

**$\beta$ -ASPARTAME**

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# **$\beta$ -ASPARTAME**

## **Introduction**

Over the past 20-30 years, significant advances have taken place in understanding the chemistry, occurrence and safety of  $\beta$  amino acids and dipeptides. A wide variety of  $\beta$ -aspartyl dipeptides occur naturally in the human diet and in human tissues or fluids. Like other dietary aspartyl dipeptides, aspartame can rearrange to form the  $\beta$ -aspartyl form. This supplement contains seven articles, each in some way relevant to the safety of  $\beta$ -aspartyl dipeptides, particularly  $\beta$ -aspartylphenylalanine methyl ester ( $\beta$ -APM) and  $\beta$ -aspartylphenylalanine ( $\beta$ -AP). The articles discuss the natural occurrence of  $\beta$ -aspartyl dipeptides, the chemistry of  $\beta$ -APM and  $\beta$ -AP formation, the results of safety studies with  $\beta$ -APM and the metabolism of  $\beta$ -APM and  $\beta$ -AP.

The published literature demonstrates that  $\beta$ -aspartyl dipeptides are common human dietary components, that  $\beta$ -AP is a normal constituent of human serum and urine and that no adverse effects have been associated with the consumption of  $\beta$ -aspartyl dipeptides. Metabolism studies in humans have shown that  $\beta$ -APM undergoes complete first-pass metabolism in the gut to form  $\beta$ -AP and its metabolites (e.g., phenylalanine, aspartate, etc.). Numerous pharmacology and toxicology studies were done that confirm the safety of  $\beta$ -APM. The pharmacology studies were standardized primary screening studies used to assess the effects on the central nervous system, gastrointestinal system, cardiovascular and renal systems and a variety of other pharmacological parameters. There were no significant effects due to  $\beta$ -APM in these studies. In addition, standard acute, short term, long term, developmental and genetic toxicity studies were done. The only  $\beta$ -APM related effects in these studies were maternotoxicity in the rabbit teratology study when 750 mg/kg/day was dosed as a suspension by gavage and a decrease in body weight gain (associated with a decreased food consumption) in F1 male rats after weaning in the two generation reproduction study at 750 mg/kg/day. No effects were observed in any of these

studies at 500 mg/kg/day.

The amount of  $\beta$ -APM and  $\beta$ -AP formed from the use of aspartame in foods is quite low due to the relatively small amounts of aspartame used to sweeten foods and the small percentage that is formed under actual commercial conditions. The intake of aspartame in the United States has been studied using survey results obtained by the Marketing Research Corporation of America (MRCA). The combined estimated intake of both  $\beta$ -APM and  $\beta$ -AP, at the mean and 90th percentile, 14 day average are 0.02 and 0.05 mg/kg/day. Thus, the no observed effect level of 500 mg/kg/day is approximately 10,000 times higher than the estimated 90th percentile estimated total  $\beta$ -AP isomer intake.

In conclusion, the articles published in this supplement discuss the chemistry and safety of  $\beta$ -aspartyl dipeptides. The results of studies with  $\beta$ -APM add to the published literature that demonstrates that  $\beta$ -aspartyl dipeptides are safe and common dietary peptides.



## **THE OCCURRENCE AND SAFETY OF $\beta$ -DIPEPTIDES IN FOOD**

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**Running Title:**  $\beta$ -Dipeptides in Nutrition and Biology

**Key Words:**  $\beta$ -dipeptides, succinimide, protein catabolism, carnosine, isoaspartyl-,  
isoalanyl-, dietary protein

## ABSTRACT

$\beta$ -Aspartyl dipeptides are common and abundant naturally occurring components of the human diet and of animal and human tissues. They are produced by the intramolecular rearrangement of  $\alpha$ -aspartyl dipeptides formed during protein digestion and possibly are synthesized by mammalian enzymes. The total intake of  $\beta$ -aspartyl dipeptides is not known; however, the quantity of some dipeptides certain foods has been examined.  $\beta$ -Aspartyl dipeptides are natural components of the human body; at least 14  $\beta$ -aspartyl dipeptides and 6  $\beta$ -aspartyl tripeptides have been identified in human urine. There are no reports of adverse effects associated with the consumption of  $\beta$ -aspartyl dipeptides.

The high intensity sweetener, can also rearrange to a  $\beta$ -aspartyl dipeptide. Because only small quantities are formed, the consumption of  $\beta$ -L-aspartylphenylalanine methyl ester ( $\beta$ -APM) is very low. Metabolism studies with  $\beta$ -APM have shown that it is hydrolyzed in the gut and that only small quantities of the  $\beta$ -aspartyl dipeptide,  $\beta$ -aspartylphenylalanine, are absorbed. This dipeptide is a normal component of human plasma and urine. Numerous studies were conducted to confirm the safety of  $\beta$ -APM. The results demonstrate that  $\beta$ -APM is not toxic, embryotoxic, teratogenic, mutagenic, clastogenic or carcinogenic at doses or concentrations greatly exceeding those relevant to human exposure. These results confirm the published literature that shows that  $\beta$ -aspartyl dipeptides, and  $\beta$ -aspartylphenylalanine particularly, are safe.



## INTRODUCTION

Aspartame and its metabolites have been extensively studied for over 20 years and found to be extremely safe [Hattan, 1988]. Two aspartame rearrangement products, diketopiperazine and  $\beta$ -aspartylphenylalanine methyl ester ( $\beta$ -APM) were also extensively evaluated.

$\beta$ -Aspartame ( $\beta$ -APM) is formed in small amounts during the manufacture and use of aspartame [Witt, 199\_].  $\beta$ -APM is rapidly hydrolyzed in the gut to  $\beta$ -AP, 10% of which is absorbed; no  $\beta$ -APM reaches the circulation [Marietta et al., 199\_]. In animal studies including tests for mutagenesis, clastogenesis, teratogenesis, acute-, subchronic-, and chronic toxicity and carcinogenicity,  $\beta$ -APM has also been shown to be safe [Hattan, 1988; Allen et al., 199\_; Mayhew et al., 199\_]. Although little was known of  $\beta$ -dipeptide chemistry and biology 25 years ago when the early studies of aspartame metabolism were begun, the ubiquitous and innocuous nature of  $\beta$ -aspartyl-dipeptides is now evident from the published literature.

$\beta$ -Dipeptides are common, naturally occurring components of the diet. Two types of  $\beta$ -dipeptides, distinguished by the presence of either  $\beta$ -alanyl or  $\beta$ -aspartyl residues, have been reported in the literature. Research spanning over 30 years has shown that both  $\beta$ -dipeptide types are formed, degraded, and excreted by animals, including man. The goal of the review is to examine the occurrence of  $\beta$ -aspartyl dipeptides.

## CHEMISTRY

$\beta$ -Aspartyl dipeptides are composed of two amino acids linked by an amide bond; the aspartyl group is in the  $\beta$ -configuration. The nomenclature for  $\beta$ -peptides is reviewed in Griffith [1986].  $\beta$ -Aspartyl dipeptides are thought to be formed in three ways: rearrangement of free  $\alpha$ -aspartyl dipeptides to the  $\beta$ -form, hydrolysis of proteins and polypeptides that contain  $\beta$ -aspartate amino acids, or through conjugation of asparagine with another amino acid (Table 1).

The formation of  $\beta$ -aspartyl dipeptides in free dipeptides is a spontaneous intramolecular cyclization reaction that converts the  $\alpha$ - to the  $\beta$ -form [John and Young, 1954; Swallow and Abraham, 1958; Bryant et al., 1959; Bernhard et al., 1962; Aswad, 1984; Murray and Clark, 1984; Meinwald et al., 1986; Clarke, 1987; Gaines and Bada, 1987; Geiger and Clarke, 1987; Stamp and LaBuza, 1989]. Cyclization leading to  $\beta$ -amino acid formation is more rapid in peptides than in proteins because of the greater flexibility of the smaller peptide molecules [Clarke, 1987]. In this reaction, 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 the  $\beta$ -aspartyl dipeptide. As reviewed by Wright [1991], deamidation of asparaginyl residues preceding glycine, serine, and alanine residues in peptides and proteins is quite common and has been termed "the  $\beta$ -aspartyl shift mechanism". Such rearrangements apparently occur via a succinimide intermediate [Hill et al., 199\_; Yukawa, 199\_].

$\beta$ -Aspartyl groups may also occur in protein and the digestion of such protein may generate  $\beta$ -aspartyl dipeptides. Enzyme digests of collagen and gelatin are known to contain  $\beta$ -aspartyl dipeptides and orally administered gelatin to humans increases the urinary excretion of  $\beta$ -aspartyl glycine (Table 2). 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.

Denaturation of protein is thought to promote cyclization and  $\beta$ -amino acid formation, although cyclization is more rapid in peptides than proteins.  $\beta$ -Amino acids are present in di- and tripeptides liberated by the actions of proteases on a wide variety of proteins such as hemoglobin [Haley, 1966], ribonuclease [Haley, 1967], pepsin [Haley, 1966], fibrin [Haley, 1966] and both native collagen [Haley, 1966; Pisano, 1966] and collagen as contained in gelatins [Pisano, 1966]. However, because  $\beta$ -isomerization occurs spontaneously even under the tightly controlled conditions of peptide synthesis [Tam et al., 1988], isomerization may have occurred in the liberated di- and tripeptides and not in the protein per se. In any case, larger peptides and proteins have been shown to contain  $\beta$ -amino acid residues and the succinimide intermediate [Yamada et al., 1985; Luo et al., 1987; Ota et al., 1987; Voorter et al., 1988; Shapira et al., 1988; Johnson et al., 1988].

$\beta$ -Aspartyl dipeptides can also be formed by synthetic enzymes. In vivo synthesis involves enzymes specific for the  $\beta$ -aspartyl dipeptides. An efficient system for the uptake of  $\beta$ -aspartate and glycine followed by synthesis of  $\beta$ -aspartyl-glycine exists in ganglion of the mollusk Aplysia California [McCaman and Stetzler, 1984 and 1985]. 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 [Armould, 1986; Tanaka et al., 1978]. The most efficient route involves the combination of free asparagine as the aspartyl donor with a second amino acid as acceptor. The reported relative reaction rates with various acceptors were 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.

## DIETARY SOURCES OF $\beta$ -DIPEPTIDES

$\beta$ -Aspartyl dipeptides exist in the food as such or are produced during the digestion of protein; however, little is known regarding dietary intake rates.  $\beta$ -Aspartyl-dipeptides are present in plants [Kasai et al., 1981], although  $\beta$ -glutamyl-dipeptides are more ubiquitous in plants than  $\beta$ -dipeptides [Gejyo et al., 1978]. For example, levels of  $\beta$ -aspartyl-aspartic acid and  $\beta$ -aspartyl-glutamic acid were reported to be 4  $\mu$ moles/kg in asparagus shoots [Kasai et al., 1981]. Although free  $\beta$ -aspartyl dipeptides are present in animal blood, levels in uncooked, undigested meat are not available. The  $\beta$ -aspartyl dipeptides derived from dietary protein catabolism occur spontaneously from  $\beta$ -dipeptides and, to a lesser extent, the proteolysis of  $\beta$ -amino acid containing proteins. Putatively, protein-derived  $\beta$ -dipeptides have been measured in fluids of the gastrointestinal tract and urine.

## LEVELS OF $\beta$ -DIPEPTIDES IN HUMAN URINE AND SERUM

Because  $\beta$ -aspartyl dipeptides are primarily derived from the " $\beta$ -aspartyl shift reaction" in proteins and peptides, they have been found where proteins and peptides are present. At least 14  $\beta$ -aspartyl-dipeptides and 6  $\beta$ -aspartyl tripeptides have been identified in human urine [Buchanan, 1962b; Pisano et al., 1966; Dorer et al., 1966; Tanaka and Nakajima, 1978; Lou, 1975; Burton et al., 1989]. The urinary excretion rates of various  $\beta$ -di- and tripeptides in humans are listed in Table 3.

Tanaka [1978] has calculated that the total excretion of known acidic  $\beta$ -aspartyl-dipeptides in healthy adults is about 150-250  $\mu$ mol/day. In addition,  $\beta$ -Asp-His excretion was reported to be about 30-60  $\mu$ mol/day [Pisano, 1966]. This falls far short of accounting for the 1,444-1,887  $\mu$ mol/day of bound aspartic acid, one-fourth of which is in the form of asparagine, that appears in the urine [Tanaka and Nakajima, 1978]. Two  $\beta$ -dipeptides have been measured in human serum. The serum levels of  $\beta$ -Asp-Gly were 800  $\mu$ g/ml and serum levels of  $\beta$ -Asp-Phe, a rearrangement

product of aspartame were 5 ng/ml in normal volunteers [Ogawa et al., 1985; Burton et al., 1989].

Data regarding urinary excretion patterns of  $\beta$ -dipeptides in patients undergoing intravenous hyperalimentation provides evidence for the dual contribution of both the diet and endogenous sources (Table 2). 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  $\beta$ -Asp-Gly [Tanaka et al., 1978; Burton et al., 1989]. 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 [Monro, 1981].

### METABOLISM OF DIETARY AND ENDOGENOUS $\beta$ -ASPARTYL DIPEPTIDES

Based on metabolism data on  $\beta$ -APM,  $\beta$ -aspartyl dipeptides are extensively metabolized in the gut and only a small fraction are absorbed intact. Studies shown that  $\beta$ -AP is slowly, passively absorbed from the gastrointestinal tract, but  $\beta$ -AP is rapidly absorbed [Marietta, 199\_]; Haley, 1970b]. In contrast to  $\beta$ -AP which was hydrolyzed by enzymes in the liver, intestinal mucosa, and cecal contents,  $\beta$ -AP was degraded only by cecal contents and pure cultures of Escherichia coli B [Lipton, 1991]. Intravenously administered  $\beta$ -AP, like  $\beta$ -Asp-Gly, was eliminated intact via the urine [Marietta, 199\_]. Although mammalian tissues (liver, muscle, kidney, brain) do possess a  $\beta$ -aspartyl peptidase of low specific activity, it is thought to be located in the cytoplasm as a free enzyme and thus separated from circulating  $\beta$ -aspartyl dipeptides.

$\beta$ -Asp-Gly has received attention in the infectious disease literature [Welling and Groen, 1978;

Welling et al., 1980; Welling, 1982; Welling et al., 1985] due to its rapid metabolism by gut bacteria [Haley, 1968; Haley, 1970a] but presumably not mammalian tissue [Haley, 1970b; Dorer, 1968]. A probable major source of  $\beta$ -Asp-Gly are foods rich in collagen (e.g., meat 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  $\mu$ moles/g feces) of  $\beta$ -Asp-Gly in humans [Welling et al., 1985]. At this level,  $\beta$ -Asp-Gly generation by protein digestion in the gut would be expected to range from 90 to 150  $\mu$ moles/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 [Welling, 1982] that the  $\beta$ -aspartyl-dipeptides of  $\beta$ -ser,  $\beta$ -ala, and  $\beta$ -gln were also present in feces from antibiotic treated patients.

The kidneys play an important role in the elimination of  $\beta$ -aspartyl dipeptides. Buchanan has shown that intravenously administered radiolabeled  $\beta$ -Asp-Gly was almost completely excreted into the urine unchanged [Buchanan, 1962a]. In contrast,  $\beta$ -Asp-Gly was rapidly hydrolyzed and the constituent amino acids reutilized.

## SUMMARY

The conversion of aspartame to  $\beta$ -Aspartame and  $\beta$ -Asp-Phe involves a rearrangement reaction common in biology.  $\beta$ -Aspartyl dipeptides are present in human tissue and adverse effects associated with  $\beta$ -aspartyl dipeptides have been published.

## REFERENCES

- Allen JL, Guy RC, Chengelis CP, Semler DE, Dickie BC, Hjelle JJ, Kotsonis FN (199\_)  
General toxicology studies with  $\beta$ -Aspartame
- Arnould JM (1986) Repartition de la carcinine ( $\beta$ -alanyl-histamine) *chez les crustacés*  
*Decapodes*. Biochem System Ecol 14: 431-433
- Aswad DW (1984) Stoichiometric methylation of porcine adrenocorticotropin by protein  
carboxyl methyltransferase requires deamidation of asparagine 25: Evidence for methylation  
at the alpha-carboxyl group of atypical L-isoaspartyl residues. J Biol Chem 259: 10714-  
10721
- Bernhard SA, Berger A, Carter JH, Katchalski E, Sela M, Shalitin Y (1962) Co-operative  
effects of functional groups in peptides: I. Aspartyl-serine derivatives. J Am Chem Soc 84:  
2421-2434
- Bryant PM, Moor RH, Pimlott PJ, Young GT (1959) Amino-acids and peptides: Part XIV.  
Further studies on the synthesis of aspartyl-peptides. J Chem Soc 3868-3873
- Buchanan DL, Haley EE, Markiw RT (1962a) Occurrence of  $\beta$ -aspartyl and  $\gamma$ -glutamyl  
oligopeptides in human urine. Biochemistry 1: 612-619
- Buchanan DL, Haley EE, Markiw RT, Peterson AA (1962b) Studies on the *in vivo* metabolism  
of  $\alpha$ - and  $\beta$ -aspartylglycine-1-C. Biochemistry 1: 620-623
- Burton EG, Schoenhard GL, Hill JA, Schmidt RE, Hribar JD, Kotsonis FN, Oppermann JA  
(1989) Identification of N- $\beta$ -L-aspartyl-L-phenylalanine as a normal constituent of human  
plasma and urine. J Nutr 119: 713-721
- Clarke S (1987) Propensity for spontaneous succinimide formation from aspartyl and  
asparaginyl residues in cellular proteins. Int J Peptide Protein Res 30: 808-821
- Dorer FE, Haley EE, Buchanan DL (1966) Quantitative studies of urinary  $\beta$ -aspartyl  
oligopeptides. Biochemistry 5: 3236-3240
- Dorer FE, Haley EE, Buchanan DL (1968) The hydrolysis of  $\beta$ -aspartyl peptides by rat tissue.  
Arch Biochem Biophys 127: 490-495
- Gaines SM, Bada JL (1987) Reversed-Phase high-performance liquid chromatographic  
separation of aspartame diastereomeric decomposition products. J Chromatogr 389: 219-225
- Geiger T, Clarke S (1987) Deamidation, isomerization, and racemization at asparaginyl and  
aspartyl residues in peptides: Succinimide-linked reactions that contribute to protein  
degradation. J Biol Chem 262: 785-794

- Gejyo F, Kinoshita Y, Ito G, Ikenaka T (1978) Identification of  $\beta$ -aspartylglycine in uremic serum and its toxicity. *Contr Nephrol* 9: 69-77
- Griffith OW (1986) Beta-Amino Acids: Mammalian metabolism and utility as alpha-amino acid analogues. *Ann Rev Biochem* 55: 855-878
- Haley EE, Corcoran BJ, Dorer FE, Buchanan DL (1966)  $\beta$ -Aspartyl peptides in enzymatic hydrolysates of protein. *Biochemistry* 5: 3229-3235
- Haley EE, Corcoran BJ (1967)  $\beta$ -Aspartyl peptide formation from an amino acid sequence in ribonuclease. *Biochemistry* 6: 2668-2672
- Haley EE (1968) Purification and Properties of a  $\beta$ -aspartyl peptidase from *Escherichia coli*. *J Biol Chem* 243: 5748-5752
- Haley EE (1970a) Beta-Aspartyl Peptidase. *Methods Enzymol* 19: 730-736
- Haley EE (1970b)  $\beta$ -Aspartyl peptidase from rat liver. *Methods Enzymol* 19: 737-741
- Hattan D (1988) Toxicological and nutritional evaluation of low nutrient density foods. In: Walker R, Quattrucci E (eds.) *Nutritional and Toxicological Aspects of Food Processing*, Taylor and Francis, New York, NY, pp 275-291
- Hill JB, Hribar JD, Liu NWK (199\_) Mechanistic aspects of the conversion of  $\alpha$ -aspartyl-phenylalanine methyl ester (Aspartame) to the  $\beta$ -isomer
- John WD, Young GT (1954) Amino-acids and peptides: Part XII. Alpha- and Beta-L-aspartyl L-valine. *J Chem Soc* 2870-2873
- Johnson BA, Murray ED, Clarke S, Glass DB, Aswad DW (1987) Protein carboxyl methyltransferase facilitates conversion of atypical L-isoaspartyl peptides to normal L-aspartyl peptides. *J Biol Chem* 262: 5622-5629
- Kasai T, Hirakuri Y, Sakamura S (1981) Aspartyl and glutamyl peptides and the acidic cysteine derivatives in asparagus (*Asparagus officinalis*) shoots. *Agric Biol Chem* 45: 433-437
- Lipton WE, Li Y-N, Younoszai MK, Stegink LD (1991) Intestinal absorption of aspartame decomposition products in adult rats. *Metabolism* 40: 1337-1345
- Lou MF (1975) Isolation and identification of L- $\beta$ -aspartyl-L-lysine and L- $\gamma$ -glutamyl-L-ornithine from normal human urine. *Biochemistry* 14: 3503-3508



- Luo S, Liao CX, McClelland JF, Graves DJ (1987) Formation of a cyclic imide in aspartyl or asparaginyl glycyl peptides induced by heating in the dry state. *Int J Peptide Protein Res* 29: 728-733
- Marietta MP, Burton EG, Burns TS, Schoenhard GL, Stargel WW, Oppermann JA, Tschanz C (199\_) A summary of the absorption, distribution, metabolism and elimination of  $\beta$ -aspartame and its free acid,  $\beta$ -aspartyl-phenylalanine, in the rat, dog, rabbit, monkey and man
- Mayhew DA, Piper CE, Noveroske JW, Kotsonis FN (199\_) Evaluation of the genetic and developmental toxicity of L- $\beta$ -aspartyl-L-phenylalanine-L-methyl ester
- McCaman MW, Stetzler J (1984) Identification of an acidic dipeptide,  $\beta$ -Aspartylglycine, in the CNS of Aplysia. *J Neurochem* 43: 1375-1384
- McCaman MW, Stetzler J (1985) Synthesis of the novel dipeptide  $\beta$ -Aspartylglycine by Aplysia ganglia. *J Neurochem* 45: 983-986
- Meinwald YC, Stimson ER, Scheraga HA (1986) Deamidation of the asparaginyl-glycyl sequence. *Int J Peptide Protein Res* 28: 79-84
- Monro HN (1981) Nutrition and Ageing. *British Med Bull* 37: 183-88
- Murray ED Jr., Clark S (1984) Synthetic peptide substrates for the erythrocyte protein carboxyl methyltransferase: Detection of a new site of methylation at isomerized L-aspartyl residues. *J Biol Chem* 259: 10722-10732
- Ogawa T, Oka Y, Sasaoka K (1985) Free amino acids and related compounds in seeds and sprouts of winged bean *Psophocarpus tetragonolobus*. *J Food Science* 50: 1503-1504
- Ota IM, Ding L, Clarke S (1987) Methylation at specific altered aspartyl and asparaginyl residues in glucagon by the erythrocyte protein carboxyl methyltransferase. *J Biol Chem* 262: 8522-8531
- Pisano JJ, Prado E, Freedman J (1966)  $\beta$ -Aspartylglycine in urine and enzymic hydrolyzates of proteins. *Arch Biochem Biophys* 117: 394-399
- Shapira R, Wilkinson KD, Shapira G (1988) Racemization of individual aspartate residues in human myelin basic protein. *J Neurochem* 50: 649-654
- Stamp JA, LaBuza TP (1989) Mass spectrometric determination of aspartame decomposition products: Evidence for  $\beta$ -isomer formation in solution. *Food Additives and Contaminants* 6: 397-414

- Swallow DL, Abraham EP (1958) Formation of  $\epsilon$ -(Aminosuccinyl)-lysine from  $\epsilon$ -aspartyl-lysine from Bacitracin A, and from the cell walls of Lactobacilli. *Biochem J* 70: 364-373
- Tam JP, Riemen MW, Merrifield RB (1988) Mechanisms of aspartimide formation: The effects of protecting groups, acid, base, temperature and time. *Peptide Research* 1: 6-18
- Tanaka T, Nakajima T (1978) Isolation and identification of urinary  $\beta$ -aspartyl-dipeptides and their concentrations in human urine. *J Biochem* 84: 617-625
- Tanaka T, Hirai M, Nakajima T (1978) Partial purification and characterization of an enzyme involved in the formation of  $\beta$ -aspartyl-dipeptides in rat kidney. *J Biochem* 84: 11471-1153
- Voorter CEM, de Haard-Hoekman WA, van den Oetelaar PJM, Bloemendal J, de Jong WW (1988) Spontaneous peptide bond cleavage in aging alpha-crystallin through a succinimide intermediate. *J Biol Chem* 263: 19020-19023
- Welling GW, Groen G (1978)  $\beta$ -Aspartylglycine, a substance unique to caecal contents of germ-free and antibiotic-treated mice. *Biochem J* 175: 807-812
- Welling GW, Groen G, Tuinte JHM, Koopman J, Kennis HM (1980) Biochemical effects on germ-free mice of association with several strains of anaerobic bacteria. *J Gen Microbiol* 117: 57-63
- Welling GW (1982) Comparison of methods for the determination of  $\beta$ -aspartylglycine in fecal supernatants of leukemic patients treated with antimicrobial agents. *Journal of Chromatography* 232: 55-62
- Welling GW, Helmus G, de Vries-Hospers HG, Tonk RHJ, van der Waaij D, Haralambie E, Linzenmeier G. (1985) Rationale for use of  $\beta$ -aspartylglycine as indicator of colonization resistance. *Biomedical Science* 155-158
- Wright HT (1991) Nonenzymatic deamidation of asparaginyll and glutaminyll residues in proteins. *Critical Reviews in Biochemistry and Molecular Biology* 26: 1-51
- Yamada H, Ueda T, Kuroki R, Fukumura T, Yasukochi T, Hirabayashi T, Fujita K, Imoto T (1985) Isolation and characterization of 101  $\beta$ -lysozyme that possesses the  $\beta$ -aspartyl sequence at aspartic acid-101. *Biochemistry* 24: 7953-7959
- Yukawa T. (199\_) Mechanism of aspartame degradation reaction

**TABLE 1**  
**SOURCES AND FORMATION OF  $\beta$ -DIPEPTIDES**

PROTEIN  $\xrightarrow{\text{Proteolysis}}$   $\alpha$ -DIPEPTIDE  $\xrightarrow{\text{Rearrangement}}$   $\beta$ -DIPEPTIDES  
from diet  
or tissue

$\beta$ -amino acid +  $\alpha$ -amino acid  $\xrightarrow{\text{Conjugation}}$   $\beta$ -DIPEPTIDES

PROTEIN  $\xrightarrow{\text{Proteolysis}}$   $\beta$ -DIPEPTIDES  
containing  
 $\beta$ -amino acids

TABLE 2

**URINARY EXCRETION OF BETA-ASPARTYL-GLYCINE**  
( $\mu$ moles/day)

Reference	Normal Diet	Restricted	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).

Data from Buchanan (29,30) includes both males and females whereas data from Tanaka (31) includes only males.

\* denotes recalculation from  $\mu$ mol/g creatinine to  $\mu$ mol/day

**TABLE 3**  
**URINARY LEVELS AND EXCRETION RATES FOR  $\beta$ -DIPEPTIDES**

Dipeptide	Daily Loss ( $\mu$ mol/day)	Reference <sup>d</sup>	( $\mu$ moles/g creatinine) <sup>a</sup>		
			Males	Females	Children
-Asp-Gly <sup>c</sup>	108	Buchanan	---	---	---
	(44-66) <sup>b</sup>	Tanaka	44.4	61.4	83.7
-Asp-His	30-60	Pisano	---	---	---
-Asp-Ala	33.6	Buchanan	---	---	---
	(11-31) <sup>b</sup>	Tanaka	11.0	20.7	25.3
-Asp-Asn	26.0	Buchanan	---	---	---
-Asp-Glu	(10-34) <sup>b</sup>	Tanaka	10.0	23.0	20.4
-Asp-Gln	13.0	Buchanan	---	---	---
-Asp-Lys	(9.6-18) <sup>b</sup>	Lou	12.1	9.6	14.8
-Asp-Ser	72.0	Buchanan	---	---	---
	(8.8-20) <sup>b</sup>	Tanaka	9.9	13.6	14.8
-Asp-Asp	(4.3-13.6) <sup>b</sup>	Tanaka	4.3	9.1	18.4
-Asp-Thr	27.0	Buchanan	---	---	---
	(3.9-8.7) <sup>b</sup>	Tanaka	3.9	5.8	1.32
-Asp-Gly-Ala	7.2	Buchanan	---	---	---
-Asp-Phe	2.7	Burton	1.87 <sup>e</sup>	---	---
-Asp-Gly-Ala	2.2	Buchanan	---		
$\beta$ -Di- and Tri-peptides identified in urine, but not qualified					
-Asp-Met		Tanaka	---	---	---
		Buchanan	---	---	---
-Asp-(leu, ileu, val, phe)		Tanaka	Identified but not quantified		
		Buchanan			
-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 based on average creatinine values listed above.

c For reference, urinary excretion of -aspartyl-glycine in healthy adults is 9.4  $\mu$ mol/day.

d References: Buchanan (29,30), Tanaka (31), Pisano (15), Lou (32), and Burton (28).

e Data from group of males and females; data shown is a 24 hour collection; if first a.m. urine catch is used, then a calculated value of 2.57  $\mu$ mol  $\beta$ -asp-phe/g creatinine is obtained.



**THE STABILITY OF ASPARTAME AND ITS  
CONVERSION PRODUCTS IN WET BEVERAGE SYSTEMS**

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**Key Words:** Aspartame, aspartylphenylalanine,  $\beta$ -aspartame,  $\beta$ -dipeptide, conversion products

## ABSTRACT

A study was carried out to monitor the behavior of aspartame and its transformation to a number of its conversion products. The systems included in the study were a series of simulated carbonated beverages ranging in pH from 2.55 to 4.37. The temperatures studied were 5°, 20°, 30°, and 40°C. Analytical methodology was developed to assay all the known conversion products and also to monitor the formation of any unknown or unexpected products. In this study, the rate of formation and quantities of the various conversion products were determined. A complete mass balance of the initial aspartame was obtained. No significant unknown materials were found. In the one case examined in this study, the flavor of the beverage had no effect upon the rate of aspartame conversion or the quantities of specific materials formed and a complete mass balance was also obtained.



## INTRODUCTION

Aspartame (N-L- $\alpha$ -aspartyl-L-phenylalanine, 1-methyl ester) is a sweetener manufactured by The NutraSweet Company. Currently aspartame (marketed as NutraSweet® brand sweetener) is used as a tabletop sweetener, in a very large variety of food products, and beverage products including carbonated soft drinks. To determine the effect that typical and extreme market beverage conditions have on the integrity of aspartame ( $\alpha$ -APM) in carbonated beverages, a stability study was initiated which was intended to simulate retail beverage market conditions covering a reasonable range of pH, temperature and time. The primary goals of the study were to achieve mass balance of the original  $\alpha$ -APM through quantitative analysis of  $\alpha$ -APM and its known conversion products, to determine that there is no significant formation of any other compounds, and determine the  $\alpha$ -APM chemical conversion pathway and any trends regarding the kinetics of  $\alpha$ -APM conversion. In order to achieve these objectives, it was necessary to develop analytical methods with sufficient sensitivity to quantitate  $\alpha$ -APM and its related conversion products when present at 0.1% of the initial aspartame concentration. This report discusses the information generated through 52 weeks of the study.

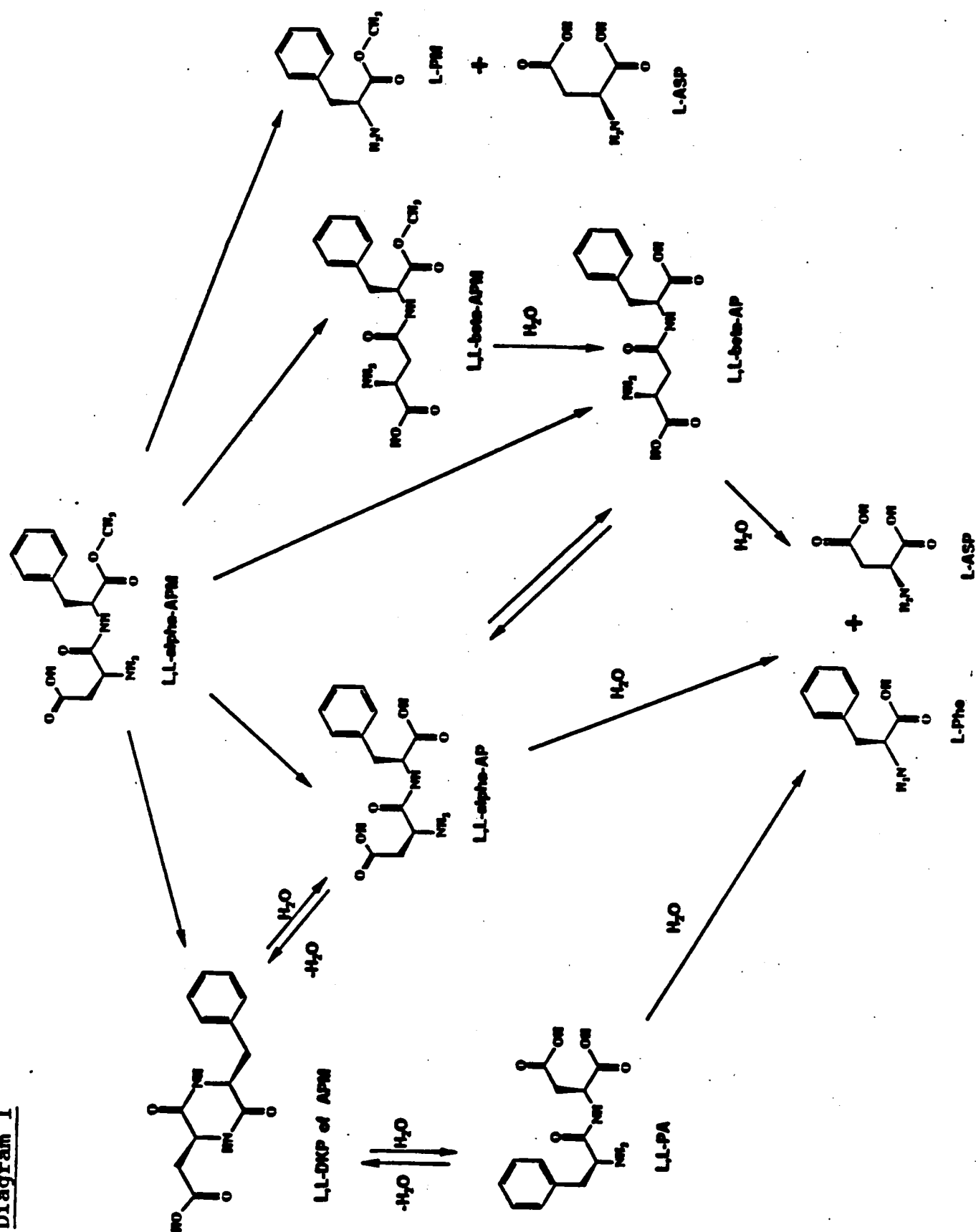
There is a large amount of background information available concerning the characteristics of aspartame and its conversion products in a variety of food systems (Beck, 1978; Vetsch, 1985; Homler, 1991). The most definitive work is the current study utilizing conditions present in the preparation and marketing of carbonated beverages. The conversion products of aspartame under these conditions are known (Scherz et al., 1983; Prudel and Davidkova, 1985; Tsang et al., 1985; Neiryneck and Nollet, 1988; Saito et al., 1989; Hayakawa, 1990). These conversion products are typical of other food products containing aspartame and are as follows:

**Compound**

- |                  |   |
|------------------|---|
| 1. $\alpha$ -APM | N-L- $\alpha$ -aspartyl-L-phenylalanine, 1-methyl ester |
| 2. $\alpha$ -AP  | N-L- $\alpha$ -aspartyl-L-phenylalanine                 |
| 3. $\beta$ -APM  | N-L- $\beta$ -aspartyl-L-phenylalanine, 1-methyl ester  |
| 4. $\beta$ -AP   | N-L- $\beta$ -aspartyl-L-phenylalanine                  |
| 5. DKP           | Diketopiperazine of -APM                                |
| 6. PA            | N-L-phenylalanyl-L-aspartic acid                        |
| 7. L-Phe         | L-phenylalanine   |
| 8. PM            | L-phenylalanine methyl ester                            |
| 9. L-Asp         | L-aspartic acid   |
| 10. MeOH         | Methanol  |

The overall scheme by which these materials are formed is shown in the following diagram [Diagram 1, Prodoliet and Bruehlhart, 1993].

Diagram 1



These reactions can be placed into one of the following categories.

1. Conversion of aspartame into its cyclic diketopiperazine derivative (DKP) formed by elimination of methanol.
2. Cleavage of the diketopiperazine derivative into either one of the two possible dipeptide derivatives, aspartyl phenylalanine (AP) or phenylalanylaspartic acid (PA).
3. Hydrolysis of the peptide methyl esters to the free carboxylic acid derivatives. Both the and isomers of aspartame can undergo this transformation.
4. Conversion of the  $\alpha$ -isomers into the  $\beta$ -derivatives. This conversion occurs for both aspartame (APM) and aspartylphenylalanine (AP).
5. Further hydrolysis of the various dipeptide derivatives into its various amino acid components, phenylalanine (and its methyl ester) and aspartic acid.

## MATERIALS AND METHODS

### Analytical Methods

In order to meet the objectives of the study, it was necessary to ensure that adequate and proper analytical methodologies existed to measure the known components and be able to detect the presence of any other materials. Due to the nature of the compounds of interest and the current state of analytical technology, it was apparent that High Performance Liquid Chromatography (HPLC) was the method of choice for the quantitation of most of the components. HPLC has been proven to be a highly sensitive technique, and affords the necessary selectivity and

flexibility to quantitatively monitor many compounds with similar structure and properties [Verzella and Mangia, 1985; Verzella et al., 1985; Stamp and Labuza, 1989]. For the volatile methanol, gas chromatography (GC) was utilized and L-aspartic acid was quantitated by amino acid analysis (AAA) because of its lack of a chromophore in the UV range. An HPLC method was developed which enabled an adequate determination of eight materials that were known to be present in the beverage systems. Specific conditions developed for the HPLC assay are as follows.

Column:	Ultrasphere Octyl 25 cm x 4.6 mm
Mobile Phase:	15% CH <sub>3</sub> CN/85% 0.18 N NaH <sub>2</sub> PO <sub>4</sub> , 0.02 N Heptane Sulfonate, Na, pH = 2.2
Flow:	1.5 mL/min
Temperature:	Column 40°C
Detection:	UV, 210 nm at 0.2 AUFS
Injection:	20 µl

#### Gas Chromatography

Methanol was determined by packed-column gas chromatography preceded by ion-exchange sample clean-up. Beverage samples were prepared for analysis using Amberlite IR-120 ion exchange resin, sodium form (Rohm and Haas). The resin was conditioned prior to use with 5 mM phosphoric acid and air dried. A 10 ml aliquot of the degassed beverage sample was mixed with 1 gram of the Amberlite in a 25 x 150 mm screw capped culture tube and shaken mechanically for 20 minutes. The mixture was allowed to stand until separation of the resin and supernatant liquid occurred and the GC sample drawn directly from the supernate.

Gas chromatography was performed under the following conditions:

Chromatograph:	Hewlett-Packard 5880
Column:	Porapak Q (80/100 mesh), 6' X 0.25" (2 mm i.d.), glass
Oven Temperature:	105°C
Carrier Gas:	Helium
Carrier Flow Rate:	50 ml/minute
Injection Volume:	5 µl
Injection Port Temperature:	115°C
Detector:	Flame Ionization
Detector Temperature:	225°C
Air Flow Rate:	400 ml/minute
Hydrogen Flow Rate:	40 ml/minute
Detector Attenuation:	1 pAmp/cm

Analyses were done typically under isothermal conditions, except when the sample beverages contained additional flavorings. For flavored samples, a post-run oven temperature program was employed.

Oven Program	105°C for 8 minutes
	105°C to 215°C at 50°C/minute
	215°C for 10 minutes

This method for methanol was found to be linear from 0.8 to 80 mg/l with a precision of 4.26% RSD (N=12) and a recovery of 104.2% (N=12).

Amino Acid Analysis

L-Aspartic acid (Asp) levels in the beverage samples were determined by a standard Carlo Erba amino acid analysis method. The method used in Ninhydrin derivatization reaction.

Instrumental conditions were as follows:

Apparatus:	Carlo Erba 3A29
Column:	3AR/6/DC/20 25 cm x 4.6 mm
Column Temperatures:	40°C initial 50°C for 18 minutes 64°C final
Eluent:	pH 3.5 citrate buffer solution
Reagent:	30 ml/hour
Reagent Flow Rate:	20 ml/hour
Injection Volume:	50 µl
Detector Range:	0.5 AUFS

The citrate buffer solution consisted of 0.15M sodium citrate, 2% (v/v) ethanol. 0.2% (v/v) thioglycol and 0.01% (v/v) buffer preservative (Pierce Chemical Co., Rockford, IL) and 9.5% (v/v) concentrated hydrochloric acid. The ninhydrin derivatization solution consisted of 0.06M ninhydrin, 75% (v/v) methylcellosolve, 25% (v/v) of 4M sodium acetate buffer solution (Pierce) and 2mM stannous chloride dihydrate (solution prepared under nitrogen). This method for L-aspartic acid determination was linear from 8 to 40 mg/l with a precision of 0.70% RSD (N=18) with recover of 99%.

Liquid Chromatography

Aspartame and its impurities were determined by reversed-phase ion-pairing HPLC. The impurities monitored for included N-L- $\beta$ -aspartyl-L-phenylalanine, 1-methylester ( $\beta$ -APM), N-L- $\alpha$ -aspartyl-L-phenylalanine ( $\alpha$ -AP), N-L- $\beta$ -aspartyl-L-phenylalanine ( $\beta$ -AP), N-L-Phenylalanyl-L-aspartic acid (PA), L-phenylalanine (Phe), L-phenylalanine, 1-methylester (PM) and 3,6-dioxo-5-(phenylmethyl)-2-piperazineacetic acid (DKP). Instrumental conditions were as follows:

Chromatograph:	Waters 590 pump
	Waters 712 WISP autoinjector
	Kratos 757 UV/VIS detector
Column:	Altex Ultrasphere C8 25 cm x 4.6 mm
Column Temperature:	40°C
Eluent:	15% (v/v) acetonitrile
	85% (v/v) 0.02 M heptanesulfonic acid (Kodak) buffered with 0.20 M sodium phosphate/phosphoric acid to pH 2.2
Eluent:	1.5 ml/minute
Injection Volume:	20 $\mu$ l
Detection:	210 nm at 0.2 AUFS

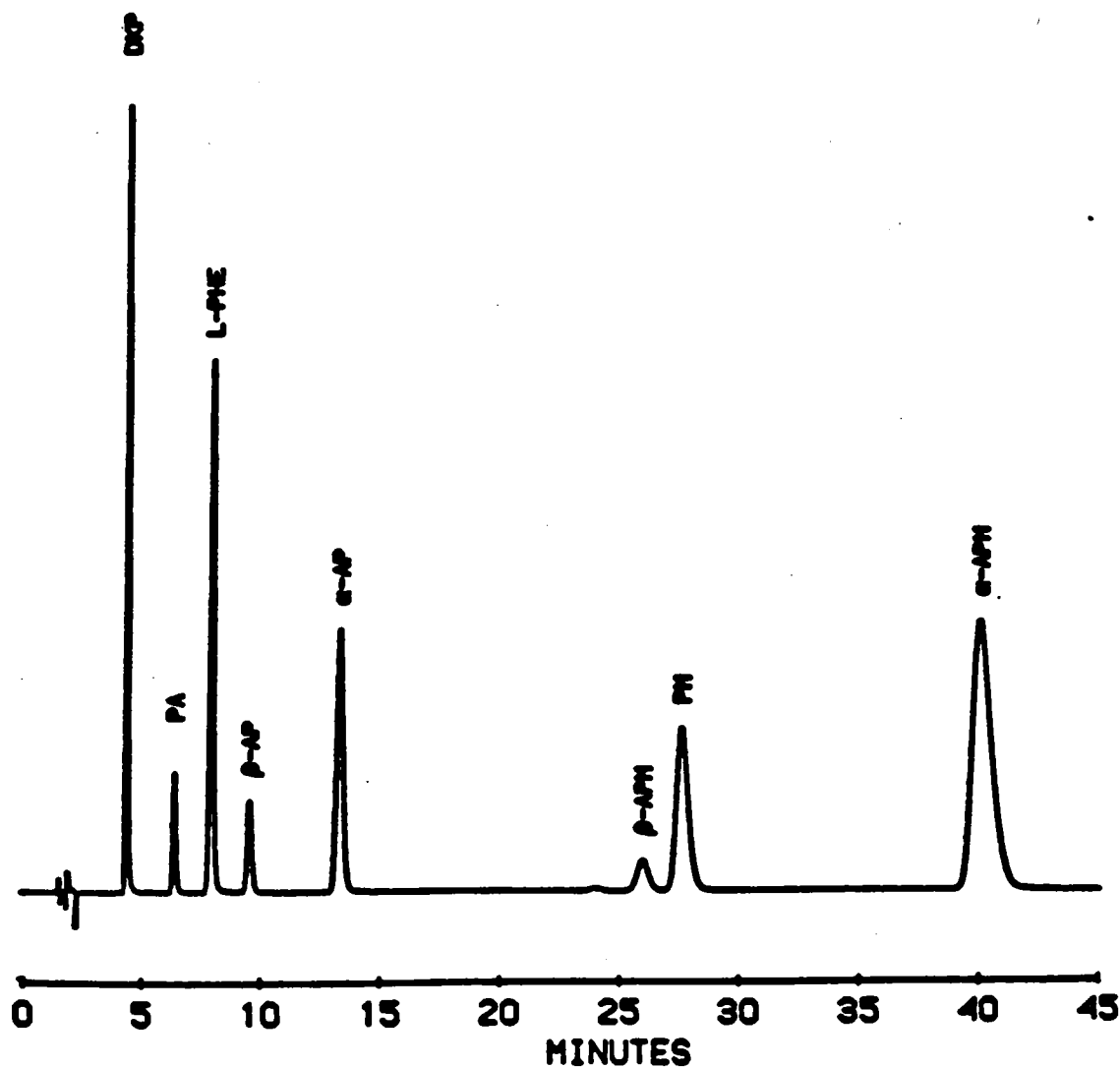
The validation parameters for the eight analytes were as follows:

Analyte	Linearity (mg/l)	Precision (% RSD)
$\alpha$ -APM	140 - 700	1.4
$\beta$ -APM	1 - 50	1.9
$\alpha$ -AP	40 - 200	1.4
$\beta$ -AP	1 - 50	1.2
L-Phe	40 - 200	2.4
DKP	40 - 200	1.5
PA	1 - 50	0.9
PM	50 - 250	1.8



The typical chromatogram of the various known conversion products is shown below. As can readily be seen, all eight components are readily distinguishable and separated efficiently from the other components.

