EVALUATION OF CROSSLINKED SOLUBLE N-(2-HYDROXYPROPYL)METHACRYLAMIDE COPOLYMERS AS A POTENTIAL DRUG CARRIER

by

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ABBREVIATIONS

ASG	Asialoglycoprotein
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
DIVEMA	Divinyl ether and maleic anhydride copolymer
EDTA	Ethylenediaminetetraacetic acid
GPC	Gel permeation chromatography
GSH	Glutathione
HMDA	Hexamethylenediamine
HPMA	N-(2-Hydroxypropyl)methacrylamide
HRP	Horseradish peroxidase
IgA	Immunoglobulin A
IgG	Immunoglobulin G
LDL	Low density lipoprotein
MTX	Methotrexate
NAP	p-Nitroaniline
PBS	Phosphate buffered saline
PVP	Polyvinylpyrrolidone
TCA	Trichloroacetic acid

ABSTRACT

Soluble crosslinked hydroxypropyl(methacrylamide) copolymers have been studied in the rat, both in vitro and in vivo, in order to assess their suitability as drug carriers in a targetable drug delivery system.

The 10 radiolabelled copolymers used were of $\bar{M}w$ 31,000 ->400,000, and their crosslinks contained oligopeptides potentially degradable by lysosomal enzymes.

In vitro studies included the uptake and intracellular degradation of copolymers of different molecular sizes by the rat visceral yolk sac and the everted rat jejunal sac. The rate of uptake of copolymer by the yolk sac decreased with increasing molecular size, but molecular size had no effect on the extent of intracellular degradation of the copolymer. The use of metabolic and lysosomal enzyme inhibitors demonstrated that uptake of copolymers was by pinocytosis and that lysosomal thiol-proteinases were at least partially responsible for the degradation observed.

Copolymers were pinocytosed and partially degraded by the rat intestine cultured in vitro and were also translocated to a small extent across the tissue. Uptake and translocation were both inhibited at 15^{o}C. Accumulation of copolymer by intestinal tissue increased with increasing copolymer size.

Following intravenous administration to rats, larger copolymer fractions were retained in the circulation and did not readily accumulate in any tissue. Copolymer crosslinks were stable in plasma.

The incorporation of a galactosamine side-chain into the copolymer resulted in rapid clearance of copolymer from the

bloodstream and accumulation by the liver. Targeting of this copolymer to the liver was also achieved, at lower rates, following intraperitoneal and subcutaneous, but not following oral administration.

Both unmodified copolymer and copolymer containing galactosamine were virtually cleared from the body within 32 days.

Rat liver lysosomal enzymes were isolated and incubated with the different copolymers. The rate of crosslink cleavage was dependent on the length and/or structure of the oligopeptide sequence.

CHAPTER 1

GENERAL INTRODUCTION

1.1 DRUG ADMINISTRATION - THE NEED FOR IMPROVEMENT

Most low molecular weight therapeutic agents diffuse readily into all cell types, and are excreted rapidly from the body. Consequently, administration of such agents, whether orally or parenterally involves frequent and repeated doses in order to maintain plasma drug levels within the desired therapeutic range. A typical pattern of plasma levels as a function of time, following a single oral dose is shown in fig1.1a, and is characterized by an initial increase in concentration, which is dependent upon the rate at which the drug becomes available to the system, and a subsequent decrease in concentration following cellular uptake of the drug, excretion, or other transport mechanisms. Fig1.1b shows the typical blood profile observed following intravenous administration of a drug, where the whole dose is introduced directly into the bloodstream and subsequently distributed as before. Difficulties in administration can occur when there is a minimum effective level of drug, or a maximum safe level, or both, and careful consideration must be given to the regular timing and quantity of drug administered in order to maintain plasma levels within the desired therapeutic range. Patient compliance is often poor, and this can lead to ineffective drug therapy or increased risk of toxic side-effects.

Optimization of drug delivery to maximise the therapeutic effect and minimise side-effects of a given drug could, in theory, be achieved by the control of blood levels, cellular uptake, metabolism and distribution of the drug within the body. Such control would allow delivery of the drug to the target tissue in the correct amount at an optimal rate to achieve the desired response.

RELATIONSHIP BETWEEN DRUG CONCENTRATION IN PLASMA AND TIME FOLLOWING a) ORAL ADMINISTRATION, AND b) INTRAVENOUS ADMINISTRATION

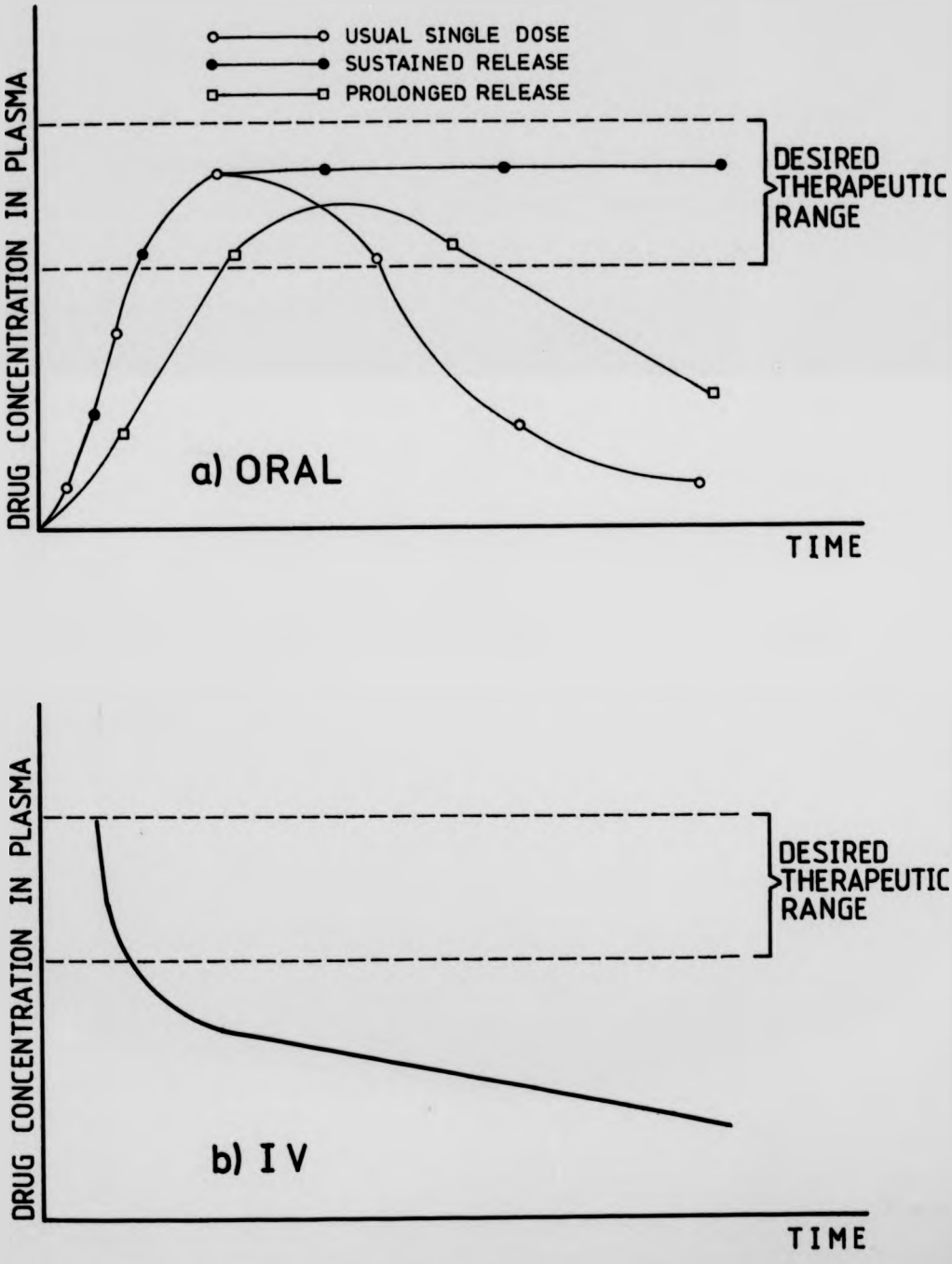


FIGURE 1.1

The need to improve drug administration has become apparent over the last 20-30 years, and over the last few years a number of systems have been proposed which aim to extend the period of time over which a therapeutic level of drug is maintained in the circulation (section 1.2). Other proposed systems aim to concentrate drug in the target tissue by the use of a 'homing device' attached to the drug (section 1.3).

This project involves the use of soluble poly N-(2-hydroxypropyl)methacrylamide copolymers as potential drug carriers in a soluble targetable drug delivery system, which aims to enhance drug uptake by the target tissue, prevent rapid excretion of the drug and to minimise non-specific side-effects due to uptake of the drug by normal tissue.

1.2 CONTROLLED DRUG RELEASE SYSTEMS

A variety of systems have been developed in which drugs are encapsulated or embedded within structures prior to their administration. Once inside the body, the drug is slowly released from its carrier. The ultimate goal for such systems is to achieve sustained release of the drug, where the rate of drug release just equals the rate of drug removal from the body by all processes over the desired time period (fig 1.1a). True sustained release preparations are, unfortunately, very difficult to produce, although prolonged action dosage forms can be achieved and offer many advantages over conventional dosage forms. Such advantages include the employment of less total drug, reduction in local and systemic side-effects, increased efficiency in treatment and less inconvenience for the patient (Ballard 1978).

It is unfortunate that the terminology used in this field of research is confused and often misleading. For the purpose of this thesis, the term 'controlled release' will be used to encompass all methods used to modify the release of drugs from carrier systems.

Controlled drug delivery systems have been designed by a number of workers, and the variety of materials, methods and routes of administration employed is diverse. For these reasons only a few specific examples of the documented systems will be discussed.

1.2.1 Enteric Coating

Enteric coating is one of the oldest methods employed to improve oral drug delivery. This can be achieved by coating tablet, capsule or granulate dosage forms with acid-resistant

polymer films which are impermeable to gastric secretions, but soluble at a higher pH, thereby releasing the drug in the small intestine. This, in theory, protects the drug from gastric degradation, protects the gastric mucosa from potentially damaging drugs (which could cause nausea and vomiting), and improves bioavailability through increased drug absorption in the small intestine. Enteric coatings are usually composed of fats, fatty acids, waxes, shellac or cellulose acetate phthalates (Ottenbrite 1980). Unfortunately, although gastric protection can be achieved, drug release in the intestine is both retarded and erratic (Sjögren & Bogentoft 1981). Orally administered multiple unit dose forms have also been described, where the preparations disintegrate in the stomach into a multitude of microencapsulated crystals, pellets or granules. These smaller units can penetrate physical barriers more easily and become distributed throughout the gastrointestinal tract (Bechgaard 1981).

1.2.2 Diffusion-controlled Systems

Diffusion-controlled reservoir systems consist of a core of drug contained within membranes, capsules, microcapsules, liposomes or hollow fibres. The development of a number of membrane-enclosed reservoir devices has proved very successful and commercially available products include Ocusert, which is applied to the conjunctiva and consists of ethylene vinyl acetate membrane-enclosed pilocarpine for the treatment of glaucoma (Stewart and Novak 1978), Progestasert, a birth control device consisting of an ethylene vinyl acetate membrane containing progesterone which is implanted in the uterus and is effective for one year (Place and Pharriss 1974), and transdermally administered scopolamine for the treatment of motion sickness (Chandrasekaran and Shaw 1980).

In most of these systems, diffusion of the drug through the polymer is the rate-limiting step. Polymers are widely used in this category e.g. silicone rubber, hydrogels and ethylene vinyl acetate copolymer, and drug diffusion takes place either through pores in the polymer structure, in the case of microporous membranes, or solution-diffusion transport in non-porous homogenous polymer films i.e. between the polymer chains.

Diffusion-controlled systems can also take the form of a solid polymeric matrix impregnated with drug. Again, drug diffusion through the polymer is the rate-limiting step, but in the case of matrices the drug is uniformly distributed throughout the polymer, and consequently drug release from these devices is not constant with time.

Advantages of matrix systems are ease of fabrication, and the ability to achieve the release of macromolecules. Langer et al (1980) demonstrated release of insulin from implanted ethylene vinyl acetate discs in diabetic rats over a period of 24 days, and also studied the release of other macromolecules from polymer matrices in vivo, including ribonuclease A (Mw 14,000), bovine serum albumin (Mw 68,000) and IgG (Mw 166,000). He proposed that the release of macromolecules takes place via diffusion of the macromolecules through channels in the matrix.

Swelling controlled systems have also been developed where the drug is initially dissolved or dispersed in a polymer solution. The solvent is then evaporated, leaving the drug incorporated within the polymer matrix. Following implantation, fluid penetrates the matrix and the polymer swells into a rubbery state which allows the drug contained in it to diffuse outward (Graham and McNeill 1984).

1.2.3 Magnetically-controlled Systems

Magnetically-controlled systems involve the dispersion of both drug and small magnetic beads within a matrix or emulsion. On exposure to an oscillating external magnetic field, drug is released at a much higher rate than in the absence of magnetic beads. Sugibayashi et al (1982) demonstrated sustained drug release and targeting of magnetic albumin microspheres containing adriamycin to the lung by magnetic means. Enhancement of antitumour effects of adriamycin on AH7974 lung metastasis in rats was observed. The mechanism responsible for the sustained release of drug from the microspheres is unclear, however, and the metabolism of systemically administered ferrite is not understood and requires further investigation.

1.2.4 Osmotic Pumps

Another novel system for optimizing drug levels in the body is the employment of osmotic pumps. Theeuwes (1980) and Eckenhoff (1981) have designed a number of preprogrammed osmotically powered pumps which contain a reservoir which can be filled with a drug suspension by a conventional syringe and filling tube. The filled pump can be implanted or administered orally or rectally, but when in contact with an aqueous environment, water moves across the membrane into the osmotic compartment and displaces an equivalent volume of drug formulation. The pumps have been shown to be precise and allow accurate control over the rate of release when used over 12 and 24 hour duration for oral and rectal administration in clinical studies.

1.2.5 Biodegradable Systems

The drug-release mechanisms discussed so far have generally been insoluble, non-degradable polymeric systems which are often used in the form of implants. Biodegradable drug delivery systems have the obvious advantage that surgical removal following implantation is not necessary since the soluble degradation products are absorbed by the body.

Biodegradable systems employ both natural and synthetic drug carriers (natural and synthetic macromolecules as biodegradable drug carriers are discussed in section 1.5). In the case of solid degradable polymeric systems, the term "bioerodible" is often used, and these may take the form of a monolithic matrix or the polymer may envelope a drug-rich reservoir, or be in the form of syringe-injectable microcapsules. The difference between non-erodible and erodible systems is that, while the polymer phase in non-erodible systems remains unchanged and drug is released by diffusion, the polymer phase in erodible systems decreases with time, allowing the drug to escape as the surrounding polymer is eroded. Heller (1980) has described three dissolution mechanisms in bioerodible systems and these are summarized in fig1.2. The first mechanism involves the use of water-soluble polymers insolubilized by degradable crosslinks. The crosslinks are hydrolytically unstable and the matrix is highly hydrophilic and completely permeated by water.

Heller investigated the release of bovine serum albumin from a hydrogel of this type and found that the rate of release was dependent on crosslink concentrations. At low crosslink concentrations the hydrogel swelled extensively and albumin was released very rapidly. The reverse was true for high crosslinker concentrations.

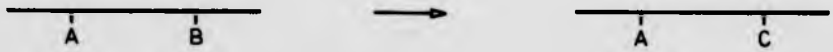
SCHEMATIC REPRESENTATION OF POLYMER DEGRADATION MECHANISMS (from HELLER and BAKER, 1980)

MECHANISM 1

TYPE 1

TYPE 2

MECHANISM II



A REPRESENTS A HYDROPHOBIC SUBSTITUENT

B—C REPRESENTS HYDROLYSIS, IONIZATION OR PROTONATION OF PENDANT GROUP

MECHANISM III

KEY
● DENOTES A HYDROLYTICALLY UNSTABLE BOND

FIGURE 1.2

The second mechanism described by Heller involves water-insoluble polymers which become soluble as a consequence of hydrolysis, ionization or protonation of pendant groups. Because no actual cleavage of the polymeric structure takes place, the solubilization does not result in any significant changes in molecular weight of the polymer.

The third mechanism includes all water-insoluble polymers that undergo hydrolytic backbone cleavage and are solubilized by conversion to small, water-soluble molecules. This category includes polylactic acid or lactic/glycolic acid copolymers, polyorthoesters, poly(ε-caprolactone) and polyamino acids (Pitt et al 1981).

Drug release from bioerodible systems is often complicated since hydrolytic erosion can occur in two different ways. Heterogeneous erosion is the term used to describe surface erosion, which is much preferred since drug release from devices that maintain constant geometry will be at a fairly constant rate. Homogeneous erosion takes place at a uniform rate throughout the matrix, which remains essentially intact until all parts reach a critical stage at which point the matrix dissolves. Bulk erosion of this nature may occur in addition to surface erosion and also diffusion, thereby making the prediction of drug release rates from such systems extremely difficult.

1.3 LYSOSOMOTROPIC DRUG DELIVERY

The drug release systems so far discussed have involved the controlled release of free drug into the circulation from various devices which are usually contained within the body.

Another approach to the problem of drug administration is by the use of soluble macromolecules as carriers of chemically bound drugs. Such systems enable the transport of the drug-carrier complex directly to cells following its introduction into the circulation.

The attachment of low molecular weight drugs to macromolecules prevents indiscriminate uptake of the drug into cells, since the complex is too large to diffuse through cell membranes. Cellular uptake of macromolecules is restricted to a mechanism known as endocytosis. Following endocytic uptake by cells, the complex is delivered to lysosomes, where free active drug is released.

The term 'lysosomotropic' has been used by de Duve et al (1974) to describe all substances that are taken up selectively into lysosomes, irrespective of their chemical nature or mechanism of uptake. The attachment of a low molecular weight drug to a macromolecule therefore transforms the drug into a lysosomotropic agent. Substances proposed as lysosomotropic drug-carriers include soluble synthetic polymers, nanoparticles, liposomes and natural macromolecules.

1.3.1 Endocytosis

Endocytosis is a term that encompasses both phagocytosis and pinocytosis. Both processes are energy-dependent and involve the invagination and subsequent internalization of regions of

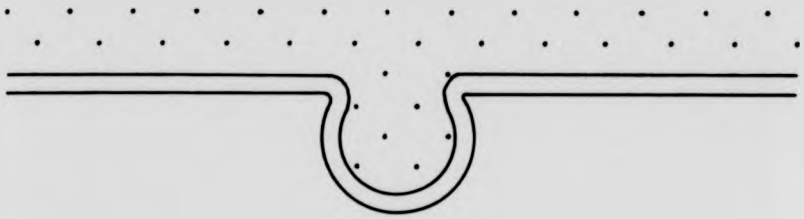
the plasma membrane of the cell to form intracellular membrane-bounded vesicles which contain extracellular fluid, dissolved solutes and substances bound to the plasma membrane at the site of invagination. These vesicles may ultimately fuse with lysosomes, which are membrane-bounded organelles containing acid hydrolases capable of digesting many natural macromolecules.

Phagocytosis generally refers to the adsorptive interiorization of particles of greater than approximately 1.0 μm in diameter by specialised phagocytic cells. This adsorption to the cell membrane may be the result of specific interaction between the particle and a specific receptor present on the plasma membrane, or it may be a non-specific phenomenon, as occurs in the binding of polystyrene beads (Steinman et al 1983). Phagocytosis is the feeding mechanism employed by many unicellular organisms e.g. Amoeboe, and the ability of macrophages to phagocytose invading micro-organisms plays an important role in animal defence systems.

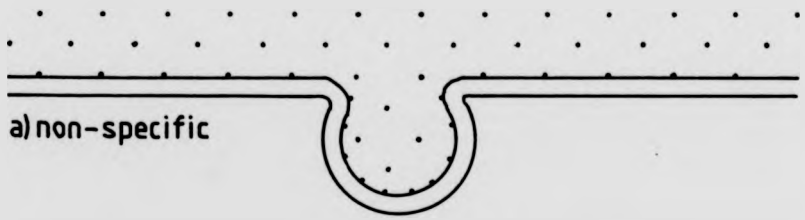
Pinocytosis is an on-going process of membrane internalization and extracellular fluid uptake common to most, if not all, cell types, and gives rise to vesicles 0.1-1 μm in diameter. It is the only mechanism by which most soluble macromolecules can enter cells. Pinocytosis can capture solutes either in the fluid-phase only or by an adsorptive mechanism (fig1.3). Fluid-phase pinocytosis is the term used when molecules present in the extracellular fluid enter the cell with no interaction with the cell membrane. When a molecule interacts with the cell membrane, molecular uptake is said to be through adsorptive pinocytosis. This interaction, too, may be in a non-specific way, such as the cationic binding of ferritin to anionic sites on the cell membrane or it may be receptor-

CELL SURFACE EVENTS IN PINOCYTOSIS

FLUID-PHASE PINOCYTOSIS



ADSORPTIVE PINOCYTOSIS



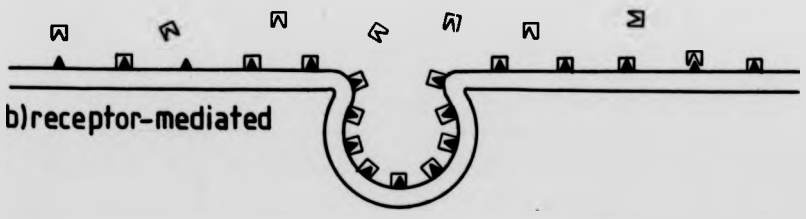


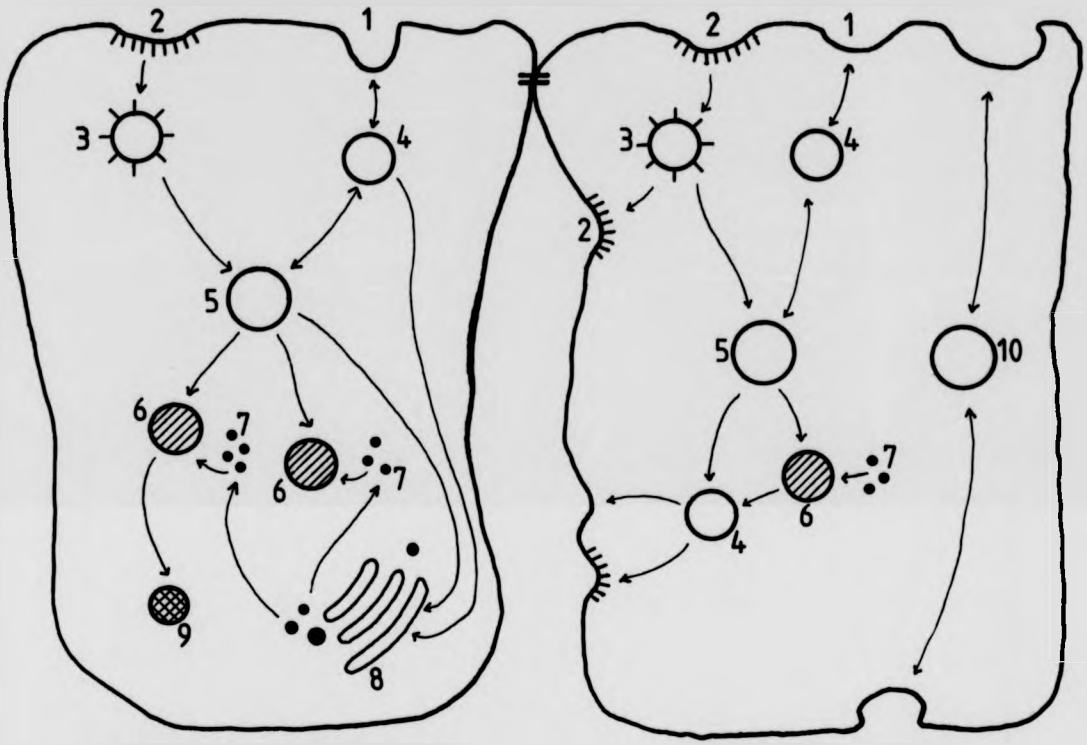
FIGURE 1.3

mediated. Receptor-mediated pinocytosis involves the interaction between a receptor component present on the cell membrane and a specific moiety present on the molecule to be internalized. Molecules known to be taken up by receptor-mediated pinocytosis include lysosomal enzymes, polypeptide hormones such as epidermal growth factor, insulin, plasma transport proteins e.g. low density lipoprotein and α_2-macroglobulin (Besterman et al 1983).

Figure 1.4 shows the membrane-bounded structures involved in the endocytic lysosomal pathway and also shows another membranous organelle, the Golgi apparatus, which is involved in primary lysosome formation, the formation of secretory vesicles, and also other intracellular vesicular pathways such as translocation of proteins and possibly membrane recycling.

Many molecules which bind to specific receptors at the cell surface prior to pinocytosis are found to be clustered within differentiated regions of the plasma membrane known as coated pits. The coat is composed of the protein clathrin (Mw 180,000) and is situated on the cytoplasmic side of the plasma membrane. Studies by Helenius et al (1980), and Anderson, Goldstein et al (1977) on viruses and LDL respectively, have shown that coated pits transform to clathrin coated vesicles of 0.1-0.2 μm in diameter on internalization of plasma membrane. Coated vesicles were first noted by Roth and Porter in oocytes (1964) and by Fawcett in reticulocytes (1964). In 1969 Kanaseki and Kadota noted that these coats, when seen in thin section, resemble a basket composed of hexagonal and pentagonal arrays. Clathrin coated vesicles have also been implicated in the transport of secretory products (Palade 1975 and 1982) and membrane proteins (Rothman, Pettegrew and Fine 1980) from the

SCHEMATIC REPRESENTATION OF SOME ENDOCYTIC PATHWAYS



FLUID AND ADSORPTIVE PINOCYTOSIS

TRANSCELLULAR TRANSPORT

KEY	
1. membrane invagination	6. secondary lysosomes
2. coated pit	7. primary lysosomes
3. coated vesicle	8. golgi complex
4. non-coated vesicle	9. residual body
5. endosome	10. transcellular vesicles

FIGURE 1.4

endoplasmic reticulum to the Golgi, the transport of lysosomal enzymes from the Golgi to the lysosomes (Friend and Farquhar 1967, Bainton and Farquhar 1970), the transport of membrane components from the Golgi to the cell surface (Rothman, Pettegrew and Fine 1980) and the recovery of membrane from the cell surface and its recycling to the Golgi complex (Ottosen, Courtoy and Farquhar 1980). The exact function of the clathrin coat, which does not appear to surround fluid-phase pinocytic vesicles, remains unclear.

Clathrin-coated vesicles are not permanent features of receptor-mediated endocytic pathways, however, since within seconds to minutes after the formation of the vesicle, the coat is lost and larger smooth surfaced vesicles are observed which appear to be formed by fusion of smaller pinocytic vesicles. This fusion is also reported to occur in fluid-phase pinocytosis. These larger vesicles have been termed endosomes by Helenius et al (1983) or receptosomes by Pastan and Willingham (1983). They appear to be acidic, pre-lysosomal compartments, free from enzyme activity, less dense than lysosomes, and showing a greater density of ligand (endocytosed material) than the plasma membrane. Helenius has proposed that the acidification of endosomes is due to the activity of a H^+-ATPase similar to that believed to operate in lysosomes and secretory granules, and that the resultant low pH would cause dissociation of the receptor-ligand complexes, generating free receptors for return to the cell surface without requiring transit through the perilous environment of the lysosome.

Intracellular vesicles are believed to pinch off from endosomes and migrate towards the perinuclear region of the cell,

ultimately fusing with lysosomes. The exposure of the vesicular contents to lysosomal enzymes results in the catabolism of any degradable material. The small molecules produced as a result of degradation escape across the lysosomal membrane and are utilised or released by the cell, whereas any large non-biodegradable material remains trapped in the lysosome. This material may be released by the cell through exocytosis, which may be described as the reverse of endocytosis, or it may remain as residual bodies within the cell.

Since direct evidence for the recycling of endocytosed membrane was put forward by Mellman et al 1980, and by Schneider, de Duve and Trouet in 1979, a considerable amount of interest has been taken in the vacuolar system of the cell, and a number of different hypotheses have been proposed by many authors to explain membrane recycling, cell-surface receptor recycling and the role of coated vesicles and endosomes.

Although the precise intracellular events leading to lysosomal hydrolysis of pinocytosed material is at present not precisely defined, many studies on the uptake of macromolecules by various cell types have helped to identify some of the factors which govern the rate at which pinocytosis takes place. The rate of uptake of a molecule by fluid-phase pinocytosis is dependent upon both the concentration of the extracellular material and the rate of vesicle formation by the tissue, since there is no interaction of the molecule with the cell membrane. The rate of vesicle formation, however, is dependent on temperature. Duncan and Lloyd (1978) demonstrated a substantial reduction in the rate of pinocytosis of ^{125}I-labelled polyvinylpyrrolidone by rat visceral yolk sacs by lowering the temperature by only 7^{o}C to 30^{o}C, and pinocytosis is completely inhibited at 4^{o}C.

Adsorptive pinocytosis may be receptor-mediated or non-specific in nature. The rate of uptake of a macromolecule by receptor-mediated pinocytosis will be determined by the specificity of the receptor-ligand interaction and also the number of receptors present on the cell surface at any given time. Adsorption to the cell membrane, whether specific or not, results in a greater concentration of the molecule on the cell surface during vesicle formation and cellular uptake of that molecule will therefore be at a higher rate than a molecule present at the same concentration entering the cell by fluid-phase pinocytosis.

Various physical parameters also govern the rate at which macromolecules can enter cells, such as charge, hydrophobicity, and size. It is generally agreed that cationic groups, when present on the surface of a macromolecule, bind to the negatively charged areas of plasma membrane, giving rise to enhanced pinocytic uptake by cells. There is, however, conflicting evidence with regard to possible stimulation of vesicle formation by cationic molecules. Shen and Ryser (1968, 79) maintain that basic polyamino acids, in particular poly-L-lysine markedly enhances the uptake of ^{131}I-BSA in Sarcoma S18011 tumour cells, and that negatively charged poly-L-glutamic acid decreased albumin uptake. Pratten et al (1978) concluded that poly-L-lysine, poly-L-ornithine, poly-L-glutamic acid and a number of dextran derivatives all failed to affect greatly the rate of pinocytic uptake of ^{125}I-PVP by rat visceral yolk sacs or rat peritoneal macrophages, thereby indicating no change in the rate of vesicle formation, although they agreed that cationic molecules entered cells by adsorptive pinocytosis.

The hydrophobicity of a macromolecule will also affect its uptake by pinocytosis. Hydrophobic molecules will generally bind to hydrophobic groups present on the lipid cell membrane. The binding of formaldehyde denatured BSA to yolk sac membranes (Livesey and Williams 1977) is probably due to the denaturation process resulting in the exposure of hydrophobic groups normally buried within the structure of the albumin which bind to hydrophobic groups on the yolk sac surface. Recent work by Duncan et al (1984) using HPMA copolymers bearing different concentrations of tyrosinamide residues has shown that uptake of the copolymers by rat yolk sacs increased with increasing tyrosinamide content. This can be explained by increasing hydrophobicity with increasing tyrosinamide content resulting in increased capacity for non-specific adsorptive pinocytosis.

The size of a macromolecule will also determine its rate of pinocytic uptake by cells. Cells of the reticulo-endothelial system have been shown to pinocytose larger molecules preferentially. Kooistra et al (1980) cross-linked ribonuclease with dimethyl suberimidate to form dimers and larger polymers without affecting the charge of the protein, and found that the larger molecules were taken up at a much higher rate than the monomers. Ryser in 1968 also found that larger polyornithine macromolecules are taken up more readily than smaller ones ($\bar{M}w$ range 4,000 to 200,000) in Sarcoma S180 11 cells, although since the molecules used were basic, size would also determine the number of positive charges capable of interacting simultaneously with the cell surface, and therefore the effect of size cannot be separated from the effect of charge. More recently, Duncan et al (1981) have demonstrated a positive correlation between molecular size and pinocytic uptake of ^{125}I-PVP in macrophages (cells of the RE

system), but rat visceral yolk sacs were shown to preferentially pinocytose the smaller molecular weight fractions.

1.3.2 Macromolecules as Drug Carriers

De Duve et al (1974) have suggested that lysosomotropic therapy may be beneficial in many diseases, including lysosomal storage diseases, silicosis, gout, bacterial infections, parasitoses, and also for cancer chemotherapy. If a drug is bound to a macromolecule in such a way that the complex is stable in the bloodstream, but not in the acidic, hydrolytic environment of the lysosome, active drug will be released in the lysosome, and if small enough will traverse the lysosomal membrane and be able to act within the cytoplasm.

Natural Macromolecules

Trouet et al (1972) pointed out that the intracellular concentration of drugs when attached to digestible natural macromolecular carriers is dependent on the pinocytic activity of the cells and on the digestive potential of their lysosomes, and given that many tumour cells have high endocytic activity, cancer chemotherapy could be improved by lysosomal therapy, providing that the drug itself was resistant to lysosomal inactivation. Trouet used daunorubicin, an effective chemotherapeutic agent, but also one which displays serious toxic side-effects, non-covalently bound to a polymeric DNA carrier, in an attempt to improve drug therapy and reduce general toxicity. To begin with, DNA-daunorubicin complexes were incubated with lysosomal extracts at pH 3.5 and pH 5.5. Results demonstrated dissociation of the complex at acid pH, the degradation of the DNA carrier and also drug activity. Intraperitoneal injections of the complexes into DBA/2 or NMRI

mice showed less toxicity of daunorubicin when attached to DNA than when administered as free drug. When mice were inoculated intraperitoneally with L1210 leukaemia cells, and subsequently treated with either free daunorubicin or DNA-daunorubicin at non-lethal doses, the survival curves were comparable. However, since the drug-carrier complex was shown to be less toxic than free drug, higher doses could be administered, and the period of survival was increased. Trouet found that the route of administration of both L1210 cells and drug-carrier complex was of great importance. Intraperitoneal administration of DNA-daunorubicin following intravenous injection of L1210 cells was less effective than if both were administered via the same route. DNA was stated to be an unsuitable carrier because of probable antigenicity and later work revealed that the non-covalent linkage of daunorubicin to DNA was not stable in the bloodstream. Adriamycin, another anthracycline used in cancer chemotherapy, was shown to be much more stable in the bloodstream when complexed with DNA, and was also shown to improve survival times and reduce toxicity (Trouet et al 1979). Covalent linkage of drugs to macromolecular carriers, giving rise to complexes completely stable in the bloodstream, was described by Masquelier et al (1980). This was achieved by linking daunorubicin or adriamycin to proteins and polypeptides using the aminosugar group of the anthracycline to obtain a peptidic linkage with carboxylic side chains of the carriers. Daunorubicin was coupled to bovine serum albumin using carbodiimide as a coupling agent, but it was found that in order to achieve enzymatic hydrolysis of the conjugate within the lysosome, a spacer sequence, composed of up to four aminoacids, was required. It was found, using lysosomal

hydrolases, that the greater the length of the spacer between the drug and the carrier, the greater the enzymatic cleavage of drug from the carrier. Trouet et al (1981) have found that alternating sequences of alanine and leucine form suitable peptidic spacers for drug attachment, providing good water-solubility and demonstrating lysosomal hydrolysis when BSA is conjugated to daunorubicin. When these conjugates were injected intraperitoneally in mice following intraperitoneal inoculation with L1210 cells, results demonstrated high therapeutic activities with a high proportion of long-term survivors after 30 days.

Other lysosomotropic agents used by Trouet's group as drug carriers include liposomes of various sizes and composition. They have entrapped daunorubicin and vincristine in multi-lamellar, unilamellar, positively and negatively charged liposomes, but have found no chemotherapeutic advantage in the use of liposomes. The disadvantages of liposomes as drug carriers include blood instability and the very high uptake of liposomes by the liver, lung, spleen and other cells of the recticuloendothelial system, thus precluding the selective uptake of drug by other tissues and organs (Trouet et al 1980).

Chu and Whiteley (1977) also used BSA as a macromolecular drug carrier, but covalently bound methotrexate (MTX) using carbodiimide. MTX-BSA was found to be as effective as free MTX in the treatment of L1210 cells in mice, and a higher, more prolonged serum concentration and a decreased rate of excretion of BSA-MTX than free MTX was noted. These observations indicate that BSA, as might be expected of a serum protein, is taken up slowly by cells by fluid-phase pinocytosis, rather than rapidly by adsorptive pinocytosis,

and that the size of the conjugate exceeds the renal threshold which is therefore retained in the bloodstream for a prolonged period of time.

Chu and Whiteley (1977) also covalently linked MTX to dextran derivatives of various molecular weights ($\bar{M}w$ 10,000-150,000). The conjugates were found to remain in the circulation for longer, and to produce significant levels of MTX within the L1210 cells, but were ineffective antitumour agents. A possible explanation for the ineffectiveness of the dextran-MTX is that the drug linkage was unsuitable for intralysosomal enzymatic hydrolysis, and that free active drug was therefore not released.

Targeting of Macromolecules

The ability not only to restrict drug uptake to pinocytosis, but also to restrict pinocytosis largely to a particular cell type can be achieved by the use of known receptor recognition systems. Receptor-mediated pinocytosis takes place more rapidly than fluid-phase pinocytosis, and this leads to rapid clearance of the macromolecule-drug conjugate from the bloodstream with accumulation of drug in the target tissue.

There are a limited number of such cell recognition systems known in which carbohydrate moieties participate. These include the recognition of galactose-glycoproteins by hepatocytes (Ashwell and Morrell 1974), mannose/acetylglucosamine terminating glycoproteins by Kupffer cells of the liver (Stahl et al 1978), and phosphomannose residues by fibroblasts (Natowicz et al 1979).

Fuime et al (1981) achieved hepatocyte targeting of antiviral drugs coupled to galactose-terminating glycoproteins

desialylated fetuin (isolated from foetal calf serum) and lactosaminated serum albumin in mice. Unfortunately, naturally occuring glycoproteins, such as fetuin, can be obtained in only relatively small amounts. Furthermore, the use of lactosaminated serum albumin as a drug carrier, though easily obtained in large amounts, is questionable because of its possible immunogenicity. Immunological studies on lactosaminated serum albumin are currently taking place.

Targeting of lysosomotropic drug carriers has been achieved by the use of immunoglobulin G (IgG) either as a carrier itself, or as a targeting moiety attached to an intermediate carrier. In order to achieve selectivity in cancer chemotherapy, it is possible to raise monoclonal antibodies against a specific tumour receptor. Arnon (1981) and Ghose et al (1981) have described the production of antibodies to several tumour systems. The results reported by both groups indicate that antibodies may serve as carriers for anticancer drugs to tumour cells but that the main difficulty anticipated is the preparation of suitable antibodies which will be specific only for the tumour cells and will not interact with normal tissue. The authors also point out that the antibodies used should be immunospecifically purified to avoid the presence of high amounts of irrelevant IgG molecules.

Unusual receptors are expressed by certain tumours and therefore allow specific antibody production (Connors 1981). However, these receptors are often present on the cell surface at very low concentrations, and in such cases it might not be possible to target sufficient drug to the cell to destroy it. A possible solution to this problem is the use of toxins rather

than conventional drugs. The conjugation of antibodies to various toxins has been reviewed by Edwards et al (1981). Several groups of workers have attached extremely potent natural cytotoxic agents, such as diphtheria toxin, abrin and ricin to antibodies. The advantage conferred on such a complex is that a much smaller quantity of drug is necessary to destroy the cell, and this is particularly useful where receptors are present in low concentrations. Unfortunately, the glycoproteinous nature of such toxins can interfere with the targeting and binding, and hence the specificity of antibody conjugates to cells. Various strategies have been devised in order to eliminate this problem. A further complication of the use of antibodies as drug carriers is the chemistry of the conjugation procedure. The crosslinking of two proteins is difficult, and the exact location of the bound drug on the IgG molecule is often unknown. It is naturally desirable to ensure that binding of the drug does not occur on the Fab fragments of the antibody, which are essential for receptor recognition. Furthermore, solubility problems arise on direct conjugation to antibody of high levels of hydrophobic anticancer agents. To facilitate improved drug binding, and drug loading, with intralysosomal release of active drug, it is possible to bind the drug to the antibody by means of an intermediate carrier.

Poznansky and Bhardwaj (1981) have described a model for enzyme therapy by chemically crosslinking 1,4-glucosidase, homologous albumin and IgG raised against isolated rat hepatocytes in order to lower hepatocyte glycogen content in Type 11 glycogenesis (Pompe's disease). Also, dextran has been used as an intermediate carrier of an alkylating agent using

antitumour IgG (Rowland 1977) and has been shown to be effective in suppression of tumour growth in mice.

Trouet et al (1981) suggest that although antibodies are the most obvious candidates for drug targeting, there are two problems which could preclude their use as drug carriers. Firstly, the receptor-mediated uptake of antibodies might not lead to lysosomal hydrolysis of the conjugate and drug release, since it has been observed that in many tissues IgGs are selectively transported across the cells intact i.e. without fusion with lysosomes. Secondly, the reported release of some tumour antigens into the bloodstream would lead to antibody-antigen complex formation and subsequent non-specific endocytosis by cells of the reticuloendothelial system. Preliminary results by various workers using antibodies as drug carriers, or targeting moieties have, however, proved promising in many cases.

Soluble Synthetic Macromolecules

The use of soluble synthetic polymers as drug carriers offers some advantages over natural macromolecules. Polymers can be 'tailor-made' for a specific purpose, the possibilities in design and structural variations being much greater than in natural macromolecules. Ringsdorf (1975) proposed a model which demonstrates the many chemical possibilities of design in synthetic polymeric carriers (fig1.5), enabling the synthesis of macromolecules with the desired biological properties. The polymer may be degradable, or non biodegradable, hydrophilic or lipophilic, drugs may be attached permanently, or temporarily, by use of a spacer group. Targeting residues may be specific e.g. antibodies, or non-

SYNTHETIC POLYMERIC DRUG DELIVERY SYSTEM (AFTER RINGSDORF, 1975)

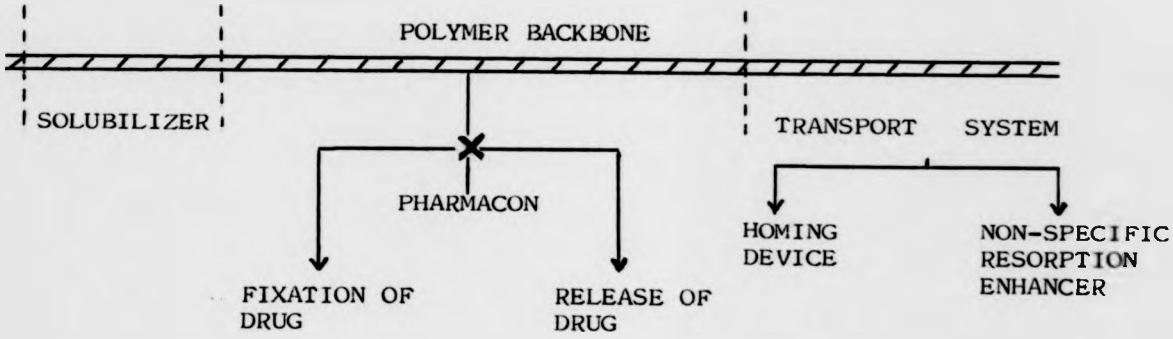


FIGURE 1.5

specific e.g. polyelectrolytes. By careful design, therefore, polymer conjugates can be synthesized with prolonged action, retarded excretion, reduced toxicity and resulting in the required body distribution of drug.

Vinyl polymers e.g. PVP, cationic e.g. poly(L-lysine) and anionic polymers e.g. divinylether-maleic anhydride copolymer (DIVEMA) and others have been proposed as potential drug carriers (Zaharko et al 1979). The effect of charge on polymeric uptake has been studied extensively by Shen and Ryser (1978, 1981) who found that poly-L-glutamic acid, a polyanion, inhibited pinocytosis, but that polycations, in particular poly(L-lysine), enhanced pinocytosis. When methotrexate was covalently linked, using a triglycine spacer, to poly(L-lysine) ($\bar{M}w$ 60,000), the conjugate entered Chinese Hamster Ovary (CHO) cells forty times faster than the free drug. Furthermore, CHO cells resistant to MTX because of a genetic defect in drug transport, demonstrated uptake of conjugate two hundred times faster than the free drug, causing at least 90% inhibition of cell growth. Shen and Ryser (1981) have also developed a pH sensitive cis-aconityl spacer between daunomycin and poly(L-lysine), which has been shown to release free active daunomycin with a hydrolysis half-life of less than three hours at pH 4 and more than ninety-six hours at pH 6 or higher.

The ability of polycations to bind cell membranes in a non-specific way, however, may preclude their use as selective drug carriers in vivo.

1.4 N-(2-HYDROXYPROPYL)METHACRYLAMIDE - A SOLUBLE SYNTHETIC DRUG DELIVERY SYSTEM

A number of considerations must be taken into account when selecting a polymer for use in drug delivery systems. Ottenbrite (1980) listed several criteria for polymers to be used in medicine. They must have:

High purity

Chemical, physical and mechanical properties to meet the proposed function

Ease of fabrication

High stability

Sterilizability

Furthermore, tissue and blood compatibility are essential requirements for polymers to be used in biological systems. Polymers should be non-immunogenic, non-allergenic, non-carcinogenic, non-irritating, nor should they cause acute or delayed toxicity.

The hydrophilic polymer N-(2-hydroxypropyl)methacrylamide was proposed as a blood plasma expander by Kopeček and Bažilová in 1973. The polymer was shown to be compatible with blood and other living tissues. It was shown to be non-toxic, apyrogenic, not to elicit antibody formation (Kopeček et al 1973, Kopeček 1982), nor activate the lymph nodes (Korčáková et al 1976). However, since HPMA is a synthetic polymer which is not degraded in the organism, the problem of possible accumulation of the polymer within the body was investigated. Previous studies, using non-degradable PVP as a blood plasma expander had demonstrated accumulation of the polymer in cells of the reticuloendothelial system, giving rise to the formation of 'foamy' cells in the spleen, lymph nodes, bone marrow, liver, lungs and thymus. The consequences of such deposition have not

been resolved. Some authors assume the condition to be temporary, and others regard it as irreversible, causing a decrease in phagocytic activity of the tissues (Ravin et al 1952, Šprincl et al 1976). Šprincl et al, in 1976, fractionated HPMA (then under the working name of Duxon), to produce samples of $\bar{M}w$ 11,000-91,000. These were injected into rabbits and the elimination and retention of each sample investigated over a period of six months. It was found that polymers of a mean molecular weight below the renal threshold (approx. 50,000 for dextran) were not significantly accumulated by the animals over the experimental period, but were excreted predominantly by the kidney.

The evaluation of soluble HPMA copolymers as potential drug carriers has been the subject of a collaborative project between Professor J.B. Lloyd and Dr. R. Duncan at the University of Keele, and Dr J. Kopeček and co-workers at the Czechoslovak Academy of Sciences in Prague, for the past six years.

The drug delivery system conforms to the model proposed by Ringsdorf (section 1.3 fig 1.5), and consists of a hydrophilic copolymeric backbone to which drugs, targeting moieties and other residues, if required, can be attached. The detailed structures of the copolymers used in this study will be discussed in Chapter 3, but in all cases their synthesis involves the reaction of N-(2-hydroxypropyl)-methacrylamide with p-nitrophenyl esters of N-methacryloylated amino acids to produce copolymers containing oligopeptide sidechains which can be designed to be susceptible to enzymatic hydrolysis. These sidechains are usually di-, tri- and tetrapeptides which are suitable for drug attachment and the binding of targeting residues.

Where hydrolysis of a sidechain is required for drug release, it is necessary that the degradable bond should originate in the amino acid specific for the given enzyme. Copolymers containing oligopeptide sidechains of different amino-acid sequences have been synthesized using p-nitroanilides as terminal drug analogues, and some have been shown to be susceptible to hydrolysis by various enzymes. Kopeček (1981) demonstrated chymotrypsin catalysed hydrolysis of p-nitroanilides of phenylalanine and tyrosine from sidechains of HPMA copolymers and also noted an increase in susceptibility to chymotrypsin attack with an increasing spacing of the phe/tyr residue from the copolymer backbone. This latter observation can be attributed to greater spacing allowing more efficient binding of the substrate to the active site of the enzyme. Efficient binding is naturally also dependent upon specific interactions between the amino acids present within the substrate and discrete regions within the active site. Consequently both the amino acid sequence and the number of amino acids present in a given oligopeptide sidechain will determine the overall activity of an enzyme towards that particular substrate (Schechter and Berger 1967, 1968). In the case of chymotrypsin, the presence of L-phenylalanine, L-tyrosine or L-leucine as a terminal residue on the sidechain is necessary for enzymatic cleavage, and the rate of cleavage is a function of its spacing from the polymer backbone. A hydrophobic residue in the penultimate position has been shown to further enhance the rate of degradation.

Studies on twenty two HPMA copolymers, each containing a different, potentially degradable sidechain, were carried out by Duncan and Lloyd (1980) using mixed lysosomal enzymes derived from rat liver. Four of the sidechains were digested

by the enzymes, liberating a terminal p-nitroaniline residue. Further studies by Duncan et al (1982) in the presence of the thiol glutathione, demonstrated hydrolysis of twelve of the sixteen sidechains investigated, to release p-nitroaniline. The addition of thiol-proteinase inhibitor leupeptin to the incubation medium caused total or partial inhibition of degradation in all but one case, indicating the involvement of lysosomal thiol-proteinases, at least in part, in the degradation of these sidechains.

Duncan et al (1981) incubated four ^{125}I-labelled HPMA copolymers ($\bar{M}w$ 50,000) each containing a different oligopeptide sidechain with rat visceral yolk sacs in vitro. All copolymers were found to be taken up by fluid-phase pinocytosis and three of the substrates were hydrolysed by the tissue, resulting in the release of radiolabelled low molecular weight sidechain degradation products back into the medium. The thiol-proteinase inhibitor leupeptin, when added to the medium, did not affect the rate of pinocytosis, but inhibited the degradation of the copolymers to varying degrees, depending on sidechain composition.

Other in vitro studies on HPMA copolymers have shown that oligopeptide sidechains are stable in rat plasma and rat serum (Kopeček, 1984).

Studies are currently in progress which aim to design oligopeptide sidechains in HPMA copolymers which are specific to lysosomal enzymes (such as Cathepsin L), are stable in the bloodstream, and result in controlled rate of drug release within lysosomes.

HPMA copolymers bearing galactose residues have been targeted to the liver following intravenous injection in rats. Copolymers, containing both galactose residues and potentially degradable sidechains terminating in a ^{125}I-labelled tyrosine residue, were cleared rapidly from the bloodstream and 69% of the recovered radioactivity was found to be contained within the liver one hour after administration (Duncan et al 1983). This may be attributed to receptor-mediated pinocytosis of the modified copolymer by liver hepatocytes (section 1.3). Five hours after administration, this level had dropped to 9%, indicating cleavage inside the liver and release of the radio-labelled tyrosine residue from the liver during that period of time.

From the experiments described, it is apparent that HPMA copolymers can be taken up by cells by fluid-phase pinocytosis with resultant liberation of a drug analogue, and that it is possible to target the copolymers to certain tissues by the attachment of specific moieties to the polymer backbone.

The size of the drug carrier is an important consideration. Ideally, the molecule should be of a molecular weight high enough to prevent rapid excretion by the kidney i.e. above the renal threshold, but low enough to permit pinocytic uptake by a range of cell types. Cells of the reticuloendothelial system preferentially capture larger molecules, whereas some epithelial cells preferentially pinocytose smaller macro-molecules (Duncan et al 1981).

It is very desirable that the polymer is eventually excreted from the body. An unfortunate problem encountered with synthetic polymers, as previously described, is the possible toxic accumulation of non-degradable molecules within the

lysosomes of cells. If a non-degradable polymer conjugate of molecular weight higher than the renal threshold (approx. 60,000) is administered, the polymer is retained within the circulation until it is endocytosed by cells, where it will accumulate following drug release. Following exocytosis or cell death, the polymer may again be released into the circulation, but will still be 'trapped' in the body unless it is eventually transported into the intestine via the liver.

On the other hand, if a polymer of molecular weight lower than the renal threshold is administered, rapid clearance of the conjugate from the body will occur through glomerular filtration, and efficient drug delivery will not be achieved.

An ingenious potential solution to this problem is the cross-linking (below the gel point) of relatively short polymer chains using oligopeptides containing enzymatically degradable bonds and diamines. These crosslinks, like the sidechains used for drug attachment, should be stable in the circulation, but susceptible to enzymatic hydrolysis within lysosomes following pinocytosis of the macromolecule. The resultant copolymer chains would still be too large to permit their passage across the lysosomal membrane, but their release back into the circulation would follow exocytosis or cell death. The polymer chains would then be of sufficiently low molecular weight to be excreted by the kidney.

Soluble crosslinked copolymers have been synthesized by Kopeček et al and certain crosslinks have been shown to be cleaved by papain, chymotrypsin and trypsin (Rejmanová et al 1981, Ulbrich et al 1980, 81). Again, the structure of the oligopeptide sequences must be chosen so as to correspond to

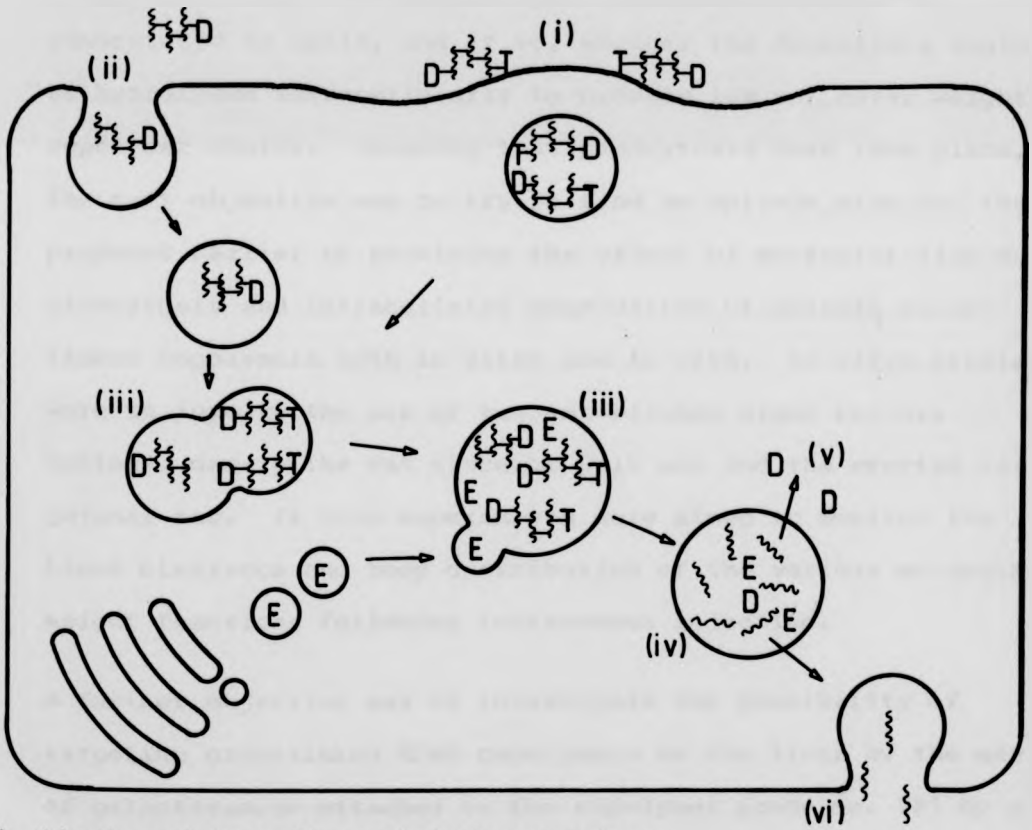
that of the active site of the enzyme responsible for the degradation. The rate of degradation by these enzymes was again found to be dependent upon the number of amino-acids present in the oligopeptide sequence. Extension of the oligopeptide sequence by one amino acid residue (from tripeptide to tetrapeptide) was found to increase the extent of degradation of the crosslinks significantly. Steric factors which may impede degradation of crosslinks in addition to those described for sidechains, include the possible conformational changes of the oligopeptide sequences when attached to polymer coils at both ends, and the presence of two or more polymer coils impeding access of the enzyme active site to the bond to be cleaved. Results, however, indicate up to 100% cleavage of certain crosslinks containing tetrapeptidic on incubation with chymotrypsin (Rejmanová et al 1981).

Preliminary in vivo experiments on soluble crosslinked copolymers were carried out by Kopeček et al (1981). A high molecular weight HPMA fraction was administered intravenously to rats, urine was subsequently collected and lower molecular weight HPMA fractions were qualitatively detected using GPC. The average molecular weight of the copolymer detected in the urine was close to that of the aminolysed polymeric precursor of the crosslinked HPMA copolymer used.

A schematic diagram to show the cellular events involved in the uptake and intracellular hydrolysis of the proposed drug delivery system following parenteral administration is shown in fig 1.6.

SCHEMATIC REPRESENTATION OF THE PINOCYTIC CAPTURE AND INTRACELLULAR HYDROLYSIS OF A SOLUBLE CROSSLINKED POLYMERIC DRUG CARRIER

KEY	
D	SOLUBLE CROSSLINKED COPOLYMERS BEARING DRUG (D) contains no targeting moiety and enters cells slowly by fluid-phase pinocytosis
T D	CONTAINS TARGETING MOIETY (T) enters cells rapidly by receptor-mediated pinocytosis
E	ENZYMES PRESENT IN LYSOSOMES



(i) binding of targeting moiety to receptors

(ii) membrane invagination to form a pinocytic vesicle

(iii) vesicle fusion

(iv) lysosomal degradation of crosslinks and polymer drug linkages

(v) release of drug

(vi) exocytosis with release of low molecular weight polymer chains

FIGURE 1.6

1.5 AIMS OF CURRENT STUDY

Soluble synthetic polymers are often both polydisperse and not biodegradable. The copolymers used in this study (kindly synthesized by P. Rejmanová and J. Kopeček) contained potentially degradable crosslinks, and one copolymer (code no. 39) had been fractionated to produce five samples of known mean molecular weight and low polydispersity.

One of the primary aims of this project was to ascertain whether or not soluble crosslinked copolymers could be pinocytosed by cells, and if so, whether the crosslinks could be hydrolysed intracellularly to produce low molecular weight copolymer chains. Assuming that pinocytosis does take place, the next objective was to try to find an optimum size for the proposed carrier by examining the effect of molecular size on pinocytosis and intracellular degradation of soluble crosslinked copolymers both in vitro and in vivo. In vitro studies were to involve the use of two established organ culture systems, namely the rat visceral yolk sac and the everted rat jejunal sac. In vivo experiments were aimed to monitor the blood clearance and body distribution of the various molecular weight fractions following intravenous injection.

A further objective was to investigate the possibility of targeting crosslinked HPMA copolymers to the liver by the use of galactosamine attached to the copolymer (code no. 78) by a diglycyl spacer. The targeting of galactosamine containing macromolecules to the liver has been described previously (Duncan et al 1983) following intravenous administration of a potential carrier, but very little is known about the precise movement of macromolecules containing a targeting moiety when administration is by other routes. The possibility of targeting

a crosslinked copolymer to the liver by intravenous, intraperitoneal, subcutaneous and oral routes was therefore of interest, and also the comparison of body distribution with a control copolymer (code no. 79) of approximately the same molecular weight, crosslink structure and polydispersity, but lacking the targeting moiety.

A further aim was to demonstrate complete clearance of the copolymers from the bodies following administration, in order to prove that the partially degradable copolymers are actually released from the lysosomes and are able to pass through the kidney glomerulus and be excreted in the urine.

The involvement of lysosomal enzymes has been proposed to be responsible for any degradation observed. The final objective was to prove that crosslinks contained in the copolymers are actually cleaved by the incubation of crosslinked copolymers with lysosomal enzymes with resultant isolation of lower molecular weight copolymer chains.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals

N-(2-Hydroxypropyl) methacrylamide copolymers	Kindly donated by Dr J Kopeček, Czechoslovak Academy of Sciences, Prague, Czechoslovakia
N,N^1-dityrosyl HMDA	
Calibration copolymers	
^{125}I-PVP (preparation IM.33P)	Radiochemical Centre, Amersham, Bucks.
[^{125}I] iodide (preparation IMS.30)	
Tissue culture medium (TC 199)	GIBCO, Bio-Cult Ltd, Paisley, Scotland
Heat inactivated calf serum (CS07)	Wellcome Reagents, Beckenham, Kent
Bovine serum albumin	Sigma (London) Chemical Company, Poole, Dorset
2,4-Dinitrophenol	
L-Phenylalanyl-L-tyrosine	
Adenosine triphosphate	
Sephadex G-15-120	
Sepharose CL-4B-200	
Sepharose CL-6B-200	
Leupeptin	Peptide Institute Inc., Osaka, Japan
Folin & Ciocalteu's reagent	Fisons Scientific Apparatus, Leics.
Chloroform	
Anaesthetic ether (diethyl ether)	May & Baker Ltd, Dagenham
Oxygen/Carbon dioxide (95:5)	British Oxygen Co Ltd, Manchester
Carbon dioxide 100%	

All other chemicals, unless otherwise stated, were of analytical grade.

2.1.2 Equipment

Assay of Radioactivity	
5136 Auto-Gamma Scintillation Spectrometer	Packard Instrument Ltd, Caversham, Berks.
3ml Disposable tubes	Luckhams Ltd, Burgess Hill, Sussex
Centrifuges	
MSE Mistral 4L	
MSE HS 18	MSE Instruments, Crawley, Sussex
MSE Superspeed 50	
MSE Bench Top	
Electrophoresis Equipment	
Shandon Electrophoresis Apparatus Model U 77	Shandon Scientific Co., Willesden, London
Bio-Rad Power Pack Model 500	Bio-Rad Laboratories Ltd, Watford, Herts.
Spectrophotometer	
Cecil CE 373	Cecil Instruments Ltd, Milton, Cambs.
Column Chromatography	
Fraction Collector Model 270	Chem Lab, Horn Church, Essex
Chromatography Columns (C-series)	Pharmacia Fine Chemicals, Uppsala, Sweden
Sephadex G-25-PD-10 Prepacked disposable columns	
Peristaltic Pumps	LKB Instruments Ltd, South Croyden, Surrey
Knauer Differential Refractometer (51)	Roth Scientific Co Ltd, Farnborough, Hants.
Chart Recorder	Magnus Scientific

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Assay of Radioactivity	
5136 Auto-Gamma Scintillation Spectrometer	Packard Instrument Ltd, Caversham, Berks.
3ml Disposable tubes	Luckhams Ltd, Burgess Hill, Sussex
Centrifuges	
MSE Mistral 4L	
MSE HS 18	MSE Instruments, Crawley, Sussex
MSE Superspeed 50	
MSE Bench Top	
Electrophoresis Equipment	
Shandon Electrophoresis Apparatus Model U 77	Shandon Scientific Co., Willesden, London
Bio-Rad Power Pack Model 500	Bio-Rad Laboratories Ltd, Watford, Herts.
Spectrophotometer	
Cecil CE 373	Cecil Instruments Ltd, Milton, Cambs.
Column Chromatography	
Fraction Collector Model 270	Chem Lab, Horn Church, Essex
Chromatography Columns (C-series)	Pharmacia Fine Chemicals, Uppsala, Sweden
Sephadex G-25-PD-10 Prepacked disposable columns	
Peristaltic Pumps	LKB Instruments Ltd, South Croyden, Surrey
Knauer Differential Refractometer (51)	Roth Scientific Co Ltd, Farnborough, Hants.
Chart Recorder	Magnus Scientific

In Vivo Equipment

Rat Metabolic Cages	North Kent Plastic Cages Ltd, Dartford, Kent
Michel Wound Clips (7.5mm)	A R Horwell, London
Potter-Elvejhem Teflon/Glass Homogeniser	Tri-R Instruments Inc., New York

2.2 ESTIMATION OF TISSUE PROTEIN

An estimation of the total protein content of both yolk sacs and jejunal sacs was carried out according to the method of Lowry et al. (1951). Duplicate samples (0.1ml) of tissue solution were pipetted into test tubes followed by 0.4ml of 1N sodium hydroxide and 0.5ml of distilled water. A calibration curve was constructed by using a standard solution of albumin (1mg/ml). A series of six dilutions was set up, in triplicate, consisting of albumin concentrations 0, 0.05, 0.10, 0.15, 0.20 and 0.25mg/ml. 1N sodium hydroxide and distilled water were added to the tubes as described in Table 2.1. Folin A (5.0ml) was added to all the tubes and 20 min later 0.5ml of Folin B was added and thoroughly mixed using a Whirlimixer. The colour was allowed to develop at room temperature for at least 45 min and then read on a Cecil CE 373 spectrophotometer at 750nm against distilled water. A calibration curve was constructed and used to read off the protein content (mg) of the 0.1ml tissue sample. Values were multiplied by a factor of 50, 250 or 500 to give the total tissue protein.

TABLE 2.1 SODIUM HYDROXIDE AND DISTILLED WATER ADDITIONS TO TUBES FOR CALIBRATION CURVE IN LOWRY PROTEIN ESTIMATION

Tube	Protein Standard (1mg/ml) (ml)	Distilled Water (ml)	1N Sodium Hydroxide (ml)
1	0	0.50	0.5
2	0.05	0.45	0.5
3	0.10	0.40	0.5
4	0.15	0.35	0.5
5	0.20	0.30	0.5
6	0.25	0.25	0.5

FOLIN REAGENTS

FOLIN A

2% anhydrous sodium carbonate	100ml
1% copper sulphate	1.0ml
1% sodium potassium tartrate	1.0ml

FOLIN B

Folin Ciocalteau's reagent	1.0ml
Distilled water	1.0ml

2.3 ^{125}I-LABELLING OF HPMA COPOLYMERS

All copolymers and the crosslinking agent N,N^{1}-dityrosyl HMDA were labelled with [^{125}I] iodide according to the Chloramine T method originally described by Hunter et al. (1963). The modification used by Williams et al. (1971) was followed here. Copolymer (20mg) was dissolved in 9.0ml phosphate buffer (Na_2HPO_4; 6.804g per litre and $KH_2PO_4 12H_2O$; 7.098g per litre) at pH 8.0 and placed on ice. 1 mCi of Na^{125}I (10µl) was then added and the mixture stirred for 2 min. 4ml of a 1mg/ml solution of Chloramine T was then added and after stirring for 8 min the reaction was stopped with sodium metabisulphite (3ml of a 2mg/ml solution). Solid potassium iodide (100mg approx.) was added to displace free [^{125}I] iodide during the subsequent dialysis.

A small sample of the preparation was reserved in order to assess the efficiency of the labelling procedure and the remainder was dialysed against 1% NaCl for 72h (2 changes daily) to remove excess [^{125}I] iodide. The efficiency of labelling and the amount of contaminating [^{125}I] iodide present at any time was assessed using low voltage paper electrophoresis (section 2.4).

2.4 ESTIMATION OF FREE $[^{125}I]$ IODIDE IN RADIOLABELLED COPOLYMER PREPARATIONS BY LOW VOLTAGE PAPER ELECTROPHORESIS

The method described by Sargent (1969) was followed. Barbitone buffer, pH 8.6 was prepared (1.84g of barbitone and 10.3g of barbitone sodium in 1 litre of distilled water) and the electrophoresis tank filled to a depth of approximately 2cm. Whatman No. 1 paper was cut into strips 30cm in length and 10cm wide. The central 20cm was pencil marked into 40 x 0.5cm strips and the fifth strip from one end was marked for the origin. The papers (up to four strips at any time) were then dipped into the barbitone buffer, blotted and placed across the supporting bars in the electrophoresis tank with the origin close to the cathode and each end submersed in the buffer compartment. The restraining bars were fixed into position, and current (400V, 2mA) passed through the strips for 15 min to allow equilibration.

Radiolabelled copolymers were applied to the premarked origins on the paper strips using 20µl microcaps. It has been found that when placed vertically in a solution, these microcaps fill to a volume of approximately 12µl by capillary action. To avoid contamination of rubber bulbs with radioactive solutions, it was therefore decided to apply 2 x 12µl samples to each paper strip. A similar sample was applied to a strip 5cm x 0.5cm of Whatman No. 1 paper which was then transferred to a Luckhams tube, pressed to the base of the tube using a glass rod and sealed.

The electrophoresis was run at 400V, 2mA for 35 min. The papers were then removed and cut into 0.5cm strips sequentially at the premarked pencil lines. The small strips were then transferred to labelled Luckhams tubes, pressed down

to the base of the tubes, which were then sealed and assayed for radioactivity. By running standards of known composition against the experimental solutions and plotting radioactivity (corrected for background) against strip number, it was possible to identify the composition of experimental solutions by comparing the position of the peaks obtained. Measurement of the radioactivity in each peak and comparison with the total radioactivity in the sample applied gave an estimate of the percentage of each component peak.

This technique was used routinely to estimate the percentage of contaminating free $[^{125}I]$ iodide in radiolabelled copolymer preparations, and also to estimate the labelling efficiency of copolymer samples following ^{125}I-labelling of HPMA copolymers using Chloramine T.

2.5 COLUMN CHROMATOGRAPHY

Column chromatography was used for the detection of low molecular weight degradation products, for molecular weight determination and for the separation of ^{125}I-labelled copolymer from contaminating free $[^{125}I]$ iodide.

Prepacked disposable columns containing G-25 gel filtration medium were used to separate ^{125}I-labelled copolymers from $[^{125}I]$ iodide. The surface of the gel contained in these columns is protected by a plastic scinter which prevents the gel drying out, and maintains an even bed surface. Columns were 8cm in length, internal diameter 16mm and bed height 5cm. Each column was supported over a suitable receptacle and the gel bed equilibrated with approximately 25ml of phosphate buffer at pH 8.0. The sample was made up to 2.5ml final volume in buffer and applied to the column. The resultant 2.5ml of buffer eluted was discarded. The high molecular weight components were then eluted with 3.5ml of buffer solution in 0.5ml fractions and assayed for radioactivity. Contaminating $[^{125}I]$ iodide remained in the gel, and the column was discarded.

Sephadex G-15 gel filtration medium was normally used for the detection of low molecular weight degradation products (up to 1500 Mw). The required quantity of Sephadex powder was placed in a beaker containing excess solvent (sodium acetate 0.02M, sodium azide 0.05% buffer). The process of swelling was achieved by placing the beaker in a boiling water bath for 1h, which also served to deaereate the buffer. The slurry was then left to cool. The column was mounted on a laboratory stand and adjusted to a vertical position to ensure even packing. The outlet tubing was sealed and the column one third filled with

buffer. The slurry was then agitated and poured into the column. The outlet tubing was then opened and buffer allowed to drip from the column into a suitable vessel. When the gel settled, a quantity of the resultant head of clear buffer solution was removed, the top few centimetres of gel stirred, more gel slurry applied and allowed to settle. This was repeated until the desired bed height was attained. The column was then packed down by running buffer through the gel overnight at the desired constant flow rate (usually 20ml/h) using a peristaltic pump. Columns were kept moist and out of direct sunlight to prevent cracking. Initially, a column of approximate length 45cm, internal diameter 2.5cm and bed height 30cm was used. However, this chromatography column proved to be larger than necessary to achieve a good separation of peaks, and took up to 9h to elute, owing to the adsorption of ^{125}I-tyrosine molecules on the tightly crosslinked gel, which took longer to elute than predicted from their molecular size. Also, because of this adsorption, the elution volume was increased, and the applied sample diluted to such an extent that counting for radioactivity became difficult with copolymers that demonstrated low labelling efficiency or when total counts applied were low for other reasons e.g. serosal fluids from jejunal sacs. For these reasons, smaller G-15 chromatography columns were subsequently used, of column length 20cm, internal diameter 16mm and bed height 15cm.

Before sample application, most of the buffer was removed from above the gel surface by suction, using a piece of flexible silicone rubber tubing attached to a 10ml syringe. The remaining buffer was allowed to drain away. Samples (usually 1ml) were then carefully layered on top of the drained bed surface, and allowed to drain into the bed before replacing

2-3ml of the buffer solution. When this, too, had drained away, approximately 5ml of buffer solution was carefully layered on top of the bed surface. Care was taken to prevent diffusion of the sample into the buffer and to avoid disturbing the bed surface, which could lead to uneven separated bands and poor resolution. The eluate was collected in 1 or 2ml aliquots using Luckhams tubes mounted in a fraction collector, each tube accumulating fluid for 3 or 6 minutes respectively. The tubes were then assayed for radioactivity and an elution profile prepared by plotting counts obtained against fraction number.

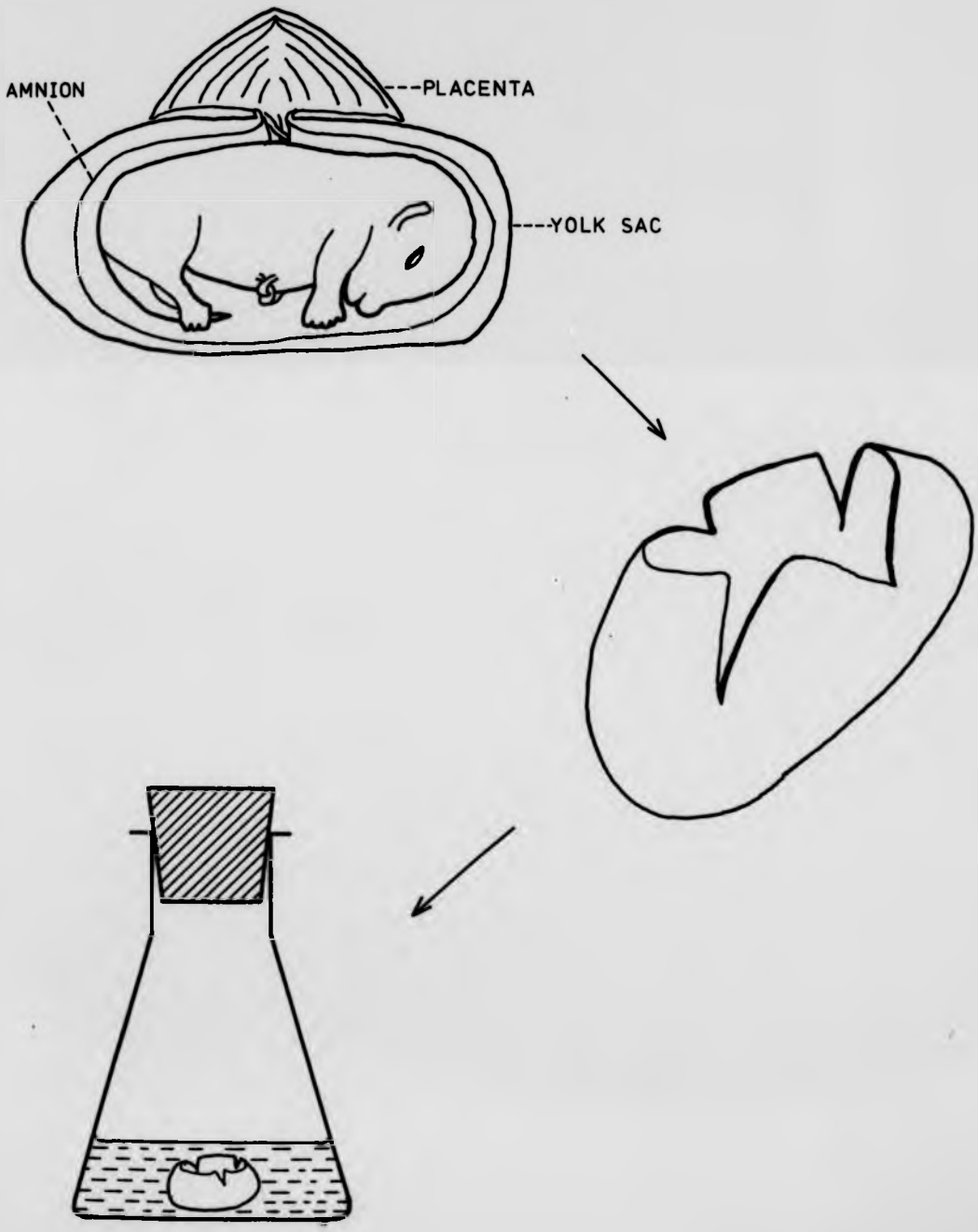
For molecular weight determination, a mixture of Sepharose 4B and Sepharose 6B (50:50) gel filtration media was used. By mixing in this way, it is possible to increase the fractionation range and therefore to determine molecular weights between 1×10^4-5×10^6 (Sepharose 6B 1×10^4-1×10^6, Sepharose 4B 3×10^4-5×10^6). For molecular weight distribution determinations of ^{125}I-labelled copolymers (Chapter 8) a column of internal diameter 16mm and bed height 60-65cm was used. Sepharose CL gel is supplied pre-swollen as a thick slurry in distilled water, and was therefore diluted in buffer (0.2M Tris/HCl, 0.5M NaCl pH 8.0), placed in a Buchner flask, and deaereated using a suction pump. Columns were packed and operated, and samples applied, in exactly the same way as described above.

2.6 MEASUREMENT OF PINOCYTOSIS BY RAT VISCERAL YOLK SAC IN CULTURE

The method used for the culture of rat visceral yolk sacs was as described by Williams et al. (1975 a, b). Yolk sacs were obtained from an inbred colony of Wistar rats in order to minimise genetic variation. Pairs of rats were mated overnight in grid cages. If a sperm plug was detected underneath the grid of the cage the following morning, pregnancy was timed from midnight on the night of mating. After 17.5 days, pregnant rats were asphyxiated with carbon dioxide, the uterus removed and placed in a Petri dish containing medium 199 (supplemented with 10% calf serum) that had previously been warmed to 37°C. Conceptuses were exposed on making a single longitudinal cut through the uterine wall, and each placental cap was gently prised free using open fine scissors. The placental caps were removed, the cut edge of the yolk sac enlarged by a single cut and the fetuses dissected free. The yolk sacs were placed in another Petri dish containing fresh medium, and any remaining amniotic tissue was carefully removed.

The yolk sacs were incubated either singly, or in groups of 6-10, in sterile, detergent-free 50ml Erlenmeyer flasks containing 9.0ml of medium 199 supplemented with 10% heat-inactivated calf serum which had previously been gassed with a mixture of 95% oxygen and 5% carbon dioxide, and sealed with a sterile silicone rubber bung. Flasks were placed in a water bath maintained at 37°C with a shaker attachment set to a stoke of approximately 3.4cm at a frequency of 100±5 strokes per minute. After a preincubation period of 15-45 min, 1ml aliquots of culture medium containing a solution of ^{125}I-labelled substrate was added to each flask. The flasks were then regassed and stoppered.

SCHEMATIC REPRESENTATION OF THE DISSECTION OF A 17·5-DAY RAT YOLK SAC FOR ORGAN CULTURE (from WILLIAMS et al 1975)



Yolk sacs were removed at various times over the incubation period (5-12h), washed three times for 2 min in up to 100ml ice-cold 1% sodium chloride to remove any extracellular substrate, and placed in either 5ml or 50ml volumetric flasks, depending on the number of yolk sacs present in each culture flask. The flasks containing yolk sacs were made up to 5ml or 50ml with 1N sodium hydroxide and left to dissolve at 37^{o}C for 2h, mixing at half hourly intervals. Duplicate 1ml samples of the resultant digest were transferred to Luckhams tubes and assayed for radioactivity (section 2.8) and protein content (section 2.2) using the method of Lowry et al. (1951).

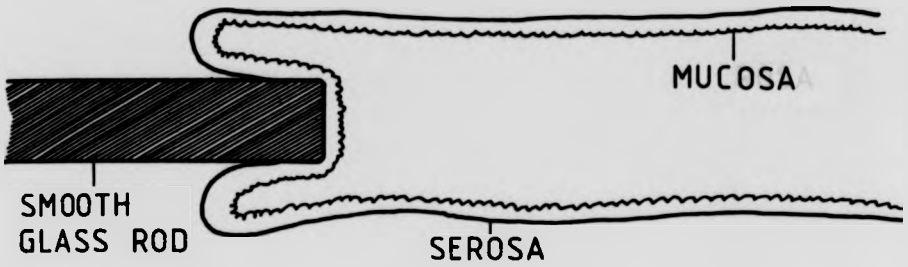
Two 1ml samples of culture medium were pipetted into disposable 3ml (Luckhams) tubes, stoppered and stored at 4^{o}C until assayed for radioactivity (see section 2.8). When studying the possible degradation of a substrate, a further two 1ml samples of culture medium were collected in the same way, but stored at -20^{o}C prior to Sephadex G-15 column chromatography (sections 2.5 and 2.9).

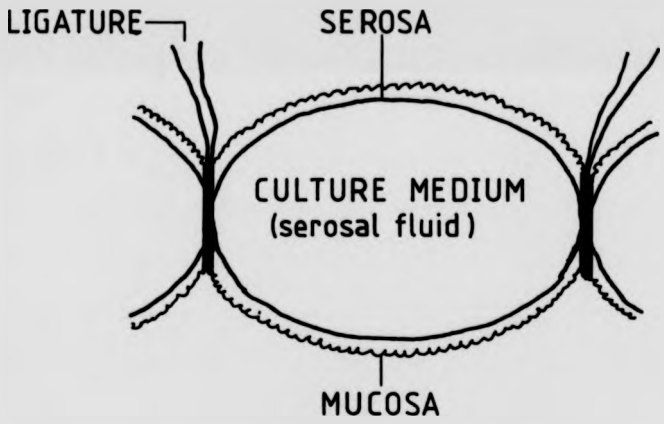
2.7 MEASUREMENT OF PINOCYTOSIS BY RAT JEJUNAL SAC IN CULTURE

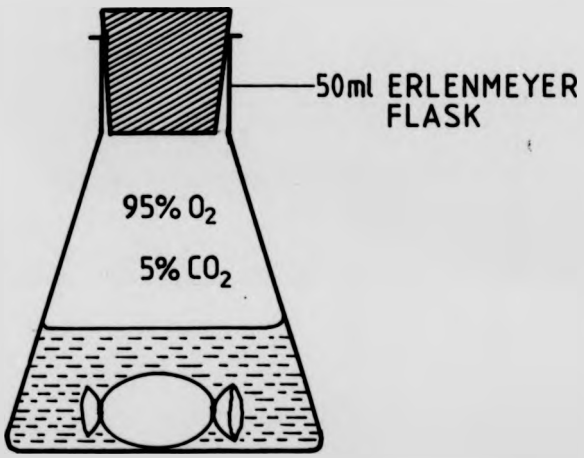
The method used for the preparation of everted intestinal sacs from adult rat jejunum was essentially that described by Wilson and Wiseman (1954), and modified by Bridges and Woodley (1980).

Intestinal tissue was obtained from adult male Wistar rats of 200-300g body weight. Animals had free access to food and water before all experiments. Each animal was sacrificed by cervical dislocation, the abdomen opened by a midline incision, and the jejunum removed by cutting across the region of the ligament of Treitz (duodenal-jejunal junction) and the ileo-jejunal junction, where the intestine is loosely bound to the posterior wall of the abdomen. The jejunum was washed out immediately with oxygenated medium 199 containing 10% heat-inactivated calf serum and 1mM ATP at $4^{o}C$, delivered from a 10ml syringe, and placed in a rectangular dissection trough (60cm x 3cm) containing the same incubation medium. All medium was gassed with a mixture of 95% O_2 and 5% CO_2 prior to use. (The addition of 10% calf serum and 1mM ATP has been shown to be necessary for the maintenance of tissue viability and structural integrity throughout the incubation period (Bridges 1980)). The ileal end of the jejunum was attached to one end of a notched glass rod (45cm x 2.0mm diam.) with a ligature and the jejunum was everted by rolling the proximal half onto the rod. The everted intestine was then slipped off the rod and replaced in the dissection trough. As far as possible, the glass everting rod was immersed in the incubation medium present in the trough throughout this procedure. After discarding the end portions, approximately 35cm of intestine was straightened carefully without stretching and one end was

SCHEMATIC REPRESENTATION OF THE TECHNIQUE FOR THE PREPARATION OF EVERTED JEJUNAL SACS







held in artery clamps. Oxygenated incubation medium (4ml) at 4°C was allowed to flow into the sac at the other end from a gravity feed pipette, after which this end, too, was sealed using artery clamps. The resultant large sac was divided into approximately 8 x 3cm sacs by ligatures, taking care to exclude Peyers patches (gut associated lymphoid tissue) which may have interfered with uptake studies. Each sac was then separated and incubated singly, as described for yolk sacs (section 2.6), but for an incubation period of 1.5h in all cases and with the addition of 1mM ATP to the culture medium.

Following incubation, each gut sac was removed, gently blotted, held vertically by forceps over a 30ml Universal container, and snipped at the base with fine scissors in order to collect incubation medium present in the serosal space. The volume of the serosal fluid was measured using a 1ml syringe. Any sacs that had leaked were discarded. Serosal fluids were transferred to disposable 3ml (Luckham) tubes, stoppered, assayed quickly for radioactivity and then stored at -20°C prior to column chromatography (section 2.5). Empty sacs were washed four times in approximately 30ml of ice-cold 1% NaCl to remove extracellular substrate and placed in 25ml volumetric flasks. Two 1ml samples of culture medium were pipetted into Luckhams tubes and stored at 4°C until assayed for radioactivity. When studying possible degradation of a substrate, a further three 1ml samples of medium were collected in the same way, but stored at -20°C until assayed. Gut sacs were made up to 25ml with 1N sodium hydroxide and assayed for both radioactivity and protein in the same way as that described for yolk sacs (sections 2.2 and 2.6) except that values for 0.1ml protein were multiplied by a factor of 250 to give total gut sac protein.

2.8 QUANTITATION OF THE PINOCYTIC UPTAKE OF A NON-DEGRADABLE SUBSTRATE BY THE YOLK SAC AND JEJUNAL SAC

The following method of quantitation of pinocytic uptake was used for both yolk sac and gut tissues.

Following incubation, 1ml samples of tissue solution (yolk sac or jejunum), and the corresponding samples of culture medium were assayed for radioactivity with a gamma counter and the total tissue protein estimated (sections 2.2, 2.6 and 2.7). The accumulation of substrate at each time interval was expressed as a clearance, defined as the volume of culture medium (µl) whose contained substrate was captured per mg of tissue protein. The following equation was used:-

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

where U = uptake (µl/mg protein)

Y = total tissue radioactivity (counts per min corrected for background)

M = radioactivity per µl of culture medium (counts per min corrected for background)

P = protein content (mg).

