Intestinal amino acid absorption in development

Thesis

INTESTINAL AMINO ACID ABSORPTION IN DEVELOPMENT

by

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Abstract

1 An *in vitro* preparation was employed to investigate uptake of a number of neutral and basic amino acids by the developing rat small intestine.

2 The kinetics of uptake of these substrates were determined from birth to maturity and changes described in the affinity for and rate of uptake by the jejunum particularly around the time of weaning. All the amino acids had a higher affinity for uptake and were accumulated to a greater extent by the jejunum during the first three weeks of life as compared with older animals. Similar kinetic profiles for certain amino acids suggested that they shared common uptake systems.

3 The specificity of neutral and basic amino acid uptake was examined developmentally by employing inhibition kinetics. The results suggested that while distinct neutral and basic uptake systems were present at birth, there was some functional overlap : neutral amino acids were able to use basic systems and vice versa. After three weeks of age, this picture changed and the uptake systems were much more defined.

4 Uptake of amino acids was found to depend to a large extent on energy derived from oxidative phosphorylation even at birth. This energy expenditure was directed toward maintaining a Na^+ gradient across the enterocyte membrane and co-transport of neutral amino acids with Na^+ thus promoted accumulation. The basic amino acids, however, were found not to be dependent on the existence of such an ion gradient but they did require the presence of

Na^+ ions for carrier binding, and their uptake demanded energy expenditure at some stage.

5 Precociously inducing the appearance of a functionally distinct absorptive cell was found to change the kinetics of basic amino acid uptake in the jejunum, and promote such specificity of uptake as was seen in older animals.

6 Changes in the developing small intestine itself have led to problems in the expression of results and these have been discussed at length in terms of their relation to these and other findings.

7 These results on the development of amino acid uptake systems are discussed in the context of morphological changes that occur postnatally in the small intestine, with particular reference to the appearance of a functionally distinct absorptive cell around the time of weaning, and are related to the physiological requirements of the neonatal animal.

For Kiki, my family and friends

When one is very young and knows a little,
mountains are mountains, water is water
and trees are trees.

When one has studied and has become sophisticated,
mountains are no longer mountains, water is no longer water
and trees are no longer trees.

When one thoroughly understands,
mountains are again mountains, water is water
and trees are trees.

Zenrin poem

What seems Chance is merely ignorance on the part of the beholder. If he knew enough, he'd see that things could not have happened otherwise.

Kurt Vonnegut Jr.

CONTENTS

SYMBOLS AND ABBREVIATIONS

V	Uptake rate
Vmax	Maximal uptake rate
S	Substrate concentration
I	Inhibitor concentration
K_m	Affinity constant
K_i	Inhibitor constant
K_D	Diffusion constant
T/M	Concentration ratio (tissue : medium)
%I	Percentage inhibition
p	Probability
SEM	Standard error of mean
n	Number of experiments/observations
a_1 and a_o	Regression coefficients
s_1 and s_o	Standard errors of regression coefficients
r^2	Coefficient of determination
Day 1	Day of birth
ECS	Extracellular space
L-lys	L-lysine
L-arg	L-arginine
L-leu	L-leucine
L-val	L-valine
L-phe	L-phenylalanine
L-met	L-methionine
DNP	Dinitrophenol
GABA	4-amino-n-butyric acid
cAMP	Cyclic adenosine monophosphate
ATPase	Adenosine triphosphatase
ATP	Adenosine triphosphate

CHAPTER 1

General Introduction

Amino acids, the end products of protein digestion, are important substrates for particular cell processes. Cells use amino acids as the building blocks for protein synthesis which in turn maintain the cells integrity, modify the permeability of the limiting plasma membrane, perform catalytic functions as enzymes and probably "store" environmental information. Amino acids also perform "messenger" roles as neurotransmitters in the nervous system and as small peptides (e.g. hormones) are involved in longer term regulatory functions. Similarly, they can be used as substrates for gluconeogenesis and ultimately yield stores of high energy phosphates for cellular function. In the developing animal, amino acids are critical for growth, there being an enormous net increase in protein synthesis at this time.

Nearly half of the naturally occurring amino acids are "essential" to the mammalian diet; the rest can be manufactured within the cells. In the neonatal animal, the cellular machinery for such manufacture is not yet fully available and this adds two more amino acids to the list of essentials. The uptake processes for amino acids lie within or on the villus lining cells in the small intestine, cells which not only transport amino acids but also concentrate them using a mechanism that requires energy expenditure. Such concentrative uptake means that amino acids can be continuously transported from very low luminal concentrations into the blood. While much is known of these transport mechanisms in the adult, the situation in the neonate is less clear. Halvor Christensen remarked a few years ago in a conference on membranes in growth and differentiation that "our understanding of

the development of transport systems is in a relatively primitive state".

In the young rat the small intestine can absorb protein intact, in addition to amino acids, up until the third week of life. At this time, coincident with weaning, the small intestine undergoes dramatic changes, which result in a population of enterocytes that are no longer able to absorb significant amounts of macromolecules, and the gut now exhibits a mature profile of enzyme activities. It has been suggested that the enterocytes produced at this time are a new population of cells arising from the crypts of Lieberkuhn and that their appearance is under the control of adrenal hormones.

The work described here is an attempt to investigate, developmentally, the uptake processes in the jejunum for a number of essential amino acids which contain representatives of both basic and neutral classes, with a particular focus on the time of weaning and the appearance of an absorptive cell type performing de novo functions. Chapter 2 discusses the structural and functional changes that the small intestine undergoes in development. Chapter 3 describes the methodology used and the everted jejunal segment preparation. Chapter 4 presents evidence in support of this in vitro technique coupled with studies on the maturing small intestine which reveal the many problems encountered in developmental physiology. The kinetics of amino acid uptake in the developing jejunum are described in Chapter 5 and this is extended in Chapter 6 to reveal the specificities of the membrane uptake systems for particular amino acids. Chapter 7 investigates the role of co-transported ions in amino acid uptake and attempts to identify the energy requiring

process involved in substrate accumulation. Lastly, the effect of precociously inducing the appearance of a new population of enterocytes on amino acid uptake is described in Chapter 8.

While each of the four main results Chapters has a separate introduction and discussion, the main findings are carried over into successive Chapters and drawn together at the end in a final discussion.

CHAPTER 2

Structural and Functional Development of the Small Intestine

Mammals obtain food as complex raw materials, salts and vitamins from the external environment and the alimentary canal transfers both food and water, via a circulatory system, throughout the body. The structure of the entire digestive tract can be histologically classified, the intestinal wall consisting essentially of four concentric layers:

1) Mucosa - of epithelium, lamina propria and muscularis mucosa

2) Submucosa - loose connective tissue and nerve plexus

3) Muscularis externa - of opposed (circular and longitudinal) smooth muscle layers

4) Serosa - flattened endothelial cells continuous with the mesentery attaching the intestine to the body wall

The small intestine can be divided into three regions, duodenum, jejunum and ileum, which are histologically and functionally distinct. Proximally the intestine is thicker and more vascular and the surface area is some four times greater than in the ileum. The morphology of the mucosal layer provides an enormous enlargement of the absorptive surface (some 600 times). Infoldings of the mucosa form villi with a core of connective tissue, capillaries and lymphatics (lamina propria). In addition, the single layer of columnar enterocytes lining the villi have a microvillus structure or brush border. Around the base of each villus, and with epithelium continuous with that of the villus, are several simple, narrow indentations, the crypts of Lieberkuhn, which are more abundant in the proximal intestine.

The number of villi increase up to the third month of life, while the number of crypts doubles. The ratio of crypts to villi varies with species, averaging some 13:1 in the rat. Interspersed with the regular enterocytes are mucous or goblet cells which provide a protective secretion; occassional enterochromaffin cells and Paneth cells are seen in the crypts. Transport in vivo takes place across the whole layer of columnar cells which separates two fluid compartments of different compositions and includes at least two barriers arranged in series, the mucosal brush border and the basal (serosal) membrane.

At birth, the mammal is transferred from a parasitic existence to one which requires regulation and adaptation. The evidence of successful development is manifest in postnatal growth which in turn is dependant upon adequate nutrition and efficient intestinal absorption. In most mammals there is an added complication for the intestine in dealing with a dietary change at weaning. Consequent with this switch from suckling milk to ingesting solid food, the intestine undergoes a number of structural alterations which result in the functional maturity associated with the adult structure as described above.

Morphological changes in development

The physiological development of the small intestine is essentially similar in all mammals but the timing of developmental events varies between species due to differences in gestation and lactation (cf. guinea pig and rat). The events described here are relevant to rats and mice. In all mammals prenatal intestinal developmental is

such that, at birth, milk can be digested and absorbed.

What is immediately striking about the neonatal rat small intestine is the colour change proximo-distally. The distal portion is distinctively yellow in colour which seems to disappear around 20-25 days of age. The colour can be seen directly on the mucosal wall and colloid granules have been described in the enterocytes by Cornell and Padykula (1965). Similar colour changes have been observed in fetal human and guinea pig intestine (due to the presence of meconium) and in neonatal puppies and rabbits (Koldovsky, 1969). The functional significance of this phenomenon is unclear.

The length of the small intestine related to body weight decreases dramatically during the first postnatal week (Hruby, 1959) but reaches 75% of the adult length at 25 days old. Miller (1971) divides the postnatal growth pattern into three distinct phases in terms of length and weight gains: preweaning, adolescent and adult. Relative to body weight, wet weight of the small intestine increases slowly prior to weaning and then rapidly accelerates (5% of body weight). Similarly, Herbst and Sunshine (1969) have noted a relative increase in jejunal/ileal weights around weaning. In the adult the intestine represents some 1.4% of body weight (similar to the neonatal level). The wall of the small intestine also thickens around weaning (Hruby, 1959) which appears to be a structural change rather than an increase in water content (see Chapter 4). Swallowing has been observed in the foetus and rhythmic intestinal movements are present at birth but

propulsive motility develops postnatally, reaching 75% efficiency by weaning.

Morphologically, the intestinal mucosa of the neonate shows a high level of structural development, (see Plates 1-5), though there is little connective tissue present in the wall (Koldovsky, 1969). The formation of crypts and villi begins in utero. Villi are the result of folding which begins near the pylorus and produces large, regular and parallel invaginations. These invaginations spread to other parts of the intestine and break up into villi due to irregular growth first in the jejunum and later the ileum. Crypts appear at birth as irregular regions of thickening or depression of the epithelium and Hilton (1902) believed that crypts and villi developed independantly.

Postnatally, villi multiply, increase in height and size (by a factor of 3) and change from a "finger shape" into a flatter "button shape". Villus height levels out at weaning, (see Plates 8 and 9), and crypts appear as continuations of the villi bases. The muscularis mucosa is not present at birth but begins to appear around 10 days of age and lymph nodes become abundant after weaning. After birth, continual proliferation of enterocytes occurs in the crypts, and cells migrate from there onto and along the villi, eventually being extruded from the tips into the intestinal lumen (see Plates 10 and 11).

Villus cell differentiation and kinetics

The epithelial cells that line the villus fall into four main categories. Cheng and Leblond (1974), after an exhaustive histological study, have put forward a unitarian theory for their origin from the crypt base.

1) Columnar cells (enterocyte or "chief cells")

These are present throughout the small intestine, increase in size (by some 30%) postnatally and are characterised by the microvillar arrangement of their luminal membranes. The enterocytes arise by division in the crypts and differentiate as they pass up the crypts. By the time these cells migrate along the villus (some 36 hours) they are fully differentiated, non dividing and perform the major absorptive function of the intestine.

Enterocytes are tall, tightly packed and form complex interdigitations laterally with adjacent cells, (see Plates 6 and 7). The cytoplasm is rich in mitochondria and rough endoplasmic reticulum. The jejunum and duodenum are composed of 95% columnar cells while the ileum contains some 90% of the total cell number. The crypts in the proximal gut are 50% deeper than those of the ileum and it is mitotic pressure that promotes enterocyte migration. However, some columnar cell mitoses give rise to other cell types:

2) Mucous cells

These arise from crypt cells and migrate to the villus tips with a turnover time of 72 hours, (see Plates 12 and 13).

3) Entero-endocrine cells

These arise from crypt base columnar cells, are extremely granular and migrate to the tip with a turnover time of 96 hours.

4) Paneth cells

These again arise from crypt base columnar cells but do not divide or migrate. They are very granular and have a long turnover time of 15 days.

The enterocyte is the major cell of the small intestine performing specialised functions through its brush border, an extremely labile structure with rapid protein turnover (Billington and Nayudu, 1976). Indeed, many of the properties of the brush border in neonatal animals are different from those in adults. Many studies have been made of the kinetics and control of intestinal cell division and migration. Herbst and Sunshine (1969) found that in rat, 15 days after birth, the small intestine showed a striking increase in relative weight, increase in crypt depth and an elevation of the mitotic and labelling index of crypt cells. The rate of migration of enterocytes along the villus in suckling rats is much slower (some 100 hours) than that in the adult animal (36 hours), but increases during weaning to adult rates. Concomitantly, there are changes in enzyme activities which appear to be under hormonal control (enzymes and hormonal influences will be discussed further in the following Sections). Lipkin (1973) has reviewed the factors involved in the proliferation and differentiation of gastrointestinal cells. Crypt cells undergo some 2 divisions and migrate at a velocity of 1 cell position per hour. Patterns of changing enzyme activities characterise the normal growth and maturation of the enterocytes. Cells differentiating and migrating from the crypts to the villus tips show increasing activities of adenosine deaminase and nucleoside phosphorylase which lead to the

synthesis of key nucleic acid precursor molecules as well as to other metabolic activities.

Recently, Kapadia and Baker (1976) investigated the influence of alterations in villus architecture on villus surface area and the relationship, at different ages, between the villus surface area and the crypts. The number of villi increased around weaning by 15% and continued to increase until 3 months of age. Despite changing shape, villus surface area changed very little after two weeks of age, though surface area was considerably greater proximally than distally (by a factor of 2) due to the presence of much higher villi proximally. Kapadia and Baker suggest that the alteration in villus shape is due to hypertrophy of the lamina propria rather than depressed cell production by the crypts.

Enzyme activity in the developing gut

In contrast to the adult, the diet of the suckling animal is relatively low in total carbohydrates and those present are not the ones generally found in the adult diet. Lactase is the major carbohydrate of milk and high activities of lactase are detectable in the mucosa three days before birth. Lactase activity peaks in the first postnatal week and then declines to adult values at weaning (Doell and Kretchmer, 1962). In contrast, the α-glucosidases (sucrase, maltase) are low in suckling rat mucosa and rise suddenly in the third week, reaching adult values by the end of weaning.

Fat is important in the neonatal diet and the mucosa can esterify fatty acids from birth (Koldovsky, 1969) but due to the low activity of pancreatic lipase until weaning, it is unlikely that much

esterification occurs pre-weaning. The major route of fat absorption is considered by some to be by pinocytosis and transport is directly into the lymphatics fats having undergone some lysosomal digestion. Similarly in the mature human newborn infant, only pancreatic amylase shows a low activity, whereas other digestive enzymes increase rapidly with age. Most intestinal absorptive functions develop during early pregnancy and are at adult levels in the newborn (Shmerling, 1976).

Evidently, the development of intestinal function involves primary changes in the cells of the crypts. de Both, van Dongen, van Hofwegen, Keulemans, Visser and Galjaard (1974) investigated the maturational processes that enterocytes undergo on their journey from the crypts to the villus tips. de Both et al showed that the activity of nonspecific esterases, mainly localised in the endoplasmic reticulum, increases when the cells migrate along the crypt. Alkaline phosphatase (a microvillus marker) activity was absent in the crypt but increased along the villus. Similar results were found for α-glucosidase and lactate dehydrogenase. However, the specific activity of enzymes bound to mitochondria or lysosomes were similar in both crypt and villus. Moog (1971) has demonstrated the marked increase in alkaline phosphatase activity during the third postnatal week. The distribution of this enzyme within the brush border is suggestive of some digestive/absorptive role. Similarly non-specific esterases show marked increases around weaning (Koldovsky, 1969). However, lysosomal hydrolases fall sharply after weaning possibly associated with "closure" (see next Section).

Even though there is a change in activity of many enzymes of glycolysis and digestion around weaning, some enzyme activities remain constant, indicating that there is not a general increase of all metabolic activities (Srivastava and Hubscher, 1968).

In contrast to these hydrolytic enzymes involved in absorption, de Both, van der Kamp and van Dongen (1975) examined the enzymes involved in nucleotide synthesis. Hypoxanthine phosphoribosyl transferase (involved in nucleotide conversion) activity was twice as high in the villus than in the crypt cells, whereas in contrast, thymidine-kinase (specific for proliferating tissues) showed a much higher activity in crypt cells.

There remains one enzyme which plays a major part in intestinal function, namely the Na^+, K^+-dependant, adenosinetriphosphatase (ATPase). The location of this enzyme in the mucosa has been a matter of histochemical dispute for some time, due mainly to the variation in techniques for separating enterocytes from underlying tissue and fraction cross-contamination. The enterocyte has a polarity of function, absorbing substrates on the luminal side and transporting them into the circulatory system on the serosal side. Much evidence has occured which favours coupling of Na^+ to substrate transport such that Na^+ ions enter the cell down a concentration gradient which is maintained by a pump on the serosal membrane. Fujita and Nakao (1973) isolated the brush borders from intestinal mucosa and assayed both for Na^+, K^+-ATPase and sucrase. With successive purification, ATPase activity disappeared while sucrase activity remained. Consequently they further fractionated the baso-lateral membranes and found the relative specific activity ratio of

apical to basolateral membrane was 22:1 for Na^+, K^+-ATPase and 1:180 for alkaline phosphate, an extensive regional differentiation of cell membrane. Charney, Gots and Giannella (1974) traced the activity of Na^+, K^+-ATPase in enterocytes as they migrated up the villi. Again, Charney et al. found more than 85% of the activity in a plasma membrane fraction devoid of brush border but also discovered another gradient of activity which increased from crypt to villus tip. A similar decrease in activity was seen from jejunum to ileum. Thus the 60% greater absorption of Na^+ and water in the jejunum corresponds with a 60% higher level of enzyme activity.

However, Luppa, Remke, Muhle, Dehmer and Muller (1975) have described the localisation of Na^+, K^+-ATPase in isolated brush borders of rat small intestine mucosa, and checked the purity of their fractions both morphologically and enzymically. The ATPase seemed to be localised deep within the brush border region. Similarly, Lacombe, Mitjavila and Carrera (1976) describe Na^+, K^+-ATPase activity in both brush border and basolateral membranes.

Plate 1

Light micrograph of villus from proximal jejunum of 3 day old rat, stained with haematoxylin and safranin. The brush border can be clearly seen and the enterocytes contain vacuoles just above the large nuclei.

X 100

Plate 2

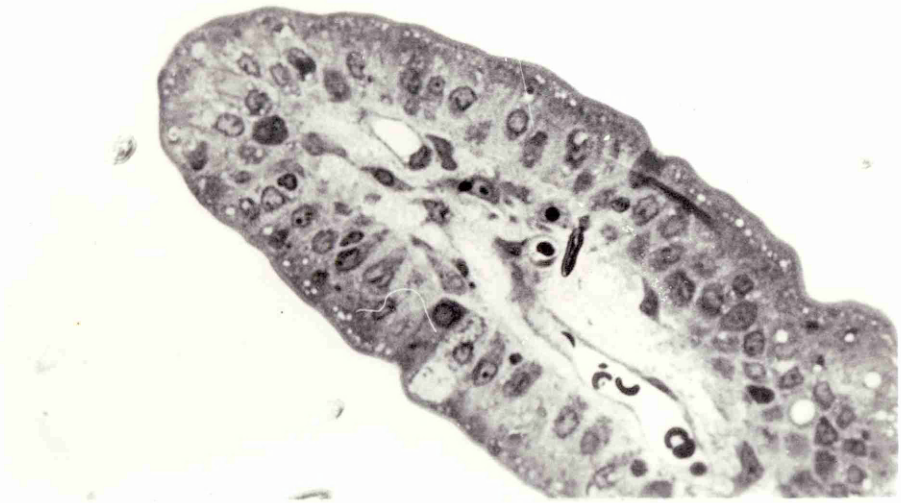
Electronmicrograph of two adjacent enterocytes from a field of Plate 1 above. Just beneath the microvilli (mv) vacuole (v) formation can be seen. The lateral cell membranes are intricately folded (f), the cytoplasm contains many dense bodies (db) among the mitochondria (m) and very large nuclei (n).

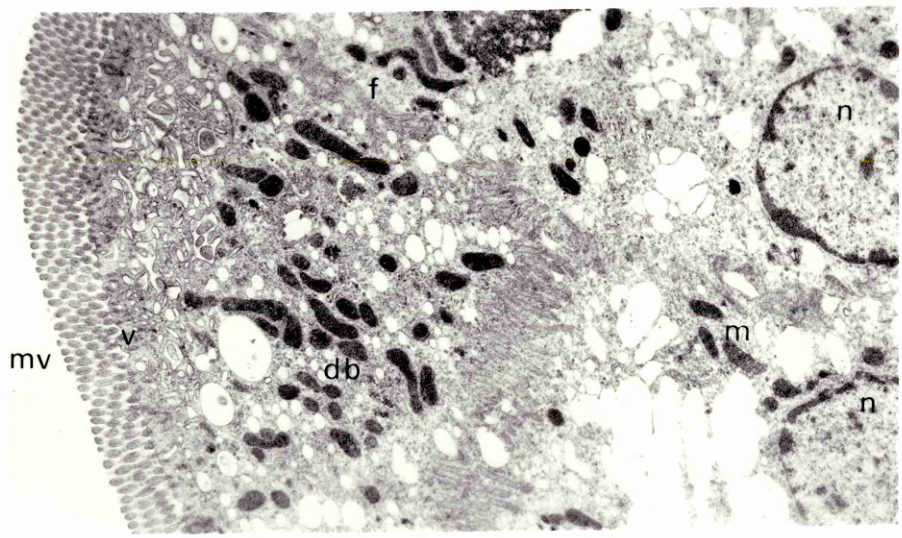
X 10000

Plate 3

Scanning electronmicrograph of brush border of 3 day old rat jejunum. The villus tip has been knocked off in the preparation of the sample, leaving a cross section view of the villus. The microvilli are densely packed and vacuoles can clearly be seen in the cell cytoplasm.

X 10000

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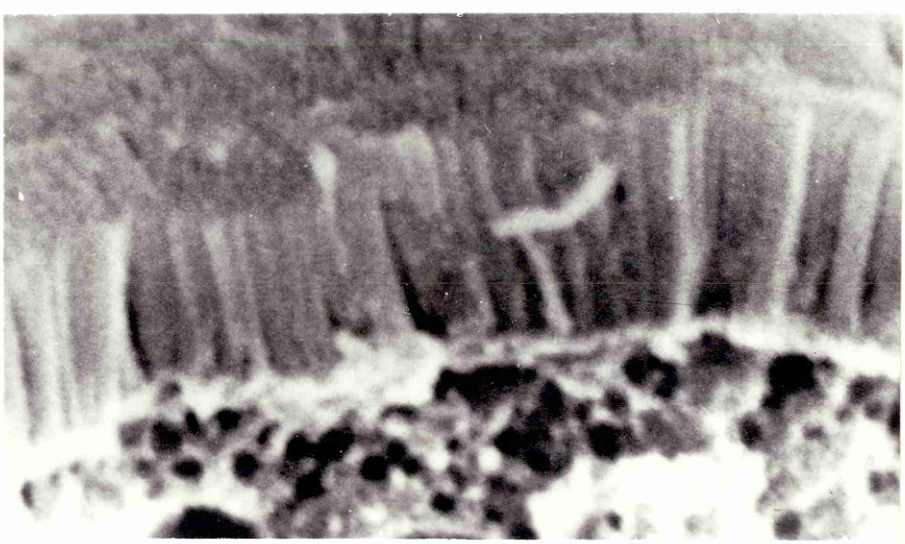


Plate 4

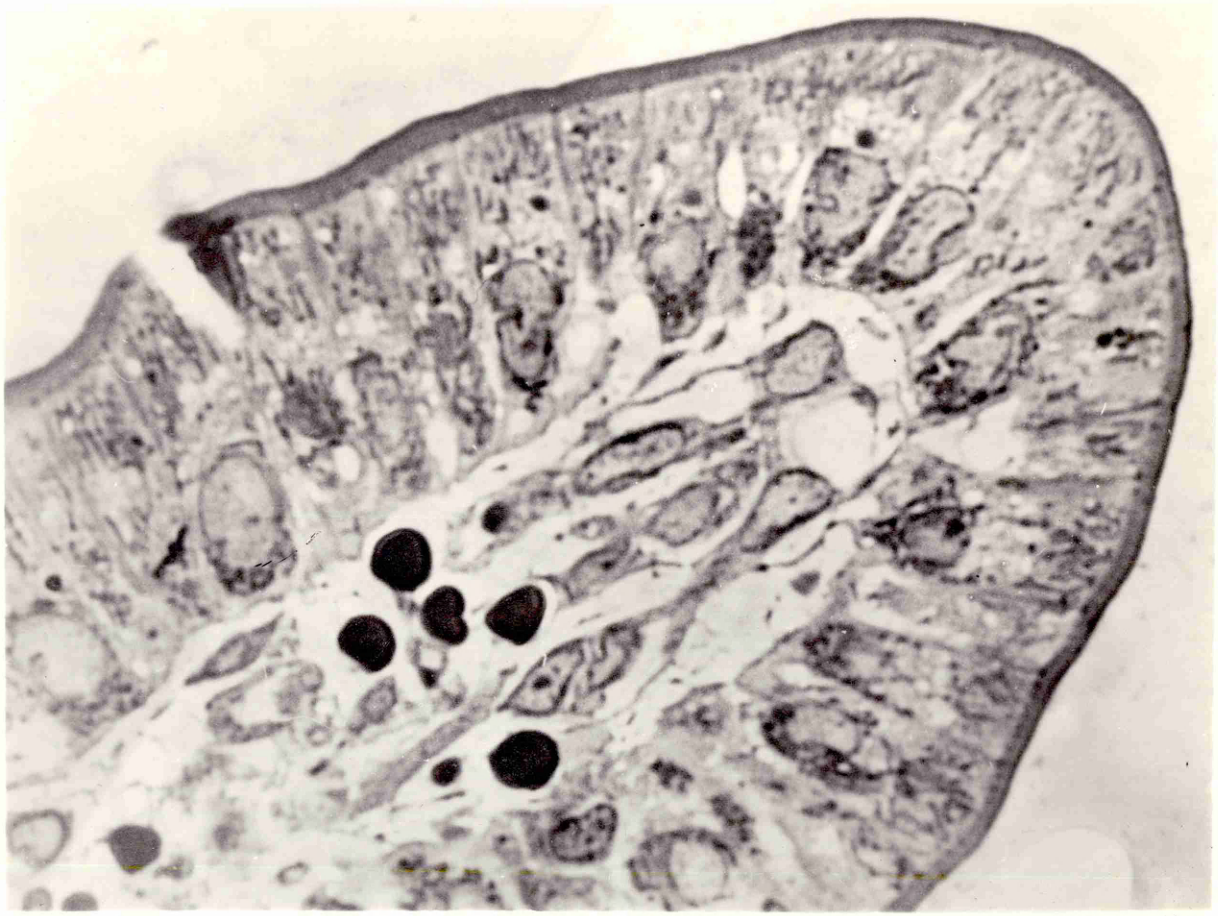
Light micrograph of 10 day old rat proximal jejunum stained with haematoxylin and safranin.

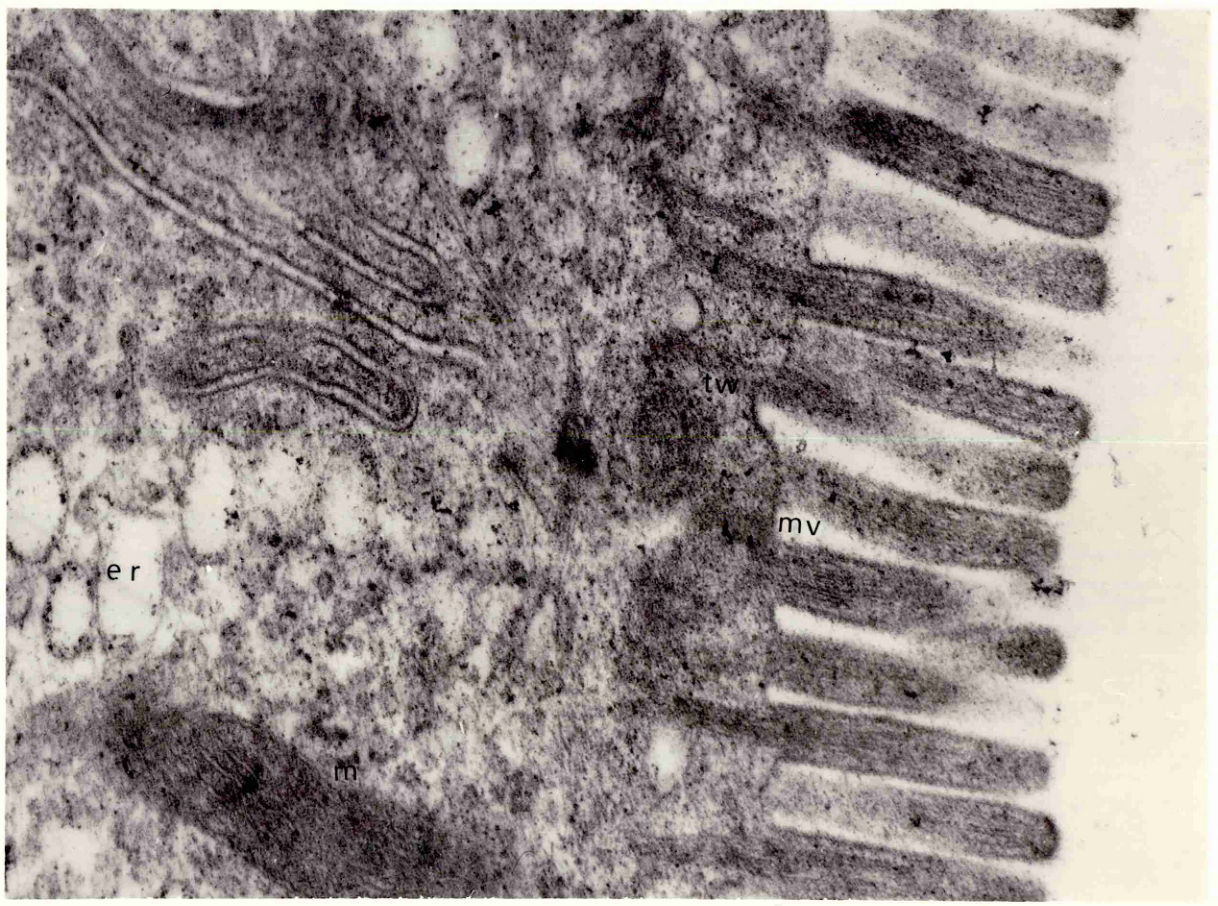
X 250

Plate 5

Electronmicrograph of brush border field from Plate 4 above. The filamentous structure of the microvillus (mv) core can be clearly seen, the microvilli being covered with fragments of glycocalyx, and there are large numbers of endoplasmic reticulum fragments (er) and mitochondria (m) just beneath the terminal web (tw).

X 15000




tw
mv
er
m

Absorption of protein and intestinal "closure" at weaning

Intestinal protein absorption can occur in three ways; intact, in the form of small peptides or in fundamental amino acid units. The latter is well documented in the adult and the last few years has seen an escalating literature on peptide absorption (for review, see Matthews, 1975). During or after uptake the peptides are almost all hydrolysed to their constituent amino acids, a very small amount of a few dipeptides appearing in the blood, and so virtually all the original protein reaches the blood as free amino acids (Wiseman, 1974).

In neonates, whose milk diet contains considerable amounts of protein, the picture of absorption is quite different. Luminal digestion of protein is minimal and macromolecules, including proteins, can be absorbed from the intestine intact. The proteolytic enzymes of the stomach and pancreas are low during the first two postnatal weeks and increase dramatically during the weaning period (Furihata, Kawachi and Sugimura, 1972; Jones, 1972). Similarly, peptidase activities of the mucosa are lower during suckling (Koldovsky, 1969).

In rats, there is good evidence to suggest that protein is absorbed by pinocytosis (Clark, 1959), a process which appears in the intestine just before birth (Orlic and Lev, 1973) and remains high until weaning. The role of protein absorption in the newborn is associated with the transfer of passive immunity from mothers' colostrum to the young which in the rat occurs mainly postnatally. Only immunoglobulins of the IgG class are transported whereas other milk proteins and immunoglobulins of other classes are apparently

digested (Brambell, 1970). The transfer of IgG in the rat is very selective (Rodewald, 1976b) and occurs via specific, saturable membrane binding sites which initiate pinocytosis and the formation of large supra-nuclear vacuoles. Baintner and Veress (1970) found that occurrence of these vacuoles diminished in the ileum between the 18th and 21st days.

The site of antibody absorption remains unresolved. Daniels (1972) found that the transport of IgG increased from the rat duodenum to the ileum by a factor of 4, and Jones (1972) found IgG activity limited to the distal gut. Studies with polyvinylpyrrolidone (PVP) an inert macromolecule also suggest that the distal intestine is mainly concerned (Clarke and Hardy, 1969). However, evidence presented by Rodewald (1973) indicates that the proximal intestine transmits antibodies to the circulation and that distal absorption is non-specific. Further, studies by Morris and Morris (1974 a, b) and Morris (1975) suggest that PVP is an unsuitable indicator of antibody absorption and that the proximal intestine is the major site, despite there being an absence of vacuolated cells. Recently Jones (1976) has again examined protein transport and found more specificity of uptake in the proximal intestine.

The specificity of IgG transport suggests a receptor binding mechanism in or on the enterocytes. Jones and Waldmann (1972) observed that isolated brush border retained selective binding of IgG and recently Balfour and Jones (1976) have isolated an IgG specific receptor from human placental cell membranes. Binding appears to be a pH dependent process (at pH 6.5 but lost at pH 7.4) and Rodewald (1976 a, b) suggests this pH change may be important

in the discharge of bound IgG into the circulation, ensuring unidirectional transfer. Daniels (1971) looked at the competition between neutral amino acid and IgG transport and found them largely independent. Similarly, Donnelly (1971) and Bamford and Donnelly (1974) investigated the effect of charged and neutral amino acids on IgG transport. Neither neutral nor acidic amino acids affected IgG transport but the basic amino acids lysine and arginine significantly stimulated protein absorption. These results suggest that in the neonatal gut, amino acid and protein transport are distinct processes, IgG possibly complexing with a membrane receptor rather than bound to a soluble carrier protein. The stimulation of protein transport by basic amino acids could be a result of stimulus of pinocytosis by positively charged solutes, interacting with the glycocalyx (Donnelly and Bamford, 1976).

Protein absorption declines dramatically around the third postnatal week in the rat, corresponding with a decreased vacuolation of the enterocytes which Clarke and Hardy (1969) describe as a replacement of cell type . What are the factors involved in this intestinal "closure" at weaning? Walker, Cornell, Davenport and Isselbacher, (1972) found that presenting intact proteins to the terminal surface of everted intestinal sacs from adult rats resulted in greater uptake than in neonates. This might suggest that "closure" is due to the increase in proteolytic enzymes around weaning. However, a number of other mechanisms appear to be operating. Delayed weaning of young rats, through deprivation of solid food, results in delayed closure (Daniels, 1972), though closure still occurs some 3 days later. Halliday (1959) found that closure could be induced early by injection of cortisone acetate, the levels of glucocorticoid being

low until 18 days postpartum (Daniels, Hardy, Malinowska and Nathanielsz, 1972). The injection of a large dose of cortisone acetate early in life resulted also in a decline in PVP uptake and was associated with the progressive displacement of vacuolated cells from the villi (Daniels, Hardy, Malinowska and Nathanielsz, 1973). Morris and Morris (1974) suggest that cortisone acetate slows release of material from the stomach and that this may contribute to a decrease in protein absorption. Corticosterone, rather than cortisone, is the major glucocorticoid in the neonatal rat and the latter shows no increase in level around weaning. Morris and Morris (1976) could find no effect of corticosterone inducing precocious replacement of the enterocytes in the distal region.

Clarke and Hardy (1969) demonstrated that during natural closure, the time course in the decline of PVP uptake (some 72 hours) corresponded well with the replacement time of villus enterocytes, and Daniels et al (1973) suggest that the crypts of Lieberkuhn may produce a new cell type when blood steroids are above a certain threshold. Indeed, bilateral adrenalectomy delays closure by some 3 or 4 days (Daniels, Hardy and Malinowska, (1973), though it does not permanently prevent it, and the subsequent closure is associated with identical histological changes in the villi. At present, the exact control of closure defies explanation though diet and the adrenal glands both play significant roles. What is of interest is the changing enterocyte function during the third postnatal week, the time course of closure being suggestive of the replacement of immature cells lining the villi with a population of enterocytes that possess different transport abilities through changing membrane properties.

Regulation of intestinal development

The changes in the structure and function of the gut around weaning are dramatic and largely irreversible. Control of development may involve either intrinsic or extrinsic factors, or even the synergistic activity of both.

The effect of delayed dietary change has already been alluded to. Could a change from high fat/low sugar to low fat/high sugar trigger the striking elevations in alkaline phosphatase, sucrase and maltase, at the same time depressing lactase and pinocytotic activity? It seems unlikely. Premature weaning does not alter enzyme activities, although depriving young rats of solid food has been shown to delay closure. Another extrinsic control may be the colonisation of the developing gut with bacteria which occurs around closure. Germ free rat studies are inconclusive; development of enzyme activities appears similar (Reddy and Wostmann, 1966). Interestingly, the turnover times of enterocytes in adult germ free rats was identical with that in the sucklings (Lesher, Walburg and Sacher, 1964) and bacterial invasion of the formers' gut reduced cell transit time to normal adult levels (Khoury, Flock and Hersh, 1969).

The previous section on closure strongly suggests adrenocortical involvement. Apart from the effect of glucocorticoids on protein absorption and pinocytotic activity, administration of exogenous corticoids causes precocious appearance of sucrase, maltase and alkaline phosphatase coupled with the disappearance of

sulphatase (Henning and Kretchmer, 1973). Adrenalectomy, as has been mentioned, will delay closure but only for a few days, and ACTH, while affecting sucrase and alkaline phosphatase levels (Moog, 1971) does not affect macromolecule absorption (Daniels, Hardy and Malinowska, 1973). Lactase appears to be unaffected by exogenous steroids and Yeh and Moog (1974) suggest an involvement of the thyroid as thyroidectomy and hypophysectomy result in a prolongation of lactase activity. The action of thyroxine (which increases normally around Day 10) appears to be a cooperative one with glucocorticoids, which in turn appear to have a synergistic effect with dietary factors on the intestinal changes around weaning. Injection of thyroxine in young rats also promotes closure of PVP uptake (Chan, Daniels and Thomas, 1973); this may be effected through increased steroid production in such animals (Malinowska, Chan, Nathanielsz and Hardy, 1974). Gastrin has also been implicated in the ontogenic development of the small intestine as this hormone increases around weaning, is stimulated by the presence of solid food in the stomach and trophically affects intestinal tissue (Lichtenberger and Johnson, 1974).

A number of hormones (thyroid, pancreatic, adrenal) affect the absorptive, metabolic and digestive functions of the mature small intestine (for review, see Levin, 1969), and their activities could well be mediated through cyclic nucleotides. Robberecht, Deschodt-Lanckman and de Neef (1974) have proposed that the relative levels of cAMP and cGMP integrate the responses of tissues to various stimulators. The rat small intestine has high levels of cGMP and guanylate cyclase particularly in villus cells (in the brush border).

While adenylate cyclase is higher in crypt cells, activity has been found in the basolateral brush border membrane (Quill and Weiser, 1975). These authors suggest that the different membrane locations for adenylate and guanylate cyclases in the enterocyte reflect integrated effects on membrane transport processes. Thus specific substrate proteins have been identified in the brush border membrane for cyclic GMP-dependent phosphorylation, which suggests a mechanism whereby cGMP might change the properties of transport systems exclusively at the luminal surface (de Jonge, 1976). Conversely cAMP, which regulates ion and fluid transport in the intestine, acts on the contraluminal membrane unless levels of it are stimulated by infection (e.g. cholera), when it can act luminally. The question of hormonal influences on absorption in the developing gut will be taken up again in Chapter 8.

This action of hormones does not deny the possibility that developmental changes in intestinal morphology are intrinsic and simply potentiated by hormone action. In fact, the occurrence of a normal pattern of closure after adrenalectomy and solid food deprivation suggests this. Ferguson, Gerskowitch and Russell (1973) demonstrated that sucrase activity in fetal intestine which had been implanted into adults appeared on cue at a postnatal age of 14 days and that cortisone caused precocious appearance of sucrase. This intrinsic maturation probably involves the appearance of cytoplasmic glucocorticoid receptors (see Chapter 8). Using an organ culture technique, DeRitis, Falchuk and Trier (1975) have also shown that fetal jejunum can undergo substantial differentiation <u>in vitro</u> in the absence of hormonal and metabolic influences.

Plate 6

Group of villi from 20 day old rat distal jejunum, stained with haematoxylin and safranin.

X 150

Plate 7

Electronmicrograph of enterocyte tight junction from 15 day old rat proximal jejunum. Tight junction (tj), intermediate junction (ij) and desmosome (d) can all be clearly seen. To the right of the junction is a large invagination of the apical membrane which contains vacuoles.

X 25000

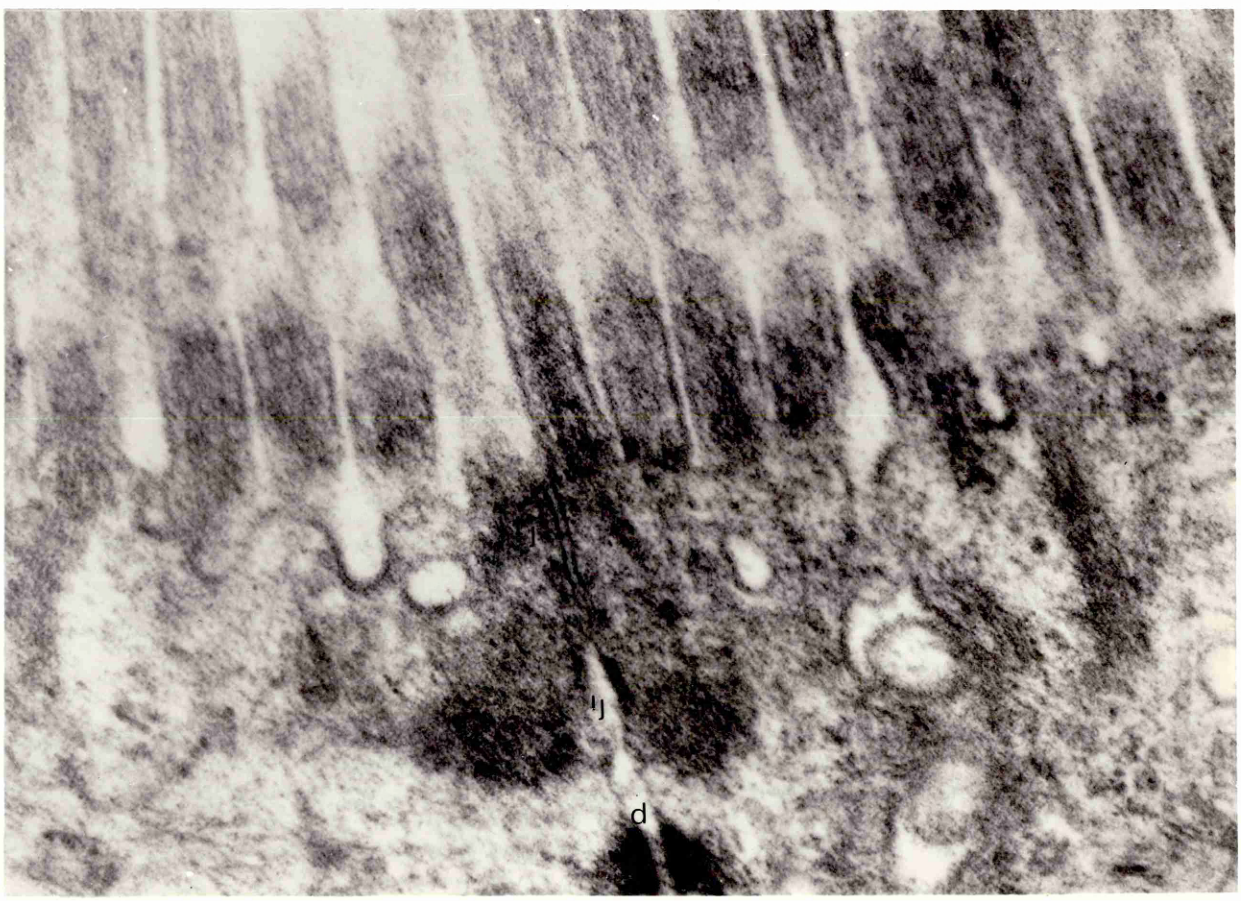

d

Plate 8

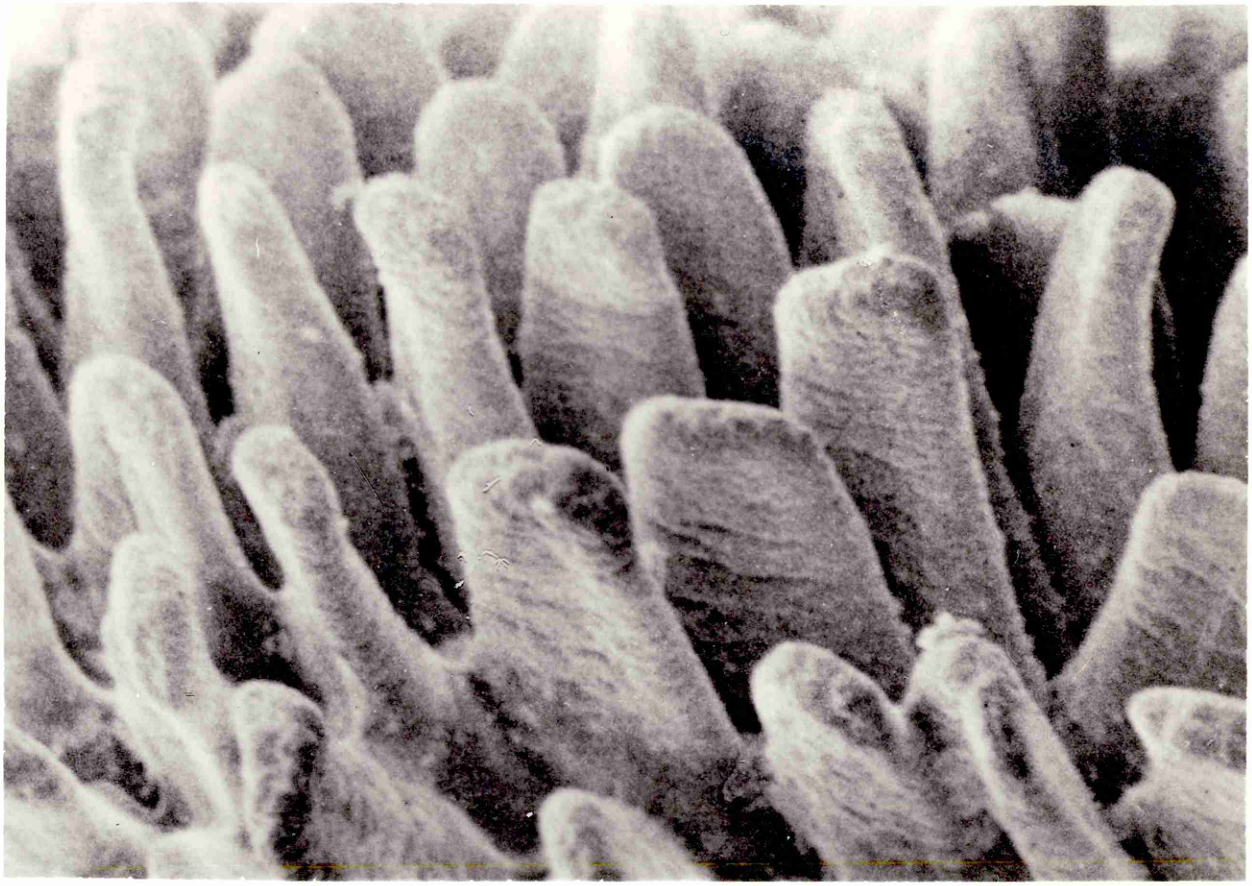
Scanning electronmicrograph of group of villi from 20 day old rat proximal jejunum. Villi are clearly wedge shaped.

X 100

Plate 9

Scanning electronmicrograph of group of villi from 20 day old rat lower jejunum. Here the villi are much rounder in shape.

X 200



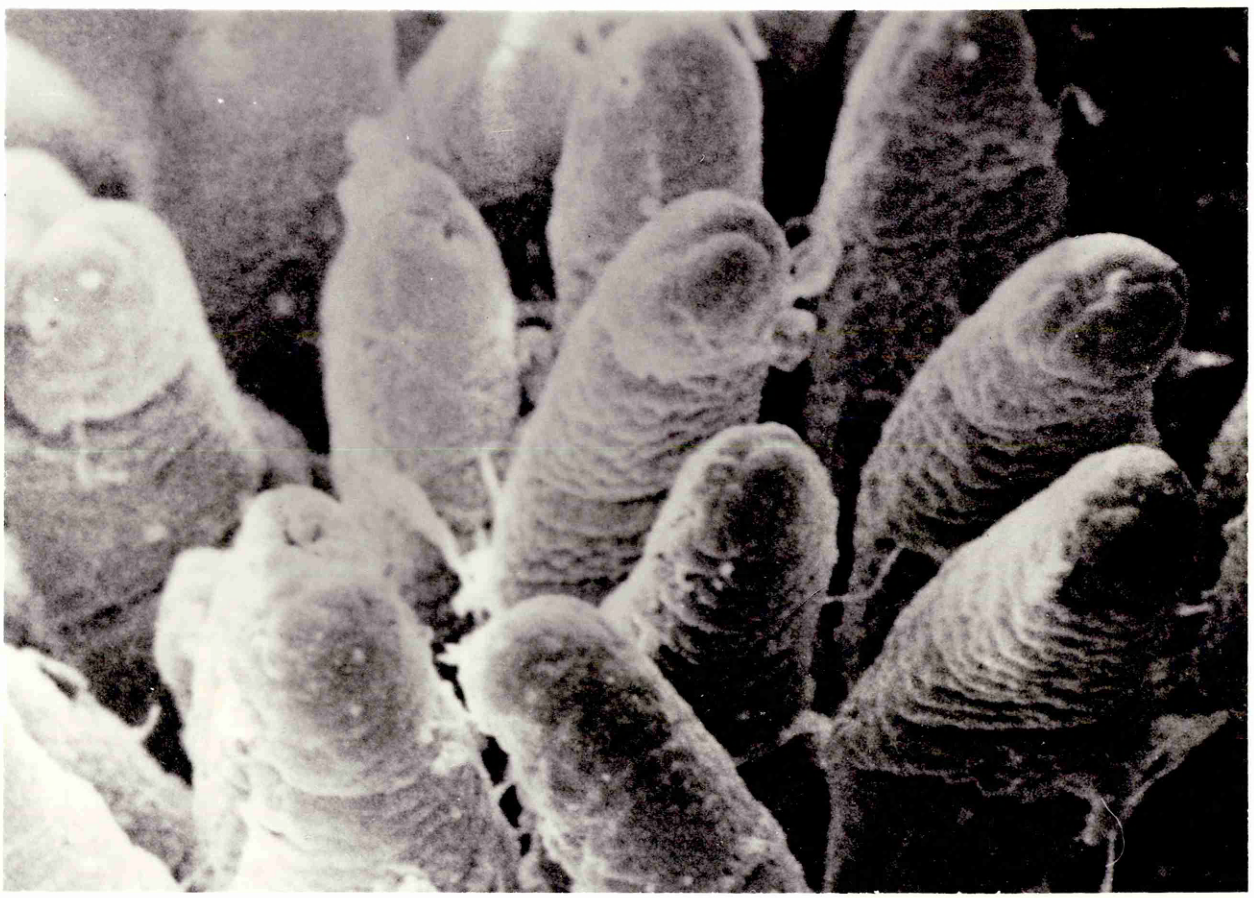


Plate 10

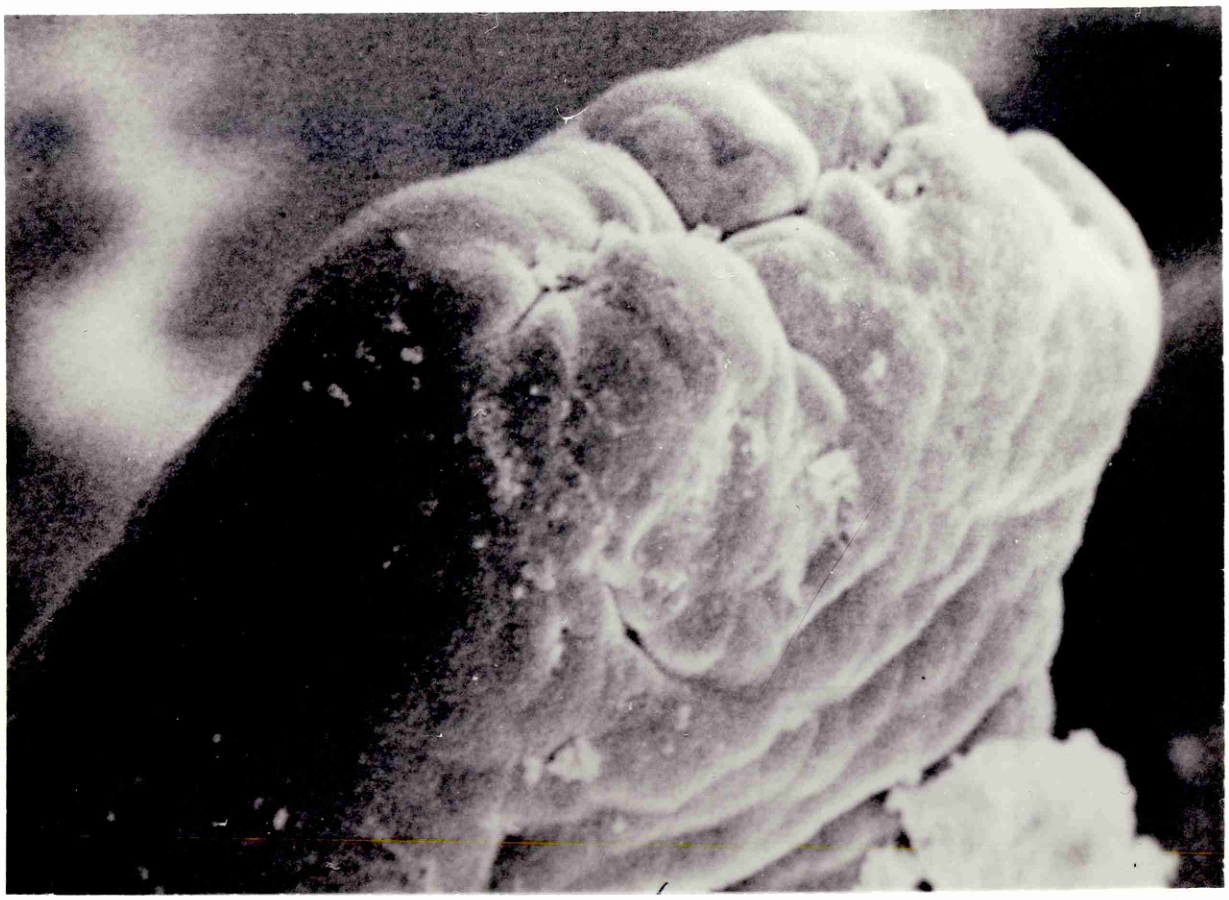
Scanning electronmicrograph of villus tip from 20 day old rat duodenum. The surface is thrown into interconnecting ridges (sulci) and the slight disruption at the tip probably represents normal shedding of epithelial cells. The large body in the bottom right of the field is probably mucous.

X 700

Plate 11

Scanning electronmicrograph of villus interior from 20 day old rat distal jejunum. The villus surface in clearly patterned and the brush border can be seen on the upper surface. Cell boundaries and nuclei are visible inside the villus adjacent to the central lamina propria.

