A STUDY OF MACROMOLECULE ABSORPTION *IN VITRO*

BY THE SMALL INTESTINE OF ADULT RATS

by

JANINE F. BRIDGES, B.Sc.

A Thesis submitted to the University of Keele in partial fulfilment of the requirements for the Degree of Doctor of Philosophy

Biochemistry Research Laboratory,
Biological Sciences Department,
University of Keele.

April, 1980

C O N T E N T S

ABBREVIATIONS

Abbreviations in this thesis comply with the policy of the Biochemical Journal.

ABSTRACT

A method has been developed for the study of macromolecular uptake by the everted sacs of adult rat intestine *in vitro*. The method incorporated a preparation procedure which minimized temporary hypoxia in the tissue. The complex medium TC 199 containing 10% calf serum and 1mM ATP was used as the incubation medium. This medium was shown to have the following advantages over the simple medium traditionally used for the incubation of everted intestinal sacs (i.e. Krebs-Henseleit Ringer).

i) Tissue viability and structural integrity was maintained for longer periods of incubation time (up to 2h).

ii) The absorptive surface was presented with a large variety of substrates therefore macromolecules were not taken up solely because they were the only nutrient available.

A method of calculating results was employed which allowed quantitation of the uptake of different macromolecules, gave an indication of the uptake mechanism and facilitated direct comparison between other cellular systems.

Using the developed method, uptake studies showed that the non-degradable macromolecule [^{125}I]-PVP was taken up into the tissue and accumulated in the serosal fluid linearly with respect to time up to 2h at rates of 0.74 and 0.12 µl/h/mg protein. Uptake was dependent on substrate concentration, was temperature sensitive and could be inhibited by glycolytic and oxidative phosphorylation inhibitors. This evidence indicated fluid phase pinocytosis to be the mechanism of uptake.

Uptake studies using the degradable macromolecule HRP as substrate showed that the protein was accumulated in the tissue and serosal fluid linearly with time up to 2h at rates of 11.95 and 0.11 µl/h/mg protein. HRP was taken up by the tissue about 16 times faster than [^{125}I]-PVP but the rates of accumulation in the serosal space were similar for both

macromolecules. This meant that a large proportion (about 99.1%) of the HRP taken up into the tissue was degraded intracellularly.

For both macromolecules, only about 0.07% of the substrate present in the system was transported to the serosal space. The functional significance of these small quantities of macromolecule which are transported across adult intestine is discussed.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 The Morphology of the Epithelium of the Mammalian Small Intestine

Any substance to be absorbed from the intestinal lumen into the circulation of the body must pass across the epithelium of the gut. Equally, substances to be transferred to the lumen of the gut have to pass through the epithelial lining or are secreted by the cells of the epithelium. The necessity to deal with absorption, secretion and excretion alike in maintaining nutrition and homeostasis of the organism requires a morphologically complex organ which contains various highly differentiated cell types.

With the introduction of electron microscopy, the structure of the small intestinal mucosa has been thoroughly investigated.

1.1.1 Organisation and general structure of the small intestinal mucosa

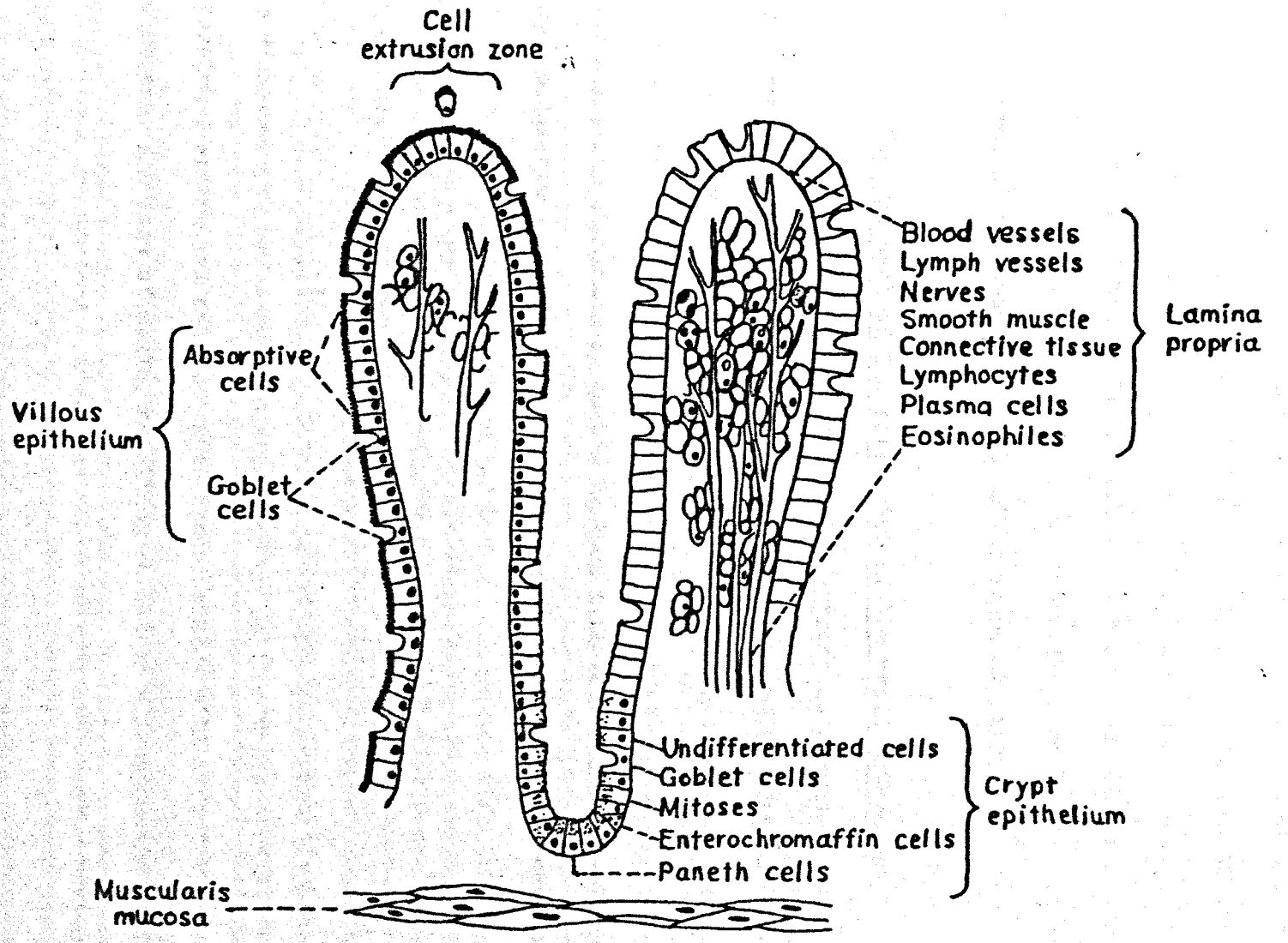
The mucosa of the small intestine may be divided into three layers (Fig. 1(i)). The deepest of these, the muscularis mucosae, consists of a thin sheet of smooth muscle generally 3-10 cells thick, which separates the mucosa from the submucosa.

The middle layer, the lamina propria, is a continuous sheet of connective tissue which together with the epithelium forms the villi and crypts. The lamina propria normally contains a variety of cell types including fibroblasts, macrophages, plasma cells, lymphocytes, eosinophils and mast cells as well as noncellular connective tissue elements. Blood and lymph vessels, unmyelinated nerve fibres and strands of smooth muscle are also regularly present.

The lamina propria provides important structural support for the intestinal epithelium and its vascular and nervous elements and there is evidence that the lamina propria may help the body combat harmful foreign substances and organisms that penetrate the intestinal epithelial barrier (Ottaway *et al.*, 1979).

The third layer of the intestinal mucosa consists of a continuous

FIGURE 1(i)



Schematic diagram of 2 villi and a crypt to illustrate the histological organization of the mucosa of the small intestine.

sheet of a single layer of columnar epithelium which covers both the villi and crypts. The epithelial cells are only separated from the lamina propria by the epithelial basement membrane which lies between the cellular elements of the lamina and the basal plasma membrane of the epithelial cells. Absorptive, goblet and a few enterochromaffin cells cover the surface of the villi and goblet, enterochromaffin and Paneth cells line the crypts. The presence of the villi normally increases the surface area of the absorptive epithelium about eightfold.

1.1.2 Cell renewal of the epithelium of the small intestine

The epithelium of the gastrointestinal tract is a dynamic, actively proliferating tissue which renews itself rapidly and regularly. The crypts have been identified as the site for cell proliferation and the absorptive cells differentiate during migration from crypts onto villi. Cell migration continues up to the villous tips (extrusion zones) from which cells are eventually shed (Trier, 1967). The mean generation time for a jejunal cell in rat is 12h and the mean migration time (the time required for cells to migrate from the proliferative region of the crypts to the villus tip) is 1-3 days. The rate of formation and extrusion of villous epithelial cells may be altered together or separately, resulting in an alteration of general architecture as well as the morphology of the individual epithelial cells. Nutritional states will affect the balance of renewal and loss of cells, resulting in either a lengthening or a shrinking of the villi. Radiation or antimitotic drugs stop cell production in the crypts while cell loss continues, resulting in a progressive shrinkage of the villi (Trier, 1967).

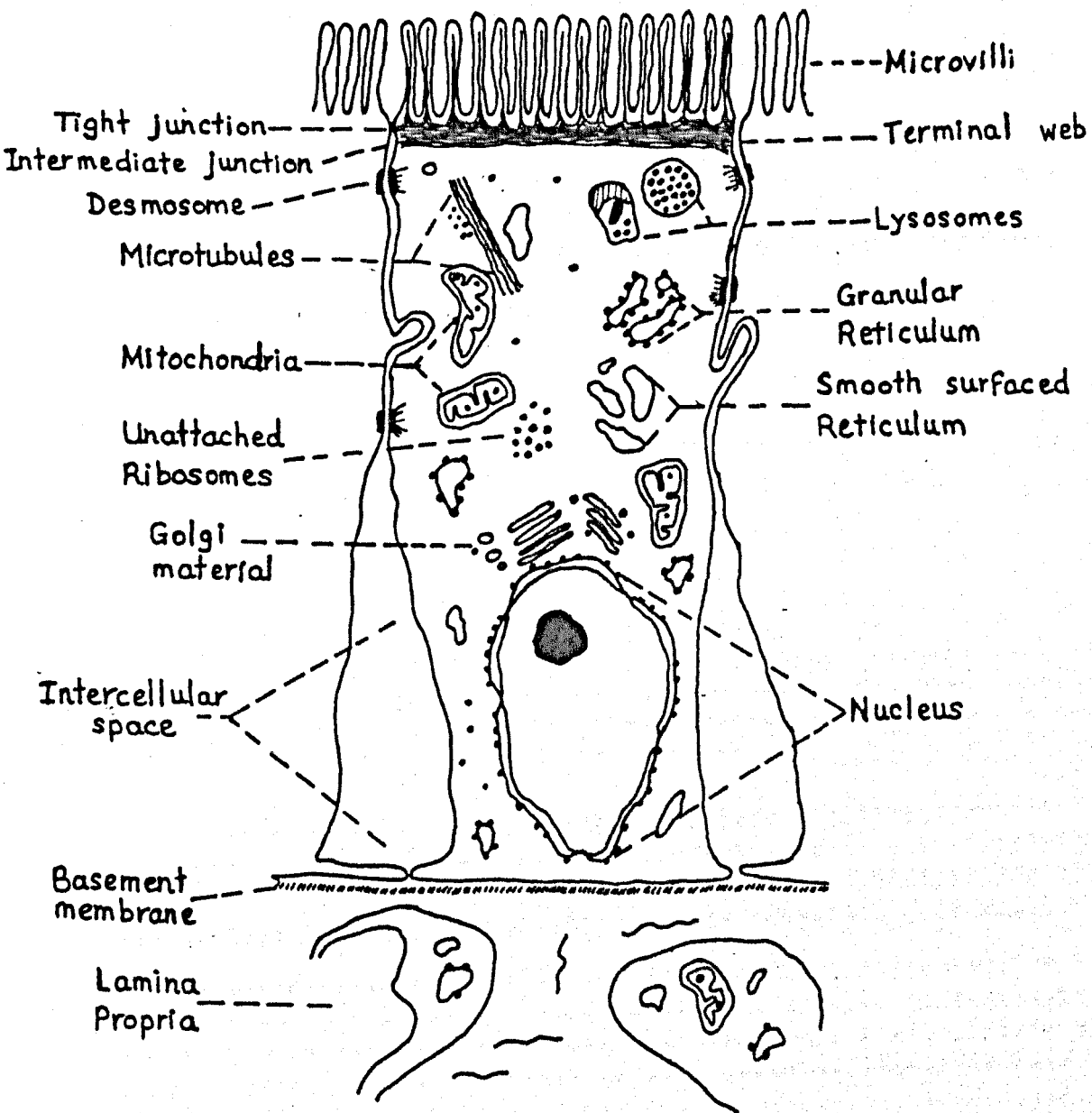
Continued cell proliferation has been used as an indication of tissue viability in organ culture. The migration rate of the small intestinal epithelium in culture can be measured quantitatively by following the progress of migration up the villi of epithelial cells pulse-labelled during DNA synthesis with ^{3}H-labelled thymidine (Hirondell *et al.*, 1975).

Alternatively monitoring of crypt-cell division by electron-microscopy gives a qualitative indication of continued cell proliferation (Lepper and Mailman, 1977).

1.1.3 Fine structure of intestinal epithelial cells

The absorptive cells or enterocytes cover the villi with a continuous sheet which is broken only at the extrusion zones of the cells on the villous tip. Fig. 1(ii) is a schematic diagram of an intestinal adsorptive cell. Mammalian enterocytes are tall, columnar or enterocyte cells with basally located nuclei, while the Golgi apparatus, the endoplasmic reticulum, mitochondria and lysosomes are located in the apical part of the cell. The cell base rests on the basement membrane, a fibrillary structure of glycoprotein. The apical plasma membrane shows the brush border arrangement typical of many epithelial cells. The microvilli of the entercyte brush border average about 1 μm in length and 0.1 to 0.2 μm in width and are strengthened by a bundle of actin fibrils which are rooted in the terminal web of the cell. The outer layer of the brush border membrane is covered with the "fuzzy" coat or glycocalyx which appears to be firmly attached to the membrane. Evidence suggests that the glycocalyx consists of glycoprotein synthesised in the Golgi and that it is continuously renewed (Forstner, 1969). It has been suggested that the glycocalyx may serve as a relatively impenetrable barrier against substances in the gut lumen e.g. bacteria and other foreign materials (Ito, 1965), that it plays an important role in selective binding of substance prior to their absorption into the cell (Fawcett, 1964) or that it might even be an important site for the digestion of nutrients prior to their adsorption (Ugolev, 1974).

Adjacent enterocytes are closely apposed and locked together. The so-called junctional complex consists of a tight-junction where the membranes actually appear to fuse, an intermediate zone, and a number of



Schematic diagram of a villous absorptive cell.

FIGURE 1(ii)

desmosomes, thickened areas beneath the cell membrane. The cells show frequent infoldings in adjacent lateral plasma membranes which might allow for expansion of individual cells as the need arises. Whether or not tight junctions can open and what can pass through is still under some dispute, it seems likely though that at least some water and sodium may pass through (Machen et al., 1972, Schults and Frizzell, 1972).

The lateral cell membrane may be seen adjacent or widely separated. There is evidence that this space opens up during absorption to expose the lateral cell membrane (Creamer, 1974).

1.2 Macromolecular Transport in Neonatal Intestine

The neonatal mammalian small intestine has a capacity to ingest macromolecules by an endocytic mechanism (Clarke, 1959; Jaques, 1969; Brambell, 1970; Rodewald, 1976). The initial event in this process is an interaction between large molecules within the intestinal lumen and components of the microvillous membrane of intestinal absorptive cells (adsorption). When sufficient concentration of molecules comes in contact with the cell membrane invagination occurs and small vesicles are formed. The uptake is energy-dependent since invagination can be inhibited with metabolic inhibitors of both glycolysis and oxidative phosphorylation (Iecce, 1966a and 1966b). After invagination, macromolecules migrate within membrane-bound vesicles (pinosomes) to the supranuclear region of the cell where the vesicles fuse with lysosomes to form large vacuoles (secondary lysosomes). Within these structures, intracellular digestion can occur. Any ingested macromolecules which escape breakdown migrate to the basal-lateral surface of the cell to be deposited in the intercellular space by reverse endocytosis (Brambell, 1966). Very little is known about those conditions of adsorption which can inhibit or facilitate invagination, nor is the mechanism of lysosomal migration to the site of vesicle formation clearly understood. The general mechanism of uptake, intracellular transport and intercellular transport of macromolecules are shown in Fig. 1(iii).

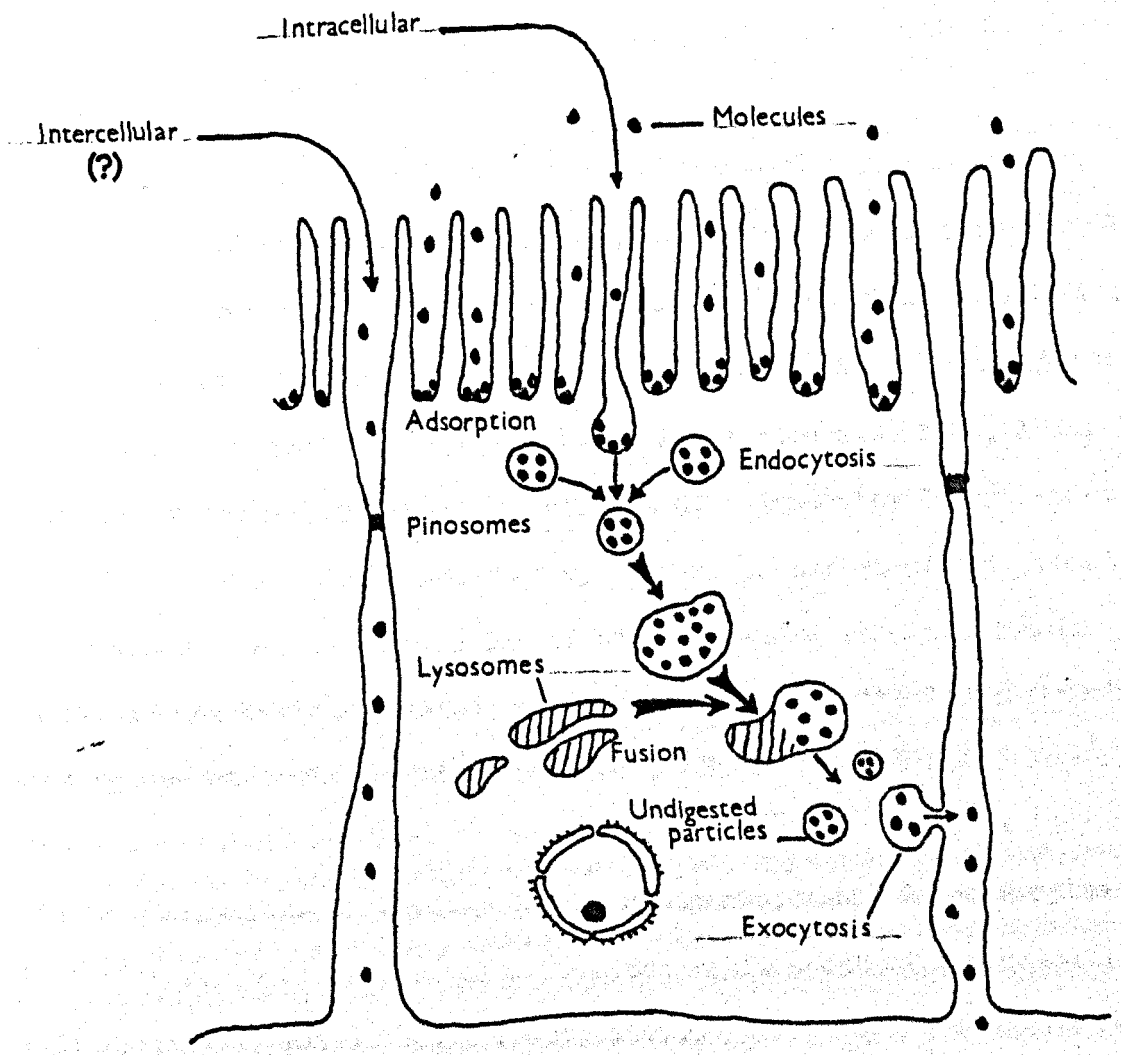
1.2.1 Gamma globulin (IgG) uptake

Intestinal uptake and transport of proteins has been most extensively studied in animals that acquire passive immunity either in part or entirely from the passage of maternal antibodies across the small intestinal tract (Brambell, 1966; Morris, 1968; Bamford, 1966; Rodewald, 1970, 1973, 1976; Jones and Waldmann, 1972; Burton and Smith, 1976). Animals such as ruminants, which receive all their maternal immunoglobulins in the postpartum

FIGURE 1(iii) General mechanisms for the uptake and transport of macromolecules by neonatal intestine

(1) Intracellular uptake. After adsorption and endocytosis by the microvillous membrane, macromolecules are transported in small vesicles and larger pinosomes. Intracellular digestion occurs when lysosomes combine with the pinosomes. Intact molecules which remain after digestion may be deposited in the intercellular space by a reverse endocytosis (exocytosis).

(2) Intercellular uptake. Alternatively macromolecules may cross the 'tight junction' barrier between cells and diffuse into the intercellular space.



General mechanisms for the uptake and transport of macromolecules by neonatal intestine.

FIGURE 1(iii)

period, have a short period of increased permeability to all macromolecules coming in contact with the intestinal mucosa (Morris, 1968). After this period of chanced uptake, which lasts only a few days, the intestinal tract "closes" to prevent further bulk passage of proteins. "Closure" is related to a morphological and functional maturation of small intestinal cells in situ (Patt, 1977).

In contrast to the bulk transport of macromolecules in ruminants, animals such as rodents, which derive passive immunity in part from the intrauterine transport of maternal antibodies (Ab) and in part from the post partum intestinal uptake of immunoglobulins, have a prolonged period of selective transport of IgG (Bamford, 1966; Rodewald, 1970, 1973, 1976; Jones and Waldmann, 1972). The concept of selectivity in transport is supported by the observation that uptake and transport of IgG proteins is much greater than the absorption of other proteins such as albumin (Halliday and Kekwick, 1960). Furthermore, among the IgG's absorbed, homologous rat IgG's are taken up more readily than are heterologous Ab's from other animal species. The period of enhanced transport in rats ceases abruptly at 20 days. Unlike ruminants, there is good evidence that closure in rodents results from the replacement of the neonatal epithelium with mature cells (Patt, 1977).

Morphological studies during the period of enhanced absorption and afterwards demonstrate that the ultrastructural appearance of intestinal epithelial cells changes from the primitive cell of the new-born (containing multiple membrane invaginations and vesicles in the apical cytoplasm) to the characteristic appearance of an adult intestinal epithelial cell (with its typical microtubular cytoplasmic network) (Clark, 1959; Rodewald, 1973). Moreover, the enzyme content of the intestinal microvillous membrane changes in the adult rodent compared with the new-born (Moog, 1953). These differences in the cellular characteristics of neonatal versus adult rats are cited as evidence that maturation of the small intestine results in the

closure of IgG transport. Early closure can be demonstrated by administering corticosteroids (Morris and Morris, 1976). The mechanism for steroid induced closure is probably related to the early maturation of intestinal cells since certain membrane enzymes present in mature intestine can be induced by use of steroids (Moog, 1953 and 1955).

1.2.2 Mechanism of selective transport of γ-globulins

Selective transport of IgG by the neonatal rat intestine represents a unique process of macromolecular transport (Fig. 1(iva). Several recent studies have shown that the mechanism of transport is still in dispute (Rodewald, 1970; 1973 and 1976; Jones and Waldman, 1972; Wild, 1973; Moxon and Wild, 1976). Brambell (1966) first theorized that IgG's transported across yolk sac and intestinal epithelium were protected from intracellular lysosomal digestion because of their attachment to specific receptor sites in the microvillous membrane. When membrane vesicles (containing both IgG attached to the receptor sites and other proteins free within the vesicles) combined with lysosomes, unattached proteins were broken down whereas attached IgG was unaffected. These proteins could then be transported out of the epithelial cells into the circulation. Studies by Sonada et al. (1973) have supported this hypothesis.

At variance with this hypothesis, two other mechanisms have been suggested to account for selective transport of IgG. It has been claimed that both homologous and heterologous immunoglobins bind equally well to membrane components of epithelial and yolk sac endoderm cells, indicating a lack of specific receptors and that selective degradation of proteins may be affected by differential action of lysosomal cathepsins (Hemmings and Williams, 1974). It has also been suggested that selection may be effected by specific transport in "coated" pinocytotic vesicles (Wild, 1975). This latter idea is based on evidence that phagolysosomes do not exocytose their contents and that coated vesicles are the only vesicle

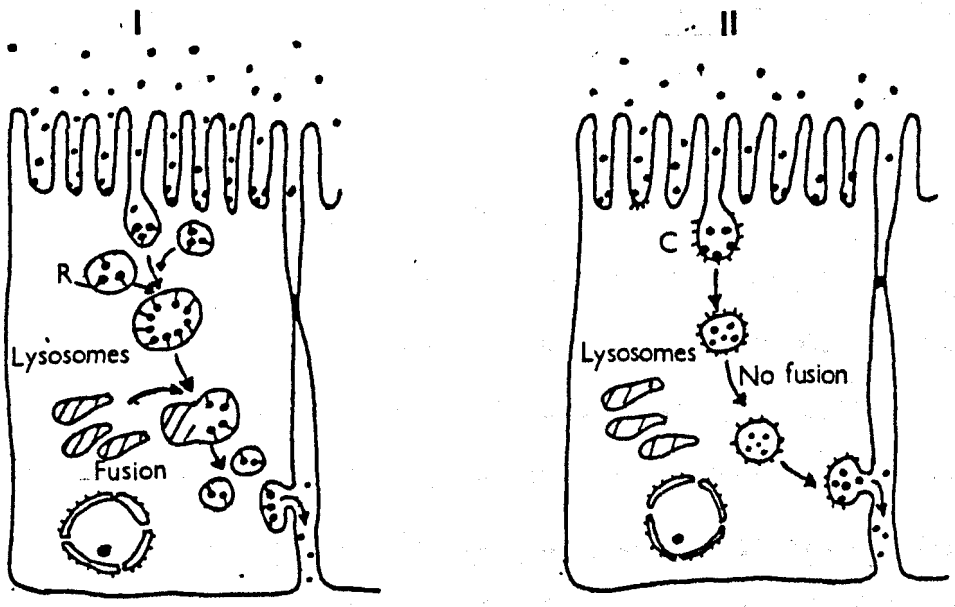
FIGURE 1(iv) Mechanisms of macromolecular absorption by the neonatal mammalian intestine

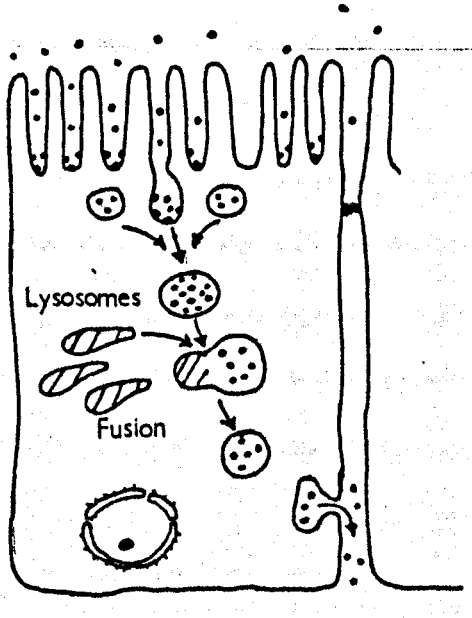
a) Selective transport of maternal IgG in colostrum occurs in the jejunum of new-born rats via either specific receptors (R) present on the microvillous membrane or receptors then coated vesicles (C). IgG is presumed to be protected from intracellular lysosomal digestion either because of attachment to the receptor site (I) or because the coated vesicles do not fuse with lysosomes (II). In either case IgG is transported in large quantities out of the cell.

b) Non-selective uptake and transport of other macromolecules occurs throughout the small intestine of most neonatal animals. Immature intestinal absorptive cells engulf large quantities of macromolecules. After intracellular digestion by lysosomes, very small quantities are deposited in the intercellular space.

(After Walker and Isselbacher, 1974)

a) Selective transport





b) Non-selective transport

Mechanisms of macromolecular absorption by the neonatal mammalian intestine.

FIGURE 1(iv)

type to be seen in confluence with the basal and lateral plasmalemma (Moxon and Wild, 1976). Whatever the case Rodewald (1973) has shown that specialized transport of IgG is localized to the jejunum and that after closure the selective uptake process ceases.

1.2.3 Uptake of other macromolecules

Although considerably less is known about the intestinal transport of macromolecules other than IgG during the neonatal period, several classic studies have shown that immature small intestine can ingest other proteins and macromolecules (Fig. 1 (iv)b) (Clarke, 1959; Graney, 1968; Orlic and Lev, 1973a; 1973b). Uptake also occurs by a pinocytotic mechanism and probably exists throughout the small intestine. In a morphological study Clarke (1959) demonstrated that proteins and colloidal materials administered orally to suckling rats and mice were ingested by columnar absorptive cells of the jejunum and ileum. Ingested macromolecules were found to enter large vacuoles in the supranuclear region, a characteristic morphological feature of immature intestinal cells. However, in another study with ferritin in suckling rats Graney (1968) suggested that the ileum may be the preferred location for transport. Despite this controversy, no specific receptor sites for macromolecular attachment to cellular membranes have been demonstrated, suggesting that the uptake of macromolecules is non-selective and therefore more likely to occur throughout the small intestine. Only trace amounts of macromolecules are transported from the intracellular space into the circulation compared to the much larger quantities of IgG absorbed by a more selective process. Transport also appears to be proportional to the concentration of macromolecules coming in contact with the mucosal surface (Walker et al., 1972).

Using neonatal everted gutsacs, Walker et al. (1972) showed that only trace amounts of horseradish peroxidase (HRP) were transported into serosal fluid but these quantities increased in proportion to the HRP concentration

in the incubation media. Walker et al. (1972) also suggested that absorption was the same in the jejunum and ileum. Rodewald (1973) suggested that ileal transport of IgG was as non-selective as that for all other proteins since a quantitative measure of transport was similar to levels reported for HRP by Walker et al. (1972). It was assumed from morphological studies, which showed excessive uptake of macromolecules by absorptive cells during the neonatal period and minimal transport of macromolecules out of the cells, that lysosomal activity must be increased (Walker and Isselbacher, 1974). Although no direct measurement of lysosomal enzyme levels has been reported to support this conclusion, Kraehenbuhl and Campiche (1969), using histochemical staining techniques to measure acid phosphatase activities have demonstrated increased lysosomal activity after the ingestion of macromolecular proteins in neonatal pigs, rats and rabbits. This morphological evidence supports the hypothesis that lysosomes effect macromolecular transport.

In summary, the neonatal intestine of many mammalian species, including man, is permeable to IgG and other macromolecules. Uptake of IgG in certain species is selective and dependent on either a specific receptor site or uptake into 'coated' vesicles allowing for the increased transport of these immunoglobulins into the circulation. Other macromolecules are taken up by a non-selective pinocytotic process, but transported in much smaller quantities into the circulation because they are subjected to considerable intracellular breakdown.

1.3 Macromolecular Transport in Mature Intestine

The uptake and transport of IgG markedly decreases when the neonatal small intestinal epithelial cell membrane matures (Bamford, 1966; Rodewald, 1973). At the time of closure, there is also morphological and physiological evidence for a decreased capacity of absorptive cells to ingest other macromolecules (Clarke, 1959). Based on these observations, it has been assumed macromolecular absorption ceases entirely with epithelial cell membrane maturation. However, reports have appeared indicating that at least small, nutritionally insignificant amounts of intact macromolecules may be transmitted across the mature mammalian gut in both man and experimental animals.

The early experiments on the passage of macromolecules into the body from the digestive tract have been well reviewed in articles by Wells and Osborne (1911), Meltwer and Kriz (1925), Watzer (1927), Ratner and Gruehl (1934) and Alexander *et al.* (1936). These give a detailed survey of the subject up until 1935.

Probably the most convincing technique used to demonstrate the passage of antigens into the body was direct or indirect anaphylaxis, in which men or experimental animals were challenged by the feeding of antigens. Ratner and Gruehl (1934) concluded from their experiments that antigens entered the blood stream directly from the gastro-intestinal tract. Between 1930 and 1935 direct passage of entire protein molecules was clearly demonstrated in man as well as in guinea pig, dog, rat and rabbit. Papers later than 1930 give accurate accounts of the state of the molecules after their absorption, the factors which regulate their entry, some degree of measurement of the quantities absorbed and the routes followed by these substances before they entered the general circulation. Recently some of these points have been clarified as follows.

Murlin *et al.* (1937) showed that the absorption of insulin from Thiry-Vella loops of the jejunum of dogs, as assessed by reduction of blood sugar, was a real phenomenon. Smyth *et al.* (1964) studied the absorption of proteolytic

enzymes labelled with radioactive iodide in rabbits. Similar experiments on the absorption of bromelain showed that this molecule was absorbed intact in man (Miller and Opher, 1964). Ravin et al. (1960) found that the absorption of bacterial endotoxin (Escherichia coli O11 B4) from the gastrointestinal tract occurred in normal rabbits. Gruskay and Cooke (1955) demonstrated permeability of the intestinal tract to intact proteins (egg albumin) in healthy children. Bernstein and Ovary (1968) were unable to confirm this although they showed a significant transfer of the hapten d-E-bis-DNP-L-lysine (M.W. 478) through the intestinal mucosa and Bockman and Winborn (1966) demonstrated transport of horse-spleen ferritin across ligated intestinal segments in normal adult hamsters.

Cornell et al. (1971), Warshaw et al. (1971) and Walker et al. (1972) provided direct morphological and physiological evidence for macromolecular transport using an enzyme marker horseradish peroxidase (HRP). This enzyme can be detected histochemically at both light and electron microscopic levels; (Graham and Karnovsky, 1966). It can also be measured quantitatively in small concentrations (Maehly and Chance, 1954). After intraluminal injection of peroxidase into ligated segments of jejunum and ileum, HRP was found adsorbed to the apical surface membranes and within membrane-bound cytoplasmic canalicular, vesicular and vacuolar structures. Absorbed HRP was noted in the intercellular spaces between adjacent absorptive cells, traversing the basement membrane and in the spaces of the lamina propria (Cornell et al., 1971). From these studies the authors concluded that HRP can be taken up by a pinocytic mechanism and transmitted to the extracellular space of the lamina propria. The mechanism was thought to be similar to that described for neonatal intestine. In a parallel in vivo experiment the ability of the rat small intestinal mucosa to transmit macromolecules from its lumen to mesenteric lymph and portal venous blood was determined by means of a quantitative study of HRP

absorption, (Warshaw et al. (1971), Warshaw et al. (1975)). After infusion of HRP into jejunum through an indwelling catheter, small but significant amounts of protein tracer were consistently transmitted across the gut into intestinal lymph and portal blood. The uptake of HRP into absorptive cells and HRP transmission into intercellular spaces and the lamina propria was the same as that reported in the ligated loop experiments.

To study the mechanism more precisely, the transport of HRP into the serosal fluid of everted small intestinal sacs from neonatal and adult rats was measured (Walker et al., 1972). From these studies Walker et al. (1972) concluded that HRP uptake was an energy dependent process similar to the pinocytic process noted in neonatal animals.

Support for these studies came from Worthington et al. 1974, 1975 and 1976. Using serological and morphological procedures these workers showed that bovine serum albumen (BSA), HRP, Horse-spleen ferritin (mol. wt. 650,000) and adenovirus particles (mol. wt. 1,000,000) were taken up from ligated loops of adult rat small intestine. Electron microscopy revealed tracer-particles of the latter three macromolecules to be present in vesicles and lysosome like bodies. These authors also reported that the number of macromolecule-containing vesicles increased in protein deficient rats.

Loehry et al. (1970) and Kingham et al. (1976), in their work on perfused rabbit intestine showed macromolecules such as polyvinyl pyrrolidone (PVP, mol. wt. 33,000) and inulin (mol. wt. 5,000) were able to pass both from the intestinal lumen to the portal blood and in the opposite direction. These workers showed that permeability diminished with increased molecular weight and postulated the existance of pores in the intestinal mucosa which allowed passage of macromolecules. The greater the number of pores, the more permeable the membrane. Also the greater the pore-size, the less selective the membrane becomes. Tagesson

et al. (1978) also reported the rate of passage of macromolecules (dextrans in this case) to be inversely proportional to their molecular weight but did not speculate as to the mechanism of uptake.

Rhodes and Karnovsky (1971) reported that after surgical trauma to the small intestine of adult guinea pigs, HRP was noted to diffuse intercellularly through the tight junctions of epithelial cells and was not taken up in demonstrable quantities by absorptive cells. This suggested that macromolecules may be transmitted by an intercellular as well as intracellular process.

1.3.1 Quantification of macromolecular uptake in adult intestine

Various authors have tried to quantitate absorption of macromolecules by the intestine. Only approximate values are available. Gruskay and Cooke (1955) reported that 0.002% of egg albumin fed to normal children was found in their serum. Thomas and Parrott (1974) found serum levels of 1-10 ng/ml BSA in normal rats which had been given 25 mg of protein orally; i.e. 0.001% of the administered dose in the entire blood. Similar experiments in mice showed a slightly wider range of values. Swarbrick et al. (1979) showed that of an orally administered 25 mg dose of ovalbumin, up to 0.004% (1 µg/ml) could be found in the blood whereas Andre et al. (1974) reported blood levels as high as 400 µg/ml (1.6%) for a 25 mg dose. Warshaw et al. (1974 and 1977) have shown in the rat that up to 2% of a dose of labelled albumin is absorbed, more than half into the portal vein and the rest into the mesenteric lymphatics. These workers point out that at the highest concentrations they used in their experiments (500 mg/kg body weight), several mg of protein were absorbed, and that weight for weight the dose was only equivalent to that of a glass of milk for a human toddler (Warshaw et al., 1974). In a recent report of their experiments in rats, Hemmings and Williams (1978) have added a new dimension to this debate. They reported that up to 5% of a dose of ingested protein

(including IgG, gliadins and haemoglobin) were found in the bloodstream of the animal concerned. By measuring radioactivity in the animals carcass, they claimed that between 12% and 46% of ingested material was found in tissues as protein bound material (with a mol. wt. greater than 10,000). Of this, more than 20% retained the antigenic characteristics of the original protein. A similar high value for absorbed protein has been reported by one other group (Morris and Morris, 1976). On the basis of these findings Hemmings and Williams (1978) suggested that about half of the protein load delivered to the small intestine of a rat is not broken down to amino acids and peptides but freely passes intact or as large molecular weight fragments, through the enterocytes and into the systemic circulation. These large molecules are then distributed to the tissues and cells and broken down. If this is correct, macromolecular absorption has nutritional as well as immunological implications.

1.3.2 Substances which interfere with macromolecular uptake

Antigen absorption across the gut mucosa is reduced by the presence of IgA-class, and to a lesser extent IgM-class antibodies in the mucosa or at the mucosal surface (Andre et al., 1974; Bazin, 1976; Walker, 1978). Since the factors which control transmucosal passage of antigen are poorly understood, the way in which antibody interferes with this transport is unknown, William and Gibbons (1972) observed a definite decrease in adhesion of the bacteria Streptococcus viridans after exposure to secretory antibodies. They concluded that the secretory antibodies block specific binding sites on the bacterial cell wall and thereby interfere with bacterial adherence to epithelial surfaces. Additional studies have shown interference in the attachment of Vibrio cholerae to intestinal mucosa by secretory IgA antibodies (Fubura and Freter (1973).

Intestinal antibodies can also protect against the effects of toxic bacterial by-products. Pierce and Cowans (1975) showed that secretory

antitoxins complexing with cholera toxin can prevent toxin binding to receptors on intestinal microvillus membranes and thereby interfere with the activation of adenylate cyclase, a step in the active fluid secretion associated with toxigenic diarrhoea. Similarly, intestinal antibodies were shown to interfere with the uptake of non-viable antigens introduced directly into the gastrointestinal tract (Walker et al., 1972). These workers reported that intestinal antigens become rapidly associated with antibodies present in the glycocalyx. Antigen-antibody formation at that site appears to prevent migration of antigen to the cellular membrane surface and thereby to interfere with endocytosis.

The opposite effect was found by Bockmann and Winborn (1966) who described increase of antigenic absorption in hamsters immunized with ferritin. This might indicate local trapping of antigen by specific antibodies binding antigen in the gut wall (Walker et al., 1974).

A number of non-immunological factors exist, present either within the intestinal lumen or on the intestinal mucous surface, which are also important as defence mechanisms. These factors include peristaltic movements, goblet cell release of mucus, pancreatic enzymes, and indigenous flora, Walker (1975) reported that antigen, when trapped at the enterocyte surface in an antigen/antibody complex was rendered more susceptible to degradation by pancreatic enzymes. More recently, Walker et al., 1977, showed that antigen/antibody complex formation within the intestinal lumen triggered the release of goblet-cell mucus which in turn can interfere with further antigen penetration.

Clinically, both IgA deficiency (Kaufman and Hobbs, 1970) and chronic pancreatic insufficiency (Wallwork et al., 1974) have been associated with allergy and it has been suggested that the pathological mechanism in gastrointestinal and other allergic diseases may be due to excessive stimulation of the immune system by antigen which crosses the mucous membrane (Editorial, Lancet, 1975). Indeed low serum levels of IgA have been noted

in healthy infants at the age of 3 months who later became atopic (Taylor et al., 1973) and in children with acute gastritis who later became hypersensitive to cows milk (Harrison et al., 1976). However, the theory that transmucosal antigen uptake is the primary abnormality in allergy has not been supported by experimental work.

1.3.3 Uptake of particles

Volkeimer et al, (1977) have differentiated the resorption phenomena (diffusion, active transport and pinocytosis) already described from the persorption phenomena which is entry of particles into the body. They have studied transport of particles of 7-110 microns diameter (pollen powder, corn, maize, potato starch grain, lycodin spores and metallic iron particles). The tested species were rats, guinea pigs, hens, rabbits, dogs and man. In the normal digestive mucosa, they found these markers to be localized at all places where the epithelium was unstratified but mainly at the extrusion zones at the tips of the villi in the jejunum. Pollen, starch grains and spores were found in the subepithelial mucosal layers as well as in the lymphatic vessels and the venous blood vessels. In normal man, 70 starch grains per 10 ml venous blood were detected after ingestion of 200 g raw oat flakes, i.e. about 20,000 starch grains for the complete blood volume. The largest persorbed particles were sand grains of 150 microns diameter; 40-70 microns diameter starch grains or spores were persorbed in constant amounts. According to these authors, persorbed particles seem to leave the bloodstream in the urine, pulmonary alveoli, cerebrospinal fluid, peritoneal cavity, milk, bile and by macrophage phagocytosis. This persorption phenomenon has been confirmed by a number of other authors, e.g. Schlewinski et al. (1971) in studies on phages and bacteria in mice, but Carter and Collins (1974) found no evidence of persorption of Salmonella in mice.

1.4 Macromolecular Uptake in Other Cellular Systems

Endocytosis is a temperature and energy dependent function that regulates the uptake of exogenous molecules from the cells environment via plasma-membrane derived vesicles and vacuoles. Both soluble (pinocytosis) and particulate (phagocytosis) substances may be interiorized. Although most, if not all, eukaryotic cells demonstrate these primitive functions, they are particularly prominent in leucocytes, macrophages, capillary endothelial and thyroid epithelial cells, yolk sac and oocytes. The subject has been well reviewed recently by Silverstein et al. (1977); Edelson and Cohn (1978) and Pratten et al. (1979). From these studies it is evident that some generalizations can be made about the endocytic mechanisms.

Most investigators use the term phagocytosis to describe the uptake of large particles i.e. over 100 nm diameter. The term pinocytosis is used to describe the vesicular uptake of everything else from small particles (lipoproteins, ferritin, colloids), to soluble macromolecular (enzymes, hormones, antibodies, toxins). So the terms phago- and pinocytosis are probably accurate in distinguishing between particle vs. fluid containing vacuoles but it remains to be determined if these two processes differ in their initiation and metabolic requirements.

During pinocytosis, substances may be captured in 2 possible ways (Jaques, 1969). They may be taken up in solution (fluid phase pinocytosis), or alternatively , they can be carried into the cell attached to the invaginating plasma membrane (adsorptive pinocytosis). Thus substrates with no affinity for the membrane are taken up solely in solution whereas substrates with membrane affinity can be taken up either membrane bound with total exclusion of fluid or by a combination of fluid and adsorptive modes. These mechanisms are shown diagrammatically in Fig. 1(v). In fluid phase endocytosis, uptake is directly related to the concentration of solute in the extracellular fluid, whereas in adsorptive pinocytosis uptake in

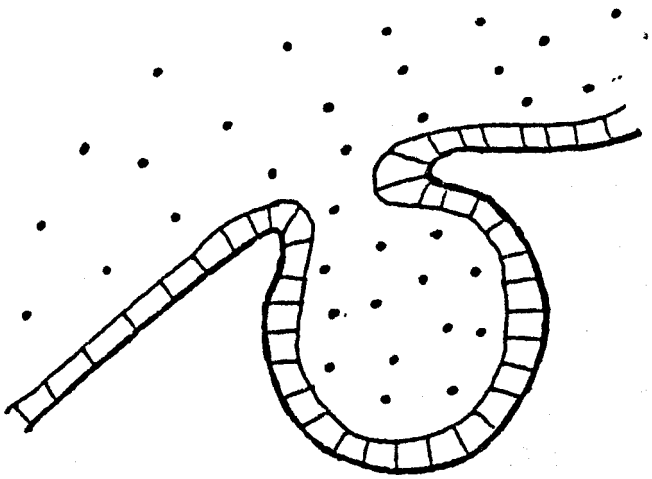
FIGURE 1(v) Possible mechanisms of pinocytic ingestion of substrates

a) Solute entering in fluid phase only.

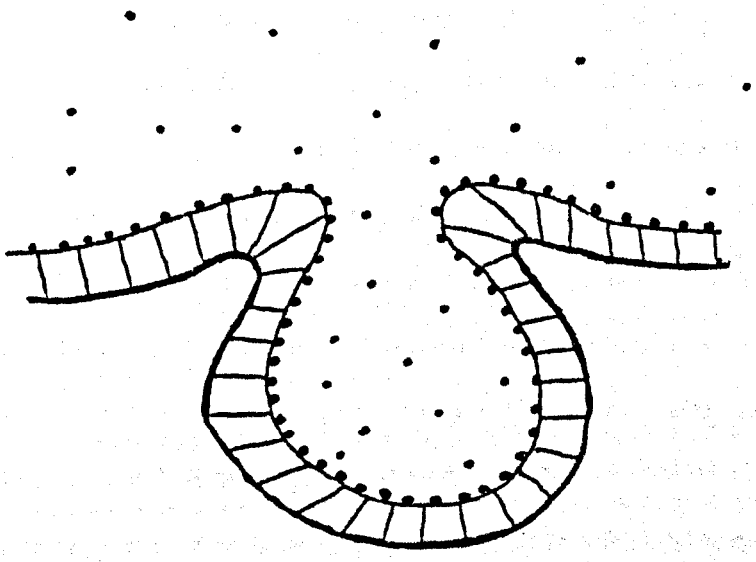
b) Ingestion of solute both by adsorption and in the fluid phase.

c) Solute ingestion by adsorption to the plasma membrane.

Fluid endocytosis



Mixed endocytosis



Adsorptive endocytosis

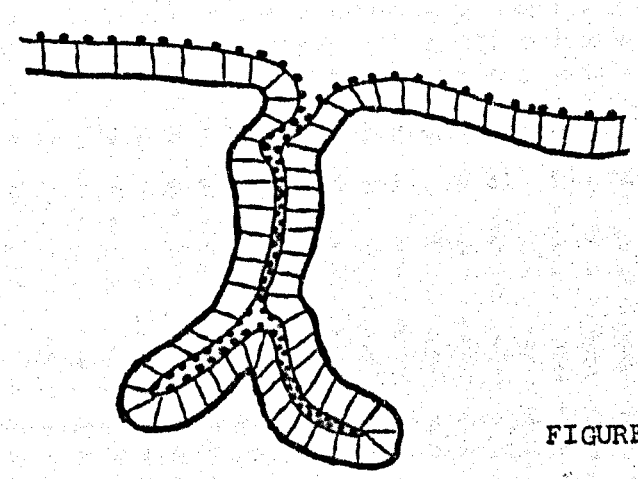


FIGURE 1 (v)

addition depends on the number, affinity and function of the cell surface binding sites.

Endocytic vacuoles assume a wide variety of shapes and sizes in electron micrographs (Fig. 1(vi)). In phagocytosis the cell surface applies closely to the particle, so that the initial phagosome usually corresponds in size and shape to thae of the particle (Fig. 1(vi)a). Pinocytic vesicles probably arise by many mechanisms. Fusion of membrane folds and invaginations of spherical, tubular or cup-like structures (Fig. 1(vi)b and c) all occur. These different forms may result from differences in the stimuli that initiate the endocytic process or from specializations of the membranes of cells in which they occur. Of considerable interest is the coated vesicle (Fig. 1(vi)c). The cytoplasmic surface of many endocytic vesicles is smooth, but electron micrographs show that most cell types contain an additional population of vesicles in which regularly spaced, short bristles protrude into the cytoplasm. From initial descriptions (Roth and Porter, 1964; Fawcett, 1965) it was postulated that these coated structures were involved in the selective uptake of proteins and particles by adsorptive pinocytosis. Recent work on lipoprotein uptake in human fibroblasts (Goldstein et al., 1979) and maternal protein in oocytes (Roth et al., 1976) has supported and extended this idea. According to Pearse (1976) the major protein which makes up 'coat' on the vesicle has a molecular weight of 180,000 and is named 'clathrin'.

The formation of phagocytic and pinocytic vesicles involves the expenditure of metabolic energy and the movement and fusion of membranes. Hence both these processes are affected by temperature and metabolic inhibitors. Particles bind to the surfaces of phago- and pinocytosing cells at $0^{\circ}C$ but are not ingested unless the temperature of the incubation medium exceeds some critical threshold ($18-21^{\circ}C$ - Silverstein et al., 1977). In this respect phagocytosis and adsorptive pinocytosis differ from fluid-phase pinocytosis. In the latter formation of pinocytic vesicles is not detectable at $0^{\circ}C$ but Steinman et al. (1974) have shown

FIGURE 1(vi) Endocytic vesicles may assume many shapes and sizes as they arise from the plasma membrane

a) A phagocytic vacuole - the cell surface applies closely to the incoming particle.

b) and c) Pinocytic vacuoles. In thin sections the cytoplasmic aspect of the vacuole may be smooth (b) or coated with tiny bristles (c).

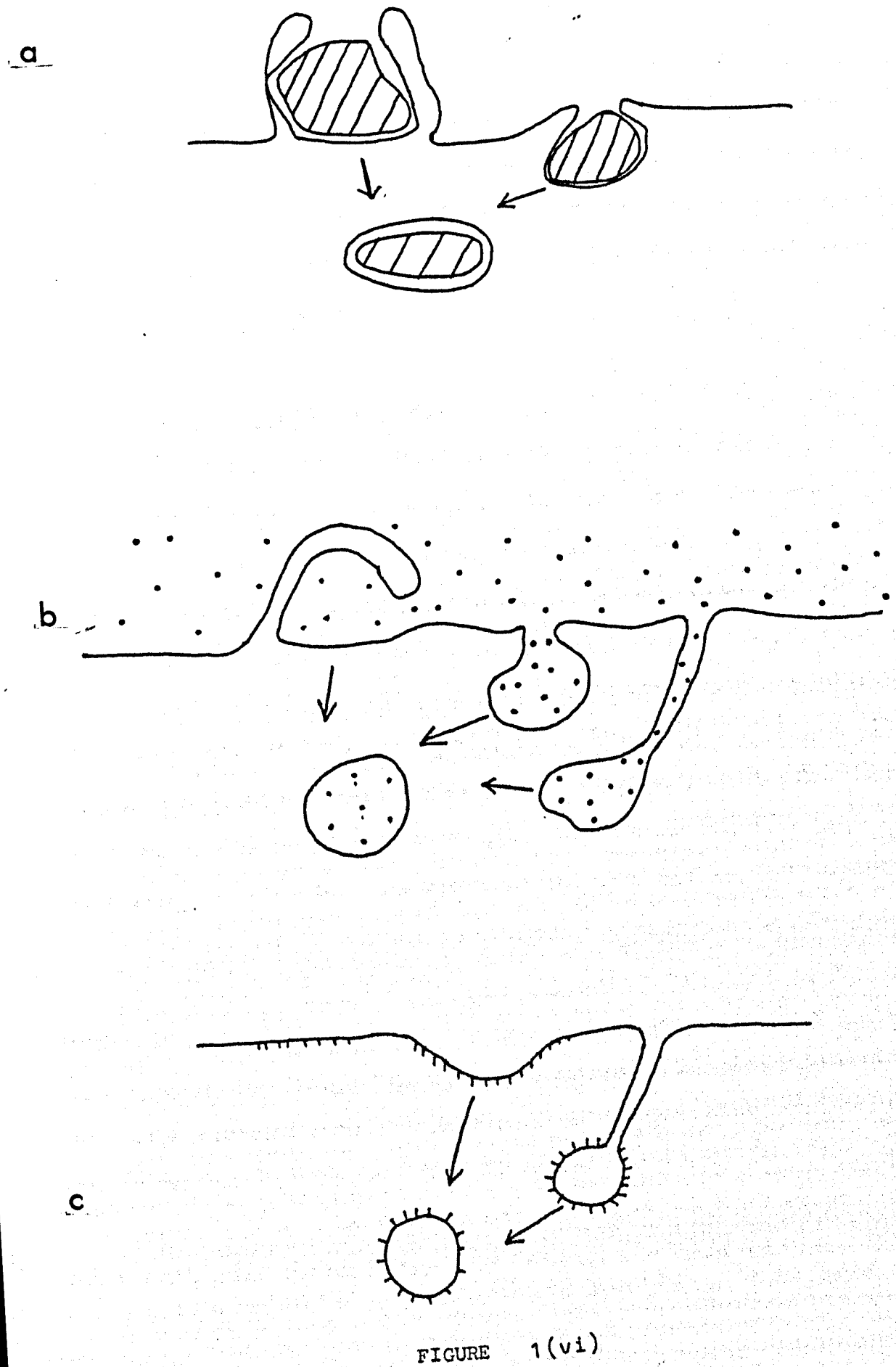


FIGURE 1(vi)

that the rate of solute uptake by fluid-phase pinocytosis is directly proportional to the incubation temperature from 2-38°C. Karnovsky et al. (1975) showed that phagocytosis could be inhibited by glycolytic inhibitors such as NaF or iodoacetic acid, but not inhibitors of oxidative metabolism (e.g. cyanide, dinitrophenol antimycin). On the other hand Steinman et al. (1974) found that fluid phase pinocytosis was dependent upon metabolic energy and that this energy could be derived from either glycolytic or oxidative metabolism. To date, however, the energy sources have not been identified.

1.4.1 Quantitation of pinocytosis

Light microscopy was used initially to enumerate phase-lucent pinocytic vesicles in mammalian cells (Lewis, 1931; Cohn, 1966) and pinocytic channels in amoebae (Holter, 1959; Chapman-Andresen, 1963). When electron microscopy first suggested that most cells contain submicroscopic pinocytic vesicles (Palade, 1956; Bennet, 1956), a variety of electron-dense probes - colloidal gold and thorium (Fedorto et al., 1968; Cohn et al., 1966; Gordon et al., 1965; Odor et al., 1956), ferritin (Farquhar et al., 1960), peroxidases (Graham and Karnovsky, 1966) - were introduced into the extracellular space. The presence of these probes within vesicles confirmed their origin at the cell surface, and demonstrated further that incoming vesicles fuse with independently labelled lysosomes both in vitro (Cohn et al., 1966) and in vivo (Straus, 1964). One difficulty with some of these probes is that they can adsorb to the cell surface and so labelling of an apparently intracellular pinocytic vesicle may really represent extracellular binding to a surface invagination, a fold or even an outgoing vesicle. Therefore most particulate electron-dense tracers are not useful for quantitative work, so a variety of radiolabelled and enzymatically active markers have been used instead.

Again, the question of binding vs interiorization must be adequately controlled.

According to Silverstein et al. (1977) at the start of a quantitative study, the type of pinocytosis occurring (fluid phase or adsorptive) should be assessed. The amount of solute accumulated by fluid phase uptake should vary linearly with the concentration of marker in the extracellular fluid and should increase continuously with time as long as the cell is not digesting and/or releasing the label. Adsorptive uptake shows saturation with increasing loads and may exhibit a rapid early binding phase. Formation of pinocytic vesicles and hence fluid phase uptake is not detectable at 0°C (Steinman et al., 1974), whereas adsorption to cell surface binding sites occurs at 0°C (e.g. Goldstein et al., 1974).

If adsorptive uptake is documented, several features can be characterized quantitatively. Binding studies at different ligand concentrations will characterize the number and affinity of binding sites. Inhibition of surface binding by using a competing ligand may help differentiate between the amount of label entering in the fluid phase vs that adsorbed to the vesicle membrane.

Markers which have been used to measure fluid phase uptake include [^{3}H]-labelled sucrose (Wagner et al., 1971) [^{3}H]-labelled inulin (Bowers et al., 1972), and [^{125}I]-polyvinylpyrrolidone (Williams et al., 1975). The latter is particularly favoured since it is reputedly chemically inert and resistant to digestion by lysosomal enzymes. Also favoured as a digestable protein marker is HRP (Straus, 1964) since its assay is easy, inexpensive, very sensitive (nanogram amounts), not complicated by non-specific binding and can be performed after very short exposure periods. Moreover, its intracellular route can be followed cytochemically (Steinman et al 1976).

Several materials have been shown quantitatively to exhibit features of adsorptive uptake in vitro including IgG and yolk proteins in oocytes (Roth et al., 1976), ribonuclease and other proteins in large amoebae

(Holter, 1959; Chapman-Andresen, 1963), lysosomal enzymes (Bach et al., 1972; Von Figura et al., 1974), colloidal gold in rabbit macrophages Gosselin (1967), altered bovine serum albumin in rat yolk sac (Williams et al., 1975), polycation-polyanion complexes in mouse macrophages (Seljelid et al., 1973), lectin (Edelson and Cohn, 1974), viruses (Silverstein and Dales, 1968), and low density lipoproteins by human fibroblasts (Goldstein et al., 1979).

1.4.2 The concept of a universal endocytic index

It is of great importance when studying pinocytosis that the comparison of results should be facilitated by the use of units that take account of the effect of inter-experimental variables. Similarly, most attempts to relate the pinocytic activities of different tissues are defeated by the widespread use of non-comparable units.

The concept of Endocytic Index (EI) as proposed by Williams et al. (1975) answers these criticisms, Endocytic Index is defined as the volume of culture medium (µl) whose substrate content is internalized by unit quantity of tissue in unit time. The following expression is used for the calculation of uptake:

$$A = \frac{Y}{M \times P} \qquad (1)$$

Where: A is the quantity of radioactive substrate or chemical marker accumulated in a given time.

Y is the total radioactivity or chemical marker in the whole tissue or in the serosal fluid (corrected for background or residual concentration).

M is the radioactivity or amount of chemical marker per microlitre (µl) of incubation medium (corrected for background or residual concentration).

P is the protein content in milligrams (mg) of the tissue.

Expressing uptake in this way has a number of advantages. It eliminates variability arising within an experiment from variations in the amount of radioactivity or chemical marker added to each culture vessel, normalizes for the effects of variation in specific radioactivity or specific enzymic activity of substrates and corrects for the differing amount of tissue or numbers of cells present. Direct numerical comparison of rates of uptake, both in experiments with the same radioactive substrate or chemical matter and in experiments with different substrates thus becomes possible. Uptake by different cell types can also be compared, assuming the culture conditions employed are similar. Quantitative data of pinocytic rates in various cell types are summarized in Table 1.

Although units of Endocytic Index are µl per unit tissue per hour this does not imply that an equivalent volume of fluid is actually ingested. In the case where a substrate enters a cell entirely in the fluid phase, the volume of fluid taken up is the same as that indicated by the EI. However, in the case of a substrate that enters mainly absorbed to the membrane, the actual volume of fluid ingested will be far smaller than the calculated EI. In such cases it may be helpful to calculate uptake in terms of nanograms (ng) of substrate internalized per unit of tissue per unit time in addition to EI. In this way it is possible to estimate the concentration of a substrate accumulated within the cell.

1.4.3 Differentiation between adsorptive and fluid-phase pinocytosis in terms of Endocytic Index (EI)

According to Pratten *et al.* (1980) the following criteria may be used to establish the mode of uptake of a substrate

(i) The magnitude of the EI can give a good indication of the mechanism of capture. A low EI (between 0.4 - 2.1 µl/h/mg protein) is likely to indicate that the uptake is entirely fluid phase. A higher EI is most

likely to be a function of the degree of membrane adsorption.

(ii) In the case of a substrate that enters entirely in the fluid phase, an increase in concentration in the extracellular medium is without effect on uptake expressed in terms of µl per unit of tissue per hour although uptake expressed in ng per unit of tissue per hour increases linearly with concentration. A substrate entering by adsorptive pinocytosis, however, does so in a concentration-dependent manner. As the concentration increases, the EI increases until the sites to which the substrate binds become saturated, then the EI will decrease and tend to the fluid-phase value.

(iii) Because the binding of substrate to the cell surface does not require a metabolic energy supply or the cytoskeletal system, inhibitors and low temperatures should be without effect on the binding component of the association of substrate with cells. Therefore a persistence of "uptake" in the presence of metabolic inhibitors or low temperatures may be interpreted as an indication of an adsorptive mechanism.

TABLE 1 Quantitative Data of Rates of Pinocytosis in Various Cell Types

Values have been calculated by the authors from published data. Figures in parenthesis have been derived by taking 10^6 cells as equivalent to 80 µg protein

CELL TYPE	TRACER (CONCENTRATION IF STATED)	CALCULATED ENDOCYTIC INDEX µl/mg protein/h	µl/10^6 cells/h	REFERENCE
Unicellular animals				
Amoeba proteus	^{125}I-dBSA	30-100	(13.69-45.66)	Chapman-Andressen (1977)
Acanthamoeba castellanii	^{131}I-HSA (1mgml)	(3.23)*	1.47	
	[^{3}H]Inulin (0.9mM)	(7.88)*	3.6	Bowers & Olszewski
	[^{14}C]Leucine (3.8mM)	(5.47)*	2.5	(1972)
	[^{14}C]Glucose	(4.12)*	1.88	
Mononuclear Phagocytes				
Rat peritoneal macrophages	^{125}I-PVP (10µg/ml)	(0.43)	0.034	
	[^{14}C]Sucrose (1µg/ml)	(0.25)	0.020	Pratten et al. (1977)
	†Colloidal [^{198}Au]gold (8 µg/ml)	(15.13-104.12)	1.21-8.33	
	†^{125}I-dBSA (10 µg/ml)	(5.5-13.0)	0.44-1.04	

TABLE 1 contd.

CELL TYPE	TRACER (CONCENTRATION IF STATED)	CALCULATED ENDOCYTIC INDEX µl/mg protein/h	CALCULATED ENDOCYTIC INDEX µl/10^6 cells/h	REFERENCE
Mouse peritoneal macrophages	[^{3}H]Haemoglobin	(3.75)	0.3	Ehrenreich & Cohn (1968)
	HRP (2 µg/ml)	(0.56)	0.045	Steinman & Cohn (1972b)
	HRP (anti HRP) aggregates (2 µg/ml)			
	Added directly	(132.75)	10.62	
	stored 30 min 37°C	(164.06)	13.12	Steinman & Cohn (1972b)
	2 days 4°C	(484.37)	38.75	
	4 days 4°C	(853.12)	68.25	
	7 days 4°C	(1337.50)	107.00	
	HRP (1mg/ml)	0.56	(0.045)	
	Endotoxin-stimulated cells HRP (1 mg/ml)	0.85	(0.068)	Edelson et al. (1975)
	Thioglycollate-stimulated cells HRP (1mg/ml)	1.90	(0.152)	
	Colloidal [^{198}Au]gold 0.1-1.0 µCi)			
	10% Fetal Calf Serum	(48.75)	3.9	Davies et al. (1973)
	50% Fetal Calf Serum	(111.25)	8.9	

TABLE 1 contd.

CELL TYPE	TRACER (CONCENTRATION IF STATED)	CALCULATED ENDOCYTIC INDEX µl/mg protein/h	µl/10^6 cells/h	REFERENCE
Liver Cells				
Rat Parenchymal Cells	^{125}I-Asialofetuin (10^{-7}M)	(700)*	320	Tolleshaug et al. (1977)
	^{125}I-dHSA (20 µg/ml)	(18.24)*	8.3	Nilsson & Berg (1977)
Rat Non-Parenchymal Cells	^{125}I-Asialogetuin (10^{-7}M)	(55)	4.4	Tolleshaug et al. (1977)
	[^{3}H]sucrose	(0.13)	0.01	
	^{131}I-albumin	(3.75)	0.30	Munthe-Kaas (1977)
	Colloidal [^{198}Au]gold (1.75 µg/ml)	(25)	2.0	
	^{125}I-d HSA (25 µg/ml)	(1250)	100.0	Nilsson & Berg (1977)
Chang strain human liver cells	[^{3}H]sucrose (2×10^{-5}mM)	(173.75)	13.9	Wagner et al. (1971)
Fibroblasts				
Fibroblasts (L cells)	HRP (1 mg/ml)	0.08	(0.035)*	Steinman et al. (1974)
Fibroblasts (L929 cells)	^{125}I-HSA (50 µg/ml)	10.2	(4.64)*	Shen & Ryser (1978)
	^{125}I-human serum albumin-polylysine	112.4	(51.14)*	

TABLE 1 contd.

CELL TYPE	TRACER (CONCENTRATION IF STATED)	CALCULATED ENDOCYTIC INDEX µl/mg protein/h	µl/10^6 cells/h	REFERENCE
Fibroblasts				
Normal Human Skin Fibroblasts	^{125}I-LDL (5–250 µg/ml)	33.06–1.42	(15.09–0.65)*	
	^{125}I-HDL (1–250 µg/ml)	1.38–0.32	(0.63–0.15)*	Miller et al. (1977)
	[^{14}C]sucrose (1.88 µg/ml)	0.19	(0.086)*	
	β-N-acetylglucosaminidase	5.80	(2.65)*	Von Figura et al. (1978)
	β-glucuronidase	27.00	(12.33)*	
Sarcoma - 180 cells	^{131}I-HSA (0.7 mg/ml)	0.046	(0.021)*	
	Soluble ferritin (5.4 mg/ml)	0.398	(0.182)*	Petitpierre-Gabathuler & Ryser (1975)
	Aggregated ferritin (5.4 mg/ml)	2.29	(1.044)*	
Chinese hamster cells	^{35}S-Chondroitin Sulphate No serum	0.25	(0.11)*	Saito & Uzman (1971)
	10% FCS	0.104	(0.05)*	
3T3 cells	[^{14}C]sucrose	(0.107)*	0.049	Davies & Ross (1978)
	HRP	(0.059)*	0.027	
Normal human fibroblasts	^{125}I-labelled albumin-[^{14}C]sucrose	17.6	(8.04)*	Pittman & Steinberg (1978)

TABLE 1 contd.

CELL TYPE	TRACER (CONCENTRATION IF STATED)	CALCULATED ENDOCYTIC INDEX µl/mg protein/h	µl/10^6 cells/h	REFERENCE
Aorta Blood Vessels				
Monkey Smooth Muscle Cells	[^{14}C]sucrose	(0.75)	0.06	
	HRP	(0.69)	0.055	Davies & Ross (1978)
Monkey Endothelial Cells	[^{14}C]sucrose (serum factor dependent)	(1.86–0.625)	0.15–0.05	
Pig Smooth Muscle Cells	^{125}I-PVP (30 µg/ml)	0.082–0.123	(0.007–0.01)	Leake & Bowyer (1977)
Pig Endothelial cells	^{125}I-PVP (30 µg/ml)	0.034	(0.003)	
Trophoblast				
Human trophoblast Cells	^{125}I-labelled haemoglobin (5–12µg/ml)			
	No serum	(53.75)	4.30	Contractor & Krakauer (1976)
	Serum	(46.00)	3.68	
Yolk sac				
	^{125}I-PVP (3 µg/ml)	1.71	(0.14)	Williams et al. (1975a)

TABLE 1 contd.

CELL TYPE	TRACER (CONCENTRATION IF STATED)	CALCULATED ENDOCYTIC INDEX µl/mg protein/h	µl/10^6 cells/h	REFERENCE
Yolk sac				
Rat yolk sac	[^{14}C]sucrose (0.1 µg/ml)	2.04	(0.16)	Roberts et al. (1977)
	†Colloidal [^{198}Au]gold (1 µg/ml)	1.45–4.83	(0.12–0.39)	
	^{125}I-BSA (1 µg/ml) (Untreated)	4.8	(0.38)	
	^{125}I-dBSA (1 µg/ml)			
	Treatment:			Moore et al. (1977)
	Frozen −20°C	8.9	(0.71)	
	Acetic acid pH 3.5	16.8	(1.34)	
	Acetic acid pH 3.0	23.4	(1.87)	
	Acetic acid pH 2.5	14.5	(1.16)	
	Formaldehyde pH 10	65.0	(5.20)	
	Buffer pH 10	8.10	(0.65)	
	Urea pH 5.5	73.3	(5.86)	
	^{125}I-labelled Orosomucoid (Untreated)	6.1	(0.49)	

TABLE 1 contd.

CELL TYPE	TRACER (CONCENTRATION IF STATED)	CALCULATED ENDOCYTIC INDEX µl/mg protein/h	µl/10^6 cells/h	REFERENCE
Yolk sac				
Rat yolk sac	^{125}I-labelled denatured Orosomucoid			
	Treatment:			
	Urea pH 5.5	5.5	(0.44)	
	Buffer pH 10	4.8	(0.38)	
	Formaldehyde pH 10	2.2	(0.18)	
	^{125}I-labelled Asialo-orosomucoid	12.5	(1.0)	
	Invertase (44 units/ml)	1.14	(0.09)	Brown & Segal (1978).

* Fibroplast conversion factor used: 455 µg protein = 10^6 cells

† Batch-dependent

1.5 Methods for the Quantitative Measurement of Intestinal Uptake *in vitro*

The need for carrying out quantitative studies under precisely defined conditions coupled with the technical ease of isolation of the intestine, has led to innumerable investigations *in vitro* using preparations where blood and lymph flow are absent.

The real break-through came in 1949 when Fisher and Parsons appreciated that the physiological activity of excised intestinal mucosa depended on a continuous supply of oxygen to the absorbing cells. They circulated oxygenated saline through the lumen in order to oxygenate the intestinal mucosa. In practise, it is not easy to supply oxygen to the cells of the intestinal mucosa from the lumen simply by filling the segment with fluid into which oxygen is bubbled. The escaping gas carried fluid away with it and in small animals such as rats, gas bubbles occupy much of the intestinal lumen volume. Also in the case of the unstirred contents of the intestinal lumen, the diffusion of material from the central core of this fluid to the immediate vicinity of the brush border of the absorbing cells, a distance of about 3mm in the jejunum of the adult rat, would take many minutes to reach a steady state. These problems can be overcome by everting intestinal segments so that the mucosal cells can be directly exposed to an oxygenated, stirred incubation medium. The problems of diffusion equilibrium in such systems are reduced to those of diffusion across any unstirred fluid layer entrapped in mucus adjacent to absorbing cells and intervillous space.

The everted sac technique was first introduced in 1954 by Wilson and Wiseman and has been extensively used since. The transport of substrate into the sac contents can be followed without the need for elaborate apparatus to circulate fluid through the intestinal lumen. The technique is described in detail in Section 3.2.

1.5.1 Objections to the use of everted intestinal sacs

There are objections to the use of everted sacs of small intestine. The sac may leak and differing degrees of distention will produce differing degrees of unfolding of the villous surface. However, the greatest objections stem from the evidence of ultrastructural and biochemical changes which occur in the tissue when everted intestinal sacs are incubated *in vitro*.

