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COMMITTEE ON TOXICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

DIKETOPIPERAZINE DERIVATIVE OF ASPARTAME (DKP)

Introduction

1. The Committee on Toxicity has been reviewing aspartame at the request of the Food Advisory Committee, following MAFF's survey on sweetener intake in 1989. This review has considered a number of areas including new toxicological data generated since the 1982 COT Report and a submission from Dr Millstone regarding the validity of the old toxicity studies on aspartame carried out by Searle. As a result of this review an ADI has been set for aspartame of 40mg/kg bw/day and a draft statement has been produced on the conclusions to date.

2. Aspartame can breakdown to form a diketopiperazine derivative (DKP), particularly in aqueous applications, and therefore the COT is also being asked to review the available data on DKP. This is particularly relevant at this time as new uses are being proposed for aspartame, which could give rise to products with higher levels of DKP than have been seen to date, as well as extending the range of products in which aspartame could be present. These proposed new uses include the adoption of microencapsulation techniques so as to enable aspartame to be used in baked goods. The COT is now being asked to review the data available on DKP and to consider whether it should set an ADI for DKP (as well as for aspartame) and if so, what the ADI should be.

3. In the 1982 Report the COT considered the data then available on DKP, including metabolism studies, short and long term studies in the mouse and the rat on DKP alone, a long term study in the rat on a combination of aspartame and DKP (3:1), mutagenicity studies, teratology and reproduction studies, and pharmacology studies. The relevant section of the 1982 COT Report is attached as Annex 1 for information. Industry was made aware that the COT was reviewing the data on aspartame and DKP and the Secretariat has received a submission from NutraSweet summarising the data available on DKP - see Annex 2. Data on potential levels of DKP in various foods sweetened with aspartame and dietary intake estimates have been prepared by MAFF -see Annex 3.

NUTRASWEET SUMMARY

4. The summary cites no new toxicity studies carried out since the publication of the COT Report in 1982. However new data are presented on:

- the natural occurrence of other diketopiperazine compounds in a range of food items
- the metabolism and excretion of DKP
- intake calculations for DKP

Toxicity studies

5. No studies other than those seen previously by the COT in its 1982 review have been made available. Both JECFA and the SCF have set an ADI for DKP of 7.5 mg/kg bw/day, based on a no-effect-level of 750 mg/kg bw/day in the 2-year rat study using DKP alone, in which an increase in benign uterine polyps was observed at the mid and high dose levels of 1,500 and 3,000 mg/kg bw/day. However a Japanese study incorporating a single dose level of 4,000 mg/kg bw/day of a mixture of aspartame and DKP (3:1) showed no such increase in uterine polyps thus giving a no-effect-level for DKP of 1,000 mg/kg bw/day. The US-FDA has stated that in the long term rat study on DKP alone, the uterine polyps seen at the 1,500 mg/kg bw/day level were spontaneous lesions and that those seen at the 3,000 mg/kg bw/day level may have been a non-specific effect of the large dose of DKP administered. Thus the FDA set an ADI of 30 mg/kg bw/day based on a no-effect-level of 3,000 mg/kg bw/day in this study. Lower no-effect-levels established in other studies were not used to set an ADI as they were the result of a lack of any effects at any of the dose levels used in those studies and thus represent only the highest dose tested.

Occurrence of other DKP derivatives in various foods

6. It has been shown that a variety of cyclic dipeptide derivatives can be found in many protein-rich foods at levels up to 100 mg/kg and that many of these dipeptides contain phenylalanine. Thus the diketopiperazine derivative of aspartame is not unique and many similar compounds are likely to be ingested in the average diet (see introduction to the NutraSweet Submission).

Metabolism and excretion of DKP

7. Data provided to the COT for the 1982 Report showed that DKP was poorly absorbed, that it was not biotransformed by mammalian enzyme systems and that it was rapidly excreted via the urine. Approximately only 4% of an orally administered dose was absorbed intact in man, whereas approximately 50% was converted in the gastrointestinal tract to aspartic acid and phenylalanine. It was assumed that this conversion was by the gut microflora as it did not occur in germ-free rats (Ranney, 1972 and 1974).

8. New data on the metabolism and excretion of DKP following ingestion of aspartame or DKP, which have not previously been seen by the COT, are presented in the NutraSweet summary. The studies by Stegink and co-workers are summarised in greater detail by the Secretariat in Annex 4 to this paper.

9. Aspartame was shown to be hydrolysed to its constituent amino acids prior to entering the portal blood in pigs, with aspartyl-phenylalanine an important intermediary. DKP dosing had no effect on portal blood amino acids indicating that in this species it was not metabolised to its constituent amino acids within the gut. Following administration of 200 mg/kg bw aspartame (containing 1.1% or 2.2 mg/kg bw DKP) to 6 normal human adults, DKP concentrations in plasma were below the limit of detection of 1μg/l. Total urinary excretion of DKP in the 24 hours after dosing amounted to 4.8% of the dose of DKP administered, with some

44% of this being excreted in the first 4 hours.

10. Work by Stegink and co-workers in both normal subjects and in those heterozygous for PKU indicated that repeated administration of aspartame (hourly for 8 hours) produced elevations in plasma phenylalanine and tyrosine concentrations that were within, or only just above, normal post-prandial limits. Plasma DKP concentrations were generally below the limit of detection following the administration of aspartame or placebo. However DKP was detected in some subjects during the placebo phase of the study, indicating that the DKP that is formed from aspartame is also a naturally occurring dietary and/or endogenous substance. Administration of DKP itself resulted in a small rise in plasma DKP concentrations and some 5% of the total dose of DKP was excreted in the urine within 24 hours of dosing.

Intake of DKP

11. NutraSweet has submitted data indicating that levels of DKP found in foods currently sweetened with aspartame are in the range of less than 0.3% up to 14% of the initial aspartame concentration. Thus, using the maximum levels of aspartame given in the proposed EC Directive on Sweeteners, non-alcoholic drinks (sweetened with 600 mg/l aspartame) may contain approximately 60 mg/l DKP and desserts (sweetened with 1,000 mg/kg aspartame) may contain approximately 100 mg/kg DKP.

12. Annex 3 contains data from MAFF including calculations of existing intakes of DKP in various subgroups of the population. The development of an encapsulation technique to allow aspartame to be used in baked goods could increase DKP intake by 0.4 and 0.5 mg/kg bw/day in children and adults respectively. This could lead to a total maximum intake of 2.1 and 1.6 mg/kg bw/day in children and adults respectively. However this is still well below a possible ADI figure of 7.5 or 10 mg/kg bw/day.

Discussion

13. There are no new toxicological data on DKP that have not already been seen by the COT at its previous review. However new data are available which indicate that the DKP that is formed from aspartame may also be a normal dietary constituent and/or endogenous substance. Furthermore evidence is now available to show that in both normal subjects and in those heterozygous for PKU, DKP is very poorly absorbed after oral administration and that material that is absorbed is excreted unchanged in the urine. Even after repeated administration of aspartame (hourly for 8 hors) DKP was not detectable in plasma.

