PEPTIDES CAN BE UTILIZED AS AMINO ACID SOURCES FOR PROTEIN ACCRETION AND CELL PROLIFERATION BY CULTURED ANIMAL CELLS

BY

Yuanlong Pan

Dissertation submitted to the Graduate Faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Animal Science

APPROVED:

K. E. Webb, Jr., Chairman

R. M. Akers

P. K. Bender

J. H. Herbein

E. A. Wong

August, 1993
Blacksburg, VA

PEPTIDES CAN BE UTILIZED AS AMINO ACID SOURCES FOR PROTEIN ACCRETION AND CELL PROLIFERATION IN CULTURED ANIMAL CELLS

by

Yuanlong Pan

Committee Chairman: K.E. Webb, Jr.
Animal Science

(ABSTRACT)

Twenty two methionine-containing di- to octapeptides were evaluated for their ability to serve as methionine sources to support protein accretion and cell proliferation in C_2C_{12} myogenic, MAC-T mammary epithelial and ovine myogenic satellite cells. Factors in serum that may be involved in regulating peptide utilization was investigated using MAC-T cells. Growth of MAC-T cells was studied in the presence of methionine-containing dipeptides with 6% desalted adult animal serum from chickens, horses, humans, pigs or rabbits. Serumal peptidase activities on the twenty two methionine-containing peptides were examined in cell-free, methionine-free Dulbecco's modified Eagle's medium supplemented with 6% fetal bovine serum. The cell cultures were incubated for 72 h at 37°C in a humidified environment of 90% air : 10% CO_2 for C_2C_{12} and ovine satellite cells or 95% air : 5% CO_2 for MAC-T cells. The basal medium contained methionine-free Dulbecco's modified Eagle's medium supplemented with 6% desalted animal serum or one of the following serumal factors: .4% bovine serum lipids, 1% chemically defined lipid concentrate, bovine insulin (1 ug/mL), or 3% low protein serum replacement (LPSR-1). Treatment media tested included basal medium or basal media supplemented with L-methionine or one of the methionine-containing peptides. Cell cultures incubated with the basal media for 72 h were characterized by decreased cell number

and decreased protein content compared with initial cultures. All the methionine-containing peptides (with the exception of glycylmethionine and prolylmethionine for C_2C_{12} cells), regardles of chain length, were able to support protein accretion with responses ranging from 29 to 123% of that of free L-methionine. The DNA contents of ovine satellite cell cultures indicated that cell proliferation occurred in the presence of all the methionine-containing peptides with responses ranging from 45 to 144% of the L-methionine response. Bovine insulin and lipids were not effective in promoting peptide utilization by MAC-T cells. However, the LPSR-1 facilitated the utilization of methionine-containing peptides in C_2C_{12} and MAC-T cells. In the cell-free, methionine-free Dulbecco's modified Eagle's medium, peptidases could release all the methionine residues from the tetra- to octapeptides during 24 h of incubation and 42 to 70% of the methionine residues from the di- and tripeptides tested. The results demonstrated that cultured animal cells possess the ability to utilize methionine-containing peptides as methionine sources for protein accretion and cell proliferation, but serumal peptidases are at least partially responsible for the observed responses.

ACKNOWLEDGEMENTS

I would like to extend my sincere appreciation to Dr. K.E. Webb, Jr. for his guidance, encouragement, inspiration, assistance and friendship throughout my graduate training and in the preparation of this manuscript.

I would also like to express my appreciation to other members of my advisory committee, Dr. R.M. Akers, Dr. P.K. Bender, Dr. J.H. Herbein, and Dr. E.A. Wong for their guidance, assistance, and friendship during my graduate study and in the preparation of this manuscript.

Sincere thanks are extended to Don Shaw, Kris Lee, Pat Boyle, and James Matthews for their friendship and assistance in laboratory analyses and in the collection of ovine skeletal muscle samples.

I greatly appreciate Ms Andrea Gay for facilitating my application for the John Lee Pratt Fellowship and the John Lee Pratt Animal Nutrition Programs for its financial support throughout my graduate education at Virginia Tech.

I also want to take this opportunity to thank my parents, Mr. Guangyan Pan and Mrs Deyin Luo, my sisters, and brothers for their support and encouragement during my graduate education at Virginia Tech.

Especially, I wish to extend my deepest appreciation to my wife, Xiaohong Zhou, for her unbounded patience, persistent and enthusiastic support, and uncountable sacrifice throughout my graduate training at Virginia Tech.

TABLE OF CONTENTS

LIST OF TABLES

Tables.

LIST OF FIGURES

Figures

Chapter I
Introduction

Utilization of peptide-bound amino acids as nitrogen sources appears to be a universal phenomenon in living species ranging from unicellular organisms to complicated higher animals and plants (Higgins and Payne, 1977; Payne, 1980; Matthews, 1991a). Koeln et al. (1993) reported that about 70% of the amino acid flux across the gastrointestinal tract of fed calves was in the form of peptides and about 90% of the peptide-bound amino acids entering the portal blood passed through the liver. On the contrary, about 83% of the free amino acids entering the portal vein were extracted by the liver. When expressed as a percentage of the total plasma amino acid pool, peptide-bound amino acids account for 52 to 78% in ruminants (DiRienzo, 1990; Seal and Parker, 1991; Koeln et al., 1993), 10% in man (Christensen et al., 1947), 9 to 51% in rat (Asatoor et al., 1978, Seal and Parker, 1991) and 11 to 14% in guinea pigs (Gardner et al., 1983). The functions of these circulating peptides are still uncertain, so is the peptide-utilizing ability of animal tissues. Muscle and mammary tissues play important roles in meat and milk production and methionine, as an essential amino acid, occupies a unique position in protein synthesis. So, it was of particular interest to investigate whether these two tissues possess the ability to utilize small methionine-containing peptides as sources of methionine for protein synthesis.

Chapter II

Literature Review

Peptide-bound Amino Acid Pool in Blood Plasma

Christensen et al. (1947) quantified the plasma peptide-bound amino N by measuring the difference in amino N from deproteinized normal human plasma before and after acid hydrolysis ("difference procedure") and found that, in normal man, the plasma contained .51 mg/dL peptide amino N (about 10% of the total plasma amino N). Asatoor et al. (1978) used the "difference procedure" to investigate the peptide-bound amino N in the blood plasma of fasting rats and found that plasma peptide amino acids accounted for 9% of the total plasma amino N. In guinea pigs 11 to 14% of the total plasma amino N was shown to be in the form of peptides by Gardner et al. (1983). Seal and Parker (1991) observed a much greater proportion of peptides in blood plasma. They found that 51.4% of the plasma amino N was present in peptides in fractions isolated from the plasma obtained by cardiac puncture of rats by reverse-phase HPLC. They observed that peptides with four to eleven amino acid residues accounted for the largest fraction, followed by dipeptides.

McCormick and Webb (1982) reported that plasma peptide-bound amino acids accounted for about 78% of the total plasma amino N in both fed and unfed calves. These results were confirmed by Danilson et al. (1986), DiRienzo (1990), Seal and Parker (1991) and Koeln et al. (1993). Sheep appear to also have a high proportion of the plasma amino N present in the form of peptides. Seal and Parker (1991) reported 77.8% of the total plasma amino N in sheep was present in the form of peptides.

At present, there is little information about the origin of these plasma peptides. There are at least three potential sources of these plasma peptides: products of endogenous protein degradation and (or) absorption of peptides of dietary origin and (or) synthesis of peptides by tissues and release into plasma. Peptidemia was shown to occur after surgery, trauma, and in certain types of shock where protein catabolism was increased (Christensen et al., 1947), These results suggest that plasma peptides may partially come from endogenous protein degradation at least under certain adverse conditions.

Peptide Absorption by the Gastrointestinal Tract

It is a well established phenomenon that intestinal absorption of small peptides (di- to tripeptides) plays an important role in the assimilation of the products of dietary protein digestion (Webb, 1986; Matthews, 1987, 1991a). These absorbed peptides are widely presumed to be completely hydrolyzed before passage into the bloodstream. Results from a number of experiments indicate that significant amounts of small peptides may escape hydrolysis and enter the circulation.

Gastrointestinal Absorption of Specific or Biologically Active Peptides. Perry et al. (1967) found that after a meal of chicken breast (containing high levels of carnosine and anserine), both carnosine and anserine were detected in human urine, indicating the absorption of intact carnosine and anserine by the gastrointestinal tract. Significant absorption into the blood of ß-aspartyl di- and tripeptides were observed in the rat (Dorer et al., 1968). Boullin et al. (1973) studied the absorption of six dipeptides into the mensenteric blood of rats and reported that all the peptides (carnosine, glycylglycine, glycylproline, prolylglycine, glycyl-D-phenylalanine, and glycylphenylalanine) did appear in the mensenteric blood and that the extent of appearance

of the peptides in the blood was inversely related to their rates of hydrolysis by mucosal homogenates. After oral administration into rats, several mucosal hydrolysis-resistant dipeptides containing one or two D-amino acids were detected in the urine and the excretion was the most extensive for the peptide that was most resistant to mucosal hydrolysis (Asatoor et al., 1973). Hagen and Jones (1987) administered glutathione to rats via stomach tube and detected an increase in blood plasma glutathione.

By using everted sacs of rat small intestine and rectum, it was clearly demonstrated that tyrosyl-D-alanlylglycine crossed the intestinal wall and appeared on the serosal side intact (Heading et al., 1979). Hagen and Jones (1987) reported that radiolabelled-glutathione was transported intact from the lumen of perfused jejunum into the portal vein by a Na^+-dependent, carrier-mediated process.

Lipophilicity (Hydrophobicity) and size seem to be the major factors that affect the absorption of many molecules. So a relatively small, lipophilic (hydrophobic) and hydrolysis-resistant peptide is more likely to be absorbed faster than a larger, hydrophilic and digestible one (Gardner and Wood, 1989). A lipid-soluble, fungal toxin, cyclochloritine, was shown to be absorbed by simple diffusion (Matthews, 1991a). Gardner and Wood (1989) summarized several studies and reported that several biologically active peptides including vasopressin, SMS 201-995 (an octapeptide), Luliberin (a decapeptide), Thrroliberin (a tripeptide) and insulin have been showed to be absorbed by the gastrointestinal tract intact into the circulation.

Gastrointestinal Absorption of Peptide-bound Amino N. Gardner et al. (1983) infused a partial enzymic digest of casein into the duodenum of guinea pigs over 30 min. They

collected blood samples from the mesenteric vein before and after the infusion. Their results showed that after infusion, the peptide-bound amino N was increased from 46 to 440 umol/liter for the whole blood and from 137 to 377 umol/liter for the plasma. After infusion, the peptide-bound amino N was 10.8% of the whole blood and 5.3% of the plasma amino N, respectively. For the blood samples taken from the mesenteric vein before infusion, 6.9 and 2.4% of the amino N was in the form of peptide-bound amino N for plasma and whole blood, respectively. The results indicate that in the guinea-pig, the gastrointestinal tract released both free and peptide-bound amino acids into the mesenteric vein, and 10 to 15% of the whole blood amino N existed as small peptides.

In ruminants, Koeln et al. (1993) reported that in fed calves, 71% of the flux of amino acids across the gastrointestinal tract was accounted for by peptide amino acids and most (93%) of the peptide-bound amino acids in the portal blood passed through the liver, entering the hepatic vein. On the contrary, most (83%) of the free amino acids in the portal vein were removed by the liver. This significant flux of peptide-bound amino acids across the gastrointestinal tract may account, at least partially, for the high concentrations of blood peptide amino acids observed firstly by McCormick and Webb (1982) and confirmed by DiRienzo (1990), Seal and Parker (1991), and Koeln et al. (1993).

Peptide Absorption Sites of the Gastrointestinal Tract

Stomach. The main end products of gastric digestion are very large polypeptides (molecular weights of several thousands) and the production of oligopeptides and free amino acids under normal physiological conditions is minimal. It seems, therefore, that it is very improbable

that there is appreciable absorption of protein or its digestion products from the stomach (Matthews, 1991a). This concept may be true for monogastric animals, but not for ruminants. In fact, after the administration of proteins into the rumen, large concentrations of free amino acids and peptides were observed in the rumen fluid (Annison, 1956). Results from several studies indicated that the nature of the dietary proteins affect the production of peptides in the rumen (Chen et al., 1987a; Broderick et al., 1988). Russell et al. (1983) reported that in vitro incubation of rumen bacteria with casein produced large amounts of small peptides. Chen et al. (1987b) reported that ruminal bacteria preferentially utilized peptides containing hydrophilic amino acids. Therefore, due to the unique multiple compartments of the ruminant stomach and the dynamic action of ruminal microorganisms and microbial digestion, the end products of the digestion of dietary protein include abundant free and small peptide-bound amino acids that are potentially available for stomach absorption.

DiRienzo (1990) studied the flux of free and peptide-bound amino acid across the intestine and stomach in sheep and calves. In sheep, the fluxes of amino acids and peptides across the intestine were 36.47 and 52.01 g/d, respectively. Sheep stomach had very limited ability to absorb free amino acids (5.2 g/d), but a large flux (308.4 g/d) of peptide amino acids was observed across the sheep stomach area. Similar results were obtained in calves. The flux of peptide-bound amino acids across the stomach accounted for 77% of total amino acid flux across the portal-drained viscera (stomach and small intestine) in both sheep and calves. This study was the first to characterize this unique role of the ruminant stomach in the assimilation of dietary protein.

The ability of ovine ruminal and omasal epithelial tissues to absorb L-methionine, methionylglycine and carnosine was investigated in vitro (Matthews, 1991b; Matthews and Webb, 1993). The results indicated that the mucosal tissues of the rumen and omasum had the ability to absorb L-methionine and the dipeptides. The omasal epithelium had a much greater ability than ruminal epithelium to absorb L-methionine and the dipeptides when the absorption rates were expressed on an equal tissue weight basis. These in vitro studies demonstrate that ruminant stomach does possess the ability to absorb both amino acids and dipeptides.

Small Intestine. Agar et al. (1953) found that, in rats, small amounts of glycylglycine and trace amounts of leucylglycine were transported from the mucosal to the serosal side of the intestinal wall. Wiggans and Johnston (1959) reported that, during the absorption of di- to tetraglycine by the rat small intestine in vitro, some glycylglycine was detected in the serosal side. Newey and Smyth (1959) reported the appearance of intact glycylglycine and DL-alanyl-DL-alanine in the serosal fluid of the sacs of everted rat small intestine.

Lis et al. (1972) reported that, in rats, the site of maximal absorption of a mixture of small peptides was in the proximal third of the small intestine, while that of the corresponding amino acid mixture was in the middle third. Rubino and Guandalini (1977) observed that the influx of glycylphenylalanine and glycylproline was maximal in the jejunum, and that of glycine and phenylalanine in the ileum.

Schedl et al. (1979) studied the kinetics of glycylsarcosine, leucine, and glycine into rings of everted hamster small intestine and found that, at all concentrations, glycylsarcosine was absorbed more rapidly in the jejunum than in the ileum, and the amino acids were

absorbed more rapidly in the ileum than in the jejunum. Based on available experimental results, Matthews (1991a) concluded that the site of maximal absorption for peptides is more proximal than that for amino acids, and the bulk of the protein digested is absorbed in the proximal and mid small intestine.

Large Intestine. For a long time, it was a widely-accepted assumption that the colon is unable to absorb the products of protein digestion, and a number of early studies appeared to support this assumption (Matthews, 1991a). However, Evered and Nunn (1968) reported that valine was concentrated by rat colonic mucosa and that amino acids could cross the colonic wall. Batt and Schachter (1969) found that, in newborn rats, colonic tissue could concentrate proline. Mediated transport of amino acids by colonic mucosa was also observed in hens (Lind et al. 1980), and in rats (Ardawi, 1986).

Few experiments have been conducted to study the absorption of small peptides by large intestine. Heading et al. (1978) reported that tyrosylglycine and tyrosyl-D-alanine which are resistant to hydrolysis could cross the wall of the rectum in vitro. Liversidge (1989) reported that the transport of peptide drugs across the rectum wall is simple, passive diffusion with no active transport system involved.

In birds, protein digestion continues to occur in the cecum (Neshelm and Carpenter, 1967), this should result in the accumulation of the products of protein digestion (free amino acids and peptides) in the lumen. Calonge et al. (1990) studied the possibility of peptide transport in chicken proximal cecum and rectum using isolated epithelial cells from these two large intestinal segments as experimental models. Their results indicated that the cecal and rectal epithelial cells of the chicken could concentrate

glycylsarcosine by a process which is partially inhibited by either the absence of Na^+ or in the presence of amiloride. An inward proton-gradient stimulated the uptake of glycylsarcosine by both cell types, showing that chicken proximal cecum and rectum have an active transport system that is similar to that in the small intestine of the chicken and other animals.

Mechanisms of Peptide Absorption by the Gastrointestinal Tract

Stomach. In monogastric animals, because the gastric digestion mainly produces very large polypeptides, it is unlikely that any significant absorption of either free amino acids or peptides occurs in the gastric region (Matthews, 1991a). Ovine ruminal and omasal epithelial tissues have been shown to absorb L-methionine, carnosine, and L-methionylglycine by non-mediated diffusion (Matthews, 1991b; Matthews and Webb, 1993).

Small Intestine. The first direct evidence of active transport of peptides by the small intestine was obtained by Addison et al. (1972). In their experiment, a hydrolysis-resistant dipeptide, glycylsarcosine, was used to show that hamster small intestine could concentrate glycylsarcosine against a concentration gradient in vitro. Later they showed that the transport of the hydrolysis-resistant glycylsarcosine and glycylsarcosylsarcosine was saturable in the small intestine (Addison et al., 1975a,b). They also reported that the tetrapeptide, glycylsarcosylsarcosylsarcosine, was very poorly taken up, not concentrated, and not inhibited by other peptides. Adibi and Morse (1977) studied the absorption of di- to hexaglycine by human jejunum and found uptake of di- and triglycine but no uptake of tetraglycine or higher peptides.

Smithson and Gray (1977) found that rat small intestine did not uptake glycylleucylglycylglycine.

A number of experiments have accumulated to support the observation that peptides can be absorbed more rapidly than the corresponding free amino acids (Craft et al., 1968; Matthews et al., 1968; Matthews, 1975). This suggests that, in the small intestine, a separate transport system exists for the uptake of peptides. Sigrist-Nelson (1975) used membrane vesicles to show that glycylleucine was transported intact across rat brush border membrane by a mediated mechanism inhibited by other peptides but not amino acids. This provides direct evidence that the transport systems for peptides are distinct from those for free amino acids in the brush border membrane of the small intestine.

Much evidence suggests that multiple peptide transport systems exist in the small intestine. Himukai and Hoshi (1980) and Himukai et al. (1982) showed that, in the guinea pig ileum, glycylglycine and glycylglycylglycine did not inhibit the uptake of glycylleucine although the K_t values of glycylglycine and glycylleucine were similar and thus concluded that glycylglycine and glycylleucine were transported by two different systems. In rat small intestine glycylleucine was shown to be transported by two systems, one system was shared by glycylproline and prolylglycine (Cheeseman and Johnston, 1982). Similar evidence was also reported by Matthews and Burston (1983, 1984). Despite evidence to support the hypothesis that the brush border membrane contains more than one transport system, it is still premature to classify the transport systems (Matthews, 1991a).

The role of Na^+ in peptide absorption has been a controversial issue, because only partial inhibition of peptide transport by Na^+ depletion was reported in a number of experiments (Addison et al., 1975b; Himukai et al., 1978

Cheeseman, 1980; Ganapathy and Radhakrishnan, 1980). On the other hand, the stimulating effect of dipeptides on the transport of Na^+ was observed in human intestine (Hellier et al., 1973; Silk et al., 1975) and in guinea pig small intestine (Himukai et al., 1983). So these two categories of studies indicate that a Na^+ gradient played a certain role in peptide absorption and its role is different from that in amino acid absorption in the small intestine. Ganapathy and Leibach (1983, 1985) proposed a proton-gradient hypothesis of intestinal transport of peptides. In this model the Na^+/H^+ exchanger in the brush border membrane of small intestine secretes H^+ into the lumen in exchange for an inward flux of Na^+ down the Na^+ concentration gradient across the brush border membrane, generating an inward proton gradient across the brush border membrane. The resulting inward proton gradient stimulates peptide-proton cotransport. The inward Na^+ gradient is maintained by the Na^+/K^+-ATPase located in the basolateral membrane. This model can explain the partial dependence of peptide uptake on the Na^+ gradient that is responsible for the creation of an inward proton gradient via Na^+/H^+ exchanger. This model has been supported by a number of in vitro experiments using brush border membrane vesicles (BBMV). Ganapathy and Leibach (1983, 1984) reported that the transport of glycylsarcosine, L-carnosine, and glycyl-L-proline was stimulated by a proton gradient across the BBMV of rabbit small intestine and was inhibited by other dipeptides. Takuwa et al. (1985) employed the membrane vesicle technique and found that the uptake of glycylglycine by rabbit intestinal brush border membrane was an active and proton dependent transport system. Shimada and Hoshi (1986) reported an active and proton-dependent peptide transport system in small intestine BBMV of Triturus.

There is convincing evidence that an inward directed proton gradient exists across the brush border membrane of

the small intestine under physiological conditions. Lucas et al. (1975, 1976) and Lucas (1983) reported that, both in vivo and in vitro, the pH in the close vicinity of the brush border membrane was between 5.5 and 6.0, while the intracellular pH of intestinal epithelial cells was 7.0 (Kurtin and Charney, 1984).

Molecular techniques have made it possible to investigate and elucidate both physiological and pathological phenomena of transport at the subcellular level. Miyamoto et al. (1991) studied the peptide transporter in rabbit small intestine at the mRNA level, using Xenopus oocyte expression cloning system. On the fourth day after injection of the poly(A)+ mRNA purified from the mucosal cells of small intestine into the oocytes, the uptake of glycylsarcosine was increased by nearly 3-fold and the uptake was significantly greater in the presence of a proton gradient. This study showed for the first time that the mucosal cells of rabbit small intestine contained the mRNA encoding an active, proton-dependent peptide transport system.

The effect of molecular structure on the uptake of peptides by the small intestine has been extensively studied and this topic has been reviewed by Matthews and Payne (1980). Briefly, blocking either the amino terminal group or carboxy terminal group reduces or abolishes affinity for transport, peptides with ß-amino groups have lower affinity than peptides with α-amino groups, peptides with L-amino acid residues have much higher affinity than corresponding peptides with D-amino acid residues, peptides with lipophilic side-chains increase affinity for uptake and the substrates for active uptake by small intestine are limited to di- and tripeptides.

Bertloot et al. (1981) reported that uptake of glycylphenylalanine by BBMV from mouse intestine increased

linearly with the increased dipeptide concentration in the incubation buffer and the uptake was only partially (up to 43%) inhibited by other di- or tripeptides. The uptake of glycylphenylalanine was not affected by free amino acids. So the authors concluded that the transport of glycylphenylalanine by BBMV from mouse intestine was characterized by both passive and facilitated diffusion systems. Non-mediated diffusion was also observed in monkey small intestine in vitro (Radhakrishnan, 1977), in hamster jejunum in vitro (Burston et al., 1980; Matthews and Burston, 1984), and in rabbit BBMV (Woottoon, 1986). Although there is no in vivo data available for estimating the significance contributed by the uptake of peptides through this route under physiological conditions, these in vitro studies clearly show that uptake of peptides through non-mediated diffusion is possible in the small intestine.

In general, small peptides (di- and tri-) are mainly absorbed in the small intestine by active, proton-dependent transport systems that are not shared by free amino acids and non-mediated diffusion may also be a component of the peptide absorption system in small intestine.

Large Intestine. Liversidge (1989) reported that the transport of peptide drugs across the rectum wall is simple passive diffusion with no active transport system involved. In birds, Calonge et al. (1990) reported that the cecal and rectal epithelial cells of chicken could concentrate glycylsarcosine by a process which is partially inhibited by the absence of Na^+ or the presence of amiloride. An inward proton gradient stimulated the uptake of glycylsarcosine by both cell types. So this study shows that chicken proximal cecum and rectum have an active transport system which is similar to that in the small intestine of chicken and other animals. In hindgut fermenters, such as rabbits, pigs and horses, considerable fermentation occurs in the relatively

well-developed caecum or colons (Penry and Jumars, 1987). So it will be very interesting to see whether the caecum or colon in these animals also have a peptide transport system similar to that in the cecum and rectum of chicken.

Peptide Metabolism by Individual Tissues in Animals

Evidence of Peptide Utilization by Tissues. Adibi et al. (1977) intravenously administered glycylleucine and glycylglycine into rats and blood samples were taken before and at intervals after the injection to detect the dipeptide concentrations in the blood. The results indicated that, before injection, the blood samples did not contain these two dipeptides and after injection, high concentrations of both peptides were detected in the blood plasma. The concentrations diminished rapidly and after 15 min these two dipeptides disappeared from the blood completely. Urine analysis showed that only trace amounts of both peptides were secreted into the urine. This excluded the possibility of renal excretion as a major mechanism for the disappearance of these peptides from the blood. One report (Krzysik and Adibi, 1977) indicated that plasma had no peptidase activity against these two peptides and whole blood peptidases did not hydrolyze glycylglycine, and moderately hydrolyzed glycylleucine. But liver, intestine, kidney, and muscle all had cytoplasmic peptidase activity against these two dipeptides, and peptidase activity was many time greater in kidney and small intestine than in liver and muscle. These results suggest that the uptake and utilization by tissues may be responsible for the disappearance of these peptides from the plasma.

Injection of radiolabeled nutrient with subsequent examination of the distribution of the radioactivity has been a useful approach to studying the metabolism of nutrients in vivo. Krzysik and Adibi (1979) injected

radiolabelled free glycine or glycylglycine into the venous blood of rats, collected samples of blood and tissues (brain, intestinal mucosa, kidney, liver, lung, muscle, and pancreas) at 5 and 30 min after injection for the analysis of radioactivity in amino acid pools and protein fractions. At 5 min, only the amino acid pools in pancreas, kidney, and liver had radioactivity higher than plasma. At 30 min, the greatest radioactivity appeared in the amino acid pools of pancreas and kidney and least in those of muscle and brain. The radioactivity of the amino acid pool in kidney was greater after injection of labeled glycylglycine than after labeled glycine. The labeling in muscle was less after glycylglycine injection. On the other hand, at 5 min, there was only trace labeling of proteins in plasma, muscle and brain. At 30 min, the radioactivity in the proteins of plasma, muscle, and brain was much lower than that of other tissues. This study demonstrated that animal tissues can utilize diglycine as a glycine source for protein synthesis with different utilization ability. Albers et al. (1988) reported that, after a bolus injection of alanylglutamine and glycyltyrosine in healthy men, both peptides were rapidly cleared from the plasma and the half-lifes were similar for the two dipeptides (3.8 min for alanylglutamine; 3.4 min for glycyltyrosine). Peptide clearance from the plasma was accompanied by a prompt equimolar increase in the concentrations of the corresponding free amino acids in the plasma. The corresponding amino acids in the plasma reached a plateau 60 s after the termination of the injection. This prompt appearance of constituent free amino acids in the plasma suggests that most of the hydrolysis may occur in the plasma and plasma peptidases may play a significant role in the clearance of these two peptides. Stehle et al. (1991) investigated the whole-body autoradiography in rats after intravenous bolus injection of L-alanyl-C^{14}-glutamine. Five

minutes after the bolus injection, the visceral organs (liver, kidney, intestine, and lung) and all exocrine glands (salivary glands and pancreas) were highly labelled. At 30 min after injection, The central nervous system, heart, skeletal muscle, and pancreas were highly labelled, but liver, kidney, and lung had less radioactivity than at 5 min after injection. The radioactivity in kidney, liver, lung, and intestine was even less at 180 min after injection than at 30 min after injection. But the radioactivity in skeletal muscle remained high at 180 min after injection. These findings indicate that all the tissues can utilize radiolabelled glutamine from the intravenously injected glutamine dipeptide and skeletal muscle plays an important role in utilizing glutamine from the dipeptide.

Many experiments have been conducted to study peptide utilization under conditions of constant infusion and total parenteral nutrition (TPN). Adibi and Johns (1983) intravenously infused triglycine and trileucine for 3 d into baboons fed an adequate diet, and found that infusion of triglycine resulted in a small increase in plasma glycine concentration and that a small concentration of this tripeptide appeared in plasma. During the infusion, both diglycine and triglycine were detected in the plasma, but their concentrations were much smaller than the rise in plasma glycine concentration. Urinary loss of diglycine and triglycine was very small. Infusion of trileucine did not increase plasma and urine excretion of leucine, both tileucine and dileucine were not detectable in the plasma or urine. These results showed that the tripeptides tested were utilized under conditions of constant infusion.

A mixture of dipeptides as the only amino N for TPN was tested in baboons by Steinhardt et al. (1984) and Vazquez et al. (1986). The major nutritional parameters (body weight gain, nitrogen balance, urinary creatinine, and 3-methyl-

histidine) were similar between the infusion of a peptide mixture and a corresponding amino acid mixture for both experiments. In addition, metabolic parameters such as plasma concentrations of insulin, glucose, triglycerides, and cholesterol were not different. Total urinary loss of dipeptides was only 1.3% of the infused amount. Most of the infused dipeptides were cleared rapidly and their plasma concentrations were hardly detectable. These two experiments indicate that peptide amino acids can be utilized by tissues as efficiently as corresponding free amino acids as amino N sources under long term TPN conditions.

Because of cytoplasmic peptidase activity, it is essentially impossible to detect the accumulation of peptides within any tissues after these peptides are injected or infused into the blood stream. Adibi et al. (1977) injected glycylsarcosine, which is resistant to hydrolysis, into rats. the clearance of glycylsarcosine from the plasma was slow, and intact glycylsarcosine was found in all tissues (renal cortex, intestinal mucosa, skeletal muscle, and liver) Only the renal cortex contained the dipeptide at a concentration several times higher than the plasma concentration. This experiment provided direct evidence that peptides in the plasma could be taken up intact by tissues and then hydrolyzed within the tissue into corresponding amino acids for protein synthesis.

Mechanisms of Peptide Utilization by Muscle. The possible mechanisms by which tissues utilize circulating peptides include hydrolysis in plasma, hydrolysis by peptidases in the capillary bed of the tissues, hydrolysis by membrane-bound peptidases in tissues, and intact uptake by the tissue with subsequent cytoplasmic hydrolysis (Adibi et al., 1977; Krzysik and Adibi, 1977; Adibi et al., 1986; Lochs et al., 1986; Furst et al., 1987). Skeletal muscle is the tissue with the largest protein depot and its unique

role in amino acid metabolism is obvious. Therefore, understanding the peptide utilization mechanism in this tissue has been an active research area. Adibi and Morse (1981) employed isolated rat diaphragm to study the mechanism of glycine and diglycine utilization by muscle. The results showed that glycine was taken up by the muscle against a concentration gradient, but diglycine was not. This result suggests that the diaphragm has an active transport system for free glycine, but not for diglycine.

A more thorough investigation was conducted by means of perfused hindquarters of rats and measurement of peptidase activities of sarcolemmal vesicles (Raghunath et al., 1990). Progressive clearance of glycylglycine and glycylleucine from the perfusion medium was detected over the 60 min of perfusion and the disappearance of glycylleucine was almost five times greater than that of glycylglycine. Neither of the two dipeptides appeared in the muscle. But this did not rule out the possible entry of the peptides intact with rapid cytoplasmic hydrolysis, because muscle cytoplasmic peptidases could hydrolyze these two peptides (Krzysik and Adibi, 1977). To overcome the hydrolysis problem, two hydrolysis-resistant peptides, glycylsarcosine and glycylproline, which have been shown to be the substrates of both renal and intestinal peptide transport systems, were used in the perfusion study. The results indicated that the clearance of glycylsarcosine from the perfusion medium was very slow but the clearance of glycylproline from the perfusion medium was even faster than that of glycylglycine. Again no accumulation of either peptide was detected in the muscle. This study showed that peptidases of cytoplasmic origin in the perfusion medium could hydrolyze both glycylleucine and glycylproline and that sarcolemmal membrane-bound peptidases could cleave only glycylglycine and glycylleucine. Hundal and Rennie (1988) reported that

skeletal muscle contained extracellular membrane-bound aminopeptidase activity against alanylglutamine and did not possess peptide transporter activity. These experiments suggest that, unlike intestine and kidney, skeletal muscle appears to lack an active transport system for peptide uptake. Hydrolysis by membrane-bound, capillary bed-associated, and maybe secreted cytoplasmic peptidases and subsequent absorption of the released amino acids are the major mechanisms for the peptide uptake in this tissue. Non-mediated diffusion may be a minor approach of peptide uptake in muscle.

Uptake and metabolism of L-carnosine by rat tissues including diaphragm was studied with an in vitro incubation procedure (Nutzenadel and Scriver, 1976). The results showed that the uptake of carnosine by diaphragm was saturable and Na^+-dependent. Since L-carnosine has several physiological functions in muscle (Rodwell, 1990), it is not surprising that muscle has specific transport for the uptake of this peptide.

Mechanisms of Peptide Utilization by the Gastrointestinal Tract. Although the absorption of peptides, especially in the small intestine, has been studied extensively, there are fewer reports on the possible mechanisms of utilization of circulating peptides. Adibi et al. (1977) injected a hydrolysis-resistant dipeptide, glycylsarcosine, into the rat and found that this dipeptide appeared in renal cortex, liver, small intestinal mucosa, and muscle. Only renal cortex concentrated this dipeptide. On the other hand, Addison et al. (1972) reported that small intestinal mucosa could actively transport the dipeptide, glycylsarcosine, from the intestinal lumen to serosal fluid. These two experiments suggest that the small intestinal mucosa handles circulating and luminal glycylsarcosine by completely different mechanisms. In totally enterectomized

rats, the plasma half-life of glycylglycine was the same as that in control rats (Adibi and Krzysik, 1977). This suggests that the ability of clearing circulating peptides by animals does not depend on one single tissue.

Mechanisms of Peptide Utilization by Liver. Amino acids and peptides of dietary origin are transported by the portal vein into the liver before they enter the arterial circulation, so the magnitude of peptide utilization by the liver has an important effect on the amount of the peptides in the arterial blood. Koeln et al. (1993) reported that, in fed calves, 71% of amino N flux across the gastrointestinal tract was in the form of peptides and most (93%) of the peptide-bound amino acids in the portal blood left the liver. To the contrary, most (83%) of the free amino acids in the portal vein were removed by the liver. This significant flux of peptide-bound amino acids across the liver suggests that this organ prefers free amino acids to peptide-bound amino acids for its metabolic needs.

Compared with free amino acids, peptide-bound amino acids have several advantages (Adibi, 1987a). First, more amino N can be stored per unit volume of body fluid in peptides than in free amino acids. Second, some amino acids (tyrosine) are poorly soluble, but peptides containing these amino acids are water soluble. Finally, some amino acids (glutamine and cysteine) are unstable in free forms, but the corresponding peptides are very stable. Therefore, it is nutritionally beneficial for the animals to absorb more peptide-bound amino acids than free amino acids (especially for those amino acids that are poorly soluble and unstable). But once the peptide-bound amino acids and also free amino acids are absorbed into the blood, it does make sense that tissues utilize the poorly soluble and unstable free amino acids first. Some evidence has shown that peptides may be the means of transport of some amino acids between specific

organs or tissues. For example, erythrocytes need a continuous supply of glutamate to maintain the intracellular concentration of glutathione, but human erythrocytes are impermeable to glutamate. To get the needed glutamate, human erythrocytes take up a-gltutamyl-peptides through saturable transport systems and then hydrolyze these intact peptides within the cytosol to produce glutamate needed for the synthesis of glutathione (King and Kuchel, 1985).