The most popular incubation medium for *in vitro* studies on everted intestinal sacs has been the balanced salt solution, Krebs Ringer (Krebs and Henseleit, 1932; Krebs, 1950) and the ultrastructural evaluation of tissue incubated in this medium has been well documented. Levine *et al.* (1970) examined intestine which had been excised, everted and cultured for up to 2h in Krebs-Henseleit phosphate buffer pH 7.4 containing 1g glucose per litre. Histological examination showed that while eversion produced no change in tissue morphology, even a short incubation period (5 min.) produced a loss of structural integrity. After 1 h there was total disruption of the epithelial border, with intact structures present only in the crypts. This morphologic change in the everted sac during incubation was found to be directly related to accumulation of fluid. Plattner *et al.* (1970) observed quantitative and qualitative changes of structure during incubation periods up to 3 h in Krebs-Henseleit buffer and found that cell membrane ruptures appeared at the cell bases after 1 h. They concluded that fluid absorption involved whole cells rather than any particular compartment and suggested that the tonicity of "physiological buffers" used for incubation media should be adjusted to the same tonicity as that of the epithelial cells themselves rather than to that of the blood plasma. This would minimise fluid uptake by epithelial cells. More recently Lepper and Mailman (1977) in an electron microscope evaluation of everted jejunal sacs and jejunal sacs incubated for 30 min in Krebs Ringer showed that cellular organelles appeared unaffected by eversion and incubation processes. However intercellular "lakes" appeared after

eversion and increased in size and number with incubation time. Detachment of the basal cell membrane from the basal membrane also occurred during incubation and Lepper and Mailman suggested that fluid uptake was responsible for this and the appearance of intercellular lakes.

Besides ultrastructural changes, biochemical changes have been shown to occur when everted intestinal sacs are incubated in minimal media, such as Krebs-Ringer. Bronk and Leese (1973) and more recently Faelli et al. (1976) showed that during the manipulation before incubation and incubation itself, the adenine nucleotide concentration of everted sacs decreased slowly. This depletion was thought to be due to leakage from the cells although cellular integrity was not impaired (Jaspar and Bronk, 1968).

1.6 Concluding Remarks

In recent years it has become more accepted that at least small quantities of macromolecules can be taken up intact into the epithelial cells of the intestinal mucosa of adult mammals. However, there are many areas of this subject that are poorly understood.

Most of the work on macromolecular uptake by adult intestine has been carried out by Walker and his group and is described in detail in Section 1.3. It is unclear from their morphological studies using the marker protein HRP whether the vesicles containing HRP seen in adult epithelial cells at both light and electron microscope levels are endocytic or secretory vesicles. The latter could be in the process of extruding HRP which had diffused into the tissue at the extrusion zones, a site reputed to facilitate such infusion by Volkeimer et al. (1977). Their physiological studies using an in vitro everted-sac technique again with HRP as a marker macromolecule gave little support to their theory that the mechanism of macromolecular entry into adult epithelial cells was an endocytic one as tissue uptake of HRP was not quantified and equated with other endocytic systems. Only the accumulation of HRP in the serosal fluid was recorded and the rate of accumulation was assumed to be linear with time although no experimental evidence was put forward to support this. Distortion of their uptake data may have occurred in two ways. (i) the use of Krebs-Ringer as an incubation medium has been shown to severely affect tissue integrity even after short incubation periods (Levine et al., 1970; Plattner et al., 1970; Lepper and Mailman, 1977). (ii) it is not clear if steps were taken to exclude any Peyers patches from the prepared sacs. Recent evidence suggests that this gut-associated lymphoid tissue shows a high endocytic capacity (Owen, 1977; Brown, 1978). Thus if Peyers patches are present in intestinal sacs, any macromolecular uptake data obtained may reflect uptake by the lymphoid tissue rather than the intestinal epithelium.

Therefore it is still unclear whether the mechanism of uptake of macromolecules is an active endocytic one, as Walker et al. (1972) suggest or a passive diffusion through pores, as Loehry et al. (1970) suggest.

Finally, still very much in dispute is the actual quantities of macromolecule that are taken up into the tissue and that pass intact through it (see Section 1.3.1). This is mainly because most of the experiments carried out to date were carried out in vivo and the blood serum levels of macromolecule were quantified seemingly without taking into account any tissue uptake (particularly by the liver) which may have occurred.

In order to answer some of these questions, an in vitro technique must be devised which maintains tissue viability and cellular integrity throughout the preparation and incubation period and allows calculation of results in such a way that macromolecular uptake by adult intestine can be quantified and directly compared with other endocytotic systems. Therefore the aims of the work in this thesis were to:

(i) Develop an in vitro system using everted intestinal sacs from adult rats which would maintain the tissue viability and cellular integrity as near to the physiological state as possible,

(ii) Use the developed method study the mechanism of macromolecular uptake and quantify the amounts taken up in such a way as to be directly comparable to other endocytotic systems. For this, the results were calculated by the method of Williams et al. (1975).

CHAPTER TWO

MATERIALS AND METHODS

2.1 Intestinal Tissue

Intestinal tissue was obtained from adult male rats of the Wistar strain about 6 months old, 200 $\pm$ 35 g in weight. Animals were kept in conditions of permanent springtime lighting and had free access to food and water before each experiment.

2.2 Preparation and Incubation of Jejunal Sacs from Adult Rat

The materials and methodology used for the preparation and incubation of everted jejunal sacs from adult rats are described in Chapter 3.

2.3 Radioisotopes

[^{125}I]iodide (preparation IMS 4, 5 mCi in 0.5 ml) and ^{125}I-labelled poly(vinyl-pyrrolidone) [^{125}I-PVP: average molecular weight 30 - 40,000, preparation 1M . 33P] were purchased from the Radiochemical Centre, Amersham, Bucks, U.K.

2.4 Chemicals

Single strength tissue culture medium 199 (preparation TC 20, containing penicillin and streptomycin) and heat-inactivated calf serum (preparation CS Ø7) were obtained from Wellcome Reagents Ltd., Beckenham, Kent. L-tyrosine-hydrochloride, L-histidine-hydrochloride, poly (vinyl-pyrrolidone), glucose oxidase (Type 1) horseradish peroxidase (HRP - Type 11) and o-dianisidine dihydrochloride were purchased from the Sigma Chemical Company Ltd., Surrey, U.K. Bovine serum albumin (BSA, preparation 0142t) was purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. Oxygen/Carbon dioxide (95:5) from British Oxygen Ltd., Manchester, U.K. All other chemicals, unless otherwise stated,

were of analytical grade.

Recipes for Tc medium 199 and Krebs Ringer medium can be found in Appendix I.

2.5 Protein Estimation

The estimation of protein was performed by the method of Lowry et al. (1951) using duplicate 0.1 ml samples of the gut-sac Solution, prepared as described in Section 3.2.2. Extinction was measured at 750 nm in a Cecil 'CE 272' spectrophotometer (Cecil Instruments Ltd., Cambridge, England) using a 5 ml cuvette of 1 cm pathlength. A standard curve was obtained using bovine serum albumen at a concentration of 0 - 0.25 mg/ml.

2.6 Estimation of Glucose by Glucose Oxidase

Glucose was estimated by the method of Lloyd and Whelan (1969).

a) Glucose oxidase reagent:

The following were dissolved in 100 ml of Tris-glycerol-phosphate buffer (36.3 g Tris, 50 g NaH_2PO_4 H_2O, 400 ml glycerol made up to 1000 ml with water pH 7.0), glucose oxidase 30 mg, HRP 3 mg, o-dianisidine dihydrochloride 10 mg. The reagent has a shelf-life of several weeks when stored at 4°.

b) Procedure:

To 1 ml of test solution containing 0 - 100 µg/ml of glucose, 2 ml glucose oxidase reagent was added, mixed well and incubated for 30 min at 37°. To stop the reaction and simultaneously develop the pink colour, 4 ml of 5M hydrochloric acid were added. After mixing, the extinction at 525 nm was measured using a 5 ml glass cuvette of 1 cm pathlength in a Cecil 'CE 272' spectrophotometer. A standard graph was obtained using 1 ml of glucose standard solutions containing 0 - 100 µg/ml. Over this

range the concentration of glucose showed a linear relationship with extinction at 525 nm.

2.7 Preparation of Tissue for Histology

Routine methods of tissue preparation and staining described by Culling (1974) were adapted for the histology of fresh and cultured intestine.

a) Reagents

Zenker's fluid compound fixative: A stock solution of Zenker's fluid was prepared which contained 25 g mercuric chloride, 12.5 g potassium dichromate and 5 g sodium sulphate made up to 500 ml with water. For an active fixative glacial acetic acid (1 : 20 v/v) was added immediately before use.

Lugol's iodine: Ig iodine, 2 g potassium iodide dissolved in 5 ml water then made up to 100 ml.

Sodium thiosulphate: 0.5% w/v.

Ethanol solutions: 30%, 50%, 70%, 90% and 100% v/v.

Ethanol/Chloroform solutions: 3:1, 2:2, 1:3 v/v.

Paraplast, 56^{o} melting point, xylene, chloroform, DPX mountant, albumin/glycerine solution (BDH Ltd., Poole, England).

Acid water: 1% v/v hydrochloric acid in water.

Erhlich's Haematoxylin (Gurr, Searle Ltd., High Wycombe, Bucks, England)

Eosin: 1% w/v in 90% ethanol.

b) Procedure

Fixing: Tissue was fixed in Zenker's fluid for 4 h, washed overnight in running tap-water and then rinsed in 1% v/v Lugol's iodine solution for 1 h with 3 changes of solution. Finally the tissue was washed in thiosulphate solution.

Embedding: To dehydrate, the tissue was ascended through 30%, 50%

70%, 90% and two changes of 100% ethanol for 15 min each. The tissue was then passed through the ethanol/chloroform mixtures 3:1, 2:2, 1:3 and two changes of absolute chloroform for 15 min each, and embedded in Paraplast at 58° for 30 min. The wax was changed and after a further 45 min the tissue was embedded in fresh wax, poured into a mould and cooled as quickly as possible in an iced water bath.

Sectioning: Blocks were trimmed and sectioned using a Reichert microtome (Shandon Instrument Co., London, U.K.) at 7μ per section with a knife angle of 7°. After floating out, sections were fixed on slides pre-heated with albumin/glycerin and dried at 37° overnight.

Staining: Sections were hydrated by ascending through xylene (two changes) 100% ethanol (two changes) and one change each of 90%, 70% and 50% ethanol for 2 min each. Sections were stained in Ehrlich's Haematoxylin (10 min), rinsed alternately in distilled water and acid water to differentiate, then "blued" in running tap water for 15 min. Sections were then ascended through 50%, 70% and 90% ethanol (2 min each), counterstained in Eosin (1 min) and dehydrated in two changes of 100% ethanol (2 min each). Finally sections were cleared in two changes of xylene (2 min each) and mounted under coverslips in DPX mountant.

2.8 Preparation of Tissue for Electron Microscopy

Tissue was prepared for electron microscopy using standard techniques adapted for the intestine after the methods of Lepper and Mailman (1977) and Webster (1979 - personal communication).

a) Reagents:

Glutaraldehyde fixative, 3% glutaraldehyde in 0.1 m sodium cacodylate buffer pH 7.3, containing 2 mM magnesium chloride ($MgCl_2$).

Osmium tetroxide fixative, 1% osmium tetroxide (OSO_4) in 0.1 M cacodylate buffer pH 7.3 containing 2 mM $MgCl_2$.

Resin, Spurr's resin (Spurr, 1969).

b) Procedure

Tissue was immersed in glutaraldehyde fixative and allowed to stand for 15 min before being diced into cubes of side about 1mm. This procedure (after Lepper and Mailman, 1977) allowed the tissue to be divided into small pieces with a minimum of damage. After dicing the cubes were placed in fresh fixative for 2 h. The small cubes of tissue were then rinsed in cacodylate buffer, two changes of 5 and 10 minutes, postfixed in O_sO_4 for 1 h and rinsed again in buffer, two changes of 5 min each. The tissue cubes were dehydrated in changes of 30% (w/v), 60% and 90% acetone, 10 minutes each and 100% acetone, three changes of 10 minutes each, then placed in resin. The resin was changed after 30 min, 12 h and 24 h respectively. Finally the tissue was embedded in Spurr's resin.

Thin pale gold-to-silver sections were cut on a Reichert Ultramicrotone OMU 2 (Shandon Instrument Co., London) with a glass knife, stained with uranyl acetate - lead citrate stain and examined in a Phillips EM 200 electron microscope at 60 kV.

2.9 Sodium Dodecyl Sulphate (SDS) Disc-Gel Electrophoresis

SDS polyacrylamide disc gel electrophoresis was performed using a multiphasic buffer system calculated from theory by Jovin et al. (1971) and modified for SDS by Neville (1971). Upper and lower gels were 3.2 x 6.25 and 11.1 x 0.9 respectively (Hjerton, 1962). The first numeral (T) denotes the total weight of monomer (acrylamid N, N' - methylene bisacrylamide) per 100 ml of solvent; the second numeral (C) denotes the amount of bisacrylamide expressed as a percentage (w/w) of the total amount of monomer.

2.9.1 Preparation of gels

a) Reagents

Upper reservoir buffer, 0.04 m boric acid, 0.041 m Tris, 0.1% SDS; pH 8.64.

Upper gel buffer, 0.027 m sulphuric acid, 0.054 m Tris; pH 6.1, (running pH 8.64).

Lower gel buffer, 0.031 m hydrochloric acid, 0.424 m Tris; pH 9.18, (running pH 9.5).

Lower reservoir buffer, same as lower gel buffer.

Catalysts:

For upper gel: 0.3% N, N, N', N' - tetramethyl ethylenediamine (TEMED), 0.2% ammonium persulphate in upper gel buffer.

For lower gel: 0.3% TEMED, 0.1% ammonium persulphate in lower gel buffer.

For upper gel solution: 6 g acrylamide, 0.4 g bisacrylamide in 100 ml of upper gel buffer.

For lower gel solution: 22 g acrylamide, 0.2 g bisacrylamide in 100 ml of lower gel buffer.

b) Procedure

After de-aeration of the solutions, one part of lower gel solution was mixed with one part of the appropriate catalyst solution and poured into precision bore glass tubes which were stoppered at one end, to within 2.5 cm from the top. The tubes were held in an upright position and 1 cm of water was layered onto the surface of the gel solution. After polymerization (about 20 min) the water was removed with a Pasteur pipette, the top of the gel rinsed once with upper gel solution and then the upper gel solution (one part upper gel solution and one part catalyst solution) layered on top of the lower gel (1.5 cm) 1 cm of water was layered on top and polymerization allowed to proceed (about 20 min). The final gels had a dimension of 7.5 x 0.5 cm.

2.9.2 Staining of Polyacrylamide Gels for Protein

The method of Weber and Osborn (1969) using Coomasie Brilliant Blue R 250 was used for protein staining in polyacrylamide gels.

Immediately after electrophoresis, the gels were extruded from the glass tubes and placed in staining solution (1.25 g Coomassie Brilliant Blue in 454 ml 50% methanol, 46 ml glacial acetic acid solution) for 2 hours. The gels were rinsed with water and placed in destaining solution (75 ml glacial acetic acid, 50 ml methanol, 875 ml water) and destained for several days with frequent changes of destaining solution.

2.10 Column Chromatography of Macromolecules

Column chromatography was performed using the Sephadex system described by Curling (1970). This method utilizes the ability of Sephadex columns to fractionate molecules according to molecular weight, the elution curve being recorded by individual fractions.

a) Reagents

Gels: Gel for separation of [^{125}I]-PVP — Sephadex G-25-80

Gel for separation of HRP — Sephadex G-75-40

Eluants: [^{125}I]-PVP eluant: 0.02 M sodium acetate with 0.02% w/v sodium azide as antimicrobial agent, pH 7.2.

HRP eluant: 0.05 M phosphate buffer with 0.02% w/v sodium azide, pH 6.0.

b) Procedure

(i) [^{125}I]-PVP: 4 cm^3 samples were applied to a column of Sephadex G-25-80 (60 x 3.5 cm) and eluted with sodium acetate buffer, flow rate 0.8 ml/min, at room temperature. The eluted radioactivity was assayed on a gamma scintillation counter and plotted against fraction number. Between runs, the column was eluted with buffer containing non-radioactive PVP (2 µg/ml) to displace any residual [^{125}I]-PVP.

(ii) HRP: 4 cm samples were applied to a column of Sephadex G-75-40 (57 x 3.5) and eluted with phosphate buffer, flow rate 0.36 ml/min, at 4° The eluted HRP was assayed by the method described in Section 7.4 and plotted against fraction number.

CHAPTER THREE

PREPARATION AND INCUBATION OF EVERTED JEJUNAL SACS FROM ADULT RATS

3.1 Introduction

In 1972, Walker *et al.* published an *in vitro* method for the study of macromolecular uptake by adult rat intestine. This method utilized the everted intestinal sac technique of Wilson and Wiseman (1954) and was used as a starting point for the present study. The method is described in detail in Section 3.2. Differences in equipment and reagents make it difficult to copy published methods satisfactorily in one's own laboratory. For this reason several experiments were performed following the published methods as closely as possible, with the exception that a non-degradable radioactive macromolecule [^{125}I]-PVP was used as the substrate. The following sections in this Chapter show the results obtained with the published methods, and how these methods were subsequently modified to yield the procedure used routinely for the work on macromolecular uptake.

3.2 Preparation of Everted Intestinal Sacs from Adult Rat Jejunum Using the Method of Wilson and Wiseman (1954)

3.2.1 Intestinal tissue

Source of intestinal tissue was described in Section 2.1.

3.2.2 Preparation of Tissue

Each animal was killed by cervical dislocation, the abdomen opened by a midline incision, and the entire small intestine washed through *in situ* with a solution of 0.9% w/v NaCl containing 0.3% w/v glucose. The jejunum was 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 back wall of the abdomen. The mesentary was then gently removed from the intestine manually.

To evert the intestine, a glass rod was used to push the ileal end of the gut into the gut lumen until it appeared at the duodenal opening of the intestine, and eversion was completed by rolling the proximal half of the intestine onto the rod. The everted intestine was then slipped off the glass rod and placed in glucose-saline at room temperature in a flat petri-dish. A length of everted intestine 3 cm long was tied off at one end by a thread ligature and a second ligature was placed loosely around the other end. A blunt needle, attached to a 1 ml syringe (tuberculin type) was introduced into the intestinal lumen and a loose ligature pulled tight over the needle. An oxygenated sample of the incubation medium (0.5 ml) was then injected into the sac, and as the needle was withdrawn the ligature was tied tight. In preparation of intestinal sacs, care was taken to exclude any Peyer's patches (gut associated lymphoid tissue) which may have interfered with uptake studies.

Each sac was placed in a sterile Erlenmeyer flask (50 ml) containing 9.0 ml of pre-gassed incubation medium. The air in each flask was displaced by a mixture of oxygen and carbon dioxide (95:5) and the vessel re-stoppered with a sterile silicone bung. Flasks were placed in a

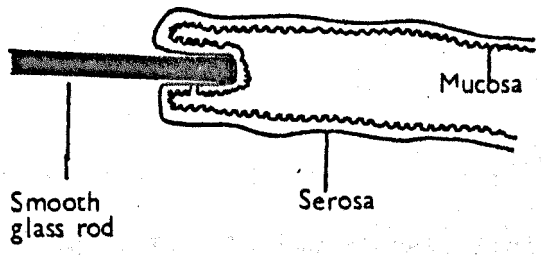
Unitemp water bath (Baird and Tatlock Ltd.) maintained at 37 ± 0.1°C with a stroke of 4 cm at a frequency of 90 ± 3 strokes per minute. After a 5 min pre-incubation period [^{125}I]-PVP (batch no. 156 BA) was introduced into each flask as a solution in 1.0 ml of incubation medium at 37°C to give a final flask concentration of 2 µg/ml. The flasks were then re-gassed, stoppered and incubation commenced. Fig. 3.1 shows a schematic representation of this method. After each period of incubation, gutsacs were removed drained and the volume of serosal fluid measured using a tuberculin-type syringe. Any sacs which had leaked were discarded. Empty sacs were agitated in six changes of ice-cold 0.9% NaCl (20 ml) to remove extracellular substrate. Each sac was placed in a graduated flask (2.5 ml) and dissolved in 1 m NaOH at 37°C for 1 h. Samples of this solution were assayed for protein by the method of Lowry et al. (1951) - see Section 2.5.

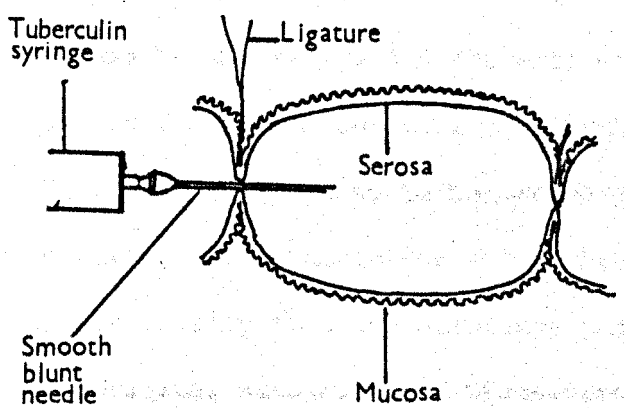
3.2.3 Assay of incubation medium, gut tissue and serosal fluid for radioactivity

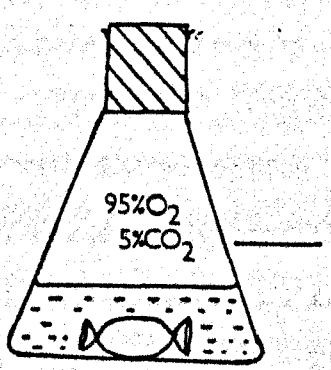
After solubilization, duplicate samples of gut-sac solution (1.0 ml) and the corresponding culture medium (1.0 ml) were placed in 3 ml disposable plastic tubes (LP 3, Luckham Ltd., Burgess Hill, Sussex) and the radioactivity measured using a gamma spectrometer. The serosal fluid samples (i.e. sac contents) were placed in 3 ml LP 3 tubes and made up to 1 ml with distilled water for counting. This procedure removed any volume-dependence of the counting efficiency.

3.2.4 Calculation of accumulation of [^{125}I]-PVP in the tissue and serosal fluid

The accumulation of [^{125}I]-PVP was calculated by the method of Williams et al. (1975) described in Section 1.3.6.







Schematic representation of the technique for the preparation of everted intestinal sacs.
(After Wilson & Wiseman 1954)

FIGURE 3(i)

3.2.5 Results

Figs. 3(ii) and 3(iii) show the results of three similar experiments in which eight sacs from a single rat were incubated in Krebs Improved Ringer II, pH 7.4 (Krebs, 1950 - see Appendix I) containing 1 g/litre glucose with 2 µg/ml [^{125}I]-PVP as substrate. Incubations were terminated at intervals up to 2h.

These experiments showed a removal of [^{125}I]-PVP from the mucosal fluid and transfer of this to the serosal fluid. A large part of the substrate transferred was retained in the sac wall (gut tissue uptake). The rate of uptake to the tissue and the rate of appearance of substrate in the serosal fluid was constant over the incubation period.

As [^{125}I]-PVP is believed to be non-degradable, the substrate was assumed to be intact after incubation with intestinal sacs. However there was the possibility that the substance was being deiodinated. Samples of the original substrate and transferred substance were therefore examined by column chromatography.

Eight everted intestinal sacs from the same animal were incubated by the above method for 2 h with 2 µg/ml [^{125}I]-PVP batch no. 156BA as substrate. The gutsacs were removed, drained, washed and dissolved in 1.0 m sodium hydroxide (25 ml). Samples of medium, tissue homogenate and serosal fluid (4 ml) were subjected to Sephadex column chromatography as described in Chapter 2.10 with the exception that a 57 x 1.5 column (Pharmacia Fine Chemicals, Sweden) was used. A sample of stock [^{125}I]-PVP 2 µg/ml in incubation medium, was incubated for 2 h at 37^{o}C in the absence of intestinal sacs and used as a control. The results are shown in Figs. 3(ii) - 3(vii) inclusive and Table 3.1.

3.2.6 Conclusions

Expressed as µl/h/mg protein, a plot of accumulation of substrate against incubation period (h) showed an increase with time for both tissue

FIGURE 3(ii) Accumulation of radioactivity by intestinal sacs incubated in the presence of [^{125}I]-PVP (2 μg/ml). Each point is derived from data on a single intestinal sac.

FIGURE 3(ii)

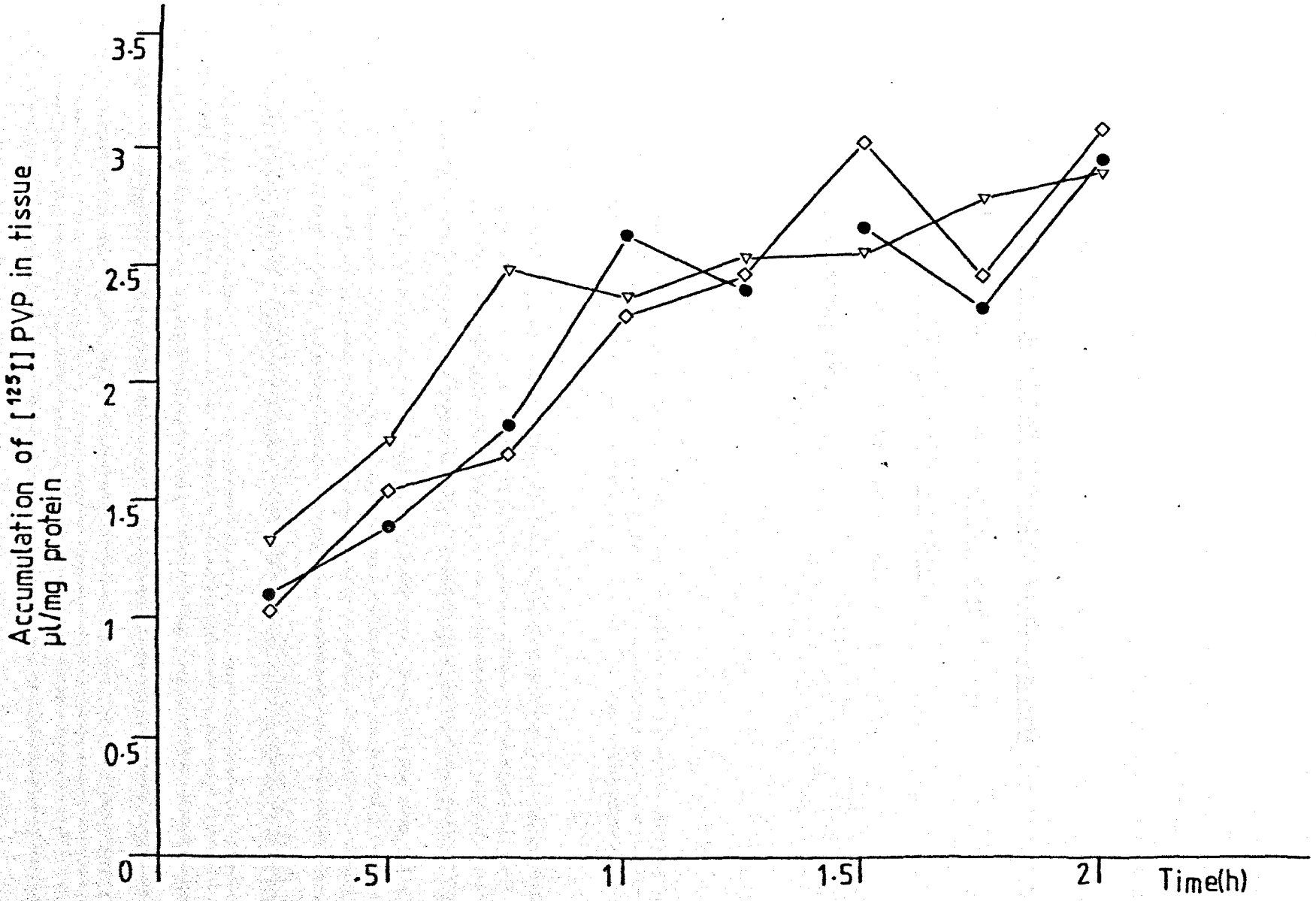

Accumulation of [125I] PVP in tissue µl/mg protein
3·5
3
2·5
2
1·5
1
0·5
0
·5
1
1·5
2
Time(h)

FIGURE 3(iii) Accumulation of radioactivity in the serosal fluid of intestinal sacs incubated in the presence of [^{125}I]-PVP (2μg/ml).

Each point is derived from data on a single intestinal sac.

FIGURE 3(iii)

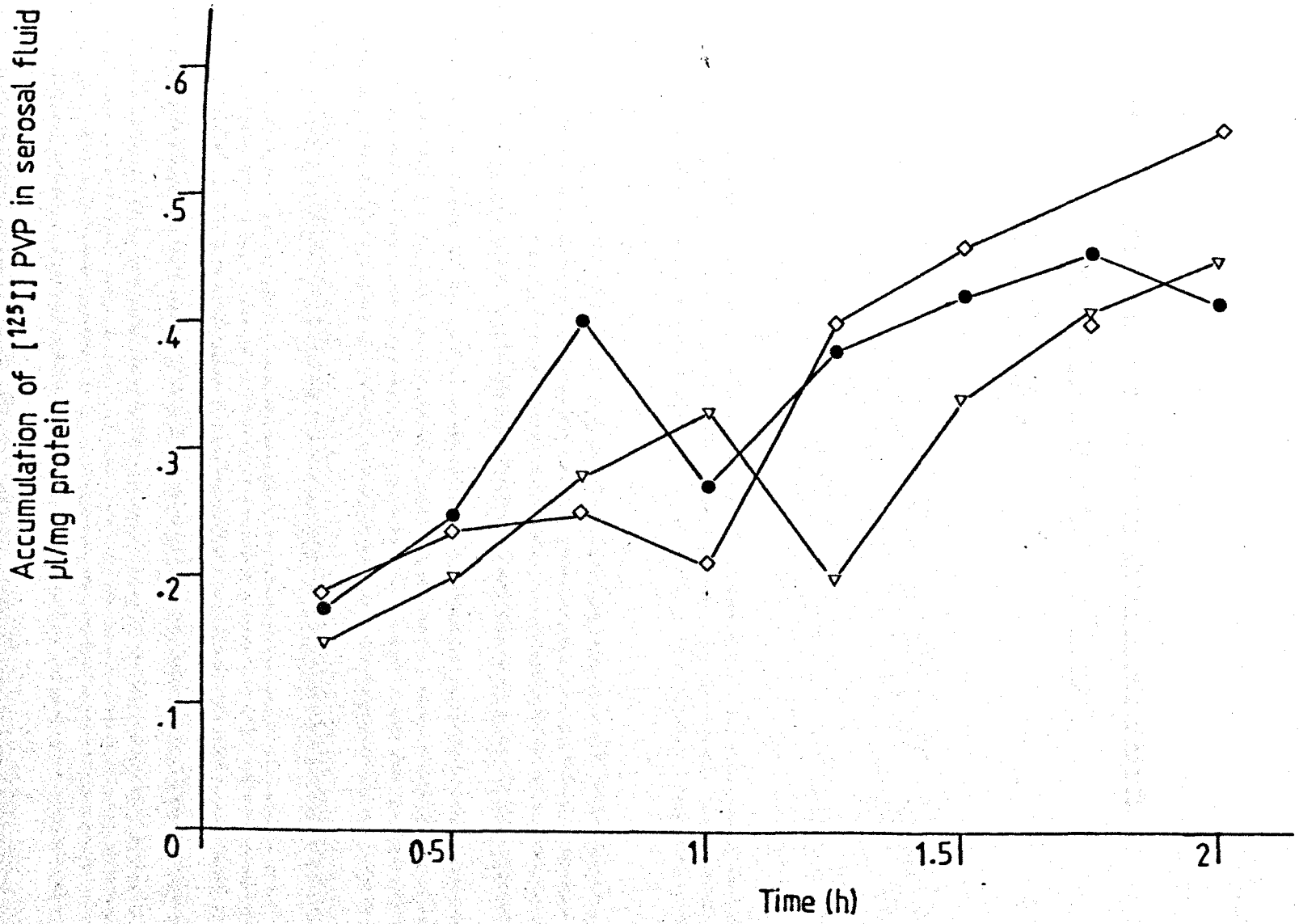

Accumulation of [125I] PVP in serosal fluid µl/mg protein
.6
.5
.4
.3
.2
.1
0
0·5
1
1.5
2
Time (h)

FIGURE 3(iv) Sephadex G 25-80 chromatography of incubation medium incubated for 2h with [^{125}I]-PVP (2µg/ml) present

FIGURE 3(iv)

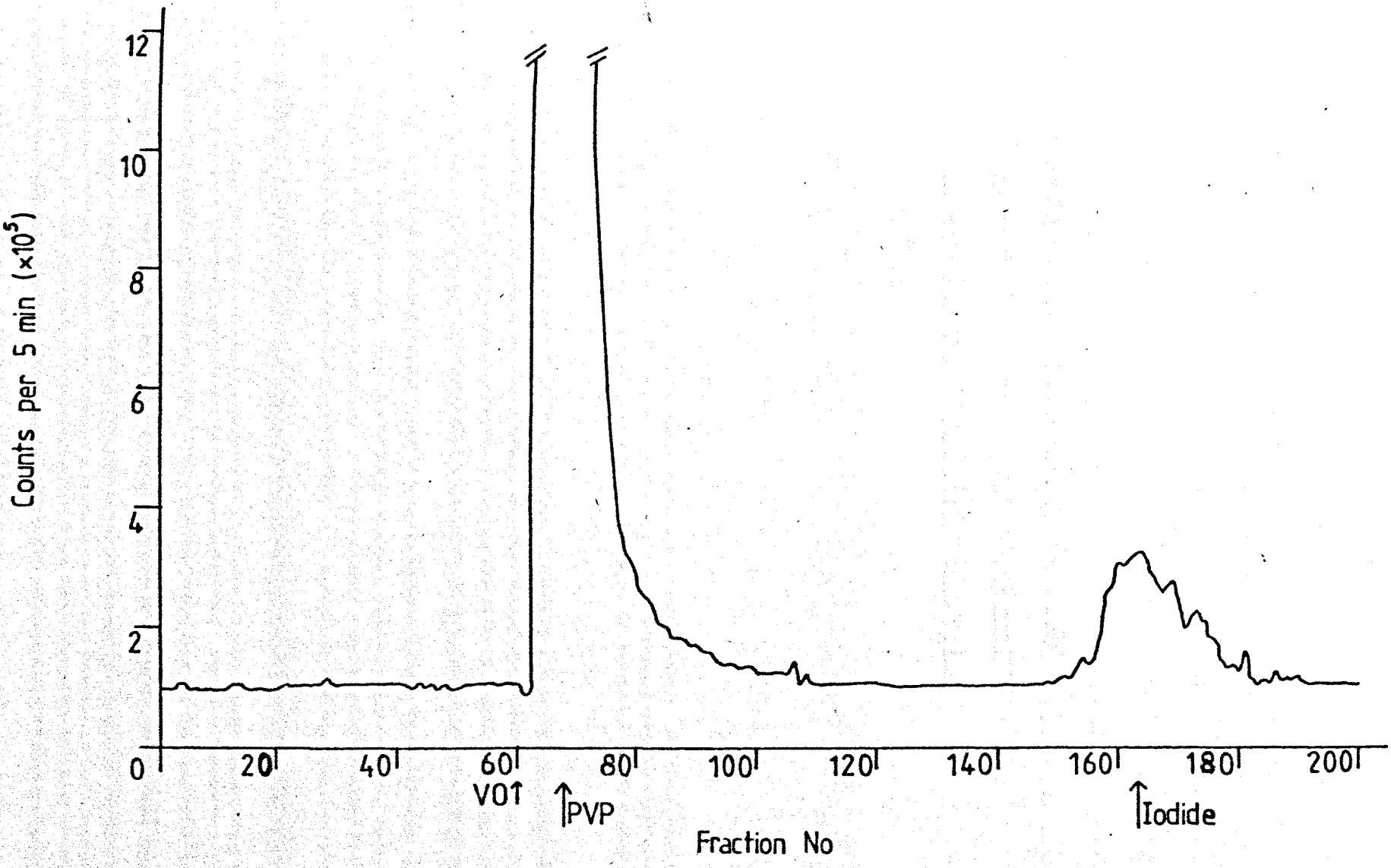

Counts per 5 min (×10⁵)
12
10
8
6
4
2
0
20
40
60
80
100
120
140
160
180
200
VOT
PVP
Iodide
Fraction No

FIGURE 3(v) Sephadex G 25 - 80 chromatography of medium incubated for 2h in the presence of everted intestinal sacs with [^{125}I]-PVP (2µg/ml) as substrate.

FIGURE 3(v)

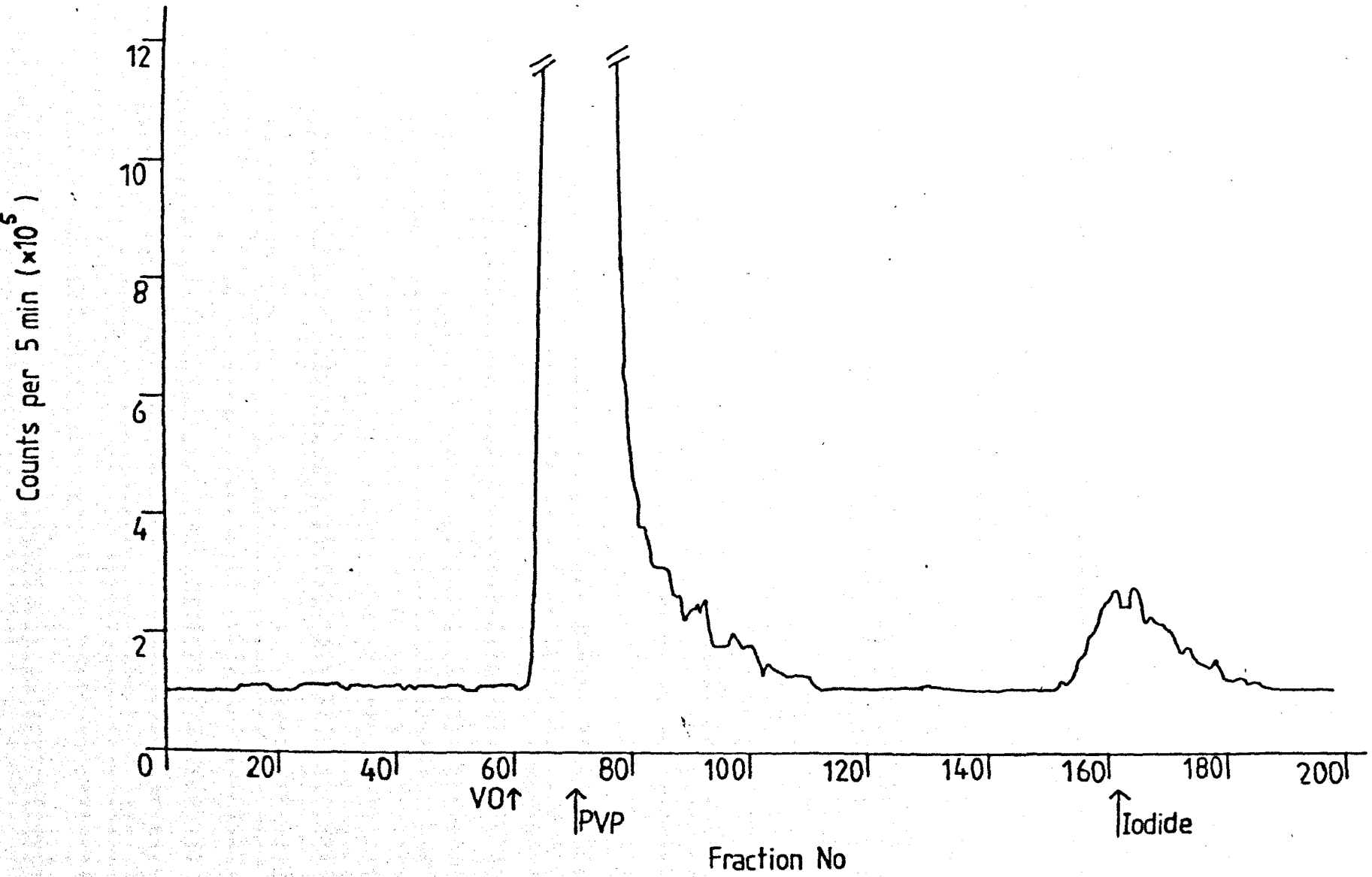

Counts per 5 min (×10^5)
12
10
8
6
4
2
0
20
40
60
80
100
120
140
160
180
200
VO↑
↑PVP
↑Iodide
Fraction No

FIGURE 3(vi) Sephadex G 25-80 of homogenate of everted intestinal sacs incubated for 2h with [^{125}I]-PVP (2µg/ml) as substrate

FIGURE 3(vi)

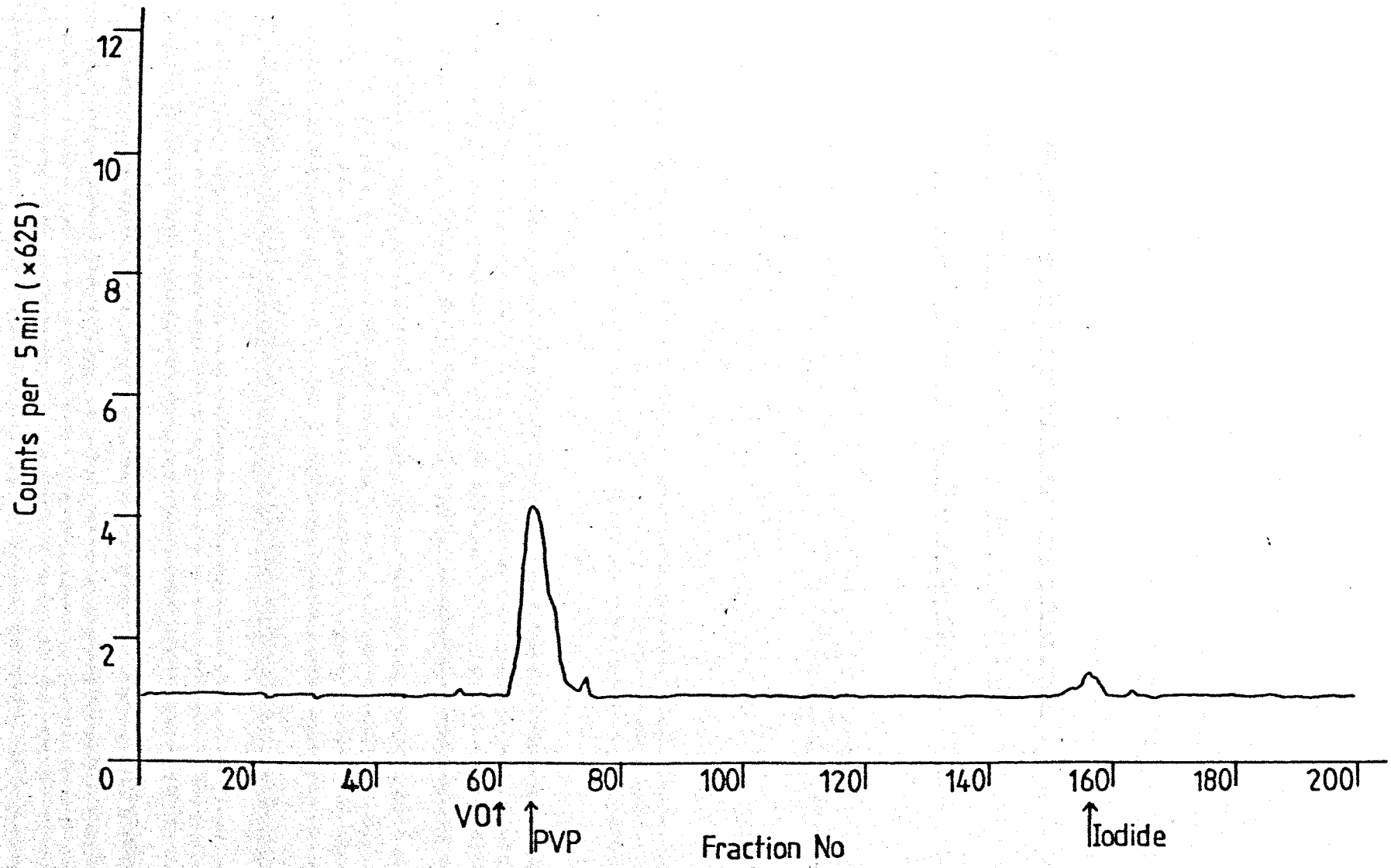

Counts per 5min (×625)
12
10
8
6
4
2
0
20
40
60
80
100
120
140
160
180
200
VOt
PVP
Fraction No
Iodide

FIGURE 3(vii) Sephadex G 25-80 chromatography of the serosal fluid from everted intestinal sacs incubated for 2h with [^{125}I]-PVP (2μg/ml) as substrate

FIGURE 3(vii)

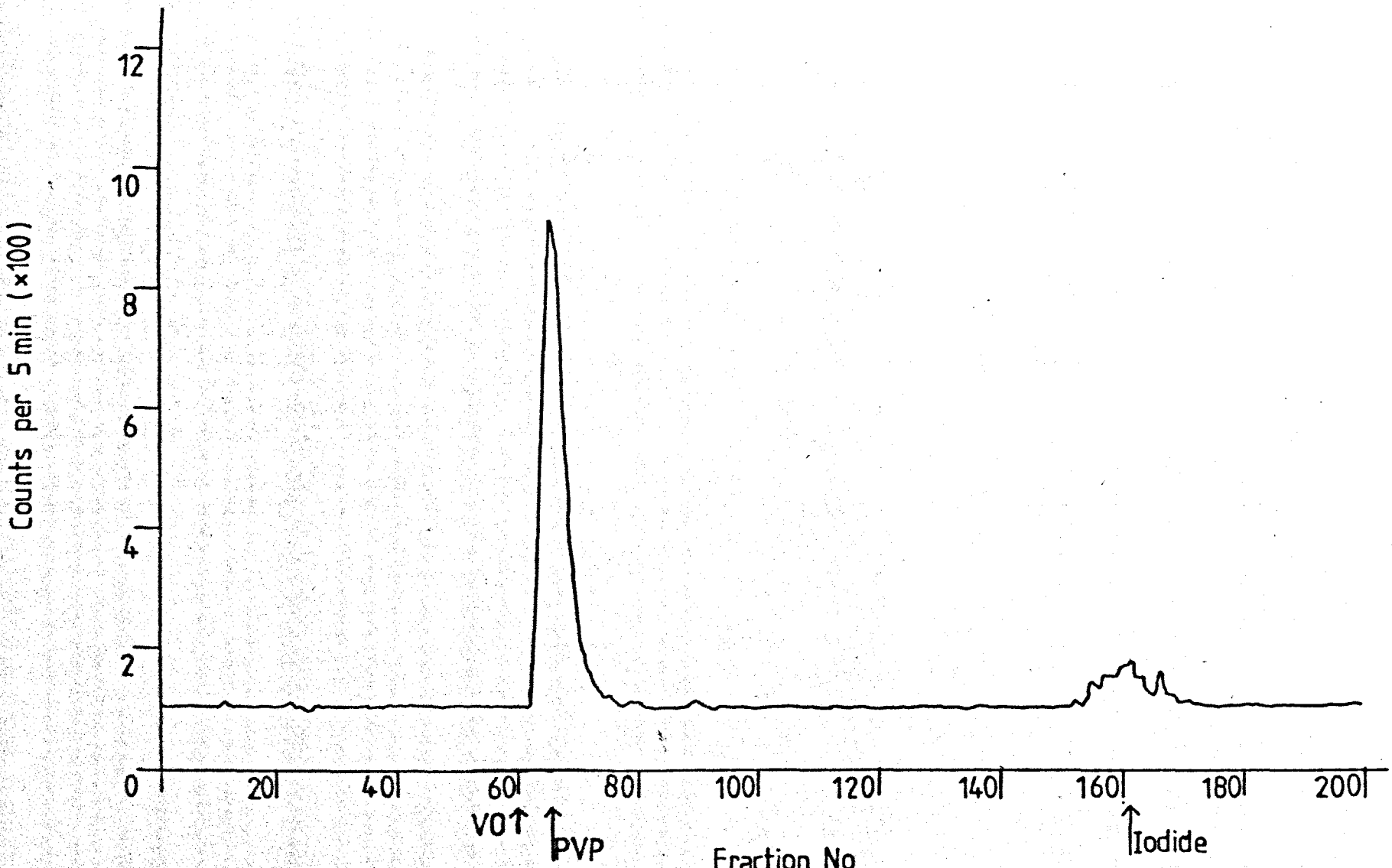

Counts per 5 min (×100)
12
10
8
6
4
2
0
20
40
60
80
100
120
140
160
180
200
V0
PVP
Fraction No
Iodide

TABLE 3.1

Percentage Recoveries of Radioactive Species after Sephadex G 25–80 Column Chromotography

	% recovery	% of that recovered [^{125}I]-PVP	% of that recovered Iodide	% residual
Control Sample 2 µg/ml [^{125}I]-PVP incubated for 2 h	95.44	99.07	0.93	-
2 µg/ml [^{125}]-PVP incubated for 2h in presence of intestinal sacs	86.56	99.01	0.91	0.08
Tissue homogenate of intestinal sacs incubated with 2 µg/ml [^{125}I]-PVP	83.57	94.07	5.9	0.03
Serosal fluid from intestinal sacs incubated with 2 µg/ml [^{125}I]PVP	85.45	81.14	17.19	0.67

uptake and appearance in the serosal fluid. The Sephadex G 25 - 80 column chromatography revealed that the majority of the radioactivity that passed into the tissue and accumulated in the serosal fluid was in the form of [^{125}I]-PVP but it was unclear whether deiodination of substrate occurred during transfer. This question is further investigated at a later stage (see Section 5).

Cellular debris in the mucosal fluid at the end of the incubation period suggested tissue breakdown occurred during the experiment. This could be due to one or a combination of several steps in the procedure. For example, the tissue preparation time i.e. the incubation of the isolated tissue was long (about 30 min) hence tissue hypoxia is likely to have occurred. Also the rate of oscillation of the water bath could have caused actual physical damage to the tissue during incubation. With any in vitro system it is essential that the tissue structure and viability be maintained as near to the physiological state as possible. Therefore it was decided to examine the affects of some of the steps in the preparation and incubation procedure on viability and integrity of the tissue to determine the optimum conditions in which macromolecular transport was to be investigated. In subsequent experiments an incubation time of 2 h was used. This was postulated (Rhodes and Karnovsky, 1971) as being the maximum time allowable for maintenance of tissue integrity after surgical trauma. The effects of the extension of incubation time on tissue viability is investigated further in Section 4.

3.3 Removal, Eversion and Preparation of Intestine

Preparation of intestine by the method previously described took about 30 min per experiment from the death of the animal to the placing of the sacs in the incubation medium. The appearance of cellular debris in the mucosal (dissection) fluid during preparation and an increase in the mean diameter of the intestine from 2.3 to 3.8 mm after eversion was observed. This suggested that tissue damage was occurring during preparation. Therefore procedure was designed to reduce both handling of the tissue and preparation time.

3.3.1 Procedure

Each animal was killed by cervical dislocation, the abdomen opened by a midline incision and the jejunum removed and placed immediately in oxygenated incubation medium kept at 4°C. While suspended in incubation medium the intestine was washed out with oxygenated incubation medium delivered from a gravity-feed pipette. The intestine was then placed in a rectangular dissection trough (60 cm x 3 cm) containing incubation medium at 4°C and everted by attaching the ileal (smallest diameter) end to one end of a notched glass rod (30 cm long 2.0 mm diameter) with a ligature and carefully rolling the proximal half onto the rod. The everted intestine was slipped off the glass rod and replaced in the dissection trough. This operation was carried out with the glass everting rod held horizontally and, as far as possible, immersed in the incubation medium in the trough.

Discarding the end portions, a 28 cm length of intestine was straightened manually without stretching and held in artery clamps. This length of intestine was tied off 0.25 cm from one end by a thread ligature and a second ligature was placed loosely 0.25 cm from the other end. A fine-nosed gravity-feed pipette (5 ml) was introduced into the intestinal lumen and the loose ligature pulled tight over the nose. An

oxygenated sample of the incubation medium (4 ml) was allowed to flow into the sac, the pipette withdrawn and the ligature tied tight. This large sac was divided into 8 x 3 cm sacs using artery clamps of 0.5 cm clamp-width, ligatured and each sac separated. The sacs were then incubated as described previously in Section 3.2.

3.3.2 Results

128 sacs were prepared in this way and the mean serosal volume was measured immediately. The mean serosal volume was found to be 0.42 ± 0.09 ml. In subsequent experiments, any sac which had less than 0.42 ± 0.09 ml at the end of the incubation period was considered to have leaked and was discarded.

Preparation of tissue by this method took about 10 min from the death of the animal to the placing of the sacs in the incubation medium and oxygenation of the dissection medium was maintained throughout the preparation time. There was no cellular debris in the mucosal fluid after preparation and the mean diameter appeared little changed after eversion (from 2.5 mm to 2.85 mm). Histological studies revealed no alteration in gross morphology after manipulation (Pl 1 and 2).

3.3.3 Conclusion

Tissue preparation by this technique reduces preparation time, allows adequate oxygenation throughout the preparation period and maintains tissue integrity. It is for these reasons that in all future experiments this technique was used for the preparation step.

PLATE 1

Mucosa from the jejunum of a normal adult male rat.

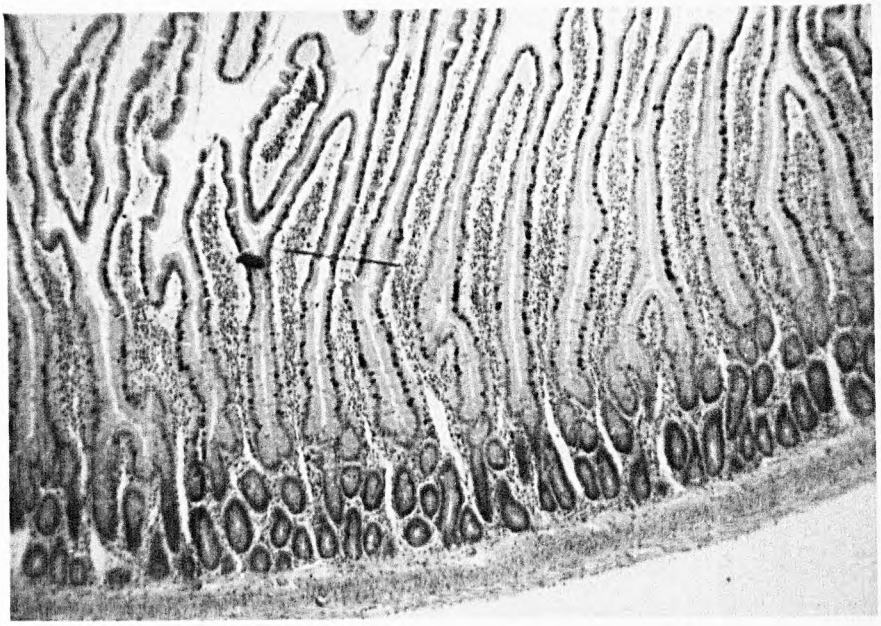
Haematoxylin and eosin.

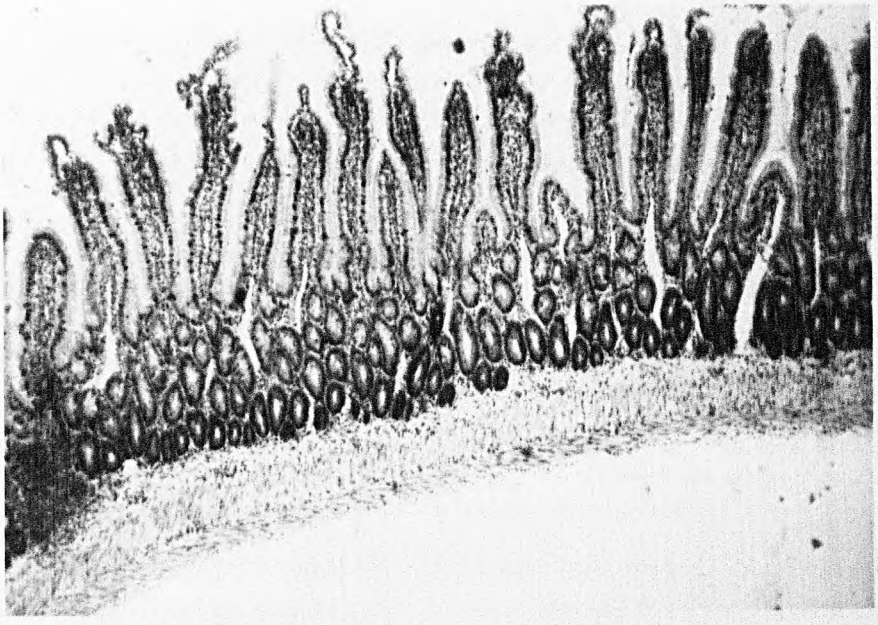
Magnification x156.

PLATE 2

Mucosa from the jejunum of an adult male rat demonstrating the normal appearance of the tissue following the mechanical manipulation of eversion for preparation of the closed sac.

Magnification x156.





3.4 Shaker Speed of Water Bath

It was not possible to follow the description of incubation as performed by Walker *et al.* (1972) as the water baths available differed from the incubator used by these workers. The optimal condition for incubation and oscillation of the tissue using the available water baths had to be determined.

Sacs were prepared by the method described in the preceeding section (3.3) and were incubated at different oscillation frequencies, stroke 3.4 cm in a Unitemp water bath (Baird and Tatlock Ltd.) at 37°C. The appearance of cellular debris in the medium was taken as an indication of tissue breakdown. The results are shown in Table 3.2.

From the results it can be seen that the oscillation frequencies above seventy strokes/min caused premature tissue breakdown. An oscillation frequency of 50 ± 3 strokes/min. was chosen for subsequent experiments. This frequency was thought to be the best to ensure adequate stirring of substrate while maintaining tissue integrity for the required incubation periods.

TABLE 3.2

Shaker Speed of Water Bath

Oscillation speed Stroke 4 cm	Time of First Indication of Tissue Breakdown (h)	No. Sacs
100	0.50	4
90	0.67	4
80	0.92	4
70	1.48	4
60	2	4
50	2	4
40	2	4
30	2	4

3.5 Washing of Sacs to Remove Non-Absorbed Substrate

After incubation, some extracellular substrate adhered to the tissue and this experiment was designed to determine the number of washings needed to remove it.

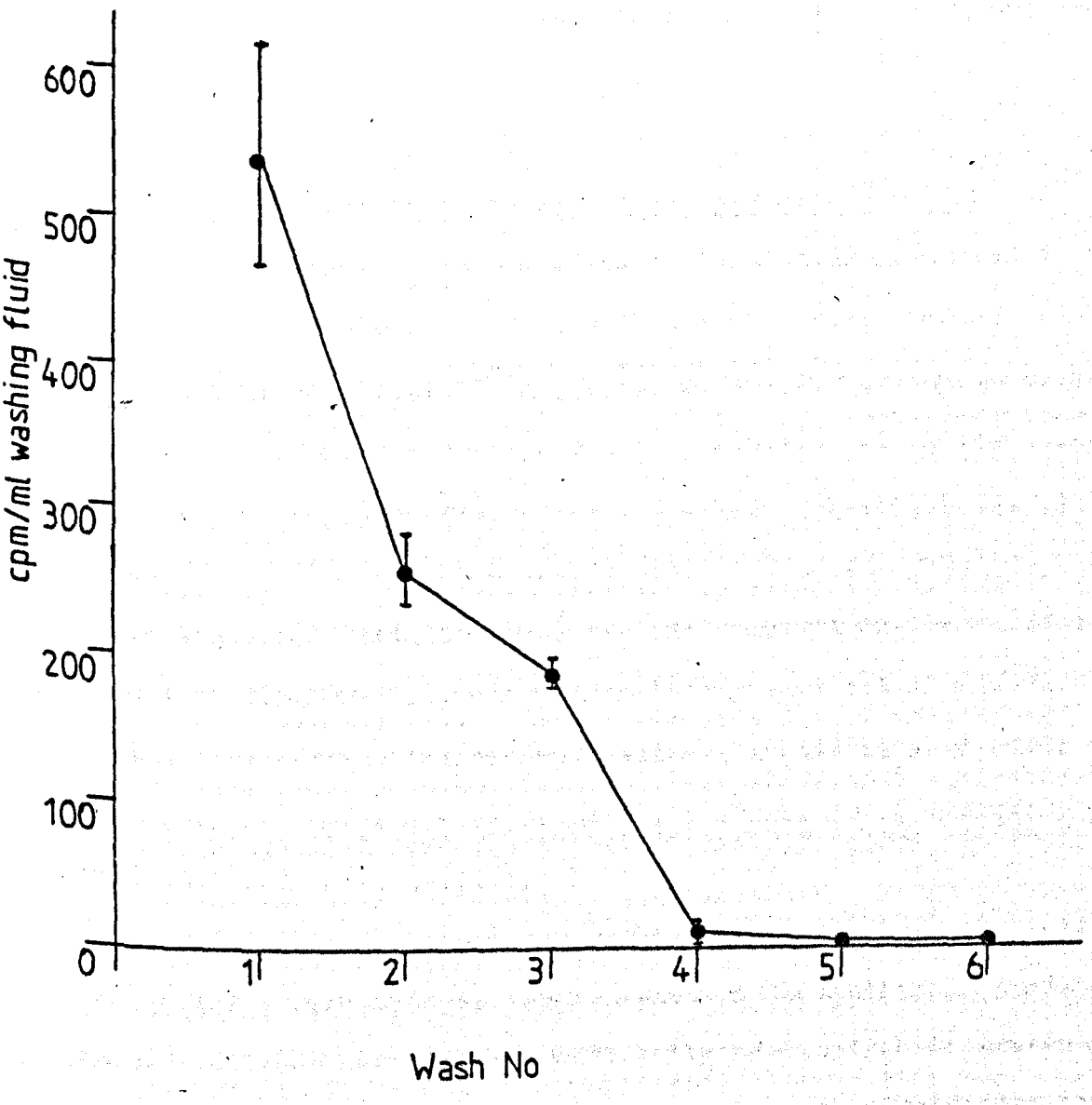
Jejunal sacs were prepared by the method described in Section 3.3 and incubated for 2 h in Krebs Improved Ringer II pH 7.4 containing 2 µg/ml ^{125}I -PVP, batch no. 156 BA, as substrate. After incubation, sacs were removed, drained and washed six times for 2 min in changes of ice-cold sodium chloride (NaCl, 20 ml, 0.9% w/v). Duplicate samples (1 ml) of the washing solutions were counted for radioactivity.

The results are shown in Fig. 3(viii). The plot shows radioactivity (in cpm corrected for background) with respect to wash number. Each point is the mean of four separate gut-sac washings.

The results show that a large proportion of extracellular radioactive substrate can be removed by the fourth wash and no further radioactivity was removed by subsequent washes. In all subsequent experiments the gutsacs were washed four times for 2 min in changes of ice-cold saline. Washing was carried out in ice-cold saline to prevent further metabolic activity by the tissue, hence any further uptake of material.

FIGURE 3(viii) The washing procedure required to remove extracellular [^{125}I]-PVP from everted intestinal sacs after incubation

FIGURE 3(viii)



3.6 Standard Method for the Preparation and Incubation of Everted Jejunal Sacs from Adult Rats

Sacs were prepared as described in Section 3.3. Assay of samples for protein and radioactivity were carried out as described in Sections 2.5 and 3.2.3 respectively. The modifications to the method regarding shaker speed and washing procedure were incorporated. Results were calculated by the method of Williams et al. (1975) using an ICL 4130 computer. The program used in the calculation of results is given in Appendix II. Unless otherwise stated, all further experiments were carried out using these conditions.

3.6.1 Reproductivity of results using the standard method

Figs 3(ix) and (x) show the results of a typical experiment in which eight gutsacs from the same rat were incubated in Krebs Improved Ringer II pH 7.4 containing 2 μg/ml [^{125}I]-PVP for up to 2 h. The rates of substrate uptake into the tissue and appearance in the serosal fluid were constant over this period. The results of eight similar experiments were each subjected to a linear-regression analysis and Table 3.3 indicates the correlation coefficient, the slope and the intercept on the ordinate axis of the computer-fitted regression line through each set of experimental points. The slope of the regression-line gives the rates of uptake (EI) from the incubation medium by the tissue and the rate of appearance of substrate in the serosal fluid.

An analysis of covariance was applied to the eight sets of data in Table 3.3. The test employed measures whether the statistical variations within each experiment differ significantly from variations between each experiment. If it is shown that these two measures of variation are not significantly different, then it may be concluded that the experimental results are drawn from a homogeneous experimental population.

The analysis was employed for accumulation into the tissue and serosal

FIGURE 3(ix) Accumulation of radioactivity by everted intestinal sacs incubated in the presence of [^{125}I]-PVP (2μg/ml).

Each point is derived from data on a single intestinal sac.

FIGURE 3(ix)

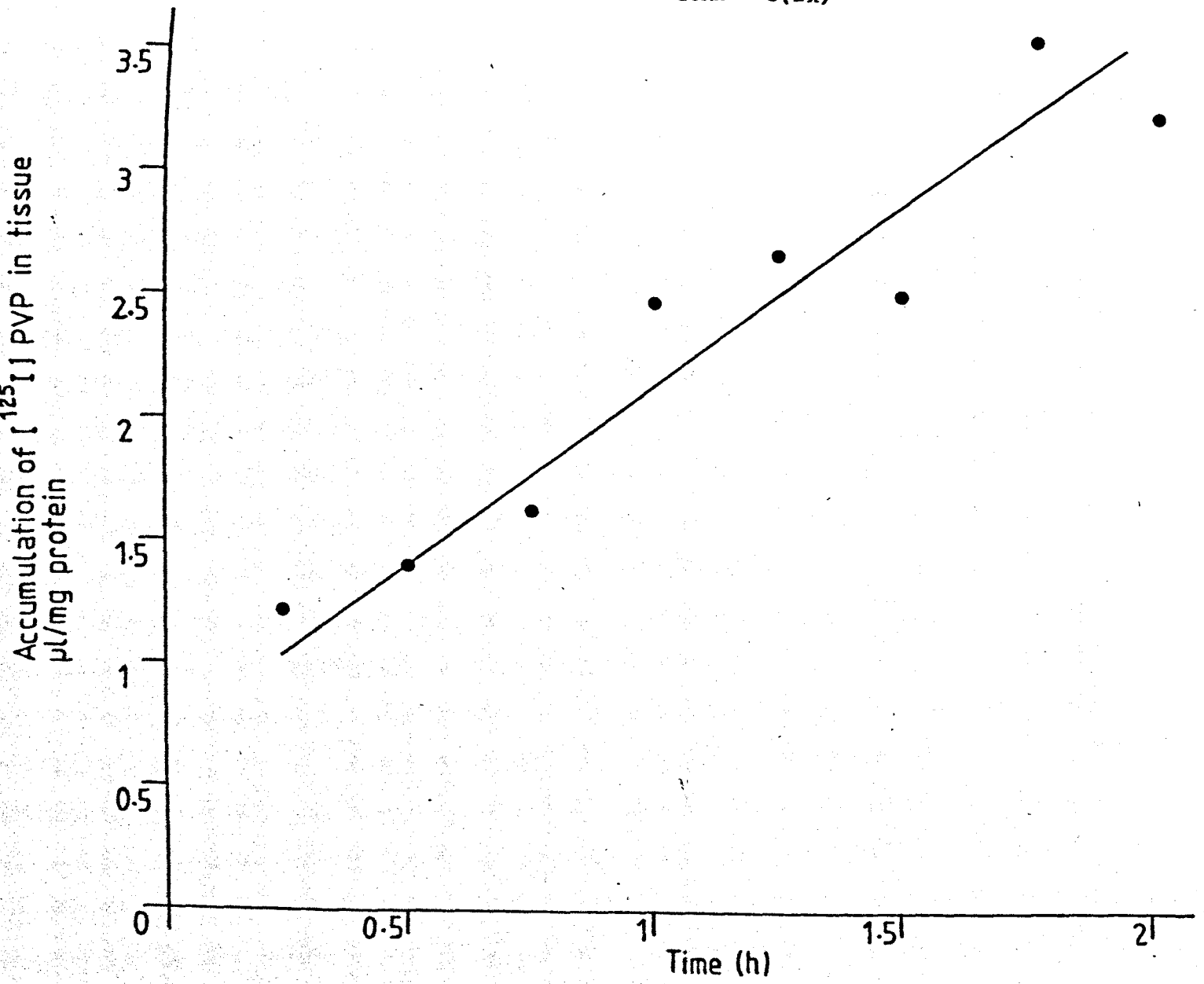

Accumulation of [125I] PVP in tissue µl/mg protein
3·5
3
2·5
2
1·5
1
0·5
0
0·5
1
1·5
2
Time (h)

FIGURE 3(x) Accumulation of radioactivity in the serosal fluid of everted intestinal sacs incubated in the presence of [^{125}I]-PVP.

Each point is derived from data on a single intestinal sac.

FIGURE 3(x)

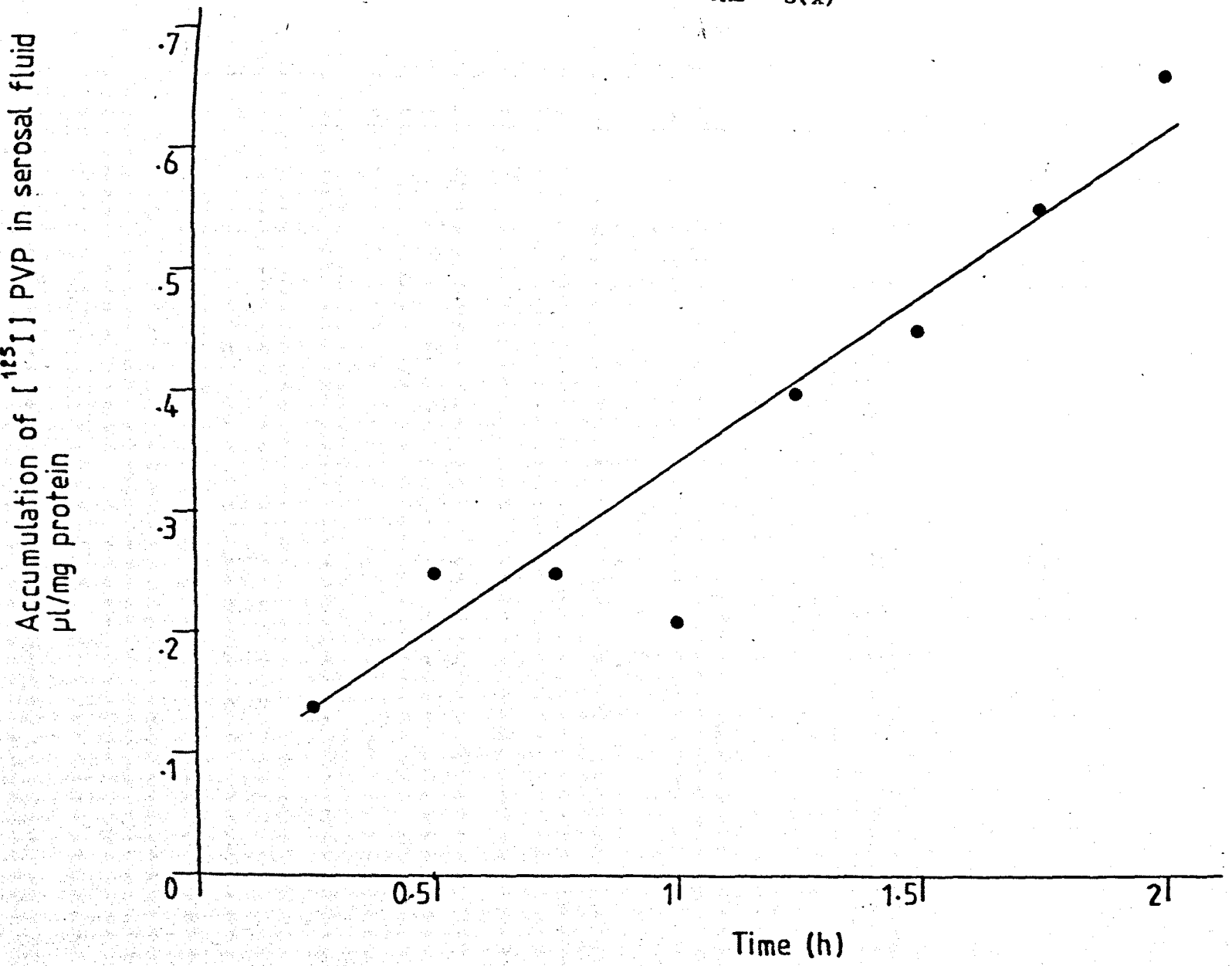

Accumulation of [125I] PVP in serosal fluid µl/mg protein
·7
·6
·5
·4
·3
·2
·1
0
0·5
1
1·5
2
Time (h)

TABLE 3.3

Correlation Coefficient, Slope and Intercept on Ordinate Axis for the Regression Lines through each Set of Data from Eight Separate Experiments

Experiment No.	Tissue Uptake			Accumulation in Serosal Fluid		
	Correlation Coefficient	Slope µl/h/mg protein	Intercept on Ordinate Axis µl/mg protein	Correlation Coefficient	Slope µl/h/mg protein	Intercept of Ordinate Axis µl/mg protein
1	0.89	0.73	0.42	0.80	0.15	0.040
2	0.89	0.64	0.45	0.72	0.08	0.060
3	0.90	0.71	1.32	0.73	0.12	0.100
4	0.94	0.90	1.41	0.74	0.09	0.190
5	0.86	0.68	1.18	0.71	0.14	0.070
6	0.90	1.32	0.64	0.90	0.17	0.008
7	0.93	0.80	0.48	0.94	0.27	-0.02
8	0.97	0.47	0.68	0.77	0.09	0.16

fluid and in both cases there was no significant difference in the variation at the 5% level. Hence the experiments were shown to be from a homogeneous population allowing data from different experiments to be pooled to give a single overall characteristic of uptake into tissue or rate of appearance of substrate in serosal fluid.

