

Summary of Published Works on Beta-Dipeptides in Humans

November 10, 1986

Sources of information:

Computer-aided searches of the published scientific literature were performed on four national data bases:

Food Science and Technology Abstracts, 1969-1986

MEDLINE, 1966-1986

Agricola (National Library of Agriculture data base), 1970-1986

Commonwealth Agricultural Bureaux (CAB) Abstracts, 1973-1986.

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SUMMARY OF PUBLISHED WORKS ON BETA-DIPEPTIDES IN HUMANS

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Introduction

Purpose of Report:

To review the published literature on beta-dipeptides in humans with special emphasis on beta-aspartyl-phenylalanine.

This review of the published literature was conducted with the initial intent of collecting the tissue levels of beta-dipeptides in humans. Secondly, the handling of beta-dipeptides was to be reviewed based on the published works. For the most part, studies of beta-dipeptides have not been conducted and reported in a manner that is easily retrievable from the literature. From conversations with experts in various fields I am lead to conclude that these experiments probably have not been conducted and reported.

Thus, this review is representative of the information currently available to the general scientific community. Thorough studies of the relative importance of exogenous versus endogenous beta-dipeptides, especially beta-aspartyl dipeptides have not been found in the literature. Indeed, the role of beta-aspartyl dipeptides in human physiology is unknown. This is also true for the carnosine family of beta-dipeptides that has been extensively studied.

Sources of information:

Computer-aided searches of the published scientific literature were performed on four national data bases:

Food Science and Technology Abstracts, 1969-1986

MEDLINE, 1966-1986

Agricola (National Library of Agriculture data base), 1970-1986

Commonwealth Agricultural Bureaux (CAB) Abstracts, 1973-1986.

In addition, general inquiries were made of:

Drs. Bietz, Wolf, and Wang (senior scientists at the Northern Regional Research Center, USDA in Peoria) concerning plant-derived dietary protein and amino acids, and

Mr. Richard Meyers, head of Clinical Chemistry at the Regional Reference Laboratory, St. Francis Medical Center, Peoria, concerning the measurement of beta-amino acids and -peptides in human fluids and tissues.

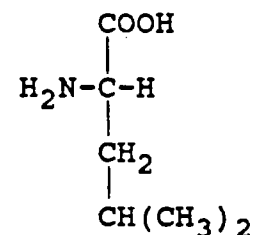
SUMMARY OF PUBLISHED WORKS ON BETA-DIPEPTIDES IN HUMANS

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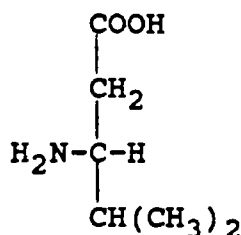
1. Chemistry of Beta-dipeptides

1.a. Nomenclature

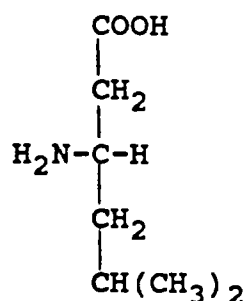
Beta-dipeptides are two amino acid containing chemical compounds. The amino acids are covalently linked together by an amide bond. These compounds are unusual in that they contain a beta-amino acid. Beta-amino acids are those amino acids where the amino group is covalently linked to the beta carbon instead of the alpha carbon (see footnote for nomenclature)(1). Alpha-amino acids are the predominate amino acid species in nature.