Lochs et al. (1986) investigated the mechanism of dipeptide clearance from the perfusion medium in rat livers and found that hydrolysis-resistant dipeptides added to the perfusion medium did not disappear from the medium after incubation, while hydrolysis-susceptible peptides were not detectable in the tissue. A significant correlation was found between the clearance rates of hydrolysis-susceptible dipeptides from the perfusion medium and plasma membrane-bound peptidase activity. These results suggest that rat livers lack active transport systems for dipeptides but hydrolysis of peptides by plasma membrane-bound peptidases may be the major mechanism of peptide utilization by liver. But this experiment does not completely rule out the existence of such transport systems, because hydrolysis-susceptible peptides may be taken up intact then hydrolyzed within the cells. To ascertain this possibility, Lombardo et al. (1988) studied the uptake of dipeptides or their constituent amino acid (alanine) by rat liver plasma membrane vesicles. An active transport system for alanine was observed in the plasma membrane vesicles, but no uptake of either glycylalanine or glycylproline by the plasma membrane vesicles was found in the presence of a Na^+ gradient or a H^+ gradient. In the same experiment, the effects of N- and C-terminal amino acid residues on the rate of peptide clearance from the medium was studied in perfused rat livers. Glycylleucine, phenylalanylleucine, and

arginylleucine had similar disappearance rates, and alanylleucine had a greater rate of disappearance than these three peptides. Leucylalanine had a greater rate of disappearance than leucylglycine. A significant relationship was found between the disappearance rates of peptides and the plasma membrane-bound peptidase activity. It is also noteworthy that a high concentration of leucine in the medium was shown to inhibit the membrane-bound peptidase hydrolysis by 50%. This suggests that the ability of liver to assimilate peptides from the blood by means of membrane-bound hydrolysis may be subject to regulation by other nutrients (such as leucine) present in the blood. Although the perfusion medium also contained peptidase activity against all the peptides tested, there was no significant correlation between the disappearance rates of peptides and the peptidase activity of the perfusion medium. These results clearly show that rat liver plasma membrane has the ability to actively take up free amino acids but not peptides. This suggests that hydrolysis of peptides by plasma membrane-bound peptidases and subsequent absorption of released free amino acids by mediated transport systems are the major mechanism of peptide utilization by liver. Fürst et al. (1987) indicated that the hydrolysis of peptides by peptidases of the hepatic capillary bed also played some role in peptide utilization by liver.

Another interesting phenomenon is that both perfused liver and muscle can release cytoplasmic peptidases into the perfusion medium during the perfusion presumably independent of any damage to plasma membrane. If this is a physiological function of liver and muscle, it will be interesting to find out the destination and function of these secreted peptidases of cytoplasmic origin in vivo.

Mechanisms of Peptide Utilization by Kidney. A significant portion of circulating amino N has been shown to

exist in small peptides in normal man (Christensen et al., 1947), in rats (Asatoor et al., 1978; Seal and Parker, 1991), in guinea pigs (Gardner et al., 1983), in calves (McCormick and Webb, 1982; Danilson et al., 1986; Seal and Parker, 1991; Koeln et al., 1993) and in sheep (Seal and Parker, 1991) The kidneys are mainly involved in excreting most of the end-products of metabolism and controlling the concentrations of most of the constituents in the body fluids (Guyton, 1986). The permeability of the glomerular membrane is 100 to 500 times as great as that of the ordinary capillary, and substances with a molecular weight of 5,000 Da or less can readily pass through the glomerular membrane and enter the Bowman's capsule lumen (Guyton, 1986). This means that all the circulating peptide-bound amino acids enter the Bowman's capsule. But the urinary loss of peptides appears to be very small (Adibi and Johns, 1983, Steinhardt et al., 1984). This suggests that the kidneys have the ability to reabsorb most of the peptides in the glomerular filtrate during the formation of urine.

Results from an in vitro study indicated that rat kidney cortex slices took up L-carnosine intact against a concentration gradient, the uptake mechanism was saturable and partially (50%) inhibited by the absence of Na^+ (Nutzenadel and Scriver, 1976). This suggests that the kidneys have active transport systems for the uptake of carnosine. Adibi et al. (1977) injected glycylsarcosine into rat blood and found this peptide appeared in all the tissues studied, but the concentration of this dipeptide in kidney cortex was several times higher than that of plasma. Adibi and Krzysik (1977) reported that glycylsarcosine accumulated more in the muscle, liver, and intestinal mucosa of nephrectomized rats than those of control rats and that glycylglycine and glycylleucine were not detectable in tissues of control rats, but were detectable in tissues of

nephrectomized rats. These findings imply that the kidneys play an important role in the utilization of circulating peptides.

The use of membrane vesicles eliminates the problem of cytoplasmic hydrolysis, and makes it possible to detect the accumulation of peptides within the vesicles. Ganapathy and Leibach (1983) studied the role of a pH gradient in dipeptide transport in renal and intestinal BBMV from rabbits. The results indicated that an inward proton gradient stimulated the uptake of glycylproline by 70% in small intestinal BBMV and by 108% in renal BBMV. So these results indicate that the mechanism of peptide uptake by renal brush border membrane is active, and proton driven.

Compelling evidence has appeared to show the existence of an inward proton gradient across the renal brush border membrane in vivo. Kleinman et al. (1980) reported that the intracellular pH of renal tubular cells was 7.7 to 7.5. But the pH of the tubular lumen was about 6.9 (Burg and Green, 1977). A Na^+/H^+ antiport system existed in both intestinal and renal brush border membranes. This antiport is responsible for generating the inward proton gradient across the brush border membrane by pumping protons out of the cells in response to an influx of Na^+ (Ganapathy and Leibach, 1983).

Several recent studies (Silbernagl et al., 1987; Daniel et al., 1991; Skopicki et al., 1991) provide convincing evidence that two distinct peptide transport systems exist in the renal brush border membrane. One system is characterized by its high affinity and low capacity, and another system by its low affinity and high capacity.

Daniel et al.(1992) used isolated BBMV from rat kidney cortex to study the factors that determine the substrate affinity for the peptide/proton symporter systems. Free amino acids, including glutamine, did not inhibit the uptake

of glycylglutamine by the membrane vesicles. The interaction between peptides for the transport system was competitive. Both free N- and C-terminal groups are essential for a peptide to have a high affinity for the transport system. Dipeptides with the amino group in the α-position had much greater affinity than the dipeptides with the amino group in the ß-position. The affinity of a dipeptide or tripeptide for the transport system increased with increased hydrophobicity of the side chains of the amino acid residues. Both basic and acidic amino acid residues as either the N- or the C-terminus significantly reduced the affinity of the dipeptides. Dipeptides with L-amino acid residues had much higher affinity than dipeptides with D-amino acid residues. Triglycine had a slightly lower affinity than diglycine, but tetraglycine had a very low affinity. This suggests that peptides with four or more residues are not the usual substrates of the peptide transport system. These results indicate that most of the features of peptides which serve as a substrate for a high affinity renal transporters were similar to these for the intestinal peptide transport system.

The very low affinity and extensive hydrolysis of tetrapeptides by the renal brush border membrane indicated that hydrolysis may be the major mechanism by which tetra- and larger peptides are reabsorbed in the form of amino acids or di- and tripeptides released from the hydrolysis (Kenny, 1977).

Mechanism of Peptide Utilization by Brain. Abraham et al. (1964) studied the uptake of dipeptides, carnosine and homocarnosine, by rat brain slices and found that carnosine uptake was concentrative in the brain slices, but homocarnosine uptake was not.

Roles of Blood in the Clearance of Circulating Peptides. A diverse range of exopeptidases, including

prolidase, has been characterized within the mature human erythrocyte but there is no protein turnover in these cells. This means that peptide substrates for these peptidases are exclusively of extracellular origin and erythrocytes may play a role in the clearance of circulating peptides This speculated function by erythrocytes was supported by the discovery of a peptide-transport system in mature human erythrocytes (King et al., 1983; King and Kuchel, 1984). Glycylproline is one of the three major iminodipeptides excreted in excess in the urine of patients with prolidase deficiency (Isemura et al., 1979), so it is of particular interest to find out whether the peptide transport system in mature human erythrocytes could take up glycylproline for the hydrolysis by cytosolic prolidase. King and Kuchel (1984) provided the first evidence that mature human erythrocytes did have the ability to uptake intact glycylproline through a saturable membrane-transport system and suggested that the influx of glycylproline in human erythrocytes was much higher than that in human kidney. Vandenberg et al. (1985) showed that mature human erythrocytes could transport tyrosylglycylglycine and glycylglycylglycine via a saturable transport system and the transport of glycylglycylglycylglycine across the membrane was much lower than that of the tripeptide. These findings further support the speculation that erythrocytes are involved in the clearance of circulating peptides (di-, tri- and tetrapeptides).

Glutathione exists in human erythrocytes at a relatively constant concentration of 2 to 3 mmol/liter and is synthesized in erythrocytes from its constituent amino acids. The erythrocyte plasma membrane has transport systems for glycine and cysteine, but human erythrocytes are completely impermeable to glutamate (King and Kuchel, 1985). King and Kuchel (1985) reported that human erythrocytes

could transport α-L-glutamylalanine and alanine-α-L-glutamate through saturable membrane transport systems and the cytosolic peptidases of human erythrocytes have the ability to hydrolyze these two peptides. These results suggest that human erythrocytes may transport α-glutamyl-dipeptides via the peptide transport system and then hydrolyze the intact peptides within the cytosol to supply the glutamate for glutathione synthesis.

Hydrolysis of peptides by plasma peptidases may also be responsible for the clearance of circulating peptides. Piez et al. (1960) dialysed or gel-filtered human serum and then kept the processed serum samples at -20°C or 37°C for various times. Free amino acids were determined from samples treated with different temperatures. The results indicated that after 3-d of storage, samples stored at -20°C showed only negligible amounts of free amino acids, but samples stored at 37°C showed significant amounts of free amino acids (ranging from 4 uM of aspartic acid to 256 uM of alanine). For the dialyzed human serum samples, after 3-d storage at 37°C, the concentrations for valine, methionine, isoleucine, leucine, threonine, phenylalanine, lysine, histidine, and arginine were 88, 29, 22, 180, 83, 59, 112, 43, and 106 uM, respectively. At the same time, significant concentrations of non-essential amino acids also accumulated in the serum samples. These findings indicate that human serum contains the peptidases which are able to release significant amounts of essential and non-essential amino acids from serum proteins. Adibi and Johns (1984) investigated the relationship between plasma peptidase activity and half-life of seven dipeptides, showing that the dipeptide half-lives were inversely related to the peptidase activity. These findings demonstrate that plasma peptidases are involved in the hydrolysis of circulating peptides. To the contrary, Krzysik and Adibi (1977) reported that, in rat

plasma, there was modest peptidase activity against glycylleucine, but no peptidase activity against glycylglycine. Peptidase activities against these two peptides were found in other tissues, such as liver, kidney, intestine, and muscle. These two dipeptides appeared to be cleared from the plasma within 15 min without significant urinary loss in rats (Adibi et al., 1977). Therefore, the clearance of circulating peptides is not totally dependent on the plasma peptidases, and other tissues are also involved in the removal of circulating peptides.

Peptide Utilization by Animal Cells in Culture

In general, animal cell cultures will not grow satisfactorily in media containing amino acids but no proteins or peptides, but the reasons are not well elucidated (Matthews and Payne, 1975). Pickart and Thaler (1973) identified tripeptides (glycylhistidyllysine and glycyllysylhistidine) in human serum which prolonged the survival of normal liver cells and stimulated growth in neoplastic liver. Amborski et al. (1970) found that Baco-Peptone or Proteos Peptone in serum-free medium helps the maintenance of beating chicken embryo heart cells.

Eagle (1955) employed a deficient medium supplemented with 5% dialyzed human serum for Hela cells or 1% dialyzed horse serum for mouse fibroblast cells to study the effects of substituting glycylleucine, leucylglycine, glycylisoleucine, glycyl-DL-phenylalanine, DL-phenylalanylglycine, leucyltyrosine, and glycyltyrosine for leucine, isoleucine, phenylalanine, and tyrosine on cell proliferation in mouse fibroblasts and Hela cultures. All the dipeptides tested were used by the two cell lines as sources of corresponding essential amino acids. In the mouse fibroblasts, the peptide concentration required for maximal cell multiplication was higher than or similar to the amino acid concentration for maximal proliferation, at low

concentrations (1 mM and 20 mM), glycyl-DL-phenylalanine promoted greater cell proliferation than DL-phenylalanylglycine. At the concentration of 100 mM, glycylleucine resulted in better proliferation than leucylglycine. Similar results were obtained in the Hela cell cultures.

Grahl-Nielsen et al. (1974) studied the effects of free lysine, di-, tetra-, hepta-, and decalysine on the cell proliferation in an intestinal cell line (RPMI No.2402) derived from the Syrian hamster with the lysine-free basal medium supplemented with 1% dialyzed calf serum. The results indicated that only dilysine was utilized as effectively as free lysine, growth decreased with increased chain length of the peptides and the duration of the lag and logarithmic growth periods increased in proportion to the chain length of the peptides. They failed to detect these lysine peptides within the cells and serum peptidase activity against decalysine. Attempts to adapt the cells to proliferate at optimum rates over a period of 4 wk indicated that the ability of the cells to utilize decalysine as a lysine source was not inducible.

Glutamine is unstable as a free amino acid. Parenteral amino acid solutions usually do not contain this amino acid. Under certain disease conditions, maintaining nitrogen balance may need an exogenous supply of this amino acid. This problem can be overcome by the use of stable glutamine-containing peptides (Adibi, 1987). Brand et al. (1987) studied the possibility of supplying glycyl-L-glutamine as a glutamine source for cultured rat thymocytes in cultures and found that the dipeptide could substitute for glutamine to support both resting and proliferating thymocytes. But to support equal rates of DNA synthesis, a 6-fold higher concentration of the dipeptide was needed in the cultures compared with free glutamine.

A proline-auxotrophic ovary cell line (CHO-K1) was used as an experimental model for studying the availability of proline-containing peptides as proline sources for protein accretion and cell proliferation (Emmerson and Phang, 1993). Four proline-dipeptides (glycylproline, glycylhydroxylproline, phenylalanylproline, and methionylproline) were tested in this experiment The results indicated that all the proline-peptides except glycylhydroxylproline were able to promote the growth of CHO-K1 cells at similar rates as in proline-free medium supplemented with 5% fetal bovine serum.

In general, the above-described cell culture experiments show that peptide-bound amino acids can be utilized by cultured animal cells tested as corresponding amino acid sources for protein synthesis and cell proliferation. These limited studies only tested a small number of established cell lines and peptides, and the media used all contained animal serum. To elucidate the nutritional and physiological function of the huge number of small peptides (di, tri) at the cellular level, much more work remains to be done.

Peptide Metabolism in Bacteria

Bacteria can be classified into two categories: Gram-negative and Gram-positive. The cell envelope of Gram-negative bacteria consists of a cell wall (comprising an outer membrane with a peptidoglycan layer beneath it) and the cytoplasmic membrane (inner membrane, IM). A periplasmic space also exists between the cell wall and IM (Braun and Hantke, 1974; Costerton et al., 1974). The cell wall of Gram-positive bacteria lacks an outer membrane but has a integument of peptidoglycan instead, and the typical periplasmic space does not exist in this category of bacteria (Rogers et al., 1978). Most of the peptide utilization studies have been focused on members of the

Enterobacteriaceae, Pseudomonadaceae, and Bacteroidaceae in the Gram-negative bacteria and members of the Streptococcaceae and Lactobacillaceae in the Gram-positive bacteria (Payne, 1980a).

Utilization of Peptides by Bacteria. Kihara and Snell (1952) studied the effects of L-alanine peptides on the growth of L. casei. The findings indicated that the growth rates differed significantly in the presence of different L-alanine peptides and possibly reflected the different intracellular peptidase activities against various peptides. For instance, L-leucyl-L-alanine was superior to L-alanyl-glycine in supporting growth of L-casei, and intracellular hydrolysis showed that the hydrolysis of L-leucyl-alanine was faster than L-alanyl-L-leucine. So the conclusion was that the most rapidly hydrolyzed peptide was the most effective in supporting growth in L. casei. The results also indicated that L-leucyl-L-alanine was better than L-alanyl-leucine and glycyl-L-alanine was better than L-alanyl-glycine in promoting growth. These results showed that dipeptides with the same amino acid composition but different residue sequence had different effects on the growth of L. casei. Kihara and Snell (1952) also reported that the growth-promoting effect of free L-alanine was much lower than L-alanine peptides and the speculated reason was that D-alanine inhibited the transport of the L-isomer, but did not affect the transport of L-alanine peptides which supplied the free L-alanine after intracellular hydrolysis. Similar results were reported in serine peptides and L. delbrueckii 9649 (Prescott et al., 1953).

Peters et al. (1952) found that, in L. delbrueckii, histidine peptides were much better than free histidine in promoting growth, and the reason was that L. delbrueckii could not effectively transport free histidine, but

efficiently took up histidine peptides which underwent intracellular hydrolysis to supply the free histidine.

Kihara et al. (1952) showed that in S. faecalis, tyrosine-containing dipeptides surpassed free tyrosine in growth-stimulating ability in the presence of B6 because, under these condition, functional tyrosine decarboxylase destroyed free tyrosine, but not tyrosine peptides. In the absence of B6, tyrosine peptides were utilized as effectively as or less effectively than free tyrosine because, in this case, tyrosine decarboxylase was not functional. A positive correlation was also observed in this experiment between intracellular hydrolysis and growth-promoting effects of peptides.

Gilvarg and Katchalski (1965) studied the utilization of peptides by E. coli. The growth rates were similar in the presence of free lysine, dilysine, and trilysine and the growth was lower with tetralysine supplementation and stopped in the presence of penta- and decalysine. Similar responses were obtained in the presence of di- to hexaarginine. Quite different results were observed in glutamic oligopeptides (di- to septa-), only free and diglutamic acid supported growth. Hydrolysis studies showed that intracellular peptidases could convert pentalysine into free lysine. This suggests that impermeability of membranes to pentalysine is the real cause of nutritional ineffectiveness of this peptide.

The utilization of peptides by ruminal bacteria has been actively investigated. Pittman and Bryant (1964) showed that the presence of peptides was essential for the growth of B. ruminicola and N from free amino acids was not utilized by this bacterium. Pittman et al. (1967) reported that B. ruminicola could not take up radiolabeled proline, glutamate, or valine, but rapidly took up radiolabeled proline from peptides and incorporated it into cell

proteins. More rapid growth was observed in the presence of peptides than in the presence of ammonia alone (Wright, 1967). Chen et. al. (1987b) showed that mixed ruminal bacteria took up peptide amino N much faster than free amino N, and metabolized hydrophilic peptides faster than hydrophobic peptides. Better growth-promoting effects of peptides on mixed ruminal bacterial cultures were also observed by Argyle and Baldwin (1989). Broderick et al. (1988) showed that mixed ruminal bacteria could take up a range of di- and tripeptides in vitro.

Mechanism of Peptide Transport in Bacteria Leach and Snell (1959) reported that the uptake rate of radiolabeled L-alanyl-glycine was 10 times that of free glycine and more glycine was accumulated from the peptide than free glycine. No uptake competition was found between labeled peptide and free amino acid. This suggested that the uptake mechanisms for peptide and free amino acid were distinct from each other.

Payne and Gilvarg (1968a) isolated a mutant from E. coli that lost its ability to uptake tri- and tetralysine, but retained the ability to transport dilysine. This indicates that E. coli has distinct transport systems for dipeptides and oligopeptides. Payne (1968) reported that a mutant from E. coli selected for its inability to transport a specific oligopeptide lost its ability to transport a number of other oligopeptides, while in the wild type strain, these oligopeptides competed with each other for the transport. This suggests that oligopeptides share a common transport system. The findings also showed that the growth response of a glycine auxotroph of E. coli (M-123) to glycyl-DL-serine was affected by the presence of nonglycine oligopeptide, but the growth response of M-123 to triglycine was severely depressed in the presence of dilysine. Thus it appears that the transport system for dipeptides has no

affinity for oligopeptides, but that for oligopeptides has some affinity for dipeptides.

Kihara and Snell (1955) summarized three possible mechanisms with which peptide-bound amino acids surpass corresponding free amino acids in growth-promoting ability. First, the transport of a free amino acid, but not that of peptide-bound form, may be inhibited by an antagonistic amino acid. Second, a free essential amino acid may be destroyed before being utilized for protein synthesis, but a peptide-bound amino acid is not. Third, the transport of a free essential amino acid may be less efficient than that of its peptides.

The enteric bacteria can utilize peptides as sole sources of both carbon and N. Salmonella typhimurium and E. coli have three genetically distinct transport systems for peptides, the dipeptide permease, tripeptide permease, and the oligopeptide permease (Higgins, 1984; Hiles et al., 1987). The dipeptide permease, which is still poorly characterized, is relatively specific for dipeptides and may also transport a number of tripeptides. The tripeptide permease transports di- and tripeptides and strongly prefers tripeptides with hydrophobic amino acids. The oligopeptide permease handles almost any peptide with up to five or six amino acid residues. (Payne, 1968; Gibson et al., 1984; Higgins and Gibson, 1986).

The gene encoding the dipeptide permease was mapped to 80 min on the chromosome of S. typhimurium (Higgins and Gibson, 1986). The dipeptide permease, transported dipeptide against a concentration gradient, had a broad side chain specificity for dipeptides, but had a high affinity for dipeptides with hydrophobic amino acid residues such as leucine, methionine, and valine (Yang et al., 1977).

Gibson et al. (1984) reported that, in S. typhimurium, two loci, tppA and tppB, were responsible for a functional

tripeptide permease, and locus tppA encoded the positive regulator of locus tppB. Locus tppB encoded the tripeptide permease. The transcription of tppB was specifically induced by anaerobiosis or exogenous leucine and the tripeptide permease had the highest affinity for uncharged tripeptides, especially those with hydrophobic amino acid residues (Jamieson and Higgins, 1984, 1986).

The molecular basis of the oligopeptide permease has been characterized in detail for S. typhimurium and E. coli. The oligopeptide permease has been shown to be coded by four genes (oppA, oppB, oppC and oppD). These genes were organized as an operon and were cotanscribed from oppA to oppD (Hogarth and Higgins, 1983). Higgins and Hardie (1983) further indicated that the oppA gene encoded a periplasmic protein which served as the initial peptide-binding site during the peptide transport. In 1987, Hiles et al. identified another gene, oppF, as the fifth essential component of the oligopeptide permease, and concluded that the five proteins, oppABCDF, were the only proteins for the oligopeptide permease. The expression of the oligopeptide permease has been shown to be constitutive, and the components of the permease were identical between E. coli and S. typhimurium (Hiles et al., 1987).

Substrate requirements for the oligopeptide transport systems have been actively studied. A free amino group is essential (Gilvang and Katchalski, 1965, Payne, 1968; Guyer et al., 1986). The terminal carboxyl group is not vital (Payne and Gilvarg, 1968a), but modification of the carboxyl terminus reduces the peptide affinity for the transport system (Payne and Gilvarg, 1968a, Guyer et al., 1986). The oligopeptide transport system transports substrate based on the hydrodynamic volume of the oligopeptides rather than amino acid residues (Payne, 1968; Payne and Gilvarg, 1968b). The oligopeptide transport system requires α-linked peptides

(Payne, 1972) and prefers L-isomers at the first two residues (Matthews and Payne, 1980). Guyers et al. (1986) studied the binding affinity of purified periplasmic oligopeptide-binding protein from E. coli and found that peptides with hydrophobic and polar side chains had similar binding affinity for the oligopeptide-binding protein, but peptides with ionic side chains had very poor binding affinity.

Passive diffusion is believed to play a minor role in peptide transport (Payne, 1975). However under conditions of a high exogenous concentration of diglycine, an E. coli. mutant with a defective transport system for diglycine could accumulate labeled diglycine (Kessel and Lubin, 1963). This suggests that passive diffusion can be an alternative approach by which bacteria take up peptides.

Annison (1956) reported that both free amino acids and peptides were detected in the rumen fluid after the infusion of protein into the rumen. This suggests that hydrolysis of peptides by extracellular peptidases with subsequent uptake of free amino acids is another mechanism by which bacteria utilize peptides as N sources.

In general, like animals and plants, many species of bacteria are able to utilize both free amino acids and peptide-bound amino acids. The utilization of peptide-bound amino acids depends on two distinct categories of mechanisms. The peptides can be hydrolyzed by extracellular or periplasmic peptidases, and the released free amino acids then are taken into the bacteria by the amino acid permeases. The second approach is that the peptides are transported into the bacteria via peptide permeases (transport systems) and hydrolyzed intracellularly (Sussman and Gilvarg, 1971).

Metabolism of Peptides by Yeast.

The yeast cell envelope consists of an outer cell wall and an inner cell membrane, between which exists a periplasmic space (Matile et al., 1969). It has been shown that there were no extracellular or periplasmic peptidase activities against a number of peptides (Becker et al., 1973). This has greatly facilitated the study of utilization and transport of peptides by yeast.

Utilization of Peptides by Yeast. Becker et al. (1973) reported that a methionine auxotroph, S. cerevisiae, grew on methionylmethionine, methionylmethionylmethionine, and methionylglycylmethionylmethionine, but not on glycylmethionylglycine. However, this mutant could grow in the presence of high concentrations of glycylmethionylglycine. Intracellular peptidase activities against methionylmethionylmethionine and glycylmethionylglycine were similar. These findings suggested that a transport system with low affinity for glycylmethionylglycine rather than an absence of intracellular peptidase was responsible for the poor utilization of glycylmethionylglycine. Studies on three lysine auxotroph mutants showed that none of the three lysine auxotroph mutants grew in the presence of glycyllysine, lysylglycine or di- to octalysine, but these mutants contained intracellular peptidases against all the lysine peptides tested. This indicated that the lack of a transport system for these peptides was the cause of the lack of growth on lysine oligopeptides. Marder et al. (1977) studied the utilization of leucine- and lysine-containing peptides by a leucine and lysine auxotroph from Saccharomyces cerevisae and showed that the auxotroph could grow on leucylleucine, leucylleucylleucine, and glycylleucine, but did not grow in the presence of glycylleucylglycine or glycylleucylglycylleucine. Studies on lysine peptides indicated that lysylglycine and lysylleucine

could promote growth, but di-, tri-, tetra-, and pentalysine did not. Hydrolysis studies showed that intracellular peptidases could release either free leucine or lysine from all the peptides tested. This suggested that the failure of supporting growth by some of the leucine and lysine peptides was due to the impermeability or poor permeability of the cell membrane to these peptides.

Mechanism of Peptide Transport by Yeast Marder et al. (1977) reported that high concentrations of phenylalanine completely abolished the growth promoting effects of free leucine on a leucine- and lysine-requiring auxotroph from S. cerevisiae, but did not affect the growth promoting abilities of leucine-containing di- and tripeptides. Competition studies indicated that methionylmethionine, methionylmethionylmethionine, and alanylalanine inhibited the growth of the auxotroph on leucylleucine, leucylleucylleucine, lysylleucine, and lysylglycine. These findings clearly showed that a carrier-mediated transport system was involved in the entry process of di- and tripeptides, and the transport system was not shared by free amino acids.

Becker and Naider (1977) showed that, in a methionine auxotroph from S. cerevisiae, the uptake of trimethionine was saturable, dependent on temperature and pH, and affected by several inhibitors of metabolic energy coupling. The transport system could transport peptides with methionine, alanine, and leucine residues but peptides with solely lysyl residues had very low affinity for the transport system. Marder et al. (1977) showed that peptides containing methionine competed strongly with the utilization of leucylleucine and leucylleucylleucine, but peptides containing alanine were poor competitors and glycine and lysine peptides did not affect the utilization of leucine peptides. These findings imply that the transport system for

leucine peptides had a high affinity for peptides with hydrophobic amino acid residues.

Naider et al. (1974) investigated the utilization of methionine peptides by a methionine auxotroph of S. cerevisiae. Peptides (di- to tetra-) with the methionine residue at the C-terminus promoted better growth than peptides with other amino acid residues at this terminus. Pentamethionine supported the growth, so did some methionine peptides with blocked terminal amino or carboxyl group. Some tri- and tetrapeptides with glycine at the C-terminus failed to support the growth.

In general, yeasts have the transport system to take up intact peptides, the transport system is distinct from that for free amino acids, can handle peptides up to five amino acid residues, and prefers peptides with hydrophobic amino acids. But it is still unclear whether yeast has distinct systems to handle di-, tri-, and oligopeptide.

Metabolism of Peptides by Fungi.

Studies on peptide transport and utilization have been focused on the Ascomycete fungus Neurospora crassa, this species has the ability to hydrolyze some extracellular peptides (Payne, 1980b). Therefore, blocking the influx of the free amino acid of interest is the essential step to eliminate the effect of extracellular hydrolysis (Wolfinbarger and Marzluf, 1974).

Utilization of Peptides by Fungi. Wolfinbarger and Marzluf (1974) reported that four different leucine auxotrophs of N. crassa failed to grow on ten leucine-containing dipeptides (leucylleucine, leucylglycine, glycylleucine, leucylphenylalanine, phenylalanylleucine, histidylleucine, leucyl-ß-alanine, glycyl-D-leucine, methionylleucine, and lysylleucine) except leucylleucine in two leucine auxotrophs (leu-1 and leu-2). The tripeptides (leucylleucylleucine, leucylglycylglycine,

glycylleucyltyrosine, and glycylalanylleucine), except leucylglycylglycine, promoted growth of all the leucine auxotrophs as efficiently as free leucine. Excessive free L-phenylalanine completely inhibited the growth of these leucine auxotrophs on free leucine, but had no effects on the growth on leucine-containing dipeptides. Excessive L-phenylalanine also completely inhibited the growth of two leucine auxotrophs (leu-1 and leu-2) on leucylleucine, this showed that the exceptional utilization of leucylleucine by leu-1 and leu-2 was due to the extracellular hydrolysis of the dipeptide with the subsequent absorption of free leucine. So the results indicated that the four leucine auxotrophs only had a transport system for leucine tripeptides.

Wolfinbarger and Marzluf (1974) reported that one mutant (cys-3) failed to grow on free L-methionine, or methionine-containing dipeptides in the presence of excessive free L-phenylalanine. Excessive L-phenylalanine did not affect the growth of cys-3 on methionine-containing tripeptides, this indicates that methionine-containing tripeptides can be taken up intact by cys-3 and hydrolyzed inside the cell to supply the free methionine. Studies on peptide utilization by a lysine auxotroph (lys-1) showed that high concentrations of L-arginine inhibited the growth of lys-1 on free lysine, lysine-dipeptides, and lysine-tripeptides by 100%, 100%, and 80%, respectively. This means that extracellular hydrolysis with subsequent absorption of free lysine is the major mechanism for the observed utilization of lysine-containing di- and tripeptides by lys-1 auxotroph.

Mechanisms of Peptide Transport by Fungi. Growth studies by Wolfinbarger and Marzluf (1974) showed that the presence of competing amino acids completely inhibited the growth of amino acid auxotrophs on free amino acids and

dipeptides, but not on tripeptides containing these amino acids. This indicated the existence of a transport system for tripeptides distinct from that for free amino acids. Wolfinbarger and Marzluf (1975a,b) reported that only one oligopeptide transport system existed in N. crassa, and this transport system transported peptides with hydrodynamic volume smaller than trileucine. The transport system was constitutive and noninducible, required a free amino group for transport, and did not transport either free amino acids or dipeptides.

Peptide Metabolism in Higher Plants

Peptide Utilization in Higher Plants. Several early reports indicated that higher plants could utilize or take up dipeptides (Plummer and Kethley, 1964; Bollard, 1966; Salonen and Simola, 1977). For instance, Plummer and Kethley (1964) reported that the pitcher-leaves of the carnivorous plant *S. flava* could take up three dipeptides (DL-alanyl-DL-aspartate, DL-alanyl-DL-leucine, and DL-alanyl-DL-methionine) intact, and DL-alanyl-DL-leucine was absorbed more rapidly and remained intact longer within the leaves than the other two dipeptides But in this study, the mechanism of peptide uptake was not examined.

A study on the localization of peptidases in germinating barley conducted by Mikola and Kolehmainen (1972) indicated that the scutellum contained high activities of both the alkaline peptidases and acid carboxypeptidases. Based on this, Mikola and Kolehmainen (1972) suggested that peptides may be transported intact from the starchy endosperm into the scutellum where the peptides are hydrolyzed and the resulting amino acids are transported to the growing tissue of the embryo.

Burston et al. (1977) studied the uptake of glycylsarcosine, glycylsarcosylsarcosine, and glycylsarcosylsarcosylsarcosine by barley scutella in vitro.

The scutella could concentrate both the di- and tripeptides, but the uptake of the tetrapeptide was very small. The uptake of glycylsarcosine was inhibited by 35% in the presence of glycylglycine, but was not affected by the presence of glycine. The scutella could also rapidly take up glycine and glycylglycine and uptake of glycine from glycylglycine was four times as rapid as that of equimolar glycine. So these findings show that barley scutella can absorb di- and tripeptides via an active transport system which is not accessible to corresponding free amino acids, and tetrapeptides are not the substrates of this active transport system. Higgins and Payne (1977a) also reported intact peptide uptake by scutellum.

Peptide Transport in Higher Plants. The peptide uptake in barley scutella has been relatively well characterized, while the peptide uptake in other plants or in the other parts of the barley remains to be elucidated. The evidence for active transport of peptides by barley scutella was obtained by using hydrolysis-resistant glycylsarcosine and glycylsarcosylsarcosine (Burston et al. 1977; Higgins and Payne, 1977a; Sopanen et al. 1977; Higgins and Payne, 1978). In all these experiments, isolated scutella could concentrate glycylsarcosine and glycylsarcosylsarcosine in vitro.

Results from several experiments (Higgins and Payne, 1977b; Sopanen et al., 1977, 1978) indicated that removal of Na^+ from the incubation medium had no effect on uptake of glycylsarcosine or glycylglycine, and an acidic (pH 3.8-4.0) environment could support the maximal uptake of glycylsarcosine or glycylglycine. So it is likely that a H^+ gradient is the driving force for the active uptake of peptides in barley scutella.

As for the maximum size of peptides taken up by the scutellum, there are conflicting reports. Burston et al.

(1977) reported that the uptake of glycylsarcosylsarcosylsarcosine by the scutella was not concentrative and very small. But Sopanen et al. (1977) showed that tetraglycine moderately inhibited the uptake of glycylsarcosine. Higgins and Payne (1978) found that, for di- to pentaglycine, the scutella could only take up di- and triglycine, but for di- to penta-alanine, the scutella took up all the peptides (di- to penta-alanine) more rapidly than equimolar free alanine with the uptake of tetra-alanine being the most rapid. These findings indicate that scutella can take up peptides of up to five amino acid residues.

A number of reports indicated that, as in animals and microorganisms, peptide uptake by the scutella of germinating barley is distinct from that of free amino acids. It has been shown that glycine or leucine have no inhibitory effects on the uptake of glycylsarcosine or glycylglycine, but glycylglycine, glycylglycylglycine, and glycylglycylglycylglycine did inhibit the uptake of glycylsarcosine (Burston et al. 1977; Sopanen et al., 1977, 1978). Sopanen et al.(1978) also showed that dipeptides with basic amino acids, acidic amino acid residues, and proline residues all inhibited the uptake of glycylglycine. This suggests that the peptide transport system is similar to that of animals in that the transport system does not take up peptides according to their charges.

Walker-Smith and Payne (1983, 1984a) reported that transport of peptides by the scutellar membrane of germinating barley was inhibited by thiol and dithiol-specific reagents and dithiothreitol could reverse the inactivation caused by these reagents. This indicates that the peptide transport system contains a redox-sensitive, dithiol-dependent protein. Walker-Smith and Payne (1984b) showed that reagents that abolish trans-membrane proton gradients all effectively inhibited the uptake of

glycylphenylalanine by barley scutella. This result supports the hypothesis that peptide uptake in scutella is proton-dependent.

Summary of Literature Review

Utilization of peptide-bound amino acids as amino acid sources appears to occur in a wide range of living species including microorganisms, higher animals, and higher plants.

Microorganisms have two distinct mechanisms to assimilate peptide-bound amino acids. The first approach involves extracellular hydrolysis of peptides with subsequent uptake of free amino acids and the second approach is that peptides are transported into the cells intact via peptide permeases and then are hydrolyzed intracellularly. Three different peptide transport systems, the dipeptide permease, the tripeptide permease, and the oligopeptide permease, have been identified in enteric bacteria. Yeasts have peptide transport system to take up peptides with up to five amino acid residues. But it is still uncertain whether yeasts have distinct systems to handle di, tri-, and oligopeptides. Fungi appear to possess one peptide transport system specific for tripeptides.

