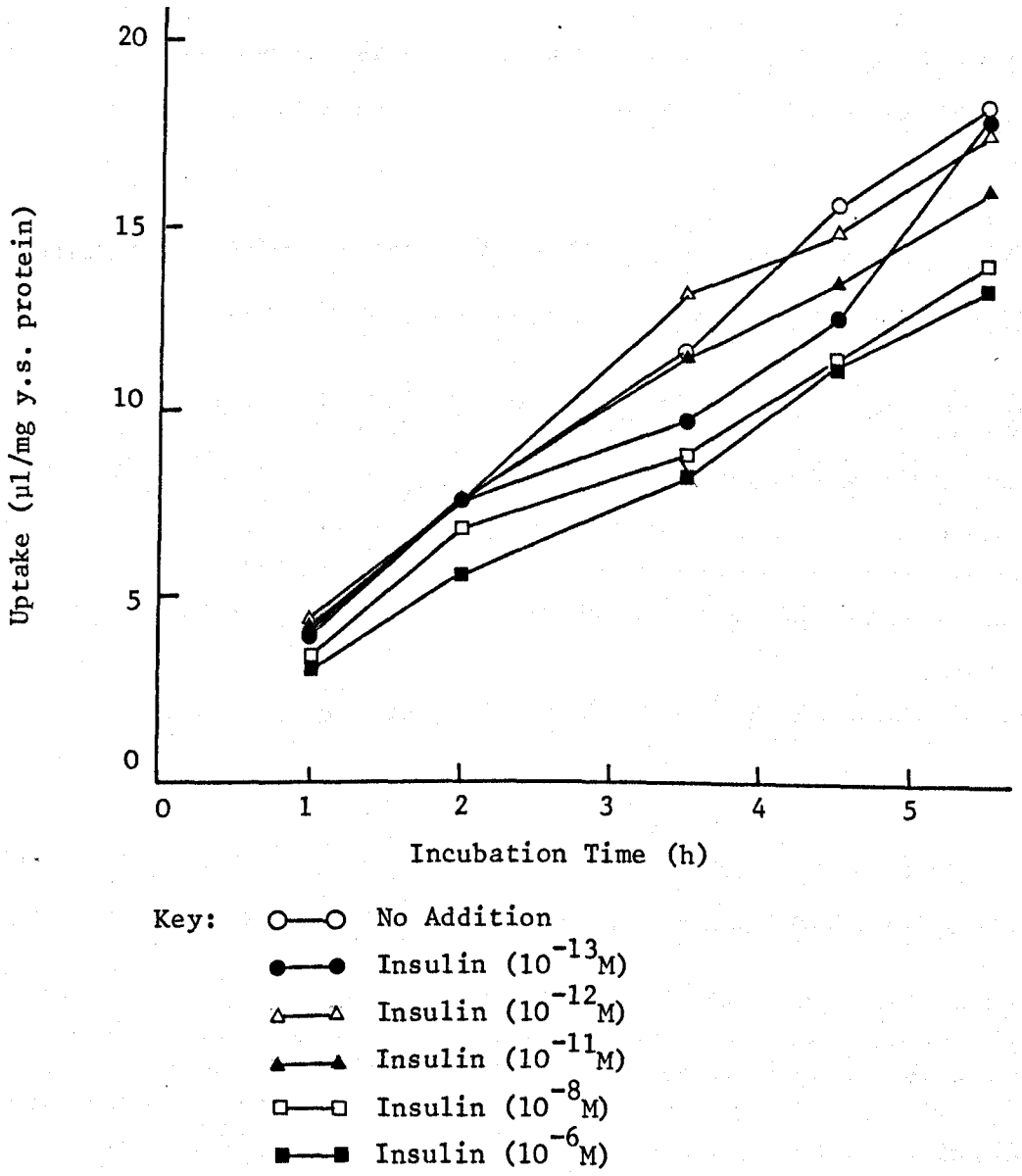
to r^2, it follows that uptake of ^{125}I-PVP (which is ingested in the fluid phase) will be more affected than uptake of ^{125}I-fdBSA (which is ingested almost entirely in the adsorptive phase). A 10% decrease in the pinosome radius would be expected to decrease fluid phase uptake to 73% of the control rate, whereas adsorptive uptake would fall to 81% of the control rate.

The rate of uptake of ^{125}I-fdBSA in the absence of serum (77 ± 2 µl/mg yolk-sac protein per h) was much less than was reported in Section 4.3.2, Table 4.3.2b (355 µl/mg yolk-sac protein per h). This was simply due to the fact that two different batches of ^{125}I-fdBSA were used for these two different sets of experiments. After labelling the protein with [^{125}I]iodide, the radiolabelled protein is denatured by exposure to formaldehyde solution (see Section 2.2) for a period of 72h, but the degree of denaturation is never constant. This is the reason for different batches of ^{125}I-fdBSA being taken up at different rates by the rat yolk sac (but always at a constant rate within one batch). This finding is consistent with the observations reported by Ibbotson & Williams (1979).

Following uptake of ^{125}I-fdBSA by the yolk sacs, the release of acid-soluble digestion products into the medium was measured and was found to be linear with time (results not shown in detail); the rate of their release is shown in Table 5.3.1b, and was found to be approximately the same as the mean E.I. for each insulin concentration studied. The quantity of radioactivity that became associated with the tissue (T.A.R. value) was approximately the same for each insulin concentration investigated, and showed no tendency to rise progressively during the incubation period (Figure 5.3.1d). The mean T.A.R. values (approximately 25 µl/mg yolk-sac protein) are reported in Table 5.3.1b. The mean time that the protein was associated with the tissue before its release as

acid-soluble fragments (the disposal time), after different periods of incubation, are shown in Figure 5.3.1e. As with the T.A.R. values, the mean disposal times (slightly over 0.3h) were unaffected by the presence of insulin. Mean disposal times for the protein in the presence of each insulin concentration differed little (Table 5.3.1b).

Figure 5.3.1a Effect of Insulin on the Uptake of ^{125}I-PVP by Yolk Sacs Incubated in Serum-Free Medium



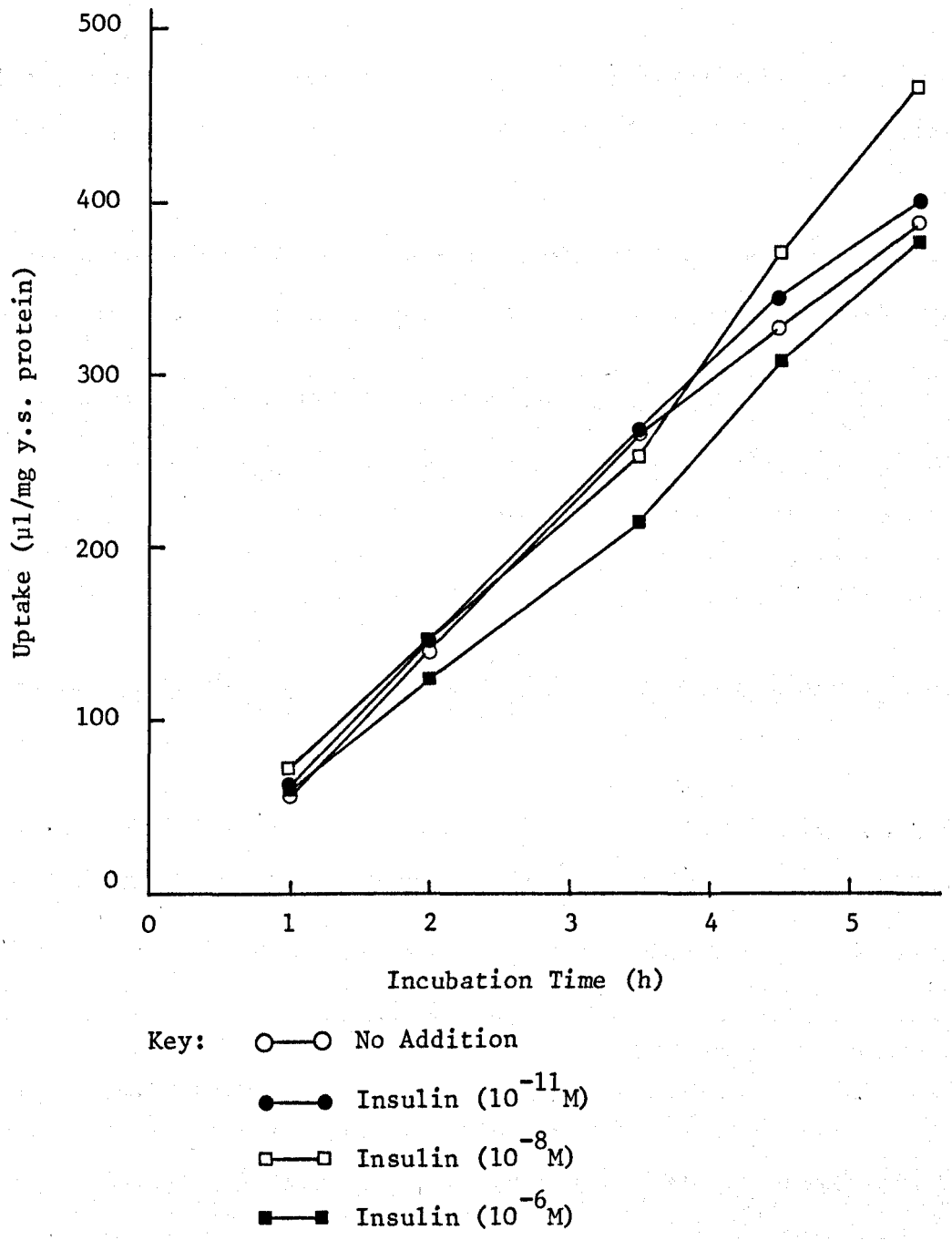
The method used was as described in Section 5.2. ^{125}I-PVP was present at a concentration of 2 µg/ml; insulin was added 20 min prior to this substrate. Each uptake plot shows mean values from 3 to 11 separate experiments; individual S.E.M. values (not shown) are less than 1.5. These results are summarized in Table 5.3.1a.

Table 5.3.1a Summary of the Effect of Different Concentrations of Insulin on the Uptake of ^{125}I-PVP by Yolk Sacs Incubated in Serum-Free Medium

These data summarize the results shown in Figure 5.3.1a. Each Endocytic Index (E.I.) is expressed as µl/mg yolk-sac protein per h, and is given with its Correlation Coefficient (Corr. Co.) in parentheses.

Insulin Conc. (M)	E.I.(Corr.Co.) (µl/mg y.s. protein per h)	Mean E.I.± S.E.M. (µl/mg y.s. protein per h)	Mean E.I. as % of Control (%)
No Addition (Control)	3.34 (0.978) 2.45 (0.973) 2.99 (0.990) 2.78 (0.975) 3.63 (0.983) 3.82 (0.996) 2.38 (0.957) 2.18 (0.975) 3.44 (0.983) 3.99 (0.992) 3.63 (0.981)	3.15 ± 0.19	100.0 ± 6.0
10^{-13}	1.86 (0.949) 2.92 (0.958) 3.83 (0.990)	2.87 ± 0.57	91.1 ± 18.1
10^{-12}	3.28 (0.981) 3.70 (0.991) 2.19 (0.965) 3.80 (0.955)	3.24 ± 0.37	102.8 ± 11.8
10^{-11}	2.46 (0.882) 2.56 (0.984) 1.96 (0.952) 2.91 (0.983) 2.48 (0.957) 3.71 (0.990) 3.60 (0.995)	2.81 ± 0.24	89.2 ± 7.9
10^{-8}	2.44 (0.969) 3.45 (0.992) 1.34 (0.859) 1.87 (0.878)	2.28 ± 0.45	72.4 ± 14.3
10^{-6}	2.19 (0.992) 2.36 (0.943) 1.79 (0.970) 2.72 (0.961) 2.31 (0.967)	2.27 ± 0.15	72.1 ± 4.8

Figure 5.3.1b Effect of Different Concentrations of Insulin on the Uptake of ^{125}I-fdBSA by Yolk Sacs Incubated in Serum-Free Medium



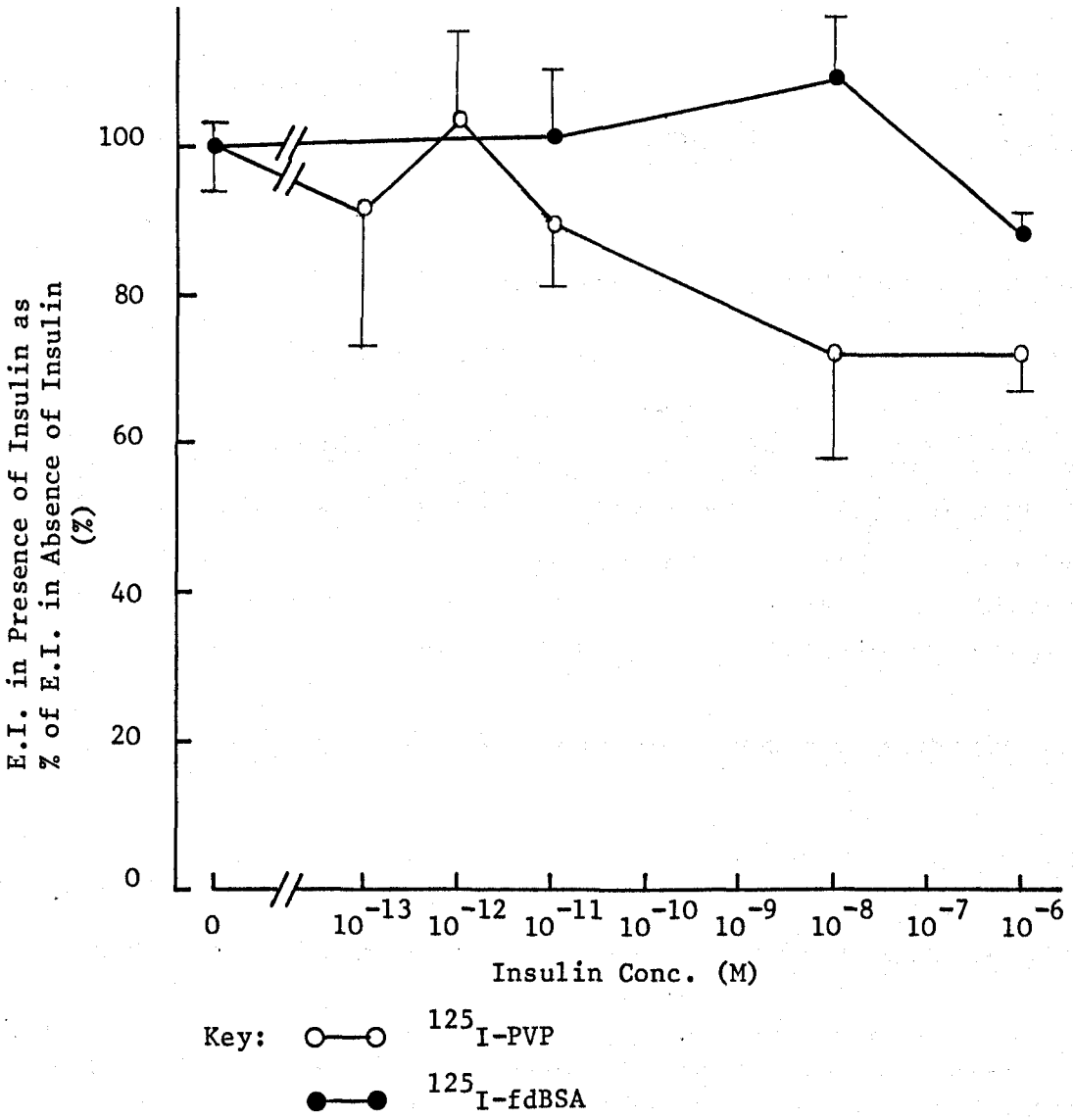
The method used was as described in Section 5.2. ^{125}I-fdBSA was present at a concentration of 1 µg/ml; insulin was added 20 min prior to this substrate. Each uptake plot shows mean values from 4 to 7 separate experiments; individual S.E.M. values (not shown) are all less than 30. These results are summarized in Table 5.3.1b.

Table 5.3.1b Summary of the Effect of Different Concentrations of Insulin on the Uptake of ^{125}I-fdBSA by Yolk Sacs Incubated in Serum-Free Medium

These data summarize the results shown in Figures 5.3.1b, d and e. Each Endocytic Index (E.I.) is expressed as µl/mg yolk-sac protein per h, and is given with its Correlation Coefficient (Corr. Co.) in parentheses. The relative effect of each insulin concentration on the E.I., expressed as a percentage of the control value (no addition of insulin), is given in parentheses beneath each mean E.I. value (± S.E.M.). The rate of release of acid-soluble radioactivity (Sol. Rel.) from the tissue is expressed in µl/mg yolk-sac protein per h), and the mean tissue-associated radioactivity value (T.A.R.) is expressed as µl/mg yolk-sac protein. The relative effect of each insulin concentration on the T.A.R. value, expressed as a percentage of the control value, is given in parentheses beneath each mean T.A.R. value. The mean disposal time was calculated from (mean T.A.R./mean E.I.) and has the units of h.