Conclusions

14. The Committee will wish to consider the following:-

(i) whether it is possible to conclude that there are no particular health concerns arising from the levels of DKP that are found in foods as a result of the current range of uses of aspartame;

(ii) whether the possible extension of the range of products in which aspartame could be used has any implicatins for human health;

(iii) whether it should set an ADI for DKP, as well as that already set for aspartame of 40 mg/kg bw/day, and if so, what the ADI should be.

Secretariat
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13. GROUP A

i. Aspartame

Extensive data have been submitted to support the safety-in-use of aspartame including data on metabolism, short- and long-term toxicity, carcinogenicity, mutagenicity and reproduction studies. In addition it is known that, on storage and in certain foods, aspartame breaks down to a diketopiperazine derivative (DKP) by hydrolysis and cyclisation. Some food sweetened with aspartame might contain DKP at levels up to 5% of the amount of aspartame added; the DKP derivative has also been subjected to extensive toxicological testing.

In general the data were satisfactory although the results of some long-term studies in the rat with aspartame and its DKP derivative did initially give some cause for concern. In a study with the DKP derivative there appeared to be a treatment-related increase in the incidence of uterine endometrial polyps. However following re-evaluation of the histological material by a group of independent pathologists it was concluded that the polyps were non-neoplastic in nature being formed during the natural ageing process in the rat and that the observed incidence was consistent with the spontaneous incidence for the strain of rats used.

A recent long-term rat study with aspartame alone and together with its DKP derivative (3:1), at levels of up to 10% of the diet has shown dose-related increases in urinary calcium levels and mineralisation of the renal pelvis, with females being more affected than males. Although information on the mineral levels in the basic diet fed to the animals is not available, we consider it probable that the levels of calcium and phosphorus exceeded those recommended[a]. It is clear from other studies that the inclusion in diets of high concentrations of substances (eg lactose, or chemically modified starches[b]) that are not readily broken down to easily absorbable derivatives tends to enhance calcium absorption and urinary excretion. We regard the occurrence of pelvic nephrocalcinosis in rats in such circumstances as being mainly a laboratory artefact, attributable to excessive intakes of calcium, phosphorus and the test material and we do not regard it as predictive of toxic risk for man.

The results of one long-term rat study with aspartame were consistent with an increased incidence of intracranial neoplasms in the treated animals; however, the increase was not dose- or sex-related and the overall incidence was within the range previously encountered in untreated animals of the same strain. Furthermore no such increase was seen in two subsequent long-term studies with aspartame, one incorporating *in utero* exposure. Following detailed consideration of these data we are of the opinion that the lesions were not associated with the dietary administration of aspartame. We have also considered the possibility that ingestion of aspartame, alone or together with glutamate, may contribute to mental retardation, brain damage or undesirable effects on neuroendocrine regulatory systems. It is pertinent to note that studies have indicated that the metabolism of aspartame in man is similar to that of phenylalanine and aspartic acid, and that studies in man, involving adults and children, both normal subjects and those heterozygous for phenylketonuria have indicated no untoward effects at levels up to one order of magnitude greater than those anticipated from the intake of the sweetener in a normal diet.

Following detailed consideration of all the toxicological data we see no objection to the use of aspartame in food. However if the technological limitations on the use of aspartame in aqueous solutions should be overcome then the situation would need to be reviewed. (References 10–23).

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Annex 2 to
TOX/91/39

REVIEW OF ASPARTYL PHENYLALANINE DIKETOPIPERAZINE (DKP)

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REVIEW OF ASPARTYL PHENYLALANINE DIKETOPIPERAZINE (DKP)

I. EXECUTIVE SUMMARY

Aspartame, like other dipeptides in foods, can cyclize to form a 2,5-dioxopiperazine or diketopiperazine. The specific cyclized product of aspartame is aspartyl phenylalanine diketopiperazine (DKP). The 2,5-dioxopiperazines are the most ubiquitous peptide derivatives found in nature. They are found in most protein-rich foods such as cocoa, cheese, protein and casein hydrolysates, and in roasted malts used in brewing.

DKP has undergone extensive safety testing including in vitro genotoxicity studies; acute, subchronic and chronic toxicity and oncogenicity studies; reproduction and teratology studies; and pharmacology and metabolism studies. These studies indicate that DKP does not produce adverse effects, even at doses several orders of magnitude greater than actual human exposure.

The Joint Expert Committee on Food Additives (JECFA) and the Scientific Committee for Foods (SCF) established a No Observable Effect Level (NOEL) of 750 mg DKP/kg/day and an Acceptable Daily Intake (ADI) of 7.5 mg/kg/day. The U.S. Food and Drug Administration (FDA) established a NOEL of 3,000 mg/kg/day and thus an ADI of 30 mg/kg/day.

The projected consumption rate of DKP from all aspartame-sweetened foods at the 90th percentile, 14-day average (all ages, users only) is 0.56 mg/kg bw/day. This value is 15-50 times less than the existing ADIs.

Thus, there are no safety or consumption rate concerns associated with the ingestion of DKP in aspartame-sweetened foods.

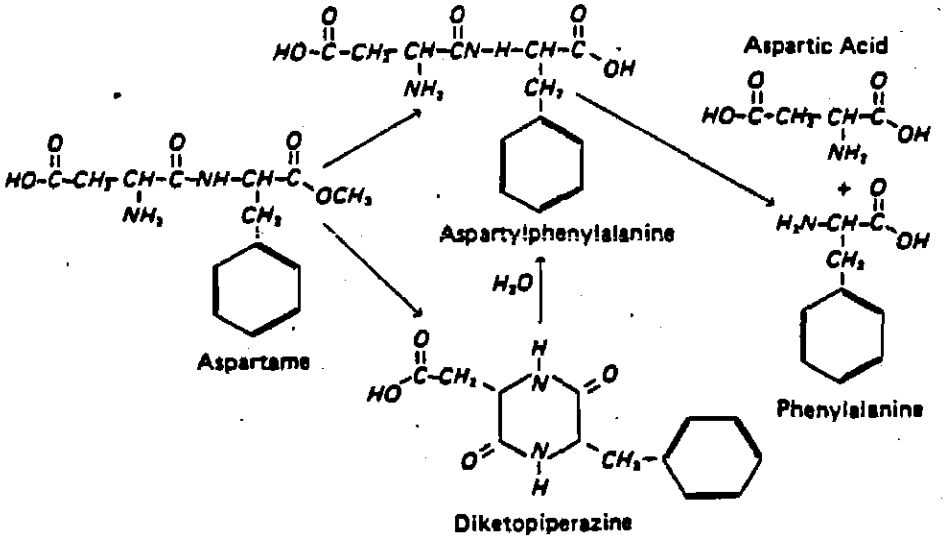
II. INTRODUCTION

The 2,5-dioxopiperazines (or diketopiperazines) are the most ubiquitous peptide derivatives found in nature. The cyclic dipeptide derivatives were described as early as 1849 (Bopp, 1849) and are found in most protein-rich foods such as cocoa, cheese, protein and casein hydrolysates, and in roasted malts used in brewing. For example, 10 cyclic dipeptide derivatives have been identified in cocoa powder alone (Van Der Greef et al, 1987). Moreover, both simple and highly modified cyclic dipeptide derivatives have been isolated from microbial cultures and certain derivatives have been isolated from mammalian tissues and fluids (Sammes et al, 1975; Peterkofsky et al, 1982; Prasad, 1989). The simple cyclic dipeptide derivatives are formed by a non-enzymatic cyclization of dipeptides and are generally stable at 37°C, apparently not degraded by tissue enzymes, and excreted in the urine. The vast majority of cyclic dipeptides are physiologically inert. Only histidyl proline diketopiperazine, a presumed catabolic product of the peptide hormone TRH, has been found to be physiologically active (Peterkofsky et al, 1982; Prasad, 1989).