### HPLC Chromatogram



In addition to the HPLC method discussed above, a GC method for the determination of methanol and an amino acid procedure for the determination of aspartic acid was also developed.

The analytical methods were validated and are summarized below.

Assay	Compound	Recovery	Standard Deviation (Accuracy)	MDQ** (Precision)
HPLC	$\alpha$ -APM	100.5%	1.4%	0.4 mcg/mL
	$\beta$ -APM	100.2%	1.9%	0.3 mcg/mL
	$\alpha$ -AP	100.2%	1.4%	0.2 mcg/mL
	$\beta$ -AP	100.8%	1.2%	0.1 mcg/mL
	DKP	100.4%	1.5%	0.1 mcg/mL
	L-Phe	102.1%	2.4%	0.1 mcg/mL
	PM	101.3%	1.8%	0.3 mcg/mL
	PA	99.1%	0.9%	0.1 mcg/mL
GC	MeOH	104.2%	4.3%	0.8 ppm
AAA	L-Asp	99.0%	0.7%	0.04 mcg/mL

\*\* MDQ = Minimum detectable quantity. Estimate was based on the response calculation at two times the noise level.

## STUDY CONDITIONS

A series of five different simulated carbonated beverages were prepared and used in the study. The pH range was chosen to approximate the pH values that are most frequently encountered in commercial beverages. The five simulated beverages used in the study are described below.

## COMPOSITION

pH	3.52*	2.55	3.19	3.48	4.37
Aspartame**	0.50***	0.50	0.50	0.50	0.50
80% Phosphoric Acid	--	0.83	0.83	--	--
Citric Acid	0.96	--	--	0.96	0.96
Sodium Citrate	0.28	--	0.46	0.28	1.20
Sodium Benzoate	0.20	--	0.20	0.20	0.20
(Preservative)					
Lemon-Lime Flavor	--	--	--	0.95	--

\* pH values listed are the pH values at time zero.

\*\* Concentration of each component given in units of grams/liter.

\*\*\* The approximate initial concentration of  $\alpha$ -APM

Four of the five studies contained aspartame, the appropriate buffer to control pH and a preservative. A fifth study also contained a lemon-lime flavor. This flavor was typical of that used by the beverage industry to produce a lemon-lime beverage. The purpose of this additional study was to determine whether the flavor had any effect upon the conversion rate and the specific amounts of the various conversion products. The beverages were stored at temperatures of 5°, 20°, 30° and 40°C. Samples were removed at various times and assayed. The sampling points were chosen by use of the Arrhenius principle based on a pseudo first order kinetic conversion model.

## RESULTS/DISCUSSION

In the following discussion a number of the more pertinent observations and conclusions are reviewed. The main items are the disappearance rates of aspartame under the various conditions of the study, the formation rate and the total amounts of the primary conversion products, the impact of flavor upon the conversion rate, and the mass balance or material accountability. As expected and known from previous studies [Scott, 1974; Furda et al., 1975; Harper, 1975; Mazur, 1976; Homler, 1984; Prudel et al., 1986; Gaines and Bada, 1988] the rate of conversion of aspartame to other products is dependent upon both pH and temperature. These effects are shown in Graphs 1 and 2. Graph 1 indicates the percent of aspartame remaining at various time periods for the studies employing an initial pH of 2.55, 3.52, and 4.37. The temperature for this graph is 20°C. As seen from the graph, aspartame is converted to its conversion products more rapidly at lower pH values, i.e. more acidic conditions. A similar correlation for the effect of pH is found at other temperature values examined in the study. This rate of conversion has been found to be acceptable for commercial beverages.

The effect of temperature is shown in Graph 2 for the quantity of aspartame present. For illustrative purposes, the study utilizing a pH of 3.52 is shown. As expected the conversion of

aspartame to the other products show a definite temperature effect. A similar effect is shown at the other pH systems used in the study.

As noted earlier two of the primary conversion products of aspartame in food products is the product of internal cyclization, diketopiperazine, DKP, and the methyl ester hydrolysis product,  $\alpha$ -aspartylphenylalanine,  $\alpha$ -AP. The formation of these products is also dependent upon both pH and temperature. Graphs 3 and 4 include the formation of DKP and  $\alpha$ -AP at the three pH values of 2.55, 3.52, and 4.37 at a temperature of 20°C.

The formation rates of both DKP and AP are generally consistent with disappearance of aspartame. This is, their rate of formation increases at lower pH values and increased temperatures.

As shown in the overall flow diagram of the various conversion products, Diagram 1, the diketopiperazine derivative when formed is subsequently hydrolyzed to the two possible dipeptide compounds, aspartylphenylalanine,  $\alpha$ -AP, and phenylalanylaspartic acid, PA.

Therefore, the concentration of DKP would reach a maximum and then decreased due to further degradation. However, this maximum (i.e., ~30-32%) has been achieved only when all the aspartame has disappeared.

A similar situation exists with  $\alpha$ -AP in that it can be subsequently hydrolyzed to its constituent amino acid components, aspartic acid and phenylalanine. Therefore, it also reaches a maximum value and then decreases over extended periods of time as the secondary reaction occurs.

The other primary conversion products are the  $\beta$ -compounds,  $\beta$ -aspartylphenylalanine,  $\beta$ -AP, and the methyl ester,  $\beta$ -APM. It has been shown independently that  $\alpha$ -AP can be converted to  $\beta$ -AP and that  $\alpha$ -APM can be converted to  $\beta$ -APM (Lawrence and Iyengar, 1987; Stamp and

Labuza, 1989). For illustrative purposes the total amount of  $\beta$ -AP and  $\beta$ -APM are combined on Graphs 5-8. Graph 5 illustrates the relationship between the total amount of the two  $\beta$ -compounds at pH values of 2.55, 3.52, and 4.37. This graph includes the data for the 20°C study.

As seen from the graphs, pH and temperature have only slight effects upon the total amount of the  $\beta$ -derivatives formed. In the 20°C study shown in Graph 5, there is only a slight increase in the total amount of  $\beta$ -derivatives as pH decreases.

Another interesting consideration is the comparison of the total quantity of  $\beta$ -compounds with the quantity of aspartame remaining in the solution. This correlation is shown in Graphs 6 and 7 at two temperatures, 20° and 40°C, for all five systems used in the study. As seen from the graph, the  $\beta$ -compounds reach a maximum value of approximately 15-25% depending upon the specific condition. However, these quantities would be obtained only upon complete disappearance of the aspartame. When approximately 50% of the aspartame has been converted, the total amount of  $\beta$ -compounds is approximately 10 to 15%, depending upon the specific pH of the study. This is important because this is the approximate level of aspartame required to maintain an acceptable beverage for consumption. A limited series of random and aged commercial beverages were also analyzed and the above relationship was consistently found.

The effect of temperature upon the presence of the  $\beta$ -derivatives is shown in Graph 8. As expected, the maximum quantities are formed at the higher temperatures, consistent with the disappearance of aspartame.

As with the  $\alpha$ -AP, DKP, and PA, the  $\beta$ -derivatives will also undergo further hydrolysis over an extended period of time to its component amino acids, aspartic acid and phenylalanine.

As stated earlier, one study was carried out to determine whether the presence of flavor had any effect upon the rate of reaction and amount of individual components formed. Two beverage systems were prepared at a pH of approximately 3.5 and were identical in all aspects except that one system contained a lemon-lime flavor. Data on the two systems were obtained and a comparison made. Graph 9 shows data at 40°C that the rate of aspartame disappearance, the rate of formation and quantities of conversion products are essentially identical in both systems. Therefore, the presence of the lemon-lime flavor does not influence aspartame and its conversion products. The specific conditions shown in Graph 9 are for 40°C but the same conclusion is obtained at the other temperatures employed.

## MASS BALANCE

A key objective of the study was to ensure that the analytical methodology allowed us to obtain a complete accounting of all materials, the aspartame added to the beverage at time zero and its conversion products, that is, obtain a complete material balance.

The analysis examines both the actual amount of L-Phe-containing material and this amount relative to the initial amount (mole balance closure). The amount of L-Phe-containing material is defined to be the sum (in units of micromoles/milliliter) of the following compounds:  $\alpha$ -APM,  $\alpha$ -AP,  $\beta$ -APM,  $\beta$ -AP, DKP, PM, L-Phe, and PA.

The mole balance closure at a given time and temperature condition for a given study is defined as:

$$100 \times \frac{\text{amount of L-Phe-containing material}}{\text{initial amount of L-Phe-containing material}}$$

The mole balance closure is a traditional and well-understood measure of quantification of

material, and it is useful because its reference point of 100% is independent of amounts or concentrations that may vary from study to study or laboratory to laboratory.

The mole balance closures ranged from 96 percent to 104 percent over all five stability studies.

The ranges and average closures for each study are as follows:

pH Study	Number of Time-Temperature Points	Mole Balance Closure (%)		
		Minimum	Average	Maximum
3.52	25	97.8	100.5	103.3
2.55	26	97.9	100.8	102.2
3.19	25	96.8	99.8	102.5
3.48	24	96.6	100.0	104.4
(with flavor)				
4.37	22	95.9	99.8	103.9

In addition to the mole balance closure calculations above, the HPLC chromatograms were examined to ensure that no significant peaks of unexpected or unusual materials were present. It was found that no unusual materials were present. In addition, a number of samples were examined by thin layer chromatography as an additional analytical test to confirm the above observations.

## CONCLUSION

Results of this study have shown that a complete material balance and accounting of aspartame and its conversion products is obtained. These results are found when only a small amount of aspartame has been converted and also when all of the aspartame has been converted. The products formed are known and well-characterized. The analytical methodology is, therefore, suitable for measuring aspartame and its conversion products and studying the kinetics of its degradation.

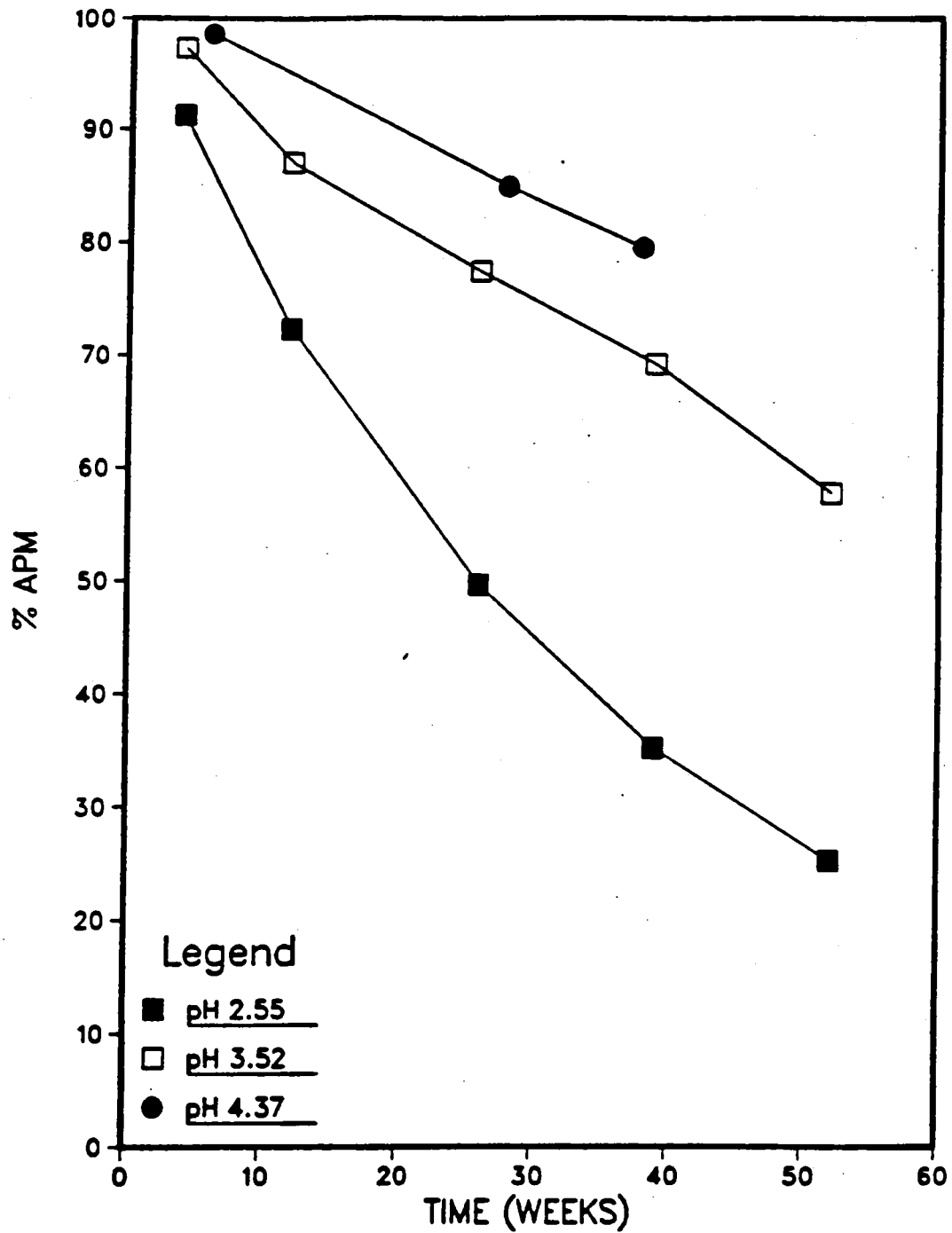
## REFERENCES

- Beck CI (1978) Application potential for aspartame in low calorie and dietetic foods. In: Inglett GE (ED) Low Calorie and special dietary foods, CRC Press, Chapter 5, Boca Raton, FL, 59-114
- Furda I, Malizia PD, Kolor MG, Vernieri PJ (1975) Decomposition products of l-aspartyl-l-phenylalanine methyl ester and their identification by gas-liquid chromatography. J Agric Food Chem 23: 340-343
- Homler BE (1984) Properties and stability of aspartame. Food Technol 38: 50-55
- Langguth P, Alder R, Merkle HP (1991) Studies on the stability of aspartame (i): specific and reproducible hplc assay for aspartame and its potential degradation products and applications to acid hydrolysis of aspartame. Pharmazie 46(3): 188-192
- Lawrence JF, Iyengar JR (1987) Liquid chromatographic determination of beta-aspartame in diet soft drinks, beverage powders and pudding mixes. J Chromatogr 404: 261-266
- Locock RA, Pagliaro LA, Michetti R, Sluchinski D (1986) The analysis and stability of aspartame. Clin Invest Med 9: 86
- Motellier S, Wainer IW (1990) Direct stereochemical resolution of aspartame stereoisomers and their degradation product by high-performance liquid chromatography on a chiral crown ether based stationary phase. J Chromatogr 516(2): 365-373.
- Neiryneck W, Nollet L (1988) Determination of the stability of aspartame in soft drinks by reversed-phase liquid chromatography. Belg J Food Chem Biotechnol 43(3): 83-88
- Noda K, Iohara T, Hirano Y, Hayabuchi H (1991) Stability of l-aspartyl-l-phenylalanine methyl ester, a peptide sweetener, in aqueous solutions of various fruit juices. J Home Econ Jpn 42(8): 691-6955
- Ozol T (1986) Stability of aspartame in artificial syrups. Acta Pharm Turc 28: 125-130
- Prudel M, Davidkova E (1985) Determination of the decomposition products of usal in model systems and determination of dioxopiperazine in soft drinks by hplc. Nahrung 29(4): 381-389
- Prudel M, Davidkova E, Davidek J, Kminek M (1986) Kinetics of decomposition of aspartame hydrochloride (usal) in aqueous solutions. J Food Sci 28: 1393-1415
- Saito K, Horie M, Hoshino Y, Nose N, Nakazawa H, Fujita M (1989) Determination of diketopiperazine in soft drinks by high performance liquid chromatography. J Liq Chromatogr 12(4): 571-582

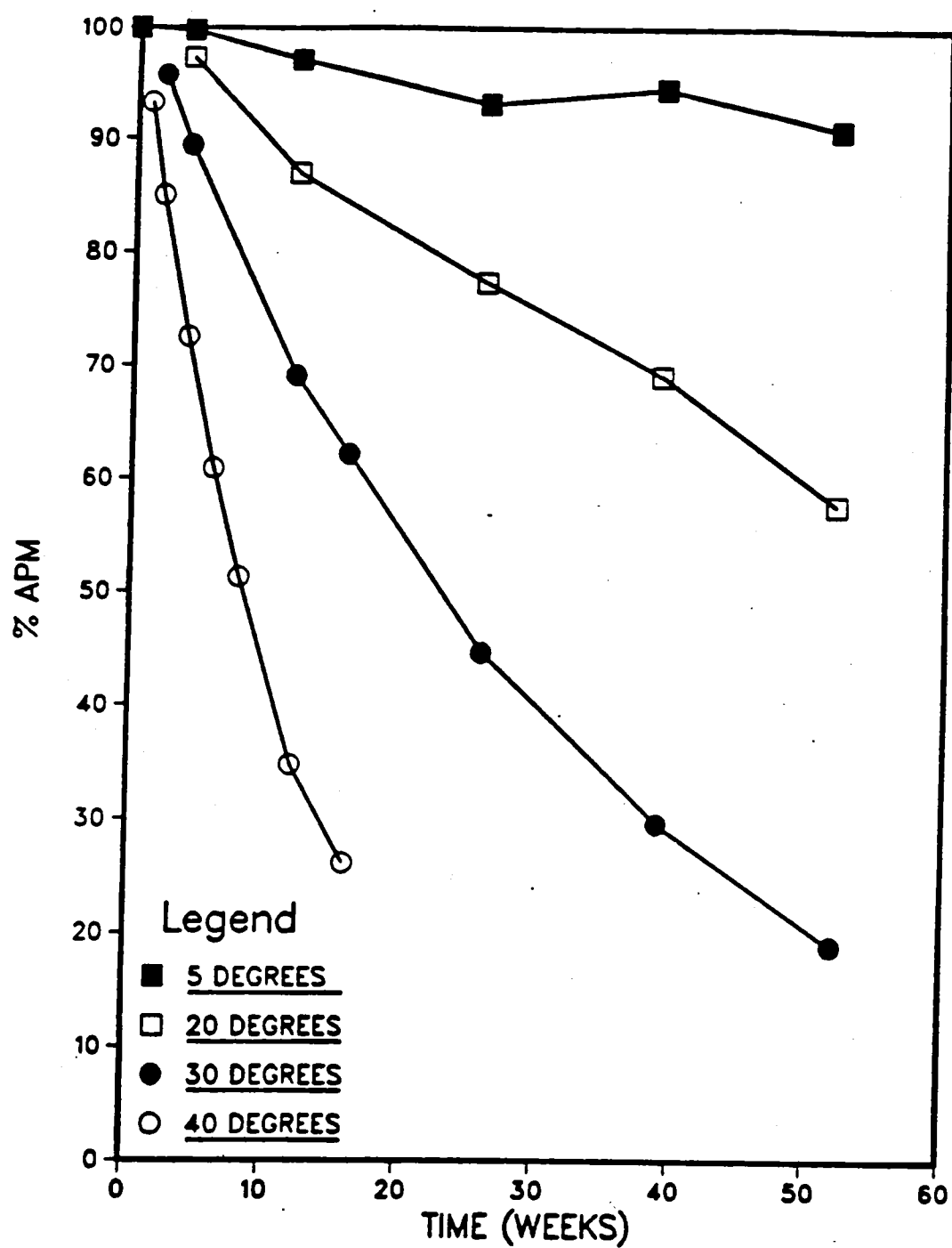


- Sanyude S, Locock R, Pagliaro L (1991) Stability of aspartame in water-organic solvent mixtures with different dielectric-constants. *J Pharm Sci* 80(7): 674-676.
- Stamp JA, Labuza TP (1989) Mass spectrometric determination of aspartame decomposition products: Evidence for B-isomer formation in solution. *Food Addit Contam* 6(4): 397-414
- Tateo F, Triangeli L, Panna E, Berte F, Verderio E (1988) Stability and reactivity of aspartame in cola-type drinks. *Frontiers of Flavor* 217-231
- Tsoubeli M, Labuza T (1991) Accelerated kinetic-study of aspartame degradation in the neutral pH range. *J Food Sci* 56(6): 1671-1675.
- Tuncel T, Araman A (1989) Stability of aspartame in some diet products marketed in Turkey. *Acta Pharm Turc* 31(2): 61-66
- Witt J (1986) The stability of aspartame and its conversion products in wet beverage systems. *International Aspartame Workshop Proceedings, Marbella, Spain, Nov 17-21, 1986, International Life Sciences Institute, Nutrition Foundation, Washington, D.C.*

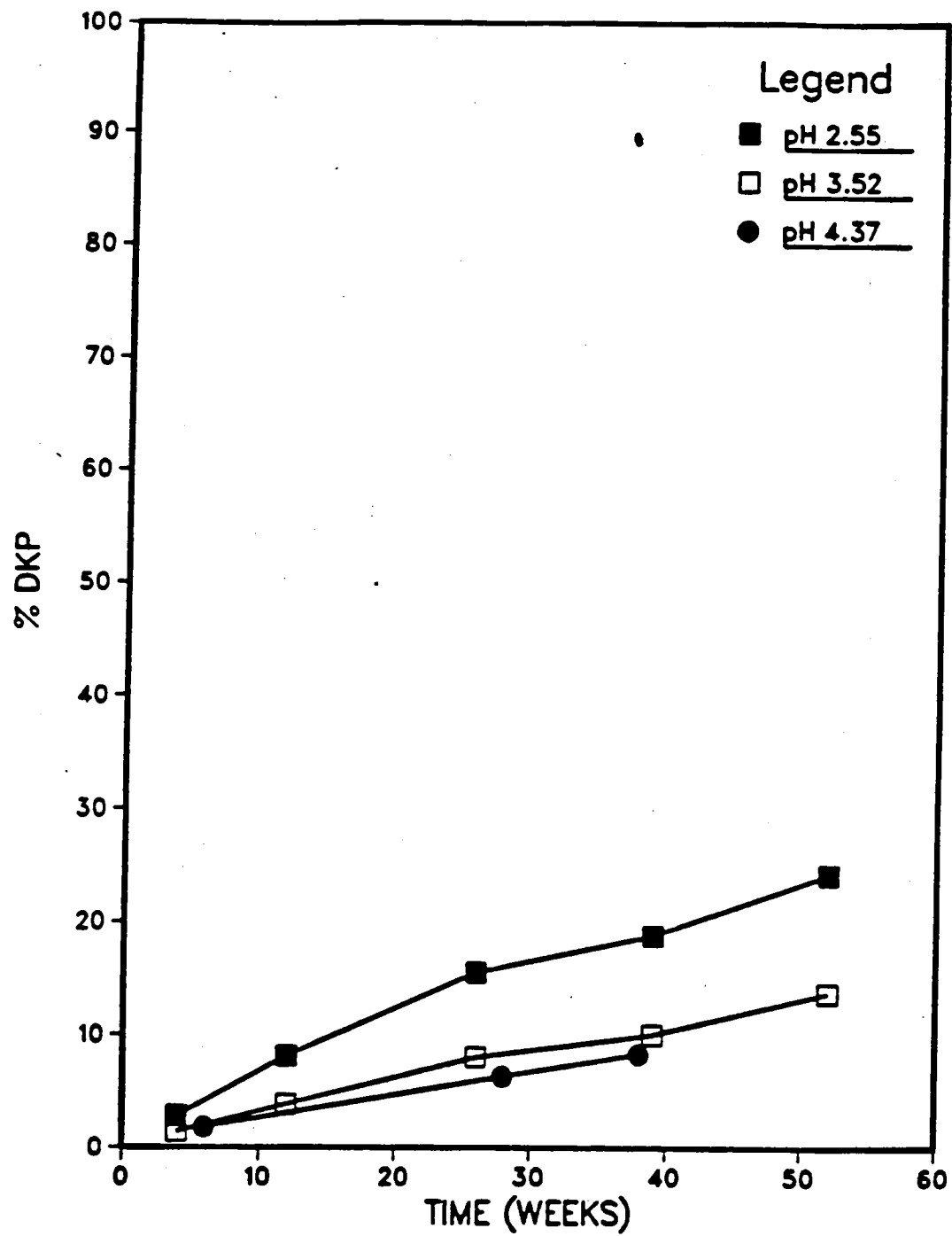
GRAPH 1: EFFECT OF pH  
AT 20°C



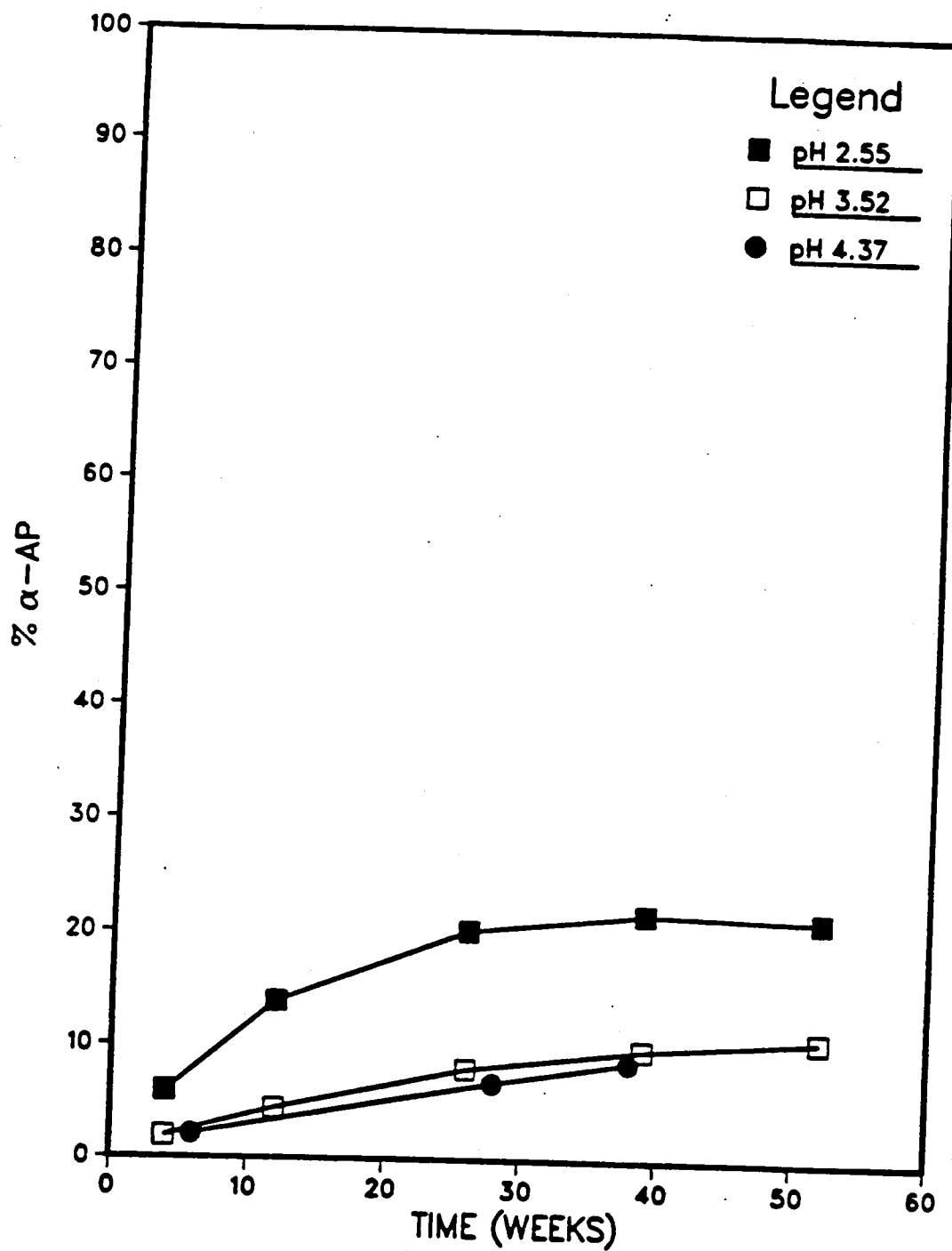
GRAPH 2: EFFECT OF TEMPERATURE  
AT pH 3.52



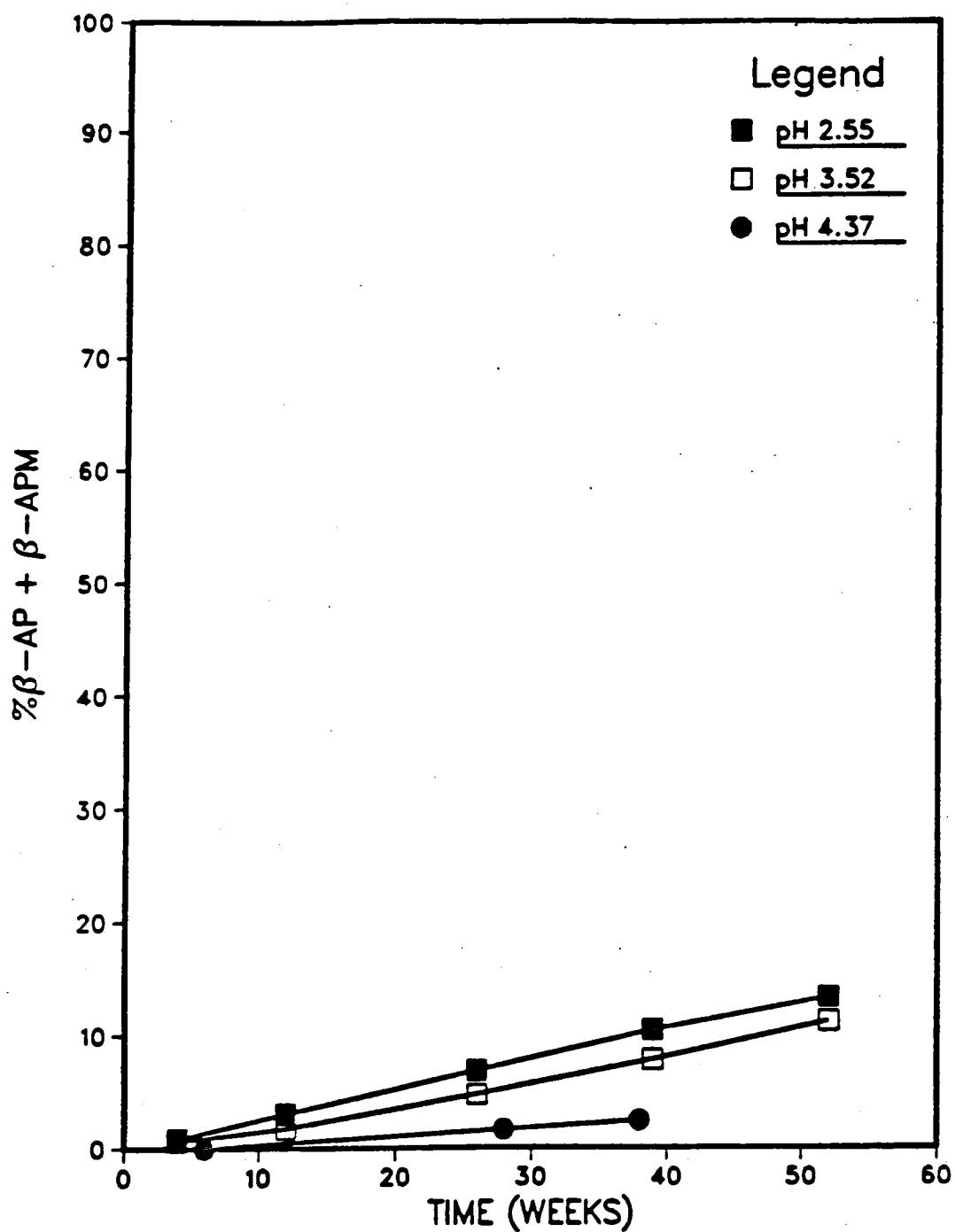
GRAPH 3: pH EFFECT ON DKP FORMATION  
AT 20°C



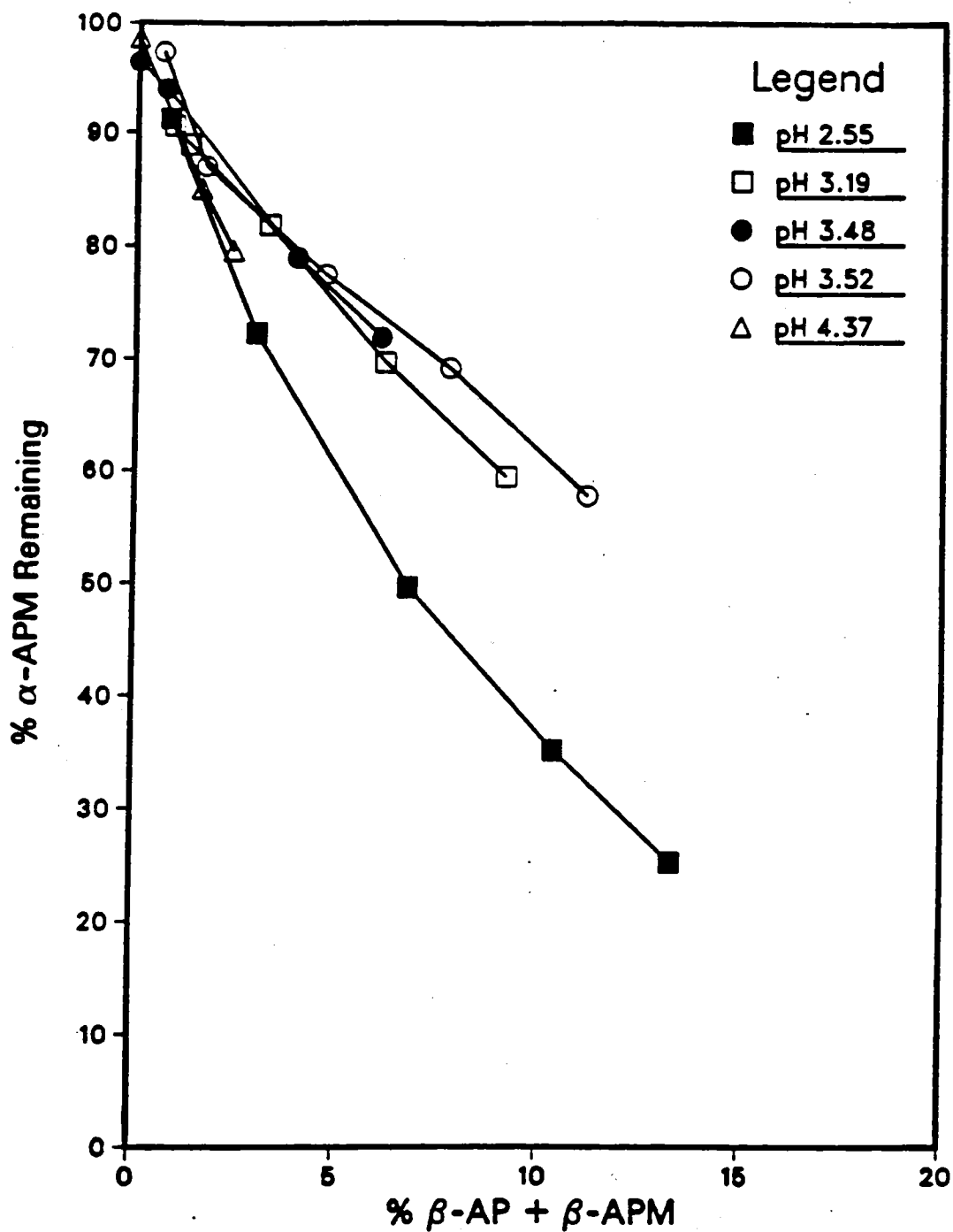
GRAPH 4: pH EFFECT ON  $\alpha$ -AP FORMATION  
AT 20°C



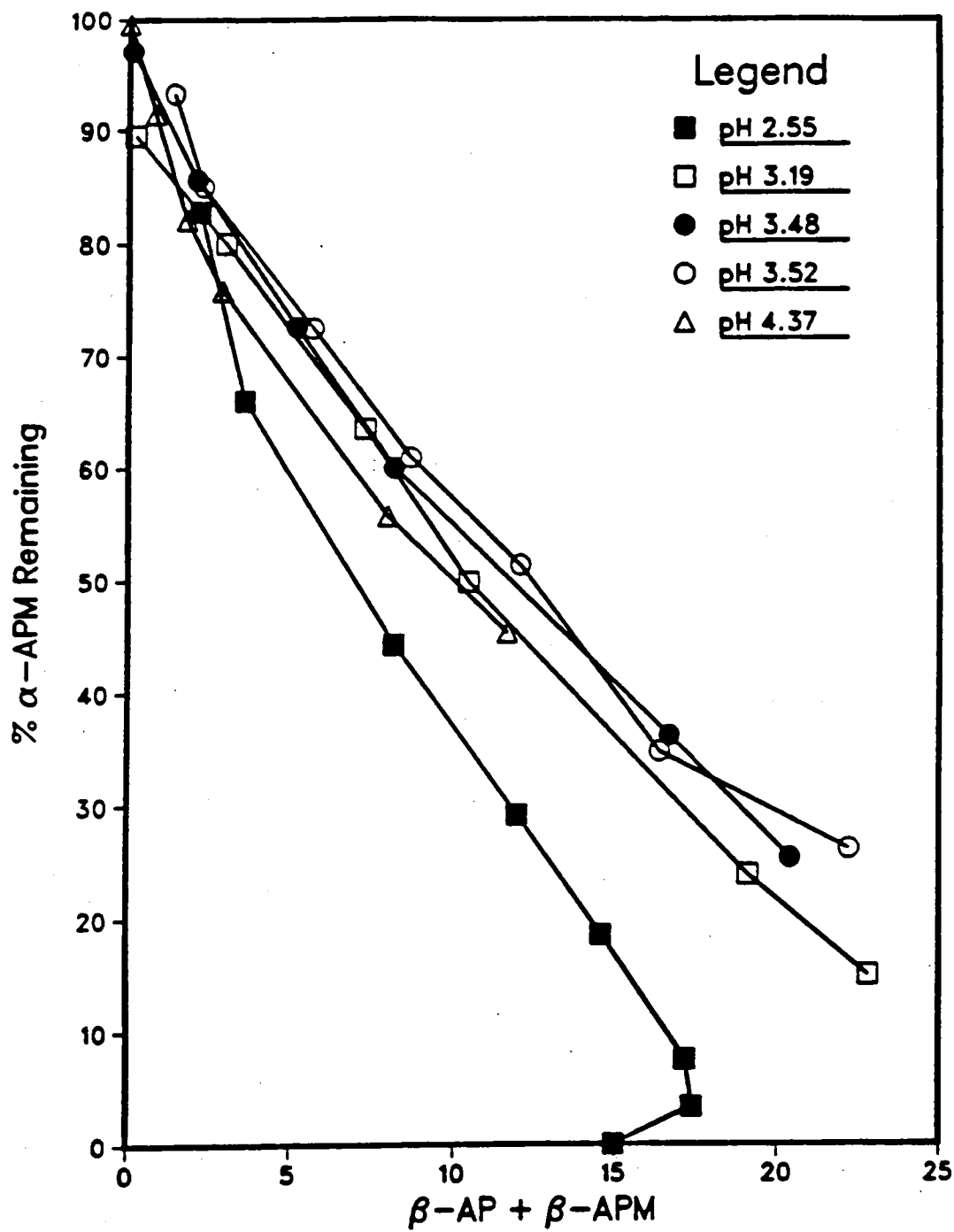
GRAPH 5:  $\beta$ -AP AND  $\beta$ -APM FORMATION  
AT 20°C



GRAPH 6: %  $\alpha$ -APM vs. %  $\beta$ -AP +  $\beta$ -APM  
FORMATION AT 20°C

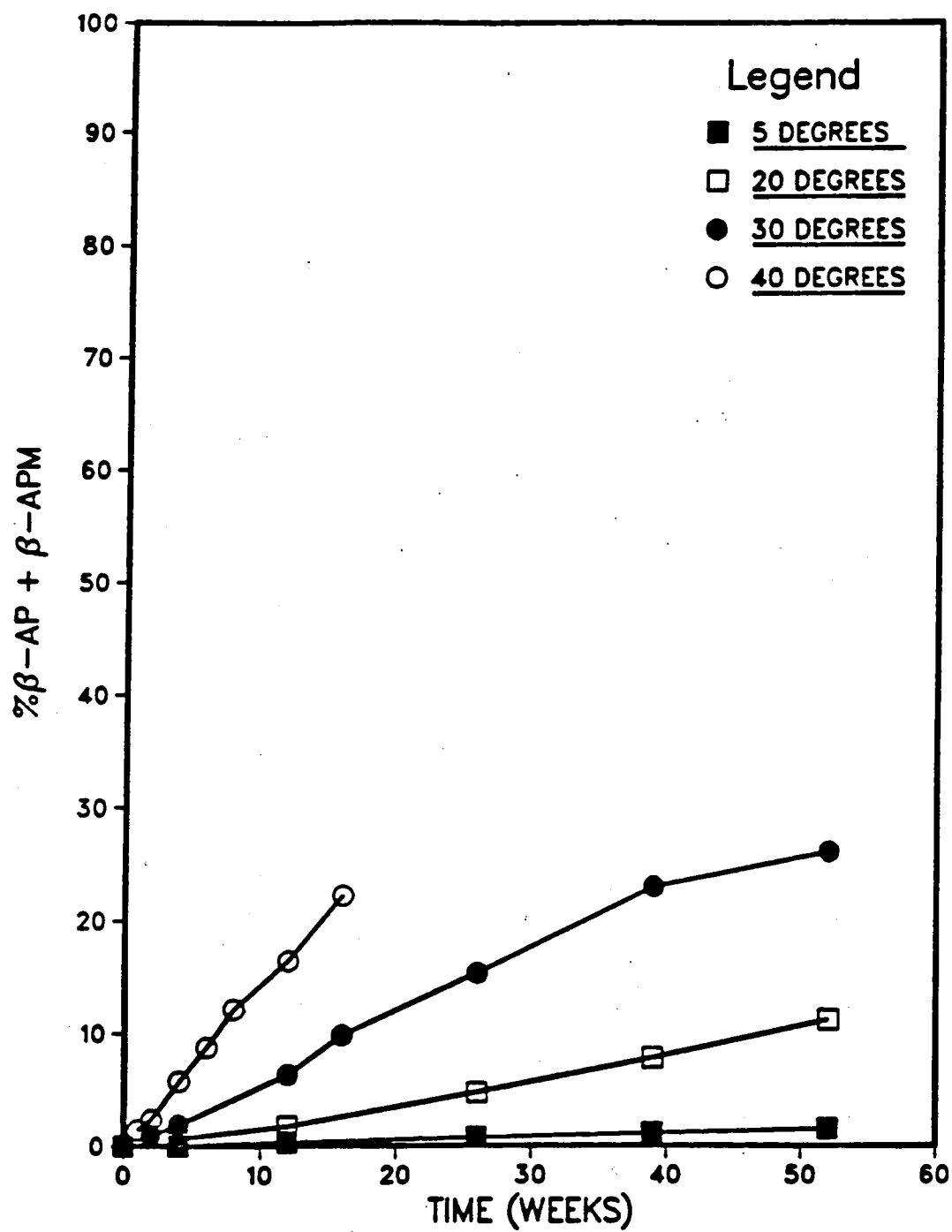


GRAPH 7: %  $\alpha$ -APM vs. %  $\beta$ -AP +  $\beta$ -APM  
AT 40°C

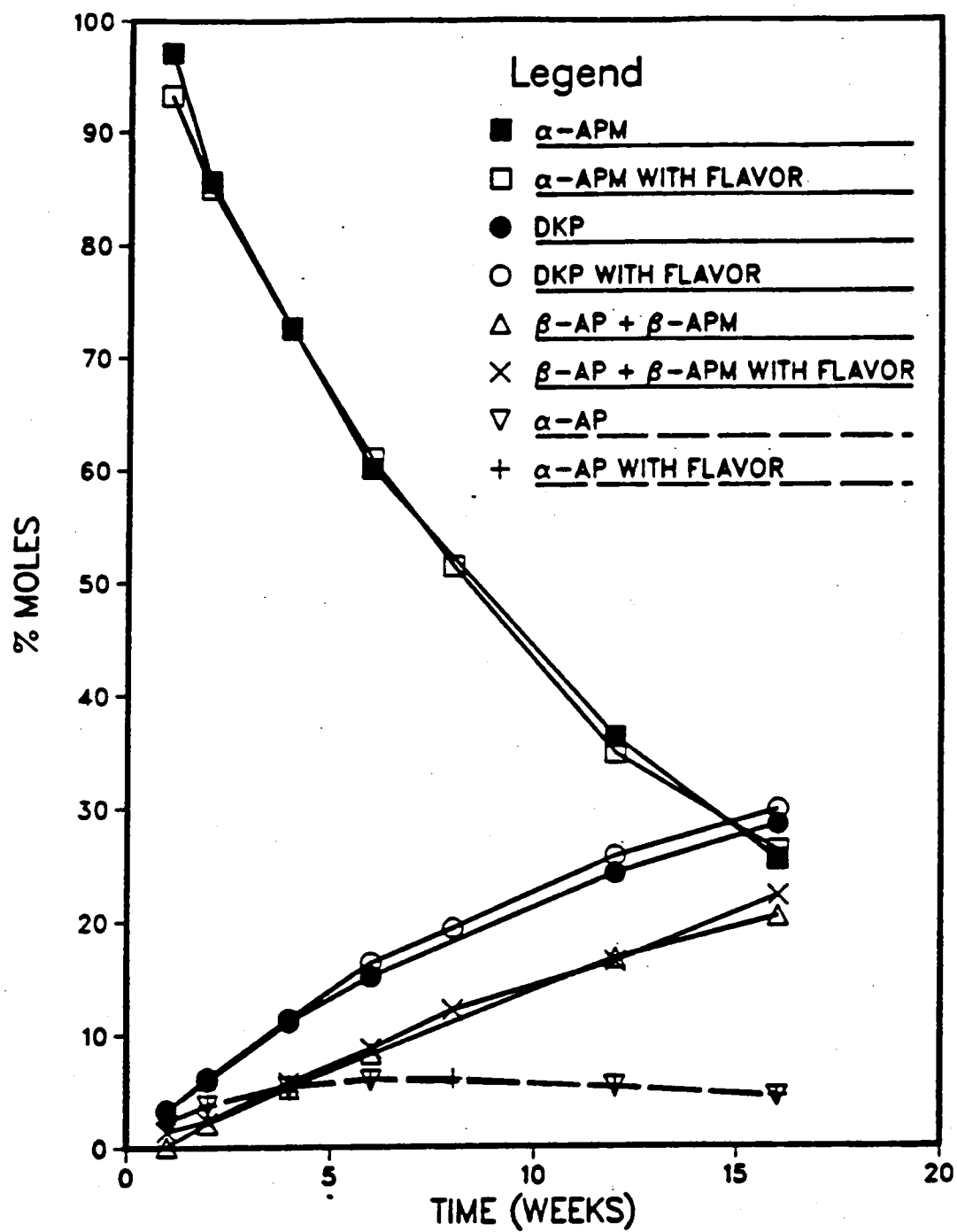




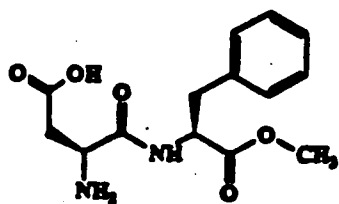
GRAPH 8:  $\beta$ -AP AND  $\beta$ -APM FORMATION  
AT pH 3.52



