PEPTIDES AS AMINO ACID SOURCES FOR THE SYNTHESIS OF SECRETED PROTEINS BY MAMMARY TISSUE EXPLANTS AND CULTURED MAMMARY EPITHELIAL CELLS

by

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in

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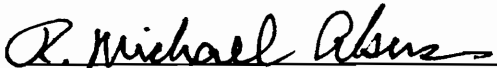

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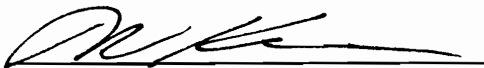

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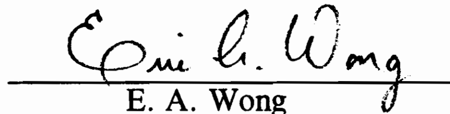

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(ABSTRACT)

Methionine- and lysine-containing di- to octapeptides were evaluated for their ability to serve as methionine and lysine sources respectively for the synthesis of secreted proteins. Mammary tissue explants from lactating (10 to 11 d) CD-1 mice and cultured bovine mammary epithelial cells (MAC-T) were used as experimental models. Explants and cultured cells were incubated at 37°C in a humidified atmosphere of 90% air/10% CO_2 and 95% air/5% CO_2, respectively, for 1 to 24 h in Dulbecco's modified Eagle's medium containing hormones, ^{3}H-leucine, and methionine or lysine substrate in either free or peptide-bound form. The ability of methionine and lysine substrates to promote incorporation of ^{3}H-leucine into secreted proteins was quantified. Mouse mammary explants were able to utilize methionine and lysine from all peptides tested except the lysyl octapeptide. All the methionyl peptides were at least as effective as free methionine in promoting ^{3}H-leucine incorporation into secreted proteins. Most methionyl di- and tripeptides promoted 15 to 76% greater ($P < .05$) ^{3}H-leucine incorporation than did free methionine. A negative correlation ($r = -.89$, $P < .01$) was detected between the rate of ^{3}H-leucine incorporation and the number of amino acid residues in the peptides. The incorporation of ^{3}H-leucine promoted by some methionyl dipeptides was reduced ($P < .05$) in the presence of a 200-fold higher concentration of glycylsarcosine or carnosine.

Incorporation of ^{3}H-leucine promoted by lysyl peptides ranged from 91 to 117% of the incorporation promoted by free lysine. MAC-T cells were also able to utilize methionine from all di- and tripeptides studied. The ability of the peptides to promote ^{3}H-leucine incorporation varied with experimental conditions. For cells allowed to grow/differentiate for 3 or 8 d, incorporation of ^{3}H-leucine promoted by peptides ranged from 67 to 85% and 86 to 110% of the incorporation promoted by free methionine, respectively. The effect of extracellular matrix on the utilization of peptide-bound methionine by MAC-T cells was also examined. Generally, there was no difference in ^{3}H-leucine incorporation/DNA promoted by methionyl dipeptides in MAC-T cells cultured on matrigel, collagen, laminin, or fibronectin coated or uncoated plates. These results suggest that peptides can serve as sources of amino acids for the synthesis of secreted proteins by both lactating mammary explants and cultured mammary epithelial cells. Mouse mammary explants appear to have a greater ability to utilize peptide-bound methionine than to utilize peptide-bound lysine. Mediated transport of some methionyl peptides may be involved in the peptide utilization by the mammary explants. More extensive utilization of peptides by MAC-T cells following a longer (3 vs 8 d) growth/differentiation period may indicate that some maturation process(s) in the cells was necessary for the most effective utilization of peptides.

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Chapter I

INTRODUCTION

The ability of the gastrointestinal tract to absorb small (di- and tri-) peptides has been recognized as an important biological phenomenon (Matthews, 1991a; Webb et al., 1992). Intact peptides may appear in the circulation following absorption (Adibi, 1971; Boullin et al., 1973; Gardner, 1975; Gardner et al., 1983;). Studies have demonstrated that peptide-bound amino acids constitute about 65 to 78% of the blood plasma amino acid pool in ruminants (McCormick and Webb, 1982; DiRienzo, 1990; Seal and Parker, 1991; Koeln et al., 1993) and 52% in rats (Seal and Parker, 1991). These results indicate that circulating peptide-bound amino acids are an important amino acid pool in these animals.

Evidence showing that peptides comprise an important part of the amino acid pool in blood has led to interest in finding out whether tissues can utilize peptide-bound amino acids. Results from a number of studies are challenging the long-time belief that proteins must be completely hydrolyzed to amino acids prior to uptake by tissues. Studies have demonstrated the clearance of peptides from blood plasma, which implies the utilization of peptides by the tissues (Adibi and Krzysik, 1977; Adibi et al., 1977; Adibi and Morse, 1982; McCormick and Webb, 1982; Adibi et al., 1986; Lochs et al., 1986; Danilson et al., 1987a; Lochs et al., 1988, 1992; Abumrad et al., 1989; Koeln et al., 1993). Direct evidence of tissue utilization of peptides has been observed *in vivo* studies (Krzysik and Adibi, 1979; Daabees and Stegink, 1978) and *in vitro* (Brand et al., 1987; Roth et al., 1988b; 1993; Pan, 1993; Christie and Butler, 1994).

The mammary gland is an important nutrient consumer during lactation. Plasma free amino acids have been generally considered to be the major amino acid

source for milk protein synthesis. It is not clear whether peptides also can serve as substrates for milk protein production. Wonsil (1993) found that there was only nominal extraction of peptide-bound amino acids from blood plasma by the lactating bovine mammary gland. In contrast, it was reported that goat mammary gland could utilize intravenously infused dipeptides for milk protein synthesis (Backwell et al., 1994). It is not known whether the different results are due to species differences or because of variable experimental conditions. Because there has been limited information regarding nutritional utilization of peptides by mammary gland, the role of peptide-bound amino acids in milk protein synthesis remains to be understood.

The present study was designed to investigate the availability of peptides as amino acid sources for milk protein synthesis. If peptides do contribute to the mammary amino acid supply, it is also of interest to examine the magnitude of peptide versus free amino acid utilization and to identify some of the factors that may influence the utilization of peptide-bound amino acids by the mammary gland.

Chapter II

LITERATURE REVIEW

Indirect Evidence of Peptide Utilization by Animal Tissues

Interest in the ability of animal tissues to utilize peptide-bound amino acids has grown. A large body of data has been accumulated from *in vivo* and *in vitro* experiments. These studies have provided several lines of indirect evidence suggesting the possibility that utilization of peptide-bound amino acids by tissues can occur in both monogastric and ruminant animals.

First, a number of studies have demonstrated the absorption of peptides from the gastrointestinal tract and the trans-mucosal passage of intact peptides from the gastrointestinal tract into the circulation. These circulating peptides are exposed to the same environment as are circulating free amino acids which are known to serve as fundamental substrates for tissue metabolism. Second, results from studies indicate that peptide-bound amino acids comprise an important part of the blood amino acid pool. This suggests that tissues are living in an environment in which there is easy access to peptide-bound amino acids. Finally, studies have shown the removal of intravenously infused non-bioactive peptides by a number of tissues which indicates the potential nutritional usage of these circulating peptides.

Evidence implying tissue utilization of peptides addressed above is presented in more detail in the following text.

Trans-Mucosal Passage of Intact Peptides in the Small Intestine. As early as 1953, Agar et al. reported that isolated rat small intestine could transfer glycylglycine and leucylglycine from the intestinal lumen to the serosal side. Although only very small amounts of intact peptides were shown to make this trans-mucosal passage, this study revealed the potential of the intestine to absorb intact peptides. Intestinal

absorption of the same two glycyl dipeptides was further studied using both *in vitro* and *in vivo* methods (Newey and Smyth, 1956). The authors also found small amounts of these dipeptides in the fluid carried through the intestinal wall and into the blood. Another early study demonstrated the appearance of intact DL-alanyl-DL-alanine in addition to glycylglycine in the serosal fluid of everted intestinal sacs of rats (Newey and Smyth, 1959). Later studies revealed that, besides di- and tripeptides, bioactive oligopeptides could also cross the intestinal wall and end up in the circulation intact (Fricker et al., 1991; Goff et al., 1991; Drewe et al., 1993).

The amount of intact peptides absorbed across the intestinal wall appears to vary with the peptide source (Gardner, 1978). Gardner (1982) studied the passage of intact peptides across isolated rat small intestine during luminal perfusion with partial digests of casein or soy proteins or with solutions containing single peptides. He found that 14 to 30% of the peptides in the partial protein digest passed intact across the intestinal wall. When single peptide solutions were perfused, about 19% of glycyl-D-phenylalanine, 6% of glycyl-L-phenylalanine, and 2% of L-leucylglycine were passed intact through the wall. An *in vivo* study showed that 9 to 34% of peptides appeared in mesenteric blood following infusion of a partial casein digest into the intestinal lumen of guinea pigs (Gardner et al., 1983). This group also reported that more than 14% of carnosine (L-ß-alanyl-L-histidine) in the diet appeared in human urine after a meal containing carnosine, indicating that at least this amount of carnosine was absorbed intact into blood (Gardner et al., 1991).

The rate of peptide absorption differs from one site to another in the small intestine. It was observed that, in an *in vivo* study, human jejunum and ileum had greater potentials to absorb glycylglycine and glycylleucine than did the duodenum (Adibi, 1971). Intact glycylglycine but not glycylleucine was detected in blood plasma following absorption. Fricker et al. (1991) reported that rat and dog jejunum have a

much greater ability than the ileum to absorb a somatostatin analogue octapeptide after oral administration. The intact peptide rapidly appeared in the blood and showed bioactivity.

The absorption of intact peptides across the small intestinal wall appears to be related to the low rate of peptide hydrolysis in the intestinal lumen and in the cytoplasm of enterocytes. The trans-mucosal passage of several peptides was compared by using everted sacs prepared from the small intestine of rats (Wiggans and Johnston, 1959). In this study, only intact glycylglycine was observed to cross the intestinal wall. It was suggested that the resistance of this peptide to hydrolysis on the mucosal side of the intestine aided the trans-mucosal accumulation. Similar results were obtained in an *in vivo* study with humans (Adibi, 1971). Heading et al. (1979) also noticed the relationship between the rate of peptide hydrolysis and passage of intact peptides across the intestinal wall.

Although it seems to be easier for slowly hydrolyzed peptides to pass intact through the intestinal wall, there is no correlation between the rate of peptide hydrolysis and the appearance of intact peptides in the circulation. Boullin et al. (1973) reported that all of six dipeptides studied were detected in the plasma taken from the mesenteric vein of rats following infusion of these peptides into the intestinal lumen. The hydrolysis rate of glycylglycine by intestinal mucosal homogenate was 2- to 6-fold greater than the hydrolysis rate of glycyl-D-phenylalanine and carnosine, but the plasma concentration of glycylglycine was also two to four times greater than the other two dipeptides. This indicates that, in addition to the rate of hydrolysis, other factor(s) also may affect the trans-mucosal passage of intact peptides by the small intestine.

Results from an *in vitro* study with rats show that the passage of intact tyrosine-containing di- and tripeptides across the small intestinal wall was dependent on

mucosal peptide concentration (Heading et al., 1978, 1979). Gardner (1982) reported that the intact peptides crossing the intestinal wall increased up to 15-fold when the intestinal perfusion concentration of partial protein digest increased by fivefold.

Trans-Mucosal Passage of Intact Peptides in the Large Intestine and Stomach. Peptide absorption was reported to occur in the large intestine (Calonge et al., 1990). Heading et al. (1978, 1979) demonstrated the transport of intact tyrosine-containing di- and tripeptides across the rectal wall of rats *in vitro*. The rectum has also been utilized as a site for the delivery of bioactive peptides into the circulation in medical therapy (Liversidge, 1989). However, the ability of the large intestine to absorb peptides is small compared with the small intestine. It has been observed that intact peptides crossed the wall of the small intestine twice as rapidly as across the large intestine under the same experimental conditions (Heading et al., 1978, 1979).

The stomach is generally considered as having limited ability to absorb amino acids. However, recent studies indicate that this assumption may not be valid in ruminants. DiRienzo (1990) quantified the *in vivo* flux of both free and peptide-bound amino acids across the stomach and intestines of calves and sheep during a 1-h feeding period. The flux of peptide-bound amino acids across the stomach accounted for approximately 77% of total (both free and bound) amino acid flux across the portal-drained viscera in both calves and sheep. This result was the first to suggest the possibility of the stomach as a site for absorption of intact peptides into the circulation.

The ability of rumen and omasum to absorb intact peptides across the mucosal tissues was confirmed by an *in vitro* study (Matthews, 1991b). It was found that carnosine and methionylglycine were transferred intact across both ruminal and omasal epithelia. The omasal epithelium had a greater ability to absorb the

dipeptides than did the ruminal epithelium. These two forestomach compartments also possess the ability to absorb free methionine (Matthews and Webb, 1993).

Recently, Seal and Parker (1993) measured the concentration of low molecular weight peptides (smaller than 1,500 Da) in the mesenteric and portal plasma of calves. They found that there was an increasing net absorption of peptide-bound amino acids between the mesenteric and portal vein (1.76 and 3.02 $mmol \cdot min^{-1}$, respectively), but no increase in the absorption of free amino acids was observed between the two veins (3.02 versus 2.03 $mmol \cdot min^{-1}$). They suggested that there was a net absorption of low molecular weight peptides from large intestinal and stomach tissues.

Peptide-Bound Amino Acid Pool in Blood. Peptide-bound amino acids comprise an important part of the blood amino acid pool. Several studies have reported that peptide-bound amino acids accounted for 65 to 78% of arterial total plasma amino acids or 59 to 70% of arterial total blood amino acids (including free amino acids in erythrocytes) in calves (McCormick and Webb, 1982; Danilson et al., 1987a, 1987b; Seal and Parker, 1991; Koeln et al., 1993). Approximately 65 to 86% of amino acids appearing in the portal plasma were peptide-bound in calves (DiRienzo, 1990; Seal and Parker, 1991). In the blood of sheep, peptide-bound amino acids comprised about 78% of arterial total plasma amino acids (Seal and Parker, 1991) and 73 to 90% of portal total plasma amino acids (DiRienzo, 1990; Seal and Parker, 1991).

Results from an early study showed that about 9% of amino acids were in peptide form in the blood plasma of rats (Asatoor et al., 1978). Seal and Parker (1991) observed a much greater proportion of peptides in rat blood. They found that about 52% of amino acids in both arterial and portal plasma were peptide-bound. In guinea pigs, the plasma and whole blood draining the intestine (mesenteric venous)

contained, respectively, 11 to 14% and 26 to 31% of amino acids present in peptide form. The percentage of peptide-bound amino acids in plasma of normal humans varied from 8 to 30% (Christensen et al., 1947).

The molecular mass of the circulating peptides varies. Seal and Parker (1991) reported that the major portion of peptides in the blood plasma of calves and sheep was pentapeptides and above. The second largest group of circulating peptides in the ruminants was dipeptides. A similar result was obtained by Koeln et al. (1993). They found that the largest flux of circulating peptides across the gastrointestinal tract of calves was associated with peptides having molecular size from 500 to 1,500 Da and dipeptides accounted for the second largest peptide flux. However, the size of circulating peptides in non-ruminant animals appears to be different. Dipeptides comprise the largest portion of circulating peptides in the blood plasma of rats (Seal and Parker, 1991).

The origin of these circulating peptides has not been clearly defined. As discussed in previous sections, peptides of dietary origin which escape hydrolysis in the gastrointestinal tract and in the mucosal cells may appear in the circulation. Some of the peptides in the blood may be the degradation products resulting from the turnover of tissue proteins or the synthetic products of some tissues.

Peptide Clearance from Blood Plasma. The existence of peptide-bound amino acids as an important blood amino acid pool has led researchers to question the metabolism of circulating peptides. Now, studies have demonstrated the removal of peptides from blood during interorgan transport, which implies the utilization of peptides by animal tissues. Adibi et al. (1977) observed that intravenously injected glycylleucine, glycylglycine, and a hydrolysis-resistant peptide, glycylsarcosine, were rapidly cleared from the plasma of rats. Glycylsarcosine and the constituent amino acids from the other two glycine-containing dipeptides were recovered in kidney,

intestine, liver, and muscle with different quantities. This study was the first to reveal the possibility that tissues can utilize peptide-bound amino acids.

When calves were fed a hay-corn-soy diet or purified diets with the nitrogen supplied only by soy proteins, peptides were removed from the plasma by the tissue of hindlimbs. When urea was the sole nitrogen source, no peptide removal was observed by hindlimb tissue (McCormick and Webb, 1982; Danilson et al., 1987a). Results from another study (Koeln et al., 1993) conducted in the same laboratory showed a small negative peptide flux in the plasma across the liver of calves, indicating the removal of some circulating peptides by liver tissue.

The role of liver, kidney, muscle, and extrahepatic splanchnic tissues of dogs in the removal of intravenously infused glycylleucine and glycylglycine from blood plasma was investigated in an in vivo study (Lochs et al., 1988). The authors found that all of the organs examined participated in the removal of these dipeptides from the plasma, but their roles varied. Liver, kidney, muscle, and extrahepatic splanchnic organs accounted for the disappearance of 25, 24, 12, and 10% of the infused glycylleucine and 13, 37, 18, and 11% of the infused glycylglycine, respectively. The disappearance of the peptides from the plasma during passage across an organ was mainly the result of assimilation by the organ. Hydrolysis in blood plasma accounted for only a very small fraction of the peptide disappearance (about 5 and .5% of total infused glycylleucine and glycylglycine, respectively). This plasma hydrolase activity is much lower than the activity in the cytosol of the tissues (Krzysik and Adibi, 1977). Urinary loss of both dipeptides and their constituent amino acids was less than .5%.

Liver, kidney, muscle, and extrahepatic splanchnic tissues of conscious dogs can also remove intravenously infused alanylglutamine and glycylglutamine from blood plasma (Abumrad et al., 1989). These organs accounted for more than 90% of the clearance of glycylglutamine and two-thirds of alanylglutamine clearance. The

disappearance of these dipeptides from the circulation was predominantly the result of tissue hydrolysis and/or uptake. Blood plasma hydrolysis was too low to contribute significantly to *in vivo* clearance of the two glutamine-containing dipeptides (Hübl et al., 1989). Kidney, muscle, and splanchnic tissues of postabsorptive and briefly starved human subjects also participated in clearance of glycylglutamine from the plasma (Lochs et al., 1992). Although neither splanchnic nor muscle removal of glycylglutamine was affected by starvation, removal by the kidney was significantly reduced.

A rapid removal of glutathione (τ-glutamyl-cysteinyl-glycine) from blood by the lactating mammary gland of cows was observed (Pocius et al., 1981). Since there is shortage of cysteine and glutamate in blood for synthesis of milk proteins (Clark et al., 1977, 1978), glutathione may provide the part of the cysteine and glutamate needed.

Abraham et al. (1964) observed the uptake of carnosine by rat brain slices. They also found that other dipeptides could decrease the uptake of carnosine by the brain slices. Recent studies demonstrated the removal of some peptides from blood plasma by brain (Banks et al., 1992, 1993). These results suggest that brain also may possess the ability to utilize peptides nutritionally.

Summary. Peptides of dietary origin can be absorbed intact across the gastrointestinal tract into the circulation. In addition to peptide absorption in the small intestine, the large intestine and the forestomach of ruminants also appear to have the ability to absorb intact peptides across their walls into blood. An important portion of blood plasma amino acids is present in peptide form. These circulating peptide-bound amino acids can be removed by various tissues during interorgan transport, indicating the nutritional significance of these peptides.

Direct Evidence of Peptide Utilization by Animal Tissues

Direct evidence of tissue utilization of peptides has been demonstrated in both *in vivo* and *in vitro* studies. A wide range of tissues and cells appear to possess the ability to utilize peptide-bound amino acids. This implies that it is important to understand the metabolism of this form of important nutrients by individual tissues.

Peptide Utilization Reported from in Vivo Studies. Krzysik and Adibi (1979) demonstrated that, in rats, intravenously injected ^{14}C-glycylglycine was as effective as ^{14}C-glycine in being incorporated into tissue proteins of pancreas, liver, intestinal mucosa, kidney, lung, and brain. The only exception was muscle where the labeling of proteins was lower after ^{14}C-glycylglycine injection than after ^{14}C-glycine injection. This is probably because the transport of dipeptides is less efficient than that of amino acids in this tissue (Adibi and Morse, 1981) and also because muscle is less active in dipeptide hydrolysis than other tissues (Krzysik and Adibi, 1977). Furthermore, they also reported that oxidation of injected glycine (labeling of expired CO_2) was enhanced when glycine was given in the form of ^{14}C-glycylglycine.

Direct evidence of tissue utilization of peptides also has been reported by Amberger et al. (1983). Three hours after intravenously infusing ^{14}C-alanylglutamine into rats, 11, 7, and 2% of the label was recovered in the proteins of liver, muscle, and kidney, respectively. Approximately half of the given label was observed in CO_2. The urinary loss was only about .7%. Similar results were obtained in rats following the infusion of alanyltyrosine (Daabees and Stegink, 1978).

Stehle et al. (1991) reported that 30 min after a bolus injection of radioisotope-labeled alanylglutamine into the tail vein of rats, 27 and 7% of the label was recovered in skeletal muscle and liver, respectively. The remainder of the label appeared in kidney, pancreas, heart, lung, and brain.

There also has been evidence reported suggesting that the mammary gland of goats can utilize intravenously infused glycylleucine and phenylalanylleucine as sources of leucine for the synthesis of milk proteins (Backwell et al., 1994).

Utilization of Peptides by Cultured Animal Cells. More direct evidence of tissue utilization of peptides comes from the study of cultured animal cells. So far, at least seven different established mammalian cell lines have been shown to possess the ability to utilize peptides as amino acid sources.

The ability of seven dipeptides to serve as the sources of phenylalanine, leucine, isoluecine, and tyrosine for the proliferation of mouse fibroblasts and human HeLa (uterine cervical carcinoma) cells was examined (Eagle, 1955). Normal cell growth and proliferation could be obtained by using a dipeptide to substitute for a corresponding amino acid. The concentrations of the dipeptides necessary for maximal cell proliferation varied and ranged from lower to higher than the concentrations of the corresponding amino acids.

The ability of di-, tetra-, hepta-, and decalysine to substitute for free lysine in the growth of hamster intestinal cells in culture was investigated by Grahl-Nielsen et al. (1974). Dilysine was approximately equivalent to lysine in fulfilling the lysine requirement for cell growth. The utilization of tetra-, hepta-, and decalysine as a lysine source gradually diminished as peptide size increased. It was suggested that the uptake of the larger peptides appeared to be the growth rate-limiting step because no delay of peptide hydrolysis inside the cells was observed.

Glycylglutamine can substitute for glutamine in rat thymocyte culture (Brand et al., 1987). When the concentration of glycylglutamine was sixfold higher than free glutamine in the medium, an equal rate of ^{3}H-thymidine incorporation into DNA of proliferating thymocytes was achieved in these two treatments. Glutamine-containing

dipeptides were also as effective as free glutamine in promoting the proliferation of cultured human hematopoietic cells (Roth et al., 1988b).

A proline-auxotrophic Chinese hamster ovary cell line was used to study the ability of proline-containing dipeptides to serve as a source of proline for cell growth (Emmerson and Phang, 1993). When the concentration of glycylproline, methionylproline, or phenylalanylproline was only one-fifth of the concentration of free proline, an equal cell number was obtained after 3 d of cultivation.

Pan (1993) studied methionine-containing peptides as substitutes for free methionine in the cultivation of rat and ovine myogenic cells and bovine mammary epithelial cells. He found that the cultured cells possessed the ability to utilize methionine-containing peptides as methionine sources for protein accretion and cell proliferation. He also observed that there was serumal peptidase activity in the culture medium, but that this was not capable of providing enough free methionine to account for the responses observed.

Summary. Animal tissues and cells appear to possess the ability to utilize peptide-bound amino acids for proteins synthesis and energy metabolism. In some instances, amino acids in peptide form are preferred over those in free form to meet the requirements of cell growth.

Mechanisms of Uptake of Peptide-Bound Amino Acids by Animal Tissues

The mechanism(s) by which animal tissues take up peptides from the extracellular environment has not been well defined because of the complexities of tissue and cell types and animal differences. Four mechanisms have been suggested as possibilities of how animal cells assimilate peptide-bound amino acids (Ugolev et al., 1990; Matthews, 1991a; Skopicki et al., 1991; Daniel et al., 1992; Minami et al., 1992). They are (1) uptake of intact peptides via carrier mediated transport system(s)

followed by intracellular hydrolysis, (2) uptake of intact peptides by simple diffusion followed by intracellular hydrolysis, (3) hydrolysis of peptides during their translocation across cell membranes, and (4) extracellular hydrolysis of peptides followed by absorption of the constituent amino acids by amino acid carriers. The first two possibilities would require the presence of cytosolic peptide hydrolases and the latter two would require the presence of membrane-bound peptidases. Cytosolic and membrane-bound peptide hydrolases may be quite different (Heizer et al., 1972; Raghunath et al., 1990).

Uptake from the Lumen of the Small Intestine. Early experiments found that peptides were absorbed more rapidly than their corresponding free amino acids from the small intestine (Matthews et al., 1968; Matthews, 1975). This indicates that different mechanisms are involved in the absorption of peptides and free amino acids. Later studies confirmed the observation and suggested that peptide and amino acid absorptions in the small intestine are distinctly separate physiological processes involving different transport proteins (Ganapathy and Radhahrisknan, 1980; Ganapathy et al., 1981).

Ganapathy and Leibach (1983), using brush border membrane vesicles of rabbit small intestine, demonstrated that pH affected peptide transport. They found that the presence of an inward H^+ gradient accelerated uphill transport of dipeptides across the brush border membrane. They suggested that H^+ was the driving force for intact peptide transport and was cotransported with the dipeptide into the enterocytes. The Na^+/H^+-antiport existing on the plasma membrane may mediate the uphill efflux of H^+ coupled to the downhill influx of Na^+ to maintain the H^+ gradient across the brush border membrane. This may explain the partial dependence of peptide transport on Na^+ observed in experiments with intact small intestine (Matthews, 1975). The Na^+/K^+-ATPase present at the basolateral membrane of the

enterocytes maintains the inward Na^+ gradient with an expenditure of ATP. Therefore, the H^+-coupled peptide transport is an active transport.

The hypothesis that a H^+-coupled and Na^+-related peptide transport was present in the small intestine was confirmed in a series of studies. Takuwa et al. (1985b), Shimada and Hoshi (1986), and Said et al. (1988) also found a H^+-dependent peptide transport system in the small intestine. The Na^+ transport was stimulated by glycylglycine in everted sacs, sheet preparations, and brush border membrane vesicles isolated from guinea pig ileum (Himukai et al., 1983).

Results from a recent study further confirmed the existence of a peptide-H^+ cotransporter in the small intestine of rabbits by expression of the transporter in *Xenopus laevis* oocytes (Miyamoto et al., 1991). It was observed that the oocytes injected with the poly A mRNA isolated from the intestinal mucosal cells had nearly a threefold increase in glycylsarcosine uptake compared with the uptake by water-injected oocytes. The expressed peptide transporter showed a dependence on a transmembrane H^+ gradient for maximal activity.

The small peptide transport system in the small intestine of rabbit contained a protein with an apparent molecular weight of 127,000 Da (Kramer et al., 1990a,b). This protein was directly involved in the uptake of small peptides. Brush border membrane-bound peptidases, like aminopeptidase N and dipeptidylpeptidase IV, were not involved in this transport system.

There may be more than one peptide transport system in the small intestine. The transport of glutathione by rabbit intestinal brush border membrane vesicles was found to be Na^+-independent, pH-dependent, and specially activated by mono- and divalent cations, in particular by Ca^{2+} (Vincenzini et al., 1989). This result indicated the possible occurrence of another carrier mediated transporter.

Results from several studies suggest that some peptides can be hydrolyzed by membrane-bound peptidase(s) on the brush border membrane. The released free amino acids are subsequently absorbed by the enterocytes via amino acid transport mechanisms. Adibi et al. (1975) found a high intraluminal concentration of free leucine when trileucine was perfused into the small intestine of healthy human subjects and an inhibition of leucine assimilation from the tripeptide when isoleucine was included in the perfusate. Rosen-Levin et al. (1980) reported that, in the small intestine of rats, leucylglycylglycine and dileucine were absorbed both intact and in the form of the constituent amino acids. They speculated that the hydrolysis of the peptides was by membrane-bound peptidase(s) and suggested that many di- and tripeptides with one or more bulky side chains may likely have access to both intact absorption and surface hydrolysis.