Numerous studies have identified phenylalanine containing cyclic dipeptides in natural products or processed protein (Sammes et al, 1975). A phenylalanine containing cyclic dipeptide, aspartyl phenylalanine diketopiperazine (DKP), is also formed from aspartame (APM). The following is a review of the extensive safety studies done with DKP and the regulatory status and consumption rates of DKP.

III. OCCURRENCE IN ASPARTAME-CONTAINING PRODUCTS

The rate of DKP formation from aspartame is dependent upon the chemistry of the food system and the conditions of food storage. DKP formation is dependent on pH, temperature, and time. Generally, the rate of DKP formation is faster at a higher pH (Homler, 1984; Gaines and Bada, 1987, 1988). The cyclization of aspartame to DKP is illustrated below.



The amount of DKP in aspartame-containing products has been determined for several food categories (e.g. beverages, tabletop sweetener, baked goods, confections, frozen dessert, yogurt) using actual and prototype products. DKP levels in food categories, discussed in Section VII, ranged from <0.3 to 14% of the initial aspartame concentration. The DKP levels that were obtained from studies at The NutraSweet Company are consistent with those reported and/or

predicted by others (Gaines and Bada, 1988; Graves and Luo, 1987; Hayakawa et al, 1990; Neirynck and Nollet, 1988; Prudel et al, 1986; Saito et al, 1989; Tuncel and Araman, 1989; Tsang et al, 1985).

IV. PHARMACOLOGY AND METABOLISM

Pharmacology Studies

A series of studies, indicated in Table 1, were done to determine potential pharmacological effects of DKP (Cook, 1972; Nutting, 1972). Studies included examination of the effects of DKP on appetite in rats, gastric juice volume, acidity or proteolytic activity, gastric ulceration, blood pressure, heart rate, pressor response to angiotensin, nicotine-induced arrhythmias, and blood coagulation. In addition, a number of studies were done to determine whether DKP had anticonvulsant, analgesic, anticholinergic, or analgesic activity. Other pharmacological screening tests included those to assess diuretic activity, blood glucose, antihistamine activity, and ganglionic blocking activity. The effects of DKP on various hormonally dependent target tissues were also evaluated to determine whether DKP had estrogenic, progestational, androgenic, myotrophic, or glucocorticoid activity or antagonizes these activities. Finally, the potential anti-inflammatory and immunosuppressive activity of DKP were assessed. There were no meaningful adverse effects of DKP in these studies.

Recently, Kreutz and colleagues (1990) reported that a direct injection of DKP into rat brain apparently gives rise to a slight, transient increase in brain dopamine release. Results from exposure in this manner are not directly relevant to assessing the safety of food additives. These investigators did not observe

effects when DKP was administered orally (Kaakkala and Wurtman, 1991, personal communication). Thus, the results of the pharmacology studies and the safety studies, discussed below, indicate that DKP does not cause any meaningful effects on the gastrointestinal, endocrine, immune, cardiovascular and central nervous systems.

Metabolism Studies

Studies with animals and humans indicate that DKP is poorly absorbed, not biotransformed by mammalian enzymes and rapidly eliminated in the urine (Ranney, 1972). Based on urinary excretion data, only approximately 4% of an orally administered dose is absorbed intact in humans. Approximately 50% of the oral dose in humans is converted in the gut to aspartic acid and phenylalanine. This conversion is thought to occur by gut microbes because little or no metabolism of DKP occurred following oral administration to germ-free rats (Ranney, 1974).

Recently, Cho et al (1987) determined DKP plasma and urine levels in humans given 2.2 mg DKP/kg bw orally. No DKP was detected in plasma (i.e., <1 μg/ml). The estimated 90th percentile, 14-day average, all ages, users only, consumption rate of DKP is 0.56 mg/kg bw/day (see Section VII). Thus, when a single oral dose is given that is four times the estimated 90th percentile consumption rate, there are no detectable plasma levels of DKP in humans.

Two other human studies were done to determine whether repeated ingestion of an unsweetened DKP-containing beverage would result in accumulation of DKP in plasma. Because DKP is metabolized by gut microbes to phenylalanine, both

normal human subjects (Study N02-84-02-075) and phenylketonuria heterozygotes (Study N12-85-02-054) were studies. Subjects were given approximately 2 mg DKP/kg bw each hour for eight hours (i.e., approximately 16 mg/kg bw over seven hours). As was observed in the acute single dose study above, urinary excretion accounted for only 2-5% of the total dose. Furthermore, plasma DKP concentrations reached a plateau after four doses.

V. SAFETY STUDIES

Short-term Tests

The genotoxic potential of DKP was evaluated in the Ames test (SA1378 and SA1384), the Host-Mediated assay in mice (PT1095S73) and rats (PT1029H72), the Dominant Lethal Mutation assay in rats (PT1008S72) and an in vivo Cytogenetics assay in rats (PT1027H72). DKP was not mutagenic in the Ames test and was negative in the in vivo studies at dosages up to 8 g/kg bw/day.

Acute and Subchronic Toxicity Studies

Acute toxicity was examined in mice, rats and rabbits given doses up to 5,000 mg DKP/kg bw by gavage (SA2479). No deaths or signs of toxicity were observed within seven days of treatment. A gavage two week mouse study (PT0885S70) and gavage two week (PT0884S70) and dietary five week rat studies (PT972S71) were also done. No adverse effects due to DKP were observed in mice or rats at the highest doses given of 1 or 6 g/kg bw/day, respectively.

Chronic Toxicity and Carcinogenicity Studies

The carcinogenicity of DKP was determined in two-year dietary studies in the mouse (PT985H73) and rat (PT988S73). In addition, a rat chronic toxicity/carcinogenicity study was done with APM and a 3:1 mixture of APM and DKP in the diet (Ishii et al, 1981 and Ishii, 1981). In the mouse study, DKP was given at doses of 0, 0.25, 0.5, and 1.0 g/kg bw/day. In the rat study, DKP was given at doses of 0, 0.75, 1.5, and 3.0 g/kg bw/day. In the study by Ishii et al (1981), APM was given at doses of 2 and 4 g/kg bw/day and APM-DKP (3:1) was given at a dose of 4 g mixture/kg bw/day (i.e., 1 g DKP/kg bw/day). Routine evaluations in these studies included body weights, food consumption, appearance and behavior, clinical pathology, opthalmic examinations, organ weights and gross and microscopic pathology of all major organs and tissues.

