Appendix I to TOX/79/47

ASPARTAME - BIOLOGICAL STUDIES

I. BIOCHEMICAL STUDIES

i. PHARMACOLOGY (Studies on aspartame and its diketopiperazine (DKP))

There were no effects in rats on appetite at 200 mg/kg, gastric secretion at 225 mg/kg, bovine pepsin inhibition in vitro at 143 µg/ml, pancreatic lipase inhibition at 1.25 mg/ml and gastric ulcer formation in rats at approximately 200 mg/kg. Cardiovascular studies in anaesthetised dogs showed a transient increase in blood pressure upon iv administration of 5 mg/kg aspartame (but not with DKP at this level). In unaesthetised dogs, no effects were noted in blood pressure or heart rate after oral administration of up to 200 mg/kg of aspartame or DKP. Furthermore, neither compound possessed in vitro blood anticoagulant activity. No effects were noted in rats on pressor responses to angiotensin after iv administration of 10 mg/kg of material and in the isolated rabbit heart, the sweetener was unable to affect aconitine-induced ventricular arrhythmia. In a range of studies on the CNS in mice, no effects were noted at the 200 mg/kg level (single dose, oral administration) in antidepressant, anticonvulsant and anticholinergic activities, hexobarbital hypnosis and motor inco-ordination, and at the 100 mg/kg level (highest dose administered) on analgesic activity; in rats no behavioural effects were noted after a single dose of 200 mg/kg. Other pharmacological studies showed that in rats, both compounds at oral doses of 100 mg/kg were inactive as diuretic agents and failed to affect blood glucose levels; after nine days oral administration of up to 200 mg/kg to hypercholesterolemic rats, bodyweight gain and serum cholesterol were not affected, neither compound possessed anti-acetyl-choline or antihistamine activity in vitro and studies on the peripheral nervous system in rats and mice at up to 2g/kg had no adverse effect.

A wide variety of studies were undertaken to assess the potential side effects of both aspartame and DKP on the endocrine system and on hormonally dependent target tissues. Aspartame failed to display any oestrogenic, progestational, androgenic, myotropic or glucocorticoid activity after oral administration and adverse effects were not noted on the pituitary ovarian axis, in addition, the sweetener did not antagonise normal physiologic responses to an oestrogen, progestogen and androgen. Aspartame also did not significantly affect rat or hamster fertility and was devoid of any anti-inflammatory or immuno-suppressive activity. DKP elicited similar results under identical test conditions to aspartame with the exception of minimal anti-inflammatory activity when orally administered at 55 mg/day to rats.

(Searle Project Appendix 7).

ii. METABOLISM (Studies on aspartame and DKP)

Studies on the pharmacokinetics and metabolism of aspartame were carried out in rats, mice, dogs, rabbits, rhesus monkeys and man. The methyl group is hydrolysed in rats and monkeys by esterases in the intestine, absorbed and then metabolised in the one-carbon pool of the body. The hydrolysis rate in the monkey was found to be slower than that of the rat. Administration of aspartame, radiolabelled in the aspartic acid moiety, indicated that the compound was metabolised in a similar manner to that of free L-aspartic acid-14C, with 60-70% of 14C being excreted in expired air and the remainder incorporated into body protein. Similar studies in experimental animals and man, with the phenylalanine (phe) moiety labelled, indicated that the compound was metabolised in a likewise manner to that of free L-phe-14C; however the rate of absorption of natural phe was found, in subsequent animal studies, to be more rapid than that of radiolabelled aspartame. The difference was thought to be due to the need for prior cleavage of the dipeptide bond in the gut before absorption of the resultant amino acids could occur. Comparative studies in man on the metabolism of aspartame-phe-14C and phe-14C were not performed; however the metabolism and pharmacokinetics of aspartame-phe-14C in man and monkey were found to be similar
in all aspects. Studies in rabbits indicated that the rates of absorption and digestion of aspartame were slower than in the other animal species investigated.