Ugolev and Iezuetova (1982) suggested that some peptides could be hydrolyzed during translocation across the brush border membrane and the resulting amino acids were directly transported from the site of hydrolysis into the cytosol in the small intestine. Recently, they reported the existence of this enzyme-transport complex in isolated small intestine of mice, rats, and guinea pigs using the anoxic criterion (Ugolev et al., 1990). According to this criterion, the active transport of intact peptides or free amino acids is oxygen-dependent. In contrast, the transport of peptides by the enzyme-transport complex is not dependent on oxygen. Thus, the comparison of tissue accumulation of corresponding amino acids in the intestine incubated with either peptides or constituent amino acids under both aerobic and anaerobic conditions will distinguish the peptide transport mediated by the enzyme-transport complex from active peptide transport and free amino acid transport. In this study, they found that anoxia did not affect tissue accumulation of the corresponding amino acids from a number of di- and tripeptides. The results indicate that these

peptide-bound amino acids were transported into the enterocytes through the enzyme-transport complex. Indirect evidence also suggested that glycylproline, in some instances, was hydrolyzed in brush border membrane followed by direct transport of constituent amino acids into enterocytes (Wiseman, 1977).

Absorption of some peptides may be increased with increasing mucosal peptide concentration, and the absorption may or may not be partially inhibited by other peptides, indicating that both simple and facilitated diffusion were involved in peptide absorption in the small intestine (Radhakrishnan, 1977; Bertloot et al., 1981; Matthews and Burston, 1984; Woottoon, 1986).

Recently, a glucose-dependent peptide transport, the paracellular pathway, was observed in the small intestine of the hamster (Atisook and Madara, 1991). Isolated intestine was perfused with recirculating buffer containing a labeled hemepeptide (molecular weight 1,900 Da) or horseradish peroxidase (molecular weight 40,000 Da) with or without glucose. The authors found that the hemepeptide selectively appeared in the paracellular space where the tight junctions were dilated when the intestinal epithelium was exposed to glucose. No peptide was detected in the paracellular space when glucose was absent from the perfusate. Horseradish peroxidase could not penetrate the epithelium, either exposed or unexposed to glucose. A previous study revealed that the dilation of tight junctions of the intestinal epithelium was elicited by the action of the Na^+-glucose cotransporter (Atisook et al., 1990). Therefore, it was suggested that the paracellular peptide pathway was regulated during Na^+-activated glucose absorption and that this pathway may particularly contribute to meal-related oligopeptide absorption.

Uptake from the Lumen of the Large Intestine and Stomach. The mechanism(s) of peptide uptake in the large intestine of mammals appears to differ from the small intestine. Transfer of di- and tripeptides across the wall of the isolated rectum of rats

was not affected by replacement of Na^+ with K^+ in the incubation buffer (Heading et al., 1978, 1979). However, the transport of peptides across the wall was partially prevented by the absence of Na^+ in comparable small intestinal experiments. The Na^+-independent transport of peptides in the rectum, compared with the partially Na^+-dependent peptide transport of the small intestine, suggests the absence of active uptake of peptides in the rectum. It appears that simple passive diffusion is involved in rectal peptide transport (Liversidge, 1989).

There may be species differences in peptide transport in the large intestine. Epithelial cells isolated from the proximal cecum and rectum of chicks had the same characteristics as the cells from the jejunum in the transport of glycylsarcosine (Calonge et al., 1990). The authors found that the transport of glycylsarcosine in the cecal and rectal cells was partially Na^+-dependent and sensitive to extracellular pH as in the jejunal cells. The dipeptide uptake was greater at pH 6 than at pH 7.4. The cellular accumulation of the dipeptides was reduced by amiloride, a well-known inhibitor of Na^+/H^+-antiport. Therefore, they suggested that peptide transport in the large intestine of chicks was the same as in the small intestine which was H^+-coupled and Na^+/H^+-antiport involved active transport.

The absorption of carnosine and methionylglycine by isolated ruminal and omasal epithelia of sheep was linearly dependent on substrate concentration (Matthews, 1991b). Since the absorption was non-saturable in the presence of increasing substrate concentration, it was suggested that non-mediated diffusion was involved in the transport of the dipeptides.

Uptake from the Lumen of Renal Cortex tubules. The proximal tubular epithelial cells of the renal cortex possess an apical brush border membrane structure. It has been demonstrated that the brush border membrane of the renal cortex cells also contains a H^+-coupled, active peptide transporter as is observed in

the small intestine (Ganapathy et al., 1981; Ganapathy and Leibach, 1983; Takuwa et al., 1985a). Di- and tripeptides but not tetrapeptides were transferred by the active transporter (Tirwppathi et al., 1991).

It is not distinctly clear whether the H^+-coupled peptide transporters in the brush border membrane of both intestine and kidney share the same properties. Results from recent studies suggest the existence of two dipeptide transporters, the high affinity (K_t = 44.1 ± 11.2 μM)/low capacity (V_{max} = .41 ± .03 nmol·mg protein^{-1}·5 s^{-1}) and low affinity (K_t = 2.62 ± .50 mM)/high capacity (V_{max} 4.04 ± .80 nmol·mg protein^{-1}·5 s^{-1}) transporters, in the renal brush border membrane of rats (Daniel et al., 1991). Both transporters reacted to a transmembrane H^+ gradient, but the high affinity transporter appeared to be far more sensitive to changes in the H^+ gradient than the low affinity transporter. In the absence of a H^+ gradient, only the low affinity system was operational, but with a reduced transport capacity. The authors noticed that glycylglutamine and glycylalanine were transported by both transporters. They also observed that, in the presence of a pH gradient, transport of each dipeptide appeared to require cotransport of one H^+ to serve as the driving force. The 1:1 ratio between H^+ and dipeptide flux was more apparent for the high affinity than for the low affinity transporter. However, the H^+-coupled peptide transport in the small intestine showed a H^+ to peptide ratio of about 2:1 (Hoshi, 1986; Abe et al., 1987). Skopicki et al. (1991) also observed the co-existence of high affinity/low capacity and low affinity/high capacity peptide transporters in the renal brush border membrane of rabbit.

Uptake from the Circulation. Our understanding of uptake of peptide-bound amino acids from the blood by tissues is relatively poor compared with our understanding of the mechanisms of peptide transport in the brush border

membranes of the intestine and kidney. It appears that, according to the limited information available, mechanisms differ among tissues and among peptides.

Perfused rat liver assimilated glycyl dipeptide-bound amino acids following extracellular hydrolysis (Lochs et al., 1986). The hydrolysis was achieved by enzymes either located on the plasma membrane or released from the cytosol. The amino acid residues released as the result of dipeptide hydrolysis then were transported into the liver cells by amino acid carriers. It was further demonstrated that the glycyl dipeptides could not be transported intact by isolated rat liver plasma membrane vesicles (Lombardo et al., 1988). However, the disappearance of homologous alanyl (2 to 4) and glycyl (2 to 6) peptides as well as leucine-containing dipeptides from liver perfusion medium was correlated with peptide hydrolase activity of liver plasma membrane. Certain amino acid residues, such as alanine, enhanced hepatic clearance of peptides from the perfusion buffer by increasing their affinity as substrates for plasma membrane peptide hydrolases (Lombardo et al., 1988).

Recently, results from two studies showed that isolated rat hepatocytes can take up a hydrophilic, cationic peptide with renin inhibitory activity by a combination of diffusion and carrier-mediated processes (Ziegler and Seeberger, 1993; Seeberger and Ziegler, 1993). The carrier-mediated transport was energy-dependent, membrane potential-dependent, and Na^+-independent. Other peptides could not inhibit the hepatocellular uptake of this bioactive peptide.

Adibi and Morse (1981) studied the accumulation of the hydrolysis-resistant peptide, glycylsarcosine, in isolated rat muscle. They found that muscle could not take up this dipeptide against a concentration gradient, suggesting that simple diffusion was involved in the peptide transport. Studies using sarcolemmal vesicles of skeletal muscle and organ perfusion techniques revealed that the assimilation of peptide-bound amino acids by muscle tissue varied among different dipeptides (Raghunath et

al., 1990). Two mechanisms appeared to be involved; the major one was hydrolysis of peptides by membrane-bound peptide hydrolases followed by uptake of the constituent amino acids by amino acid transport systems, and the minor one was uptake of intact peptides by simple diffusion or other transport mechanisms.

Kidney has been shown to play an important role in removal of circulating peptides (Adibi and Krzysik, 1977; Adibi and Morse, 1982; Abumrad et al., 1989). Some circulating peptides end up in the glomerular filtrate and are taken up by the brush border membrane of the cortex cells. Others are directly extracted from the basolateral membrane of renal cells. Lash and Jones (1983, 1984) found that basal lateral membrane vesicles from rat kidney take up intact glutathione and glutathione disulfide by a Na^+-dependent active transport process. About two Na^+ were cotransported with each glutathione. It was suggested that the Na^+/K^+-ATPase on the cell membrane was coupled to this transport process by maintaining the cellular Na^+ gradient. In contrast, glutathione, in the renal cortex tubule lumen, was hydrolyzed by brush border membrane-bound τ-glutamyltransferase and other peptidases and the resulting amino acids were taken up by amino acid transporters on the brush border membrane (Inoue and Morino, 1985)

Uptake of carnosine by rat brain slices is saturable and can be reduced up to 75% by other dipeptides (Abraham et al., 1964). Since the uptake also was concentrative and dependent on the presence of oxygen and glucose, the authors suggested that an active transport mechanism was involved.

Peptide Competition. Although at least four mechanisms by which tissues assimilate peptide-bound amino acids have been demonstrated, the types of peptides to be transported are far more numerous than the types of transport systems. Therefore, a large number of different peptides must share a common transport process. Studies have shown that, like the transport of amino acids, transport of one

peptide can be inhibited by another (Matthews et al., 1979; Taylor et al., 1980; Silbernagl et al., 1987). The inhibitory effects during peptide transport appear to be due to the competition of peptides for the peptide binding site on the transporter (Kramer et al., 1990a, b).

The transport of ^{14}C-glycyl-L-proline into intestinal and renal brush border membrane vesicles of rabbits was reduced by about 70 and 50%, respectively, in the presence of an 800-fold higher concentration of unlabeled glycylproline or L-histidyl-L-proline (Ganapathy et al., 1981). But L-leucylglycine and carnosine only reduced the transport of ^{14}C-glycyl-L-proline into the intestinal and renal vesicles by about 20% under the same experimental conditions. In addition, glycylglycine was totally ineffective as an inhibitor. The authors suggested that the low inhibitory capacity of carnosine and the non-inhibitory property of glycylglycine were probably due to their low affinity for the dipeptide transport system. L-leucylglycine was extensively hydrolyzed by the membrane-bound peptidases in this study. This was suggested to be one of the reasons for its low inhibitory capacity because the constituent amino acids had shown no effect on the peptide transport. Results from another study showed that, in addition to dipeptides, tripeptides also could compete with glycyl-L-proline during transport across the intestinal brush border membrane of rats (Said et al., 1988).

Skopicki et al. (1991) found that the transport of glycyl-L-proline into renal brush border membrane vesicles by the high affinity/low capacity transporter could be inhibited by the presence of glycylglycine, but glycylglycine showed no effect on the transport of glycyl-L-proline mediated by the low affinity/high capacity transporter. In contrast, carnosine could only inhibit the transport of glycyl-L-proline mediated by the low affinity transporter but not the high affinity transporter. However, carnosine transport across the intestinal brush border membrane of rodents was reduced

significantly in the presence of glycylglycine (Addison et al., 1974) and, conversely, glycylglycine transport was inhibited significantly in the presence of carnosine (Iseki et al., 1989).

Uptake of glycylglutamine by renal brush border membrane vesicles of rats was studied in the presence of other glycine-containing dipeptides (Daniel et al., 1991). All eight of the dipeptides tested inhibited uptake of glycylglutamine. The inhibition ranged from 63 to 86%. A similar range of inhibition also was observed in the uptake of glycylglutamine by human intestinal brush border membrane vesicles in the presence of other di- and tripeptides (Minami et al., 1992).

The competition between two peptides during transport is a complicated process and may not be just a simple reverse relation in every case. Himukai et al. (1982) observed that, in guinea pig small intestine, glycylleucine inhibited uptake of glycylglycine, but glycylglycine could not inhibit uptake of glycylleucine. Matthews and Burston (1983) found that, in hamster jejunum *in vitro*, leucylleucine could inhibit mediated uptake of intact glycylsarcosine completely, but glycylsarcosine could not cause complete inhibition of mediated uptake of intact leucylleucine. Glycylsarcosine could, however, cause complete inhibition of mediated uptake of intact valylvaline, which in turn could cause complete inhibition of mediated uptake of intact leucylleucine. It was suggested that the competition of peptides might be complicated by allosteric effects.

Peptide Structure. The uptake of peptide-bound amino acids by tissues can be affected by peptide structure. Several factors seem to affect the transport process. Burtson et al. (1972) found that amino acid composition influenced peptide absorption in the isolated small intestine. When buffer containing glutaminyltyrosine was incubated, glutamic acid was absorbed nearly twice as rapidly as when buffer containing glutaminylmethionine was used. Results from another study show that the

rate of H^+-coupled active transport of tyrosylprolylphenylalanine across the renal brush border membrane of rats was about sixfold greater than the transport of tyrosylproline (Tiruppathi et al., 1991). However, the mediated uptake of both tyrosylproline and tyrosylprolylphenylalanine by the brush border membrane vesicles was inhibited almost to the same extent by the presence of other di- or tripeptides. The results from the peptide competition experiment suggested a common transporter was responsible for the transport of the tyrosine-containing di- and tripeptides. Therefore, the different rate of uptake between tyrosylproline and tyrosylprolylphenylalanine was probably due to the difference of one amino acid residue. They also found, in contrast, that a tetrapeptide, tyrosylprolylphenylalanylproline, could not be taken up by the H^+-coupled transport system.

Location of an amino acid in either the N- or C-terminal position also has an important influence on absorption in some instances. Burston et al. (1972) reported that lysine was absorbed much more rapidly when it was present as lysylglycine compared with glycyllysine.

Several structural features of peptides which may determine their affinity for the high affinity type transporter of rat kidney brush border membrane have been studied (Daniel et al., 1992). To determine whether the free amino group of the N-terminal amino acid had to be located in the α-position, the relative uptake of ß-alanylglycine and ß-asparaginylglycine with their corresponding α-amino dipeptide configurations were compared. The uptake of dipeptides with the amino group in the α-position was about 100-fold greater than the uptake of dipeptides with the amino group in the ß-position. They found that the sterospecificity of peptides also affected peptide uptake. Peptides with L-amino acid residues had much higher affinity for the transporter than peptides with the D-isomer. They also noticed the

effect of peptide backbone chain length on their uptake by comparing the transport of homogeneous di- to tetrapeptides. Peptides with four amino acid residues had much lower uptake rates than the corresponding di- and tripeptides. However, di- and tripeptides with the same homogeneous amino acid residues had very similar affinity for the transporter.

Summary. More than one mechanism of uptake of peptide-bound amino acids appears to exist in a single tissue. The transport of one peptide in one type of tissue is not necessary the same as in another type of tissue. Peptide transport is influenced by its structure. Peptides sharing the same transport process compete with each other during transport across the cell membrane.

Factors in Blood That May Influence Peptide Utilization

As discussed in the first section of this chapter, the clearance of circulating peptides during interorgan transport indicates the uptake of peptide-bound amino acids by tissues. However, it is unknown whether other factors also may contribute to the removal of peptides from the plasma and if so, to what extent. Erythrocytes and blood plasma hydrolases have been considered as possible factors which may be involved in the clearance of circulating peptides.

Erythrocytes. Erythrocytes have been demonstrated to be an important vehicle of interorgan amino acid transport (Felig et al., 1973; Heitmann and Bergman, 1980; McCormick and Webb, 1982; Danilson et al., 1987b). However, it is completely unknown whether the cells also play a role in transport of circulating peptide-bound amino acids among tissues. The cytoplasm of erythrocytes contains considerable hydrolase activity against peptides, the function of which remains unknown (Adams et al., 1952a,b). One possibility could be that it is involved in the hydrolysis of circulating peptides. If so, there must be peptide transport across the cell membrane.

In the early 1980's several studies were conducted by Kuchel's group to investigate the uptake of di- and tripeptides in human erythrocytes using the proton nuclear magnetic resonance (NMR) spectroscopic technique (King et al., 1983; King and Kuchel, 1984, 1985; Vandenberg et al., 1985). They found that extracellular di- and tripeptides, but not tetrapeptides, entered the erythrocytes by a saturable transport system and that the peptides were rapidly hydrolyzed to free amino acids in the cells. Later, Young et al. (1987) reported a conflicting result that human erythrocytes had a very low ability for dipeptide transport. This subsequently was confirmed by Kuchel's group (Kuchel et al., 1987). They found no evidence of high capacity glutamyl-dipeptide uptake by human erythrocytes with improved NMR spectroscopic methodology.

Recently, Odoom et al. (1990) studied the uptake of nine different dipeptides by human erythrocytes with proton NMR spectroscopic techniques. They found that the uptake occurred by simple diffusion. According to their calculation, the diffusive fluxes of circulating peptides into human erythrocytes were too small for these cells to play a significant role in circulating peptide metabolism under normal physiological conditions. Similar results were obtained by comparing the transport of dipeptides and their corresponding free amino acids into human erythrocytes (Lochs et al., 1990). This study showed that the cell uptake of free amino acids was 5- to 13-fold greater than that of the dipeptides. The transport of the dipeptides into the cells appeared to be either by simple diffusion or by a carrier system which had a very weak affinity for the dipeptides. It was suggested that erythrocyte uptake did not appear to play any appreciable role in clearance of circulating peptides.

Peptidases. Blood plasma contains hydrolase activity against peptides (Piez et al., 1960). However, it is not certain to what extent the circulating peptides are

hydrolyzed and if there is a relationship between plasma peptide hydrolysis and tissue utilization of peptide-bound amino acids.

Peptidase activity against glycylleucine and glycylglycine was measured in various tissues of rats (Krzysik and Adibi, 1977). The authors found that blood plasma had no detectable peptidase activity against glycylglycine and a modest activity against glycylleucine. But other tissues had much greater peptidase activity against the two dipeptides. Moreover, intravenously injected glycylleucine and glycylglycine rapidly disappeared from the plasma and the corresponding amino acids increased in the various tissues (Adibi et al, 1977). These results indicated that hydrolysis in plasma did not account for disappearance of glycylglycine and only partially for the disappearance of glycylleucine, suggesting an important involvement of other tissues in the metabolism of circulating peptides. Lochs et al.(1988) also reported that liver, muscle, gut, and kidney removed about 72% of intravenously infused glycylleucine and 82% of infused glycylglycine in the conscious dog. Plasma hydrolysis only accounted for about 5 and .5% of the disappearance of infused glycylleucine and glycylglycine, respectively.

Other studies also revealed that plasma hydrolysis of intravenously infused glutamine- and tyrosine-containing dipeptides only accounted for a very small portion of the disappearance of peptides from blood plasma (Hübl et al., 1989; Abumrad et al., 1989; Druml et al., 1991).

The half-life of circulating peptides in the plasma is closely related to their molecular structure (Adibi et al., 1986). Dipeptides with glycine on the N-terminal end were more resistant to plasma hydrolase activity than peptides without a N-terminal glycyl residue. Similar results also were obtained by Hübl et al.(1989).

Summary. Erythrocytes do not play an important role in the removal of circulating peptides from the blood plasma. Serumal peptidase activity also only

accounts for limited clearance of peptides from the circulation. Therefore, the disappearance of peptide-bound amino acids from the plasma during interorgan transport is due mainly to tissue uptake.

Advantages of Peptides as Amino Acid Sources

Besides the fact that peptide-bound amino acids comprise an important part of the plasma amino acid pool, there are advantages to animals and humans to utilize peptides as amino acid sources. These possible advantages are based on the following considerations: (1) the solubility of amino acids in blood plasma, (2) the stability of amino acids, and (3) the osmolarity of amino acid solutions used in parenteral nutrition.

Solubility. Amino acids usually have limited solubility in aqueous solution. Some amino acids, like tyrosine and cystine, are poorly soluble in water (Jakubke and Jeschkeit, 1977; Daabees and Stegink, 1978; Albers et al., 1988; Stehle et al., 1988; Druml et al., 1991). However, the solubility of these amino acids can be increased if they exist in the form of peptides containing charged amino acids (Grant, 1992). Amino acids in small peptide form are frequently more soluble than in the free form (Stehle et al., 1982). For example, bis-L-alanyl-L-cystine and bis-L-glycyl-L-cystine have 5,000-fold greater solubility than does free cystine (Stehle et al., 1988). Eleven tyrosine-containing di- and tripeptides were observed to be 10- to 1,500-fold more soluble than free tyrosine (Daabees and Stegink, 1978; Stehle et al., 1982). Therefore, alanyltyrosine and glycyltyrosine have been considered to be valuable substrates to provide a tyrosine source in parenteral nutrition (Daabees and Stegink, 1978 and 1979; Neuhäuser et al., 1986; Albers et al., 1988 and 1989; Druml et al., 1989 and 1991). Most amino acids are two- to fourfold more soluble when linked as C-terminal amino acid residues of glycyl-dipeptides (Adibi, 1987a). In general, a peptide is

soluble if the total charges including the charge properties of the α-amino and carboxyl groups are 20% of the total number of amino acid residues in the peptide (Grant, 1992).

Stability. Some amino acids are unstable in solution when they exist in the free form. The most unstable amino acids concerned in nutrition are methionine, cysteine, and glutamine (Adibi, 1987a; Abumrad et al., 1989; Grant, 1992; Adibi et al., 1993). The main problem with the sulfur-containing amino acids occurs because of their susceptibility to oxidation. Methionine is easily oxidized to methionine sulfoxide or methionine sulfone depending on whether one or two oxygen atoms are bonded to the sulfur atom (Walker et al., 1974). Cysteine is readily oxidized to cystine and the later is nearly insoluble in water (Rudman et al., 1981). Glutamine can be intramolecularly cyclized by interaction of its side chain with the α-amino group to form toxic pyroglutamic acid (Stehle et al., 1982; Stehle and Fürst, 1987; Grant, 1992) or decomposed to form toxic ammonia (Adibi et al., 1993). However, these unstable amino acids play important roles in body metabolism. Methionine is an essential amino acid and occupies a unique position in protein synthesis. Cysteine is generally considered to be a nonessential amino acid because it can be readily synthesized from methionine via the transsulfuration pathway (Irwin and Hegsted, 1971). However, this biosynthetic pathway is not fully developed in premature and newborn infants (Sturman et al., 1970; Gaull et al., 1972; Pohlandt, 1974) and is impaired in certain liver diseases (Rudman et al., 1981; Chawla et al., 1984). Recent studies suggest that glutamine is an essential nutrient for rapidly proliferating cells, such as enterocytes and lymphocytes (Newssholme et al., 1988; Souba, 1991), the principal substrate for renal ammoniagenesis (Welbourne, 1987), and a promoter of muscle protein synthesis (Maclennan et al., 1987; Wu and Thompson, 1990). These results indicate that a sufficient supply of these amino acids is important for keeping normal body

functions and metabolism in animals and humans, although the instability of these amino acids prevents their efficient utilization.

One way to increase the efficiency of utilization of the unstable amino acids is to increase their stability. It appears that the stability of some amino acids can be improved when they occur in peptide form (Adibi, 1978b). Peptide-bound glutamine is stable in solution (Thierfelder and Von Cramm, 1919; Stehle et al., 1982, 1984; Roth et al., 1988b). L-alanyl-L-glutamine was not affected by heat sterilization (120 to 140°C, 20 to 30 min) at various pHs, whereas, under the same conditions, 35 to 95% of free glutamine was converted to pyroglutamic acid (Stehle et al., 1982, 1984; Roth et al., 1988b). Therefore, alanylglutamine and glycylglutamine are considered as efficient and safe glutamine sources in parenteral nutrition (Amberger et al., 1983; Albers et al., 1988, 1989; Roth et al., 1988a, 1988b; Abumrad et al., 1989; Hübl et al., 1989; Stehle et al., 1989; Adibi et al., 1993). Cystine-containing short-chain peptides are not only highly soluble but also stable to heat sterilization (Stehle et al., 1988). Since cystine is readily converted to cysteine in animal body, these peptides can compensate for the instability of free cysteine. Recently, two stable cysteine-containing dipeptides have been synthesized (Stehle et al., 1990). There is no information available concerning the stability of peptide-bound methionine versus free methionine. However, an amino acid, in general, is expected to be less reactive when occurring in peptide form than in the free form because its active α-amino and/or carboxyl group(s) is(are) incorporated into peptide bond(s). Seal and Parker (1991) found that 68 to 94% of methionine was bound in low molecular weight peptides in steer and sheep portal and peripheral blood.

The stability of peptides is influenced by the composition of peptides. Studies revealed that glycine at the N-terminus is generally superior to other amino acids for minimizing dipeptide hydrolysis in plasma (Adibi et al., 1986; Stehle et al., 1988). The

plasma half-life of glycylglutamine is four or twelve times longer than alanylglutamine in rats and humans, respectively (Adibi et al., 1986; Hübl et al., 1989).

Osmolarity. The osmolarity of amino acid solutions for intravenous or parenteral administration is of a great concern in clinical nutrition. In parenteral nutrition, sometimes, a more invasive and complicated central vein catheterization has to be used instead of a peripheral vein procedure. The principal reason for the use of a central vein is the need for administration of hypertonic solutions, mostly a hypertonic amino acid solution (Adibi, 1987a, 1987b). The main advantage of using short-chain peptides as additional or alternative substrates for amino acids in parenteral nutrition is the lower osmolarity of the peptide-containing solutions (Fürst et al., 1990). Several studies have shown that partial or total substitution of amino acids with peptides in parenteral nutrition has no side effects. Adibi and Johns (1984) reported that under the conditions of total parenteral nutrition, the parameters of protein nutrition were not affected by substituting the glycine and leucine components of a commercially available amino acid solution with triglycine and trileucine. Another study showed that a dipeptide mixture, used as the source of amino acids for total parenteral nutrition in the baboon, was as effective as a corresponding amino acid mixture in maintaining positive nitrogen balance (Steinhardt et al., 1984). Similar results were obtained from a study on man (Grimble et al., 1988).

Summary. The advantages of peptides as amino acid sources are the following: (1) some peptides have a greater solubility than corresponding free amino acids; (2) amino acids in peptide form usually are more stable in aqueous solution than in free form; (3) with peptides, amino acid supply can be increased without changing plasma osmolarity which is of great concern in parenteral nutrition.

Chapter III

OBJECTIVES

The mammary glands of lactating animals have a tremendous demand for amino acids to meet the needs for tissue metabolism and for synthesis of milk proteins. How these demands for amino acids are met becomes a question to be answered in light of our knowledge about circulating peptides and free amino acids. Therefore, the overall objective of the research presented here was to elucidate the ability of peptide-bound amino acids to substitute for free amino acids in the synthesis of secreted proteins by mammary gland. Specific objectives included:

1) To determine the ability of methionine-containing and lysine-containing di- to octapeptides in the promotion of synthesis of secreted proteins and cell proliferation.

2) To evaluate the effect of amino acid position in dipeptides on the utilization of peptides in the synthesis of secreted proteins and on cell proliferation.

3) To examine the effects of peptide competition and peptidase inhibition on the utilization of peptides in the synthesis of secreted proteins.

4) To compare the ability of mouse mammary tissue explants to utilize lysine-containing peptides in the promotion of synthesis of secreted proteins with that of methionine-containing peptides.

5) To estimate the potential role of extracellular peptide hydrolysis on the utilization of peptides

6) To investigate the effect of cell maturation on the utilization of peptide-bound methionine for the synthesis of secreted proteins.

7) To assess the role of extracellular matrix on cell proliferation and synthesis of secreted proteins promoted by methionine-containing peptides.