There were no meaningful changes in behavior or survival in any of the above studies. In the mouse study, no meaningful changes occurred in body weights, food consumption or clinical laboratory data; and no treatment-related gross or microscopic findings were observed. In the rat study with DKP alone, consistent decreases in mean body weights compared to control values occurred only in the 3 g/kg bw/day group. The effect on mean body weights was probably due to caloric dilution of the diet by DKP since its concentration ranged from 4.1 to 7.7% of the diet at weeks 13 and 52. Slight decreases in serum total cholesterol, that were not considered meaningful, were observed in the rat study with DKP alone at 3 g/kg bw/day and in the rat study with APM-DKP at 4 g mixture/kg bw/day. In the rat study with DKP alone (PT988S73), a slight but statistically significant increase in uterine endometrial polyps was also observed in the 1.5 and 3 g/kg bw/day dose groups. No other treatment-related gross or

microscopic findings were observed in either the rat study with DKP alone or the rat study with APM-DKP mixture. Detailed pathologic evaluations established that the uterine polyps were localized normal benign endometrial hyperplasia that would not become malignant (see discussion in Section VI). Furthermore, the incidence of polyps in the mid- and high-dose groups was within the range of the spontaneous incidence of this lesion in various rat strains (Tarone et al, 1981; Goodman et al, 1979; Takaki et al, 1989; Rao et al, 1990; Charles River, 1985). Moreover, the incidence of uterine polyps was not increased in either the rat study with APM-DKP mixture (Ishii et al, 1981) or the mouse study with DKP (PT985H73) at the highest doses of DKP given of 1 g DKP/kg bw/day.

In addition to the above carcinogenicity studies, 26 and 56 week studies were done in mice to assess the effects of urinary bladder implants of DKP (PT1032ot72 and PT1034ot73, respectively). There was no significant increase in bladder neoplasia.

In conclusion, DKP is neither carcinogenic nor toxic in mice or rats at doses of at least 1 and 3 g DKP/kg bw/day, respectively.

Reproduction and Teratology Studies

A Segment I diet admix study (PT996S72) was done in rats to assess the effects of DKP on mating, fertility, gestation, lactation, and on early and late stages of fetal development. DKP was given in the diet (except during the mating period when it was given by gavage) at intended dosages of 0.5, 1.0, and 2.0 g/kg bw/day to both sexes before and during mating and to females during gestation and lactation. Treatment with DKP had no effect on parental survival, food

consumption, mating performance, and fertility rates or paternal body weight. There were intermittent decreases in maternal body weights at the high dose during gestation and lactation. There were no effects on survival or any other signs of toxicity in the offspring. Thus, there were no adverse effects on reproduction, no fetal malformations, and no effects on neonatal growth and survival at dosages of at least 2 g/kg bw/day.

Two Segment II teratology studies were done in rats with DKP. In one study, DKP was given in the diet at doses of 0.5, 1.0 and 2.0 g/kg bw/day (PT997S72). There were no effects on maternal food consumption or body weight and no embryotoxic or teratogenic effects. In the second study, APM-DKP mixture (3:1 ratio) was given in the diet at doses of 1, 2 and 3 g of mixture/kg bw/day (PT1001H72). Thus, doses of DKP were 0.25, 0.50 and 0.75 g/kg bw/day. There were no effects on maternal food consumption or body weight and no embryotoxic or teratogenic effects. The results of the two rat teratology studies indicate that DKP is neither embryotoxic nor teratogenic at doses of at least 2 g/kg bw/day.

Two Segment II teratology studies were also done in rabbits with DKP. In one study, DKP was administered by intubation at doses of 0.5, 1.0 and 2.0 g/kg bw/day (PT 1003H72). The vehicle or test article suspensions were administered twice daily separated by four hours. Daily dosing volumes were 20, 5, 10 and 20 ml/kg bw for the control, low-dose, mid-dose and high-dose groups, respectively. There were no effects on maternal food consumption or body weights and no embryotoxic or teratogenic effects in low- and mid-dose rabbits. The incidence of deaths in the high-dose rabbits precluded any meaningful interpretation of

results. Deaths were observed in all groups: control (6/21), low-dose (4/21), mid dose (6/22) and high-dose (19/21). The deaths were not considered DKP related. They were generally accompanied by necropsy findings indicative of intubation error or trauma (i.e., respiratory complications and infections, gastric bolus and gastric perforations). Marked decreases in mean food consumption (i.e., compared to control and pretreatment values) and body weights were also observed in high-dose rabbits. The marked reduction in maternal food consumption was probably caused by forced intubation of large volumes of suspension. The results of this study indicate that DKP is neither embryotoxic nor teratogenic in rabbits at doses of at least 1 g/kg bw/day.

A Segment II rabbit study was also done by gavage with an APM-DKP mixture (3:1 ratio) at doses of 1, 2 and 3 g of mixture/kg bw/day (PT1002H72). Thus, DKP doses were 0.25, 0.50 and 0.75 g/kg bw/day. The vehicle or mixture suspensions were administered as divided doses separated by four hours. Control, low-, mid- and high-dose rabbits received 20, 6.6, 13.3 and 20 ml/kg bw/day, respectively. There were no embryotoxic or teratogenic effects. Deaths, which were not dose dependent, were observed in all groups including the control group. Many deaths were accompanied by clinical signs of respiratory distress (e.g., labored respiration, wheezing) and/or necropsy findings of respiratory complications and infections. Mid- and high-dose rabbits also had marked decreases in maternal food consumption (i.e., compared to control and/or pretreatment values) and decreases in maternal and fetal body weight. In the above Segment II rabbit study done with DKP alone (PT1003H72) and a Segment II rabbit study done with APM (1201), forced administration of large volumes of dosing suspension twice daily to rabbits also severely reduced maternal food intake. Reductions in

maternal food intake have been reported to cause reduced fetal body weight, malformations and abortions (Matsuzawa et al, 1981; Clark et al, 1986). Moreover, in the Segment II rabbit study done with DKP alone, at doses where there was no effect on maternal food consumption (i.e., 0.5 and 1.0 g DKP/kg bw/day), there were also no effects on maternal or fetal body weights. Therefore, the decreased maternal and fetal body weights observed were most likely caused by decreases in maternal food consumption. In summary, the results of the two rabbit teratology studies indicate that DKP is neither embryotoxic nor teratogenic at doses of at least 1 g DKP/kg bw/day.