The regression line for the 64 experimental points (Fig. 3(xi) and (xii)) of the eight experiments had a slope of 0.78 $\pm$ 0.09 µl/h/mg protein for tissue uptake and 0.14 $\pm$ 0.02 µl/h/mg for accumulation in the serosal fluid.

FIGURES 3(xi) and 3(xii) Accumulation of [^{125}I]-PVP by the intestinal tissue (3(xi)) and in the serosal fluid (3 (xii)) of everted intestinal sacs incubated in the presence of [^{125}I]-PVP (2 µg/ml).

The 64 points in each plot are derived from eight separate experiments, eight intestinal sacs per experiment. Each point corresponds to data from a single intestinal sac.

FIGURE 3(xi)

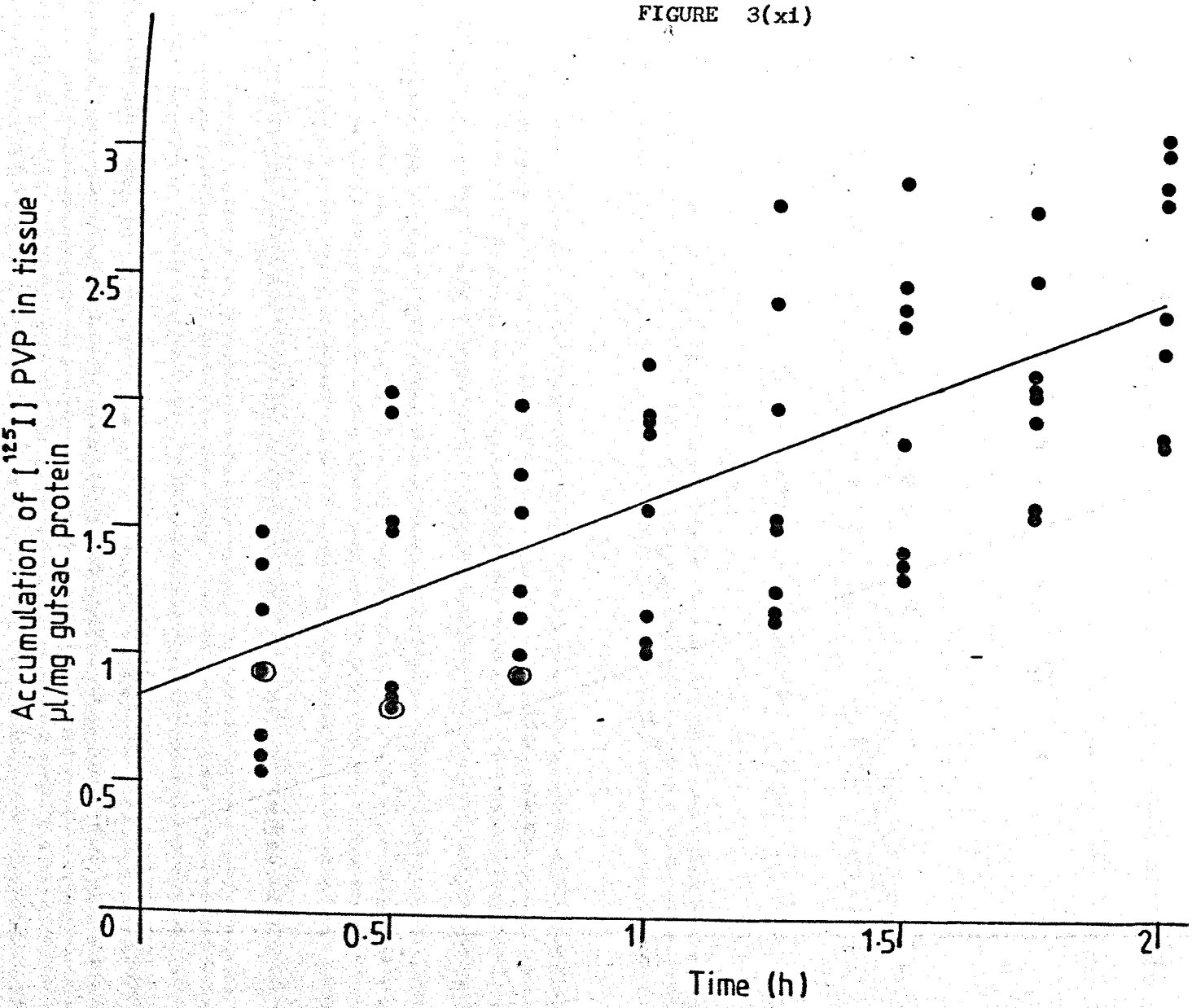
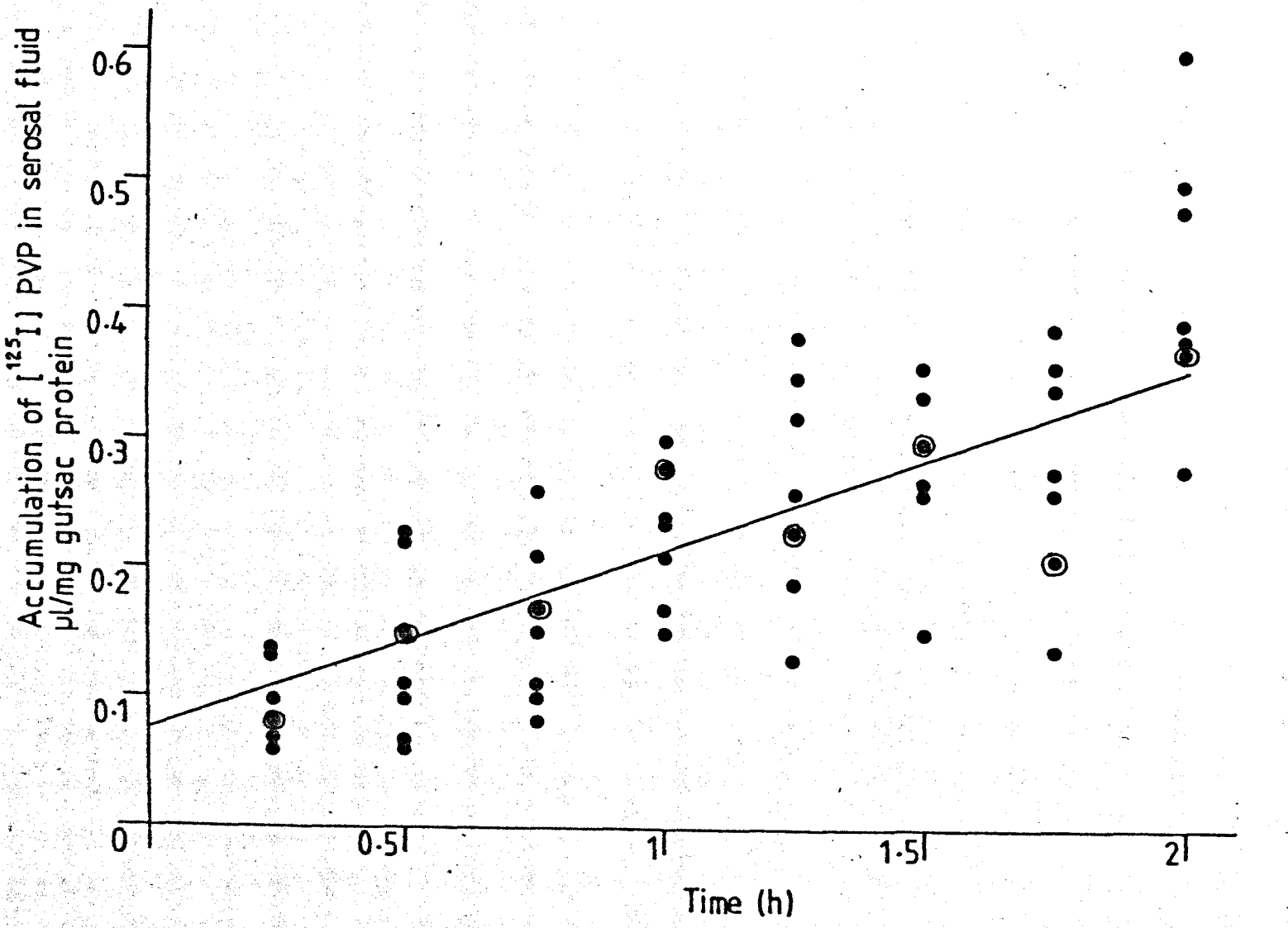

Accumulation of [125I] PVP in tissue
µl/mg gutsac protein
3
2·5
2
1·5
1
0·5
0
0·5
1
1·5
2
Time (h)

FIGURE 3(xii)


Accumulation of [125I] PVP in serosal fluid µl/mg gutsac protein
0·6
0·5
0·4
0·3
0·2
0·1
0
0·5
1
1·5
2
Time (h)

3.7 Discussion

The method described by Walker *et al.* (1972) could not be duplicated exactly in this laboratory. The method was therefore examined step-by-step and subsequently modified for routine use using the available equipment.

The dissection time was reduced from 30 min to 10 min resulting in no cellular debris appearing in the mucosal fluid at the end of the preparation period. Fisher and Parsons (1949) believed that the key feature in preparation of isolated intestine was continuous oxygenation of tissue. However it is not possible to maintain continuous oxygenation during the setting up of everted sacs. Fisher and Gardner (1974) showed that a delay of 15 min between excision and perfusion was detrimental to subsequent absorption of glucose and water though damage was slowly reversible. They concluded that precautions to eradicate temporary hypoxia were highly desirable to avoid suboptimal absorption and experimental variability. Thus the rapidity of the modified method reduced temporary hypoxia to a minimum.

Physical tissue damage was reduced by optimising the shaking speed of the water baths.

Rates of appearance of substrate in tissue and serosal fluid were constant over the incubation period and a plot of accumulation of substrate against incubation time (h) yielded a straight line for tissue and serosal substrate accumulation. Williams *et al.* (1975) termed the rate of substrate accumulation calculated in this way the endocytic index (EI), the units being µl/mg tissue protein/h of incubation. When results from sets of data were pooled, an analysis of covariance showed that the experimental results were drawn from a homogeneous experimental population. This indicated that the data from different experiments could be pooled and a single overall EI used to show rate of uptake tissue or rate of appearance of substrate in serosal fluid.

CHAPTER FOUR

EFFECT OF INCUBATION MEDIA ON [^{125}I]-PVP UPTAKE AND TISSUE VIABILITY

4.1 Introduction

Ryser (1968) put forward some criteria which must be fulfilled before a model culture system can furnish meaningful results. One of the main criteria was that in any investigation of macromolecular uptake, the method must preserve the integrity of the cells since lysis will obviously distort any uptake data gathered. In this respect, the incubation medium is of great importance and must be selected so that throughout the time-course of the experiment the structure of the incubated tissue is maintained as closely as possible to that of fresh tissue.

Using the technique described in Section 3.6, the effects of different media on viability and tissue integrity during dissection and incubation were assessed. The rate of accumulation of [^{125}I]-PVP in different media was determined and the medium which gave the greatest reproducibility of results ascertained. Once the best culture medium for use in this system had been determined, viability experiments were carried out under the conditions in which macromolecular transport was to be investigated.

4.2 Uptake Patterns of [^{125}I]-PVP : 2 h Incubations

4.2.1 Method

Everted intestinal sacs from adult rats were incubated in Krebs Improved Ringer II pH 7.4, Tc medium 199 and Tc medium 199 plus 10% v/v calf serum (CS) using the standard method (Section 3.6). [^{125}I]-PVP (batch no. 157BA) at a concentration of 2 µg/ml was used as substrate and incubations were terminated at regular intervals up to 2 h. The pH of the culture medium was measured before the start of each incubation (but after re-gassing) and after each sample had been taken. A Pye-Unicam pH meter model 292 was used.

4.2.2 Results

The results of this experiment are shown in Fig. 4(i) and (ii). Each plot is the result of 4 similar experiments. In each experiment eight sacs from the same rat were incubated in the culture medium under test. Table 4.1 indicates the correlation coefficients and slopes of the computer fitted regression lines through the averages of each set of experimental points.

The pattern of uptake is similar for all media. Rate of appearance of substrate in the tissue was Krebs Ringer > Tc medium 199 alone > Tc medium 199 plus 10% CS. Accumulation of substrate in the serosal fluid was similar in all cases. Cellular debris in the mucosal fluid at the end of incubation was as follows: Krebs Ringer > Tc medium 199 > Tc medium 199 plus 10% CS. pH fluctuated only $\pm$ 0.1 pH unit throughout the incubation period no matter which medium was used.

4.2.3 Conclusion

Uptake of [^{125}I]-PVP into the tissue and appearance in the serosal fluid were linear with respect to time. This was the case for all media used. Generally, the more complex the medium the less tissue accumulation

FIGURE 4(i) Uptake of [^{125}I]-PVP by the tissue of everted intestinal sacs which were incubated in different media for up to 2 h.

Each plot is the mean of 4 similar experiments ± S.E. for each point.

Key to the diagrams is as follows:-

Tc 199 containing 10% CS. ▼—▼

Krebs Improved Ringer II. ♦—♦

Tc 199 only. O—O

FIGURE 4(i)

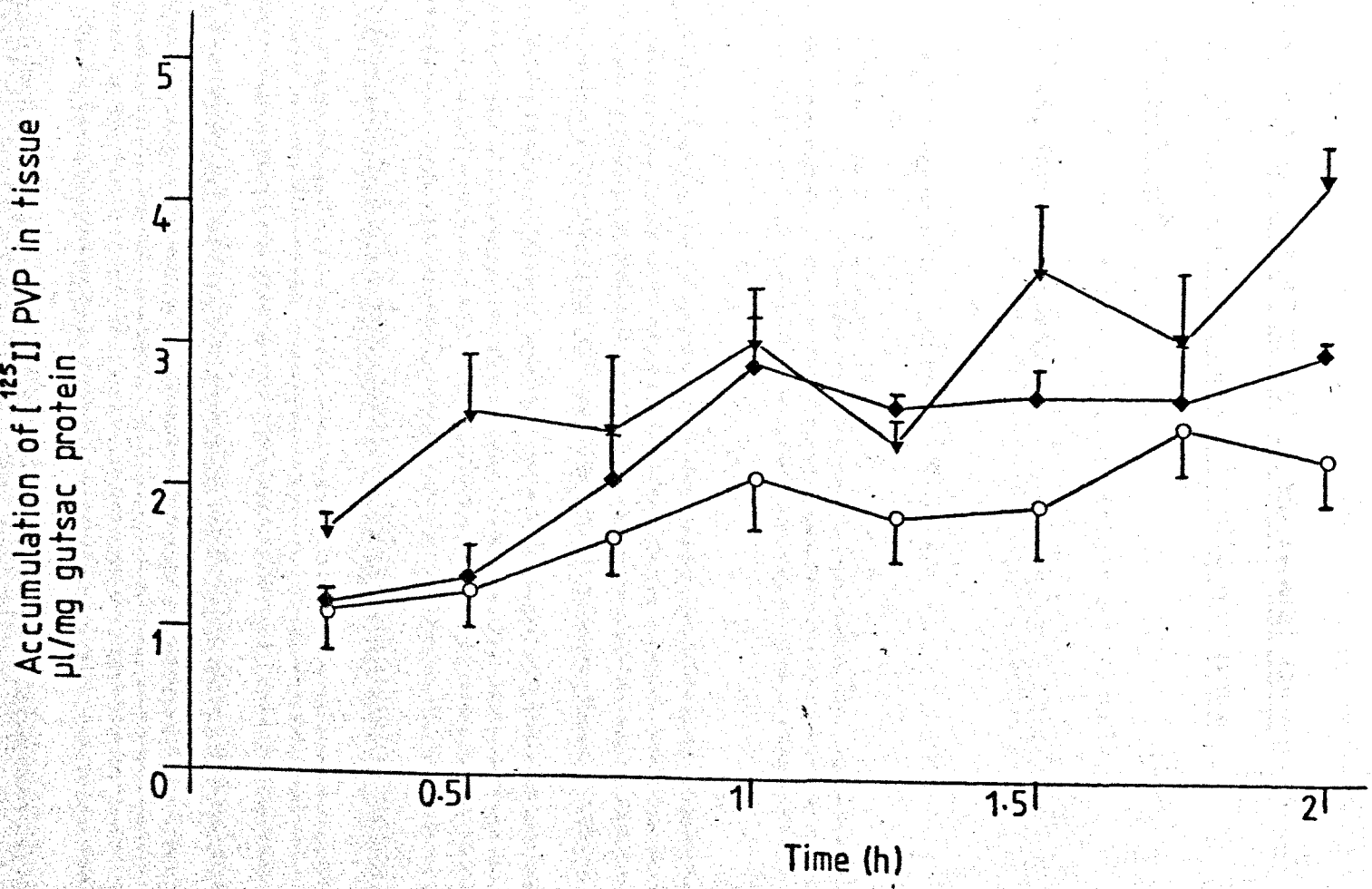

Accumulation of [125I] PVP in tissue µl/mg gutsac protein
0
1
2
3
4
5
0·5
1
1·5
2
Time (h)

FIGURE 4(ii) Accumulation of [^{125}I] -PVP in the serosal fluid of intestinal sacs incubated in different media for up to 2 h.

Each plot is the mean of four similar experiments ± S.E. for each point.

Tc 199 containing 10% CS. ▼——▼

Krebs Improved Ringer II. ♦——♦

Tc 199 only O——O

FIGURE 4(ii)

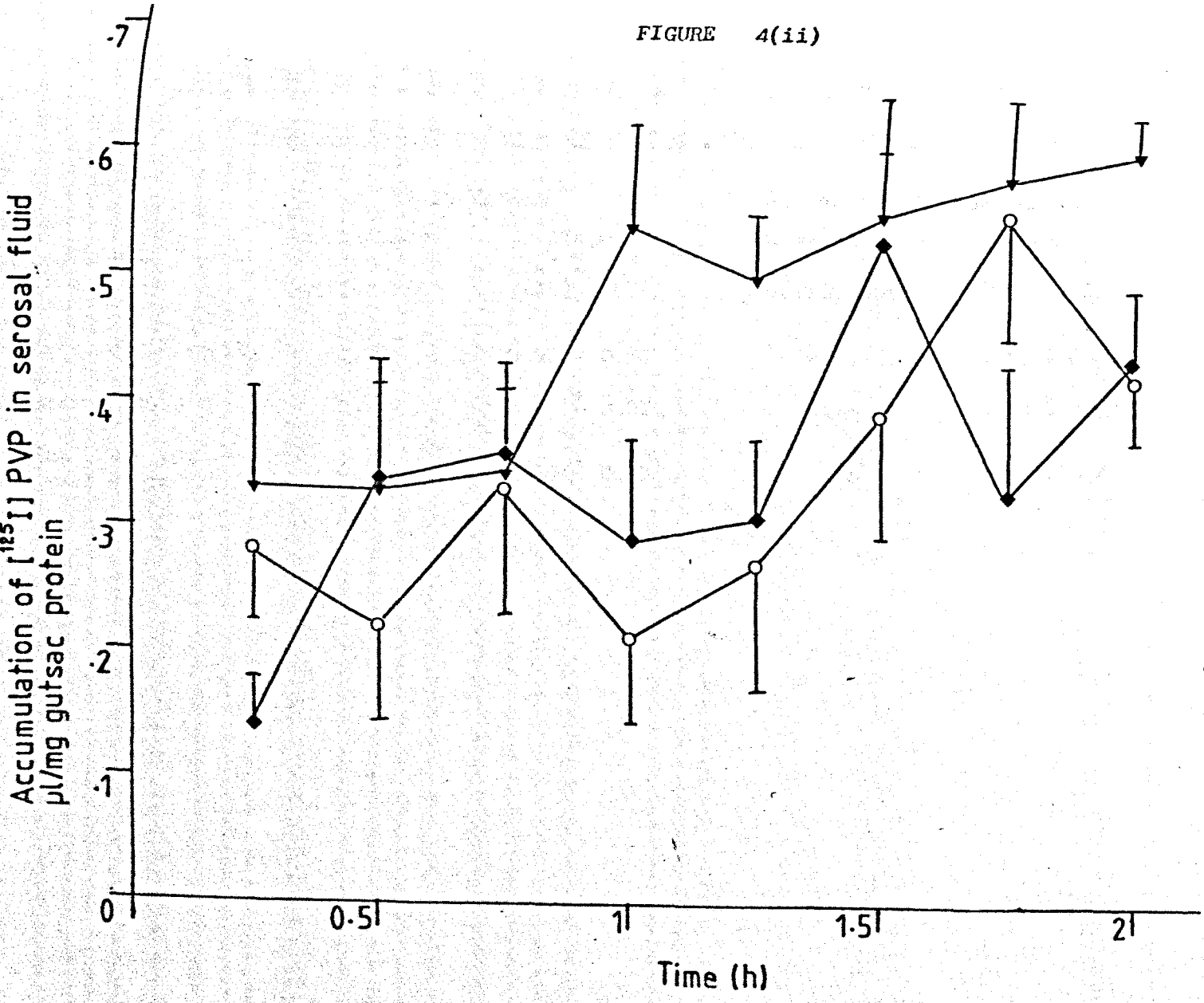

Accumulation of [125I] PVP in serosal fluid µl/mg gutsac protein
·7
·6
·5
·4
·3
·2
·1
0
0·5
1
1·5
2
Time (h)

TABLE 4.1

Uptake (EI) of [^{125}I]-PVP Substrate into Tissue and Appearance in the Serosal Fluid of Gut Sacs Cultured in vitro up to 2 h in Different Media

Culture medium	Tissue Uptake		Appearance in Serosal Fluid		No. of Experiments
	Correlation Coefficient	Slope µl/h/mg (EI) - SE	Correlation Coefficient	Slope µl/h/mg ± SE	
Krebs Improved Ringer II	0.77	0.93 ± 0.08	0.66	0.13 ± 0.04	4
Tc medium 199	0.90	0.70 ± 0.07	0.73	0.14 ± 0.02	4
Tc medium 199 + 10% v/v calf serum	0.90	0.59 ± 0.10	0.88	0.13 ± 0.08	4

of substrate occurred although substrate accumulation in the serosal fluid was similar for all media. Tissue viability, indicated by cellular debris in the mucosal fluid at the end of the incubation period was better in Tc medium 199 than in Krebs-Ringer. The addition of 10% CS to the Tc medium 199 enhanced viability but gave rise to a decreased rate of [^{125}I]-PVP uptake to the tissue although accumulation of substrate in the serosal fluid remained the same. More variability between individual experiments also occurred when CS was present in the medium. pH was maintained constant throughout the incubation period by all media.

4.3 Uptake Patterns of [^{125}I]-PVP : 3.5 h and 5 h Incubations in Different Media

4.3.1 Method

Everted intestinal sacs from adult rats were prepared incubated and assayed as described in Chapter 3.6. Incubations were carried out in Krebs Improved Ringer II, pH 7.4, Tc medium 199 and Tc medium 199 plus 10% calf serum for up to 5 h. Further incubations were carried out in Tc 199 and Tc 199 plus 10% calf serum for up to 3.5 h. [^{125}I]-PVP (batch no. 157BA, 2 µg/ml) was used as substrate in both sets of experiments. Accumulation of substrate was plotted against time for each set of data and the related EI calculated.

The pH of the culture medium was recorded before the start of each incubation and after each sample had been taken.

4.3.2 Results

Figs 4(iii) and (iv) show the results of incubations carried out in different media up to 5 h. Each plot is the result of four similar experiments, eight sacs from the same rat being incubated per experiment. Table 4.2 indicates correlation coefficients and slopes of computer-fitted lines through each set of experimental points.

Figs. 4(v) and (vi) show plots of incubations carried out in Tc medium 199 and Tc medium 199 plus 10% calf serum. Each plot is the result of six similar experiments, eight intestinal sacs per experiment. Table 4.3 indicates correlation coefficients and slopes for these experiments.

Accumulation of substrate both in the tissue and serosal fluid seemed steady up to 5 h for intestinal tissue incubated in Tc medium 199 alone and Tc medium 199 plus 10% calf serum although after 2 h there was a loss of linearity indicated by larger error bars. With Krebs-Ringer as incubation medium tissue uptake was steady up to about 3 h but then a rapid influx of substrate occurred. This coincided with a rapid influx of substrate into

FIGURE 4(iii) Uptake of [^{125}I]-PVP by the tissue of everted intestinal sacs which were incubated in different media for up to 5 h.

Each plot is the mean of four similar experiments ± S.E. for each point.

Key to diagram is as follows:

Tc 199 containing 10% CS. ▼——▼

Krebs Improved Ringer II ♦——♦

Tc 199 only. O——O

FIGURE 4(iii)

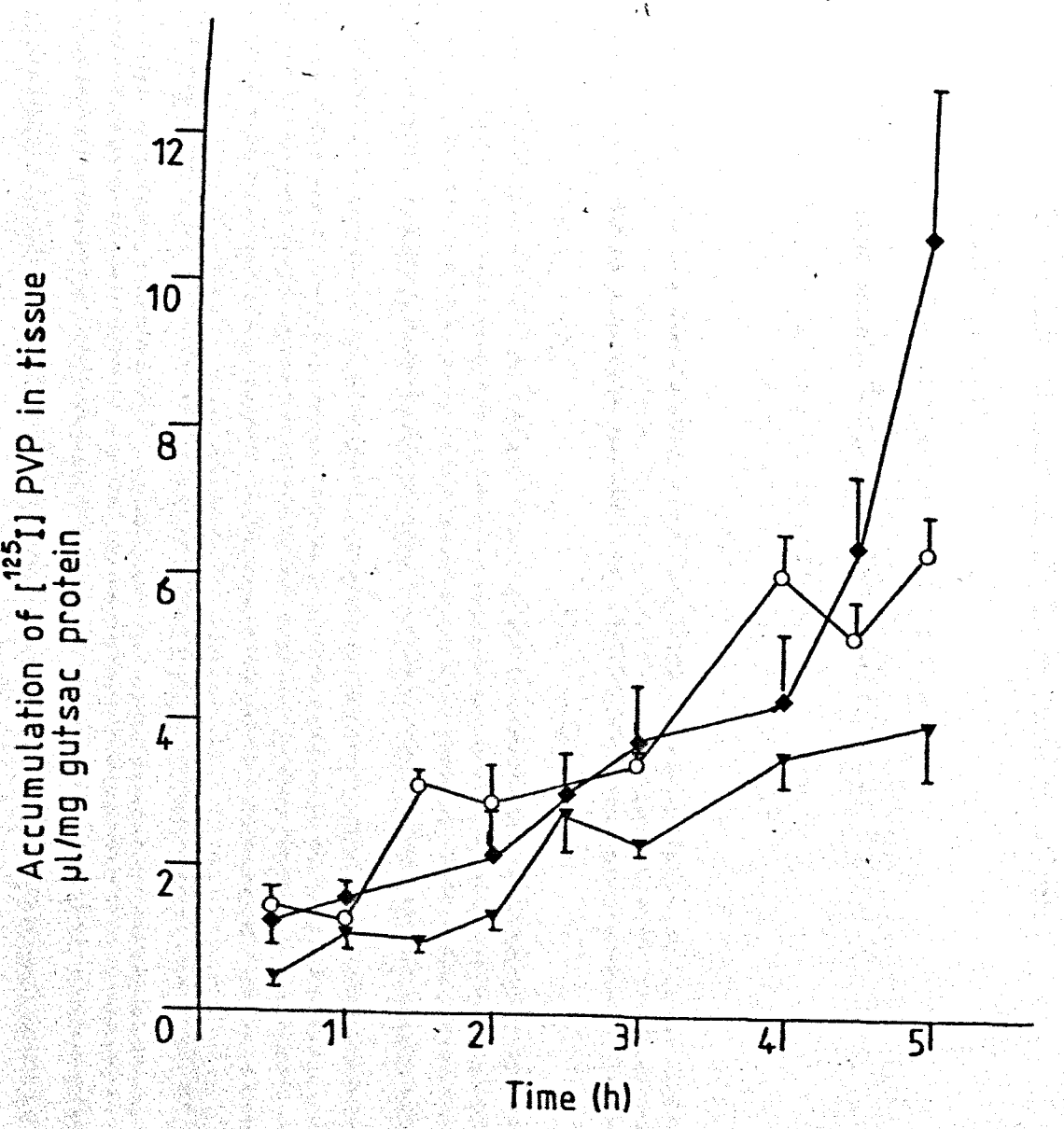

Accumulation of [125I] PVP in tissue
µl/mg gutsac protein
0
2
4
6
8
10
12
1
2
3
4
5
Time (h)

FIGURE 4(iv) Accumulation of [^{125}I]-PVP in the serosal fluid of intestinal sacs incubated in different media for up to 5 h.

Each plot is the mean of four similar experiments ± S.E. for each point.

Key to diagram is as follows:

Tc 199 containing 10% CS. ▼——▼

Krebs Improved Ringer II. ♦——♦

Tc 199 only O——O

FIGURE 4(iv)

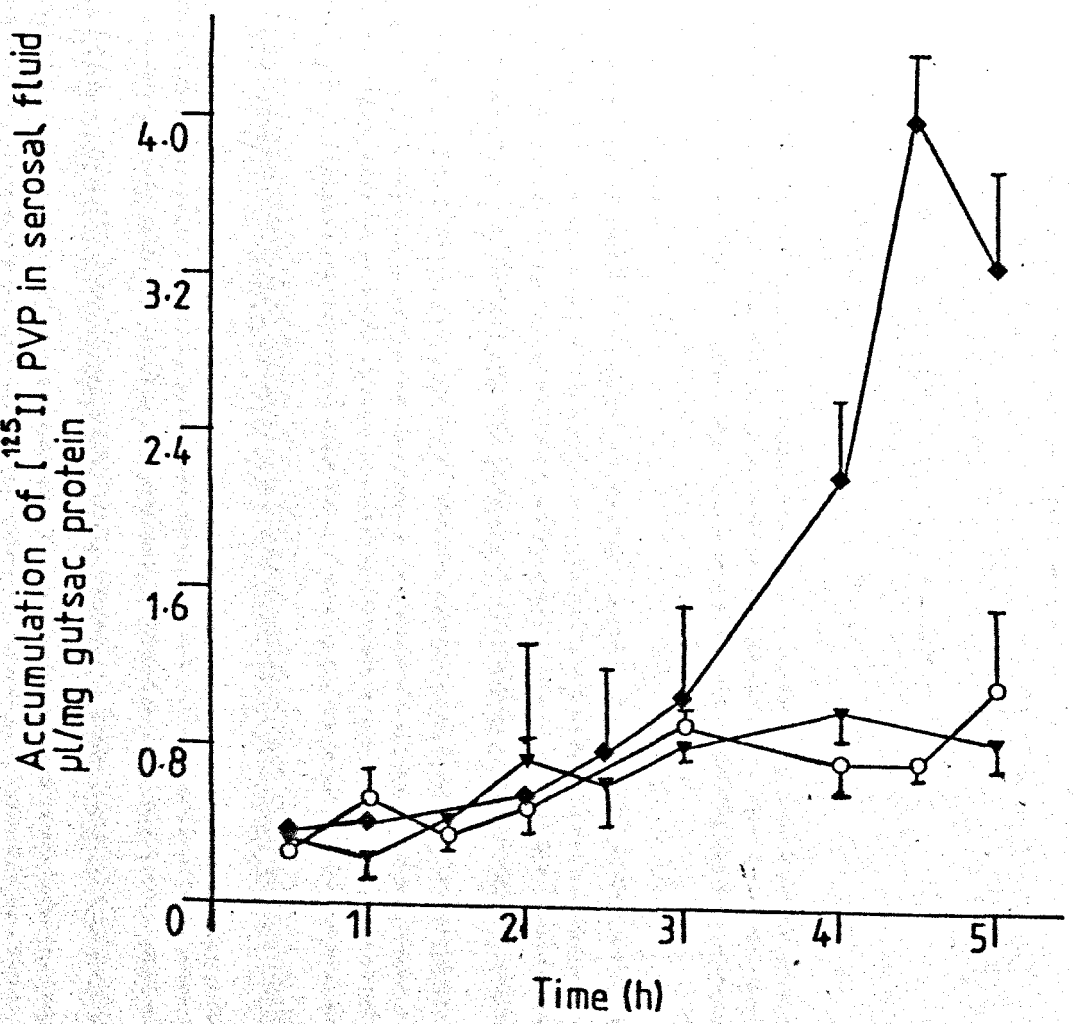

Accumulation of [125I] PVP in serosal fluid µl/mg gutsac protein
4·0
3·2
2·4
1·6
0·8
0
1
2
3
4
5
Time (h)

FIGURE 4(v) Uptake ± S.E. of [^{125}I]-PVP by the tissue of everted intestinal sacs incubated for up to 3.5 h in different media

Key to diagram is as follows:-

Tc 199 containing 10% CS. ▼——▼

Tc 199 only. O——O

FIGURE 4(v)

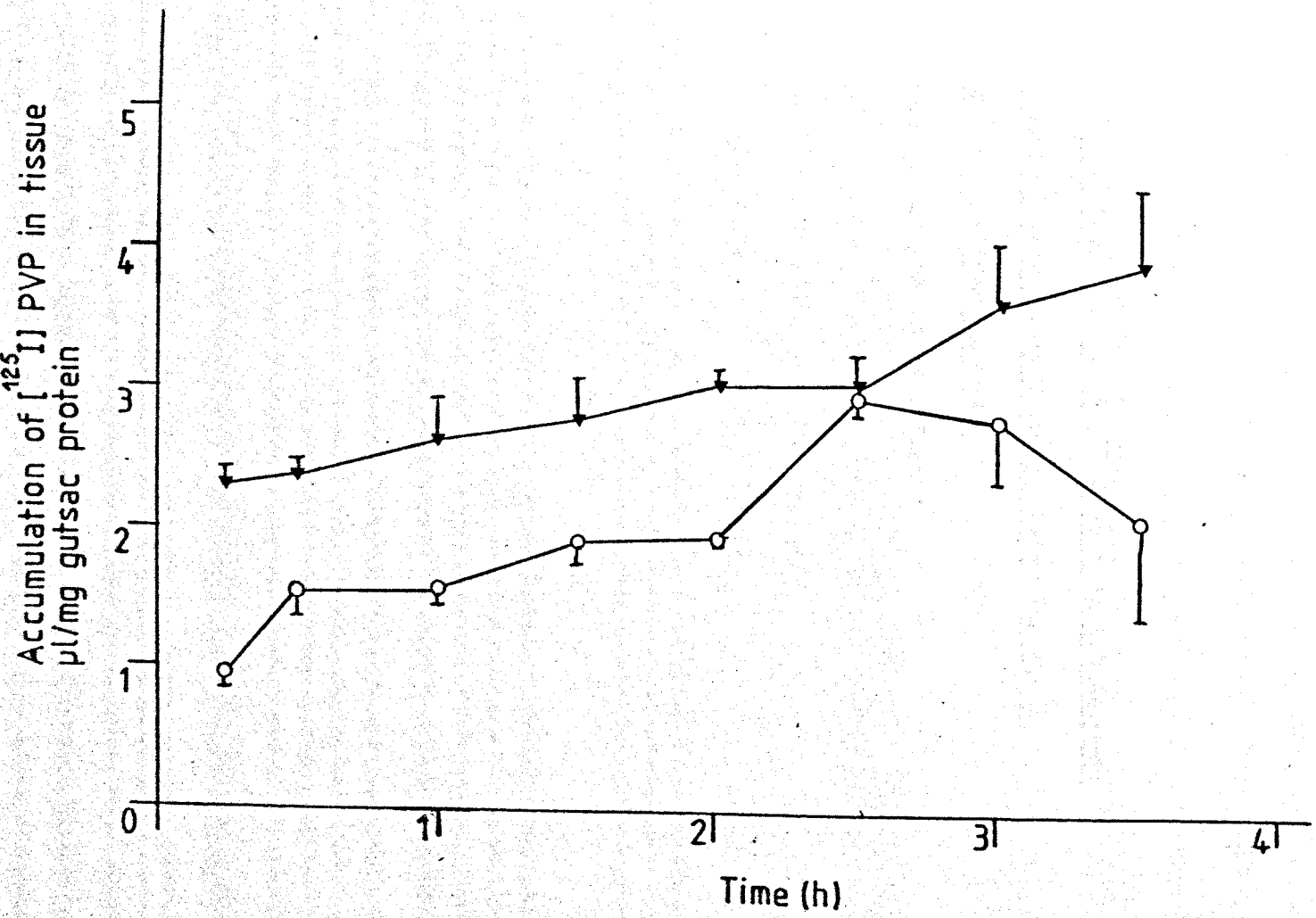

Accumulation of [125I] PVP in tissue µl/mg gutsac protein
0
1
2
3
4
5
1
2
3
4
Time (h)

FIGURE 4(vi) Accumulation $\pm$ S.E. of [^{125}I]-PVP in the serosal fluid of intestinal sacs incubated for up to 3.5 h in different media

Key to diagram is as follows:

Tc 199 containing 10% CS. ▼——▼

Tc 199 only. O——O

FIGURE 4(vi)

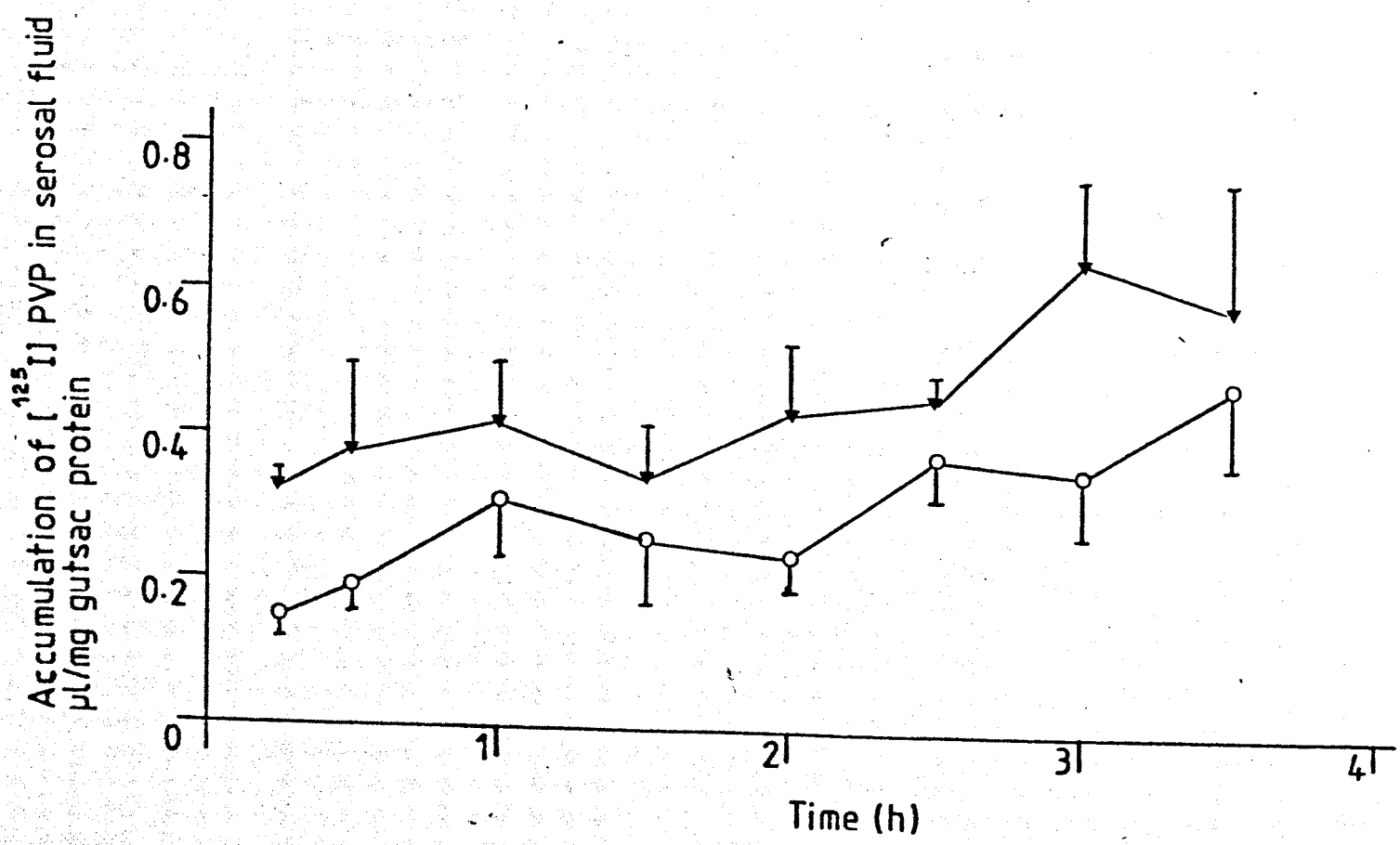

Accumulation of [125I] PVP in serosal fluid
µl/mg gutsac protein
0·8
0·6
0·4
0·2
0
1
2
3
4
Time (h)

TABLE 4.2

Uptake of [^{125}I]-PVP in µl/h/mg protein into the Tissue and Appearance in the Serosal Fluid of Gut Sacs Cultured in vitro up to 5 h in Different Culture Media

Culture medium	Tissue Uptake		Appearance in Serosal Fluid		No. of Experiments
	Correlation Coefficient	Slope (EI) µl/h/mg ± SE	Correlation Coefficient	Slope (EI) µl/h/mg - SE	
Krebs Improved Ringer II	0.86	2.46 ± 1.44	0.68	0.68 ± 0.11	4
Tc medium 199	0.88	0.92 ± 0.28	0.87	0.12 ± 0.07	4
Tc medium 199 + 10% calf serum	0.92	0.79 ± 0.30	0.85	0.17 ± 0.10	4

TABLE 4.3

Accumulation of [^{125}I]-PVP in µl/h/mg protein into the Tissue and Serosal Fluid of Gut Sacs Incubated up to 3.5 h in Tc Medium 199 with and without 10% Calf Serum

Culture Medium	Tissue Uptake		Appearance in Serosal Fluid		No. of Experiments
	Correlation Coefficient	Slope ± SE µl/h/mg	Correlation Coefficient	Slope ± SE µl/h/mg	
Tc 199 only	0.81	0.72 ± 0.07	0.92	0.10 ± 0.07	6
Tc 199 + 10% Calf Serum	0.97	0.46 ± 0.08	0.89	0.13 ± 0.10	6

the serosal fluid. Uptake plots for tissue incubated up to 3.5 h in Tc medium 199 and Tc medium 199 plus 10% calf serum (Figs. 4(v) and (vi) and Table 4.3) showed more clearly that after 2 h incubation there was more variability between individual experiments. Addition of calf serum to the culture medium seems to decrease the rate of uptake of [^{125}I]-PVP to the tissue but not the appearance in the serosal fluid (Table 4.3).

Gross tissue breakdown occurred with intestinal sacs incubated in Krebs-Ringer II medium after 2 h. After 2 h the tissue was pale and the mucosa ragged. Sacs incubated in Tc medium 199 alone showed signs of tissue breakdown (e.g. appearance of debris in the mucosal fluid) after 2.25 h but gross tissue breakdown did not occur until 2.5 h. Sacs incubated in Tc 199 plus 10% calf serum appeared pink with no stripping of the mocosa after 2 h. Cellular debris occurred in the mucosal fluid after 2.5 h and gross tissue breakdown followed quickly after 2.75 h.

In all cases, pH fell during incubation, changes being 1.5, 1.2 and 0.8 pH units for Krebs-Henseleit Ringer, Tc medium 199 and Tc medium 199 plus 10% calf serum respectively after 5 h incubation.

4.3.3 Conclusions

From these experiments it appears that whichever medium is used, extension of the incubation period beyond 2 h leads to gross tissue breakdown and more variability between individual experiments, with a resulting loss of linearity in the uptake plots. The rapid influx of substrate which occurred when tissue was incubated beyond 3 h in Krebs Ringer medium is indicative of cytolysis of the intestinal epithelium according to Walker *et al.* (1972).

The presence of calf serum in Tc medium 199 slightly depresses tissue uptake of substrate and gives rise to a greater variability between experiments. However presence of calf serum in the medium enhances tissue viability. pH fall is less when calf serum is present in the medium, probably due to the buffering capacity of the serum proteins present. When

the pH fall over 5 h is compared with that over 2 h (see Section 4.2.2) most of the fall in pH occurs after 2 h whichever medium is used. This pH drop is thought to be an indication of cell lysis (Dean, 1977).

Taking these points into consideration, incubation time must not exceed 2 h and the best culture medium to sustain tissue viability throughout the experimental period is Tc medium 199 plus 10% calf serum. Despite the slight loss in experimental reproducibility it was felt that maintenance of tissue viability throughout the 2 h incubation period was of prime importance. Therefore Tc medium 199 plus 10% CS was chosen as incubation medium for future experiments.

4.4 Effects of Different Concentrations of ATP on Uptake of [^{125}I]-PVP

As outlined in Section 1.5.1, manipulation and incubation of intestinal tissue for *in vitro* incubation resulted in loss of adenine nucleotides, particularly ATP, from the tissue (Bronk and Leese, 1973). Faelli *et al.* (1976) postulated that the nucleotide loss was due to leakage from the cells, although structural integrity was not impaired (Jaspar and Bronk, 1968). Therefore in this experiment, exogenous ATP was added to the incubation medium in varying amounts (1 mM - 5 mM) in order to assess any effects on tissue viability and reproducibility of results.

4.4.1 Method

Incubations of everted jejunal sacs from adult male rats were carried out as described in Chapter 3.6 with 2 µg/ml [^{125}I]-PVP (batch no. 158BA) as substrate. Experiments were carried out in Tc medium 199 plus 10% calf serum containing 0, 1, 2 and 5 mM ATP. Incubations were terminated at intervals up to 2h, accumulation of substrate was plotted against time for each set of data and the related EI calculated.

4.4.2 Results

The results from these experiments are shown in Fig. 4(vii) and (viii) and Table 4.4. Each plot is the result of six similar experiments, eight sacs from the same rat per experiment.

When exogenous ATP was added to the medium, at the 1 or 2 mM concentrations the rate of uptake of [^{125}I]-PVP into the intestinal tissue increased, the rate of accumulation of substrate in the serosal fluid decreased and the intercept on the ordinate axis for the regression lines through each set of tissue uptake data was lower. At the 5 mM concentration of ATP, however, tissue uptake resembled that of the control. When 1 or 2 mM ATP was present in the medium, reproducibility of the experiments was enhanced both for tissue uptake and accumulation of substrate in the serosal fluid. This was indicated by an increased correlation coefficient and decreased standard error (SE) of

FIGURE 4(vii) Plots of tissue uptake ± S.E. of [^{125}I]-PVP by intestinal sacs incubated in the presence and absence of ATP as follows:

No ATP

1mM ATP

2mM ATP

5mM ATP

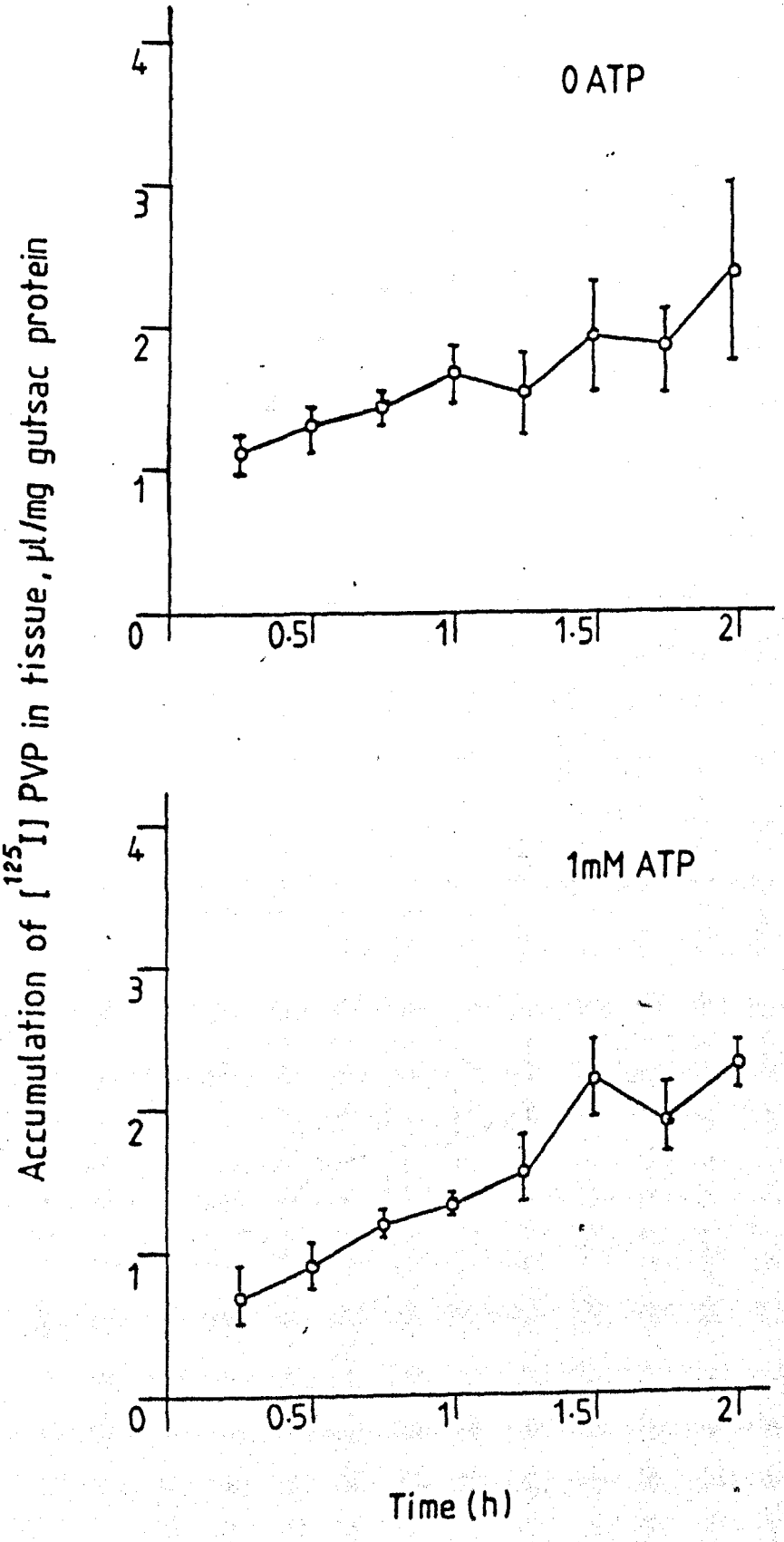

0 ATP
1mM ATP
Accumulation of [125I] PVP in tissue, µl/mg gutsac protein
Time (h)
0
1
2
3
4
0.5
1
1.5
2

FIGURE 4(vii)

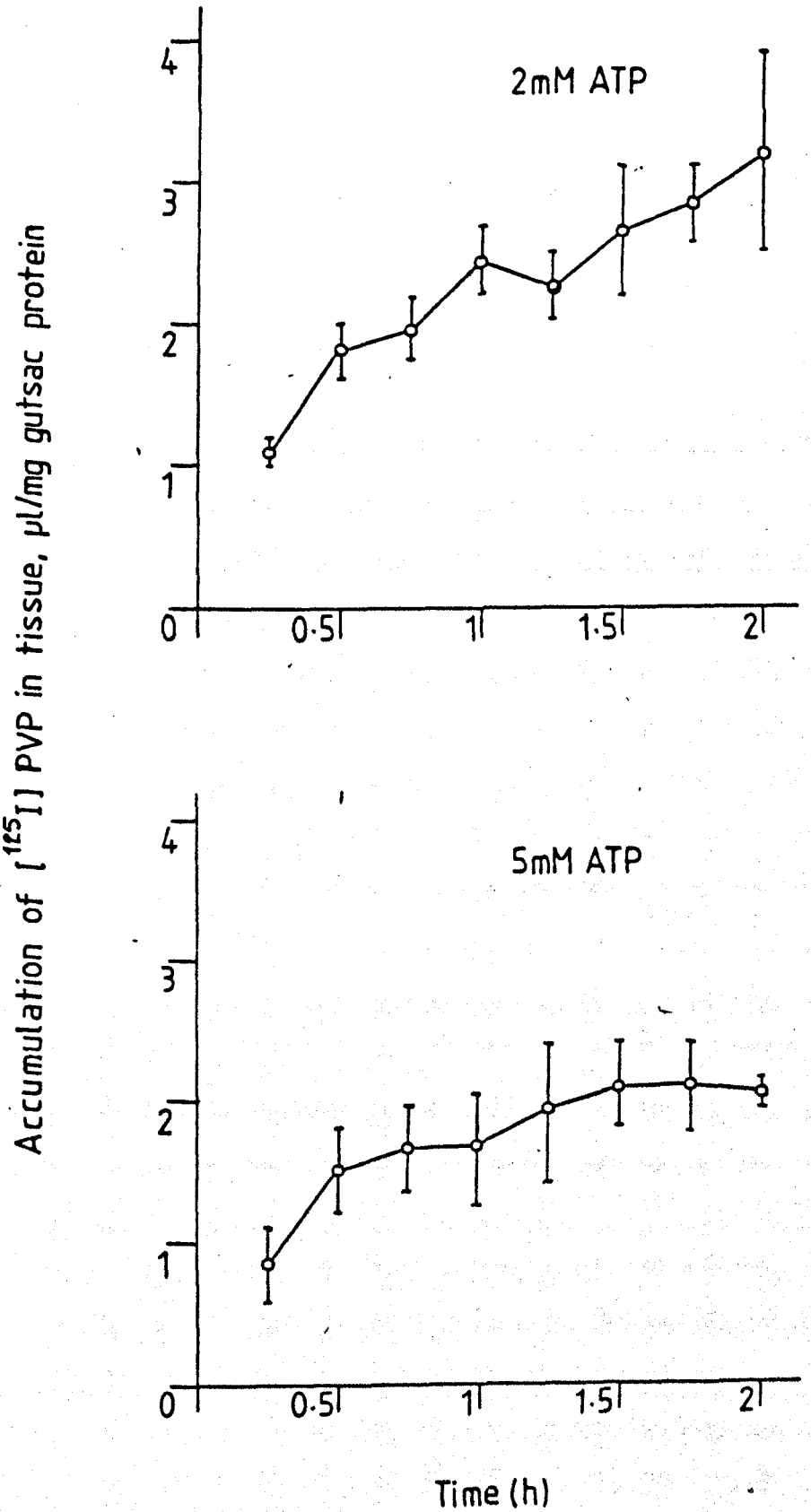

2mM ATP
5mM ATP
Accumulation of [125I] PVP in tissue, µl/mg gutsac protein
Time (h)
0
0·5
1
1·5
2
1
2
3
4

FIGURE 4(vii)

FIGURE 4(viii) Plots of the accumulation ± S.E. of [^{125}I]-PVP in the serosal fluid of intestinal sacs incubated in the presence and absence of ATP as follows:

No ATP

1mM ATP

2mM ATP

5mM ATP

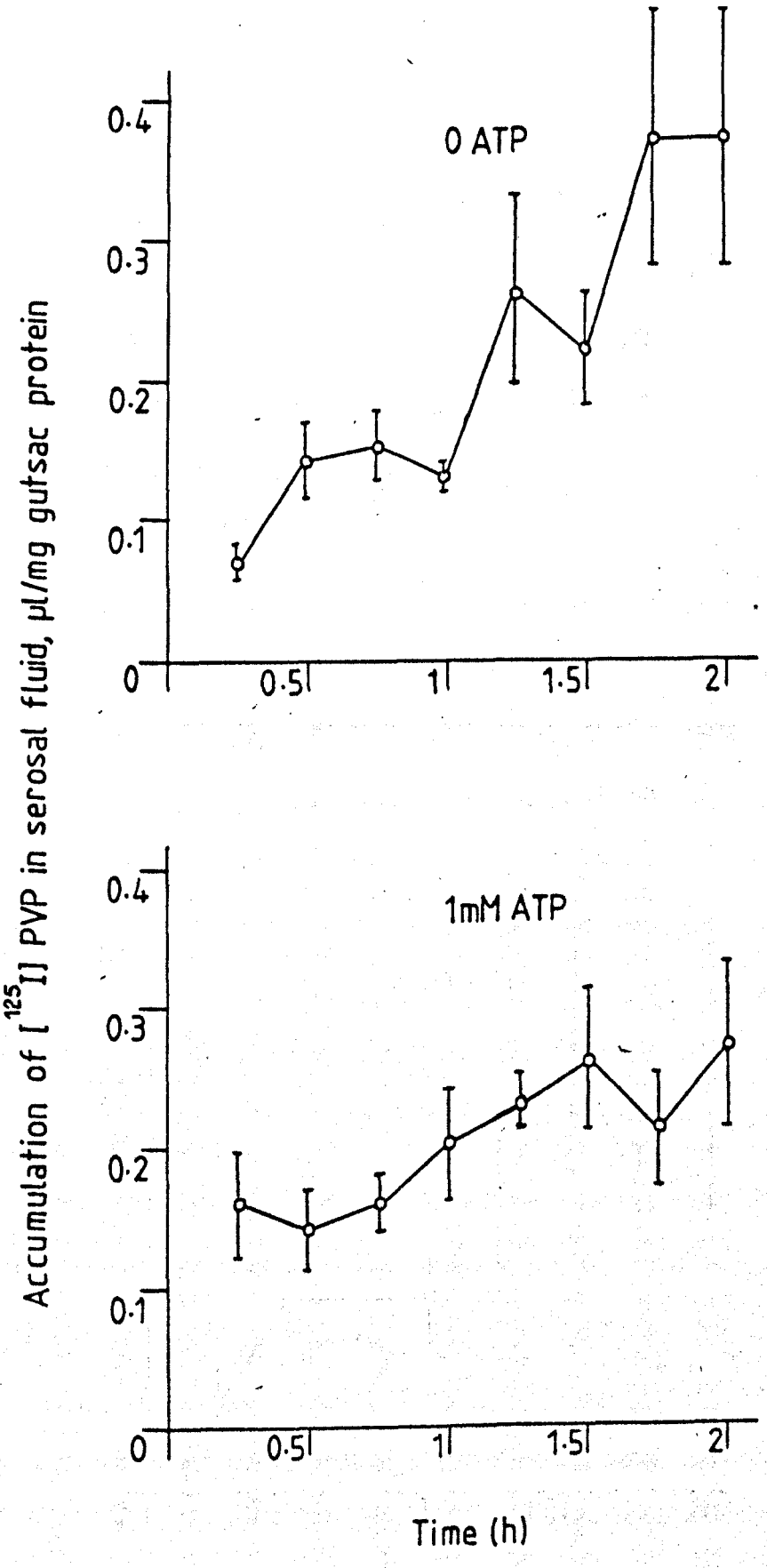


FIGURE 4(viii)

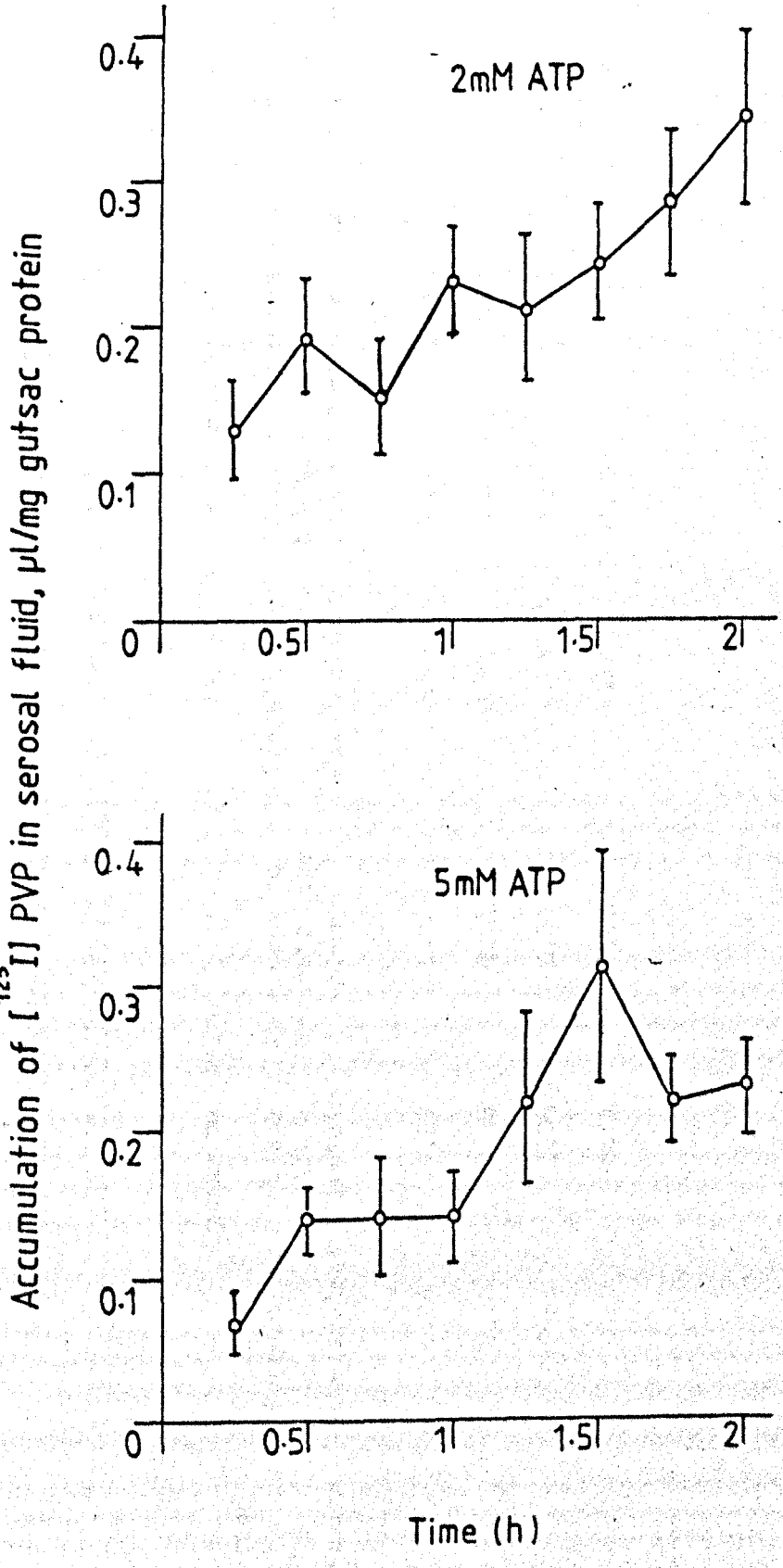


FIGURE 4(viii)

TABLE 4.4

Correlation Coefficient, Slope and Intercept on the Ordinate Axis for the Regression Lines through each Set of Data

Concentration ATP in Culture Medium	Uptake to Tissue			Serosal Fluid Accumulation			No. of Experiments
	Correlation Coefficient	Slope µl/h/mg Protein	Intercept on Ordinate Axis µl/mg	Correlation Coefficient	Slope µl/h/mg Protein	Intercept on Ordinate Axis	
0	0.94	0.59	1.00	0.90	0.15	0.04	6
1	0.96	0.89	0.51	0.87	0.07	0.13	6
2	0.96	1.05	0.90	0.93	0.10	0.11	6
5	0.88	0.61	1.05	0.82	0.10	0.07	6

the mean EI. However, addition of 5 mM ATP to the medium did not improve reproducibility compared to the control (no ATP) and a pH fall in the medium was observed during incubation. This was taken as an indication of cellular damage.

4.4.3 Conclusions

As addition of exogenous ATP to the medium improved reproducibility, it was considered justified that ATP be included in the incubation medium for future experiments. Medium containing a concentration of 1 mM ATP was subsequently, used routinely.

4.5 Viability Studies

Once a standard *in vitro* technique had been decided upon, it was necessary to test fully the viability and cellular integrity of the tissue under the experimental conditions. Three methods for monitoring gut sac integrity during the period of incubation were used.

a) Sacs were monitored for the active transport of D-glucose and L-methionine during the entire incubation period.

b) Sacs were examined by light and electron microscopy for morphologic integrity.

c) Oxygen uptake of sacs was monitored during the entire incubation period.

D-glucose was chosen as substrate because it is known to be transferred against a concentration gradient (Fisher and Parsons, 1949) and this has been used as a criterion of tissue viability in *in vitro* preparations. The choice of L-methionine as substrate was determined by the evidence for its absorption against a concentration gradient *in vitro* by adult rat intestine (Wiseman, 1954) and the observation of Matthews and Wilson (1953) that no transamination occurred during transport.

4.5.1 Method

a) Transport of glucose and L-methionine

Jejunal sacs were prepared as described in Chapter 3.6 and incubated with equal concentrations of D-glucose (1 mg/ml) in the initial mucosal and serosal fluids. Incubations were carried out using Tc medium 199 containing 10% CS and 1 mM ATP as incubation medium, experiments being terminated at intervals up to 2 h. After each incubation period, sacs were removed and drained. Incubation medium and serosal fluid samples were assayed for glucose as described in Section 2.6. L-methionine uptake was monitored by the autoanalytical method of McGale *et al.* (1977). Jejunal sacs were prepared and incubated with equal concentrations of

L-methionine (30 µg/ml) in the initial medium and serosal fluids and samples obtained as described above. Directly after collection, samples were deproteinized with 3% w/v sulphosalicylic acid, centrifuged (24,000 g, 4°C, 20min) and the supernatant analysed. L-methionine analysis was carried out using a dual-column six-sample amino-acid analyser and integrator (Model JLC 6AH: Jeol Ltd.; 1418, Nakagami, Tokyo, Japan).

b) Microscopy

For light microscopy jejunal sacs were prepared and incubated as described in Section 3.6 using Tc medium 199 containing 10% CS and 1 mM ATP as incubation medium. Samples were taken at intervals up to 2 h and gut sacs fixed in Zenkers fluid, dehydrated and embedded in Paraplast. Sections were stained with haematoxylin and eosin. The procedure is described in detail in Section 2.8.

Jejunal sacs were prepared and incubated in the same way for electron microscopy. Fixing, staining and sectioning were then carried out as described in Section 2.8. Where indicated, 0.2 m cacodylate buffer pH 7.3 was used in the fixative.

c) Measurement of oxygen uptake

The oxygen uptake by six everted jejunal sacs was measured during incubation using a Rank-type electrode (MSE Spectroplus). Incubations were carried out in the reaction vessel of the electrode (5 ml capacity) using Tc medium 199 containing 10% CS and 1 mM ATP as incubator medium. The medium was saturated by gassing with 95:5 O_2:CO_2 for 2 min at 37°C and this was used to set the recorder to 100% saturation. Changes in oxygen content of the medium during incubations were recorded directly on a chart recorder (Servoscribe RG 541.2).

4.5.2 Results

a) Glucose and L-methionine uptake

The results obtained from twelve incubations, four sacs per incubation,

with equal concentrations of D-glucose in the initial medium and serosal fluids are shown in Table 4.5. Glucose concentrations are expressed as µg/ml. All sacs showed a removal of glucose from the medium. The ratio of glucose concentration in the serosal fluid to that in the medium was greater than unity for all sacs. This concentration gradient increased as the incubations progressed.

Table 4.6 shows the results obtained from incubations of jejunal sacs with equal concentrations of L-methionine in the initial medium and serosal fluids. The uptake of L-methionine against a concentration gradient exhibited a similar pattern to that of D-glucose as the serosal/medium concentration ratio increased as incubation progressed. These results indicated that the transfer of both D-glucose and L-methionine increased with time and that a concentration gradient could be maintained throughout the incubation period.

b) Microscopy

The results from light and electron microscopy are represented by plates 3 - 20. The magnification is shown beneath each plate.

i) Light microscopy

Plates 3 - 10 show representative sections from fresh and incubated intestine. The tissue from which these sections were prepared was as follows:

Plates 3, 4; excised "fresh" intestine

Plates 5, 6; excised, everted intestine

Plates 7, 8; intestine incubated for 1 h

Plates 9, 10; intestine incubated for 2 h

There was no evidence of morphological change in tissue produced by the mechanical manipulations of eversion. The gross appearance of the tissue did not change markedly up to 1 h after incubation. On removal from the incubation medium after 1 h the tissue appeared pink with no stripping of the mucosa. Histological examination revealed no interstitial oedema

of villi. There appeared no loss of cytoplasmic detail and submucosal structures were intact. After 2h incubation, on removal from the incubation medium the tissue appeared paler pink but even so the mucosa looked to be intact. Histologically there appeared a little interstitial oedema in places and the cytoplasmic detail was less clear. However the overall appearance of the tissue did not differ greatly from that of "fresh" tissue.

ii) Electron Microscopy

Plates 11 - 20 show representative sections from fresh and incubated intestine, the key to the plates is as follows:

Plates 11, 12, 13; excised "fresh" intestine, mid-villus region

Plate 14; excised, everted intestine, mid-villus region

Plates 15, 16, 17; intestine incubated for 1.5 h, mid-villus region.

Plate 18; intestine incubated for 1.5 h, mid-villus region, 0.2 m cacodyate buffer pH 7.3

Plates 19, 20; intestine incubated for 1.5 h, extrusion zone (villus tip) region

Plates 21, 22; intestine incubated for 1.5 h, crypt region.

After eversion, the ultrastructure of all cells was the same as that of cells from freshly excised tissue. The microvilli, junctional complexes, mitochondria and cellular integrity all appeared normal. After 1.5 h incubation and fixation in 0.1 m cacodylate buffer, the tip and crypt cells appeared normal and the crypt cells were still dividing (Pl. 22). However, cells from the mid-villus region did not appear entirely normal. In these cells the cellular integrity, microvilli and junctional complexes were all normal but the mitochondria and nuclei were swollen. There was also evidence of small inter and intra-cellular lakes. When incubated intestine was fixed in 0.2 m cacodylate buffer, the tip and crypt cells were shrunken, but the mid-villus cells appeared normal.

c) Oxygen uptake

A number of jejunal sacs were prepared and oxygen uptake monitored continuously up to 2 h. Fig. 4 (ix) shows the results for normal sacs.

TABLE 4.5

Glucose Concentration Gradients Developed by Jejunal Sacs from Adult Rats Incubated up to 2 h in Tc Medium 199 Containing 10% Calf Serum and ATP

Time (h)	No. Sacs	Medium Fluid glucose conc$^{n}_{s}$ ± SE µg/ml	Serosal Fluid glucose conc$^{n}_{s}$ ± SE µg/ml	Glucose Final Concentration Ratio Serosal/Medium
0	-	562.5 ± 16.90	562.5 ± 16.90	1
0.5	12	522.5 ± 5.6	534.4 ± 13.7	1.02
1.0	12	450.1 ± 28.3	520.0 ± 23.3	1.16
1.5	12	357.5 ± 33.1	465.6 ± 20.9	1.30
2.0	12	269.4 ± 57.5	424.4 ± 26.8	1.58

TABLE 4.6

L-methionine Uptake Patterns Expressed as Serosal/Mucosal Concentration Ratios

Time (h)	No. Sacs	Medium L-methionine Conc$^{n}_{.}$ ± SE µg/ml	Serosal L-methionine Conc$^{n}_{.}$ ± SE µg/ml	Serosal/Medium Ratio
0	8	26.70 ± 1.36	26.70 ± 1.36	1
1.0	8	24.70 ± 0.91	56.40 ± 2.47	2.28
2.0	8	25.20 ± 0.66	64.90 ± 0.35	2.58

PLATE 3

Mucosa from the jejunum of a normal adul' male rat.