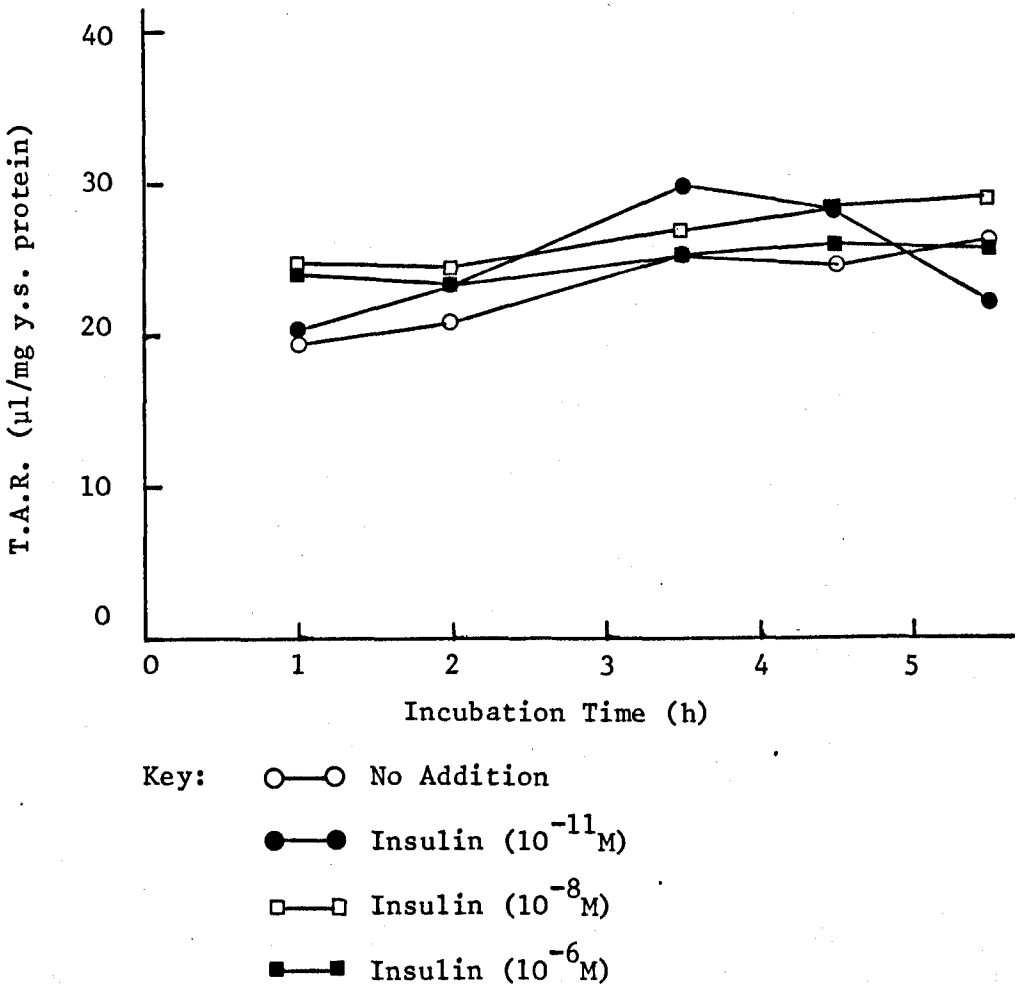
Insulin Conc. (M)	E.I.(Corr.Co.) (µl/mg y.s. protein/h)	Mean E.I. ± S.E.M. (µl/mg y.s. protein/h)	Rate of Sol. Rel. (µl/mg y.s. protein/h)	Mean T.A.R. (µl/mg y.s. protein)	Mean Disposal Time (h)
No Addition (Control)	72.21 (0.942) 76.36 (0.986) 85.07 (0.984) 80.52 (0.978) 73.02 (0.990)	77.44 ± 2.41 (100.0 ± 3.1)	72.47	23.23 (100.0)	0.30
10^{-11}	89.15 (0.991) 70.50 (0.982) 90.91 (0.990) 61.15 (0.981)	77.93 ± 7.25 (100.6 ± 9.4)	75.55	25.68 (110.1)	0.33
10^{-8}	84.23 (0.949) 86.76 (0.981) 97.98 (0.981) 69.66 (0.989)	84.66 ± 5.82 (109.3 ± 7.5)	86.37	26.05 (111.7)	0.31
10^{-6}	66.20 (0.973) 57.92 (0.974) 73.15 (0.997) 74.35 (0.980) 76.80 (0.981) 62.29 (0.990) 62.86 (0.981)	68.37 ± 2.56 (88.3 ± 3.3)	70.43	24.76 (106.2)	0.36

Figure 5.3.1c Effect of Different Concentrations of Insulin on the Relative Endocytic Indices of ^{125}I-PVP and ^{125}I-fdBSA by Yolk Sacs Incubated in Serum-Free Medium



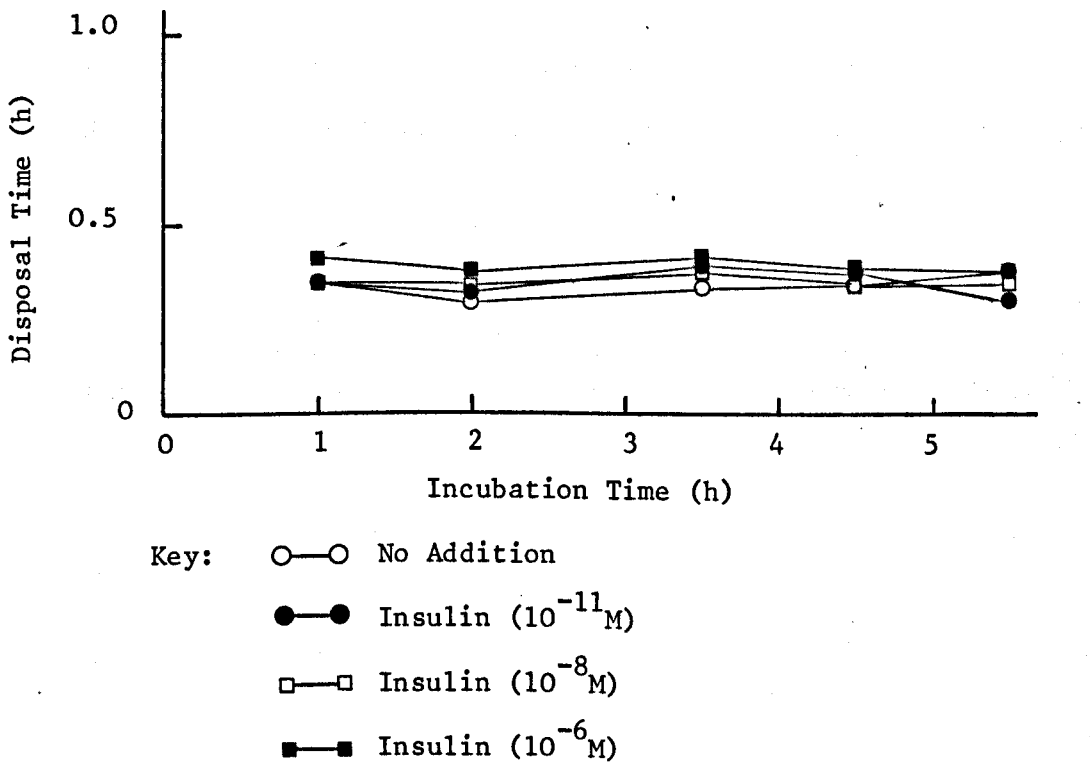
Each point represents the mean E.I. of the substrate at a particular insulin concentration expressed as a percentage of the mean control E.I. in the absence of insulin. These results are from the data summarized in Tables 5.3.1a and b.

Figure 5.3.1d Effect of Insulin on the Quantity of Radioactivity that becomes Associated with Yolk-Sac Tissue during Uptake of ^{125}I-fdBSA in Serum-Free Medium



The quantity of radioactivity that becomes associated with yolk-sac tissue (tissue-associated radioactivity, T.A.R.) is expressed as mean values; individual S.E.M. values (not shown) are less than 3. These results are from the experiments shown in Figure 5.3.1b.

Figure 5.3.1e Effect of Different Concentrations of Insulin on the Disposal Time of ^{125}I-fdBSA after Uptake by Yolk Sacs in Serum-Free Medium



Disposal time is calculated as (T.A.R. divided by the rate of uptake up to a given incubation time), and represents the mean time ^{125}I-fdBSA is associated with the tissue before acid-soluble fragments are released. These results are from the experiments shown in Figure 5.3.1b.

5.3.2 Effects of Cycloheximide and Puromycin on the Uptake of ^{125}I-PVP and ^{125}I-fdBSA by Yolk Sacs

The effects of the two antibiotic protein synthesis inhibitors, cycloheximide and puromycin, on the rates of fluid-phase and adsorptive pinocytosis in the rat yolk sac were investigated using the radiolabelled substrates ^{125}I-PVP and ^{125}I-fdBSA. The effects of these two agents (both at the concentration of 10^{-4}M) on the uptake of ^{125}I-PVP with time are shown in Figure 5.3.2a. Puromycin had no effect upon the rate of uptake of this fluid-phase marker, whereas cycloheximide had an inhibitory effect. Table 5.3.2a shows the Endocytic Index of ^{125}I-PVP under the different conditions. In the absence of any agent, the mean control E.I. was 2.95 ± 0.12 µl/mg yolk-sac protein per h, and in the presence of puromycin (10^{-4}M) the mean E.I. was 3.04 µl/mg yolk-sac protein per h (or 103 ± 7% of the control value). The presence of 10^{-4}M cycloheximide reduced the mean E.I. to 59 ± 8% of the control value.

The effects of cycloheximide and puromycin on the time course of uptake of ^{125}I-fdBSA is shown in Figure 5.3.2b. The effects of these two agents on uptake of this substrate were the same as their effects on ^{125}I-PVP; at the concentration tested, puromycin had no effect on the rate of uptake of the protein, but cycloheximide had an inhibitory effect. The values of the Endocytic Indices for individual determinations are reported in Table 5.3.2b. The mean E.I. in the absence of any agent was 74 ± 1 µl/mg yolk-sac protein per h, and in the presence of 10^{-4}M puromycin was 97 ± 5% of this control value. Addition of cycloheximide to the incubation medium resulted in a reduction of the mean E.I. to 51 ± 2% of the control value.

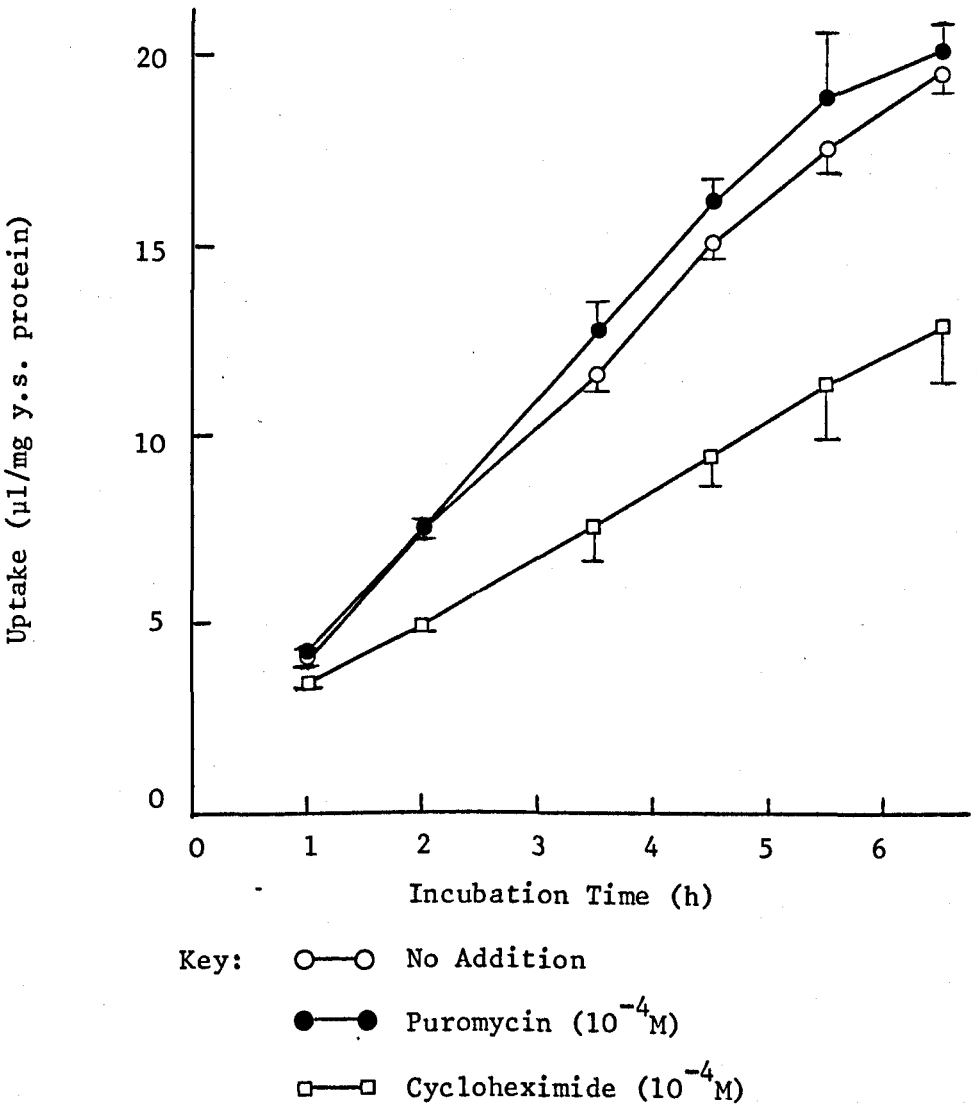
The production of acid-soluble fragments following intralysosomal digestion of the internalized ^{125}I-fdBSA was found to be linear with

time (results not shown in detail), and the rate of this release (as shown in Table 5.3.2b) was similar to the mean E.I. for each set of conditions. The quantity of radioactivity associated with the yolk-sac tissue at different time points during the incubation are illustrated in Figure 5.3.2c. Table 5.3.2b shows that the mean T.A.R. value in the absence of any agent and in the presence of puromycin were virtually the same, being 24 and 22 µl/mg yolk-sac protein respectively. In the presence of 10^{-4}M cycloheximide, the mean T.A.R. value fell to 51% of the control value; this fall corresponds exactly to the fall in mean Endocytic Index. The mean time during which the substrate is associated with the tissue before its release into the medium as acid-soluble fragments (the disposal time) was unaffected by either agent during the whole of the incubation period (see Figure 5.3.2d and Table 5.3.2b).

The ability of the yolk-sac tissue to recover its pinocytic capacity in serum-free medium following previous exposure to cycloheximide (10^{-4}M) was investigated, and the results are shown in Table 5.3.2c. During an initial exposure to cycloheximide, over a period of 2.5h, the rate of uptake of ^{125}I-PVP was reduced to 75% of the control value in the absence of inhibitor (this reduction was 15% less than was reported in Table 5.3.2a; this difference may be attributable to the smaller number of determinations in these recovery experiments). Washing the yolk sacs in incubation medium at 37°C had virtually no effect upon the rate of uptake of ^{125}I-PVP for a further 2.5h in the control experiments (3.69 and 3.45 µl/mg yolk-sac protein per h before and after washing, respectively), and the rate of uptake of ^{125}I-PVP by yolk sacs that had been washed following exposure to cycloheximide was 106% of the control value. Therefore the inhibitory effect of 10^{-4}M cycloheximide upon the rate of fluid-phase pinocytosis in the rat yolk sac is fully reversible

after exposure for a period of 2.5h.

Figure 5.3.2a Effect of Cycloheximide and Puromycin on Uptake of ^{125}I-PVP by Yolk Sacs Incubated in Serum-Free Medium



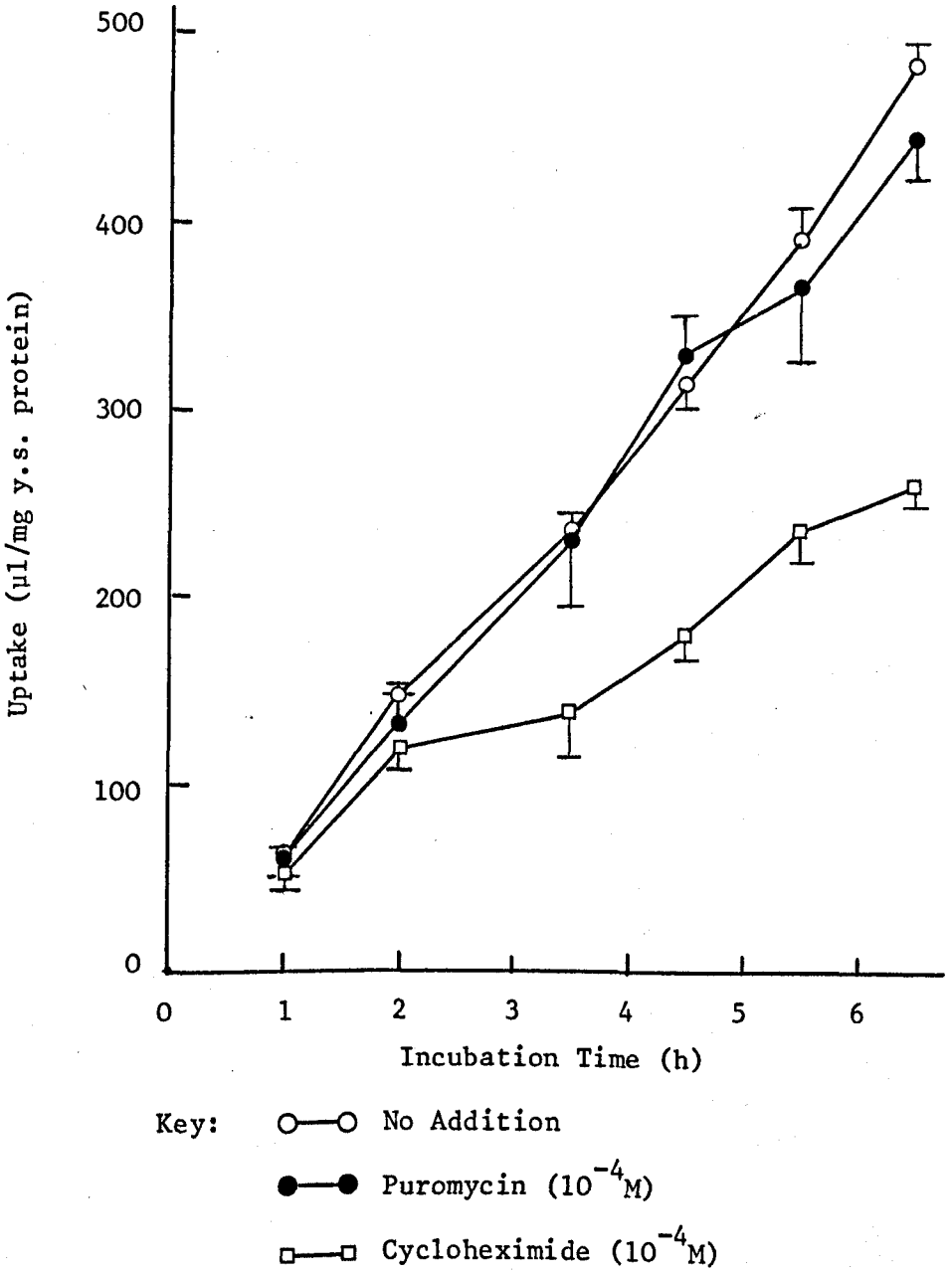
The method used was as described in Section 5.2. ^{125}I-PVP was present at a concentration of 2 µg/ml; either cycloheximide or puromycin were added 20 min prior to this substrate. Each uptake plot shows mean values (± S.E.M.) from 6 to 13 separate experiments. These results are summarized in Table 5.3.2a.