Chapter IV

UTILIZATION OF PEPTIDE-BOUND METHIONINE FOR THE SYNTHESIS OF SECRETED PROTEINS BY MAMMARY TISSUE EXPLANTS FROM LACTATING MICE

ABSTRACT

Mammary tissue explants from lactating (10 to 11 d) CD-1 mice were used to study the ability of methionine-containing di- to octapeptides to substitute for free methionine as a source of this amino acid for the synthesis of secreted proteins. Explants were incubated at 37°C in a humidified atmosphere of 90% air and 10% CO_2 for 1 h in Dulbecco's modified Eagle's medium containing ^{3}H-leucine and either no methionine, L-methionine, or one of 23 methionine-containing peptides. The ability of methionine substrates to promote incorporation of ^{3}H-leucine into secreted proteins was quantified. Mammary tissue explants were able to utilize methionine from all peptides tested. All the peptides were at least as effective as free methionine in promoting ^{3}H-leucine incorporation into secreted proteins. Most di- and tripeptides promoted 15 to 76% greater ($P < .05$) ^{3}H-leucine incorporation than did free methionine. Dipeptides with methionine either at the N- or C-terminus showed the same ability to serve as a methionine source. There was a negative correlation ($r = .89$, $P < .01$) was detected between the rate of ^{3}H-leucine incorporation promoted by peptides and the number of amino acid residues in the peptides. The incorporation of ^{3}H-leucine promoted by some dipeptides was reduced ($P < .05$) in the presence of a 200-fold higher concentration of glycylsarcosine or carnosine. The results indicate that peptide-bound methionine can serve as a source of methionine for the synthesis of secreted proteins by lactating mammary tissue. Mediated transport of some peptides is probably involved in peptide utilization.

(Key Words: Mammary, Tissue Culture, Peptide, Enkephalin, Amino Acids, Mouse)

Introduction

It is known, now, that a relatively large portion of blood amino acids is in the form of peptides (DiRienzo, 1990; Seal and Parker, 1991; Koeln et al., 1993). Large amounts of plasma peptides are removed during interorgan transport, which implies the potential nutritional significance of circulating peptides (McCormick and Webb, 1982; Danilson et al., 1987a, Lochs et al., 1988). Direct evidence of tissue utilization of peptide-bound amino acids also has been reported from both *in vivo* and *in vitro* studies (Krzysik and Adibi, 1979; Brand et al., 1987; Emmerson and Phang, 1993).

Free amino acids in plasma generally have been considered as the major source of these nutrients for milk protein synthesis. Wonsil (1993) found that there was efficient extraction of free amino acids and only nominal extraction of peptide-bound amino acids from blood plasma by lactating bovine mammary gland. However, Backwell et al. (1994) reported that goat mammary gland could utilize intravenously infused dipeptides for milk protein synthesis. It is not known whether the different results are due to species differences or variable experimental conditions. Because there has been limited information accumulated regarding the nutritional utilization of peptides by the mammary gland, the role of peptide-bound amino acids in milk protein synthesis remains to be determined. The present study was designed to investigate the availability of peptides to serve as methionine sources for the synthesis of secreted proteins by lactating mammary tissue.

Materials and Methods

Medium. Methionine-free Dulbecco's modified Eagle's medium (DMEM) was prepared by combining 584 mg of L-glutamine[1], 52.5 mg of L-leucine[1], 148 mg of L-lysine · HCl[1], 3.7 g of sodium bicarbonate[2], 10 mL of antibiotic-antimycotic solution[3], and 1 pack (9.2 g) of deficient DMEM powder[4] in an 1 L volumetric flask. The mixture was dissolved in deionized water that was made using a water purification system[5] and was diluted to volume and adjusted to pH 7.18. The solution then was sterilized by filtration through a .20 μm membrane filter unit[6]. The sterilized medium had a pH of about 7.35.

Tissue Collection and Pre-incubation. Lactating (10 to 11 d) CD-1 mice were anesthetized by intrathoracic injection of xylazine[7] (2.2 μg/g body weight) and the bodies were cleaned with a paper towel wetted with 75% ethanol. The mice then were sacrificed by exsanguination in a laminar flow hood[8] pre-cleaned with 75% ethanol. The abdominal skin was cut open and mammary tissues in the thorax, abdominal, and inguinal locations were removed and put into methionine-free DMEM. The tissue was cut into 1 to 2 mm^3 explants in a 100 mm sterile petri dish with a surgical blade and the explants were washed five times with methionine-free DMEM. In order to deplete intracellular methionine, the explants then were pre-incubated at 37°C in a humidified atmosphere of 90% air and 10% CO_2 for 2 h in 6-well culture plates[9] (12 explants/well) containing 1.5 mL of methionine-free DMEM

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[1] Sigma Chemical Co., St. Louis, MO
[2] GIBCO, Grand Island, NY
[3] Cat. No. 600-5240AG, GIBCO, Grand Island, NY
[4] Cat. No. D4655, Sigma Chemical Co., St. Louis, MO
[5] Milli-Q Reagent Grade, Cat. No. ZD5211584, Millipore, Bedford, MA
[6] Cat. No. 09-740-24A, Fisher Scientific Product Corporation, Pittsburgh, PA
[7] Mobay Corporation, Animal Health Division, Shawnee, KA
[8] Purifier™ Clean Bench, Labconco Corporation, Kansascity, MI
[9] Cat. No. 08-772-1B, Fisher Scientific Product Corporation, Pittsburgh, PA

supplemented with 5 μg insulin[10], .5 μg hydrocortisone[11], 1 μg 17-ß-estradiol[12], and .1 μg prolactin[13]/mL medium..

Peptide Utilization. Following pre-incubation, the medium was removed and the explants were further incubated for 1 h in 1.5 mL of methionine-free DMEM supplemented with all the hormones as in the pre-incubation medium, 1 μCi L-[4,5-^{3}H]leucine[14] (^{3}H-leucine)/mL medium, and either no methionine, L-methionine[1], or one of the 23 methionine-containing peptides[1] (Table 4.1). All methionine-containing dipeptides in pairs (with methionine in N- or C-terminal) and tripeptides, which are constituted with L-α-amino acid residues, available from Sigma Chemical Co., were examined. Additionally, methionine-containing enkephalin peptides were chosen to represent longer peptides. Response to free methionine concentration was quantified by incubating tissue explants in media containing 0, 12.5, 25, 50, 100, and 200 μmol/L L-methionine. Incubations were terminated by collection of the medium. The explants were blotted on paper towels and weighed. Based upon the results of the resulting response curve (Figure 4.1), the concentration of methionine, either in free form or in peptide-bound form, in the incubation medium was chosen to be 30 μM.

Peptide Hydrolysis. To determine if there was hydrolysis of peptides in the incubation medium prior to uptake by the explants, the pre-incubated explants were incubated as above except that no free methionine or peptide was included in the medium. Following a 1-h incubation, the medium was collected and pooled. Methionine-containing peptides (30 μM) were added to 1 mL of the conditioned medium and incubated for an additional 1 h. The incubation was stopped by mixing one part of the medium with one part of HPLC internal standard containing .1 mM

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[10] Cat. No. I6634, Sigma Chemical Co., St. Louis, MO
[11] Cat. No. H0888, Sigma Chemical Co., St. Louis, MO
[12] Cat. No. E2758, Sigma Chemical Co., St. Louis, MO
[13] Cat. No. NIDDK-oPRL-19, National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases, Baltimore, MD
[14] Cat. No. 20036, ICN Biomedicals, Inc., Irvine, CA

norleucine. The mixture was immediately filtered through 10,000 MW cut off membrane filters[15]. The concentration of free methionine in the filtrate was determined by HPLC with a Waters Pico-Tag column (3.9 X 300 mm)[16] at a wavelength of 254 nm and 45°C in a flow rate of 1 mL/min. The eluent I for the HPLC procedure contained 70 mM sodium acetate, 2.5% acetonitrile and .025% ethylenediamine tetraacetic acid (EDTA) solution (10 mM) in deionized water. Eluent II consisted of deionized water, acetonitrile and methanol in a ratio of 40:45:15.

Effect of Peptide Competition. The pre-incubated explants were incubated for 1 h with methionine-free DMEM containing all the hormones as in the pre-incubation medium, ^{3}H-leucine, 30 μM of dipeptide, and with or without 6 mM of hydrolysis-resistant peptide, glycylsarcosine[1] or carnosine[1] (L-ß-alanyl-L-histidine). The dipeptides studied here were L-phenylalanyl-L-methionine, L-methionyl-L-phenylalanine, L-prolyl-L-methionine, L-methionyl-L-proline, L-seryl-L-methionine, and L-methionyl-L-serine.

Effect of Peptidase Inhibitor. The pre-incubated explants were incubated for 1 h with methionine-free DMEM containing all the hormones as in the pre-incubation medium, ^{3}H-leucine, 30 μM of serylmethionine, with or without 121 μM of a cytosolic peptidase inhibitor, p-hydroxymercuribenzoate (PHMB)[17] (Heizer et al., 1972; Raghunath et al., 1990).

Quantification of Secreted Proteins. The secreted proteins in the medium were precipitated with 5% trichloroacetic acid overnight at 4°C followed by centrifugation at 16,000 *X* g. The resulting protein pellets were washed three times by repeated suspension in 5% trichloroacetic acid followed by centrifugation at the same speed.

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[15] Cat. No. UFC3 LGC NB, Millipore, Bedford, MA
[16] Waters Millipore Corp., Milford, MA
[17] Cat. No. H3133, Sigma Chemical Co., St. Louis, MO

The proteins were solubilized in Scintigest[18]/deionized water mixture (1:2 ratio) in a 50°C water bath for at least 4 h and then were mixed with scintillation cocktail, Scintiverse LC[18]. The radioactivity of the ^{3}H-leucine labeled proteins was determined by liquid scintillation counting[19].

Statistical Analysis. The variance of incorporation rates of ^{3}H-leucine into secreted proteins promoted by different concentrations of L-methionine was partitioned into linear and quadratic components using the general linear model procedure (SAS, 1990). The effects of peptide treatments were also analyzed by general linear model procedure (SAS, 1990). When a significant F-value was obtained, means of different treatments were compared using Duncan's multiple range test at $\alpha = .05$. Relationships between the rate of ^{3}H-leucine incorporation promoted by the peptides and the extent of peptide hydrolysis or the back bone chain length of peptides was calculated using correlation and regression procedures (SAS, 1990).

Results

In this study, ^{3}H-leucine was used as a marker to evaluate the ability of methionine substrates to promote synthesis of secreted proteins. This is because milk proteins are rich in leucine that cannot be synthesized by tissues. If protein synthesis occurs, then leucine incorporation will occur. So, a small fraction of the leucine in the incubation medium was present as ^{3}H-leucine.

Effect of Medium L-Methionine Concentration. In this preliminary experiment, mammary tissue explants were incubated in media containing 0 to 200 μM L-methionine. The analysis of variance clearly showed that methionine concentration

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[18] Fisher Scientific Product Corporation, Pittsburgh, PA

[19] Beckman LS 5000TA Liquid Scintillation Counter, Beckman Instrument . Nuclear System Operations, CA

had an effect ($P < .0005$) on the incorporation of ^{3}H-leucine into secreted proteins. The incorporation of ^{3}H-leucine into secreted proteins increased linearly with methionine concentration up to 50 μM, and no further increase was observed at higher concentrations of L-methionine (Figure 4.1).

Utilization of Di- and Tripeptides. Mammary tissue explants were able to utilize methionine from all 17 di- and tripeptides studied (Figure 4.2A). However, differences ($P < .0001$) were observed among these peptides in their ability to promote incorporation of ^{3}H-leucine into secreted proteins. Eleven of these peptides promoted 15 to 76% greater ($P < .05$) incorporation of ^{3}H-leucine into secreted proteins than did free methionine, with dipeptides containing either valine or serine showing the highest ^{3}H-leucine incorporation. The remaining six peptides were not different ($P > .05$) from free methionine in promoting ^{3}H-leucine incorporation. Tissue explants incubated in methionine-free medium (negative control) also showed some incorporation of ^{3}H-leucine into secreted proteins. However, the incorporation rate was lower ($P < .05$) than that promoted by L-methionine and all the peptides.

Fourteen of the seventeen peptides formed seven pairs (methionylvaline and valylmethionine, methionylserine and serylmethionine, methionylalanine and alanylmethionine, methionylglycine and glycylmethionine, methionylproline and prolylmethionine, methionylphenylalanine and phenylalnylmethionine, methionylleucine and leucylmethionine) such that the constituent amino acids were the same in each pair with the methionyl residue being either in the N- or C-terminal position. There was no difference ($P > .05$) between the two peptides of each pair in promoting ^{3}H-leucine incorporation, except the two pairs containing alanine and proline residues (Figure 4.2A).

Partial hydrolysis of all di- and tripeptides was observed in conditioned medium after a 1-h incubation (Figure 4.2B). The extent of the hydrolysis varied with

peptides, but was not correlated ($r = .03$, $P < .91$) with the rate of ^{3}H-leucine incorporation promoted by the peptides.

Utilization of Tetra- to Octapeptides. Mammary tissue explants also were able to utilize methionine from all of the six methionine-containing tetra- to octapeptides (Figure 4.3A). The methionyl residue in these peptides was located either in the C-terminal position or in the interior of the peptide chains. The methionine-containing tetrapeptide (GGFM) promoted a greater ($P < .05$) incorporation of ^{3}H-leucine into secreted proteins than did L-methionine. The other five methionine-containing peptides showed the same ability ($P > .05$) as L-methionine in promoting ^{3}H-leucine incorporation.

To determine whether the incorporation of ^{3}H-leucine was due to the bioactivity of these methionine-containing enkephalin peptides, two similar bioactive but methionine-free enkephalin peptides, YGGF and YGGFLK, were included in this study (Figure 4.3A). YGGF differs from the methionine-containing enkephalins, GGFM and YGGFM, by only one amino acid residue, and YGGFLK has also only one residue different from YGGFMK. The two methionine-free peptides showed no difference ($P > .05$) from the negative control in promoting ^{3}H-leucine incorporation, which was lower ($P < .05$) than that promoted by methionine-containing peptides or L-methionine.

The methionine-containing tetra- to octapeptides were partially hydrolyzed in the conditioned medium (Figure 4.3B). The extent of the hydrolysis varied among the peptides. There was no correlation ($r = .06$, $P < .92$) between the rate of ^{3}H-leucine incorporation promoted by the peptides and the extent of peptide hydrolysis.

Effect of Peptide Chain Length. A negative correlation ($r = -.89$, $P < .01$) was observed between the rate of ^{3}H-leucine incorporation promoted by the peptides and peptide backbone chain length (Figure 4.4). Thus, about 80% ($R^2 = .80$) of the

difference in the rate of ^{3}H-leucine incorporation among the peptides can be accounted for by the backbone chain length of the peptides. A regression analysis showed that ^{3}H-leucine incorporation decreased linearly with peptide backbone chain length: for each increase of amino acid residue on the peptide chain, there is a decrease of about 7 units (% of methionine treatment) (slope = -7.18 ± 1.607) of ^{3}H-leucine incorporation on average.

Effect of Peptide Competition. To determine whether carrier mediated peptide transport was involved in the peptide utilization, hydrolysis-resistant peptides, glycylsarcosine and carnosine, were used to examine if they could influence protein synthesis by competing with the methionine-containing dipeptides during transport. When the concentration of glycylsarcosine or carnosine was 200-fold higher than the tested dipeptides in the incubation medium, the incorporation of ^{3}H-leucine promoted by the methionine-containing dipeptides with either a proline or a serine residue was reduced ($P < .05$; Figure 4.5). Incorporation of ^{3}H-leucine promoted by the methionine-containing dipeptides with a phenylalanine residue was not affected by the presence of either glycylsarcosine or carnosine (Figure 4.5).

Effect of Peptidase Inhibitor. To study whether the explants take up intact peptides or whether the peptides are hydrolyzed by membrane-bound peptidases during translocation across the cell membrane, a cytosolic peptidase inhibitor, PHMB, was used to examine its effect on the synthesis of secreted proteins. PHMB is reported to be capable of inhibiting cytosolic but not membrane-bound peptidase activity in intestinal and muscle cells (Heizer et al., 1972; Raghunath et al., 1990). In the presence of PHMB, the incorporation of ^{3}H-leucine promoted by L-methionine was reduced by about 30% (Figure 4.6). The incorporation of ^{3}H-leucine promoted by serylmethionine was reduced by about 60% in the presence of PHMB. If we consider the effect of PHMB on ^{3}H-leucine incorporation promoted by L-methionine

as background, there was still about a 30% reduction of ^{3}H-leucine incorporation promoted by serylmethionine due to the presence of PHMB.

After measuring the effect of PHMB on ^{3}H-leucine incorporation promoted by serylmethionine, the effect of different PHMB concentrations on the inhibition of ^{3}H-leucine incorporation promoted by serylmethionine was examined (Figure 4.7). It was observed that the incorporation of ^{3}H-leucine promoted by the dipeptide decreased linearly with medium PHMB concentration up to 200 μM. No further inhibition was observed at higher PHMB concentrations.

Discussion

The assumption underlying the present experimental design is that animal tissues cannot synthesize methionine or leucine which are required for the synthesis of milk proteins (Mepham, 1987). Thus, the ^{3}H-leucine labeled proteins can only be synthesized using the methionine in either free or peptide-bound form and the leucine supplied in the medium.

However, it was observed, in this study, that the tissue explants incubated in methionine-free (negative control) medium also synthesized a certain amount of ^{3}H-leucine labeled, secreted proteins. The methionine utilized in the synthesis of these ^{3}H-leucine labeled proteins may be from the turnover of non-secreted milk proteins, from the turnover of tissue proteins, or from an intracellular free methionine pool. Because all of the tissue explants were pre-incubated in methionine-free medium to deplete intracellular methionine, the methionine utilized in the synthesis of ^{3}H-leucine labeled proteins in methionine-free medium would most likely come from the turnover of proteins. It has been reported that a tissue with an insufficient amino acid supply has a much higher protein turnover rate than when there is a sufficient amino

acid supply (Waterlow et al., 1978). Therefore, we cannot treat the ^{3}H-leucine incorporation in the negative control as a background effect and simply subtract this amount of incorporation from that of peptide or free methionine treatments.

In the present study, the response curve of ^{3}H-leucine incorporation into secreted proteins promoted by different concentrations of free methionine showed a quadratic effect (Figure 4.1). The saturation of ^{3}H-leucine incorporation at around 50 μM methionine indicates the maximum cell ability for synthesis of secreted proteins under the present experimental conditions. Thus, subsequent experiments were designed to allow each peptide to completely express their ability to serve as a methionine source. Therefore, according to this response curve, the concentration of methionine, either as free or as peptide-bound methionine in the incubation medium was chosen to be 30 μM to study the effects of methionine-containing peptides on the synthesis of secreted proteins.

The present results show that methionine-containing di- to octapeptides can substitute for free methionine for the synthesis of secreted proteins in lactating mammary tissue *in vitro*. Although partial hydrolysis of all peptides was observed to occur in conditioned medium, the extent of such partial hydrolysis could not account for the responses observed in ^{3}H-leucine incorporation into secreted proteins. This is because the preliminary experiment (Figure 4.1) showed a linear increase of ^{3}H-leucine incorporation with medium free methionine concentrations up to 50 μM. Therefore, methionine released from the partial hydrolysis of peptides present at a concentration of 30 μM should not stimulate the same or even higher rate of ^{3}H-leucine incorporation compared with 30 μM L-methionine. Furthermore, there was no correlation between the rate of ^{3}H-leucine incorporation promoted by the peptides and the extent of peptide hydrolysis. The partial hydrolysis of peptides could be caused by the activity of peptidases secreted with milk proteins (Jenness, 1974)

and/or released from damaged cells. Since the explants were thoroughly washed prior to pre-incubation, there was little chance for having peptidase activity from damaged cells.

Direct and indirect evidence suggesting the possibility of tissue utilization of peptide-bound amino acids has been reported to occur in muscle, liver, gut, kidney, pancreas, and lung (Krzysik and Adibi, 1979; McCormick and Webb, 1982; Danilson et al., 1987a; Lochs et al., 1986, 1988; Abumrad et al., 1989; Koeln et al., 1993). Peptide-bound amino acids also have been used to substitute for the corresponding free amino acids to fulfil the growth requirement of cultured mammalian cells (Brand et al., 1987; Emmerson and Phang, 1993; Pan, 1993). However, information regarding the utilization of peptide-bound amino acids by the mammary gland is limited. Pocius et al. (1981) reported a rapid removal of glutathione (τ-glutamylcysteinylglycine) from blood by lactating bovine mammary gland. Since there was a shortage of cysteine and glutamate in blood for synthesis of milk proteins (Clark et al., 1977, 1978), it was suggested that glutathione could provide the amount of cysteine and glutamate needed. Recently, Backwell et al. (1994) found that the ^{13}C-phenylalanine and ^{13}C-leucine from intravenously infused glycyl-L-[1-^{13}C]-phenylalanine and glycyl-L-[1-^{13}C]-leucine were incorporated into milk caseins of goats. The present study further demonstrates that a wide range of peptides can serve as methionine sources for the synthesis of secreted proteins by lactating mouse mammary tissue. Some small peptides were even more efficient than free methionine in promoting the protein synthesis.

Among the dipeptides evaluated in the present study, there were seven pairs and each pair differed by having the methionyl residue in either the N- or C-terminal position. For five of the seven pairs, the peptides within each pair showed no difference in their ability to promote ^{3}H-leucine incorporation. This indicates, at least

for these peptides, that the location of the methionine residue is not an important factor determining the effectiveness of these peptides as methionine substrates for the synthesis of secreted proteins by mammary explants from the lactating mice. In this connection, Pan (1993), using the same methionyl dipeptides, found that dipeptides with a N-terminal methionine were generally better than dipeptides with a C-terminal methionine in promoting protein accretion in cultured myogenic cells. Fagle (1955) also reported that glycyl-DL-phenylalanine was preferred to DL-phenylalanylglycine to substitute for phenylalanine in promoting the proliferation of cultured mouse fibroblast cells. The inconsistency of observed results may be due to tissue and/or peptide differences.

Mechanisms involved in the metabolism of peptides by tissues have not been clearly elucidated. However, it is generally agreed that peptide hydrolase activity is associated with tissue metabolism of peptides. This is because there is no evidence for direct utilization of intact peptides. Therefore, the rate of peptide hydrolysis will influence the efficiency of tissue utilization of peptide-bound amino acids. In the present study, most pairs of the dipeptides showed no difference in the rate of hydrolysis in the conditioned medium (Figure 4.2B). The hydrolases against the peptides in the conditioned medium could be of cytoplasmic origin. This indicates that there was probably no difference in intracellular hydrolysis of the two dipeptides in each pair.

In the present study, methionine from di- to octapeptides was utilized at least as effectively as free methionine for the synthesis of secreted proteins by the mammary explants. However, the results show that the efficiency of the peptide-bound methionine as a source of methionine decreased with increasing numbers of amino acid residues in the peptide chain. For almost all previous investigations concerning tissue utilization of peptide-bound amino acids, dipeptides were the only

substrates studied. Only one study examined the effect of peptide chain length on their utilization (Grahl-Nielsen et al., 1974). It was observed that the utilization of di-, tetra-, hepta-, and decalysine, as a lysine source for intestinal cell growth in culture, diminished gradually as peptide size increased. Intravenously injected tri- and tetrapeptide-bound amino acids were assimilated by the liver and kidney of rat (Adibi and Morse, 1982) and a very small amount of hexapeptides were removed from a perfusion medium by isolated rat liver (Lombardo et al., 1988), thus providing indirect evidence for tissue utilization. Overall, it appears that small peptides have more potential nutritional significance than the oliopeptides. One may, however, speculate that having a mixture of peptides of different sizes may provide a steady and stable supply of amino acids to tissues.

Although the methionine-containing tetra- to octapeptides used here have been reported to have morphine and opiate agonist activities and are produced in brain and adrenal medulla (Hughes et al., 1975; Malfroy et al., 1978; Stern et al., 1979; Gubler et al., 1982), similar bioactive, but methionine-free enkephalin peptides failed to stimulate ^{3}H-leucine incorporation into secreted proteins. These results suggest that the tetra- to octapeptides studied here have no bioactive effect on mammary protein synthesis and that the ^{3}H-leucine incorporation promoted by these peptides was due to their being a source of methionine.

Transport of peptide-bound amino acids across cell membranes may be involved in tissue utilization of peptides. Four mechanisms have been suggested as possibilities of how enterocytes and renal cortex tubular cells absorb peptide-bound amino acids across brush border membranes (Ugolev et al., 1990; Matthews, 1991a; Skopicki et al., 1991; Daniel et al., 1992). They are (1) uptake of intact peptides via carrier mediated transport systems followed by intracellular hydrolysis, (2) uptake of intact peptides by simple diffusion followed by intracellular hydrolysis, (3) hydrolysis

of peptides during their translocation across cell membranes, and (4) extracellular hydrolysis of peptides via plasma membrane-bound peptidases followed by the absorption of the constituent amino acids by amino acid carriers. Although these mechanisms of peptide uptake across brush border membranes have been suggested, the mechanisms by which tissues assimilate peptide-bound amino acids from the circulation are poorly understood. It has been reported that extracellular hydrolysis of peptides by membrane-bound peptidases followed by uptake of the constituent amino acids is one mechanism for liver and muscle tissue assimilating peptide-bound amino acids in vitro (Adibi and Morse, 1981; Lochs et al., 1986; Lombardo et al., 1988; Raghunath et al., 1990). Some peptides may also be transported into muscle cells by simple diffusion (Raghunath et al., 1990). There has been no information regarding the mechanism by which mammary tissue takes up peptide-bound amino acids. According to the results of the present study, it appears that mediated transport may be involved in uptake of at least some dipeptides by mouse mammary explants. Hydrolysis-resistant dipeptides, glycylsarcosine and carnosine, are frequently used to study peptide transport (Ganapathy et al., 1981; Matthews, 1991a). The reduced transport of other di- or tripeptide in the presence of glycylsarcosine or carnosine provides evidence for carrier mediated peptide transport, because it is assumed under these conditions that the hydrolysis-resistant peptide is competing with the other peptide for binding to and subsequent translocation by the transporter. The incorporation of ^{3}H-leucine promoted by the methionyl dipeptides with either serine or proline residues was reduced in the presence of glycylsarcosine or carnosine in the present study. This suggests that the 200-fold higher concentration of the hydrolysis-resistant peptide had a concentration advantage to occupy the transport system. This in turn affected the utilization of the methionyl dipeptide by blocking its uptake.

Another concern regarding the uptake of peptides by mammary explants is whether the peptides are transported intact or hydrolyzed during translocation across the cell membrane. One way to answer this question is to examine whether intracellular peptidases have any effect on the utilization of the peptide-bound methionine. It has been reported that cytosolic and brush border membrane-bound peptide hydrolases are different in intestinal cells (Heizer et al., 1972). The sulfhydryl blocking reagent, PHMB, inhibits the cytosolic peptidase activity of both intestinal and muscle cells, but has no effect on membrane-bound peptidase activity (Heizer et al., 1972; Raghunath et al., 1990). In the present study, the incorporation of ^{3}H-leucine into secreted proteins promoted by serylmethionine was reduced ($P < .05$) by about 60% in the presence of PHMB. The PHMB also caused a reduction ($P < .05$) in ^{3}H-leucine incorporation promoted by L-methionine by about 30%. Protein synthesis includes polypeptide modification that involves proteinase activity. One example is cleavage of the initial methylated methionine from the N-terminal of polypeptide chains. Some of these proteinases could be targets of PHMB. Therefore, the reduction of ^{3}H-leucine incorporation promoted by L-methionine in the presence of PHMB may be due to the inhibition of intracellular proteinase activity involved in protein synthesis per se. If we consider this inhibited proteinase activity as background, there was still about an additional 30% reduction of ^{3}H-leucine incorporation when serylmethionine was the substrate. This suggests that serylmethionine may be transported intact into the mammary cells.