Haematoxylin and eosin.

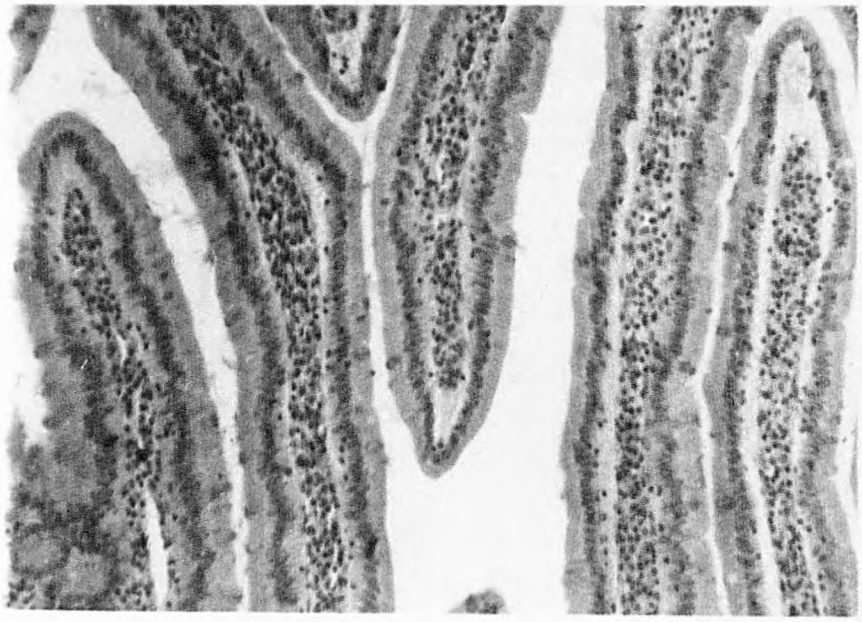
Magnification x 390.

PLATE 4

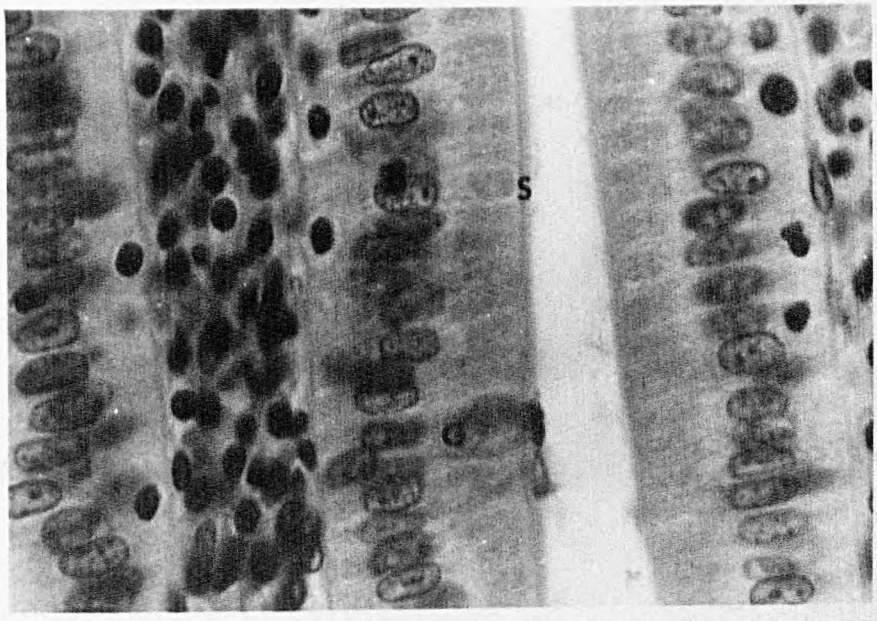
High magnification light micrograph of epithelial cells from the mid-portion of a normal adult rat jejunal villus.

Many absorptive cells and a goblet cell packed with mucous granules (G) are seen. The striated border (S) and cell nuclei can be readily identified in the absorptive cells. Haematoxylin and eosin.

Magnification x1950.



3



4

PLATE 5

Jejunum of an adult male rat demonstrating the normal appearance of the villi tips and mid-villus regions of the tissue following the mechanical manipulation of eversion

Haematoxylin and eosin.

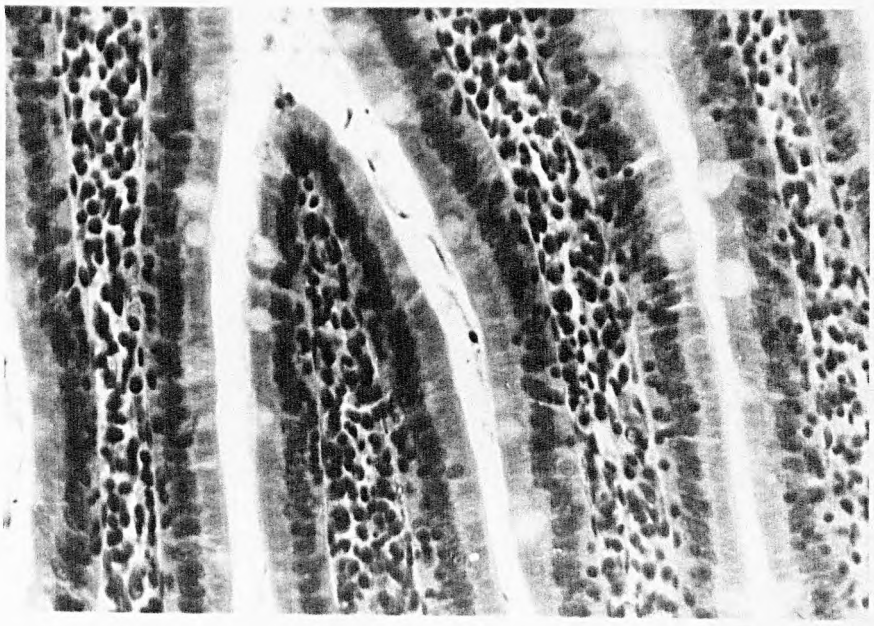
Magnification x780

PLATE 6

High magnification light micrograph of epithelial cells from the mid portion of an adult rat jejunal villus demonstrating the normal appearance of the absorptive cells. The striated border (S) and cell nuclei (N) after eversion.

Haematoxylin and eosin.

Magnification x1950.



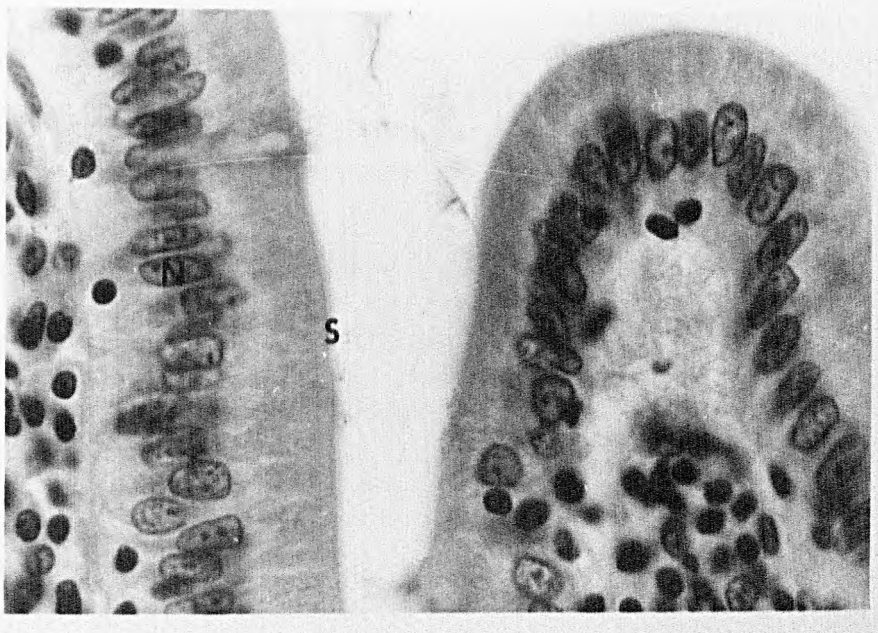

N
S

PLATE 7

Rat small intestine showing the normal appearance of the tissue following eversion and incubation at 37°C for 1 h. Haematoxylin and eosin.

Magnification x156.

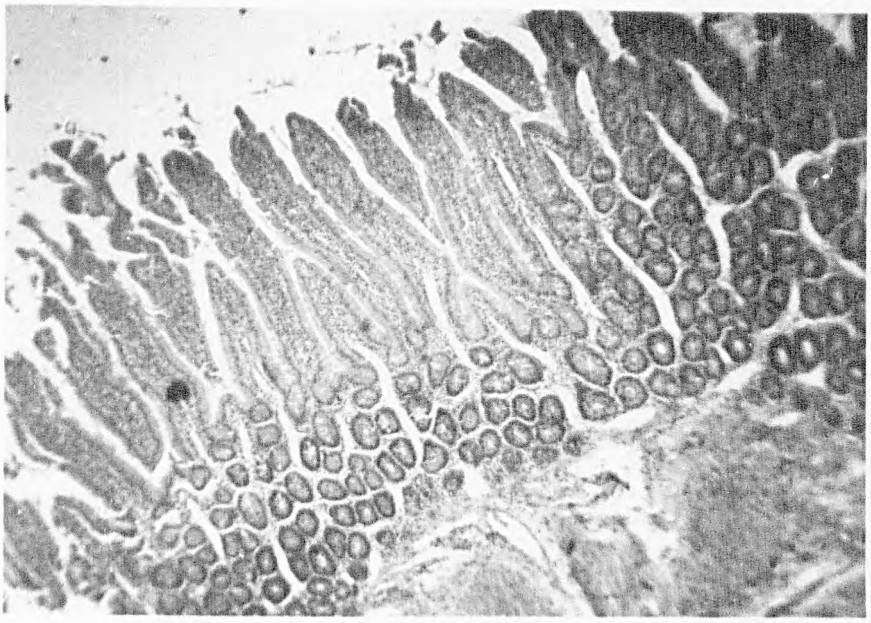
PLATE 8

High magnification light micrograph of epithelial cells from the mid-portion of adult rat jejunal villi after eversion and incubation at 37°C for 1 h.

Note the normal appearance of the absorptive cells and the undischarged goblet cells (G).

Haematoxylin and eosin.

Magnification x3900.



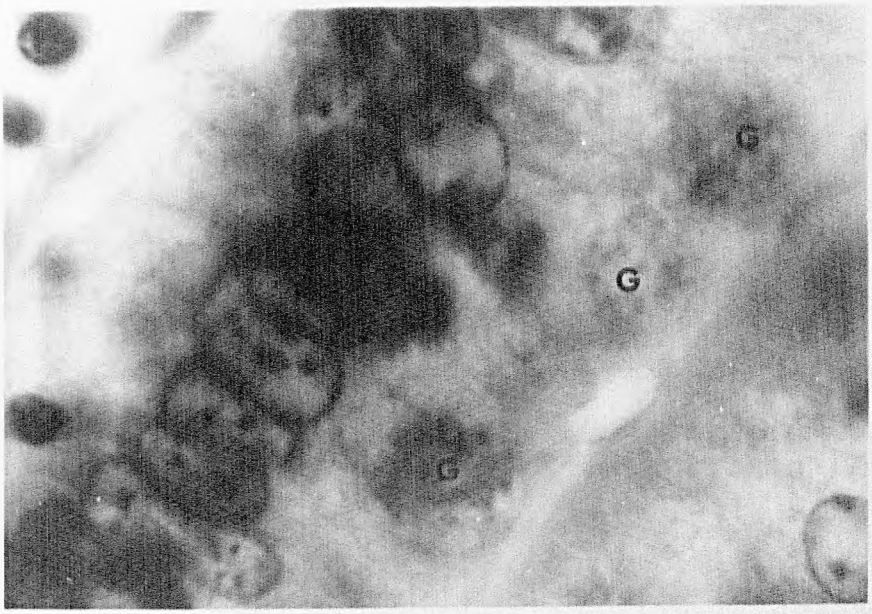

G
G
G

PLATE 9

Jejunum of an adult male rat demonstrating the appearance of the tissue following eversion and incubation at 37°C for 2 h.

Note the slight oedema of the lamina propria (L). Haematoxylin and eosin.

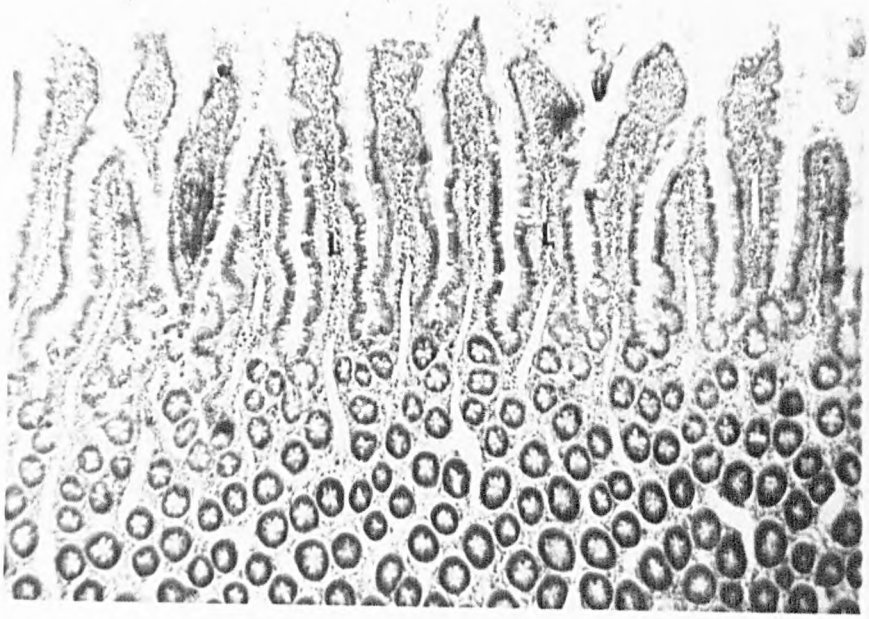
Magnification x156.

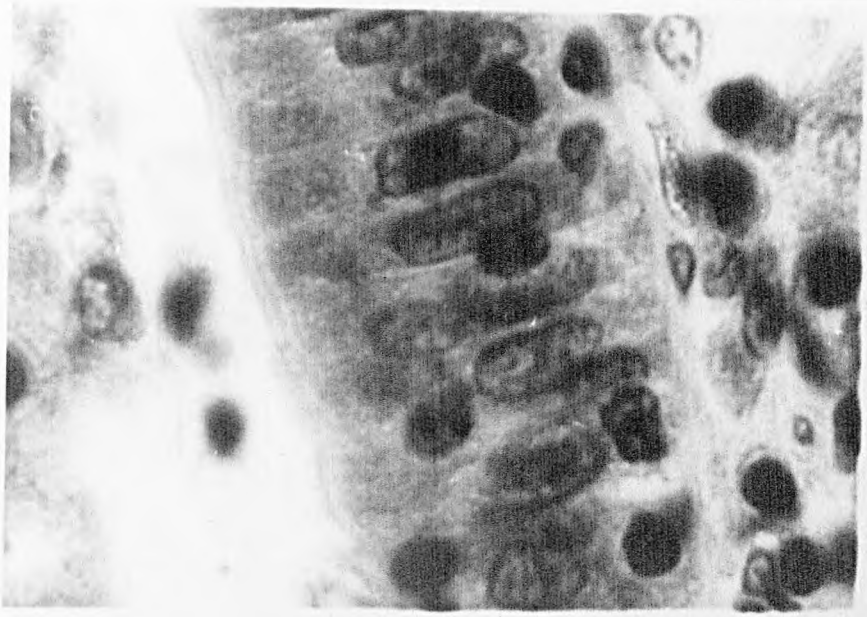
PLATE 10

High magnification light micrograph of the mid-portion of an adult rat villus following eversion and incubation at 37°C for 2 h.

Note the normal appearance of the absorptive cells. Haematoxylin and eosin.

Magnification x3900



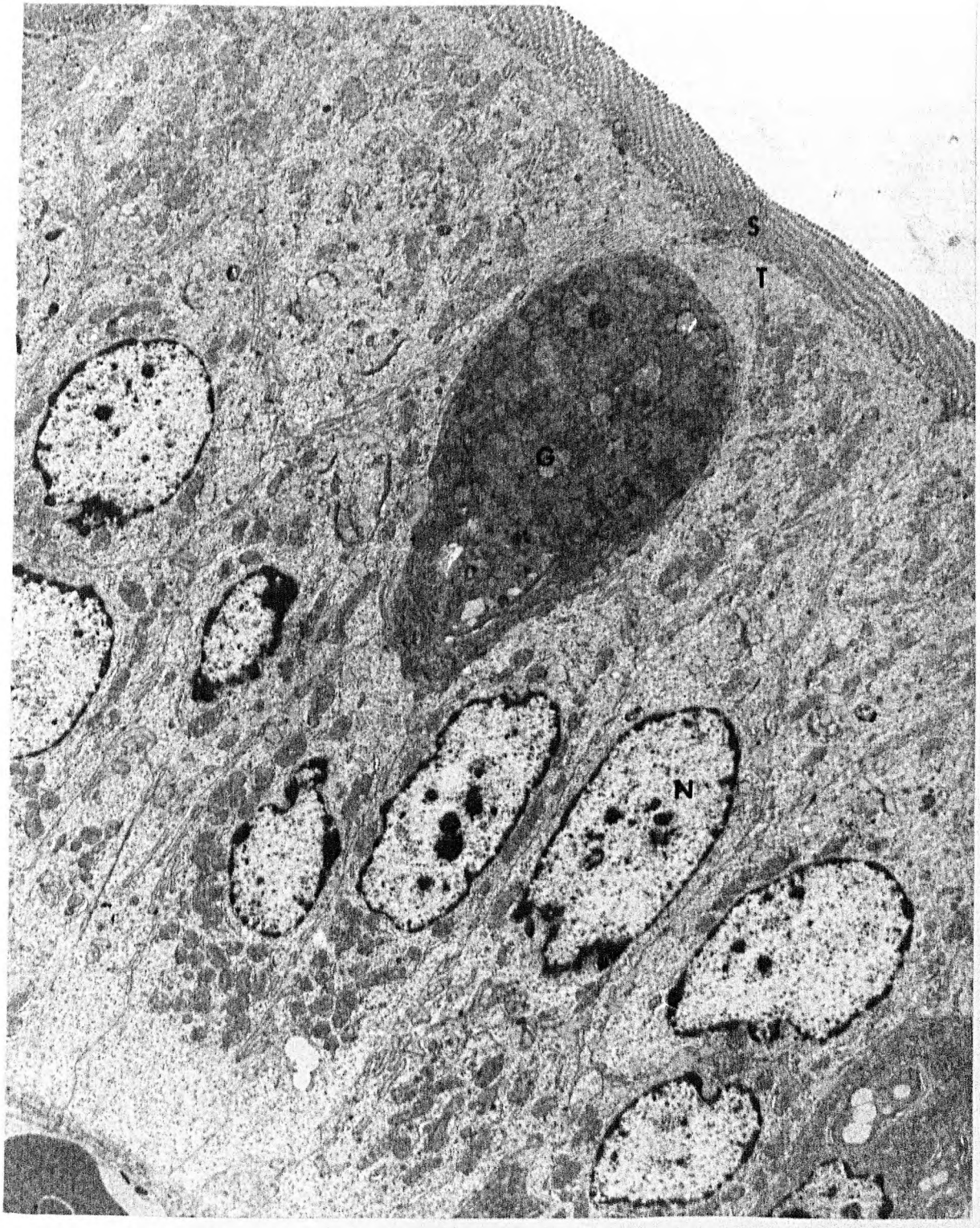


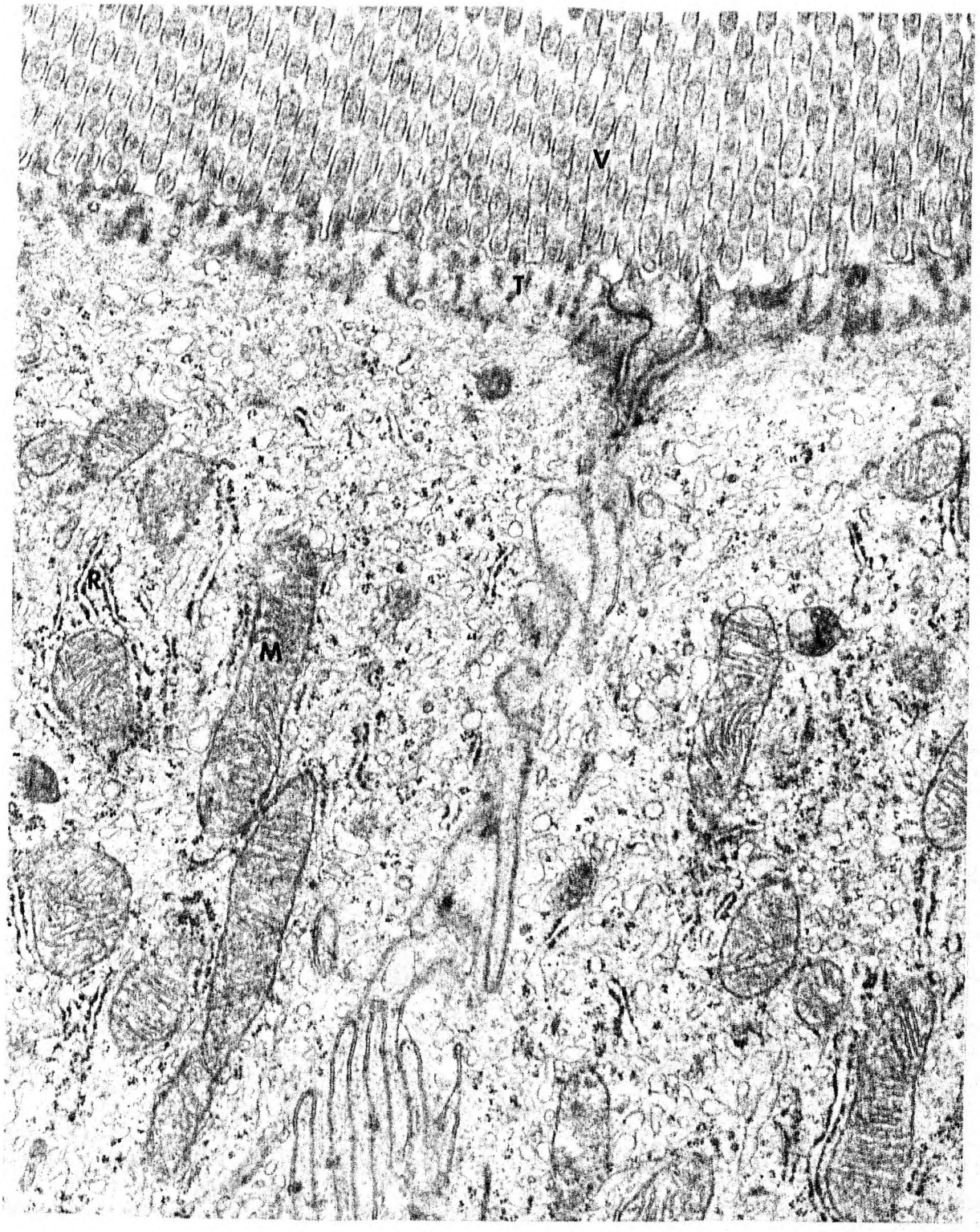
PLATES 11 - 13 Electron Micrographs of Freshly Excised Tissue

PLATE 11 Magnification 4,436. An overview of several intestinal cells located in the middle region of a single villus. Many absorptive cells and a goblet cell packed with mucus granules (G) are seen. The striated border (S), terminal web (T) and cell nuclei (N) can be identified in the absorptive cells.

PLATE 12 Magnification 25,050 shows the apical third of 2 adjacent epithelial cells shown in the previous plate. V, microvilli; T, terminal web; M, mitochondrion; L, lysosome; R, granular endoplasmic reticulum.

PLATE 13 Magnification 72,360 shows in detail the junctional complexes between 2 adjacent absorptive cells. Three types of junctional complex are clearly demonstrated: the tight junction, (T), the intermediate junction, (I) and desmosome (D).


S
T
G
N


V
J
R
M

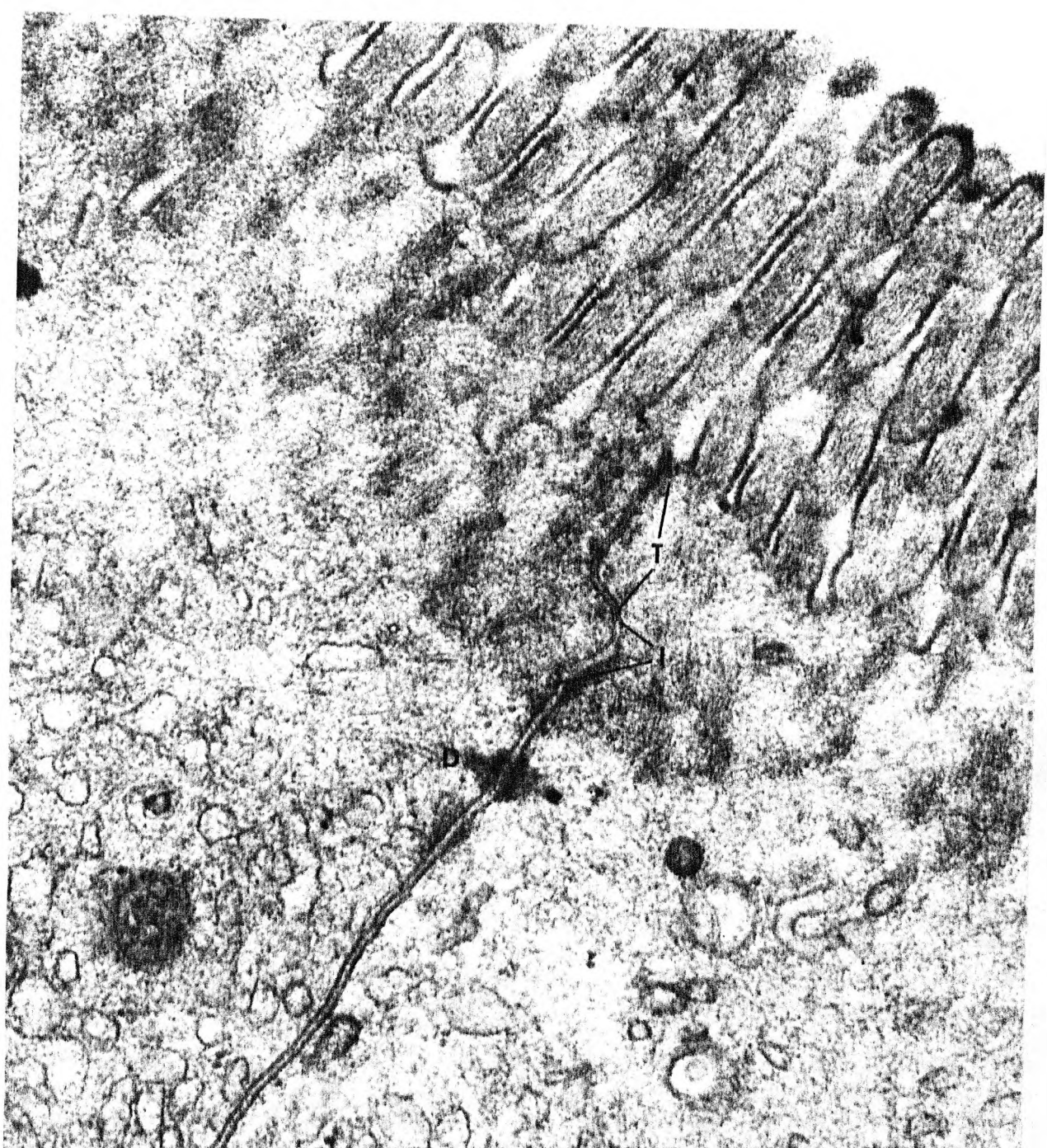

T
I
D

PLATE 14

Magnification 25,050, shows the apicial regions of absorptive cells from excised, everted intestine. General absorptive cell morphology including the microvilli (V), and junctional complex (J) appear normal.

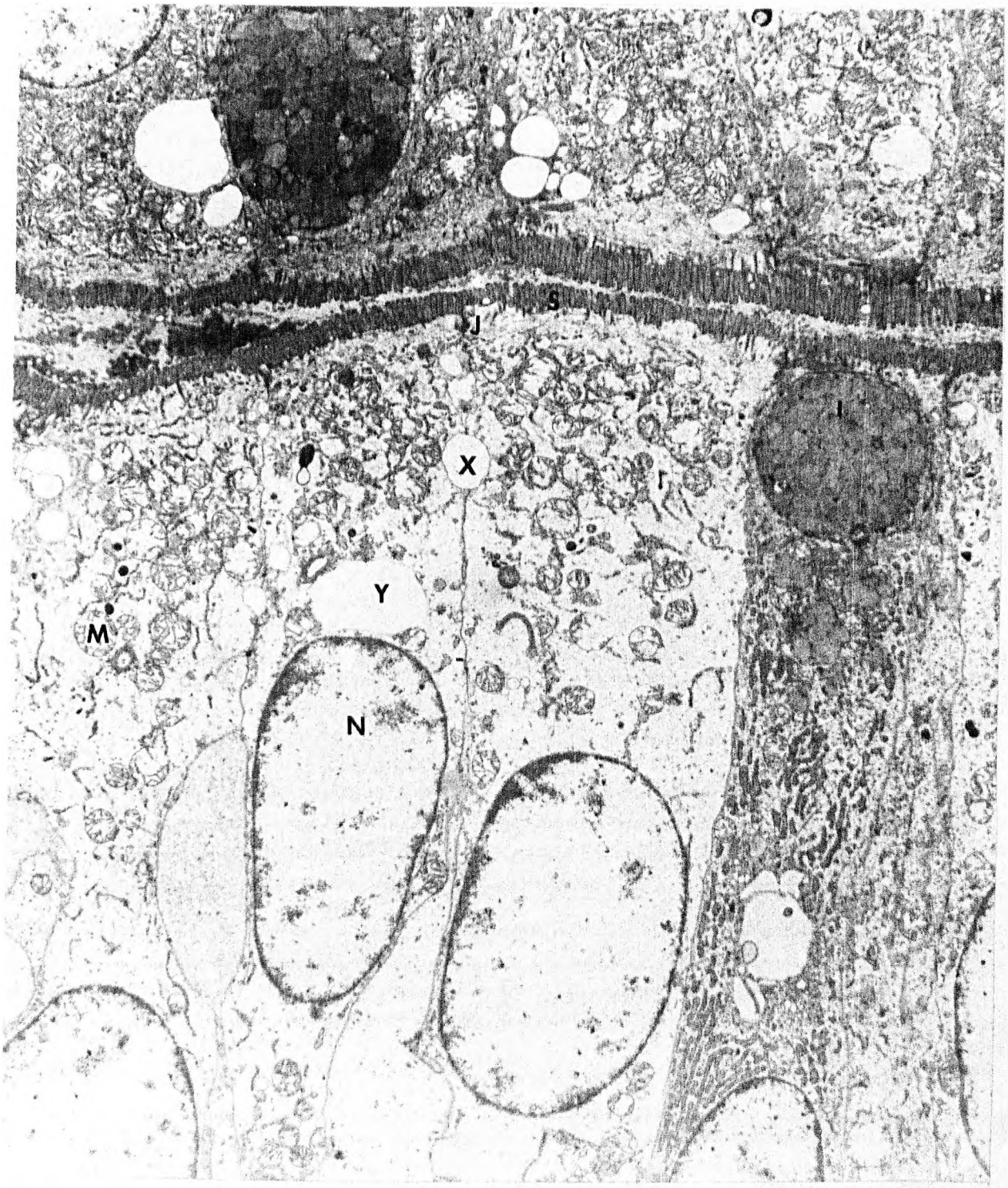
PLATES 15 - 17 Electron Micrographs of Everted Intestine Incubated for 1.5 h in Tc medium 199 Containing 10% CS and 1mM ATP.

The fixative used for electron microscopy was 0.1M glutaraldehyde/sodium cacodylate buffer pH 7.3.

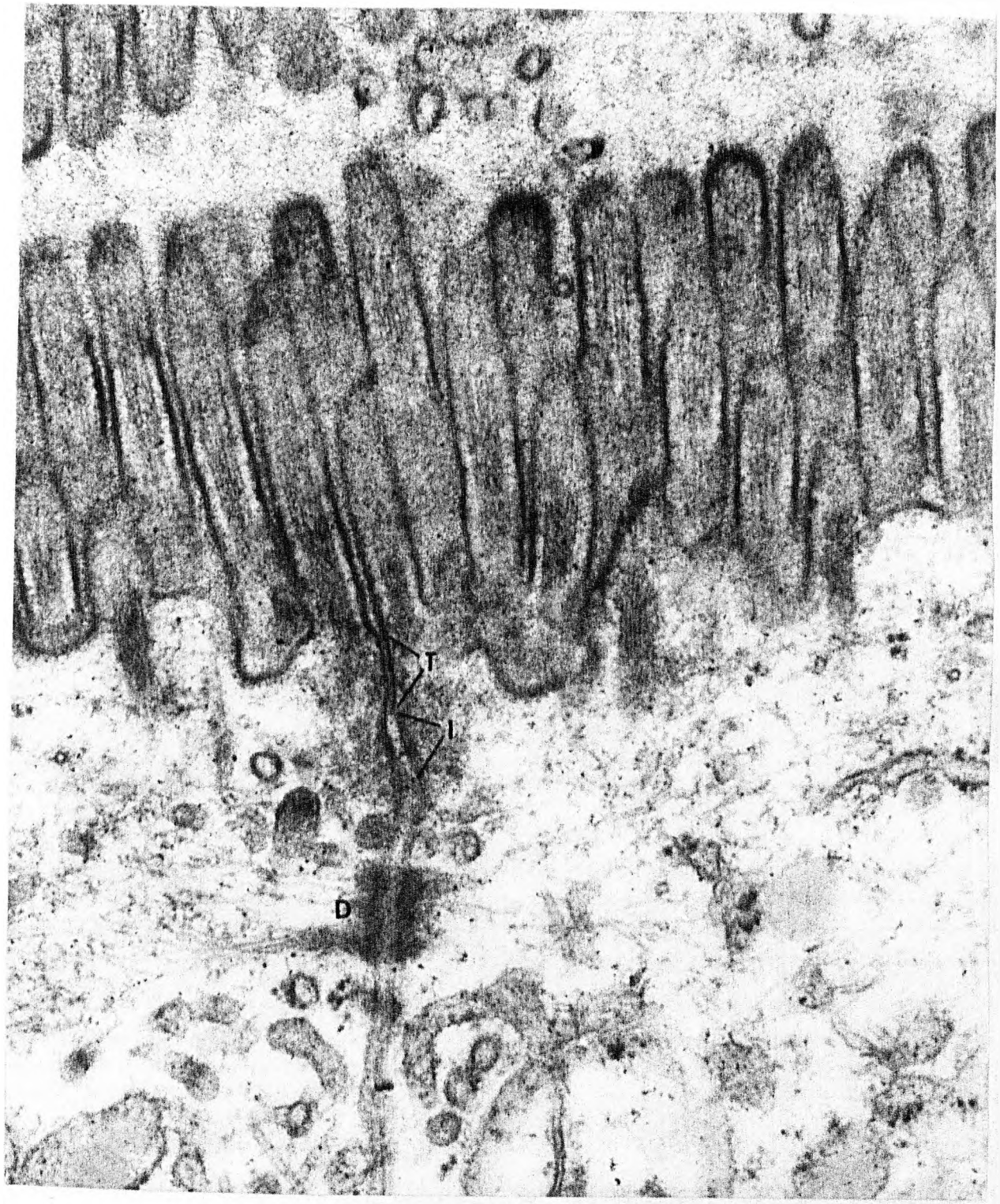
PLATE 15 Magnification 4,463, shows several intestinal cells located in the mid-villus region. The striated border (S), junctional complexes (J) goblet cell and cellular integrity appear normal. The mitochondria (M) and nuclei (N) appear swollen and there is also evidence of small intercellular (X) and intracellular (Y) lakes.

PLATE 16 Magnification 72,360, shows the apical region of two adjacent absorptive cells shown in the above plate. The three types of junctional complexes: the tight junction (T), the intermediate junction (I) and desmosome (D) all appear normal.

PLATE 17 Magnification 65,000, shows more clearly the swollen mitochondria (M) intracellular lake (Y) and intercellular lake (X) seen in the previous plate.



15


T
I
D

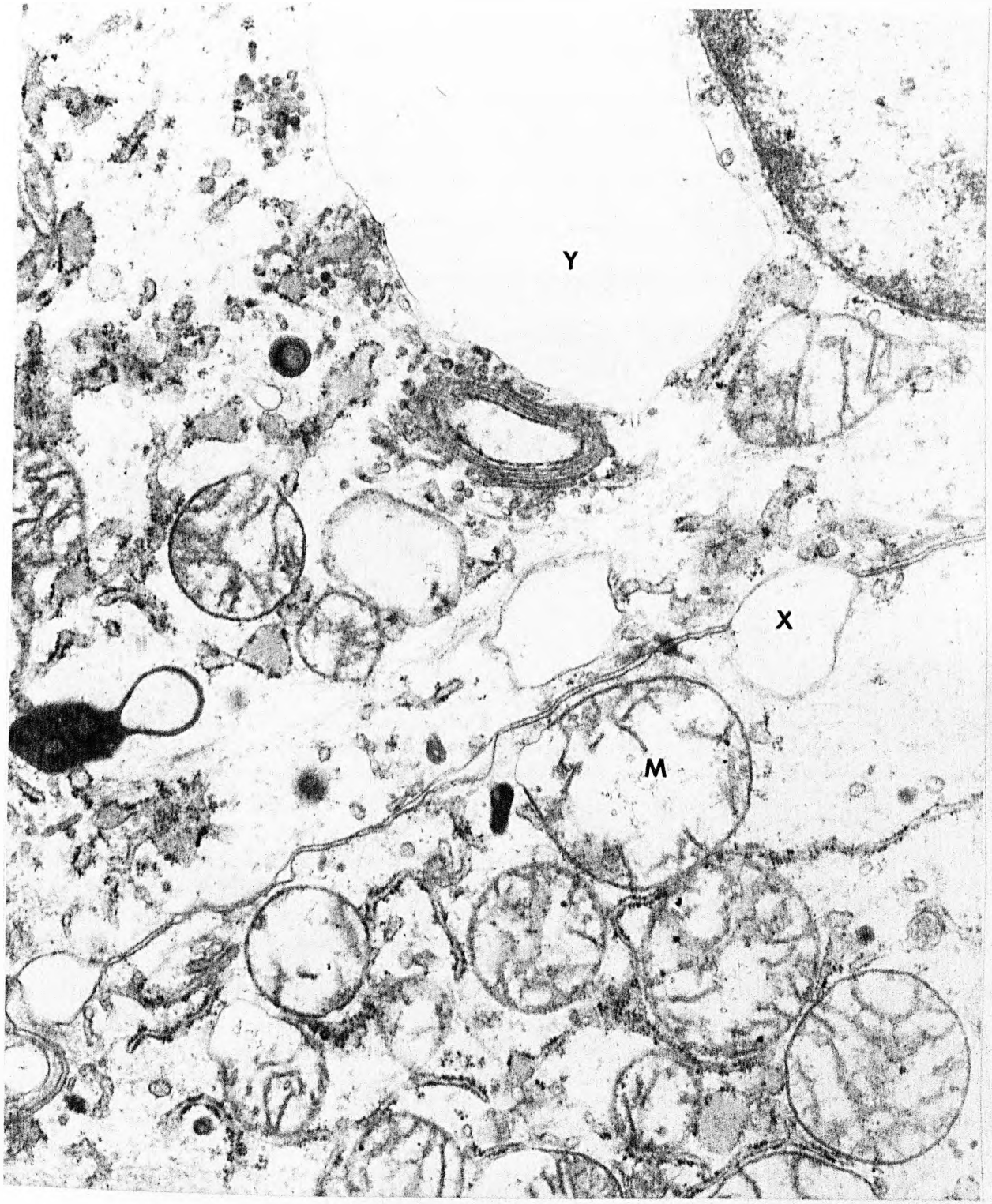
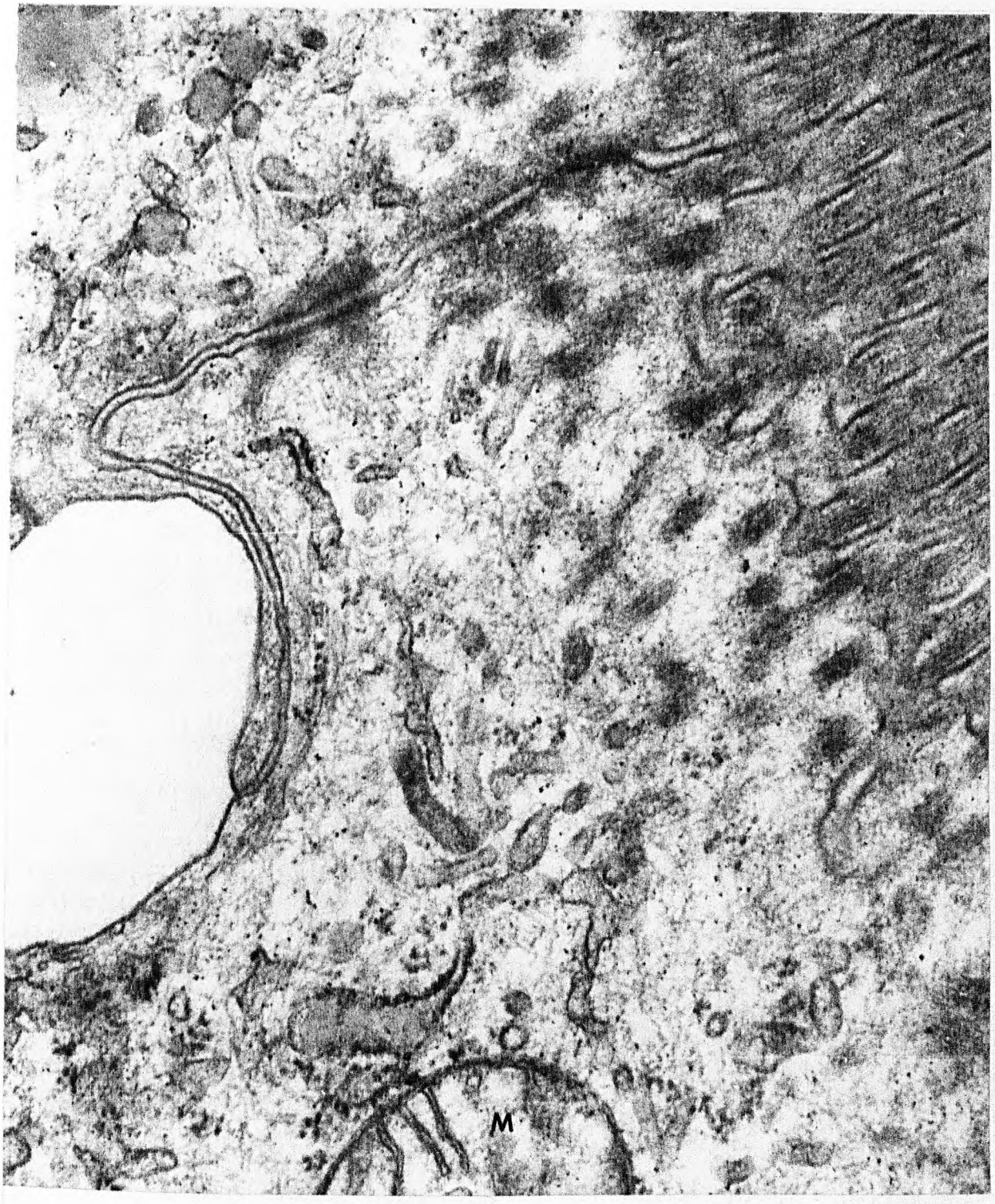

Y
X
M

PLATE 18

Magnification 72,360. An Electron micrograph of everted intestine incubated for 1.5 h in Tc medium 199 containing 10% CS and 1mM ATP. The fixative used was 0.2M glutaraldehyde/sodium cacodylate buffer pH 7.3.

This plate shows the junctional complex and surrounding cytoplasm of two adjacent epithelial cells in the mid-villus region. The cytoplasm appears dense and the visible mitochondrion (M) appears normal.


M

PLATES 19 - 22 Electron Micrographs of Cells Located at the Crypts and Villi Tips of Intestinal Tissue Incubated for 1.5 h in Tc medium 199 Containing 10% CS and 1mM ATP. The fixative used for electron microscopy was 0.1M glutaraldehyde/sodium cacodylate pH 7.3.

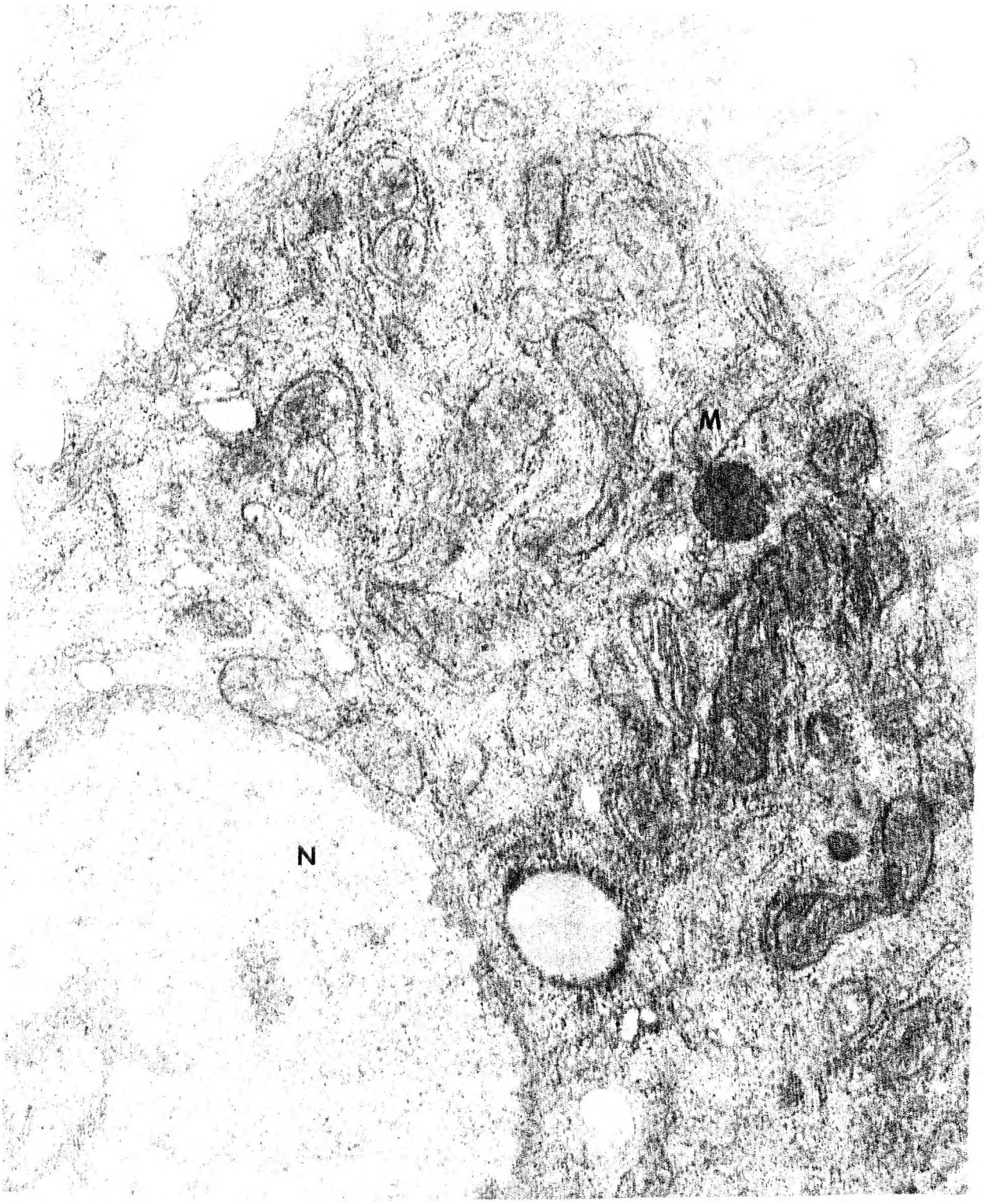
PLATE 19 Magnification 25,050, shows cells located at the tip of a single villus. The mitochondria (M) and nucleus (N) appear normal. According to Creamer (1974) the "extrusion zones" found at the villi tips are the only sites where a gap may be regularly found between cells. Note the extensive plications of adjacent lateral cell membranes.

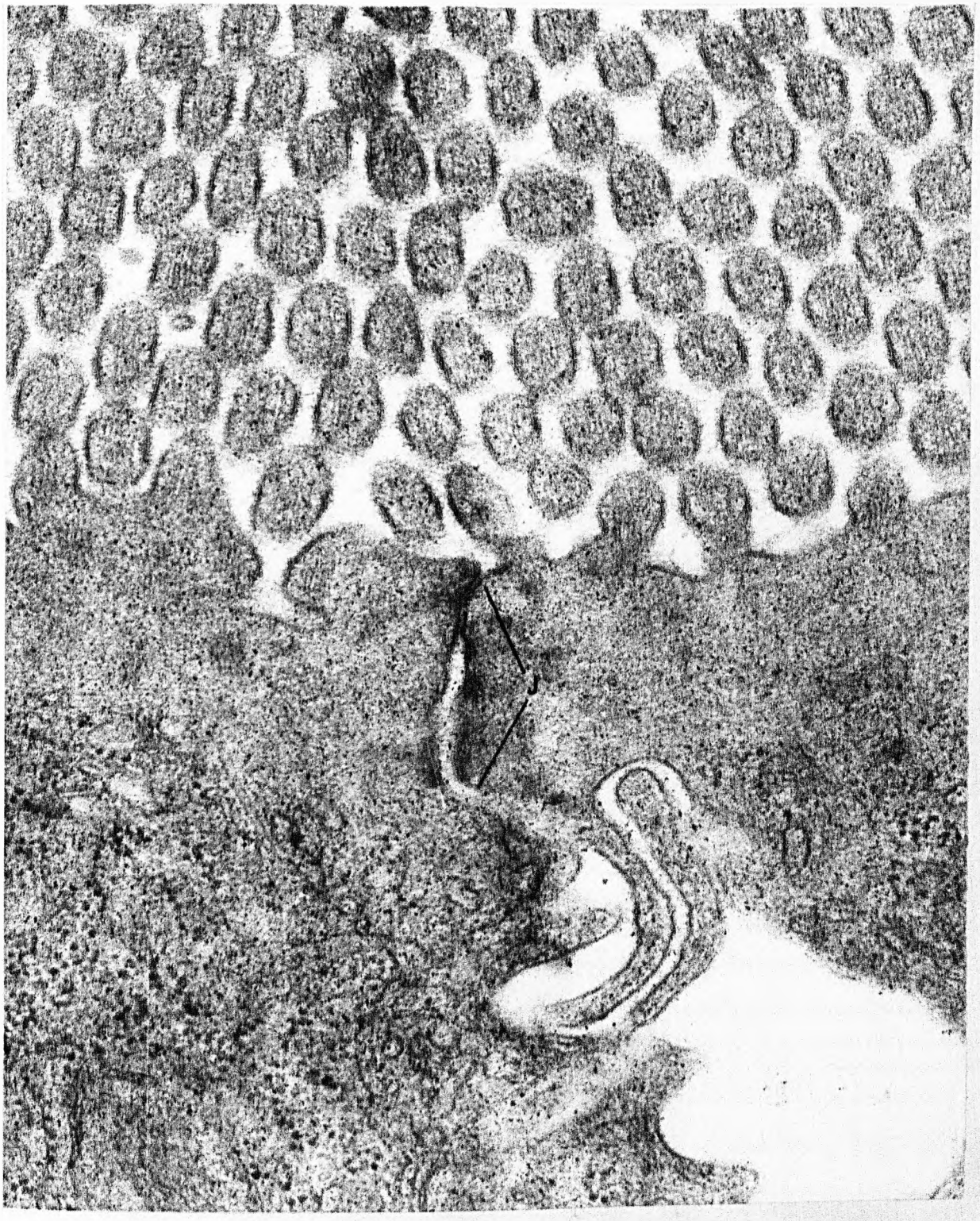
PLATE 20 Magnification 72,360, shows the junctional complexes (J) between two adjacent cells at the "extrusion zone" to be intact.

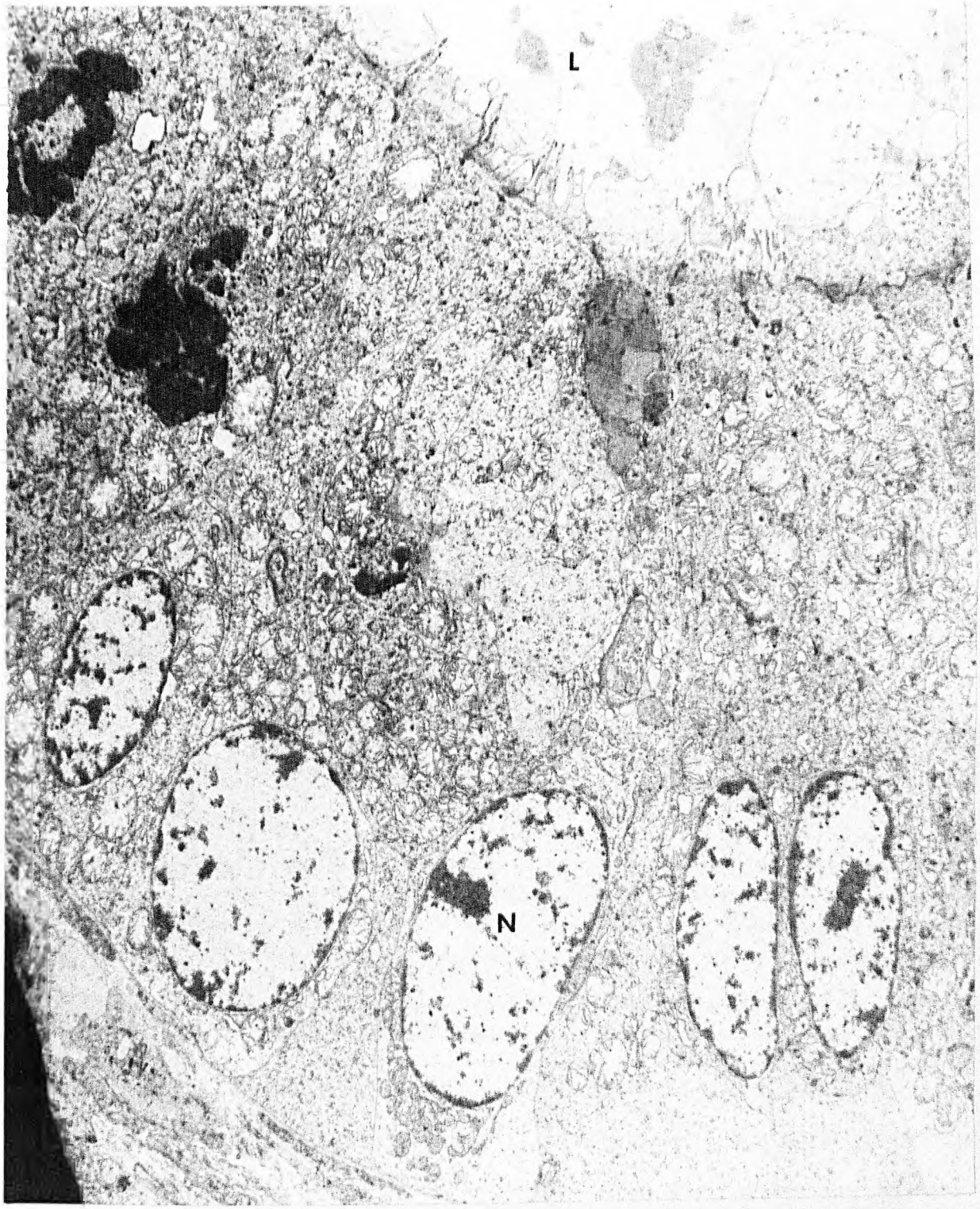
PLATE 21 Magnification 4,463, shows several undifferentiated crypt cells. The crypt lumen (L) is in the upper portion of the micrograph. Mitochondria, ribosomes and elements of endoplasmic reticulum are distributed throughout the cytoplasm. The nuclei (N) of the cells are also evident.

PLATE 22 Magnification 14,900, is a higher power micrograph of the dividing cells situated in the top left hand corner of the previous plate. This plate shows 2 undifferentiated crypt cells in mitosis. Both cells are probably in late telophase. Although the chromosomal masses (C) are present in both cells, each

PLATE 22 conts. cell is already enclosed by a complete plasma membrane. The spindle pole (SP) and spindle (S) of the left hand cell can be clearly seen.


M
N




L
N

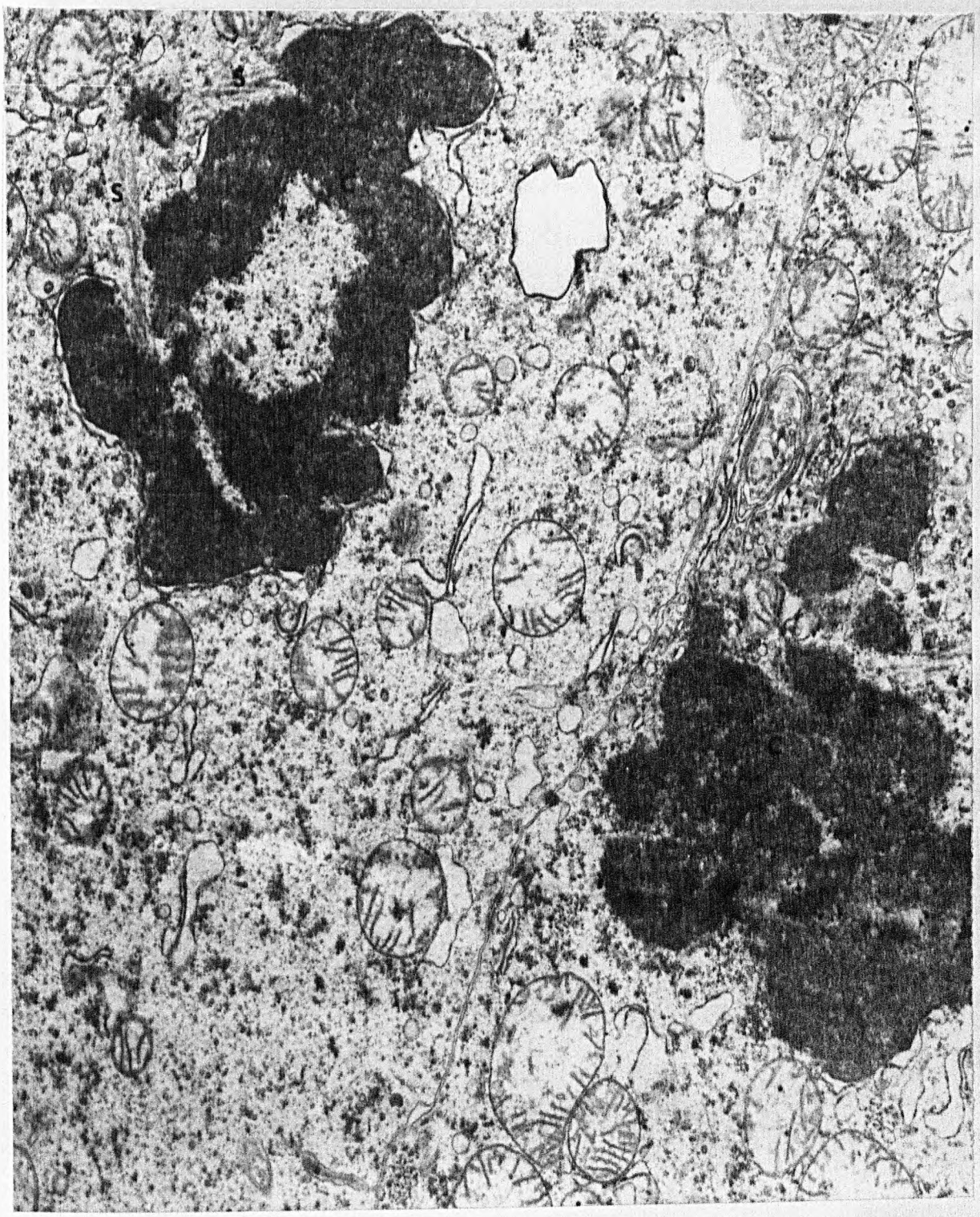
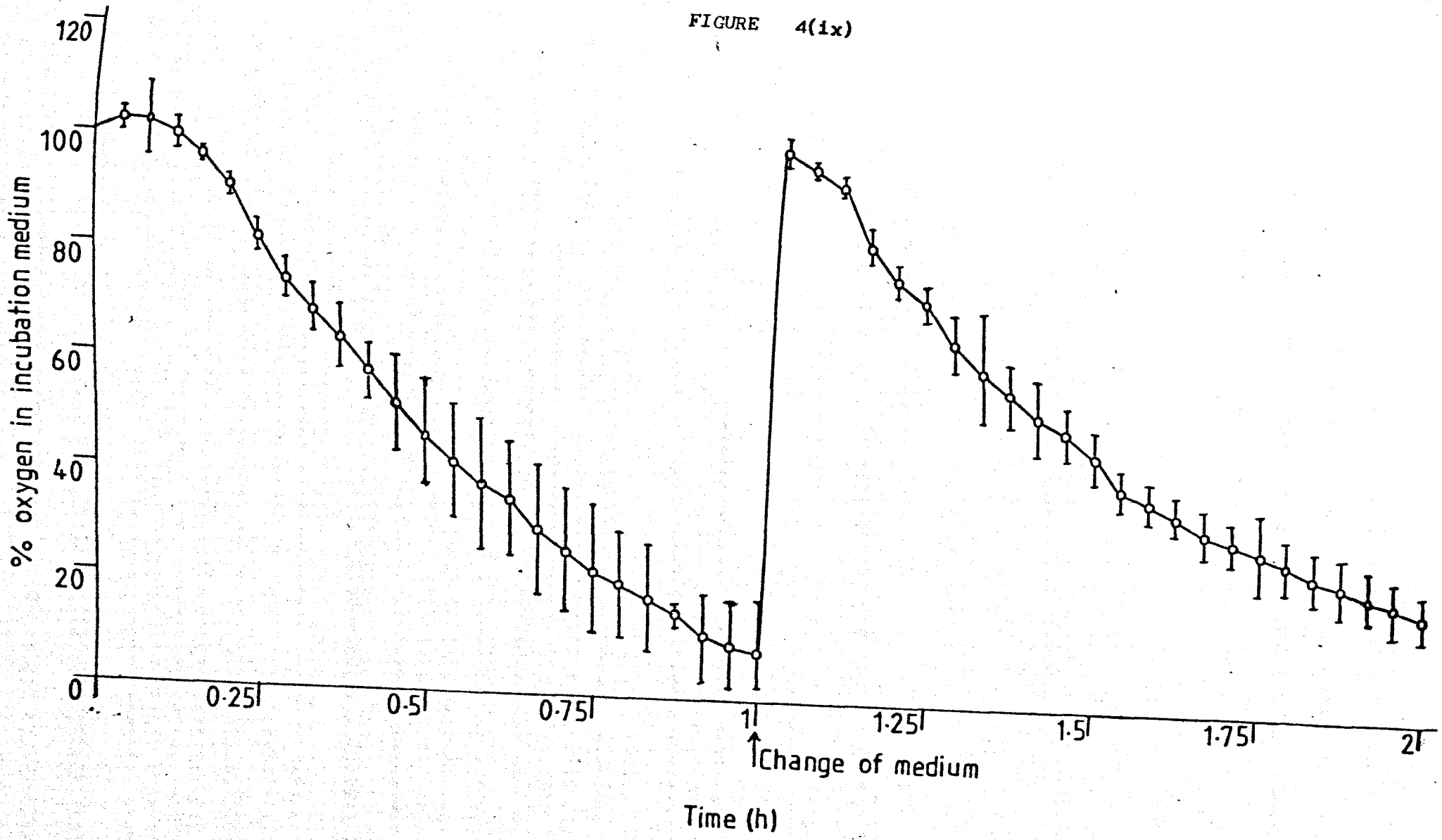

S
C

FIGURE 4(ix) Oxygen uptake by everted intestinal sacs incubated for up to 2 h in Tc medium 199 containing 10% CS and 1mM ATP

Each point is the average ± S.E. obtained from six intestinal sacs.

FIGURE 4(ix)


120
100
80
60
40
20
0
% oxygen in incubation medium
0·25
0·5
0·75
1
1·25
1·5
1·75
2
Change of medium
Time (h)

After an initial lag phase (4 min) all sacs showed a steady oxygen consumption up to 1 h when the oxygen content of the incubation medium was depleted. If the deoxygenated medium was replaced with fresh, oxygenated medium, oxygen consumption continued at a similar rate up to 2 h.

4.5.3 Conclusions

Glucose uptake showed an increasing serocal/mucosal fluid concentration ratio with respect to time up to 2 h. Glucose was, therefore, accumulated against a concentration gradient indicating no leakage of sacs had occurred. L-methionine showed a similar pattern.

Histological examination showed that the preparation procedure did not alter the gross tissue morphology and tissue incubated up to 2 h in the chosen incubation medium retained its structural integrity well. Electron microscopy revealed that the preparation procedure had no effect on the ultrastructural morphology of any epithelial cells. After incubation, tip and crypt cell morphology remained normal but the mitochondria and nuclei of the mid-villus epithelial cells became susceptible to osmotic changes in the cacodylate fixative.

The consistent rate of oxygen uptake by the jejunal sacs showed that the sacs were viable throughout the experimental period. Fischer and Parsons (1949) pointed out that adequate oxygenation was essential for the full activity of tissue, incubated *in vitro*, therefore to ensure adequate oxygenation it was thought justifiable to re-gas the incubation medium after 1 h of incubation. This additional gassing was included in all future experiments.

4.6 Discussion

For incubations up to 2 h, the results indicated that as the incubation medium used became more complex, the rate of tissue accumulation of [^{125}I]-PVP decreased while rates of accumulation of substrate in the serosal fluid did not differ appreciably no matter which medium was used (Krebs Improved Ringer II is a basic medium, Tc medium 199 a complex medium; for medium constituents see Appendix 1).

The decrease in tissue uptake exhibited by intestinal sacs incubated in the complex media may be due to two factors: i) tissue viability was better in these media which would minimize substrate diffusion into the tissue due to cytolysis, ii) complex media such as Tc medium 199 contain substances which could compete with the substrate for uptake.

Tc medium 199 simulates *in vivo* physiological conditions more closely than Krebs Ringer in that a large variety of substances are presented at the absorptive surface for uptake. Therefore macromolecules will not be ingested solely because they are the only substrate available. It is for this reason, and the fact that tissue viability was maintained better in Tc medium 199, especially when 10% CS was present, that Tc medium 199 containing 10% CS was chosen as the incubation medium for all future experiments.

Extension of the incubation period beyond 2 h led to greater variability between individual experiments and gross tissue breakdown. Therefore for future experiments, the incubation time was restricted to 2 h.

In Tc medium, 199 and 10% CS, tissue viability was good but reproducibility of uptake data was reduced compared with that obtained from experiments performed in the absence of CS. The reasons for this loss of reproducibility may lie with the chemical composition of [^{125}I]-PVP itself. Weese (1949) showed PVP to have coiled chain-like molecules. Like proteins it contains $-\overset{O}{\overset{\|}{C}}-N$

groups which can attract other molecules by the electrostatic attraction of polar nitrogen and carboxyl oxygen atoms. The macromolecular structure is shown in Fig. 4(x).

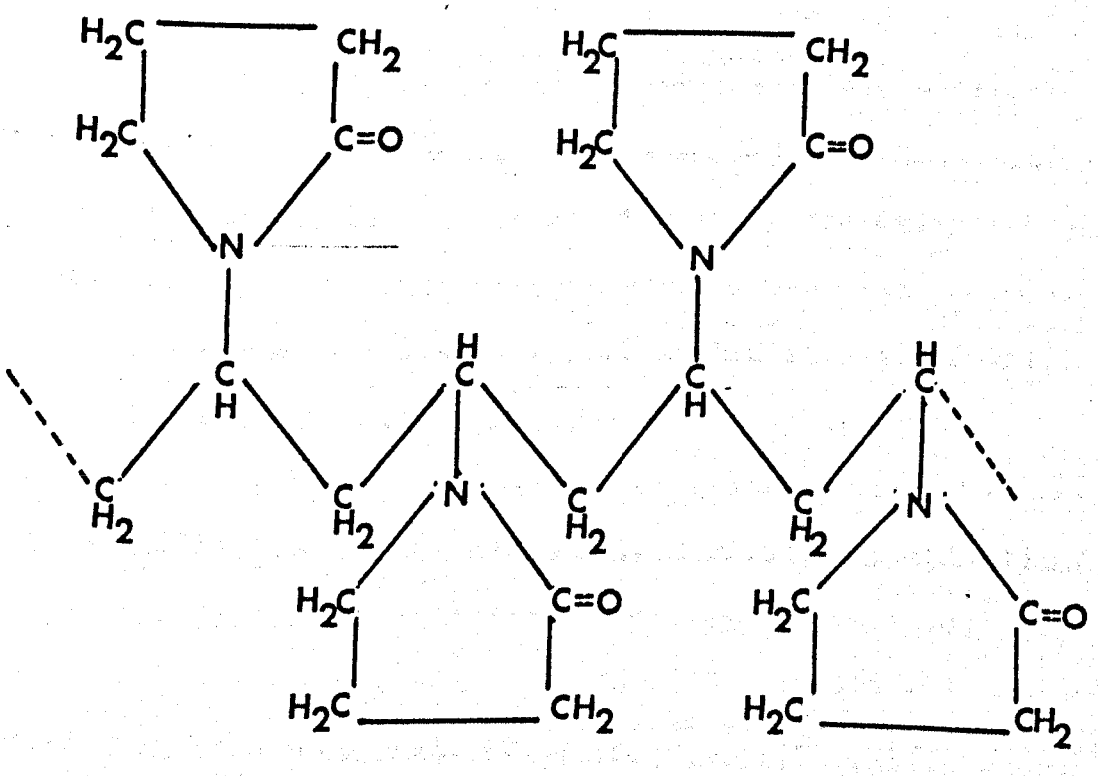
The site of the [^{125}I]-label is not known. Thus it is possible that [^{125}I]-PVP could be binding to serum proteins or other appropriately charged groups present in CS, altering the size of the PVP molecule. Macromolecules of varying size may be accumulated at different rates, hence loss of reproducibility would occur. In support of this Millard (1977 - personal communication) showed by TCA protein precipitation that more [^{125}I]-PVP could be precipitated from solution in the presence of CS (about 85%) than when [^{125}I]-PVP was present alone. This suggested interaction of CS and [^{125}I]-PVP in some way which made [^{125}I]-PVP more susceptible to precipitation.

Because each batch of [^{125}I]-PVP may vary it was thought prudent that all experiments and controls in any one series be carried out with the same batch of [^{125}I]-PVP.

When exogenous ATP (up to 2 mM) was added to the Tc medium 199 as well as 10% CS, tissue uptake increased serosal fluid accumulation decreased and reproducibility for both tissue and serosal fluid accumulation improved. One explanation is that the [^{125}I]-PVP has a high affinity for the highly polar ATP molecules, particularly the P-O groups, hence binding of PVP to other molecules is prevented, reducing macromolecular size and tissue binding and improving reproducibility.