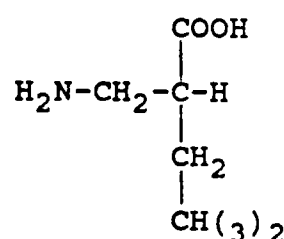
α -Leucine



β -Leucine



β -Homoleucine



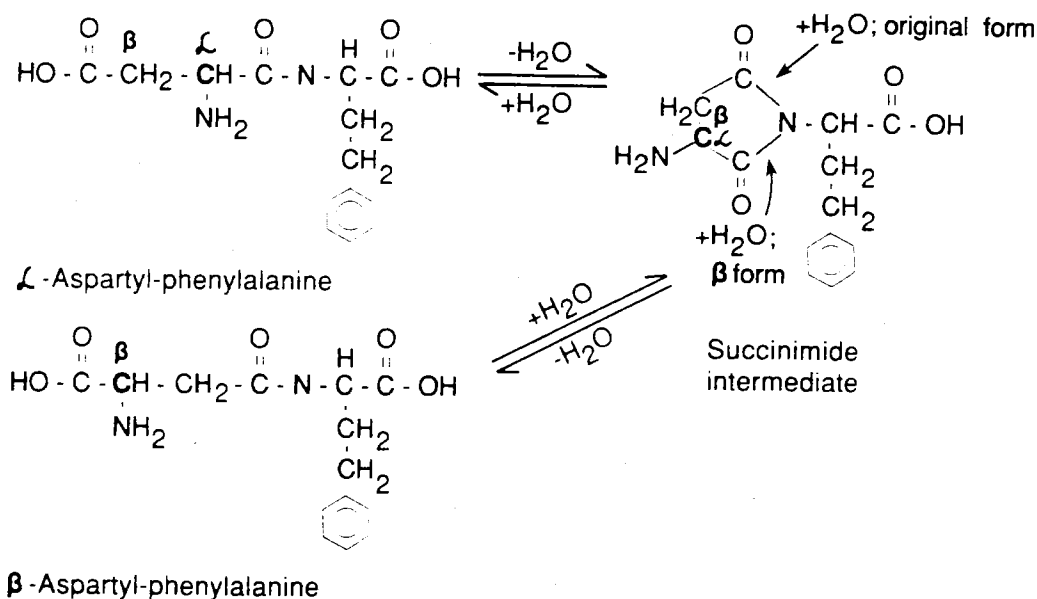
3-Amino-2-(2-methylpropyl)-propanoic acid

"As illustrated above, three different beta-amino acids are plausible analogues of an alpha-amino acid. In type I analogues the alpha-amino group is "moved" to the beta-carbon whereas in type II or III analogues an extra methylene group is inserted between the original carboxyl and amino groups. Type I and II analogues are named systematically as 3-amino derivatives of the parent acid but are frequently referred to by the nomenclature shown. Note that there is some redundancy (beta-leucine = beta-homoleucine, etc); in such cases the type I name is preferred. Alpha-substituted-beta-alanines (type III analogues) are named systematically as substituted propanoic acids or, less commonly, as 2-aminomethyl derivatives of the parent acid (e.g. 2-aminomethyl-4-methylpentanoic acid in the example shown). With the exception of beta-proline (3-carboxypyrrolidine), alpha-substituted-beta-alanines are not named by reference to the homologous alpha-amino acid. The prefix "iso" is, however, used with some alpha-substituted-beta-alanines. Isoserine and isocysteine are alpha-hydroxy- and alpha-mercapto-beta-alanine, respectively; isothreonine is beta-amino-alpha-hydroxybutanoic acid. Many di- and trisubstituted-beta-alanines bear trivial names related to the plant, microorganism, or antibiotic in which they were discovered.

"Both alpha- and beta-substituted-beta-alanines generally have at least one chiral carbon and thus occur in R- and S-configurations. Although all of the protein L-alpha-amino acids except cysteine are S-enantiomers, the configurationally analogous beta-amino acids may be R or S. The enantiomers shown [above], all analogues of L-alpha-leucine, are R-beta-leucine, S-beta-homoleucine, and R-3-amino-2-(2-methyl)propyl-propanoic acid. The D and L designations common to alpha-amino acids are frequently applied to type I and II analogues by evaluating the configuration at the beta-carbon as if it were the alpha-carbon. The D and L designations are less clearly assigned with type III analogues. R-beta-aminoisobutyrate (AiB), the type III analogue of L-alanine, is, for example, referred to as D-beta-AiB. Correspondingly, in the sense illustrated [above], S- or L-B-AiB is an analogue of D-alanine". Taken from an article written by O. Griffith published in Annual Reviews of Biochemistry (1).

