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HOGAN & HARTSON L.L.P.
Columbia Square
555 Thirteenth Street, N.W.
Washington, DC 20004-1109
202/637-5600

M E M O February 29, 2000

TO: Linda Kahl, Ph.D.

FROM: Martin J. Hahn

☐ For your information

☒ As you requested

☒ For your review and comment

☐ For your files

☒ Other

Dr. Kahl: Enclosed are an original color and two color copies of the GRAS Notification we provided to you last week, as well as a new GRAS Exemption Claim letter.

These color copies should replace the black and white documents filed with you last week. The Appendices 4, 5 and 6 remain unchanged and so I have not provided new copies.

Please let us know if you have any questions.

HOGAN & HARTSON
L.L.P.

MARTIN J. HAHN
PARTNER
(202) 637-5926
MJHAHN@HHLAW.COM

February 29, 2000

COLUMBIA SQUARE
555 THIRTEENTH STREET, NW
WASHINGTON, DC 20004-1109
TEL (202) 637-5600
FAX (202) 637-5910

2000 MAR -1 P 2:53

BY HAND DELIVERY

Linda Kahl, Ph.D.
Office of Premarket Approval (HFS-215)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
200 C Street SW
Washington, D.C. 20204

Re: GRAS Exemption Claim for ARASCO® (arachidonic acid-rich single-cell oil) and DHASCO® (docosahexaenoic acid-rich single-cell oil) as Sources of ARA and DHA in Infant Formula

Dear Dr. Kahl:

We are submitting this GRAS notification on behalf of our client, Martek Biosciences Corporation. This notification contains data and information that establish that DHASCO® and ARASCO®, single-cell oil sources of docosohexaenoic acid (DHA) and arachidonic acid (ARA), are GRAS when used in term infant formulas at levels up to 1.25% each of total dietary fat at a ratio of 1:1 to 1:2 (DHASCO:ARASCO). DHA and ARA are important components found in breast milk and the proposed use levels are comparable to the levels and ratios found in breast milk.

DHASCO and ARASCO may very well be the most extensively studied new infant formula ingredients. The studies summarized in this notification provide broad and compelling preclinical and clinical data establishing that DHASCO and ARASCO are GRAS sources of DHA and ARA on the basis of scientific procedures. Numerous regulatory authorities around the world, including the United Kingdom, France and the Netherlands have reviewed the data and concluded that DHASCO and ARASCO are safe and suitable sources of DHA and ARA for use in infant formulas. DHASCO/ARASCO-supplemented infant formulas,

BRUSSELS BUDAPEST* LONDON MOSCOW PARIS* PRAGUE* WARSAW
BALTIMORE, MD COLORADO SPRINGS, CO DENVER, CO LOS ANGELES, CA McLEAN, VA NEW YORK, NY ROCKVILLE, MD
\\\DC - 61954/7 - #1011667 v4
*Affiliated Office

at levels noted in this Notification, have been available outside the U.S. for over three years.

In addition, Martek convened an Expert Panel to review the available data and information on DHASCO and ARASCO. The following individuals comprise the Expert Panel: Joseph Borzelleca, Ph.D.; Gary Flamm, Ph.D.; Bruce German, Ph.D.; Walter Glinsman, M.D.; David Kritchevsky, Ph.D.; David Bechtal, Ph.D. After reviewing the available data, the Expert Panel concluded in its December 1999 report that DHASCO and ARASCO are GRAS as sources of DHA and ARA in infant formula when provided at a maximum level of 2.5% each of total dietary fat, a use level that is twice as high as that recommended in this notification.

In accordance with the criteria set forth in the GRAS notification proposed regulation found at 62 Fed. Reg. 18938, 18961 (1997), Martek submits the following information as part of its GRAS exemption claim.

Name and Address of Notifier: Martek Biosciences Corporation, 6480 Dobbin Road, Columbia, Maryland 21045.

Common or Usual Name of the Substances: The common or usual name for DHASCO is "docosohexaenoic acid rich oil." The common or usual name for ARASCO is "arachidonic acid rich oil."

Applicable Conditions of Use: DHASCO and ARASCO are intended to be used as sources of DHA and ARA in infant formulas for use by term infants. The maximum use level for these oils will be 1.25% each of total dietary fat and the ratio of their use will be from 1:1 to 1:2 (DHASCO:ARASCO).

Basis for GRAS Determination: Martek has determined after reviewing the extensive data base of information supporting the safety of DHASCO and ARASCO that they are GRAS ingredients on the basis of scientific procedures.

Availability of Data: The data and information that are the basis for the notifier's GRAS determination are available for the Food and Drug Administration's (FDA) review and copying at reasonable times at the law offices of Hogan & Hartson, L.L.P., 555 13th Street N.W., Washington DC 20004 or will be sent to FDA upon request.

GRAS Exemption Claim: The uses of DHASCO and ARASCO as sources of DHA and ARA in term infant formulas, at a maximum use level of 1.25% each of total dietary fat and at a ratio of 1:1 to 1:2 (DHASCO:ARASCO), are exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act (the act) because Martek has determined that such uses are GRAS.

* * * * *

We enclose an original and two copies of this notification for your review. If you have any questions, please contact me at the above phone number and address.

Sincerely,

Martin J. Hahn

Enclosures

cc (w/o encl.): Alan M. Rulis, Ph.D.
George H. Pauli, Ph.D.

Martek Biosciences Corporation
GRAS Notification for the use of
DHASCO and ARASCO
(single cell sources of DHA and ARA)
as Sources of the LCPUFAs in Infant Formulas
February 18, 2000

2000 MAR -1 P 2:54

EXECUTIVE SUMMARY

This notification establishes that DHASCO® and ARASCO® are generally recognized as safe (GRAS) sources of docosahexaenoic acid (DHA) and arachidonic acid (ARA), when added to infant formulas at a level of up to 1.25% each of total dietary fat at DHA:ARA ratio of 1:1 to 1:2. For infants, this use level is equivalent to 0.625% of dietary calories, about 75 mg of the oil/kg body wt/day, or about 30 mg DHA or ARA/kg body wt/day. These values are well within the normal range of DHA and ARA levels found in human milk.