The metabolites of aspartame-phe-14C found in the plasma were the same (both quantitatively and qualitatively) in all animal species studied. 86-94% of the radioactivity was associated with complex phenylalanine containing substances and 2-3% exhibited similar mobilities on TLC to those of phenylalanine and tyrosine. The plasma half-life in dogs after oral administration of aspartame-phe-14C was approximately 12 days.

Under certain conditions, small amounts of aspartame can be transformed by demethylation and cyclization to DPK. Small amounts of this product were found in foods containing aspartame and hence its metabolism in rats, rabbits, monkeys and man was studied. DPK-phe-14C was found to be poorly absorbed from the gut and the 14C then rapidly excreted in the urine. DPK is absorbed by the body unchanged and radiotracer techniques revealed that its major metabolite was phenylacetylglutamine, (a metabolite associated with phenylalanine metabolism); however phenylacetylglutamine was not formed when DPK was injected iv into monkeys and it was thought therefore that the metabolite arose from bacterial degradation of DPK in the gut. Further studies with germ-free rats showed that only a small amount of the metabolite was formed in the urine. A third metabolite of DPK was identified, in the faeces of monkeys (after oral administration), as phenylacetic acid. Both phenylacetylglutamine and phenylacetic acid were identified in man in the urine and faeces respectively as the major metabolites of DPK.

In vivo and in vitro studies were performed to investigate the potential of DPK (a cyclic amide) to react with dietary nitrite and form nitrosamines. DPK failed to react with nitrite under conditions in which piperidine (a secondary amine) reacted with nitrite to form N-nitrosopiperidine.

Neither aspartame nor DPK stimulated or inhibited hepatic enzyme activities in model in vivo and in vitro studies, but liver phenylalanine hydroxylase was inhibited by both compounds in a dose-related manner. This effect was also observed when animals were fed diets which were supplemented with equimolar amounts of phenylalanine; however a similar supplementation in vivo in the monkey did not affect plasma phenylalanine or tyrosine levels. Further studies showed that the inhibition was due to absolute changes in activity rather than a shift in the known circadian rhythm of the enzyme.

The effects of dietary aspartame on maternal and fetal phenylalanine were studied in rabbits. The feeding of diets containing 6% aspartame to pregnant rabbits resulted in a maximal increase in maternal plasma and tyrosine levels on day 9 of gestation followed by a return to normal values by day 20. Fetal/maternal plasma amino-acid ratios were not significantly different in control and treated groups; however, as the increase in tyrosine levels in maternal plasma exceeded those of phenylalanine the phenylalanine/tyrosine ratio fell; this indicated that most probably, the phenylalanine hydroxylase activity was unaffected by treatment. In an in vitro study with maternal phenylalanine hydroxylase, the findings in the above rabbit study were verified.

It was thus concluded that the metabolism of aspartame in man was similar to that of phenylalanine and aspartic acid. (Searle Project Appendices 8, 9, 10, 10A).
## II ACUTE TOXICITY

<table>
<thead>
<tr>
<th>Species</th>
<th>Sex</th>
<th>Route</th>
<th>LD$_{50}$ (mg/kg)</th>
<th>Reference (Appendix)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat—Charles River</td>
<td>Male</td>
<td>Ig</td>
<td>&gt; 5000</td>
<td>Searle (12)</td>
</tr>
<tr>
<td>Rat—Charles River</td>
<td>Male</td>
<td>Iv</td>
<td>&gt; 100</td>
<td>Searle No. 1179 S74 (12A)</td>
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<td>Searle (12)</td>
</tr>
<tr>
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<td>Male</td>
<td>Ip</td>
<td>&gt; 1000</td>
<td>Searle (12)</td>
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<tr>
<td>Rabbit—New Zealand</td>
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<td>&gt; 5000</td>
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<td>White Luenberg</td>
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<tr>
<td>Dog—Beagle</td>
<td>Male</td>
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<td>&gt; 100</td>
<td>Searle No. 1178 S74 (12B)</td>
</tr>
</tbody>
</table>
III SUB-ACUTE TOXICITY STUDIES