In the present study, most di- and tripeptides promoted a greater incorporation of ^{3}H-leucine into secreted proteins than did free methionine. This could result from different rates of uptake between peptide-bound methionine and free methionine. It is known that carrier mediated peptide transport is different from amino acid transport in the brush border membrane (Matthews, 1991a). The influx of

certain amino acids into enterocytes is more rapid in small peptide form than in free form (Adibi, 1971; Matthews, 1975). The greater incorporation of ^{3}H-leucine promoted by these peptides in the present study suggests that these methionine-containing peptides may be transported into the mammary cells more rapidly than free methionine. An alternative interpretation of the greater ^{3}H-leucine incorporation promoted by some peptides compared to free methionine could involve intracellular compartmentation or channeling of amino acid sources. There is evidence that amino acids in different intracellular pools or from different sources are used differently for protein synthesis (Schneible et al., 1981; Tanaka and Ichihara, 1983; Sivaram and Murray, 1990). Amino acid incorporation into proteins may be coupled with transport (Deutscher, 1984). It may be possible that free amino acids and peptide amino acids are channelled differently for incorporation into proteins.

The different abilities among the peptides studied here to promote ^{3}H-leucine incorporation may be due to different efficiencies of transporting these peptides across mammary cell membranes. Different peptide transport systems have been identified in the brush border membranes of enterocytes and the renal cortex tubular cell membrane and peptides differ in affinities for the different transporters (Daniel et al., 1991; Matthews, 1991a; Skopicki et al., 1991; Minami et al., 1992). Also, peptides sharing the same transporter do not necessarily have the same affinity for the transporter (Daniel et al., 1992). It may also be possible that hydrolysis efficiencies of different peptides are not the same as the peptides are hydrolyzed by either cytosolic or membrane-bound peptidases. Peptidases from different tissues or from the same tissue but from a different cellular compartment show variable hydrolase activities against different peptides or even to the same peptide (Heizer et al., 1972; Krzysik and Adibi, 1977; Lochs et al., 1986; Lombardo et al., 1988; Raghunath et al., 1990).

In conclusion, di- to octapeptide-bound methionine can serve as a source of methionine for the synthesis of secreted proteins by lactating mouse mammary tissue. Most di- and tripeptide-bound methionine appears to be more efficient than free methionine in promoting synthesis of secreted proteins. This implies an important potential nutritional significance of small peptides.

Implications

The present study shows that some methionyl di- and tripeptides are more efficient than free methionine in promoting synthesis of secreted proteins by mammary tissue from the lactating mice. If the results observed here represent the *in vivo* situation and if delivery of intact peptides to the mammary gland can be manipulated, supplementing the methionine need of lactating animals with peptide-bound methionine in the form of small peptides may become practical.

Table 4.1. Methionine-containing peptides studied

Peptide	Abbreviation[a]
L-alanyl-L-methionine	AM
L-methionyl-L-alanine	MA
L-phenylalanyl-L-methionine	FM
L-methionyl-L-phenylalanine	MF
Glycyl-L-methionine	GM
L-methionyl-glycine	MG
L-leucyl-L-methionine	LM
L-methionyl-L-leucine	ML
L-prolyl-L-methionine	PM
L-methionyl-L-proline	MP
L-seryl-L-methionine	SM
L-methionyl-L-serine	MS
L-valyl-L-methionine	VM
L-methionyl-L-valine	MV
L-methionyl-L-methionine	MM
L-methionyl-L-alanyl-L-serine	MAS
L-methionyl-L-leucyl-L-phenylalanine	MLF
des-Tyr-methionine enkephalin (Gly-Gly-Phe-Met)	GGFM
[Ala^2]-methionine enkephalin (Tyr-Ala-Gly-Phe-Met)	YAGFM
methionine enkephalin (Tyr-Gly-Gly-Phe-Met)	YGGFM
methionine enkephalin-Lys (Tyr-Gly-Gly-Phe-Met-Lys)	YGGFMK
methionine enkephalin-Arg-Phe (Tyr-Gly-Gly-Phe-Met-Arg-Phe)	YGGFMRF
methionine enkephalin-Arg-Gly-Leu (Tyr-Gly-Gly-Phe-Met-Arg-Gly-Leu)	YGGFMRGL
des-Met^5-methionine enkephalin (Tyr-Gly-Gly-Phe)	YGGF
leucine enkephalin-Lys (Tyr-Gly-Gly-Phe-Leu-Lys)	YGGFLK

[a] One-letter abbreviations for amino acids. A, alanine; F, phenylalanine; G, glycine; K, lysine; L, leucine; P, proline; R, arginine; S, serine; V, valine; Y, tyrosine.

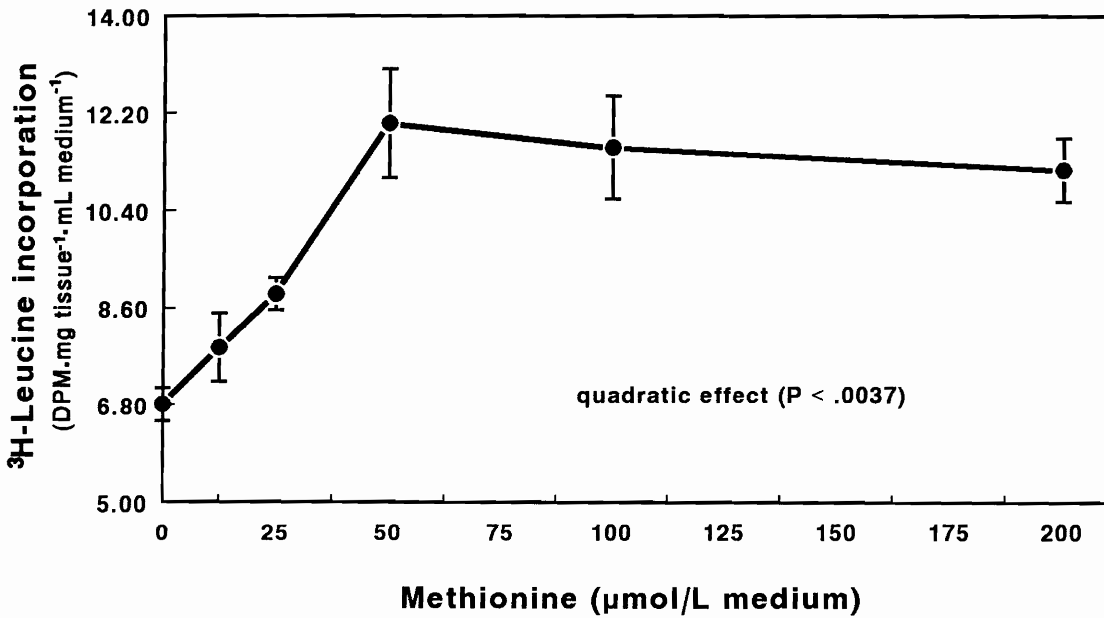

Figure 4.1. Incorporation of ^{3}H-leucine into secreted proteins by mammary explants from lactating mice promoted by different concentrations of L-methionine in the incubation medium. Values represent means ± SE (n = 6). The response showed a quadratic effect (P = .0037).

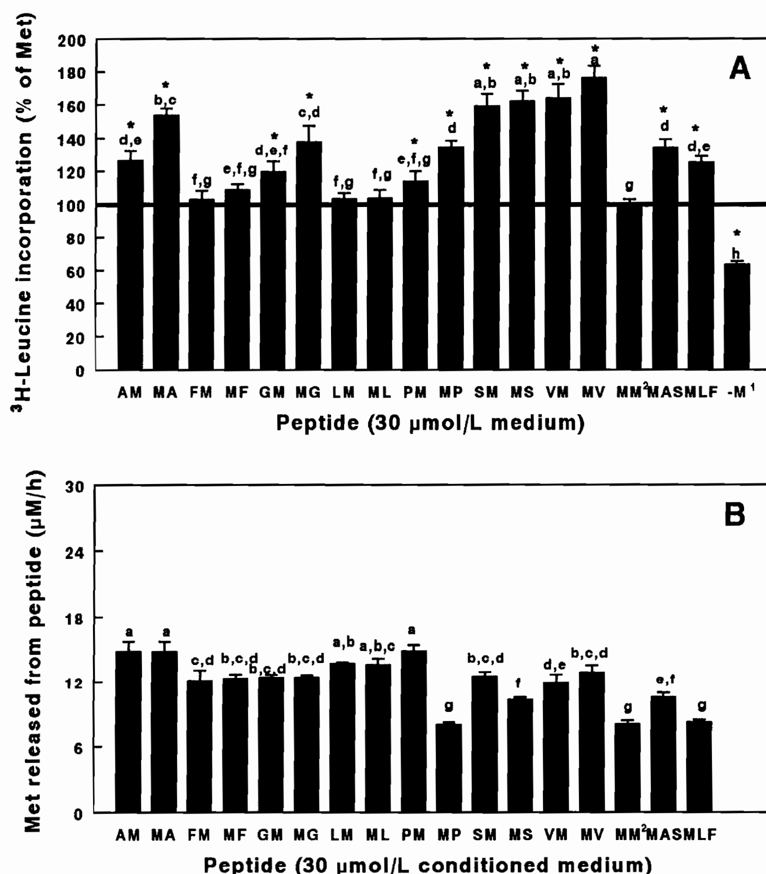

Figure 4.2. A. Incorporation of ^{3}H-leucine into secreted proteins promoted by methionine-containing di- and tripeptides by mammary explants from lactating mice. Values represent means ± SE (n = 12, two repeated experiments) which are expressed as the percentage of ^{3}H-leucine incorporation promoted by L-methionine. The horizontal line indicates the amount of ^{3}H-leucine incorporation promoted by L-methionine. Bars lacking a common letter differ (P < .05). Means (*) differ from L-methionine treatment (P < .05). [1] -M, no methionine substrate in the incubation medium (negative control). [2] MM, 15 μmol/L medium. B. Hydrolysis of methionine-containing di- and tripeptides in conditioned medium. Values represent means ± SE (n = 6).

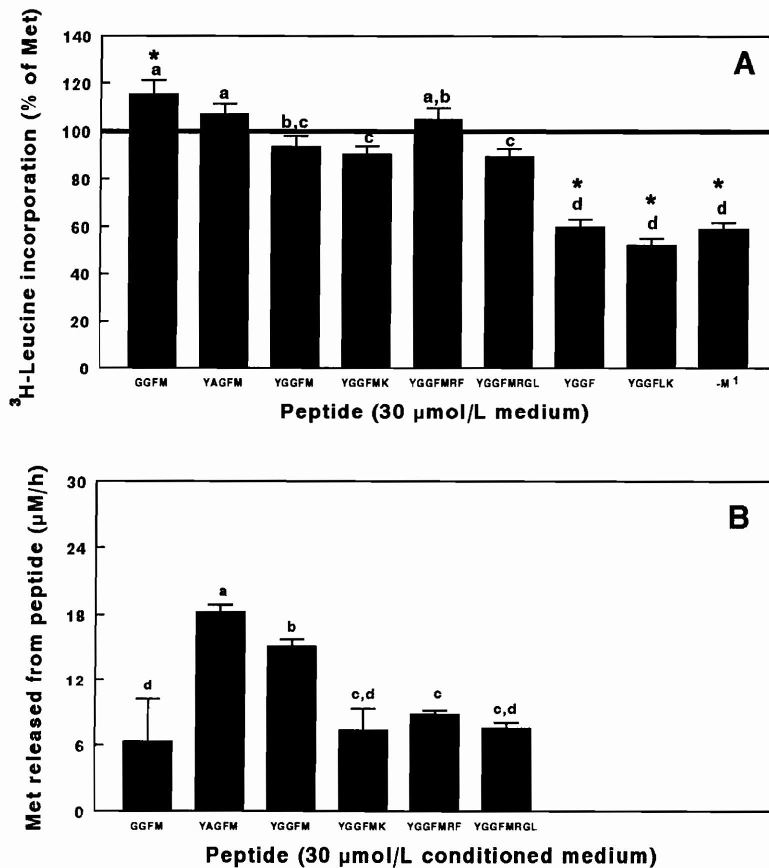

Figure 4.3. A. Incorporation of ^{3}H-leucine into secreted proteins promoted by methionine-containing and methionine-free enkephalin peptides by mammary explants from lactating mice. Values represent means ± SE (n = 12, two repeated experiments) which are expressed as the percentage of ^{3}H-leucine incorporation promoted by L-methionine. The horizontal line indicates the amount of ^{3}H-leucine incorporation promoted by L-methionine. Bars lacking a common letter differ (P < .05). Means (*) differ from L-methionine treatment (P < .05). [1] -M, no methionine substrate in the incubation medium (negative control). B. Hydrolysis of methionine-containing enkephalin peptides in conditioned medium. Values represent means ± SE (n = 6).

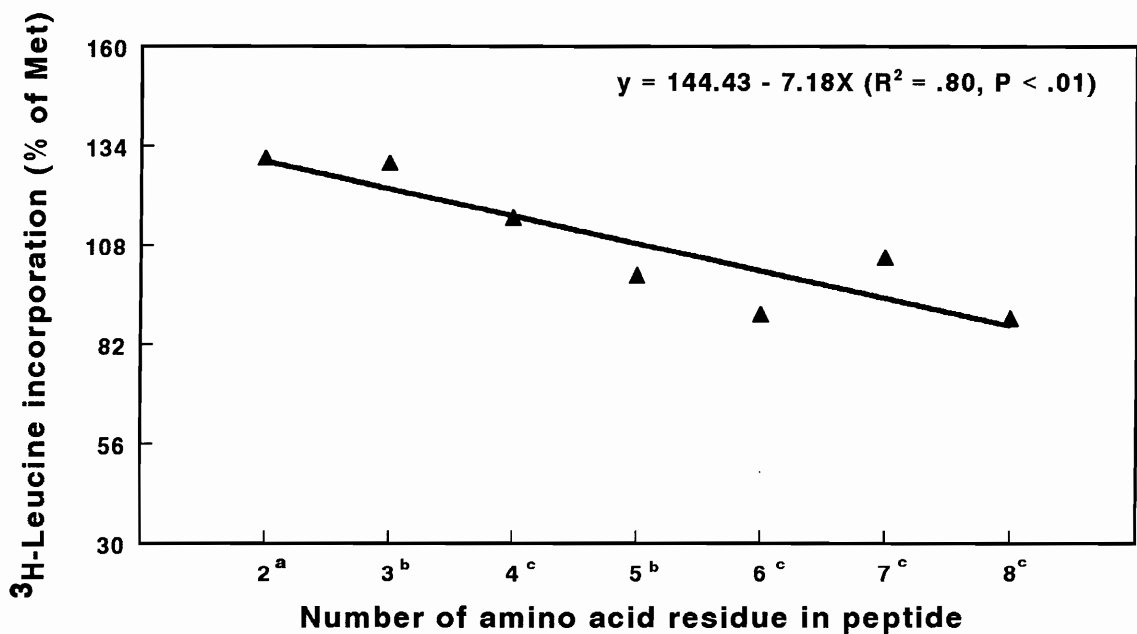

Figure 4.4. Relationship between rate of ^{3}H-leucine incorporation promoted by methionine-containing peptides and number of amino acid residues in the peptides.
[a] Value represents the mean of fifteen dipeptides with n = 12 in each treatment.
[b] Values represents the mean of two tripeptides or two pentapeptides with n = 12 in each treatment. [c] Value is the mean of one peptide with n = 12.

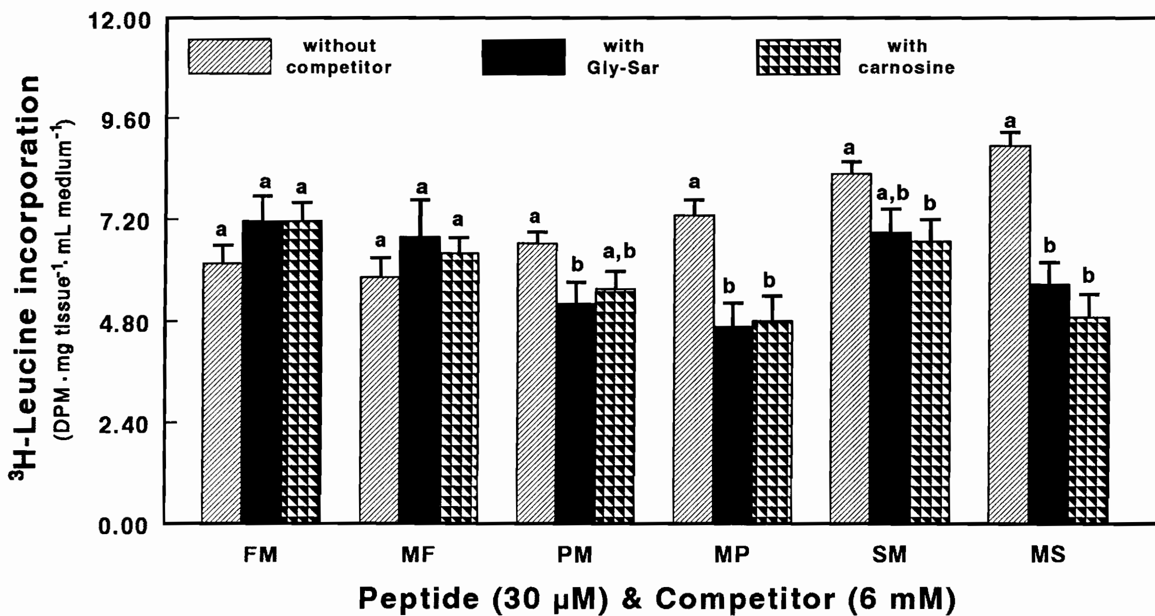

Figure 4.5. Incorporation of ^{3}H-leucine into secreted proteins promoted by methionine-containing dipeptides in the presence of hydrolysis-resistant dipeptides. Values represent means ± SE (n = 8, two repeated experiments). Bars lacking a common letter within each group differ (P < .05).

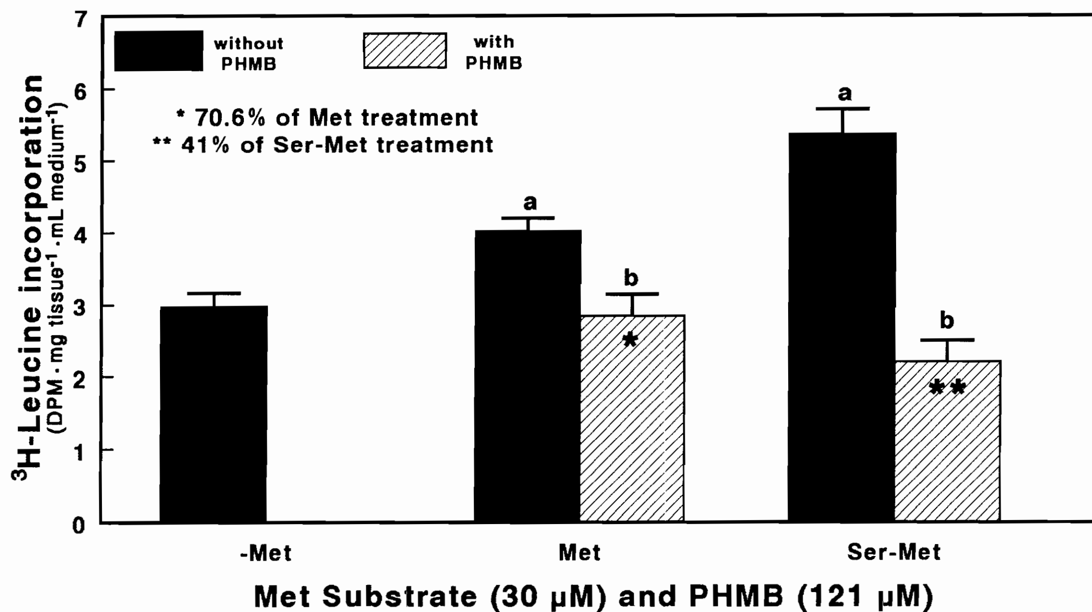

Figure 4.6. Incorporation of ^{3}H-leucine into secreted proteins promoted by serylmethionine in the presence of a cytosolic peptidase inhibitor, p-hydroxymercuribenzoate (PHMB). Values represent means ± SE (n = 6). Bars lacking a common letter within each group differ ($P < .05$).

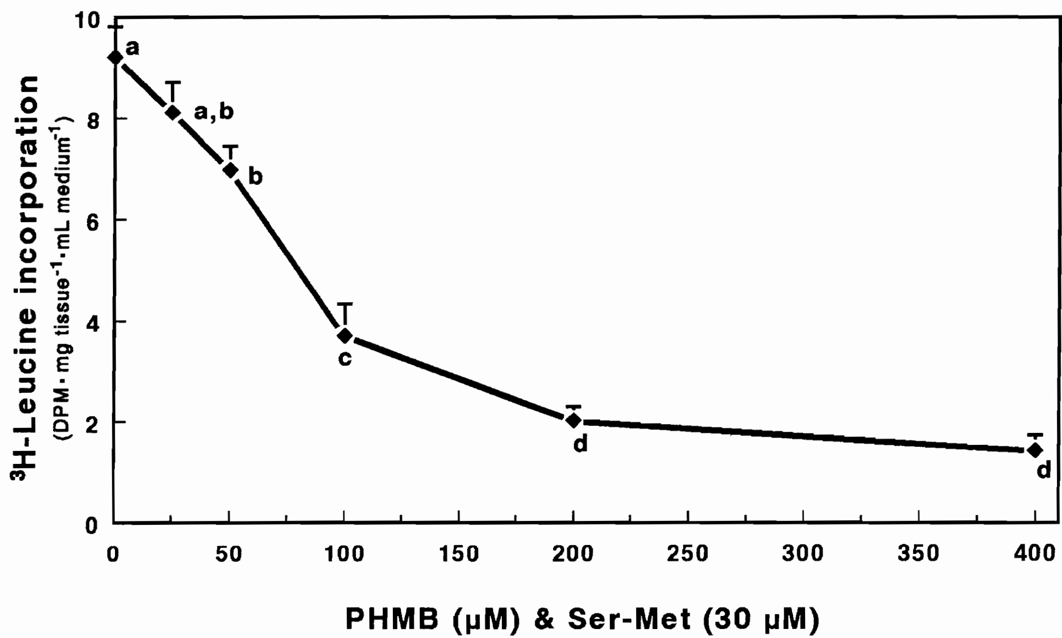

Figure 4.7. Incorporation of ^{3}H-leucine into secreted proteins promoted by serylmethionine in the presence of different concentrations of PHMB. Values represent means ± SE (n = 6). Means lacking a common letter differ ($P < .05$).

Chapter V

UTILIZATION OF PEPTIDE-BOUND LYSINE FOR THE SYNTHESIS OF SECRETED PROTEINS BY MAMMARY TISSUE EXPLANTS FROM LACTATING MICE

ABSTRACT

Twenty-three lysine-containing di- to octapeptides were evaluated for their ability to substitute for free lysine in the synthesis of secreted proteins by mammary tissue explants from lactating (10 to 11 d) CD-1 mice. The explants were incubated at 37°C in a humidified atmosphere of 90% air and 10% CO_2 for 80 min in Dulbecco's modified Eagle's medium containing hormones, ^{3}H-leucine, and one of the lysine substrates in either free or peptide-bound form. The incorporation of ^{3}H-leucine into secreted proteins promoted by lysine substrates was quantified. Mammary explants were able to utilize lysine from all peptides tested except the octapeptide. These peptides were as efficient ($P > .05$) as free lysine in promoting ^{3}H-leucine incorporation into secreted proteins. The incorporation of ^{3}H-leucine promoted by these peptides ranged from 91 to 117% of that promoted by free lysine. All tri- to octapeptides, except glycylhistidyllysine, did not stimulate ^{3}H-leucine incorporation into secreted proteins in a methionine-free medium, suggesting that the responses observed when these peptides were incorporated into a lysine-free medium were due to the presence of the lysine residue and not due to any bioactivity of the peptides per se. The results suggest that a wide range of peptide-bound lysine can serve as a source of lysine for the synthesis of secreted proteins by lactating mammary tissue.
(Key Words: Mammary, Tissue Culture, Peptide, Amino Acid, Mouse, Lysine)

Introduction

Results from a number of studies are now challenging the long time belief that proteins must be completely hydrolyzed to amino acids prior to delivery to and/or entry into tissues. There is evidence showing that skeletal muscle, liver, gut, kidney, pancreas, and lung may have the ability of utilizing peptides as sources of amino acids (Krzysik and Adibi, 1979; McCormick and Webb, 1982; Danilson et al., 1987a; Abumrad et al., 1989). Results presented in Chapter IV reveal that lactating mouse mammary explants can utilize a wide range of methionyl peptides as a source of methionine for the synthesis of secreted proteins. Backwell et al. (1994) further confirmed the ability of goat mammary gland to utilize peptide-bound amino acids for milk protein synthesis *in vivo*.

It has been reported that lysine is one of the most limiting essential amino acids for milk protein synthesis in cows (Spires et al., 1975; Clark et al., 1977). The relative concentration of arterial plasma free lysine, in addition to methionine, was only about half of their concentration in milk (Clark et al., 1978). Bickerstaffe et al. (1974) observed that the uptake of both free lysine and free methionine from blood plasma was less than their output in milk proteins. This suggests the possible involvement of other blood lysine sources in milk protein synthesis. It has been observed that about 73 to 77% of blood plasma lysine or 70% of whole blood lysine was bound to peptides in calves and sheep (Seal and Parker, 1991; Koeln et al., 1993). Peptide-bound lysine accounted for about 30% of total blood plasma lysine in rats (Seal and Parker, 1991). These results indicate that circulating lysyl peptides may serve as a source of lysine for milk production. However, no information regarding the utilization of peptide-bound lysine is available to support this assumption. Therefore, the purpose of this study was to evaluate the ability of lysyl peptides to

substitute for free lysine in the synthesis of secreted proteins by lactating mammary tissue.

Materials and Methods

Media. Lysine-free Dulbecco's modified Eagle's medium (DMEM) was prepared by combining 584 mg of L-glutamine[1], 52.5 mg of L-leucine[1], 6 mg of L-methionine[1], 3.7 g of sodium bicarbonate[2], 10 mL of antibiotic-antimycotic solution[3] and 1 pack (9.2 g) of deficient DMEM powder[4] in an 1 L volumetric flask. The mixture was dissolved in deionized water that was made using a water purification system[5] and was diluted to volume and adjusted to pH 7.18 with 1 N HCl. Methionine- and lysine-free DMEM was prepared in the same way except that L-methionine was omitted. The prepared solutions then were sterilized by filtration as described in Chapter IV. The pH of the medium raised to about 7.35 after sterilization.

Tissue Collection and Pre-incubation. Mammary tissue explants from lactating (10 to 11 d) CD-1 mice were prepared as described in Chapter IV. The explants were thoroughly washed five times with lysine-free or methionine- and lysine-free DMEM. To deplete intracellular lysine and/or methionine, the explants were pre-incubated at 37°C in a humidified atmosphere of 90% air and 10% CO_2 for 3 h and 40 min in 6-well culture plates[6] (12 explants/well) containing 1.5 mL of lysine-free or methionine-

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[1] Sigma Chemical Co., St. Louis, MO
[2] GIBCO, Grand Island, NY
[3] Cat. No. 600-524-AG, GIBCO, Grand Island, NY
[4] Cat. No. D4655, Sigma Chemical Co., St. Louis, MO
[5] Milli-Q Reagent Grade, Cat. No. ZD5211584, Millipore, Bedford, MA
[6] Cat. No. 08-772-1B, Fisher Scientific Product Corporation, Pittsburgh, PA

and lysine-free DMEM supplemented with 5 μg insulin[7], .5 μg hydrocortisone[8], 1 μg 17-ß-estradiol[9], and .1 μg prolactin[10]/mL medium.

Peptide Utilization. Following pre-incubation, the medium was removed and the explants pre-incubated in lysine-free DMEM were further incubated for 80 min in lysine-free DMEM containing all the hormones as in the pre-incubation medium, 1 μCi L-[4,5-^3H]leucine[11] (^3H-leucine)/mL medium, and L-lysine or one of the 23 lysyl peptides[1] (Table 5.1). Response to free lysine concentration was quantified by incubating tissue explants in media containing 0, 60, 120, 180, 240, and 300 μM L-lysine. The incubation was terminated by collecting the incubation medium. The explants then were blotted on paper towels and weighed. Based upon the results of the resulting response curve (Figure 5.1), the concentration of lysine, either in free or peptide form in the medium, was chosen to be 70 μM.

Peptide Hydrolysis. To examine if the peptides were hydrolyzed prior to uptake by the explants, explants pre-incubated in lysine-free DMEM were further incubated for 80 min in fresh pre-incubation medium. The incubation media were collected and pooled. One of the lysine-containing peptides (70 μM) was added to 1 mL of the conditioned medium and incubated for an additional 80 min. Following the incubation, free lysine in the medium was detected by HPLC as described previously (Chapter IV).