DHA and ARA are long-chain polyunsaturated fatty acids (LCPUFA) that are found naturally in breast milk. DHA and ARA are considered important for the developing infant because these long chain polyunsaturated fatty acids (LCPUFAs) are the primary building blocks for the brain and retina. DHASCO and ARASCO are oils extracted from single cell organisms that are rich in DHA and ARA. DHASCO oil is a triglyceride, produced by the algae *Crypthecodinium cohnii* and is standardized with high oleic sunflower oil to contain 40% by weight of DHA. ARASCO oil, also a triglyceride, is produced by the fungus *Mortierella alpina* and also is standardized with high oleic acid sunflower oil to contain 40% by weight of ARA. In both cases, these oils contain DHA and ARA in triglyceride structures that are chemically equivalent to those delivered to infants from mother's milk.

Martek convened a panel of esteemed experts to review the available data on DHASCO and ARASCO (hereinafter referred to as the "Expert Panel") 1/ A copy of the Expert Panel's report has been submitted to FDA and can be found in the food master file number 636. After reviewing the available data and information regarding the importance of DHA and ARA in the infant diet, the Expert Panel unanimously agreed:

1) that there is a deficiency in the DHA and ARA status in infants fed formula not fortified with DHA and ARA;

2) that this is reflected in a decreased blood and other tissue (*e.g.*, brain) levels of DHA and ARA; and

3) that this deficiency contributes to the visual and neurological deficits observed in formula-fed compared with breast-fed infants.

1/ The following individuals comprise the Expert Panel: Joseph Borzelleca, Ph.D; Gary Flamm, Ph.D.; Bruce German, Ph.D.; Walter Glinsman, M.D.; David Kritchevsky, Ph.D.; David Bechtal, Ph.D.

In making these conclusions regarding the importance of DHA and ARA in the infant diet, the Expert Panel noted that numerous independent scientific bodies from around the world have recommended the addition of DHA and ARA to infant formulas. For example, the Food and Agriculture Organization (FAO) of the World Health Organization (WHO) reviewed the available data and recommended in 1994 that infant formulas be fortified with "40 mg of ARA and 20 mg of DHA per kg per day." (84). The Expert Panel also considered the findings in the 1998 Report prepared by the Life Science Research Organization (LSRO) of the American Society for Nutritional Sciences. The LSRO Panel is one of the few expert panels that failed to recommend fortification with DHA and ARA after reviewing the available data. The LSRO Panel did acknowledge, however, the importance of the LCPUFAs in the infant diet and recommended that this issue be reevaluated in the near future as new studies emerge. The Expert Panel convened by Martek reviewed studies not considered by the LSRO Panel and concluded that the weight of the evidence supported the addition of DHA and ARA to infant formulas to address the deficiency found in the infant formulas commercially available in the United States.

In addition to recognizing the importance of DHA and ARA in the infant diet, the Expert Panel also carefully reviewed the published and unpublished data from studies involving the use of DHASCO and ARASCO. The presence of DHA and ARA in breast milk establishes that these LCPUFAs are GRAS. The focus of this notification, and of the Expert Panel's review, therefore, is not on whether DHA or ARA are GRAS, but on whether the data establish that DHASCO and ARASCO are GRAS sources of DHA and ARA. After reviewing the available data, the Expert Panel concluded that DHASCO and ARASCO are GRAS as sources of DHA and ARA in infant formula when provided at a maximum level of 2.5% each of total dietary fat. The level found to be GRAS by the Expert Panel is twice as high as that proposed for use in this notification.

The Expert Panel considered numerous factors in its assessment of the GRAS status of DHASCO and ARASCO, including the chemical composition and manufacturing processes for these oils. Both oils are manufactured by a controlled fermentation process, followed by oil extraction and purification using methods common to the vegetable oil industry. All ingredients used in the processing of the oils are either food grade, or of higher quality, and the entire process meets current Good Manufacturing Practices for foods. The oils undergo rigorous analytical and quality assurance testing and meet well-defined product specifications prior to release. DHASCO and ARASCO do contain other common saturated and monounsaturated fatty acids found in human milk. Minor nonsaponifiable fractions of the oils have been characterized and contain primarily cholesterol-related sterols, which can be found in other food sources. The studies conducted on the oil and the biomass, from which the oil is extracted, establish the safety of these oils as sources of DHA and ARA.

Data also establish that DHASCO and ARASCO are absorbed in a manner consistent with other dietary triglycerides and that their supplementation in the infant formula will normalize blood DHA and ARA levels to those found in breast fed babies. The DHA and ARA are distributed throughout the body and are found at the highest levels in brain, retina, testes, and heart. DHA and ARA can be catabolized completely to CO2 and H2O, but the catabolic rate is slower than with other dietary fatty acids. This is necessary in order to maintain DHA and ARA levels in the rapidly expanding neurological tissues of infants and children.

In addition, ARA, but not DHA, serves as a precursor molecule to the omega-6 class of eicosanoids, which are well recognized as stimulators of immune function. Studies have shown that small amounts of DHA can be retroconverted to eicosapentaenoic acid (EPA) in humans, although accumulation of EPA is negligible at doses of DHASCO used for infant supplementation. Although oxidation of these highly unsaturated fatty acids in blood or tissues has been raised as a potential concern, studies have shown that DHA, in particular, activates antioxidant defense systems in the body and may actually protect against oxidation of polyunsaturated fatty acids. Animal and human studies have confirmed that supplementation with DHA and ARA protects, rather than accentuates oxidative damage. This is consistent with the finding that infants who receive breast milk (containing DHA and ARA) are more protected from Necrotizing Enterocolitis (NEC) than infants fed formulas without DHA and ARA.