(i) **Species** : Mouse

**Strain** : HA(ICR)

**Group Size** : 5♂ + 5 ♀

**Route** : Dietary administration

**Dose/Dietary Levels** : 0, 3, 5, 13 g/kg/day

**Duration of Treatment** : 4 weeks

**RESULTS**

**Appearance and Behaviour** : Monitored daily; no adverse effects

**Growth** : Measured on days 0, 3, 7, 14, 21 and 28 of treatment. No adverse effects.

**Food and Water Consumption** : Measured as above for growth; no adverse effects

**Haematology** : Not performed

**Serum Analyses** : Not performed

**Urine Studies** : Not performed

**Organ Weights** : Not measured

**Post-mortem observations** : 3 males + 2 females (controls) + all animals of high dose group. Gross observations only. Mucosa of stomach, duodenum and jejenum of treated animals were heavily coated with clear viscous fluid.

**Other Observations** : None

**No-untoward-effect level** : 13 g/kg

**Reference** : Searle Project No. 815 S59 (Appendix 13)
<table>
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<th>Species</th>
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<tr>
<td>Strain</td>
<td>Charles River CD</td>
</tr>
<tr>
<td>Group Size</td>
<td>5♂ + 5♀</td>
</tr>
<tr>
<td>Route</td>
<td>Dietary administration</td>
</tr>
<tr>
<td>Dose/Dietary Levels</td>
<td>0, 2, 4, 10g/kg/day</td>
</tr>
<tr>
<td>Duration of Treatment</td>
<td>4 weeks</td>
</tr>
</tbody>
</table>

**RESULTS**

- **Appearance and Behaviour**: Monitored daily; no adverse effects
- **Growth**: Measured on days 0, 3, 7, 14, 21 and 28 of treatment; no adverse effects.
- **Food and Water Consumption**: Measured as for growth; food intake decreased significantly after weeks 2 and 3 of administration in females receiving 10g/kg.
- **Haematology**: Not performed
- **Serum Analyses**: Not performed
- **Urine Studies**: Not performed
- **Organ Weights**: Not measured
- **Post-mortem observations**: 3 males + 2 females of controls + all animals of high dose group. Gross observations only. Mucosa of stomach, duodenum and jejunum of treated animals were heavily coated with a clear viscous fluid.
- **Other Observations**: None
- **No-untoward-effect level**: 10g/kg
- **Reference**: Searle Project No 814969 (Appendix 14)
IV. SHORT-TERM TOXICITY STUDIES

i. Species : Rat
   Strain : Charles River CD
   Weight range : Males 363-425g; females 223-306g
   Group Size : 10♂ + 10♀
   Route : Dietary administration
   Dose/Dietary Levels : 0, 5, 125 mg/kg/day
   Duration of Treatment : 8 weeks

RESULTS

Appearance and Behaviour : Monitored weekly; no adverse effects
Growth : Monitored weekly; no adverse effects
Food and Water Consumption : Monitored weekly; no adverse effects
Haematology : Erythrocyte and total and differential white cell counts, microhaematocrit and haemoglobin levels and coagulation and prothrombin time after 1 and 2 months. No adverse effects.
Serum Analyses : No adverse effects on BUN, glucose, SGPT, SAP, bilirubin, protein content, Na, K, Ca, Cl, CO₂; SGOT levels reduced in a dose-related manner in both sexes.
Urine Studies : No adverse effects in appearance, pH specific gravity, protein, glucose, ketones, bilirubin and microscopic examination of sediment.
Organ Weights : Only change observed was statistically significant increase in relative liver weight in males given 125 mg/kg.
Histopathology : Chronic murine pneumonia was observed in several animals/group. No adverse effects attributable to treatment.
Other observations : Ophthalmoscopy on all rats prior to and at end of treatment; no adverse effects.
No-untoward-effect level : 125 mg/kg.
Reference : Searle Project No PT 719H68 (Appendix 15).
Species: Dog
Strain: Beagle
Bodyweight range: 7.2 - 13.2 kg
Group Size: 2♂ + 2♀
Route: Oral (gelatin capsule)
Dose/Dietary Levels: 0, 5, 125 mg/kg/day
Duration of Treatment: 8 weeks