Table 5.3.2a Summary of the Effect of Cycloheximide and Puromycin on the Uptake of ^{125}I-PVP by Yolk Sacs Incubated in Serum-Free Medium

These data summarize the results shown in Figure 5.3.2a. Each Endocytic Index (E.I.) is expressed as µl/mg yolk-sac protein per h, and is given with its Correlation Coefficient (Corr. Co.) in parentheses.

Agent	E.I.(Corr. Co) (µl/mg y.s. protein per h)	Mean E.I. ± S.E.M. (µl/mg y.s. protein per h)	Mean E.I. as % of Control (%)
No Addition (Control)	3.05 (0.964) 2.65 (0.987) 2.68 (0.977) 3.09 (0.977) 2.39 (0.962) 3.04 (0.988) 3.37 (0.989) 2.40 (0.993) 3.06 (0.993) 3.84 (0.994) 3.34 (0.978) 2.45 (0.973) 2.99 (0.990)	2.95 ± 0.12	100.0 ± 4.1
Puromycin (10^{-4}M)	2.86 (0.968) 2.35 (0.977) 3.22 (0.981) 2.89 (0.972) 3.87 (0.961) 3.04 (0.959)	3.04 ± 0.20	103.1 ± 6.8
Cycloheximide (10^{-4}M)	2.43 (0.945) 1.40 (0.964) 1.23 (0.987) 1.35 (0.947) 1.46 (0.970) 2.48 (0.986)	1.73 ± 0.23	58.6 ± 7.8

Figure 5.3.2b Effects of Cycloheximide and Puromycin on Uptake of ^{125}I-fdBSA by Yolk Sacs Incubated in Serum-Free Medium



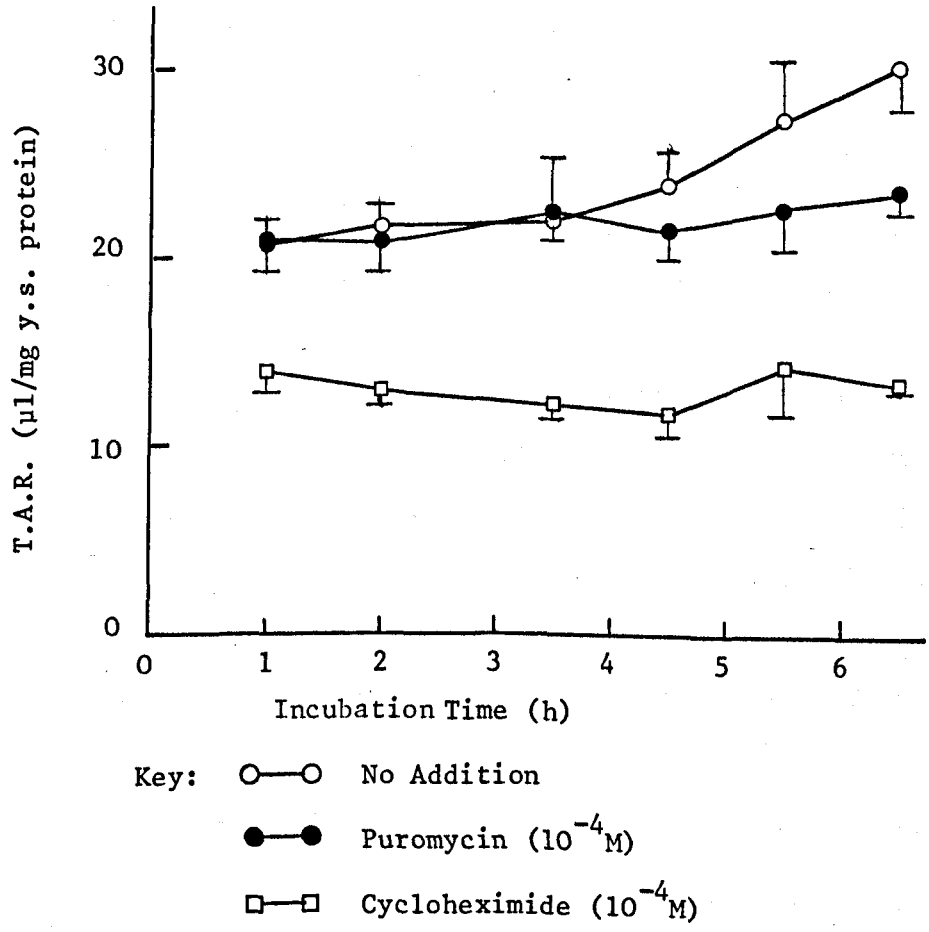
The method used was as described in Section 5.2. ^{125}I-fdBSA was present at a concentration of 1 µg/ml; either cycloheximide or puromycin was added 20 min prior to this substrate. Each uptake plot shows mean values (± S.E.M.) from 3 to 9 separate experiments. These results are summarized in Table 5.3.2b.

Table 5.3.2b Summary of the Effects of Cycloheximide and Puromycin on the Uptake of ^{125}I-fdBSA by Yolk Sacs Incubated in Serum-Free Medium

These data summarize the results shown in Figures 5.3.2b, c and d. The abbreviations used are as in Table 5.3.1b. The relative effect of either cycloheximide on puromycin on the E.I. or T.A.R. value, expressed as a percentage of the control value (no addition of agent), is given in parentheses beneath each mean E.I. and mean T.A.R. value, respectively.

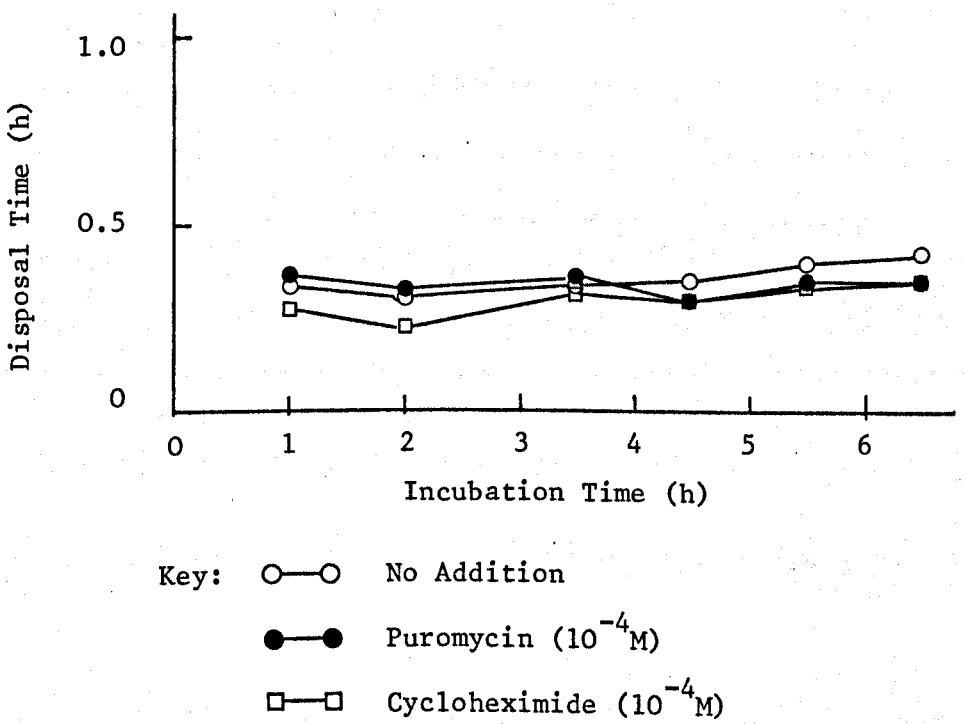
Agent	E.I.(Corr.Co) (µl/mg y.s. protein/h)	Mean E.I. (± S.E.M.) (µl/mg y.s. protein/h)	Rate of Sol. Rel. (µl/mg y.s. protein/h)	Mean T.A.R. (µl/mg y.s. protein)	Mean Disposal Time (h)
No Addition (Control)	71.21 (0.965) 68.89 (0.985) 68.64 (0.980) 78.52 (0.989) 76.51 (0.991) 77.53 (0.951) 71.91 (0.882) 72.21 (0.942) 76.36 (0.986)	73.53 ± 1.25 (100 ± 1.7)	72.06	24.26 (100.0)	0.33
Puromycin (10^{-4}M)	72.79 (0.993) 57.27 (0.971) 79.21 (0.977) 80.90 (0.985) 61.85 (0.951) 73.82 (0.956)	70.97 ± 3.87 (96.5 ± 5.3)	69.25	21.98 (90.6)	0.31
Cyclo-heximide (10^{-4}M)	34.78 (0.949) 39.28 (0.935) 37.48 (0.981)	37.18 ± 1.31 (50.6 ± 1.8)	36.50	12.44 (51.3)	0.33

Figure 5.3.2c Effect of Cycloheximide and Puromycin on the Quantity of Radioactivity that becomes Associated with Yolk-Sac Tissue during Uptake of ^{125}I-fdBSA in Serum-Free Medium



The quantity of radioactivity that becomes associated with yolk-sac tissue (tissue-associated radioactivity, T.A.R.) is expressed as mean values (± S.E.M.). These results are from the experiments shown in Figure 5.3.2b.

Figure 5.3.2d Effect of Cycloheximide and Puromycin on the Disposal Time of ^{125}I-fdBSA after Uptake by Yolk Sacs in Serum-Free Medium



Disposal time is calculated as (T.A.R. divided by the rate of uptake up to a given incubation time), and it represents the mean time ^{125}I-fdBSA is associated with the tissue before acid-soluble fragments are released. These results are from the experiments shown in Figure 5.3.2b.

Table 5.3.2c Uptake of ^{125}I-PVP by Yolk Sacs Incubated in Serum-Free Medium Following Previous Exposure to Cycloheximide

The method used was as described in Section 5.2. Briefly, the rate of uptake of ^{125}I-PVP (2 µg/ml) was monitored over 2.5h in the absence or presence of cycloheximide (10^{-4}M) in serum-free medium; cycloheximide was added 20 min prior to this substrate. The yolk sacs were washed, and then reincubated in serum-free medium for a further 2.5h in the presence of ^{125}I-PVP alone. Mean uptake rates were calculated from the slopes of mean uptake vs. time plots.

Agent in Initial Incubation	No. of Determinations	Mean Uptake Rates: Before Washing µl/mg y.s. protein per h	Before Washing As % of Control	After Washing µl/mg y.s. protein per h	After Washing As % of Control
No Addition (Control)	5	3.69	(100.0)	3.45	(100.0)
Cyclo-heximide (10^{-4}M)	3	2.76	(74.8)	3.66	(106.1)

5.3.3 Effect of Bacitracin on the Uptake of ^{125}I-PVP and ^{125}I-fdBSA by Yolk Sacs

The influence of the dodecapeptide antibiotic bacitracin on the rate of fluid-phase pinocytosis, as measured by the rate of uptake of ^{125}I-PVP, is shown in Figure 5.3.3a. Addition of bacitracin at a concentration of 0.01 mg/ml (7×10^{-6}M) to the serum-free medium 199 caused a slight inhibition of uptake, and progressively increasing the concentration of this agent to 10.0 mg/ml (7×10^{-3}M) led to a progressive fall in the uptake of ^{125}I-PVP with time; uptake was always virtually linear with time. The rates of uptake of ^{125}I-PVP in the presence of different amounts of bacitracin are reported in Table 5.3.3a. The mean control E.I. (± S.E.M.) in the absence of bacitracin was 3.23 ± 0.17 µl/mg yolk-sac protein per h. The presence of bacitracin at a concentration of 0.01 mg/ml (7×10^{-6}M) resulted in a fall in the mean E.I. to 81 ± 2% of the control value. An inhibition of uptake to 50% of the control value was achieved at a concentration of 1.0 mg/ml (7×10^{-4}M), and at the highest concentration used (10.0 mg/ml, 7×10^{-3}M) the mean E.I. had fallen to 15 ± 2% of the control value.

The effect of different concentrations of bacitracin on the rate of adsorptive pinocytosis in the rat yolk sac was followed by measuring the rate of uptake of ^{125}I-fdBSA. A range of bacitracin concentrations from 0.1 mg/ml to 10.0 mg/ml (7×10^{-5}M to 7×10^{-3}M) was investigated, and these results are shown in Figure 5.3.3b. This agent inhibited uptake of ^{125}I-fdBSA in a dose-dependent manner, the lowest bacitracin concentration used having only a small effect upon uptake, whereas the highest concentration, caused uptake to virtually cease. The Endocytic Indices from all the individual experiments are shown in Table 5.3.3b. The mean control E.I. of ^{125}I-fdBSA, in the absence of bacitracin, was 337 ± 41 µl/mg yolk-sac protein per h. The E.I. fell to 50% of the

control value in the presence of 1.0 mg/ml bacitracin; this degree of inhibition for this concentration of agent is the same as was found for the uptake of ^{125}I-PVP. However, the effect of the highest bacitracin concentration on the uptake of ^{125}I-fdBSA was much greater than was found with ^{125}I-PVP; this finding is illustrated more fully in Figure 5.3.3c. [The mean control E.I. of ^{125}I-fdBSA in serum-free medium was much higher than was found in Section 5.3.1, Table 5.3.1b and in Section 5.3.2, Table 5.3.2b. This is because a new batch of ^{125}I-fdBSA was prepared for these experiments with bacitracin; this variable is more fully discussed in Section 5.3.1.]

The release of acid-soluble radioactivity into the incubation medium following uptake and digestion of the protein by the tissue is shown in Figure 5.3.3d to be linear with time. The rate of this release (as reported in Table 5.3.3b) was similar to the rate of uptake of the protein for concentrations of bacitracin up to 5.0 mg/ml; at a bacitracin concentration of 10.0 mg/ml there was no detectable release of acid-soluble radioactivity up to 5h.

The effect of bacitracin on the quantity of radioactivity that became associated with the yolk-sac tissue during the incubation (T.A.R.) is shown in Figure 5.3.3e. In the absence of bacitracin the T.A.R. remained constant during the entire incubation period, as expected for a steady-state situation between incoming protein from pinocytosis and outgoing digestion products following contact of the protein with the lysosomal system. In the presence of 0.1 mg/ml bacitracin the mean T.A.R. appeared to rise slightly during the incubation period, and in the presence of 1.0 mg/ml bacitracin this increase in T.A.R. with time was quite marked. Under these three conditions the mean T.A.R. values at 1h were very similar (so one may compare them directly), and then they diverged with increase in incubation time, presumably because

bacitracin is exerting some kind of progressive effect. In the presence of higher concentrations of bacitracin the uptake of ^{125}I-fdBSA was very much lower, and therefore the initial T.A.R. values were much lower; this means that these data cannot be compared directly with those above. Nevertheless, even when the rate of uptake of the protein was very low (at 5 mg/ml bacitracin), one can still detect a steady rise in T.A.R. values with time; the inhibition of uptake in the presence of a concentration of 10.0 mg/ml bacitracin was so great that it was impossible to determine whether there was any progressive tissue accumulation.

The mean time during which the radiolabelled substrate, ^{125}I-fdBSA, is associated with the tissue before its release as acid-soluble fragments (the disposal time) is shown in Figure 5.3.3f. In the absence of bacitracin the disposal time is constant during the entire incubation period, and has a value of approximately 0.3h; this compares well with the values found in Table 5.3.1b and in Table 5.3.2b. The disposal time in the presence of the lowest bacitracin concentration used (0.1 mg/ml) was very similar to that of the control. As the concentration of bacitracin was further increased the magnitude of the disposal time at any point during the incubation increased. Moreover, the effect of a given concentration of bacitracin appeared to increase progressively with time, as reflected in the effect on disposal time. Such an effect is indicative of a progressive accumulation of active inhibitor within the lysosomal system rather than it reaching a steady-state value within 5h. Although Figure 5.3.3f clearly shows that the increase in disposal time with increase in incubation time to be virtually linear in the presence of 1.0 and 5.0 mg/ml bacitracin, in the presence of 10.0 mg/ml bacitracin the data were more scattered. This may reflect the toxic nature of these very high concentrations of bacitracin (see below).