A large number of safety studies have been conducted using DHASCO and ARASCO oils, including acute, subchronic, developmental and reproductive toxicology studies in rats and *in vitro* mutagenicity and clastogenicity assays with bacterial and mammalian cells. All studies were modeled after FDA Redbook guidelines and conducted at GLP-compliant laboratories. The study results must be evaluated relative to well known effects of supplementation with high doses of polyunsaturated fatty acids (PUFAs) in order to distinguish between PUFA-related effects and effects due to the sources of DHA and ARA themselves. None of the thirty-two toxicological studies undertaken indicated any toxigenicity related to these oils (summarized in Appendix 5). The No Adverse Effect Levels (NOAELs) in these studies corresponded to the highest doses tested.

Studies conducted in twelve different animal species, including nonhuman primates, have provided a large base of experience and extensive safety data with DHASCO and ARASCO oils (summarized in Appendix 1). None of the reports (published or unpublished) have suggested any toxicological or safety issues associated with the use of these oils. At least fourteen well-controlled clinical studies involving about 1,500 infants (700 infants receiving the supplemented formula) have confirmed that DHASCO and ARASCO increase circulating levels of DHA and ARA in preterm and term infants (summarized in Appendix 2). Significant improvements in growth, visual acuity and mental acuity have also been reported in infant groups supplemented with these oils and no adverse events have been reported.

Some, although not all, of the animal toxicology studies found modest increases in liver and spleen weights with very high doses of the oils that were considered statistically significant. Increased liver and spleen weights were not found consistently across the studies and the increased weights were within the normal range for liver and spleen. The modest increase in liver and spleen weights was not accompanied by abnormal histology or serum enzyme levels, which would have been expected if the increase was attributable to a toxicological effect. Moreover, when the liver and spleen weights were assessed relative to other organ weights (such as the brain), the weight changes in almost all instances were no longer apparent.

Modest increases in liver and spleen weights is a well-known effect in mammals given large doses of LCPUFAs of any source (*e.g.*, fish oils). The modest increases in liver and spleen weights are, therefore, considered to be an LCPUFA effect, and not an effect unique to the DHASCO and ARASCO. To further support the safety of DHASCO and ARASCO, studies have been conducted on the biomass in an attempt to assess whether there could be any component in

the biomass other than the purified oil (the subject of this Notification), that may be of toxicological concern. These studies consistently found the ARASCO and DHASCO biomasses to be nontoxigenic.

In addition, twenty-nine separate, well-controlled clinical intervention studies using DHASCO and/or ARASCO have been conducted on adults or children with no reported adverse effects of the treatment (summarized in Appendix 3). Two such studies with particular emphasis on safety and bioavailability were conducted by the U.S. Department of Agriculture using high doses of either ARASCO (3 g/day) or DHASCO (15 g/day) with healthy adult volunteers. The preclinical and clinical studies conducted with these oils further support their use as a safe dietary source of DHA and ARA.

The safety of DHASCO and ARASCO as sources of DHA and ARA is further supported by the highly controlled conditions under which they would be used. DHASCO and ARASCO would be added to infant formulas at levels and ratios comparable to that found in breast milk. The proposed fortification levels and ratios are consistent with the WHO/FAO recommendations and with the recommendations of other independent scientific panels. Because infant formulas are manufactured under strict controls, the levels and ratios of DHASCO and ARASCO proposed in this notification will not be exceeded.

The use of DHASCO and ARASCO in commercial infant formulas around the world is yet further evidence of the safety of these oils as sources of DHA and ARA. These oils have now been in commercial use in infant formulas in over 60 countries including the United Kingdom, France and Israel, at levels in accord with WHO/FAO guidelines. These infant formulas have been marketed for as long as three years with no reported adverse findings attributable to the DHASCO and ARASCO. One of these formulas in extensive use around the world is a Low Birth Weight (LBW) formula that is given to infants under strict supervision by doctors. This LBW formula has been introduced in 56 countries and has been provided to an estimated 100,000 LBW babies of most races, cultures and both sexes with no adverse events reported by the physicians. In addition, over 40 million capsules have been sold as dietary supplements to an estimated 250,000 individuals, primarily in the United States, with no significant adverse events reported to the Company. The large numbers of individuals (infants through adults) who have consumed the DHASCO or ARASCO oils as commercial products or in clinical trials with no adverse effects provides additional support for the establishment of GRAS status for these products at use levels that can commonly be obtained in the diet.

Based upon the historical presence of DHA- and ARA-containing triglycerides in human milk, the substantial equivalence of DHASCO and ARASCO triglycerides to those triglycerides found in human milk, and a critical evaluation and analysis of the preclinical and clinical information available on DHASCO and ARASCO, the data establish that these oils are GRAS on the basis of scientific procedures for use in supplementing the diets of infants at levels of 1.25% of dietary fat (0.625% of energy or up to 75 mg DHASCO (or ARASCO) per kg body weight per day). This corresponds to a DHA or ARA level of 0.50% of dietary fat, 0.25% of energy, or up to 30 mg DHA or ARA per kg body weight per day.

1 DHA AND ARA ARE GRAS INGREDIENTS AND DHASCO AND ARASCO ARE GRAS SOURCES OF THESE LCPUFAS

1.1 DHA and ARA are GRAS Because They are Components in Breast Milk

From an evolutionary point of view, breast milk represents the optimal source of nutrition for the human infant and it is often referred to as the "gold standard." Infant formulas are the sole source of nutrition for a human infant and should, therefore, be as nutritionally balanced as human milk. DHA and ARA are found in human milk in low, but significant quantities. The DHA and ARA content of human milks from 65 published reports around the world are given in Table 1. It is clear that the DHA content of human milk is quite variable ranging from 0.06% to 1.4% of total fat and has been shown to be dependent on the dietary DHA intake of the mother (1). Mothers with diets low in fish and other sources of DHA, but otherwise high in fat (*e.g.*, a typical Western diet), have breast milk DHA levels on the low end of the range. Women from the United States, for example, have among the lowest levels of DHA in their breast milk compared to worldwide averages (Figure 1).