RESULTS
Appearance and Behaviour: Monitored daily; no adverse effects
Growth: Monitored weekly; no adverse effects
Food and Water Consumption: Monitored weekly; no adverse effects
Haematology: No adverse effects attributable to treatment in erythrocyte and total and differential white cell counts, haematocrit, haemoglobin, coagulation and prothrombin time.
Serum Analysis: No adverse effects on sugar, BUN, BSP retention, SGPT, SGOT, SAP, Na, K, Cl, Ca, Protein, CO₂, bilirubin.
Urine Studies: No adverse effects on pH, specific gravity, sugar, acetone, protein, bilirubin, occult blood, or microscopic examination of the sediment.
Organ Weights: There was a dose-related increase in relative spleen weight in females only; no other changes were observed.
Histopathology: No adverse effects noted in a wide range of tissues and organs examined.
Other Observations: Ophthalmoscopy on all dogs prior to and at the end of treatment; no adverse effects.
No-untoward-effect level: 125 mg/kg
Reference: Searle Project No P-T 720H68
Species: Rat
Strain: Charles River CD (weanling rats)
Group Size: $5^2 + 5^2$
Route: Dietary administration
Dose/Dietary Levels: 0 (controls), 5% L-phenylalanine, 9% aspartame
Duration of Treatment: 9 weeks

RESULTS
Appearance and Behaviour: Monitored twice weekly for first 4 weeks, then at weekly intervals; no adverse effects
Growth: Monitored as above; bodyweights were reduced in both treatment groups, and to a greater extent in males.
Food and Water Consumption: Monitored as above; food consumption was reduced in a manner similar to that of rate of bodyweight gain.
Haematology: Monitored for PCV, haemoglobin, erythrocyte and total and differential white cell counts, prothrombin time after 9 weeks; no adverse effects.
Serum Analyses: After 9 weeks no adverse effects on BUN, uric acid, SAP, bilirubin or sugar; decrease in GPT in both male treatment groups and decrease in Ca and Cl in males given aspartame.
Urine Studies: No adverse effects on specific gravity, pH, occult blood, protein, glucose, ketones or microscopic examination of the sediment.
Organ Weights: No adverse effects were noted in several organs with the exception of a slight increase in absolute and relative thyroid weights in both male treatment groups.
Histopathology: No treatment-related changes were observed.
Other Observations: None.
No-untoward-effect level: 9% aspartame ($\leq 6.7$ g/kg/day).
Reference: Searle Project No 847570 (Appendix 17).
iv. Species: Rat
Strain: Long-Evans - 21 days old
Group Size: 32♂ + 32 ♀
Route: Dietary administration
Dose/Dietary Levels: 0 (control), 2.5 or 5.0 g/kg/day L-phenylalanine or 4.5 or 9.0 g/kg/day aspartame
Duration of Treatment: 13 weeks

RESULTS
Appearance and Behaviour: Observed daily for survival. All other observations made when bodyweights were recorded. During days 66-86 of treatment the rats were subjected to 3 types of behavioural study:

a. 12♂ + 12 ♀ rats selected for determination of general activity levels.

b. 20♂ + 20 ♀ rats selected for evaluation of learning of conditioned avoidance response in a two-way shuttle box.

c. 8♂ + 8 ♀ rats selected for evaluation of non-discriminated avoidance response (Sidman).