There is some dispute as to whether exogenous ATP can contribute directly to the energy requirements of the cell. Bronk and Leese (1973) showed that ADP could contribute directly, but ATP could only contribute indirectly. They also demonstrated the presence of adenylate kinase (myokinase) in the medium after <u>in vitro</u> incubations. This enzyme catalyses the reaction:-

$$AMP + ATP \leftrightarrow ADP + ADP$$



Structure of PVP

FIGURE 4(x)

and is thought to leak from the tissue during incubation. Bronk et al. (1973) postulated that ADP formed in this way could fulfil part of the tissues energy requirements in vitro. On the other hand Stewart (1978) showed that although ATP undergoes hydrolysis at the microvillar membrane surface to yield, progressively, ADP, AMP and adenine, at low concentrations of nucleotide (less than 3 mM) there is carrier mediated transport of ATP and phosphorylation products. Hence ATP is taken up intact and contributes directly to the cells energy requirements. Above 3 mM, ATP and its hydrolysates appear to be cytotoxic. Thus when exogenous ATP is added to the medium it may contribute directly or indirectly to the energy available to the intestinal cells or simply prevent further diffusion of the cells own ATP. Whatever the case, the increased reproducibility produced by its addition to the incubation medium justifies its use in future experiments. The results in this study supported the findings of Stewart (1978) in that the higher concentration of ATP used (5 mM) appeared to be cytotoxic to the tissue.

The reason for the reduction in the rate of accumulation of substrate in the serosal fluid when exogenous ATP was present is unclear from this study. The mechanism of transfer of the substrate macromolecule from the tissue to the serosal space may be involved and this is discussed further in Section 9.

Once a standard incubation method and medium had been elucidated, several methods of assessment of tissue viability were employed. Both D-glucose and L-methionine were accumulated against a concentration gradient. These gradients could be maintained up to 2 h which indicated that no leakage of sacs had occurred in this time. The amounts of glucose which disappeared from the medium were considerably greater than the amount transferred to the serosal fluid. This observation agrees with the findings of Wilson (1956) who found that about 75% of the glucose which disappears from the medium is converted to lactic acid; this leaves less

than 25% to be transferred and measured as serosal accumulation. The uptake pattern of L-methionine was also examined in the system. No L-methionine disappeared from the medium whereas the amount in the serosal fluid increased, thus a concentration gradient was formed and maintained up to 2 h. That no L-methionine seemed to disappear from the medium suggested it was being replenished, possibly by amino-acid release from digested calf serum proteins. This would also explain the increased concentration gradient observed in this study compared to that obtained by Wiseman (1955), i.e. 2.28 and 1.18 respectively. Digestion of calf serum proteins could have been by enzymes attached to the brush border (Gray, 1975) and/or membrane contact digestion (Ugolev, 1955).

Light microscopy showed that tissue incubated for up to 2 h retained its gross structural integrity well. Electron microscopy revealed that after 1.5 h incubation, the ultrastructure of all cell types was normal. The evidence of cell division in the crypts suggested that the structural integrity was actively maintained. However after incubation, the mitochondria and nuclei of the mid-villus epithelial cells became more susceptible to osmotic effects from the fixative buffer. This suggested that in this region of the villi, the osmotic pressure of the cellular organelles had changed due to incubation, though not enough to disrupt the cell structure. The osmolarity of these organelles may have increased as they became more susceptible to fluid uptake from the fixative buffer. According to Plattner et al. (1970) cellular fluid absorption is responsible for changes in cellular structure and cell membrane ruptures during in vitro incubations. Tc medium 199 is a commercial medium designed to match the tonicity of most cells. It is probable that the tonicity of this medium more closely matches that of the cells than does Krebs Ringer hence cellular fluid uptake due to osmotic differences between cell and medium is reduced and in the case of the mid-villus organelles, even reversed! There is also some evidence to suggest that the intestinal mucosa is preferentially nourished from the lumen rather than the bloodstream (Hirschfield and

Kern, 1969). If this is the case, mucosal nutrition would be more complete in the complex Tc medium 199 than in the simpler Krebs Ringer medium, hence tissue viability and structural integrity may be enhanced in this way.

Oxygen uptake by jejunal sacs was constant over the 2 h incubation period. This suggested that the sacs were viable, although it was thought prudent to introduce a re-gassing stage at 1 h into the incubation procedure.

In summary, the primary aim of the investigations carried out in this chapter was to determine whether an *in vitro* preparation of intestine of adult rats could be useful in examining the transport of macromolecules. An *a priori* consideration of such a preparation suggests that certain conditions must be satisfied if the results of macromolecular transport are to have any value; the tissues of the intestinal wall must be viable throughout the experimental period and there must be complete integrity of the wall separating mucosal and serosal compartments. The histology, glucose transport and methionine transport as well as the oxygen uptake data confirmed these criteria in this system.

CHAPTER FIVE

[^{125}I]-PVP UPTAKE IN EVERTED AND NONEVERTED GUTSACS AND THE KINETICS OF [^{125}I]-PVP UPTAKE BY EVERTED INTESTINAL SACS

5.1 Introduction

By definition, the transport of a molecule across a membrane is passive if the rate of transport is directly proportional to the electrochemical or concentration gradient. The proportionality constant, the permeability constant, depends on the nature of the molecule and the structure of the membrane. Consequently permeability measurements yield information about permeation mechanisms and membrane structure. Active transport is defined as the energy dependent transport of solute against concentration and electrical gradients.

In this section, the accumulation in the tissue and serosal fluid of the non-degradable macromolecule [^{125}I]-PVP was assessed in the presence and absence of a concentration gradient using everted and non-everted intestinal sacs to determine whether uptake was an active process.

Once an active process had been implicated the magnitude of the EI can give a good indication of the mechanism of accumulation (Pratten *et al.*, 1979 - see Section 1.4.3). In the case of a substrate that enters entirely in the fluid phase, an increase in concentration in the extracellular medium is without effect on uptake expressed as µl per unit of tissue per hour, although uptake expressed as ng increases linearly with concentration. A substrate entering by adsorption, however, does so in a concentration dependent manner. As the substrate concentration increases the EI (µl/h/mg protein) increases. Once the sites to which it binds become saturated, the EI will begin to decrease. Therefore the mechanism of macromolecular accumulation by adult intestine was assessed by increasing the substrate concentration in the medium and noting the effect on uptake expressed both in µl and ng/h/mg protein. This is also described in this section.

5.2 Uptake of [^{125}I]-PVP Against a Concentration Gradient in Everted and Non-Everted Jejunal Sacs

5.2.1 Method

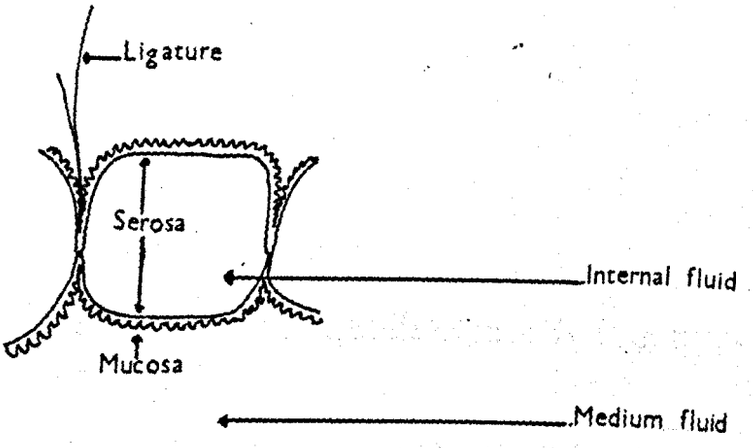
Everted and non-everted jejunal sacs were incubated in Tc medium 199 containing 10% CS and 1 mM ATP (see Chapter 3.6 for method). To ensure adequate oxygenation of non-everted sacs during incubation, a small bubble of oxygen was introduced into the fluid inside the sacs. [^{125}I]-labelled PVP (Batch no. 159 BA) at a concentration of 2 µg/ml was used as substrate. Unlabelled PVP of the same average molecular weight (PVP - 40, average molecular weight 33,000) and concentration (2 µg/ml) was placed in the fluid inside the sacs to equilize the concentration of PVP on both sides of the intestine. Incubations were terminated at regular intervals up to 2 h. Radioactivity in the medium, internal fluid and gut sac tissue was assessed and the protein content of each gut sac determined. Accumulation of substrate was plotted against time for each set of data and the related EI calculated. Fig. 5(i) shows a diagrammatic representation of these experiments.

It is possible that during incubation of [^{125}I]-PVP substrate with intestinal sacs, some de-iodination of substrate may occur. To examine this possibility, dialysed [^{125}I]-PVP (48 h in 0.9% NaCl) was incubated in the absence and presence of everted intestinal sacs. The character of the radioactive species present in the medium serosal fluid and intestinal tissue after 2 h was investigated by Sephadex G 25 column chromatography (see Section 2.10). Tissue and serosal fluid accumulation of free [^{125}I]-iodide (substrate concentration 2 µg/ml) was assessed using the everted intestinal sacs and the standard incubation procedure.

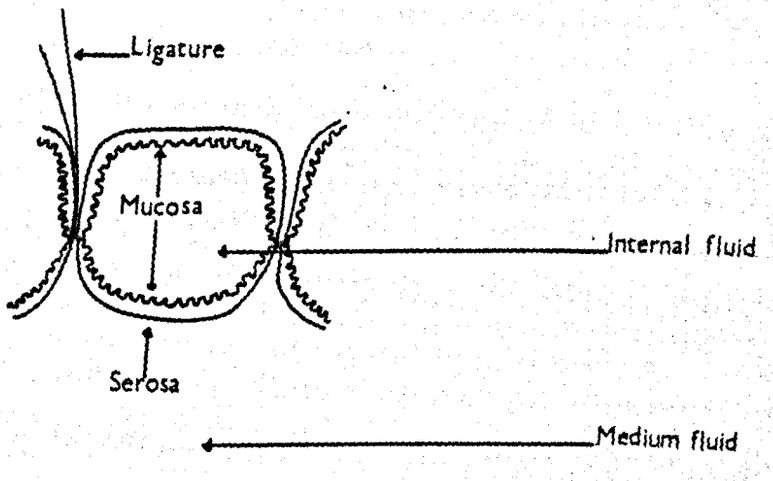
5.2.2 Results

Figs. 5(ii) and 5(iii) show the rates of accumulation of substrate in the tissue and sac internal fluid respectively for everted and noneverted

a) Everted



b)Non-everted



Diagrammatic representation of everted and non-everted intestinal sacs.

FIGURE 5(i) a and b

FIGURE 5(ii) Accumulation of radioactivity ± S.E. in the tissue of:

a) Everted intestinal sacs with no PVP in the internal fluid.

b) Everted intestinal sacs with PVP (2 µg/ml) in the internal fluid.

c) Non-everted intestinal sacs with no PVP in the internal fluid.

d) Non-everted intestinal sacs with PVP (2 µg/ml) in the internal fluid.

In all cases [^{125}I]-PVP (2 µg/ml) was used as substrate.

Broken lines indicate computer-fitted regression lines.

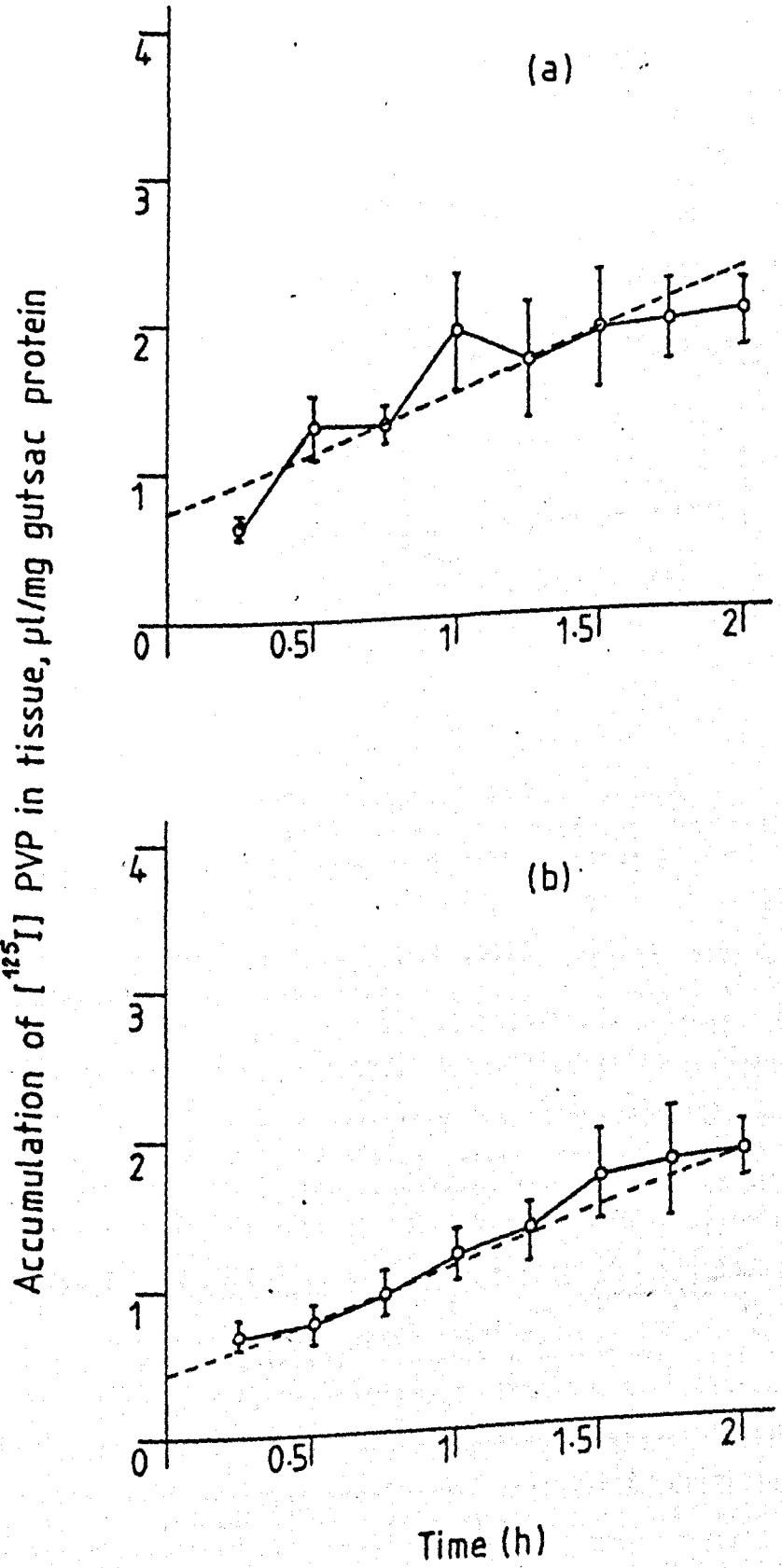


FIGURE 5(ii) a and b

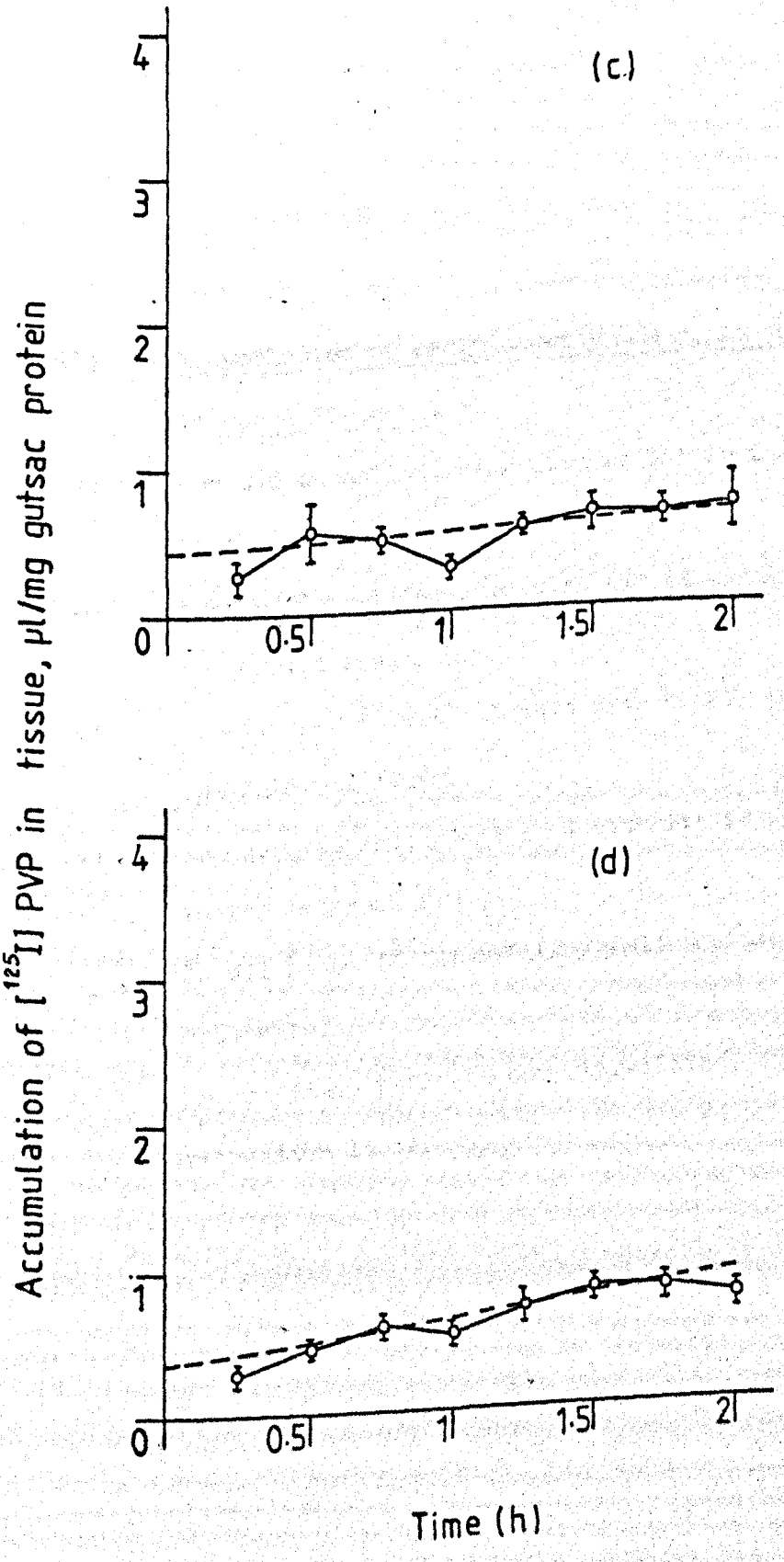


FIGURE 5(ii) c and d

FIGURE 5(iii) Accumulation of radioactivity ± S.E. in the internal fluid of:

a) Everted intestinal sacs with no PVP in the internal fluid.

b) Everted intestinal sacs with PVP (2 µg/ml) in the internal fluid.

c) Non-everted intestinal sacs with no PVP in the internal fluid.

d) Non-everted intestinal sacs with PVP (2 µg/ml) in the internal fluid.

In all cases [^{125}I]-PVP (2 µg/ml) was used as substrate.

Broken lines indicate computer-fitted regression lines.

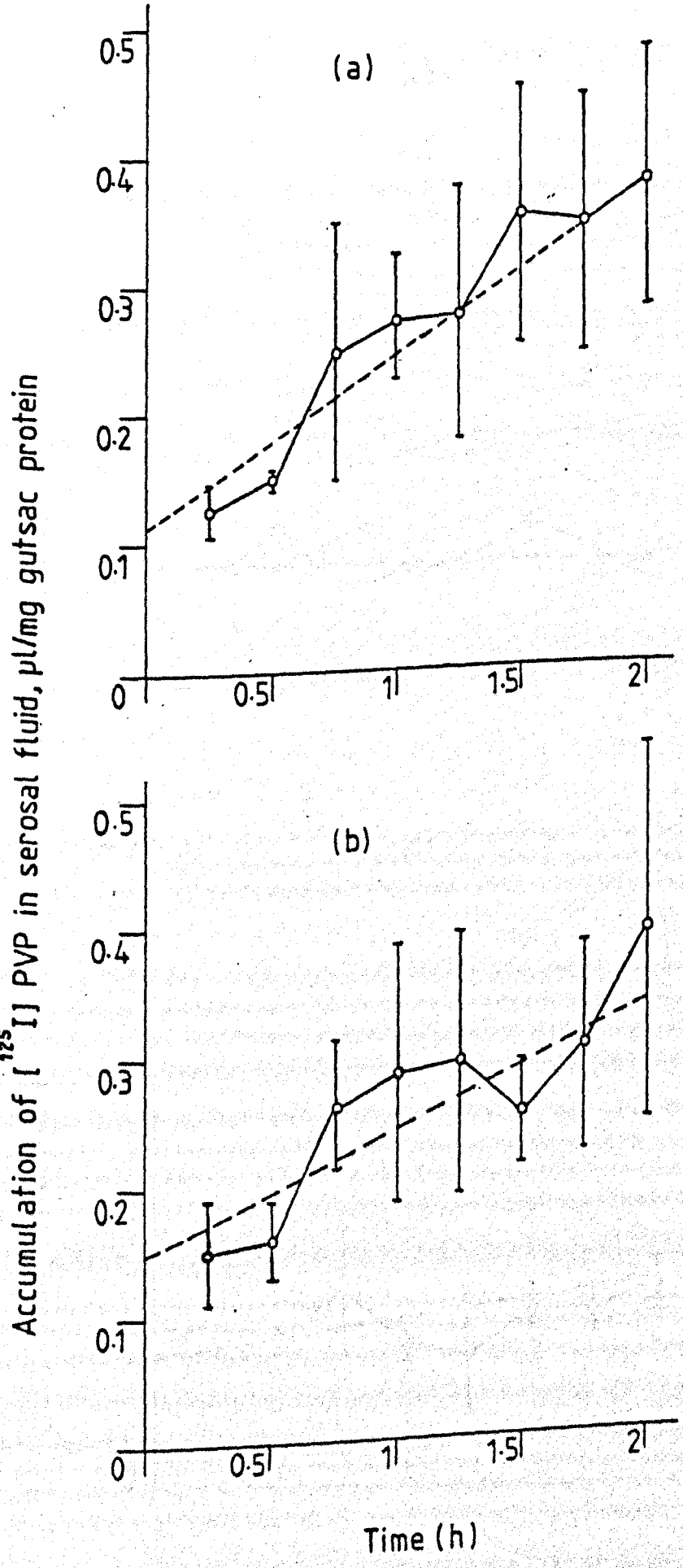


FIGURE 5(iii) a and b

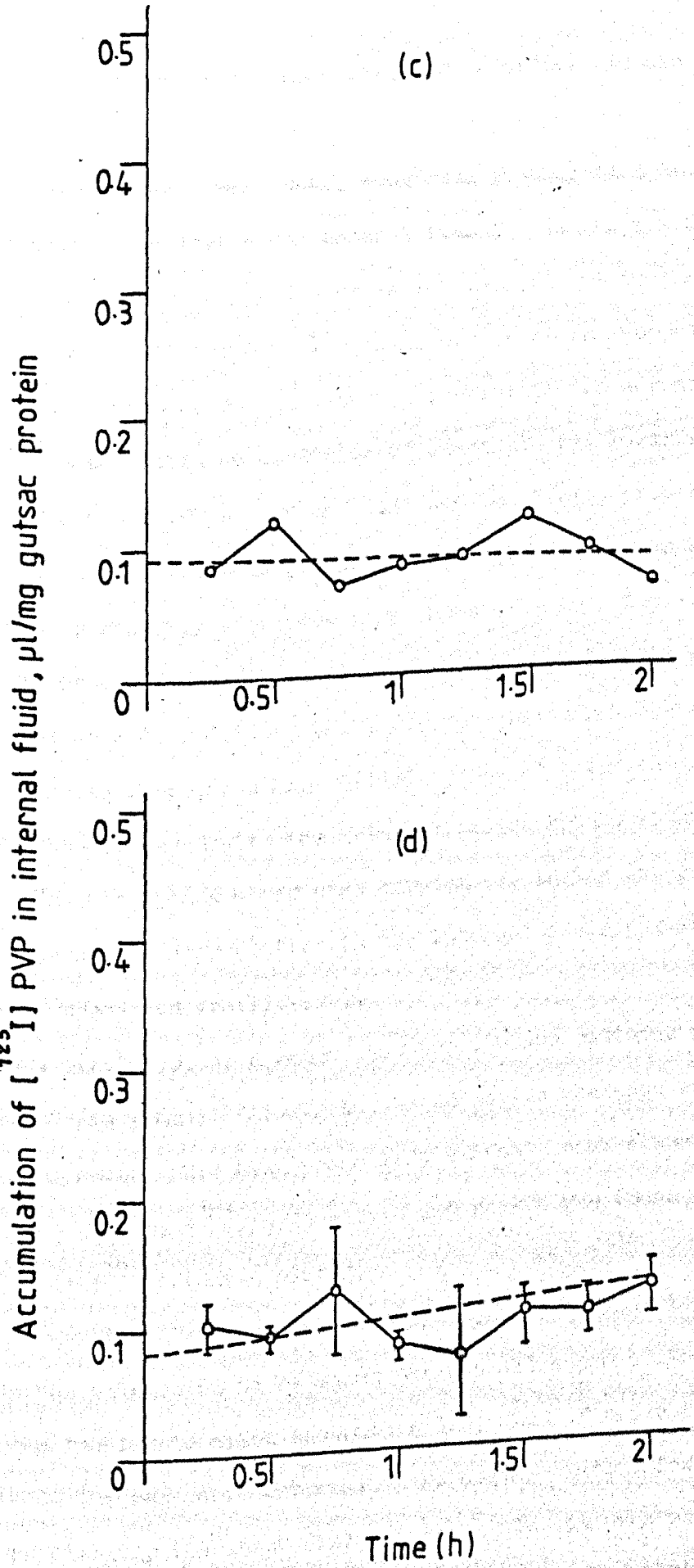


FIGURE 5(iii) c and d

gut sacs. The mean EI's and correlation coefficients are shown in Table 5.1.

When everted sacs were used, substrate accumulation increased linearly with time for both tissue and serosal fluid. The EI's for tissue uptake were similar whether non-radioactive PVP was present in the serosal fluid or not (0.69 $\pm$ 0.09 and 0.76 $\pm$ 0.09 μl/h/mg protein respectively). However, when non-radioactive PVP was present in the serosal fluid, the rate of serosal fluid accumulation of substrate was lower than when no PVP was present (0.07 $\pm$ 0.02 and 0.12 $\pm$ 0.03 μl/h/mg protein respectively).

The substrate accumulation patterns obtained when non-everted sacs were used were appreciably different from those obtained with everted sacs. Substrate accumulation did not increase perceptively with time for either the tissue or internal fluid (N.B. for non-everted sacs the internal fluid is the mucosal fluid, see Fig. 5(i)b). The EI's for tissue uptake were reduced when compared with the EI's for tissue uptake obtained using everted sacs. In fact, non-everted sacs without PVP in the internal fluid showed no nett substrate accumulation in the internal fluid (exhibited by a negative regression coefficient).

Figs. 5(iv)a, b, c, d and e show the elution patterns from Sephadex G 25 - 80 of dialysed [^{125}I]-PVP incubated in the presence of everted intestinal sacs. Two main peaks were evident, peak A was present in the void volume, where [^{125}I]-PVP eluted and peak B was found in the same position as [^{125}I]-iodide eluted.

The mean total recovery for [^{125}I]-labelled radioactivity from the columns was calculated to be about 58% for all experiemnts. This recovery value was too low to allow accurate calculation of separate radioactive species in the recovered material. Percentage recoveries could only be improved slightly (to about 60%) by pre-eluting columns with cold PVP. Qualitatively, the graphs (Figs. 5(v)a - e) indicated that no deiodination took place when the dialysed [^{125}I]-PVP was incubated in the absence or

TABLE 5.1

Accumulation of Substrate in Tissue and Internal Sac Fluid of Everted and Non-Everted Intestinal Sacs Incubated for 2 h with [^{125}I] -PVP as Substrate

Form of Experiment	No. Expts.	Slope of tissue accumulation of radioactivity µl/h/mg protein	Correlation Coefficient	Slope of internal fluid accumulation of radioactivity µl/h/mg protein	Correlation Coefficient
Everted gut sacs no non-radioactive PVP in internal fluid	4	0.76 ± 0.07	0.97	0.12 ± 0.03	0.80
Everted gut sacs 2 µg/ml non-radioactive PVP in internal fluid	10	0.69 ± 0.09	0.98	0.07 ± 0.02	0.73
Non-everted gut sacs no non-radioactive PVP in internal fluid	4	0.07 ± 0.05	0.71	-0.004 ± 0.012	-0.17
Non-everted gut sacs 2 µg/ml non-radioactive PVP in internal fluid	10	0.11 ± 0.03	0.72	0.03 ± 0.01	0.78

FIGURE 5(iv) Sephadex G 25 - 80 column chromatography of:

a) Dialysed [^{125}I]-PVP (2 µg/ml)

b) Dialysed [^{125}I]-PVP (2 µg/ml) incubated for 2 h at 37°C in the absence of intestinal sacs.

c) The radioactivity present in the medium after dialysed [^{125}I]-PVP (2 µg/ml) had been incubated for 2 h at 37°C in the presence of everted intestinal sacs.

d) The radioactivity taken up by the tissue of everted intestinal sacs incubated for 2 h at 37°C with dialysed [^{125}I]-PVP (2 µg/ml) as substrate.

e) The radioactivity present in the serosal fluid of everted intestinal sacs incubated for 2 h at 37°C with dialysed [^{125}I]-PVP (2 µg/ml) as substrate.

FIGURE 5(iv)a

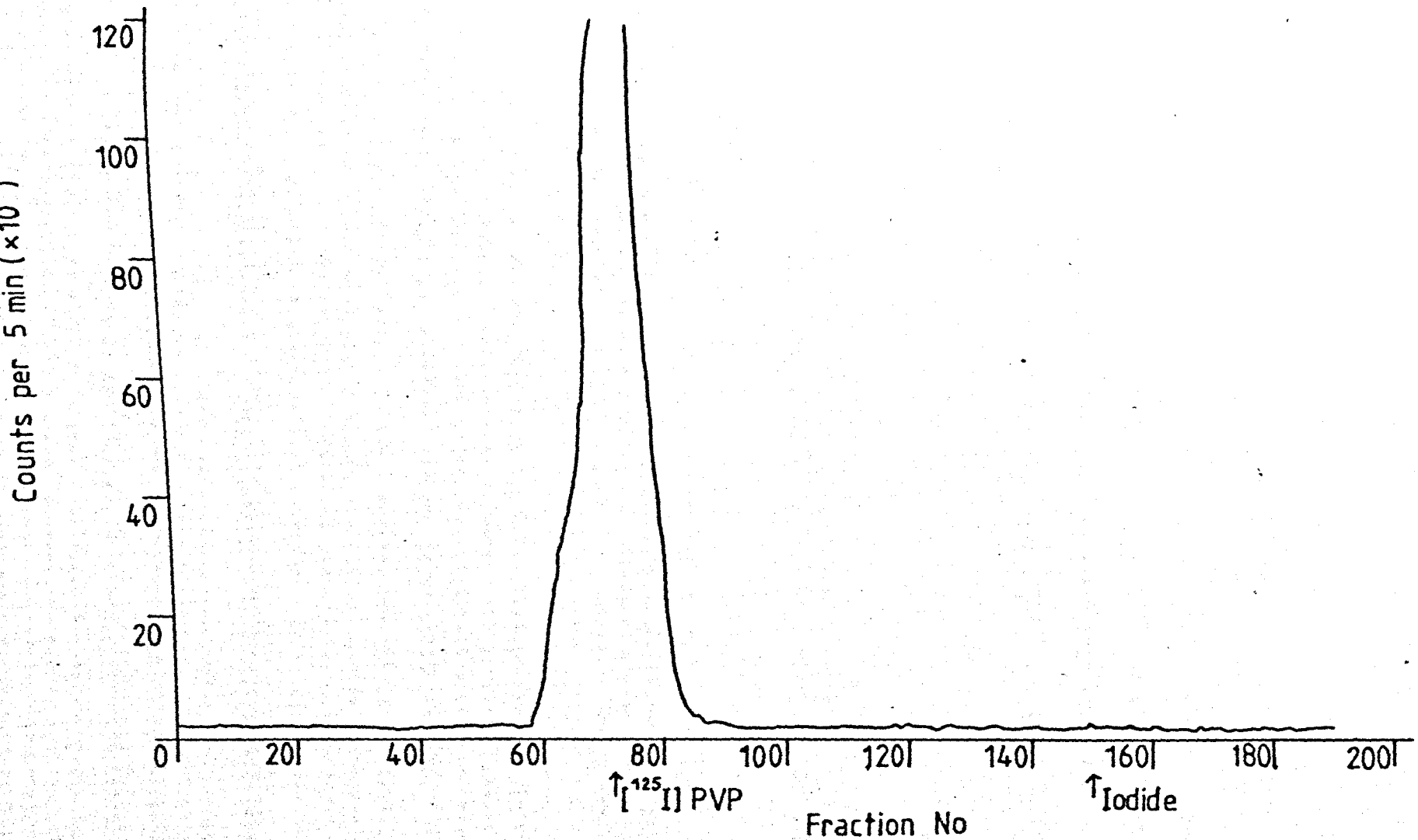

120
100
80
60
40
20
Counts per 5 min (×10³)
0
20
40
60
80
100
120
140
160
180
200
↑[¹²⁵I] PVP
↑Iodide
Fraction No

FIGURE 5(iv)b

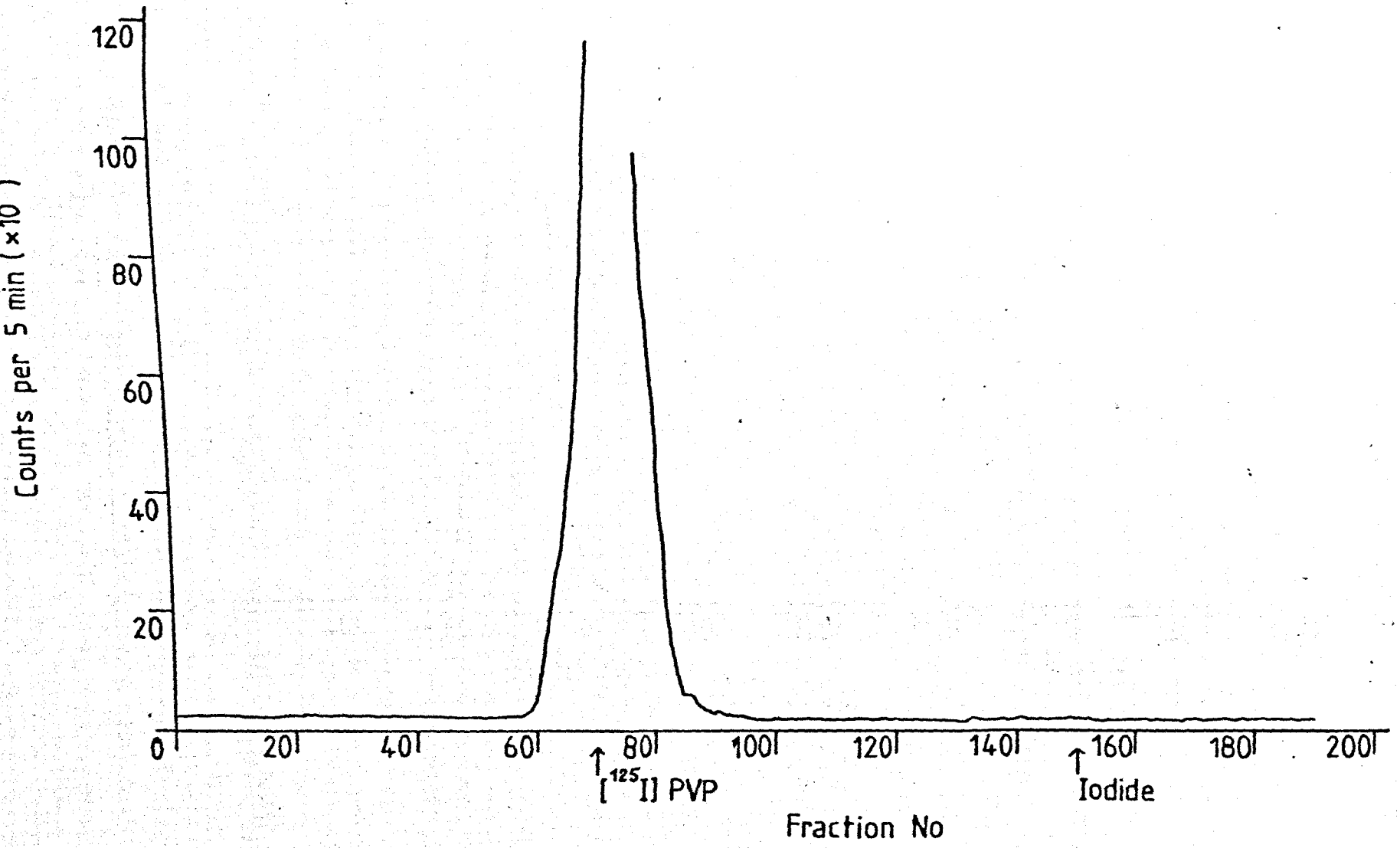

120
100
80
60
40
20
Counts per 5 min ($\times 10^3$)
0
20
40
60
80
100
120
140
160
180
200
[^{125}I] PVP
Iodide
Fraction No

FIGURE 5(iv)c

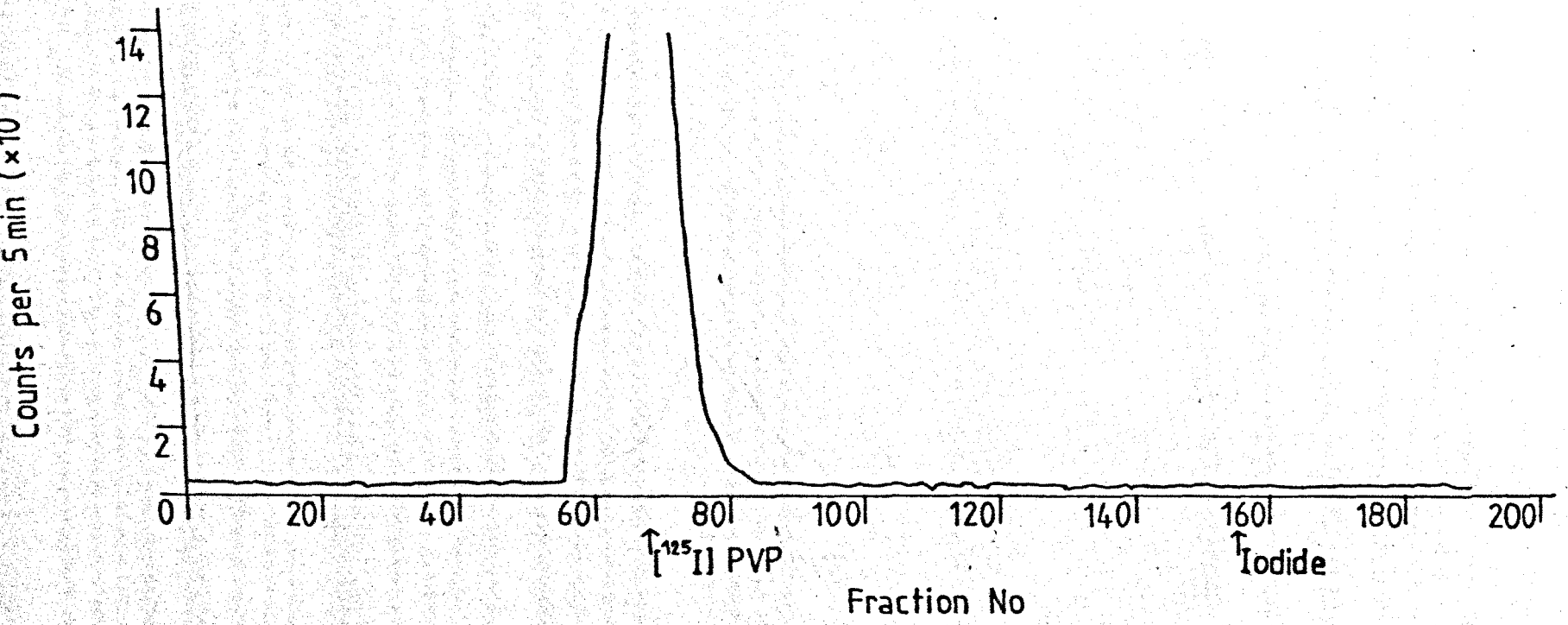

Counts per 5 min (×10³)
14
12
10
8
6
4
2
0
20
40
60
80
100
120
140
160
180
200
↑[¹²⁵I] PVP
↑Iodide
Fraction No

FIGURE 5(iv)d

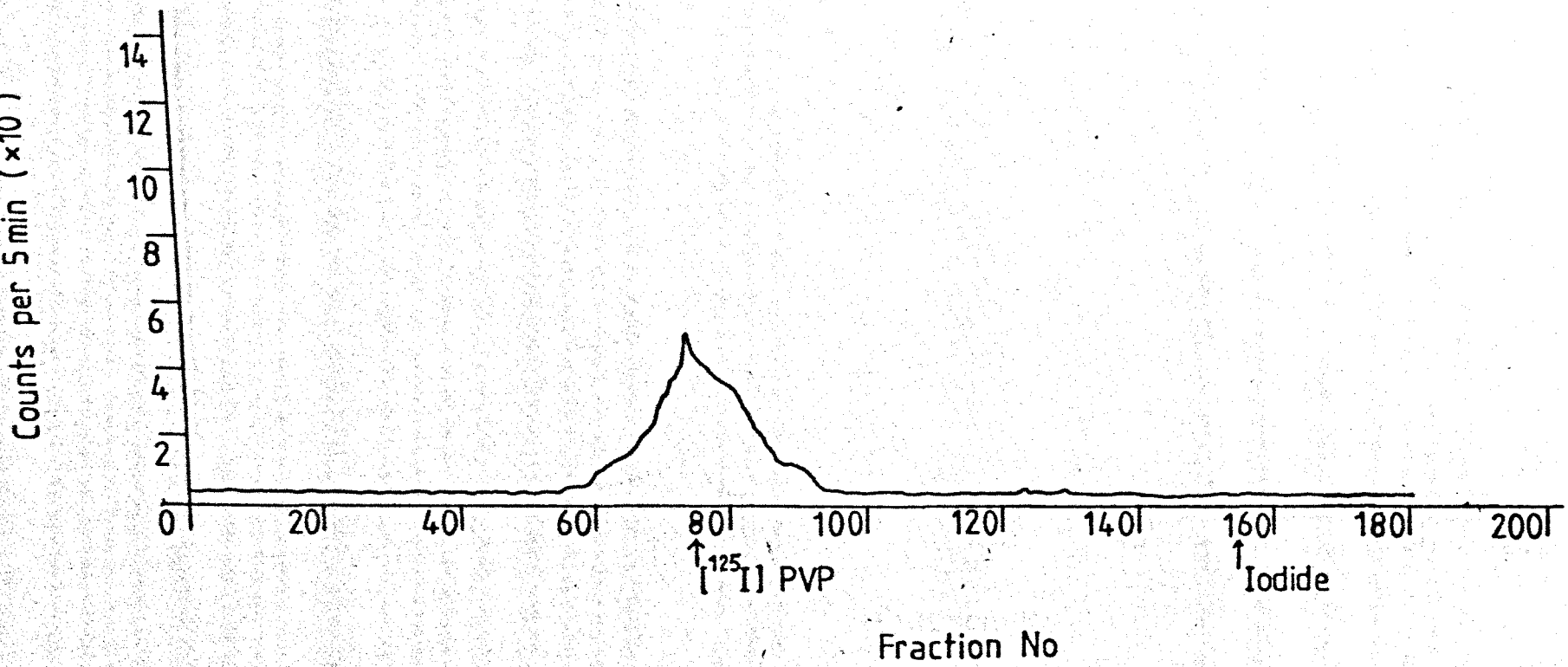

Counts per 5 min (×10³)
14
12
10
8
6
4
2
0
20
40
60
80
100
120
140
160
180
200
↑ [¹²⁵I] PVP
↑ Iodide
Fraction No

FIGURE 5(iv)e

Counts per 5 min (×10³)
14
12
10
8
6
4
2
0
20
40
60
80
100
120
140
160
180
200
[125I] PVP
Iodide
Fraction No

presence of intestinal sacs.

[^{125}I]-Iodide (Fig. 5(v)) showed uptake patterns different from those of [^{125}I]-PVP for both tissue uptake and accumulation in the serosal fluid. For tissue uptake the EI for iodide was twice as great as that of PVP (1.54 against 0.74). The rate of iodide transport through the tissue to the serosal space was about 33 times greater than that of PVP (2.35 and 0.07). Accumulation of iodide in the tissue and serosal space was rapid and linear at first then levelled out after about 0.75 h in the tissue and 1.25 h in the serosal space.

5.2.3 Conclusions

When the intestine was everted, tissue and serosal fluid accumulation increased with time in the absence and presence of PVP in the internal fluid. This suggested that [^{125}I]-PVP was accumulated against a concentration gradient. The rate of tissue accumulation of [^{125}I]-PVP was similar in the presence and absence of PVP inside the sacs although serosal fluid accumulation was reduced in the presence of PVP. With non-everted intestine and PVP inside, a slight linear accumulation of radioactivity was observed in the tissue and internal fluid though no nett uptake of radioactivity was observed in the absence of PVP inside.

The results obtained using dialysed [^{125}I]-PVP as substrate suggested no de-iodination occurred during incubation in the presence and absence of everted sacs though it must be stressed that these results were only qualitative.

[^{125}I]-iodide uptake was different from that of [^{125}I]-PVP in that iodide uptake was greater and levelled out after a time.

FIGURE 5(v) Accumulation of free [^{125}I]-iodide ± S.E. in the tissue and serosal fluid of everted intestinal sacs incubated for 2 h in the presence of [^{125}I]-iodide (2 µg/ml) compared with the accumulation of [^{125}I]-PVP

Key to diagrams as follows:

Uptake of [^{125}I]-iodide ▼——▼

Uptake of [^{125}I]-PVP ▽——▽

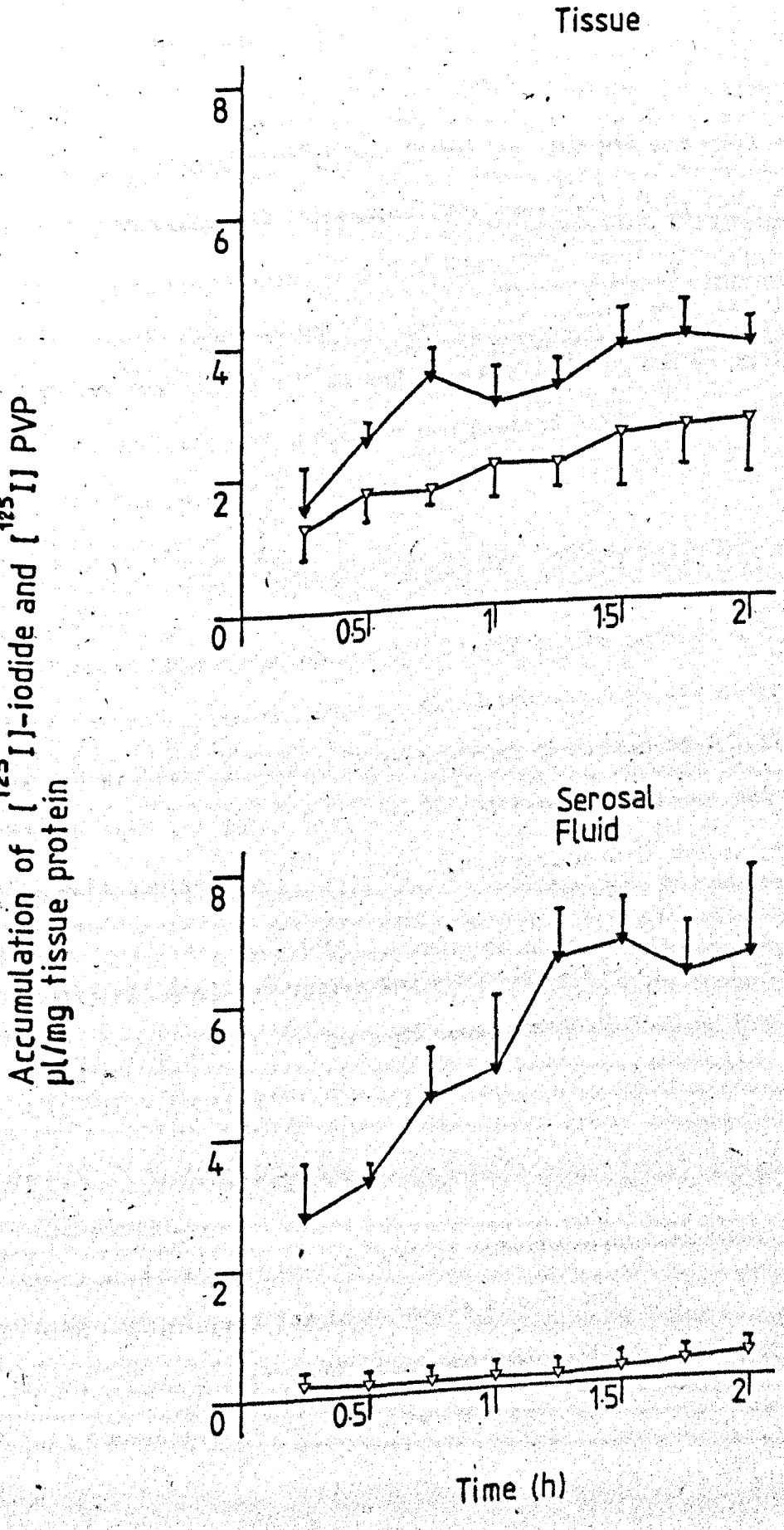

Tissue
Serosal Fluid
Accumulation of [125I]-iodide and [125I] PVP µl/mg tissue protein
0
2
4
6
8
0.5
1
1.5
2
Time (h)

FIGURE 5(v)

5.3 The Effects of Different Concentrations of Substrate [^{125}I]-PVP on Substrate Accumulation

5.3.1 Method

In similar experiments [^{125}I]-PVP (Batch no. 160 BA) was used as substrate at seven different concentrations, i.e. 0.25, 0.5, 2.0, 5.0, 10.0 and 20.0 µg/ml of medium. Everted intestinal sacs were prepared and incubated as described in Section 3.6. Incubations were carried out in Tc medium 199 containing 10% CS and 1 mM ATP and terminated at intervals up to 2 h. Accumulation of substrate was plotted against time for each set of data and the related EI calculated.

5.3.2 Results

With different concentrations of [^{125}I]-PVP in the medium, accumulation of substrate in the tissue and serosal fluid proceeded at a constant rate up to 2 h. Fig. 5(vi) and Table 5.4 show that the rates of [^{125}I]-PVP transport both into the intestinal tissue and serosal fluid do not change appreciably with increasing [^{125}I]-PVP concentration over the range investigated.

Fig. 5(vii) shows tissue uptake and accumulation in the serosal fluid expressed as ng/h/mg protein. In both cases, accumulation increased linearly with concentration.

5.3.3 Conclusions

The endocytic index was independent of substrate concentration within the range 0.25 - 20.0 µg/ml for both tissue uptake and accumulation in the serosal fluid. Uptake expressed in ng per unit of tissue per hour increased linearly with concentration over this range.

FIGURE 5(vi) Uptake of [^{125}I]-PVP ± S.E. expressed as Endocytic Index (E.I. - µl/h/mg tissue protein) plotted against substrate concentration

Key to diagram as follows:-

Tissue uptake ▼——▼

Accumulation in the serosal fluid ▽——▽

Broken lines indicate computer-fitted regression lines.

FIGURE 5(vi)

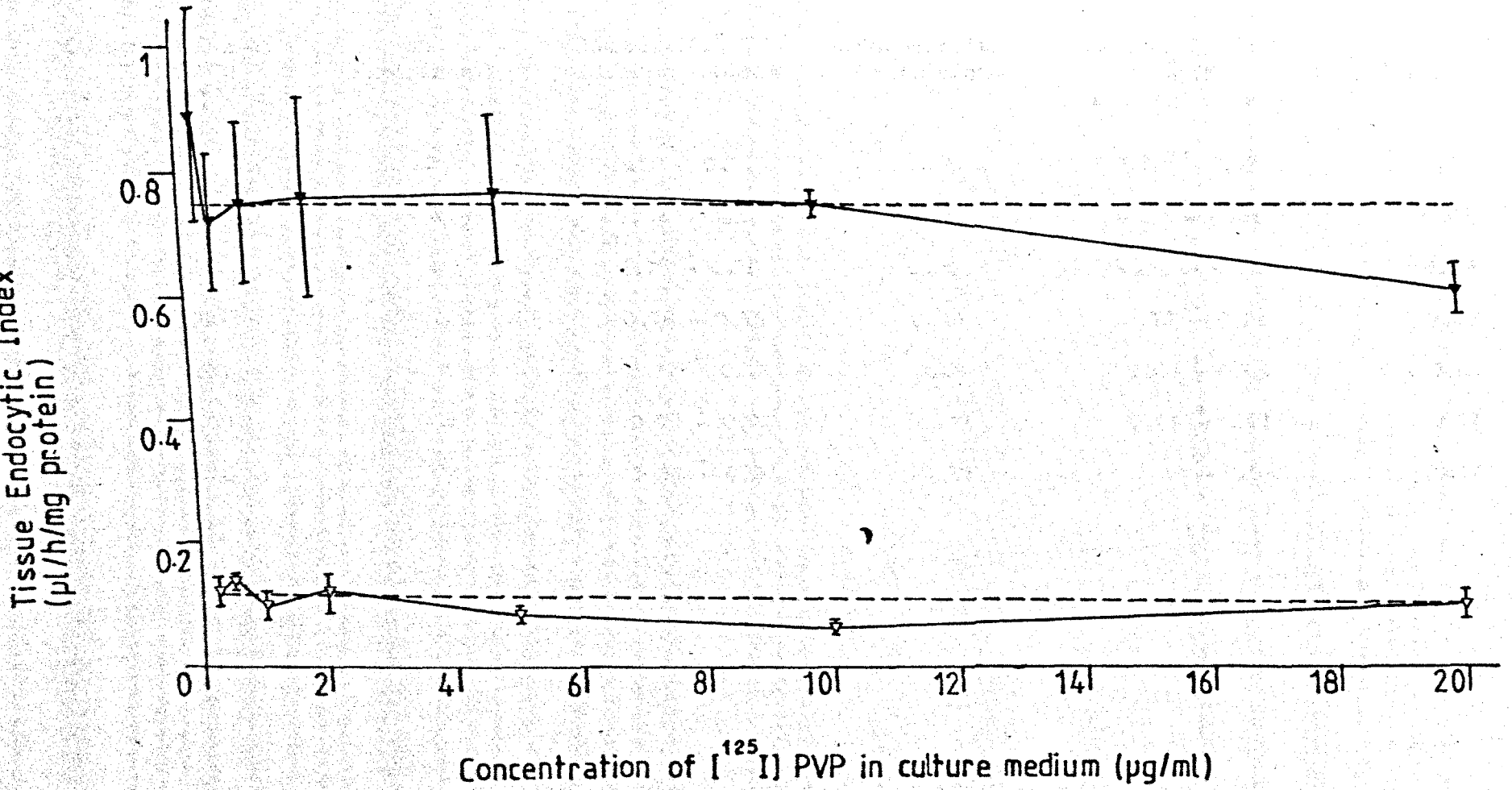

Tissue Endocytic Index
(µl/h/mg protein)
1
0·8
0·6
0·4
0·2
0
2
4
6
8
10
12
14
16
18
20
Concentration of [125I] PVP in culture medium (µg/ml)

TABLE 5.2

Endocytic Indices of [^{125}I]-PVP at Different Concentrations of [^{125}I]-PVP in the Medium

Concentration of [^{125}I]-PVP µg/ml	No. Experiments	Tissue EI ± SE µl/h/mg protein	Correlation Coefficient	Serosal Fluid EI ± SE µl/h/mg protein	Correlation Coefficient
0.25	8	0.89 ± 0.17	0.90	0.12 ± 0.02	0.87
0.50	8	0.72 ± 0.11	0.96	0.13 ± 0.01	0.88
1.00	8	0.75 ± 0.13	0.86	0.10 ± 0.02	0.79
2.00	8	0.76 ± 0.16	0.90	0.12 ± 0.03	0.88
5.00	4	0.77 ± 0.12	0.99	0.08 ± 0.01	0.80
10.00	4	0.75 ± 0.02	0.87	0.06 ± 0.01	0.82
20.00	4	0.61 ± 0.04	0.83	0.09 ± 0.02	0.96

FIGURE 5(vii) Uptake of [^{125}I]-PVP $\pm$ S.E. expressed as ng/h/mg tissue protein plotted against substrate concentration.

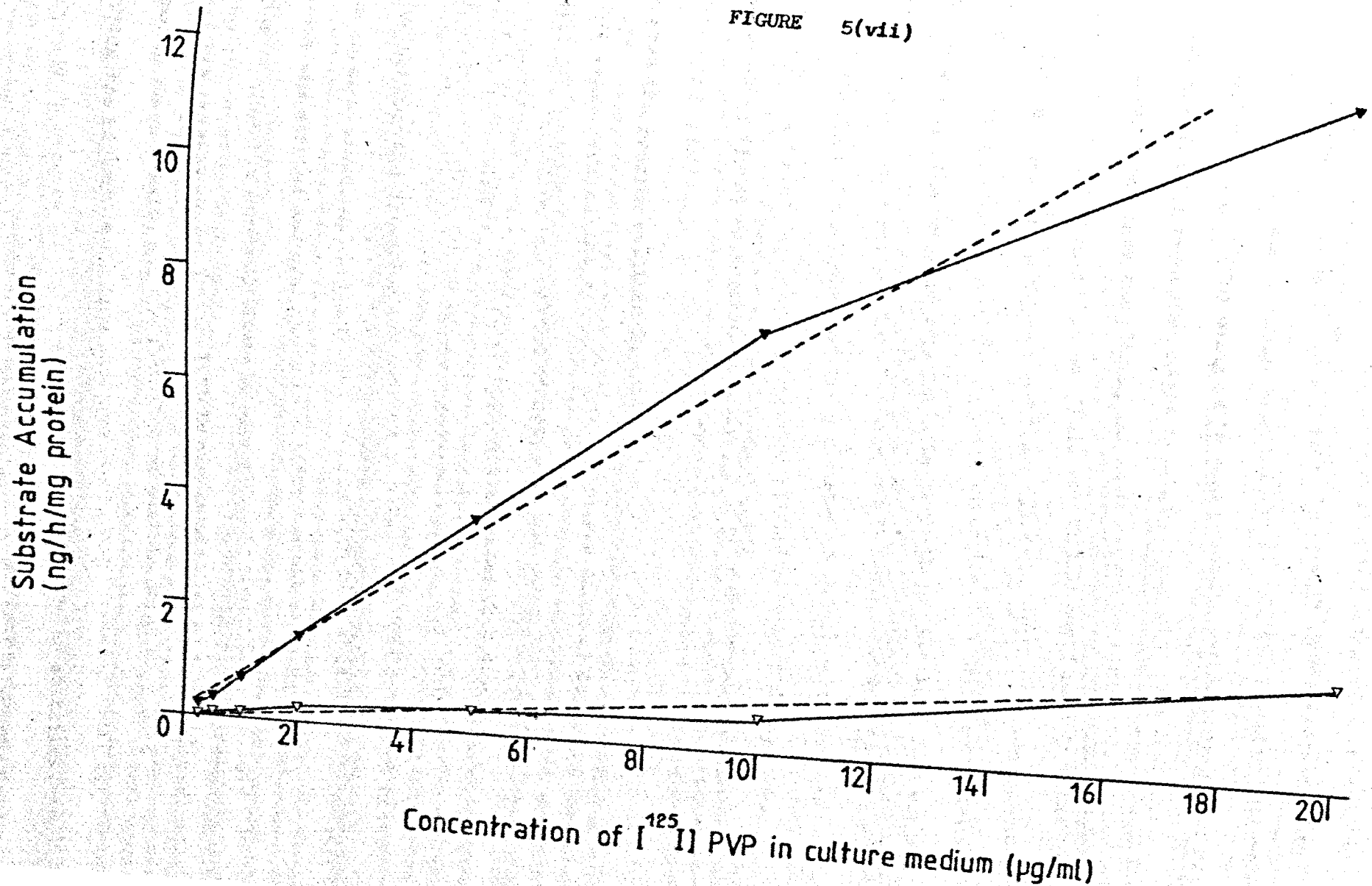
Key to diagram as follows:

Tissue Uptake ▼——▼

Accumulation in the serosal fluid ▽——▽

Broken lines indicate the computer-fitted regression lines.

FIGURE 5(vii)


Substrate Accumulation
(ng/h/mg protein)
0
2
4
6
8
10
12
2
4
6
8
10
12
14
16
18
20
Concentration of [125I] PVP in culture medium (µg/ml)

5.4 Discussion

The results in this Chapter suggest that the intestine can transfer macromolecules such as [^{125}I]-PVP from the mucosal to the serosal fluid, but very little macromolecular material can pass in the opposite direction.

Loehry *et al.* (1970) using an *in vivo* perfusion method showed that PVP with an effective molecular weight of 33,000 could pass both from the intestinal lumen into the plasma, and in the opposite direction. Hampton & Rosario (1967) showed that HRP (mol. wt. 40,000) could pass from the blood capillaries into the gut lumen and later Walker *et al.* (1974) demonstrated that HRP could also pass in the opposite direction. A detailed review of these and similar works in this field is given in Section 1.3. To explain these uptake phenomena two theories were put forward. Loehry *et al.* (1970) implicated that pores of large diameter (> 0.8nm) were involved and that macromolecules passed by passive diffusion in both directions. Walker *et al* (1974) postulated an active, energy dependent endocytic process was involved.

From the results outlined in this Chapter it can be seen that the tissue uptake rate of the macromolecule [^{125}I]-PVP differed depending upon whether the intestine was everted or not. If diffusion through pores was the mode of uptake, then it is generally accepted that tissue accumulation would not occur to any appreciable extent and would be similar whether the intestine was everted or not. This was not found to be the case. Tissue uptake increased linearly with time when the intestine was everted and could even take place against a concentration gradient. When not everted, tissue uptake was appreciably less and only when PVP was present in the internal fluid was uptake linear with time. Considering these results and the fact that no visible pores have been found using the highest resolution electron microscopy (Creamer, 1974) it is reasonable to suppose that tissue uptake at least may be by an active process. While tissue accumulation may be an active mechanism, transfer

of [^{125}I]-PVP from the tissue to the serosal fluid may occur simply by passive diffusion, as has been demonstrated for other actively absorbed substrates such as glucose and some amino acids (Crane et al. 1961). In an attempt to clarify the mechanism, a graph was plotted of tissue uptake of substrate against accumulation in the serosal space and a linear regression analysis was applied to the data (Fig. 5viii).. A linear relationship was recorded but the regression coefficient was low (0.62). A least squares fit analysis was then applied to the data to see if a relationship could be described by any other type of graph e.g. a hyperbola, but a linear graph was found to be the best fit. These analyses showed that the substrate accumulation in the serosal space was related to the tissue accumulation. That the regression coefficient was low may be due to the inaccuracy of the serosal space substrate accumulation data. It is apparent from graphs of accumulation of substrate in the serosal space vs. time that variation exists between experiments, indicated by fairly large error bars. This variation is likely to be a result of radioactive counting inaccuracies due to the low activity of the samples. In a laboratory with no great pressure on counting facilities, this could be remedied by extending the counting time for the serosal fluid samples.

Using Sephadex G 25 - 80 column chromatography, qualitatively it appeared that no de-iodination during uptake and transport of [^{125}I] -PVP. The total recovery of radiolabel was calculated to be only about 58% of the applied radioactivity and this was due to an amount of activity "sticking" on the column. Elution of dialysed (iodide free) [^{125}I] -PVP and [^{125}I] -iodide separately showed that the percentage recovery of [^{125}I] -PVP was low (about 60%) and that of [^{125}I] -iodide high (about 90%). This suggested that most of the unrecovered activity from the experimental samples consisted of [^{125}I] -PVP. Therefore the qualitative results gave a good indication of the state of [^{125}I] -PVP after incubation, i.e. that no de-iodination had taken place. Low-voltage electrophoresis would have

FIGURE 5(viii) Plot of tissue uptake of [^{125}I]-PVP (µl/h/mg) tissue protein against accumulation of [^{125}I]-PVP in the serosal fluid (µl/h/mg tissue protein). The plot is the result of 10 similar experiments, eight intestinal sacs per experiment.

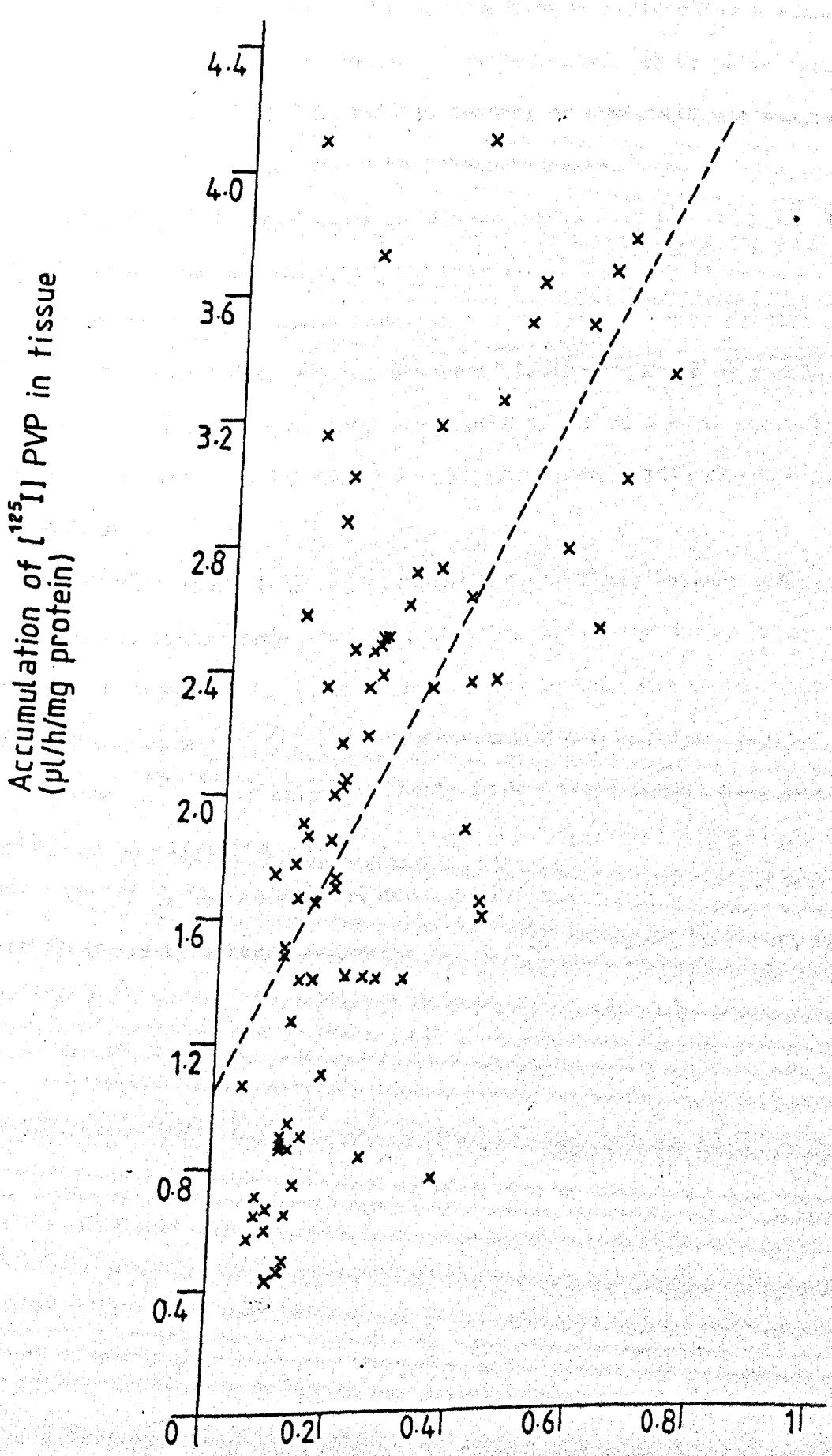


FIGURE 5(viii)

been a better method for accurate quantitation of radioactive species present before and after incubation. Unfortunately at the time these experiments were performed in this laboratory no equipment was available to concentrate the samples prior to electrophoresis.

The pattern of [^{125}I] -iodide uptake indicated that the rate of iodide accumulation in the serosal space was more rapid than the tissue uptake (about twice as much). These findings are similar to those of Wiseman (1964) who concluded that jejunal uptake of iodide occurred by simple diffusion. If this is the case, the levelling off of iodide accumulation in the serosal space may be due to equilibrium between this and the iodide in the medium.

The endocytic index (µl/h/mg protein) was shown not to vary with an increase in substrate concentration for either tissue uptake or accumulation in the serosal space. Also uptake in ng/h/mg protein was shown to be directly proportional to the substrate concentration. These results suggested that [^{125}I] -PVP enters entirely in the fluid phase, i.e. not be adsorption to the membrane and neither stimulates nor inhibits its own uptake. The significance of this and a comparison between the results obtained here and in other endocytotic systems is discussed in detail in Section 9.2.1.

CHAPTER SIX

THE EFFECTS OF METABOLIC INHIBITORS AND TEMPERATURE ON THE UPTAKE OF [^{125}I]-PVP BY EVERTED JEJUNAL SACS

6.1 Introduction

Movement of substrates across a membrane against a concentration gradient requires an expenditure of metabolic energy. Thus use of metabolic inhibitors such as azide or iodoacetate which markedly decrease the production of energy in the cell, greatly inhibit active transport. Free diffusion and facilitated diffusion mechanisms are not affected. Similarly, low temperatures have been shown to affect active transport processes.

In this study, representative inhibitors of cellular glycolysis and oxidative phosphorylation were used to determine the energy requirements for [^{125}I]-PVP absorption in the adult rat small intestine. Concentrations of inhibitor that were known to decrease the active transport of L-histidine - ^{14}C (Walker et al., 1971) were used in subsequent absorption experiments. Sodium fluoride (NaF) was used as an inhibitor of glycolysis and 2,4-dinitrophenol (2.4-D.N.P.) and sodium azide (NaN_3) were used as inhibitors of oxidative phosphorylation. The sites and mechanisms of action are as follows:-

a) Sodium fluoride, an inhibitor of anaerobic glycolysis. Sodium fluoride forms magnesium fluorophosphate with the Mg^{2+} cofactor of the enzyme enolase, abolishing the enzymes action. Enolase catalyses the reaction 2-phosphoglyceric acid $\xrightarrow{\text{enolase}}$ phosphoenolpyruvic acid, the penultimate step in the glycolytic sequence.

b) Sodium azide, an inhibitor of the electron transport chain. Of the electron transport cytochromes, only a_3 is capable of reducing oxygen. The N_3 ion binds to cytochrome a_3 and prevents re-oxidization.

c) 2.4 Dinitrophenol, an inhibitor of oxidative phosphorylation. 2.4 Dinitrophenol prevents the exchange of the oxygen of water for that of the terminal phosphate of ATP or phosphoric acid. The exact mechanism is unclear.

Besides the effects of the above mentioned metabolic inhibitors, the

effects of a range of temperatures (2 - 42°C) on [^{125}I]-PVP absorption was also determined.

6.2 The Effect of Various Metabolic Inhibitors on the Uptake of [^{125}I]-PVP by Everted Jejunal Sacs

In 1956 Wiseman observed that in *in vitro* experiments involving everted intestinal sacs, metabolic inhibitors such as KCN in concentrations up to 1 x 10^{-3}M had little or no effect unless placed both in the medium and serosal fluid. Therefore this practise was adopted for the experiments carried out in this study.

In previous experiments, exogenous ATP in the concentration of 1mM has been used as a standard addition to the incubation medium. Although any contribution by exogenous ATP to the energy requirements of intestinal tissue *in vitro* is questionable it was excluded from the medium in standard experiments involving the use of metabolic inhibitors. However using certain inhibitors, a comparison was drawn between substrate accumulations in the presence and absence of exogenous ATP in an attempt to resolve the question as to whether exogenous ATP was an energy source or not.