1.b. Chemical and enzymatic formation *in vivo* and *in vitro*

Beta-dipeptides are formed in two ways: as a rearrangement of alpha-dipeptides to the beta form or as a direct product of a conjugation of one beta-amino acid (usually beta-alanine or -aspartate) with one alpha-amino acid (usually histidine or glycine). Although several authors suggest that an intramolecular cyclization reaction is involved in the conversion of the alpha to the beta-forms (2-4), I have yet to find a concise chemical proof of this proposed mechanism. Under this proposed mechanism, dipeptides containing alpha-aspartyl and alpha-asparagyl residues cyclize with loss of water or ammonia, respectively, and then reopen (with the addition of water) to give only the beta-aspartyl-dipeptide (5).



In the example illustrated above, α -aspartyl-phenylalanine is spontaneously converted to its β -form through a succinimide intermediate. Such α to β conversions have been reported to occur under acidic, neutral and basic conditions *in vitro* (2-5).

It has also been suggested that the β forms are more stable chemically than the α forms (6); again, no physical chemical proofs of this have been found to date. It is clear that the β -forms are "chemically" stable in physiologic fluids; however, they may be hydrolyzed by enzymes present in these fluids.

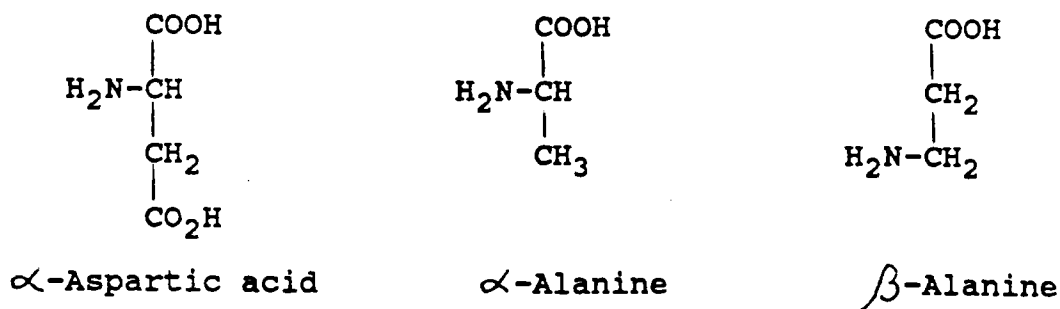
With the single exception of the enzyme lysozyme (7), β -amino acids have not been proven to be present in proteins. They are present in di- and tri-peptides liberated by the actions of proteases on a wide variety of proteins such as hemoglobin (8), ribonuclease (9), pepsin (8), fibrin (8) and both native collagen (8,10) and collagen as contained in gelatins (10).

Beta-dipeptides and free beta-amino acids are also formed by the synthetic activity of enzymes that compose certain metabolic pathways. *In vivo* synthesis involves enzymes specific for the beta-aspartyl- or beta-alanyl-dipeptides. An efficient system for the uptake of alpha-aspartate and glycine followed by synthesis of beta-aspartyl-glycine exists in ganglion of the mollusk *Aplysia californica* (11, 12). However, the enzymes involved in this synthesis have not yet been defined. In rat kidney, an enzyme having properties similar to known asparaginases is capable of forming beta-aspartyl-dipeptides in two ways (13). The most efficient route involves the combination of free asparagine as the aspartyl donor with a second amino acid as acceptor (Gly>> Ala=Ser> Leu=Asp=Thr> Glu=Val). The enzyme can also utilize other beta-Asp-dipeptides (-Glu=-Asp>> -Ser> -Thr> -Val> Leu) as donors of the beta-Aspartyl group to other amino acid acceptors.

The beta-alanyl-dipeptides are formed via an ATP-dependent enzyme reaction. The most common of these is the enzyme carnosine synthetase which combines beta-alanine- and histidine. Gamma-aminobutyrate (yielding homocarnosine) and beta-amino-*n*-butyrate can substitute for beta-alanine as substrate. In the second position, histidine can be replaced by L-ornithine and to a lesser degree by L-lysine, D,L-1,4 diaminobutyrate, and 1-methylhistidine. Carnosine synthetase has been found in a wide range of tissues including muscle, red blood cells, and brain; it is particularly high in olfactory bulbs of the mouse (1).

1.c Products of Beta-dipeptide hydrolysis

The beta-alanyl-dipeptides are degraded by enzymes present in the gut and in various tissues to give beta-alanine and the second amino acid. Beta-alanine is found in human serum, milk, and urine. Carnosinase is widely distributed in the tissues.