This calculation was carried out using the computer programme shown in Appendix 1.

With a non-degradable substrate, such as ^{125}I-PVP, uptake was consistently found to be linear with time. A best-fit regression line was used to describe the relationship and a correlation coefficient calculated.

2.9 QUANTITATION OF THE PINOCYTIC UPTAKE OF A DEGRADABLE SUBSTRATE BY THE YOLK SAC AND JEJUNAL SAC

In experiments using degradable substrates, tissue uptake was often found to be non linear with time. In such cases, uptake/time plots were prepared from a series of experiments using mean uptake values at each time interval, and subsequent experiments performed to assess the extent of degradation of any substrate incubated over a given time period.

Radiolabelled copolymer 74 and 39 fractions contained potentially degradable sequences which included ^{125}I-tyrosine residues. It was therefore possible to identify ^{125}I-labelled low molecular weight degradation products present in the medium when these copolymers were incubated with either yolk sac or jejunal sacs, and so assess the total pinocytic uptake by these tissues.

This was achieved by incubating 6-10 yolk sacs in a single flask (section 2.6) for 5h, or 2 gut sacs in a single flask for 1.5h (section 2.7) with ^{125}I-labelled copolymers and estimating tissue accumulation of substrate as described in section 2.8. Samples of culture medium (1ml) were assayed for radioactivity and then applied to a Sephadex G-15 column (section 2.5). Elution with 0.05M sodium acetate yielded 1ml fractions which were counted for radioactivity. Columns were calibrated with blue-Dextran, ^{125}I-labelled HPMA copolymer, $[^{125}I]$ iodide and 3-iodo-L-tyrosine, and other radiolabelled molecules where deemed necessary. The extent of degradation was calculated from the elution profiles by expressing the amount of radioactivity present in peaks corresponding to low molecular weight degradation products as a percentage of the total radioactivity recovered from the column. This percentage

was expressed in terms of µl of culture medium per mg of tissue protein.

The true pinocytic uptake of the degradable substrate was then obtained by summing the values for tissue accumulation and for degradation product (both in terms of µl/mg protein) over the period of incubation, in the case of yolk sacs i.e.

True Pinocytic Uptake = Tissue Accumulation + Degradation Products in Culture Medium

(µl/mg protein) (µl/mg protein) (µl/mg protein)

In the case of jejunal sacs

True Pinocytic Uptake = Tissue Accumulation + Serosal Accumulation + Degradation Products in Culture Medium

(µl/mg protein) (µl/mg protein) (µl/mg protein) (µl/mg protein)

The quantitation of transport of ^{125}I-labelled substrates across the jejunum (serosal accumulation) is discussed in section 2.10.

2.10 QUANTITATION OF TRANSPORT OF ^{125}I-LABELLED HPMA COPOLYMERS ACROSS THE RAT JEJUNUM

The transport of molecules across the rat jejunum was calculated using the equation

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

where U = the quantity of radioactive substrate accumulated in the serosal fluid in a given time (µl/mg protein)

Y = the total radioactivity in the serosal fluid (counts per min corrected for background)

M = radioactivity per µl of culture medium (counts per min corrected for background)

P = protein content (mg)

In experiments using ^{125}I-PVP, a non-degradable substrate, the values obtained represented the transport of the intact macromolecule across the gut, and uptake was found to be linear with time. A best fit regression line was used to describe the relationship and a correlation coefficient calculated.

When using degradable substrates, the values obtained using the equation represented both transport of macromolecules and also transport of low molecular weight degradation products. Accumulation/time plots were found to be linear with time, but further experiments were necessary in order to identify and quantify the proportion of low molecular weight degradation products. This was achieved by Sephadex G-15 column chromatography (section 2.5).

2.11 IN VIVO METHODS

2.11.1 Intravenous Injections

Male Wistar rats of 200-250g body weight were used. A rat, under light ether anaesthesia, was placed on its back and a small incision of approximately 1.5mm was made in the left groin. The left femoral vein was carefully exposed by removal of surrounding tissue. Radiolabelled substrate (0.1ml) was injected into the vein with a sterile disposable syringe fitted with a 25 gauge needle. Following injection, a swab was held over the area to staunch any bleeding.

2.11.2 Intraperitoneal Injections

Male Wistar rats of 250-350g body weight were used for these experiments. Up to 0.5ml of radiolabelled substrate was taken into a sterile disposable syringe fitted with a 25 gauge needle. Each rat, under light ether anaesthesia, was placed on its back and injected by pinching the skin of the abdomen, lifting and injecting the solution through the skin into the peritoneal cavity. Care was taken not to penetrate the gut or any other organ. The animals were allowed to recover and placed in metabolic cages.

2.11.3 Subcutaneous Injections

Male Wistar rats of 200-250g body weight were used. Animals were placed under light ether anaesthesia and laid on the abdomen prior to injection. Radiolabelled substrate (0.1ml) was injected subcutaneously at the back of the neck. Animals were then allowed to recover and placed in metabolic cages.

2.11.4 Oral Administration

Male Wistar rats of 200-250g body weight were used. Each animal was placed under light ether anaesthesia and laid on its

back prior to administration of copolymer. Up to 0.5ml of radiolabelled substrate was taken into a sterile disposable 1.0ml syringe fitted with narrow gauge flexible silicone rubber tubing. The tubing was carefully guided through the mouth, down the oesophagus and the substrate administered directly into the stomach. Animals were then allowed to recover and placed in metabolic cages.

2.11.5 Collection and Analysis of Blood from Experimental Rats

Following oral administration or injection of radiolabelled copolymers (intraperitoneal, intravenous or subcutaneous), rats were held under ether anaesthesia when the collection of blood samples were undertaken. A small incision was made in the right foot pad and samples of blood were collected in two 50µl heparinised tubes (Hawkesley & Son Ltd., Lancing, Sussex.). Bleeding was quenched by a tourniquet. Further blood samples were taken by temporarily loosening the tourniquet. Each blood sample was dispersed in 1ml distilled water contained in a 3ml disposable Luckhams tube, stoppered, and assayed for radioactivity.

2.11.6 Collection and Analysis of Radioactive Tissue Samples

Following intravenous or subcutaneous injection of radiolabelled copolymers and the collection of blood samples, rats were sacrificed by cervical dislocation whilst under ether anaesthesia. The liver, lungs, kidneys and spleen were removed, quickly rinsed in 1% NaCl (approximately 20ml), blotted and weighed. Between 5 and 30ml of distilled water was added to each tissue (dependent on tissue weight) and each was homogenised in a Potter-Elvejhem Teflon/glass homogeniser (Tri-R Instruments Inc., New York) at 3,500rpm (speed setting 3.5). The final volume of each suspension was noted and duplicate samples (1ml) from each tissue were assayed for

radioactivity.

At the end of the experimental period following intraperitoneal injections, rats were sacrificed by chloroform asphyxiation (since cervical dislocation or prolonged ether anaesthesia may cause internal bleeding) and the recovery of residual radioactive substrate present within the peritoneal cavity was attempted. Each rat was placed on its back and a flap of skin reflected from the abdomen, taking care not to puncture the body wall. 10ml of 1% NaCl was injected into the peritoneal cavity from a sterile disposable syringe fitted with a 25 gauge needle, avoiding the penetration of any organs. The abdomen was then massaged for 1 min. The liquid was then withdrawn from the peritoneal cavity by penetrating the body wall at the side of the flap with a Pasteur pipette placed at an angle of about 30^{o} to the bench. An average recovery of approximately 7ml of the injected saline solution was made. The solution was then centrifuged in an MSE bench-top centrifuge for 10 min at 1,000rpm (200G). The supernatant was poured off and the remaining pellet (consisting predominantly of peritoneal macrophages) was resuspended to a final volume of 1ml and assayed for radioactivity. Two 1ml samples of the supernatant were transferred to Luckhams tubes and assayed for radioactivity, and two further 1ml samples were stored at $-20^{o}C$ prior to column chromatography. Counts for both supernatant and cells were corrected to 10ml. The collection and analysis of liver, lung, kidneys and spleen was essentially as described above, with the exception that these organs were washed more thoroughly (3 x 20ml 1% NaCl) in order to remove any radioactivity that may have adsorbed to the tissues following injection into the peritoneal cavity. In addition to the organs previously mentioned, the entire small intestine was removed, rinsed through with 10ml 1% NaCl using a plastic

disposable syringe, washed and treated exactly as above. Gut washings were also retained and assayed for radioactivity.

Rats subjected to oral administration of radiolabelled copolymer were sacrificed by cervical dislocation following ether anaesthesia. Liver, lungs, kidneys, spleen and small intestine were removed and analysed as described above.

In all experiments where recovery following administration of radiolabelled copolymers was allowed, each rat was placed in a metabolic cage and allowed free access to food and water until the end of the experiment. Total urine and faeces were collected in all cases. The volume of urine was noted, two 1ml samples were assayed for radioactivity, and whenever possible three 1ml samples were stored at -20°C prior to column chromatography. Faeces samples were weighed, homogenised and duplicate 1ml samples assayed for radioactivity.

2.12 PREPARATION OF TRITOSOMES

Tritosomes were prepared according to the method described by Trouet et al (1974).

Five male Wistar rats of approximately 400g body weight were injected intraperitoneally with Triton WR 1339 (20% w/v in saline - 1ml/100g rat body weight) whilst under light ether anaesthesia. The animals were allowed to recover and left for three days with free access to food and water, and were starved overnight on day 3-4.

On day 4 the animals were sacrificed, their livers removed and placed in 0.25M sucrose on ice. All following experimental procedures were carried out at $4^{o}C$.

The livers were washed, blotted, chopped with scissors and sieved. The total liver weight was noted. The tissue was suspended in 0.25M sucrose (5ml/g tissue) and homogenised (3 passes) using a teflon/glass homogeniser. The resultant suspension was centrifuged in an MSE 4L centrifuge for 10 min at 1,650 rpm. The supernatant was stored on ice, and pellets resuspended in 0.25M sucrose (3ml/g tissue) and centrifuged for 10 min at 1,400 rpm. Supernatants were pooled and centrifuged at 17,000 rpm for 10 min in an MSE HS 18 centrifuge. Again, pellets were washed by resuspending in 0.25M sucrose (5ml/g tissue) and centrifuged at 17,000 rpm for 10 mins. Supernatants were this time discarded and the pellets were resuspended in 45% sucrose (1ml/g tissue). Approximately 10ml of the suspension was placed in each of three centrifuge tubes, and approx. 7ml of 34.5% sucrose followed by approx. 4ml of 14.3% sucrose were layered above. The tubes were balanced and subsequently centrifuged at 25,000 rpm for 2h in an MSE

Superspeed 50 centrifuge.

Tritosomes, visible as a turbid band of material, were recovered from the interface between the 34.5% and the 14.3% sucrose. Tritosomes were removed carefully, pooled, and 1ml aliquots taken and stored at -20^{o}C. Approximately 16ml total volume of tritosomes were recovered.

2.13 ESTIMATION OF CROSSLINK CLEAVAGE OF HPMA COPOLYMERS

The estimation of the amount (%) of cleaved bonds was carried out as described by Bohdanecký et al (1974), and is based on Stockmeyer's conclusions (1944). The calculation involves the determination of the average molecular weight and number average of the polymer precursor (section 3.4), of the crosslinked polymer, and of the polymer obtained following enzymatic cleavage.

The relationship between these values is as follows

$$\bar{X}n = \bar{Y}n \quad (1 - \gamma/2) \qquad (1)$$

$$\bar{X}w = \bar{Y}w \quad (1 - \gamma\, Yw/Yn) \qquad (2)$$

$$\gamma = \bar{Y}n.\varsigma \qquad (3)$$

where $\bar{Y}w, \bar{Y}n$, respectively = the weight average and number average of the polymer precursor

$\bar{X}w, \bar{X}n$ = the weight average and number average of the crosslinked or cleaved polymer

γ = the crosslinking index i.e. the number of crosslinked units per primary chain

ς = the network density i.e. the molar fraction of the crosslinked units.

Equation (2) holds for high $\bar{Y}w$ and small ς

The amount of cleaved bonds (%) =

$$100 - \frac{\text{crosslinking density of cleaved polymer}}{\text{crosslinking density of crosslinked polymer*}} \times 100 \qquad (4)$$

* i.e. the crosslinked copolymer before enzymatic cleavage.

Bohdanecký et al (1974) obtained a simpler relationship for the calculation of cleaved bonds by rearranging (4) assuming that

a) polymer precursors contain a small amount of comonomers with reactive groups (in this case approx. 2% mol).

b) the difference between the average weight of the monomer unit in the precursor and the crosslinked polymer is negligible

c) it is therefore possible to introduce the average weight of the monomer unit (150).

On making these assumptions the equation can be simplified to

$$\text{The \% of cleaved bonds} = 100 - \frac{1 - \frac{\bar{M}w \text{ aminolysed precursor}}{\bar{M}w \text{ cleaved polymer}}}{1 - \frac{\bar{M}w \text{ aminolysed precursor}}{\bar{M}w \text{ crosslinked polymer}}} \times 100$$

The calculation of the average molecular weight of copolymer following enzymatic cleavage is described in Chapter 8.

CHAPTER 3

CHARACTERIZATION OF COPOLYMERS

3.1 INTRODUCTION

This chapter describes the copolymers used in the present study, and reports experiments carried out to ascertain the suitability of the copolymers for radioiodination and their subsequent use in a variety of in vitro and in vivo experimental methods.

All soluble poly N-(2-hydroxypropyl)methacrylamide copolymers used in this study were synthesized and donated by Dr J Kopeček and Dr P Rejmanová of the Institute of Macromolecular Chemistry, Czechoslovak Acadamy of Sciences, Prague, Czechoslovakia. The synthesis, structures and characteristics of the copolymers used are discussed in sections 3.2 and 3.5, and the information presented in those sections, unless otherwise stated, was received from the Institute of Macromolecular Chemistry in Prague.

3.2 SYNTHESIS AND STRUCTURES OF SOLUBLE CROSSLINKED HPMA COPOLYMERS

All the polymeric materials used in this study were derived from initial reactive copolymers (polymer precursors) synthesized by copolymerization of N-(2-hydroxypropyl) methacrylamide (HPMA) with p-nitrophenyl esters of either N-methacryloylated oligopeptides or N-methacryloylated amino acids (Rejmanová et al 1977, Kopeček et al 1977). The content of the reactive groups was only several % mol. in all cases. For example, the preparation of the polymer precursor used in the synthesis of copolymers 39 and 74 is by the copolymerization of HPMA with p-nitrophenyl ester of N-methacryloylglycylglycyl-phenylalanine (shown in fig 3.1).

To prepare polymers with degradable crosslinks, the copolymers of HPMA p-nitrophenyl ester of N-methacryloylated oligopeptides were reacted with hexamethylenediamine (HMDA). This procedure results in the formation of crosslinks, intramolecular cyclization and free NH_2 groups. In the case of copolymer 39 the crosslinking agent was N,N^1-dityrosyl HMDA, and the ratio of polymer-bound ONp groups to diamine NH_2 groups was 1:1. Reaction conditions were chosen so that the degree of crosslinking did not exceed the gel point. 14.7% of the initial side-chains (P-Gly-Gly-Phe-ONp) were connected by intermolecular crosslinks (section 2.13), the rest formed intramolecular bridges or were aminolysed with 1-amino-2-propanol. The crosslinking procedure is shown in fig 3.2.

Copolymer 74 was prepared from the same polymer precursor, which was reacted with tyrosineamide to form a copolymer with sidechain composition P-Gly-Gly-Phe-TyrNH_2.

SYNTHESIS OF HPMA COPOLYMER PRECURSOR P-Gly-Gly-Phe-ONp

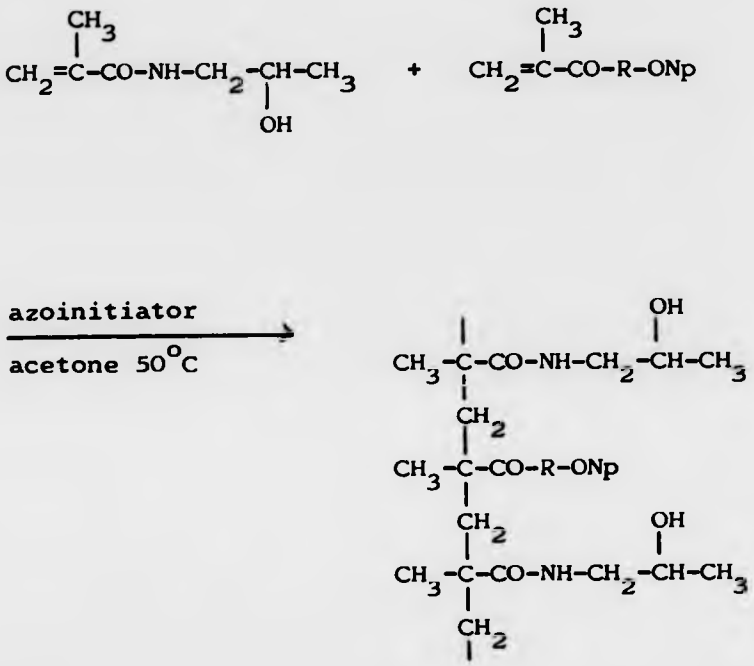
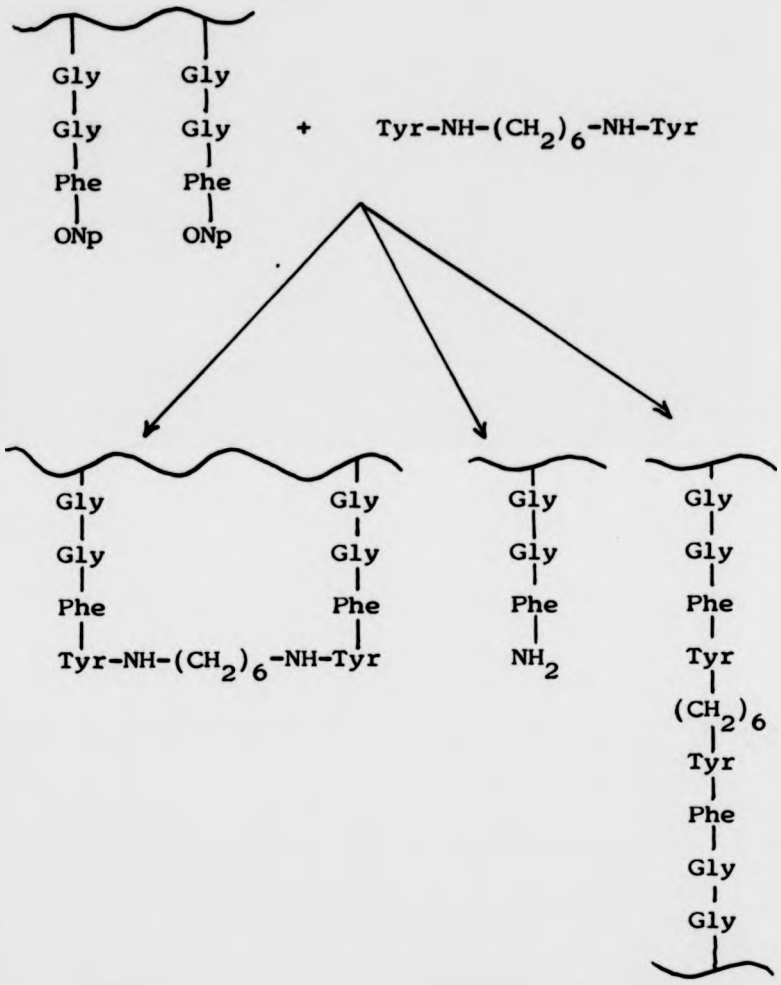


FIGURE 3.1

SYNTHESIS OF CROSSLINKED COPOLYMER 39



Intramolecular cycle | Aminolysed sidechain | Crosslink

FIGURE 3.2

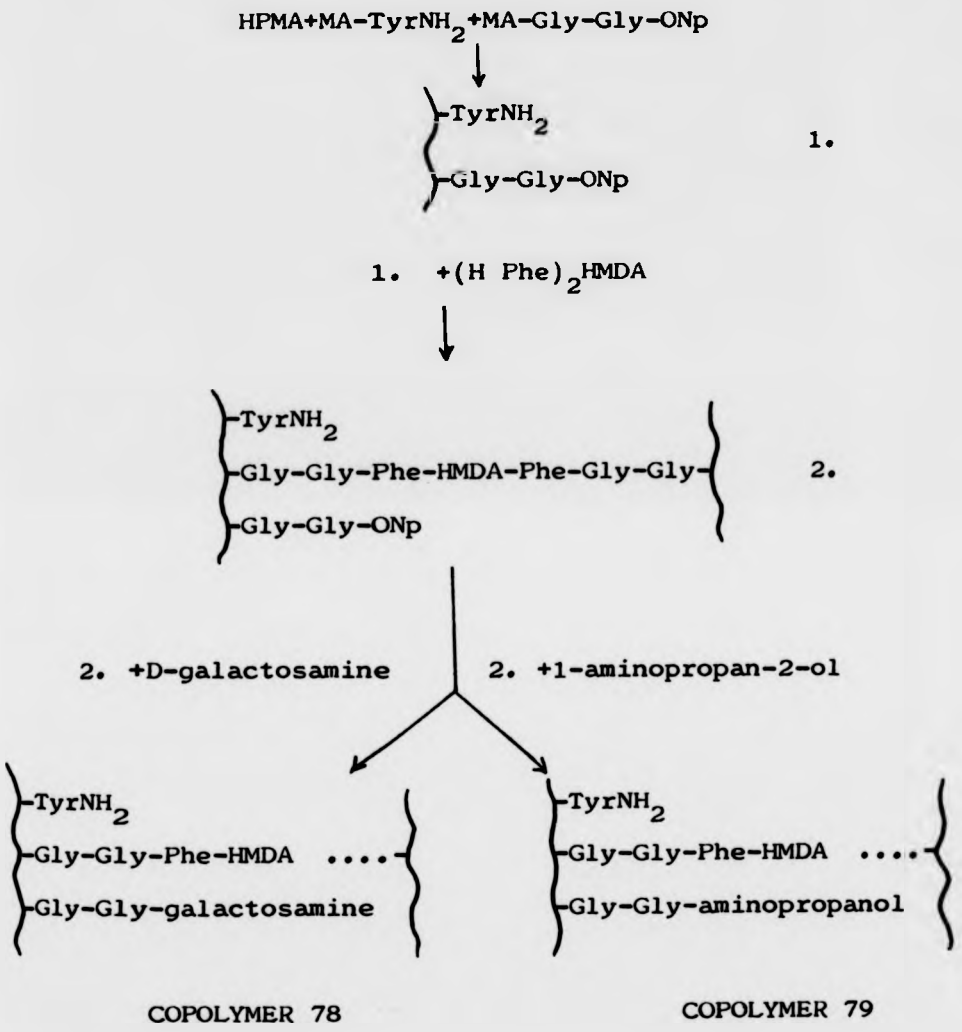
The general scheme for the preparation of crosslinked copolymers 78 and 79 is shown in fig 3.3.

Copolymer 68 is of a similar structure to copolymer 79, but reaction conditions were employed to give a greater density of crosslinks.

The structures of the crosslinks contained in copolymers 39, 68, 78 and 79 are shown in Table 3.1. The sidechain composition of copolymer 74 is also shown in Table 3.1.

This table is reproduced for quick reference on the last page of this thesis.

SYNTHESIS OF SOLUBLE CROSSLINKED COPOLYMERS 78 AND 79



HPMA = Poly N-(2-hydroxypropyl)methacrylamide

MA-TyrNH$_2$ = N-methacryloyltyrosinamide

MA-Gly-Gly-ONp = N-methacryloylglycylglycine p-nitrophenolester

(H Phe)$_2$HMDA = N,N^1-bis(H-Phe)hexamethyldiamine

FIGURE 3.3

3.3 RADIOLABELLING EFFICIENCIES OF COPOLYMERS USING CHLORAMINE T

Each copolymer was radiolabelled using Chloramine T (section 2.3) on several occasions. The efficiency of labelling was estimated on each occasion by calculating the percentage of the total $\left[^{125}I\right]$ iodide used which became bound to the tyrosine residues contained within the copolymer during the reaction. This estimate was carried out by subjecting a small amount (28 µl) of the reaction mixture to paper electrophoresis (section 2.4). The remainder was dialysed against 1% NaCl to remove the excess free $\left[^{125}I\right]$ iodide.

The labelling efficiencies of the copolymer preparations radiolabelled throughout this project are summarised in Table 3.2. From these results it can be seen that copolymers that contain the tyrosine residue attached directly to the polymer backbone (68, 78, 79) show consistently lower labelling efficiencies than those where the tyrosine residue is present as the fourth amino acid of a crosslink (copolymer 39 fractions). This could be the result of steric influences during the labelling procedure, where the tyrosine residues present in the crosslinks are more accessible to the $\left[^{125}I\right]$ iodide than those close to the polymer backbone.

TABLE 3.2

THE EFFICIENCY OF RADIOLABELLING COPOLYMERS USING CHLORAMINE T

POLYMER CODE NO.	LABELLING EFFICIENCY (%) 1	2	3	4	5	6 *
39 (unfractionated)	66	43	47	45	47	
39a	56	56	45			
39b	57	61	27			
39c	56	42	40			
39d	53	46	56			
39e	39	21	41	29	45	
74	28	28				
68	16	16	12	8	16	12
78	19	17	11			
79	15	25	18			
N,N^1-dityrosyl-HMDA	45	96				

* Numbers 1-6 denote separate occasions on which copolymers were radioiodinated.

3.4 CHARACTERISTICS OF CROSSLINKED COPOLYMERS AND COPOLYMER 74

Polymers are generally composed of molecules of similar monomer units, but of various chain lengths. The diversity of molecular sizes is further increased on crosslinking polymer precursors. It is therefore necessary to describe polymers in terms of average molecular weight and to give an indication of the distribution of polymer sizes. The polydispersity of a polymer preparation can be indicated by $\bar{M}w/\bar{M}n$ i.e. average molecular weight divided by the average number of molecules. The average molecular weight and $\bar{M}w/\bar{M}n$ of each copolymer used are shown in Table 3.3.

In order to study the possible effect of molecular size on the pinocytic uptake of polymers by different tissues, copolymer 39 was fractionated, in Prague, using Sepharose 6B gel permeation chromatography to give five samples whose characteristics are shown in Table 3.3. It was not possible to estimate the size of copolymer 39a, which eluted largely in the void volume of the column used. For this reason, neither was it possible to estimate the polydispersity of the preparation.

Copolymer 74 (P-Gly-Gly-Phe-TyrNH_2) was synthesized from the same polymeric precursor as copolymer 39 and it can be seen from Table 3.3 that this copolymer and copolymer 39 fractions b-e all show a low polydispersity, four of them giving a value of less than 2. This was a desirable feature for the purpose of the proposed study.

Copolymers 39 (unfractionated), 68, 78 and 79 all contain a broader range of molecular sizes, as indicated by the $\bar{M}w/\bar{M}n$ values.

TABLE 3.3 CHARACTERISTICS OF CROSSLINKED COPOLYMERS AND COPOLYMER 74

POLYMER CODE NO.	$\bar{M}w$	$\bar{M}w/\bar{M}n$	γ	ρ ($\times 10^{-3}$)	% OF CROSSLINKED SIDECHAINS	$\bar{M}w$ PRECURSOR	$\bar{M}w/\bar{M}n$ PRECURSOR
39 (un)	190,000	6.80	0.518	3.885	14.70	32,100	1.60
39a	> 400,000					32,100	1.60
39b	400,000	2.50			17.00	32,100	1.60
39c	155,000	1.90			14.00	32,100	1.60
39d	110,000	1.70			13.00	32,100	1.60
39e	34,000	1.80			1.10	32,100	1.60
74	31,000	1.50				32,100	1.60
68	171,000	5.80	0.669	6.100	14.90	21,500	1.31
78	73,800	3.05	0.541	4.940	12.00	21,500	1.31
79	64,300	2.74	0.509	4.640	11.30	21,500	1.31

γ = the crosslinking index i.e. the number of crosslinked units per primary chain.
ρ = the network density i.e. the molar fraction of crosslinked units.

Radiolabelled copolymer preparations were subjected to Sepharose 4B/6B column chromatography (column length 60cm, 16mm internal diameter, flow rate 20ml/h) in order to observe the molecular weight distribution patterns. The results are shown in figs 3.4-3.13. These elution profiles compared extremely well with those obtained in Prague for the same (unlabelled) compounds using Sepharose 4B/6B chromatography and detection with a Knauer Differential Refractometer. This comparison demonstrated that molecules of all sizes within a preparation were radiolabelled to a similar extent, and therefore that a true representation of the molecular weight distribution of all samples could be obtained using radiolabelled copolymer preparations.

FIGURE 3.4

SEPHAROSE 4B/6B COLUMN CHROMATOGRAPHY OF ^{125}I-LABELLED COPOLYMER 39

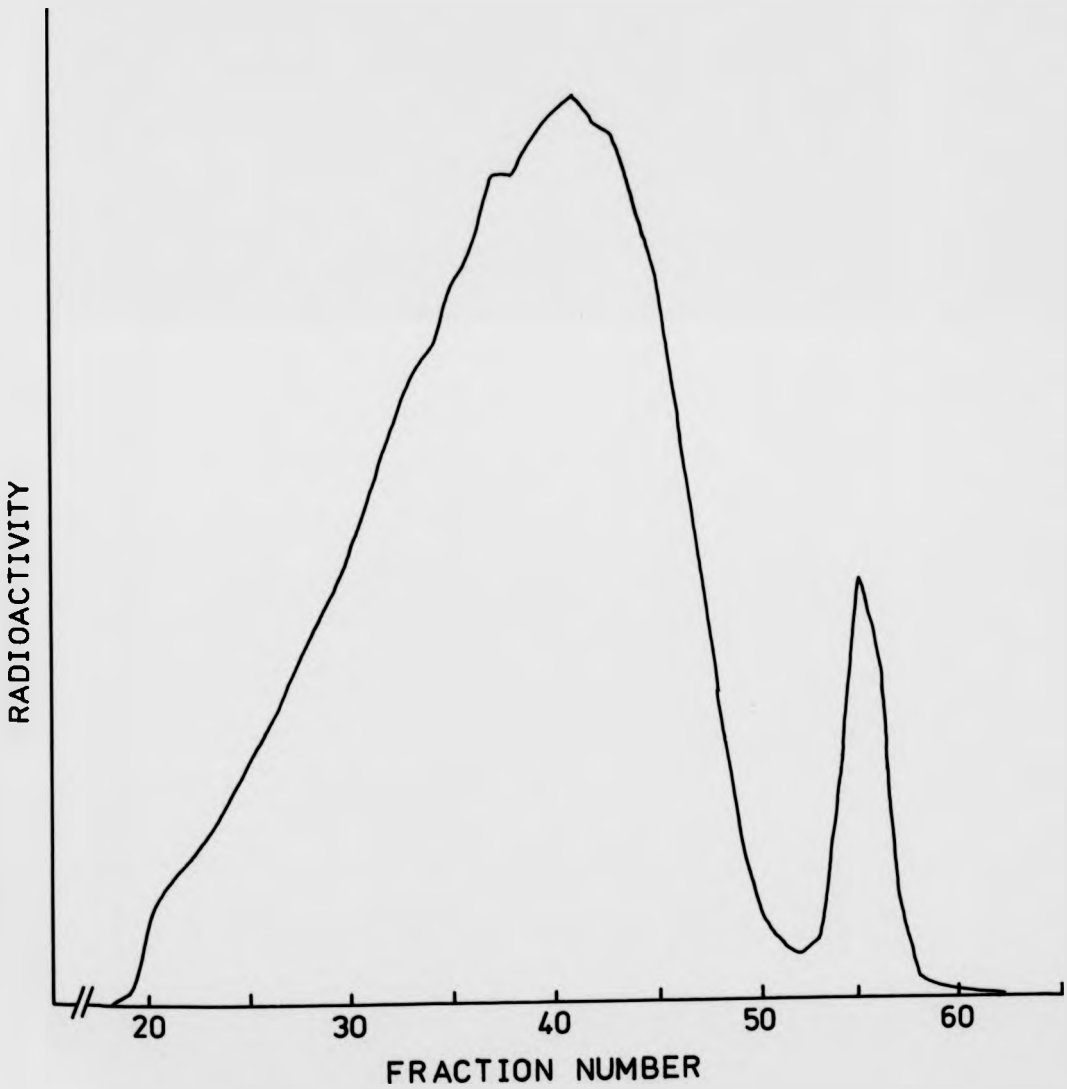


FIGURE 3.4

SEPHAROSE 4B/6B COLUMN CHROMATOGRAPHY OF ^{125}I-LABELLED COPOLYMER 39

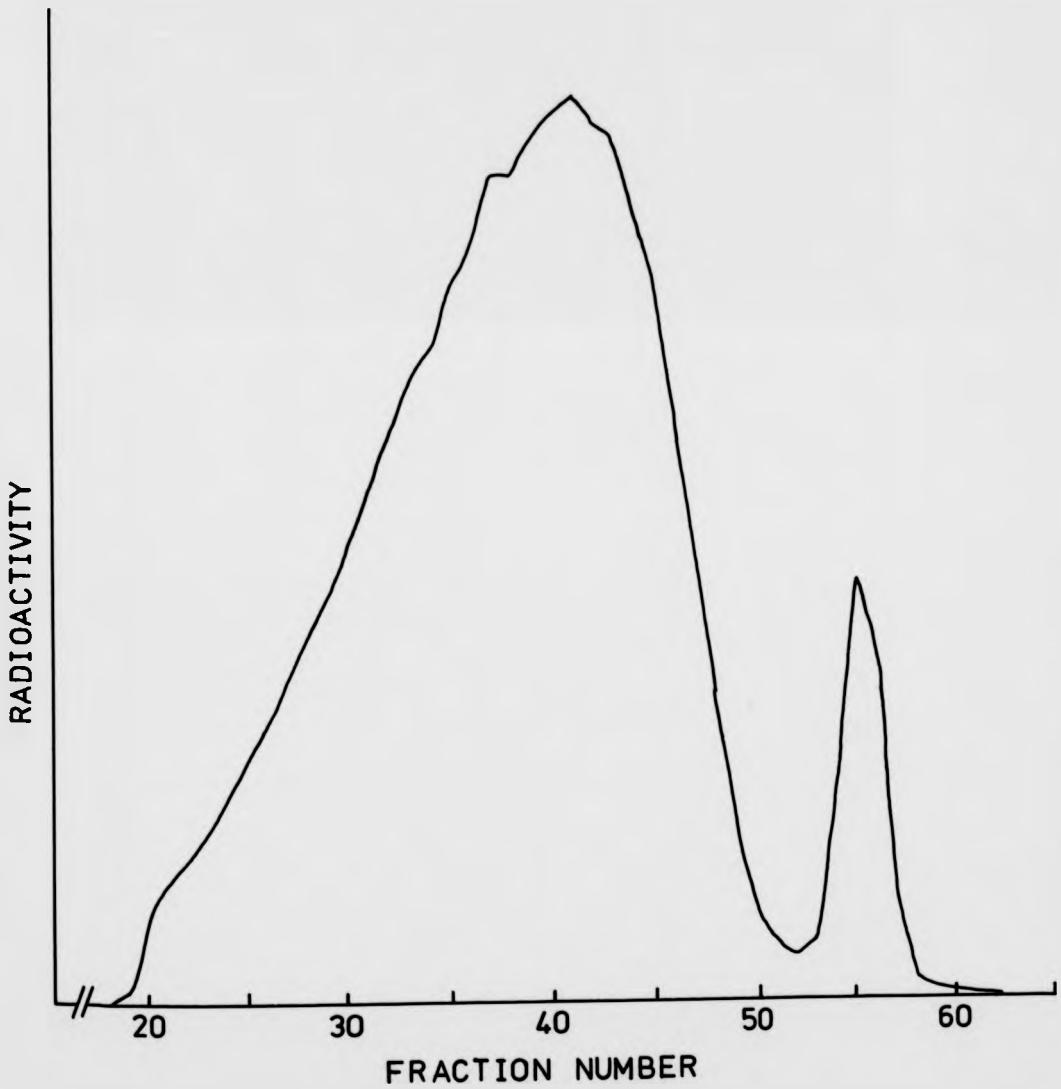


FIGURE 3.5

SEPHAROSE 4B/6B COLUMN CHROMATOGRAPHY OF ^{125}I-LABELLED COPOLYMER 39a

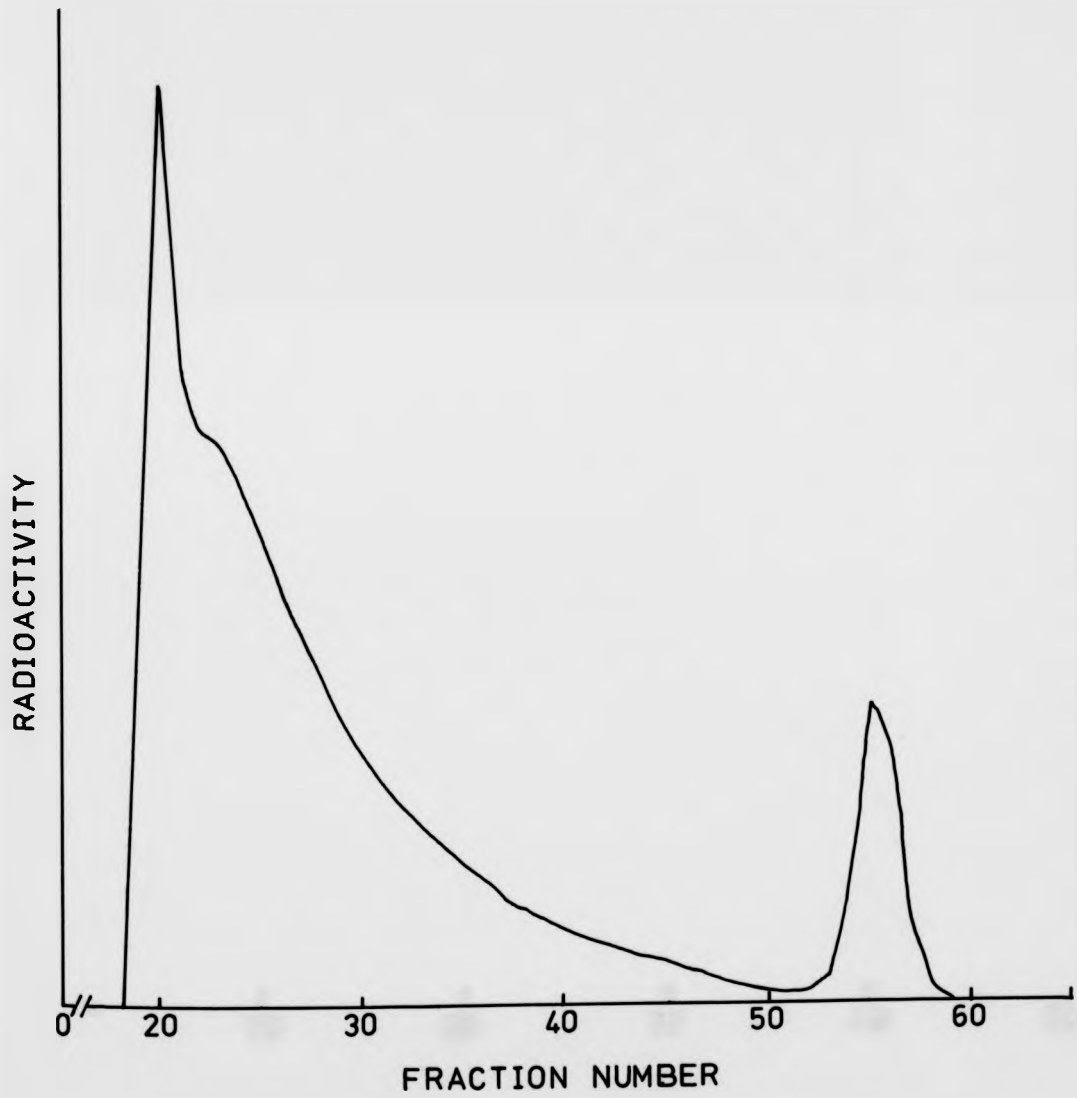


FIGURE 3.6

SEPHAROSE 4B/6B COLUMN CHROMATOGRAPHY OF ^{125}I-LABELLED COPOLYMER 39b

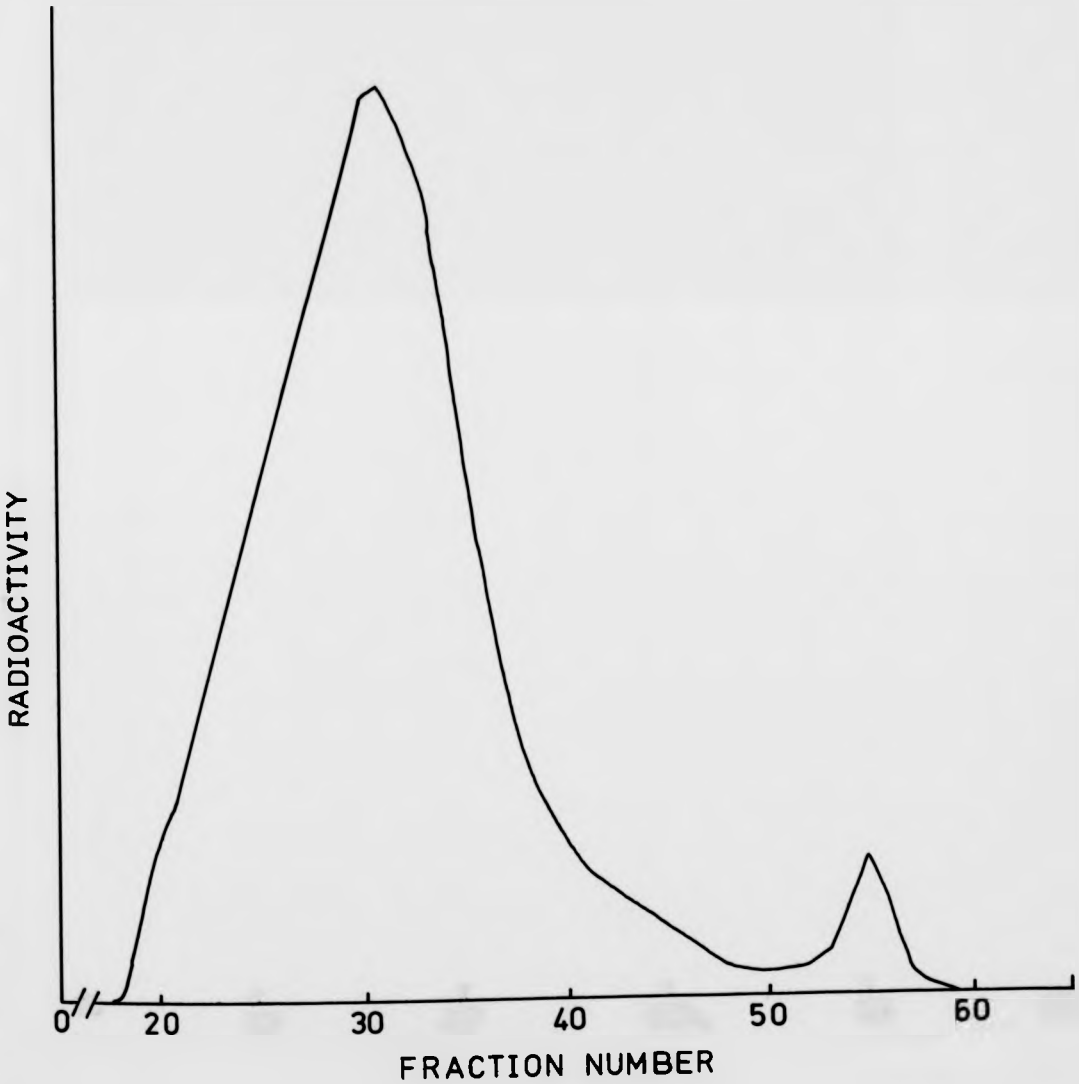


FIGURE 3.7

SEPHAROSE 4B/6B COLUMN CHROMATOGRAPHY OF ^{125}I-LABELLED COPOLYMER 39c

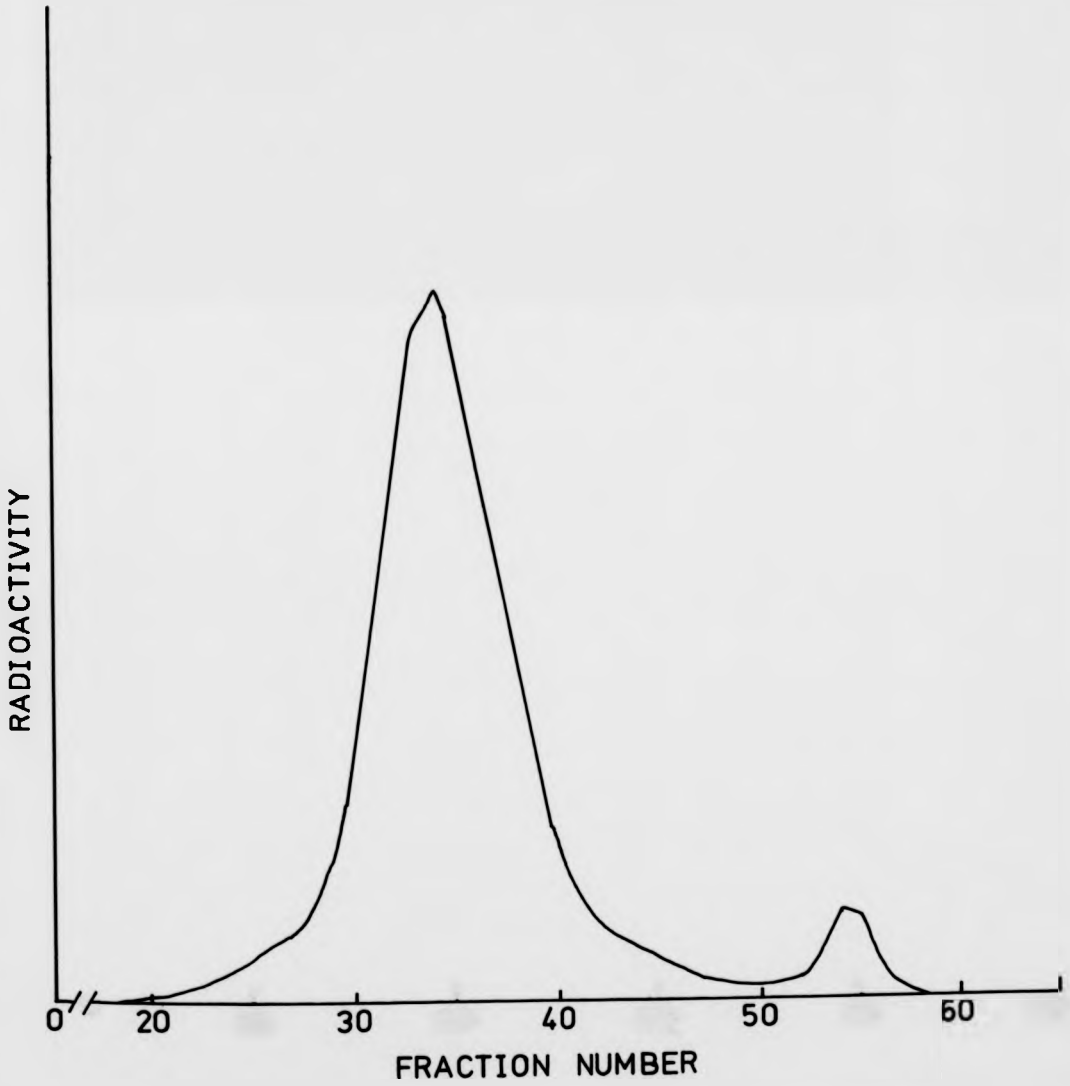


FIGURE 3.8

SEPHAROSE 4B/6B COLUMN CHROMATOGRAPHY OF ^{125}I-LABELLED COPOLYMER 39d

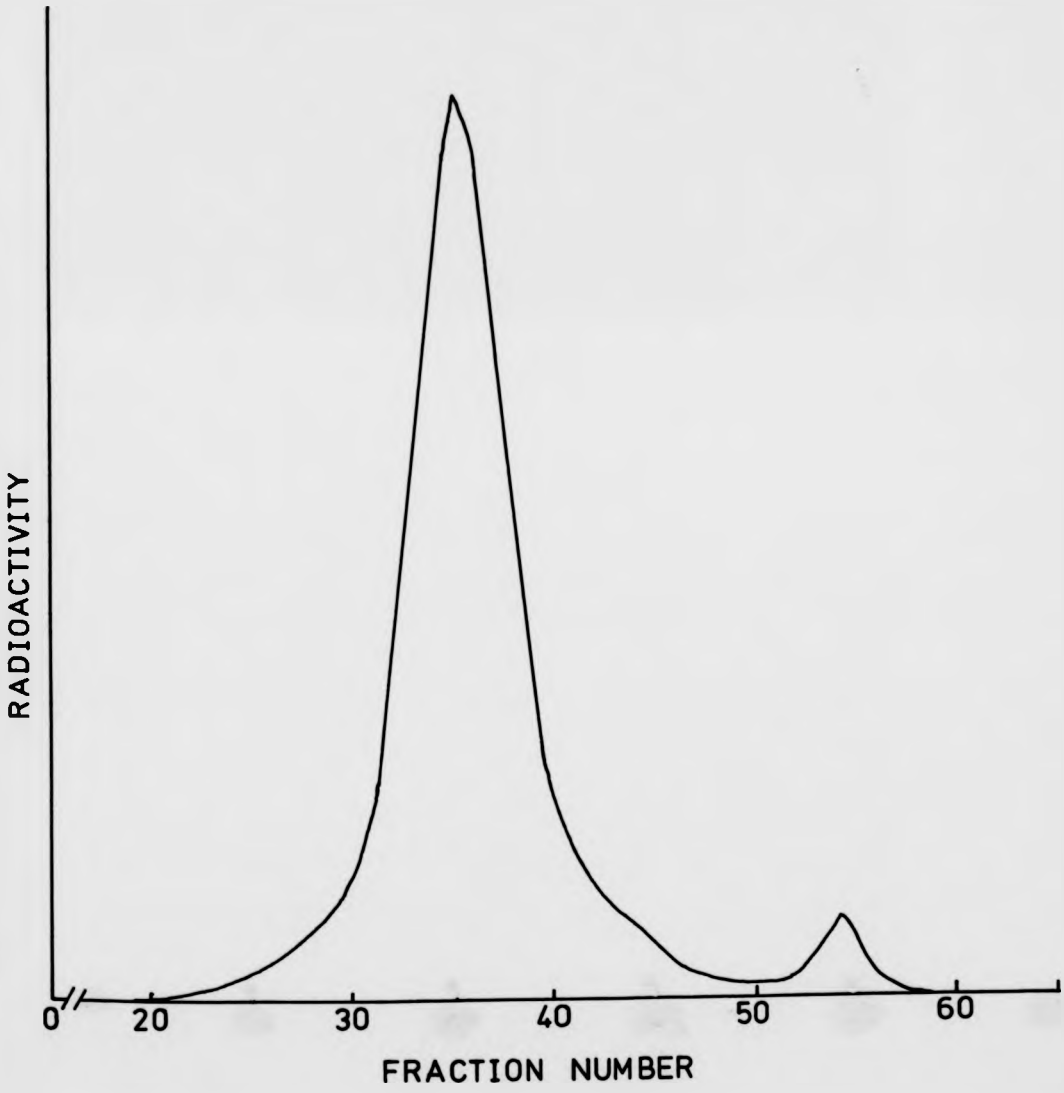


FIGURE 3.9

SEPHAROSE 4B/6B COLUMN CHROMATOGRAPHY OF ^{125}I-LABELLED COPOLYMER 39e

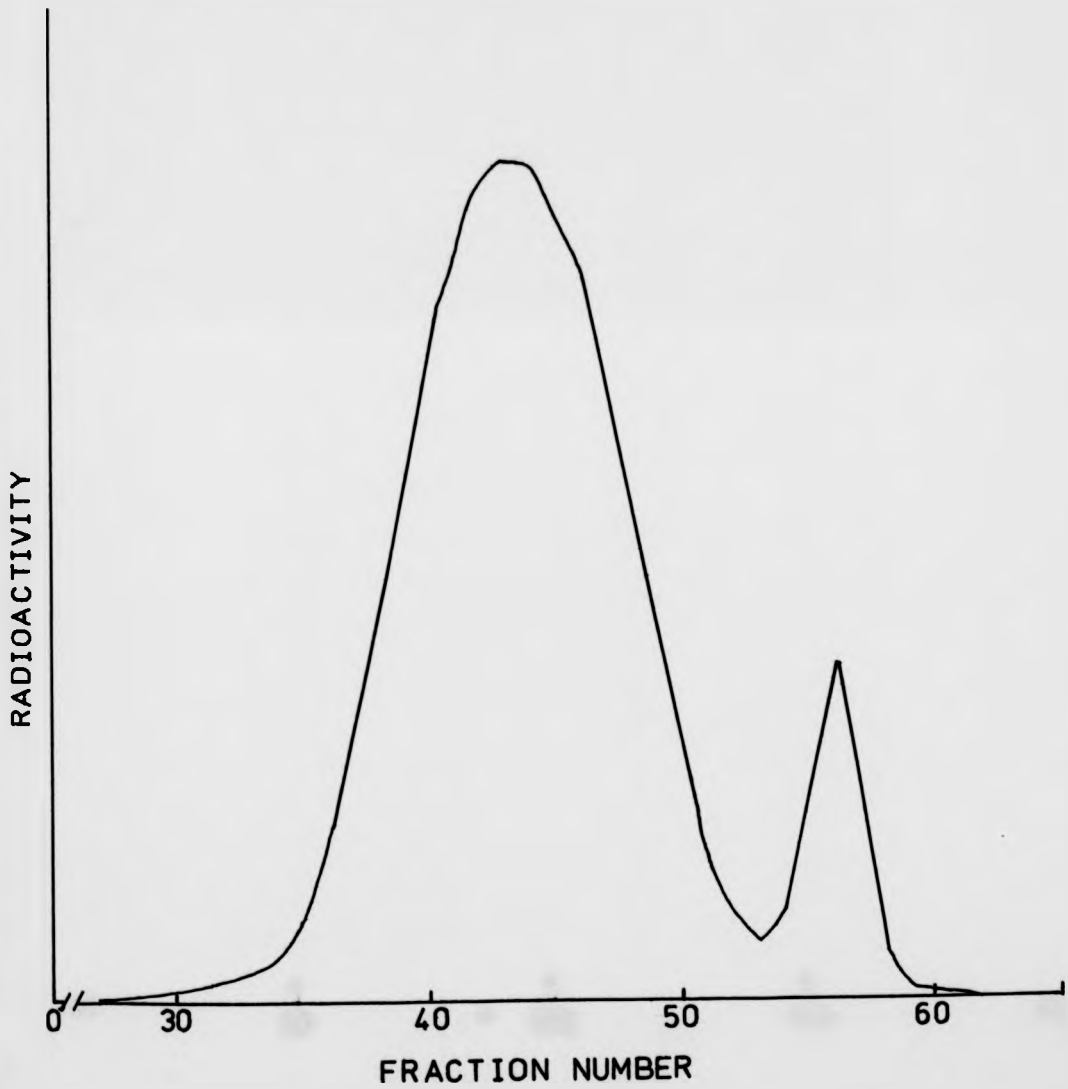


FIGURE 3.10

SEPHAROSE 4B/6B COLUMN CHROMATOGRAPHY OF ^{125}I-LABELLED COPOLYMER 74

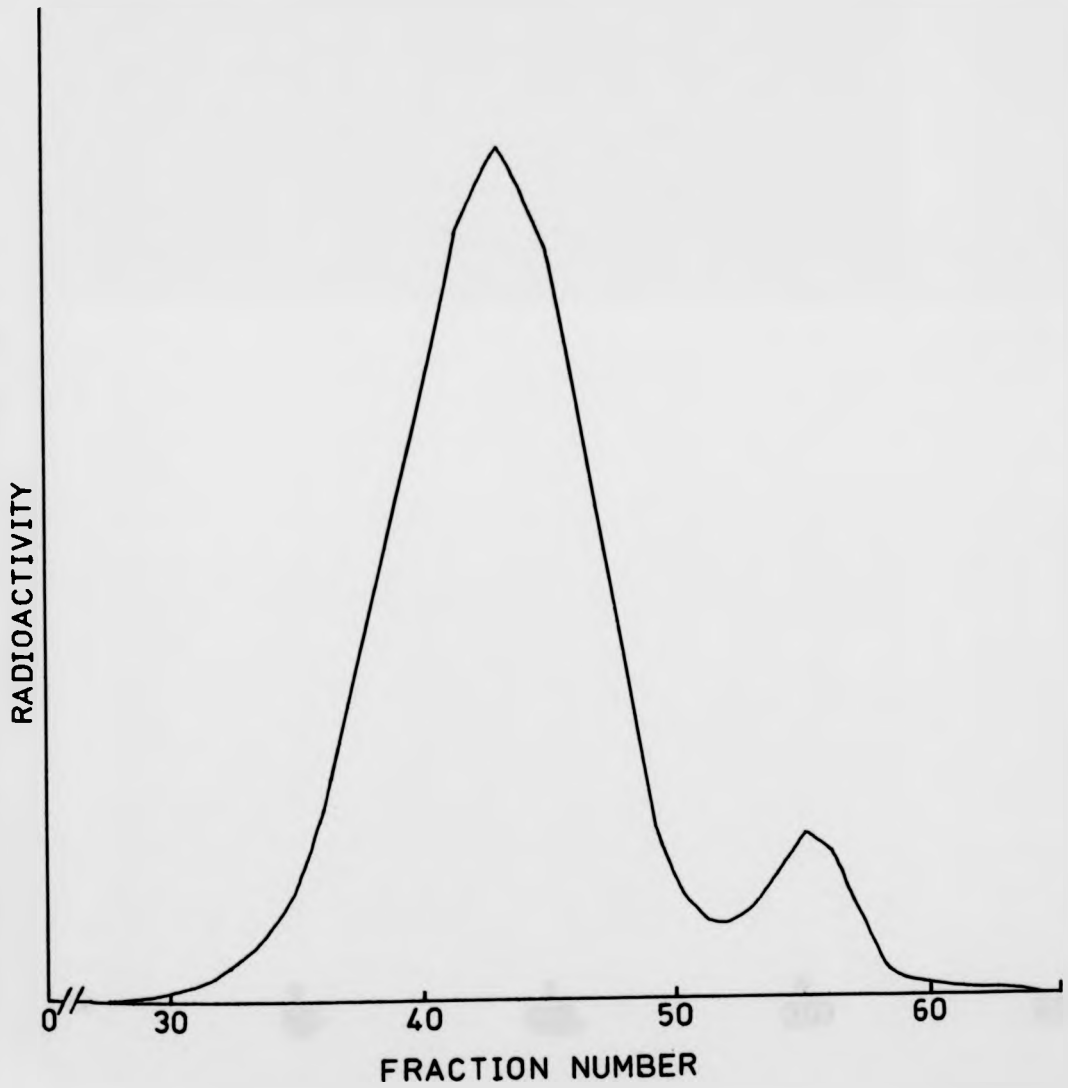


FIGURE 3.11

SEPHAROSE 4B/6B COLUMN CHROMATOGRAPHY OF ^{125}I-LABELLED COPOLYMER 68

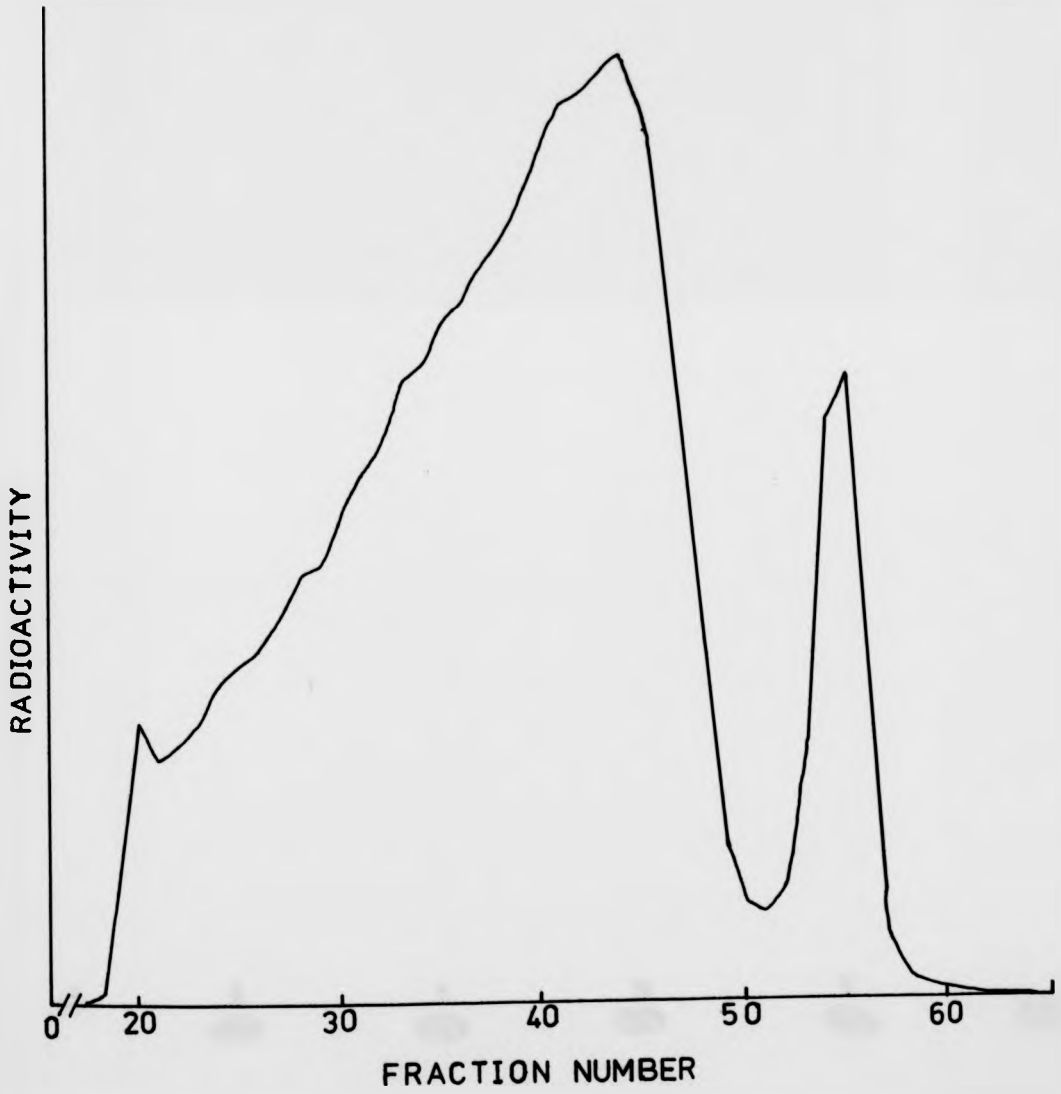


FIGURE 3.12

SEPHAROSE 4B/6B COLUMN CHROMATOGRAPHY OF ^{125}I-LABELLED COPOLYMER 78

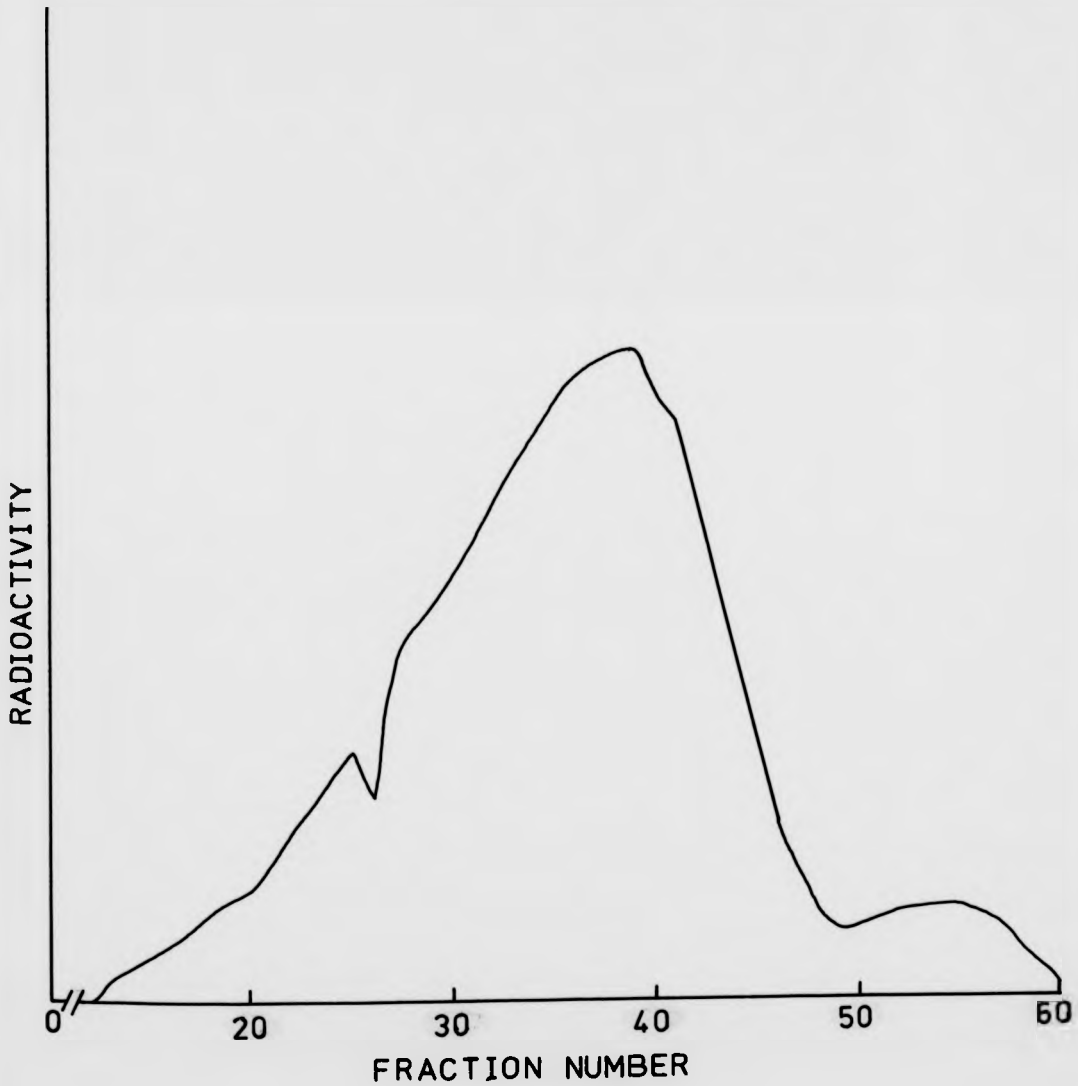
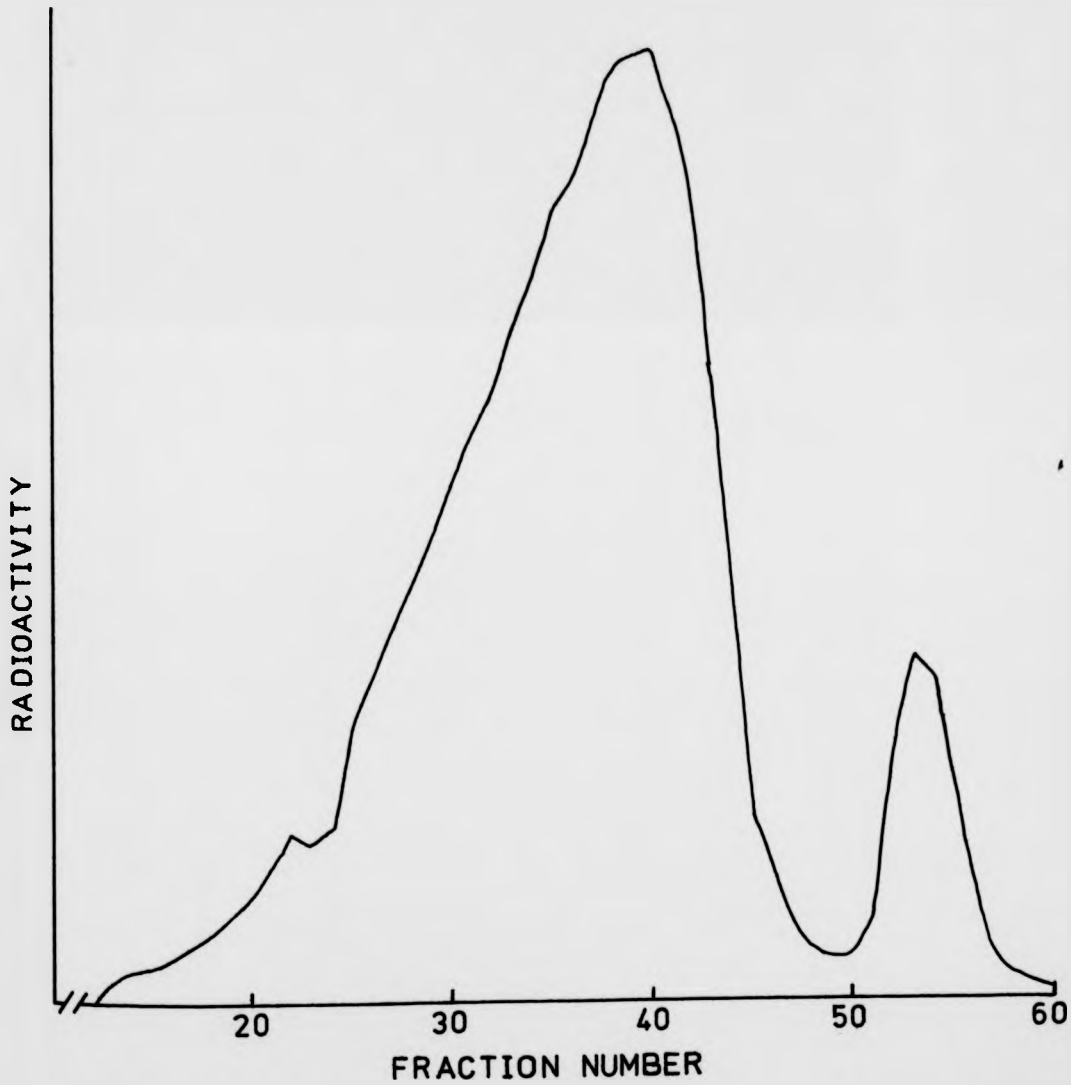


FIGURE 3.13

SEPHAROSE 4B/6B COLUMN CHROMATOGRAPHY OF ^{125}I-LABELLED COPOLYMER 79



3.5 THE STABILITY OF RADIOLABELLED COPOLYMER 39 PREPARATIONS AT 4°C

Following radioiodination, copolymer preparations were dialysed to remove excess $[^{125}I]$ iodide, and stored at 4°C prior to use. In order to assess the stability of the label during storage, preparations were periodically checked for free $[^{125}I]$ iodide using paper electrophoresis. A typical result (copolymer 39c after 4 weeks of storage) is shown in fig 3.14 and all results obtained for copolymer 39 fractions over a six week period are summarized in Table 3.4. It can be seen that the free $[^{125}I]$ iodide levels increase by $< 1\%$ per week during storage. This was an acceptable amount for the proposed studies, although care was taken to dialyse preparations periodically in order to remove free $[^{125}I]$ iodide prior to use following long periods of storage.

FIGURE 3.14

LOW VOLTAGE PAPER ELECTROPHORESIS OF ^{125}I-LABELLED COPOLYMER 39c FOLLOWING STORAGE AT 4°C FOR FOUR WEEKS

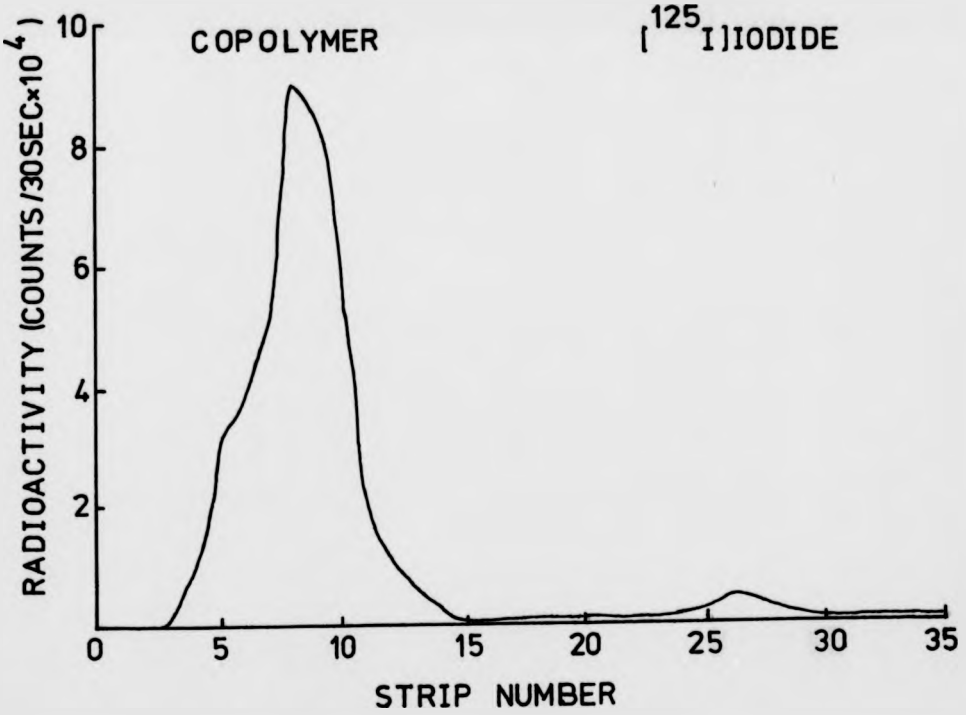


TABLE 3.4

STABILITY OF ^{125}I-LABELLED COPOLYMER 39 FRACTIONS ON STORAGE AT 4°C OVER A SIX WEEK PERIOD

POLYMER CODE NO.	% $[^{125}I]$ IODIDE CONTENT OF PREPARATIONS AFTER		
	1 WEEK	4 WEEKS	6 WEEKS
39 (un)	0.62	1.53	1.80
39a	1.06	2.90	2.97
39b	1.07	2.62	3.66
39c	2.90	3.62	5.10
39d	2.18	3.80	5.90
39e	1.92	4.61	5.05

3.6 THE STABILITY OF RADIOLABELLED COPOLYMER PREPARATIONS IN TISSUE CULTURE MEDIUM (MEDIUM 199 WITH 10% CALF SERUM) AT 37°C

Since almost all experiments performed using organ culture systems involved the incubation of copolymers at 37°C in medium Tc199 containing 10% calf serum, experiments were carried out to test the stability of the [^{125}I] label of the copolymer under those conditions. The release of [^{125}I] iodide and/or [^{125}I] iodotyrosine was also monitored.

All copolymers used for organ culture experiments were therefore assessed for free [^{125}I] iodide both before and after incubation for 5h in 10 ml of Tc199 containing 10% calf serum (62.5 µg/ml), using Sephadex G-15 column chromatography. A typical elution profile following incubation of copolymer 39a is shown in fig 3.15 and results are summarized in Table 3.5.

From the results, it is apparent that a very small increase in free [^{125}I] iodide occurs during a 5h incubation period (2% or less) and that no [^{125}I] iodotyrosine is released from the preparations under the experimental conditions.

Copolymer 39e was tested under the same experimental conditions for 12h. During this period there was an increase of 3.36% in total free [^{125}I] iodide, but [^{125}I] iodotyrosine was not released from the preparation under the experimental conditions in the absence of tissue.

From these results, it was concluded that the copolymer preparations were suitable for use under the given experimental conditions.

SEPHADEX G-15 COLUMN CHROMATOGRAPHY OF ^{125}I-LABELLED COPOLYMER 39a IN MEDIUM 199 (+10% CALF SERUM) FOLLOWING INCUBATION AT 37°C FOR 5H

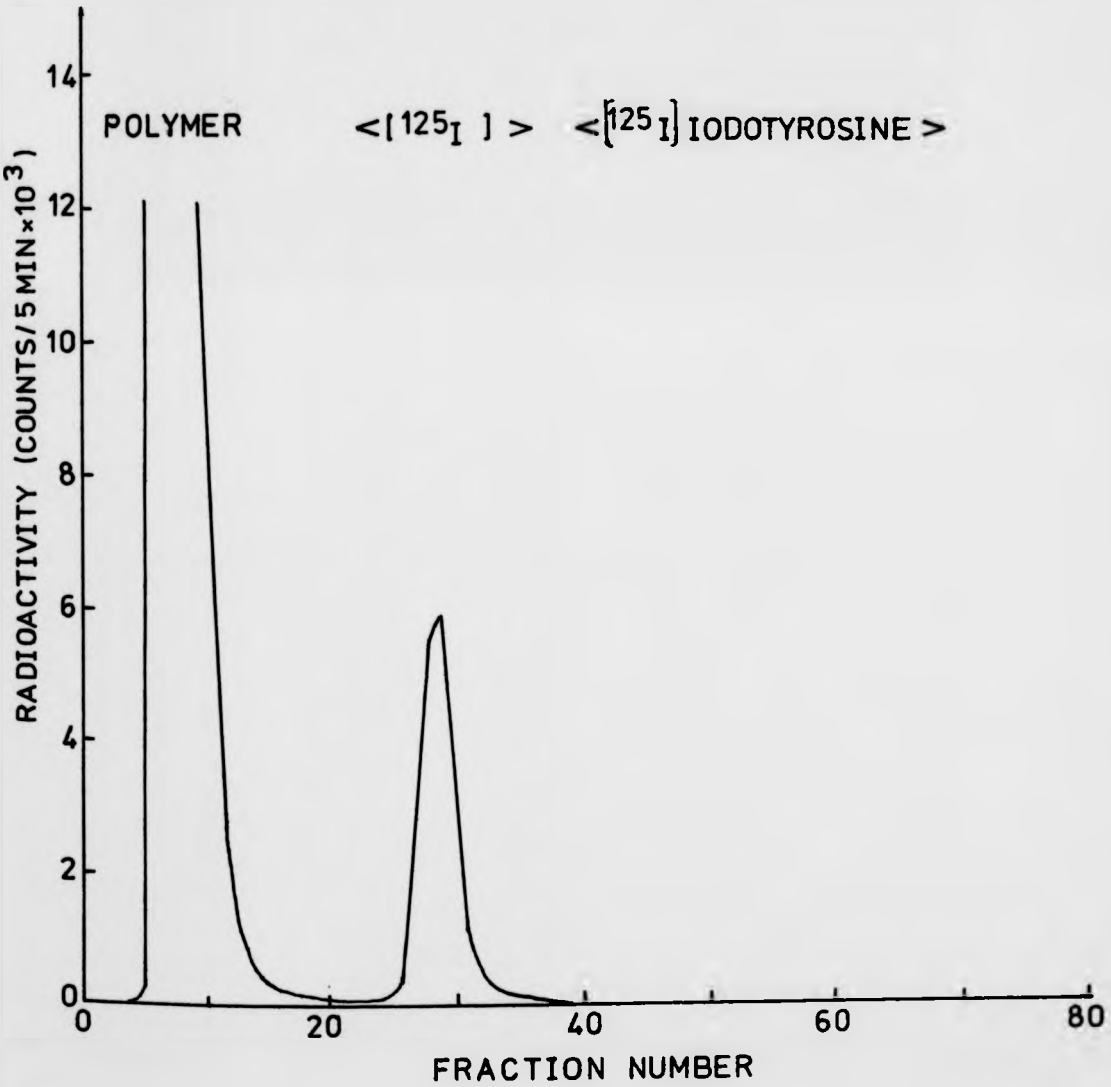


FIGURE 3.15

TABLE 3.5 STABILITY OF COPOLYMERS ON INCUBATION IN CULTURE MEDIUM (MEDIUM 199 CONTAINING 10% CALF SERUM) AT 37°C OVER A 5H PERIOD

POLYMER CODE NO.	% $[^{125}I]$ IODIDE BEFORE INCUBATION	% $[^{125}I]$ IODIDE FOLLOWING INCUBATION	% $[^{125}I]$ IODOTYROSINE
39a	2.80	4.56	0.00
39b	4.39	4.89	0.00
39c	5.00	5.49	0.00
39d	6.22	7.20	0.00
39e	1.80	3.80	0.00
74	2.00	2.10	0.00
68	3.70	3.90	0.00

3.7 THE STABILITY OF RADIOLABELLED COPOLYMER PREPARATIONS IN RAT PLASMA

It is important to establish that crosslink cleavage takes place only within lysosomes, and not in the circulation. Also, it is necessary that the radiolabel remains attached to ^{125}I-labelled copolymers in the bloodstream in order to monitor levels of radioactivity effectively.

An in vitro method was used to investigate the stability of copolymers to be used in in vivo studies i.e. ^{125}I-labelled copolymers 39, 78 and 79 in plasma.

Radiolabelled copolymer (62.5 µg in 0.1 ml.05M phosphate buffer pH 8.0) was added to tubes containing 1 ml of rat plasma, and incubated for 0, 1, 5 and 24h at 37°C. Following incubation, plasma proteins were precipitated with 0.5 ml 20% trichloroacetic acid (TCA), the tubes centrifuged at 2,000g for 20 min, and the resultant supernatant applied to a Sepharose 4B/6B column (length 60cm, I.D. 16mm, flow rate 20 ml/h).

No differences were observed in the molecular weight distributions of copolymers before and following incubation with rat plasma. A typical elution profile is shown in fig 3.16 and results are summarized in Table 3.6.

Sepharose 4B/6B chromatography does not discriminate between the smaller molecules i.e. $[^{125}I]$ iodide and $[^{125}I]$ iodotyrosine. The second peak present in the elution profiles at t=0 is due to the presence of $[^{125}I]$ iodide in the preparation. Any increase in the size of the second peak during the experimental period could be due to the release of $[^{125}I]$ iodide or $[^{125}I]$ iodotyrosine from the copolymer. From the results presented, it can be seen that no significant increase in the

SEPHAROSE 4B/6B CHROMATOGRAPHY OF RAT PLASMA CONTAINING ^{125}I-LABELLED COPOLYMER 78 FOLLOWING 24H INCUBATION AT 37°C

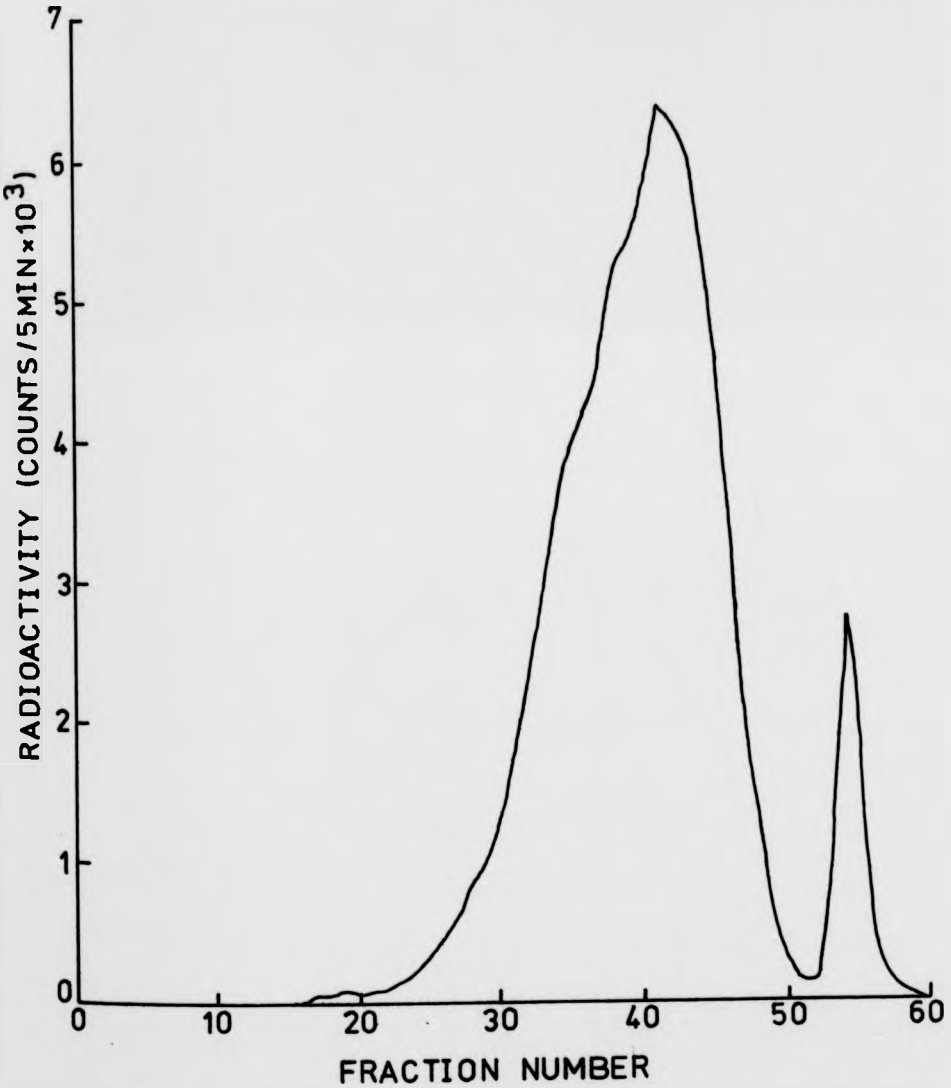


FIGURE 3.16

TABLE 3.6 SEPHAROSE 4B/6B CHROMATOGRAPHY OF ^{125}I-LABELLED COPOLYMERS AFTER INCUBATION IN RAT PLASMA AT 37°C

SUBSTRATE	POLYMER (1ST PEAK) (% TOTAL COUNTS FROM COLUMN)				L.M.W. (2ND PEAK) (% TOTAL COUNTS FROM COLUMN)			
	t=0	1h	5h	24h	t=0	1h	5h	24h
39	100.00	94.98	94.85	93.27	0.00	5.20	4.43	3.96
78	92.21	91.13	91.83	92.09	6.53	7.44	7.12	7.01
79	87.91	87.36	87.15	87.24	11.07	11.41	11.22	11.01

second peak of radioactivity is detected for polymers 78 and 79 during a 24h incubation with plasma. This indicates that neither $[^{125}I]$ iodide or $[^{125}I]$ iodotyrosine is released during that period. It can therefore be concluded that administration of polymer containing no free iodide to rats in vivo for up to 24h, will result in the recovery of blood radioactivity that represents copolymer and not $[^{125}I]$ iodide or $[^{125}I]$ iodotyrosine. This copolymer may be crosslinked or may have had its crosslinks cleaved following pinocytosis, digestion and exocytosis by cells.