6.2.1 Methods

Everted jejunal sacs were prepared and incubated as described in Section 3.6 [^{125}I]-PVP was used as substrate in a concentration of 2µg/ml, Batch nos. 169 - 172 BA being used throughout the series of experiments. (The batch no. used in each experiment is given in the results tables.) Tc medium 199 plus 10% CS was used as the incubation medium for all the experiments. The metabolic inhibitors and concentrations used were as follows - DNP 2 x 10^{-5}, 10^{-4}, 10^{-3}M ; NaF 10^{-5}, 10^{-4}, 10^{-3}M ; NaN_3, 10^{-5}, 10^{-4}, 10^{-3}M. NaF and NaN_3 were used together, both in a concentration of 10^{-4}M. DNP, 2 x 10^{-5} and 10^{-4}M was used in the absence and presence of 1mM ATP in the medium. For each experiment, a control was carried out where no inhibitor was present.

Substrate accumulation was plotted against time for each set of data

and the related Endocytic Index calculated in µl/h/mg protein. Rate of substrate accumulation was also expressed as a percentage of that of the control.

6.2.2 Results

1) 2.4 DNP The results (Table 6.1) indicate that in the absence of ATP, the lowest concentration of 2.4 DNP used inhibited the substrate accumulation in tissue and serosal fluid only slightly (3.85 and 6.67% inhibition respectively). However, at the higher concentrations used (10^{-4} and 10^{-3}M) both tissue uptake and accumulation in the serosal fluid increased with increasing inhibitor concentration.

When exogenous ATP (1mM) was present in the medium, similar results were obtained i.e. 2×10^{-5}M 2.4 DNP appeared to inhibit substrate accumulation in the tissue and serosal fluid only slightly (20.27 and 6.67% inhibition respectively). At 10^{-4}M, 2.4 DNP, rates of accumulation in tissue and serosal fluid increased.

2) NaF Table 6.2 shows the effects of the inhibitor NaF on the uptake of the [^{125}I]-PVP substrate. A NaF concentration of 1.2×10^{-5}M inhibited substrate accumulation in tissue and serosal fluid slightly (14.44 and 1.54% inhibition) and the presence of 10^{-4}M NaF produced a greater degree of inhibition (42.03 and 15.38% inhibition). At the highest concentration (10^{-3}M NaF) substrate accumulation increased in both tissue and serosal fluid.

3) NaN_3 The effects of different concentrations of NaN_3 on [^{125}I]-PVP accumulation are shown in Table 6.3. As the concentration of inhibitor was increased, the accumulation of substrate in tissue and serosal fluid decreased progressively. Maximum inhibition reached were about 44 and 25% at a NaN_3 concentration of 10^{-3}M.

4) NaF and NaN_3 A combination of NaF and NaN_3, each in a concentration of 10^{-4}M, produced percentage inhibitions similar to those obtained for

the same concentration of each individual inhibitor. Accumulation of substrate in tissue and serosal fluid was inhibited by about 40 and 29% respectively.

From Fig. 6(i) the effects of different inhibitors, expressed as a percentage of uninhibited values, can be compared. The most potent inhibitor was NaN_3 which inhibited rates of substrate accumulation in both tissue and serosal fluid at all the concentrations used and gave the greatest percentage inhibition when inhibitors of the same concentration were compared. It should be noted that no inhibitor produced more than 50% inhibition.

TABLE 6.1

The Effects of the Metabolic Inhibitor 2.4 DNP on the Uptake of [^{125}I]-PVP in the Presence and Absence of 1mM ATP in the Incubation Medium (PVP - Batch No. 171BA)

Concn. of ATP (mM)	Concn. of Inhibitor (M)	Tissue EI ± SE µl/h/mg protein	% of Control	∴ % Inhibition	Serosal EI ± SE µl/h/mg protein	% of Control	∴ % Inhibition	No. of Experiments
0	0 (control)	0.84±0.13	-	-	0.26± 0.13	-	-	5
0	2×10^{-5}	0.73±0.13	86.90± 15.48	13.10	0.25± 0.06	96.15	3.85	8
0	1×10^{-4}	1.01±0.14	120.23± 16.67	-20.23	0.30± 0.07	115.38± 26.92	-15.38	8
0	1×10^{-3}	1.26±0.13	150.00± 15.48	-50.00	0.35± 0.07	134.62± 26.92	-34.62	6
1	0 (control)	0.74±0.14	-	-	0.15± 0.04	-	-	4
1	2×10^{-5}	0.59±0.08	79.73± 10.81	20.27	0.14± 0.03	93.33± 20.00	6.67	6
1	1×10^{-4}	0.79±0.08	106.75± 10.81	-6.75	0.17± 0.04	113.33± 26.67	-13.33	6

TABLE 6.2

The Effect of NaF on the Uptake of [^{125}I]-PVP in the Absence of 1mM ATP in the Incubation Medium

Concn. of Inhibitor (M)	Batch No. of [^{125}I]-PVP	No. of Experiments	Tissue EI ± SE µl/h/mg protein	% of Control	% Inhibition	Serosal EI ± SE µl/h/mg protein	% of Control	% Inhibition
0 (Control)	171 BA	4	0.90±0.17	-	-	0.26±0.08	-	-
1.2×10^{-5}	171 BA	4	0.77±0.04	85.56 ± 4.44	14.44	0.23-0.07	88.46 ±26.92	11.54
1×10^{-3}	171 BA	4	1.25±0.12	138.89 ±13.33	-38.89	0.39±0.08	150.00 ±30.76	-50.00
0 (Control)	172 BA	4	0.69±0.16	-	-	0.13±0.03	-	-
1×10^{-4}	172 BA	4	0.49±0.12	57.97 ±13.33	42.03	0.11±0.01	84.62 ± 3.85	15.38

TABLE 6.3

The Effects of NaN_3 on the Uptake of [^{125}I]-PVP in the Absence of Exogenous ATP

Also indicated is the effect on rate of substrate accumulation of a combination of the inhibitors NaF and NaN_3 each at a concentration of 1×10^{-4}M

Concn. of Inhibitor(s) (M)	Batch No. of [^{125}I]-PVP	No. of Expts.	Tissue EI ± SE µl/h/mg protein	% of Control	% Inhibition	Serosal EI ± SE µl/h/mg protein	% Control	% Inhibition
0 (Control)	171 BA	4	1.05± 0.26	-	-	0.24±0.10	-	-
1×10^{-5}	171 BA	7	1.03± 0.26	98.10 ± 24.76	1.9	0.20±0.04	83.33± 16.67	16.67
1×10^{-4}	171 BA	8	0.64± 0.12	60.95 ± 11.43	39.05	0.19±0.02	79.17± 8.33	20.83
1×10^{-3}	171 BA	8	0.59± 0.05	56.19 ± 4.76	43.81	0.18±0.06	75.00± 25.00	25.00
0 (Control)	172 BA	4	0.69± 0.11	-	-	0.24±0.10	-	-
1×10^{-4} NaF) 1×10^{-4} NaN_3	172 BA	4	0.41± 0.08	59.42 ± 11.59	40.58	0.17±0.06	70.83± 25.00	29.17

FIGURE 6(i) Comparison of the effects of metabolic inhibitors on

a) Tissue uptake of [^{125}I]-PVP.

b) Accumulation of [^{125}I]-PVP in the serosal fluid.

Results are expressed as a percentage of control (uninhibited) values and are $\pm$ S.E.

FIGURE 6(1)a

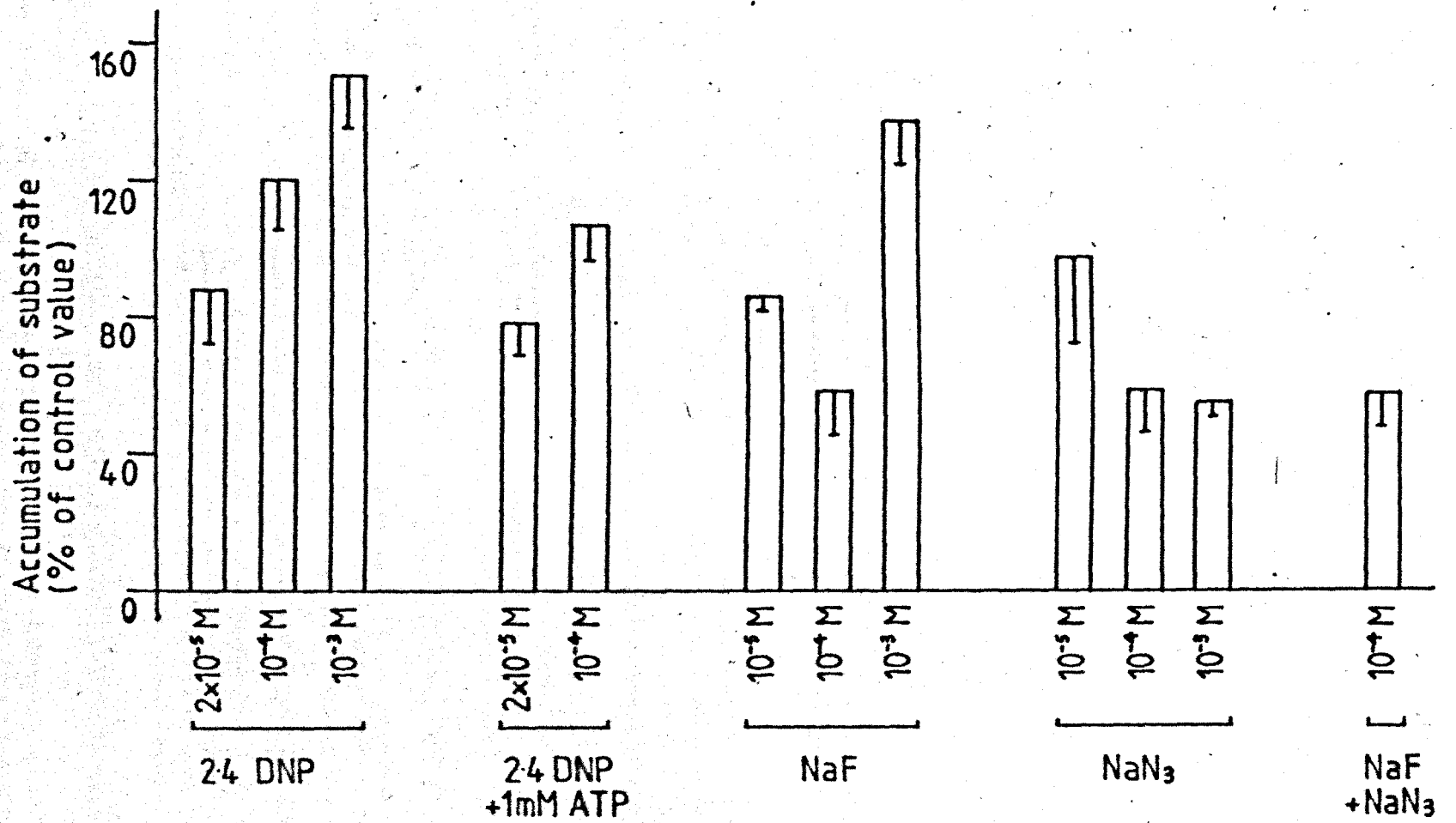
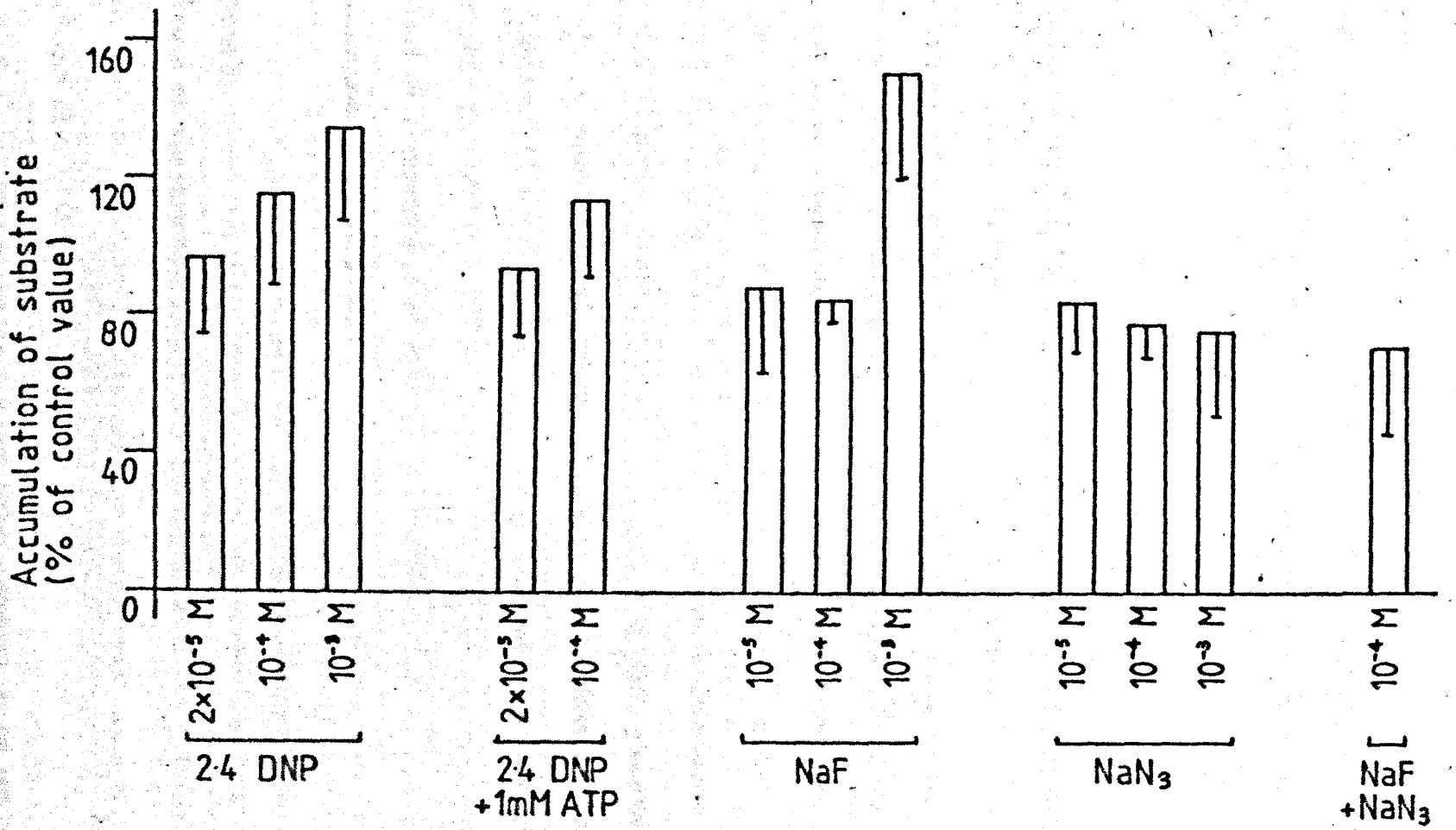

Accumulation of substrate (% of control value)
160
120
80
40
0
2x10⁻⁵M
10⁻⁴M
10⁻³M
2·4 DNP
2·4 DNP +1mM ATP
NaF
NaN₃
NaF +NaN₃

FIGURE 6(i)b


Accumulation of substrate (% of control value)
160
120
80
40
0
2×10⁻⁵ M
10⁻⁴ M
10⁻³ M
2·4 DNP
2×10⁻⁵ M
10⁻⁴ M
2·4 DNP +1mM ATP
10⁻⁵ M
10⁻⁴ M
10⁻³ M
NaF
10⁻⁵ M
10⁻⁴ M
10⁻³ M
NaN₃
10⁻⁴ M
NaF +NaN₃

6.3 The Effect of Temperature on the Uptake of [^{125}I]-PVP by Everted Jejunal Sacs

Rates of fluid phase uptake of macromolecules in other cell systems have been shown to be strongly temperature dependent (Steinman et al., 1974). In this study, the effect of different temperatures on the rates of accumulation of [^{125}I]-PVP were studied to determine whether such temperature dependency exists for the uptake of this macromolecule in adult intestine.

6.3.1 Method

The effects of varying the incubation temperature on the rate of uptake of [^{125}I]-PVP by everted jejunal sacs was investigated using incubation temperatures between 2 - 42°C. Sacs were incubated for up to 2h in Tc medium 199 containing 10% CS and 1mM ATP with [^{125}I]-PVP (Batch 161 BA) at a concentration of 2µg/ml as substrate. The experimental regime was the same as that described in Section 3.6 except that incubations were executed at 2, 10, 20, 30, 37 and 42°C. For all temperatures below 20°C a temperature probe (Grant Instruments Ltd., Cambridge, England) was used to lower the temperature of the water bath. Accumulation of substrate was plotted against time for each set of data and the related Endocytic Index calculated in µl/h/mg protein. Rate of substrate accumulation was also expressed as a percentage of that of the control, i.e. a percentage of the rate of accumulation at 37°C.

6.3.2 Results

The effects of varying the incubation temperature on the pattern of tissue uptake and accumulation of substrate in the serosal fluid are shown in Table 6.4 and Fig. 6(ii).

In the case of tissue uptake at 2°C the rate appeared to be quite high (0.78 ± 0.14 µg/h/mg protein) but at 10°C, 20°C and 30°C tissue uptake was

only about 50% of that of the control at 37°C. Tissue uptake at 42°C was found to be about twice that of the control at 37°C.

In the case of accumulation of substrate in the serosal fluid, increasing the temperature from 2 - 42°C caused an increase in the rate of accumulation. At 42°C, a rapid influx of substrate to the serosal fluid was observed. According to Walker *et al.* (1972) this is indicative of extensive cytolysis of intestinal tissue.

TABLE 6.4

The Effect of Temperature on the Uptake of [^{125}I] -PVP

Temperature °C	No. of Experiments	Tissue-Uptake µl/h/mg protein ± SE	% of Control	Serosal-fluid accumulation µl/h/mg protein ± SE	% of Control
2°	4	0.78 ± 0.14	108.33	0.04 ± 0.01	30.77
10°	4	0.39 ± 0.17	54.17	0.06 ± 0.01	46.15
20°	4	0.34 ± 0.13	47.22	0.08 ± 0.01	61.54
30°	4	0.33 ± 0.12	45.83	0.09 ± 0.01	69.23
37°	4	0.72 ± 0.08	100.00	0.13 ± 0.02	100.00
42°	4	1.32 ± 0.31	183.33	0.69 ± 0.22	530.77

FIGURE 6(ii) Effect of temperature on the accumulation of [^{125}I]-PVP (expressed as EI - µl/h/mg tissue protein) in:

a) Tissue

b) Serosal fluid

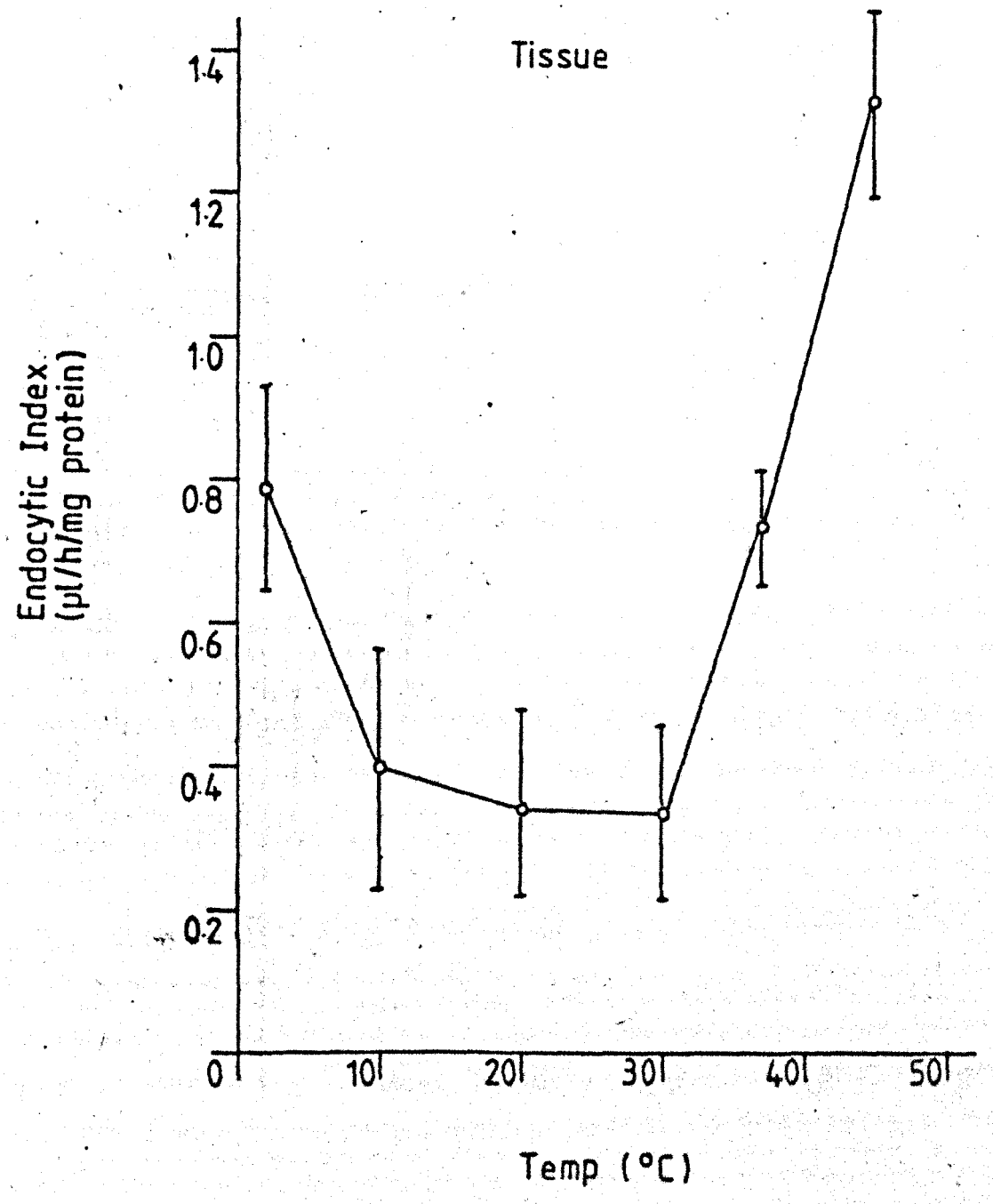

Tissue
Endocytic Index
(µl/h/mg protein)
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
10
20
30
40
50
Temp (°C)

FIGURE 6(ii)a

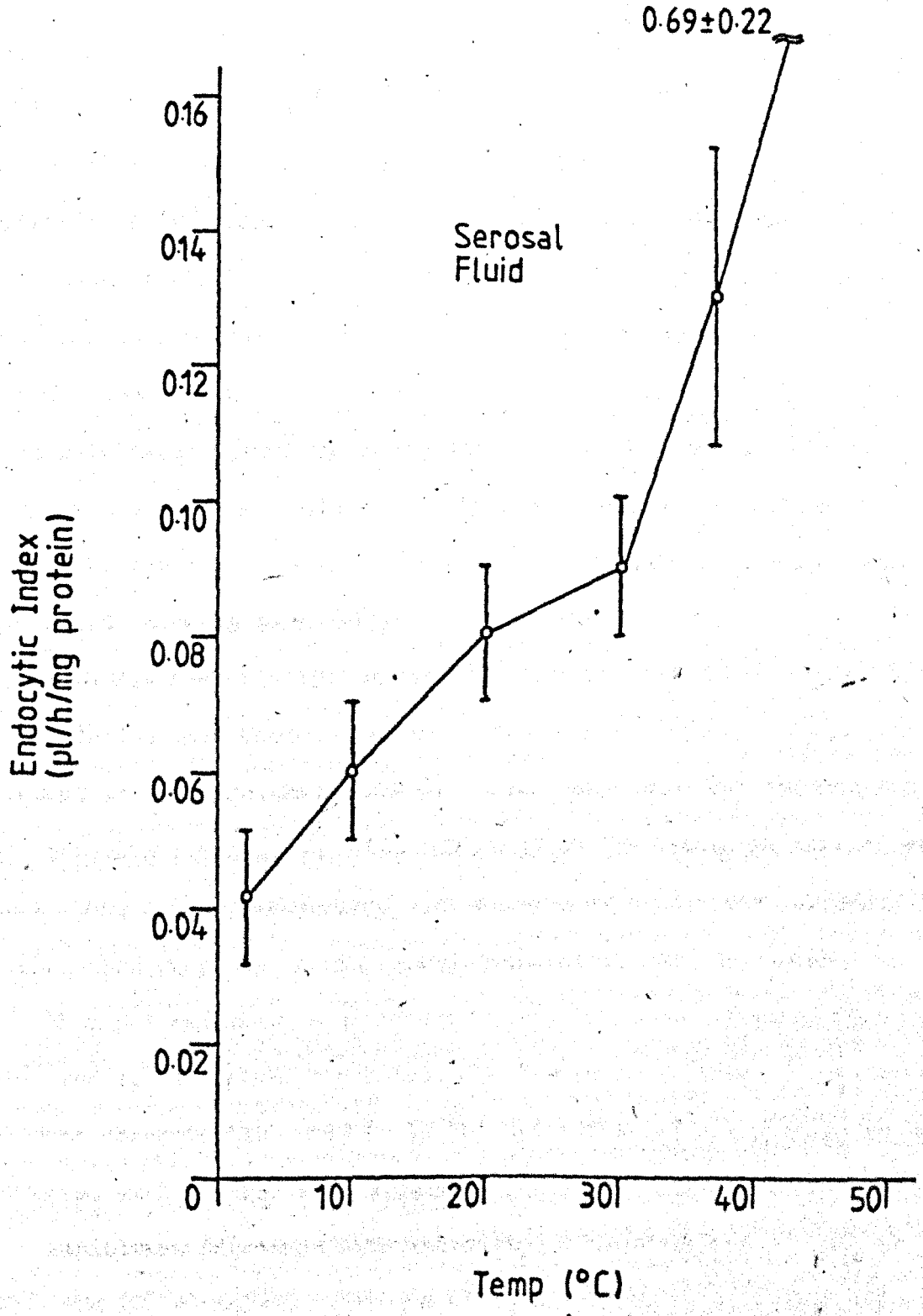

0·69±0·22
Serosal Fluid
Endocytic Index (µl/h/mg protein)
0·16
0·14
0·12
0·10
0·08
0·06
0·04
0·02
0
10
20
30
40
50
Temp (°C)

FIGURE 6(ii)b

6.4 Discussion

The results showed that at the lowest concentration (2×10^{-5}M) of the inhibitor 2.4 DNP there was an inhibitory effect on the rate of accumulation of substrate in both tissue and serosal fluid. At higher inhibitor concentrations (10^{-4}M and above) rates of accumulation of substrate increased over those of the control. For the accumulation of substrate in the serosal fluid, the effects are similar to those observed by Walker *et al.* (1972) who used the same concentrations of 2.4 DNP but with HRP as substrate. They postulated that at concentrations of DNP greater than 10^{-4}M cytolysis occurs due to the cytotoxicity of the inhibitor. This cytolysis allows influx of substrate to the serosal fluid. (They did not monitor tissue uptake.) This explanation may account for the increased substrate accumulation observed at inhibitor concentrations of 10^{-4}M and above in this study.

With 2.4 DNP (2×10^{-5} and 10^{-4}M) similar patterns of substrate accumulation were observed whether exogenous ATP (1mM) was absent or present in the incubation medium. This suggested that ATP did not render the intestinal epithelium more resistant to the cytotoxic effects of the inhibitor, but no conclusions can be drawn as to whether exogenous ATP contributes directly to the energy requirements of the tissue.

When NaF was used as an inhibitor, at the lower concentrations (1.2×10^{-5} and 10^{-4}M) inhibition increased with concentration. However at the highest concentration used ($\times 10^{-3}$M) cytotoxic effects similar to those observed with 2.4 DNP were inferred.

Inhibition increased with increasing concentrations of NaN_3 ($\times 10^{-5}$, 10^{-4} and 10^{-3}M) and no cytotoxic effects were observed within this range. In this system cytotoxicity of inhibitors was as follows, 2.4 DNP > NaF > NaN_3. The fact that a combination of inhibitors, (NaF and NaN_3 each used in a concentration $\times 10^{-4}$M) did not produce any more or less inhibition than was produced by either substance singly when present in the same

concentration is discussed later in this section.

To some degree, the results obtained with metabolic inhibitors agree with those obtained by Wiseman (1956) in his work on active amino acid transport. He observed that, even in concentrations up to 10^{-3}M, KCN and other inhibitors produced a maximum of 50% inhibition. This was also seen to be the case for inhibitors used in this study; no substance inhibited the rate of substrate accumulation by more than 50%. Barry et al. (1961) and later Browne et al (1977) postulated that this phenomenon was due to energy for active transport being derived from more than one source by the enterocytes of the rat small intestine. In the rat, several possible sources of energy have been implicated. One was the metabolism of glucose mainly by aerobic glycolysis or the pentose phosphate pathway; the other, metabolism of non-carbohydrates by the citric acid cycle (Barry et al, 1961). Presumably, then, once one of the processes was inhibited by a particular inhibitor, energy could be produced by another process. Therefore complete inhibition could not be accomplished at any one time, not even with a combination of inhibitors such as NaF and NaN_3. Although glycolysis and oxidative phosphorylation could be inhibited by a combination of these two substances, according to Barry et al (1961) energy production could still proceed via the pentose phosphate pathway.

The results obtained from the study of the effects of different temperatures on [^{125}I]-PVP uptake showed that between 10 and 30°C, tissue uptake was reduced to about 50% of that of the control value at 37°C. At the temperature higher than the physiological normal (42°C) tissue uptake was about twice that of the control. Accumulation of [^{125}I]-PVP in the serosal fluid was shown to be strongly temperature-dependent and rate of accumulation increased with temperature from 2 to 42°C. These results, and the apparently anomalous result obtained for tissue uptake at 2°C, can be explained in terms of changes in membrane fluidity which occur at different temperatures. This is discussed in detail in Section 9.2.1.

In this section, rates of accumulation of [^{125}I]-PVP both in tissue and serosal fluid were shown to be decreased appreciably in gutsacs incubated with either appropriate concentrations of metabolic inhibitors or at temperatures lower than the physiological normal. These observations suggest that [^{125}I]-PVP is taken up to adult intestine by an energy dependent process. These findings are compared with macromolecular uptake processes in other cell types in Section 9.2.1.

CHAPTER SEVEN

ADAPTATION OF THE GENERAL *IN VITRO* TECHNIQUE FOR THE ASSESSMENT OF UPTAKE OF THE PROTEIN HORSERADISH PEROXIDASE (HRP)

7.1 Introduction

In the previous sections, the pattern of uptake of the non-degradable macromolecule ^{125}I -PVP has been described. Non-degradable macromolecules are useful for the production of information regarding uptake mechanisms without having the complexities of degradation clouding the issue. It is unlikely that any such macromolecules would be encountered in the physiological state but large degradable macromolecules particularly proteins, would be. Therefore the next step in the study of macromolecular uptake using the *in vitro* method described in Section 3.6, was to elucidate the patterns of tissue and serosal fluid accumulation of a degradable macromolecule.

Previous studies in the field of macromolecular uptake have employed various degradable protein markers, some radiolabelled, e.g. ^{125}I -BSA (Walker et al. 1976). The main objection to the use of radiolabelled protein markers is the uncertainty of whether the radioactivity measured represents the intact molecule. According to Straus (1969) there are criteria which must be fulfilled by a potential macromolecular marker protein before it can be used as such. These are as follows: the marker must be easily detectable quantitatively in small samples, the marker must possess some property, e.g. such as enzymic activity, which would be lost should any degradation occur, the molecule must not be present normally in any significant quantity that would interfere with its assay in the tissue under test.

Straus (1969) ascertained that a substance which fulfils all these criteria is horseradish peroxidase (HRP). The advantages of using HRP as a marker protein are related to its enzymic nature. Due to its catalytic activity, very low concentrations of protein can be detected *in situ* by either light or electron microscopy and the amounts taken up can be measured quantitatively in small tissue samples. Since most animal cells do not contain significant peroxidase activity, the enzyme activity detected is that of the protein marker. Its high molecular weight of 40,000

facilitates its use as a macromolecule.

Taking these points into account, it can be seen that the general method described in Section 3.6 must be adapted for use with the degradable protein marker HRP. In this section, these adaptations are described together with a more rapid technique for the detection of HRP.

7.2 SDS Gel-electrophoresis of Different Commercial Preparations of HRP as a Check for Purity

In the use of enzymic marker proteins it is important to ensure that the activity of the preparation used is due solely to the enzyme under test and not to any contamination which may be present. For this reason, the commercially produced HRP available for this study was checked for homogeneity using SDS gel electrophoresis.

7.2.1 Methods

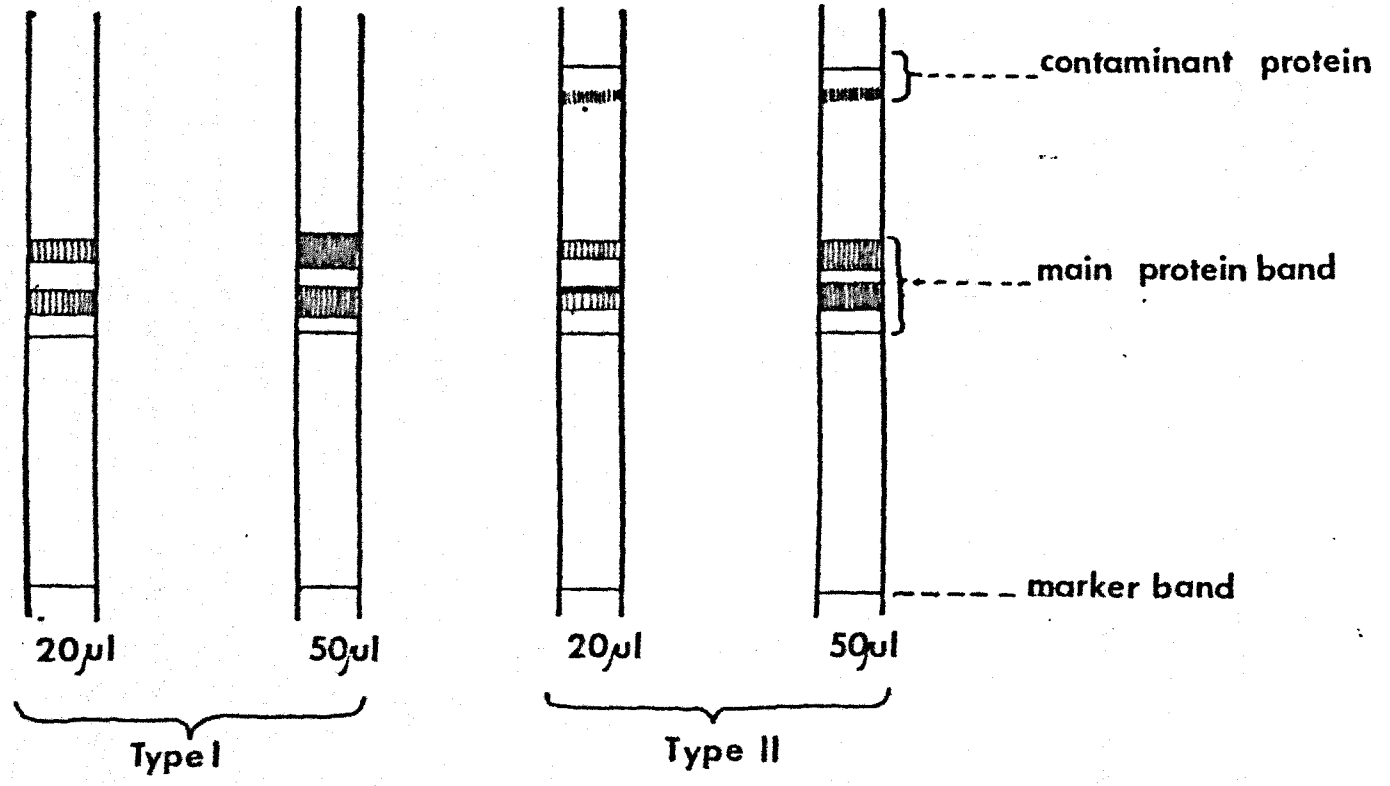
SDS gels for electrophoresis were prepared as described in Section 2.9. HRP samples of type I and type II peroxidase (Sigma Chemical Co., Surrey) were dissolved in water to a final concentration of 1 mg/ml. Of each of these solutions duplicate samples of 20 µl and 50 µl were taken for electrophoresis. To each sample, 50 µl of bromophenol blue and a few crystals of sucrose were added and dissolved prior to layering the sample on top of the polyacrylamide gel. The sucrose ensured that the protein solution sank immediately to the surface of the gel. Electrophoresis was then performed in a Shandon disc-gel electrophoresis apparatus (model: Analytical). Electrophoresis commenced at a constant current of 3 mA per tube until the bromophenol marker dye reached the running gel (about 5 min.) and then was completed at a constant current of 2 mA per tube until the marker dye reached to within 1 cm from the bottom of the gel.

Immediately after the completion of electrophoresis, each gel was extruded from the glass tube and stained for protein as described in Section 2.9.2.

7.2.2 Results

A diagrammatic representation of the gels after staining is given in Fig. 7(i). From the gels it can be seen that the type II peroxidase contained two more protein bands than the type I peroxidase. Because

FIGURE 7(i)



Diagrammatic representation of the results of SDS gel-electrophoresis of HRP types I and II

these protein bands were so far removed from the main protein bands, it is likely that the former were due to contaminants rather than isoenzymes.

7.2.3 Conclusion

According to the manufacturer (Sigma Chemical Company Ltd.) the three distinct areas which made up the main protein band are due to isoenzymes of HRP (electrophoretically distinct bands of enzymes with the same function). Therefore the purest preparation was the type I peroxidase and this was used in future experiments.

It is perhaps appropriate to note here that the type I peroxidase was not the most pure preparation commercially available. It was, however, the purest preparation available which could be used economically.

7.3 A Continuous Assay Method for Detection of HRP in Tissue and Serosal Fluid Samples

7.3.1 Introduction

Maehly & Chance (1954) developed a continuous sampling technique for HRP estimation. The principle of the method is as follows:-

$$\underset{\text{hydrogen peroxide}}{H_2O_2} + \text{o-dianisidine} \xrightarrow{\text{peroxidase}} H_2O + \underset{\text{(orange colour)}}{\text{oxidised o-dianisidine}}$$

The orange colour of oxidised o-dianisidine was measured spectrophotometrically at 460nm. The method is described in detail below.

7.3.2 Methods

a) Reagents:

Reaction mixture, 0.003% v/v hydrogen peroxide (H_2O_2, from a stock of 30% v/v) in 0.05M phosphate buffer pH 6.0. For each ml of solution 25 µl of an aqueous solution of o-dianisidine dihydrochloride (10 mg/ml) were added immediately before use.

b) Procedure:

The continuous assay method of Maehly & Chance (1954) was used to determine the progress curves of HRP. The enzyme was assayed as follows: 0.1 ml of test solution was mixed with 2.9 ml of the reaction mixture. The rate of extinction at wavelength 460 nm was determined. Controls were in the absence of enzyme and with heat denatured enzyme. Solutions of HRP (type I, Sigma Chemical Co.) of concentration 0.1, 0.2, 0.3, 0.4 and 0.5 µg/ml were assayed. HRP solutions were made up in

i) 0.05M phosphate buffer pH 6.0.

ii) Tc medium 199 + 10% CS and 1mM ATP, pH 7.3.

Enzyme activity was measured at half minute intervals up to 15 min. and the progress curves plotted.

7.3.3 Results

Fig. 7(ii) shows the progress curves for HRP. When the enzyme was dissolved in 0.05M phosphate buffer pH 6.0, for all concentrations of enzyme used the graphs indicated a low rate of reaction which decreased as the reaction proceeded. In contrast when the enzyme was dissolved in Tc medium 199 containing 10% CS and 1mM ATP, for all enzyme concentrations the graphs (Fig. 7(iii)) indicated a high, linear rate of reaction up to 15 min. This was confirmed by the high regression coefficients obtained from computer fitted lines.

Assays carried out in the complete absence of enzyme and in the presence of denatured enzyme revealed no change in extinction at 460 nm when samples were left to stand at room temperature for up to six hours.

7.3.4 Conclusion

The continuous assay technique used for HRP as described above presents an effective method for the measurement of the activity of the enzyme. When the enzyme is diluted in Tc medium 199 containing 10% CS and 1mM ATP the rate of reaction is linear for a long period. Thus the initial velocity of reaction can be easily calculated. Nevertheless, for a large number of samples this assay would prove unsuitable due to the length of time required for each sample assay (about 15 min). However the long linear rate of reaction can be utilized in constructing a discontinuous assay method.

Provided that the incubation period of the enzyme is within the linear portion of the progress graph, samples of enzyme can be incubated for a fixed time, the reaction stopped; either by lowering the pH or denaturing the enzyme, and the samples analysed. Using HRP concentrations between 0 - 0.5 μg/ml, the incubation period of the enzyme with its reaction mixture must be about 10 min, so that the incubation period falls within the linear portion of the graph. Also an incubation period of 10 min would allow the overall maximum extinction at 460 nm attained to

FIGURE 7(ii) Progress curves from the continuous assay of HRP catalysed reactions.

HRP was dissolved in 0.05m phosphate buffer pH 6.0. Concentrations of HRP and the computed regression coefficients for each plot were as follows:-

a) 0.1 µg/ml reg. coeff. 0.97

b) 0.2 µg/ml reg. coeff. 0.97

c) 0.3 µg/ml reg. coeff. 0.98

d) 0.4 µg/ml reg. coeff. 0.97

e) 0.5 µg/ml reg. coeff. 0.91

FIGURE 7(11)

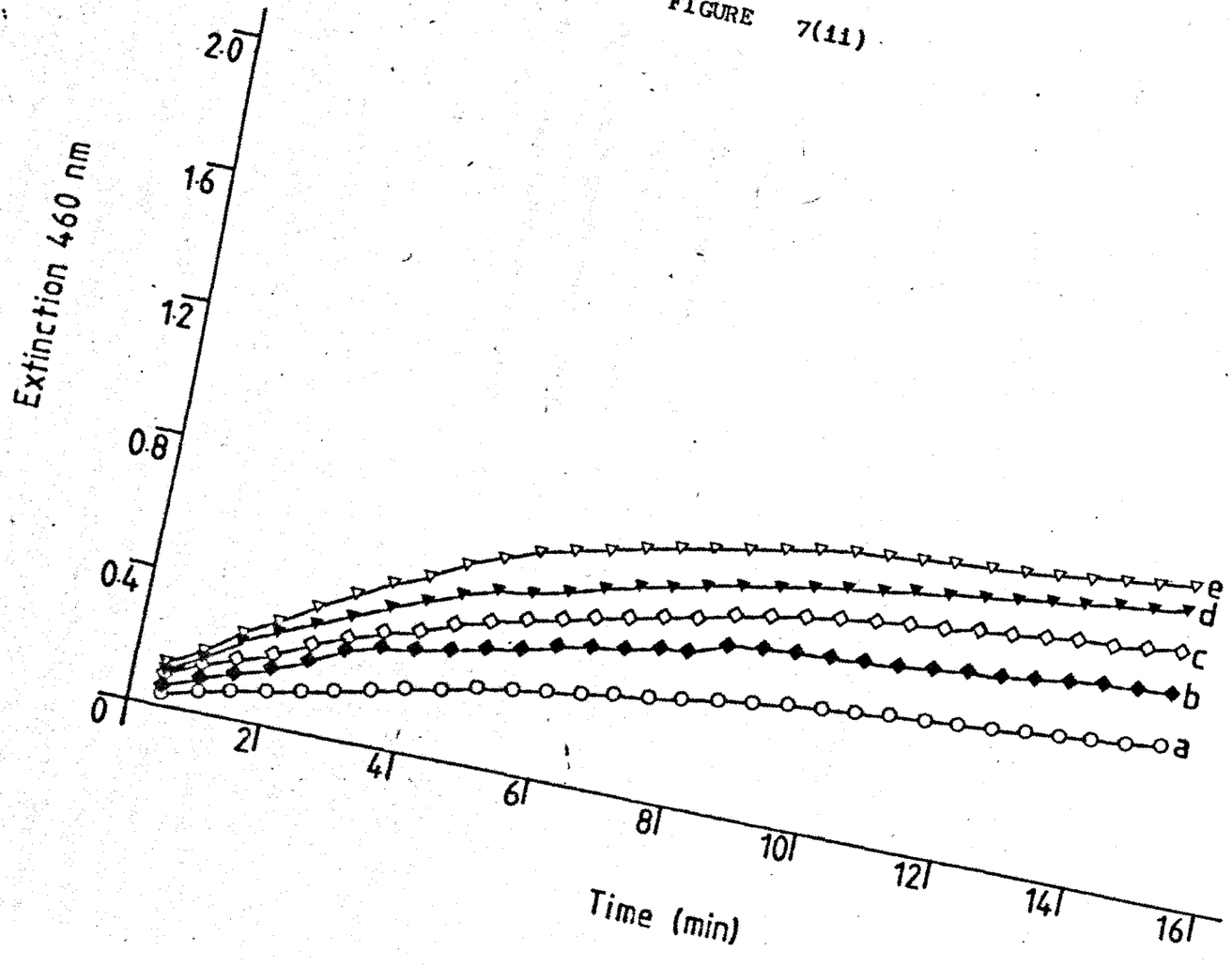

Extinction 460 nm
2·0
1·6
1·2
0·8
0·4
0
2
4
6
8
10
12
14
16
Time (min)
a
b
c
d
e

FIGURE 7(iii) Progress curves from the continuous assay of HRP catalysed reactions.

HRP was dissolved in Tc medium 199 containing 10% CS and 1mM ATP. Concentrations of HRP used and the computed regression coefficients for each plot were as follows:

a) 0.1 µg/ml reg. coeff. 1.00

b) 0.2 µg/ml reg. coeff. 1.00

c) 0.3 µg/ml reg. coeff. 0.99

d) 0.4 µg/ml reg. coeff. 0.99

e) 0.5 µg/ml reg. coeff. 0.99

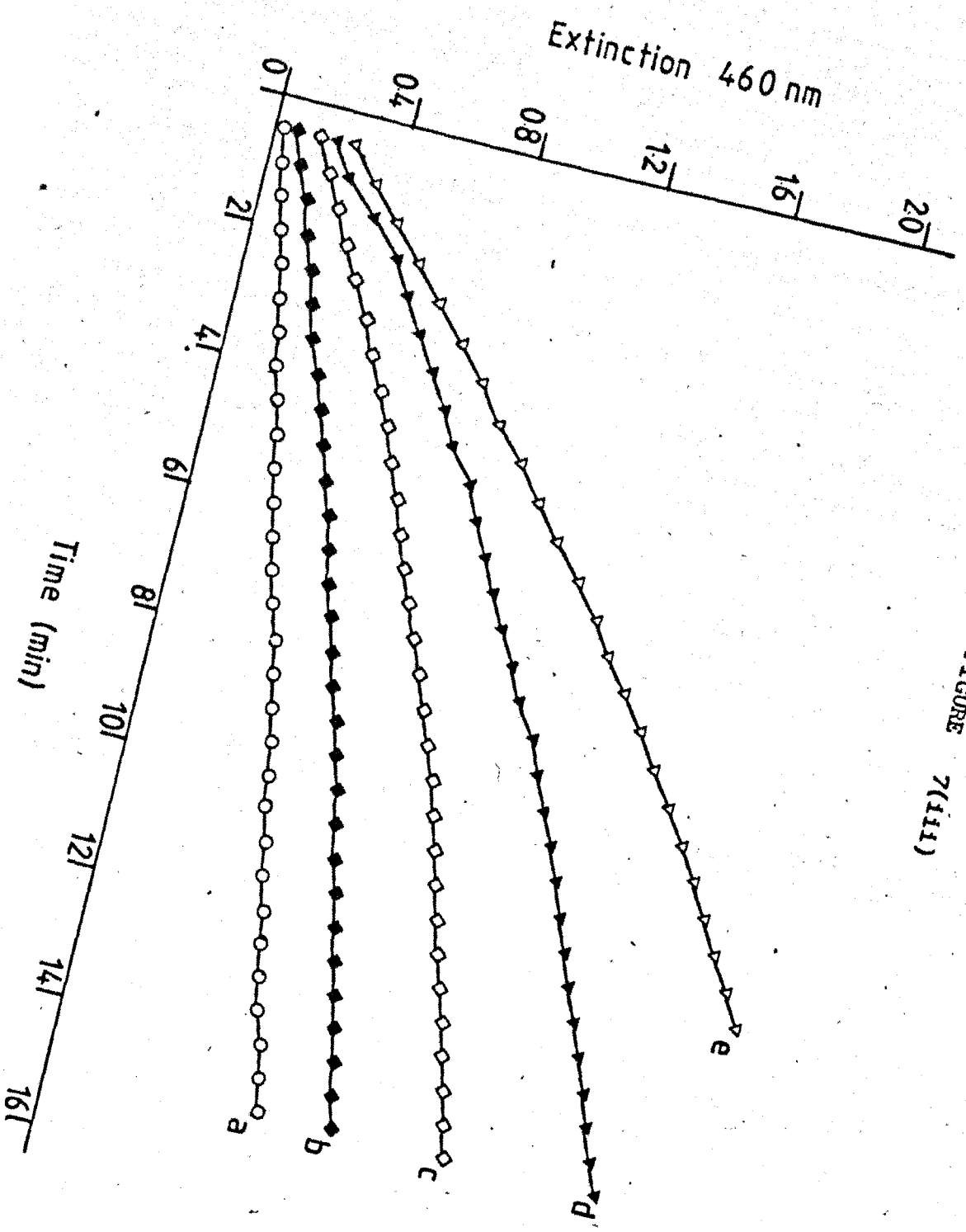


FIGURE 7(iii)

FIGURE 7(iii)

fall well within the measurement range of the spectrophotometer used. Taking these points into account a discontinuous assay method was carried out as described in the next section (7.4).

7.4 A Discontinuous Assay Method for HRP

7.4.1 Introduction

The pH range which HRP was found to be catalytically active, was found to be between pH 5.0 and 7.0 with an optimum at pH 6.0. At any pH above or below this range progressive irreversible denaturation of the enzyme occurs and the reaction ceases. Therefore the quickest and easiest method of stopping at HRP catalysed reaction would be to rapidly increase or decrease the pH. In the following discontinuous assay method, the pH was lowered rapidly by the addition of 5M hydrochloric acid pH 1.0 to give a final solution of pH 1.2. The addition of hydrochloric acid had a twofold affect, it stopped the reaction and simultaneously developed a pink colour which was subsequently read at 525 nm. Preliminary experiments showed that when the reaction was stopped in this way, no further reaction occurred up to 3h.

7.4.2 Method

a) Reagents

Reaction mixture, as described in Section 7.3.2.

5M hydrochloric acid pH 1.0.

b) Procedure

To duplicate 0.1 ml samples of test solution was added 2.9 ml of the reaction mixture from a rapid delivery pipette to facilitate mixing. The reaction was allowed to proceed for exactly 10 min at room temperature. To stop the reaction and simultaneously develop the pink colour, 4 ml of 5m hydrochloric acid were added. After mixing the extinction at 525 nm was measured. A standard graph was obtained with HRP standard solutions containing 0 - 0.5 µg/ml of enzyme. The standard graph was obtained with HRP solutions dissolved in Tc medium 199 containing 10% CS and 1mM ATP.

7.4.3 Results

Fig. 7(iv) shows that a linear relationship exists between enzymatic

FIGURE 7(iv) Plot of extinction 525nm against concentration of HRP from the discontinuous assay method for HRP

Computed slope and regression coefficient were 1.090 and 0.997 respectively.

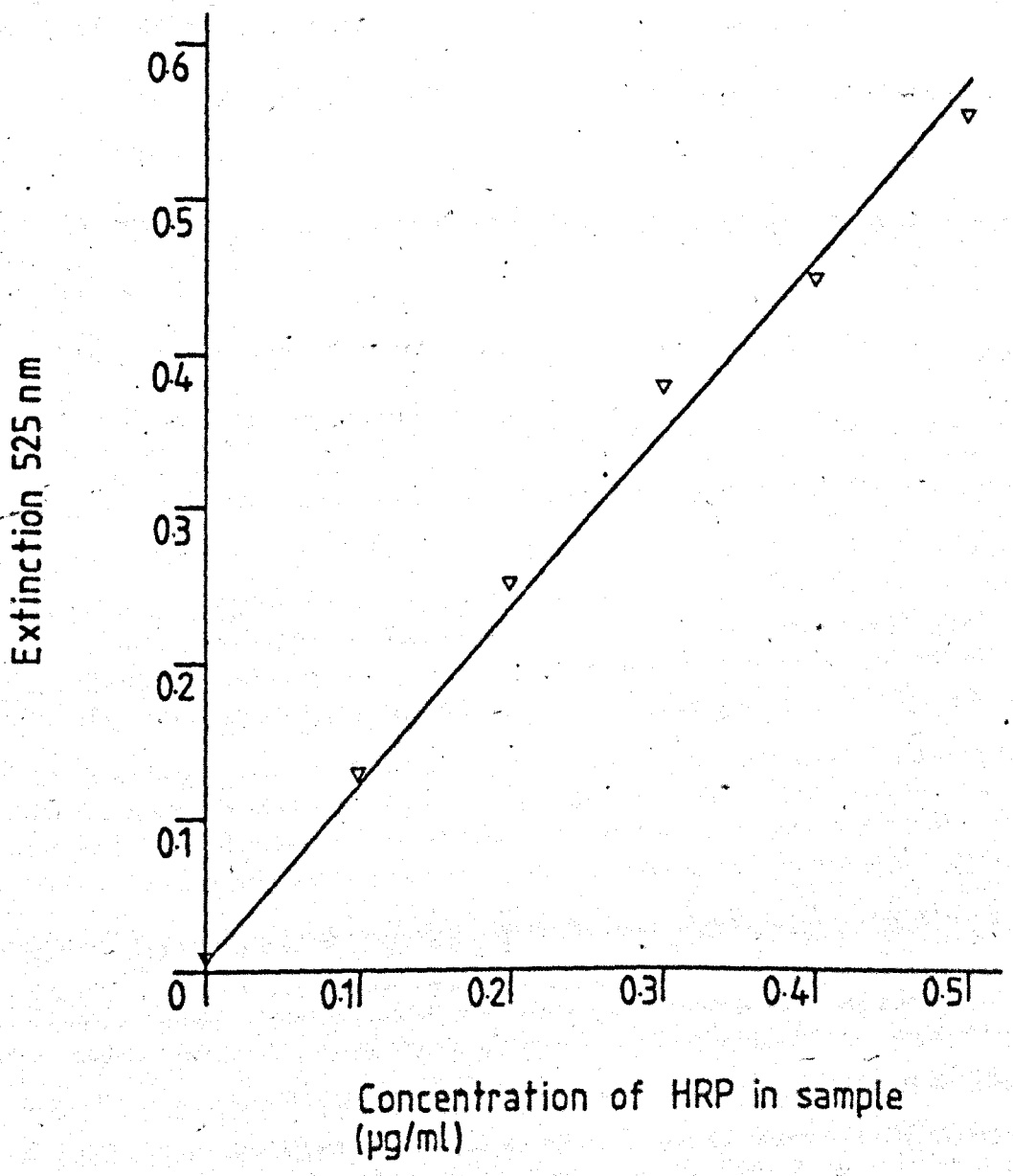

0·6
0·5
0·4
0·3
0·2
0·1
0
0·1
0·2
0·3
0·4
0·5
Extinction 525 nm
Concentration of HRP in sample
(µg/ml)

FIGURE 7(iv)

activity and enzyme protein content, reflected by the high regression coefficient of the computer-fitted line when HRP samples were initially dissolved in Tc medium 199 containing 10% CS and 1mM ATP.

7.4.4 Conclusions

The discontinuous assay described above provides a rapid, accurate method for the assay of large numbers of samples. However, for the assay of HRP by this method, two points must be observed.

a) HRP samples must be dissolved in Tc medium 199 containing 10% CS and 1mM ATP just prior to assay for optimum enzyme activity.

b) All samples must be serially diluted to fall within the range 0 - 0.5 µg/ml.

The application of this method to the measurement of HRP uptake by intestinal sacs is described in Section 7.7.

7.5 Preparation of Tissue for the Assay of HRP

7.5.1 Introduction

In order to monitor the tissue accumulation of [^{125}I]-PVP, the intestinal sac tissue was solubilized in 1M NaOH (25ml) prior to assay (see Section 3.6). Obviously, this method of tissue solubilization would not be possible for the assay of tissue accumulation of HRP as the enzyme would be denatured. Therefore, conditions for the solubilization of the tissue which maintained enzyme activity using the available equipment had to be determined. The most rapid and efficient method of tissue disruption available was by homogenization. A homogenization medium was chosen which could preserve the activity of the enzyme while preventing its degradation by other enzymes released during the cell disruption process e.g. lysosomal enzymes. Most lysosomal enzymes have an acid pH optimum between 3.0 - 5.5 (Dean, 1977). Thus a suspending medium of 0.05M phosphate buffer pH 8.0 was chosen as being the most suitable. The optimal conditions for tissue homogenization using the available homogenizer (Virtis) were determined and the effects that the homogenization procedure had on HRP activity were assessed.

7.5.2 Method

Freshly excised, everted jejunal sacs were used in a total volume of 0.05 phosphate buffer pH 8.0 of 25 ml. Homogenization was carried out as follows using a "Virtis" homogenizer (Virtis Research Equipment, Gardiner, New York, U.S.A.) at setting 6 in a 50 ml flask with two blades attached at right angles to each other. All following operations were carried out at 4^{o}.

1) 15 seconds
2) 30 seconds
3) 45 seconds
4) 60 seconds

Under these conditions, homogenization was seen to be complete after 30

FIGURE 7(v) Comparison of enzyme activity between HRP homogenized for 30 secs in the absence (a) and presence (b) of intestinal sacs

Computed slope and regression coefficient of each graph were as follows:

a) 1.10 slope ; 0.999 reg. coeff.

b) 0.96 slope ; 0.995 reg. coeff.

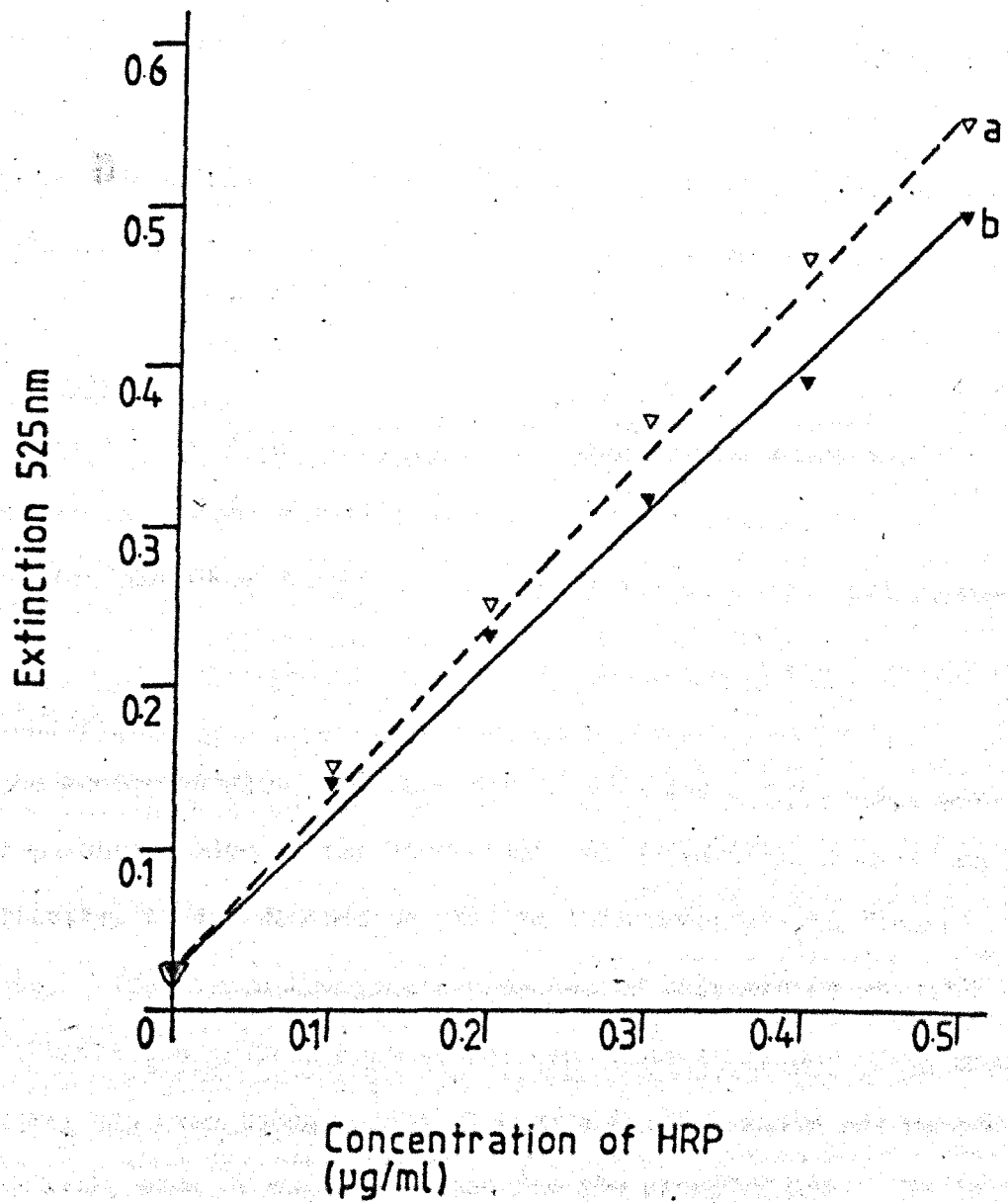

0·6
0·5
0·4
0·3
0·2
0·1
0
Extinction 525nm
0·1
0·2
0·3
0·4
0·5
a
b
Concentration of HRP
(µg/ml)

FIGURE 7(v)

seconds. Therefore in all subsequent experiments, sacs were homogenized as described above for 30 seconds.

To test the effects of the above homogenization procedure on the activity of HRP, two assays were carried out. In the first assay 0, 25, 50, 75, 100 and 125 µg/ml HRP were each diluted in 0.05M phosphate buffer pH 8.0 (25 ml) and homogenized for 30 seconds with an everted jejunal sac using the "Virtis" homogenizer as described above. A 1 ml sample was then obtained from each homogenate, diluted in 10 ml of Tc medium 199 containing 10% CS and 1 mM ATP and assayed for enzyme activity as described in Section 7.3.2. For a control, 0 - 125 µg/ml HRP were serially diluted in Tc medium 199 to give a standard in the range 0 - 0.5 µg/ml enzyme concentration. This was also assayed as described in Section 7.3.2.

7.5.3 Results

The results are shown in Fig. 7(v). There is no appreciable difference in the enzyme activity of HRP samples subjected to the homogenization procedure compared to the activity of enzyme not homogenized.

7.5.4 Conclusions

As the homogenization procedure described above provides a rapid method of solubilization of the tissue with no appreciable loss of marker enzyme activity, it was decided to utilize this procedure in future experiments. The tissue homogenate produced by this method was also used to estimate the protein contents of each intestinal sac after samples for HRP assay had been taken. A 1 ml sample of homogenate was removed from each flask, some of which was used for the assay of HRP. To the remaining solution in each flask was added 5M NaOH (1 ml) to bring the NaOH molarity of the flask solution to that required for protein solubilization (about 0.2M). The solution in the flask was then assayed for protein as described in Section 2.5.

7.6 Derivation of the Washing Procedure Required to Remove Extracellular HRP Adhering to the Tissue after Incubation

7.6.1 Introduction

In 1972 Walker et al. described the uptake of the macromolecular marker protein HRP and in their study Walker et al used the initial substrate concentration of 10 mM (400 µg/ml). The same substrate concentration was used in the investigations of HRP uptake in this study.

7.6.2 Method

Everted jejunal sacs were prepared and incubated as described in Section 3.6, with 400 µg/ml HRP as substrate for about 10 min. Sacs were then removed and washed in up to 6 changes of 0.9% w/v NaCl (20 ml). Samples of the washing solution were taken and assayed as described in Section 7.4.

7.6.3 Results

The results are summarized in Fig. 7(vi). Each point is the average obtained from samples of washings of 4 separate intestinal sacs. The graph shows no enzyme to be present in the fifth and sixth wash solutions.

7.6.4 Conclusion

For subsequent experiments a washing procedure of 5 changes, (20 ml each), of 0.9% NaCl was used to remove any extracellular HRP adhering to the tissue.

FIGURE 7(vi) The washing procedure required to remove extracellular HRP from everted intestinal sacs after incubation

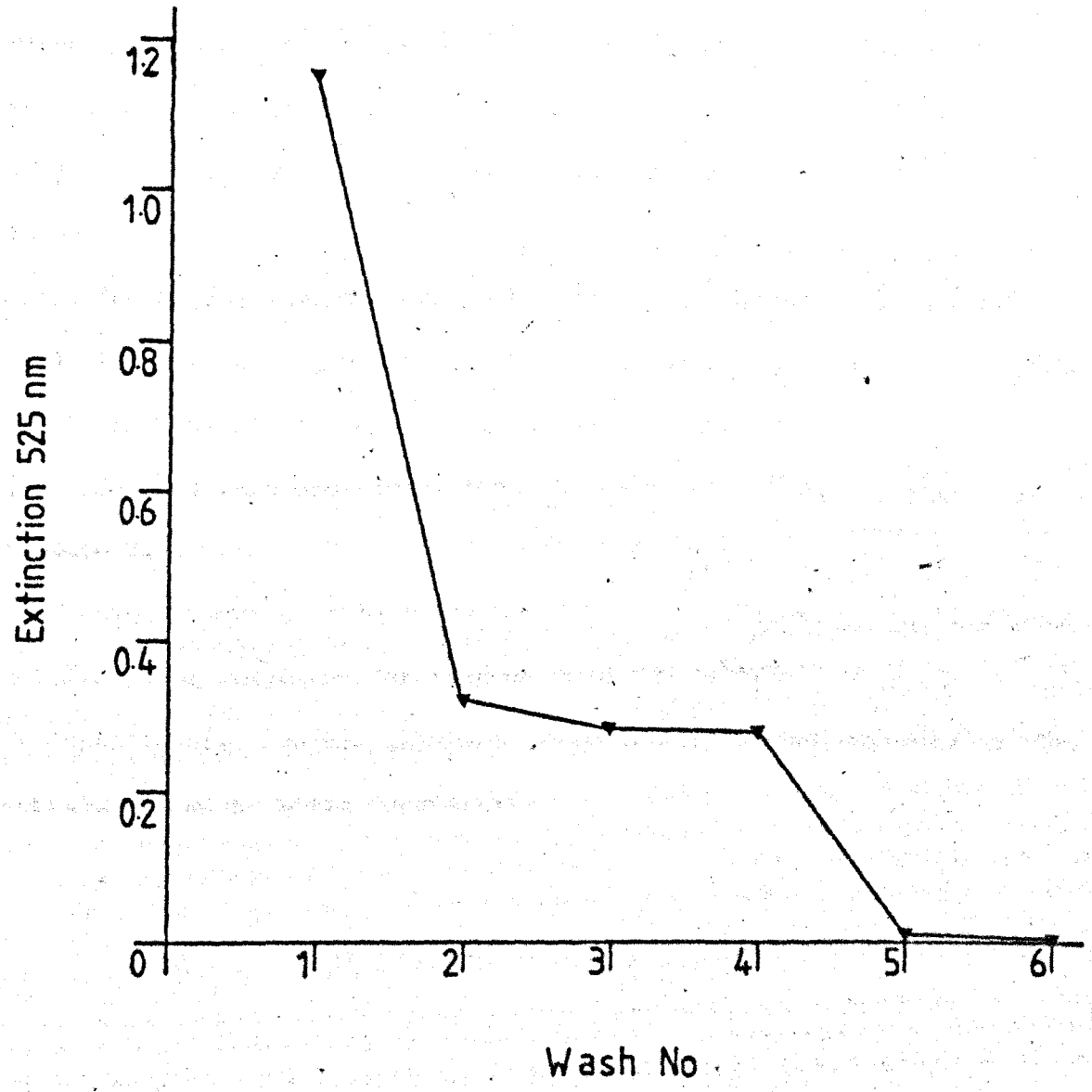


FIGURE 7(vi)

7.7 Standard Method for the Study of Macromolecular Uptake using the Enzymic Marker Protein HRP

The general method was as described in Section 3.6 with the following exceptions:-

(i) Samples were diluted to fall within the range 0 - 0.5 µg/ml. This meant for an original substrate concentration of 400 µg/ml, medium, tissue homogenate and serosal fluid samples had to be diluted 1000, 2 and 20 times respectively.

(ii) The tissue washing procedure described in Section 7.6 was incorporated.

(iii) The tissue was homogenized by the method described in Section 7.5.

(iv) Prior to assay, HRP samples were dissolved in Tc medium 199 containing 10% CS and 1mM ATP to ensure optimum enzyme activity.

(v) Samples were assayed by the discontinuous method described in Section 7.4.

Results were calculated by the method of Williams *et al* (1975) using a G.E.C. 4082 computer; the program used for calculating results is given in Appendix II. Unless otherwise stated, all further experiments were carried out under these conditions.

7.8 Discussion

By the continuous assay, the HRP catalysed reaction showed a linear rate of reaction when the enzyme was dissolved in Tc medium 199 but not when solubilized in Phosphate buffer. This was probably due to the presence of cofactors in Tc medium 199 which were necessary for the optimum activity of the enzyme. The higher rates of reaction seen in the presence of Tc medium 199 supported this theory.

Described in this section is a method for assessing the uptake of HRP. This method was subsequently used to characterize the uptake patterns of HRP in the adult rat intestine as described in Section 8.

CHAPTER EIGHT

THE EFFECTS OF DIFFERENT CONCENTRATIONS ON HRP UPTAKE

8.1 Introduction

Studies on the uptake of degradable substances have the added complication that catabolism of the substrate may occur. In the intestine it is possible that catabolism can occur extracellularly because of the presence of pancreatic proteases and/or brush border peptidases or intracellularly by lysosomal degradation. Consequently it is necessary to monitor substrate degradation in addition to tissue uptake and accumulation of substrate in the serosal fluid to determine, if possible, the site or sites of degradation.

The units of Endocytic Index are sufficiently versatile to describe the uptake of degradable substrates and as shown by Pratten et al. (1979) use of E.I. can give an indication of the mechanism of macromolecular accumulation (see Section 1.4.3). Therefore in this study the uptake of the degradable macromolecule HRP by everted adult rat intestine was measured using the method described in Section 7.7. Using this technique, the basal rates of tissue uptake and accumulation of substrate in the serosal fluid were determined. Concurrently the rate of overall degradation was determined in an attempt to identify the site of degradation. Secondly, the HRP taken up to tissue and accumulated in the serosal fluid was compared with that of stock HRP by column chromatography. Finally the effects of increasing the medium concentration of HRP were also investigated.

8.2 The Uptake of HRP by Adult Rat Jejunum

8.2.1 Method

Everted jejunal sacs were prepared and incubated as described in Section 7.7, for up to 1.75 h in Tc medium 199 containing 10% CS and 1mM ATP with 400 µg/ml HRP as substrate. The rates of HRP uptake into the tissue and accumulation in the serosal fluid were measured and the E.I.'s (µl/h/mg protein) calculated. The total amount of HRP present in the system at any one time was also calculated and plotted against time. Total amount of HRP was obtained by summing the medium and tissue amounts with that in the serosal fluid, expressed in µl/h/mg protein for comparative purposes.

8.2.2 Results

Figs. 8(i)a and b show the results from eight separate incubations, eight sacs per incubation. Each point corresponds to data from a single intestinal sac. An analysis of covariance similar to that described in Section 3.6.1 was applied to this data. For both tissue uptake and accumulation of HRP in the serosal fluid there was no significant difference in the variance at the 5% level, indicating that interexperimental variation was no greater than intraexperimental variation. Hence it was legitimate to pool the uptake data from similar experiments to give a single overall plot (Figs. 8(ii)a and b.

The rates of tissue uptake and accumulation in the serosal fluid of HRP were 11.99 and 0.11 µl/h/mg protein. By a least squares fit analysis tissue uptake was found to be linear with time (regression coefficient 0.94) but accumulation of substrate in the serosal fluid was found not to be linear with time (reg. coeff 0.42).

A plot of the amount of HRP per mg of tissue protein against time (Fig. 8(iii)) showed that the total amount of HRP in the system decreased as the incubation progressed at a rate equivalent to 18.74 µl/h/mg protein.