No adverse effects noted in general appearance or behaviour. Aspartame-treated rats tended to have a slightly higher general activity than controls, but the difference was not significant. No adverse effects were noted in conditioned responses in groups given 2.5 g/kg/day L-phenylalanine or 4.5 g/kg/day aspartame, but at the higher levels, significant decreases in avoidance were observed. No adverse effects were noted in the Sidman test at any treatment level.

Growth: Bodyweights recorded twice weekly for 2 weeks, weekly for 5 weeks and fortnightly thereafter. Full data not reported; on day 86 dose-related reductions were noted in all treatment groups.
<table>
<thead>
<tr>
<th>Food and Water Consumption</th>
<th>Measured at same time as bodyweights. Results not reported.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survival</td>
<td>Several animals died during the course of the study, but mortality was neither dose nor treatment-related.</td>
</tr>
<tr>
<td>Serum Analyses</td>
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<td>Haematology</td>
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<tr>
<td>Urine Studies</td>
<td>Not performed</td>
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<td>Organ Weights</td>
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<tr>
<td>Histopathology</td>
<td>Not performed</td>
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<tr>
<td>Other Observations</td>
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<tr>
<td>No-untoward-effect level</td>
<td>2.5 g/kg</td>
</tr>
<tr>
<td>Reference</td>
<td>Searle Project No (Appendix 23)</td>
</tr>
</tbody>
</table>
Species and Strain: Rat - Charles River CD. (The animals were selected from the F₂ₐ litters of the 2-generation reproduction study.

Age at Start of Study: 1 day

Group Size: 20♂ + 20♀

Route: Maternal milk: in addition, the mothers diets, which contained the treatment agent, were accessible to the litters.

Dose/Dietary Levels: 0, 2, 4 g/kg/day (mothers diet)

Duration of Treatment: 21 days

RESULTS

Appearance and Behaviour: Observed regularly; no treatment-related effects

Growth: Bodyweights recorded on all animals killed by design. No treatment-related effects

Haematology: Performed on 5♂ + 5♀ prior to death on days 5, 15 and 21; haematocrit, haemoglobin, erythrocyte and total and differential white cell counts. Only effect noted was in total leucocyte count which was significantly decreased in males on day 15 and females on day 21 from both treatment groups.

Serum Analyses: Performed on 5♂ + 5♀ prior to death on days 15 and 21; fasting blood sugar, BUN, total protein, bilirubin, GPT, AP. No adverse effects noted.

Urine Studies: Not performed

Organ Weights: Not performed

Gross Observations: 5♂ + 5♀ killed after periods of 24 hours, 5, 15 and 21 days and subjected to autopsy together with any rats found dead or killed in extremis. No adverse effects noted.

Histopathology: Heart, liver, lungs, bladder and kidney examined. All organs unremarkable except the kidney where nuclear hypertrophy and nuclear vesiculation were noted in males and females (given 4 g/kg aspartame) on days 15 and 21. The severity was greater on day 15. These changes were also noted in one control male on day 15.

No-untoward-effect level: 2 g/kg

Reference: Searle Project No P-T 893H71 (Appendix 22)
vi. Species and Strain: Rhesus monkey. Macaca mulatta.

Group Size: 2-3 animals

Route: In milk, from age of 1-9 days

Dose levels: 1, 3 or 4-6 g/kg/day - No conventional controls

Duration of Treatment: 29-30 weeks for low dose group and 52 weeks for medium and high dose groups.

RESULTS

Appearance and Behaviour: Observed daily: from day 218, animals in medium and high dose groups exhibited seizure activity in which the convulsions were described as grand mal and occurred inconsistently though usually when the animal was being handled. Animals from these 2 groups also became infected with Shigella during the study for which they were treated. One monkey from the high dose group died on day 300 but the cause of death was not determined.

Growth: Bodyweights recorded daily; weight gain in all animals was comparable with the exception of one monkey from the low dose group which gained approximately half that of the others - this animal showed evidence of "physical deficiencies" at birth. Head circumference and body length were recorded at 4-weekly intervals; again all values were comparable with the exception of the one monkey from the low dose group.