Copolymer 39, on the other hand, released small molecules (5.2% of the total amount of radioactivity present) within the first hour of incubation in rat plasma. This radioactivity could be free $[^{125}I]$ iodide or $[^{125}I]$ iodotyrosine, or both, since no free $[^{125}I]$ iodide was detected in the preparation at the beginning of the experiment. This value does not increase beyond the first hour of incubation. The experiment was performed once only because of the difficulty involved in obtaining large volumes of rat plasma. It is therefore possible that the column chromatography result given for t=0, which indicated that no $[^{125}I]$ iodide was present in the preparation, was questionable. However, since the release of small molecules was confined to the first hour of incubation with no subsequent release, and the first time-point in subsequent in vivo studies was 1h, it could be assumed that increases in low molecular weight substances recovered after that time would be due to the degradation of copolymer 39 crosslinks by lysosomal enzymes to yield free $[^{125}I]$ iodotyrosine.

3.8 CONCLUSIONS

1. All the soluble HPMA copolymers subjected to radioiodination were labelled to a sufficient degree to enable adequate counts to be detected to give meaningful results in the proposed experimental methods.

2. In excess of 94% of the radioactive label remained attached to copolymers during six weeks storage at 4°C. The release of free $[^{125}I]$ iodide from preparations at a rate of < 1% per week was considered acceptable, provided that dialysis of ^{125}I-labelled copolymers to remove excess free $[^{125}I]$ iodide was performed after prolonged storage prior to their use in biological experiments.

3. All radiolabelled copolymers were found to be stable (i.e. no release of $[^{125}I]$ iodotyrosine and insignificant increases in free $[^{125}I]$ iodide) in medium Tc199 containing 10% calf serum at 37°C for 5h.

4. All radiolabelled copolymers were found to be stable in rat plasma from 1-24h at 37°C and were therefore acceptable for use in proposed in vivo experiments.

CHAPTER 4

PINOCYTIC UPTAKE AND INTRACELLULAR DEGRADATION OF ^{125}I-LABELLED HPMA COPOLYMERS BY THE RAT VISCERAL YOLK SAC IN VITRO

4.1 INTRODUCTION

This chapter reports experiments on the pinocytosis and intracellular handling of crosslinked polymers. The specific questions investigated are the following:

1. Are crosslinked copolymers pinocytosed by cells?
2. If so, is the mechanism responsible fluid-phase pinocytosis, or adsorptive pinocytosis?
3. If pinocytosis does take place, is the rate of uptake dependent on the size of the copolymer?
4. Are crosslinked copolymers hydrolysed within the lysosomes?
5. If so, is the extent of degradation dependent on the size of the copolymer?

Quantitative studies on pinocytosis can be achieved by both in vitro and in vivo methods. In vivo methods are less satisfactory in that interpretation is complicated by the differing rates of pinocytic uptake of substrates into different tissues, the clearance of low molecular weight substrates by the kidney, and the possible metabolism of radiolabelled substrates over the experimental period. For reasons such as these, it is better to commence studies on pinocytosis of a given substrate using a well documented, reliable in vitro method.

The rat visceral yolk sac has been used extensively as an in vitro model system for the quantitative study of pinocytosis. The method, which has been described in Chapter 2, was published in 1975 by Williams, Lloyd and coworkers, and is used routinely for investigations on fluid-phase pinocytosis, adsorptive pinocytosis, and intralysosomal protein degradation.

The yolk sac is a highly pinocytic tissue which plays an important role in histotrophic embryonic nutrition in the rat, particularly during the period between implantation of the embryo into the uterine wall and the formation of a fully functional chorio-allantoic placenta (Freeman et al 1981). Until the 16th day of gestation, the yolk sac is composed of parietal and visceral yolk sac tissue (fig 4.1), after which the parietal yolk sac membrane is shed, leaving the visceral yolk sac in direct contact with the uterine cavity.

The visceral yolk sac is comprised of three layers of cells (fig 4.2) . The outer visceral endoderm of columnar epithelial cells demonstrate an extensive microvillous structure at the apical membrane, and rest on a basement membrane. Secondly, the mesenchymal layer, which rests on the serosal basement membrane, contains scattered mesenchymal cells interspersed with vitelline blood capillaries. Finally, a thin layer of mesothelial cells are present which line the extra-embryonic coelom.

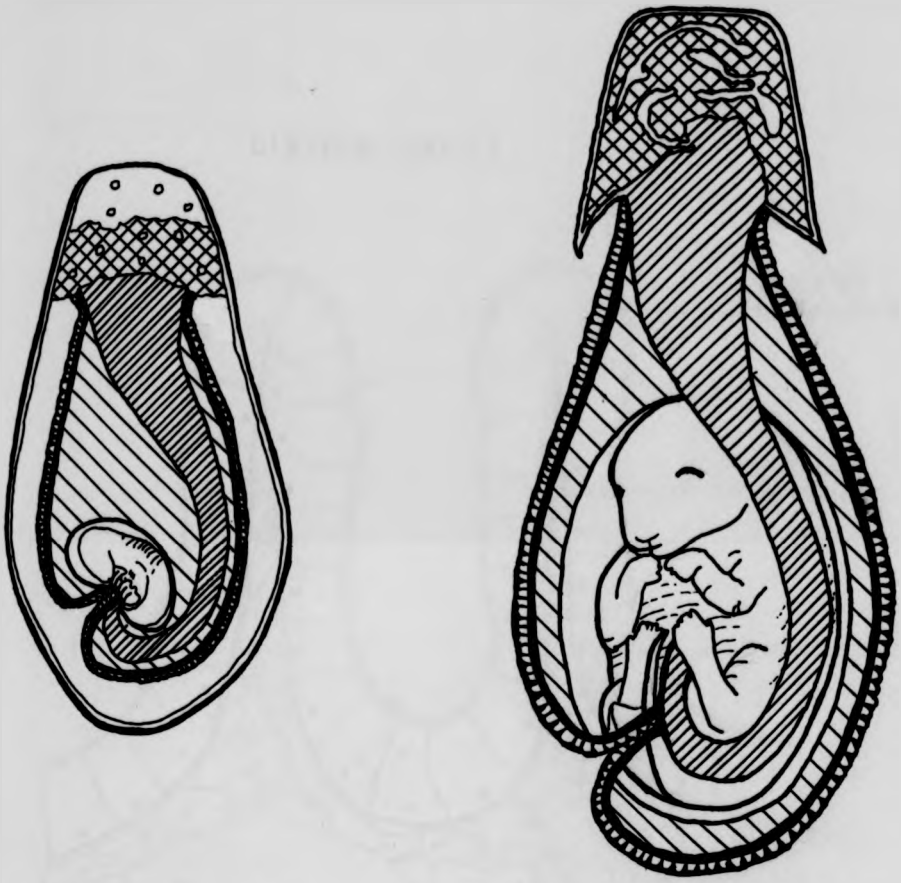
It is the microvillous epithelial cells found on the outer surface of the yolk sac which are in contact with the uterine cavity and which are responsible for the pinocytosis of macromolecules derived from the uterus. These molecules are degraded within lysosomes to provide smaller nutrients for the growing embryo.

The 17.5 day yolk sac is easy to maintain in organ culture for periods of time up to 12h without loss of structural integrity or pinocytic capacity. The yolk sac cultured in vitro has been used to study the pinocytic uptake of various proteins including ^{125}I-labelled bovine serum albumin (BSA), hen egg-white lysozyme, bovine pancreatic ribonuclease A and

THE DEVELOPMENT OF FOETAL MEMBRANES IN THE RAT

FIGURE 4.1

(From BECK and LLOYD, 1968)



10 TO 16 DAYS

16 DAYS TO TERM

KEY

Chorioallantoic placenta

Umbilical cord

Extra-embryonic coelom

Parietal yolk sac

Visceral yolk sac

DIAGRAMMATIC SECTION THROUGH THE RAT YOLK SAC

(from IBBOTSON 1977)

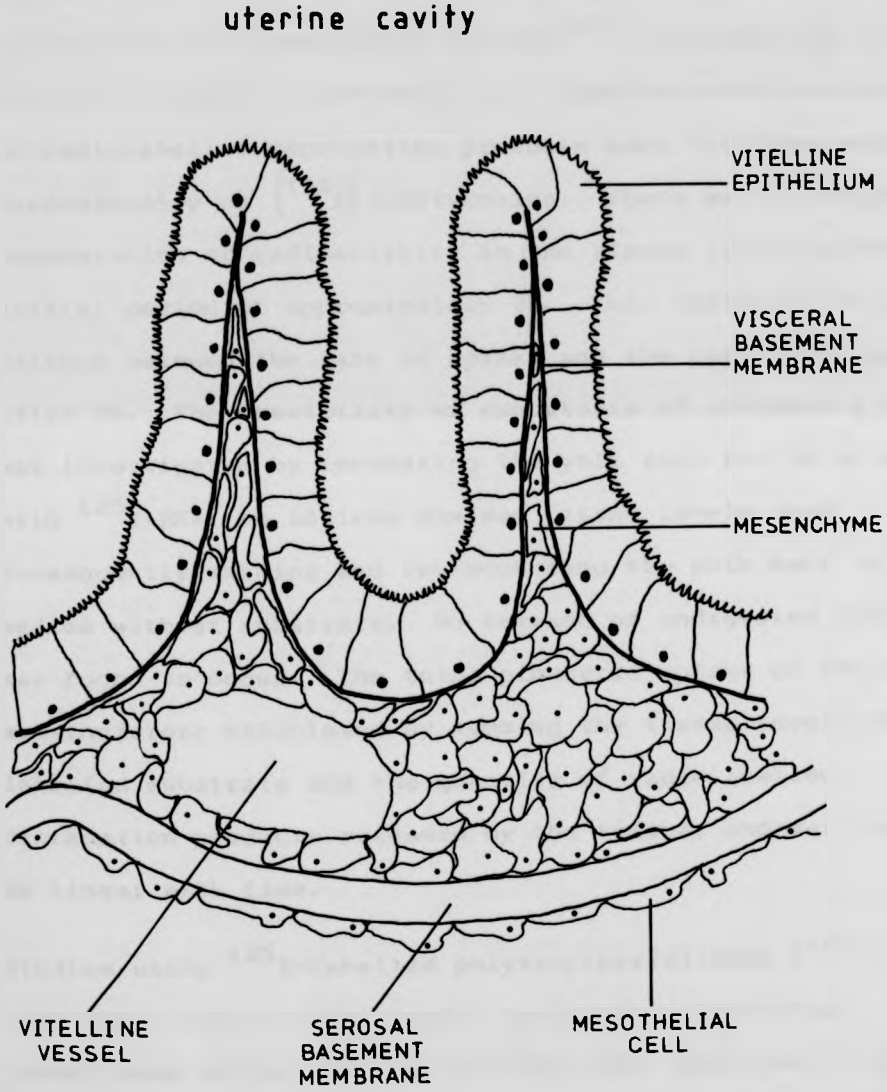


FIGURE 4.2

bovine insulin (Livesey and Williams 1977 , Forster 1982). The rate of pinocytic uptake was different for each protein, but all substrates demonstrated rapid uptake, indicating some degree of adsorption of substrate to the plasma membrane prior to vesicle formation. Williams et al (1975b) have shown that pinocytosis of formaldehyde-treated ^{125}I-labelled BSA by yolk sacs is followed by intracellular digestion with the release of radiolabelled degradation products back into the medium, predominantly as [^{125}I] iodotyrosine. There was no progressive accumulation of radioactivity in the tissue itself after an initial period of approximately 2h. This indicated an exact balance between the rate of uptake and the rate of degradation after 2h. The possibility of exocytosis of undigested protein was investigated by incubating the yolk sacs for 3h in medium with ^{125}I-BSA, to achieve maximum tissue levels, and subsequently washing and re-incubating the yolk sacs in fresh medium without substrate. No release of undigested protein was found to occur. The total pinocytic uptake of the protein was therefore calculated by summing the tissue levels of labelled substrate and the quantity of radiolabelled degradation products released by the tissue, and was found to be linear with time.

Studies using ^{125}I-labelled polyvinylpyrrolidone (^{125}I-PVP) have shown this to be a useful marker for fluid-phase pinocytosis in the 17.5 day rat yolk sac (Williams et al 1975a). ^{125}I-PVP is a non-degradable polymer which has been shown to accumulate within lysosomes following pinocytic uptake by cells (Roberts et al 1977). Uptake of this substrate is linear with time, and the rate shows minimal inter-experiment variation. The rate of exocytosis of ^{125}I-PVP is insignificant when compared to its rate of pinocytosis (< 1% per hour of that

pinocytosed) (Williams et al 1975a). It was therefore chosen as a control with which to compare uptake values obtained for pinocytosis of ^{125}I-labelled crosslinked HPMA copolymers.

As discussed in Chapter 1, many factors govern the rate at which a substrate is pinocytosed by cells. These include non-specific effects such as the size, charge and hydrophobicity of the molecule, and also the presence of moieties that may interact with cell-surface receptors, giving rise to enhanced uptake by receptor-mediated pinocytosis. These factors have been explored using synthetic polymers as substrates. HPMA copolymers of $\bar{M}w$ 30,000 consisting of a single copolymer chain and containing oligopeptide side chains have been shown to be pinocytosed by the rat visceral yolk sac and to enter cells at a rate similar to that observed with ^{125}I-PVP of $\bar{M}w$ 40,000 (Duncan et al 1981). This finding indicates that HPMA copolymers of that size do not interact with the cell membrane but are taken up by fluid-phase pinocytosis.

Duncan et al (1981) also examined the effect of molecular size of ^{125}I-PVP on its pinocytosis by rat visceral yolk sacs and rat peritoneal macrophages in vitro. Polymers of four mean molecular weights of 50,000, 84,000, 700,000 and 7,000,000 were used, and it was shown that macrophages preferentially captured the very high molecular weight fractions, whereas yolk sacs captured the lowest molecular weight preparations most rapidly. From this study, it was apparent that these cells, at least, can specifically select substrate of a preferred size from a range of otherwise identical molecules during fluid-phase pinocytosis.

The first experiments in the present work were to ascertain whether crosslinked HPMA copolymers could be pinocytosed by cells. Accordingly, copolymer 39 (crosslink structure P-Gly-Gly-Phe-Tyr-HMDA-Tyr-Phe-Gly-Gly-P) (see Fig 3.2) was radiolabelled and studied using the rat visceral yolk sac system. The possibility of the size of the molecule having an effect on its rate of uptake was also investigated by fractionating copolymer 39 (using Sepharose 6B chromatography) to produce five samples of $\bar{M}w$ 34,000, 110,000, 150,000, 400,000 and >400,000, before radioiodination and subsequent uptake studies.

Crosslinked copolymer 39 contains tyrosine residues within the potentially degradable crosslink structure. Rejmanová et al (1981) have shown that the crosslinks contained within this copolymer are cleaved by chymotrypsin in vitro, and preliminary in vivo experiments carried out by the same group in 1981 indicate crosslink cleavage following intravenous administration with resultant excretion of copolymer chains of lower molecular weight in the urine of a test animal. From these findings, it would be expected that if the ^{125}I-labelled copolymer fractions were pinocytosed by the yolk sac, intracellular hydrolysis of crosslinks might yield radiolabelled degradation products of low molecular weight, which could be detected in the culture medium. Experiments were therefore carried out to detect any degradation products in the culture medium following incubation of ^{125}I-labelled copolymer 39 fractions with yolk sacs.

It is necessary to demonstrate that radioactivity associated with tissue following culture with radiolabelled substrates is due to pinocytic uptake by the tissue, and not due to the

substrate binding to the cell surface without internalization taking place. Furthermore, it is also possible that any degradation products detected in the culture medium could be the result of extracellular hydrolytic events. Using metabolic and enzyme inhibitors, Duncan et al (1981) demonstrated pinocytic uptake of certain HPMA copolymers containing ^{125}I-labelled tyrosine residues in potentially degradable oligopeptide side-chains and the release of [^{125}I] iodotyrosine back into the culture medium following incubation with yolk sacs. Uptake was completely inhibited by 2,4-dinitrophenol (50 μg/ml), a known metabolic inhibitor, and thiol-proteinase inhibitor leupeptin (40 μg/ml) caused varying degrees of inhibition of lysosomal hydrolysis depending on side-chain composition. These inhibition studies proved that both pinocytosis and intracellular hydrolysis were taking place, and that the observed results were not due to extracellular events. Inhibition studies of this type were carried out using ^{125}I-labelled crosslinked copolymers.

Copolymer 74 ($\bar{M}w$ 31,000) was prepared from the same polymeric precursor as copolymer 39, but contains side-chains (rather than crosslinks) of the sequence P-Gly-Gly-Phe-TyrNH_2. Since copolymer 39e ($\bar{M}w$ 34,000) was of similar molecular weight to copolymer 74, uptake studies on both substrates were carried out in order to compare the extent of degradation when the same oligopeptide sequence was present in the form of side-chains, rather than crosslinks or intramolecular cycles.

Copolymer 68 ($\bar{M}w$ 171,000 - crosslink structure P-Gly-Gly-Phe-HMDA-Phe-Gly-Gly-P) was designed in order to trace the fate of the copolymer backbone, rather than the potentially degradable crosslinks contained within it. Since

the degradability of crosslinks and side-chains depends on both steric and structural factors, and the likelihood of enzymatic cleavage increases with increasing distance from the copolymer chain (Kopeček 1982 - see Chapter 1), it appeared unlikely that residues in close proximity to the polymer chain would be released by enzymatic hydrolysis. Consequently, in copolymer 68, tyrosine residues are attached directly to the polymer chain, in the hope of preventing enzymatic cleavage.

The lysosomal membrane is impermeable to molecules of molecular weight 200-300 (Reijngoud and Tager 1977). In view of this, any pinocytic uptake of copolymer 68 would result in the accumulation of radiolabel within lysosomes, since the copolymer chains with attached [^{125}I] iodotyrosine would be too large to pass through the lysosomal membrane, unlike free [^{125}I] iodotyrosine released from tyrosine-containing degradable crosslinks or side-chains.

Copolymer 68 was subsequently radioiodinated, and pinocytic uptake experiments performed using the yolk sac system.

Experiments were also performed to attempt to demonstrate crosslink cleavage within lysosomes following pinocytic uptake of ^{125}I-labelled copolymer 68. This was done by comparing the molecular weight distribution of the applied radiolabelled preparation with the distribution of radio-labelled material recovered from homogenised yolk sac tissue after incubation with the copolymer.

4.2 RESULTS

4.2.1 Pinocytic Uptake and Degradation of Copolymer 39 Fractions by the Yolk Sac: The Effect of Molecular Size

The accumulation of copolymer 39 fractions by the yolk sac was measured at various times over a 5h incubation period (at 37°C). Yolk sacs were incubated singly in all cases, and radiolabelled copolymer (2.5 µg/ml) was added to each flask at the beginning of each experiment.

Concurrent control experiments were performed using ^{125}I-PVP as substrate.

Figures 4.3 - 4.8 show progress curves for the tissue accumulation of radioactivity for each copolymer fraction (39a-e) and the unfractionated copolymer 39. Figure 4.9 combines 4.3 - 4.8. Results are expressed for each time point in µl/mg protein (Chapter 2, sections 8 and 9) as a mean ± S.E. of the uptake values obtained from 6-14 yolk sacs.

These values represent the levels of radioactivity associated with the tissue at any given time. They do not necessarily represent the total pinocytic uptake of the substrate. If digestion of substrate has occurred within the tissue following pinocytosis, with the subsequent release of radiolabelled degradation products back into the medium, the true rates of pinocytosis will in fact be higher than are apparent from figs. 4.3 - 4.8.

Inspection of Figs. 4.3 -4.8 shows that uptake is, to a first approximation, linear with time. Making that assumption, rates of uptake can be calculated and these are given in Table 4.1. There is clearly a marked decrease in the rate of accumulation with increasing molecular weight.

TISSUE ACCUMULATION OF ^{125}I-LABELLED HPMA COPOLYMER 39 BY RAT VISCERAL YOLK SAC

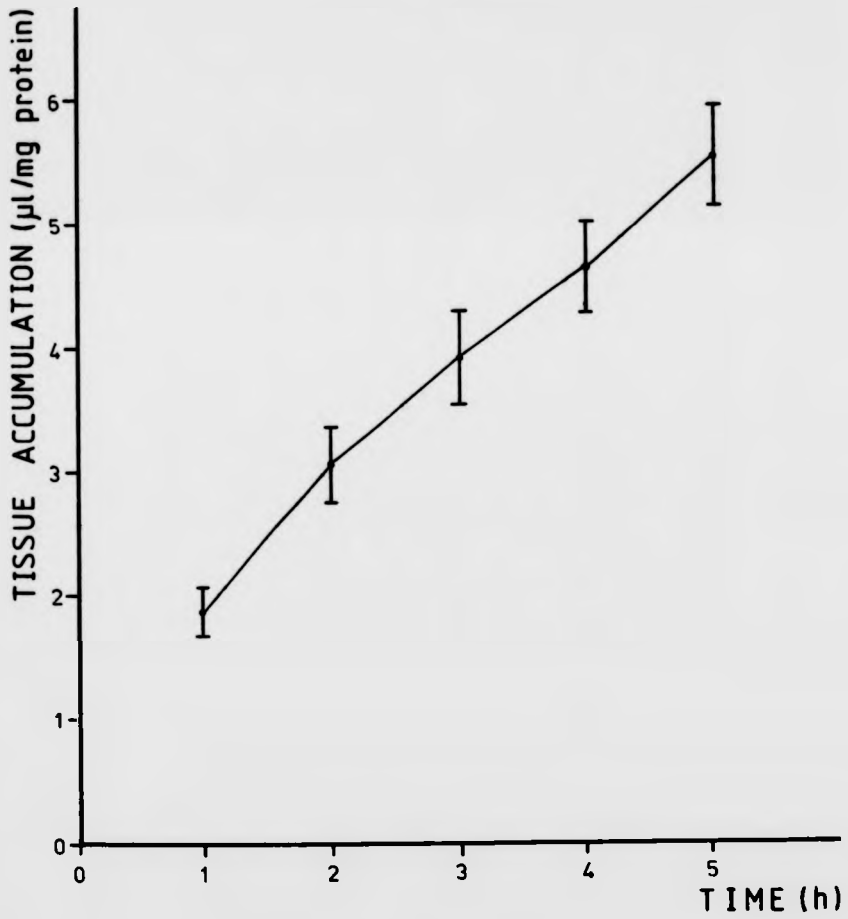


FIGURE 4.3

TISSUE ACCUMULATION OF ^{125}I-LABELLED H P M A COPOLYMER 39a RAT VISCERAL YOLK SAC

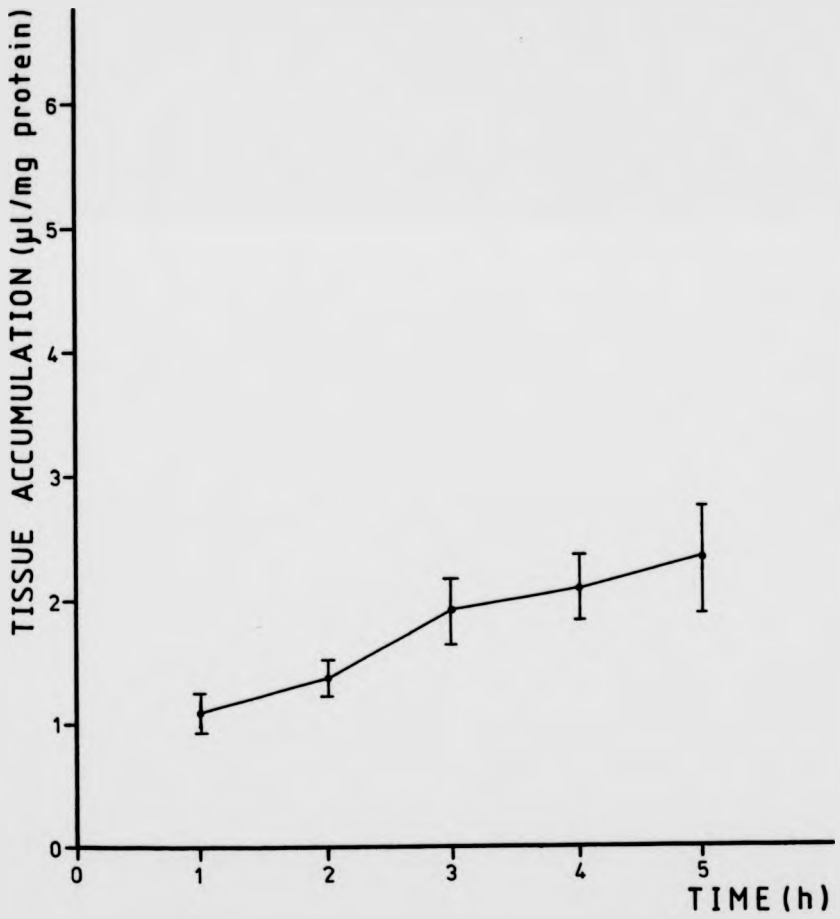


FIGURE 4.4

TISSUE ACCUMULATION OF ^{125}I-LABELLED H P M A COPOLYMER 39b BY RAT VISCERAL YOLK SAC

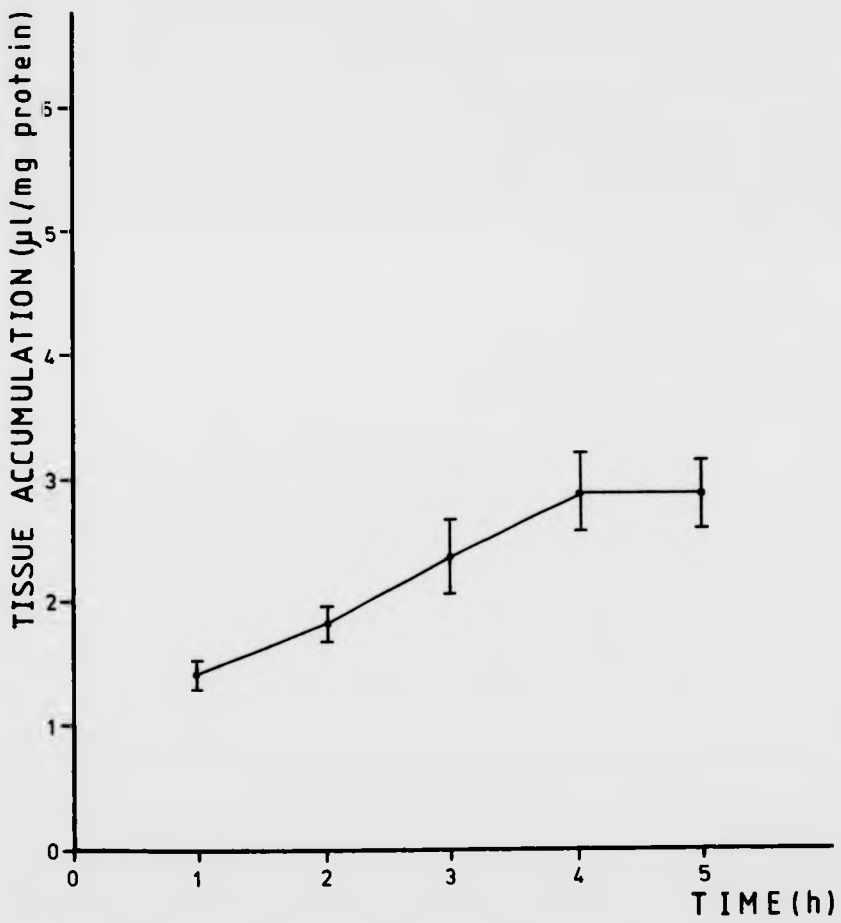


FIGURE 4.5

TISSUE ACCUMULATION OF ^{125}I-LABELLED H P M A COPOLYMER 39c BY RAT VISCERAL YOLK SAC

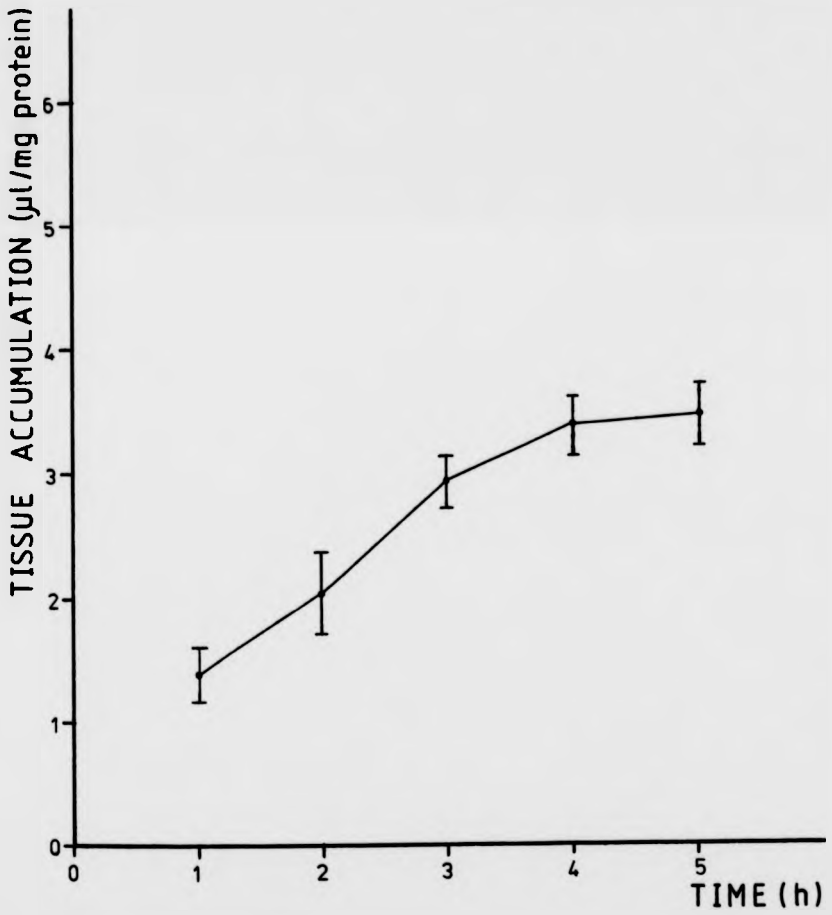


FIGURE 4.6

TISSUE ACCUMULATION OF ^{125}I-LABELLED H P M A COPOLYMER 39d BY RAT VISCERAL YOLK SAC

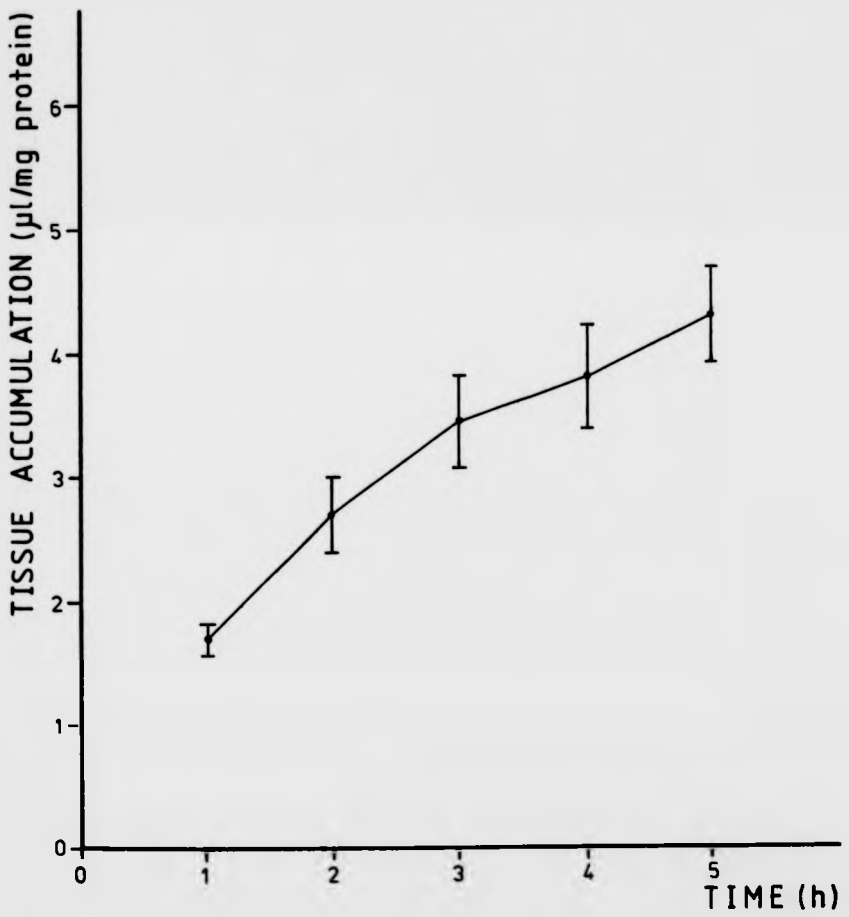


FIGURE 4.7

TISSUE ACCUMULATION OF ^{125}I-LABELLED HPMA COPOLYMER 39e BY RAT VISCERAL YOLK SAC

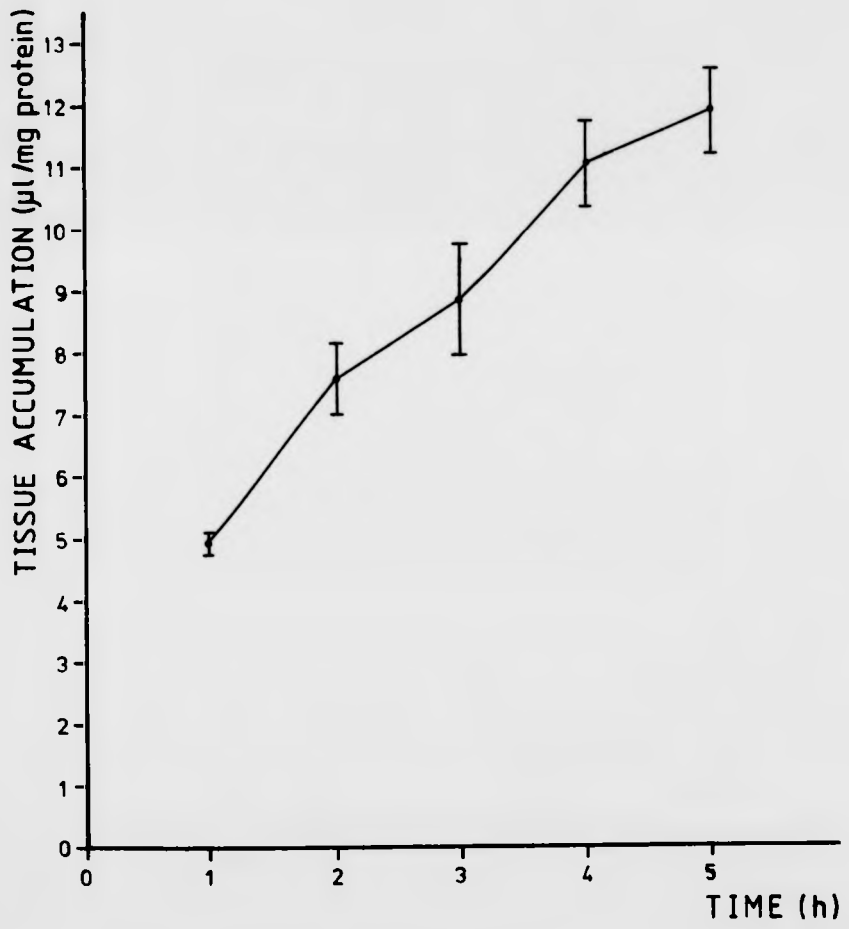
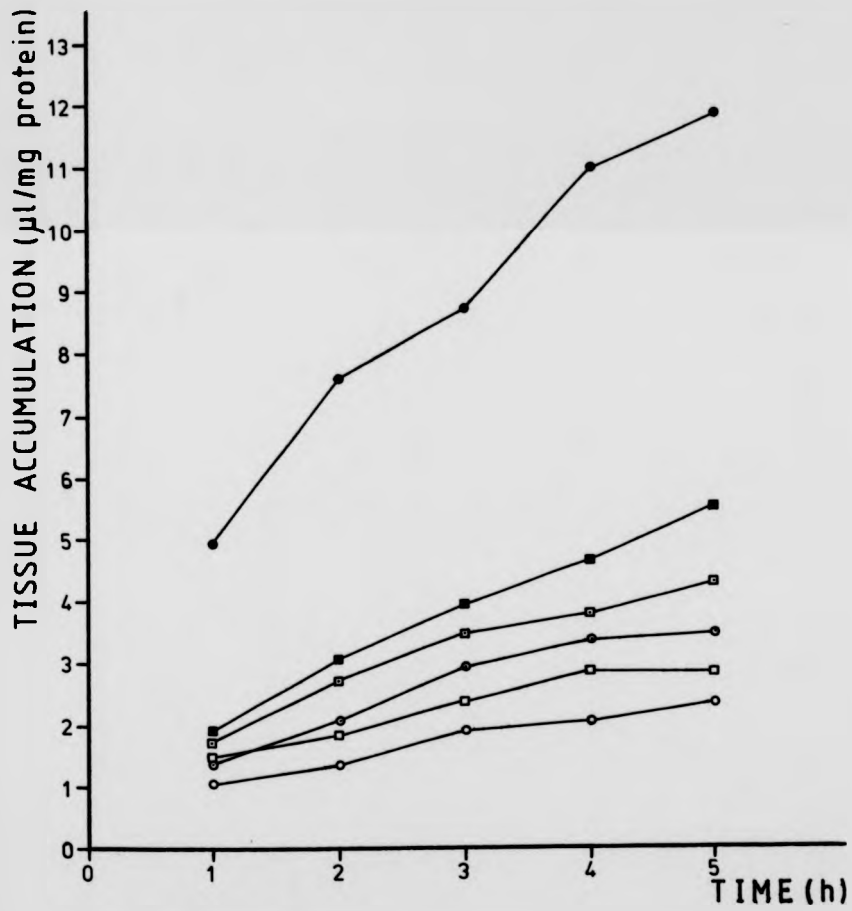


FIGURE 4.8

TISSUE ACCUMULATION OF ^{125}I-LABELLED H P M A COPOLYMER FRACTIONS BY RAT VISCERAL YOLK SAC



KEY	
DATA POINT	COPOLYMER
■	39
○	39a
□	39b
◐	39c
◧	39d
●	39e

FIGURE 4.9

TABLE 4.1 TISSUE ACCUMULATION OF ^{125}I-LABELLED COPOLYMER 39 BY RAT VISCERAL YOLK SACS

POLYMER/FRACTION	$\bar{M}w$	NO. OF EXPTS PERFORMED	RATES OF ACCUMULATION + SE (µl/mg protein/hr)	MEAN % MATCHED PVP CONTROL
39	190,000	5	0.88 ± 0.12	38.90
39a	>400,000	3	0.33 ± 0.13	20.60
39b	400,000	3	0.40 ± 0.13	23.00
39c	155,000	3	0.55 ± 0.12	26.90
39d	110,000	3	0.63 ± 0.12	32.90
39e	34,000	3	1.74 ± 0.20	84.10
PVP	~ 40,000	7	1.79 ± 0.16	

Table 4.1 and fig 4.8 show that tissue accumulation of copolymer 39e, the fraction with the lowest $\bar{M}w$, is approximately the same as that measured for the fluid-phase marker ^{125}I-PVP in matched control experiments (Table 4.1, fig. 4.10). All other fractions, and the unfractioned copolymer 39 showed consistently lower rates of uptake.

To investigate the possible degradation of ^{125}I-labelled copolymer 39 by yolk sacs and release of radiolabelled digestion products from the tissue, 6-10 yolk sacs were incubated with radiolabelled substrate (125 µg/ml) in a single flask for 5h at 37°C. Tissue accumulation of radioactivity was measured, and samples (1ml) of culture medium were applied to a Sephadex G-15 column and eluted with 0.02M sodium acetate in order to detect any ^{125}I-labelled low molecular weight degradation products (section 2.5 and 2.9). Results of the column chromatography are shown in fig. 4.11. It can be seen that in all cases two further peaks eluted after the polymer and the [^{125}I] iodide peak; these were not present in the culture medium before incubation (fig. 4.12) and must therefore represent degradation products. The third peak was found to correspond in position to a ^{125}I-tyrosine marker. Attempts were made to identify the smaller, fourth peak by applying other possible degradation products as markers, namely ^{125}I-labelled phenylalanine-tyrosine, 3, 5-di [^{125}I] iodo-L-tyrosine and ^{125}I-labelled N,N'-dityrosyl HMDA (the crosslinking agent used for synthesis of the copolymer). Fig. 4.11 shows that these three compounds all elute in approximately the same position as the fourth peak.

PINOCYTIC UPTAKE OF ^{125}I-PVP BY RAT VISCERAL YOLK SAC

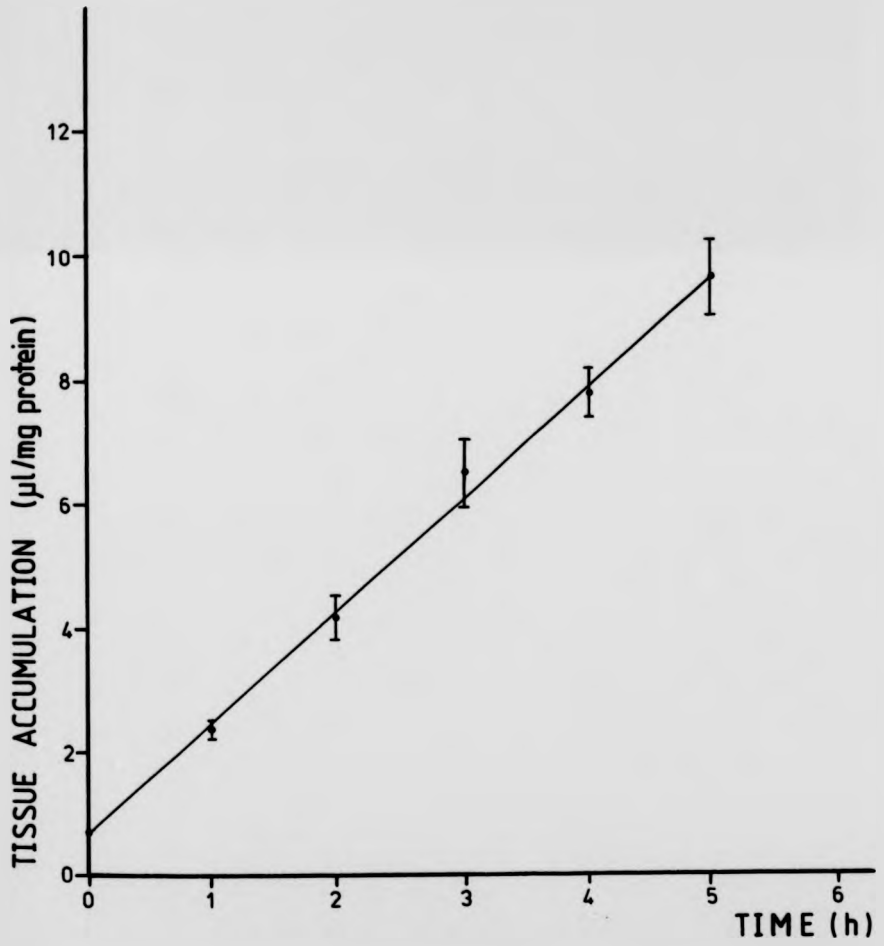
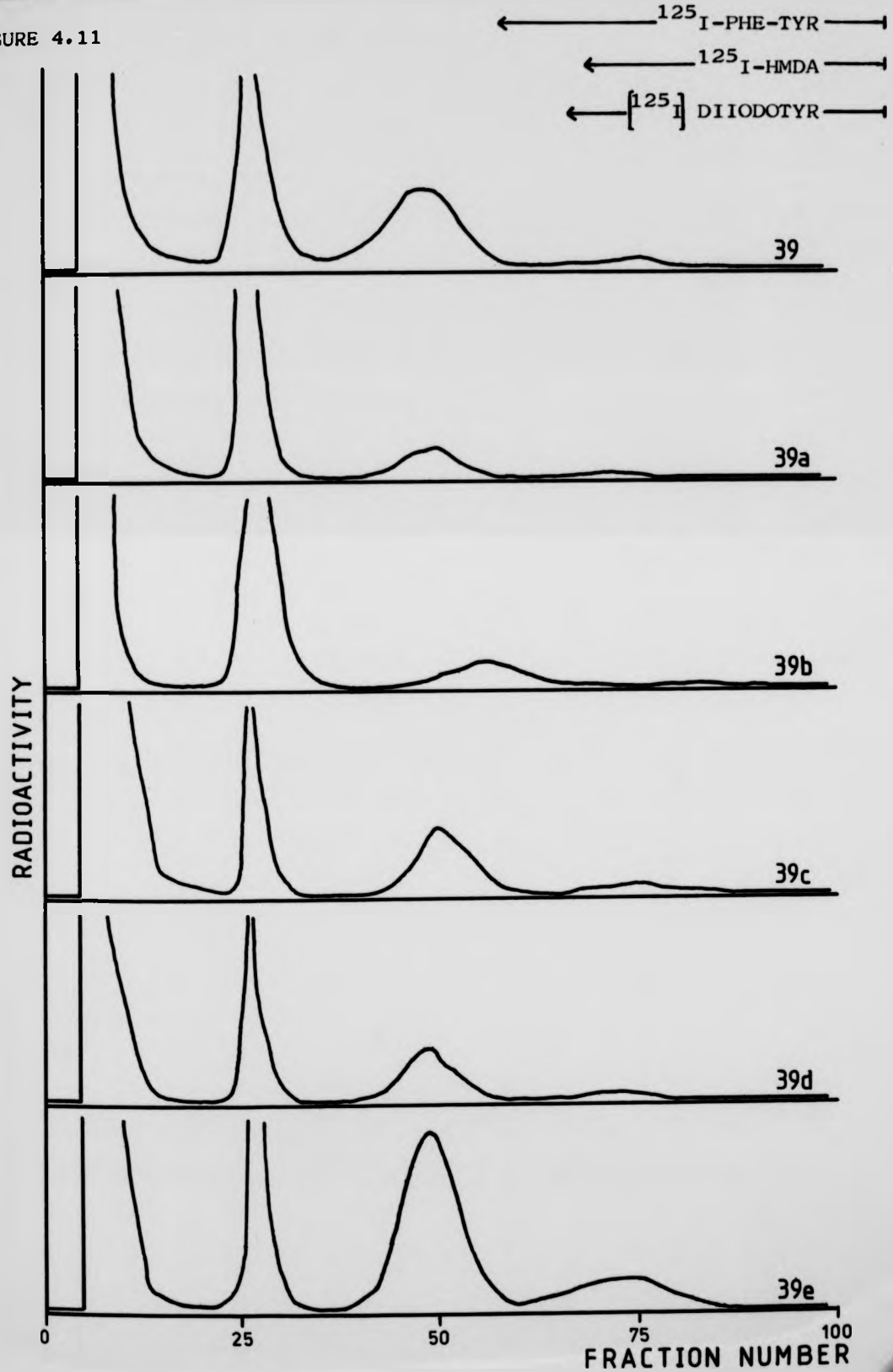


FIGURE 4.10

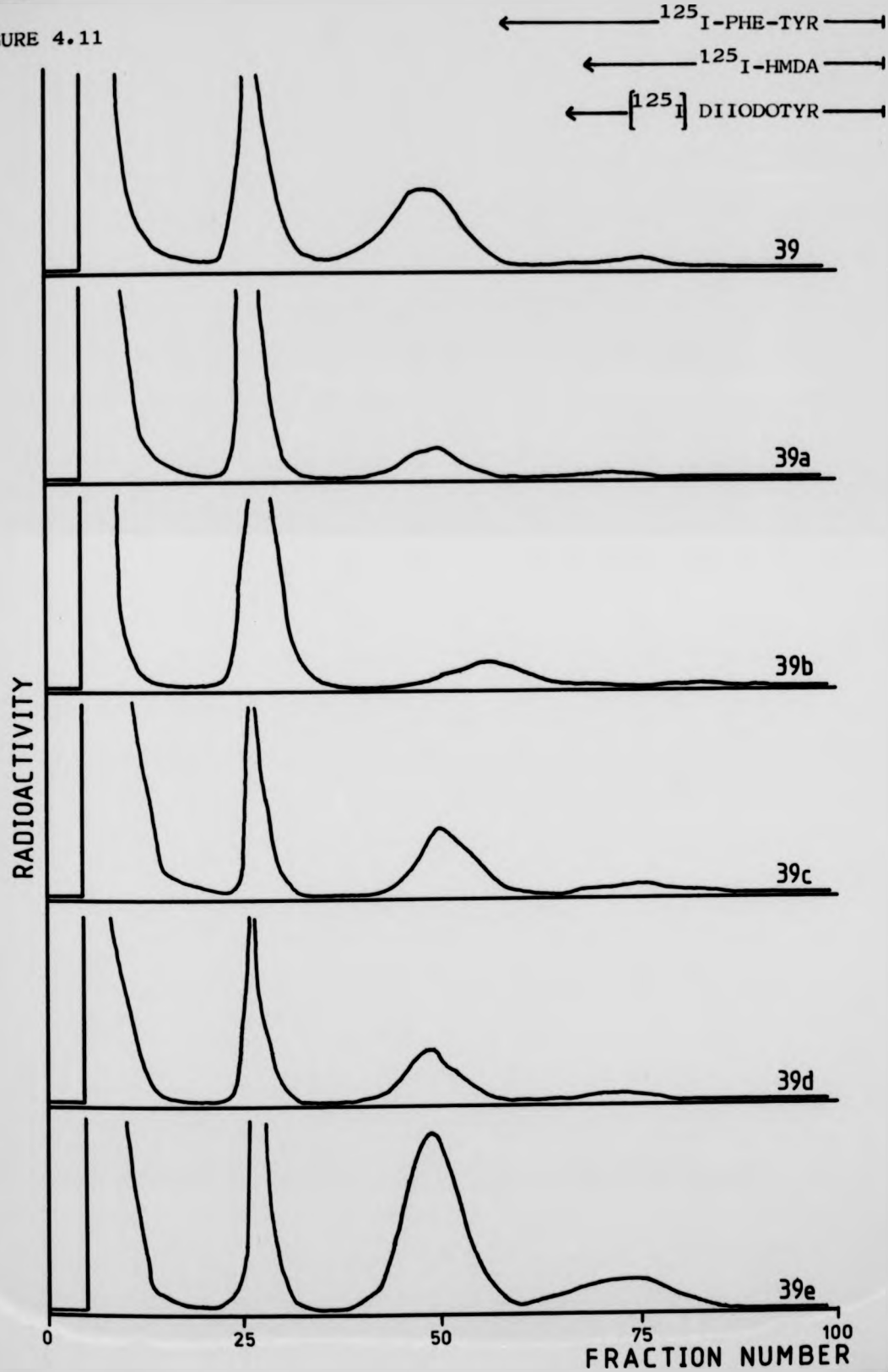
SEPHADEX G-15 CHROMATOGRAPHY OF CULTURE MEDIUM FOLLOWING A 6H INCUBATION OF COPOLYMER 39 FRACTIONS WITH RAT VISCERAL YOLK SACS

FIGURE 4.11



SEPHADEX G-15 CHROMATOGRAPHY OF CULTURE MEDIUM FOLLOWING A 5H INCUBATION OF COPOLYMER 39 FRACTIONS WITH RAT VISCERAL YOLK SACS

FIGURE 4.11



The extent of degradation was calculated from the elution profiles by expressing the amount of radioactivity in peaks 3 and 4 as a percentage of the total recovered from the column. This value was used to calculate the total amount of radiolabelled digestion products released by the yolk sacs, which was then expressed in terms of µl of culture medium per mg of yolk sac protein. Tissue accumulation of radiolabelled substrate was also assessed and the sum of these two values (in µl/mg protein) gives the total pinocytic uptake of copolymer over the 5h period i.e.

True pinocytic uptake (µl/mg protein) = Tissue accumulation (µl/mg protein) + Degradation Products in culture medium (µl/mg protein)

Tables 4.2 and 4.3 and Fig. 4.13 show a summary of the data obtained from the degradation experiments. From these results it is apparent that total pinocytic uptake does increase with decreasing molecular weight, as indicated by the tissue accumulation experiments. However, despite the differences in uptake, molecular weight appears to have no effect on the extent to which the copolymers are degraded since, with all fractions, approximately 40% of the total uptake is degraded during a 5h incubation period.

Enzymatic cleavage of the crosslink P-Gly-Gly-Phe-Tyr-HMDA-Tyr-Phe-Gly-Gly-P to produce free tyrosine residues must involve the hydrolysis of at least two covalent bonds. One of these must be the link between tyrosine and hexamethylene diamine. To assess the extent of tyrosine release from the crosslinking agent N,N'-dityrosyl HMDA, and in an attempt to identify the nature of the small second peak of radioactive

SEPHADEX G-15 CHROMATOGRAPHY OF CULTURE MEDIUM CONTAINING ^{125}I-LABELLED COPOLYMER 39 (unfractionated) BEFORE INCUBATION WITH YOLK SACS

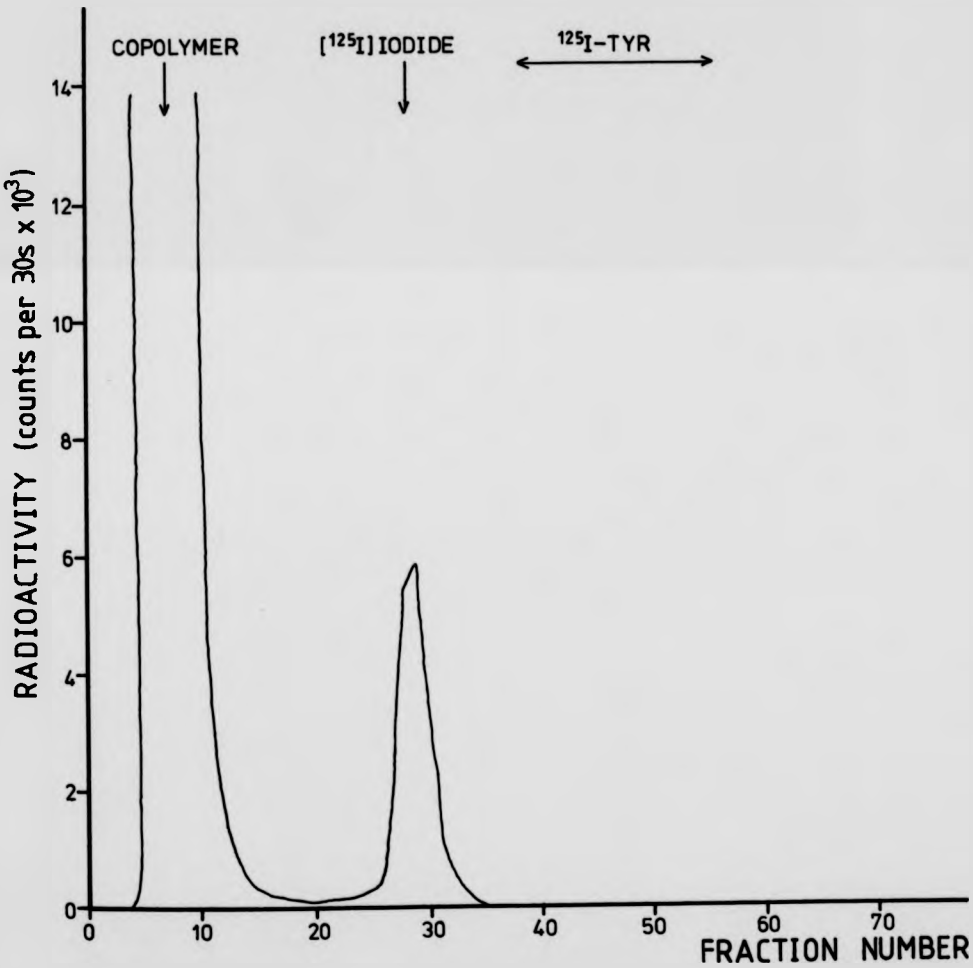


FIGURE 4.12

TABLE 4.2 SEPHADEX G-15 COLUMN CHROMATOGRAPHY OF CULTURE MEDIUM FOLLOWING INCUBATION OF YOLK SACS WITH ^{125}I-LABELLED COPOLYMER 39

(Results expressed as a % of Radioactivity applied)

COPOLYMER	LENGTH OF INCUBATION (h)	TOTAL YOLK SAC PROTEIN mg	SUBSTRATE CONC. (µg/ml)	PEAK 1 (COPOLYMER)	PEAK 2 (^{125}I)	PEAK 3 (^{125}I-Tyr)	PEAK 4 (UNKNOWN)	RECOVERY OF TOTAL RADIOACTIVITY APPLIED
39	5	85.99	125	82.40	3.66	2.61	0.53	90.08
39a	5	76.80	125	92.78	5.15	0.80	0.24	99.30
39b	5	64.60	125	87.28	6.06	0.96	0.22	95.04
39c	5	79.30	125	89.30	2.13	1.69	0.44	94.10
39d	5	64.90	125	78.20	1.86	1.40	0.40	82.30
39e	5	84.97	125	80.28	5.49	4.63	1.43	92.42
							MEAN RECOVERY	92.21

TABLE 4.3 PINOCYTIC UPTAKE AND DEGRADATION OF COPOLYMER 39 AFTER 5H INCUBATION WITH YOLK SACS

POLYMER CODE NO.	TISSUE ACCUMULATION (µl/mg protein)	DEGRADATION PRODUCTS (ul/mg protein) PEAK 3 (^{125}I-Tyr)	PEAK 4 (UNKNOWN)	TOTAL	TOTAL UPTAKE (µl/mg protein)	% DEGRADED
39	4.81	3.03	0.62	3.65	8.47	43.16
39a	1.78	1.04	0.32	1.36	3.14	43.30
39b	2.13	1.49	0.35	1.84	3.97	46.29
39c	3.86	2.13	0.44	2.69	6.55	41.10
39d	3.95	2.17	0.40	2.79	6.74	41.35
39e	8.18	5.45	1.68	7.13	15.31	46.60

PINOCYTIC UPTAKE AND DEGRADATION OF COPOLYMER 39 FRACTIONS AFTER 5h INCUBATION WITH RAT VISCERAL YOLK SACS

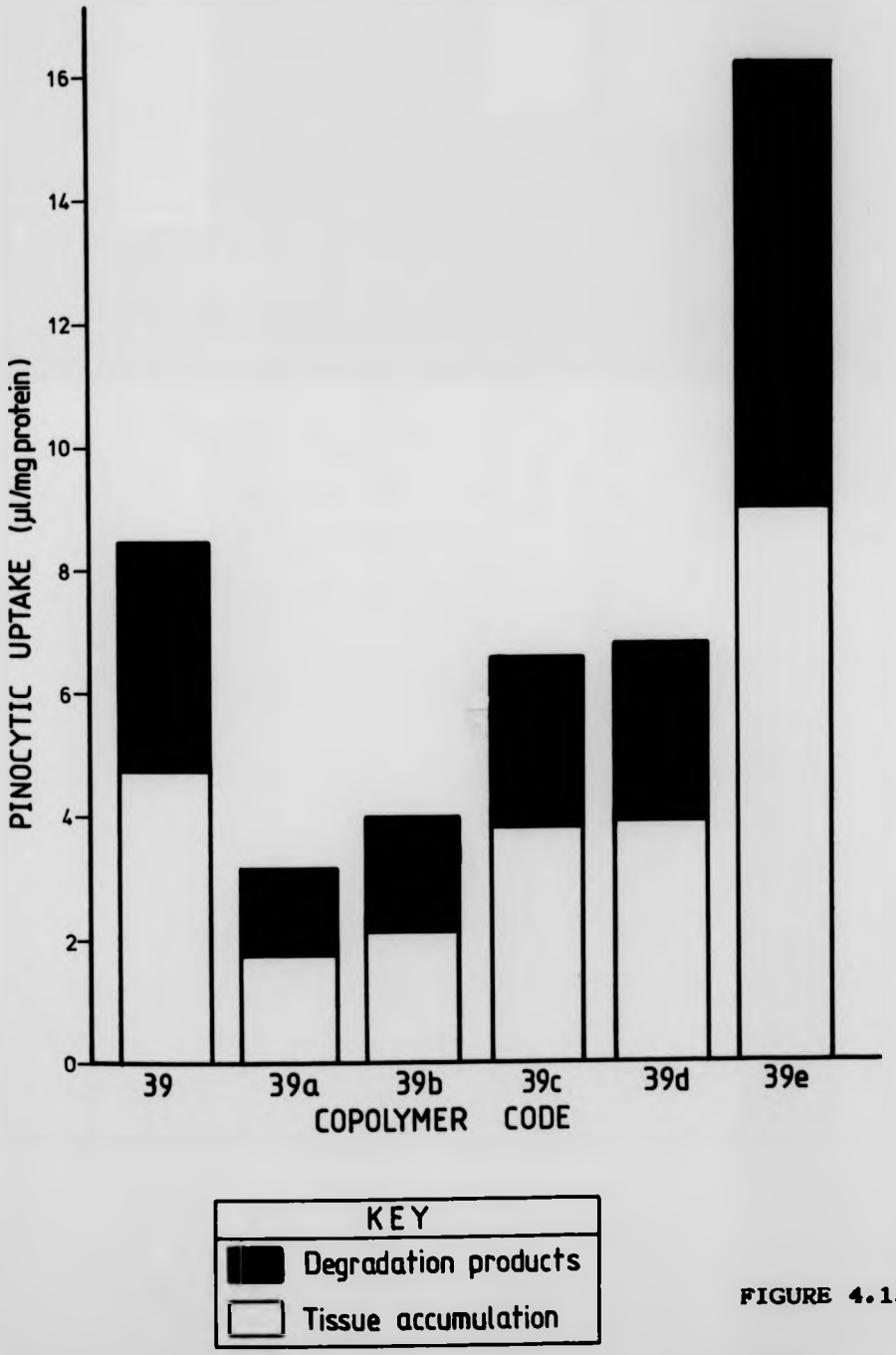


FIGURE 4.13

degradation product, the crosslinking agent N,N'-dityrosyl HMDA was radiolabelled and used as a substrate in the yolk sac system. Results of uptake and degradation experiments are summarized in fig 4.14. Matched ^{125}I-PVP controls were carried out concurrently.

Analysis of the results revealed no progressive accumulation of radioactivity in the yolk sac tissue, probably indicating that [^{125}I]-N,N'-dityrosyl HMDA does not enter yolk sac tissue by pinocytosis, but simply diffuses into the tissue and is degraded readily, as shown in fig. 4.15. The possible sites of degradation within the cell have not been investigated.

The elution profile shown in fig. 4.15 shows, again, a peak of radioactivity which elutes after [^{125}I] iodotyrosine following incubation with yolk sacs (5h). This peak may either be residual undegraded [^{125}I]-N,N'-dityrosyl HMDA, or it may represent partially degraded substrate i.e. tyrosyl HMDA.

4.2.2 Pinocytic Uptake and Degradation of ^{125}I-labelled Copolymer 74 by Yolk Sacs

Tissue accumulation of copolymer 74 over 5h was estimated as described in section 4.2.1. The experiment was performed three times and in each case the rate decreased with time, as shown in fig. 4.16. Sephadex G-15 column chromatography of culture medium (1ml) following incubation of ^{125}I-labelled copolymer 74 with yolk sacs for 5h revealed a peak which co-eluted with [^{125}I] iodotyrosine and a further peak, as observed with copolymer 39 fractions following incubation with yolk sacs. Total uptake and degradation of copolymer 74 was calculated as explained in 4.2.1 and results are shown in Table 4.4, along with values obtained for copolymer 39e, which are included for comparison.

TISSUE ACCUMULATION OF ^{125}I-LABELLED N,N'-DITYROSYL-HMDA AND ^{125}I-PVP BY YOLK SACS OVER A 5h INCUBATION PERIOD

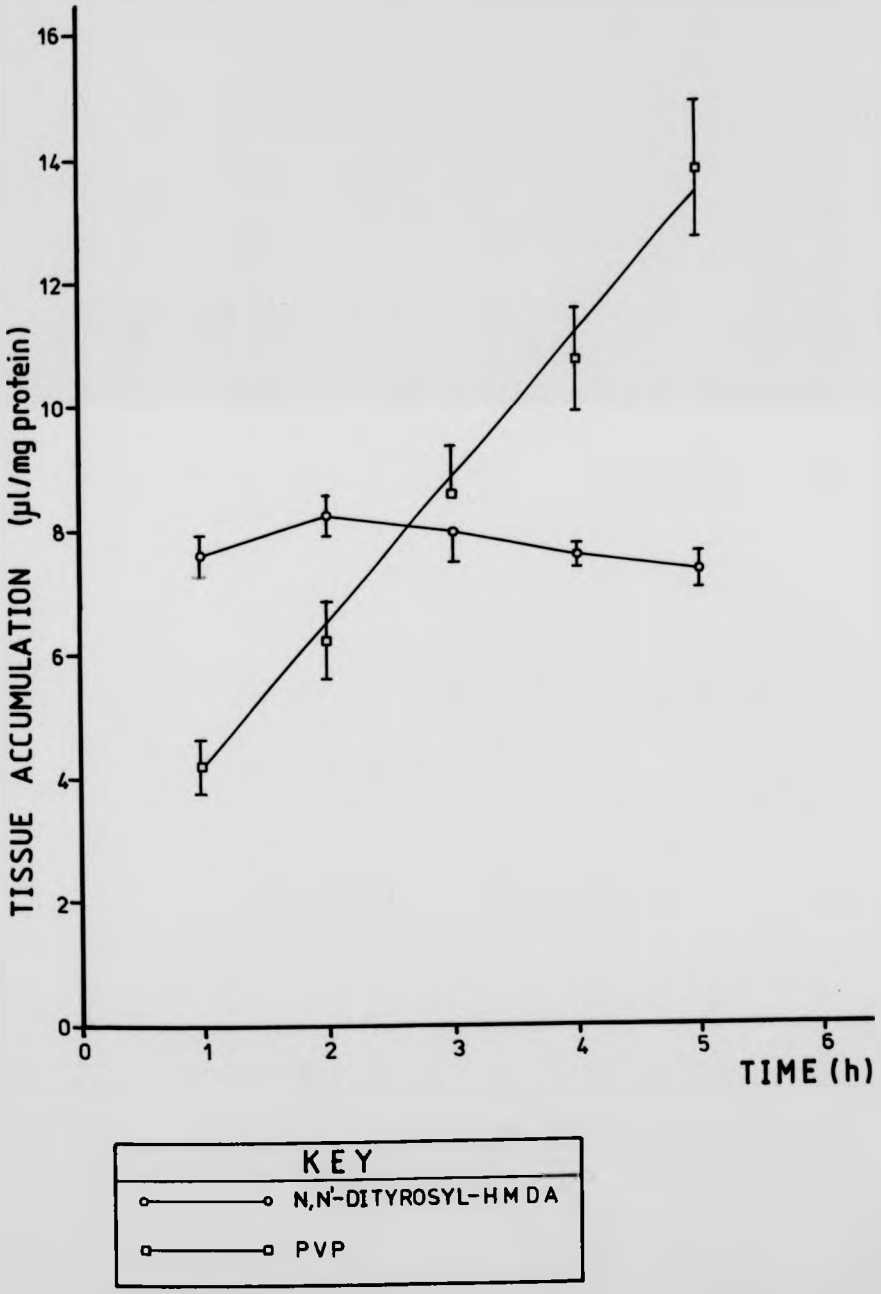


FIGURE 4.14

SEPHADEX G-15 CHROMATOGRAPHY OF MEDIUM CONTAINING ^{125}I-LABELLED N,N-DITYROSYL-HMDA AFTER 5h INCUBATION AT 37°C WITH YOLK SACS (+10% CALF SERUM)

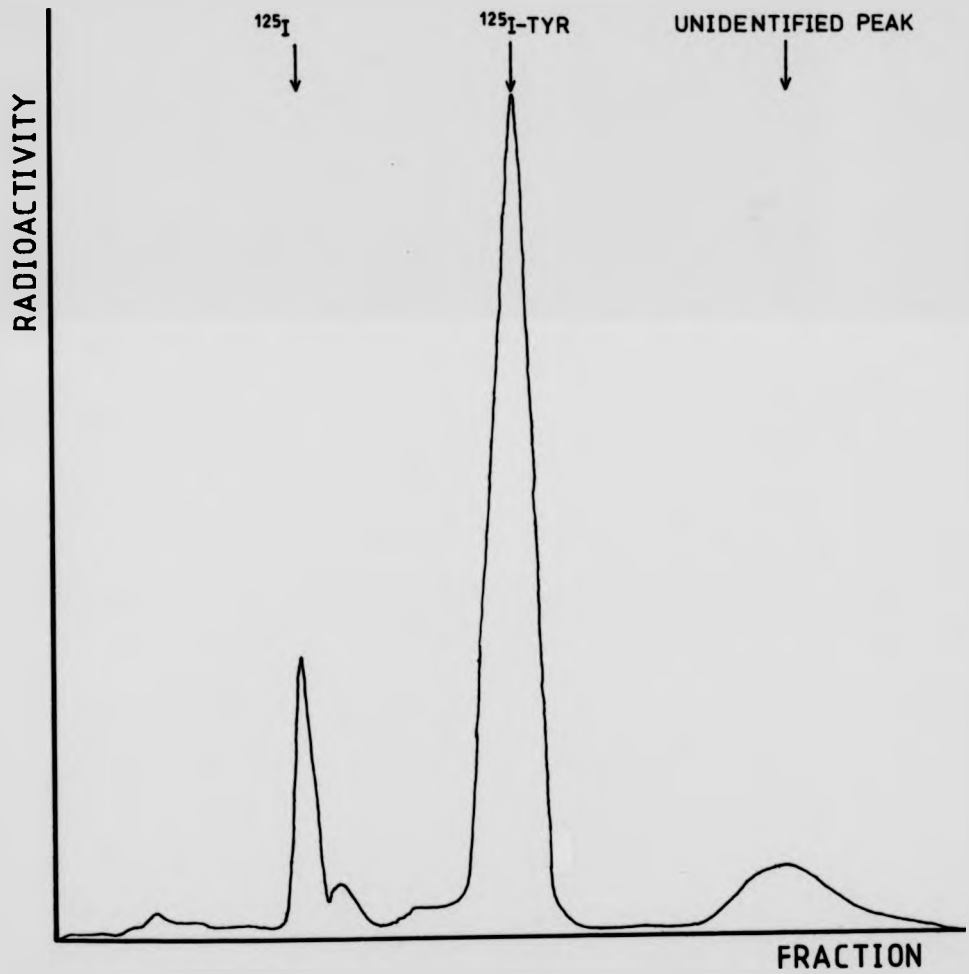


FIGURE 4.15

FIGURE 4.16

TISSUE ACCUMULATION OF ^{125}I-PVP AND COPOLYMER 74 BY RAT VISCERAL YOLK SAC

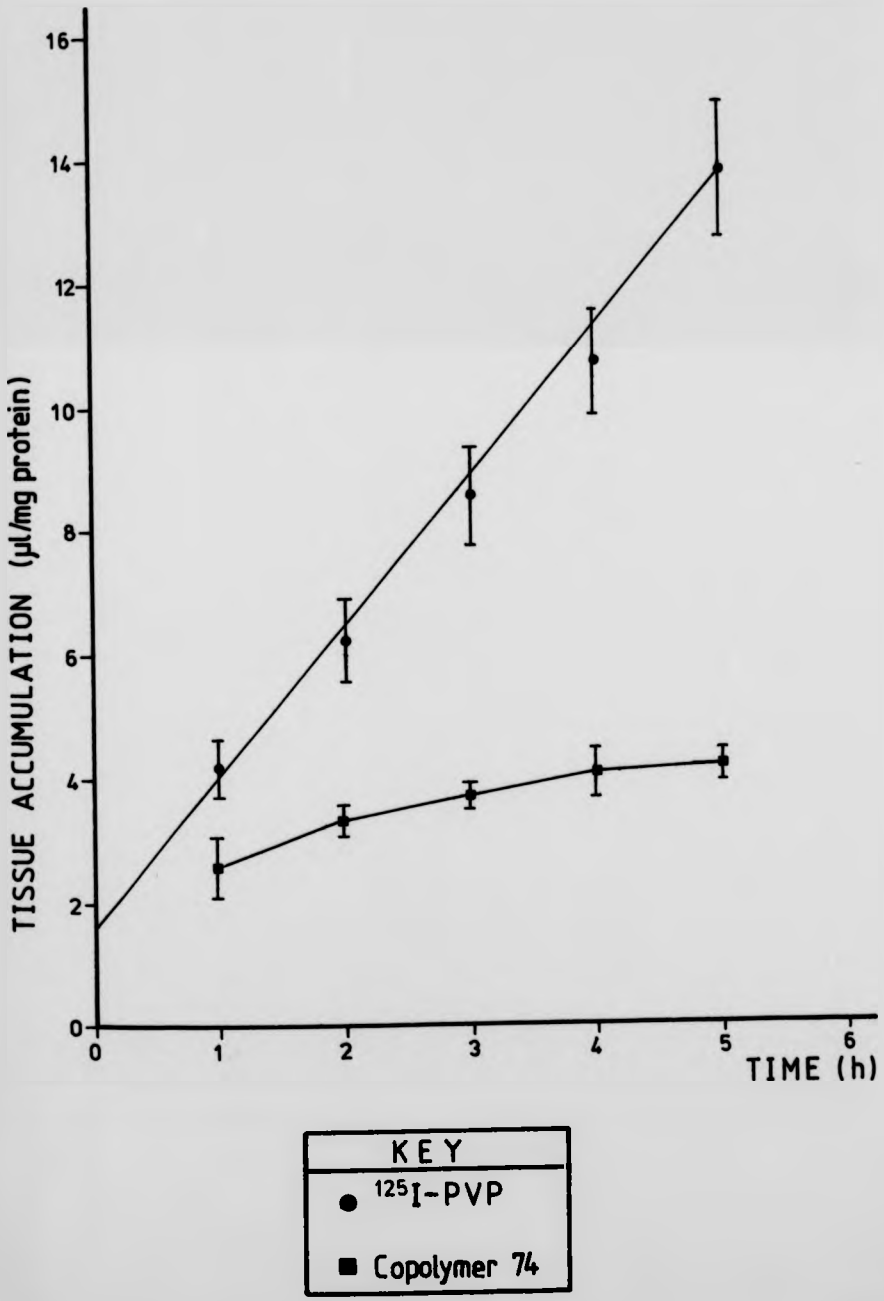


TABLE 4.4

PINOCYTIC UPTAKE AND DEGRADATION OF COPOLYMER 74 OVER A 5H INCUBATION

POLYMER CODE NO.	TISSUE ACCUMULATION (µl/mg protein)	DEGRADATION PRODUCTS (µl/mg protein) PEAK 3	PEAK 4	TOTAL	TOTAL UPTAKE (µl/mg protein)	% DEGRADED
39e	8.17	5.45	1.68	7.13	15.31	46.60
74	4.56	8.49	3.02	11.51	16.07	71.60
	4.56	7.80	2.86	10.66	15.31	69.60

It is attractive to suppose that the unidentified peak observed to elute in the same position on chromatography of culture medium in which yolk sacs have been incubated with either copolymer 39, copolymer 74 or N,N'-dityrosyl HMDA are the same molecule. A comparison of the structures of the three substrates would suggest that [^{125}I]diiodotyrosine is the likely constituent of the peak, since copolymer 74 (P-Gly-Gly-Phe-TyrNH_2) does not contain N,N'-dityrosyl HMDA, and N,N'-dityrosyl HMDA does not contain phenylalanine.

4.2.3 Pinocytic Uptake and Degradation of Copolymer 39e over a 12h Incubation Period

Since the extent of degradation of all the copolymer 39 fractions was shown to be virtually the same (40% of the total uptake) after 5h incubation with yolk sacs, an experiment was performed to follow the pinocytic uptake and degradation of copolymer 39 over a prolonged incubation period. Fraction 39e was chosen because of its comparatively high rate of uptake.

The maximum incubation period for yolk sacs in medium 199 supplemented with 10% calf serum at 37^{o}C is 12h, after which time the tissue begins to degenerate (Williams et al 1975a, Duncan et al, unpublished). The experiment was performed as described in the previous sections. Each flask (containing 10 yolk sacs) was incubated with copolymer 39e (125 µg/ml), for a different time period. Sample times of 3, 5, 8 and 12h were chosen, and care was taken to gas the flasks periodically with 95% O_2/5% CO_2 in order to preserve the tissue for as long as possible.

1ml samples of culture medium from each flask were applied to a Sephadex G-15 column and eluted with sodium acetate (0.02M) as described previously. Table 4.5 and fig. 4.17 summarize the results of this experiment. It can be seen from fig. 4.17 that after 12h the yolk sac is still pinocytosing and digesting substrate. The results also show that the extent of degradation of the copolymer is slowly increasing with time; this is a consequence of the fact that tissue levels of undegraded copolymer level off after the first 3-5h.

4.2.4 The Effect of 2,4-Dinitrophenol and Leupeptin on the Uptake and Degradation of Copolymer 39e by the Yolk Sac

Pinocytic uptake and degradation experiments using copolymer 39e were performed with addition of 2, 4-dinitrophenol (50 µg/ml) or leupeptin (40 µg/ml) to the incubation medium.

The experiments were carried out exactly as described in the previous sections. The incubation period for all experiments was 5h.

The results of the experiments are summarised in figs. 4.18, 4.19 and Table 4.6. It can be seen that the addition of 2, 4-dinitrophenol to the incubation medium at the beginning of the experiment results in a greatly decreased uptake and subsequent degradation of the substrate, demonstrating that tissue accumulation does represent true pinocytosis. The decreased level of degradation products indicates that degradation does not occur when pinocytosis is inhibited and is an intracellular event and not the result of degradation in the culture medium by secreted enzymes.

The results obtained using leupeptin show an undiminished

PINOCYTIC UPTAKE AND DEGRADATION OF COPOLYMER 39e BY YOLK SACS OVER 12h

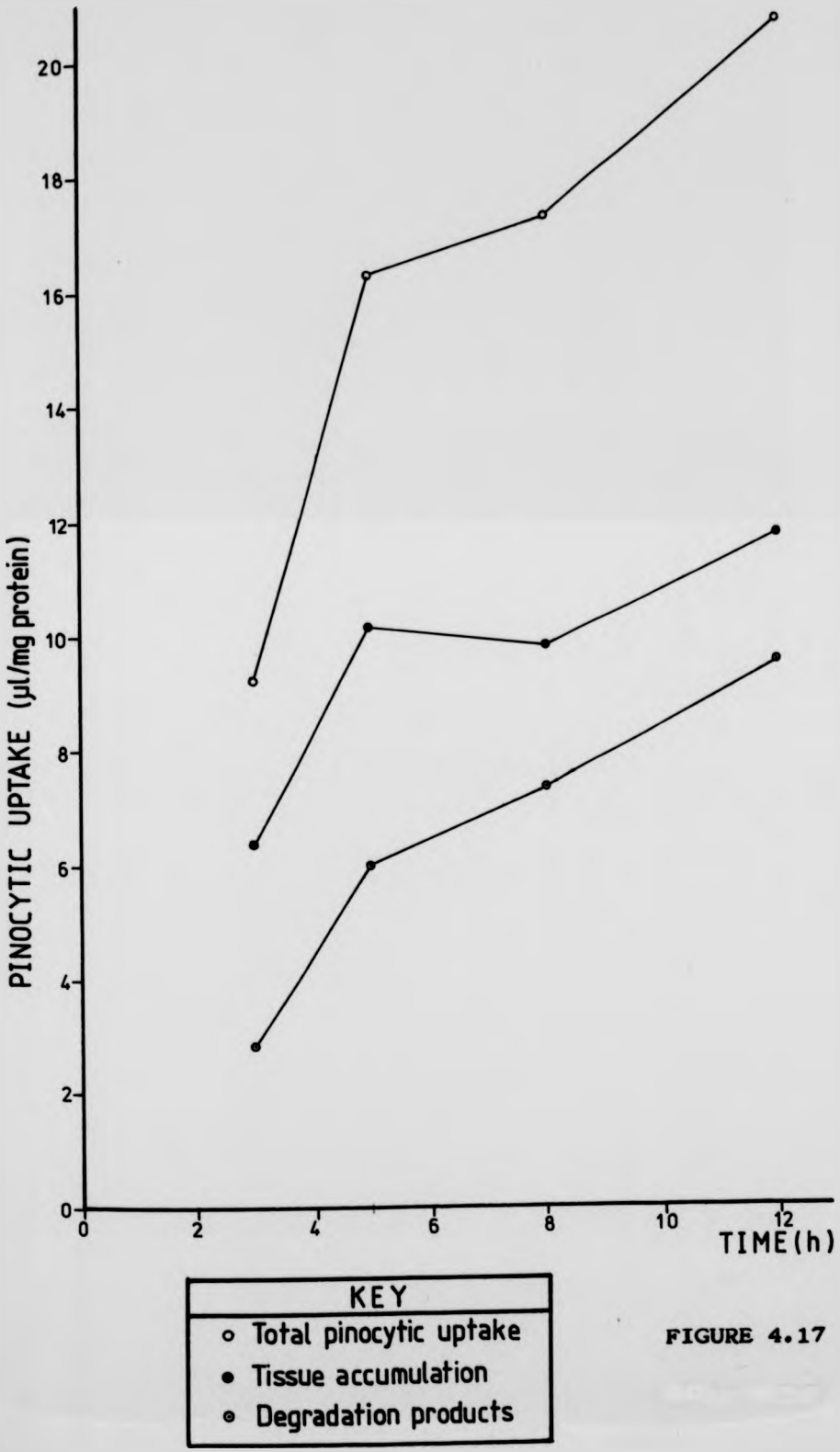


FIGURE 4.17

TABLE 4.5 PINOCYTIC UPTAKE AND DEGRADATION OF ^{125}I-LABELLED COPOLYMER 39e BY YOLK SACS OVER A 12H INCUBATION PERIOD

SUBSTRATE CONC. (µg/ml)	INCUBATION PERIOD (h)	TISSUE ACCUMULATION µl/mg/inc. period	DEGRADATION PRODUCTS (µl/mg)			TOTAL UPTAKE	% DEGRADED
			^{125}I-Tyr	UNKNOWN	TOTAL		
125	3	6.40	2.45	0.41	2.87	9.27	31.00
125	5	10.19	5.30	0.77	6.03	16.26	37.10
125	8	9.90	6.18	1.25	7.43	17.33	42.90
125	12	11.18	7.56	2.02	9.58	20.76	46.15

SEPHADEX G-15 CHROMATOGRAPHY OF CULTURE MEDIUM FOLLOWING A 5h INCUBATION WITH YOLK SACS

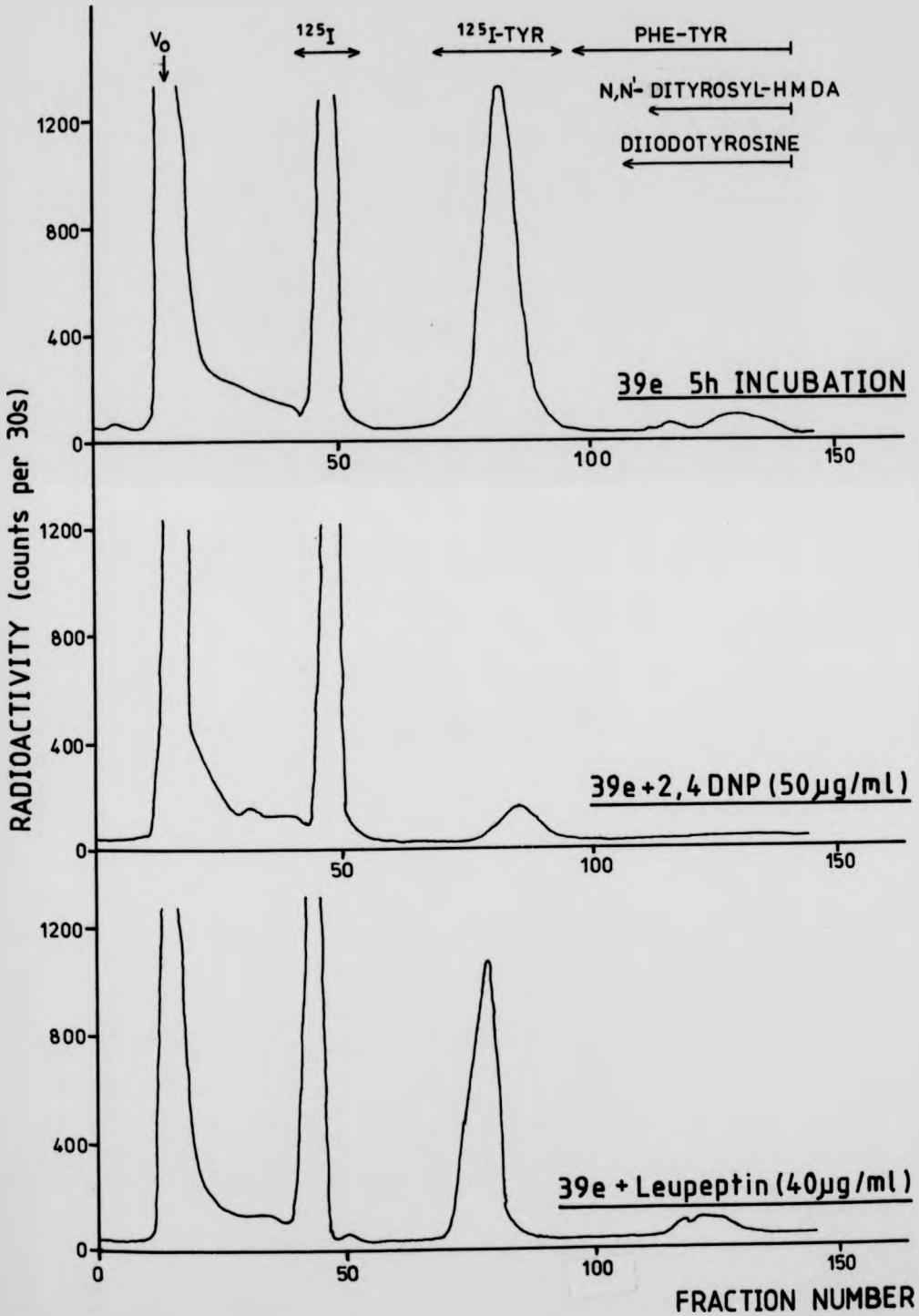


FIGURE 4.18

UPTAKE AND DEGRADATION OF COPOLYMER 39e AFTER 5h INCUBATION IN THE PRESENCE AND ABSENCE OF INHIBITORS

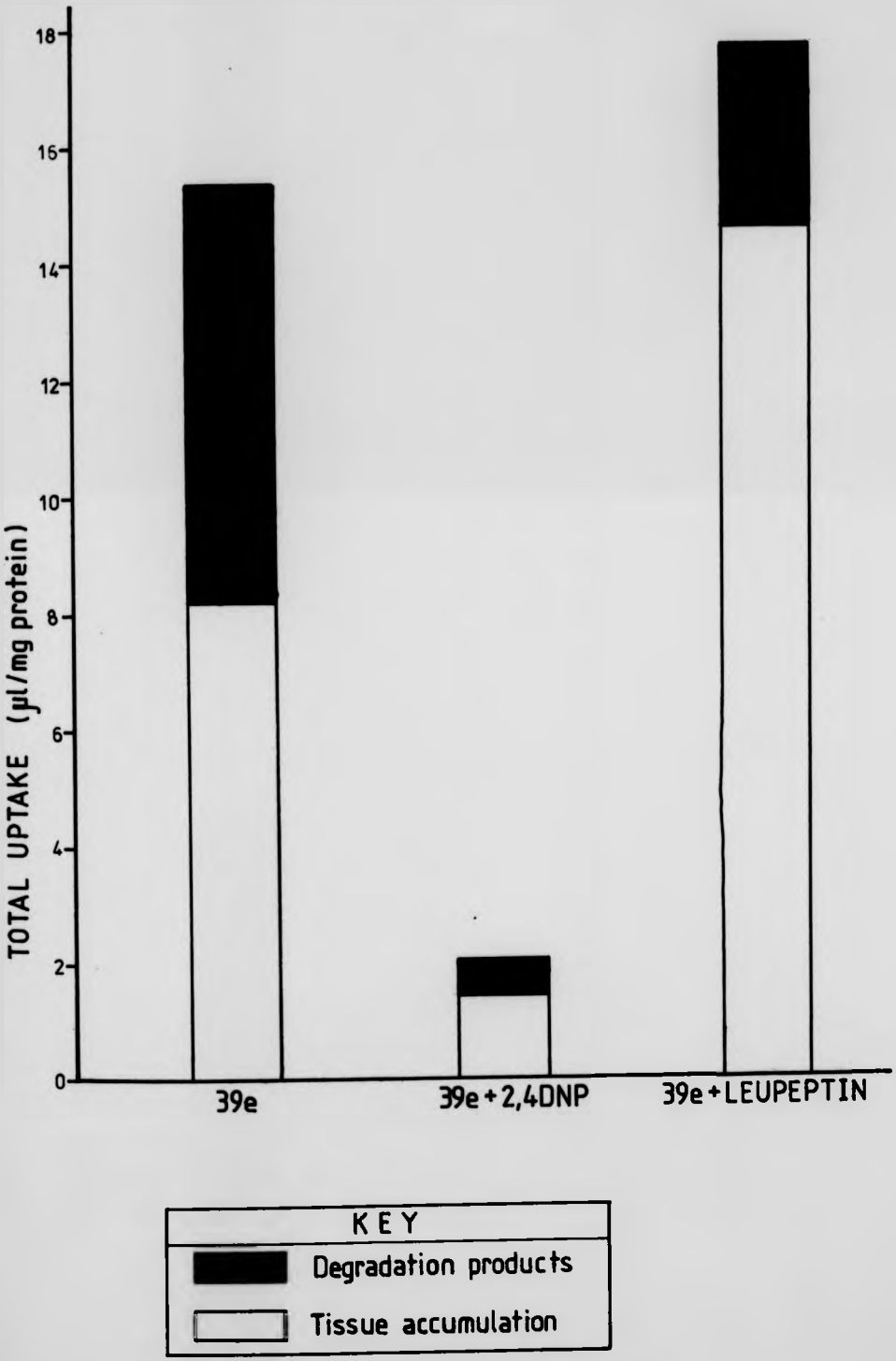


FIGURE 4.19

TABLE 4.6 PINOCYTIC UPTAKE AND DEGRADATION OF COPOLYMER 39e BY YOLK SACS IN THE PRESENCE AND ABSENCE OF INHIBITORS AFTER A 5H INCUBATION

SUBSTRATE	INHIBITOR	YOLK SAC ACCUMULATION (µl per mg protein)	DEGRADATION PRODUCTS (TOTAL) (µl per mg protein)	TOTAL UPTAKE	% DEGRADED
39e	-	9.04	7.15	16.19	44.20
39e	2, 4-DNP (50 µg/ml)	1.43	0.63	2.06	30.05
39e	LEUPEPTIN (40 µg/ml)	14.63	3.10	17.73	17.70

total uptake value, but a reduced level of degradation. This indicates that in the presence of leupeptin the yolk sac is capturing copolymer 39e at the normal rate and that its digestion in the lysosomes is inhibited.

