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GLYCYL-L-LEUCINE TRANSPORT BY THE RAT SMALL INTESTINE

by

GORDON A. JOHNSTON

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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THE RAT SMALL INTESTINE

submitted by Gordon A. Johnston
in partial fulfilment of the requirements for the degree of Master of Science.

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Supervisor

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June, 1982

In Memory of My Father

ABSTRACT

The transport of the dipeptide glycyl-L-leucine across the brush border membrane of the male rat proximal intestinal enterocyte was studied using everted rings. The movement of substrates was followed using: [^{14}C]-L-leucine; glycyl-[^{3}H]-L-leucine and [^{14}C]-glycyl-L-leucine.

The influx of L-leucine from a solution of the free amino acid and from the peptide glycyl-L-leucine displayed saturation kinetics as substrate concentrations increased, indicating that a mediated process was involved in the observed uptake. Transfer of L-leucine from the dipeptide was found to occur at a faster rate and reach higher intracellular concentrations than from free solution and unlike uptake as the free monomer never reached levels above that in the incubation medium. Replacement of medium Na^+ with choline reduced the transport of peptide bound L-leucine far less than it did uptake from free solution suggesting that dipeptide influx is less dependent on the cation.

Saturation of the free amino acid system with excess cold L-leucine (20 mM) reduced the transport of L-leucine from glycyl-L-leucine by approximately fifty percent, to levels not significantly different from those determined in the absence of Na^+ ($p<0.05$). The replacement of medium Na^+

in the presence of excess free L-leucine, had no effect on the remaining transfer of peptide bound L-leucine. This data suggests that L-leucine originating from the dipeptide is taken up into the enterocyte by two pathways. One route which is available to L-leucine liberated from glycyl-L-leucine by hydrolysis at the brush border corresponds to the well characterized, Na^+ dependent free amino acid transport system; the second route is an intact peptide uptake mechanism and Na^+ independent. This conclusion gained support from the observation that 10 mM L-alanyl-β-naphthylamide (ANA), a potent brush border hydrolase inhibitor, reduced peptide bound L-leucine uptake to levels not significantly different from the Na^+ free determinations with or without 20 mM free L-leucine ($p<0.05$).

The transfer of L-leucine from glycyl-L-leucine was greater than the glycine uptake. This additional transport of L-leucine was abolished by the removal of medium Na^+, by the presence of 20 mM free L-leucine and by the addition of 10 mM ANA. That is when the contribution made to the influx of L-leucine from the dipeptide by surface hydrolysis followed by the preferential uptake of free L-leucine was eliminated, transfer of both moieties became equal. It was concluded therefore, that glycine transport from glycyl-L-leucine is accomplished exclusively via the intact peptide

system. This notion was verified by the demonstration that peptide bound glycine influx was unaffected by either Na^+ removal or the presence of 20 mM cold free L-leucine.

The alkaloid harmaline, a reputed inhibitor of Na^+ dependent transport mechanisms, was found to have nonspecific inhibitory effects on both free amino acid and dipeptide transport at concentrations in excess of 2.5 mM.

The transport of L-leucine from glycyl-L-leucine, in the presence of 20 mM cold free L-leucine, was competitively inhibited by glycyl-L-proline, although only partially. Calculations showed that infinitely high concentrations of glycyl-L-proline would fail to inhibit 44 percent of intact glycyl-L-leucine transport. This implies the existence of at least two pathways for the transport of intact glycyl-L-leucine, only one of which is shared with glycyl-L-proline.

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INTRODUCTION

It is known that the intraluminal phase of protein digestion, carried out by activated pancreatic proteases, yields a complex mixture of free amino acids and small peptides and that the peptides predominate (Adibi et al., 1981). Further, the existence of a distinct mediated transport system for the transfer of di- and tripeptides into the intestinal enterocyte is now accepted (Matthews et al., 1975, 1980). There appears to be confusion however, as to the nature of this mediated peptide transport system.

Early reports on peptide uptake indicated that the process could be reduced with metabolic inhibitors and by the removal of medium Na^+ (Newey and Smyth, 1960; Rubino et al., 1971) suggesting an active, Na^+ dependent mechanism. Subsequent investigations utilizing poorly hydrolyzed but transported peptides claim to have demonstrated intracellular accumulation of these substrates as well as the Na^+ dependency of their uptake (Matthews et al., 1972, 1974, 1979). Other studies using more common peptides however, have succeeded in demonstrating only a partial Na^+ dependency of the influx mechanism which varies according to the peptide used (Rubino et al., 1971; Ganapathy et al., 1979). Cheeseman and Parsons (1976), provided an explanation for this partial Na^+ dependency through a study of dipeptide transport into the frog small intestine. They concluded that

the transport of glycyl-L-leucine was a consequence of two routes of uptake available to the constituent amino acids. Some forty percent of glycine and L-leucine, initially presented to the intestinal mucosa as the dipeptide, was shown to enter via the free amino acid uptake system following their liberation by surface hydrolysis. The remainder entered via an intact peptide system. It was found that it was the free amino acid route which was Na^+ dependent while the peptide route was not. Evidence exists indicating that a variety of other peptides are also transported via both the free amino acid system following hydrolysis and via an intact peptide route (Fern et al., 1969; Cheng et al., 1970; Adibi, 1971; Rosen-Levin et al., 1980). With the contribution made to peptide transport by free amino acid system eliminated the uptake of glycyl-L-leucine, leucyl-L-glycine and carnosine have been found to be Na^+ independent and non-concentrative (Cheeseman and Parsons, 1976; Cheeseman, 1980).

Clearly, an investigation of peptide transport cannot be properly carried out without eliminating the free amino acid uptake from surface hydrolysis. In the present work, the experiments were designed to follow the transport of glycyl-L-leucine (using radio-labelled peptide) into everted rings of rat small intestine with the free amino acid uptake from surface hydrolysis either completely or partially blocked by one of three separate methods. Initially the free amino acid uptake system was saturated with 20 mM cold free

L-leucine; next the surface hydrolysis was reduced by the use of an aminopeptidase inhibitor L-alanyl-β-naphthylamide; and finally an attempt was made to eliminate the Na^+ dependent free amino acid uptake through the use of the alkaloid harmaline at concentrations reported to inhibit Na^+ dependent transport mechanisms.

The study was concluded with an investigation of the specificity of the intact peptide transport system to determine whether more than one uptake route is available to the intact peptide.

ABBREVIATIONS

In an effort to simplify the text of this work the names of the amino acids, whether occurring singly or in the form of a peptide, have been abbreviated to the first three letters of their complete name (with the single exception of tryptamine which is shortened to Trp). Note that unless otherwise indicated all amino acids and their corresponding peptides are considered to be in the 'L' configuration. Below is a key to the abbreviations employed listed in alphabetical order.

AMINO ACIDS

Abbreviation	Full Name
Ala	L-alanine
Arg	L-arganine
Asp	L-asparagine
Glu	L-glutamic acid
Gly	glycine
His	L-histidine
Hyp	hydroxyproline
Leu	L-leucine
Met	L-methionine
Phe	L-phenylalanine
Pro	L-proline
Sar	sarcosine
Trp	L-tryptamine
Tyr	L-tyrosine
Val	L-valine

PEPTIDES

Abbreviation	Full Name
Ala-Phe	L-alanyl-L-phenylalanine
Asp-Gly	L-asparagnyl glycine
Asp-Phe	L-asparagnyl-L-phenylalanine
β Ala-Gly	β alanyl-glycine
β Ala-His	β alanyl-L-histidine
Glu-Glu	L-glutamyl-L-glutamic acid
Gly-Gly	glycyl glycine
Gly-Leu	glycyl-L-leucine
Gly-Met	glycyl-L-methionine
Gly-Pro	glycyl-L-proline
Gly-Sar	glycyl sarcosine
Gly-Trp	glycyl-L-tryptophan
Gly-Val	glycyl-L-valine
Leu-Ala	L-leucyl-L-alanine
Leu-Gly	L-leucyl glycine
Leu-Leu	L-leucyl-L-leucine
Leu-Phe	L-leucyl-L-phenylalanine
Lys-Lys	L-lysyl-L-lysine
Met-Met	L-methionyl-L-methionine
Pro-Hyp	L-prolylhydroxy proline
Pro-Gly	L-prolyl glycine
β Ala-Gly-Gly	β alanylglycyl glycine
Gly-Gly-Gly	glycylglycyl glycine
Gly-Sar-Sar	glycylsarcosyl sarcosine
Leu-Gly-Gly	L-leucylglycyl glycine
Met-Gly-Met	L-methionylglycyl methionine
Ala-Gly-Gly-Gly	L-alanylglycylglycylglycine
Gly-Leu-Gly-Gly	glycyl-L-leucylglycylglycine
Leu-Gly-Gly-Gly	L-leucylglycylglycylglycine

The uptake of an amino acid from a solution of that amino acid is notated by the abbreviated name of the amino acid followed in brackets by the solution from which it originated. For example, the uptake of free L-leucine (Leu) from a solution of free Leu is indicated as Leu (Leu). The uptake of an amino acid from a peptide is similarly notated by the abbreviated name of the amino acid followed in brackets by the abbreviated name of the peptide form which it originated. For example, the uptake of L-leucine (Leu) from the dipeptide glycyl-L-leucine is written as Leu (Gly-Leu).

Gly (Gly-Leu) indicates the uptake of glycine from glycyl-L-leucine.

HISTORICAL REVIEW

In the early 1800's it was believed that whole protein was absorbed by the intestine with no chemical change whatsoever. This "Liebig theory" was shown to be inadequate by Salvioli (1880) and Hofmeister (1881) who demonstrated that peptone left in the lumen of a "living" intestine "disappeared" or was so changed as to no longer yield a biuret reaction. The feeding experiments of Plósz and Maly, working independently, showed that peptones could replace protein as food (1874).

Following this and other works it became generally accepted toward the end of the 19th century, that protein was broken down by proteolytic enzymes of the gut to peptones and proteoses which being water soluble were then absorbed by a specific activity of the intestinal enterocyte (Reid, 1899). This theory gained general acceptance (with notable dissenters such as Salvioli, Funk, Heidenhain and Shore) despite the failure of researchers to isolate peptones from the blood stream and the demonstration by several investigators that amino acids could be isolated from the contents of the lumen during normal digestion. Kölliker and Müller, (1856) first identified leucine and tyrosine in the

lumen of the intestine but attached no importance to their discovery. Kühn (1867) identified the proteolytic enzyme trypsine and subsequently demonstrated that it was able to break down fibrin introduced into the duodenum releasing appreciable quantities of leucine and tyrosine. Kühn concluded, however, that proteid so completely hydrolyzed could not be absorbed by the intestine and therefore that such complete hydrolysis was unnatural. Although the work of Kölliker et al. and Kühn was repeated by several workers (Schmidt and Mulheim, 1879; Sheridan Lea, 1890) it was not until Neumeister (1897) that the significance of the presence of free amino acids in the itestinal lumen was appreciated. Neumeister felt that this finding pointed to the further breakdown of peptones during the digestive process and that free amino acids might be absorbed by the intestine.

New light was shed on the situation by the discovery of Conheim (1901) that the intestine of octopus possessed a hydrolytic enzyme which consistently broke down peptones to their constituent amino acids. Conheim believed that only one enzyme was responsible and named it erepsin. Further, " . . . he was able to isolate leucine, tyrosine, lysine, histidine and arginine . . ." (Cathcart, 1912) from the octopus intestine.

Kutscher and Seeman (1901) isolated several amino acids from the intestine of a dog fed a protein meal; peptones and proteoses were not detected. These workers, in agreement with the conclusions of Neumeister (1897) suggested that amino

acids were the normal end products of protein digestion and as such were absorbed by the intestine and used by tissues for resynthesis into proteins.

Over the next few years several workers were able to establish that protein in the intestinal lumen was broken down beyond the peptone stage to its constituent free amino acids and peptides in the course of normal digestion (Abderhalden, 1912; Van Slyke et al., 1910; London, 1910, 1912; Conheim, 1912, 1913). Further it was shown that amino acids whether fed or injected intravenously could be utilized in metabolism (Loewi, 1902; Henderson et al., 1903; Buglia, 1912).

Although it was well accepted that protein digestion proceeded to the peptide and amino acid stage in the intestinal lumen and was absorbed as such there was reluctance to accept the concept of amino acids being taken into the blood and transported to tissues in this form. This reluctance stemmed from the failure of many workers to identify amino acids in the bloodstream of animals following a protein meal. As a result, Abderhalden and several others continued to hold the view that though proteins may undergo hydrolysis to amino acids in the lumen and be absorbed in this form they must be resynthesized to plasma proteins within the mucosal cells before being passed on to the blood (Abderhalden, 1912).

Through the development and application of more sensitive quantitative methods for the detection of amino

acids, Delaunay, and Van Slyke and Meyer (1911-12) largely resolved the controversy, laying to rest the resynthesis theory. These workers demonstrated a rise in amino nitrogen in the blood of dogs following a meat meal. Folin and Denis (1912) substantiated these findings by showing a rise in non-protein nitrogen in the portal blood of cats following intraintestinal injections of glycocoll. Definitive proof that amino acids were in the blood of dogs after a protein meal was provided by Abel, Rowntree and Turner (1913) who, utilizing a vivi-diffusion technique, were able to isolate, purify and identify several amino acids from such animals. Abderhalden (1912) verified these findings using a similar technique.

The accepted theory to around 1914, as to the fate of a protein meal was summarized by Van Slyke: "Ingested proteins are hydrolzyed in the digestive tract setting free most, if not all, of their amino acids. These are absorbed into the blood stream, from which they rapidly disappear as the blood circulates through the tissues." (Van Slyke and Meyer, 1913).

It should be noted here that to this time the possibility of peptide absorption from the intestine had not been discounted. Indeed, most investigators during this period observed that proteins were broken down in the gut to mixtures of amino acids and peptides (Van Slyke and Meyer, 1912, 1913; Cathcart and Leathes, 1906; Abderhalden, 1912; London, 1910; Kutscher and Seeman, 1901; Conheim et al.,

1912). The idea that peptides might be absorbed by the intestine and hydrolyzed during the uptake process or subsequent to it was discussed by Van Slyke in his 1913 review. He reasoned that " . . . normal digestion proceeds in the intestinal lumen and wall until most, if not all, of the proteolytic products are reduced to the stage of free amino acids" and suggests further that peptides might undergo hydrolysis " . . . either before or after entering the intestinal wall . . ." (Van Slyke, 1917) to free amino acids.

During the ensuing years there developed what is now referred to as the classical hypothesis of protein absorption. The evolution of this theory is difficult to trace but during the 1920's, 30's and on into the 50's it was believed that proteins were completely hydrolyzed to amino acids in the lumen of the intestine and absorbed in this form. Certainly one of the chief contributing factors to this theory was the concept held during this period that protein digestion products were absorbed from the lumen into the bloodstream by simple diffusion. It seemed logical " . . . that before leaving the intestinal lumen these products should first be reduced to free amino acids, their most diffusable form" (Matthews et al., 1975). The idea that amino acids entered the bloodstream from the intestinal lumen by simple diffusion arose largely from the failure of researchers to demonstrate a difference in the rate of uptake of stereoisomers of several amino acids. These experiments involved observing the rates of absorption of mixtures of 'L'

and 'D' configuration amino acids (Johnston and Lewis, 1928; Wilson and Lewis, 1929; Chase and Lewis, 1934; Bolton and Wright, 1937). In addition Kratzner (1944) reported that the rate of absorption of amino acids into chick intestine was inversely proportional to their molecular size.

Evidence already in the literature at this time, suggesting the active nature of amino acid (and peptide) transport by the intestine, was apparently ignored.

Reid (1896, 1899) studied the transport of peptones in an in vivo preparation of loops of dog ileum. He demonstated that reducing the blood supply (a procedure which would have reduced diffusion) to a loop of gut had no effect on peptone absorption but that atropine poisoning of the gut wall did reduce peptone uptake. Reid concluded therefore, that peptone absorption was a process involving an active "assimilation" by the epithelial cells of the ileum.

Also overlooked was the excellent work of Höber and Höber (1937). These workers studied the physical characteristics of amino acid absorption into loops of rat intestine. Their results led them to conclude that "Amino acids are absorbed at a rate higher than could be expected from their calculated molecular volume . . . ." They also found that " . . . the percentage absorption of amino acids falls off with rising molarity" which is incompatible with a diffusion model for amino acid uptake. "Their (amino acids) absorption is not like diffusion, but is a process complicated by the presence of an accelerating factor . . ."

(Hober and Hober, 1937).

It was not until 1951 with the work of Gibson and Wiseman that the simple diffusion theory was finally rejected. These researchers utilized tied off loops of rat small intestine and showed clearly that the `L` isomers of some thirteen different amino acids disappeared more rapidly than their `D` isomers. Wiseman (1952) using the same technique showed that while the `L` isomers of alanine, phenylalanine, methionine, histidine and isoleucine could be transported against a concentration gradient, the `D' isomers could not. This absorptive process was also shown to be oxygen dependent.

Wilson and Wiseman (1953, 1954) developed the everted sac technique in 1953 and used it to demonstrate that the presence of one `L` amino acid could inhibit the entry of another into the intestinal enterocyte. This early work stimulated a series of investigations characterizing the competition between amino acids for entry into the intestinal cells which continue today. This work has lead to the conclusion that there exist specific energy requiring pathways in the enterocytes for the transfer of amino acids from the lumen into the absorptive cell.

Although this and similar work dispelled belief in the simple diffusion theory of amino acid absorption, it surprisingly served to reinforce belief in the classical theory of protein absorption. It was reasoned that if there exists specific active mechanisms for amino acid transport

then protein must be broken down to this level before absorption could possibly take place.

Dent and Shilling (1949) lent support to this so called classical hypothesis through their examination of dogs' blood utilizing the then new technique of two dimensional paper chromatography. They showed that there was a substantial rise in the amino acid levels of both portal and jugular samples following a protein meal. Christensen et al. (1947, 1949) provided further evidence in favor of complete protein degradation to amino acids when they failed to detect any rise in peptides in the peripheral blood of man after the ingestion of gelatin. It is interesting to note that these workers did find a slight rise in plasma peptides following a protein meal in dog but dismissed the findings as insignificant. Subsequently many other workers demonstated that dietary protein was absorbed into the blood mainly as free amino acids in several different species (Parshin and Rubel, 1951; Denton et al., 1954; Wheeler and Morgan, 1958; Newey and Smyth, 1959). To most investigators at this time the fact that protein entered the portal blood stream only as free amino acids was proof enough that degradation of a protein meal in the intestine must be to the amino acid stage. Apparently the idea that protein digestion products might be absorbed into mucosal cells in one form and enter the blood stream in another had been forgotten.

Evidence had been accumulating over the previous years which was inconsistent with the classical theory of protein

degradation and absorption and which it seems was ignored until Fisher's interjection in 1954.

Cajori (1933) in a study of peptone absorption from Thiry-Vella loops in dogs found that the rate of disappearance of the peptone was much faster than could be accounted for by the hydrolytic activity of the luminal enzymes. He suggested therefore, that there must be hydrolytic activity intimately associated with the mucosal cells and/or perhaps peptones were absorbed as such and hydrolyzed to amino acids within the cells. Earlier studies also had questioned the adequacy of the proteolytic activity of the intestinal lumen to account for the rapidity of peptone absorption (Dunn and Lewis, 1921) and observed that peptones appeared to be taken up by the intestine much more quickly than corresponding mixtures of completely hydrolyzed protein (Nolf, 1907; Messerli, 1913).

Fisher (in a critique of the classical theory) (1954, 1967) cited this earlier work emphasizing that the time required for the complete digestion of protein was a matter of days and that it was likely therefore, that peptides entered the intestinal enterocyte. He pointed out that there must be a distinction made between the uptake of substrate from the lumen into the serosa and passage from the serosa to the blood stream and therefore experiments showing a rise in amino acids in the blood stream following a protein meal did not negate the possibility of peptide absorption from the lumen.

One of the earliest studies of peptide transport was that of Agar, Hird and Sidhu in 1953. These workers looked at the absorption of Gly-Gly, Gly-Gly-Gly and Leu-Gly into the perfused rat intestine, a preparation developed by Fisher and Parsons (1949). They demonstrated that while most of the peptides were hydrolyzed before entering the blood stream trace amounts of both Gly-Gly and Leu-Gly appeared on the serosal side. Further they were able to show that the Gly-Gly appearing on the serosal side was the result of intestinal transport of the dipeptide and not the resynthesis from free Gly.

The comments of Fisher as to the inadequacy of the evidence supporting the classical hypothesis of protein absorption and the results of Agar's et al. work prompted further study of peptide transport. Newey and Smyth (1957, 1959) studied the uptake of Gly-Tyr, Gly-Leu and Gly-Gly into everted intestine of rat and into loops of dog intestine; the latter being an in vivo preparation. They found, as had Agar et al., that most of the dipeptides transferred appeared on the serosal side as free amino acids and that only in the case of Gly-Gly did significant amounts of intact dipeptide appear in the blood or serosal fluids. They did comment it was possible that: " . . . unchanged peptide . . . " could " . . . enter the mucosal cells from the intestinal lumen." (Newey and Smyth, 1959).

The findings of Newey and Smyth were verified the same

year by Wiggans and Johnston (1959) who utilized the Wiseman preparation of everted sacs of rat intestine. They observed that intact Gly-Gly did appear on the serosal side of the gut, following introduction of the dipeptide to the mucosal surface. They suggested that Gly-Gly might be more resistant to hydrolysis than other peptides thereby allowing for its intact transport across the mucosal barrier.

It was the work of Newey and Smyth from 1960 to 1964 which served to clarify the role of peptide transport in protein digestion and brought about the eventual abandonment of the classical theory of protein absorption. Their 1960 study traced the absorption of several dipeptides (Gly-Gly, Gly-Leu, Gly-Trp, Gly-Try, Leu-Gly) by an in vitro preparation of everted rat small intestine and an in vivo perfusion of rat intestinal lumen. It was shown that the peptidase activity in both the in vitro and in vivo mucosal solutions was inadequate to account for the rate of dipeptide hydrolysis and disappearance observed. This lead Newey and Smyth to suggest that protein in the intestinal lumen undergoes partial hydrolysis to peptides of different sizes which are then absorbed into the enterocytes and hydrolyzed therein to amino acids.

The work was continued with a detailed study of Gly-Gly uptake into in vivo and in vitro preparations of rat small intestine (Newey and Smyth, 1962). They found Gly-Gly to be taken up into the absorptive cells intact and subsequently hydrolyzed intracellularly. This finding lent support to

their previous conclusions (Newey and Smyth, 1960). They demonstrated further that the rate of entry of Gly-Gly was approximately the same as that for free Gly, despite the larger size of the molecule and that the dipeptide's transport was via a saturable mechanism which was inhibited by anoxia and 2, 4 - dinitrophenol. The authors did not consider this as sufficient evidence to conclude that Gly-Gly entrance into the intestine was via a mechanism distinct from that utilized by free Gly.

Crane and Neuberger (1960) used ^{15}N-labelled whole protein in their study of protein digestion in man. They showed that a maximum concentration of ^{15}N-labelled amino acids appeared in the systemic blood between 30 and 50 minutes after the ingestion of the protein, indicating that the digestion and absorption process occurs more rapidly than had previously been assumed. These investigators concluded therefore, that protein must enter the mucosal cells largely as intact peptide, following an initial rapid intraluminal hydrolysis " . . . by cooperation of pepsin, trypsin and chymotrypsin", therein be hydrolyzed to free amino acids and subsequently be released in this form into the portal blood." (Crane et al., 1960).

The inadequacy of the classical hypothesis was emphasized by Milne (1964) who reported that the genetic disorders of Cystinuria and Hartnup's disease were demonstrable in the intestine as well as the kidney. Both disorders are characterized by the failure of the intestine

(or kidney) to absorb certain essential free amino acids, however, patients with the defect display no signs of protein malnutrition following a protein meal. An explanation of the phenomena is not possible based on a theory that protein is absorbed only as free amino acids from the intestine.

Still, at this time, there was a reluctance by the majority of investigators to accept the idea that peptides could enter the mucosal cells of the intestine. This resistance arose, it would appear, from the inability of Newey and Smyth and others to determine precisely the extent of the contribution made by intact peptide transport and cellular hydrolysis in protein digestion and absorption. Also a factor was the work of Ugolev (1964, 1966) whose research on sugar absorption demonstrated that disaccharides are hydrolyzed on the surface of the brush border membrane (surface digestion) with the resulting monosaccharides being taken up by the enterocyte. This sequence of events was assumed to apply to the transport of peptides and was especially attractive since it did not require the consideration of a new mechanism for the transport of protein. The amino acids released from the peptide by surface hydrolysis would be absorbed by the well known and extensively studied free amino acid transport system. The acceptance of this hypothesis was supported by the demonstration of Nachlas et al. (1960) that leucine aminopeptidase is only found in the brush border region of the intestine and probably on its surface and by the

contention, held by many, that Newey and Smyth's work was done with an uncommon and atypical peptide.

Interest in peptide transport and its significance to protein digestion waned after 1962 but was rekindled with the findings of Matthews et al. (1968) and Adibi et al. (1968).

These investigators, working independently, demonstrated that amino acids are taken up into the intestine more rapidly from di and tri peptides than from a solution of the equivalent free amino acids.

Matthews et al. (1968) using an in vivo preparation of tied loops of rat intestine showed that Gly uptake from Gly-Gly and Gly-Gly-Gly was more rapid than from a solution of the free amino acid. This work served to verify Newey and Smyth's observations on Gly-Gly absorption as well as their conclusion that the peptide must be transported into the absorptive cells of the rat intestine intact.

Adibi et al. (1968) used a constant in situ perfusion technique to examine the relative rates of absorption of Gly-Gly and Gly-Leu and their constituent amino acids into the jejunum of man. They found that the uptake rates of amino acids from the dipeptides was greater than from mixtures of the equivalent amino acids. These workers considered that their results " . . . suggest the existence of another entry mechanism, besides the free amino acid carrier, for the transport of the products of protein digestion in the intestine." (Adibi and Phillips, 1968).

Matthews et al. (1969) extended their observations on

peptide absorption into in vivo loops of rat intestine, to include mixed di and tri peptides of Gly and Met. They found the mechanism of transport for Met-Met and Gly-Met to be saturable and the absorption of all amino acids to be faster from the peptides than from the equivalent free amino mixtures, in all cases. This investigation also noted that the competitive inhibutory effect of Met on Gly transport, normally seen in mixtures of the two free amino acids, was avoided when the mixed peptides, Gly-Met, Met-Gly and Met-Gly-Met were presented to the intestinal mucosa.

Over the next several years the kinetic advantage of amino acid uptake into intestinal tissue from peptides over free amino acids became well established in several different species and for a wide variety of peptides (Adibi et al., 1971, 1974; Cheng et al., 1971; Lis et al., 1971; Cheeseman et al., 1974, 1976; Matthews et al., 1974; Lane et al., 1975). According to Fisher, who in 1967 stated: "If it could be shown that a peptide left the intestine more rapidly than did an equimolar mixture of its constituent amino acids, we should have proof of peptide absorption whatever appeared on the other side of the mucosa", the above studies demonstrate that intact peptide transport into the intestine does occur.

The work of Asatoor et al. (1970) provided additional very strong evidence for a distinct peptide transport mechanism as well as an indication of the nutritional significance of this sytem. Their investigation involved a study of the uptake of β-alanine, L-histidine and the

dipeptide β-alanyl-L-histidine by the intestine of a patient with Hartnup's disease. This inherited disease (outlined previously in this section) is characterized by the inability of the jejunum and renal tubules to absorb certain neutral amino acids including L-histidine. Using an oral tolerance test it was shown that while the unaffected amino acid β-alanine was readily absorbed, L-histidine was not. However, when these amino acids were administered as the dipeptide β-alanyl-L-histidine (carnosine) both were found to be absorbed normally. Some investigators at this time suggested that a dipeptide made up of one affected and one unaffected amino acid would be transported via the pathway of the unaffected amino acid. Asatsoor et al. (1970b), however, demonstrated that the affected amino acid, phenylalanine, was readily transported when presented to the defective intestine as di-phenylalanine.

Analogous results have been obtained from a study of patients with cystinuria, a disease in which the intestine of patients cannot absorb the amino acids ornithine, lysine, arginine and cystine (Hellier et al., 1970, 1972). As in Hartnup patients, the affected free amino acids were readily transported when administered in the form of a dipeptide.

These results strongly indicate that dipeptides are transported intact into the intestinal enterocyte via a mechanism distinct from that utilized by free amino acids. Also the fact that Hartnup and cystinuric patients do not show signs of protein malnutrition suggests that amino acid

absorption in the form of small peptides is of major nutritional significance.

The renewed interest in the study of peptide absorption into intestinal tissues, stimulated by the aforementioned observations, has yielded additional evidence supporting the concept of a distinct peptide transport mechanism. The following is a brief summary of the more important of these findings together with representative examples.

It has been shown repeatedly that the competition for a transport mechanism normally exhibited between free amino acids can be reduced or completely avoided when the same amino acids are constitutents of a peptide (Matthews et al., 1969; Chung et al., 1970, 1979; Rubino et al., 1971; Adibi, 1971; Cheeseman et al., 1974, 1976). Cheeseman and Parsons (1974, 1976) studied the transport of Leu and Gly and Gly-Leu into the vascular bed of an in vivo preparation of perfused frog intestine. They showed that while the transfer of Gly was greatly inhibited by the presence of Leu, with Gly-Leu in the intestinal lumen the transfer of Gly was equal to that of Leu. There was no dipeptide evident in the portal blood.

There appears to be no competition between peptides and amino acids for a transport mechanism when the complication of superficial hydrolysis is taken into account (Rubino et al., 1971; Cheeseman et al., 1974; Sigrist-Nelson, 1975). Sigrist-Nelson used radio active labels to study the uptake of Gly, Leu, Gly (Gly-Leu) and Leu (Gly-Leu) into isolated rat intestinal brush border membranes vesicles. Gly and Leu

present in the incubation medium at 40 times the concentration of Gly-Leu failed to inhibit the transport of either labeled species of the dipeptide. Conversely, Gly-Leu at 40 times the concentration of Gly and Leu had no effect on the uptake of either free amino acid.

Several groups have demonstrated that when the enterocytes of the intestine are exposed to a concentration of constituent amino acids that is sufficiently high to saturate the free amino acid transport system, further uptake of these amino acids is observed upon the addition of the peptide (Adibi, 1971, 1974; Crampton et al., 1973; Cheeseman et al., 1974, 1976; Ward and Boyd, 1980, 1982). The previously mentioned study of Cheeseman and Parsons (1976) demonstrated that when the peptide Gly-Leu was added to the frog intestinal lumen in the presence of saturating concentrations of Gly and Leu (10 mM), further transfer of the constituent amino acids into the vascular bed occurred. This additional transfer, which the authors suggest must have entered the intestine via an intact peptide route, was shown to be saturable. Other 'saturation' studies are discussed later in this section.

Other observations which support the notion of an independent peptide transport system have been discussed under separate headings, these include: the different effect the removal of medium Na^+ has on peptide and free amino acid uptake, the competition for transport exhibited by peptides and the demonstration of intact peptide transport.

Presently it is regarded as established fact that intestinal protein digest is absorbed by the mucosal cells both as free amino acids and as small peptides in nutritionally significant amounts. In order to more fully outline the present understanding of the peptide absorptive mechanisms however, a consideration of the hydrolysis of protein together with the hydrolases responsible is necessary.

The intraluminal hydrolysis of protein is accomplished chiefly through the actions of pancreatic endopeptidases (trypsin, chymotrypsin and elastase) and exopeptidases (principally carboxypeptidases A and B). A trace of aminopeptidase activity is also associated with pancreatic secretions (Gray and Cooper, 1971; Kim, 1977; Matthews et al., 1980).

Studies carried out in man, dog and rat indicate that the main product of the hydrolysis of dietary protein during the intraluminal phase of digestion is a complex mixture of free amino acids and small peptides, with the peptides predominating (Nixon and Mawer, 1970 a, b; Crampton et al., 1971; Adibi et al., 1973; Chung et al., 1979). The finding that peptide concentrations are usually much larger than free amino acids both in the jejunum and ileum establish the importance of peptides as a substrate for absorption sites (Adibi et al., 1981). Estimates of the mean chain length of the peptides are few but suggest a range of from two amino acid residues to as large as six (Chen et al., 1962; Adibi et

al., 1973).

Since peptide hydrolase activity has been shown to be very high in the intestinal epithelium but very slight in pancreatic secretions and intraluminal contents plus the fact that mainly free amino acids appear in the portal blood, the epithelium is considered to be the site of final peptide hydrolysis (Josefsson and Sjostrom, 1966; Peters, 1970; Kim, 1977; Matthews, 1975, 1980; Adibi et al., 1981). Subcellular fractionation studies have revealed that most of the peptide hydrolase activity associated with the intestinal epithelium is located in both the brush border membrane and the cytoplasmic fractions (Josefsson et al., 1966; Rhodes et al., 1967; Hezier et al., 1969; Peters, 1970; Kim et al., 1972, 1974).