Food Consumption: Milk consumption was reduced in the high dose group, such that the average intake of aspartame was 3.6g/kg/day. Food consumptions in the other groups were comparable.

Haematology: Monitored for haematocrit, haemoglobin, erythrocyte and total and differential white cell counts after 3, 6, 9 and 12 months of treatment; no adverse effects.

Serum Analyses: Monitored after 3, 6, 9 and 12 months for BUN, uric acid, GOT, AP, bilirubin, glucose, Ca, inorganic phosphate, cholesterol and protein; no adverse effects. Phenylalanine and tyrosine levels were measured twice weekly for 13 weeks, weekly for 17 weeks and
Serum Analyses (continued): for the first few days thereafter and their results were compared with those of a group of monkeys fed 2-25 g/kg/day of L-phenylalanine. The amino acid levels were very low in the group given 1 g/kg aspartame; levels in the medium and high dose groups were comparable with those of the treated controls.

Urine Studies: Specific gravity, pH, occult blood, protein, glucose, ketones, bilirubin and phenylketone levels were measured after 3, 6, 9 and 12 months; no adverse effects were noted with the exception of a significant increase in the excretion of phenylketones in the medium and high dose groups.

Post-Mortem Studies: No post-mortem observations were made as the animals were not killed at the end of the study.

Other Observations: Monkeys of the medium and high dose aspartame groups were maintained on a powdered similac diet for 3 months after cessation of treatment; no further convulsions were observed.

No-untoward-effect level: 1 g/kg

Reference: Searle Project No 856070 (Appendix 19)
V LONG-TERM TOXICITY (CARCINOGENICITY) STUDIES

i. SPECIES AND STRAIN: Hamster - Golden Syrian (Weanling)

GROUP SIZE: 70♂ + 70♀ (controls), 35♂-35♀ (treatment groups).
ROUTE AND DOSE LEVELS: Dietary administration providing 0, 1, 2, 4 or 12 g/kg/day. (High dose group given 6g/kg/day during weeks 1-14, 8g/kg/day during weeks 15-18, 10g/kg/day during weeks 19-22 and 12g/kg/day from week 23 onwards.)
DURATION OF TREATMENT: 46 weeks.

RESULTS

SURVIVAL: Monitored daily. The whole colony became infected by a disease thought to be "wet tail" soon after the study commenced; the study was terminated when 80% mortality had been reached in the control and low dose female groups after 46 weeks. (The study had originally been designed to last 104 weeks.)

APPEARANCE AND BEHAVIOUR: Observed at weekly intervals for 4 weeks, fortnightly intervals for a further 10 weeks and then every 4 weeks for the remainder of the study; no adverse effects attributable to treatment.

GROWTH: Bodyweights measured at same time intervals as observations on appearance and behaviour; rate of bodyweight gain was similar in control and treated groups.

FOOD AND WATER CONSUMPTION: Measured at same time intervals as growth; values for all treatment groups comparable with those of controls.

HAEMATOLOGY: PCV, haemoglobin, erythrocyte, total and differential white cell and platelet counts measured after 14, 26 and 45 weeks of treatment, reticulocyte counts after 14 and 26 weeks and prothrombin time and activated PTT after 45 weeks, all on approximately ⅓ of the animals per group. No adverse effects were noted.

SERUM ANALYSES: Monitored after weeks 26 and 45 for BUN, SAP, SGPT, bilirubin, glucose, sodium, potassium and calcium; no adverse effects.

URINE STUDIES: Samples collected at end of treatment period and analysed for specific gravity, pH, occult blood, protein, glucose, ketones, bilirubin, phenylketones and microscopic examination of sediment; no adverse effects.

ORGAN WEIGHTS: No adverse effects noted in absolute or relative weights of the major organs.