Effect of Bioactive Peptides. The tri- to octapeptides used in the present study have been reported to be bioactive in various tissues (Table 5.2). To examine whether these peptides had bioactive effects on mammary protein synthesis, mammary explants pre-incubated in a methionine- and lysine-free medium were incubated for

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[7] Cat. No. I6634, Sigma Chemical Co., St. Louis, MO
[8] Cat. No. H0888, Sigma Chemical Co., St. Louis, MO
[9] Cat. No. E2758, Sigma Chemical Co., St. Louis, MO
[10] Cat. No. NIDDK-oPRL-19, National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases, Baltimore, MD
[11] Cat. No. 20036, ICN Biomedicals, Inc., Irvine, CA

80 min in methionine- and lysine-free DMEM containing all the hormones as in the pre-incubation medium, ^{3}H-leucine, and 70 μM of L-lysine or one of the lysyl peptides, or 70 μM of L-lysine plus 70 μM of L-methionine.

Quantification of Secreted Proteins. Secreted proteins in the incubation medium were precipitated with 5% trichloroacetic acid overnight at 4°C followed by centrifugation at 16,000 *X* g. The resulting protein pellets were washed and the radioactivity of the ^{3}H-leucine labeled secreted proteins was quantified as described in Chapter IV.

Statistical Analysis. The data analysis procedure was the same as described previously (chapter IV). In summary, the general linear model procedure (SAS, 1990) was used to analyze the effects of different treatments. The means of different treatments were compared using Duncan's multiple range test at $\alpha = .05$.

Results

Effect of L-Lysine Concentration. Mammary tissue explants were incubated in media containing 0 to 300 μM of L-lysine to determine an optimal concentration of lysine substrate for peptide utilization experiments. The results showed that medium L-lysine concentration had an effect ($P < .001$) on the incorporation of ^{3}H-leucine into secreted proteins. However, the response showed a tendency towards a quadratic effect ($P < .056$). The incorporation of ^{3}H-leucine increased linearly over a range of L-lysine concentration from 0 to about 180 μM, and then was followed by a plateau (Figure 5.1).

Utilization of Di- and Tripeptides. Mammary explants were able to utilize lysine from all of the fifteen di- and tripeptides studied for the synthesis of secreted proteins (Figure 5.2A). These lysyl peptides generally were similar to free lysine in promoting

^3H-leucine incorporation into secreted proteins. The incorporation of ^3H-leucine promoted by glycylhistidyllysine was about 17% greater ($P < .05$) than that promoted by L-lysine. The other peptides were not different ($P > .05$) from L-lysine in promoting ^3H-leucine incorporation which ranged from 91 to 108% of the incorporation promoted by L-lysine. Within each of the three peptide pairs, aspartyllysine and lysylaspartate, glycyllysine and lysylglycine, valyllysine and lysylvaline, location of the lysyl residue at either the N- or C-terminal position did not affect ($P > .05$) ^3H-leucine incorporation (Figure 5.2A). Mammary explants incubated in lysine-free medium (negative control) also showed some incorporation of ^3H-leucine into secreted proteins (Figure 5.2A). But the incorporation rate was lower ($P < .05$) than the incorporation promoted by all of the peptides or by L-lysine.

All of the peptides were partially hydrolyzed in the conditioned medium after 80 min of incubation (Figure 5.2B). The extent of hydrolysis varied ($P < .0001$) among the peptides. However, no correlation ($r = .03$, $P < .93$) was detected between the rate of ^3H-leucine incorporation promoted by the peptides and the extent of peptide hydrolysis.

Utilization of Tetra- to Octapeptides. Mammary explants were able to utilize lysine from all of the oligopeptides studied except for the octapeptide (Figure 5.3A). Among the tetra- to hexapeptides, location of the lysyl residue at either the N- or C-terminal end or positioned in the middle of the chain appeared not to influence ($P > .05$) ^3H-leucine incorporation into secreted proteins. The ^3H-leucine incorporation rate with the octapeptide was similar ($P > .05$) to that in the negative control which was lower ($P < .05$) than the incorporation promoted by all other oligopeptides and by L-lysine.

Partial hydrolysis of all the oligopeptides in the conditioned medium was observed after 80 min incubation (Figure 5.3B). The extent of hydrolysis varied (P <

.0001) among the peptides, but it was not correlated ($r = .55$, $P < .16$) with the rate of ^{3}H-leucine incorporation promoted by the peptides.

Effect of Peptide Chain Length. There was an inverse relationship between the rate of ^{3}H-leucine incorporation promoted by lysine from the peptides and the number of amino acid residues in the peptides ($r = -.80$, $P < .057$; Figure 5.4).

Effect of Bioactive Peptides. In this study, mammary explants incubated in a methionine-free medium containing one of the peptides with various reported bioactivities produced lower ($P < .05$) ^{3}H-leucine incorporation into secreted proteins than did explants incubated in a methionine- and lysine-containing medium (Figure 5.5). All of the peptides, except glycylhistidyllysine, did not differ ($P > .05$) from the negative control (medium containing L-lysine but without methionine) in influencing ^{3}H-leucine incorporation in methionine-free medium. In the absence of medium methionine, glycylhistidyllysine promoted a greater ($P < .05$) ^{3}H-leucine incorporation than did other bioactive peptides and the negative control.

Discussion

In the present study, the incorporation of ^{3}H-leucine into secreted proteins increased linearly with increasing medium free lysine concentration up to 180 μM (Figure 5.1). The subsequent plateau could be due to the saturation of tissue ability to synthesize secretory proteins. To allow lysyl peptides be able to show their possible differences in promoting ^{3}H-leucine incorporation into secreted proteins, 70 μM was chosen as the concentration of lysine substrate for peptide utilization experiments.

The present results show that all of the lysyl peptides, except the octapeptide, are as efficient as free lysine in promoting the synthesis of secreted proteins. These results further indicate that mouse lactating mammary tissue, in addition to utilizing

methionyl peptides as sources of methionine (Chapter IV), also possesses the ability to utilize lysine bound in different peptides (di- to hexa-) for the synthesis of secreted proteins. Since all of the lysyl and methionyl peptides studied contain a wide range of other amino acids in the peptide chains, this implies that those peptide-bound amino acids also may be directly utilized as amino acid sources by mammary tissue. This speculation is supported by the results from *in vivo* studies by Steinhardt et al. (1984) and Grimble et al. (1988), who found that peptide mixtures as the sources of amino acids in total parenteral nutrition were as effective as the corresponding amino acid mixture in maintaining positive nitrogen balance in both baboons and man.

Partial hydrolysis of all peptides in conditioned medium was observed in the present study. However, the extent of hydrolysis could not account for the responses observed in the synthesis of ^{3}H-leucine labeled secreted proteins promoted by the peptides. Incorporation of ^{3}H-leucine into secreted proteins increased linearly with increasing free lysine concentration in the medium up to 180 μM (Figure 5.1). However, only part of the 70 μM of lysyl peptides was hydrolyzed after 80 min incubation in conditioned medium, but lysyl peptides promoted a similar or greater ^{3}H-leucine incorporation than did free lysine present at a concentration of 70 μM. Therefore, although utilization of free lysine released from hydrolyzed peptides could occur, the synthesis of ^{3}H-leucine labeled secreted proteins promoted by peptides was mainly due to the utilization of peptide-bound lysine.

Consistent with the results from the study of methionyl peptides (Chapter IV), the present results also show that dipeptides with the same amino acid composition, but differing only in lysine position (N- or C-terminal) have similar potencies as lysine sources for protein synthesis by mammary tissue. These results further confirm that lactating mouse mammary tissue, in utilizing peptides as amino acid sources, differs from cultured mouse myogenic cells and fibroblasts where protein accretion in both

of these cell types is influenced by the position of the needed amino acid in dipeptides (Fagle, 1955; Pan, 1993).

All of the tri- and longer peptides used in the present study have been reported to have various bioactivities in different tissues (Table 5.2). The experiment conducted with these peptides is based on the assumption that protein synthesis is limited by the absence of exogenous methionine because methionine is an essential amino acid. Therefore, if a bioactive peptide can stimulate protein synthesis in a methionine-free medium, this peptide has a bioactive effect on the process of protein synthesis separate from simply serving as a supply of lysine, and vice versa. In this experiment, all of these peptides, except glycylhistidyllysine, failed to stimulate the incorporation of ^{3}H-leucine into secreted proteins in a methionine-free medium, indicating that they do not have a bioactive effect on protein synthesis in mammary tissue explants under the present experimental conditions. Thus, the increased rate of ^{3}H-leucine incorporation promoted by these peptides in methionine-containing medium is due to the supply of the lysine source. Glycylhistidyllysine promoted a greater ^{3}H-leucine incorporation compared with the negative control and other bioactive peptides in a methionine-deficient medium, thus suggesting that this tripeptide can stimulate protein synthesis in a way other than or in addition to providing a source of lysine. However, the incorporation of ^{3}H-leucine promoted by glycylhistidyllysine in the methionine-free medium was lower than that in the complete medium (Figure 5.5), but this tripeptide promoted a greater incorporation of ^{3}H-leucine than did free lysine in methionine-containing medium (Figure 5.2A). These results suggest that the bioactive effect of glycylhistidyllysine on mammary protein synthesis may be minor compared to its ability to serve as a lysine source for protein synthesis under the present experimental conditions. Additionally, these results indicate that all of the peptides, except glycylhistidyllysine, had no bioactive

effect on the synthesis of secreted proteins by mammary explants in a methionine-free medium.

The evidence for mammary tissue being able to utilize bioactive peptides to meet its amino acid needs from the present study is in agreement with an earlier observation reported by Pocius et al. (1981). They observed a rapid removal of bioactive peptide, glutathione (τ-glutamylcysteinylglycine), from blood by the lactating mammary gland of cows. Since there is a shortage of cysteine and glutamate in blood for the synthesis of milk proteins (Clark et al., 1977, 1978), it was suggested that glutathione probably provided the amount of cysteine and glutamate needed. The present results and others indicate that peptides, including bioactive peptides, in the circulation can be directly utilized by mammary tissues as amino acid sources under *in vivo* conditions. It is known that blood contains different bioactive peptides with sizes ranging from di- to oligopeptides (Pickart and Thaler, 1979). Peptide-bound amino acids constitute about 65 to 78% of the blood plasma amino acid pool in ruminants and 52% in rats (McCormick and Webb, 1982; DiRienzo, 1990; Seal and Parker, 1991; Koeln et al., 1993). A large portion of the circulating peptide-bound amino acids are in oligo-form (Seal and Parker, 1991; Koeln et al., 1993) which may represent the bioactive group. Thus, animal tissues are living in an environment in which there is an easy access to peptide-bound amino acids.

In the present study, the rate of ^{3}H-leucine incorporation promoted by the peptides appears to decrease with increasing number of amino acid residues in the peptides. However, the overall differences among the peptides or between peptides and free lysine in promoting protein synthesis are not as pronounced as those observed in the comparisons among the methionyl peptides or between methionyl peptides and free methionine (Chapter IV). This may be due to different rates of uptake of peptide-bound lysine and uptake of peptide-bound methionine by the

mammary tissue. The outer surface of the plasma membrane is negatively charged. Lysine, bearing a positive charge at physiological pH, may bind non-specificially to the membrane, thus slowing the process of transporting lysine substrate across the cell membrane (McGivan et al., 1977). Another explanation may be the possible involvement of a relatively larger portion (compared with methionyl peptide study, Chapter IV) of lysine of intracellular origin in the synthesis of ^{3}H-leucine labeled proteins. The reutilization of intracellular lysine, in turn, may mask the variability of mammary tissue to utilize different extracellular lysine substrates for the synthesis of secreted proteins. The intracellular lysine may come from degraded, non-secreted milk proteins. It has been observed that small amounts of newly synthesized casein are degraded, even in the presence of prolactin which has been found to have a significant effect in preventing casein degradation, in mammary tissue from the goat in the middle stage of lactation (Wilde and Knight, 1986). Lysine content in milk proteins is about threefold higher than methionine (Clark et al., 1977). If milk protein degradation occurs in the mammary tissue, a much greater portion of lysine will be generated intracellularly than the amount of methionine generated. In a previous study (Chapter IV), the amount of secreted proteins produced in methionine-free medium (negative control) was about 60% of that produced in methionine-containing medium. The ratio of secreted proteins produced in lysine-free to lysine-containing media is about .70 even with increased pre-incubation time to deplete intracellular lysine. These results suggest that degradation of non-secreted milk proteins may occur under the present experimental conditions.

In conclusion, lactating mouse mammary tissue possesses the ability to utilize peptide-bound lysine for the synthesis of secreted proteins. A wide range of lysyl peptides, including different bioactive peptides, can serve as potential sources of lysine.

Implications

The present results show that a wide range of lysine-containing peptides can be used as lysine sources for the synthesis of secreted proteins by lactating mammary tissue. Since a large portion of blood lysine is present in peptide form in both ruminants and non-ruminant animals and since milk proteins are rich in lysine, the utilization of circulating peptide-bound lysine for milk protein synthesis may actually be the *in vivo* situation. The present results also showed that glycylhistidyllysine not only can serve as a lysine source for lactating mammary tissue but also may stimulate synthesis of secreted proteins through its bioactive effect on this tissue. If peptides can be protected from hydrolysis in the gastrointestinal tract and blood, supplementing this or other such peptides in the diet may result in increased milk protein production by the mammary gland.

Table 5.1. Lysine-containing peptides studied

Peptide	Abbreviation[a]
L-aspartyl-L-lysine	DK
L-lysine-L-aspartate	KD
glycyl-L-lysine	GK
L-lysyl-glycine	KG
L-valyl-L-lysine	VK
L-lysly-L-valine	KV
L-glutamyl-L-lysine	EK
L-arginyl-L-lysine	RK
L-lysyl-L-phenylalanine	KF
L-lysyl-L-lysine	KK
L-lysyl-L-leucine	KL
glycyl-L-histidyl-L-lysine	GHK
L-lysyl-L-tryptophyl-L-lysine	KWK
L-lysyl-L-tyrosyl-L-lysine	KYK
L-thronyl-L-seryl-L-lysine	TSK
Pro-Phe-Gly-Lys	PFGK
Val-Thr-Lys-Gly	VTKG
Asp-Leu-Trp-Gln-Lys	DLWQK
L-lysyl-L-glutamyl-L-glutamyl-L-alanyl-L-glutamic acid	KEEAE
Lys-Arg-Gln-His-Pro-Gly	KDQHPG
neuromedin N (Lys-Ile-Pro-Tyr-Ile-Leu)	KIPYIL
leucine enkephalin-Lys (Tyr-Gly-Gly-Phe-Leu-Lys)	YGGFLK
Val-His-Leu-Thr-Pro-Val-Glu-Lys	VHLTPVEK

[a] One-letter abbreviation for amino acids. A, alanine; D, aspartate; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine, L, leucine; P, proline; Q, glutamine; R, arginine; S, serine; T, thronine; V, valine; W, tryptophan; Y, tyrosine.

Table 5.2. Reported bioactivity of the lysine-containing peptides studied

Peptide[a]	Bioactivity	Reference
GHK	liver cell growth factor	Pickart et al., 1973, 1979
KWK	binds to DNA	Maurizot et al., 1978
	induce nicks in phage circular DNA	Pierre and Laval, 1981
KYK	induce nicks in phage circular DNA	Pierre and Laval, 1981
TSK	antireproductive	Sigma Chemical Co.
PFGK	uncertain	Sigma Chemical Co.
VTKG	uncertain	Sigma Chemical Co.
DLWQK	uncertain	Sigma Chemical Co.
KEEAE	thymosin $_{-1}$ fragment 23-27	Sigma Chemical Co.
KDQHPG	thyrotrophin-releasing hormone	
	precursor peptide fragment 1-6	Jackson et al., 1985
KIPYIL	neurotensin like peptide	Sigma Chemical Co.
YGGFLK	opiate agonist activity	Sigma Chemical Co.
VHLTPVEK	N-terminal region of ß-chain	
	of sickle-cell hemoglobin	Ingram, 1957

[a] One-letter abbreviation for amino acids. A, alanine; D, aspartate; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine, L, leucine; P, proline; Q, glutamine; S, serine; T, thronine; V, valine; W, tryptophan; Y, tyrosine.

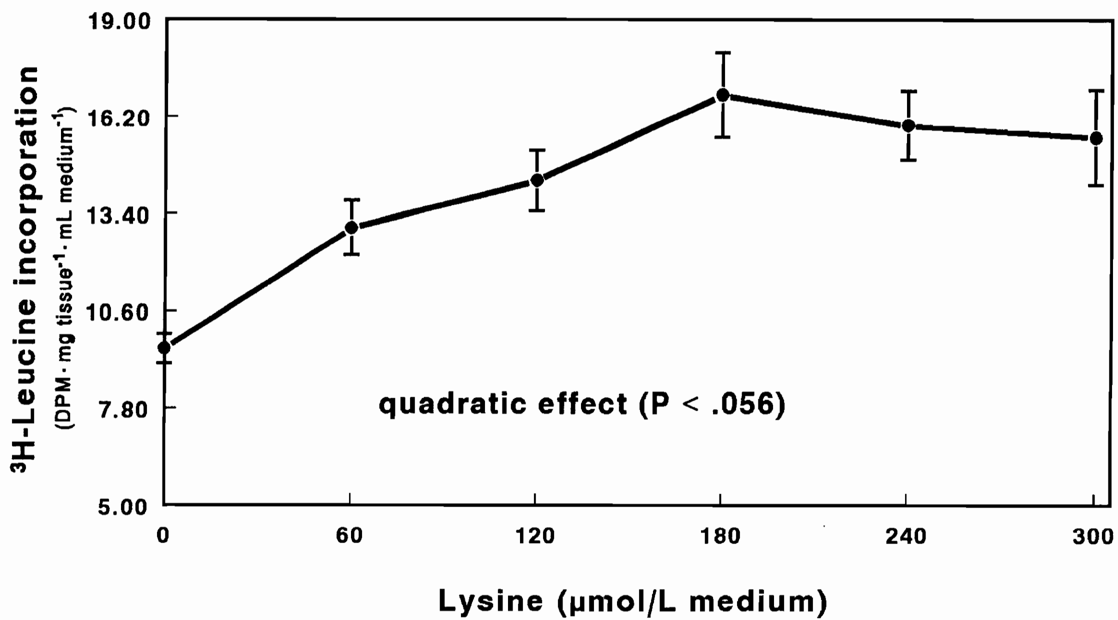

Figure 5.1. Incorporation of ^{3}H-leucine into secreted proteins by mammary explants from lactating mice promoted by different concentrations of L-lysine. Values represent means ± SE (n = 6).

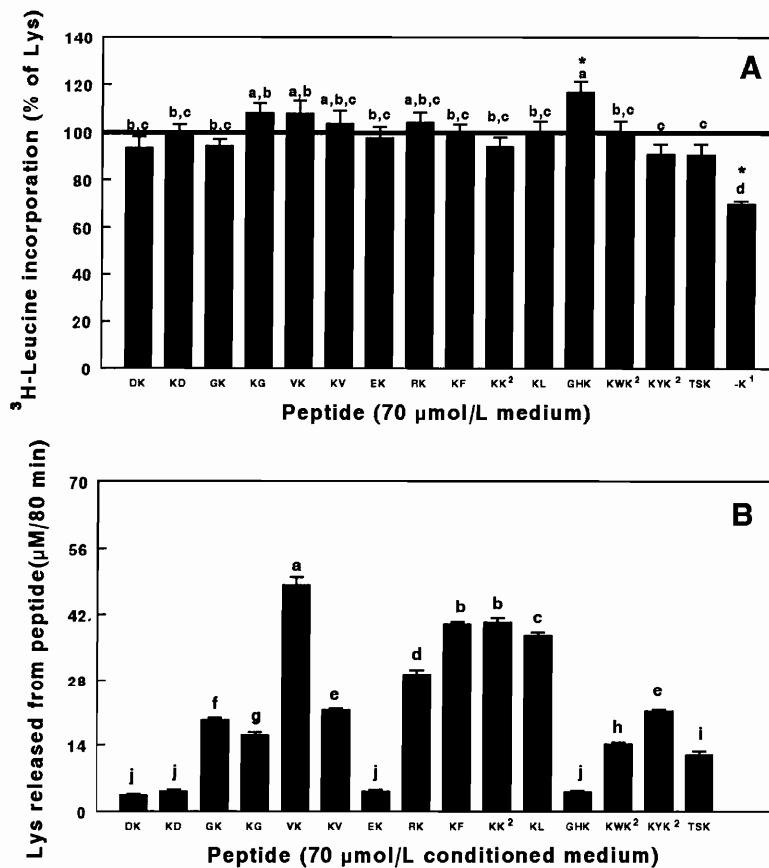

Figure 5.2. A. Incorporation of ^{3}H-leucine into secreted proteins promoted by lysine-containing di- and tripeptides by mammary explants from lactating mice. Values represent means ± SE (n = 12, two repeated experiments) which are expressed as the percentage of ^{3}H-leucine incorporation promoted by L-lysine. The horizontal line indicates the amount of ^{3}H-leucine incorporation promoted by L-lysine. Bars lacking a common letter differ (P < .05). Means (*) differ (P < .05) from L-lysine treatment. [1] -K, no lysine substrate in the incubation medium (negative control). [2] KK, KWK, KYK, 35 μmol/L medium. B. Hydrolysis of lysine-containing di- and tripeptides in conditioned medium. Values represent means ± SE (n = 6).

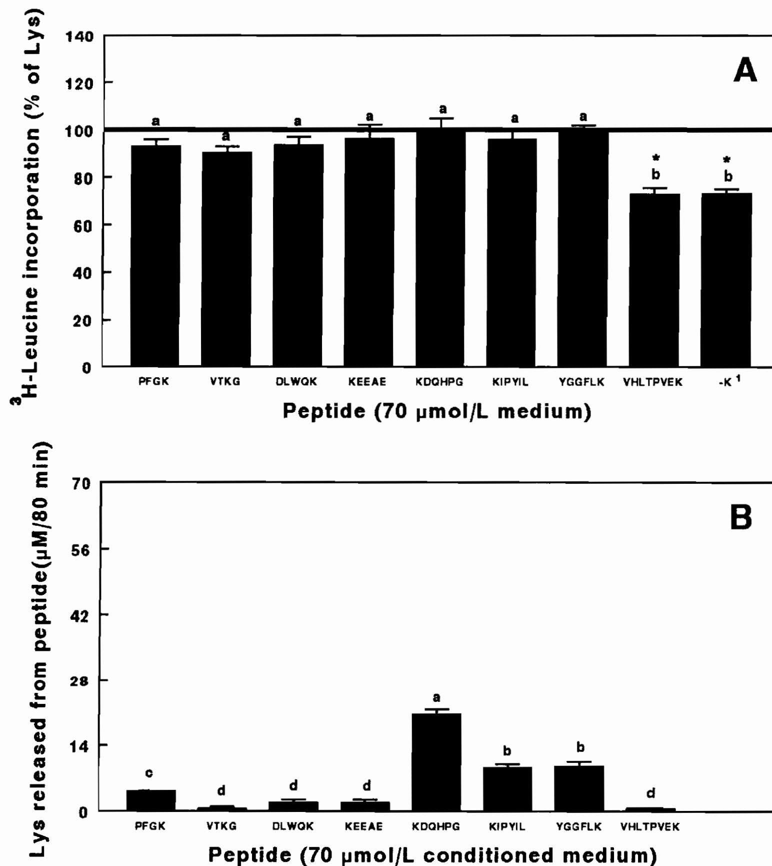

Figure 5.3. A. Incorporation of ^{3}H-leucine into secreted proteins promoted by lysine-containing tetra- to octapeptides by mammary explants from lactating mice. Values represent means $\pm$ SE (n = 12, two repeated experiments) which are expressed as the percentage of ^{3}H-leucine incorporation promoted by L-lysine. The horizontal line indicates the amount of ^{3}H-leucine incorporation promoted by L-lysine. Bars lacking a common letter differ ($P < .05$). Means (*) differ ($P < .05$) from L-lysine treatment. [1] -K, no lysine substrate in the incubation medium (negative control). B. Hydrolysis of lysine-containing tetra- to octapeptides in conditioned medium. Values represent means $\pm$ SE (n = 6).

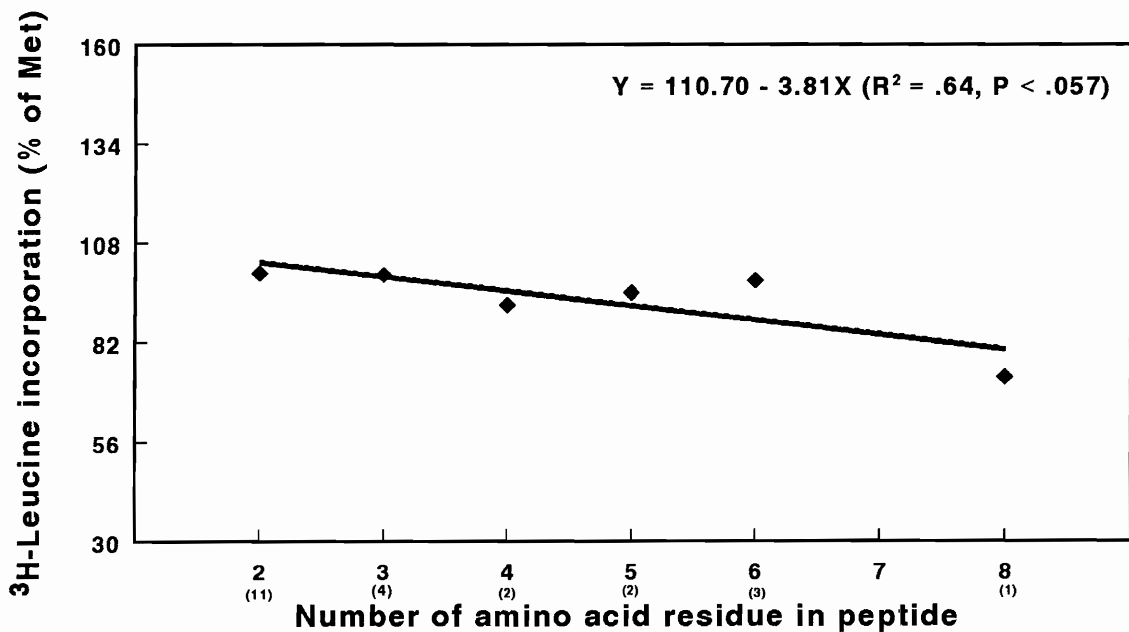

Figure 5.4. Relationship between rate of ^{3}H-leucine incorporation promoted by lysine-containing peptides and the number of amino acid residues in the peptides.
[a] Numbers in parentheses are the number of peptides of each chain length evaluated and for each peptide n = 12.

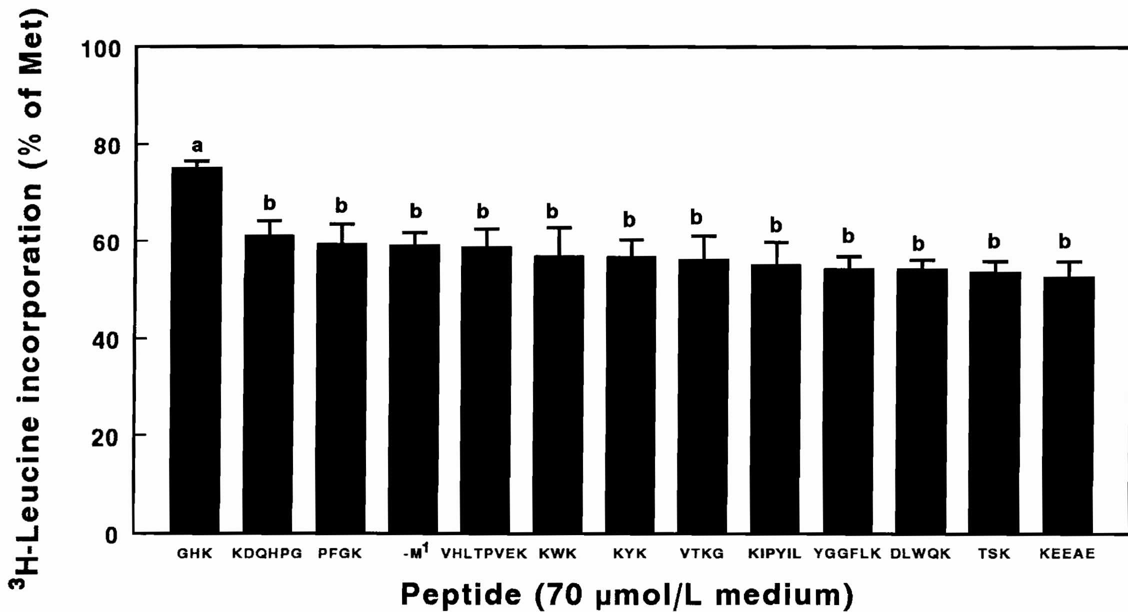

Figure 5.5. Effect of lysine-containing peptides known to be bioactive on ^{3}H-leucine incorporation into secreted proteins in a methionine-free medium. Values represent means ± SE (n = 6) which are expressed as percentage of ^{3}H-leucine incorporation promoted by L-methionine. All means are lower ($P < .05$) than the methionine treatment. Bars lacking a common letter differ ($P < .05$). [1] -M, no methionine but with 70 μM L-lysine in the incubation medium (negative control).