X 1000



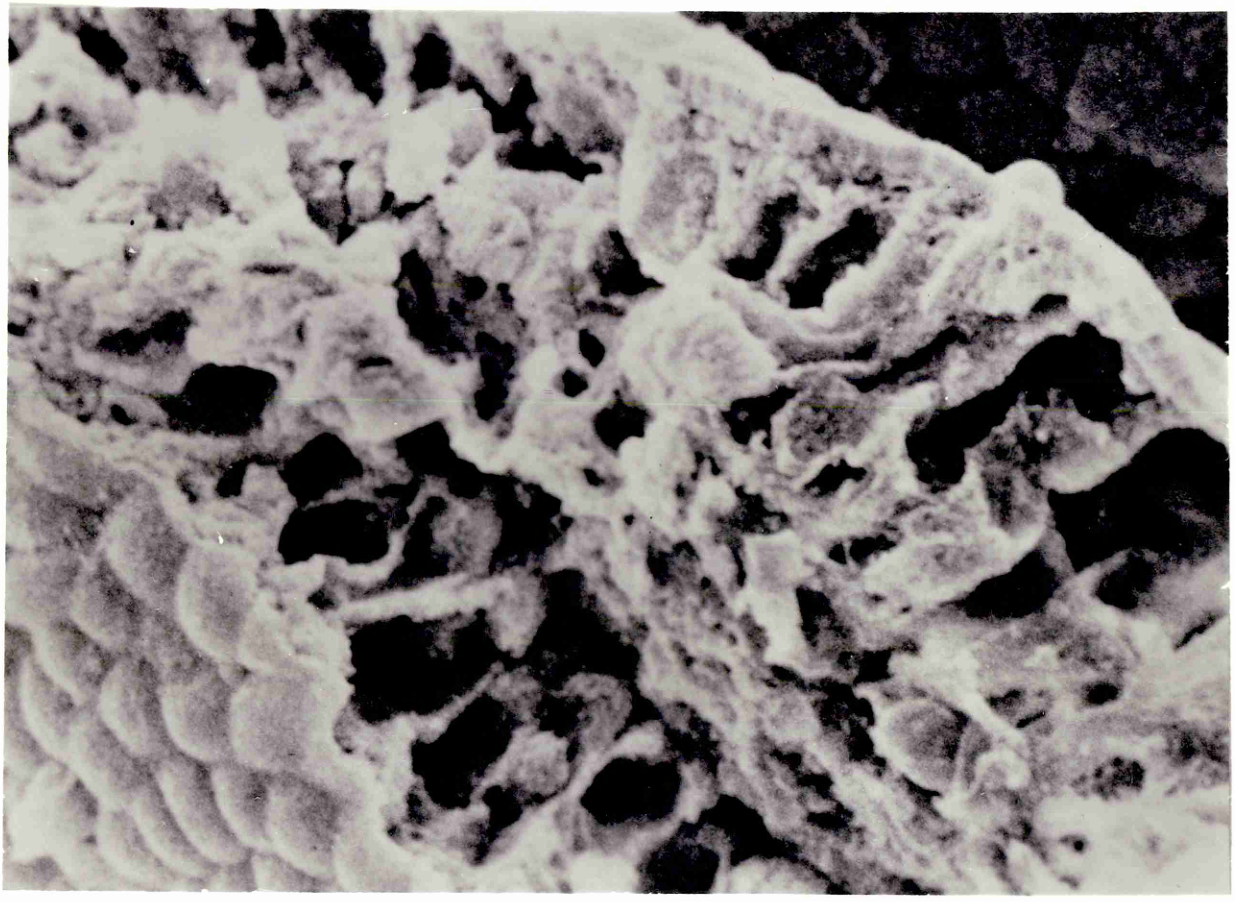


Plate 12

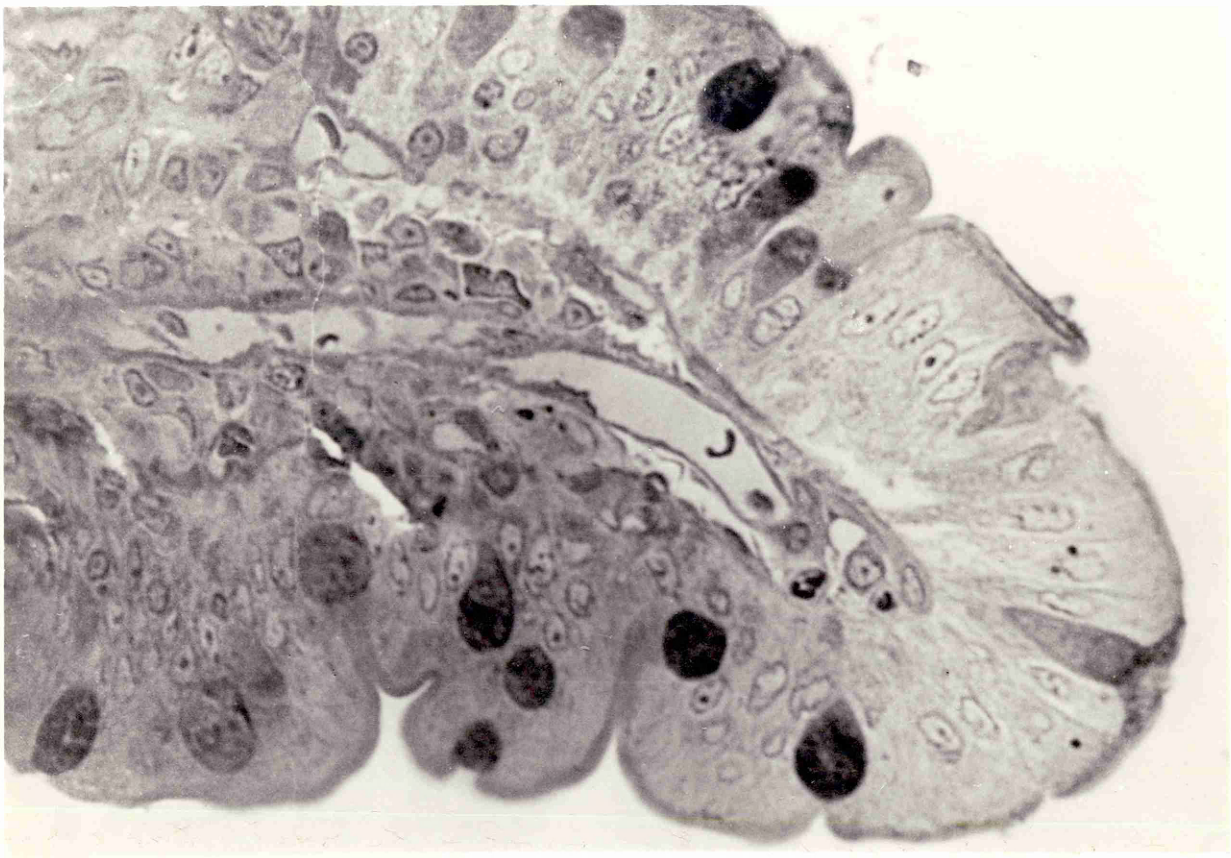
Light micrograph of villus tip from 30 day old rat lower jejunum stained with haematoxylin and safranin. The dense goblet cells stand out from the enterocytes and the central blood vessels with red blood cells can be clearly seen.

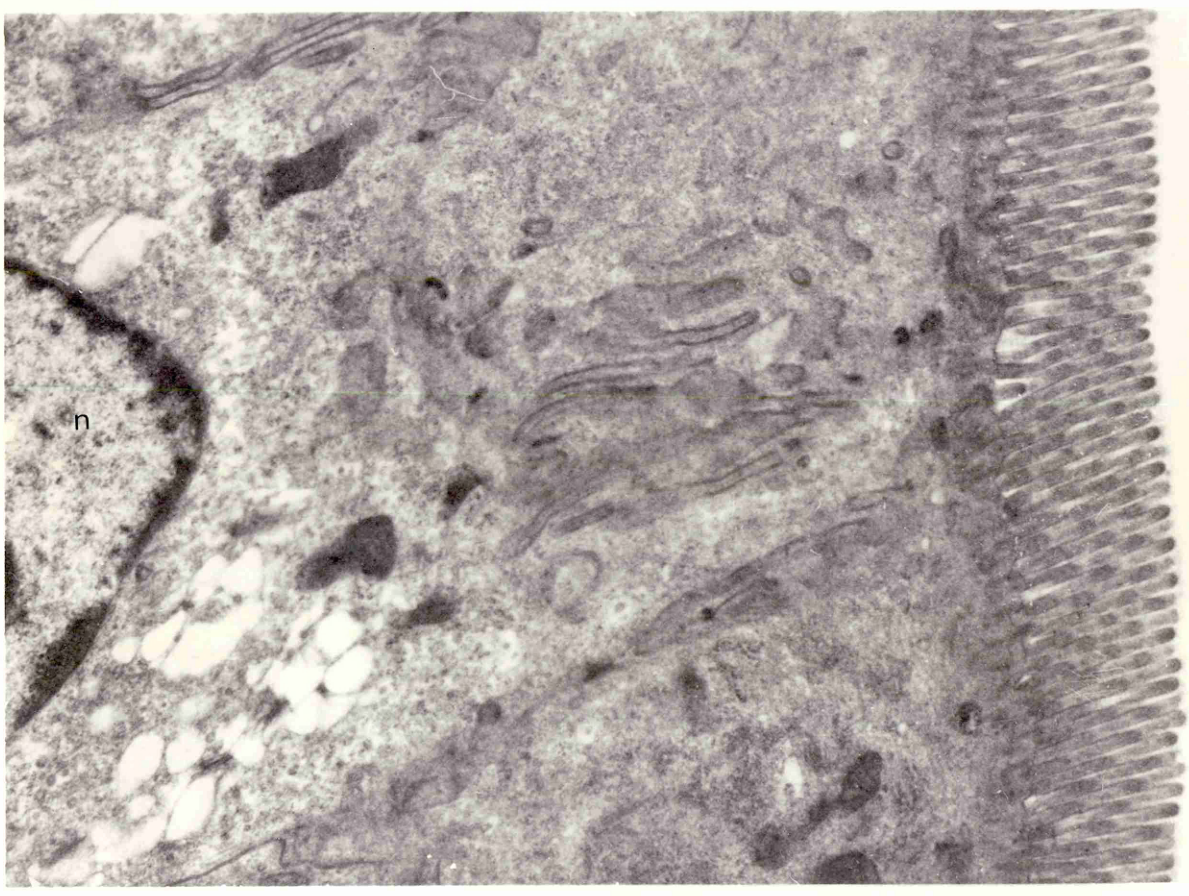
X 150

Plate 13

Electronmicrograph of field from Plate 12 above. Vacuoles are no longer evident in the apical cytoplasm at this age, and the number of dense bodies is reduced. A network of tubules can be seen in the cytoplasm above the large distal nucleus (n).

X 2500




n

CHAPTER 3

Methodology and the Validation of an "*in vitro*" Technique

Tracing the development of the technology used to study intestinal uptake, accumulation and efflux of solutes is a major study in itself. Leaving aside *in vivo* techniques (recently reviewed by Smyth 1974), it is worth considering the various *in vitro* preparations and so formulate the reasoning behind the methodology adopted here. *In vitro* studies were initiated by Waymouth Reid in 1901 who isolated bits of intestine. Subsequent development of these techniques is well illustrated by the elegant studies of Crane *et al* (1976) using liposomes that have been impregnated with an isolated hexose transport protein and Kinner and Murer (1976) who have successfuly fractionated brush-border membrane vesicles. The development of *in vitro* preparations can be divided into two parts: the use of whole intestine and subsequently the reductionist approach leading to isolated fractions of intestinal wall.

After a series of unsuccessful attempts in the 1930s to develop a viable *in vitro* preparation. Fisher and Parsons (1949) realised that the vital requirement was to oxygenate the mucosa. Wilson and Wiseman (1954) extended Fisher and Parsons technology and produced the classic "everted sac", a robust preparation so successful that it survives today, although with numerous modifications (see Parsons, 1968). The success of gut eversion lies in the ability to thoroughly oxygenate the mucosa by exposing the villi to a large volume of medium, and the ease of tracing transported solute into the very small serosal compartment. Despite a number of advantages, such as the ability to control experimental conditions and to observe regional differences in transport, serious disadvantages have been reported. Eversion changes intestinal permeability, increasing passive transport and decreasing active ion absorption (Baker,

Watson, Long and Wall, 1969). The presence of serosal muscle layers complicates transport into the serosal compartment, acting as an accumulating store or even a barrier to solutes (Crane and Mandelstam, 1960). Finally, histological evidence suggests that the structural integrity of the preparation is progressively lost on incubation, and after one hour at 37°C there is total disruption of the epithelial border (Levine, McNary, Kornguth and Leblanc, 1970).

Agar, Hird and Sidhu (1954) introduced 1-2mm long intestinal rings as a preparation more readily adaptable for short term studies of solute accumulation (Robinson, 1966), but these have the major disadvantage that the separation of mucosal/serosal compartments is lost and thus form a common pool from which substances are taken and again returned. Bronk and Parsons (1965) have used extensively a mucosal slice preparation which has the benefits of speed and of being free from muscle layer complications, but still suffers from the same "single compartment" problem as rings. However, Bronk and Leese (1974) conclude after ten years experience with the mucosal slice that this preparation may come closer to providing the normal in vivo environment of the mucosal cells, because in the intact animal the basal pole of the enterocyte is always exposed to substrates from the blood.

A number of methods describing the isolation of epithelial cells from varying areas of the intestine from a number of species have been reported (e.g. rat - Perris, 1966; chick - Kimmich, 1970; ox rumen - Weekes, 1974). Cell separation techniques invariably include enzyme digestion and/or mechanical disruption steps which can result in a cell preparation of questionable integrity and viability, and of

neccessity a loss of polarity.

Miller and Crane (1961) introduced the isolated brush border preparation, which has been successfully exploited and extended (Murer and Hopfer, 1974; Murer, Hopfer and Kinne, 1976; Sigrist-Nelson 1975), resulting in the identification and separation of specific transport proteins/glycoproteins (Crane, Malathi and Preiser, 1976) which have been inserted into liposomes with demonstration of some transport activity (Semenza, Tannenbaum, Toggenburger and Wahlgren 1976).

Barry, Dikstein, Matthews and Smyth (1961) demonstrated with the "everted sac" that an electrical potential is associated with the transport of glucose, and later Baillien and Schoffeniels (1962) showed that certain amino acids also caused increased trans-intestinal potential differences. Since then, strips of gut mounted in Ussing-type chambers have been extensively employed to study the ion-dependencies of solute transport, particularly by Schultz and Curran at Harvard (see review by Schultz and Frizzell 1975) and in human biopsy material (Turnberg et al (1976). The benefits of this technique are that electrical measurements can be made in parallel with isotopic flux experiments, fluxes can be measured in both directions on adjacent pieces of gut and the fluid environment presented to both mucosal and serosal compartments can be rapidly modified (James and Smith, 1976; Henin and Smith, 1976).

The everted intestinal segment - an in vitro technique for amino acid absorption

The small intestine of the new-born rat is an extremely delicate organ. Attempts to evert intestine from very young rats have been made (Donnelly, 1971; Ingham, 1972) but the amount of handling required and thinness of the gut wall necessitate lengthy preparation times which can result in damaged tissue. In both cases above, the intestine was finally cut into small segments.

Critical to any *in vitro* preparation is the length of time the tissue is without its blood supply until placed into a suitable physiological medium. The reasoning adopted here was to reduce this period, which required therefore a preparation that was quick to isolate with minimum handling.

Preparation

Adult female Wistar rats, strain CFHB, bred in our own colony were isolated after mating. Litter size was standardised at 12 ± 1 (mixed sex) throughout all experiments, this being an average litter size for our colony. Rat pups were kept in the colony with mother until required or until weaned (normally at 28 days of age) and given food and water *ad lib*. Animals were on a 12h light : 12h dark cycle. Day of birth was routinely designated DAY ONE for all experimental purposes. Animals were removed from mother one hour before the experiment, to clear the intestine of food. All absorption experiments were carried out between 12 noon and 4 pm.

Young animals (under 30 days of age) were killed by decapitation. Older rats were first stunned by a blow on the neck and then killed by cervical transection. The small intestine was exposed by a mid-line abdominal incision, cut at the duodeno-jejunal flexure, gently stripped from the mesentery, removed by cutting at the ileo-caecal junction and transferred into 0.9% saline. The central fifth was isolated, divided and each half transferred to moist filter paper on a cutting table. Using fine curved scissors, the length of intestine was slit longitudinally (avoiding any stretching or damage to the mucosa). The intestine was then quickly washed very gently in saline to remove any adhering ingesta, blotted on saline damp paper and cut into small segments 5-10mm long, directly into the incubation medium. Conveniently these small gut segments spontaneously evert on incubation and the fuzzy appearance of the villi can be seen clearly. The time from death to commencement of incubation did not exceed 2 minutes. All segments used were taken from the central fifth of the intestine i.e. jejunum, and their origin (proximal or distal) carefully noted. Segments were routinely mixed for experiments.

Incubations were carried out in 10ml of medium in stoppered 150ml conical flasks in a Gallenkamp shaking water bath (80 oscillations/min) at 37°C for 15 min unless otherwise stated. At the end of incubation, segments were removed, briefly washed in saline containing 5mM cold amino acid to displace adhering radioactive substrate, drained and transferred to capped vials. Each segment was then rapidly and thoroughly homogenised in 5ml 0.32M sucrose in a motor-driven teflon-glass homogeniser (1000 rev/min:6 strokes). Aliquots were taken for scintillation counting and protein estimation.

The incubation medium used throughout these experiments was the bicarbonate saline of Krebs and Henseleit (1932). This saline was freshly prepared each day and equilibriated with 95% O_2 : 5% CO_2 before use. Glucose was present at a concentration of 10mM unless otherwise stated, and amino acids as described in the text.

Chemicals

The following ^{14}C amino acids were obtained from the Radiochemical Centre, Amersham, U.K:

L - (U - ^{14}C) valine

L - (U - ^{14}C) leucine

L - (1 - ^{14}C) methionine

L - (U - ^{14}C) phenylalanine

L - (U - ^{14}C) arginine monohydrochloride

L - (U - ^{14}C) lysine monohydrochloride

4 amino - n - U - ^{14}C butyric acid (GABA)

Cold amino acids and routine chemicals were obtained from:

Sigma, London Chemical Co. U.K.

BDH, Poole, Dorset, U.K.

Protein estimation

Protein was determined on portions of tissue homogenates by the method of Lowry et al (1951) and the colour read in a Pye Unicam SP6-500 UV - Spectrophotometer at 500 nm.

^{14}C estimation

Liquid scintillation spectrometry at ambient temperature was carried out on aliquots of tissue homogenate (0.4ml). The scintillant used was a solution of 6g/l PPO (2, 5-diphenylenazole) in 50/50 (v/v) toluene/2-methoxy ethanol (the latter necessary to take up water in the sample). Estimations were made in a Beckman 150 liquid scintillation counter and quench corrections performed by internal standardisation of representative samples. Samples were counted until a 3% accuracy was achieved, at an efficiency of 85%; background was subtracted from each sample.

CHAPTER 4

Developmental Studies with Everted Intestinal Segments

Results and conclusions drawn from *in vitro* studies are only of value if the preparation remains functionally and morphologically intact, behaving in a way that is comparable with the *in vivo* situation.

Scanning electron micrographs of segments taken from rats of various ages (Plates 14-19) show the effect of incubation on the tissue. The preparation maintains good morphology over the incubation period despite the mechanical buffeting effects of shaking (for details see plate legends).

Viability of everted segments

1) Oxygen consumption

Using everted segments of neonatal rat ileum, Donnelly (1971) has shown that oxygen uptake is linear for at least one hour of incubation (the maximum period used in this study) and that the thickness of the tissue is within limits for adequate oxygenation.

2) Hexose requirement

Glucose (10mM) was present in all incubations as Smyth (1974) has pointed out that tissue deterioration with incubation was greater in the absence of a metabolisable hexose.

Table 4.1 shows the uptake of an amino acid (L-phenylalanine) by jejunal segments from 18 day old rats, in the presence and absence of glucose.

TABLE 4.1 Accumulation of L-phenylalanine in jejunal segments from 18 day old rats.

V = uptake (n moles/mg. protein/15 min)

T/M = concentrative uptake

n = number of observations

Substrate	Condition	V	T/M	% Stimulation
L-phenylalanine (1mM)	no glucose	38.83 (4) ± 5.15	4.20	-
	+ 10mM glucose	90.03 (8) ± 10.47	9.71	130%

Even in the absence of a metabolisable sugar, L-phenylalanine is actively accumulated. However, glucose stimulates uptake of the amino acid by providing more energy to the process in the form of ATP. In vivo, energy would not be a limiting factor for amino acid uptake, and thus glucose was included in the medium.

3) Sacs and segments

Accumulation of amino acid into everted jejunal sacs and segments was compared in young rats (see Table 4.2).

The extent of accumulation and time course of uptake of L-lysine was similar in either preparation. This suggests that the everted gut segment demonstrates similar characteristics of amino acid accumulation to the classical everted sac preparation which has been

extensively employed in the study of amino acid uptake *in vitro*.

TABLE 4.2

Time course of uptake of L-lysine (1 mM) in jejunal sacs and segments from 17 day old rats - a comparison.

Values represent mean L-lysine uptake ± SEM into tissue in n moles/mg protein with the number of experiments in brackets.

	time (min)			
	5	10	15	20
Sacs	20.81 ± 2.99 (4)	34.00 ± 5.75 (4)	-	-
Segments	15.57 ± 1.52 (16)	29.78 ± 2.45 (16)	-	24.30 ± 6.54 (4)

4) Characteristics of uptake into segments

To further confirm the validity of the *in vitro* preparation, time course studies of amino acid uptake were made. The effects of substrate concentration on uptake, the extent of concentrative uptake (active transport) of substrates, and the contribution of diffusion in total tissue absorption were also examined.

Figures 4.1a and 4.1b show the absorption of six amino acids (at a concentration of 1 mM) by segments of the small intestine from rats at three stages of development. At this concentration, uptake of substrate is linear over the first 15 minutes after which the concentration of substrate in the tissue rapidly reaches a "steady-state" (saturation).

Figure 4.2 shows the effect of varying substrate concentration (L-lysine) on uptake. It is evident that while uptake into the tissue is linear over 15 minutes for the lower substrate concentrations, at 10 mM the tissue reaches a steady state somewhat earlier. From these experiments, a 15 minute period of incubation was chosen for subsequent kinetic determinations (Chapter 5). This time period represents the upper end of the initial rate of substrate uptake and can thus be used to determine the kinetic values of Vmax and K_m. Similarly after 15 minutes incubation the tissue is rapidly approaching a steady state and so the concentration ratio of T/M can be determined. A problem arises in the choice of this time period, namely that with incubation for 15 minutes at high concentrations the tissue has then reached a steady state. However determinations of the kinetic parameters K_m and Vmax were made over a wide concentration range which emphasised the lower concentrations (0.1 mM to 5 mM).

Figure 4.3 indicates that uptake of L-lysine is a saturable process which exhibits first order kinetics. Intestinal segments also show concentrative uptake of amino acids (see Figure 4.4), the concentration of substrate in the tissue being well above that of the incubation medium (T/M $>$ 1). The extent of active transport into segments depends upon the substrate and varies in some cases with age. These age related changes in transport characteristics are discussed at length in Chapter 5.

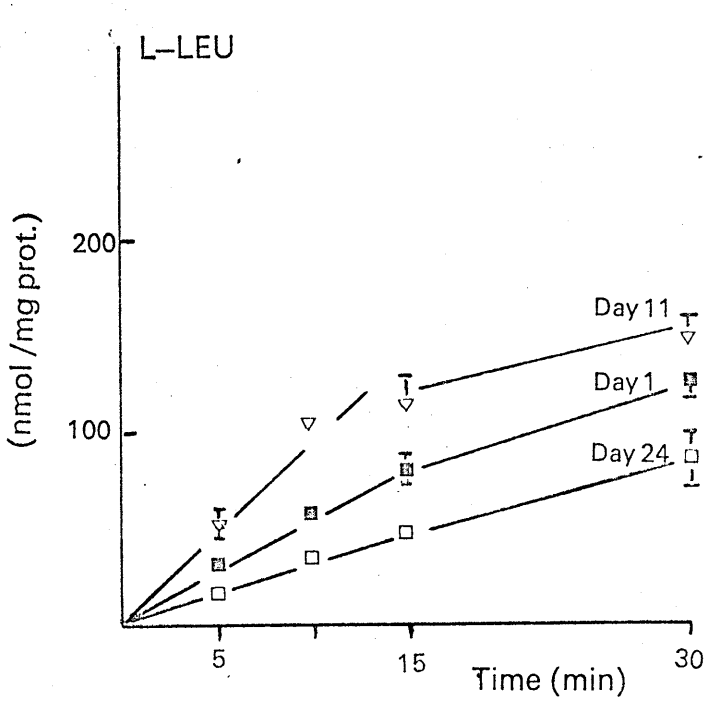
Lastly, Figure 4.5 shows the result of incubation of segments at 37°C and 4°C. Cold incubation results in a 90% decrease in L-lysine accumulation and the T/M ratio never approaches unity. Entry of L-lysine under these conditions is by diffusion alone, low temperature inhibiting the operation of the energy dependant mechanism of active transport.

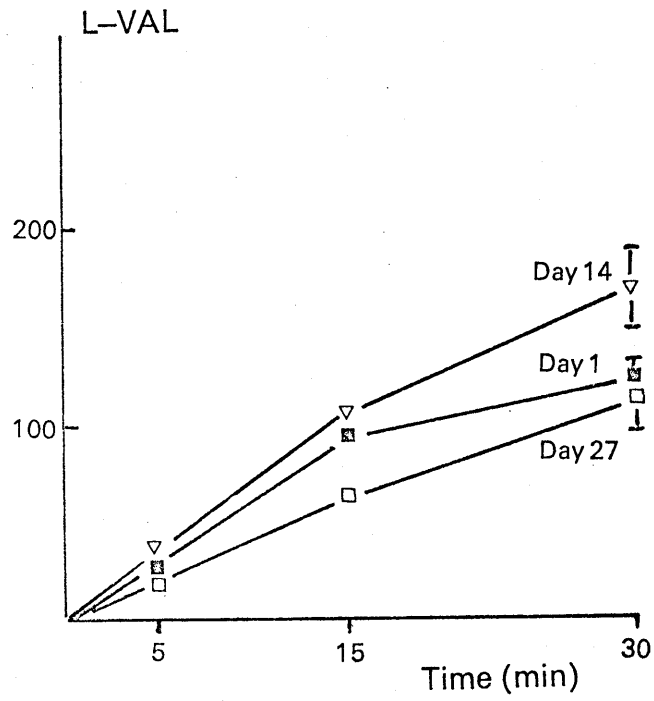
Figure 4.1a

Time course of uptake of L-leucine, L-valine and L-methionine at three developmental age points.

Substrate concentration, 1 mM

Values (n moles/mg protein) are means of 4 experiments $\pm$ SEM.


L–LEU
(nmol /mg prot.)
200
100
Day 11
Day 1
Day 24
5
15
30
Time (min)


L–VAL
200
100
Day 14
Day 1
Day 27
5
15
30
Time (min)

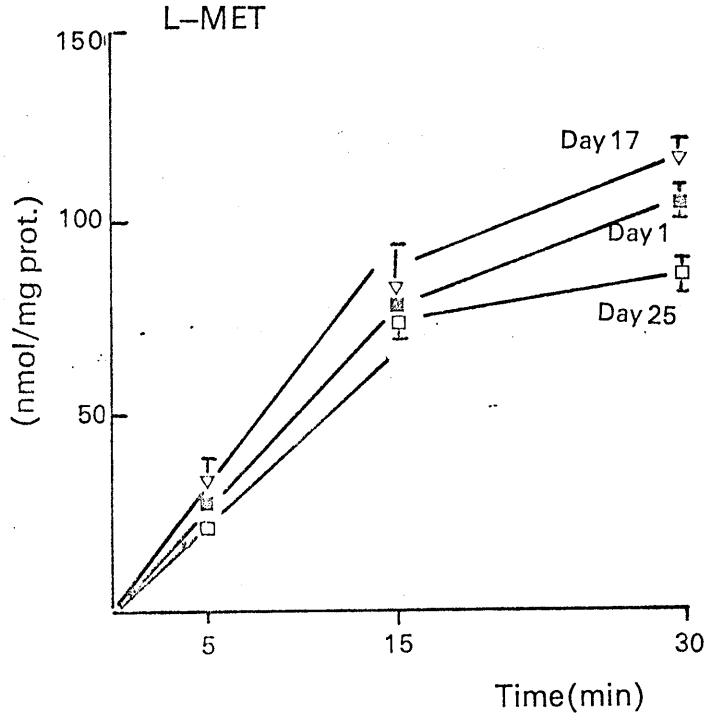

L–MET
150
100
50
(nmol/mg prot.)
Day 17
Day 1
Day 25
5
15
30
Time(min)

Figure 4.1b

Time course of uptake

of L-lysine, L-arginine

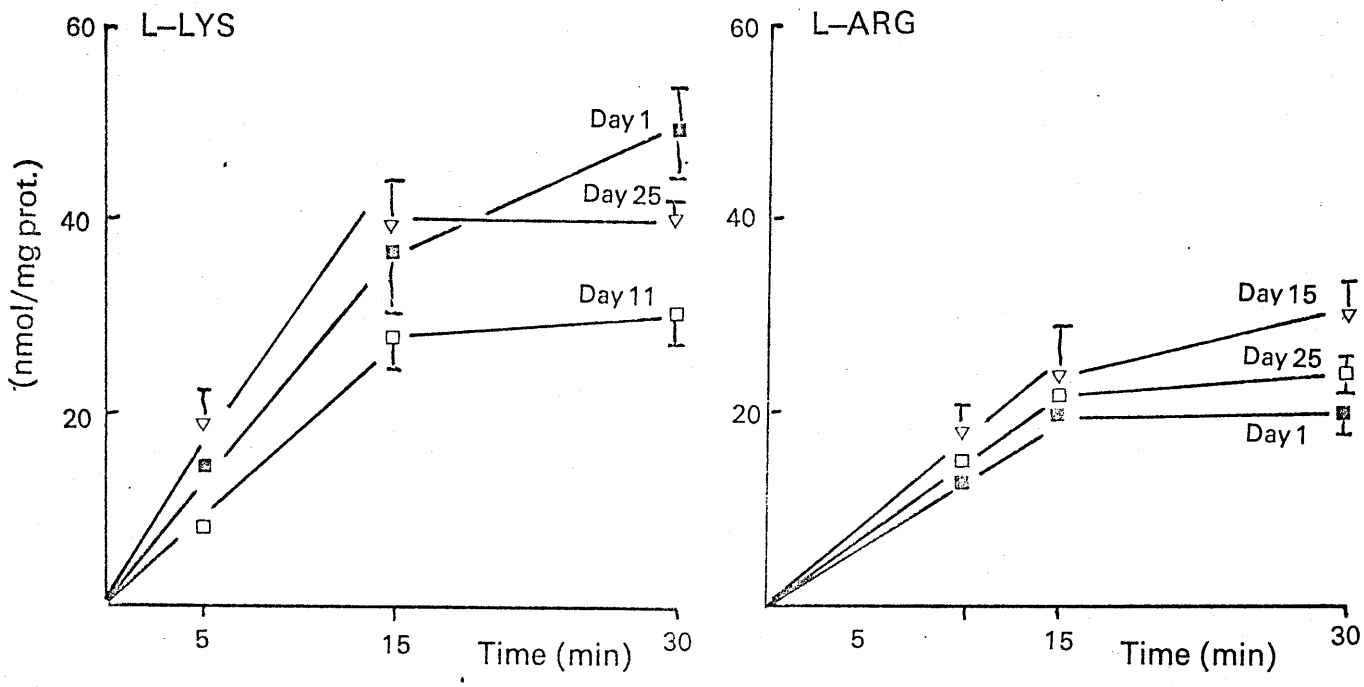
and L-phenylalanine

at three developmental age points.

Substrate concentration, 1 mM

Values (n moles/mg protein) are

means of 4 experiments $\pm$ SEM.


L–LYS
60
40
20
(nmol/mg prot.)
Day 1
Day 25
Day 11
5
15
30
Time (min)
L–ARG
60
40
20
Day 15
Day 25
Day 1
5
15
30
Time (min)

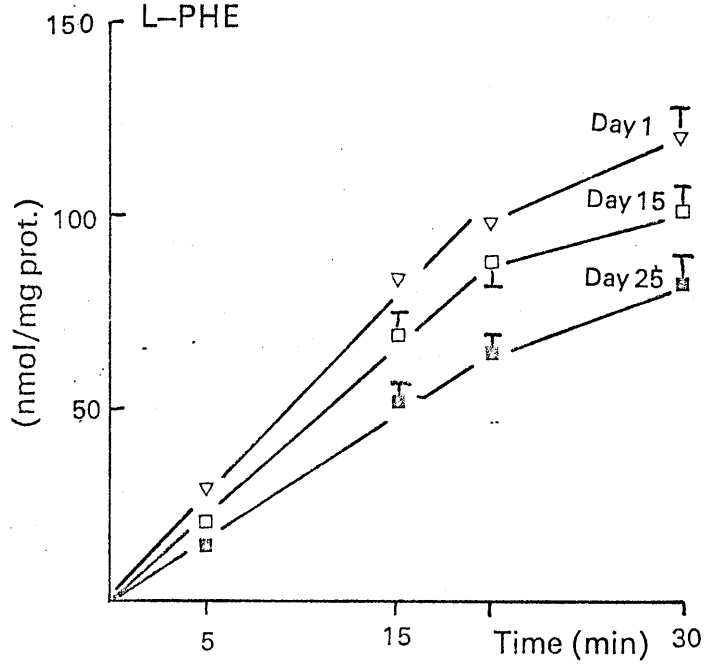

L–PHE
150
100
50
(nmol/mg prot.)
Day 1
Day 15
Day 25
5
15
30
Time (min)

Figure 4.2

Time course of uptake of L-lysine into 15 day old rat intestinal segments at three concentrations of substrate.

Uptake in n moles/mg protein ± SEM and represents a mean of 4 experiments.

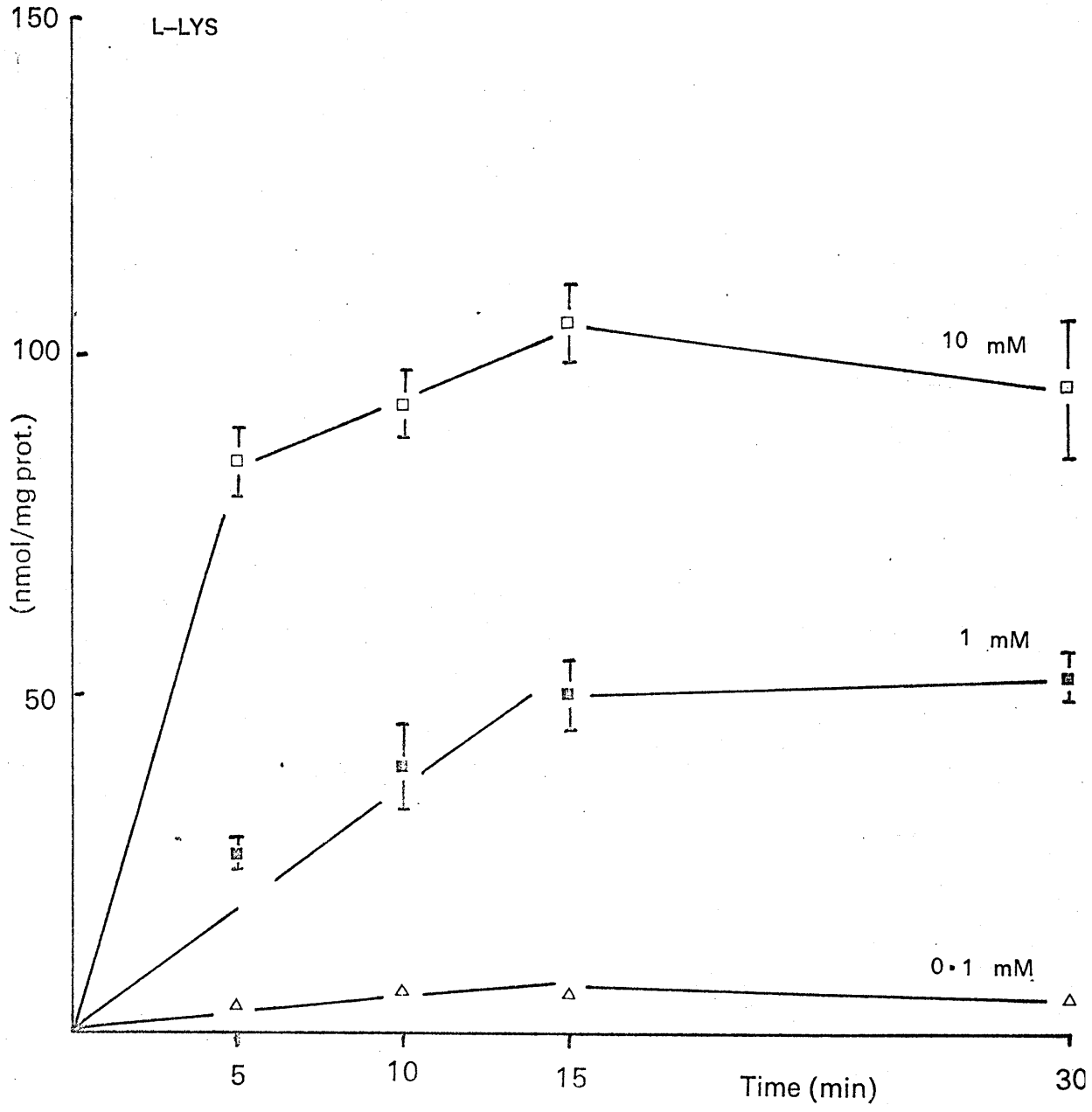
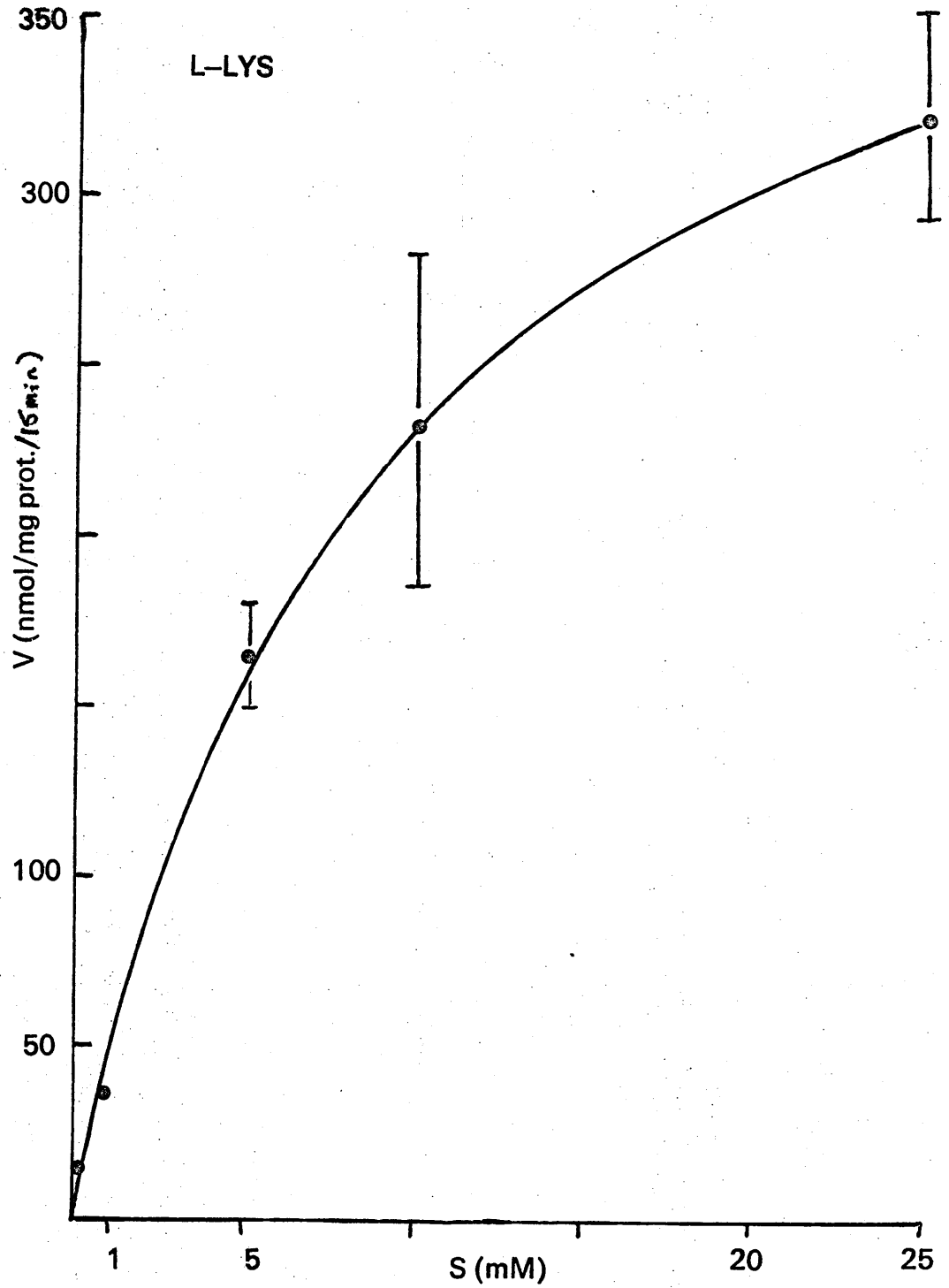

150
100
50
L-LYS
(nmol/mg prot.)
10 mM
1 mM
0·1 mM
5
10
15
30
Time (min)

Figure 4.3 Kinetics of L-lysine uptake in everted jejunal segments from 14 day old rats.

Bars indicate standard errors of the means of at least 8 observations at each point.


L-LYS
350
300
100
50
V (nmol/mg prot./15min)
1
5
S (mM)
20
25

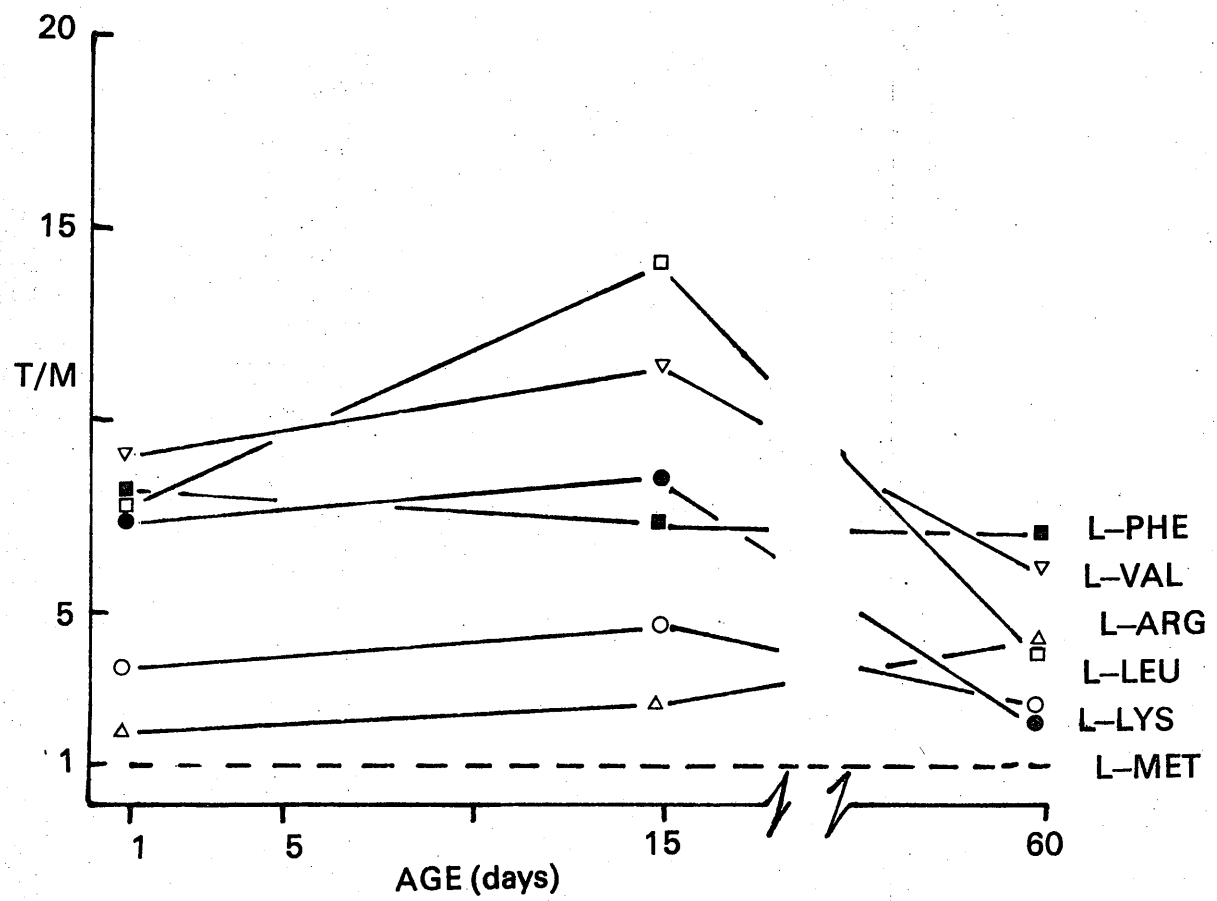


Figure 4.4 Extent of concentrative uptake of six "essential" amino acids in everted jejunal segments from young rats of various ages.

T/M is the ratio of tissue:medium concentration of substrate (medium contains 1mM substrate).
Incubation time 15min.

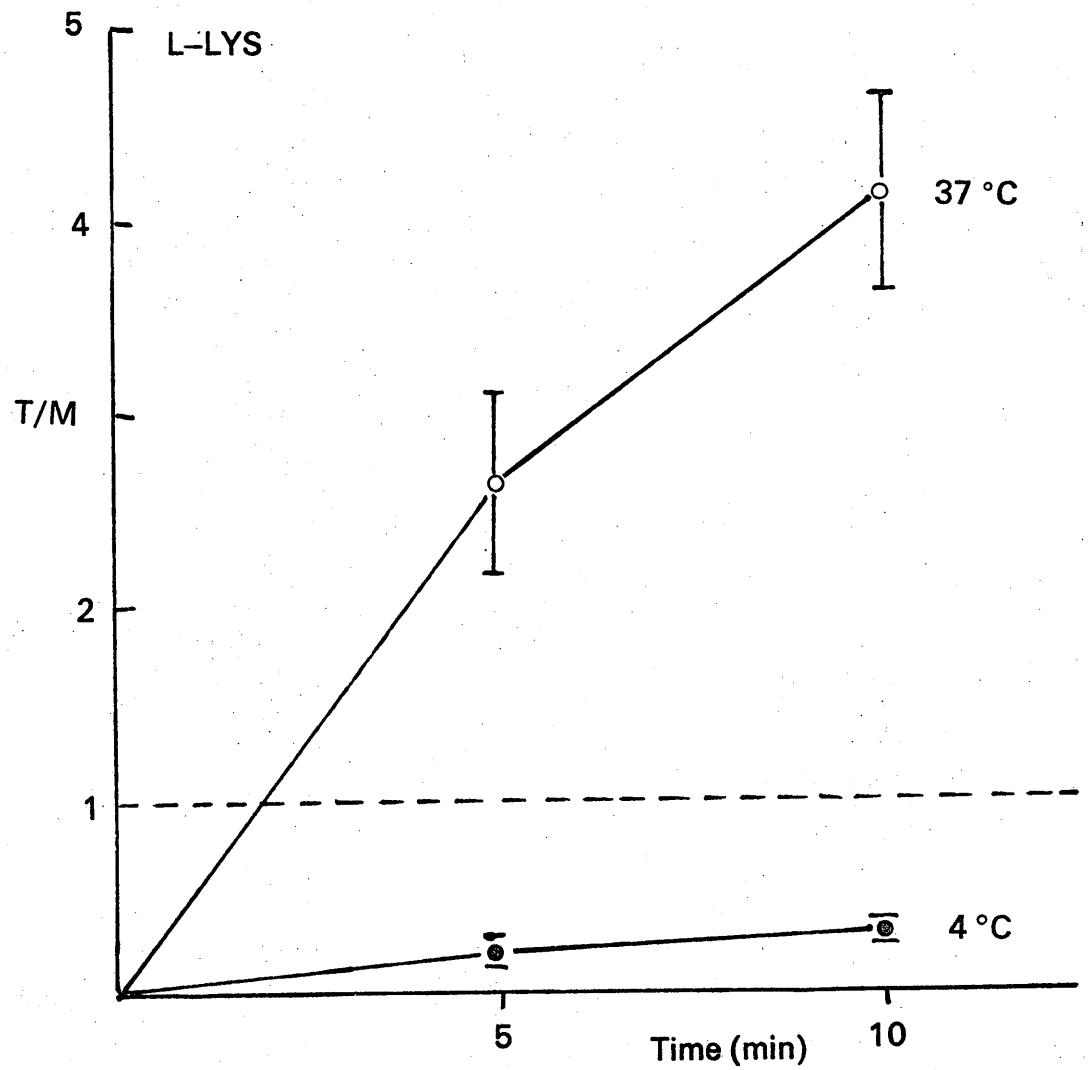


Figure 4.5 Effect of temperature on time course of uptake of L-lysine (1mM) in everted jejunal segments from 16 day old rats.

T/M is the extent of concentrative uptake. Bars indicate the standard errors of the means of at least 4 observations at each point.

5) Uptake of γ amino $\wedge$ butyric acid (GABA)

GABA is a naturally occurring W-amino acid. It is not transaminated by rat tissue homogenates nor is it actively accumulated by the adult rat jejunum (Evered and Hargreaves, 1973). None of the W-group (β-alanine; DL-β-aminoisobutyric acid; δ-aminovaleric acid) are actively transported in kidney cortex or intestine but β-alanine is actively accumulated in the brain.

In experiments with neonatal rat jejunum, GABA uptake shows a similar time course to L-leucine and L-lysine but a T/M ratio greater than one is never achieved (Figure 4.6). Plotting uptake as a function of increasing concentration demonstrates that GABA uptake is linear; the lack of saturation indicates that uptake is by simple diffusion (Figure 4.7), and no concentrative uptake is evident ($T/M < 1$). These characteristics of GABA uptake are seen in jejunal segments from rats of a number of ages (Figure 4.8).

The results from these experiments agree with Evered and Hargreaves (1973), and suggest that the intestinal segment preparation is behaving in vitro in predictable ways. The segments take up and accumulate amino acids, exhibit saturation kinetics for uptake and demonstrate that absorption is an energy dependant process. The jejunal segments have no specific uptake system for GABA which appears to be transported by diffusion alone.

Radio labels as markers of transport

As in all methods which use isotopic tracers, it is essential that the labelled atom remains bound to the substrate whose fate is being determined. Metabolism in the tissue will cleave the labelled atom;

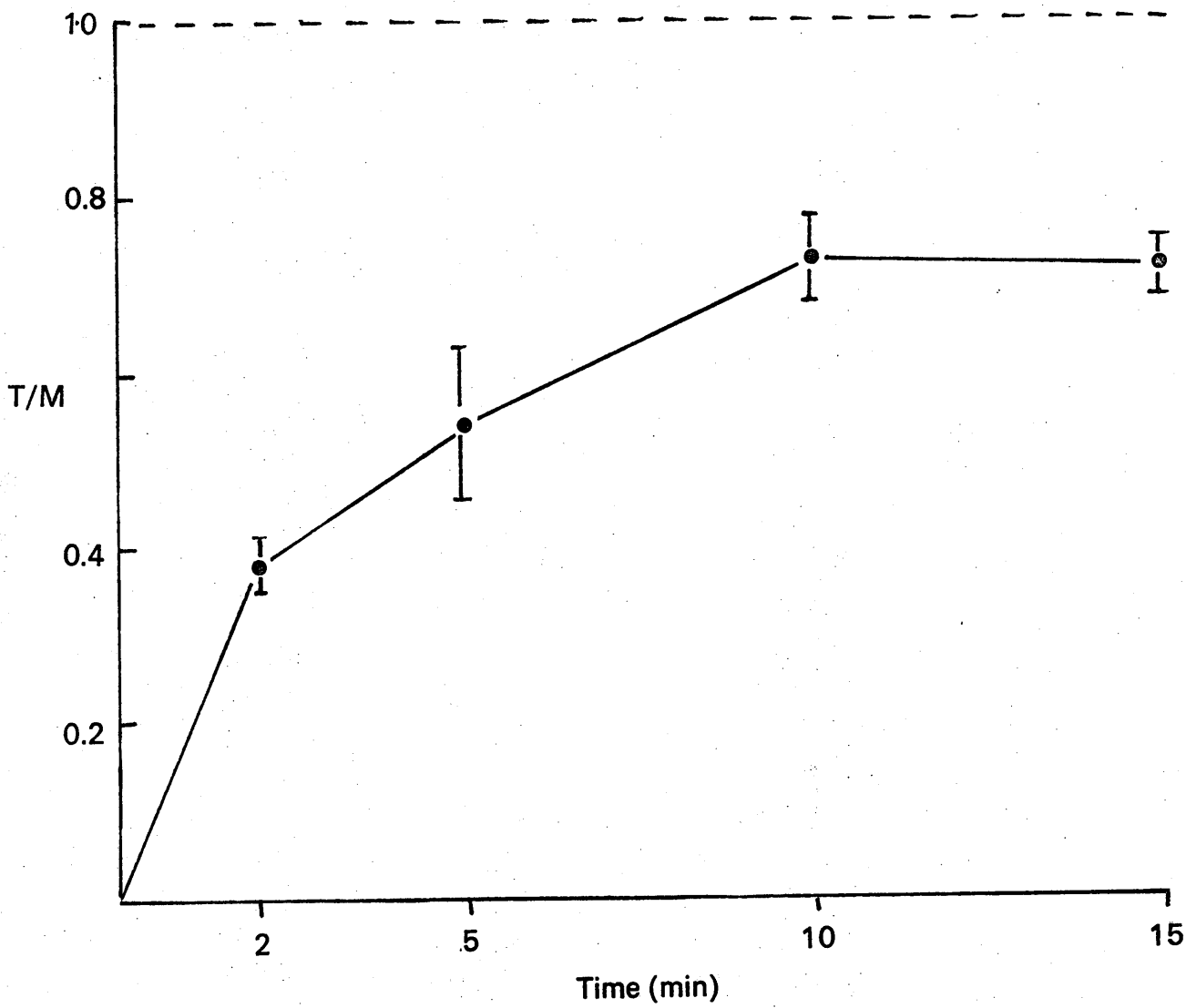


Figure 4.6 Time course of uptake of GABA (1mM) in everted jejunal segments from 5 day old rats.

T/M indicates extent of concentrative uptake. Bars indicate the standard errors of the means of at least 4 observations at each point.