To determine the optimal level of DHA in breast milk we must consider the diets to which our species evolved. Such Paleolithic diets were thought to contain much more DHA and much less total fat than the typical Western diet (2). The DHA content of breast milk, therefore, would have been much higher than it is today. Thus, an estimated optimal level of DHA in the breast milk would likewise be much higher than it is in the United States today.

Table 1. Breast milk DHA and ARA levels from women around the world.

Author/year	*Reference*	*wt % DHA*	*wt % ARA*	*Country*
Finley, et al. (1985)	(3)	0.06	0.29	USA
Harris, et al. (1984)	(4)	0.1	0.4	USA
Putnam, et al. (1982)	(5)	0.1	0.6	USA
van der Westhuizen, et al. (1988)	(6)	0.1	1	S. Africa
Sas, et al. (1986)	(7)	0.1	0.5	Hungary
Spear, et al. (1992)	(8)	0.11	0.54	USA
Sanders and Reddy (1992)	(9)	0.14	0.32	UK (vegan)
Maurage, et al. (1998)	(10)	0.14	0.24	France
Auestad, et al. (1997)	(11)	0.15	0.48	USA
Spear, et al. (1992)	(8)	0.15	0.58	USA
Okolska, et al. (1983)	(12)	0.15	1.56	Poland
Dotson, et al. (1992)	(13)	0.16	0.53	USA
Jackson, et al. (1994)	(14)	0.16	0.56	USA
Harzer, et al. (1983)	(15)	0.16	0.39	Germany & UK
Carlson, et al. (1986)	(16)	0.19	0.59	USA
Francois, et al. (1998)	(17)	0.2	0.5	USA
van der Westhuizen, et al. (1988)	(6)	0.2	0.6	S. Africa
Innis, et al. (1994)	(18)	0.2	0.5	Canada
Bitman, et al. (1983)	(19)	0.21	0.58	USA
Henderson, et al. (1998)	(20)	0.21	0.52	USA
Drury and Crawford (1990)	(21)	0.21	0.6	Hungary
Makrides, et al. (1995)	(22)	0.21	0.4	Australia

Author/year	Reference	wt % DHA	wt % ARA	Country
Makrides, et al. (1996)	(1)	0.21	0.41	Australia
Koletzko, et al. (1988)	(23)	0.22	0.36	Germany
Bitman, et al. (1983)	(19)	0.23	0.6	USA
Sanders, et al. (1978)	(24)	0.23	0.72	UK (vegan)
Genzel-Boroviczeny, et al. (1997)	(25)	0.23	0.45	Germany
Bitman, et al. (1983)	(19)	0.24	0.55	USA
Beijers and Schaafsma (1996)	(26)	0.24	0.31	Netherlands
Genzel-Boroviczeny, et al. (1997)	(25)	0.24	0.48	Germany
Martin, et al. (1993)	(27)	0.24	0.36	France
Carnielli, et al. (1998)	(28)	0.26	0.48	Netherlands
Foreman-van Drongelen, et al. (1996)	(29)	0.26	0.52	Netherlands
van Beusekom, et al. (1993)	(30)	0.26	0.47	Netherlands
Muskiet, et al. (1987)	(31)	0.27	0.6	Tanzania
Specker, et al. (1987)	(32)	0.29	0.69	USA
Hall (1979)	(33)	0.29	0.19	UK
Yu, et al. (1998)	(34)	0.29	0.46	Sweden
Sanders and Reddy (1992)	(9)	0.3	0.38	UK
Jansson, et al. (1981)	(35)	0.3	0.4	Sweden
Villacampa, et al. (1982)	(36)	0.3	0.57	Spain
Cherian and Sim (1996)	(37)	0.3	0.4	Canada
Clandinin, et al. (1997)	(38)	0.3	0.54	Canada
Babin et al. (1999)	(39)	0.31	0.5	France
Kaila, et al. (1999)	(40)	0.31	0.35	Finland
Rueda, et al. (1998)	(41)	0.32	0.52	Panama
Billeaud, et al. (1997)	(42)	0.32	0.52	France
Chardigny, et al. (1995)	(43)	0.32	0.5	France
Gibson, et al. (1981)	(44)	0.32	0.4	Australia
FAO/WHO RECOMMENDATIONS FOR FULL TERM INFANTS*	(45)	**0.33**	**0.65**	
de la Presa-Owens, et al. (1998)	(46)	0.34	0.5	Spain
Ogunleye, et al. (1991)	(47)	0.34	0.56	Nigeria
Beijers and Schaafsma (1996)	(26)	0.34	0.37	Netherlands
ISSFAL 1999 RECOMMENDATIONS FOR FULL TERM INFANTS	(48)	**0.35**	**0.50**	
Sanders and Reddy (1992)	(9)	0.37	0.35	UK
Guesnet, et al. (1993)	(49)	0.37	0.45	France
Rueda, et al. (1998)	(41)	0.38	0.69	Spain
Prentice, et al. (1989)	(50)	0.39	0.31	The Gambia
Luukkainen, et al. (1994)	(51)	0.39	0.37	Finland
de Lucchi, et al. (1988)	(52)	0.4	0.8	Spain
Jacobs, et al. (1996)	(53)	0.4	0.6	Netherlands
van Beusekom, et al. (1993)	(30)	0.4	0.5	Dominica
Clandinin, et al. (1981)	(54)	0.4	0.5	Canada
Innis, et al. (1988)	(55)	0.4	0.7	Canada
Muskiet, et al. (1987)	(31)	0.41	0.56	Surinam
Horby Jorgensen, et al. (1996)	(56)	0.43	0.47	Sweden
Muskiet, et al. (1987)	(31)	0.43	0.71	Curacao
Luukkainen, et al. (1995)	(51)	0.48	0.54	Finland
Drury and Crawford (1990)	(21)	0.49	0.57	Thailand
Innis, et al. (1990)	(57)	0.5	0.8	Canada
Ogunleye, et al. (1991)	(47)	0.53	0.36	Japan

Author/year	Reference	wt % DHA	wt % ARA	Country
Fidler, et al. (1998)	(58)	0.55	0.77	Germany
Rocquelin, et al. (1998)	(59)	0.55	0.44	Congo
Boersma, et al. (1991)	(60)	0.56	0.58	St. Lucia
Sanders, et al. (1978)	(24)	0.59	0.54	UK
Kneebone, et al. (1985)	(61)	0.71	0.64	Malaysia
Kneebone, et al. (1985)	(60)	0.9	0.47	Malaysia
Kneebone, et al. (1985)	(60)	0.9	0.57	Malaysia
Koletzko, et al. (1991)	(62)	0.93	0.82	Nigeria
Innis, et al. (1988)	(55)	1.4	0.6	Canada

* Fatty acid recommendations given in mg/kg body weight and were converted to weight % fat assuming that term infants consume 110 kcal/kg body wt/day and that 50% of calories in formula are from fat.