The ability of yolk-sac tissue to recover its pinocytic capacity following exposure to different concentrations of bacitracin was investigated using the fluid-phase marker ^{125}I-PVP, and the results are shown in Figure 5.3.3g and Table 5.3.3c. During the initial uptake phase (2.5h) in the presence of different amounts of bacitracin, increasing the concentration of agent increased the inhibition of uptake; the mean uptake rates over this shorter period of uptake are similar to the mean Endocytic Indices reported in Table 5.3.3a for uptake over a 5h period. After exposure of the yolk sacs to bacitracin they were washed in warmed (37^{o}C) medium and then reincubated in bacitracin-free medium, and the mean rates of uptake of ^{125}I-PVP over the 2.5h recovery period, are also reported in Table 5.3.3c. [In these experiments the mean uptake rate for a given bacitracin concentration was calculated from the mean uptake plot, and not calculated as a mean of the rates from individual uptake plots. This was because the data tended to be quite scattered following the washing procedure, and it was therefore decided that a rate calculated from the mean uptake plot would be more accurate. Data obtained during the initial 2.5h incubation phase was treated in the same manner to allow for direct comparisons with the recovery phase.] Recovery of the pinocytic capacity of the tissue was virtually complete following exposure to bacitracin concentrations of up to 1.0 mg/ml. At the higher concentration of 5.0 mg/ml the recovery was slight, but at the highest concentration investigated (10.0 mg/ml) there was no recovery of the pinocytic capacity of the tissue as measured by uptake of ^{125}I-PVP.

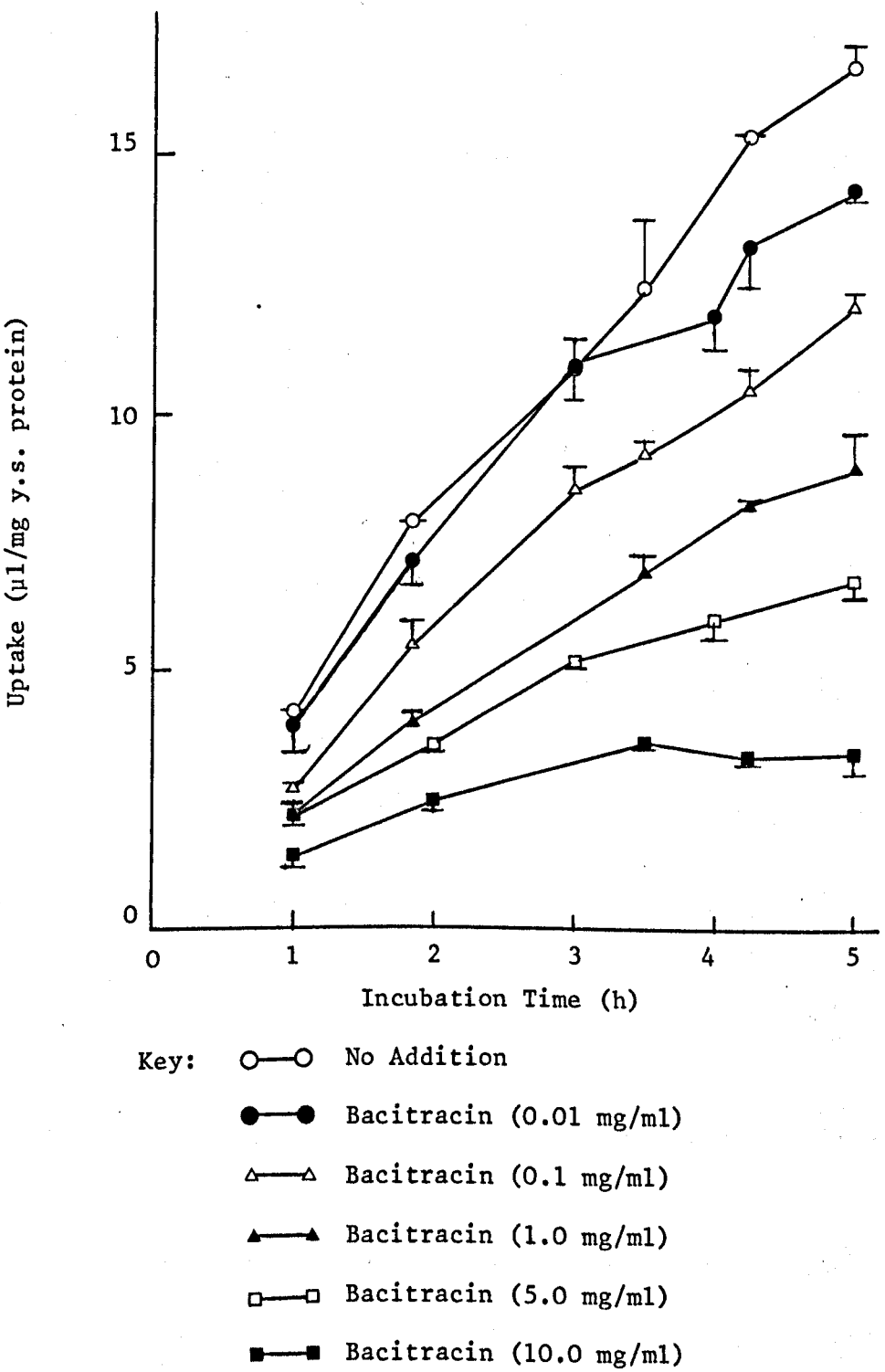
Since high concentrations of bacitracin appear to irreversibly affect the ability of the yolk sac to pinocytose ^{125}I-PVP, experiments were performed to look for evidence of damage to the tissue under these conditions. Pinocytosis of ^{125}I-PVP by the yolk sac results in its

accumulation within the lysosomal compartment of the cells. The rate of loss of this substrate from the tissue in control incubations is usually observed to be minimal, being less than 1% of the accumulated substrate/mg yolk-sac protein per h (Williams et al., 1975a). Yolk sacs were therefore incubated for 2.5h in the presence of ^{125}I-PVP and high concentrations of bacitracin, washed, and then reincubated in substrate-free medium which still contained the same high concentrations of bacitracin. The results obtained are shown in Figure 5.3.3h. Increasing the concentration of bacitracin increased the percentage release of ^{125}I-PVP from the tissue, and the rates of release of the substrate are reported in Table 5.3.3d. [The rate of release of ^{125}I-PVP was calculated from the linear part of the plots between the time points 0.8h and 2.5h, since during the first part of this reincubation period the large release of radioactivity probably arises from incomplete washing; this has been fully discussed in Section 4.3.1.] The mean total uptake of ^{125}I-PVP during the 2.5h "loading phase" is reduced in the presence of bacitracin because of its inhibitory action upon pinocytosis. Nevertheless, in spite of the small accumulation of ^{125}I-PVP, during this initial phase, the absolute amount of ^{125}I-PVP released during the reincubation phase, in ng released/mg yolk-sac protein per h, is greater in the presence of the highest concentration of bacitracin. To permit a more direct comparison between these values, the results are also expressed as a percentage of the tissue-associated radioactivity released. In the presence of 5.0 mg/ml bacitracin this rises from 0.88% (the control value) to 2.36% released/mg yolk-sac protein per h, and to 3.91%/mg yolk-sac protein per h in the presence of 10 mg/ml bacitracin. (This is equivalent to a 2.7- and 4.4-fold increase in release of accumulated substrate over the control value respectively.) These data suggest some damage to the tissue in the presence of high concentrations of

bacitracin.

[A preliminary report of these findings has been made (Forster & Williams, 1981).]

Figure 5.3.3a Effect of Different Concentrations of Bacitracin on the Uptake of ^{125}I-PVP by Yolk Sacs Incubated in Serum-Free Medium



The method used was as described in Section 5.2. ^{125}I-PVP was present at a concentration of 2 µg/ml; bacitracin was added 20 min prior to this substrate. Each uptake plot shows mean values (±S.E.M.) from 3 to 4 separate experiments. These results are summarized in Table 5.3.3a.

Table 5.3.3a Summary of the Effect of Different Concentrations of Bacitracin on the Uptake of ^{125}I-PVP by Yolk Sacs Incubated in Serum-Free Medium

These data summarize the results shown in Figures 5.3.3a and c. Each Endocytic Index (E.I.) is expressed as µl/mg yolk-sac protein per h, and is given with its Correlation Coefficient (Corr. Co.) in parentheses.

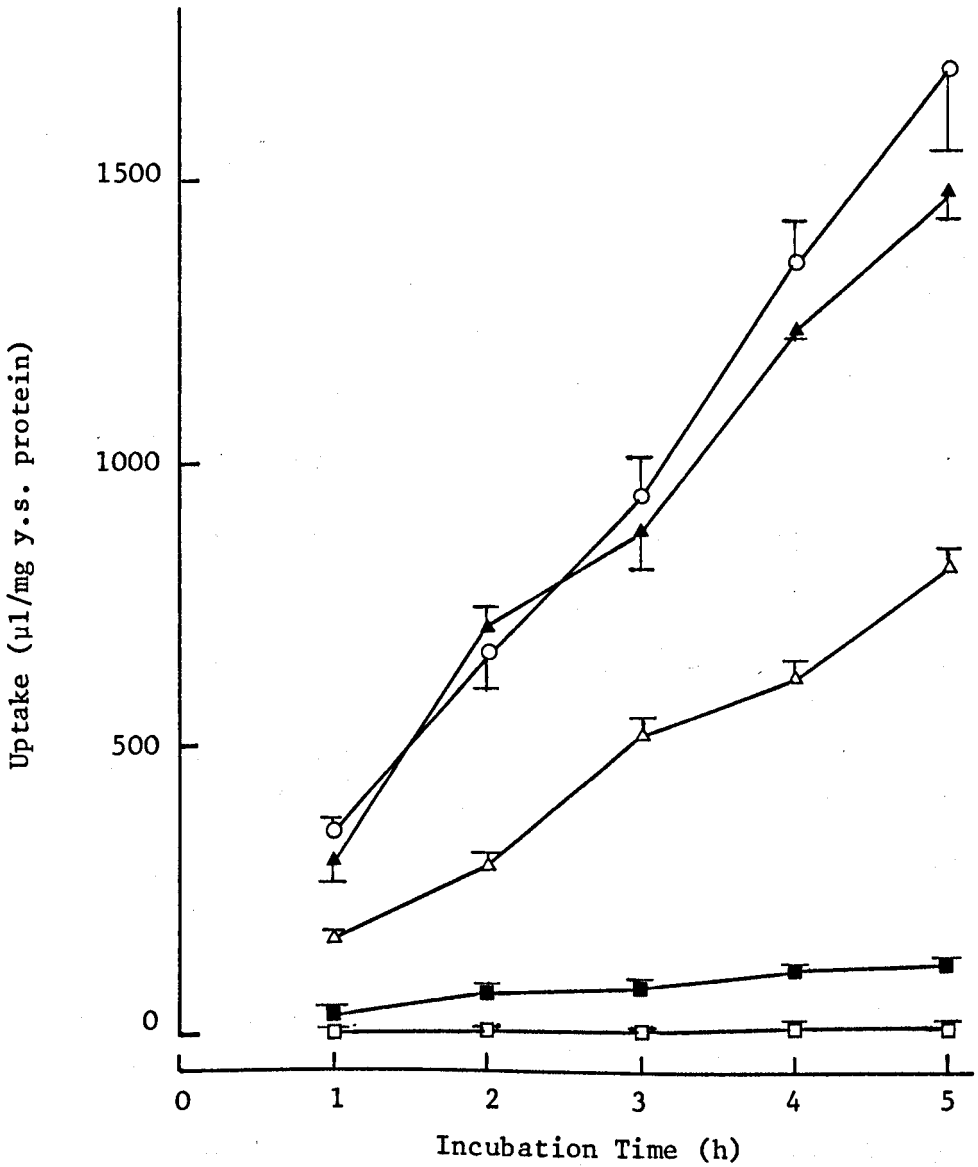
Bacitracin Conc. (mg/ml)	E.I.(Corr. Co.) (µl/mg y.s. protein per h)	Mean E.I. ± S.E.M. (µl/mg y.s. protein per h)	Mean E.I. as % of Control (%)
No Addition (Control)	3.56 (0.991) 2.98 (0.967) 3.15 (0.964)	3.23 ± 0.17	100.0 ± 5.3
0.01	2.68 (0.992) 2.55 (0.984) 2.44 (0.979) 2.71 (0.965)	2.60 ± 0.06	80.5 ± 1.9
0.1	2.28 (0.996) 2.41 (0.984) 2.34 (0.975)	2.34 ± 0.04	72.4 ± 1.2
1.0	1.74 (0.995) 1.22 (0.888) 1.89 (0.976)	1.62 ± 0.20	50.2 ± 6.2
5.0	1.16 (0.972) 1.30 (0.978) 1.01 (0.943)	1.16 ± 0.08	35.9 ± 2.5
10.0	0.55 (0.901 0.58 (0.955) 0.35 (0.956)	0.49 ± 0.07	15.2 ± 2.2

Table 5.3.3a Summary of the Effect of Different Concentrations of Bacitracin on the Uptake of ^{125}I-PVP by Yolk Sacs Incubated in Serum-Free Medium

These data summarize the results shown in Figures 5.3.3a and c. Each Endocytic Index (E.I.) is expressed as µl/mg yolk-sac protein per h, and is given with its Correlation Coefficient (Corr. Co.) in parentheses.

Bacitracin Conc. (mg/ml)	E.I. (Corr. Co.) (µl/mg y.s. protein per h)	Mean E.I. ± S.E.M. (µl/mg y.s. protein per h)	Mean E.I. as % of Control (%)
No Addition (Control)	3.56 (0.991) 2.98 (0.967) 3.15 (0.964)	3.23 ± 0.17	100.0 ± 5.3
0.01	2.68 (0.992) 2.55 (0.984) 2.44 (0.979) 2.71 (0.965)	2.60 ± 0.06	80.5 ± 1.9
0.1	2.28 (0.996) 2.41 (0.984) 2.34 (0.975)	2.34 ± 0.04	72.4 ± 1.2
1.0	1.74 (0.995) 1.22 (0.888) 1.89 (0.976)	1.62 ± 0.20	50.2 ± 6.2
5.0	1.16 (0.972) 1.30 (0.978) 1.01 (0.943)	1.16 ± 0.08	35.9 ± 2.5
10.0	0.55 (0.901 0.58 (0.955) 0.35 (0.956)	0.49 ± 0.07	15.2 ± 2.2

Figure 5.3.3b Effect of Different Concentrations of Bacitracin on the Uptake of ^{125}I-fdBSA by Yolk Sacs Incubated in Serum-Free Medium



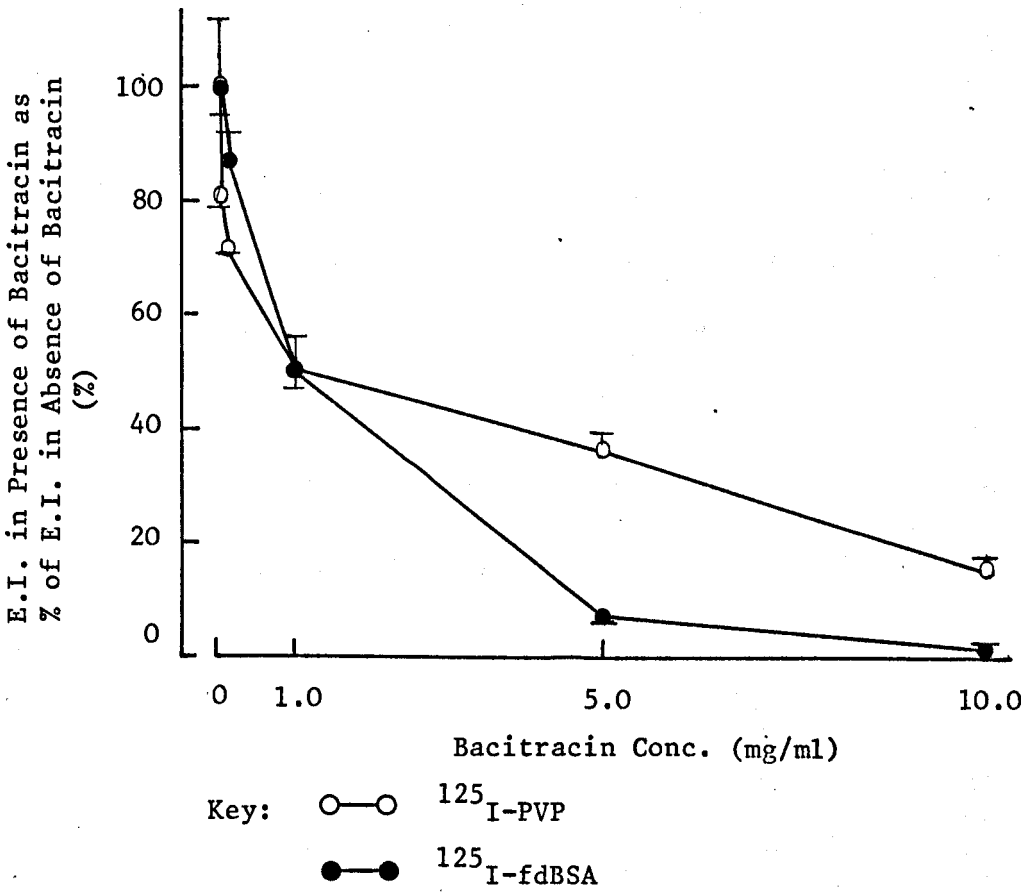
The method used was as described in Section 5.2. ^{125}I-fdBSA was present at a concentration of 1 µg/ml; bacitracin was added 20 min prior to this substrate. Each uptake plot shows mean values (± S.E.M.) from 3 to 5 separate experiments. These results are summarized in Table 5.3.3b.