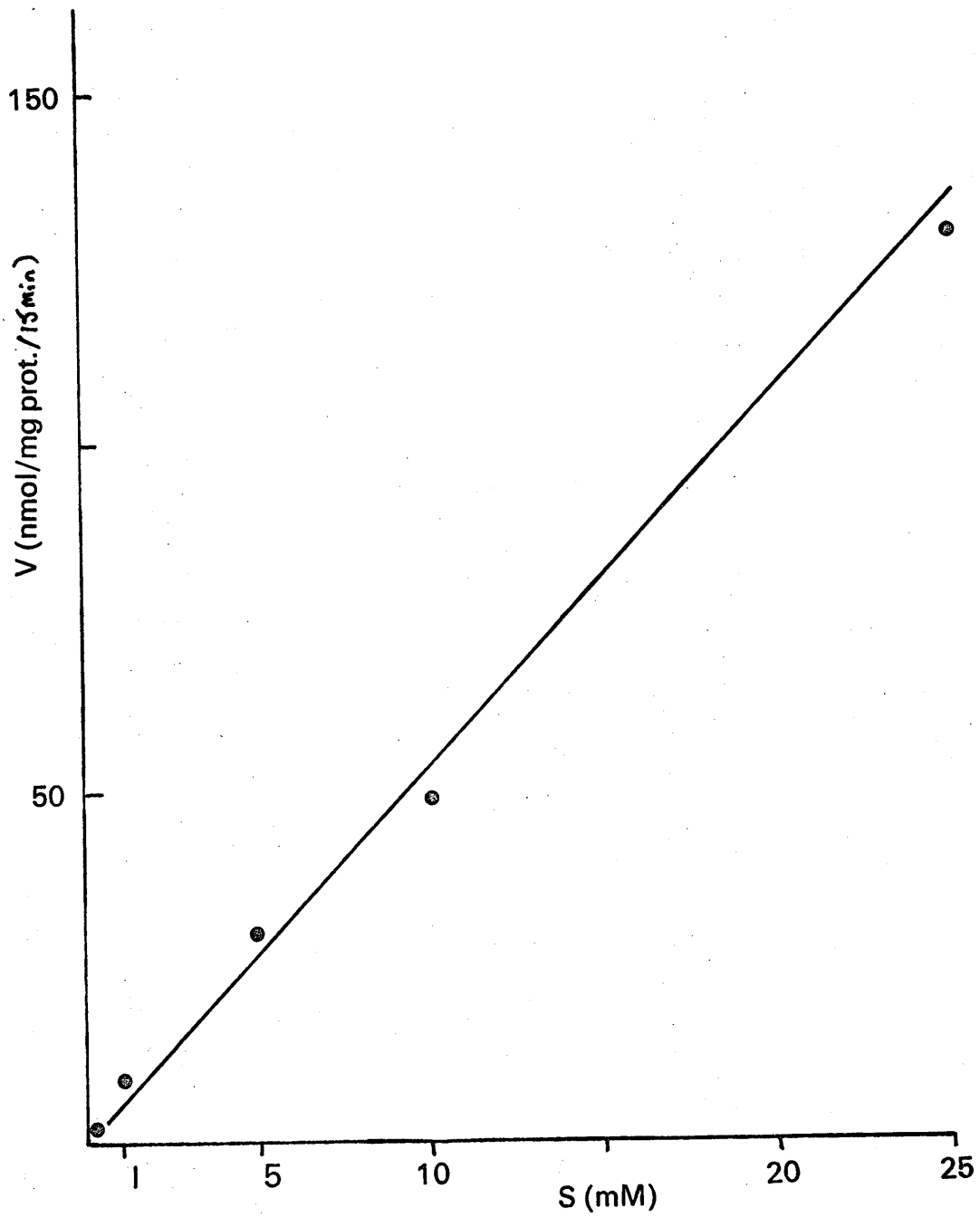
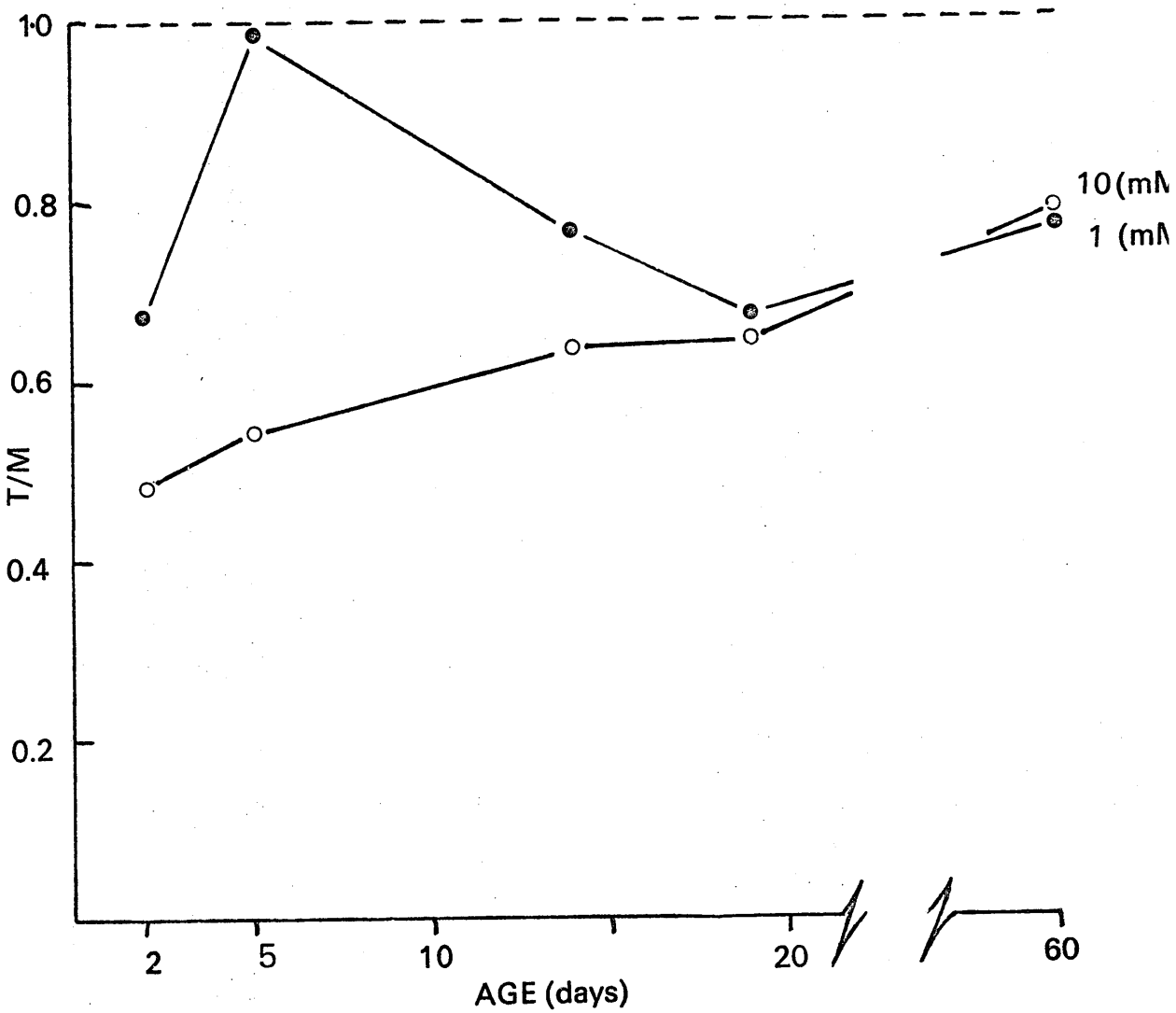


Figure 4.7 Kinetics of GABA uptake in everted jejunal segments from 5 day old rats.

Uptake (V) = nanomoles substrate/mg. protein/15 min. Means of at least 4 observations at each point.

Figure 4.8 Concentrative uptake of GABA in everted jejunal segments from rats of various ages.

T/M = extent of concentrative uptake. Means of at least 4 observations at each point. 15 min. incubation


1·0
0.8
0.6
0.4
0.2
T/M
10 (mM
1 (mM
2
5
10
20
60
AGE (days)

this is particularly critical with some hexose sugars and acidic amino acids (White and Landau, 1965; Spencer and Zamcheck, 1962). Similarly, the choice of label is important. ^{3}H compounds are less desirable as transport markers than ^{14}C, as the ^{3}H atom is very labile and can exchange with the tissue water (Robinson, 1966).

Using thin-layer chromatographic techniques, Daniels (1970) has demonstrated that L-methionine is transported across the everted gut sac unchanged. Larsen et al (1964) have shown that only alanine, of all the neutral and basic amino acids they studied, was changed when transported across rat everted sacs. Robinson (1966) has reported that L-phenylalanine is accumulated in gut rings without undergoing metabolism. Donnelly (1971), using small rings of intestine from young rats, found that only L-methionine was changed during absorption, oxidation of substrate occurring to a very small extent to give methionine sulphoxide which could well have been the result of tissue evaporation during preparation for chromatography and therefore artefactual. Thus it seems that little metabolism occurs of the neutral and basic amino acids whose accumulation is to be measured in this study.

Fate of ^{14}C label - subcellular fractionation

In any differentiating tissue such as the developing gut, protein synthesis is occurring at a relatively high rate, the extent of synthesis changing with age. Amino acids, being the building blocks for protein manufacture, could therefore be incorporated into cellular and subcellular fractions as they accumulate within the free amino acid pool inside the cell. The extent of incorporation into protein cannot be estimated by the chromatographic techniques

described previously. Therefore, a series of experiments were performed to investigate the fate of ^{14}C labelled amino acids in gut segments after incubation as a function of age.

The sub-cellular fractionation proceedure is described in Figure 4.9. Aliquots of all fractions were counted and protein content determined to calculate sequential recoveries. The data for ^{14}C-L-lysine accumulation is presented in Table 4.3; the results are expressed as the counts associated with the two major protein fractions as % of the starting homogenate. It is obvious that the extent of incorporation of L-lysine into structural protein is extremely small and changes little with age or length of time of incubation. Thus at least 95% of the accumulated amino acid is located in the free amino acid pool.

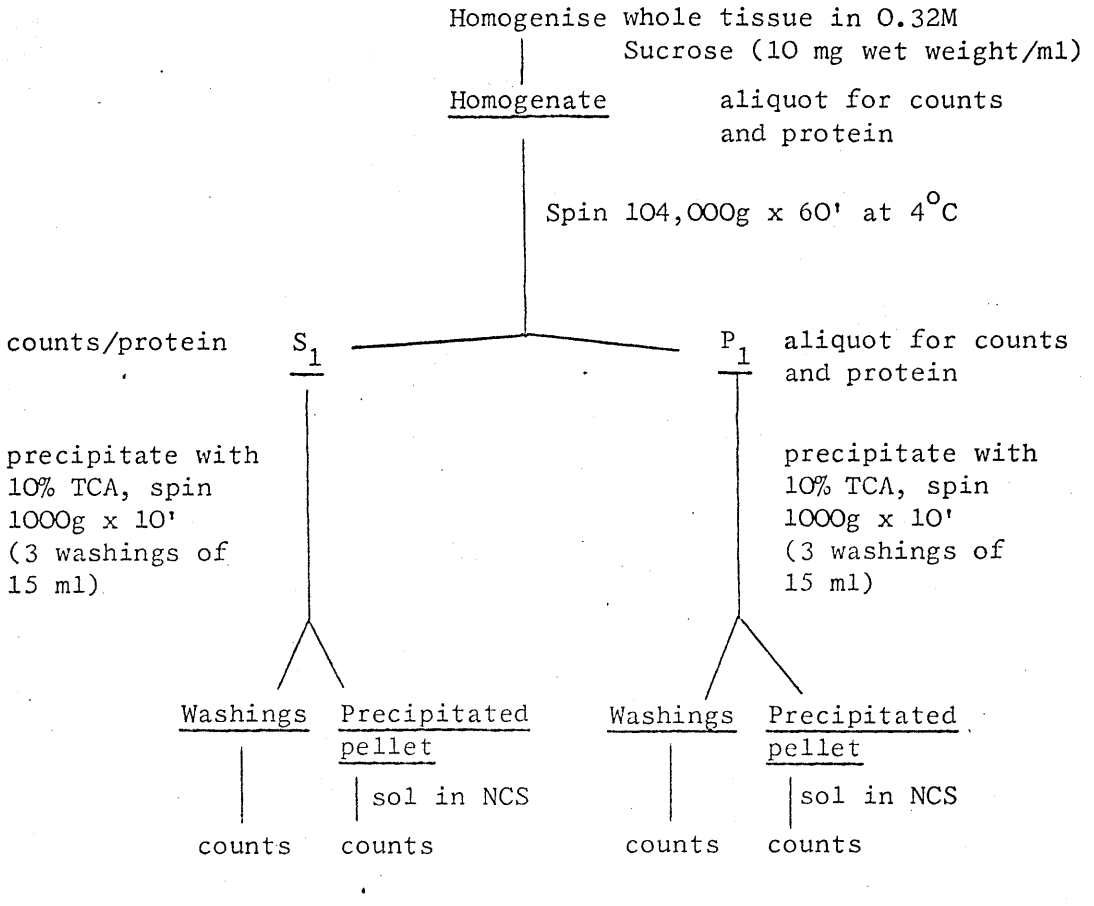


Figure 4.9. Sub-cellular fractionation of intestinal segments.

Homogenate = initial protein

P_1 = insoluble protein of high molecular weight

P_1 ppt = TCA insoluble protein

S_1 = soluble protein of low molecular weight

S_1 ppt = TCA insoluble protein

S_1 wash = TCA soluble protein (free amino acid pool)

AGE (DAYS)		H Homogenate counts % H	P_1 Insoluble Protein counts % H	S ppt Acid Insoluble Protein counts % H	S w Free Amino Acid Pool counts % H
1	15'	100%	1.4%	3.0%	Counts > 95% of Homogenate
	60'		1.4%	4.8%	
6	15'	100%	2.0%	3.2%	
	60'		3.4%	7.6%	
11	15'	100%	0.8%	1.5%	
	60'		1.2%	2.9%	
25	15'	100%	2.6%	2.4%	
	60'		2.4%	3.0%	
60	15'	100%	2.3%		
	60'		4.0%		

Table 4.3 Subcellular fractionation of L-lysine accumulation (1mM) into intestinal segments from rats of various ages.

Plate 14

Scanning electronmicrograph o roup of villi from 3 day old rat jejunum.

X 200

Plate 15

Scanning electronmic graph of villus tips from 3 day old rat jejunum fter 15min incubation in Krebs Ringer bicarbonate. While the main body of the villus shows good eservation, the tips are beaded in appearan indicating the beginnings of disruption.

X 700

Plate 16

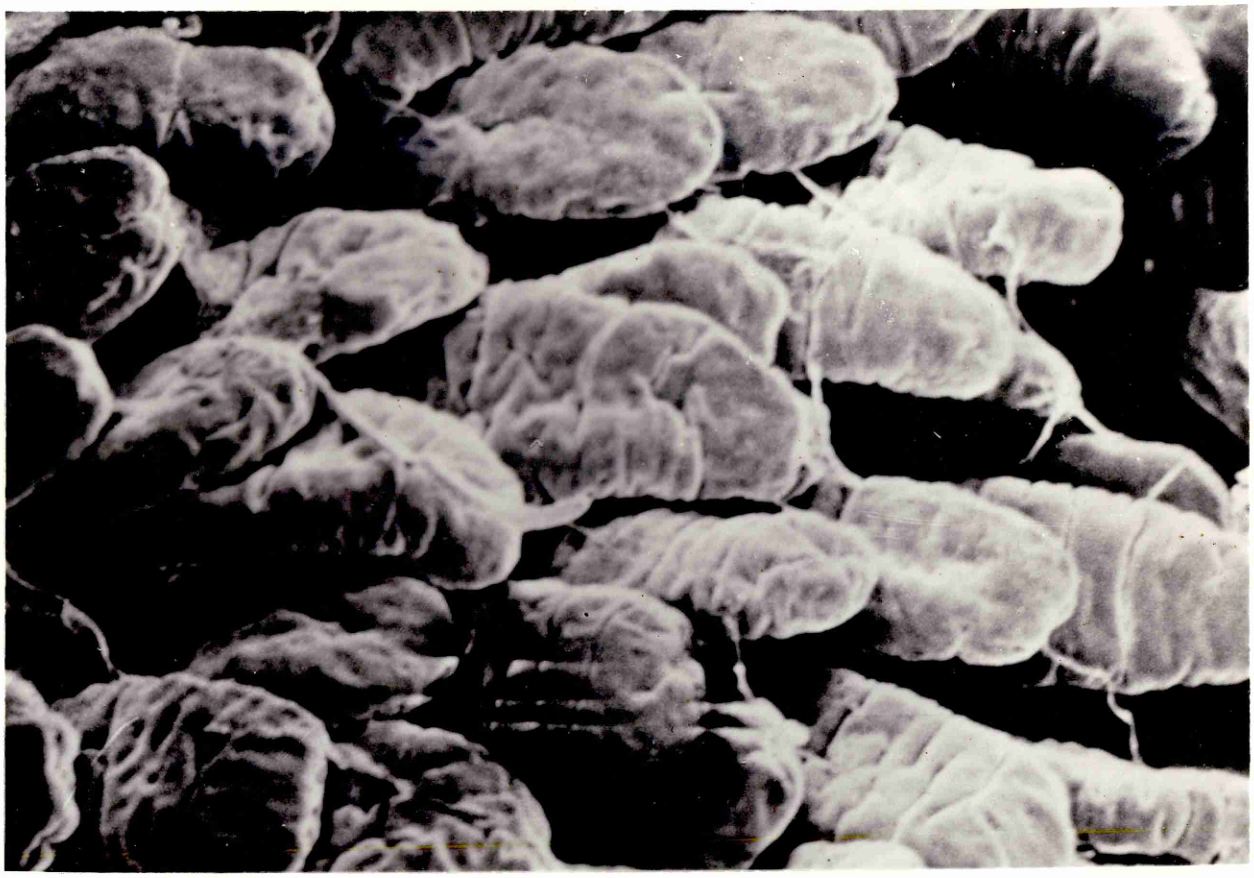
Scanning electronmicrograph of group of villi from 16 day old rat jejunum.

X 100

Plate 17

Scanning electronmicrograph of villi from 16 day old rat jejunum after 15min incubation in Krebs Ringer bicarbonate. The preservation at this age seems good after incubation. The villi are dotted with mucus secretions from the goblet cells.

X 400



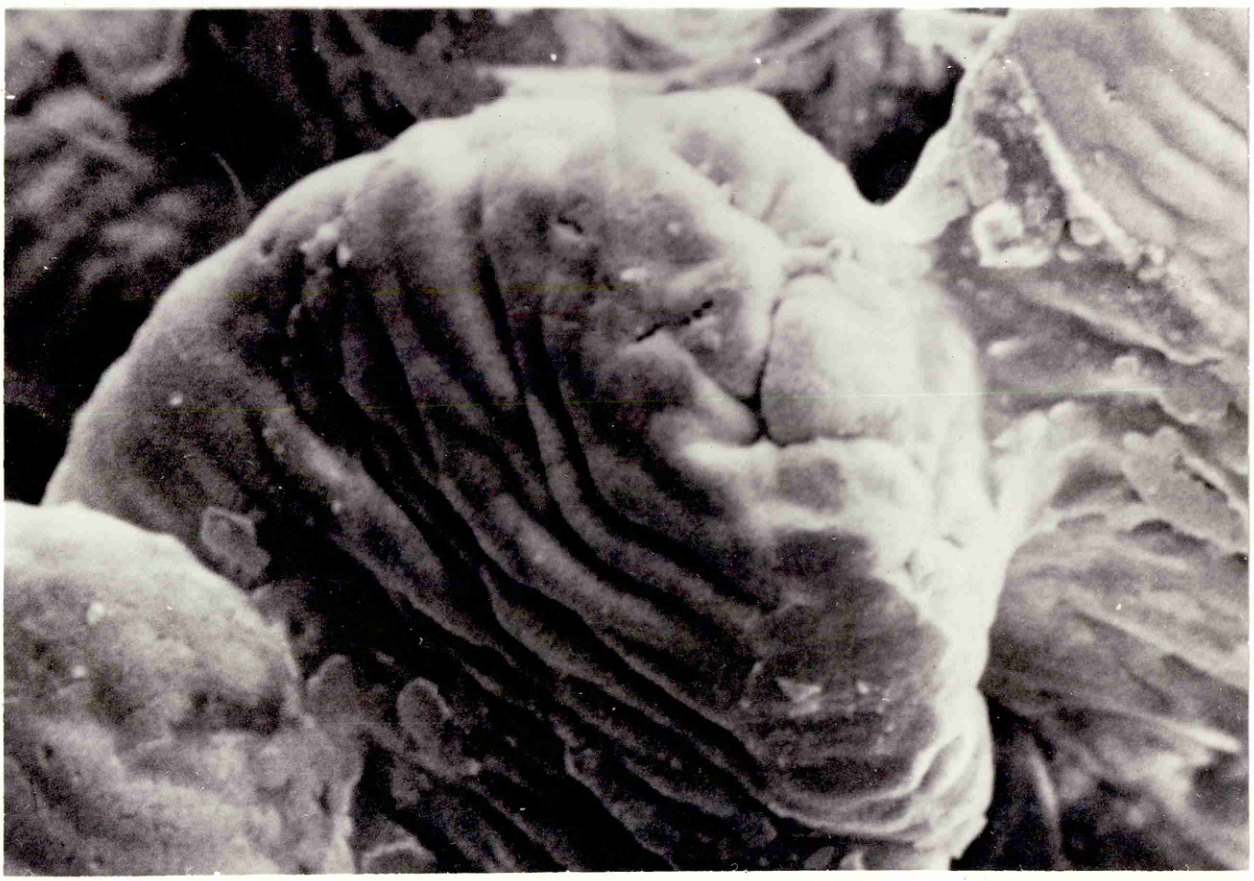


Plate 18

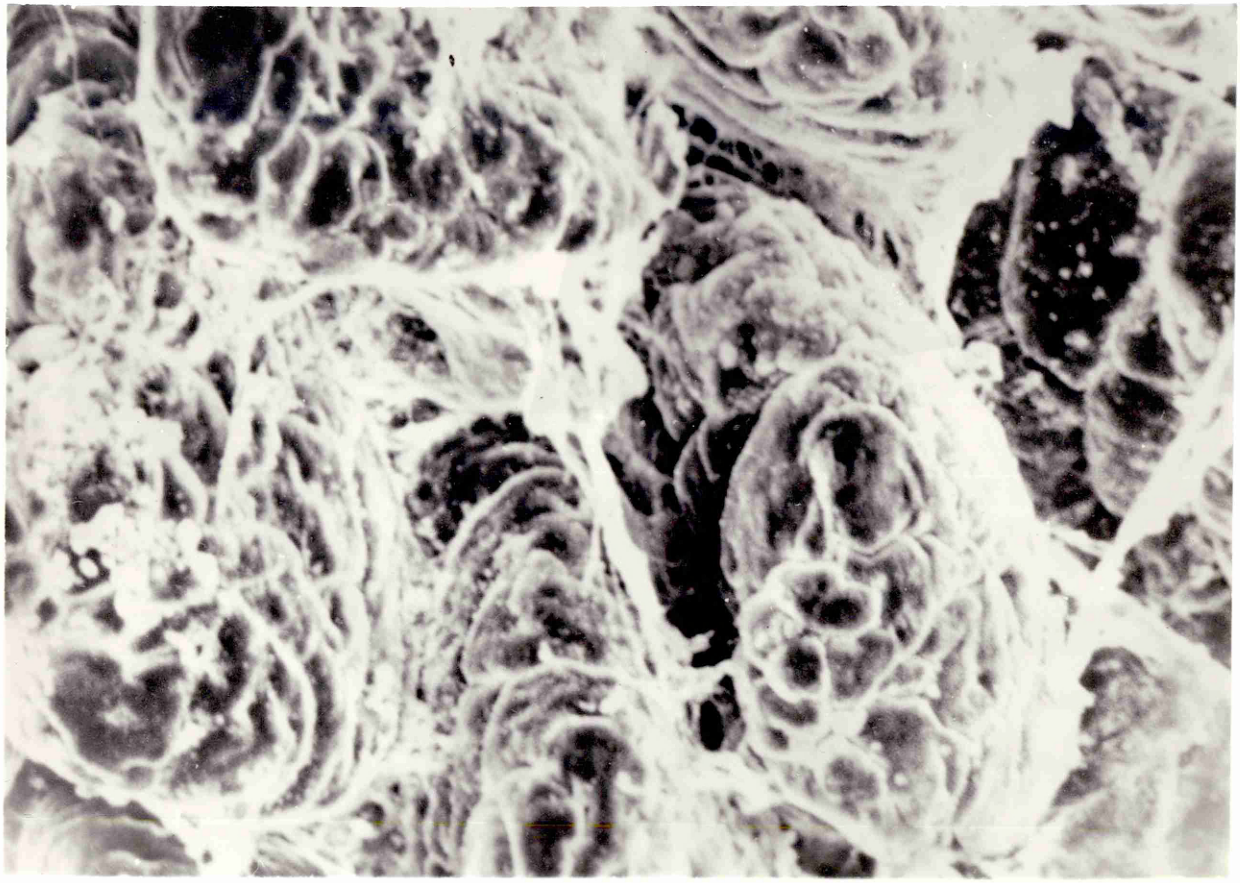
Scanning electronmicrograph of 25 day old rat jejunum. The villi are covered with strands of mucus.

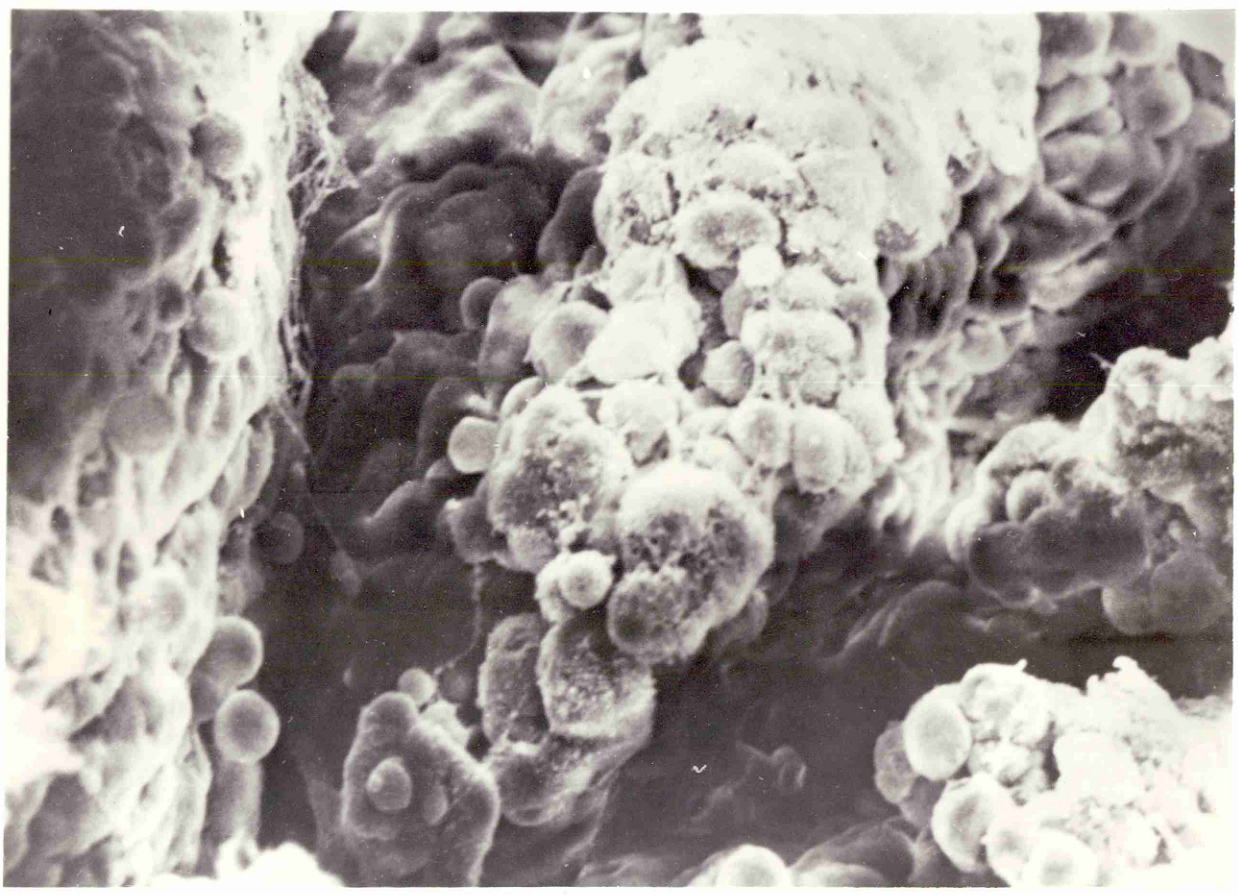
X 200

Plate 19

Scanning electronmicrograph of 25 day old rat jejunum after 15min. incubation in Krebs Ringer bicarbonate. The beaded appearance of the villus tips indicates that some disruption has occurred.

X 400





Problems of expressing absorption in a developing tissue

Developmental studies are beset with problems because the tissue is undergoing morphological change from day to day. This makes the expression of results difficult in terms of relating to a specific tissue unit. A series of experiments were carried out to look at the frequently used parameters of absorption studies and how these changed in the development of the small intestine.

1) Protein content related to tissue wet weight

Protein was estimated in gut segments from rats of various ages and expressed as % wet tissue weight (see Figure 4.10). It is evident that the amount of protein varies in the developing jejunum. Over the first two postnatal weeks, protein content increases in the gut probably as a result of increasing cell density along the villi. The protein concentration then drops and remains stable up to 4 weeks postnatally. The sudden fall may be the result of increasing surface area of the gut or the proliferation of serosal muscle layers with a high water content. In the adult jejunum protein comprises some 11% of tissue wet weight.

2) Tissue water

Accumulation studies often express the uptake of substrate in terms of a tissue concentration. Tissue water is assumed to be 80% of the initial wet weight of intestine from adult rats. In the developing intestine, Miller (1971) found that tissue water (as % wet weight) varied with age and body weight, being 76% up to 25 days old, 80% up to 60 days old and 76% in the adult. Tissue water also varied regionally along the small intestine, the proximal intestine

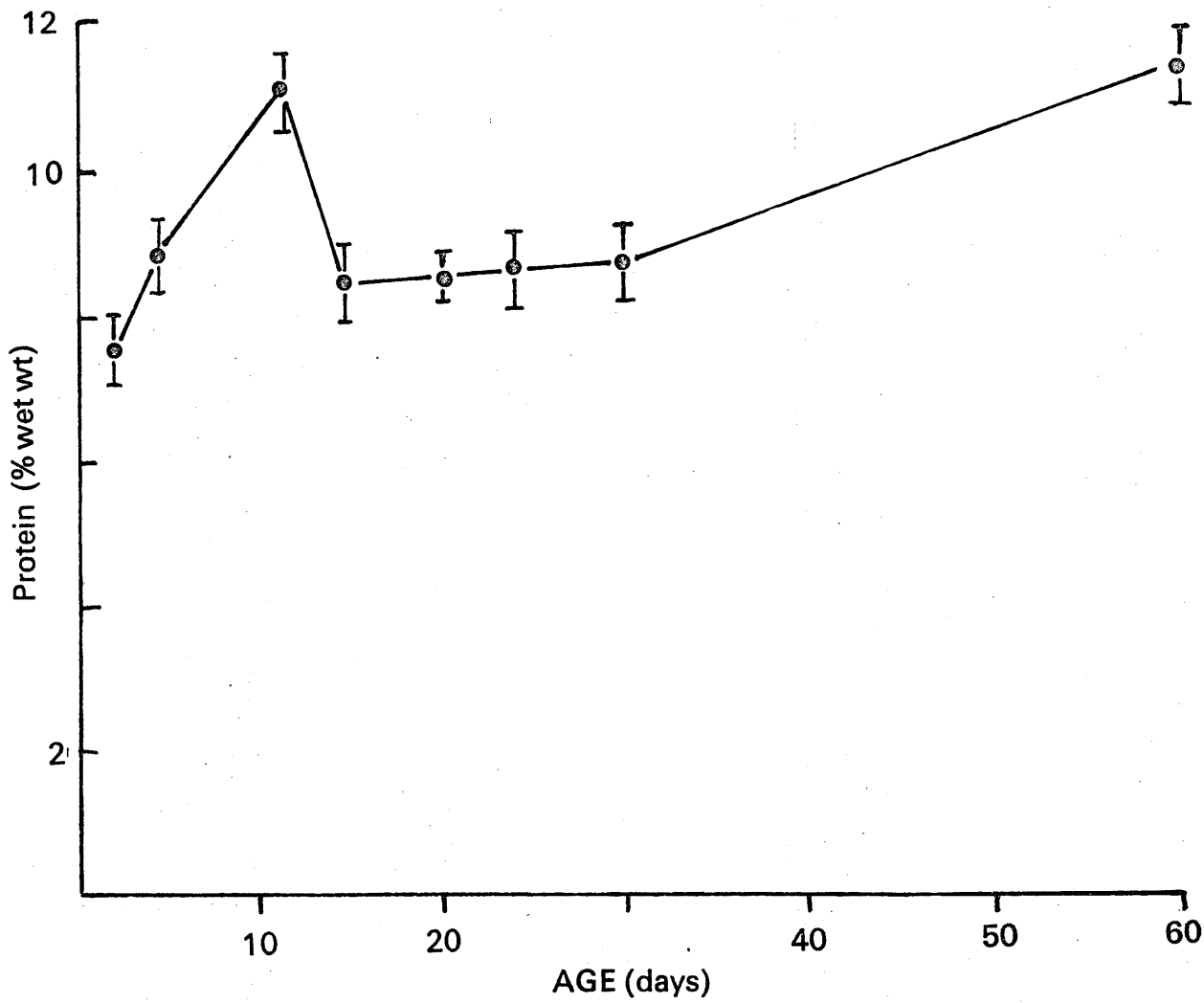


Figure 4.10 The protein content of jejunal tissue during development.

Bars indicate standard errors of the means of at least 8 observations at each point.

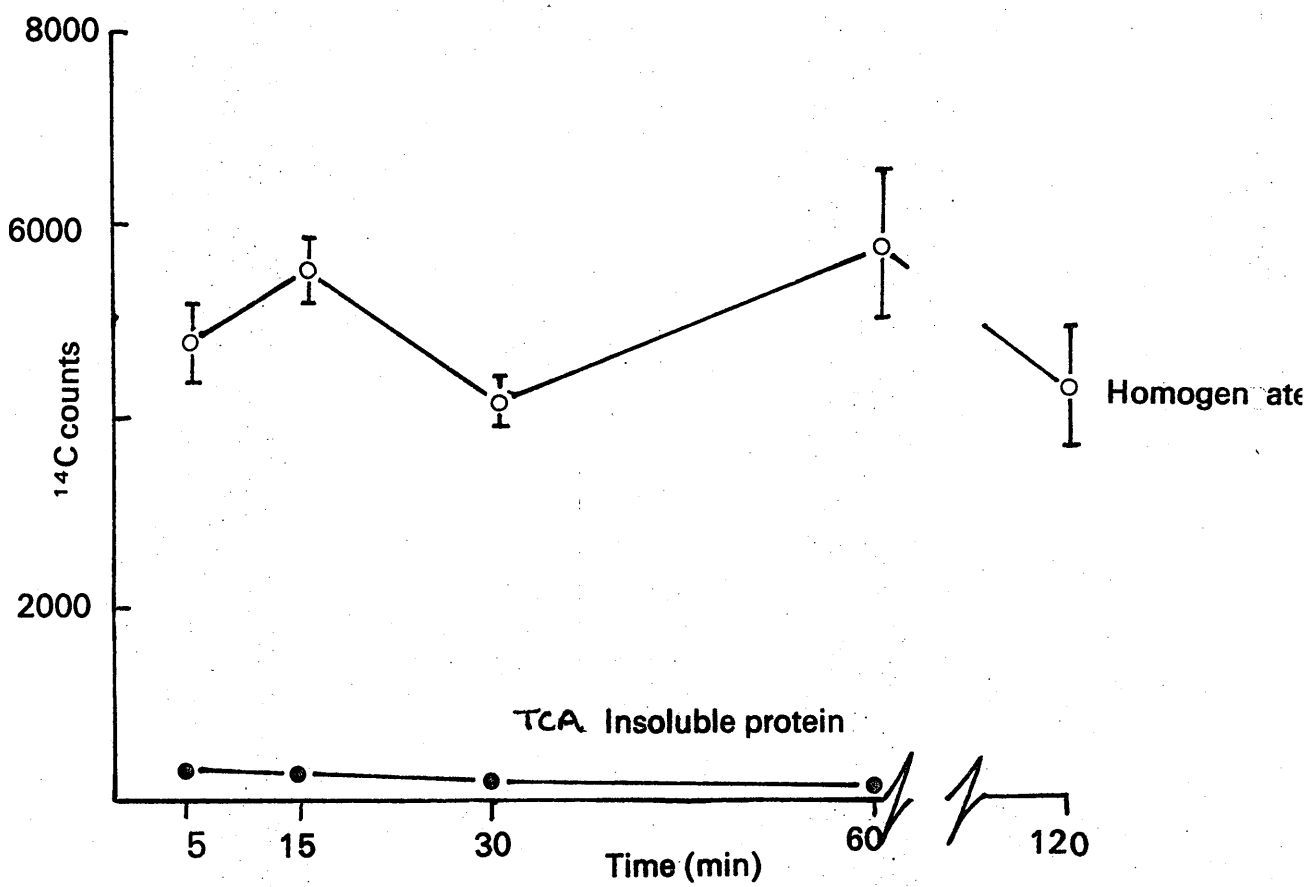


Figure 4.11 Time course of equilibration of ^{14}C hydroxymethyl inulin in jejunal segments from 15 day old rats.

^{14}C counts associated with whole tissue homogenate and TCA insoluble protein. Bars indicate standard errors of means of at least 4 observations at each point.

containing more water than the distal areas. Batt and Schacter (1969) assumed that tissue water was 86% of final wet weight and Fondacaro, Nathan and Wright (1974) used the figure of 80%. For the purpose of calculating tissue concentration here, Miller's results were used, and a figure of 78% ($\pm$ 2) initial wet weight was adopted throughout the entire age range.

Ingham (1972) measured fluid uptake during incubation of everted segments and found that final wet weights were at most only 5% more than initial wet weights. This very small increase may be the result of tissue-medium osmolarity acclimatisation or fluid uptake associated with substrate accumulation. Using the ratio of tissue fluid : dry weight Ingham also showed that everted gut segments were not significantly damaged during even quite long incubations (40 min), as there was no increase in the ratio. However, long incubations of tissue from very young rats ($<$ 7 days old) did result in tissue damage.

3) Extracellular space in the developing gut

In a developing tissue, undergoing phases of mitosis, the extracellular space (ECS) is bound to change. The rate of uptake of a substrate will obviously depend on the storage capacity of the tissue, and changes in transport in development might thus simply indicate the variation in cell packing density rather than changing membrane transport characteristics.

The determination of ECS, as accurately as possible, is vital for the determination of intracellular substrate concentration. Such a determination is not easy to perform, both because it is difficult

to find a substance that easily equilibrates with the ECS (but does not enter the cell) and because the methods by which the determination is made must be as standardised as possible, not damaging the tissue and leading to results stable in time. Inulin, a polyfructosan of molecular weight 5000, is commonly used to estimate ECS, but Marlow and Sheppard (1970) have reported that in some cases, inulin enters cells. The gut from young laboratory animals is capable of absorbing large molecules and cellular uptake of inulin is therefore possible.

Gut segments (prepared in the usual way) were incubated in Krebs bicarbonate containing ^{14}C hydroxymethyl inulin (carboxylic acid ^{14}C inulin has been shown to be a poor tracer by Marlow and Sheppard). To examine the possibility of inulin entry into cells, half the segments were rinsed in cold inulin and homogenised (controls) while the other half (washed) were subjected to a McIlwain tissue chopper and extensively washed through with cold inulin. This treatment, it was hoped, would release the extracellular inulin but at the same time leave most of the cells intact. From the results (Table 4.4) it seems that extensive washing reduces the counts associated with the tissue and little gets into cells.

Table 4.4

Everted gut segments from 17 day old rat incubated in hydroxymethyl ^{14}C inulin (10 µCi/10ml KRB ≡ 83 µmolar) for 30 min.

	^{14}C Counts	% Control
Control	4540 ± 917 (4)	-
Washed	695 ± 177 (4)	15%

The number of counts inside the cells did not exceed 15% at any age, showing that the neonatal intestine is not more "open" to inulin.

In a further series of experiments, gut segments were incubated in ^{14}C inulin for varying periods of time up to 2h. After each incubation, the tissue was rinsed in cold inulin, homogenised and subcellular fractionation carried out (as before, Figure 4.9) to trace the fate of the ^{14}C label. From the results in Figure 4.11 it can be seen that inulin equilibrates rapidly between the incubation medium and the tissue. After two hours, the uptake of ^{14}C inulin is similar to that after 5 minutes. Subcellular fractionation reveals that less than 6% of the inulin present is found associated with the soluble protein fraction. No counts were found in the insoluble protein. The amount of inulin associated with protein similarly does not increase with length of incubation and thus inulin seems to fulfill the requirements of an ECS marker, even in the developing intestine.

To examine the volume of ECS in the developing intestine, everted segments from rats of various ages (from standardised litters) were incubated in ^{14}C inulin, rinsed in cold inulin, homogenised and counted. The results are expressed as the volume of ECS (Tissue concentration : mucosal concentration) % - see Figure 4.12. There is little change in ECS in the development of the jejunum, except for a slight decrease between 10 and 25 days of age, which could well be the cause of the decrease in tissue water content at this time (as described by Miller, 1971). Using this technique, the ECS of the adult jejunum is some 25% which lies within the range of values (20-30%) described by Esposito, Faelli and Capraro (1972) using

in vivo techniques. Esposito and Csaky (1974) have surveyed a number of markers for ECS determination in the isolated everted small intestine and found ^{14}C inulin and ^{14}C PEG (polyethylene glycol) to be the most suitable and representative. They point out that the intestinal ECS is composed of two distinct compartments; a serosal compartment which includes the intercellular and subepithelial spaces and a mucosal compartment. Both were found to be of equivalent size, and combined represented 17% of the water of the tissue which they described as a low estimate.

If you assume that the concentration of substrate in the incubation fluid $(S)_o$ is the same as that in the extracellular fluid (ECF), then:

$$(S)_o \times (ECF) = \text{amount of substrate in ECF}$$

and

$$\text{(Total substrate accumulated)} - \text{(amount in ECF)} = \frac{\text{Intracellular amount}}{\text{Intracellular water}}$$

$$\text{Intracellular water} = \text{Total tissue water} - \text{volume extracellular fluid}$$

Thus:

$$\frac{\text{Intracellular amount}}{\text{Intracellular water}} = \text{Intracellular concentration}$$

However, it is a big assumption that the substrate concentration in the medium and in the ECS is the same. Also, the enterocytes will have a different substrate concentration to the serosal muscle layers and there may be substrate compartmentation within the cells.

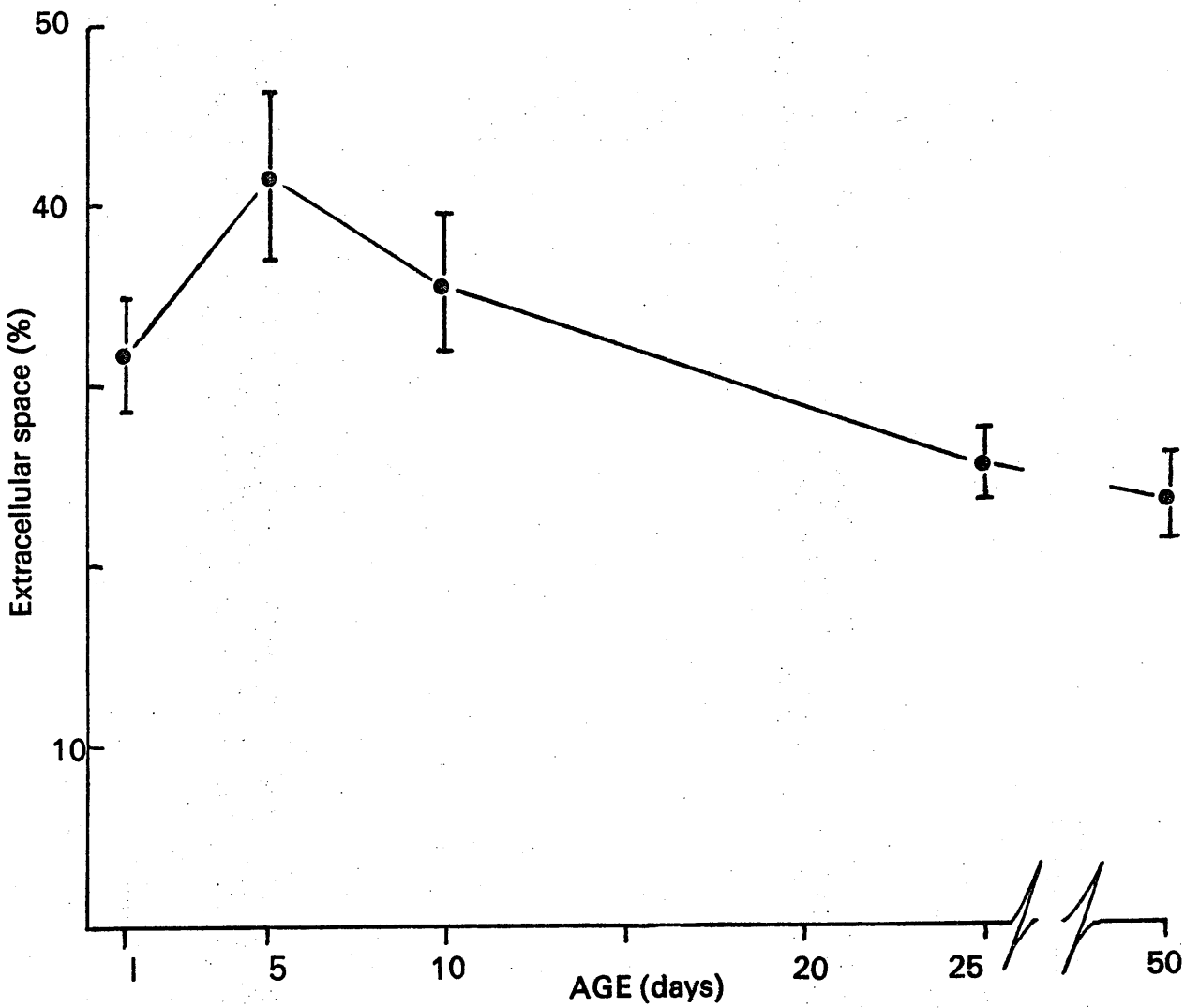


Figure 4.12 Extracellular space changes in the developing rat jejunum. (% total tissue water).

Bars indicate standard errors of the means of at least 4 observations at each point.

4) Serosal tissue and the "sink" effect

The isolated intestinal segment is a heterogenous tissue sample of basically two compartments, one absorptive (the mucosal villi) and one non-absorptive (the submucosal muscle and connective tissue mass). Techniques have already been discussed which measure accumulation in isolated mucosal tissue, but it is not possible to accurately correct for this heterogeneity in the whole everted segment. Robinson (1966) has pointed out that since there exists a barrier to free diffusion through the sub-mucosal layers to the intestinal muscle, it is probable that the concentration in the muscular layer never greatly exceeds that of the medium. If it did, then the substrate would simply diffuse out again into the medium. Robinson found that accumulation of L-phenylalanine and L-arginine by dog intestinal muscular tissue was unaffected by incubation.

Deren (1968) has described the doubling in thickness of the mouse intestinal wall between days 19 and 22 postnatally which represents an increase mainly in the serosal compartment. To investigate substrate uptake into this non-absorptive compartment, an amino acid which is accumulated to a great extent by the gut (L-valine) was selected. Substrate concentrations over a wide range were chosen to determine the extent of diffusion.

Figure 4.13 shows the contribution of diffusion to substrate uptake. The tissue becomes saturated at a concentration of 10 mM and extrapolating back from the higher concentration of substrate yields a straight line which will be virtually parallel to a line representing the diffusion component (Neame and Richards, 1972). Calculations of this diffusion component reveal that diffusion

contributes 10% to the values of substrate uptake at 10 mM. For subsequent kinetic determinations, 10 mM was fixed as the highest concentration value used (see Chapter 5), and thus while the subsequently reported uptake values contain a diffusion component this never exceeds 10%. This value for the diffusion component in total substrate uptake determined graphically compares well with the value obtained from low temperature incubation studies (see Figure 4.5).

Comparing the values for the diffusion component in rats of 15 and 28 days of age (Figures 4.13 a and b) it is evident that the increase in the non-absorptive serosal compartment (which occurs around day 20) does not form an appreciable sink for accumulated substrate: diffusion still represents only 10-11% of total substrate uptake at 10 mM in the 28 day old animals. However, the increase in protein content due to this non-absorptive tissue mass will affect the expression of kinetic results (see Chapter 5).

A rather crude measure of serosal protein was made by scraping off the mucosal layer from intestinal segments of rats during development (see Figure 4.14). The increase in the serosal compartment can be seen during the fourth postnatal week, and is similar to the increase found in the developing mouse intestine (Deren, 1968).

Figure 4.13

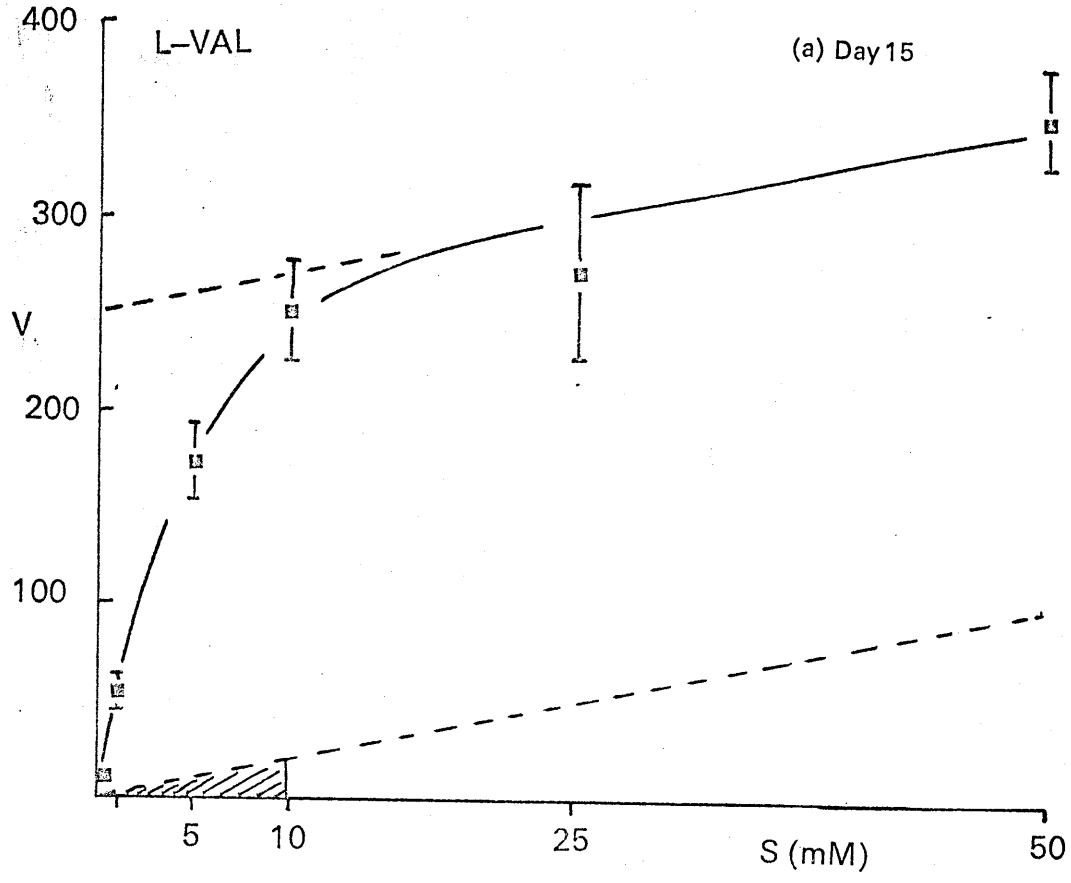
Uptake of L-valine into a) 15 day old and b) 28 day old rat intestinal segments over a wide concentration range.

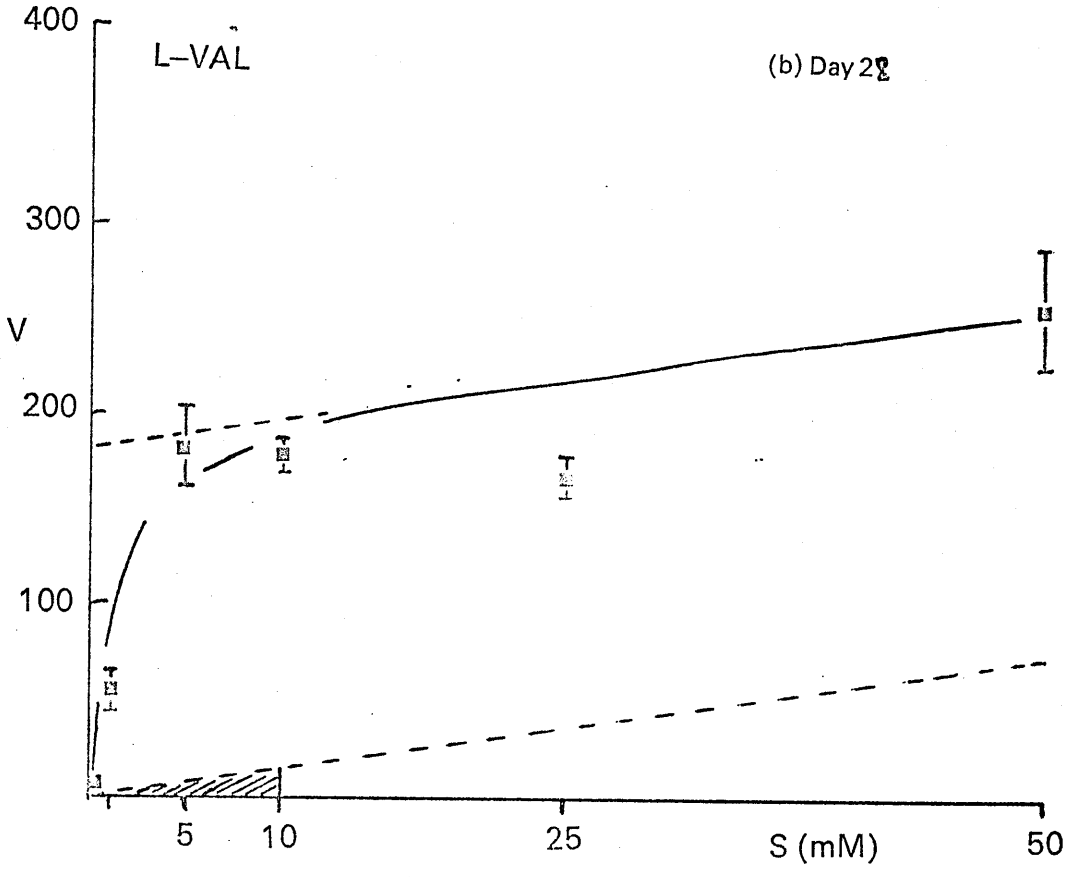
V = uptake in n moles/mg protein/15 min

S = concentration of L-valine in mM

Values of uptake are the means $\pm$ SEM of 4 experiments

Dotted line represents diffusion component in total uptake estimated by extrapolation from high concentration values.


400
L–VAL
(a) Day 15
300
V
200
100
5
10
25
S (mM)
50


400
L–VAL
(b) Day 2
300
V
200
100
5
10
25
S (mM)
50

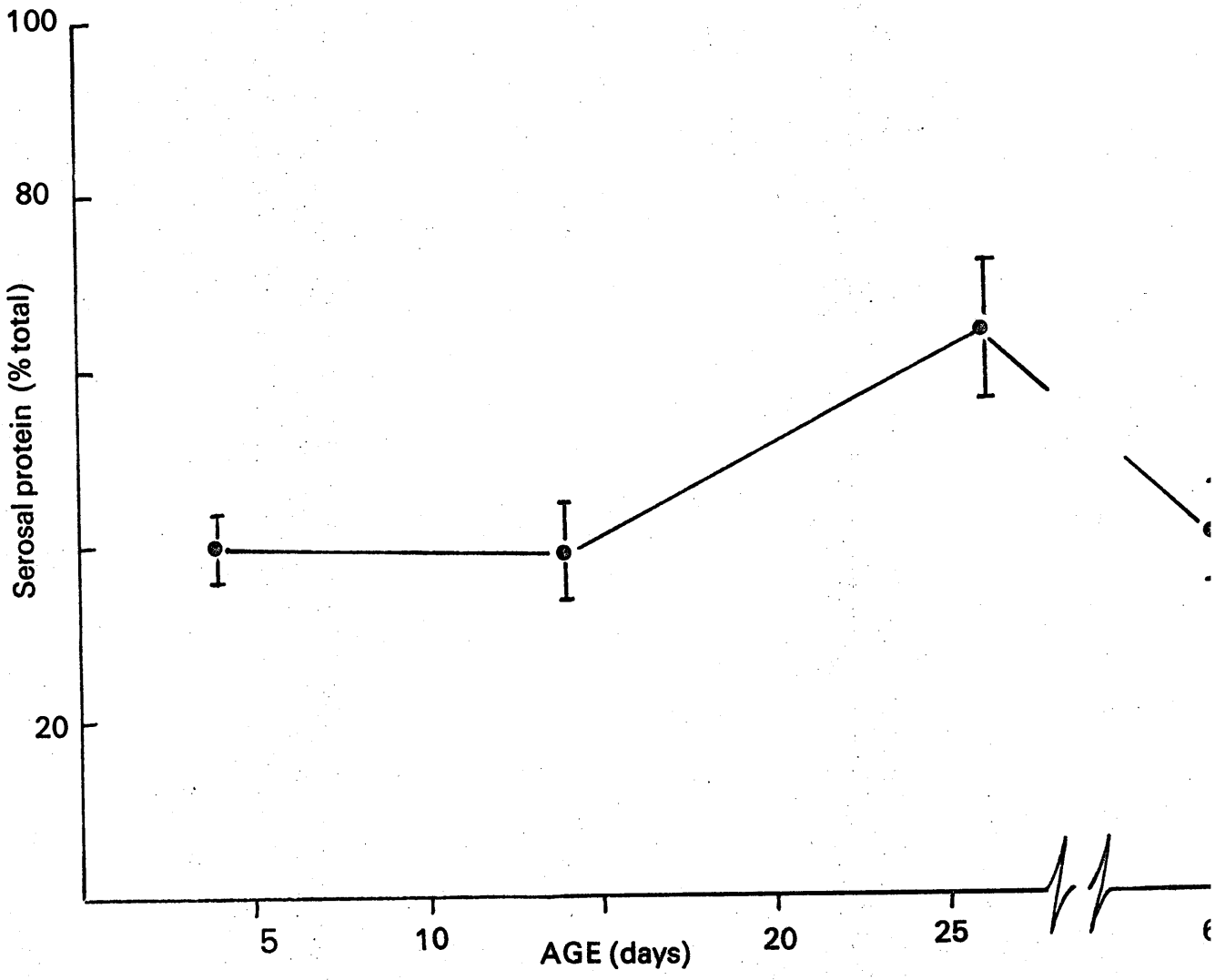


Figure 4.14 Contribution of serosal layers to total protein of jejunum and the changes in development.