Brush border peptidases are considered to be active against peptides of from two to eight amino acid residues. They hydrolyze peptide bonds sequentially from the NH_2 -terminal end of the peptide and are therefore referred to as amino-oligopeptidases (Kim, 1977; Adibi et al., 1981). The brush border peptidases have been found to be distinct from those in the cytoplasm of the villous cells with the activity of each depending on the amino acid composition and chain length of the peptide substrate presented. About ten percent of the total cellular activity against dipeptides is associated with the brush border membrane, the remaining activity being found in the cytoplasmic fraction. With tripeptides as the substrate, 60 percent of the peptidase

activity is associated with the brush border and the majority of the activity against tetrapeptides is found in this fraction. Peptides made up of more than four amino acid residues appear to be hydrolyzed exclusively by brush border amino-oligopeptidases (Robinson, 1963; Josefsson et al., 1966; Hezier et al., 1969; Kim et al., 1972, 1974, 1977). Cytoplasmic amino-peptidase activity is mainly associated with di- and tripeptidase activities while amino-oligopeptidase activity probably represents the majority of the aminopeptidase activity of the intestinal brush border membrane. There are several lines of evidence suggesting that the peptidases found in the brush border membrane and those in the cytosol are distinct groups of enzymes. The zymograms obtained from these two groups of enzymes differ. The patterns obtained from the brush border fraction show few bands, are homogeneous and appear not to depend on the substrate used. On the other hand zymograms obtained with cytosolic enzymes are characterized by heterogeneous multiple band patterns which vary according to the substrate used. It is interesting that the zymograms produced from cytosolic enzymes obtained from various organs of the rat, (liver, kidney, small intestine) are virtually identical (Kim et al., 1977). Kim postulates therefore, that the peptidases of the cytoplasm do not vary between organs, and that these enzymes ". . . may play a more general role in cellular metabolism." (Kim, 1977). Zymogram patterns obtained from brush border peptidases of the small intestine have been found to be

unique to this organ (Kim et al., 1977). Wojnarowska and Gray (1975) noted the different specific activities displayed by intestinal brush border hydrolases as compared to their renal counterparts and suggested that perhaps the intestinal membrane peptidases are organ specific. The studies of Norén et al. (1977) however, show that there are similarities between the "intestinal brush border peptidases and the corresponding particulate enzymes from other tissues, a finding which is also valid for cytosolic dipeptidases." (Norén et al., 1977). These workers suggest that the role played by brush border peptidases in digestion depends on their localization in a particular organ and not on differentiation of enzymes for a specific function. Continued work in the area will clarify this point. Brush border enzymes have been shown to be more heat stable than cytosolic enzymes and unlike the peptidases of the cytoplasm are uninhibited by p-hydroxymercuri-benzoate. It has been assumed for some time that di- and tripeptides containing Pro are hydrolyzed exclusively in the cytoplasm as peptidase activity against these Pro containing peptides has been shown to be absent from the brush border membranes of man and rat (Fujita et al., 1972; Kim et al., 1977; Adibi et al., 1981). Recently however, Ganapathy et al. (1981) reported significant Gly-Pro hydrolyzing activity in highly purified brush border membrane vesicles of kidney and intestine in rabbit, dog and pig (Ganapathy et al., 1981).

Isolation and purification of two amino-oligopeptidases

of the brush border of rat intestine has been accomplished by several groups (Wojnarowska and Gray, 1975; Kim and Brophy, 1976; Kim, 1977; Gray and Santiago, 1977; Kania et al., 1977). The enzymes isolated while not being identical between groups were all found to be high molecular weight integral transmembrane glycoproteins with two domains. A hydrophillic moiety of the protein extends out of the membrane bilayer (representing as much as 80 percent of the molecule) being oriented toward the external surface of the microvillus membrane. This portion includes the active site as well as the total sugar content of the enzyme. A smaller hydrophobic peptide sequence is integrated into the lipid matrix thereby anchoring the protein to the membrane (Kim et al., 1976; Louvard et al., 1975; Adibi et al., 1981). The hydrophoibic portion of the peptidase has been shown to span the microvillus membrane with a portion situated at the inner face of the membrane (Louvard et al., 1976). As stated by Wojnarowska and Gray:

> "These peptide hydrolases are strategically located at the intestinal lumen cell interface and possess biochemical characteristics making them ideally suited to play a pivotal role in the final stage of protein digestion." (Wojnarowska and Gray, 1975)

The isolated enzymes were shown to hydrolyze a wide variety of peptides (including Gly-Leu) containing neutral or basic amino acids all requiring a free α-amino acid and L-configuration of the amino acids at the NH_2-terminal and COOH-terminal ends. The rate of peptide hydrolysis by brush

border peptidases is influenced by the chain length of the substrate. Wojnarowska and Gray (1975) noted that the major enzyme they isolated (oligopeptidase II) hydrolyzed tri- and tetra-peptides at a faster rate than dipeptides. The detailed study of Kania et al. (1977) also working with amino-oligopeptidase II found that for each series of peptides tested, the addition of a third amino acid residue increased both the affinity of the substrate for the enzyme and the rate of hydrolysis. In the case of the Phe peptides, the addition of a fourth residue lowered the Km but brought about no further increase in the rate of hydrolysis over that found for the tripeptide. Other factors determining the efficiency of the hydrolysis of peptides is discussed later in this section.

The intracellular hydrolysis of small peptides is accomplished by the aminopeptidases of the cytosol. The great majority of the cytosol peptidase activity is against di- and tripeptides although Chung et al. (1979) presents evidence for the intact transport of a tetrapeptide into rat small intestine in vivo which is apparently hydrolyzed within the mucosal cells. Most, if not all, di- and tripeptides can be hydrolyzed by both brush border and cytosol peptidases and as in the brush border, peptides are hydrolyzed in the cytoplasm from the NH_2 -terminal end. There are several dipeptidases in the cytoplasm of the intestinal mucosal cells each with different although overlapping substrate specificities but some have an exceptionally broad range of

substrates.

A true dipeptidase with a wide substrate specificity has been isolated and purified from the intestine of pig (Norén et al., 1971, 1973) and of monkey (Das et al., 1973). This enzyme was found to be active against Gly-Leu (and hence has been named Gly-Leu dipeptidase) as well as nearly all the neutral dipeptide substrates tested with the exception of Gly-Gly, Gly-Pro, Gly-His and some dipeptides composed of basic or acidic amino acids. Investigators consider that Gly-Leu dipeptidase is responsible for most of the intracellular dipeptidase activity of the intestine (Josefsson et al., 1977; Adibi et al., 1981).

A cytoplasmic aminotripeptidase has been isolated which has a broad substrate specificity for tripeptides with a free α-NH_2 group, a free α-COOH group and L-configurations of the first two amino acid residues (Adibi et al., 1981). This enzyme differs from the brush border amino-oligopeptidases in that it does not hydrolyze peptides larger than three amino acid residues long and will hydrolyze tripeptides with a Pro at the NH_2 -terminus.

Peptide Molecular Structure as it Affects Uptake and Hydrolysis by the Intestinal Musocsal Cells

Although there still exists a need for a systematic study of the subject, several investigations have demonstrated that the molecular structure of a peptide

influences its affinity for the transport site as well as the degree of hydrolysis it undergoes at the intestinal brush border.

Sterioisomerism

The peptide transport system of the small intestine favors peptides made up of L-amino acids. Peptides containing D-amino acids appear to be transported via the same system as those made up of L-amino acids but at a reduced rate. Also the presence of D-amino acids reduces the rate of hydrolysis by the peptidases of the intestinal epithelium.

The fact that the introduction of a D-amino acid residue reduces the rate of transport of a dipeptide was first shown by Burston et al. (1972). They noted that Gly-D-Val was transported very slowly by rings of everted rat gut and that hydrolysis of the dipeptide was also reduced which was evidenced by the appearance of intact peptide in the intracellular fluid.

Cheeseman and Smyth (1972, 1973) found the D-Leu-Gly transport into everted sacs of rat ileum while being slow, occurred via a saturable mechanism apparently shared by L-Leu-L-Ala. They reported that D-Leu-Gly was hydrolyzed only slightly by homogenates of the small intestine. Additional evidence that peptides containing D-amino acid residues are transported by the same carrier system as those made up of L-amino acid residues was provided by Addison et

al. (1975). These workers observed that Gly-D-Leu, which was poorly transported, inhibited the uptake of Gly-Sar-Sar though much more weakly than did L-Gly-L-Leu. The presence of D-amino acids apparently reduces affinity for the transport system.

The study carried out by Asatoor et al. (1973) on the transport and hydrolysis of dipeptides containing D-amino acid residues remains the most thorough to date. They observed that L-Ala-L-Phe and L-Leu-L-Leu were taken up into loops of tied rat small intestine at rates many times greater than those of their D-D and L-D isomers although the L-D isomers were taken up more quickly than the D-D isomers. The major portion of the absorption of both D-Ala-D-Phe and D-Leu-D-Leu was concluded to occur in the ileum since the rates of uptake were much greater there than in the jejunum. Hydrolysis of D-Ala-D-Phe, D-Leu-D-Leu and Gly-D-Trp was slight, all of these dipeptides appearing intact in significant quantities in the urine of the test animals. Finally, in the jejunum the rate of absorption of Gly, D-Ala, D-Leu-D-Phe, D-Trp, D-Ala-D-Phe, D-Leu-D-Leu and Gly-D-Trp was shown to decrease with the increasing molecular weights suggesting a large simple diffusive component to their uptake. This relationship was not found in the ileum.

Recently Boyd and Ward (1982) completed a microelectrode study of several aspects of oligopeptide transport into the small intestine of the mudpuppy (Necturus maculosus). As part of this work they showed that while the entry of L-Leu-

L-Leu was associated with a depolarization of the intestinal cell membrane, the exposure of the mucosal cells to the D-D form of the peptide produced only a very slight alteration in membrane potential. The authors interpret this to mean that the L-L configuration of the peptide was transported much more readily than the D-D form.

Amino Acid Side Chains

Very little information is available in the literature regarding the influence amino acid side chain structure has on peptide transport.

Adibi et al. (1974) found that Gly-Leu and Leu-Gly were absorbed better than Gly-Gly into human jejunum. They interpreted this data as an indication that longer chain amino acids whether in the amino terminal or carboxyl terminal positions, increases a dipeptide's affinity for the carrier system (Adibi et al., 1981).

Kania et al. (1977) carried out a kinetic analysis of the major rat intestinal surface membrane aminopeptidase (amino-oligopeptidase II) with oligopeptide substrates. Their results indicate that a bulky side chain on the NH_2-terminal amino acid of a peptide increases the affinity of the substrate for the enzyme as well as increasing hydrolytic rates. However, no decrease in Km with increasing length of the side chains on the carboxyl terminal amino acids of peptides could be demonstrated. The rate of the hydrolysis of peptides with Leu as the NH -terminal amino acid was shown

to incrase proportionally as the length of the side chains of the COOH-terminal amino acid increased. This effect was not observed with other peptides.

Amino Terminal Group

Evidence in the literature suggests that any substitution of the amino terminal group of dipeptides results in greatly reduced affinity for transport sites. Acetylation of the NH -terminal group of Gly-Gly reduced this peptide's ability to inhibit the uptake of Gly-Pro (Rubino et al., 1971), β-Ala-His (Addison et al., 1974), and Gly-Sar-Sar (Addison et al., 1975). The methylation of the NH_2-terminal group (Sar-Gly) resulted in poor uptake and hydrolysis (Burston et al., 1972) as well as failure to inhibit the transport of Gly-Sar-Sar (Addison, 1975). Das and Radhakrishnan (1975) concluded that the N-terminal, α-amino group is important in determining uptake since N-benzyloxycarbonyl-Gly-Leu was found to be a poor inhibitor of Gly-Leu transport. Also Amaya-F et al. (1976) demonstrated that N, N-di (1-deoxy-2-ketosyl)-Gly-Leu was not taken up by an in vivo preparation of rat small intestine, nor was this dipeptide hydroyzed to any extent by Leu-aminopeptidase.

Kania et al. (1977) has shown that the affinity of dipeptides for the major rat intestinal surface membrane aminopeptidase as well as their rate of hydrolysis may vary according to the amino acid residue at the NH_2 -terminal position. They found that the Leu group provided a "marked

kinetic advantage over most of the NH_2 -terminal amino acids tested." This advantage was most notable when Leu dipeptide hydrolytic rates and affinities were compared to those of Gly and Val dipeptides, the former having hydrolytic coefficients one to two orders of magnitude higher than the latter. This data is compatible with the observation that Leu-Gly hydrolysis is greater in frog intestine than is that of Gly-Leu (Cheeseman et al., 1976).

Carboxyl Terminal Group

Addison et al. (1974, 1975) were able to show that the methylation of the carboxyl groups of Gly-Gly and Gly-Gly-Gly eliminated the ability of these peptides to inhibit Gly-Sar-Sar and β-Ala-His absorption into hamster jejunum in vitro. This same study found that the esterification of Asp-Phe to produce Asp-Phe-OMe significantly reduced its ability to inhibit Gly-Sar-Sar absorption (Addison et al., 1975). Although based solely on competition studies many authors have concluded that the amidation and/or esterification of the carboxyl group of dipeptides reduces their affinity for transport mechanism (Addision, 1974, 1975; Matthews, 1975, 1980; Adibi et al., 1981).

The rate of hydrolysis of dipeptides has been shown to be an order of magnitude higher when their COOH-terminal residue is Leu or Phe than when it is Gly (Kania et al., 1977).

Elongation of the Peptide Chain and Substitution of the Peptide Bond

Studies have shown that β-Ala-His, β-Asp-Gly and β-Ala-Gly-Gly, while being poorly hydrolyzed are transported into hamster jejunum in vitro and that this transport can be inhibited by various other peptides which have an α-amino acid (Matthews, 1974; Addision et al., 1974 a, b, 1975). This has led to the conclusion that the elongation of the peptide length by the insertion of an 'extra' carbon between the NH_2 -terminal group and the peptide bond effects hydrolysis of the dipeptide but not its affinity for the peptide transport mechanism. However, the insertion of two 'extra' carbons, a γ linkage, appears to be incompatible with dipeptide transport. Burston et al., (1972) observed that γ-Glu-Glu was transported very poorly into rat small intestine and was hydrolyzed slowly, intact peptide appearing in the intracellular fluid.

Addison et al. (1972, 1974, 1975) through their studies on Gly-Sar uptake have concluded that methylation of the nitrogen of the peptide bond of a dipeptide appears to reduce hydrolysis but not transport. It is interesting to note that Gly-Sar as well as Gly-Sar-Sar are the only peptides which have been demonstrated to be actively transported into intestinal tissue.

Although the dipeptide, Gly-Pro has a substituted peptide bond, its transport has been dmonstrated by several workers (Rubino et al., 1971; Addison et al., 1975; Das and

Radhakrishnan, 1975; Ganapathy et al., 1979, 1980). It would appear that Gly-Pro is hydrolyzed more slowly than many dipeptides an observation which has been attributed to the substituted peptide bond (Matthews et al., 1975, 1980).

A large proportion of the studies carried out to determine the influence molecular structure has on peptide transport and hydrolysis have been done in hamster using Gly-Sar and Gly-Sar-Sar. Obviously there is a need for further work on this subject utilizing other species and more common peptides.

Hydrolysis of Peptides

It is now well established that di- and tripeptide uptake is the result of three processes: (1) hydrolysis by luminal enzymes followed by absorption of the liberated free amino acids; (2) hydrolysis by membrane bound aminopeptidases followed by absorption of the liberated free amino acids and (3) intact absorption followed by intracellular hydrolysis (Adibi, 1971; Matthews, 1975, 1980). Intra-luminal hydrolysis appears to play only a very minor role (Kim et al., 1977) so the major question is the relative importance of surface hydrolysis followed by free amino absorption and intact peptide uptake. Estimates of the relative importance of these two processes has revealed that it is likely to vary from peptide to peptide.

Fern et al. (1969) estimated the surface hydrolysis of Leu-Leu, Leu-Gly, Gly-Leu and Gly-Gly by everted rings of rat

jejunum in vitro by determining the percentage inhibition of [^{14}C] Leu uptake caused by each dipeptide. They reasoned that it was the free amino acids released by surface hydrolysis which would bring about the observed inhibition of Leu uptake. The results indicated that at 1 mM concentration of the dipeptides, Leu-Leu was 50 percent hydrolyzed, and both Leu-Gly and Gly-Leu were about 35 percent hydrolyzed. They noted that Gly-Gly was hydrolyzed much more slowly and intact dipeptide was found in the tissue.

Both Cheng et al. (1970) and Caspary (1973) have utilized the snake venom L-amino oxidase in their studies of dipeptide uptake into everted rings of intestine in vitro. The enzyme which destroys L-amino acids by oxidative deamination but has no effect on dipeptides was shown to reduce Met (Met-Met) transport by slightly more than 50 percent and to reduce Met (Met-Gly) and Met (Gly-Met) to even lower levels while Gly transport was unaffected (Cheng et al., 1970). Caspary found that the enzyme approximately halved the uptake of Met (Met-Met), Leu (Gly-Leu), Leu (Leu-Gly) and Leu (Leu-Leu). These authors suggested that the reduction in uptake of these dipeptides caused by the L-amino oxidase was probably a result of the destruction of the amino acids liberated by superficial hydrolysis and that the remaining uptake was via a process insensitive to the enzyme, i.e., intact dipeptide transport. Also since only free amino acids were demonstrable in the intracellular fluid these results indicate that the final hydrolysis of these

dipeptides must occur at a site which is inaccesible to the enzyme (intramembrane or intracellular hydrolysis).

Adibi's (1971) investigation of the disappearance rates of Gly-Leu and Gly-Gly from the jejunum and ileum of man yields results which differ from the above. The presence of a saturating concentration of isoleucine produced only a 13 percent reduction in Leu (Gly-Leu) influx suggesting that the major portion of Gly-Leu transport into the mucosal cell is via an intact transport route. The uptake of Gly-Gly was completely unaffected by the presence of saturating concentrations of free Leu. Since Leu has been shown to have a much higher affinity for a carrier shared with Gly (Cheeseman and Parsons, 1976; Himukai and Hoshi, 1979) this observation indicates that Gly-Gly influx must be exclusively as the intact dipeptide.

Cheeseman and Parsons (1974, 1976) perfused the small intestine of the frog (Rana pipiens) with either Gly-Leu or Leu-Gly in the presence of a saturating concentration of free Leu and looked at the appearance of Gly and Leu in the vascular bed. With the free amino acid transport system saturated the authors assumed that any further transfer of amino acids from either of the dipeptides would have to be via the intact transport route. From this procedure they estimated that 60 pecent of Gly-Leu and Leu-Gly transport was the result of intact peptide absorption and therefore that about 40 percent of these dipeptides presented to the intestinal mucosa underwent superficial hydrolysis.

The saturation technique was utilized by Crampton et al., (1973) in their investigation of Met-Met and Gly-Gly absorption into tied loops of rat intestine in vivo. With the free Met uptake system saturated with 100 mM free Met, it can be calculated from their data that intact Met-Met transport accounted for some 60 percent of the observed uptake in the most proximal loops studied (duodenum and upper jejunum); about 80 percent in the following two loops (lower jejunum and upper ileum) and approximately 50 percent in the more distal loops (lower ileum). The absorption of Gly-Gly was determined to involve the uptake of intact dipeptide exclusively.

More recently Rosen-Levin et al. (1979) found that the disappearance of Leu-Gly-Gly and Leu-Leu from the perfused small intestine of rat in vivo was reduced by 38 and 51 percent respectively in the presence of 20 mM Gly-Pro. These workers considered this reduction to be due to the blocking of intact peptide transport by Gly-Pro and that the remaining uptake of Leu and Gly was via the free amino acid transport system following their liberation from the peptide by surface hydrolysis. Additional evidence was obtained through the use of either 0.1 mM phthalimido or Ala-β-naphthylamide (10 mM) both of which inhibit brush border aminopeptidases without affecting intact transport. In the presence of one of these inhibitors Leu-Gly-Gly transport was reduced by 64 percent and Leu-Leu uptake by 49 percent. This reduction was considered to be due to the inhibition of surface hydrolysis

thereby allowing only intact transport to take place. These results indicate that for Leu-Gly-Gly uptake surface hydrolysis followed by the uptake of free amino acids plays a slightly greater role in assimilation than intact transport while for Leu-Leu transport both processes are of equal importance.

Himukai and Hoshi (1979) studied the uptake of Leu (Gly-Leu) and Gly (Gly-Leu) into isolated everted segments of guinea pig intestine. They noted that Leu (Gly-Leu) influx was about twice that of Gly (Gly-Leu) and also that the saturation of the free amino acid uptake system reduced Leu (Gly-Leu) uptake by 46 to 64 percent but had no effect on Gly (Gly-Leu) transport. They concluded that Leu (Gly-Leu) uptake proceeds approximately equally via both the intact peptide and free amino acid transport systems while Gly (Gly-Leu) influx occurs via an intact peptide transport mechanism exclusively.

Yasumoto et al. (1980) were able to reduce Gly-Leu influx into isolated epithelial cell of rat intestine by 50 percent using a potent aminopeptidase inhibitor, bestatin, as well as demonstrate intracellular intact Gly-Leu in the presence of the inhibitor. This was taken by the authors as proof that Gly-Leu transport is mediated by the two processes of intact transport followed by hydrolysis and hydrolysis preceeding transport and that they contribute almost equally in the uptake of Gly-Leu.

Maximum Size of Transported Peptides

Matthews et al. (1968) studied the uptake of di-, tri- and tetraglycine into tied loops of rat intestine and found that Gly uptake was more rapid from each of the peptides than from a solution of the free amino acid. They concluded that all of the oligopeptides must therefore be transported intact. The following year Matthews et al. (1969) determined that both Met and Gly were transported more rapidly when presented as oligopeptides of Met alone or as the mixed dipeptide. Trimethionine was the largest peptide used.

In apparent contrast to the above work Peters et al. (1970) found only free Gly and Gly-Gly in the plasma and lymph of rats following intra-duodenal infusion of high concentrations of Gly and Gly oligopeptides (up to and including tetra-Gly). Since Peters et al. previous work (1968) indicated that most dipeptidase activity of the rat enterocyte was in the cytosol and that longer peptides were cleaved to free amino acids or dipeptides, it was concluded that only free Gly and Gly-Gly were transported into the enterocyte.

Edwards (1970) utilized tied loops of rat small intestine in vivo and demonstrated that Gly-Gly and Gly-Gly-Gly inhibited β-Ala-His uptake but had no effect on Leu-Gly transport. This was considered evidence that tri-Gly was transported via a route shared with β-Ala-His but not Leu-Gly. Both Addison et al. (1975) and Sleisenger et al.

(1976) showed that the poorly hydrolyzed di- and tri-peptides Gly-Sar and Gly-Sar-Sar were transported into rings of everted guinea pig intestine in vitro. They found, however, that the tetra peptide Gly-Sar-Sar-Sar was not taken up into the intestinal rings except very slowly and suggested therefore, that tri-peptides may be the largest peptides transported.

Adibi and Morse (1977) found that of the peptides of Gly studied only di- and tri-Gly were transported intact into the absorptive cells of human jejunum and everted rings of rat small intestine. The disappearance of hexa-, penta- and tetra-Gly was attributed to their being hydrolyzed by brush border amino-oligopeptidases into smaller peptides and free-Gly and absorbed as such. Smithson and Gray (1977) demonstrated that the tetrapeptide Gly-Leu-Gly-Gly was not transported intact into either an in vivo or in vitro preparation of rat jejunum. In contrast to these findings Chung et al. (1979) have reported that 50 percent of the transport of the tetrapeptide Leu-Gly-Gly-Gly into rat intestine is via an intact peptide route not shared by Leu-Gly or Leu-Gly-Gly. They showed that L-alanine which caused no inhibition of Leu (Leu-Gly) or Leu (Leu-Gly-Gly) uptake reduced Leu (Leu-Gly-Gly-Gly) transport by 50 percent. Also the use of 20 mM L-Alanyl-β-naphthylamide which inhibits brush border aminopeptidase activity had little effect on Leu (Leu-Gly-Gly-Gly) absorption. Burston et al. (1979) investigated the uptake of Leu-Gly-Gly-Gly as well as

Ala-Gly-Gly-Gly into rat and hamster everted intestinal rings and could find no evidence for intact transport of these tetrapeptides.

While Gly-Gly and Gly-Gly-Gly were found to cause large and persisting depolarizations of the intestinal enterocyte membrane of mudpuppy, the tetrapeptide, Gly-Gly-Gly-Gly, had no effect whatsoever on the membrane resting potential (Boyd and Ward, 1982). It was assumed therefore, that the tetrapeptide was not transported ". . . suggesting an upper limit of three amino acid residues for intestinal oligopeptide transport" (Boyd and Ward, 1982).

The Nature of the Mediated Transport of Peptides

The process of peptide transport displays saturation kinetics as well as specificity for the substrate transported (Matthews, 1975, 1980). It is therefore considered established that the mechanism responsible is mediated transport. What is less clear is whether this mediated system is active or passive. An active transport process may be defined as ". . . the energy-requiring movement of a metabolite or an inorganic ion across a membrane against a gradient of concentration" (Lehninger, 1975). In order to establish that peptide transport is the result of an active uptake mechanism therefore requires the demonstration that peptides are accumulated or concentrated within the enterocyte and also that this uptake is dependent on metabolic energy.

As early as 1962 Newey and Smyth showed that peptide transport into the small intestine was apparently inhibited by anoxia and metabolic inhibitors but more direct proof of active transport was difficult. The demonstration of intact peptide within the intestinal enterocyte accumulating against its concentration gradient is made difficult by the rapidity with which most small dipeptides are hydrolyzed within the mucosal cells. In an effort to circumvent this problem, Addision et al. (1972) studied the uptake of a readily transported but poorly hydrolyzed peptide, Gly-Sar. They found that this dipeptide was accumulated intact against an electrochemical gradient into rings of everted hamster intestine. Further they demonstrated that this accumulation was significantly inhibited by anoxia, 2, 4-dinitrophenol and by the absence of medium Na^+ . These workers found very similar results in their study of the tripeptide Gly-Sar-Sar uptake into everted hamster intestinal rings and concluded that the uptake of both Gly-Sar and Gly-Sar-Sar must be via an active transport system (Addision et al., 1974).

Matthews et al. (1974) looked at the uptake of β-Ala-His (carnosine), a dipeptide which is hydrolyzed even more poorly than Gly-Sar, into rings of everted hamster jejunum. Their results indicate that like Gly-Sar, carnosine was concentrated by the intestinal rings, the uptake being saturable and apparently conforming to Michaelis-Menton type kinetics. Carnosine transport was reduced by anoxia, metabolic inhibitors and the replacement of medium Na^+ with

tris, choline and K^+ .

Addision et al. (1975) examined the transport of the poorly hydrolyzed tripeptide β-Ala-Gly-Gly into hamster intestinal rings. They found that while its uptake was inhibited by anoxia and metabolic inhibitors accumulation of the peptide could not be demonstrated.

The work of Cheeseman and Parsons (1976) and Cheeseman (1977, 1980) suggests, in contrast to the aforementioned investigations, that peptide transport in the intestine is via a passive mediated mechanism. The uptake of both Leu (Gly-Leu) and Leu (Leu-Gly) into an in vivo perfusion preparation of the frog (R. pipiens) intestine reached much lower tissue concentrations than did the constituent free amino acids during the stop flow portion of the procedure (Cheeseman and Parsons, 1976). This observation was considered evidence in favor of a nonconcentrative mechanism for the transport of these dipeptides. Subsequently, Cheeseman (1977, 1980) using the same in vivo preparation verified the extremely poor accumulation of Leu (Gly-Leu) and further (in sharp contrast to Matthews' findings) that carnosine was not concentrated within the enterocytes during the one hour stopped vascular flow to levels above that in the incubation medium. This latter observation was repeated using everted tissue rings in vitro. Commenting on these observations Cheeseman (1980) points out that when intestinal enterocytes have an intact vascular flow, i.e., in vivo preparations, substrates are cleared rapidly and accumulation

never occurs. Also the rapid hydrolysis which most peptides undergo in the cytosol of the mucosal cells creates a natural downhill gradient from the lumen into the enterocyte. Hence entry of peptides into the absorptive cells need not be active.

The Na^+ -coupled transport of amino acids into the intestine is now accepted. The role of Na^+ in the transport of peptides however, is still a contentious issue. Those who maintain that peptide transport is an active process envisage the coupling of Na^+ to peptide transport as the source of energy, driving the substrate uphill against its electrochemical gradient, as Na^+ diffuses into the enterocyte down its concentration gradient maintained by the (Na^+ -K^+)-ATPase. The elucidation of the part played by Na^+ in peptide transport is important in understanding the mechanism and is discussed separately in the following section.

Na^+ Dependency of Peptide Transport

In 1971, Rubino et al. noted that replacement of medium Na^+ with choline reduced the uptake of Gly (Gly-Pro) into rabbit ileal mucosa in vitro by decreasing the apparent Vmax of the process whereas the absence of the cation reduced free Gly transport by increasing the Kt. They found also that the appreciable influx of Gly (Gly-Pro) remaining in the near absence of Na^+ was too large to be attributed to simple diffusion.

The investigations outlined in the previous section

(Active Transport of Peptides) studying the transport of Gly-Sar, Gly-Sar-Sar, carnosine and β Ala-Gly-Gly into hamster jejunum in vitro found all of these peptides to be inhibited, to varying degrees, by the removal of medium Na^+. The authors in each case concluded therefore, that peptide transport is Na^+ dependent (Addision et al., 1972, 1974, 1975; Matthews, 1974).

More recently Ganapathy and Radhakrishnan (1979) made use of the drug harmaline in their effort to determine the role played by Na^+ in the translocation of Gly-Leu and Gly-Pro across the intestinal membrane of strips of monkey ileum. Harmaline, a reputed inhibitor of Na^+ dependent transport mechanisms, was found to reduce Gly-Pro uptake by about 35 percent and Gly-Leu influx by 57 percent. The simple removal of Na^+ from the incubation medium had very similar effects. These investigators concluded that while the transport of dipeptides is at least partially dependent on medium Na^+, the significant transport remaining in the absence of Na^+ must occur via a mediated mechanism since it was inhibited by the presence of other dipeptides.

As noted earlier the transport of dipeptides into mudpuppy intestine is rheogenic (Boyd and Ward, 1982). The authors point out that the dipeptides studied (Leu-Leu, Gly-Pro and carnosine) are all uncharged at the pH of the medium employed (7.25) indicating that the influx of the dipeptides themselves did not depolarize the intestinal cells but that dipeptide transport must be associated with the flux

of an ionic species. While this data is consistent with the theory of the Na^+ -coupled transport of dipeptides, it was found that unlike free amino acid influx, dipeptides persist in producing electrical effects even in the absence of medium Na^+. Boyd and Ward (1982) suggest that the rapid intracellular hydrolysis of dipeptides might maintian a concentration gradient sufficient to drive Na^+ -coupled peptide entry even with Na concentrations at very low levels.

In contrast to the above studies there are reports in the literature which claim to demonstrate the Na^+ independence of peptide uptake into intestinal tissue.

As discussed previously Cheeseman and Parsons (1974, 1976) in their study of the uptake of Gly-Leu and Leu-Gly by the frog small intestine showed that with the free amino acid transport system saturated with free Leu and Gly, additional influx of both these amino acids occurred following the introduction of either Gly-Leu or Leu-Gly into the lumen. They reasoned that this additional transport must have been via an intact peptide route and demonstrated it to be unaffected by the removal of Na^+ from the luminal solutions. These observations lead Cheeseman and Parsons to propose a model for dipeptide absorption into the small intestine which suggests that Leu (Gly-Leu) or Leu (Leu-Gly) can enter the intestinal enterocyte via two distinct routes: one being an intact peptide transport system is Na^+ independent and non-concentrative and another corresponding to the usual free

amino acid uptake system which is known to be Na^+ dependent and capable of accumulating its substrates. The latter route would be utilized by amino acids freed from dipeptides during surface hydrolysis. Cheeseman (1977, 1980) extended the above observations demonstrating that β-Ala-His (carnosine) was taken up into the anuran small intestine via a Na^+ independent mechanism.

Sigrist-Nelson (1975) studied the transport of Gly, Leu and Gly-Leu into isolated brush border membranes of rat small intestine. Although she reached no conclusion as to the role of Na^+ in Gly-Leu transport, it was noted that Na^+ replacement had a lesser effect on the dipeptide's uptake than on free Leu absorption.