Chapter VI

UTILIZATION OF PEPTIDE-BOUND METHIONINE FOR THE SYNTHESIS OF SECRETED PROTEINS BY CULTURED BOVINE MAMMARY EPITHELIAL CELLS

ABSTRACT

A bovine mammary epithelial cell line (MAC-T) was used to study the ability of 17 methionine-containing di- and tripeptides to substitute for free methionine in the synthesis of secreted proteins. MAC-T cells were plated at 100,000 cells/well (24-well plate) and allowed to grow for 3 or 8 d. The cells were then incubated in methionine-free Dulbecco's modified Eagle's medium supplemented with hormones, ^{3}H-leucine, and L-methionine or one of the methionyl peptides for 3, 6, or 24 h. The ability of methionine substrates to promote incorporation of ^{3}H-leucine into secreted and cell proteins was quantified. Effect on cell proliferation was determined by measuring DNA content. Cells were able to utilize methionine from all peptides tested. Most peptides were as efficient as free methionine in promoting ^{3}H-leucine incorporation into proteins. Ability of the peptides to promote protein synthesis varied with incubation time. Generally, differences among peptides or between peptides and free methionine were greater at 3 and 6 h of incubation compared with 24 h of incubation. For cells allowed to grow for 3 or 8 d, incorporation of ^{3}H-leucine into secreted proteins promoted by peptides after 3 h incubation ranged from 58 to 85% and 65 to 110% of the incorporation promoted by free methionine, respectively. Peptides did not influence the proliferation of cells in culture for 8 d. The results suggest that di- and tripeptide-bound methionine can serve as a source of methionine

for the synthesis of secreted proteins by MAC-T cells. The cells with longer growth period (8 vs 3 d) appear to have a greater ability to utilize the peptides.

(Key Words: Mammary, Cell Culture, Peptide, Amino Acids, Bovine)

Introduction

Peptide-bound amino acids comprise an important part of the blood amino acid pool in ruminants. Studies have shown that about 65 to 78% of total arterial plasma amino acids are in peptide form in calves and sheep (McCormick and Webb, 1982; Danilson et al., 1987a; Seal and Parker, 1991; Koeln et al., 1993). A relatively large portion of the circulating peptides may originate from dietary proteins (DiRienzo, 1990; Seal and Parker, 1991; Koeln et al., 1993). In addition to the small intestine, the forestomach and large intestine of ruminants also possess the ability to transport intact peptides across the mucosa into the circulation (DiRienzo, 1990; Matthews, 1991; Seal and Parker, 1993). Thus, peptide absorption may constitute the primary source of absorbed amino acids in ruminants and circulating peptide-bound amino acids are probably an important amino acid supply for the tissues.

Mammary tissue is a major consumer of amino acids in lactating animals. It has been observed that several types of free amino acids in blood plasma are present in quantities that are insufficient to account for their output in milk proteins in cows (Bickerstaffe et al., 1974; Clark et al., 1977, 1978). This indicates that other circulating forms of amino acids, including possibly peptide-bound amino acids, may at least be supplementary sources of amino acids for milk protein synthesis in these animals. However, no direct evidence regarding nutritional utilization of peptides by the bovine mammary gland is available. Therefore, the present study was designed to examine the ability of a wide range of methionyl peptides to serve as sources of

methionine for the synthesis of secreted and cell proteins by a bovine mammary epithelial cell line (MAC-T). The differentiation of MAC-T cells was stimulated by prolactin and the synthesis of ß-casein mRNA by these cells appeared to be stimulated by increasing cell culture time in the presence of prolactin (Huynh et al., 1991). Therefore, the present study was also designed to examine whether the cells grown in media supplemented with lactogenic hormones for different times had different ability to utilize these methionine-containing peptides.

Materials and Methods

Media and Buffer. Dulbecco's modified Eagle's medium (DMEM) was prepared by combining 3.7 g of sodium bicarbonate[1], 10 mL of antibiotic-antimycotic solution[2], 1 mL of gentamicin reagent solution[3], and 1 pack (1 L package) of DMEM powder[4] in an 1 L volumetric flask. The mixture was dissolved in deionized water prepared using a water purification system[5], and diluted to volume. Methionine- and leucine-free DMEM was prepared by combining 584 mg of L-glutamine[6], 148 mg of L-lysine · HCl[6], 3.7 g of sodium bicarbonate, 10 mL of antibiotic-antimycotic solution, 1 mL of gentamicin reagent solution and 1 pack (9.2 g) of deficient DMEM powder[7] in an 1 L volumetric flask and diluted to volume with deionized water. Dulbecco's phosphate buffered saline (DPBS) was prepared by adding deionized water to DPBS powder[8] (without including the calcium package) to a volume of 1 L. All of the prepared solutions were adjusted to pH 7.18 with 1 N HCl and sterilized by filtration

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[1] GIBCO, Grand Island, NY
[2] Cat. No. 600-5240AG, GIBCO, Grand Island, NY
[3] Cat. No. 600-5710AD, GIBCO, Grand Island, NY
[4] Cat. No. 430-2800EB, GIBCO, Grand Island, NY
[5] Cat. No. ZD5211584, Millipore, Bedford, NY
[6] Sigma Chemical Co., St. Louis, MO
[7] Cat. No. D4655, Sigma Chemical Co., St. Louis, MO
[8] Cat. No. 450-1500EB, GIBCO, Grand Island, NY

with a .20 μm membrane filter unit[9]. The sterilized media and buffer had a pH of about 7.35.

Cell Growth. Bovine mammary epithelial cells (MAC-T) were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The cells were plated at 100,000 cells/well (24-well plate[10]) containing 1 mL of DMEM supplemented with 10% fetal bovine serum[11] (FBS) and placed in the incubator overnight to allow the cells to attach to the plate. After seeding, the medium was discarded and each well was washed with 1 mL of DPBS. Then the cells were allowed to grow in 1 mL of DMEM containing 1 μg insulin[12] and 1 μg prolactin[13]/mL medium, .1 μg dexamethasone[14], and 5% of FBS. The growth DMEM was changed at 24-h intervals.

Peptide Utilization. After growth, the medium was removed and each well was washed with DPBS. The cells were then pre-incubated for 24 h in methionine- and leucine-free DMEM supplemented with all the hormones as in the growth DMEM plus .8 mM leucine[6] to deplete intracellular methionine. Following pre-incubation, the medium was replaced by DPBS to wash the cells. The cells were then incubated in methionine- and leucine-free DMEM supplemented with all the hormones as in the growth DMEM, .2 mM L-leucine, 1 μCi L-[4,5-^{3}H]leucine[15] (^{3}H-leucine)/well, and 6 ĭM of L-methionine[6] or one of the 17 methionyl peptides[6] (Table 6.1). All of the methionine-containing dipeptides (in pairs with methionine at either the N- or C-terminal position) and tripeptides, which were composed of L-α-amino acids and which were available from Sigma Chemical Co., were examined. The 6 μM concentration of methionine substrates and the 24-h pre-incubation time were chosen

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[9] Cat. No. 09-740-24A, Fisher Scientific Product Corporation, Pittsburgh, PA
[10] Cat. No. 3047, Becton Dickinson Company, Lincoln Park, NJ
[11] Cat. No. 200-6140AJ, GIBCO, Grand Island, NY
[12] Cat. No. I6634, Sigma Chemical Co., St. Louis, MO
[13] Bovine prolactin (NIDDK-bPRL), National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases, Baltimore, MD
[14] Cat. No. D1756, Sigma Chemical Co., St. Louis, MO
[15] Cat. No. TRK.683, Amersham International Plc., Buckinghamshire, England

based upon the response of the incorporation of ^{3}H-leucine into proteins to free methionine concentration in the medium (Figure 6.1) in a preliminary experiment. This preliminary experiment was conducted by incubating cells that had grown for 8 d in media containing 0, 3.125, 6.25, 12.5, 25, 50, 100, or 200 μM L-methionine for 24, 48, or 72 h.

Cell Growth and Incubation Time Comparison. The effect of cell growth was evaluated by measuring ^{3}H-leucine incorporation into secreted and cellular proteins by cells that were allowed to grow for 3 or 8 d. The previously grown cells were incubated with the various substrates for 3 h. The effect of length of incubation time was evaluated by allowing cells to grow for 8 d then incubating them with the various substrates for 3, 6, or 24 h.

Peptide Hydrolysis. The potential for peptide hydrolysis prior to uptake by the cells was estimated. The 8-d cells were pre-incubated for 24 h. The cells, after washing with DPBS, were incubated for another 3 h in fresh pre-incubation DMEM supplemented with 6 μM L-methionine. The incubation media then were collected and pooled. Each of the methionyl peptides (6 μM) was added to 1 mL of the conditioned medium. The conditioned medium with or without the peptides was incubated for an additional 3 h. After the incubation, free methionine in the medium was measured by HPLC as described in Chapter IV. The amount of free methionine found in the conditioned medium incubated without any of the peptides was considered as background and was subtracted from the quantity of methionine observed following incubation of peptides in the conditioned medium.

Harvest of Cells and DNA Analysis. Following incubation, the cells were washed with DPBS and dissociated from the plate by incubation at 37°C with .5 mL of .25% trypsin[16]. Subsequently, the trypsin activity was stopped by adding .5 mL of

[16] Cat. No. 610-5095AE, GIBCO, Grand Island, NY

double-concentrated PBS (.1 M Na_2HPO_4, 4 M NaCl, and .004 M $Na_2EDTA\text{-}2H_2O$) to each well. The cell solution was sonicated for 30 s using a sonic dismembrator[17]. An aliquot of the sonicated cell solution was used for determining DNA content as a measure of cell proliferation and the remaining cell solution was used for quantifying ^{3}H-leucine labeled proteins in the cells. The DNA content was determined using a fluorometric method (Labarca and Paigen, 1980). Each sample contained 1,850 μL of PBS, 50 μL of sonicated cell solution, and 100 μL of .002% fluorochrome[18]. The fluorescence of the samples was immediately measured using a fluorometer[19]. The DNA content of each unknown sample was calculated according to a standard curve which was obtained by measuring the fluorescence of a series of known DNA concentrations prepared with calf thymus DNA[20].

Quantification of Secreted and Cell Proteins. ^{3}H-leucine labeled, secreted proteins in the incubation medium and proteins in the sonicated cell solution were precipitated with 5% trichloroacetic acid and quantified by liquid scintillation counting as described previously (Chapter IV).

Statistical Analysis. The effects of L-methionine concentration and peptides on promoting ^{3}H-leucine incorporation into proteins was analyzed by the general linear model procedure (SAS, 1990). Means of different peptide treatments were compared using Duncan's multiple range test at $\alpha = .05$ (SAS, 1990). The paired t test (Kleinbaum et al., 1987) was applied to analyze the difference of ^{3}H-leucine labeled proteins per unit of DNA promoted by peptides between cells grown for 3 and 8 d.

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[17] Model 300, Fisher Scientific Product Corporation, Pittsburg, PA
[18] Hoechst 33258, Cat No. B2883, Sigma Chemical Co., St Louis, MO
[19] TKO 100 DNA Fluorometer, Hoefer Scientific Instruments, San Francisco, CA
[20] Cat. No. D1501, Sigma Chemical Co., St. Louis, MO

Results

The ^{3}H-leucine labeled proteins in the cells contained non-secreted proteins and structural proteins. Here, it is defined as "cell proteins".

Effect of L-Methionine Concentration and Pre-incubation Time. MAC-T cells, grown for 8 d and starved of methionine for 24, 48, or 72 h, were incubated in media containing 0 to 200 μM of L-methionine for 24 h. After 24, 48, or 72 h of starvation, medium methionine concentration influenced ($P < .01$) the incorporation of ^{3}H-leucine into both secreted and cell proteins (Figure 6.1A). Incorporation of ^{3}H-leucine into secreted proteins increased with increasing medium L-methionine concentration up to about 12.5 to 25 μM and then plateaued as indicated by the quadratic effect ($P < .01$). A similar response (quadratic effect, $P < .01$) was observed for the incorporation of ^{3}H-leucine into cell proteins (Figure 6.1).

The rate of ^{3}H-leucine incorporation into the secreted proteins promoted by different concentrations of L-methionine was about 24 to 46% greater than the negative control in cells starved of methionine for 24 h (Table 6.2). Extending the starvation time to 48 or 72 h did not change the response pattern of ^{3}H-leucine incorporation (Figures 6.1B and 6.1C). Moreover, increasing starvation time to 48 or 72 h did not improve ^{3}H-leucine incorporation rate compared with cells starved for 24 h (Table 6.1). In fact, cell numbers were reduced approximately one-half by increasing starvation time from 24 to 72 h (Figure 6.2). The L-methionine concentration appeared to have no influence ($P < .01$) on cell proliferation as indicated by the similar DNA contents of cells harvested from medium containing different concentrations of methionine (Figure 6.2).

Effect of Cell Growth Time. The number of the cells resulting from a growth period of 3 d was only about two-thirds of the number of cells that resulted from an 8-

d growth period as indicated by DNA contents (Table 6.3). Cell number differences resulted in a difference in the total amount of ^{3}H-leucine incorporation into proteins at the two growth times. Therefore, the ability of peptides to promote ^{3}H-leucine incorporation in the 3- and 8-d cells was compared, in terms of response patterns, with the relative incorporation promoted by free methionine, and on per unit of DNA basis.

Methionine substrates had an effect ($P < .0003$) on the DNA content of cells allowed to grow for 3 d. Only one peptide, methionylmethionine, stimulated an increased number of cells compared with the negative control (Table 6.3). The number of cells incubated with all other peptides and free methionine was not different ($P > .05$) from that of the negative control. In 8-d cells, the amount of DNA varied among substrates but none of the differences were statistically significant (Table 6.3).

Cells with different growth times showed differences in the pattern of response to the methionine substrates. When cells were allowed to grow for 3 d and then incubated with peptides for 3 h, the incorporation of ^{3}H-leucine into secreted proteins promoted by all of the peptides, except 6 μM methionylmethionine, was lower ($P < .05$) than that promoted by L-methionine (Figure 6.3A). Among these peptides, glycylmethionine, prolylmethionine, and methionylleucylphenylalanine were not different ($P > .05$) from the negative control in promoting ^{3}H-leucine incorporation into secreted proteins. However, when cells were allowed to grow for 8 d prior to being incubated with peptides for 3 h, ^{3}H-leucine incorporation into secreted proteins promoted by all of the peptides, except methionylproline, prolylmethionine, and methionylalanine, was either the same ($P > .05$) or greater ($P < .05$) than the incorporation promoted by L-methionine (Figure 6.4A). Only one peptide, methionylproline, showed no difference ($P > .05$) from the negative control

in promoting ^{3}H-leucine incorporation into secreted proteins. For cells allowed to grow for 3 or 8 d, incorporation of ^{3}H-leucine into secreted proteins promoted by peptides ranged from 58 to 85% and 65 to 110% of the incorporation promoted by an equal molar amount of free methionine, respectively.

When the incorporation of ^{3}H-leucine into secreted proteins was expressed as a percentage of the incorporation promoted by L-methionine, all of the peptides, except methionylproline, showed a greater ($P < .05$) ability for promoting ^{3}H-leucine incorporation in 8-d cells than in 3-d cells (Figure 6.5).

By using the paired-T test it was observed that the total amount of ^{3}H-leucine labeled proteins per unit of DNA promoted by all of the peptides, except methionylphenylalanine, was greater ($P < .0005$) in 8-d cells than in 3-d cells (Table 6.3). However, with individual T tests, only eight of the peptides promoted a greater ($P < .05$) ^{3}H-leucine incorporation per unit DNA in 8-d cells compared with the 3-d cells (Table 6.3).

The incorporation of ^{3}H-leucine into cell proteins promoted by the different methionine substrates showed a response similar to the response of the corresponding secreted proteins in both 3- and 8-d cells (Figures 6.3B, 6.4B). The incorporation rate, however, was about 9- and 11-fold greater in cell proteins than in secreted proteins in 3- and 8-d cells, respectively.

Effect of Incubation Time. All of the cells used in these comparisons were allowed to grow for 8 d prior to incubation with methionyl peptides for 3, 6, or 24 h. The response patterns and the ability of peptides to promote ^{3}H-leucine incorporation into secreted proteins, relative to free methionine, varied with incubation time.

Several difference were observed. First, relative differences among peptides or between peptides and free methionine in promoting ^{3}H-leucine incorporation,

generally, appeared to be greater in cells incubated with peptides for 3 or 6 h (Figures 6.4A, 6.6A) than in cells incubated for 24 h (Figure 6.7A). The range of ^{3}H-leucine incorporation into secreted proteins promoted by peptides was about 65 to 110%, 83 to 127%, and 86 to 100% of the incorporation promoted by an equal molar amount of free methionine in cells incubated with peptides for 3, 6, and 24 h, respectively. Second, methionylproline was not different ($P > .05$) from the negative control in promoting ^{3}H-leucine incorporation into secreted proteins during 3 or 6 h of incubation (Figures 6.4A, 6.6A). This dipeptide, however, promoted a greater ($P < .05$) ^{3}H-leucine incorporation into secreted proteins than did the negative control after 24 h of incubation (Figure 6.7A).

When the incorporation of ^{3}H-leucine into secreted proteins promoted by methionyl peptides was expressed as a percentage of the incorporation promoted by L-methionine, a greater ($P < .05$) ^{3}H-leucine incorporation was observed for about half of the peptides for the 3 and 6 h incubation compared with cells incubated for 24 h (Figure 6.8).

The pattern of ^{3}H-leucine incorporation in response to different methionine substrates between cell proteins and the corresponding secreted proteins was similar for all of the three incubation times (Figures 6.4B, 6.6B, and 6.7B). However, the incorporation rate in the cell proteins was about 11-, 9-, and 5-fold greater than that in corresponding secreted proteins after 3, 6, or 24 h of incubation, respectively. The DNA contents of cells (data not shown) incubated with different methionine substrates were not different ($P > .05$) from the negative control in all of the three incubation times.

Peptide Hydrolysis. All of the peptides were partially hydrolyzed in conditioned medium after 3 h of incubation (Table 6.2). The extent of hydrolysis varied ($P < .0001$) among peptides. No correlation ($r = .38$, $P < .13$) was found between the rate

of ^{3}H-leucine incorporation into secreted proteins promoted by the peptides and the degree of peptide hydrolysis under the same experimental conditions.

Discussion

In the present study, medium free methionine concentration had a quadratic effect on the incorporation of ^{3}H-leucine into both secreted and cell proteins. A maximum synthesis of ^{3}H-leucine labeled proteins was achieved at about 12 μM free methionine concentration, which is likely indicative of the upper limit of cells ability for protein synthesis under the present experimental conditions. Therefore, 6 μM was chosen as the concentration of methionine substrate for subsequent experiments so that each methionyl peptide would have the opportunity to completely express the ability to serve as a methionine source. Because increasing cell starvation time for methionine from 24 to 72 h did not improve the rate of ^{3}H-leucine incorporation into proteins promoted by free methionine, but increasing starvation time caused a decrease in the number of living cells, subsequent experiments were designed to pre-incubate cells for 24 h to deplete intracelluar methionine.

All of the 17 methionyl di- and tripeptides were utilized by bovine mammary epithelial cells as methionine sources for the synthesis of secreted and cell proteins. The ^{3}H-leucine labeled cell proteins, generally, showed a similar response pattern as the secreted proteins to methionine substrates. It also was observed that, when incubation time increased from 3 to 24 h, the ratio of ^{3}H-leucine labeled secreted proteins to cell proteins decreased from 1:11 to 1:5, indicating that a large portion of the cell proteins are proteins awaiting secretion. Most of the methionine-containing peptides were as efficient as free methionine in promoting protein synthesis. These results are consistent with our previous results (Chapters IV and V) which revealed

that a wide range of methionyl and lysyl peptides can substitute for free methionine and lysine for the synthesis of secreted proteins by lactating mouse mammary tissue. The ability of ruminant mammary epithelial cells to utilize peptide-bound amino acids for protein synthesis also is supported by a recent *in vivo* study which shows that the mammary gland of goats can utilize intravenously infused glycylleucine and phenylalanylleucine as a source of leucine for the synthesis of milk casein (Backwell et al., 1994).

It has been observed that the relative concentration of blood plasma free methionine to total plasma amino acids is only about half of its relative concentration in milk proteins (Clark et al., 1978). The ratio of uptake of free methionine from blood plasma to its output into milk proteins ranges from .55 to .93 in cows of different breeds with various daily milk production (Bickerstaffe et al., 1974). These results suggest that other blood methionine sources may also contribute to milk protein synthesis. Recently, results from some studies show that about 36 to 83% and 94% of total arterial plasma methionine is bound as peptides in calves and sheep, respectively (Seal and Parker, 1991; Koeln et al., 1993). These results suggest that, *in vivo*, methionyl peptides, in addition to free methionine, may actually be a source of methionine for milk protein synthesis by ruminant animals.

All of the peptides studied in the present study were partially hydrolyzed in the conditioned medium after 3 h of incubation. However, the extent of peptide hydrolysis did not account completely for the responses observed in the synthesis of ^{3}H-leucine labeled proteins when methionyl peptides were used as methionine sources. This conclusion is reached based on the following reasons. First, there was no correlation between the rate of ^{3}H-leucine incorporation into proteins promoted by the peptides and the extent of peptide hydrolysis. Second, it has been observed that ^{3}H-leucine incorporation into proteins increased linearly with increasing medium

free methionine concentration up to about 12 μM. Thus, methionine released from partial hydrolysis of 6 μM peptides should not stimulate a similar or even greater ^{3}H-leucine incorporation than 6 μM free methionine. The partial hydrolysis of peptides may be caused by the activity of cytosolic peptidases which were secreted with milk proteins (Jenness, 1974).

Cell growth time had an effect on the utilization of peptide-bound methionine for the synthesis of proteins in the present study. Cells cultured for 8 d prior to incubation with peptides showed a relatively greater ability to utilize peptide-bound methionine for protein synthesis compared with cells cultured for 3 d. These differences may be related to cell maturation status. It has been observed that the lactogenic hormones, prolactin, cortisone, and insulin, can stimulate mammary epithelial cell differentiation and initiation of mammary function, producing α-lactalbumin (Ray et al., 1981). The production of α-lactalbumin increased with increasing cell culture time in the presence of lactogenic hormones. Results from another study also show that lactogenic hormones can stimulate the differentiation of mammary tissue from cows in early pregnancy (Collier et al., 1977). MAC-T cells retain their ability to differentiate and secrete milk specific proteins (Huynh et al., 1991). Under the present experimental conditions, cells cultured with the lactogenic hormones for 8 d could be in a more differentiated stage than cells cultured for 3 d. The more differentiated cells (8 d) may possess a more fully developed process for taking up peptide-bound methionine compared with the less differentiated cells (3 d). It has been observed that the mechanism and capacity of transport of some nutrients changes with the development of tissues. For example, the transport of bile salts and calcium in sucking rats occurs by a diffusion process, but it changes to active transport in adult animals (Ghishan et al., 1980; Little and Lester, 1980; Barnard et al., 1985). Results from a recent study show that, in cultured intestinal epithelial cells, the

expression of neutral endopeptidase and dipeptidylpeptidase IV, which are abundant in the brush border membranes of intestine and kidney and may be involved in the uptake of peptide-bound amino acids, is positively correlated with the degree of cell differentiation (Jalal et al., 1992).

The present results show that incubation time influence the rate of ^{3}H-leucine incorporation into proteins. Most of the peptides promoted up to about 27% greater ^{3}H-leucine incorporation into secreted proteins than did free methionine after 3 or 6 h of incubation. In comparison, peptides incubated for 24 h showed a much smaller difference from free methionine in promoting ^{3}H-leucine incorporation into proteins. One explanation for the higher incorporation rate promoted by most of the peptides compared with free methionine after 3 or 6 h of incubation may be due to the more efficient uptake of peptide-bound methionine than free methionine. It is well known now that mediated peptide transport differs from amino acid carrier systems (Ganapathy and Radhahrisknan, 1980; Matthews, 1991). Some peptides are absorbed more rapidly than their corresponding free amino acids in the small intestine (Matthews et al., 1968; Matthews, 1975). Thus, in a relatively short incubation period, the amount of ^{3}H-leucine labeled proteins synthesized will greatly depend on the speed of methionine uptake either in free or peptide form. If the speed of uptake of methionine substrates exceeds the speed of protein synthesis, methionine substrates of extracellular origin will accumulate in the cytoplasm as incubation time increases. At that point, differences in rate of uptake of peptide-bound methionine and free methionine will not influence the rate of ^{3}H-leucine incorporation into proteins. This may explain the relative consistency in ^{3}H-leucine incorporation promoted by most of peptides and free methionine after 24 h of incubation.

Generally, differences among peptides in promoting ^{3}H-leucine incorporation into proteins also were greater at 3 and 6 h of incubation compared with 24 h of

incubation. This could be caused by different efficiencies of uptake of these peptides by the mammary epithelial cells. Different peptide transport mechanisms have been recognized (Rosen-Levin et al., 1980; Bertloot et al., 1981; Ganapathy and Leibach, 1983; Ugolev et al., 1990). Also, peptides sharing the same transport process may be transported with differing efficiencies (Daniel et al., 1992). However, differences among these methionyl peptides in promoting ^{3}H-leucine incorporation diminished after increasing incubation time to 24 h. This may also be explained by the same rationale presented in the previous paragraph.

The proliferation of cells allowed to grow for 8 d was not influenced by different methionine substrates. This may have resulted from two or more possible reasons. First, cells on plastic can only grow into a monolayer. The cell DNA content increased about 18-fold after 8 d in culture. Thus, there may be no extra space for cell growth. Second, differentiated cells usually have limited ability for proliferation. If these 8-d cell cultures were in an advanced stage of differentiation compared with 3-d cell cultures, we may not expect to see distinct cell growth activity. In cells allowed to grow for 3 d, although only one methionyl peptide stimulated a statistically greater DNA content compared with negative control, there was a tendency for differences in DNA content to exist among different treatments. We may see a more clear effect of different methionine substrates on cell proliferation with increasing incubation time.

In conclusion, cultured bovine mammary epithelial cells can utilize methionine bound in the form of a wide range of di- and tripeptides for the synthesis of secreted proteins. Cells with longer growth time appear to have a greater ability to utilize peptide-bound methionine, indicating that this ability of cells may develop with cell differentiation.

Implications

The present results show that a wide range of small peptide-bound methionine can be utilized as efficiently as free methionine for the synthesis of secreted proteins by mammary epithelial cells. Since small peptides are usually absorbed more rapid than the corresponding free amino acids, supplementation of methionine needs for dairy animals can be accomplished by supplying methionine in small peptide form which may result in increased milk protein production by the mammary gland.