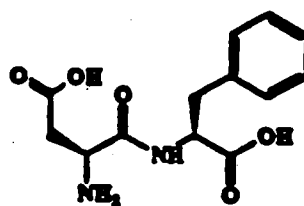
# GRAPH 9: EFFECT OF FLAVOR AT 40°C



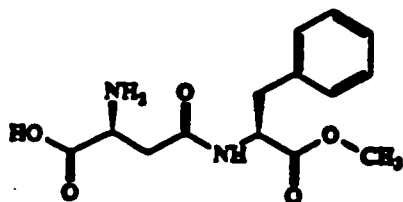
# Structures



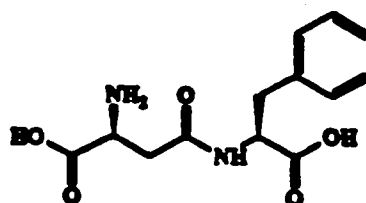
**L,L-alpha-APM**



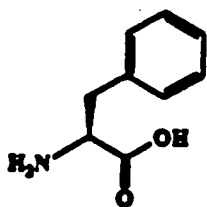
**L,L-alpha-AP**



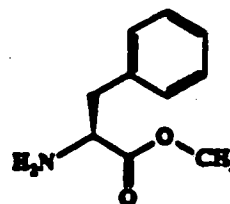
**L,L-beta-APM**



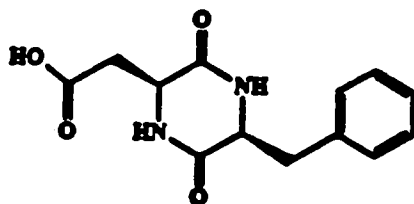
**L,L-beta-AP**



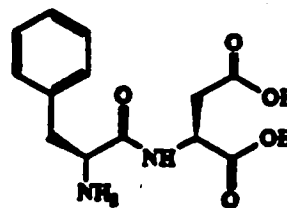
**L-Phe**



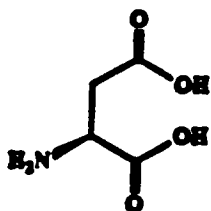
**L-PM**



**L,L-DKP of APM**



**L,L-PA**



**L-ASP**

**CH<sub>3</sub>OH**

**MeOH**

KINETIC REPORT FOR APH AND ITS CONVERSION PRODUCTS IN BEVERAGES JANUARY 30, 1967

STUDY NUMBER 8202: AT INITIAL PH-3.52, WITH PRESERVATIVE (.2000 GRAMS/LITER) BUFFERED WITH CITRIC ACID (.9610 GRAMS/LITER) AND SODIUM CITRATE (.2000 GRAMS/LITER)

CONCENTRATIONS ARE MEANS OF SIX MEASUREMENTS EXPRESSED IN MICROMOLES PER MILLILITER  
NOTE: VALUES BELOW DETECTION LIMITS WERE SET TO ZERO BEFORE AVERAGING

TEMP	WEEKS	ALPHA-APH	BETA-APH	ALPHA-AP	BETA-AP	DEP	PH	L-PH2	PA	L-ASP	MEQR
5	0	1.719	0.000	0.003	0.000	0.002	0.000	0.000	0.000	0.000	0.013
5	4	1.716	0.001	0.012	0.000	0.006	0.000	0.000	0.000	0.000	0.034
5	12	1.669	0.006	0.024	0.000	0.013	0.000	0.000	0.000	0.001	0.073
5	26	1.601	0.011	0.044	0.001	0.022	0.000	0.001	0.000	0.000	0.098
5	39	1.626	0.010	0.065	0.002	0.030	0.016	0.004	0.000	0.000	0.536
5	52	1.565	0.023	0.083	0.003	0.040	0.008	0.002	0.000	0.011	0.130
20	4	1.672	0.010	0.032	0.000	0.024	0.006	0.000	0.000	0.026	0.280
20	12	1.496	0.024	0.076	0.006	0.066	0.021	0.003	0.000	0.169	0.384
20	26	1.330	0.054	0.138	0.027	0.138	0.042	0.013	0.000	0.094	0.130
20	39	1.187	0.080	0.169	0.054	0.172	0.060	0.027	0.001	0.152	0.687
20	52	0.991	0.100	0.186	0.092	0.236	0.062	0.041	0.002	0.153	0.653
20	78	0.752	0.117	0.195	0.179	0.336	0.074	0.080	0.003	0.015	0.114
20	81	0.741	0.119	0.202	0.189	0.348	0.076	0.000	0.000	0.097	0.343
30	2	1.645	0.013	0.038	0.001	0.039	0.017	0.004	0.000	0.166	0.629
30	4	1.537	0.027	0.065	0.006	0.072	0.029	0.000	0.000	0.215	0.057
30	12	1.187	0.063	0.124	0.046	0.198	0.070	0.026	0.001	0.205	1.070
30	16	1.070	0.093	0.130	0.075	0.247	0.085	0.041	0.002	0.400	1.323
30	26	0.768	0.115	0.130	0.148	0.346	0.100	0.089	0.004	0.403	1.320
30	37	0.510	0.147	0.119	0.250	0.425	0.108	0.151	0.009	0.025	0.114
30	52	0.327	0.136	0.094	0.312	0.496	0.091	0.215	0.014	0.000	0.296
30	78	0.118	0.114	0.054	0.407	0.573	0.060	0.347	0.026	0.000	0.469
30	81	0.105	0.110	0.052	0.415	0.586	0.057	0.366	0.027	0.161	0.572
40	1	1.602	0.015	0.037	0.009	0.055	0.026	0.001	0.000	0.275	0.759
40	2	1.461	0.030	0.064	0.010	0.105	0.040	0.007	0.000	0.320	0.071
40	4	1.246	0.062	0.094	0.035	0.194	0.080	0.025	0.001	0.000	
40	6	1.047	0.081	0.104	0.069	0.279	0.108	0.049	0.002	0.000	
40	8	0.802	0.103	0.104	0.104	0.332	0.122	0.076	0.004	0.000	
40	12	0.598	0.111	0.090	0.172	0.441	0.133	0.143	0.000	0.000	
40	16	0.450	0.154	0.077	0.220	0.512	0.130	0.205	0.012	0.000	



KINETIC REPORT FOR APH AND ITS CONVERSION PRODUCTS IN BEVERAGES

STUDY NUMBER 8203: AT INITIAL PH=2.55

JANUARY 30, 1987

SUPPLIED WITH PHOSPHORIC ACID (.8300 GRAMS/LITER)

CONCENTRATIONS ARE MEANS OF SIX MEASUREMENTS EXPRESSED IN MICROMOLES PER MILLILITER

NOTE: VALUES BELOW DETECTION LIMITS WERE SET TO ZERO BEFORE AVERAGING

TIME	WEEKS	ALPHA-APH	BETA-APH	ALPHA-AP	BETA-AP	DAP	PH	L-PHE	PA	L-ASP	MEAN
5	0	1.690	0.000	0.011	0.000	0.004	0.000	0.000	0.000	0.000	0.035
5	4	1.650	0.000	0.030	0.000	0.012	0.000	0.000	0.000	0.000	0.000
5	12	1.603	0.007	0.083	0.001	0.024	0.010	0.001	0.000	0.010	0.008
5	26	1.489	0.010	0.152	0.002	0.046	0.017	0.002	0.000	0.015	0.193
5	39	1.386	0.016	0.207	0.004	0.059	0.030	0.007	0.000	0.022	0.254
5	52	1.313	0.019	0.259	0.007	0.079	0.028	0.007	0.001	0.030	0.343
20	4	1.541	0.011	0.100	0.001	0.047	0.024	0.002	0.000	0.000	0.000
20	12	1.220	0.035	0.235	0.016	0.136	0.065	0.015	0.002	0.075	0.359
20	26	0.839	0.054	0.341	0.061	0.262	0.101	0.052	0.007	0.127	0.634
20	39	0.595	0.060	0.369	0.114	0.310	0.110	0.080	0.014	0.169	0.820
20	52	0.427	0.057	0.361	0.167	0.409	0.110	0.134	0.023	0.230	1.056
20	78	0.204	0.036	0.300	0.263	0.514	0.110	0.228	0.045	0.320	1.202
20	81	0.194	0.035	0.296	0.270	0.524	0.110	0.239	0.040	0.339	1.263
30	2	1.452	0.015	0.110	0.003	0.070	0.045	0.004	0.000	0.044	0.210
30	4	1.263	0.032	0.101	0.012	0.136	0.081	0.014	0.002	0.000	0.000
30	9	0.919	0.050	0.251	0.047	0.245	0.127	0.046	0.006	0.145	0.695
30	12	0.690	0.072	0.276	0.093	0.340	0.156	0.090	0.014	0.222	0.000
30	16	0.407	0.070	0.262	0.139	0.397	0.164	0.129	0.020	0.347	1.117
30	26	0.227	0.053	0.210	0.231	0.508	0.164	0.252	0.045	0.429	1.272
30	39	0.077	0.030	0.134	0.309	0.531	0.134	0.362	0.077	0.072	0.210
40	1	1.400	0.019	0.100	0.010	0.102	0.071	0.002	0.001	0.000	0.000
40	2	1.115	0.041	0.165	0.020	0.105	0.120	0.025	0.003	0.129	0.616
40	4	0.740	0.071	0.205	0.067	0.321	0.190	0.079	0.011	0.224	0.020
40	6	0.494	0.080	0.194	0.124	0.430	0.226	0.150	0.022	0.362	0.951
40	8	0.315	0.075	0.165	0.173	0.479	0.234	0.216	0.035	0.355	1.159
40	12	0.126	0.055	0.112	0.235	0.537	0.210	0.352	0.062	0.519	1.206
40	16	0.055	0.033	0.080	0.261	0.547	0.175	0.463	0.084	0.632	1.412
40	26	0.000	0.000	0.051	0.247	0.506	0.094	0.667	0.115	0.635	0.000

JANUARY 30, 1967

KINETIC REPORT FOR APH AND ITS CONVERSION PRODUCTS IN BEVERAGES

STUDY NUMBER 8213; AT INITIAL PH-3.19, WITH PRESERVATIVE (.2000 GRAMS/LITER)  
BUFFERED WITH PHOSPHORIC ACID (.8300 GRAMS/LITER) AND SODIUM CITRATE (.4600 GRAMS/LITER)

CONCENTRATIONS ARE MEANS OF SIX MEASUREMENTS EXPRESSED IN MICROMOLES PER MILLILITER  
NOTE: VALUES BELOW DETECTION LIMITS WERE SET TO ZERO BEFORE AVERAGING

TEMP	WEEKS	ALPHA-APH	BETA-APH	ALPHA-AP	BETA-AP	DAP	PH	L-PHE	PA	L-ASP	MEQN
5	0	1.737	0.000	0.007	0.000	0.004	0.000	0.000	0.000	0.000	0.000
5	6	1.677	0.002	0.024	0.000	0.012	0.000	0.000	0.000	0.000	0.000
5	26	1.615	0.013	0.070	0.001	0.033	0.009	0.001	0.000	0.008	0.105
5	30	1.585	0.015	0.091	0.002	0.043	0.022	0.019	0.000	0.012	0.127
5	52	1.473	0.033	0.131	0.008	0.078	0.023	0.004	0.000	0.017	0.176
5	70	1.455	0.037	0.166	0.011	0.086	0.044	0.007	0.000	0.020	0.247
20	6	1.573	0.013	0.061	0.001	0.043	0.017	0.001	0.000	0.000	0.000
20	8	1.544	0.019	0.074	0.003	0.056	0.022	0.002	0.000	0.000	0.000
20	16	1.421	0.043	0.130	0.014	0.114	0.040	0.009	0.000	0.000	0.000
20	26	1.209	0.069	0.172	0.038	0.160	0.050	0.020	0.001	0.066	0.369
20	30	1.031	0.089	0.207	0.072	0.227	0.083	0.049	0.003	0.094	0.509
20	52	0.794	0.116	0.221	0.133	0.296	0.089	0.065	0.005	0.144	0.655
20	70	0.573	0.121	0.210	0.228	0.406	0.119	0.120	0.008	0.194	0.855
30	2	1.582	0.015	0.057	0.002	0.056	0.026	0.002	0.000	0.024	0.107
30	4	1.458	0.031	0.080	0.009	0.101	0.046	0.007	0.001	0.000	0.000
30	6	1.352	0.047	0.114	0.010	0.139	0.072	0.012	0.001	0.000	0.000
30	8	1.223	0.066	0.129	0.033	0.178	0.077	0.020	0.001	0.092	0.331
30	16	0.977	0.110	0.163	0.102	0.321	0.115	0.060	0.004	0.000	0.000
30	26	0.553	0.131	0.147	0.204	0.411	0.129	0.129	0.010	0.219	0.870
30	30	0.319	0.127	0.116	0.289	0.498	0.140	0.221	0.010	0.284	1.068
30	52	0.146	0.114	0.078	0.363	0.543	0.101	0.306	0.030	0.400	1.246
40	1	1.554	0.000	0.051	0.003	0.074	0.037	0.003	0.000	0.030	0.121
40	2	1.389	0.030	0.084	0.014	0.135	0.071	0.011	0.001	0.000	0.000
40	4	1.102	0.077	0.115	0.050	0.250	0.120	0.037	0.002	0.140	0.391
40	6	0.865	0.094	0.119	0.080	0.322	0.142	0.068	0.004	0.204	0.533
40	12	0.416	0.124	0.090	0.207	0.487	0.168	0.184	0.014	0.347	0.896
40	16	0.260	0.137	0.070	0.259	0.559	0.157	0.203	0.021	0.407	1.005

## KINETIC REPORT FOR APH AND ITS CONVERSION PRODUCTS IN BEVERAGES

STUDY NUMBER 8214: AT INITIAL PH-3.48, WITH FLAVOR AND PRESERVATIVE (.2000 GRAMS/LITER)  
 BUFFERED WITH CITRIC ACID (.9610 GRAMS/LITER) AND SODIUM CITRATE (.2800 GRAMS/LITER)

CONCENTRATIONS ARE MEANS OF SIX MEASUREMENTS EXPRESSED IN MICROMOLES PER MILLILITER  
 NOTE: VALUES BELOW DETECTION LIMITS WERE SET TO ZERO BEFORE AVERAGING

TEMP	WEEKS	ALPHA-APH	BETA-APH	ALPHA-AP	BETA-AP	DEP	PH	L-PHE	PA	L-ASP	MEQN
5	0	1.729	0.000	0.005	0.000	0.003	0.000	0.000	0.000	0.000	0.005
5	6	1.696	0.000	0.017	0.000	0.009	0.000	0.000	0.000	0.000	0.005
5	26	1.652	0.009	0.048	0.001	0.025	0.005	0.001	0.000	0.007	0.069
5	38	1.634	0.012	0.066	0.001	0.032	0.015	0.014	0.000	0.009	0.096
5	52	1.530	0.024	0.094	0.005	0.052	0.014	0.002	0.000	0.012	0.132
5	78	1.543	0.020	0.121	0.007	0.064	0.041	0.006	0.000	0.017	0.202
20	6	1.666	0.000	0.031	0.000	0.021	0.001	0.000	0.000	0.000	0.000
20	8	1.624	0.010	0.042	0.001	0.030	0.010	0.001	0.000	0.000	0.000
20	26	1.363	0.048	0.129	0.021	0.119	0.037	0.010	0.000	0.040	0.239
20	38	1.241	0.063	0.164	0.044	0.166	0.060	0.031	0.000	0.059	0.362
20	52	1.001	0.096	0.101	0.090	0.236	0.063	0.039	0.001	0.093	0.494
20	78	0.925	0.112	0.203	0.163	0.324	0.096	0.077	0.004	0.132	0.695
30	2	1.627	0.011	0.041	0.001	0.042	0.017	0.001	0.000	0.010	0.002
30	4	1.530	0.024	0.066	0.006	0.077	0.032	0.004	0.000	0.000	0.000
30	6	1.438	0.034	0.089	0.013	0.111	0.042	0.008	0.000	0.060	0.256
30	8	1.332	0.049	0.101	0.023	0.137	0.053	0.013	0.000	0.000	0.000
30	16	1.060	0.086	0.137	0.072	0.253	0.081	0.044	0.002	0.002	0.002
30	26	0.772	0.110	0.142	0.157	0.350	0.102	0.091	0.005	0.150	0.609
30	38	0.520	0.132	0.122	0.237	0.434	0.113	0.159	0.009	0.224	0.892
30	52	0.303	0.145	0.087	0.315	0.492	0.092	0.221	0.016	0.302	1.085
30	78	0.110	0.100	0.055	0.410	0.508	0.079	0.374	0.027	0.380	1.333
40	1	1.679	0.000	0.039	0.002	0.056	0.025	0.002	0.000	0.025	0.113
40	2	1.480	0.027	0.064	0.009	0.102	0.047	0.007	0.000	0.000	0.000
40	4	1.256	0.056	0.092	0.034	0.193	0.082	0.022	0.001	0.090	0.319
40	6	1.030	0.078	0.102	0.064	0.260	0.103	0.045	0.002	0.142	0.434
40	12	0.625	0.124	0.091	0.165	0.410	0.135	0.129	0.007	0.255	0.759
40	16	0.439	0.138	0.076	0.214	0.493	0.130	0.202	0.011	0.325	0.978



JANUARY 30, 1987

KINETIC REPORT FOR APH AND ITS CONVERSION PRODUCTS IN BEVERAGES

STUDY NUMBER 8219: AT INITIAL PH=4.37, WITH PRESERVATIVE (.2000 GRAMS/LITER) BUFFERED WITH CITRIC ACID (.9610 GRAMS/LITER) AND SODIUM CITRATE (1.200 GRAMS/LITER)

CONCENTRATIONS ARE MEANS OF SIX MEASUREMENTS EXPRESSED IN MICROMILES PER MILLILITER

NOTE: VALUES BELOW DETECTION LIMITS WERE SET TO ZERO BEFORE AVERAGING

TIME	WEEKS	ALPHA-APH	BETA-APH	ALPHA-AP	BETA-AP	DP	PH	L-PHE	PA	L-ASP	MOON
5	0	1.749	0.000	0.003	0.000	0.003	0.000	0.000	0.000	0.000	0.026
5	6	1.754	0.000	0.011	0.000	0.007	0.000	0.000	0.000	0.000	0.052
5	26	1.699	0.003	0.033	0.000	0.020	0.000	0.000	0.001	0.000	0.060
5	30	1.673	0.004	0.044	0.000	0.025	0.007	0.007	0.001	0.000	0.086
5	52	1.621	0.007	0.065	0.000	0.039	0.000	0.000	0.000	0.003	0.123
5	70	1.635	0.010	0.086	0.002	0.052	0.017	0.005	0.004	0.005	0.000
20	6	1.724	0.000	0.037	0.000	0.032	0.001	0.001	0.000	0.000	0.011
20	20	1.407	0.020	0.119	0.009	0.110	0.000	0.005	0.000	0.011	0.262
20	30	1.390	0.025	0.152	0.017	0.145	0.012	0.010	0.000	0.010	0.285
20	52	1.301	0.035	0.101	0.030	0.108	0.009	0.015	0.000	0.026	0.379
20	70	1.162	0.053	0.233	0.059	0.268	0.025	0.031	0.004	0.035	0.489
30	2	1.653	0.004	0.029	0.000	0.032	0.000	0.001	0.000	0.000	0.047
30	4	1.601	0.001	0.049	0.002	0.060	0.001	0.002	0.000	0.000	0.000
30	6	1.570	0.012	0.070	0.004	0.089	0.000	0.003	0.000	0.000	0.192
30	8	1.475	0.016	0.083	0.007	0.109	0.012	0.005	0.000	0.015	0.329
30	16	1.303	0.034	0.139	0.026	0.207	0.016	0.019	0.000	0.045	0.700
30	26	1.096	0.050	0.100	0.044	0.302	0.020	0.037	0.001	0.067	0.082
30	36	0.885	0.055	0.200	0.114	0.303	0.026	0.060	0.002	0.100	1.133
30	52	0.654	0.066	0.194	0.103	0.465	0.013	0.093	0.006	0.010	0.000
30	70	0.401	0.072	0.161	0.323	0.644	0.045	0.165	0.000	0.000	0.000
40	1	1.742	0.000	0.029	0.000	0.045	0.001	0.001	0.000	0.000	0.239
40	2	1.508	0.011	0.051	0.003	0.079	0.009	0.003	0.000	0.020	0.316
40	4	1.435	0.021	0.005	0.011	0.150	0.017	0.010	0.000	0.000	0.000
40	6	1.325	0.029	0.114	0.023	0.220	0.019	0.021	0.001	0.002	0.002
40	12	0.974	0.060	0.146	0.070	0.364	0.024	0.056	0.002	0.002	0.002
40	16	0.792	0.081	0.150	0.124	0.460	0.022	0.095	0.003	0.005	0.791

KINETIC REPORT FOR APM AND ITS CONVERSION PRODUCTS IN BEVERAGES

JANUARY 30, 1987

STUDY NUMBER 8202: AT INITIAL PH=3.52, WITH PRESERVATIVE (.2000 GRAMS/LITER) BUTTERED WITH CITRIC ACID (.9610 GRAMS/LITER) AND SODIUM CITRATE (.2800 GRAMS/LITER)

CONCENTRATIONS EXPRESSED AS THE PERCENT OF APM INITIALLY PRESENT

NOTE: VALUES BELOW DETECTION LIMITS WERE SET TO ZERO BEFORE AVERAGING

TEMP	WEEKS	ALPHA-APM	BETA-APM	ALPHA-AP	BETA-AP	DP	PH	L-PHE	PA	L-ASP	MDM
5	0	100.0	0.0	0.2	0.0	0.1	0.0	0.0	0.0	0.0	0.7
5	4	99.8	0.0	0.7	0.0	0.4	0.0	0.0	0.0	0.1	2.0
5	12	97.1	0.3	1.4	0.0	0.7	0.0	0.0	0.0	0.0	4.3
5	26	93.2	0.7	2.6	0.0	1.3	0.0	0.0	0.0	0.0	5.7
5	39	94.6	1.0	3.0	0.1	1.0	1.0	0.3	0.0	0.4	31.2
5	52	91.0	1.3	4.8	0.2	2.4	0.5	0.1	0.0	0.6	
20	4	97.3	0.6	1.9	0.0	1.4	0.4	0.0	0.0		
20	12	87.0	1.4	4.4	0.3	3.0	1.2	0.2	0.0	1.9	0.0
20	26	77.4	3.1	8.0	1.6	0.0	2.4	0.7	0.0	2.0	16.3
20	39	69.1	4.6	9.0	3.2	10.0	3.5	1.6	0.1	9.0	22.3
20	52	57.7	5.0	10.8	5.4	13.7	3.6	2.4	0.1	5.5	7.6
20	70	43.7	6.8	11.4	10.4	19.6	4.3	4.8	0.2	0.0	40.0
20	81	43.1	7.0	11.7	11.0	20.2	4.4	5.1	0.3	0.9	30.0
30	2	95.7	0.0	2.2	0.1	2.3	1.0	0.1	0.0	0.9	6.6
30	4	89.4	1.5	3.0	0.3	4.2	1.7	0.2	0.0		
30	12	65.1	3.7	7.2	2.6	11.5	4.1	1.5	0.1	5.6	19.9
30	16	62.2	5.4	8.0	4.4	14.4	4.9	2.4	0.1		
30	26	44.7	6.7	8.0	0.6	20.1	5.8	5.2	0.3	9.7	36.6
30	39	29.7	8.5	6.9	14.5	24.7	6.3	0.0	0.5	12.5	49.9
30	52	19.0	7.9	5.5	18.2	20.9	5.3	12.5	0.0	16.6	62.2
30	70	6.9	6.6	3.2	23.7	33.3	3.5	20.2	1.5	23.3	77.0
30	81	6.1	6.4	3.1	24.1	30.1	3.3	21.3	1.6	23.5	76.0
40	1	93.2	0.9	2.2	0.5	3.2	1.5	0.0	0.0	1.5	6.6
40	2	85.0	1.7	3.7	0.6	6.1	2.0	0.4	0.0	3.0	
40	4	72.5	3.6	5.5	2.1	11.3	5.1	1.4	0.1	5.1	17.2
40	6	60.9	4.7	6.0	4.0	16.3	6.3	2.9	0.1	0.7	26.1
40	8	51.3	6.0	6.0	6.1	19.3	7.1	4.4	0.2	9.3	33.3
40	12	34.8	6.4	5.2	10.0	25.7	7.7	0.3	0.4	10.0	44.1
40	16	26.2	0.9	4.5	13.3	29.8	0.0	11.9	0.7	19.1	50.7

KINETIC REPORT FOR APH AND ITS CONVERSION PRODUCTS IN BEVERAGES

STUDY NUMBER 0203: AT INITIAL PH=2.55

BUFFERED WITH PHOSPHORIC ACID (1.0300 GRAMS/LITER)

CONCENTRATIONS EXPRESSED AS THE PERCENT OF APH INITIALLY PRESENT

NOTE: VALUES BELOW DETECTION LIMITS WERE SET TO ZERO BEFORE AVERAGING

JANUARY 30, 1987

TEMP	WEEKS	ALPHA-APH	BETA-APH	ALPHA-AP	BETA-AP	DOP	PH	L-PHE	PA	L-ASP	MBON
5	0	100.0	0.0	0.7	0.0	0.2	0.0	0.0	0.0	0.0	2.1
5	4	90.1	0.0	2.3	0.0	0.7	0.0	0.0	0.0	0.0	0.6
5	12	94.0	0.4	4.9	0.0	1.4	0.6	0.0	0.0	0.6	3.2
5	26	80.1	0.6	9.0	0.1	2.7	1.0	0.0	0.0	0.9	11.4
5	39	82.0	0.9	12.3	0.3	3.5	1.0	0.4	0.0	1.3	15.0
5	52	77.7	1.1	15.3	0.4	4.7	1.7	0.4	0.0	1.8	20.3
20	4	91.2	0.7	5.9	0.1	2.0	1.4	0.1	0.0	0.0	0.0
20	12	72.2	2.1	13.9	0.9	0.1	3.0	0.9	0.1	4.4	21.2
20	26	49.6	3.2	20.2	3.6	15.5	6.0	3.1	0.4	7.5	37.5
20	39	35.2	3.6	21.0	6.0	10.0	7.0	5.2	0.0	10.0	40.5
20	52	25.3	3.4	21.4	9.9	20.2	7.0	8.0	1.4	10.1	62.5
20	78	12.1	2.1	17.7	15.6	30.4	6.5	13.5	2.7	19.4	75.9
20	81	11.5	2.1	17.5	16.0	31.0	6.5	14.1	2.0	20.1	74.7
30	2	85.9	0.9	6.5	0.2	4.2	2.7	0.3	0.0	2.6	12.9
30	4	74.7	1.9	10.7	0.7	0.0	4.0	0.8	0.1	0.0	0.0
30	8	54.1	3.4	14.0	2.8	14.5	7.5	2.7	0.4	0.6	0.6
30	12	40.3	4.2	16.3	5.5	20.1	9.2	5.3	0.0	13.1	41.1
30	16	29.0	4.1	15.5	8.2	23.5	9.7	7.6	1.2	0.0	0.0
30	26	13.4	3.1	12.4	13.6	30.1	9.7	14.9	2.6	20.5	64.1
30	39	4.5	1.8	8.0	10.3	31.4	7.9	21.4	4.6	25.4	75.3
40	1	82.0	1.1	6.4	1.1	6.0	4.2	0.1	0.0	4.3	12.9
40	2	66.0	2.4	9.7	1.2	11.0	7.6	1.5	0.2	7.7	0.0
40	4	44.3	4.2	12.1	4.0	19.0	11.7	4.7	0.7	13.3	34.4
40	6	29.2	4.7	11.5	7.3	25.7	13.6	8.9	1.3	21.4	49.0
40	8	10.4	4.4	9.0	10.2	20.3	13.0	12.0	2.1	31.0	54.2
40	12	7.5	3.3	6.6	13.9	31.0	12.4	20.0	3.6	30.7	60.6
40	16	3.3	2.0	4.7	15.4	32.4	10.4	27.4	5.0	37.4	76.1
40	26	0.0	0.4	3.0	14.6	29.9	5.6	39.5	6.0	37.6	83.5

JANUARY 30, 1967

KINETIC REPORT FOR APH AND ITS CONVERSION PRODUCTS IN BEVERAGES

STUDY NUMBER 8213: AT INITIAL PH-3.19, WITH PRESERVATIVE (.2000 GRAMS/LITER)  
BUFFERED WITH PHOSPHORIC ACID (.8300 GRAMS/LITER) AND SODIUM CITRATE (.4600 GRAMS/LITER)

CONCENTRATIONS EXPRESSED AS THE PERCENT OF APH INITIALLY PRESENT  
NOTE: VALUES BELOW DETECTION LIMITS WERE SET TO ZERO BEFORE AVERAGING

TEMP	WEEKS	ALPHA-APH	BETA-APH	ALPHA-AP	BETA-AP	DOP	PH	L-PHE	PA	L-ASP	PROH
5	0	100.0	0.0	0.4	0.0	0.2	0.0	0.0	0.0	0.0	0.0
5	6	94.6	0.1	1.4	0.0	0.7	0.0	0.0	0.0	0.0	0.0
5	26	93.0	0.7	4.0	0.1	1.9	0.5	0.1	0.0	0.5	6.1
5	30	91.3	0.0	5.2	0.1	2.4	1.3	1.1	0.0	0.7	7.3
5	52	84.0	1.9	7.5	0.5	4.0	1.3	0.2	0.0	1.0	10.1
5	70	83.0	2.2	9.6	0.6	5.0	2.5	0.4	0.0	1.6	16.2
20	6	90.6	0.0	3.5	0.1	2.5	1.0	0.1	0.0	0.0	0.0
20	0	80.9	1.1	4.3	0.2	3.2	1.2	0.1	0.0	0.0	0.0
20	16	81.0	2.5	7.5	0.0	6.6	2.3	0.5	0.0	0.0	0.0
20	26	69.6	4.0	9.9	2.2	9.2	3.3	1.1	0.1	3.0	21.3
20	30	59.4	5.1	11.9	4.1	13.1	4.0	2.0	0.2	5.4	29.3
20	52	45.7	6.7	12.7	7.7	17.1	5.1	3.7	0.3	8.3	37.7
20	70	33.0	7.0	12.5	13.1	23.4	6.0	6.0	0.5	11.2	49.2
30	2	91.1	0.9	3.3	0.1	3.2	1.5	0.1	0.0	1.4	6.1
30	4	84.0	1.0	5.0	0.5	5.0	2.7	0.4	0.0	0.0	0.0
30	6	77.0	2.7	6.5	1.1	8.0	4.2	0.7	0.0	0.0	0.0
30	8	70.4	3.0	7.4	1.9	10.2	4.4	1.2	0.1	9.3	19.0
30	16	51.7	6.3	9.4	5.9	18.5	6.6	3.9	0.2	0.0	0.0
30	26	31.8	7.6	9.5	11.0	23.7	7.4	7.4	0.6	12.6	50.5
30	30	18.4	7.3	6.7	16.6	20.7	8.0	12.7	1.0	16.4	61.5
30	52	8.4	6.6	4.5	20.9	31.3	5.0	17.6	1.7	23.0	71.0
40	1	89.5	0.0	2.9	0.2	4.2	2.1	0.2	0.0	2.2	7.0
40	2	80.0	2.2	4.8	0.8	7.0	4.1	0.6	0.0	0.0	0.0
40	4	63.5	4.4	6.6	2.9	14.4	6.9	2.2	0.1	6.1	22.5
40	6	49.8	5.4	6.9	5.1	18.5	8.2	3.9	0.2	11.7	30.7
40	12	23.9	7.2	5.2	13.9	20.0	9.7	10.6	0.0	20.0	51.6
40	16	15.0	7.9	4.1	14.9	32.2	9.0	16.3	1.2	23.4	62.5

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KINETIC REPORT FOR APH AND ITS CONVERSION PRODUCTS IN BEVERAGES

STUDY NUMBER 0214: AT INITIAL PH=3.40, WITH FLAVOR AND PRESERVATIVE (.2000 GRAMS/LITER) BUTTERED WITH CITRIC ACID (.9610 GRAMS/LITER) AND SODIUM CITRATE (.2000 GRAMS/LITER)

CONCENTRATIONS EXPRESSED AS THE PERCENT OF APH INITIALLY PRESENT  
NOTE: VALUES BELOW DETECTION LIMITS WERE SET TO ZERO BEFORE AVERAGING

TEMP	WEEKS	ALPHA-APH	BETA-APH	ALPHA-AP	BETA-AP	DP	PH	L-PHE	PA	L-ASP	MBON
5	0	100.0	0.0	0.3	0.0	0.2	0.0	0.0	0.0	0.0	0.3
5	6	98.1	0.0	1.0	0.0	0.5	0.0	0.0	0.0	0.0	0.0
5	26	95.5	0.5	2.0	0.0	1.4	0.3	0.1	0.0	0.4	4.0
5	38	94.5	0.7	3.8	0.1	1.8	0.0	0.0	0.0	0.5	5.5
5	52	88.5	1.4	5.5	0.3	3.0	0.0	0.1	0.0	0.7	7.6
5	70	89.2	1.6	7.0	0.4	3.7	2.4	0.4	0.0	1.0	11.7
20	6	96.4	0.0	1.8	0.0	1.2	0.0	0.0	0.0	0.0	0.0
20	8	93.9	0.6	2.4	0.1	1.8	0.6	0.0	0.0	0.0	0.0
20	26	78.9	2.8	7.5	1.2	6.9	2.1	0.6	0.0	2.3	15.0
20	38	71.0	3.6	9.5	2.5	9.6	3.5	1.0	0.0	3.4	20.0
20	52	57.9	5.5	10.5	5.2	13.1	3.6	2.3	0.1	5.4	20.6
20	70	47.7	6.5	11.8	9.4	18.7	5.6	4.5	0.2	7.6	40.2
30	2	94.1	0.7	2.4	0.1	2.4	1.0	0.1	0.0	1.0	4.0
30	4	88.5	1.4	3.8	0.4	4.5	1.8	0.2	0.0	0.0	0.0
30	6	83.2	2.0	5.1	0.7	6.4	2.4	0.5	0.0	0.0	0.0
30	8	77.0	2.8	5.9	1.3	7.9	3.1	0.7	0.0	3.5	14.9
30	16	61.3	5.0	7.9	4.2	14.6	4.7	2.5	0.1	9.1	39.8
30	36	14.7	6.4	8.2	9.1	20.7	5.9	5.3	0.3	13.0	51.6
30	18	10.5	7.6	7.0	13.7	25.1	6.6	9.2	0.5	17.5	62.0
30	52	17.5	8.4	5.0	18.2	28.5	5.3	12.0	0.9	22.0	77.1
30	70	6.8	6.2	3.2	23.7	34.0	4.6	21.6	1.6	1.4	6.5
40	1	97.1	0.0	2.3	0.1	3.3	1.5	0.1	0.0	0.0	0.0
40	4	85.6	1.6	3.7	0.5	5.9	2.7	0.4	0.0	0.0	0.0
40	4	72.6	3.2	5.3	2.0	11.1	4.0	1.3	0.1	9.3	18.4
40	6	60.0	4.5	5.9	3.7	15.1	6.0	2.6	0.1	0.2	25.1
40	12	36.2	7.2	5.3	9.5	24.2	7.8	7.5	0.4	10.7	43.9
40	16	25.1	8.0	4.4	12.4	28.5	7.5	11.7	0.6	10.0	56.6

KINETIC REPORT FOR AMI AND ITS CONVERSION PRODUCTS IN BEVERAGES

JANUARY 30, 1987

STUDY NUMBER 8215; AT INITIAL PH=4.37, WITH PRESERVATIVE (.2000 GRAMS/LITER)  
BUFFERED WITH CITRIC ACID (.9610 GRAMS/LITER) AND SODIUM CITRATE (1.200 GRAMS/LITER)

CONCENTRATIONS EXPRESSED AS THE PERCENT OF AMI INITIALLY PRESENT  
NOTE: VALUES BELOW DETECTION LIMITS WERE SET TO ZERO BEFORE AVERAGING

TEMP	WEEKS	ALPHA-AMI	BETA-AMI	ALPHA-AP	BETA-AP	EXP	PH	L-PHE	PA	L-ASP	MDH
5	0	100.0	0.0	0.2	0.0	0.2	0.0	0.0	0.0	0.0	1.5
5	6	100.3	0.0	0.6	0.0	0.4	0.0	0.0	0.0	0.0	1.5
5	26	97.2	0.2	1.9	0.0	1.1	0.0	0.0	0.0	0.1	3.0
5	38	95.7	0.3	2.5	0.0	1.4	0.0	0.4	0.0	0.0	3.9
5	52	92.7	0.4	3.7	0.0	2.3	0.0	0.0	0.0	0.1	4.9
5	78	93.5	0.6	4.9	0.1	2.9	1.0	0.3	0.2	0.3	7.1
20	6	98.6	0.0	2.1	0.0	1.8	0.1	0.0	0.0	0.0	0.0
20	20	95.0	1.1	6.8	0.5	6.3	0.5	0.3	0.0	0.6	13.0
20	38	79.5	1.4	0.7	1.0	8.3	0.7	0.6	0.0	1.0	16.3
20	52	74.4	2.0	10.6	1.7	10.8	0.5	0.9	0.0	1.5	21.7
20	78	66.5	3.0	13.3	3.4	15.3	1.5	1.0	0.2	2.0	27.9
30	2	96.8	0.2	1.6	0.0	1.9	0.0	0.0	0.0	0.4	2.7
30	4	91.5	0.0	2.0	0.1	3.4	0.0	0.1	0.0	0.0	0.0
30	6	89.0	0.7	4.0	0.2	5.1	0.5	0.2	0.0	0.0	0.0
30	8	84.3	0.9	4.8	0.4	6.2	0.7	0.3	0.0	0.0	0.0
30	16	78.5	1.9	7.9	1.5	11.0	0.9	1.1	0.0	0.0	11.0
30	26	62.7	2.9	10.3	3.6	17.2	1.1	2.1	0.1	2.6	30.2
30	38	50.6	3.1	11.4	6.5	21.9	1.5	3.9	0.1	3.0	40.5
30	52	37.4	3.0	11.1	10.5	26.6	0.0	5.3	0.4	3.6	50.4
30	78	22.9	4.1	9.2	18.5	36.8	2.6	9.4	0.5	0.0	64.0
40	1	99.6	0.0	1.6	0.0	2.6	0.1	0.0	0.0	0.6	4.6
40	2	91.1	0.6	2.9	0.2	4.5	0.5	0.2	0.0	0.0	0.0
40	4	82.1	1.2	4.9	0.6	6.6	1.0	0.6	0.0	1.6	13.6
40	6	75.8	1.6	6.5	1.3	12.6	1.1	1.2	0.0	2.3	18.0
40	12	55.7	3.5	8.4	4.5	20.0	1.4	3.2	0.1	4.6	35.7
40	16	45.3	4.6	8.6	7.1	26.0	1.2	5.4	0.2	5.4	45.2



**MECHANISTIC ASPECTS OF THE CONVERSION OF  
 $\alpha$ -ASPARTYLPHENYLALANINE METHYL ESTER (ASPARTAME) TO THE  
 $\beta$ -ISOMER**

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**Keyword:** Aspartame, aspartimidophenylalanine methyl ester,  
rearrangement, intermediate, O<sup>18</sup> labeling.



## ABSTRACT

Aspartame is known to rearrange in solution and thereby lose its sweetness during prolonged storage. In this study, the molecular mechanism of this rearrangement was explored.

L-Aspartimido-L-phenylalanine methyl ester was identified as the most likely intermediate in the rearrangement of aspartame to  $\beta$ -L-aspartyl-L-phenylalanine methyl ester in solution. This intermediate was synthesized and characterized. The mechanism of the rearrangement was probed by mass spectrometry of equilibrated samples of O<sup>18</sup> carboxyl-labeled aspartame and a novel mass spectral fragmentation was confirmed by examination of O<sup>18</sup> carboxyl labeled  $\beta$ -L-aspartyl-L-phenylalanine methyl ester. From these findings,  $\beta$ -aspartame is formed from via a succinimide intermediate in a manner consistent with the "beta-aspartyl shift" mechanism, commonly found in biology.

## INTRODUCTION

Aqueous solutions of the dipeptide sweetener  $\alpha$ -L-aspartyl-L-phenylalanine methyl ester (aspartame;  $\alpha$ -APM) are known to lose potency over time [Stegink and Filer, 1984]. This phenomenon has been studied quantitatively in dilute, mildly acidic solution and has been found to be the result of cyclization to 5(S)-benzyl-3,6-dioxopiperazine-2(S)-acetic acid, hydrolysis to a  $\beta$ -L-aspartyl-L-phenylalanine and the component amino acids, and transformation to  $\beta$ -L-aspartyl-L-phenylalanine methyl ester ( $\beta$ -APM) [Witt, 199\_].

The cyclization of  $\alpha$ -aspartyl peptides to aspartimides is a well documented side reaction in aspartyl peptide synthesis [Perseo *et al.*, 1986]. This reaction has been observed under basic, neutral and acidic conditions, but occurs only in strongly acidic media (such as the stomach) when the  $\beta$ -carboxyl is not esterified or otherwise activated [Ondetti *et al.*, 1968; Schon and Kisfaludy, 1979]. The aspartimide of  $\alpha$ -APM, L-aspartimido-L-phenylalanine methyl ester (AIPM, Figure 1 compound *i*) has recently been detected in solutions of the dipeptide held at pH 3 and 100°C for six hours. It has been suggested that this compound may undergo hydrolysis at the  $\alpha$  carbonyl to generate  $\beta$ -APM [Stamp and LaBuza, 1989]. Hydrolytic opening of aspartimides under basic conditions is known to favor formation of the  $\beta$  or isoaspartyl peptide [Baba *et al.*, 1973; Igano *et al.*, 1981; Yang and Merrifield, 1976; McFadden *et al.*, 1986; Murray *et al.*, 1986]. Under carefully controlled conditions, AIPM can be converted preferentially to  $\alpha$ -L-aspartyl-L-phenylalanine or its methyl esters in base or in the presence of Lewis acids [Takahashi and Takemoto, 1989] (Figure 1, Pathway A).

An alternative (Figure 1, Pathway B) is suggested by the selective acid hydrolysis of peptides at aspartyl residues. This has been attributed to acceleration of hydrolysis by attack of the  $\beta$  carboxyl on the amide bond [Partridge and Davis, 1950; Battersby and Robinson, 1955]. The

intermediate *ii* can then rearrange in concerted fashion to  $\beta$ -APM or by dissociation and recombination of a tight ion pair. An anhydro species obtained by dehydration of *ii* has been detected in the anhydrous, thermal decomposition of aspartame [Graves and Luo, 1987].

To differentiate these pathways and determine the most probable mechanism for the  $\alpha$  to  $\beta$  conversion of aspartame in dilute acidic solution, we have synthesized AIPM (*i*), examined its behavior under these conditions, and also the behavior of isotopically labeled aspartame.