Gly-Gly transport into isolated ileum of guinea pig was found to be reduced only slightly following the total replacement of Na^+ in the incubation medium, whereas Gly uptake was abolished (Himukai and Hoshi, 1978). These authors noted that the omission of Na^+ caused a small increase in the Kt for Gly-Gly transport but the Vmax of the process was unaffected. The fact that the Vmax was unchanged suggested to these workers that superficial hydrolysis with subsequent transport of the liberated monomer may not be involved in the transport of Gly (Gly-GLy). The following year Himukai and Hoshi (1979) observed that while Gly (Gly-Leu) uptake into isolated guinea pig ileum was unaffected by Na^+ replacement with D-mannitol, Leu (Gly-Leu) transport was reduced by 50 percent. This discrepancy, they

reasoned, arises from the fact that Gly (Gly-Leu) uptake occurs only via the intact peptide transport route while Leu (Gly-Leu) absorption utilizes both the intact mechanism which is Na^+ independent plus the Na^+ dependent free amino acid system following surface hydrolysis and the release of free Leu. Only this latter process is eliminated by Na^+ removal.

Ganapathy et al., (1980) studied the primary translocation step in the uptake of Gly-Pro into purified intestinal and renal brush border vesicles. These membrane vesicles were shown to be devoid of (Na^+ -K^+)-ATPase and cytoplasmic components, thereby simplifying the interpretation of the results. The determinations showed that Gly-Pro transport was the same from NaCl and KCl solutions into both intestinal and renal vesicles. Further, it was found that while L-Ala influx was stimulated by the introduction of a Na^+ gradient across the brush border membrane of both preparations, the transport of Gly-Pro was unaffected. In sharp contrast to Ganapathy and Radhakrishnan's (1979) findings, the addition of harmaline (2 mM) to the incubation medium had no effect on Gly-Pro transport into renal or intestinal vesicles either in the presence or absence of Na^+. The authors concluded that "This work clearly demonstrates that the dipeptide transport into intestinal and renal brush border vesicles is totally Na^+ independent" (Ganapathy et al., 1980).

Specificity of Peptide Transport Route(s)

The interpretation of the results as well as the design

of the many experiments carried out in an effort to determine the multiplicity of the peptide uptake system has divided investigators to two groups. One group suggests that there is but one route available for intact peptide transport while the other proposes the existence of at least two and perhaps as many as three separate uptake systems.

Much of the confusion in this area has arisen from the failure of several workers to take into account the complicating factors of superficial hydrolysis, experienced by most peptides to varying degrees, when designing their experiments and interpreting the results therefrom. Clearly such an oversight makes it impossible to determine whether the competitive inhibition observed is a result of interactions between the peptides themselves or between their constituent amino acids released by hydrolases at the membrane surface.

The evidence for and against the existence of multiple pathways for peptide transport consists of demonstrations that one peptide does or does not inhibit the uptake of another peptide. If it is to be convincingly proven that peptides share the same uptake route their mutual inhibition must be shown to be of a competitive nature.

Rubino et al. (1971) provided evidence for the uptake of peptides via one common carrier system with their demonstration that Gly (Gly-Pro) uptake into rabbit ileal mucosa in vitro was inhibited by six other dipeptides and a tripeptide. This inhibition was shown to be competitive,

however only in the case of Leu-Leu.

Das and Radhakrishnan (1975) carried out a study on the inhibition of Leu (Gly-Leu) and Gly (Gly-Leu) uptake into monkey intestine in vitro by a wide spectrum of dipeptides. All of the 38 dipeptides tested inhibited both Leu and Gly uptake from Gly-Leu to varying degrees. The competitive nature of the inhibition was shown for only four of the 38 dipeptides. It should be noted that no correction was made for superficial hydrolysis effects in this work. The authors state that their results suggest strongly that there is a dipeptide uptake system in monkey intestine with an extremely broad specificity.

In an effort to avoid the complicating factor of brush border surface hydrolysis, a series of investigations have been carried out utilizing poorly hydrolyzed peptides such as di-, tri- and tetrapeptides of Gly and Sar as well as the dipeptide carnosine (β-Ala-His). All of these experiments have used the in vitro preparation of everted rings of hamster jejunum. Addision (1974a) demonstrated that Gly-Sar-Sar was inhibited by di- and tripeptides of Gly and Met. Carnosine uptake was shown to be inhibited by several peptides made up of neutral amino acids. The effects of Gly-Pro were found to be competitive (Addision, 1974b). The dipeptides Lys-Lys and Glu-Glu failed to inhibit carnosine influx, nor did they inhibit the uptake of each other which is compatible with the existence of a separate pathway for the uptake of dipeptides made up of neutral amino acids and

those made up of basic or acidic amino acids. The inhibition of the influx of Gly-Sar-Sar and β-Ala-Gly-Gly by a series of di- and tripeptides (including Lys-Lys and Glu-Glu) was demonstrated by Addision et al. (1975). They concluded that di- and tripeptides share a common uptake mechanism but that peptides made up of basic or acidic residues would appear to have a low affinity for this system since the inhibition of Gly-Sar-Sar uptake by Lys-Lys and Glu-Glu was slight. Sleisenger et al. (1976) provided additional evidence in support of a common transport system for di- and tripeptides with the demonstration that Gly-Sar was inhibited by Gly-Sar-Sar in a competitive way and vice versa. The inhibitory effect of Glu-Glu on Gly-Sar uptake into hamster jejunum in vitro was investigated in some detail by Matthews et al. (1978). Incubations were carried out at a pH of five in order to reduce surface hydrolysis of Glu-Glu to a minimum. It was shown that at a constant concentration of Glu-Glu, Gly-Sar uptake was inhibited in a competitive manner and also that a constant concentration of Gly-Sar had the same effect on Glu-Glu transport. Taylor et al. (1979) obtained analogous results with Gly-Sar and Lys-Lys. The conclusion was that the neutral dipeptide Gly-Sar, the acidic dipeptide Glu-Glu and the basic dipeptide Lys-Lys all share the same uptake mechanism.

Several studies exist which are at odds with the concept of a single carrier for peptide transport in the intestine. Edwards (1970) found that carnosine absorption into loops of

rat gut in vivo was inhibited by di- and tri-Gly but not by Leu-Ala. Leu-Ala uptake, while being inhibited by a number of dipeptides was not reduced in the presence of carnosine, β-Ala-Gly nor di- or tri-Gly. This data suggests the presence of at least two transport mechanisms for oligopeptides in the rat intestine. Gupta and Edwards (1976) added to the above observations noting that neither Leu-Gly or β Ala-His (carnosine) representative members of two distinctly different transport groups, inhibited the uptake of Pro-Hyp into loops of rat intestine in vivo. Since Pro-Hyp was shown to be inhibited by other prolyl dipeptides these authors concluded that this was evidence for the existence of at least three separate "carrier protein systems in the rat small gut", exemplified by Leucyl, Alanyl and Propyl derivatives.

Lane et al. (1975) looked at the uptake of Pro-Gly and Gly-Pro into rat jejunum in vivo and found that while 40 mM Pro-Gly failed to inhibit 10 mM Gly-Pro uptake, 40 mM Gly-Pro did inhibit 10 mM Pro-Gly transport significantly. Since Rubino (1971) also found that Pro-Gly did not effect Gly-Pro uptake, Lane et al. suggested that these dipeptides might be handled by different uptake systems. These results and their interpretations are consistent with those of Edwards (1970) and Gupta and Edwards (1976) who found that Leu-Ala uptake into the rat small intestine in vivo, was inhibited by Gly-Pro but not Pro-Gly, and also concluded that the two Pro dipeptides are transported by separate routes.

Gly-Leu influx across the mucosal border of guinea pig ileum in vitro was shown not to be inhibited by the presence of di- or tri-Gly but Gly-Leu was found to inhibit di-Gly uptake by 46 percent, the inhibition being of a mixed type (Himukai and Hoshi, 1979). The authors concluded that Gly-Gly and Gly-Leu are transported by separate systems with their respective binding sites being in such close proximity that the binding of Gly-Leu to its binding site might cause a deformation of the Gly-Gly site and thereby reduce the efficiency of Gly-Gly binding and subsequent transport. Gly-Gly binding, apparently has no effect on the Gly-Leu site.

The Boyd and Ward (1982) micro-electrode study, outlined previously, found that the depolarization of the enterocyte membrane associated with carnosine transport was abolished by the presence of Leu-Leu, suggesting that these dipeptides share a common transport mechanism. It was discovered, however, that even at high concentrations, carnosine failed to totally inhibit the depolarization caused by Leu-Leu influx. Since these observations were made in the presence of a saturating concentration of free Leu (thereby eliminating the complication of surface hydrolysis) these authors postulated the existence of two transport systems for peptide transport both of which can be utilized by Leu-Leu but only one of which is shared with carnosine. Further, it appears from this study, that tri-Leu shares the same transport route with di-Leu since no further depolarization was observed when the tripeptide was introduced into the

incubation medium ". . . in the presence of a higher (10 mM) concentration of the dipeptide" (Boyd and Ward, 1982).

METHODS AND MATERIALS

1. Animals

Male Spreague-Dawley rats with a weight range of 250 to 300 grams were used. The rats were maintained with free access to food and water up to the time of sacrifice.

2. Experimental Procedure

This project utilized a modification of the ring technique as described by Crane and Mandelstam (1960). The rat was killed with a blow to the head and an incision into the abdominal cavity made along the median line. The small intestine was severed distal to the ligament of Treitz, pulled free of the adhering mesentery and flushed out with normal saline (at room temperature). Next the small intestine was freed from the body by cutting it at the ileocaecal junction. The small intestine was then transferred to a bowl containing Normal saline (at room temperature), washed of adhering blood and everted using a stainless steel rod, outer diameter of 2 mm. The everted intestine was placed in a trough, again containing normal saline at room temperature and the middle fifth was excised. Normally the length of the everted gut measured slightly over one metre in length so that the middle fifth removed fell between the 40 and 60 cm. marks. The middle fifth of the

intestine was chosen based on the available evidence suggesting that, in rat, maximal absorption of peptides occurs in the proximal ileum (Matthews, 1975, 1980). The procedure outlined here would most likely succeed in isolating the proximal to mid ileum. The excised section of small intestine was then cut into small rings (1 - 2 mm in thickness) with a pair of sharp, fine scissors and collected in a reservoir (50 mls) of Krebs ringer solution which was at room temperature.

When determinations were carried out in test solutions containing Na^+, the rings were allowed to equilibrate in the Krebs for five minutes. In the experiments in which choline was used to replace Na^+ in the test solutions, the rings were preincubated in the Krebs for thirty minutes.

The reservoir was continually gassed with a 95 percent O_2 - five percent CO_2 mixture for the experiments carried out in the presence of NA^+ and with 100 percent O_2 for those experiments in which Na^+ was replaced with choline, due to the different buffer systems employed in each.

From the reservoir the rings were transferred to 12, 16 x 125 mm test tubes, each containing 1 ml of a test solution together with the radio actively labelled marker. Normally each everted gut yielded some 70 rings and therefore routinely five rings were placed in each of the 12 tubes.

These tubes were incubated in a water bath at 37° C and gassed vigorously with either the 95 percent O_2 - 5 percent CO_2 mixture or the 100 percent O_2 depending upon the buffer

system employed.

Termination of the incubation period in each test solution was accomplished by emptying the tubes into a filter funnel connected to a vacuum. The rings were then immediately washed with ice cold choline chloride (154 mM) and transferred to a separate filter paper dampened with choline chloride.

The wet weight of each group of rings was determined on a torsion balance (VEREENIGDE DRAADFABRIEKEN NIJMEGEN HOLLAND) after which the rings were placed in a drying oven (PRECISION MODEL 18) at 100° C for 60 minutes. The dry weight of each group of rings was then determined on the same torsion balance.

Extraction of the tissue was accomplished by immersion of each of the 12 groups of rings into 300 micro litres of 0.05 N nitric acid for one hour. Two hundred micro-litre aliquots of each of these groups of extracts were taken for liquid scintillation counting.

3. Solutions

The Krebs ringer solution containing Na^+ consisted of the following: (expressed in millimoles per litre) NaCl, 118; KCl, 4.7; $CaCl_2$, 1.25; KH_2PO_4 , 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25. The sodium free phosphate ringer solution was of the following composition: Choline Chloride, 118mM; K_2HPO_4 , 1.2; KH_2PO_4 , 0.85; $MgSO_4$, 1.20; and $CaCl_2$, 2.5, KCl 3 mM.

4. Materials

L-leucine, L-proline, glycyl-L-leucine, leucyl-L-leucine, glycyl-L-glycine, prolyl-L-glycine and leucyl-L-alanine were supplied by the Sigma Chemical Co. (St. Louis, Missouri). The aqueous counting scintillant cocktail (ACS) was obtained from Amersham Corp. (Arlington Heights, Illinois) as were the L-[3,4,5 - ^{3}H(N)] leucine, L-[^{14}C] leucine, and the [1 - ^{14}C] glycyl-L-leucine. The [^{14}C] inulin was from New England Nuclear (Lachine, Quebec).

5. Scintillation Counting

Two hundred micro-litre aliquots of the acid tissue extract were added directly to the scintillant for counting. No deproteinization was necessary. Quenching was measured from the channels ratio using an external standard. Counting was performed in a Beckman LS 3133T liquid scintillation counter.

6. Synthesis of Glycyl-L-[^{3}H] Leucine

All chemicals for this synthesis were supplied by Sigma Chemical Co. (St. Louis, Missouri).

N-tert-BOC-glycine, Ø.875 g, was activiated with Ø.63 g of N-hydroxyl succinimide which had been dissolved in 1Ø ml of ethyl acetate. These reactants were cooled on ice. 1.Ø3 g of dicyclohexyl-carbodimide dissolved in 5 ml of ethyl acetate was then added and the mixture stirred and cooled for 12 hours.

The resulting white precipitate was filtered and washed with 5Ø mls of ethyl acetate. The solution was kept and evaporated to dryness. The white residue was recovered and suspended in a small volume of ether. This was filtered, leaving a white powder (BOC-glycine-OSu) which was tested for purity by a melting point determination (155-16Ø° C).

To an aqueous solution of 5 m Ci of L-[3,4,5 - $^{3}H(N)$] leucine and $NaHCO_3$ powder was added an equal volume of dioxane to yield a one percent solution of bicarbonate. To this, 1ØØ mg of the activated BOC-glycine was added and the reactants were left stirring overnight.

The solution was evaporated to dryness and the residue dissolved with 1Ø mls of ethyl acetate. This solution was deccanted into a separating funnel and 5 mls of Ø.1 N HCl was added. The organic phase containing the product was separated and washed repeatedly with ethyl acetate. The acetate washings were then pooled and evaporated to dryness.

The BOC group was removed from the glycine by the addition of 2 mls of trifluoroacetic acid and after two hours the TFA was evaporated off. To ensure complete removal of the acid further drying of the residue was carried out using a N_2 stream. The final product was resuspended in water and the pH adjusted to 7.4 with NaOH.

Identification of the final product was accomplished through a thin layer of chromatography on silica gel using two solvent systems; isobutanol-acetic acid-water (12:3:5 v/v) and propanol-water (8:2 v/v). The radioactivity was

found to be associated with the spot corresponding to glycyl-L-leucine at a level indicating better than 99 percent purity.

7. Extracellular Space Determination

The volume of the extracellular fluid (ECS) in the intestinal tissue rings was estimated utilizing [^{14}C] inulin, a marker reported in the literature to freely equilibrate within the extracellular space and not penetrate the cell membrane (Esposito and Csaky, 1974; Dinda and Beck, 1977).

Everted intestinal rings were incubated with the [^{14}C] inulin marker for 2, 5, 10, 15, 25 and 30 minutes in solutions of varying composition.

It was found necessary to determine the extracellular fluid volume for the tissue rings under five distinct conditions each of which had the effect of changing the ECS and each of which would be utilized in this study. The varying conditions refers to the varying composition of the test solutions and/or the time the tissue was preincubated in this solution. The five conditions were:

i) Krebs bicarbonate ringer: Pre-incubation of five minutes

ii) Krebs Choline ringer: Choline used to substitute for Na^+ in which the tissue was preincubated for 30 minutes

iii) The same as in ii) but the tissue was preincubated for five minutes

iv) The same as i) but with the addition of 5 mM harmaline and no preincubation with harmaline

v) The same as ii) but with 5 mM harmaline added and no preincubation with the harmaline

The extracellular fluid volume was calculated by comparing the disintegrations (D.P.M.s) associated with 200 μ l aliquots of acid used to extract the everted intestinal rings, with the disintegrations (D.P.M.s) associated with corresponding 200 μl standards. Extracellular fluid volumes were expressed as micro litres per gram dry weight of tissue.

The results of the five extracellular space determinations are represented as Figures 1 to 5. The graphs, in all five cases, indicate an apparent failure of the [^{14}C] inulin to reach equilibrium with the extracellular (ECS) fluid after 30 minutes incubation. Evidence in the literature however, indicates "that a complete equilibration of [^{14}C] inulin with the EC fluid occurs as early as five minutes after the beginning of the incubation" (Dinda et al., 1977) and that the observed progressive increase in calculated extracellular space was a result of a steady increase in extracellular fluid containing [^{14}C] inulin "and not from an incomplete equilibration of inulin in the EC fluid " (Dinda et al., 1977).

Further, previous work in this field indicates that [^{14}C] inulin to be one of the more satisfactory markers for extracellular space available to date (Esposito et al., 1979;

Dinda et al., 1977; Esposito and Csaky, 1974).

Since, in this study, incubation periods of less than five minutes were employed, it was necessary to approximate the extracellular spaces to be used in calculating the uptake of substrate into the tissue rings, during these shorter incubation times. This was accomplished by extrapolating the straight part of the curves (beyond five minutes) of graphs 1 - 5 back to their intersection with the y axis. The extracellular space for incubation times of less than five minutes were then read from this extrapolated line. Graphs 1 - 5 have these lines included.

8. Calculation and Expression of Results

The amount of substrate assumed to be absorbed into the enterocyte of the intestinal rings was calculated by comparing the amount of the radio-labelled marker in the tissue extracts with that in a known volume of its corresponding standard. The appropriate extracellular substrate, estimated from the ECS determined for each sample from the standard curves (Figures 1-5), was subtracted to give the intracellular amount and was expressed as μmoles per gram dry weight of tissue per minute.

A determination of the intracellular substrate concentration was possible using the average wet to dry tissue weight ratio measured for each sample. The ratio enabled an estimate to be made of the millilitres of water present per gram of tissue. From this figure the appropriate

ECS was subtracted to yield the amount of intracellular water present in each gram of tissue. The intracellular concentration of substrate was determined as μmoles per gram dry wt/millilitre intracellular water per gram of tissue and expressed as millimolar.

9. Statistical Methods

Statistical comparisons of the groups of data obtained under the various conditions employed in this study were made using a nested design of analysis of variance (Snedecor and Cochran, 1967). A five percent level of probability was employed as being significant; hence, if $p < 0.05$, the data groups were considered to be significantly different.

RESULTS

1. Leucine Uptake

The following are the results of the 'in vitro' experiments (everted intestinal rings) performed to investigate the characteristics of free leucine and in particular glycyl-L-leucine transport into the rat small intestine.

A. Leucine Uptake Against Time

Figure 6 represents graphically the data obtained from the incubation of intestinal rings in 1 mM Leu over increasing periods of time both in the presence and absence of Na^+.

The graphs in Figure 6 demonstrate that leucine uptake was reasonably linear over the first five minutes of incubation both with and without Na^+ and that in both situations the uptake of the substrate plateaued.

In the presence of Na^+ the uptake of Leu was much more rapid than in the absence of the cation and in the former case the epithelial cells were able to accumulate the substrate to a level substantially above the 1mM concentration of Leu in the medium reaching intracellular concentrations of 6.5 mM after 30 minutes of incubation. Replacement of Na^+ with choline abolished the ability of the enterocytes to concentrate the free amino acid above

that in the test solutions, intracellular concentrations reaching a maximum of 0.91 mM after 30 minutes.

B. Leucine Uptake Versus Leucine Concentration

In order to further describe the characteristics of leucine uptake into the enterocyte of the rat small intestine, a series of experiments were carried out in which rings of everted rat gut were incubated for one minute in test solutions containing a range of concentrations of the substrate with and without Na^+ . The results of these determinations are represented graphically in Figure 7.

A one minute incubation was chosen for this series of experiments based upon the observations of Leu uptake against time (Figure 5). The uptake of Leu at one minute appeared to be linear in nature so that any factors affecting Leu transport in this region of the curve should have been clearly delineated. This shorter incubation period should have reduced the extent of backflux of the Leu from the epithelial cells into the medium with the result that the data obtained reflected primarily Leu uptake. Further short incubation times reduce the loss of peptidases from the brush border and cytosol.

As Figure 7 shows Leu uptake was far more rapid and substantial in the presence of Na^+ than in its absence. Further, in the presence and absence of Na^+ , Leu uptake was a saturable function of the increasing concentrations of the substrate, saturating at about 20 mM.

i) Kinetics

The apparent Michaelis constants were derived for Na dependent Leu (Leu) transport into rings of everted rat intestine. The Na^+ dependent Leu (Leu) transport was calculated by subtracting the uptake of Leu determined in the absence of Na^+ from that obtained in the presence of Na^+. The resulting values were plotted as substrate concentration over velocity of the reaction versus substrate concentration (Morris, 1978).

The resulting graph together with the calcualted Vmax (14.0 μmoles g dry wt.$^{-1}$ min.$^{-1}$) and Kt (3.3 mM) are presented as Figure 8.

2. Glycyl-L-Leucine Uptake

Having established some of the characteristics of free Leu uptake into the rat small intestine, a series of experiments designed to describe Gly-Leu transport into the rat intestinal enterocyte were undertaken.

A. Leu (Gly-Leu) Uptake Over Time, ± Na

Figure 9 illustrates the data obtained from incubating intestinal rings in test solutions containing 1 mM Gly-Leu at various incubation times in the presence and absence of Na^+.

As with the uptake studies of Leu (Leu), Leu (Gly-Leu) uptake proceded in a reasonably linear fashion for the first ten minutes of incubation both in the presence and the absence of Na^+.

In the presence of Na^+, Leu (Gly-Leu) uptake was much more rapid than in the studies where choline replaced Na^+,

also the epithelial cells were able to accumulate the substrate to a level above that in the medium (1 mM), only when Na^+ was present. Leu (Gly-Leu) intracellular concentrations reached levels of 5.5 mM after 30 minutes in the presence of Na^+ but in the absence of the cation the intracellular concentration for the same incubation period fell to 0.70 mM.

As seen with the Leu (Leu) studies, the uptake of Leu (Gly-Leu) did plateau whether Na^+ was present or not.

B. Leu (Gly-Leu) Uptake Versus Gly-Leu Concentration

The Uptake of Leu (Gly-Leu) was studied further by incubating everted intestinal rings in test solutions of increasing concentrations of the substrate, Gly-Leu, for one minute. As in the free leucine experiments the one minute incubation period was chosen since the uptake of Leu (Gly-Leu) against time studies revealed linearity of uptake at this time and that at this relatively short incubation time backflux of leucine from the enterocyte was predicted to be slight.

The data obtained from these determinations is presented in Figure 10 and Table I. In the presence of Na^+ the uptake of Leu (Gly-Leu) proceeded at a faster rate than in the absence of the cation. Both with and without Na^+, the uptake of the peptide was a saturable function of the concentration of the substrate. This saturation occurred at approximately 20 mM in both cases. The removal of Na^+ reduced the maximal uptake of Leu (Gly-Leu)

to slightly less than 50 percent of the control maximum.

Note that the removal of Na^+ reduced the uptake of Leu (Gly-Leu) by about 50 percent as compared to an almost 80 percent reduction of Leu (Leu) uptake in the absence of the cation.

Clearly these results suggest that leucine entry into the rat small intestine was less sensitive to Na^+ when presented as the peptide, Gly-Leu, than when presented as a free amino acid.

i) Kinetics

The apparent Michaelis constants for Leu (Gly-Leu) uptake were determined as follows. Firstly the kinetics of Na^+ dependent Leu (Gly-Leu) transport were analyzed. This Na^+ dependent transport was taken to be the difference between the uptake of Leu (Gly-Leu) determined in the presence of Na^+ and that determined in Na^+'s absence. The resulting data was plotted as substrate concentration over velocity of the reaction versus substrate concentration and appears as Figure 11. Note that the Vmax (12.3μ moles g dry wt.$^{-1}$ min^{-1}) and Km (3.8 mM) of this Na dependent Leu (Gly-Leu) uptake is similar to the Vmax 14.0 and Km 3.3 determined for the Na^+ dependent Leu (Leu) uptake (Figure 7).

Secondly the kinetics of Na^+ independent Leu (Gly-Leu) uptake were examined. The Na^+ independent Leu (Gly-Leu) uptake was taken to be the transport of Leu (Gly-Leu) determined in the absence of Na^+. This data was also plotted as [S]/V versus [S] and the Michaelis constants

calculated from the graph. The results of this plot appear as Figure 12 together with the Vmax (19.3 μmoles g dry wt.$^{-1}$ min.$^{-1}$) and Km (8.7 mM) of Na^+ independent Leu (Gly-Leu) transport.

Note that the Na^+ independent Leu (Gly-Leu) system has a much higher Vmax and Km than either the Na^+ dependent Leu (Gly-Leu) (Figure 10) or the Na^+ dependent Leu (Leu) (Figure 7) systems.

C. Saturation of the Free Leucine Transport System

The Leu (Leu) uptake versus Leu concentration studies revealed that the transport system handling this free amino acid saturated at 20 mM Leu. In an attempt to eliminate the free amino acid system, thereby leaving the peptide system as the only possible entry route for Leu (Gly-Leu), this saturation characteristic was exploited.

Initially rings of everted rat small intestine were incubated in test solutions containing 20 mM Leu in the presence of Na^+ for one minute. The uptake of Leu (Leu) under these conditions is indicated by bar 'A' of the histogram (Figure 13).

To verify the saturation of the free leucine system the above experiment was repeated but the concentration of Leu in the medium was increased to 25 mM. The results of this determination are represented as bar 'B' of the histogram (Figure 13). Note that with the 25 mM solutions there was no additional uptake of Leu over and above that obtained with a substrate concentration of 20 mM. The free Leu transport

system was therefore assumed to be saturated.

D. Leu (Gly-Leu) Uptake from 5 mM Gly-Leu in the Presence of 20 mM Leu

Everted rings of rat gut were incubated in solutions containing 5 mM Gly-Leu, 20 mM Leu and Na^+ for one minute. The data obtained is represented as bar 'C' of the histogram (Figure 13).

There was a substantial uptake of Leu above that obtained from either the 20 mM or 25 mM free Leu test solutions. This additional Leu uptake was assumed to have entered via the Gly-Leu intact peptide transport system exclusively, since 20 mM leucine had been shown to saturate the free Leu uptake mechanism completely.

E. Leu (Gly-Leu) Uptake Versus the Concentration of Gly-Leu Plus 20 mM Leu; $\pm Na^+$.

The characterization of the intact Gly-Leu transport mechanism was begun by observing the uptake of Leu (Gly-Leu) into rings of everted rat intestine over a range of concentrations of the peptide in the presence of 20 mM free Leu, during an incubation period of one minute. The experiments were carried out both in the presence and the absence of medium Na^+ .

The data obtained from this series of experiments is presented in Table I.

The removal of Na^+ , in the presence of 20 mM Leu had no significant effect on the uptake of Leu (Gly-Leu) ($p>0.05$). It would appear that the intact transport of Gly-Leu is not

dependent on the presence of medium Na^+ .

F. Leu (Gly-Leu) Uptake Versus Time in the Presence of 10 mM L-Ala-β -naphthylamide (ANA)

ANA has been demonstrated to be an effective inhibitor of the membrane peptidases responsible for the surface hydrolysis of peptides in the rat small intestine (Chung et al., 1979; Rosen-Levin et al., 1980).

The agents effectiveness was initially tested by incubating intestinal rings in 1 mM Gly-Leu solutions, in the presence of 10 mM ANA and Na^+ , for increasing periods of time. A 10 mM concentration of ANA was used since this was the maximum concentration soluble in water.

The results of these determinations are presented in Figure 14. This Figure also includes, for comparitive purposes, a graph of the results of Leu (Gly-Leu) transport versus time in the absence of Na^+ experiments (Figure 6).

The uptake of Leu (Gly-Leu) determined over time, in the presence of ANA was not significantly different from that observed simply in the absence of Na^+ ($p>0.05$).

G. Leu (Gly-Leu) Versus the Concentration of Gly-Leu in the Presence of 10 mM ANA and Na^+ .

The effect of ANA was studied further by incubating intestinal rings in solutions containing a 10 mM concentration of the peptidase inhibitor plus increasing concentrations of Gly-Leu with Na^+ for one minute. The resulting data appears in Table I.

The uptake of Leu (Gly-Leu) in the presence of 10 mM ANA

was not significantly different from either Leu (Gly-Leu) determined in the absence of medium Na^+ or Leu (Gly-Leu) uptake in the presence of 20 mM Leu and the absence of Na^+ ($p>0.05$). However, an analysis of variance between Leu (Gly-Leu) plus 10 mM ANA and Leu (Gly-Leu) plus 20 mM Leu in the presence of Na^+ revealed a significant difference at the five percent level of probability. Since there was no significant difference found between Leu (Gly-Leu) uptake plus 20 mM Leu and Leu (Gly-Leu) uptake in the absence of Na^+ ($p>0.05$), a student t test was employed to determine any significant differences between each data point for Leu (Gly-Leu) transport plus 20 mM Leu and Leu (Gly-Leu) uptake plus 10 mM ANA. Only two of the six points proved to be significantly different.

H. Gly (Gly-Leu) Uptake Versus the Concentration of Gly-Leu Plus Na^+

Utilizing the marker[^{14}C] Gly-L-Leu, the uptake of Gly (Gly-Leu) was studied by incubating intestinal rings for one minute in solutions containing a range of concentrations of Gly-Leu plus Na^+ .

The results of this work are presented in Table II.

Note that the uptake of Gly (Gly-Leu) reached about half the levels attained by Leu (Gly-Leu) uptake over the same concentration range, in the presence Na^+ and is not significantly different from the uptake of Leu (Gly-Leu) plus 20 mM free Leu ($p>0.05$).

I. Gly (Gly-Leu) Uptake Versus the Concentration of Gly-Leu in the Presence of 20 mM Leu and Na^+

The effect of a saturating concentration of free Leu on the uptake of Gly (Gly-Leu) was studied by incubating everted rings of rat intestine in solutions containing 20 mM free Leu, Na^+ and a range of concentrations of Gly-Leu, for one minute. The resulting data from this series of experiments is presented in Table II.

There is no significant difference between Gly (Gly-Leu) uptake in the presence of 20 mM Leu and Na^+ and Gly (Gly-Leu) uptake in the presence of Na^+ (control) ($p>0.05$). The addition of 20 mM Leu had no effect on Gly (Gly-Leu) transport.

J. Gly (Gly-Leu) Uptake Versus the Concentration of Gly-Leu in the Absence of Na^+

The Na^+ dependency of Gly (Gly-Leu) uptake was investigated by incubating intestinal rings in solutions containing a range of Gly-Leu concentrations, in the absence of Na^+ for one minute. The results of these determinations are presented in Table II.

There is no significant difference between Gly (Gly-Leu) uptake in the presence or absence of Na^+ ($p>0.05$).

3. Harmaline Study

Harmaline is a psychotomimetic alkaloid with the reputed ability to reduce Na^+ dependent solute transport. The drug was applied to the study of peptide transport in the

following series of experiments.

A. Leu (Leu) Uptake Versus the Concentration of Harmaline Plus Na^+

Initially the effectiveness of harmaline (HCl) was tested by incubating intestinal rings for one minute in solutions containing 1mm Leu, Na^+ and increasing concentrations of harmaline.

The results are presented graphically in Figure 15.

The data indicates that as the harmaline concentration was increased, the uptake of Leu (Leu) diminished. Note that the harmaline was effective at reducing Leu (Leu) uptake at very low concentrations.

B. Leu (Gly-Leu) Versus the Concentration of Harmaline Plus Na^+

In a similar series of experiments to those just described, everted gut rings were incubated for one minute in test solutions containing 1mM Gly-Leu, Na^+ and increasing concentrations of harmaline.

The results of this work, represented graphically as Figure 15, demonstrate a gradual reduction in the uptake of Leu (Gly-Leu) as the concentration of harmaline was increased. It is evident from Figure 15 that harmaline did not reduce the uptake of Leu (Gly-Leu) to the same extent as it did Leu (Leu). Also, as in the free Leu study, harmaline inhibited the uptake of Leu (Gly-Leu) even at the lowest concentrations used.

C. Leu (Leu) Uptake Versus the Concentration of Harmaline in the Absence of Na^+

Harmaline's effectiveness in the absence of Na^+ was studied by incubating everted intestinal rings for one minute in solutions containing 1mM Leu and increasing concentrations of harmaline. In this series of experiments choline was used to replace Na^+.

The graph constructed from the results of this work is included in Figure 15.

Note that harmaline had no effect on Leu (Leu) uptake until its concentration reached 2.5 mM. With concentrations of harmaline above 2.5 mM the reduction of Leu (Leu) uptake was highly significant ($p<0.05$). Harmaline therefore did inhibit Leu (Leu) uptake in the absence of Na^+.