4.2.5 Pinocytic Uptake and Degradation of Copolymer 68 by the Yolk Sac

Tissue accumulation of copolymer 68 by yolk sacs over 5h was estimated as described above. The experiment was repeated 5 times with matched ^{125}I-PVP controls and the results showed a linear uptake as shown in fig. 4.20.

Radiolabelled low molecular weight degradation products were not expected to be released from this substrate. To verify this, experiments to detect such products were carried out as described previously. After incubation, samples of the medium were applied to a Sephadex G-15 column, but, as shown in fig. 4.21, no degradation products were detected.

The absence of degradation products appearing in the medium is not, however, an indication that crosslinks are not cleaved. The crosslink structure contained in copolymer 68 (P-Gly-Gly-Phe-HMDA-Phe-Gly-Gly-P) is potentially biodegradable but, since the crosslink does not include a radiolabelled tyrosine residue, cleavage cannot be demonstrated by the detection of radiolabelled degradation products. The radiolabelled tyrosine residues remain attached to the copolymer backbone which is too large to traverse the lysosomal membrane. Crosslink cleavage might be detected, however, by comparing the molecular weight distribution of radiolabelled copolymer before and after incubation with yolk sacs. This requires the chromatography of polymers present within the yolk sac tissue and preliminary experiments

TISSUE ACCUMULATION OF COPOLYMER 68 BY RAT VISCERAL YOLK SACS

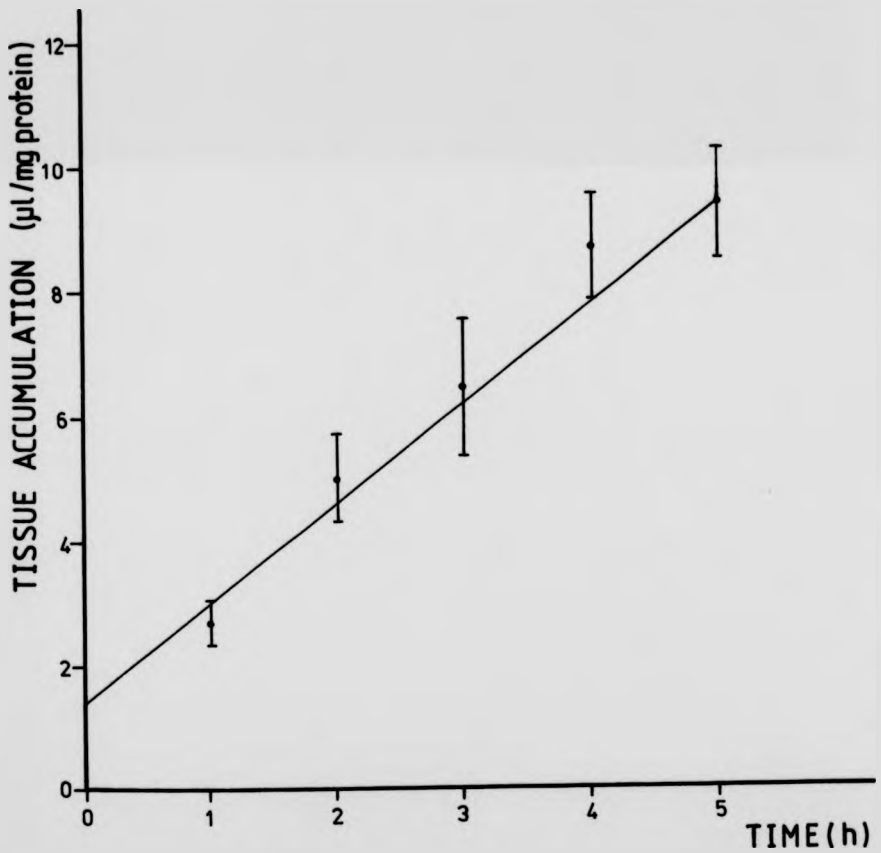


FIGURE 4.20

SEPHADEX G-15 CHROMATOGRAPHY OF CULTURE MEDIUM FOLLOWING 5h INCUBATION OF ^{125}I-LABELLED COPOLYMER 68 WITH YOLK SACS

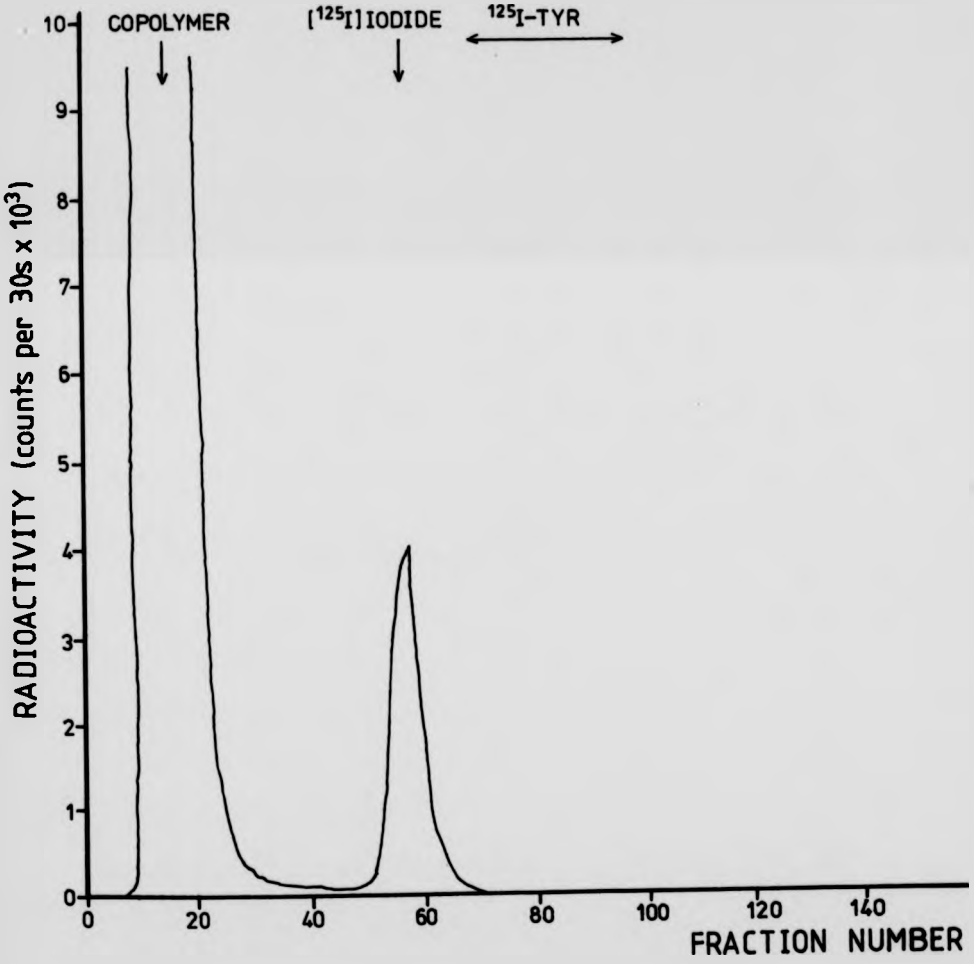


FIGURE 4.21

were necessary in order to optimize the preparation of tissue samples prior to chromatography.

Ten yolk sacs were incubated in a single Erlenmeyer flask containing 11ml medium 199 supplemented with 10% calf serum. ^{125}I-Labelled copolymer 68 (125 µg/ml) was added at the beginning of the experiment and 1ml of the medium was removed immediately. This was applied to a Sepharose 4B/6B column (section 2.5) and the molecular weight distribution of the copolymer in the medium determined (fig. 4.22). After 5h incubation at 37^{o}C the yolk sacs were removed and homogenised in distilled water.

Before application of the homogenate to the Sepharose 4B/6B column it was necessary to centrifuge the sample. Several possible methods of pre-treatment of homogenate were tried before centrifugation to recover the maximum amount of radioactivity from the homogenate in the supernatant:

1) No further treatment. Homogenates were made up to 10ml with distilled water, and duplicate 1ml samples assayed for radioactivity.
2) TCA precipitation of protein. 0.1ml calf serum + 0.5ml TCA was added to duplicate 1ml samples of homogenate before centrifugation.
3) 0.2% final concentration of Triton X100 was added to duplicate 1ml samples of homogenate in order to disrupt lysosomal membranes before centrifugation.
4) Both treatments 2 and 3 were carried out on duplicate 1ml samples of homogenate before centrifugation.

After centrifugation, the radioactivity present in both supernatant and pellet was expressed as a percentage of the

original amount of radioactivity present in a 1ml sample of homogenate.

The same procedure was carried out with various controls at the same time.

1) 10 yolk sacs previously incubated for 5h in medium with no substrate. An equivalent amount of substrate was added after these yolk sacs were homogenised.
2) 10 yolk sacs that had not been incubated. Substrate was added to the homogenate as in 1).
3) No yolk sacs. Substrate was added to 10ml water only.

Results obtained are shown in Table 4.7. It can be seen that there is very little difference between the methods used. It was decided to use Triton X100 (0.2% final concentration) since the percentage radioactivity remaining in the pellet after centrifugation in samples incubated with ^{125}I-labelled copolymer 68 was slightly lower than the other methods.

A sample of this supernatant was applied to the same Sepharose 4B/6B column, and the molecular weight distribution of the ^{125}I-labelled copolymer recovered from the yolk sacs observed. Fig. 4.22 shows the distribution of radiolabelled substrate in the medium together with that recovered from the yolk sacs. The starting material has one broad peak of radioactivity corresponding to the distribution of the copolymer, followed by a sharper, smaller peak which was found to coelute with $[^{125}I]$ iodide. The radioactivity recovered from the yolk sac homogenate, however, shows two polymer peaks, one corresponding to low and one corresponding to high molecular weight material. There is clearly an absence of copolymer of medium range molecular weight. The small peak in the higher molecular

TABLE 4.7 EVALUATION OF METHODS FOR LIBERATION OF ^{125}I-LABELLED COPOLYMER FROM YOLK SAC LYSOSOMES

% RADIOACTIVITY

TREATMENT		CONTROL YOLK SACS NOT INCUBATED	CONTROL YOLK SACS INCUBATED WITHOUT SUBSTRATE	CONTROL WATER ONLY + 10% SERUM	YOLK SACS INCUBATED WITH COPOLYMER 68 (1)	YOLK SACS INCUBATED WITH COPOLYMER 68 (2)
None	SUPERNATANT	91.30	85.60	85.60	85.20	74.30
	PELLET	14.70	17.50	16.10	17.90	23.00
TCA + Calf Serum	S	70.80	58.50	88.80	92.70	60.80
	P	40.70	38.60	19.60	20.30	32.80
0.2% TRITON X100	S	87.70	89.40	92.30	88.90	85.40
	P	11.90	14.80	9.80	11.90	8.90
TRITON + TCA	S	87.20	87.70	97.10	98.40	85.70
	P	21.90	21.70	13.10	13.10	15.00

SEPHAROSE 4B/6B CHROMATOGRAPHY OF MEDIUM CONTAINING COPOLYMER 68 AND YOLK SAC HOMOGENATE AFTER 5h INCUBATION OF YOLK SACS WITH COPOLYMER 68

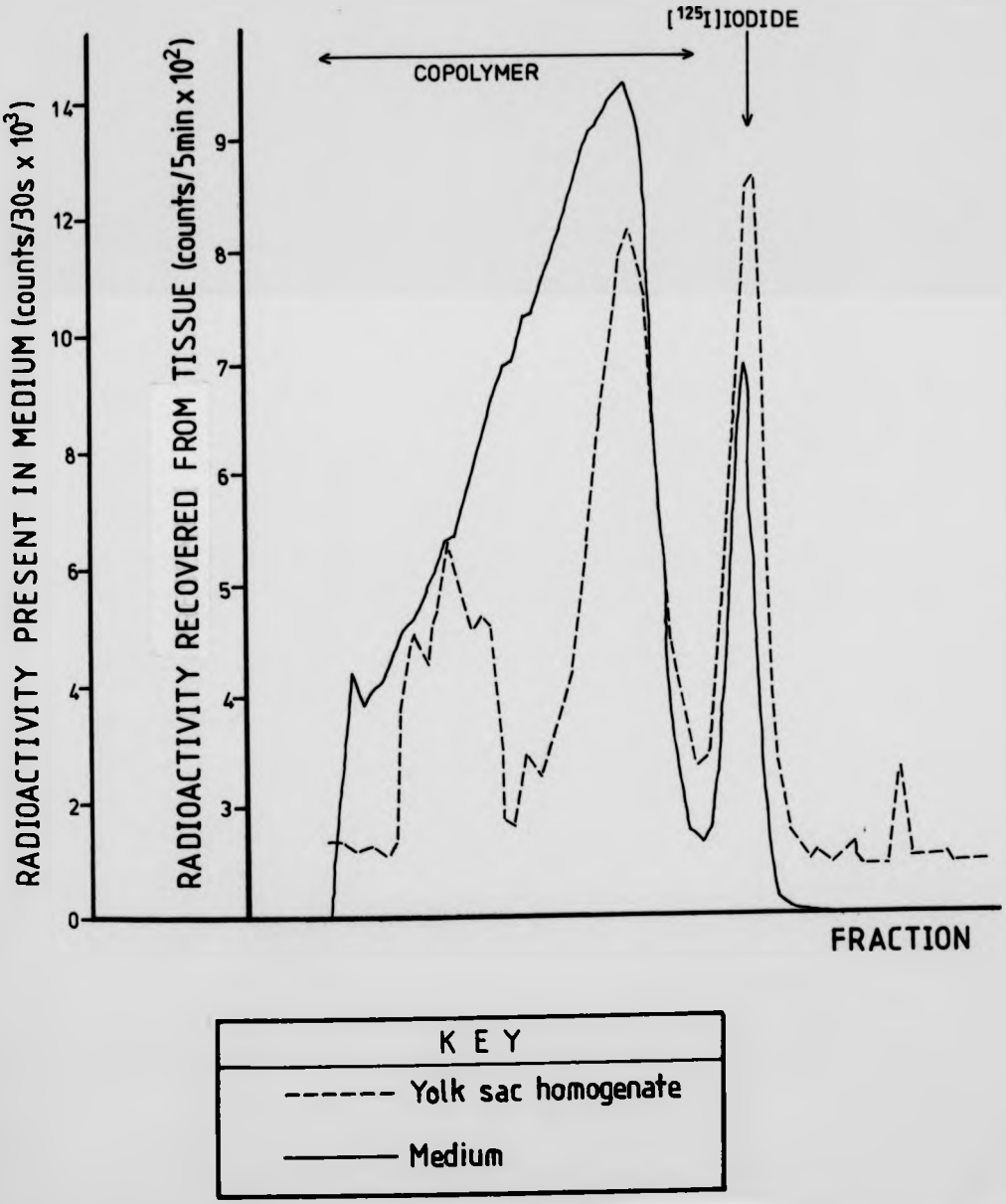


FIGURE 4.22

weight range could possibly be due to radiolabelled material binding to protein still present in the homogenate supernatant, and the second, larger peak represents lower molecular weight polymer chains which could be the product of crosslink cleavage of larger molecules. Unfortunately, owing to the poor labelling efficiency of this particular copolymer, it was not possible to repeat this experiment.

The results do not, however, demonstrate conclusively cross-link cleavage by lysosomal enzymes, for two reasons:

1) It is possible that the treatment of yolk sacs with Triton X100 after incubation could have caused crosslink cleavage. This possibility is eliminated by later experiments (see Chapters 7 and 8).
2) It is possible, in view of the uptake and degradation studies using the copolymer 39 fractions described earlier, that the molecular weight distribution of polymer obtained with material derived from yolk sacs represents preferential capture of low molecular weight material during pinocytosis and is not indicative of cleavage products.
   This possibility was investigated further and is discussed in section 4.2.6.

4.2.6 Preferential Uptake of Lower Molecular Weight Copolymer 39 fractions by the Yolk Sac

The following experiment was carried out in order to observe whether or not yolk sacs preferentially pinocytosed material of a given size, when presented with a polydisperse preparation.

Unfractioned copolymer 39 was used, since uptake of any fraction could be detected by changes in the molecular weight distribution of copolymer in the medium and the lower molecular weight radiolabelled degradation products would elute much later.

10 yolk sacs were incubated for 12h in a single Erlenmeyer flask containing 11ml medium 199 supplemented with 10% calf serum and gassed periodically with 95% O_2/5% CO_2. A 1ml sample was removed immediately after adding 62.5 µg/ml ^{125}I-labelled copolymer 39 at the beginning of the experiment. The sample was applied to a Sepharose 4B/6B column (section 2.5), and the molecular weight distribution of the medium at the start of incubation plotted.

A 1ml sample of the medium after 12h incubation with yolk sacs was applied to the same column. The results are shown in fig. 4.23 and Table 4.8.

There is no observable difference in the shape of the curve in the first 34 fractions, indicating that there is little, if any, uptake of higher molecular weight fraction of the copolymer.

There is a 10% reduction in the total count after incubation of fraction nos. 35-49. This coincides almost exactly with the molecular weight distribution of copolymer 39e, the smallest fraction ($\bar{M}w$ 34,000) as seen in fig. 4.23.

The increase in fraction nos. 50-57 is almost entirely due to ^{125}I-tyrosine release since the increase in percentage of $[^{125}I]$ iodide concentration over a 12h period of incubation is $<2.00\%$ (Chapter 3, section 6).

SEPHAROSE 4B/6B CHROMATOGRAPHY OF CULTURE MEDIUM BEFORE AND FOLLOWING A 12h INCUBATION OF YOLK SACS WITH ^{125}I-LABELLED COPOLYMER 39

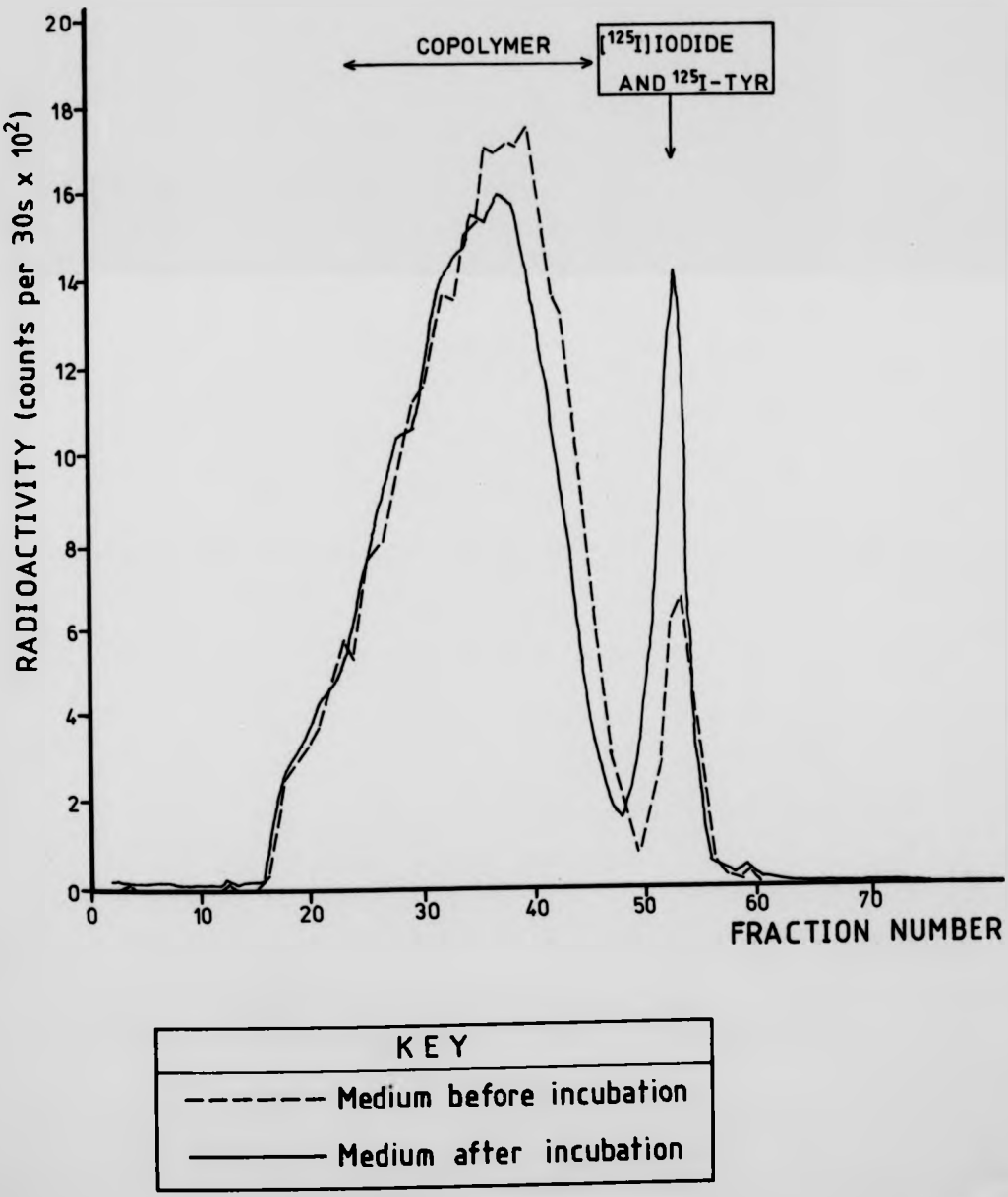


FIGURE 4.23

TABLE 4.8

SEPHAROSE 4B/6B CHROMATOGRAPHY OF ^{125}I-LABELLED COPOLYMER 39 IN MEDIUM 199 + 10% CALF SERUM BEFORE AND FOLLOWING A 12H INCUBATION WITH YOLK SACS

FRACTION NOS.	% OF TOTAL COUNT BEFORE INCUBATION	% OF TOTAL COUNT AFTER INCUBATION
16-34	39.90	40.50
35-49	52.00	42.00
50-57	7.47	15.86
	99.37	98.36

4.3 DISCUSSION

The rate of uptake of the crosslinked copolymer 39 by yolk sac tissue was clearly dependent upon the molecular size of the copolymer used. From fig. 4.9, it can be seen that there is a marked decrease in rate of tissue accumulation as the mean molecular weight of the sample increases. This result is consistent with the findings made by Duncan et al (1981) on the effect of molecular size of ^{125}I-PVP on its pinocytosis by the rat visceral yolk sac. The rate of tissue accumulation of copolymer 39e ($\bar{M}w$ 34,000) was 84.1% of matched ^{125}I-PVP ($\bar{M}w$ approx. 40,000) control experiments, (an accepted measure of the rate of fluid-phase pinocytosis). Fractions with higher mean molecular weights are taken up more slowly, the largest, fraction 39a ($\bar{M}w$ >400,000), only accumulating in the tissue at 20.6% the rate of matched control ^{125}I-PVP experiments. Unfractioned copolymer 39 ($\bar{M}w$ 190,000), which contains a large proportion of lower molecular weight fractions, gives a tissue accumulation value of 38.9% of a matched control.

Measuring the tissue radioactivity does not, however, take into account the possibility of copolymer uptake with subsequent degradation by the tissue and the release of low molecular weight radiolabelled degradation products back into the culture medium. Using each of the fractions, Sephadex G-15 chromatography of the culture medium after 5h incubation with yolk sacs demonstrated the presence of a peak of radioactivity which co-eluted with [^{125}I] iodotyrosine, indicating degradation of copolymer fractions by the yolk sac tissue. This peak was not present in the radiolabelled preparations prior to use, nor did it appear if the copolymers were

incubated in the absence of yolk sacs (Chapter 3). A further peak, eluting after [^{125}I] iodotyrosine was also noted, with all fractions, after incubation of copolymers with yolk sacs, and this was provisionally identified as [^{125}I]-diiodotyrosine rather than other possible degradation products such as ^{125}I-labelled phenylalanyl-tyrosine or ^{125}I-labelled dityrosyl-HMDA. It is however, possible that other degradation products might be partially responsible for the production of this small peak of radioactivity.

The detection of degradation products in the medium after incubation with yolk sacs means that the tissue accumulation plots do not represent total pinocytic uptake of copolymers by yolk sacs, but only the radioactivity remaining in the tissue at any given time. Total pinocytic uptake is given by summing the value obtained for accumulation by the tissue and the value for degradation. Exocytosis of undegraded copolymer following its uptake by the tissue should also be accounted for when calculating total uptake values, but this was not investigated in the present study since Williams (1975) found that the rate of exocytosis of both ^{125}I-BSA and ^{125}I-PVP was negligible on incubation of these substrates with rat yolk sacs.

The total pinocytic uptake of copolymer 39 fractions after 5h incubation with yolk sacs also demonstrated a size-dependence, with uptake increasing with decreasing mean molecular weight. Degradation of copolymer fractions was shown not to be size dependent : all copolymer fractions were degraded to around the same extent (Table 4.3). Approximately 40% of the total uptake of all copolymer fractions is degraded within the 5h incubation period.

Duncan and Kopeček (1984) suggest that the preferential uptake of lower molecular weight ^{125}I-PVP fractions by yolk sac tissue can be explained by considering the ultrastructure of this tissue. Newly forming pinocytic residues originate at the base of microvilli which are present on the apical region of the cell. This microvillous area acts as a barrier or sieve which restricts the entry of molecules larger than approximately Mw 100,000. The results presented support this suggestion, since copolymer 39e ($\bar{M}w$ 34,000) is of a smaller size than the proposed threshold, and enters the tissue at a much higher rate than the other fractions, the larger molecules being taken up at a rate proportional to the mean molecular weight of the fraction.

Although non-crosslinked copolymer 74 ($\bar{M}w$ 31,000) is of a similar size as copolymer 39e, the rate of tissue accumulation was much lower, but experiments performed to detect degradation of copolymer 74 showed that approximately 70% of the total uptake had been degraded during the 5h incubation period, and that total uptake was virtually the same. These data indicate that degradation of copolymer sidechains is much more rapid than copolymer crosslinks of the same amino acid sequence. This is probably due to greater accessibility of the susceptible bond in copolymer 74 to enzymatic cleavage, since this is in the form of a sidechain. Also, in copolymer 39e it is necessary that at least two bonds must be cleaved to release radiolabelled degradation products from crosslinks or intramolecular cycles. It is interesting to note that copolymer 74 is degraded to approximately double the extent of copolymer 39e.

Extended incubation of copolymer 39e with yolk sacs revealed that the tissue was still accumulating substrate after 12h, and that the rate of degradation of the copolymer was slowly increasing with time. This experiment indicated that tissue levels of undegraded copolymer were beginning to level off, but unfortunately it was not possible to extend the experimental period further, since yolk sac tissue begins to degenerate after 12h in vitro (Williams et al 1975). The pattern of uptake and degradation observed during this experiment was unlike that described by Williams following pinocytic studies formaldehyde-denatured ^{125}I-labelled bovine serum albumin in rat yolk sacs, where the levels of radioactivity in the tissue became constant after a short initial period of approximately 2h. Pinocytosis of ^{125}I-labelled BSA was shown to be adsorptive in nature, the rate of uptake ranging from 11.1 to 80.8 µl/mg/h, and the rate of degradation, again indicated by the release of [^{125}I]iodotyrosine was extensive (approximately 80% of the total uptake after 5h). One obvious difference between these experiments is that HPMA copolymers enter the tissue much more slowly by fluid-phase pinocytosis, resulting in a total pinocytic uptake after 12h which is less than that observed in the first hour of uptake of membrane bound ^{125}I-labelled BSA. Because of these lower levels of intracellular copolymers, and in view of the high rates of degradation achieved by lysosomal enzymes during protein degradation, one might expect HPMA copolymers to be degraded quickly. One possible reason for the slower rates of degradation observed might be steric hindrance due to the presence of non-degradable copolymer chains which might prevent enzymes from gaining access to the appropriate site.

Accumulation of copolymer chains within lysosomes would be expected since molecules of molecular weight greater than approximately 220 cannot cross the lysosomal membrane (Reijngoud and Tager 1977). Release of copolymer chains of a molecular weight low enough to be excreted by the kidney would take place following exocytosis or cell death.

The introduction of 2-4-dinitrophenol, a known uncoupler of oxidative phosphorylation, at 50 µg/ml into the culture medium caused considerable inhibition of uptake of copolymer 39e by yolk sacs. The concentration used has previously been shown to be inhibitory and non-toxic to cells in this and other experimental systems (Duncan et al 1979 ; Bowers 1977 ; Cohn 1966 and Munthe-Kaas 1977). Both the accumulation of radioactivity by the tissue and the appearance of $[^{125}I]$-iodotyrosine in the medium were virtually abolished, thereby indicating that both uptake and degradation take place through pinocytosis of copolymer by the yolk sac.

Leupeptin, a peptide aldehyde that inhibits the thiol-dependent lysosomal proteinases (Barrett 1977), when added to the culture medium at a concentration of 40 µg/ml, demonstrated reduced degradation of copolymer 39e. Pinocytic uptake of copolymer 39e was approximately the same both in the presence and absence of inhibitor, but a proportion representing only 18% of the total uptake was degraded in the presence of leupeptin, in contrast with 44% in the absence of inhibitor. Lysosomal thiol-proteinases are therefore partly responsible for the degradation of copolymer 39e observed after incubation with yolk sacs.

Copolymer 68 is a more densely crosslinked preparation than copolymer 39, and contains shorter crosslinks, which are also potentially degradable. The tissue accumulation of ^{125}I-labelled copolymer 68 was found to be linear with time, and there was no $[^{125}I]$ iodotyrosine released into the medium following incubation with yolk sacs. These data confirmed that it is possible to trace the fate of the copolymer chains in the yolk sac, since the radiolabelled tyrosine residue remained attached to the copolymer backbone throughout the experimental period. This unfractionated copolymer has a mean molecular weight of 171,000 and total uptake values after 5h (11.4 µl/mg) compared with unfractionated copolymer 39 ($\bar{M}w$ 190,000) after 5h (8.5 µl/mg) taking into consideration the difference in mean molecular weights.

Sepharose 4B/6B chromatography of yolk sac tissue homogenized after incubation with copolymer 68 revealed that most of the radiolabelled copolymer contained within the tissue was in the form of lower molecular weight fractions of the original preparation. This could indicate crosslink cleavage by enzymes within the tissue, but the results could also be explained by crosslink cleavage during preparation of the tissue for chromatography, or by the preferential uptake of lower molecular weight fractions by the tissue during incubation.

Preferential uptake of lower molecular weight copolymer 39 fractions by the yolk sac was demonstrated by comparing the molecular weight distribution of the unfractionated copolymer 39 in medium before and after incubation with yolk sacs for 12h. A 10% reduction in the total counts was observed in the

region of the curve corresponding to the lower molecular weight fraction 39e, with a corresponding increase of 8% in the second peak due to the release of [^{125}I] iodotyrosine back into the culture medium following uptake of copolymer by the tissue. There was little or no change in the molecular weight distribution in areas of the curve corresponding to larger copolymer fractions, indicating little, if any, uptake of these molecules.

In summary, the data obtained from the experiments performed using the rat visceral yolk sac have shown that ^{125}I-labelled crosslinked HPMA copolymers are captured by cells at a rate consistent with uptake by fluid-phase pinocytosis, and the rate of uptake is dependent on the mean molecular weight of the copolymer used. From the range of sizes presented, the yolk sac tissue selects against copolymers of higher molecular weight. Pinocytic uptake of copolymers is followed by degradation of oligopeptide sequences by lysosomal enzymes after fusion of pinocytic vesicles with lysosomes. Lysosomal thiol-proteinases are partly responsible for this intracellular degradation of copolymers.

From the experiments performed using yolk sac tissue, it was not possible to prove conclusively that crosslink cleavage took place to yield copolymer chains of lower mean molecular weight, since lower molecular weight fractions were preferentially pinocytosed. Copolymer 39 was synthesized from copolymer chains containing sidechains which were subsequently crosslinked (Chapter 3). Only 14% of the initial sidechains were connected by crosslinks, the rest formed intramolecular cycles or remained as sidechains. Sidechains contained no tyrosine residues and were therefore not radio-

labelled. Under the circumstances, degradation products detected could have, in all cases, been due to enzymatic hydrolysis of bonds present in intramolecular cycles contained within the preparations, rather than crosslinks.

5.1 INTRODUCTION

Various experimental systems have been used to quantitate endocytosis. Pratten et al (1980) have described three main types of cells that have been used for such studies, namely unicellular animals such as Amoeba and Tetrahymena; mammalian cells e.g. mononuclear phagocytes, fibroblasts and liver cells; and vertebrate organ cultures.

Chapter 4 described the pinocytic uptake of soluble crosslinked HPMA copolymers by the rat visceral yolk sac, and it was observed that the rate of uptake of copolymers increased with decreasing molecular size of the substrate, and that the copolymers were partially degraded within lysosomes during the experimental period.

Another organ which can be used to quantitate pinocytosis is the rat intestine. Like the rat visceral yolk sac, the intestinal epithelium, and in particular the jejunum, has a microvillous apical plasma membrane which is highly absorptive in nature.

In this chapter, the pinocytic uptake of crosslinked copolymers by the everted rat jejunal sac is described, and a comparison is made between the results obtained from the studies of the two organ culture systems i.e. the rat visceral yolk sac and the everted rat jejunal sac. Specifically, the questions investigated are the following:-

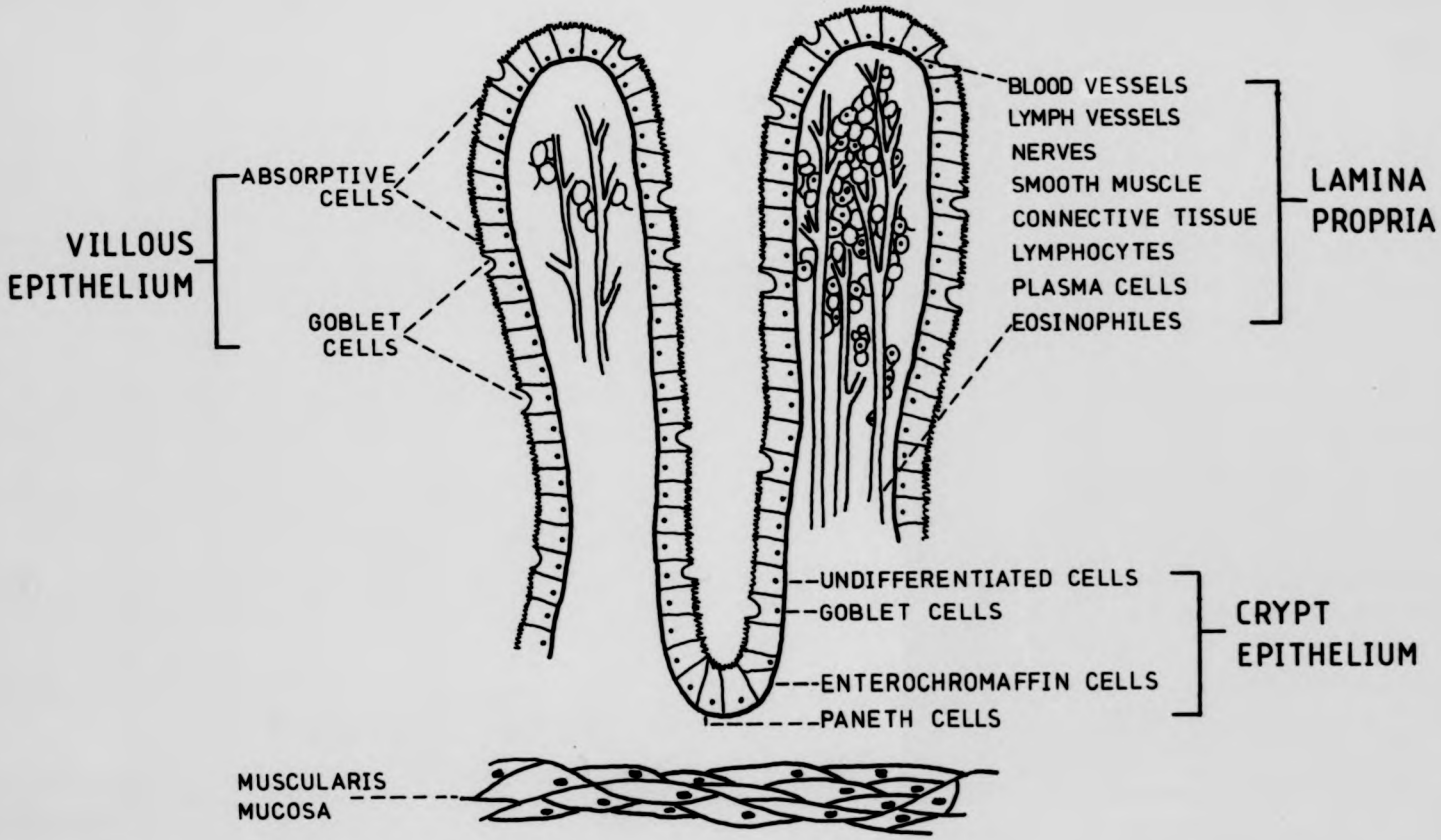
1. Are crosslinked copolymers pinocytosed by the rat jejunal epithelial cells?
2. If so, does this occur by fluid-phase pinocytosis, as in the yolk sac, or is the uptake adsorptive in nature?

CHAPTER 5

PINOCYTIC UPTAKE, INTRACELLULAR DEGRADATION AND TRANSPORT OF ^{125}I-LABELLED HPMA COPOLYMERS BY THE RAT JEJUNAL SAC IN VITRO

3. If pinocytosis does take place, is the rate of uptake in this tissue, too, dependent on the size of the copolymer?
4. Are crosslinked copolymers transported across the jejunal tissue i.e. from the mucosal to the serosal surface?
5. Are crosslinked copolymers hydrolysed within the lysosomes, and if so, is the extent of degradation dependent on the size of the copolymer?

The small intestine is a much thicker tissue than the visceral yolk sac, consisting of a strongly muscular tube with an outer longitudinal muscle layer, and an inner circular muscular layer. Adjacent to the circular layer lies a bed of vascular connective tissue, the submucosa, and finally, adjacent to the lumen lies the intestinal mucosa. The mucosa may be divided into three layers (Fig. 5.1). The deepest layer, the muscularis mucosae, is a thin sheet of smooth muscle which separates the mucosa from the submucosa. The middle layer, the lamina propria, is a continuous sheet of connective tissue which, together with the epithelium, forms the villi and the crypts. The lamina propria provides structural support for the intestinal epithelium and contains a variety of cell types including fibroblasts, macrophages, plasma cells, lymphocytes, mast cells and eosinophils. Also present are blood and lymph vessels, nerve fibres, smooth muscle strands and non-cellular connective tissue elements. The third layer of the intestinal mucosa consists of a continuous sheet of a single layer of columnar epithelial cells which covers the villi and the crypts. Absorptive, goblet and some enterochromaffin cells cover the surface of the villi and goblet, enterochromaffin and Paneth cells line the crypts. The presence of the villi increases the surface area of the absorptive epithelium about eightfold. The epithelial cells, in turn, have a microvillous apical



SCHEMATIC DIAGRAM OF TWO VILLI AND A CRYPT TO ILLUSTRATE THE HISTOLOGICAL ORGANIZATION OF THE MUCOSA OF THE SMALL INTESTINE (From BRIDGES 1980)

FIGURE 5.1

membrane which further increases the surface area available for absorption (Fig. 5.2). This apical membrane is often termed the brush border, and is known to contain a variety of hydrolytic enzymes (Bruce 1983).

The intestinal epithelium is a highly active tissue which renews itself every 2-3 days. Cell proliferation occurs in the crypts and the absorptive cells differentiate and mature during their migration from the crypts to the tips of the villi, from which cells are eventually shed (Trier 1967). The rate of cell renewal and loss, and the height of the villi, can be affected by a number of factors, such as certain nutritional states, or the use of radiation or antimitotic drugs (Trier 1967).

With the exception of the tips of the villi, where old cells are shed, the epithelial cells, or enterocytes, are closely apposed and locked together by junctional complexes comprising tight junctions where membranes from adjacent cells appear to fuse, intermediate zones, and a number of desmosomes which are thickened areas beneath the cell membrane (Fig. 5.2). These junctional complexes are believed to be impermeable to macromolecules. Most studies suggest that uptake of macromolecules takes place by endocytosis and innumerable E.M. studies using electron dense substrates such as horseradish peroxidase (HRP) have demonstrated the presence of pinocytic vesicles within the columnar epithelial cells.

Quantitative studies on pinocytosis have been undertaken using both the neonatal and adult mammalian intestine, and much work has been centred on the pinocytic uptake of IgG from the gut lumen and its transport across the tissue into the circulation

SCHEMATIC DIAGRAM OF A VILLOUS ABSORPTIVE CELL (modified from BRIDGES 1980)

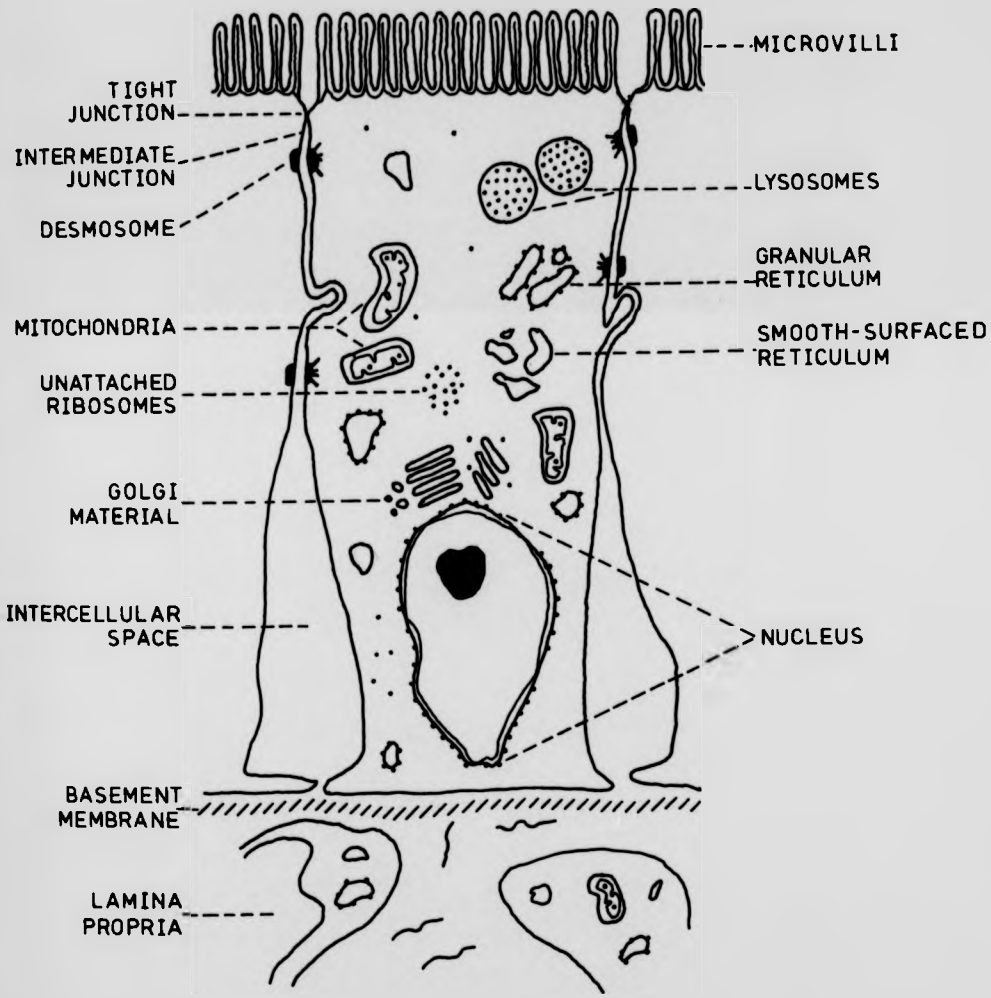


FIGURE 5.2

in neonatal mammals, which are known to acquire passive immunity from the transfer of maternal antibodies present in colostrum (Brambell 1966, Rodewald 1980). The species studied by various workers include the rabbit, rat and pig. The mechanism of uptake of IgG in the neonatal intestine is believed to be receptor-mediated pinocytosis.

Increased permeability to all macromolecules coming into contact with intestinal mucosa has been demonstrated in immature individuals of many species (Walker 1981). A few days after birth, when immunological and nonimmunological host defences develop, and intestinal epithelial cells mature functionally and morphologically, the uptake of macromolecules decreases. This phenomenon is known as closure.

Comparatively few studies have been carried out on the uptake and transport of macromolecules across the adult intestine. Walker (1981) provided morphological and physiological evidence for pinocytosis of macromolecules by the intestine, using an enzyme marker, horseradish peroxidase (HRP). This enzyme can be detected histochemically at both light and electron microscopic levels and can also be radiolabelled and measured quantitively in very small concentrations. Following intraluminal injection of HRP into ligated segements of jejunum and ileum, the substrate was found adsorbed to the apical surface membranes and within membrane-bound cytoplasmic vesicles. Extracellular HRP was present in the intercellular spaces between adjacent absorptive cells (following exocytosis from the baso-lateral membrane), traversing the basement membranes and in the spaces of the lamina propria. Walker (1981) reported further evidence by other workers for the movement of macromolecules such as insulin, haptens and

larger antigens which were transported in sufficient quantity to evoke a biological response.

Most previous studies have been carried out by injection of substrate directly into the lumen of a ligated portion of intestine in situ, with subsequent removal and examination of the tissue. The present study, however, was carried out according to the method of Bridges and Woodley (section 2.7) using everted sacs of adult rat jejunum incubated in vitro. This method, improved from that of Wilson and Wiseman (1954) involves the use of everted segments (3-4cm) of jejunum which are filled with culture medium and tied off at both ends before total immersion in culture medium. (The use of everted sacs facilitates the necessary oxygenation of the intestinal mucosa during the experimental period.)

Previous objections to the use of everted sacs centre around the possibility that the sacs may leak and that differing degrees of distention will produce differing degrees of unfolding of the villous surface. A further important objection stems from evidence of ultrastructural and biochemical changes which occur in the tissue when everted sacs are cultured in vitro (Levine et al 1970, Plattner et al 1970).

The method described by Bridges (1980) employs modified procedures which show that tissue viability and structural integrity can be maintained over a 2h incubation period. This was achieved by reducing the tissue preparation time and by the use of complex medium TC 199 containing 10% calf serum and 1mM ATP, as opposed to the traditional basic Krebs-Henleit Ringer medium used by other workers. Viability studies by Bridges included the use of light and electron microscopy (E.M.)

studies, the monitoring of oxygen consumption of the tissue, and sacs were also monitored for the active transport of D-glucose and L-methionine across the tissue into the serosal fluid during the incubation period. Both D-glucose and L-methionine were accumulated against a concentration gradient, and the finding that these gradients could be maintained for up to 2h indicated both tissue viability and that no leakage of sacs had occurred in that period of time.

Studies by Bridges (1980) and Beahon (1981), using non-degradable ^{125}I-PVP have shown this to be taken up into the tissue in a linear fashion by rat jejunal sacs, as in rat visceral yolk sacs, but at a lower rate (0.74 µl/h/mg protein as opposed to 1.71 µl/h/mg protein in the yolk sac). Uptake was proportional to substrate concentration, was temperature sensitive, and could be partially inhibited by inhibitors of glycolysis or oxidative phosphorylation. This evidence indicated fluid-phase pinocytosis to be the mechanism of uptake. ^{125}I-PVP was therefore again chosen as a control with which to compare uptake values obtained for the pinocytosis of ^{125}I-labelled HPMA copolymers by everted jejunal sacs.

^{125}I-PVP was also shown by Bridges (1980) to accumulate in the serosal fluid of the everted sac linearly with respect to time up to 2h at a rate of 0.12 µl/mg protein/hr. Bridges proposed that ^{125}I-PVP had traversed the tissue following its pinocytosis by absorptive epithelial cells.

Pinocytic uptake studies using a degradable substrate were carried out by Bridges (1980) and Beahon (1981). Both authors used ^{125}I-labelled horseradish peroxidase (HRP) as a substrate in the everted sac system, and found a linear accumulation of radioactivity with time (up to 2h) in both the tissue and the

serosal fluid. Bridges noted that this substrate entered the tissue about sixteen times faster than ^{125}I-PVP but that tissue uptake increased with substrate concentration up to 50 µg/ml, after which uptake became independent of substrate concentration. This work was stated to be of a preliminary nature, but the author suggested that the results are indicative of substrate undergoing adsorptive pinocytosis. Accumulation of ^{125}I-HRP into the serosal fluid took place at a similar rate to that observed for ^{125}I-PVP.

Bridges (1980) also considered the possibility of substrate degradation. A comparison of the rate of total substrate disappearance and the combined rate of tissue and serosal accumulation of HRP revealed that the substrate disappeared at a greater rate than could be accounted for by the measured uptake values. Bridges concluded that a large proportion of the HRP taken into the tissue, or adsorbed onto the membrane, was degraded.

In order to ascertain whether crosslinked copolymers could be pinocytosed by this tissue, and transported to some extent into the serosal fluid, copolymer 39 (crosslink structure P-Gly-Gly-Phe-Tyr-HMDA-Tyr-Phe-Gly-Gly-P, $\bar{M}w$ 190,000) was again radiolabelled and incubated with everted rat jejunal sacs. The possible effect of molecular size of the copolymer on the rate of jejunal tissue accumulation of substrate was also investigated using radiolabelled fractionated copolymer 39 of mean molecular weights 34,000, 110,000, 150,000, 400,000 and >400,000.

The potentially degradable crosslinks contained within ^{125}I-labelled copolymer 39 contain radiolabelled tyrosine

residues which were shown (Chapter 4) to be liberated in the culture medium following hydrolysis by lysosomal enzymes present in the rat visceral yolk sac tissue. In the present study experiments were again carried out, using column chromatography, to detect low molecular weight radiolabelled degradation products in both the external medium and the serosal fluid following incubation of ^{125}I-labelled copolymer 39 fractions with everted jejunal sacs.

In Chapter 4, inhibition studies were described which proved that crosslinked copolymers were pinocytosed by the rat visceral yolk sac and degraded intracellularly. The work carried out by Bridges (1980), and others previously, has demonstrated that complete inhibition of uptake of macromolecules by the small intestine using metabolic inhibitors has not been achieved.

Bridges (1981) did show, however, that ^{125}I-PVP uptake into both the tissue and the serosal fluid was reduced by approx. 50% when experiments were carried out at temperatures between 10 and 30^{o}C. Since this method of pinocytic inhibition equalled the maximum observed by Bridges using metabolic inhibitors, pinocytic uptake experiments on copolymer 39 fractions (a and e) were carried out at low temperature (15^{o}C) to observe whether the uptake values obtained at 37^{o}C were the result of an active, pinocytic process, or the binding of substrate to the tissue without subsequent internalization.

Copolymer 68 ($\bar{M}w$ 171,000, crosslink structure P-Gly-Gly-Phe-HMDA-Phe-Gly-Gly-P) contains a tyrosine residue, which can be radiolabelled, close to the copolymer backbone. Unlike copolymer 39, this copolymer can be used to trace the fate of

the copolymer backbone following intralysosomal degradation of the potentially degradable crosslinks contained within it (Chapters 3 and 4). Copolymer 68 was therefore radiolabelled and used as a substrate in the present system in order to compare patterns of tissue accumulation of radioactivity with the yolk sac system and to observe the possible transport of macromolecular material into the serosal fluid.

The final experiment performed using the everted jejunal sac was an attempt to disprove the possibility of leakage of macromolecules across the tied ends of the sac into the serosal fluid. Although this possibility had been eliminated by Bridges, the present experiment aimed to demonstrate that the experimental method was reproducible. The method employed involved the use of loops of intestine, the cut ends of which were not immersed in the culture medium. The substrate used for this experiment was unfractionated ^{125}I-labelled copolymer 39, and column chromatography of the serosal fluid was carried out at the end of the experimental period in order to identify the nature of the radioactivity accumulated within it.

5.2 RESULTS

5.2.1 Tissue Accumulation of Copolymer 39 Fractions by the Everted Jejunal Sac : The Effect of Molecular Size

The accumulation of copolymer 39 fractions by everted jejunal sacs was measured at various times over a 1.5h incubation period at 37°C. Sacs were incubated singly and radiolabelled copolymer (125 µg/ml) was added to each flask at the beginning of each experiment. Duplicate samples were taken at each time point for each experiment. Concurrent control experiments were performed using ^{125}I-PVP as substrate.

Figures 5.3-5.8 show progress curves for the tissue accumulation of radioactivity for each copolymer fraction (39a-e) and the unfractionated copolymer 39. Fig. 5.9 combines figs. 5.3-5.8. Results for each time point are expressed as a clearance value (µl/mg protein); (see Chapter 2, sections 8 and 9). Each value is given as a mean ± S.E. of the uptake values obtained from 4-8 sacs. ^{125}I-PVP was used as a fluid-phase marker in matched control experiments and results showed a linear uptake as shown in fig. 5.10.

Figures 5.3-5.9 show that the tissue accumulation of the copolymers is approximately linear with time. Rates of uptake were calculated for each of the copolymer fractions and are shown in Table 5.1.

From the results presented, there is an increase in the rate of accumulation of substrate within the tissue with increasing molecular size. ^{125}I-PVP ($\bar{M}w$ 40,000) enters the tissue at a rate similar to that observed for copolymer 39e ($\bar{M}w$ 34,000), but in this tissue, the larger molecules associate with the tissue at a faster rate. This is clearly demonstrated by

TISSUE ACCUMULATION OF ^{125}I-LABELLED H P M A COPOLYMER 39a BY RAT JEJUNAL SAC

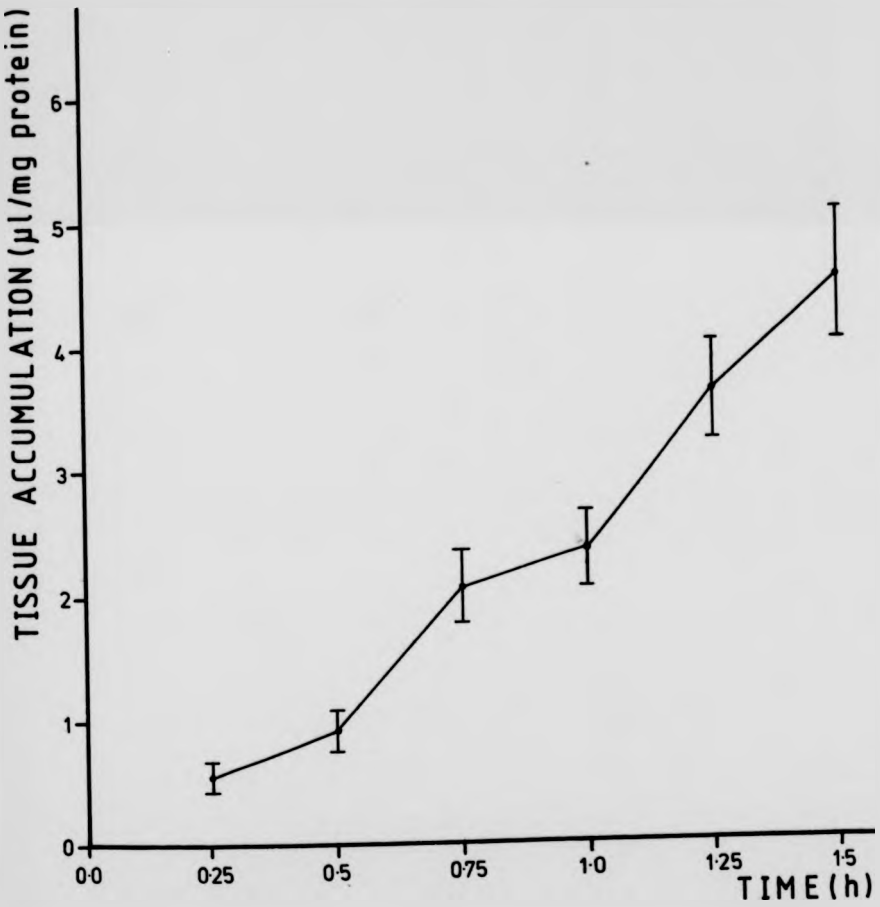


FIGURE 5.3

TISSUE ACCUMULATION OF ^{125}I-LABELLED H P M A COPOLYMER 39b BY RAT JEJUNAL SAC

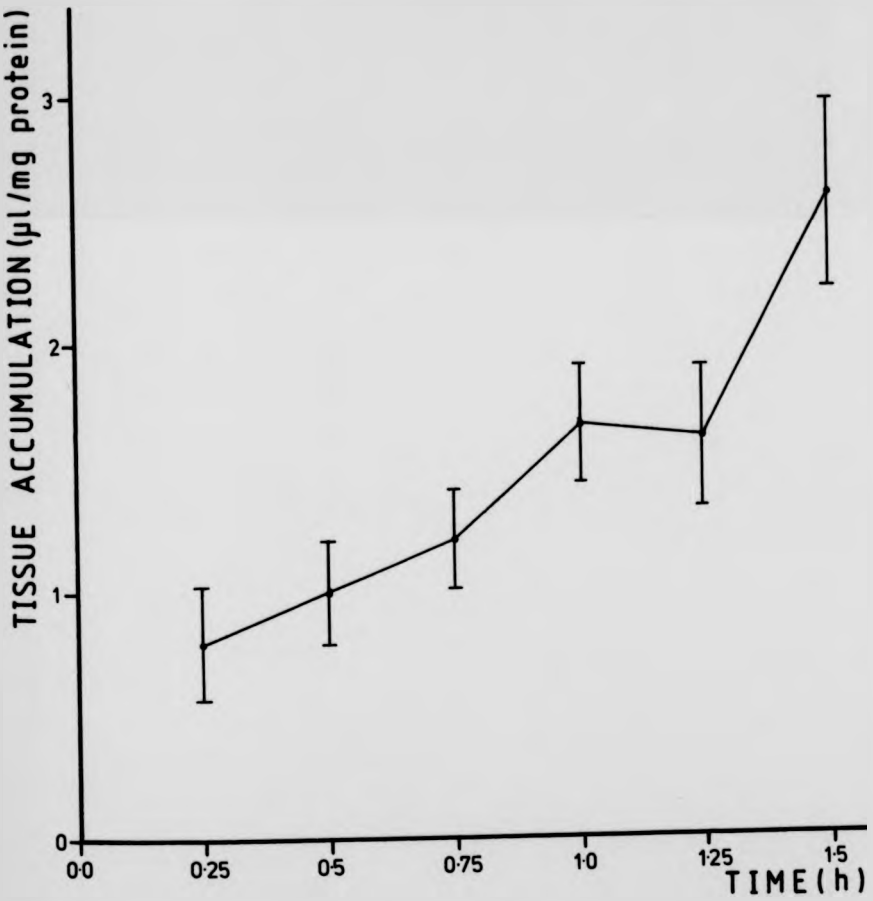


FIGURE 5.4

TISSUE ACCUMULATION OF ^{125}I-LABELLED H P M A COPOLYMER 39c BY RAT JEJUNAL SAC

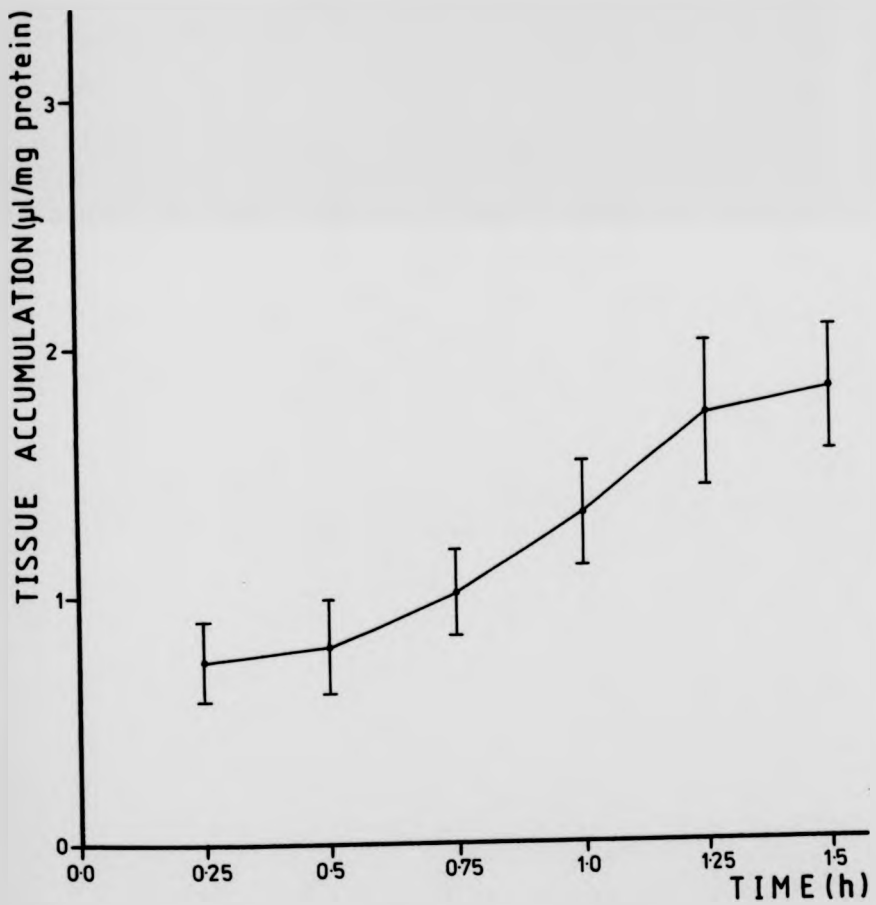


FIGURE 5.5

TISSUE ACCUMULATION OF ^{125}I-LABELLED H P M A COPOLYMER 39d BY RAT JEJUNAL SAC

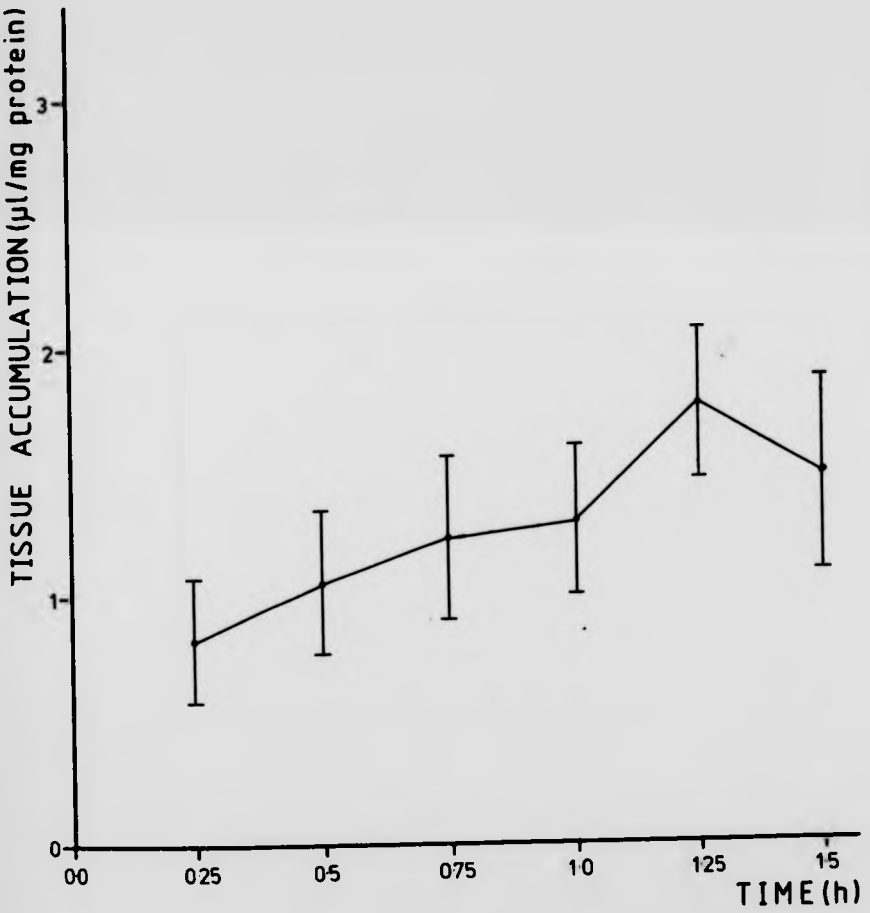


FIGURE 5.6

TISSUE ACCUMULATION OF ^{125}I-LABELLED H P M A COPOLYMER 39e BY RAT JEJUNAL SAC

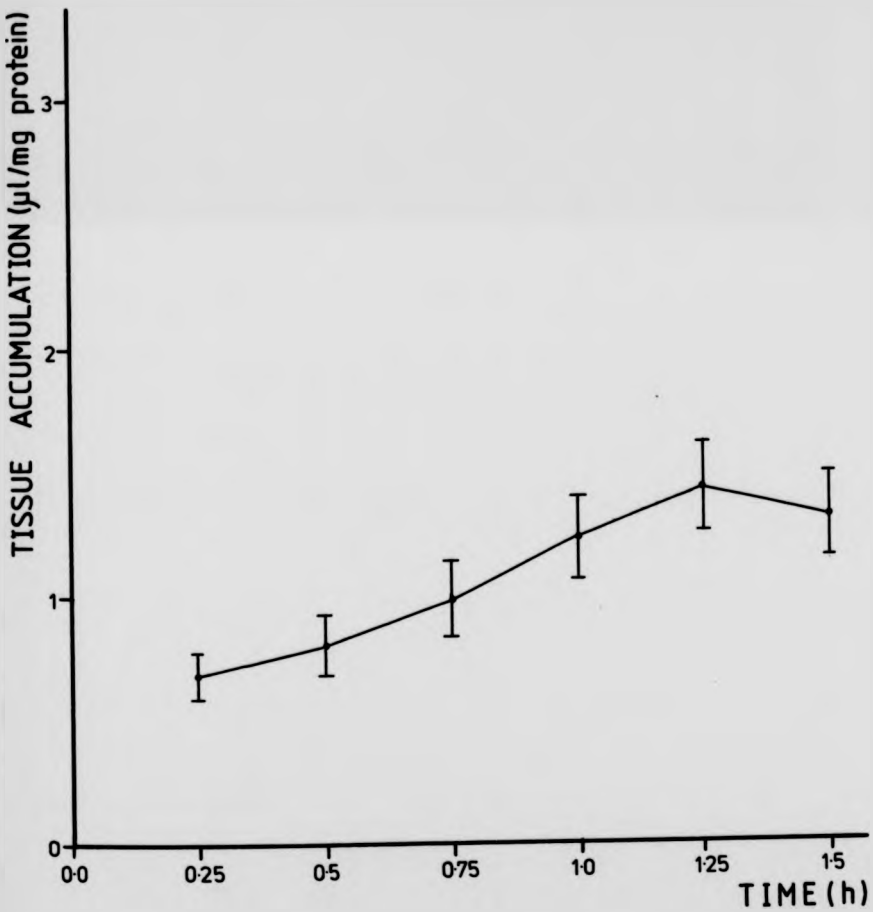


FIGURE 5.7

TISSUE ACCUMULATION OF ^{125}I-LABELLED H P M A COPOLYMER 39 BY RAT JEJUNAL SAC

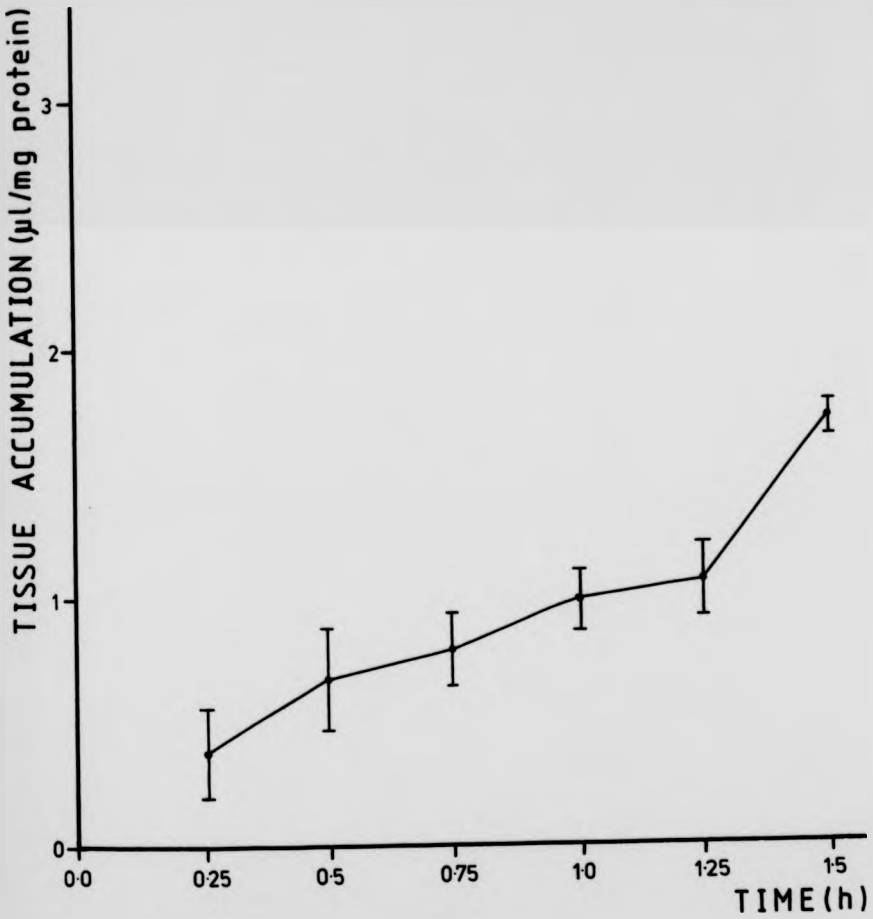
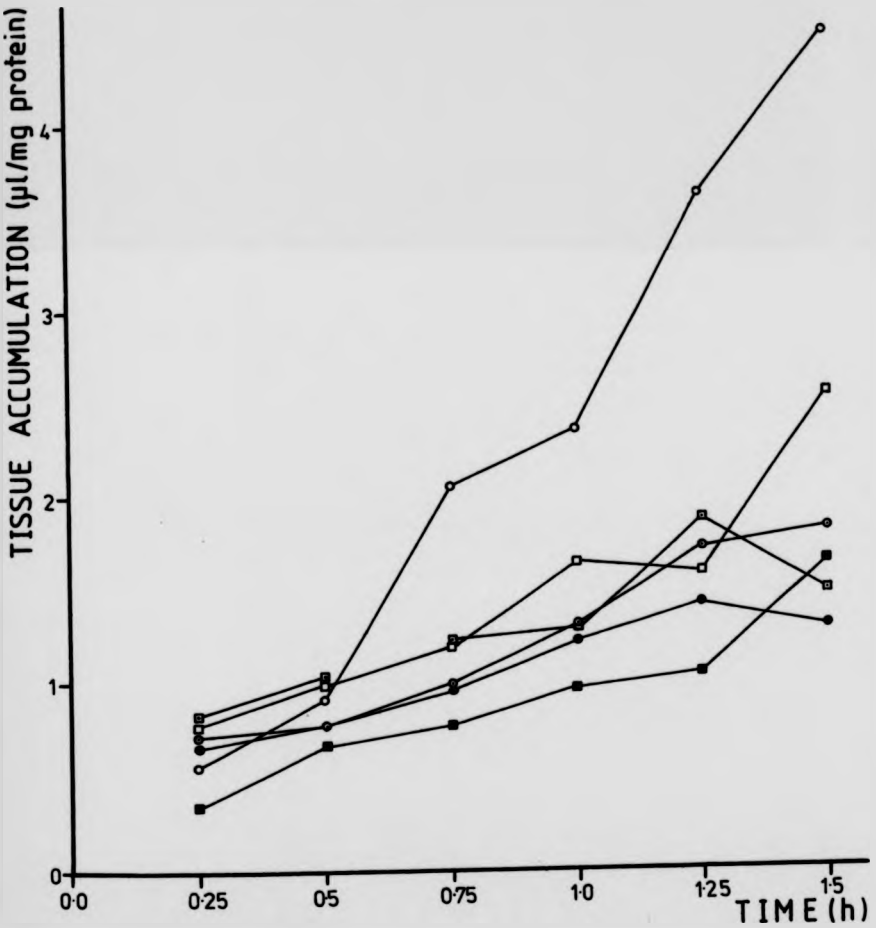


FIGURE 5.8

TISSUE ACCUMULATION OF ^{125}I-LABELLED H P M A COPOLYMER FRACTIONS BY RAT JEJUNAL SAC



KEY	
DATA POINT	COPOLYMER
■	39
○	39a
□	39b
●	39c
▣	39d
●	39e

FIGURE 5.9

TISSUE ACCUMULATION OF ^{125}I-PVP
BY THE RAT JEJUNAL SAC

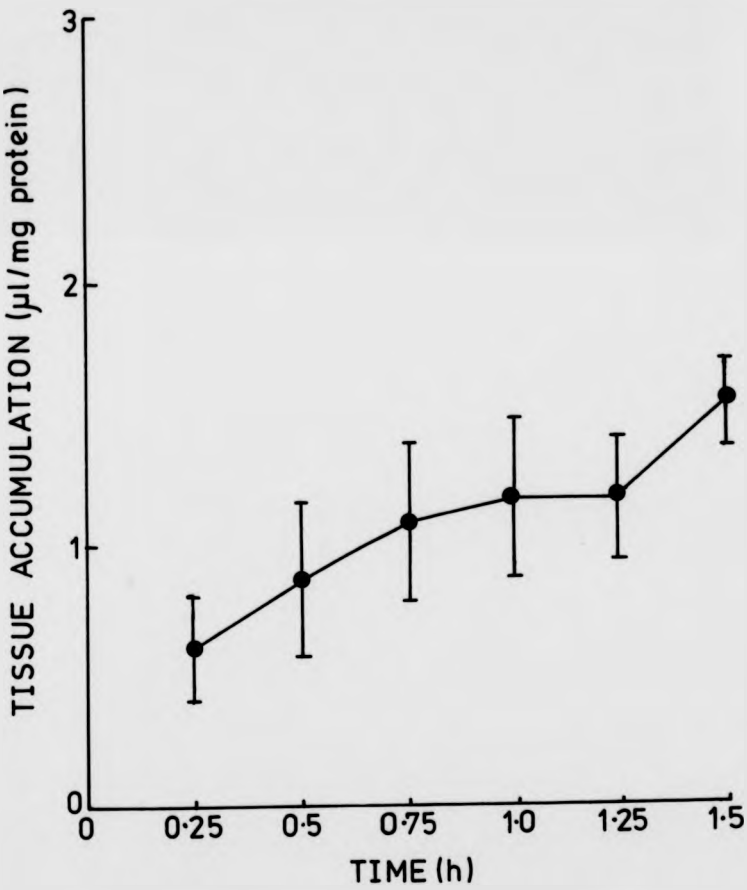


FIGURE 5.10

TABLE 5.1

TISSUE ACCUMULATION OF ^{125}I-LABELLED COPOLYMER 39 BY RAT JEJUNAL SACS

POLYMER CODE NO.	$\bar{M}w$	NO. OF EXPERIMENTS PERFORMED	RATES OF ACCUMULATION + SE (µl/mg protein/hr)	% PVP CONTROL
39	190,000	2	0.86	130.30
39a	>400,000	4	3.22 ± 0.36	489.40
39b	400,000	4	1.29 ± 0.24	196.00
39c	155,000	4	0.98 ± 0.22	149.10
39d	110,000	4	0.80 ± 0.17	121.10
39e	34,000	4	0.61 ± 0.12	92.50
PVP	~ 40,000	4	0.66 ± 0.21	100.00

the values obtained for the largest fraction ($\overline{Mw}$ >400,000), which is taken up approximately five times faster than ^{125}I-PVP.

5.2.2 Accumulation of Radioactivity in the Serosal Fluid of Jejunal Sacs Incubated with Copolymer 39 Fractions

Following the 1.5h incubation period of everted jejunal sacs with copolymer 39 fractions (section 5.2.1), serosal fluid was recovered from the sacs (section 2.7), and assayed for radioactivity. Figs. 5.11-5.16 show the progress curves for the accumulation of radioactivity in the serosal fluids for each copolymer fraction (39a-e) and the unfractionated copolymer 39. Again, results are expressed for each time point in µl/mg protein as a mean ± S.E. of the values obtained for 4-8 sacs. Fig. 5.17 shows figs. 5.11-5.16 combined and the accumulation of ^{125}I-PVP in the serosal fluid is shown in fig. 5.18.

The figures demonstrate that radioactivity is translocated across the intestine and that the values obtained for the serosal accumulation of substrate are lower than those obtained for the tissue in all cases. It is also apparent from the figures that the rate of accumulation of radioactivity in the serosal fluid is not dependent upon the molecular size of the copolymer.