The potential for DKP to cause embryotoxic and teratogenic effects was also assessed by Lederer et al (1985). In this study, rats were given DKP in the diet at concentrations of 0.3, 1.0, and 3.0% (i.e., doses of approximately 0.2, 0.8, and 2.3 g/kg bw/day) immediately after mating through day 20 of gestation when pups were delivered by caesarian section. The authors concluded that there was a decrease in the total number of implantations in the 1 and 3% DKP dose groups; however, the data were not adjusted by the authors to reflect the number of dams evaluated (i.e., the 1 and 3% DKP dose groups were smaller). In fact, when adjusted for the number of dams, the number of implantations/dam and the number of embryos/dam were not affected. Thus, there were no effects on maternal body weight and no evidence of embryotoxicity or teratogenicity in this study. These results further demonstrate that doses of approximately 2 g DKP/kg bw/day are neither embryotoxic nor teratogenic.

A Segment III rat study (PT1011H72) was done to evaluate the effects of DKP on parturition and perinatal and postnatal growth and survival. DKP-containing diets were provided from gestation day 14 through weaning of the offspring. Maternal doses were approximately 0.67, 1.3, and 2.5 g/kg bw/day. DKP did not affect maternal appearance, behavior, body weight or body weight gain. Furthermore, there were no effects on gestation indices, litter size, number of live births, or offspring appearance, behavior, body weight or growth. Thus, doses of DKP of at least 2.5 g/kg bw/day did not affect parturition or perinatal and postnatal growth and survival.

In summary, results from Segment I, Segment II and Segment III studies indicate that DKP does not influence reproduction or produce teratogenic or embryotoxic effects at doses of at least 1 to 2.5 g DKP/kg bw/day.

Clinical Studies

DKP has been given to humans as a single dose of 2.2 mg/kg bw and as repeated doses of approximately 2 mg/kg bw every hour for eight hours (see metabolism section). There were no adverse effects in these studies. In addition, since DKP is a minor component of aspartame, it has also been evaluated in numerous clinical studies done with aspartame. For example, the aspartame used in a 6 month study done with aspartame at 75 mg/kg bw/day (Leon et al, 1989) contained 0.56% DKP. Thus, a daily dose of 0.42 mg DKP/kg bw/day was given in this study. There were no treatment-related adverse effects. Similarly, when APM was given to subjects alleged to be hypersensitive to APM, no reproducible hypersensitivity reactions were observed at a dose of 3,610 mg over 150 minutes (Garriga et al, 1991). The dose of DKP in this study was approximately

0.34 mg/kg bw. Thus, as a component of APM, DKP has been shown to be safe at various doses in numerous subpopulations including children, adolescents, adults, diabetics, obese subjects, etc. (Butchko and Kotsonis, 1989).

VI. REGULATORY APPROVALS

In 1980, the Joint Expert Committee on Food Additives (JECFA) of the Codex Alimentarius of the Food and Agriculture Organization/World Health Organization set the Acceptable Daily Intake (ADI) of DKP at 7.5 mg/kg body weight. The Scientific Committee for Foods (Commission of the European Committees, 1985) has also independently set the same ADI. The ADI was based on the two-year rat study done with DKP where there were statistically significant increases in uterine polyps in the mid- and high-dose groups (i.e., 1.5 and 3.0 g DKP/kg bw/day, respectively) but not in the low-dose group (i.e., 750 mg/kg bw). At the time of their 1980 evaluation, JECFA did not have available the results from a two-year rat study by Ishii et al (1981) done with aspartame and a mixture of APM and DKP. No increased incidence of uterine polyps was observed in this study at the highest dose given of 1 g DKP/kg bw/day (i.e., 4 g APM-DKP mixture/kg bw/day).

The U.S. Food and Drug Administration (FDA) approved aspartame for use in beverages in July 1983 (Fed. Reg., 1983). At that time, the FDA evaluated DKP safety studies including the chronic toxicity and carcinogenicity studies in mice and rats. In order to resolve any concerns regarding the apparent increased incidence of uterine polyps, the FDA reviewed interpretations of the study results from four groups: 1) The FDA Pathology Group; 2) The Massachusetts Institute

of Technology Pathology Group; 3) The Armed Forces Institute of Pathology; and, 4) The G. D. Searle Pathology Group. These four groups concluded that the polyps were non-neoplastic benign proliferations that would not undergo malignant transformation. Furthermore, it was concluded that the uterine polyps were a localized form of endometrial hyperplasia that occurs spontaneously in aging rats. The FDA concluded that the polyps were spontaneous lesions in the 1.5 g/kg bw/day group and may also represent a non-specific effect of the large amount of DKP administered at 3 g/kg bw/day. Therefore, the FDA concluded that the no-effect level was 3 g/kg bw/day. Using a standard one hundred-fold safety factor, the ADI for DKP was 30 mg/kg bw/day.

VII. CONSUMPTION AND THE ACCEPTABLE DAILY INTAKE

The consumption rate of DKP can be estimated from the amount of DKP in products and the consumption rates of aspartame in the various food categories. The amount of DKP in both commercial and prototype products has been determined by The NutraSweet Company. Studies done with prototype products utilized typical storage conditions as well as extreme conditions of temperature, pH, and storage time. The estimated average levels of DKP at retail for various food categories are in Table 2. The 90th percentile, 14-day average (all ages, users only) consumption rates for aspartame were estimated using data from the Marketing Research Corporation of America (MRCA). The APM consumption rates from MRCA data are similar to those of The Ministry of Agriculture, Fisheries and Food (1987).

Carbonated beverages randomly selected at retail had DKP levels that were approximately 4 to 5% of the initial APM concentration. Using the 90th percentile, 14-day average of APM consumption for carbonated beverages of 2.2 mg/kg bw/day, the estimated consumption rate of DKP in carbonated soft drinks is only 0.11 mg/kg bw/day. Summation of 90th percentile, 14-day average consumption for all product categories including baked goods yields a total DKP consumption rate of 0.56 mg/kg bw/day (Table 3). The mean estimated intake rate is 0.25 mg/kg bw/day.

The 90th percentile consumption rate is an overestimate of actual intake because it assumes a 100% market penetration for additional food categories and that consumption rates for individual categories are additive. Nonetheless, this estimate is still 15-50 times less than the present ADIs.

VIII. TABLES

TABLE 1

PHARMACOLOGY STUDIES WITH DKP

Gastrointestinal System

- Appetite Inhibition in Rats
- Effects on Gastric Secretion in Rats
- Pepsin Inhibition In Vitro
- Pancreatic Lipase Inhibition In Vitro
- Effects on Gastric Ulceration in Rats

Cardiovascular System

- Effects on Blood Pressure in Anesthetized Dogs Following Intravenous Administration
- Effects on Blood Pressure and Heart Rate Following Oral Administration in Unanesthetized Normotensive Dogs
- Inhibition of the Pressor Response to Angiotensin in Rats
- Antiarrhythmic Activity Using the Isolated Rabbit Heart
- Effects on Blood Coagulation In Vitro

Central Nervous System

- General Effects in Mice
- Antidepressant Activity in Mice
- Effects on Hexobarbital Hypnosis in Mice
- Effects on Motor Coordination in Mice
- Anticonvulsant Activity in Mice
- Analgesic Activity in Mice
- Central Anticholinergic Activity in Mice
- Effects on Behavior in Rats