D. Leu (Leu) Uptake Versus the Concentration of Leu Plus 5 mM Harmaline in the Presence and Absence of Na^+

The nature of the inhibitory effect of harmaline on Leu (Leu) uptake was investigated by incubating intestinal rings in solutions containing 5 mM harmaline and increasing concentrations of Leu for one minute. This procedure was carried out in the presence and absence of Na^+.

A harmaline concentration of 5 mM was chosen based on the observations made previously in this section (experimental series (i)) that a significant effect of the inhibitor was not obvious at lower concentrations.

The results of the two series of experiments outlined

above are presented graphically in Figure 16. Included with these reults is a control curve of Leu (Leu) versus Leu concentration plus Na^+.

Note that, in the presence of Na^+, the addition of 5 mM harmaline reduced the uptake of Leu (Leu) only slightly below the control levels. Further note that the removal of Na^+, in the presence of 5 mM harmaline, caused a dramatic reduction in the uptake of Leu (Leu). Clearly 5 mM harmaline had not eliminated Na^+ dependent Leu uptake to any significant degree ($p>0.05$).

E. Leu (Gly-Leu) uptake Versus the Concentration of Gly-Leu Plus 5 mM Harmaline in the Presence of Na^+

This series of experiments was designed to further study the nature of harmaline inhibition of Leu (Gly-Leu) uptake.

Everted rings of rat small intestine were incubated in solutions containing 5 mM harmaline plus increasing concentrations of Gly-Leu for one minute, in the presence of Na^+. The resulting data is presented in Table I.

Leu (Gly-Leu) uptake was reduced by 5 mM harmaline to approximately the same level as that observed by removing Na^+ and by the addition of 20 mM Leu or 10 mM ANA.

F. Gly (Gly-Leu) Uptake Versus the Concentration of Harmaline in the Presence of Na^+

The effect of harmaline on Gly (Gly-Leu) uptake was determined by incubating rings of everted rat gut in solutions containing 1 mM Gly-Leu, Na^+, and increasing concentrations of harmaline, for one minute. The results of

these experiments are presented graphically in Figure 17. Also included in Figure 17, is the graph of data obtained from the determinations of Leu (Leu) uptake versus harmaline concentration in the absence of Na^+.

Harmaline had no effect on Gly (Gly-Leu) uptake until its concentration reached 2.5 mM. As the inhibitor concentration was increased above 2.5 mM to a maximal 10 mM, the inhibition became highly significant ($p \leq 0.05$).

G. Gly (Gly-Leu) Uptake Versus the Concentration of Gly-Leu in the Presence of 5 mM Harmaline and Na^+

The effect of harmaline upon the uptake of Gly (Gly-Leu) was studied further by incubating everted rings of intestine in test solutions containing increasing concentrations of Gly-Leu, 5 mM harmaline and Na^+. The results of this experiment are presented in Table II.

Harmaline (5mM) reduced Gly (Gly-Leu) uptake to levels significantly lower than those determined for Gly (Gly-Leu) uptake plus Na^+ (control) over the same concentration range of substrate ($p < 0.05$).

4. Competition Studies

In an effort to more fully characterize the intact peptide transport utilized by Gly-Leu, the effect of other peptides on Gly-Leu uptake was studied.

A. Leu (Gly-Leu) Versus the Concentration of Gly-Pro in the Presence of 20 mM Leu and Na^+

The effect of Gly-Pro on Leu (Gly-Leu) transport was

determined by incubating everted rat intestinal rings for one minute in solutions containing 1 mM Gly-Leu, increasing concentrations of Gly-Pro and 20 mM Leu. All the competition studies were carried out in the presence of Na^+. The results of this experiment are presented graphically in Figure 18 and in Table III.

As the graph shows, Gly-Pro inhibited the transport of Leu(Gly-Leu) at all the concentrations used. A maximal inhibition of about 57 percent was achieved at a Gly-Pro concentration of 20 mM.

The efficacy of Gly-Pro as an inhibitor of Leu (Gly-Leu) uptake was investigated further by plotting the data depicted in Figure 18, as Jc/Jc-Ji vs. 1/[I]. Jc and Ji represent the uptake of Leu (Gly-Leu) measured in the presence and absence of Gly-Pro respectively and [I] is the concentration of Gly-Pro used (Inui and Christensen, 1966). This graph appears as Figure 19.

The data when plotted in this manner yielded a straight line with a y-intercept of 1.78, which is significantly different from 1.0. The inverse of the y-intercept indicates the percentage uptake that would be inhibited by an infinitely high Gly-Pro concentration (Inui and Christensen, 1966) and corresponds to 56 percent. This value is the same percent inhibition observed at the 20 mM Gly-Pro concentration used in these determinations (Figure 18). It would appear that Gly-Pro is capable of only partially inhibiting the uptake of Leu (Gly-Leu) even at infinitely

high concentrations.

The nature of the partial inhibition of the uptake of Leu (Gly-Leu) by Gly-Pro was determined by first subtracting the unaffected portion of Leu (Gly-Leu) transport (44 percent) from the uptake of the substrate determined for each concentration of inhibitor in Figure 18. The resulting data was replotted as 1/V vs. [I], where V is the uptake of Leu (Gly-Leu) sensitive to Gly-Pro and I is the concentration of Gly-Pro used. This plot appears as Figure 20. The fact that a straight line was obtained indicates that Gly-Pro competetively inhibits the sensitive portion of Leu (Gly-Leu) transport (Neame and Richards, 1972).

B. Leu (Gly-Leu) Uptake Versus Concentrations of Proline Plus 20 mM Leu

To ensure that the observed inhibition of Leu (Gly-Leu) uptake by Gly-Pro was not a consequence of the Pro moiety, the following experiment was performed.

Everted rings of rat small intestine were incubated for one minute in solutions containing 1 mM Gly-Leu and increasing concentrations of Pro.

The resulting data, included in Table III, indicates that proline had no effect on reducing Leu (Gly-Leu) uptake ($p>0.05$).

C. Leu (Gly-Leu) Uptake Versus the Concentration of Gly-Leu in the Presence of 20 mM Leu and 10 mM Gly-Pro

This experiment was designed to obtain additional

information on the nature of the inhibitory effect of Gly-Pro on Leu (Gly-Leu) transport. Due to a concern that the osmolality of the test solutions employed in these studies were becoming too high, it was decided to use a 10 mM Gly-Pro concentration. The dipeptide did produce a near maximal inhibitory effect at this concentration (Figure 18).

Intestinal rings were incubated in solutions containing 20 mM Leu, 10 mM Gly-Pro and increasing concentrations of Gly-Leu, for one minute. The resulting data is presented in Table III.

Figure 21 represents the data as a Woolf plot ([S]/V vs. [S]). Also included in Figure 21 is a Woolf plot of the data obtained from the control experiment; Leu (Gly-Leu) uptake versus substrate concentration plus 20 mM Leu and Na^+. The presence of Gly-Pro appears to have had little effect on the Vmax of Leu (Gly-Leu) transport while significantly increasing the Michaelis constant of the process. These results would indicate that Gly-Pro competetively inhibits Leu (Gly-Leu) intact transport and therefore verifies the conclusions drawn from experiment (i) of this section.

D. Leu (Gly-Leu) Uptake Versus the Concentration of Gly-Leu in the Presence of 10 mM ANA and 10 mM Gly-Pro

The experiment just described ((iii) of this section) was repeated utilizing 10 mM ANA instead of 20 mM Leu to eliminate the contribution of the free Leu transport system to Leu (Gly-Leu) uptake. The results of this work are

presented in Table III.

The uptake of Leu (Gly-Leu) under the conditions of this experiment are not significantly different from Leu (Gly-Leu) uptake determined in experiment (C) ($p>0.05$). It is evident therefore that 10 mM ANA and 20 mM Leu were equally effective in eliminating the contribution made to Leu (Gly-Leu) total transport, by the surface hydrolysis of Gly-Leu followed by the preferential uptake of free Leu.

E. The Effect of Various Dipeptides on the Uptake of Leu (Gly-Leu) in the Presence of 20 mM Leu and 10 mM Gly-Pro

Everted rings of rat intestine were incubated in test solutions containing 1 mM Gly-Leu, 20 mM Leu, 10 mM Gly-Pro and a 10 mM concentration of either Gly-Gly, Pro-Gly, Leu-Gly, Leu-Leu or Leu-Ala for one minute. The results of these determinations appear in Table IV.

Two of the dipeptides tested, Leu-Leu and Leu-Ala, inhibited the uptake of Leu (Gly-Leu) under the conditions of this experimental series. Only the Leu-Leu inhibition proved to be significant.

F. Gly (Gly-Leu) Uptake Versus the Concentration of Leu-Leu in the Presence of 20 mM Gly-Pro

The nature of Leu-Leu inhibition of Gly-Leu uptake in the presence of 20 mM Gly-Pro was investigated, utilizing the marker [^{14}C] Gly-Leu.

Intestinal rings were incubated for one minute, in test solutions containing 1 mM Gly-Leu, 20 mM Gly-Pro and

increasing concentrations of Leu-Leu. The 20 mM Leu was not required in this experiment since, as shown earlier Gly (Gly-Leu) enters the enterocyte exclusively via the peptide route. The results of this experimental series are presented in Figure 22.

The graph of the data from this determination shows Leu-Leu to have no effect on Gly (Gly-Leu) uptake until a critical concentration of Leu-Leu was reached which suggests other than a competitive inhibition.

G. Leu (Gly-Leu) Uptake Versus the Concentration of Gly-Leu in the Presence of 20 mM Leu and 10 mM Leu-Leu

The complexity of the interaction of Leu-Leu with Gly-Leu transport was studied further with the following determination.

Rings of everted rat gut were incubated for one minute in solutions containing 20 mM Leu, 10 mM Leu-Leu and increasing concentrations of Gly-Leu. Figure 23 is a graphical presentation of the data resulting from this experiment. Included in Figure 23 is a control curve showing the uptake of Leu (Gly-Leu) versus the concentration of Gly-Leu plus 20 mM Leu.

From a comparison of the two curves of Figure 23, it is evident that Leu-Leu had a significant inhibitory effect on the uptake of Leu (Gly-Leu) ($p<0.05$).

A kinetic analysis of the data presented in Figure 23 was accomplished by plotting both sets of results as a Woolf

plot ([S]/V vs. [S]). The resulting curves are found in Figure 24.

Figure 24 reveals that the presence of 10 mM Leu-Leu effected a substantial decrease in the Vmax of Leu (Gly-Leu) uptake while significantly increasing the apparent Km. Clearly then, Leu-Leu had a mixed inhibitory effect on Leu (Gly-Leu) uptake, the exact nature of which will require further study.

DISCUSSION

1. Studies on the Na^+ Dependency of Peptide Transport

The observed 50 percent reduction in Leu (Gly-Leu) influx into everted rat intestinal rings following Na^+ replacement in the incubation medium (Figure 10) tends to support the findings of Cheeseman and Parsons (1974, 1976) and Himukai and Hoshi (1980) as well as their suggestion that Gly-Leu transport occurs equally via two routes, only one of which is dependent of Na^+ (See LITERATURE REVIEW; Na^+ Dependency of Dipeptide Transport).

The kinetic constants calculated for the Na^+ dependent portions of Leu (Leu) and Leu (Gly-Leu) transport (Figures 8 and 11) were found to be similar (K_t :Leu (Leu) 3.3 mM, Leu (Gly-Leu) 3.8 mM; Vmax:Leu (Leu) 14.0 μmoles g dry wt.$^{-1}$ min.$^{-1}$ Leu (Gly-Leu) 12.3 μmoles g dry wt.$^{-1}$ min.$^{-1}$) indicating that it is possible that the two processes share a common pathway. On the other hand the K_t and Vmax calculated for the Na^+ independent portion of Leu (Gly-Leu) influx (Figure 12), were quite different (K_t 8.7 mM; Vmax 19.3 μmoles g dry wt.$^{-1}$ min.$^{-1}$) suggesting that this process occurs via a pathway distinct from the free amino acid transport system. If, as indicated by the work of Cheeseman et al. (1974, 1976) and Himukai et al. (1980), the Na^+ independent portion of Leu (Gly-Leu) does represent intact peptide transport, the kinetic constants

determined above are in agreement with the general trend reported in the literature indicating that peptide transport might be characterized by a higher K_t and Vmax than that of free amino acids (Matthews et al., 1968, 1969; Adibi et al., 1974).

With the exception of this general observation a comparision between the absolute values determined for the apparent kinetic constants in this work with those reported in the literature has not been attempted. Thomson and Dietschy (1980) have determined that the type of in vitro preparation used to study transport has a ". . . profound influence on the apparent kinetics of the active solute transport process." They conclude therefore, that it is not valid to compare the ". . . kinetic constants derived from different in vitro tissue preparations." Several studies have also pointed out that failure to correct for the resistance of the large unstirred layers inherent in in vitro and in vivo intestinal preparations can distort the apparent kinetics of mediated transport systems. It is probable that the unstirred water layer in the everted ring preparation used in this study was large, even though there was an effort made to reduce it by vigorous bubbling of the test solutions. The K_t and Vmax values calculated are therefore most likely an overestimation (Winne, 1972; Wilson et al., 1974; Thomson, 1979; Thomson et al., 1980). Despite this inaccuracy it may still be valid to compare the kinetic constants determined with the same preparation, in an attempt

to observe variations as a result of experimental 'manipulations' (Thomson et al., 1980).

In the presence of a saturating concentration of free Leu (20 mM) additional uptake of Leu, into everted rings of rat intestine, was observed following the addition of Gly-Leu to the test solution (Figure 13). Since the free amino acid system was shown to be completely saturated by a 20 mM concentration of cold Leu (Figures 7 and 13), the further uptake of Leu must have been via a pathway not shared by free Leu and had to originate from the dipeptide. These observations strongly suggest that the intact dipeptide uptake mechanism utilized by Gly-Leu was isolated from the free amino acid system by saturating the latter with free Leu.

The influx of Leu from 1 mM Gly-Leu, following the addition of 20 mM cold Leu to the test solutions was reduced to about half of the control uptake observed in the presence of Na^+ (Table I). If it is assumed that the Leu (Gly-Leu) entering the intestinal enterocyte with the free amino acid system saturated does so in the form of intact Gly-Leu it would appear that as much as half the observed influx of Leu (Gly-Leu) occurs via an intact peptide transport system. Further the transport observed in the presence of saturating Leu was found to be unaffected by the replacement of medium Na^+ with choline ($p<0.05$) and was not significantly different from Leu (Gly-Leu) transport determined simply in the absence of the cation ($p<0.05$). A kinetic analysis of Leu (Gly-Leu)

transport in the presence of 20 mM Leu yielded kinetic constants (K+ 8.5 mM; Vmax 20 μmoles g dry wt.$^{-1}$ min.$^{-1}$) similar to those calcualted for the Na^+ independent portion of Leu (Gly-Leu) control studies (K+ 8.7 mM; Vmax 19.3 μ moles g dry wt.$^{-1}$ min^{-1}). Either the addition of 20 mM Leu or the removal of Na^+ evidently unmasks the same Na^+ independent, intact dipeptide transport system.

The reduction in Leu (Gly-Leu) influx caused by saturating the free amino acid transport system indicates also that half of Leu uptake from the dipeptide must occur via this route and therefore that about 50 percent of the Gly-Leu presented to the ileal mucosa undergoes superficial hydrolysis prior to being transported. The liberated free Leu is then taken up by its well characterized amino acid transport route. Further evidence in support of this conclusion was found through the application of an inhibitor of surface hydrolysis.

Kania (1977) has shown that L-Ala-β-naphthylamide (ANA) has a much greater affinity for the aminopeptidases of the intestinal brush border membrane than dipeptides with Gly as the NH_2 -terminal amino acid, as well as a hydrolytic rate far in excess of the variety of dipeptides they tested. This compound has therefore been successfully used as an inhibitor of surface hydrolysis in studies of dipeptide transport into the small intestine (Chung et al., 1979; Rosen-Levin, 1979). In this investigation the presence of ANA (10 mM) in the medium reduced the rate of uptake of Leu (Gly-Leu) to

approximately 50 percent of control values and to levels not significantly different from those observed in the presence of saturating Leu and the absence of Na^+ ($p>0.05$) or simply in the absence of Na^+ ($p>0.05$) (Table I).

The three methods employed to eliminate the contribution made to Leu (Gly-Leu) transport by surface hydrolysis (removal of Na^+ from the medium, saturation with free Leu and inhibition of surface hydrolases) reduced uptake to levels not significantly different from one another ($p>0.05$) and corresponded to approximately 50 percent of control levels (Table I). These results are consistent with the findings of both Cheeseman and Parsons (1976) and Himukai and Hoshi (1980) that Leu (Gly-Leu) transport into the small intestine occurs via two systems; one, the preferential transport of Leu liberated from Gly-Leu following surface hydrolysis by the Na^+ dependent and concentrative free amino acid system and two, the uptake of intact dipeptide by a nonconcentrative, saturable and Na^+ independent mechanism.

Gly (Gly-Leu) uptake into everted rat intestine, in the presence of Na^+, was approximatley half that of Leu (Gly-Leu) control determinations over a wide range of Gly-Leu concentrations (Table II). This observation was made for Gly-Leu transport into monkey intestine (Das et al., 1975; Ganapathy et al., 1979), guinea pig intestine (Himukai et al., 1980) and rat jejunum (Fern et al., 1969). Das et al. (1975) attributed this preferential uptake of Leu from Gly-Leu partly to a greater efflux of Gly over Leu from the

enterocyte into the lumen and partly to a difference in the uptake rates of Leu and Gly from free solution following surface hydrolysis. Both Fern et al. (1969) and Himukai et al. (1980) attributed this same phenomena entirely to the preferential transport of liberated Leu following surface hydrolysis of Gly-Leu at the brush border membrane. The latter conclusion gains support from the observation that the efflux of Gly and Leu from the intestinal enterocyte have been shown to be equal (Fern et al., 1969; Himukai et al., 1980) and by the much smaller K_t value of Leu (1.5 mM) than Gly (27 mM) reported by Himukai and Hoshi (1975). The findings of this study tend to support this explanation. There was no significant difference ($p>0.05$) found between Gly (Gly-Leu) transport (Table II) and that observed for Leu (Gly-Leu) uptake measured in the presence of either 20 mM cold Leu (in the presence or absence of Na^+) or 10 mM ANA or simply in the absence of medium Na^+ (Table I). In other words, when the contribution made to Leu (Gly-Leu) influx by surface hydrolysis followed by the preferential absorption of the liberated Leu is eliminated the transfer of Leu (Gly-Leu) and Gly (Gly-Leu) across the intestinal membrane become equal. It is also apparent from these observations that Gly (Gly-Leu) transport into rings of everted rat intestine must occur exclusively via intact peptide transport. Indeed, when 20 mM free Leu was added to the medium during a determination of Gly (Gly-Leu) uptake over a concentration range of the substrate, there was no significant reduction in

transfer of Gly (Table II; $p>0.05$). In view of the much greater affinity for the free amino acid carrier exhibited by Leu over Gly, an excess of Leu in the incubation medium would have inhibited any portion of Gly (Gly-Leu) transport occurring via the free amino acid uptake system after surface hydrolysis and a reduction in total Gly (Gly-Leu) transport would have been observed. The kinetic constants determined for Gly (Gly-Leu) transport (K_t 7.83 mM; Vmax 17.8 μmoles g dry wt.$^{-1}$ min.$^{-1}$) indicate further that only the intact peptide transport route is involved since the values closely match those determined for the Na^+ independent portion of Leu (Gly-Leu) transport (K_t 8.7 mM; Vmax 19.3 μmoles g dry wt.$^{-1}$ min.$^{-1}$) and Leu (Gly-Leu) uptake in the presence of 20 mM free Leu (K_t 8.5 mM; Vmax 20.0 μmoles g dry wt.$^{-1}$ min.$^{-1}$).

Finally, in support of the studies with Leu (Gly-Leu) it was found that Gly (Gly-Leu) uptake was not significantly affected by the replacement of medium Na^+ with choline ($p<0.05$), again suggesting that intact Gly-Leu transport is not Na^+ dependent.

The Na^+ independent nature of dipeptide transport has also been demonstrated in an in vivo preparation of frog intestine (Cheeseman et al., 1976), isolated rabbit intestinal brush border membrane vesicles (Ganapathy et al., 1980) and guinea pig intestine in vitro (Himukai et al., 1980). The Na^+ independency of dipeptide uptake has therefore been established in four different animal species using a variety of preparations.

The demonstration that intact Gly-Leu transport in Na^+ independent is consistent with the observation that Leu (Gly-Leu), in this study, was not accumulated within the rat enterocyte to concentrations above that in the medium (see: Results; Leu (Gly-Leu) Uptake Versus Time in the Absence of Na^+). It would appear therefore, that intact Gly-Leu transport into the rat intestine is via a facilitated mechanism. This conclusion was also reached by Cheeseman and Parsons (1976) and Cheeseman (1980) who found intact Gly-Leu and carnosine uptake into the frog small intestine to be via a non-accumulative, Na^+ independent route.

2. Harmaline Study

Harmaline (3,4-dihydro-7-methoxy-1-methyl-9-pyrid [3,4-bis] indole) is a psychotomimetic alkaloid, extracted from a Colombian liana and belongs to the lysergic and diethylamide-like class of drugs.

The earliest study utilizing this drug, showed it to be an inhibitor of the (Na^+ + K^+)-ATPase system in several membrane ATPase preparations (Canessa et al., 1973). Canessa concluded that the drug competetively inhibited the binding of Na^+ ions to the phosphorylated intermediate of the (Na^+ + K^+)-ATPase reaction in the rat brain. Since this initial observation by Canessa several workers have verified her findings in a wide variety of tissue and enzyme preparations

(Dunn and Hunt, 1975; Robinson, 1975; Sepulveda et al., 1976; Ehrenfeld and Romeu, 1977; Sarmarzija, 1977; Sousa and Grosso, 1977; Lea and Ashley, 1981).

In 1974, harmaline's ability to inhibit sugar and amino acid transport into guinea pig intestinal rings was noted (Sepulveda and Robinson, 1974). Subsequent published studies by these and other authors have presented convincing evidence that in addition to harmaline's inhibitory action on the Na^+ pump, the drug also competes with Na^+ for its binding site on the carrier molecule at the membrane surface. These workers maintain that harmaline's ability to inhibit solute transport is due to this latter action and is not secondary to its inhibition of the ($Na^+ + K^+$)-ATPase system (Sepulveda and Robinson, 1974; 1975; 1976; 1978; Alvarado et al., 1979).

Since a specific inhibitor of Na^+ dependent amino acid transport at the level of the carrier would provide an additional method of isolating the peptide system enabling a comparison with the Leu saturation and ANA experimental data, an investigation of harmaline's effects was undertaken.

The initial experiments using harmaline showed that increasing concentrations of the drug did significantly reduce the uptake of Leu into everted intestine of both 1 mM free Leu and 1 mM Gly-Leu (Figure 15). The inhibition was maximal in both cases at an inhibitor concentration of 10 mM but harmaline did not reduce Leu (Gly-Leu) uptake to as low a level as it did Leu (Leu) uptake. This observation fits the hypothesis that Gly-Leu uptake is made up of two components

only one of which is Na^+ dependent (the free amino acid transport system) and therefore harmaline sensitive.

The Na^+ free determinations of Leu (Leu) uptake in the presence of increasing concentrations of harmaline (Figure 15) however, suggest that harmaline has inhibitory effects on solute transport into the intestinal enterocyte which are in addition to its inhibition of Na^+ binding. These results show that when present in concentrations in excess of 2.5 mM harmaline significantly reduced Leu (Leu) uptake in Na^+ free rivers.

At harmaline concentrations below 2.5 mM the drug had no effect on Gly (Gly-Leu) transport which serve to verify the findings of the Leu saturation and ANA experiments (previously described), demonstrating the Na^+ independence of this process. However, concentrations of harmaline above 2.5 mM had a significant inhibitory effect (Figure 17). Further, a harmaline concentration of 5 mM was shown to inhibit Gly (Gly-Leu) transport over a wide range of substrate concentrations by an average of 33 percent as compared to control levels (Table I).

Harmaline then, has been shown in these experiments to inhibit free amino acid uptake in the absence of medium Na^+ and also to inhibit the Na^+ independent process of Gly (Gly-Leu) uptake when present in concentrations above 2.5 mM. Neither of these observations is consistent with the theory that harmaline inhibits amino acid transport solely through competition with Na^+ at the membrane carrier site.

Recently it has been demonstrated that harmaline is able to penetrate into the cells of muscle fibres and actually be accumulated intracellularly against its concentration gradient to levels substantially above that in the bathing medium (Lea and Ashley, 1981). These results ". . . are found to be consistent with the hypothesis that extracellular harmaline can penetrate the cell membrane and act on the Na^+ pump at the interior facing site" (Lea and Ashley, 1981).

Samarzija et al. showed that harmaline's effects may not be limited to the (Na^+ - K^+)-ATPase system. During an investigation of the action of 5 mM harmaline on rat proximal tubular solute and water transport, these workers concluded that the drug inhibited both the (Na^+ - K^+)-ATPase and Mg^+ - ATPase systems of peritubular cell membrane fractions "as well as the HCO_3 stimulated ATPase of a brush border membrane fraction" (Samarzija, 1977). Further, the data from their work suggests that harmaline's inhibitory effect on the (Na^+ - K^+)-ATPase could not ". . . simply result from a competition between harmaline and Na^+ " (Samarzija, 1977).

The transport of free Leu was studied in the presence of 5 mM harmaline over a concentration range of the substrate both in the presence and absence of Na^+ (Figure 16). A 5 mM harmaline concentration was chosen based on the observation that at this level the drug induced a near maximal inhibition of Leu from a 1 mM solution of free Leu (Figure 15) and also on reports in the literature suggesting that higher concentrations would produce secondary effects on the

enterocyte (Sepulveda and Robinson, 1976).

Harmaline's ability to inhibit Leu (Leu) uptake was shown to diminish as the substrate concentration was increased in the presence of Na^+ (Figure 16). At a Leu concentration of 1 mM, 5 mM harmaline reduced Leu (Leu) uptake (as compared to control uptakes) by as much as 30 percent. This inhibition fell to a low of 14 percent at Leu concentrations of 20 mM. A kinetic analysis of this data indicates that the presence of harmaline caused a decrease in the Vmax of Leu (Leu) uptake while inducing an increase in the apparent Km. This indicates that harmaline inhibited Leu (Leu) transport in a noncompetitive manner. This is not unexpected since none of the theories as to the drugs mode of action suggest direct competition with the substrate and harmaline. The removal of Na^+ from this preparation reduced the uptake of free Leu to levels approximately 50 percent lower than the 5 mM harmaline alone could effect (Figure 16), indicating the considerable portion of Na^+ dependent Leu (Leu) transport unaffected by this relatively high concentration of the drug.

An explanation for harmaline's failure to lower leucine uptake to the same extent as Na^+ removal may lie in the fact that physiological Na^+ concentrations (143 mM) were used. Many of the previous studies utilizing harmaline were done at much lower Na^+ levels and it is well established in the literature that as Na^+ concentrations are increased harmaline inhibition can be reduced and eventually abolished

(Alvarado et al., 1979). It is possible, as suggested in a discussion of Samarzija's (1977) negative results with harmaline, that Na^+ concentrations as high as 143 mM may be sufficient ". . . to counteract any possible harmaline effects. . ." in some experimental preparations (Alvarado, 1979).

On the basis of the observations made on the inhibition of Leu (Leu) and Gly (Gly-Leu) uptake by harmaline it is possible to explain the ability of the drug to reduce Leu (Gly-Leu) transport to levels similar to those in the Na^+ free, Leu saturation and ANA experiments (Table I). The drug exerted two simultaneous inhibitory effects on Leu (Gly-Leu) absorption, one being an incomplete inhibition of the Na^+ dependent uptake of free Leu liberated by surface hydrolysis and the other a nonspecific inhibition of the Na^+ independent intact transport of Gly-Leu. The sum of these two effects, it would appear, inhibited total Leu (Gly-Leu) uptake fortuitously to the same degree as did the simple removal of Na^+, Leu saturation or the use of ANA.

While harmaline may be useful at or below a concentration of 2.5 mM to indicate whether or not Na^+ is involved in a given transport process, it cannot be used at high enough concentrations to eliminate Na^+ dependent transport due to the drugs nonspecific intracellular effects.

In light of the characteristics of harmaline demonstrated here, the work of Ganapathy and Radhakrishnan

(1979; see LITERATURE REVIEW: Na^+ Dependency of Peptide Transport) must be viewed with caution. These authors, through the use of harmaline, claim to have demonstrated the Na^+ dependency of Gly-Leu uptake into monkey intestine in vitro. They observed that both Leu (Gly-Leu) and Gly (Gly-Leu) influx to be significantly reduced in the presence of 4 mM harmaline. Maximal effect was obtained only after a ten minute preincubation of the intestinal tissue with the drug.

It is most likely that the reduction of dipeptide observed in the above procedure was a result not only of the inhibition of Na^+ -dependent processes but also of the nonspecific intracellular inhibitory effects induced by a harmaline concentration as high as 4 mM. Further incubating the tissue for ten minutes in harmaline would, in all likelihood, result in intracellular accumulation of the drug, as shown to occur in muscle fibres (Lea and Ashley, 1980), wherein concentrations even higher than 4 mM could exert a nonspecific inhibition of cellular metabolism and secondarily peptide transport.

Ganapathy et al. (1980); see LITERATURE REVIEW: Na^+ Dependency of Peptide Transport) demonstrated harmaline's ineffectiveness at inhibiting dipeptide transport into intestinal and renal brush border membrane vesicles from rabbit, which lacked a $(Na^+ - K^+)$-ATPase system. Apparently when the target for harmaline's nonspecific inhibitory effects is removed it no longer is able to

disrupt Na^+ -independent processes.

3. Competition Study

In the presence of a saturating concentration of cold Leu (20 mM), Gly-Pro was found to inhibit Leu from Gly-Leu (1 mM) by only 57 percent at the highest inhibitor concentration used (Figure 18). This partial inhibition was shown to be competitive in nature by the two methods outlined in Figures 20 and 21. A replotting of this data as outlined by Inui and Christensen in 1966 (Figure 19) revealed that an infinitely high concentration of Gly-Pro would fail to inhibit 44 percent of Leu (Gly-Leu) transport, suggesting that this portion of Leu (Gly-Leu) influx proceeds via an intact uptake system not shared by Gly-Pro. That is, it would appear, from this investigation that intact Gly-Leu has two routes of entry into the intestinal enterocyte; one which is used by both Gly-Leu and Gly-Pro and another which is available only to Gly-Leu. The above procedure was repeated using L-alanyl naphthylamide (10 mM) in place of 20 mM cold Leu to eliminate the contribution of surface hydrolysis to Leu (Gly-Leu) transport. The fact that the results obtained were not significantly different ($p>0.05$) verifies the effectiveness of ANA as an inhibitor of the brush border amino-peptidases which hydrolyze Gly-Leu (Table III).

Similarly Rosen and Levin (1979) were able to block only 70 percent of Leu-Leu influx into rat intestine through the simultaneous application of Gly-Pro (20 mM) and ANA (10 mM).

Although these workers did not analyze this aspect of their data, they alluded to the possibility that there exists in the rat intestine a ". . . third mechanism of assimilation of peptides that is not blocked by Gly-Pro. . ." (Rosen-Levin, 1979). Boyd and Ward (1982) observed that while Leu-Leu could completely abolish the depolarization caused by carnosine influx into mudpuppy intestine, even high concentrations of carnosine could not totally abolish the depolarization induced by Leu-Leu transport. Since the effects were seen in the presence of a saturating concentration of free Leu, the authors concluded that two pathways must exist for intact Leu-Leu absorption only one of which is shared by carnosine.

The finding that there may exist more than one intact transport route available to Gly-Leu is in agreement with the several studies suggesting that peptide uptake occurs via at least two uptake mechanisms (see LITERATURE REVIEW: Competition Studies). The investigations indicating that a single transport system handles most, if not all, peptide absorption from the gut are largely unsatisfactory. Das and Radhakrishnan's work (1975) showed that all 38 dipeptides tested inhibited Leu (Gly-Leu) and Gly (Gly-Leu) uptake into monkey intestine which they considered as strong evidence for the existence of a single wide spectrum dipeptide uptake system. However, the inhibition was shown to be competitive in the case of only four of the dipeptides. Furthermore, this kinetic analysis was performed by determining the effects

of the four dipeptides on the influx of Leu (Gly-Leu) with no compensation for the complication of surface hydrolysis. These investigators also carried out a limited study of the inhibitory effects of five dipeptides on the influx of Leu (Gly-Leu) into human intestine in vitro and again no correction was made for surface hydrolysis. It is interesting however, that Gly-Pro (10 mM), which is poorly hydrolyzed by human brush border aminopeptidases (Kim et al., 1977), was able to bring about only a 44 percent reduction in Gly-Leu (0.5 mM) uptake. No kinetic treatment of this data was reported. Matthew's groups have studied the effects of various peptides on the uptake of peptides of Gly and Sar (Addison et al., 1975; Sleisenger et al., 1976; Matthews et al., 1978; Taylor et al., 1979). Although the use of these peptides (Gly-Sar, Gly-Sar-Sar, etc.) reduces the complication of surface hydrolysis it is unclear, to date, whether or not such atypical peptides reflect the behavior of the vast majority of peptides which do undergo superficial hydrolysis. Certainly the technique employed in the latter two studies (Matthews et al., 1978; Taylor et al., 1979) of lowering medium pH from 7.4 to 5.0 in order to reduce aminopeptidase activity of the brush border, creates unphysiological conditions which likely compromise the normal functioning of transport systems.