Bars indicate standard errors of the means of at least 8 observations at each point.

Expression of results

The only measure of substrate transport that can be made with the everted intestinal segment is that of accumulation inside the tissue. This means that, unlike the everted sac, tracing transport across the gut is not possible but, as has been mentioned, transport measurements with the everted sac are suspect because of the interference of serosal muscle layers.

Throughout this work, the uptake of amino acid by intestinal segments is expressed in nanomoles absorbed per mg. protein. Weighing of tissues was avoided for reasons of damage and delay. However, protein content can be related to wet weight of the tissue using Figure 4.10. The calculation of uptake is based on the specific activity of the incubation medium:

$$\frac{\text{counts/mg protein}}{\text{counts/nmole substrate}} = \text{n moles absorbed}$$

Strictly speaking, absorption is dependent on the surface area rather than the weight of the tissue sample, but measurements of intestinal surface area are difficult and Robinson (1966) has found that uptake and weight of samples correlate well.

It is possible also to determine the mean concentration of the amino acid throughout the entire segment. It has been shown that tissue water is 78% of the wet weight in development and thus a ratio T/M (tissue concentration : mucosal concentration) can be used to express the extent of concentrative uptake inside the jejunum. Similarly, intracellular concentration can be calculated from the size of the extracellular space.

The precision of the experiments depends on a number of steps. Scintillation counting accuracy was pre-set to 3%. Rats were taken from standardised litter sizes and position of segments randomised to reduce variability. In general 4-8 parallel samples have been taken from a single rat for every incubation condition, and these have been combined with results of duplicate experiments using different rats. The number of observations (n) is given with each set of results and these have been averaged to give a mean value $\pm$ the standard error of the mean. Students 't' test has been employed where indicated and differences were taken to be significant when values of $p < 0.05$ were obtained. More specific problems of interpreting developmental transport data will be dealt with at length in the relevant Chapters.

CHAPTER 5

Kinetics of Amino Acid Absorption in Development

Introduction

The milk diet of the neonate contains much protein (including immunoglobulins), some of which is absorbed intact while the rest is hydrolysed into smaller peptides and amino acids. In the neonatal rat, gastric glands do not appear until Day 18 and stomach proteolytic activity is low until Day 21 (Koldovsky, 1969). Similarly Koldovsky (1969) reports that breakdown of casein in the rat jejunum is low until Day 21 when activity rises to a peak at Day 60. This means that the concentration of free amino acids in the intestinal lumen must be fairly low until the time of weaning.

The naturally occurring amino acids number 20, have molecular weights in the range of 90-250 daltons and most are of the L- configuration. All are required for protein synthesis in animal tissues but some can be synthesised in the body from others by transamination. The vital group of amino acids which are dietary "essentials" for man and most mammals include leucine, isoleucine, valine, threonine, methionine, phenylalanine, tryptophan and lysine. In neonates, histidine and arginine are also essential for growth (see Table 5.1). It is possible, though, to replace most of the essential amino acids (save lysine and threonine) in the diet of growing rats by the corresponding α-ketoacids (Walser, 1976).

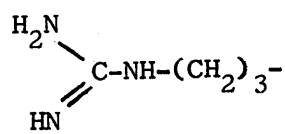
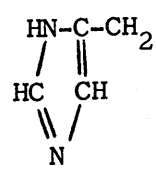
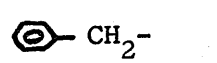
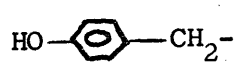
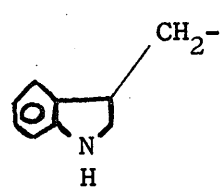
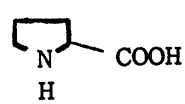
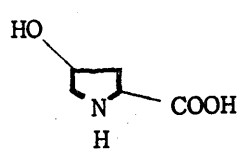
The plasma amino acid pool is extremely heterogenous and represents only some 10% of the total free pool. While protein digestion barely affects peripheral blood amino acid levels, there exists a circadian periodicity. Developmentally, plasma levels of particular amino acids change around weaning, which may be reflected in other tissues such as the brain where there is an increase in the free amino acid pool around 20 days post-natally (Miller, 1970). These changes have been shown to be important in terms of neural protein synthesis which

is at a high level at this time, the free amino acid pool stabilising polysomal RNA (Munro, 1968).

The problem of transporting amino acids into cells depends upon their hydrophilic nature, in contrast to the predominantly hydrophobic nature of the limiting plasma membrane. Advancing technology has provided new tools for the examination of membrane structure and has demonstrated the enormous extent of fluidity and lability within the membrane. Fifty years ago, the picture of the membrane was that of a static lipid bilayer coated with protein. In the last decade, this picture has changed dramatically. Firstly, the lipids are extremely mobile, exchanging laterally (in the 'cis' direction) some 10^6 times/second, and the hydrophobic tails are very flexible, particularly in the centre. Many types of phospholipid have been identified with different head groups which are important for membrane bound enzyme activation e.g. Na, K-ATPase. There is also evidence for marked asymmetry between the outer and inner leaflets of the lipid bilayer. Proteins are responsible for membrane fluidity, being built in very asymmetrically; some are deep within the lipid matrix (intrinsic) while others are more superficial (extrinsic). In general, proteins possess great mobility, being able to diffuse both in a 'cis' direction and also rotate about an axis perpendicular to the plane of the membrane ('trans' direction). It is this inherent fluidity in membrane structure that permits exchange of information and material between the cell and the surrounding environment (Singer and Nicholson, 1972; Bretscher and Raff, 1975).

TABLE 5.1

The principal amino acids (E = essential)

Name		(R)-C(H)(NH$_2$)-COOH	M.W.	
Glycine		H-	75.1	neutral
Alanine		CH_3-	89.1	neutral
Valine	E	$(CH_3)_2CH$-	117.1	neutral
Leucine	E	$(CH_3)_2CH-CH_2$-	131.2	neutral
Isoleucine	E	$C_2H_3-CH(CH_3)$-	131.2	neutral
Serine		CH_2OH-	105.1	neutral
Threonine	E	CH_3-CHOH-	119.1	neutral
Aspartic acid		$HOOC-CH_2$-	133.1	acidic
Glutamic acid		$HOOC-CH_2-CH_2$-	147.1	acidic
Lysine	E	$H_2N(CH_2)_4$-	146.2	basic
Ornithine		$H_2N(CH_2)_3$-	132.2	basic
Arginine	(E)		174.2	basic
Histidine	(E)		155.2	neutral
Phenylalanine	E		165.2	neutral
Tyrosine			181.2	neutral
Tryptophan	E		204.2	neutral
Cysteine		$HSCH_2$-	121.2	basic
Methionine	E	$CH_3-S-(CH_2)_2$-	149.2	neutral
		Formula		
Proline			115.1	neutral
Hydroxyproline			131.1	neutral

Modes of transport

Transfer across biological membranes is postulated as being of two main types : diffusion and carrier transport. Diffusion involves movement entirely under the control of physical forces, is without direct interaction with the membrane, and its rate is proportional directly to the concentration of the solute. Diffusing molecules will thus pass down a concentration gradient, into and out of tissues, until a steady state is reached. Numerous factors will alter the rate of transfer of molecules across a membrane e.g. temperature, electrical charge, size and shape of solute, solubility and surface area of membrane available. In an experimental system, these factors are assumed constant and incorporated into a single factor, K_D the diffusion constant.

Carrier transport describes transfer which occurs at a rate higher than could be attributed to diffusion alone, and at the same time is saturable at high concentrations. Carrier transport can itself be subdivided into 1) equalising transport (facilitated transfer) in which the concentration of solute either side of a membrane are the same at equilibrium, but the rate of transport declines with increasing concentration (tissue medium concentration, i.e. T/M, never exceeds unity), and 2) concentrative (active) transport where the concentrations inside and outside the cell are different at equilibrium (T/M always greater than one).

Kinetics of amino acid uptake

The primary event in the mediated transport of a solute across a membrane is the reversible combination of a molecule of the substrate with a specific membrane moieity or carrier. The mode of attachment and nature of membrane carriers will be considered later. If the

number of molecules increase (by raising the substrate concentration), more carriers become filled until saturation occurs and transport is then maximal. This process of transport across the membrane can then be described kinetically as:

$$S + E \underset{k-1}{\overset{k_1}{\rightleftharpoons}} ES \underset{k-2}{\overset{k_2}{\rightleftharpoons}} E + S_1 \qquad (1)$$

where S and S_1 are the same substrate but on opposite sides of the membrane, E represents the carrier and ES the substrate-carrier complex: k's denote rate constants for both the loaded and unloaded condition of the carrier. Loaded sites may move faster than unloaded ones and thus produce an unequal distribution of carrier sites each side of the membrane. This equation (1) also describes the interaction of an enzyme with its substrate where E converts S into a product (P). In most instances this kinetic analogy extends only to initial absorption events and does not necessarily imply an enzymic step.

If transport is measured under initial rate conditions the process follows as does an enzymic reaction, Michaelis-Menten kinetics (Michaelis and Menten, 1913).

$$V = \frac{Vmax\ S}{S + K_m} \qquad (2)$$

A plot of V, the velocity of uptake, against S, the substrate concentration, will result in a rectangular hyperbola from which both Vmax, the maximum velocity of uptake (or number of carrier sites), and K_m (the affinity constant which is equal to the concentration at which half of the available carriers are filled) can be estimated (Figure 5.1).

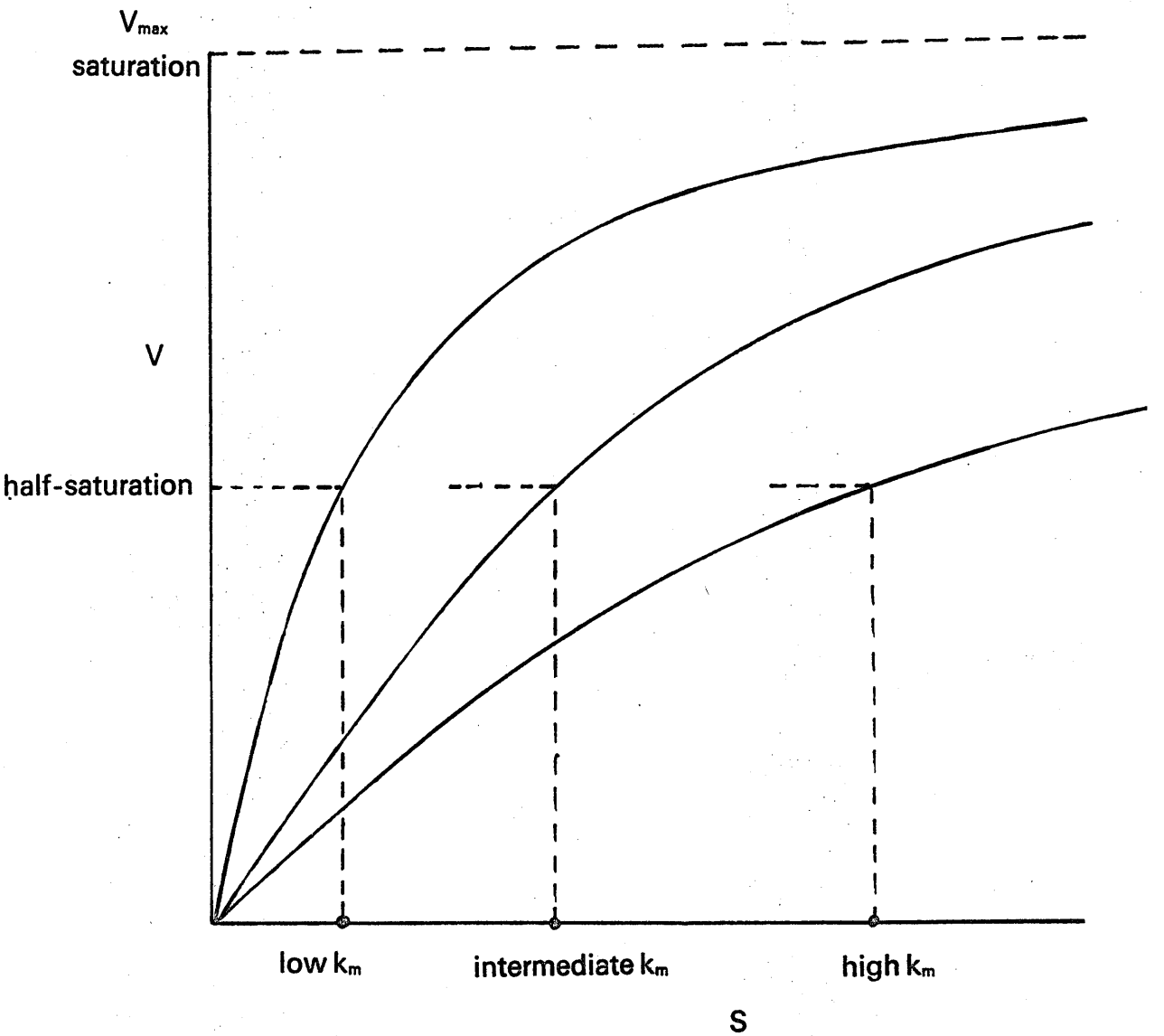


Figure 5.1 Plot of V against S showing the kinetic relationship between Vmax and K_m.

Amino acid transport (among a variety of other substances) in the intestine, and in a number of other tissues, follows Michaelis-Menten kinetics, as will be demonstrated in this Chapter, which describes the affinity of the carrier sites on the absorbing enterocyte (K_m) and the number of such sites (Vmax). Estimations of these from inspection of simple data plots are not rigorous enough and a number of alternatives have been employed which rearrange such plots to give a straight line. If the experimental results do not conform to a straight line, then either a two-carrier system is in operation or transfer cannot be accounted for solely in terms of Michaelis-Menten kinetics.

There are three graphical methods of linear transformation commonly used which have all been utilized here. What follows is a brief description of their derivation: a fuller account can be found in Neame and Richards (1972).

1) 1/V against 1/S

Lineweaver and Burk (1934) suggested that the Michaelis equation could be transformed into the following relationship:

$$\frac{1}{V} = \frac{K_m}{Vmax} \cdot \frac{1}{S} + \frac{1}{Vmax} \qquad (3)$$

and a plot of 1/V against 1/S is therefore linear. Vmax and K_m can be calculated from the reciprocal of the y and x intercepts (see Figure 5.2a).

2) S against S/V

Hanes (1932) obtained this plot simply by multiplying equation (2) by S.

$$\frac{S}{V} = \frac{S}{Vmax} + \frac{K_m}{Vmax} \qquad (4)$$

Plotting S against S/V results in a straight line from which Vmax and K_m can again be calculated (see Figure 5.2b).

3) V against V/S

Here the original expression (equation 2) has been rearranged by Hofstee (1959) into:

$$V = Vmax - \frac{V}{S} \cdot K_m \qquad (5)$$

When V is plotted against V/S, a straight line with negative slope is achieved with Vmax the y intercept and K_m the slope of the line (see Figure 5.2c).

Clearly, these three algebraic expressions are simply re-arrangements of the original Michaelis-Menten equation.

Dowd and Riggs (1965) compared the estimation of kinetic constants from all three linear transformations on simulated data and arrived at a number of interesting conclusions based on the variance of their results. They found that the Lineweaver-Burk plot (3) gave a deceptively good fit, even with unreliable experimental values and if used without adequate weighting, it can result in large errors. The double reciprocal plot is by far the most common. As for the other two plots, (Hanes, 4; Hofstee, 5), Dowd and Riggs found little to choose between them save that plotting V against V/S is slightly preferable, particularly if the value of V is likely to be large.

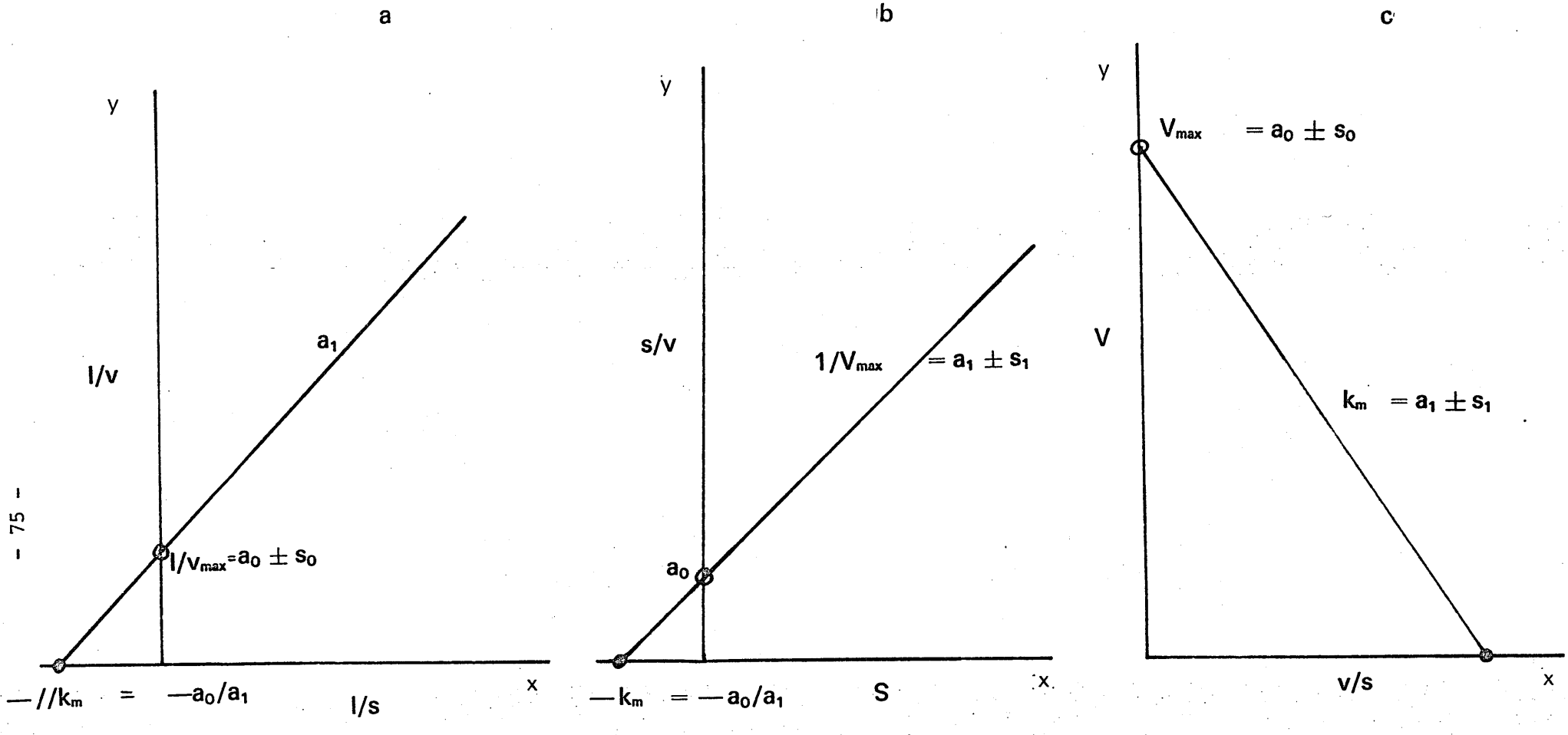


Figure 5.2 Three linear transformations of the Michaelis-Menten expression;
a) Lineweaver and Burk (1934)
b) Hanes (1932)
c) Hofstee (1959)
Calculation of Vmax and K_m are indicated together with the regression coefficients from linear regression analysis.

Kinetics of accumulation of essential amino acids in development

The requirements of specific amino acids has been described, requirements which are particularly acute in the developing organism. A number of studies have been made of the kinetics of amino acid transport in the developing intestine of the chick (Lerner et al, 1976), rabbit (Deren et al, 1965), guinea pig (Butt and Wilson, 1968), rat (Reiser and Christiansen, 1969) and pig (Smith et al, 1976), using a range of techniques and substrates.

In the rat, active transport of amino acids from the gut is already present before birth and changes in kinetics have been described around weaning, coinciding with the appearance of a cell type on the villus that has different absorptive properties, namely impermeability to large macromolecules. Transport of amino acids appears to decrease after weaning (Donnelly, 1971; Fondacaro and Nathan 1971; Batt and Schachter, 1969) but kinetic studies were either not performed or were inadequate (see earlier discussions). Similarly, the problems of expressing the results were not dealt with adequately. For example, the influence of non-absorbing muscle tissue in diluting uptake rates was revealed by Fondacaro, Nathan and Wright (1974) but they did not report any kinetic analysis of uptake over this period.

This Chapter describes the kinetics of uptake of a number of essential amino acids from the intestine from birth to maturity, with particular reference to the period around weaning. Essential amino acids were chosen as they are interesting from a nutritional and developmental viewpoint, and contain both neutral and basic types.

Problems of uptake expression and kinetic analysis are considered to be of paramount importance in the interpretation of transport data.

Results

Experimental design

The technique has been described earlier. Radioactive substrate of such a high specific activity was present so that there was an almost negligible addition to the cold substrate concentration in the incubation medium. 10 mM glucose was present throughout and the intestinal segments (from animals ranging from 1 to 60 days of age) were incubated for 15 minutes.

Six amino acids were chosen which are all essential in the diet:

L-lysine and L-arginine (diamino-monocarboxylic - BASICS)

L-leucine and L-valine (monoamino-monocarboxylic - NEUTRALS, with aliphatic side chains)

L-methionine (NEUTRAL with a sulphur containing side chain)

L-phenylalanine (NEUTRAL with a cyclic side chain)

These substrates were considered to be of particular interest because they are essential amino acids, they represent a good cross section of the various classes of amino acids containing both neutral and basic representatives and moreover the four neutral substrates are purported in the adult rat intestine to be transported by at least four different systems. Chapter 6 looks in detail at the interactions between these selected substrates. In the young rat no assumptions of specific carrier systems were made but the six substrates were selected to maximise the possibilities of identifying specific transport systems.

Transfer rates were obtained over as wide a range of concentrations as possible in order to minimise unreliability which can result from choosing concentrations below or above the K_m of a particular substrate (Neame and Richards, 1972).

The expression of results and related problems

Some of the general problems concerning the expression of amino acid uptake *in vitro* in a developing tissue such as the neonatal gut have already been alluded to, and have also been reviewed by Deren (1968). The major problem is the choice of a standard means of expression. There are a number of choices: tissue wet weight, tissue dry weight, protein, tissue water, and perhaps surface area. The first three are all directly related, and have been shown to change in the developing small intestine (see Chapter 4). Tissue water is a measure of both the internal and external compartments and is obviously related to tissue wet weight. The last one, surface area, is most difficult to measure reliably.

Each of these parameters have been used in previous developmental studies but little attention has been devoted to the implications of their use. In this work, uptake has been expressed in two ways: 1) amount of amino acid accumulated/mg protein/15 min and 2) concentration in the tissue as a ratio of the mucosal concentration (T/M).

Using the first measure, the non-absorbing muscle mass is obviously included in the protein expression (see Figure 4.14) and as will be seen, the doubling of the serosal tissue weight around weaning certainly affects extent but not rate of uptake. The degree of concentrative uptake is based upon a measure of the total tissue

water and has not been manipulated to yield intracellular concentration by assuming extracellular concentration. Diffusion contributes to amino acid accumulation and thus these values of uptake (V), as indicated in the following expression:

$$V = \frac{Vmax\ S}{S + K_m} + K_D\ S$$

K_D, the diffusion constant, can be calculated by a number of methods (Neame and Richards, 1972). At low substrate concentrations, inward diffusion will be negligible and as the tissue concentration rises can reverse so that diffusion is outwards. At high concentrations of substrate (above the maximal uptake rate), inward diffusion will be significant and can be calculated from the linear portion of a data plot (see Figure 4.13).

The values of uptake (V) described here have not been corrected for diffusion because the diffusion component measured with an extensively accumulated substrate (L-valine) showed a contribution of at most 10% over the concentration range used here (0.1 - 10 mM) (see Figure 4.13). For the determination of Vmax and K_m in this work the highest substrate concentration point (25 mM) has been omitted. This diffusion component appeared to be unaffected by changes in the serosal thickening during development. Similarly experiments using GABA have shown that diffusion does not change with age (see Figure 4.8).

Also, the linear plot S/V against S has a property not shared by the other two, namely that even if a diffusion component is not first subtracted from the total transfer, the resulting line still cuts the abscissa at $-K_m$.

For each substrate concentration at each age, the uptake is given as the mean (with its standard error) of a fixed number

weighting is essential so that linear regression can be applied to the transformed data in the three kinetic plots. Linear regression provides the regression coefficients (a_o and a_1) together with their standard errors (s_o and s_1) and the coefficient of determination (r^2) which measures the "goodness of fit" of the regression line. These terms are shown in the kinetic plots in Figure 5.2 as they were used in the determination of Vmax and K_m. Linear regression was performed on the means of weighted data, (i.e. keeping 'n' constant) in order to regulate the standard error on each mean.

Wilkinson (1961) and Neame and Richards (1972) have described algebraic methods of determining the standard error of Vmax and K_m from data plots, but these methods, to be statistically valid, would have to be applied to each individual data point.

Amino acid absorption in development

Neutral amino acids

i) L-leucine (Figures 5.3 and 5.4)

Data plots for the accumulation of L-leucine by intestinal segments of varying age are shown in Figure 5.3. It is evident from Figure 5.3a that the jejunum will concentrate L-leucine at birth: if the 25mM point is disregarded, T/M is 3 at 5mM and 1.5 at 10mM, indicating that the concentration inside the tissue is maximal at 15mM. The shape of the hyperbolae are essentially similar up to 15 days of age where there is a peak in leucine uptake. By 24 days of age (Figure 5.3f) the curve of uptake is distinctly flattened, as it is at 60 days. The T/M ratio reveals that the jejunum accumulates leucine to a concentration of only 5-10mM.

The results of linear transformation of the data plots can be seen in Table 5.2, where Vmax and K_m have been calculated from all three kinetic plots for comparison. The results of Vmax and K_m from the Hofstee plot are shown as a function of age in Figure 5.4. The coefficient of determination is better than 0.85 in most cases and the "goodness of fit" of the line is again indicated in the standard errors of the regression coefficients (Vmax and K_m). K_m and Vmax change little up to weaning, though there appears to be a trend of increasing Vmax from day 1 to day 15. There is no significant difference in K_m over this period. Post-weaning, there is an apparent marked decline in Vmax, and over the period 15-24 days of age Vmax declines by 50%. Correspondingly, the value of K_m increases, although the size of the errors on the regression coefficients preclude significance.

ii) L-valine (Figures 5.5 and 5.6)

The neonatal jejunum concentrates L-valine at birth (Figure 5.5a) and the T/M ratio peaks at 2 weeks of age (Figure 5.5d). The extent of uptake then declines to an adult level post-weaning. Figure 5.6 plots the Vmax and K_m of L-valine accumulation in the developing jejunum. There is a trend for Vmax to increase before weaning, and then decrease to a neonatal level by 4 weeks of age. K_m changes little until weaning when there is a trend for the membrane carrier affinity to decline with age.

Comparing Figures 5.4 and 5.6 for these two neutral amino acids, the trends in increasing Vmax before weaning and increasing K_m post weaning are similar. The values of Vmax at birth (some 200 nanomoles substrate/mg protein/15 min) and post weaning are very

similar, as are the values of K_m (birth = 1.0mM; 60 days of age = 2.0mM).

iii) L-phenylalanine (Figures 5.7 and 5.8)

Figure 5.7a reveals that the neonatal jejunum accumulates this neutral amino acid with a cyclic side chain to the extent of some 10mM prior to weaning. With age, the data plot flattens considerably and the Vmax decreases (Figure 5.8). Affinity for transport decreases with age; this is most marked around the time of weaning.

iv) L-methionine (Figures 5.9 and 5.10)

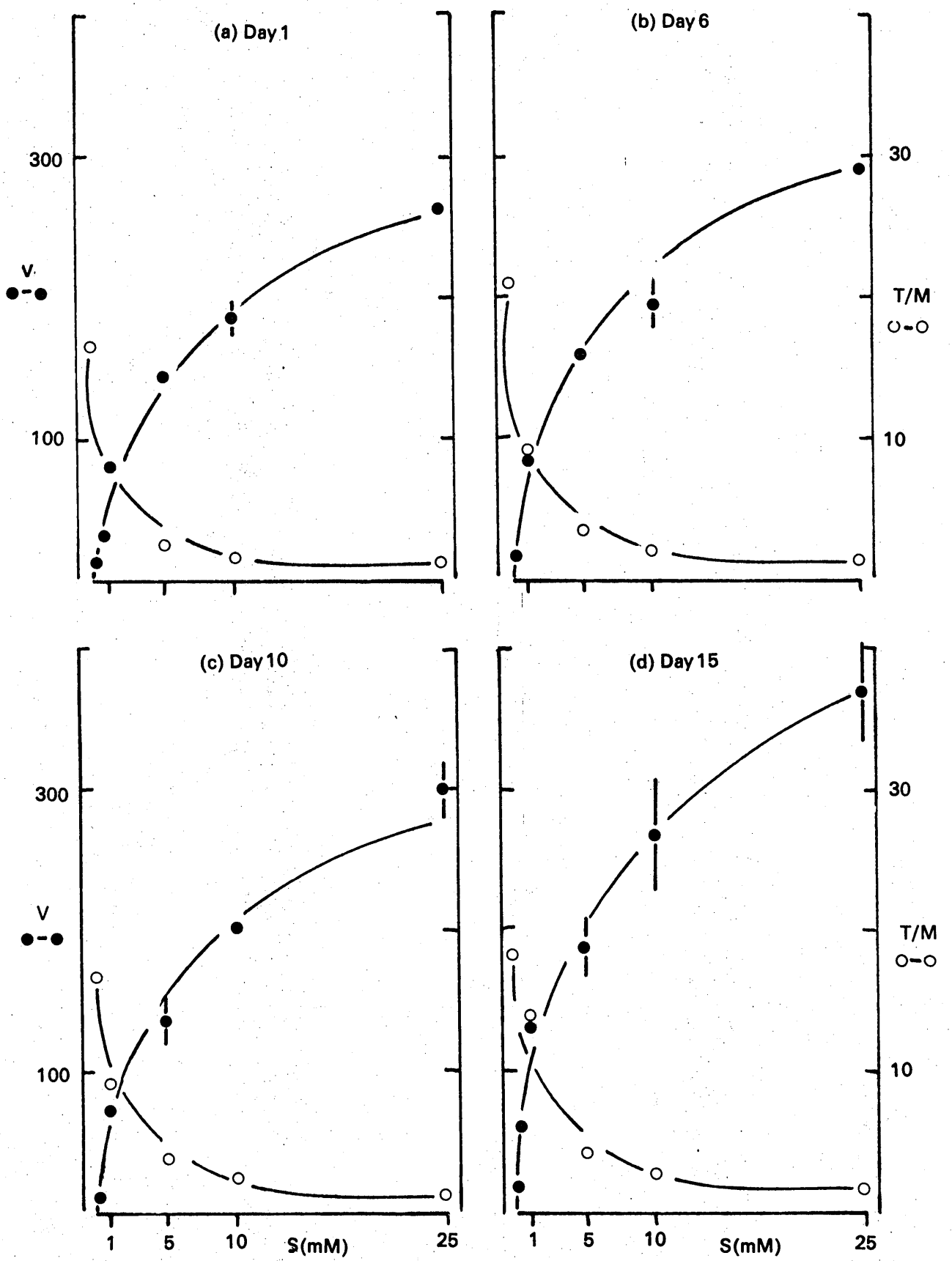
This neutral amino acid is again accumulated to a high extent from birth (Figure 5.9a). The extent of uptake decreases in the third postnatal week as seen in the T/M ratio (Figure 5.9e). Vmax peaks around day 11 (Figure 5.10) and then declines post-weaning. Similarly, K_m shows a marked increase (lower affinity) after weaning.

Reviewing the developmental changes in Vmax and K_m of all four neutral amino acids, the trends are very similar for all substrates. Vmax, or the number of carrier sites available to the substrate, increases up to 14 days of age and then apparently declines markedly around the time of weaning. As will be discussed later, this decline in Vmax could well be artefactual, simply a result of the expression of accumulation. On the other hand, K_m (carrier affinity) is relatively similar up to weaning and then increases (i.e. decreasing affinity). The actual values of Vmax for each neutral amino acid all lie in the same range at each age point and similarly K_m values are around 1mM at birth (with the exception of L-phenylalanine) and 3.5mM post weaning. The marked similarities

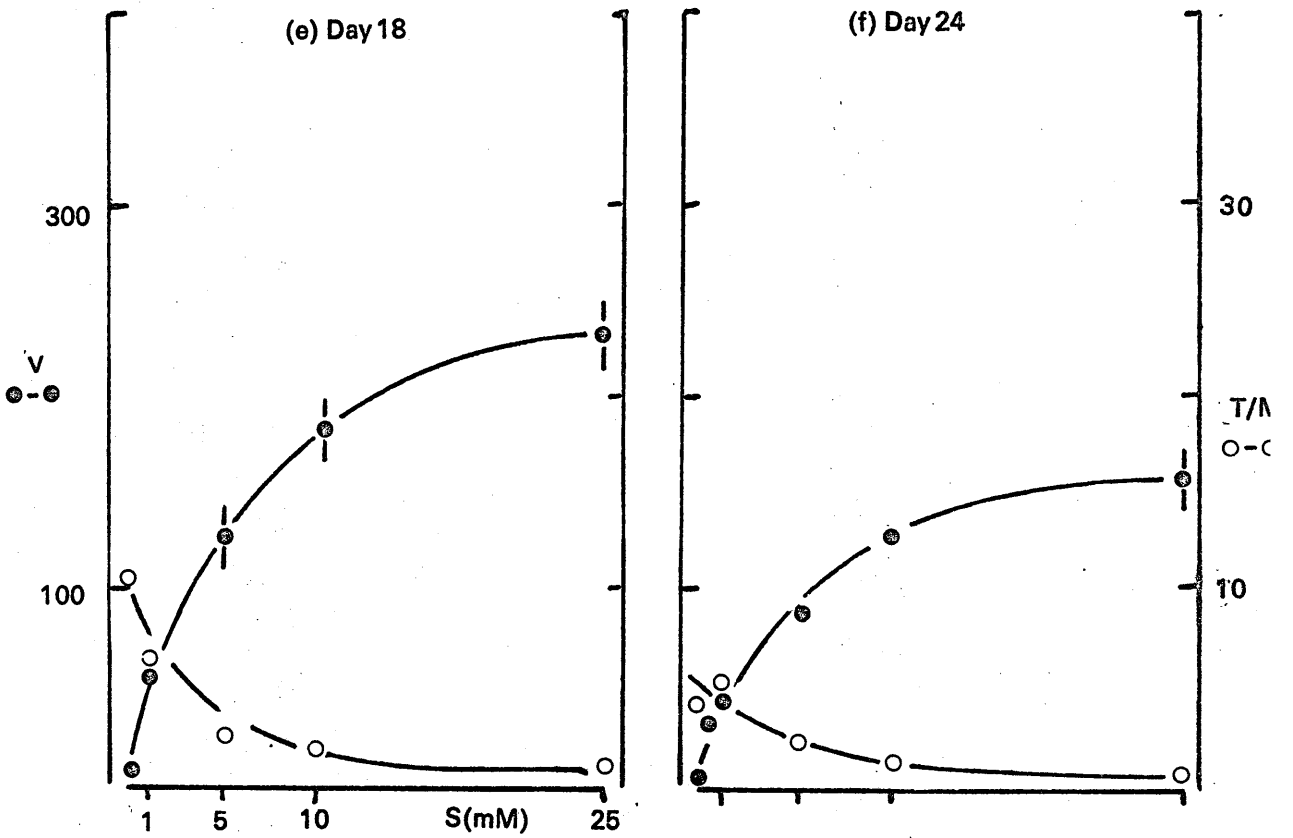
Figure 5.3 Kinetics of L-leucine uptake in everted jejunal segments from rats of various ages.

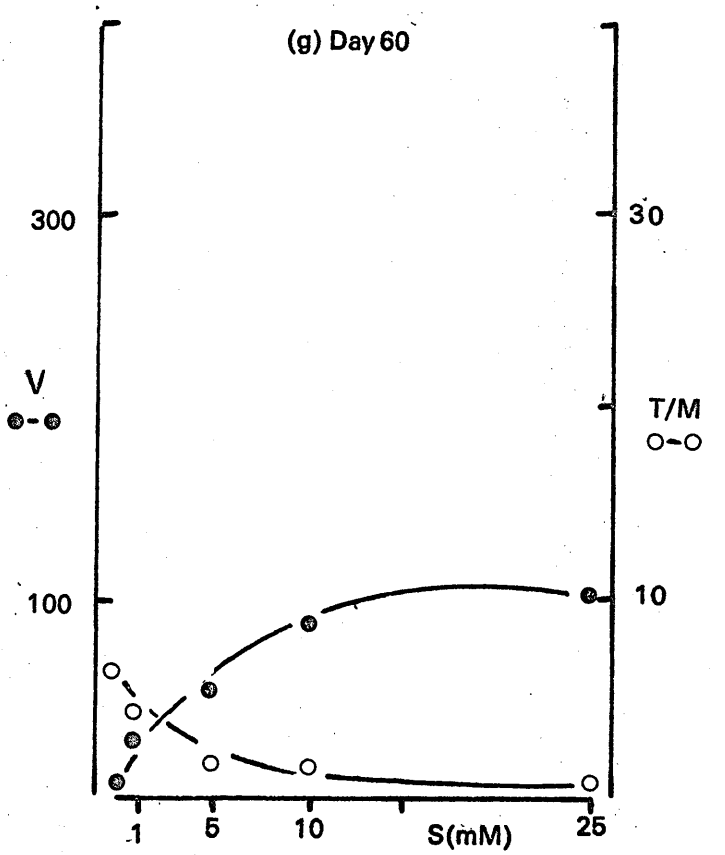
V = nanomoles substrate accumulated/ mg. protein/15 min. T/M = concentrative uptake. Bars indicate standard errors of the means of 4 observations at each point, where these extend beyond the symbols.

L–LEU



L-LEU


(e) Day 18
(f) Day 24
300
100
V
30
10
T/M
1
5
10
S(mM)
25


(g) Day 60
300
100
V
30
10
T/M
1
5
10
S(mM)
25

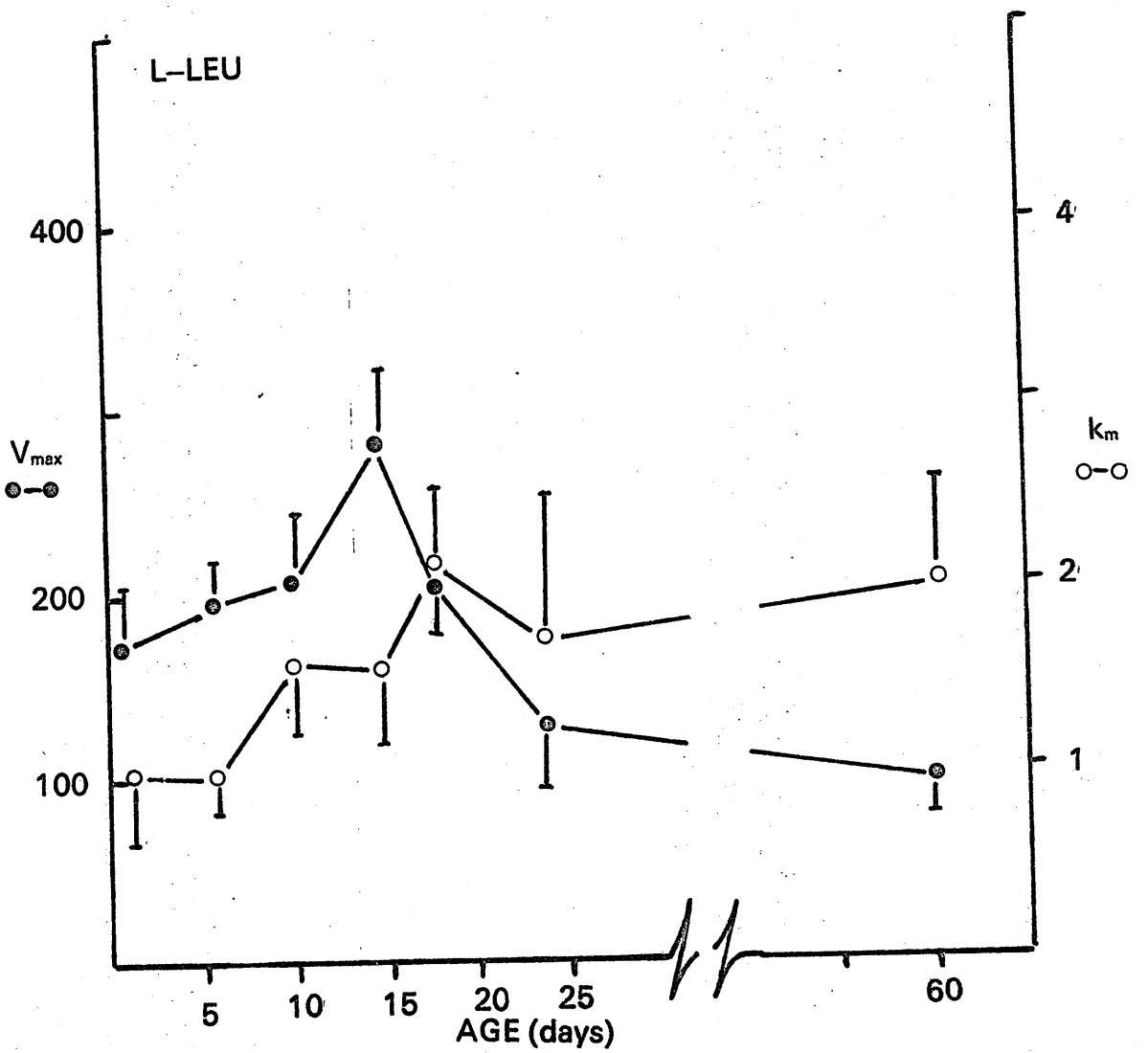


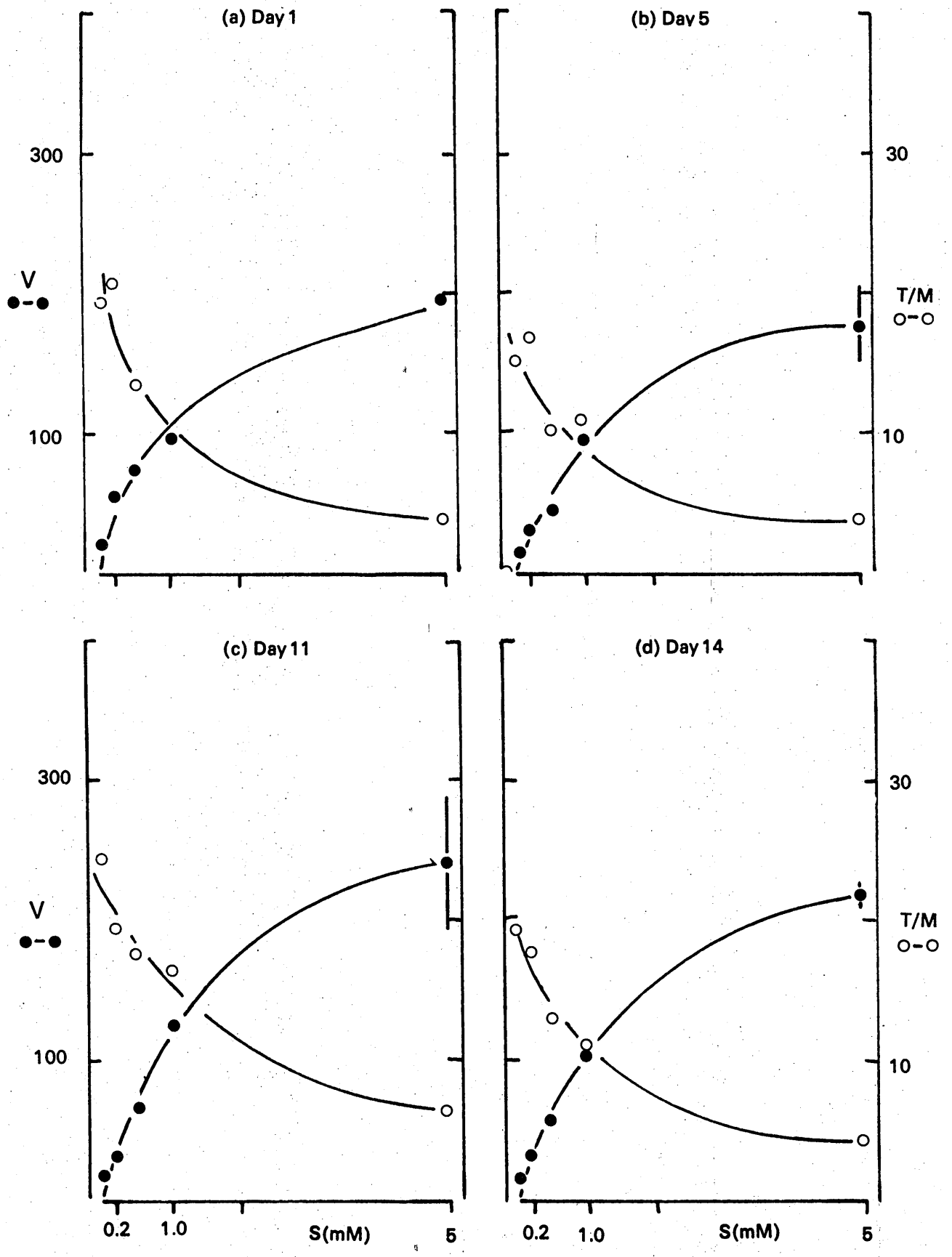
Figure 5.4 Vmax and K_m values for L-leucine uptake in the developing jejunum, calculated by linear regression of Hofstee plots from Figure 5.3.

Bars represent standard errors of the regression coefficients.

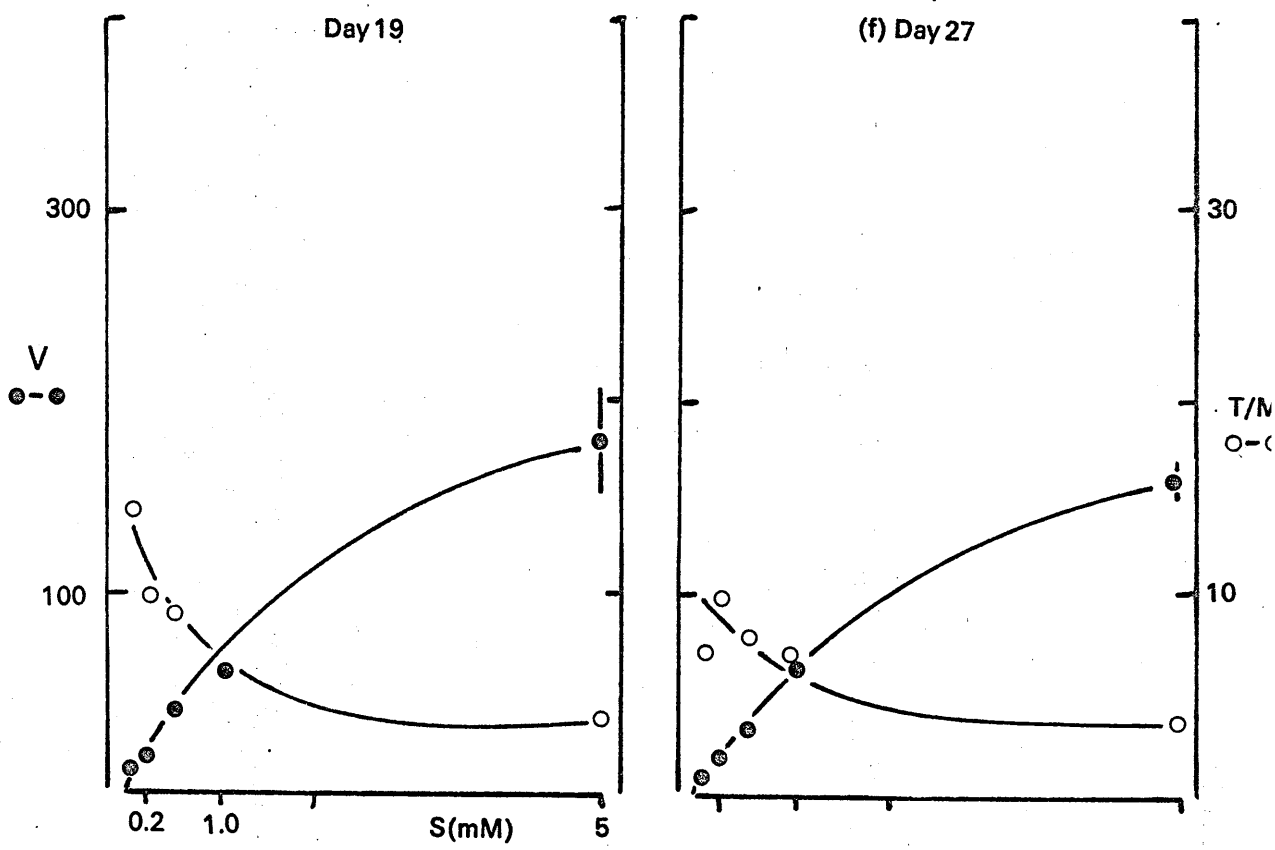
Figure 5.5 Kinetics of L-valine uptake in everted jejunal segments from rats of various ages.

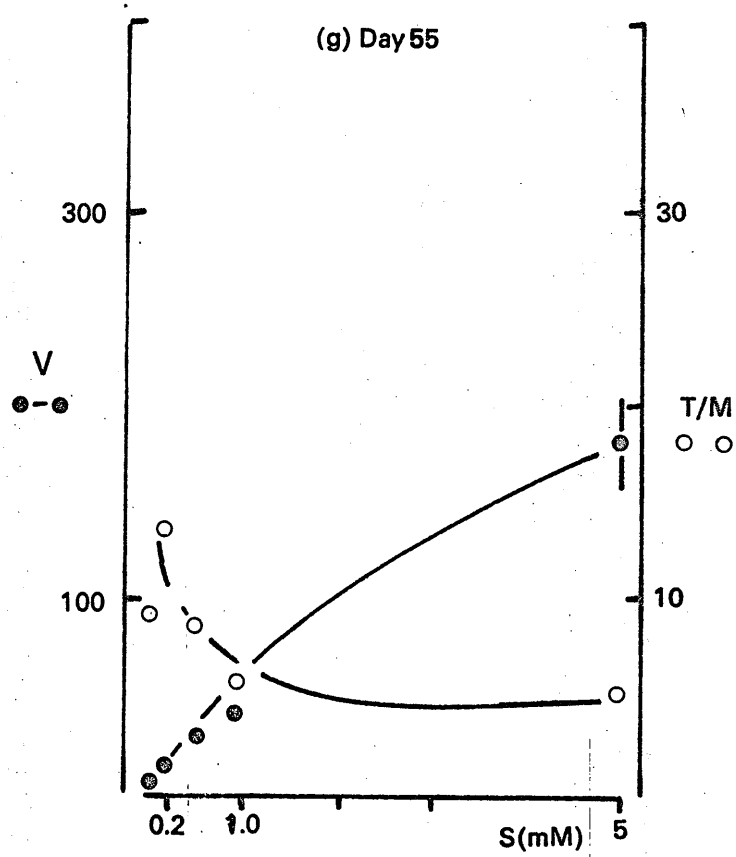
V = nanomoles substrate accumulated/ mg. protein/15 min. T/M = concentrative uptake. Bars indicate standard errors of the means of 4 observations at each point, where these extend beyond the symbols.