Miscellaneous

- Diuretic Activity in Rats
- Effects on Blood Glucose in Rats
- Effects on Body Weight Gain and Blood Cholesterol in Hypercholesterolemic Rats
- Antiacetylcholine Activity In Vitro
- Antihistamine Activity In Vitro
- Autonomic Ganglionic Blockade
- Effect of Dietary Administration on Serum Levels of Glucose, Insulin, Triglycerides, Free Fatty Acids and Cholesterol in Rats
- Anti-inflammatory Tests in Rats

TABLE 2

DKP CONTENT IN COMMERCIALLY AVAILABLE OR PROTOTYPE FOODS

FOOD CATEGORY--	DKP CONTENT (% of aspartame initially present)
• Beverages Carbonated Juices Teas Wines	4.5
• Tabletop Sweetener	<0.3[a]
• Baked Goods	9.9[a]
• Confections Soft Candy Hard Candy	 8.9[a] 5.6[a]
• Frozen Dessert	3.5[a]
• Yogurts	9.2[a]
• Others[b]	14.0[c]

[a] Based on prototype products stored under relevant marketplace conditions.

[b] Includes: Breakfast cereals, chewing gum, dry mixes, chewable vitamins, cookie fillings, frozen cheesecake, frozen fruit, frozen fruit toppings, frozen dairy and non-dairy toppings, frosting and fillings, fruit spreads, fruit toppings, and fruit syrups.

[c] Worst-case estimate based on DKP concentrations in representative prototype (1.6-13.7%).

TABLE 3

APM AND DKP CONSUMPTION RATES

	DKP CONTENT[a] (% of aspartame initially present)	APM CONSUMPTION[b] (mg/kg bw/day)	DKP CONSUMPTION (mg/kg bw/day)
CURRENT APM CONSUMPTION IN THE U.S.	5%	2.2	0.11
PROJECTED U.S. CONSUMPTION OF:			
• Baked Goods	10%	2.8[c]	0.28
• Confections			
Soft Candy	9%	1.4[c]	0.13
Hard Candy	6%	0.6[c]	0.04
		TOTAL	0.56[d]

[a] These values are provided as projected estimates of DKP content in foods at retail. The 5% value of DKP for current APM consumption in the US was chosen because of the large contribution that beverages make to the overall current consumption of aspartame in the U.S. (>80%).

[b] Annualized MRCA survey data July 1988 - June 1989, 90th percentile, 14-day average for all ages, eaters only.

[c] Estimated intake values are based on average use levels of aspartame of 2,000 ppm for baked goods, 2,500 ppm soft candy and 3,000 ppm for hard candy. These values are provided as estimates of consumption based on current consumption of applicable foods as determined by the MRCA Survey data base. These estimates are overestimates as they assume 100% market penetration of aspartame-containing products.

[d] The total value represents the sum of the 90th percentile, 14-day average values for current use and of the projected uses of baked and confectionery products. This value is an overestimate as it is unlikely that 90th percentile users of beverages will also be a 90th percentile users of baked goods and confections.

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Annex 3 to
TOX/91/39

DIKETOPIPERAZINE

INTRODUCTION

1. The diketopiperazine derivative of aspartame (5-benzyl-3,6-dioxo-2-piperazineacetic acid, DKP) is the major breakdown product of aspartame in foodstuffs (Figure 1). DKP is one of a related group of cyclic dipeptide derivatives; other derivatives in this group have been found at levels up to 100 mg/kg in roasted malt for brewing (Sakamura et al, 1978) and in cocoa powder (Pickenhagen and Dietrich, 1975).

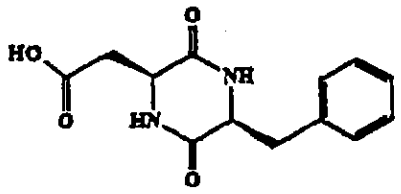

Figure 1 - The diketopiperazine derivative of aspartame (DKP)

DIKETOPIPERAZINE FROM THE BREAKDOWN OF ASPARTAME

2. A large amount of information has been generated concerning the characteristics of aspartame and its breakdown products under a wide range of conditions of temperature and pH. A general model for the degradation of aspartame has been proposed (Prudel et al, 1986), and the major reactions which occur under conditions for the storage of foodstuffs are detailed in Figure 2. Mass balance analysis and other analytical techniques have indicated that no undefined compounds are generated on the degradation of aspartame under these conditions (Prudel et al, 1986; Dever et al, 1986; Stamp and Labuza, 1989; Witt, 1990).

3. The most definitive research on the degradation of aspartame in individual food systems has been carried out on carbonated beverages (Homler, 1984; Tsang et al, 1985; Saito et al, 1989; Witt, 1990), one of the major dietary sources of aspartame in the UK (MAFF Food Surveillance Paper No. 29). This research indicates that, under normal storage conditions for these products (20-25°C and pH 2.8-3.4), the half-life of