In the present work the Gly-Pro insensitive portion of Leu (Gly-Leu) uptake was isolated by the addition of 20 mM cold Leu and 10 mM cold Gly-Pro to the incubation medium and

the effect of 10 mM concentrations of several dipeptides on Leu (Gly-Leu) uptake via this second intact peptide route determined (Table IV). Only Leu-Leu proved to have a significant inhibitory effect and this inhibition was found to be noncompetitive (Figure 22). The interaction of Leu-Leu and Leu (Gly-Leu) transport was tested further by determining the effect of Leu-Leu (10 mM) on increasing concentration of Gly-Leu in the presence of saturating cold Leu (20 mM). Under these conditions only the free amino acid system was unavailable for Leu (Gly-Leu) transport with both intact peptide systems (if indeed two exist as suggested by the data) unaffected. As indicated in Figure 23, Leu-Leu did inhibit Leu (Gly-Leu) uptake and an analysis of this effect showed the inhibition to be of a mixed type or noncompetitive. A possible explanation of this phenomena is that the binding site for intact Leu-Leu transport is very close to the binding site for intact Gly-Leu transport not shared by Gly-Pro. The binding of Leu-Leu to its binding site may therefore cause a conformational change of the Gly-Leu binding site thereby reducing the efficiency of its function. This allosteric hinderance effect has been suggested by Himukai et al. (1980) as an explanation of the 'mixed type' inhibition exerted by Gly-Leu on Gly-Gly uptake into guinea pig small intestine.

Clearly further investigations are required to determine the nature of the possible second intact transport route for Leu (Gly-Leu).

FIGURE 1

Extracellular space (ECS, expressed as μlitres per gram of dry tissue) in rings of everted rat intestine after incubating the tissue for different lengths of time with [^{3}H]-inulin in Krebs bicarbonate ringer solution containing 1 mM glycyl-L-leucine. The rings of gut were incubated for five minutes in the Krebs ringer containing 1 mM glycyl-L-leucine prior to the ECS determinations. The broken line indicates the extrapolation of the linear portion of the curve back to the ordinate used for estimating the ECS for incubation periods of less than five minutes. Ordinate: ECS in μlitres of water per gram of dry tissue. Abscissa: incubation time in minutes. Values are presented as $\pm$S.E.

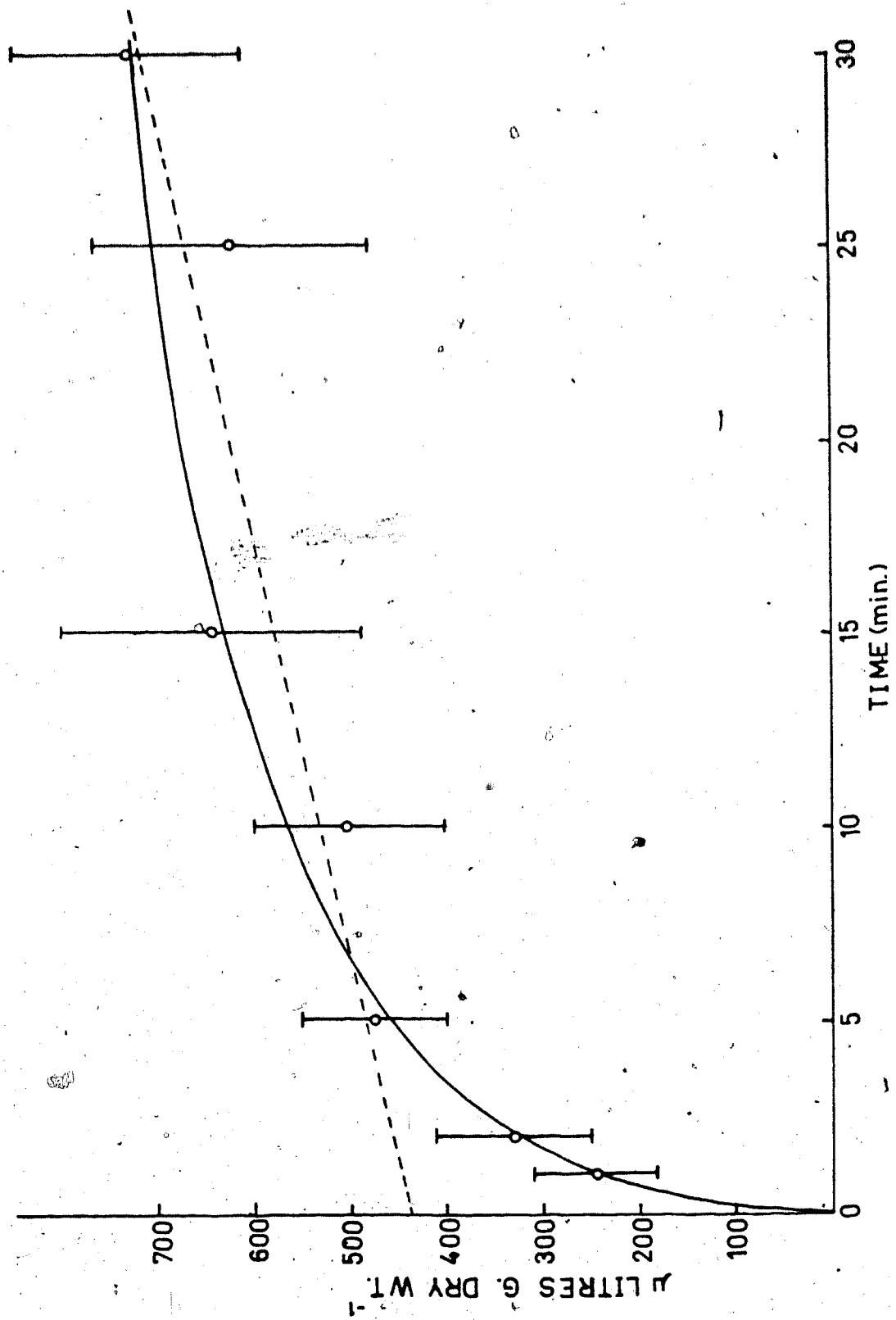

μ LITRES G. DRY WT.$^{-1}$
100
200
300
400
500
600
700
0
5
10
15
20
25
30
TIME (min.)

FIGURE 2

Extracellular space (ECS, expressed as μlitres of water per gram of dry tissue) in rings of everted rat intestine after incubating the tissue for different lengths of time with [^{3}H]-inulin in Na^+ free choline ringers. The rings of gut were incubated in the Na^+ free choline ringers for 30 minutes prior to the ECS determinations. The broken line indicates the extrapolation of the linear portion of the curve back to the ordinate used for estimating the ECS for incubation periods of less than five minutes. Ordinate: ECS in μlitres per gram of dry tissue. Abscissa: incubation time in minutes. Values are presented as means $\pm$ S.E.

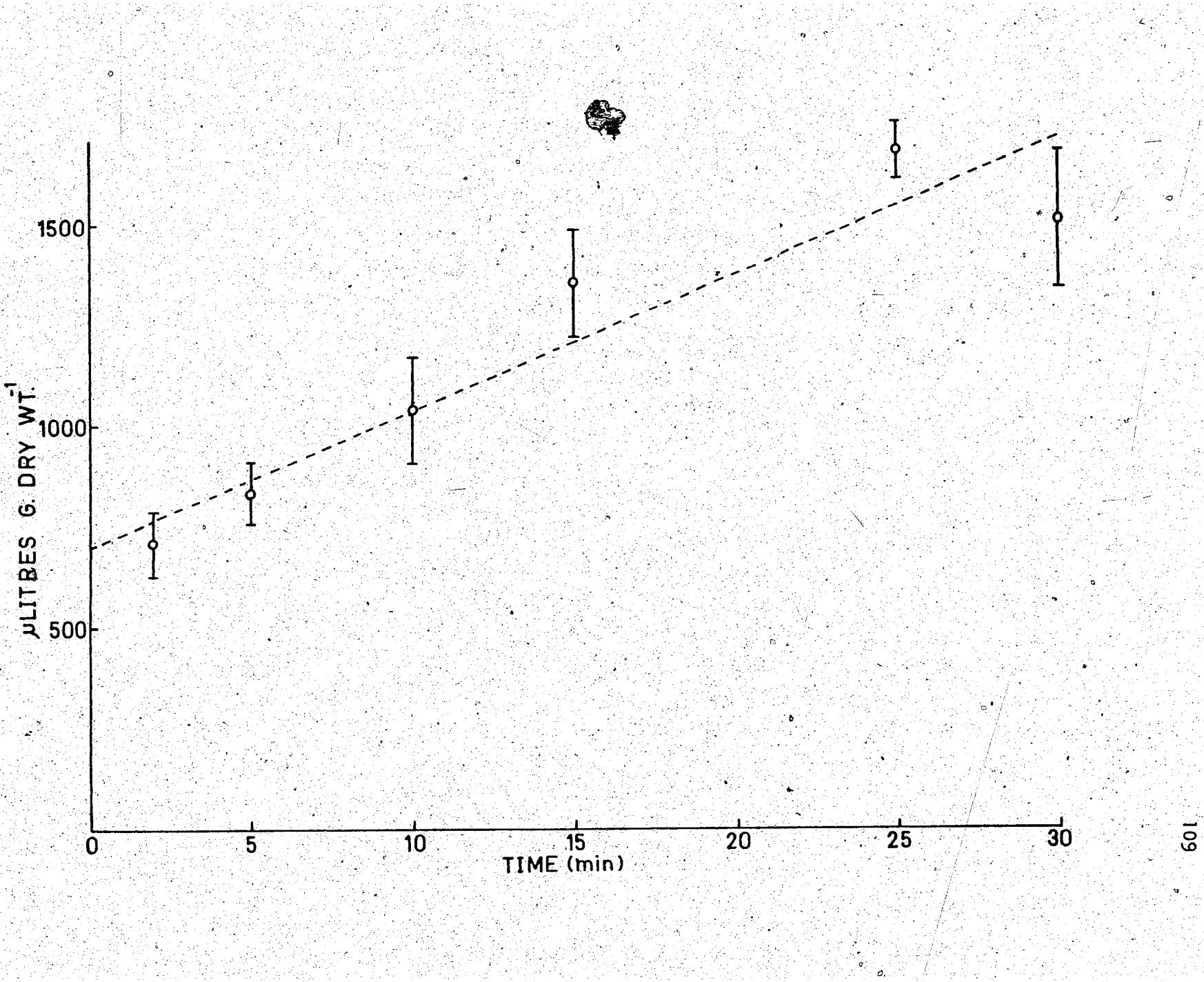

1500
1000
500
µLITRES G. DRY WT.$^{-1}$
0
5
10
15
20
25
30
TIME (min)

FIGURE 3

Extracellular space (ECS, expressed as μlitres per gram of dry tissue) in rings of everted rat intestine after incubating the tissue for different lengths of time with [^{3}H]-inulin in Na^+ free choline ringers. The rings of gut were incubated in the Na^+ free choline ringers for five minutes prior to the ECS determinations. The broken line indicates the extrapolation of the linear portion of the curve back to the ordinate used for estimating the ECS for incubation periods of less than five minutes. Ordinate: ECS in μlitres per gram of dry tissue. Abscissa: incubation time in minutes. Values are presented as means $\pm$ S.E.

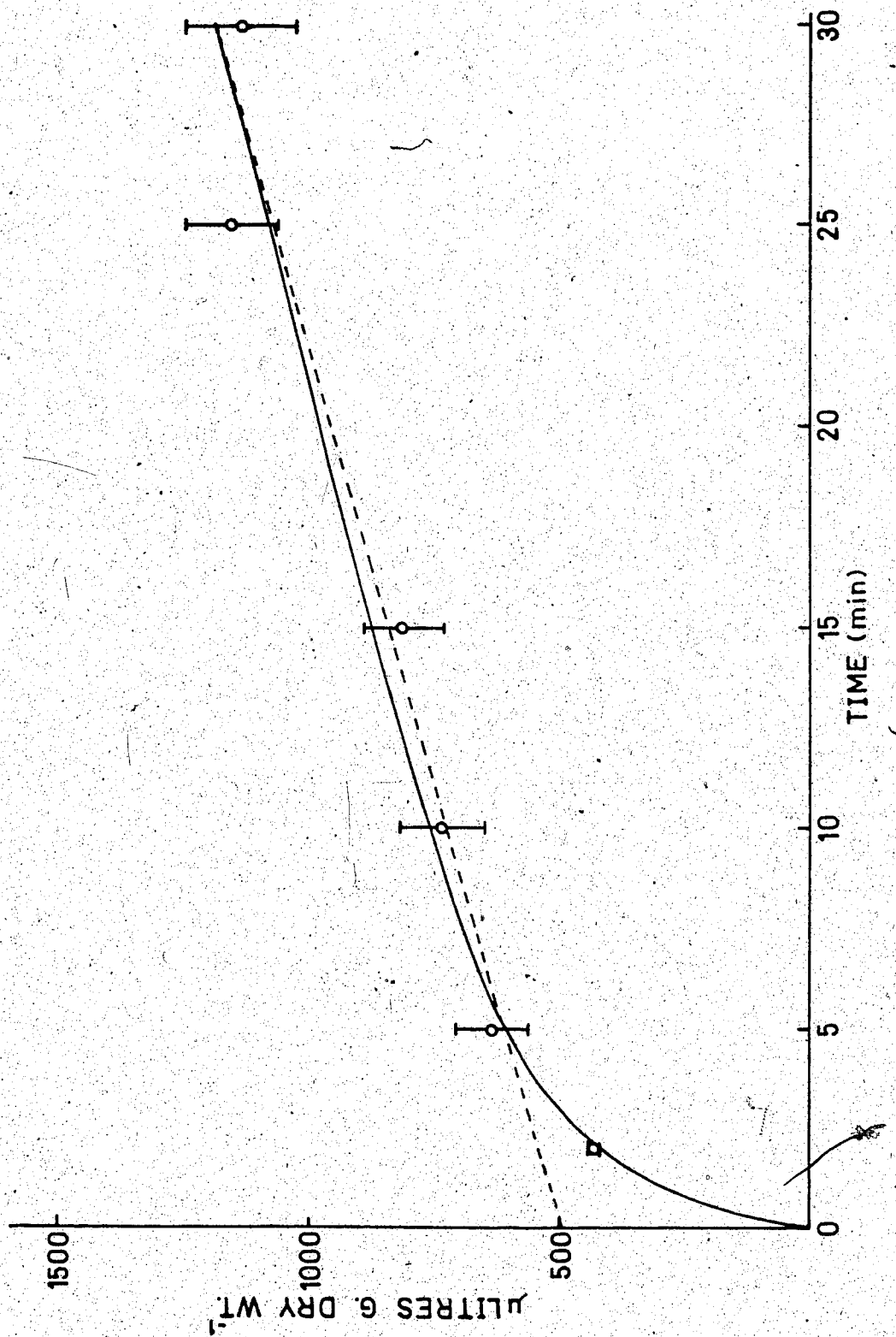
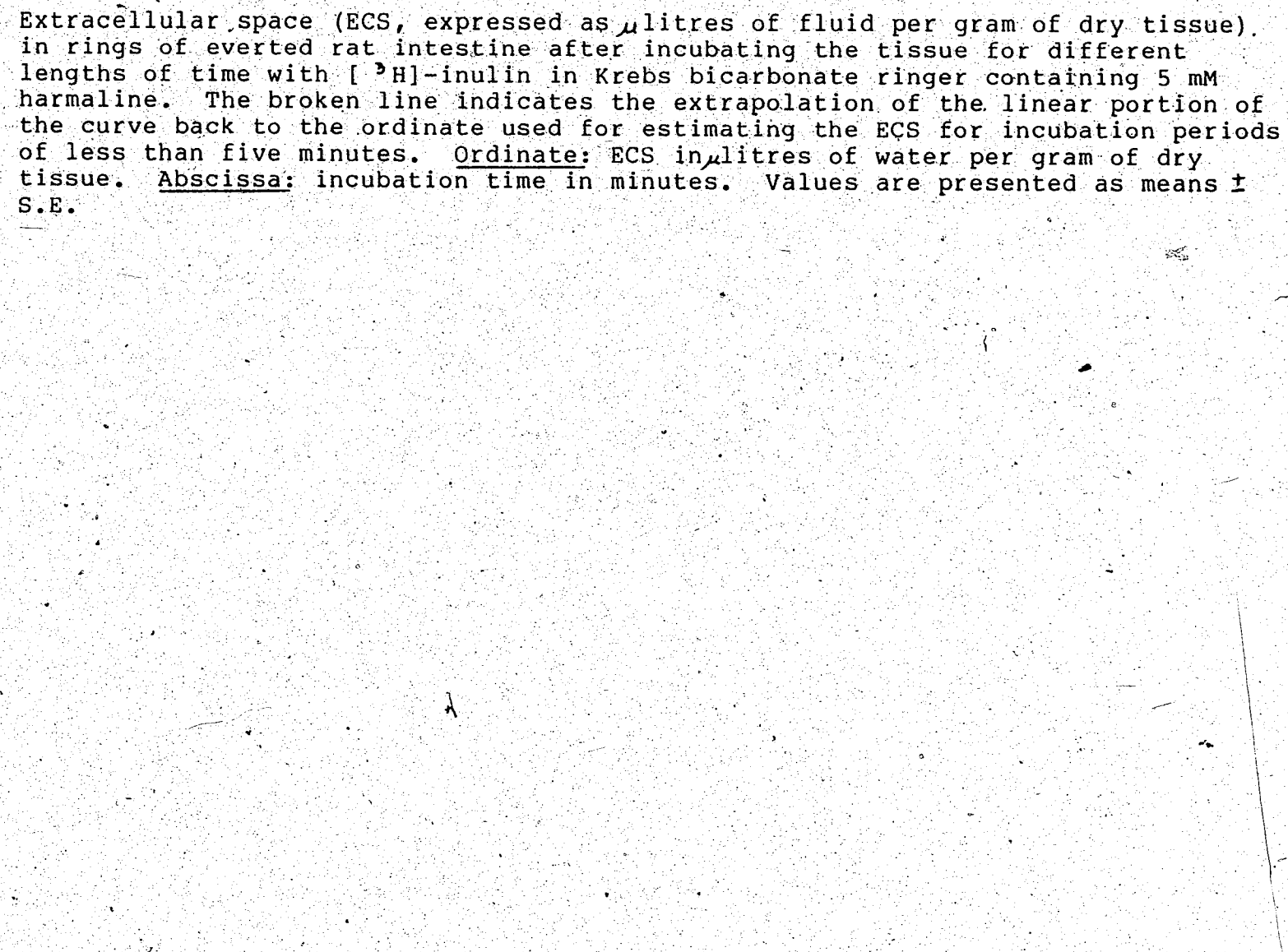

μLITRES g. DRY WT.-1
1500
1000
500
0
5
10
15
20
25
30
TIME (min)

FIGURE 4



Extracellular space (ECS, expressed as μlitres of fluid per gram of dry tissue) in rings of everted rat intestine after incubating the tissue for different lengths of time with [^{3}H]-inulin in Krebs bicarbonate ringer containing 5 mM harmaline. The broken line indicates the extrapolation of the linear portion of the curve back to the ordinate used for estimating the ECS for incubation periods of less than five minutes. Ordinate: ECS in μlitres of water per gram of dry tissue. Abscissa: incubation time in minutes. Values are presented as means $\pm$ S.E.

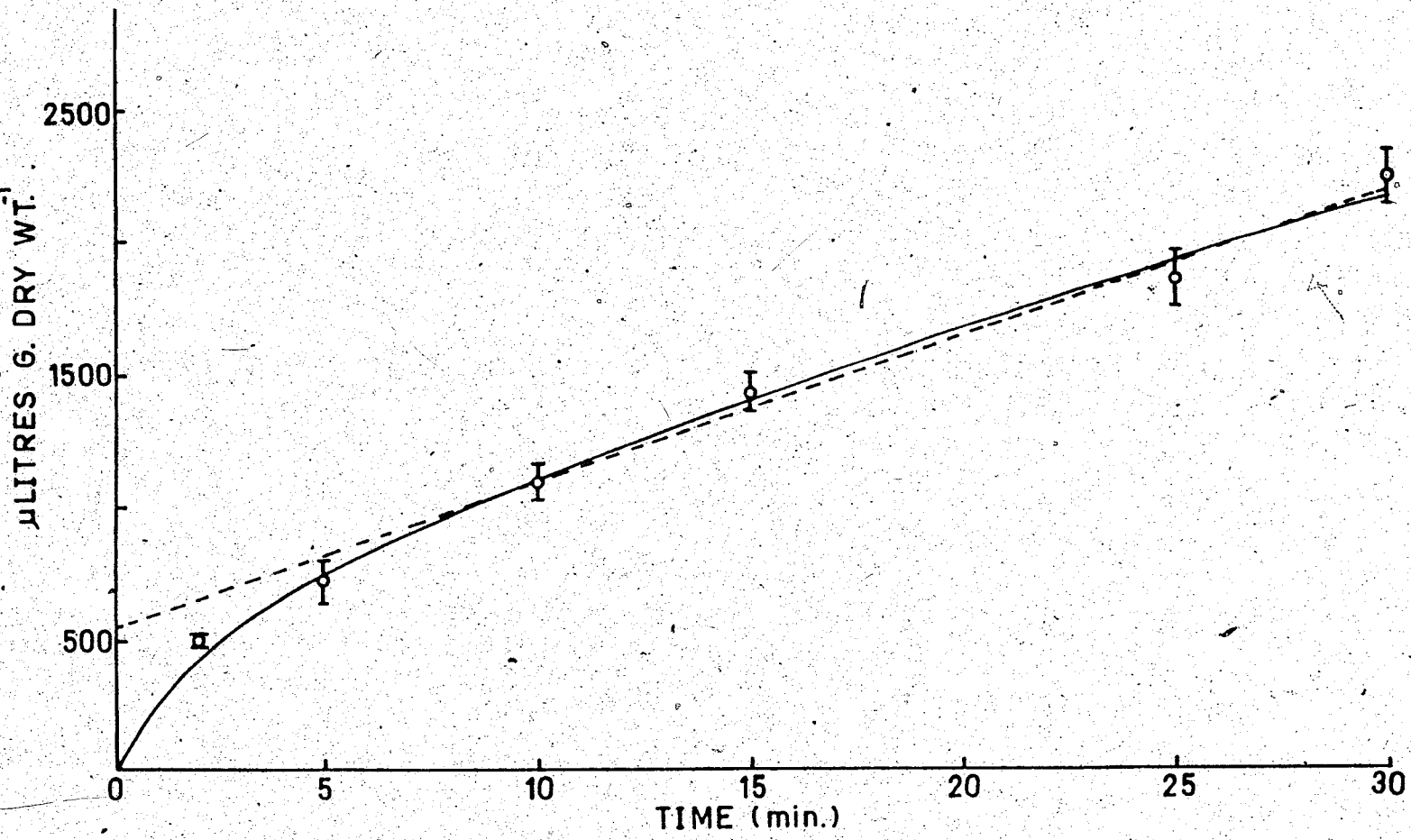

2500
1500
500
µLITRES G. DRY WT.⁻¹
0
5
10
15
20
25
30
TIME (min.)

FIGURE 5

Extracellular space (ECS, expressed as μlitres per gram of dry tissue) in rings of everted rat intestine after incubating the tissue for different lengths of time with [^{3}H]-inulin in Na^+ free choline ringers containing 5 mM harmaline. The broken line indicates the extrapolation of the linear portion of the curve back to the ordinate used for estimating the ECS for incubation periods of less than five minutes. Ordinate: ECS in μlitres of water per gram of dry tissue. Abscissa: incubation time in minutes. Values are presented as means $\pm$S.E.

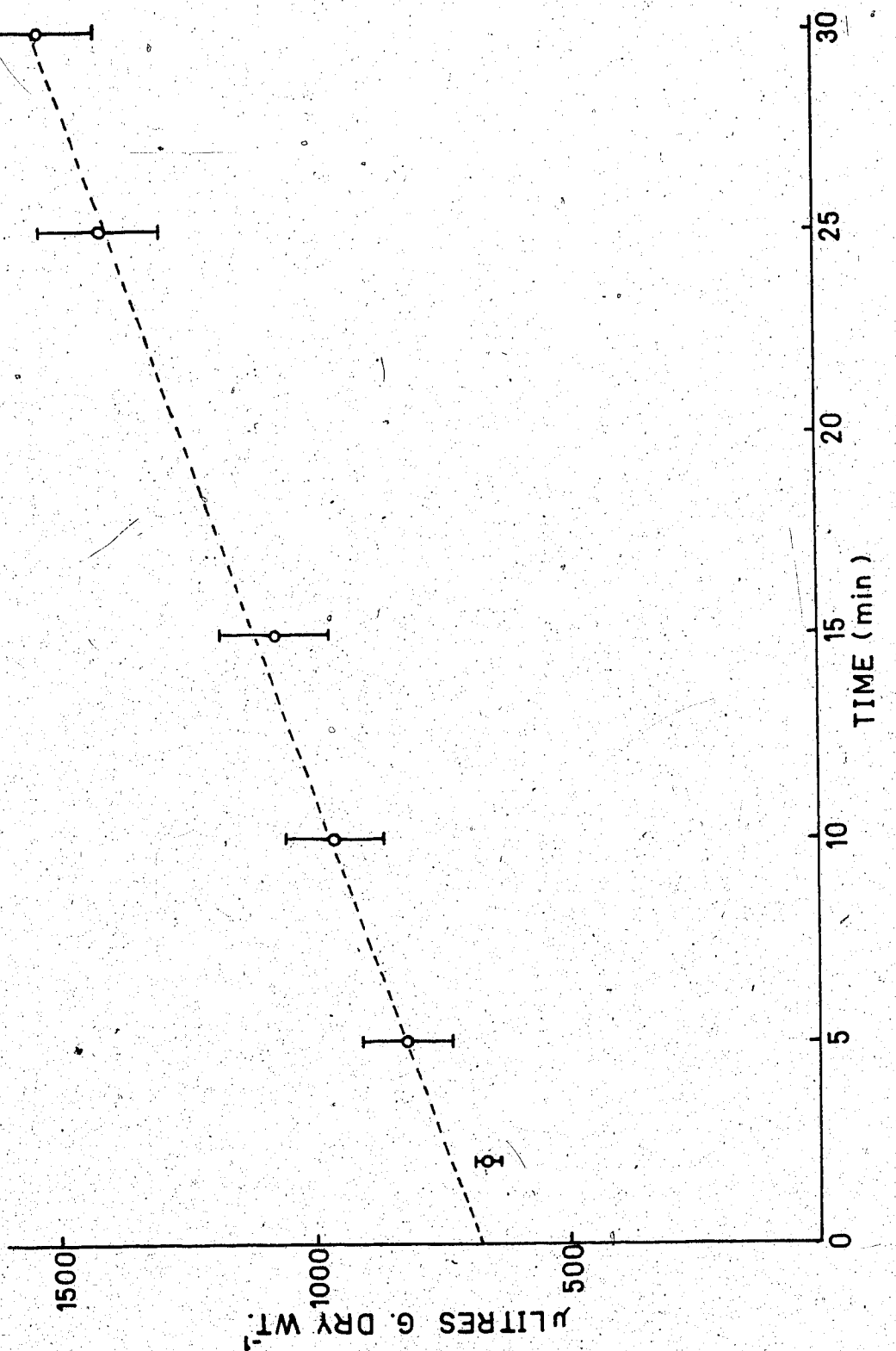
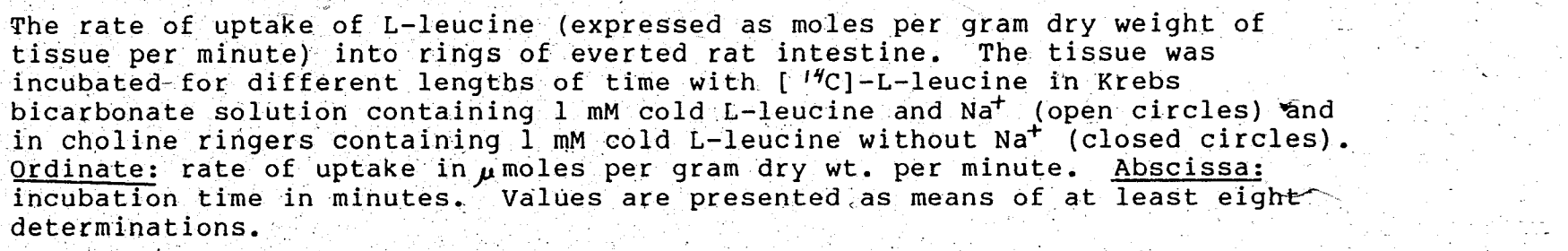

1500
1000
500
0
5
10
15
20
25
30
TIME (min)
μLITRES G. DRY WT.-1

FIGURE 6



The rate of uptake of L-leucine (expressed as moles per gram dry weight of tissue per minute) into rings of everted rat intestine. The tissue was incubated for different lengths of time with [^{14}C]-L-leucine in Krebs bicarbonate solution containing 1 mM cold L-leucine and Na^+ (open circles) and in choline ringers containing 1 mM cold L-leucine without Na^+ (closed circles). Ordinate: rate of uptake in μmoles per gram dry wt. per minute. Abscissa: incubation time in minutes. Values are presented as means of at least eight determinations.

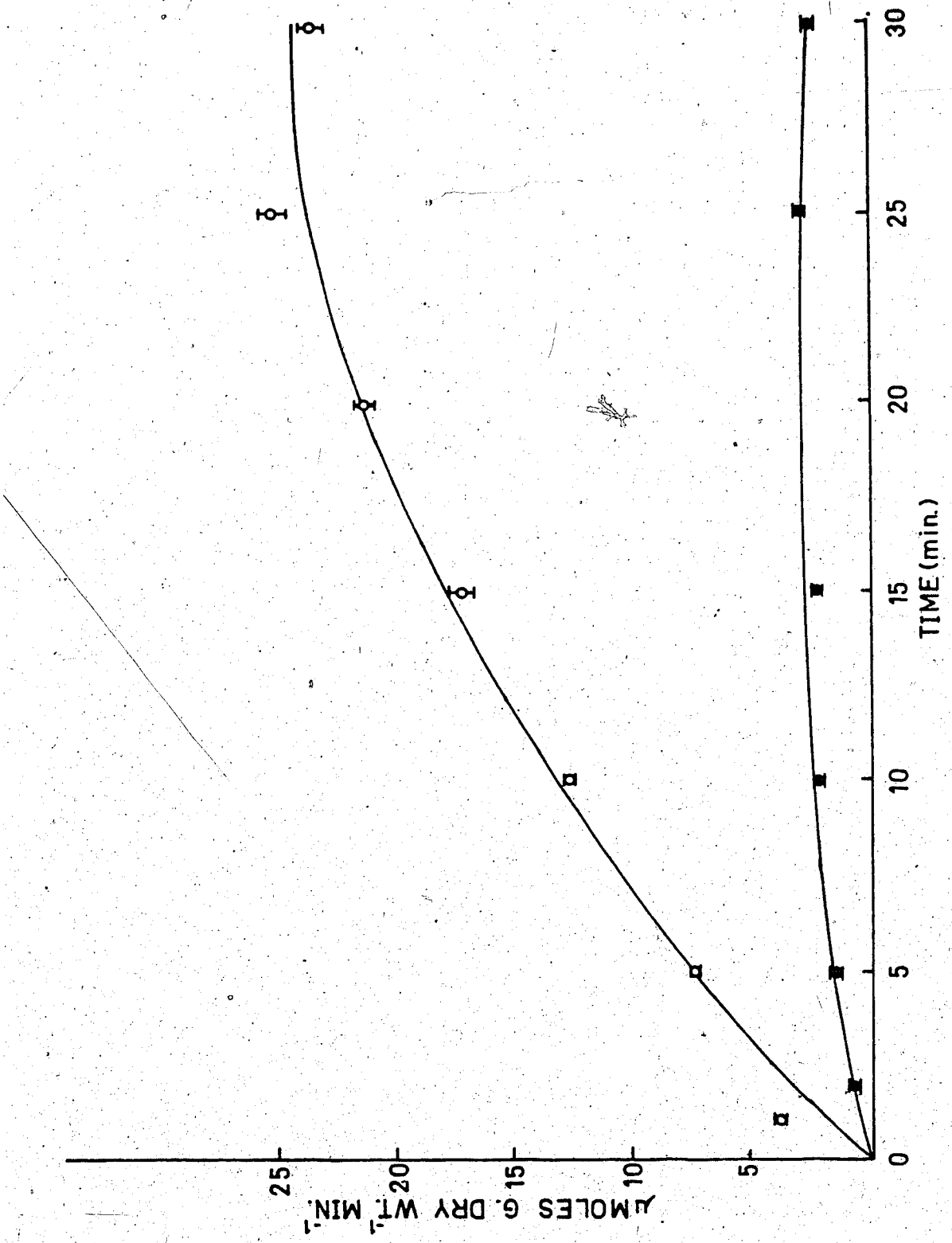
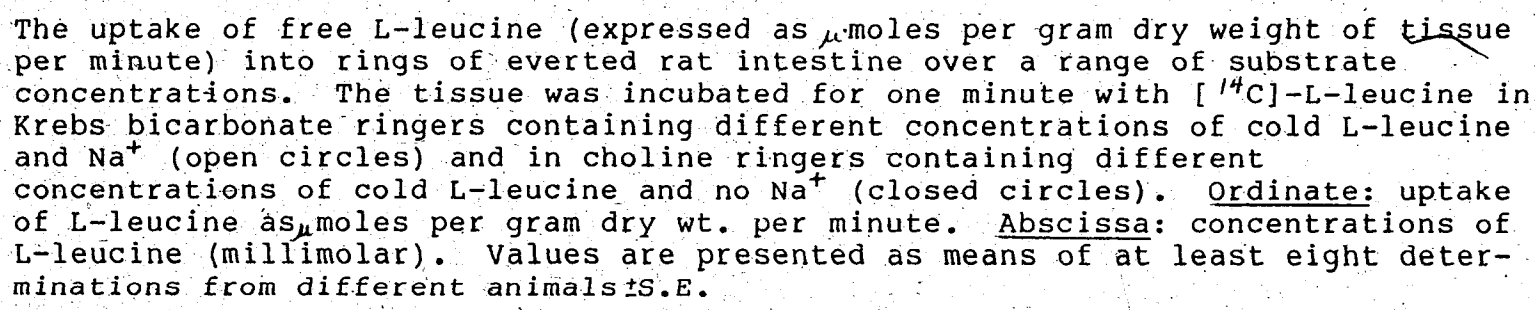

μMOLES G. DRY WT.⁻¹ MIN.⁻¹
25
20
15
10
5
0
5
10
15
20
25
30
TIME (min.)