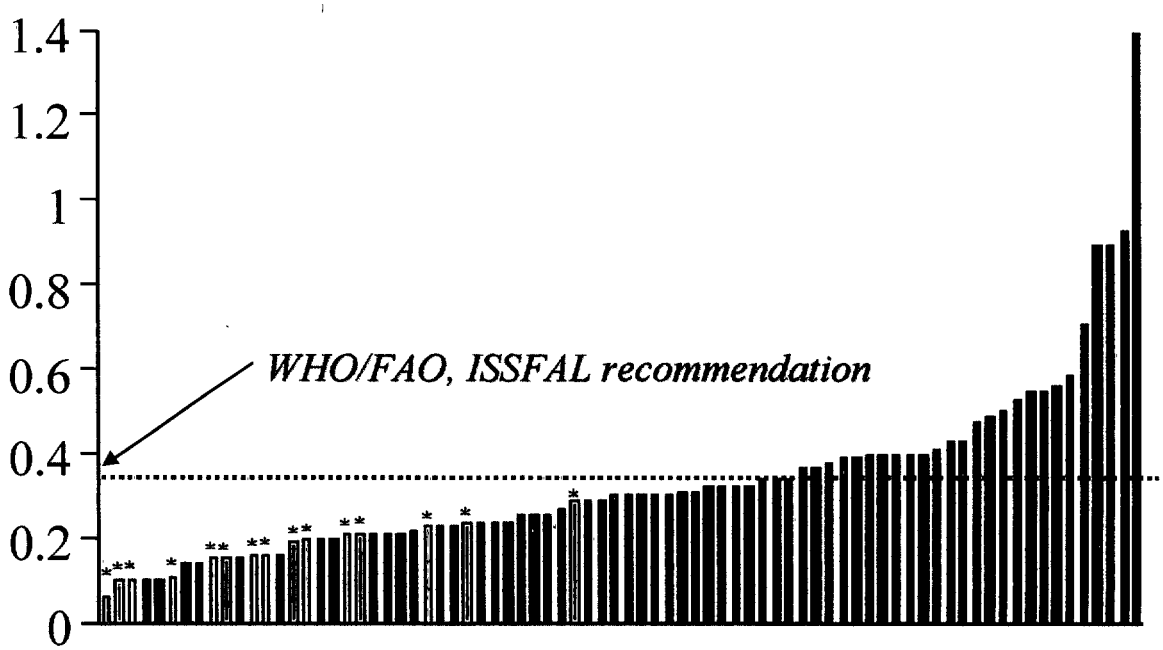

Figure 1. DHA content (% of total milk fat) from the 65 separate references in Table 1 arranged in ascending order. * indicates values from the United States.

1.2 The DHA and ARA in DHASCO and ARASCO are Structurally Similar to the DHA and ARA in Breast Milk.

DHA and ARA are found in both triglycerides and phospholipids in human milk. However, breast milk fat is primarily triglyceride (*ca.* 98%), with only about 1% phospholipid, and 1% nonsaponifiable fats such as cholesterol and phytosterols (63). Although the DHA level in the phospholipid fraction of breast milk is generally higher than in the triglyceride fraction (1.4% *vs.* 0.2% respectively) (63), there is substantially less of the phospholipid fraction in the breast milk. The vast majority of DHA in breast milk, therefore, is in the triglyceride, rather than the phospholipid, form (*i.e.,* it is estimated that 6.6% of the DHA in breastmilk is a phospholipid while 93.3% is triglyceride).

The lowest DHA content of a triglyceride molecule is 33% (*i.e.,* one DHA per three fatty acids on the triglyceride). The DHA molecule may be on the outside position of the triglyceride (sn-1 or sn-3) or on the inside position (sn-2) (see Figure 2). If it is on the sn-1 or sn-3 position, it will be cleaved by the infant's pancreatic lipases and enter the gut wall as a free fatty acid. If it is on the sn-2 position, it may be absorbed as the sn-2 monoglyceride.

DHASCO oil contains about 40-50% DHA by weight. The most abundant triglyceride molecule in DHASCO has only one DHA per triglyceride as is the case for breast milk. However, the possibility exists for two DHA molecules to exist on the same triglyceride in DHASCO (Figure 2). For those triglycerides that contain two DHA molecules, the DHA will still be absorbed either as the free fatty acid or as the monoglyceride (in a fashion identical to that of the DHA-triglyceride from mother's milk) after processing by the baby's lipase in the gut. The same holds true for the ARA in ARASCO which also may be present in a form with two ARA molecules on the triglyceride although the most common triglyceride structure will have only one ARA (Figure 2).