FIGURE 8(i) Accumulation of HRP in tissue (8(i)a) and serosal fluid (8(ii)b) of everted jejunal sacs incubated for 1.75 h with HRP (400 µg/ml) as substrate

The 64 points in each plot are derived from eight similar experiments, eight intestinal sacs per experiment. Each point corresponds to data from a single intestinal sac.

FIGURE 8(i)a

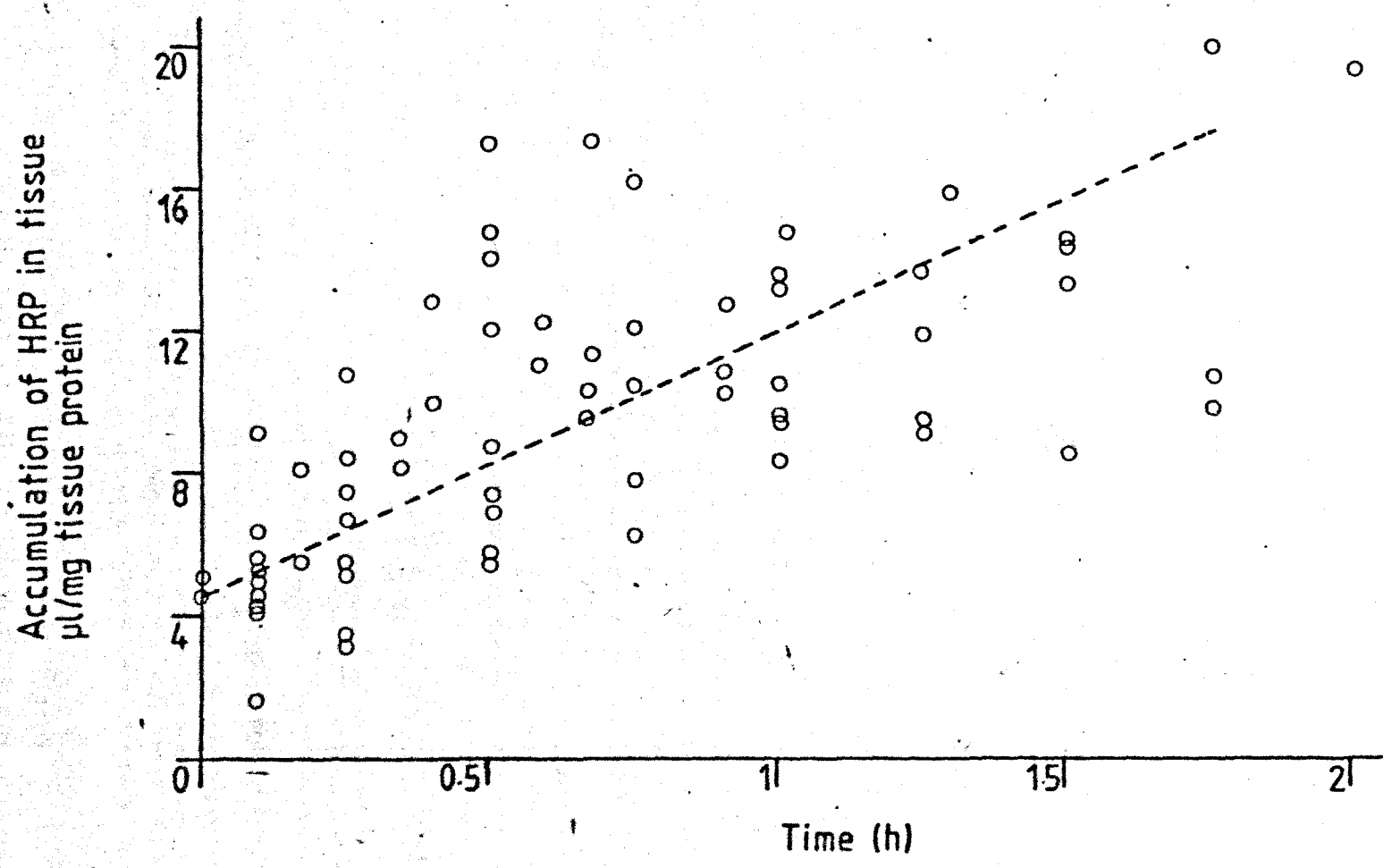

Accumulation of HRP in tissue
µl/mg tissue protein
20
16
12
8
4
0
0.5
1
1.5
2
Time (h)

FIGURE 8(i)b

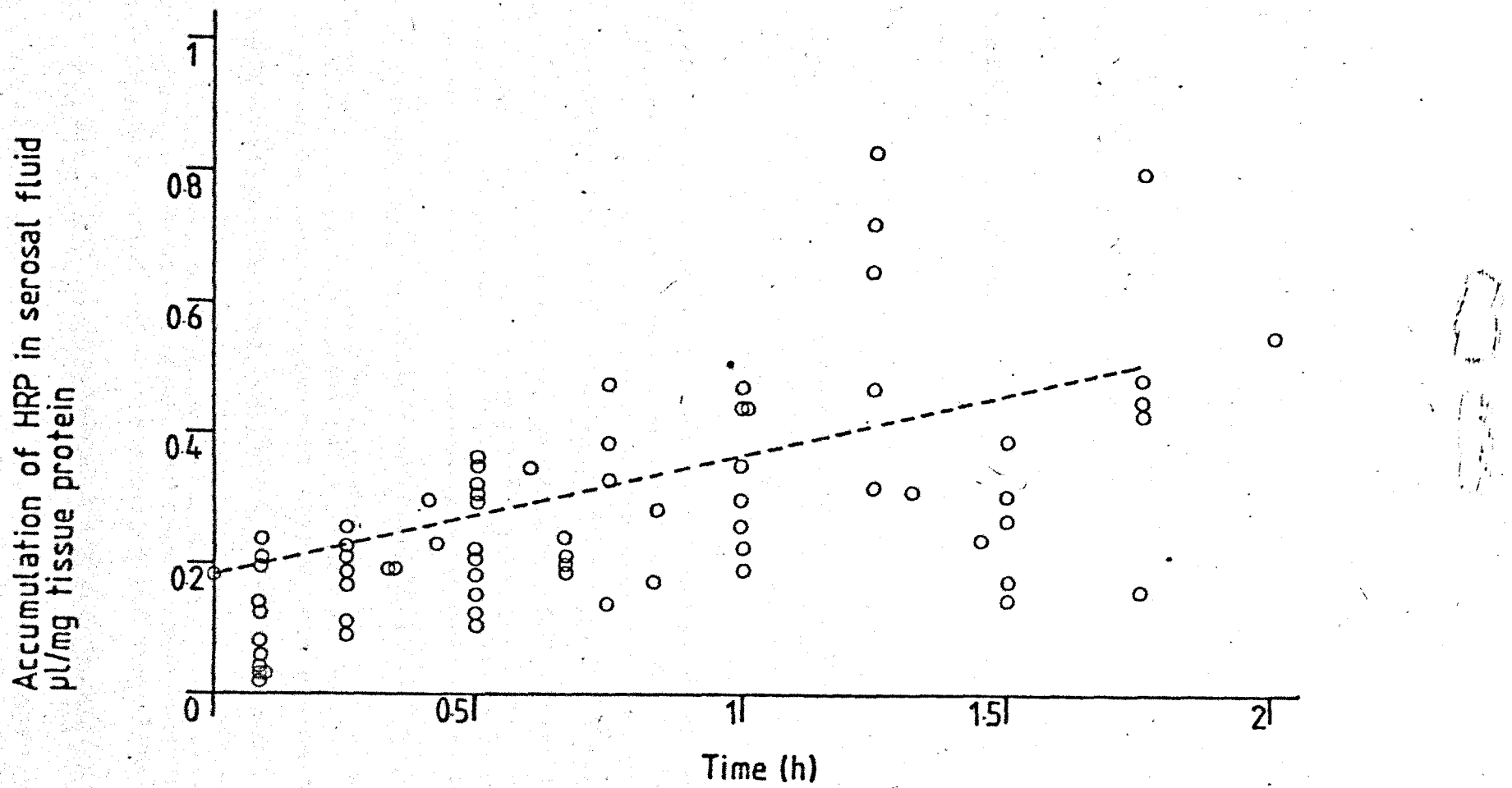

Accumulation of HRP in serosal fluid
µl/mg tissue protein
1
0.8
0.6
0.4
0.2
0
0.5
1
1.5
2
Time (h)

FIGURE 8(ii) Accumulation of HRP ± S.E. in tissue (8(ii)a) and serosal fluid (8(ii)b) of everted intestinal sacs incubated for 1.75 h with HRP (400 µg/ml) as substrate.

Each point is the mean of those obtained from eight similar experiments.

FIGURE 8(ii)a

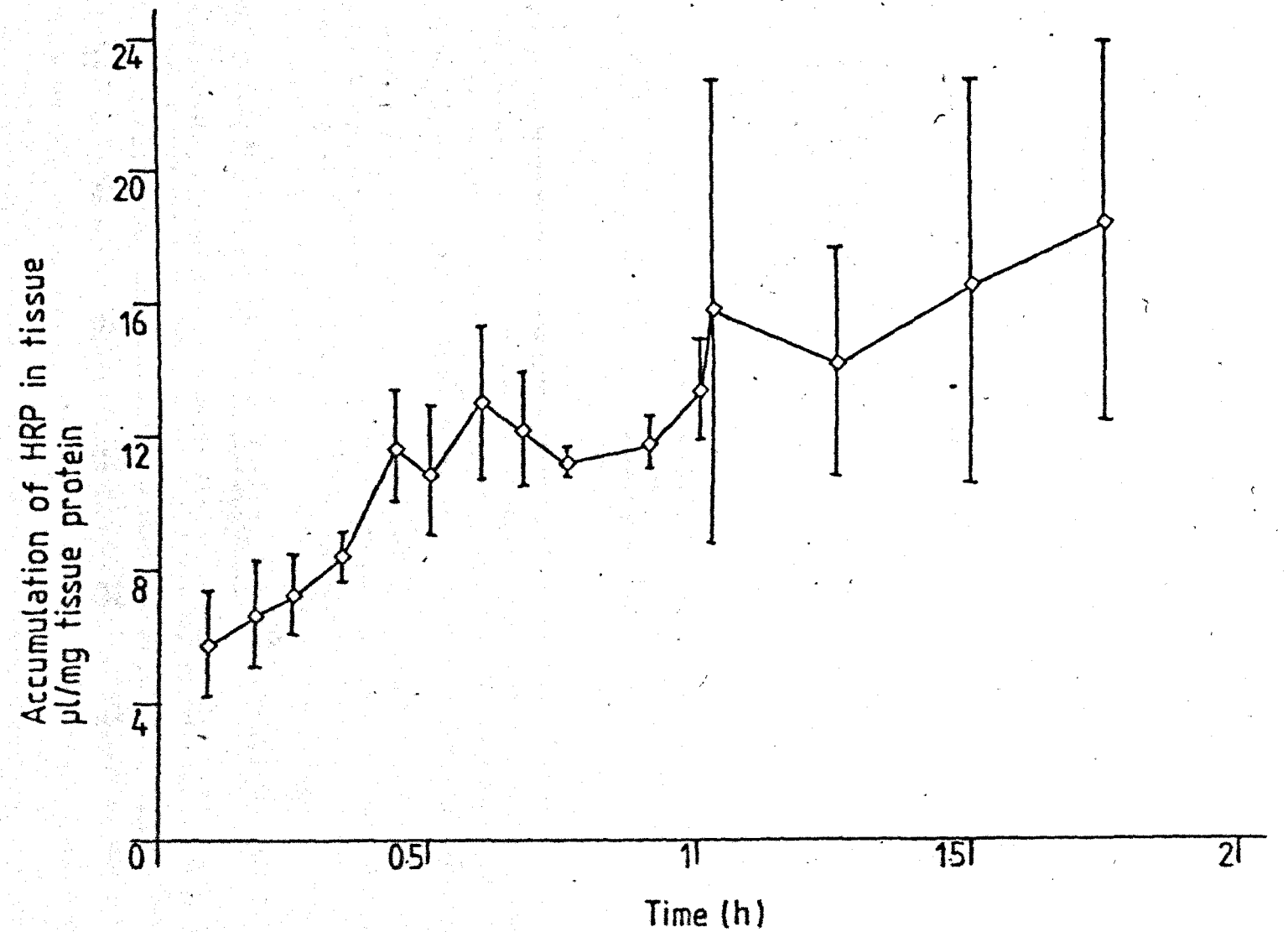

Accumulation of HRP in tissue
µl/mg tissue protein
24
20
16
12
8
4
0
0.5
1
1.5
2
Time (h)

FIGURE 8(ii)b

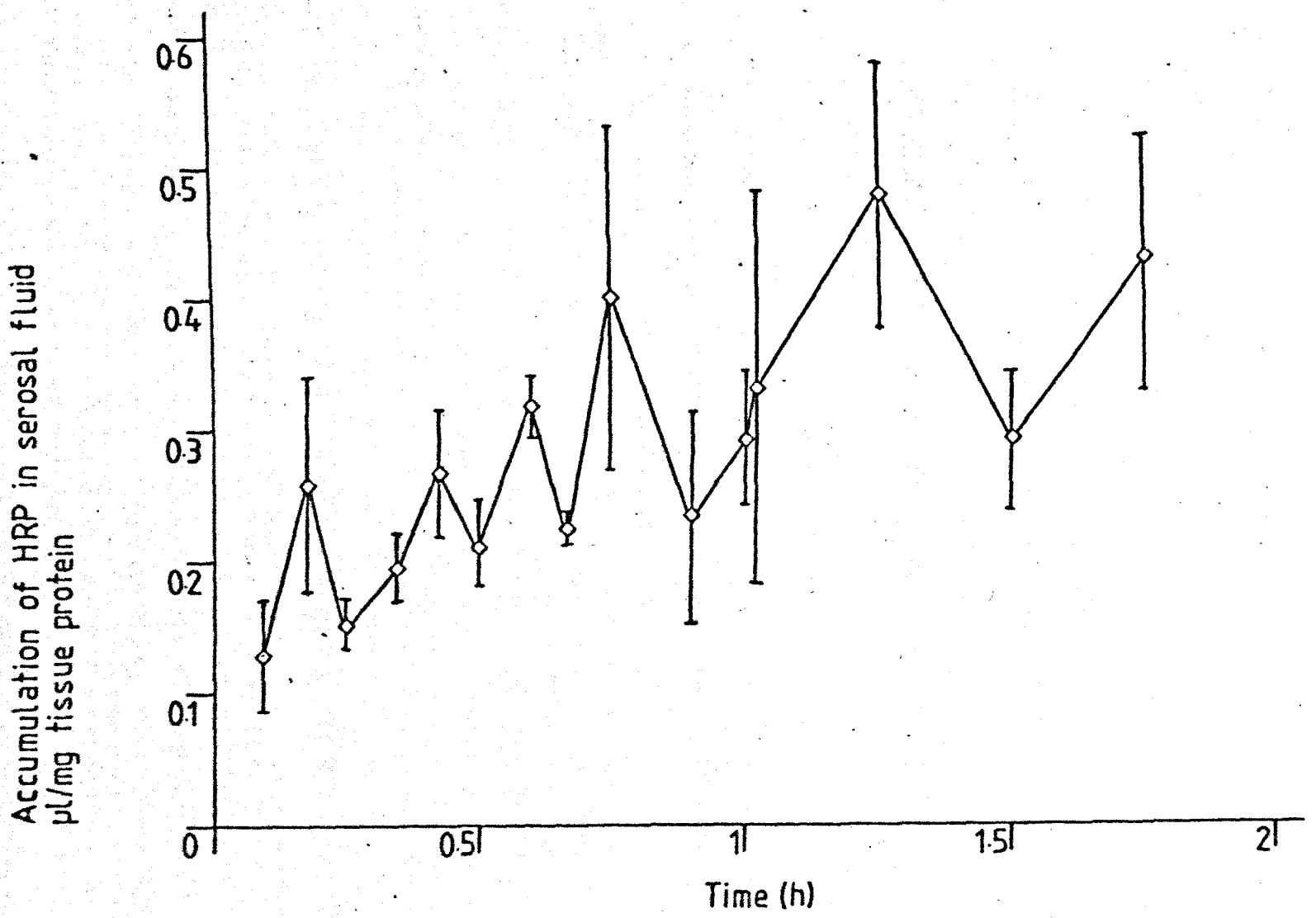
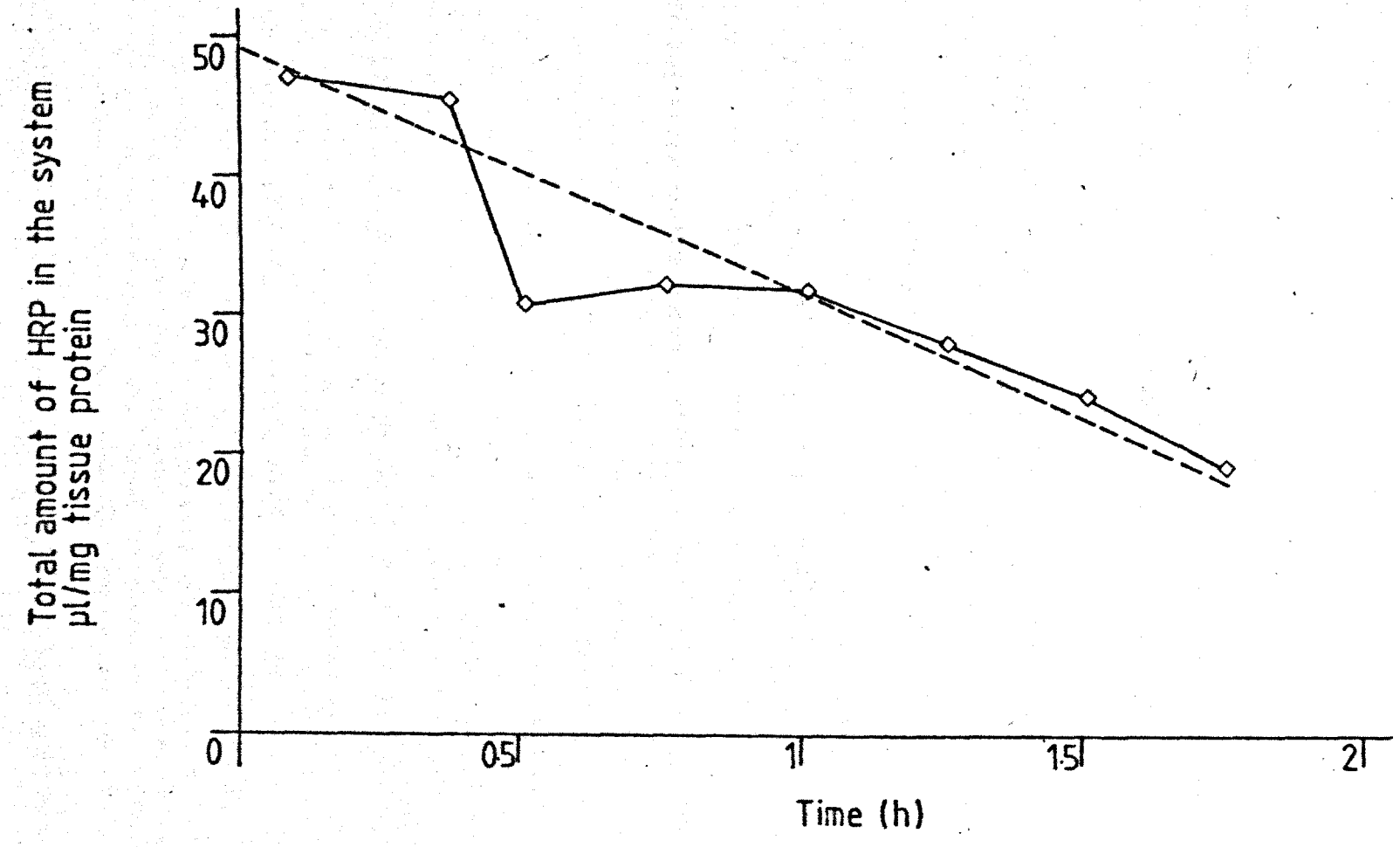

Accumulation of HRP in serosal fluid
µl/mg tissue protein
0·6
0·5
0·4
0·3
0·2
0·1
0
0·5
1
1·5
2
Time (h)

FIGURE 8(iii) Total amount of HRP in the system expressed as µl/mg tissue protein plotted against incubation time (h) during experiments in which everted intestinal sacs were incubated for up to 1.75 h with HRP (400 µg/ml) as substrate.

The plot is the mean of eight separate experiments.

Computed slope and regression coefficient are -18.74 µl/h/mg tissue protein and -0.87 respectively.

FIGURE 8(iii)


Total amount of HRP in the system
µl/mg tissue protein
50
40
30
20
10
0
0.5
1
1.5
2
Time (h)

The tissue content of HRP (µl/mg protein) was plotted against amount of HRP in the serosal fluid (µl/mg protein) shown in Fig. 8(iv). A least squares fit analysis carried out on this data revealed that there was no linear relationship between tissue uptake and accumulation of HRP in the serosal fluid.

8.2.3 Conclusions

The plots of accumulation of HRP against incubation period showed an increase with time for both tissue uptake and accumulation in the serosal fluid but only the tissue uptake was shown to be linear with time.

Total disappearance of HRP from the system was found to be the equivalent of 18.74 µl/h/mg protein and when this is compared with the combined tissue uptake and accumulation of HRP in the serosal fluid (12.06 µl/h/mg protein) it is evident that the substrate disappeared at a faster rate than can be accounted for by tissue uptake alone.

There was no linear relationship between tissue uptake and accumulation of substrate in the serosal fluid.

FIGURE 8(iv) Plot of tissue accumulation of HRP (µl/h/mg tissue protein) against accumulation of HRP in the serosal fluid (µl/h/mg tissue protein).

The data was obtained from eight similar experiments, eight intestinal sacs per experiment. Each point is data derived from one intestinal sac. Computed regression coefficient was 0.46.

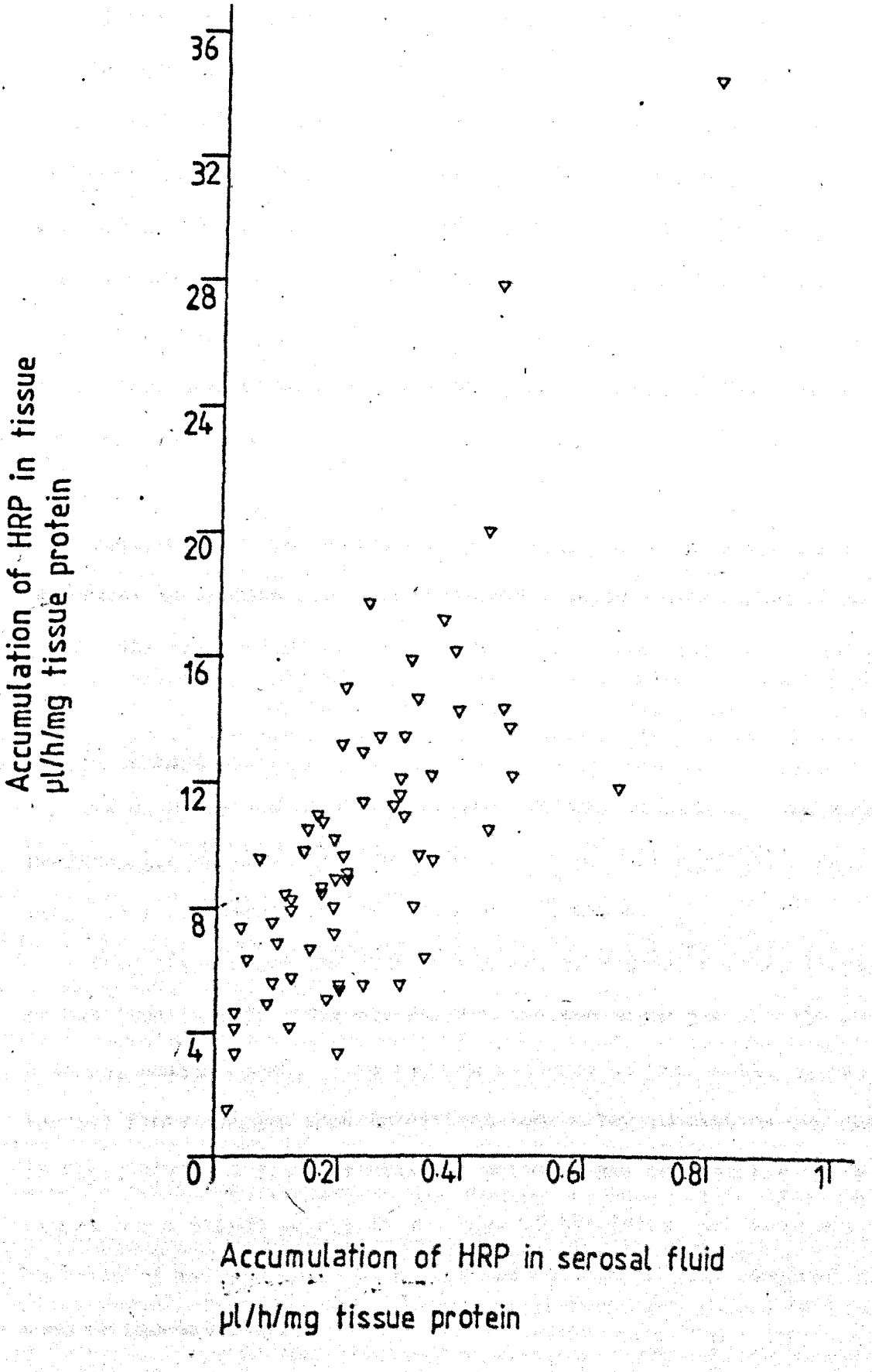

Accumulation of HRP in tissue
µl/h/mg tissue protein
36
32
28
24
20
16
12
8
4
0
0·2
0·4
0·6
0·8
1
Accumulation of HRP in serosal fluid
µl/h/mg tissue protein

FIGURE 8(iv)

8.3 Chromatography of HRP Samples Before and After Incubation with Everted Intestinal Sacs

8.3.1 Method

Everted intestinal sacs were incubated for 1.75 h with 400 µg/ml HRP as substrate as described in Section 7.7. Samples of the original medium and the medium tissue and serosal fluid after incubation were taken and processed as follows: original and incubated medium samples (4 ml) were applied to the column without further treatment; tissue samples were homogenized as described in Section 7.5, freeze dried overnight and the volume made up to 4 ml with Tc medium 199 containing 10% CS and 1mM ATP. The reconstituted samples were then applied to the column. Serosal samples were pooled and a final volume of 4 ml was applied to the column.

Sephadex G 75-80 column chromatography was carried out for HRP as described in Section 2.10 and fractions assayed as described in Section 7.4. The void volume of the column was estimated with Blue Dextran.

8.3.2 Results

Figs. 8(v)a - d show the elution patterns obtained. The total recovery for HRP was found to be about 93% of that applied. Each graph is a representative of 3 separate elutions.

Fig. 8(v)a shows the elution pattern of the unincubated sample of HRP (400 µg/ml). Clearly evident are two major peaks followed by a third, smaller peak. The elution patterns of medium tissue and serosal fluid samples from intestinal sacs after incubation are shown in Figs. 8(v) b - d. The observed patterns are essentially the same except for a slight change in the size of the third peak observed in the case of medium HRP. This peak was reduced in size compared to that of incubated HRP.

FIGURE 8(v) Sephadex G 75 - 80 column chromatography of:

a) Stock unincubated HRP, 400 µg/ml.

b) Medium incubated for 1.75 h with HRP (400µg/ml) present.

c) Homogenate of everted intestinal sacs incubated for 1.74 h with HRP (400 µg/ml) as substrate.

d) Serosal fluid from everted intestinal sacs incubated for 1.75 h with HRP (400 µg/ml) as substrate.

FIGURE 8(v)a

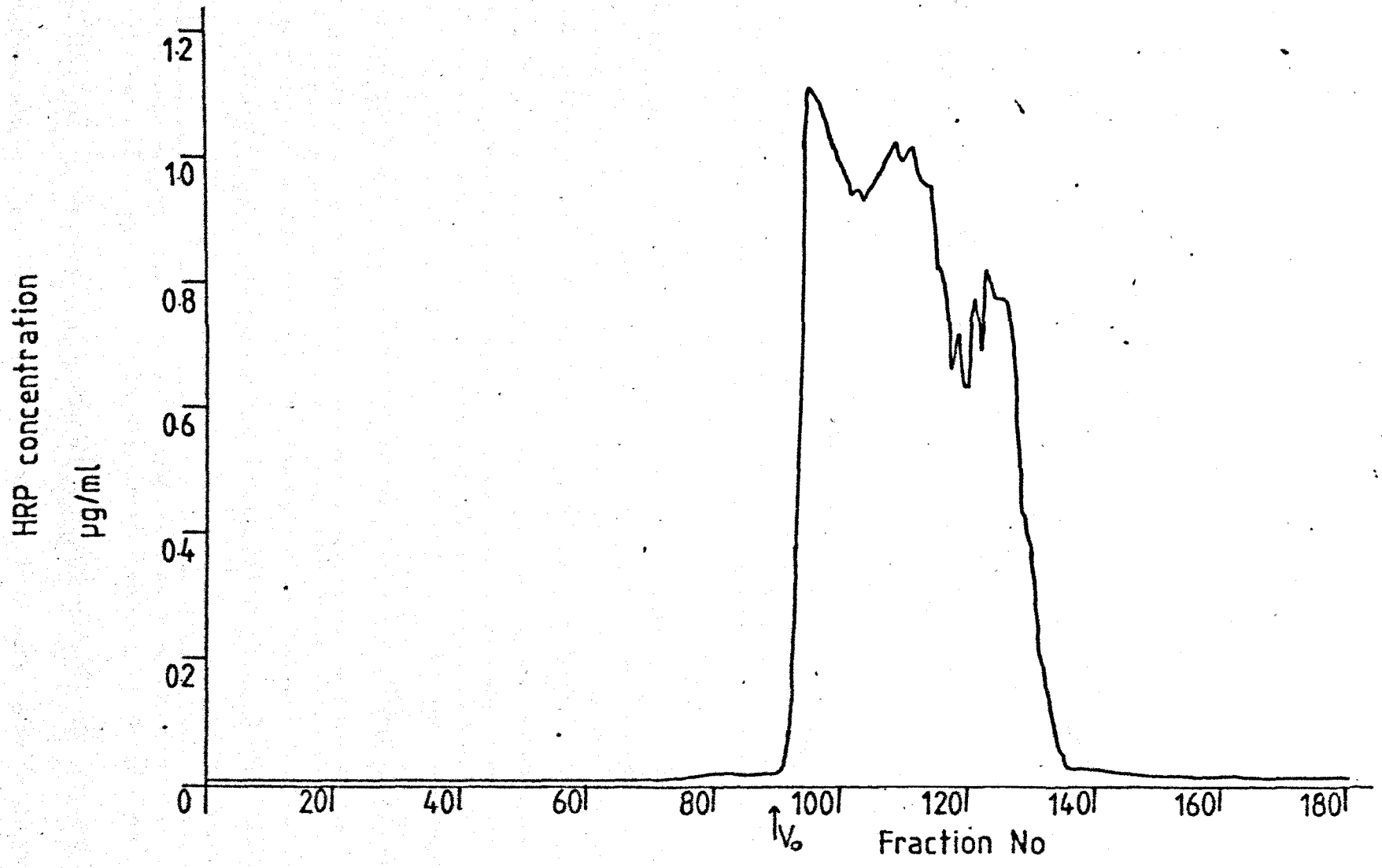

HRP concentration
µg/ml
1·2
1·0
0·8
0·6
0·4
0·2
0
20
40
60
80
100
120
140
160
180
V_o
Fraction No

FIGURE 8(v)b

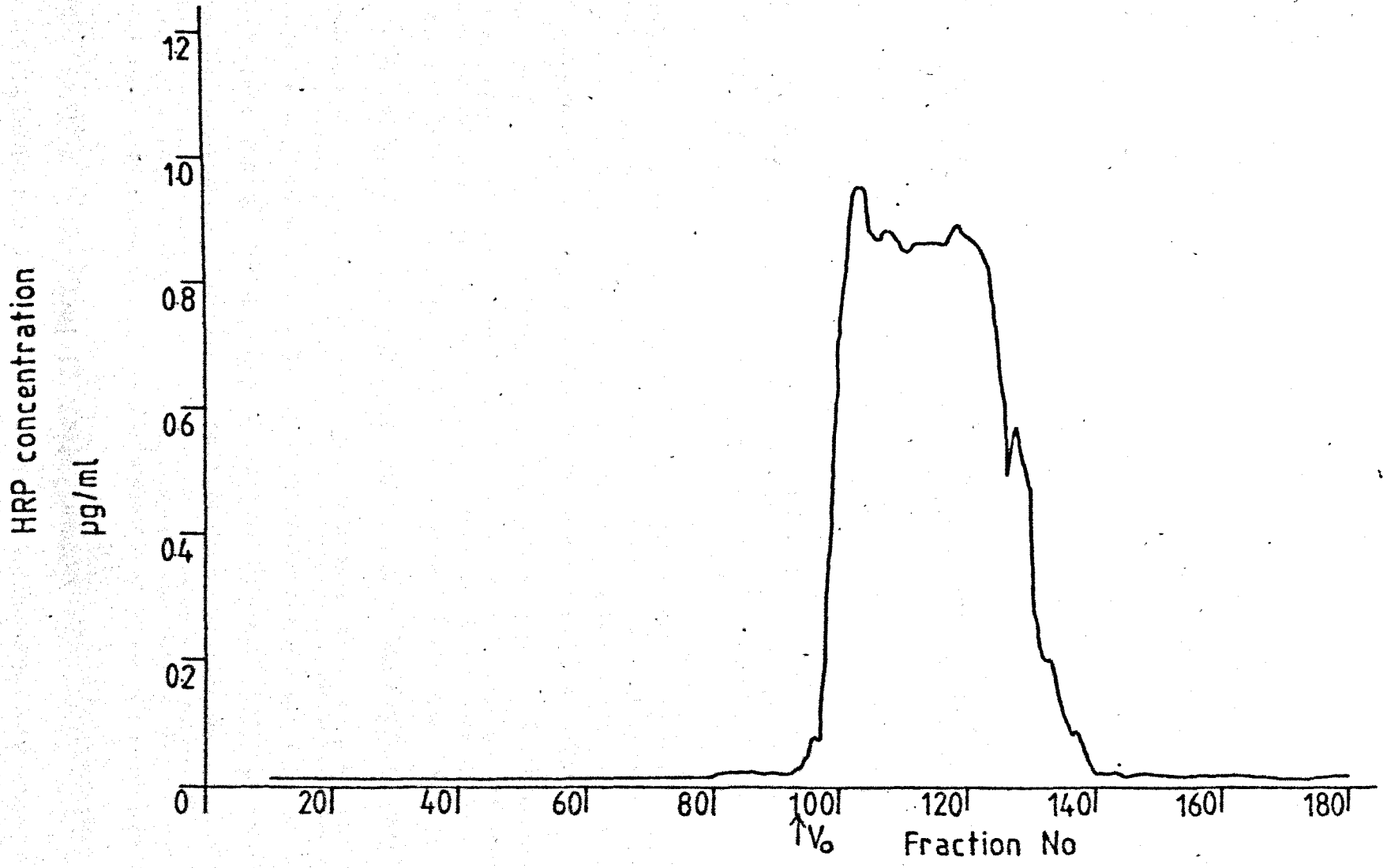

HRP concentration
µg/ml
12
10
08
06
04
02
0
20
40
60
80
100
120
140
160
180
Vo
Fraction No

FIGURE 8(v)c

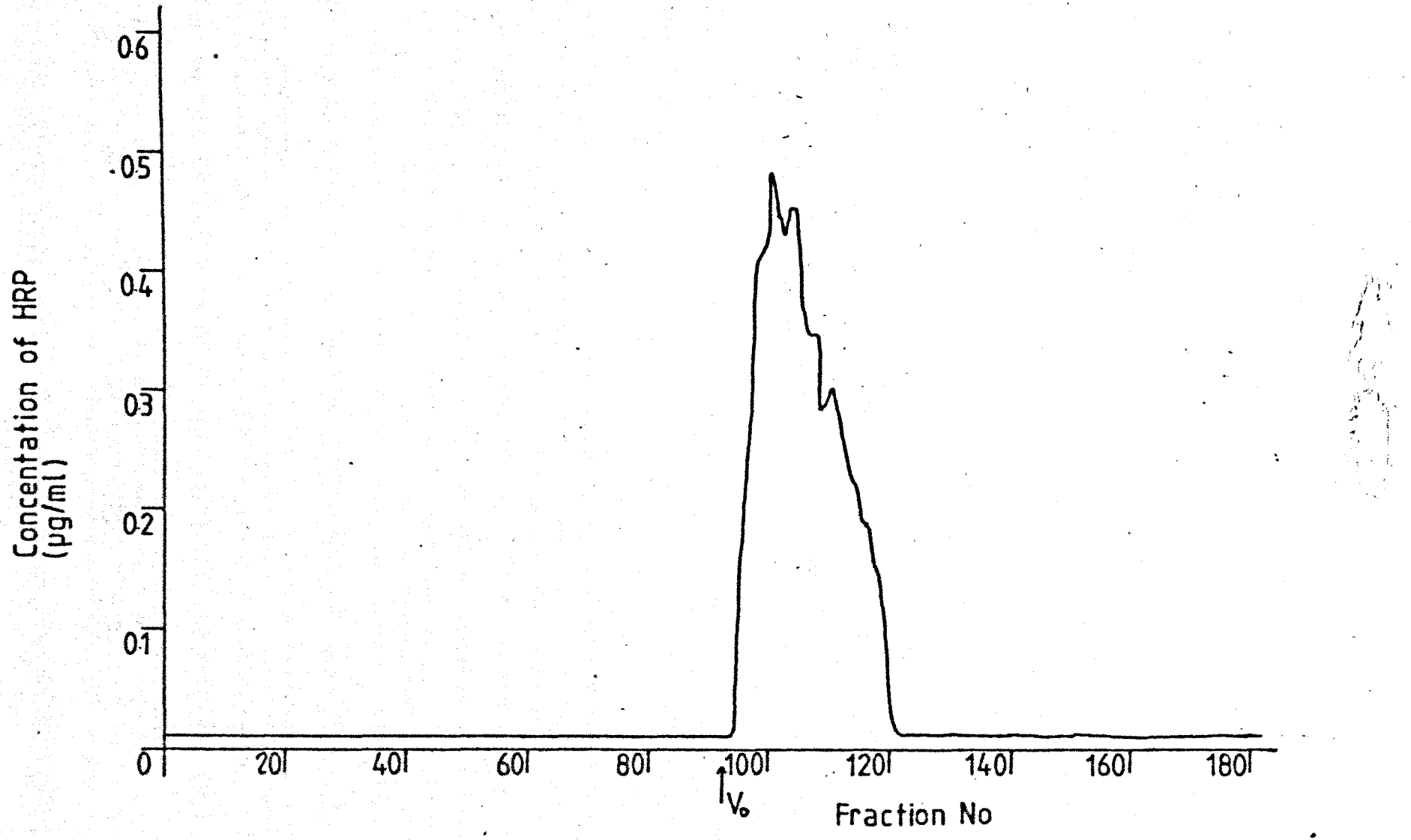
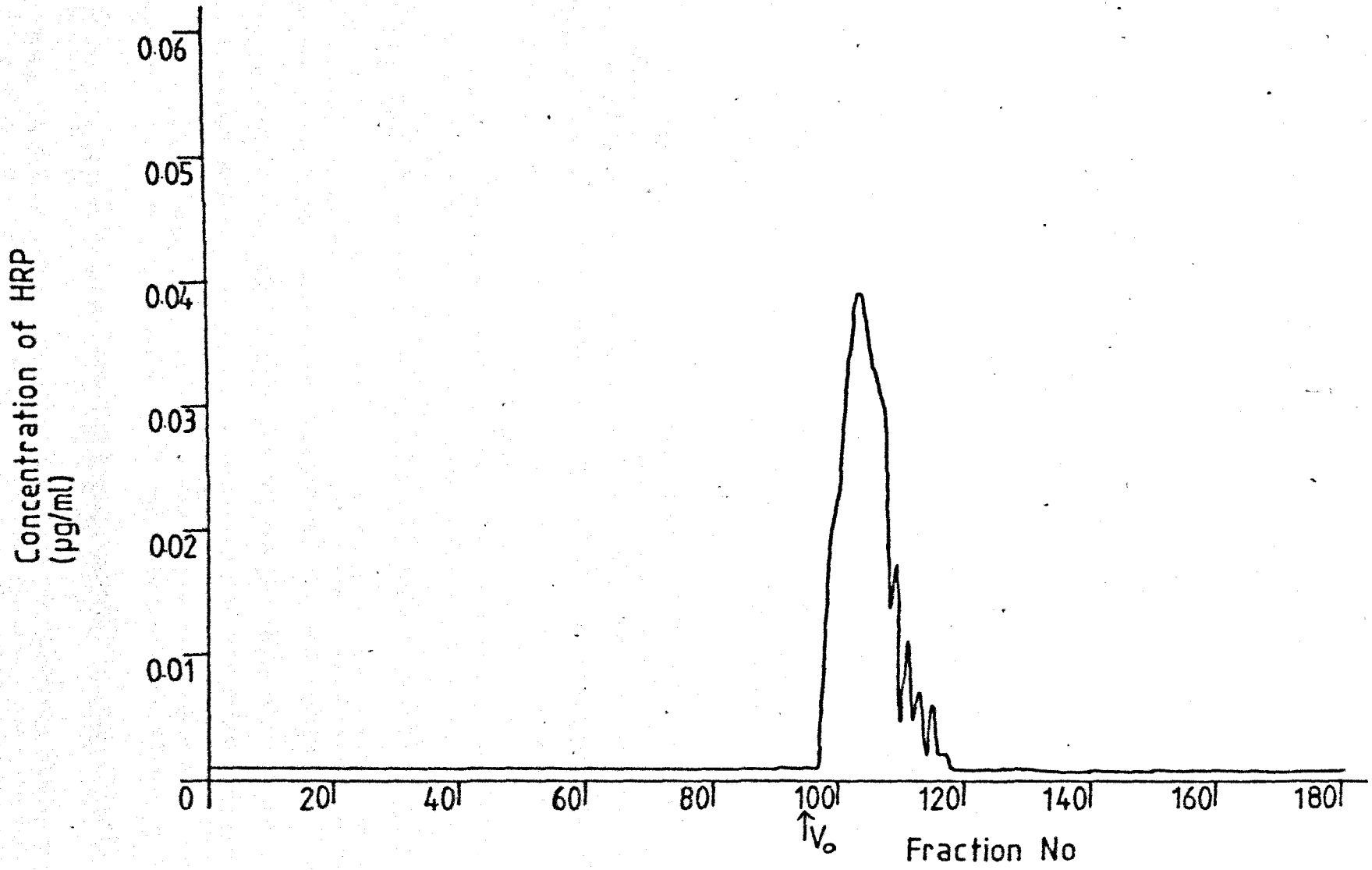

0.6
0.5
0.4
0.3
0.2
0.1
Concentation of HRP (µg/ml)
0
20
40
60
80
100
120
140
160
180
V_o
Fraction No

FIGURE 8(v)d


0·06
0·05
0·04
0·03
0·02
0·01
Concentration of HRP (µg/ml)
0
20
40
60
80
100
120
140
160
180
V_o
Fraction No

8.3.3 Conclusion

The incubation of HRP with intestinal sacs results in a decrease in the medium content of low molecular weight HRP. As the tissue and serosal fluid samples contained HRP similar to that in the medium, the disappearance of the low molecular weight HRP may be due to degradation.

8.4 The Effects of Different Concentrations of HRP on HRP Uptake

8.4.1 Method

Everted intestinal sacs were prepared and incubated for up to 1.75 h in Tc medium 199 containing 10% CS and 1mM ATP as described in Section 7.7. Incubations were carried out using different concentrations of HRP substrate as follows: 5, 10, 20, 30, 50, 100, 200 and 400 µg/ml. The rates of tissue uptake, accumulation of substrate in the serosal fluid and total substrate degradation were calculated (E.I. - in µl/h/mg protein) and plotted against substrate concentrations (µg/ml). Uptake and degradation in ng/h/mg protein were calculated from the E.I.'s and plotted against substrate concentration.

8.4.2 Results

Figs. 8(vi)a, b and c rates of tissue uptake, accumulation in the serosal fluid and total substrate degradation of HRP expressed as E.I. (µl/h/mg protein) and plotted against substrate concentration. Each point is the mean of four separate experiments, eight intestinal sacs per experiment. Rates of tissue uptake and accumulation of HRP in the serosal fluid showed an initial increase of rate of uptake with substrate concentration up to 30 and 50 µg/ml, then the rates decreased and finally levelled out between 100 - 400 µg/ml. The plot of rate of substrate degradation in µl/h/mg protein showed an increase rate of substrate degradation occurred with substrate concentration up to 100 µg/ml. At 50 µg/ml however, a small trough occurred in the graph which indicated that at this concentration, degradation did not adhere to the pattern described by the rest of the graph.

Figs 8(vii)a, b and c showed rates of tissue uptake, accumulation in the serosal fluid and total substrate degradation expressed in µg/h/mg protein plotted, against substrate concentration (µg/ml). The tissue uptake graph described a hyperbolic curve. Accumulation of HRP

FIGURE 8(vi) Plots of tissue uptake of HRP (8(vi)a) and accumulation of HRP in the serosal fluid (8(vi)b) expressed as µl/h/mg tissue protein (E.I.) against substrate concentration. Each point is the mean ± S.E. of four similar experiments, eight intestinal sacs per experiment.

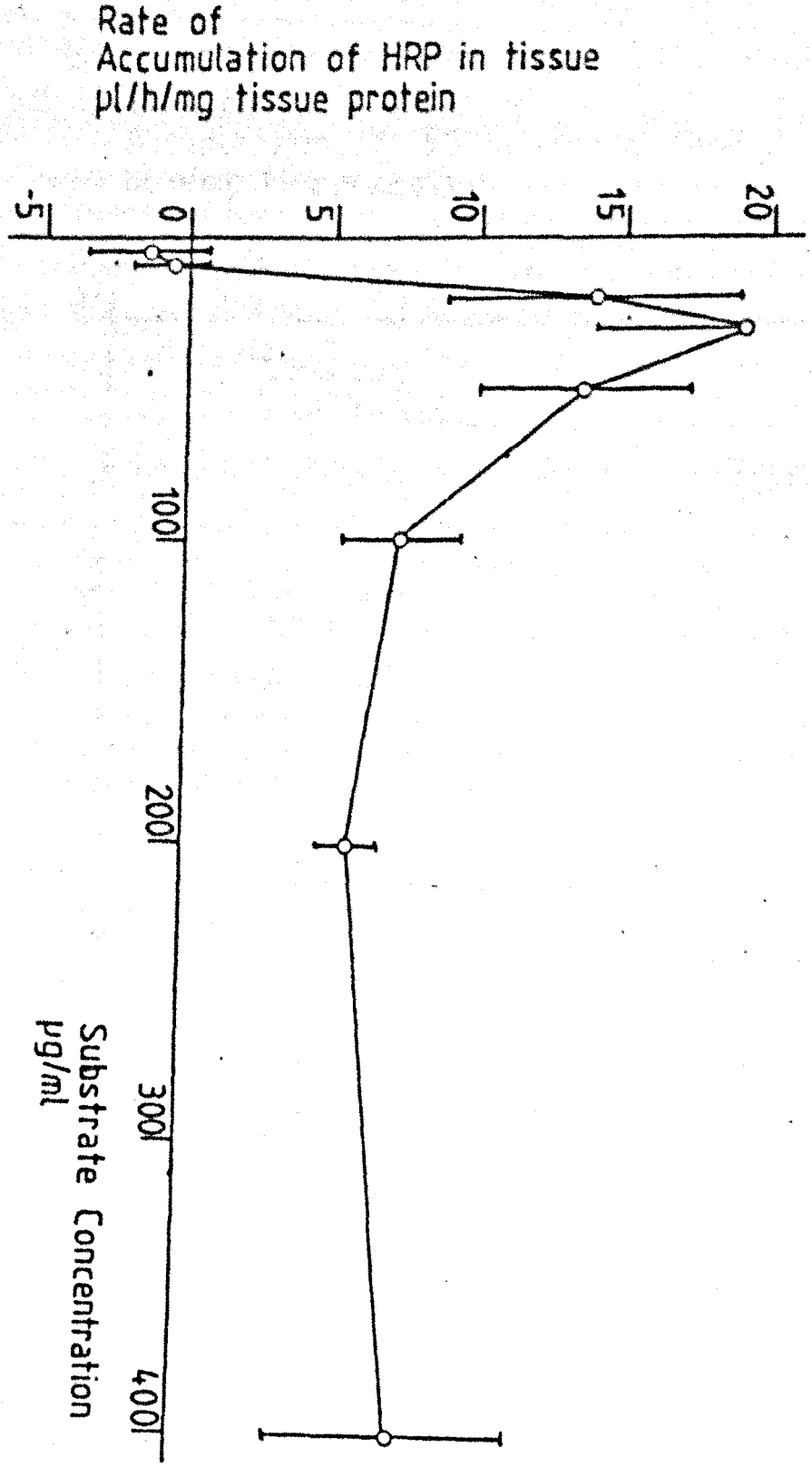

Rate of
Accumulation of HRP in tissue
µl/h/mg tissue protein
-5
0
5
10
15
20
100
200
300
400
Substrate Concentration
µg/ml

FIGURE 8(v1)a

FIGURE 8(v1)b

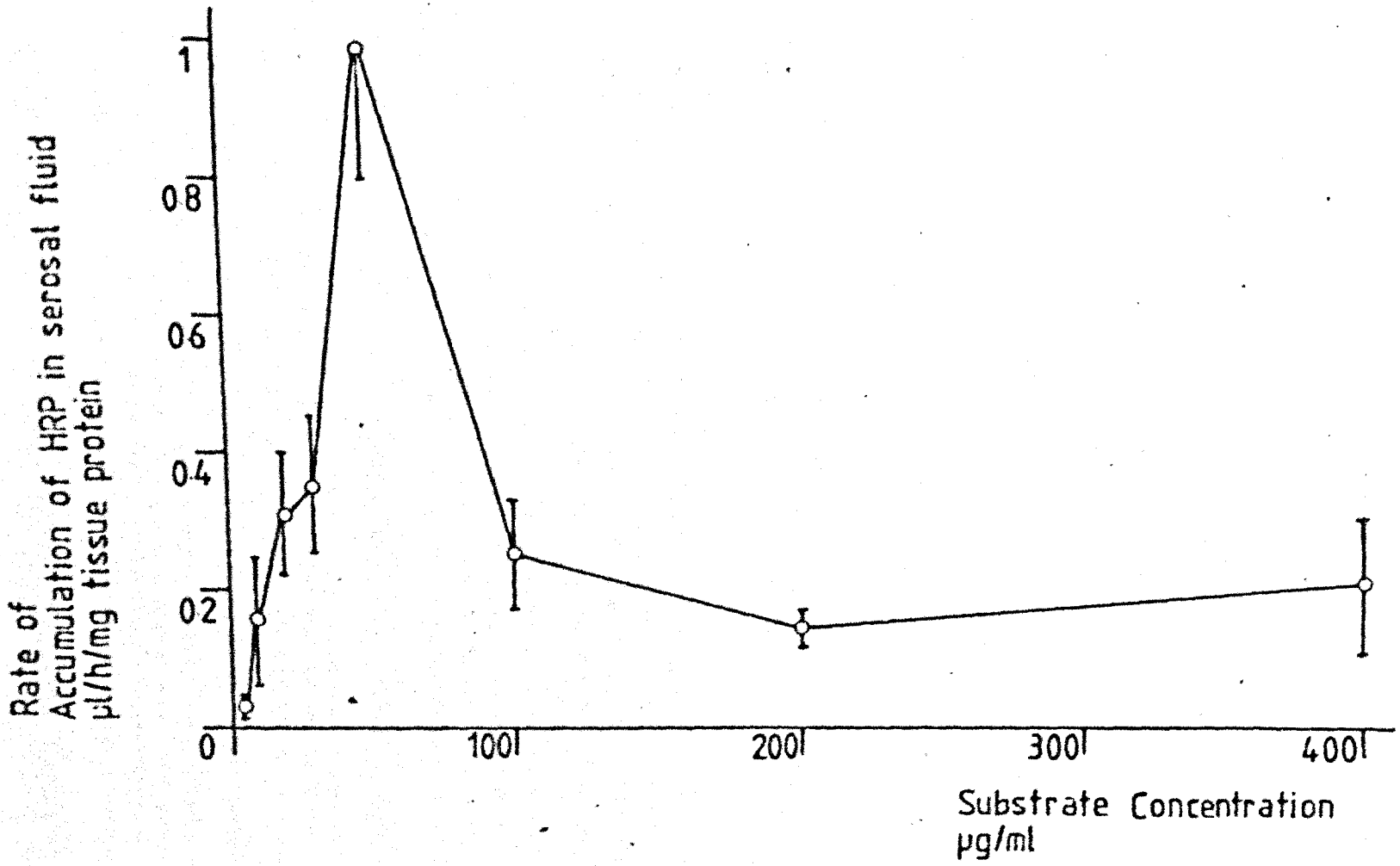

Rate of
Accumulation of HRP in serosal fluid
µl/h/mg tissue protein
1
0·8
0·6
0·4
0·2
0
100
200
300
400
Substrate Concentration
µg/ml

FIGURE 8(vi)c Plots of the rate of degradation of HRP in the system (expressed as µl/h/mg protein) against substrate concentration.
Each point is the mean ± S.E. of four similar experiments, eight intestinal sacs per experiment.

FIGURE 8(vi)c

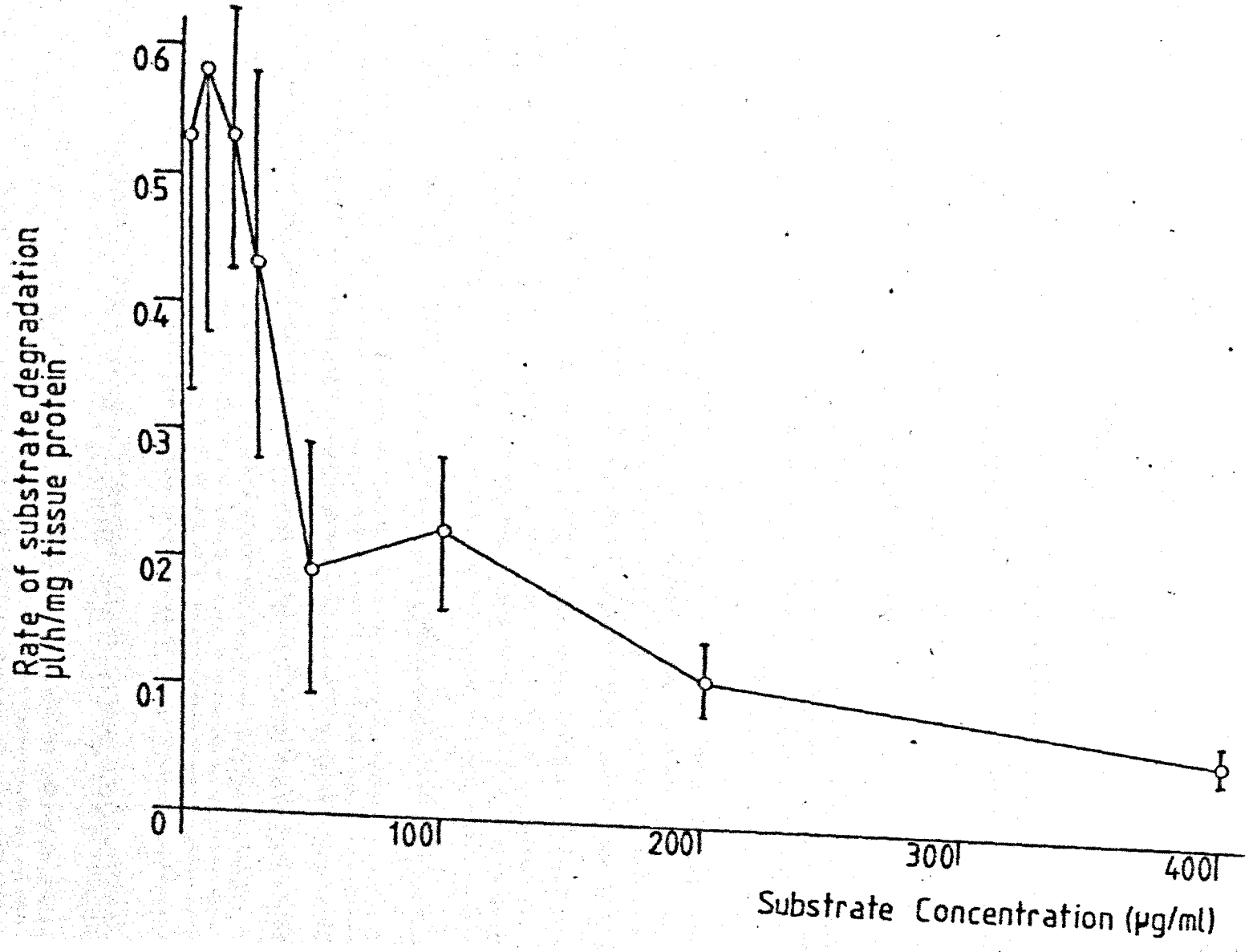

Rate of substrate degradation
µl/h/mg tissue protein
0.6
0.5
0.4
0.3
0.2
0.1
0
100
200
300
400
Substrate Concentration (µg/ml)

FIGURE 8(vii) Plots of tissue uptake of HRP (8(vii)a) and accumulation of HRP in the serosal fluid (8(vii)b) expressed as ng/h/mg tissue protein against substrate concentration.

Each point is the mean ± S.E. of four similar experiments, eight intestinal sacs per experiment.

FIGURE 8(vii)a

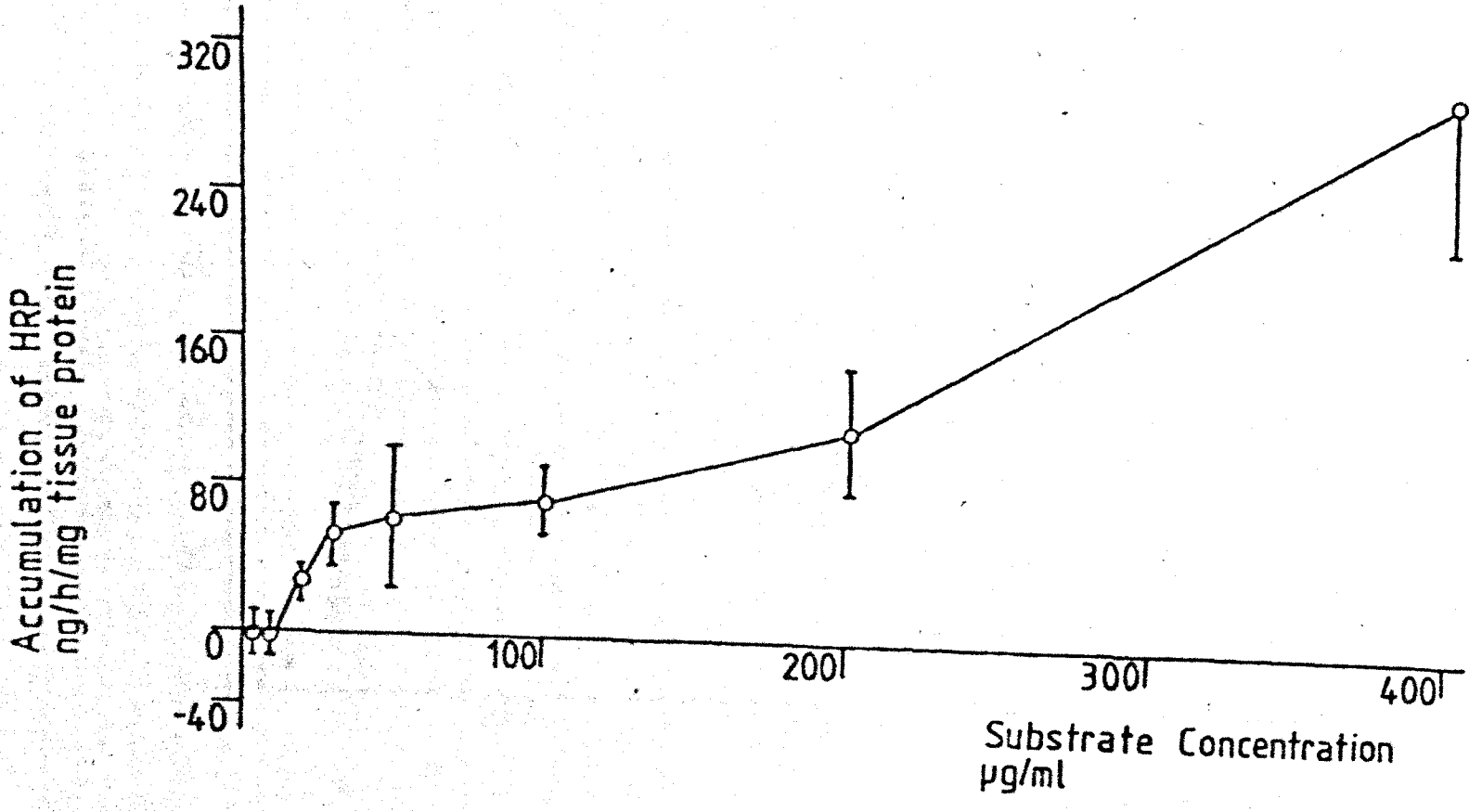


FIGURE 8(vii)b

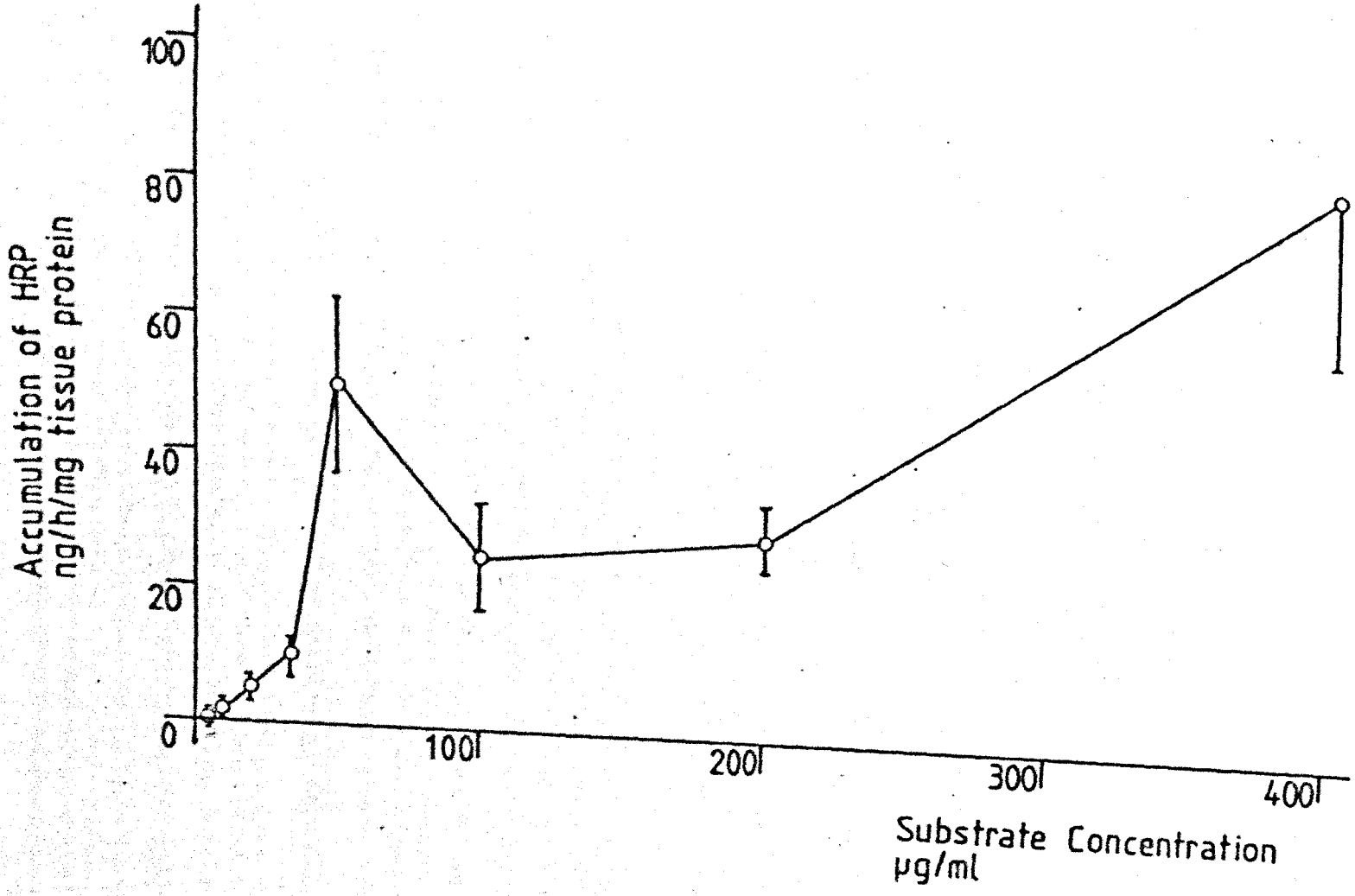
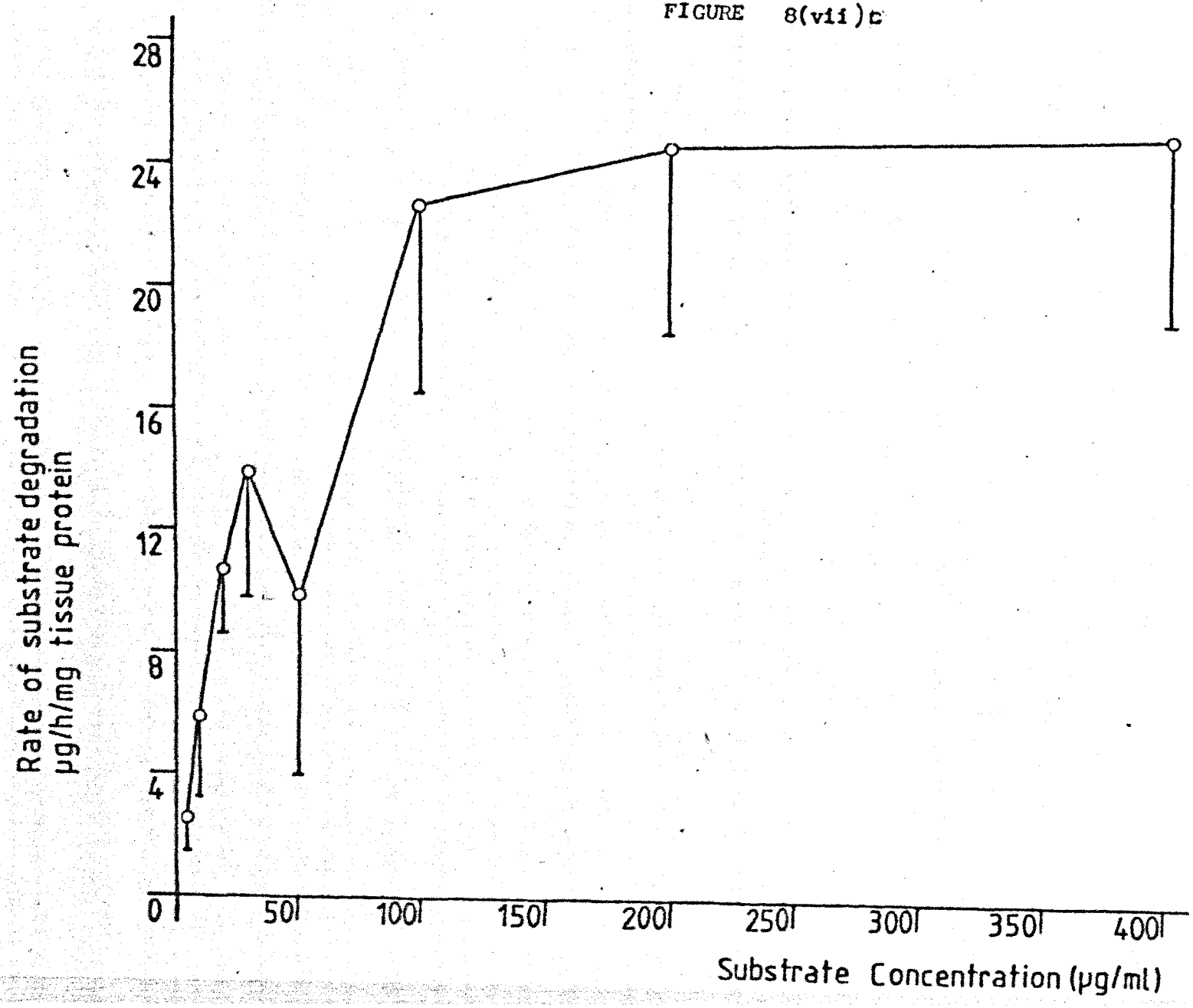


FIGURE 8(ii)c Plot of the rate of degradation of HRP in the system (expressed as ng/h/mg protein) against substrate concentration.

Each point is the mean ± S.E. of four similar experiments, eight intestinal sacs per experiment.

FIGURE 8(vii)c



in the serosal fluid exhibited a similar pattern except a peak occurred at 50 µg/ml. The graph of substrate degradation showed that the rate of degradation in µg/h/mg protein increased progressively up to 100 µg/ml then levelled out.

8.4.3 Conclusions

The endocytic index (E.I.) was dependent upon substrate concentration within the range 5 - 50 µg/ml but between 100 - 400 µg/ml the E.I. became independent of substrate concentration. This was the case for both tissue uptake and accumulation of substrate in the serosal fluid. Uptake expressed in ng per mg of tissue protein per hour increased with substrate concentration but the increase was not linear.

Rate of substrate degradation in µl/h/mg protein was dependent upon substrate concentration in the range 5 - 50 µg/ml, then became independent of substrate concentration. Degradation expressed as ng per unit of tissue protein per hour again increased with concentration but was not linear.

8.5 Discussion

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

The results in Section 8.4 indicated that binding of HRP to the tissue occurred. The E.I.'s were dependent on substrate concentration up to 50 µg/ml for both tissue uptake and substrate accumulation in the serosal fluid, after this (between 100 - 400 µg/ml) the E.I.'s became independent of substrate concentration. Uptake in µg/h/mg protein exhibited a non-linear relationship with substrate concentration. According to Pratten *et al.* (1979) and Silverstein *et al.* (1977) these results are indicative of a substrate undergoing adsorptive endocytosis. It is likely that HRP entered the cells by adsorptive pinocytosis in a concentration independent manner until the sites to which it bound became saturated, then the E.I. decreased. As the substrate concentration rose, the binding component of uptake became less significant and E.I. tended to the fluid phase and became independent of substrate concentration.

A comparison of the rate of total substrate disappearance and the combined rate of tissue uptake and accumulation of substrate in the serosal fluid (18.74 and 12.06 µl/h/mg protein) revealed that the substrate disappeared at a greater rate than could be accounted for by intestinal uptake and transport. This indicated that some substrate degradation had occurred.

According to Matthews (1977) in the intestine *in vivo* three types of protein digestion are recognised. (i) Intraluminal hydrolysis, defined as hydrolysis within the fluid in the intestinal lumen. This is carried out by pancreatic proteases. For *in vitro* preparations this has been termed intra medium hydrolysis (Matthews, 1977). (ii) Cellular hydrolysis. This applies when hydrolysis is not in the lumen and is

associated with the intestinal mucosa. Hydrolysis can be carried out by peptidases associated with the plasma membrane of the brush border or by the action of pancreatic proteases adsorbed in the brush border region. (iii) Intracellular hydrolysis, which occurs either in the cytosol or in some organelle within the cell e.g. lysosomes.

In vitro, due to the absence of intramedium hydrolysis, protein degradation is restricted to the latter two processes. According to Kim (1977), in the rat cellular hydrolysis plays an important role in the digestion of dietary proteins and this process if carried out mainly by amino-oligopeptidases in the brush border membrane. If this is the case, then it is possible that at least some of the degradation of HRP observed in this study occurred by the method described by Kim (1977).