Table 5.3.3b Summary of the Effect of Different Concentrations of Bacitracin on the Uptake of ^{125}I-fdBSA by Yolk Sacs Incubated in Serum-Free Medium

These data summarize the results shown in Figures 5.3.3b, c and d. The abbreviations used are as in Table 5.3.1b.

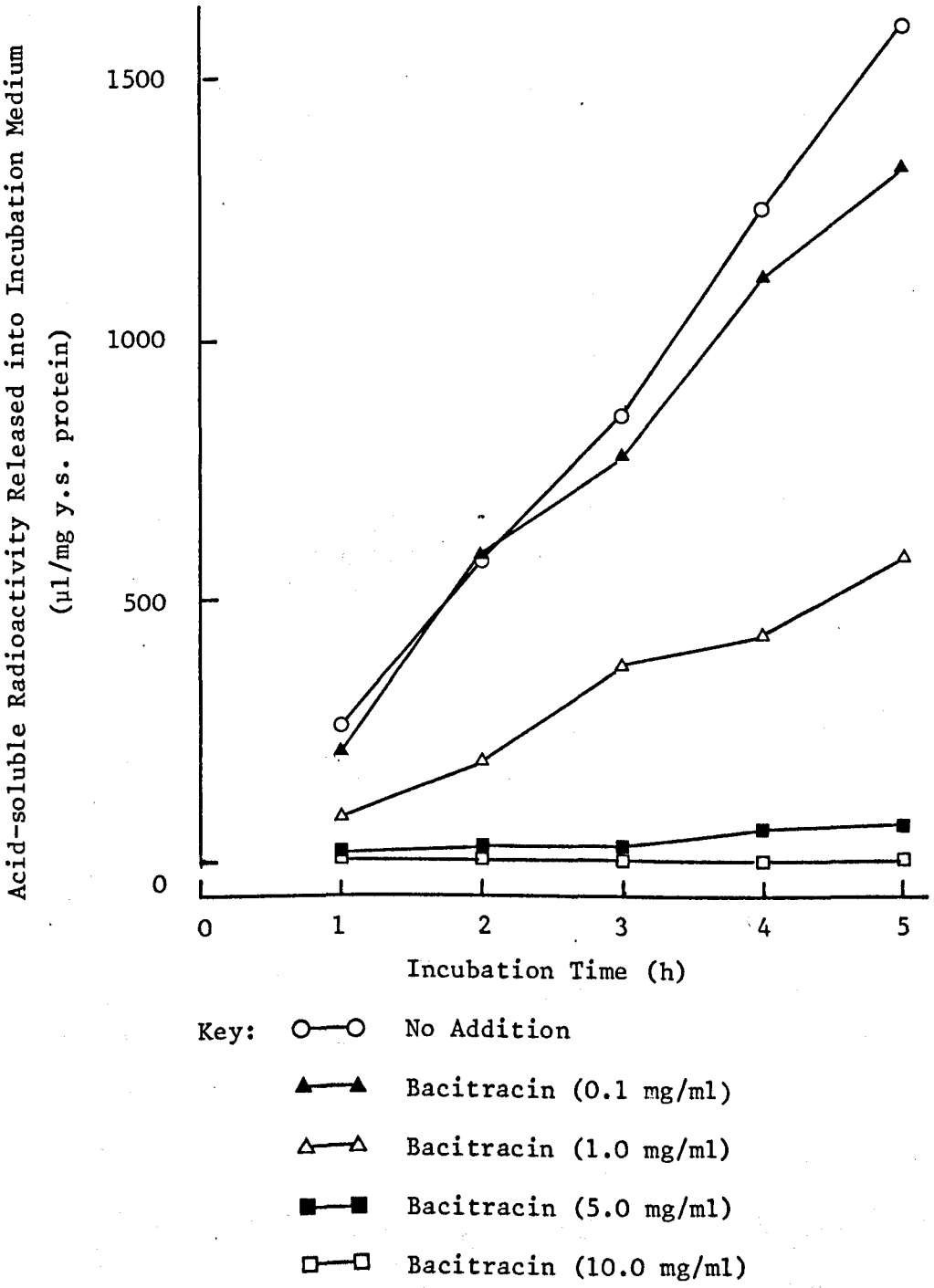
Bacitracin Conc. (mg/ml)	E.I.(Corr.Co.) (µl/mg y.s. protein/h)	Mean E.I. ± S.E.M. (µl/mg y.s. protein/h)	Mean E.I. as % of Control (%)	Rate of Sol. Rel. (µl/mg y.s. protein per h)
No Addition (Control)	450.47 (0.962) 410.96 (0.961) 245.08 (0.978) 319.20 (0.925) 259.39 (0.977)	337.0 ± 40.7	100.0 ± 12.1	335.6
0.1	282.87 (0.967) 273.03 (0.955) 327.90 (0.997)	294.6 ± 16.9	87.4 ± 5.0	278.6
1.0	173.95 (0.980) 171.65 (0.969) 165.65 (0.983) 150.46 (0.969)	165.4 ± 5.3	49.1 ± 1.6	123.5
5.0	22.46 (0.964) 21.97 (0.918) 29.66 (0.969)	24.7 ± 2.5	7.3 ± 0.7	14.7
10.0	2.80 (0.867) 2.49 (0.891) 5.14 (0.962)	3.5 ± 0.8	1.0 ± 0.2	0.2

Figure 5.3.3c Effect of Different Concentrations of Bacitracin on the Relative Endocytic Indices of ^{125}I-PVP and ^{125}I-fdBSA by Yolk Sacs Incubated in Serum-Free Medium



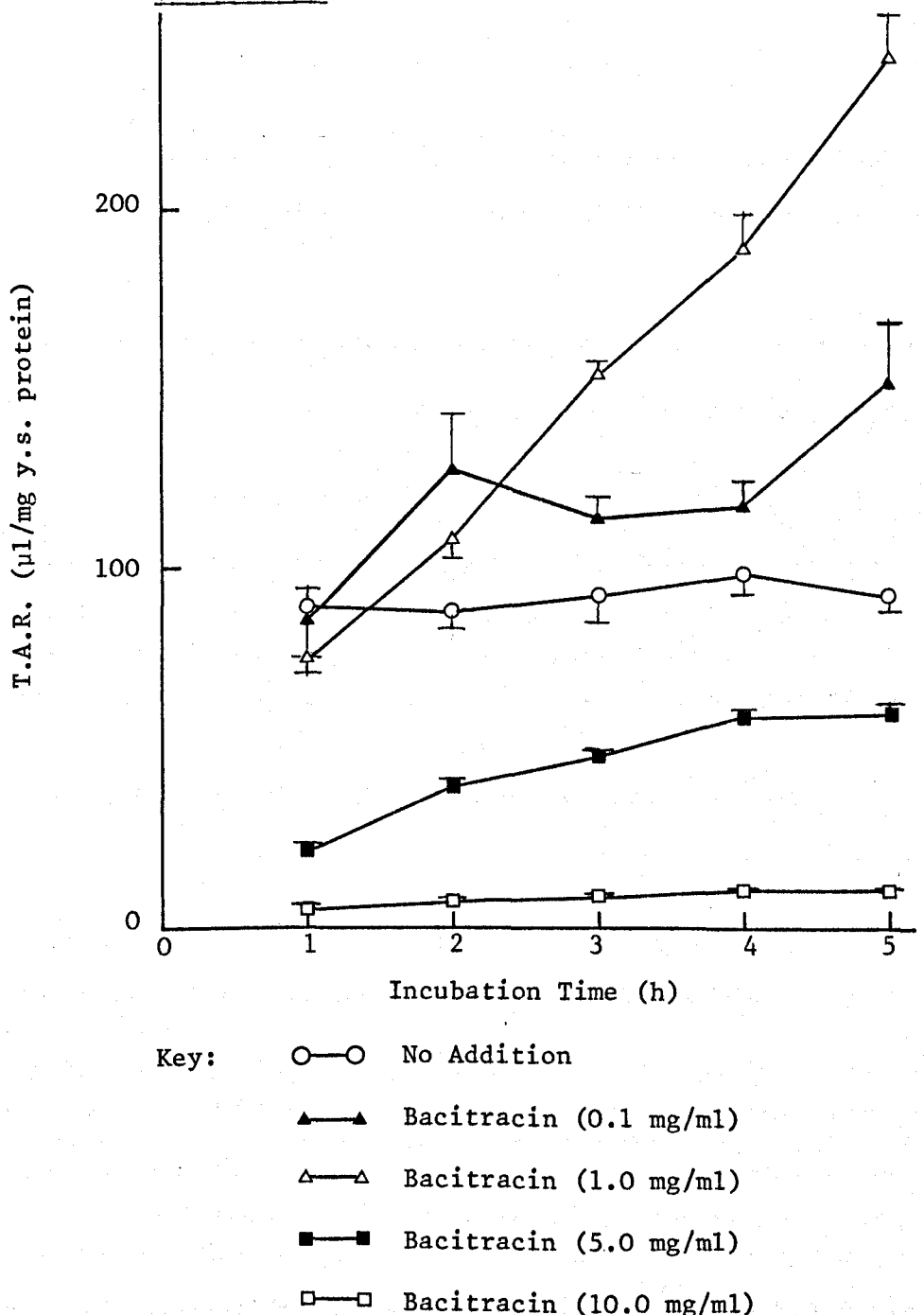
Each point represents the mean E.I. (± S.E.M.) of the substrate at a particular bacitracin concentration expressed as a percentage of the mean control E.I. in the absence of bacitracin. These results are from the data summarized in Tables 5.3.3a and b.

Figure 5.3.3d Effect of Different Concentrations of Bacitracin on the Release of Acid-Soluble Radioactivity into the Incubation Medium by Yolk Sacs during Uptake of ^{125}I-fdBSA



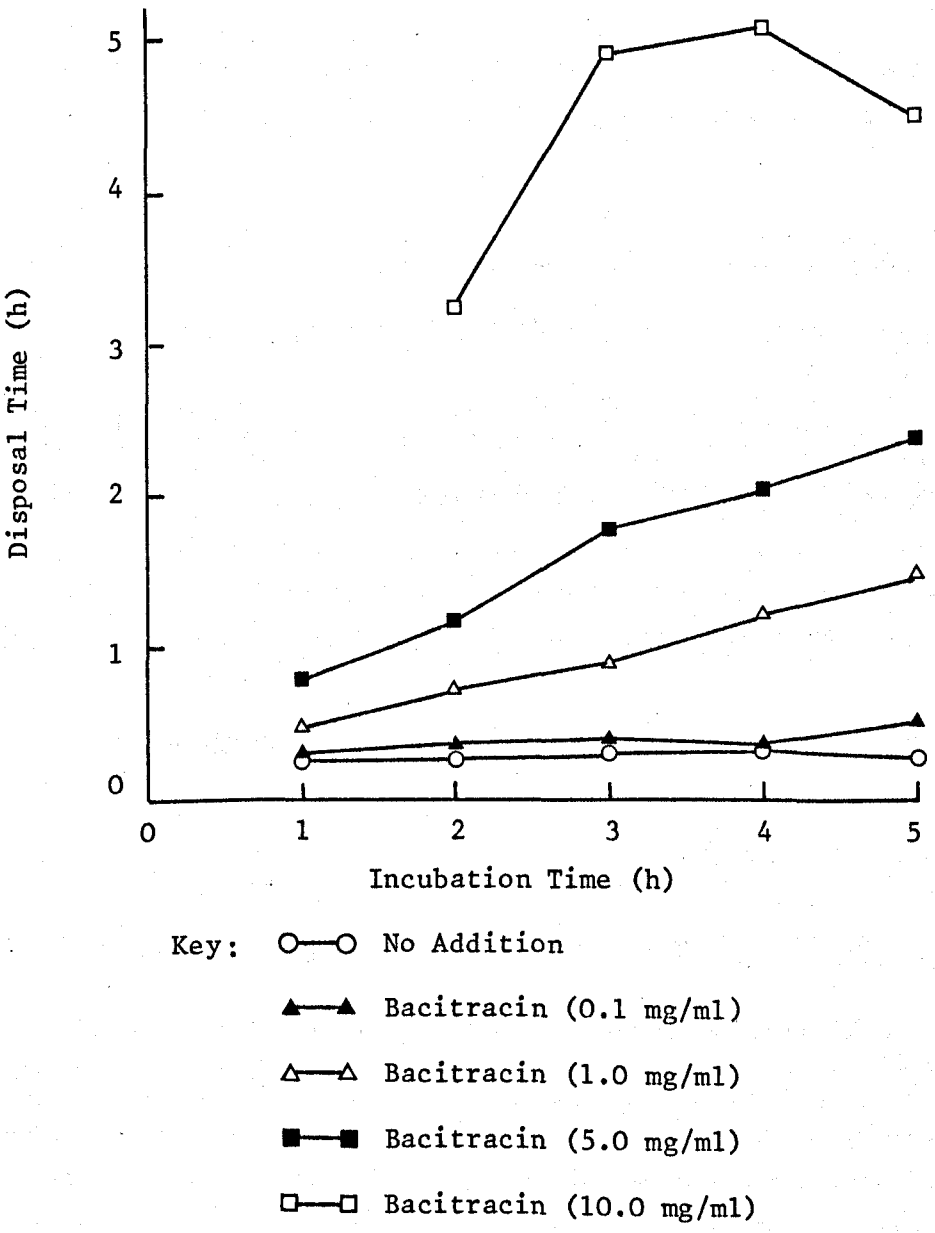
The acid-soluble radioactivity released into the incubation medium is expressed as mean values. These results are from the experiments shown in Figure 5.3.3b, and are summarized in Table 5.3.3b.

Figure 5.3.3e Effect of Different Concentrations of Bacitracin on the Quantity of Radioactivity that becomes Associated with Yolk-Sac Tissue during Uptake of ^{125}I-fdBSA in Serum-Free Medium



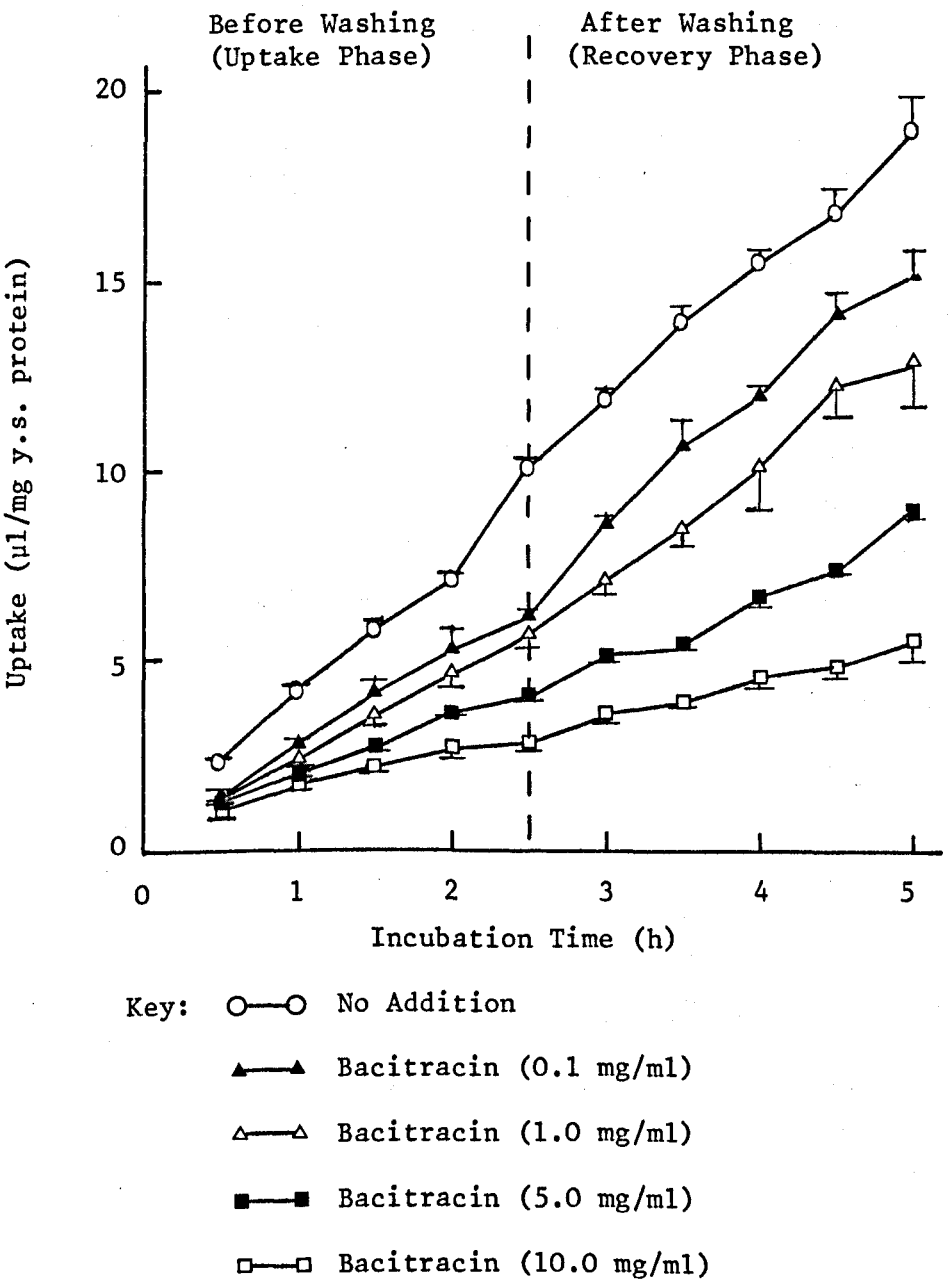
The quantity of radioactivity that becomes associated with yolk-sac tissue (tissue-associated radioactivity, T.A.R.) is expressed as mean values (± S.E.M.). These results are from the experiments shown in Figure 5.3.3b.