FIGURE 7



The uptake of free L-leucine (expressed as μmoles per gram dry weight of tissue per minute) into rings of everted rat intestine over a range of substrate concentrations. The tissue was incubated for one minute with [^{14}C]-L-leucine in Krebs bicarbonate ringers containing different concentrations of cold L-leucine and Na^+ (open circles) and in choline ringers containing different concentrations of cold L-leucine and no Na^+ (closed circles). Ordinate: uptake of L-leucine as μmoles per gram dry wt. per minute. Abscissa: concentrations of L-leucine (millimolar). Values are presented as means of at least eight determinations from different animals ± S.E.

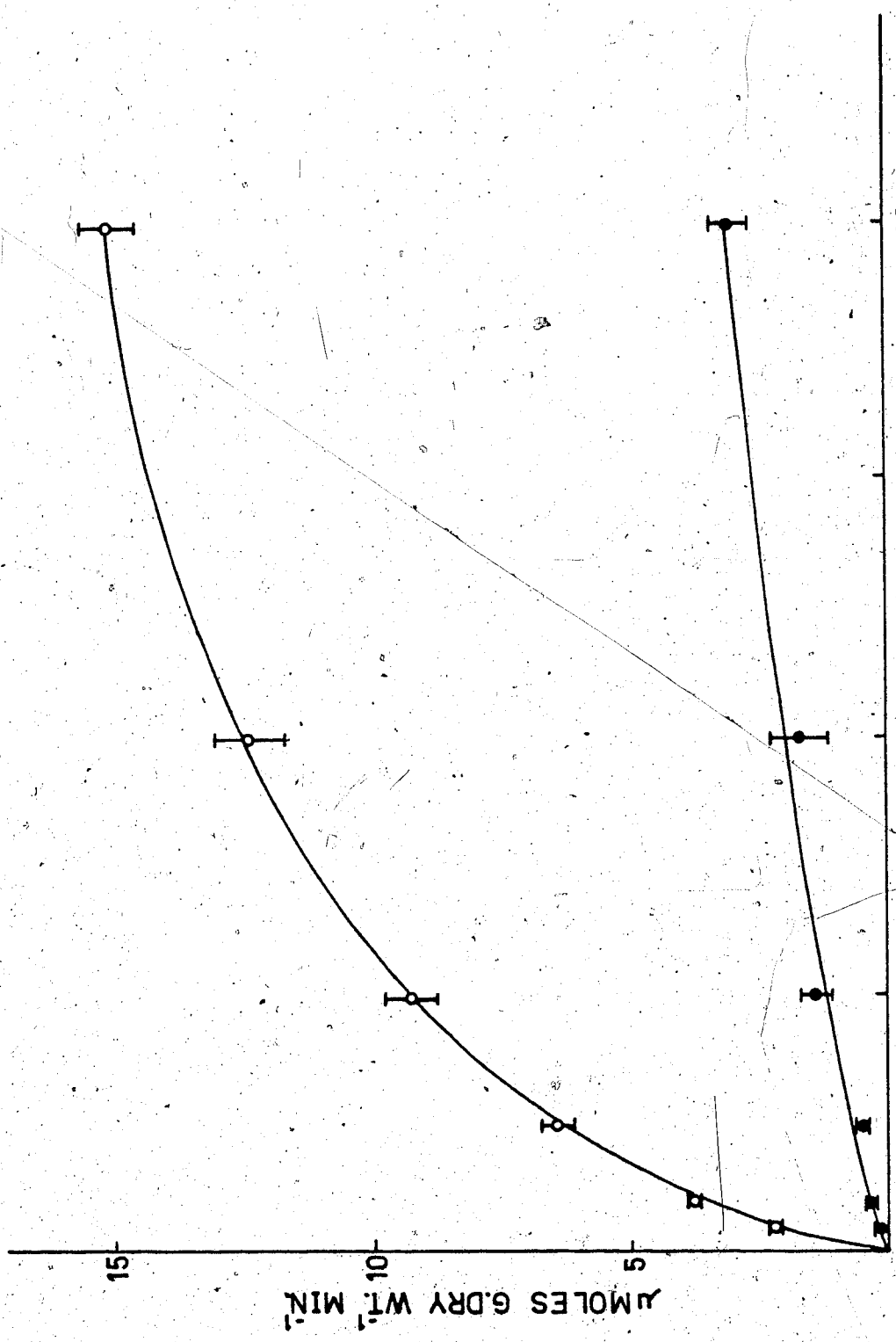
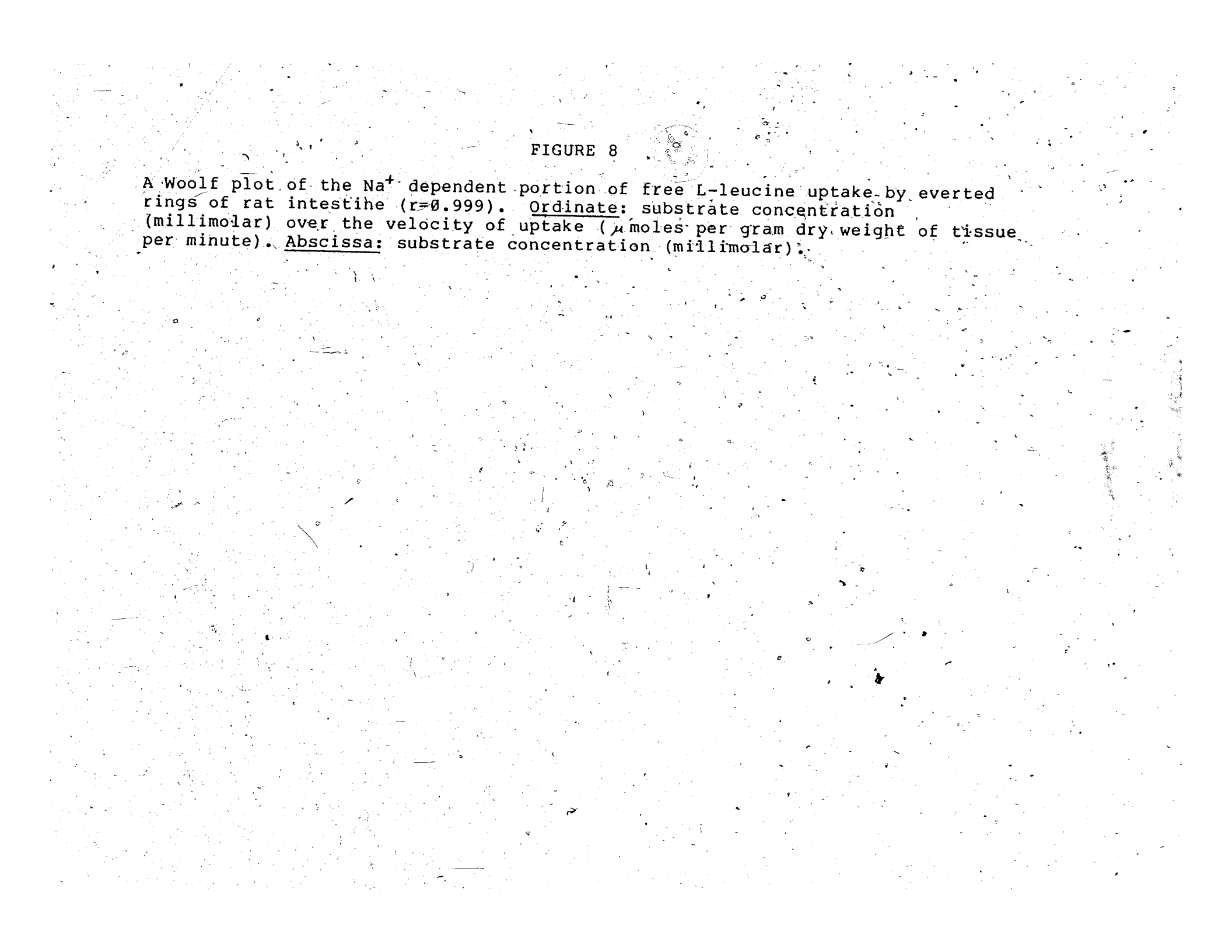

μMOLES G.DRY WT⁻¹ MIN⁻¹
15
10
5
0
5
10
15
20
L'EUCINE CONC. (millimolar)

FIGURE 8



A Woolf plot of the Na^+ dependent portion of free L-leucine uptake by everted rings of rat intestine (r=0.999). Ordinate: substrate concentration (millimolar) over the velocity of uptake (μmoles per gram dry weight of tissue per minute). Abscissa: substrate concentration (millimolar).

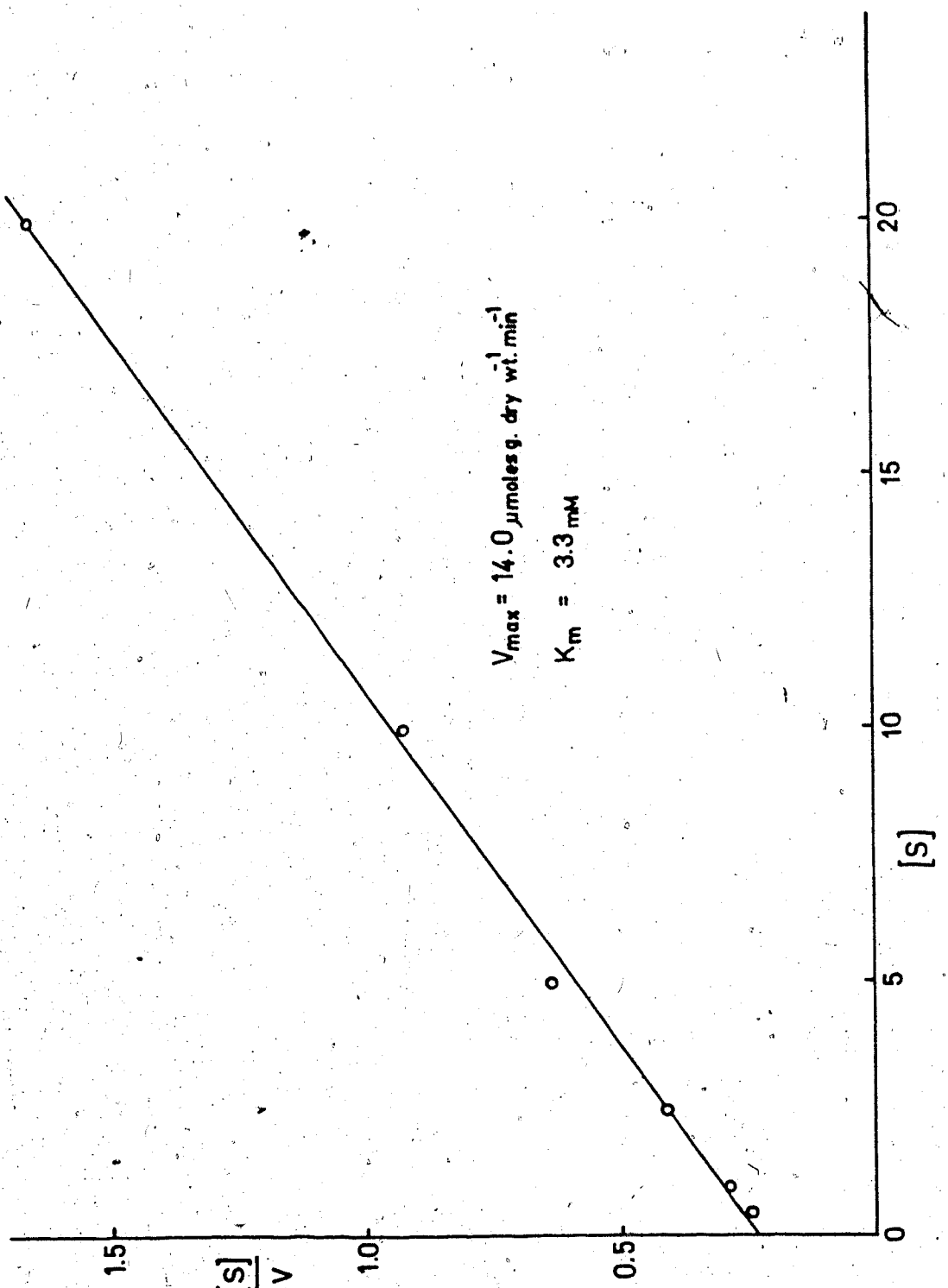

V_{max} = 14.0 µmoles g. dry wt.$^{-1}$ min^{-1}
K_m = 3.3 mM
[S]/V
[S]
0
5
10
15
20
0.5
1.0
1.5

FIGURE 9

The rate of uptake of L-leucine (expressed as μmoles per gram dry weight of tissue per minute) from glycyl-L-leucine into rings of everted rat small intestine. The tissue was incubated for different lengths of time with glycyl-[^{3}H]-L-leucine in Krebs bicarbonate ringer containing 1 mM cold glycyl-L-leucine and Na^+ (open circles) and in choline ringers containing 1 mM cold glycyl-L-leucine and no Na^+ (closed circles). Ordinate: rate of uptake in moles per gram dry weight of tissue per minute. Abscissa: incubation time in minutes. Values are presented as means of at least eight determinations from different animals $\pm$ S.E.

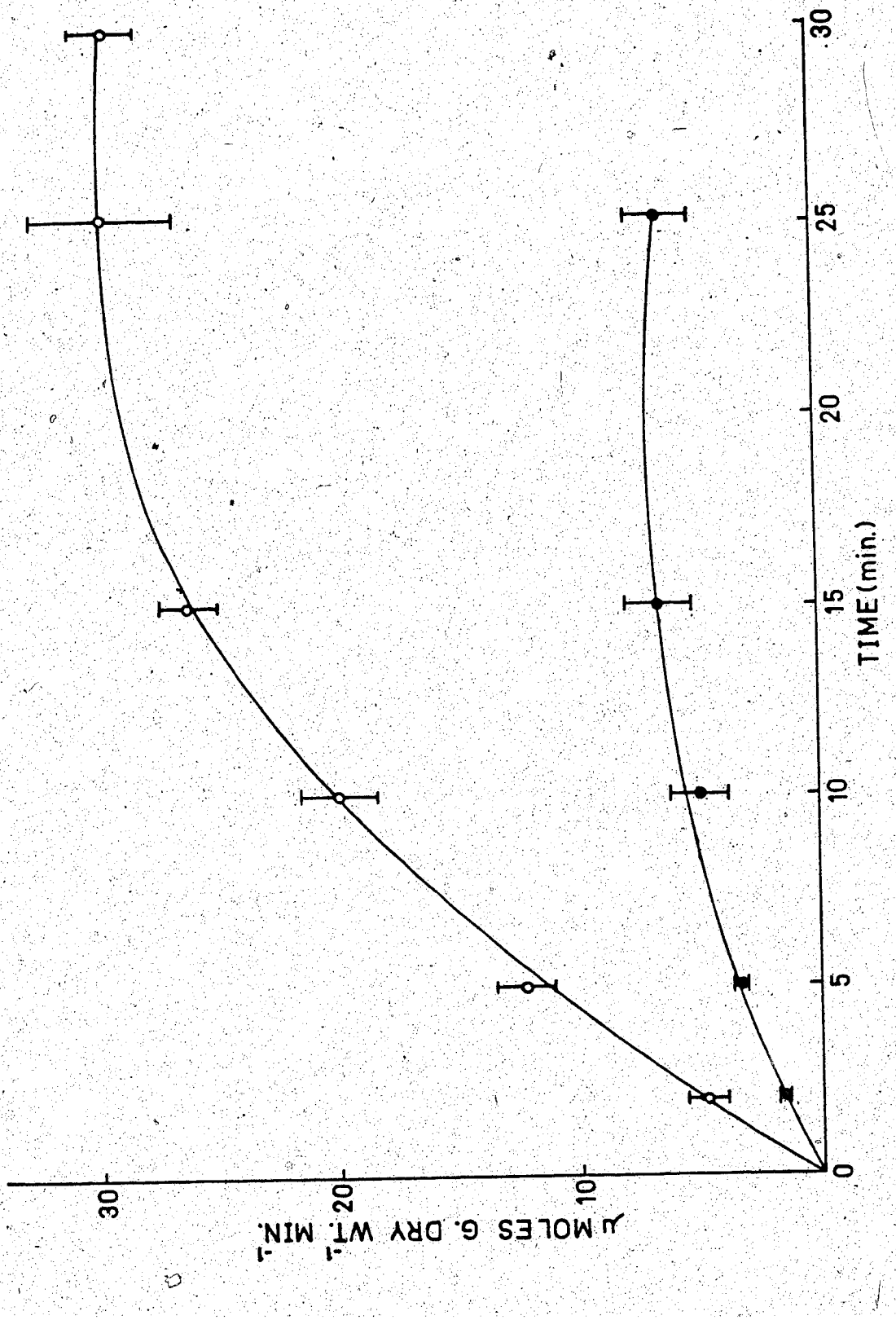

μ MOLES G. DRY WT. MIN.
-1
-1
30
20
10
0
5
10
15
20
25
30
TIME (min.)

FIGURE 1Ø

The uptake of L-leucine from glycyl-L-leucine expressed as μ moles per gram dry weight of tissue per minute) into rings of everted rat small intestine over a range of substrate concentrations. The tissue was incubated for one minute with glycyl-[^{3}H]-L-leucine in Krebs bicarbonate ringers containing different concentrations of cold glycyl-L-leucine and Na^+ (open circles) and in choline ringers containing diferent concentrations of cold Gly-Leu and no Na^+ (closed circles). Ordinate: uptake of L-leucine from glycyl-L- leucine expressed as μ moles per gram dry weight of tissue per minute. Abscissa: concentrations of glycyl-L-leucine (millimolar). Values are presented as means of at least ten determinations from different animals $\pm$ S.E.

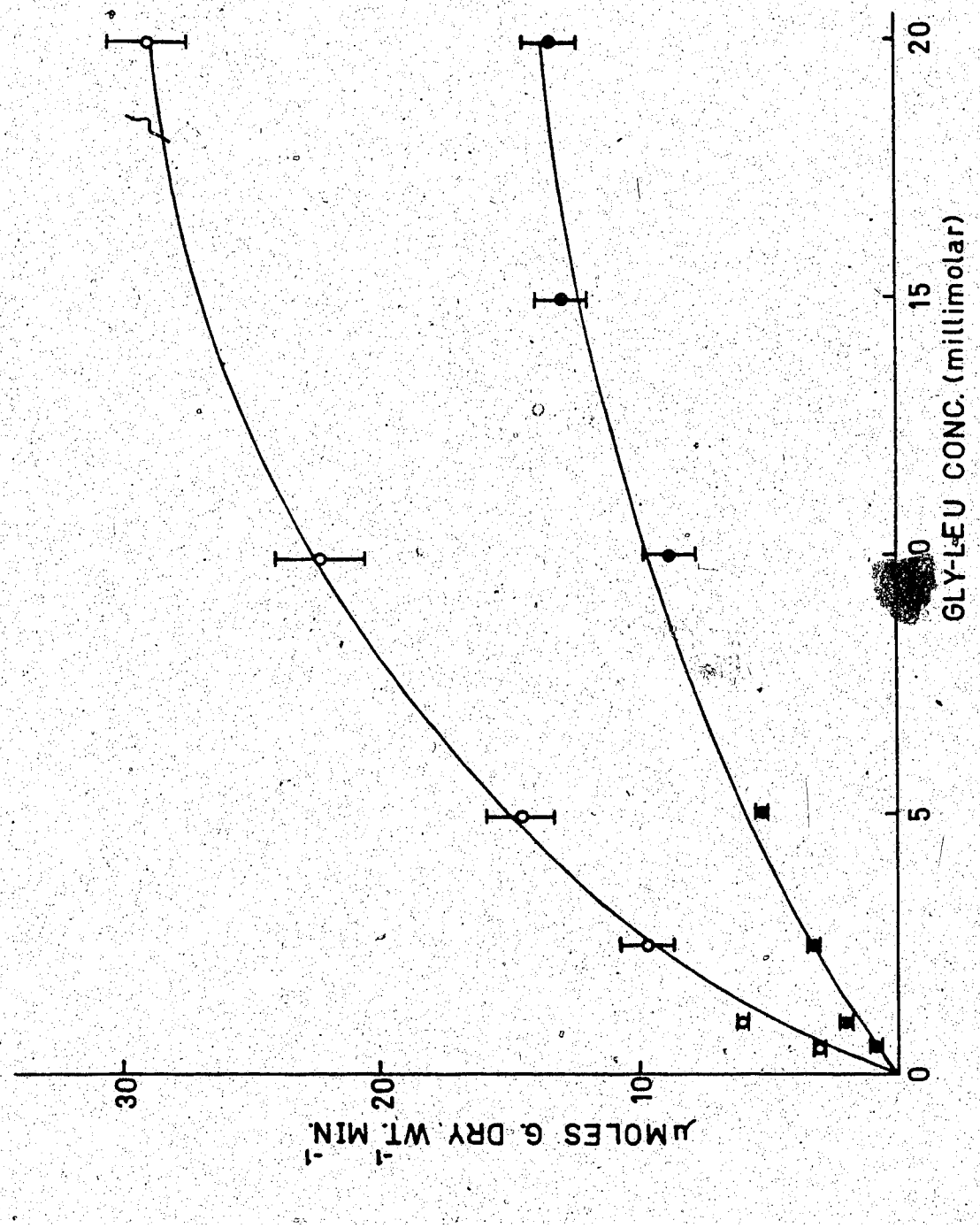

μMOLES G. DRY WT. MIN.$^{-1}$
30
20
10
0
0
5
10
15
20
GLY-L-LEU CONC. (millimolar)

FIGURE 11

A Woolf plot of the Na^+ dependent portion of L- leucine uptake from glycyl-L-leucine into rings of everted rat small intestine (r=Ø.996). Ordinate: substrate concentration (millimolar) over the velocity of uptake (μ moles per gram dry weight of tissue per minute). Abscissa: substrate concentration (millimolar).

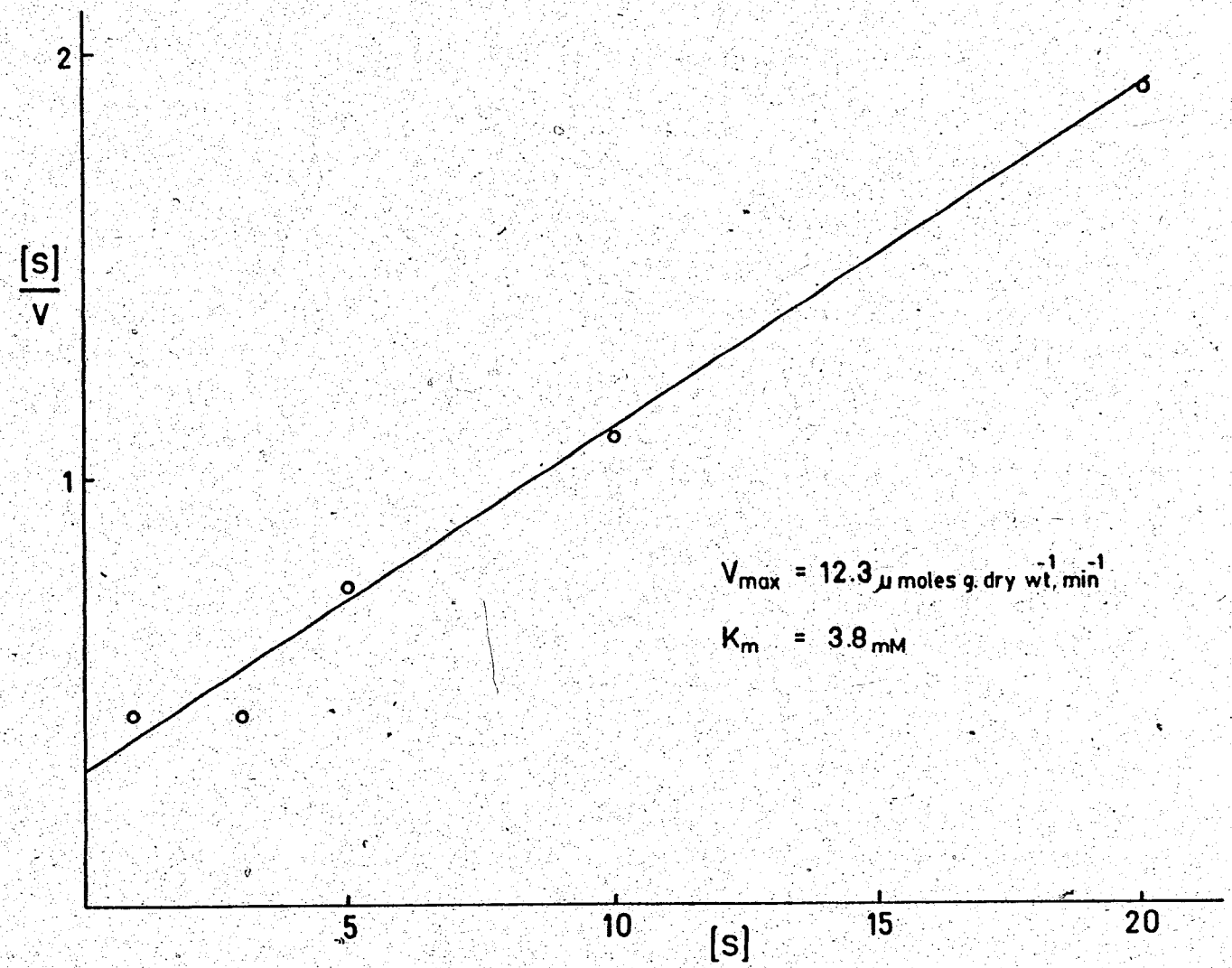

2
1
[S]
V
5
10
15
20
[S]
V_{max} = 12.3 μ moles g. dry wt^{-1} min^{-1}
K_m = 3.8 mM

FIGURE 12

A Woolf plot of the Na^+ independent portion of L- leucine uptake from glycyl-L-leucine into rings of everted rat small intestine (r=Ø.973). Ordinate: substrate concentration (millimolar) over the velocity of uptake (μmoles per gram dry weight of tissue per minute). Abscissa: substrate concentration (millimolar).

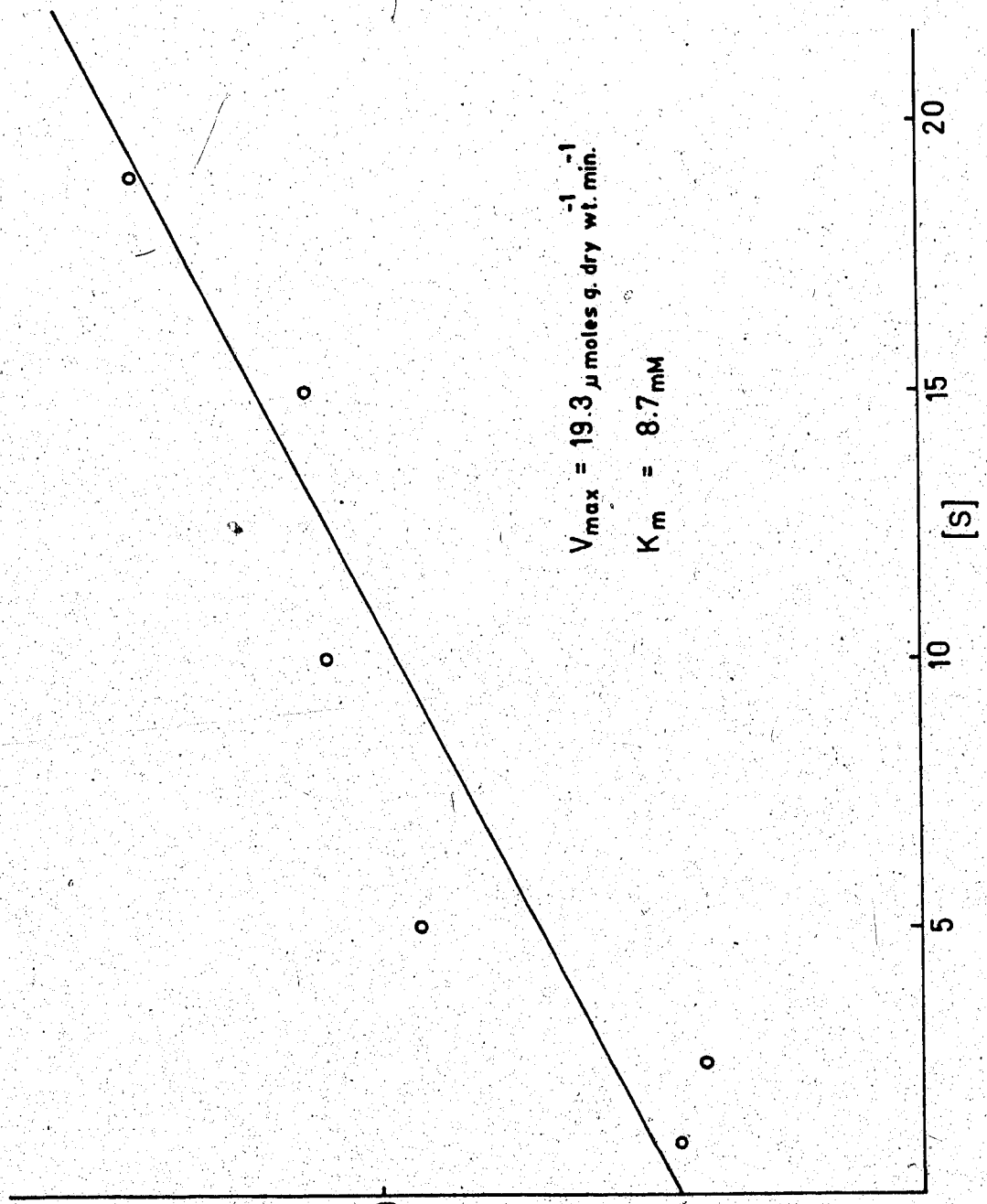

$\frac{[S]}{V}$
1.0
5
10
15
20
[S]
V_{max} = 19.3 μ moles g. dry wt.$^{-1}$ min.$^{-1}$
K_m = 8.7 mM

FIGURE 13

The uptake of labelled L-leucine (expressed as moles per gram dry weight of tissue per minute) from 20 mM cold free L-leucine (Bar A), 25 mM cold free L-leucine (Bar B) and 5 mM cold glycyl- L-leucine (Bar C). Everted rings were incubated for one minute in each experiment. Each bar of the histogram represents the mean of at least eight determinations in different animals S.E.