In contrast, the products of beta-aspartyl-dipeptides are alpha- aspartate and the second amino acid. Hydrolysis of the dipeptide with the addition of water across the amide bond yields a dicarboxylic acid; alpha and beta-amino aspartate are equivalent.

Beta-aspartyl peptidases have been reported in *E. coli* (14, 15) and liver, kidney, brain, lung, skeletal muscle and heart muscle (6, 16). There is a difference in substrate specificity between the liver and bacterial enzymes (See Table 1.c.). Note that beta-Asp-Phe is hydrolyzed by the bacterial enzyme.

 Table 1.c
 RELATIVE SUBSTRATE SPECIFICITIES OF BETA-ASPARTYL PEPTIDASES

<i>Escherichia coli</i>		Rat Liver	
Substrate	Percent Maximum	Substrate	Percent Maximum
β -Asp-LEU	100	β -Asp-GLY	100
-SER	82	β -Asp-Gly-Gly	95
-MET	68	-MET	82
-VAL	56	-LEU	65
-GLN	48	-SER	56
-PHE	38*	β -Asp-Gly-Ala	55
-ALA	33	-ALA	51
-ILE	19	Leucylglycine	50
-THR	18	Glycylalanine	40
-ASN	10	Glycylglycine	40
-GLY	0	-ILE	37
α -ASP-PHE	0	-THR	29
β -Ala-HIS	0 [Carnosine]	-VAL	28

Results in each column are relative to the best substrate (100%) for that enzyme source. Bacterial enzyme did not degrade tripeptides.

Table compiled from data contained in references 6, 14-16.

2. Natural Abundance of beta-Dipeptides in Humans

Beta-Dipeptides are endogenous chemicals found in both plants and animals. This section describes the natural abundance of beta-di- and tri-peptides in humans. In section 3, the sources of beta-dipeptides in humans are discussed.

To date, data on the physiologic levels of two families of beta-dipeptides have been reported: beta-aspartyl- and beta-alanyl-dipeptides.

References to beta-aspartyl-phenylalanine in tissues fluids were not found. It was identified, but not quantified in pooled human urine (See below).

2.a. Tissue pools of beta-dipeptides

The largest single pool of beta-dipeptides in mammals is the carnosine (beta-Ala-His) found in skeletal muscle (approx. 1.5-4 umol/g in rats; 100 umol/g tissue in pigs). It is also found in relatively high concentrations in the olfactory bulbs. Although the physiologic role of carnosine is unknown, carnosine has been implicated in muscle contraction (17) and neurotransmission (18-20). It has a beneficial effect on wound healing (21) and is depleted during infection (22). Carnosine can also be viewed as a storage form of both beta-alanine and histidine.

References to human tissue levels of the beta-aspartyl-dipeptides have not been found.

A single report of human serum beta-Asp-Gly levels (0.08 umol/dl; 8 uMolar) was found (23).

The urinary excretion rates of various beta-di- and tripeptides are listed in Table 2.1.

TABLE 2.1

URINARY LEVELS AND EXCRETION RATES FOR BETA-DIPEPTIDES

<u>Dipeptide</u>	<u>Daily loss (umol/day)</u>	<u>reference^d</u>	<u>(umoles/g creatinine)^a</u>		
			<u>males</u>	<u>females</u>	<u>children</u>
β -Asp-Gly ^c	108 (44-66) ^b	Buchanan Tanaka	---- 44.4	---- 61.4	---- 83.7
β -Asp-His	30-60	Pisano	----	----	----
β -Asp-Ala	33.6 (11-31) ^b	Buchanan Tanaka	---- 11.0	---- 20.7	---- 25.3
β -Asp-Asn	26.0	Buchanan	----	----	----
β -Asp-Glu	(10-34) ^b	Tanaka	10.0	23.0	20.4
β -Asp-Gln	13.0	Buchanan	----	----	----
β -Asp-Lys	(9.6-18) ^b	Lou	12.1	9.6	14.8
β -Asp-Ser	72.0 (9.9-20) ^b	Buchanan Tanaka	---- 9.9	---- 13.6	---- 14.9
β -Asp-Asp	(4.3-13.6) ^b	Tanaka	4.3	9.1	18.4
β -Asp-Thr	27.0 (3.9-8.7) ^b	Buchanan Tanaka	---- 3.9	---- 5.8	---- 13.2
β -Asp-Gly-Gly	7.2	Buchanan	----	----	----
β -Asp-Gly-Ala	2.2	Buchanan	----	----	----

Beta-Di-and Tripeptides identified in urine, but not quantified.