Table 6.1. Hydrolysis of methionine-containing peptides in conditioned medium

Peptide (6 μM)	Abbreviation	Methionine released[1] (μM/3 h)
L-alanyl-L-methionine	AM	2.3 ± .25[e,f,g]
L-methionyl-L-alanine	MA	1.8 ± .18[f,g]
L-phenylalanyl-L-methionine	FM	2.9 ± .16[c,d,e,f]
L-methionyl-L-phenylalanine	MF	3.2 ± .25[c,d,e]
Glycyl-L-methionine	GM	.5 ± .23[h,i]
L-methionyl-glycine	MG	2.0 ± .19[f,g]
L-leucyl-L-methionine	LM	3.9 ± .44[c]
L-methionyl-L-leucine	ML	3.8 ± .38[c,d]
L-prolyl-L-methionine	PM	5.1 ± .39[b]
L-methionyl-L-proline	MP	.2 ± .24[i]
L-seryl-L-methionine	SM	1.3 ± .08[g,h]
L-methionyl-L-serine	MS	1.8 ± .72[f,g]
L-valyl-L-methionine	VM	2.1 ± .33[e,f,g]
L-methionyl-L-valine	MV	2.7 ± .90[d,e,f]
L-methionyl-L-methionine	MM	6.2 ± .70[a]
L-methionyl-L-methionine[2]	MM[2]	3.8 ± .36[c,d]
L-methionyl-L-alanyl-L-serine	MAS	4.1 ± .41[b,c]
L-methionyl-L-leucyl-L-phenylalanine	MLF	3.2 ± .91[c,d,e]

[1] Values represent means ± SE (n = 6). Means lacking a common letter differ ($P < .05$).

[2] MM, 3 μM

Table 6.2. Incorporation of ^{3}H-leucine into secreted and cell proteins by MAC-T cells starved for methionine for 24, 48, and 72 h as influenced by L-methionine concentration in the medium

Medium methionine (μM)	Length of starvation					
	24 h		48 h		72 h	
	Secreted protein	Cell protein	Secreted protein	Cell protein	Secreted protein	Cell protein
	---------------Percentage of negative control (DPM/well)----------------					
0	100	100	100	100	100	100
3.125	124±3.8	121±1.3	102±1.6	116±2.1	116±2.8	25±1.5
6.25	135±3.3	136±2.6	105±1.3	130±2.5	118±3.0	140±3.1
12.5	146±5.7	163±3.2	109±2.2	142±2.6	133±3.2	167±5.9
25	135±3.1	175±3.3	112±1.3	139±2.7	122±2.7	164±5.1
50	138±2.7	176±3.1	114±4.0	139±2.5	123±5.5	167±4.4
100	140±3.0	172±1.3	113±0.9	143±3.2	130±3.2	162±2.6
200	143±3.1	165±5.7	113±2.1	148±2.3	148±9.9	186±10.9

Table 6.3. Ratio of ^{3}H-leucine incorporation into proteins promoted by different methionine substrates to cell DNA content

Methionine substrate (6 μM)	DNA (μg/well cell) 3 d[2]	8 d[3]	SEM	P[4]	Total protein[1] (DPM/μg DNA) 3 d	8 d[5]	SEM	P[6]
AM	23.0[a,b,c]	40.0	2.56	.005	1042	1191	86	.274
MA	23.7[a,b,c]	35.1	2.78	.033	903	1263	160	.192
FM	24.5[a,b]	33.4	2.11	.030	822	1287	91	.105
MF	19.6[d]	41.4	1.55	.001	1114	1032	57	.358
GM	21.4[b,c,d]	37.5	2.11	.003	799	1116	72	.026
MG	23.6[a,b,c]	41.7	1.28	.001	885	989	50	.201
LM	20.4[c,d]	41.3	2.79	.003	1123	1144	102	.890
ML	21.2[c,d]	38.7	2.32	.003	968	1091	85	.352
PM	19.4[d]	33.3	1.10	.001	937	1189	22	.001
MP	22.4[a,b,c,d]	36.8	2.05	.004	816	844	77	.805
SM	20.8[c,d]	36.8	1.04	.001	949	1282	52	.006
MS	22.4[a,b,c,d]	33.0	.56	.001	896	1360	53	.002
VM	20.7[c,d]	33.5	2.02	.006	1054	1494	96	.023
MV	20.6[c,d]	37.8	1.77	.001	1019	1150	64	.205
MM	24.8[a]	35.5	1.82	.009	1115	1367	80	.075
MM[7]	24.4[a,b]	39.2	2.58	.010	900	1173	88	.079
MAS	23.4[a,b,c]	39.1	1.47	.001	997	1186	41	.023
MLF	22.5[a,b,c,d]	34.3	2.09	.010	900	1380	119	.032
M	21.0[c,d]	39.8	1.37	.001	1285	1149	78	.273
-M	21.2[c,d]	30.8	2.09	.023	769	1028	59	.027

[1] Secreted proteins plus cell proteins
[2] Means lacking a common letter within the column differ ($P < .05$).
[3] Analysis of variance shows no difference within the column ($P < .08$).
[4] Probability of individual T test
[5] Paired-T test shows that 8-d cells incubated with all of the peptides, except methionylphenylalanine, synthesized more ($P < .0005$) ^{3}H-leucine labeled proteins per unit of DNA than did 3-d cells.
[6] Probability of individual T test
[7] MM, 3 μM

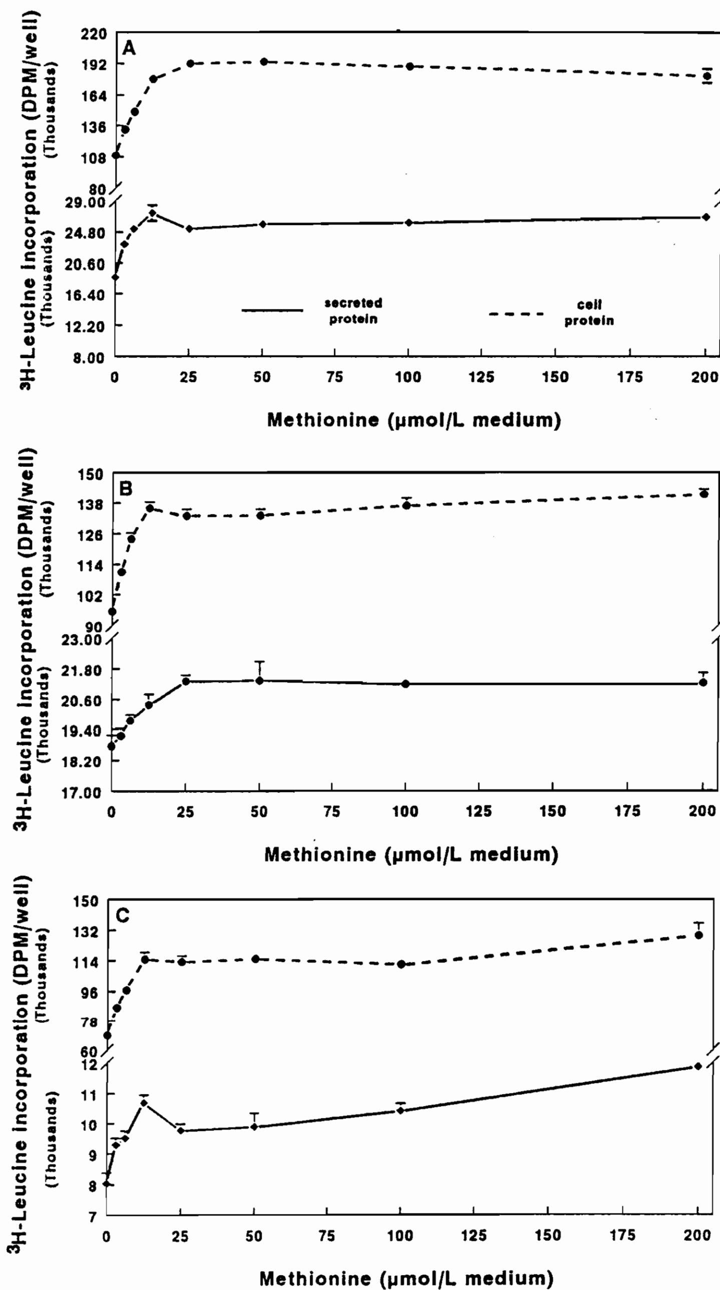

Figure 6.1. Incorporation of ^{3}H-leucine into secreted and cell proteins promoted by different concentrations of L-methionine by MAC-T cells pre-starved for methionine for 24 (A), 48 (B), or 72 (C) h. Prior to starvation, cells were allowed to grow for 8 d and then following starvation, cells were incubated with different L-methionine concentrations for 24 h. Values represent means ± SE (n = 6). The response to changing methionine concentration was quadratic ($P < .01$) in all cases.

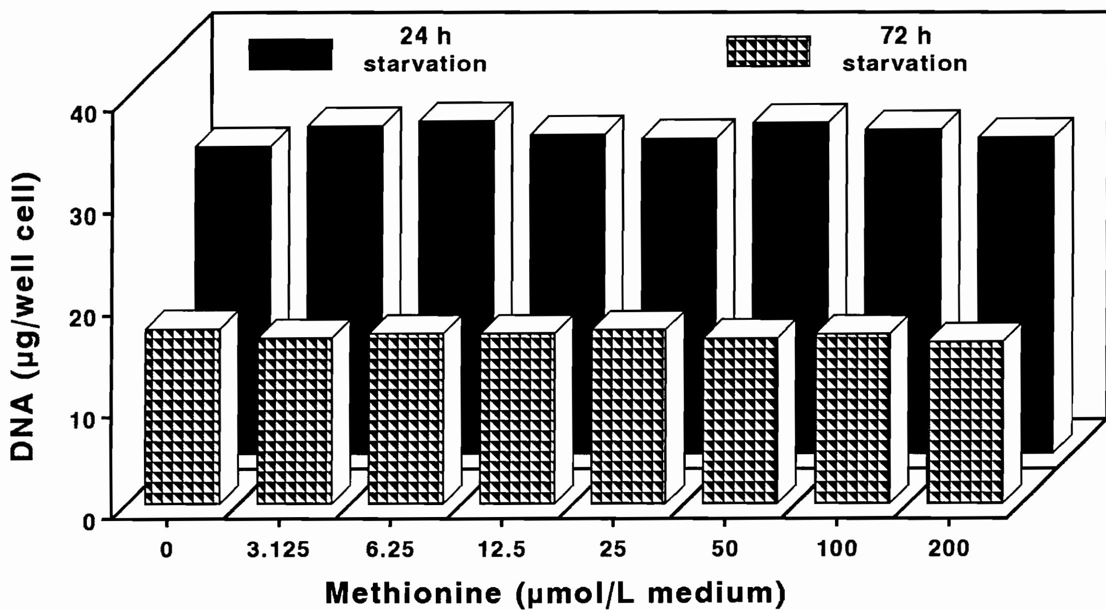

Figure 6.2. DNA content of MAC-T cells cultured in medium containing different concentrations of L-methionine. Cells were allowed to grow for 8 d and then were starved of methionine for 24 or 72 h prior to being incubated in the presence of varying methionine concentrations for 24 h. Values represent means ± SE (n = 6). DNA content was not influenced by methionine concentration at either 24 ($P < .49$) or 72 h ($P < .60$).

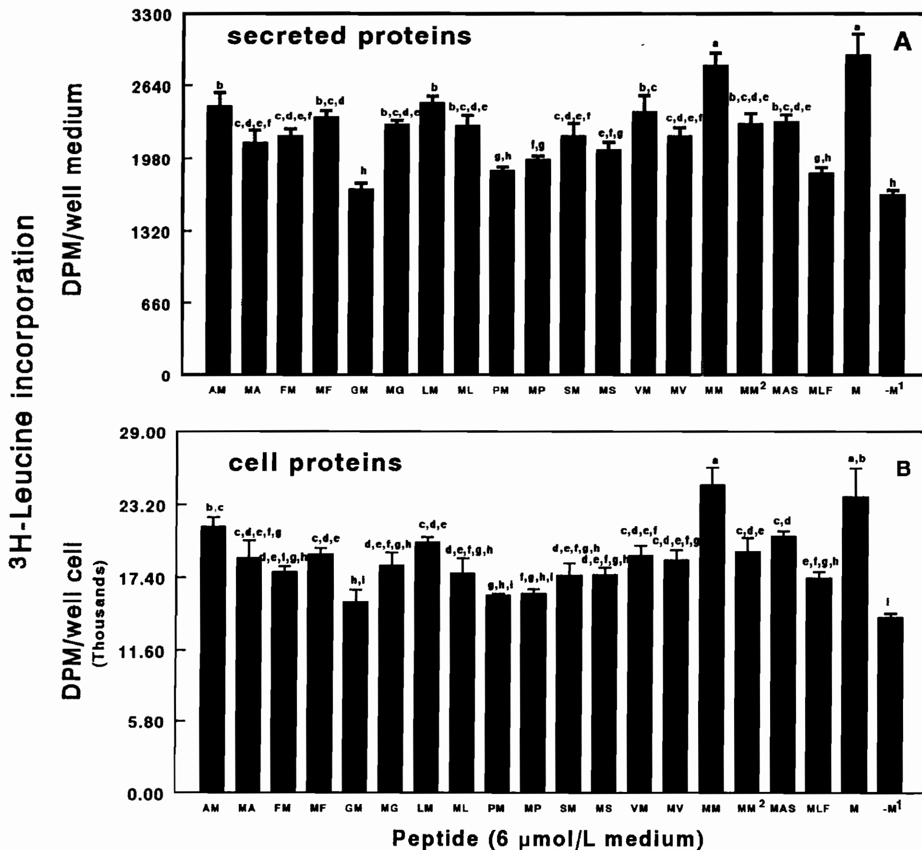

Figure 6.3. Incorporation of ^{3}H-leucine into secreted and cell proteins promoted by methionine-containing peptides in MAC-T cells after 3 d of growth and 3 h of incubation with peptides. Values represent means ± SE (n = 6). Bars lacking a common letter differ ($P < .05$). [1] -M, no methionine substrate in the incubation medium. [2] MM, 3 μmol/L medium.

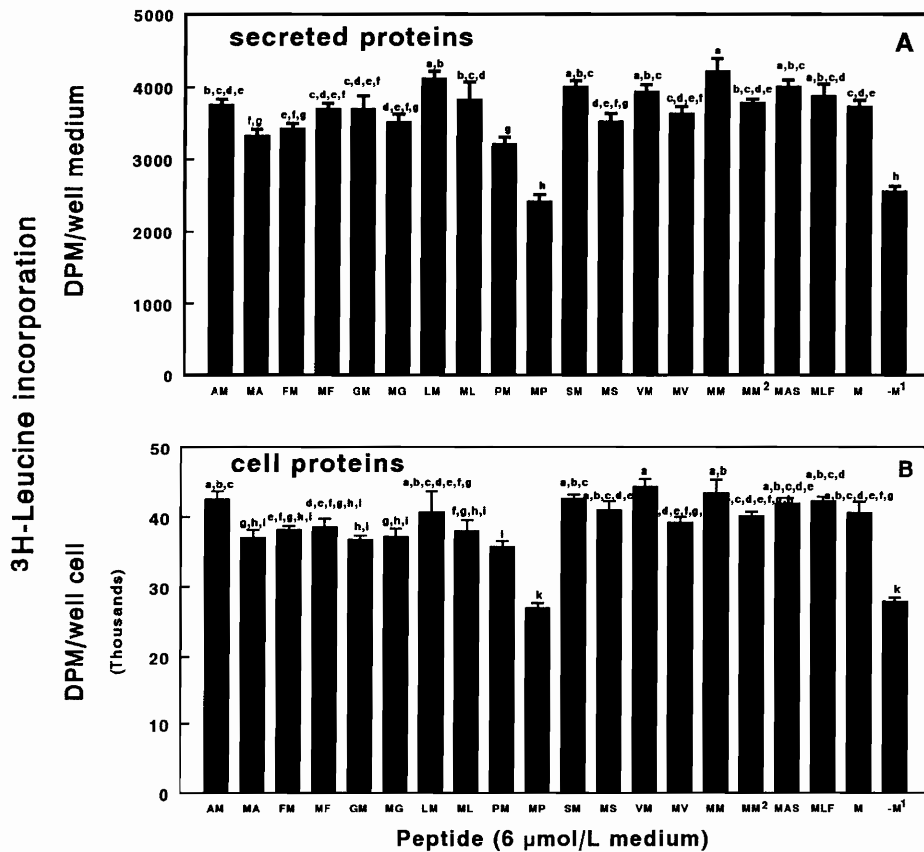

Figure 6.4. Incorporation of ^{3}H-leucine into secreted and cell proteins promoted by methionine-containing peptides in MAC-T cells after 8 d of growth and 3 h of incubation with peptides. Values represent means ± SE (n = 6). Bars lacking a common letter differ (P < .05). [1] -M, no methionine substrate in the incubation medium (negative control). [2] MM, 3 μmol/L medium.

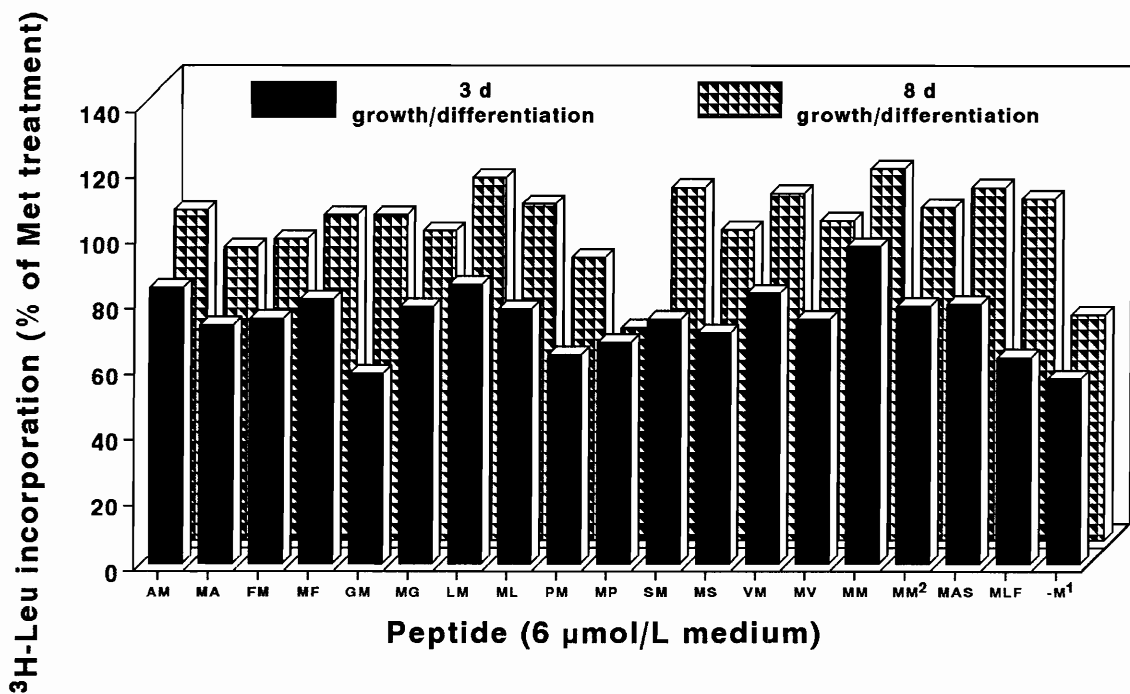

Figure 6.5. Effect of cell growth time on the utilization of peptide-bound methionine for the synthesis of ^{3}H-leucine labeled, secreted proteins by MAC-T cells. The cells were allowed to grow for 3 or 8 d prior to incubation with peptides for 3 h. Values represent means ± SE (n = 6) which are expressed as the percentage of ^{3}H-leucine incorporation promoted by L-methionine. The incorporation rate in all the treatments, except the one containing methionylproline, was greater ($P < .05$) in 8-d cells than in 3-d cells. [1] -M, no methionine substrate in the incubation medium (negative control). [2] MM, 3 μmol/L medium.

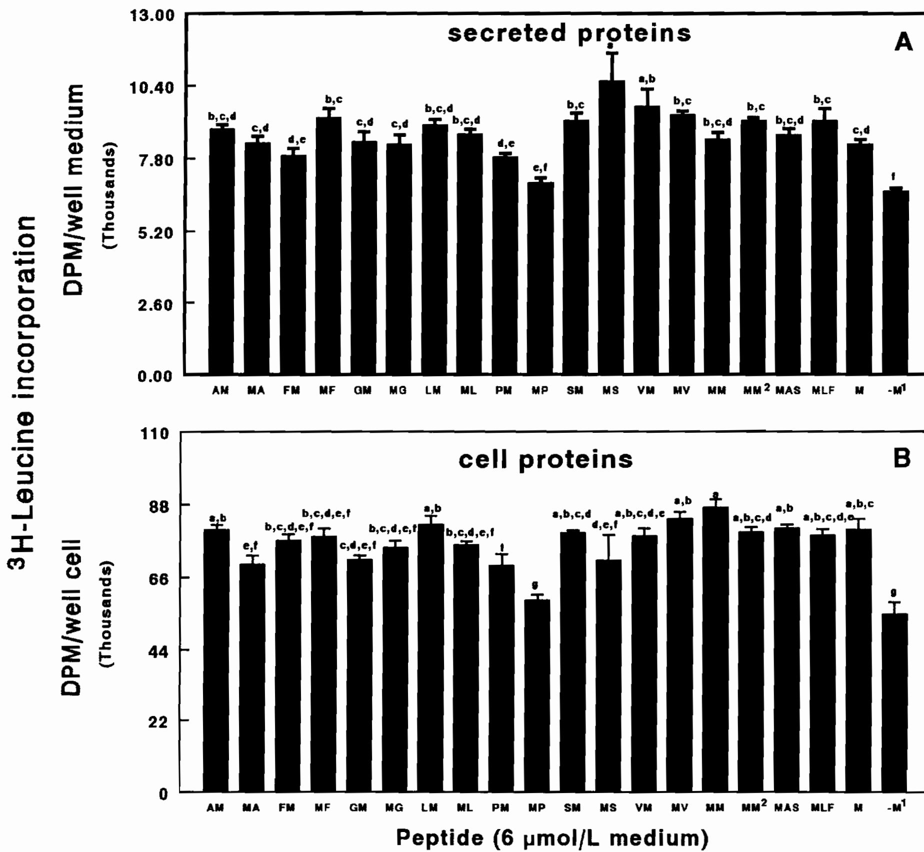

Figure 6.6. Incorporation of ^{3}H-leucine into secreted and cell proteins promoted by methionine-containing peptides in MAC-T cells after 6 h of incubation. The cells were allowed to grow for 8 d prior to 6 h of incubation. Values represent means ± SE (n = 6). Bars lacking a common letter differ ($P < .05$). [1] -M, no methionine substrate in the incubation medium (negative control). [2] MM, 3 μmol/L medium.

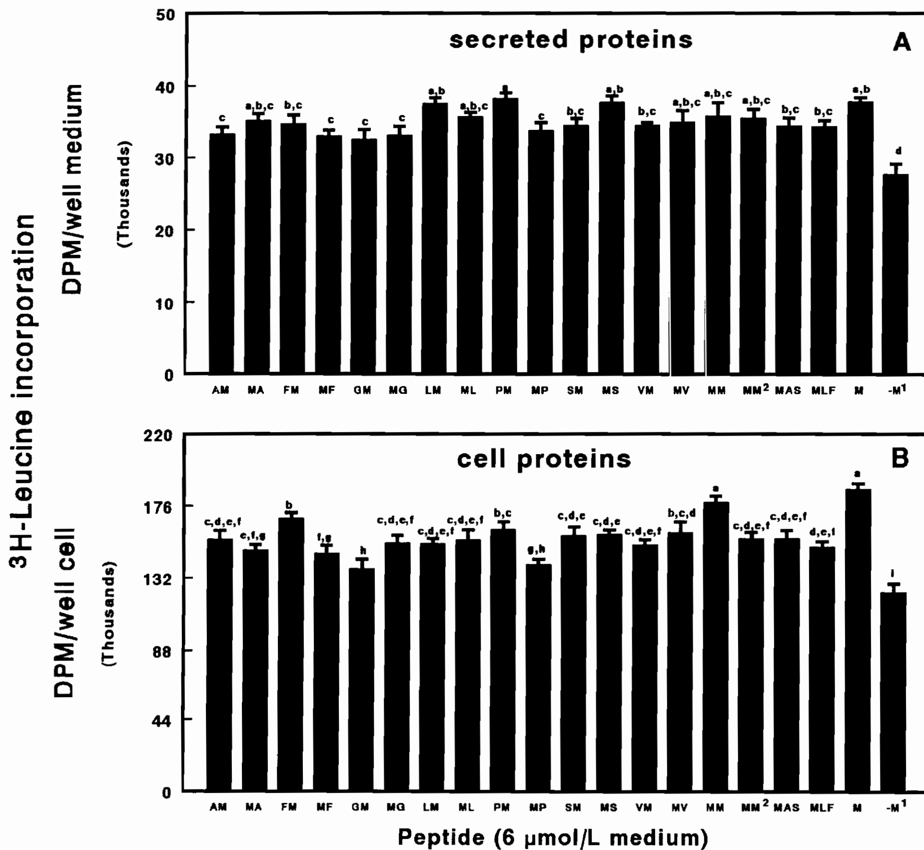

Figure 6.7. Incorporation of ^{3}H-leucine into secreted and cell proteins promoted by methionine-containing peptides in MAC-T cells after 24 h of incubation. The cells were allowed to grow for 8 d prior to 24 h of incubation with methionyl peptides. Values represent means ± SE (n = 6). Bars lacking a common letter differ ($P < .05$).
[1] -M, no methionine substrate in the incubation medium (negative control).
[2] MM, 3 μmol/L medium.

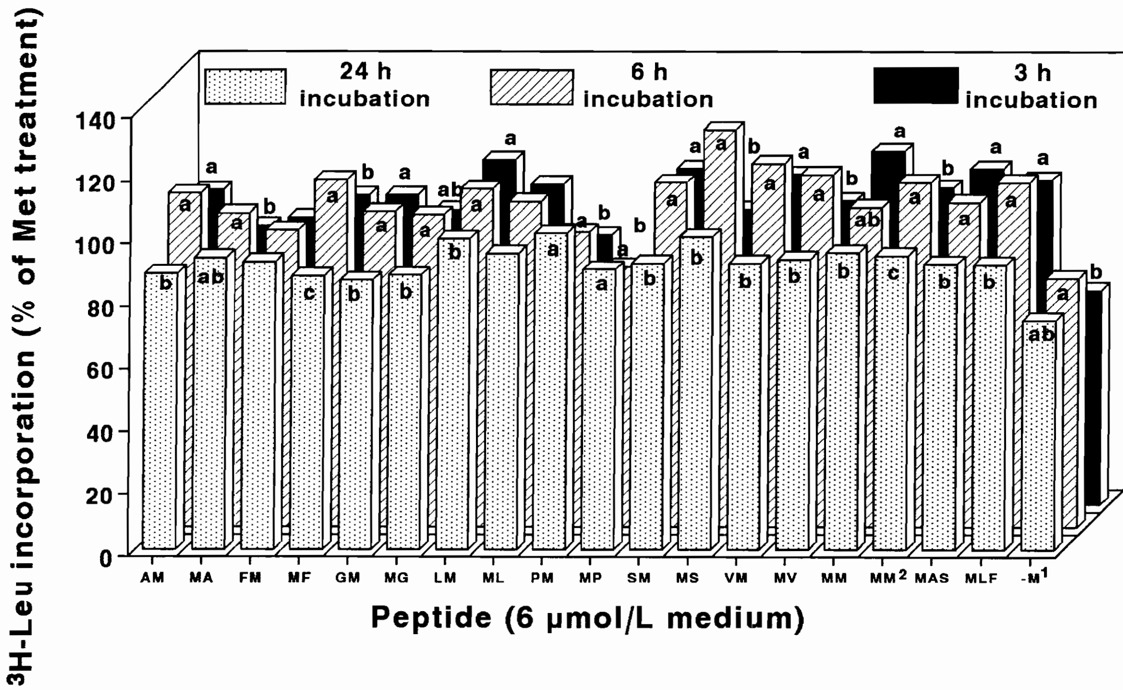

Figure 6.8. Effect of incubation time on the utilization of peptide-bound methionine for the synthesis of ^{3}H-leucine labeled secreted, proteins by MAC-T cells. The cells were allowed to grow for 8 d prior to incubation treatment. Values represent means ± SE (n = 6) which are expressed as the percentage of ^{3}H-leucine incorporation promoted by L-methionine. Bars lacking a common letter within each peptide group differ ($P \leq .05$). [1] -M, no methionine substrate in the incubation medium (negative control). [2] MM, 3 μmol/L medium.