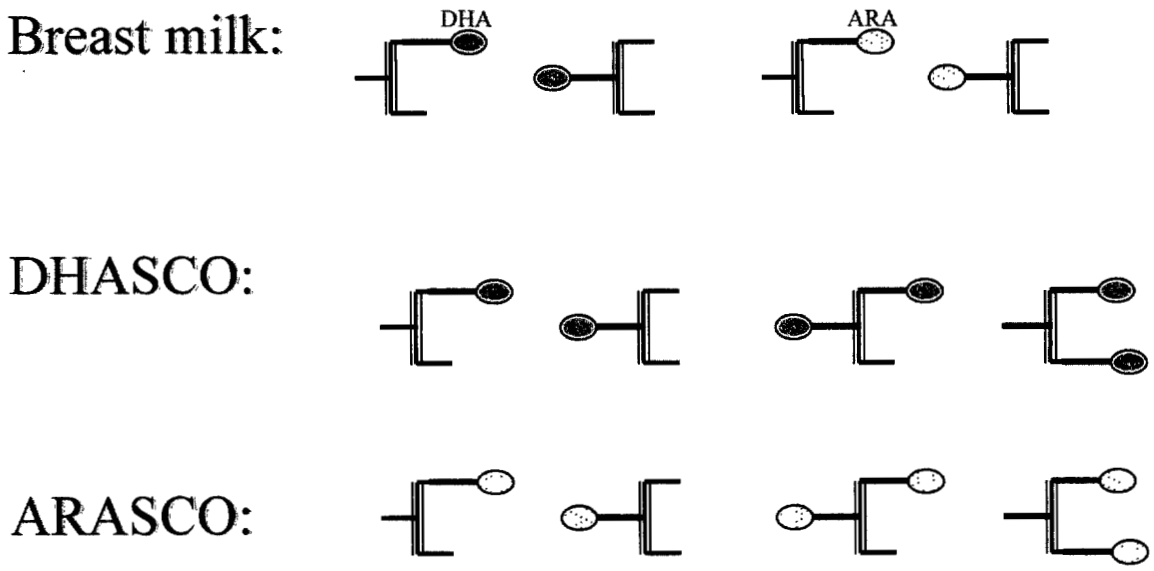

Figure 2. Triglyceride structures in human breast milk and DHASCO and ARASCO prior to cleavage in the gut of an infant by lipases.

2 THE IMPORTANCE OF DHA AND ARA IN INFANT NUTRITION

Before assessing the safety of DHASCO and ARASCO, it is useful to review the importance of dietary DHA and ARA from any source for infants. The Martek Expert Panel unanimously agreed that there is a biochemical deficiency in infants receiving formulas with no supplemental DHA and ARA relative to infants being fed their own mother's milk -- the gold standard for infant nutrition. This biochemical deficiency is reflected in abnormally low levels of circulating DHA and ARA in the blood of unsupplemented formula-fed babies relative to breast-fed babies.

There also is clinical evidence to suggest that there may be both short term and long term detrimental consequences as a result of these low levels of DHA and ARA.

Over the past twenty years, there have been a large number of retrospective studies comparing the neurological outcomes of breast-fed and formula-fed infants. A recent meta-analysis of the most relevant of these studies has indicated that there is a consistent 3-4 IQ point advantage to the breast-fed infants even after the contributions of all other recognized confounding factors had been removed (64). A summary of the data from this paper indicates a consistency across the studies in the meta-analysis (Figure 3a) as well as a dose response in terms of duration of breast feeding (Figure 3b). This latter observation implicates some factor in the source of nutrition as being a causative agent for the improved intelligence scores later in life. Several researchers have speculated that LC-PUFAs, or specifically DHA, may be that factor (65, 66). Breast-fed babies, however, are getting many nutrients from the breast milk besides DHA and ARA, and one can argue that, based on these data alone, the specific contribution of DHA and ARA to improved long term IQ is inconclusive. One observation, however, is very clear and consistent: infants who are provided standard infant formulas (*i.e.,* without supplemental DHA and ARA) have significantly lower levels of DHA in the cell membranes in their blood and brain relative to breast-fed babies.

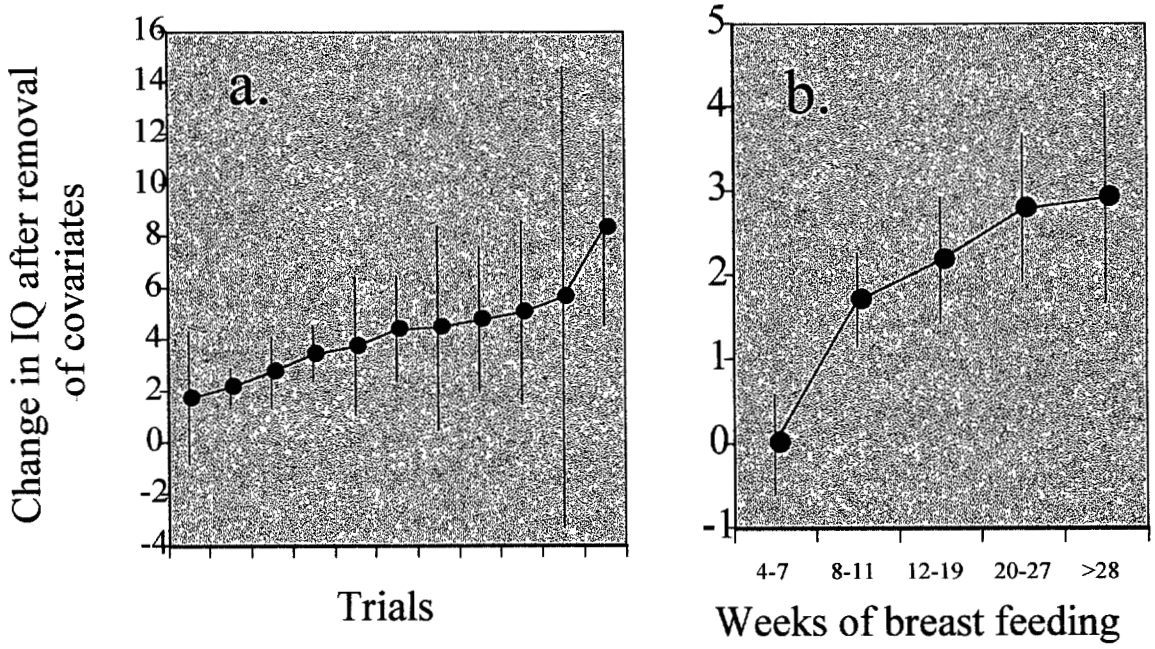

Figure 3. Meta-analysis of a group of studies comparing the neurological outcome of breast-fed vs. formula-fed infants. (a) differences in IQ of children who were breast-fed as babies vs. a formula-fed group after the contributions of various influential covariates have been removed; and (b) the effect of duration of breast-feeding on the IQ difference between breast-fed and formula-fed infants. Data is from Anderson *et al* (65)