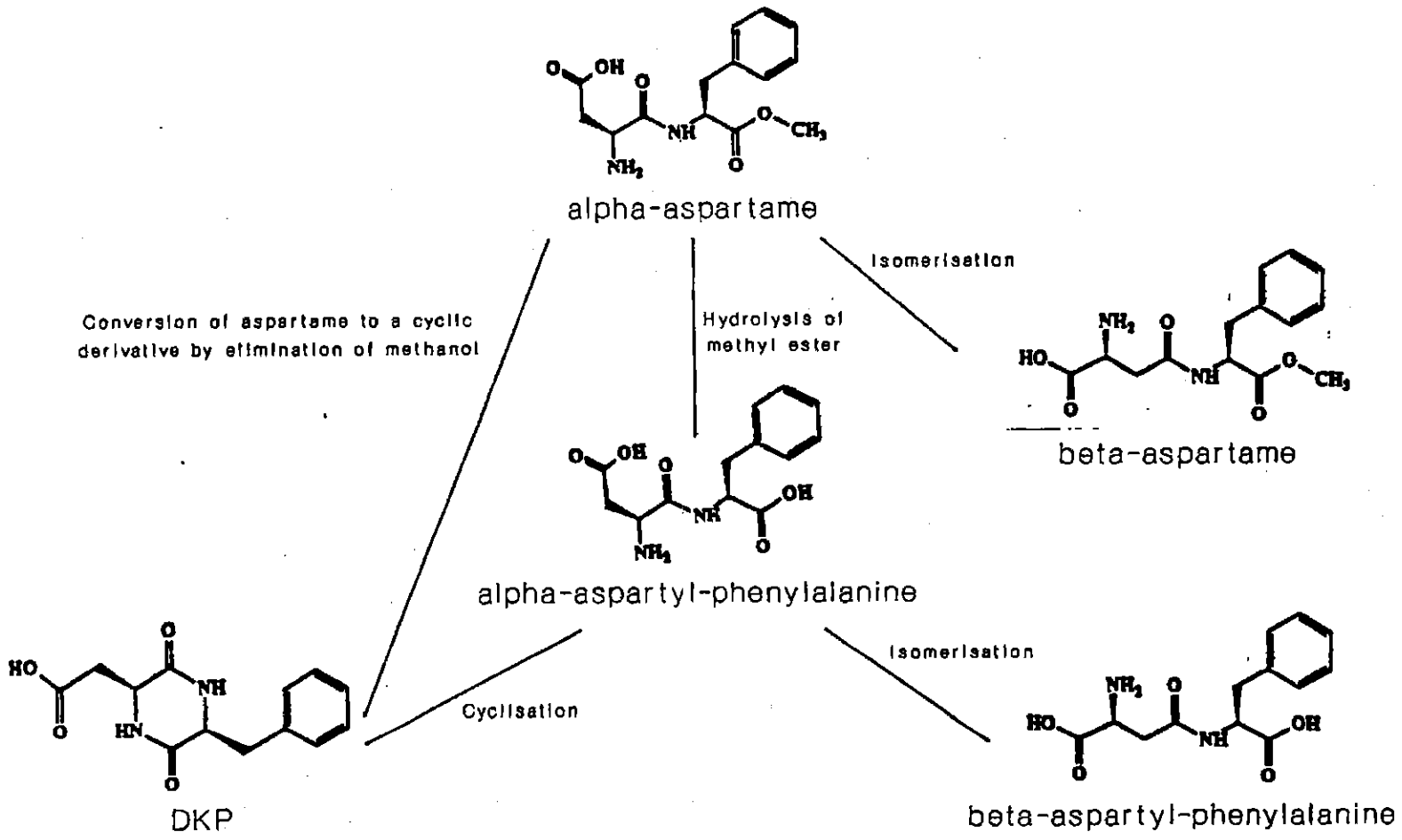

Figure 2

The major degradation products of aspartame under conditions of storage of foodstuffs (Prudel et al,1986)

aspartame is 32-48 weeks and DKP is the major breakdown product. The proportion of degraded aspartame accounted for by DKP is relatively constant at 40% under all conditions where the beverage would remain sweet enough to be organoleptically acceptable. The majority of the remainder is accounted for by ß-aspartame and aspartylphenylalanine, as indicated in Table 1.

Table 1 - Compounds generated on the breakdown of aspartame in carbonated beverages, under conditions where the beverage remains acceptably sweet.

Breakdown product	% of total degradation products
DKP	40
ß-aspartame) ß-aspartylphenylalanine)	20-30
α-aspartylphenylalanine	5-20
α- and ß- phenylalanylaspartic acid	<2
phenylalanine methyl ester	<2
phenylalanine and aspartic acid	<2

4. The other major dietary source of aspartame is table-top sweetener products. Aspartame is very stable in such dried products; storage of prototype table-top sweetener tablets under likely retail conditions led to <0.3% of the aspartame content degrading to DKP.

5. At pH values above those found in carbonated beverages, the ratio of DKP to aspartylphenylalanine formed on the breakdown of aspartame increases (Prudel et al, 1986). Diet yoghurt and other diet desserts, typically at pH 4.2 and above, will therefore contain a higher proportion of DKP as a percentage of the total degradation products. However, in the case of diet yoghurts, the additional breakdown of aspartame to constituent amino acids may occur as a result of microbial metabolism where aspartame is added to the yogurt before fermentation (Keller et al, 1991).

6. Aspartame is not sufficiently heat-stable to be used in baked goods. However, NutraSweet has developed an encapsulation technique whereby the aspartame is protected during the baking process. Large-

scale introduction of products formulated with encapsulated aspartame is not envisaged before 1992; although the formulation appears to comply with current food and food additive regulations, the company has agreed that it will not market the product in the UK until this use has been considered by the FAC. NutraSweet has supplied MAFF with details of the DKP content of baked goods sweetened with aspartame, both freshly made and upon storage. The pH of the cake batters used in the prototype products was 6.6-7.2, and DKP accounted for 55-110% of the apparent proportion of degraded aspartame in these products. This concurs with the results of Prudel et al, who found that in the pH range 6.1-7.2, DKP was practically the only degradation product formed on the breakdown of aspartame in aqueous solutions.

PURITY SPECIFICATIONS FOR ASPARTAME

7. Specifications for aspartame have been defined in the UK (The Sweeteners in Food Regulations, SI 1983 No. 1211) and by JECFA (FAO Food and Nutrition Paper 19). Each set of purity criteria specifies that the DKP content of aspartame used in foods should be not more than 1.5%. This represents an additional, albeit small, potential source of DKP in addition to that formed on the breakdown of aspartame during manufacture and storage of foodstuffs.

POTENTIAL DKP LEVELS IN FOOD PRODUCTS

8. The levels of DKP which would arise from storage of food products sweetened with aspartame that are currently on the UK market are given in Table 2. Table 3 summarises information received from NutraSweet regarding the DKP levels determined after storage of prototype baked products using encapsulated aspartame.

DIETARY INTAKE ESTIMATES

9. Data on dietary intakes will be presented following the Committee's discussion of the safety data.

Table 2 - Potential DKP levels in food products currently on the UK market.

Food product	Storage conditions	DKP (% of initial aspartame content)
Carbonated diet soft drinks	24 weeks at 20°C	11.5 (a)
Table-top sweeteners	-	1.5 (b)
Diet desserts	4 weeks at 4°C	11.5 (c)
Powdered instant beverages	24 weeks at 21°C	1.6 (c)
Confectionery	7-9 weeks at 21°C	up to 16.6 (c)

Notes: (a) Data from CE Adams, US Dept. of Agriculture, in 'A White Paper - the Safety of Aspartame'.

(b) As breakdown of aspartame in these products is minimal (paragraph 6), it is assumed that the maximum likely DKP content is that specified in purity criteria (paragraph 7).

(c) Unpublished data submitted by NutraSweet.

Table 3 - DKP levels on baking and storage of baked goods formulated with encapsulated aspartame (unpublished data submitted by NutraSweet).

Baked goods	Storage conditions	DKP (% of initial aspartame content)
Cheesecake	15 days at 7°C	4.2
Plain biscuits	12 weeks at 21°C	3.6
Sponge cake	14 days at 21°C	14.8
Chocolate cake	15 days at 21°C	39.6
Fig rolls	12 weeks at 21°C	1.4
Lemon pie filling	13 days at 21°C	4.4

Secretariat

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DIKETOPIPERAZINE - DIETARY INTAKE ESTIMATES

1. A model for the estimation of dietary intake by children has been derived using data on:

i the actual UK consumption of aspartame by the general population and members of the diabetic population; and

ii the relative importance of different food types as vehicles for aspartame intake for different groups within these populations.

These data are available through the MAFF surveys of sweetener intake (Food Surveillance Paper No 29: Intakes of Intense and Bulk Sweeteners in the UK 1987-1988), which provide food intake data for different age groups in the populations. It is assumed that levels of breakdown of aspartame to DKP in different food types are as given in Table 2 of this Annex – see page 5 ; these levels correspond to those expected from typical commercial storage conditions. Estimates of median and extreme consumption of DKP using this model are summarised in Table 1. In the case of the consumers with the highest intake of aspartame, diabetic children aged 2-9, the potential maximum intake of DKP is 1.7 mg/kg bodyweight/day.