Peptide utilization in higher plants is observed in the pitcher-leaves of the carnivorous plants and barley scutella. The peptide transport system in barley scutella appears to concentrate di- to pentapeptides and to depend on a proton gradient across the membrane.

A peptide-bound amino acid pool exists in the blood plasma of higher animals. Peptide transport systems have been found in the brush border membrane of the intestine and kidney of higher animals. These systems are active and proton-dependent and handle di- and tripeptides. The peptide transport systems are at least partially responsible for the observed flux of peptide amino acids across the gastrointestinal tract. The uptake of peptides by the rumen

and omasum accounts for 77% of total amino acid flux across the portal-drained viscera in ruminants. The peptide transport systems in kidney may be involved in the reabsorption of di- and tripeptides in the glomerular filtrate during the formation of urine. Several mechanisms have been shown to be responsible for the utilization of circulating peptides by tissues, including hydrolysis in blood, hydrolysis by tissue capillary bed-associated and membrane-bound peptidases, and intact uptake by the tissues with subsequent intracellular hydrolysis.

Chapter III
Objectives

The overall objective of this study was to elucidate the utilization of peptide-bound amino acids as amino acid sources for protein accretion and cell proliferation at the cellular level, using cultured C_2C_{12} myogenic (mouse skeletal muscle cell line), MAC-T mammary epithelial (bovine mammary epithelial cell line), and ovine myogenic satellite cells as experimental models. Specific objectives were as follows:

1) To determine the availability of methionine in di- to octapeptides for protein accretion and cell proliferation.
2) To determine the effect of methionine position in dipeptides on protein accretion and cell proliferation.
3) To determine the effect of peptide chain length (tri- to octapeptides) on protein accretion and cell proliferation.
4) To investigate the relationship between dipeptide hydrophobicity and protein accretion.
5) To determine whether cultured C_2C_{12} and (or) MAC-T cells are able to accumulate protein and (or) DNA in the presence of methionine-containing peptides in a serum-free medium with and without the supplementation of lipids, insulin, or low protein serum replacement (LPSR-1).
6) To determine whether cultured MAC-T cells are able to accumulate protein and (or) DNA in the presence of methionine-containing dipeptides and desalted adult animal sera from several species

Chapter IV
Methionine-containing Peptides as Methionine Sources for Protein Accretion in Cultured C_2C_{12} AND MAC-T Cells

ABSTRACT

Twenty two methionine-containing di- to octapeptides were evaluated for their ability to be a source of methionine to support protein accretion in C_2C_{12} myogenic and MAC-T mammary epithelial cells. The cell cultures were incubated for 72 h at 37°C in a humidified environment of 90% air : 10% CO_2 for C_2C_{12} cells or 95% air : 5% CO_2 for MAC-T cells. The basal medium contained methionine-free Dulbecco's Modified Eagle's Medium and 6% desalted fetal bovine serum. Treatments included basal medium, the basal medium supplemented with one of the 22 methionine-containing peptides, or the basal medium supplemented with free L-methionine. Cell cultures incubated with the basal medium alone for 72 h were characterized by decreased cell number, decreased protein content, and cell detachment compared with initial cultures. Methionine-containing peptides with the exception of glycylmethionine and prolylmethionine in C_2C_{12} cells were able to support protein accretion with responses ranging from 29.1% to 123.3% of the response of L-methionine. Dipeptides with methionine at the N-terminus promoted greater ($P < .0001$) protein accretion than dipeptides with methionine at the C-terminus. Stimulation of protein accretion by seven pairs of dipeptides with methionine at either the C-terminus or the N-terminus was correlated with the hydrophobicity of the dipeptides. These results indicate that C_2C_{12} myogenic and MAC-T mammary epithelial cells have the ability to utilize methionine-containing peptides as sources of methionine to support protein accretion.

(Key Words: Methionine, Peptide, Protein, Myogenic, Mammary)

Introduction

Utilization of peptide-bound amino acids as nitrogen sources has been shown to be a universal phenomenon in species ranging from unicellular organisms to higher animals and plants (Higgins and Payne, 1977; Payne, 1980c; Matthews, 1991a). Evidence continues to accumulate in support of the concept that absorption of intact small peptides from the gastrointestinal tract constitutes a major form by which end products of dietary protein digestion enter the blood. When expressed as a percentage of the total plasma amino acid pool, peptide-bound amino acids account for 52 to 78% in ruminants (DiRienzo, 1990; Seal and Parker, 1991; Koeln et al., 1993), 10% in man (Christensen et al., 1947), 9 to 51% in rats (Asatoor et al., 1978, Seal and Parker, 1991) and 11 to 14% in guinea pigs (Gardner et al., 1983). However, there is little information about the fate of the absorbed and circulating small peptides or peptide-utilizing capacity of tissues. It was of particular interest to investigate whether muscle and mammary tissues possess the ability to utilize small methionine-containing peptides as sources of methionine for protein synthesis.

In the present study, cultured C_2C_{12} myogenic (Yaffe and Saxel, 1977) and MAC-T mammary epithelial cells (Huynh et al., 1991) were used as experimental models to determine 1) the availability of methionine di- to octapeptides for protein accretion, 2) the effect of methionine position in dipeptides on protein accretion, 3) the effect of peptide chain length (tri- to octapeptides) on protein accretion, and 4) the relationship between dipeptide hydrophobicity and protein accretion.

Materials and Methods

Medium Preparation. The methionine-free Dulbecco's modified Eagle's medium (MFDMEM) was prepared by adding glutamine (58.4 mg/100 mL medium)[1] and 1% (v/v) antibiotic-antimycotic solution[2] to deficient Dulbecco's modified Eagle medium[3]. The resulting medium was sterilized by filtration[4]. Fetal bovine serum (FBS)[5] was desalted by gel filtration chromatography with a Sephadex G-25M desalting column[6]. The basal medium contained MFDMEM plus 6% desalted FBS (dFBS).

Treatment media consisted of the basal medium supplemented with either free L-methionine[7] (6 uM, C_2C_{12}; 15 uM, MAC-T) or one of the 22 methionine-containing peptides (15 dipeptides, 2 tripeptides, 1 tetrapeptide, 1 pentapeptide, 1 hexapeptide, 1 septapeptide, and 1 octapeptide, see Table 4.1 for the abbreviations and hydrophobicity values) at concentrations that were equivalent to L-methionine in methionine content.

The growth medium used to maintain and propagate C_2C_{12} myogenic cells consisted of Dulbecco's modified Eagle's medium (DMEM)[8], 1% (v/v) antibiotic-antimycotic solution and 15% (v/v) FBS. The growth medium for MAC-T mammary epithelial cells was composed of DMEM, 1% antibiotic-antimycotic solution and 10% FBS.

Cell Culture Procedure. The C_2C_{12} and MAC-T cells were plated at 10,000 cells per 35 mm dish[9] and 40,000 cells per well (24-well plate)[10], respectively. The C_2C_{12} cells were

---

[1]Sigma Chemical Co., St. Louis, MA
[2]Cat. No. 600-5240AG, GIBCO, BRL, Inc., Grand Island, NY
[3]Cat. No. 320-1970AJ, GIBCO, BRL, Inc., Grand Island, NY
[4]Cat. No. 09-730-218, Nalge Company, Rochester, NY
[5]Cat. No. 230-6140AG, GIBCO, BRL, Inc., Grand Island, NY
[6]Pharmacia LKB Biotechnology, Piscataway, NJ
[7]Sigma Chemical Co., St. Louis, MO
[8]Cat. No. 380-2430AJ, GIBCO, BRL, Inc., Grand Island, NY
[9]Fisher Scientific, Pittsburgh, PA
[10]Fisher Scientific, Pittsburgh, PA

incubated in growth medium at 37°C in a humidified environment of 90% air, 10% CO_2. The MAC-T cells were incubated in growth medium at 37°C in a humidified environment of 95% air, 5% CO_2. The cultures were incubated for 24 h, then the growth medium was replaced by MFDMEM and the cells were incubated for another 24 h. Subsequently, the MFDMEM medium was removed, then starved cells were incubated with one of the treatment media for 72 h. The treatment media were changed at 24-h intervals.

Harvest and Analytical Procedures. After 72 h of incubation, the cultures were washed twice with cold Dulbecco's Phosphate Buffered Saline (D-PBS)[11] and then were dissolved by treatment with .4 mL or .8 mL .5 N NaOH overnight at room temperature. The resulting solutions were neutralized with .04 mL or .08 mL 5 N HCl and the protein contents were determined by the enhanced bicinchoninic (BCA)[12] assay (Smith et al., 1985).

Methionine-containing Peptides Twenty two methionine-containing peptides (di to octapeptides)[13] were utilized in this study. The purity of the methionine-containing peptides was determined by HPLC with a Waters Pico-Tag column (3.9 * 300 mm)[14] at a wavelength of 254 nm. Eluent 1 consisted of deionized water, 70 mM sodium acetate, 2.5% acetonitrile and .025% ethylenediamine tetraacetic acid (EDTA) solution (10 mM) and eluent 2 contained 40% deionized water, 45% acetonitrile and 15% methanol. The flow rate was 1 mL per min.

The methionine-containing peptides (15 uM) were added to 15 mL centrifuge tubes containing 12 mL of the MFDMEM supplemented with 6% dFBS and the mixed solutions were

---

[11] GIBCO, BRL, Inc., Grand Island, NY
[12] Pierce, Rockford, IL
[13] Sigma Chemical Co., St. Louis, MO
[14] Waters Millipore Corp., Milford, MA

filtered through ultrafree MC filters[15] with a 10,000 Da cutoff to remove large molecules. An aliquot of 40 uL of the resulting filtrate was dried under vacuum and derivitized with reagent containing phenylisothiocyanate (PITC), methanol, triethylamine and deionized water and dried under vaccum again (Cohen et al., 1989). Finally 80 uL diluent containing 5% acetonitrile in phosphate buffer was added to the dried samples, and 50 uL of the resulting solution was subject to HPLC analysis.

L-Methionine Standard Curve For Protein Accretion. Prior to the peptide utilization experiment, preliminary studies were conducted to determine the effect of free L-methionine concentrations (0 to 50 uM) in the medium on protein accretion in both C_2C_{12} and MAC-T cells. The response curves (Figures 4.1 and 4.2) were used to determine the concentrations of both L-methionine and methionine-containing peptides to be used in the peptide utilization experiments.

Hydrophobicity Values of Methionine-Containing Dipeptides. The hydrophobicity values (Table 1) of methionine-containing dipeptides were calculated as the average of the values of the two constituent amino acids according to the Fauchere scale (Von Heijne, 1987; Daniel et al., 1992).

Statistical Analysis. Treatments were replicated in three to eight wells or dishes. Data were analyzed by the GLM procedure of SAS (1989) with culture dish or well as the experimental unit. The ANOVA model was:

$$Y_{ij} = \mu + \alpha_i + E_{ij}$$

Where:

Y_{ij} = jth protein response from the ith treatment

μ = overall mean

[15]Waters Millipore Corp., Milford, MA

α_i = effect of ith treatment

E_{ij} = error component associated with the jth protein response from the ith treatment

Means of different peptide treatments were compared using Duncan's means separation test at an alpha value of .05.

Results and Discussion

HPLC analysis demonstrated that none of the methionine-containing peptides had detectable amounts of free methionine. Observed growth responses to methionine-containing peptides are thus interpreted as being due to the presence of methionine in the peptides and not free methionine. The HPLC results also verified the effectiveness of the desalting process in removing both free methionine and methionine-containing molecules smaller than 10,000 Da from FBS.

Cultured cells were sensitive to the methionine concentrations in the medium. Cells incubated with the basal medium alone for 72 h were characterized by decreased cell number, decreased protein content, and cell detachment. Protein accretion increased linearly over an range of L-methionine concentrations from 0 to approximately 12 uM for C_2C_{12} cells (Figure 4.1) and from 0 to approximately 30 uM for MAC-T cells (Figure 4.2), and then plateaued. Based upon these results, 6 and 15 uM were chosen as methionine concentrations for the treatment media for C_2C_{12} and MAC-T cells, respectively.

Expressed as a percentage of the response to free L-methionine (Figure 4.3), growth of C_2C_{12} cells differed due to type of dipeptide (11% to 108%). Methionylmethionine, methionylvaline, and leucylmethionine, were utilized as efficiently as free L-methionine. Prolylmethionine and glycylmethionine were poorly utilized by C_2C_{12} cells. Remaining peptides were utilized at a rate of about 62 to 86% of the rate of free methionine, except for

methionylserine, serylmethionine, and valylmethionine which were utilized at 26 to 43% of the rate of free methionine.

The results presented in Figure 4.4 indicate that all the methionine-containing dipeptides were able to support protein accretion of cultured MAC-T cells with the response ranging from 35% to 122% of the free L-methionine growth response. Methionylvaline, methionylleucine, methionylmethionine, and leucylmethionine supported greater ($P < .05$) protein accretion than did free L-methionine. Phenylalanylmethionine , methionylphenylalanine, alanylmethionine, methionylalanine , methionylserine, and methionylglycine were utilized as effectively as free L-methionine. Glycylmethionine and prolylmethionine were the least utilized peptides in MAC-T cells. These results show that C_2C_{12} myogenic and MAC-T mammary epithelial cells have the ability to discriminatively utilize methionine-containing dipeptides. Because all the peptides were tested at concentrations that were equivalent in methionine content to L-methionine alone, hydrolysis of dipeptide with subsequent uptake of free methionine was unlikely to be the sole mechanism for the utilization of methionylvaline, methionylleucine, methionylmethionine, and leucylmethionine which were observed to elicit a greater protein accretion ($P < .05$) than free methionine in MAC-T cells. This suggests that uptake of intact peptides was a possible mechanism of peptide utilization in MAC-T cells. Taylor et al. (1950) showed that in E. coli, methionylmethionine was equal to free L-methionine in supporting growth; glycylmethionine, methionylglycine, and methionyltyrosine were less efficient. In yeast, a methionine auxotroph (G1333) could grow on nine methionine-containing dipeptides including methionylmethionine, glycylmethionine, phenylalanylmethionine, alanylmethionine, valylmethionine, methionylalanine, methionylphenylalanine, methionylglycine,

and methionylglutamine, but the growth response varied among peptides with methionylglutamine being the poorest substrate (Naider et al., 1974). Wolfinbarger and Marzluf (1974) reported that three methionine auxotrophs of fungi (N. crassa) utilized methionylmethionine and methionylleucine well, but the growth response was very poor in the presence of glycyl-DL-methionine, methionylglycine, methionylhistidine, and histidylmethionine. In addition to above studies, reports have been presented to show that peptides containing other essential and nonessential amino acids can be utilized as sources of these amino acids in a wide range of species. For instance, E. coli can utilize peptides containing phenylalanine and tyrosine (Simmonds et al., 1947a), leucine (Simmonds et al., 1947b), lysine (Gilvarg and Katchalski, 1965), arginine (Gilvarg and Katchalski, 1965), glutamic acid (Gilvarg and Katchalski, 1965), and proline (Simmonds and Fruton, 1948).

By contrast, relatively fewer investigations have been made to ascertain the nutritional functions of small peptides in animals at the cellular level. Eagle (1955) reported that in the presence of dialyzed animal serum both Hela cells and mouse fibroblasts could utilize dipeptides containing leucine, isoleucine, phenylalanine, and tyrosine as corresponding amino acid sources to support growth. The peptide concentrations required for maximal cell proliferation were higher than or equal to those of the corresponding amino acids for maximal growth. Cultured animal cells also have been shown to utilize lysine-containing peptides as evidenced in works with a small intestine epithelial cell line (RPMI No. 2402) (Grahl-Nielsen et al., 1974), on glutamine-containing dipeptide in rat thymocytes (Brand et al., 1987), and on proline-containing dipeptides in a proline-auxotroph ovary cell line (Emmerson and Phang, 1993). Therefore, current results are

consistent with the concept that peptide-bound amino acids can be utilized by organisms as corresponding amino acid sources.

To determine whether the position of the methionine residue affects the utilization of methionine-containing dipeptides, seven methionine-X and seven X-methionine dipeptides were compared for effects on protein accretion. Methionine-X dipeptides appeared to promote greater ($P < .05$) protein accretion than X-methionine dipeptides in muscle and mammary cells (Figure 4.5). Naider et al. (1974) also found that methionine position in dipeptides affected growth response of a yeast methionine auxotroph (G1333), but X-methionine peptides supported greater growth than methionine-X peptides. Furthermore, they showed that intracellualr peptidases preferred methionine-X dipeptides to X-methionine peptides. This unexpected result suggested that the difference in growth responses was due to the different uptake rates rather than different intracellular hydrolysis rates. In Hela cells and mouse fibroblasts (Eagle, 1955), glycyl-DL-phenylalanine and glycylleucine promoted greater cell proliferation than DL-phenylalanylglycine and leucylglycine. Kihara and Snell (1952) reported that leucylalanine and glycylalanine supported greater growth of L. casei than alanylleucine and alanylglycine. These findings demonstrate that dipeptides with the same amino acid compostion but different sequences may have significantly different growth-supporting abilities. This may be due to different rates of uptake or intracellular hydrolysis rates of peptides.

The protein accretion of C_2C_{12} with tri- to octapeptides are presented in Figure 4.6. Values ranged from 40% to 123% of the free L-methionine response. Oligopeptides with six and seven amino acid residues were utilized as effectively as free methionine, and the octapeptide promoted

greater protein accretion than free methionine. The data in Figure 4.7 show that the tripeptide, methionylalanylserine, and the oligopeptides ranging from penta- to octapeptides supported growth of MAC-T cells as well as free L-methionine and the tetrapeptide, gylcylglycylphenylalanylmethionine, was even better ($P < .05$) in promoting protein accretion in MAC-T cells. These findings indicate that MAC-T mammary epithelial cells utilized tri- to octapeptides to a greater extent than C_2C_{12} myogenic cells. Grahl-Nielsen et al. (1974) reported that, in the presence of 1% dialyzed calf serum, a hamster small intestinal cell line could grow on di-, tetra-, hepta-, and decalysine, but that the growth responses decreased with the increased chain length. There was no detectable peptidase activity against decalysine in the calf serum, indicating that intact uptake of oligolysine was the possible mechanism for the observed growth in the cultured small intestine cells. Koeln et al. (1993) reported that peptide-bound amino acid flux across the gastrointestinal tract of calves was 2.5-fold to 7.2-fold higher than the free amino acid flux, and that oligopeptides with molecular weights ranging from 500 to 1,500 Da (4 to 11 amino acids) accounted for the largest portion of the peptide-bound amino acid flux across the portal-drained viscera (PDV). Seal and Parker (1991) showed that oligopeptides with 4 to 11 amino acid residues comprised the largest fraction of plasma peptide-bound amino acids, followed by dipeptides in sheep, calves, and rats. These in vivo studies indicate that high concentrations of circulating oligopeptides are available for animal tissues to utilize. This suggests that it is likely that animal tissues should have developed some mechanism(s) to utilize the circulating peptides as amino acid sources. The utilization of oligopeptides also has been observed in other species. A methionine auxotroph of S. cerevisiae (Naider et

al. 1974) could grow on methionine-containing oligopeptides up to five amino acid residues (pentamethionine) in length. Taylor et al. (1950) showed that a methionineless mutant of E. coli could utilize methionine-containing tripeptides (methionylglycylglycine and glycylmethionylmethionine). Three methionine auxotrophs grew in the presence of methionine-containing oligopeptide with up to four amino acid residues (Wolfinbarger and Marzluf, 1974).

The results presented in Table 4.2 show that the protein accretion in both cell lines was moderately related to the hydrophobicity of the dipeptides tested. Specifically, for seven x-methionine peptides, there was a linear relationship ($P < .0001$) between peptide hydrophobicity (or x amino acid hydrophobicity) and protein accretion in both cell lines. The efficiency with which the dipeptides were utilized for protein accretion increased with increasing hydrophobicity. For seven methionine-x peptides, a quadratic response ($P < .0001$) in C_2C_{12} cells was obtained between the peptide hydrophobicity (or x amino acid hydrophobicity) and protein accretion with the greatest protein accretion occurring at .60 on the Fauchere hydrophobicity scale (Figure 4.8). With the seven methionine-x peptides, a linear response ($P < .0035$) in MAC-T cells was observed between peptide hydrophobicity (or x amino acid hydrophobicity) and protein accretion. Protein accretion increased with increased hydrophobicity. These findings indicate that these cells have developed the ability to utilize hydrophobic peptides somewhat more readily than hydrophilic peptides. Gardner and Wood (1989) reported that hydrophobic and mucosal hydrolysis-resistant peptides are more likely to be absorbed faster than hydrophilic and easily-hydrolyzed peptides. Adibi and Soleimanpour (1974) have shown that the dipeptide transport system in human jejunum has a higher affinity for dipeptides

with hydrophobic amino acids. Burston and Matthews (1990), however, did not find any relationship between the affinity for transport and the hydrophobicity of peptides in the hamster jejunum, suggesting that the peptide transport system of the small intestine may have a broad specificity for peptide substrates. A considerable portion of the circulating peptide-bound amino acids may come from gastrointestinal absorption (Gardner et al., 1983; Koeln et al., 1993). This means that the peptides released by the gastrointestinal tract determine the chemical properties of the circulating peptides. Daniel et al. (1992) found that the oligopeptide/H^+ symporter in the renal brush border membrane possesses a higher affinity for di- and tripeptides with hydrophobic amino acids. The permeability of the glomerular membrane is 100 to 500 times as great as that of the ordinary capillary, and substrates with a molecular weight of 5,000 Da or less readily can pass through the glomerular membrane and enter the Bowman's capsule lumen (Guyton, 1986). This means that circulating peptide-bound amino acids can enter the Bowman's capsule. But the urinary loss of peptides is very small (Adibi and Johns, 1983; Steinhardt et al., 1984). So the major function of the symporter is to reabsorb peptides of plasmic origin from the glomerular filtrate during the formation of urine, and the high affinity for hydrophobic peptides may suggest that the majority of the reabsorbed peptides (from the glomerular filtrate) of plasma origin are relatively hydrophobic. If this speculation is true, it means that animal tissues, such as skeletal muscle and mammary gland, are normally exposed to relatively hydrophobic peptides in the circulation and have developed the ability to utilize hydrophobic peptides better than hydrophilic peptides.

Results from the present study show that cultured myogenic and mammary epithelial cells can flourish on

methionine-containing peptides of up to at least eight amino acid residues in length in the presence of 6% dFBS. Krzysik and Adibi (1979) showed that radioactivity from injected radiolabeled glycylglycine appeared in both the amino acid pool and the protein pool of the skeletal muscle of rats. Although they did not investigate the mechanism of peptide utilization by the skeletal muscle, the results clearly indicate that skeletal muscle can utilize dipeptides in the blood for protein synthesis. Adibi and Morse (1981) concluded that the skeletal muscle had no active transport system for glycylglycine, and the observed utilization of this dipeptide was due to diffusion and subsequent intracellular hydrolysis. Raghunath et al. (1990) reported that hydrolysis of peptides by sarcolemmal membrane-bound peptidases with subsequent uptake of released free amino acids was the major mechanism of peptide utilization by skeletal muscle. The findings in the current experiment are consistent with the concept that skeletal muscle has the ability to utilize circulating peptides for protein synthesis. There is little information on peptide utilization in the mammary gland. One report indicated that methionine-containing peptides could promote greater production of secreted proteins by cultured tissue explants of mouse mammary glands (Wang et al., 1993). The findings in the present study also indicate that mammary epithelial cells possess the ability to utilize peptides for protein accretion. Further investigations are needed to elucidate the mechanisms behind these responses.

Implications

The C_2C_{12} myogenic and MAC-T mammary epithelial cells appear to be able to utilize small methionine-containing peptides as sources of methionine to support protein accretion. Dipeptides with methionine at the N-terminus appear to be preferred substrates. Hydrophobicity or some

related characteristic of dipeptides is also moderately related to the efficiency with which these dipeptides are used to promote protein accretion. Results showed that cell culture may be a useful approach for evaluating the ability of a tissue to utilize peptides as sources of amino acids for protein synthesis. Further investigations are needed to ascertain the mechanisms responsible for the observed C_2C_{12} and MAC-T cell growth responses.

Table 4.1. Peptides examined and hydrophobicity values of selected dipeptides.

Symbol	Peptide	Hydrophobicity value
MA	Methionylalanine	1.06
AM	Alanylmethionine	1.06
MF	Methionylphenylalanine.	.32
FM	Phenylalanylmethionine	.32
MG	Methionylglycine	1.215
GM	Glycylmethionine	1.215
ML	Methionylleucine	.365
LM	Leucylmethionine	.365
MP	Methionylproline	.97
PM	Prolylmethionine	.97
MS	Methionylserine	1.235
SM	Serylmethionine	1.235
MV	Methionylvaline	.605
VM	Valylmethionine	.605
MM	Methionylmethionine	.60
MAS	Methionylalanylserine	
MLF	Methionylleucylphenylalanine	
GGFM		
YGGFM		
YGGFMK		
YGGFMRF		
YGGFMRGL		

A = alanine, F = phenylalanine, G = glycine, K = lysine, L = leucine, P = proline, R = arginine, S = serine, V = valine, Y = tyrosine

Table 4.2. Relationship between methionine-dipeptide hydrophobicity and protein accretion in C_2C_{12} and MAC-T cells

Cell line	Dipeptide	Linear[a]	r^2	Quadratic[a]	r^2
C_2C_{12}	X-Methionine	.0001	.42	.0001	.47
C_2C_{12}	Methionine-X	.0128	.10	.0001	.43
MAC-T	X-Methionine	.0001	.57	.0001	.57
MAC-T	Methionine-X	.0035	.28	.0106	.31

[a]Probability of a linear or quadratic effect.

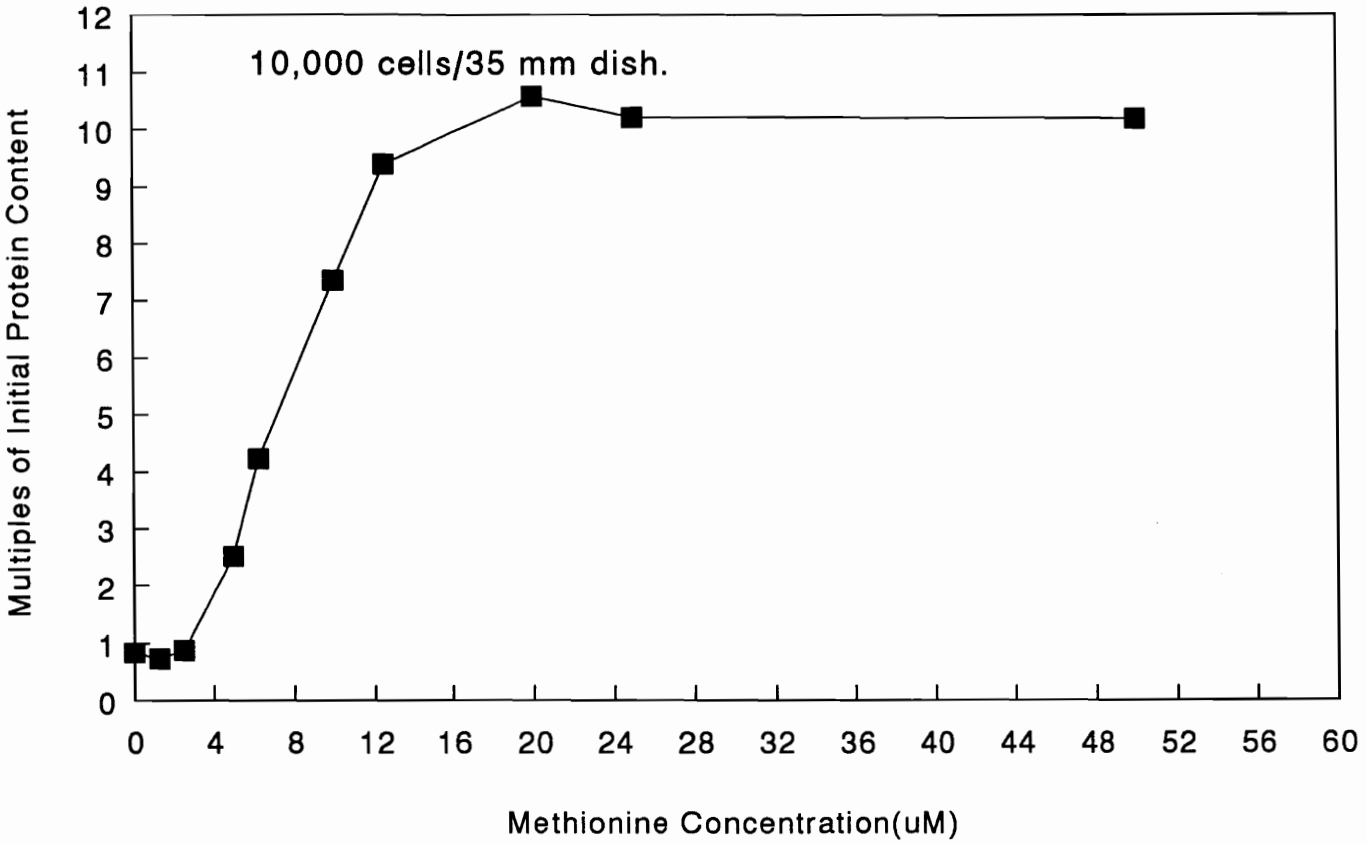


Figure 4.1. Methionine standard curve for protein accretion in C2C12 myogenic cells

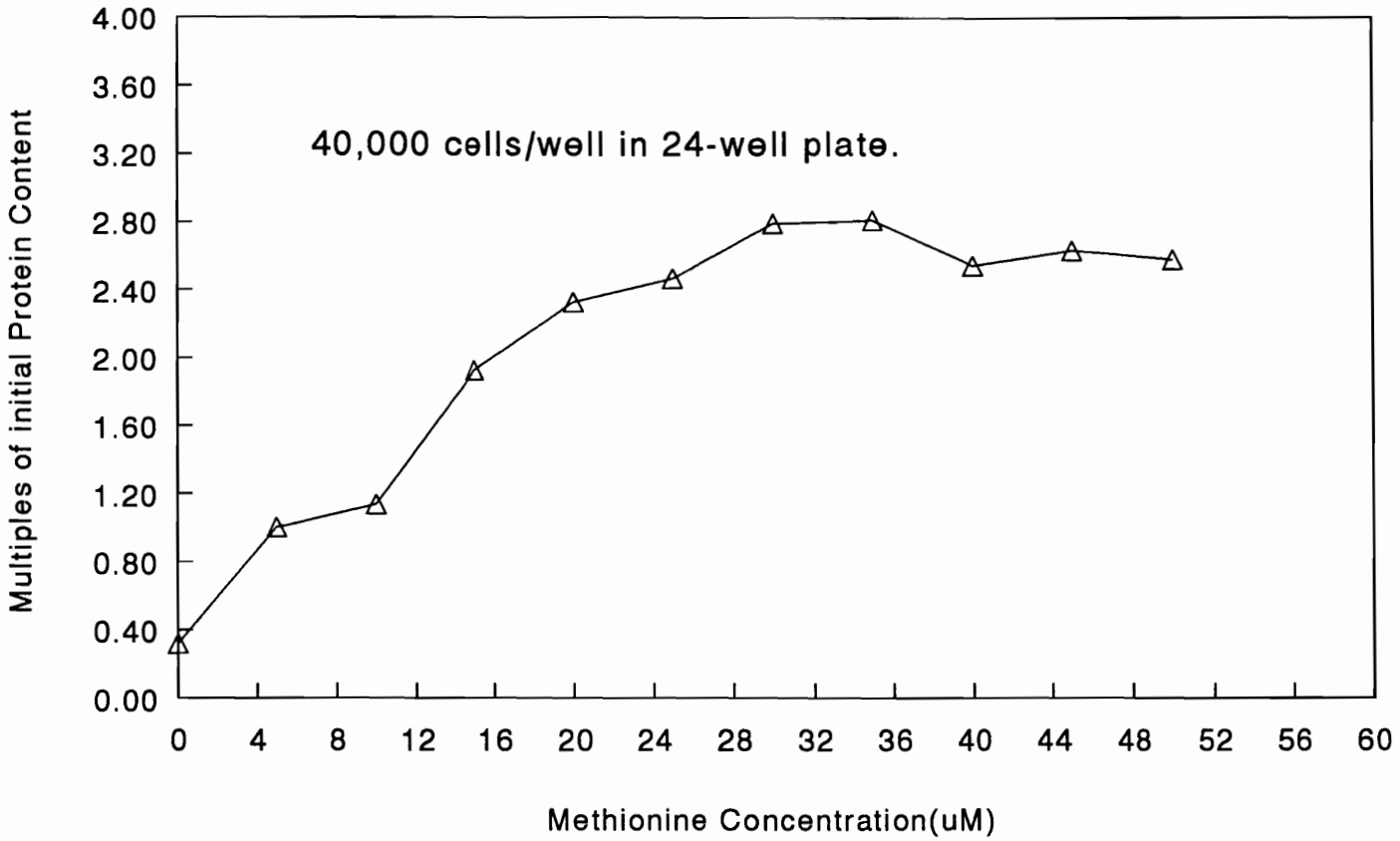


Figure 4.2. Methionine standard curve for protein accretion in MAC-T cells

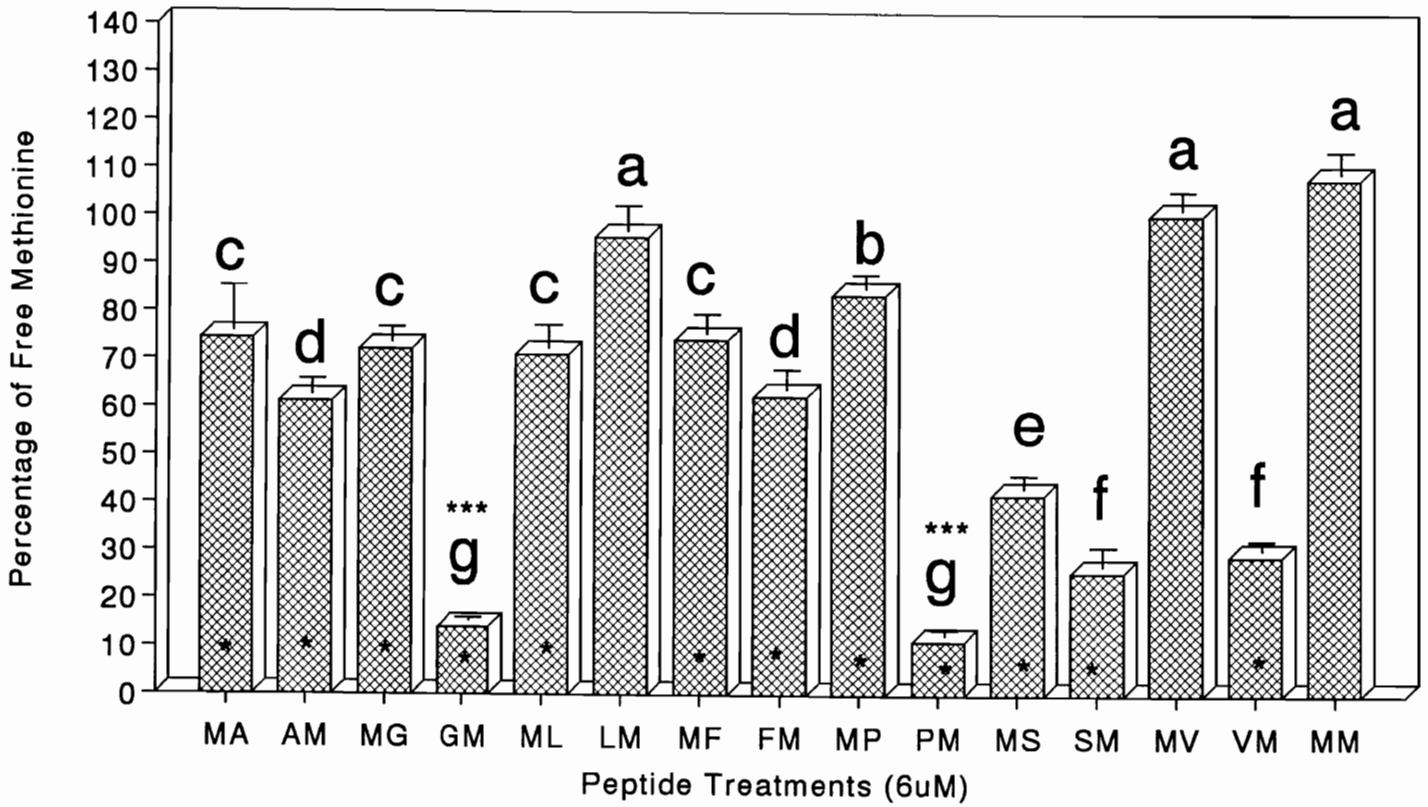


Figure 4.3. Effect of methionine-dipeptide on protein accretion in C2C12 cells. Bars (means+SE) with different letters differ (P < .05). n = 9. * Different from met (P < .05). *** Not different from initial level.