β -Asp-Met	Tanaka	identified, but not quantified.
β -Asp-(leu, ileu, val, phe)	Buchanan	"
β -Asp-Gly-(pro, val, Asn, Glu)	Buchanan	"

^a The average excretion rate for creatinine is 1-1.5 g/day.

()^b Estimated daily excretion in adults bases on average creatinine values listed above.

^c For reference, urinary excretion of alpha-aspartyl-glycine in healthy adults is 9.4 umol/day.

^d References: Buchanan (25, 26), Tanaka (24), Pisano (10), and Lou (27).

At LEAST 14 beta-aspartyl-dipeptides have been identified in HUMAN urine. Six beta-aspartyl-tripeptides have also been identified.

Tanaka (24) has calculated that the total excretion of acidic beta-aspartyl-dipeptides in healthy adults is about 150-250 umol/day. In addition, beta-Asp-His was reported by Pisano (10) to be about 30-60 umol/ day. This falls far short of accounting for the 1,444-1,887 umol/day of bound aspartic acid, one-fourth of which is in the form of asparagine, that appears in the urine. Thus, there are many aspartate containing peptides yet to be defined in the normal urine.

The level of each beta-deptide in tissues and physiologic fluids is dependent on their rates of formation and degradation. Definitive reports on the rates of formation and degradation of beta-dipeptides (with the exception of beta-Ala-His: carnosine) have not been found in the literature. For dipeptides that are "apparently" not degraded by human tissue and are stable in physiologic solutions, an estimate of levels can be obtained. For those beta-dipeptides that are degraded by human tissues, definition of their levels in tissues or fluids would not *per se* provide information as to their turnover (formation and degradation).

The interpretation of tissue levels is also of interest and open to discussion. Do relatively higher levels of a beta-dipeptide indicate a role in normal physiological function or the presence of a pool of poorly handled metabolic products or dietary molecules?

3. Sources of Beta-dipeptides

Humans produce beta-dipeptides as part of their normal metabolism. In addition, beta dipeptides are obtained from ingested materials (foods, fluids, drugs) that themselves contain such dipeptides or their precursors. The digestion of proteins to dipeptides primarily results in the formation of beta-aspartyl-dipeptides via the isomerization of the alpha-form. In contrast, the majority of the other beta-dipeptides (beta-alanyl-dipeptides) are products of concerted biochemical pathways, primarily carnosine metabolism. Beta-aspartyl-dipeptides can also be formed enzymatically.

3.a. Beta-Dipeptides derived from protein

3.a.1. Beta-Dipeptides derived from dietary protein

The beta-dipeptides derived from protein catabolism occur via a non-enzymatic chemical reaction involving the corresponding alpha-dipeptides. Peptides containing aspartate or asparagine readily undergo an intramolecular cyclization which ultimately yields the beta-form of aspartate (see section 1.b.); this accounts for the relative abundance of the beta-aspartyl dipeptides in protein digests. Putatively protein-derived beta-dipeptides have been measured in fluids of the gastrointestinal tract and urine.

Because enzyme digests of collagen and gelatin are known to contain beta-aspartyl-dipeptides and because orally administered gelatin to humans increases the urinary excretion of beta-aspartyl-glycine, it is reasonable to conclude that beta-aspartyl-dipeptides formed by the action of digestive enzymes in the gut are absorbed from the gastrointestinal tract into the blood stream. However, with the exception of carnosine, absorption of beta-dipeptides from the gut has not been demonstrated.

It is likely (although not proven) that protein turnover in human tissues also

contributes to the urinary pools of beta-asp-dipeptides because patients receiving only intravenous hyperalimentation (primarily glucose and minerals) also excrete these dipeptides via the urine [see Table 3.a.1].