Figures 5.11-5.17 show the progressive accumulation of radioactivity in the serosal fluid with time, but do not indicate the nature of the radiolabelled molecules transported. In order to determine whether macromolecules had been transported across the jejunal sacs, or if the radioactivity represented low molecular weight degradation products or iodide, column chromatography was carried out on serosal fluids following incubation with each of the copolymer fractions using

ACCUMULATION OF ^{125}I-LABELLED HPMA COPOLYMER 39a IN THE SEROSAL FLUID OF RAT JEJUNAL SAC

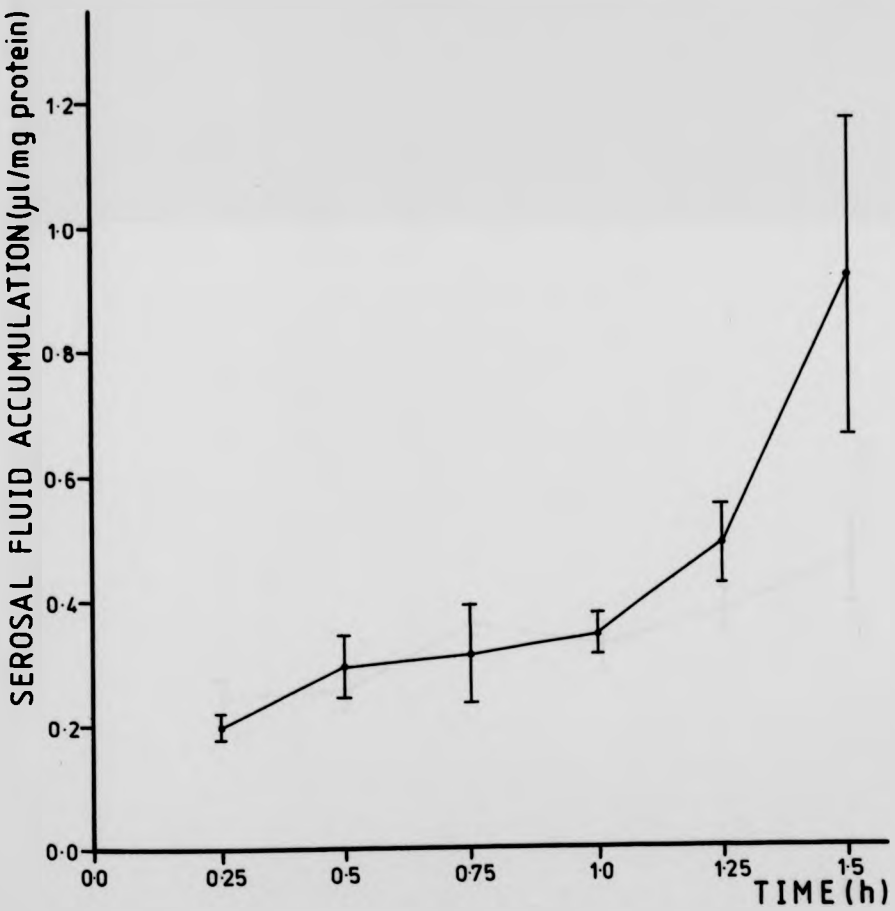


FIGURE 5.11

ACCUMULATION OF ^{125}I-LABELLED H P M A COPOLYMER 39b IN THE SEROSAL FLUID OF RAT JEJUNAL SAC

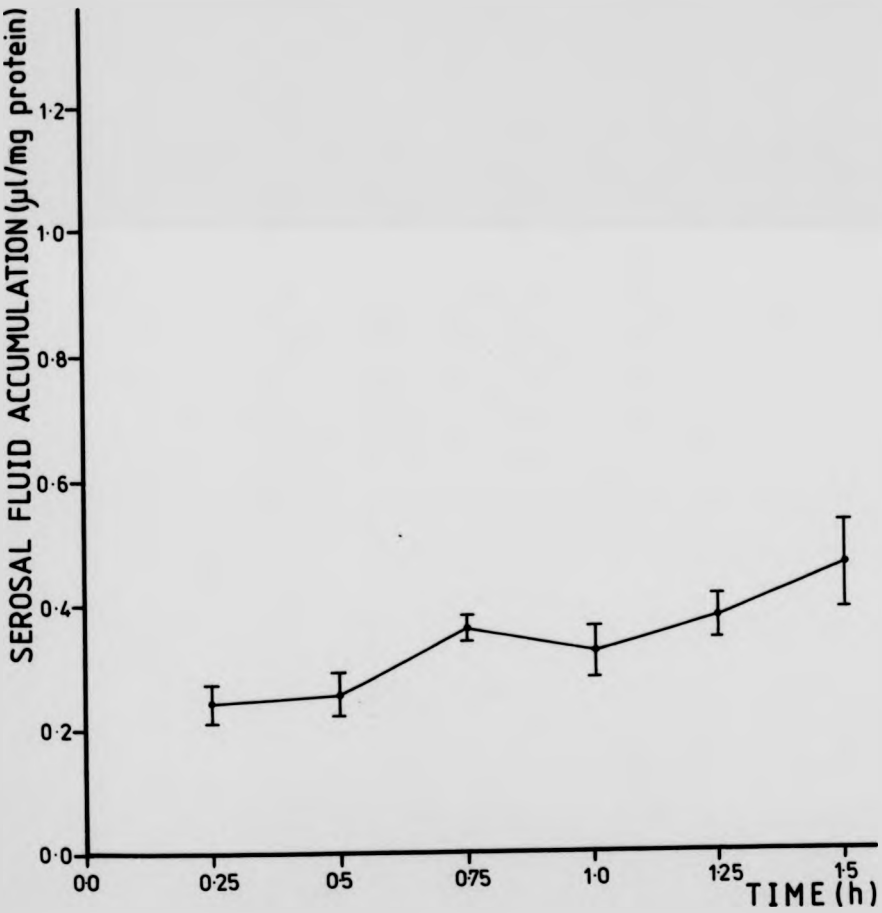


FIGURE 5.12

ACCUMULATION OF ^{125}I-LABELLED H P M A COPOLYMER 39c IN THE SEROSAL FLUID OF RAT JEJUNAL SAC

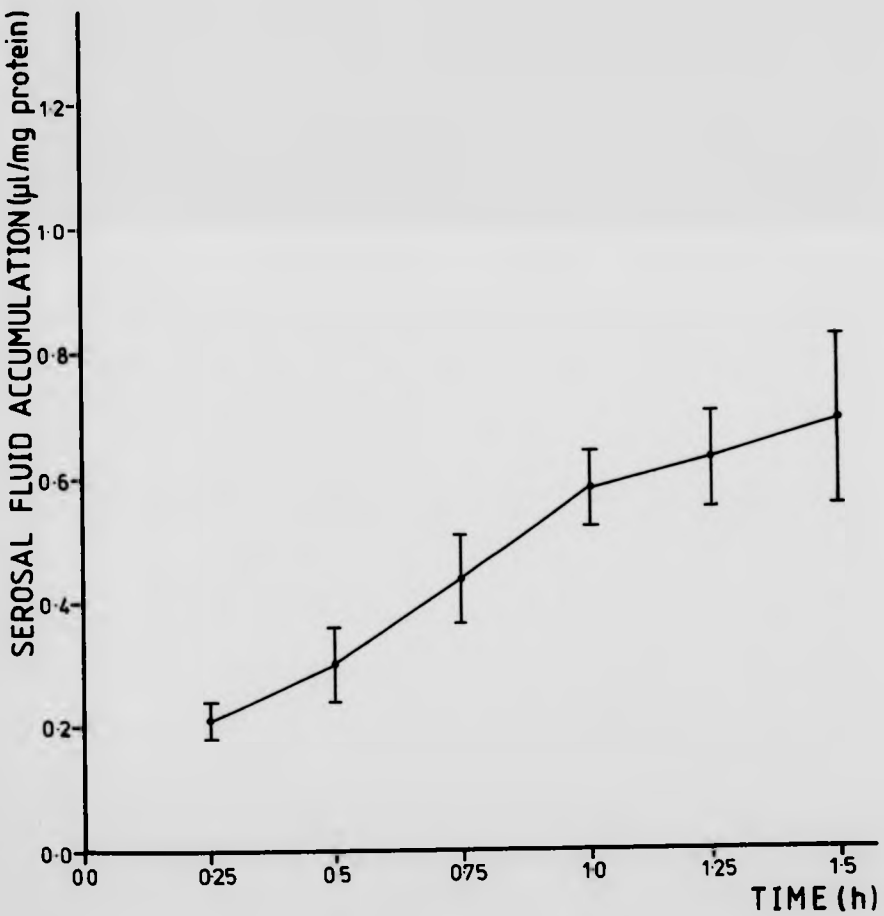


FIGURE 5.13

ACCUMULATION OF ^{125}I-LABELLED H P M A COPOLYMER 39d IN THE SEROSAL FLUID OF RAT JEJUNAL SAC

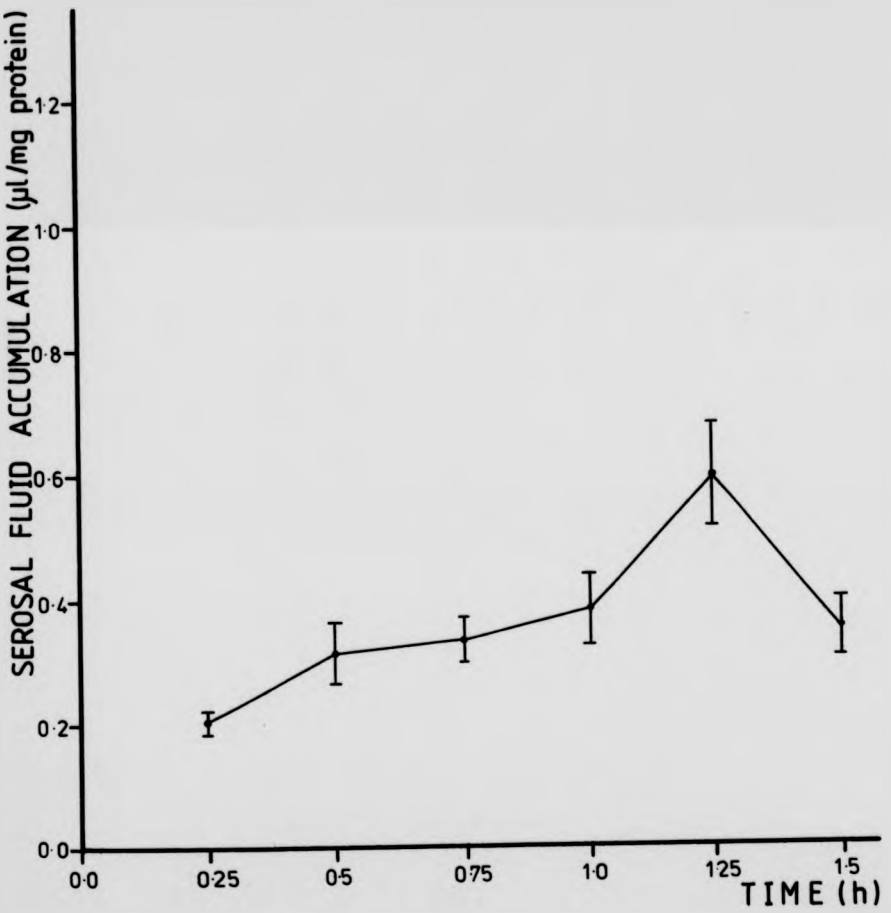


FIGURE 5.14

ACCUMULATION OF ^{125}I-LABELLED HPMA COPOLYMER 39e IN THE SEROSAL FLUID OF RAT JEJUNAL SAC

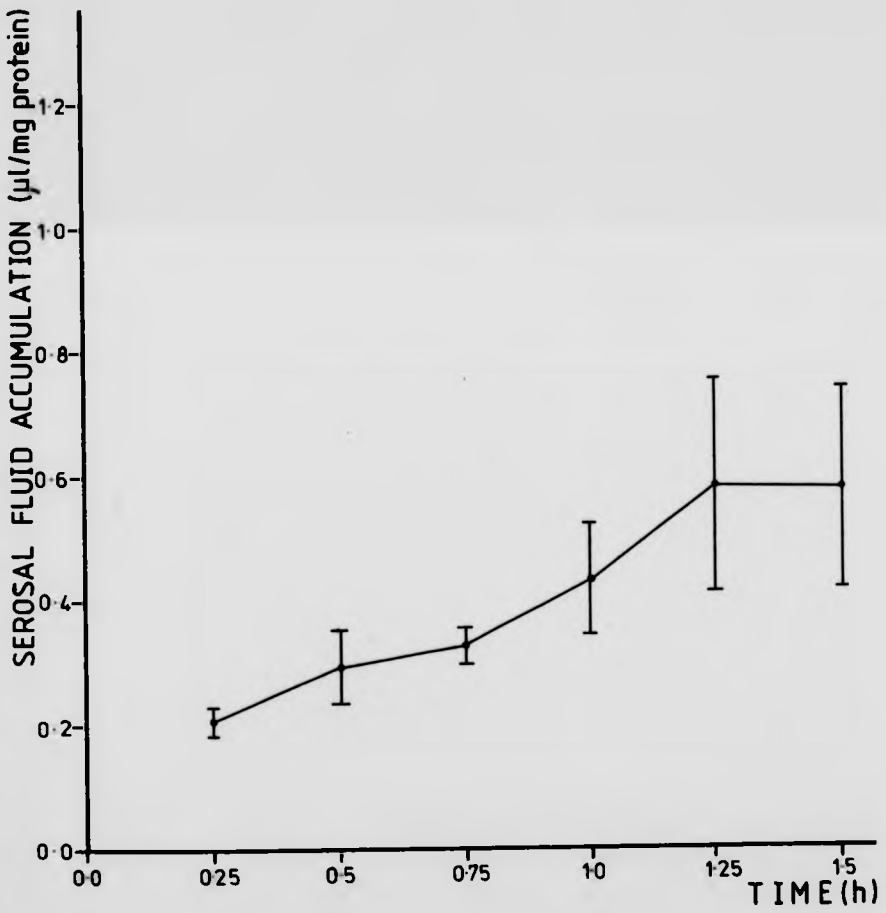


FIGURE 5.15

ACCUMULATION OF ^{125}I-LABELLED H P M A COPOLYMER 39
IN THE SEROSAL FLUID OF RAT JEJUNAL SAC

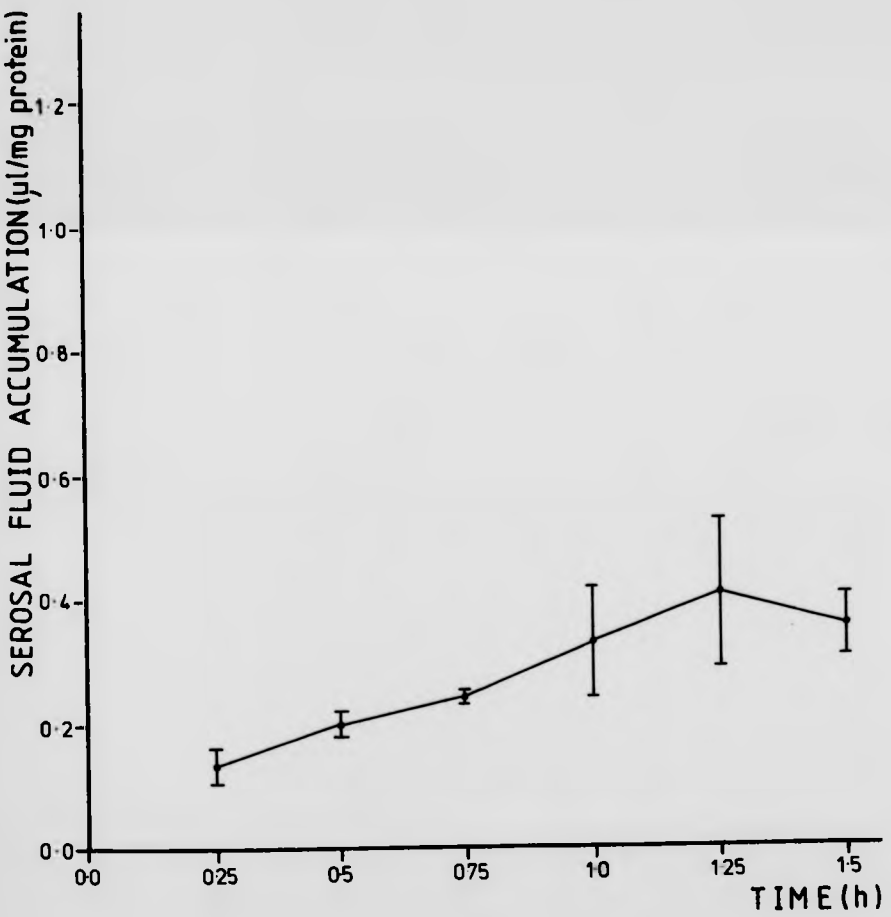
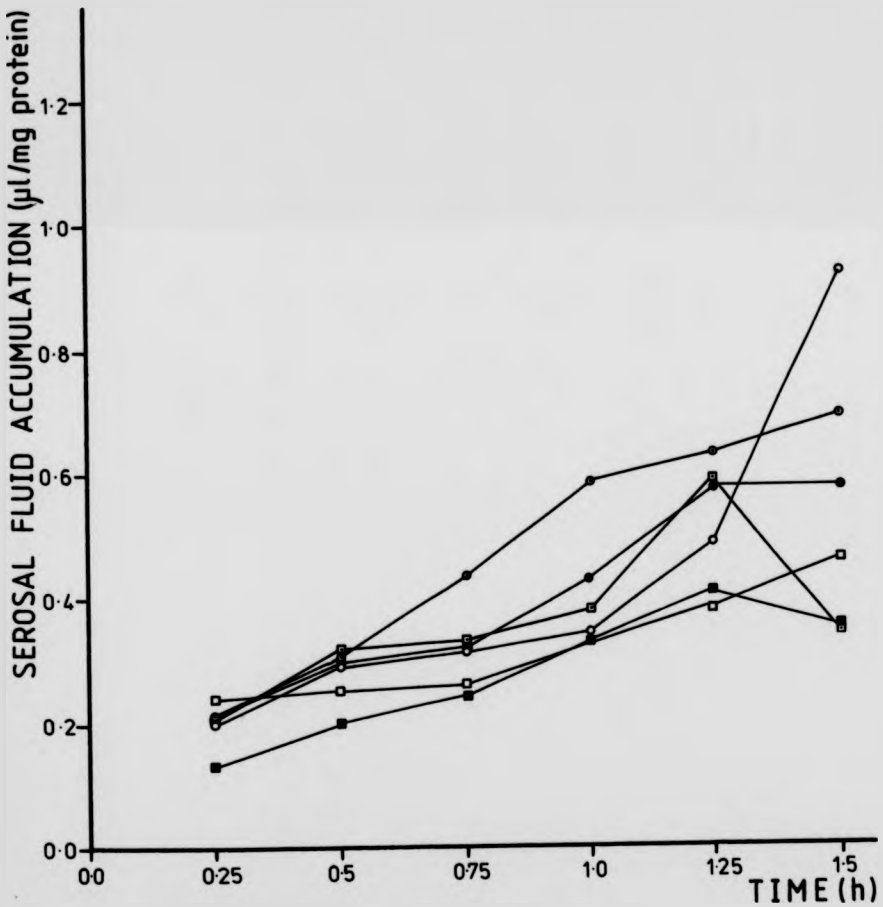


FIGURE 5.16

ACCUMULATION OF ^{125}I-LABELLED H P M A COPOLYMER FRACTIONS IN THE SEROSAL FLUID OF RAT JEJUNAL SAC



KEY	
DATA POINT	COPOLYMER
■	39
○	39a
□	39b
●	39c
▣	39d
●	39e

FIGURE 5.17

ACCUMULATION OF ^{125}I-PVP IN THE SEROSAL FLUID OF THE RAT JEJUNAL SAC

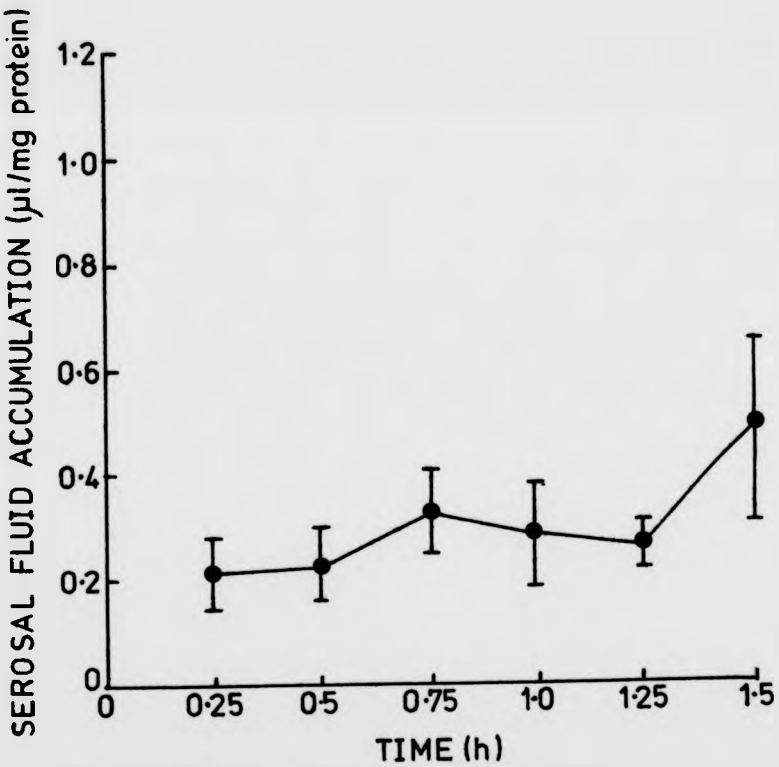


FIGURE 5.18

disposable Sephadex G-25 PD 10 columns (section 2.5). A typical elution profile is shown in fig. 5.19 and the results are summarized in Table 5.2.

Between 40-64% of the radioactivity recovered from the serosal fluids was found to be present in the first peak (fig. 5.19 and Table 5.2), indicating the movement of macromolecules across the intestine. The amount translocated was independent of the molecular size of the polymeric substrate. It was not possible, however, to identify the component(s) of the second peak of radioactivity (fig. 5.19) using Sephadex G-25 PD 10 columns. Further chromatography experiments were therefore carried out using Sephadex G-15 in order to identify radiolabelled degradation products. Because the total radioactivity present in the serosal fluids was so low in all cases, G-15 columns were prepared of bed volume 15 ml and bed height 25 cm in order to minimise the elution volume following application of serosal samples. A typical elution profile is shown in fig. 5.20 and from this it can be seen that two peaks elute after the polymer peak. The second and third peaks were found to co-elute with $[^{125}I]$ iodide and $[^{125}I]$-iodotyrosine respectively, indicating the presence of low molecular weight radiolabelled polymer degradation products in the serosal fluid. The results from duplicate experiments are shown in Table 5.3 and the value for each peak is expressed as a percentage of the total radioactivity recovered from the serosal fluid in each case.

On comparing Tables 5.2 and 5.3, it is apparent that the values presented for the proportion of polymer present in the serosal fluid, particularly in the case of copolymer 39b,

SEPHADEX G-25 CHROMATOGRAPHY OF SEROSAL FLUID FOLLOWING INCUBATION OF ^{125}I-LABELLED COPOLYMER 39e WITH JEJUNAL SACS

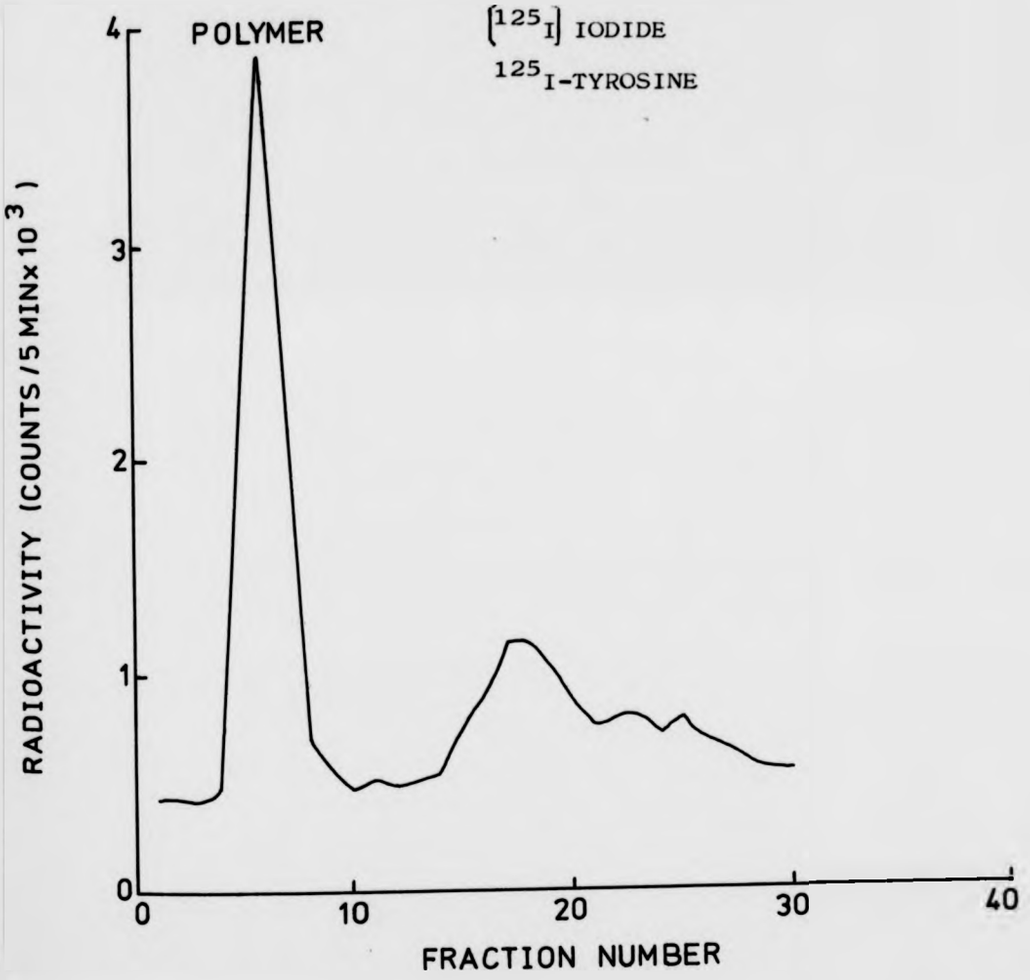


FIGURE 5.19

TABLE 5.2

SEPHADEX G-25 COLUMN CHROMATOGRAPHY OF SEROSAL FLUID FOLLOWING INCUBATION OF COPOLYMER 39 FRACTIONS WITH EVERTED JEJUNAL SACS FOR 1.5H

POLYMER CODE NO.	POLYMER (1ST PEAK) %	L.M.W. (2ND PEAK) %
39a	59	41
39b	40	60
39c	64	36
39d	50	50
39e	40	60

Results are expressed as the % of the total counts recovered from the column.

SEPHADEX G-15 CHROMATOGRAPHY OF SEROSAL FLUID FOLLOWING INCUBATION OF ^{125}I-LABELLED COPOLYMER 39c WITH JEJUNAL SACS

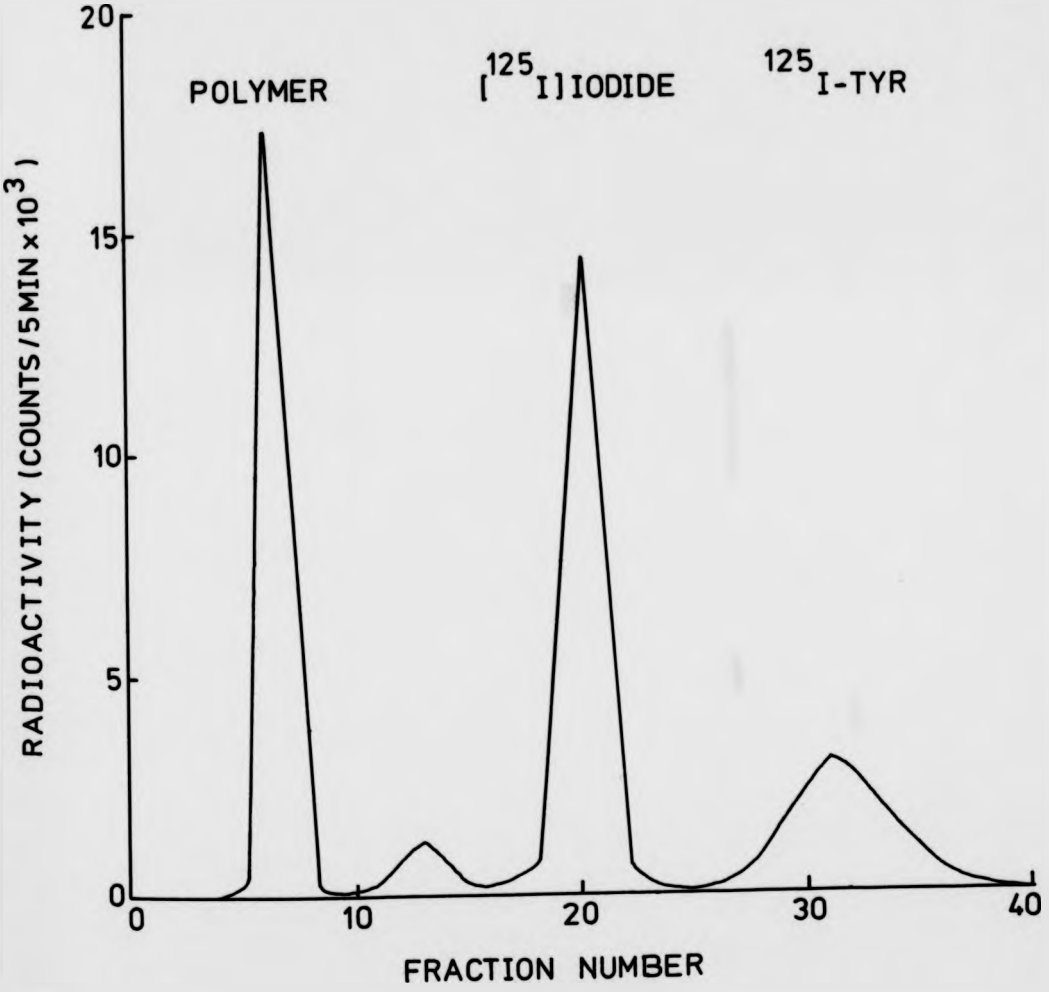


FIGURE 5.20

TABLE 5.3

SEPHADEX G-15 COLUMN CHROMATOGRAPHY OF SEROSAL FLUID FOLLOWING 1.5H INCUBATION WITH POLYMER 39 AND FRACTIONS

POLYMER CODE NO.	SEROSAL FLUID			% $[^{125}I]$ IODIDE IN MEDIUM (a)
	% POLYMER	% $[^{125}I]$ IODIDE	% ^{125}I-TYR	
39	60.32	15.63	18.06	2.49
	54.00	20.89	7.79	2.42
39a	37.04	42.63	17.51	4.85
	28.64	20.48	19.88	4.76
39b	11.06	74.99	17.23	11.00
	12.24	74.71	11.69	12.86
39c	63.78	19.68	13.93	6.99
	65.80	18.87	10.92	7.12
39d	39.42	36.61	21.86	3.30
	42.95	40.08	16.30	3.38
39e	40.81	25.00	30.58	6.19
	30.55	39.38	25.88	5.73

Results are expressed as a % of the radioactivity recovered from the column.

(a) These values represent the levels of free $[^{125}I]$ iodide present in the outer culture medium at the beginning of each experiment and are included for comparison.

differ to some extent. This can be explained by considering the quantity of free [^{125}I] iodide present in the external medium at the beginning of each experiment (see column 4, Table 5.3). The greater the quantity of free [^{125}I] iodide present in the outer medium, the large the peak due to [^{125}I] iodide in the serosal fluid, giving rise to a larger proportion of the total radioactivity present. Consequently, the proportion of radiolabelled polymer present appears to be less than that reported when using G-25 (Table 5.2). The radiolabelled copolymer preparations used in earlier experiments utilizing G-25 chromatography did not contain so much free [^{125}I] iodide since they had not been stored for long periods at 4^{o}C (Chapter 3), and the proportion of radiolabelled polymer present in the serosal fluid is consequently higher, and probably represents a more accurate picture of the true cellular events. G-25 chromatography, however, gave no indication of the proportions of [^{125}I] iodide and [^{125}I] iodotyrosine present in the serosal fluid following incubation with copolymer fractions. The possible routes of entry of the radiolabelled species to the serosal fluid is discussed in section 5.2.3.

5.2.3 Total Pinocytic Uptake of Copolymer 39 Fractions by the Rat Jejunal Sac

The presence of [^{125}I] iodotyrosine in the serosal fluid following incubation of jejunal sacs with radiolabelled copolymer fractions was described in section 5.2.2. This indicated that some degradation of copolymer had occurred during the 1.5h incubation period, but, in order to calculate the total pinocytic uptake of copolymers by this tissue, it is also necessary to investigate the possible release of

radiolabelled degradation products from the tissue (following pinocytosis) back into the culture medium. In order to do this, two jejunal sacs were incubated in each flask with radiolabelled substrate (125 µg/ml) for 1.5h at 37°C. The tissue was assayed for radioactivity and protein content and samples (1 ml) of culture medium were applied to a Sephadex G-15 column and eluted with 0.02M sodium acetate in order to detect any ^{125}I-labelled low molecular weight degradation products (sections 2.5 and 2.9). Fig. 5.21 shows an elution profile typical of all those obtained, and Table 5.4 summarises the results of these experiments.

This particular series of experiments was carried out concurrently (in duplicate) with those described in the previous section describing column chromatography of the serosal fluid following incubation of jejunal sacs with ^{125}I-labelled copolymer 39 fractions. It can be seen from Table 5.4 that the lower percentage of polymer recovered from the column for copolymer 39b was due to the high level of free [^{125}I] iodide in the pre-culture medium. Table 5.4 shows that very little [^{125}I] iodotyrosine was recovered from the culture medium in all cases over the 1.5h incubation period. The highest value of 0.62% (1.26 µl/mg protein) was observed for copolymer 39e, but the values obtained as a whole do not indicate that the extent of degradation is dependent on the molecular size of the substrate.

From these experiments, however, we cannot conclude that this low level of copolymer degradation is due to enzymatic hydrolysis within lysosomes following pinocytosis of copolymers with subsequent release of [^{125}I] iodotyrosine from cells back

SEPHADEX G-15 CHROMATOGRAPHY OF CULTURE MEDIUM FOLLOWING INCUBATION OF ^{125}I-LABELLED COPOLYMER 39c WITH JEJUNAL SACS

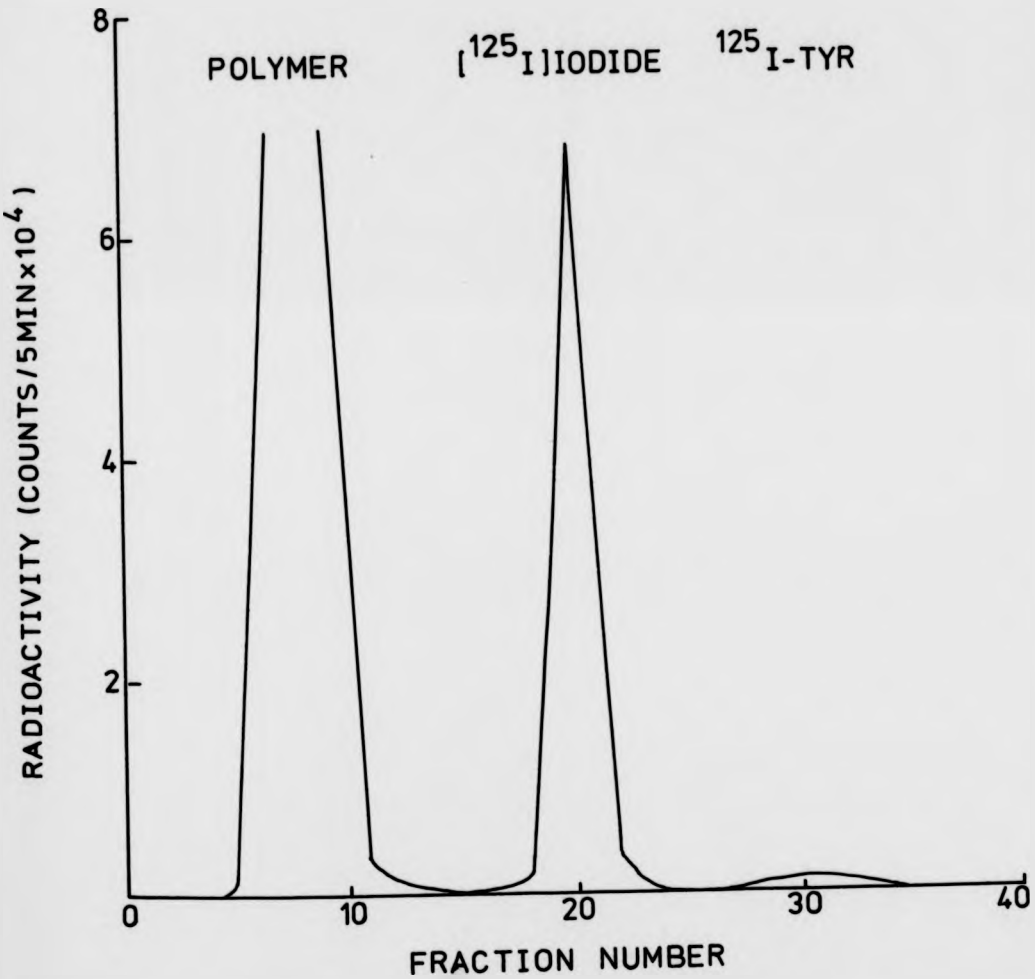


FIGURE 5.21

TABLE 5.4

DEGRADATION OF POLYMER 39 & FRACTIONS.

SEPHADEX G-15 COLUMN CHROMATOGRAPHY OF MEDIUM SAMPLES

AFTER 1.5H INCUBATION WITH GUT SACS

Polymer Code No.	% recovery of radioactivity	% Polymer	% [^{125}I] Iodide	% ^{125}I-tyr	^{125}I-tyr (µl/mg/1.5h)
39	100.00	97.24	2.49	ND	
	100.00	97.38	2.42	0.11	0.22
39a	100.00	94.34	4.85	0.35	0.77
	98.99	90.21	4.76	0.21	0.41
39b	90.37	74.90	11.00	0.30	0.83
	94.55	81.21	12.86	0.26	0.69
39c	95.31	85.55	6.99	0.20	0.43
	100.00	91.95	7.12	0.36	0.56
39d	100.00	96.28	3.30	0.27	0.60
	100.00	95.84	3.38	0.25	0.62
39e	100.00	93.27	6.19	0.41	0.96
	100.00	93.31	5.73	0.62	1.26

into the medium, since the tissue possesses an array of hydrolytic enzymes on the apical membrane of its epithelial cells (the brush border), some of which are shed from the tissue into the gut lumen in vivo (Bossman & Haschen 1983). It is therefore possible that enzymes may be shed from the brush border into the external culture medium by everted jejunal sacs.

Further experiments were carried out using 'conditioned' medium, to examine this possibility. Two everted jejunal sacs were incubated in each flask for 1.5h at 37°C in the absence of radiolabelled substrate on the assumption that any enzymes released during this period would be shed into the culture medium. The sacs were then removed from the medium, and labelled substrate (125 μg/ml) added and incubated for a further 1.5h. Samples of medium (1 ml) were then applied to the same Sephadex G-15 column and eluted with 0.02M sodium acetate. The results of the column chromatography of this conditioned medium are shown in Table 5.5, and when compared with Table 5.4, it can be seen that in all cases the levels of $[^{125}I]$ iodotyrosine detected in the conditioned medium are equal to, or even slightly greater than, those obtained on incubation of the radiolabelled copolymers with jejunal sacs. It can be concluded therefore that if intracellular degradation of copolymers takes place, it does not lead to the release of degradation products back into the external medium over a 1.5h incubation period. Since it has been shown that the copolymers are stable in medium at 37°C (Chapter 3), the low levels of $[^{125}I]$ iodotyrosine detected in the external medium must be due to extracellular degradation of copolymers by enzymes released by the tissue, or present

TABLE 5.5

EXTRACELLULAR DEGRADATION OF POLYMER 39 & FRACTIONS.
SEPHADEX G-15 COLUMN CHROMATOGRAPHY OF MEDIUM SAMPLES
AFTER 1.5H INCUBATION IN CONDITIONED MEDIUM

Polymer Code No.	% recovery radioactivity	% Polymer	% $[^{125}I]$ iodide	% ^{125}I-tyr	^{125}I-tyr (µl/mg/1.5h)
39	94.00	97.97	1.58	0.23	0.46
	100.00	97.66	1.96	0.25	0.45
39a	97.70	94.30	4.80	0.34	0.66
	85.70	93.40	5.70	0.54	0.87
39b	92.50	86.80	12.60	0.27	0.53
	91.00	75.60	11.00	0.25	0.60
39c	100.00	89.99	6.70	0.39	0.76
39d	89.50	80.24	2.17	0.31	0.53
	97.10	92.53	2.52	0.30	0.55
39e	98.10	95.06	4.03	0.56	1.18
	97.50	91.30	4.79	0.64	1.16

at the cell surface during the experimental period.

The fact that [^{125}I] iodotyrosine is present in the external medium following incubation of copolymers with jejunal sacs gives rise to the possibility that the [^{125}I] iodotyrosine present in the serosal fluid may have been translocated from the external medium across the tissue by a mechanism other than pinocytosis, possible by active transport or simple diffusion. Furthermore, the apparently high levels of free [^{125}I] iodide in the serosal fluid may have been actively transported across the tissue, or cleaved from radiolabelled molecules by an iodinase and transported, or it also may have diffused across the tissue.

In an attempt to establish the possible routes of entry of the different radiolabelled molecules into the serosal fluid, calculations were performed which compared the number of radioactive counts present in each peak present in the serosal fluid following incubation with the copolymer fractions, with the counts present in an equivalent volume of culture medium surrounding the jejunal sacs. For example, the total number of radioactive counts due to [^{125}I] iodide present in the medium at the beginning of an experiment to determine uptake and transport of copolymer 39b was 73,996 cpm in 10 ml (11% of the total counts present). The volume of the serosal fluid was approximately 0.8 ml. One would therefore expect to find up to 5920 cpm present in the serosal fluid due to the diffusion of iodide. The actual number of counts recovered due to [^{125}I] iodide was in fact less than this value (2402 cpm or 36.20% of the expected value). Table 5.6 summarises the results of these calculations on the contents of the serosal fluids, and results are expressed as a percentage of the value

TABLE 5.6

SEPHADEX G-15 COLUMN CHROMATOGRAPHY OF SEROSAL FLUID
(Values expressed as % of radioactivity present in an equivalent volume of external culture medium)

POLYMER CODE NO.	% OF COUNTS PRESENT IN EQUIVALENT EXTERNAL MEDIUM		
	POLYMER	[^{125}I] IODIDE	^{125}I-TYR
39 (un)	0.41	4.40	115.09
	1.63	23.04	188.72
39a	1.09	23.59	137.30
	1.52	20.75	429.08
39b	0.89	18.36	63.01
	0.84	32.60	235.86
39c	0.67	30.18	99.18
	0.69	29.70	172.00
39d	1.08	25.72	229.66
	1.26	34.21	169.70
39e	1.97	18.17	338.77
	0.85	18.16	109.72

expected for equilibration as described above.

Table 5.6 shows that the quantity of iodide present in the serosal fluid in all cases is less than that present in an equivalent volume of external culture medium (4-34%). This observation suggests that the mechanism of transport is probably diffusion. This finding in conjunction with Table 5.3 illustrates the need to check the levels of contaminating $[^{125}I]$ iodide in radiolabelled preparations prior to use when stored for periods longer than one month (Chapter 3).

From Table 5.6 it can be seen that, in all cases, the concentration of polymer in the serosal fluid represents $< 2\%$ of the concentration in the outer medium. These values are much lower than those observed for $[^{125}I]$ iodide and $[^{125}I]$-iodotyrosine. The only possible routes which could account for this movement are leakage and vesicular transport, since it may safely be assumed that macromolecules are too large to diffuse across the tissue (Chapter 1). Leakage through the tied ends of the sacs was investigated by Bridges (section 5.1), and subsequent experiments (section 5.2.6) in the present study support the finding that everted jejunal sacs do not leak. It is therefore reasonable to conclude that macromolecules are transported across the mucosa by pinocytosis followed by exocytosis, and subsequently move across the tissue either intracellularly (by pinocytosis) or intercellularly.

It is apparent from Table 5.6 that $[^{125}I]$ iodotyrosine is present (in all but one case) at a greater concentration in the serosal fluid than in the outside medium following incubation with jejunal sacs for 1.5h. Since the

$[^{125}I]$ iodotyrosine in the outer medium can be accounted for by extracellular or brush border enzymatic cleavage, it seems likely that the levels detected in the serosal fluid are transported across the tissue following copolymer degradation within the tissue. If the $[^{125}I]$ iodotyrosine present in the serosal fluid was the result of active transport of free $[^{125}I]$ iodotyrosine generated by extracellular or brush border enzyme activity, one would expect the levels in the outer medium to be less than those observed on using conditioned medium.

Further experiments using G-15 column chromatography of serosal fluids following incubation with freshly labelled copolymer preparations were not carried out, since the Sephadex G-15 chromatography using small columns proved both difficult and time-consuming. Sephadex G-25 column chromatography (see earlier) was carried out using fresh preparations, and the results obtained for transport of macromolecules were therefore a more accurate representation of the true levels transported, since a low concentration of $[^{125}I]$ iodide was present in the radiolabelled copolymer preparations.

Given the assumptions discussed above, the total pinocytic uptake of copolymers by jejunal sacs over the experimental period is given by the sum of the tissue and serosal accumulation of copolymer, and any degradation products released following lysosomal hydrolysis of copolymer. Since it is probable that the only intracellular degradation that takes place results in the accumulation of $[^{125}I]$ iodotyrosine in the serosal fluid, pinocytic uptake is taken to be the sum

of the accumulation of radiolabelled substrate in the jejunal tissue, and that transported into the serosal fluid. On making this assumption, it must be noted that values given for the serosal accumulation of radioactivity include those counts due to the presence of iodide. The calculations necessary to correct for this error are complicated and hardly significant in terms of the possible differences which would be observed in total pinocytic uptake values where the majority of the pinocytosed substrate is present in the tissue after a 1.5h incubation period.

Table 5.7 shows the pinocytic uptake of ^{125}I-labelled copolymer 39 fractions by the jejunal sac, and the values obtained for ^{125}I-PVP control experiments. The results of all experiments were subjected to a linear regression analysis and Table 5.8 indicates the correlation coefficient and the slope of the regression line (the rate of uptake) for each substrate. The sum of the rate of uptake from the incubation medium by the tissue and the rate of appearance of substrate in the serosal fluid is also shown for each substrate in Table 5.7, and it can be seen that because the rates of accumulation of substrate in the serosal fluid are low, the total rates of pinocytic uptake of copolymer 39 by this tissue resemble those given for tissue accumulation alone. There is an increase in the rate of pinocytosis with increasing molecular size by everted rat jejunal sacs.

TABLE 5.7

UPTAKE OF ^{125}I-LABELLED POLYMER 39 AND FRACTIONS BY ADULT RAT INTESTINE AFTER 1.5H INCUBATION

Polymer	Tissue Accumulation (µl/mg) (T)	Serosal Fluid Accumulation (µl/mg) (Total) (S)	Total (T + S) (µl/mg)
39a	4.51 ± 0.55	0.93 ± 0.25	5.44
39b	2.60 ± 0.37	0.47 ± 0.07	3.07
39c	1.84 ± 0.25	0.70 ± 0.14	2.54
39d	1.51 ± 0.39	0.33 ± 0.05	1.84
39e	1.32 ± 0.16	0.58 ± 0.16	1.90
39	1.37	0.45	1.82
PVP	1.53 ± 0.17	0.48 ± 0.18	2.01

TABLE 5.8

RATE OF UPTAKE OF ^{125}I-LABELLED COPOLYMER FRACTIONS AND ^{125}I-PVP BY THE EVERTED JEJUNAL SAC

POLYMER CODE NO.	$\bar{M}w$	TISSUE ACCUMULATION (µl/mg/h)	CORRELATION COEFFICIENT	SEROSAL ACCUMULATION (µl/mg/h)	CORRELATION COEFFICIENT	TOTAL RATE OF ACCUMULATION (T+S) (µl/mg/h)
39	190,000	0.86	0.85	0.29	0.78	1.15
39a	>400,000	3.22 ± 0.36	0.99	0.49 ± 0.13	0.87	3.71
39b	400,000	1.29 ± 0.24	0.94	0.17 ± 0.01	0.94	1.46
39c	150,000	0.98 ± 0.22	0.98	0.41 ± 0.09	0.99	1.15
39d	110,000	0.80 ± 0.17	0.86	0.19 ± 0.02	0.68	0.87
39e	34,000	0.61 ± 0.12	0.95	0.33 ± 0.12	0.98	0.94
PVP	~ 40,000	0.66 ± 0.21	0.96	0.15 ± 0.01	0.74	0.81

5.2.4 The Effect of Temperature on the Pinocytic Uptake of Copolymer 39 Fractions by the Jejunal Sac

Pinocytic uptake experiments using copolymer fractions 39a and 39e were performed exactly as described in previous sections, but at 15°C, with control experiments using the same substrates at 37°C. The incubation period for all experiments was 1.5h. Results are expressed for each time point in µl/mg protein as a mean of the uptake values obtained from four jejunal sacs. The experimental results are summarised in figs. 5.22 and 5.23 and Table 5.9. From Table 5.9 it can be seen that at 15°C the uptake of copolymer 39a ($\bar{M}w$ >400,000) by the tissue is only 13% of that observed at 37°C for the same substrate, and that serosal accumulation of radioactivity is only 26% of the 37°C values. Copolymer 39e ($\bar{M}w$ 34,000) enters the tissue and serosal fluid at 52% and 33% respectively the normal rate at 15°C. These results show that uptake is a metabolic process which cannot proceed efficiently at reduced temperatures and precludes the possibility that the progressive accumulation of substrate by the tissue at 37°C is due to substrate binding to the surface of the tissue without subsequent internalization.

5.2.5 Pinocytic Uptake of Copolymer 68 by the Rat Jejunal Sac

The experimental procedure to determine the pinocytic uptake of radiolabelled copolymer 68 ($\bar{M}w$ 171,000) was exactly as described above. Jejunal sacs were incubated with substrate (125 µg/ml) for 1.5h. The experiment was performed three times with matched ^{125}I-PVP controls and the result of both tissue and serosal accumulation of the copolymer is shown in fig. 5.24. The rates of uptake of radioactive substrate by the tissue and into the serosal fluid were 0.97 and 0.35 µl/mg/h

FIGURE 5.22

ACCUMULATION OF ^{125}I-LABELLED COPOLYMER 39a BY THE RAT JEJUNAL SAC AT 15°C AND 37°C

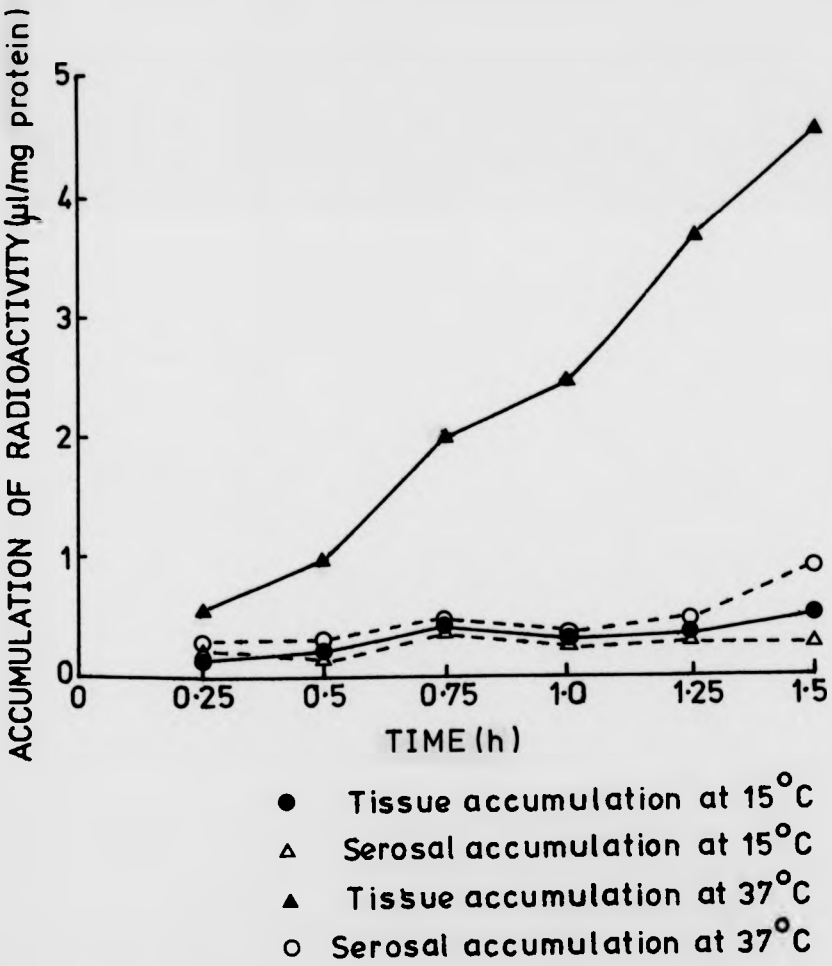


FIGURE 5.23

ACCUMULATION OF ^{125}I-LABELLED COPOLYMER 39e BY THE RAT JEJUNAL SAC AT 15°C AND 37°C

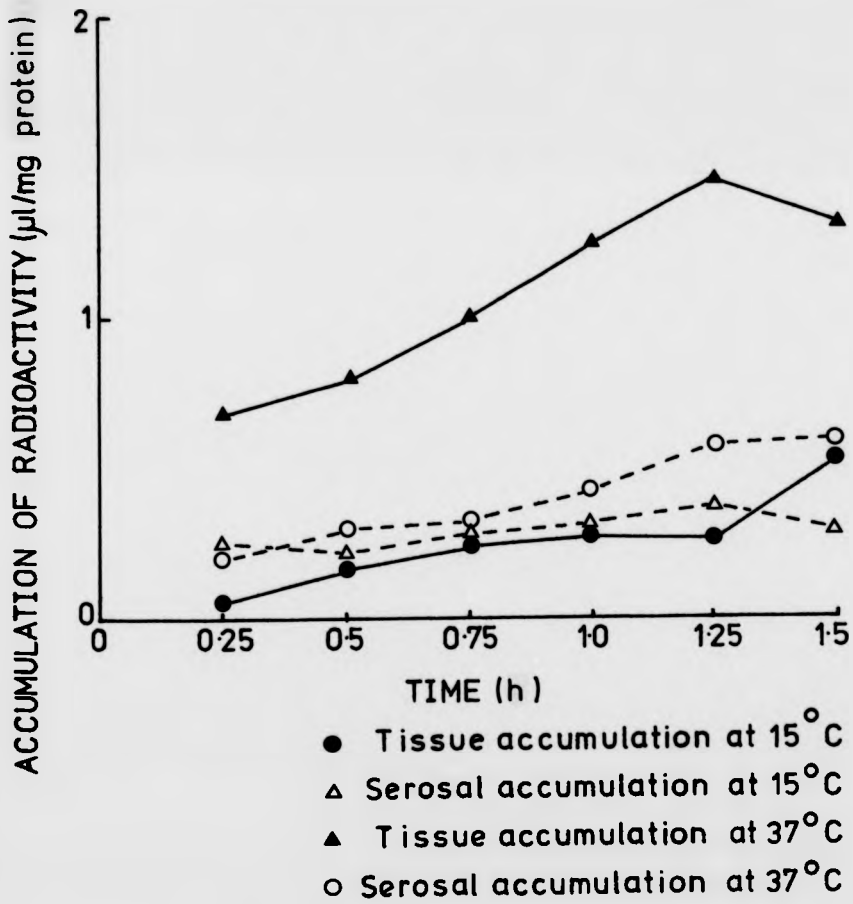


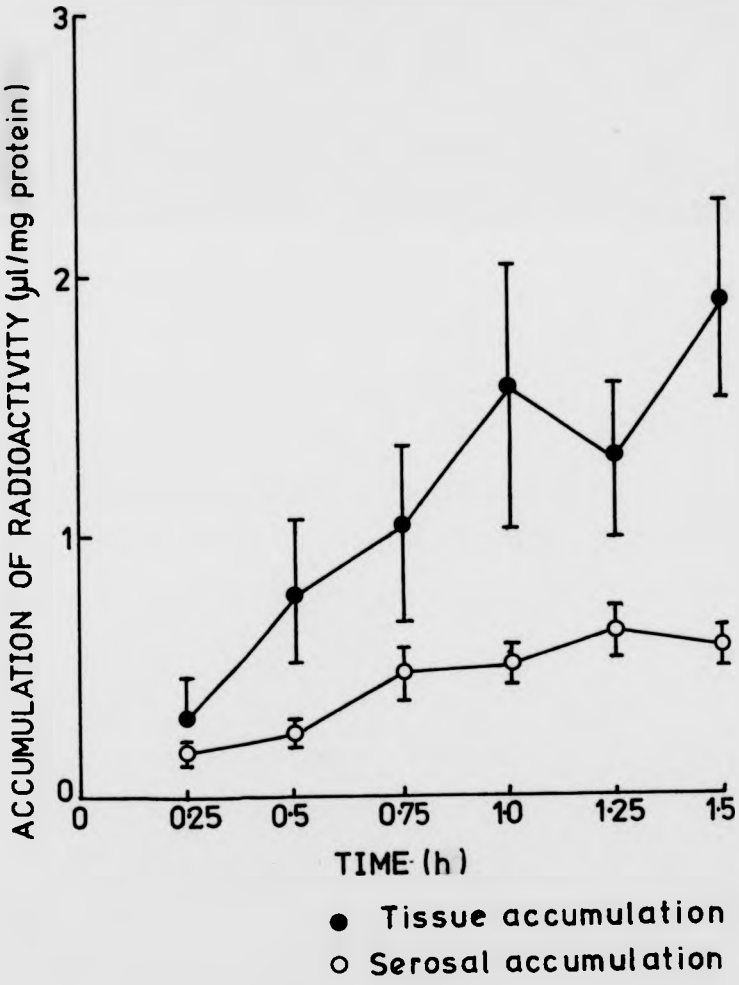
TABLE 5.9

THE UPTAKE OF COPOLYMERS 39a AND 39e BY JEJUNAL SACS AT 15°C

POLYMER CODE NO.	TISSUE ACCUMULATION (µl/mg/h)	% OF 37°C CONTROL	SEROSAL ACCUMULATION (µl/mg/h)	% OF 37°C CONTROL
39a	0.42	13	0.13	26
39e	0.32	52	0.11	33

FIGURE 5.24

ACCUMULATION OF ^{125}I-LABELLED COPOLYMER 68 BY THE RAT JEJUNAL SAC



respectively, giving a total rate of uptake of 1.31 µl/mg/h. This value is similar to the rate obtained for unfractionated copolymer 39 (1.82 µl/mg/h) which is of approximately the same average molecular weight (190,000) and polydispersity. In fact, the slightly larger copolymer (39) has a slightly higher rate of uptake which is to be expected in view of the substrate size effect in this tissue (section 5.2.1).

Previous experiments using the rat visceral yolk sac (section 4.2.5) have shown that [^{125}I] iodotyrosine is not released from the copolymer backbone following pinocytic uptake of this substrate.

Since the radiolabelling efficiency of this copolymer was consistently low (section 3.3), the number of radioactive counts recovered from the serosal fluid was correspondingly low for the substrate concentration used in these experiments. For this reason, the substrate was dialysed to remove contaminating [^{125}I] iodide immediately before use in uptake experiments, and radioactive counts present in the serosal fluid following incubation of copolymer 68 with jejunal sacs were assumed to be due to radiolabelled macromolecules whose crosslinks may or may not have undergone cleavage during their transport across the tissue. Since [^{125}I] iodotyrosine is not released from this substrate, and free [^{125}I] iodide levels, in theory, should be insignificant, this seemed a reasonable assumption to make. Both Sephadex G-15 (to detect iodide) and Sepharose 4B/6B (to detect changes in molecular weight distribution) ideally should have been carried out on serosal fluids, but the necessary dilution of the samples to achieve separation of peaks would have been too great for the radioactive counts present to provide meaningful results.

5.2.6 Pinocytic Uptake of Copolymer 39 by Loops of Jejunal Tissue

This experiment was carried out to prove that macromolecules actually pass across the intestine, rather than gaining entrance to the serosal fluid through the tied ends of the jejunal sacs, which remain immersed in the culture medium during the experimental period.

Three everted jejunal sacs were prepared as described previously (section 2.7), but on this occasion were of approximately 10 cm in length (rather than 3-4 cm). Each sac was placed in a culture flask containing medium as shown in fig. 5.25, with the tied ends of the sacs suspended by cotton and remaining above the culture medium throughout the experimental period. Each flask was then incubated for 1.5h at 37°C with 125 µg/ml of ^{125}I-labelled copolymer 39 (unfractionated) exactly as described in earlier experiments.

The results were expressed in µl/mg protein giving the total pinocytic uptake over 1.5h and are shown in Table 5.10. Also shown in Table 5.8 for comparison are the average results obtained from previous experiments on incubation of sacs with ^{125}I-labelled unfractionated copolymer 39.

Sephadex G-25 column chromatography of the serosal fluids from two loops of tissue revealed that 34.77% and 22.2% of the radioactivity present in the serosal fluid was attached to macromolecules.

It must be stressed that this experiment shows only that macromolecules are transported across the rat jejunum, and that the actual quantities given do not represent the true

SCHEMATIC REPRESENTATION OF THE TECHNIQUE USED FOR THE INCUBATION OF ^{125}I-LABELLED COPOLYMER 39 WITH LOOPS OF JEJUNAL TISSUE

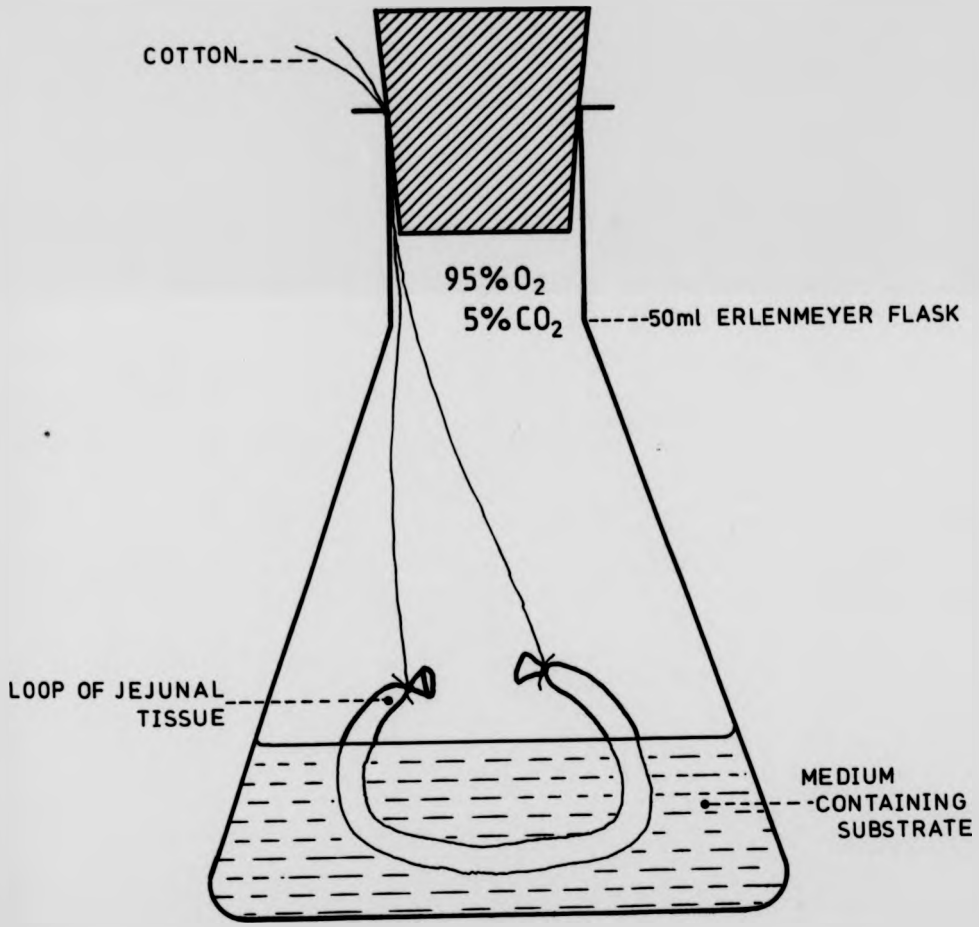


FIGURE 5.25

SCHEMATIC REPRESENTATION OF THE TECHNIQUE USED FOR THE INCUBATION OF ^{125}I-LABELLED COPOLYMER 39 WITH LOOPS OF JEJUNAL TISSUE

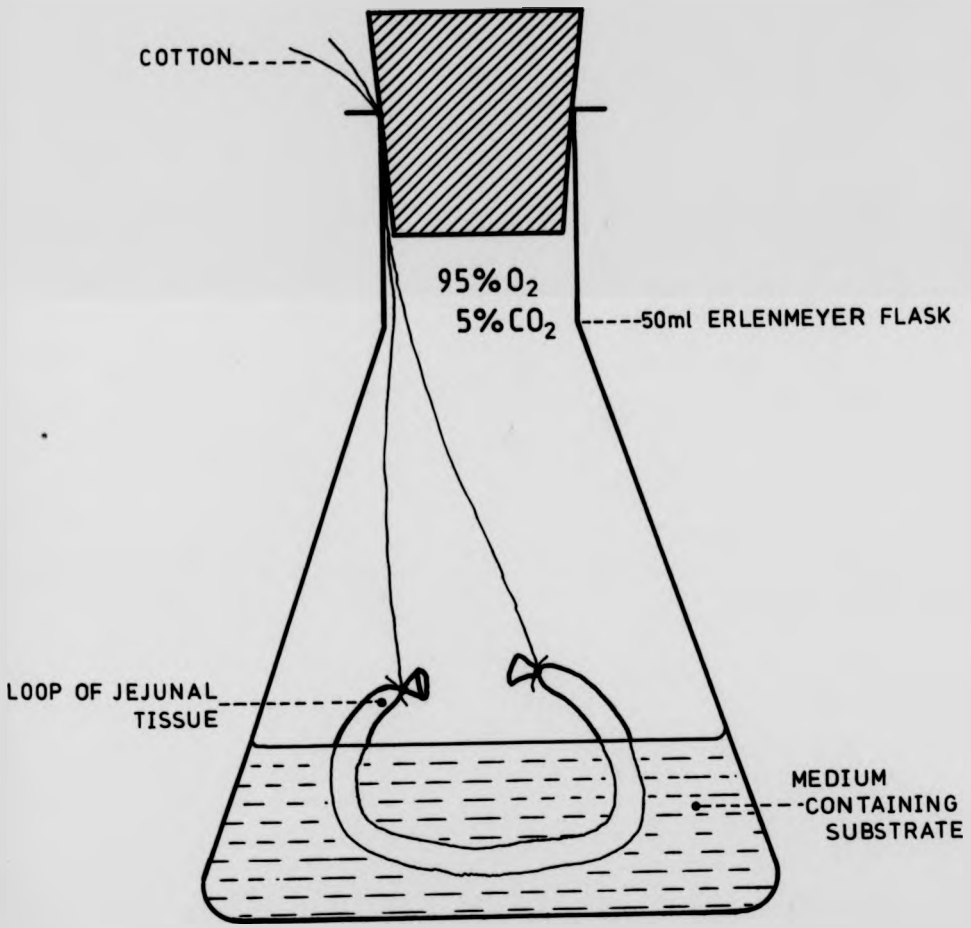


FIGURE 5.25

TABLE 5.10

PINOCYTIC UPTAKE OF ^{125}I-LABELLED COPOLYMER 39 BY LOOPS OF JEJUNAL TISSUE

JEJUNAL TISSUE	TISSUE ACCUMULATION (µl/mg/1.5h)	SEROSAL ACCUMULATION (µl/mg/1.5h)	TOTAL UPTAKE (µl/mg/1.5h)
Loop 1	0.66	0.36	1.02
Loop 2	0.51	0.18	0.68
Loop 3	0.76	0.25	1.01
Sac	1.38 $\pm$ 0.16	0.45 $\pm$ 0.01	1.82

rates of uptake, since only approximately 50% of the tissue was in contact with the medium containing the substrate during the experimental period. The results are expressed in terms of µl/mg protein contained in the whole sac, and are therefore a gross underestimate of the true uptake values.

5.3 DISCUSSION

The rate of uptake of the crosslinked copolymer 39 by jejunal tissue was dependent upon the size of the copolymer used. There was an increase in the rate of tissue accumulation with larger mean molecular weight of the copolymers. This was clearly demonstrated in the case of copolymer 39a ($\bar{M}w$ >400,000), where the rate of tissue accumulation was 489% of matched ^{125}I-PVP ($\bar{M}w$ 40,000) control experiments (Table 5.11 and fig. 5.9). ^{125}I-PVP is an accepted marker of fluid-phase pinocytosis both in the rat jejunum and the rat visceral yolk sac. With the exception of copolymer 39e ($\bar{M}w$ 34,000), a slightly smaller molecule than ^{125}I-PVP, it can be seen from Table 5.11 that all copolymer fractions enter the tissue at a higher rate than the fluid-phase substrate. This observation is the opposite of that observed using the same substrates in the rat visceral yolk sac system (the results of which are also shown in Table 5.11) where all larger copolymer fractions enter the tissue at a lower rate than ^{125}I-PVP.

Table 5.11, however, only represents the rate of accumulation of substrate by the tissue during the experimental period. It does not account for substrate which has been pinocytosed by the tissue and degraded by lysosomal enzymes with subsequent release of low molecular weight radiolabelled degradation products, or the transport of substrate across the tissue following its uptake by cells (in the case of the everted jejunal sacs).

Sephadex G-15 chromatography of the external culture medium following incubation of each of the fractions with jejunal sacs for 1.5h did not reveal the presence of any low molecular

TABLE 5.11

TISSUE ACCUMULATION OF ^{125}I-LABELLED POLYMER 39 FRACTIONS & POLYMER 68 IN RAT VISCERAL YOLK SACS AND ADULT RAT JEJUNUM

		Yolk sac			Jejunum		
Polymer code	$\bar{M}w$	No. of experiments	Rate of accumulation (µl/mg/h)	% PVP control	No. of experiments	Rate of accumulation (µl/mg/h)	% PVP control
39	190,000	5	0.88 ± 0.12	49.40	2	0.86	130.30
39a	>400,000	3	0.33 ± 0.13	18.50	4	3.22 ± 0.36	489.40
39b	400,000	3	0.40 ± 0.13	22.30	4	1.29 ± 0.24	196.00
39c	155,000	3	0.55 ± 0.12	30.80	4	0.98 ± 0.22	149.10
39d	110,000	3	0.63 ± 0.11	35.00	4	0.80 ± 0.17	121.10
39e	34,000	3	1.74 ± 0.20	97.15	4	0.61 ± 0.12	92.50
PVP	~ 40,000	7	1.79 ± 0.15	100.00	4	0.66 ± 0.21	100.00
68	171,000	5	1.71 ± 0.16	95.64	3	1.01	152.68

weight degradation products attributable to hydrolysis by lysosomal enzymes with subsequent release of [^{125}I] iodotyrosine. By using conditioned medium, it was discovered that the small amount of [^{125}I] iodotyrosine present (1 µl/mg/1.5h or less) was the product of extracellular hydrolysis of susceptible bonds by enzymes present in the culture medium during the experiment. This finding also excluded the possibility that brush border enzymes present on the apical membranes of the enterocytes were responsible for the degradation observed.

All the crosslinked copolymer 39 fractions were translocated to some extent into the serosal fluid during the 1.5h incubation period (fig. 5.17), but the rate of transfer of radioactivity was independent of copolymer molecular weight, and all rates were much lower than those observed for the uptake into the tissue.

The total pinocytic uptake by the jejunal sac was therefore estimated by summing the radioactivity present in the tissue with that transported into the serosal fluid, or the total rate by summing the individual rates. Table 5.12 shows a summary of the total rates of uptake by the rat visceral yolk sac and the rat jejunum, and it can be seen that, when degradation by the yolk sac and serosal transfer by the jejunal sac has been accounted for, the patterns observed from the tissue levels remain the same i.e. in the yolk sac, the rate of pinocytosis increases with decreasing molecular size and in the jejunal sac the rate of pinocytosis increases with increasing molecular size. It is also apparent that ^{125}I-PVP enters the jejunum at half the rate it enters the yolk sac and that copolymer 39d ($\bar{M}w$ 110,000) is taken up at a similar rate by both tissues.

TABLE 5.12

RATES OF UPTAKE OF ^{125}I-LABELLED HPMA COPOLYMERS BY RAT VISCERAL YOLK SACS AND RAT JEJUNUM

		YOLK SAC		JEJUNUM			
POLYMER CODE NO.	$\bar{M}w$	RATE OF[a] UPTAKE	% OF[b] MATCHED CONTROL	RATE OF[c] TISSUE ACCUMULATION	RATE OF TRANSFER[d] INTO THE SEROSAL FLUID	TOTAL RATE[e] OF UPTAKE	% OF MATCHED CONTROL
39	190,000	1.27 (5)	71	0.86 (2)	0.29	1.15	142
39a	>400,000	0.48 (3)	27	3.22 (4)	0.49	3.71	458
39b	400,000	0.57 (3)	32	1.29 (4)	0.17	1.46	180
39c	150,000	0.79 (3)	44	0.98 (4)	0.41	1.15	142
39d	110,000	0.90 (3)	50	0.80 (4)	0.19	0.99	122
39e	34,000	2.50 (3)	140	0.61 (4)	0.33	0.94	116
PVP	~ 40,000	1.79 (7)	-	0.66 (4)	0.15	0.81	-

[a]Total uptake (µl/mg protein/h) assuming a rate of intracellular degradation equivalent to 44.09% of uptake per 5h. Number of experiments shown in parentheses.

[b]Rate of uptake expressed as a percentage of the rate of accumulation of ^{125}I-labelled PVP measured in matched control experiments.

[c,d]Rates of tissue accumulation by intestine and transfer to serosal fluid expressed in terms of µl/mg protein/h. Number of experiments shown in parentheses.

[e]Sum of the rates of tissue accumulation and serosal transfer (µl/mg protein/h).

TABLE 5.12

RATES OF UPTAKE OF ^{125}I-LABELLED HPMA COPOLYMERS BY RAT VISCERAL YOLK SACS AND RAT JEJUNUM

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39d	110,000	0.90 (3)	50	0.80 (4)	0.19	0.99	122
39e	34,000	2.50 (3)	140	0.61 (4)	0.33	0.94	116
PVP	~ 40,000	1.79 (7)	-	0.66 (4)	0.15	0.81	-

[a] Total uptake (µl/mg protein/h) assuming a rate of intracellular degradation equivalent to 44.09% of uptake per 5h. Number of experiments shown in parentheses.

[b] Rate of uptake expressed as a percentage of the rate of accumulation of ^{125}I-labelled PVP measured in matched control experiments.

[c,d] Rates of tissue accumulation by intestine and transfer to serosal fluid expressed in terms of µl/mg protein/h. Number of experiments shown in parentheses.

[e] Sum of the rates of tissue accumulation and serosal transfer (µl/mg protein/h).

The reason why the two morphologically similar absorptive epithelial tissues from the same species should behave in opposite ways remains obscure, but it is apparent that where the yolk sac discriminates against larger molecules, the rat intestine does not. The higher rates of uptake of larger molecules by the jejunal sac must be due to an adsorptive interaction between the substrate and the enterocyte membrane which intensifies with increasing molecular weight.

Studies by Duncan et al (1981) on the effect of molecular size of ^{125}I-PVP on its pinocytosis by rat visceral yolk sacs and rat peritoneal macrophages showed that these two cell types could discriminate between molecules of the same substrate with different molecular weight. Macrophages captured the highest mean molecular weight preparation of ^{125}I-PVP more rapidly whereas the rate of capture by the yolk sac decreased with increasing molecular weight (Chapter 4).

The possibility that the differences in rates of uptake with increasing substrate size in macrophages could be attributable to a substrate-induced change in the rate of vesicle formation was disproved by observing the rate of ^{125}I-PVP ($\bar{M}w$ 40,000) uptake in the presence of unlabelled preparations of various molecular sizes. Duncan et al postulated that the very large ($\bar{M}w$ 700,000 and 7,000,000) ^{125}I-PVP molecules have some affinity for the macrophage cell surface that is not manifest in their smaller counterparts, and that it was possible that the affinity of the vinylpyrrolidone residue for the plasma membrane is so slight as to be ineffective until the molecule can provide a sufficient number of attachment sites. This suggestion could perhaps also explain the interaction between

certain residues present on crosslinked HPMA copolymers and the enterocyte membrane.

The rate of transfer of radioactivity into the serosal fluid is much lower than that observed for pinocytic uptake of substrate into the tissue. This is probably due to the fact that the substrate molecules do not only have to cross the intestinal epithelium, but also several other layers of cells (including two muscle layers) and basement membranes, each of a different nature (section 5.1). The yolk sac similarly consists of several layers of cells, but is by no means as thick as the intestinal tissue (section 4.1). Jacques (1976) suggested that transport is the result of several selection steps at different levels, each of which must be overcome before the molecule can reach the serosal surface. It is impossible, however, to extrapolate the results obtained for transport across the intestine to an in vivo situation, where endocytosed material would presumably enter the blood circulation or lymphatic system and be transported to remote organs rather than traverse the whole intestinal tissue to the extent observed.

Sephadex chromatography of the serosal fluid revealed that the radioactivity recovered was due to the presence of ^{125}I-labelled copolymers, [^{125}I] iodide and [^{125}I] iodotyrosine. The presence of radiolabelled copolymers indicated that some macromolecule transport had occurred across the intestine and that potentially degradable oligopeptide crosslinks or intermolecular bridges within the molecules had somehow escaped enzymatic hydrolysis. Alternatively, hydrolysis of the crosslink could have taken place in one position only, resulting in the radiolabelled tyrosine residue remaining

attached to a polymer chain via an oligopeptide sidechain. A further possibility is the pinocytic transport across the enterocytes (and possibly subsequent layers of tissue) via a non-lysosomal route (diacytosis). This finding is consistent with observations made by Bridges (1980) and Beahon (1981) on the transport of degradable ^{125}I-HRP macromolecules across everted jejunal sacs.

[^{125}I] Iodotyrosine was found to be present in the serosal fluids following incubation of sacs with all copolymer 39 fractions, in quantities greater than could be accounted for by simple diffusion across the tissue. This probably indicates that pinocytosed copolymers were degraded to some extent by lysosomal enzymes present in the tissue and suggests a unidirectional flow of degradation products away from the gut lumen (mucosal surface). This would make sense in physiological terms, when the degradation products of macromolecules ingested by enterocytes might be passed on to the blood circulation or to other cells deeper in the tissue for utilization in anabolic processes. [^{125}I] iodotyrosine is not, however, re-incorporated into newly synthesized proteins but is excreted. It is also quite possible that a unidirectional flow of degradation products could occur in the yolk sac tissue in order to supply the growing embryo with a source of amino acids, but it would not be possible to demonstrate this in the 'open' yolk sac system, where both surfaces of the yolk sac tissue are in contact with the same culture medium.

For two reasons, it was not possible to prove, or disprove, the occurrence of pinocytic uptake and lysosomal degradation of crosslinked HPMA copolymers by the rat jejunal sac by

carrying out experiments which employed metabolic and proteinase inhibitors e.g. 2,4-dinitrophenol and leupeptin (section 4.2.4). Firstly, as discussed in section 5.1, studies by other workers have shown that it is not possible to totally inhibit uptake by this tissue using metabolic inhibitors. Bridges (1980) found that various concentrations of 2,4-dinitrophenol (which abolishes pinocytosis in the rat visceral yolk sac), sodium fluoride and sodium azide did not produce more than 50% inhibition of uptake of ^{125}I-PVP by jejunal sacs. Secondly, in order to prove that degradation of a given substrate takes place in the lysosomes, experiments using e.g. the lysosomal thiol-proteinase inhibitor, leupeptin, are used which aim to detect a reduction in the amount of degradation taking place within lysosomes. The levels of radioactivity representing degradation product in the serosal fluid were, however, too low to enable significant differences to be observed from experiments which employed leupeptin.

The experiments carried out at low temperature (15^{o}C) did show significant differences in the uptake of copolymers 39a and 39e when compared to those carried out at 37^{o}C. Copolymer 39a was taken up at 13% the rate observed at 37^{o}C into the tissue and 26% of the normal rate into the serosal fluid. Copolymer 39e values were 52% and 33% of the 37^{o}C values for the tissue and serosal accumulation. These results, together with figs. 5.22 and 5.23 show that the uptake values obtained at 37^{o}C do not represent substrate progressively binding to the tissue over the incubation period, without membrane internalization, since this could equally well occur at 15^{o}C. The reduced levels observed at 15^{o}C, particularly with copolymer 39a, clearly demonstrate that the mechanism

responsible for the uptake and transport of macromolecules by the intestine is a temperature-dependent metabolic process.

The pinocytosis of copolymer 68 ($\bar{M}w$ 171,000) by the jejunal sac gave a total rate of uptake of 1.31 µl/mg/h. This value was similar to that observed for copolymers of similar average molecular size (Table 5.11). As discussed previously, this copolymer also contains potentially degradable crosslinks, but the radiolabel is situated close to the copolymer backbone and therefore is an indicator of the presence of the copolymer chain rather than of crosslink presence/cleavage, which is the case in copolymer 39. It was assumed, therefore, that radioactivity present in the serosal fluid following incubation of copolymer 68 with jejunal sacs, was due to the presence of macromolecules, since $[^{125}I]$ iodotyrosine is not released from this copolymer (Chapters 3 and 4), and the copolymer preparation used was essentially free of contaminating $[^{125}I]$ iodide.

The transport of macromolecules across the jejunal tissue was demonstrated using loops of intestine. This experiment disproved the possibility that all macromolecules entered the serosal fluid through the cut ends of the sacs which are tied off with cotton. The experiment did not aim to give accurate quantitative measures of translocation rates, since approximately 50% of the tissue, though included in the tissue protein determination, was not in contact with the substrate. Three separate experiments, however, indicated transfer of radioactivity into the serosal fluid, and Sephadex G-25 chromatography carried out on two of the samples indicated that 34.77% and 22.2% of the radioactivity was due to the

presence of macromolecules, the rest being due to [^{125}I] iodide and [^{125}I] iodotyrosine.

In summary, the data obtained from the experiments performed using the rat everted jejunal sac have shown that ^{125}I-labelled crosslinked HPMA copolymers are taken up by the tissue at a rate that is dependent on the mean molecular weight of the copolymer used. The lowest molecular weight fraction ($\bar{M}w$ 34,000) is taken up at a rate consistent with fluid-phase pinocytosis, and increasing molecular size results in adsorption to the cell membrane with resulting increase in uptake with high molecular weight fractions. Uptake of HPMA copolymers is by a mechanism dependent upon metabolic energy and/or membrane fluidity and is most probably by pinocytosis (rather than intercellular transport). HMPA copolymers move across the intestine into the serosal fluid at a lower rate which is independent of molecular size. A small amount of degradation of all copolymer 39 fractions is observed, some of which takes place extracellularly. The degradation products observed in the serosal fluid are probably the result of intracellular lysosomal degradation with subsequent release of degradation products across the baso-lateral membrane of the enterocytes, resulting in the eventual appearance on the serosal side of the tissue.

From the experiments performed using everted jejunal sacs, and rat visceral yolk sacs (Chapter 4, section 3), it was not possible to prove conclusively that crosslink cleavage took place to yield copolymer chains of lower mean molecular weight.

CHAPTER 6

BLOOD CLEARANCE AND ORGAN DEPOSITION OF ^{125}I-LABELLED HPMA COPOLYMERS FOLLOWING INTRAVENOUS ADMINISTRATION TO RATS

6.1 INTRODUCTION

The in vitro experiments reported in previous chapters have demonstrated that soluble crosslinked HPMA copolymers are pinocytosed by cells and that the crosslinks are degraded to some extent by lysosomal enzymes present within the tissues. An effect of size of the polymer on its rate of uptake by cells has also been observed. In the yolk sac, pinocytic uptake of the substrate is seen to increase with decreasing molecular weight, and the jejunal sac shows a preferential uptake of higher molecular weight fractions. From these results, it is apparent that morphologically similar cell types do not behave in a similar fashion.

In order to study the pinocytic uptake of crosslinked HPMA copolymers of differing average molecular weights by different tissues within the body, in vivo experiments were subsequently undertaken. This chapter reports the blood clearance and organ deposition of ^{125}I-labelled crosslinked copolymer fractions of different molecular weights following intravenous injection to rats. The possibility of targeting crosslinked polymers to hepatocytes by the incorporation of galactosamine residues into the polymer structure is also investigated (see Chapter 1).

The clearance from the bloodstream of any macromolecule, particularly one with little affinity for cell membranes such as the HPMA copolymers described so far, is governed to a large extent by its size (Kooistra et al 1980). Molecules of less than approximately Mw 40,000 are removed rapidly from the bloodstream by glomerular filtration. The exact renal threshold value is dependent on the shape and charge of the

molecule, and in the case of polymers the flexibility of the polymer coil may also play a role in the passage across the glomerular barrier (Rypàček et al 1982).

Rogoeczi (1976) demonstrated an essentially biphasic pattern of clearance of ^{125}I-PVP ($\bar{M}w$ 50,000) from the bloodstream of rabbits, with an initial rapid clearance of the polymer due to the ultrafiltration of smaller molecules by the kidney followed by a slower rate of clearance due to tissue uptake of the larger molecules.

Rogoeczi (1976) also demonstrated that ^{125}I-PVP possessed no affinity for plasma proteins: he incubated it with human plasma and compared the dispersity of the preparation before and after incubation, using gel permeation chromatography. Similar studies have been carried out on crosslinked HPMA copolymers (section 3.7) using rat plasma, and the results demonstrate that this polymer, too, has no affinity for plasma proteins.

In vivo experiments using ^{125}I-PVP ($\bar{M}w$ 33,000) as a marker for fluid-phase pinocytosis have been carried out by Munniksma et al (1980) in order to determine rate constants of fluid-phase pinocytosis of liver, spleen, parenchymal and sinusoidal liver cells (hepatocytes and Kupffer cells). Rate constants of 1.2 and 1.8 ml of plasma per gram of tissue weight per day were calculated for liver and spleen respectively, and Kupffer cells were observed to pinocytose about nine times as much PVP as parenchymal cells. Subcellular fractionation of liver showed that PVP is mainly localized in the lysosomes following pinocytic uptake.

The effect of molecular size on blood clearance and organ deposition has been studied by a number of workers. Kooistra et al (1980) found that rat sinusoidal liver cells (Kupffer cells - of the reticuloendothelial system) demonstrated preferential uptake of large basic proteins following in vivo studies using lysozyme, ribonuclease and lactic dehydrogenase. Illum et al (1982) studied the effect of particle size on the blood clearance and organ deposition of intravenously administered colloidal particles and showed that small (1.27 μm diameter) polystyrene microspheres were taken up by sinusoidal cells of the liver, while large polystyrene particles (15.8 μm diameter) were mechanically deposited in the capillary beds of the lungs.

The elimination and retention of ^{14}C-labelled non-degradable Poly N-(2-hydroxypropyl)methacrylamide (Duxon - see Chapter 1) by rabbits following intravenous administration was investigated by Sprincl et al (1976). Five polymer fractions ($\bar{M}w$ 11,000, 28,000, 33,000, 63,000 and 91,000) of Gaussian molecular weight distribution were used in the study and it was found that polymers of lower molecular weights (11,000 and 28,000) were eliminated from the body via the kidney. The higher molecular weight polymers were found to be deposited in the organs of the reticuloendothelial system, particularly the spleen and lymph nodes.

Rypàček et al (1982) also investigated the blood clearance and renal excretion of another 'inert' synthetic polymer, N(2-hydroxyethyl)aspartamide (PHEA) in rabbits and mice. Polymer fractions ($\bar{M}w$ 11,000-32,000) were injected intravenously into rabbits and demonstrated increased renal excretion with

decreasing average molecular weight of the polymer.

The in vivo studies described above (Sprincl 1976, Rypáček 1982) have employed soluble synthetic polymers ranging from $\bar{M}w$ 11,000 to Mw 91,000. The HPMA copolymer 39 fractions (crosslink structure P-Gly-Gly-Phe-Tyr-HMDA-Tyr-Phe-Gly-Gly-P) described in previous chapters are of $\bar{M}w$ 34,000->400,000, and have a narrow polydispersity. In order to observe the effect of polymer size on its clearance from the bloodstream and to examine the possibility of different patterns of organ deposition on administration of polymers of different sizes, radiolabelled copolymer 39 fractions and copolymer 74 (the aminolysed polymeric precursor of copolymer 39) were injected intravenously to rats and their subsequent fate examined. ^{125}I-PVP, a known in vitro and in vivo marker of fluid-phase pinocytosis, was used as a control substrate.

It has been shown previously (Chapters 4 and 5) that experiments performed in vitro using copolymers 39 and 74 demonstrate hydrolysis of susceptible bonds following uptake of copolymer by cells, with resultant liberation of radiolabelled low molecular weight degradation products. Detection of these ^{125}I-labelled degradation products was achieved following Sephadex G-15 column chromatography (section 2.5) of culture medium after each experiment. It is not possible, however, to determine the possible degradation of crosslinks or sidechains from small blood samples (50 µl) following administration of ^{125}I-labelled copolymers 39 and 74 in vivo.

Radiolabelled copolymer 68 ($\bar{M}w$ 171,000, crosslink structure P-Gly-Gly-Phe-HMDA-Phe-Gly-Gly-P) contains a radioiodinated tyrosine residue attached to the copolymer backbone which is

not susceptible to hydrolysis by enzymes (Chapter 4). This copolymer was also administered intravenously to rats for comparison with the unfractionated copolymer 39 ($\bar{M}w$ 190,000). If the blood clearance patterns and organ deposition of the two copolymers proved similar, this would probably indicate that the radiolabel was still attached to the copolymer in both cases, and that degradation of oligopeptide sequences present in copolymer 39 had not taken place to any significant extent. Alternatively, if extensive degradation of copolymer 39 did take place over the proposed experimental period, one might expect a different rate of blood clearance and pattern of organ deposition due to a more rapid cellular uptake of smaller radiolabelled copolymer degradation products.

The role of carbohydrates in the recognition and uptake of glycoproteins from the blood circulation has been the subject of a number of studies (Ashwell and Morrell 1974, Schlesinger et al 1980, Jourdian et al 1981, Pitha et al 1981, Shepherd et al 1983), and has been discussed in Chapter 1. One such recognition system is the uptake of galactose-exposing proteins by mammalian hepatocytes. The incorporation of galactosamine into sidechains of ^{125}I-labelled tyrosine-containing HPMA copolymers ($\bar{M}w$ 25,000) has been shown to enhance the pinocytic uptake of the polymer from the bloodstream into the liver in the rat (Duncan et al 1983). With a control HPMA copolymer bearing 1-aminopropan-2-ol in place of the galactosamine, 10.11% of the recovered radioactivity was present in the liver after 1h, whereas 68.95% of the recovered radioactivity was found in the liver following intravenous administration of the copolymer bearing galactosamine after 1h. This finding is presumably due to the receptor-mediated pinocytosis of

galactose-bearing macromolecules by liver hepatocytes (Chapter 1). For both copolymers approximately 20% of the recovered radioactivity was found in the kidneys.