Chapter VII

EFFECT OF EXTRACELLULAR MATRIX ON THE UTILIZATION OF PEPTIDE-BOUND METHIONINE FOR THE SYNTHESIS OF SECRETED PROTEINS BY BOVINE MAMMARY EPITHELIAL CELLS

ABSTRACT

The ability of bovine mammary epithelial cells cultured on different extracellular matrices to utilize peptide-bound methionine for synthesizing secreted proteins was compared with that of cells cultured on a plastic surface. Some differences were observed among cells under different culture conditions. Cells cultured on collagen, laminin, or fibronectin coated plates tended to proliferate to a greater extent than cells cultured on plastic. Cells cultured on basement membrane matrix gel (matrigel) had the lowest proliferating ability among all the cultures. Also, cells grown on matrigel showed less ability than cells grown on plastic to utilize free methionine for protein synthesis. Cells cultured on all the extracellular matrices, in general, were similar to cells cultured on plastic in their ability to utilize dipeptide-bound methionine for synthesis of secreted proteins. These results suggest that, although extracellular matrices may stimulate cells and cause differing degrees of differentiation compared with cells cultured on plastic, these matrices did not improve the ability of cells to utilize peptide-bound methionine for synthesis of secreted proteins.

(Key Words: Mammary, Cell Culture, Dipeptide, Methionine, Extracellular Matrix, Bovine)

Introduction

Milk protein synthesis occurs in differentiated mammary epithelial cells. Mammary epithelial cells cultured on extracellular matrices, that are components of the basement membrane, are often better than those cultured on plastic surfaces at expressing specific mammary functions (Lee et al., 1985; Huynh et al., 1991). The interactions between cells and extracellular matrices are important for cell differentiation and organization (Hay, 1981; Wicha et al., 1982; Haeuptle et al., 1983). Several extracellular matrices, laminin, fibronectin, collagen, and basement membrane matrix, have been reported to stimulate the differentiation of cultured epithelial cells (Kleinman et al., 1984; Li et al., 1987). Results from a previous study (Chapter VI) revealed that bovine mammary epithelial cells cultured on a plastic surface can utilize methionine bound in a wide range of di- and tripeptides for the synthesis of secreted proteins. Increasing the growth time of cells in the presence of lactogenic hormones improves the ability of cells to utilize methionyl peptides for protein synthesis presumably as a result of more advanced differentiation of the cells. It is not known, however, whether similar results would be obtained if the cells were cultured on extracellular matrices that bear more resemblance than a plastic surface to *in vivo* conditions. Thus, the present study was designed to evaluate the ability of cells cultured on different extracellular matrices to utilize peptide-bound methionine to substitute for free methionine in the synthesis of secreted proteins.

Materials and Methods

Media and Buffer. Dulbecco's modified Eagle's medium (DMEM), methionine- and leucine-free DMEM, and calcium-free Dulbecco's phosphate buffered saline

(DPBS) were prepared as described in Chapter VI. The media and buffer had a pH about 7.35.

Cell Cultivation. Bovine mammary epithelial cells (MAC-T) were cultured at 37°C in a humidified atmosphere with 95% air and 5% CO_2. The cells were plated at 100,000 cells/well (24-well plate) containing 1 mL of DMEM supplemented with 10% fetal bovine serum[1] and incubated overnight to allow the cells to attach to the plate. The 24-well plastic plates were either uncoated[2] or commercially coated with one of the following extracellular matrices: rat tail collagen (type I) [3], human fibronectin[4], mouse laminin[5], or basement membrane matrix gel (matrigel)[6] from Engelbreth-Holm-Swarm murine tumor. After seeding, the cells were allowed to grow for 8 d followed by a pre-incubation period to deplete intracellular methionine as described previously (Chapter VI). The cells were then incubated for 3 h in methionine- and leucine-free DMEM supplemented with all the hormones as in the growth DMEM, .2 mM L-leucine[7], 1 μCi L-[4,5-^{3}H]leucine[8] (^{3}H-leucine)/well, and 6 μM of L-methionine[7], L-methionyl-L-serine[7], or L-methionyl-L-proline[7]. Methionylserine was shown to be as efficient as free methionine in promoting synthesis of secreted proteins by the mammary epithelial cells cultured on a plastic surface (Chapter VI). Similarly, methionylproline was shown to be a poor methionine source in a 3-h incubation period in the previous study (Chapter VI).

Harvest of Cells and Analytical Procedures. The cells cultured on uncoated plastic plates and on plates coated with collagen, fibronectin, or laminin were harvested with .25% trypsin as described in Chapter VI. The cells cultured on

[1] Cat. No. 200-6140AJ, GIBCO, Grand Island, NY
[2] Cat. No. 3047, Becton Dickinson Company, Lincoln Park, NJ
[3] Cat. No. 40408, Becton Dickinson Labware, Bedford, MA
[4] Cat. No. 40411, Becton Dickinson Labware, Bedford, MA
[5] Cat. No. 40412, Becton Dickinson Labware, Bedford, MA
[6] Cat. No. 40433, Becton Dickinson Labware, Bedford, MA
[7] Sigma Chemical Co., St. Louis, MO
[8] Cat. No. TRK.683, Amersham International Plc., Buckinghamshire, England

matrigel were washed in 1 mL of DPBS and dissociated from the gel by dissolving the gel at 37°C with 1 mL of dispase[9] for 2 h. The cell suspension then was centrifuged at 2,000 *x* g. The supernates were discarded and the cells were resuspended in 1 mL of PBS. All of the cell suspensions were sonicated and DNA contents were determined by a fluorometric method (Chapter VI). The ^{3}H-leucine labeled secreted proteins in the incubation medium were quantified as previously described (Chapter VI).

Statistical Analysis. The variance of amount of ^{3}H-leucine labeled, secreted proteins synthesized by cells cultured on different extracellular matrices and supplemented with different methionine substrates were analyzed using the general linear model procedure (SAS, 1990). The means of different treatments were compared using Duncan's multiple range test at $\alpha = .05$ (SAS, 1990).

Results

Incorporation of ^{3}H-leucine into secreted proteins by MAC-T cells supplemented with different methionine substrates was influenced by the matrices tested (Table 7.1). Cells grown on laminin, generally, produced a greater ($P < .05$) amount of ^{3}H-leucine incorporation than did cells cultured on other substrata regardless of methionine substrates. In contrast, cells grown on matrigel showed a lesser ability to utilize all of the methionine substrates for synthesizing proteins in comparison to other matrices.

Also, incorporation of ^{3}H-leucine into secreted proteins by the cells cultured on different matrices was affected by the methionine substrates (Figure 7.1). Methionylserine and free methionine promoted greater ^{3}H-leucine incorporation ($P < .05$) than did methionylproline and the negative control (no methionine) in cells

[9] Cat. No. 40235, Becton Dickinson Labware, Bedford, MA

cultured on all of the substrata except matrigel. The amount of ^{3}H-leucine labeled proteins synthesized by cells grown on plastic, collagen, or fibronectin, supplemented with methionylproline, was not different ($P > .05$) from the respective negative controls. However, methionylproline promoted a greater ($P < .05$) ^{3}H-leucine incorporation into secreted proteins than did the negative control in cells cultured on laminin. Methionine substrates were not different from the negative control in promoting ^{3}H-leucine incorporation into secreted proteins by cells cultured on matrigel.

Larger numbers of cells were harvested from the wells coated with collagen, laminin, and fibronectin than from wells coated with matrigel or from uncoated plastic wells as indicated by DNA content (Figure 7.2). In contrast, different methionyl substrates showed no difference ($P > .05$) from the negative control in maintaining the number of cells cultured on the same substratum (Figure 7.2).

Several different features emerged when the incorporation of ^{3}H-leucine into secreted proteins was compared on the basis of DNA (equal number of cells) content (Figure 7.3). First, cells grown on the plastic surface showed a similar ($P > .05$) or greater ($P < .05$) ability than the cells cultured on other substrata to utilize all of the methionine substrates for the synthesis of secreted proteins. Second, cells grown on matrigel had a similar ability ($P > .05$) as cells grown on the other four substrata to utilize methionylserine and methionylproline for the synthesis of ^{3}H-leucine labeled, secreted proteins. This same culture showed the lowest ability to utilize free methionine among all of the cell cultures. Moreover, cells cultured on laminin and fibronectin showed a tendency to utilize methionylproline for protein synthesis, while cells grown on plastic and collagen could not use this dipeptide.

Discussion

It has been recognized that the basement membrane plays an important role in the regulation of differentiation of mammary epithelial cells. However, the effect of an individual extracellular matrix on the expression of mammary function in cultured mammary epithelial cells has not been clearly defined.

Laminin is a major basement membrane protein which has been shown to influence the growth and differentiation of different cultured epithelial cells including mammary epithelial cells (Kleinman et al., 1984). Fibronectin, a glycoprotein, is also a component of the basement membrane and it alters cell morphology, growth, differentiation, and migration *in vitro* (Hynes and Yamada, 1982; Kleinman et al., 1984). Li et al. (1987) observed that primary mouse mammary epithelial cells cultured on laminin had 1.5- to 4-fold increases in ß-casein mRNA, while cells cultured on fibronectin showed no changes in morphology and ß-casein gene expression compared with cells cultured on a plastic surface. However, an established mouse mammary epithelial cell line cultured on laminin or fibronectin, although forming a dome-like structure, showed about equal ability in the synthesis of ß-casein mRNA to the cells cultured on plastic (Medina et al., 1987). In the present study, cells cultured on a plastic surface in the presence of either methionylserine or free methionine, synthesized a similar or even greater amount of secreted proteins than did an equal number of cells cultured on laminin or fibronectin and supplemented with the same methionine sources. The results indicate that, although laminin and fibronectin stimulated cell proliferation, these extracellular matrices did not improve the ability of individual cells to utilize either methionylserine or free methionine for protein synthesis compared with cells grown on a plastic surface under the present experimental conditions. Laminin and fibronectin appeared to slightly improve the

ability of cells to utilize methionylproline, relative to the utilization of other methionine substrates, compared to plastic and collagen. This implies that cells cultured on laminin and fibronectin may be slightly different from cells cultured on the plastic surface or on collagen.

Mouse mammary epithelial cells cultured on floating type I collagen gel showed an increase in the secretion of ß-casein (Li et al., 1987). However, attached collagen gel stimulated cell proliferation (Richards et al., 1983), but had limited ability to influence the differentiation of mammary epithelial cells (Tonelli and Sorof, 1982; Lee et al., 1985). In the present study, cells cultured on collagen-coated plates appeared to be similar to the cells cultured on attached collagen gel in that collagen stimulated cell proliferation, but it did not improve the ability of the cells to utilize either peptide-bound or free methionine for the synthesis of secreted proteins. These results suggest that type I collagen may have little influence on cell differentiation under the present experimental conditions.

Basement membrane matrix from Engelbreth-Holm-Swarm tumor cells has been shown to stimulate a high degree of morphological differentiation and ß-casein gene expression in cultured mammary epithelial cells (Li et al., 1987; Barcellos-Hoff et al., 1989; Aggeler et al., 1991). In the present study, bovine mammary epithelial cells cultured on gel formed with basement membrane matrix (matrigel) showed the lowest ability to proliferate when compared with cells cultured on other substrata. This may indicate that there were differences in the degree of cell differentiation in the various culture conditions, because differentiated cells usually have a limited ability for proliferation. However, in spite of the difference in proliferation, the cells cultured on the matrigel showed the same ability as an equal number of cells cultured on plastic to utilize peptide-bound methionine for the synthesis of secreted proteins. These same cells, however, did not utilize free methionine as well as did an equal

number of cells cultured on plastic or on other matrices. These results further indicate that there were differences between the cells cultured on matrigel and cells cultured on other substrata.

In conclusion, cells cultured on different extracellular matrices were somewhat different from cells cultured on a plastic surface which may indicate differences in degree of cell differentiation between the cells on extracellular matrices and cells on plastic. However, in general, cells cultured on each extracellular matrix showed about the same relative ability as cells cultured on plastic to utilize peptide-bound methionine for the synthesis of secreted proteins.

Implications

The present results show that cells cultured on extracellular matrices, especially on matrigel, may be in a more advanced stage of differentiation than the cells cultured on a plastic surface. However, cells on matrigel showed the same ability as an equal number of cells cultured on plastic in utilizing peptide-bound methionine for synthesis of secreted proteins. These results suggest that evaluation of peptides as amino acid substrates can be accomplished in MAC-T cells cultured on a plastic surface.

Table 7.1 Total incorporation of ^{3}H-leucine into secreted proteins by MAC-T cells as influenced by methionine substrates and culture substrata[1]

Methionine substrates (6 μM)	Substrata				
	Plastic[2]	Matrigel	Collagen	Laminin	Fibronectin
	DPM/well medium				
No Met[3]	2084 ± 242 ^{a,II}	1140 ± 132 ^{b,I}	2129 ± 133 ^{a,II}	2390 ± 47 ^{a,IV}	1965 ± 107 ^{a,III}
Met	2697 ± 92 ^{b,I}	1192 ± 284 ^{c,I}	2717 ± 67 ^{b,I}	3359 ± 77 ^{a,I}	2927 ± 53 ^{b,I}
Met-Pro	1932 ± 85 ^{b,II}	1180 ± 374 ^{b,I}	2017 ± 128 ^{b,II}	2610 ± 88 ^{a,III}	1994 ± 72 ^{b,III}
Met-Ser	2780 ± 187 ^{ab,I}	1771 ± 184 ^{c,I}	2471 ± 71 ^{b,I}	3035 ± 41 ^{a,II}	2507 ± 84 ^{b,II}

[1] Values represent mean ± SE (n = 6).
[2] Means lacking a common number within each column differ ($P < .05$).
[3] Means lacking a common letter within each row differ ($P < .05$).

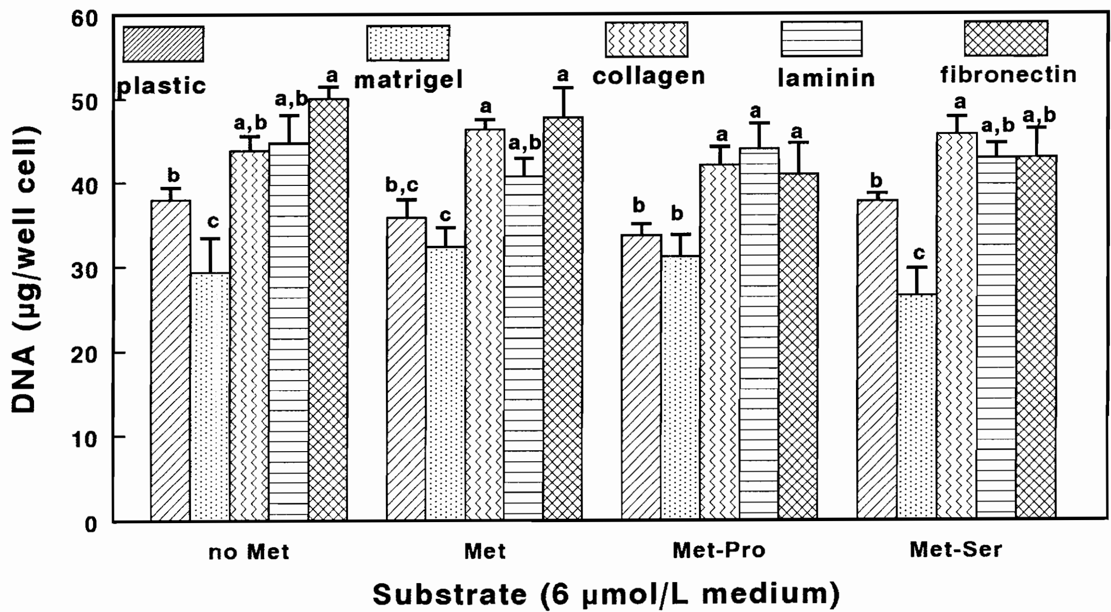

Figure 7.1. DNA content of MAC-T cells cultured on different substrata and incubated with medium containing different methionine substrates. Values represent means ± SE (n = 6). Bars lacking a common letter within each methionine substrate group differ ($P < .05$). There was no difference ($P > .05$) within each substratum group.

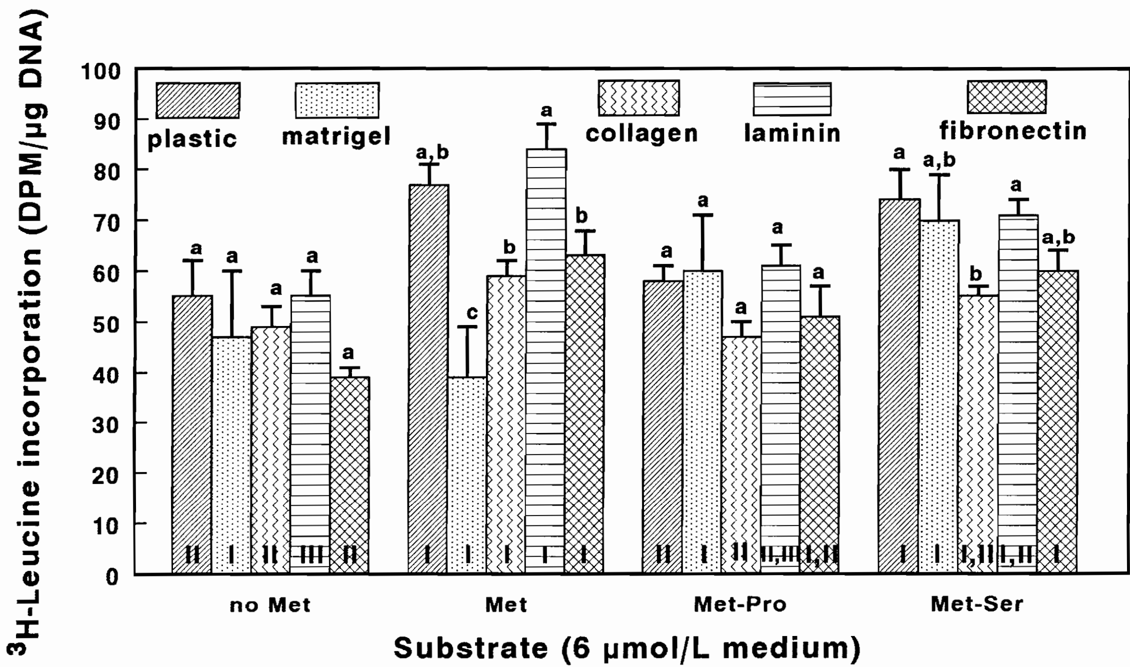

Figure 7.2. ^{3}H-leucine incorporation into secreted proteins/μg DNA by MAC-T cells as influenced by methionine substrates and culture substrata. Values represent means ± SE (n = 6). Bars lacking a common letter within a methionine substrate group or lacking a common number within each substratum group differ (P < .05).

Chapter VIII

EPILOGUE

The primary significance of the results from the present research is the demonstration that both mammary explants from lactating mice and bovine mammary epithelial cells possess the ability to utilize peptide-bound amino acids for the synthesis of secreted proteins. These results are complementary to previous reports that a large portion of blood amino acids exist in peptide form (McCormick and Webb, 1982; DiRienzo, 1990; Seal and Parker, 1991; Koeln et al., 1993), which implies that, *in vivo*, peptide-bound amino acids may be an important supply of amino acids for the mammary gland and other body tissues.

Bioactive peptides may represent a proportion of the blood peptides. Oligopeptides with various reported bioactivities were observed to be able to serve as amino acid sources for the synthesis of secreted proteins by mammary tissue in the present study. These results indicate that, in animals with high milk production, circulating bioactive peptides may serve as a supplemental source of amino acids for the mammary gland when the animals are in negative balance. These results also suggest that tissue utilization of oligopeptide-bound amino acids may be a way for the animal body to deposit inactivated, bioactive peptides or to inactivate extra bioactive peptides. Thus, it may be necessary to reconsider the metabolism of circulating peptides.

This study also, for the first time, revealed that a wide range of methionine- and lysine-containing peptides of various sizes with different amino acid compositions can serve as methionine or lysine sources for the mammary tissue. Furthermore, most of the peptides were at least as efficient as the corresponding free amino acids for the synthesis of secreted proteins by the mammary tissue. The practical importance of this finding is that a new way of supplementing the amino acid needs of the animals

may be suggested. Supplementation of amino acids in the diet in the form of peptides may improve milk protein production, because some amino acids can be absorbed more rapidly from the gastrointestinal tract when they occur in peptide form (Matthews, 1975) and also because peptide-bound amino acids are usually more stable and sometimes more soluble than corresponding free amino acids (Adibi, 1978b; Stehle et al., 1982).

However, further studies are required for a better understanding of the utilization of peptide-bound amino acids by the mammary gland. We already know, from the present study, that there are differences among peptides and between peptides and corresponding free amino acids in promoting synthesis of secreted proteins. Further study of the synthesis of specific milk proteins may help us to find peptides that are advantageous in stimulating the synthesis of some specific milk proteins.

In the animal's body, circulating peptides, like circulating free amino acids, are dynamic. The types or sizes of circulating peptides may change with different dietary proteins. Peptides may compete with or stimulate each other to serve as amino acid sources for tissues. Therefore, the use of different mixtures of peptides to substitute for corresponding amino acids for mammary tissue *in vitro* would help us to understand better the utilization of peptide-bound amino acids for milk protein production.

Peptide transport across cell membranes is one of the major factors influencing the efficiency of peptides as amino acid sources for tissues. Mediated peptide transport appears to occur in mouse mammary tissue according to the present study. But it is not known whether other transport process(es) may also be involved. Further studies of the effects of compounds known to inhibit membrane-bound

peptidase activity, cations, or pH changes on milk protein synthesis would provide more knowledge of peptide transport in the mammary gland.

Hormones play an important role in the regulation of mammary function. The increasing milk production stimulated by growth hormone may be the direct action of this hormone on mammary tissue or/and may be mediated by insulin-like growth factor (IGF). This is because although growth hormone receptor mRNA was detected in mammary tissue (Glimm et al., 1990; Hauser et al., 1990; Jammes et al., 1991) but no growth hormone receptor has been identified in this tissue (Gertler et at., 1984; Akers, 1985; McFadden et al., 1990). Additionally, high affinity IGF receptors have been characterized on mammary plasma membrane (Dehoff et al., 1988; Collier et al., 1989; Hadsell et al., 1990). IGF-I can promote milk protein production by stimulating the expression of the ß-casein gene (Prosser et al., 1987; Duclos et al., 1989). It follows that increased milk protein synthesis will be accompanied by an increased requirement for amino acids. It will be of particular interest to learn if IGF can stimulate the utilization of peptide-bound amino acids by the mammary gland compared with corresponding amino acids.

In summary, peptides appear to be an important source of amino acids for the mammary gland. Much remains unclear about the process of peptide utilization. Therefore, there will be a long way to go before we may apply this knowledge into the every day practice of nutritional management of animals.

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APPENDIX

Statistical Analysis Examples

Example 1. Analysis of variance of incorporation rates of ^{3}H-leucine into secreted proteins promoted by different concentrations of L-methionine by general linear models procedure of SAS

General Linear Models Procedure

Dependent Variable: MDPM (medium DPM)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	12	344048981.25	28670748.44	10.89	.0001
Error	35	92176338.23	2633609.66		
C. Total	47	436225319.48			

R-Square	C.V.	Root MSE	MDPM Mean
.79	6.54	1622.84	24792.73

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Methionine	7	322672428.65	46096061.24	17.50	.0001
Replication	5	21376552.60	4275310.52	1.62	.1796

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Methionine	7	322672428.65	46096061.24	17.50	.0001
Replication	5	21376552.60	4275310.52	1.62	.1796

Contrast	DF	Contrast SS	Mean Square	F Value	Pr > F
Linear	1	1290726.03	1290726.03	.49	.4885
Quadratic	1	86889166.47	86889166.47	32.99	.0001

Example 2. Analysis of variance of incorporation rates of ^{3}H-leucine into secreted proteins promoted by different methionine substrates using general linear models procedure of SAS

General Linear Models Procedure

Dependent Variable: MDPM (medium DPM)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	24	97301196.03	4054216.50	5.38	.0001
Error	95	71607409.96	753762.21		
C. Total	119	168908605.99			

R-Square	C.V.	Root MSE	MDPM Mean
.58	10.07	868.19	8625.51

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treatment	19	91136414.49	4796653.39	6.36	.0001
Replication	5	6164781.54	1232956.31	1.64	.1580

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	19	91136414.49	4796653.39	6.36	.0001
Replication	5	6164781.54	1232956.31	1.64	.1580

Example 3. Comparison of means of different methionine substrate treatments using Duncan's multiple range test of SAS

General Linear Models Procedure

Duncan's Multiple Range Test for Variable: MDPM (medium DPM)

Á = .05 df = 95 MSE = 753762.2

Means with the same letter are not significantly different

			Mean	N	Treatment
		A	10552.2	6	MS
	B	A	9654.2	6	VM
	B	C	9359.3	6	MV
	B	C	9233.0	6	MF
	B	C	9163.2	6	MLF
	B	C	9162.0	6	MM*
	B	C	9154.0	6	SM
D	B	C	8981.2	6	LM
D	B	C	8864.5	6	AM
D	B	C	8656.7	6	ML
D	B	C	8637.3	6	MAS
D	B	C	8495.0	6	MM
D		C	8386.5	6	GM
D		C	8342.7	6	MA
D		C	8303.3	6	MG
D		C	8299.0	6	M
D		E	7892.7	6	FM
D		E	7838.2	6	PM
	F	E	6919.5	6	MP
	F		6615.8	6	-M

Example 4. Analysis of correlation between the rate of ^3H-leucine incorporation into secreted proteins promoted by methionyl peptides and the extent of peptide hydrolysis (free methionine released) by correlation procedure of SAS

Correlation Analysis

2 'VAR' Variables: PMDPM* Methionine

Variable	N	Mean	Std Dev	Sum	Minimum	Maximum
PMDPM	17	130.78	24.67	2223.30	97.37	175.86
Methionine	17	11.99	2.30	203.77	8.00	14.92

Pearson Correlation Coefficients / Prob > |R| under Ho: r = 0 / N = 17

	PMDPM	Methionine
PMDPM	1.0000 .0	.0285 .9137
Methionine	.0285 .9137	1.0000 .0

* PMDPM, percentage of medium DPM of free methionine treatment

Example 5. Regression analysis of the rate of ^{3}H-leucine incorporation into secreted proteins promoted by methionyl peptides and the number of amino acid residues in the peptides

Model: PMDPM = Residue (number of amino acid residue)
Dependent Variable: PMDPM (percentage of medium DPM of free methionine treatment)

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	1444.04	1444.04	19.97	.0066
Error	5	361.51	72.30		
C. Total	6	1805.55			

Root MSE	8.50	R-Square	.80
Dep Mean	108.52	Adj R-sq	.76
C.V.	7.48		

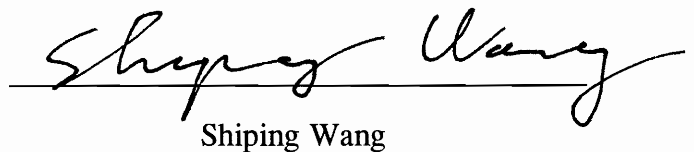
Parameter Estimates

Variable	DF	Parameter Estimate	Standard Error	T for Ho: Parameter=0	Prob > lTl
Intercept	1	144.43	8.65	16.69	.0001
Residue	1	-7.18	1.61	-4.47	.0066

VITA

Shiping Wang, daughter of Zixiao Wang and Zhanzhi Shi, was born May 9, 1955, in Wuhan, Hubei, The People's Republic of China. She completed her undergraduate training from Huazhong Agricultural University in Wuhan in Veterinary Medicine in July, 1977. After graduation, She worked as a teaching assistant in Huazhong Agricultural University until December, 1983. In January, 1984, She began her graduate study in University of Edinburgh, Scotland and received a Master of Philosophy degree in Veterinary Anatomy in January, 1986. She, then, worked as a research associate in the School of Veterinary Medicine, University of California at Davis until July, 1986. She went back to China and worked as a lecturer in Huazhong Agricultural University for about five years. She initiated her Ph.D. program at Virginia Tech in May, 1991. Throughout this program of study, she was supported by the John Lee Pratt Animal Nutrition Program.

She is a member of Gamma Sigma Delta, the Honor Society of Agriculture.



Shiping Wang