Figure 5.3.3f Effect of Different Concentrations of Bacitracin on the Disposal Time of ^{125}I-fdBSA after Uptake by Yolk Sacs in Serum-Free Medium



Disposal time is calculated as (T.A.R. divided by the rate of uptake up to a given incubation time), and it represents the mean time ^{125}I-fdBSA is associated with the tissue before acid-soluble fragments are released. These results are from the experiments shown in Figure 5.3.3b.

Figure 5.3.3g Recovery of the Capacity to Ingest ^{125}I-PVP by Yolk Sacs Incubated in Serum-Free Medium following Exposure to Different Concentrations of Bacitracin



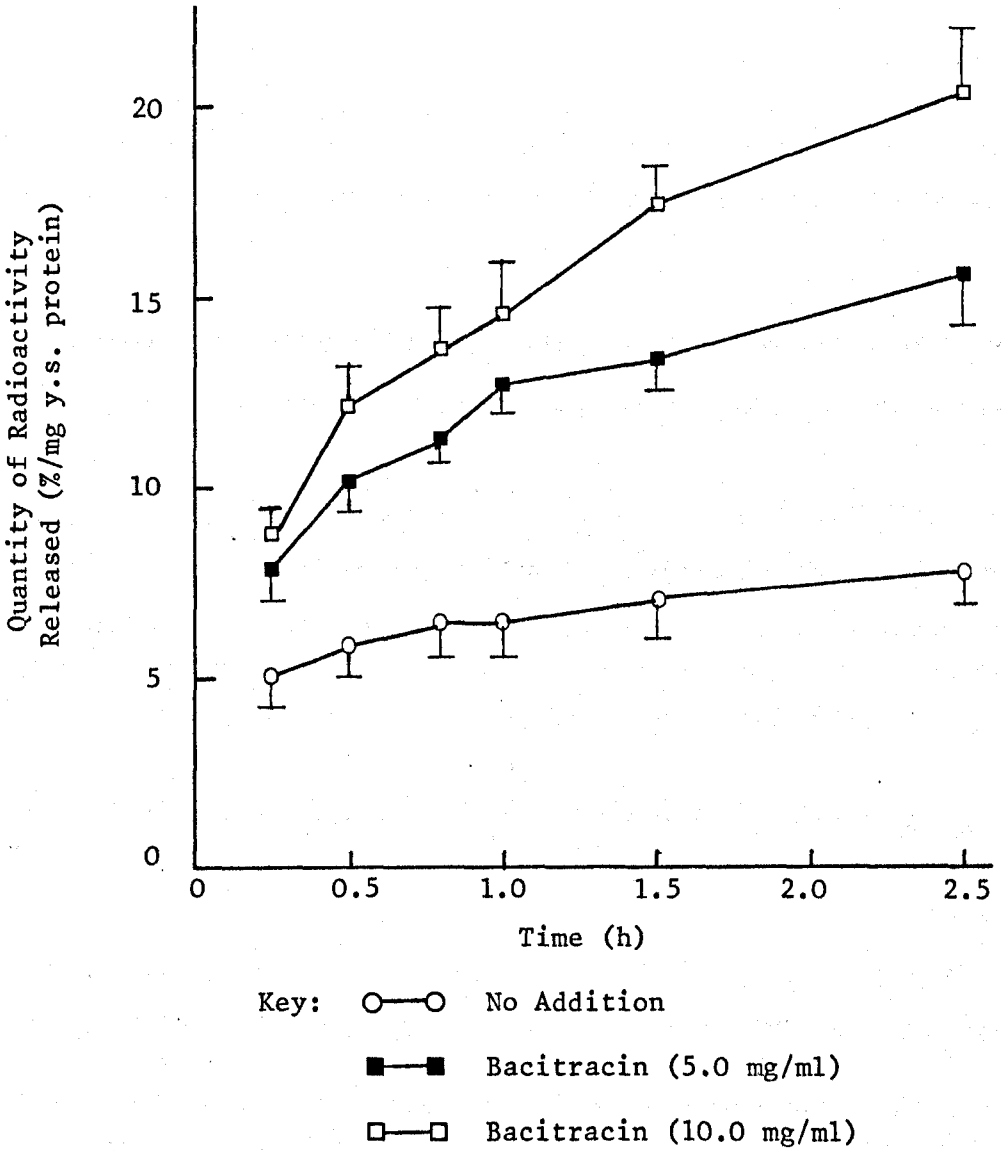
The method used was as described in Section 5.2. Briefly, the rate of uptake of ^{125}I-PVP (2µg/ml) was monitored over 2.5h in the absence or presence of different concentrations of bacitracin in serum-free medium; bacitracin was added 20 min prior to this substrate. The yolk sacs were then washed, and reincubated in serum-free medium for a further 2.5h in the presence of ^{125}I-PVP alone. Each uptake/recovery plot shows mean values (± S.E.M.) from 2 to 5 separate experiments. These results are summarized in Table 5.3.3c.

Table 5.3.3c Summary of the Recovery of Uptake of ^{125}I-PVP by Yolk Sacs Incubated in Serum-Free Medium following Exposure to Different Concentrations of Bacitracin

These data summarize the results shown in Figure 5.3.3g. Mean uptake rates and correlation coefficients (shown in parentheses) for each bacitracin concentration were calculated from the gradients of these plots.

Bacitracin Conc. (mg/ml)	No. of Determinations	Mean Uptake Rates: Before Washing/Uptake µl/mg y.s. protein per h	Before Washing/Uptake As % of Control	After Washing/Recovery µl/mg y.s. protein per h	After Washing/Recovery As % of Control
No Addition (Control)	5	3.69 (0.990)	100.0	3.45 (0.996)	100.0
0.1	3	2.31 (0.997)	62.6	3.32 (0.994)	96.2
1.0	3	2.11 (0.999)	57.2	3.07 (0.989)	89.0
5.0	2	1.40 (0.995)	37.9	1.92 (0.977)	55.7
10.0	2	0.94 (0.989)	25.5	1.00 (0.992)	29.0

Figure 5.3.3h Effect of High Concentrations of Bacitracin on the Release of ^{125}I-PVP from Yolk Sacs Incubated in Serum-Free Medium



The method used was as described in Section 5.2. Each release plot represents the quantity of radioactivity released with time from yolk sacs that had previously been incubated in the presence of ^{125}I-PVP. Each experiment point is a mean value (± S.E.M.) from 6 separate experiments.

Table 5.3.3d Summary of the Effect of High Concentrations of Bacitracin on the Release of ^{125}I-PVP from Yolk Sacs Incubated in Serum-Free Medium

These data summarize the results shown in Figure 5.3.3h. Each rate of release of radioactivity was calculated from the gradients of these plots between time points 0.8 and 2.5h. Results are expressed both as ng of ^{125}I-PVP released and as a percentage of the radioactivity associated with the tissue at the start of the reincubation period.

Bacitracin Conc. (mg/ml)	Mean Total Uptake (ng/ mg y.s. protein)	^{125}I-PVP Released (ng/mg y.s. protein per h)	Radioactivity Released (%/mg y.s. protein per h)
No Addition (Control)	23.65	0.21	0.88
5.0	12.36	0.29	2.36
10.0	7.61	0.38	3.91

5.4 Discussion

A number of hormones are known to influence the degradation of endogenous proteins in certain cell types in vitro, and a collaborative study was undertaken to investigate the effects of insulin, a known inhibitor of the degradation of endogenous proteins in many cell types (see Section 5.1), on both the rate of pinocytosis (data reported in Section 5.3.1) and on the rate of endogenous protein degradation in the rat yolk sac (unpublished data compiled by F.J. Ballard and S.E. Knowles, reported in Table 5.4a). The effect of insulin on the rate of fluid-phase pinocytosis in the rat yolk sac was not very marked over the concentration range used. [There was no detectable inhibition of pinocytosis up to a concentration of 10^{-11}M insulin, but by 10^{-6}M the rate of uptake of ^{125}I-PVP had fallen to approximately 70% of the control value in the absence of insulin.] Strangely, the effect of this hormone on the rate of uptake of ^{125}I-fdBSA was even less pronounced, with no inhibition of uptake up to 10^{-8}M insulin, and only a slight indication of inhibition of adsorptive pinocytosis at 10^{-6}M insulin. A possible explanation of this effect was advanced in Section 5.3.1. With ^{125}I-fdBSA as substrate, there was no evidence of accumulation of the radiolabelled-protein within the tissue (Figure 5.3.1d), indicating no detectable net inhibition of proteolysis by this agent. Likewise, the presence of insulin (10^{-11} to 10^{-6}M) had no effect on the rate of degradation of the endogenous protein of rat yolk sacs (Table 5.4a, data from F.J.B. & S.E.K.). This lack of response to insulin is atypical of many cell types; for example, Hopgood et al. (1980) demonstrated that hepatocyte monolayers were sensitive to inhibition of endogenous proteolysis by insulin at a concentration of 10^{-8}M, and Libby & Goldberg (1981) demonstrated that 10^{-5}M insulin could inhibit the degradation of cell

protein to 40% of the control value in cultured muscle cells from 11-day old chick embryos.

The response of the yolk sac to the insulin agonist, glucagon, has already been investigated (Brown & Segal, 1977; Livesey, 1978). The latter showed that the rate of uptake of ^{125}I-PVP by rat yolk sacs was inhibited in a dose-dependent manner by glucagon over the concentration range 10^{-11} to 10^{-6}M. Only slight inhibition of uptake was detected at the lower concentrations used, but in the presence of 10^{-6}M glucagon uptake fell to only approximately 30% of the control value. At this high concentration, the rate of uptake of ^{125}I-fdBSA was also inhibited, to 40% of the control value. However, glucagon (over the same concentration range) had no effect on the degradation of endogenous protein (F.J.B. & S.E.K., unpublished data). This lack of response of endogenous protein degradation in yolk sacs to the presence of glucagon (at these concentrations) is again atypical of many cell types (Hopgood et al., 1980). Clearly, only the pinocytic activity of the yolk sac is affected by these two hormones, and the tissue is more responsive to the presence of glucagon.

Antibiotics are another group of substances of which some have been found to influence rates of endogenous protein degradation in different cell types in vitro. Probably one of the most well examined is cycloheximide which, in addition to acting as an inhibitor of protein synthesis, also inhibits proteolysis within some cell types. This antibiotic was chosen, along with another antibiotic protein-synthesis inhibitor, puromycin, to see if they influence pinocytosis and rates of endogenous protein degradation within the rat yolk sac. (This investigation too was a collaborative study with F.J.B. and S.E.K.). Data on the rates of pinocytosis are reported in Section 5.3.2. and the

data provided by F.J.B. and S.E.K. are to be found in Table 5.4b. At a concentration of 10^{-4}M, puromycin was found to affect neither the rate of fluid-phase pinocytosis nor the rate of adsorptive pinocytosis in the rat yolk sac, and had no effect upon the intra-lysosomal degradation of ^{125}I-fdBSA following its uptake by the tissue. However, puromycin (at the same concentration) was found to inhibit degradation of endogenous proteins to 80% of the control value (see data provided by F.J.B. and S.E.K., Table 5.4b) in yolk sacs incubated in Minimal Essential Medium.

In contrast to puromycin, cycloheximide was found to inhibit the rate of fluid-phase pinocytosis in yolk sacs to 60% of the control value at a concentration of 10^{-4}M. At this concentration a similar degree of inhibition of the rate of uptake of ^{125}I-fdBSA was observed, the mean Endocytic Index falling to 50% of the control value. The quantity of radioactivity associated with the tissue during the incubation of the protein with yolk sacs, in the presence of cycloheximide, also fell to 50% of the control value, and was constant throughout the incubation period. These results suggest that cycloheximide influences only the rate of pinocytosis, the normal rate-limiting step in the digestion of exogenous proteins by the yolk sac. There was no evidence of any inhibition of the degradative capacity of the yolk sac by this antibiotic; this is in agreement with the observation of Freikopf-Cassel & Kulka (1981), that cycloheximide (4×10^{-4}M) has no effect upon the rate of degradation of ^{125}I-labelled BSA, introduced into cultured hepatoma cells by erythrocyte ghost-mediated injection. However, cycloheximide (10^{-4}M) was found to inhibit the degradation of endogenous proteins in rat yolk sacs, both in Minimal Essential Medium and in Earle's Salts, to approximately 80% of the control value (see Table 5.4b); this is the same degree of inhibition as was found

with 10^{-4}M puromycin. Since cycloheximide had no effect upon the degradation of the pinocytosed ^{125}I-fdBSA, then presumably this lysosomal site of proteolysis is independent from the sensitive endogenous-proteolytic site. Inhibition of proteolysis of endogenous proteins by cycloheximide has also been found in many other cell types, for example, Hopgood et al. (1980) with hepatocyte monolayers, and Amenta et al. (1978) with rat embryo fibroblasts. Examination of a range of concentrations of both of these antibiotics may have yielded more information, but the concentration chosen for this brief study was known to be effective in other cell types.

Both of these antibiotics, cycloheximide and puromycin, have consistent effects on the degradation of endogenous protein in virtually all cell types, and the recovery, following removal of these inhibitors from extracellular medium, is known to be rapid. Many workers (e.g. Ballard, 1977) have argued that this suggests the need for a short half-life protein for rapid protein degradation. The object of the work reported in this chapter was to expand this proposal by suggesting that if the effect on endogenous protein degradation is mediated by a putative protein that is essential to the process of autophagy, then the protein may be a membrane component rather than some lysosomal enzyme . It is therefore likely that such a putative protein may also influence pinocytosis, another membrane-related process. Although the data collected on the effect of cycloheximide on pinocytosis in yolk sacs supports this suggestion, the absence of any effect of puromycin on pinocytosis clearly refutes this proposal. Presumably the effect of cycloheximide on pinocytosis in the rat yolk sac is independent of its role as an inhibitor of protein synthesis.

The third antibiotic examined in this study was bacitracin. Its effects upon pinocytosis in yolk sacs are reported in detail in

Section 5.3.3. The concentrations used were greater than those of insulin and the other two antibiotics, the lowest concentration of bacitracin investigated being higher than the largest insulin concentration, and the range was extended well beyond those of both cycloheximide and puromycin. Bacitracin was found to inhibit the rate of fluid-phase pinocytosis, as determined by the rate of uptake of ^{125}I-PVP, in a dose-dependent manner. At concentrations of up to 1.0 mg/ml ($7x10^{-4}$M), the inhibition of uptake induced by bacitracin was fully reversible. [It is interesting to note that the degree of inhibition of ^{125}I-PVP uptake in the presence of $7x10^{-4}$M bacitracin and 10^{-4}M cycloheximide (both fully reversible) was 50% of the control value in the absence of any agent in each case.] At higher concentrations of bacitracin the inhibition of uptake was not fully reversible, and evidence of damage to the tissue was found under these conditions (see Figure 5.3.3h and Table 5.3.3d).

Bacitracin also inhibited the adsorptive uptake of ^{125}I-fdBSA by yolk sacs in a dose-dependent manner over the concentration range 0.1 mg/ml ($7x10^{-5}$M) to 10.0 mg/ml ($7x10^{-3}$M). The relative inhibition of uptake of this protein,in the presence of bacitracin concentrations in excess of 1 mg/ml, was always greater than that of ^{125}I-PVP. A possible explanation of this effect is that bacitracin is a hydrophobic compound and competes with the radiolabelled protein for hydrophobic binding sites (Livesey & Williams, 1982) on the surface of the yolk sac (see Section 3.1). During the course of the incubation of ^{125}I-fdBSA in the presence of bacitracin, it was found that the quantity of radioactivity associated with the yolk-sac tissue increased with time, and that the length of time that the protein was associated with the tissue before being released as acid-soluble radioactive fragments (the disposal time) also increased with the duration of the

incubation (Figure 5.3.3f.). This progressive accumulation of ^{125}I-fdBSA by the tissue with time indicates that intralysosomal proteolysis is being inhibited, and suggests that bacitracin can either inhibit the degradative capacity of the lysosomal enzymes so that intralysosomal digestion becomes rate-limiting or that it prevents pinosome-lysosome fusion. Since bacitracin is known to inhibit various proteolytic enzymes (Mäkinen, 1972) the former explanation seems more likely.

Since the completion of this work, another group (studying fibroblasts in culture) has reported on the effects of bacitracin on endocytosis (van Leuven & Cassiman, 1981 and van Leuven et al., 1981). They found that bacitracin inhibited the receptor-mediated endocytosis of ^{125}I-labelled α_2Macroglobulin-trypsin complexes. In the presence of 0.1 mg/ml bacitracin endocytosis decreased to 80% of the control value (compared with 87 ± 5% in the yolk sac), 50% inhibition was achieved at a concentration of 0.25 mg/ml, and a concentration of 1.0 mg/ml decreased the rate of uptake to 20% of the control value. Bacitracin at this last concentration (1.0 mg/ml) only inhibited pinocytosis in the yolk sac to 50% of the control value, thus fibroblasts appear to be more sensitive to this agent than yolk sacs. From measuring kinetic parameters of the receptor-mediated endocytosis in fibroblasts, van Leuven et al. concluded that bacitracin inhibited endocytosis of ^{125}I-labelled α_2Macroglobulin-trypsin complexes in a competitive fashion. They also examined the effect of this antibiotic on the intracellular half-life of these internalized ^{125}I-labelled α_2Macroglobulin-trypsin complexes. Under control conditions they reported the half-life of the complexes as 2.1h. In the continuous presence of 0.25 mg/ml bacitracin the intracellular half-life was 2.5-2.7h. Their comment on this finding was that bacitracin does not appreciably affect

intracellular degradation of endocytosed ^{125}I-labelled α_2 Macroglobulin-trypsin complexes. Grinde & Seglen (1980) also reported that bacitracin, at a concentration of 2mM (approx. 3 mg/ml), had no significant effect upon the degradation of endogenous [^{14}C]valine-labelled proteins in isolated hepatocytes in culture. However, the data of Ballard et al. (1980) show that, in osteogenic sarcoma cells, bacitracin at a concentration of 1.0 mg/ml inhibited endogenous protein degradation by 7 %, and at a concentration of 10.0 mg/ml, inhibited degradation of endogenous protein by 43%. The same paper also showed that, in the same cell type, bacitracin (1.0 mg/ml) inhibited the rate of degradation of exogenous ^{125}I-labelled insulin by almost 90%. The effects of bacitracin appear to vary somewhat according to cell type. In summary, the antibiotic inhibits pinocytosis in both fibroblasts and the yolk sac, and inhibits the degradation of endocytosed, radiolabelled proteins in osteogenic sarcoma cells and yolk sacs, but van Leuven et al. (1980) claim no such effect in fibroblasts. The effect of bacitracin on the rate of endogenous protein degradation also varies between cell types; Grinde & Seglen (1980) found no effect in isolated hepatocytes and Ballard et al. (1980) found an inhibitory effect in osteogenic sarcoma cells.