Copolymers 78 and 79 are crosslinked copolymers prepared from the same polymeric precursor ($\bar{M}w$ 21,500) and contain the same crosslink structure (P-Gly-Gly-Phe-HMDA-Phe-Gly-Gly-P) with tyrosineamide attached to the polymer backbone to enable radioiodination of the copolymer. Both are of similar $\bar{M}w$ (copolymer 78 73,800 and copolymer 79 64,300) and polydispersity (3.05 and 2.74 respectively). Copolymer 78 contains galactosamine (2 mol %) attached to the copolymer backbone via a diglycyl spacer, and copolymer 79 contains 1-aminopropan-2-ol in place of galactosamine.

These polymers were prepared (section 3.2), radiolabelled (section 2.3) and administered intravenously to rats in order to observe the effect of the galactosamine moiety on the pinocytic uptake of crosslinked copolymers i.e. polymers of Mw higher than the renal threshold, by the liver. Since copolymer 79 is similar to copolymer 78 in all respects except for the absence of galactosamine, any differences observed in the blood clearance and organ deposition of the two polymers must be attributable to the presence of the sugar moiety.

6.2 RESULTS

6.2.1 Blood Clearance and Organ Deposition of Copolymer 39 Fractions and Copolymer 74 in the Rat Following Intravenous Administration: The Effect of Size

Following injection of radiolabelled copolymers (125 µg in 0.1 ml phosphate buffer pH 8.0) into the femoral vein, duplicate 50 µl blood samples were taken at various time intervals over a period of one hour and assayed for radioactivity. At the end of each experiment, the animals were sacrificed and the liver, lungs, kidneys and spleen removed and assayed for radioactivity (section 2.11.6). Experiments using each substrate were repeated at least twice.

Figures 6.1-6.6 show the blood clearance of the different fractions and of the unfractionated copolymer 39. Figure 6.7 combines figures 6.1-6.6. Figure 6.8 shows the clearance of the polymeric precursor (copolymer 74) from the rat bloodstream and fig 6.9 that of the ^{125}I-PVP control. Results are expressed as a percentage of the value obtained two minutes following intravenous administration, since it was not possible in practice to obtain a value for t=0. Furthermore, to express the results as a percentage of the dose administered requires a value for the total blood volume and also must assume that 100% of the dose enters the circulation.

From these results it can be seen that the clearance of radiolabelled copolymer from the bloodstream is related to the average molecular weight of the preparation. Blood levels of the three largest fractions (39a, b and c $\bar{M}w$ >400,000, 400,000 and 150,000) after one hour were in excess of 90% of the

CLEARANCE OF ^{125}I-LABELLED COPOLYMER 39a FROM THE RAT BLOODSTREAM FOLLOWING INTRAVENOUS ADMINISTRATION

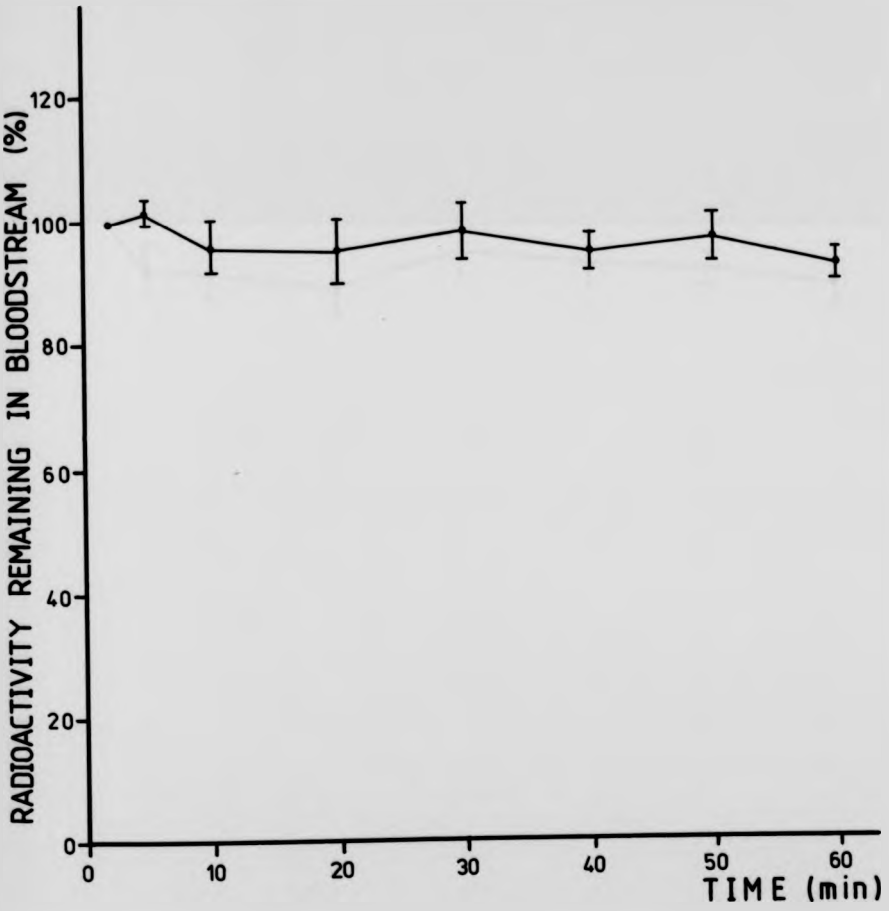


FIGURE 6.1

CLEARANCE OF ^{125}I-LABELLED COPOLYMER 39b FROM THE RAT BLOODSTREAM FOLLOWING INTRAVENOUS ADMINISTRATION

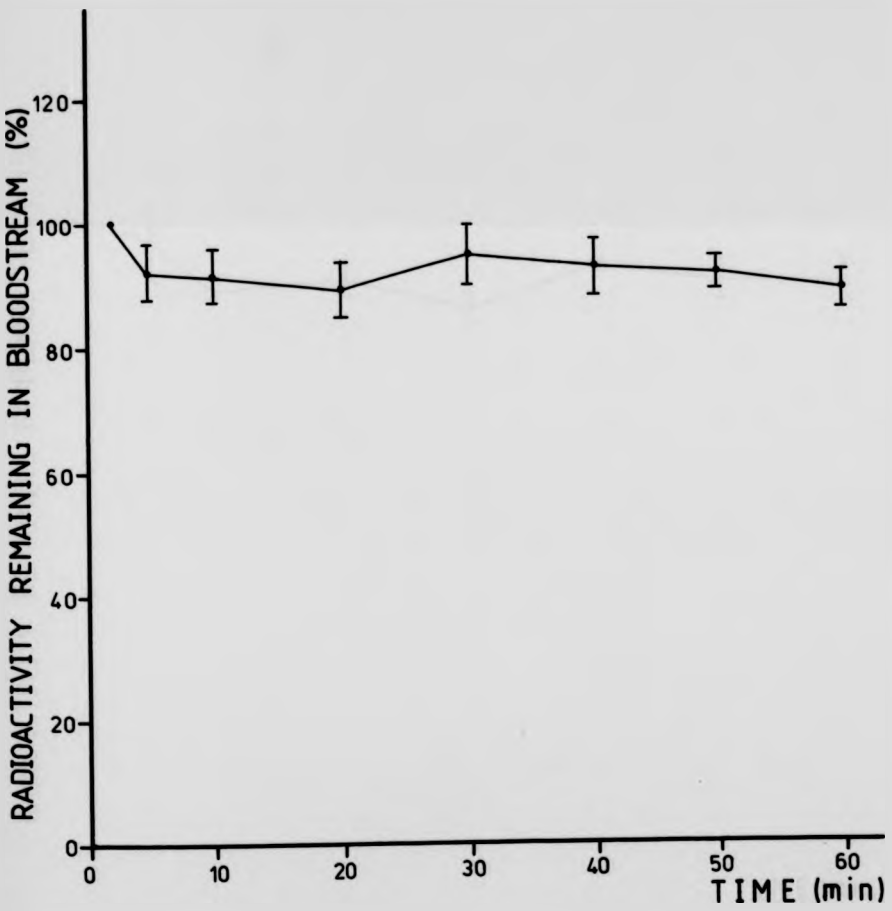


FIGURE 6.2

CLEARANCE OF ^{125}I-LABELLED COPOLYMER 39c FROM THE RAT BLOODSTREAM FOLLOWING INTRAVENOUS ADMINISTRATION

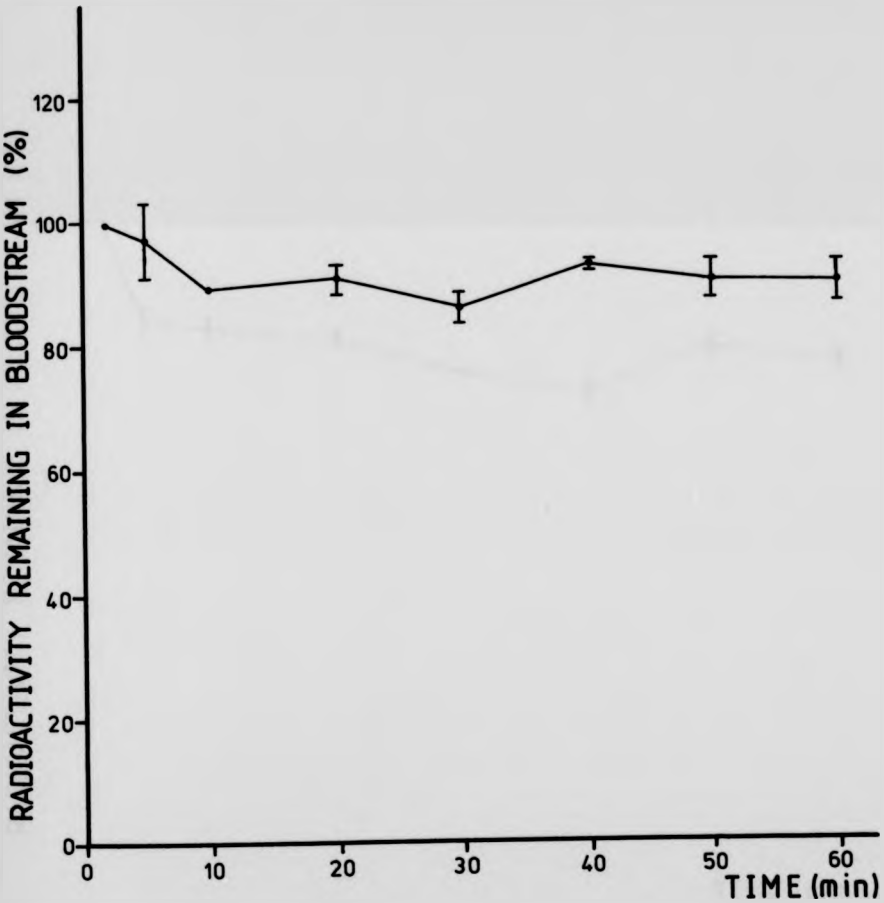


FIGURE 6.3

CLEARANCE OF ^{125}I-LABELLED COPOLYMER 39d FROM THE RAT BLOODSTREAM FOLLOWING INTRAVENOUS ADMINISTRATION

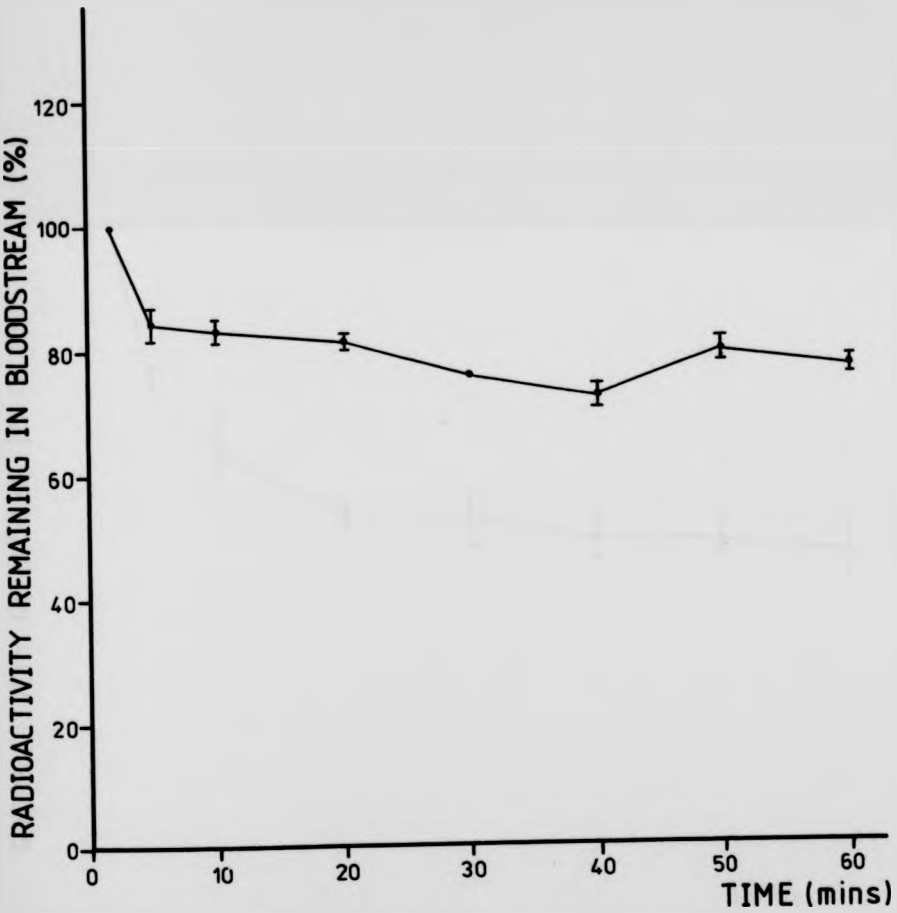


FIGURE 6.4

CLEARANCE OF ^{125}I-LABELLED COPOLYMER 39d FROM THE RAT BLOODSTREAM FOLLOWING INTRAVENOUS ADMINISTRATION

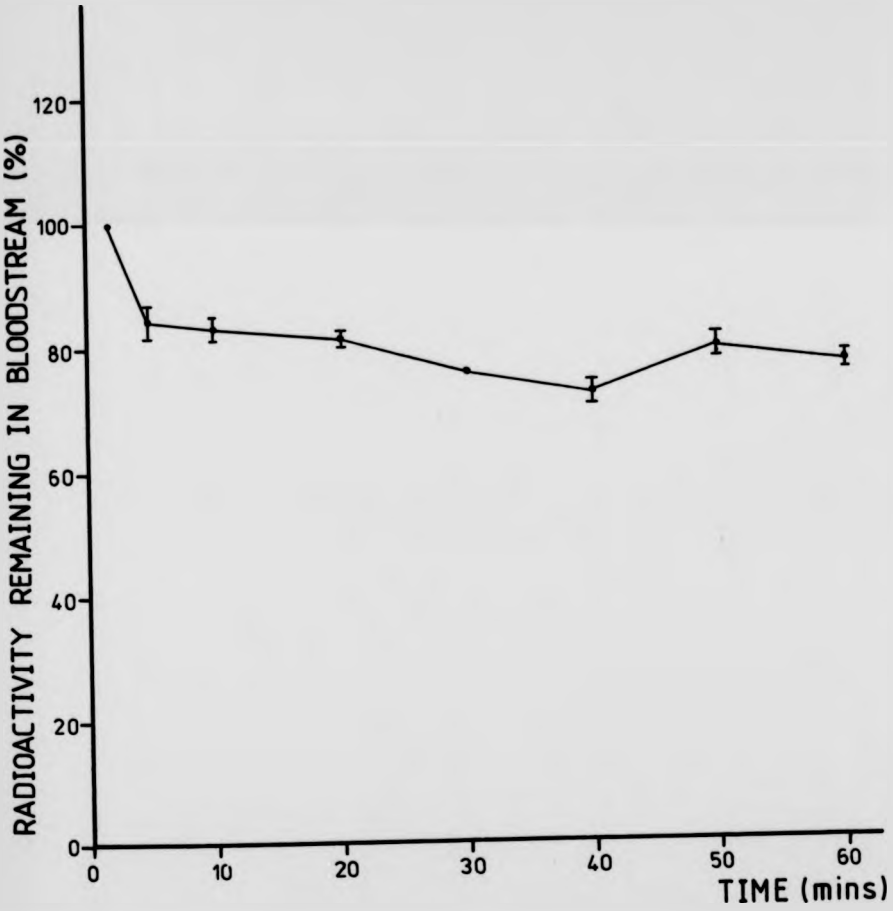


FIGURE 6.4

CLEARANCE OF ^{125}I-LABELLED COPOLYMER 39e FROM THE RAT BLOODSTREAM FOLLOWING INTRAVENOUS ADMINISTRATION

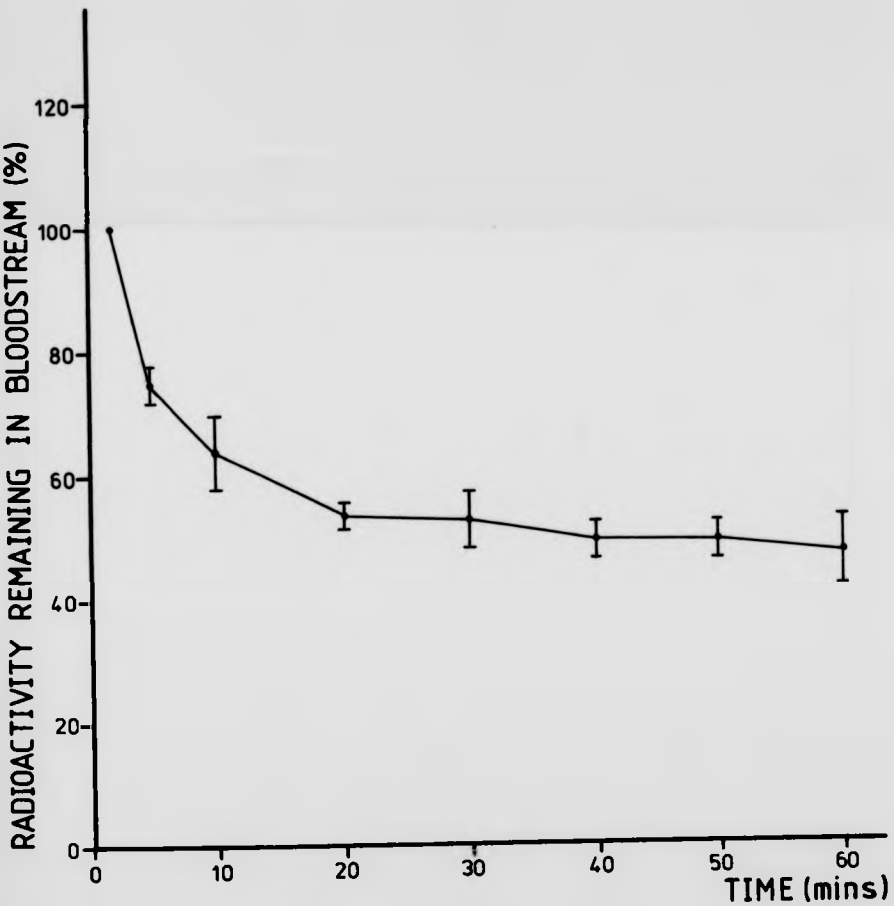


FIGURE 6.5

CLEARANCE OF ^{125}I-LABELLED COPOLYMER 39 FROM THE RAT BLOODSTREAM FOLLOWING INTRAVENOUS ADMINISTRATION

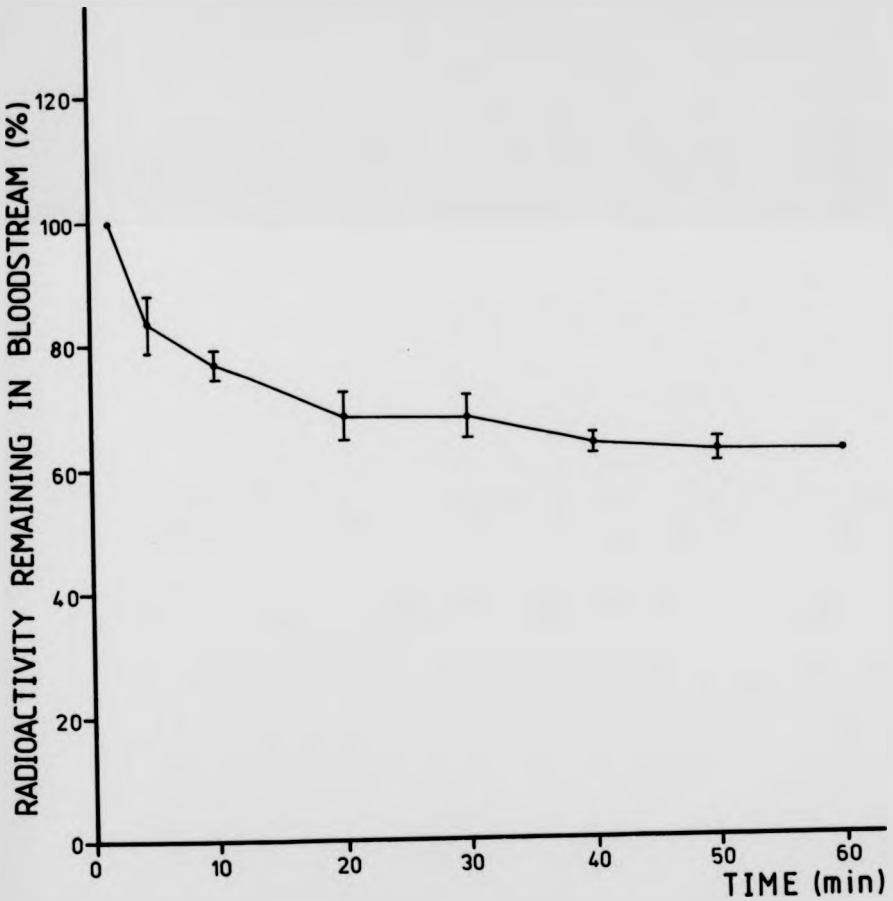
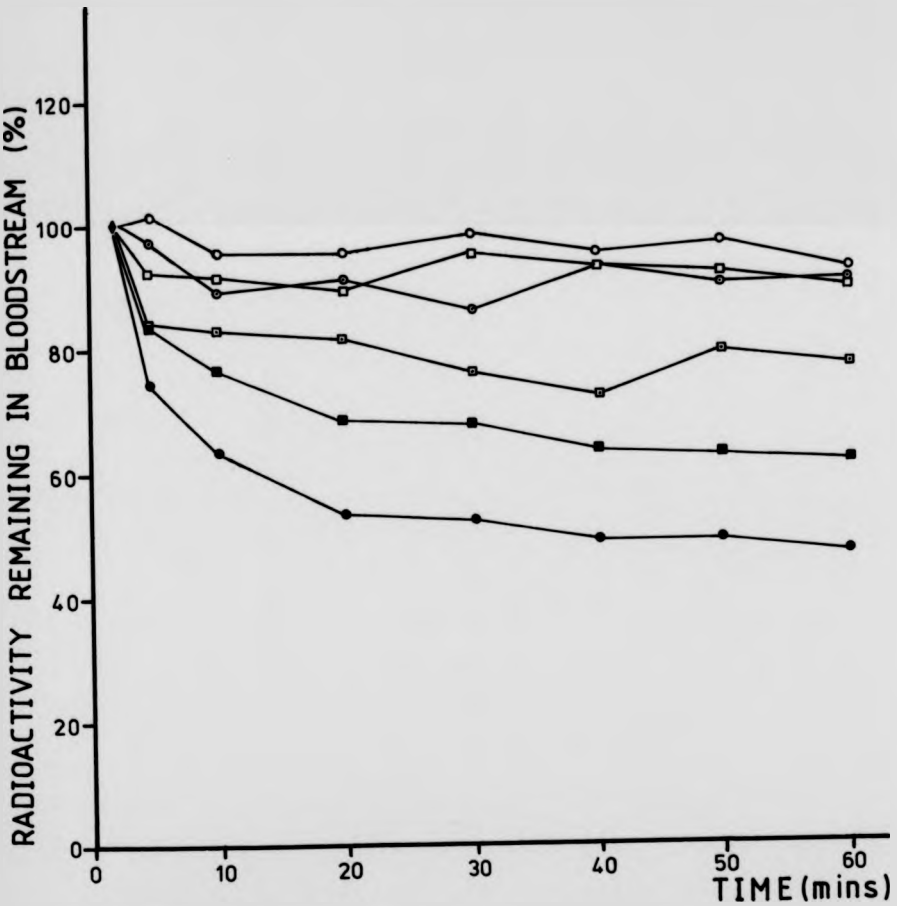


FIGURE 6.6

CLEARANCE OF ^{125}I-LABELLED COPOLYMER FRACTIONS FROM THE RAT BLOODSTREAM FOLLOWING INTRAVENOUS ADMINISTRATION



KEY	
DATA POINT	COPOLYMER
■	39
○	39a
□	39b
●	39c
⊡	39d
●	39e

FIGURE 6.7

CLEARANCE OF ^{125}I-LABELLED COPOLYMER 74 FROM THE RAT BLOODSTREAM FOLLOWING INTRAVENOUS ADMINISTRATION

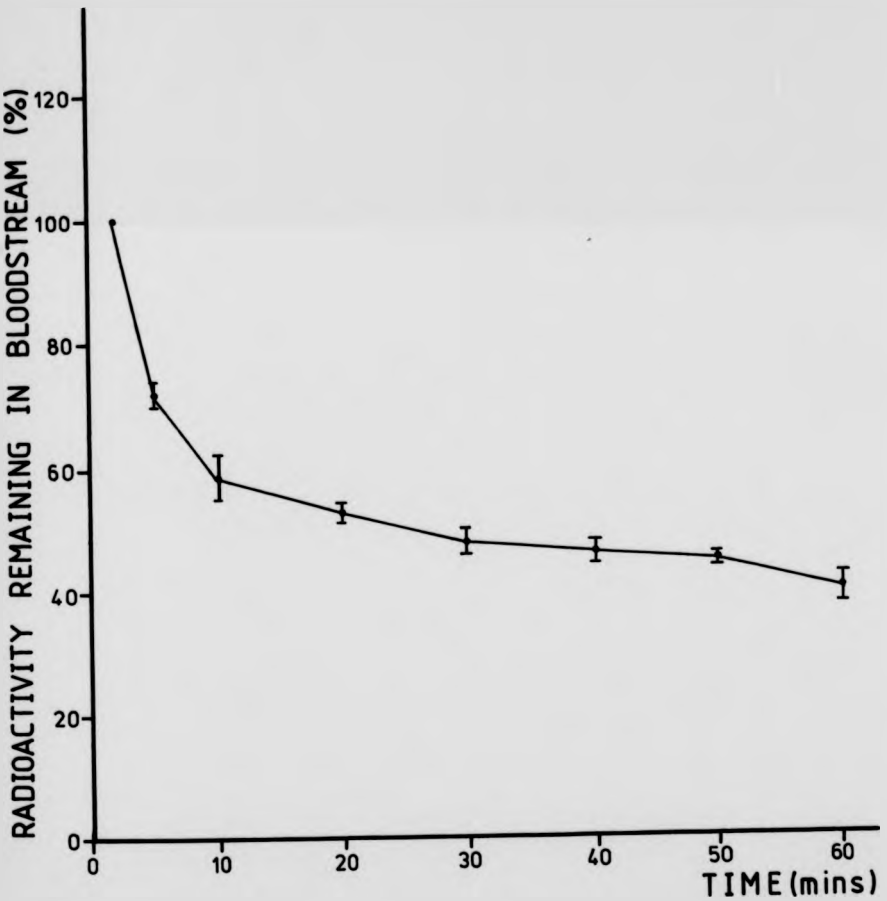


FIGURE 6.8

CLEARANCE OF ^{125}I-LABELLED PVP FROM THE RAT BLOODSTREAM FOLLOWING INTRAVENOUS ADMINISTRATION

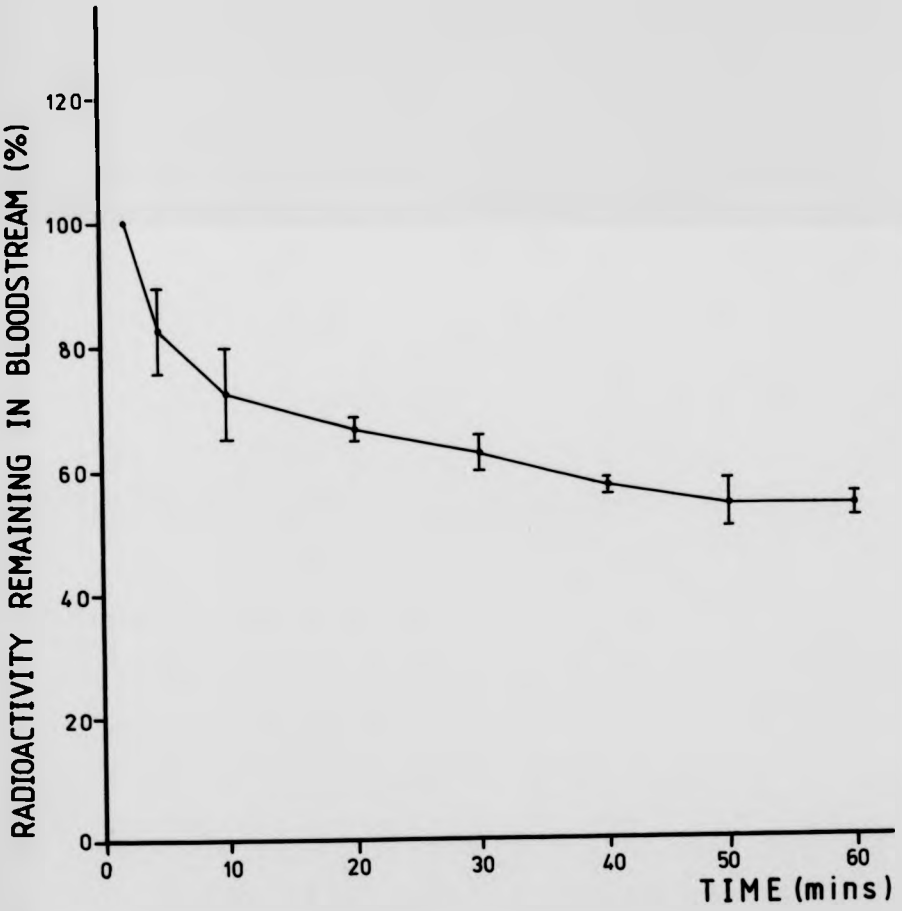


FIGURE 6.9

2 min value, indicating a very slow rate of clearance. Copolymer 39d, $\bar{M}w$ 110,000, demonstrates a more rapid clearance over the first five minutes following administration, after which there is little further clearance over the rest of the experimental period with 77.5% of the 2 min value remaining in the bloodstream after one hour. This observation is consistent with the removal of smaller polymers contained within the preparation by glomerular filtration. Copolymer 39e ($\bar{M}w$ 34,000) and the unfractionated copolymer 39 (which contains a large proportion of low molecular weight fractions) show a more pronounced clearance over the first ten minutes of the experimental period, followed by a gradual decrease in radioactivity present in the bloodstream resulting in 47.2% (for 39e) and 62.4% (for the unfractionated copolymer) of the initial values still present in the bloodstream after one hour.

This pattern of more rapid clearance with decreasing molecular weight is apparent again on administration of copolymer 74 ($\bar{M}w$ 31,000) which is cleared quickly with only 40% of the initial value present in the bloodstream at the end of the experiment (fig 6.8). Finally, ^{125}I-PVP ($\bar{M}w$ 40,000) (fig 6.9) is cleared at a rate consistent with its average molecular weight, showing similar, but slightly higher levels than copolymer 39e ($\bar{M}w$ 34,000).

Table 6.1 summarises the amounts of radioactivity recovered from the tissues one hour after intravenous administration of the various fractions. The total in the blood was calculated by multiplying the concentration of radioactivity in the blood by the blood volume, estimated by the formula of Everett et al (1956), i.e. 55.6 µl blood per gram body weight. Dose recoveries were calculated by expressing the total radioactivity

TABLE 6.1 BODY DISTRIBUTION OF ^{125}I-LABELLED COPOLYMER 39 FRACTIONS AND COPOLYMER 74 1 HOUR FOLLOWING INTRAVENOUS ADMINISTRATION TO RATS

POLYMER CODE NO.	% RADIOACTIVITY RECOVERED FROM				
	LIVER	LUNGS	KIDNEYS	SPLEEN	BLOOD
PVP	4.45 ± 0.40	1.59 ± 0.23	12.29 ± 3.98	0.36 ± 0.05	54.20 ± 2.02
39	4.96 ± 0.56	2.95 ± 0.65	4.44 ± 0.54	0.38 ± 0.09	87.25 ± 1.80
39a	4.52 ± 1.24	3.00 ± 1.51	1.14 ± 0.12	0.35 ± 0.09	90.99 ± 2.64
39b	5.02 ± 0.74	1.63 ± 0.17	1.33 ± 0.29	0.53 ± 0.17	91.50 ± 1.15
39c	4.41 ± 0.83	2.73 ± 1.10	2.10 ± 0.31	0.37 ± 0.10	90.39 ± 1.99
39d	4.71 ± 0.71	2.26 ± 0.64	2.33 ± 0.71	0.37 ± 0.10	90.35 ± 1.92
39e	5.68 ± 0.63	3.45 ± 0.83	12.16 ± 2.16	0.53 ± 0.10	78.17 ± 2.62
74	7.97 ± 1.26	2.02 ± 0.31	18.87 ± 0.00	0.41 ± 0.00	70.75 ± 1.58

recovered from the experiment as a percentage of the dose administered and were almost always in excess of 90%. It can be seen that the unfractionated copolymer 39, fraction 39e, copolymer 74 and ^{125}I-PVP are all found in a higher percentage in the kidneys. This is presumably due to ultrafiltration, since all contain a significant number of small molecules, and are cleared from the bloodstream rapidly. Table 6.1 also shows that between 4 and 8% of the radioactivity recovered is found in the liver, and between 1 and 3% in the lung.

In order to establish whether the values presented in Table 6.1 were the consequence of tissue uptake of copolymers by the various organs or were due to the presence of residual blood which contained the copolymers, control experiments were performed (once only) to estimate the body distribution of radiolabelled copolymers 2 minutes following intravenous administration, on the assumption that uptake into the various organs would be less after 2 minutes than one hour. Table 6.2 shows the results of these experiments, from which it can be seen that similar levels of radioactivity are recovered from the lungs, liver and spleen 2 minutes and 1 hour following intravenous administration. This finding suggests that the levels of radioactivity shown in Table 6.1 for those organs are largely due to the presence of residual blood.

The levels of radioactivity present in the kidney after 2 minutes do however increase with decreasing average molecular weight and this finding is more pronounced after 1 hour. This indicates a very rapid clearance of small molecules in the first few minutes following intravenous administration. The percentage of the unfractionated copolymer in the kidney after 2 minutes is higher than that noted after 1 hour. This could

TABLE 6.2 BODY DISTRIBUTION OF ^{125}I-LABELLED COPOLYMER 39 FRACTIONS AND COPOLYMER 74 2 MINUTES FOLLOWING INTRAVENOUS ADMINISTRATION TO RATS

POLYMER CODE NO.	% RADIOACTIVITY RECOVERED FROM				
	LIVER	LUNGS	KIDNEYS	SPLEEN	BLOOD
39	8.95	4.15	12.09	1.32	73.48
39a	5.85	3.42	1.78	0.11	88.83
39b	5.11	2.26	1.46	0.19	90.98
39c	1.47	1.47	0.58	1.01	95.47
39d	4.77	1.60	1.39	0.26	91.98
39e	5.83	5.19	6.38	0.46	82.18
74	8.88	2.86	9.79	1.30	77.16

indicate the rapid passage of smaller molecules into the kidney following introduction of the copolymer into the bloodstream, with subsequent passage into the bladder during the experimental period. This would result in a lower level of radioactivity in the kidney at the end of the 1 hour experiment. The contents of the bladder were not assayed for radioactivity. It must be noted, however, that the 2 minute experiment was performed once only and that this observation was noted only on administration of the unfractionated copolymer. All fractions derived from this copolymer, including the smallest copolymer, 39e ($\overline{Mw}$ 34,000), showed a higher percentage of recovered radioactivity in the kidney after 1 hour than after 2 minutes.

Everett et al (1956) proposed a method for estimating organ blood volumes. Using their values, the data of Table 6.1 were revised to subtract blood-borne radioactivity. The result is shown in Table 6.3 and indicates virtually no accumulation of radioactivity in the liver, lungs and spleen. This confirms the earlier surmise that the levels of radioactivity shown in Table 6.1 for those organs are largely due to residual blood. The levels of radioactivity recovered from the kidney (after correcting for blood) do, however, indicate that smaller polymer molecules are being filtered out of the blood by the organ.

6.2.2 Blood Clearance and Organ Deposition of Copolymer 68 in the Rat Following Intravenous Administration

The quantitation of blood clearance and body distribution of copolymer 68 over a 1 hour experimental period following intravenous administration to rats was carried out exactly as described in the previous section. The blood clearance

TABLE 6.3 BODY DISTRIBUTION OF ^{125}I-LABELLED COPOLYMER 39 FRACTIONS AND COPOLYMER 74 1 HOUR FOLLOWING INTRAVENOUS ADMINISTRATION TO RATS (VALUES CORRECTED FOR BLOOD-BORNE RADIOACTIVITY)

POLYMER CODE NO.	% RADIOACTIVITY RECOVERED FROM				
	LIVER	LUNGS	KIDNEYS	SPLEEN	BLOOD
39	0.00	0.00	4.20 ± 0.06	0.00	95.80 ± 0.06
39a	0.00	0.00	0.00	0.00	100.00
39b	0.00	0.00	0.42 ± 0.02	0.00	99.58 ± 0.02
39c	0.00	0.00	0.93 ± 0.34	0.05 ± 0.04	99.02 ± 0.33
39d	0.00	0.00	1.44 ± 0.50	0.00	98.56 ± 0.58
39e	0.00	0.00	15.21 ± 1.61	0.06 ± 0.04	84.74 ± 1.57
74	0.00	0.00	19.97 ± 0.25	0.02 ± 0.01	80.02 ± 0.32

(fig 6.10) shows the mean ± S.E. of four experiments and the organ distribution of recovered radioactivity is shown in Table 6.4. From these results it is apparent that this unfractionated copolymer enters the kidney, with little accumulation elsewhere. Again, this is most probably due to the ultrafiltration of smaller molecules present in the polydisperse preparation. The clearance of copolymer 68 from the bloodstream is, however, slightly slower than that observed for unfractionated copolymer 39. The reason for this is not known, but one possibility is that copolymer 68, a more densely crosslinked copolymer, could contain a smaller proportion of lower molecular weight fractions than copolymer 39, resulting in a less rapid initial loss of copolymer from the bloodstream (figs 6.6 and 6.10). Sepharose 4B/6B elution profiles of the two copolymers (section 3.4, figs 4 and 11) appear to support this suggestion.

6.2.3 Blood Clearance and Organ Deposition of Copolymers 78 and 79 Following Intravenous Administration to Rats: Targeting to the Liver

Copolymers 78 and 79 were administered to rats as described previously and blood levels monitored for 1 hour. The body distribution of radioactivity was estimated after 1 hour and the results of triplicate experiments are shown in fig 6.11 and Table 6.5. Fig 6.11 shows that the copolymer containing galactosamine residues (copolymer 78) is cleared more rapidly from the bloodstream than the control copolymer (79). From Table 6.5 it is clear that the radiolabelled copolymer 78 has been efficiently captured by the liver, where 60.42% of the recovered radioactivity is found to be present 1 hour following intravenous administration, in contrast with only 7.48% with the control polymer. Little accumulation of copolymers 78 and

79 is seen in the lungs and spleen. Both copolymers are present in the kidneys, but a greater amount (12.35%) is present following administration of copolymer 79, than copolymer 78 (3.76%). The reason for this is probably the ultrafiltration of smaller molecules, and although both preparations contain approximately the same proportion of lower molecular weight fractions, it is likely that smaller copolymer 78 molecules will contain galactosamine residues which could be removed from the circulation by the liver (through receptor-mediated pinocytosis by hepatocytes) even before encountering the kidney glomerulus. This suggestion is supported by the values shown in Table 6.6 for the organ deposition of copolymers 78 and 79 2 minutes following intravenous administration, where it can be seen that 21.55% of the recovered radioactivity is found in the liver in the case of copolymer 78, and only 8.29% (not correcting for blood) in the case of copolymer 79. The smaller molecules present in the copolymer 79 preparation, which contain no galactosamine residues and therefore have little or no affinity for cell membranes, would be filtered out in the normal way and be present in the kidneys in greater quantity.

Table 6.5 also shows the results of the same experiments after correcting for radioactivity present in residual blood within the organs. Again, it is apparent that the radioactivity present in the liver 1 hour following intravenous administration of the control copolymer 79 (7.48%) is largely due to residual blood, whereas, in the case of the galactosamine containing copolymer 78, almost 60% of the recovered radioactivity is still found to be present in the liver after correcting for blood content.

CLEARANCE OF ^{125}I-LABELLED COPOLYMER 68 FROM THE RAT BLOODSTREAM FOLLOWING INTRAVENOUS ADMINISTRATION

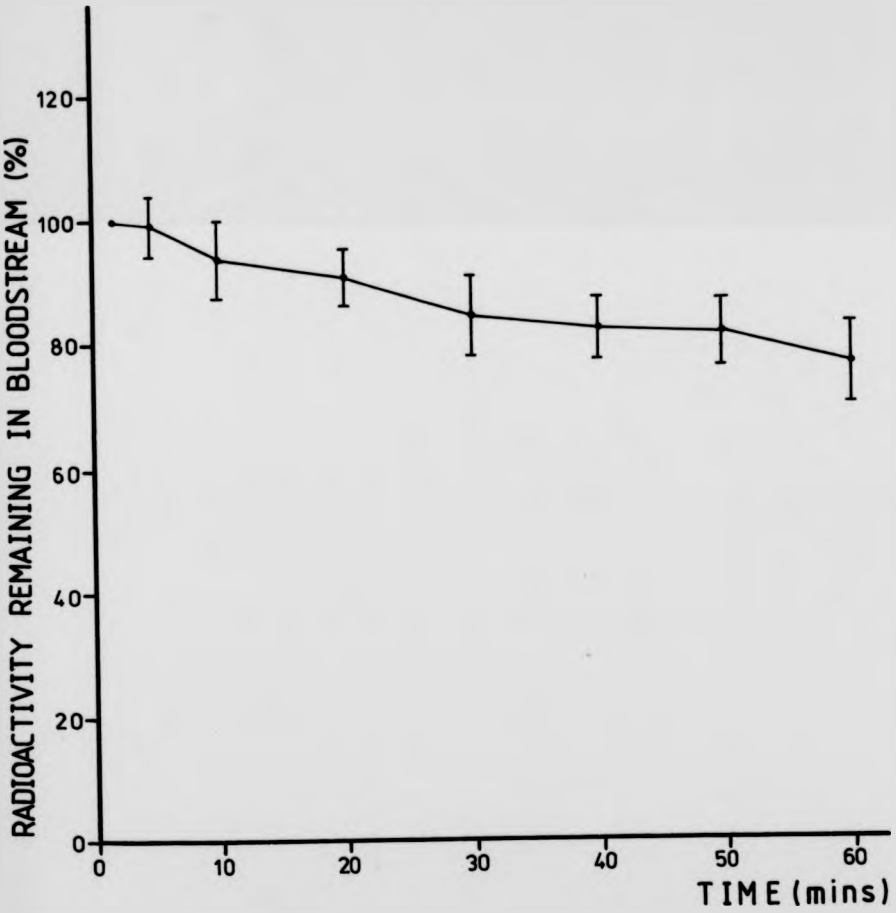
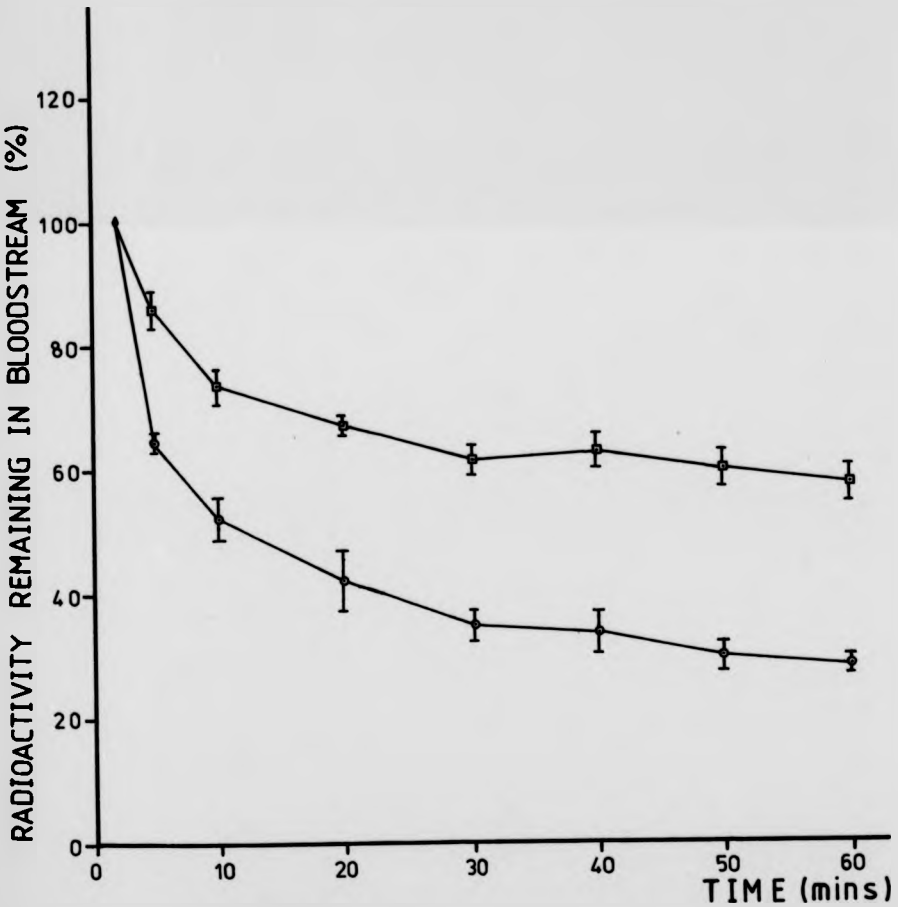


FIGURE 6.10

TABLE 6.4 BODY DISTRIBUTION OF ^{125}I-LABELLED COPOLYMER 68 1 HOUR FOLLOWING INTRAVENOUS ADMINISTRATION TO RATS

POLYMER CODE NO.	% RADIOACTIVITY RECOVERED FROM				
	LIVER	LUNGS	KIDNEYS	SPLEEN	BLOOD
68 (not correcting for blood)	8.37 ± 1.07	5.62 ± 0.70	10.88 ± 3.00	0.60 ± 0.06	75.53 ± 3.88
68 (correcting for blood)	0.00	0.90 ± 0.37	11.40 ± 3.69	0.01 ± 0.01	87.68 ± 3.92

CLEARANCE OF ^{125}I-LABELLED COPOLYMERS 78 AND 79 FROM THE RAT BLOODSTREAM FOLLOWING INTRAVENOUS ADMINISTRATION



KEY	
DATA POINT	COPOLYMER
•	78
▫	79

FIGURE 6.11

TABLE 6.5 BODY DISTRIBUTION OF ^{125}I-LABELLED COPOLYMERS 78 AND 79 1 HOUR FOLLOWING INTRAVENOUS ADMINISTRATION TO RATS

POLYMER CODE NO.	% RADIOACTIVITY RECOVERED FROM				
	LIVER	LUNGS	KIDNEYS	SPLEEN	BLOOD
78 (not correcting for blood)	60.42 ± 1.05	1.65 ± 0.47	3.76 ± 0.70	0.39 ± 0.13	33.78 ± 1.67
78 (correcting for blood)	59.18 ± 1.35	0.22 ± 0.13	3.54 ± 0.76	0.17 ± 0.09	36.89 ± 1.80
79 (not correcting for blood)	7.48 ± 1.14	2.95 ± 1.03	12.35 ± 3.59	0.78 ± 0.29	76.54 ± 5.73
79 (correcting for blood)	0.00	0.47 ± 0.38	12.81 ± 4.38	0.25 ± 0.20	86.46 ± 4.90

TABLE 6.6

BODY DISTRIBUTION OF ^{125}I-LABELLED COPOLYMERS 78 AND 79 2 MINUTES FOLLOWING INTRAVENOUS ADMINISTRATION TO RATS

POLYMER CODE NO.	% RADIOACTIVITY RECOVERED IN LIVER	LUNG	KIDNEY	SPLEEN	BLOOD
78	21.55	5.92	7.88	0.27	64.39
79	8.29	2.99	4.86	0.46	83.40

6.3 DISCUSSION

The rate of clearance of radiolabelled copolymer 39 fractions from the rat bloodstream following intravenous administration was dependent upon the average molecular weight of the preparation used. The unfractionated copolymer 39 and copolymers of an average molecular weight of less than 150,000 were cleared from the bloodstream to a greater extent than the larger fractions over the one hour experimental period.

This finding is consistent with results published previously by other workers using soluble synthetic polymers of different average molecular weights (Sprinčl 1976 and Rypàček 1982), and also confirms that low molecular weight fractions do not bind to plasma proteins (which would effectively increase their molecular weight and give rise to a different pattern of blood clearance). The rapid initial clearance of smaller molecules can be explained by filtration through the kidney glomerulus (Tables 6.1 and 6.3) and the subsequent slower clearance rate is presumably due to endocytosis of different sizes of copolymer by a range of cell types and/or movement of the radiolabelled molecules from the circulation into intercellular spaces. The clearance after the first 20 minutes or so is very slow in all cases (figs 6.1-6.10), and hardly apparent in the three largest fractions.

It is possible that very large molecules contained within the preparations would ultimately be phagocytosed by cells of the reticuloendothelial system (e.g. Kupffer cells), although the largest copolymer fractions 39a and b, show little or no accumulation in organs containing cells of the RE system (liver, lungs, spleen) over the experimental period. It cannot be

assumed, however, that prolonged experiments would show similar results.

[^{125}I]-Iodotyrosine is not released from radiolabelled copolymer 68 following pinocytic uptake by cells (section 4.2.5). The copolymer backbone therefore retains its radiolabel throughout the experimental period, whether or not the unlabelled crosslinks contained within the molecule are degraded. The radioactivity levels recorded from the bloodstream and tissues must therefore denote the presence of macromolecules at all times. The blood clearance and body distribution of radioactivity following intravenous administration of ^{125}I-labelled copolymer 68 was very similar to that observed for the unfractionated copolymer 39 over the same experimental period. Had copolymer 39 been degraded extensively, with resultant release of radiolabelled low molecular weight degradation products from the degraded crosslinks, the pattern of radioactivity recovered from the blood and tissues would probably be different (section 6.1). Since the observations for the two unfractionated copolymers are similar, it appears that little degradation of copolymer 39 occurs during the 1 hour experimental period.

The blood clearance and organ deposition of ^{125}I-labelled copolymer 78 following intravenous administration to rats was markedly different from all other copolymers used in this study including its control (copolymer 79), showing rapid blood clearance and copolymer deposition in the liver. This can be attributed to the incorporation of galactosamine residues into the copolymer, leading to the receptor-mediated pinocytosis of the copolymer by rat liver cells as described by Duncan et al (1983).

CHAPTER 7

ORGAN DEPOSITION OF ^{125}I-LABELLED HPMA COPOLYMERS FOLLOWING INTRAPERITONEAL, SUBCUTANEOUS AND ORAL ADMINISTRATION TO RATS

7.1 INTRODUCTION

The blood clearance and organ deposition of ^{125}I-labelled crosslinked HPMA copolymers following intravenous administration to rats was reported in Chapter 6. This chapter investigates the fate of soluble crosslinked HPMA copolymers following different routes of administration to rats (intraperitoneal, subcutaneous and oral). The specific questions asked are:-

1. How long does it take for soluble crosslinked HPMA copolymers to migrate from the various sites of administration?
2. Is it still possible to target crosslinked copolymers to the liver using routes of administration other than the intravenous one? (Chapter 6)
3. Does the route of administration of crosslinked copolymers affect the organ distribution of radioactivity?
4. Can crosslinked copolymers be cleared from the body?

The possible movements of polymers within the body are depicted schematically, using a compartmental model, in fig 7.1. Also included in the figure are the points of entry of copolymer following the different routes of administration used in this study. It can be seen from fig 7.1 that the proposed routes of administration result in the introduction of polymer into different body compartments.

The intravenous route (used in experiments described in Chapter 6) involves the entry of polymer directly into the blood circulation, resulting in the rapid transport of molecules to all vascularized areas of the body. Molecules of a molecular weight below that of the renal threshold are filtered from the plasma by the kidney glomerulus and excreted in the urine. Those molecules that are retained in the circulation may either

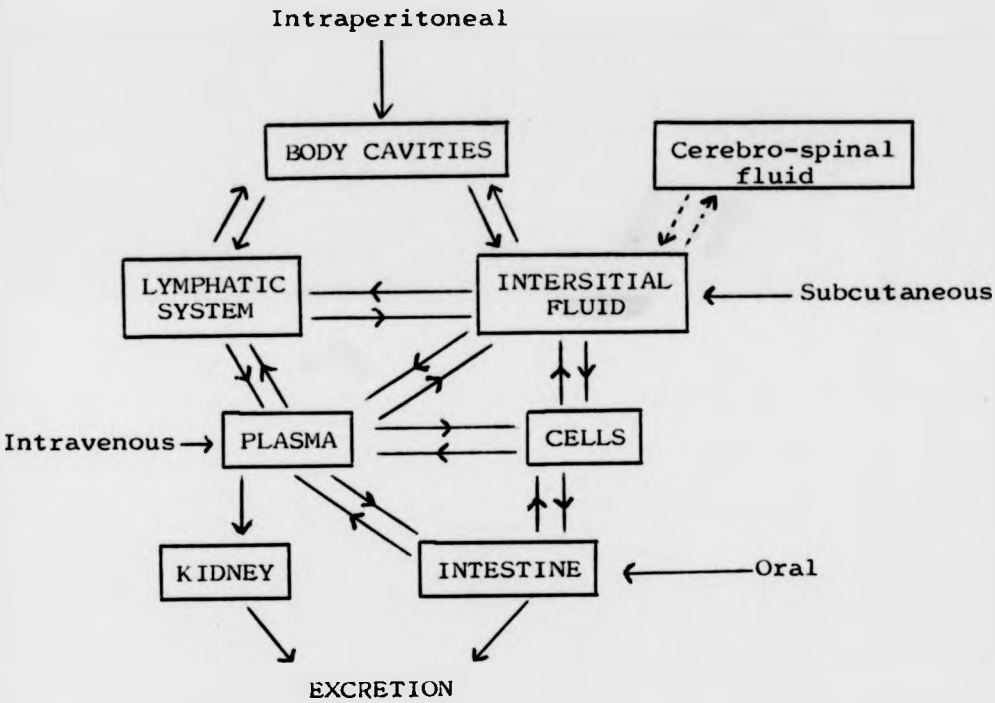


FIGURE 7.1

The Possible Fates of Synthetic Polymers In Vivo.

undergo passage across cells of the blood vessels,contained within membrane bounded vesicles (diacytosis), or they may pass between the cells, depending on the permeability of the vascular tissue in question. Clearance of polymers from the blood circulation into interstitial fluid across capillary walls takes place by diacytosis, since capillary endothelial cells are joined together by relatively impermeable cell junctions (Simionescu et al 1975). Transendothelial passage or passage between cells may, however, take place in capillaries of the sinusoidal type where gaps are found between endothelial cells,and these intercellular spaces allow free communication with the interstitial fluid. Such sinusoidal capillaries are found in the liver, spleen, bone marrow and lymph nodes (Drobnik and Rypáček 1984). The vascular system of the brain, on the other hand, is relatively impermeable to macromolecules, diacytosis is reduced, and endothelial cells are tightly joined together (Rapoport 1980).

Subcutaneous administration of copolymer results in the deposition of copolymer into the interstitial fluid (fig 7.1) and consequently, migration of polymer from the site of injection is less rapid than following intravenous administration. When present in the interstitial fluid, macromolecules may be pinocytosed by adjacent cells or be drained from this compartment either into venous capillaries (where pores are present) and hence be transported to all vascularized areas, or pass through the lymphatic system. Larger molecules are believed to be drained preferentially via the lymphatic system (Szabó et al 1973). The lymphatic vessels gradually converge into two large trunks which empty into the large veins near to the heart, thus transporting the polymer into the blood circulation. The distribution of the polymer then proceeds as

described above.

Intraperitoneal administration deposits the copolymer into the peritoneal cavity. Movement of macromolecules from this compartment is hindered by the serose, which lines the inner surface of the cavity, and is reported to be slow and take place mainly in the lymphatic capillaries (Noronha-Blob 1977) which are more permeable than blood capillaries to large molecules (Drobnik and Rypàček 1984). Movement into the interstitial fluid can also take place. Ultimately, molecules may be transported into the blood circulation and distributed throughout the body.

It has been reported that polymers are not transported across the intestine into the blood circulation in significant quantities (Pitha 1981, Weishenker 1978, Drobnik and Rypàček 1984). Oral administration of copolymers was, nevertheless, undertaken in view of the previous finding that soluble crosslinked HPMA copolymers are transported across the rat jejunum in vitro in small quantities (Chapter 5).

The crosslinked HPMA copolymers used in this study were polydisperse preparations 39 (crosslink structure P-Gly-Gly-Phe-Tyr-HMDA-Tyr-Phe-Gly-Gly-P), 78 and 79 (both of crosslink structure P-Gly-Gly-Phe-HMDA-Phe-Gly-Gly-P). The characteristics of these copolymers are shown in section 3.4. All three copolymers have been shown to be stable in rat plasma over 1-24 h (section 3.7).

^{125}I-Labelled copolymer 39 enters cells by fluid-phase pinocytosis i.e. with little or no interaction with cell membranes. It contains a radiolabelled tyrosine residue within its degradable crosslinks. Copolymer 79 also enters cells by

fluid-phase pinocytosis but contains the radiolabelled tyrosine residue attached directly to the copolymer backbone, not in its potentially degradable crosslink. The organ distribution of radioactivity following administration of copolymer 79 will therefore denote the presence of copolymer chains, whereas that of copolymer 39 will denote the presence of either crosslinks or crosslink degradation products (section 4.2.1).

Copolymer 78 is of a similar structure to copolymer 79 but contains galactosamine, and has been shown to accumulate rapidly in the liver following intravenous administration to rats (section 6.2.3). Because it interacts with specific cell membranes, it shows a different body distribution of copolymer from that of its control (copolymer 79). The administration of copolymer directly into the blood circulation does, however, allow easy access of copolymer to all vascularized areas, and the liver contains more blood at any time than any other organ in the body. It was of interest, therefore, to observe whether copolymer 78 was still able to accumulate in the liver following administration to other, more remote, sites of the body.

Body distributions of radioactivity were studied following administration of these three substrates via the different routes over periods up to 24 h. Preliminary experiments (employing intraperitoneal administration) were also carried out to observe whether or not crosslinked copolymers 78 and 79 could eventually be totally eliminated from the body following uptake of the polymers by cells and intralysosomal cleavage of crosslinks to produce copolymer chains small enough to be excreted from the body. (The importance of body clearance of copolymer following its administration was discussed in Chapter 1).

7.2 RESULTS

7.2.1 Organ Deposition of ^{125}I-labelled Copolymers Following Intraperitoneal Administration to Rats

Male Wistar rats (250-350g) were injected intraperitoneally with 125 µg of ^{125}I-labelled copolymer (39, 78 or 79) in PBS as described in section 2.11.2 and placed, if necessary, into metabolic cages. After 2 minutes, 1h, 2.5h or 24h, animals were anaesthetized and duplicate 50 µl blood samples were taken and assayed for radioactivity (section 2.11.5). The animals were then sacrificed and the peritoneal cavity washed with 10 ml of normal saline, as described in section 2.11.6, in order to recover copolymer remaining in the cavity. Organs were removed, homogenised, and assayed for radioactivity. Experiments for each time point, for each substrate, were carried out in triplicate. Table 7.1 shows the percentage of the administered dose that was recovered at the end of each experiment. Results from each experiment are expressed as a percentage of the recovered radioactivity.

The organ distribution of radioactivity following intraperitoneal injection of copolymer 39 is shown in Table 7.2 and fig 7.2. It can be seen that over the 24h experimental period, the radioactivity recovered from the peritoneal washings declined quite rapidly, with only 1.54% of the total recovered radioactivity remaining after 24h. Over 50% of the radioactivity recovered was found in the urine after 24h, with levels in the kidney first rising over 2.5h to 3.48% and at some point declining to give a value of 1.68% after 24h. Blood levels of radioactivity, and levels associated with the liver rose over the first 2.5h and then reamined constant.

TABLE 7.1

INTRAPERITONEAL ADMINISTRATION. DOSE RECOVERIES (%)

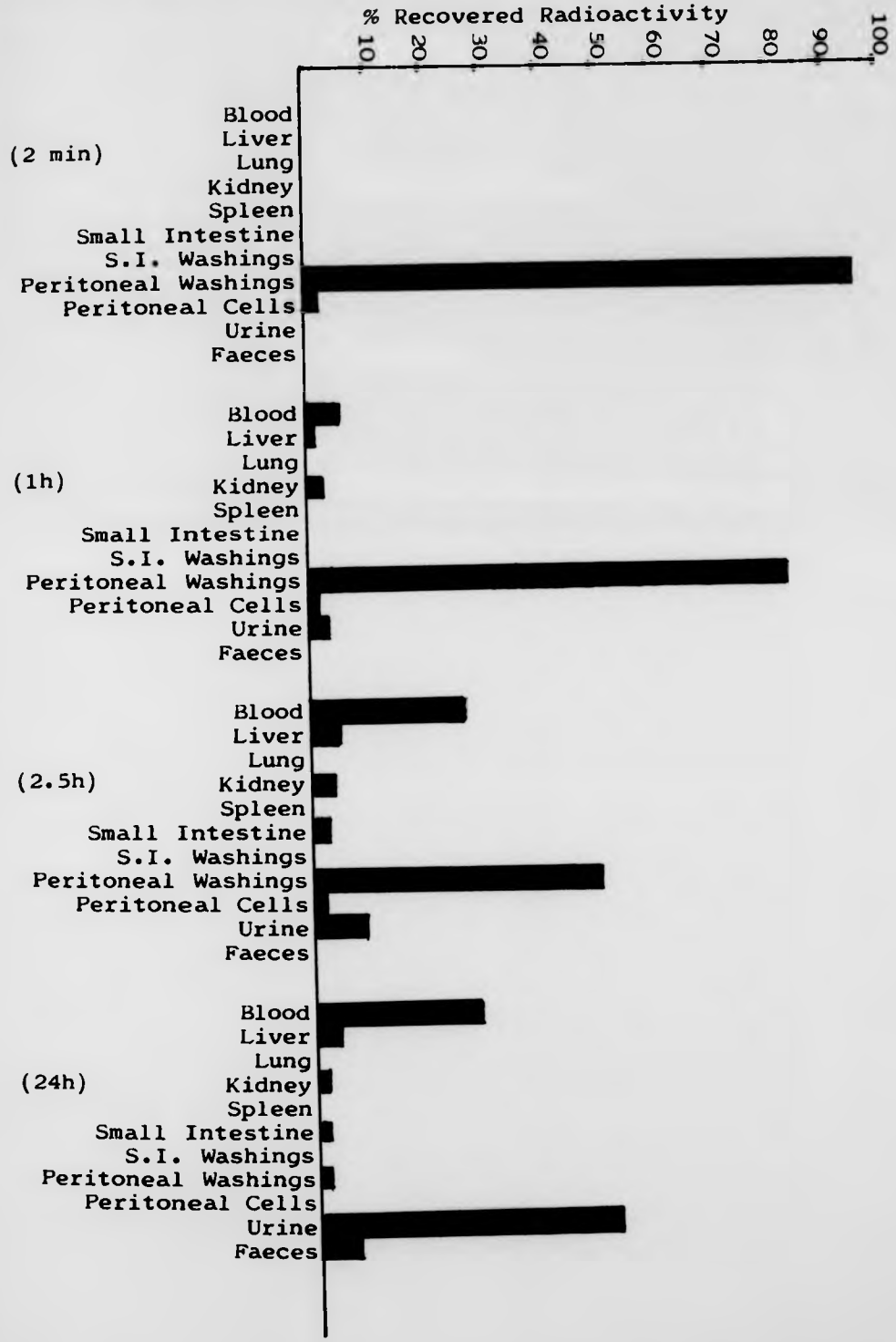
SUBSTRATE	2 min	1h	2.5h	24h
39	117.00	86.09	99.60	59.35
	64.00	54.49	76.03	79.68
	73.71	21.16	40.66	71.97
78	130.00	108.00	81.43	73.74
	66.91	108.00	100.40	68.19
	64.93	48.43	32.24	60.03
79	103.00	58.24	38.00	46.07
	63.37	51.88	33.90	40.43
	78.29	66.77	73.03	45.53

TABLE 7.2 BODY DISTRIBUTION OF ^{125}I-LABELLED COPOLYMER 39 AFTER INTRAPERITONEAL ADMINISTRATION TO RATS

Organ	2 min	1h	2.5h	24h
BLOOD	0.38 ± 0.00	6.33 ± 1.65	27.73 ± 3.65	28.72 ± 1.48
LIVER	0.37 ± 0.06	1.41 ± 0.38	4.86 ± 0.82	4.50 ± 0.61
LUNG	0.03 ± 0.01	0.21 ± 0.03	0.60 ± 0.08	0.79 ± 0.04
KIDNEY	0.10 ± 0.00	2.15 ± 0.41	3.48 ± 0.18	1.68 ± 0.13
SPLEEN	0.04 ± 0.01	0.04 ± 0.01	0.16 ± 0.04	0.24 ± 0.01
SMALL INTESTINE	0.45 ± 0.10	0.97 ± 0.15	2.48 ± 0.46	1.55 ± 0.11
S.I. WASHINGS	0.03 ± 0.02	0.38 ± 0.07	0.76 ± 0.17	0.74 ± 0.13
PERITONEAL WASHINGS	96.98 ± 0.28	83.20 ± 2.59	49.45 ± 3.46	1.54 ± 0.14
PERITONEAL CELLS	1.65 ± 0.11	1.96 ± 0.31	1.35 ± 0.11	0.11 ± 0.02
URINE	0.00	3.34 ± 2.13	9.11 ± 1.82	53.12 ± 1.28
FAECES	0.00	0.01 ± 0.01	0.01 ± 0.00	7.01 ± 0.81

FIGURE 7.2

BODY DISTRIBUTION OF ^{125}I-LABELLED COPOLYMER 39 FOLLOWING INTRAPERITONEAL ADMINISTRATION TO RATS



In order to ascertain the nature of the radioactivity recovered from the urine following intraperitoneal injection of copolymer 39, 1 ml samples of urine were applied to a Sepharose 4B/6B column and eluted as described previously (section 2.5).

A typical elution profile is shown in fig 7.3 and calculated data are presented at the end of this section, along with further discussion. Figure 7.3 shows that approximately one half of the recovered radioactivity co-elutes with copolymer, and the other half is present in the second peak, which co-elutes with both [^{125}I] iodide and [^{125}I]iodotyrosine.

The organ deposition of copolymer 79 following intraperitoneal administration (Table 7.3 and fig 7.4) shows a very similar pattern to that observed for copolymer 39, the only notable differences are that blood levels are slightly lower and urine levels are slightly higher. A typical elution profile following Sepharose 4B/6B chromatography of urine recovered from rats injected with ^{125}I-labelled copolymer 79 is shown in fig 7.5. It can be seen that two peaks of radioactivity are again present, the first co-eluting with copolymer and the second with [^{125}I] iodide. This figure shows that the radioactivity present in the urine is largely attached to the copolymer.

The experimental results following intraperitoneal administration of the galactosamine containing copolymer 78 to rats are shown in Table 7.4 and fig 7.6. It can be seen that the copolymer is cleared from the peritoneal cavity at about the same rate as copolymers 39 and 79 and that, despite administration by the intraperitoneal route, the copolymer is still able to accumulate progressively in the liver, with

TABLE 7.3 BODY DISTRIBUTION OF ^{125}I-LABELLED COPOLYMER 79 AFTER INTRAPERITONEAL ADMINISTRATION TO RATS

Organ	2 min	1h	2.5h	24h
BLOOD	2.30 ± 0.52	17.58 ± 3.73	19.79 ± 3.93	16.96 ± 0.38
LIVER	0.80 ± 0.26	2.84 ± 0.43	4.02 ± 0.95	3.96 ± 0.38
LUNG	0.12 ± 0.02	0.55 ± 0.08	1.45 ± 0.78	0.75 ± 0.06
KIDNEY	0.18 ± 0.04	2.49 ± 0.36	4.75 ± 0.98	2.29 ± 0.05
SPLEEN	0.11 ± 0.03	0.24 ± 0.06	0.20 ± 0.08	0.40 ± 0.06
SMALL INTESTINE	0.86 ± 0.21	2.16 ± 0.20	3.04 ± 0.56	1.61 ± 0.13
S.I. WASHINGS	0.33 ± 0.16	0.91 ± 0.31	3.36 ± 0.93	0.42 ± 0.07
PERITONEAL WASHINGS	93.48 ± 0.54	68.63 ± 3.49	24.63 ± 8.22	1.37 ± 0.31
PERITONEAL CELLS	1.79 ± 0.04	1.32 ± 0.16	0.93 ± 0.39	0.24 ± 0.08
URINE	0.00	3.26 ± 2.66	37.62 ± 12.57	67.23 ± 1.13
FAECES	0.00	0.02 ± 0.01	0.21 ± 0.17	4.77 ± 0.44

FIGURE 7.4

BODY DISTRIBUTION OF ^{125}I-LABELLED COPOLYMER 79 FOLLOWING INTRAPERITONEAL ADMINISTRATION TO RATS

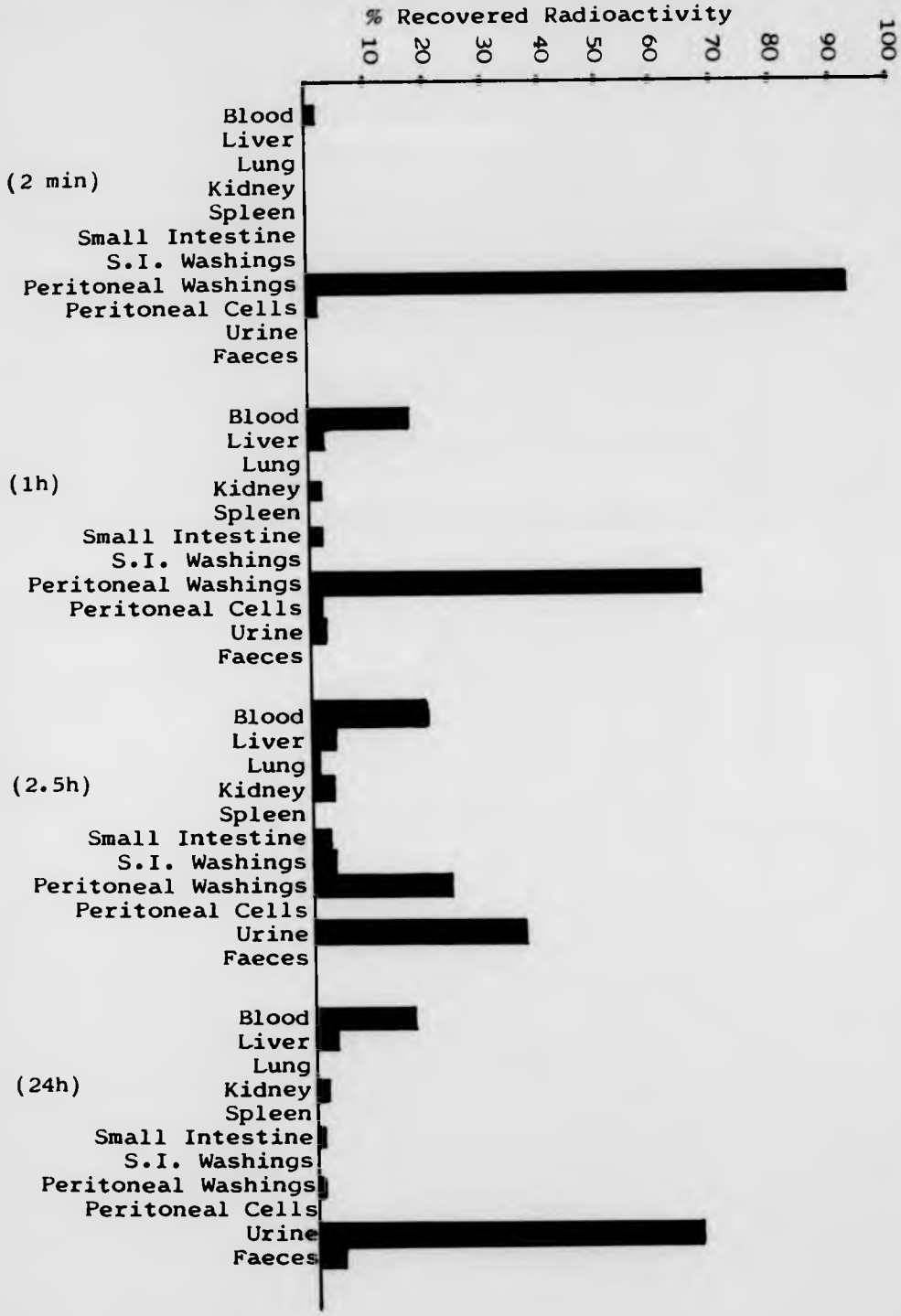
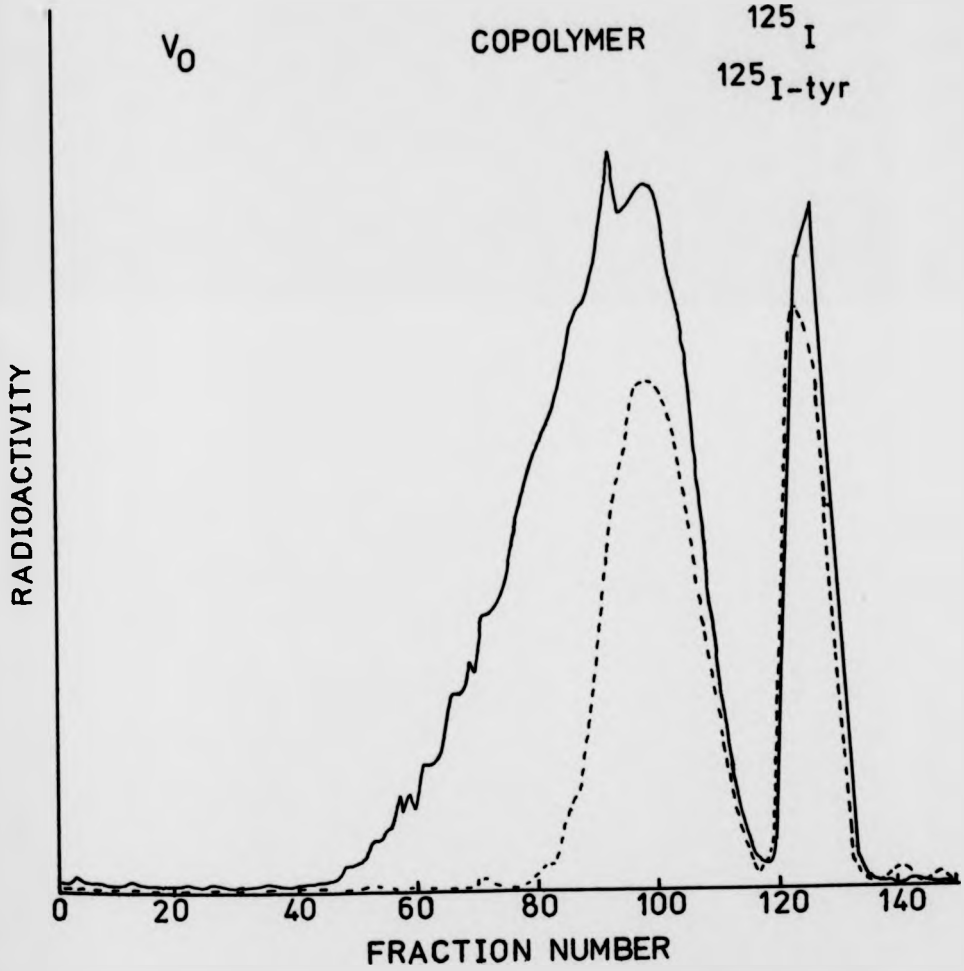


FIGURE 7.5

SEPHAROSE 4B/6B COLUMN CHROMATOGRAPHY OF ^{125}I-LABELLED COPOLYMER 79



—— Distribution of copolymer 79

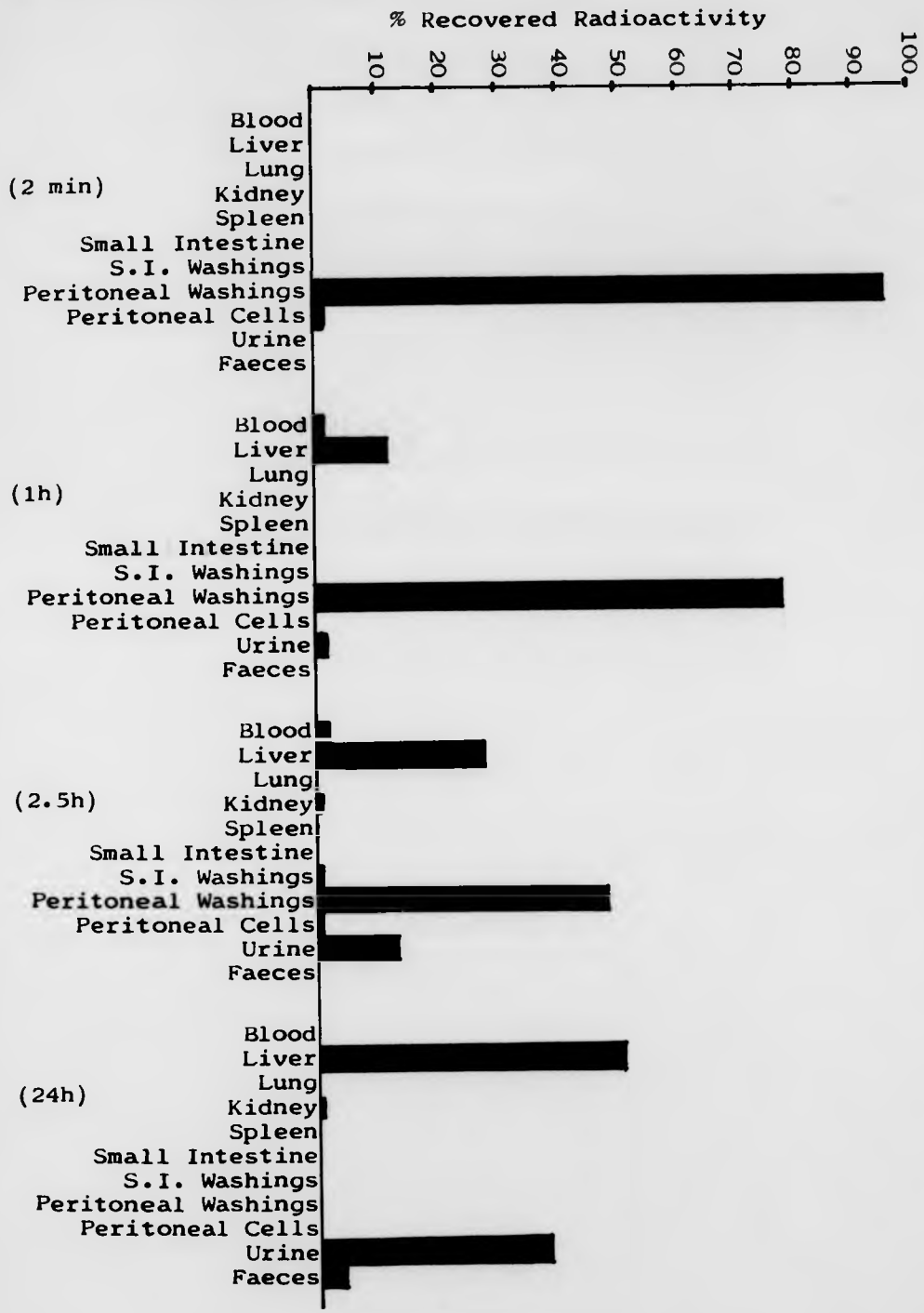
- - - Copolymer 79 in urine 24h following i.p. administration

TABLE 7.4 BODY DISTRIBUTION OF ^{125}I-LABELLED COPOLYMER 78 AFTER INTRAPERITONEAL ADMINISTRATION TO RATS

Organ	2 min	1h	2.5h	24h
BLOOD	0.20 ± 0.08	2.14 ± 0.26	2.64 ± 0.27	0.58 ± 0.30
LIVER	0.23 ± 0.05	13.39 ± 6.23	29.07 ± 3.80	51.90 ± 0.37
LUNG	0.01 ± 0.01	0.04 ± 0.00	0.10 ± 0.02	0.03 ± 0.01
KIDNEY	0.04 ± 0.01	0.68 ± 0.29	1.26 ± 0.10	1.12 ± 0.10
SPLEEN	0.02 ± 0.01	0.02 ± 0.00	0.05 ± 0.01	0.04 ± 0.01
SMALL INTESTINE	0.29 ± 0.11	0.60 ± 0.14	0.94 ± 0.19	0.72 ± 0.19
S.I. WASHINGS	0.04 ± 0.02	0.49 ± 0.11	1.53 ± 0.38	0.37 ± 0.09
PERITONEAL WASHINGS	96.88 ± 0.12	79.10 ± 7.59	49.63 ± 7.49	0.53 ± 0.09
PERITONEAL CELLS	2.30 ± 0.59	0.95 ± 0.35	1.31 ± 0.23	0.14 ± 0.02
URINE	0.00	2.57 ± 0.60	13.47 ± 3.23	39.88 ± 1.43
FAECES	0.00	0.00	0.00	4.69 ± 1.49

FIGURE 7.6

BODY DISTRIBUTION OF ^{125}I-LABELLED COPOLYMER 78 FOLLOWING INTRAPERITONEAL ADMINISTRATION TO RATS



almost 52% of the recovered radioactivity present in the organ 24h following injection. This value is approximately the same as that observed to accumulate in the liver 1h following intravenous administration of the copolymer. Presumably, the copolymer is cleared rapidly by receptor-mediated pinocytosis by liver cells following entry into the blood circulation. This would result in low blood levels, as observed throughout the experimental period.

Although this experiment illustrates the progressive accumulation of copolymer 78 in the liver, a single experiment was performed to try to ascertain whether the accumulation of copolymer took place following transport into the bloodstream, or whether it was possible that the copolymer was binding to receptors present on the outside of the liver, thereby gaining access to the organ from the peritoneal cavity.

An intact liver was removed from a rat following ligation of major blood vessels, and was incubated in medium 199 containing ^{125}I-labelled copolymer 78 at 37^{o}C for 1h. Following incubation, the liver was washed three times in ice cold saline, blotted, homogenised, and samples of homogenate assayed for radioactivity. It was found that only 0.3% of the polymer in the solution was associated with the tissue at the end of the experiment. It was therefore concluded that copolymer accumulation in the liver, following intraperitoneal injection, took place following transport into the blood and removal from the circulation by liver cells.

From Table 7.4 it is apparent that copolymer 78 does not accumulate significantly in any of the other organs investigated. 40% of the recovered radioactivity was found in the urine after

24h. An elution profile of a urine sample is shown in fig 7.7 which shows the presence of copolymer and a small amount of free [^{125}I] iodide.

Table 7.5 summarises the results of chromatography of all the urine samples and shows that, in all cases, the majority of the radioactivity present in the urine represents radiolabelled copolymer rather than low molecular weight [^{125}I] iodide or [^{125}I] iodotyrosine. It can be seen that on administration of ^{125}I-labelled copolymer 39, the percentage of the total counts recovered in the low molecular weight peak is much greater than that which could be expected from free [^{125}I] iodide alone, and must therefore represent a mixture of [^{125}I] iodide and [^{125}I] iodotyrosine crosslink degradation products. The high level of radioactivity present in the second peak from urine containing ^{125}I-labelled copolymer 79 reflects the high level of free [^{125}I] iodide present in the copolymer preparation on administration.