TABLE 3.a.1.
URINARY EXCRETION OF BETA-ASPARTYL-GLYCINE
(umoles/day)

Reference	normal diet	restricted diet	hyperalimentation
Buchanan	103 (66-200)	65 (55-70)	55 (40-84)
Tanaka	44.4-66.6*	-----	35.6 (10.4-74.2)

Results shown as Mean (range).

* denotes recalculation from umol/g creatinine to umoles/day.

Data from Buchanan (25, 26) includes both males and females whereas data from Tanaka (24) includes only males.

2.a.2. Fecal Beta-asp-gly and Antibiotic Therapy

Beta-aspartyl-glycine has received attention in the infectious disease literature due to its metabolism by gut bacteria, but presumably not human gut tissue (28-31). A probable major source of beta-asp-gly are foods rich in collagen (e.g., meats and gelatin containing foods) due to their high glycine content; approximately one-third of the amino acids present in collagen are glycine. Although levels of beta-asp-gly in fecal samples are very low (below the limits of detection using standard protocols) under normal conditions, antimicrobial therapy resulting in the loss of the normal bacterial flora of the gut leads to a substantial increase in fecal excretion (1-2 micromole/g feces) of beta-asp-gly in humans. At this level, beta-asp-gly generation by protein digestion in the gut would be expected to range from 90 to 150 micromoles/day in adults. This should be regarded as a minimum range because it does not correct for loss of this dipeptide from the gut by absorption into the blood stream. There is also a passing reference that the beta-aspartyl dipeptides of -ser, -ala, and -gln were also present in feces from antibiotic treated patients.

From the above example it is clear that bacteria play a pivotal role in the breakdown of dietary beta-dipeptides.

2.a.3. Beta-dipeptides as products of tissue degradation

Although definitive studies showing that the *in vivo* degradation of human tissue protein leads to the formation of beta-dipeptides have not been found in the literature, it is likely that this occurs. The most direct evidence for this comes from the urinary excretion patterns of beta-dipeptides in patients undergoing intravenous hyperalimentation. When dietary protein is removed as a source of beta-dipeptides, the urinary excretion of these dipeptides falls to a lower "steady state" level that appears to be unique for each patient. However, at least a portion of this "steady state" excretion of beta-dipeptides may be contributed by the actions of a kidney enzyme (a pseudo-asparaginase) that utilizes asparagine and glycine (the preferred acceptor) to yield beta-aspartyl-glycine.

Clinically, loss of muscle or tissue protein is routinely assessed by urinary

excretion patterns of creatinine, urea nitrogen, or methyl-histidine. Changes in protein levels of several grams per day are not uncommon in certain anabolic and catabolic conditions.

3.b. Beta-Dipeptides as non-protein components of meat and plants

3.b.1. Beta-Dipeptides in meats

The histidine-containing dipeptides are the principal non-protein derived beta-dipeptides. Carnosine (beta-alanyl-histidine) and anserine (beta-alanyl-1 methylhistidine) have long been known to be present in significant amounts (130 and 4 $\mu\text{mol/g}$ protein) in skeletal muscles. More recently ophidine (beta-alanyl-3 methylhistidine) has also been detected in muscle (0.1-9 $\mu\text{mol/g}$ protein).

The tissue content of these dipeptides changes as a function of age and muscle examined. Because the RATIO of these beta-dipeptides changes with the age of the animal and the animal species and because these dipeptides are stable during cooking, studies have utilized this in determining the quality and label claims of processed meats (32). For example, determinations of the amount of chicken present in lucheon meats and the source (muscle; animal species; age at slaughter) of the meat found in "tinned" hams have utilized this approach.

Carnosine is formed from beta-alanine and histidine by the ATP-dependent enzyme carnosine synthetase. It is found in its highest levels in muscle. Dietary carnosine is partially degraded during digestion and is transported across the intestinal wall and in to the blood by a specific peptide transport system.

3.b.2. Beta-dipeptides in plants

Beta-aspartyl-aspartic acid is present in very low amounts in asparagus shoots; alpha-Asp-(Asp) and -(glu) were both found to be present at 4 $\mu\text{moles/kg}$ (33). This is the only report of beta-dipeptides in plants. In contrast there are many reports of gamma-glutamyl-dipeptides in plants. Conversations with agriculutral biochemists at the Northern Regional Research Center lead me to believe that few studies have investigated the presence of beta-dipeptides in plants.