### EXPERIMENTAL CONDITIONS AND METHODS

N-Carbobenzyloxy- $\alpha/\beta$ -L-aspartyl-L-phenylalanine methyl ester (*1*, *2*): N-carbobenzyloxy-L-aspartic acid (10 g, 37.4 mmol) and 4.45 g (43.6 mmol) of acetic anhydride were combined in 15 ml of ethyl acetate and stirred for 18 hours under nitrogen. The resulting solution was evaporated to dryness under vacuum, dissolved in 80 ml of ethyl acetate, and mixed with 13.4 g (74.8 mmol) of L-phenylalanine methyl ester in 175 ml of the same solvent. The resulting mixture was stirred under nitrogen for 18 hours. The mixture was filtered and the filtrate washed twice with 70 ml of 1N hydrochloric acid followed by 70 ml of water, then extracted five times with 100 ml of 1.6% aqueous sodium carbonate. The combined extracts were acidified with concentrated hydrochloric acid followed by 70 ml of water, then extracted five times with 100 ml of 1.6% aqueous sodium carbonate. The combined extracts were acidified with concentrated hydrochloric acid and allowed to stand overnight. The white precipitate was collected by filtration, washed with water, and air dried to give 15.3 g of the  $\alpha/\beta$  dipeptide as shown by comparison with authentic samples on TLC (silica), 86:9:5 PhCH<sub>3</sub>/MeOH/HOAc; Rf( $\alpha$ ) .30, Rf( $\beta$ ) .25.

N-Carbobenzyloxy-L-aspartimido-L-phenylalanine methyl ester (*3*): To a solution containing 15.3 g (35.7 mmol) of *1* and *2* was added 7.22 g (71.4 mmol) of triethylamine and 3.65 g (35.7

mmol) of acetic anhydride. The solution was stirred for 18 hours under nitrogen. The resulting solution was diluted with 20 ml of water, extracted three times with 4 ml of toluene, and the combined extracts washed three times with 50 ml of 10% aqueous potassium bicarbonate, 50 ml of water, 50 ml of 1N hydrochloric acid, and twice with 50 ml of water, then dried over sodium sulfate. After filtration and evaporation to dryness under vacuum, 13.6 g of **3** was obtained as an oil which showed a single spot on TLC at  $R_f$  .19; silica, 86:9:5, PhCH<sub>3</sub>/MeOH/HOAc.

**L-Aspartimido-L-phenylalanine methyl ester (i)**: A solution of **3** in 140 ml of THF was hydrogenated at 60 psi over 1.4 g of 5% Pd/C at ambient temperature for 1.5 hours. The catalyst was removed by filtration and the filtrate was evaporated to dryness under vacuum. Most of the residue dissolved in ether and was decanted from a small amount of gummy oil. The ether was evaporated under vacuum and the residue placed on a Kugelrohr for 5 hours to give 7.5 g of **i** as an oil. TLC showed a major component as  $R_f$  .49 and very minor components as  $R_f$  .42 and .37. NMR (CDCl<sub>3</sub>), 1.60 (s, 2H), 2.18 (dd, 1H,  $J=18, 6$ ), 2.93 (dd,  $J=18, 8.5$ ), 3.47 (dd,  $J=10, 7$ ), 3.63 (dd,  $J=8.5, 6$ ), 3.77 (s, 3H), 5.02 (dd,  $J=10$ ), 7), 7.22 (s, 5H); IR 1785, 1745, 1715; Anal. Calcd. for C<sub>14</sub>H<sub>16</sub>N<sub>2</sub>O<sub>4</sub>: C, 60.86; H, 5.84; N, 10.14. Found: C, 60.69; H, 5.89; N, 9.55.

**Benzyl alcohol (O<sup>18</sup>)**: Benzyl bromide (2.514 g, 14.7 mmol), 1.574 g (14.7 mmol) of 2,6-lutidine, and 1 g of H<sub>2</sub>O<sup>18</sup> were mixed in 1.3 ml of THF and heated at reflux for 5 hours. Ten ml each of toluene and H<sub>2</sub>O were added and the organic layer separated and dried over Na<sub>2</sub>SO<sub>4</sub>. Solvent was evaporated under vacuum and the residue distilled with the product collected at 55°-56°C (.25 mm Hg); 251 mg, 15% of theory.

**N-Carbobenzyloxy-β-(α-benzyl (O<sup>18</sup> ester) aspartylphenylalanine methyl ester (6)**. Labeled benzyl alcohol (225 mg, 2.04 mmol) and 810 mg (1.89 mmol) of N-carbobenzyloxy-β-aspartylphenylalanine methyl ester were stirred in 4.2 ml of methylene chloride under nitrogen

while 11 mg (.09 mmol) of DMAP in .2 ml and 423 mg (2.05 mmol) of dicyclohexylcarbodiimide in 1 ml of the same solvent were added. The resulting solution was stirred at ambient temperature for 20 hours. The reaction mixture was evaporated under vacuum to give 1.7 g of an oily residue which was dissolved in 10 ml of hot ethyl acetate and filtered. Hot hexane (10 mL) was added and the solution allowed to cool to room temperature. After standing in an ice bath for 4 hours, the precipitate was collected by filtration, washed with 10 ml of cold 1:1 ethyl acetate/hexane, and air dried to give 455 mg of 6; 46% of theory. The product was homogeneous by TLC on silica, with an  $R_f$  of .73 in 85:18:1.3:6  $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}/\text{HCOOH}$ .

$\beta$ -Aspartyl ( $\text{O}^{18}$ ) phenylalanine methyl ester (7). The labeled benzyl ester (425 mg, .81 mmol) was dissolved in 50 ml of methanol and hydrogenated in a Parr shaker at 60 psi and 50°C over 43 mg 5% Pd/C for 3 hours. The catalyst was removed by filtration and the solution concentrated to about 5 mL. After cooling in an ice bath for 1 hour, the white precipitate was collected and washed with cold methanol to 171 mg of 7; 71% of theory. The product was NLT 99% pure by HPLC and was identical to authentic  $\beta$ -L-aspartyl-L-phenylalanine methyl ester by TLC, HPLC, and its NMR spectrum.

**Equilibration Study:** AIPM (100.4 mg) was dissolved in 200 ml of phosphate buffer, 7 ml samples were sealed in ampoules and stored in constant temperature chambers at 20°C and 40°C. A 100.1 mg sample of AIPM was dissolved in 200 ml of citrate buffer and treated in the same way. Ampoules were withdrawn at the indicated intervals and analyzed by HPLC on a Beckman Ultrasphere 25 cm x 4.6 mm column using a 15% acetonitrile, 85% buffer (0.18 M sodium phosphate, 0.02M heptanesulfonic acid) mobile phase at a flow rate of 1.5 mL/min. Components were detected by UV (210 nm) and identified by comparison of retention times with those of authentic samples.

The N-acetyl methyl esters of the  $\alpha$ -APM and  $\beta$ -APM were prepared for analysis by GCMS. Typically 50  $\mu$ g of each sample in methanol was acidified with methanolic HCl, evacuated to dryness, then dissolved in 50  $\mu$ l of a diazomethane solution prepared by the method described in the Aldrich Technical Information Bulletin Number AL-180. The solution was allowed to stand for five to ten minutes, then blown dry with dry nitrogen. The sample was treated with the diazomethane solution three times. The dry residue was dissolved in a dilute solution of triethylamine in dioxane (5  $\mu$ l in 1 ml), allowed to stand for five minutes, and evacuated to dryness. The resulting sample was dissolved in 250  $\mu$ l of acetic anhydride and 6  $\mu$ l of the triethylamine solution and allowed to stand at room temperature overnight. The solution was evacuated to dryness and 500  $\mu$ l of dioxane were added for analysis by GCMS.

GCMS analyses were completed on a Finnigan 4000 mass spectrometer and Incos data system using a 15 m, DBI fused silica capillary column with a 0.25 mm i.d., 0.25 micron bonded phase film thickness (J & W Scientific, Folsom, CA) and helium carrier gas. The injection port was held at 275°C and the column was programmed from 150°C to 290°C at 25°C/minute with a five minute hold. Electron impact ionization at 70 eV was used and source temperature was 200°C. The ratios of the ions monitored were determined using the MID (multiple ion detection) facility of the Incos data system. During the GCMS analysis, the eight ions shown in Table 2 were sampled approximately five times each second with a 26 millisecond ramp time over a 0.5 dalton mass window. The percentages shown have been corrected for the natural isotopes present and for possible interfering fragments by subtracting the peak percentages determined from an unlabeled sample.

## RESULTS AND DISCUSSION

AIPM was prepared from the mixture of  $\alpha/\beta$  isomers resulting from coupling

carbobenzyloxyaspartic anhydride with phenylalanine methyl ester as outlined in Scheme 1 (Figure 2).

The product was obtained as an oil after evaporation of an ether solution. The compound could not be induced to crystallize and degraded when chromatographed, but the NMR and IR spectra were consistent with the proposed structure. This material was dissolved in citrate and phosphate buffer, sealed in ampoules, and stored at constant temperature. Samples were analyzed at the intervals in Table 1.

Comparison of the data for AIPM with  $\alpha$ -APM demonstrates that the aspartimide is a possible intermediate. AIPM shows a spectrum of degradation products entirely consistent with those observed for APM. It is much more labile and therefore would not be detected in APM reaction mixtures. Finally, ring opening to  $\beta$ -APM is favored by at least 10:1 and would explain the net conversion to the  $\beta$ -form. The above mentioned observations are necessary but not sufficient to invoke the intermediacy of AIPM. To differentiate the pathways, we prepared  $\alpha$ -APM labeled with  $O^{18}$  at the  $\beta$ -carboxyl. It is apparent that the fate of the label is quite different in the proposed pathways (Scheme 2, Figure 3). In Pathway A, one-half of the label is lost during dehydration to the aspartimide and the remainder is fixed at the  $\beta$ -carboxyl in the newly formed amide bond. In Pathway B, all of the  $O^{18}$  is retained, with one-half being transferred to the  $\alpha$ -carboxyl during closure to ii and subsequent migration.

The labeled aspartame was prepared as outlined in Scheme 3 (Figure 4).

The mass spectrum of the product showed 96% incorporation of one  $O^{18}$  atom in the  $\beta$ -carboxyl function. The stability of  $O^{18}$  toward exchange with  $H_2O$  during the equilibration process was demonstrated by allowing a solution of  $\beta$ -APM\*HCl to stand in  $H_2O^{18}$  (pH 1.5-2.0) for four

weeks. The mass spectrum showed less than 6% total incorporation of  $O^{18}$ . The  $O^{18}$  labeled  $\alpha$ -APM was dissolved in citrate buffer (pH 3.4) and held at  $40^{\circ}\text{C}$  for four weeks. The  $\beta$ -APM formed was then isolated from the resulting mixture by HPLC. This was converted to the methyl ester with diazomethane and, subsequently, to the acetamide with acetic anhydride. The results from mass spectral analysis of this material and similarly treated starting material and recovered  $\alpha$ -APM ( $O^{18}$ ) are shown in Table 2.

It is apparent from the molecular ion peaks of the  $\alpha$ -APM samples that no  $O^{18}$  has been lost by exchange with the medium. Almost exactly one half of the  $O^{18}$  has been lost in the conversion of  $\alpha$ - to  $\beta$ -APM and this is precisely as expected if AIPM is the intermediate. Pathway A also requires that all of the label should be in the carbonyl and should be lost in the 144 fragment (Figure 5), but the 144/146 ratio remained about 50:50.

This is not predicted for either of the proposed pathways or any combination thereof. For all the label to reside in the  $\alpha$ -carboxyl, either both oxygens or only the O8 must migrate during the  $\alpha$  -  $\beta$  isomerization and no rational pathway for such an occurrence is apparent. Furthermore, if the labeled oxygen did somehow migrate preferentially to the  $\alpha$ -carboxyl during the reaction, it would be evenly distributed between the carbonyl and alkoxy portion of the ester following treatment with diazomethane (Figure 6). The 70/72 ratio remains unchanged at about 50:50 and all the label must, therefore, be located on the carbonyl oxygen.

The most likely explanation for these divergent observations is that the mass spectral fragmentation is not quite as simple as anticipated. An alternative fragmentation which accommodates all the experimental data is shown in Scheme 4 (Figure 7). The key feature of this pattern is stabilization of the 172/174 fragment exclusively by the alkoxy oxygen of the  $\alpha$ -aspartyl ester. Expulsion of carbon monoxide to generate the 144/146 fragment then occurs with



exclusive elimination of the carbonyl of the  $\alpha$ -aspartyl ester and transfer of the alkoxy group to the carbonyl of the former amide linkage.

To test this hypothesis,  $\beta$ -APM labeled with  $O^{18}$  in the  $\alpha$ -carboxyl was prepared in a manner exactly analogous to Scheme 3 (Figure 4) but with N-carbobenzyloxy- $\beta$ -L-aspartyl-L-phenylalanine methyl ester as starting material. Incorporation of the label was found to be 89% by mass spectrometry. This material was isolated, derivatized, and analyzed exactly as the labeled  $\alpha$ -APM. The results recorded in Scheme 4 (Figure 7) agreed perfectly with the proposed fragmentation pattern.

Based on the evidence developed in this study, conversion of  $\alpha$ - to  $\beta$ -APM occurs exclusively via the pathway in which AIPM (*i*) is an intermediate.

## REFERENCES

- Baba T, Sugiyama H, Seto S (1973) Rearrangement of .alpha.-to .beta.-aspartyl peptide with anhydrous hydrogen fluoride. *Chem Pharm Bull Japan* 21(1): 207-209
- Battersby AR, Robinson JC (1955) Studies on specific chemical fission of peptide links: Part I. The rearrangement of aspartyl and glutamyl peptides. *J Chem Soc* 259-269
- Graves DJ, Luo S (1987) Decomposition of aspartame caused by heat in the acidified and dried state. *J Agric Food Chem* 35: 439-442
- Igano K; Minotani Y, Yosida N, Kono M, Inouye K (1981) A synthesis of human proinsulin C-peptide. *Chem Pharm Bull Japan* 54(10): 3088-3094
- McFadden PN, Clarke S (1986) Chemical conversion of aspartyl peptides to isoaspartyl peptides: A method for generating new methyl-accepting substrates for the erythrocyte D-aspartyl/L-isoaspartyl protein methyltransferase. *J Biol Chem* 261(25): 11503-11511
- Murray ED Jr, Clarke S (1986) Metabolism of a synthetic L-isoaspartyl-containing hexapeptide in erythrocyte extracts: Enzymatic methyl esterification is followed by nonenzymatic succinimide formation. *J Biol Chem* 261(1): 306-312
- Ondetti MA, Deer A, Sheehan JT, Pluscec J, Kocy O (1968) *Biochemistry* 7(11): 4069-4075
- Partridge SM, Davis HF (1950) *Nature (London)* 165: 62
- Perseo G, Forino R, Galantino M, Gioia B, Malatesta V, De Castiglione R (1986) Side reactions in peptide synthesis. I. Formation of aminosuccinyl derivatives from aspartyl peptides: A known side reaction in unusual conditions. *Int J Pept Protein Res* 27(1): 51-60
- Schon I, Kisfaludy L (1979) Formation of aminosuccinyl peptides during acidolytic deprotection followed by their transformation to piperazine-2,5-dione derivatives in neutral media. *Int J Pept Protein Res* 14(5): 485-494
- Stamp JA, Labuza TP (1989) Mass spectrometric determination of aspartame decomposition products: Evidence for B-isomer formation in solution. *Food Addit Contam* 6(4): 397-414
- Stegink LD, Files LJ Jr., (eds) (1984) *Aspartame: Physiology and Biochemistry*, Marcel Dekker, Inc., NY, NY

Takahashi S, Takemoto T (1989) A process for preparation of cyclic imides and their conversion into .alpha.-aspartylphenylalanine derivatives. EP 297560 A2

Witt J (1986) Stability of aspartame and its conversion products in wet beverage systems. International Aspartame Workshop Proceedings, Marbella, Spain, Nov 17-21, 1986. International Life Sciences Institute, Nutritional Foundation, Washington, D.C.

Yang CC, Merrifield RB (1976) .beta.-Phenacyl ester as a temporary protection group to minimize cyclic imide formation during subsequent treatment of aspartyl peptides with hydrofluoric acid. J Org Chem 41(6): 1032-1041

FIGURE 1

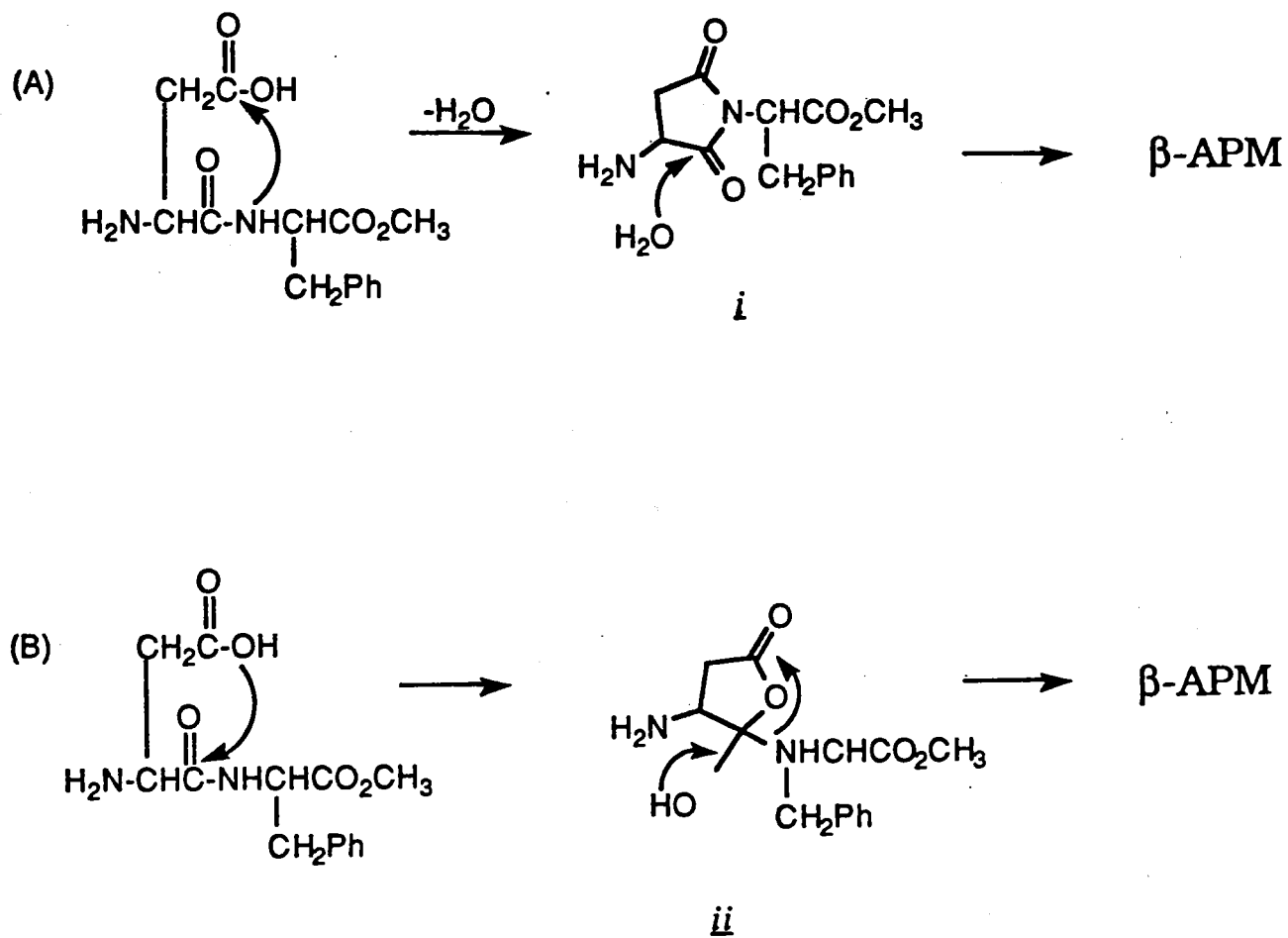
MECHANISTIC PATHWAYS FOR  $\alpha$  to  $\beta$  CONVERSION

FIGURE 2

SCHEME 1

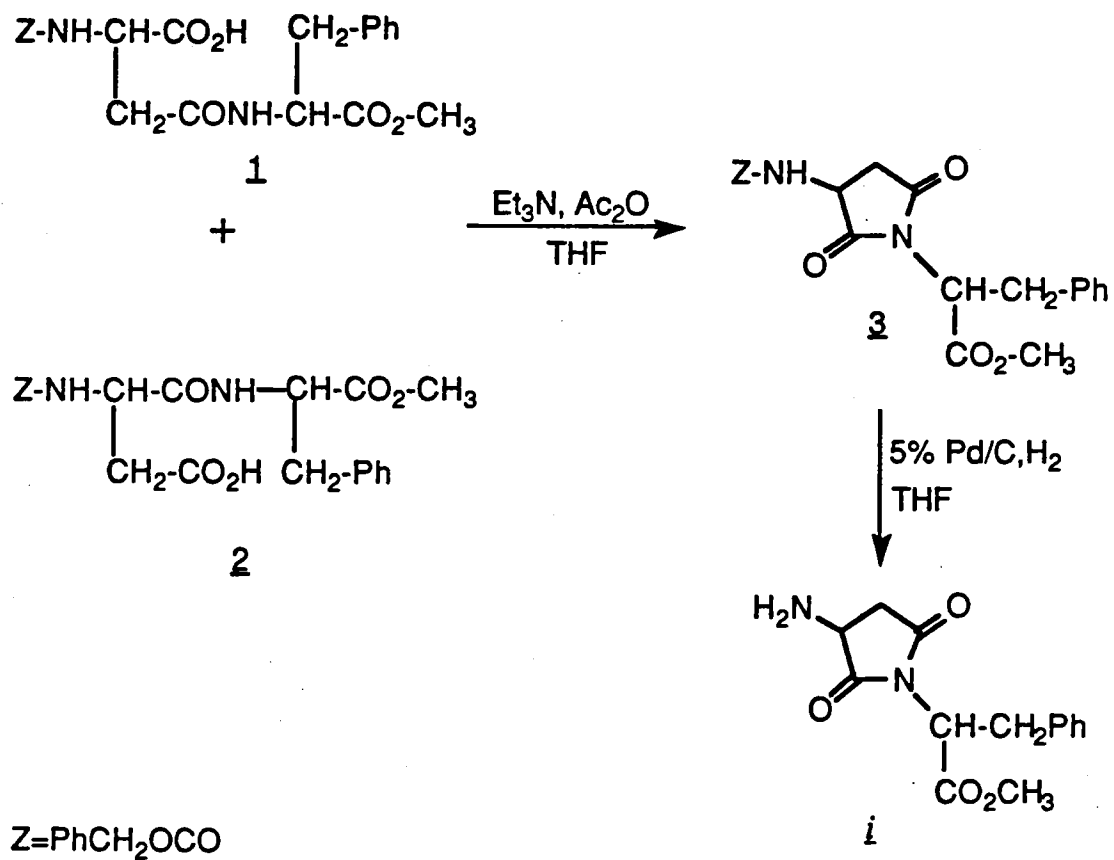


FIGURE 3

SCHEME 2

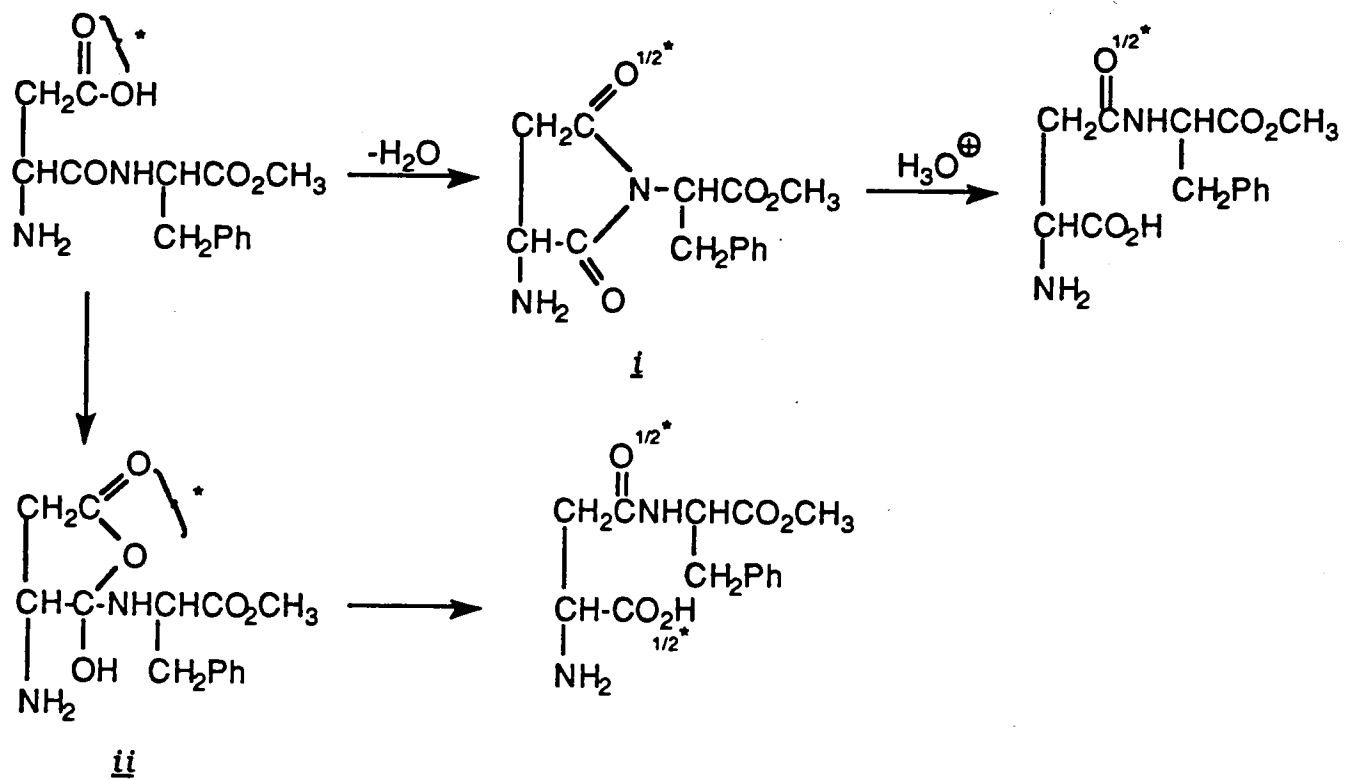


FIGURE 4

SCHEME 3

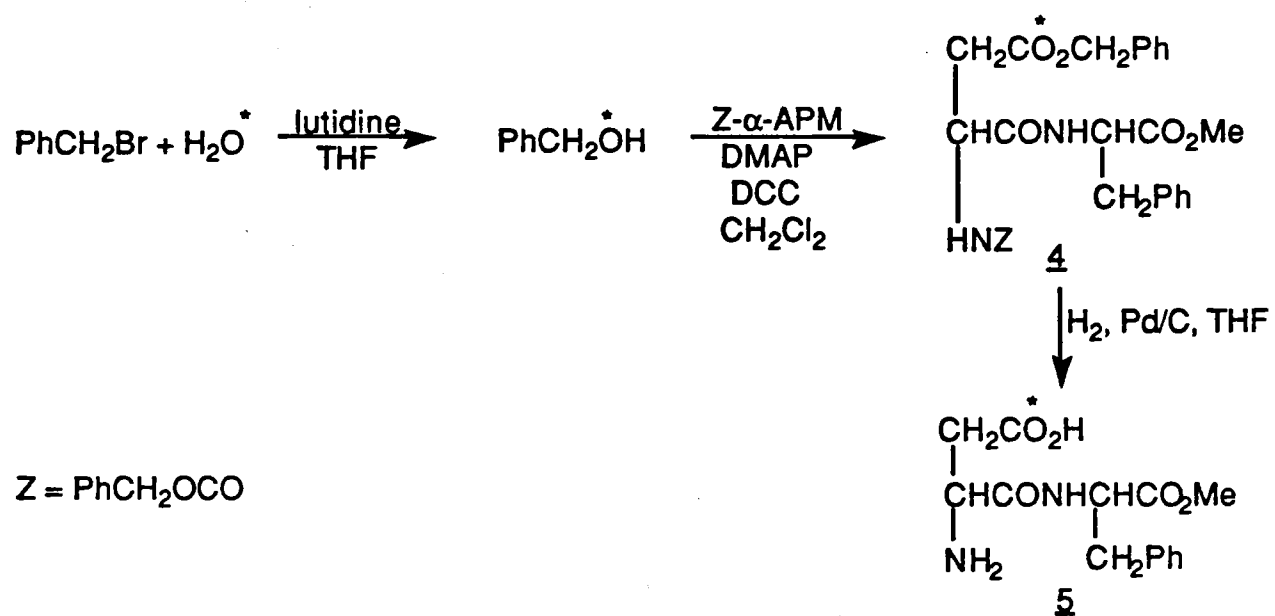


FIGURE 5

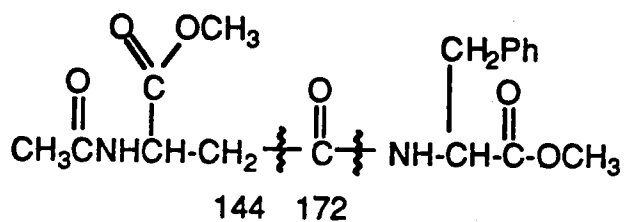


FIGURE 6

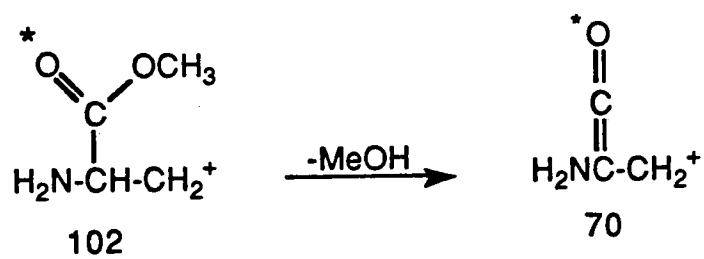
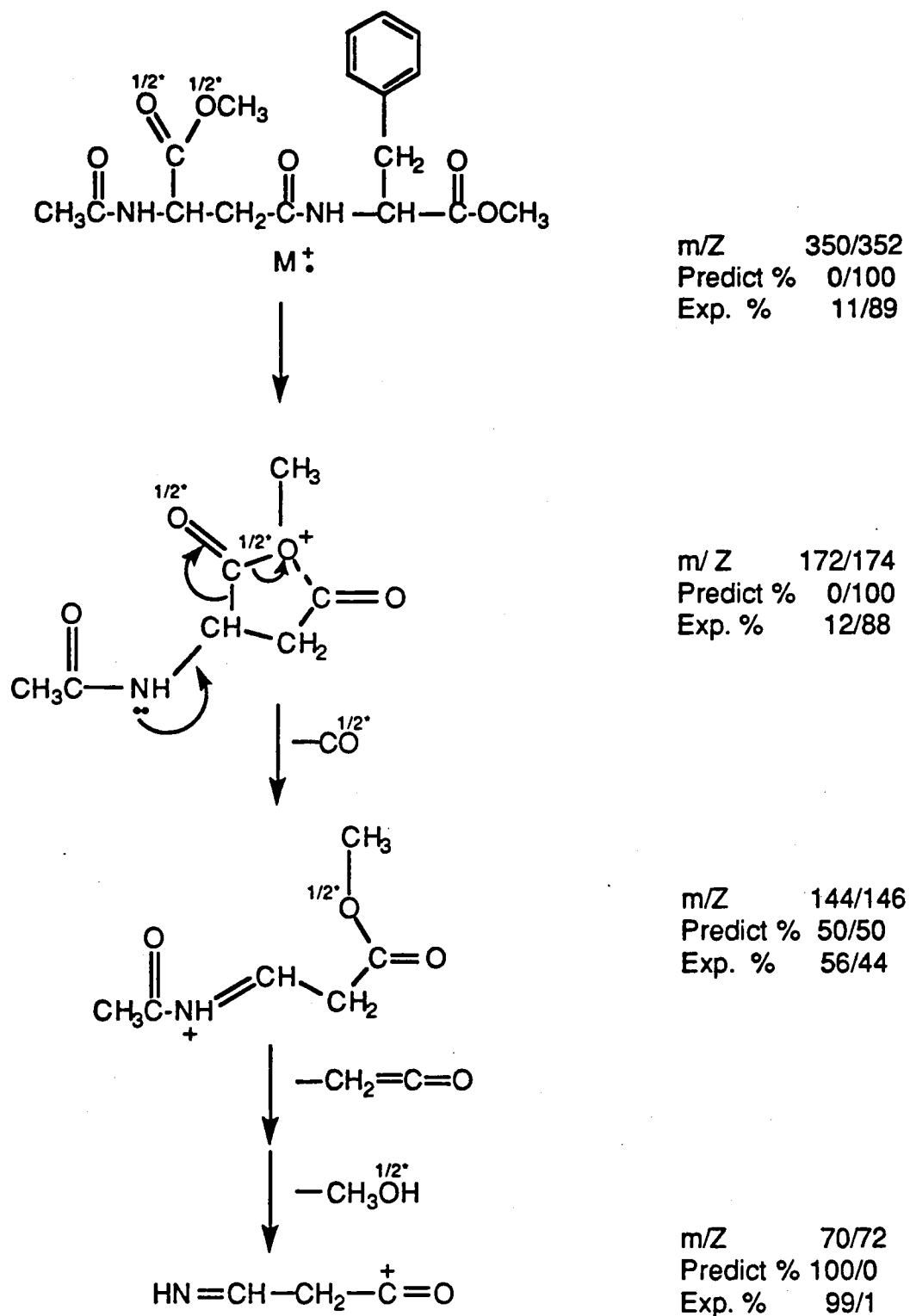




FIGURE 7

SCHEME 4



$\alpha$ -APM, pH = 3.4, citrate buffer, 40°C.

TIME	1 $\alpha$ -APM	2 $\beta$ -APM	3 $\alpha$ -AP	4 $\beta$ -AP	5 DKP	6 PM	7 PHE	8 PA	TOTAL MOLE BALANCE
1 Week % Moles	90.3	0.7	2.3	0.1	3.6	1.6	0.0	0.0	98.6%
2 Weeks % Moles	82.6	1.5	3.8	0.6	6.2	2.9	0.5	0.0	98.2%
3 Weeks % Moles	76.6	2.5	4.9	1.3	9.3	4.1	1.0	0.0	99.7%

**AIPM, pH = 3.4, citrate buffer, 40°C.**

TIME	1 $\alpha$ -APM	2 $\beta$ -APM	3 $\alpha$ -AP	4 $\beta$ -AP	5 DKP	6 PM	7 PHE	8 PA	9 AIPM	TOTAL MOLE BALANCE
1 Week % Moles	5.0	64.3	0.2	1.0	0.2	0.1	0.1		7.5	78.3%
3 Weeks % Moles	4.6	65.4		4.1		2.0			0.0	76.1%

**AIPM, pH = 3.4, citrate buffer, 20°C.**

[illegible]

**$\alpha$ -APM, pH = 2.5, phosphate buffer, 40°C.**

TIME	1 $\alpha$ -APM	2 $\beta$ -APM	3 $\alpha$ -AP	4 $\beta$ -AP	5 DKP	6 PM	7 PHE	8 PA	TOTAL MOLE BALANCE
1 Week % Moles	83.5	1.1	7.0	0.3	7.1	4.7	0.0	0.0	103.7%
2 Weeks % Moles	66.1	2.4	10.4	1.2	11.7	8.0	1.7	0.2	101.7%
3 Weeks % Moles	53.0	3.6	12.0	2.7	16.8	10.6	3.3	0.5	102.5%

**AIPM, pH = 2.5, phosphate buffer, 40°C.**

TIME	1 $\alpha$ -APM	2 $\beta$ -APM	3 $\alpha$ -AP	4 $\beta$ -AP	5 DKP	6 PM	7 PHE	8 PA	9 AIPM	TOTAL MOLE BALANCE
1 Week % Moles	5.0	55.2	0.3	4.1	0.3	0.2			21.7	86.8%
2 Weeks % Moles	6.2	66.5		10.6					5.0	88.3%

**AIPM, pH = 2.5, phosphate, 20°C.**

[illegible]

TABLE 2

	M/e	70	72	144	146	172	174	350(M+)	352
$\beta$ -APM(O <sup>18</sup> )%		54	46	54	46	54	46	52	48
$\alpha$ -APM (O <sup>18</sup> )% Recovered		53	47	7	93	9	91	6	94
$\alpha$ -APM (O <sup>18</sup> )% Starting Material		53	47	5	95	7	93	4	96



**MECHANISM OF ASPARTAME DEGRADATION REACTION**

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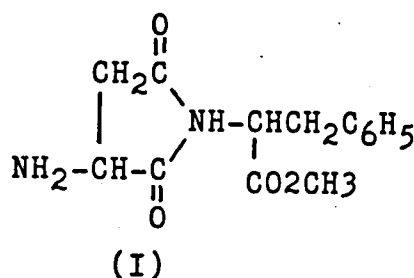
**Key Words: aspartame, degradation, kinetic**

## SUMMARY

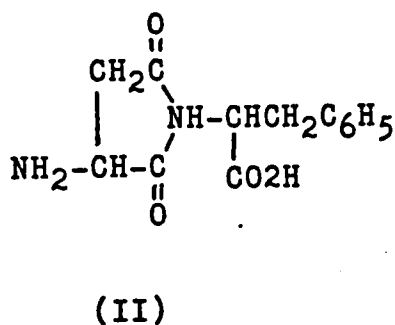
A kinetic study was conducted to clarify the pathway and the rate of the degradation of aspartame in weakly acidic aqueous solution. It was observed that aspartame primarily decomposed to diketopiperazine, aspartylphenylalanine, phenylalanine methyl ester and aspartimidophenylalanine methyl ester in a ratio of 33:40:10:17. These primary decomposition products underwent further rearrangements and hydrolyses. Based on the kinetics observed, an integrated pathway for the degradation of aspartame in soft drinks is discussed.

## ABBREVIATION

asp:	aspartic acid
APM:	$\alpha$ -aspartylphenylalanine methyl ester
$\beta$ -APM:	$\beta$ -aspartylphenylalanine methyl ester
AP:	$\alpha$ -aspartylphenylalanine
$\beta$ -AP:	$\beta$ -aspartylphenylalanine
DKP:	diketopiperazine of aspartylphenylalanine
IMIDM:	Postulated intermediate of the reaction (1) with the possible structure (I)



IMIDE: Postulated intermediate of the reaction (2) with the possible structure (II)



Phe:	Phenylalanine
PM:	Phenylalanine methyl ester



## INTRODUCTION

It is known that aspartame (APM) sweetened soft drinks lose their sweetness during long-term storage, especially at higher temperatures. Furda *et al.* [1975] observed that besides the rearrangement products L-aspartylphenylalanine (AP) and 3-carboxymethyl-6-benzyl-2,5-diketopiperazine (DKP) that the hydrolytic products aspartic acid (Asp), phenylalanine (Phe) and phenylalanine methyl ester were produced from APM when stored in water for long periods. Recently, Prudel *et al.* [1986] investigated the kinetics of APM hydrochloride decomposition in aqueous solutions. Preliminary studies in our laboratory of the degradation of APM in carbonated beverages indicated a more complex degradation reaction than had been previously reported. In this report, we describe the kinetics of APM degradation and provide a kinetic model for the loss of APM and the generation of decomposition products.

## EXPERIMENTAL

Fifty milligrams of APM was dissolved in 100 ml of 0.2 N phosphoric acid-sodium phosphate buffer adjusted to pH 3.0, 4.5, 6.0. To each of these solutions was added 50 mg of benzoic acid as a preservative. Similarly prepared were the solutions of six degradation intermediates. Each solution was incubated at a constant temperature (38.6°C or 40°C). At appropriate intervals, samples were withdrawn and analyzed by HPLC and amino acid analyzer.

## RESULTS

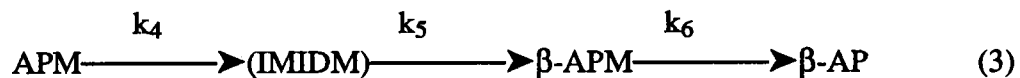
### Calculation of the Temporary Rate Constants

#### Disappearance Rate of APM and Its Degradation Intermediates

When APM was the starting material at pH 3.0 and 38.6°C, a least squares fit of  $-\ln[APM]/[APM_0]$  vs. time gave the disappearance rate constant of APM as  $1.74 \times 10^{-3}/\text{hour}$  (Figure 1). In a similar manner the temporary disappearance rate constants of other compounds were determined, and the results shown in Table 1. In the case where a good fitness was not obtained due to analytical errors, the sum of the products was used to determine the disappearance rate of the starting materials.

Rate constants of the parallel reactions in Figure 2 were determined using the equations:

$k_1/k_0 = d[AP]/d[APM]$ ,  $k_2/k_0 = d[DKP]/d[APM]$ ,  $k_3/k_0 = d[Asp]/d[APM]$ , where  $k_0$  is the disappearance rate constant of APM. However, in the case of  $APM \rightarrow \beta\text{-APM}$ , a least squares analysis of  $\beta\text{-APM}$  vs. APM did not yield a good fit suggesting the presence of an intermediate (Figure 3) [Meinwald, 1986]. Then postulating the reaction (3), rate constants  $k_4$  and  $k_5$  were determined by the graphical integration of equation (4) via personal computer where the best fitness of the data and calculated values were given on the display (Figure 4). In this way, degradation rate constants of APM and other intermediates were determined, Table 2 and 3.



$$\begin{aligned} \beta\text{-APM} = \text{APM}_0 & * \frac{k_4 k_5}{(k_5 - k_0)} * \frac{1}{(k_6 - k_0)} * [\exp(-k_0 t) - \exp(-k_6 t)] \\ & - \frac{1}{(k_6 - k_5)} * [\exp(-k_5 t) - \exp(-k_6 t)] \end{aligned} \quad (4)$$

### Determination of the Reaction Rate Constants

Because the temporary constants calculated above did not take into consideration the reverse and secondary reactions, these constants were corrected in the following way. Reaction constants which were not affected by the reverse and secondary reactions (i.e.,  $\text{PM} \rightarrow \text{Phe} + \text{CH}_3\text{OH}$  or  $\beta\text{-APM} \rightarrow \beta\text{-AP}$ ) were adopted without correction. Then according to the reaction schemes in Figure 5, differential equations 5 to 13 were set up.

$$d[\text{APM}]/dt = -(k_{82} + k_{81} + k_{85} + k_{84})[\text{APM}] + k_{48}[\text{IMIDE}] \quad (5)$$

$$d[\text{IMIDM}]/dt = k_{84}[\text{APM}] - (k_{46} + k_{49} + k_{48})[\text{IMIDM}] + k_{94}[\beta\text{-APM}] \quad (6)$$

$$d[\beta\text{-APM}]/dt = k_{49}[\text{IMIDM}] - (k_{95} + k_{97} + k_{94})[\beta\text{-APM}] \quad (7)$$

$$d[\text{DKP}]/dt = -(k_{12} + k_{13})[\text{DKP}] + k_{21}[\text{AP}] + k_{31}[\text{PA}] + k_{81}[\text{APM}] \quad (8)$$

$$d[\text{AP}]/dt = -(k_{21} + k_{25} + k_{26})[\text{AP}] + k_{12}[\text{DKP}] + k_{62}[\text{IMIDE}] + k_{82}[\text{APM}] \quad (9)$$

$$d[\text{PA}]/dt = k_{13}[\text{DKP}] - (k_{31} + k_{35})[\text{PA}] \quad (10)$$

$$d[\text{IMIDE}]/dt = k_{26}[\text{AP}] - (k_{62} + k_{67})[\text{IMIDE}] + k_{76}[\beta\text{-AP}] + k_{46}[\text{IMIDM}] \quad (11)$$

$$d[\beta\text{-AP}]/dt = k_{67}[\text{IMIDE}] - (k_{75} + k_{76})[\beta\text{-AP}] + k_{97}[\beta\text{-APM}] \quad (12)$$

$$d[\text{Asp}]/dt = k_{25}[\text{AP}] + k_{35}[\text{PA}] + k_{75}[\text{APM}] + k_{95}[\beta\text{-APM}] \quad (13)$$

By graphical integrations of the above equations via personal computer, the fittest solutions on the display were selected. The rate constants thus obtained were quite consistent with the data; typical results are shown in Figures 6 and 7. Similarly, rate constants for more neutral conditions, pH 6.0, 38.6°C were determined and are given in Figures 8 and 9.

## DISCUSSION

### First Step in APM Degradation Reaction

The rate constants for the formation of the initial degradation products of aspartame are shown in Table 4. It is noteworthy that at pH 3.0 the conversion rate of APM to IMIDM intermediate and that the proportion of the peptide fission reaction ( $\text{APM} \rightarrow \text{Asp} + \text{PM}$ ) in the first degradation reactions are substantial. These unexpected results are not observed at neutral pH nor in strongly acidic media (unpublished results) suggesting that these reactions are specific to weakly acidic solutions. According to the scheme proposed by M. Prudel [1986] for APM degradation, DKP is the secondary product of APM and is formed primarily from AP. However, the present analysis shows that DKP is formed from both APM and AP.

### The Secondary and Higher Decomposition Reactions of APM

As can be seen from Figure 8, at the pH of carbonated beverages (pH 3) APM and its degradation products are produced at a characteristic frequency. AP is one of the initial degradation products. The reaction velocities of AP and APM to cyclize to DKP are in the same order of magnitude (Table 5). It has been proposed that APM tended to cyclize to DKP due to the presence of a methyl ester [Greenstein and Winitz, 1961](Figure 10). The results of this study indicate that this is not the case in a weakly acidic condition. At neutral pH, AP does not cyclize to DKP showing that this reaction is peculiar to weakly acidic conditions. Moreover, the

reactivities of AP to IMIDE intermediate and to peptide bond fissioned products are somewhat higher than those of APM. Although the reason is not clear, it may be attributed to the presence of two carboxyl groups in AP vs. one in APM.