L–VAL



L-VAL


Day 19
300
V
100
0.2
1.0
S(mM)
5
(f) Day 27
30
T/M
10


(g) Day 55
300
V
100
0.2
1.0
S(mM)
5
30
T/M
10

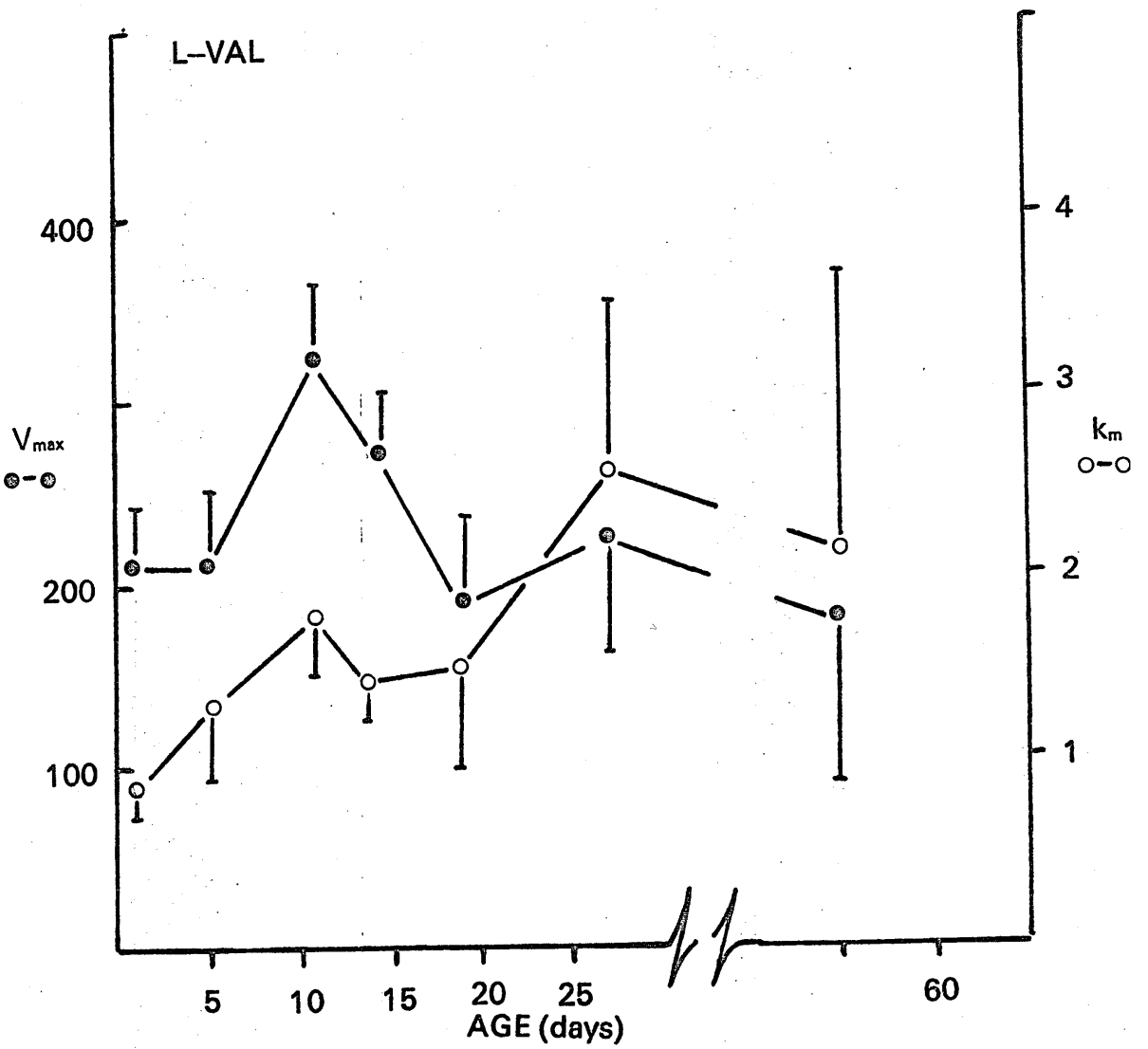


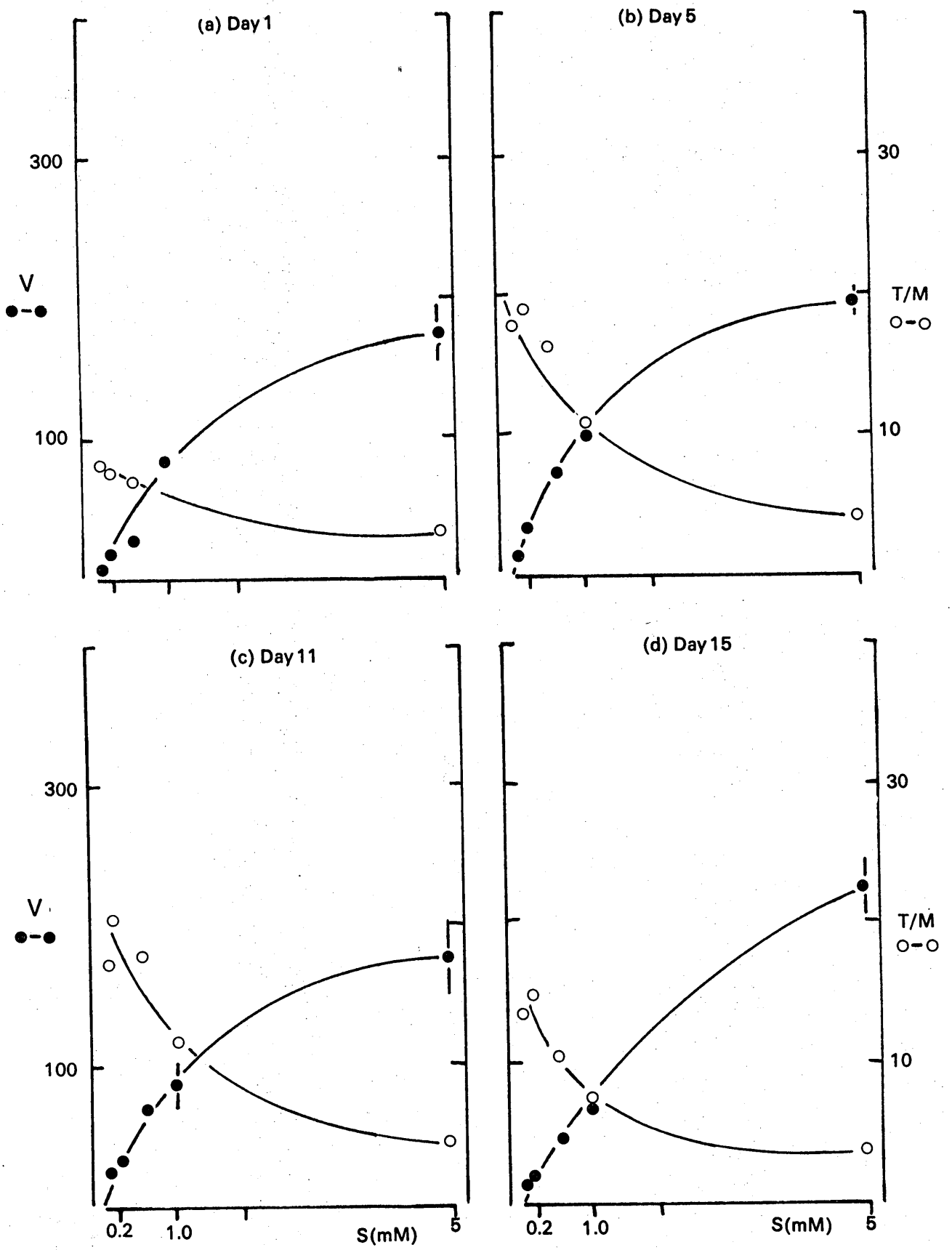
Figure 5.6 Vmax and K_m values for L-valine uptake in the developing jejunum, calculated by linear regression of Hofstee plots from Figure 5.5.

Bars represent standard errors of the regression coefficients.

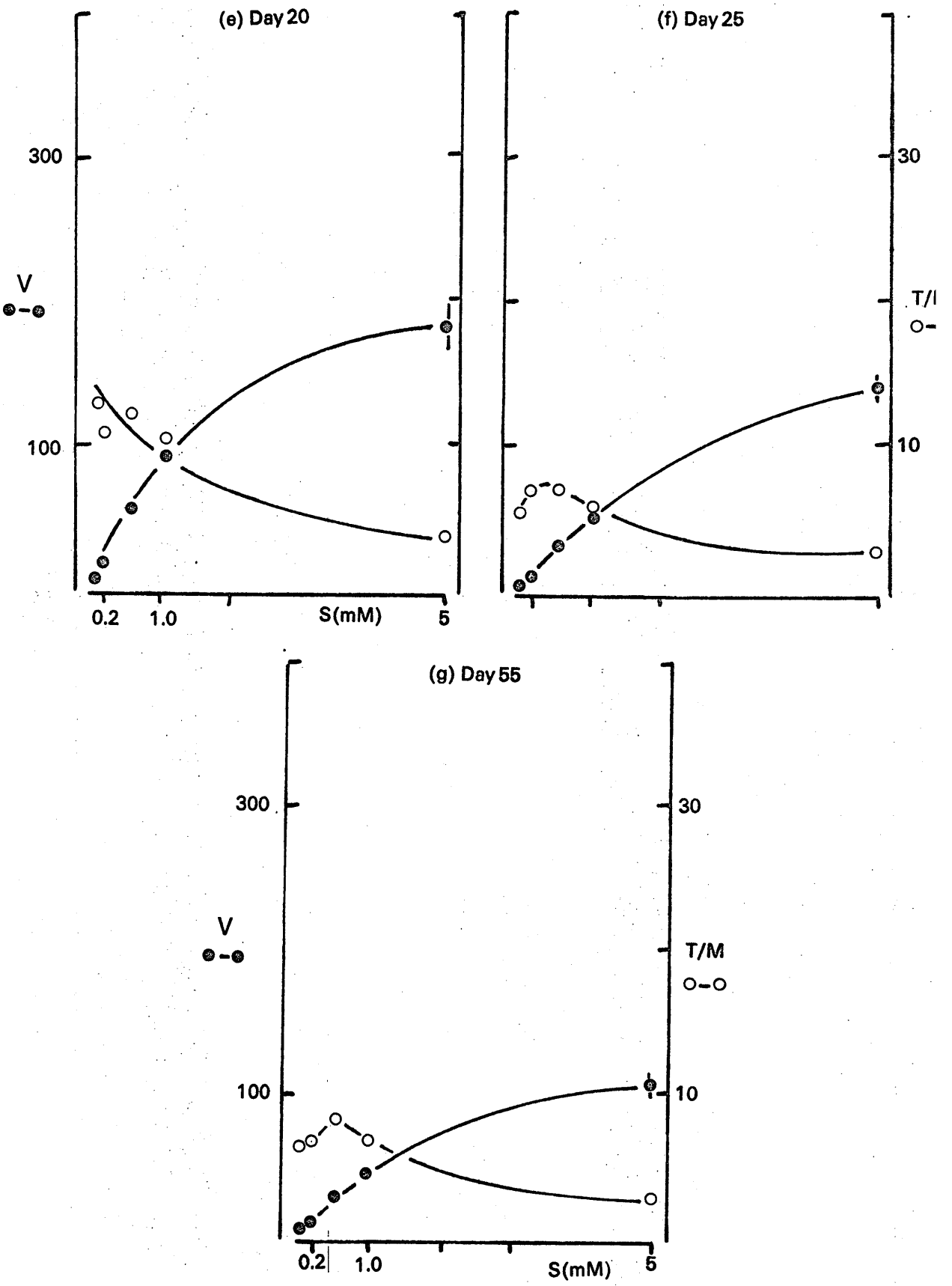
Figure 5.7 Kinetics of L-phenylalanine uptake in everted jejunal segments from rats of various ages.

V = nanomoles substrate accumulated/ mg. protein/15 min. T/M = concentrative uptake. Bars indicate standard errors of the means of 4 observations at each point, where these extend beyond the symbols.

L–PHE



L-PHE


(e) Day 20
300
V
100
0.2
1.0
S(mM)
5
(f) Day 25
30
T/
10
(g) Day 55
300
V
100
30
T/M
10
0.2
1.0
S(mM)
5

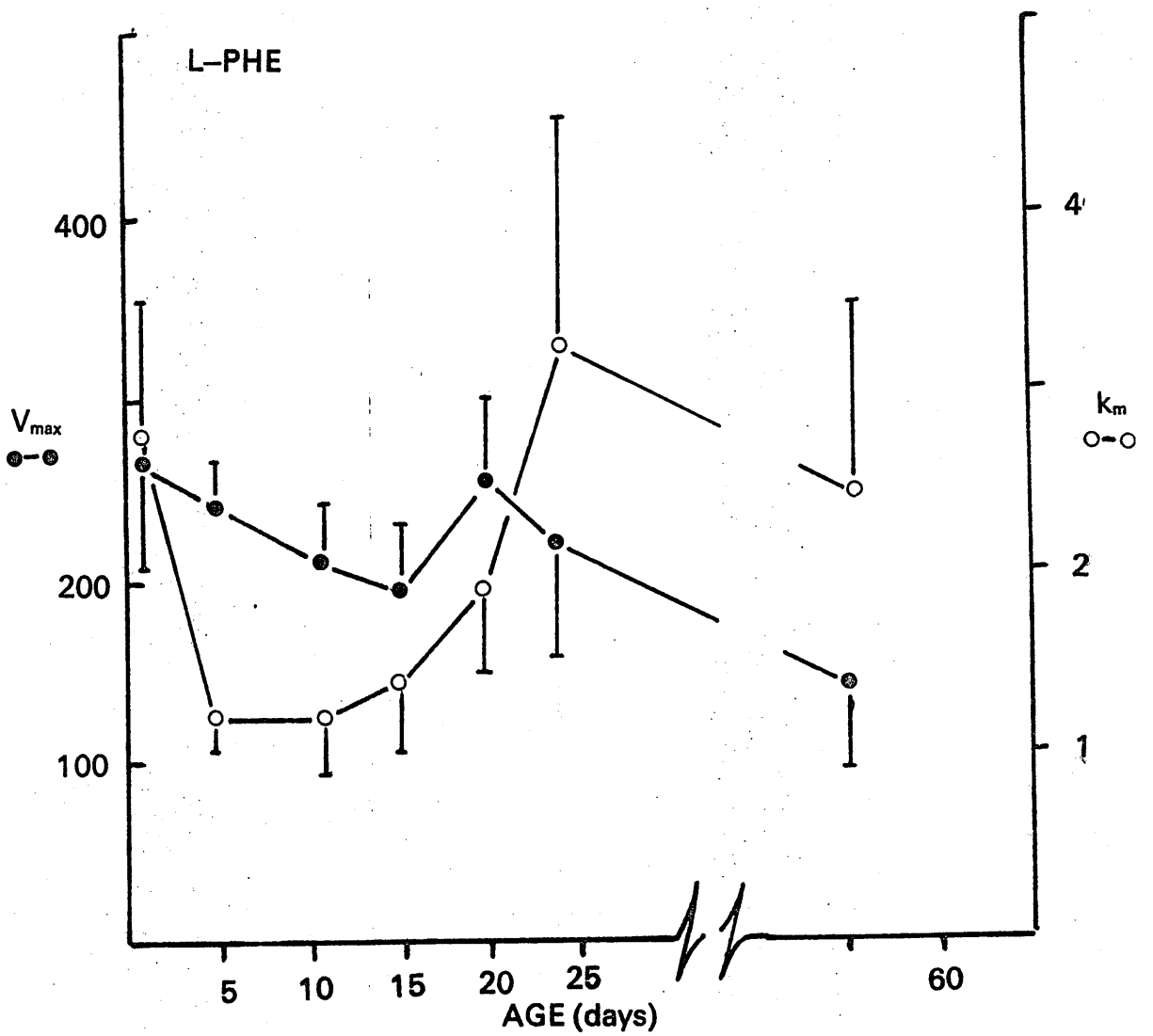


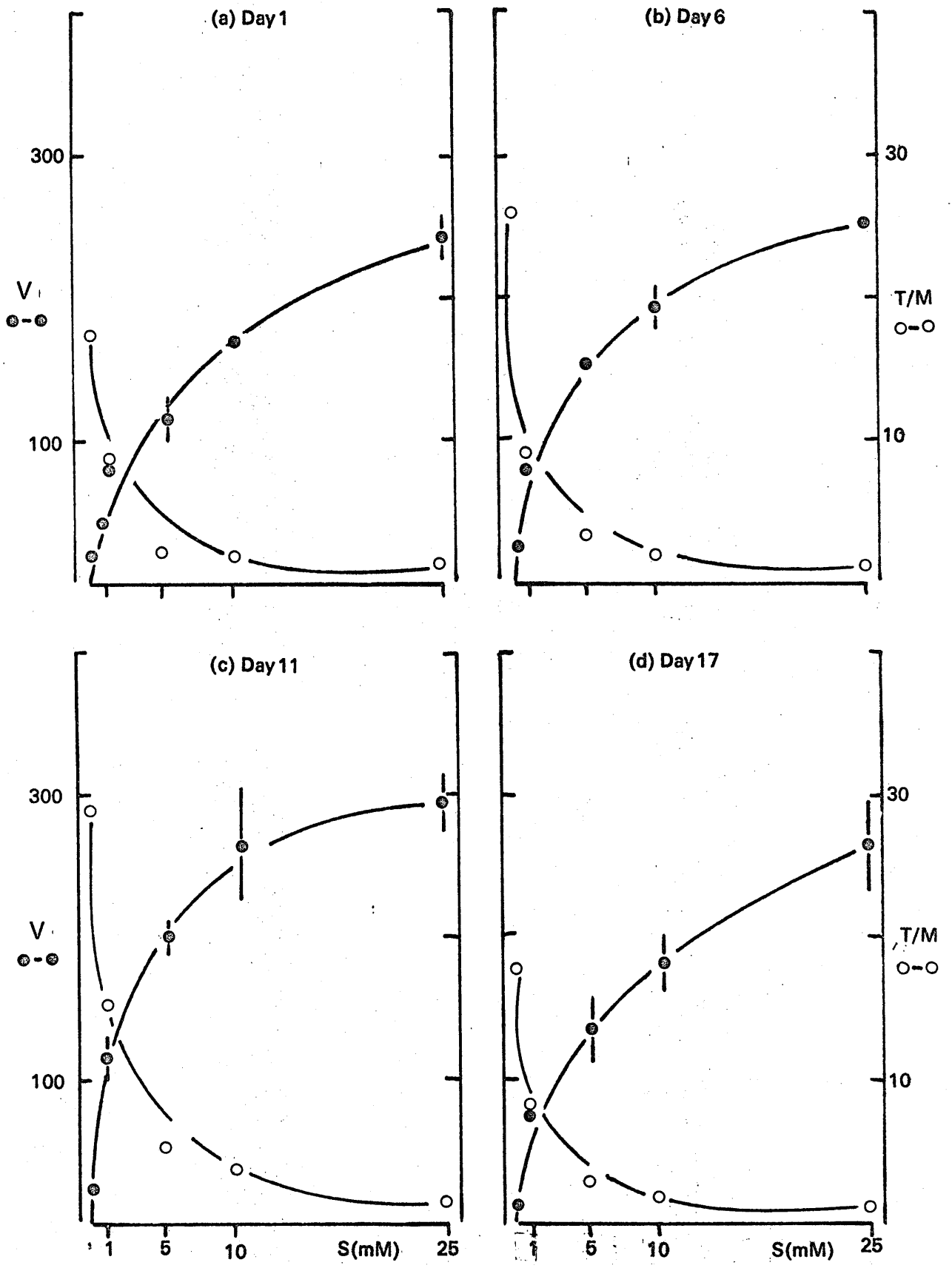
Figure 5.8 Vmax and K_m values for L-phenylalanine uptake in the developing jejunum, calculated by linear regression of Hofstee plots from Figure 5.7.

Bars represent standard errors of the regression coefficients.

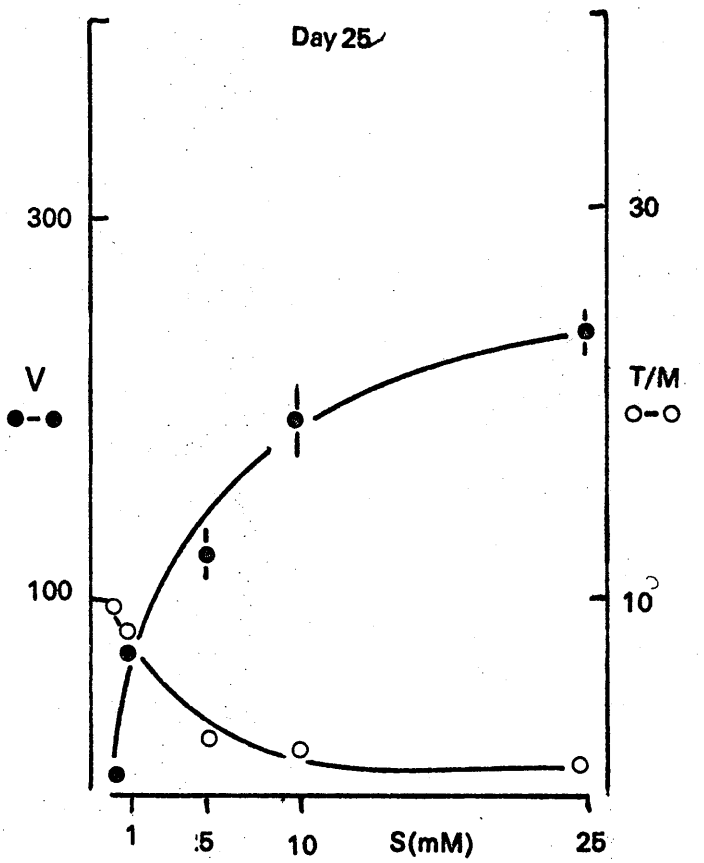
Figure 5.9 Kinetics of L-methionine uptake in everted jejunal segments from rats of various ages.

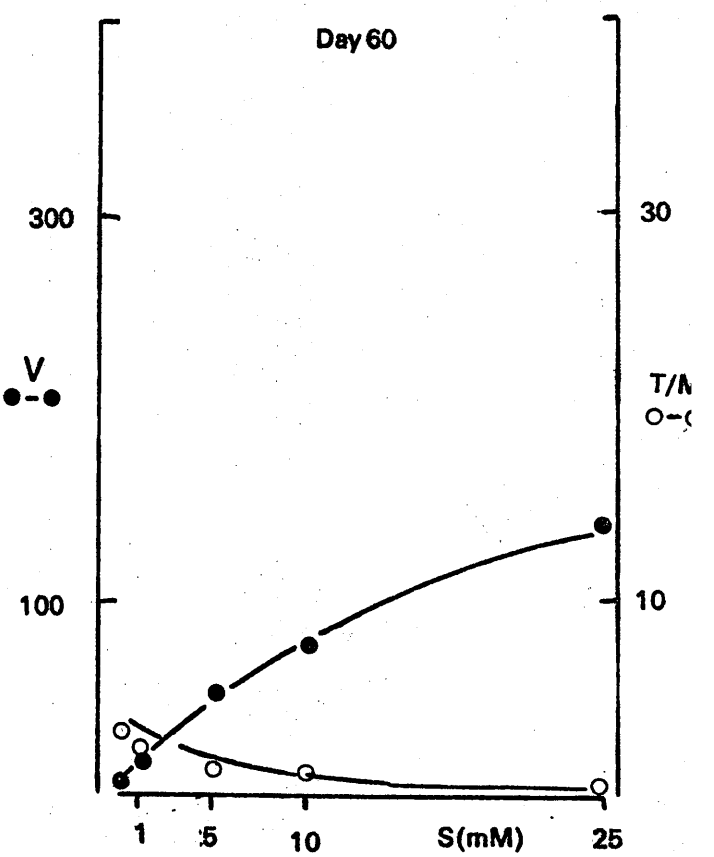
V = nanomoles substrate accumulated/ mg. protein/15 min. T/M = concentrative uptake. Bars indicate standard errors of the means of 4 observations at each point, where these extend beyond the symbols.

L–MET



L-MET


Day 25
300
100
V
30
T/M
10
1
5
10
S(mM)
25


Day 60
300
100
V
30
10
1
5
10
S(mM)
25

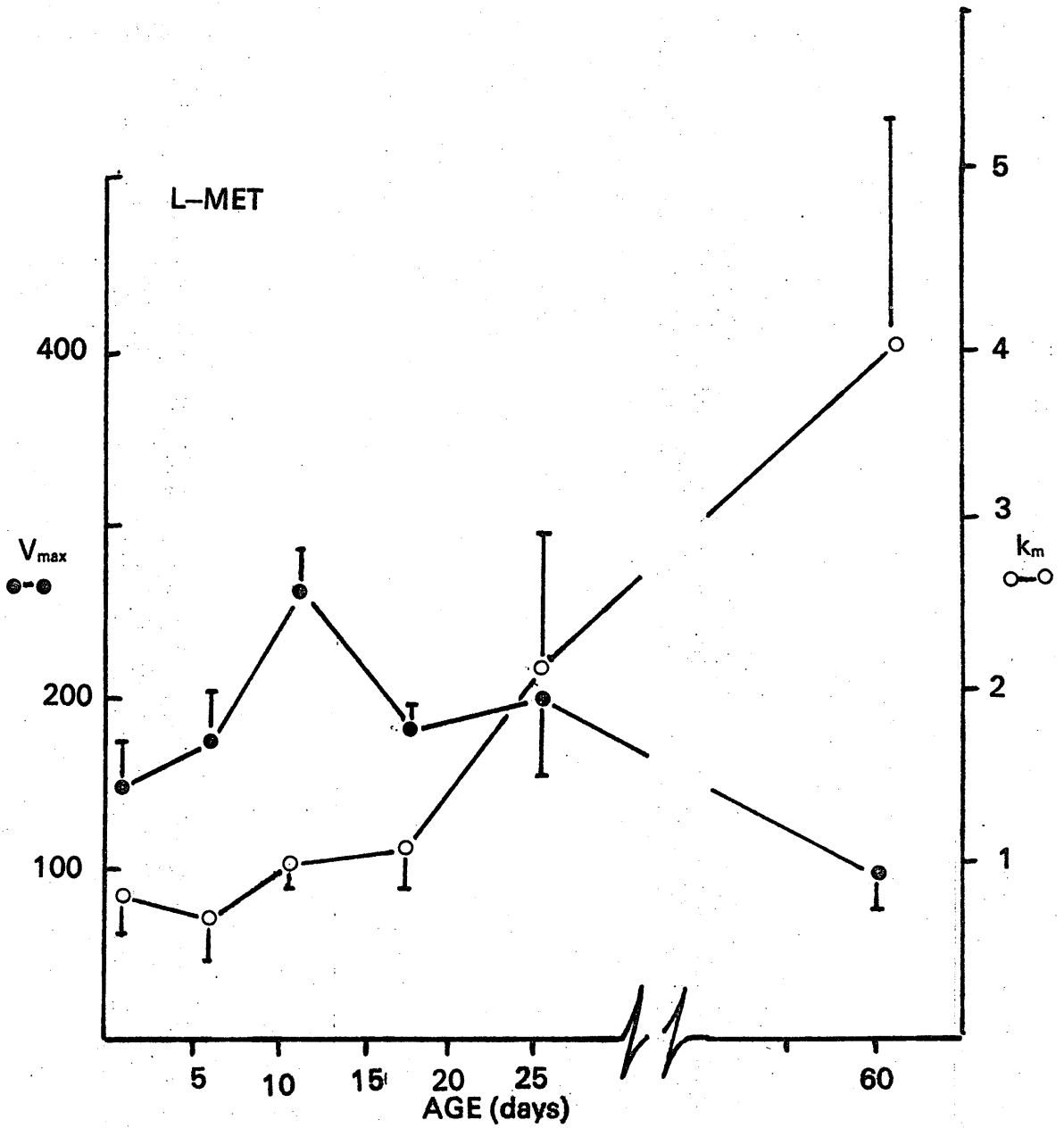


Figure 5.10 Vmax and K_m values for L-methionine uptake in the developing jejunum, calculated by linear regression of Hofstee plots from Figure 5.9.

Bars represent standard errors of the regression coefficients.

in the developmental profiles of kinetic constants for the two substrates L-leucine and L-valine has already been mentioned. Both are neutral amino acids with aliphatic side chains and would be likely to share similar carrier sites on the villi. However, L-methionine and L-phenylalanine exhibit different profiles in development, which suggests distinct carriers or perhaps overlapping sites. Carrier specificity is discussed at length in Chapter 6.

Basic amino acids

i) L-lysine (Figures 5.11 and 5.12)

The jejunum concentrates this basic amino acid from birth (Figure 5.11a) and the extent of uptake increases with age. After weaning the T/M ratio declines considerably and the curve of uptake is much flatter. Figure 5.12 reveals the apparent decrease in Vmax at the time of weaning. Similarly, there is a trend of increase in K_m after weaning.

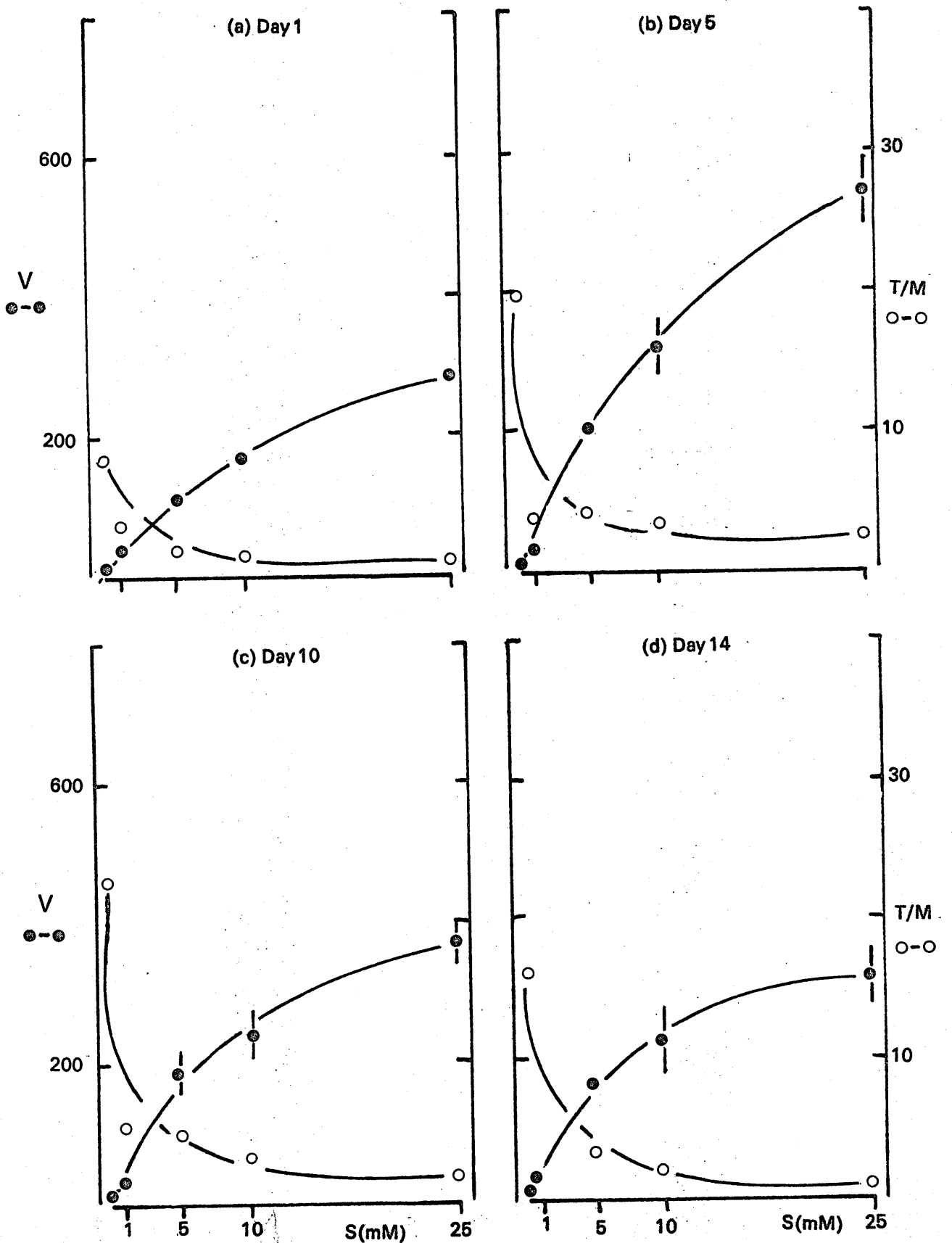
ii) L-arginine (Figures 5.13 and 5.14)

Compared with L-lysine, the jejunum accumulates L-arginine to a much lesser extent. T/M ratios in Figure 5.13 show that although the neonatal intestine accumulates L-arginine, the tissue concentration never exceeds 5mM even at peak transporting age (Figure 5.13c). The kinetics of accumulation show little variation with age, the Vmax is constant around 50 nanomoles L-arginine/mg protein/15 min and there is no sharp post-weaning decrease. However, K_m values do show a change with age, increasing in the third postnatal week.

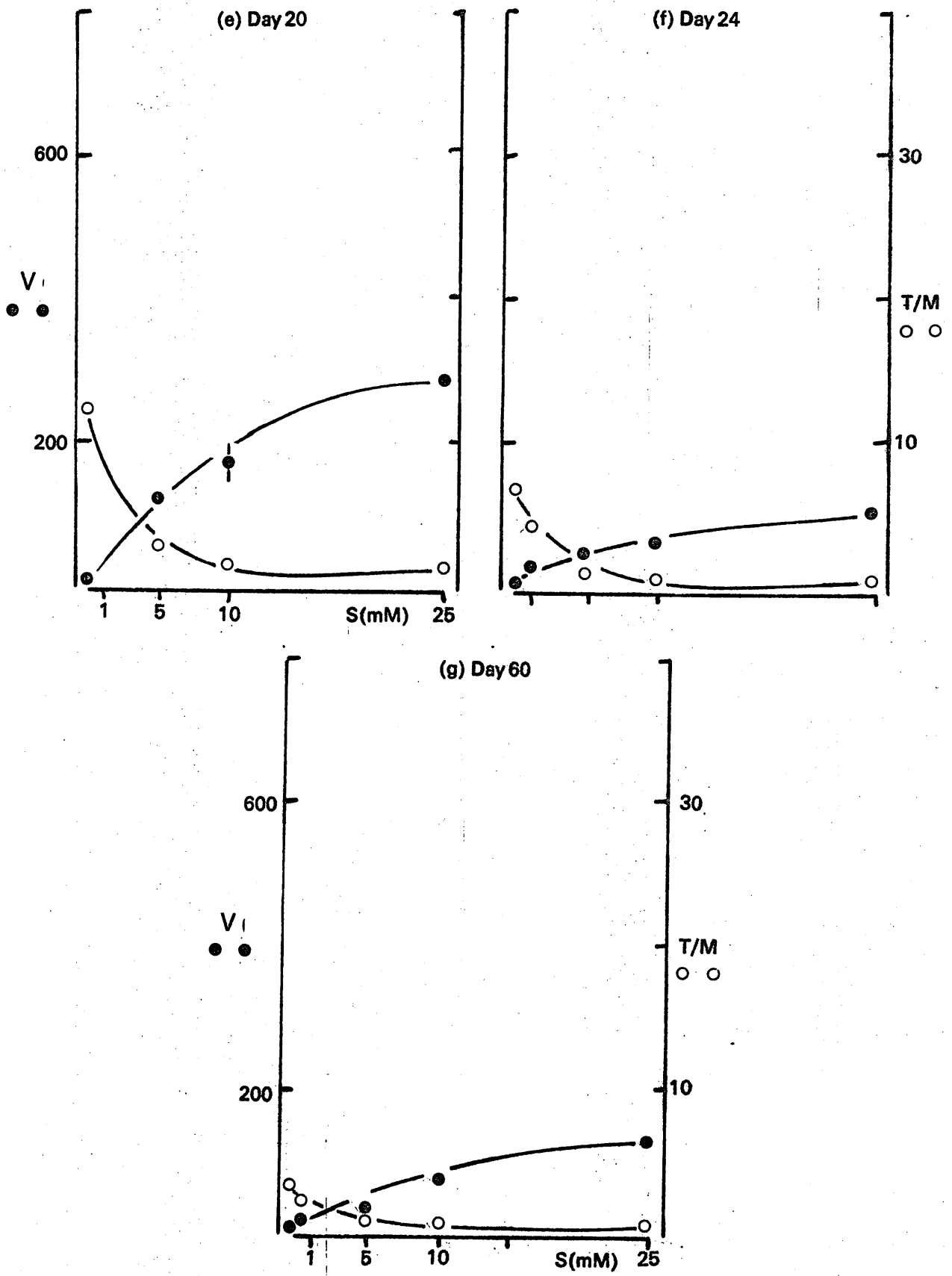
Figure 5.11 Kinetics of L-lysine uptake in everted jejunal segments from rats of various ages.

V = nanomoles substrate accumulated/ mg. protein/15 min. T/M = concentrative uptake. Bars indicate standard errors of the means of 8 observations at each point, where these extend beyond the symbols.

L–LYS



L–LYS



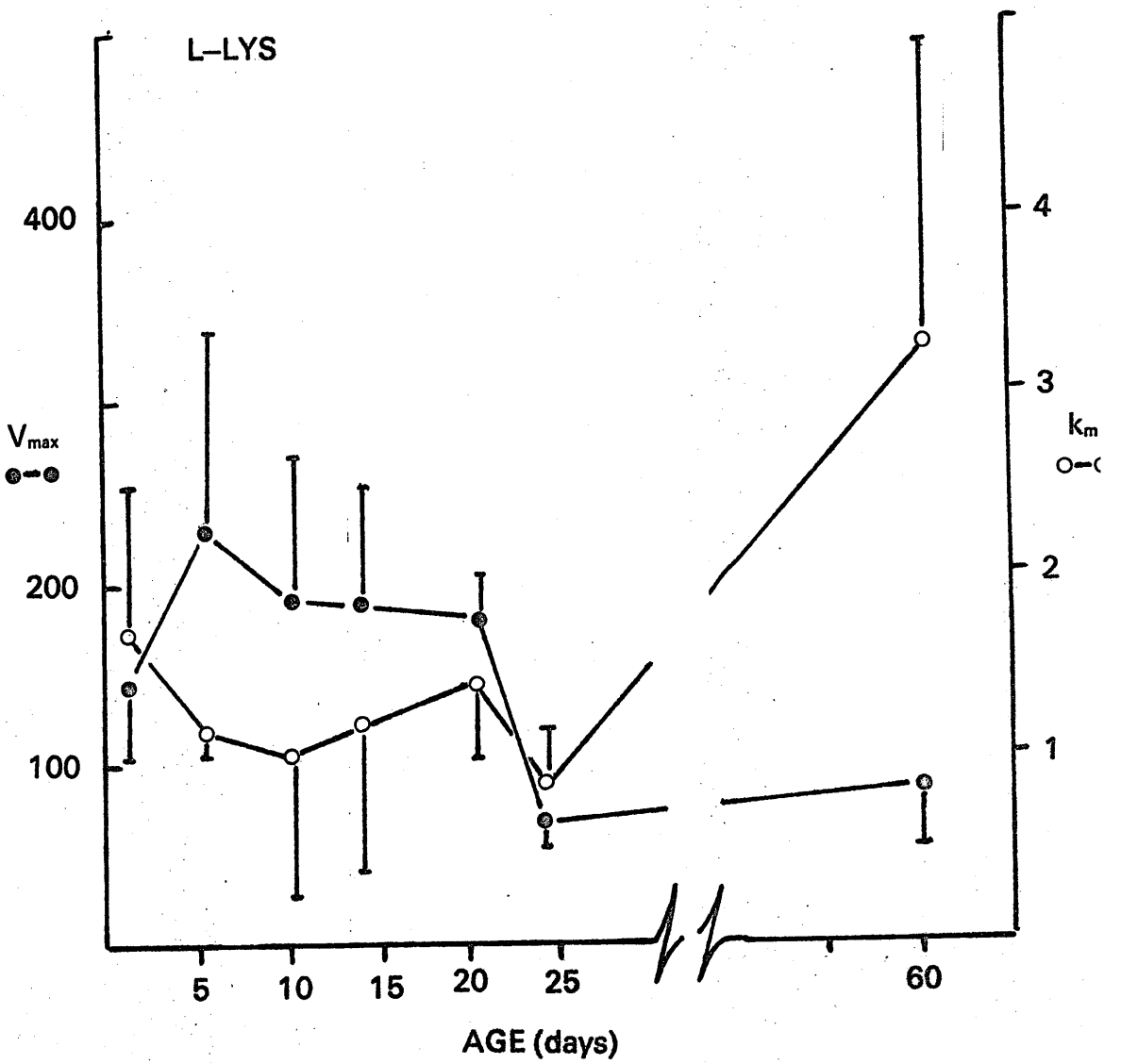


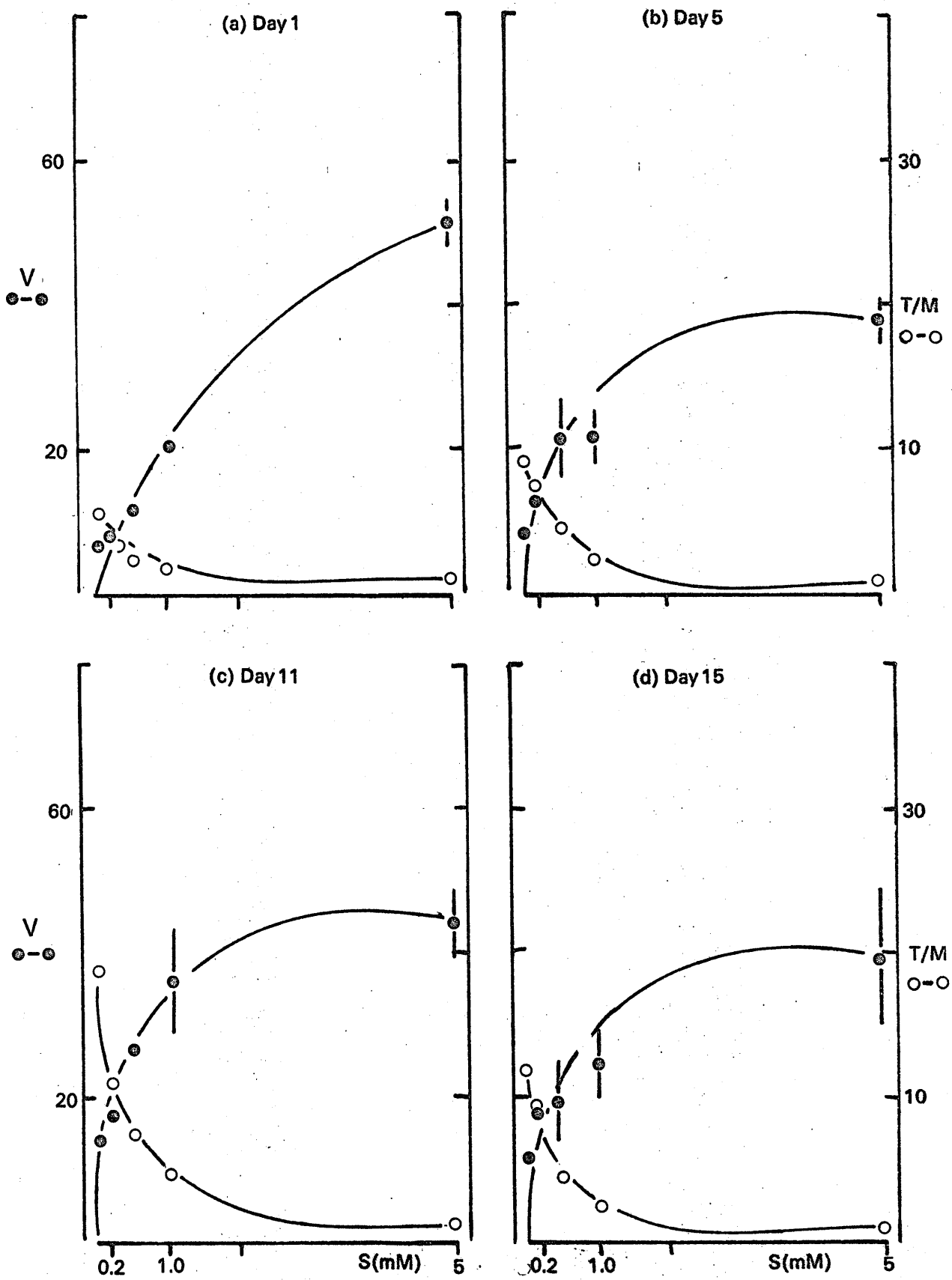
Figure 5.12 Vmax and K_m values for L-lysine uptake in the developing jejunum, calculated by linear regression of Hofstee plots from Figure 5.11.

Bars represent standard errors of the regression coefficients.

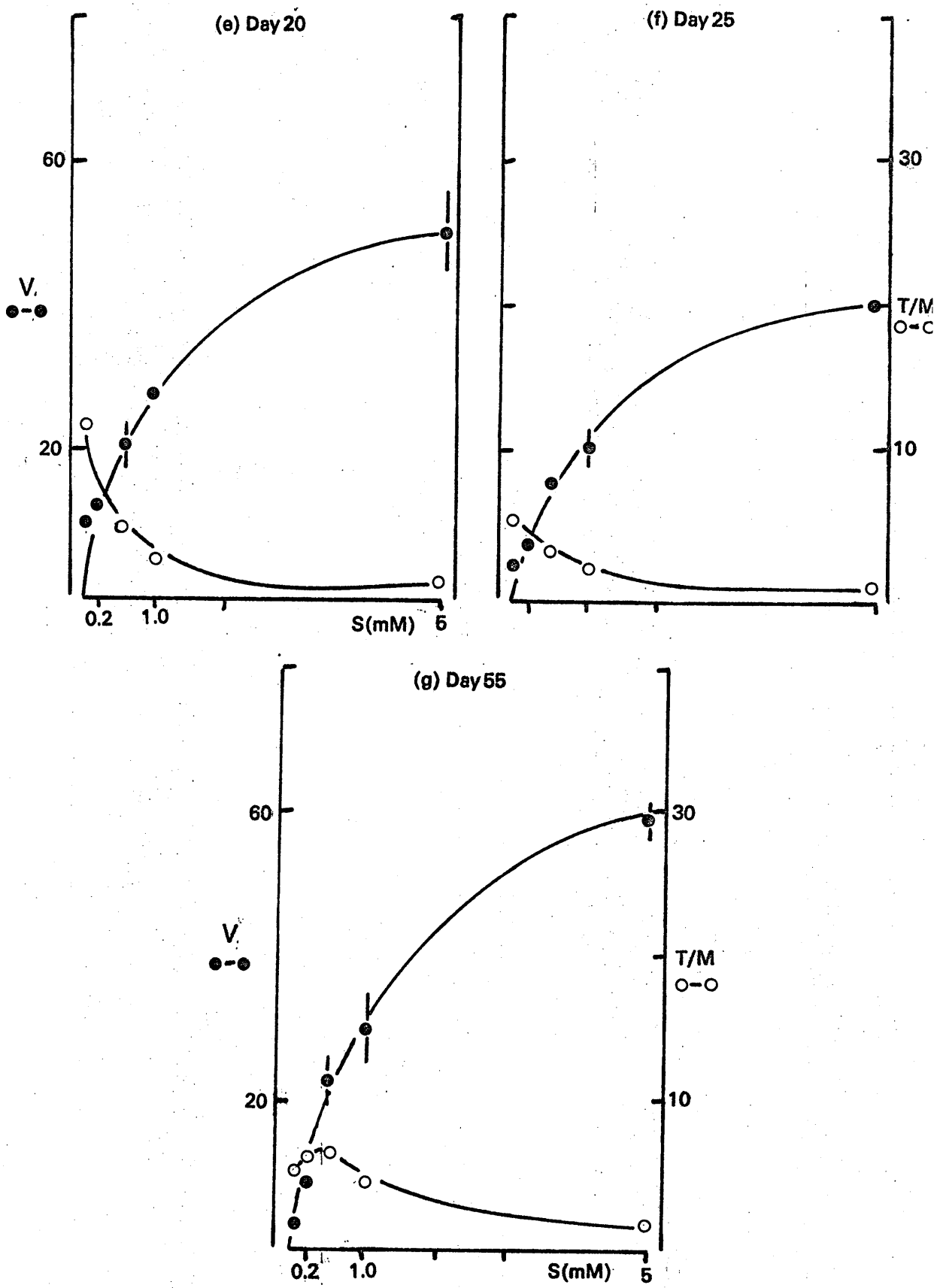
Figure 5.13 Kinetics of L-arginine uptake in everted jejunal segments from rats of various ages.

V = nanomoles substrate accumulated/ mg. protein/15 min. T/M = concentrative uptake. Bars indicate standard errors of the means of 4 observations at each point, where these extend beyong the symbols.

L–ARG



L-ARG



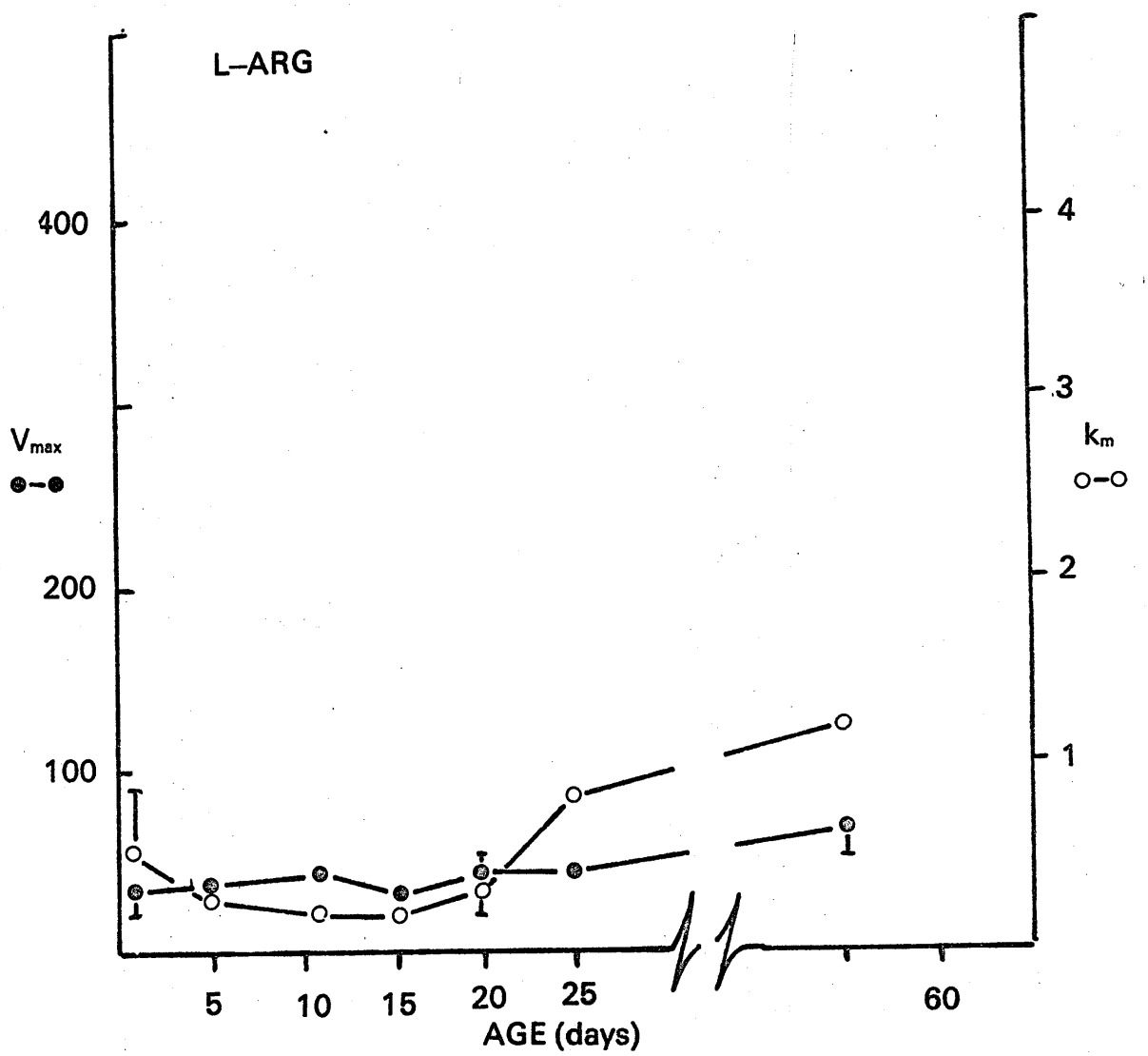


Figure 5.14 Vmax and K_m values for L-arginine uptake in the developing jejunum, calculated by linear regression of Hofstee plots from Figure 5.13.

Bars represent standard errors of the regression coefficients.

The differences in kinetic constants profiles for the two basic amino acids during postnatal development is somewhat surprising. The very low Vmax values for L-arginine suggest that carriers for this substrate are fewer in number than for L-lysine perhaps because L-lysine has neutral sites available to it for transport (see Chapter 6). Similarly, the K_m for L-arginine uptake is very low throughout development (0.5mM) and at 60 days of age the K_m still indicates high affinity absorption (1.0mM) compared with L-lysine (3.0mM). The only similarity between the two basic amino acids is a higher K_m after weaning.

A comparison of the three kinetic plots

Bearing in mind the cautionary conclusions of Dowd and Riggs (1965), the data plots were subjected to all three linear transformations and K_m and Vmax values were calculated from each (see Table 5.2). From the coefficient of determination (r^2) it is evident that constructing a straight line by either the Hanes plot (S against S/V) or the Lineweaver-Burk double reciprocal method (1/V against 1/S) is much easier than with the Hofstee plot (V/S against V). The latter emphasises points off the straight line and so r^2 values ranged widely, though the majority were 0.80 or better. This produced in turn large standard errors on some means. Nevertheless the Hofstee plot is considered more appropriate on data where V is likely to be variable, being the factor under determination, whereas S is controlled. The double reciprocal plot produces consistently low values of both Vmax and K_m, whereas the Hanes plot produces high values, both compared with values from the Hofstee method which lie somewhere between them. Despite these differences, the trends in Vmax and K_m for absorption of these neutral and basic amino acids in

TABLE 5.2 Kinetic constants derived from linear transformation of data plots by the three methods described in the text for comparison of Vmax and Km. r^2 = coefficient of determination (r^2 = 1.0 = a perfect fit).

L-leu / Day		1			6			10			15		
		Vmax	Km	r^2	Vmax	Km	r^2	Vmax	Km	r^2	Vmax	Km	r^2
v	v/s	170.13 ± 32.38	1.05 ± 0.36	0.74	195.89 ± 19.64	0.98 ± 0.18	0.93	208.12 ± 28.89	1.63 ± 0.39	0.90	278.12 ± 40.26	1.50 ± 0.36	0.85
1/v	1/s	111.11	0.59	0.93	181.82	0.89	0.99	181.82	1.35	0.99	277.78	1.82	0.99
s	s/v	217.39	1.94	0.98	222.22	1.42	0.99	243.90	2.46	0.96	303.03	1.94	0.97
L-val / Day		1			5			11			14		
v	v/s	212.01 ± 28.89	0.89 ± 0.19	0.89	212.12 ± 40.29	1.33 ± 0.36	0.82	325.69 ± 42.98	1.81 ± 0.32	0.91	272.15 ± 29.75	1.49 ± 0.22	0.94
1/v	1/s	204.08	0.88	0.99	344.83	2.72	0.97	227.27	1.20	0.99	196.08	1.06	0.99
s	s/v	250.00	1.23	0.99	238.10	1.62	0.98	333.33	1.90	0.99	303.3	1.79	0.99
L-phe / Day		1			5			11			15		
v	v/s	267.87 ± 58.97	2.81 ± 0.78	0.81	240.24 ± 24.52	1.26 ± 0.18	0.94	212.15 ± 34.65	1.26 ± 0.30	0.86	196.86 ± 35.04	1.41 ± 0.35	0.84
1/v	1/s	333.3	3.8	0.99	312.5	1.78	0.99	200	0.71	0.96	185.19	1.35	0.99
s	s/v	277.78	2.97	0.97	250	1.35	0.99	232.56	1.58	0.97	238.10	1.95	0.97
L-met / Day		1			6			11			17		
v	v/s	149.68 ± 25.06	0.85 ± 0.26	0.78	178.54 ± 27.99	0.71 ± 0.22	0.84	268.42 ± 22.63	1.19 ± 0.18	0.96	183.10 ± 17.38	1.14 ± 0.20	0.94
1/v	1/s	105.26	0.57	0.94	151.52	1.78	0.99	238.10	0.95	0.99	166.67	0.88	0.99
s	s/v	188.68	1.64	0.96	217.39	1.48	0.98	303.03	1.73	0.99	208.33	1.71	0.98
L-arg / Day		1			5			11			15		
v	v/s	37.5 ± 13.22	0.55 ± 0.35	0.46	35.98 ± 3.70	0.34 ± 0.07	0.89	43.32 ± 4.06	0.24 ± 0.05	0.84	33.10 ± 4.33	0.19 ± 0.06	0.75
1/v	1/s	20.96	0.25	0.71	30.12	0.31	0.97	37.88	1.24	0.94	30.58	0.30	0.95
s	s/v	62.5	1.24	0.83	41.67	0.55	0.99	48.08	0.37	0.99	41.67	0.49	0.98
L-lys / Day		1			5			10			14		
v	v/s	146.94 ± 38.43	1.79 ± 0.75	0.65	237.23 ± 107.33	1.22 ± 0.10	0.38	194.94 ± 74.04	1.08 ± 0.81	0.47	195.31 ± 71.63	1.27 ± 0.84	0.53
1/v	1/s	90.09	1.0	0.99	112.36	0.63	0.92	88.5	0.51	0.89	86.96	0.62	0.90
s	s/v	222.22	3.98	0.91	588.24	8.65	0.49	400	6.44	0.59	312.5	3.84	0.90

L-leu \ Day		18			24			60		
		Vmax	Km	r^2	Vmax	Km	r^2	Vmax	Km	r^2
v	v/s	201.96 ± 24.47	2.10 ± 0.42	0.93	127.04 ± 34.74	1.70 ± 0.80	0.60	90.93 ± 15.46	2.03 ± 0.56	0.87
1/v	1/s	172.41	1.75	0.99	100	1.0	0.99	88.50	2.33	0.99
s	s/v	232.56	2.88	0.98	163.93	2.95	0.96	109.89	3.14	0.93
L-val \ Day		19			27			55		
v	v/s	193.52 ± 51.92	1.56 ± 0.58	0.71	229.93 ± 62.92	2.6 ± 0.92	0.73	186.25 ± 91.47	2.19 ± 1.47	0.43
1/v	1/s	87.72	0.68	0.85	100	1.0	0.94	63.69	2.5	0.96
s	s/v	277.78	2.72	0.92	294.12	3.62	0.94	400	6.12	0.65
L-phe \ Day		20			25			55		
v	v/s	257.16 ± 45.2	1.96 ± 0.46	0.86	219.32 ± 65.8	3.30 ± 1.24	0.70	149.13 ± 49.14	2.44 ± 1.06	0.64
1/v	1/s	285.71	2.43	0.99	200	1.67	0.98	100	2.0	0.99
s	s/v	263.16	2.05	0.99	277.78	4.53	0.91	178.57	3.18	0.94
L-met \ Day					25			60		
v	v/s				199.69 ± 44.78	2.17 ± 0.76	0.73	99.87 ± 22.32	4.05 ± 1.34	0.82
1/v	1/s				149.25	1.70	0.99	66.23	2.47	0.99
s	s/v				250	3.43	0.94	126.58	6.06	0.94
L-arg \ Day		20			25			55		
v	v/s	43.94 ± 8.16	0.36 ± 0.13	0.71	45.41 ± 3.48	0.85 ± 0.10	0.96	67.82 ± 18.32	1.26 ± 0.02	0.68
1/v	1/s	34.48	0.25	0.86	41.32	0.76	0.99	50	1.0	0.97
s	s/v	58.82	0.74	0.99	49.26	1.0	0.99	50	1.50	0.90
L-lys \ Day		20			24			60		
v	v/s	181.67 ± 25.15	1.49 ± 0.37	0.94	63.96 ± 13.22	0.93 ± 0.37	0.69	84.86 ± 27.56	3.3 ± 1.65	0.67
1/v	1/s	158.73	1.41	0.99	48.54	0.70	0.95	68.49	2.68	0.99
s	s/v	204.08	2.2	0.97	77.52	1.62	0,97	116.28	6.06	0.70

the developing gut, which have been drawn from the Hofstee plots, are also mirrored in the calculations derived from the other two methods (Table 5.2).