3.c. Beta-Dipeptides derived from pharmaceuticals

In addition to endogenous and dietary sources of beta-dipeptides, the pharmaceutical industry is exploring the use of beta-amino acid containing drugs (34). These include the synthetic beta-amino acid derivatives of bradykinin, angiotensin, luteinizing hormone releasing hormone (LHRH), thyrotropin, various enkephalins, oxytocin, 14-cyclopeptides (growth hormone inhibitors), antibiotic P168 and edeine (an antibiotic). Most of these derivatives utilize beta-alanine, a common constituent of human tissues, urine, feces, and milk.

3.d. Aspartame containing soft drinks as a source of beta-dipeptides

A potential, non-classical source of beta-dipeptide is from foods or beverages containing aspartame, the methyl ester of alpha-aspartyl-phenylalanine. Tsang, Clarke, and Parrish (35) have reported that soft drinks containing aspartame do lose aspartame with storage. However, their data indicate that the major breakdown products are a diketopiperazine and alpha-aspartyl-phenylalanine, and NOT beta-aspartyl-phenylalanine. Heating of solutions containing aspartame to 100 C to simulate cooking results in racemization of aspartic acid and phenylalanine, but not formation of beta-dipeptides (36).

4. Disease-related changes in Beta-dipeptide levels

Uremia is the only condition for which beta-aspartyl-dipeptide levels have been measured and reported in serum (23). Beta-Asp-Gly levels were 5-22 fold higher in uremic patients (0.41-1.83 mg/dl) than in normal subjects (0.08 mg/dl).

This suggests that the kidney plays an important role in the elimination of beta-Asp-Gly, especially its excretion. Buchanan has shown that intravenously administered radiolabeled beta-Asp-Gly was nearly completely excreted intact via the kidneys (37). In contrast, alpha-Asp-gly was rapidly hydrolyzed and the constituent amino acids reutilized.

It should be pointed out that mammalian tissues (liver, muscle, kidney, brain) do possess a beta-aspartyl peptidase. It is of low specific activity in these tissues. In addition, this peptidase is thought to be located in the cytoplasm as a free enzyme thereby being separated from circulating beta-aspartyl dipeptides. Thus, the beta-aspartyl peptidases appear not to be of great significance in the elimination of these dipeptides.

5. Beta-dipeptides as potential neurotransmitters

Carnosine has been implicated as a neurotransmitter in mammals in the olfactory pathway (18-20). However, its role as a neurotransmitter has yet to be define.

Beta-aspartyl-glycine is rapidly formed from extracellular aspartate and glycine by ganglion of the mollusk *Aplysia californica* (11, 12). Addition of exogenous beta-Asp-Gly had no effect on several parameters commonly associated with nervous activity in these ganglia. The role of this beta-dipeptide in *Aplysia* is unknown at this time.

It is noteworthy that administration of exogenous beta-Asp-Gly to mice with compromised kidney function (pretreated with uranyl acetate) caused behavioral changes (low activity and responsivity to stimuli); two animals eventually died (23). However, the pathophysiological significance of this data is open to criticism. The LOWEST dose of beta-Asp-Gly used that caused these behavior effects resulted in serum levels that were 635-fold and 50-fold higher than in normals and uremic patients, respectively. Many substance are known to accumulate in serum during uremia; their effects were not defined.