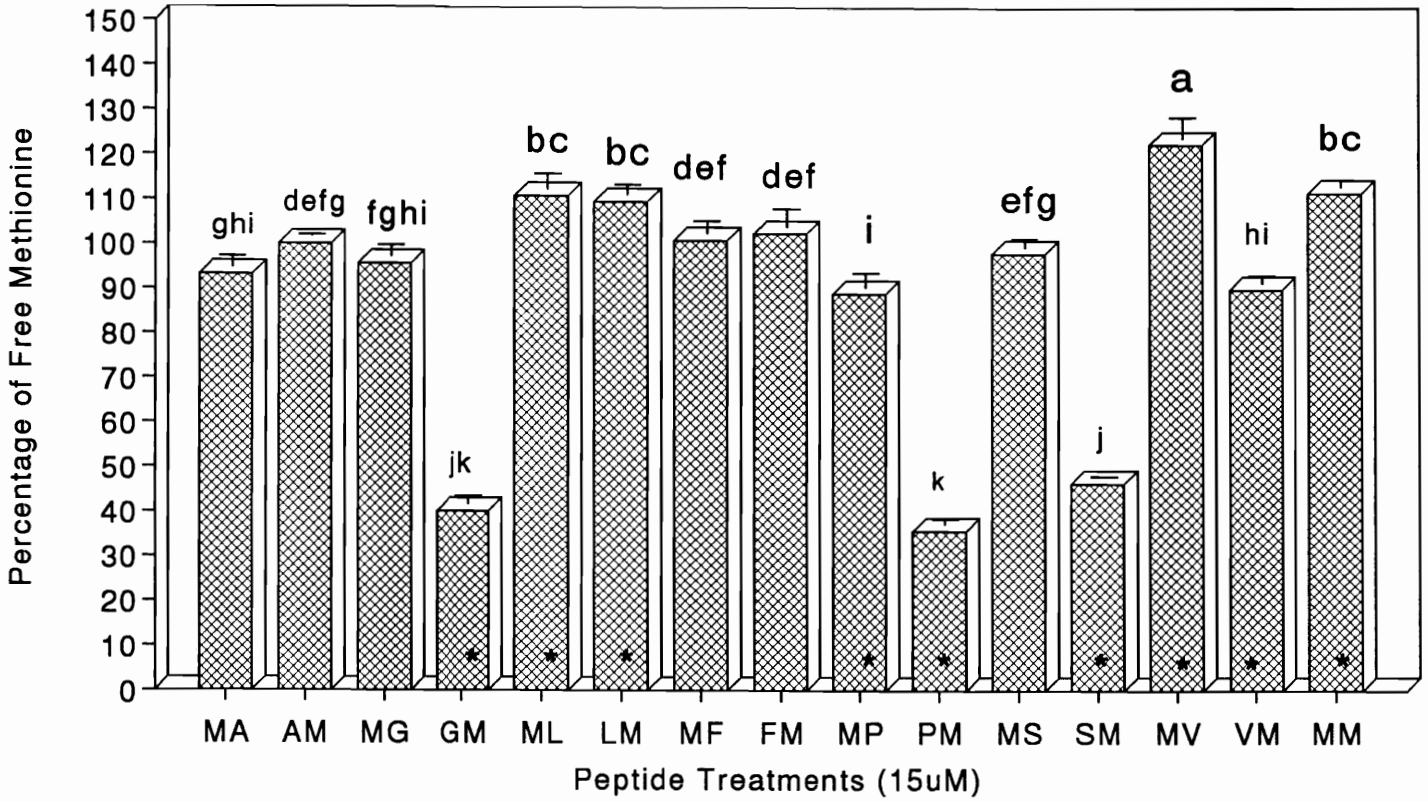


Figure 4.4. Effect of methionine-dipeptides on protein accretion in MAC-T cells. Bars (means+SE) with different letters differ ($P < .05$). * Different from L-methionine ($P < .05$). $n = 4$.

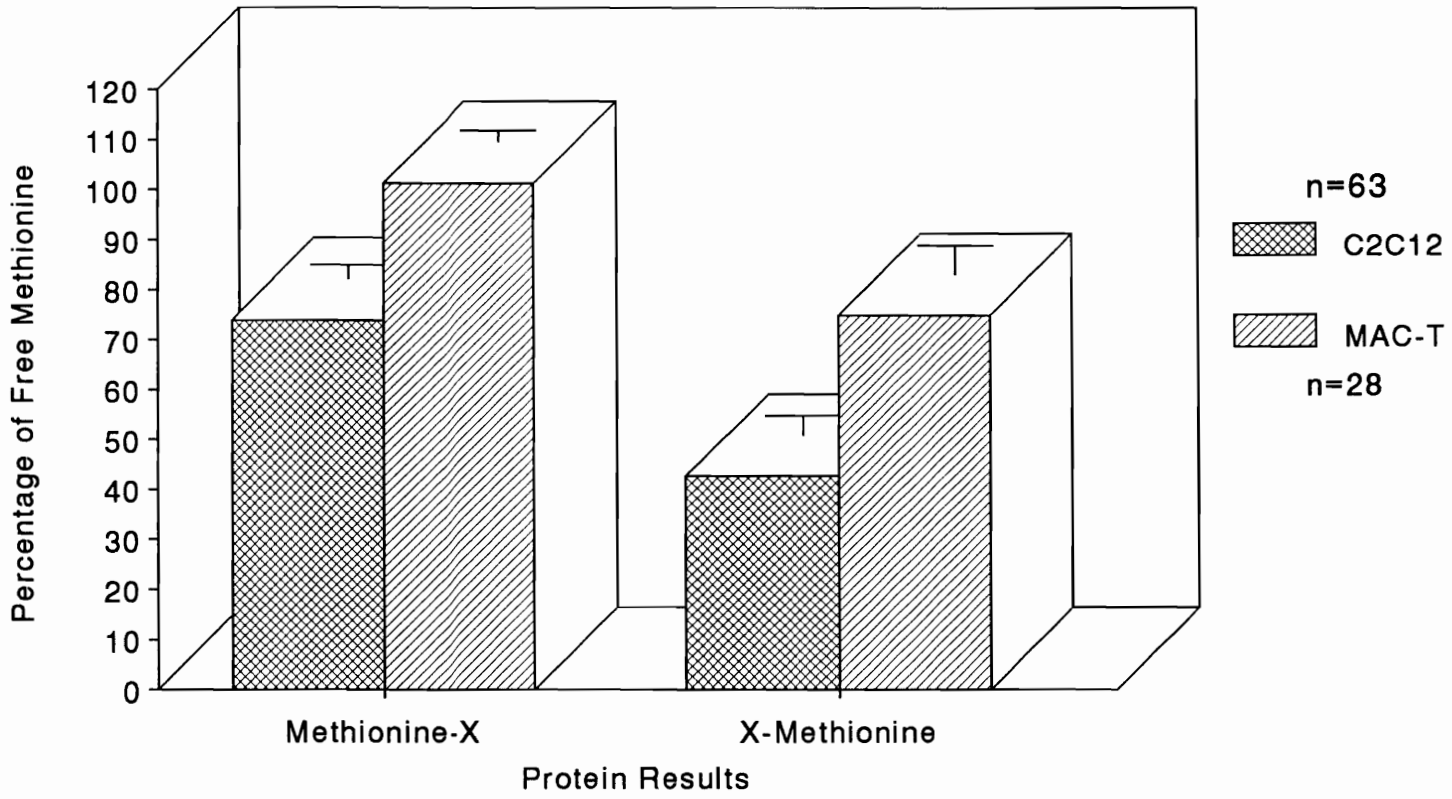


Figure 4.5. Effect of methionine position in dipeptides on protein accretion in C2C12 and MAC-T. Seven methionine-x and seven x-methionine peptides were compared. Protein accretion was greater with methionine-x peptides ($P < .05$).

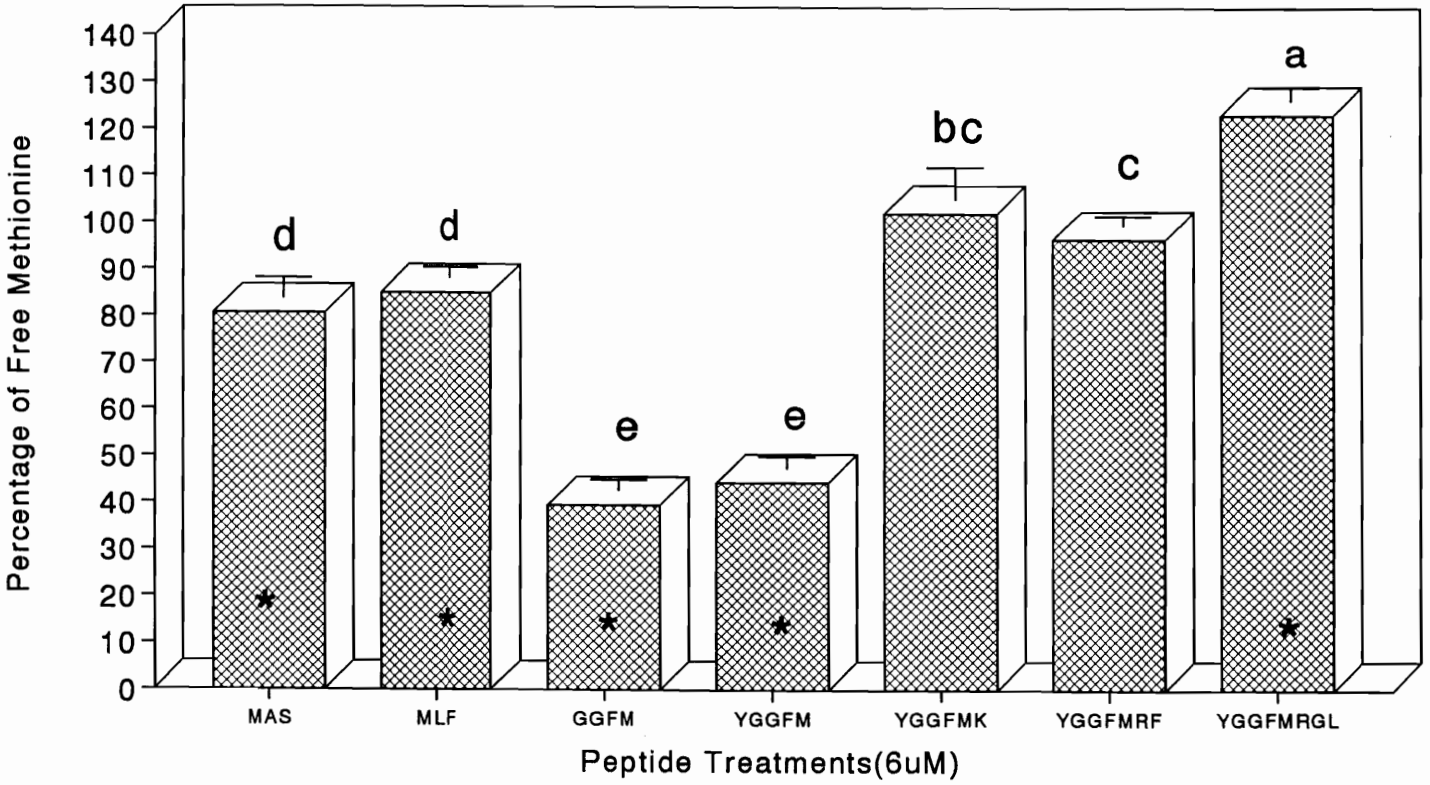


Figure 4.6. Effects of tri- to octapeptides on protein accretion in C2C12 cells. Bars (means+SE) with different letters differ ($P < .05$). * Different from methionine ($P < .05$). n = 8.

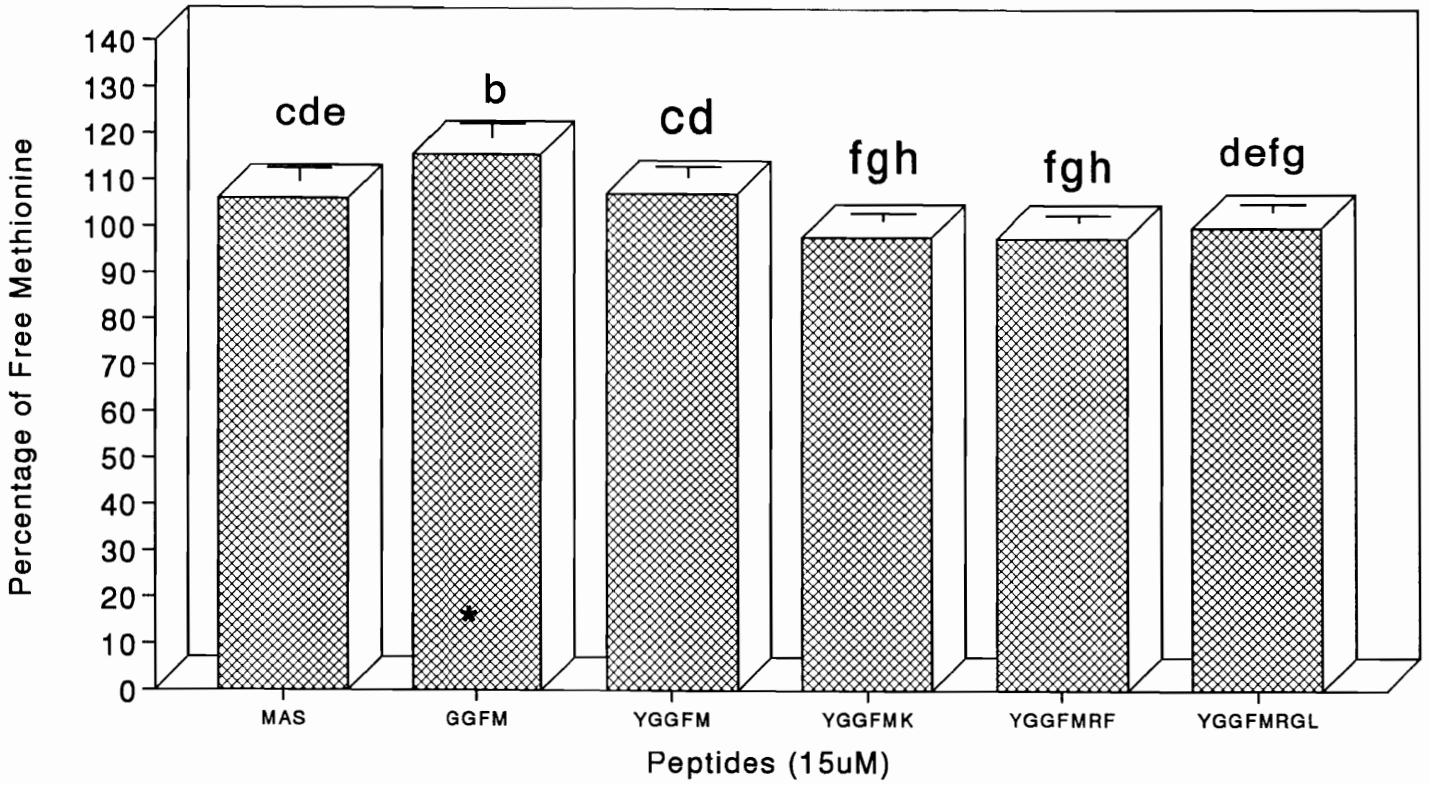


Figure 4.7. Effect of tri- to octapeptides on protein accretion in MAC-T cells. Bars (means+SE) with different letter differ ($P < .05$). * Different from L-methionine ($P < .05$). $n = 4$.

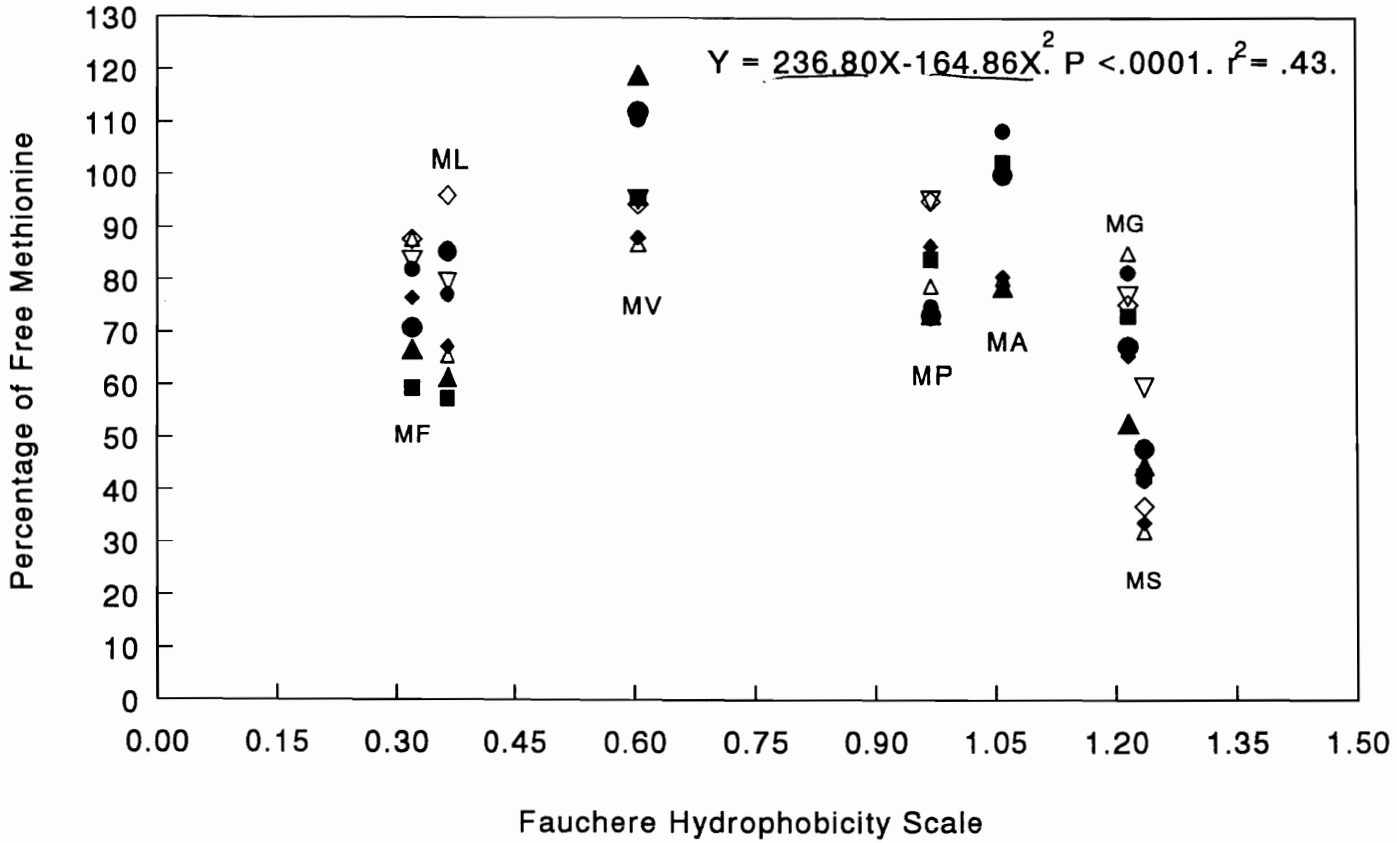


Figure 4.8. Relationship between methionine-x hydrophobicity and protein accretion in C2C12 cells

Chapter V
Serumal Factor(s) Promotes Peptide Utilization in Cultured Animal Cells

ABSTRACT

MACT-T and C_2C_{12} cells, as indicated in Chapter IV, utilized a number of methionine-containing di- to octapeptides as sources of methionine to support protein accretion and cell proliferation in the presence of 6% desalted fetal bovine serum. In the present study, serumal factors that may be involved in regulating the use of peptides as amino acid substrates for protein accretion and cell proliferation in C_2C_{12} and MAC-T cells were examined. The basal media contained methionine-free DMEM supplemented with .4% bovine serum lipids, 1% chemically defined lipid concentrate, bovine insulin (1 ug/mL), 3% low protein serum replacement (LPSR-1) or 6% desalted animal serum. Treatment media included basal media supplemented with no methionine, L-methionine, or one of the methionine-containing peptides. L-methionine promoted protein and DNA accretion ($P < .05$) in the presence of desalted animal sera, insulin, or LPSR-1. Methionine-containing peptides also promoted protein and DNA accretion($P < .05$) in the presence of desalted animal serum or LPSR-1, but not with insulin, except methionylleucine. In a cell-free medium, fetal bovine serum was shown to hydrolyze peptides to varying degrees. Results of this study indicate that animal sera contain some factor(s) that regulate(s) utilization of peptides as amino acid sources for C_2C_{12} and MAC-T cells. Peptidases may be largely responsible for the stimulatory effect serum has on peptide utilization.

(Key Word: Peptide, Myogenic, Mammary epithelial cells, Serum, Serum replacement)

Introduction

A significant portion (10% to 78%) of the plasma amino acid pool has been shown to be in the form of peptide-bound amino acids in several species of animals (Christensen et al., 1947; Gardner et al., 1983; Koeln et al., 1993). Ruminants seem to have higher plasma peptide-bound amino acids than non-ruminants (DiRienzo, 1990; Seal and Parker, 1991; Koeln et al., 1993). The functions of these circulating peptides are still uncertain. Intravenously-injected peptides were cleared from the blood and with radiolabeled or hydrolysis-resistant peptides, the clearance of injected peptides from the plasma was accompanied by the appearance of radioactivity or intact hydrolysis-resistant peptides in tissues including liver, muscle, kidney, gut, lung, brain, and pancreas (Adibi, 1987b; Stehle et al., 1991). These results suggest that peptides may serve as sources of free amino acids. Certain peptides may have various regulatory impacts on cell growth and functions. For instance, glycylhistidyllysine and glycyllysylhistidine, have been shown to prolong the survival of normal liver cells and stimulate growth in neoplastic liver (Pickart and Thaler, 1973).

Results from an earlier study (Chapter IV) indicated that C_2C_{12} myogenic and MAC-T mammary epithelial cells were able to utilize a variety of methionine-containing peptides (at least up to octapeptides) as methionine sources for protein accretion in the presence of 6% desalted fetal bovine serum. An objective of the present study was to determine whether cultured C_2C_{12} and (or) MAC-T cells are able to utilize methionine-containing peptides to support protein accretion and(or) cell proliferation in a serum-free medium with and without the supplementation of lipids, insulin, or a serum substitute. Another objective was to determine whether cultured MAC-T cells are able to utilize

methionine-containing dipeptides for protein accretion and cell proliferation in the presence of desalted adult animal sera from several species.

Materials and Methods

Methionine-containing Peptides The methionine-containing peptides[1] used in this study and their hydrophobicity values are presented in Table 4.1. The purity of these peptides was examined by HPLC analysis and the hydrophobicity values were calculated and were reported previously (Chapter IV).

Media Preparation. The methionine-free Dulbecco's modified Eagle's medium (MFDMEM) was prepared by adding glutamine (58.4 mg/100 mL medium)[2] and 1% (v/v) antibiotic-antimycotic solution[3] to deficient Dulbecco's modified Eagle medium[4]. The resultant medium was sterilized by filtration[5]. Adult animal sera[6] including human serum (HuS), horse serum (HS), porcine serum (PS), rabbit serum (RS) and chicken serum (CS) were desalted by gel filtration chromatography using a Sephadex G-25M desalting column[7]. The basal media contained MFDMEM supplemented with .4% bovine serum lipids (SL)[8], 1% chemically defined lipid concentrate (CL)[9], bovine insulin (1 ug/mL)[10], 3% low protein serum replacement (LPSR-1)[11] or 6% of one of the desalted animal sera.

The treatment media consisted of the appropriate basal medium supplemented with either free L-methionine[12] (6 uM,

---

[1]Sigma Chemical Co., St. Louis, MO
[2]Sigma Chemical Co., St. Louis, MO
[3]Cat. No. 600-5240AG, GIBCO, BRL, Inc., Grand Island, NY
[4]GIBCO, BRL, Inc., Grand Island, NY
[5]Cat. No. 09-730-218, Nalge Company, Rochester, NY
[6]GIBCO, BRL, Inc., Grand Island, NY
[7]Pharmacia LKB Biotechnology, Piscataway, NJ
[8]Sigma Chemical Co., St. Louis, MO
[9]GIBCO, BRL, Inc., Grand Island, NY
[10]Sigma Chemical Co., St. Louis, MO
[11]Sigma Chemical Co., St. Louis, MO
[12]Sigma Chemical Co., St. Louis, MO

C_2C_{12}; 15 uM, MAC-T) or one of the methionine-containing peptides at concentrations that were equivalent to L-methionine in methionine content.

The growth medium used to maintain and propagate C_2C_{12} myogenic cells consisted of Dulbecco's modified Eagle's medium (DMEM)[13], 1% (v/v) antibiotic-antimycotic solution and 15% (v/v) FBS[14]. The growth medium for MAC-T mammary epithelial cells was composed of DMEM, 1% antibiotic-antimycotic solution and 10% FBS.

Cell Culture Procedure. The C_2C_{12} and MAC-T cells were plated at 10,000 cells per well (6 well-plate)[15] and 40,000 cells per well (24-well plate)[16], respectively. The C_2C_{12} cells were incubated in growth medium at 37°C in a humidified environment of 90% air, 10% CO_2 and MAC-T cells were incubated in growth medium at 37°C in a humidified environment of 95% air, 5% CO_2. The cultures were incubated for 24 h, and then the growth medium was replaced by MFDMEM, and the cells were incubated for another 24 h. Subsequently, MFDMEM was removed from the cultures and the starved cells were then incubated with one of the treatment media for 72 h. The treatment media were changed at 24-h intervals.

Harvest and Analytical Procedures. Protein and DNA were quantified following 72 h of incubation either on separate or the same culture plate/well. When separate plates/wells were assayed, the cultures were washed twice with ice-cold Dulbecco's Phosphate Buffered Saline (D-PBS)[17] and were prepared and analyze for protein content using the enhanced bicinchoninic (BCA)[18] assay of Smith et al.(1985) as previously described (Chapter IV). Cultures for DNA

---

[13]GIBCO, BRL, Inc., Grand Island, NY
[14]GIBCO, BRL, Inc., Grand Island, NY
[15]Fisher Scientific, Pittsburgh, PA
[16]Fisher Scientific, Pittsburgh, PA
[17]Cat. No. 450-1600EB, GIBCO, BRL, Grand Island, NY
[18]Pierce (Rockford, IL)

determination were harvested and the DNA concentrations were determined by measuring the fluorescence produced by the interaction between sample DNA and the fluorochrome Hoechst 33258 (Labarca and Paigen, 1980).

When protein and DNA were quantified from the same plate/well, the cultures were washed once with D-PBS, and 500 uL of buffer (pH 7.4) containing .05 M Na_2HPO_4, 2 M NaCl, and .002 M EDTA was added to each well, then the cultures were sonicated for 15 s by a Sonic Dismembrator Model 300[19]. Then 100 uL of the sonicated sample was transferred for DNA assay as described by Labarca and Paigen (1980) and the rest of the sonicated sample was treated with 400 uL 1N NaOH overnight (or 18 h). Then the NaOH-treated sample was neutralized with 80 uL 5N HCl solution, and the neutralized sample was used for protein assay by the BCA procedure (Smith et al., 1985).

Fetal Bovine Serum Peptidase Activity. The methionine-containing peptides (15 uM) were added to 15 mL sterile centrifuge tubes containing 12 mL of the MFDMEM supplemented with 6% desalted FBS and the mixed solutions were then sealed with caps and incubated at 37°C for 24 h. Before and after the 24-h incubation, samples were taken and filtered through ultrafree MC filters[20] of with a 10,000 Da cutoff to remove large molecules. A 40 uL aliquot of the resulting filtrate was processed for HPLC analysis as described previously (Chapter IV) to detect the appearance of free methionine in the samples.

Statistical Analysis. Treatments were replicated in four wells or dishes. Data were analyzed by the GLM procedure of SAS (1989) with culture dish or well as the experimental unit. The ANOVA model was:

$$Y_{ij} = \mu + \alpha_i + E_{ij}$$

[19]Fisher Scientific, Pittsburgh, PA
[20]Waters Millipore Corp., Milford, MA

Where:

Y_{ij} = jth protein or DNA response from the ith treatment

μ = overall mean

α_i = effect of ith treatment

E_{ij} = error component associated with the jth protein response from the ith treatment

The model for LPSR-1 supplementation was: $Y_{ij} = \mu + \alpha_i + \beta_j + \Gamma_{ij} + E_{ij}$

Where:

Y_{ij} = protein or DNA content obtained from the ith substrate in the presence or absence of LPSR-1

μ = overall mean

α_i = effect of substrate i

β_j = effect of LPSR-1

Γ_{ij} = interaction between substrates and LPSR-1

E_{ij} = error component

Means of different peptide treatments were compared using Duncan's means separation test at an alpha value of .05.

Results and Discussion

In a previous study, it was determined that the supplemented peptides were not contaminated with free methionine and that the desalting process effectively eliminated free methionine from serum (Chapter IV). In the same study, it was determined that free L-methionine concentrations of 6 and 15 uM in treatment media for C_2C_{12} and MAC-T cells, respectively, were appropriate for measuring cell responses to the presence of methionine-containing peptides.

Previously it was observed that methionine-containing peptides could serve as sources of methionine to support protein accretion in cultured C_2C_{12} myogenic and MAC-T mammary epithelial cells when the peptides were added to a medium composed of MFDMEM and supplemented with 6% desalted

FBS (Chapter IV). The data presented in Table 5.1 show that MAC-T mammary epithelial cell cultures were unable to maintain their initial protein content and cell numbers during 72 h of incubation in serum-free MFDMEM. These cells were able to accumulate small amounts of protein, but did not proliferate, as indicated by similar DNA content, in the serum-free medium containing free methionine. The growth responses to methionine-containing dipeptides were quite different among peptides. The MAC-T cells failed to maintain the initial protein contents in the presence of any of the dipeptides and managed to maintain the cell numbers only in the presence of methionylleucine, leucylmethionine, methionylphenylalanine, and phenylalanylmethionine. Methionylproline, and prolylmethionine were markedly ineffective as sources of free methionine in the serum-free and methionine-free DMEM. The protein:DNA ratio remained the same as the initial ratio in the presence of free methionine or phenylalanylmethionine. All other treatments resulted in lower ($P < .05$) protein:DNA ratios.

Because it appeared that desalted FBS was necessary for efficient peptide utilization to occur, several other sera were examined for their ability to stimulate the use of methionine-containing peptides as sources of methionine for cell proliferation and protein accumulation by cultured MAC-T cells. Desalted sera from adult pigs, horses, rabbits, humans, and chickens were tested. The data for protein accumulation and cell proliferation are presented in Tables 5.2, 5.3, and 5.4 for the sera from pigs, horses, and rabbits, respectively. For all three species, cultures in MFDMEM supplemented with the desalted sera alone resulted in a reduction ($P < .05$) of both protein content and cell number in comparison to initial values. Generally, MAC-T cells cultured in MFDMEM containing 6% desalted sera from any species and any of the dipeptides accumulated ($P < .05$)

protein and increased ($P < .05$) cell number. An exception occurred with prolylmethionine. This dipeptide was not utilized in the presence of serum from the pig or horse. Only data for protein accumulation were obtained from sera from the human and chicken and these data are presented in Figures 5.1 and 5.2, respectively. Like in the other species, MFDMEM supplemented with either human or chicken serum, but with no methionine supplementation, was unable to support protein accumulation in cultured MAC-T cells. Inclusion of an amino acid substrate generally resulted in protein accumulation. Overall, however, the desalted chicken serum supported the least protein accumulation among the various substrates in comparison to other species. Whatever factor(s) that is responsible for stimulating the utilization of the amino acid substrates appears to be present in lower amounts in adult chicken serum.

We have observed that the location of methionine in dipeptides influences its use by cultured cells (Chapter IV). As in our previous observation, the present results indicate that dipeptides with methionine at the N-terminus resulted in a greater utilization than dipeptides with methionine at the C-terminus. Among the dipeptides tested, the single exception in the presence of sera from pigs, rabbits, chickens, humans and cattle was with the dipeptide pair, methionylleucine and leucylmethionine. With these dipeptides, methionine in the C-terminus generally was more favorably utilized.

It may be possible that the serumal factor(s) that facilitates the utilization of methionine from methionine-containing peptide is a hydrolase(s). Hydrolytic activity of desalted FBS against a number of methionine-containing peptides was examined in a cell-free incubation. After 24 h of incubation in the cell-free medium, essentially all of the methionine contained in alanylmethionine,

methionylphenylalanine and the enkephalin segments was released into the medium (Figure 5.3). The released methionine then could be absorbed by the cells and utilized in the same manner as if free methionine was added to the medium. The remaining di- and tripeptides were hydrolyzed less extensively with methionine release ranging from 42 to 70%. Piez et al. (1960) reported that, after 3-d storage of dialyzed human serum samples at 37°C, the concentrations of valine, methionine, isoleucine, leucine, threonine, phenylalanine, lysine, histidine, and arginine were 88, 29, 22, 180, 83, 59, 112, 43, and 106 uM , respectively. At the same time, significant amounts of nonessential amino acids also accumulated in the serum samples. This clearly shows that animal serum has hydrolytic enzymes that can release amino acids from plasma peptides. Plasma peptidase activities against a number of peptides were reported by Krzysik and Adibi (1977), Lochs et al. (1988), and Stehle and Furst (1990). Because the tetra- to pentapeptides used in the present study were methionine-enkephalin segments and because animal sera have been shown to contain aminopeptidase M, dipeptidyl carboxypeptidases, and angiotensin-converting enzyme which are involved in the hydrolysis of enkephalins in plasma (Shibanoki et al., 1991, 1992), it is not surprising to see this extent of methionine release. The observed serumal factor(s) in animal sera that facilitates the utilization of methionine-containing peptides may be the above-mentioned enkephalin-hydrolyzing enzymes and other peptidases.