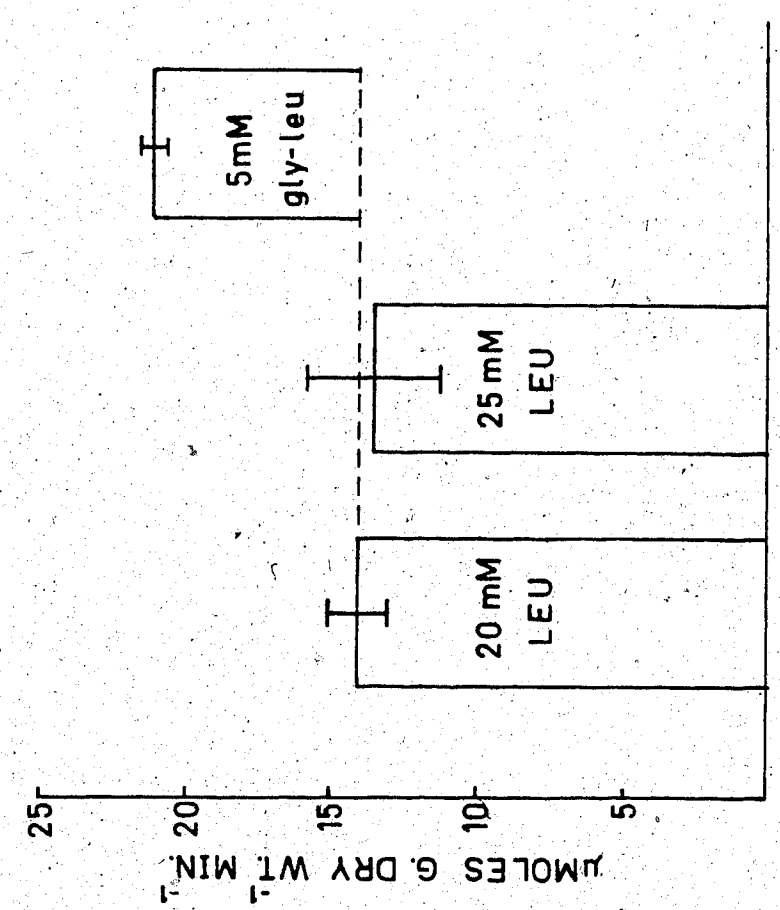
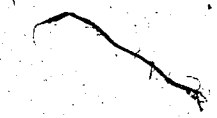

5mM
gly-leu
25 mM
LEU
20 mM
LEU
25
20
15
10
5
μMOLES G. DRY WT. MIN.-1

FIGURE 14

The rate of uptake of L-leucine from glycyl-L- leucine (expressed as μmoles per gram dry weight of tissue per minute) into everted rings of rat small intestine, in the presence of 10 mM L-Ala- naphthylamide (ANA). The tissue was incubated for different lengths of time with glycyl-[^{3}H]-L- leucine in Krebs bicarbonate ringer containing 1 mM cold glycyl-L-leucine, Na^+ and 10 mM ANA (closed circles). Ordinate: rate of uptake in μmoles per gram dry weight of tissue per minute. Abscissa: incubation time in minutes. Values are presented as means of at least four determinations from different animals $\pm$S.E. Also included in the figure, for comparative purposes, is the graph constructed from the determination of the uptake of L-leucine from glycyl-L-leucine in the absence of Na^+ (open circles and broken line) orginally Figure 9.



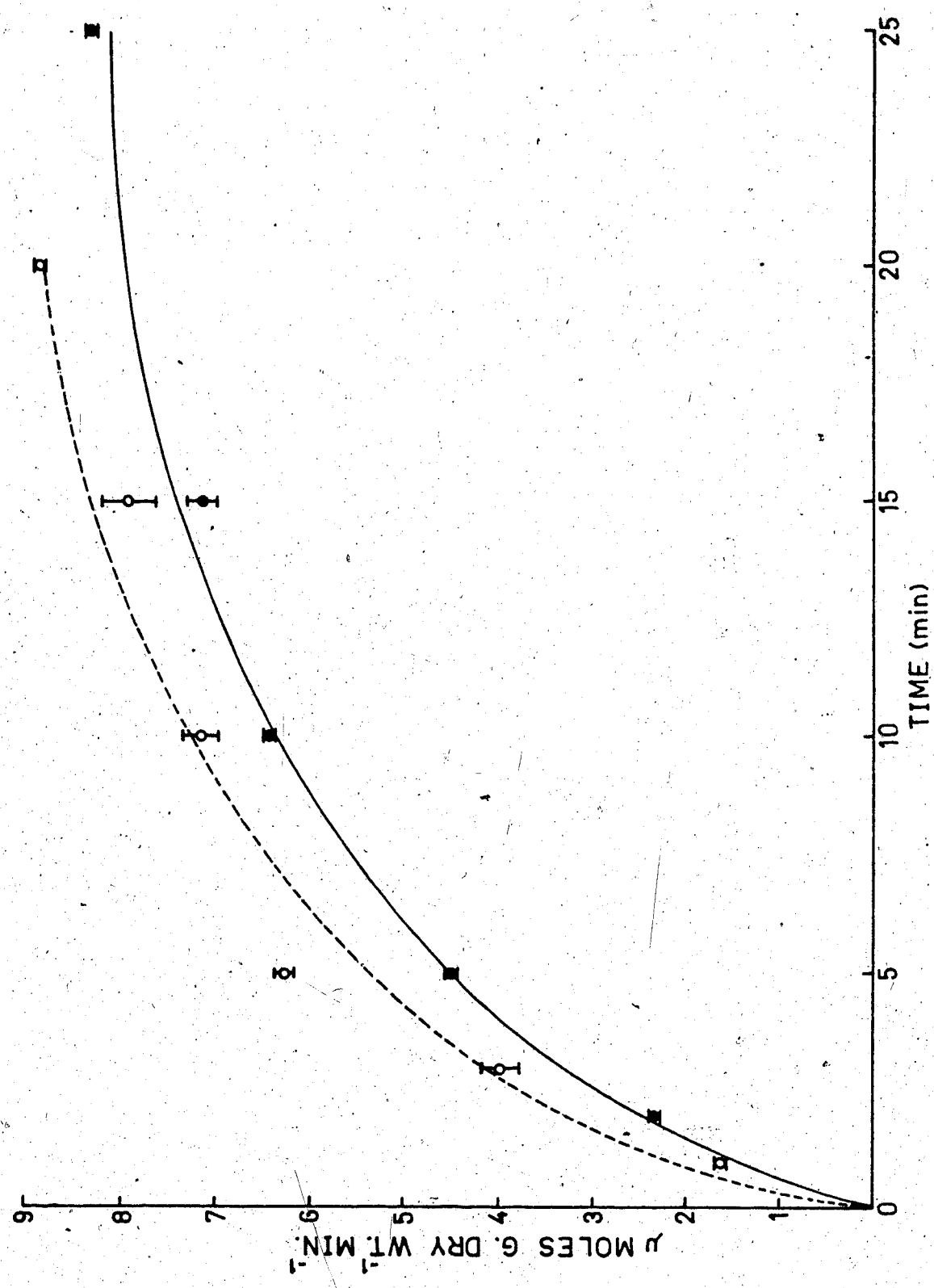

μ MOLES G. DRY WT. MIN.-1
TIME (min)
0
1
2
3
4
5
6
7
8
9
5
10
15
20
25

FIGURE 15

The uptake of L-leucine from 1 mM free L-leucine (closed circles) and from 1 mM glycyl-L-leucine (open circles) in the presence of increasing concentrations of harmaline. Everted rings of rat small intestine were incubated for one minute in each determination. Values are presented as means of at least eight determinations from different animals ± S.E.

Also represented is the uptake of L-leucine from free L-leucine under the same conditions described above but in the absence of Na^+ (open triangles). Values are presented as the means of at least ten determinations from different animals ± S.E.

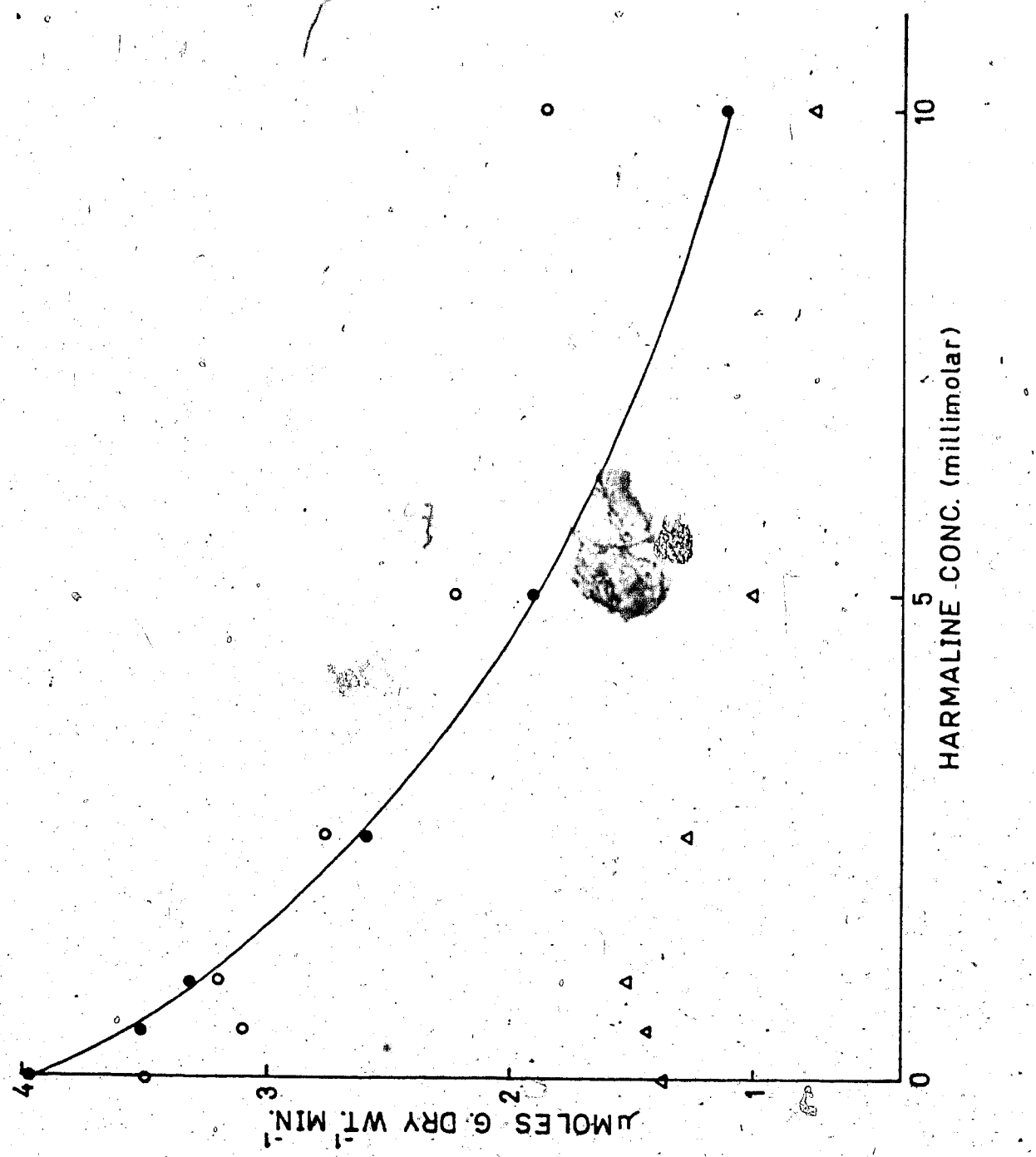

μMOLES G. DRY WT. MIN.⁻¹
0
1
2
3
4
5
10
HARMALINE CONC. (millimolar)

FIGURE 16

The uptake of L-leucine from free L-leucine (expressed as μmoles per gram dry weight of tissue per minute) into rings of everted rat small intestine over a range of substrate concentrations and in the presence of 5 mM harmaline. The tissue was incubated for one minute with [^{3}H]-L-leucine in Krebs bicarbonate ringer containing different concentrations of cold L-leucine, 5 mM harmaline and Na^+ (open circles and broken line) and in choline ringers containing different concentrations of cold L-leucine, 5 mM harmaline and no Na^+ (closed circles). Also included is a control curve for comparative purposes, for the uptake of L-leucine from free L-leucine over the same range of substrate concentrations (open circles and solid line). Ordinate: L-leucine uptake expressed as μmoles per gram dry weight of tissue per minute. Abcissa: L-leucine concentrations (millimolar). Values are presented as means of at least eight determinations from different animals $\pm$ S.E.

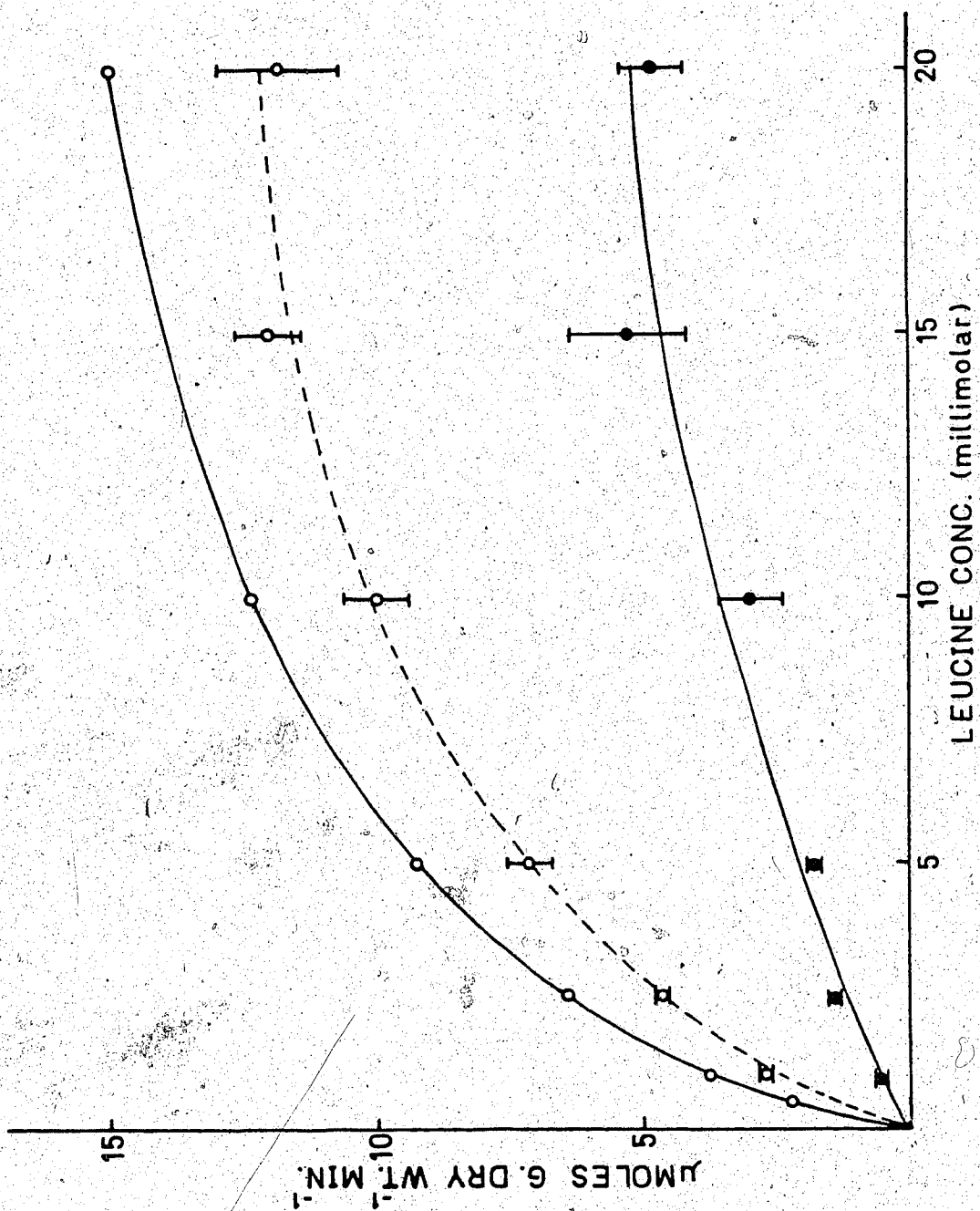
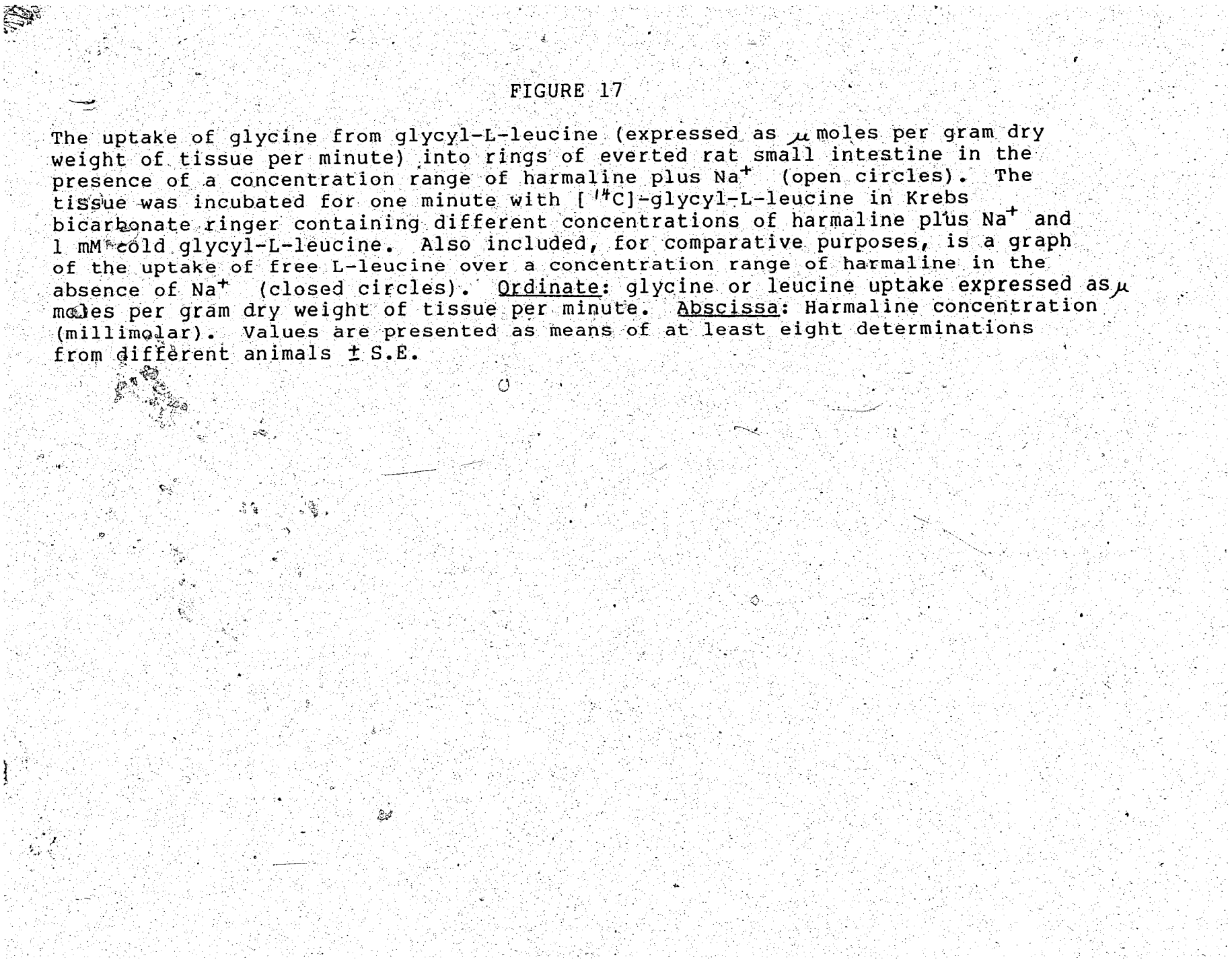

μMOLES G. DRY WT. MIN.-1
15
10
5
5
10
15
20
LEUCINE CONC. (millimolar)

FIGURE 17



The uptake of glycine from glycyl-L-leucine (expressed as μ moles per gram dry weight of tissue per minute) into rings of everted rat small intestine in the presence of a concentration range of harmaline plus Na^+ (open circles). The tissue was incubated for one minute with [^{14}C]-glycyl-L-leucine in Krebs bicarbonate ringer containing different concentrations of harmaline plus Na^+ and 1 mM cold glycyl-L-leucine. Also included, for comparative purposes, is a graph of the uptake of free L-leucine over a concentration range of harmaline in the absence of Na^+ (closed circles). Ordinate: glycine or leucine uptake expressed as μ moles per gram dry weight of tissue per minute. Abscissa: Harmaline concentration (millimolar). Values are presented as means of at least eight determinations from different animals $\pm$ S.E.

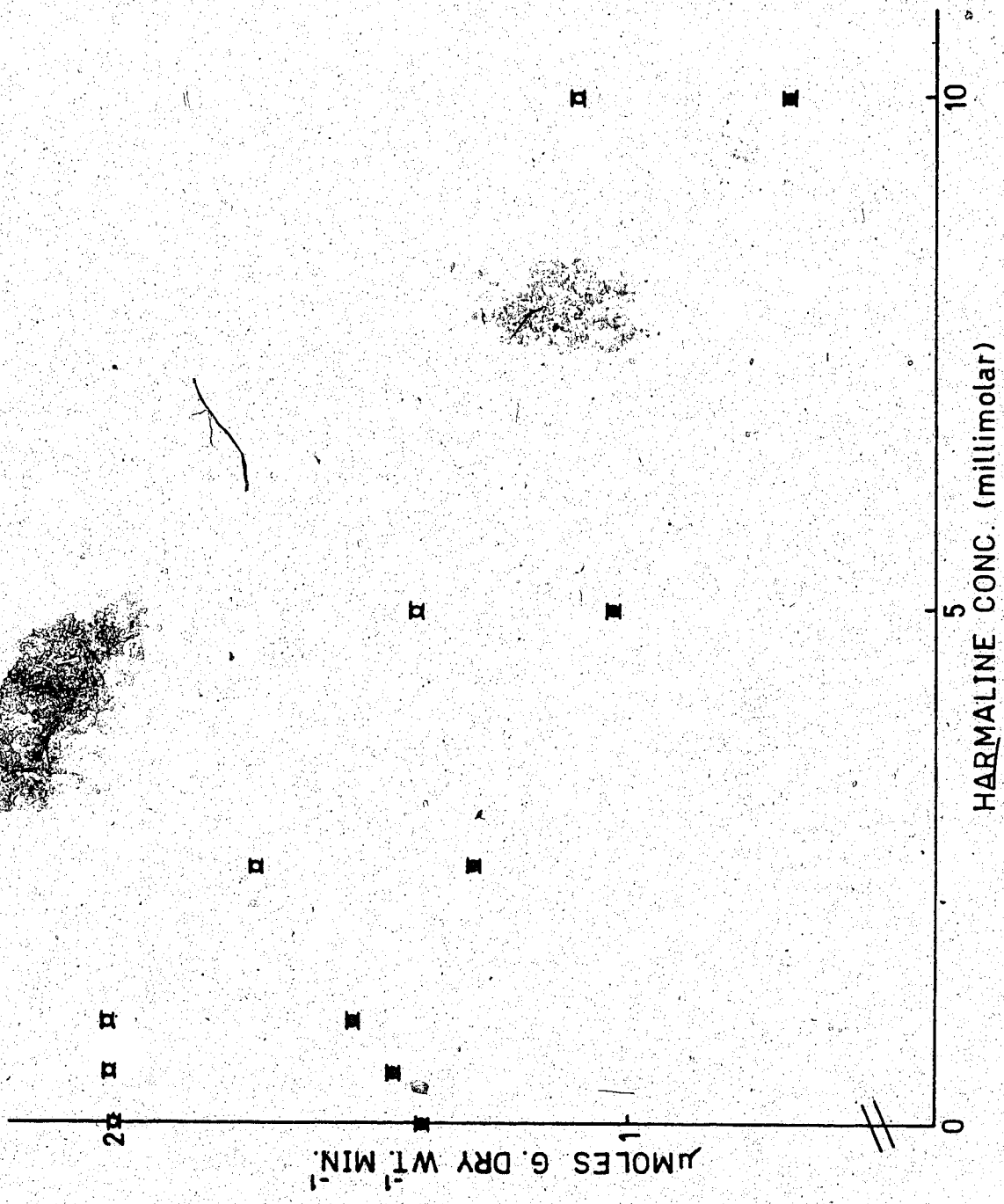
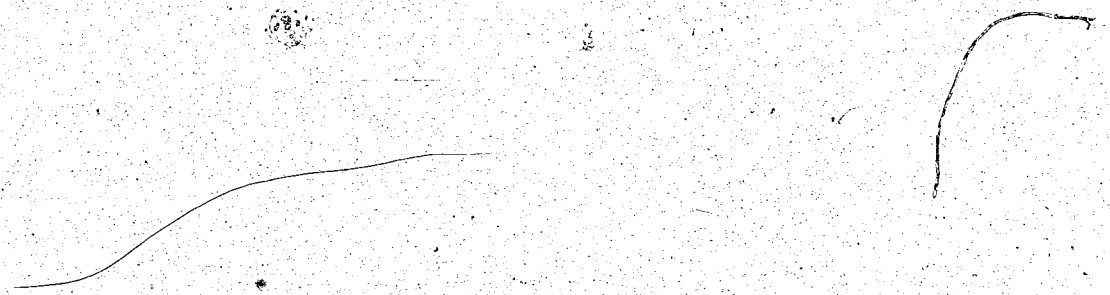

μMOLES G. DRY WT. MIN.⁻¹
2
1
0
5
10
HARMALINE CONC. (millimolar)

FIGURE 18

The uptake of L-leucine from glycyl-L-leucine (expressed as μ moles per gram dry weight of tissue per minute) into rings of everted rat small intestine in the presence of a range of concentrations of glycyl-L-proline, excess free L-leucine and Na^+. The tissue was incubated for one minute with glycyl-[^{3}H]-L-leucine in Krebs bicarbonate ringers containing different concentrations of cold glycyl-L-proline, 20 mM cold free L-leucine, Na^+ and 1 mM cold glycyl-L-leucine. Ordinate: L-leucine uptake expressed as μ moles per gram dry weight of tissue per minute. Abscissa: Glycyl-L-proline concentration (millimolar). Values are presented as means of at least eight determinations from different animals ± S.E.



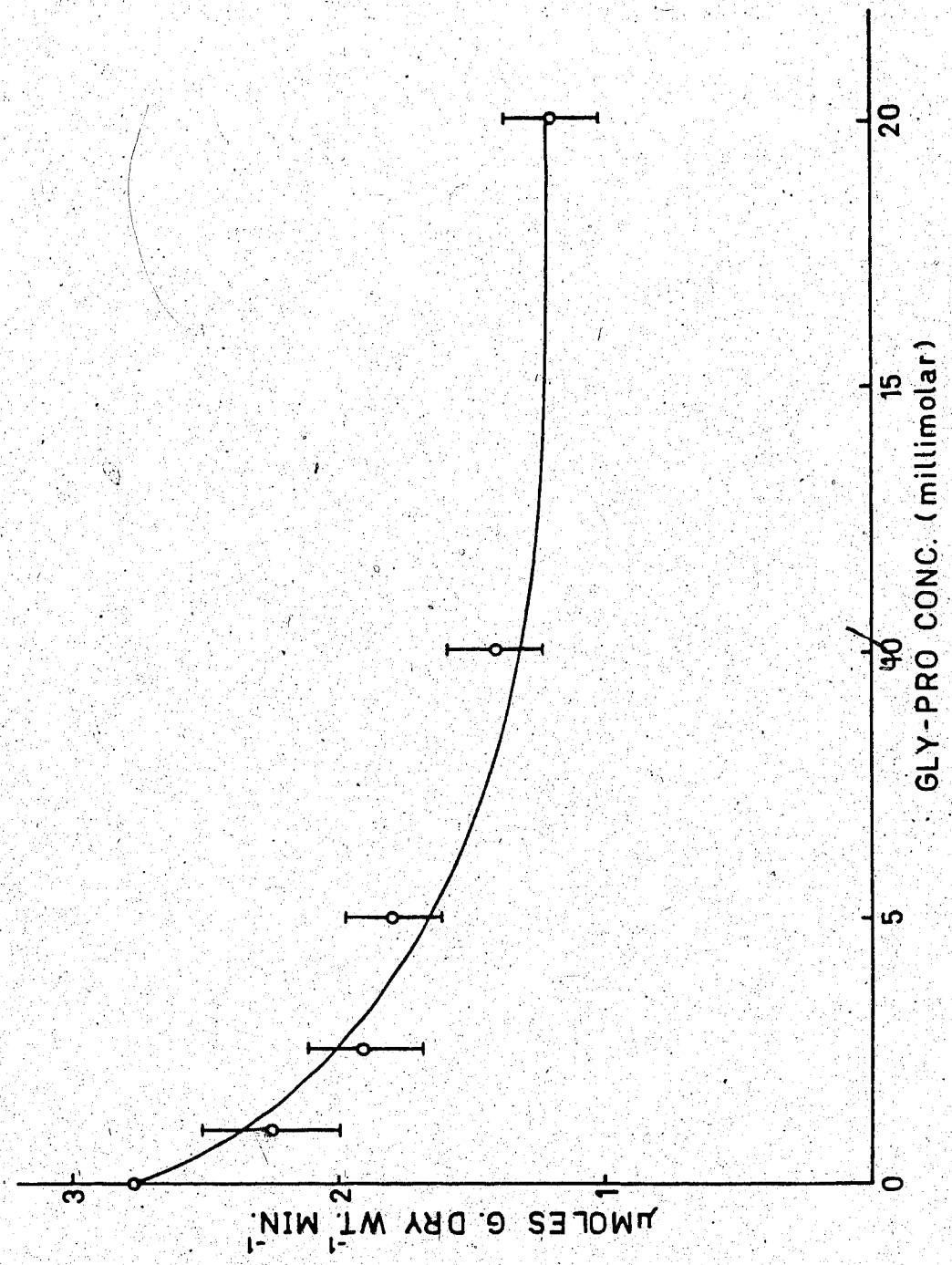

μMOLES G. DRY WT. MIN.$^{-1}$
3
2
1
0
5
10
15
20
GLY-PRO CONC. (millimolar)

FIGURE 19

An Inui and Christensen plot of the data presented in Figure 18 (r=Ø.988). Jc and Ji represent the uptake of L-leucine from glycyl-L-leucine (expressed as μ moles per gram dry weight of tissue per minute) in the presence and absence of glycyl-L-proline respectively while [I] represents the concentration of glycyl-L-proline.

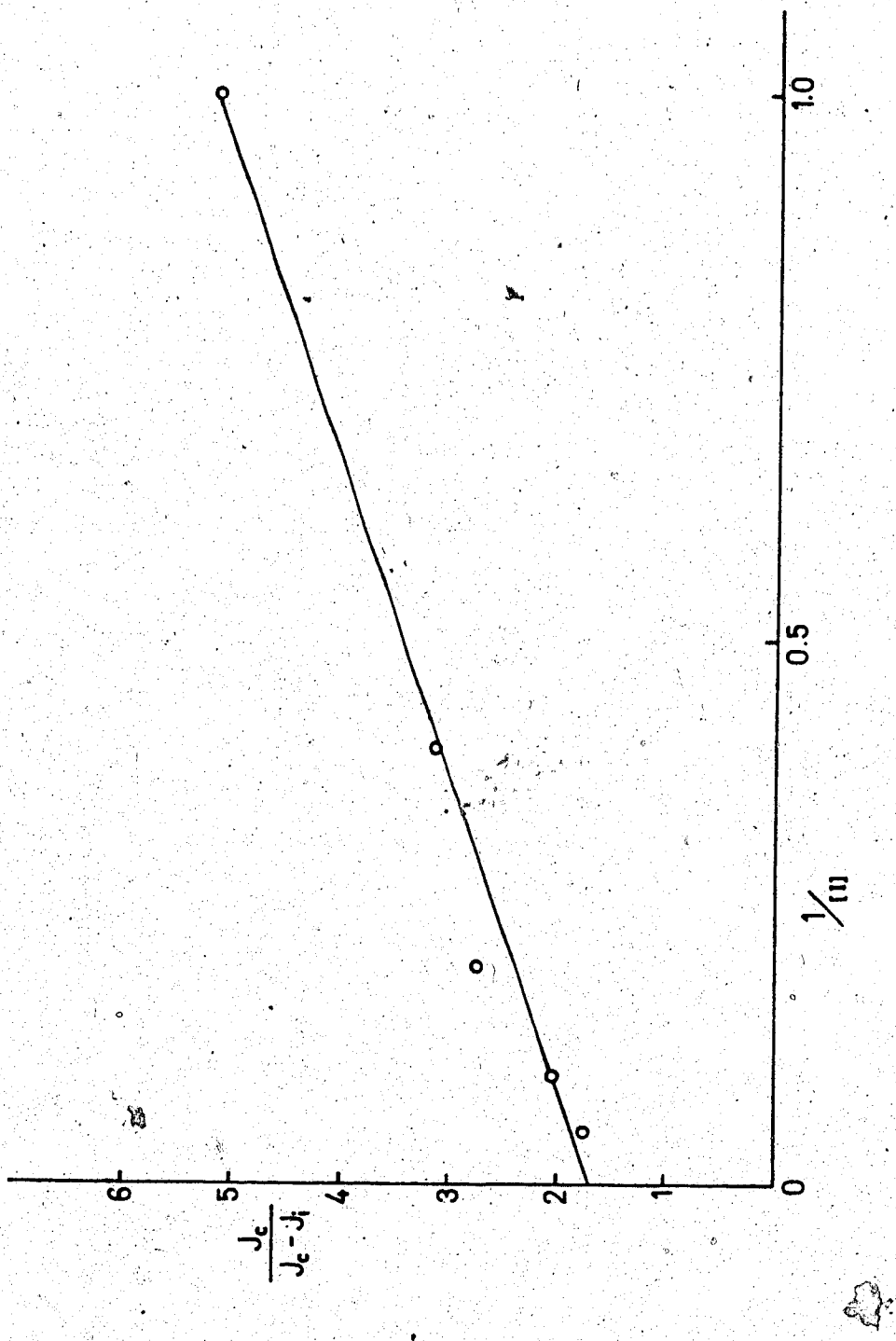

$\frac{J_c}{J_c - J_i}$
6
5
4
3
2
1
0
0.5
1.0
$1/[I]$

FIGURE 20

A plot of 1/V versus [I] where V represents the uptake of L-leucine from glycyl-L-leucine (expressed as μ moles per gram dry weight of tissue per minute) sensitive to inhibition by glycyl-L-proline and [I] indicates the concentration of glycyl-L-proline (millimolar).