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2.1 Biochemical Outcomes

Preterm and full term infants fed standard, unsupplemented formulas have a circulating DHA and ARA status (as indicated by red blood cell or plasma phospholipid levels of these fatty acids) about one-half that of breast-fed infants (10, 22, 29, 38, 67-79). Furthermore, the brain DHA levels of formula-fed infants are about one-third lower than those of breast-fed infants (80, 81). Table 2 lists 32 controlled clinical studies involving over 2,600 infants (over 1,300 received the LC-PUFA-supplemented formulas) performed over the last 15 years (17 studies with term infants and 15 studies with pre-term infants). Each study compared outcomes of standard formula-fed infants with DHA-supplemented formula-fed infants and, in most instances, also with breast-fed infants. In every study reporting blood fatty acids levels, the DHA status of the infants was returned to normal (as defined by the DHA status of breast-fed infants) when the formulas were supplemented with DHA. In all of these studies, the ARA levels were also normalized when supplemental ARA was used in the formulas as well. Of all the trials completed with DHA/ARA supplementation in Table 2, more infants have received DHASCO or ARASCO (573 infants) than any other sources of DHA and ARA (378 received egg yolk and 417 received various fish oils).

In assessing the importance of these changes in the blood and brain biochemistry in the formula-fed infant, it is crucial to more fully understand the function of DHA in the tissues of the body. Recent studies have revealed that DHA has many critical functions in the normal development and metabolism of neuronal cells. These include, but are not limited to: 1) the control of normal migration of neurons from the surface of the ventricles of the brain to the cortical plate during brain development (82); 2) the control of the normal resting potential of the neurons and cardiac cells by regulation of sodium and calcium channels (83); 3) the regulation of the packing density of certain membrane proteins such as rhodopsin in the retina (84); and, 4) the regulation of levels of certain adrenergeneric and serotonergic neurotransmitters (85, 86). With such key roles in normal neuronal development and function, it is quite plausible that abnormally low levels of this primary nutrient, DHA, during crucial periods of brain development, could lead to the long term neurological deficits observed in formula-fed infants relative to breast-fed infants (66, 87).

In many of the clinical trials listed in Table 2, the study protocols involved using the supplemented formulas only for the first 30-120 days before switching back to routine formulas. Interestingly, blood lipid assessments at one year of age have shown that the babies who had the DHA/ARA supplemented formulas only for these initial few months, still maintained their high DHA status in a fashion similar to the breast fed babies, in spite of many months on unsupplemented formulas.

2.2 Visual Outcomes

Of the 32 DHA/ARA supplementation studies shown in Table 2, thirteen analyzed visual outcomes (*e.g.,* visual acuity as measured by visual evoked potential or Teller Acuity Cards) as a primary endpoint. Nine of these studies included a breast-fed infant control group (11, 22, 69, 75, 77, 79, 88-92). In every study that reported a difference in visual acuity between breast-fed and formula-fed infants (22, 69, 75, 78) (*i.e.,* where the babies fed standard formula had a visual deficit compared to breast-fed babies -- the gold standard), this deficit was overcome by adding DHA/ARA to the infant formula. That is, the infant groups receiving DHA/ARA-supplemented formulas where DHA and ARA were provided at the same levels as found in breast milk, did not

Table 2. Thirty-two infant clinical intervention trials with DHA/ARA-supplemented formulas provided by egg yolk, fish oil, or DHASCO/ARASCO single cell oils (SCO).

Study	*Ref.*	*PUFA Source*	*Study Size*[2] *(n)*	*Suppl'd Infants (n)*	*Are Babies' Blood Lipids Normalized with DHA/ARA?*		*Are Functional Outcomes Normalized with DHA/ARA?*	
					DHA	ARA	Visual	Neurological
PRETERM STUDIES								
Koletzko (1989)	(67)	egg	29	8	yes	yes	not tested	not tested
Clandinin (1992)	(93)	fish	32	12	yes	no	not tested	not tested
Hoffman (1993)	(69)	fish	51	28	yes	no	**yes**	not tested
Carlson (1993)	(94, 95)	fish	67	33	yes	no	**yes**	**yes**
Carnielli (1994)	(70)	SCO	16	5	yes	yes	not tested	not tested
Foreman (1996)	(29)	SCO	43	15	yes	yes	not tested	not tested
Boehm (1996)	(71)	egg	41	12	yes	yes	not tested	not tested
Carlson (1996)	(96, 97)	fish	59	26	yes	no	**yes**	**yes**
Koletzko (1996)	(88)	egg	57	18	n.g.	n.g.	no difference	**yes**
Hansen (1997)	(89)	SCO	284	113	yes	yes	no difference	not tested
Clandinin (1997)	(38)	SCO	91	48	yes	yes	not tested	not tested
Vanderhoof (1997)	(98)	SCO	287	60	yes	yes	not tested	not tested
Carnielli (1998)	(28)	SCO/egg	77	38	n.g.	n.g.	not tested	not tested
Carlson (1998)	(99)	egg	119	34	yes	yes	not tested	not tested
Ryan (1999)	(100)	fish	63	31	yes	no	not tested	not tested
TERM STUDIES								
Kohn (1994)	(73)	egg	n.g.	n.g.	yes	yes	not tested	not tested
Agostoni (1995)	(74, 101)	egg	86	27	yes	yes	not tested	**yes**
Decsi (1995)	(102)	egg	22	12	yes	yes	not tested	not tested
Makrides (1995)	(22, 92)	fish	55	12	yes	no	**yes**	not tested
Carlson (1996)	(75)	egg	58	19	yes	yes	**yes**	not tested
Innis (1996)	(76)	fish	131	68	yes	no	not tested	not tested
Koletzko (1996)	(91)	egg	53	32	n.g.	n.g.	no difference	**yes**
Gibson (1997)	(77)	SCO	113	45	yes	yes	not tested	not tested
Auestad (1997)	(11, 103)	egg	197	89	yes	yes	no difference	no difference
Gibson (1997)	(77)	fish	67	40	n.g.	n.g.	no difference	not tested
Bellu (1997)	(104)	egg	123	60	n.g.	n.g.	not tested	not tested
Willatts (1998)	(105)	egg	44	21	n.g.	n.g.	not tested	**yes**
Birch (1998)	(78)	SCO	108	45	yes	yes	**yes**	**yes**[3]
Horby (1998)	(79)	fish	56	26	yes	no	no difference	not tested
Maurage (1998)	(10)	fish	83	47	yes	yes	not tested	not tested
Carlson (1999)	(90)	fish/SCO	335	223	yes	yes	no difference	no difference
Makrides (1999)	(107)	Fish/egg	146	55	Yes	Yes	not tested	not tested