The comparative reactivities of AP and  $\beta$ -AP at pH 3.0, 38.6°C are listed in Table 6. Formation of DKP is not observed from  $\beta$ -AP, which can not cyclize to DKP structurally. Moreover, peptide bond fission reaction yielding Asp and Phe from  $\beta$ -AP is quite slow compared with that from AP. This phenomenon is more marked in the case with APM and  $\beta$ -APM (Table 7).

DKP is formed from APM and AP as stated above, and also from PA (Table 8). The DKP formed was hydrolyzed to AP and PA reversibly. The reactivities of DKP toward AP and PA were found to be the same (Table 9).

#### **Reproducibility of the Experiments and Effect of Buffer**

To confirm the accuracy of the results, the experiments were repeated twice at pH 3.0 and 40°C; despite incubation at different temperatures, the results showed a very good agreement with those obtained at 38.6°C (Table 10).

In conclusion, APM behaves quite uniquely in weakly acidic condition. And we are now going to measure the reaction rate of the imide intermediate starting from aspartimide intermediate itself to obtain more accurate data. Although the reactivities of other dipeptides in weakly acidic solution has not been studied, it seems interesting to compare those with that of APM.

## REFERENCES

- Bodanszky M, Kwei JZ (1978) Side reactions in peptide-synthesis: 7. Sequence dependence in formation of aminosuccinyl derivatives from beta-benzyl-aspartyl peptides. *Int J Peptide & Protein Res* 12: 69-74
- Bodanszky M, Tolle JC, Deshmane SS, Bodanszky A (1978) Side reactions in peptide-synthesis: 6. Re-examination of Benzyl group in protection of side-chains of tyrosine and aspartic-acid. *Int J Peptide & Protein Res* 12: 57-68
- Furda I, Malizia PD, Kolor MG, Vernieri PJ (1975) Decomposition products of L-aspartyl-L-phenylalanine methyl ester and their identification by gas-liquid chromatography. *J Agr Food Chem* 23: 340-343
- Greenstein JP, Winitz M (eds) (1984) *Chemistry of the amino acids*, Vol. 2, John Wiley & Sons, Inc., New York, NY
- Meinwald YC, Stimson ER, Scheraga HA (1986) Deamidation of the asparaginyglycyl sequence. *Int J Peptide & Protein Res* 28: 79-84
- Prudel M, Davidkova E, Davidek J, Kminek M (1986) Kinetics of decomposition of aspartame hydrochloride (Usal) in aqueous solutions. *J Food Sci* 51: 1393-1415

**TABLE 1**  
Temporary Disappearance Rate Constants of the Starting Materials  
pH 3.0, 38.6°

Starting Material	APM	DKP	AP	PM	β-APM	PA	β-AP
Rate Constants*	174	8.2	154	164	73.4**	62.6**	15

\*  $k = 1/\text{hr} \times 10^{-5}$

\*\* Determined using the sum of the products

**TABLE 2**  
Temporary Rate Constants of APM Degradation at  
pH 3.0, 38.6° ( $k = 1/\text{hr} \times 10^{-5}$ )

Starting Material	PRODUCT								
	APM	DKP	AP	PM	IMIDM	β-APM	PA	Phe	β-AP
APM	174	75.3	54.6	18.6	40	--	--	--	--
DKP	--	8.23	2.67	--	--	--	4.22	--	--
AP	--	57	154	--	--	--	--	33.9	--
PM	--	--	--	164	--	--	--	156	--
β-APM	1.6	--	--	--	--	--	--	--	71.8
IMIDM	--	--	--	--	--	950	--	--	--
PA	--	28.4	0.37	--	--	--	--	33.8	5
β-AP	--	--	5.17	--	--	--	--	8.83	15.0

The numeral shows the formation rate constants of the product from the starting material in the left column. And when the product and starting material is the same, the numeral shows the decreasing rate constant.

**TABLE 3**  
Temporary Rate Constants of APM Degradation at  
pH 6.0, 38.6° ( $k = 1/\text{hr} \times 10^{-5}$ )

Starting Material	PRODUCT							
	APM	DKP	AP	PM	$\beta$ -APM	PA	Phe	$\beta$ -AP
APM	4320	4320	162	0	--	--	--	--
DKP	--		0	--	--	0.16	--	--
AP	--	--	0	--	--	--	33.9	--
PM	--	--	--	4420	--	--	4420	--
$\beta$ -APM	1.6	--	--	--	253	--	--	241
PA								
$\beta$ -AP	--	--	0	--	--	--	0	0

The numeral shows the formation rate constants of the product from the starting material in the left column. And when the product and starting material is the same, the numeral shows the decreasing rate constant.

**TABLE 4**  
Comparison of the First Degradation of APM  
( $k = 1/\text{hr} \times 10^{-5}$ )

		pH 3.0	pH 6.0
APM	AP	30 (33%)	160
	DKP	75 (40%)	4200
	IMDM	32 (17%)	0
	Asp + PM	19 (10%)	0



**TABLE 5**  
**Comparison of the Reactivity of APM and Asp-Phe**  
**pH 3.0, 38.6° ( $k = 1/\text{hr} \times 10^{-5}$ )**

	from APM	from AP
to DKP	75	57
to IMIDM (E)	32	110
to Asp+Phe or PM	19	34

**TABLE 6**  
**Comparison of the Reactivity of APM and  $\beta$ -AP**  
**pH 3.0, 38.6° ( $k = 1/\text{hr} \times 10^{-5}$ )**

	from APM	from $\beta$ -AP
to Asp+Phe	34	8
to IMIDE	110	80
to DKP	57	--

**TABLE 7**  
**Peptide Bond Fission Reaction**  
**pH 3.0, 38.6° ( $k = 1/\text{hr} \times 10^{-5}$ )**

	from APM	from $\beta$ -APM
to Asp+Phe + PM	19	0

**TABLE 8**  
**Rate Constants of DKP Formation**  
 pH 3.0, 38.6° ( $k = 1/\text{hr} \times 10^{-5}$ )

from APM	from AP	from PA
75	57	28

**TABLE 9**  
**Rate Constants of DKP Ring Opening**  
 pH 3.0, 38.6° ( $k = 1/\text{hr} \times 10^{-5}$ )

to AP	to PA
5	5

**TABLE 10**  
**Reproductivity of the Experiments**  
 pH 3.0, 38.6° ( $k = 1/\text{hr} \times 10^{-5}$ )  
**Temporary Rate Constants of APM Degradation**

	APM	DKP	AP	PM	$\beta$ -APM+ $\beta$ -AP	PA	Phe
38.6°	176	75.3	54.6	18.6		--	--
40°	198	87.2	58.1	22.1	34.8	--	--
40°	196	87.2	56.9	21.1	34.3	--	--

The numeral shows the temporary decreasing constant of APM and the formation constants of products from APM.



## **GENERAL TOXICOLOGY STUDIES WITH $\beta$ -ASPARTAME**

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**Key Words:**  $\beta$ -aspartame,  $\beta$ -dipeptide,  $\beta$ -aspartyl-dipeptide,  $\beta$ -aspartylphenylalanine, acute toxicity, subchronic toxicity, chronic toxicity, pharmacology, mice, rats, dogs

## ABSTRACT

$\beta$ -aspartame (N-L- $\beta$ -aspartyl-L-phenylalanine methyl ester;  $\beta$ -APM) is formed in small quantities from aspartame in solution, and is also found in low concentrations in aspartame. To confirm the safety of  $\beta$ -APM, acute short-term (28 days) and long-term (26 weeks) toxicity studies were done, and the genotoxicity and carcinogenic potential was evaluated. Acute gavage toxicity studies were done in rats and mice at dosages of 0 and 5000 mg  $\beta$ -APM/kg. In these acute studies, there were no adverse effects related due to  $\beta$ -APM. Dosages tested in the 4- and 26-week rat and dog studies were approximately 250, 500, and 1000 mg  $\beta$ -APM per kg body weight. In either the short-term or long-term rat or dog studies, there were no  $\beta$ -APM related changes in clinical observations, body weights, food consumption, electrocardiograms, ophthalmic examinations, hematology, clinical chemistry, or urinalysis parameters. In addition, there were no  $\beta$ -APM related changes in organ weights, organ weight ratios, or gross or microscopic findings. Pharmacokinetic analysis of plasma samples from the 26-week rat and 4- and 26-week dog studies showed that  $\beta$ -aspartylphenylalanine, the  $\beta$ -APM metabolite, was detected at all dosage levels; plasma levels were proportional to dosage; and there was no indication of accumulation in plasma following repeated doses. Additional studies showed that  $\beta$ -APM did not produce effects in a variety of pharmacology screening tests that examined the central nervous system, gastrointestinal tract, and cardiovascular and renal systems.

In addition, there is compelling evidence that  $\beta$ -APM is not carcinogenic. First,  $\beta$ -APM undergoes rapid and complete first-pass metabolism to  $\beta$ -AP, a normal constituent of human plasma and urine. Second,  $\beta$ -APM was not genotoxic in various in vitro and in vivo tests. Finally, no carcinogenic effects were observed in several studies with aspartame in which  $\beta$ -APM and  $\beta$ -AP were minor constituents.

In conclusion, there were no  $\beta$ -APM related changes in the rat and dog following repeated dosages of up to 1000 mg/kg for 26 weeks. In addition,  $\beta$ -APM is not carcinogenic.

## INTRODUCTION

$\beta$ -dipeptides are naturally occurring substances found in both plants and animals [Carnegie, *et al.*, 1982; Kasai, *et al.*, 1981; Drey, 1985]. These compounds are either normal components of our diets, formed from the protein digestion, and/or synthesized in vivo from amino acids [Pisano *et al.*, 1966; Kasai *et al.*, 1980]. A number of  $\beta$ -aspartyl dipeptides have been identified as normal constituents in human urine [Kakimoto and Armstrong, 1961; Buchanan *et al.*, 1961]. For example,  $\beta$ -aspartylphenylalanine ( $\beta$ -AP), has been isolated and identified in human plasma and urine [Burton *et al.*, 1989]. There are no published reports that  $\beta$ -aspartyl dipeptides produce toxicity or play a role in human disease [Hjelle *et al.*, 1993].

$\beta$ -aspartame (N-L- $\beta$ -aspartyl-L-phenylalanine, 1-methyl ester,  $\beta$ -APM) is formed in small amounts from aspartame in solution and is present in very low concentrations in manufactured aspartame (Figure 1) [Tsang, *et al.*, 1985; Witt, 1986; Witt, 1993]. The results of beverage stability studies indicate that  $\beta$ -APM formation depends on temperature, pH and time [Witt, 1986].

Although  $\beta$ -aspartyl dipeptides are considered safe in the published literature, acute short-term and long-term toxicity studies were conducted to confirm the safety of  $\beta$ -APM. Specifically, the acute oral toxicity was determined in mice and rats, and 4- and 26-week diet admix studies were in rats and dogs. The results of the acute and 4-week studies have been discussed previously [Hattan *et al.*, 1987]. This manuscript provides detailed descriptions of the acute and 4-week studies and also presents the results of the long-term toxicity studies. Finally, the evidence demonstrating  $\beta$ -APM is not carcinogenic is reviewed.

## METHODS

**Acute Studies:** The acute toxicity of  $\beta$ -APM was determined in male and female mice and rats. In acute studies, Crl:CD-1(ICR)BR mice and Crl:CD(SD)BR rats, obtained from the Portage, Michigan facility of Charles River Laboratories, were used. At study initiation, mice were approximately 5 weeks old and rats were approximately 8 to 10 weeks old. The animals were housed in suspended stainless steel, screen bottom cages; mice were housed two or three to a cage and rats were housed grouped by sex. Mice and rats were fed ad libitum Certified Rodent Chow #5002 and Rodent Chow #5001, respectively. Mice were fasted for 4 hours before dosing and rats were fasted overnight before dosing. Water was provided ad libitum from an automatic water system. Healthy animals were randomly assigned to the test and control groups (10 animals/sex/group) and placed in temperature (72F  $\pm$  3F) and humidity (50%  $\pm$  20%) controlled rooms with a 12-hour light/dark cycle.  $\beta$ -APM was suspended in 0.5% (w/v) methylcellulose and 0.1% (v/v) polysorbate 80 in distilled water at a concentration of 250 mg/ml. The  $\beta$ -APM suspensions were thoroughly stirred during dosing and samples were analyzed to confirm concentration and homogeneity. The mice and rats were given single gavage doses of the vehicle or 5000 mg  $\beta$ -APM/kg body weight. Animals were observed daily for 14 days. Body weights were recorded on the dosing days, and on Days 7 and 14. At the end of the observation period, all animals were killed and subjected to a gross necropsy.

**Short-Term and Long Term Toxicity Studies:** Specific design features of the short-term and long term rat and dog toxicity studies are presented in Table 1. The animals were provided  $\beta$ -APM in the diet for 4 or 26 weeks. At the end of the 26-week studies, a satellite group of animals was given control diet for a 4-week recover period. The dosages in these studies were 0, 250, 500 and 100 mg/kg/day. Rats and dogs were individually housed in stainless steel cages in temperature (72  $\pm$  5F) and humidity (25%) controlled rooms with a 12-hour light/dark cycle. Tap



water was available ad libitum. Fresh diets containing  $\beta$ -APM were prepared at least once weekly. Sampling and analysis of the diet admixes confirmed that the  $\beta$ -APM diets were homogeneous and at the appropriate concentrations.

Healthy animals were assigned to dosage groups by a randomized blocking procedure based on body weights. Statistics were performed on quantitative data, separated by sex, for the 4- and 26-week studies. The statistical analyses on the data consisted of a standard one way analysis of variance (ANOVA) [Winer, 1971], and homogeneity of variance tests either by the Bartlett-Box [Box, 1949] or Levene's [Levene, 1960] method. In general, if the F-test among groups was significant, two-tailed t-tests of each treated group versus the control group were performed using the pooled error term from the one-way analysis of variance. All group comparisons found to be statistically significant at the 5% level or less were reported. Pharmacokinetic analysis (e.g. Cmax, Tmax) of data included normalization of values that were tested for dose, sex and day effects using analysis of variance techniques.

In the rat studies, fasted animals were anesthetized with ether and blood was collected from the abdominal aorta (4-week study) or orbital sinus (26-week study) for clinical pathology determinations.

In the 4-week dog study, samples of venous blood (jugular) for clinical pathology were collected before feeding on Day 1 and after feeding on Days 12, 15, and 28. In the 26-week dog study, samples of venous blood and urine were collected before the start of dosing, and immediately before feeding during Weeks 8, 17, 26, and 30 of the study.

The blood parameters measured in the studies were as follows: white blood cell count (WBC), red blood cell count (RBC), hematocrit (HCT), hemoglobin (HGB), red blood cell indices,

platelet count (PLT), activated partial thromboplastin time (APTT) (dogs only), prothrombin time (PT), differential smear evaluation, alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea (UN), creatinine (CT) (dogs only), glucose (GLUC), alkaline phosphatase (ALP), cholesterol (CHOL), total bilirubin (TBILI), sodium (Na), potassium (K), chloride (Cl), calcium (Ca), total protein (TPRO), albumin (ALB), globulin (G) (calculated), and A/G ratio (calculated). The urinary parameters measured were as follows: pH, refractive index/specific gravity, glucose, bilirubin, protein, ketones, occult blood, urobilinogen, microscopic examination of the centrifuged sediment, and volume (in the 4-week rat and both 26-week studies).

Plasma samples obtained in the 4-week dog study and both 26-week studies for pharmacokinetic determinations were analyzed for N-L--aspartyl-L-phenylalanine, the free acid of  $\beta$ -APM by high performance liquid chromatography [Burton *et al.*, 1989] to confirm systemic exposure.

Rats were killed at the end of the studies following ether anesthesia and blood collection. Dogs were killed by an overdose of sodium pentobarbital and gross necropsies were performed. Major organs were weighed and in general, the following tissues were sampled for microscopic examination: adrenal, aorta, bone marrow, bone (sternum and/or femur), brain, epididymis, esophagus, eye, gallbladder (dogs only), heart, duodenum, jejunum, ileum, cecum, cervix, colon, rectum, kidney, lacrimal/Harderian gland, larynx, liver, lung, lymph node, mammary gland (females only), ovary, pancreas, peripheral nerve, pituitary gland, prostate, salivary gland, seminal vesicle, skeletal muscle, skin, spinal cord, spleen, stomach, testis, thymus, thyroid gland, parathyroid, tongue, trachea, urinary bladder, uterus, and vagina. The testes were fixed in Bouin's solution and the eyes in Zenker's solution, all other tissues were fixed in buffered 10% formalin. After fixation, tissues were embedded in paraffin, sectioned and stained with hematoxylin and eosin and examined microscopically for any abnormalities.

The potential pharmacological effects of  $\beta$ -APM on the central nervous system, cardiovascular and renal systems and the gastrointestinal systems were studied in standard pharmacology models. In addition, the effects of  $\beta$ -APM on platelet aggregation, cyclooxygenase activity, slow reacting substance biosynthesis, 5-lipoxygenase activity, and pancreatic lipase activity were evaluated using standard in vitro pharmacology methods.

## RESULTS

### Acute Oral Toxicity Study in the Mouse

None of the animals died. Watery stools, which are common findings in rats administered large amounts of test article suspension by gavage, were observed in 7 of 10 female animals within 4 hours after the 5000 mg/kg dosage; these effects were not observed 24 hours after dosing. There were no  $\beta$ -APM related clinical observations in the males, changes in body weights, or gross pathologic findings in any animals.

### Acute Oral Toxicity Study in the Rat

None of the animals died. At 5000 mg/kg, watery stools were observed in 5 of 10 male and 10 of 10 females within 4 hours after dosing. Yellow staining of the genital area was observed in 2 of 10 males and 4 of 10 females 24 hours after dosing. No clinical signs were observed 48 hours after dosing. There were no other  $\beta$ -APM related clinical observations, any  $\beta$ -APM related changes in body weights, or gross pathologic findings in any animals.

### Four Week Dietary Admix Study in the Rat

The  $\beta$ -APM dosages were 250, 500 and 1,000 mg/kg/day. No animals died during treatment and

no treatment related clinical observations, or treatment related changes in body weights, food consumption, ophthalmic examinations, hematology, serum chemistry, or urinalysis parameters were observed. Furthermore, there were no treatment related changes in organ weights or organ weight ratios and there were no gross or microscopic findings.

#### **Four Week Dietary Admix Study in the Dog**

The actual  $\beta$ -APM dosages 250, 500 and 1,000 mg/kg. No animals died and there were no treatment related changes in clinical observations, body weights, food consumption, electrocardiograms, ophthalmic examinations, or hematology, clinical chemistry, and urinalysis parameters. Furthermore, there were no treatment related changes in organ weights or organ weight ratios, and no gross or microscopic findings related to  $\beta$ -APM dosing.

Mean maximum plasma  $\beta$ -AP concentrations for the 1000 mg/kg dosage group for Days 1, 15 and 28 were 28.2, 31.6, and 23.6 g/ml, respectively. Plasma concentrations were approximately proportional to dosage. Steady state plasma levels of  $\beta$ -AP were attained by Day 15. These results demonstrate there is no dose accumulation or apparent change in pharmacokinetics after repeated  $\beta$ -APM dosing.

#### **Twenty-six Week Dietary Admix Study in the Rat**

The  $\beta$ -APM dosages were 250, 500 and 1,000 mg/kg/day. Four animals died prior to the end of the study: one control female, one low dose male, and two high dose males; the low dose male died accidentally and the control female died from unknown causes. Of the two high dose animals that died, one had oral malocclusion due to overgrowth of teeth; this animal had low food consumption and lost 16% of its body weight the week before it died. The other high dose animal was found dead after the Week 13 ophthalmic examinations and probably died accidentally. There were no treatment related changes in clinical observations, body weights

(Figure 2), food consumption, ophthalmic examinations, or hematology (Table 2), clinical chemistry (Table 3), and urinalysis parameters. Further, no treatment related changes in organ weights or organ weight ratios were observed and there were no gross or microscopic findings related to  $\beta$ -APM dosing.

Mean plasma  $\beta$ -AP concentrations for each dosage group at the three sampling intervals are presented in Table 6. Plasma  $\beta$ -AP concentrations were detectable at all dosages and were approximately proportional to dose at each sampling interval. Plasma  $\beta$ -AP concentrations were similar between sexes at all intervals. These data demonstrate no dose accumulation or apparent changes in pharmacokinetics following repeated daily  $\beta$ -APM dosing in rats.

#### Twenty-six Week Dietary Admix Study in the Dog

The dosages were 250, 500 and 1000 mg/kg/day. No animals died and there were no  $\beta$ -APM related changes in clinical observations, body weights (Figure 3), food consumption, electrocardiograms, ophthalmic examinations, or hematology (Table 4), clinical chemistry (Table 5), and urinalysis parameters. There were no  $\beta$ -APM related changes in organ weights or organ weight ratios, and no gross or microscopic findings related to  $\beta$ -APM dosing.

Mean maximum plasma  $\beta$ -AP concentrations for each dosage group at all sampling time points are presented in Table 7. Plasma  $\beta$ -AP concentrations of for the low, medium and high dosages, were approximately proportional to dosage. Steady state plasma levels of  $\beta$ -AP were apparently attained on Study Day 1 and mean maximum concentrations ranged from 21.5 to 29.4  $\mu$ g/ml in both sexes at Weeks 1, 12 and 26. These data demonstrate there is no dose accumulation or apparent changes in pharmacokinetics as a result of repeated daily administration of  $\beta$ -APM.

#### Other Studies

The possible effects of  $\beta$ -APM on various organ and pharmacological systems was evaluated in

standardized screening assays. an oral dosage of 60 mg  $\beta$ -APM/kg was tested in a variety of pharmacological assays in the mouse, rat and dog. A concentration of  $10^{-4}$ m  $\beta$ -APM was also evaluated in a number of in vitro assay systems.  $\beta$ -APM had no pharmacologic effects on the central nervous system, gastrointestinal tract, the cardiovascular and renal systems, and in a number of standard in vitro systems used to detect effects on platelet aggregation, cyclooxygenase activity, slow reacting substance biosynthesis, 5-lipoxygenase activity, and pancreatic lipase activity.

## DISCUSSION

The results of the acute and 4-week studies have been reported previously by FDA. These toxicology study results confirm the literature that show that  $\beta$ -aspartyl dipeptides are safe. The only findings in the acute, subchronic and chronic toxicity studies with  $\beta$ -APM were transient watery stools and yellow staining of the genital area in the rodent acute studies at 5000 mg/kg. These clinical signs are commonly observed in rodents following gavage dosing of high dosages of materials in suspension and were not specifically related to treatment with  $\beta$ -APM. In the short-term and long-term studies, there were no meaningful changes in body weights and food consumption. There were no  $\beta$ -APM related effects on clinical observations, clinical pathology, organ weights, and gross and microscopic pathology after dosages of 1000 mg/kg/day.

Plasma concentrations in the 26-week rat and the 4- and 26-week dog studies demonstrated that  $\beta$ -AP was detected at all dosage levels and also increased proportionately with dosage. There was no evidence of dose accumulation or changes in pharmacokinetics as a result of repeated daily administration of  $\beta$ -APM. The lack of toxicity associated with  $\beta$ -APM is not surprising given its simple structure and its metabolism.  $\beta$ -APM is rapidly and completely metabolized to  $\beta$ -AP, a small portion of which is absorbed but the majority of which is further hydrolyzed to

aspartate and phenylalanine.

There is strong evidence that  $\beta$ -APM and  $\beta$ -AP are not carcinogenic. First,  $\beta$ -APM has been shown to lack mutagenic or clastogenic activity in a number of tests designed to detect mutagenic activity, DNA damage, or chromosomal aberrations [Mayhew *et al.*, 1993]. Second,  $\beta$ -APM and its metabolite  $\beta$ -AP were not embryotoxic, fetotoxic, or teratogenic in rabbit teratology studies and did not affect reproductive performance or postnatal survival and development of progeny in rat multigeneration studies at dosages as high as 750 mg/kg/day [Mayhew *et al.*, 1993]. These data demonstrate  $\beta$ -APM has no effect on DNA. Finally,  $\beta$ -APM and  $\beta$ -AP were constituents in the APM toxicological samples that were extensively tested during APM toxicity testing including in the longer-term rodent carcinogenicity tests. For example, a 2-year rat carcinogenicity study [Ishii *et al.*, 1981]. Aspartame contained 0.089% (w/w)  $\beta$ -APM and 0.025% (w/w)  $\beta$ -AP. In this study, the aspartame dosages were as high as 4 g/kg/day. Consequently, total  $\beta$ -isomer ( $\beta$ -APM and  $\beta$ -AP) dosage was approximately 4.6 mg/kg/day for 110 weeks. There was no evidence of any effect of treatment on tumor incidence in this study. The combined estimated intake of both  $\beta$ -APM and  $\beta$ -AP, at the mean and 90th percentile, 14-day average are 0.020 and 0.051 mg/kg/day. The amount of total  $\beta$ -APM and  $\beta$ -AP in beverages used in this estimated intake is 1.85% of the initial APM. This value was used based on analysis of products obtained randomly in the marketplace and stability study results in mock products.

A variety of toxicology studies with  $\beta$ -APM were done including two generation reproduction studies, teratology studies, and 26-week subchronic toxicity studies in rats and dogs.<sup>93,94</sup> The NOEL in these studies was 500 mg/kg/day. This NOEL is approximately 10,000 times higher than the estimated 90th percentile, 14-day average, users only total  $\beta$ -AP isomers intake rate. The chronic toxicity in dogs and rats and carcinogenicity in mice and rats of  $\beta$ -APM and  $\beta$ -AP have also been tested, not as individual components but as constituents in the APM toxicological

samples tested. Chemical analysis of representative APM lots tested in toxicology studies done by G.D. Searle and Company showed that 0.12% w/w was  $\beta$ -APM and 0.13% w/w was  $\beta$ -AP. Therefore, the total daily dosage of the  $\beta$ -AP isomer constituent in the chronic toxicity and carcinogenicity studies at the APM NOAEL of 4,000 mg/kg/day was 10 mg/kg/day (i.e., 0.25% w/w times 4,000 mg/kg/day). This dosage is approximately 200 times the estimated daily intake of total  $\beta$ -AP isomers of 0.051 mg/kg/day based on commercial product tests, stability data, and recent MRCA APM intake surveys. These toxicity test data add to the published literature that shows  $\beta$ -AP is a natural component of human plasma and urine and demonstrate that  $\beta$ -APM and  $\beta$ -AP are safe. The lack of a carcinogenic effect of  $\beta$ -AP is consistent with the finding that  $\beta$ -AP is an endogenous component of human blood and urine [Burton *et al.*, 1989].

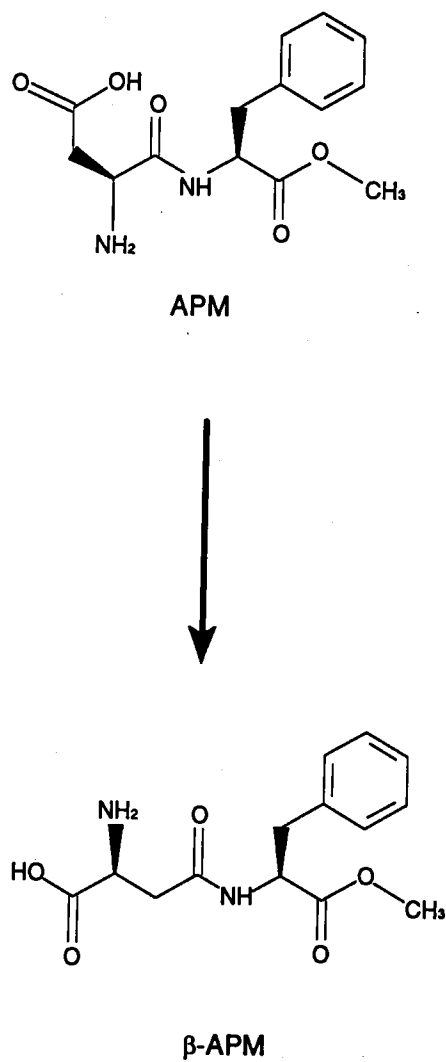
In conclusion,  $\beta$ -APM did not produce toxicity in long-term studies in rats and dogs at dosages up to 1000 mg/kg/day and did not produce adverse pharmacologic effects. In addition,  $\beta$ -APM and  $\beta$ -AP are not carcinogenic based on the absence of genotoxicity or effects on somatic germ cell expression and the lack of oncogenic effects when  $\beta$ -APM and  $\beta$ -AP were constituents in aspartame tested in 2-year carcinogenicity studies.



## REFERENCES

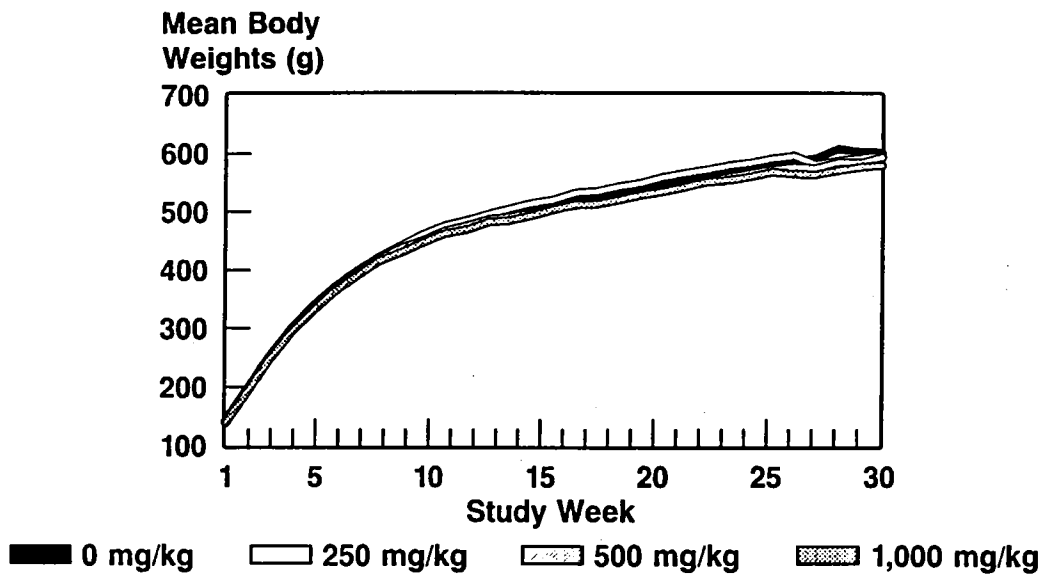
- Buchanan DL, Haley EE, Markiw RT (1962a) Occurrence of  $\beta$ -aspartyl and  $\gamma$ -L-glutamyl oligopeptides in human urine. *Biochemistry* 1(4): 612-620
- Buchanan DL, Haley EE, Markiw RT, Peterson AA (1962b) Studies on the *in vivo* metabolism of  $\alpha$ - and  $\beta$ -aspartylglycine-L-C<sup>14</sup>. *Biochem* 1: 620-623
- Carnegie PR, Hee KP, Bell AW (1982) Ophidine ( $\beta$ -Alanyl-L-3-methylhistidine, "Balentine") and other histidine dipeptides in pig muscles and tinned hams. *J Sci Food Agric* 33: 795-801
- Clarke S (1987) Propensity for spontaneous succinimide formation from aspartyl and asparaginy residues in cellular proteins. *Int J Peptide Protein Res* 30: 808-821
- Gaines SM, Bada JL (1987) Reversed-phase high-performance liquid chromatographic separation of aspartame diastereomeric decomposition products. *J Chromatogr* 389: 219-225
- Geiger T, Clarke S (1987) Deamidation, isomerization, and racemization at asparaginy and aspartyl residues in peptides: Succinimide-linked reactions that contribute to protein degradation. *J Biol Chem* 262: 785-794
- Hjelle JJ, Tschanz C, Kotsonis F (199\_) The occurrence and safety of -dipeptides in food
- Kada T, Hirano K, Shirasu Y (1980) Screening of environmental chemical mutagens by the rec-assay system with *Bacillus subtilis*. In: de Serres FJ, Hollaender A (eds) *Chemical Mutagens: Principles and methods for their source*, Plenum Press, NY, NY
- Kakimoto Y, Armstrong MD (1961)  $\beta$ -L-aspartyl-L-histidine, a normal constituent of human urine. *J Biol Chem* 236: 3280-3282
- Lou MF (1975) Isolation and identification of L-beta-aspartyl-L-lysine and L-gamma-glutamyl-L-ornithine from normal human urine. *Biochemistry* 14(15): 3503-3508
- McCaman MW, Stetzler J (1984) Identification of an acidic dipeptide,  $\beta$ -aspartylglycine, in the CNS of *Aplysia*. *J Neurochemistry* 43: 1375-1384
- Noveroske JW, Chmielewski G (1985a) A range-finding study of SC-19129 (once daily) in pregnant rabbits. G. D. Searle & CO
- Noveroske JW, Chmielewski G (1985b) A range-finding study of SC-19129 (twice daily) in pregnant rabbits. G. D. Searle & CO

- Pisano JJ, Prado E, Freedman, J. (1966)  $\beta$ -Aspartylglycine in Urine and Enzymatic Hydrolyzates of Proteins. Archives of Biochemistry and Biophysics 117: 394-399
- Tanaka T, Nakajima T (1978) Isolation and identification of urinary  $\beta$ -aspartyl dipeptides and their concentrations in human urine. J Biochem 84: 617-425
- Tesh JM (1968) Ph.D. Thesis, University of Liverpool
- Wilson JG (1965) Embryological Considerations in Teratology. In: Wilson JG, Warkany J (eds) Teratology: Principles and Techniques, University of Chicago Press, pp 251-277

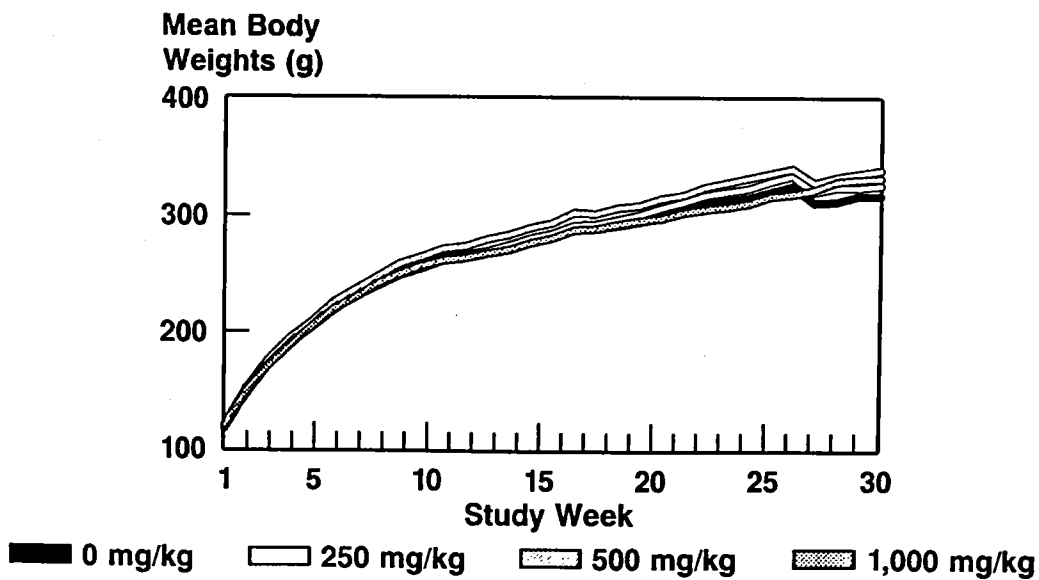
**Figure 1****Structures and Pathway of Conversion of  
APM to  $\beta$ -APM.**

## Figure 2

### Mean Body Weights Twenty-Six Week Rat Study with $\beta$ -Aspartame Male

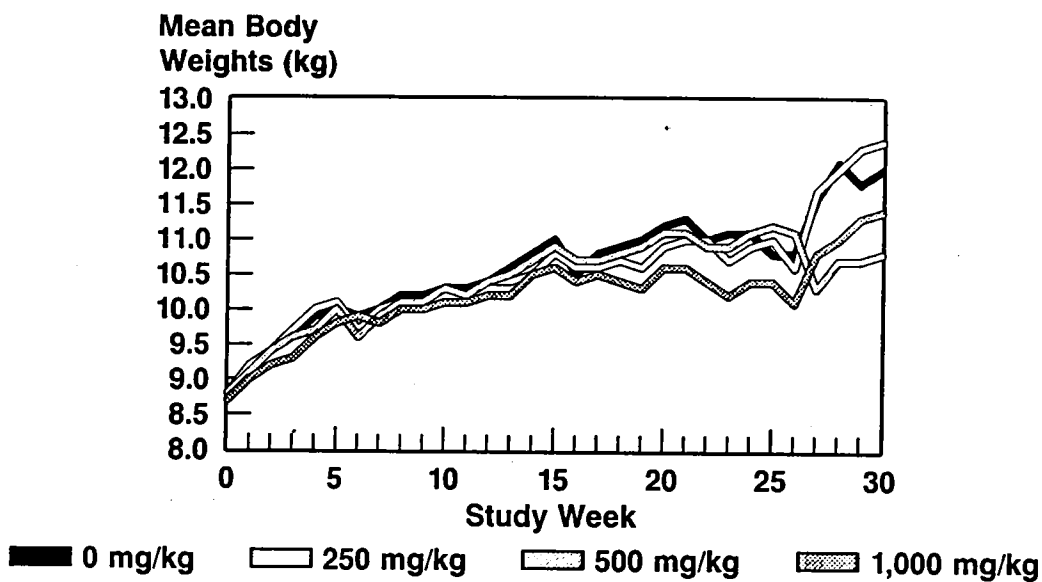


### Female



## Figure 3

### Mean Body Weights Twenty-Six Week Dog Study with $\beta$ -Aspartame Male



### Female

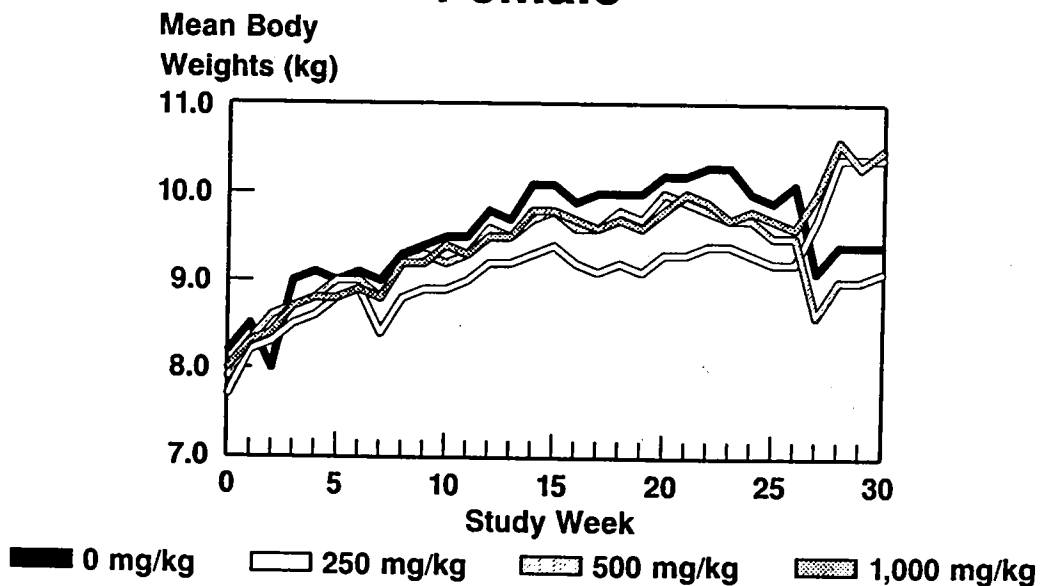


TABLE 1

**BETA-ASPARTAME SUBCHRONIC & CHRONIC DIET ADMIX TOXICITY STUDIES  
IN RATS AND DOGS**

**EXPERIMENTAL DESIGN**

	4-WEEK RAT	26-WEEK RAT	4-WEEK DOG	26-WEEK DOG
<b>DESIGN</b>				
Species/Strain	Rat/CD	Rat/CD	Dog/Beagle	Dog/Beagle
Source	Charles River Labs (Wilmington, MA)	Charles River Labs (Wilmington, MA)	Hazleton Research Products, Inc. (Cumberland, VA)	Hazleton Research Products, Inc. (Cumberland, VA)
Acclimation	13-14 days	13-14 days	>6 weeks	11 weeks
Dosing Duration	4-weeks plus a 4-week reversal period	26-weeks plus a 4-week reversal period	4-weeks	26 weeks plus a 4-week reversal period
Purina Certified Chow	5002	5002	5007	5007
Frequency of Dosing	ad libitum	ad libitum	2 hours/day plus 1 hour/day basal diet	3 hours/day
Dosage (mg/kg/day)	0, 250, 500, 1000	0, 250, 500, 1000	0, 250, 500, 1000	0, 250, 500, 1000
Animal/Sex/Group	10 plus 5 (0, 1000 only) for reversal	20 plus 10 for reversal plus 8 for PK group	3	4 plus 2 for reversal
Age at Start	4-5 weeks	5 weeks	7-8 months	8-10 months
Weight at Start	86-139 g	108-157 g	7-14 kg	6-11 kg
<b>STUDY OBSERVATIONS</b>				
General Observations	Daily	Twice daily	Daily	Twice Daily
Physical Examinations	Pretreatment then weekly	Pretreatment then weekly	Pretreatment & on days 12 & 28	Pretreatment weeks 8, 17, 26, & 30
Body Weights	Pretreatment & days 1, 3, 6, 8, 10, 15, 22, 28, 36, 43, 50, & 56.	Pretreatment then weekly	Pretreatment & days 7, 14, 21, 29, & 30.	Pretreatment & weekly
Food Consumption	Same as body weights	Weekly	Pretreatment then daily	Pretreatment then weekly

TABLE 1

**BETA-ASPARTAME SUBCHRONIC & CHRONIC DIET ADMIX TOXICITY STUDIES  
IN RATS AND DOGS**

**EXPERIMENTAL DESIGN**

	4-WEEK RAT	26-WEEK RAT	4-WEEK DOG	26-WEEK DOG
<b>STUDY OBSERVATIONS (Continued)</b>				
Ophthalmic Examinations	Day 27	Pretreatment & weeks 13, 25 & 30	Day 26	Pretreatment & weeks 16, 25 & 30
Electrocardiographic Exams <sup>a</sup>	Not Done	Not Done	Pretreatment & days 12 & 28	Pretreatment & weeks 8, 17, 26 & 30
Water Consumption (24 hours)	Not Done	Not Done	Week 4	Not Done
Clinical Chemistry and Hematology	Week 5	Pretreatment & weeks 9, 17, 27 & 31	Pretreatment & days 12, 15 <sup>b</sup> & 28	Pretreatment & weeks 8, 17, 26 & 31
Urinalysis	Day 28 or 29	Pretreatment & weeks 9, 17, 27 & 31	Pretreatment & weeks 2 & 4	Pretreatment & weeks 8, 17, 26 & 31
Pharmacokinetic Blood Collections	Not Done	Weeks 2, 13 & 26	1, 2, 4, 6 & 24 hours after initiation of feeding	Before & 3, 5, 7 & 24 hours after initiation of feeding on day 1
Feed Consumption	Same as body weights	Weekly	Pretreatment then daily period on days 1, 15 & 28	Pretreatment then weekly & during weeks 12, 25 & 26.
<b>AUTOPSY &amp; HISTOPATHOLOGY</b>				
Necropsy and Tissue Preservation	All animals	All animals	All animals	All animals
Organ Weights	All main group animals killed by design	All animals killed by design	All animals	All animals
Extent of Histopathology	All tissues from control & 1000 mg/kg main group animals	All tissues from control & 1000 mg/kg main group animals	All animals	All animals

PT = Pretreatment

a = leads II, aVL and V<sub>10</sub> in the 4-week dog and leads I, II, III, aVR, aVL, aVF, CV<sub>5</sub>RL, CV<sub>6</sub>LU, and V<sub>10</sub> in the 26-week dog study.

b = Samples analyzed for urea and total bilirubin concentrations

TABLE 2

Twenty-Six Week  $\beta$ -APM Rat Study, Mean Hematology Data for Week 27<sup>a</sup>

DOSAGE GROUP	HGB (g/dl)	HCT (L/L)	RBC (X10E12/L)	WBC (X10E9/L)	PLATELET (X10E9/L)	PT (seconds)
0 mg/kg						
Male						
mean	12.5	43.7	8.46	4.6	1,456	13.3
sd	0.95	2.78	0.564	1.42	210.3	1.56
n	20	20	20	20	20	20
Female						
mean	12.6	44.0	8.06	2.0	1,226	15.1
sd	1.25	3.97	0.686	0.64	262.6	6.24
n	19	19	19	19	19	16
250 mg/kg						
Male						
mean	12.4	43.3	8.46	3.8	1,515	13.0
sd	0.90	2.86	0.762	1.44	262.5	1.77
n	19	19	19	19	19	19
Female						
mean	12.6	44.2	8.09	2.2	1,343	14.7
sd	1.02	3.12	0.609	0.75	269.9	5.31
n	20	20	20	20	20	19
500 mg/kg						
Male						
mean	12.8	44.6	8.77	3.7	1,373	14.2
sd	0.76	2.86	0.603	0.73	243.6	3.96
n	20	20	20	20	20	18
Female						
mean	12.4	43.5	7.97	2.4	1,371	14.4
sd	0.92	2.67	0.537	1.44	335.9	5.28
n	20	20	20	20	20	17
1000 mg/kg						
Male						
mean	12.4	42.5	8.29	3.9	1,505	14.0
sd	1.19	4.59	1034	1.10	354.9	3.09
n	18	18	18	18	18	18
Female						
mean	12.5	43.4	8.14	2.2	1,201	14.6
sd	1.51	4.59	0.838	0.85	300.8	3.36
n	19	19	19	19	19	19