The effect of non-absorptive muscle tissue on kinetic plots

From Figure 4.14 it is evident that the serosal layers double in thickness over the period of the third and fourth postnatal weeks. However, the proportion of substrate in this muscle "sink" remains the same over weaning. The implication of the serosal thickening is that a larger proportion of measurable protein is non-absorptive post-weaning than pre-weaning. This problem in developmental studies is inescapable but can be side-stepped for kinetic purposes.

It is clear that in the jejunum prior to weaning, for every mg. protein measured, 60% represents mucosal protein. After weaning, only 35% represents absorptive tissue protein (see Figure 4.14). Thus, to compare uptake in terms of nanomoles substrate/mg protein, values of V after 25 days of age must be corrected by a factor of 1.7 for each substrate concentration. It is obvious that multiplying through by a constant will shift the data plot in a vertical direction (i.e. raise the Vmax) but will not change the rate of uptake with regard to increasing concentration. The K_m will be little affected. To prove this point, uptake data for two amino acids L-valine and L-lysine) after the time of weaning have been replotted taking into account this correction factor (see Table 5.3).

TABLE 5.3

Uptake data corrected for non-absorptive serosal protein increase after weaning and Vmax and K_m calculated from the new Hofstee plot.

	V (no correction)			V^1 (V replotted with correction per serosal protein)		
	Vmax	K_m	r^2	Vmax	K_m	r^2
27 day L-valine	229.93 ±62.92	2.61 ±0.92	0.73	391.35 ±107.07	2.61 ±0.92	0.73
24 day L-lysine	63.96 ±13.22	0.93 ±0.37	0.69	108.73 ±22.47	0.93 ±0.37	0.69

The increase in serosal mass at this period of development thus produces an artefactually abrupt decrease in Vmax when compared with values prior to the weaning age, whatever way V is expressed (wet weight, dry weight etc). However the calculated values of K_m are unaffected by this increase in non-absorptive serosal tissue.

The importance of this point will be raised again in the discussion that follows, where reported post-weaning changes in amino acid absorption are re-examined.

Discussion

The results described here indicate that the small intestine of the neonatal rat can accumulate amino acids against a concentration gradient from birth; in some cases to a very great extent (e.g. L-valine, L-leucine and L-lysine). Donnelly (1971) has described the active absorption of L-leucine but not L-lysine in foetal rat intestine and suggests this may be due to the differential development of neutral and basic amino acid transport systems. Deren, Strauss and Wilson (1965) found that intestinal rings from foetal rabbits were capable of accumulating L-valine, L-methionine and L-lysine, and the degree of accumulation increased with foetal development. In contrast, betaine was not actively accumulated until a later foetal stage. In the guinea pig, which is born in a very advanced state of development, the intestine can actively accumulate all the amino acids (with the exception of betaine) by 40 days of gestation, with a marked surge in transport capacity at birth (Rosenberg, 1966; Butt and Wilson, 1968; Wilson, 1971). Similarly, chick embryonic intestine can actively accumulate glycine 4 days before hatching (Holdsworth and Wilson, 1967; Hudson and Levin, 1968).

In the neonatal rat, proteolytic activity in the stomach and small intestine is low until weaning (Koldovsky, 1969), corresponding with the change in diet from milk (9% w/v of protein) to solid food (21% w/v protein). Consequently, the concentration of free amino acids in the gut lumen is low during the first two postnatal weeks, until the large scale ingestion of solid food around weaning, when luminal free amino acid levels would be expected to rise. The ability of the neonatal rat jejunum to actively accumulate essential amino acids increases with age and appears to peak around day 14. Kinetic analysis reveals that the K_m for both neutral and basic substrates is low during this period (around 1mM), membrane carriers having a high affinity for amino acids and being able to transport substrates at a high rate from low luminal concentrations. The amount of protein (as % wet weight) increases during this period and appears to be confined to mucosal tissue. The observed increase in Vmax could therefore be due to the increasing number of carrier sites on the jejunal absorptive surface which is expanding at this time.

During the third postnatal week, morphological changes occur in the small intestine. Cell division in the crypts increase and the migration rate of these cells up the villi is doubled (Herbst and Sunshine, 1969). The changing properties of these enterocytes (shown by their impermeability to macromolecules) suggest they possess a functionally different membrane. The transport of amino acids before and after weaning exhibit different properties. The extent of accumulation markedly declines, T/M ratios are lower and the Vmax in all six substrates studied here is reduced. Similarly, K_m values are higher suggesting that the enterocyte carrier sites now have a lower affinity for their substrates. These results suggest the possibility of a "new", functionally distinct population of enterocytes.

On re-examination of the postweaning decrease in L-methionine accumulation, Fondacaro, Nathan and Wright (1974) found that the expression of results included a rapidly increasing non-absorptive component, which artificially lowered transport rates. When the mucosa alone was considered, they found no difference between 15 and 30 day old rats in terms of L-methionine accumulation. Similarly, the decline in Vmax directly after weaning has been described here as a result of the increasing muscle mass, but this does not explain the lower values of uptake at 60 days of age. This decline in gut transport around weaning in the rat has been reported for a number of substrates e.g. amino acids (Donnelly, 1971; Fitzgerald, Reiser, Johnson and Christiansen, 1969; Batt and Schachter, 1969) and sugars (Ingham, 1972).

Correcting for muscle mass affects Vmax, but does not change K_m. In each of the six amino acids studied here, K_m was higher after

weaning by a factor of two (day 15-24). The lower affinity of the transport systems on the enterocytes would explain the real decline in uptake seen in older rats. Alternatively, it is possible that the cell population after weaning possesses more specific carriers, thus reducing the number of sites available for transport of individual amino acids. This question of specificity of uptake in development is further examined in Chapter 6.

Declining rates of amino acid transport with age have been described in other species. Butt and Wilson (1968) found that in the guinea pig intestine, transport of betaine, sarcosine, proline, glycine, valine and lysine was lower in the adult than in the neonate. Smith and James (1976) found that the newborn pig colon is able to concentrate methionine: after 4 days of age, though, the Vmax was only 25% of that at birth and the K_m had increased from 0.33mM to 10mM; by day 10 there was no net transport (James and Smith, 1976).

The values of K_m determined here in the adult small intestine (60 days of age) correspond well with those found by Larsen, Ross and Tapley (1964) using everted sacs, except for L-lysine which was considerably higher. Carrier affinity for neutral amino acids is some two to three times lower than for L-arginine, and transport varies with the different intestinal regions. For neutral and basic amino acids, the area of maximal transport is in the region of lower jejunum - upper ileum. This area of the small intestine is chiefly implicated in substrate absorption (sugars, vitamins and fats). In addition, a regional variation in oxygen uptake is present in the gut, decreasing proximo-distally and this has been correlated with mucosal cell mitochondrial activity. This regional

variation in mitochondrial activity is present in the neonatal gut at weaning (Bamford and Holmes, 1971).

The kinetic studies described in this Chapter suggest that there is a correlation between amino acid accumulation and the appearance of a functionally distinct population of enterocytes along the villi at the age of weaning. Comparisons of K_m for uptake of the six essential amino acids indicate that the membrane of this new enterocyte probably has a different array of carrier sites, while the decline in Vmax in the gut of older animals may be due to fewer carrier sites or to an increase in their substrate specificity. To try and resolve these questions, carrier specificity for amino acids was examined in the developing jejunum (Chapter 6), the nature of the driving force required for active accumulation investigated (Chapter 7) and an attempt made to induce these developmental changes in amino acid absorption around weaning by promoting the premature appearance on the villi of the "new" cell type (Chapter 8).

CHAPTER 6

Competition between Amino Acids for Uptake in Development

Introduction

The kinetic studies of amino acid absorption in the developing small intestine, described in the preceeding Chapter, reveal that there is a shift in the characteristics of uptake as the rat matures, particularly around weaning. The number of amino acid carrier sites on the enterocytes appears to decrease after weaning and the affinity of these sites for their substrates similarly decreases. This observation suggests the possibility that with the appearance during the third postnatal week of a functionally distinct enterocyte lining the villi, these absorptive cells carry arrays of membrane carriers that are more highly specialised. Increased specificity of transport sites would mean that less were available to a given substrate, resulting in slower accumulation within the tissue. Alternatively, one could speculate that the number of carriers present, while inherently specific, may become generally fewer in number. To test the first hypothesis, the specificity of amino acid absorption in the jejunum was investigated from birth until post-weaning and the results are presented here.

The kinetics of amino acid uptake into a variety of tissues suggests that there is present in a cell's membrane a population of carrier sites that recognise, transport and then release substrate. As the number of substrate moieties increase outside the cell, these carrier sites become progressively filled until the population is saturated, and the substrate is then being transported at a maximal rate. The use of the phrase "carrier site" in this context does not preclude the possibility of a system of specific, charged pores in the membrane which would similarly explain the kinetic evidence, nor does

the use of Michaelis-Menten kinetics in transport imply the existence of an enzyme system, though such a system has been invoked by a number of people for amino acid transport in the kidney, brain and small intestine (namely γ-glutamyl transpeptidase). These points will be discussed in Chapter 7.

Kinetics of inhibition

The transport of substrates across cell membranes requires firstly recognition, and secondly binding (reversible) with the carrier site. An indication of this binding step has been determined in the previous Chapter by calculating the K_m or affinity of carriers in the developing jejunum for particular neutral and basic amino acids. These determination were made in the presence of one substrate (S) alone. In the presence of a second substrate (I) the rate of carrier transport of (S) may be altered in a number of ways:

1) interference with the rate of attachment of (S) to its carrier sites - competitive inhibition

2) interference with the metabolism of the cell upon which transport of (S) depends - non-competitive inhibition

In the first instance, presence of (I) will affect K_m of (S) but not Vmax; in the second case Vmax of (S) will be affected by (I) but not K_m.

If both (S) and (I) are transported (which as we have seen is the case for the six essential amino acids described), the rate of transport of substrate in the presence of inhibitor can be described thus:

$$V = \frac{Vmax\ (S)}{(S) + K_m\ (1 + \frac{(I)}{K_i})} \qquad (1)$$

This equation is also used to describe competitive inhibition in enzyme kinetics and is identical with the Michaelis-Menten equation:

$$V = \frac{Vmax\ \ S}{K_m + S} \qquad (2)$$

except that the K_m here has been replaced by an "apparent K_m" i.e. $K_m\ (1 + (I)/K_i)$. The K_m of (S) is thus increased in value by a factor $(I)/K_i$.

The value of the experimentally determined K_i of a substrate acting as an inhibitor should be the same as the K_m of that substrate when transported alone. This is not always the case and the observed differences are then interpreted as evidence for transport by more than one carrier. Also, the effect of an inhibitor will depend on the relationship between the K_m of (S) and the K_m of (I) i.e. K_i. The lower the value of K_m of (I) compared with K_m of (S) the greater should be its inhibitory effect: conversely if K_m of (I) is high enough, no inhibitory effect of (S) would be detectable.

In addition to affecting uptake of (S), (I) can also inhibit efflux, and the kinetic picture becomes extremely complicated. Efflux has so far been largely disregarded, but it can lead to substantial error when calculating kinetic constants of uptake. Yet another possibility is that the presence of (I) can activate the uptake of (S) by altering carrier sites so that (S) fits more readily. This will be obvious in the case of Na^+ ions effecting amino acid uptake (to be described in the next Chapter). If one amino acid stimulates

the uptake of another, then increasing the concentration of (I) would result in a lower K_m of (S) leaving Vmax unchanged; alternatively if (S) becomes attached to (I) when it is being transported, it would then acquire another entry route and so Vmax would be increased.

Non-competitive inhibition can be seen in Chapter 7 which describes the effect of metabolic inhibitors on amino acid uptake. In these instances lack of, or interruption with, the cells' metabolic machinery reduces the rate of concentrative uptake by disabling the movement or production of carrier sites.

A number of kinetic methods are available which describe substrate competition (see Neame and Richards, 1972); in essence these are variations on the kinetic themes described in the previous Chapter. In this study, the algebraic method of Dixon and Webb (1964) has been adopted to explore competitive inhibition. K_i can be determined by means of a "velocity ratio" which relates the unhibited rate of transfer to the inhibited rate, provided inhibition is competitive. The rate is determined in each case using the same concentration of (S) and the values inserted into the equation:

$$\frac{V}{V_{(I)}} = \frac{(S) + K_m \left(1 + \frac{(I)}{K_i}\right)}{(S) + K_m} \qquad (3)$$

which has been derived simply by dividing the Michaelis-Menten equation (2) by the inhibition equation (1) and cancelling out Vmax.S.

In (3) V and $V_{(I)}$ are the rates of uptake in the absence and presence of competitive inhibitor (I) respectively and K_i can be determined thus:

$$K_i = \frac{V_{(I)}}{V - V_{(I)}} \cdot \frac{K_m (I)}{(S) + K_m} \qquad (4)$$

The various K_m values of the six substrates under study have already been determined at various ages of development, though obviously their reliability will affect the accuracy of the K_i values determined.

Specificity of membrane carriers - an overview

Amino acids are small molecules which share a common structure (see Table 5.1) and can be broadly classified into three classes : acidic, neutral and basic, depending on the ratio of carboylic to amino groups. The neutral amino acids can further be sub-divided into five groups:

a) those with aliphatic side chains e.g. valine, leucine
b) those with sulphur containing side chains e.g. methionine
c) those with cyclic side chains e.g. phenylalanine
d) those with side chains containing hydroxylic groups e.g. serine
e) Imino acids e.g. proline

a) to d) above are separated on the basis of their R- groups, but unlike these, imino acids do not have the imino and carboxyl groups attached to the same α-carbon atom.

The choice of amino acids in this study therefore incorporates examples of the basic class (lysine and arginine) and three groups of the neutral class (a, b and c above).

Using the types of kinetic studies described above, carrier sites in a range of tissues have been described which are specific for certain classes or groups of amino acids. Such work has led to descriptions of the nature and structure of specific carrier sites and eventually to the isolation of membrane components which specifically bind substrate. Isolation of membrane carriers will be discussed more fully later.

Cori (1926) was perhaps the first to record competition (between glycine and alanine) in the absorption of amino acids by the rat *in vivo*, and with the development of *in vitro* techniques, detailed studies of substrate interaction emerged. Wiseman (1955) described, in the hamster small intestine, a special transporting mechanism common to the L-forms of the neutral class which all competed for this mechanism, while the basic and acidic amino acids did not appear to be involved in active transport nor did they affect neutral substrate transport. Uptake of basic amino acids is now well known but the ability of the small intestine to actively transport the acidic class is clouded by the rapid transamination that these amino acids undergo. Broadly speaking, five pathways for L-amino acids have been described in the intestine as being functionally discrete (Wiseman, 1974).

1) A carrier system for the *neutral* amino acids, unaffected by basics or acidics.

2) A carrier system for the *basic* group which can also be used by *cysteine* and possibly *leucine* and *tryptophan*.

3) A mechanism specific for *imino* acids e.g. *proline*, *sarcosine* etc., possibly used also by *alanine* and *leucine*, while proline can use the

first pathway.

4) A mechanism in the rat for glycine and proline distinct from 1) and 3).

5) A mechanism distinct from 1) that actively transports valine, leucine and isoleucine.

Alvarado (1966) has suggested that these may all represent one polyfunctional carrier capable of binding at different sites any actively transported amino acid.

Obviously, the situation in the gut lumen involves a mixture of substrates all competing for uptake. Using in vivo loops of rat small intestine, Delhumeau (1962) has described the pattern of absorption of amino acids from an equimolar mixture. Cysteine, methionine, arginine, isoleucine, tryptophan, leucine and valine were faster than phenylalanine, proline, tyrosine, lysine, alanine, histidine, serine, glycine and threonine, while aspartate and glutamate were only slowly absorbed. This pattern is very similar to that seen by Adibi and Gray (1967) in the human jejunum.

Discrete pathways for amino acid uptake have been described in other cells and tissues. Christensen (1975) has reported four transport systems in the Ehrlich ascites tumour cell (Gly, A, Asc and L) for neutral amino acids. Lajtha (1974) details seven systems (with some overlap) for amino acid transport in the brain, namely: acidic, large basic, small basic, small neutral, w-amino, (e.g. GABA), proline type and large neutral (including the aromatics), and suggests that influx and efflux specificities are different. In peripheral nerve, Wheeler (1975) lists a similar array of transport systems. Stewart (1975)

has investigated the specificity of uptake of amino acids into shellfish gill and describes acidic, basic and three neutral sites, a similar array to that in the small intestine.

In addition to inhibition kinetics, three other lines of research have led to the identification of specific membrane carriers : studies with mutant cell lines, genetically deficient in transport pathways (see review by e.g. Oxender, 1975); inborn errors of amino acid metabolism (see Csaky, 1975); and investigations of the development of transport systems in the maturing organism. The latter are few and far between but will be discussed fully later as they relate to the results now presented, and to the developmental changes in amino acid transport kinetics described earlier.

Results

Everted jejunal segments were used throughout. Rats of three age points were selected: just postnatal (1/2 days old), pre-weaning (14/15 days old) and post-weaned (24/26 days old). These three age points were considered to be of the most interest for the development of transport specificity. The concentration of the substrate (S) was set at 0.5mM in all cases except for L-arginine (1mM). The test amino acid (I) was present at a concentration of 5mM in all cases. Experimental procedure was identical to that previously described and the results are expressed for (S) and (S + I) in nanomoles (S) accumulated/mg. protein/15 min. Inhibition is expressed as (S) - (S + I)/(S) %, and the results analysed statistically using the unpaired, two-tailed Students' 't' test, with significance (p) set at 5% ($p < 0.05$). The K_i of the test amino acid was determined where appropriate using the method of Dixon and Webb (1964) described

earlier in equation (4); K_m values of each amino acid are derived from Table 5.2. The experimental design is a one-way analysis of inhibition resulting in interaction between all amino acids at each age studied.

Problems of expression of results

The presence of an increasing mass of muscle tissue in the developing jejunum does not complicate inhibition experiments as comparisons are being made within age groups rather than between them. The results are expressed as total accumulation of (S) and no measure has been made of diffusion. However, the extent of diffusion into jejunal segments with an extracellular concentration of 0.5mM is negligible and has been ignored.

Inhibition of uptake can be either competitive or non-competitive as has been described earlier. In vitro the preparation has only a limited energy supply, though 10mM glucose was present in the incubation medium throughout. Sugar/amino acid interactions have been reported in the small intestine, which may complicate the results observed here; interaction is thought to be of a non-competitive nature. The use of the Dixon and Webb method for calculating K_i assumes that inhibition is competitive. Where K_i does not agree with K_m of (I), this suggests either partially competitive inhibition or the possibility of multicarrier transport. In addition Bronk and Leese (1974) indicate that absorption from a mixture of amino acids involves a degree of energy competition.

Amino acid uptake specificity in development

1) Early postnatal

Table 6.1 shows the results of two substrate incubations and the effect of (I) on (S) in the 1-2 day old rat jejunum. The vertical column contains the values of uptake for amino acid alone, and the horizontal columns, uptake in the presence of a number of inhibitors. Four of the five additional substrates significantly inhibited L-lysine uptake; L-arginine > L-leucine > L-methionine ≡ L-valine ($p < 0.001$). L-phenylalanine had no significant effect on L-lysine accumulation. L-leucine > L-arginine > L-valine inhibited L-methionine accumulation, but L-phenylalanine was again without effect. L-valine had no effect on L-arginine uptake but L-leucine and particularly L-phenylalanine, actually significantly increased the uptake of this basic amino acid. L-leucine > L-phenylalanine significantly decreased L-valine uptake and similarly L-phenylalanine inhibited L-leucine accumulation.

2) Pre-weaning

Table 6.2 shows a similar set of experiments for 14-15 day old rats. L-arginine and L-leucine inhibited L-lysine uptake by 60% or more and L-methionine and L-phenylalanine by 30-40%. At this age L-valine had not the inhibitory effect seen in day old rats, but L-phenylalanine (while having no effect in day old animals), significantly inhibited uptake in 15 day olds.

L-leucine > L-valine ≡ L-phenylalanine > L-arginine all significantly ($p < 0.001$) depressed L-methionine uptake by over 50%. Inhibition was much greater than in 1 day old tissue and

L-phenylalanine was now effective.

L-valine inhibited L-arginine uptake by 30% but L-leucine and L-phenylalanine did not activate L-arginine uptake as they did on Day 1.

L-leucine and L-phenylalanine had similar inhibitory effects on L-valine accumulation as on Day 1 (some 70%) and L-phenylalanine again depressed L-leucine uptake.

3) Post-weaning

Table 6.3 shows the interaction of substrates in the 4 week old rat and possibly represents to a large extent the adult situation. L-arginine inhibited L-lysine uptake by more than 70% and both L-valine and L-leucine had slight but significant effects (some 30%). L-methionine and L-phenylalanine were without effect. As with the 15 day old animal, L-leucine $>$ L-valine $\equiv$ L-phenylalanine $\gg$ L-arginine all significantly depressed L-methionine uptake.

L-valine slightly inhibited L-arginine uptake and L-leucine and L-phenylalanine had slight stimulating effects as with day old animals. Both L-leucine and L-phenylalanine inhibited L-valine accumulation but now L-phenylalanine had no effect on L-leucine uptake.

The effect of age on substrate-substrate inhibition is summarised in Table 6.4 which also shows the values of K_i of (I) determined from the K_m of (S).

A number of points arise from this Table:

1) Neither L-leucine nor L-phenylalanine inhibit L-arginine uptake at any age. If anything there is a slight stimulatory effect.

2) L-methionine, and to a slight extent L-phenylalanine, significantly inhibit L-lysine uptake before weaning but have no effect after this period. Similarly L-phenylalanine decreases L-leucine uptake before but not after weaning.

3) L-phenylalanine inhibition of L-methionine and L-valine inhibition of L-arginine increases around the third postnatal week.

4) L-arginine, L-valine and L-leucine inhibit L-lysine uptake from birth; similarly L-arginine, L-valine, L-leucine inhibit L-methionine uptake from birth; and L-leucine and L-phenylalanine both inhibit L-valine uptake from birth.

The K_i of an amino acid (I) acting as a fully competitive site inhibitor should theoretically be the same as the K_m of (I) when transported alone provided only one carrier site is involved. Table 6.5 compares K_m and K_i values of the six amino acids. Vmax values are also inserted for comparison. If Vmax is a measure of the number of carrier sites on the membrane available to a given substrate, the substrates which share a similar transport system should have similar values of Vmax.

Basic class (L-lysine and L-arginine)

L-arginine inhibits L-lysine from birth with a K_i very similar to its own K_m. However, Vmax values of these two basic substrates are very dissimilar until after weaning, L-lysine being more readily accumulated before weaning. L-methionine and L-phenylalanine inhibit L-lysine uptake until weaning but their K_i values are very much

higher than their K_m, indicating that inhibition is probably only partially competitive. Interestingly, L-arginine also inhibits L-methionine uptake but again the K_i is much greater, indicating that probably different sites are involved. L-valine and L-leucine both inhibit L-lysine uptake, and L-valine to a certain extent affects L-arginine accumulation, but K_i values of the two aliphatic neutral substrates are not in the range of their K_m values.

This pattern suggests that L-lysine and L-arginine compete for a common basic carrier site from birth. The high value of Vmax for L-lysine early in development may indicate that it can also use other sites, for example those for L-methionine and L-phenylalanine before weaning and also the aliphatic neutral system. L-valine can affect L-arginine uptake later in development but has a low affinity for this basic system compared with L-arginine. The stimulation of L-arginine uptake by L-leucine and L-phenylalanine can be explained by inhibition of L-arginine efflux by intracellular levels of the neutral amino acids, an effect which has also been described by Robinson (1968).

Neutral class

1) S-group (L-methionine)

L-methionine interferes with L-lysine uptake early in development but has no effect after weaning. All of the other three neutral substrates inhibit L-methionine uptake and, while their K_i values are not similar early on in development, after weaning they all appear to be using the same site.

2) Cyclic (L-phenylalanine)

L-phenylalanine competes fully for the L-valine and L-leucine sites, and the L-methionine site.

3) Aliphatic (L-valine and L-leucine)

Both of these neutral amino acids affect the L-lysine system but not fully competitively, K_i values indicating that they have a much lower affinity for the basic system. L-leucine and L-valine are fully competitive inhibitors for the same site from birth and both affect L-methionine uptake with K_i values in their K_m range later in development.

The Vmax values for all neutral amino acids are very similar to each other throughout development. L-leucine and L-valine are very closely matched indicating a common system, and so are the Vmax ranges of L-methionine and L-phenylalanine. After weaning, uptake of the neutral class is much faster than the accumulation of the basic substrates and the former have correspondingly higher values of K_m.

TABLE 6.1

Competition for uptake of amino acids into everted jejunum segments from early postnatal rats (Day 1/2). Uptake expressed as nanomoles/mg protein/15 min. Values represent means ± SEM

n = number of experiments and significance of inhibition determined by a two-tailed Students 't' test

% I = percentage inhibition

In all cases (except L-arginine) the control amino acid (radioactive) was present at a concentration of 0.5mM and the inhibiting amino acid (I) at 5.0mM.

5mM INHIBITOR (I)		L-METHIONINE n=6			L-ARGININE n=6			L-VALINE n=6			L-LEUCINE n=6			L-PHENYLALANINE n=6		
0.5mM (S)	control	+ I	% I	p <	+ I	% I	p <	+ I	% I	p <	+ I	% I	p <	+ I	% I	p <
L-LYSINE n = 10	24.93 ± 1.14	13.30 ± 0.96	46.65	0.001	5.94 ± 0.48	76.21	0.001	13.43 ± 1.39	46.13	0.001	8.5 ± 0.72	65.90	0.001	19.38 ± 4.97	22.26	NS
L-METHIONINE n = 8	40.56 ± 6.55				22.22 ± 3.67	45.22	0.05	24.07 ± 3.12	40.66	0.05	16.75 ± 1.63	58.70	0.01	27.34 ± 2.81	32.59	NS
(1mM) L-ARGININE n = 6	16.79 ± 1.67							17.70 ± 1.88	NIL	NS	24.33 ± 2.22	* 44.91	0.05	34.39 ± 1.52	* 104.82	0.001
L-VALINE n = 4	49.5 ± 3.16										12.42 ± 0.48	74.91	0.001	18.79 ± 1.55	62.04	0.001
L-LEUCINE n = 2	45.22 ± 3.91													23.99 ± 3.01	46.95	0.01

TABLE 6.2

Competition for uptake of amino acids into everted jejunum segments from pre-weaning rats (Day 14/15). Uptake expressed as nanomoles/mg protein/15 min. Values represent means ± SEM

n = number of experiments and significance of inhibition determined by a two-tailed Students 't' test

% I = percentage inhibition

In all cases (except L-arginine) the control amino acid (radioactive) was present at a concentration of 0.5mM and the inhibiting amino acid (I) at 5.0mM.

5mM INHIBITOR (I)		L-METHIONINE n=6			L-ARGININE n=6			L-VALINE n=6			L-LEUCINE n=6			L-PHENYLALANINE n=6		
0.5mM (S)	control	+ I	% I	p<	+ I	% I	p<	+ I	% I	p<	+ I	% I	p<	+ I	% I	p<
L-LYSINE n = 10	25.94 ± 2.57	17.83 ± 2.31	31.26	0.05	8.09 ± 1.77	68.81	0.001	20.76 ± 2.65	19.97	NS	11.10 ± 0.48	57.21	0.001	15.64 ± 2.11	39.71	0.01
L-METHIONINE n = 8	47.35 ± 2.63				21.57 ± 1.27	54.45	0.001	20.40 ± 0.83	56.92	0.001	15.17 ± 0.57	67.96	0.001	20.40 ± 1.06	56.92	0.001
(1mM) L-ARGININE n = 6	56.48 ± 3.78							38.73 ± 3.96	31.43	0.01	44.08 ± 4.55	21.95	NS	55.34 ± 2.32	NIL	NS
L-VALINE n = 4	80.88 ± 5.34										18.91 ± 0.79	76.62	0.001	26.11 ± 1.24	67.72	0.001
L-LEUCINE n = 2	78.85 ± 10.09													24.61 ± 1.23	68.79	0.01

TABLE 6.3

Competition for uptake of amino acids into everted jejunal segments from post-weaning rats (Day 24/26). Uptake expressed as nanomoles/mg protein/15 min. Values represent means ± SEM

n = number of experiments and significance of inhibition determined by a two-tailed Students 't' test

% I = percentage inhibition

In all cases (except L-arginine) the control amino acid (radioactive) was present at a concentration of 0.5mM and the inhibiting amino acid (I) at 5.0mM

5mM INHIBITOR (I)		L-METHIONINE n=6			L-ARGININE n=6			L-VALINE n=6			L-LEUCINE n=6			L-PHENYLALANINE n=6		
0.5mM (S)	control	+ I	% I	p <	+ I	% I	p <	+ I	% I	p <	+ I	% I	p <	+ I	% I	p <
L-LYSINE n = 10	14.06 ± 0.84	12.13 ± 1.13	13.73	NS	3.79 ± 0.32	73.04	0.01	10.69 ± 0.62	23.97	0.01	9.83 ± 0.28	30.09	0.001	15.09 ± 0.64	NIL	NS
L-METHIONINE n = 8	26.96 ± 1.38				16.58 ± 1.09	38.50	0.001	12.51 ± 0.59	53.60	0.001	9.78 ± 0.96	63.72	0.01	11.70 ± 0.24	56.60	0.001
L-ARGININE n = 6	33.41 ± 2.44							23.00 ± 1.31	31.16	0.01	39.76 ± 2.45	* 19.01	NS	44.66 ± 0.98	* 33.67	0.01
L-VALINE n = 4	57.93 ± 6.72										13.18 ± 1.02	77.25	0.001	26.04 ± 1.53	37.79	0.01
L-LEUCINE n = 2	32.51 ± 1.34													23.01 ± 6.55	29.22	NS

TABLE 6.4

The development of specific amino acid transport systems in the rat jejunum from birth until post-weaning, and the kinetics of inhibition

K_m and K_i of inhibitory amino acids in mM; - = No inhibition; ↑ = Increased uptake

(I) INHIBITOR		L-METHIONINE			L-ARGININE			L-VALINE			L-LEUCINE			L-PHENYLALANINE		
AGE		1/2	15/16	24/26	1/2	15/16	24/26	1/2	15/16	24/26	1/2	15/16	24/26	1/2	15/16	24/26
(S) SUBSTRATE																
L-LYSINE	K_m	$0_{\pm}85$ $\pm$ 0.26	$1_{\pm}14$ $\pm$ 0.20	-	$0_{\pm}55$ $\pm$ 0.35	$0_{\pm}19$ $\pm$ 0.06	$0_{\pm}85$ $\pm$ 0.10	$0_{\pm}89$ $\pm$ 0.19	-	$2_{\pm}6$ $\pm$ 0.92	$1_{\pm}05$ $\pm$ 0.36	$1_{\pm}50$ $\pm$ 0.36	$1_{\pm}70$ $\pm$ 0.80	-	$1_{\pm}41$ $\pm$ 0.35	-
	K_i	4.47	7.89		1.22	1.63	1.20	4.56		10.31	2.02	2.68	7.56		5.45	
L-METHIONINE	K_m				$0_{\pm}55$ $\pm$ 0.35	$0_{\pm}19$ $\pm$ 0.06	$0_{\pm}85$ $\pm$ 0.10	$0_{\pm}89$ $\pm$ 0.19	$1_{\pm}49$ $\pm$ 0.22	$2_{\pm}6$ $\pm$ 0.92	$1_{\pm}05$ $\pm$ 0.36	$1_{\pm}50$ $\pm$ 0.36	$1_{\pm}70$ $\pm$ 0.80	-	$1_{\pm}41$ $\pm$ 0.35	$3_{\pm}30$ $\pm$ 1.24
	K_i				3.81	2.91	6.49	4.60	2.63	3.52	2.21	1.64	2.31		2.63	3.12
L-ARGININE	K_m							-	$1_{\pm}49$ $\pm$ 0.22	$2_{\pm}6$ $\pm$ 0.92	↑	-	-	↑	-	↑
	K_i								1.74	5.08						
L-VALINE	K_m										$1_{\pm}05$ $\pm$ 0.36	$1_{\pm}50$ $\pm$ 0.36	$1_{\pm}70$ $\pm$ 0.80	$2_{\pm}81$ $\pm$ 0.78	$1_{\pm}41$ $\pm$ 0.35	$3_{\pm}30$ $\pm$ 1.24
	K_i										1.07	1.14	1.24	1.96	2.29	3.42
L-LEUCINE	K_m													$2_{\pm}81$ $\pm$ 0.78	$1_{\pm}41$ $\pm$ 0.35	-
	K_i													3.83	1.70	

le 6.5: kinetic parameters and the development of amino acid carrier systems. Vmax in nmoles/protein/15 min, Km and Ki in mM.

					Ki when acting as inhibitor of:					
		Age	Vmax	Km	L-Lys	L-Arg	L-Met	L-Phe	L-Val	L-Leu
sic	L-Lysine	1	146.94 ± 38.43	1.79 ± 0.75						
		15	195.31 ± 71.63	1.27 ± 0.84						
		25	63.96 ± 13.22	0.93 ± 0.37						
	L-Arginine	1	37.5 ± 13.22	0.55 ± 0.35	1.22		3.81			
		15	33.10 ± 4.33	0.19 ± 0.06	1.63		2.91			
		25	45.41 ± 3.48	0.85 ± 0.10	1.20		6.49			
ıtral group	L-Methionine	1	149.68 ± 25.06	0.85 ± 0.26	4.47					
		15	183.10 ± 17.38	1.14 ± 0.20	7.89					
		25	199.69 ± 44.78	2.17 ± 0.76	-					
ıtral :lic	L-Phenylalanine	1	267.89 ± 58.97	2.81 ± 0.78	-	-	-		1.96	3.83
		15	196.86 ± 35.04	1.41 ± 0.35	5.45	-	2.63		2.29	1.70
		25	219.32 ± 65.8	3.30 ± 1.24	-	-	3.12		3.42	-
ıtral phatic	L-Valine	1	212.01 ± 28.89	0.89 ± 0.19	4.56	-	4.60			
		15	272.15 ± 29.75	1.49 ± 0.22	-	1.74	2.63			
		25	229.93 ± 62.92	2.60 ± 0.92	10.31	5.08	3.50			
	L-Leucine	1	170.13 ± 32.88	1.05 ± 0.36	2.02	-	2.21		1.07	
		15	278.12 ± 40.26	1.50 ± 0.36	2.68	-	1.64		1.14	
		25	127.04 ± 34.74	1.70 ± 0.80	7.56	-	2.31		1.24	

Discussion

The interaction between neutral and basic amino acids for uptake in the developing jejunum presents a complex picture, but a number of observations can be made.

The basic class are transported by specific sites which are present at birth. While all the neutral amino acids affect L-lysine uptake to some extent, the picture is one of increasing K_i of neutral substrates with age such that after weaning the inhibitory effect of neutral amino acids is much decreased and suggests the presence of a more specific basic carrier system at this time. The basic group have a very high affinity for this system (K_m around 1mM). There is some evidence that both L-lysine and L-arginine might be able to utilise some neutral carrier sites, which would explain the extensive accumulation of L-lysine early in development. Conversely, the lower affinity of L-lysine for the basic system could also explain its higher uptake compared with L-arginine, which has a higher affinity and lower uptake.

The neutral class presents a more complex picture. There appears to be a system for the aliphatic group (L-valine and L-leucine) which is present at birth, and which may also be used by L-phenylalanine. All three also appear to be able to use those carriers that transport L-methionine, particularly later in development; there is a marked decrease in their K_i values around weaning. In order to unravel the specificity of amino acid uptake, these observations will first be discussed with regard to other developmental studies and then "inserted" into the picture seen in the mature intestine.

Few developmental studies of amino acid carrier systems have been made. Donnelly (1971) reported that in the young rat ileum (of unspecified age) L-leucine uptake was inhibited by L-phenylalanine and L-methionine to a great extent, but less so by L-alanine, L-histidine and L-lysine. L-proline (though transported - see Batt and Schachter, 1969) was without effect, which suggested a separate carrier system. L-leucine and L-lysine also inhibited L-histidine. Donnelly suggests that these results for the neonatal rat ileum favour a common carrier for L-alanine, L-methionine and L-leucine, and a separate system for L-lysine, while L-histidine overlaps both of these systems, similar to the separate basic and neutral systems proposed here for the young rat jejunum and re-inforcing the overlap found for L-lysine.

Fitzgerald, Reiser, Johnson and Christiansen (1969) found that the transport patterns of L-valine, L-lysine and glycine in the rat small intestine showed distinct variation in development, which suggested separate transport systems for neutral, basic and glycine. The occurrence of two phases (7 day and 28 day old) of intensification of cystine accumulation in rat intestinal segments observed by States and Segal (1968) may be the result of two different transport systems (Christensen, 1973). Pratt and Terner (1971) found that, in rat small intestine, transport activity for L-lysine appeared 4 days before birth, L-valine 2 days before birth and glycine at birth.

In a classic paper which perhaps initiated the study of transport system development as a tool for defining carrier sites, Deren, Strauss and Wilson (1965) describe changes in the small intestine of the foetal rabbit. Transport of L-valine, L-methionine and L-lysine was evident at the 22nd day of gestation, L-proline and glycine followed a few days later, while betaine uptake was not seen until the 26th day. Similarly, foetal guinea pig small intestine of 35 days gestation can accumulate L-valine, L-proline, L-lysine and glycine (Butt and Wilson, 1968), while sarcosine uptake is not evident until 40 days and N-dimethylglycine and betaine until 60 days gestation (just prior to birth), indicating at least three separate transport systems.

James and Smith (1976) and Smith and James (1976) describe the existence of a methionine uptake system in the newborn pig colon which subsequently disappears within a few days after birth. Colonic absorption of amino acids has been described elsewhere e.g. L-proline in rat colon (Batt and Schachter, 1969) and L-phenylalanine in dog colon (Robinson, Luisier and Mirkovitch, 1973).

Miller, Burrill and Lerner (1974) have investigated neutral amino acid transport in the chick small intestine and describe seven discrete components of three major categories: 'U'-shared with both L-leucine and glycine, α -shared with L-leucine but not glycine and β -unshared with leucine. The other components are of narrower specificities within the three groups. In a later paper Lerner, Burrill, Sattelmeyer and Janicki (1976) looked at the developmental patterns of these intestinal transport mechanisms and found regional differences.

Developmental changes in specificity have also been described for amino acid transport by the rat pancreas (Cheneval and Johnstone, 1974), rat kidney (Baerloch , Scriver and Mohyuddin, 1971) and rat liver (Christensen and Clifford, 1963). Similarly there are changes in sugar transport specificity in development (Deren, 1968; Ingham, 1972).

The results of uptake inhibition in the post-weaned rat largely reflect the adult situation, in which some neutral amino acids can use more than one carrier system. L-lysine and L-arginine are transported by the same system. This basic carrier seems also to be used by L-leucine and possibly L-valine. Reiser and Christiansen (1969) found that L-valine was a partial competitive inhibitor of L-lysine uptake in everted intestinal sacs of the rat, and explained their finding in terms of allosteric modification of the basic carrier when L-valine was bound to a specific but closely associated site. Similarly Larsen, Ross and Tapley (1964) found that L-leucine inhibited L-lysine uptake in the rat and Johns and Bergen (1973) report the same finding in the sheep. That these aliphatic neutral amino acids have in addition a system distinct from the dibasics (as seen here) is evidenced also by the clinical condition of cystinuria where the dibasic carrier is absent (Hellier, Holdsworth and Perrett, (1973).

L-methionine, L-phenylalanine, L-valine and L-leucine all appear from these results to share a common carrier in the 4 week old jejunum. L-methionine and L-phenylalanine have been shown to share a common carrier in the rabbit ileum (Preston, Schaeffer and Curran, 1974), and Bartsocas, Thier and Crawford (1974) have demonstrated the

inhibition of L-methionine by L-valine in the rat jejunum. Christensen (1975) has described four of the principal transport systems (Gly; A; ASC and L) for neutral α amino acids in a number of tissues. All four neutral substrates studied here are transported by both 'A' and 'L' systems, the latter being described as Na^+-independent (see Chapter 7). Daniels (1970) has examined neutral substrate transport in rat intestine in detail and describes two systems: a methionine system with affinity for the more lipid-soluble neutrals, and a sarcosine system with affinity for small, water soluble amino acids. Young and Ellory (1977) have recently described a specific neutral amino acid transport system in sheep erythrocytes which also has a significant affinity for dibasic amino acids.

It is now well known that the small intestine (as well as the kidney, brain and erythrocyte) will absorb small peptides in addition to amino acids (see reviews by Matthews, 1975; Hellier and Holdsworth, 1975). However, the transport systems for peptides appear to be independent of those involved with amino acid uptake in the small intestine (Sigrist-Nelson, 1975; Cheeseman and Smyth, 1975; Gardner, 1976). In the hamster jejunum, Addison, Burston, Payne, Wilkinson and Matthews (1975) have presented evidence for active transport of tripeptides which share a common carrier with dipeptide uptake (Sleisenger, Burston, Dalrymple, Wilkinson and Matthews, 1976). In the frog intestine, Cheeseman and Parsons (1976) suggest that peptide uptake into the mucosa is Na^+-independent.

Interaction between sugars and amino acids in the small intestine has received much attention in recent years and it must be considered as in all experiments reported here, glucose was present at a concentration of 10mM.

Two distinct hypotheses have been published applicable to membrane preparations. Robinson and Alvarado (1971) proposed a "polyfunctional" carrier in the brush border membrane for sugars and amino acids. Mutual influence would be mediated by allosteric effects on the carrier kinetics. In contrast, Semenza (1971) amongst others has suggested that the flow of one substrate changes the force which determines the flow of the other i.e. specific carriers, but interaction by transport-dependent localised changes of the Na^+ concentration in the vicinity of the brush border. While much evidence supports the second hypothesis in vitro, Murer, Sigrist-Nelson and Hopfer (1975) suggest that in some cases (e.g. the intact cell) mutual inhibition of sugar and amino acid uptake could be an expression of energy competition. Hardcastle and Daniels (1973) suggest that the observed inhibitory effect of galactose on L-leucine transport in everted sacs of rat jejunum was a non-competitive event, but Bolufer, Larralde and Penz (1975) working in vivo suggest that a similar interaction they themselves found was not energy dependent.

The brush border contains an array of systems that transport organic substrates, some specific, others shared. The results described here suggest not only that there is a quantitative change in the number of particular amino acid carriers along the villi with the appearance of a new cell type during the third postnatal week, but also that the specificity of the carriers changes around this time. Before the mechanisms that could induce these maturational changes are discussed, we must briefly consider evidence for the nature of such membrane "carriers"; this subject has been reviewed extensively elsewhere (Lin, 1971; Christensen, 1972; Oxender, 1974).

Inhibition experiments have revealed the specificity of particular carriers and have led to theories concerning the chemical nature of substrate-carrier binding. Specific amino acid carriers not only transport the L- forms but also the D-enantiomorphs albeit in most cases with lower affinity (Daniels, Newey and Smyth, 1969). In some cases the transport of D-amino acids may be active (Nakamura, Yasumoto, Sugiyama and Mitsuda, 1974). This has also been found with neonatal rat small intestine (Donnelly, 1971). The non-polar character of the side chain of neutral amino acids is important with regard to affinity of the carrier system. The change in the side chain decreases the affinity of the carrier. The degree of solubility of the side chain in the lipid phase of the membrane may also be important for the attachment of NH_3 and COOH groups and of α-hydrogen to the active sites of the carrier (Kotyk and Janacek, 1975). Daniels, Dawson, Newey and Smyth (1969) have emphasised that the lipid-water partition coefficient determines the chemical specificity of the neutral amino acid.

Much evidence (genetic disorders, mutants lacking a transport system) suggests that membrane proteins are involved in substrate binding and transport; no other membrane component possesses such selective behaviour, and amino acid transport is sensitive to protein synthesis inhibitors e.g. puromycin (Phang, Valle, Fisher and Granger, 1975). However, as Kotyk and Janacek (1975) point out, the protein involvement could be as a binding enzyme and the mobile carrier could itself be lipid (carrier mobility is considered in Chapter 7). Guidotti (1976) has recently reviewed the five membrane transport systems in eukaryotic organisms that are well characterised. All five are transmembrane oligomeric glycoproteins and Guidotti suggests this may be the general

structure of all transport systems.

Ugolev (1974) makes an interesting case for the close association of digestive enzymes on the brush border with transport sites, and presents evidence for a quaternary structure of enzyme-carrier complex with allosteric interactions. This is an intriguing idea, and may explain the transport changes observed here around weaning as being a result of the rise in hydrolytic enzyme activity at this time.

The physical ability of a specific membrane component to bind substrate has been used as a tool for isolating carrier systems. In many cases, non-transported analogues of a specific substrate are manufactured and used as carrier "lockers". If the analogue is bound irreversibly and labelled radioactively, it is possible to locate specific areas of membrane associated with transport. This technique has enabled the isolation of specific carrier proteins for some amino acids from bacteria (Kotyk and Janacek, 1975) and to a lesser extent from higher organisms. The size of very diverse carriers are remarkably similar, some 30,000 daltons. Faust and Shearin (1974) have reported the isolation of a small protein (55,000 daltons) from the mucosal brush border of hamster jejunum that has both D-glucose and L-histidine binding sites. However as Sigrist-Nelson and Hopfer (1974) point out the binding of D-glucose could well be to disaccharidase in the membrane. More recently, Crane, Malathi and Preiser (1976) and Semenza, Tannenbaum, Toggenburger and Wahlgren (1976) have reported the reconstitution of erythrocyte sugar transport in liposomes and significant purification of a glucose transport system.

The picture that emerges is one of a series of specific protein or glycoprotein molecules, embedded in or on the membrane, that recognise and bind specific substrates and transport them through the membrane, releasing substrate at the other side. How can the developmental changes described here and elsewhere be explained in these terms? The peak in amino acid transport in the second postnatal week is undoubtedly the result of increased manufacture of carrier sites along an "expanding" absorptive surface. The changes in affinity and transport rate at weaning could be the result of a collection of interrelated events. The production of a new cell type along the villi appears to carry with it some changes in the specificity and/or affinity of the carrier systems. These functionally distinct carrier sites may have been modified by endogenous or exogenous factors. The new cell type is thought to be under hormonal (steroid) control. Glucocorticoids may also modify the synthesis of these "mature" transport sites. Conversely, the increased hydrolytic activity of the enterocyte membrane may produce new interactions between digestion and transport. Affinity and carrier movement may again be altered by the emergence of an ion dependent process, or altered metabolic involvement with the uptake process which may now use energy from a different source.

Christensen (1973) reviews developmental changes in a number of tissues and suggests the induction of membrane transport systems by hormones. Guidotti, Gazzola, Borghetti and Franchi-Gazzola (1975) describe the regulation of neutral amino acid transport ('A' system) across particular tissues by adaptive mechanisms. Adaptive regulation is common in well established systems and is suggested to be a common property of embryonic, immature or developing tissues.

Adaptive regulation continuously adapts the efficiency of transport to the actual needs of the intracellular machinery. The time-dependent adaptive control mechanism for transport system 'A' that Guidotti et al describe involves repression-derepression of transport activity by amino acid substrates acting at the gene transcription level, and coupled to control of transport-protein breakdown (or inactivation) at the cell membrane.

To further characterise the observed changes in amino acid transport in the developing jejunum and answer some of the questions raised above, ion-substrate linked transport was examined developmentally and the source of energy used in active uptake investigated (Chapter 7). On the other hand if these changes in uptake characteristics are more strictly associated with the appearance in maturation of a new cell type, then experimental modification of this developmental event should provide the necessary evidence (Chapter 8).

CHAPTER 7

Sodium and Energy Requirements for Amino Acid Accumulation in Development

Introduction

Concentrative uptake of substrate into cells necessitates expenditure of energy in some form and at some locus; either directly at the carrier translocation step or indirectly to maintain a co-transport gradient. In 1958, Riklis and Quastel noticed that the active uptake of glucose by guinea-pig intestine was stimulated by Na^+ ions. Earlier, Christensen and Riggs (1952) had shown that active uptake of amino acids into duck erythrocytes was strongly inhibited when Na^+ was replaced by K^+ in the medium. This effect was later observed for all amino acids whose uptake generated large concentration gradients across the cell membrane (Na^+ dependent systems) but not for those who failed to establish such gradients ('L' system).

Na^+ dependent transport of amino acids and sugars has now been described in a range of tissues from many species (Parsons, 1975, Schultz and Curran, 1970). All cells, except for the erythrocytes of certain mammals (dog, cattle and sheep) have low intracellular concentrations of Na^+ ions compared with their environment, in contrast to the high intracellular level of K^+ ions. These differences are not maintained by special cation selective properties of the cell or by membrane impermeability, but by ion pumping, which utilises 30% of the cells' energy production. This ion pumping behaviour regulates the internal osmotic pressure of the cell and the Na^+ and K^+ distribution is maintained away from equilibrium. This means that in the steady state, work can be derived from the system: classically in the form of muscle contraction, nervous conduction and substrate transport.

The maintainance of such ion gradients are dependent upon two things: firstly the ion pump (Na^+, K^+-ATPase) and secondly a supply of energy in the form of ATP for use by this enzyme. In development, there is much evidence to suggest that for a number of tissue systems (e.g. brain), ionic gradients become set up as the animal matures, coincident with the appearance (synthesis) of an ATPase, which is in some cases dependent upon steroid levels. Similarly, many functions of immature tissues are known to be insensitive to anaerobic conditions, suggesting a high tissue glycolytic activity.

We have seen that the accumulation of amino acids changes in development with peak activity at two weeks of age which then decreases to adult levels. Changes in transport site numbers, affinity and specificity with maturation have been described, and linked to the appearance in the small intestine of a functionally distinct mucosal cell around weaning. In this Chapter, the existence and role of Na^+ ion gradients in amino acid accumulation in the neonatal jejunum are examined and the nature of the energy source for concentrative uptake investigated. The results are discussed in the context of the developmental changes in amino acid uptake already described and related to the current theories of the mechanisms that promote active substrate uptake.

Results

Everted jejunal segments from rats of 2, 15 and 25 days of age were used in all experiments, incubated in Krebs Ringer bicarbonate (pH 7.4) containing 10mM glucose.

1) Effect of metabolic inhibitors on amino acid uptake

2, 4-dinitrophenol (DNP) was selected as a metabolic inhibitor, at a concentration of 0.5mM. DNP inhibits ATP formation through its effect (inhibition of creatine kinase, the enzyme which phosphorylates ADP) on mitochondrial oxidative phosphorylation.

Table 7.1 shows the effects of DNP on the uptake of six amino acids (at a concentration of 0.5mM). In all cases, the presence of DNP inhibits accumulation of amino acid to a certain extent. Only the inhibited uptake of L-arginine in 2 day old rats was not significant, though approached it ($p < 0.10$). There was a slight trend for the inhibitor to be less effective on the uptake of the basic amino acids. This may result from the relatively low uptake of these substrates, such that the initial ATP levels in the preparation were sufficient to maintain amino acid accumulation. The extent of inhibition was fairly constant with age, suggesting that if glycolysis plays any part in providing ATP, it is no more important for uptake at birth. DNP inhibited uptake by only 50-60% and ATP must have been available in the jejunal segments in order to achieve tissue concentrations greater than 1.

Thus concentrative uptake of amino acids into neonatal jejunum is dependent upon ATP at some point, though results using DNP must be interpreted with caution as Stewart (1975) has described non-specific tissue membrane damage after DNP application.

2) Na^+ ions and amino acid uptake

In these experiments, NaCl in the Krebs Ringer was replaced by LiCl, thus reducing the Na^+ concentration to 25 m equivalents/litre. A

number of alternatives to Na^+ ions have been described in the literature, but all have related problems. KCl as replacement for NaCl produces a much higher inhibition of amino acid transport than anything else in the rat small intestine (Bronk and Parsons, 1966), but several factors may be of significance in its effect; it reduces not only the Na^+ gradient but also the K^+ gradient, K^+ may compete with and block the Na^+ site on the carrier and K^+ may cause membrane distruption (Schultz and Curran, 1970). Tris (2-amino-2-hydroxymethylpropane-1 : 3 diol) replacement of Na^+ produces a large increase in the electrical gradient across the cells (Barry and Egenton, 1972). Choline has been used to replace extracellular Na^+ but it can cross cell membranes and is actively transported by the rat small intestine. There is some evidence that choline could substitute for Na^+ at the carrier site.

Li^+ was adopted as the cation to replace Na^+ in this study as it is without deleterious effects. However, there is evidence that Li^+ can partially mimic the action of Na^+ for sugar uptake (Bihler and Adamic, 1967), and Bronk and Leese (1974) indicate that Li^+ can support some amino acid accumulation in mucosal slices and rings of rat small intestine.

Na^+ replacement was not complete, there still being 25 milli equivalents present in the incubation medium used here. However, Table 7.2 shows that Li^+ replacement significantly inhibits uptake of all six amino acids in 2 day old rat jejunum. Inhibition is much more marked in neutral amino acid uptake (some 90%) as compared with L-lysine and L-arginine (70% and 45% respectively), and less significant overall at the higher concentration used (5mM). Despite

Na^+ replacement, concentrative uptake of substrates is apparent (T/M = 2 for most amino acids). Table 7.3 shows similar results for 15/16 day old rat jejunal segments. Again, Na^+ replacement has less effect on the basic amino acids although it is still significant. Table 7.4 describes Na^+ replacement in 26 day old small intestine.

The marked inhibition of neutral amino acid uptake is present from birth, but though accumulation is reduced, some concentrative uptake is still evident in the presence of Li^+ (T/M about 2). Lowering the extracellular Na^+ concentration reduces basic amino acid uptake from birth though the affect is not as significant.

Hofstee plots of amino acid uptake in the presence of lowered Na^+ ions have been made and kinetic constants calculated by linear regression. These are compared with the kinetics of uptake in normal Krebs Ringer bicarbonate in Table 7.5. The plots were performed on only three concentration values, and are therefore for comparison only. It is clear that while Vmax is unaffected, lowered Na^+ affects the affinity of the substrate for the carrier. This effect is much more apparent in the neutral amino acids, large errors obscurring the situation with the two basic substrates. The lowered affinity of substrate for uptake follows a similar pattern in rats of all ages. The lowered K_m values in the presence of Na^+ ions indicates that Na^+ affects the binding of substrate to its carrier site.

3) Effects of ouabain on uptake

Accumulation of amino acids was measured directly in the presence of 1.0mM ouabain. Preliminary experiments indicated that no pre-incubation was necessary as has been reported in adult intestinal

preparations, possibly because of the lack of a thick muscular wall which would otherwise prevent access of ouabain to its reactive sites. Ouabain is a cardiac glycoside that binds specifically to Na^+, K^+-ATPase by competing with the K^+ site on the enzyme. Inhibition of this enzyme results in a rapid Na^+ equilibration and destroys the ionic gradient across the membrane.

Table 7.6 shows the effects of ouabain on amino acid uptake. No significant inhibition was evident with basic amino acid uptake from birth to post-weaning. On the other hand, all the neutral amino acids were significantly inhibited by ouabain from birth (some 50% I), although this decreased with age (20% I at day 26). The latter could be due to the increase in muscle tissue preventing ouabain access to the lateral and basal membranes of the enterocytes. Concentrative uptake is never totally abolished (T/M = 2-3 on Day 2).