The copolymer present in the urine following intraperitoneal administration of all radiolabelled preparations could represent the filtration of low molecular weight polymer fractions present in the dose administered, or filtration of partially degraded molecules which have at some point undergone pinocytosis, intralysosomal digestion and exocytosis with transport back into the bloodstream. It is probable that a combination of these two possibilities has taken place, particularly in the case of copolymer 39, which, when degraded, releases [^{125}I] iodotyrosine. The elution profile obtained from the urine sample following administration of copolymer 39 indicates the presence of such degradation products. In view of this finding, it is not possible to determine whether the

FIGURE 7.7

SEPHAROSE 4B/6B COLUMN CHROMATOGRAPHY OF ^{125}I-LABELLED COPOLYMER 78

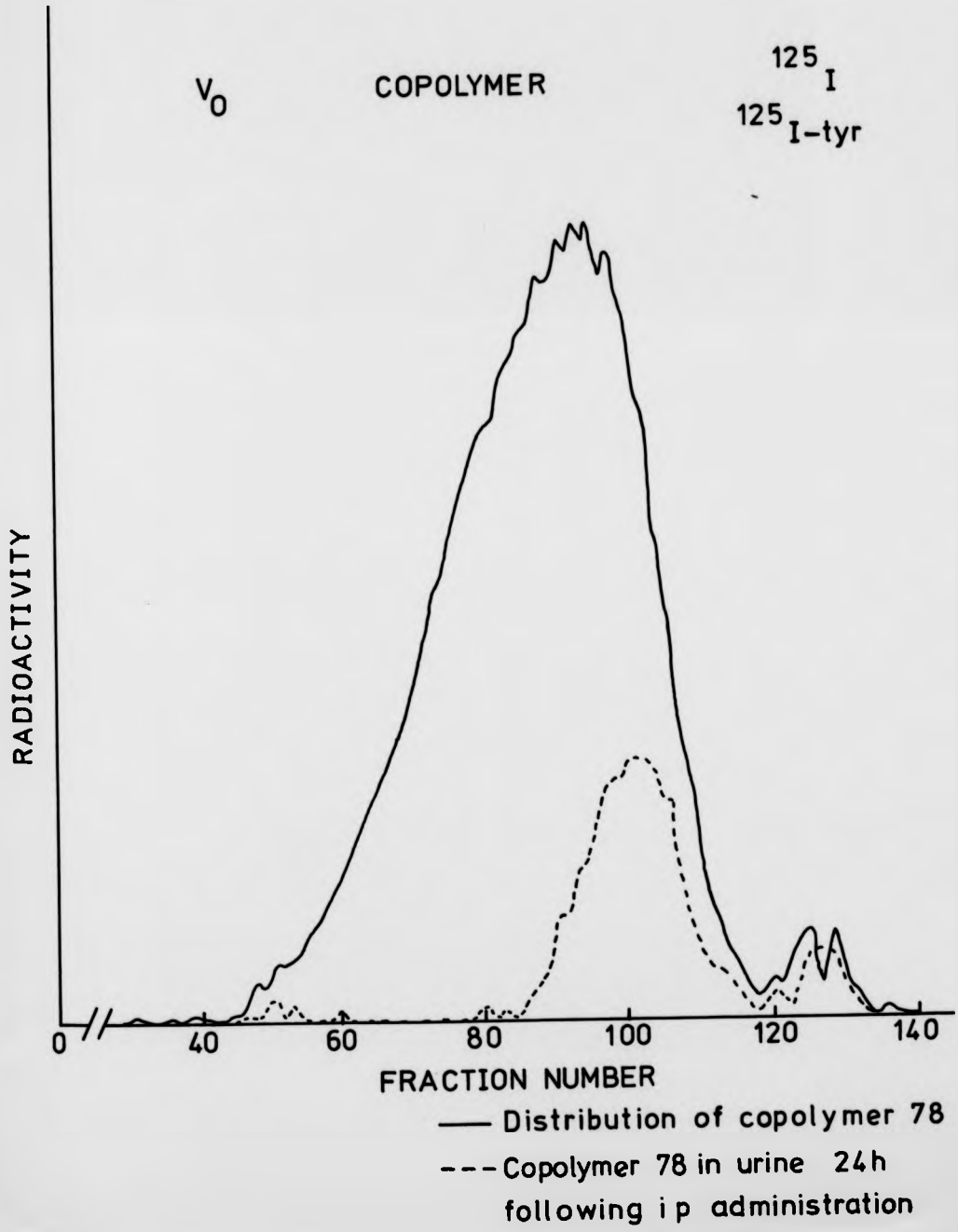


TABLE 7.5

SEPHAROSE 4B/6B CHROMATOGRAPHY OF URINE SAMPLES 24H AFTER INTRAPERITONEAL INJECTION OF COPOLYMERS

POLYMER CODE NO.	RECOVERY FROM COLUMN (%)	POLYMER (1ST PEAK)		L.M.W. PEAK (2ND PEAK)		% IODIDE IN DOSE ADMINISTERED
		% TOTAL COUNTS	% DOSE ADMINISTERED IN TOTAL URINE	% TOTAL COUNTS	% DOSE ADMINISTERED IN TOTAL URINE	
39	93.14	60.08	6.87	33.55	3.77	3.86
78	96.87	84.71	3.17	8.33	0.30	2.08
79	99.55	67.27	10.52	29.97	4.72	16.25

observed organ distribution of radioactivity on administration of ^{125}I-labelled copolymer 39 represents the presence of ^{125}I-labelled copolymer or ^{125}I-labelled crosslink degradation products. Extensive degradation of copolymer 39 seems unlikely, however, over the experimental period, in view of the similarity between the body distributions of copolymer 39 and copolymer 79, which contains radiolabelled polymer chains. The most likely explanation for the slight differences observed in the body distribution of copolymers 39 and 79 24h following intraperitoneal administration, is the difference in average molecular weight of the two copolymers (39 $\bar{M}w$ 190,000 : 79 $\bar{M}w$ 68,000). Copolymer 79 is of a lower average molecular weight and therefore contains a larger number of molecules small enough to pass through the kidney glomerulus. This results in a higher proportion of the recovered radioactivity in the urine and a lower proportion in the blood on administration of copolymer 79 than on administration of copolymer 39.

7.2.2 Organ Deposition of ^{125}I-labelled Copolymers Following Subcutaneous Administration to Rats

Male Wistar rats (200-250g) were injected subcutaneously with 125 µg of ^{125}I-labelled copolymer (39, 78 or 79) in PBS as described in section 2.11.3. Following periods of 1h, 5h and 24h, animals were anaesthetized, blood samples taken and then the animals were sacrificed as described in sections 2.11.5 and 2.11.6. Organs were removed, homogenised and assayed for radioactivity. Again, all experiments were carried out in triplicate. The percentage of the administered dose recovered at the end of each experiment was calculated and the results are shown in Table 7.6. From this table, it can be seen that although dose recoveries obtained at each time point were variable, there was an increase in dose recovery with time.

TABLE 7.6

SUBCUTANEOUS INJECTION - DOSE RECOVERIES (%)

SUBSTRATE	1h	5h	24h
39	7.19	38.51	62.90
	9.33	24.88	71.21
	11.91	17.47	66.76
78	14.28	40.32	42.50
	9.43	39.81	63.84
	14.24	32.93	56.76
79	14.68	29.10	36.27
	26.63	29.47	65.98
	14.24	24.48	65.54

The reason for this is that the organs sampled in the case of subcutaneous injection did not include the injection site, unlike previous routes of administration (peritoneal cavity and bloodstream). The dose recoveries indicate that migration of the copolymer from the site of injections is relatively slow, but after 24h dose recoveries approach those obtained following intraperitoneal injection of copolymers. Because of the low recoveries obtained following short experimental periods (1 and 5h), the data presented will include typical results expressed as a percentage of the dose administered, in addition to the usual format i.e. percentage of the recovered dose.

The body distribution of radioactivity following subcutaneous administration of ^{125}I-labelled copolymer 39 is shown in Table 7.7 and fig 7.8. It can be seen that the radioactivity recovered after 1h is largely confined to the blood, kidney and urine. After 24h the recovered radioactivity is found largely in the blood, urine and faeces, as on intraperitoneal administration of this copolymer, but a greater proportion of the recovered radiolabelled material is excreted within 24h following subcutaneous administration. Similarly, subcutaneous administration of ^{125}I-labelled copolymer 79 (Table 7.8 and fig 7.9) results in the recovery of radioactivity largely from the blood, kidney and urine within 1h, with the majority of recovered radioactivity excreted by the kidney after 24h. Again, more of the recovered radioactivity is excreted with 24h following subcutaneous injection of ^{125}I-labelled copolymer than following intraperitoneal administration of the same copolymer.

Table 7.9 and fig 7.10 show the results following subcutaneous administration of ^{125}I-labelled copolymer 78 to rats. As

TABLE 7.7

BODY DISTRIBUTION OF ^{125}I-LABELLED COPOLYMER 39 FOLLOWING SUBCUTANEOUS ADMINISTRATION TO RATS

ORGAN	% RECOVERED RADIOACTIVITY 1h	*% DOSE ADMINISTERED 1h	% RECOVERED RADIOACTIVITY 5h	*% DOSE ADMINISTERED 5h	% RECOVERED RADIOACTIVITY 24h	*% DOSE ADMINISTERED 24h
BLOOD	28.94 ± 2.53	2.47	18.12 ± 2.67	5.23	8.95 ± 1.06	5.89
LIVER	7.33 ± 1.98	0.83	3.20 ± 0.27	1.04	1.73 ± 0.15	1.23
LUNG	1.01 ± 0.21	0.11	0.65 ± 0.12	0.18	0.22 ± 0.04	0.15
KIDNEY	19.76 ± 3.12	1.95	6.31 ± 1.26	1.52	0.80 ± 0.06	0.57
SPLEEN	0.33 ± 0.07	0.04	0.13 ± 0.02	0.04	0.05 ± 0.01	0.03
URINE	42.36 ± 7.84	1.76	71.43 ± 4.26	30.34	58.19 ±12.10	50.32
FAECES	0.27 ± 0.22	0	0.15 ± 0.12	0.17	30.07 ±11.80	10.75

*Typical results

FIGURE 7.8

BODY DISTRIBUTION OF ^{125}I-LABELLED COPOLYMER 39 FOLLOWING SUBCUTANEOUS ADMINISTRATION TO RATS

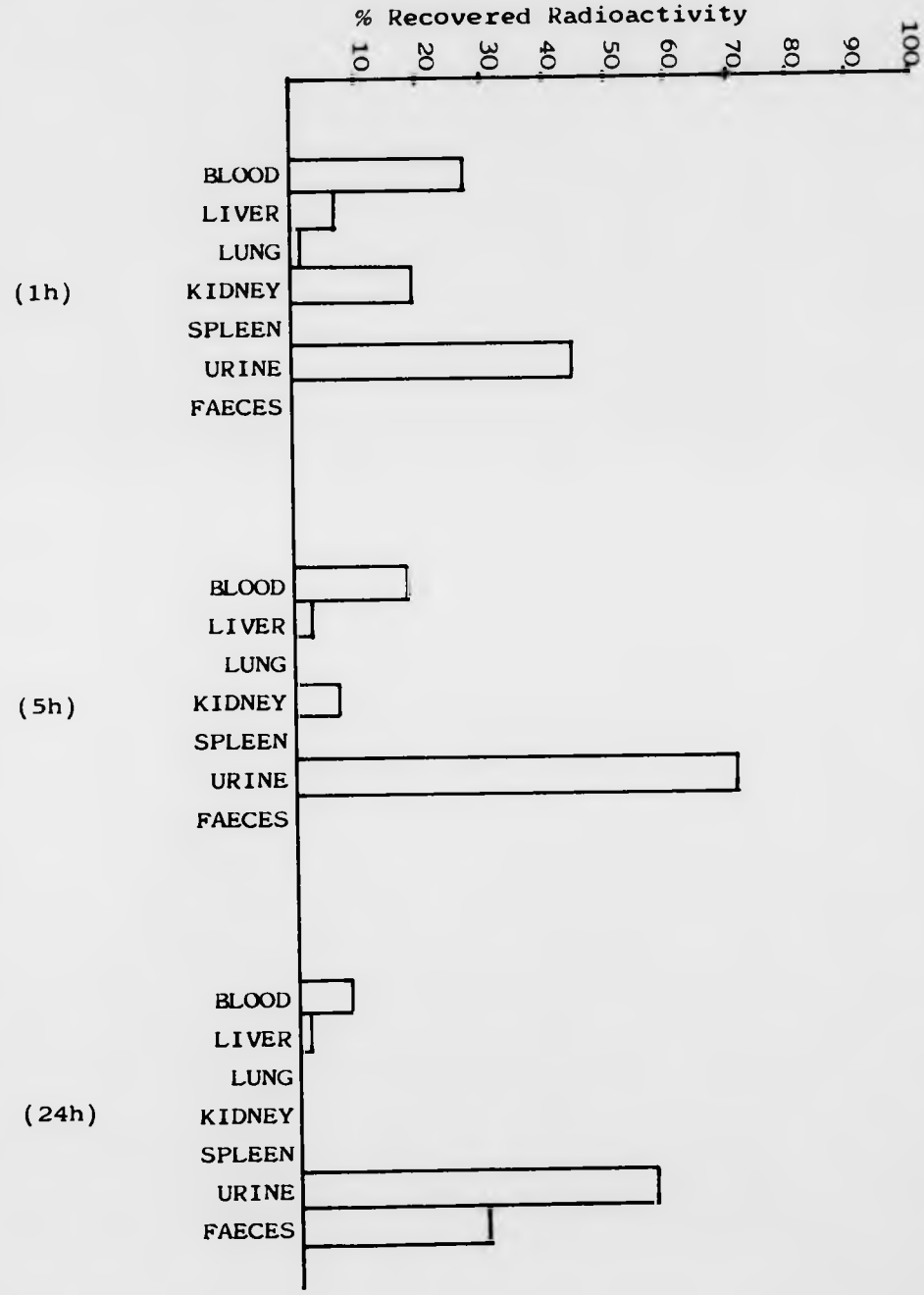


TABLE 7.8

BODY DISTRIBUTION OF ^{125}I-LABELLED COPOLYMER 79 FOLLOWING SUBCUTANEOUS ADMINISTRATION TO RATS

ORGAN	% RECOVERED RADIOACTIVITY 1h	*% DOSE ADMINISTERED 1h	% RECOVERED RADIOACTIVITY 5h	*% DOSE ADMINISTERED 5h	% RECOVERED RADIOACTIVITY 24h	*% DOSE ADMINISTERED 24h
BLOOD	30.22 ± 12.25	2.75	9.10 ± 0.29	2.60	5.82 ± 0.02	3.26
LIVER	5.95 ± 1.89	0.71	1.75 ± 0.11	0.57	1.22 ± 0.11	0.66
LUNG	1.22 ± 0.49	0.11	0.30 ± 0.04	0.11	0.17 ± 0.02	0.09
KIDNEY	13.41 ± 5.25	1.21	3.98 ± 0.08	1.21	1.40 ± 0.18	0.74
SPLEEN	0.45 ± 0.22	0.02	0.08 ± 0.01	0.02	0.06 ± 0.01	0.03
URINE	48.74 ± 20.09	9.87	84.54 ± 0.51	24.36	83.38 ± 1.17	46.64
FAECES	0	0	0.25 ± 0.20	0.21	7.95 ± 1.18	4.51

*Typical results

FIGURE 7.9

BODY DISTRIBUTION OF ^{125}I-LABELLED COPOLYMER 79 FOLLOWING SUBCUTANEOUS ADMINISTRATION TO RATS

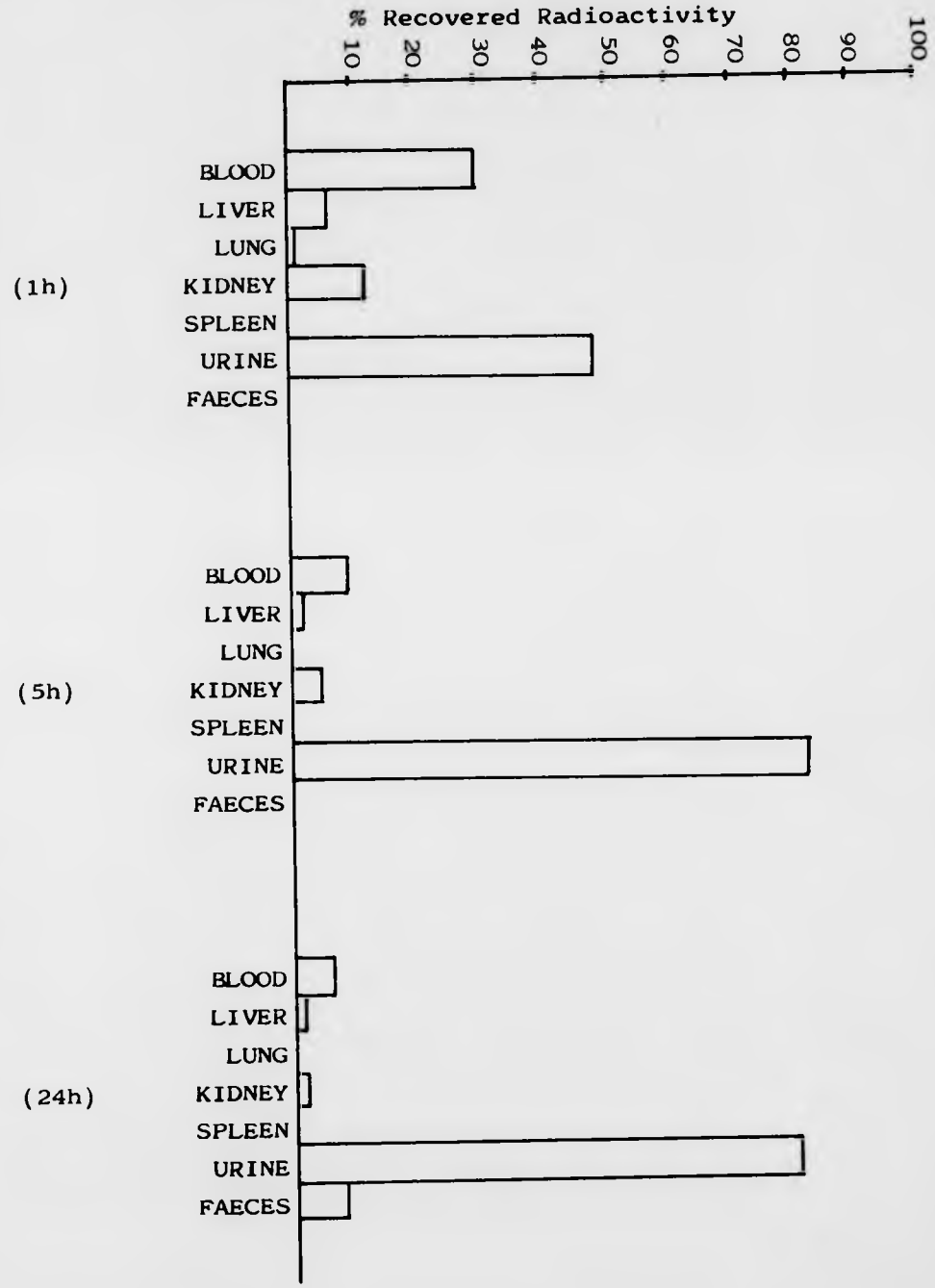


TABLE 7.9

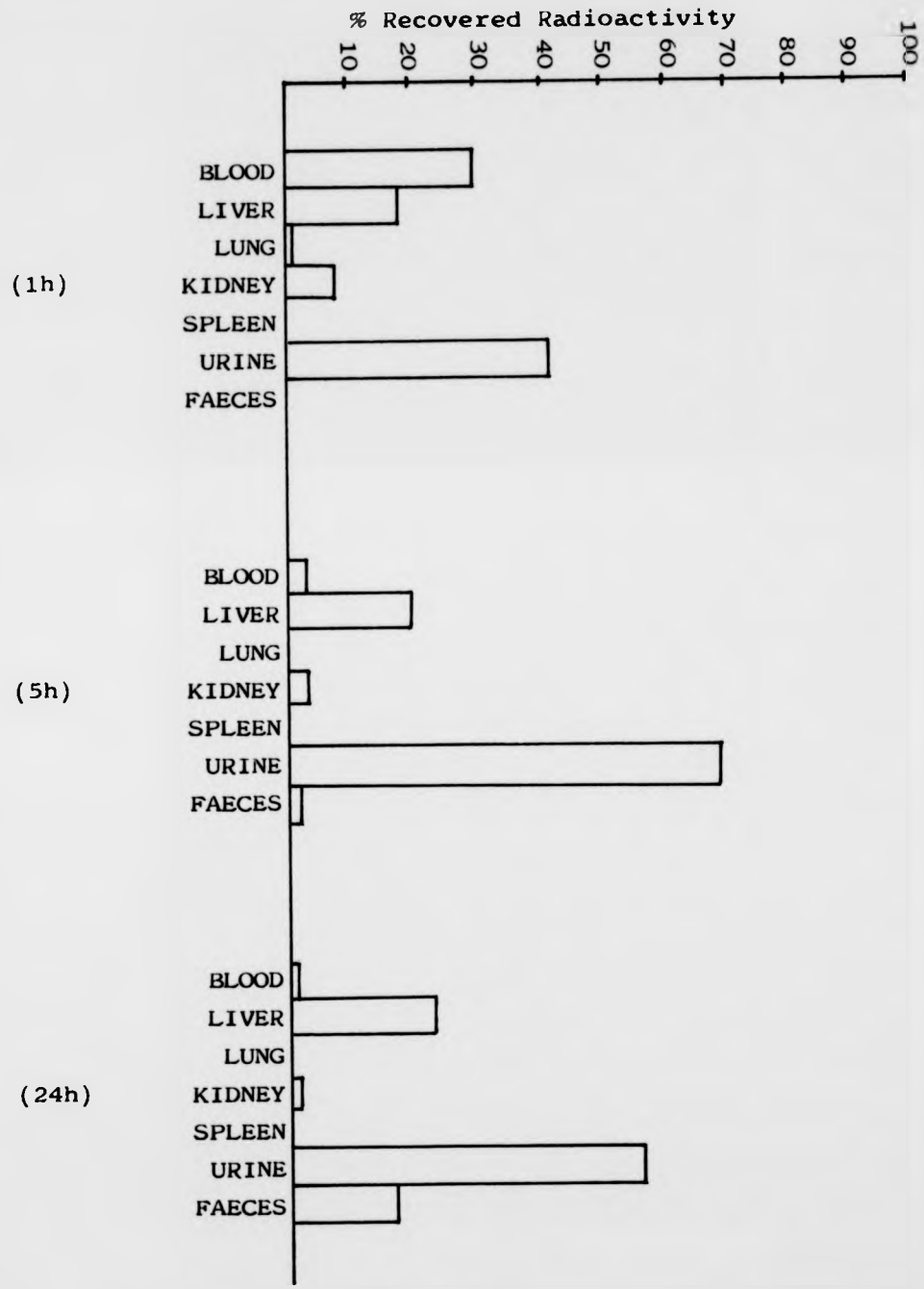
BODY DISTRIBUTION OF ^{125}I-LABELLED COPOLYMER 78 FOLLOWING SUBCUTANEOUS ADMINISTRATION TO RATS

ORGAN	% RECOVERED RADIOACTIVITY 1h	*% DOSE ADMINISTERED 1h	% RECOVERED RADIOACTIVITY 5h	*% DOSE ADMINISTERED 5h	% RECOVERED RADIOACTIVITY 24h	*% DOSE ADMINISTERED 24h
BLOOD	29.12 ± 7.82	1.96	3.47 ± 0.57	1.31	1.19 ± 0.33	0.60
LIVER	18.58 ± 0.39	2.78	20.35 ± 1.27	7.67	23.58 ± 1.03	12.93
LUNG	1.14 ± 0.30	0.07	0.27 ± 0.03	0.10	0.05 ± 0.01	0.03
KIDNEY	8.75 ± 0.65	1.04	3.96 ± 0.48	1.46	1.76 ± 0.23	0.94
SPLEEN	0.41 ± 0.14	0.01	0.16 ± 0.09	0.06	0.04 ± 0.01	0.02
URINE	41.99 ± 8.35	8.42	69.47 ± 1.59	26.17	57.08 ± 2.05	30.95
FAECES	0	0	2.54 ± 0.37	0.90	16.25 ± 2.70	8.92

*Typical results

FIGURE 7.10

BODY DISTRIBUTION OF ^{125}I-LABELLED COPOLYMER 78 FOLLOWING SUBCUTANEOUS ADMINISTRATION TO RATS



observed with copolymers 39 and 79, the radioactivity recovered after 1h is present in the blood, kidney and urine in measurable quantities. In addition, 18.58% of the recovered radioactivity is again associated with the liver. The percentage of recovered radioactivity in the liver is seen to rise very slowly over the 24h period (fig 7.10), but when results are expressed as a percentage of the dose administered (Table 7.9), it is apparent that because the dose recoveries are increasing with time, the actual quantity of polymer accumulating in the liver is increasing quite rapidly with time.

A further interesting feature in Table 7.9 is that in excess of 16% of the recovered radioactivity is present in the faeces 24h after subcutaneous injection of copolymer 78. This value is somewhat higher than that observed 24h following intraperitoneal administration of the same copolymer (4.69%) and also higher than that observed for copolymer 79 by either route (4.77% intraperitoneal and 7.95% subcutaneous).

The pattern of organ deposition and excretion of copolymers following subcutaneous administration is different from that observed following intraperitoneal administration of the copolymers in that more of the recovered radioactivity is found in the urine following subcutaneous injection in all cases. This is best illustrated after 24h where dose recoveries following both routes of administration are similar. This indicates that a greater proportion of smaller molecular weight copolymer fractions is eliminated from the body following subcutaneous injection than following intraperitoneal injection after 24h. This, in turn, could indicate either that there is a preferential movement of smaller molecules away from the site of injection following subcutaneous injection, but not (at

least not so pronounced) following intraperitoneal injection, or that copolymers undergo more degradation of crosslinks en route to the circulation following subcutaneous administration than following intraperitoneal administration.

7.2.3 Organ Deposition of ^{125}I-labelled Copolymers Following Oral Administration to Rats

^{125}I-Labelled copolymers (125 µg in PBS) were administered into the stomachs of Male Wistar rats (200-250g body weight) as described in section 2.11.4. Duplicate blood samples (50 µl) were taken 1h, 5h or 24h following administration of copolymer (section 2.11.5), after which the animals were sacrificed. Organs were removed, washed and assayed for radioactivity as described in section 2.11.6. Dose recoveries were calculated following each experiment, the results of which are shown in Table 7.10. The percentage of the dose recovered at the end of each experiment was variable, but no obvious trend with time was apparent.

The organ distribution of radioactivity recovered following oral administration of ^{125}I-labelled copolymer 39 is shown in Table 7.11 and fig 7.11. A very high proportion (42.44%) of the recovered radioactivity was associated with the small intestine 1h following administration of the copolymer. 45.34% of the recovered radioactivity was found in the caecum (+ contents) but it was not possible to determine whether this radioactivity was associated with the tissue or the material contained within it. Results presented in section 5.2.1 demonstrated the preferential uptake of high molecular weight copolymer 39 (fraction a, $\bar{M}w$ >400,000) by rat intestine in vitro. The rate of uptake of this fraction was approximately five times that of the smallest fraction 39e ($\bar{M}w$ 34,000) and the fluid-

TABLE 7.10

ORAL ADMINISTRATION - DOSE RECOVERIES (%)

SUBSTRATE	1h	5h	24h
39	55.12	114.55	76.83
	81.73	105.59	97.99
	63.96	111.70	76.28
78	83.72	76.04	78.80
	54.98	86.06	57.21
	67.89	80.76	66.67
79	53.79	74.00	45.00
	45.70	83.00	64.11
	62.52	64.72	56.40

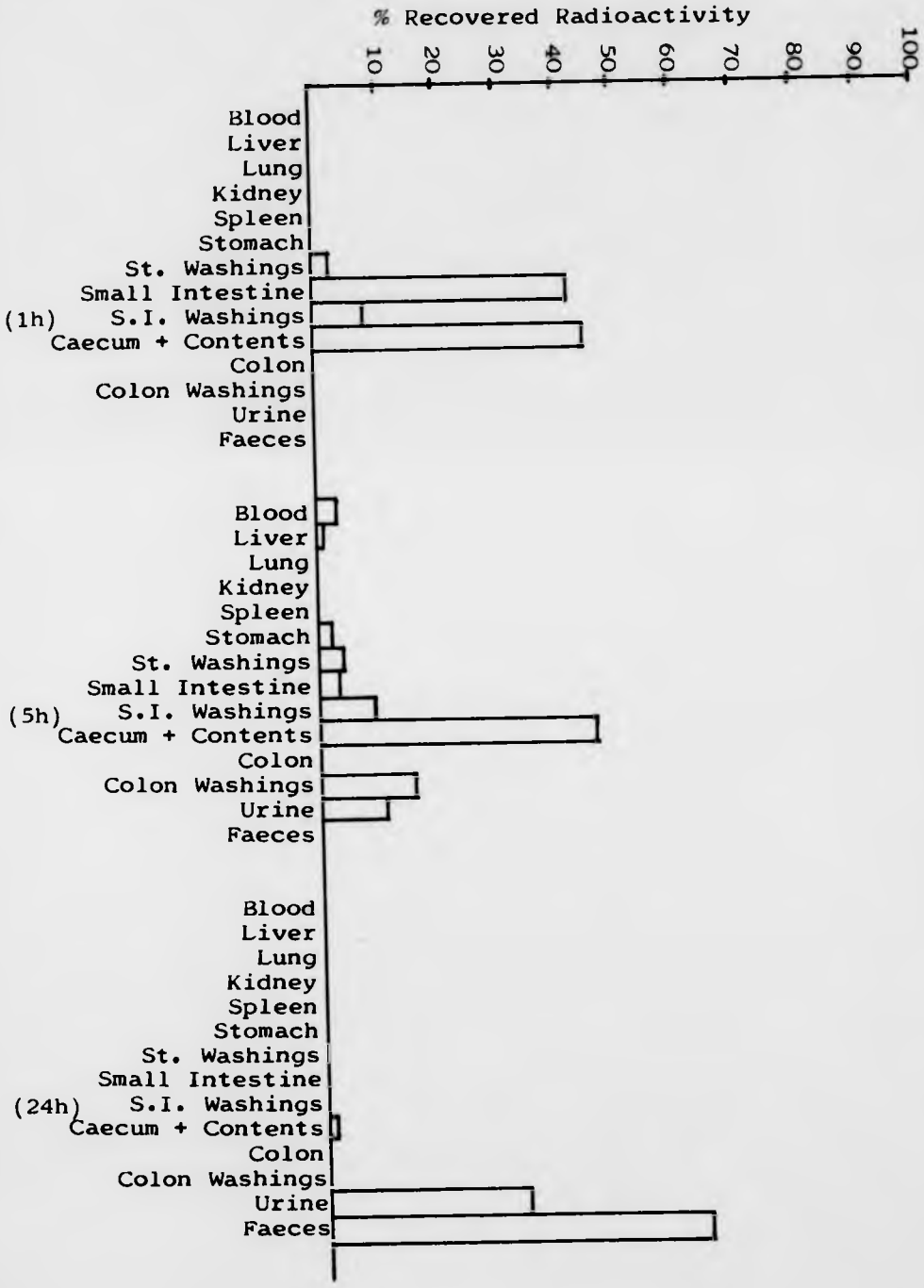
TABLE 7.11

BODY DISTRIBUTION OF ^{125}I-LABELLED COPOLYMER 39 FOLLOWING ORAL ADMINISTRATION TO RATS

POLYMER CODE NO.	ORGAN	1h	5h	24h
39	BLOOD	0.11 ± 0.03	4.68 ± 0.60	0.03 ± 0.02
	LIVER	0.33 ± 0.06	1.37 ± 0.10	0.07 ± 0.02
	LUNG	0.61 ± 0.10	0.35 ± 0.15	0.01 ± 0.01
	KIDNEY	0.15 ± 0.03	0.48 ± 0.02	0.01 ± 0.01
	SPLEEN	0.38 ± 0.07	0.09 ± 0.02	0.00
	STOMACH	0.04 ± 0.00	2.18 ± 0.10	0.10 ± 0.02
	ST. WASHINGS	2.23 ± 0.29	4.38 ± 1.18	0.48 ± 0.15
	SMALL INTESTINE	42.44 ± 4.72	3.86 ± 0.57	0.13 ± 0.04
	S.I. WASHINGS	7.97 ± 2.64	9.37 ± 3.73	0.28 ± 0.10
	CAECUM + CONTENTS	45.34 ± 2.09	46.92 ± 1.98	1.36 ± 0.75
	COLON	0.14 ± 0.03	0.89 ± 0.18	0.04 ± 0.02
	COLON WASHINGS	0.23 ± 0.04	14.48 ± 3.48	0.71 ± 0.52
	URINE	0.00	10.69 ± 0.99	32.77 ± 4.55
	FAECES	0.02 ± 0.02	0.27 ± 0.16	64.01 ± 5.56

FIGURE 7.11

BODY DISTRIBUTION OF ^{125}I-LABELLED COPOLYMER 39 FOLLOWING ORAL ADMINISTRATION TO RATS

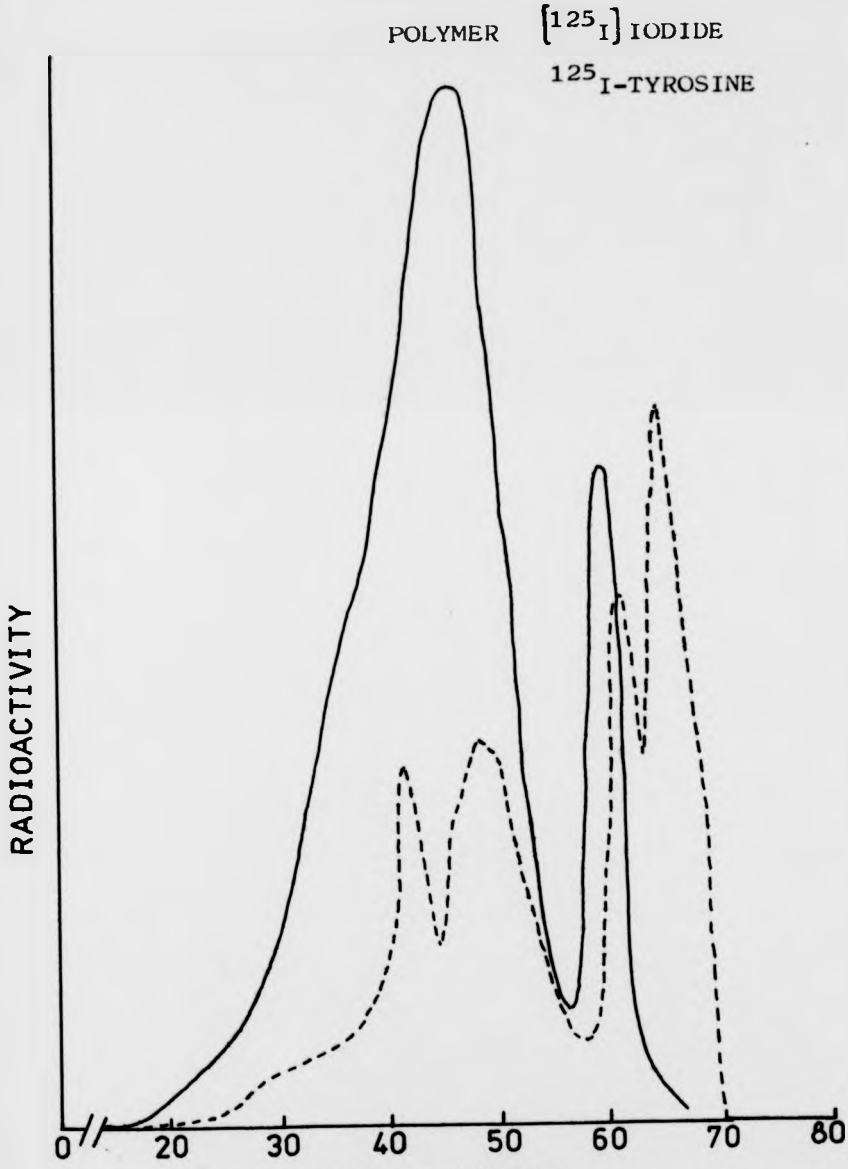


phase marker ^{125}I-PVP ($\bar{M}w$ 40,000). However, the crosslink contained in copolymer 39 (P-Gly-Gly-Phe-Tyr-HMDA-Tyr-Phe-Gly-Gly-P) has been shown to be rapidly hydrolysed by chymotrypsin (49% crosslink cleavage in 5 min) (section 1.4), which is secreted into the lumen of the small intestine. In view of this, the most likely explanation for a large proportion of this tissue-associated radioactivity seems not to be the pinocytic uptake of copolymer by the small intestine, but the rapid uptake of free [^{125}I] iodotyrosine following its release on degradation of copolymer by chymotrypsin present in the gut lumen. Results after 5h show that the intestinal tissue level had dropped to less than 4%, blood levels had risen from 0.11% to 4.68% and 10.69% of the recovered radioactivity had been excreted in the urine. Within 24h virtually all of the radioactivity recovered was present in the urine (32.77%) and faeces (64.01%).

In order to try to establish whether or not significant degradation of copolymer had taken place on its passage through the small intestine, a 1 ml sample of caecum homogenate (which included the contents) was precipitated with 20% trichloroacetic acid (TCA) to remove protein, centrifuged (2,000g for 20 min) and the supernatant applied to a Sepharose 4B/6B column (section 2.5). Material from the caecum was chosen after a 5h experimental period, rather than washings from the small intestine, because of the comparatively high level of radioactivity present in the tissue with its contents at that time (45.34%). The elution profile obtained is shown in fig 7.12: similar levels of radioactivity elute in two peaks, one of which co-elutes with ^{125}I-labelled copolymer, and the other with [^{125}I] iodide and [^{125}I] iodotyrosine. This shows that the copolymer has been degraded, since the polymer peak present

FIGURE 7.12

SEPHAROSE 4B/6B COLUMN CHROMATOGRAPHY OF ^{125}I-LABELLED COPOLYMER 39



— Distribution of copolymer 39

---Copolymer 39 recovered from the caecum following oral administration

co-elutes with the lower molecular weight fractions in copolymer preparation, and a greater proportion of radioactivity is present in the second peak than in the copolymer preparation, indicating the presence of radiolabelled degradation products. Although these results do not indicate whether copolymer or $[^{125}I]$ iodotyrosine, or both, are taken up by intestinal tissue, they do show that $[^{125}I]$ iodotyrosine was released in the gut lumen.

The body distribution of radioactivity following oral administration of copolymer 79 is shown in Table 7.12 and fig 7.13. Since the radiolabel is attached to the copolymer chain and is not removed by enzymes, the observed 7% of the recovered radioactivity observed in the bloodstream after 1h may represent some translocation of macromolecules across the small intestine into the circulation. The possibility that this radioactivity represents free $[^{125}I]$ iodide, at least in part, cannot, however, be discounted. Over 2% of the radioactivity was recovered from the liver after 1h, but the remainder of the copolymer was largely confined to the stomach (6.94%) the small intestine (7.46%) and their contents. After 5h, blood levels drop to 1.9% and the polymer can be seen to have moved further down the intestinal tract, located mainly in the caecum and colon contents. Within 24h virtually all the radioactivity has been eliminated from the body, although only approximately half the amount of radioactivity is recovered in the urine (18%) than on administration of copolymer 39 (33%).

Sepharose 4B/6B column chromatography of TCA-precipitated caecum homogenate (fig 7.14) was carried out in order to observe any change in the molecular weight distribution of the radiolabelled copolymer. The elution profile indicates that

TABLE 7.12

BODY DISTRIBUTION OF ^{125}I-LABELLED COPOLYMER 79 FOLLOWING ORAL ADMINISTRATION TO RATS

POLYMER CODE NO.	ORGAN	1h	5h	24h
79	BLOOD	7.38 ± 3.28	1.90 ± 0.71	0.98 ± 0.27
	LIVER	2.10 ± 0.28	0.21 ± 0.05	0.05 ± 0.01
	LUNG	0.26 ± 0.03	0.06 ± 0.02	0.00
	KIDNEY	0.55 ± 0.16	0.08 ± 0.02	0.01 ± 0.01
	SPLEEN	0.07 ± 0.01	0.02 ± 0.01	0.00
	STOMACH	6.94 ± 2.95	0.53 ± 0.05	0.15 ± 0.10
	ST. WASHINGS	54.08 ± 9.35	8.40 ± 3.70	0.98 ± 0.67
	SMALL INTESTINE	7.46 ± 1.71	1.91 ± 0.53	0.71 ± 0.56
	S.I. WASHINGS	20.45 ± 0.45	10.26 ± 4.66	1.09 ± 0.81
	CAECUM + CONTENTS	0.29 ± 0.07	48.94 ± 3.25	3.28 ± 1.66
	COLON	0.17 ± 0.05	0.54 ± 0.12	0.06 ± 0.01
	COLON WASHINGS	0.28 ± 0.11	14.99 ± 4.00	2.46 ± 0.97
	URINE	0.00	4.15 ± 0.44	18.04 ± 2.25
	FAECES	0.00	8.01 ± 6.50	72.21 ± 1.40

FIGURE 7.13

BODY DISTRIBUTION OF ^{125}I-LABELLED COPOLYMER 79 FOLLOWING ORAL ADMINISTRATION TO RATS

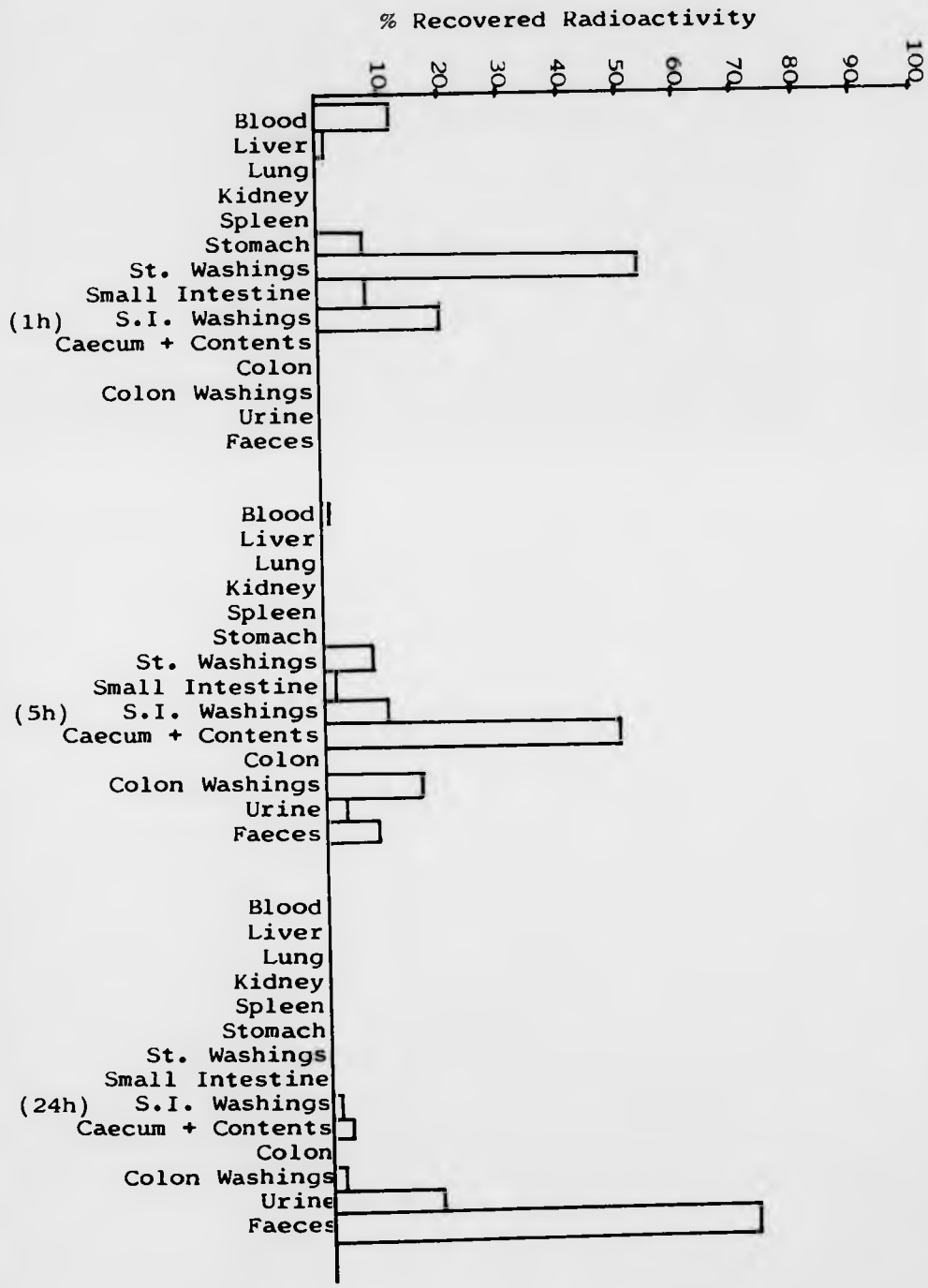
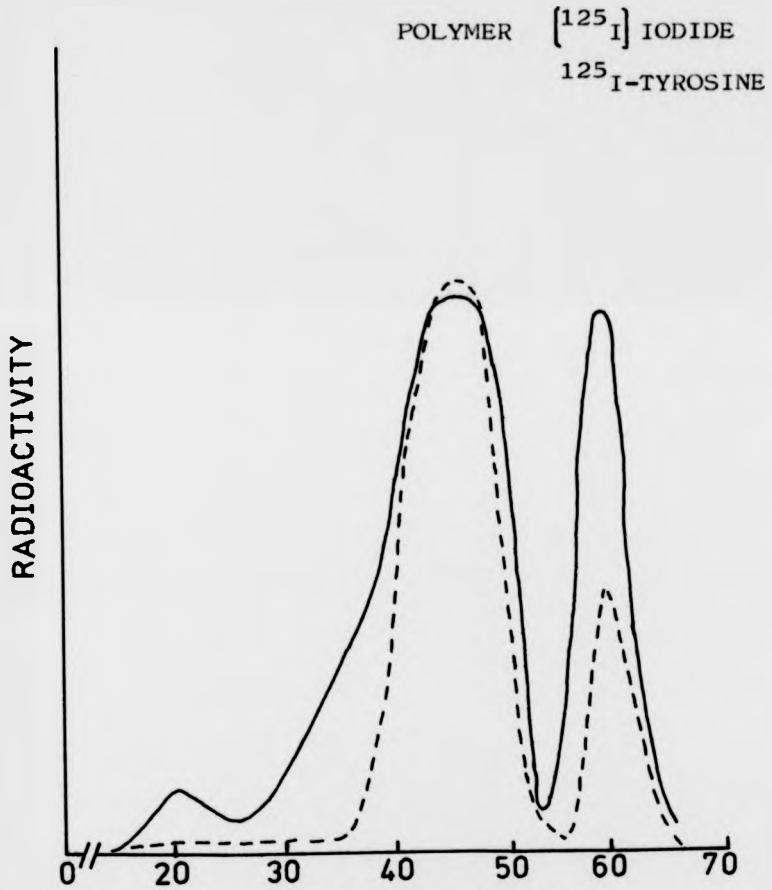


FIGURE 7.14

SEPHAROSE 4B/6B COLUMN CHROMATOGRAPHY OF ^{125}I-LABELLED COPOLYMER 79

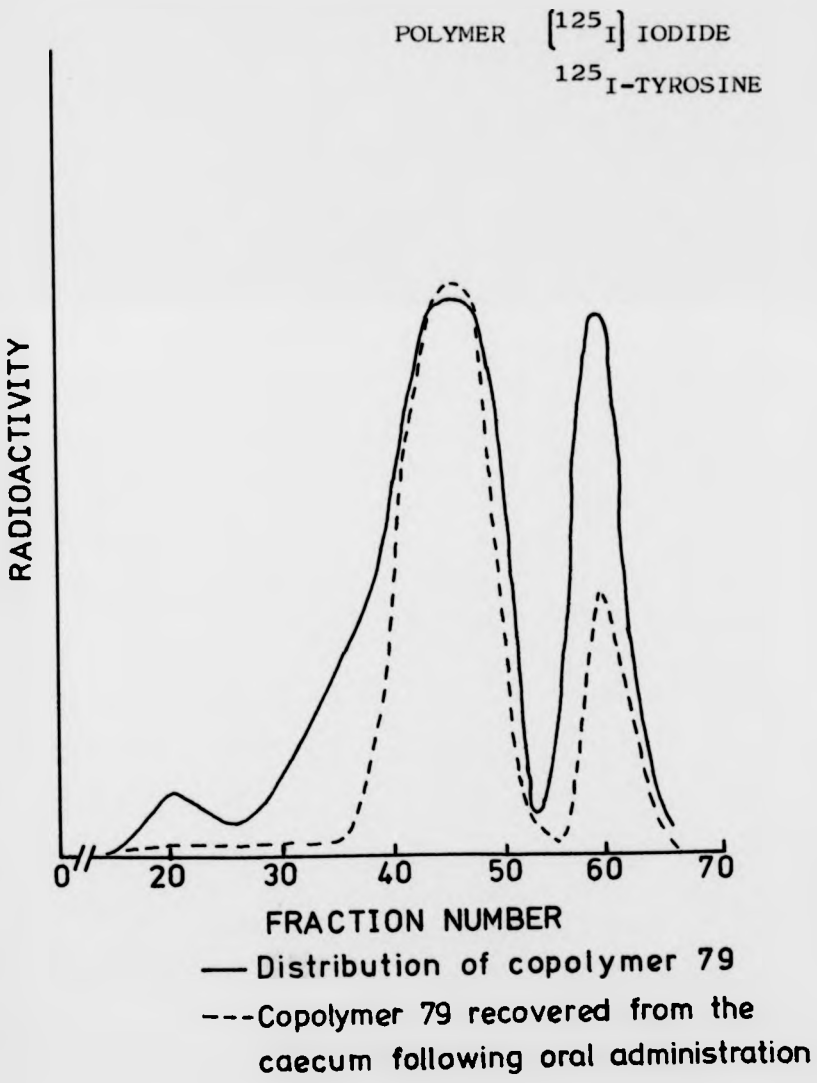


— Distribution of copolymer 79

--- Copolymer 79 recovered from the caecum following oral administration

FIGURE 7.14

SEPHAROSE 4B/6B COLUMN CHROMATOGRAPHY OF ^{125}I-LABELLED COPOLYMER 79



some copolymer crosslink degradation has occurred during the passage of the copolymer through the small intestine and into the caecum, since the resultant polymer peak appears to co-elute with the smaller molecular weight fractions present in the original copolymer preparation.

The organ distribution of ^{125}I-labelled copolymer 78 following its oral administration to rats is shown in Table 7.13 and fig 7.15. It can be seen that, like copolymer 39, a large proportion of the recovered radioactivity (65.81%) is associated with the tissue of the small intestine 1h following administration. A major difference between the two copolymer structures, however, is the position of the radiolabelled tyrosine residue, which is present in the degradable crosslink of copolymer 39, but is attached to the polymer chain of copolymer 78. After 5h however, the body distributions of copolymers 39 and 78 are very different. Unlike the distribution of radiolabel on using copolymer 39, copolymer 78 is still largely confined to the intestinal tract, with very little elsewhere, and the level of radioactivity associated with the small intestine has dropped to just over 1% of the recovered radioactivity. Within 24h, in excess of 80% of the recovered radioactivity is found in the faeces, and only 7% in the urine. These findings demonstrate that the copolymer could not have been pinocytosed by the small intestine, despite the levels noted 1h following oral administration, since one would expect to find significant levels of radioactivity in the blood, kidney, urine and perhaps the liver following the passage of copolymer across the intestine. Instead, the distribution of radioactivity recovered 24h following oral administration of copolymer 78 is similar to that observed for the control copolymer (79) which does not contain galactosamine, and is not

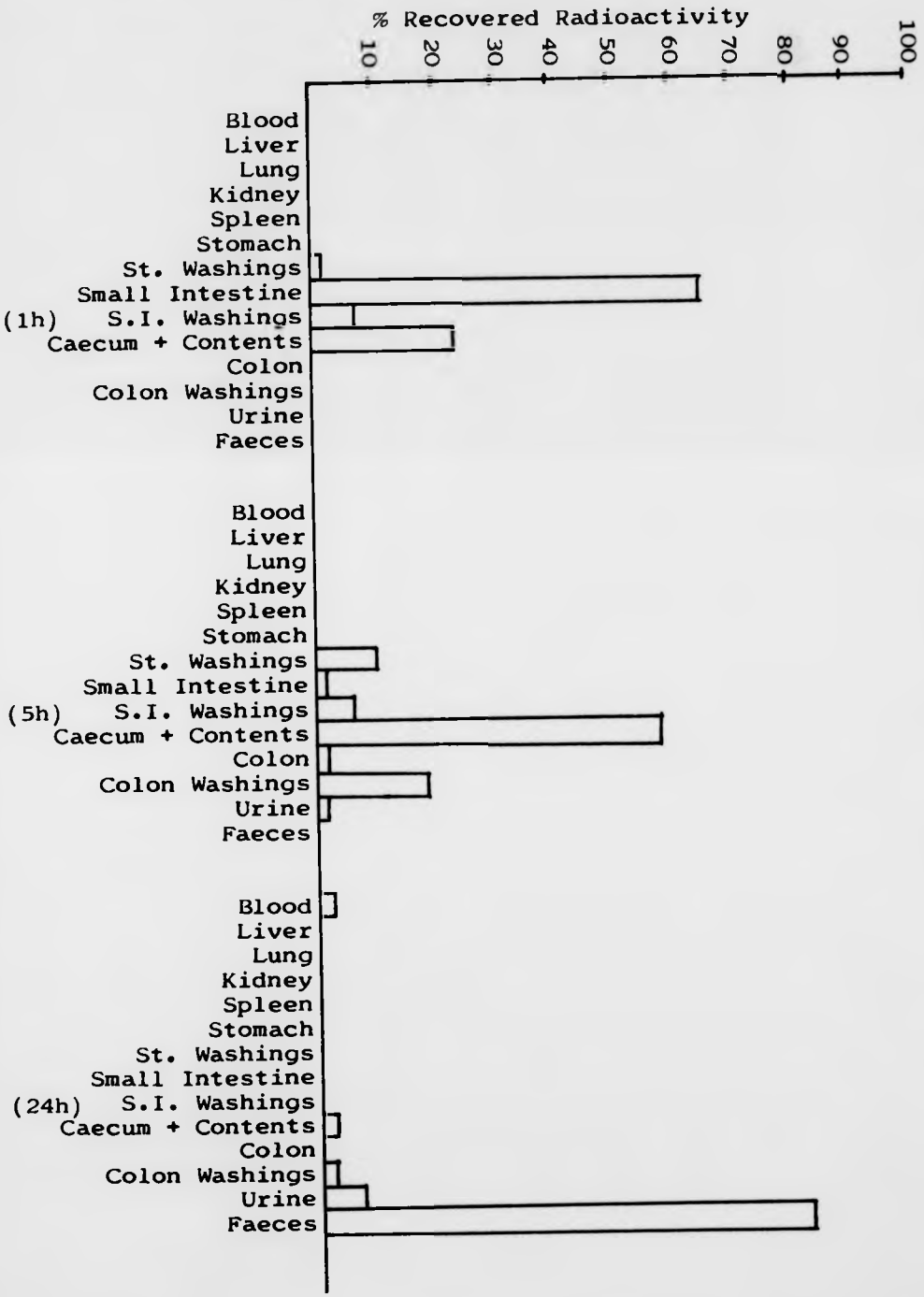
TABLE 7.13

BODY DISTRIBUTION OF ^{125}I-LABELLED COPOLYMER 78 FOLLOWING ORAL ADMINISTRATION TO RATS

POLYMER CODE NO.	ORGAN	1h	5h	24h
78	BLOOD	0.64 ± 0.11	0.63 ± 0.28	2.68 ± 0.43
	LIVER	0.11 ± 0.02	0.05 ± 0.03	0.05 ± 0.02
	LUNG	0.17 ± 0.06	0.01 ± 0.00	0.02 ± 0.01
	KIDNEY	0.06 ± 0.01	0.02 ± 0.01	0.00
	SPLEEN	0.08 ± 0.02	0.01 ± 0.00	0.01 ± 0.01
	STOMACH	0.04 ± 0.01	0.17 ± 0.02	0.01 ± 0.01
	ST. WASHINGS	2.22 ± 0.34	10.50 ± 1.87	0.09 ± 0.04
	SMALL INTESTINE	65.81 ±10.48	1.17 ± 0.22	0.04 ± 0.02
	S.I. WASHINGS	7.33 ± 2.38	7.38 ± 2.06	0.17 ± 0.07
	CAECUM + CONTENTS	23.05 ± 9.23	57.94 ± 7.92	2.83 ± 1.07
	COLON	0.06 ± 0.01	2.32 ± 1.27	0.04 ± 0.00
	COLON WASHINGS	0.20 ± 0.01	18.07 ± 3.97	2.61 ± 1.86
	URINE	0.01 ± 0.01	1.46 ± 0.90	7.31 ± 0.91
	FAECES	0.23 ± 0.12	0.26 ± 0.19	84.13 ± 2.07

FIGURE 7.15

BODY DISTRIBUTION OF ^{125}I-LABELLED COPOLYMER 78 FOLLOWING ORAL ADMINISTRATION TO RATS



associated with the small intestine following oral administration.

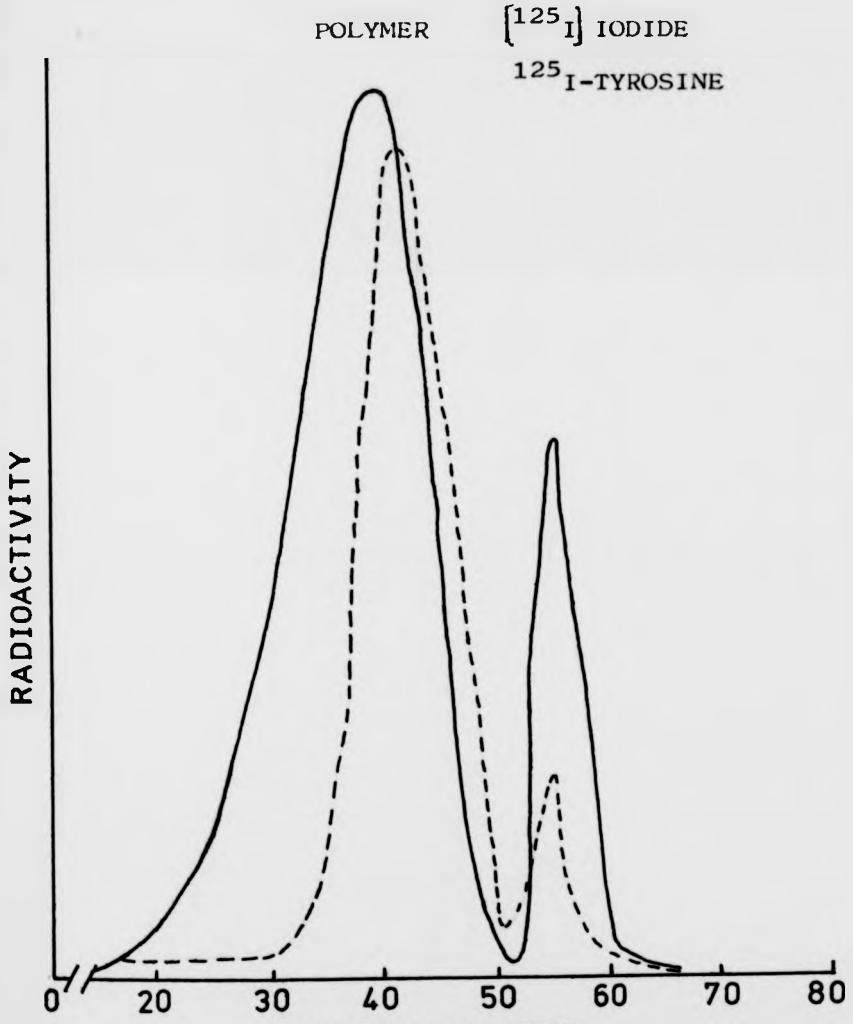
These results can be explained by the hypothesis that the galactose-containing copolymer bound to receptors present on the surface of cells of the small intestine, but that subsequent internalization of copolymer did not take place to any significant extent. Either copolymer was displaced by galactose, or similar moieties, present in the food subsequently eaten by the animals, or enzymes present on the microvillous brush border of the enterocytes (section 5.1) or the gut lumen, were able to hydrolyse a bond present in the linkage of the galactose to the polymer (P-Gly-Gly-galactosamine). Each of these possibilities would result in the body distribution pattern observed i.e. a temporary association of copolymer with cells of the small intestine, after which the radiolabelled copolymer continues its passage through the alimentary tract.

Sepharose 4B/6B column chromatography of TCA-precipitated caecum homogenate was carried out following oral administration of ^{125}I-labelled copolymer 78 as described previously and the elution profile (fig 7.16) shows results very similar to those observed using copolymer 79. Copolymer recovered from the caecum appears to be of a lower molecular weight than the preparation administered, indicating some degree of crosslink cleavage by enzymes present in the gut lumen.

It is clear from results presented that significant translocation of copolymer across the intestine does not take place, and that it is not possible to target copolymer 78 to the liver following oral administration.

FIGURE 7.16

SEPHAROSE 4B/6B COLUMN CHROMATOGRAPHY OF ^{125}I-LABELLED COPOLYMER 78

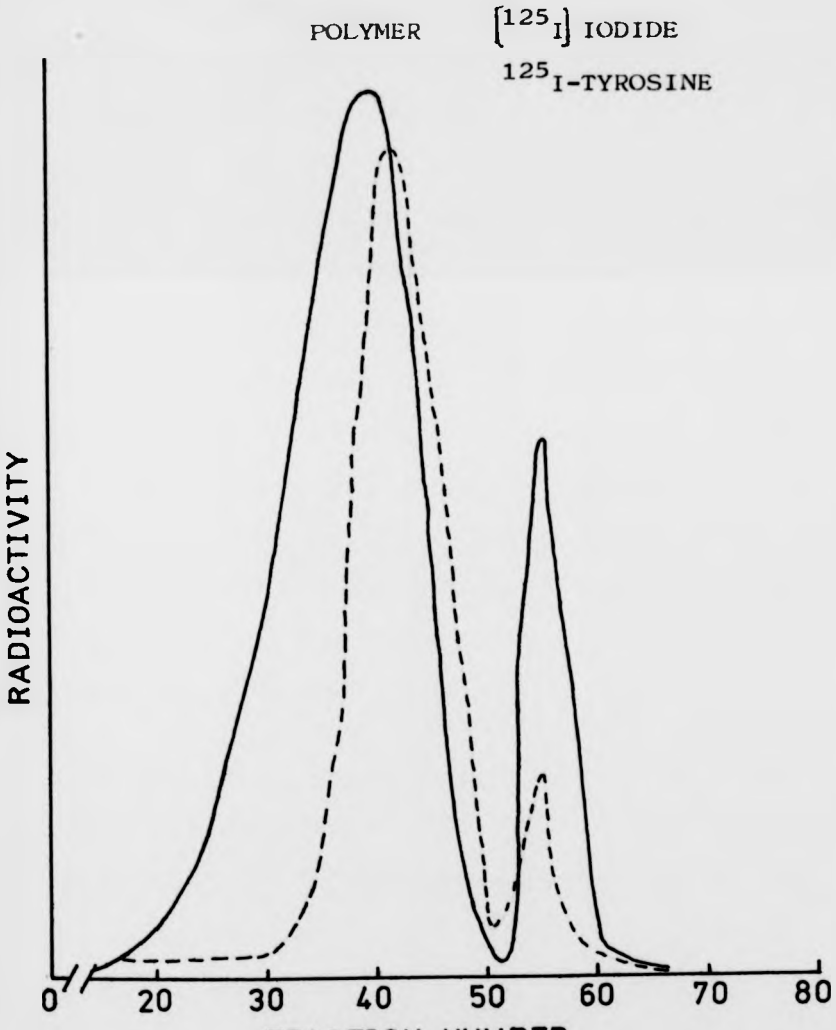


—Distribution of copolymer 78

---Copolymer 78 recovered from the caecum following oral administration

FIGURE 7.16

SEPHAROSE 4B/6B COLUMN CHROMATOGRAPHY OF ^{125}I-LABELLED COPOLYMER 78



—Distribution of copolymer 78

---Copolymer 78 recovered from the caecum following oral administration

7.2.4 Uptake of ^{125}I-labelled Copolymers 78 and 79 by Rat Jejunal Sacs In Vitro

In view of the finding that ^{125}I-labelled copolymer 78 interacts with intestinal cell membranes 1h following its oral administration to rats, this copolymer and control copolymer 79 (which contains no galactosamine) were studied using the everted rat intestinal sacs exactly as described in Chapter 5 to see whether this observation could be reproduced in vitro. Since it was suspected that copolymer 78 bound to the tissue through the galactosamine moiety but was not pinocytosed, the experiment was carried out at both 37°C and 15°C. If copolymer 78 bound to cells, one would expect a high association of the copolymer with the tissue at low temperature, but little, if any, interaction between copolymer 79 and the cell membranes under the same conditions. If the copolymers were pinocytosed by the tissue at 37°C, one would expect a greater uptake of copolymer 78 by receptor-mediated pinocytosis than copolymer 79 which should enter the tissue in the fluid-phase at a rate proportional to the molecular size of the copolymer ($\bar{M}w$ 63,800). The results of the experiments are shown in figs 7.17, 7.18 and Table 7.14.

Clearly, the in vitro experiments do not confirm the observations in vivo. No significant difference in uptake or binding of the two substrates is apparent at 37°C or 15°C. The results support previous findings (section 5.2.4) that values at 15°C are lower than those noted at 37°C. Neither copolymer shows affinity for the enterocyte cell membrane, and uptake values at 37°C are similar to those observed for lower molecular weight copolymer 39 fractions (section 5.2.1). Copolymer 79, which does not contain galactosamine, appears

FIGURE 7.17

ACCUMULATION OF ^{125}I-LABELLED COPOLYMER 79 BY THE EVERTED RAT JEJUNAL SAC

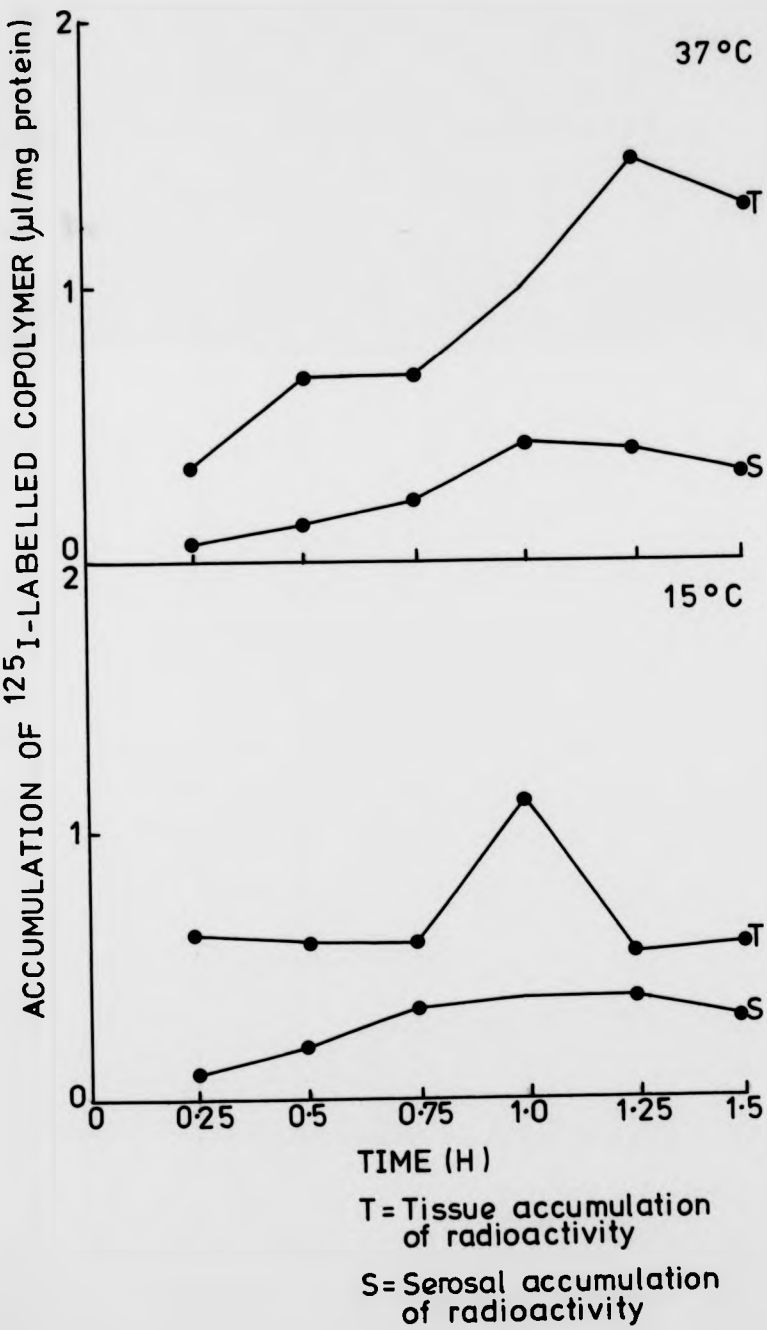
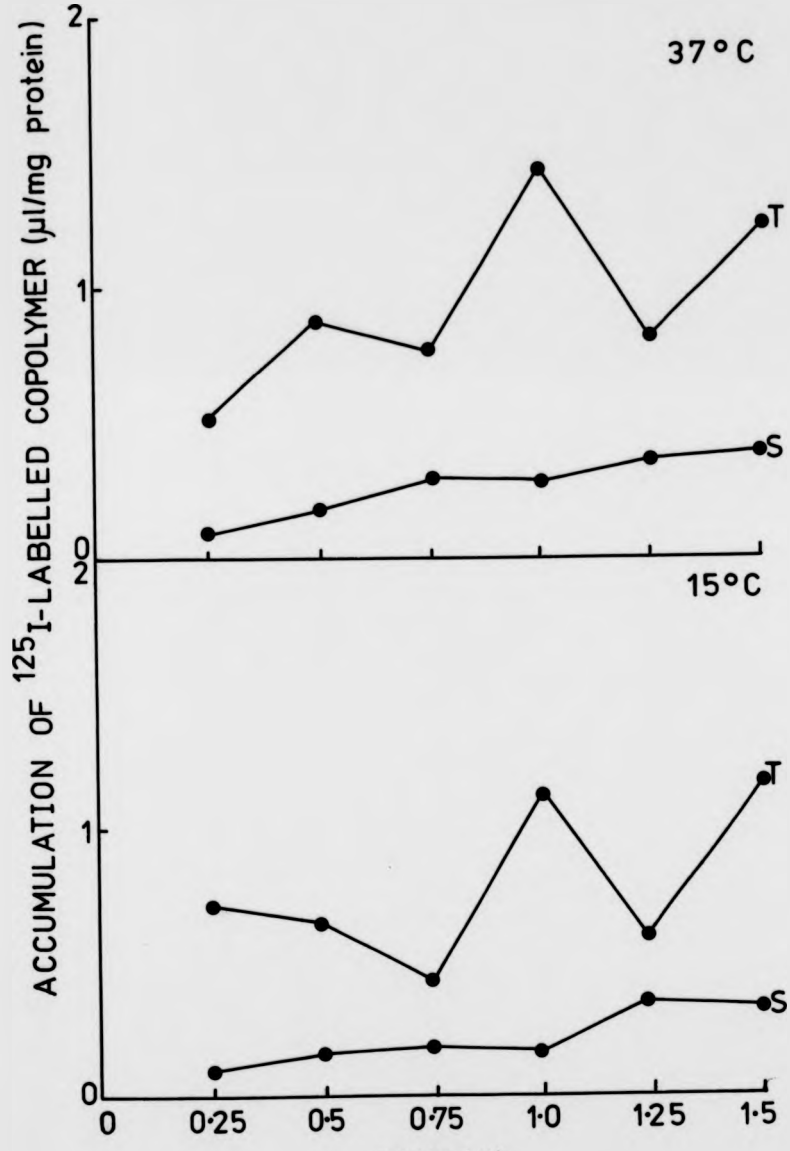


FIGURE 7.18

ACCUMULATION OF ^{125}I-LABELLED COPOLYMER 78 BY THE EVERTED RAT JEJUNAL SAC



T = Tissue accumulation of radioactivity

S = Serosal accumulation of radioactivity

TABLE 7.14

UPTAKE OF ^{125}I-LABELLED COPOLYMERS 78 AND 79 BY RAT JEJUNAL SACS IN VITRO AT 15°C AND 37°C

POLYMER CODE NO.	RATE OF TISSUE ACCUMULATION (µl/mg protein/h)	RATE OF SEROSAL ACCUMULATION (µl/mg protein/h)	T + S (µl/mg/h)
78 (15°C)	0·339	0·180	0·519
78 (37°C)	0·452	0·214	0·666
79 (15°C)	0·034	0·184	0·218
79 (37°C)	0·880	0·265	1·145

to enter the tissue at a slightly higher rate than copolymer 78, the exact opposite to the expected result. Although the experiment was performed once only, using two jejunal sacs for each time point, it can be safely concluded that copolymer 78 does not bind significantly to the tissue.

It is possible that the galactosamine receptors may be present in a different region of the small intestine and not in the jejunum. Other possible explanations for this finding are that the receptors were damaged or their conformations altered during preparation of the tissue e.g. through disturbing the mucus layer which coats the intestinal epithelium or under the experimental conditions e.g. pH. If either of the two explanations are responsible for the observed differences between the in vivo and in vitro experiments performed using these substrates, the experimental results reported in Chapter 5 may not represent the findings one would make on repeating those experiments in vivo.

7.2.5 Body Clearance of ^{125}I-labelled Copolymers 78 and 79 Following Intraperitoneal Administration to Rats

Two male Wistar rats (each weighing 250g) were used for this preliminary experiment. Each was injected intraperitoneally with ^{125}I-labelled copolymer 78 or 79 (125 µg) as described in section 2.11.2 and placed into a metabolic cage. Urine and faeces were collected regularly and assayed for radioactivity. After 32 days, the animals were sacrificed exactly as described in section 2.11.6 following collection of blood samples (section 2.11.5). The organ distributions of radioactivity are shown in Table 7.15 and the recoveries of radioactivity from the urine and faeces are shown in figs 7.19 and 7.20.

TABLE 7.15

BODY DISTRIBUTION OF RADIOACTIVITY 32 DAYS FOLLOWING INTRAPERITONEAL ADMINISTRATION OF ^{125}I-LABELLED COPOLYMERS 78 AND 79

ORGAN	78 % DOSE ADMINISTERED	78 % RECOVERED RADIOACTIVITY	79 % DOSE ADMINISTERED	79 % RECOVERED RADIOACTIVITY
BLOOD	2.30	2.29	2.53	2.65
LIVER	1.11	1.11	0.33	0.35
LUNG	0.05	0.05	0.05	0.06
KIDNEY	0.16	0.16	0.08	0.09
SPLEEN	0.07	0.07	0.09	0.09
STOMACH	0.03	0.03	0.04	0.05
ST. WASHINGS	0.05	0.05	0.02	0.02
SMALL INTESTINE	0.16	0.16	0.12	0.13
S.I. WASHINGS	0.06	0.06	0.03	0.03
CAECUM + CONTENTS	0.06	0.06	0.03	0.03
COLON	0.08	0.08	0.06	0.06
COLON WASHINGS	0.07	0.07	0.01	0.01
PERITONEAL WASHINGS	0.09	0.09	0.05	0.05
PERITONEAL CELLS	0.01	0.01	0.00	0.00
URINE	62.12	61.76	81.42	85.24
FAECES	34.15	33.96	10.62	11.11
TOTAL	100.57	100.00	95.51	100.00

FIGURE 7.19

RECOVERY OF ^{125}I-LABELLED COPOLYMER 79 FROM URINE AND FAECES FOLLOWING I.P. ADMINISTRATION

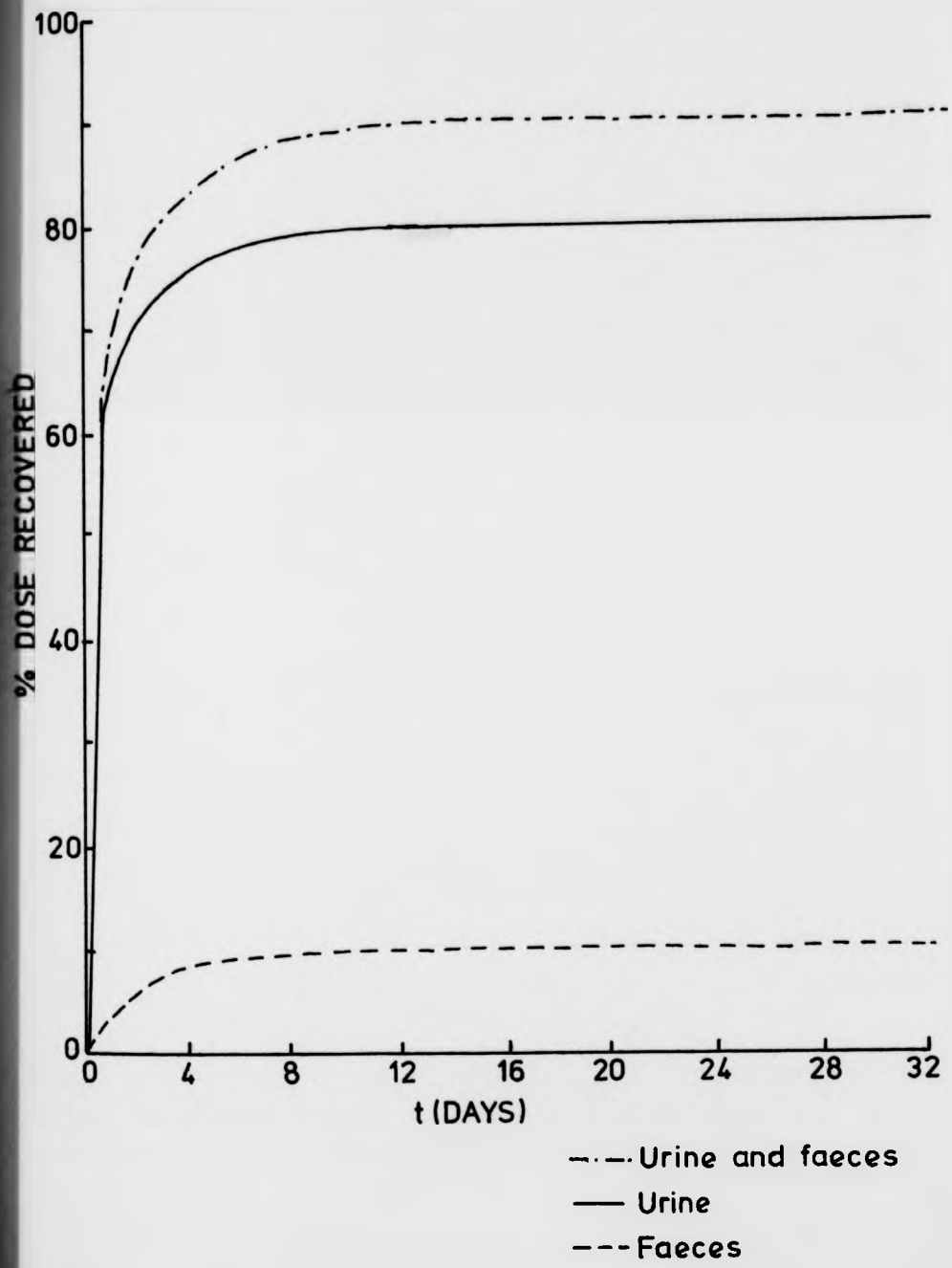


FIGURE 7.19

RECOVERY OF ^{125}I-LABELLED COPOLYMER 79 FROM URINE AND FAECES FOLLOWING I.P. ADMINISTRATION

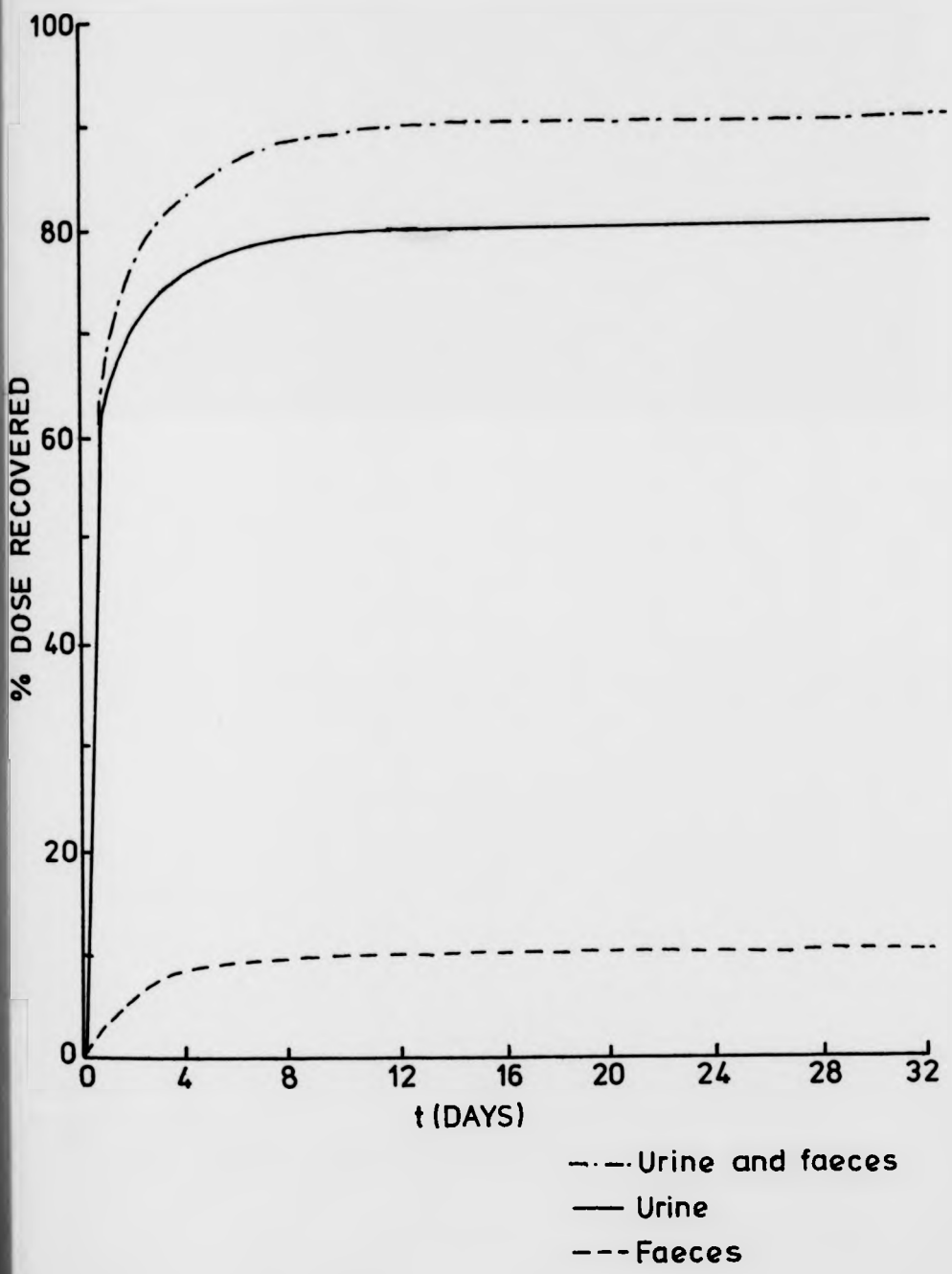


FIGURE 7.20

RECOVERY OF ^{125}I-LABELLED COPOLYMER 78 FROM URINE AND FAECES FOLLOWING I.P. ADMINISTRATION

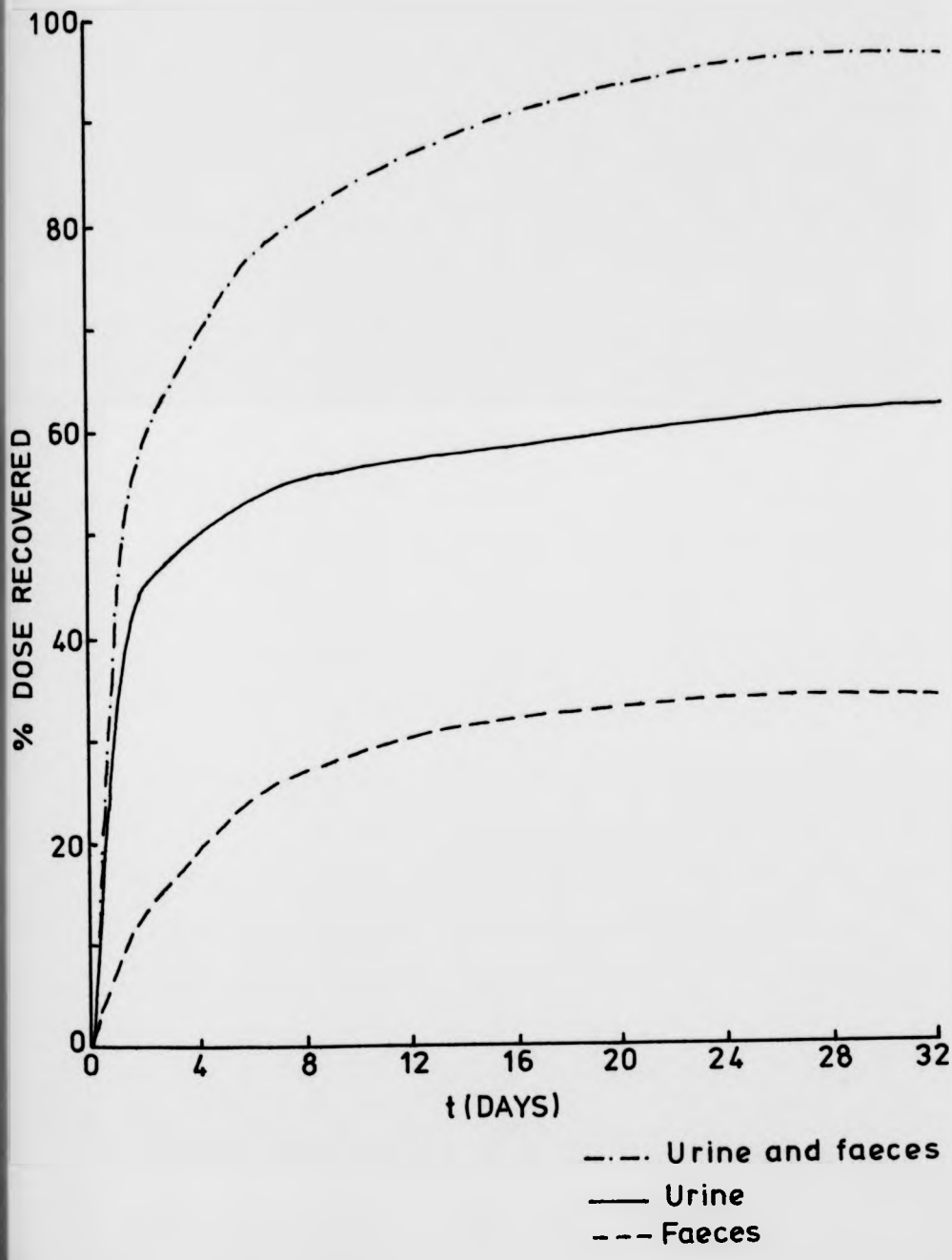
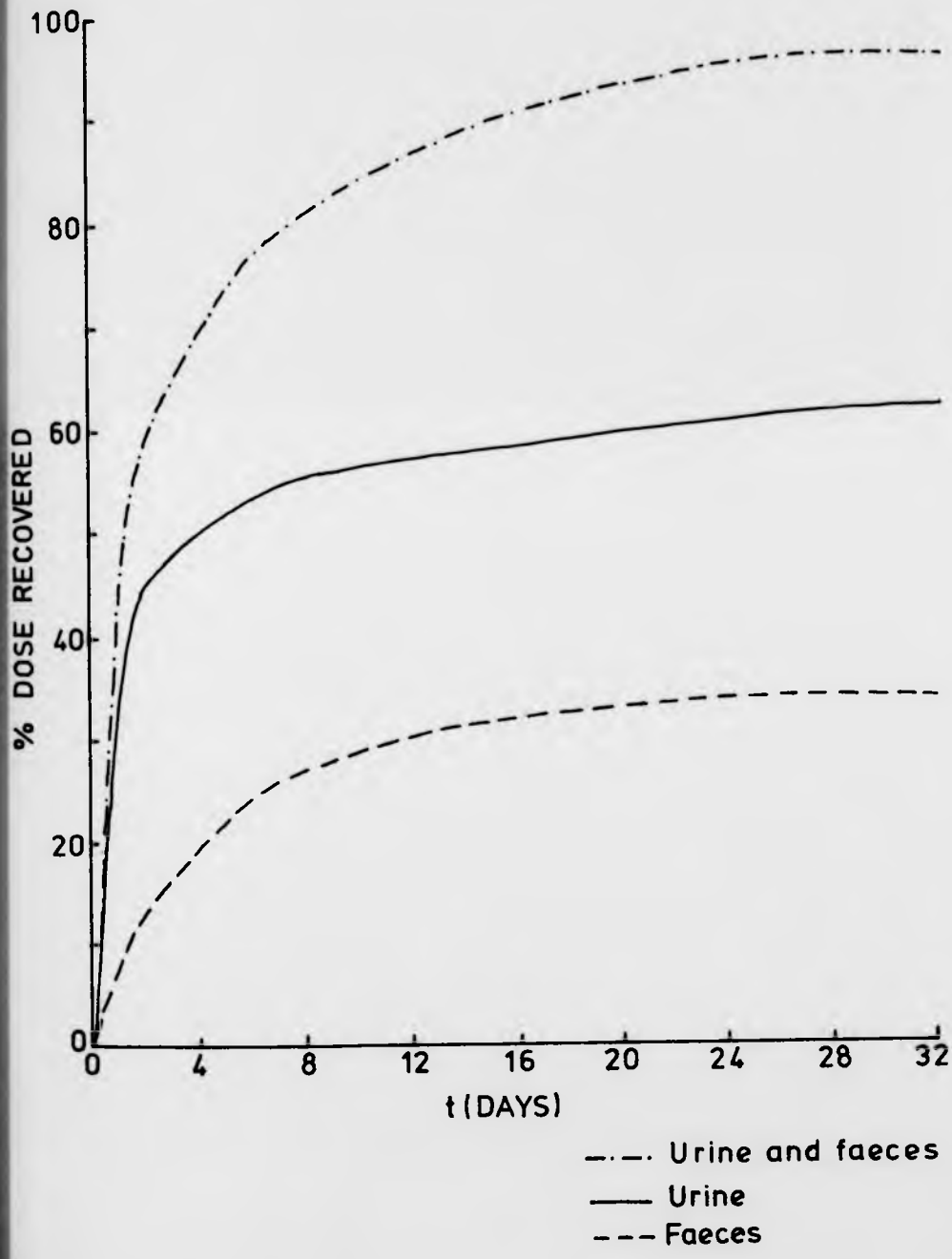


FIGURE 7.20

RECOVERY OF ^{125}I-LABELLED COPOLYMER 78 FROM URINE AND FAECES FOLLOWING I.P. ADMINISTRATION



The percentage of the dose recovered from the animal injected with copolymer 78 was 100.60% with 96.27% of the dose recovered in the urine and faeces. 2.29% of the recovered radioactivity was still present in the blood and only 1.11% remained in the liver.