At the beginning of this chapter, two hypotheses were proposed to relate the rate of pinocytosis to the rate of endogenous protein degradation within a tissue (see Section 5.1). One suggestion was that a fall in the intracellular levels of amino acids acts as a trigger to increase autophagy or to increase the rate of pinocytosis in a tissue, so that a fall in the rate of capture of exogenous protein may induce an increase in the rate of endogenous protein degradation; this suggestion was called the "nutritional hypothesis". The second proposal was that, since pinocytosis and autophagy are both membrane-

related processes, any agent interfering with pinocytosis may also be expected to interfere with endogenous protein turnover at the stage of uptake into the lysosomal system, and this suggestion was called the "membrane-mediated hypothesis". The effects of all the agents used in this chapter on pinocytosis and proteolysis in the rat visceral yolk sac are summarized in Table 5.4c, alongside their reported effects on some other cell types. The effect of insulin is to suppress the rate of degradation of endogenous proteins in all cell types other than the rat yolk sac and the rat pulmonary macrophage. The effect of glucagon is to stimulate proteolysis in many cell types, but again this agent had no effect on the degradation of either endogenous or exogenous proteins in the rat yolk sac. [The effects of these hormones on pinocytosis do not appear to have been investigated in other tissues, preventing comparison between the yolk sac and other tissues.] Thus the data from investigations with these hormones support neither hypothesis. The effect of puromycin on the rat yolk sac again did not support either of the two hypotheses, and no recent data from other cell types could be located in the literature. The inhibitory effect of cycloheximide on the rate of degradation of endogenous proteins in hepatocyte monolayers and rat embryo fibroblasts was consistent with the effect in the yolk sac; an inhibitory effect was also observed for pinocytosis in the yolk sac. This possibly supports a "membrane-related" hypothesis. The effect of bacitracin is to suppress both pinocytosis and proteolysis in most cell types shown in Table 5.4c, and again may be taken as an indication of some common control mechanism. [Unfortunately no data are available on the effect of this antibiotic on the rate of endogenous protein degradation in the rat yolk sac.]

In summary, not only does the yolk sac behave atypically compared with other tissues, but there is no coherent pattern of evidence to

strongly support either the "nutritional hypothesis" or the "membrane-related hypothesis". Clearly more data, from many cell types, are needed to establish the mechanisms by which pinocytosis and autophagy are controlled and any possible relation between the two.

Table 5.4a Effect of Different Concentrations of Insulin on Endogenous Protein Degradation in Yolk Sacs (F.J. Ballard & S.E. Knowles: Unpublished Data)

The method used was as described in Section 5.2. The tissue was incubated in Minimal Essential Medium for a 1.5h preincubation period in the presence of the same concentration of insulin as was used during the degradation period; degradation of endogenous radiolabelled proteins was monitored for a total of 4h following the preincubation period.

Insulin Conc. (M)	No. of Determinations	Mean Total Protein Degradation (%)	Mean Degradation as % of Control (%)
No addition (Control)	5	12.78	100
10^{-11}	5	13.09	102
10^{-10}	5	13.03	102
10^{-9}	5	12.57	98
10^{-8}	5	12.72	100
10^{-7}	5	12.69	99
10^{-6}	5	13.24	104

Table 5.4b *Effect of Cycloheximide and Puromycin on Endogenous Protein Degradation in Yolk Sacs (F.J. Ballard & S.E. Knowles: Unpublished Data)*

The method used was as described in Section 5.2. The tissue was incubated either in Minimal Essential Medium (M.E.M.) or Earle's Salts (E.S.). Sometimes a 1.5h preincubation period in the presence of the same concentration of agent as was used during the degradation period was incorporated into the experiment, and the degradation of endogenous radiolabelled proteins was monitored for a total of 2 or 4h following the optional preincubation period.

Agent (10^{-4}M)	No. of Determ-inations	M.E.M. or E.S.	Pre-incubation for 1.5h	Mean Total Protein Degrad. in 2h (%)	Mean Degrad. as % of Control in 2h (%)	Mean Total Protein Degrad. in 4h (%)	Mean Degrad. as % of Control in 4h (%)
Control	3	M.E.M.	No	-	-	16.31	100
Cyclohex.	3	M.E.M.	No	-	-	14.36	88
Control	4	M.E.M.	No	-	-	16.03	100
Cyclohex.	3	M.E.M.	No	-	-	12.84	70
Control	3	M.E.M.	Yes	-	-	15.93	100
Cyclohex.	3	M.E.M.	Yes	-	-	10.96	69
Control	4	E.S.	No	-	-	16.69	100
Cyclohex.	4	E.S.	No	-	-	13.59	81
Control	3	E.S.	Yes	-	-	14.97	100
Cyclohex.	3	E.S.	Yes	-	-	12.21	82
Control	3	M.E.M.	Yes	9.78	100	-	-
Cyclohex.	4	M.E.M.	Yes	7.75	79	-	-
Control	3	M.E.M.	Yes	-	-	15.84	100
Cyclohex.	4	M.E.M.	Yes	-	-	12.04	76
Control	4	M.E.M.	No	-	-	15.12	100
Puromycin	4	M.E.M.	No	-	-	12.41	82

Table 5.4c The Effects of Various Agents on Pinocytosis and Protein Degradation in Different Tissues

Agent	Effects Observed in Rat Visceral Yolk Sac			Effects Observed in Other Tissues		
	Pinocytosis	Degradation of Exogenous Proteins	Degradation of Endogenous Proteins	Pinocytosis	Degradation of Exogenous Proteins	Degradation of Endogenous Proteins
Insulin	Slight suppression (Section 5.3.1)	No Effect (Section 5.3.1)	No Effect (Table 5.4a)	-	-	Suppression of Proteolysis/Autophagy in:- i) Chick embryo muscle cells (Libby & Goldberg, 1981 ii) Rat embryo fibroblasts (Amenta & Brocher, 1980a) iii) Rat hepatocyte monolayers (Hopgood et al., 1980) iv) Rat Liver (Pfeifer, 1977; Marzella et al., 1980) v) Normal and transformed cell lines (Ballard et al., 1980; Gunn et al., 1980) No Effect in:- i) Rabbit pulmonary macrophages (Woodside & Massaro, 1979)
Glucagon	Suppression (Livesey, 1978; Brown & Segal, (1977)	No Effect (Livesey; 1978)	No Effect (F.J.B. & S.E.K., unpublished data)	-	-	Enhancement of Proteolysis/Autophagy in:- i) Rat hepatocyte monolayers (Hopgood et al., 1980) ii) Rat liver (Shelburne et al., 1973; Schworer & Moritmore, 1979)
Puromycin	No Effect (Section 5.3.2)	No Effect (Section 5.3.2)	Suppression (Table 5.4b)	-	-	-
Cycloheximide	Suppression (Section 5.3.2)	No Effect (Section 5.3.2)	Suppression (Table 5.4b)	-	-	Suppression of Proteolysis in:- i) Rat embryo fibroblasts (Amenta et al., 1978) ii) Rat hepatocyte monolayers (Hopgood et al., 1980)
Bacitracin	Suppression (Section 5.3.3)	Suppression (Section 5.3.3)	-	Suppression of Pinocytosis / Receptor Clustering in:- i) Chinese hamster ovary cells (Maxfield et al., 1979; Davies et al., 1980; Dickson et al., 1981) ii) Fibroblasts (Van Leuven & Cassiman, 1981; Van Leuven et al., 1981) iii) BALB/c3T3 cells (Maxfield et al., 1979; Davies et al., 1980; Dickson et al., 1981)	Suppression in:- i) Osteogenic sarcoma cells (Ballard et al., 1980) No Effect in:- i) Fibroblasts (Van Leuven & Cassiman, 1981; Van Leuven et al., 1981)	Suppression of Proteolysis in:- i) Osteogenic sarcoma cells (Ballard et al., 1980) No Effect in:- i) Hepatocytes (Grinde & Seglen, 1980)

CHAPTER 6

GENERAL DISCUSSION

General Discussion

The main aims of this project were to examine the effects of certain factors on the pinocytic activity of the rat visceral yolk sac *in vitro* and also to investigate their effects on the rates of uptake of proteins and on the rates of degradation of the proteins after pinocytic capture. The purpose of this discussion is firstly to emphasize the main points from the discussion sections of the individual chapters and then to try to draw some general conclusions.

The first factor examined was the influence of changes in the physical structure of a protein molecule on its rate of uptake by the tissue. Although the uptake of simple proteins by the rat yolk sac has now been studied quite extensively [Williams *et al.*, 1975b; Moore *et al.*, 1977; Agarwal & Moore, 1979; Ibbotson & Williams, 1979; Livesey & Williams, 1979; Livesey & Williams, 1981; Kooistra & Williams, 1981; Kooistra *et al.*, 1981; Livesey & Williams, 1982], and the effects of chemical modifications and of size and charge differences between proteins analyzed in detail, little information has been gained on the influence of minor changes in protein structure (of the kind which may be expected to occur *in vivo*) on the rates of uptake and digestion of proteins. Four bovine pancreatic ribonuclease molecules were chosen for this investigation (see Chapter 3): ribonuclease A was chosen as it is a polypeptide chain of 124 amino acids; ribonuclease B is a glycosylated form of this simple protein which proffers a simple oligosaccharide subunit; ribonuclease S is a modified version of the ribonuclease A molecule resulting from cleavage by subtilisin between amino acid residues 20 and 21 with the two fragments remaining bound together non-covalently; ribonuclease S protein is the ribonuclease S molecule after removal of the eicosapeptide. This part of the project produced some questions as well as some answers. An interesting result was that "nicking" of a single peptide bond was found to greatly increase the rate of

uptake of the radiolabelled protein by the yolk sac (from comparing the rates of uptake of ribonucleases A and S, see Section 3.3.1) which gave support to the suggestion of Dice & Goldberg (1976) that limited extracellular proteolytic cleavage may be a sufficient signal to ensure the rapid removal of proteins from the circulation. This part of the study also showed no evidence of a mannose receptor on the yolk sac (since the rates of uptake of ribonucleases A and B were the same, see Section 3.3.1), furthermore the presence of the simple oligosaccharide present on ribonuclease B did not appear to mask any binding sites on the simple protein core.

Once associated with the yolk-sac tissue, it was expected that the radiolabelled proteins would be degraded exclusively intralysosomally. [Livesey & Williams (1979) have already provided a strong argument for formaldehyde-denatured ^{125}I-labelled BSA being degraded entirely intralysosomally within the rat yolk sac, and, with the exception of glucagon, other proteins also seem to be degraded only within lysosomes.] However, a study of the effect of 2,4-DNP on the rate of uptake of the radiolabelled ribonucleases (see Section 3.3.2), led to the observation that the production of acid-soluble radioactivity following incubation of yolk sacs with either ^{125}I-RNase S or Spr in the presence of 2,4-DNP was greater than could be accounted for by a change in pinocytic rate alone. The "inflated" rates of production of acid-soluble radioactivity with these substrates were caused by proteolytic activity that was neither initially associated with the medium nor released into the medium from the tissue (see Section 3.3.3). A yolk-sac homogenate was found to degrade all the radiolabelled ribonucleases with maximal activity at pH 4-5. However, limited degradative capacity towards ribonucleases other than ribonuclease A was present at neutral pH [this was found neither with formaldehyde-denatured ^{125}I-labelled BSA nor with other

^{125}I-labelled proteins (Livesey, 1978)]. These observations are consistent with, although not direct evidence for, proteolytic activity being present on the surface of the yolk sac that would lead to the production of acid-soluble radioactivity from ^{125}I-RNases S and Spr in the absence of pinocytosis by the yolk sac. (Some suggestions for further investigation of these observations are discussed in Section 3.4.) Since the degradation of ^{125}I-RNases A and B in the presence of 2,4-DNP can be linked to the pinocytic activity of the tissue it is assumed that these two ribonucleases are degraded intracellularly. The presence of the oligosaccharide affords no protection against degradation by intracellular enzymes.

The second of the two factors which could potentially influence the pinocytic activity of the rat visceral yolk sac in vitro is the composition of the incubation medium. Some of the more complex media contain not only inorganic salts and glucose, but also a wide variety of other components such as vitamins, nucleotides, amino acids, and antibiotics. It is not known which components are essential for yolk-sac survival and function. The addition of serum further increases the complexity of the incubation medium. It is important to try to gain some insight into how these constituents influence pinocytosis and the subsequent digestion of internalized proteins. In studies of such factors it is desirable to use a radiolabelled protein whose behaviour has previously been extensively studied in the yolk-sac incubation system, and whose site of degradation is well established. The protein substrate chosen for these studies was therefore formaldehyde-denatured ^{125}I-labelled BSA (^{125}I-fdBSA).

Addition of increasing amounts of serum to the medium 199 in which yolk sacs were incubated led to a fall in the rates of pinocytosis (see Section 4.3.2); the effect of the serum on the rate of adsorptive uptake

of ^{125}I-fdBSA was proportionally greater than the effect on the rate of fluid-phase pinocytosis, presumably due to competition between the radiolabelled protein and non-labelled serum proteins for binding sites on the surface of the yolk sac. Increasing the amount of serum in the extracellular medium also led to an accumulation of the radiolabelled substrate within the tissue during the course of the incubation. Assuming that this was not a result of inhibition of pinosome-lysosome fusion, the increased intralysosomal half-life of ^{125}I-fdBSA may again arise from competition, but this time between the radiolabelled substrate and non-labelled serum proteins for the lysosomal enzymes. An alternative suggestion is that, following digestion of exogenous proteins, an increase in the concentrations of amino acids in an intracellular pool (following digestion of large amounts of protein) may act as a signal to switch off lysosomal proteolysis, and may also be envisaged as a possible signal for a reduction in the pinocytic rate of the tissue. If such a homeostatic nutritional mechanism operates, then access to large amounts of protein in the extracellular medium may also reduce the need for degradation of endogenous proteins. This suggestion is compatible with observations of the behaviour of many cell types in vitro (see Section 4.1), and, following a collaborative study with F.J. Ballard and S.E. Knowles, the same may operate in the rat yolk sac (see Section 4.4). Since serum has such a profound effect on fundamental cellular processes, its presence must always be noted and borne in mind when interpreting in vitro data. The removal of serum enables culture conditions to be better defined since, being a biological product, the precise composition of the serum probably varies between batches derived from different animals. However, it is not always possible to avoid this complication by simply omitting serum from the culture medium since some cell types cannot be incubated or cultured successfully if

serum is not present in the medium. To negate the need for the presence of serum in incubation media, some groups of workers have been investigating the possibility of replacing the serum with single proteins (e.g. serum albumin) or with milk. Thus, Steimer & Klagsbrun (1981) have examined the effects of growing certain fibroblastic cells in a medium supplemented with bovine milk. They found that some cell types (the non-transformed cell lines) did not grow at all in milk-supplemented medium, and that other cell types (transformed cells) survived by growing as large clusters in suspension instead of being attached to the culture dishes; addition of fibronectin was found to reverse some of these adverse effects. Sereni & Baserga (1981) found that, for a variety of cell lines, when supplementing media with milk instead of serum, in order to get a good plating efficiency calf serum still had to be added to a final concentration of 0.5% to the medium containing milk. They underline the economic advantages of using milk in place of serum as a culture-medium supplement, but this change in procedure raises the problem as to whether milk (like serum) affects fundamental cellular processes such as pinocytosis and protein degradation in vitro.

Removal of certain components from a complex medium resulted in no change in the pinocytic capacity of the yolk-sac tissue (for a comparison of results in Earle's Salts and Minimal Essential Medium with those in medium 199, see Section 4.3.1). Although it is not known what precise ingredients of these media are responsible for pinocytosis in the rat yolk sac, it appears that the rates of uptake of the fluid-phase marker, ^{125}I-PVP, measured in all these defined media, are compatible with the yolk sac exhibiting its maximum rate of pinocytosis under all these incubation conditions, hence not all of the low molecular weight ingredients of medium 199 can be absolutely essential for pinocytic activity.