Table 1 - Estimated intake of DKP by the general UK population and the UK diabetic population

		General population DKP intake (mg/kg bw/day)		Diabetic population DKP intake (mg/kg bw day)	
		Median	Maximum	Median	Maximum
Children	2- 5	0.09	0.2	0.5	1.7
	6- 9	0.03	0.1		
	10-14	0.04	0.1	0.4	1.2
Adults	15-19	0.04	0.2	0.5	0.7
	20-24	0.06	0.2	0.2	0.4
	25-34	0.03	0.2	0.3	1.1
	35-54	0.02	0.2	0.2	0.7
	55-64			0.1	0.8

2. Baked goods formulated with encapsulated aspartame are not currently on sale in the UK. Such products would provide a new dietary source of DKP. This contribution to intake may be modelled using data

on the actual intake of sweetened baked goods (biscuits, fruit pies, buns, cakes and pastries) from:

i a nutritional survey of over 2000 adults in the UK in 1986-87 (Gregory et al (1990) The Dietary and Nutritional Survey of British Adults, HMSO); and

ii a recent pilot study of the dietary behaviour of pre-school children aged 1.5 to 4.5 years.

These data are combined with figures for the degradation of aspartame in prototype baked goods, supplied by NutraSweet, to yield estimates for DKP intake. If baked goods were sweetened with aspartame at 1700 mg/kg, the maximum levels permitted in the proposed EC Directive on Sweeteners, this model indicates that extreme (97.5th percentile) consumers of baked goods who preferentially consume products sweetened with aspartame would have an intake of DKP from this source of 0.4 mg/kg bodyweight/day in the case of pre-school children, and 0.5 mg/kg bodyweight/day in the case of adults. Baked goods sweetened with aspartame are therefore likely to be a major source of DKP for those individuals that consume them.

Additives Branch I
Food Science Division I
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SECRETARIAT SUMMARY OF NEW DATA ON METABOLISM AND EXCRETION OF DKP

1. Burgert, Merrick, Coon, Takeuchi and Stegink (1985). Am J Clin Nutr 41 p867.

1.1 This paper examined the intestinal metabolism of aspartame, DKP and phenylalanine methyl ester (a second breakdown product of aspartame) in young pigs. Aspartame given as an oral bolus dose of 0.38 mmol/kg bw produced an increase in portal blood levels of phenylalanine, tyrosine and aspartate whereas DKP at an equivalent dose had no such effects. It was concluded that aspartame was hydrolysed to its constituent amino acids before entering the portal blood and that aspartylphenylalanine was an important intraluminal intermediate in aspartame metabolism. DKP was not hydrolysed to its constituent amino acids within the gut.

2. Cho, Coon and Stegink (1987). Fd Chem Toxicol 25 p499-504

2.1 This work was carried out some time ago before significant amounts of DKP itself were available and it was therefore administered in the form of the 1.1% DKP impurity that was present in aspartame. Plasma and urine concentrations of DKP were measured in samples obtained from 6 normal adults (3 male and 3 female) ingesting 200 mg/kg bw of aspartame (and thus 2.2 mg/kg bw of DKP). The DKP concentrations in plasma were below the limit of detection of 1µg/ml at all time intervals up to 24 hours post dosing. The total amount of DKP excreted in the urine in the 24 hour period after dosing was approximately 4.8% of the dose administered, with 44% of this amount in the period 0-4 hours. The study was conducted before the marketing of aspartame and therefore subjects were not exposed to aspartame or DKP in their normal diets.

3. Stegink et al (1984). Unpublished data from NutraSweet

3.1 Six healthy normal adults received each of three treatment regimens in a crossover manner with at least 1 week between treatments:

- 1 8oz serving of unsweetened beverage/hour for 8 hours
- 1 8oz serving of unsweetened beverage containing 600 mg aspartame/hour for 8 hours
- 1 8oz serving of unsweetened beverage containing 150 mg DKP/hour for 8 hours

Each aspartame dose was equivalent to 1-1.5 l of normal beverage sweetened with aspartame and each DKP dose was equivalent to >1 l of aspartame-sweetened beverage that had undergone complete degradation.

3.2 The aims of the study were:

- to determine whether aspartame caused a significant rise in plasma phenlyalanine concentrations
- to determine whether DKP administration resulted in the detection of DKP in the plasma or urine
- to obtain profiles of amino acids, DKP, methanol and formate in plasma and urine after repeated administration of aspartame and DKP
- to compare the ratios of plasma phenylalanine concentration to the sum of the concentrations of the other large neutral amino acids across the treatment groups.

3.3 Aspartame caused increases in phenylalanine and tyrosine concentrations, which reached a plateau after 5 doses; these were within the limits for normal post-prandial concentrations. Aspartate and glutamate concentrations were not significantly altered after treatment. Concentrations of the other large neutral amino acids and also those of methanol and formate were not altered by aspartame ingestion. The ratios of peak phenylalanine and tyrosine concentrations to the sum of the other large neutral amino acids were elevated with respect to the placebo treatment but were not greater than the values expected under normal dietary conditions.

3.4 Most plasma DKP concentrations were below the limit of detection following treatment with aspartame or placebo. However the persistent presence of DKP in one individual in the placebo arm of the study would indicate that the DKP formed from aspartame is a naturally occurring dietary or endogenous compound. DKP ingestion caused a small increase in plasma DKP concentrations which reached a plateau after 4 doses. DKP was also detectable in the urine in the period 0-24 hours after administration of DKP with some 5% of the total dose administered being recovered over this time. DKP had no effect on plasma amino acid concentrations, nor on blood methanol or formate concentrations or plasma large neutral amino acid ratios.

4. Stegink et al (1985). Unpublished information from NutraSweet

4.1 The design of this study in subjects heterozygous for PKU was identical to that above for normal subjects. Aspartame produced an increase in plasma phenylalanine levels which reached a plateau after 6 doses. These levels were only just above normal post-prandial concentrations for PKU subjects and were well below levels of concern:-

phenylalanine level in μmol/dL	
baseline	6.95 ± 0.92
plateau after aspartame	12.6 ± 3.44
post-prandial in PKU subjects	132 ± 25

Tyrosine concentrations were only slightly elevated, and concentrations of other amino acids were unaffected as were blood methanol and formate levels.

4.2 Most plasma DKP concentrations were below the limit of detection after aspartame or placebo treatments. However the presence of DKP in the placebo stage in some instances would indicate that this is a naturally occurring dietary and/or endogenous substance. DKP treatment produced a small increase in plasma DKP levels which reached a plateau after 4 doses. DKP was also detectable in the urine after DKP treatment with approximately 2% of the total dose of DKP being recovered. DKP ingestion did not produce any effect on plasma amino acid concentrations, on blood methanol or formate levels or on urinary concentrations of formate. Furthermore there were no effects on plasma large neutral amino acid ratios.