One preliminary experiment (Figures 5.4 and 5.5) indicated that, contrary to the results observed in the presence of 6% desalted FBS (Chapter IV), both methionylglycine and glycylmethionine failed to serve as methionine sources for MAC-T cells in the presence of bovine insulin, though free methionine promoted protein accretion

and cell proliferation in the presence of insulin. A subsequent experiment was conducted to investigate the ability of MAC-T cells to utilize other methionine-containing dipeptides in the presence of bovine insulin. The results are shown in Table 5.5. The MAC-T cells, in the absence of animal serum, increased their protein mass and DNA content in the presence of free methionine and bovine insulin ($P < .05$), indicating that insulin had both cell proliferation and anabolic effects on cultured MAC-T mammary epithelial cells in the presence of this free amino acid. This was consistent with the observation that insulin had mitogenic effect on mammary epithelial cells (Baumrucker and Stemberger, 1989). Kasuga et al. (1981) proposed that insulin exhibits its mitogenic effect via the receptor of insulin-like growth factor-I (IGF-1). Because the insulin concentration used in current experiment was very high (1 ug/mL), it is possible that the response elicited by insulin was due to the binding of insulin to IGF-1 receptor. Contrary to its growth-promoting effects in the presence of free methionine, insulin was essentially ineffective in promoting either protein accumulation or cell proliferation in MAC-T cells in the presence of the methionine-containing dipeptides tested. With the exception of methionylleucine that resulted in a slight increase ($P < .05$) in protein content and cell number above initial levels, the response to all other peptides in the presence of insulin was either the maintenance or loss of protein and DNA in comparison of starting levels. Leucylmethionine, methionylphenylalanine, and phenylalanylmethionine, in the presence of insulin, were able to support the maintenance of initial protein and DNA levels. This means that some protein synthesis was taking place (insulin alone was lower), but cell proliferation was not occurring. A second group of relatively hydrophilic peptides including methionylproline, prolylmethionine,

methionylvaline, and valylmethionine were unable to support maintenance of initial levels of either protein or cell number in the presence of insulin. Insulin exhibits anabolic effects on protein metabolism and stimulates the uptake of neutral amino acids in several tissues including skeletal muscle and the non-ruminant mammary gland (Anderson and Rilema, 1976; Granner, 1990). The present results also show that MAC-T mammary epithelial cells can grow well on free methionine in the presence of bovine insulin. Differences in protein contents between the absence and presence of insulin were also observed in methionylleucine (24.98 vs 36.90 ug/well), leucylmethionine (16.34 vs 27.08 ug/well), methionylphenylalanine (21.60 vs 28.37 ug/well) MAC-T cells. Insulin had no effect on the growth of MAC-T cells with glycylmethionine, methionylglycine , phenylalanylmethionine, methionylproline, prolylmethionine, methionylvaline, or valylmethionine in the culture medium. To the contrary, fetal bovine serum had stimulatory impacts on the growth of MAC-T cells on all above-mentioned dipeptides (Chapter IV). While there was some effect on protein synthesis, bovine insulin seems to have limited stimulating effects on the utilization of the methionine-containing dipeptides tested. Because the insulin concentration used in the present study was very high (1 ug/mL), it is likely that insulin-like growth factors are not among the serumal factors of fetal bovine serum that promote the utilization of methionine-containing dipeptides. It appears that there may be some relationship between the observed responses and dipeptide hydrophobicity. The peptides in the second group above are much less hydrophobic than the other dipeptides tested. The specific nature of their relationship is not apparent. These results are consistent with our earlier report that, in MAC-T cells, protein accretion increased with increased hydrophobicity of methionine-containing dipeptides (Chapter

IV). Several reports have appeared to suggest that significant amounts of relatively hydrophobic peptides may exist in the circulation as sources of the corresponding free amino acids. First, it was shown that the dipeptide transport system in human jejunum had higher affinity for dipeptides with hydrophobic amino acids. Gardner and Wood (1989) reported that hydrophobic and mucosal-hydrolysis resistant peptides are more likely to be absorbed faster than hydrophilic and hydrolysis-susceptible peptides. Daniel et al. (1992) showed that the oligopeptide/H^+ symporter in the brush border membrane had a higher affinity for di- and tripeptides with hydrophobic amino acids. The major function of the symporter is to reabsorb peptides of plasmic origin from the glomerular filtrate during the formation of urine. The high affinity for hydrophobic di- and tripeptides may imply that the bulk of the peptides of plasmic origin in the glomerular filtrate are relatively hydrophobic. Therefore, it is reasonable that animal cells, such as cultured MAC-T cells, have developed the ability to utilize hydrophobic peptides better than hydrophilic ones. In terms of protein contents of the cultures after 72 h of incubation, methionine dipeptides with the methionine residue at the N-terminus resulted in greater ($P < .05$) protein contents than those with methionine residues at the C-terminus (Table 5.5). Dipeptides with the same amino acid compositions had similar effects on DNA content of cultures, indicating comparable cell proliferation rates. It appears, therefore, that cell proliferation may have been more important than protein synthesis.

Any culture medium supplemented with an
presumably contains essential fatty acids fr
serum that are necessary for normal cell grc
serum-free DMEM supplemented with bovine ins
essential fatty acids and because cell prol

appeared to be impaired more than protein synthesis, it was felt that an essential fatty acid deficiency may be responsible for the observed responses. Adding neither .4% of a bovine serum lipid mixture nor .1% of a chemically defined lipid concentrate in combination with insulin resulted in protein accumulation above initial levels in the presence of methionylproline or prolylmethionine (Figure 5.6). The addition of the bovine serum lipid mixture did result in a lower net protein synthesis in the presence of free methionine. Therefore, it appears that essential fatty acids were not the limiting factor for the growth of MAC-T cells on methionine-containing dipeptides in response to insulin stimulation.

The growth of MAC-T cells on several methionine-containing di-, tri-, tetra- and pentapeptides was evaluated in the presence of LPSR-1. The LPSR-1 contains growth-enhancing factors and carrier proteins and its components include albumin, transferrin, insulin, other hormones and growth factors, vitamins, attachment factors, and soybean trypsin inhibitor. The actual composition is proprietary information of the manufacturer. Several cell lines including human lung fibroblasts, kidney cells (MDCK), Buffalo Green Monkey (BGM) kindey cells and Vero 76 cells (African green monkey kidney cells) were shown to grow in medium supplemented with LPSR-1 (Candal et al., 1989). Results from the present study (Table 5.6) showed that the inclusion of LPSR-1 in the medium resulted in protein accretion and cell proliferation ($P < .0001$) where substrate was present. The serumal factor(s) that facilitates the utilization of methionine-containing peptides appears also to be present in LPSR-1. As in several previous experiments, the methionine-containing peptides examined in this experiment were used to varying degrees as sources of methionine for protein accretion and cell proliferation.

The effect of levels of addition of LPSR-1 to the culture medium was examined in a second cell line, C_2C_{12} myogenic cells (Table 5.7). There was a direct relationship between level of LPSR-1 and protein accretion. Adding 3% LPSR-1 to the medium resulted in a greater ($P < .0001$) protein accretion than adding 1% LPSR-1. Prolylmethionine and the tetra- and pentapeptides were not utilized as well as other substrates.

We have observed that fetal bovine serum contains some factor(s) that facilitate the utilization of methionine-containing di- to octapeptides (Chapter IV). Results from the present study demonstrate that adult animal sera from humans, horses, chickens, pigs, and rabbits all can promote the utilization of methionine-containing dipeptides with few exceptions. In the presence of any of the five sera, methionylphenyalanine and methionylvaline promoted the greatest protein accretion and prolylmethionine was the least utilized dipeptide. There were some differences in the growth responses of the MAC-T cells on the same dipeptides in the presence of different animal sera, suggesting some species differences may exist.

Implications

Some factor(s) present in animal serum facilitates the utilization of peptides by cultured C_2C_{12} myogenic and MAC-T mammary epithelial cells. This factor(s) appears to be present in FBS as well as sera from adult rabbits, pigs, chickens, horses, and humans. By themselves, neither insulin nor serum lipids are able to facilitate peptide utilization. It appears likely that peptidases are at least partially responsible for the stimulatory role serum has on peptide utilization. Because, especially in ruminants, there can be high concentrations of circulating peptides as a result of absorption, understanding their utilization will enhance the ability to improve animal productivity.

Table 5.1. Protein and DNA contents of MAC-T cells in the presence of methionine dipeptides in serum free methionine deficient DMEM

Treatment	Protein[a]	DNA[a]	Protein:DNA[a]
	--------ug/well-----		
Initial	28.1^{d}	1.9^{c}	14.5^{cd}
MFDMEM[b]	8.9^{i}	1.2^{f}	7.7^{h}
MFDMEM + M	31.8^{c}	2.0^{c}	15.9^{c}
MFDMEM + ML	25.0^{e}	2.1^{c}	11.9^{e}
MFDMEM + LM	16.3^{g}	1.7^{de}	9.9^{efgh}
MFDMEM + MF	21.6^{f}	1.9^{cd}	11.6^{ef}
MFDMEM + FM	23.2^{ef}	1.9^{cd}	12.4^{de}
MFDMEM + MP	8.7^{i}	1.2^{f}	7.4^{h}
MFDMEM + PM	8.1^{i}	.9^{g}	9.2^{fgh}
MFDMEM + MV	15.3^{g}	1.6^{e}	10.3^{efg}
MFDMEM + VM	12.2^{h}	1.6^{e}	7.8^{gh}
SE	.9	.1	.8

[a]Each value represent the mean of four observations.
[b]Serum-free, methionine free Dulbecco's modified Eagle' s medium.
[c,i]Means within the same column lacking common superscripts differ ($P < .05$).

Table 5.2. Protein and DNA contents of MAC-T cells in the presence of methionine dipeptides in methionine free DMEM supplemented with desalted adult porcine serum

Treatment	Protein[a]	DNA[a]	Protein:DNA[a]
	------ug/well--------		
Initial	45.2[e]	2.4[h]	18.9[d]
PS[b]	31.4[f]	1.6[j]	19.8[d]
PS + M	81.9[c]	5.4[c]	15.2[e]
PS + ML	49.5[e]	2.8[g]	17.8[d]
PS + LM	62.0[d]	3.6[e]	17.5[de]
PS + MF	84.2[c]	5.5[c]	15.3[e]
PS + FM	58.6[d]	3.0[fg]	19.8[d]
PS + MP	59.4[d]	3.2[f]	18.6[d]
PS + PM	45.6[e]	2.0[i]	22.9[c]
PS + MV	84.2[c]	4.7[d]	17.8[d]
PS + VM	65.3[d]	3.6[e]	18.1[d]
SE	2.8	.1	.8

[a]Each value represents the mean of four observations.
[b]PS = 6% desalted adult porcine serum.
[c,j]Means within the same column lacking common letters differ ($P < .05$).

Table 5.3. Protein and DNA contents of MAC-T cells in the presence of methionine dipeptides in methionine free DMEM supplemented with desalted adult horse serum

Treatment	Protein[a]	DNA[a]	Protein:DNA[a]
	------ug/well------		
Initial	27.0^{f}	2.4^{g}	11.2^{gh}
HS[b]	15.6^{g}	1.6^{h}	10.1^{h}
HS + M	64.0^{d}	4.0^{d}	16.0^{ef}
HS + ML	67.7^{cd}	3.9^{d}	17.2^{def}
HS + LM	65.4^{d}	3.3^{ef}	20.3^{c}
HS + MF	68.5^{cd}	4.2^{d}	16.3^{def}
HS + FM	65.2^{d}	4.3^{d}	15.1^{f}
HS + MP	71.9^{c}	4.8^{c}	15.1^{f}
HS + PM	22.2^{f}	1.8^{h}	12.7^{g}
HS + MV	64.8^{d}	3.5^{e}	18.6^{cd}
HS + VM	55.4^{e}	3.0^{f}	18.4^{cde}
SE	2.1	.1	.8

[a]Each value represents the mean of four observations.
[b]HS = 6% desalted adult horse serum.
[c,g]Means within the same column lacking common letters differ ($P < .05$).

Table 5.4. Protein and DNA contents of MAC-T cells in the presence of methionine dipeptides in methionine free DMEM supplemented with desalted adult rabbit serum

Treatment	Protein[a]	DNA[a]	Protein:DNA[a]
	-------ug/well------		
Initial	45.2^{f}	2.4^{h}	18.9^{cd}
RS[b]	28.6^{g}	1.4^{i}	20.9^{c}
RS + M	89.2^{cd}	6.1^{c}	14.6^{e}
RS + ML	61.1^{e}	3.4^{fg}	18.0^{d}
RS + LM	66.6^{e}	4.2^{e}	16.0^{ef}
RS + MF	94.0^{c}	6.4^{c}	14.6^{f}
RS + FM	62.3^{e}	3.0^{g}	20.5^{c}
RS + MP	64.4^{e}	3.7^{f}	17.4^{de}
RS + PM	59.5^{e}	3.1^{g}	19.2^{cd}
RS + MV	91.8^{c}	5.4^{d}	17.1^{de}
RS + VM	79.7^{d}	4.2^{e}	18.9^{cd}
SE	3.5	.1	.6

[a]Each value represents the mean of four observations.
[b]RS = 6% desalted adult rabbit serum.
[c,l]Means within the same column lacking common letters differ ($P < .05$).

Table 5.5. Protein and DNA contents of MAC-T cells in the presence of methionine dipeptides in methionine free DMEM supplemented with bovine insulin

Treatment	Protein[a]	DNA[a]	Protein:DNA[a]
	--------ug/well------		
Initial	28.1^{de}	1.9^{d}	14.5^{d}
Insulin	8.2^{h}	1.1^{fg}	7.7^{fg}
Insulin + M	57.0^{b}	2.8^{b}	20.8^{b}
Insulin + ML	36.9^{c}	2.2^{c}	16.6^{c}
Insulin + LM	27.1^{de}	2.1^{cd}	13.3^{d}
Insulin + MF	28.4^{d}	2.0^{cd}	14.3^{d}
Insulin + FM	25.7^{e}	1.9^{d}	13.7^{d}
Insulin + MP	11.4^{g}	1.1^{fg}	10.2^{e}
Insulin + PM	7.6^{h}	1.0^{g}	7.4^{g}
Insulin + MV	15.5^{f}	1.5^{e}	10.3^{e}
Insulin + VM	12.8^{f}	1.3^{ef}	9.7^{ef}
SE	.8	.1	.7

[a] Each value represents the mean of four observations.
[b,h] Means within the same column lacking common letters differ ($P < .05$).

Table 5.6. Changes in protein and DNA contents of MAC-T cell cultures in response to methionine substrates and the addition of 3% LPSR-1

Treatment				
Medium[a]	LPSR-1[bdf]	Substrate[cef]	Protein	DNA
			-------ug/well------	
MFDMEM	-	-	-18.9	1.4
MFDMEM	+	-	-8.2	1.1
MFDMEM	-	M	18.0	.4
MFDMEM	+	M	72.4	1.8
MFDMEM	-	ML	12.0	-.4
MFDMEM	+	ML	42.9	.6
MFDMEM	-	LM	15.5	.2
MFDMEM	+	LM	55.6	1.3
MFDMEM	-	MP	-7.5	1.1
MFDMEM	+	MP	54.0	.9
MFDMEM	-	PM	-14.1	.9
MFDMEM	+	PM	3.8	.9
MFDMEM	-	MAS	33.1	.8
MFDMEM	+	MAS	81.8	3.2
MFDMEM	-	GGFM	-13.8	1.1
MFDMEM	+	GGFM	27.3	.1
MFDMEM	-	YGGFM	-15.1	1.3
MFDMEM	+	YGGFM	27.9	.1
		SE	3.6	.1

[a]Methionine free Dulbecco's modified Eagle's medium.
[b]Low protein serum replacement (LPSR-1).
[c]M = methionine, ML = methionylleucine, LM = leucylmethionine, MP = methionylproline, pM = prolylmethionine, MAS = methionylalanylserine, GGFM = glycylglycylphenylalanylmethionine, YGGFM = tyrosylglycylglycylphenylalanylmethionine.
[d]LPSR-1 differed from no LPSR-1 ($P < .0001$).
[e]Substrates differed ($P < .0001$).
[f]LPSR-1*substrate interaction ($P < .0001$).
[g]The initial contents for protein and DNA were 45.1 and 2.4 ug/well respectively.

Table 5.7. Changes in protein of C2C12 cell cultures in response to methionine substrates and the additions of 1% or 3% LPSR-1

Treatment			
Medium[a]	LPSR-1[beg]	Substrate[cfg]	Protein[d]
	%		ug/well
MFDMEM	1	-	-5.4
MFDMEM	3	-	-1.4
MFDMEM	1	M	10.3
MFDMEM	3	M	32.7
MFDMEM	1	ML	2.8
MFDMEM	3	LM	3.0
MFDMEM	3	LM	12.9
MFDMEM	1	MP	2.7
MFDMEM	3	MP	13.0
MFDMEM	1	PM	-5.5
MFDMEM	3	PM	-3.5
MFDMEM	1	MAS	4.3
MFDMEM	3	MAS	18.4
MFDMEM	1	GGFM	-4.8
MFDMEM	3	GGFM	-1.4
MFDMEM	1	YGGFM	-4.3
MFDMEM	3	YGGFM	.8
		SE	2.2

[a]Methionine free Dulbecco's modified Eagle's medium.
[b]Low protein serum replacement.
[c]M = methionine, ML = methionylleucine, LM = leucylmethionine, MP = methionylproline, PM = prolylmethionine, MAS = methionylalanylserine, GGFM = glycylglycylphenylalanylmethionine, YGGFM = tyrosylglycylglycylphenylalanylmethionine.
[d]The initial protein level was 13.42 ug/well.
[e]Levels of LPSR-1 differed ($P < .0001$).
[f]Substrates differed ($P < .0001$).
[g]LPSR-1*substrate interaction ($P < .0003$).

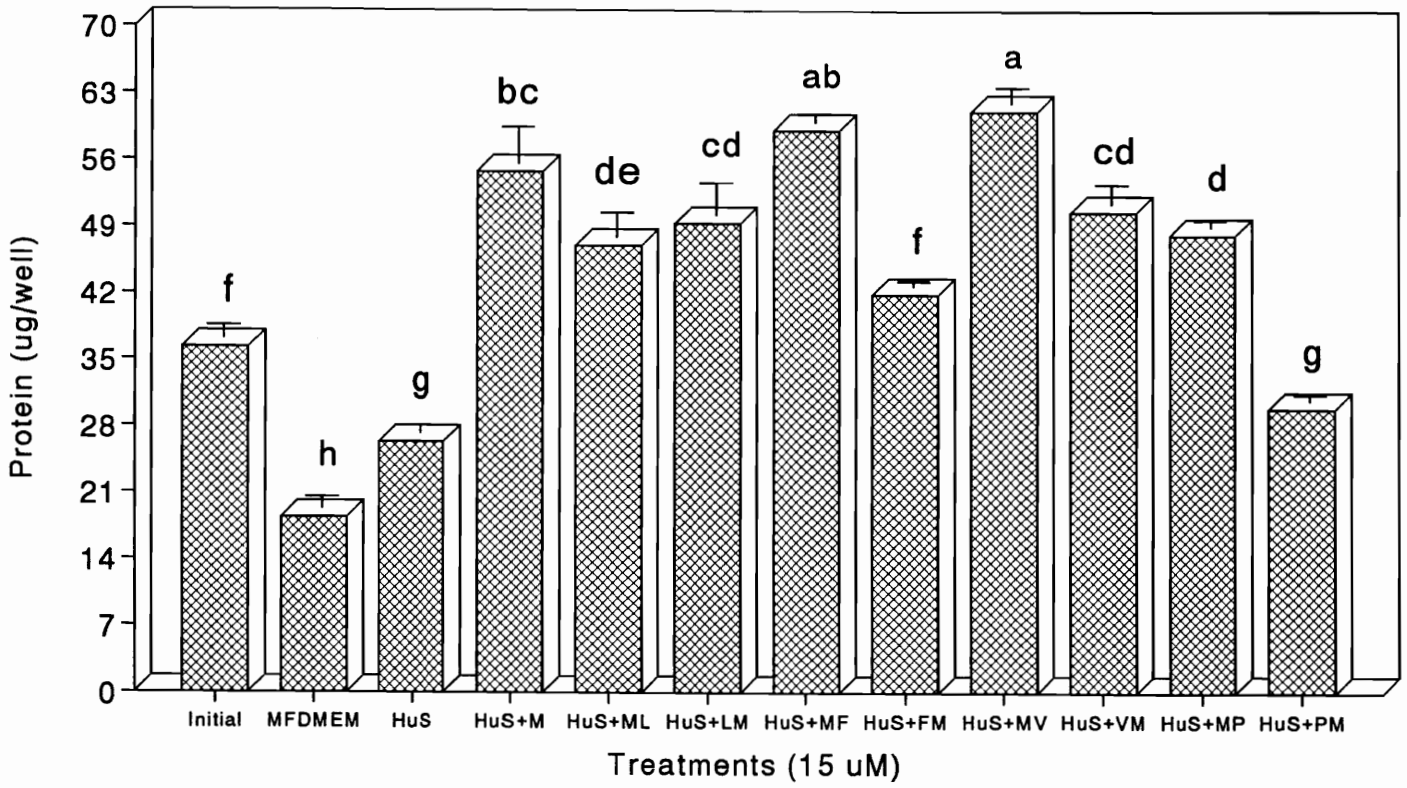


Figure 5.1. Effect of methionine dipeptides on protein accretion in MAC-T cells in response to desalted human serum. MFDMEM = methionine-free DMEM. HuS = human serum. Bars (means+SE) lacking common letters differ ($P < .05$). n = 4.

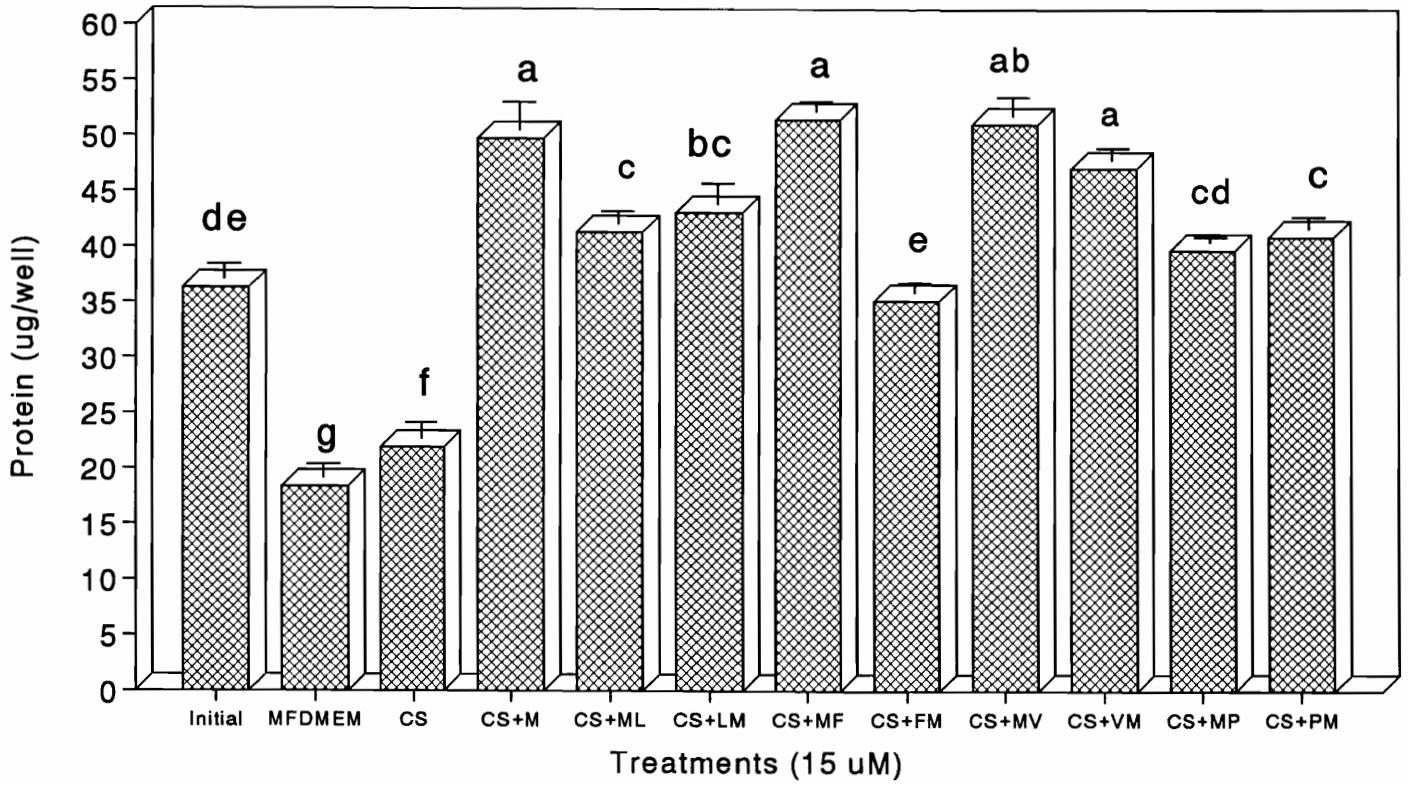


Figure 5.2. Effect of methionine dipeptides on protein accretion in MAC-T cells in response to chicken serum. MFDMEM = methionine-free DMEM. CS = chicken serum. Bars (means + SE) lacking common letters differ ($P < .05$). n = 4.

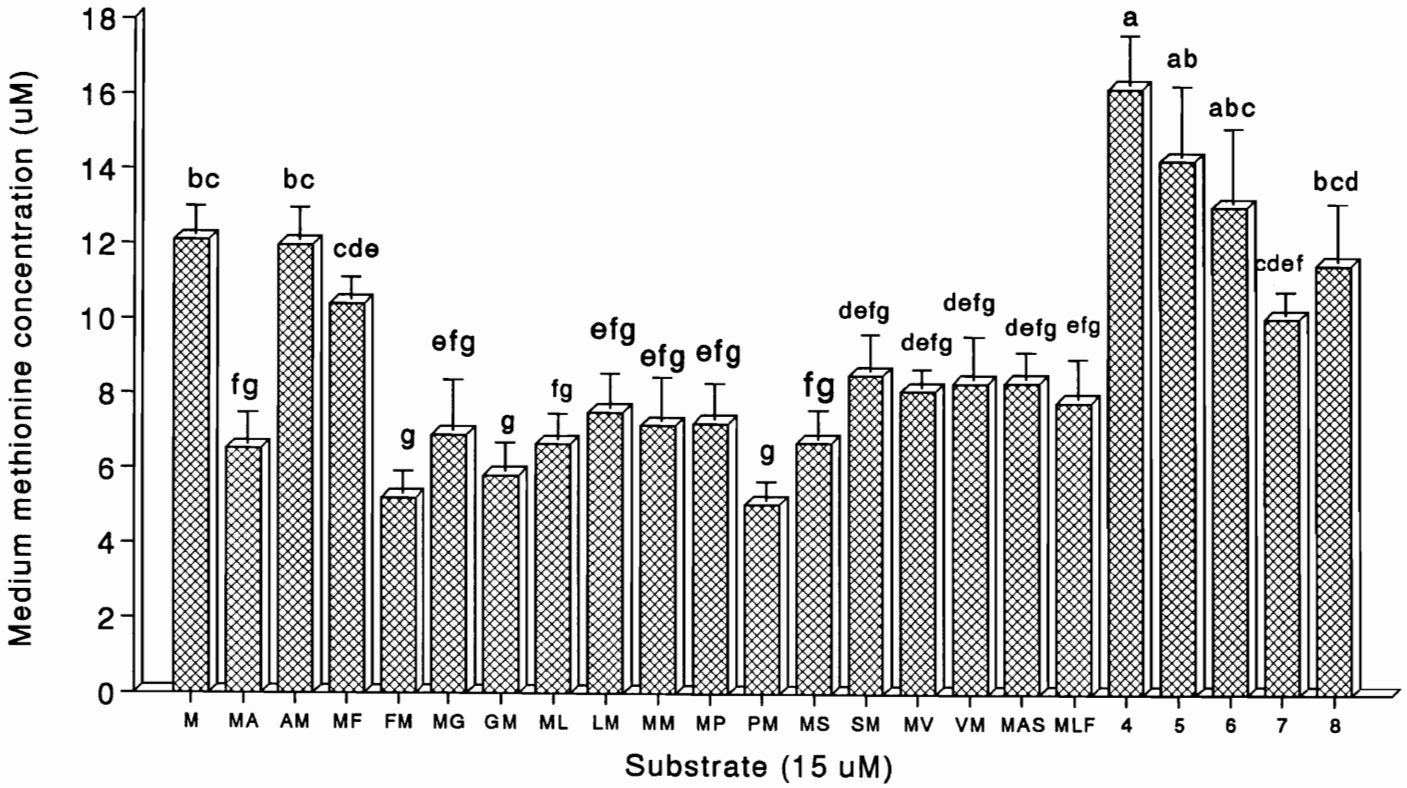


Figure 5.3. Medium free methionine concentrations resulting from hydrolysis of methionine-containing di- to octapeptides by 6% desatlted fetal bovine serum in methionine-free DMEM after 24 h incubation. 4 to 8 = tetra- to octa-enkephalin segments.

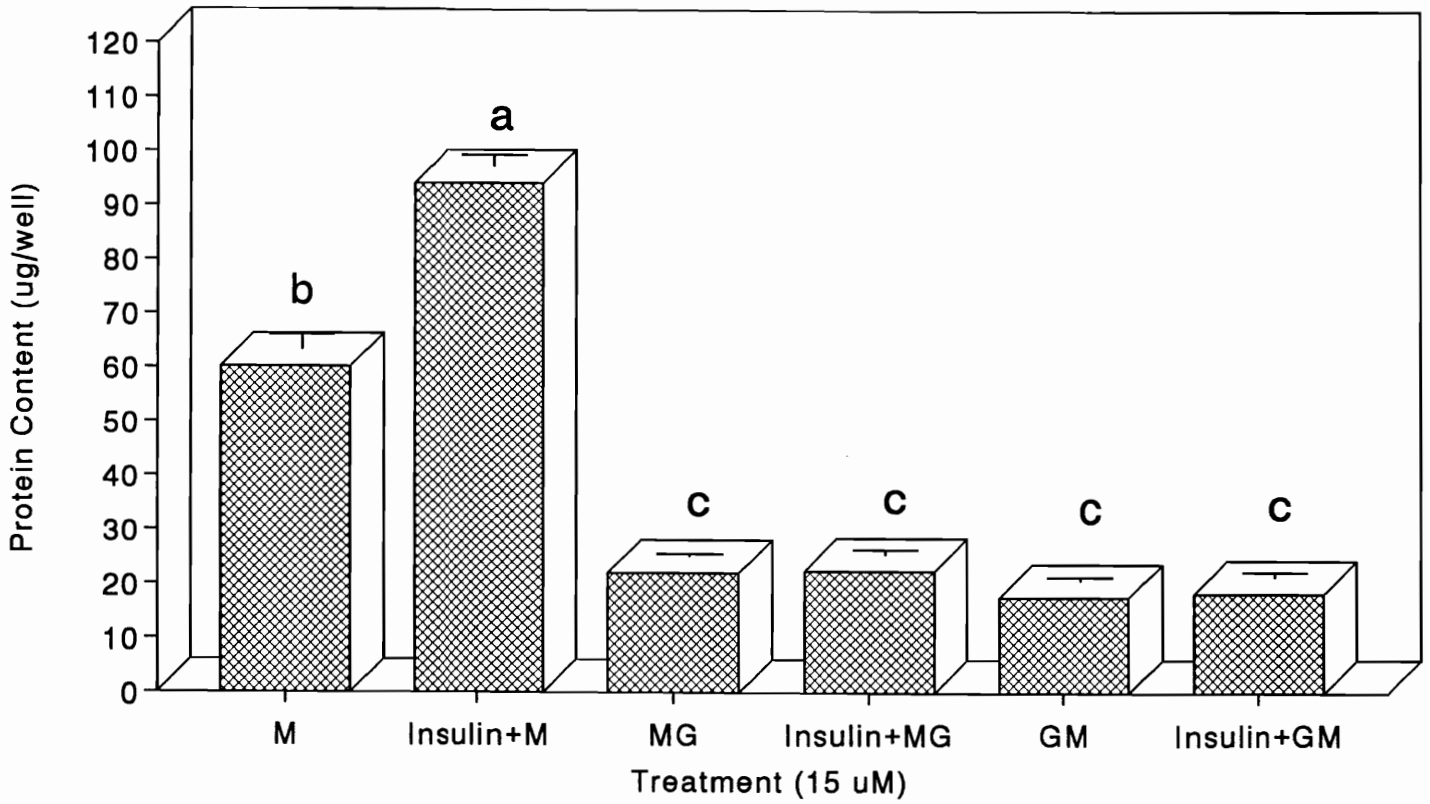


Figure 5.4. Effect of glycylmethionine and methionylglycine on protein accretion in MAC-T cells in the presence of insulin. Bars (means + SE) with different letters differ ($P < .05$). n = 4.

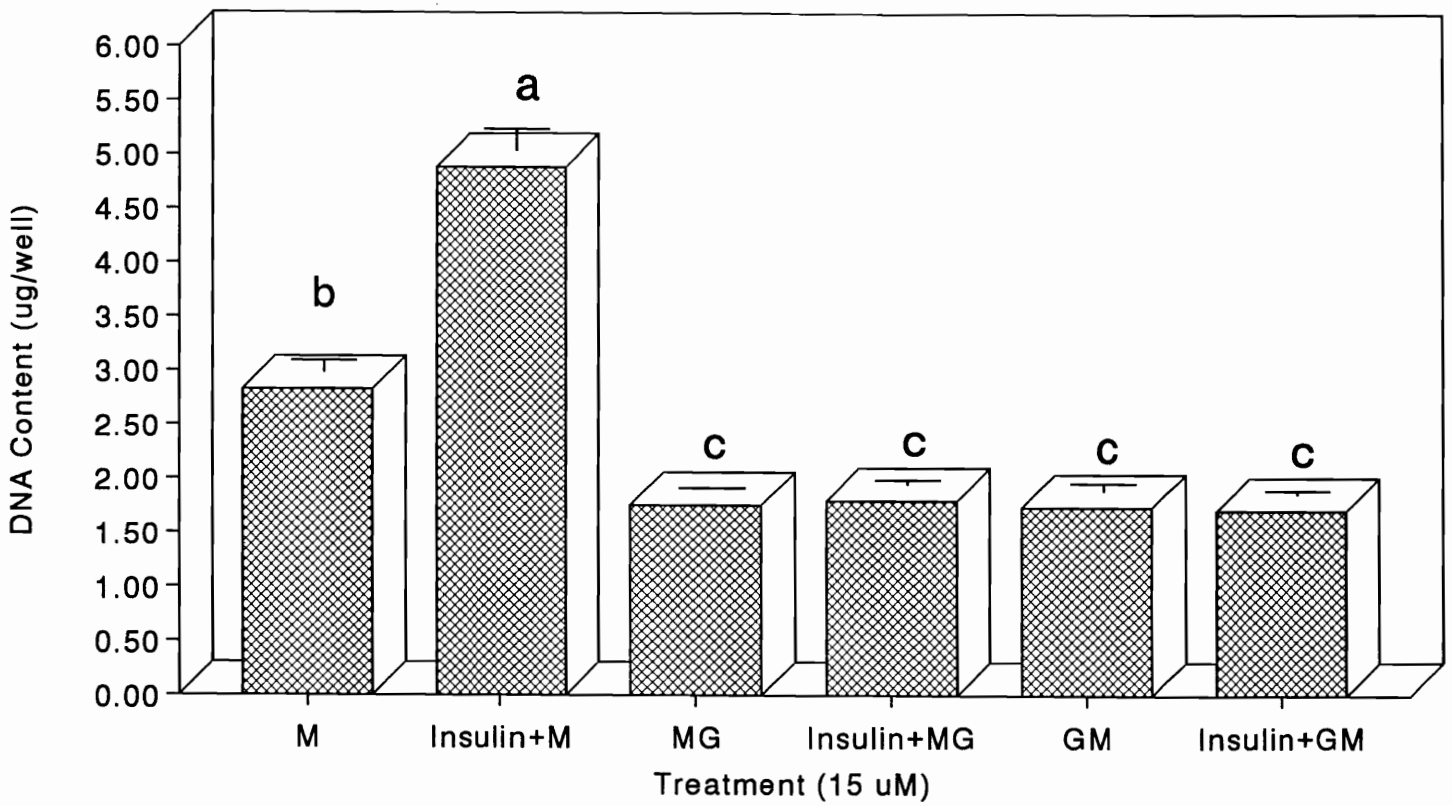


Figure 5.5. Effect of glycylmethionine and methionylglycine on DNA accretion in MAC-T cells in the presence of insulin. Bars (means + SE) with different letters differ ($P < .05$). $n = 4$.