Of the HRP that was taken up into the tissue only a very small amount was transported to the serosal fluid (0.91%). Also the amount of HRP accumulated in the serosal space was not directly related to the amount in the tissue (Fig. 8(iii)). This suggested that some factor prevented the direct transfer of intact HRP from the tissue to the serosal fluid and this factor was likely to have been the degradation of HRP by lysosomal enzymes.

From the results, a mechanism of HRP uptake can be postulated. The macromolecule bound to the membrane where some portion was degraded, the rest was internalized, mainly by adsorptive endocytosis, and fusion with lysosomes led to further degradation by the lysosomal enzymes. This allowed only a small amount (less than 0.066% of the original substrate) to pass to the serosal fluid. Obviously, much more work is required to confirm this hypothesis.

CHAPTER NINE

GENERAL DISCUSSION

9.1 Preparation and Incubation of Intestinal Sacs from Adult Rat Jejunum

A method has been developed for the investigation of macromolecular uptake in adult rat intestine using everted jejunal sacs. While the method was based on that of Walker et al. (1972) using the everted intestinal sac technique of Wilson and Wiseman (1954) there were a number of important differences.

The preparation procedure was devised to reduce preparation time which had the advantage of minimizing temporary hypoxia. According to Fischer and Parsons (1949) and Fischer and Gardner (1974) this is essential in preparation of isolated intestine. The speed of the shaking procedure was reduced after the demonstration that high shaking speeds produced tissue damage. Tissue uptake, in addition to accumulation of substrate in the serosal fluid, was measured and a sampling technique used by which the rate of uptake could be accurately determined. The results were calculated in such a way that experimental variability arising from differences in the amount and specific activity of radioactivity or chemical marker and the quantity of tissue present were normalized. The method of expression of results (after Williams et al., 1975) allowed direct numerical comparison of uptake with different substrates and cell types.

Most importantly, the use of a complex medium such as Tc medium 199 containing 10% CS and 1 mM ATP was found to be highly necessary for the maintenance of tissue viability and structural integrity throughout the incubation period. The viability of the incubated intestinal sacs was assessed by glucose and methionine accumulation and oxygen consumption. Structural integrity was assessed by light and electron microscopy and appeared better than that of sacs prepared by different methods and incubated for shorter or equivalent periods of time (Plattner et al., 1970; Lepper and Mailman, 1977).

The use of complex medium had the added advantage that the absorptive surface was presented with a large variety of substances. A study of macromolecular uptake under such conditions is nearer to the physiological state in that the macromolecule is not taken up solely because it is the only nutrient available.

Compared with the method of Walker *et al.* (1972) the method described in this thesis was superior and resulted in tissue which showed better viability and structural integrity for longer periods of incubation. Using this method it has been possible for the first time to accurately study the kinetics of macromolecular uptake by adult small intestine and to make valid observations on the mechanism of uptake.

9.2 Mechanism of Macromolecular Uptake by the Adult Rat Intestine

As far as is known, the work presented in this thesis is the first major quantitative assessment of macromolecular uptake by adult intestine *in vitro*. The reason for this lack of quantitative study may be largely due to the dogma that macromolecular uptake ceases completely in mammals early in the neonatal stage of development, i.e. after "closure". Therefore to assess the character of macromolecular uptake in the adult comparison must be made with that of neonatal intestine and other cell types.

The sequence of events in the uptake of soluble macromolecules (pinocytosis) are outlined in detail in Section 1.2 and the differentiation between fluid phase and adsorptive pinocytosis is outlined in Section 1.4.3. It is with these processes that macromolecular uptake by adult rat intestine is now compared.

9.2.1 The uptake mechanism of [^{125}I]-PVP by adult rat intestine

Using the technique developed in this study, useful information was obtained which clarified the mechanism of macromolecular uptake in adult rat intestine.

When [^{125}I]-PVP was used as substrate, the macromolecule was shown to accumulate progressively in a linear fashion in both tissue and serosal fluid. Taking an average of the experimental values obtained the Endocytic Indices (E.I.'s) for tissue uptake and accumulation of [^{125}I]-PVP in the serosal fluid were 0.74 $\pm$ 0.04 and 0.12 $\pm$ 0.03 µl/h/mg protein. Expressed thus, uptake was shown not to vary with increasing substrate concentration for either tissue uptake or accumulation of [^{125}I]-PVP in the serosal fluid. However uptake expressed as ng/h/mg protein was seen to be directly proportional to substrate concentration (see Section 5.3).

Temperature affected the uptake of [^{125}I]-PVP as follows:-

For tissue uptake, below 30°C substrate accumulation decreased to about 50% of 'normal' (at 37°C) except at 2° where tissue accumulation appeared greater than that of 'normal'. However accumulation of substrate in the serosal fluid increased with incubation temperature from 2 - 42°C although at 42°C a rapid influx of substrate was observed.

Metabolic inhibitors decreased uptake of [^{125}I]-PVP to varying degrees. At very low concentrations (2×10^{-5}M) the oxidative phosphorylation inhibitor 2.4 . DNP decreased both tissue uptake and accumulation of substrate in the serosal fluid, though was cytotoxic at higher concentrations. The glycolytic inhibitor NaF decreased both tissue uptake and accumulation of substrate in the serosal fluid progressively with concentration up to 1×10^{-4} M but was cytotoxic above this. The electron transport inhibitor NaN_3 (up to 10^{-3} M) progressively inhibited accumulation both in tissue and serosal fluid and exhibited no cytotoxic effects at the concentrations used.

Comparing the results obtained in this study with the criteria for endocytic uptake outlined in Sections 1.2 and 1.3.7 the uptake patterns of [^{125}I]-PVP by adult rat intestine match those observed for fluid phase pinocytosis. The progressive linear uptake and independence of uptake from substrate concentration together with the low E.I. obtained for tissue uptake are similar to those observed for [^{125}I]-PVP uptake in other cell systems (Pratten et al., 1979 - see Table 1.1).

Certainly [^{125}I]-PVP accumulation in both tissue and serosal fluid was demonstrated in this study to be a temperature-dependent process. Accumulation of substrate in the serosal fluid exhibited the classic linear increase in accumulation with temperature described for fluid phase pinocytosis by Silverstein et al. (1974). Tissue uptake did not show a linear response although temperatures lower than 30°C caused decreased substrate uptake. This can be explained in one of two ways. Either pinocytosis carried on at a basal rate at temperatures below 30°C

or the behaviour of the mucosal membrane at low temperatures gave these effects. Allison and Davies (1974) postulated that energy independent micropinocytosis could take place with vesicles being transported across the cytoplasm of cells by Brownian motion. This seems unlikely to have happened in this study as the accumulation of [^{125}I]-PVP in the serosal fluid exhibited linearity with temperature. (If micropinocytosis was responsible for macromolecular transport the accumulation of substrate in the serosal fluid would be expected to follow the same pattern as that of tissue uptake.)

At physiological temperatures the fatty acyl chains of lipid molecules in bilayer membranes such as the plasma membrane of the intestinal brush border are in a relatively disordered fluid state. The transition from the fluid to the rigid state occurs as the temperature falls below the 'melting' or phase transition temperature (Stryer, 1975). This transition temperature depends on the length of the fatty acyl chains and on their degree of saturation. In short, membrane fluidity is enhanced by the presence of double bonds in fatty acyl chains (unsaturated fatty acyl chains) and by their short chain length. According to Stryer (1975) a high proportion of unsaturated residues prevents the membrane from becoming too rigid at low temperatures. Allison and Davies (1974) observed that when the intestinal plasma membrane becomes rigid, it invaginates and traps substrate in infoldings which may or may not form vesicles, but which cannot be transported through the cytoplasm even if formed. This is likely to have happened in the entrapment of [^{125}I]-PVP at temperatures below 30^{o}. The intestinal membrane became rigid due to the presence of a large proportion of saturated fatty acyl chains and trapped [^{125}I]-PVP which was not transported and not washed off. This seems particularly likely if the exaggerated tissue uptake at 2^{o}C is noted. At this temperature, membrane rigidity and folding, and subsequently substrate entrapment,

would be at a maximum. In the cellular systems where a linear increase with temperature is observed for pinocytic uptake, there is probably a high proportion of unsaturated fatty acyl chains in the plasma membrane of the cells which render the membrane less susceptible to rigidity at low temperatures.

At temperatures above 37°C membrane proteins are progressively denatured, the membrane becomes increasingly fluid and membrane disruption occurs. The membrane can no longer function as a selective barrier and rapid influx of substrate may occur. This phenomena was observed at 42°C in this study.

Cohn (1966) showed that agents that depressed either glycolysis and/or aerobiosis reduced the rate of pinocytosis depending upon the predominant pathway in the cell examined. In this study, the uptake and transport of [^{125}I]-PVP was reduced by glycolytic and oxidative phosphorylation inhibitors. This also was an indication that [^{125}I]-PVP was being endocytosed by fluid-phase pinocytosis rather than by adsorption which is not affected by metabolic inhibitors (Steinman et al., 1974). Inhibition of tissue uptake of [^{125}I]-PVP compared favourably with inhibition of fluid-phase pinocytosis reported in other cellular systems using a similar concentration of the same inhibitor. Cohn (1966) using mouse macrophages found 5×10^{-4}M 2.4. DNP and 1×10^{-4}M NaF reduced pinocytosis to about 20% and 50% of the control value while Steinman (1974) found 1×10^{-3} M NaN_3 reduced pinocytosis to 57% of the control value in L-strain fibroblasts. These latter two values are similar to the reduction in tissue uptake to about 58% and 56% of the control value found in this study although the 2.4. DNP concentrations used by Cohn (1966) proved to be cytotoxic to adult rat intestine.

The reason for the reduction in pinocytosis by inhibitors is unclear. Kamovsky et al. (1975) postulated that inhibitors indirectly lowered the ATP content of the cells, thus inhibiting pinocytosis. However Steinman et al. (1974) showed inhibition could be caused without a corresponding

decrease in ATP levels. Taken together, these findings are consistent in the view that pinocytosis is dependent upon metabolic energy and that this energy can be derived from either glycolysis or oxidative metabolism. However these findings do not prove conclusively that ATP is the energy source for pinocytosis, in fact to date this issue remains unresolved.

In summary [^{125}I]-PVP has been shown to be endocytosed by fluid-phase pinocytosis. The transport of [^{125}I]-PVP across the tissue to the serosal space will be discussed in more detail later in this section. It is perhaps appropriate here to mention that these findings do not agree with those of Hardy and Clarke (1969) and Loehry et al. (1970 and 1976). Hardy and Clarke (1969) used [^{125}I]-PVP K60 mean molecular weight 160,000 as substrate and found that macromolecular uptake ceased completely after 'closure'. It should be noted that the macromolecule used by Hardy and Clarke (1969) was much larger than that used in this study. It may be that above a certain molecular weight or size pinocytosis ceases and gives way instead to the slower process of phagocytosis as reported for the adult rat intestine by Volkeimer et al. (1977). In this respect, it would be appropriate to carry out in vitro experiments with a molecular weight range of [^{125}I]-PVP substrates to see if or when a "cut off" point is reached. It is more likely, however, that the method used by Hardy and Clarke (1969) for detection of [^{125}I]-PVP (autoradiography) was simply not sensitive enough to detect the small amounts of [^{125}I]-PVP taken up by the tissue after "closure". (In this study tissue uptake of [^{125}I]-PVP was found to be only 0.44% of that in the medium after 2h, equivalent to 88 ng/mg protein after 2 h).

Loehry et al. (1970) and (1976) used a molecular weight range of [^{125}I]-PVP substrate between 8,000 and 80,000 in cannulated jejunum of rabbit. They found that the larger the molecule, the smaller the rate of uptake. Experiments performed using [^{125}I]-PVP mol. wt. 33,000

indicated that the molecule could pass at the same rate from the gut lumen to the capillaries and in the opposite direction. In this study [^{125}I]-PVP was able to pass from gut lumen to serosal space but little or no macromolecule passed in the opposite direction, the rate of uptake did not increase with increasing substrate concentration and uptake of [^{125}I]-PVP was reduced by metabolic inhibitors and low temperature. All these results contradict the pore hypothesis in that rate of [^{125}I]-PVP uptake would be unaffected by any of these if it were simply diffusing through the tissue. Creamer (1974) also pointed out that no pores had been found by extensive electron microscope study.

9.2.2 The mechanism of uptake of HRP by adult rat intestine

Using the degradable marker protein HRP as substrate produced a tissue uptake plot (in µl/mg protein) which increased linearly with time while the plot of accumulation of substrate in the serosal fluid increased monotonically with time but was not linear. An average of the E.I.'s obtained experimentally for tissue uptake and accumulation of substrate in the serosal fluid were 11.95 $\pm$ 2.1 and 0.11 $\pm$ 0.04 µl/h/mg protein.

Uptake expressed as µl/h/mg protein was shown to increase initially with substrate concentration, then decrease (see Section 8) for both accumulation in tissue and serosal fluid. Uptake expressed as ng/h/mg protein increased monotonically, but was not directly proportional to substrate concentration.

Although this work is only preliminary and must be extended by observing uptake over a temperature range and in the presence of metabolic inhibitors the results (i.e. high E.I. and pattern of uptake in response to different substrate concentrations - see Section 8.6) are consistent with those observed for adsorptive pinocytosis. Adsorptive pinocytosis involves the binding of a substrate to the exterior of the

cell. Then the substrate and plasma membrane to which it is attached are internalized. The degree of binding depends on the composition of the plasma membrane and the chemical nature of the substrate. The areas of plasma membrane to which the substrate molecules bind have been termed the receptors or binding sites and these may be lipid protein or their glycosylated forms in composition. They may be highly specific, with affinity for one or a group of molecules or they may accept a very wide range of types of molecule (non-specific receptors). The recognition systems for these receptors have been studied most extensively in those cell types where adsorptive pinocytosis has been shown to have great functional significance, e.g. human fibroblasts (Goldstein et al., 1979 review), rat visceral yolk sac (Ibbotson et al., 1978) and neonatal intestine (Morris, 1974). From these studies, the function of adsorptive pinocytosis has become recognised as an important and general mechanism by which some animal cells take up biologically important macromolecules intact from the external fluid. Proteins taken up by this mechanism include plasma transport proteins, certain polypeptide hormones and γ-globulins. The latter is responsible for maternofoetal transmission of passive immunity either prenatally (via the yolk sac) or postnatally (via neonate intestine) and it is on the uptake of this macromolecule that most studies have been done. Several theories have emerged concerning the mechanism of uptake and transfer of intact protein macromolecules (see Section 1.2.2).

Brambell (1966) first postulated that γ-globulins transported across yolk sac endoderm and neonatal intestine were protected from intracellular lysosomal digestion because of their attachment to specific receptors sites in the microvillous membranes of these organs. When the membrane vesicles containing both γ-globulins attached to receptor sites and other proteins free within the vesicles combined with lysosomes, the unattached proteins were broken down whereas the attached immunoglobulins were unaffected. These undegraded proteins could then be transported out of

the cells to the embryo or circulation. It has also been claimed (Hemmings and Williams, 1974) that homologous and heterologous proteins bind equally well to membrane components of yolk sac and neonatal gut indicating a lack of specific receptors and that selective degradation of proteins may be effected by the differential action of lysosomal cathepsins. Transport of non-digested protein can then occur. It has also been suggested (Moxon and Wild, 1976) that selection may be affected by specific transport in coated vesicles. Proteins destined for transport across the cell attach to specific receptors at sites on the apical plasma membrane where coated vesicles form. Consequently they are segregated from other protein molecules which enter the cell non-selectively via the apical tubular cannalicular system and accumulate in macropinocytic vesicles. These subsequently fuse with lysosomes and all the protein they contain is digested. Coated vesicles do not fuse with lysosomes because of their 'coat' supposedly prevents membrane contact and fusion.

An extension of this theory was put forward by Goldstein et al. (1979) from their studies on human fibroblasts. They postulated the existance of a non-specific receptor mediated pinocytosis where substances were first bound to cell surface receptors in specialized regions called coated pits which invaginate to form coated vesicles. These can then fuse with lysosomes or be transported across the cell to exocytose non-degraded material at the basal membrane. The fate of the internalized material depends upon cell type. It is in terms of these latter hypotheses that HRP uptake can be most easily explained.

The non-specific receptor hypothesis outlined by Goldstein et al. (1979) implicates LDL (low density lipoprotein) receptors as the non-specific receptors (LDL receptors are so-called because they were originally demonstrated to bind and internalize cholesterol, now it is thought they bind a much wider range of substances). This LDL-receptor

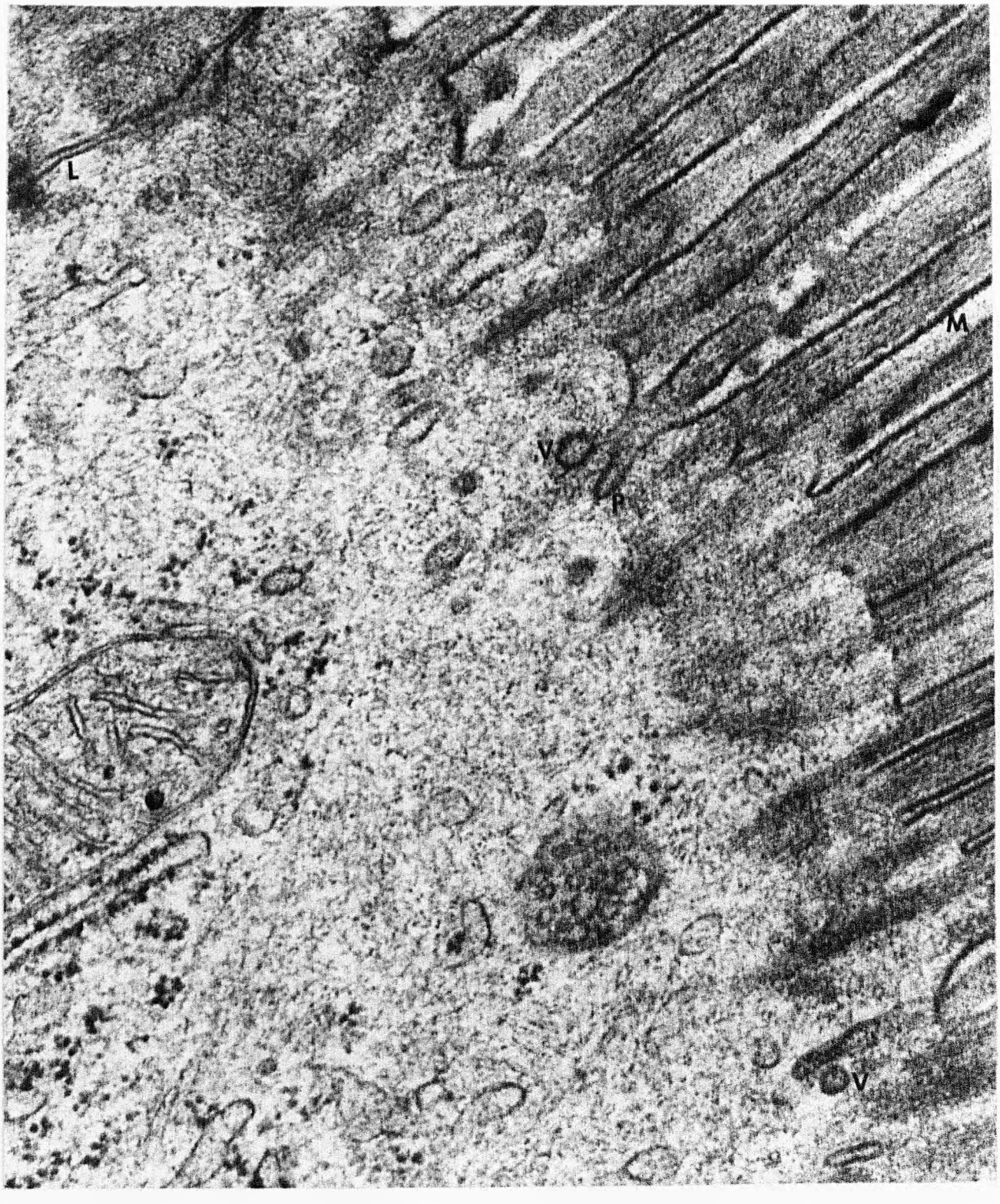
endocytosis is thought to operate in the following way. LDL-receptors on the cell surface bind to a macromolecular substrate then migrate laterally in the plane of the membrane until they reach a coated pit. When the coated pit invaginates to form a coated vesicle, the receptor and bound substrate are carried with it. These coated vesicles can either fuse with lysosomes and their contents are degraded or be transported across the cell to exocytose their contents at the basal membrane. The fate of the coated vesicles depends on cell type. In fibroblasts all the protein is degraded, in yolk sac and neonatal intestine the substrate can be transported intact. Receptors that enter the cells in coated vesicles are not destroyed when vesicles fuse with lysosomes, but Goldstein et al. (1979) suggest that the receptors return to the surface again to cluster in pits and be recycled.

In this study HRP exhibited a pattern of uptake very similar to that of adsorptive pinocytosis, i.e. uptake was shown to be saturable and subsequently to revert to a fluid phase endocytosis (see Section 8.4). The observation (Fig. 8(vi)) that uptake was saturable indicated receptors were involved. The linear uptake pattern showed that the process was ongoing, i.e. the receptors must have been recycled. Most of the HRP adsorbed by and taken into the tissue was digested presumably by enzymes attached to the brush border and/or lysosomal enzymes (see Section 8.5). This meant that only a small proportion escaped intact from lysosomal digestion or a small proportion of vesicles did not fuse with lysosomes (coated vesicles?). From the available data it is difficult to speculate as to the exact character of the uptake mechanism but coated vesicles can be shown to exist in normal adult rat intestine (see Plate 23). These vesicles are indistinguishable from the ones demonstrated in neonatal rat intestine by Rodewald (1973) and in fibroblasts by Goldstein et al. (1979). If, as some authors suggest (Brambell, 1970, Wild, 1973) specific immunoglobulin receptors as well as

PLATE 23

Magnification 16,500, shows the apical cytoplasm of two adjacent absorptive cells from the freshly excised intestine of an adult rat.

It appears that the plasma membrane (M) covering the microvilli is wider than the lateral plasma membrane (L). A pit-like indentation (P), formed by the apical plasma membrane is projecting into the web region. Vesicles (V) enclosed by a membrane similar to those of the microvillous membrane is present in the terminal web.


L
M
V
P
V

non-specific receptors exist in the neonatal gut, then it is possible that at "closure" only the specific uptake ceases and the non-specific processes (non-specific receptor mediated and fluid phase pinocytosis persist in adulthood).

Perhaps it is appropriate to mention here that a non-specific receptor mechanism of macromolecular uptake in adult rats would help explain the phenomena observed by Walker *et al.* (1975), Walker *et al.* (1976a, b & c), Siefert *et al.* (1977), Tolo *et al.* (1977), Walker (1978), Pierce (1978) and Lake *et al.* (1979). All these workers reported that penetration of antigenic macromolecules was reduced when the experimental animals used had been pre-immunized orally or parentally. Walker (1975) postulated that this effect was due to secretory antibody (predominantly secretory IgA) binding to the antigen (Ag) in the gut lumen and preventing absorption. Tolo *et al.* (1977) went further and suggested that mucosal antibody (Ab) retarded penetration of its specific Ag but enhanced penetration of unrelated macromolecules. This effect can be explained in terms of a non-specific receptor. An Ag binds to its specific Ab in the gut lumen, which stops the Ag binding to a receptor and also reduces competition for receptors therefore allowing other macromolecules to be taken up in greater amounts.

Obviously, this area needs much further investigation and the *in vitro* technique outlined in this study provides a reproducible method for the task.

9.3 Comparison of the Mechanism of Uptake of the Non-Degradable Macromolecule [^{125}I]-PVP with that of the Degradable Macromolecule HRP

If averages of the Endocytic Indices (E.I.'s) for accumulation of [^{125}I]-PVP into the tissue and serosal fluid are taken, the percentage uptake values with respect to the original substrate concentration can be calculated (assuming an average gut sac protein content of 30 mg). This was done as follows:- For the mean E.I.'s of 0.74 $\pm$ 0.04 and 0.12 $\pm$ 0.03 µl/h/mg protein; total uptake values in ng after 2 h were 103.2 $\pm$ 5.58 and 14.4 $\pm$ 3.6 ng. Therefore of the total substrate present in the system (20 µg) 0.52 $\pm$ 0.03% accumulated in the tissue throughout the course of the experiment and of this 13.95 $\pm$ 3.49% passed into the serosal space (i.e. 0.072 $\pm$ 0.018% of the total substrate). A similar calculation can be performed for HRP uptake using the mean values of tissue uptake and accumulation in the serosal fluid of 11.95 $\pm$ 2.10 and 0.11 $\pm$ 0.04 µl/h/mg protein. Here, of the total substrate (4.0 mg) 7.24 $\pm$ 1.27% was adsorbed by and taken up into the tissue and of this 0.91 $\pm$ 0.34% passed to the serosal fluid (i.e. 0.066 $\pm$ 0.024% of the total substrate).

According to Pratten et al. (1979 - see Section 1.4.3) the magnitude of the E.I. itself can give a good indication of the mechanism of uptake. Substances which enter cells at a low rate, giving a small E.I. (between 0.4 and 2.1 µl/h/mg protein) are likely to be taken up by fluid phase pinocytosis. An higher E.I. is most likely to be a function of adsorptive pinocytosis. Therefore it is likely that [^{125}I]-PVP is taken up by fluid phase pinocytosis. This is substantiated by its low E.I. for tissue uptake (0.74 $\pm$ 0.04 µl/h/mg protein) and the effects of different substrate concentrations, temperature and metabolic inhibitors (see Section 9.2.1) On the other hand, HRP is likely to be taken up by adsorptive pinocytosis. This is substantiated by the high E.I. for

tissue uptake (11.95 ± 2.10 µl/h/mg protein) and the effects of different substrate concentrations (see Section 9.2.2) Although this is strong evidence for adsorptive pinocytosis being the mechanism of uptake for HRP, it could be further confirmed by noting the effects of temperature and metabolic inhibitors.

It is interesting to note that while the E.I.'s for tissue uptake of [^{125}I]-PVP and HRP are so different, the E.I.'s for accumulation of the substances in the serosal fluid are similar (0.12 ± 0.03 and 0.11 ± 0.04 µl/h/mg protein). These represent only 0.072 ± 0.018 and 0.06 ± 0.024% of the total substrate present in the system. This means that most of the HRP being taken up by adsorptive pinocytosis is digested either by enzymes adsorbed to the brush border (Kim, 1977) or by lysosomal enzymes (Dean, 1977). Perhaps the most interesting feature is that despite the different mechanisms of tissue uptake for the two substrates - one non-digestible ([^{125}I]-PVP) and one digestible (HRP) - about the same amount is transported across the tissue and deposited in the serosal space. This fact may be very important for the explanation of the function of macromolecular uptake by adult intestine. This is discussed in detail in Section 9.5.

9.4 Method of Transport of Macromolecules across the Intestine

Preliminary data on the mechanism of transfer of the non-degradable macromolecule [^{125}I] -PVP across the intestine indicated that a linear relationship existed between tissue accumulation and accumulation of substrate in the serosal fluid (see Section 5.4). In the case of the degradable macromolecule HRP there was no linear relationship between tissue accumulation and accumulation in the serosal fluid. If the values for percentage substrate transferred from the tissue to the serosal fluid are examined (see Section 9.3) it can be seen that for [^{125}I] -PVP about 14% was transferred whereas for HRP only 0.91% was transferred. From this limited data it is difficult to postulate the mechanism of transfer of macromolecules from the tissue to the serosal fluid. However this data does suggest that some factor prevented the direct transfer of intact HRP from the tissue to the serosal fluid. This factor may have been the intracellular digestion of HRP by, e.g. lysosomes.

From the work carried out on other cellular systems, two main theories have emerged as to the mechanism of transfer of endocytosed material across cells: i) microtubules are responsible for the transport of endocytic vesicles across cells.

ii) Actinomycin filaments are responsible for the transport of endocytic vesicles across cells.

Evidence for either process is scanty and conflicting. Moxon and Wild (1976) postulated that coated pinocytic vesicles were transported into and at least part way across cells via a microtubular system.

Electron micrographs of mouse peritoneal macrophages (Reaven and Axeline, 1973) and human polymorphonuclear leukocytes (Stossel, 1977) showed microtubules permeating the region of the subplasmalemma microfilaments adjacent to particles undergoing ingestion. Compounds such as colchicine, which depolarizes the cytoplasmic microtubules, at

most cause a small reduction in pinocytic rate (Silverstein et al., 1977). Colchicine and related alkaloids have other effects upon cellular physiology one of which is to alter cytoplasmic organization. In the presence of these compounds, pinosomes which usually move directly towards the cell's Golgi region move more randomly about the cytoplasm.

The suggestion that pinocytosis may involve a cellular contractile mechanism arises from its association with peripheral membrane movement or 'ruffling' (Silverstein et al., 1977), energy dependence (Cohn, 1966) and the observation of thin filaments attached to pinocytic vesicles in the cytoplasm (Caslay-Smith, 1969). These filaments are thought to be a form of actinomyosin. The effects of cytochalasin B on the uptake of solutes and small molecules have been used as a measure of the role of an actinomysin contractile system in pinocytosis. Cytochalasin is reported to inhibit the uptake of [^{3}H]-sucrose by Chang liver cells (Wagner et al., 1971) and peroxidase by mouse macrophages (Steiman et al., 1974) indicating that an actinomysin system may be involved in fluid phase pinocytosis. However cytochalasin is reported to have no effect on the uptake of ferritin or colloidal gold by mouse macrophages (Allison and Davies, 1974) which suggests that the actinomysin system may not be involved in adsorptive pinocytosis.

In the absence of additional data it is difficult to assess the mechanism of transport of macromolecules across adult rat intestine other than to say that it probably involves fusion with the lysosomes. However it may be interesting in future experiments to test the effects of both colchicine and cytochalasin B on macromolecular transport in adult rat intestine in order to pinpoint similarities and differences between this and other cell systems.

9.5 Functional Significance of Macromolecular Uptake by the Adult Intestine

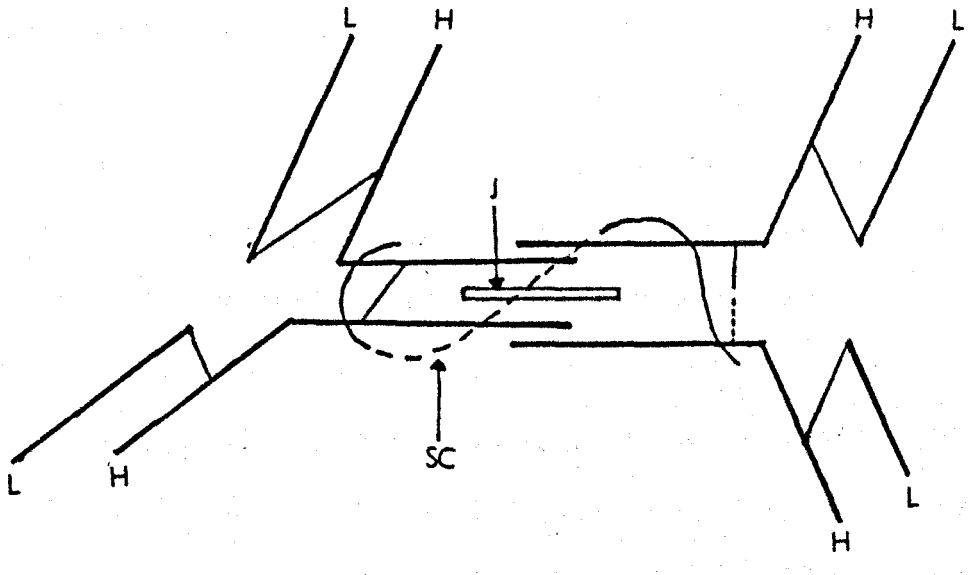
The quantity of macromolecules passing intact into the serosal space is so small (about 0.07% of the substrate in the system - see Section 9.4) as to be nutritionally insignificant but their uptake may be important in other ways. Rothman *et al.* (1975a and b) suggest that an enteropancreatic circulation of digestive enzymes occurs.

If an excess of pancreatic enzyme is secreted into the intestine, Rothman *et al.* (1975a and b) proposed that a large percentage of this can be conserved intact by recycling involving macromolecular protein absorption by the intestine. There is much controversy about the feasibility of such an enteropancreatic circulation and even if other factors could be discounted (such as inactivation by plasma inhbitiors, e.g. α_1 - antitrypsin, α_1 - antichymotrypsin and pancreatic inhibitors) the macromolecular uptake rate would be too slow for the process to be functionally worthwhile. A more likely explanation is that macromolecular uptake and transport in the adult serves the same function as that in the neonate, i.e. immunological. Bazin (1976) suggested that the adult alimentary tract served as a surveillance point from which the immunological system could monitor antigens (macromolecules capable of eliciting antibody formation). Bazin's theory is corroborated by several examples of immunization by antigen given orally. Ehrlich (1891) showed antibodies occurred in mice which had been given ricin orally. Davies (1922) observed the appearance of coproproteins (anti-Shigella agglutinins) in patients suffering from bacillary dysentry one and a half days after the onset of symptoms, at the time when patients had no serum antibodies. Indeed many publications have shown that immunization by the oral route usually leads to stimulation of the immunological system and to the appearance of specific antibodies in the intestinal

secretion and serum (Heremans, 1968; Bazin, 1976 review). The origin of these antibodies is now generally accepted as being the lymphocytes (immunocytes) which are found in abundance in the intestinal mucosa (Ferguson, 1976). Some are concentrated in nodules (e.g. lymphoid tissues of the nasopharynx, Peyers Patches and appendix) and others are scattered in the intestinal mucosa where they form the lymphoid jacket or cylinder separated from the gut contents by the intestinal epithelia. The most common antibody secreted by these cells is secretory IgA. Secretory IgA is a dimeric molecule, molecular weight 380 - 400,000, composed of 4 α heavy chains and 4 light chains joined by J chains to the secretory component (Fig. 9(i).

For antigens to be immunogenic they must leave the intestinal lumen and traverse the intestinal epithelia. Therefore it is possible that pinocytic uptake of macromolecules may serve the function of sampling antigens from the gut lumen. Some support for this theory can be found in the gut-physiology itself in that the greatest number of Peyers Patches are found in the mid to lower jejunal region. Here any luminal digestion which was going to occur would have done so hence any macromolecules left are likely to be digestion resistant and potentially antigenic. Thus the Peyers Patches would be optimally placed to sample these potential antigens and formulate an immune response against them. Also Walker et al. (1972) suggested that it was in this region that maximum macromolecular absorption occurred. The various experiments in this thesis demonstrated that absorption of intact macromolecules by adult rat intestine seemed to be physiological as the organ maintained its normal ultrastructure.

Certainly there is ample evidence that antigen within the lumen of the gut can stimulate an immune response locally in the lamina propria and systemically throughout the body. If it is conceded that both major lymphoid cell types, the T cells (Thymus derived lymphocytes) and B cells



Model for secretory IgA (SC, secretory component; H, heavy chain; L, light chain; J, J chain.)

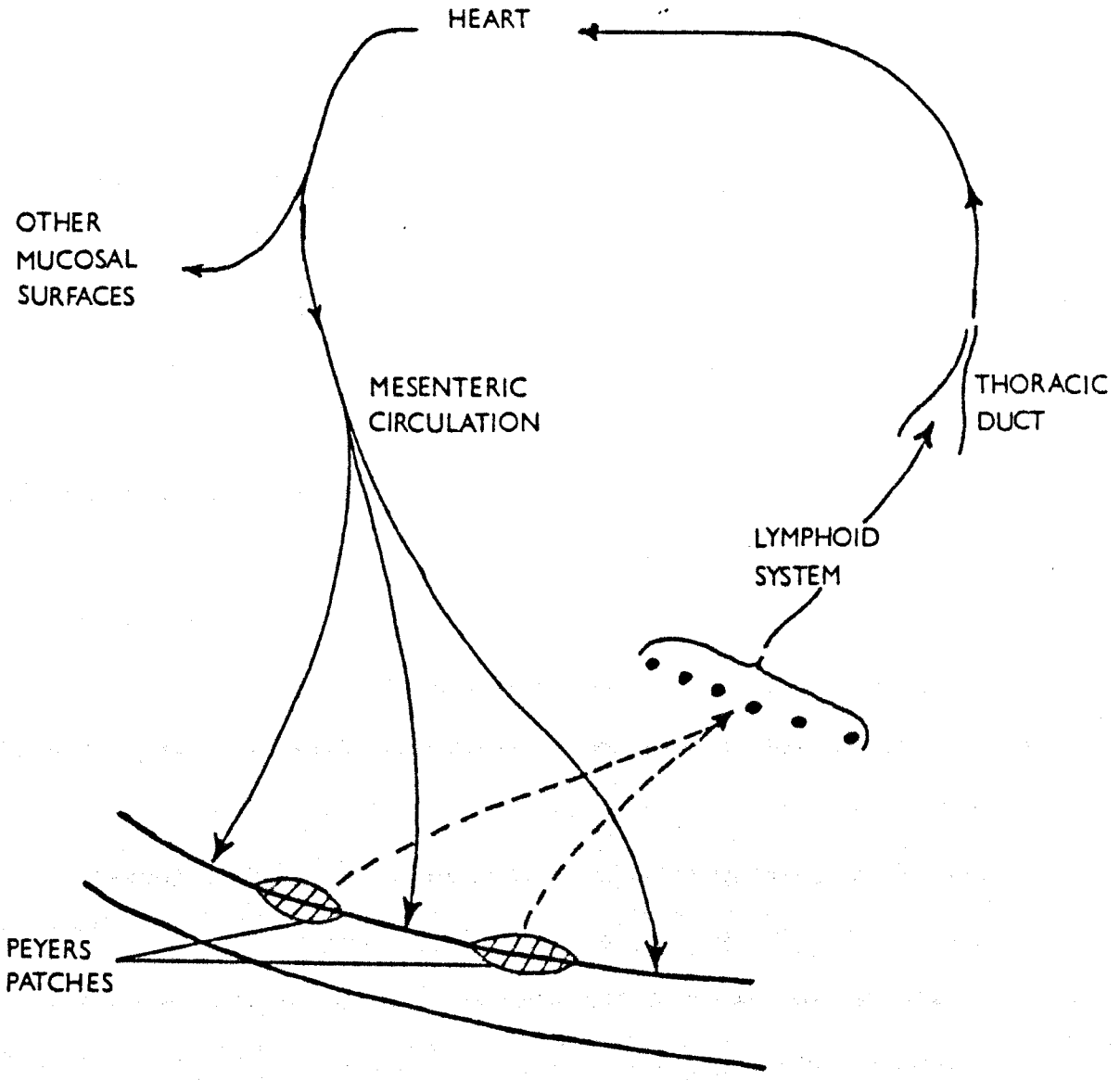
FIGURE 9(i)

(Bone marrow derived plasma cells) within the intestinal mucosa are carrying out immine responses, the next problem is, where does primary sensitization occur? Many authors have considered it likely that sensitization takes place in the lamina propria and that effector cells arise in situ. However Ferguson and Parrott (1972) showed that effector cells were present in completely antigen free grafts of foetal gut and concluded effector cells must have migrated into the grafts from circulating blood. Parrot (1976) later postulated that this is the normal way in which the gut mucosa is populated by lymphoid cells. Furthermore she concluded that initiation of a primary response needed a sufficient traffic of lymphocytes so that the limited number of cells with appropriate immunological potential could be selectively retained. Such circumstances only normally prevail in organized lymphoid tissues. Pierce (1978) went on to postulate a circulation of enteric immunocytes involving the gut-associated lymphoid tissue known as Peyers Patches and summarized in Fig. 9(ii). Pierce (1978) suggested that effective encounter of antigen with Peyers patch lymphoid tissue leads to blast transformation. Cellular division and subsequent migration of IgA immunoblasts, first to mesenteric lymph nodes then through the thoracic duct to the systemic circulation follow. Hence IgA cells generated from a small amount of gut can disseminate through the host. The vast majority return to the lamina propria of the bowel, where they rapidly assume the morphology of mature plasma cells and have an average life span of about five days. The presence in the mucosa or uptake of antigen against which their antibody is directed is one known 'determinant' of the selective "homing" of these cells to the intestine. Therefore it seems likely that primary triggering of the immune response occurs in the Peyers Patches whereas maintenance of immunological protection is carried out in the mucosa itself.

Although macromolecular antigens are able to traverse the adult intestinal mucosa, the vast majority of adults show no ill-effects as a

FIGURE 9(ii) Circulation of enteric immunocytes.

Immunoblasts originate in Peyers Pathces or mesenteric lymph nodes, migrate through the thoracic duct and enter the systemic circulation. Homing is predominantly to the intestine but possibly also to other mucosal surfaces. In the lamina propria, immunoblasts appear as plasma cells.



Diagrammatic representation of the circulation of enteric immunocytes. (After Pierce,N. 1978)

FIGURE 9(ii)

result of this phenomenon. However, in man at least, when increased quantities of antigenic or toxic substances gain access to the body because of an alteration in the intraluminal digestive process or because of a defect in the mucosal barrier, macromolecular absorption may be increased to pathological proportions. Walker (1975) postulated that certain factors may predispose to abnormal or pathologic transport of macromolecules:

i) Selective IgA deficiency

ii) Alteration in mucosal barrier, increased adherence of Ag, inflammation, ulceration

iii) Lysosomal dysfunction

iv) Abnormal intraluminal digestion: achlorhydria, pancreatic insufficiency.

The factors listed above are meant to be representative not comprehensive. The gastrointestinal diseases possibly associated with Ag absorption are gastrointestinal allergy, inflammatory bowel disease, coeliac disease, toxigenic diarrhoea, chronic hepatitis, nectrolizing enterocolitis and autoimmune diseases. Since the evidence to support the hypothesis that intestinal permeability to Ag is involved in pathogenesis of human disease is largely indirect, these comments are highly speculative and obviously much more work is needed in this field. to provide direct evidence.

9.6 To the Future

Over the last ten years evidence for macromolecular uptake by adult intestine has accumulated enormously, but many areas remain to be investigated. Techniques described in this thesis show that the methodology for adequate quantitation is available and investigations can be carried out in a way that will give a comprehensive understanding of the physiological functions of this process.

Although preliminary results already indicate that some protein uptake may be receptor mediated, this must be confirmed (see Section 8.5) and the pattern of uptake for many diverse substances determined. The uptake of some substances by discrete types of vesicle remains hypothesis and this could be elucidated by electron microscope studies of HRP uptake employing cytochemical procedures to map the location of HRP in the cell. The role of macromolecular size and structure in the uptake process could be examined by using the same macromolecule over a molecular weight range, and different molecules of known diverse structures. The rate and mechanism of uptake of antigenic macromolecules could be determined before and after oral and parenteral immunization. Finally very little known about the mechanism of Ag trapping in Peyers Patches. According to the electron microscope study of Owen (1977) the mechanism of uptake is pinocytic, but this needs to be quantified and compared with that of macromolecular uptake in normal villous intestine to see if, indeed, macromolecular uptake is greater in the Peyers Patches. The developed method described in this thesis lends itself easily to such a study in that sacs can be produced which contain Peyers Patches.

All this would contribute to the understanding of how the adult intestine handles macromolecules and in particular antigenic macromolecules, and ultimately may lead to a better understanding of disease states associated with macromolecular uptake.

APPENDIX ONE

INCUBATION MEDIA

KREBS-HENSELEIT IMPROVED RINGER II

(Krebs, 1950)

Composition at Working Strength

Inorganic Salts	g/l
NaCl	5.54
KCl	0.35
$MgSO_4,7H_2O$	0.29
$CaCl_2$	0.28
KH_2PO_4	0.16
$NaHCO_3$	2.1
Glucose	1.0
L-pyruvic acid	0.43
L-fumaric acid	0.62
L-glutamic acid	0.72

Tc MEDIUM 199

(Morgan, Morton and Parker, 1950)

Composition at Working Strength

Inorganic Salts	mg/l
NaCl	6800.0
KCl	400.0
$CaCl_2$	200.0
$MgSO_4.7H_2O$	200.0
$NaH_2PO_4.2H_2O$	150.0
$Fe(NO_3)_3.9H_2O$	0.72

Amino Acids	
l-arginine hydrochloride	70.0
l-histidine hydrochloride	20.0
l-lysine hydrochloride	70.0
dl-phenylalanine	50.0
dl-methionine	30.0
dl-serine	50.0
dl-threonine	60.0
dl-leucine	120.0
dl-isoleucine	40.0
dl-valine	50.0
dl-glutamic acid	150.0
dl-aspartic acid	60.0
dl-alanine	50.0
l-proline	40.0
l-hydroxyproline	10.0
glycine	50.0
dl-tryptophane	20.0
l-tyrosine	40.0
l-cystine	20.0
l-cysteine hydrochloride	0.1

Lipid Sources	
Cholesterol	0.2
Tween 80	5.0

Vitamins	
Anaurine hydrochloride	0.01
Riboflavin	0.01
Pyridoxine hydrochloride	0.025
Pyridoxal hydrochloride	0.025
Nicotinic acid	0.025
Nicotinamide	0.025
Calcium pantothenate	0.01
Inositol	0.05
p-amino-benzoic acid	0.05
Choline chloride	0.5
Ascorbic acid	0.05
d-biotin	0.01
Folic acid	0.01
Menaphthone	0.01
Calciferol	0.1
Vitamin A acetate	0.1
a-Tocopherol phosphate	0.01

Nucleic acid derivatives	
Adenine hydrochloride	5.1
Guanine hydrochloride	0.3
Xanthine	0.3
Hypoxanthine	0.3
Thymine	0.3
Uracil	0.3
Adenylic acid	0.2
Adenosine triphosphate	5.0
Ribose	0.5
Deoxyribose	0.5

Miscellaneous	
Sodium acetate (hydrated)	84.0
Glucose	1000.0
Phenol red	10.0
Glutathione	0.05
l-glutamine	100.0

In Tc 20 Only

Sodium bicarbonate ($NaHCO_3$)	1100
Penicillin	200,000 units
Streptomycin	100,000 µg

APPENDIX TWO

COMPUTER PROGRAMMES

```
 10 REM ......................
 20 REM HRP PROGRAM APRIL 1979
 25 REM ......................
 30 DIM C(2),Y(2),R(2)
 40 C(1)=10 : C(2)=250
 50 PRINT : PRINT "HRP Program" : PRINT
 60 PRINT "Type the concentration:";
 70 INPUT K
 80 PRINT : PRINT "Type the name of the data file:";
 90 INPUT F1$
100 PRINT "And the names of the results files-"
110 PRINT "     1) For Uptake Through:";
120 INPUT F2$
130 PRINT "     2) For Uptake Into:";
140 INPUT F3$
150 FILES #3,F1$,(EOF370)
160 FILES #4,F2$,#5,F3$
170 PRINT #4, : PRINT #5,
180 FILES #4,F2$,#5,F3$ : SCRATCH #4 : SCRATCH #5
190 W5=0
200 GOSUB  260
210 K=1
220 RESET #3
230 W5=1
240 GOSUB  260
250 STOP
260 PRINT
270 PRINT "Time"," Uptake"," Uptake","Protein"
280 PRINT ""," Through"," Into"
290 PRINT
300 INPUT #3,T,M,Y(1),Y(2),P
310 P1=P*M
320 FOR I=1 TO 2
330 R(I)=(C(I)*Y(I)/P1)*K
340 NEXT I
350 IF W5=1 THEN  PRINT #4,T;",";R(1) : PRINT #5,T;",";R(2)
360 PRINT T,R(1),R(2),P
370 GOTO  300
380 REM END OF DATA
390 PRINT
400 RETURN
```

```
0005  REM .....................
0010  REM GUTSAC PROGRAM VN 2
0015  REM .....................
0020  DIM H(16),I(16),K(16,2),L(16),R(16,2)
0030  PRINT "Non-digestible Program" : PRINT
0040  PRINT "Expt. No. (Digits only) =";
0050  INPUT Z
0060  PRINT "Background in CPM=";
0070  INPUT A
0080  PRINT "Conc of PVP used, in ug/ml =";
0090  INPUT P
0100  PRINT "Counting time for each ml of medium, secs =";
0110  INPUT C
0120  PRINT "Counting time for each ml of GS solution, secs =";
0130  INPUT D
0140  PRINT "No. of points in plot =";
0150  INPUT G
0155  PRINT
0160  PRINT "Is the data in a file (Y or N)?";
0170  INPUT A1$
0180  IF A1$='N' THEN F0=0:GOTO 230
0190  PRINT "Name of file?";
0200  INPUT A1$
0210  FILES #8,A1$,(EOF650)
0220  F0=1:T5=0
0230  FOR X=1 TO G
0240  IF F0=0 THEN INPUT H(X),I(X),K(X,1),K(X,2),L(X) :GOTO 270
0250  INPUT #8, H(X),I(X),K(X,1),K(X,2),L(X),
0260  T5=T5+1
0270  NEXT X
0280  PRINT "Are the results to be stored (Y or N)?";
0290  INPUT A1$
0300  IF A1$='N' THEN F0=0:GOTO360
0310  PRINT : PRINT "Name of file?";
0320  INPUT A1$
0330  FILES #9,A1$:PRINT #9,
0340  FILES #9,A1$:SCRATCH #9
0350  F0=1
0360  FOR I=1 TO 2
0370  FOR X=1 TO G
0380  LET M=(I(X)*60/C)-A
0390  LET Q=((K(X,I)*60/D)-A)*(2.0+23.0*(I-1))
0400  LET N=M+Q/20
0410  LET R(X,I)=(Q*1000)/(N*L(X))
0420  IF I=1 THEN  440
0430  IF F0=1 THEN PRINT USING '4#.4#,","',#9,H(X),R(X,1),R(X,2)
0440  NEXT X
0450  NEXT I
0460  IF F0=1 THEN PRINT USING '4#.4#,","',#9,-1,Z
0470  FOR K=1 TO 2
0480  PRINT
```

```
0490  PRINT
0500  PRINT "Incubation Time ";"  Protein in GS","     Uptake","       Uptake"
0510  PRINT "     (Hours)    ";" ","                      Through       Into
0520  FOR X=1 TO G
0530  PRINT USING '5S,2#.2#,9S,4#.4#,8S,4#.4#,8S,4#.4#',H(X),L(X),R(X,1),R(X,2)
0540  NEXT X
0550  IF K=2 THEN  610
0560  FOR I=1 TO 2
0570  FOR J=1 TO G
0580  LET R(J,I)=R(J,I)*P
0590  NEXT J
0600  NEXT I
0610  PRINT
0620  PRINT
0630  NEXT K
0640  STOP
0650  REM **PREMATURE EOF ON DATA FILE**
0660  PRINT
0670  PRINT USING '"**END OF DATA AFTER ",##',T5;
0680  PRINT " POINTS**"
0690  PRINT "**RUN ABANDONED**"
0700  STOP
```

REFERENCES

ALEXANDER, H.L., SHIRLEY, K. & ALLEN, D. (1936) J. Clin. Invest., 15, 163.

ALLISON, A.C. & DAVIES, P. (1974) In Transport at the Cellular Level. Society for Experimental Biology Symposium, XXVIII, pp. 419–446. Cambridge University Press.

ANDRE, C., LAMBERT, R., BAZIN, H. & HEREMANS, J.F. (1974) J. Immunol. 4, 701.

BACH, G., FRIEDMAN, R., WEISSMANN, B. & NEUFIELD, E.F. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 85–90.

BAMFORD, D.R. (1966) Proc. R. Soc. Lond. (Biol.) 166, 30–47.

BARRY, B.A., MATTHEWS, J. & SMYTH, D.H. (1961) J. Physiol., 157, 279.

BAZIN, H. (1976) In: Immunological Aspects of the Liver and Gastrointestinal Tract. (FERGUSON, A. & MACSWEEN, R.N.M. eds.), p. 33–82, University Park Press, Baltimore.

BENNETT, H.S. (1956) J. Biophys. Biochem. Cytol. Suppl. 2, 85–98.

BERSTEIN, I.D. & OVARY, Z. (1968) Int. Arch. Allerg. 33, 521–527.

BINGHAM, J.K., NEWEY, H. & SMYTH, D.H. (1966) Biochim. Biophys. Acta, 120, 314.

BOCKMAN, D.E. & WINBORN, W.B. (1966) Anat. Rec. 155, 603–622.

BORGSTRÖM, B. (1974) In: Biomembranes (SMYTH, D.H. ed.) Vol. 4B, pp. 555–621, Plenum Press, London and New York.

BOWERS, B. & OLSZEWSKI, T.E. (1972) J. Cell Biol. 53, 681–694.

BRAMBELL, F.W.R. (1966) Lancet, 2, 1087.

BRAMBELL, F.W.R. (1966) Lancet, 2, 1087–1093.

BRAMBELL, F.W.R. (1970) In: The Transmission of Passive Immunity from Mother to Young. Amsterdam, 197.

BRIDGES, J.F. & WOODLEY, J.F. (1979) 2nd Brambell Symposium. Elsevier, Amsterdam. In Press.

BRONK, J.R. & LEESE, H.J. (1973) J. Physiol. 235, 183–196.

BROWNE, J.L., SANDFORD, P.A. & SMYTH, D.H. (1977) Proc. Roy. Soc. Lond. (B), 195, (1119), 307–321.

BROWN, W.R. (1978) Gastroenterol, 75, 129-138.

BROWN, W.R., ISOBE, K., NAKANE, P.K. & PACINI, B. (1977) Gastroenterol. 73, 1333-1339.

BURTON, K.A. & SMITH, M.W. (1977) J. Physiol. 270, (2) 473-488.

CARTER, P.B. & COLLINS, F.M. (1974) J. Exp. Med. 139, 1189.

CHAPMAN-ANDRESEN, C. (1962) Trav. Lab. Carlsberg. 33, 73-264.

CLARKE, S.L. (1959) Biophys. Biochem. Cytol. 5, 41-50.

CLARKE, R.M. & HARDY, R.N. (1969) J. Physiol. 204, 113-125.

COHN, Z.A. (1966) J. Exp. Med. 124, 557-571.

CORNELL, R., WALKER, W.A. & ISSELBACHER, K.J. (1971) Lab. Invest. 25, 42-48, 1971.

CRANE, R.K., MILLER, D., & BIHLER, I. (1961) In: Membrane Transport and Metabolism (KLEINZELLER, A. & KOTYK, A. Eds.), pp. 439-450, Czechoslovak Academy of Science Press, Prague.

CREAMER, B. (1974) In: Intestinal Absorption, (SMYTH, D.H. Ed.) Biomembranes Vol. 4A, pp. 1-43, Plenum Press, London and New York.

CULLING, C.F.A. (1974) Handbook of Histopathological and Histochemical Techniques, 3rd edn., Butterworth, London.

CURLING, J.M. (1970) In: Experiments in Physiology and Biochemistry 3, (KERKUT, G.A. Ed.) pp. 417-484. Academic Press, London, New York.

DAVIES, A. (1922) Lancet ii, 1009.

DEAN, R. (1977) Lysosomes, p. 21, Studies in Biology No. 84, Edward Arnold, Camelot Press, Southampton.

EDELSON, P.J. & COHN, Z.A. (1978) Cell Surface Reviews, 5, 387-407.

EDITORIAL (1978) Lancet (ii), 715-717.

FAELLI, A., ESPOSITO, G. & CAPRARO, V. (1976) Biochim. Biophys. Acta. 455, 759-766.

FARQUHAR, M.G. & PALADE, G.E. (1960) J. Biophys. Biochem. Cytol 7, 297-304.

FAWCETT, D.W. (1964) Anat. Rec. 148, 370-375.

FAWCETT, D.W. (1965) J. Histochem. Cytochem. 13, 75-91.

FEDORKO, M.E., HIRSCH, J.G. & COHN, Z.A. (1968) J. Cell Biol. 38, 392-402.

FERGUSON, A. & PARROTT, D.M.V. (1972) Clin. Exp. Immunol. 12, 477.

FISCHER, R.B. & GARDNER, M.L.G. (1974) J. Physiol. 241, 211-234.

FISHER, R.B. & PARSONS, D.S. (1949) J. Physiol. 110, 281-293.

FORSTNER, G.G. (1969) Am. J. Med. Sci., 258, 172-177.

FUBARA, E.S. & FRETER, R. (1973) J. Immunol, 111, 395.

GOLDSTEIN, J.L., ANDERSON, R.G.W. & BROWN, M.S. (1979) Nature 279, 679-685.

GORDON, G.B., MILLER, L.R. & BENSCH, K.G. (1965) J. Cell. Biol. 25, pp. 41-55.

GOSSELIN, R.E. (1967) Fed. Proc. 26, 987-93.

GRAHAM, R.C. & KARNOVSKY, M.L. (1966) Histochem. Cytochem. 14, 291-302.

GRANEY, D. (1968) J. Anat. 123, 227-254.

GRAY, G.M. & WOJNAROWSKA, F. (1975) Biochem. Biophys. Acta 403, 147-160.

GRUSKAY, F.L. & COOKE, R.E. (1955) Paediatrics, 16, 763.

HALLIDAY, R. & KEKWICK, R.A. (1960) Proc. Roy. Soc. Lond. (Biol.) 183, 279-286.

HAMPTON, J.C. & ROSARIO, B. (1967) Anat. Rec. 159, 159-170.

HARRISON, M., KILBY, A., WALKER-SMITH, J.A., FRANCE, N.E. & WOOD, C.B.S. (1976) Br. Med. J. (ii), 1501.

HECHL, G. & WEESE, H. (1943) Münch med. Wochschr. 90, No. 1, 11-15.

HEMMINGS, W.A. & WILLIAMS, E.W. (1974) I.R.C.S. 2, 1450.

HEMMINGS, W.A. & WILLIAMS, E.W. (1974) Proc. Roy. Soc. Biol., 187, 209-219.

HEMMINGS, W.A. & WILLIAMS, E.W. (1978) Gut, 19, 715.

HEREMANS, J.F. (1968) Curr. Top. Microbiol. Immunol. 45, 131.

HETTWER, J.P. & KRIZ, R.A. (1925) Am. J. Physiol. 73, 539.

HIRONDEL, C.L., DOE, W.F. & PETERS, T.J. (1975) Clin. Sci. Mol. Med. 50, 425–429.

HIRSCHFIELD, J.S. & KERN, S. (1969) J. Clin. Invest., 48, 1224–1229.

HJERTEN, S. (1962) Arch. Biochem. Biophys. Suppl. 1, 147.

HOLTER, H. (1959) Int. Rev. Cytol. 8, 481–504.

HUGON, J.S. & BORGERS, M. (1968) J. Histochem. Cytochem. 16, (4), 229–236.

ITO, S. (1965) J. Cell Biol. 27, 475–491.

JAQUES, P.J. (1969) In: Lysosomes in Biology and Pathology (DINGLE, J.T. & FELL, H.B., Eds.) Vol. 2, pp. 395–420. Amsterdam: North Holland.

JASPER, D.K. & BRONK, J.R. (1968) J. Cell Biol. 38, 277–291.

JONES, E.A. & WALDMANN, T.A. (1972) J. Clin. Invest. 51, 2916–2927.

JOVIN, T.K., DANTE, M.L. & CHRAMBACH, A. (1971) "Multiphasic buffer systems output." Federal Scientific and Technical Information, United States Department of Commerce, Springfielx, Virginia.

KARNOVSKY, M.L., LAZDINS, J., SIMMONS, S.R. (1975) In: Mononuclear Phagocytes in Immunity, Infection and Pathology. (VAN FURTH, R. Ed.), pp. 423–439. Oxford, Blackwell.

KAUFMANN, H.S. & HOBBS, J.R. (1970) Lancet (ii), 1061.

KIM, Y.S. (1977) In: Peptide Transport and Hydrolysis, Ciba Foundation Symposium 50, (ELLIOTT, K.E. & O'CONNOR, M. Eds.), pp. 151-171, Elsevier, North Holland. Biomedical, Amsterdam.

KINGHAM, J.G.C., WHORWELL, P.J. & LOEHRY, C.A. (1976) Gut, 17, 354–361.

KRAEHENBUHL, J.P. & CAMPICHE, M.A. (1969) J. Cell Biol. 42, 345–365, 1969.

KREBS, H.A. (1950) Biochim. Biophys. Acta. 4, 249.

KREBS, H.A. & HENSELEIT, K. (1932) Hoppe-Seyler's Z. Physiol. Chem. 210, 33–66.

LAKE, A.M., BLOCH, K.J., NEUTRA, M.R. & WALKER, W.A. (1979) J. Immunol. 122, (3), 834-842.

LECCE, J.G. (1966a) J. Physiol. 184, 594-604.

LECCE, J.G. (1966b) Biol. Neonat. 9, 50-61.

LE FEVRE, M.C. & JOEL, D.D. (1977) Life Sci. 21, 1403.

LEPPER, K. & MAILMAN, D.S. (1977) Life Sci. 20, 1697-1704.

LEVINE, R.R., MCNARY, W.F., KORNGUTH, R.J. & LE BLANC, R. (1970) Eur. J. Pharmacol. 9, 211-219.

LEWIS, W.H. (1931) Bull. John Hopkins Hosp. 49, 17-36.

LLOYD, J.B. & WHELAN, W.J. (1969) Analytical Biochem. 30 (3), 467-470.

LOEHRY, C.A., AXON, A.T.R., HILTON, P.J., HIDER, R.C. & CREAMER, B. (1970), Gut 11, 466-470.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951) J. Biol. Chem. 193, 265-275.

MAEHLY, A.C. & CHANCE, B. (1954) In: Method of Biochemical Analysis, Vol. 1 (GLICK, D. Ed.) pp. 357-364, New York, John Wiley & Sons, Inc.

MACHEN, T.E., ERLIJ, D. & WOODING, F.B.P. (1972) J. Cell Biol. 54, 302-321.

MATTHEWS, D.M. (1977) Gastroenterol. 73, 1267-1279.

MATTHEWS, D.M. (1977) In: Peptide Transport and Hydrolysis, Ciba Foundation Symposium 50, (ELLIOTT, K.E. & O'CONNOR, M. Eds.), pp. 353-355, Elsevier, North-Holland Biomedical, Amsterdam.

MATTHEWS, D.M. & LASTER, L. (1965) Am. J. Physiol. 208, 593-600.

MATTHEWS, D.M. & WISEMAN, G. (1953) J. Physiol. 120, 55P.

MILLER, J.H. & OPHER, A.W. (1964) Exp. Med. Surg. 22, 277.

MOOG, F. (1953) Exp. Zool. 124, 329-337.

MOOG, F. & THOMAS, E.R. (1955) Endocrinol., 56, 187-196.

MORRIS, I.G. (1968) In: Handbook of Physiology, Alimentary Canal (CODE, C.F. Ed.) Vol. 2, pp. 1491-1512 Washington D.C., American Physiological Society.

MORRIS, I.G. (1974) In: Biomembranes Vol., 4A, (SMYTH, D.H. Ed.) pp. 483-541, Plenum Press, London and New York.

MORRIS, B. & MORRIS, R. (1976) J. Physiol. 254, 389-403.

MORRIS, B. & MORRIS, R. (1976) J. Physiol. 255(3), 619-634.

MOXON, L. & WILD, A.E. (1976) Cell Tiss. Research, 171, 175-193.

MURLIN, J.R., TOMBOULIAN, R.L. & PIERCE, H.B. (1937) Am. J. Physiol., 120, 733.

NEVILLE, D.M. (1971) J. Biol. Chem. 246, 6328.

ODOR, D.L. (1956) J. Biophys. Biochem. Cytol. Suppl. 2, 105-108.

ORLIC, D. & LEV, R. (1973) J. Cell Biol. 56, 106-119.

ORLIC, D. & LEV, R. (1973b) Gastroenterol. 65, 60-68.

OTTAWAY, C.A., ROSE, M.L. & PARROTT, D.M. (1979) In: International Review of Physiology, Gastrointestinal Physiology III, (CRANE, R.K. Ed.) Vol. 19, pp. 323-356, University Park Press, Baltimore.

OWEN, R.L. (1977) Gastroenterol. 72, 440-451.

PALADE, G.E. (1956) J. Biophys. Biochem. Cytol. Suppl. 2, 85-98.

PARROTT, D.M. (1976) In: Immunological Aspects of the Liver and Gastrointestinal Tract (FERGUSON, A. & MACSWEAN, R.N.M. Eds.) pp. 1-32. University Park Press, Baltimore.

PATT, J.A. (1977) Biol. Rev. 52, 411-429.

PEARSE, B.M.F. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 1255-1259.

PEARSE, B.M.F. (1978) J. molec. Biol. 126, 803-812.

PIERCE, N.F. & GOWANS, J.L. (1975) J. Exp. Med. 142, 1550-1563.

PLATTNER, H., KLIMA, J., MEHNART, A. & BERGER, H. (1970) Virchows Arch. Abt. B. Zellpath. 6, 337-349.

PRATTEN, M.K.P., DUNCAN, R. & LLOYD, J.B. (1980) In: Coated Vesicles (OCKLEFORD, C. & WHYTE, A. Eds.) p. 179-218, Cambridge University Press.

RATNER, B. & GRUEHL, H.L. (1934) J. Clin. Invest, 13, 517.

RAVIN, H.A., ROWLEY, D., JENKINS, C. & FINE, J. (1960) J. Exp. Med. 112, 783.

RHODES, R.S. & KARNOVSKY, M.J. (1971) Lab. Invest. 25, 220-229.

RODEWALD, R.D. (1970) J. Cell Biol. 45, 635-640.

RODEWALD, R. (1973) J. Cell Biol. 58, 189-211.

RODEWALD, R. (1976) J. Cell Biol. 71, 666-671.

RODEWALD, R. (1976) In: Materno-foetal Transmission of Immunoglobulins (HEMMINGS, W.A. Ed.), pp. 137-155, Cambridge University Press.

ROTH, T.F., CUTTING, J.A. & ATLAS, S.B. (1976) J. Supramol. Struct. 4, 527-548.

ROTH, T.F. & PORTER, K.R. (1964) J. Cell Biol. 20, 313-332.

ROTHMAN, S.S. & GOTZE, H. (1975a) Nature 257, 607.

ROTHAMN, S.S. & LIEBOW, C. (1975b) Science 189, 472.

RYSER, H.J.P. (1968) Science 159, 390-396.

RYSER, H.J.-P. (1970) In: Proceeding of the 4th International Congress of Pharmacology Vol. 3, pp. 99-132 Basel: Schwabe.

SCHLEWINSKI, E., GRABEN, N., FUNK, J., SAHM, E. & RAETTIG, H. (1971) Zbl. Bakt. Hyg. I. Abt. Orig. A. 218, 93.

SCHULTE, S.G. & CURRAN, P.F. (1970) Physiol. Rev. 50, 637-718.

SCHULTZ, S.G. & FRIZZELL, R.A. (1972) Gastroenterol. 63, 161-176.

SELJELID, R., SILVERSTEIN, S.C. & COHN, Z.A. (1973) J. Cell Biol., 57, 484-498.

SIEFERT, J., RING, J., STEININGER, J. & BRENDEL, W. (1977) Nutr. Metab. 21, (Suppl. 1), 256-258.

SILVERSTEIN, S.C. & DALES, S. (1968) J. Cell Biol. 36, 197-230.

SILVERSTEIN, S.C., STEINMAN, R.M. & COHN, Z.A. (1977) Ann. Rev. Biochem., 46, 669-722.

SMYTH, R.D., BRENNAN, R. & MARTIN, G.J. (1964) Exp. Med. Surg. 22, 46.

SONODA, S., SHIGEMATSU, T. & SCHLAMOWITZ, M. (1973) J. Immunol. 10, 1682-1692.

STEINMAN, R.M. (1976) In: In Vitro Methods in Cell Mediated and Tumor Immunity, (BLOOM, B.R. & DAVID, J.R. Eds.), PP. 379-386. New York, Academic Press.

STEINMAN, R.M., SILVER, J.M. & COHN, Z.A. (1974) J. Cell Biol. 63, 949-969.

STEWART, S.O. (1978) Nutr. Reviews 36, (10).

STRAUS, W. (1964) J. Cell Biol. 20, 497-507.

STRAUS, W. (1969) In: Meth. Archieum. exp. Path., (BAJUSZ, E. & JASMIN, G. Eds.) Vol. 4, pp. 54-91, Karger Press, Basel/New York.

STRYER, L. (1975) In: Biochemistry pp. 248-249. San Francisco: W.H. Freeman.

SWARBRICK, E.T., STOKES, C.R. & SOOTHILL, J.F. (1979) Gut, 20, 121-125.

TAGESSON, C., SJODAHL, R. & THOREN, B. (1978) Scand. J. Gastroenterol. 13, (5), 519-524.

TAYLOR, E.W. (1970) Cytobios 7-8, Vol. 2, 193-197.

TAYLOR, B., NORMAN, A.P., ORGEL, H.A., STOKES, C.R., TURNER, M.W. & SOOTHALL, J.F. (1973) Lancet (ii), 111.

THOMAS, H.C. & PARROTT, D.M.V. (1974) Immunology, 27, 631-642.

TOLO, K., BRANDTZAEG, P. & JONSEN, J. (1977) Immunology 33, 733-743.

TRIER, J.S. (1968) In: Hand Book of Physiology (CODE, C.F. Ed.) Section 6, Vol. III, pp. 1125-1177. American Physiological Society, Washington.

TRIER, J.S. & RUBIN, C.E. (1965) Gastroenterol. 49, 594-603.

WALKER, W.A. (1975) Antigen absorption from the small intestine and Gastrointestinal Disease. Ped. Clin. N. America 22 (4) 731-746.

WALKER, W.A., ABEL, S.N., UKE, M. & BLOCH, K.J. (1976) J. Immunol. 117, (3), 1028-1032.

WALKER, W.A., CORNELL, R., DAVENPORT, L.M. & ISSELBACHER, K.J. (1972) J. Cell Biol. 54, 195-205.

WALKER, W.A. & ISSELBACHER, K.J. (1974) Uptake and transport of macromolecules by the intestine. Possible role in clinical disorders. Gastroenterol. 67, 531-550.

WALKER, W.A. & ISSELBACHER, K.J. (1977) New Engl. J. Med. 297, 767.

WALZER, M. (1927) J. Immunol. 14, 143-149.

WAGNER, R., ROSENBERG, M. & ESTENSEN, R. (1971) J. Cell Biol. 50, 804-817.

WARSHAW, A.L., BELLINI, C.A. & WALKER, W.A. (1977) Am. J. Surg. 133, 55.

WARSHAW, A.L., WALKER, W.A. & CORNELL, R. (1971) Lab. Invest. 25, 675-684.

WEBSTER, P. (1979) Personal communication.

WEESE, H. (1949) FIAT Rev 62, 224.

WELLS, H.G. & OSBORNE, T.B. (1911) J. Infect. Dis. 8, 66.

WILD, A.E. (1973) In: Lysosomes in biology and pathology (DINGLE, J.T. Ed.) Vol. 3, pp. 169-215, North Holland Biomedical Press, Amsterdam.

WILD, A.E. (1975) Phil. Trans. B. 271, 395-407.

WILLIAMS, K.E. & IBBOTSON, G.E. (1979) In: Protein Trnasmission through Living Membranes (HEMMINGS, W.A. Ed.) pp. 185-195, Elsevier, North Holland Biomedical Press.

WILLIAMS, K.E., KIDSTON, E.M., BECK, F. & LLOYD, J.B. (1975) J. Cell Biol., 64, 113-134.

WILLIAMS, R.G. & GIBBONS, R.J. (1972) Science 177, 697.

WILSON, T.H. (1956) J. Biol. Chem. 22, 751-757.

WILSON, T.H. & WISEMAN, G. (1954) J. Physiol. 123, 116-125.

WISEMAN, G. (1953) J. Physiol. 120, 63-72.

WISEMAN, G. (1955) J. Physiol. 127, 414-422.

WISEMAN, G. (1956) J. Physiol, 133, 626-630.

WISEMAN, G. (1974) In: Biomembranes (SMYTH, D.H. Ed.) Vol. 4A, pp. 363-483, Plenum Press, London and New York.

WORTHINGTON, B.S., BOATMAN, E.S. & KENNY, G.E. (1974) Am. J. Clin. Nutr. 27, 276–286.

WORTHINGTON, B.S. & ENWONWU, C. (1975) Am. J. Dig. Dis. 20, (8), 750–763.

WORTHINGTON, B.S. & SYROTUCK, J. (1976) J. of Nutr. 106, 20–32.

UGOLER, A.M. (1974) In: Biomembranes (SMYTH, D.H. Ed.) Vol. 4A, pp. 285–363, Plenum Press, London and New York.

VOLKHEIMER, G. (1977) Adv. N.E.E.E. in Pharmacol. and Clin. 14, 163–187.