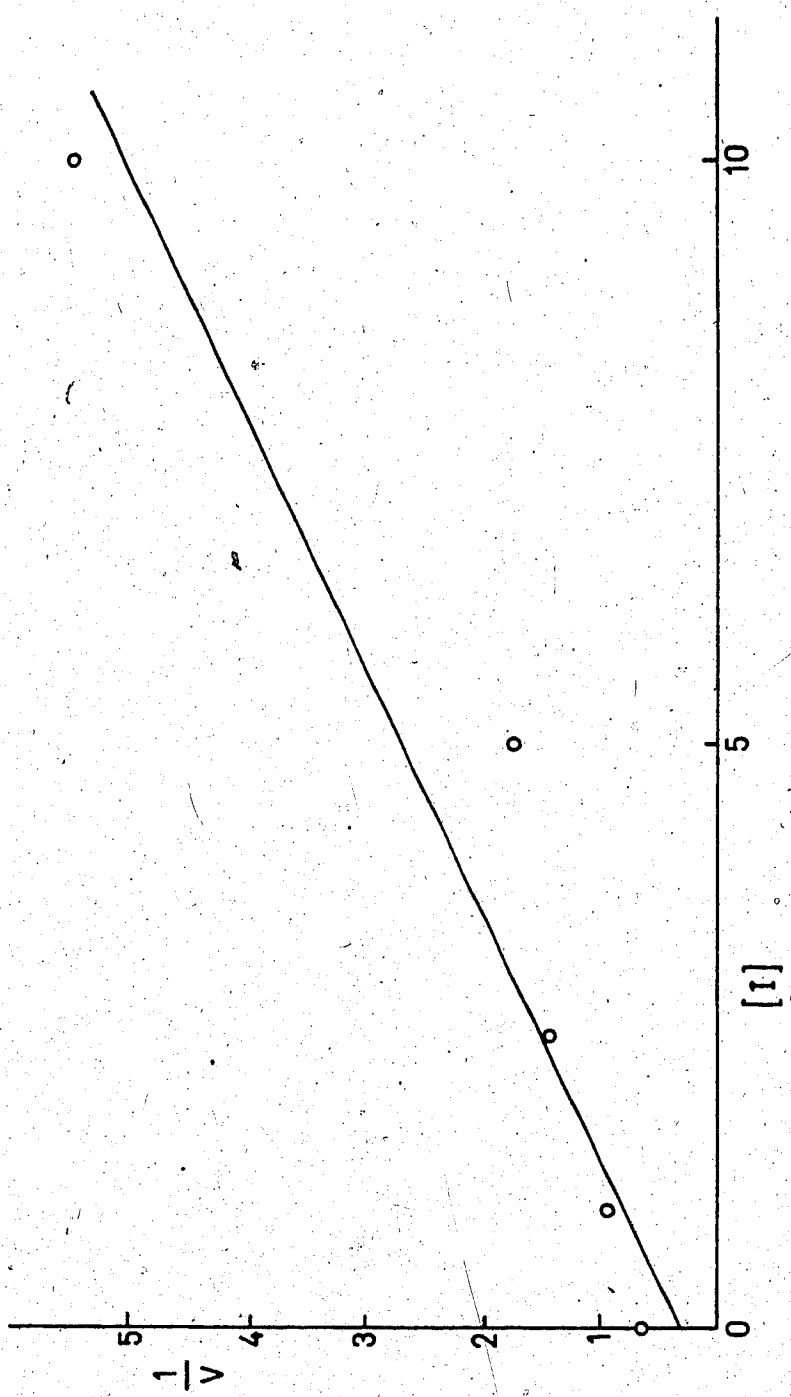
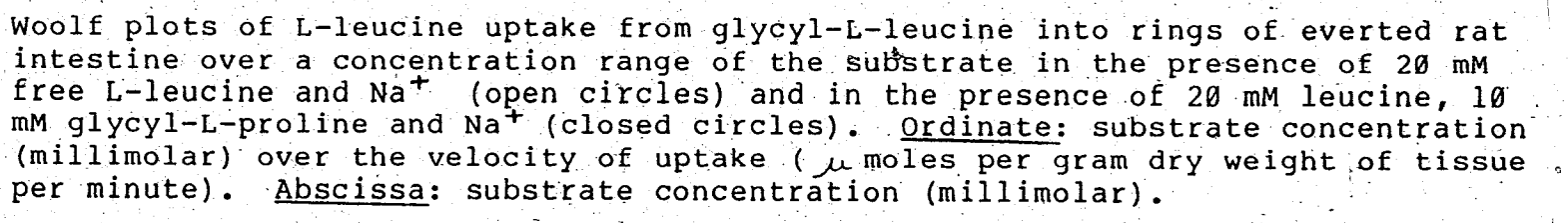

1/v
0
1
2
3
4
5
0
5
10
[I]

FIGURE 21



Woolf plots of L-leucine uptake from glycyl-L-leucine into rings of everted rat intestine over a concentration range of the substrate in the presence of 20 mM free L-leucine and Na^+ (open circles) and in the presence of 20 mM leucine, 10 mM glycyl-L-proline and Na^+ (closed circles). Ordinate: substrate concentration (millimolar) over the velocity of uptake (μ moles per gram dry weight of tissue per minute). Abscissa: substrate concentration (millimolar).

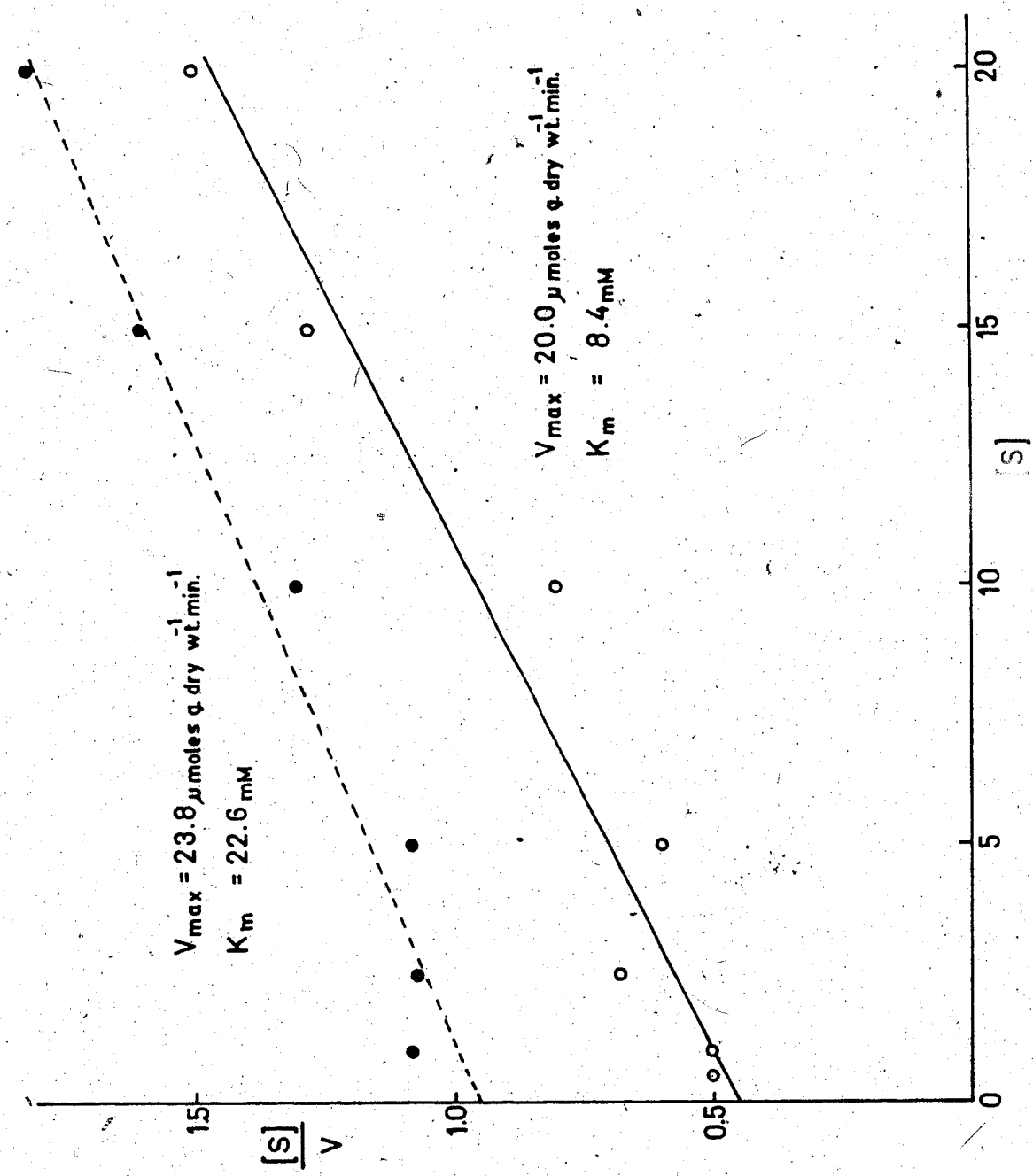

V_{max} = 23.8 μ moles q. dry wt.$^{-1}$ min.$^{-1}$
K_m = 22.6 mM
V_{max} = 20.0 μ moles q. dry wt.$^{-1}$ min.$^{-1}$
K_m = 8.4 mM
[S]/V
[S]
0
5
10
15
20
0.5
1.0
1.5

FIGURE 22

The uptake of glycine from glycyl-L-leucine (expressed as μ moles per gram dry weight of tissue per minute) into rings of everted rat small intestine in the presence of glycyl-L-proline and a range of concentrations of L-leucyl-L-leucine plus Na^+ . The tissue was incubated for one minute with [^{14}C]-glycyl-L-leucine in Krebs bicarbonate ringers containing 10 mM cold glycyl-L-proline, Na^+ , 1 mM cold glycyl-L-leucine and different concentrations of cold L-leucyl-L-leucine. Ordinate: glycine uptake expressed as μ moles per gram dry weight of tissue per minute. Abscissa: L-leucyl-L-leucine concentration (millimilar). Values are presented as means of at least eight determinations from different animals $\pm$S.E.

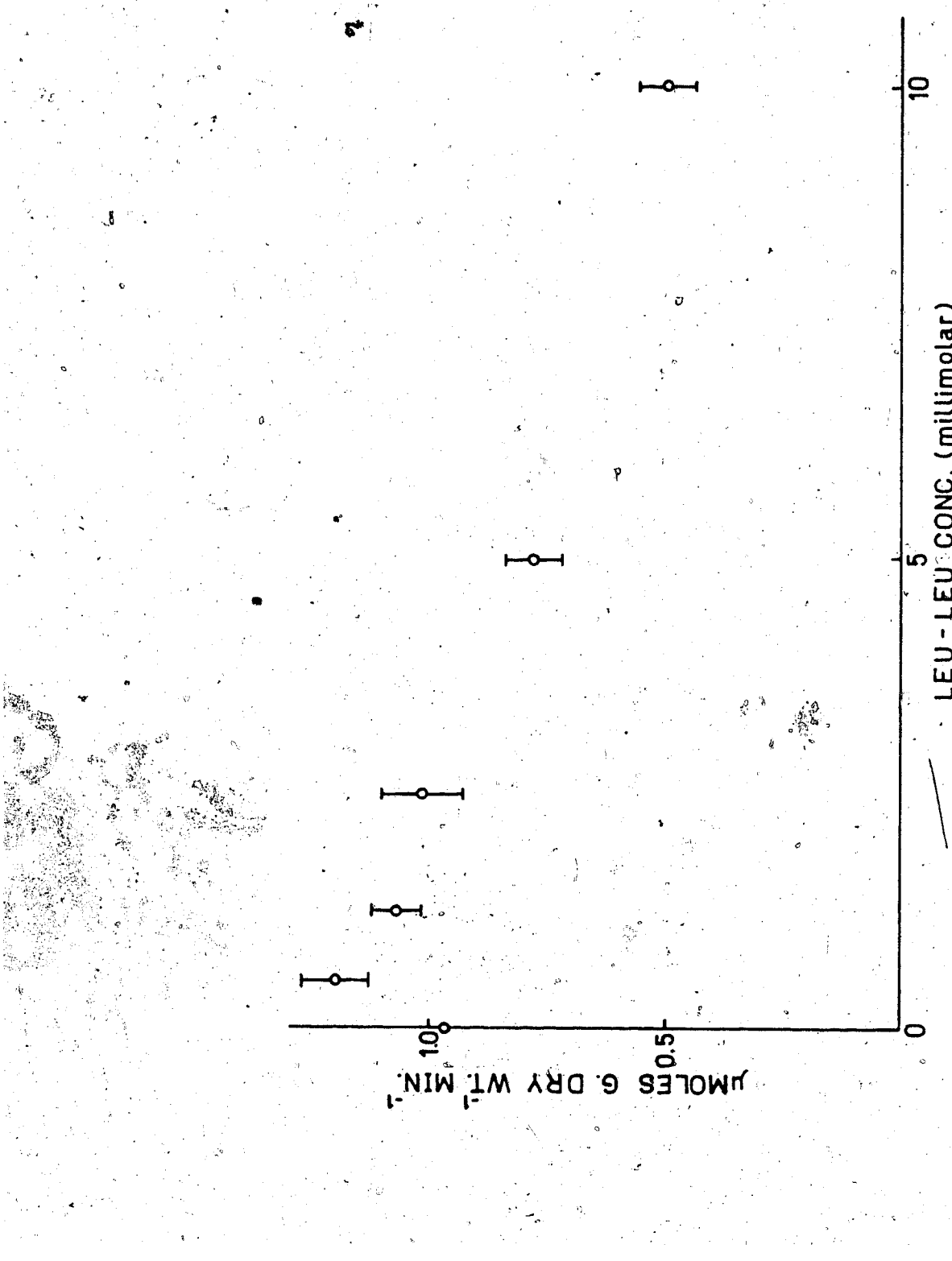
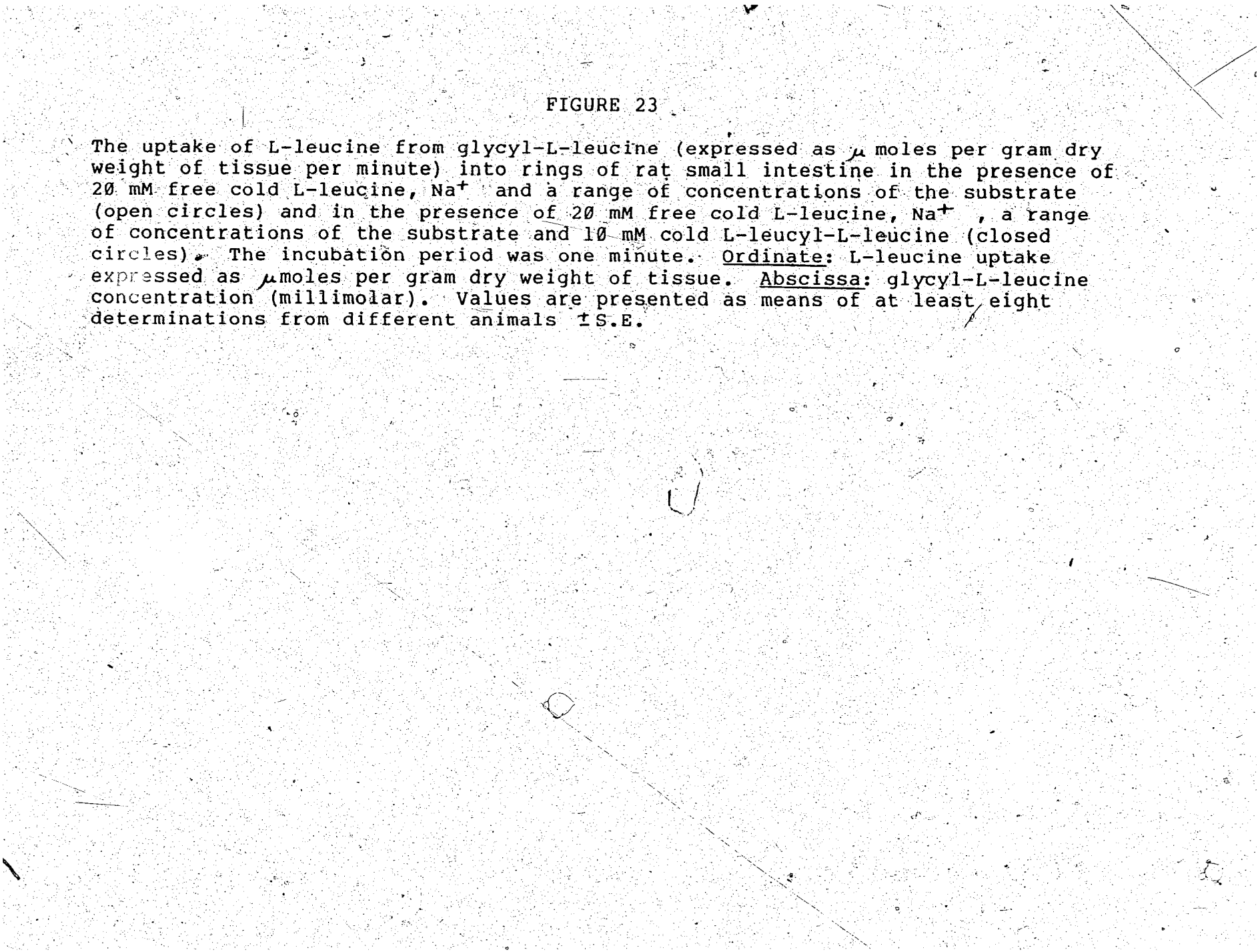

μMOLES G. DRY WT. MIN.⁻¹
1.0
0.5
0
5
10
LEU - LEU CONC. (millimolar)

FIGURE 23



The uptake of L-leucine from glycyl-L-leucine (expressed as μ moles per gram dry weight of tissue per minute) into rings of rat small intestine in the presence of 20 mM free cold L-leucine, Na^+ and a range of concentrations of the substrate (open circles) and in the presence of 20 mM free cold L-leucine, Na^+, a range of concentrations of the substrate and 10 mM cold L-leucyl-L-leucine (closed circles). The incubation period was one minute. Ordinate: L-leucine uptake expressed as μmoles per gram dry weight of tissue. Abscissa: glycyl-L-leucine concentration (millimolar). Values are presented as means of at least eight determinations from different animals $\pm$S.E.

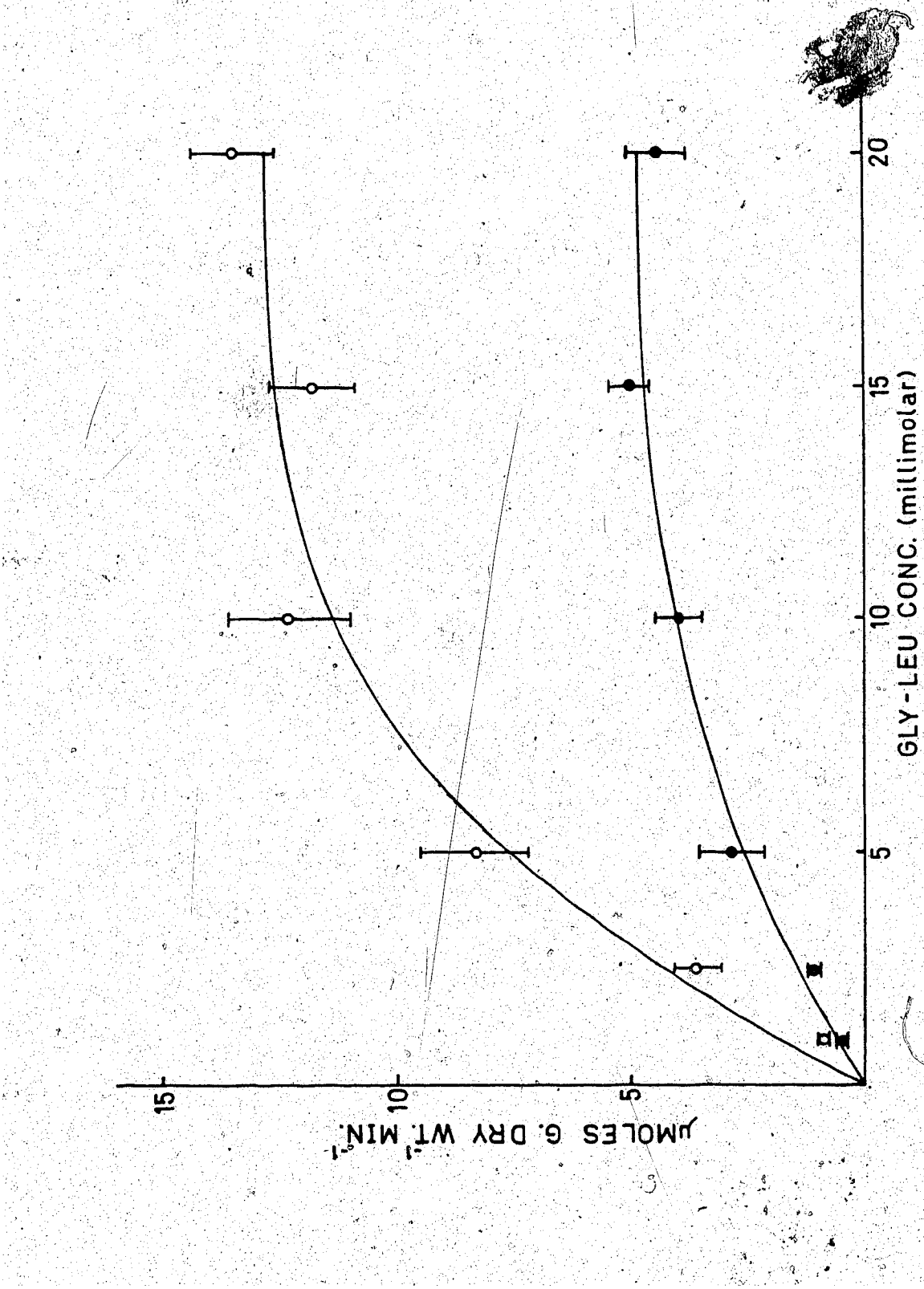
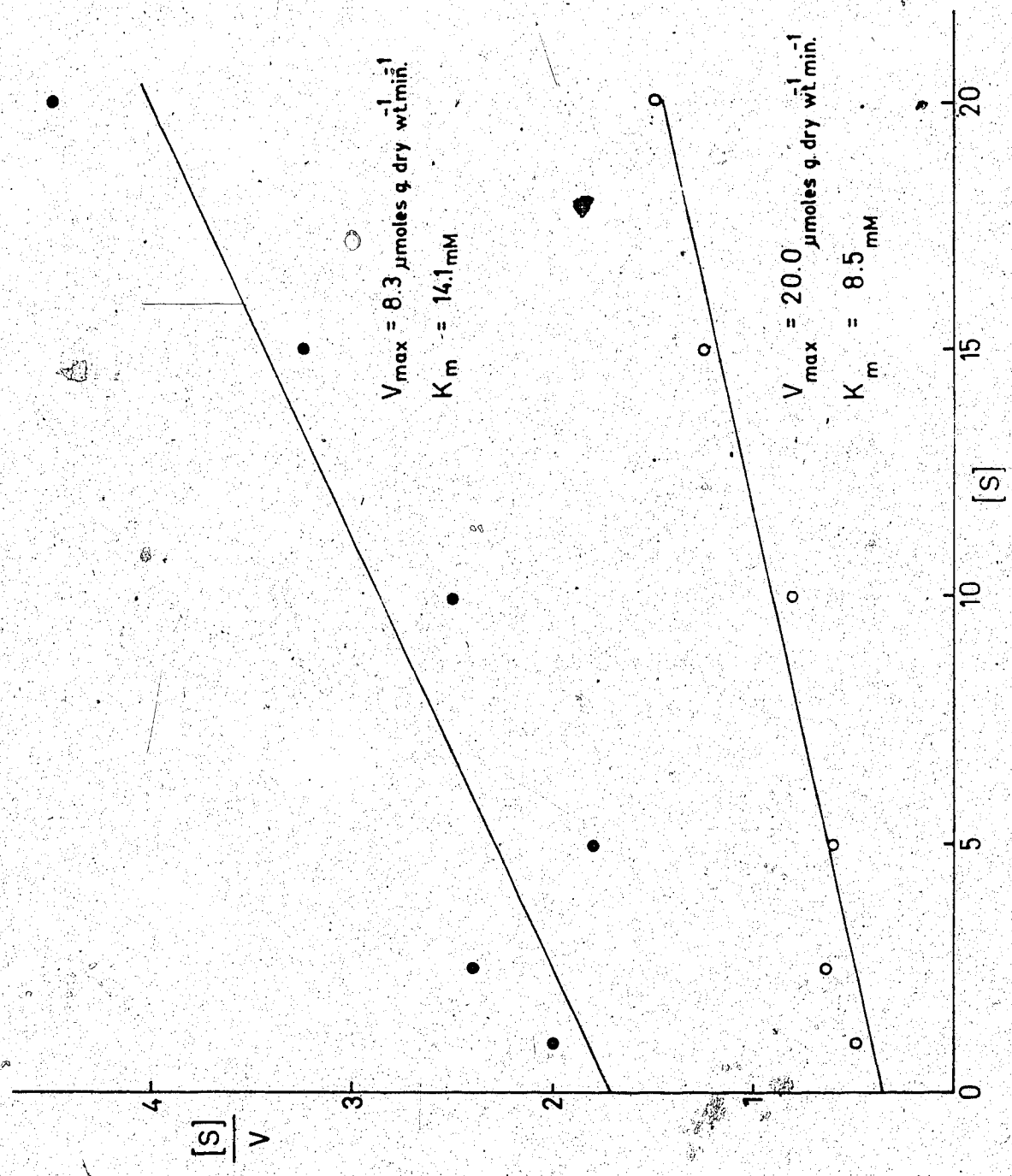

μMOLES G. DRY WT. MIN.⁻¹
15
10
5
5
10
15
20
GLY-LEU CONC. (millimolar)

FIGURE 24

Woolf plots of L-leucine uptake from glycyl-L-leucine into rings of everted rat intestine over a concentration range of the substrate in the presence of 20 mM cold free L-leucine and Na^+ (open circles) and in the presence of 20 mM cold free L-leucine, 10 mM L-leucyl-L-leucine plus Na^+ (closed circles). Original data presented in Figure 23. Ordinate: substrate concentration (millimolar) over the velocity of uptake (μ moles per gram dry weight of tissue per minute). Abscissa: substrate concentration (millimoles/litre).


V_{max} = 8.3 μmoles g. dry wt^{-1} min^{-1}
K_m = 14.1 mM
V_{max} = 20.0 μmoles g. dry wt^{-1} min^{-1}
K_m = 8.5 mM
[S]/V
[S]
0
5
10
15
20
1
2
3
4

Gly-Leu Con. (mM)	0.5	1.0	2.5	5.0	10.0	15.0	20.0	N
With sodium	2.97 (0.24)	6.00 (0.44)	9.65 (1.00)	14.49 (1.29)	22.24 (1.79)	--	28.82 (1.67)	8
Sodium-free	2.18 (0.15)	2.20 (0.23)	3.83 (0.30)	5.38 (0.46)	9.12 (0.77)	13.20 (1.11)	13.73 (1.09)	14
+20 mM Leucine with sodium	1.00 (0.10)	1.97 (0.13)	3.66 (0.41)	8.33 (0.95)	12.33 (1.15)	11.75 (0.85)	13.45 (0.81)	8
+20 mM Leucine without sodium	1.18 (0.19)	2.03 (0.30)	4.11 (0.53)	6.59 (0.97)	11.98 (2.20)	--	12.23 (1.51)	8
+10 mM ANA	--	1.41 (0.09)	3.07 (0.16)	5.06 (0.29)	9.04 (0.77)	9.96 (1.14)	12.34 (1.34)	8
+5 mM Harmaline with sodium	1.13 (0.09)	2.36 (0.18)	5.37 (0.21)	7.88 (0.50)	13.74 (0.77)	--	16.13 (1.97)	8

TABLE I

UPTAKE OF LEUCINE FROM GLYCYL-L-LEUCINE

Everted rings were incubated for one minute under various conditions as indicated. Values are the means of N determinations. Values in parentheses are the standard errors of the means. Mannitol was used as an osmotic control.

Gly-Leu Conc. (mM)	1.0	2.5	5.0	10.0	15.0	20.0	N
With sodium	2.21 (0.09)	4.46 (0.25)	6.52 (0.53)	8.85 (0.85)	--	12.04 (1.55)	12
Sodium-free	1.8 (0.15)	3.73 (0.27)	5.73 (0.57)	8.36 (0.86)	--	10.72 (1.15)	12
+20 mM Leucine with sodium	2.39 (0.07)	4.70 (0.22)	7.02 (0.25)	10.25 (1.06)	13.47 (1.51)	14.77 (0.85)	8
+5 mM Harmaline with sodium	1.75 (0.11)	2.86 (0.16)	4.22 (0.22)	5.76 (0.27)	8.17 (0.74)	8.83 (0.75)	12

TABLE II

UPTAKE OF GLYCINE FROM GLYCYL-L-LEUCINE

Everted rings were incubated for one minute under the various conditions as indicated. Values are the means of N determinations and the values in parentheses are the standard errors of the means. Mannitol was used as an osmotic control.

								N
Gly-Pro Conc. (mM)	0.0 2.75 (0.34)	1.0 2.23 (0.26)	2.5 1.91 (0.16)	5.0 1.78 (0.17)	10.0 1.39 (0.16)	15.0 --	20.0 1.18 (0.16)	4
Pro Conc. (mM)	--	1.0 1.25 (0.30)	2.5 1.33 (0.18)	5.0 1.62 (0.16)	10.0 1.39 (0.08)	15.0 1.47 (0.27)	20.0 1.61 (0.22)	4
Gly-Leu Conc. (mM) +20 mM Leu and 10 mM Gly-Pro, with sodium		1.0 0.93 (0.18)	2.5 2.34 (0.20)	5.0 4.63 (0.74)	10.0 7.71 (1.37)	15.0 9.39 (0.58)	20.0 10.94 (3.27)	8
+10 mM ANA and 10 mM Gly-Pro, with sodium		0.85 (0.08)	3.28 (1.09)	4.18 (0.90)	7.17 (2.28)	9.12 (1.61)	--	8

TABLE III

UPTAKE OF LEUCINE FROM GLYCYL-L-LEUCINE

Everted rings were incubated in solutions containing 1 mM glycyl-L-leucine and different concentrations of glycyl-L-proline or proline as indicated.

Also everted rings were incubated in solutions containing various concentrations of glycyl-L-leucine under the conditions indicated. All incubations were for one minute. Values are means of N determination. Values in parentheses are the standard error of the means. Mannitol was used as the osmotic control.

Control	GlyGly	ProGly	LeuGly	LeuLeu	LeuAla
1.Ø3	1.13	1.18	1.Ø3	Ø.64*	Ø.87
(Ø.19)	(Ø.19)	(Ø.Ø4)	(Ø.Ø5)	(Ø.Ø6)	(Ø.Ø5)

TABLE IV

EFFECT OF VARIOUS DIPEPTIDES ON THE UPTAKE OF 1 mM GLYCYL-L-LEUCINE IN THE PRESENCE OF 2Ø mM FREE LEUCINE AND 1Ø mM GLYCYL-L-PROLINE

Everted rings were incubated for one minute in the additional presence of 1Ø mM dipeptide as indicated. Values are the mean of eight determinations and the values in parentheses are the standard error of the mean. Mannitol was used as an osmotic control.

*Indicates $p<Ø.Ø5$ compared to the control.

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