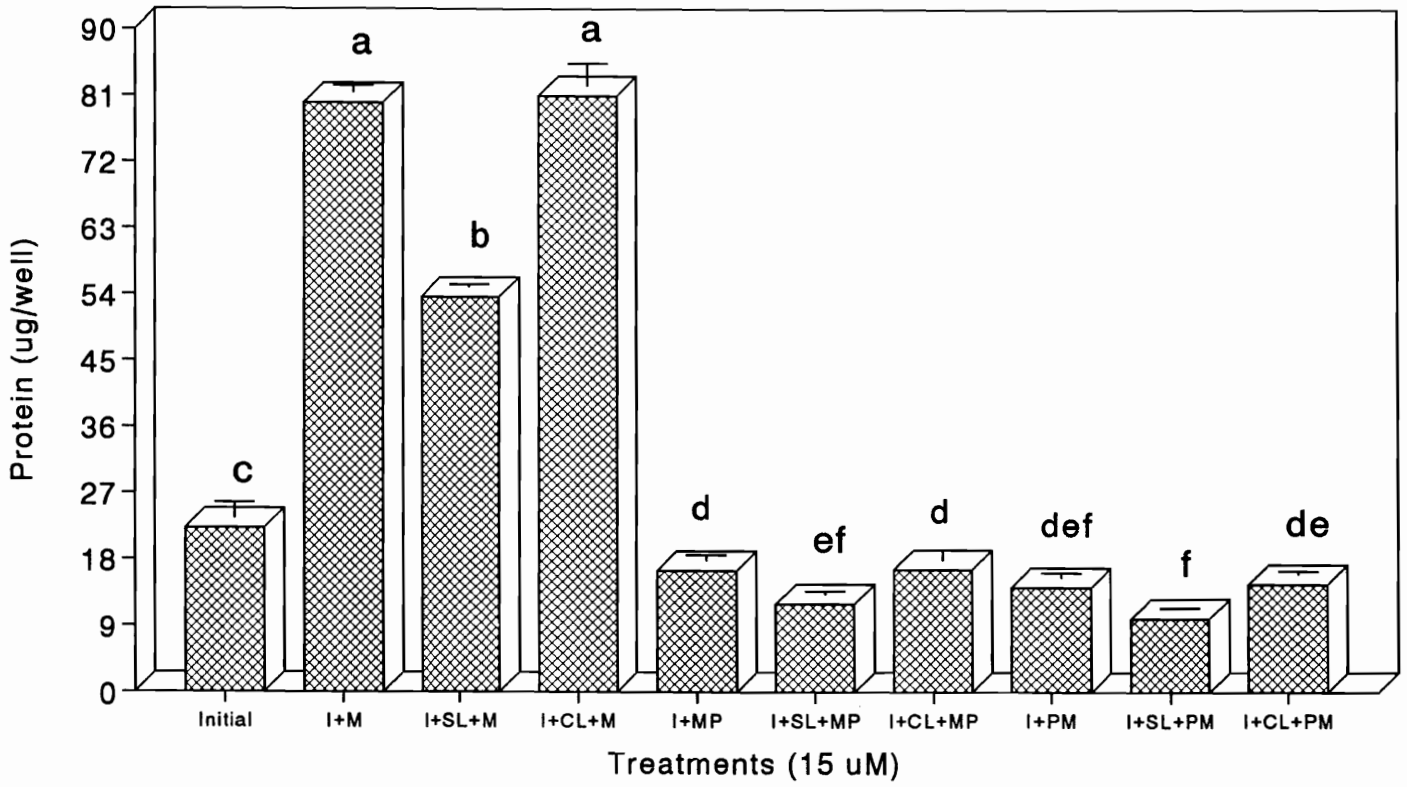


Figure 5.6. Effects of lipid supplementation on the growth of MAC-T cells on methionine dipeptides. I = insulin. SL = bovine serum lipids. CL = chemically defined lipid concentrates. Bars (means+SE) lacking common letters differ ($P < .05$). n = 4.

Chapter VI
Utilization of Peptide-bound Amino Acids as Amino Acid Sources for Protein Accretion and Cell Proliferation in Primary Cultures of Ovine Skeletal Muscle

ABSTRACT

Ruminants have been shown to have high concentrations of peptide-bound amino acids in the circulation. Earlier studies in our laboratory showed that a myogenic cell line (C_2C_{12}) developed from mouse skeletal muscle and a mammary epithelial cell line (MAC-T) developed from bovine mammary epithelial tissues are able to utilize peptides as amino acid sources. In the present study, primary cultures of ovine myogenic satellite cells were evaluated for their ability to use peptide-bound methionine as a source of methionine for protein accretion and cell proliferation. The basal medium contained methionine-free DMEM supplemented with 6% desalted fetal bovine serum. Treatment media included the basal medium supplemented with no methionine, methionine or one of 22 methionine-containing peptides. No protein or DNA accretion was observed in the presence of basal medium alone. Growth responses to all the peptides were obtained with protein and DNA accretion ranging from 49 to 107%, and from 45 to 144% of the corresponding methionine response, respectively. These results indicate that the ovine myogenic satellite cells possess the ability to utilize methionine-containing peptides as methionine sources for protein accretion and cell proliferation.

(Key Words: Methionine, Peptide, Ovine, Satellite cells, Protein accretion, Cell proliferation)

Introduction

High concentrations of peptide-bound amino acids were observed in the blood plasma of calves (McCormick and Webb, 1982; Seal and Parker, 1991; Koeln et al., 1993). Koeln et al. (1993) reported that, in fed calves, about 70% of the

amino acid flux across the gastrointestinal tract was in the form of peptides and only about 10% of the peptide-bound amino acids entering the portal blood were removed by the liver. On the contrary, most (83%) of the free amino acids entering the portal vein were extracted by the liver. DiRienzo (1990) reported that the flux of peptide-bound amino acids across the stomach region of the gastrointestinal tract accounted for 77% of the total amino acid flux across the portal-drained viscera in both fed sheep and calves. Little is known about the fate of these circulating peptide-bound amino acids. Earlier we showed that MAC-T cells, a cell line originally developed from bovine mammary epithelial tissue, and C_2C_{12} cells, a myogenic cell line developed from mice, possess the ability to utilize methionine-containing di- to octapeptides for protein accretion and cell proliferation (Chapter IV). Because of the importance of muscle protein synthesis in meat animal production, it is of interest to investigate whether skeletal muscle of ruminants has the ability to utilize methionine-containing di- to octapeptides.

Therefore, in the present study, primary cultures of ovine myogenic satellite cells were used to investigate whether ovine skeletal muscle possess the ability to utilize methionine-containing di- to octapeptides for protein accumulation and cell proliferation.

Materials and Methods

Methionine-containing Peptides The twenty two methionine-containing peptides (di to octapeptides)[1] tested in this study and the hydrophobicity values of the dipeptides are presented in Table 4.1. The purity of these peptides was determined by HPLC analysis and the

[1]Sigma Chemical Co., St. Louis, MO

hydrophobicity values were calculated as previous described (Chapter IV).

Medium and Vessel Preparation. The preincubation medium for ovine satellite cells was composed of Dulbecco's modified Eagle's medium (DMEM)[2] supplemented with 10% horse serum (HS)[3], 1% antibiotic-antimycotic solution[4], and .1% gentamicin solution[5] (Dodson et al., 1986). The growth medium for ovine satellite cells contained DMEM supplemented with 15% HS, 1% antibiotic-antimycotic solution, and .1% gentamicin solution (Dodson et al, 1986). The differentiation medium for the cells contained DMEM supplemented with 1% HS, 1% antibiotic-antimycotic solution, and .1% gentamicin solution (Dodson et al, 1990). The freezing medium consisted of DMEM supplemented with 20% HS, 10% dimethyl sulfoxide (DMSO)[6], 1% antibiotic-antimycotic solution, and .1% gentamicin (Dodson et al., 1990). Prior to use, culture flasks (80 cm^2)[7] were coated with basement membrane matrix[8] to facilitate the attachment of isolated satellite cells. About 1 mL of thawed basement membrane matrix was added to 9 mL of cold DMEM (2 to 4^oC) supplemented with 1% antibiotic-antimycotic solution and mixed thoroughly. The 1:10 diluted matrix was added to the flasks so that the entire growth area was covered. The flasks were kept in a tissue culture hood[9] for 1 h, then the unbound material was aspirated and the flasks were rinsed gently with serum-free DMEM plus 1% antibiotic-antimycotic solution. The vessels were then ready for use.

---

[2]Cat. No. 380-2430AJ, GIBCO, BRL, Inc., Grand Island, NY
[3]Cat. No. 230-6050AG, GIBCO, BRL, Inc., Grand Island, NY
[4]Cat. No. 600-5240AG, GIBCO, BRL, Inc., Grand Island, NY
[5]Cat. No. 600-5710AD, Gibco, BRL, Inc., Grand Island, NY
[6]Cat. No. D-8779, Sigma Chemical Co., St. Louis, MO
[7]Cat. No. 1-71196, Marsh Biomedical Products, Rochester, NY
[8]Cat. No. 40234, Collaborative Biomedical Products, Bedford, Mass
[9]Labconco Co., Kansas City, MO

Methionine-free Dulbecco's modified Eagle's medium (MFDMEM) was prepared by adding glutamine (58.40 mg/100 mL medium)[10] and 1% (v/v) antibiotic-antimycotic solution to deficient Dulbecco's modified Eagle medium[11]. The resulting medium was sterilized by filtration[12]. Fetal bovine serum (FBS)[13] was desalted by gel filtration chromatography in a Sephadex G-25M desalting column[14]. The basal medium contained MFDMEM plus 6% desalted FBS (dFBS).

Treatment media consisted of the basal medium supplemented with either free L-methionine[15] (8 uM) or one of the 22 methionine-containing peptides (15 dipeptides, 2 tripeptides, 1 tetrapeptide, 1 pentapeptide, 1 hexapeptide, 1 septapeptide, and 1 octapeptide, see Table 4.1 for the abbreviations and hydrophobicity values) at concentrations that were equivalent to L-methionine in methionine content.

Isolation of Myogenic Satellite Cells. Satellite cells were isolated from the semimembranosus and semitendanosus muscles of four lambs (1 to 5 mo old) by a modification of the procedure described by Dodson et al. (1986). All procedures used in the isolation of muscle and the harvest of cells were conducted aseptically. Semimembranosus and semitendinosus muscles were removed from anesthetized (pentobarbital) lambs immediately after exsanguination. The muscle tissues were immersed in ice-cold Dulbecco's Phosphate Balanced Saline (D-PBS)[16] and transported to a tissue culture hood as soon as possible for the isolation of satellite cells. After removal of excessive connective tissue, the muscles were cut into small strips and then the

---

[10]Sigma Chemical Co., St. Louis, MA
[11]Cat. No. 320-1970AJ, GIBCO, BRL, Inc., Grand Island, NY
[12]Cat. No. 09-730-218, Nalge Company, Rochester, NY
[13]Cat. No. 230-6140AG, GIBCO, BRL, Inc., Grand Island, NY
[14]Pharmacia LKB Biotechnology, Piscataway, NJ
[15]Sigma Chemical Co., St. Louis, MO
[16]Cat. No. 450-1600EB, Sigma Chemical Co., St. Louis, MO

strips were passed through a small, sterile meat grinder. The ground muscle was incubated with pronase E[17] (2 mg/mL d-PBS) for 1 h at 37°C in a water bath with agitation every 10 min. After the incubation, the mixture was centrifuged at 1,500 x g for 12 min. The supernatant was discarded. The pellet was suspended in D-PBS and then centrifuged at 500 x g for 10 min to pellet tissue debris (this process was repeated once). The resultant supernatant was collected and centrifuged at 1,500 x g for 10 min to pellet the satellite cells. The resulting pellet was suspended in preincubation medium and then the cell suspension was incubated at 37°C, 90% air, 10% CO_2 in a humidified environment for 1 h to remove fibroblasts. After preincubation, the cell suspension was centrifuged at 1,500 x g for 6 min to pellet the satellite cells again, and the resulting pellet was suspended in freezing medium. Aliquots of 2 mL of the cell suspension were transferred into 2 mL cryogenic vials[18]. The vials were put into a Nalgene Cryo 1°C Freezing Container[19] and then precooled at -70°C overnight. The precooled vials were then stored in liquid nitrogen until used. Satellite cells isolated from the four lambs were stored separately.

Culture Procedure for Myogenic Satellite Cells. A stored cell suspension from one of the four lambs was thawed and then plated on a 80 cm^2 culture flask coated with basement membrane matrix. The isolated satellite cells were grown in 80 cm^2 flasks for 5 to 6 d at 37°C, 90% air, 10% CO_2 in a humidified environment. The growth medium was changed every 12 h within the first 48 h and then every 24 h. The cells were then released by .05% trypsin solution and the enzyme action was stopped by the addition of growth medium. The resulting cells were counted by a hemacytometer,

[17]Cat. No. P-5147, Sigma Chemical Co., St. Louis, MO
[18]Cat. No. 03-374-6, Fisher Scientific, Pittsburgh, PA
[19]Cat. No. 5100-0001, Fisher Scientific, Pittsburgh, PA

suspended in growth medium, and seeded in 12-well plates[20] for peptide utilization evaluations.

Myogenic Ability of the Isolated Cells. To verify the myogenic ability of the isolated cells, isolated cells stored in liquid nitrogen were thawed and then cultured in the growth medium for 5 to 6 d until confluence. The cultures then were incubated with the differentiation medium for 3 d. The myogenic ability of the isolated cells was determined by observing the formation of myotubes.

L-Methionine Standard Curve For Protein Accretion. Experiments were conducted to determine the effect of free L-methionine concentrations in the medium (0 to 50 uM) on protein accretion in the primary cultures of the ovine skeletal muscle at different cell densities (10,000 and 20,000 cells /well). The response curve generated was used to determine the cell density and the concentration of both L-methionine and methionine-containing peptides to be used in the peptide utilization studies.

Peptide Utilization By Primary Cultures. The ovine satellite cells obtained from the above propagation were suspended in growth medium, plated at 20,000 cells per well (12-well plate), incubated at 37^{o}C, 90% air, 10% CO_2 in a humidified environment for 24 h, then the growth medium was replaced by MFDMEM, and the cultured cells were incubated for another 24 h. Subsequently, the MFDMEM was removed from the cultures and the starved cells were incubated with one of the treatment media for 72 h. The treatment media were changed at 24-h intervals.

Harvest and Analytical Procedures. After 72 h of incubation, the cultures were washed twice with ice-cold D-PBS. The protein and DNA contents were analyzed either from a sample of the same well or from samples taken from

[20]Cat. No. 08-757-16B, Fisher Scientific, Pittsburgh, PA

separate wells. When protein and DNA were analyzed from separate samples, cultures for protein assay were dissolved by treatment with .4 or .8 mL of .5 N NaOH overnight. The resulting solutions were neutralized with .04 or .08 mL of 5 N HCl and the protein contents were determined by the enhanced bicinchoninic (BCA)[21] assay (Smith et al., 1985). Cultures for DNA determination were harvested and the DNA concentrations were determined by measuring the fluorescence that resulted from the interaction between DNA and the fluorochrome Hoechst 33258 (Labarca and Paigen, 1980).

When protein and DNA were determined from the same sample, 500 uL of buffer (pH 7.4) containing .05 M Na_2PO_4, 2 M NaCl and .002 M EDTA were added to each well, then the cultures were sonicated for 15 s by a Sonic Dismembrator[22]. A portion (100 uL) of the sonicated sample was transferred for DNA assay as described by Labarca and Paigen (1980) and the remainder was treated with 400 uL 1N NaOH overnight (or 18 h). The NaOH-treated sample was neutralized by .08 mL of 5N HCl solution, and the neutralized sample was used for protein assay by the enhanced BCA procedure (Smith et al., 1985).

Statistical Analysis. Treatments were replicated in four wells. Satellite cells from each lamb were examined separately. The protein and DNA results were analyzed as a randomized complete block design. The model was:

$$Y_{ij} = \mu + \alpha_i + \beta_j + E_{ij}$$

Where:

Y_{ij} = protein or DNA content obtained from the ith treatmet in the jth animal

μ = overall mean

α_i = effect of treatment i

β_j = effect of animal j

[21]Pierce (Rockford, IL)
[22]Fisher Scientific, Pittsburgh, PA

E_{ij} = error component asociated with the ith treatment in the jth animal

Data were analyzed by the GLM procedure of SAS (1989) with animals as blocks and culture wells as the experimental units to test the peptide treatment effects. Means of different peptide treatments were compared using Duncan's means separation test at an alpha value of .05.

Results and Discussion

The growth of myogenic satellite cells in a series of methionine concentrations is presented in Figure 6.1. At both seeding cell densities, cultured cells were sensitive to the methionine concentrations in the medium. Maximal protein accretion was obtained at methionine concentrations of approximately 25 uM for both initial cell densities. Protein accretion for the initial cell density of 20,000 was almost twice that for a cell density of 10,000. The relatively higher protein contents resulted in more accurate estimation of protein by the BCA assay, therefore, an initial cell density of 20,000 with a substrate concentration of 8 uM was chosen for subsequent peptide utilization estimates.

Data presented in Figure 6.2 indicate that cultured myogenic cells were able to utilize all the methionine-containing dipeptides tested for protein accretion with responses ranging from about 49 to 95% of the response for free methionine. Only one dipeptide, alanylmethionine, was as effective as methionine in supporting protein accretion. Prolylmethionine and glycylmethionine were the least utilized peptides. In our earlier report (Chapter IV), we also showed that glycylmethionine and prolylmethionine were poorly utilized by the C_2C_{12} myogenic cell line developed from mouse skeletal muscle and the MAC-T cell line developed from bovine mammary epithelial tissues. It appears that there is some similarity in the utilization of methionine-

containing dipeptides among the three cell types, differences also are obvious. For instance, leucylmethionine, methionylvaline, and methionylmethionine were utilized as effectively as methionine by C_2C_{12} myogenic cells and better than methionine by MAC-T mammary epithelial cells, but the growth response of satellite cells to these three dipeptides was about 75% of the methionine response. This suggests that a different ability of peptide utilization may exist among different tissues or even species. This phenomenon has been suggested previously. Lochs et al. (1988) investigated the utilization of glycylleucine and glycylglycine by different tissues of dogs in vivo. Their results indicated that all of the tissues examined (liver, muscle, kidney, and gut) utilized both peptides. However, tissues differed considerably in the utilization of the same dipeptides. Liver, kidney, muscle, and gut extracted 25, 24, 12, and 10% of the infused amount of glycylleucine, respectively. In the case of glycylglycine, kidney played the most important role in the clearance of this peptide (37%), followed by muscle (18%), liver (15%), and gut (11%).

Data showing the effects of methionine-containing dipeptides on the proliferation of myogenic satellite cells are presented in Figure 6.3. One dipeptide, alanylmethionine, resulted in greater cell proliferation ($P < .05$) than methionine, and methionylphenylalanine was as effective as free methionine. The remaining dipeptides promoted cell proliferation rates ranging from 45 to 85% of the response for free methionine. We are not aware of any report indicating the presence of an active transport system for dipeptides in skeletal muscle. In an earlier study in our lab (Chapter V), we showed that 6% desalted FBS could hydrolyze methionine-containing dipeptides to varying degrees, suggesting that hydrolytic enzymes in the serum are

at least partially responsible for the observed growth in cultured cells. Hydrolysis of the peptides by plasma, membrane-bound, and capillary bed-associated peptidases with the subsequent uptake of the free amino acids has been proposed as the major mechanism for the utilization of peptide-bound amino acids by tissues (Adibi and Morse, 1981; Furst et al., 1987; Raghunath et al., 1990). Nutzenadel and Scriver (1976) showed that the diaphragm of rats could take up carnosine via a saturable and Na^+-dependent system. Since carnosine has several physiological functions in muscle (Rodwell, 1990), it is not surprising that muscle has specific transport system for this dipeptide.

Protein accretion was observed to differ within several pairs of dipeptides having the same amino acid compositions (Figure 6.2). In the case of peptides composed of methionine with phenylalanine, glycine,or proline, having methionine at the N-terminus resulted in greater ($P < .05$) protein accretion than having methionine at the C-terminus. Conversely, preferential utilization ($P < .05$) occurred in the presence of C-terminal methionine when dipeptides were composed of alanine, or serine plus methionine. Essentially the same pattern of response was observed among the same pairs of dipeptides for cell proliferation (Figure 6.3). Molecular structure of dipeptides with the same amino acid composition apparently affects both protein accretion and cell proliferation. These effects are likely due to the rates at which methionine from the dipeptides become available as a source of methionine for protein synthesis, which, in turn, influences cell proliferation. Our earlier studies (Chapters IV and V) showed that, in the presence of 6% desalted serum from fetal bovine, adult humans, pigs, horses, chickens, or rabbits, dipeptides with the same amino acid composition but different molecular structures also affected protein accumulation and (or) cell proliferation

differently in C_2C_{12} and (or) MAC-T cells. Dipeptides with the same amino acid composition but different residue sequences have been shown to affect protein accretion in a number of organisms (Kihara and Snell, 1952; Eagle, 1955; Naider et al., 1974).

Peptides with more than two amino acid residues can also serve as sources of their constituent amino acids. The data presented in Figure 6.4 show that cultured myogenic satellite cells are able to utilize methionine-containing tri- to octapeptides to meet their requirements for methionine for protein accretion with the responses ranging from 66 to 108% of the free methionine response. A tetrapeptide, glycylglycylphenylalanylmethionine was utilized better ($P < .05$) than free methionine in supporting protein accretion. The penta- and hexapeptides examined were utilized as efficiently as methionine in promoting protein accretion. The remaining peptides were less effectively used than methionine. Cell proliferation rates also varied among these longer peptides (Figure 6.5). The tetra-, penta-, and hexapeptides examined promoted greater ($P < .05$) cell proliferation than did free methionine. The octapeptide was as effective as methionine, and the remaining peptides were less effective ($P < .05$) than methionine. Our earlier study (Chapter IV) indicated that C_2C_{12} myogenic and MAC-T mammary epithelial cells were able to utilize tri- to octapeptides as methionine sources for protein accretion. Grahl-Nielsen et al. (1974) showed that, in the presence of 1% dialyzed calf serum, a cell line developed from the small intestine of hamster was able to grow in the presence of di-, tetra-, hepta-, and decalysine, but the growth responses decreased with increasing chain length. They observed no detectable peptidase activity against the decalysine. In our earlier study (Chapter V), we showed that the utilization of methionine-containing tetra- to octaenkephalin segments by

MAC-T mammary epitheliial cells as a source of methionine was entirely dependent on the presence of 6% desalted FBS in the culture medium, and 6% desalted FBS was able to release all the methionine residues from the tetra- to octa-methionine-enkephalins within a 24 h incubation in cell-free medium. Although we did not identify the enzyme(s) responsible for the observed hydrolysis of enkephalins, animal sera have been shown to contain enkephalin-hydrolyzing enzymes including aminopeptidase M, dipeptidyl carboxylpeptidase, and angiotensin converting enzyme (Shibanoki et al., 1991, 1992). Therefore, the observed growth of myogenic satellite cells on these methionine-containing enkephalin segments is likely at least partially due to the utilization of free methionine released from the hydrolysis of these segments by serum-associated hydrolytic enzymes. Protein accretion and cell proliferation rate associated with particular peptides that exceed the rate observed with free methionine are likely not due entirely, if at all, to the rate of hydrolysis of the peptides by peptidases. In these cases, getting methionine to the site of protein synthesis probably also involves transporting the peptides across membranes. It seems reasonable to infer from the data collected with cultured cells that the variation observed in the utilization of the peptides examined may be due to a combination of transport and hydrolytic events.

The data for the effects of methionine-containing di- to octapeptides on protein:DNA ratios are presented in Figures 6.6 and 6.7. After 72 h of incubation, the protein:DNA ratio resulting from the presence of free methionine was similar to initial protein:DNA ratio, and most of the peptides appeared to result in protein:DNA ratios similar to that of free methionine. But the protein:DNA ratios were reduced in the presence of valylmethionine and hexa- to octapeptides. These results

suggest some peptide-bound amino acids may influence cell functions differently from corresponding free amino acids. In fact, Amborski et al. (1970) reported that Baco-Peptone or Proteos Peptone in a serum-free medium helped to maintain the beating of chicken embryo heart cells. Two tripeptides (glycylhistidyllysine and glycyllysylhistidine) isolated from human serum have been shown to prolong the survival of normal liver cells and stimulate growth in neoplastic liver (Pickart and Thaler, 1973). These findings suggest that, in addition to serving as amino acid sources, circulating peptides may have other physiological functions.

Our earlier studies (Chapters IV and V) showed that, in the presence of either FBS or bovine insulin, protein accretion in C_2C_{12} and (or) MAC-T cells was related to the hydrophobicity of the methionine-containing dipeptides tested regardless of the methionine position in the dipeptides. Data presented in Table 6.1 show that, in the primary cultures of ovine myogenic satellite cells, protein accretion and cell proliferation were related to the hydrophobicity of dipeptide with methionine at the N-terminus. This again suggests that hydrophobicity or related properties of the dipeptides tested affects their utilization by cultured animal cells. Hydrophobicity of the dipeptides with methionine at the C-terminus had less impact on the protein accretion in the primary cultures of ovine myogenic cells.

The results of the present study demonstrate that the primary cultures of isolated ovine myogenic satellite cells possess the ability to utilize methionine-containing di- to octapeptides as methionine sources for both protein accretion and cell proliferation with varied responses among peptides. Molecular structure of the methionine-containing dipeptides with the same amino acid composition affects the availability of the dipeptides as methionine sources. This

is consistent with the concept that peptide-bound amino acids can serve as amino acid sources for protein synthesis in animals.

Implications

The growth of cultured ovine myogenic satellite cells was sensitive to the concentrations of methionine in the medium. In the presence of 6% desalted FBS, cultured ovine myogenic satellite cells were able to utilize methionine-containing di- to octapeptides for both protein accumulation and cell proliferation. Some peptides, especially tetra- to octapeptides, were utilized as effectively as or even better than free methionine. Results from the present study clearly show that cultured ovine myogenic satellite cells are able to utilize peptide-bound amino acids as sources of their constituent amino acids. Because high concentrations of peptides have been shown to be present in the circulation of ruminants, these peptides may serve as amino acid sources for tissues (such as skeletal muscle) that possess the ability to utilize peptides. Further research is needed to ascertain the mechanisms responsible for the observed growth responses.

Table 6.1. Relationship between methionine dipeptide hydrophobicity and protein accretion in ovine myogenic satellite cells

Dipeptide	Response	Linear[a]	r^2	Quadratic[a]	r^2
Methionine-X	Protein	.0008	.10	.0003	.14
Methionine-X	DNA	.0001	.13	.0003	.14
X-Methionine	Protein	.41	.006	.71	.006
X-Methionine	DNA	.73	.001	.93	.001

[a]Probability of a linear or quadratic effect.

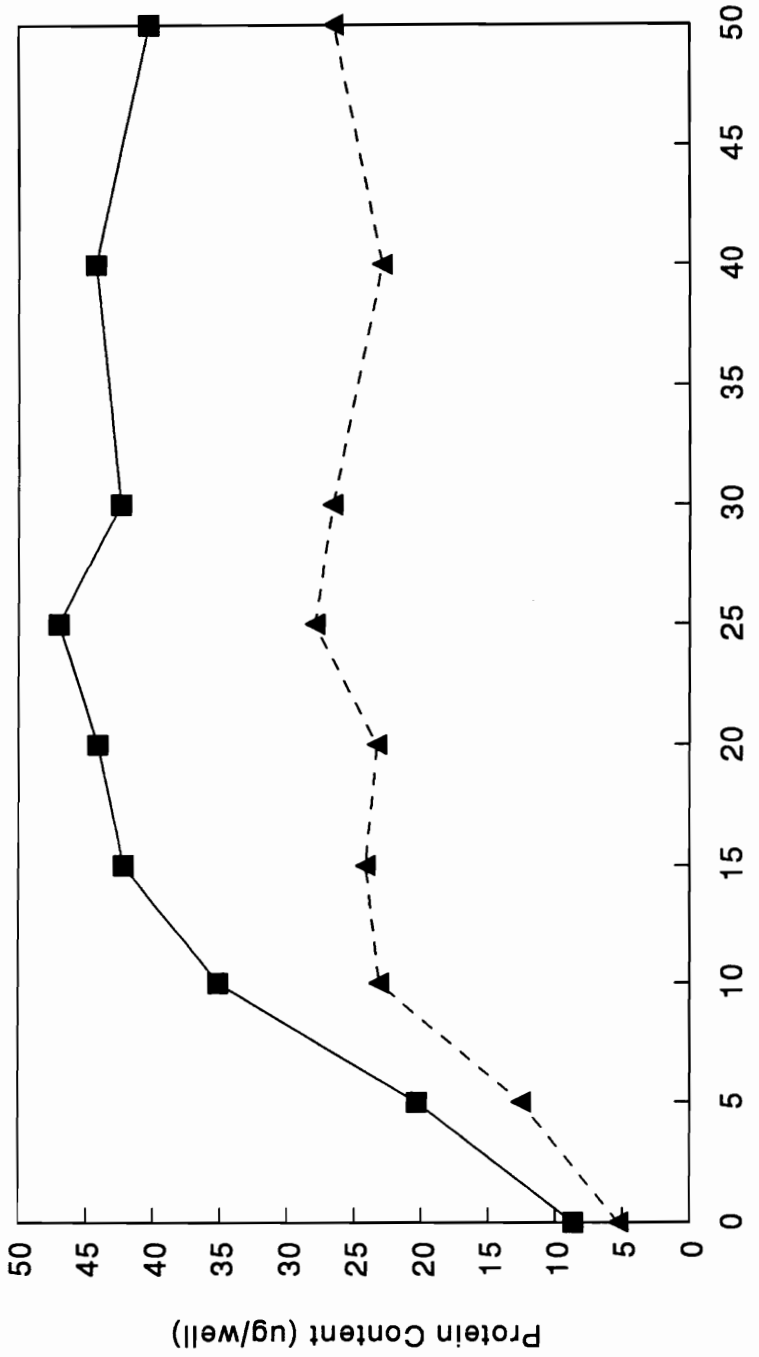


Figure 6.1. Methionine standard curve for protein accretion in primary cultures of ovine skeletal muscle

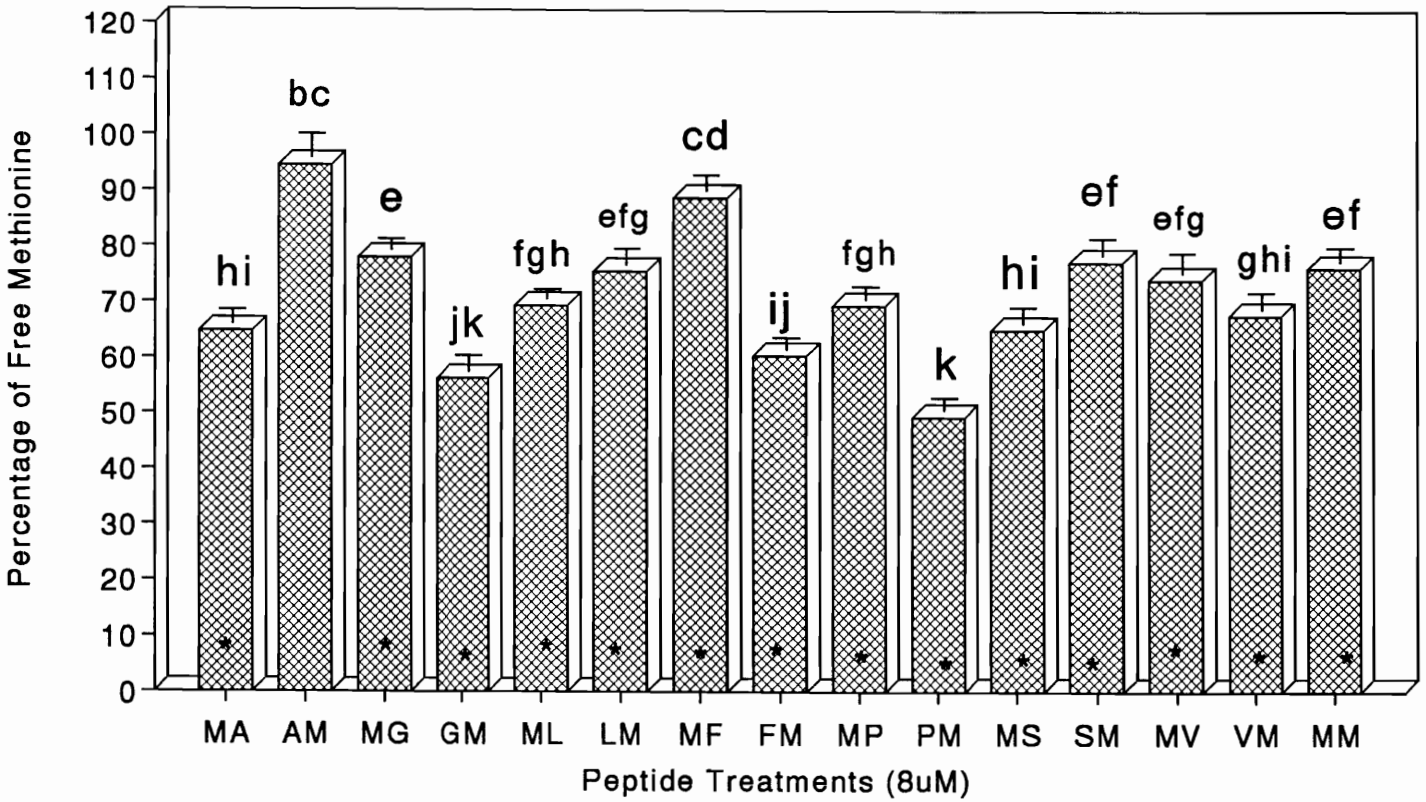


Figure 6.2. Effect of methionine dipeptide on protein accretion in primary cultures of ovine skeletal muscle. Bars (means+SE) with different letters differ (P < .05). *Different from free methionine (P < .05). n = 16.

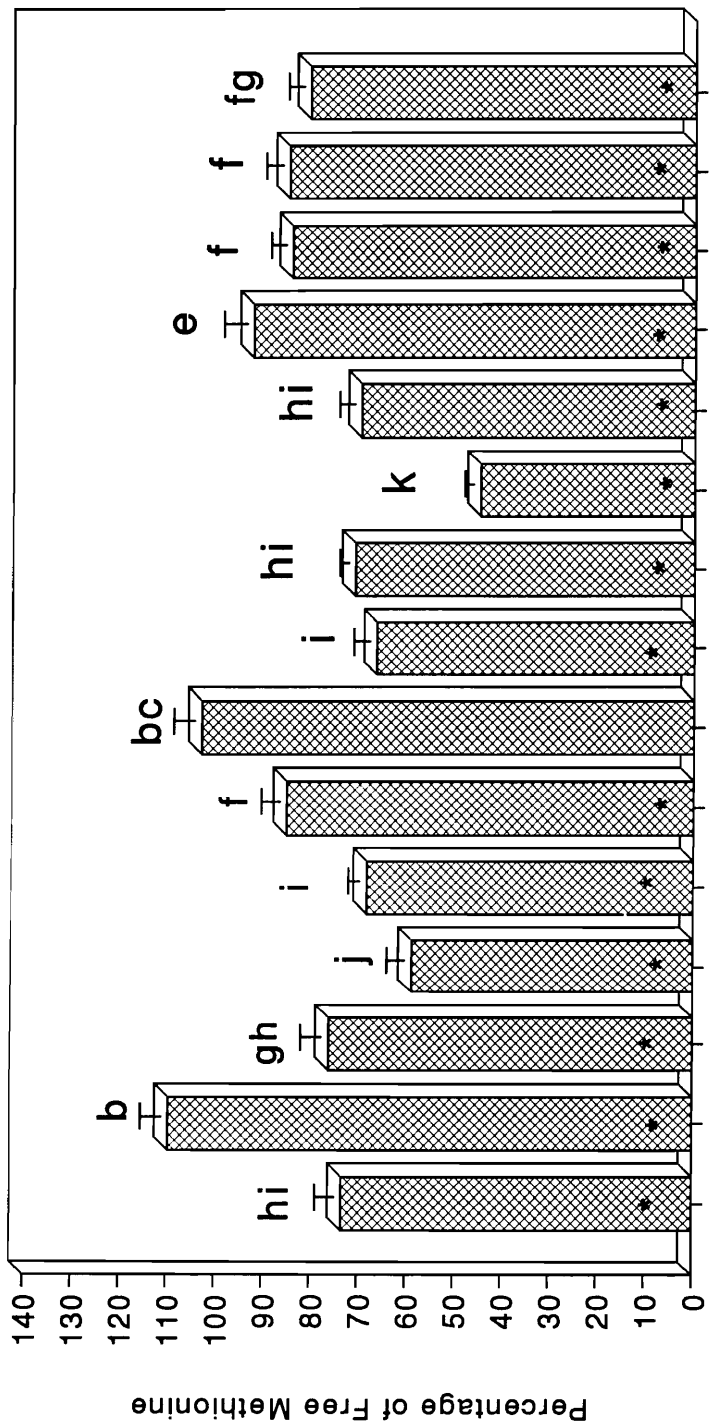


Figure 6.3. Effect of methionine dipeptide on DNA accretion in primary cultures of ovine skeletal muscle. Bars (means+SE) with different letters differ ($P < .05$). * Different from free methionine ($P < .05$). n = 16.