<sup>a</sup> Values are the mean, standard deviation (sd) and group size (n) as indicated.  
No statistically significant differences were found



TABLE 3

Twenty-Six Week  $\beta$ -APM Rat Study, Mean Clinical Chemistry Data for Week 27<sup>a</sup>

DOSAGE GROUP	ALT (U/L)	AST (U/L)	ALP U/L	CHOL (mmol/L)	TBILI (mcmol/L)	GLUC (mmol/L)	UN (mg/dL)	TPRO (g/L)	ALB (g/dL)	Na (mmol/L)	K (mmol/L)	Cl (mmol/L)	Ca (mmol/L)
0 mg/kg													
Male													
mean	28	116	54	11.5	0.3	108.8	13.4	7.3	2.9	140	4.8	107	10.0
sd	7.6	25.3	11.0	11.5	0.08	9.11	1.41	0.29	0.18	1.1	0.24	1.1	0.31
n	20	20	20	20	20	20	20	20	20	20	20	20	20
Female													
mean	25	103	25	62	0.4	103.6	14.7	7.7	3.5	140	4.3	108	10.2
sd	5.8	35.2	9.1	11.0	0.10	10.95	2.45	0.49	0.40	1.3	0.32	1.6	0.37
n	19	19	19	19	19	19	19	19	19	19	19	19	19
250 mg/kg													
Male													
mean	32	126	48	46	0.3	106.9	13.4	7.1	2.8	140	4.8	108	9.8
sd	14.1	34.5	7.9	11.7	0.13	13.42	1.54	0.32	0.26	1.2	0.22	1.3	0.32
n	19	19	19	19	19	19	19	19	19	19	19	19	19
Female													
mean	38*	128	28	69	0.4	105.0	14.9	7.8	3.6	140	4.2	108	10.2
sd	27.4	58.7	9.9	17.8	0.10	12.39	2.84	0.57	0.36	1.4	0.48	2.3	0.35
n	20	20	20	20	20	20	20	20	20	20	20	20	20
500 mg/kg													
Male													
mean	36	138	50	53	0.3	110.8	13.3	7.1	2.9	140	4.8	108	9.8
sd	31.5	83.5	15.8	16.8	0.08	11.99	1.66	0.30	0.17	1.5	0.25	1.8	0.39
n	20	20	20	20	20	20	20	20	20	20	20	20	20
Female													
mean	33**	109	29	67	0.4	104.3	15.3	7.7	3.5	140	4.0	107	10.3
sd	9.5	21.4	14.1	24.7	0.14	16.03	3.12	0.58	0.52	1.2	0.37	1.6	0.44
n	20	20	20	20	20	20	20	20	20	20	20	20	20
1000 mg/kg													
Male													
mean	40	137	57	54	0.3	107.5	14.2	7.1	2.9	141	4.8	107	10.0
sd	24.0	69.0	13.8	20.0	0.08	14.41	4.13	0.33	0.22	1.8	0.34	1.4	0.38
n	18	18	18	18	18	18	18	18	18	18	18	18	18
Female													
mean	26	106	30	57	0.3	108.8	15.7	7.4	3.3	140	4.3	108	9.9*
sd	6.3	20.7	10.5	14.2	0.10	19.10	2.48	0.49	0.34	1.5	0.37	1.7	0.31
n	20	20	20	20	20	20	20	20	20	20	20	20	20

<sup>a</sup> Values are the mean, standard deviation (sd) and group size (n) as indicated\* statistically different from control at  $p < 0.05$ \*\* statistically different from control at  $p < 0.01$

TABLE 4

Twenty-Six Week  $\beta$ -APM Dog Study, Mean Hematology Data for Week 26<sup>a</sup>

DOSAGE GROUP	HGB (g/dl)	HCT (L/L)	RBC (X10E12/L)	WBC (X10E9/L)	PLATELET (X10E9/L)	APTT (seconds)	PT (seconds)
0 mg/kg							
Male							
mean	15.2	48.2	8.34	7.4	411	10.3	7.6
sd	.63	2.02	.478	1.58	74.0	.56	2.52
n	6	6	6	6	6	6	6
Female							
mean	15.2	51.0	8.90	8.4	434	11.8	6.5
sd	.86	2.69	.589	1.06	43.1	.37	.20
n	6	6	6	6	6	6	6
250 mg/kg							
Male							
mean	15.8	49.3	8.91	8.1	373	11.8	7.2
sd	.39	1.55	.350	1.24	38.2	1.19	1.84
n	6	6	6	6	6	6	6
Female							
mean	16.0	54.2	9.29	7.0	365	12.8	7.2
sd	1.00	3.23	.729	.80	61.1	1.24	2.06
n	6	6	6	6	6	6	6
500 mg/kg							
Male							
mean	15.0	49.0	8.53	8.6	362	12.2	6.7
sd	1.03	3.40	.588	2.04	43.5	1.74	.41
n	6	6	6	6	6	6	6
Female							
mean	14.9	49.2	8.38	7.0	418	11.6	7.0
sd	1.03	3.27	.666	.93	52.2	.41	1.90
n	6	6	6	6	6	6	6
1000 mg/kg							
Male							
mean	14.9	47.8	8.54	7.1	406	11.3	6.5
sd	.89	2.93	.470	.42	64.0	.38	.49
n	6	6	6	6	6	6	6
Female							
mean	14.8	49.6	8.74	7.8	417	11.7	6.4
sd	.44	1.69	.303	1.07	45.6	.41	.32
n	6	6	6	6	6	6	6

<sup>a</sup> Values are the mean, standard deviation (sd) and group size (n) as indicated  
No statistically significant differences were found

TABLE 5

Twenty-Six Week  $\beta$ -APM Dog Study, Mean Clinical Chemistry Data for Week 26<sup>a</sup>

DOSAGE GROUP	ALT (U/L)	AST (U/L)	ALP (U/L)	CHOL (mmol/L)	TBILI (mcmol/L)	GLUC (mmol/L)	UN (mg/DL)	ALB (g/L)	TPRO (g/L)	Na (mmol/L)	K (mmol/L)	Cl (mmol/L)	Ca (mg/dL)	CREAT (mcmol/L)
0 mg/kg														
Male														
mean	27	20	32	105	.3	104.8	11.1	3.3	6.5	143	5.0	111	10.4	.9
sd	3.8	2.5	5.2	16.8	.10	7.24	.87	.23	.37	1.2	.15	1.8	.29	.08
n	6	6	6	6	6	6	6	6	6	6	6	6	6	6
Female														
mean	27	20	33	112	.4	103.8	9.9	3.5	6.4	143	4.6	110	10.8	.8
sd	6.8	3.8	12.5	15.4	.40	5.48	.78	.15	.44	1.3	.18	1.4	.46	.12
n	6	6	6	6	6	6	6	6	6	6	6	6	6	6
250 mg/kg														
Male														
mean	29	23	30	112	.3	103.0	11.4	3.6	6.7	142	4.9	111	10.5	1.0
sd	6.3	7.8	10.2	19.6	.14	4.26	1.45	.19	.40	.8	.29	1.0	.22	.08
n	6	6	6	6	6	6	6	6	6	6	6	6	6	6
Female														
mean	26	17	32	139	.2	100.7	11.0	3.7	6.2	143	4.8	110	10.6	1.0
sd	3.1	1.8	10.3	27.2	.05	2.75	2.69	.18	.37	1.7	.36	1.3	.25	.11
n	6	6	6	6	6	6	6	6	6	6	6	6	6	6
500 mg/kg														
Male														
mean	26	18	33	95	.2	114.3	11.5	3.4*	5.7	143	4.6	111	10.4	1.0
sd	2.1	2.1	11.1	17.6	.05	11.72	2.24	.18	.37	.9	.29	1.0	.35	.10
n	6	6	6	6	6	6	6	6	6	6	6	6	6	6
Female														
mean	27	18	40	135	.2	104.0	12.3	3.6	6.1	143	4.5	109	10.6	1.0**
sd	3.2	1.8	13.7	37.7	.06	6.05	2.09	.15	.27	1.9	.11	1.0	.22	.10
n	6	6	6	6	6	6	6	6	6	6	6	6	6	6
1000 mg/kg														
Male														
mean	33	17	40	144	.2	108.2	11.3	3.4	6.1	143	4.9	111	10.4	1.0
sd	3.0	1.4	7.3	25.6	.05	3.66	2.18	.21	.40	1.3	.20	2.0	.25	.11
n	6	6	6	6	6	6	6	6	6	6	6	6	6	6
Female														
mean	29	18	37	156	.3	107.5	13.3*	3.4	6.2	143	4.6	110	10.3	1.0
sd	4.9	2.4	16.8	33.1	.18	5.32	2.05	.19	.33	.8	.10	.8	.37	.08
n	6	6	6	6	6	6	6	6	6	6	6	6	6	6

<sup>a</sup> Values are the mean, standard deviation (sd) and group size (n) as indicated\* statistically different from control at  $p < 0.05$ \*\* statistically different from control at  $p < 0.01$

TABLE 6

Plasma Concentrations of  $\beta$ -APM, During the Twenty-Six Week Dietary Admix Toxicity Study in the Rat

Treatment Group	Sex	$\beta$ -AP Plasma Concentrations (mcg/ml) <sup>a</sup>		
		Week 2	Week 13	Week 26
250 mg/kg	Male	0.602 $\pm$ 0.035	0.814 $\pm$ 0.073	1.11 $\pm$ 0.13 <sup>b</sup>
	Female	0.609 $\pm$ 0.034	0.777 $\pm$ 0.079	1.00 $\pm$ 0.06
500 mg/kg	Male	1.31 $\pm$ 0.10	1.21 $\pm$ 0.17	1.34 $\pm$ 0.20
	Female	1.35 $\pm$ 0.10	1.15 $\pm$ 0.27	1.54 $\pm$ 0.19
1000 mg/kg	Male	3.06 $\pm$ 0.21	3.92 $\pm$ 0.22	3.22 $\pm$ 0.09
	Female	2.49 $\pm$ 0.12	3.04 $\pm$ 0.48	2.72 $\pm$ 0.35

<sup>a</sup> Values are the means  $\pm$  standard errors of the means (SEM) for 8 animals per sex and 16 animals for both sexes combined on Week 2, and of 6 animals per sex and 12 animals for both sexes combined on Week 13 and Week 26, unless otherwise indicated.

<sup>b</sup> Value is the mean  $\pm$  SEM of 4 animals.

\* statistically different from control at  $p < 0.05$

\*\* statistically different from control at  $p < 0.01$

TABLE 7

Maximum Observed Plasma Concentrations (C<sub>max</sub>) of  $\beta$ -AP  
During the Twenty-Six Week Dietary Admix Toxicity Study in the Dog<sup>a</sup>

Treatment Group	Sex	$\beta$ -AP Plasma Concentrations (mcg/ml) <sup>a</sup>		
		Day 1	Day 83 C <sub>max</sub> (mcg/ml) <sup>a</sup>	Day 182
250 mg/kg	Male	5.03 $\pm$ 0.48	6.48 $\pm$ 0.56	6.87 $\pm$ 0.68
	Female	5.47 $\pm$ 0.48	7.45 $\pm$ 0.59	6.51 $\pm$ 0.57
500 mg/kg	Male	11.1 $\pm$ 1.40	13.9 $\pm$ 1.40	12.7 $\pm$ 1.80
	Female	10.4 $\pm$ 0.80	11.8 $\pm$ 2.50	16.9 $\pm$ 0.80
1000 mg/kg	Male	23.2 $\pm$ 1.40	21.5 $\pm$ 1.60	27.7 $\pm$ 1.70
	Female	24.9 $\pm$ 1.60	24.4 $\pm$ 1.50	29.4 $\pm$ 0.90

<sup>a</sup> Value is the mean  $\pm$  SEM of 6 animals.

\* statistically different from control at  $p < 0.05$

\*\* statistically different from control at  $p < 0.01$



**EVALUATION OF THE GENETIC AND DEVELOPMENTAL TOXICITY OF  
L- $\beta$ -ASPARTYL-L-PHENYLALANINE-L-METHYL ESTER**

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**Key Words:**  $\beta$ -aspartame,  $\beta$ -dipeptide,  $\beta$ -aspartyl dipeptide,  $\beta$ -aspartylphenylalanine, mutagenesis, chromosomal aberrations, micronucleus test, reproductive effects, teratology, salmonella, Chinese hamster ovary cells, mice, rats, rabbits.

## ABSTRACT

$\beta$ -Aspartyl dipeptides are common components of the human diet. A very minor source of dietary  $\beta$ -aspartyl dipeptides is from the use of the dipeptide sweetener aspartame (L- $\alpha$ -aspartyl-L- $\alpha$ -phenylalanine-L-methyl ester). Aspartame can rearrange in solution in small quantities to L- $\beta$ -aspartyl-L- $\alpha$ -phenylalanine-L-methyl ester ( $\beta$ -APM). Studies were conducted with  $\beta$ -APM to confirm the safety in genetic and developmental toxicity studies.  $\beta$ -APM was tested in the Ames Salmonella test, Escherichia Coli, mutagenicity assay; the CHO/HGPRT mutagenesis test, the Bacillus subtilis Rec-assay, in an assay for chromosomal aberrations in CHO cells, and in the mouse erythrocyte micronucleus test for chromosomal breakage. These assays were all negative. Also,  $\beta$ -APM did not produce adverse effects in a two-generation reproductive study in rats with a teratology phase at dosages up to 750 mg/kg and in a teratology study in rabbits at dosages up to 750 mg/kg. Thus,  $\beta$ -APM was not genotoxic in in vitro or in vivo and did not produce effects on reproductive performance or developmental parameters in a two-generation rat study with a teratology phase or in a rabbit teratology study at dosages up to 750 mg/kg/day. This dosage is far above the probable human intake of total  $\beta$ -isomers (i.e.,  $\beta$ -APM and  $\beta$ -AP) at the 90th percentile, 14-day average of 0.05 mg/kg/day.



## INTRODUCTION

$\beta$ -Aspartyl dipeptides occur naturally in both plants and animals [Tanaka *et al.*, 1978; Kasai *et al.*, 1981; Carnegie *et al.*, 1982; McCaman and Stetzler, 1984; Hjelle *et al.*, 199\_]. Although amino acids in nature occur predominantly in the  $\alpha$ -form, dipeptides containing one amino acid in which the amino group is covalently linked to the  $\beta$  carbon ( $\beta$ -dipeptides) are also abundant. In humans,  $\beta$ -dipeptides are formed via normal metabolic pathways as well as acquired in the diet [Pisano *et al.*, 1966]. Two families of  $\beta$ -dipeptides exist: (1)  $\beta$ -alanyl dipeptides, typified by carnosine which occur in the greatest abundance; and (2)  $\beta$ -aspartyl dipeptides, typified by  $\beta$ -aspartyl-glycine which are formed predominantly as products of protein degradation [Hjelle, *et al.*, 199\_]. In the latter, aspartyl or asparaginyl residues undergo a spontaneous intramolecular cyclization of the  $\alpha$  form which can produce the  $\alpha$ - or  $\beta$ - form upon hydrolysis [Clarke, 1987; Geiger and Clarke, 1987]. At least 14  $\beta$ -aspartyl dipeptides have been identified in human urine [Kakimoto and Armstrong, 1961; Buchanan, 1962a & b; Lou, 1975; Tanaka and Nakajima, 1978, Burton *et al.*, 1991]. The total excretion of acidic  $\beta$ -aspartyl dipeptides is reported to be about 150-200  $\mu$ mol/day [Tanaka and Nakajima, 1978].

A minor dietary source of  $\beta$ -aspartyl dipeptides are products containing the dipeptide sweetener aspartame (L- $\alpha$ -aspartyl-L- $\alpha$ -phenylalanine-L-methyl ester). In solution, aspartame can undergo a rearrangement, like that for dietary protein and peptides, to produce L- $\beta$ -aspartyl-L- $\alpha$ -phenylalanine-L-methyl ester ( $\beta$ -APM) [Gaines and Bada, 1987].

A number of genetic and developmental toxicity studies with  $\beta$ -APM were conducted. This manuscript provides descriptions of the results of a battery of standard genetic toxicology studies utilizing the Salmonella typhimurium, Echerichia Coli and

CHO/HGPRT mutagenesis assays, the Rec-assay for DNA damage, the test for chromosomal aberrations in CHO cells, and the mouse erythrocyte micronucleus assay (that detects chromosomal breakage). In addition, developmental toxicity studies with  $\beta$ -APM in a two-generation rat reproduction study with a teratology phase and a teratology study in rabbits are reported.

## MATERIALS AND METHODS

### Chemicals

$\beta$ -APM was produced by The NutraSweet Company (Deerfield, IL); all other chemicals were from commercial suppliers. The identity and purity of the  $\beta$ -APM was determined before and after each study. The negative control for the genetic toxicity studies was dimethylsulfoxide (DMSO). Positive controls in the Salmonella/microsome assay in the absence of activation were sodium azide for strains TA100 and TA1535, 9-aminoacridine for strain TA1537, and 2-nitrofluorene for strains TA98 and TA1538; 2-anthramine was the positive control for all strains in the presence of activation. Other positive controls were ethylmethane sulfonate (EMS) in the absence of activation and 9,10-dimethyl-1,2- $\beta$ -benzanthracene (DMBA) in the presence of activation for the CHO/HGPRT mutagenesis assay; mitomycin C (MMC) in the absence of activation and cyclophosphamide (CP) in the presence of activation for the chromosomal aberration assay in CHO cells; and CP for the mouse erythrocyte micronucleus test.

### Animal husbandry

Mice used in the in vivo micronucleus assay were housed five per cage, and rats and rabbits used in the developmental studies were housed in individual stainless steel cages unless otherwise indicated; all were given free access to municipally supplied tap water. Mice were given Agway Prolab R-M-H 300 food. Rats were provided ground Labsure

Laboratory Animal Diet No. 2. Rabbits were given 150 g of certified Purina Rabbit Chow #5322 per day. Animals were housed in rooms maintained at  $74^{\circ}\pm 5^{\circ}\text{F}$ ,  $72^{\circ}\pm 5^{\circ}\text{F}$ , and  $65^{\circ}\pm 5^{\circ}\text{F}$  for mice, rats and rabbits, respectively. All animals were maintained on a 12-hour light/dark schedule with the exception of the rabbit teratology study (14-hour light/10-hour dark).

### Genetic Toxicity Studies

Bacterial strains. The Salmonella typhimurium strains TA98, TA100, TA1535, TA1537, and TA1538 used for this study were obtained from Dr. Bruce Ames, University of California, Berkeley, CA. Master vials were stored at  $-80^{\circ}\text{C}$  (10% DMSO added) and working plates were prepared monthly and stored at  $4^{\circ}\text{C}$ . Daily cultures were established from colonies on working plates and grown overnight in Oxoid nutrient broth. The strains were checked before assays to ensure that they were sensitive to crystal violet (rfa mutation) and that TA98 and TA100 contained the R-factor (ampicillin resistance). The Escherichia coli strain, WP2uvrA, was also used to evaluate the mutagenicity of  $\beta$ -APM. Bacillus subtilis strains H17Rec and M45Rec<sup>-</sup> were used in the Rec-assay.

Cell cultures. Chinese hamster ovary (CHO) cells used for the HGPRT assay were obtained from Dr. Samuel Latt, Children's Hospital Medical School, Boston, MA, who originally obtained the line from Dr. Arthur Pardee at the Sydney Farber Cancer Center, Boston, MA. Working and experimental cultures were maintained in F12 medium (without hypoxanthine) containing 5% dialyzed fetal bovine serum; the exposure medium was supplemented with HEPES buffer (20mM). CHO cells used at passage 13 for the chromosomal aberration assay were obtained from Dr. Sheila Galloway, Litton Bionetics, Kensington, MD. Working cultures were maintained in McCoy's 5A medium plus 10% fetal bovine serum. Both types of CHO cells were screened for the absence of mycoplasma contamination, and the cultures for the mutagenesis assay were tested to

insure that the spontaneous mutation frequency was acceptably low. Master vials of each culture were then stored at  $-70^{\circ}\text{C}$ .

Metabolic activation systems. In vitro assays were conducted in the absence and presence of metabolic activation systems which utilized mixtures containing the 9000 x g supernatant fraction (S9) of rat liver homogenates prepared from Aroclor 1254-induced (500 mg/kg) Sprague-Dawley rats. For the Salmonella/microsome mutagenesis assay, the S9 mixture consisted of 3% rat liver S9 in a cofactor mixture of 8 mM  $\text{MgCl}_2$ , 33 mM KCl, 5 mM glucose-6-phosphate, 4 mM NADP, and 100 mM  $\text{Na}_2\text{HPO}_4$  at pH 7.4. The S9 mixture was prepared before the assay and maintained at  $4^{\circ}\text{C}$  until use; then, 0.5 ml of the mixture was added to the appropriate plates. For the CHO assays, the S9 fraction was combined with a solution of cofactors and serum-free culture medium (F12 or McCoy's 5A) and maintained at  $4^{\circ}\text{C}$  until use. The final concentrations in each flask were 4.5 mg/ml isocitric acid (trisodium salt), 2.4 mg/ml NADP, and 20  $\mu\text{l/ml}$  S9 fraction.

Samonella microsome mutagenesis assay. Triplicate plates of all five strains were used for the mutagenesis assay. Concentrations were chosen on the basis of results from the range-finding assay. The following components were added sequentially to 2 ml aliquots of molten top agar containing 50  $\mu\text{M}$  biotin and 50  $\mu\text{M}$  histidine: 0.1 ml of a test sample or control sample, 0.1 ml overnight bacterial culture, and, for the activated portion of the assay, 0.5 ml S9 mix. The contents of the tubes were mixed and plated on petri dishes containing minimal medium bottom agar. After two days of incubation at  $37^{\circ}\text{C}$ , the plates were scored for visible colonies.

CHO/HGPRT mutagenesis assay. Range-finding experiments delineated the concentrations used in subsequent mutagenesis assays, and toxicity and mutagenicity studies were conducted concurrently to confirm the toxicity of each concentration used in

assays with and without activation. Based on the range-finding studies, cells were seeded at a density of approximately  $1.5 \times 10^6$  in T-75 flasks and grown for one day before being exposed to  $\beta$ -APM and the controls. Duplicate flasks were prepared for the  $\beta$ -APM concentrations and the positive and negative controls. Exposure times were 4 and 16 hours for activated and nonactivated assays, respectively. The cells were replated for toxicity evaluation and for expression of the mutant phenotype immediately after removal of the chemicals in the nonactivated assay and the day following removal of the chemicals in the activated assay. All flasks were maintained independently throughout the expression period of at least 7 days duration, and the cells were replated as necessary to maintain their maximum growth rate (usually every 2-3 days). Following expression, the cells were plated at  $2 \times 10^5$  per dish (usually 6 dishes per flask) in medium containing 2  $\mu$ g/ml 6-thioguanine (6TG) for mutant selection, and 200 cells from each flask were plated in two petri dishes in medium without 6TG to determine cloning efficiency of the cells. After at least 1 week the resulting colonies were fixed with methanol stained with Giemsa, and counted. Only colonies containing at least 50 cells were counted.

Rec-assay. The Rec-assay was done using the method of Kada *et al.* (1976, 1980). The strains H17Rec<sup>+</sup> and M45Rec<sup>-</sup> were grown overnight in B<sub>2</sub> broth and stored frozen. The culture was thawed on the day of the experiment and streaked on a broth agar plate. A paper disk containing  $\beta$ -APM was placed over the starting point of the streaks and subsequently circulated at 37°C for 20 hours. The length of the inhibition zone was then measured as an index of DNA damage.

In vitro chromosomal aberration assay in CHO cells. In a range-finding study there was no effect of  $\beta$ -APM on the cell cycle of CHO cells. Thus, the concentrations tested in the chromosomal aberration assays were based on the solubility limit of  $\beta$ -APM. CHO cells were seeded at a density of  $1.5 \times 10^6$  cells/ml in plastic T-75 flasks the day before

exposures. For assays with and without S9, duplicate flasks were used for each of eight concentrations of  $\beta$ -APM and for the positive controls; two sets of duplicate flasks were used for the negative controls. In the absence of activation, cells were exposed for eight hours in McCoy's 5A medium supplemented with 10% fetal bovine serum, 20 mM HEPES buffer, and 1% penicillin-streptomycin solution. In the presence of activation, cells were exposed for two hours in serum-free medium followed by a growth period of eight hours in the medium supplemented with serum. Vinblastine sulfate (final concentration 0.26 g/ml) was added to the medium 2 - 2.5 hours before the cells were harvested by treatment with trypsin and concentration by centrifugation. The cells were then suspended in hypotonic solution (0.03 M KCl and 0.01 M sodium citrate) for 12 minutes at 37°C, fixed three times in 3:1 methanol:acetic acid, dropped on glass slides, air dried, then stained for approximately five minutes at room temperature in 5% Giemsa.

For cytogenetic analysis, 100 metaphase cells from each of the duplicate flasks of the three highest concentrations of  $\beta$ -APM and the positive controls and one set of the negative controls were analyzed, at 100 x magnification, for the presence of the following types of chromosomal aberrations: chromatid and chromosome breaks, chromatid deletions, fragments, acentric fragments, translocations, quadriradials, interstitial deletions, triradials, dicentric, pulverized chromosomes, cells with greater than one pulverized chromosomes, pulverized cells, complex rearrangements, rings, double minute chromosomes, and uncoiled chromosomes. Gaps and endoreduplicated chromosomes were recorded but were not included in the aberration frequency. The results were expressed as the percent of cells with aberrations and as the number of aberrations per cell.

Micronucleus assay in mice. Seven-week old male and female CD-1 mice (Charles River, Wilmington, MA) were quarantined for 7 days before use in assays. The mice were 56 days old at the time of dosing, and their body weight ranged from 23.3 to 34.6 g. Each

mouse was identified by ear punch and randomly assigned to a dose group. Mice were housed five/cage and all animals in a cage received the same treatment. The mice received two gavage doses at an interval 24 hours apart of either  $\beta$ -APM; dimethyl sulfoxide (DMSO), the negative control; or cyclophosphamide (CP), the positive control. They were killed by cervical dislocation approximately 24 hours later. Bone marrow cells were aspirated from both femurs into syringes containing approximately 1 ml of fetal bovine serum, pooled, and centrifuged. The supernatant was removed leaving a very small drop of serum on the pellet. Then the pellet was gently mixed to ensure a homogeneous sample. A small drop of the suspension mixture was spread in a thin smear on each of four slides, which were allowed to dry overnight. Then 2 of the 4 slides were stained in Camco Quick stain (methyl alcohol: Wright-Giemsa stain combination) for 15 seconds; the remaining 2 slides were stored unstained in case they were needed. After drying, the slides were mounted with coverslips using Pro-Texx.

Mice were randomly assigned to test groups and the animal number was used as a coding number for slide analysis. For each animal, 1000 polychromatic erythrocytes (PCEs) were counted for the presence of micronuclei. The frequency of micronucleated cells per animal was expressed as the number of micronucleated PCEs per 1000 PCEs counted, and were evaluated using the Student's t-test. The ratio of PCEs to normochromatic erythrocytes (NCEs), i.e., PCE/NCE in 200 erythrocytes for each mouse, was also recorded. Data from each sex were analyzed separately.

## **Developmental Toxicity Studies**

### **Teratology Evaluation in Rabbits**

Based on the results of a dose range-finding study, the following study was conducted. Estrus of sixty sexually mature (18-24 weeks, 2.70-3.66 kg) New Zealand White rabbits (Ranch Rabbits, Sussex, England) was synchronized by i.v. injection of 25 I.U. luteinizing hormone (Pregnyl) shortly after arrival. After three weeks of acclimatization, females were artificially inseminated (Gestation Day 0) in random order with pooled semen from proven bucks, and then injected with 50 USP units of chorionic gonadotropin via the marginal ear vein to ensure ovulation. Females were allocated to each of four treatments in order of insemination so that those inseminated on any one day were evenly distributed between groups.  $\beta$ -APM was administered orally as a suspension in 0.5% methylcellulose (w/v) and 0.1% polysorbate 80 (w/v) in distilled water at dosage levels of 250, 500, and 750 mg/kg/day on Days 6 through 19 of gestation. The control group received vehicle at the same volume of 4 ml/kg. Animals were weighed and examined daily for signs of reaction to treatment. Animals that aborted were killed by intraperitoneal injection and examined for numbers of corpora lutea and implantation sites. On Day 29, surviving females were killed for examination of ovaries and uterine contents; numbers of corpora lutea, implantation sites, resorption sites, and live or dead fetuses were recorded. Fetuses were weighed and inspected for external abnormalities. Following examination of the viscera, approximately one-third of each litter was decapitated and the heads fixed in Bouin's fluid for free-hand serial sectioning. Eviscerated fetuses were processed for skeletal evaluation [Tesh, 1968].

### **Two-Generation Rat Reproduction Study**

**Animals.** Male (140-181 g) and female (185-220 g) rats of the Charles River CD strain (Kent, England) in the F<sub>0</sub> generation were allowed to acclimatize on standard diet (Labsure



Laboratory Animal Diet No. 2, Croyden, Surrey, England) for five and three days, respectively. Prior to treatment, animals of each sex were divided into groups by body weight (5 g range) and allocated to five groups by random selection from each weight range in rotation. F<sub>0</sub> and F<sub>1</sub> generations were housed in polypropylene cages with mesh floors and suspended over absorbent paper. During gestation and lactation, females were placed singly in shoebox cages provided with autoclaved wood shavings. All animals were killed by carbon dioxide inhalation except culled offspring which were euthanized by ip injection of pentobarbital.

Breeding procedure. A vaginal smear was taken daily from each female for ten days prior to pairing to assess regularity and duration of the estrus cycle. Each female was paired with a male of the same treatment group until spermatozoa were detected (Gestation Day 0) from a vaginal smear prepared each morning, and the time interval between pairing and mating (pre-coital) was recorded. Each pairing consisted of seven-day cohabitation periods, which was extended for an additional seven days if mating had not occurred. Sperm-positive females were returned to single housing. Males of each parental generation were killed when the majority of females had littered successfully.

Dietary exposure. Appropriate concentrations of diet were prepared weekly and duplicate samples of each batch were analyzed for actual concentration. Food consumption was recorded weekly for each generation throughout the study, except during mating. Prior to weekly diet preparation, dietary concentrations were calculated for each sex to obtain the specified dose level, using body weights and food consumption of the previous week (mean g food/rat/day x ppm  $\beta$ -APM/mid-week rat weight). Exceptions were: (1) during mating, males received the diet prepared for the female of the pair, and (2) during the last week of lactation, concentrations were not re-adjusted due to unquantified diet consumption by the litter.

Experimental Design. Based on the results of dose range-finding studies, groups of 34 male and 34 female Charles River CD strain Sprague Dawley rats were offered the appropriate dietary admix to give 0, 250, 500, and 750 mg  $\beta$ -APM/kg body weight/day. This generation ( $F_0$ ) received the appropriate diet beginning 71 and 15 days prior to mating until termination for males and females, respectively. Each female was paired with a male receiving the same diet until detection of spermatozoa (Gestation Day 0). On Day 20 of gestation, 12 females from each group were killed for the teratology evaluation of the  $F_0$  generation. The remaining 22 females were allowed to deliver and raise the  $F_1$  litters to Day 21 post partum. All  $F_1$  offspring were assessed for physical development, auditory and visual function, and behavioral parameters. Following weaning, 40 males and 40 females were randomly selected from each treatment group to investigate reproductive performance of the  $F_1$  parental generation; 2 males and 2 females were selected from each litter, where possible. Unselected offspring were killed after completion of observations for macroscopic physical abnormalities. After the selected progeny had been exposed to the experimental diets for ten weeks (13-14 weeks of age), pairs were mated (avoiding littermates) as described for the  $F_0$  generation. On Day 20 of gestation, 20 females in each group were killed for standard teratology evaluation. The remaining 20 females of each group were allowed to raise the  $F_2$  generation. Females producing a litter were killed on Day 21 post partum; mated females not producing a litter were killed on Day 25 post coitum. The  $F_2$  generation was killed after weaning for determination of external and internal macroscopic abnormalities.

Parental data. Each animal was examined daily throughout the study for clinical signs. Males were weighed weekly until termination; females were weighed weekly until paired, and on Days 0, 6, 13, and 20 post coitum. From Day 20 post coitum, females were examined three times a day for onset and progress of parturition and for adverse clinical

signs post partum, and the duration of gestation was recorded. Reproductive performance of females was measured as regularity and length of estrus, precoital interval, and the following indices: gestation index ( $\# \text{live litters} \times 100 / \# \text{pregnant}$ ), conception rate ( $\# \text{pregnant} \times 100 / \# \text{mating}$ ), fertility index ( $\# \text{pregnant} \times 100 / \# \text{paired}$ ), and mating index ( $\# \text{mated} \times 100 / \# \text{paired}$ ). At termination, all parental animals were subjected to necropsy, including a detailed examination of the cranial, thoracic, abdominal and pelvic cavities, and their viscera. The external and cut surfaces of the organs and tissues were examined, abnormalities were recorded, and specified tissue samples were retained in fixative.

Teratology data. Selected mated females of the  $F_0$  and  $F_1$  generations killed on Gestation Day 20 were examined for the following: number of corpora lutea in each ovary, number of implantation sites, number of early and late resorption sites, number and distribution of live and dead fetuses in each uterine horn, weight and sex of individual fetuses, placenta weights, and abnormalities of the reproductive tract. The fetuses were examined for external anomalies. Half of the fetuses were examined for visceral abnormalities, then fixed and processed for subsequent skeletal examination [Tesh, 1968]. The remaining fetuses were fixed in Bouin's for free hand sectioning and tissue examination [Wilson, 1965].

Litter data. All litters ( $F_1$ ,  $F_2$ ) were counted and observed approximately 24 hours after birth and pups were toe-marked for identification. Individual offspring were weighed and sexed on Days 1, 4, 14, and 21 post partum. Mortality and evidence of treatment-related clinical signs were recorded daily. Using a random selection procedure, litters were reduced to eight on Day 4 post partum to give four of each sex, when possible. Culled offspring and those found dead were examined externally and internally for abnormalities, and specimens of the tissues involved were retained.  $F_1$  litters were examined for physical development, auditory and visual function, and behavioral parameters (activity and

learning). The time of physical development was assessed individually by noting the days of onset and completion of the following: pinna unfolding, hair growth, tooth eruption, eye opening, and testicular descent or vaginal opening. After weaning at Day 21 post partum, progeny were examined for auditory function by using the startle response to a sudden noise. Visual function was assessed by examination of pupil closure in response to bright light, as well as by visual placing response (i.e., movement of forelimbs in the placing response when the pup was lowered toward a metal grid). At this time each litter was separated by sex and placed in clear cages for measurement of activity. A system of infrared light sources and detectors was utilized to measure litter activity for 12 night hours. The time taken for each littermate to swim through a water-filled Y-maze was used to evaluate learning ability. Six successive trials were recorded for a maximum of 60 seconds, and maintained improvement in swimming time was used as an indication of learning. The following indices were calculated: sex ratio, live birth index ( $\# \text{live offspring Day 1} \times 100 / \# \text{offspring born}$ ), lactation index ( $\# \text{live offspring Day 4} \times 100 / \# \text{live offspring before weaning}$ ), viability index ( $\# \text{live offspring Day 4} \times 100 / \# \text{live offspring Day 1}$ ), plus mean activity scores and swimming times.

Statistical evaluation. Intergroup differences in body weight, body weight change, food consumption, swimming time, reproductive organ weights, and fetal weights were tested by two-tailed Student's t-test if variability by ANOVA was significant. Corpora lutea count, implantation count, resorption counts, precoat interval, pre- and post-implantation loss (%), litter size, post-implantation survival index, live birth index, viability index, lactation index, gestation length, physical activity scores and sex ratio were subjected to the Mann-Whitney U test where appropriate. Differences with an associated probability of  $p < 0.05$  were considered statistically significant.

## RESULTS AND DISCUSSION

### Salmonella/microsome mutagenesis assay

$\beta$ -APM was evaluated in two independent Ames tests. In the first assay, concentrations up to 20 mg/plate were evaluated. In the second assay,  $\beta$ -APM was tested from 0.01 mg/plate to 20 mg/plate in the range-finding assay with no evidence of toxicity; however, the test article was not completely soluble in stock solutions at 200 mg/ml which was used for the highest dose. Therefore, 8.5 mg/plate was used as the highest concentration. Analytical characterization of the test sample revealed that the intended concentrations were achieved. As summarized in Tables 1 and 2, negative and positive control values were in the appropriate ranges, no toxicity in response to  $\beta$ -APM was observed, and there was no evidence of a positive response in any strain (TA98, TA100, TA1535, TA1537, or TA1538) in the Salmonella mutagenesis assays following exposure to  $\beta$ -APM in the absence or presence of metabolic activation.

### E. Coli mutagenesis assay

$\beta$ -APM was tested at concentrations up to 20 mg/plate. The data are presented in Table 2. The number of revertants observed for the negative and positive control materials were in the acceptable ranges and there was no indication of cytotoxicity.  $\beta$ -APM did not cause an increase in revertants in any strain in the absence or presence of a metabolic activation system.

### B. Subtilis Rec-assay

$\beta$ -APM was tested at concentrations up to 200  $\mu$ g/ml. The results are presented in Table 3. The results for the negative and positive control materials were within the acceptable range.  $\beta$ -APM was negative in this assay at all concentrations tested.

**CHO/HGPRT mutagenesis assay**

$\beta$ -APM was tested at concentrations up to 1 mg/ml in the range-finding assay with no evidence of toxicity; therefore, the concentrations used in the CHO/HGPRT mutagenesis assays were 0.1 mg/ml to 2.0 mg/ml (the solubility limit of  $\beta$ -APM in DMSO).

Analytical assay of test solutions demonstrated the intended test concentration levels were achieved. As summarized in Table 4, negative and positive control values were in the appropriate ranges, there was no toxicity in response to  $\beta$ -APM and no evidence of a positive response was observed in the CHO/HGPRT mutagenesis assay following exposure of cells to  $\beta$ -APM (up to 2 mg/ml) in the absence or presence of metabolic activation.

**In vitro chromosomal aberration assay in CHO cells**

$\beta$ -APM was tested at concentrations up to 1 mg/ml in the range-finding assay with no evidence of toxicity or cell cycle delay; therefore, eight concentrations of  $\beta$ -APM, up to 2.0 mg/ml (the solubility limit of  $\beta$ -APM in DMSO), were used in the chromosomal aberration assays. The three highest test sample concentrations and the negative and positive control samples, in the absence and presence of metabolic activation, were analyzed. As shown in Table 5, negative control aberration frequencies, in the absence and presence of activation, were acceptably low, and both positive controls induced significant increases in aberration frequencies. Aberration frequencies in response to  $\beta$ -APM were the same as observed for negative controls, under both activation conditions; therefore, there was no evidence of a positive response chromosomal aberrations in CHO cells following exposure to  $\beta$ -APM (up to 2 mg/ml) in the absence or presence of metabolic activation.

**Micronucleus assay in mice**

Male and female mice were dosed with 500, 750, and 1000 mg/kg  $\beta$ -APM. The results of the micronucleus induction assay are summarized in Table 6. The percent PCEs in the

positive control mice, the percent PCEs at each dose level of  $\beta$ -APM (up to 1000 mg/kg) were similar to values obtained for the negative controls. The average number of micronucleated PCEs ranged from 1.2 to 3.0 per 1000 PCEs for the animals dosed with  $\beta$ -APM. The average numbers of micronucleated PCEs found in the male and female negative control animals were 2.4 and 1.0 per 1000 PCEs, respectively. The positive control, CP, induced significant increases in the number of micronucleated PCEs in both sexes. A Student's t-test analysis comparing each dosage of  $\beta$ -APM and the positive control, CP, to the negative control showed significant increases for CP, but no significant effect of  $\beta$ -APM for micronucleus induction in either sex.

#### **Teratology study in rabbits**

The results of this study are summarized on Table 7. There were no teratogenic, embryotoxic or fetotoxic effects at any dose; however, there was maternotoxicity at 750 mg/kg/day as evidenced by deaths and decreased food consumption. These effects were not evident at 500 mg/kg/day, which is 2000 times the estimated maximum dietary consumption of the  $\beta$ -isomers. Furthermore, maternotoxicity was less pronounced in other exploratory studies when the same amount of  $\beta$ -APM was given by gavage in divided rather than single doses, as was done in the above study (Noveroske and Chmielewski, 1985 a,b).

#### **Two-generation rat reproduction study**

During the two generation study, group mean dosages for both males and females were within 90% of intended values throughout the study with the exception of two more variable intervals in the F<sub>1</sub> generation (Weeks 1 and 3 after weaning for all F<sub>1</sub> treatment groups and Week 1 of lactation, F<sub>1</sub> females). In both intervals, animals consumed a different amount of food than estimated from the previous week.

In the F<sub>0</sub> generation, general condition, body weight gain, food intake, and food conversion efficiency were not significantly affected. Estrous cycles, mating performance and fertility were also not affected by treatment. Litter examination at Day 20 post coitum and fetal evaluations at necropsy and after fetal processing revealed no adverse treatment related effects. Gestation length, litter size, viability and growth of offspring (F<sub>1</sub> animals) to weaning were similar in all groups. Offspring development and behavioral performance showed no adverse treatment-related effects. Terminal necropsy of culled and surplus offspring and adults revealed no treatment related abnormalities. Analysis of reproductive organ weights of parental animals (F<sub>0</sub>) showed no treatment related effects. In the F<sub>1</sub> generation, general condition and body weight were not significantly affected by treatment. However, body weight gain was slightly, but significantly reduced (5 to 6 %) in males after weaning at 750 mg/kg/day. This decrease was accompanied by a slight reduction (3 to 5 %) in food intake. Body weight gain and food consumption were not affected in females. Estrous cycles, mating performance and fertility were Similar in all groups. Litter examination at Day 20 post coitum and fetal evaluation at necropsy and after processing revealed no adverse treatment-related effects. Gestation length, litter size, offspring (F<sub>2</sub> generation) viability and growth were similar in all groups. Necropsy of F<sub>1</sub> adults and F<sub>2</sub> offspring revealed no abnormalities that were related to treatment. In summary, there were no treatment-related effects in F<sub>0</sub> or F<sub>1</sub> or F<sub>2</sub> animals other than the slight decrease in body weight gain in F<sub>1</sub> males after weaning at 750 mg/kg/day that was associated with a decrease in food consumption. The results of this study are summarized on Table 8-11.



## CONCLUSIONS

$\beta$ -APM is formed in small quantities during the manufacturing process or during liquid storage of aspartame. Animal studies indicate that most of the  $\beta$ -APM is metabolized to its constituent amino acids and methanol, however, a small amount (approximately 5 to 10%) is absorbed as  $\beta$ -aspartylphenylalanine.  $\beta$ -Aspartylphenylalanine, like a number of other  $\beta$ -Aspartyl dipeptides, is a natural endogenous substance. Standard developmental and genetic toxicity studies were done with  $\beta$ -APM. The only findings in these studies was maternotoxicity in the rabbit teratology study when 750 mg/kg/day was given as a single administration and a decrease in body weight gain in F<sub>1</sub> males after weaning in the two-generation rat study at 750 mg/kg/day that was associated with a decrease in food consumption. There were no effects at 500 mg/kg/day.