In summary, uptake of the six neutral and basic amino acids studied here is dependent upon oxidative phosphorylation from birth, though concentrative uptake can still occur in its absence. Uptake is also contingent upon the presence of Na^+ ions in the incubation medium which promotes binding of substrate to its carrier (certainly for neutral though not definite for basic amino acids). The inhibition of neutral amino acid uptake with ouabain indicates that accumulation from birth is dependent also upon the existence of a Na^+ gradient across the cell membrane and that an enzyme (Na^+, K^+-ATPase) which maintains this gradient is present early in development. Basic amino acids are not affected by the loss of such an ionic gradient, although they are inhibited (albeit to a lesser extent) by lowering the Na^+

TABLE 7.1

The effect of 2, 4-DNP on amino acid uptake

into jejunal segments from rats of various age

Uptake in nanomoles/mg protein/15 min; Values are means $\pm$ SEM

2, 4-DNP at concentration of 0.5mM

n = 8 for control and 4 for DNP at each point

% I = percentage inhibition

2 tailed Students 't' test

NS = not significant

* = $p < 0.05$

** = $p < 0.01$

*** = $p < 0.001$

SUBSTRATE		AGE (days)								
		2			15			25		
0.5mM		CONTROL	+ DNP	% I	CONTROL	+ DNP	% I	CONTROL	+ DNP	% I
L-LYSINE	Uptake	30.28 ± 2.78	16.08 ± 1.20	46.90	19.13 ± 2.61	11.90 ± 0.68	37.79	12.60 ± 0.62	8.38 ± 0.75	33.49
	T/M	5.82	3.09	***	4.17	2.59	*	2.81	1.87	***
L-ARGININE	Uptake	21.29 ± 1.32	15.83 ± 2.55	25.65	31.96 ± 4.41	18.79 ± 2.44	41.21	19.64 ± 0.99	12.40 ± 1.63	36.86
	T/M	4.09	3.04	NS	6.96	4.09	*	4.38	2.76	**
L-VALINE	Uptake	76.33 ± 6.74	34.41 ± 2.49	54.92	56.10 ± 2.69	23.35 ± 3.63	58.38	33.34 ± 2.00	15.61 ± 2.48	53.18
	T/M	14.68	6.62	***	12.22	5.09	***	7.43	3.48	***
L-LEUCINE	Uptake	46.50 ± 2.48	25.10 ± 2.99	45.68	48.46 ± 4.71	33.16 ± 4.19	31.57	43.68 ± 3.06	22.32 ± 2.87	48.90
	T/M	8.94	4.83	***	10.56	7.22	*	9.74	4.98	***
L-METHIONINE	Uptake	48.58 ± 2.71	32.51 ± 4.89	33.08	41.84 ± 3.25	16.10 ± 2.62	61.52	31.34 ± 3.29	12.47 ± 1.38	60.21
	T/M	9.34	6.25	*	9.12	3.51	***	6.99	2.78	***
L-PHENYLALANINE	Uptake	45.23 ± 2.12	20.60 ± 5.84	54.46	48.78 ± 5.90	17.29 ± 0.74	64.58	47.29 ± 1.46	24.12 ± 1.13	49.00
	T/M	8.70	3.96	**	10.63	3.77	***	10.54	5.38	***

TABLE 7.2

Uptake of amino acids in 2 day old jejunal segments -

effect of low Na^+ (25mM) (NaCl replaced by LiCl)

Results are the means $\pm$ SEM of at least 4 observations at each point

p = significance using 2-tailed Students 't' test

% I = percentage inhibition

SUBSTRATE	mM	n moles/mg. protein/15 min		T/M		C : low Na^+ n moles % I	p <
		CONTROL	Low Na^+	CONTROL	Low Na^+		
L-LYSINE	0.1	9.18 ± 1.05	2.81 ± 0.36	8.83	2.70	69.39	0.001
	0.5	30.28 ± 2.78	7.85 ± 1.18	5.82	1.51	74.08	0.001
	5.0	104.21 ± 5.22	46.44 ± 4.32	2.01	0.89	55.44	0.001
L-ARGININE	0.1	6.94 ± 0.18	3.82 ± 0.46	6.67	3.67	44.96	0.001
	0.5	21.29 ± 1.32	11.83 ± 0.74	4.09	2.28	44.43	0.001
	5.0	52.05 ± 3.46	54.03 ± 3.97	1.00	1.04	-	NS
L-VALINE	0.1	20.59 ± 1.03	2.04 ± 0.32	19.80	1.96	90.09	0.001
	0.5	76.33 ± 6.74	9.19 ± 1.76	14.68	1.77	87.96	0.001
	5.0	179.40 ± 5.77	76.11 ± 11.97	3.45	1.46	58.58	0.001
L-LEUCINE	0.1	16.82 ± 1.11	1.90 ± 0.26	16.17	1.83	88.70	0.001
	0.5	46.50 ± 2.48	7.65 ± 1.37	8.94	1.47	83.55	0.001
	5.0	143.23 ± 8.82	67.27 ± 11.94	2.75	1.29	53.06	0.001
L-METHIONINE	0.1	17.66 ± 2.86	1.67 ± 0.33	16.98	1.61	90.60	0.001
	0.5	48.58 ± 2.71	9.55 ± 0.78	9.34	1.84	80.34	0.001
	5.0	118.88 ± 13.8	58.78 ± 4.85	2.29	1.13	50.56	0.005
L-PHENYLALANINE	0.1	8.64 ± 0.51	2.42 ± 0.50	8.31	2.33	71.99	0.001
	0.5	45.23 ± 2.12	10.39 ± 1.07	8.70	2.00	77.03	0.001
	5.0	173.63 ± 17.71	77.70 ± 9.65	3.34	1.49	55.25	0.005

TABLE 7.3

Uptake of amino acids in 15/16 day old jejunal segments -

effect of low Na^+ (25mM) (NaCl replaced by LiCl)

Results are the means $\pm$ SEM of at least 4 observations at each point

p = significance using 2-tailed Students 't' test

% I = percentage inhibition

SUBSTRATE	mM	n moles/mg. protein/15min		T/M		C : Low Na^+ n moles % I	p <
		CONTROL	Low Na^+	CONTROL	Low Na^+		
L-LYSINE	0.1	7.62 ± 1.57	2.14 ± 0.39	8.30	2.33	71.92	0.05
	0.5	19.13 ± 2.61	7.35 ± 1.33	4.17	1.60	61.58	0.005
	5.0	123.96 ± 0.29	64.58 ± 6.95	2.70	1.41	47.90	0.001
L-ARGININE	0.1	11.76 ± 1.53	3.53 ± 0.83	12.81	3.85	69.98	0.005
	0.5	21.49 ± 3.11	11.47 ± 1.39	4.68	2.50	46.63	0.05
	5.0	51.18 ± 9.99	52.96 ± 5.34	1.12	1.15	-	NS
L-VALINE	0.1	21.60 ± 0.82	1.69 ± 0.22	23.53	1.84	92.18	0.001
	0.5	56.10 ± 2.69	7.42 ± 0.68	12.22	1.62	86.77	0.001
	5.0	383.00 ± 76.71	65.70 ± 6.21	8.34	1.43	82.58	0.05
L-LEUCINE	0.1	16.32 ± 2.5	1.97 ± 0.35	17.78	2.15	87.93	0.001
	0.5	48.46 ± 4.71	9.06 ± 1.47	10.56	1.97	77.18	0.001
	5.0	169.85 ± 17.54	55.72 ± 7.8	3.70	1.21	67.19	0.001
L-METHIONINE	0.1	12.95 ± 0.18	1.47 ± 0.21	14.11	1.60	88.65	0.001
	0.5	41.84 ± 3.25	6.38 ± 0.77	9.12	1.39	84.75	0.001
	5.0	138.50 ± 13.96	48.39 ± 7.05	3.02	1.05	65.06	0.001
L-PHENYLALANINE	0.1	14.05 ± 1.19	2.05 ± 0.42	15.31	2.23	85.41	0.001
	0.5	48.78 ± 5.90	6.72 ± 1.15	10.63	1.46	86.22	0.001
	5.0	174.67 ± 20.34	62.13 ± 8.82	3.81	1.35	64.43	0.001

TABLE 7.4

Uptake of amino acids in 26 day old jejunal segments -

effect of low Na^+ (25mM) (NaCl replaced by LiCl)

Results are the means $\pm$ SEM of at least 4 observations at each point

p = significance using 2-tailed Students 't' test

% I = percentage inhibition

SUBSTRATE	mM	n moles/mg. protein/15 min		T/M		C : Low Na^+ n moles % I	p <
		CONTROL	Low Na^+	CONTROL	Low Na^+		
L-LYSINE	0.1	6.36 ± 0.36	2.52 ± 0.16	7.07	2.80	60.38	0.001
	0.5	12.60 ± 0.62	7.21 ± 0.37	2.80	1.60	42.78	0.001
	5.0	79.33 ± 0	38.32 ± 2.24	1.76	0.85	51.70	0.001
L-ARGININE	0.1	6.04 ± 0.90	1.36 ± 0.09	6.71	1.51	77.48	0.001
	0.5	15.01 ± 1.69	5.21 ± 0.35	3.34	1.16	65.29	0.001
	5.0	37.28 ± 3.0	20.56 ± 2.78	0.83	0.46	44.85	NS
L-VALINE	0.1	7.0 ± 1.23	1.36 ± 0.12	7.78	1.51	80.57	0.005
	0.5	33.34 ± 2.01	5.37 ± 0.44	7.41	1.19	83.77	0.001
	5.0	153.01 ± 13.49	42.59 ± 2.91	3.40	0.95	72.17	0.001
L-LEUCINE	0.1	12.68 ± 0.18	2.08 ± 0.11	14.09	2.31	83.60	0.001
	0.5	43.68 ± 3.06	8.34 ± 0.45	9.71	1.85	80.91	0.001
	5.0	148.56 ± 9.48	65.01 ± 2.09	3.30	1.45	56.24	0.001
L-METHIONINE	0.1	9.44 ± 0.89	1.42 ± 0.11	10.49	1.58	84.96	0.001
	0.5	31.34 ± 3.29	6.22 ± 0.41	6.96	1.38	80.15	0.001
	5.0	120.74 ± 12.44	42.39 ± 1.71	2.68	0.94	64.89	0.001
L-PHENYLALANINE	0.1	5.84 ± 0.81	1.46 ± 0.07	6.49	1.62	75.00	0.001
	0.5	30.13 ± 1.41	5.42 ± 0.21	6.70	1.20	82.01	0.001
	5.0	145.50 ± 4.95	49.68 ± 2.98	3.23	1.10	65.86	0.001

TABLE 7.5

The effect of lowered Na^+ levels on the kinetics of amino acid uptake in rat jejunual segments of varying age

Kinetic constants have been calculated from linear regression of Hofstee plots, r^2 indicates the coefficient of determination, on only 3 pairs of values for each substrate at each age

Vmax in nanomoles/mg protein/15 min $\pm$ standard error of the regression coefficient

K_m in mM $\pm$ standard error of the regression coefficient

SUBSTRATE		AGE (days)					
		2		15/16		26	
		Na^+	Low Na^+	Na^+	Low Na^+	Na^+	Low Na^+
L-LYSINE	Vmax	126.54 ± 21.70	55.49 ± 27.26	135.47 ± 79.60	115.44 ± 90.45	72.24 ± 45.80	45.85 ± 19.42
	K_m	1.36 ± 0.34	2.06 ± 1.41	1.84 ± 1.55	5.55 ± 5.40	1.13 ± 1.13	1.89 ± 1.12
	r^2	0.94	0.68	0.58	0.51	0.50	0.74
L-ARGININE	Vmax	58.21 ± 5.70	66.95 ± 20.95	46.73 ± 13.12	68.55 ± 19.66	38.16 ± 7.60	28.56 ± 3.09
	K_m	0.77 ± 0.12	1.81 ± 0.78	0.33 ± 0.18	2.00 ± 0.78	0.57 ± 0.19	2.08 ± 0.30
	r^2	0.98	0.84	0.77	0.87	0.90	0.98
L-VALINE	Vmax	213.81 ± 5.83	297.40 ± 86.42	448.58 ± 259.93	272.19 ± 135.09	262.14 ± 19.57	101.88 ± 48.41
	K_m	0.93 ± 0.04	14.91 ± 4.77	2.23 ± 1.76	16.53 ± 8.98	3.55 ± 0.33	7.80 ± 4.34
	r^2	1.00	0.91	0.62	0.77	0.99	0.76
L-LEUCINE	Vmax	154.99 ± 35.61	189.83 ± 122.06	194.07 ± 46.35	126.73 ± 5.64	184.58 ± 25.75	158.71 ± 68.33
	K_m	0.89 ± 0.32	10.32 ± 7.59	1.18 ± 0.42	6.40 ± 0.34	1.43 ± 0.28	7.94 ± 3.99
	r^2	0.89	0.65	0.89	1.00	0.96	0.80
L-METHIONINE	Vmax	127.03 ± 19.30	141.15 ± 58.56	164.90 ± 28.24	139.31 ± 37.53	151.12 ± 31.08	104.43 ± 17.94
	K_m	0.66 ± 0.16	7.43 ± 3.63	1.25 ± 0.31	9.74 ± 2.99	1.61 ± 0.46	7.50 ± 1.50
	r^2	0.94	0.81	0.94	0.91	0.92	0.96
L-PHENYLALANINE	Vmax	264.97 ± 57.39	212.53 ± 58.57	220.86 ± 33.35	100.10 ± 91.58	265.84 ± 47.08	108.74 ± 92.05
	K_m	2.68 ± 0.77	9.04 ± 2.86	1.56 ± 0.33	4.95 ± 5.77	4.17 ± 0.92	7.62 ± 7.69
	r^2	0.92	0.91	0.96	0.42	0.95	0.50

TABLE 7.6

The effect of ouabain (1.0mM) on amino acid uptake into jejunal segments from rats of various age

Uptake in nanomoles/mg protein/15 min; Values are means $\pm$ SEM

n = 8 (control); 4 (ouabain) at each point

% I = percentage inhibition

- = no inhibition

2 tailed Students 't' test

NS = not significant

* = $p < 0.05$

** = $p < 0.01$

*** = $p < 0.001$

SUBSTRATE		AGE (days)								
		2			15			25		
0.5mM		CONTROL	+ OUABAIN	% I	CONTROL	+ OUABAIN	% I	CONTROL	+ OUABAIN	% I
L-LYSINE	Uptake	30.28 ± 2.78	29.00 ± 3.32	-	19.13 ± 2.61	16.38 ± 4.31	-	12.60 ± 0.62	11.29 ± 0.70	-
	T/M	5.82	5.58	NS	4.17	3.57	NS	2.81	2.52	NS
L-ARGININE	Uptake	21.29 ± 1.32	21.13 ± 1.13	-	31.96 ± 4.41	20.76 ± 2.83	35.04	19.64 ± 0.99	16.38 ± 1.04	16.60
	T/M	4.09	4.06	NS	6.96	4.52	NS	4.38	3.65	NS
L-VALINE	Uptake	76.33 ± 6.74	22.56 ± 1.86	70.44	56.10 ± 2.69	36.63 ± 3.90	34.71	33.34 ± 2.00	25.26 ± 2.66	24.24
	T/M	14.68	4.34	***	12.22	7.98	**	7.43	5.63	*
L-LEUCINE	Uptake	46.50 ± 2.48	27.10 ± 2.07	41.72	48.46 ± 4.71	24.63 ± 3.36	49.17	43.68 ± 3.06	38.86 ± 3.08	11.03
	T/M	8.94	5.21	***	10.56	5.37	**	9.74	8.66	NS
L-METHIONINE	Uptake	48.58 ± 2.71	22.06 ± 0.43	54.59	41.84 ± 3.25	22.40 ± 1.33	46.46	31.34 ± 3.29	21.24 ± 0.37	32.23
	T/M	9.34	4.24	***	9.12	4.88	***	6.99	4.74	*
L-PHENYLALANINE	Uptake	45.23 ± 2.12	19.07 ± 0.99	62.26	48.78 ± 5.90	30.97 ± 4.57	36.51	47.29 ± 1.46	36.77 ± 4.02	22.25
	T/M	8.70	3.67	***	10.63	6.75	*	10.54	8.20	*

concentration in the medium. Thus the neutral amino acid systems in the jejunum of the young rat which have been described are Na^+ dependent and require the expenditure of energy by the cell to maintain a Na^+ gradient. The basic system , while apparently Na^+ influenced, does not require an ionic gradient though somehow ATP is utilised in the uptake of these amino acids.

Discussion

In some way, amino acid transport in the rat jejunum is both dependent upon Na^+ ions and a supply of ATP from birth. The question arises as to whether the energy required is directly for carrier translocation or is used in maintaining a Na^+ gradient down which amino acids can travel and thus accumulate.

Glycolysis in the newborn is a major adaptation to the rigours of birth and the transient exposure to anoxia. Wilson and Lin (1960) found that intestinal sacs of 3 day old neonatal rabbit were capable of actively transporting L-tyrosine anaerobically. By 7 days of age this capacity decreased while aerobic dependence increased, and anaerobic transport was lost by 7 weeks. Donnelly (1971) showed that L-leucine transport in 1 day old rat ileum was inhibited by DNP but active uptake still occurred. Similarly States and Segal (1968) found that L-cystine was accumulated anaerobically in 1 week old rat gut. Merrill, Spring and Tousmis (1967) describe large glycogen masses in the mucosal cells of the foetal guinea pig which may act as an energy source. These stores disappear at the same time as mitochondria appear. In the rat, Cornell and Padykula (1969) describe large numbers of mitochondria in the foetal ileum (5 days before birth) and so aerobic potential is high at birth. The results here

describe generally a 50% or so reduction in uptake at 2 days of age, though concentrative uptake is not abolished. The fact that active uptake was not abolished by DNP at any age probably reflects the tissues capacity of ATP, sufficient (with that from the glycolytic breakdown of glucose in the medium) to sustain a degree of uptake. Luisier and Robinson (1975) also suggest that glucose is able to provide energy for transport by glycolysis in the adult rat (particularly the jejunum) but not in the guinea pig.

Na^+ ions are also somehow involved in amino acid uptake right from birth. States and Segal (1968) showed that transport in the young rat gut was less sensitive to Na^+, though this has not been observed here. What is clear is that Li^+ ions do not inhibit basic amino acid transport to the same extent as the neutral substrates, suggestive of a Na^+-independent transport system. This is backed up to a large extent by the finding that ouabain has no effect on basic amino acid uptake (see Table 7.6). While these amino acids do not depend on the presence of a Na^+ gradient across the mucosal cell membrane, Na^+ still appears to affect uptake. The role of this cation in basic amino acid uptake does not become clear from the Hofstee plots of uptake in the absence of Na^+; neither K_m nor Vmax appear to be affected to any extent (see Table 7.5).

The Na^+ energy link is much clearer with the neutral amino acids. Ouabain significantly reduces uptake of these amino acids, though never inhibits active uptake. Rosenberg (1966) found that the activity of Na, K-ATPase reached fully maturational levels at birth in the guinea-pig: coincident with the fully-functional development of active transport systems for amino acids. Cornell and Padykula

(1969) have demonstrated the presence of this enzyme in the neonatal intestine, and the results here with ouabain strongly suggest it is present in the jejunum at birth. In a recent review of membrane transport, Martonosi (1975) describes the developmental changes in Na^+, K^+-ATPase in a range of tissues. In the rat, activity in a number of tissues increases after birth, though the profile for intestinal tissue is not described. Ingham (1972) showed that active sugar transport is present in foetal rat jejunum and so, by inference, Na, K^+-ATPase must be present in the gut at this time.

Na^+ independent transport of L-lysine in rat intestine was described by Munck and Schultz (1969). However, transmural transport had a considerable dependence upon Na^+. Their findings are reflected in the results of this work.

The role of Na^+ ions in solute transport has a weight of evidence in its favour (reviewed by Schultz and Curran, 1970). The hypothesis is that the carrier site is capable of combining with free Na^+ and substrate, resulting in a ternary complex which is more stable than the carrier amino acid complex alone. Absence of Na^+ causes a substantial depression of substrate influx, but maximal rate can be achieved by raising the substrate concentration. The lowered affinity of amino acid for its carrier in the low Na^+ condition has been observed here. The increased Na^+ concentration inside the cell is then regulated by activity of the sodium pump.

Measurement of transmural potential differences (P.D.) has provided much support for the Na^+-gradient hypothesis first put forward by Crane (1962) for sugar transport. Schultz and Zalusky (1965) showed that in the rabbit ileum, amino acids produced an increase in the

potential difference across the intestinal wall, and ouabain inhibited this effect. These findings have been confirmed in a number of species, including man, and suggest that hexoses have a separate transport system (see Wiseman, 1974). Nakamura et al (1974) report that certain D-amino acids also produce potential differences in the rat small intestine. Interestingly, Kohn, Smyth and Wright (1968) were unable to demonstrate a change in P.D. across the rat small intestine with L-lysine or L-arginine. However, Munck (1970) explained this in terms of a large co-flux of Cl^- ions in the rat jejunum which negated any rise in P.D.

The direct involvement of energy in carrier translocation has been suggested by a number of workers (Kimmich, 1970; Christensen, 1973), who have reported amino acid transport against a Na^+ gradient. However, Bronk and Leese (1974) found that the accumulation of substrates by mucosal cells was independent of the concentration of ATP. Similarly Murer and Hopfer (1974) found that vesicles of brush border membrane (not containing ATPase) could accumulate alanine in the presence of Na^+. Thus the critical factor for transport is the presence of a Na^+ gradient. However, Hardcastle (1974) has reported an increased permeability effect due to ATP chelating divalent cations.

The presence of Na, K-ATPase in the basolateral membranes of the mucosal cells is now well established (Chapter 2) although it was not demonstrated for some years initially. However the evidence for this enzyme's activity as being the only sink for metabolic energy is questionable. Transport in many tissues is reduced by ouabain though never eliminated, as we have seen here. Robinson (1970) has

suggested that a second enzyme system is involved in bulk Na^+ flow through the intestine, possibly the cytochrome system.

Cyclic AMP (cAMP), which is abundant in intestinal tissue, has also been implicated in solute transport. Theophylline, which raises cAMP (by inhibiting its breakdown) stimulates neutral and basic amino acid transport in the rat jejunum (Kinzie, Ferrendelli and Alpers, 1973) and also stimulates NaCl secretion. Similarly, dibutyryl cAMP enhances neutral substrate accumulation. Burrill, Satelmeyer and Lerner (1974), with similar work on chick intestine, suggest a model whereby cAMP activates a Na^+ sensitive protein kinase, which in turn phosphorylates a membrane element (carrier?), thus enhancing carrier translocation. This model necessitates a direct involvement of ATP in carrier translocation which Burrill, Satelmeyer and Lerner (1976) have subsequently reported for neutral amino acid uptake in the chick. Recently, Leese, Prendergast and Read (1976) have examined the effect of theophylline on glucose and fluid transport across the rat jejunum. They describe increased sugar uptake in the tissues with theophylline as being the result of diminished salt absorption due to high cAMP levels. The intercellular spaces are thus very small and so there is less wash out of the glucose inside the cells. These authors did not observe any effect of cAMP mediated secretion on the coupled influx of sodium and glucose at the brush border.

Finally, two other theories put forward to explain substrate transport are worth considering here. Mitchell (see review, 1976) has suggested that coupling between enzymatic reactions and membrane transport might be due to enzymes incorporated in such a way that the very process of reaction performs transport across the membrane.

Mitchell's chemi-osmotic coupling theory has found application in the interpretation of transport in the phosphotransferring system in bacteria, and extensively in terms of oxidative phosphorylation. Christensen (1975) has expanded the ideas of proton gradients to encompass plasma membranes. He suggested that such gradients of H^+ or cations could be stored in a membrane after ATP cleavage and thus facilitate secondary active transport. Na^+/H^+ antiport has been invoked to describe transepithelial electrolyte transport in the small intestine and recently Murer, Hopfer and Kinne (1976) have described such a system in the brush border membrane. Addition of Na^+ causes expulsion of protons which thus stimulates the uptake of Na^+. Conversely, Garcia-Sancho, Sanchez and Christensen (1977) have recently described uptake of acidic amino acids into the ascites tumor cell in terms of cotransport with H^+. Reducing pH stimulates L-glutamate transport by activating a Na^+ independent system ('L').

The tripeptide glutathione (γ-glutamyl-cysteinyl-glycine) is found in large amounts in liver, kidney and intestine. Meister (1973) has proposed a role for glutathione in the membrane transport of amino acids in the kidney which also incorporates the idea of ATP being required for the reformation of the active substrate carrier. The hypothesis involves glutathione and membrane bound γ-glutamyl transpeptidase which can utilise all the common amino acids except proline. The so formed γ-glutamyl peptides are then transported and released intracellularly as free amino acids by the action of γ-glutamyl cyclotransferase. ATP is required for the synthesis of glutathione and the two enzyme steps. It is suggested that γ-glutamyl transpeptidase is located close to a pore in the membrane

also used as an amino acid binding site, with groups specific for attachment of α-amino and α-carboxyl groups. Proline being an imino acid does not have free amino and carboxyl groups and so would not associate with this membrane binding site.

Young, Ellory and Tucker (1975), working with erythrocytes from sheep with a low glutathione content, found that there was a diminished uptake of amino acids. They concluded, however, that this membrane defect was not a consequence of low glutathione or the high levels of basic amino acids associated with these cells. Low uptake of cysteine was suggested as the primary cause for diminished glutathione in these cells (Young, Ellory and Tucker, 1976).

Orlowski (1976) has recently re-evaluated the role of glutathione in transport processes. Some cells are deplete and yet still transport amino acids. γ-glutamyl transpeptidase is highly concentrated in the brush border membrane of the rat small intestine (Curthoys and Shapiro, 1975). In addition it is villus-specific and inversely proportional to the cell levels of glutathione, which Cornell and Meister (1976) suggest is evidence for a high rate of usage of glutathione in villus tip cells. As the cells migrate from the crypts to the villus tips, γ-glutamyl transpeptidase is synthesised in concert with increasing amino acid absorptive ability.

The results from the experiments described here suggest only that oxidative phosphorylation is important for amino acid uptake from birth and that Na^+ ions are somehow integral to either carrier substrate binding (basic amino acids and neutrals) or carrier translocation (neutral substrates). The results with ouabain strongly implicate the use (by neutral amino acids) of Na^+ gradients across

the intestine which appear to be present early in life. The links between energy and transport can be made at many points in the uptake process : for carrier translocation, for Na^+ translocation or to generate other ion gradients. The precise mechanism (or more likely, series of mechanisms) as yet remains obscure despite a gallery of attractive theories.

CHAPTER 8

Glucocorticoids and Amino Acid Absorption in Development

Introduction

Glucocorticoids have been implicated in the structural and functional changes observed in the rat small intestine at weaning; stimulating the mitotic activity of the crypts and so increasing the migration rate of cells along the villi (Herbst and Sunshine, 1969). Coupled with the appearance of these cells there is, at this time, a marked decrease in the ability of the enterocytes to absorb macromolecules ("closure"), an increase in the activity of sucrase, maltase and alkaline phosphatase, and changes in membrane transport systems for particular substrates (Koldovsky, 1969).

Results reported here suggest that there are changes in carrier affinity and specificity for amino acids as the jejunum develops, and that these are particularly significant during the third postnatal week, the time of weaning. These findings strongly suggest that the "new cell type" emerging from the crypts has a functionally distinct plasma membrane with arrays of carrier systems more typical of the mature gut.

Daniels et al (1973) have shown that the injection of glucocorticoids into the rat early in life results in the progressive displacement of vacuolated cells from the villi, these being rapidly replaced by new cells from the crypts (Herbst and Sunshine, 1969). If the changes in amino acid uptake around weaning described here are a function of the appearance of this new cell type, then steroid action on the crypt cells should result in altered characteristics of amino acid uptake.

Results

Circulating levels of glucocorticoid hormones in the rat are low until 18 days postpartum (Daniels et al, 1972), the major rat glucocorticoid being corticosterone. Daniels et al (1973) found that injection of cortisone acetate at Day 12 resulted in total "closure" of the enterocytes to PVP uptake by Day 16. Similarly, adrenalectomy delays "closure" until Day 24.

Half the rats of mixed, standardised litters were injected subcutaneously with 2.5mg of cortisone acetate (Cortistab - Boots Pharmaceuticals) on the 12th day of age, ear punched for recognition and left with mother. Four days later, both injected animals and their littermates were removed and everted jejunal segments prepared in the usual way. Uptake of the two basic amino acids L-lysine and L-arginine was looked at in detail as they seemed developmentally interesting from the previous results (see Chapters 5 and 6).

Figure 8.1 shows the effects of cortisone acetate on body weight. Striking reductions are evident two days after glucocorticoid injection; this effect on body weight persists throughout development. Table 8.1 compares the effects of cortisone acetate on the amount of protein in jejunal segments from two typical experiments. It is obvious that, while glucocorticoids severely reduce body weight, there is no effect on the protein content of the small intestine. Thus uptake can be expressed in terms of mg protein with some confidence (see also Plates 20 and 21).

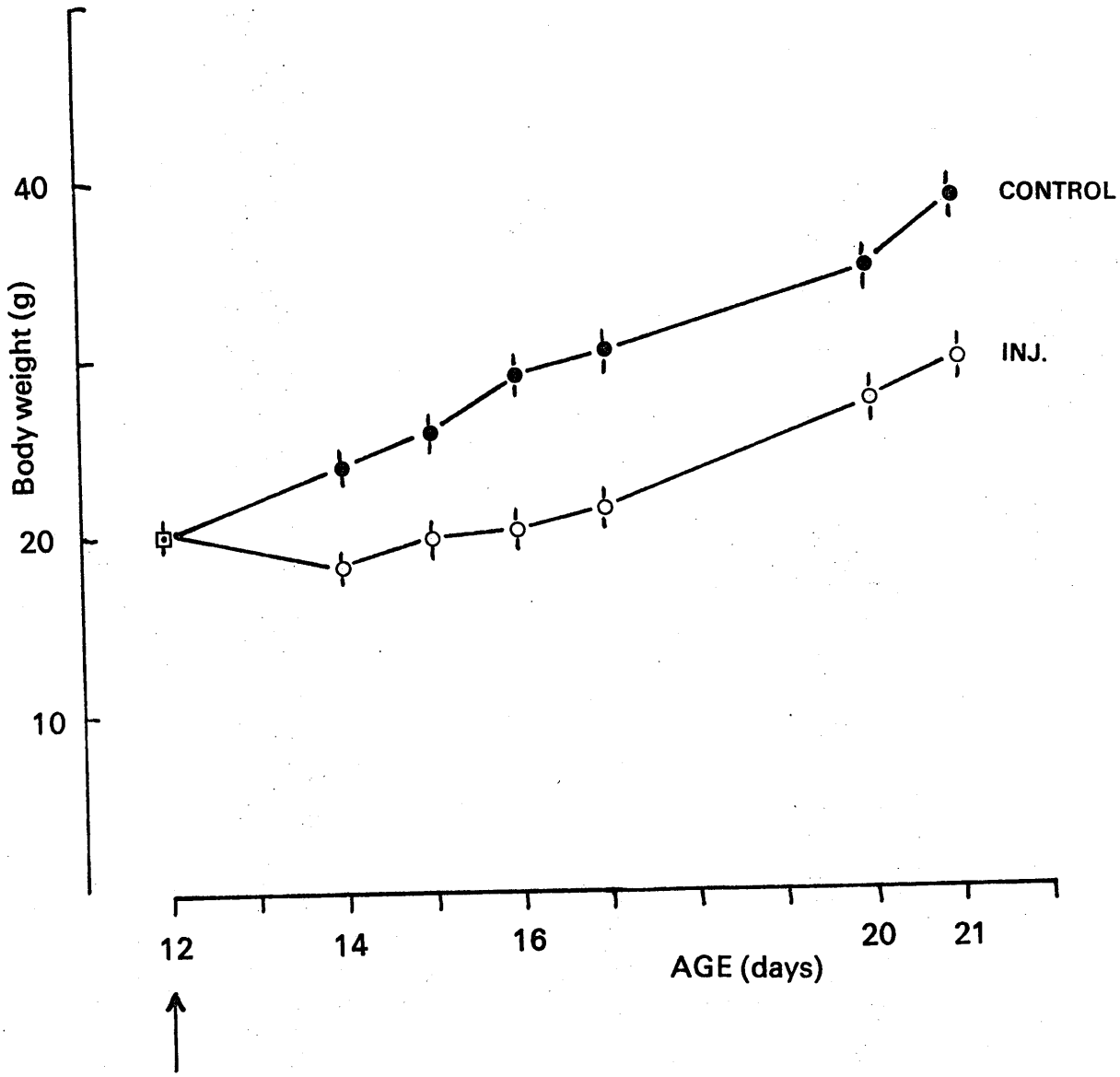


Figure 8.1 Effects of cortisone acetate injected in 12 day old rats on body weight gains with maturation.

Bars represent standard errors of the means of 8 observations for controls and 6 for injected, at each point.

Plate 20

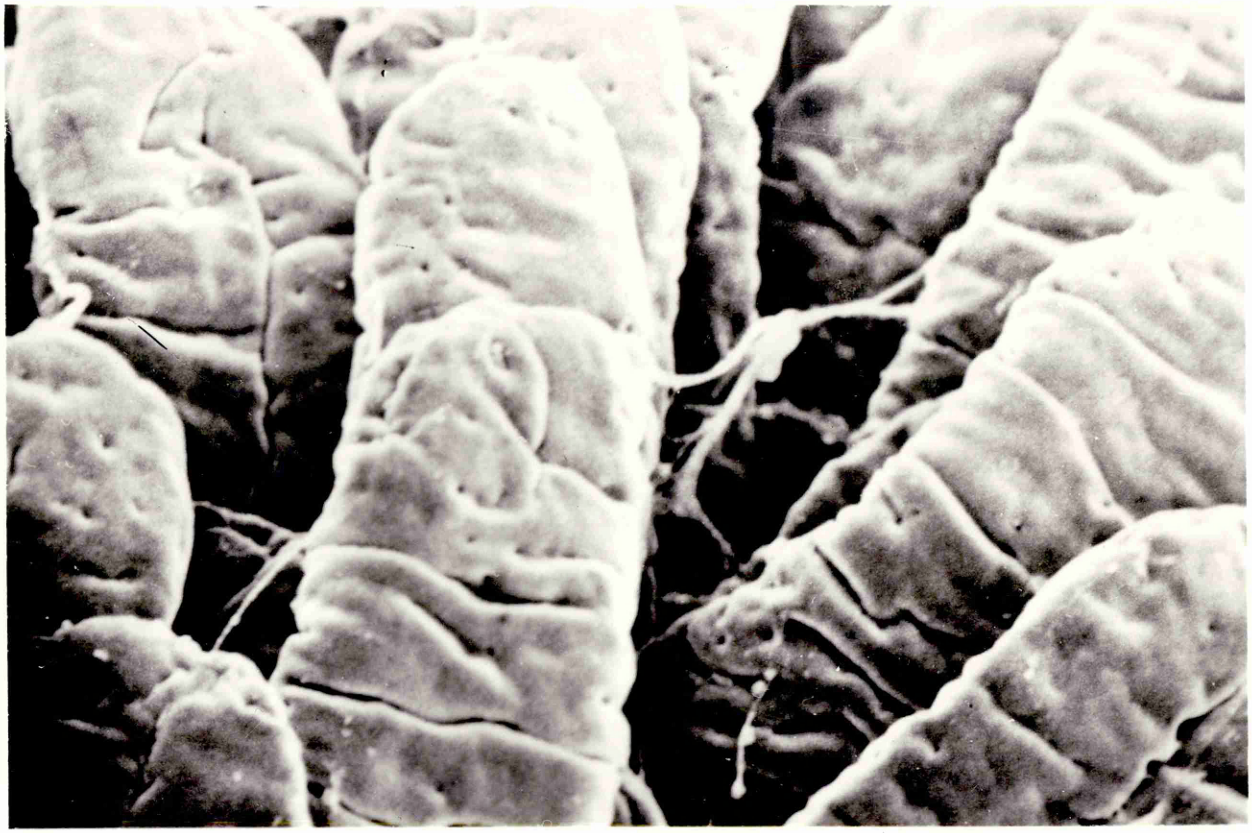
Scanning electronmicrograph of group of villi from 16 day old rat jejunum. The pores which are clearly seen studded over the villi probably represent the openings of the goblet cells. Strands of mucous can be seen between the villi.

X 200

Plate 21

Scanning electronmicrograph of group of villi from 16 day old rat jejunum. This animal had been injected 4 days previously with cortisone acetate. No obvious deformation of the villi has resulted from treatment.

X 400



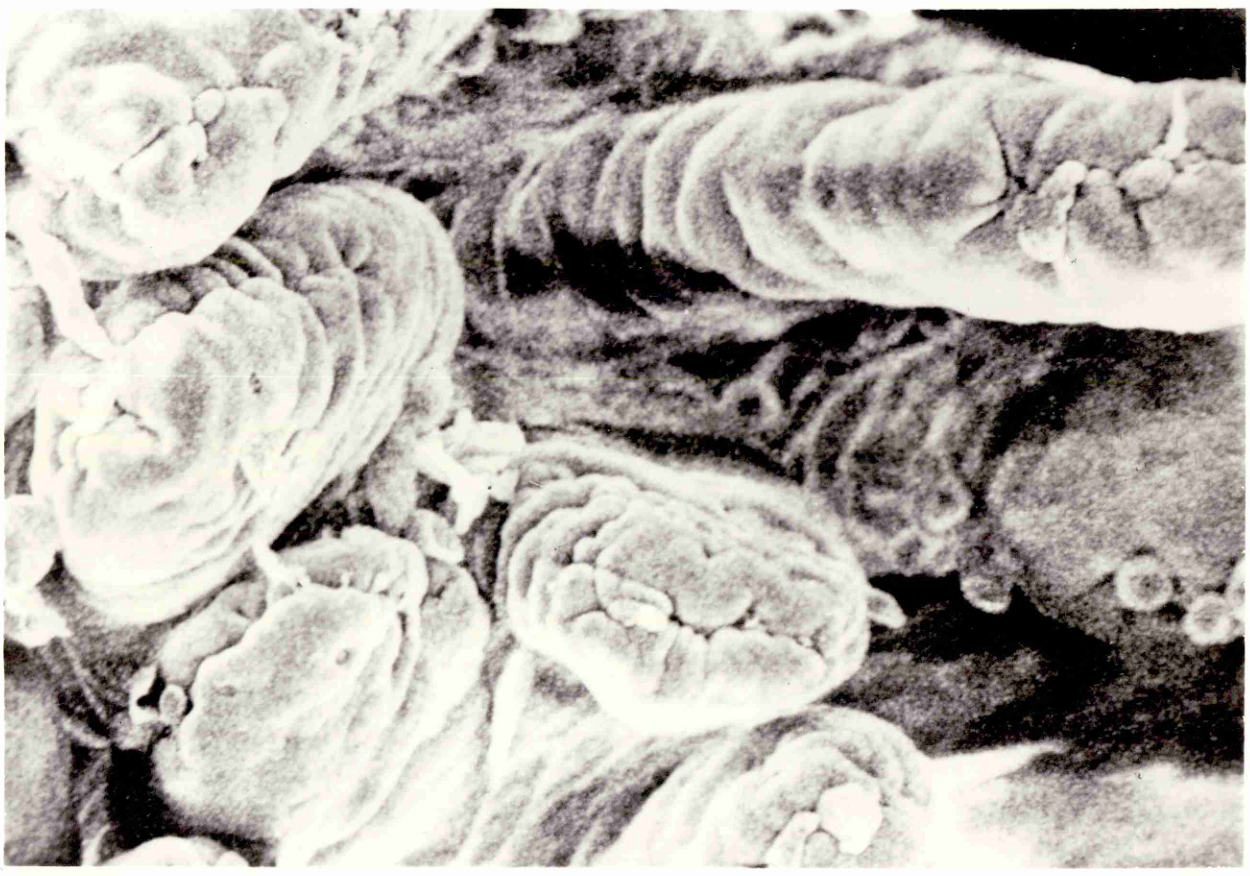


Table 8.1 Effects of cortisone acetate injected in 12 day old rats on protein content of jejunal segments prepared four days later on Day 16.

Jejunal Segments (mg protein/segment)	
INJECTED	CONTROL
5.14 ± 0.25 (n=32)	5.15 ± 0.17 (n=32)

1) Effects of cortisone acetate on kinetics of basic amino acid uptake

Figure 8.2 shows the uptake of L-lysine and L-arginine in jejunal segments from normal 16 day old rats and those injected 4 days previously with cortisone acetate. Uptake of L-lysine in injected rats is half that of control littermates and saturates at 1mM while control uptake has not saturated at this concentration. Uptake of L-arginine in injected rats is below that of controls at low concentrations but saturation occurs at 1mM in both. Injected animals still exhibit active uptake of basic amino acids but to a far lesser extent.

Hofstee plots of this data are shown in Figure 8.3 and the kinetics of uptake in injected and control rats are well contrasted in the case of L-lysine. While the K_m is apparently unaffected by the administration of glucocorticoids, the Vmax of L-lysine is markedly reduced, though L-arginine does not show such a change (see Table 8.2).

Figure 8.2 Kinetics of uptake of a) L-lysine and b) L-arginine in everted jejunal segments from 16 day old rats; comparison between control animals and those injected 4 days previously with 2.5mg cortisone acetate.

V = nanomoles substrate accumulated/ mg. protein/15 min. T/M = concentrative uptake.

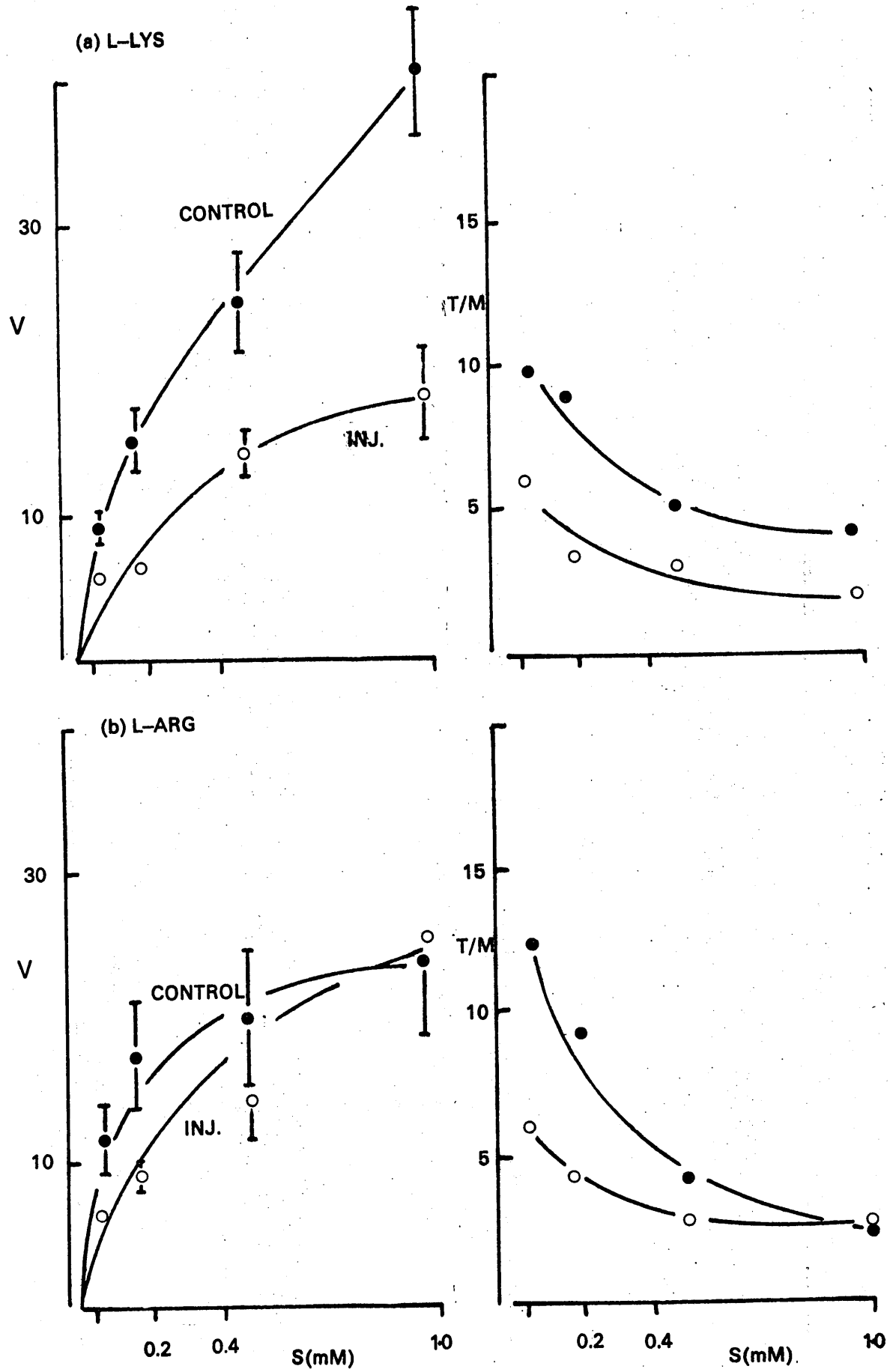
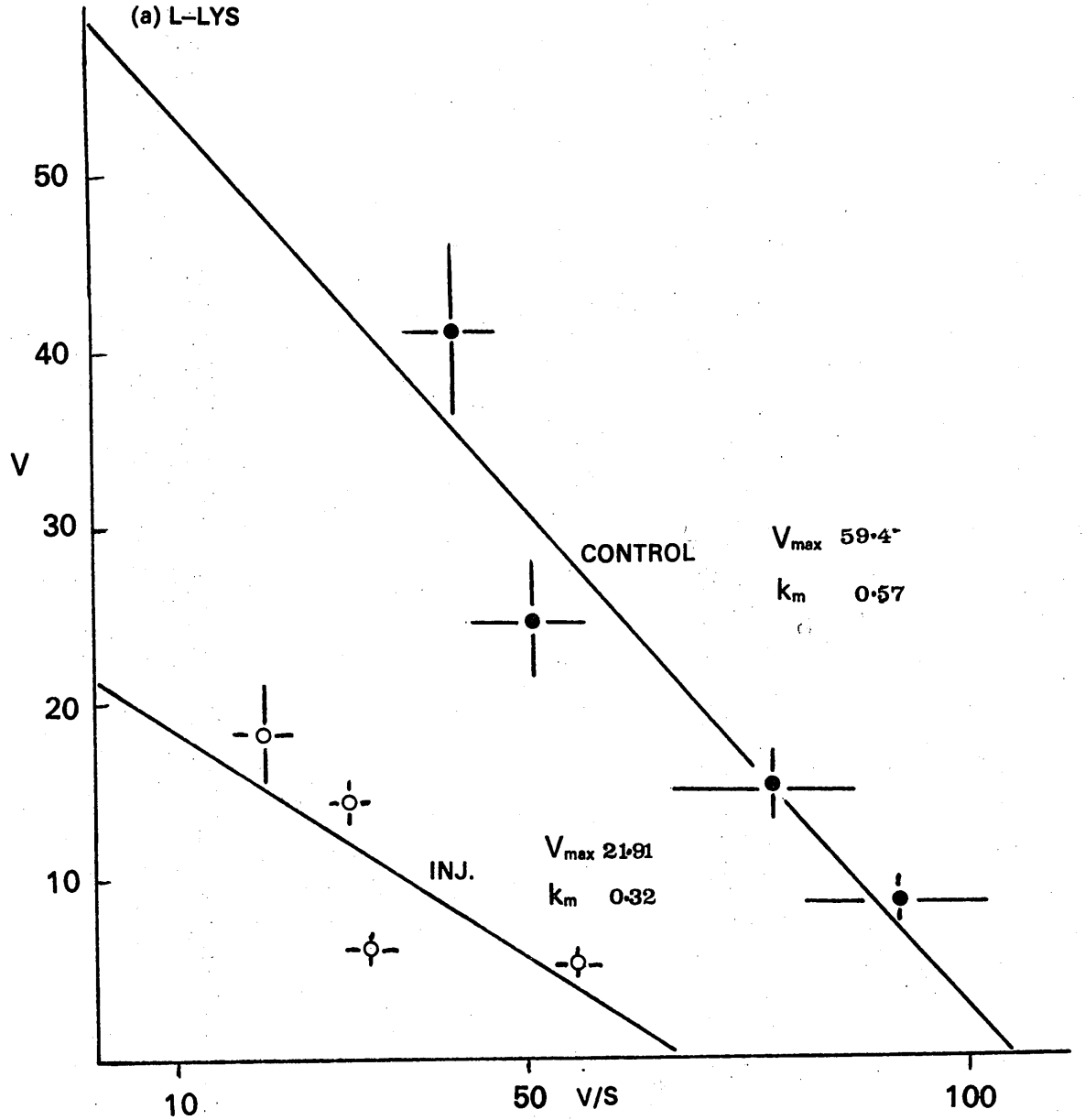

(a) L-LYS
CONTROL
INJ.
V
30
10
T/M
15
10
5
(b) L-ARG
CONTROL
INJ.
V
30
10
T/M
15
10
5
0.2
0.4
1·0
S(mM)
0.2
0.4
1·0
S(mM)

Figure 8.3 Hofstee plots of data in Figure 8.2 to calculate values of Vmax and K_m for a) L-lysine and b) L-arginine uptake in 16 day old rat jejunum.


(a) L-LYS
50
40
V
30
20
10
CONTROL
V_{max} 59·4
k_m 0·57
INJ.
V_{max} 21·91
k_m 0·32
10
50 V/S
100

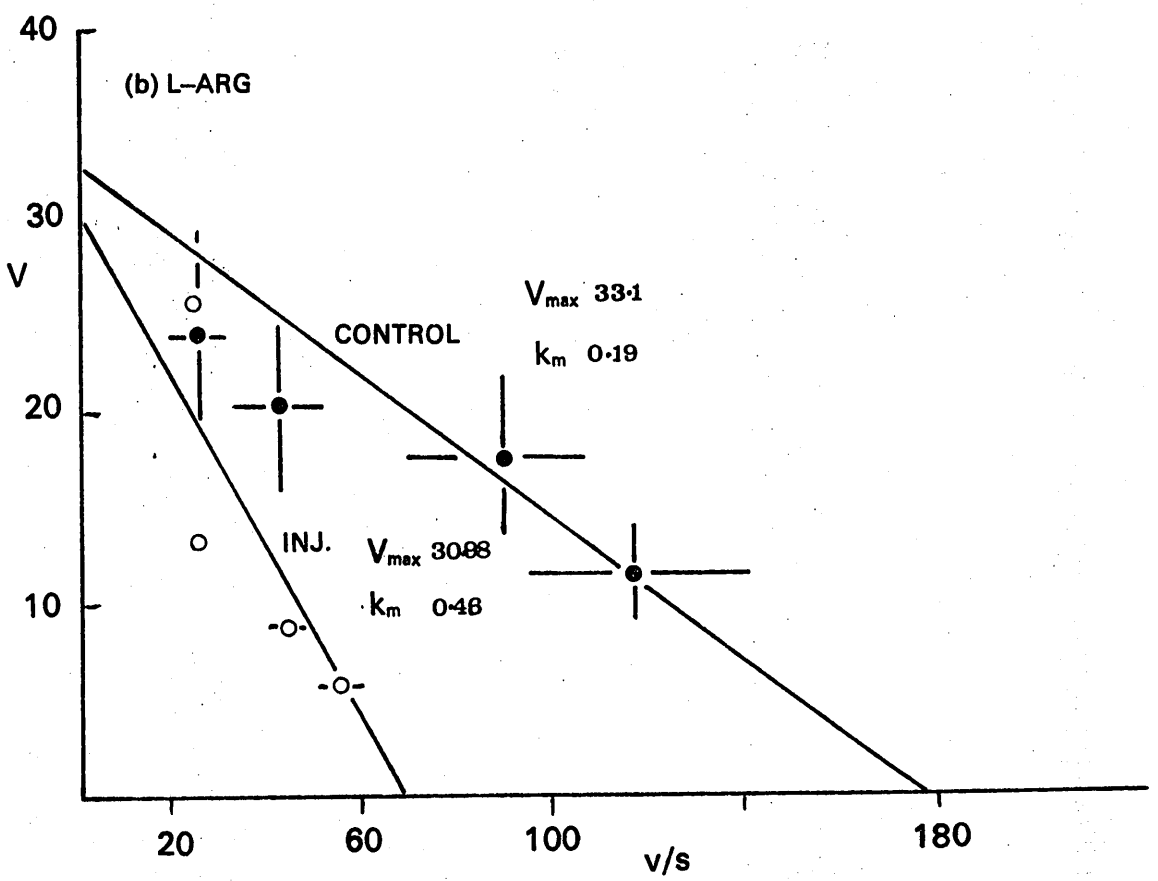

40
(b) L-ARG
30
V
20
10
CONTROL
V_{max} 33·1
k_m 0·19
INJ.
V_{max} 30·88
k_m 0·46
20
60
100
180
v/s

Table 8.2 Kinetic constants derived from Figure 8.3 : comparison of injected with control animals.

Vmax = nanomoles/mg protein/15 min. ± standard error of the regression coefficient

K_m = mM ± standard error of the regression coefficient. r^2 = coefficient of determination

	16 DAY INJ			16 DAY CON		
	Vmax	K_m	r^2	Vmax	K_m	r^2
L-LYSINE	21.91 ± 6.41	0.32 ± 0.18	.62	59.42 ± 9.93	0.57 ± 0.15	.88
L-ARGININE	30.98 ± 9.99	0.46 ± 0.25	.64	33.10 ± 4.33	0.19 ± 0.06	.75

INJ = injected animals

CON = littermate controls

The reduction in the uptake of L-lysine in injected animals may possibly result from the early appearance of the "new" cell type on the villi, with carriers that are more substrate specific.

2) Effects of cortisone acetate on the specificity of carrier systems

The uptake of L-lysine and L-arginine was measured in the presence and absence of 4 neutral amino acids. All experiments were carried out using jejunal segments from rats of 16 days old which had been injected with cortisone acetate on day 12, and the results are shown in Table 8.3.

Both L-leucine and L-methionine significantly inhibit L-lysine uptake by some 40%, but concentrative uptake is not abolished. Only L-valine affected L-arginine uptake and to a lesser extent (25%). In Table 8.4 the K_i of these inhibitors (calculated as before from the Dixon and Webb algebraic method) are compared in glucocorticoid treated rats and normal pre- and post-weaned rats. L-arginine is inhibited by L-valine in all three cases, but the K_i in injected animals is in the direction of that for post-weaned rats. Whereas in normal 16 day old rat jejunum L-valine appears to be a fully competitive inhibitor of L-arginine uptake, the situation in the 26 day old and injected animals is one which suggests only partial competition, and the possibility exists that the induced cell population has a more specific system for L-arginine.

How does this theory stand as regards L-lysine? L-methionine, L-leucine and L-phenylalanine all inhibit L-lysine uptake in normal 16 day old rats. The picture for injected animals is

TABLE 8.3

Inhibition of basic amino acids by neutrals in jejunal segments from 16 day old rats injected with cortisone acetate on Day 12

V = mean accumulation in nanomoles of test substrate/mg protein/15 min ± SEM

T/M = concentration ratio

p = significance using unpaired Students 't' test

% I = percentage inhibition

\- = no inhibition

n = number of experiments

INHIBITOR I 5mM	L-METHIONINE n=6				L-LEUCINE n=6				L-VALINE n=6				L-PHENYLALANINE n=6			
0.5mM TEST	V	T/M	%I	p <	V	T/M	%I	p <	V	T/M	%I	p <	V	T/M	%I	p <
L-LYSINE V 14.60 ± 1.16 T/M 3.18 n = 8	8.96 ± 0.59	1.95	39%	0.001	8.44 ± 0.75	1.84	42%	0.001	11.71 ± 0.92	2.55	20%	NS (0.1)	15.59 ± 0.87	3.40	-	NS
L-ARGININE V 23.27 ± 1.56 T/M 5.07 n = 8	22.00 ± 1.86	4.79	-	NS	22.42 ± 1.35	4.88	-	NS	17.40 ± 1.26	3.79	25%	0.05	20.00 ± 0.63	4.36	-	NS

Table 8.4 Inhibition of basic amino acids by neutrals. K_i values determined from Table 8.3 and compared with values from pre-weaned and post-weaning control rats.