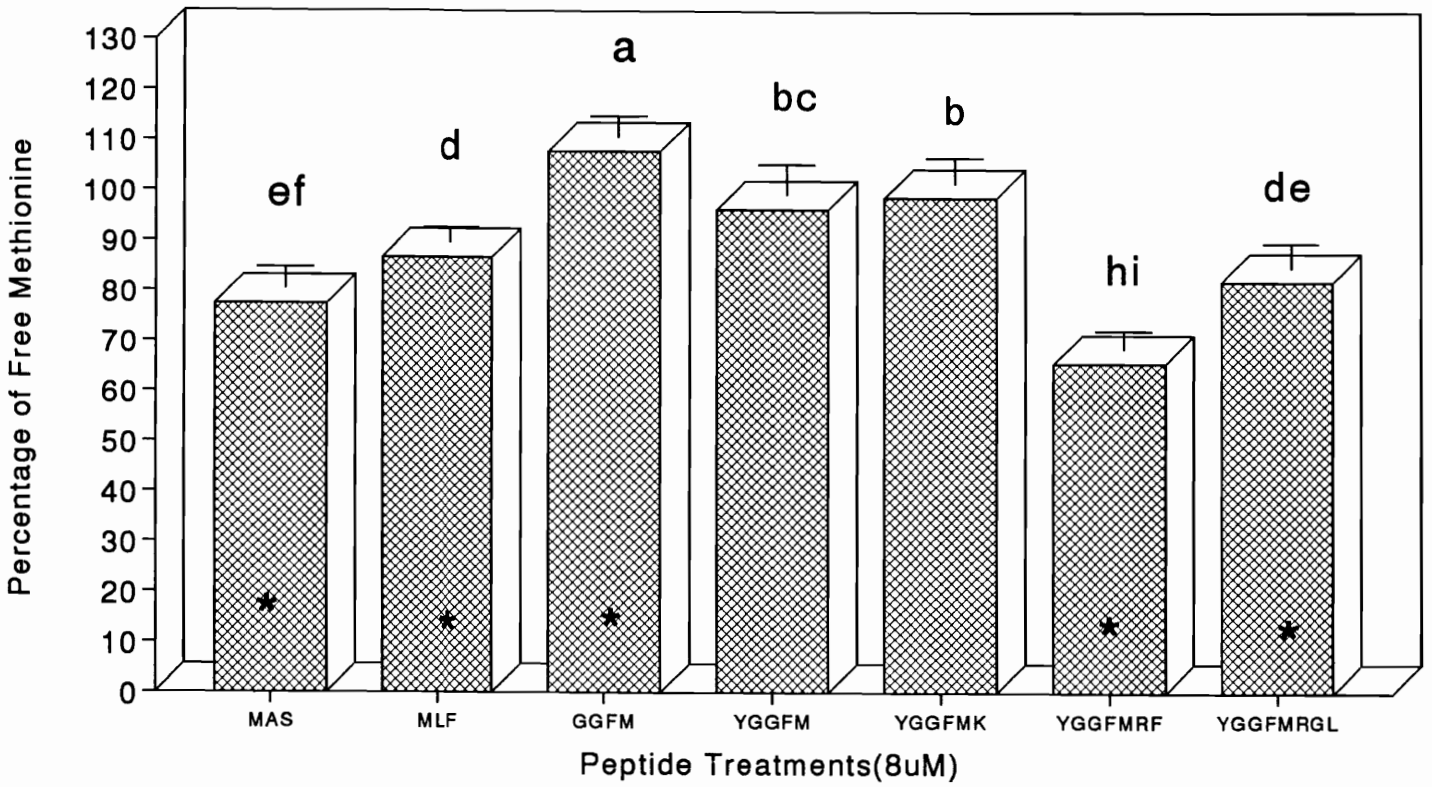


Figure 6.4. Effects of tri- to octapeptides on protein accretion in primary culture of ovine skeletal muscle. Bars (means+SE) with different letters differ ($P < .05$). * Different from free methionine ($P < .05$). n = 16.

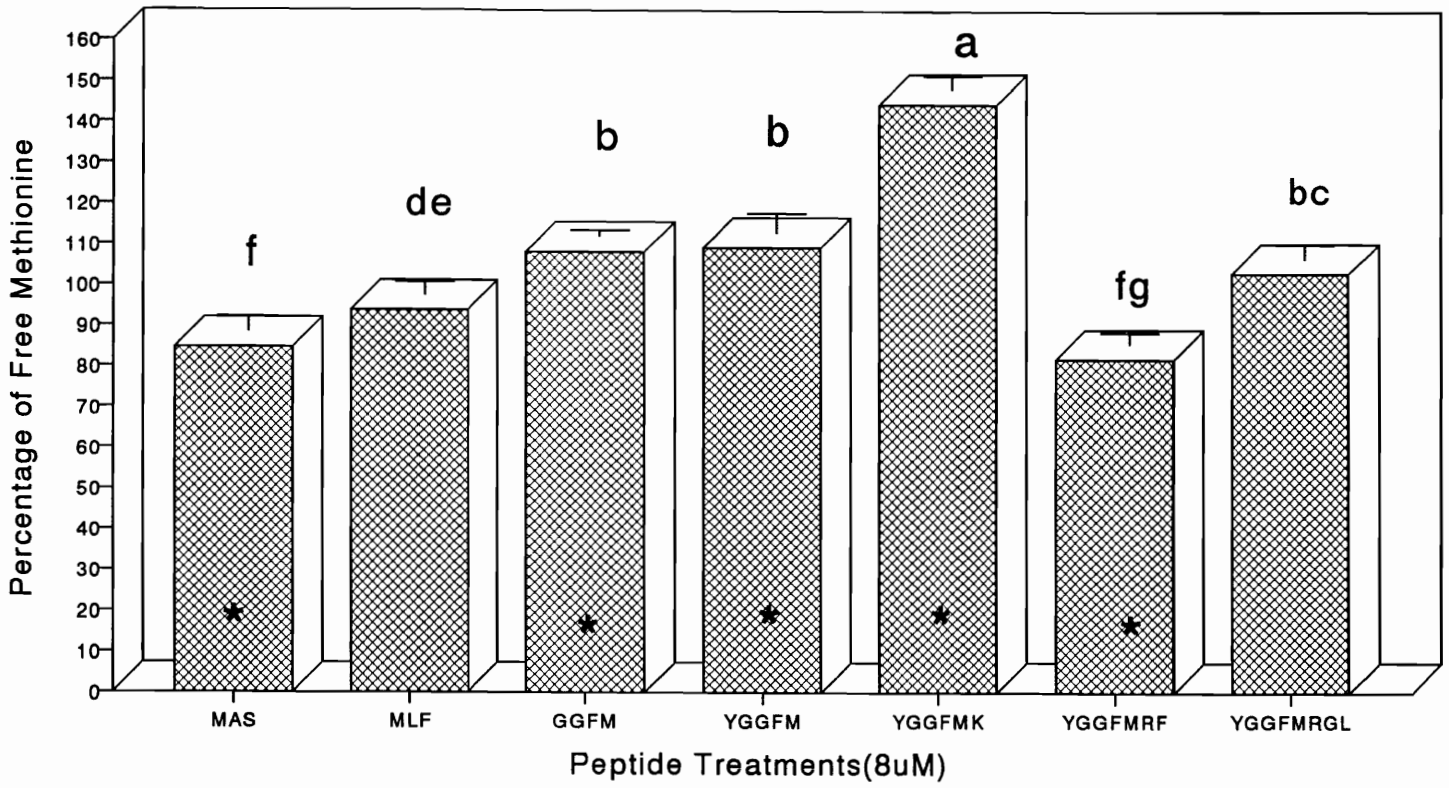


Figure 6.5. Effects of tri- to octapeptides on DNA accretion in primary culture of ovine skeletal muscle. Bars (means+SE) with different letters differ ($P < .05$). * Different from free methionine ($P < .05$). n = 16.

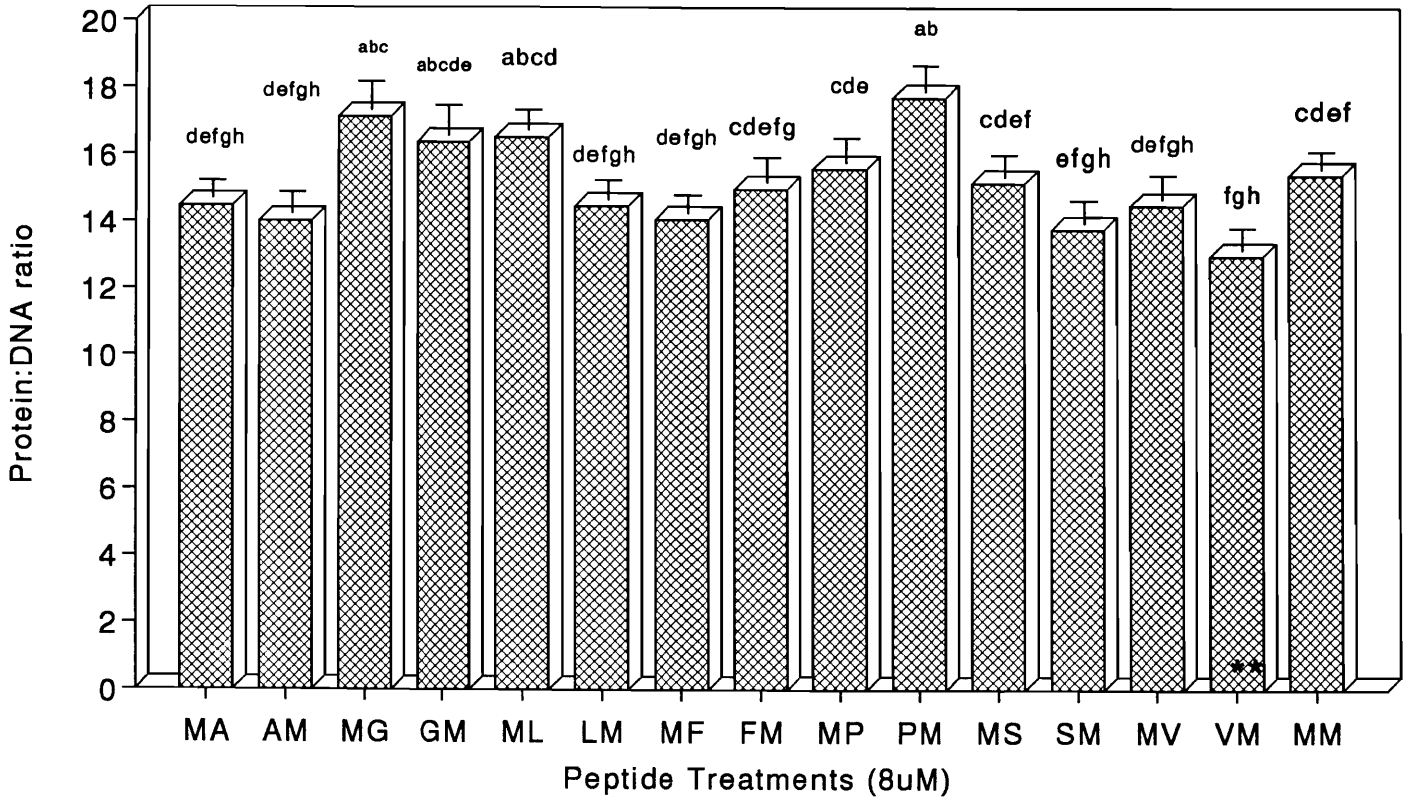


Figure 6.6. Effect of methionine dipeptide on protein:DNA ratio in primary cultures of ovine skeletal muscle. Bars (means+SE) with different letters differ ($P < .05$). ** smaller than methionine response ($P < .05$). n = 16.

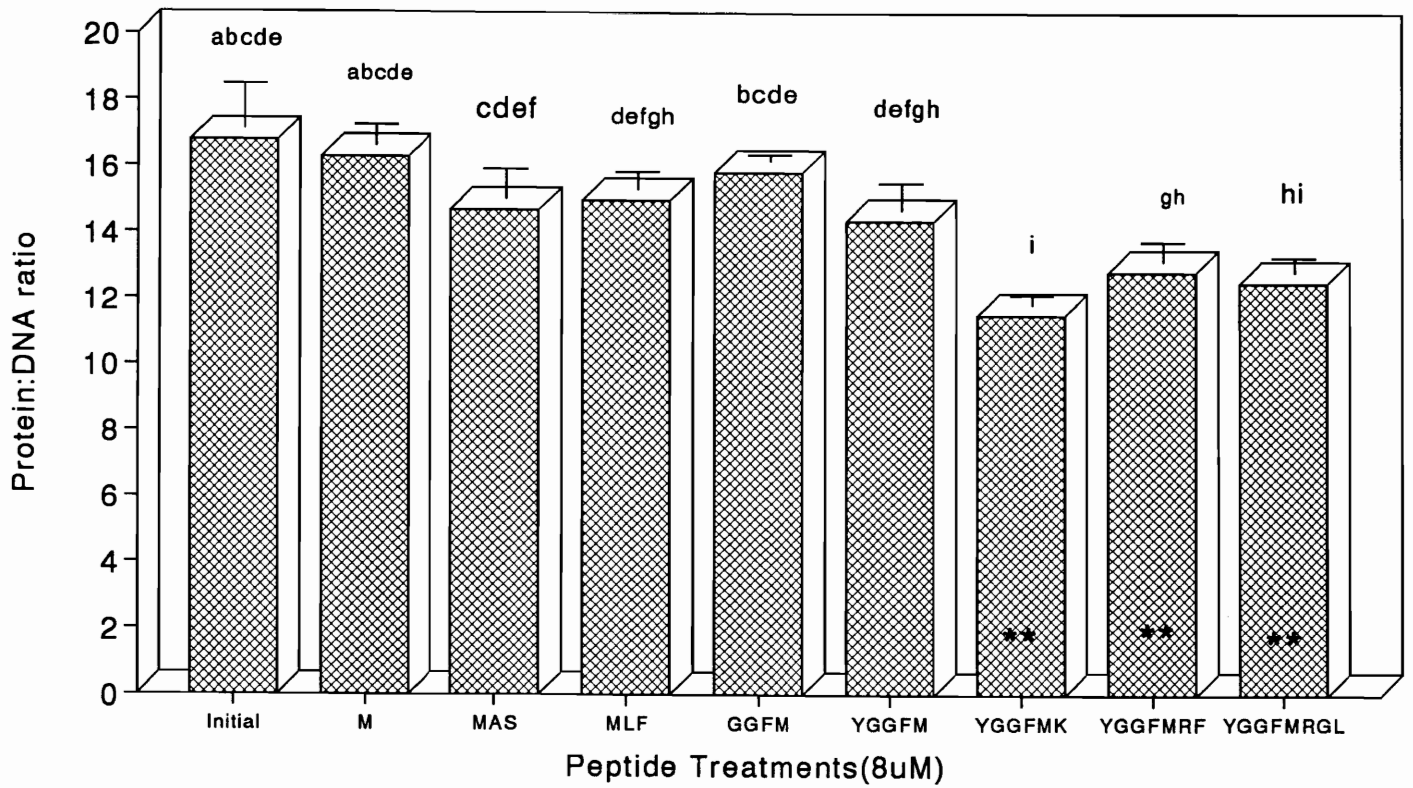


Figure 6.7. Effects of tri- to octapeptides on protein:DNA ratio in primary cultures of ovine skeletal muscle. Bars (means+SE) with different letters differ ($P < .05$). **Smaller than methionine response ($P < .05$). n = 16.

Chapter VII

Epilogue

There are free and peptide-bound amino acid pools in the blood. The gastrointestinal tract and kidneys have proton-driven, active transport systems to get peptide-bound amino acids absorbed into the blood and to prevent these peptides from being lost during urine formation. This suggests that animals possess certain systems to maintain these peptides within the body for some purpose. Serving as sources of amino acids appears to be at least one function of these circulating peptides (Krzysik and Adibi, 1977; Adibi, 1987; Albers et al., 1988). Certain peptides may have various regulatory impacts on cell growth and other functions (Amborski et al. 1970; Pickart and Thaler, 1973).

Compared with free amino acids, peptide-bound amino acids are more stable and soluble and can store more N per volume of blood (Adibi, 1987). These features make peptide-bound amino acids a better storage form of amino acids within the animal body. Hydrolysis of circulating peptides may be regulated by the concentration of free amino acids (Lombardo et al., 1988). Constant utilization of free amino acids in blood by tissues may decrease the blood concentrations of free amino acids and promote the hydrolysis of circulating peptides to replenish the diminished free amino acid pool. This mechanism can avoid the presence of excessive free amino acids after meals, minimize the energy cost for the disposal of excessive free amino acids, and stabilize the free amino acid pool between meals. If this is true, maximizing the flux of peptide-bound amino acids across the gastrointestinal tract after meals may improve the amino acid supply for the relatively rapid growth in young growing animals and for the increased requirements by lactating mammary glands.

In the current experiments, only a limited number of peptides and tissues (cells) were examined. Therefore, more research is needed to investigate whether cultured myogenic and mammary epithelial cells from other species and whether other cell types can also utilize these methionine-containing peptides and other peptides for protein accretion and cell proliferation.

Kidney and intestinal cells have peptide transport systems, so it would be very interesting to investigate whether these cells can utilize peptide-bound amino acids and whether there is any different effects of peptide supplementation on protein synthesis and proliferation of these cells when the peptides are added to the culture media either on the brushborder membrane side or basolateral side.

Both free and peptide-bound amino acids are present in the blood of animals in vivo, it will be interesting to investigate the effects of coexistence of both peptide-bound amino acids (from the same peptide or a mixture of peptides) and free amino acids on protein accretion and cell proliferation of cultured animal cells.

Certain peptides have been shown to exhibit specific effects on liver cells (Pickart and Thaler), therefore, it is possible that the growth of other tissues is also specifically affected by certain peptides Different tissues in vivo have been shown to have different abilities to utilize amino acids from same dipeptides (Lochs et al., 1988). It will be of particular interest to study the peptide-utilizing abilities among tissues and the specific effects of peptides on individual tissues so that protein accretion and cell proliferation of individual tissues can be manipulated by supplying different peptides in the diets.

Intestinal absorption of peptides of dietary origin has been shown to play an important role in the assimilation of the end products of dietary protein digestion. However,

little has been known about the regulation of the peptide absorption by hormones and other nutrients. Understanding this may improve protein digestion and absorption by the gastrointestinal tract.

In general, peptide-bound amino acids appear to play an important role in supplying amino acids to tissues for protein accretion and cell proliferation. But much work will be needed to elucidate the peptide-utilizing ability of animal tissues and the effects of peptides on cell growth and other physiological processes of individual tissues.

Literature Cited

Abraham, D., J.J. Pisano and S. Udenfriend. 1964. Uptake of carnosine and homocarnosine by rat brain slices. Arch. Biochem. and Biophy. 104:160.

Abumrad, N. N. and B. Miller. 1983. The physiologic and nutritional significance of plasma free amino acid levels. J. Parent. Ent. Nutr. 7:163.

Addison, J. M. D. Burston and D. M. Matthews. 1972. Evidence for active transport of the dipeptide glycylsarcosine by hamster jejunum in vitro. Clin. Sci. 43:907.

Addison, J.M., D. Burston, J.A. Dalrymple, D.M. Matthews, J.W. Payne, M.H.Sleisenger and S. Wilkinson. 1975a. A common mechanism for transport of di and tripeptides by hamster jejunum in vitro. Clin. Sci. Mol. Med. 49:313.

Addison, J.M., D. Burston, J.W. Payne, S. Wilkinson and D.M. Matthews. 1975b. Evidence for active transport of tripeptides by hamster jejunum in vitro. Clin. Sci. Mol. Med. 49:305.

Adibi, S. A. and E. L. Morse. 1977. The number of glycine residues which limits intact absorption of glycine oligopeptides in human jejunum. J. Clin. Invest. 60:1008.

Adibi, S. A. and E. L. Morse. 1981. Mechanism of diglycine accumulation in muscle. J. Lab. Clin. Med. 97:583.

Adibi, S.A. 1987a. Utilization of intravenously administered peptides: Influence of molecular structure. In Adibi, S.A., W. Fekl, P. Furst and M. Oehmke (Ed.) Dipeptide As New Substrates in Nutrition Therapy. p. 69. Karger, New York.

Adibi, S.A. and B.A. Johns. 1983. Utilization of intravenously infused tripeptides in baboons: Effect on plasma concentration and urinary excretion of amino acids. Metabolism 32:103.

Adibi, S.A. and B.A. Krzysik,. 1977. Effect of nephrectomy and enterectomy on plasma clearance of intravenously administered dipeptides in rats. Clin. Sci. Mol. Med. 52:205.

Adibi, S.A., and B.A. Johns. 1984. Partial substitution of amino acids of a parenteral solution with tripeptides:

effects on parameters of protein nutrition in baboons. Metabolism. 33:420.

Adibi, S.A., and M.R. Soleimanpour. 1974. Functional characterization of dipeptide transport system in human jejunum. J. Clin. Invest. 53:1368.

Adibi, S.A., B.A. Krzysik and A.L. Drash. 1977. Metabolism of intravenously administered dipeptides in rats: Effects on amino acid pool, glucose concentration and insulin and glucagon secretion. Clin. Sci. Mol. Med. 52:193.

Adibi, S.A., G.A. Paleos and E.L. Morse. 1986. Influence of molecular structure on half-life and hydrolysis of dipeptides in plasma: importance of glycine as N-terminal amino acid residue. Metabolism. 35:830-836.

Adibi, S.A.. 1987b. Experimental basis for use of peptides as substrates for parenteral nutrition: A review. Metabolism. 36:1001.

Agar,W.T., F.J.R. Hird and G.S. Sidhu. 1953 The active absorption of amino acids by the intestine. J. Physiol. 121:255.

Albers, A., J. Wernerman, P. Stehle, E. Vinnars and P. Furst. 1988. Availability of amino acids supplied intravenously in healthy man as synthetic dipeptides: kinetic evaluation of L-alanyl-L-glutamine and glycyl-L-tyrosine. Clin. Sci. 75:463.

Amborski, R.L., M. Dietrich and G.F. Amborski. 1970. Effect of peptides on animal cells in culture. I. Maintenance of beating heart cells. Exp. Cell Res. 63:427.

Anderson, L.D. and J.A. Rilema. 1976. Effects of hormones on protein and amino acid metabolism in mammary-gland explants of mice. Biochem. J. 158:355.

Annison, E. F. 1956. Nitrogen metabolism in the sheep. Biochem. J. 64:705.

Ardawi, M. S .M. 1986. The transport of glutamine and alanine into rat colonocytes. Biochem. J. 238:131-135.

Argyle, J.L. and R.L. Baldwin. 1989. Effects of amino acids and peptides on rumen microbial growth yields. J. Dairy Sci. 72:2017.

Asatoor, A.M. A. Chadha, M. D. Milne and D. I. Prosser. 1973. Intestinal absorption of stereoisomers of dipeptides in the rat. Clin. Sci. Mol. Med. 45:199-212.

Asatoor, A.M., M.R. Bending, and M.D. Milne. 1978. Peptide excretion in experimental Fanconi syndrome in the rat. Clin. Sci. Mol. Med. 55:205.

Batt, E. R. and D. Schachter. 1969. Developmental pattern of some intestinal transport mechanisms in newborn rat and mice. Am. J. Physiol. 216:1064-1068.

Baumrucker, C.R. and B.H. Stemberger. 1989. Insulin and insulin-like growth factor-I stimulate DNA synthesis in bovine mammary tissue in vitro. J. Anim. Sci. 67:3503.

Becker, J.M. and F. Naider. 1977. Peptide transport in yeast: Uptake of radioactive trimethionine in Saccharomyces cerevisiae. Arch. Biochem. Biophy. 178:245-255.

Becker, J.M., F. Naider and E. Katchalski. 1973. Peptide utilization ib yeast: studies on methionine and lysine auxotrophs of Saccharomyces cerevisiae. Biochim. et Biophy. Acta. 291:388-397.

Berteloot, A., A.H. Khan and K. Ramaswamy. 1981. Characteristics of dipeptide transport in normal and papain-treated brush border membrane vesicles from mouse intestine. Biochem. Biophy. Acta. 649:179-188.

Bollard, E.G. 1966. A comparative study of the ability of organic nitrogenous compounds to serve as sole sources of nitrogen fro the growth of plants. Plant Soil. 25:153.

Boullin, D. J., R. F. Crampton, C. E. Heading and D. Pelling. 1973. Intestinal absorption of dipeptides containing glycine, phenylalanine, proline, Ô-alanine or histidine in the rat. Clin. Sci. Mol. Med. 45:849.

Brand, K., J. von Hintzenstern, K. Langer and W. Fekl. 1987. Pathways of glutamine and glutamate metabolism in resting and proliferating rat thymocytes: Comparison between free and peptide-bound glutamine. J. Cell. Physiol. 132:559.

Braun, V. and K. Hantke. 1974. Biochemistry of bacteria cell envelopes. Annu. Rev. Biochem.. 43:89.

Broderick, G. A. and R. J. Wallace. 1988. Effects of dietary nitrogen source on concentrations of ammonia, free amino acids and fluorescamine-reactive peptides in the sheep rumen. J. Anim. Sci. 66:2233.

Broderick, G.A., R.J. Wallace and N. McKain. 1988. Uptake of small neutral peptides by mixed rumen microorganisms in vitro. J. Sci. Food Agric. 42:109.

Burg, M. and N. Green. 1977. Bicarbonate transport by isolated perfused rabbit proximal convoluted tubules. Am J. Physiol. 233:F307.

Burston, D., and D.M. Matthews. 1990. Kinetics of influx of peptides and amino acids into hamster jejunum in vitro: physiological and theoretical implications. Clin. Sci. 79:267.

Burston, D., T.C. Marrs, M.H. Sleisenger, T. Sopanen, and D.M. Matthews. 1977. Peptidases in germinating barley grain: properties, localization and possible functions. In Elliot, K. and M. O'Connor (Eds) Peptide transport and Hydrolysis, Ciba Foundation Symposium. p:79. Associated Scientific Publ. Amsterdam

Burston,D., E. Taylor and D. M. Matthews. 1980. Kinetics of uptake of lysine and lysyllysine by hamster jejunum in vitro. Clin. Sci. 59:285.

Calonge, M.L., A. Ilundain and J. Bolufer. 1990. Glycylsarcosine transport by epithelial cells isolated from chicken proximal cecum and rectum. Am. J. Physiol. 258:G660.

Candal, F. J., G. Marchetti, M. Davis, V. George and E.W. Ades. 1989. Mammalian tissue culture growth, viral replication and cultivation using serum replacement factor. Proc. Soc. Exp. Biol. Med. 192:1.

Cheeseman, C.I. 1980. The transport of dipeptides by the small intestine. Can. J. Physiol. Pharmacol. 58:1326.

Cheeseman, C.I. and G. Johnston. 1982. Glycyl-L-leucine transport in the rat small intestine. Can. J. Physiol. Pharmacol. 60:1177.

Chen, G., C.J. Sniffen and J. B. Russel. 1987a. Concentration and estimated flow of peptides from the rumen of dairy cattle: Effects of protein quantity, protein solubility and feeding frequency. J. Dairy Sci. 70:983.

Chen, G., H.J. Strobel, J.B. Russel and C. J. Sniffen. 1987b. Effect of hydrophobicity on utilization of peptides by ruminal bacteria in vitro. Appl. Environ. Micro. 53:2021.

Christensen, H.N., D.G. Decker, E.L. Lynch, T.M. Mackenzie and J.H. Powers. 1947. The conjugated, non-protein, amino acids of plasma. V. A study of the clinical significance of peptidemia. J. Clin. Invest. 26:853.

Cohen, S.A., M. Meys, and T.L. Tarvin. 1989. The Pico-Tag Method. A Manual of Advanced Techniques for Amino Acid Analysis. WM02, Rev.1. Millipore Corporation, Bedford, MA.

Costerton, J.W., J.M. Ingram and K.J. Cheng. 1974. Structure and function of the cell envelope of Gram-negative bacteria. Bacteriol. Rev.. 38:87-110.

Craft, I.L., D. Geddes, C.W. Hyde, I.J. Wise and D.M. Matthews. 1968. Absorption and malabsorption of glycine and glycine peptides in man. Gut. 9:425.

Daniel, H., E.L. Morse and S.A. Adibi. 1991. The high and low affinity transport systems for dipeptides in kidney brush border membrane respond differently to alterations in pH gradient and membrane potential. J. Biol. Chem. 266:19917.

Daniel, H., E.L. Morse, and S.A. Adibi. 1992. Determination of substrate affinity for the oligopeptide/H^+ symporter in the renal brush border membrane. J. Biol. Chem. 267:9565.

Danilson, D.A., K.E. Webb,Jr. and J.H. Herbein. 1986. Transport and hindlimb exchange of peptide and serum protein amino acids in calves fed soy and urea purified diets. J. Anim. Sci. 64:1852.

DiRienzo, D. B. 1990. Free and peptide amino acid fluxes across the mesenteric and non-mesenteric viscera of sheep and calves. Ph.D Dissertation, Virginia Polytechnic Institute and State University.

Dodson, M.V., B.A. Mathison and B.D. Mathison. 1990. Effects of medium and substratum on ovine satellite cell attachment, proliferation and differentiation in vitro. Cell Differ. Develop. 29:59.

Dodson, M.V., D.C. McFarland, E.L. Martin, and M.A. Brannon. 1986. Isolation of satellite cells from ovine skeletal muscles. J. Tiss. Cult. Methods. 10:233.

Dorer, F. E., E. E. Haley and D. L. Buchanan. 1968. The hydrolysis of ß-aspartyl peptides by rat tissue. Arch. Biochem. Biophy. 127:490.

Eagle, H. 1955. Utilization of dipeptides by mammalian cells in tissue culture. Proc. Soc. Exp. Biol. Med. 89:96.

Emmerson, K.S. and J.M. Phang. 1993. Hydrolysis of proline dipeptides completely fulfills the proline requirement in a proline-auxotrophic Chinese hamster ovary cell line. J. Nutr. 123:909.

Evered, D. F. and P. B. Nunn. 1968. Uptake of amino acids by mucosa of rat colon in vitro. Europ. J. Biochem. 4:301.

Furst, P., S. Albers and P. Stehle. 1987. Stress-induced intracellular glutamine depletion: The potential use of glutamine-containing peptides in parenteral nutrition. In Adibi, S.A., W. Fekl, P. Furst and M. Oehmke (Ed) Peptides as New Substrates in Nutrition Therapy. p. 117. Karger, New York.

Ganapathy, V. and A.N. Radhakrishnan. 1980. Sodium-dependent inhibition of amino acid, and dipeptide transport by harmaline in monkey small intestine. Biochem. Pharmacol. 29:713.

Ganapathy, V. and F. H. Leibach. 1985. Is intestinal peptide transport energized by a proton gradient? Am J. Physiol. 249:G153.

Ganapathy, V. and F.H. Leibach. 1983. Role of pH gradient and membrane potential in dipeptide transport in intestinal and renal brush border membrane vesicles from the rabbit. J. Biol. Chem. 258:14189.

Ganapathy, V., G. Burckhardt and F.H. Leibach. 1984. Characteristics of glycylsarcosine transport in rabbit intestinal brush-border membrane vesicles. J. Biol. Chem. 259:8954.

Gardner, M. G. and D. Wood. 1989. Transport of peptides across the gastrointestinal tract. Biochem. Soc. Trans. 17:934.

Gardner, M. L. G., B. S. Lindbald, D. Burston and D. M. Matthews. 1983. Transmucosal passage of intact peptides

in the guinea-pig small intestine in vivo: a reappraisal. Clin. Sci. 64:433.

Gibson, M.M., M. Price and C.F. Higgins. 1984. Genetci characterization and molecular cloning of the tripeptide permease (tpp) genes of Salmonella typhimurium. J. Bacteriol. 160:122.

Gilvarg, C. and E. Katchalski. 1965. Peptide utilization in Escherichia coli. J. Biol. Chem. 240:3093.

Grahl-Nielsen, O., P. Odegaard and G.L. Tritsch. 1974. Oligopeptides as sources of indispensable amino acids for mammalian cells in culture. In Vitro. 9:414.

Granner, D. K.. 1990. Hormones of the pancreas and GI tract.In Murray, R. K., P. A. Mayes, D. K. Granner and V. W. Rodwell (Eds) Harper's Biochemistry. p:530. Prentice Hall, Englewood Cliffs, New Jersey.

Guyer, C. A., D.G. Morgan and J.V. Staros. 1986. Binding specificity of the periplasmic oligopeptide-binding protein from Escherichia coli. J. Bacteriol. 168:775.

Guyton, A.C.. 1986. Formation of urine by the kidney: glomerular filtration, tubular function and plasma clearance. In Dreibelbis, D. (Ed) Textbook of Medical Physiology. p.393. W.B. Saunders Company. Philadelphia, PA 19105.

Hagen, T. M. and D. P. Jones. 1987. Transepithelial transport of glutathione in vascularly perfused small intestine of rat. Am. J. Physiol. 252:G607.

Heading, C. E., C. S. Rogers and S. Wilkinson. 1978 Absorption of two tyrosine containing dipeptides from the small intestine and rectum of the rat. J. Physiol. 278:21P.

Heading, C. E., C. S. Rogers and S. Wilkinson. 1979. Absorption of two tyrosine containing tri-peptides from the small intestine and rectum of the rat. J. Pharm. Pharmacol. 31:39.

Hellier, M. D., C. Thirumalai and C. D. Holdsworth. 1973. The effect of amino acids and dipeptides on sodium and water absorption in man. Gut 14:41.

Higgins, C.F. and J.W. Payne. 1977a. Peptide transport by germinating barley embryos. Planta. 134:205.

Higgins, C.F. and J.W. Payne. 1977b. Characterization of active dipeptide transport by germinating barley embryos: Effects of pH and metabolic inhibitors. Planta 136:71.

Higgins, C.F. and M.M. Gibson. 1986. Peptide transport in bacteria. Methods Enzymol. 125:365.

Higgins, C.F., and J.W. Payne. 1977. Peptide transport by germinating barley embryos. Planta. 134:205.

Higgins, C.F., and J.W. Payne. 1978. Peptide transport by germinating barley embryos: uptake of physiological di- and oligopeptides. Planta. 138:211.

Higgins, C.F., and M.M. Hardie. 1983. Periplasmic protein associated with the oligopeptide permeases of Salmonella typhimurium and Escherichia coli. J. Bacteriol. 155:1434.

Higgins, C.F.. 1984. Peptide transport systems of Salmonella typhimurium and Escherichia coli. Microbiology. 17

Hiles, I.D., M.P. Gallagher, D.J. Jamieson and C.F. Higgins. 1987. Molecular characterization of the oligopeptide permease of Salmonella typhimurium. J. Mol. Biol. 193:125.

Himukai, M. and T. Hoshi. 1980. Mechanisms of glycyl-L-leucine uptake by guinea-pigs small intestine: relative importance of intact-peptide transport. J. Physiol. 302:155.

Himukai, M., A. Kameyama and T. Hoshi. 1983. Interaction of glycylglycine and Na^+ at the mucosal border of guinea-pig small intestine: a non-mutual stimulation of transport. Biochem. Biophy. Acta 732:659.

Himukai, M., A. Kano-Kameyama and T. Hoshi. 1982. Mechanisms of inhibition of glycylglycine transport by glycyl-L-leucine and L-leucine in guinea-pig small intestine. Biochem. Biophy. Acta 687:170.

Himukai, M., Y. Suzuki and T. Hoshi.1978. Differences in characteristics between glycine and glycylglycine transport in guinea pig small intestine. Jpn. J. Physiol. 28:499.