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## REFERENCES

- Buchanan DL, Haley EE, Markiw RT (1962a) Occurrence of  $\beta$ -aspartyl and  $\gamma$ -L-glutamyl oligopeptides in human urine. *Biochemistry* 1(4): 612-620
- Buchanan DL, Haley EE, Markiw RT, Peterson AA (1962b) Studies on the *in vivo* metabolism of  $\alpha$ - and  $\beta$ -aspartylglycine-L-C<sup>14</sup>. *Biochem* 1: 620-623
- Carnegie PR, Hee KP, Bell AW (1982) Ophidine ( $\beta$ -Alanyl-L-3-methylhistidine, "Balentine") and other histidine dipeptides in pig muscles and tinned hams. *J Sci Food Agric* 33: 795-801
- Clarke S (1987) Propensity for spontaneous succinimide formation from aspartyl and asparaginyl residues in cellular proteins. *Int J Peptide Protein Res* 30: 808-821
- Gaines SM, Bada JL (1987) Reversed-phase high-performance liquid chromatographic separation of aspartame diastereomeric decomposition products. *J Chromatogr* 389: 219-225
- Geiger T, Clarke S (1987) Deamidation, isomerization, and racemization at asparaginyl and aspartyl residues in peptides: Succinimide-linked reactions that contribute to protein degradation. *J Biol Chem* 262: 785-794
- Hjelle JJ, Tschanz C, Kotsonis F (199\_) The occurrence and safety of -dipeptides in food
- Kada T, Hirano K, Shirasu Y (1980) Screening of environmental chemical mutagens by the rec-assay system with *Bacillus subtilis*. In: de Serres FJ, Hollaender A (eds) *Chemical Mutagens: Principles and methods for their source*, Plenum Press, NY, NY
- Kakimoto Y, Armstrong MD (1961)  $\beta$ -L-aspartyl-L-histidine, a normal constituent of human urine. *J Biol Chem* 236: 3280-3282
- Lou MF (1975) Isolation and identification of L-beta-aspartyl-L-lysine and L-gamma-glutamyl-L-ornithine from normal human urine. *Biochemistry* 14(15): 3503-3508
- McCaman MW, Stetzler J (1984) Identification of an acidic dipeptide,  $\beta$ -aspartylglycine, in the CNS of *Aplysia*. *J Neurochemistry* 43: 1375-1384
- Noveroske JW, Chmielewski G (1985a) A range-finding study of SC-19129 (once daily) in pregnant rabbits. G. D. Searle & CO
- Noveroske JW, Chmielewski G (1985b) A range-finding study of SC-19129 (twice daily) in pregnant rabbits. G. D. Searle & CO

Pisano JJ, Prado E, Freedman, J. (1966)  $\beta$ -Aspartylglycine in Urine and Enzymatic Hydrolyzates of Proteins. Archives of Biochemistry and Biophysics 117: 394-399

Tanaka T, Nakajima T (1978) Isolation and identification of urinary  $\beta$ -aspartyl dipeptides and their concentrations in human urine. J Biochem 84: 617-425

Tesh JM (1968) Ph.D. Thesis, University of Liverpool

Wilson JG (1965) Embryological Considerations in Teratology. In: Wilson JG, Warkany J (eds) Teratology: Principles and Techniques, University of Chicago Press, pp 251-277

TABLE 1

*S. typhimurium* and *E. coli* assay of B-APM

Chemical	Concentration per Plate	Metabolic Activation	Histidine Revertant Colonies			E. coli Colonies		
			TA98	TA100	TA1535	TA1537	TA1538	WP <sub>2</sub> uvrA
DMSO		+		161	8	11	53	34
		-	35	154	4	9	20	37
Distilled water		+		169	10	9	50	39
		-	37	158	4	7	24	29
B-APM	0.01 mg	+	75	145	7	10	61	34
		-	39	152	4	8	22	34
B-APM	0.05 mg	+	84	147	6	10	52	40
		-	40	149	4	9	22	44
B-APM	0.1 mg	+	76	162	7	12	56	42
		-	34	149	6	8	19	39
B-APM	0.5 mg	+	78	150	9	13	56	32
		-	37	146	4	8	24	36
B-APM	1 mg	+	76	155	7	12	53	37
		-	41	150	5	9	25	35
B-APM	5 mg	+	68	160	11	14	54	37
		-	33	142	3	8	23	41
B-APM	10 mg	+	72	152	7	11	48	33
		-	33	142	4	9	27	36
B-APM	20 mg	+	68	162	7	11	49	37
		-	34	152	4	9	22	32

TABLE 1 (Continued)  
*S. typhimurium* and *E. coli* assay of B-APM

Chemical	Concentration per Plate	Metabolic Activation	Histidine Revertant Colonies <sup>a</sup>			E. coli Colonies	
			TA98	TA100	TA1535	TA1537	TA1538 WP <sub>2</sub> uvrA
2-Anthramine	0.5 µg	+	248	308	--	--	196
2-Anthramine	1 µg	+	560	612	--	91	656
2-Anthramine	2 µg	+	--	--	--	317	--
2-Anthramine	5 µg	+	--	--	300	--	561
2-Anthramine	10 µg	+	--	--	446	--	777
AF-2	0.02 µg	-	--	691	--	--	--
AF-2	0.05 µg	-	244	1056	--	--	441
AF-2	0.1 µg	-	308	--	--	--	1632
ENNG	2 µg	-	--	--	67	--	--
ENNG	4 µg	-	--	--	884	--	--
9-Aminoacridine	80 µg	-	--	--	--	294	--
9-Aminoacridine	100 µg	-	--	--	--	363	--
2-Nitrofluorene	2 µg	-	--	--	--	--	467
2-Nitrofluorene	5 µg	-	--	--	--	--	984

<sup>a</sup> Mean of duplicate plates

**TABLE 2**  
***Salmonella typhimurium* assay of**  
 **$\beta$ -APM**

Chemical	Concentration per Plate	Metabolic Activation	Histidine Revertant Colonies <sup>a</sup>				
			TA98	TA100	TA1535	TA1537	TA1538
DMSO	100 $\mu$ l	+	27	92	17	5	23
		-	19	116	24	6	8
$\beta$ -APM	0.4 mg	+	24	102	21	4	22
		-	15	97	18	6	12
$\beta$ -APM	1.3 mg	+	24	91	9	7	17
		-	18	87	20	4	13
$\beta$ -APM	4.3 mg	+	21	85	14	6	21
		-	15	118	21	5	10
$\beta$ -APM	6.4 mg	+	26	93	14	5	18
		-	11	103	20	6	11
$\beta$ -APM	8.5 mg	+	24	100	18	6	17
		-	17	96	15	6	9
2-Anthramine	1.0 $\mu$ g	+	2161	1118	161	375	1962
2-Nitrofluorene	10.0 $\mu$ g	-	301	-	-	-	846
Sodium azide	2.5 $\mu$ g	-	-	704	561	-	-
9-Aminoacridine	100 $\mu$ g	-	-	-	-	832	-

<sup>a</sup> Values are average numbers of his<sup>+</sup> revertants from triplicate plates.

TABLE 3

Rec-assay of B-APM

Chemical	$\mu\text{g per ml}$	$\frac{\text{Inhibition Zones (mm)}}{\text{H17(Rec}^+)$	$\frac{\text{M45(Rec}^-)$	Rec-effect
DMSO		0	0	0
Distilled water		0	0	0
Kanamycin sulfate	100	5.3	6.5	1.2
Kanamycin sulfate	200	8.3	9.3	1.0
B-APM	50	0	0	0
B-APM	100	0	0	0
B-APM	200	0	0	0
Mitomycin C	10	3.7	21.1	17.4
Mitomycin C	20	8.3	22.0	13.7



**TABLE 4**  
**CHO/hgp<sup>r</sup>t mutagenesis assay of  $\beta$ -APM**

Chemical	Concentration (mg/ml)	Metabolic Activation	Exposure Time (hours)	Relative Survival <sup>a</sup> (%)	Mutation Frequency <sup>a</sup> (X10 <sup>-6</sup> )
DMSO	1.0%	+	4	100.0	17.9
		-	16	100.0	11.7
$\beta$ -APM	0.10	+	4	140.7	16.9
		-	16	130.9	5.9
$\beta$ -APM	0.50	+	4	108.7	7.6
		-	16	90.2	18.7
$\beta$ -APM	0.75	+	4	112.2	21.5
		-	16	93.0	25.7
$\beta$ -APM	1.00	+	4	96.7	26.9
		-	16	102.4	7.5
$\beta$ -APM	1.25	+	4	95.8	18.1
		-	16	87.8	24.4
$\beta$ -APM	1.50	+	4	108.0	12.8
		-	16	89.5	7.8
$\beta$ -APM	1.75	+	4	107.1	16.0
		-	16	88.3	5.8
$\beta$ -APM	2.00	+	4	99.1	26.7
		-	16	92.8	14.0
DMBA	0.015	+	4	114.3	199.0
EMS	0.235	-	16	97.0	202.0

<sup>a</sup> Values are averages from duplicate cultures, except the solvent control values which are averages from quadruplicate cultures.

**TABLE 5**  
Chromosomal aberration assay of  $\beta$ -APM

Chemical	Concentration (mg/ml)	Metabolic Activation	Exposure Time (hours)	Number of Aberrations per Cell <sup>a</sup> (%)	% of Cells With Aberrations <sup>a</sup> (X10 <sup>-6</sup> )
DMSO	1.0%	+	2	0.000	0.0
		-	8	0.005	0.5
$\beta$ -APM	1.50	+	2	0.000	0.0
		-	8	0.005	0.5
$\beta$ -APM	1.75	+	2	0.005	0.5
		-	8	0.000	0.0
$\beta$ -APM	1.00	+	2	0.000	0.0
		-	8	0.005	0.5
CP	0.05	+	2	>0.25	22.0
MMC	0.005	-	8	>0.56	34.5

<sup>a</sup> Values are averages from duplicate cultures; 100 cells were evaluated for aberrations in each culture.

**TABLE 6**  
Micronucleus assay of  $\beta$ -APM<sup>a</sup>

Chemical	Dose per kg	Sex	% PCE <sup>b</sup> $\pm$ S.D.	MN-PCE $\pm$ S.D. <sup>c</sup>
DMSO	10 ml	M	51.6 $\pm$ 10.0	2.4 $\pm$ 1.7
		F	50.5 $\pm$ 14.1	1.0 $\pm$ 1.0
$\beta$ -APM	500 mg/kg	M	55.1 $\pm$ 5.0	1.2 $\pm$ 1.6
		F	48.6 $\pm$ 5.3	3.0 $\pm$ 2.2
$\beta$ -APM	750 mg/kg	M	51.1 $\pm$ 6.2	1.6 $\pm$ 1.3
		F <sup>d</sup>	51.0 $\pm$ 19.7	2.0 $\pm$ 2.5
$\beta$ -APM	1000 mg/kg	M	44.2 $\pm$ 4.7	1.6 $\pm$ 2.0
		F	39.5 $\pm$ 6.1	2.6 $\pm$ 1.7
CP	60 mg	M <sup>d</sup>	18.9 $\pm$ 8.3	42.0 $\pm$ 14.7
		F	19.8 $\pm$ 10.5	43.6 $\pm$ 5.6

<sup>a</sup> Values are mean  $\pm$  standard deviation for five animals, except as noted.

<sup>b</sup> % PCE=100xPCE/(NCE+PCE). Values are based on 200 erythrocytes per animal.

<sup>c</sup> MN-PCE=Micronucleated PCEs. Values are based on 1000 PCEs per animal.

<sup>d</sup> Slides for one of five animals not used due to poor distinction between PCEs and NCEs.

TABLE 7

Mean gestational parameters on day 29 for rabbits treated with  $\beta$ -APM

	mg $\beta$ -APM/kg/day			
	0	250	500	750
# pregnant females	14	13	12	9
% aborting	0	7.1	7.7	10.1
# corpora lutea/doe	12.7(2.6)	12.2(3.9)	11.2(2.7)	12.3(2.5)
# implantations/litter	10.7(3.0)	8.9(4.0)	8.5(3.1)	11.0(2.6)
# resorptions/litter				
early	0.5(0.7)	0.4(0.6)	0.7(0.8)	0.0(0.5)
late	1.1(1.0)	0.45(0.6)	0.7(0.8)	0.8(0.9)
% Pre-implantation loss/litter	15.9	23.8	22.8	10.7
% Pre-implantation loss/litter	13.6	6.7	18.0	9.3
# live fetuses/litter	9.1(2.4)	8.2(3.4)	7.2(3.2)	9.9(2.1)
Fetal weight	36.1(7.2)	40.2(6.0)	41.1(5.2)	37.2(7.3)
Placental weight, g.	5.3(1.1)	6.1(2.0)	5.9(1.3)	5.3(1.2)

TABLE 8

Mean gestational parameters on day 20 for rats treated with  $\beta$ -APM  
for two generations

mg $\beta$ -APM/kg/day				
	0	250	500	750
<b>f<sub>0</sub>-f<sub>1</sub> Generations</b>				
# pregnant females	12	12	12	12
# corpora lutea/dam	16.3 (1.3)	17.3 (2.4)	17.3 (1.8)	15.7 (2.3)
# implantations/litter	14.8 (1.7)	14.9 (2.1)	16.2 (2.0)	13.7 (3.2)
# resorptions/litter				
early	0.8 (0.9)	0.4 (0.7)	0.3 (0.6)	0.5 (0.7)
late	0.3 (0.6)	0.3 (0.6)	0.1 (0.3)	0.4 (0.7)
% Pre-implantation loss/litter	8.6 (9.4)	12.7(14.5)	7.2 (6.0)	11.2(20.3)
% Post-implantation loss/litter	7.2 (5.4)	4.9 (5.5)	2.5 (4.1)	6.5 (6.1)
<b>f<sub>0</sub>-f<sub>1</sub> Generations</b>				
# pregnant females	18	18	18	18
# corpora lutea/dam	16.4 (2.1)	16.3 (3.4)	15.8 (1.8)	15.8 (2.0)
# implantations/litter	12.8 (3.3)	14.4 (2.9)	12.7 (3.8)	13.3 (3.5)
# resorptions/litter				
early	0.7 (0.9)	0.6 (0.8)	0.6 (0.8)	1.0 (1.0)
late	0.5 (0.7)	0.1 (0.2)	0.2 (0.5)	0.1 (0.2)
% Pre-implantation loss/litter	22.5(18.0)	11.1(16.7)	19.2(24.5)	15.1(21.1)
% Post-implantation loss/litter	9.5(12.3)	5.0 (6.3)	5.9 (6.7)	8.4 (8.2)

TABLE 9

Summary of rat fetal observations ( $F_0$ - $F_1$ )  
for rats treated with  $\beta$ -APM for two generations

	mg $\beta$ -APM/kg/day			
	0	250	500	750
# fetuses (litters) examined	80(12)	83(12)	94(12)	73(12)
# male:female fetuses	35:45	36:47	50:44	39:34
Pulmonary hemorrhage	0	0	1.1(8.3)	0
Hemorrhage on lung surface	0	0	1.1(8.3)	0
Hepatic hemorrhage	10.0(50.0)	16.9(58.3)	20.2(83.3)	15.1(41.7)
Hemorrhagic peritoneal fluid	1.3 (8.3)	3.6(16.7)	1.1 (8.3)	1.4 (8.3)
Local abdominal hemorrhage	1.3 (8.3)	0	0	0
Hemorrhagic abdomen	5.0(33.3)	2.4(16.7)	0	1.4 (8.3)
Testis towards midline/cranial	0	0	2.0 (8.3)	5.1(16.7)
Hemorrhagic thyroid glands	1.2 (8.3)	0	1.1 (8.3)	0
Nasal Hemorrhage,intra-muscular	1.3 (8.3)	1.2 (8.3)	1.1 (8.3)	1.4 (8.3)
Subcutaneous hemorrhages:				
cranial	2.5(16.7)	1.2 (8.3)	2.1(16.7)	0
cervical	1.3 (8.3)	0	1.1 (8.3)	1.4 (8.3)
thoracic	1.3 (8.3)	0	1.1 (8.3)	0

TABLE 10

Mean F<sub>1</sub> litter viability in rats treated with  $\beta$ -APM

	mg $\beta$ -APM/kg/day			
	0	250	500	750
No. litters	21	22	22	22
Live birth index (%)	97	99	99	96
Viability index (%)	94	95	82	93
Litter size, day 1 post partum	13.8(1.9)	14.1(1.9)	13.0(2.8)	15.8(2.5)
Live offspring post partum				
day 1				
day 4	13.3(1.9)	13.9(1.9)	12.9(2.7)	14.0(2.4)
day 4 <sup>a</sup>	12.5(1.7)	13.1(2.1)	10.5(3.9)	13.0(2.1)
day 7	8.0(0.0)	8.0(0.0)	7.2(2.1)	8.0(0.2)
day 14	8.0(0.0)	8.0(0.2)	7.7(1.3)	8.0(0.2)
day 21	8.0(0.0)	8.0(0.2)	7.7(1.3)	7.8(0.5)
	8.0(0.0)	8.0(0.2)	7.7(1.3)	7.8(0.5)

<sup>a</sup> After litter size adjustment.

TABLE 11

Summary of rat foetal observations (F<sub>1</sub>-F<sub>2</sub>)  
for rats treated with  $\beta$ -APM for two generations

	mg $\beta$ -APM/kg/day			
	0	250	500	750
# fetuses (litters) examined	99(18)	119(18)	108(18)	105(18)
# male:female fetuses,	46:53	71:48	48:60	55:55
Slight unilateral microphthalmia, <sup>a</sup> reduced retinal layer	0	0	1.0(5.6)	0
Bilateral microphthalmia	1.0(5.6)	0	0	0
Unilateral anophthalmia, severe hydrocephaly	0	0	0	1.0(5.6)
Unilateral dilated orbital sinus	0	0	0.9(5.3)	0
Dilatation of brain ventricles	2.0(11.1)	3.4(11.1)	0	0
Hemorrhage between cerebral hemisphere, pia mater	0	0.8(5.6)	0	1.0(5.6)
Hepatic hemorrhage	10.0(50.0)	16.9(58.3)	20.2(83.3)	15.1(41.7)
Hemorrhagic peritoneal fluid	3.0(16.7)	0.8(5.6)	1.9(10.5)	1.0(5.6)
Local abdominal hemorrhage	1.0(5.6)	2.5(16.7)	0.0(5.3)	0
Hemorrhagic abdomen	6.1(27.8)	0.8(5.6)	0.0(5.3)	1.9(11.1)
Subcutaneous hemorrhage,cranial	6.1(27.8)	2.5(16.7)	2.8(15.8)	3.8(16.7)





**PHARMACOKINETICS AND METABOLISM OF  
β-ASPARTYLPHENYLALANINE METHYL ESTER IN THE RAT**

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**Key words:** β-aspartame, β-aspartylphenylalanine, pharmacokinetic, metabolism, rat

## SUMMARY

Pharmacokinetics and metabolism of  $\beta$ -aspartylphenylalanine methyl ester ( $\beta$ -APM) were studied in rats using  $\beta$ -[ $^{14}\text{C}(\text{U})$ -Phe]-APM ( $\beta$ -[ $^{14}\text{C}$ ]-APM) and compared with those of [ $^{14}\text{C}$ ]-phenylalanine ([ $^{14}\text{C}$ ]-Phe). The oral pharmacokinetics of  $\beta$ -[ $^{14}\text{C}$ ]-APM were shown to be different from those of [ $^{14}\text{C}$ ]-Phe. Bioavailability of  $\beta$ -[ $^{14}\text{C}$ ]-APM was lower than that of [ $^{14}\text{C}$ ]-Phe. Rats given  $\beta$ -[ $^{14}\text{C}$ ]-APM excreted 33% of the administered radioactivity in the urine and 10% in the expired air within 48 hours. About 25% of the [ $^{14}\text{C}$ ]-APM administered was excreted in the feces mainly as  $\beta$ -aspartylphenylalanine ( $\beta$ -AP). In contrast, [ $^{14}\text{C}$ ]-Phe was absorbed completely from the intestine and was utilized more extensively for protein synthesis and intermediary metabolism than the [ $^{14}\text{C}$ ]-Phe released from  $\beta$ -APM.

Only a small portion of the  $\beta$ -AP formed from  $\beta$ -APM in the intestine was absorbed intact. The larger portion of  $\beta$ -AP was transferred to the lower parts of the small intestine and the cecum where it was metabolized in different ways. Metabolites found in the urine were phenylacetyl glycine,  $\beta$ -AP, Phe, and three unknown substances. Phenylacetyl glycine was the main metabolite (19% recovery of the dose in the 24 hour pooled urine); the other metabolites recovered were estimated to be less than 1.6% recovery of the total dose of  $\beta$ -APM. Metabolic routes for orally administered  $\beta$ -APM in rats were proposed.

## INTRODUCTION

A number of  $\beta$ -aspartyl oligopeptides have been detected in normal human urines [Lou, 19\_\_; Dorer *et al.*, 1966; Buchanan *et al.*, 1962a]. Although  $\beta$ -aspartyl peptides promptly appeared in the urine in the same chemical form when administered intravenously to humans and rats [Buchanan *et al.*, 1962b; Dorer *et al.*, 1968], urinary excretion was small when they were given orally [Dorer *et al.*, 1968]. With respect to  $\beta$ -aspartyl peptides, the pharmacokinetics, and metabolism following oral administration of the widely used sweetener, aspartame, have been studied extensively.  $\alpha$ -Aspartyl phenylalanine methyl ester ( $\alpha$ -APM) is rapidly hydrolyzed in the gastrointestinal tract to methanol, phenylalanine (Phe), and aspartic acid which subsequently enter the systemic circulation [Opperman *et al.*, 1973; Ranney *et al.*, 1976; Opperman, 1984]. In this report, the pharmacokinetics and metabolism of orally administered  $\beta$ -[ $^{14}\text{C}$ ]-APM, radiolabeled in the Phe residue, and [ $^{14}\text{C}$ ]-Phe were studied in rats.

## MATERIALS AND METHODS

Animals: Male Sprague-Dawley rats weighing 200-280 g (Charles River Japan Co.) were used in this study.

Chemicals: L-[ $^{14}\text{C}$ (U)]-Phenylalanine (460 mCi/mmol) was obtained from New England Nuclear.  $\beta$ -[ $^{14}\text{C}$ (U)-Phe]- $\beta$ -APM (1.4 mCi/mmol) was synthesized from [ $^{14}\text{C}$ ]-Phe by Amersham International. Both labeled compounds had radiopurities of greater than 97%, as determined by thin-layer chromatography (TLC). Unlabeled  $\beta$ -aspartylphenylalanine ( $\beta$ -AP),  $\beta$ -APM, and Phe were obtained from our laboratories.

Administration: Solutions of  $\beta$ -[ $^{14}\text{C}$ ]-APM (10  $\mu\text{Ci}$ /20 mg  $\beta$ -APM/2 ml) and [ $^{14}\text{C}$ ]-Phe (10  $\mu\text{Ci}$ /10 mg Phe/2 ml) were prepared in saline. Rats were fasted overnight prior to the administration by gavage of 20 mg  $\beta$ -[ $^{14}\text{C}$ ]-APM/kg body weight and 10 mg [ $^{14}\text{C}$ ]-Phe/kg (approximately equimolar dosage). In some experiments, rats were given unlabeled  $\beta$ -APM at a dose of 500 mg/kg for the isolation of  $\beta$ -APM metabolites.

Plasma levels of radioactivity: Blood samples were collected from the vena cava of three rats, 0.5, 1, 2, 4, 6, 10, 24, and 48 hours after dosing. Aliquots of these samples were analyzed for total radioactivity, protein-associated radioactivity, and metabolites in the deproteinized plasma. Plasma protein was precipitated by adding plasma to five-fold volume of ethanol and centrifugation. This yielded a protein pellet and deproteinized plasma supernatant.

Excretion of radioactivity:  $\beta$ -[ $^{14}\text{C}$ ]-APM or [ $^{14}\text{C}$ ]-Phe was administered to three rats, each housed separately in glass metabolic cages. Urine and feces were collected for 48 hours. [ $^{14}\text{C}$ ]- $\text{CO}_2$ , present in the expired air, was trapped in a mixture of ethanolamine and methanol (1:2, v/v).

Measurement of metabolites in the plasma, urine, feces, and contents of the small intestine: After administration of  $\beta$ -[ $^{14}\text{C}$ ]-APM the following was performed: blood samples were taken at 0.5, 2, and 6 hours; feces was collected from 0-24 hours; urine was collected from 0-6 hours and 10-24 hours; the small intestine dissected after 0.5 hour to yield its contents. Feces and contents of the small intestine were suspended in saline and centrifuged to yield a supernate. Plasma was deproteinized as above. Aliquots of the fecal plasma and urine samples were applied to a TLC plate of silicagel (60F254; Merck) and developed with n-butanol-acetic acid-water (2:1:1). An autoradiogram of each plate was prepared using [ $^3\text{H}$ ]-type X-ray film (LBK Co.). For each urine sample, each radioactive spot on the plate was eluted with methanol and

the radioactivity in the eluents measured. In the other samples, radioactivity of each spot was estimated from the autoradiogram.

Identification of  $\beta$ -APM metabolite in urine: Urine of 15 rats was collected for 24 hours following the administration of unlabeled  $\beta$ -APM. The urine samples were adjusted to pH 2 with 1N HCl and extracted twice with a two-fold volume of ethyl acetate. The extracts were concentrated and applied as a band to TLC plates and developed with the solvent described above. The zone corresponding to Rf 0.7-0.8 was eluted with methanol, dried by evaporation, and dissolved in methanol. The metabolite was precipitated by adding ten-fold volume of ethyl ether to the methanol solution and purified by recrystallization with methanol-ethyl ether. The purified sample was analyzed by EI-mass spectrometry (JMS-DX-300; Japan Electronic Co.) and NMR spectrometry (JHM-GX-400; Japan Electronic Co.). The amino acid composition of the metabolite was determined by an amino acid analyzer (Type 835; Hitachi Co.) after hydrolysis in 6N HCl at 110°C for 24 hours.

Whole body autoradiography: Rats were killed by ether anesthesia and immediately frozen in acetone-dry ice 0.5, 2, and 24 hours after dose of  $\beta$ -[ $^{14}\text{C}$ ]-APM and 6 hours after a dose of [ $^{14}\text{C}$ ]-Phe. Whole body sections of 20 m thickness were prepared using an established method [Ullberg, 1954], placed in contact with X-ray films ( $^3\text{H}$ -Type) for two months, and then the films developed and fixed.

Determination of radioactivity: Aliquots of plasma, urine, the solvent in  $\text{CO}_2$ -traps, eluents from TLC plates, and fecal residue following combustion with an automatic sample oxidizer (Aloka Co.) were dissolved in scintillation solution and radioactivity determined by scintillation spectroscopy in a Tri-Carb 300 Liquid Scintillation spectrometer (Packard Co.).

## RESULTS

The time courses of total radioactivity in the plasma are shown in Fig. 1. Peak plasma radioactivity occurred 10 hours after  $\beta$ -[ $^{14}\text{C}$ ]-APM administration. Radioactivity at the peak was equivalent to 11  $\mu\text{g/ml}$  of  $\beta$ -APM. The maximum plasma concentration ( $C_{\text{max}}$ ) and the area under the curve (AUC) of plasma radioactivity observed for  $\beta$ -[ $^{14}\text{C}$ ]-APM were approximately 25% and 33%, respectively, of those observed after [ $^{14}\text{C}$ ]-Phe administration. Bioavailability of  $\beta$ -[ $^{14}\text{C}$ ]-APM was estimated to be considerably lower than that of [ $^{14}\text{C}$ ]-Phe. The half-lives of plasma radioactivity were very long in both cases.

Substantial differences were observed in the excretion patterns for  $\beta$ -APM and Phe. Of the original dose of  $\beta$ -[ $^{14}\text{C}$ ]-APM, rats excreted 33, 25, and 10% of the radioactivity in the urine, feces, and expired air, respectively. In contrast, following [ $^{14}\text{C}$ ]-Phe administration, 4% of the dose appeared in the urine, 3% in the feces, and 28% in expired air (Table 1).

The distribution of radioactivity as visualized by whole body autoradiography is shown in Figure 2. Following  $\beta$ -[ $^{14}\text{C}$ ]-APM administration, radioactivity is primarily associated with the organs of absorption and excretion, although by 24 hours small amounts of radioactivity can be observed in other organs. After 0.5 hour, radioactivity was predominately localized to the stomach and intestines with smaller amounts in the kidney cortex and liver. By 2 hours, relatively high levels of radioactivity appeared in the cecum with smaller amounts detected in the renal medulla, pancreas, and bone marrow of the thigh bone. Radioactivity levels were also increased in the liver. At 24 hours, radioactivity disappeared from the contents of the stomach and small intestine, but was still observed in the contents of the large intestine and renal medulla. Lower amounts of radioactivity were observed to be distributed in various tissues from the whole body image. For comparison, rats given [ $^{14}\text{C}$ ]-Phe showed strong radioactivity

distributed widely over the whole body image 6 hours after dosing, but little radioactivity was observed in the stomach and small intestine.

The distribution of radioactive metabolites in the contents of small intestine, feces, and plasma is shown in Table 2. By 0.5 hour, most of the original  $\beta$ -APM had been converted to  $\beta$ -AP. Almost all of the radioactivity in the feces was  $\beta$ -AP with only a trace amount associated with Phe. Radioactivity in the deproteinized fraction of the plasma was detected mainly in  $\beta$ -AP and Phe at 0.5 hour, and in two unknown metabolites (Rf; 0.74 and 0.96) after 6 hours. The total concentration of these unknown metabolites equivalent to 0.9  $\mu$ g/ml of  $\beta$ -APM at most. Plasma protein-associated radioactivity was 32, 70, and 91% of the total plasma radioactivity, 0.5, 2, and 6 hours, respectively, after  $\beta$ -[ $^{14}$ C]-APM administration. In contrast, administration of [ $^{14}$ C]-Phe resulted in a more rapid and extensive incorporation of radioactivity into the plasma protein fraction (Table 3). Radioactive metabolites detected in the urine of rats given  $\beta$ -[ $^{14}$ C]-APM were  $\beta$ -AP, Phe, and four unknown metabolites (Table 4). An unknown metabolite (RF; 0.7) exhibited the most radioactivity (19% recovery of the radioactive dose in the 24 hours pooled urine), with five other metabolites comprising less than 1.6% recovery of the total dose. It is noteworthy that no radioactivity was detected as  $\beta$ -APM in any urine sample.

A major unknown metabolite (RF, 0.74) of  $\beta$ -APM was isolated and purified from the pooled urines of 15 rats administered unlabeled  $\beta$ -APM (500 mg/kg). Fifteen milligrams of the purified metabolite (white crystal) was obtained. EI-mass spectrum of this metabolite yielded a molecular weight of 193 and indicated the presence of a benzyl residue. The  $^1$ H-NMR spectrum of this metabolite showed the presence of one benzene ring and two separate methylene groups, and  $^{13}$ C-NMR spectroscopy revealed an additional two carbonyl groups. The metabolite (1.2 mg) was hydrolyzed in 6N HCl for 24 hours at 110°C, neutralized, concentrated, and analyzed by amino acid analyzer. Glycine was the only amino acid detected and represented 35% of the



metabolite, indicating a one molar concentration of glycine in this metabolite. From the molecular weight determined by mass spectroscopy and the presence of a glycyl moiety and benzyl and carbonyl groups, the metabolite was presumed to be N-phenylacetyl glycine. This was confirmed by HPLC and NMR of an authentic sample.

## DISCUSSION

The metabolic fate of the  $\beta$ -isomer of APM has been studied extensively in rodents, primates, and humans, and has been shown to involve rapid hydrolysis of the methyl ester by intestinal esterases yielding  $\beta$ -AP which was subsequently hydrolyzed to its constituent amino acid by microvillar peptidases. The amino acids then enter the systemic circulation [Opperman *et al.*, 1973; Ranney *et al.*, 1976; Opperman, 1984]. Pharmacokinetics of the constituent amino acids of  $\beta$ -APM were similar to those of methanol, Phe, and aspartic acid when they were given orally [Opperman *et al.*, 1973; Ranney *et al.*, 1976; Opperman, 1984]. Phenylalanyl and aspartyl moieties of  $\beta$ -APM were distributed in the body of rats in a manner indistinguishable from natural Phe and aspartic acid [Matsuzawa and Ohara, 1984].

In this study, the pharmacokinetics of orally administered  $\beta$ -APM and Phe were shown to be different, which suggests that  $\beta$ -APM is metabolized differently than  $\beta$ -APM. Bioavailability of  $\beta$ -[ $^{14}\text{C}$ ]-APM was lower than that of [ $^{14}\text{C}$ ]-Phe (Fig. 1). The results of this study indicate that  $\beta$ -APM is poorly absorbed following oral administration and is rapidly hydrolyzed to  $\beta$ -AP in the intestine. A small portion of the  $\beta$ -AP formed appeared in the plasma by 0.5 hour (Table 2), but its bioavailability appeared to be small considering the low recovery of  $\beta$ -AP in the urine and rapid loss from the plasma. About 25% of the  $\beta$ -[ $^{14}\text{C}$ ]-APM dose was excreted in the feces as  $\beta$ -AP, while almost all of [ $^{14}\text{C}$ ]-Phe was absorbed from the gastrointestinal tract (Tables 1, 2). The reported low capacity of liver and other tissues of rats to hydrolyze  $\beta$ -aspartyl peptides [Dorer *et*

*al.*, 1968] might account, in part, for a smaller pool of  $\beta$ -AP-derived [ $^{14}\text{C}$ ]-Phe for protein synthesis and catabolism to [ $^{14}\text{C}$ ]- $\text{CO}_2$ .

Two hours after  $\beta$ -[ $^{14}\text{C}$ ]-APM administration, radioactivity was visualized in the cecum, a site of substantial bacterial metabolism. The predominate metabolite in plasma and urine, at this and later times, was N-phenylacetyl glycine. It is unlikely that this metabolite was produced from  $\beta$ -AP absorbed into the systemic circulation from the intestine. In work by others, only trace amounts of N-phenylacetyl glycine were produced when  $\beta$ -APM was given intravenously to rats [Matsuzawa, unpublished data].  $\beta$ -Aspartyl peptides injected systemically in rats and humans were excreted unchanged in the urine [Buchanan *et al.*, 1962; Dorer *et al.*, 1968]. It is thought that N-phenylacetyl glycine is produced via phenylacetic acid, which might be produced from Phe by the action of intestinal microflorae. Phe is known to be metabolized by the intestinal microflora to produce phenylacetic acid [Van der Heiden *et al.*, 1971]. Recently, Lipton *et al.* [1991] reported that  $\beta$ -AP was not hydrolyzed by rat intestinal mucosa, but is cleaved by intestinal microflora and pure cultures of *E. coli* to yield Phe. The  $^{14}\text{C}$ -phenylacetic acid produced would be absorbed from the intestine and conjugated with glycine to form ( $^{14}\text{C}$ -N-phenylacetyl glycine. The conjugation of phenylacetic acid or allylacetic acids with amino acids is known to be species dependent. The conjunction occurs mainly with glycine in rodents and with glutamine or taurine in primates and humans [James *et al.*, 1972a,b]. Possible metabolic routes of  $\beta$ -APM administered orally in rats are proposed as shown in Figure 2.

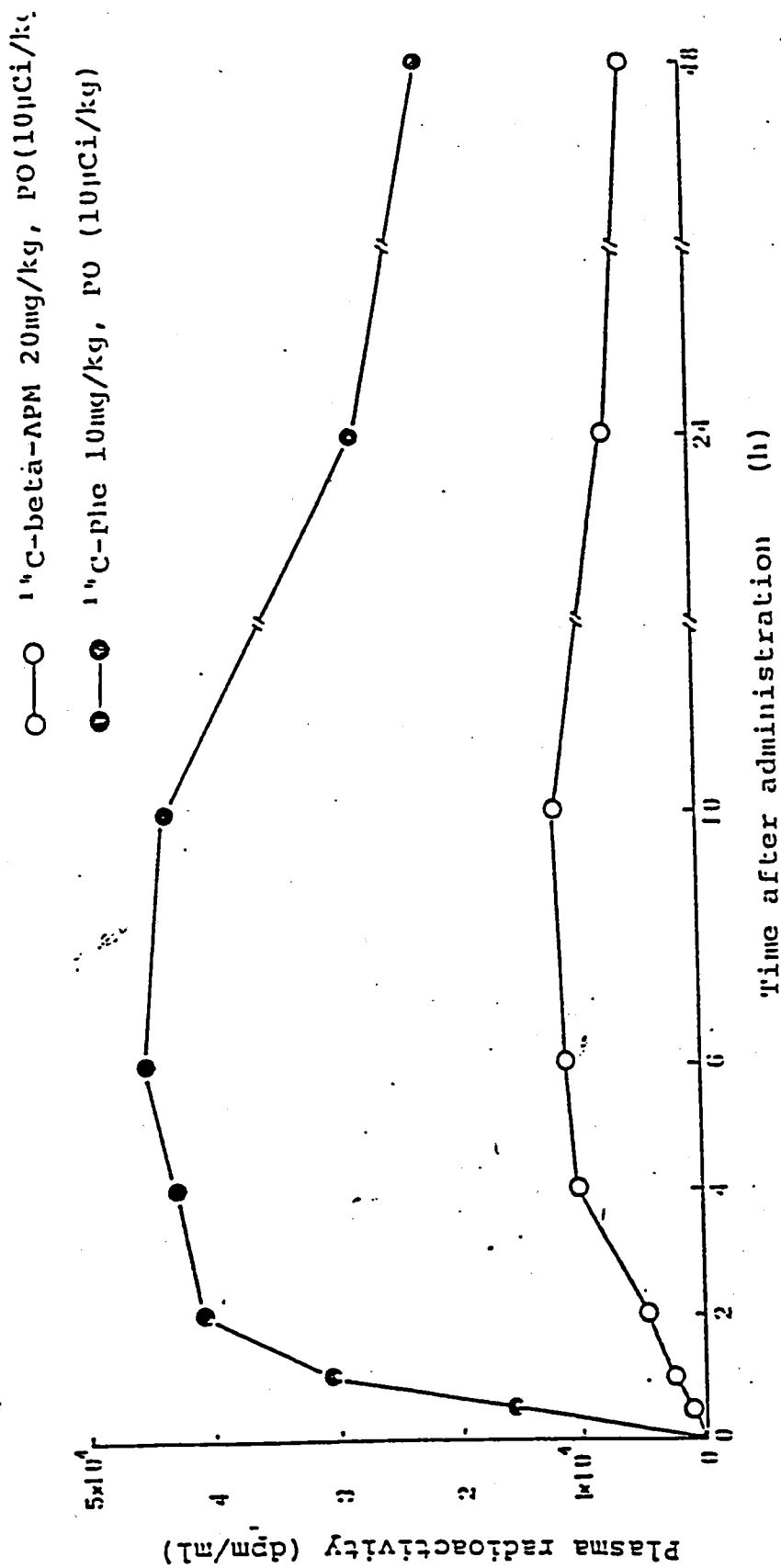
## REFERENCES

- Buchanan DL, Haley EE, Markiw RT (1962) Occurrence of beta-aspartyl and gamma-glutamyl oligopeptides in human urine. *Biochemistry* 1: 612-620
- Buchanan DL, Haley EE, Markiw RT, Paterson AA (1962) Studies on the *in vivo* metabolism of alpha-and beta-aspartyl glycine-1-<sup>14</sup>C. *Biochemistry* 1: 621-623
- Dorer FE, Haley EE, DL Buchanan DL (1966) Quantitative studies of urinary beta-aspartyl oligopeptides. *Biochemistry* 5: 3236-3240
- Dorer FE, Haley EE, Buchanan DL (1968) The hydrolysis of beta-aspartyl peptide by rat tissue. *Arch Biochem Biophys* 127: 490-495
- James MO, Smith RL, Williams RT (1972) Conjugation of phenylacetic acid in man, subhuman primates and some non-primate species. *Proc Roy Soc London Series B*, 182: 25
- James MO, Smith RL, Williams RT (1972) The conjugation of 4-chloro and 4-nitro-phenylacetic acids in man, monkey and rat. *Xenobiotica* 2: 449
- Lipton WE, Li YN, Younoszai MK, Stegink LD (1991) Intestinal absorption of aspartame decomposition products in adult rats. *Metabolism* 40: 1337
- Lou MF (1975) Isolation and identification of L-beta-aspartyl-L-lysine and L-gamma-glutamyl-L-ornithine from normal human urine. *Biochemistry* 14: 3503-3508
- Matsuzawa Y Unpublished data
- Matsuzawa Y, Ohara Y (1984) Tissue distribution of orally administered isotopically labeled aspartame in the rat. In: Stegink LD, Filer LF Jr. (eds) *Aspartame: Physiology and Biochemistry*, Chapter 8, Marcel Dekker, Inc., New York, NY, pp 161-200
- Opperman JA, Muldoon E, Ranney RE (1973) Effect of aspartame on phenylalanine metabolism in the monkey. *J Nutr* 103: 1460-1466
- Opperman JA (1984) Aspartame metabolism in animals. In: Stegink LD, Filer LJ Jr. (eds) *Aspartame: Physiology and Biochemistry*, Marcel Dekker, Inc., Chapter 7, New York, NY, pp 141-159
- Ranney RE, Opperman JA, Muldoon E, McMahan FG (1976) Comparative metabolism of aspartame in experimental animals and humans. *J Toxicol Environ Health* 2: 441-451

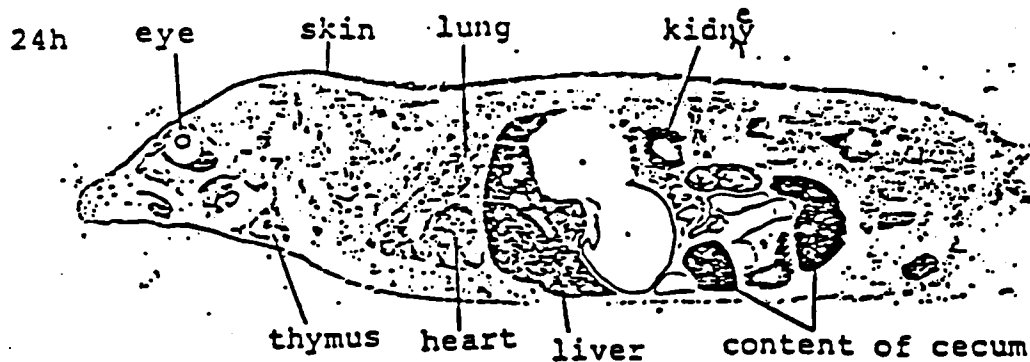
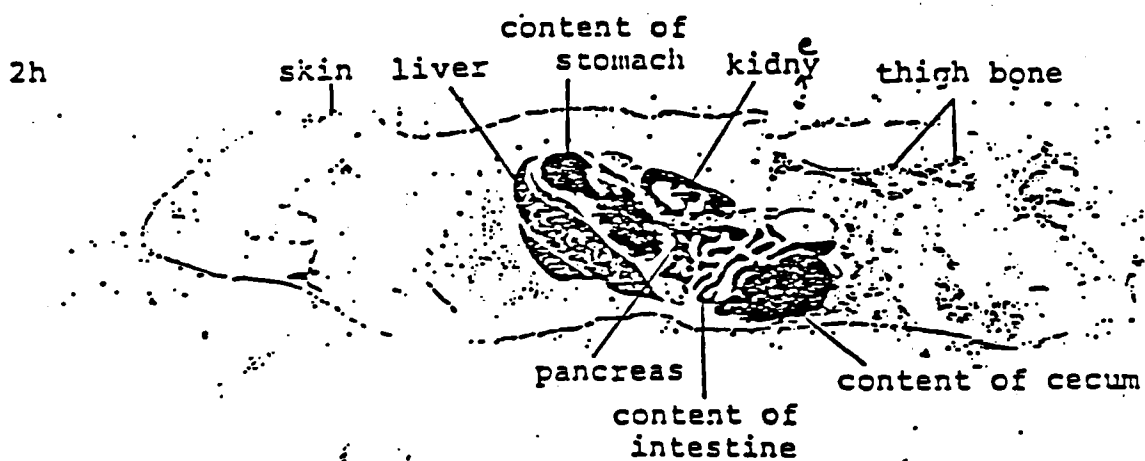
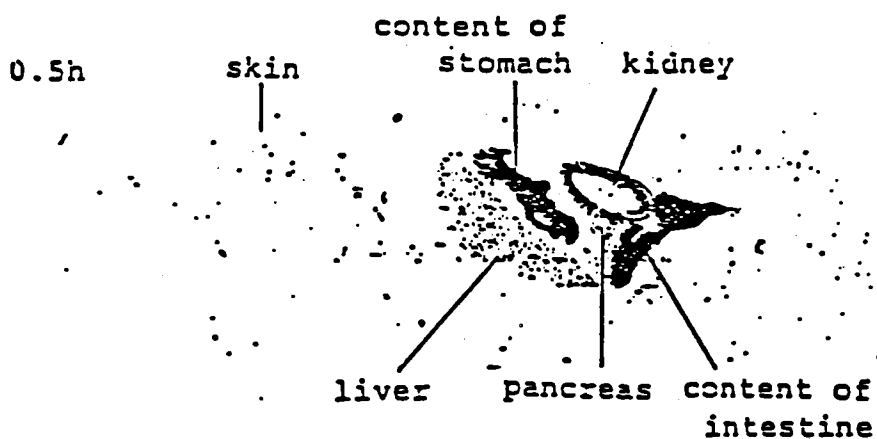
Ullberg S (1954) Studies on the distribution and fate of <sup>35</sup>S-labeled benzylpenicillin in the body. Acta Radiol Suppl 118: 1-110

Van der Heiden C, Wauters EAK, Ketting D, Duran M, Wadman SK (1971) Gas chromatographic analysis of urinary tyrosine and phenylalanine metabolites in patients with gastrointestinal disorders. Clin Chim Acta 34: 289-296

Fig. 1 Time course of plasma radioactivity in rats administered  $^{14}\text{C}$ -beta-APM or  $^{14}\text{C}$ -Phe



$^{14}\text{C}$ -beta-APM



$^{14}\text{C}$ -Phe

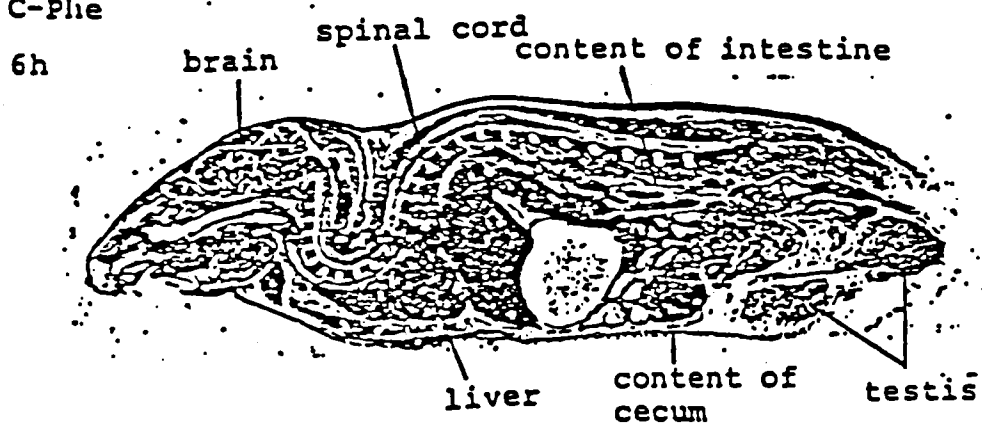


Table.1 Excretion ratio of radioactivity in urine, feces, expired air of rats orally administered  $\beta$ -[ $^{14}\text{C}$ ]-APM or [ $^{14}\text{C}$ ]-Phe

Time (hrs)	Percentage of original dose					
	$\beta$ -[ $^{14}\text{C}$ ]-APM 20 mg/kg			[ $^{14}\text{C}$ ]-Phe 10 mg/kg		
	Urine	Feces	Expired Air	Urine	Feces	Expired Air
0-2	0.7	0.0	0.3	0.9	0.0	9.9
2-6	7.0	0.0	3.2	1.5	0.0	7.3
6-10	14.9	14.8	4.1	0.6	0.9	2.3
10-24	9.1	9.4	1.4	0.8	1.4	4.5
24-48	1.7	1.2	1.1	0.5	0.6	4.5
Total	33.4%	25.4%	10.1%	4.3%	2.9%	28.2%

Table 2. Distribution of metabolite radioactivity in content of small intestine, plasma and feces by TLC after oral administration of  $\beta$ -[ $^{14}\text{C}$ ]-APM

Sample	Rf;	0.16 ( $\beta$ -AP)	0.24 (U-II)	0.29 (U-III)	0.54 (Phe)	0.71 ( $\beta$ -APM)	0.74 (U-I)	0.96 (U-IV)
Contents of small intestine at 0.5h		++++				+		
Feces during 24h		++++			T			
Plasma at 0.5h (deproteinized)		++			+			
Plasma at 6h (deproteinized)		T			T		++	+

KEY: ++++ Strong radioactivity  
 ++ Moderate radioactivity  
 + Mild radioactivity  
 T Trace radioactivity

\* Silicagel plate developing solvent  
 n-BuOH; AcOH;  $\text{H}_2\text{O}$  (2;1;1)



Table 3. Radioactivity of total plasma and plasma protein after oral administration of  $\beta$ -[ $^{14}\text{C}$ ]-APM (20 mg/10  $\mu\text{Ci/kg}$ ) or [ $^{14}\text{C}$ ]-Phe (10 mg/10  $\mu\text{Ci/kg}$ )

Dose	Time (hr)	Radioactivity (dpm/ml)			Portion of Plasma Protein (%)
		Total Plasma	Plasma Protein	Protein (%)	
$\beta$ -[ $^{14}\text{C}$ ]-APM	0.5	4380	1380	32	
$\beta$ -[ $^{14}\text{C}$ ]-APM	2.0	9290	6590	70	
$\beta$ -[ $^{14}\text{C}$ ]-APM	6.0	11200	10190	91	
[ $^{14}\text{C}$ ]-Phe	0.5	15500	10980	71	
[ $^{14}\text{C}$ ]-Phe	2.0	41400	39100	94	
[ $^{14}\text{C}$ ]-Phe	6.0	44600	43200	97	

Table 4. Distribution of metabolite radioactivity in the urine by TLC after oral administration of  $\beta$ -[ $^{14}\text{C}$ ]-APM

Sample	Recovery of radioactivity (% of dose)	Rf;	( $\beta$ -AP)	(U-II)	(U-III)	(Phe)	( $\beta$ -APM)	(U-I)	(U-IV)
									(% of dose)
0 - 6 hr (Urine)	3.14		3	6	2	1	0	83	0
10 - 24 hr (Urine)	9.29		0	1	1	2	0	83	9
0 - 24 hr (Pooled Urine)	21.70		1	1	1	2	0	89	7



**A SUMMARY OF THE ABSORPTION, DISTRIBUTION, METABOLISM  
AND ELIMINATION OF  $\beta$ -ASPARTAME  
AND ITS FREE ACID,  $\beta$ -ASPARTYLPHENYLALANINE,  
IN THE RAT, DOG, RABBIT, MONKEY AND MAN**

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**Key Words:  $\beta$ -Aspartame,  $\beta$ -Aspartylphenylalanine, Absorption, Distribution, Metabolism,  
Rat, Dog, Rabbit, Monkey, Man**

**ABSTRACT**

$\beta$ -aspartame ( $\beta$ -APM), a rearrangement product of aspartame, was completely hydrolyzed at its methyl ester bond to its free acid,  $\beta$ -aspartylphenylalanine ( $\beta$ -AP), following oral administration. While virtually no  $\beta$ -APM reached the systemic circulation intact, the oral bioavailability of  $\beta$ -AP ranged from 7 to 23% in the rat, rabbit, dog, monkey and man. Maximal plasma concentrations of  $\beta$ -AP were achieved in all species by one to six hours following the oral administration of  $\beta$ -APM or  $\beta$ -AP. Following oral administration of radiolabeled  $\beta$ -APM or  $\beta$ -AP ( $^{14}\text{C}$  in the phenylalanine moiety), approximately 90% of the radiolabel from  $\beta$ -APM was absorbed by the rat, rabbit, dog, monkey and man; 65% (rat) to 90% (rabbit and dog) of the radiolabel was absorbed following oral administration of  $\beta$ -AP. Radioactivity absorbed by the rat distributed primarily to the liver, kidneys and bladder with lowest concentrations occurred in the skeletal muscle, brain and eye. N-Acetyl- $\beta$ -AP, phenylalanine, phenylacetic acid and the glycine and glutamine conjugates of phenylacetic acid were the major metabolites observed in the rat, rabbit, dog, monkey and man following oral administration of  $\beta$ -AP. The glycine and glutamine conjugates were formed following the hydrolysis and oxidation of orally administered  $\beta$ -AP to phenylacetic acid by bacteria in the gut. The resulting phenylacetic acid was absorbed and conjugated with glycine (rat and rabbit) or glutamine (monkey and man). Following the oral administration of  $\beta$ -APM or  $\beta$ -AP, radioactivity was slowly eliminated from the plasma of the rat, rabbit and dog. The protracted clearance of the radioactivity from plasma probably was undoubtedly the result of the de novo incorporation of radiolabeled phenylalanine, resulting from the metabolism of  $\beta$ -APM or  $\beta$ -AP, into plasma and tissue proteins. Finally, the results of this study are consistent with the findings that  $\beta$ -AP is also an endogenous product in the urine and plasma of humans who have not consumed aspartame.