INHIBITORS OF → / AGE ↑	L-LYSINE			L-ARGININE		
	INJ 16	CON 16	CON 24	INJ 16	CON 16	CON 24
L-METHIONINE % I	39%	31%	NIL	NIL	NIL	NIL
L-METHIONINE K_i	5.05	7.89	-	-	-	-
L-LEUCINE % I	42%	57%	30%	NIL	NIL	NIL
L-LEUCINE K_i	4.62	2.68	7.56	-	-	-
L-VALINE % I	NIL	NIL	24%	25%	31%	31%
L-VALINE K_i	-	-	10.63	9.50	1.74	5.08
L-PHENYLALANINE % I	NIL	40%	NIL	NIL	NIL	NIL
L-PHENYLALANINE K_i	-	5.45	-	-	-	-

% I = percentage inhibition

K_i in mM

INJ = injected animals

CON = littermate controls

somewhat changed. L-phenylalanine does not inhibit L-lysine uptake now and the degree of L-leucine inhibition is markedly reduced (both characteristic of the post-weaned jejunum).

These results suggest that the observed decrease in L-lysine uptake in the small intestine after the third week of life can be simulated by early exposure of the tissue to exogenous glucocorticoids. The new population of absorptive cells thus induced have a carrier system for basic amino acids which is much more specific and defined, and cannot be used by other substrates. Alternatively basic amino acids may not be able to use carrier sites previously unspecific.

The appearance of more specific transport systems for amino acids at the time of weaning has been suggested in Chapter 6 to explain the observed changes in kinetics of uptake and substrate specificity. These results further support the concept of functionally distinct carrier sites appearing coincident with the new absorptive cell.

Discussion

The effects of early administration of glucocorticoids in the young rat are most striking. The decrease in body weight which has been described is due to a depression of bone growth and interference with protein synthesis (increased gluconeogenesis from protein). There is some evidence that corticosteroids antagonise the activity of growth hormone. The appearance of these animals shows patchy fur growth, runting and precocial eye opening. Cortisol affects the brain quite severely in neonates; mitosis is inhibited and this results in a permanent cell deficit of 20-30%. Some few days later, with the appearance of intrinsic adrenal activity, there is no mitotic inhibition.

The work by Daniels et al and other workers described in Chapter 2 suggests that the cessation of macromolecular absorption at weaning and the increase in villus cell turnover are both events that are under the control of glucocorticoids which are at increased levels in the blood at this time. Similarly we have seen from the work of Moog and Koldovsky that glucocorticoids regulate the appearance of some enzyme activities notably alkaline phosphatase, sucrase and maltase. What are the mechanisms by which glucocorticoids cause intestinal maturation?

Firstly, the changes brought about by these steroids are slow, some 72 hours, a length of time which agrees well with the new turnover time of crypt cells. Studies with sucrase support this view: the enzyme first appears in cells at the base of the villi and activity proceeds towards the tip (Herbst and Koldovsky, 1972). Similarly, vacuolated cells typical of pinocytosis are displaced from mature cells arising in the crypts (Williams and Beck, 1969) and the time period of "closure" takes some 72 hours. If the affect of glucocorticoids is on the genome causing synthesis of new proteins, it is extremely complex, being a mixture of activation and repression. Henning and Kretchmer (1973) suggest that the steroid causes the synthesis of new messenger-RNA, a classical situation, which can be blocked by the inhibitor, actinomycin.

Forstner and Galand (1976) have recently developed a method for isolating intestinal microvillus membrane from suckling rats to study the membrane changes at weaning. Administration of cortisol increased the activity of several membrane bound hydrolases and produced marked changes in the protein and glycoprotein composition

of the isolated plasma membrane. They concluded that cortisol does not alter membrane composition by inhibiting degradation of selected glycoproteins but that it stimulates the synthesis of specific membrane glycoproteins (e.g. alkaline phosphatase, sucrase and maltase) while inhibiting synthesis of others.

Henning, Ballard and Kretchmer (1975) examined the cytoplasmic fraction of intestines from neonatal rats for the presence of specific glucocorticoid - binding proteins. Using labelled dexamethasone, they showed that cytoplasmic macromolecules with high specificity for steroids were present (mainly in mucosal tissue) from late foetal life through adulthood, but concentrations were significantly higher during the first two postnatal weeks. These were not Vitamin D receptors. Pressley and Funder (1975), using a similar binding technique, have described mucosal cytoplasmic glucocorticoid receptors in the adult rat. These were found to be more abundant in the duodenum and jejunum than in the ileum.

These findings confirm the regulatory role of glucocorticoids in the maturation of the small intestine, a peak in adrenal activity coinciding with a significant concentration of receptors in the gut. It is interesting to speculate that the onset of weaning, change in diet etc. in the rat results in increased stress and adrenal activity (Brambell, 1970). The administration of glucocorticoids in these experiments could of course result in other, more direct changes in substrate transport; hormones have well documented effects on the absorptive, metabolic and digestive functions of the small intestine which must be considered.

Apart from primary actions such as the induction of crypt cell mitosis, hormones can produce functional intestinal changes by:

(a) Secondary actions on muscular movements, blood flow etc.

(b) Modifying appetite, eating times and so putting circadian rhythms out of phase.

(c) Sensitising tissues to other hormones in a synergistic way.

(For a full account see Levin, 1969). Shishova (1959) reported evidence that cortisone considerably increased amino acid absorption by normal rats, and that adrenalectomy reduced amino acid uptake in vivo. Kansal and Wagle (1975) have reported that hydrocortisone (and ACTH) treatment in rats led to the increased transport (300%) of L-histidine in the intestine in vivo. There is conflicting evidence concerning the influence of thyroid hormones. Excess thyroid hormone has no effect on amino acid uptake but hypothyroidectomy enhances absorption in the rat (see Wiseman, 1974). Syme and Levin (1976) have recently investigated the effect of altered thyroid status in the rat (induced by thyroid hormones) on intestinal electrogenic valine transfer. Hypothyroidism increased the Vmax (but not K_m) of valine uptake and thyroid hormone treatment redressed this increase. Insulin had no effect on the absorption of a number of amino acids (Akedo and Christensen, 1962) in the rat diaphragm but isolated intestine from alloxan-diabetic rats showed increased transport of amino acids, (Wiseman, 1974; Kansal and Wagle, 1975). Mainoya (1975) looked at the effect of prolactin on sugar and amino acid transport by the rat jejunum. Significant increases in jejunal absorption of

D-glucose, L-glycine and L-proline as well as of fluid and NaCl occurred *in vivo*. Mainoya concluded that the action of prolactin on intestinal transport may primarily subserve a nutritional role rather than an osmoregulatory one, and could explain the increased intestinal transport of sugar and amino acids during pregnancy and lactation (Penzes and Simon, 1968). The primary action of hormones is probably on the metabolic machinery of the enterocyte, affecting energy for active transport or regulating the activity of Na^+, K^+-ATPase. Alternatively, hormones could exert permeability effects via cyclic nucleotides which are known to be active in the absorptive mucosal cells (see Chapter 2).

The induction effects of corticosteroids on the small intestine during the third postnatal week result in the appearance of a functionally distinct enterocyte. This new cell has the ability to produce a range of hydrolytic glycoproteins on its membrane which use maltose and sucrose as substrates. These "mature" enterocytes no longer exhibit pinocytosis on a large scale, have reduced numbers of lysosomes in the cytoplasm and have a short villus "life". Coupled with these changes, the carrier systems for amino acids on the plasma membrane have changed, becoming more specific to particular substrate configurations. These changes in the small intestine can be induced by causing the premature appearance of this new cell type.

CHAPTER 9

Final Discussion

The amount of protein in the milk diet of the neonatal rat is relatively low compared with the solid food diet of the adult. Coupled with this, proteolytic activity of the gut is low until the time of weaning, which results in small concentrations of free amino acids present in the lumen of the small intestine. Despite this, the absorptive cells of the neonatal jejunum have been shown to possess mechanisms from birth which effectively accumulate amino acids, in some cases to considerable levels (e.g. the neutral amino acids).

The kinetic studies described in Chapter 5 have shown that these uptake systems have high affinities for their substrates, ensuring high rates of uptake from luminal concentrations of 1mM or less Maturation of the small intestine occurs during the first three weeks of life, involving an increase in length, in villus height and further expansion of the absorptive surface area through increasing numbers of villi. As a result, the population of carrier systems increases and accumulation of amino acids rises post-natally, reaching a peak around the second week of life.

Competition experiments (see Chapter 6) have revealed something of the recognition and binding steps, prerequisite for amino acid uptake into the cells. It appears that the carrier systems present in the jejunum of the early postnatal rat fall broadly into two classes; those involved with basic amino acid uptake (L-lysine and L-arginine) and another population concerned with the transport of neutral substrates (L-valine, L-leucine, L-methionine and L-phenylalanine). However, there appears to be some functional overlap, all the neutral amino acids being able to interfere with L-lysine uptake. Calculations

of the affinity of neutral substrates for the basic carrier have shown that the former are probably acting in a partially-competitive way, inhibiting basic substrate uptake by steric hindrance or possibly in a non-competitive fashion, depleting the cells' energy stores. A polyfunctional nature of amino acid carriers in the early postnatal jejunum would, however, explain the discrepancy between the extent of uptake of the two basic amino acids at this time, L-lysine being accumulated to a far greater extent than L-arginine, if the former was able to utilise neutral transport sites. The basic carrier has a much higher affinity for L-arginine than L-lysine and this could also explain the lower uptake of the former. The postnatal jejunum accumulates the neutral amino acids to a greater extent than either of the basic substrates, indicating a larger population of carrier sites on the villi, capable of considerable functional overlap.

Concentrative uptake of both basic and neutral amino acids can be achieved by the postnatal jejunum even in the absence of a supply of ATP from oxidative phosphorylation (see Chapter 7). This suggests that glycolysis plays an important part neonatally in supplying ATP for energy dependant processes. The energy dependant step in the uptake of neutral amino acids appears to be involved with maintaining a Na^+ gradient across the absorptive cells. Na^+ ions are clearly involved in the uptake of all amino acids studied here, but in different ways for neutral and basic substrates. Abolition of the Na^+ gradient reduces neutral uptake but does not significantly affect basic accumulation. The role of Na^+ ions in the uptake of L-lysine and L-arginine may be concerned with the binding step of amino acid to carrier.

While the population of enterocytes that line the villi of the early postnatal gut is relatively static, significant changes in crypt cell kinetics occur in the third week of life which result in faster migration and turnover of these absorptive cells. This "new" population of enterocytes demonstrates a profile of hydrolytic enzyme activity similar to that of the adult small intestine, and no longer absorbs large macromolecules such as proteins. Coincident with the appearance of these functionally distinct absorptive cells is a change in the kinetics of amino acid uptake in the jejunum. The dramatic fall in substrate accumulation around this time may be artefactual due to the developmental changes in the non-absorptive tissue of the jejunum, but certainly by 2 months of age, the gut accumulates amino acids to a lesser extent than in the early postnatal period (see Chapter 5). The affinity of the carriers for their particular substrates does reveal a real decrease, and it is tempting to postulate that the array of carriers on these "new" villus cells are functionally distinct. Obviously, with the rise in proteolytic activity and solid food diet, luminal concentrations of free amino acids are higher after weaning.

The competition experiments performed on post-weaned animals similarly suggest that the enterocytes now lining the villi have arrays of carriers that are functionally distinct (see Chapter 6). Specificity of substrate uptake is much more defined in the jejunum of post-weaned animals; there is less overlapping function of the basic and neutral amino acid systems, though the dependence of these systems on co-transported ions and energy supply retains similar characteristics (see Chapter 7).

It has been suggested that the changing crypt cell kinetics in the third week of life are under the control of glucocorticoid hormones. Precociously inducing the appearance of the "new" enterocyte population with exogenous steroids results in the small intestine demonstrating functions characteristic of the post-weaned animal, which include changes in the kinetics and specificity of amino acid uptake (see Chapter 8). Despite the caveats required when describing changing functions in a tissue undergoing a nexus of maturational events, it is clear that the small intestine undergoes dramatic structural, and consequently functional, changes around the time of weaning.

Developmental studies of amino acid uptake can, and have, revealed much about the nature of the membrane systems involved in transport. Some transport systems arise, developmentally, before others and defining the specificity of uptake, coupled with kinetic determination of substrate binding affinities, provides information from which an idea of the membrane carrier itself can be built up. Attempts have been made to isolate carrier sites from membranes, most of them fraught with the difficulties of contamination, non-specific binding and loss of activity due to separation from other intrinsic membrane structures. Despite the experimental problems which surround carrier isolation, this is the one area of research which most fascinates membrane biologists. Consequently we might understand more about the mechanisms which maintain an intracellular environment structured to the temporal requirements of the cell, and the feedback systems inherent in these mechanisms.

For the developing organism, which undergoes a sequence of particularly "critical" periods, impairment of adequate supplies of amino acids (especially the essential ones - through malnutrition or transport defects) can result in lowered plasma levels of these substrates and thus reduce the level of protein synthesis in a rapidly maturing tissue such as the brain. The consequences of such defects for the developing animal are significant and largely irreversible.

APPENDIX

MICROSCOPY

Rats of various ages (1 to 60 days) were killed by decapitation, the entire small intestine was removed and divided into four regions:

1) Duodenum
2) Upper Jejunum
3) Lower Jejunum
4) Ileum

Each region was slit longitudinally, quickly washed in 0.9% saline to remove adhering ingesta and processed for microscopy as described below.

1) Scanning Electron Microscopy

2mm lengths of intestine from each region were transferred rapidly into a gluteraldehyde/formaldehyde primary fixative (modified Karnovsky, 1965) comprising 4% paraformaldehyde and 0.5% gluteraldehyde in 55mM Cacodylate buffer. The tissues were fixed for 30 min to 2 h. depending on age (i.e. size), and then washed in cacodylate buffer. The tissues were then dehydrated through the alcohols to acetone, and placed in a Polaron critical point drying apparatus overnight (acetone being replaced with liquid CO_2 in which the tissue was left immersed). Each dried segment was then positioned carefully onto a stub using double-sided sellotape, and made electrically conductive with a few drops of colloidal silver (DAG).

The samples were then coated in a polaron sputter coating unit with gold for 5 min to ensure that there was electrical continuity between the specimen and the stub but at the same time, that the morphological contouring of the gut was still visible. Stubs were stored in a

dessicator until viewed on a Mini SEM (ISI Instruments). Photographs were taken on Kodak plus x film.

2) Preparation for a) light and b) transmission electron microscopy

Tissue samples were processed and initially fixed as described above. After washing in cacodylate buffer, samples were post-fixed in 2% osmium tetroxide in cacodylate buffer for 1 to 2 h. and again washed in cacodylate buffer. The tissues were then dehydrated through the alcohols to 100% propylene oxide and finally embedded in epoxy resin (Durcapan) in uncapped vials on a rotator. Samples were finally transferred to fresh resin in a rubber mould and polymerised in an oven at 60°C for 48 h.

The benefits of this technique involving plastic embedded material are that light micrographs can be compared directly with electron micrographs from the same block of tissue. One serious disadvantage lies in staining plastic embedded sections, as stain penetration is not as good as with wax embedded material.

a) Light Microscopy (all materials from BDH, Poole, Dorset)

Blocks were trimmed for orientation and semi-thin sections (1 μ) cut on a Reichert ultratome. Sections were floated onto glass slides and dryed on a heated block. Two staining proceedures were employed:

(i) Toluidine blue for 10 sec - 2 min.

(ii) Regaud's haematoxylin/Safranin O.

This polychrome method was modified from that of Cheng, Merzel and Leblond (1969) for mouse small intestine based on Schantz and Schecter (1965) for plastic embedded sections.

All proceedures were carried out at 90°C. Sections were mordanted for 7 min in 5% ferric ammonium sulphate, washed, and stained with 50% iron haematoxylin for 7 min. Sections were again washed and "blued" with 0.1 N sodium hydroxide for 1 min. Finally, the sections were counter stained with 1% safranin for 20 sec, washed thoroughly, dried, and coverslips applied using DPX mountant. The prepared gut sections were examined on a Zeiss Photomicroscope II and photographs taken on Kodak plus x film through a blue/green filter.

b) Transmission Electron Microscopy

Using the same blocks of tissue, thin sections (silver/gold) were cut on a Reichert ultratome, transferred to copper grids and double stained with uranyl acetate and lead citrate (Reynolds, 1963). The sections were viewed in a Corinth 275 electron microscope and photographs taken on Agfa-line film.

REFERENCES

ADDISON, J.M.; BURSTON, D.; PAYNES, J.W.; WILKINSON, S. and MATTHEWS, D.M. (1975)
Evidence for active transport of tripeptides by hamster jejunum in vitro. Clin. Sci. Mol. Med. 49 305-312.

ADIBI, S.A. and GRAY, S.J. (1967)
Intestinal absorption of essential amino acids in man. Gastroenterology. 52 837-845.

AGAR, W.T.; HIRD, F.J.R. and SIDHU, G.S. (1954)
The uptake of amino acids by the intestine. Biochim. Biophys. Acta. 14 80-84.

AKEDO, H. and CHRISTENSEN, H.N. (1962)
Nature of insulin action on amino acid uptake by isolated diaphragm. J. Biol. Chem. 237 118-122.

ALVARADO, F. (1966)
Transport of sugars and amino acids in the intestine: evidence for a common carrier. Science. 151 1010-1013.

BAERLOCH, K.E.; SCRIVER, C.R. and MOHYUDDIN, F. (1971)
Ontogeny of amino acid transport in rat kidney. Biochim. Biophys. Acta. 249 353-363.

BAILLIEN, M. and SCHOFFENIELS, E. (1962)
Action des acides amines sur la difference de potential electrique et sur le courant de court-circuitage an niveau de l'epithelium isole de l'intestine grele de la tortue greque. Archs inter. Physiol. Biochim. 70 140-142.

BAINTNER, K. and VERESS, B. (1970)
Complex changes in the suckling rat small intestine by the end of the third week of life. Experientia. 26 54-55.

BAKER, R.D.; WATSON, S.; LONG, J. and WALL, M.J. (1969)
Effects of eversion on transmural electrical properties of rat jejunum. Biochim. Biophys. Acta. 173 192-199.

BALFOUR, A.H. and JONES, E.A. (1976)
An IgG-specific receptor on human placental cell membranes. Abst. S.E.B., Nottingham.

BAMFORD, D.R. and DONNELLY, H.T. (1974)
The effect of amino acids on the intestinal absorption of IgG in the neonatal rat. Immunology. 27 151-157.

BAMFORD, D.R. and HOLMES, J.E. (1971)
Regional variation in the aerobic metabolism of intestinal mucous membrane. Comp. Biochem. Physiol. 38A 603-610.

BARRY, R.J.C.; DIKSTEIN, S.; MATTHEWS, J. and SMYTH, D.H. (1961)
Electrical potentials in the isolated intestine. J. Physiol. 155 17-18p.

BARRY, R.J.C. and EGGENTON, J. (1972)
Membrane potentials of epithelial cells in rat small intestine. J. Physiol. 227 201-216.

BARTSOCAS, C.S.; THIER, S.O. and CRAWFORD, J.D. (1974)
Transport of L-methionine in rat intestine and kidney. Pediat. res. 8 673-678.

BATT, E.R. and SCHACTER, D. (1969)
Developmental pattern of some intestinal transport mechanisms in newborn rats and mice. Am. J. Physiol. 216 1064-1068.

BIHLER, I. and ADAMIC, S. (1967)
The effect of lithium on intestinal sugar transport. Biochim. Biophys. Acta. 135 466-474.

BILLINGTON, T. and NAYUDU, P.R.V. (1976)
Studies on the brush border membrane of mouse duodenum. J. mem. Biol. 27 83-100.

BOLUFER, J.; LARRALDE, J. and PONZ, F. (1975)
Interaction between sugars and amino acids in intestinal absorption by rat in vivo. Experientia. 31 1171-1173.

de BOTH, N.J.; van DONGEN, J.M.; van HOFWEGEN, B.; KEULEMANS, J.; VISSER, W.J. and GALJAARD, H. (1974)
The influence of various cell kinetic conditions on functional differentiation in the small intestine of the rat. Devel. Biol. 38 119-137.

de BOTH, N.J.; van der KAMP, A.W.M. and van DONGEN, J.M. (1975)
The influence of changing crypt cell kinetics on functional differentiation in the small intestine of the rat. Differentiation. 4 175-182.

BRAMBELL, F.W. (1970)
"The Transmission of Passive Immunity from Mother to Young", North-Holland (Amsterdam).

BRETSCHER, M.S. and RAFF, M.C. (1975)
Mammalian plasma membranes. Nature. 258 43-49.

BRONK, J.R. and LEESE, H.J. (1974)
Accumulation of amino acids and glucose by the mammalian small intestine.
in "Transport at the Cellular Level", SEB Symp. 28, Cambridge Univ. Press.

BRONK, J.R. and LEESE, H.J. (1974)
The absorption of a mixture of amino acids by rat small intestine. J. Physiol. 241 271-286.

BRONK, J.R. and PARSONS, D.S. (1965)
The polarographic determination of the respiration of the small intestine of the rat. Biochim. Biophys. Acta. 107 397-404.

BRONK, J.R. and PARSONS, D.S. (1966)
Amino acid accumulation and incorporation in rat intestine in vitro. J. Physiol. 184 950-963.

BURRILL, P.H.; SATTELMEYER, P.A. and LERNER, J. (1974)
Effect of theophylline and Na on methionine influx in Na-depleted intestine. Biochim. Biophys. Acta. 373 265-276.

BURRILL, P.H.; SATTELMEYER, P.A. and LERNER, J. (1976)
Effects of Na depletion, theophylline and 2, 4-DNP on neutral amino acid influx and cAMP levels in chicken small intestine. Comp. Biochem. Physiol. 53C 95-99.

BUTT, J.H. and WILSON, T.H. (1968)
Development of sugar and amino acid transport by intestine and yolk sac of the guinea pig. Am. J. Physiol. 215 1468-1477.

CHAN, W.S.; DANIELS, V.G. and THOMAS, A.L. (1973)
Premature cessation of macromolecule uptake by the young rat intestine following thyroxine administration. J. Physiol. 231 112-113p.

CHARNEY, A.N.; GOTS, R.E. and GIANELLA, R.A. (1974)
Na, K-ATPase in isolated intestinal villus tip and crypt cells. Biochim. Biophys. Acta. 367 265-270.

CHEESEMAN, C.I. and PARSONS, D.S. (1976)
Role of small peptides in transfer of amino nitrogen across the wall of vascularly perfused intestine. J. Physiol. 262 459-476.

CHEESEMAN, C.I. and SMYTH, D.H. (1975)
Interaction of amino acids, peptides and peptidases in the small intestine. Proc. Roy. Soc. (Lond) B. 190 149-163.

CHENEVAL, J.P. and JOHNSTONE, R.M. (1974)
Transport of amino acids in rat pancreas in development. Biochim. Biophys. Acta. 345 17-26.

CHENG, H. and LEBLOND, C.P. (1974)
Origin, differentiation and renewal of the four main epithelial cell types in mouse small intestine. Am. J. Anat. 141 461-561.

CHENG, H.; MERZEL, J. and LEBLOND, C.P. (1969)
Renewal of Pareth cells in the small intestine of the mouse. Am. J. Anat. 126 507-526.

CHRISTENSEN, H.N. (1972)
Nature and roles of receptor sites for amino acid transport. Advances Biochem. Psychopharmacol. 4 Raven Press (N.Y.).

CHRISTENSEN, H.N. (1973)
On the development of amino acid transport systems. Fed. Proc. 32 19-28.

CHRISTENSEN, H.N. (1975)
"Biological Transport" 2nd ed. Benjamin (Mass.)

CHRISTENSEN, H.N. and CLIFFORD, J.B. (1963)
Early postnatal intensification of hepatic accumulation of amino acids. J. Biol. Chem. 238 1743-1745.

CHRISTENSEN, H.N. and RIGGS, T.R. (1952)
Concentrative uptake of amino acids by the Ehrlich cell. J. Biol. Chem. 194 57-67.

CLARK, S.L. (1959)
The ingestion of proteins and colloidal materials by columnar absorptive cells of the small intestine in suckling rats and mice. J. Biophys. Biochem. Cytol. 5 41-50.

CLARK , S.L. and HARDY, R.N. (1969)
An analysis of the mechanism of cessation of uptake of macromolecular substances by the intestine of the young rat. J. Physiol. 204 127-134.

CORI, C.F. (1926)
The absorption of glycine and dl-alanine. Proc. Soc. Exp. Biol. Med. 24 125-126.

CORNELL, J.S. and MEISTER, A. (1976)
Glutathione and γ-glutamyl cycle enzymes in crypts and villus tip cells of rat jejunal mucosa. Proc. Nat. Acad. Sci. 73 420-422.

CORNELL, R. and PADYKULA, H.A. (1965)
A cytochemical study of intestinal absorption in the postnatal rat. Anat. Rec. 151 339-351.

CORNELL, R. and PADYKULA, H.A. (1969)
A cytological study of intestinal absorption in the suckling rat. Am. J. Anat. 125 291-316.

CRANE, R.K. (1962)
Hypothesis for mechanism of intestinal active transport of sugars. Fed. Proc. 21 891-895.

CRANE, R.K.; MALATHI, P. and PREISER, H. (1976)
Transport properties of the brush border membrane : reconstitution. Abstr. FEBS Symp. On Biochemistry of Membrane Transport, Zurich.

CRANE, R.K. and MANDELSTAM, P. (1960)
The active transport of sugars by various preparations of hamster intestine. Biochim. Biophys. Acta. 45 460-476.

CSAKY, T.Z. (1975)
"Intestinal Absorption and Malabsorption", Raven Press (N.Y.).

CURTHOYS, N.P. and SHAPIRO, R. (1975)
γ-glutamyltranspeptidase in intestinal brush border membranes. FEBS letts. 58 230-233.

DANIELS, V.G. (1970)
Intestinal amino acid transfer. Ph.D. Thesis, Univ. of Sheffield.

DANIELS, V.G. (1971)
Transport of proteins and amino acids by the newborn rat intestine in vitro. J. Physiol. 58P.

DANIELS, V.G. (1972)
The effect of diet on the time of intestinal macromolecular absorption in the newborn rat. J. Physiol. 226 112-114P.

DANIELS, V.G.; DAWSON, A.G.; NEWEY, H. and SMYTH, D.H. (1969)
Effect of carbon chain length and amino group position on neutral amino acid transport systems in rat small intestine. Biochim. Biophys. Acta. 173 575-577.

DANIELS, V.G.; HARDY, R.N.; MALINOWSKA, K.W. and NATHANIELSZ, P.W. (1972)
Adrenocortical hormones and absorption of macromolecules by the small intestine of the young rat. J. Endocr. 52 405-406.

DANIELS, V.G.; HARDY, R.N. and MALINOWSKA, K.W. (1973)
The effect of adrenalectomy or pharmacological inhibition of adrenocortical function on macromolecule uptake by the newborn rat intestine. J. Physiol. 229 697-707.

DANIELS, V.G.; HARDY, R.N.; MALINOWSKA, K.W. and NATHANIELSZ, P.W. (1973)
The influence of exogenous steroids on macromolecule uptake by the small intestine of the newborn rat. J. Physiol. 229 681-695.

DANIELS, V.G.; NEWEY, H. and SMYTH, D.H. (1969)
Sterochemical specificity of neutral amino acid transfer systems in rat small intestine. Biochim. Biophys. Acta. 183 637-639.

DELHUMEAU, G.; PRATT, G.V. and GITLER, C. (1962)
The absorption of amino acid mixtures from the small intestine of the rat. J. Nutr. 77 52-60.

DEREN, J.J. (1968)
Development of intestinal structure and function.
in "Handbook of Physiology - Secn 6 - Alimentary Canal. Vol 3. Intestinal Absorption. Am. Physiol. Soc. (Washington).

DEREN, J.J.; STRAUSS, E.W. and WILSON, T.H. (1965)
The development of structure and transport systems of the foetal rabbit intestine. Devel. Biol. 12 467-486.

DIXON, M. and WEBB, E.C. (1964)
"Enzymes", 2nd ed. Longmans (London).

DOELL, R.G. and KRETCHMER, N. (1962)
Studies of the small intestine during development. Biochim. Biophys. Acta. 62 353-362.

DONNELLY, H.T. (1971)
An in vitro study of the absorption of amino acids and γ-globulin by foetal and neonatal rat intestine. Ph.D. Thesis, Queens Univ. (Belfast).

DONNELLY, H.T. and BAMFORD, D.R. (1976)
The effect of amino acids on IgG transport in the neonatal rat. in "Maternofoetal Transmission of Immunoglobulins", ed. W.A. Hemmings, Cambridge Univ. Press.

DOWD, J.E. and RIGGS, D.S. (1965)
A comparison of estimates of Michaelis-Menten kinetic constants from various linear transformations. J. Biol. Chem. 240 863-869.

ESPOSITO, G. and CSAKY, T.Z. (1974)
Extracellular space in the epithelium of rat small intestine. Am. J. Physiol. 226 50-55.

ESPOSITO, G.; FAELLI, A. and CAPRARO, V. (1972)
The extracellular space of rat intestine in vivo. Pflugers. Arch. 331 70-76.

EVERED, D.F. and HARGREAVES, B.M.C. (1973)
Uptake of W-amino acids by rat small intestine and kidney cortex in vitro. Xenobiotica. 3 753-757.

FAUST, R.G. and SHEARIN, S.J. (1974)
Molecular weight of a D-glucose and L-histidine binding protein from intestinal brush borders. Nature. 248 60-61.

FERGUSON, A.; GERSKOWITCH, V.P. and RUSSELL, R.I. (1973).
Pre- and postweaning disaccharidase patterns in isografts of foetal mouse intestine. Gastroenterology. 64 292-297.

FISHER, R.B. and PARSONS, D.S. (1949)
A preparation of surviving rat small intestine for the study of absorption. J. Physiol. 221 311-329.

FITZGERALD, J.F.; REISER, S.; JOHNSON, C.F. and CHRISTIANSEN, P.A. (1969)
The developmental pattern of sugar and amino acid transport by the postnatal rat small intestine. Clin. Res. 17 526P.

FONDACARO, J.D. and NATHAN, P. (1971)
The active transport of methionine by immature rat small intestine. Physiologist. 14 142P.

FONDACARO, J.D.; NATHAN, P. and WRIGHT, W.E. (1974)
Methionine accumulation in villi isolated from maturing rat intestine. J. Physiol. 241 751-760.

FORSTNER, G. and GALAND, G. (1976)
The influence of hydrocortisone on the synthesis and turnover of microvillous membrane glycoproteins in suckling rat intestine. Can. J. Biochem. 54 224-232.

FUJITA, M. and NAKAO, M. (1973)
Localisation of auabain sensitive ATPase in intestinal mucosa. in "Organisation of Energy Transducing Membranes", eds. M. Nakao, and L. Packer, Univ. Tokyo Press.

FURIHATA, C.; KAWACHI, T. and SUGIMURA, T. (1972)
Premature induction of pepsinogen in developing rat gastric mucosa by hormones. Biochem. Biophys. res. Commun. 47 705-711.

GARCIA-SANCHO, J.; SANCHEZ, A. and CHRISTENSEN, H.N. (1977)
Role of proton dissociation in the transport of acidic amino acids by the Ehrlich ascites tumour cell. Biochim. Biophys. Acta. 464 295-312.

GARDNER, M.L.G. (1976)
Absorption from a mixture of seventeen free amino acids by the isolated small intestine of the rat. J. Physiol. 262 563-574.

GUIDOTTI, G. (1976)
The structure of membrane transport systems. TIBS, January 1976.

GUIDOTTI, G.; GAZZOLA, G.C.; BORGHETTI, A.F. and FRANCHI-GAZZOLA, R. (1975)
Adaptive regulation of amino acid transport across the cell membrane in avian and mammalian tissues. Biochim. Biophys. Acta. 406 264-279.

HALLIDAY, R. (1959)
The effect of steroid hormones on the absorption of antibody by the young rat. J. Endocr. 18 56-66.

HANES, C.S. (1932)
Studies of plant amylases. Biochem. J. 26 1406-1421.

HARDCASTLE, P.T. (1974)
The effect of ATP on the transport of hexoses and amino acids in everted sacs of rat small intestine. Biochim. Biophys. Acta. 332 114-121.

HARDCASTLE, P.T. and DANIELS, V.G. (1973)
Interaction between hexoses and amino acids for transport in rat small intestine. Comp. Biochem. Physiol. 45A 995-999.

HELLIER, M.D. and HOLDSWORTH, C.D. (1975)
Digestion and absorption of proteins.
in "Intestinal Absorption in Man", eds. I. McColl and G.E. Sladen, Academic Press.

HELLIER, M.D.; HOLDSWORTH, C.D. and PERRETT, D. (1973)
Dibasic amino acid absorption in man. Gastroenterology. 65 613-618.

HENIN, S. and SMITH, M.W. (1976)
Electrical properties of pig colonic mucosa measured during early postnatal development. J. Physiol. 262 169-187.

HENNING, S.J.; BALARD, P.L. and KRETCHMER, N. (1975)
A study of the cytoplasmic receptors for glucocorticoids in intestine of pre- and postweaning rats. J. Biol. Chem. 250 2073-2079.

HENNING, S.J. and KRETCHMER, N. (1973)
Development of intestinal function in mammals. Enzyme. 15 3-23.

HERBST, J.J. and KOLDOVSKY, O. (1972)
Cell migration and cortisone induction of sucrose activity in jejunum and ileum. Biochem. J. 126 471-476.

HERBST, J.J. and SUNSHINE, P. (1969)
Postnatal development of the small intestine of the rat. Pediat. Res. 3 27-33.

HILTON, W.A. (1902)
The morphology and development of intestinal folds and villi in vertebrates. Am. J. Physiol. 1 459-504.

HOFSTEE, B.H.J. (1959)
Non inverted versus inverted plots in enzyme kinetics. Nature. 184 1296-1298.

HOLDSWORTH, C.D. and WILSON, T.H. (1967)
Development of active sugar and amino acid transport in the yolk sac and intestine of the chicken. Am. J. Physiol. 212 233-240.

HRUBY, L. (1959)
Postnatal development of the rat jejunoileum. Cs. Morfol. 7 390-394.

HUDSON, D.A. and LEVIN, R.J. (1968)
The ontogeny of electrical activity associated with absorption of solutes across the developing small intestine of the chick. J. Physiol. 195 369-385.

INGHAM, P.A. (1972)
An in vitro study on the intestinal absorption of sugars in the foetal and neonatal rat. Ph.D. Thesis, Queens Univ. (Belfast).

JAMES, P.S. and SMITH, M.W. (1976)
Methionine transport by pig colonic mucosa measured during early postnatal development. J. Physiol. 262 151-168.

JOHNS, J.T. and BERGEN, W.G. (1973)
Studies on amino acid uptake by ovine small intestine. J. Nutri. 103 1581-1586.

JONES, E.A. and WALDMANN, T.A. (1972)
The mechanism of intestinal uptake and transcellular transport of IgG in the neonatal rat. J. Clin. Invest. 51 2916-2927.

JONES, R.E. (1972)
Intestinal absorption and digestion of protein in the young rat during the normal and cortisone induced closure period. Biochim. Biophys. Acta. 274 412-419.

JONES, R.E. (1976)
Studies on the transmission of bovine IgG across the intestine of the young rat.
in "Maternofoetal Transmission of Immunoglobulins" ed. W.A. Hemmings, Cambridge Univ. Press.

de JONGE, H.R. (1976)
Cyclic nucleotide-dependent phosphorylation of intestinal epithelium proteins. Nature. 262 590-593.

KANSAL, V.K. and WAGLE, D.S. (1975)
Studies on the action of hormones on the intestinal transport of L-histidine. Ind. J. Physiol. Pharmac. 19 28-33.

KAPADIA, S. and BAKER, S.J. (1976)
The effects of alterations in villus shape on the intestinal mucosal surface of the albino rat. Digestion. 14 256-268.

KARNOVSKY, M.J. (1965)
A formaldehyde-glutaraldehyde fixative of high osmolality for use in E.M. J. Cell Biol. 27 137A.

KHOURY, K.A.; FLOCK, M.H. and HERSH, T. (1969)
Small intestinal mucosal cell proliferation and bacterial flora in the conventionalisation of the germ-free mouse. J. Exp. Med. 130 659-670.

KIMMICH, G.A. (1970)
Preparation and properties of mucosal epithelial cells isolated from the small intestine of the chicken. Biochemistry. 9 3659.

KINNE, R. and MURER, H. (1976)
Sidedness and coupling of transport processes in renal and intestinal epithelia. Abstr. FEBS Symp. on Biochemistry of Membrane Transport, Zurich.

KINZIE, J.L.; FERRENDELLI, J.A. and ALPERS, D.H. (1973)
cAMP-mediated transport of neutral and dibasic amino acids in jejunal mucosa. J. Biol. Chem. 248 7018-7024.

KOHN, P.G.; SMYTH, D.H. and WRIGHT, E.M. (1968)
Effects of amino acids, peptides and disaccharides on the electrical potential across rat small intestine. J. Physiol. 196 723-746.

KOLDOVSKY, O. (1969)
"Development of the Functions of the Small Intestine in Mammals and Man", Karger (Basle).

KOTYK, A. and JANACEK, K. (1975)
"Cell Membrane Transport", Plenum Press (N.Y.).

KREBS, H.A. and HENSELEIT, K. (1932)
Untersuchungen uber die Harnstoffbildung im Tierkorper.
Hoppe-Seyler's Z. Physiol. Chem. 210 33-66.

LACOMBE, C.; MITJAVILA, S. and CARRERA, G. (1976)
The action and interaction of three alimentary substances on the activity of the ATPases in enterocyte brush borders.
Life Sci. 18 1245-1254.

LAJTHA, A. (1974)
Amino acid transport in the brain.
in "Aromatic Amino Acids in the Brain", CIBA Symp. 22.

LARSEN, P.R.; ROSS, J.E. and TAPLEY, D.F. (1964)
Transport of neutral, dibasic and N-methyl substituted amino acids by rat intestine. Biochim. Biophys. Acta. 88 570-577.

LEESE, H.J.; PRENDERGAST, J. and READ, B.S. (1976)
The effect of theophylline on glucose and fluid transport across the rat jejunum. Trans. Biochem. Soc. 4 272-274.

LERNER, J.; BURRILL, P.H.; SATELMEYER, P.A. and JANICKI, C.F. (1976)
Developmental patterns of intestinal transport mechanisms in the chick. Comp. Biochem. Physiol. 54A 109-111.

LESHER, S.; WALBURG, H.E. and SACHER, G.A. (1964)
Generation time in the duodenal crypt cells of germ-free and conventional mice. Nature. 202 884-886.

LEVIN, R.J. (1969)
The effects of hormones on the absorptive, metabolic and digestive functions of the small intestine. J. Endocr. 45 315-348.

LEVINE, R.; McNARY, W.F.; KORNGUTH, P.J. and Le BLANC, R. (1970)
Histological re-evaluation of everted gut technique for studying intestinal absorption. Eur. J. Pharmacol. 9 211-217.

LICHTENBERGER, L. and JOHNSON, L.R. (1974)
Gastrin in the ontogenic development of the small intestine.
Am. J. Physiol. 227 390-395.

LIN, E.C.C. (1971)
Molecular basis of membrane transport systems.
in "Structure and Function of Biological Membranes", ed. R. Rothfield.

LINEWEAVER, H. and BURK, D. (1934)
The demonstration of enzyme dissociation constants. J. Am. Chem. Soc. 56 658-666.

LIPKIN, M. (1973)
Proliferation and differentiation of gastrointestinal cells. Physiol. Rev. 53 891-915.

LOWRY, O.H.; ROSEBROUGH, N.H.; FARR, A.L. and RANDALL, R.J. (1951)
Protein measurement with the folin phenol reagent. J. Biol. Chem. 193 65-75.

LUISIER, A.L. and ROBINSON, J.W.L. (1975)
Glycolysis as an energy source for intestinal transport of amino acids. Comp. Biochem. Physiol. 51A 105-109.

LUPPA, D.; REMKE, H.; MUHLE, W.; DETTMER, D. and MULLER, F. (1975)
Subcellular localisation of Na, K-ATPase in the brush border membrane of the mucosal cell of the rat small intestine. Acta. Biol. Med. Ger. 34 1293-1300.

MAINOYA, J.R. (1975)
Effect of prolactin on sugar and amino acid transport by the rat jejunum. J. Exp. Zool. 192 149-154.

MALINOWSKA, K.W.; CHAN, W.S.; NATHANIELSZ, P.W. and HARDY, R.N. (1974)
Plasma adrenocorticosteroid changes during thyroxine induced accelerated maturation of the neonatal rat intestine. Experientia. 30 61-62.

MARLOW, C.G. and SHEPPARD, G. (1970)
Labelled tracers of inulin for physiological measurements. Clinica. Chimica. Acta. 28 469-478.

MARTONOSI, A. (1975)
Membrane transport during development in animals. Biochim. Biophys. Acta. 415 311-333.

MATTHEWS, D.M. (1975)
Intestinal absorption of peptides. Physiol. Rev. 55 537-608.

MEISTER, A. (1973)
On the enzymology of amino acid transport. Science. 180 33-39.

MERRILL, T.G.; SPRINZ, H. and TOUSMIS, A.J. (1967)
Changes of intestinal absorptive cells during maturation. J. Ultrastruc. Res. 19 304-326.

MICHAELIS, L. and MENTEN, M.L. (1913)
Die Kinetik der Invertinwirkung. Biochem. Z. 49 333-369.

MILLER, D.L. (1971)
Rat small intestine : development, composition and effects of perfusion. Digestive Diseases. 16 247-254.

MILLER, D. and CRANE, R.K. (1961)
The digestive function of the epithelium of the small intestine. Biochim. Biophys. Acta. 52 281-293.

MILLER, D.S.; BURRILL, P. and LERNER, J. (1974)
Distinct components of neutral amino acid transport in chick small intestine. Comp. Biochem. Physiol. 47A 767-777.

MILLER, S.A. (1970)
Nutrition in the neonatal development of protein metabolism. Fed. Proc. 29 1497-1502.

MITCHELL, P. (1976)
Vectorial chemistry and the molecular mechanics of chemiosmotic coupling: power transmission by proticity. Trans. Biochem. Soc. 4 399-430.

MOOG, F. (1971)
in "Hormones and Development", eds. T. Hamburgh and E.J.W. Barrington, Meredith (New York).

MORRIS, B. and MORRIS, R. (1974a)
The effects of cortisone acetate on stomach evacuation and the absorption of ^{125}I-labelled globulins in young rats. J. Physiol. 240 79-89.

MORRIS, B. and MORRIS, R. (1974b)
The absorption of ^{125}I-labelled IgG by different regions of the gut in young rats. J. Physiol. 241 761-770.

MORRIS, B. (1975)
The transmission of ^{125}I-labelled IgG by proximal and distal regions of the small intestine of 16 day old rats. J. Physiol. 245 249-259.

MORRIS, B. and MORRIS, R. (1976)
The effects of corticosterone and cortisone on the uptake of PVP and the transmission of IgG by the small intestine of young rats. J. Physiol. 254 389-403.

MUNCK, B.G. (1970)
Interactions between lysine Na and Cl transport in rat jejunum. Biochim. Biophys. Acta. 203 424-433.

MUNCK, B.G. and SCHULTZ, S.G. (1969)
Lysine transport across isolated rabbit ileum. J. Gen. Physiol. 53 157-182.

MUNRO, H.N. (1968)
Role of amino acid supply in regulating ribosome function. Fed. Proc. 27 1231P.

MURER, H. and HOPFER, U. (1974)
Demonstration of electrogenic Na-dependent D-glucose transport in intestinal brush border membranes. Proc. Nat. Acad. Sci. 71 484-488.

MURER, H.; HOPFER, U. and KINNE, R. (1976)
Sodium/proton antiport in brush border membrane vesicles isolated from rat small intestine and kidney. Biochem. J. 154 597-604.

MURER, H.; SIGRIST-NELSON, K. and HOPFER, U. (1975)
On the mechanism of sugar and amino acid interaction in intestinal transport. J. Biol. Chem. 250 7392-7396.

NAKAMURA, V.; YASUMOTO, K.; SUGIYAMA, K. and MITSUDA, H. (1974)
D-amino acid induced transmural P.D. changes in rat small intestine. Agr. Biol. Chem. 38 2149-2155.

NEAME, K.D. and RICHARDS, T.G. (1972)
"Elementary Kinetics of Membrane Carrier Transport", Blackwell (Oxford).

ORLIC, D. and LEV, R. (1973)
Fetal rat intestinal absorption of HRP from swallowed ammiotic fluid. J. Cell Biol. 56 106-119.

ORLOWSKI, M. (1976)
Possible role of glutathione in transport processes. in "Transport Phenomena in the Nervous System", eds. G. Levi, L. Battistin and A. Lajtha, Plenum Press (N.Y.).

OXENDER, D. (1974)
Membrane transport proteins.
in "Biomembranes", 5, Plenum Press (N.Y.).

OXENDER, D. (1975)
Genetic approaches to the study of transport systems.
in "Biological Transport", ed. H.N. Christensen, Benjamin (Mass.).

PARSONS, D.S. (1968)
in "Handbook of Physiology - Alimentary Canal", Vol. 3, Ch. 64. Am. Physiol. Soc. (Washington).

PARSONS, D.S. (1975)
Multimembrane systems.
in "Biological Membranes", ed. D.S. Parsons, Oxford Univ. Press.

PENZES, L. and SIMON, C.T. (1968)
Intestinal absorption and turnover of DL-methionine during reproduction in the rat. Jap. J. Physiol. 18 288-296.

PERRIS, A.D. (1966)
Isolation of the epithelial cells of the rat small intestine. Can. J. Biochem. 44 687-693.

PHANG, J.M.; VALLE, D.L.; FISHER, L. and GRANGER, A. (1975)
Puromycin effect on amino acid transport. Am. J. Physiol. 228 23-26.

PRATT, R.M. and TERNER, C. (1971)
Development of amino acid transport by small intestine of chick embryo. Biochim. Biophys. Acta. 225 113-122.

PRESSLEY, L. and FUNDER, J.W. (1975)
Glucocorticoid and mineralocorticoid receptors in gut mucosa. Endocrinology. 97 588-596.

PRESTON, R.L.; SCHAEFFER, J.F. and CURRAN, P.F. (1974)
Structure affinity relationships of substrates for the neutral amino acid transport system in rabbit ileum. J. Gen. Physiol. 64 443-467.

QUILL, H. and WEISER, M.M. (1975)
Adenylate and guanylate cyclase activities and cellular differentiation in rat small intestine. Gastroenterology. 69 470-478.

REDDY, B.S. and WOSTMANN, B.S. (1966)
Intestinal disaccharidase activity in growing and germ-free conventional rats. Arch. Biochem. Biophys. 113 609-616.

REISER, S. and CHRISTIANSEN, P.A. (1969)
A cross inhibition of basic amino acid transport by neutral amino acids. Biochim. Biophys. Acta. 183 611-624.

REISER, S.; FITZGERALD, J.F. and CHRISTIANSEN, P.A. (1970)
Kinetics of the accelerated intestinal transport in 2 day old rats. Biochim. Biophys. Acta. 203 351-353.

REYNOLDS, E.S. (1963)
The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell. Biol. 17 208-212.

RIKLIS, E. and QUASTEL, J.H. (1958)
Effects of cations on sugar absorption by isolated guinea pig intestine. Can. J. Biochem. Physiol. 36 347-362.

de RITIS, G.; FALCHUK, Z.M. and TRIER, J.S. (1975)
Differentiation and maturation of cultured foetal rat jejunum. Develop. Biol. 45 304-317.

ROBBERECHT, P.; DESCHODT-LANCKMAN, M. and de NEEF, P. (1974)
In vivo effects of pancreozymin, secretin, vasoactive intestinal polypeptide and pilocarpine on the levels of cAMP and cGMP in the rat pancreas. FEBS Letts. 43 139-143.

ROBINSON, J.W.L. (1966)
Certain aspects of intestinal amino acid absorption. These de Doctorat, Universite de Lausanne.

ROBINSON, J.W.L. (1968)
Interaction between neutral and dibasic amino acids for uptake by rat small intestine. Eur. J. Biochem. 7 78-89.

ROBINSON, J.W.L. (1970)
The difference in sensitivity to cardiac steroids of Na, K-ATPase and amino acid transport in the intestinal mucosa of the rat and other species. J. Physiol. 206 41-60.

ROBINSON, J.W.L. and ALVARADO, F. (1971)
Interaction between the sugar and amino acid transport systems at the small intestinal brush border. Eur. J. Physiol. 326 48-75.

ROBINSON, J.W.L.; LUISIER, A.L. and MIRKOVITCH, V. (1973)
Transport of amino acids and sugars by the dog colonic mucosa. Pflugers. Arch. 345 317-326.

RODEWALD, R. (1973)
Intestinal transport of antibodies in the newborn rat. J. Cell. Biol. 58 189-211.

RODEWALD, R. (1976a)
pH dependent binding of immunoglobulins to intestinal cells of the neonatal rat. J. Cell. Biol. 71 666-670.

RODEWALD, R. (1976b)
Intestinal transport of peroxidase conjugated IgG fragments in the neonatal rat.
in "Maternofoetal Transmission of Immunoglobulins", ed. W.A. Hemmings, Cambridge Univ. Press.

ROSENBERG, I.H. (1966)
Development of foetal guinea pig small intestine. Fed. Proc. 25 456P.

SCHANTZ, A. and SCHECHTER, A. (1965)
Iron haematoxylin and safranin O as a polychrome stain for Epon sections. Stain Technology. 40 279-282.

SCHULTZ, S.G. and CURRAN, P.F. (1970)
Coupled transport of sodium and organic solutes. Physiol. Rev. 50 637-718.

SCHULTZ, S.G. and FRIZELL, R.A. (1975)
Amino acid transport by the small intestine.
in "Intestinal Absorption and Malabsorption", ed. T.Z. Csaky, Raven Press.

SCHULTZ, S.G. and ZALUSKY, R. (1965)
Interactions between active sodium transport and active amino acid transport in isolated rabbit ileum. Nature. 204 292-294.

SEMENZA, G. (1971)
On the mechanisms of mutual inhibitions among Na-dependant systems in the small intestine - a hypothesis. Biochim. Biophys. Acta. 241 637-649.

SEMENZA, G.; TANNENBAUM, C.; TOGGENBURGER, G. and WAHLGREN, L. (1976)
A possible common activation site in Na-driven transport systems: partial purification of the Na-dependant D-glucose transport system from small intestinal brush borders. Abstr. FEBS Symp. on Biochemistry of Membrane Transport, Zurich.

SHISHOVA, O.A. (1959)
Effect of ionising irradiation, adrenalectomy and cortisone on the absorption of amino acids by the intestinal tract. Biochimiya. 24 885-890.

SHMERLING, D.H. (1976)
Development of digestive and absorptive function in the human foetus. Nutr. Metabol. 20 76-79.

SIGRIST-NELSON, K. (1975)
Dipeptide transport in isolated intestinal brush border membrane. Biochim. Biophys. Acta. 394 220-226.

SIGRIST-NELSON, K. and HOPFER, U. (1974)
Intestinal transport protein. Nature. 252 422P.

SINGER, S.J. and NICHOLSON, G.L. (1972)
The fluid mosaic model of the structure of cell membranes. Science. 175 720-731.

SLEISENGER, M.H.; BURSTON, D.; DALRYMPLE, J.A.; WILKINSON, S. and MATTHEWS, D.M. (1976)
Evidence for a single common carrier for uptake of a dipeptide and a tripeptide by hamster jejunum in vitro. Gastroenterology. 71 76-81.

SMITH, M.W. and JAMES, P.S. (1976)
Amino acid transport by the helicoidal colon of the newborn pig. Biochim. Biophys. Acta. 419 391-394.

SMITH, M.W.; MUNN, E.A.; BURTON, K.A. and HENRIQUES de JESUS, C. (1976)
Functional changes in the brush border surface of the newborn pig intestine.
in "Maternofoetal Transmission of Immunoglobulins", ed. W.A. Hemmings, Cambridge Univ. Press.

SMYTH, D.H. (1974)
Methods of studying intestinal absorption.
in "Biomembranes", 4A, ed. D.H. Smyth, Plenum.

SNELL, K. and WALKER, D.G. (1973)
Gluconeogenesis in the newborn rat. Enzyme. 15 40-81.

SPENCER, R.P. and ZAMCHECK, N. (1962)
Intestinal contribution to control of metabolism. Gastroenterology. 42 732-739.

SRIVASTAVA, L.M. and HUBSCHER, G. (1968)
The effect of age on glycolytic and hexokinase activities in the mucosa of the rat small intestine. Biochem. J. 110 607-608.

STATES, B. and SEGAL, S. (1968)
Developmental aspects of cystine transport in rat intestinal segments. Biochim. Biophys. Acta. 163 154-162.

STEWART, M.G. (1975)
Studies of amino acid absorption by tissues of the bivalve mollusc Mya arenaria. Ph.D. Thesis, Queens Univ. (Belfast).

SYME, G. and LEVIN, R.J. (1976)
Effect of altered thyroid status induced by thyroid hormones, goitrogens and diet on intestinal electrogenic valine transfer. Proc. Roy. Soc. (Lond) B. 194 121-139.

TURNBERG, L.A.; ISAACS, P.E.T.; CORBETT, C.L. and RILEY, A.K. (1976)
In vitro behaviour of human jejunum and ileum : response to theophylline and acetylcholine.
in "Intestinal Ion Transport", ed. J.W.L. Robinson, MTP Press.

UGOLEV, A.M. (1974)
Membrane (contact) digestion.
in "Biomembranes", 4A, ed. D.H. Smyth, Plenum (N.Y.).

WALKER, W.A.; CORNELL, R.; DAVENPORT, L.M. and ISSELBACHER, K.G. (1972)
Macromolecular absorption. Mechanism of HRP uptake and transport in adult and neonatal rat intestine. J. Cell. Biol. 54 195-205.

WALSER, M. (1976)
Nutritional effects of nitrogen free analogues of essential amino acids. Life Sci. 17 1011-1020.

WEEKES, T.E.C. (1974)
The preparation of a viable suspension of epithelial cells from the rumen mucosa of cattle. Comp. Biochem. Physiol. 49B 407-413.

WHEELER, D.D. (1975)
Amino acid transport in peripheral nerve. J. Neurochem. 24 97-104.

WHITE, L.W. and LANDAU, B.R. (1965)
Sugar transport and fructose metabolism in human intestine in vitro. J. Clin. Invest. 44 1200-1205.

WILKINSON, G.N. (1961)
Statistical estimations in enzyme kinetics. Biochem. J. 80 324-332.

WILLIAMS, R.M. and BECK, F. (1969)
A histochemical study of gut maturation. J. Anat. 105 487-501.

WILSON, T.H. (1971)
Developmental studies on transport in the yolk sac and small intestine.
in "Intestinal Transport of Electrolytes, Amino Acids and Sugars", ed. T. Armstrong and P. Nunn, Thomas.

WILSON, T.H. and LIN, E.C.C. (1960)
Active transport by intestines of newborn rabbits. Am. J. Physiol. 199 1030-1032.

WILSON, T.H. and WISEMAN, G. (1954)
The use of sacs of everted small intestine for the study of transference of substrates from the mucosal to the serosal surface. J. Physiol. 123 116-125.

WISEMAN, G. (1955)
Preferential transference of amino acids from mixtures by sacs of everted small intestine of the golden hamster. J. Physiol. 127 414-422.

WISEMAN, G. (1974)
Absorption of protein digestion products.
in "Biomembranes", 4A, ed. D.H. Smyth, Plenum (London).

YEH, K-Y. and MOOG, F. (1974)
Intestinal lactase activity in the suckling rat : influence of hypophysectomy and thyroidectomy. Science. 182 77-79.

YOUNG, J.D. and ELLORY, J.C. (1977)
Substrate specificity of amino acid transport in sheep erythrocytes. Biochem. J. 162 33-38.

YOUNG, J.D.; ELLORY, J.C. and TUCKER, E.M. (1975)
Amino acid defect in glutathione-deficient sheep erythrocytes. Nature. 254 156-157.

YOUNG, J.D.; ELLORY, J.C. and TUCKER, E.M. (1976)
Amino acid transport in normal and glutathione deficient sheep erythrocytes. Biochem. J. 154 43-48.