Hogarth, B.G., and C.F. Higgins. 1983. Genetic organization of the oligopeptide permease (opp) locus of Salmonella

typhimurium and Escherichia coli. J. Bacteriol. 153:1548.

Hundal, H.S. and M.J. Rennie. 1988. Skeletal muscle tissue contains extracellular aminopeptidase activity against Ala-Gln but no peptide transporter. Eur. J. Clin. Invest. 18:A34.

Huynh, H.T., G. Robitaille and J.D. Turner. 1991. Establishment of bovine mammary epithelial cells (MAC-T): an in vitro model for bovine lactation. Exp. Cell Res. 197:191.

Isemura, M., T. Hanyu, F. Gejyo, R. Nakazawa, R. Igarashi, S. Matsuo, K. Ikeda and Y. Sato. 1979. Prolidase deficiency with imidodipeptiduria: a familial case with and without clinical symptoms. Clin. Chim. Acta. 93:401.

Jamieson, D.J. and C.F. Higgins. 1984. Anaerobic and leucine-dependent expression of a peptide transport gene in Salmonella typhimurium. J. Bacteriol. 160:131.

Jamieson, D.J. and C.F. Higgins. 1986. Two genetically distinct pathways for transcriptional regulation of anaerobic gene expression in Salmonella typhimurium. J. Bacteriol. 168:389.

Kasuga, M., E. Van Oberghen, S.P. Nissley, and M.M. Rechler. 1981. Demonstration of two subtypes of insulin-like growth factors by affinity crosslinking. J. Biol. Chem. 256:5305.

Kenny, A.J.. 1977. Endopeptidases in the brush border of the kidney proximal tubule. In Peptide Transport and Hydrolysis (Ciba Foundation Symposium). p.209. Elsevier, North Holland, New York.

Kessel, D. and M. Lubin. 1963. On the distinction between peptidase activity and peptide transport. Biochim. Biophy. Acta. 71:656.

Kihara, H. and E.E. Snell. 1952. Peptide and bacterial growth II. L-analine peptides and growth of Lactobacillus casei. J. Biol. Chem. 197:791.

Kihara, H. and E.E. Snell. 1955. Peptides and Bacterial Growth VII. Relation to inhibition by thienylalanine, ethionine, and canavanine. J. Biol. Chem. 212:83.

Kihara, H., O.A. Klatt and E.E. Snell. 1952. Peptide and bacterial growth III. Utilization of tyrosine and tyrosine peptides by S. faecalis. J. Biol. Chem. 197:801.

King, G.F. and P.W. Kuchel. 1984. A proton n.m.r. study of iminodipeptide transport and hydrolysis in the human erythrocyte. Biochem. J. 220:553.

King, G.F. and P.W. Kuchel. 1985. Assimilation of a-glutamyl-peptides by human erythrocytes. Biochem. J. 227:833.

King, G.F., M.J. York, B.E. Chapman and P.W. Kuchel. 1983. Proton NMR spectroscopic studies of dipeptidase in human erythrocytes. Biochim. Biophy. Res. Commun. 110:305.

Kleinman, J.G., W.W. Brown, R.A. Ware and J.H. Schwartz. 1980. Cell pH and acid transport in renal cortical tissue. Am. J. Physiol. 239:F440.

Koeln, L.L., T. S. Schlagheck, and K.E. Webb, Jr. 1993. Amino acid flux across the gastrointestinal tract and liver of calves. J. Dairy Sci. 76:(In press).

Krzysik, B. A. and S. A. Adibi. 1977. Cytoplasmic dipeptidase activities of kidney, ileum, jejunum, liver, muscle and blood. Am. J. Physiol. 233:E450.

Krzysik, B.A. and S.A. Adibi. 1979. Comparison of metabolism of glycine injected intravenously in free and dipeptide forms. Metabolism 28:1211.

Kurtin, P. and A.N. Charney. 1984. Intestinal ion transport and intracellular pH during acute respiratory alkalosis and acidosis. Am. J. Physiol. 247:G24.

Labarca, C. and K. Paigen. 1980. A simple, rapid, and sensitive DNA assay procedure. Anal. Biochem. 102:344.

Leach, F.R. and E.E. Snell. 1959. Occurrence of independent uptake mechanisms fro glycine and glycine peptides in L. casei. J. Biol Chem. 34:292.

Lind, J., B. G. Munck and O. Olsen. 1980. Effects of dietary intake of sodium chloride on sugar and amino acid transport across isolated hen colon. J. Physiol. 305:327.

Lis, M.T., D.M. Matthews and R.F. Crampton. 1972. Effects of dietary restriction and protein deprivation on intestinal absorption of protein digestion products in the rat. Brit. J. Nutrition. 28:443.

Liversidge, G. G. 1989. Rectal route of administration for peptides. Biochem. Soc. Trans. 17:942.

Lochs, H., E.L. Morse and S.A. Adibi. 1986. Mechanism of hepatic assimilation of dipeptides: Transport versus hydrolysis. J. Biol. Chem. 261:14976.

Lochs, H., P. E. Williams, E. L. Morse, N. N. Abumrad and S. A. Adibi. 1988. Metabolism of dipeptides and their constitute amino acids by liver, gut, kidney and muscle. Am. J. Physiol. 254:E588.

Lombardo, Y.B., E.L. Morse and S.A. Adibi. 1988. Specificity and mechanism of influence of amino acid residues on hepatic clearance of oligopeptides. J. Biol. Chem. 263:12920.

Lucas, M.L. 1983. Determination of acid surface pH in vivo in rat proximal jejunum. Gut 24:734.

Lucas, M.L., J.A. Blair, B.T. Cooper and W.T. Cooke. 1976. Relationship of the acid microclimate in rat and human intestine to malabsorption. Biochem. Biophy. Trans. 4:154.

Lucas, M.L., W. Schneider, F.J. Haberich, J.A. Blair. 1975. Direct measurement by pH-microelectrode of the pH microclimate in rat proximal jejunum. Proc. R. Soc. Lond. 192:39.

Manson, M.D., V. Blank, G. Brade, and C.F. Higgins. 1986. Peptide chemotaxis in E. coli involves the Tap signal transducer and the dipeptide permease. Nature. 321:253.

Marder, R., J. M. Becker and F. Naider. 1977. Peptide transport in yeast. Utilization of leucine- and lysine-containing peptides by Saccharomyces cerevisiae. J. Bacteriol. 131:906.

Matile, P., H. Moor and C.F. Robinow. 1969. Yeast CytologyIn Rose, A.H. and J.S. Harrison (Eds) The Yeast. 1:219. Academic Press, New York.

Matthews, D. M. 1987. Mechanisms of peptide transport. In S. A. Adibi, W. Fekl, P. Furst and M. Oehmkes (Ed).

Dipeptides as New Substrates in Nutrition Therapy. Karger, New York.

Matthews, D. M. and J. W. Payne. 1975. Peptides in the nutrition of microorganisms and peptides in relation to animal nutrition. In Matthews, D. M. and J. W. Payne (ED) Peptide Transport in Protein Nutrition. p1. North Holland. Amsterdam.

Matthews, D.M. 1991a. Protein Absorption: Development and Present State of the Subject. Wiley-Liss, Inc., New York.

Matthews, D.M. and D. Burston. 1983. Uptake of L-leucyl-L-leucine and glycylsarcosine by hamster jejunum in vitro. Clin. Sci. 65:177.

Matthews, D.M. and D. Burston. 1984. Uptake of a series of neutral dipeptides including L-alanyl-L-alanine, glycylglycine, and glycylsarcosine by hamster jejunum in vitro. Clin. Sci. 67:541.

Matthews, D.M. and J.W. Payne. 1980. Transmembrane transport of small peptides. Curr. Top. Membr. Transp. 14:331.

Matthews, D.M., I.L. Craft, D.M. Geddes, I.J. Wise and C.W., Hyde. 1968. Absorption of glycine and glycine peptides from the intestine of the rat. Clin. Sci. 35:415.

Matthews, D.M.. 1975. Intestinal absorption of peptides. Physiol. Rev. 55:537.

Matthews, J.C. 1991b. Absorption of carnosine and methionylglycine by sheep ruminal and omasal epithelia. MS Dissertation. Virginia Polytechnic Institute and State University.

Matthews, J.C., and K.E. Webb, Jr.. 1993. Absorption of L-methionine and L-methionylglycine by sheep ruminal and omasal epithelia. The FASEB Journal. 7:A523.

McCormick, M. E. and K. E. Webb, Jr. 1982. Plasma free, erythrocyte free and plasma peptide amino acid exchange of calves in steady state and fasting metabolism. J. Nutr. 112:276-282.

Mikola, J., and L. Kolehmainen. 1972. Localization and activity of various peptides in germinating barley. Planta. 104:167.

Miyamoto, Y., Y.G. Thompson, E.F. Howard, V. Ganapathy and F.H. Leibach. 1991. Functional expression of the intestinal peptide-proton co-transporter in Xenopus laevis oocytes. J. Biol. Chem. 266:4742.

Naider, F., J. M. Becker and E. Katzir-Katchalski. 1974. Utilization of methionine-containing peptides and their derivatives by a methionine-requiring auxotroph of Saccharomyces cerevisiae. J. Biol. Chem. 249:9.

Neshelm, M.C. and K.J. Carpenter. 1967. The digestion of heat damaged protein. Br. J. Nutr. 21:399.

Newey, H. and D. H. Smith. 1959. The intestinal absorption of some dipeptides. J. Physiol. 145:48.

Nutzenadel, W. and C. R. Scriver. 1976. Uptake and metabolism of ß-alanine and L-carnosine by rat tissues in vitro: role in nutrition. Am. J. Physiol. 230:643.

Nutzenadel, W. and C. R. Scriver. 1976. Uptake and metabolism of ß-alanine and L-carnosine by rat tissues in vitro: role in nutrition. Am. J. Physiol. 230:643.

Payne, J. W. 1968. Oligopeptide transport in E. coli: specificity with respect to side chain and distinction from dipeptide transport. J. Biol. Chem. 243:3395.

Payne, J. W. 1975. Transport of peptide in microorganisms. In Matthews, D.M. and J.W. Payne (Eds) Peptide Transport in Protein Nutrition. p.283. North Holland, New York.

Payne, J.W. 1980a. Transport and utilization of peptides by bacteria. In Payne, J.W. (Ed) Microorganisms and Nitrogen Sources. p.211. John Wiley and Sons Ltd. New York

Payne, J.W. 1980b. Transport and utilization of peptides by bacteria. In Payne, J.W. (Ed) Microorganisms and Nitrogen Sources. p.281. John Wiley and Sons Ltd. New York.

Payne, J.W. 1980c. Microorganisms and Nitrogen Sources. John Wiley and Sons Ltd., New York.

Payne, J.W. and C. Gilvarg. 1968a. The role of the terminal carboxyl group in peptide transport in Escherichia coli. J. Biol. Chem. 243:335.

Payne, J.W. and C. Gilvarg. 1968b. Size restriction on peptide utilization in Escherichia coli. J. Biol. Chem. 243:6291.

Payne, J.W.. 1972. Effects of N-methyl peptide bonds on peptide utilization by Escherichia coli. J. Gen. Microbiol. 71:259.

Penry, D.L. and P.A. Jumars. 1987. Modeling animal guts as chemical reactors. Am. Nat 129:69.

Perry,T. L., S. Hansen, B. Tischler, R. Bunting and K. Berry. 1967. Carnosinemia: a new metabolic disorder associated with neurologic disease and mental defect. New Engl. J. Med. 277:1219.

Peters, V.J., J.M. Prescott and E.E. Snell. 1952. Peptide and bacterial growth IV. Histidine peptides as growth factors for L. delbrueckii 9649. J. Biol. Chem. 202:521.

Pickart, L. and M. M. Thaler. 1973. Tripeptide in human serum which prolongs survival of normal liver cells and stimulates growth in neoplastic liver. Nature New Biol. 243:85.

Piez, K. A., V.I. Oyama, L. Levintow and H. Eagle. 1960. Proteolysis in stored serum and its possible significance in cell culture. Nature. 188:59.

Pittman, K.A. and M.P. Bryant. 1964. Peptides and other nitrogen sources for growth of Bacteroides ruminicola. J. Bacteriol. 88:401.

Pittman, K.A., S. Lakshmanan and M.P. Bryant. 1967. Oligopeptide uptake by Bacteroides ruminicola. J. Bacteriol. 93:1499.

Plummer G.L. and J.B. Kethley. 1964. Foliar absorption of amino acids, peptides and other nutrients by the pitcher plant, Sarracenia flava. Bot. Gaz. (Chicago) 125:245.

Prescott, J.M., V.J. Peters and E.E. Snell. 1953. Peptides and bacterial growth V. Serine peptides and growth of L. delbrueckii 9649. J. Biol. Chem. 202:533.

Radhakrishnan. 1977. Intestinal dipeptidases and dipeptide transport in the monkey and in man. In Elliott, K. and M. O'Connor (ED) Peptide Transport and Hydrolysis. p37. Elsevier. Amsterdam.

Raghunath, M., E.L. Morse, and S.A. Adibi. 1990. Mechanism of clearance of dipeptides by perfused hindquarters: sarcolemmal hydrolysis of peptides. Am. J. Physiol. 259:E463.

Rodwell, V.W.. 1990. Conversion of amino acids to specialized products. In Murray, R.K., P.A. Mayes, D.K. Granner and V.W. Rodwell (Ed). Harper's Biochemistry. p.307. Appleton & Lange, Norwalk, Connectcut/San Mateo, California.

Rogers, H.J., J.B. Ward and I.D.J. Burdett. 1978. Structure and growth of the walls of Gram-positive bacteria. In Stanier, R.Y., H.J. Rogers and B.J. Ward (Eds) Relations Between Structure and Function in the Prokaryotic Cell. p.139. Cambridge Univ. Press, London and New York.

Rubino, A., and S. Guandalini. 1977. Dipeptide transport in the intestinal mucosa of developing rabbits. In Elliott, K., and M. O'Connor (Ed) Peptide Transport and Hydrolysis. p61. Elservier, Amsterdam.

Russell, J. B., C. J. Sniffen and P. J. Vansoest. 1983. Effect of carbohydrate limitation on degradation and utilization of casein by mixed rumen bacteria. J. Dairy Sci. 66:763.

Salonen M.L.and L.K. Simola. 1977. Dipeptides and amino acids as nitrogen sources for the callus of Atropa belladonna. Physiol. Plant. 41:55.

SAS. 1989. SAS User's Guide: Statistics. SAS Inst. Inc., Cary, NC.

Schedl, H.P., D. Burston, E. Taylor and D.M. Matthews. 1979. Kinetics of mucosal influx of glycylsarcosine, glycine and leucine into hamster jejunum and ileum in vitro. Clin. Sci. 56:25.

Seal, C.J. and D.S. Parker. 1991. Isolation and characterization of circulating low molecular weight peptides in steer, sheep and rat portal and peripheral blood. Comp. Biochem. Physiol. 99B:679.

Shibanoki, S, S.B. Weinberger, D. Beniston, K. A. Nudelman, G. Schulteis, E. L. Bennett, M.R. Rosenzweig, K. Ishikawa and J. L. Martinez, Jr. 1991. Hydrolysis of leu-enkephalin by chick plasma in vitro. J. Pharmacol. Exp. Ther. 256:650.

Shibanoki, S., S.B. Weinberger, G. Schulteis, K. Ishikawa, J.L. Martinez, Jr.. 1992. Enkephalin hydrolysis by mouse plasma in vitro. Life Sci. 50:667.

Shimada, T.and T. Hoshi. 1986. Sodium-independent, hydrogen ion-dependent changes in membrane potential and conductance induced by dipeptides in Triturus enterocytes. Jpn. J. Physiol. 36:451.

Sigrist-Nelson, K. 1975. Dipeptide transport in isolated intestinal brush border membrane vesicles. Biochim. Biophys. Acta. 394:220.

Silbernagl, S., V. Ganapathy and F.H. Leibach. 1987. H+ gradient-driven dipeptide reabsorption in proximal tubule of rat kidney studies in vivo and in vitro. Am. J. Physiol. 253:F448.

Silk, D.B.A., P.D. Fairclough, N.J. Park, A.E. Lane, J.P.W. Webb, M.L. Clark and A.M. Dawson. 1975. A study of relations between the absorption of amino acids, dipeptides, water and electrolytes in the normal human jejunum. Clin. Sci. Mol. Med. 49:401.

Simmonds, S., and J.S. Fruton. 1948. The utilization of proline derivatives by mutant strains of Escherichia coli. J. Biol. Chem. 174:705.

Simmonds, S., E.L. Tatum, and J.S. Fruton. 1947a. The utilization of phenylalanine and tyrosine derivatives by mutant strains of Escherichia coli. J. Biol. Chem. 169:91.

Simmonds, S., E.L. Tatum, and J.S. Fruton. 1947b. The utilization of leucine derivatives by a mutant strain of Escherichia coli. J. Biol. Chem. 170:483.

Skopicki, H.A., K. Fisher, D. Zikos, R. Bloch, G. Flouret and D.R. Peters. 1991. Multiple carriers for dipeptide transport: carrier-mediated transport of glycyl-L-proline in renal BBMV. Am. J. Physiol. 261:F670.

Smith, P.K., R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, and D.C. Klenk. 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150:76.

Smithson K.W. and G. M. Gray. 1977. Intestinal assimilation of a tetrapeptide in the rat. J. Clin. invest. 60:665.

Sopanen, T., D. Burston and D.M. Matthews. 1977. Uptake of small peptides by scutellum of germinating barley. FEBS Lett. 79:4.

Sopanen, T., D. Burston, E. Taylor and D.M. Matthews. 1978. Uptake of glycylglycine by the scutellum of germinating barley grain. Plant Physiol. 61:630.

Stehle, P., and P. Furst. 1990. In vitro hydrolysis of glutamine-, tyrosine- and cystine-containing short chain peptides. Clin. Nutr. 9:37.

Stehle, P., I. Ratz and P. Furst. 1991. Whole-body autoradiography in rats after intravenous administration of L-alanyl-L-(U-14C)glutamine. Ann. Nutr. Metab. 35:213.

Steinhardt, H.J., G.A. Paleos, M. Brandl, W.L. Fekl and S.A. Adibi. 1984. Efficacy of a synthetic dipeptide mixture as the source of amino acids for total parenteral nutrition in a subhuman primate (baboon): Plasma concentration, metabolic clearance, and urinary excretion of a series of dipeptides. Gastroenterology 86:1562.

Sussman, A.J. and C. Gilvarg. 1971. Peptide transport and metabolism in bacteria. Ann. Rev. Biochem. 40:397.

Takuwa, N., T Shimada, H. Matsumoto, M. Himukai and T. Hoshi. 1985. Effect of hydrogen ion-gradient on carrrier-mediated transport of glycylglycine across brush border membrane vesicles from rabbit small intestine. Jap. J. Physiol. 35:629.

Taylor, S.P., S. Simmonds, and J.S. Fruton. 1950. Utilization of methionine derivatives by a mutant strain of Escherichia coli. J. Biol. Chem. 187:613.

Vandenberg, J.L., G.F. King and P.W. Kuchel. 1985. The assimilation of tri- and tetrapeptides by human erythrocytes. Biochim. Biophy. Acta 846:127.

Vazquez, J.A., G.A. Paleos, H.J. Steinhardt, K. Langer, M. Brandl, W.L. Fekl and S.A. Adibi. 1986. Protein nutrition and amino acid metabolism after 4 weeks of total parenteral nutrition with a mixture of 14 dipeptides: serendipitous observations on effects of sepsis in baboons. Am J. Clin. Nutr. 44:224.

Von Heijne, G. 1987. Protein sequence. In Von Heijne (Ed) Sequence Analysis in Molecular Biology. p99. Academic Press. New York.

Walker-Smith, D.J., and J.W. Payne. 1983. Peptide uptake in germinating barley embryos involves a dithiol-dependent transport protein. FEBS Lett. 160:25.

Walker-Smith, D. J. and J. W. Payne. 1984a. Characteristics of the active transport of peptides and amino acids by germinating barley embryos. Planta. 162:159.

Walker-Smith, D. J. and J. W. Payne. 1984b. Characteristics of the peptide-transport system in the scutellum of germinating barley embryos. Planta. 162:166.

Wang, S., K.E. Webb, Jr., and R.M. Akers. 1993. Methionine from peptides can be used by mammary tissue explants from lactating mice for the synthesis of secreted protein. The FASEB Journal. 7:A522 (Abstr.).

Webb, Jr., K. E. 1986. Amino acid and peptide absorption from the gastrointestinal tract. Fed. Proc. 45:2269.

Wiggans, D.S. and J.M. Johnston. 1959. The absorption of peptides. Biochim. Biophys. Acta. 32:69.

Wolfinbarger, Jr., L., and G.A. Marzluf. 1974. Peptide utilization by amino acid auxotrophs of Neurospora crassa. J. Bacteriol. 119:371.

Wolfinbarger, Jr., L. and G.A. Marzluf. 1975a. Specificity and regulation of peptide transport in Neurospora crassa. Arch. Biochem. Biophy. 171:637.

Wolfinbarger, Jr., L. and G.A. Marzluf. 1975b. Size restriction on utilization of peptides by amino acid auxotrophs of Neurospora crassa. J. Bacteriol. 122:949.

Woottoon, R.. 1986. Analysis of the transport of glycyl-L-proline into brush-border membrane vesicles from rabbit small intestine. Biochem. Soc. Trans. 14:1192.

Wright, D.E. 1967. Metabolism of peptides by rumen microorganisms. Appl. Microbiol. 15:547.

Yaffe, D. and O. Saxel. 1977. Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. Nature. 270:725.

Yang, S.L., J.M. Becker and F. Naider. 1977. Transport of 14C-Gly-Pro in a proline peptidase mutant of Salmonella typhimurium. Biochim. et Biophy. Acta. 471:135.

Appendix

Cell Culture Medium, Reagent and Vessel Preparation

Medium and Reagent Preparation for C_2C_{12} Myogenic Cells

1. The growth medium for C_2C_{12} myogenic cells contained Dulbecco's modified Eagle's medium (DMEM)[1] supplemented with 15% (v/v) fetal bovine serum (FBS)[2] and 1% (v/v) antibiotic-antimycotic solution[3].
2. The freezing medium consisted of DMEM supplemented with 15% (v/v) FBS and 10% (v/v) dimethyl sulfoxide (DMSO)[4].
3. .5% trypsin solution is prepared by adding 1 mL 2.5% trypsin solution[5] to 4 mL Dulbecco's phosphate balanced saline (D-PBS)[6].

Primary Culture Medium and Vessel Preparation.

1. The preincubation medium for ovine satellite cells was composed of DMEM supplemented with 10% (v/v) horse serum (HS)[7], 1% (v/v) antibiotic-antimycotic solution, and .1% (v/v) gentamicin solution[8] (Dodson et al., 1986).
2. The growth medium for ovine satellite cells contained DMEM supplemented with 15% (v/v) HS, 1% (v/v) antibiotic-antimycotic solution, and .1% (v/v) gentamicin solution (Dodson et al, 1986).
3. The differentiation medium for ovine satellite cells contained DMEM supplemented with 1% (v/v) HS, 1% (v/v) antibiotic-antimycotic solution, and .1% (v/v) gentamicin solution (Dodson et al, 1986).
4. The freezing medium consisted of DMEM supplemented with 20% (v/v) HS, 10% (v/v) DMSO, 1% (v/v) antibiotic-

---

[1]Cat. No. 380-2430AG, GIBCO, BRL, Inc., Grand Island, NY
[2]Cat. No. 230-6140AG, GIBCO, BRL, Inc., Grand Island, NY
[3]Cat. No. 600-5240AG, GIBCO, BRL, Inc., Grand Island, NY
[4]Cat. No. D-8779, Sigma, Chemical Co., St. Louis, MO
[5]Cat. No. 610-5090AG, GIBCO, BRL, Inc., Grand Island, NY
[6]Cat. No. 310-4190PJ, GIBCO, BRL, Inc., Grand Island, NY
[7]Cat. No. 230-6050AG, GIBCO, BRL, Inc., Grand Island, NY
[8]Cat. No. 600-5710AD, GIBCO, BRL, Inc., Grand Island, NY

antimycotic solution, and .1% (v/v) gentamicin (Dodson et al., 1990)..

5. .10% trypsin solution was prepared by adding .5 mL 2.5% trypsin solution to 12 mL D-PBS.
6. The D-PBS solution was prepared by adding D-PBS powder[9] to 900 mL deionized H_2O, adjusting the pH to 7.08 with 1 N HCl solution, and bringing the volume to 1000 mL.
7. Pronase solution was prepared by dissolving 200 mg pronase E[10] in 100 mL D-PBS (pH=7.08) and sterilizing the solution by a tissue culture filter (250 mL)[11].
8. Coating 80 cm^2 Culture Flasks[12]. 1 mL thawed basement membrane matrix[13] is added to 9 mL cold DMEM supplemented with 1% antibiotic-antimycotic solution and mixed thoroughly. The 1:10 diluted matrix is added to the flasks so that the entire growth area is covered. The flasks are kept in a tissue culture hood for 1 h, then the unbound material is aspirated, and the flasks are rinsed gently with serum-free DMEM plus 1% antibiotic-antimycotic. The vessels are now ready to use.

Culture Procedure for C_2C_{12} Myogenic Cells

1. Thaw the liquid nitrogen-frozen cultures quickly by placing the vials in a 37°C water bath.
2. Transfer .5 mL of thawed cell suspension to a 80 cm^2 culture flask containing 13 mL growth medium and incubate the cells at 37°C in a humidified environment of 90% air and 10% CO_2
3. After 8 h of incubation, change the growth medium of the cultures and continue the incubation until about 70%

---

[9]Cat. No. 450-1600EB, GIBCO, BRL, Inc., Grand Island, NY
[10]Cat. No. P-5147, Sigma, Chemical Co., St. Louis, MO
[11]Cat. No. 09-730-218, Nalge Company, Rochester, NY
[12]Cat. No. 1-71196, Marsh Biomedical Products, Rochester, NY
[13]Cat. No. 40234, Collaborative Biomedical Products, Bedford, Mass

confluency indicated by the percentage of growth area occupied by cells under a microscope.

4. When the confluence rate reaches 70%, remove the growth medium, wash the cultures with D-PBS (37°C) once and add 2.0 mL .5% trypsin to the flasks. Then keep the flasks in the incubator for 10 min at 37°C.
5. Stop trypsin action by adding 4 mL growth medium.

Count the cell density with a hemacytometer and plate cells at 1* 10^4 cells/dish in 35 mm tissue culture dishes[14] or at 5,000 cells/well in 12-well plates[15] for peptide utilization experiment.

Isolation Procedure for Ovine Satellite Cells

1. Shear the lamb about the hind limbs and tail.
2. Anesthetize the lamb (pentabarbital, 6.5 g/100 mL saline, 10 to 15 mL per lamb).
3. Place the anesthetized lamb in dorsal recumbency on a stable table.
4. Scrub the ventral and medial parts of the hind limbs with zephiran chloride (1:750 dilution)[16], and then with 70% ethanol.
5. Skinning the sterilized area, isolate the semimembranosus and semitendinosus muscles and excise the muscles immediately after exsanguination.
6. Immediately soak the excised muscle portions in cold, sterilized PBS, and transport the muscle tissue to a laminar hood.
7. Wash the muscle with sterilized PBS.
8. Trim off excess connective tissue.

---

[14]Cat. No. 08-772A, Fisher Scientific, Pittsburgh, PA
[15]Cat. No. 08-757-16B, Fisher Scientific, Pittsburgh, PA
[16]Winthrop Laboratories, Division of Sterling Drug Inc., New York, NY

9. Clip small (2.5 to 5 cm) strips of skeletal muscle from the cleaned semimembranosus muscle and place the strips into a steriled beaker containing sterilized PBS.
10. Rinse the strips three times with PBS.
11. Grind the strips in a small sterile meat grinder[17]
12. Put 60 g minced muscle into each of the sterile centrifuge bottles (250 mL capacity)[18].
13. Add 120 mL pronase (2 mg/mL; dissoled in sterile PBS without calcium and magnisium).
14. Vortex the mixture solution briefly.
15. Incubate the mixture solution for 1 h at 37 ^{o}C in a nonshaking water bath and agitate the solution every 10 min.
16. After incubation, centrifuge the mixture solution at 1,500 x g for 12 min at 25^{o}C, discard the supernate and retain the pellets.
17. Add 100 mL of sterile PBS to each of the centrifuge bottles containing the pellets.
18. Gently vortex each bottle for 30 s.
19. Centrifuge the four bottles at 500 x g for 10 min at 25 ^{o}C.
20. Pour the resultant supernate into four sterile centrifuge bottles.
21. Repeat steps 17, 18, 19, 20 once.
22. Centrifuge the bottles containing the collected supernate at 1,500 x g for 10 min at 25 ^{o}C, discard the supernate, and retain the pellets.
23. Add 30 mL DMEM (low glucose)-10% HS to each of the four bottles containing the mononucleated cell pellets, and gently vortex the bottles.
24. Aliquot 20 mL of the above cell suspension into a 15 cm dish.

---

[17]Cat. No. 358-T, Rival MFG. Co., Kansas City, MO
[18]Cat. No. 05-579-20, Fisher Scientific, Pittsburgh, PA

25. Add 10 mL DMEM-15% HS to each dish and swirl the dishes briefly.
26. Place the dishes into a humidified cell culture incubator of 95% air and 5% CO_2 at 37 ^{o}C for 1 h.
27. After incubation, transfer the contents of the dishes into a sterile beaker (at least 500 mL capacity), and then transfer 100 mL to sterile centrifuge bottles.
28. Centrifuge the bottles at 1,500 x g for 10 min at 25 ^{o}C.
29. Discard the supernates, and add 10 mL freezing medium to each of the centrifuge bottles, resuspend the cell pellet by repeated passage through a 10 mL disposable pipette[19]. Transfer 1 or 2 mL aliquots of the resulting cell suspension into cryogenic vials[20].
30. Put the vials into a Cryo 1^{o}C Freezing Container[21], and keep the container with the vials at -70 to - 80^{o}C overnight and then store the precooled vials in liquid nitrogen until use.

Culture Procedure for Ovine Satellite Cells

1. Add 1 mL of thawed cell suspension and 13 mL growth medium to one 80 cm^2 Nunc tissue culture flask coated with basement membrane matrix and place the flask into a humidified environment of 90% air, 10% CO_2 at 37^{o}C.
2. After 12 h of incubation , remove the medium and wash the culture with warm D-PBS twice and add 13 mL growth medium to the flask.
3. Change the growth medium at 12-h intervals during the first 48 h of incubation, and then shift the medium change schedule to 24-h intervals until the cultures reach 80% confluency.

---

[19]Cat. No. 13-678-11E, Fisher Scientific, Pittsburgh, PA
[20]Cat. No. 03-374-6, Fisher Scientific, Pittsburgh, PA
[21]Cat. No. 5100-0001, Fisher Scientific, Pittsburg, PA

4. Harvest the cells by using .05% trypsin without EDTA in D-PBS solution, count the cells with a hemacytometer, and seed the cells into 12-well cell culture plates at 20,000 cells per well for peptide utilization experiment.

Procedure for Desalting Animal Serum

1. Equilibrate Sephadex G-25M columns[22] with 225 mL of eluent containing 3.70 g $NaHCO_3$ per litre of deionized H_2O.
2. Use a sterile pipette[23] to add 2 mL of serum to each column and wait until an equal volume of eluent comes out of the column.
3. Add 1 mL of the eluent to each column and wait until about 1 mL of eluent comes out of the column.
4. Add 3 mL of eluent to each column and collect the desalted serum (about 3 mL) coming out of the column into a 15 mL sterile centrifuge tube[24].
5. Regenerate the column by flushing the column with 240 mL of the eluent.
6. Repeat steps 2 to 5 to desalt more serum and to refresh the column.
7. Keep the refreshed columns filled with the eluent at $4^{o}C$ until next use.
8. Filter the desalted serum through a Gelman filter[25] and store the resulting sterile desalted serum at $-20^{o}C$ until use.

---

[22]Cat. No. 17-0851-01, Pharmacia Fine Chemicals, Uppsala, Sweden
[23]Cat. No. 13-678-11D, Fisher Scientific, Pittsburgh, PA
[24]Cat. No. 05-539-1, Fisher Scientific, Pittsburgh, PA
[25]Cat. No. 09-730-218, Fisher Scientific, Pittsburgh, PA

The SAS System

General Linear Models Procedure

Dependent Variable: Protein

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Medel	10	17615.8	1761.58	101.97	.0001
Error	33	570.07	17.28		
Corrected Total	43	18185.89			

R-Squre	C.V.	Root MSE	Protein Mean
.97	7.78	4.16	53.43

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treatment	10	17615.82	1761.58	101.97	.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	10	17615.82	1761.58	101.97	.0001

The SAS System
General Linear Models Procedure
Dependent Variable: CPRO (Percentage of Free Methionine Response)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Medel	27	161713.8	5989.40	59.68	.0001
Error	360	36129.1	100.36		
Corrected Total	387	197842.91			

R-Squre	C.V.	Root MSE	Protein Mean
.82	13.64	10.02	73.44

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treatment	24	135143.64	5630.99	56.11	.0001
Animal	3	26570.19	8856.73	88.25	.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	24	135143.64	5630.99	56.11	.0001
Animal	3	26570.19	8856.73	88.25	.0001

The SAS System
General Linear Models Procedure
Dependent Variable: Protein

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Medel	19	7529.55	396.29	20.84	.0001
Error	60	1141.07	19.02		
Corrected Total	79	8670.61			

R-Squre	C.V.	Root MSE	Protein Mean
.87	89.19	4.36	4.89

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRT	1	1882.87	1882.87	99.01	.0001
SUb	9	4937.66	548.63	28.85	.0001
TRT*SUB	9	709.02	78.78	4.14	.0003

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	1	1882.87	1882.87	99.01	.0001
SUb	9	4937.66	548.63	28.85	.0001
TRT*SUB	9	709.02	78.78	4.14	.0003

Model: Protein= PHYDR (Dipeptide Hydrophobicity)
Dependent Variable: Protein

Analysis of Variance

Dependent Variable: Protein

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Medel	1	15342.98	15342.98	35.03	.0001
Error	26	11387.63	437.98		
Corrected Total	27	26730.62			

Root MSE	20.93	R-Square	.574
Dep. Mean	76.15	Adj R-sq	.558

Parameter Estimates

Variable	DF	Parameter Estimate	Standard Error	T for H0: Parameter=0	Prob > ITI
Intercept	1	129.63	9.86	13.14	.0001
PHYDR	1	-64.87	10.96	-5.92	.0001

General Linear Models Procedure
Duncan's Multiple Range Test for variable: protein
Alpha= .05 df= 33 MSE= 17.28

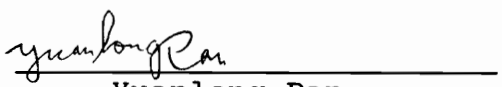
Means with the same letter are not significantly different

Duncan Group		Mean	N	Treatment
	A	71.93	4	HS + MP
B	A	68.52	4	HS + MF
B	A	67.70	4	HS + ML
B		65.39	4	HS + LM
B		65.17	4	HS + FM
B		64.82	4	HS + MV
B		63.99	4	HS + M
	C	55.44	4	HS + VM
	D	27.01	4	Initial
	D	22.16	4	HS + PM
	E	15.63	4	HS

VITA

Yuanlong Pan, son of Guangyan Pan and Deyin Luo, was born on March 17, 1964, in Lanzhou, Gansu, P.R. of China. He graduated from the high school affiliated to Gansu University of Agriculture in July, 1980. He was then admitted into Gansu University of Agriculture and majored in Veterinary Medicine from 1980 to 1984 with a Bachelor of Agriculture degree granted. In September, 1984, he began his graduate program in the field of Comparative Anatomy in the same university and received a Master's degree in Comparative Anatomy in June, 1987. He came to Virginia Tech in August, 1990 to study for a Ph.D degree in Animal Science. He received financial support from John Lee Pratt Animal Nutrition Program throughout his graduate training at Virginia Tech.

He is a member of the American Society of Animal Science.



Yuanlong Pan