## INTRODUCTION

$\beta$ -Aspartame (N- $\beta$ -L-aspartyl-L-phenylalanine, 1-methyl ester;  $\beta$ -APM) and its free acid (N- $\beta$ -L-aspartyl-L-phenylalanine;  $\beta$ -AP) products formed in low amounts in aqueous solution (Witt, 1986; Lawrence and Iyengar, 1987). Because both compounds are ingested by humans consuming aspartame, albeit in small quantities, a series of studies was undertaken to examine the absorption, distribution, metabolism and excretion of  $\beta$ -APM and  $\beta$ -AP in various species, including the rat, rabbit, dog and monkey, and man. Pharmacokinetic and metabolic studies were done with female rats, rabbits, monkeys and men receiving oral  $\beta$ -APM. Similar studies were conducted with female rats, rabbits and dogs receiving oral and intravenous  $\beta$ -AP. In addition, the absorption and metabolism of  $\beta$ -APM was studied in pregnant rats and in dogs receiving the compound in their diet. The tissue distribution of  $\beta$ -APM-related radioactivity was assessed in male rats gavaged with  $\beta$ -APM.

## MATERIALS AND METHODS

### Pharmacokinetics and metabolism of [ $^{14}$ C]- $\beta$ -APM and [ $^{14}$ C]- $\beta$ -AP in the rat.

Female Crl:CD(SD)BR rats (226-276 g), housed individually in stainless steel cages, were dosed orally and intravenously via the caudal vein with 10 mg/kg (2 ml/kg) of [ $^{14}$ C] $\beta$ -AP or orally with 10 mg/kg (2 ml/kg) of [ $^{14}$ C]- $\beta$ -APM. The dose of radiolabel from [U- $^{14}$ C]-phenylalanine- $\beta$ -AP or  $\beta$ -APM ranged from 50 to 300  $\mu$ Ci/kg. Radiochemical purity of each test article was determined with thin layer chromatography 4 hours before dosing. For one group of rats, heparinized blood samples (0.5-1.0 ml) were collected via a tail vein catheter 0.08, 0.25, 0.50, 0.75, 1, 2, 4, 6, 8, 12, 24, 32, 48, 72, 96, and 120 hours after dosing. Tubes used to collect blood from animals dosed with [ $^{14}$ C]- $\beta$ -APM also contained the esterase inhibitor, diethyl *p*-nitrophenyl phosphate. Plasma samples were either analyzed immediately or stored frozen at -70°C until analysis. Following each blood sample collection, an equivalent volume of isotonic saline was

injected into the rat via the tail vein catheter. Urine samples were collected 0-6, 6-12, 12-24, 24-48, 48-72, 72-96, and 96-120 hours after dosing. Feces were collected 24, 48, 72, 96, and 120 hours after dosing. To collect  $^{14}\text{CO}_2$ , dosed rats were placed in metabolic cages enclosed in plastic bags and expired air was collected 0-0.5, 0.5-1, 1-2, 2-3, 3-4, 4-5, 5-6, 6-7 and 24-25 hours after dosing. The expired air was drawn, by means of a partial vacuum, through a gas washer containing ethanolamine:2-methoxymethanol (1:2, v/v). Plasma and urine samples were analyzed with conventional direct liquid scintillation spectrometry and high performance liquid radiochromatography. Chromatographic conditions were similar to those described by Burton *et al.* (1989) as were the isolation of metabolites from plasma and urine and their identification using gas chromatographic/mass spectrometric analysis. Radioactive compound eluting from the HPLC system were mixed with Flo-Scint® III at a ratio of 1.0 to 5.5 ml/min in a Flo-One® mixing chamber and detected using a Model CU, Flo-One® radioactive flow detector (Radio Analytic, Inc., Tampa, FL). Counting efficiency was determined using appropriate standards. Fecal samples were combusted and trapped products were measured by liquid scintillation spectrometry. Total radioactivity in expired air was measured by liquid scintillation spectrometry of aliquots of the ethanolamine:2-methoxymethanol used to trap the radiolabeled  $^{14}\text{CO}_2$ . Areas under the plasma concentration-time curves were calculated using the trapezoidal rule (Gibaldi and Perrier, 1982). Plasma concentration curves for total radioactivity and [ $^{14}\text{C}$ ]- $\beta$ -AP were analyzed with the CSTRIP computer program (Sedman and Wagner, 1976) with initial parameters from CSTRIP then used in the NONLIN program (Metzler, *et al.*, 1974).

Tissue distribution studies following the oral administration of [ $^{14}\text{C}$ ]- $\beta$ -APM were conducted in male Long Evans rats, CrI:(LE)BR, weighing 210-257 g. Three rats per time point were sacrificed at pre-dose and 1, 3, 6, 24, 48, 96, 168, and 240 hours after the oral administration of 2 mg/kg (55  $\mu\text{Ci/kg}$ ) of [ $^{14}\text{C}$ ]- $\beta$ -APM. Urine and feces were collected every 24 hours from the rats killed at 240 hours. The following tissues were collected for analysis: adrenals, bladder, brain, eyes minus lens, eye lens, fat, femurs, heart, kidneys, large intestine, large intestinal

contents, liver, lungs, heparinized plasma, red blood cells, skeletal muscle from leg, skin from shaved back, small intestine, small intestinal contents, spleen, stomach, stomach contents, submaxillary glands, testes and thymus. Tissues for radiochemical analyses were dissected, weighted and stored frozen until analysis. Total radioactivity in red blood cells, plasma, tissues and excreta was measured by liquid scintillation spectrometry.

#### Pharmacokinetics and metabolism of [ $^{14}\text{C}$ ]- $\beta$ -APM and [ $^{14}\text{C}$ ]- $\beta$ -AP in the rabbit

Female New Zealand rabbits (2-3 kg), housed in individual metabolism cages, were dosed orally by gavage and intravenously with 10 mg/kg (2 ml/kg) of [ $^{14}\text{C}$ ]- $\beta$ -AP or orally with 10 mg/kg (2 ml/kg) of [ $^{14}\text{C}$ ]- $\beta$ -APM. The dose of radiolabel ranged from 24 to 51  $\mu\text{Ci/kg}$ . Heparinized blood samples were obtained via ear veins from the rabbits 0.08, 0.25, 0.50, 0.75, 1, 2, 4, 6, 8, 12, 24, 48, 72, 96, and 120 hours after dosing as described above for rats. Following withdrawal of blood samples up to 6 hours, an equivalent volume of normal saline was administered intravenously to the rabbits. Urine was collected in containers surrounded by dry ice 0-6, 6-12, 12-24, 24-48, 48-72, 72-96 and 96-120 hours after dosing. Feces was collected every 24 hours for up to 120 hours. Expired air was collected 0-0.5, 0.5-1, 1-2, 2-3, 3-4, 4-5, 5-6, and 6-7 hours after dosing as described above. All samples were either analyzed immediately or stored frozen at  $-70^{\circ}\text{C}$  until analysis using conventional liquid scintillation spectrometry and high performance liquid radiochromatography (HPLC) as described above.

#### Pharmacokinetics and metabolism of [ $^{14}\text{C}$ ]- $\beta$ -APM and [ $^{14}\text{C}$ ]- $\beta$ -AP in the dog

Female beagles (8.3-10.7 kg), housed in individual metabolism cages from 24 hours before dosing until the end of sample collection, were fasted from 18-24 hours before compound administration to 6 hours after dosing. The dogs were dosed orally by gavage (2 ml/kg) and intravenously (1 ml/kg) with 10 mg/kg of [ $^{14}\text{C}$ ]- $\beta$ -AP or orally with 10 mg/kg (2 ml/kg) of [ $^{14}\text{C}$ ]- $\beta$ -APM. The dose of radiolabel ranged from 10 to 25  $\mu\text{Ci/kg}$ . Each dog was dosed three times with a 4-week interval between doses. Heparinized blood samples were collected 0.25,



0.5, 1, 1.5, 2, 3, 4, 5, 7, 9, 12, 24, 48, 72, 96, and 120 hours after dosing with additional samples obtained 2 and 5 minutes after intravenous dosing as described above for rats. Urine was collected in containers surrounded by dry ice from -24-0, 0-6, 6-12, 12-24, 24-48, 48-72, 72-96 and 96-120 hours after dosing. Feces was removed from the metabolism cages at 0, 24, 48, 72, 96, and 120 hours after dosing. All samples were either analyzed immediately or stored frozen at -70°C until analysis with conventional liquid scintillation spectrometry and HPLC.

#### Pharmacokinetics and metabolism of [<sup>14</sup>C]-β-APM--APM in the monkey

Three female rhesus monkeys (*Macaca mulatta*), weighing 4.2-5.1 kg, were fasted from 16 hours prior to dosing to 7 hours after dosing. The monkeys, restrained in primate chairs prior to and for 12 hours after dosing, were transferred to individual stainless steel metabolism cages until 96 hours after dosing. Each monkey received a single, oral 10 mg/kg (24 μCi/kg) dose of [<sup>14</sup>C]-β-APM (2 ml/kg) by nasogastric intubation. Heparinized blood samples (3 ml) were collected from the saphenous or cephalic vein 0, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 48, 72, and 96 hours after dosing, as described above for rats. Fluid loss was replaced by a slow, intravenous infusion of isotonic saline administered via a cannulated cephalic vein for the initial five hours after dosing. Urine was collected via a urinary catheter from 0-6 and 6-12 hours and by free catch 12-24, 24-48, 48-72, and 72-96 hours after dosing. Feces was collected 24, 48, 72, and 96 hours after dosing. To collect expired air, a plastic helmet was placed over each monkey's head and expired CO<sub>2</sub> was drawn, by means of a partial vacuum, through and trapped by gas washers as described above for rats. Samples of expired air were collected 0-0.5, 0.5-1, 1-1.5, 1.5-2, 2-3, 3-4, 4-5, 5-6, and 6-7 hours after dosing. All samples were analyzed immediately or stored frozen at -20°C until analysis with conventional liquid scintillation spectrometry or HPLC.

#### Pharmacokinetics and metabolism of [<sup>14</sup>C]-β-APM in man

Five healthy men (18-40 years of age and 64-84 kg in weight) received single 31.9 ± 0.1 mg (mean ± SEM) oral doses of [<sup>14</sup>C]-β-APM dissolved in 48 ml of water. The dose of radiolabel

was  $164 \pm 1 \mu\text{Ci}$  (mean  $\pm$  SEM). Written informed consent was obtained from each man prior to initiation of the study. Heparinized blood samples were obtained from the men just before and 0.125, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.5, 3, 3.5, 4, 5, 6, 7, 8, 9, 10, 12, 24, 36, 48, 72, 96, 120, 144, 168, and 504 hours after dosing and processed as described above. Urine samples were obtained at -12-0, 0-1, 1-2, 2-3, 3-4, 4-8, 8-12, 12-24, and for each of six subsequent 24-hour periods. Feces were collected for the 12-hour interval preceding dosing and for each of the seven subsequent 24-hour intervals. All samples were frozen immediately and stored at  $-20^{\circ}\text{C}$  until analysis using conventional liquid scintillation spectrometry and HPLC with radiometric detection.

## RESULTS AND DISCUSSION

### Pharmacokinetics

#### Absorption

Orally administrated  $\beta$ -APM was completely hydrolyzed at its methyl ester bond prior to absorption from the gastrointestinal tract. Identical observations have been made for aspartame (Ranney and Oppermann, 1979; Oppermann, 1984). In the rabbit,  $\beta$ -APM was detected in plasma and ranged from 0.03 to 0.06  $\mu\text{g/ml}$  at 0.5 to 1 hour following oral  $\beta$ -APM (10 mg/kg). The area under the plasma concentration-time curve (AUC) calculated for  $\beta$ -APM in the rabbit was less than 2% of the AUC for  $\beta$ -AP. These data indicate that very little unchanged  $\beta$ -APM reached the systemic circulation of the rabbit.

#### Time to Maximal Plasma Concentrations ( $T_{\text{max}}$ )

Maximal plasma concentrations of total radioactivity were attained 5-9 hours after the oral administration of  $\beta$ -APM (10 mg/kg) or  $\beta$ -AP (10 mg/kg) to the rat, rabbit, dog, monkey and man (Table 1). Peak plasma concentrations of total radioactivity occurred at 5 to 6 hours in the

rabbit and man indicating these species may absorb  $\beta$ -APM and  $\beta$ -AP more rapidly than the rat, dog or monkey. Similar results were reported for  $\alpha$ -APM by Oppermann (1984). In these studies, maximal plasma concentrations of total radioactivity occurred at 3-5 hours in the dog and rat, 8-10 hours for the monkey and approximately 6 hours in man.

In contrast to the absorption of aspartame,  $\beta$ -APM and  $\beta$ -AP were not completely hydrolyzed to their constitutive amino acids prior to absorption into the systemic circulation. Maximal plasma concentrations of  $\beta$ -AP occurred 1-6 hours after the oral administration of either  $\beta$ -APM or  $\beta$ -AP to the rat, rabbit, dog, monkey and man (Table 1). In these species, maximal plasma concentrations of  $\beta$ -AP occurred 3-5 hours before the peak concentrations of total radioactivity.

#### Maximal Plasma Concentrations (C<sub>max</sub>)

Following the oral administration of  $\beta$ -APM or  $\beta$ -AP to the rat, rabbit, dog and monkey, maximal plasma concentrations of total radioactivity ranged from 5.7 to 11.8  $\mu$ g-equivalents/ml (Table 1). Highest plasma concentrations occurred in the dog (10.4 to 11.8  $\mu$ g-equivalents/ml). Concentrations of total radioactivity were dose-proportional in all species tested including man where the dose was approximately 20-fold lower (i.e. 0.4-0.5 mg/kg). Maximal plasma concentrations of  $\beta$ -AP in humans (0.38 to 2.34  $\mu$ g-equivalents/ml) followed the same pattern. Highest concentrations of  $\beta$ -AP occurred in the dog (1.56 to 2.34  $\mu$ g-equivalents/ml); concentrations in the rat, rabbit and monkey were lower and similar (0.38 to 0.73  $\mu$ g-equivalents/ml). Plasma concentrations of  $\beta$ -AP in man were again dose-proportional.

#### Bioavailability

The extent of absorption of radioactivity following the oral administration of  $\beta$ -AP and  $\beta$ -APM was determined by two approaches. First, because less than 10% of the radioactivity administered orally to the monkey and man was recovered in feces, greater than 90% of the oral dose was presumed absorbed. In the rat, rabbit and dog, the fecal excretion of radioactivity

following 10 mg/kg oral doses of  $\beta$ -APM or  $\beta$ -AP was compared with that measured following intravenous  $\beta$ -AP. Based on fecal excretion, nearly 90% of the radioactivity from orally-administered  $\beta$ -APM was absorbed by the rat, rabbit and dog (88%, 92% and 91%, respectively). The rat absorbed 65% of the radioactivity from an oral dose of  $\beta$ -AP; while the rabbit and dog absorbed more than 90% of the dose.

Based on urinary recovery data and plasma AUCs, the bioavailability of  $\beta$ -AP was 7% to 9% in rats, rabbits, dogs, monkeys and men receiving oral  $\beta$ -APM (Table 1). Following the oral administration of  $\beta$ -AP, the bioavailabilities of  $\beta$ -AP calculated using plasma AUCs were 8.4% in the rat, 22.6% in the rabbit and 15.7% in the dog. The bioavailabilities of  $\beta$ -AP in the rabbit were 7.8% after oral  $\beta$ -APM and 7.7% after oral  $\beta$ -AP when calculated from urinary recoveries of  $\beta$ -AP and N-acetyl- $\beta$ -AP, an "intact" metabolite of  $\beta$ -AP.

Plasma concentrations of radioactivity (only the rats received radiolabeled  $\beta$ -APM) and  $\beta$ -AP also were measured in pregnant rats and in male and female dogs undergoing toxicological testing. Although  $\beta$ -APM was administered in the diet and at a wide range of doses (5.8 to 950 mg/kg in the rat; 250 to 1000 mg/kg in the dog), plasma concentrations of radioactivity and  $\beta$ -AP were dose-proportional in both species (Mayhew *et al.*, 199\_; Allen *et al.*, 199\_).

### Distribution

Excluding the gastrointestinal tract, highest concentrations of  $\beta$ -APM-related radioactivity were measured in the liver, bladder and kidneys of male rats 3 to 6 hours after the received oral dose [ $^{14}\text{C}$ ]- $\beta$ -APM (2 mg/kg). Peak concentrations of radioactivity in the liver and kidneys were 3 to 4-fold higher than those measured in plasma. The lowest concentrations of radioactivity (i.e., tissue/plasma ratios less than 0.25) were measured in the brain, eye, lens of the eye and skeletal muscle. This distribution pattern of radioactivity following oral  $\beta$ -APM is similar to that observed for  $\beta$ -APM in the male rat using whole-body autoradiography (Matsuzawa and Ohara

1984).

The half-lives for the clearance of radioactivity from various tissues ranged from 3.7 days (small intestines) to minimal clearance after 10 days (brain, skeletal muscle, red blood cells). The differences in the half-lives of radioactivity in various tissues probably result largely from differences in the way the tissues themselves incorporate [ $^{14}\text{C}$ ]-phenylalanine (resulting from the metabolism of  $\beta$ -APM) into their cellular components. The clearance of radioactivity from plasma following oral doses of [ $^{14}\text{C}$ ]-L-phenylalanine and [ $^{14}\text{C}$ ]- $\alpha$ -APM to the rat, dog and monkey also is protracted (Harper, 1984).

### **Metabolism**

The metabolic pathway proposed for  $\beta$ -APM is presented in Figure 1. As discussed above, virtually no  $\beta$ -APM is absorbed into the systemic circulation with the methyl ester bond intact. However, unlike orally administered  $\alpha$ -APM, which is completely hydrolyzed to methanol and its constitutive amino acids prior to absorption,  $\beta$ -AP (resulting from the hydrolysis of  $\beta$ -APM) does reach the systemic circulation intact. Its fate is discussed below with those of the other major metabolites.

### **Plasma Metabolic Profile**

The major metabolites found in 0-6 hour plasma of the rat, rabbit, monkey and man following the oral administration of  $\beta$ -APM or  $\beta$ -AP were  $\beta$ -AP, N-acetyl- $\beta$ -AP, phenylalanine, phenylacetic acid and either the glycine (rat and rabbit) or glutamine (monkey and man) conjugate of phenylacetic acid. Tyrosine was also found in dog plasma. On the basis of total plasma radioactivity,  $\beta$ -AP and phenylacetylglutamine were the major metabolites found in man. Phenylacetylglutamine and N-acetyl- $\beta$ -AP were the predominant plasma metabolites found in the monkey.  $\beta$ -AP and phenylalanine were the major metabolites occurring in dog plasma.

$\beta$ -AP, phenylalanine, phenylacetic acid and phenylacetyl glycine were the major metabolites found in the plasma of the rat and rabbit receiving either  $\beta$ -AP or  $\beta$ -APM by gavage. They also were the major metabolites observed in the plasma of pregnant rats receiving  $\beta$ -APM in the diet. Recently, Burton *et al.* (1989) have shown that  $\beta$ -AP is a normal constituent of human plasma and urine and may result, in part, from the conjugation of asparagine and phenylalanine by a renal enzyme.

In contrast to the plasma metabolic profile observed following the oral administration of  $\beta$ -AP or  $\beta$ -APM,  $\beta$ -AP accounted for most (70-100%) of the plasma radioactivity 60 minutes after the intravenous administration of  $\beta$ -AP to the rat, rabbit and dog. Phenylalanine (1-20%) and N-acetyl- $\beta$ -AP (5%) accounted for the balance of the intravenous radioactivity.

#### Urinary Metabolic Profile

Following the intravenous administration of  $\beta$ -AP to the rat,  $\beta$ -AP and N-acetyl- $\beta$ -AP accounted for more than 90% (approximately 75% and 10-20%, respectively) of the urinary radioactivity (Table 2). A similar, although slightly different, profile occurred for the rabbit with  $\beta$ -AP, N-acetyl- $\beta$ -AP and phenylacetyl glycine accounting for about 78%, 20% and 2% of the total urinary radioactivity of the rabbit, respectively. In the dog,  $\beta$ -AP accounted for more than 99% of the intravenously administered radioactivity recovered in the urine.

In marked contrast, following oral dosing with  $\beta$ -AP or  $\beta$ -APM, the glycine (rat and rabbit) and glutamine (primates) conjugates of phenylacetic acid accounted for most (75-95%) of the radioactivity collected in the 0-12 hour urines of the rat, rabbit, monkey and man. Most of the remaining radioactivity was accounted for by N-acetyl- $\beta$ -AP (5-20%) and  $\beta$ -AP. In sharp contrast to the concentrations of  $\beta$ -AP attained in the urine following intravenous dosing with  $\beta$ -AP, less than 7% of the urinary radioactivity following oral  $\beta$ -APM or  $\beta$ -AP could be attributed to  $\beta$ -AP. The dog was an exception, however, with  $\beta$ -AP accounting for 70% of the

total urinary radioactivity and an unidentified metabolite accounting for approximately 25% of the radioactivity excreted into the urine (equivalent to approximately 5% of the oral dose of  $\beta$ -AP or  $\beta$ -APM).

The preponderance of the conjugates of phenylacetic acid in the urine of the rat, rabbit, monkey and man deserves comment. The major metabolic pathway for phenylalanine in mammals is its hydroxylation to tyrosine (Harper, 1984; Bender, 1985). The oxidation of phenylalanine to phenylacetic acid normally plays a minor role in the metabolism of phenylalanine. The large amounts of phenylacetic acid conjugates occurring in the urine of the rat, rabbit, monkey and man following the oral administration of  $\beta$ -APM and  $\beta$ -AP are presumed to be a consequence of the extensive metabolism of phenylalanine to phenylacetic acid by the intestinal flora of these species. The resulting phenylacetic acid then is absorbed, conjugated with either glycine (non-primates) or glutamine (primates and excreted into the urine (James, *et al.*, 1972). This hypothesis is supported by the absence of phenylacetic acid or its conjugates in rat and rabbit plasma following intravenous  $\beta$ -AP.

### Elimination

Intravenously administered  $\beta$ -AP was cleared rapidly from rat, rabbit and dog plasma with elimination half-lives of 0.55, 0.58 and 0.87 hours, respectively (Table 1). Following the oral administration of  $\beta$ -APM and  $\beta$ -AP to rat, rabbit and dog, the elimination half-lives for  $\beta$ -AP increased to 1.1-2.4 hours. The reason(s) underlying the differences in the elimination half-lives following oral and intravenous dosing are not clear. The  $\beta$ -AP elimination half-lives in the monkey and man were 2-6 and 1.1 hours, respectively.

The plasma elimination half-lives for total radioactivity following intravenous  $\beta$ -AP or oral  $\beta$ -APM and  $\beta$ -AP were protracted in the rat, rabbit, dog, monkey and man ranging from approximately 80-600 hours. The reason the prolonged clearance of total radioactivity is the

incorporation of [ $^{14}\text{C}$ ]-phenylalanine, resulting from the metabolism of  $\beta$ -AP or  $\beta$ -APM, into plasma and tissue proteins. For purposes of comparison, the elimination half-life of [ $^{14}\text{C}$ ]-phenylalanine administered orally to the monkey is approximately 60 hours (Oppermann, *et al.*, 1973).

The major route of excretion of radioactivity in all species was the urinary tract (Table 3). Following oral and intravenous  $\beta$ -AP or  $\beta$ -APM, 21 to 91% of the administered dose of radioactivity was recovered in the urine. This was particularly true for the rabbit which excreted approximately 70% (oral) to 90% (intravenous) of each dose of radioactivity into the urine. The rabbit also excreted less than 1% of an intravenous dose of  $\beta$ -AP and only 5-9% of an oral dose of  $\beta$ -AP or  $\beta$ -APM in the feces. In contrast, the rat, dog, monkey and man excreted lesser amounts (21 to 56%) of radioactivity into the urine. Urinary recoveries of radioactivity following the intravenous (47% of the dose) and oral (37-46% of the dose) administration of  $\beta$ -AP and  $\beta$ -APM were quite similar in the rat. Unlike the rabbit and dog, the rat excreted more radioactivity in the feces following intravenous  $\beta$ -AP, oral  $\beta$ -AP and oral  $\beta$ -APM (6, 39 and 18%, respectively). The dog excreted the least radioactivity into urine (21-27%) and the greatest amount into the feces (10-11%) following oral  $\beta$ -AP and  $\beta$ -APM. In addition, the excretory profiles (urine, feces, and breath) following oral  $\beta$ -APM were very similar in the rat, monkey and man. One possible explanation for the differences in the excretory profiles of the rat and rabbit following oral  $\beta$ -AP and  $\beta$ -APM may be differences in their capabilities to excrete metabolites of  $\beta$ -AP and  $\beta$ -APM into the bile. Another possibility is differences in the extent to which orally-administered radioactivity is absorbed by the two species.

Following the oral or intravenous administration of  $\beta$ -APM and  $\beta$ -AP, the rat, rabbit and monkey excreted approximately 4-10% of the administered radioactivity in their expired breath. The single exception was the intravenously dosed ( $\beta$ -AP) rabbit which excreted less than 1% of the radiolabel in expired air.



## SUMMARY

Regardless of species, virtually no orally administered  $\beta$ -APM reached the systemic circulation with the methyl ester intact. Unlike  $\beta$ -APM, oral  $\beta$ -APM and  $\beta$ -AP are not completely metabolized to their constitutive amino acids prior to absorption.  $\beta$ -AP was absorbed intact with an oral bioavailability of 7-16% in the rat, rabbit, dog, monkey and man. Maximal plasma concentrations of  $\beta$ -AP (0.4-0.8  $\mu\text{g/ml}$ ), which were attained 2-6 hours after oral dosing, were similar in the rat, rabbit and monkey. Higher concentrations (1.6-2.3  $\mu\text{g/ml}$ ) occurred in the dog. Based on the assumption of dose-proportionality, similar  $C_{\text{max}}$ s (calculated to be approximately 1.4  $\mu\text{g/ml}$ ) occurred in man. Maximal plasma concentrations of total radioactivity were achieved 5-8.5 hours after the oral administration of  $\beta$ -APM or  $\beta$ -AP to the rat, rabbit, dog, monkey and man. Peak concentrations of radioactivity, which range from 5.7 to 11.8  $\mu\text{g-equivalents/ml}$ , were highest in the dog (10.8-11.4  $\mu\text{g-equivalents/ml}$ ) and very similar in the rat, rabbit and monkey (5.7-9.2  $\mu\text{g-equivalents/ml}$ ). The extent of absorption of total radioactivity ranges from 65 to 95% of the dose. Plasma concentrations of radioactivity and  $\beta$ -AP in the dog and pregnant rat receiving  $\beta$ -APM in their diet were dose-proportional over a wide range of doses (5.8 to 950 mg/kg in the rat; 250 to 1000 mg/kg in the dog).

Once absorbed by the rat, total radioactivity from an oral dose of  $\beta$ -APM distributed primarily to the liver and kidneys. Peak concentrations of radioactivity occurred in the liver, kidneys and bladder 3-6 hours after dosing. Lowest concentrations of radioactivity occurred in the brain, eye lens of the eye and skeletal muscle. The metabolites of orally-administered  $\beta$ -APM and  $\beta$ -AP in the rat, pregnant rat, rabbit, dog, monkey and man are  $\beta$ -AP, N-acetyl- $\beta$ -AP, phenylalanine, phenylacetic acid and the glycine and glutamine conjugates of phenylacetic acid. The major metabolite found in the rats and rabbits after oral  $\beta$ -AP or  $\beta$ -APM is phenylacetyl-glycine. In the

monkey and man, it is phenylacetylglutamine. An unidentified metabolite has also been found in dog urine. Intravenous  $\beta$ -AP was cleared from rat, rabbit and dog plasma with an elimination half-life of about 0.5-1.6 hours. Total radioactivity was eliminated from rat, rabbit and dog plasma very slowly, however, with an elimination half-life of 80-340 hours. More than likely, the protracted clearance of total radioactivity from the plasma was due to the incorporation of [ $^{14}\text{C}$ ]-phenylalanine, resulting from the metabolism of  $\beta$ -AP or  $\beta$ -APM, into plasma and tissue proteins. Following oral  $\beta$ -APM or  $\beta$ -AP, 21-73% of the dose of radioactivity was excreted through the urinary tract, the major route of excretion for all species. In the rat, rabbit and monkey, approximately 5-10% of the radioactive dose was excreted in expired air. The remainder of the radioactivity was excreted in the feces. The rabbit, dog, monkey and man excreted 5-11% of the dose of radioactivity in the feces; the rat excreted 20-40% of radioactive dose into the feces.

## REFERENCES

- Bender DA (1985) Metabolic and pharmacological studies. In: Barrett GC (Ed) Chemistry and Biochemistry of the Amino Acids, Chapman and Hill, New York, NY pp 139-196
- Burton EG, Schoenhard GL, Hill JA, Schmidt RE, Hribar JD, Kotsonis FN, Oppermann JA (1989) Identification of N- $\beta$ -L-Aspartyl-L-Phenylalanine as a normal constituent of human plasma and urine. J Nutr 119: 713-721
- Gibaldi M, Perrier D (1982) Appendix D: Estimation of areas. In: Gibaldi M, Perrier D (eds) Pharmacokinetics, 2nd Edition, Marcel Dekker, Inc., New York, pp 445-449
- Harper AE (1984) Phenylalanine metabolism. In: Stegink LD, Filer LJ Jr. (eds) Aspartame: Physiology and Biochemistry, Marcel Dekker, Inc., New York, NY, pp 77-109
- James MO, Smith RL, Williams RD, Reidenberg M (1972) The conjugation of phenylacetic acid in man, sub-human primates and some non-primate species. Proc R Soc Lond B 182: 25-35
- Lawrence JF, Iyengar JR (1987) Liquid chromatographic determination of  $\beta$ -aspartame in soft drinks, beverage powders and pudding mixes. J Chromatogr 404: 261-266
- Matsuzawa Y, OHara Y (1984) Tissue distribution of orally administered isotopically labelled aspartame in the rat. In: Stegink LD, Filer LJ Jr. (eds) Aspartame: Physiology and Biochemistry, Marcel Dekker, Inc., New York, NY, pp 161-199
- Metzler CM, Elfring GK, McEwen AJ (1974) A package of computer programs for the pharmacokinetic modeling. Biometrics 30: 562-563
- Oppermann JA, Muldoon E, Ranney RE (1973) Metabolism of aspartame in monkeys. J Nutr 103: 1454-1459
- Oppermann JA (1984) Aspartame metabolism in animals. In: Stegink LD, Filer LJ Jr. (eds) Aspartame: Physiology and Biochemistry, Marcel Dekker, Inc., New York, NY, pp 141-159
- Ranney RE, Oppermann JA (1979) A review of the metabolism of the aspartyl moiety of aspartame in experimental animals and man. J Environ Pathol Toxicol 2: 979-985
- Sedman AJ, Wagner JG (1976) CSTRIP: A Fortran IV computer program for obtaining initial polyexponential parameter estimates J Pharm Sci 65:1006-1010

**Witt J (1986) Stability of aspartame and its conversion products in wet beverage systems.  
International Aspartame Workshop Proceedings, Marbella, Spain, Nov 17-21, 1986,  
International Life Sciences Institute, Nutrition Foundation, Washington, D.C.**

**FIGURE 1:  
STRUCTURES AND PROPOSED METABOLIC PATHWAY**

ASPARTIC ACID IS A HYPOTHETICAL METABOLITE  
WHICH WAS NOT IDENTIFIED IN THIS STUDY.

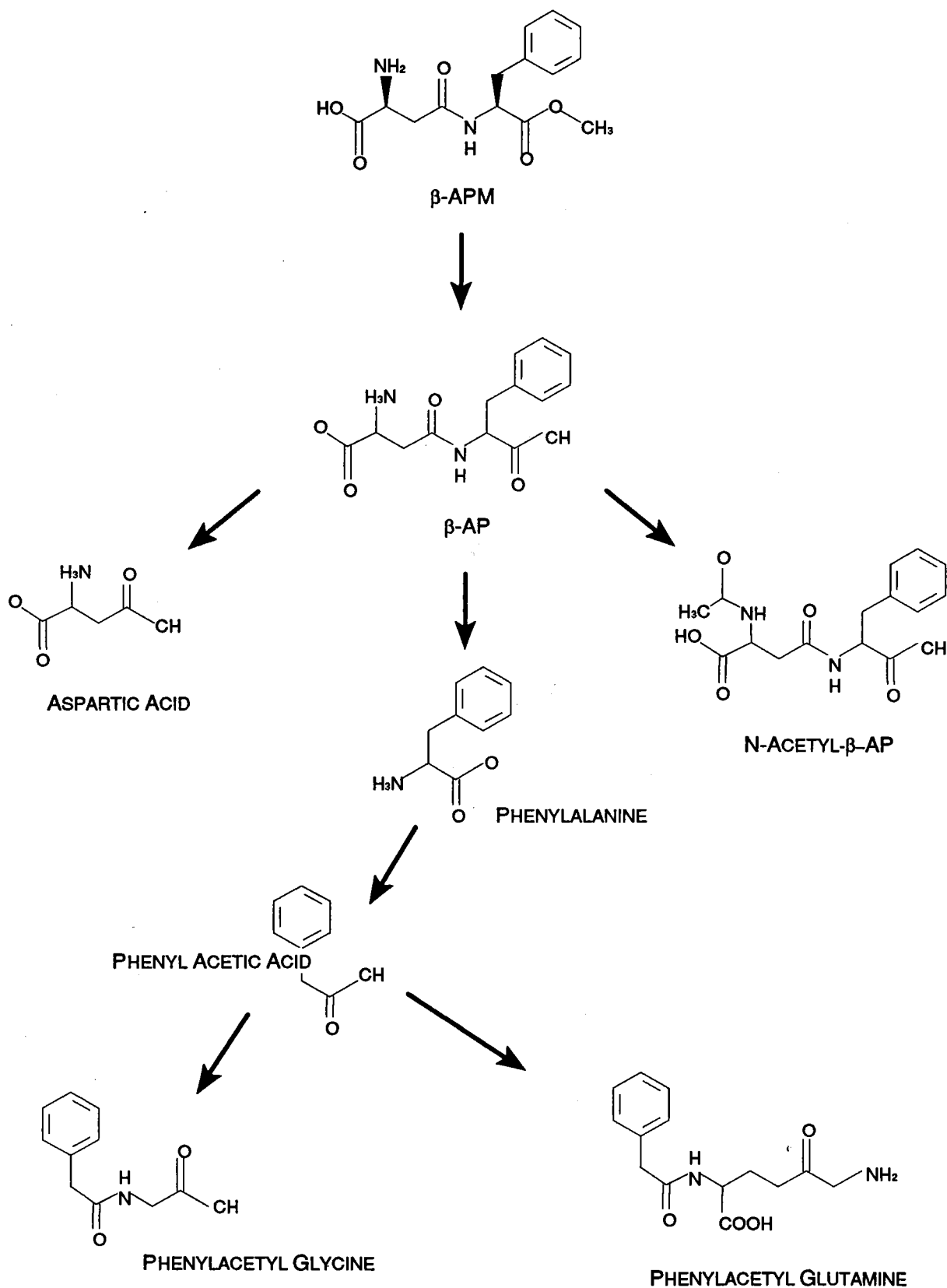


Table 1

Pharmacokinetic Parameters for Total Radioactivity and Beta-AP  
in Rats, Rabbits, Dogs, Monkeys and Men Receiving Oral or Intravenous Doses of  
Beta-AP and Beta-APM

Route of Administration	Total Radioactivity				Beta-AP		
	Tmax (hours)	Cmax ( $\mu\text{g-equiv.}$ ) ml	t 1/2 <sup>b</sup> (hours)	% of Dose Absorbed <sup>c</sup>	Tmax (hours)	Cmax ( $\mu\text{g-equiv.}$ ) ml	Bioavailability (% of Dose) <sup>d</sup>
<b>I. Rat</b>							
Beta-AP i.v.	0	112	113	-	0	104	-
Beta-AP p.o.	7	8.3	118	65.2	2	0.73	8.4
Beta-APM p.o.	7.5	9.2	85	87.9	2	0.69	7.2
<b>II. Rabbit</b>							
Beta-AP i.v.	0	48.6	77	-	0	42.4	-
Beta-AP p.o.	5	6.1	184	95.3	6	0.66	7.7 - 22.6
Beta-APM p.o.	5	5.9	148	92.2	2	0.38	7.8 - 8.5
<b>III. Dog</b>							
Beta-AP i.v.	0	69.6	55.9	-	0	65.0	-
Beta-AP p.o.	8.5	11.8	317	92	2.0	2.34	15.7
Beta-APM p.o.	8.5	10.4	338	91	1.5	1.56	8.5
<b>IV. Monkey</b>							
Beta-APM p.o.	7.3	5.7	>90	95	4	0.62	2 - 6
<b>V. Man</b>							
Beta-APM 32 mg p.o.	5.5	0.45	587	90	1.5	0.07	1.1
							7.0

<sup>a</sup> All animals, except man, received 10 mg/kg doses of Beta-APM or Beta-AP. Humans received single, oral 31.9 mg doses of Beta-APM.

<sup>b</sup> Elimination half-life.

<sup>c</sup> Calculated from fecal excretory data.

<sup>d</sup> Calculated from urinary recovery data and/or by comparison of oral and intravenous areas under the plasma concentration-time curves.  
mpa/141

Table 2

Urinary Metabolic Profiles of the Rat, Rabbit, Dog, Monkey and Men  
Receiving Oral or Intravenous Doses of Beta-AP or Beta-APM<sup>a,b</sup>

Metabolite <sup>a</sup>	Rat		Rabbit		Monkey	
	Beta-AP i.v.	Beta-AP p.o.	Beta-AP i.v.	Beta-AP p.o.	Beta-APM p.o.	Beta-APM p.o.
Beta-AP	31%	<1%	67%	2%	2.1%	1.7%
N-Ac-Beta-AP	6%	<1%	17%	4.5%	4.5%	5.9%
Phenylalanine	-	-	-	-	-	0.8%
Phenylacetylglutamine	3%	32%	2%	61%	58%	-
Phenylacetylglutamine	-	-	-	-	-	41%
Metabolite A	-	-	-	-	-	-

	Dog		Man	
	Beta-AP i.v.	Beta-AP p.o.	Beta-APM 32 mg p.o.	Beta-APM 32 mg p.o.
Beta-AP	70%	16%	14%	7.0%
N-Ac-Beta-AP	-	-	-	-
Phenylalanine	-	-	-	-
Phenylacetylglutamine	0.03%	1.11%	0.58%	-
Phenylacetylglutamine	-	-	-	31%
Metabolite A	0.65%	5.5%	5.1%	-

<sup>a</sup>Values are the percent of the dose excreted as Beta-AP, N-acetyl-Beta-AP, Phenylalanine, Phenylacetylglutamine, Phenylacetylglutamine or Metabolite A.

<sup>b</sup>All animals, except man, received 10 mg/kg doses of Beta-APM or Beta-AP. Humans received single, oral 31.9 mg doses Beta-APM.

Table 3

Urinary, Fecal and Respiratory Excretion of Radioactivity  
by Rats, Rabbits, Dogs, Monkeys and Men Receiving Oral or Intravenous Doses of  
Beta-AP or Beta-APM<sup>a,b</sup>

	Route of Administration	Urine	Feces	Breath	Total
I. Rat					
Beta-AP	i.v.	47%	6.1%	9.5%	62.6%
Beta-AP	p.o.	37%	39%	5.2%	81.2%
Beta-APM	p.o.	46%	18%	5.8%	69.8%
II. Rabbit					
Beta-AP	i.v.	91%	0.6%	0.6%	92.2%
Beta-AP	p.o.	73%	5.3%	4.3%	82.6%
Beta-APM	p.o.	67%	8.4%	4.1%	79.5%
III. Dog					
Beta-AP	i.v.	72.2%	2.9	N.D. <sup>c</sup>	75.1%
Beta-AP	p.o.	27.4%	10.2%	N.D.	37.6%
Beta-APM	p.o.	21.1%	11.2%	N.D.	32.3%
IV. Monkey					
Beta-APM	p.o.	56%	4.6%	4.8%	65.6%
V. Man					
Beta-APM	32 mg p.o.	42%	9.6%	N.D.	51.6%

<sup>a</sup> Values are the percent of the dose.

<sup>b</sup> All animals, except man, received 10 mg/kg doses of Beta-APM or Beta-AP. Humans received single, oral 31.9 mg doses of Beta-APM.

<sup>c</sup> Not determined.

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