Since serum can influence both the degradation of exogenous and endogenous proteins and the rate of pinocytosis within the rat visceral yolk sac, other substances known to influence the degradation of endogenous proteins were investigated for their effects on pinocytosis and subsequent degradation of internalized proteins. The first group of substances investigated was hormones. Glucagon had previously been shown to have an inhibitory effect on pinocytosis in the yolk sac. (Brown & Segal, 1977; Livesey, 1978), but in contrast to this the agonist of glucagon, insulin,was found to have very little effect on pinocytosis in the yolk sac until relatively high concentrations were reached (see Section 5.3.1). These two hormones have been shown to have opposite and very marked effects on rates of degradation of endogenous proteins in many cell types in vitro (see Section 5.1), but the collaborative study with F.J.B. and S.E.K. showed that these two hormones had no effect at all on the rate of degradation of endogenous proteins in rat yolk sacs (see Section 5.4). The main conclusion reached in this part of the study was that the response of rat visceral yolk sac to these hormones is not representative of most cell types that have been studied.

The other group of substances investigated for their effects on pinocytosis and proteolysis in the rat yolk sac was the antibiotics. It is important to investigate these compounds since not only do some of them influence the rate of degradation of endogenous proteins (see Section 5.1), but they are commonly added to many media to increase the shelf-life. The first two antibiotics examined, cycloheximide and puromycin, are both inhibitors of protein synthesis. From the collaborative study with F.J.B. and S.E.K. (see Section 5.4) it was found that both of these antibiotics had inhibitory effects on the rate of degradation of endogenous proteins. This led to the suggestion

that if these compounds inhibit endogenous protein breakdown via an inhibition of autophagy, then it is possible that some short-lived protein is necessary for the associated membrane events to occur. If pinocytosis, another membrane-related process, is under the influence of the same control mechanism then presumably these two substances would have the same effect upon pinocytosis. However, only cycloheximide was found to have an effect on pinocytosis in the yolk sac. From these results it can be concluded that, in the rat yolk sac, even if autophagy is under the control of some short-lived protein, pinocytosis is not.

The other antibiotic used in this study was bacitracin (see Section 5.3.3). It was found to inhibit both pinocytosis and the degradation of endocytosed ^{125}I-fdBSA in a dose-dependent manner in the yolk sac. This latter finding was in agreement with data from osteogenic sarcoma cells (Ballard et al., 1980), but contrasted with the findings in fibroblasts (van Leuven et al., 1981). However, bacitracin was reported to inhibit pinocytosis of ^{125}I-labelled $\alpha_2$macroglobulin-trypsin complexes in fibroblasts (van Leuven et al, 1981). [Bacitracin has also been shown to differ in its effects on the rate of endogenous protein degradation in different cell types (see Section 5.4).] In conclusion, the effects of the antibiotics used in this study were found to be varied, their effects being dependent upon the cell type under investigation.

In summary, from the data presented in this study, three simple but nonetheless important factors have emerged. First, a minor change in the structure of a protein (the cleaving of a single peptide bond) can be sufficient to cause rapid uptake by the yolk sac. Such a cleavage is possibly of the type that may occur in vivo, and if so, supports the suggestion of Dice & Goldberg (1976) that proteolytic "nicking" may be a signal for a protein to be removed from the circulation. Second, it appears that not all exogenous proteins are degraded entirely

within the lysosomal system of the rat yolk sac, as had previously been thought [from the study of a large number of ^{125}I-labelled simple proteins (Livesey, 1978)]. Further experimentation is required to support or refute this suggestion, but it does outline the need for caution when attempting to predict the outcome of an experiment on the basis of even a well-established general trend. [It is interesting to note that the two protein preparations that are not totally degraded within the lysosomal system are the ones produced by proteolytic cleavage with subtilisin. Possibly the preparations as marketed still contain subtilisin activity which contributes to the process of extralysosomal degradation. This possibility should not be overlooked in any future attempts to explain these anomalous results.]

The third and most overwhelming factor to emerge is the extent to which the composition of the incubation medium can influence the behaviour of the tissue. Many medium components frequently used in cell culture have very pronounced effects upon both pinocytosis and proteolysis within the tissue, and the manner in which each tissue may respond to these components is not always easy to predict.

One of the aims of this project was to try and gain evidence for a homeostatic control mechanism based on amino acid levels in cells, by looking for some coordination between the ingestion of protein from outside the cell with the rate of intracellular proteolysis. Serum had an inhibitory effect on all of these processes in the yolk sac [which agrees well with other cell types (see Section 4.1)],but there appeared to be no correlation between the influence of hormones on pinocytosis and on proteolysis, nor between the response of the yolk sac and the response of other tissues to these hormones. This raises the question of the physiological role(s) of the yolk sac. This membrane has long been considered to be important in the nutrition of the embryo before the full development of the placenta (Beck et al., 1967). More recently,

Freeman et al. (1981) have demonstrated that proteins are taken up by the yolk sac at 9 days gestation, broken down to amino acids, and then these amino acids are taken up by the embryo and inserted into its own proteins. It may be that this bulk catabolic function of the pre-placental yolk sac is conserved following the development of the placenta, and is still present at 17.5 days gestation. Another physiological role proposed for the yolk sac is that of protection of the embryo, a role most likely to be functional right up to the end of gestation. (By sequestering and degrading such substances as maternal antigens and toxic agents, the yolk sac may prevent such materials from reaching the embryonic tissues.) Ongoing, non-regulated pinocytic and proteolytic capacities may therefore be essential to the efficient functioning of the yolk sac, and so, in contrast with other cell types, the yolk sac may well be deliberately non-responsive to hormonal control. This does not, however, preclude the tissue from being responsive to basic components affecting pinocytosis, such as non-physiological metabolic inhibitors, cytoskeletal inhibitors (Duncan & Lloyd, 1978; Duncan et al., 1979), and the supply of protein from the extracellular environment (Chapter 4). Thus the yolk sac remains a valuable diagnostic tool for the investigation of such controlling factors in pinocytosis.

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APPENDIX I

Computer Program to Calculate the Uptake of a Non-Degradable Substrate

Input Data:

- H(X) Duration of the yolk-sac incubation period (h)
- I(X) Mean observed radioactivity count for 1.0 ml of incubation medium
- K(X) Mean observed radioactivity count for 1.0 ml of yolk-sac solution
- L(X) Total protein content of the yolk sac (mg)

```
0020  REM KEW 125-IPVP PROGRAM,MODIFIED NOV.,1974
0030  DIM H(15),I(15),K(15),L(15,R(15)
0060  PRINT "NON-DIGESTIBLE PROG. ENTERED"
0070  PRINT "EXPT NO. (DIGITS  ONLY)=";
0080  INPUT Z
0090  PRINT "BACKGROUND IN CPM=";
0100  INPUT A
0110  PRINT "COUNTING TIME FOR EACH ML OF MEDIUM, SECS=";
0120  INPUT C
0130  PRINT "COUNTING TIME FOR EACH ML OF YS SOLUTION, SECS=";
0140  INPUT D
0150  PRINT "NO. OF POINTS IN PLOT=";
0160  INPUT G
0165  PRINT "NAME OF DATA FILE:";
0167  INPUT F1$
0170  PRINT "NAME OF REGRESSION FILE:";
0174  INPUT F2$
0176  FILES £1,F1$,£2,F2$
0180  PRINT "NAME OF RESULTS FILE:";
0184  INPUT F3$
0186  FILES £3,F3$
0190  FOR X=1 TO G
0200  INPUT £1, H(X),I(X),K(X),L(X),
0210  NEXT X
0220  FOR X=1 TO G
0230  LET M=(I(x)*60/C)-A
0240  LET Q=((K(X)*60/D)-A)*5
0250  LET N=M+Q/20
0260  LET R(X)=(Q*1000)/(N*L(X))
0265  LET A$='2£.2£,4£.4£'
0266  LET C$='"      ",2£.2£,2£.2£,4£.4£'
0270  PRINT USING A$,£2,H(X),R(X)
0280  NEXT X
0310  PRINT £3, " "
0320  PRINT £3, " "
0330  PRINT £3, "INCUBATION TIME (HOURS)","PROTEIN IN YS","   UPTAKE"
0340  PRINT £3,
0350  FOR X=1 TO G
0360  PRINT USING C$,£3,H(X),L(X),R(X)
0370  NEXT X
0380  STOP
0010  DIM C$30
```

APPENDIX II

Computer Program to Calculate the Uptake of a Degradable Substrate

Input Data:

- H(X) Duration of the yolk-sac incubation period (h)
- I(X) Mean observed radioactivity count for 1.0 ml of incubation medium
- J(X) Mean observed acid-soluble radioactivity count for 1.0 ml of incubation medium
- K(X) Mean observed radioactivity count for 1.0 ml of yolk-sac solution
- L(X) Total protein content of the yolk sac (mg)

```
0020  REM PROTEIN PROGRAM ,REVISED OUTPUT ,NOV. 1974
0030  DIM E(12),H(12),I(12),J(12),K(12),L(12),S(12)
0040  DIM C$30
0060  PRINT "PROTEIN PROGRAM ENTERED"
0070  PRINT "EXPT. NO. (DIGITS ONLY)=";
0080  INPUT V
0090  PRINT "BACKGROUND IN CPM=";
0100  INPUT  A
0110  PRINT  "PERCENT SOLUBLES IN PREP=";
0120  INPUT  B
0130  PRINT  "COUNTING TIME MEDIUM TOTALS,SECS=";
0140  INPUT  C
0150  PRINT  "COUNTING TIME MEDIUM SOLUBLES,SECS=";
0160  INPUT  Z
0170  PRINT  "YS COUNTING TIME,SECS=";
0180  INPUT  D
0190  PRINT  "CORRECTION FACTOR FOR MEDIUM TOTALS=";
0200  INPUT  Y
0210  PRINT  "CORRECTION FACTOR FOR MEDIUM SOLUBLES=";
0220  INPUT  R
0230  PRINT  "NO. OF POINTS IN PLOT=";
0240  INPUT  G
0264  PRINT  "NAME OF DATA FILE:";
0265  INPUT  F1$
0266  PRINT  "NAME OF REGRESSION FILE:";
0267  INPUT  F2$
0269  FILES  £1,F1$,£2,F2$
0270  PRINT  "NAME OF RESULTS FILE:";
0272  INPUT  F3$
0275  FILES  £3,F3$
0277  FOR X=1 TO G
0280  INPUT £1, H(X),I(X),J(X),K(X),L(X),
0290  NEXT X
0300  FOR X=1 TO G
0310  LET M=(I(X)*60/C-A)*Y
0320  LET N=(J(X)*60/Z-A)*R
0330  LET O=N-(M*B/100)
0340  LET Q=((K(X)*60/D)-A)*5
0350  LET P=(M-N)+O/2
```

cont. ....

```
0360  LET F=((10*O)+Q)*1000
0370  LET E(X)=(Q*1000)/(L(X)*P)
0380  LET S(X)=F/(L(X)*P)
0385  LET A$='2£.2£,4£.4£'
0387  LET C$='4£.4£,4£.4£,4£.3£,4£.3£'
0390  PRINT USING A$,£2,H(X),S(X)
0400  NEXT X
0430  PRINT £3, " "
0440  PRINT £3, " "
0450  PRINT £3, " INCUBATION ","PROTEIN","MICROLITRES","    UPTAKE"
0460  PRINT £3, "TIME (HOURS)"," IN YS "," PER MG YS "
0470  FOR X=1 TO G
0480  PRINT USING C$,£3,H(X),L(X),E(X),S(X)
0490  NEXT X
0500  STOP
```

APPENDIX III

Computer Program to Calculate the Release of Radiolabelled Substrates from Yolk Sacs

This program is designed to calculate the release of both non-degradable and degradable radioiodinated substrates from yolk sacs when reincubated in substrate-free medium, after being initially incubated in substrate-containing medium. The release is expressed in two forms: as the percentage of the amount of substrate associated with the tissue at the beginning of the reincubation period which is released per h per mg yolk-sac protein, and as ng of material released per h per mg yolk-sac protein.

Input data:

- P(X) Serial number of sample
- Q(X) Duration of the reincubation period of yolk sacs (h)
- R(X) Mean observed radioactivity (total uncorrected count in time stated) in 1.0 ml of reincubation medium.
- S(X) Mean observed acid-soluble radioactivity (total uncorrected count in time stated) in 1.0 ml of reincubation medium.

```
0010  REM EXOCYTOSIS PROGRAMME, DECEMBER 1980
0020  DIM P(20),Q(20),R(20),S(20),T(20),U(20),W(20)
0030  DIM A(20),B(20),C(20)
0040%  "EXOCYTOSIS PROGRAMME ENTERED"
0050 %  "EXPERIMENT NUMBER = ";
0060  INPUT V1$
0070 % "BACKGROUND IN CPM =";
0080  INPUT D
0090  % "TOTAL VOLUME OF UPTAKE MEDIUM, ML = ";
0100  INPUT B1
0104  % "TOTAL VOLUME OF RELEASE MEDIUM, ML =";
0106  INPUT B2
0110  % "NUMBER OF SAMPLES AT EACH TIME =";
0120  INPUT K
0130  % "NUMBER OF TIMES SAMPLES WERE TAKEN =";
0140  INPUT E
0150  % "MG SUBSTRATE/ML UPTAKE MEDIUM =";
0160  INPUT Q1
0170  % "MEAN CPM PER ML UPTAKE MEDIUM =";
0180  INPUT L2
0190  % "CORRECT CPM IN 5ML Y.S. SOLUTION =";
0200  INPUT A
```

```
0210  % "MG OF Y.S.PROTEIN =";
0220  INPUT Y
0230  FOR I=1 TO 2
0240  % "DATA FOR ";
0250  IF I=2 THEN 280
0260  % "TOTALS"
0270  GOTO 290
0280  % "SOLUBLES"
0290  % "COUNTING TIME,SECS=";
0300  INPUT C(I)
0310  % "CORRECTION FACTOR =";
0320  INPUT M(I)
0330  NEXT I
0340  % "NAME OF DATA FILE =";
0350  INPUT F1$
0360  % "NAME OF RESULTS FILE =";
0370  INPUT F2$
0380  FILES £1,F1$,£2,F2$
0390  J=0
0400  L=0
0410  FOR X=1 TO E
0420  INPUT £1,P(X), Q(X),R(X),S(X)
0430  NEXT X
0440  FOR X=1 TO E
0450  F=((R(X)*60.0/C(1))-D)*M(1)
0460  J=J+F*K
0470  REM J IS COUNTS SO FAR REMOVED
0480  T(X)=J+((B2-K)*F)
0490  REM T(X) IS TOTAL COUNTS SO FAR EXOCYTOSED
0500  G=((S(X)*60.0/C(2))-D)*M(2)
0510  L=L+G*K
0520  REM L IS SOLUBLES SO FAR REMOVED
0530  U(X)=L+((B2-K)*G)
0540  REM U(X) IS TOTAL SOLUBLES SO FAR EXOCYTOSED
0550  NEXT X
0560  V=T(E)+A
0570  REM V IS TOTAL ACTIVITY TAKEN UP
0580  FOR X=1 TO E
0582  A(X)=1000*T(X)*B1*Q1/((L2*B1+V)*Y)
0584  B(X)=1000*U(X)*B1*Q1/((L2*B1+V)*Y)
0586  C(X)=A(X)-B(X)
0590  T(X)=100*T(X)/V
0600  U(X)=100*U(X)/V
0610  REM CONVERTED TO PERCENTAGES
0620  W(X)=100*U(X)/T(X)
0625  M4=100*V/(B1*L2+V)
0630  NEXT X
0631  GOTO 930
0632  % £2,
0634  % £2,
0635  % £2,"THIS IS EXOCYTOSIS EXPT CODED ";V1$
0643  M5=Q1*B1*V/((B1*L2+V)*Y)
0645  % £2,"EFFICIENCY OF UPTAKE =";M4;"%"
0646  % £2,"TOTAL UPTAKE PER MG PROTEIN (MG) =";M5
0650  % £2,
0655  % £2,"AND NOW FOR THE JUICY BITS ..."
0660  % £2,
0670  % £2,"SERIAL NO.","TIME","% ACTIVITY ","% SOLUBLES"
```

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0680  % £2,"OF SAMPLE", "(HOURS)","EXOCYTOSED","SO FAR"
0690  % £2,
0700  % £2,
0710  FOR X=1 TO E
0720  % £2,P(X),Q(X),T(X),W(X)
0730  NEXT X
0740  % £2,
0750  % £2,
0760  % £2,
0765  % £2,"MICROGRAMS","MICROGRAMS","MICROGRAMS"
0770  % £2, "PROTEIN ","SOLUBLES","INSOLUBLES"
0780  % £2,"EXOCYTOSED","EXOCYTOSED","EXOCYTOSED"
0790  % £2,"PER MG YS","PER MG YS","PER MG YS"
0800  % £2,
0805  FOR X=1 TO E
0810  % £2,A(X),B(X),C(X)
0820  NEXT X
0825  IF P3=2 THEN 900
0826  % £2,
0826  % £2,
0830  % £2,"YS INSOLUBLES =";Z1;"MICROGRAMS/MG"
0835  % £2,"YS SOLUBLES =";Y9;"MICROGRAMS/MG"
0840  Z2=Y9+B(E)
0845  Z3=Z1+C(E)
0850  Z4=100*Z2/(Z2+23)
0855  % £2,
0860  % £2,"TOTAL SOLS PRODUCTION = ";Z2;"MICROGRAMS PER MG"
0865  % £2,"TOTAL INSOLS PRODUCTION = ";Z3;"MICROGRAMS PER MG"
0870  % £2,"THIS IS ";Z4;"% SOLUBLES"
0900  STOP
0930  % "DO YOU HAVE DATA FOR YS SOLS (1FOR Y,2 FOR N)"
0935  INPUT P3
0940  IF P3=2 THEN 632
0945  %"INPUT CORRECT SOLS CPM IN WHOLE YS"
0950  INPUT Y4
0955  Y9=1000*Y4*B1*Q1/(Y*(B1*L2+V))
0960  Z1=(1000*A*B1*Q1/(Y*(B1*L2+V)))-Y9
0965  GOTO 632
```