Following injection of control copolymer 79, 95.5% of the dose was recovered at the end of the experiment and 96.35% of the recovered radioactivity was present in the urine and faeces. The blood contained 2.65% of the recovered radioactivity and 0.35% was found in the liver. On correction of liver values for blood-borne radioactivity (section 6.2), 0.76% of the recovered radioactivity was present in the liver following administration of copolymer 78, and none was present in the liver following administration of copolymer 79.

These results clearly show that crosslinked copolymers can be excreted from the body following pinocytic uptake and crosslink cleavage of larger polymers to release smaller molecules of a size below the renal threshold.

Total body clearance was not observed in either case, but this could well be achieved on extending the experimental period. The copolymers still present in the body in both cases could represent molecules too large to permit filtration by the kidney, which cannot be degraded further. This could occur if the copolymer precursor ($\bar{M}w$ 21,500) contains a few copolymer chains of a size above that of the renal threshold.

An interesting feature of figs 7.19 and 7.20 is the difference in the patterns of excretion between the two copolymers. Copolymer 79 (shown in fig 7.19) is excreted largely by the kidney, with approximately 63% of the dose recovered in the

urine in the first 24h. This level rises to 75% after 4 days after which the rate of excretion by the kidney slows down considerably with 82% of the dose recovered in the urine after 32 days. The rate of dose recovery from the faeces shows a similar pattern with 8% of the dose recovered in the first 4 days, rising to 10% after 32 days.

Copolymer 78, however, shows a slower rate of excretion of copolymer after the first 24h, with 56% of the dose recovered in the urine after 10 days rising to 63% after 32 days. Recovery from the faeces amounts to 28% after 10 days, rising to 34% after 32 days. This indicates a slower clearance of the galactose-containing copolymer from the liver, with some of the accumulated copolymer released back into the blood circulation to be excreted by the kidneys and some being released into the bile and ultimately excreted in the faeces.

7.3 DISCUSSION

Intraperitoneal administration of copolymers results in the deposition of the dose into a body cavity with a large surface area surrounded by a membrane through which the polymer must pass. Movement of polymers from the peritoneal cavity has been reported to be protracted and delayed (Noronha-Blob 1977) with large molecules remaining in the cavity for several days (Punonen and Viinamäki 1982). The results presented here, however, indicate that all three copolymers used in the study are largely cleared (approximately 99% of the recovered radioactivity) from the peritoneal cavity within 24h.

Subcutaneous injection of copolymers gives rise to a bolus of material in the interstitial fluid at the site of injection. Although movement of copolymer into the circulation possibly occurs at a similar rate to that following intraperitoneal administration, it was not practicable to attempt to recover any of the dose still present at the site of the injection at the end of each experiment and so determine the quantity of copolymer remaining. Dose recoveries 24h following subcutaneous administration approached those obtained 24h following intraperitoneal administration.

Oral administration of ^{125}I-labelled copolymers resulted in the elimination of a larger proportion of the dose after 24h than on administration of copolymers via parenteral routes.

Dose recoveries of copolymers were, in most cases, in the region of 60% 24h following administration by intraperitoneal and subcutaneous routes, and 70% 24h following oral administration. These values are only approximate, however, since they are calculated from data which include total blood

levels of radioactivity, which in turn is calculated from an estimate of rat blood volume. Such estimates of blood volumes are dependent on rat body weight, age and sex of the animals, and different values for blood volume per gram rat body weight have been reported in the literature (Millard 1979, Everett 1956). For this reason, and also the difficulty in the recovery of all metabolic waste from the animal, it is difficult to determine precisely the proportion of the dose actually remaining in the body, or excreted, at the end of the 24h experimental period. A more detailed analysis of the whole carcass to estimate residual radioactivity would help to establish the location of copolymer, and so assess the accuracy of the methods employed, but such a procedure is beyond the scope of the current study. It is possible that the proportion of the dose not accounted for may be present in low levels in many different tissues which were not examined, or may have accumulated in one or a few tissues which were not examined, or have been retained at the site of injection in the case of parenteral administration. Alternatively, the calculated blood (or urine) values could have been underestimated. In order to compare the data from the experiments, it is assumed that the distribution of the radioactivity recovered from the experiments represents the distribution of the whole dose.

Clearance of galactose-containing glycoproteins by the liver following intravenous administration has been reported by a number of workers (Regoeczi et al 1978, Schlesinger et al 1980, Quintart et al 1984 and Schiff et al 1984). Clearance of liposomes bearing surface galactose residues by rat liver hepatocytes has also been reported (Ghosh et al 1982).

Evidence for the clearance of galactose-containing copolymers by the liver following intravenous administration to rats has also been described by Duncan et al (1983) and Chapter 6 of this thesis. The work presented here shows that it is also possible to target galactose-containing HPMA copolymers to the liver following alternative routes of parenteral administration, namely by the intraperitoneal and subcutaneous routes.

Intraperitoneal administration of copolymer 78 results in 51.90% of the recovered radioactivity present in the liver after 24h, while subcutaneous administration results in 23.58% in the liver after 24h with a greater proportion of the recovered radioactivity present in the urine. Two hypotheses might be put forward to explain this difference in the quantity accumulated by the liver on using the two routes. Firstly, it must be noted that each copolymer chain present in this crosslinked copolymer (which contains 2 mol % galactosamine) may not necessarily contain both a galactosamine residue and a radiolabelled tyrosine residue, and that the likelihood that both these residues are present on each copolymer will decrease with decreasing molecular weight of the copolymer. This would result in the presence of some low molecular weight fractions containing either galactosamine and no radiolabel (which cannot be traced) or a radiolabel, but no galactosamine residue (which would not accumulate in the liver). It is therefore possible that either preferential transport of low molecular weight copolymer 78 fractions (some of which do not contain galactosamine) took place from the site of subcutaneous injection, but not following intraperitoneal injection, or that some degradation of copolymer 78 crosslinks took place en route to the liver following subcutaneous

injection, but not following intraperitoneal injection. Both possible explanations would result in the body distributions of copolymer observed, that is, less accumulation of radiolabel in the liver following subcutaneous administration of copolymer, with smaller copolymer fractions which do not contain galactosamine residues being filtered from the circulation by the kidney. There is no significant accumulation of radiolabelled copolymer 78 in any organ other than the liver following administration by any of the parenteral routes, thus indicating specific receptor-mediated uptake of this copolymer by liver cells.

It was not possible to target copolymer 78 to the liver following its oral administration to rats, since uptake of significant quantities of copolymer by the gut was not observed. The galactose-containing polymer, however, transiently bound to cells of the small intestine. This tissue association did not occur on oral administration of ^{125}I-labelled copolymer 79, which is similar to copolymer 78 in size and structure but does not contain galactosamine. This suggests that there is a receptor present in the small intestine of the rat which recognises galactosamine. This observation was also noted by Anderson et al (1980) who reported an interaction of Escherichia Coli K88 Antigen with porcine intestinal brush border membranes. The formation of such complexes was found to be inhibited by glycoproteins with terminal N-acetylgalactosamine and N-acetylglucosamine, and therefore denotes the presence of carbohydrate receptors on the brush border of the small intestine of pigs. There is no reason to suppose that rats may not also possess such receptors on the brush border of enterocytes of the small intestine.

This finding could not be reproduced in vitro, however, on incubation of ^{125}I-labelled copolymer 78 with everted rat jejunal sacs (section 7.2.4).

Copolymer 79 does not significantly accumulate in the liver, or any of the organs examined during the 24h experimental period whatever route of administration is employed. Radioactivity is largely confined to the blood, kidney and urine following parenteral administration, indicating the removal of low molecular weight copolymer fractions following their appearance in the circulation. Presumably, larger molecules remain in the circulation until pinocytosed by cells, after which digestion of crosslinks and release of lower molecular weight fractions would occur. A greater proportion of radioactivity is again recovered in the urine following subcutaneous administration than following intraperitoneal administration of the copolymer. The possible reasons for this observation have already been discussed i.e. preferential movement of low molecular weight fractions from the site of subcutaneous, but not intraperitoneal injection, or more extensive degradation of copolymer en route to the circulation following subcutaneous than intraperitoneal administration. Oral administration of this copolymer resulted in a small percentage of radioactivity in the stomach, small intestine and blood after 1h, followed by the appearance of radioactivity in the urine. This indicates the transport of some copolymer and/or free [^{125}I] iodide across the gut into the circulation with ultimate removal by the kidney. The fact that a greater proportion of the recovered radioactivity was found in the bloodstream 1h after oral administration of copolymer 79 (7.38%) than on oral administration of copolymer 78 (0.64%), which is of similar

size and structure but contained less free $[^{125}I]$ iodide (section 7.2.1), suggests that free $[^{125}I]$ iodide could be largely responsible for this observation.

The body distributions of copolymer 79 following the various routes of administration show that this copolymer has little affinity for cell membranes, giving similar body distributions of radioactivity to those observed on administration of ^{125}I-PVP (of similar $\bar{M}w$), which has been described as a fluid-phase marker for in vivo studies (Munniksma et al 1980, Kooistra et al 1979, Ose et al 1979). Copolymer 79 does not accumulate in cells of the reticuloendothelial system (i.e. cells present in the liver, spleen and lungs) over a 24h experimental period, and does not appear to enter cells by adsorptive pinocytosis.

Very little difference in body distributions of radioactivity is observed on parenteral administration of ^{125}I-labelled copolymer 79 and ^{125}I-labelled copolymer 39. This indicates that the difference in average molecular weight of the two copolymers (section 7.1) has little effect on their ultimate fates in vivo, and therefore that both copolymers can be considered to be 'fluid-phase' substrates (if little or no degradation of radiolabelled copolymer 39 crosslinks takes place).

The extent of copolymer 39 degradation following intra-peritoneal and subcutaneous routes is not known, and it is not possible to determine from the results what proportion of the recovered radioactivity is due to radiolabelled crosslink degradation products. Drobnik and Rypàček (1984) state that movement of polymers from the site of subcutaneous injection

takes place by size-dependent transport via venous or lymphatic capillaries. In view of this, the finding that the body distributions of the two copolymers (39 and 79) are similar following the same routes of administration might be interpreted that crosslinks are not extensively degraded in transit to the circulation and therefore that there is preferential movement of smaller copolymer fractions from the injection site following subcutaneous injection rather than the alternative explanation of degradation of crosslinks to give rise to smaller polymer chains.

On oral administration of ^{125}I-labelled copolymer 39, the radiolabelled crosslinks appear to be degraded substantially by enzymes present in the gut lumen, with subsequent uptake of [^{125}I] iodotyrosine degradation product by cells of the small intestine. These degradation products appear to enter the circulation and are eventually excreted by the kidney.

^{125}I-Labelled copolymers 78 and 79 contain crosslinks which also appear to be hydrolysed in the gut lumen giving rise to smaller molecular weight fractions in the lower regions of the gut.

Extended experiments following intraperitoneal injection of ^{125}I-labelled copolymers 78 and 79 largely confirmed that it is possible to clear crosslinked HPMA copolymers from the body following their pinocytic uptake by cells. Although total body clearance was not observed using either copolymer in this preliminary experiment (which lasted 32 days) 92.04% of the copolymer administered was recovered in the urine and faeces in the case of copolymer 79, and 96.27% in the case of copolymer 78. On correcting for blood-borne radioactivity

it was found that less than 1% of the dose administered was still present in the liver. Extension of the experimental period for a further few days would probably have resulted in complete body clearance of copolymers.

More than three times as much copolymer was excreted in the faeces following administration of galactose-containing copolymer 78 (34.15%) than the control copolymer 79 (10.62%). The excretion of ^{125}I-PVP from the bloodstream into the intestine following intravenous administration is reported by Drobnik and Rypàček (1984). They suggest that the fraction excreted by the intestinal pathway is less dependent on the polymer molecular weight than that excreted by the kidney, and that intestinal excretion may be a way in which large molecules that cannot be degraded or excreted by the kidney can escape from the body. The authors also suggest that some of the copolymer excreted in the faeces may be transported into the intestinal lumen with the bile, which is produced by liver hepatocytes, the rest being excreted by vesicle transport across the intestine into the lumen.

The results shown here strongly support this latter proposal by Drobnik and Rypàček, since the copolymer containing galactose (78) is rapidly accumulated by liver cells and is excreted in the faeces to a much greater extent than copolymer 79, which is not taken up significantly by the liver.

Since bile is produced by hepatocytes, this result also suggests that a significant proportion of the galactose-containing copolymer is endocytosed by hepatocytes, rather than kupffer cells of the liver which are also reported to express galactose receptors (Kolb et al 1981).

An upper limit of between Mw 6,000 and 10,000 was suggested by Rozé (1971) for biliary elimination of PVP. This limit is much lower than that observed for HPMA copolymers, since the average molecular weight of the polymeric precursor i.e. the molecular weight of the crosslinked copolymer (78 or 79) following 100% cleavage by enzymes, is $\bar{M}w$ 21,500.

Studies by Schiff et al (1984) have demonstrated that all immunoglobulin A (IgA) which enters liver cells by receptor-mediated pinocytosis and 1-4% of injected asialoglycoprotein (ASG) i.e. galactose-bearing protein, are transported from blood to bile intact, although a major fraction of the ASG is degraded in hepatic lysosomes. The authors propose that proteins pinocytosed by hepatocytes can be processed in one of three ways: they can be transported to bile, returned to the blood, or transferred intracellularly to lysosomes. They suggest that receptor-mediated pinocytosis of IgA and ASG involves two distinct intracellular routes. IgA is contained within vesicles which are transported to the bile canaliculi without fusion with lysosomes, whereas ASG is transported in vesicles which are destined to fuse with lysosomes and is therefore largely degraded. It is proposed that the distinct receptors for the two proteins are not normally present in juxtaposition on the plasma membrane and that the occasional missorting of receptor-ligand complexes gives rise to a small percentage of transcellular passage of ASG into the bile. Similarly, Limet et al (1982) have proposed different transcellular routes which escape fusion with lysosomes for the transport of intact IgA into the bile following its binding and receptor-mediated uptake by hepatocytes. From figures 7.19 and 7.20 it can be seen that the levels of

radioactivity recovered from both the urine and the faeces rise more slowly following administration of copolymer 78 than on administration of copolymer 79. This suggests that copolymer 78 is released from cells (probably the liver) both into the blood (followed by filtration by the kidney) and into the bile (followed by excretion in the faeces). It is not possible to determine whether that proportion released into the intestine is of a larger molecular weight (degraded or not) than that filtered by the kidney, which has probably been partially degraded within hepatocyte lysosomes. The transcellular route into the bile therefore cannot be identified conclusively.

The cellular events taking place within hepatocytes following receptor-mediated pinocytosis of copolymer 78 probably include the fusion of pinocytic vesicles containing copolymer with acidic endosomes (prelysosomal vesicular compartments section 1.3), resulting in receptor-ligand dissociation (Wolkoff et al 1984) and return of the receptors to the cell surface. Copolymer may then either be transported across the cell intact via specialised vesicles and released at the biliary membrane, or transported to lysosomes. Fusion of the copolymer-containing vesicle with lysosomes probably results in the degradation of copolymer crosslinks and eventual exocytosis of lower molecular weight fractions either back into the blood or into the bile canaliculi.

CHAPTER 8

CROSSLINK CLEAVAGE OF ^{125}I-LABELLED HPMA COPOLYMERS BY LYSOSOMAL ENZYMES

8.1 INTRODUCTION

The work reported thus far in this thesis has involved the administration of radiolabelled copolymers to tissues in vitro, or to animals in vivo, in order to monitor the uptake of the copolymers into cells and the resultant release of radiolabelled degradation products from cells following enzymic hydrolysis within lysosomes. This chapter describes the study of the direct interaction between radiolabelled copolymers and lysosomal enzymes in vitro. An attempt is made to quantitate the extent of cleavage of the crosslinks contained within the copolymers used throughout this study by comparison of copolymer molecular weights before and following incubation with the enzymes.

Several techniques have been employed to isolate intact lysosomes from cells derived from various organs (Dean 1977). The technique employed here involves decreasing the lysosomal density using the non-lytic detergent Triton WR-1339. The detergent is administered intraperitoneally to rats and eventually accumulates in liver lysosomes following endocytic uptake. Separation of the detergent laden 'tritosomes' is achieved by initial differential centrifugation of liver homogenate in 0.25M sucrose followed by sucrose density (discontinuous gradient) centrifugation at high speed, resulting in an interfacial band of lysosomes (Trouet et al 1974). The lysosomal membranes may subsequently be ruptured by the addition of Triton X-100, to release the lysosomal enzymes.

Lysosomes are known to contain at least sixty acid hydrolases, including various lipases, phosphatases, nucleases, sulphatases,

glycosidases, exopeptidases and endopeptidases (Barrett and Heath 1977, Bainton 1981). Collectively, they are capable of hydrolysing almost all classes of natural macromolecules, the breakdown products of which are usually available for metabolic re-use.

The enzymes of particular interest to this study i.e. those able to hydrolyse the oligopeptide sequences contained within crosslinks are the proteinases, comprising endopeptidases (hydrolases cleaving peptide bonds away from the ends of polypeptides), and exopeptidases (hydrolases cleaving peptide bonds near the end of polypeptides).

As discussed previously (Chapter 1.4), the extent of crosslink cleavage by various purified enzymes has been shown to be dependent upon both the length of the oligopeptide sequence present in the crosslink, and upon the amino acid sequence itself, which must conform to the specificity of a given enzyme to promote efficient hydrolysis. It has been shown (Kopeček 1981, Ulbrich et al 1980, 1981, Rejmanovà et al 1981) that the extension of the oligopeptide sequence by one amino acid residue (from tripeptide to tetrapeptide) leads to a pronounced increase in the rate of degradation of the oligopeptide crosslinks in HPMA copolymers. Previous studies on HPMA copolymer crosslink cleavage have been carried out in Prague using the purified enzymes chymotrypsin, trypsin and papain (Ulbrich et al 1980, 1981, Rejmanovà et al 1981). Incubation of a copolymer containing the same crosslink structure as that of copolymer 39 (P-Gly-Gly-Phe-Tyr-HMDA-Tyr-Phe-Gly-Gly-P - a tetrapeptide) with chymotrypsin showed that 45% of the crosslinks were cleaved after 5 minutes, 88% after 30 minutes, and 100% after 48 hours. Similar studies using

the crosslink structure P-Gly-Gly-Phe-HMDA-Phe-Gly-Gly-P, a tripeptide showed 22% crosslink cleavage after 48h incubation with chymotrypsin. This crosslink structure is contained within copolymers 68, 78 and 79.

The polydisperse crosslinked copolymers 39, 68, 78 and 79 were used in this study. Characteristics of the copolymers are given in Chapter 3.4, and details relevant to this study are repeated in Table 8.1.

Radiolabelled copolymer 39 contains a tetrapeptide sequence (which includes $[^{125}I]$ iodotyrosine) known to be hydrolysed completely by chymotrypsin within 48h, and partially by enzymes of the yolk sac lysosomes (Chapter 4). Radiolabelled copolymers 68, 78 and 79 contain the same tripeptide sequence within their crosslinks. Degradation of this sequence could not be detected in previous experiments since the radiolabelled tyrosine residue is attached to the copolymer backbone. This feature, however, enables detection of radiolabelled copolymer chains following crosslink cleavage. It is therefore possible, using gel permeation chromatography, to estimate the average molecular weight of each of the copolymers both before and following incubation of crosslinked polymers with lysosomal enzymes and hence estimate the extent of crosslink cleavage.

Copolymers 78 and 79 are of similar average molecular weight (~70,000) and crosslink density (see Table 8.1), their only difference being the incorporation of galactosamine in copolymer 78. Copolymer 68, on the other hand, is larger ($\bar{M}w$ 171,000) and contains a greater density of crosslinks.

Each of the four radiolabelled crosslinked copolymers were incubated with rat liver lysosomal enzymes for up to 72h in

TABLE 8.1

CHARACTERISTICS OF CROSSLINKED COPOLYMERS

POLYMER CODE NO.	$\bar{M}w$	$\bar{M}w/\bar{M}n$	γ	ρ ($\times 10^{-3}$)	% CROSSLINKED SIDECHAINS	$\bar{M}w$ PRECURSOR
39	190,000	6.80	0.518	3.885	14.7	32,100
68	171,000	5.80	0.669	6.100	14.9	21,500
78	73,800	3.05	0.541	4.940	12.0	21,500
79	64,300	2.74	0.509	4.640	11.3	21,500

γ denotes the crosslinking index i.e. the number of crosslinked units per primary chain.

ρ denotes the network density i.e. the molar fraction of crosslinked units.

order to see whether these crosslinks were cleaved.

Experiments reported in Chapter 4 described the pinocytic uptake and intracellular degradation of copolymer 39 fractions. Studies on the degradation of copolymer included the use of leupeptin, a lysosomal thiol-proteinase inhibitor, and results demonstrated a reduced degradation of the copolymer. This finding suggested the involvement of one or more lysosomal thiol-proteinases in the degradation process. To allow the full activity of these thiol-dependent enzymes to be expressed in the experiments described in this in vitro study, reduced glutathione was included in the incubation medium.

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8.2 RESULTS

Rat liver 'tritosomes' in sucrose were prepared according to the method of Trouet (1974) (see Chapter 2). Approximately 30-fold purification of a representative lysosomal enzyme (aryl sulphatase) over the initial homogenate was achieved.

Radiolabelled copolymers (125 µg in 0.1 ml phosphate buffer pH 8.0) were added to 0.6 ml of 0.2M potassium phosphate buffer pH 5.5 containing 0.2% Triton X-100, 5 m M reduced glutathione and 2 mM EDTA. At the beginning of the experiment 0.3 ml of undiluted tritosomes (approx. 2 mg protein/ml) was added to each experimental tube. The pH of the resultant mixture was found to be 5.7. Incubation took place at 37°C in stoppered tubes for periods of 0, 24, 48 and 72h for each substrate. Control experiments were carried out concurrently, in which tubes contained copolymers buffer and GSH only. The experiment was performed once only.

Following incubation, proteins were precipitated using TCA as described previously (section 4.2.5). Supernatants were stored at -20°C prior to column chromatography. ^{125}I-Labelled copolymer preparations, following TCA precipitation, and samples of supernatants following incubation of each copolymer with tritosomes for 24h (and copolymer 39 following 48h and 72h incubation periods) were applied to a Sephadex G-15 column (bed height 16 cm, I.D. 8 mm, flow rate 14 ml/h). Figure 8.1 and Table 8.2 show that after 24h incubation, approximately 30% of the radioactivity present in the supernatants recovered following incubation of copolymer 39 with tritosomes co-elutes with [^{125}I] iodotyrosine. A similar percentage was found when incubations were extended to 48 or 72h.

FIGURE 8.1

SEPHADEX G-15 COLUMN CHROMATOGRAPHY OF ^{125}I-LABELLED COPOLYMER 39 FOLLOWING INCUBATION WITH RAT LIVER TRITOSOMES

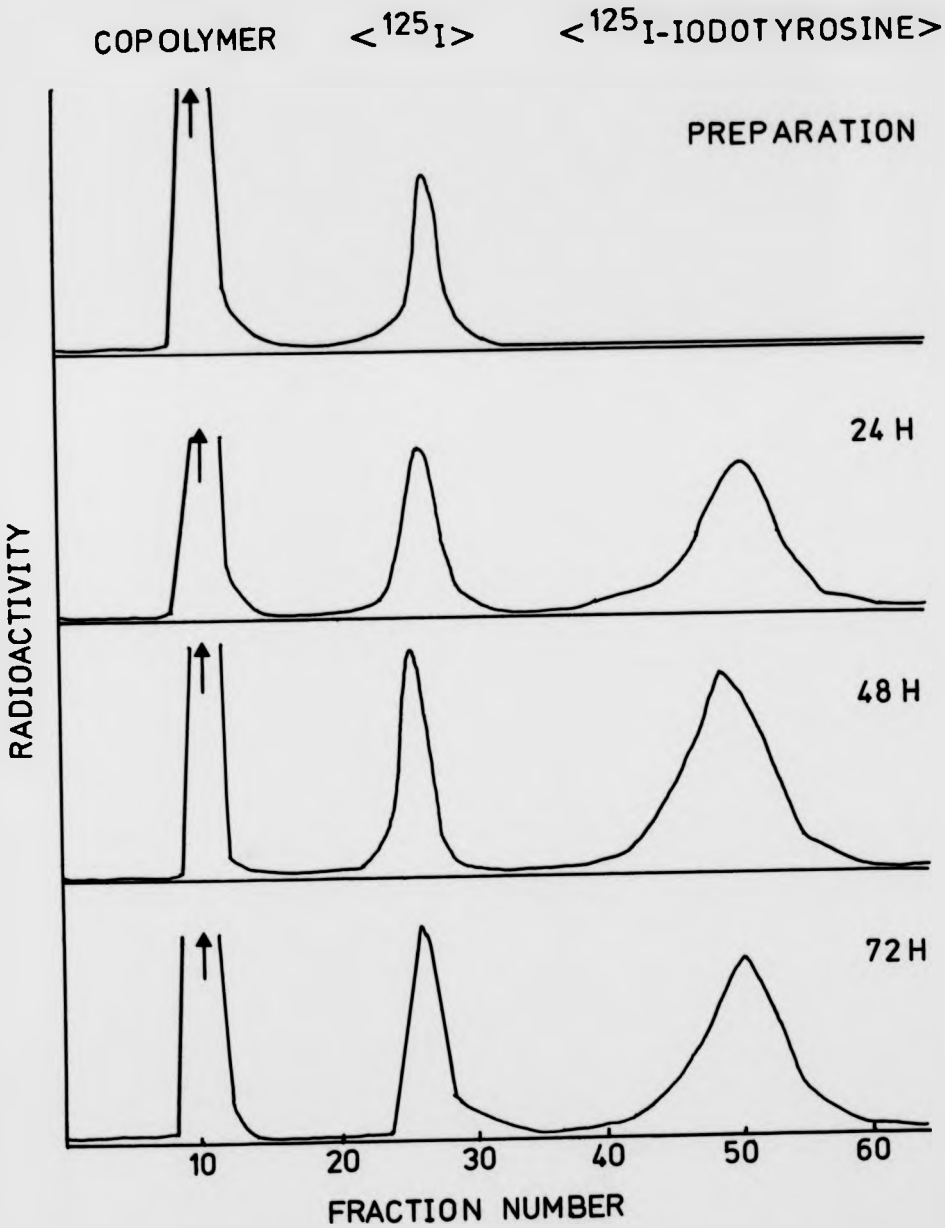


TABLE 8.2

SEPHADEX G-15 COLUMN CHROMATOGRAPHY FOR THE DETECTION OF [^{125}I] IODOTYROSINE FOLLOWING INCUBATION OF RADIOLABELLED COPOLYMERS WITH TRITOSOMES

POLYMER CODE NO.	% RADIOACTIVITY CO-ELUTING WITH [^{125}I] IODOTYROSINE AFTER (t=0) PREPARATION	24h	48h	72h
39	0	30.03	29.80	31.89
68	0	0	-	-
78	0	0	-	-
79	0	0	-	-

Copolymers 68, 78 and 79, however, showed no release of [^{125}I] iodotyrosine after 24h incubation with tritosomes (Table 8.2). Figure 8.2 shows typical elution profiles obtained from these experiments. These results support those reported in previous chapters, i.e. that lysosomal enzymes are unable to liberate [^{125}I] iodotyrosine when the residue is attached directly adjacent to the copolymer chain.

This experiment indicates the release of radiolabelled low molecular weight degradation products following incubation of copolymer 39 with lysosomal enzymes, but does not indicate whether the [^{125}I]-iodotyrosine is released from crosslinks which connect copolymer chains together, or from the hydrolysis of intramolecular cycles (Chapter 3.2). If crosslink cleavage had taken place, one would expect a lowering of the average molecular weight of the copolymer, but this would not be expected if hydrolysis was confined to intramolecular cycles.

To assess the extent of crosslink cleavage, supernatants derived from the experiment described above were applied to previously calibrated Sepharose 4B/6B columns, the details of which are shown in Table 8.3 and fig 8.3, and eluted with 0.2M Tris/HCl buffer pH 8.0 containing 0.5M NaCl. The HPMA polymers used for the calibration of the columns were all of low polydispersity ($\bar{M}w/\bar{M}n$ 1.2).

SEPHADEX G-15 COLUMN CHROMATOGRAPHY OF ^{125}I-LABELLED COPOLYMER 78 BEFORE AND FOLLOWING 24H INCUBATION WITH RAT LIVER TRITOSOMES

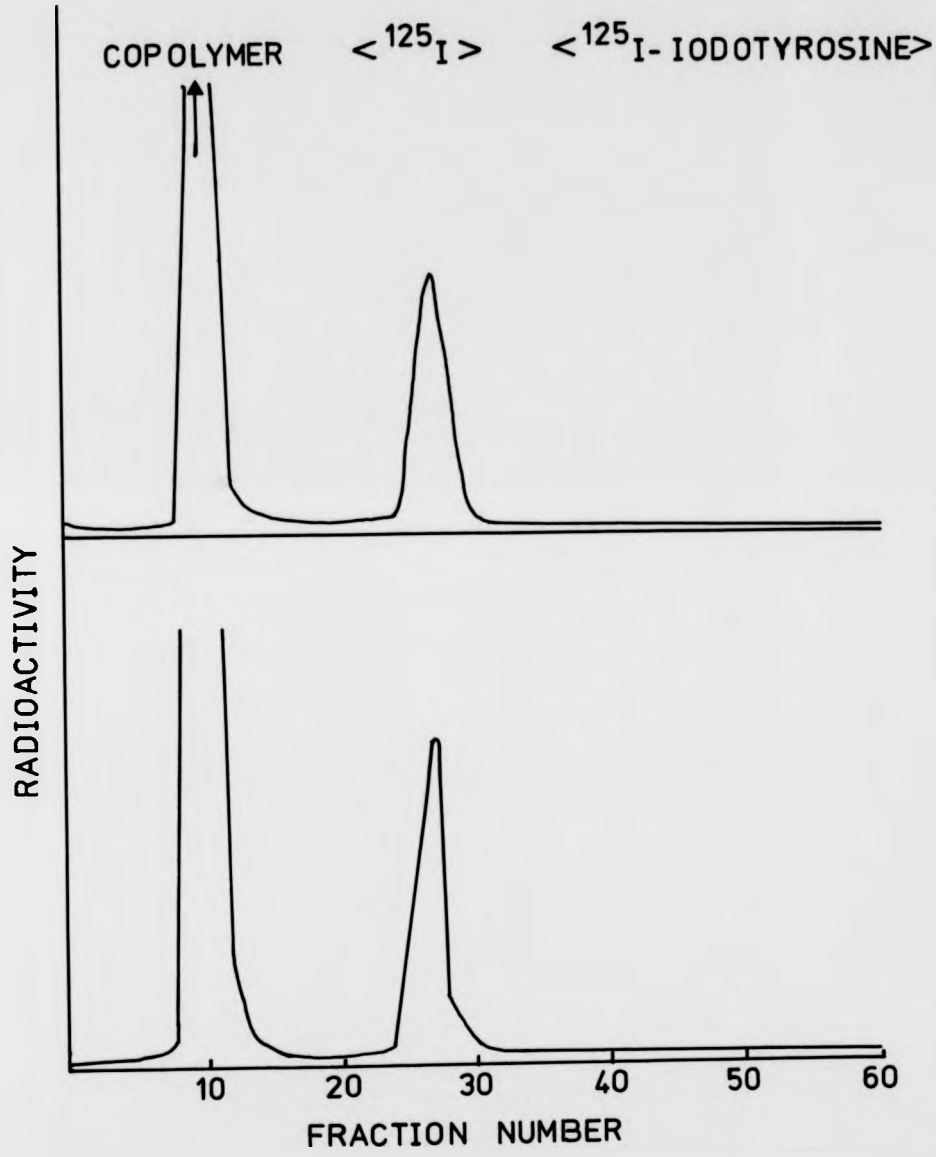


FIGURE 8.2

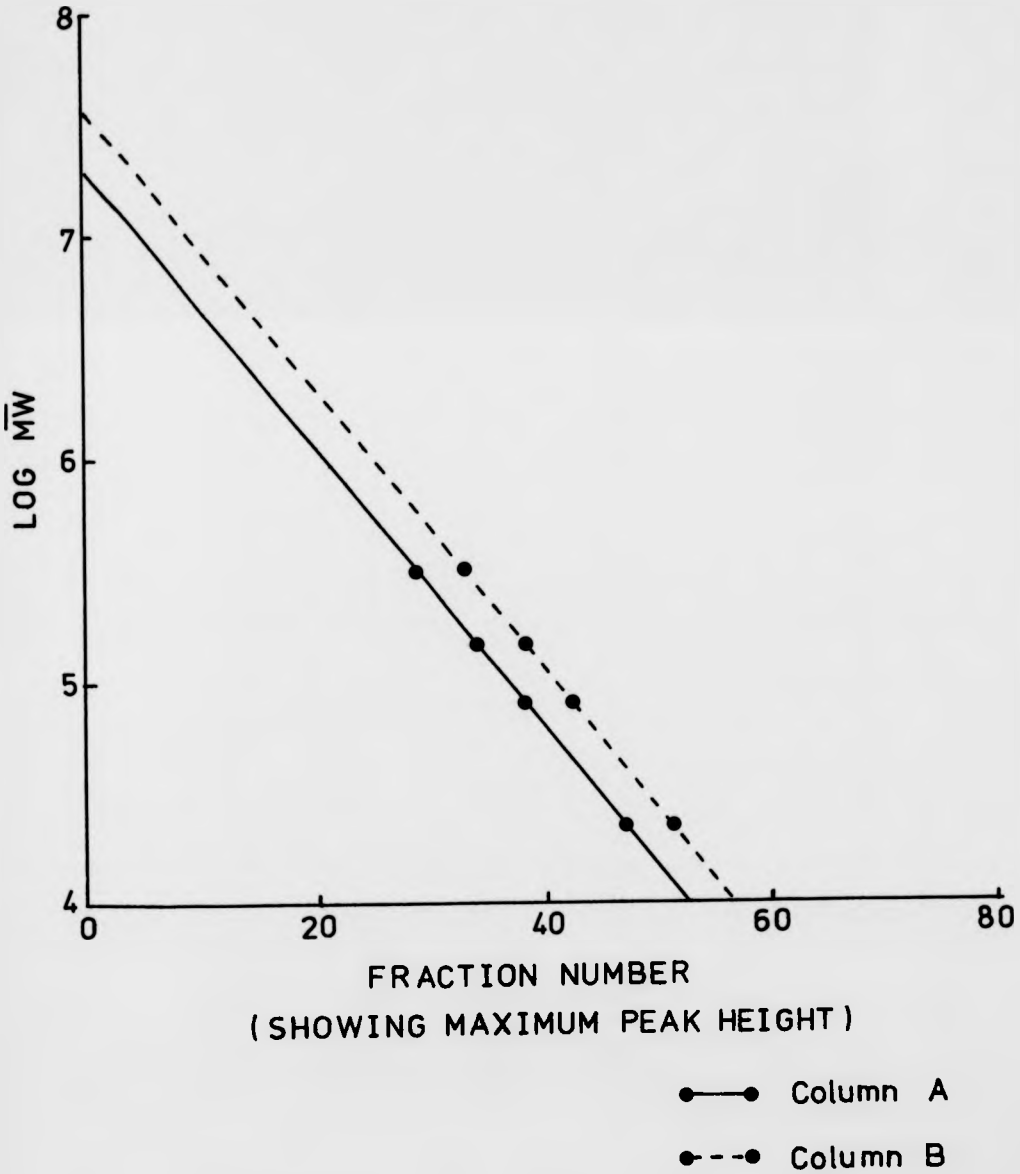
TABLE 8.3

DETAILS OF SEPHAROSE 4B/6B COLUMNS USED FOR DETERMINATION OF AVERAGE MOLECULAR WEIGHTS OF COPOLYMERS

	COLUMN A	COLUMN B
length	60 cm	65 cm
I.D.	16 mm	16 mm
flow rate	20 ml/h	20 ml/h
void volume	44 ml	50 ml
maximum peak height for 22,000 $\bar{M}w$ polymer	Fraction 47	Fraction 51
" 78,000 " "	" 38	" 42
" 143,000 " "	" 34	" 38
" 297,000 " "	" 29	" 33
regression analysis:		
constant a (intercept)	7.2903	7.5416
" b (slope)	-0.06283	-0.06283
correlation coeff.	.999	.999
column used for $\bar{M}w$ determination of copolymers	39,78	68,79

FIGURE 8.3

SEPHAROSE 4B/6B COLUMN CALIBRATION USING ^{125}I-LABELLED POLYMERS OF KNOWN MEAN MOLECULAR WEIGHT



The radioactive counts recovered from each fraction were recorded and the average molecular weight of each copolymer was calculated for each time point using the computer programme shown in Appendix 2.

The extent of crosslink cleavage was then calculated from the average molecular weight values for the copolymer precursor, the crosslinked copolymer and the copolymer following enzymatic cleavage using the formula described by Bohdanecký (1974) (section 2.13) i.e.

$$\text{The \% of cleaved bonds} = 100 - \frac{1 - \frac{\bar{M}w \text{ aminolysed precursor}}{\bar{M}w \text{ cleaved polymer}}}{1 - \frac{\bar{M}w \text{ aminolysed precursor}}{\bar{M}w \text{ crosslinked polymer}}} \times 100$$

The results, shown in figs 8.4-8.7 and Table 8.4, indicate that, in all cases, there is a decrease in the average molecular weight of copolymer following incubation with tritosomes, indicating some hydrolysis of all crosslinks by lysosomal enzymes. The values obtained in control experiments (in which copolymers were incubated for 24h in the absence of tritosomes) were taken to represent the initial average molecular weight of the preparations.

It can be seen from Table 8.4 that the values calculated for these control experiments are higher than the average molecular weights estimated in Prague for the copolymer preparations, with the exception of copolymer 39. The most likely explanation for these higher values is that different column dimensions and flow rate were employed in Prague. The Czech column was 90 cm in length and an approximate flow rate of 10-12 ml/h was used. This longer column and lower flow rate would lead to a better resolution of peaks and consequently a

FIGURE 8.4

SEPHAROSE 4B/6B CHROMATOGRAPHY OF ^{125}I-LABELLED COPOLYMER 39 ON INCUBATION WITH RAT LIVER TRITOSOMES

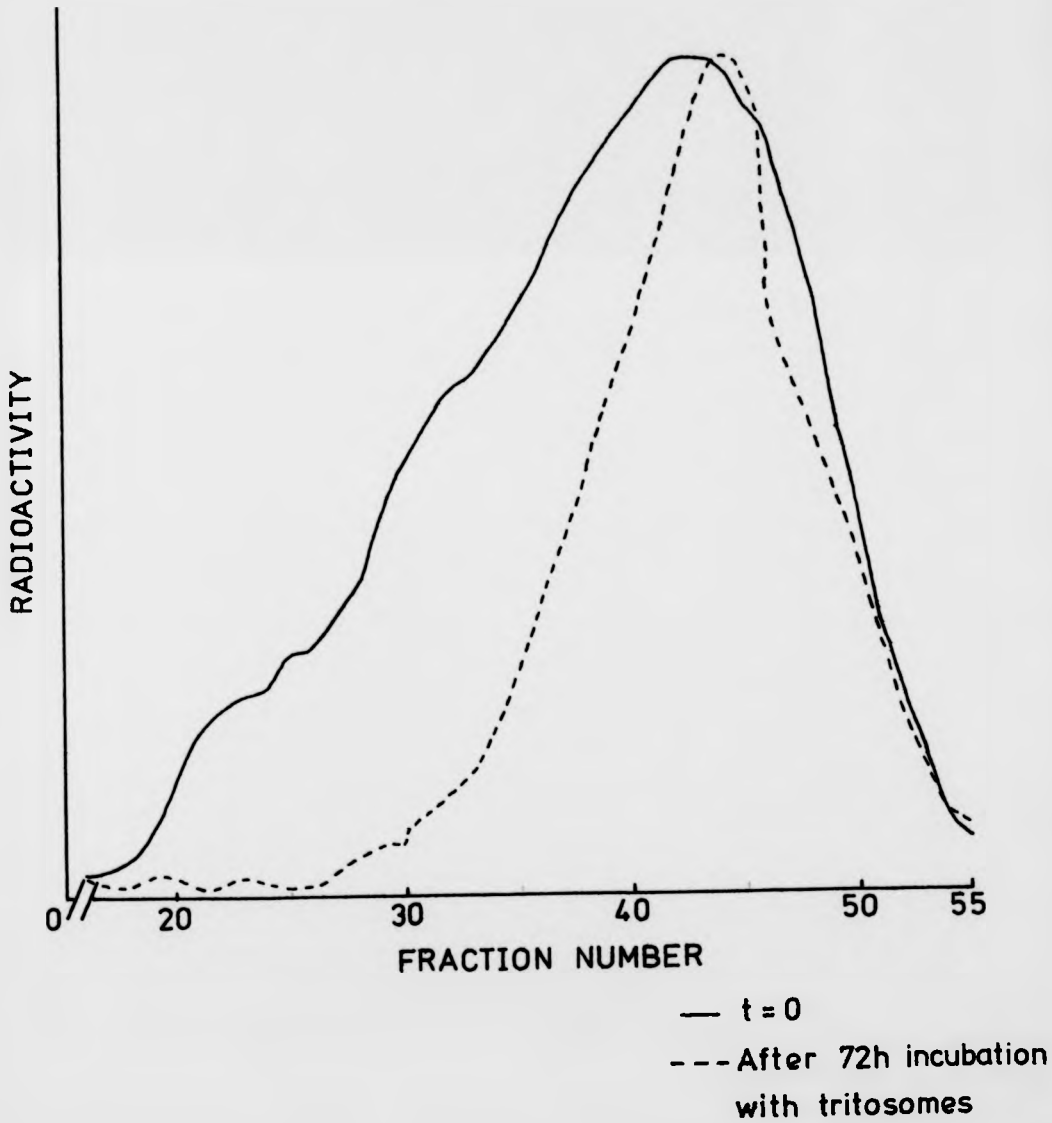
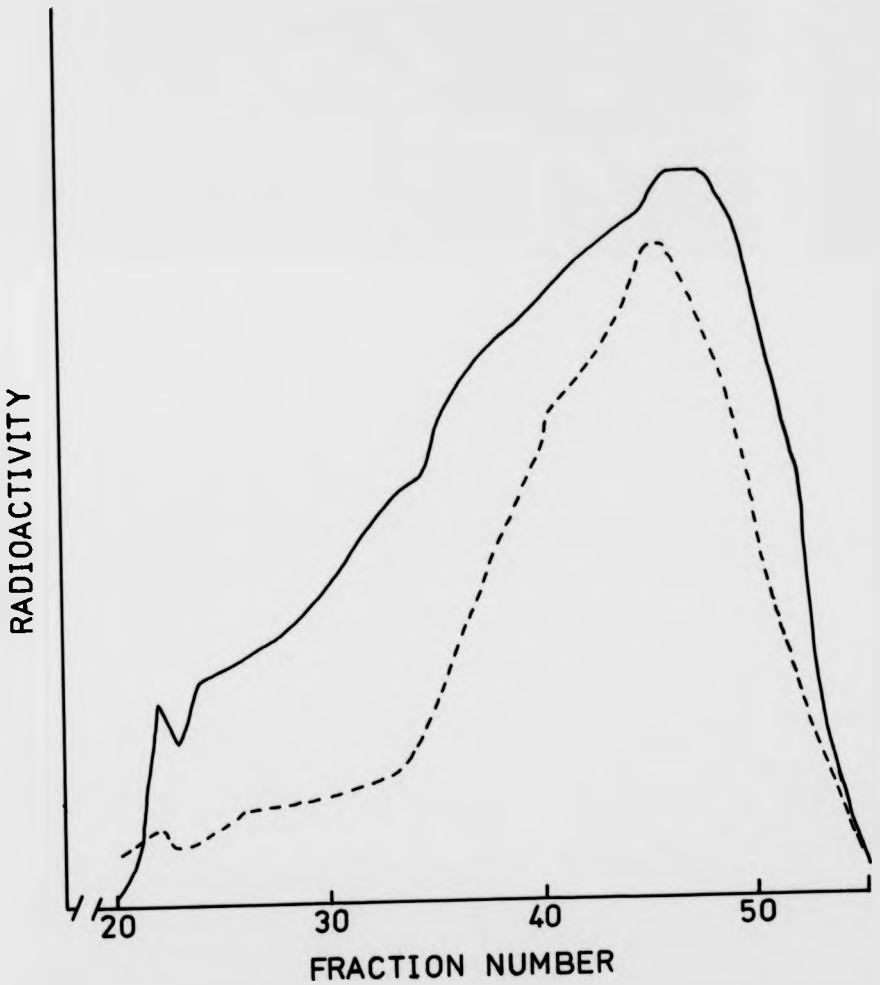


FIGURE 8.5

SEPHAROSE 4B/6B CHROMATOGRAPHY OF ^{125}I-LABELLED COPOLYMER 68 ON INCUBATION WITH RAT LIVER TRITOSOMES



— t = 0

---After 72h incubation with tritosomes

FIGURE 8.6

SEPHAROSE 4B/6B CHROMATOGRAPHY OF ^{125}I-LABELLED COPOLYMER 78 ON INCUBATION WITH RAT LIVER TRITOSOMES

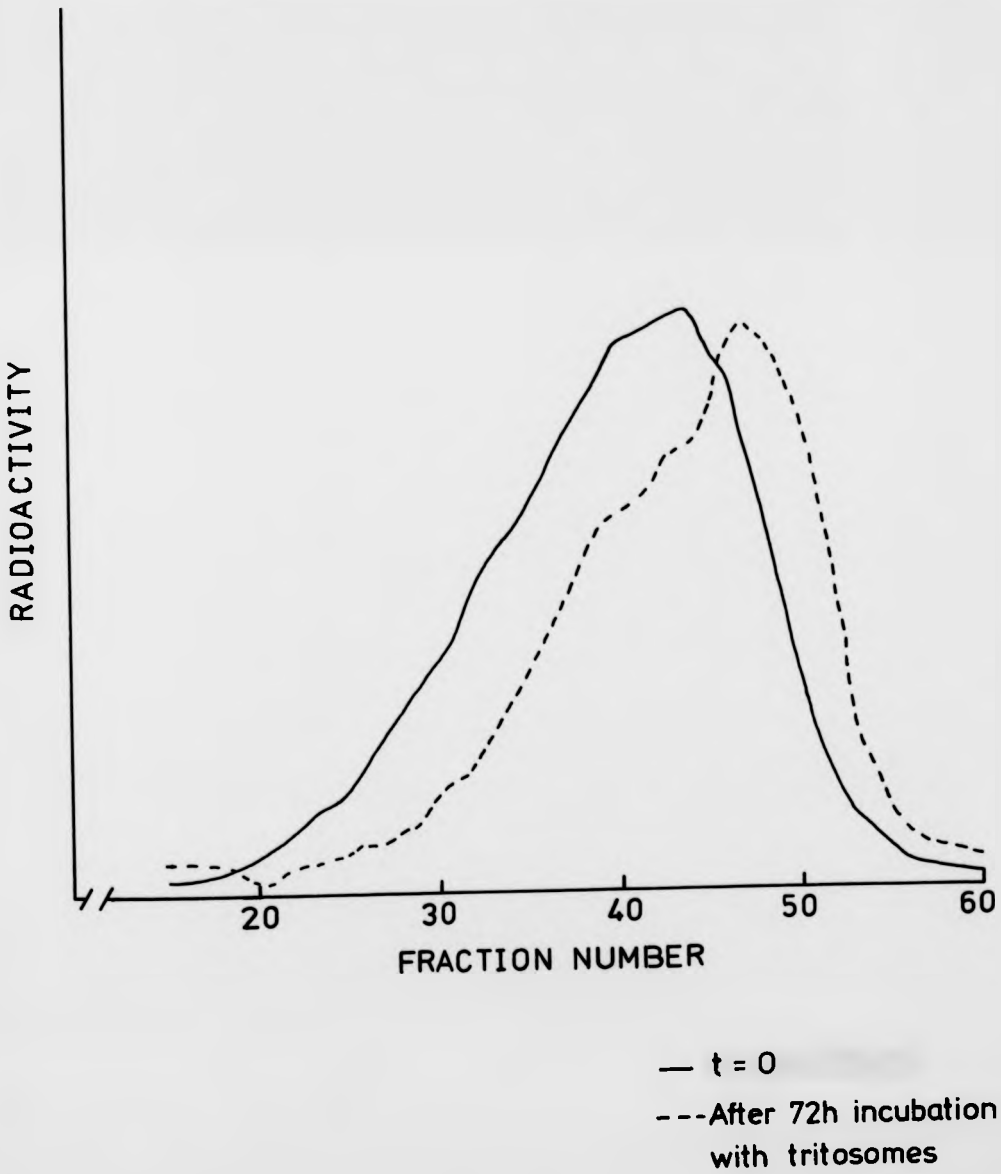


FIGURE 8.7

SEPHAROSE 4B/6B CHROMATOGRAPHY OF ^{125}I-LABELLED COPOLYMER 79 ON INCUBATION WITH RAT LIVER TRITOSOMES

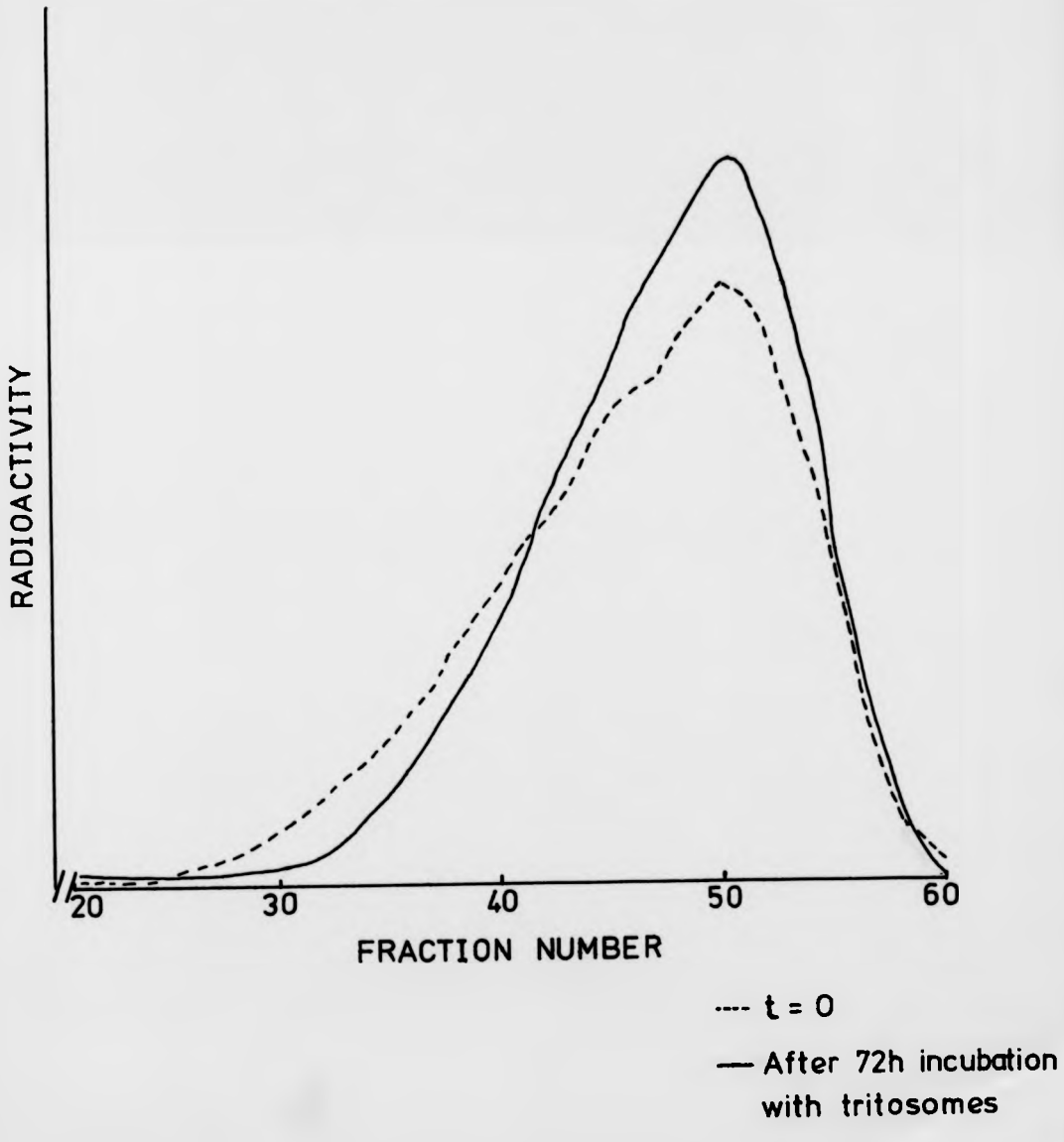


TABLE 8.4 THE AVERAGE MOLECULAR WEIGHTS OF RADIOLABELLED PREPARATIONS AND COPOLYMER FOLLOWING INCUBATION WITH TRITOSOMES

POLYMER CODE NO.	PRAGUE VALUES	CONTROLS (-TRITOSOMES)	FOLLOWING INCUBATION FOR 0h	24h	48h	72h
39	190,000	163,700	171,000	66,000	65,000	63,300
68	171,000	212,000	173,600	148,800	134,000	80,400
78	74,500	103,300	95,900	89,200	62,300	53,800
79	64,300	98,000	88,800	83,100	70,000	63,900

more accurate column calibration and molecular weight determination. For the purpose of the present study i.e. to detect differences in average molecular weights of copolymers following incubation with lysosomal enzymes, the columns employed were adequate.

It must be noted however, that the values for the average molecular weight of the polymeric precursors used in the calculation of crosslink cleavage (32,000 for copolymer 39 and 21,500 for copolymers 68, 78 and 79) were estimated using detection by differential refractometry in Prague, since it was not possible to evaluate radiolabelled precursors in the current study. In view of the fact that differences in calculated molecular weight averages occur using the different columns, it can be assumed that different (probably higher) values for the same polymeric precursor would be estimated using the shorter columns. The values presented for % crosslink cleavage (Table 8.5) must be interpreted as approximate, but they do allow comparisons between values to be made.

Each value in Table 8.5 is expressed in comparison with the appropriate control experiment, but a value for copolymer 39 at t=0 was not obtained, since the average molecular weight calculated for that copolymer at t=0 was higher than that obtained for its control (a 24h incubation of copolymer 39 in the absence of tritosomes). The difference in chromatography system would not account for the difference in average molecular weight of the copolymer 39 control which gives a value smaller, rather than larger, than the Prague value (Table 8.4). The reason for this is not known. All other samples taken at t=0 were lower than those calculated in

TABLE 8.5

THE PERCENTAGE CLEAVAGE OF ORIGINAL CROSSLINKS FOLLOWING INCUBATION WITH TRITOSOMES (EXPRESSED AS % CONTROL)

POLYMER CODE NO.	INCUBATION PERIOD t 0	24h	48h	72h
39	-	35.95	36.82	38.56
68	2.56	4.78	6.67	18.46
78	2.02	4.17	17.30	24.12
79	2.94	5.12	11.52	14.98

control experiments. This finding could be attributable to either entrapment of larger molecules in the pellet following TCA precipitation of protein with subsequent centrifugation, or extremely rapid degradation taking place between the addition of tritosomes and the addition of precipitating protein and TCA which took place directly afterwards.

Table 8.5 shows that approximately 37% of the crosslinks contained within copolymer 39 are cleaved within the first 24h on incubation with tritosomes, and this value does not increase significantly on further incubation. However, in the case of this particular polymer, this figure of 37% does not represent total crosslink cleavage, since it has already been shown that 30-32% of the total radioactivity present in the copolymer is released in the form of $[^{125}I]$ iodotyrosine degradation product. The values shown for copolymer 39 in Tables 8.4 and 8.5 represent the average molecular weight (and crosslink cleavage) of the copolymer that retains some $[^{125}I]$ iodotyrosine i.e. that which has not lost the radiolabel following liberation of the $[^{125}I]$ iodotyrosine residue from the completely hydrolysed crosslink.

A number of cleavage events could possibly result in the observed recovery of lower molecular weight copolymer 39 polymer chains still containing radiolabelled tyrosine residues. Firstly, it must be noted that the release of $[^{125}I]$ iodotyrosine from crosslinks or intramolecular cycles (section 4.2.1) requires the hydrolysis of two peptide bonds. Such cleavage events could include the following possibilities:

1. Crosslink cleavage by enzymes, with no hydrolysis of intramolecular cycles, thereby retaining radioactive

tyrosine residues within intramolecular cycles.

2. Preferential cleavage of crosslinks in larger copolymer fractions, leaving a greater proportion of smaller radiolabelled copolymer fractions.
3. Cleavage of one peptide bond contained within the crosslink resulting in a lower average molecular weight of the copolymer, but $[^{125}I]$ iodotyrosine would still be attached to an oligopeptide sidechain. Such single cleavage events could occur at one of two points in the sequence i.e.

   (i) P-Gly-Gly-Phe Tyr*-HMDA-Tyr-Phe-Gly-Gly-P

   (ii) P-Gly-Gly-Phe-Tyr* HMDA-Tyr-Phe-Gly-Gly-P

   (assuming one radiolabelled tyrosine residue per crosslink).
4. A combination of these possible events.

It is apparent that the total crosslink cleavage of copolymer 39 shown in Table 8.5 is an underestimate, since it does not take into consideration the $[^{125}I]$ iodotyrosine released during the experiment. Although it is not possible to accurately assess the extent of crosslink cleavage for this copolymer, it can be stated that maximum cleavage appears to have taken place within the first 24h.

Interpretation of the results shown in Tables 8.4 and 8.5 for copolymers 68, 78 and 79 is more straight forward, since $[^{125}I]$ iodotyrosine release does not take place during incubation of these substrates with tritosomes. As the copolymer backbone is radiolabelled, one can follow the average molecular weight of the whole preparation throughout the experiment. The crosslinks of each of the three copolymers was cleaved to a small, but similar, extent after a 24h incubation with tritosomes (Table 8.5). Further cleavage of

each polymer was observed throughout the experimental period.

Copolymer 68 is a more densely crosslinked preparation than copolymers 78 and 79 and this is reflected in the finding that only 6.67% of the crosslinks were cleaved in the first 48h. At the end of the experimental period (72h), however, copolymer 68 has been degraded to a similar extent (18.46%) as copolymer 79 (14.98%). Copolymer 78 is slightly larger than copolymer 79, but contains the same density of crosslinks, is degraded slightly more than copolymer 79 with 24.12% of the original crosslinks cleaved after 72h incubation with tritosomes.

8.3 DISCUSSION

The crosslinked copolymers used in this study are all hydrolysed to some extent by lysosomal enzymes. The results support previous reports that crosslinks containing tetrapeptide sequences are cleaved more readily than those containing tripeptide sequences, since the % of cleavage of the copolymer 39 crosslinks was greater than that achieved with copolymers 68, 78 and 79.

The maximum rate of cleavage of crosslinks contained in copolymer 39 by lysosomal enzymes was observed within the first 24h. However, it was not possible to estimate the total cleavage of crosslinks contained within copolymer 39, since the required calculation is dependent on the average molecular weights of both the initial preparation and the resultant hydrolysed copolymer. It was impossible to estimate the latter figure, since a proportion of the preparation lost its radiolabel following cleavage of [^{125}I] iodotyrosine from crosslinks and/or intramolecular cycles. However, the incubation of crosslinked copolymer 39 fraction c ($\bar{M}w$ 150,000) with the lysosomal thiol-proteinase Cathepsin B has recently demonstrated 64% cleavage of crosslinks within 72h (Cartlidge, Rejmanovà et al, in press). This experiment did not involve radiolabelled polymer, but was made possible by the detection of copolymer in the chromatography eluant using differential refractometry.

Crosslinks of copolymers 68, 78 and 79 were hydrolysed to a lesser extent and in all cases the values observed indicate that cleavage was still continuing after 72h incubation (Table 8.5), with a maximum value of 24% for copolymer 78 after 72h.

None of the polymers underwent complete crosslink cleavage during the 72h incubation period, however, and the results obtained from this study show that although a significant increase in the rate of cleavage is observed using the tetra-peptide crosslink (presumably at least partly due to easier access and a 'better fit' of the substrate in the enzyme active site - see Chapter 1.4), the specificity of the amino acid sequences for lysosomal proteinases is not optimal.

Recent work of Duncan et al (1983) has described the design of oligopeptide side-chains in HPMA copolymers to promote efficient degradation by lysosomal enzymes. HPMA copolymer side-chains were synthesized to match the known specificities of two lysosomal thiol-proteinases, Cathepsins D and L. Cathepsin D is known to cleave bonds between hydrophobic residues (Barrett 1977) and Cathepsin L has been shown to cleave bonds where hydrophobic residues occupy the P_2 and P_3 positions (Kärgel et al 1980) (terminology according to Schechter and Berger 1967). A series of different copolymers were incubated with lysosomal enzymes and it was found that sidechains of composition P-Gly-Phe-Leu-Gly-Phe-NAp and P-Gly-Gly-Phe-Leu-Gly-Phe-NAp demonstrated (respectively) $>$ 52% and $>$ 40% release of p-nitroaniline after 5h incubation. Both sidechains also released Phe-NAp, demonstrating the importance of hydrophobic amino acids in the P_3 and P_2 positions relative to the cleaved bond.

An extension of this work was carried out by Rejmanová et al (1983), who incubated copolymers containing both sidechains and crosslinks with bovine spleen Cathepsin B. From the series of sidechain structures examined, the maximum cleavage of

p-nitroaniline (-NAp) was 83%, achieved after a 48h incubation of the sidechain P-Gly-Phe-Leu-Gly-NAp with Cathepsin B. This observation was in agreement with data reported by Duncan (1983) using mixed lysosomal enzymes, and demonstrates the importance of hydrophobic residues in the P_3 and P_2 position relative to the cleaved bond on using Cathepsin B, in addition to Cathepsin L. Crosslinked copolymers used by Rejmanová (1983) were symmetrical (as in the present study) and maximum crosslink cleavage was achieved using the crosslink structure P-Gly-Phe-Tyr-Ala-Ala-HMDA.....P where 100% cleavage was observed after 24h incubation of the substrate with Cathepsin B. The structurally similar tetrapeptide copolymer P-Gly-Phe-Tyr-Ala-HMDA.....P, on the other hand, demonstrated 71% cleavage under the same experimental conditions. The initial bond cleaved by the enzyme in the pentapeptide copolymer was between the two alanine residues, and that in the tetrapeptide copolymer between alanine and HMDA i.e. in both cases with the two hydrophobic residues in positions P_3 and P_2. From these results, it is also apparent that the interaction of the amino acid present in the $P_1{}^1$ position i.e. Ala v HMDA (on the leaving group side) with the enzyme active site also plays a role in the relationship between structure and the rate of degradation of substrate.

From these recent studies by Duncan and Rejmanová, it is clear that the presence of hydrophobic residues in the P_3 and P_2 positions, relative to the bond to be cleaved, are important in order to promote efficient crosslink and sidechain cleavage by lysosomal enzymes, particularly Cathepsins B and L. The structure present in the $P_1{}^1$ position has also been shown to be important, and the extension of the amino acid sequence from tetrapeptide to pentapeptide would probably maximise crosslink cleavage by suitable lysosomal enzymes.

These recent findings indicate that the crosslinks contained in copolymers 39, 68, 78 and 79, are not the ideal structures for lysosomal hydrolysis, and therefore not the most favourable for incorporation into a drug carrier to promote maximum rate of cleavage within lysosomes. There may be circumstances, however, where the maximum rate of drug release or crosslink cleavage is not required. Since oligopeptide sidechains present on the polymer precursor are used to synthesize both crosslinks and drug linkages, it would be possible to control the rate of release of the drug by altering the length and sequence of the oligopeptide. Provided that 100% cleavage of both active drug and crosslinks is eventually achieved (see Chapter 7), both therapeutic efficiency and elimination of copolymer from the body would be possible.

CHAPTER 9

GENERAL DISCUSSION

CHAPTER 9

GENERAL DISCUSSION

A number of lysosomotropic polymeric drug carriers have been proposed which include polyphosphazenes, derivatives of poly(methacrylic acid), synthetic poly(amino acids), polyvinyl analogs of nucleic acids, copolymers of vinylpyrrolidone and others (Duncan and Kopeček 1984).

The advantages of such systems have been discussed previously (sections 1.3 and 1.4) and include ease of fabrication and the ability to design polymeric carriers to meet specific requirements. It is also possible to attach a number of moieties to the polymer backbone, thereby enabling a much higher drug loading than can be achieved with other lysosomotropic systems. Polymeric drug delivery systems, like other systems, also have their disadvantages.

One possible disadvantage of synthetic polymeric drug carriers is that of poor biocompatibility, since synthetic polymers are 'foreign' molecules in biological systems. Incompatible polymers can cause inflammatory and/or immune responses by the host, or result in adverse chemical modification of the polymer or its degradation products by the host tissues (Black 1984).

The homopolymer hydroxypropyl(methacrylamide) has been shown to be non-toxic, apyrogenic and generally shows good biocompatibility (Kopeček et al 1973, Kopeček 1981, 1984). Recent studies on the immunogenicity of hydroxypropyl(methacrylamide) copolymers and the homopolymer of HPMA using 5 different inbred strains of mice have shown that the homopolymer HPMA is non-immunogenic and that HPMA copolymers containing oligopeptide side chains are only weakly immunogenic (Říhová et al 1985). The factors influencing the immune response to HPMA copolymers are the structure of the oligopeptide side

chains, the dose of polymer administered, its average molecular weight and the presence/absence of drug models. The HPMA copolymer containing the oligopeptide side chain sequence P-Gly-Gly-OH caused a greater immune response than sequences P-Acap-Leu-HMDA, P-Acap-Phe-OH or P-Gly-Phe-Tyr-OH in all 5 strains of mice. The immune reaction was also dependent upon the size of the copolymer, with a preparation of copolymer of $\bar{M}w$ 150-200,000 resulting in a 2-5 increase of antibody producing cells in the spleen over that observed on administration of a preparation of $\bar{M}w$ 5,000. The incorporation of drug models (haptens) into the HPMA copolymer with side chain composition P-Acap-Leu-HMDA also increases the originally weak immunogenicity of the copolymer. The content of side chains (1-8.4 mol %) in the copolymer does not affect the immunological response.

These findings reported by Říhová et al (1985) indicate that small changes in polymer structure can result in large differences in the immune response of the immunized organism. Nevertheless, it was concluded from the paper that HPMA copolymers can be considered as possible drug carriers in a targetable drug delivery system.

Clearly, it would be necessary to undertake immunological studies on each proposed polymeric drug-carrier conjugate, taking polymer $\bar{M}w$ and side chain/crosslink structure into consideration, in addition to the drug and any targeting moiety incorporated into the final structure. This procedure is a necessary prerequisite in any system since antibody formation is possible even when natural molecules are modified and employed as drug carriers (Gregoriadis 1979, 1980). For

example, desialylated fetuin has been shown to be strongly immunogenic, whereas lactosaminated homologous albumin appears less likely to induce significant antibody formation (Fiume et al 1983). It should be noted, however, that if a drug-carrier conjugate is rapidly cleared from the bloodstream by receptor-mediated uptake by target cells, its duration in the circulation is short, and the possibility of any immune response is thereby reduced.

Two other problems encountered when considering the use of synthetic polymers as drug carriers are those of the polydispersity and degradability of the polymer. These problems, with respect to HPMA copolymers, have been considered in the present study.

Synthetic polymers are polydisperse by nature. If a polydisperse preparation of polymer is used as a drug carrier, the fate of the conjugate will depend, at least in part, on the effect of size of the different molecular weight fractions contained within the preparation on their rate of uptake by cells, and also on the renal threshold of the organism employed. However, the ability to synthesize and isolate different sizes of the same polymer can be an advantage, if there is an effect of size of the proposed carrier on its rate of uptake by specific cell types.

The work presented in this thesis clearly indicates the importance of size (and low polydispersity) of the proposed polymeric carrier. From the results presented in Chapters 4 and 5 it is evident that the fractions derived from polydisperse copolymer 39 are taken up by cells at very different rates than the unfractionated copolymer.

The uptake of copolymer 39 fractions by the rat yolk sac epithelium was by fluid-phase pinocytosis i.e. with no interaction with the cell membrane, and this tissue showed preferential uptake of lower molecular weight copolymer fractions (copolymer 39e $\bar{M}w$ 34,000). Uptake of copolymer 39 fraction e by everted rat jejunal sacs was also at a rate consistent with that observed for ^{125}I-PVP ($\bar{M}w$ 40,000) which is reported to enter this tissue by fluid-phase pinocytosis, but larger fractions ($\bar{M}w > 400,000$) were pinocytosed 3-4 times faster. This indicates that some degree of adsorption of larger copolymer 39 fractions to the cell membrane occurs prior to pinocytic uptake. The results suggest that it might be possible to enhance cellular uptake by certain tissues by the manipulation of copolymer size alone.

If a drug is to be targeted to a particular tissue by the attachment of an antibody or other targeting moiety to the carrier, uptake of the conjugate will take place by receptor-mediated pinocytosis following the binding of the conjugate to receptors on the cell surface. The size of the carrier may still be important, however, since results presented in Chapter 4 show that very large molecules are hardly pinocytosed at all by yolk sac epithelial cells. It is possible that the sheer bulk of a molecule may preclude, or reduce, its entry into certain cell types, despite the attachment of targeting moieties. It is therefore advisable to use molecular sizes known to be taken up readily by cells by fluid-phase pinocytosis when targeting moieties are to be attached to a drug carrier, since the rate of uptake of the conjugate by target cells will then be governed solely by cell surface receptor interaction with the conjugate, and non-specific uptake of conjugates by

non-target tissue will be minimal.

The blood clearance and body distribution of copolymer 39 fractions over 1h following intravenous administration. confirms previous reports that smaller copolymer fractions are rapidly filtered from the blood circulation by the kidney glomerulus. The renal threshold for such copolymers will therefore largely dictate the minimum size of a drug carrier which is designed to be retained in the circulation for any significant period of time.

It would appear from these studies on the effect of copolymer size that when uptake is to be by cells other than those of the reticuloendothelial system (which accumulate large molecules), a drug-carrier conjugate of molecular weight in the region of 100-150,000 would enable pinocytic uptake by most cell types, whilst preventing rapid glomerular filtration. The use of HPMA copolymers of a larger size than this could induce a greater immune response that that reported for molecules of $\bar{M}w$ 150-200,000 (Říhová et al 1985). Also, since copolymer size has been shown to be of such importance, it is necessary to ensure that the polydispersity of the polymer carrier is kept to a minimum in order to achieve the desired response.

The design of the proposed crosslinked HPMA copolymers incorporates oligopeptide sequences which it was hoped would confer limited biodegradability in biological systems. Although total degradation of the polymer would be desirable, this is not essential providing that the polymer is not allowed to accumulate within, and subsequently damage, cells. It must therefore be eventually eliminated from the body. In vitro

experiments performed in Chapters 4 and 5 demonstrated that oligopeptide sequences are hydrolysed by lysosomal enzymes with resultant release of low molecular weight degradation products, and results presented in Chapter 8 show that at least some of this degradation results in the cleavage of crosslinks (rather than inter-molecular cycles) by lysosomal enzymes. The long term in vivo experiment described in Chapter 7 shows that the resultant lower molecular weight copolymer chains are virtually cleared from rats within 32 days following intraperitoneal injection of 0.5 μg/g body weight of crosslinked HPMA copolymers 78 and 79. These results confirm that the oligopeptide crosslinks used in the study are degraded in vivo and that the problem of polymer accumulation in the body can be overcome using the proposed crosslinked carrier concept.

It is quite possible that copolymers could be cleared more rapidly if crosslinks of an optimum structure for specific lysosomal enzymes were incorporated into the carrier (Chapter 8). Unfortunately, the copolymers used throughout the study contained crosslinks which were of optimal structure for cleavage by chymotrypsin, although at the beginning of the study there was no reason to suppose that these structures could not be hydrolysed rapidly by lysosomal enzymes. The density and length of the crosslinks contained in the carrier will also determine the rate of crosslink cleavage. Rapid cleavage will be achieved using the minimum number of longer crosslinks (e.g. tetrapeptide sequences) of optimal structure for a given enzyme.

To ensure efficient uptake of drug-carrier conjugate by cells and elimination from the body, it is also important that the

polydispersity of not only the crosslinked copolymer preparation is kept to a minimum, but also that of the polymeric precursor. This will ensure that the crosslinked molecules introduced into the body are retained in the circulation (i.e. are of a size above that of the renal threshold) and that the resultant hydrolysed copolymer chains can be excreted from the body (i.e. are of a size below that of the renal threshold).

Targeting of soluble crosslinked HPMA copolymers to the liver by the incorporation of galactosamine into the structure has been achieved following intravenous, intraperitoneal and subcutaneous administration. The galactosamine-containing copolymer was also transiently associated with the brush border of the small intestine following oral administration - a finding which could, perhaps, be exploited in some useful way (delivering drugs active in the small intestine). These results show that it may be possible to administer HPMA-drug conjugates via routes other than the intravenous one. Since the appearance of copolymer is protracted and delayed following subcutaneous and intraperitoneal administration, this feature may also allow further control over the rate of drug delivery to target cells.

The polydispersity of copolymers 39, 78 and 79 led to difficulty in interpretation of some of the data obtained from in vivo experiments employing intraperitoneal and subcutaneous administration of these copolymers. Because the preparations contained molecules of various sizes it was not possible to determine whether lower molecular weight fractions excreted in the urine were non-degraded molecules which were present in the initial preparation, or fragments of copolymers which had been

partially degraded within cells. In addition, it was not possible to determine whether molecules of a particular size preferentially migrated from one body compartment to another.

The use of radiotracers such as [^{125}I] iodide are invaluable in allowing the detection of very small quantities of copolymer (and degradation products) throughout the body. Iodination of copolymers results in only very small changes in the polymer properties (Drobnik and Rypaček 1984), and therefore radiolabelled copolymers will be likely to record the true fate of the unlabelled drug carrier. Radiotracers can, however, also lead to complications in interpretation of data. It cannot be proven, for example, that the radiolabel monitored throughout the experiment is attached to the copolymer (or its degradation products). A known (usually small) quantity of free [^{125}I] iodide was present in radiolabelled copolymer preparations on administration to rats, and this must affect the body distribution of radioactivity to some extent. Also, it is possible that some free [^{125}I] iodide might be released from the copolymer during the experimental period, for example, through the action of deiodinases present in certain tissues. Correction for radioactive decay is another problem encountered during long term experiments using ^{125}I-labelled preparations. Since urine and faeces samples were collected and assayed regularly throughout the long term experiment, and the majority of the radioactivity was recovered in the first few days, corrections for decay were not made during this experiment.

Despite these, and other problems encountered during in vivo studies (Chapter 7), the results reported in Chapter 7 were remarkably reproducible and therefore do allow some conclusions to be drawn from the work undertaken, especially

where large proportions ($>$ 10%) of the recovered radioactivity are found in tissues and excreta. Such studies, though time consuming and fraught with potential sources of error, are essential in order to gain a basic understanding of the ultimate fate of the copolymers in the body.

In summary, the work presented in this thesis proves that the theoretical concept of soluble crosslinked copolymers as drug carriers (section 1.4) does work in practice. Crosslinked HPMA copolymers are stable in rat plasma and are pinocytosed by a number of different cell types. Crosslinks (and drug linkages - Duncan et al 1981) are degraded intracellularly and resultant lower molecular weight copolymer chains can be eliminated from the body. It is possible to target crosslinked copolymers to the liver by the incorporation of galactosamine into the copolymer structure.

In addition, the study has shown the importance of size of the drug carrier on its rate of cellular uptake and the ability to target the carrier to the liver following different routes of administration.

The results reported will help in the design of polymers for use in future studies in that further consideration will be given to the size, polydispersity and crosslink structure of copolymers, in addition to those of drug linkages and other targeting moieties.

As a consequence of the work presented in this study, further in vivo experiments are planned to study the effect of size of HPMA copolymers on body distribution following different routes of administration to rats. Also, following the work of Duncan

et al (1983) and the current study on the effect of attachment of galactosamine to copolymers, the effect of the incorporation of other sugar groups into HPMA copolymers on biological systems is currently being studied by other workers in the group. Other studies recently undertaken include the testing of biocompatibility of HPMA copolymers using macrophages, evaluation of the toxicity of HPMA-drug conjugates using human hepatoma cells, and the use of antibody-copolymer conjugates. Last, but not least, in vivo studies on mice inoculated with L1210 leukaemia cells using daunomycin attached to single chain HPMA copolymers via oligopeptide sequences have commenced and preliminary results show a significant increase in survival time over free drug (Duncan 1985 - personal communication).

Collectively, these results show that the use of HPMA copolymers as drug carriers in a lysosomotropic drug delivery system is indeed a feasible proposition.

To date, the primary aim of this project, and many other proposed lysosomotropic drug delivery systems, has been to improve the treatment of malignant disorders by delivering existing anti-tumour drugs to the required tissues in optimum concentrations and thereby reduce damage to normal tissues (Monsigny et al 1980, Trouet et al 1982, 1983, Baurain et al 1982). There is no reason to restrict the use of lysosomotropic drug delivery systems to this one medical application, however. Recent work on liposomes, for example, has opened up new possibilities in their use as drug carriers (Gregoriadis 1981). The stability of liposomes in plasma (Hunt 1982) and simulated intestinal environments (Rowland and Woodley 1982) has been shown to be dependent on lipid composition and much recent work

in the field has therefore been concerned with the stability of liposomes of different compositions in biological environments. As a result, liposomes can now be synthesized which are stable in the circulation, non-toxic and can also contain surface targeting moieties (Ghosh et al 1982).

However, because of their size, targeting of liposomes to cells other than those of the reticulo-endothelial system has been difficult to achieve (Poste et al 1982, 1983). This feature of liposomes nevertheless can allow their targeting to macrophages, which avidly phagocytose liposomes. Drugs can be contained within the liposomes which activate the macrophages, rendering them tumouricidal (Poste et al 1982, 1983). Alternatively, liposomes could be used to carry drugs for treating infectious diseases involving macrophages e.g. leishmaniasis, leprosy, tuberculosis (Alving 1979, 1983).

The limitations imposed by size (discussed above) are also often apparent on using other vesicular carriers such as erythrocyte ghosts (Pitt et al 1983) and microspheres (Illum and Davies 1982). Similarly, treatment of diseases involving cells of the reticulo-endothelial system could be improved by the use of drug carriers such as these.

The manipulation of copolymer size or the attachment of specific targeting moieties to macrophage receptors could also enable crosslinked HPMA copolymers to be used as targetable carriers for antibiotics, with the added advantages of covalent drug linkages and comparative ease of fabrication. Single chain HPMA copolymers containing antibiotics attached by oligopeptide side chains P-Gly-Gly-ampicillin, P-Gly-Phe-ampicillin and P-Gly-Val-Phe-ampicillin and others containing 6-amino-

penicillanic acid have already been synthesized and shown to inhibit the growth of Staphylococcus aureus 209 P (Solovskij et al 1983).

It would also be possible to use smaller, single chain copolymers as molecular bridges between drug and antibody carriers. Natural macromolecules dextran and human serum albumin, when used as intermediate carriers between drug and antibody, have demonstrated improved drug toxicity over direct coupling of drug to antibody on incubation with tumour cells in vitro (Tuskada et al 1982, Garnett et al 1983). The use of an HPMA 'bridge' would also allow increased drug loading in such a system.

Other applications of the crosslinked HPMA copolymer include that of a blood plasma expander (section 1.4) and as a carrier for replacement enzymes in the treatment of lysosomal storage diseases.

Clearly, the potential applications of HPMA copolymers in drug delivery systems are diverse and numerous.

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APPENDIX I

COMPUTER PROGRAMME TO CALCULATE THE UPTAKE OF NON-DIGESTIBLE SUBSTRATES

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

Input Data

H(X) Duration of yolk sac incubation period (h)

I(X) Mean radioactivity count per 30s for 1.0ml of incubation medium

K(X) Mean radioactivity count per 5min for 1.0ml of yolk sac solution

L(X) Total protein content of the yolk sac (mg)

COMPUTER PROGRAMME TO CALCULATE THE AVERAGE MOLECULAR WEIGHT OF COPOLYMERS

```
0010PRINT"PRAGUE PROGRAMME"
0020DIMA(90,2)
0030PRINT"ENTER CONSTANTS"
0040PRINT"'a'="
0050INPUTA
0060PRINT"'b'="
0070INPUTB
0080PRINT"ENTER FIRST PEAK COUNT-'C'"
0090INPUTC1
0100PRINT"ENTER NO. OF COUNTS"
0110INPUTC
0120MATA=ZER(C,2)
0130PRINT"ENTER PEAK HEIGHTS"
0140X=C1-1
0150FORI=1TOC
0160X=X+1
0170A(I,1)=X
0180PRINT"COUNT";X
0190INPUTA(I,2)
0200NEXTI
0210H1=0
0220D1=0
0230P1=0
0240FORI=1TOC
0250H1=H1+A(I,2)
0260S1=A+(B*A(I,1))
0270S2=10^S1
0280D1=D1+(A(I,2)/S2)
0290P1=P1+(S2*A(I,2))
0300NEXTI
0310W=P1/H1
0320N=H1/D1
0330U=W/N
0340PRINT"PEAK PARAMETERS :"
0350PRINT"COUNT VALUES","HEIGHTS"
0360MATPRINTA
0370PRINT
0380PRINT"'a'=";A
0390PRINT"'b'=";B
0400PRINT"MW=";W
0410PRINT"MN=";N
0420PRINT"U =";U
0430END
```

APPENDIX II

TABLE 3.1

COPOLYMER CROSSLINK/SIDECHAIN STRUCTURES

POLYMER CODE NO.	STRUCTURE
39	P-Gly-Gly-Phe↓-Tyr↓-HMDA↓-Tyr↓-Phe-Gly-Gly-P
74	P-Gly-Gly-Phe↓-$TyrNH_2$
68	P(-$TyrNH_2$)-Gly-Gly-Phe↓-HMDA↓-Phe-Gly-Gly-P
78	P(-$TyrNH_2$)-Gly-Gly-Phe↓-HMDA↓-Phe-Gly-Gly-P Gly-Gly-galactosamine
79	P(-$TyrNH_2$)-Gly-Gly-Phe↓-HMDA↓-Phe-Gly-Gly-P Gly-Gly-aminopropanol

↓ denotes the bonds probably hydrolysed by lysosomal enzymes.