6. Summary with illustration

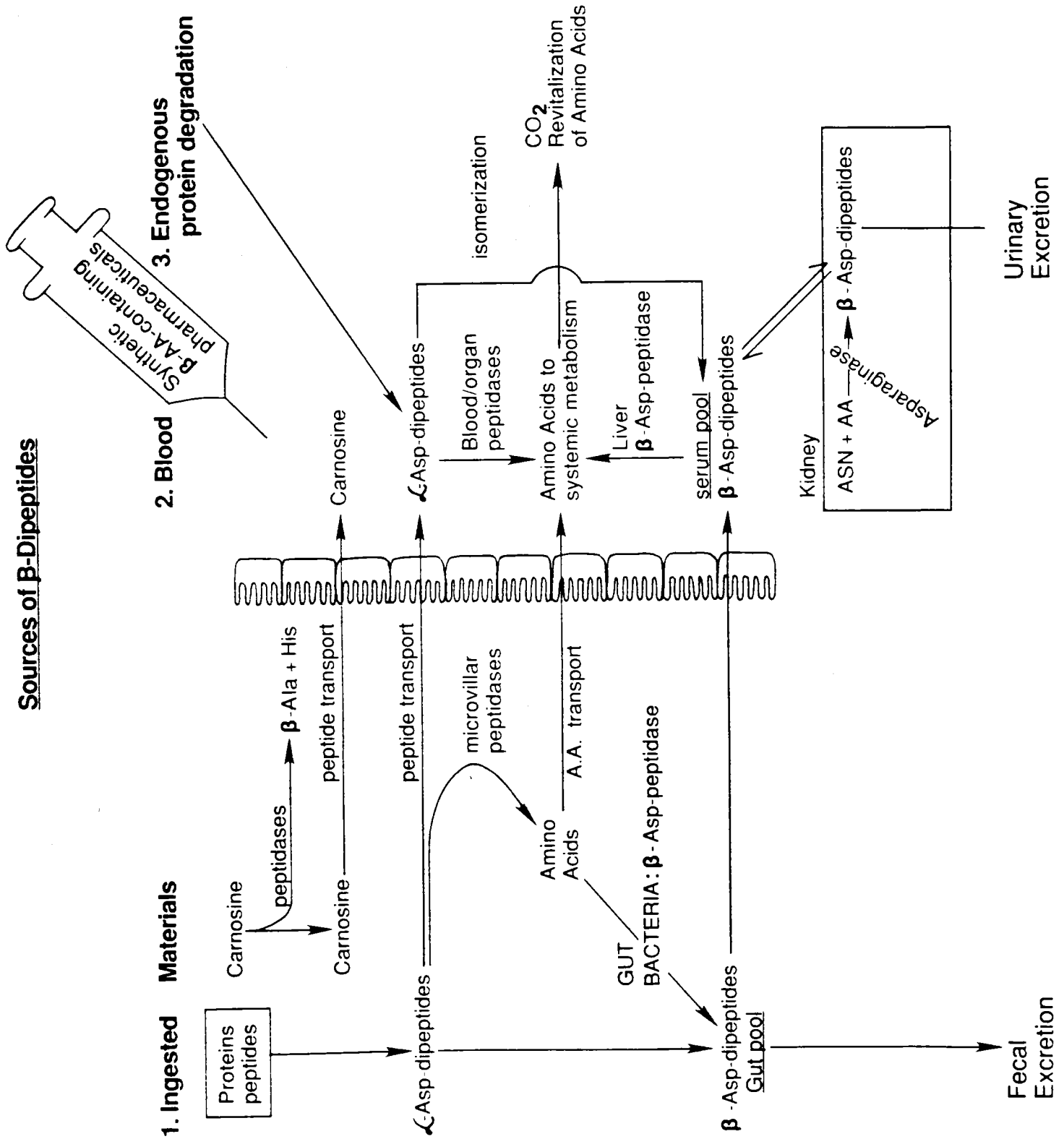
Beta-dipeptides are found in humans as products of both biochemical synthesis and rearrangement of degradative products of proteins. Two families of beta-dipeptides have been found: the beta-alanyl-dipeptides typified by carnosine are found in greatest abundance and beta-aspartyl-dipeptides typified by beta-Asp-Gly are formed predominately as products of protein degradation.

The acquisition, formation, metabolism, and excretion of beta-dipeptides is illustrated on the following page. Both families of beta-dipeptides can be degraded by enzymes present in the gut. They can also be absorbed from the gastrointestinal tract. Carnosine is transported by a peptide transport system.

Once in the body the fate of any given beta-dipeptide will be dependent on its susceptibility to hydrolysis by serum or tissue enzymes. Carnosinase is found in many tissues. Several tissues possess beta-peptidase(s) capable of degrading beta-aspartyl dipeptides. From published reports, beta-Asp-Gly is excreted essentially intact following

intravenous injection.

Sources of β -Dipeptides



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