2 Total number of infants enrolled in the study.

3 The results of the cognitive portion of the study are unpublished, but were presented at the ISSFAL/NIH workshop in Washington D.C. in April, 1999 and are in press (106).

display any visual or mental deficits, and they had significantly better visual and neurological outcomes compared to babies from the unsupplemented formula-fed groups. The magnitude of the visual improvement in one study of term infants supplemented with DHASCO and ARASCO was equivalent to "one line on an eye chart" after one-year (78).

Two additional studies (in which there was no breast milk control) showed improved visual acuity in infants fed DHA-supplemented formulas compared to infants fed standard formula. In most of the studies the formula-fed infants eventually "caught up" with the breast-fed infants when the cruder measures of visual acuity were used. However, differences were still evident even after three years of age when finer measures of visual perception, such as stereo acuity, were used (108). Among the remaining studies, no statistically significant differences were found between formula-fed and breast-fed babies and, therefore, no effect of DHA/ARA supplementation was observed.

A closer look at the experimental design in the studies not showing an improvement in the DHA/ARA supplementation group may at least in part explain the lack of statistically significant effects of DHA/ARA supplementation. For example, in one of the cases (11), only a small amount of DHA was used in the supplementation (less than one third the level of the typical recommendations outlined in section 2.7 below), and the breast milk DHA content of the nursing mothers was remarkably low compared to worldwide norms (see Table 1 and Figure 1). In another case (89), the period of supplementation was short (average one month - during hospital stay of the preterm infant). In the other cases, the sample size may simply have been too small, the variability too large, or the test metric not sensitive enough to identify any significant differences between groups. This is particularly obvious in Horby-Jorgensen *et al.* (79) where there was a clear trend to improved visual acuity, but the difference did not reach statistical significance with the small sample size.

2.3 Neurological Outcomes

Of the 32 DHA/ARA supplementation studies shown in Table 2, nine reported neurological outcomes (*e.g.*, tests for visual memory and attention, problem solving or standardized developmental tests like the Bayley Scales of Infant Development) as a primary endpoint. Six of those nine reported statistically significant improvements in neurological/cognitive outcomes in infants fed formulas supplemented with DHA or DHA/ARA compared to infants fed standard formula.

As with visual outcomes, in every study detecting a deficit in standard formula-fed infants compared to breast-fed infants, the deficit was overcome by including DHA/ARA in the infant formula, and the babies receiving the DHA/ARA-supplemented formulas performed significantly better than the infants receiving standard formula. One of the studies (11, 103) which did not show a statistically significant difference in neurological outcome between formula-fed (supplemented or unsupplemented) and breast-fed babies, as mentioned above, had only a very low level of DHA available to the babies either in the supplemented formulas or in the breast milk of the nursing mothers, and consequently, no difference was detected between any of the groups. One other study (90) showed a clear trend toward improved Bayley scores in the DHA/ARA supplemented infants, but the difference did not reach statistical significance at $p<0.05$.

New data on neurological assessments in the study by Birch and colleagues (78) was recently presented at a workshop in Washington D.C. (109) and is included in Table 2. These data (106) indicated that at the 18-month assessment of these babies, the infants fed standard formula exhibited a statistically significant seven-point deficit in developmental quotient on the Bayley Mental Development Index assessment relative to infants fed DHASCO/ARASCO-supplemented formulas. Once again, the standard formulas were shown to result in a neurological deficit that was overcome by the addition of DHASCO and ARASCO. This study is expected to be published in the near future. Once it is published, we will place a copy in our food ingredient master file, number 636, where it will be easily accessible for the agency's review.

2.4 Reports Published Since the Expert Panel Review that Failed to Show Improvements in Visual and Neurological Outcomes

Since the review of the available data and information by the Expert Panel, two additional publications appeared in the peer-reviewed literature which address the issue of DHA/ARA supplemented infant formula. Neither of these studies used DHASCO or ARASCO as sources for DHA and ARA. The studies used phospholipid sources of DHA and ARA and, in both studies, the authors reported no statistically significant improvements in visual or neurological development with the supplemented infants.

One study, by Lucas *et.al* (110), involved 309 full-term infants which were randomized and placed into two groups. The first group received a standard infant formula, while the second group received a similar formula but with the addition of 0.30% ARA and 0.32% DHA in the form of an egg phospholipid and a triglyceride fraction (detail not provided in the paper) for the first 6 months of life. About 125 babies from each group were evaluated at 18 months of age using the Bayley Scales of Infant Development (Bayley II) as the primary outcome parameter. The authors found that the infants fed the DHA/ARA-supplemented formulas performed better by 1.0 point on the Bayley MDI but that this was not statistically significant (Figure 4).

This paper has been criticized because of the unusual nature of the DHA/ARA supplementation and, unlike the 36 previous trials shown in Table 2, the authors had no measure or indication to confirm that the DHA and ARA from this novel source were even absorbed by the infants. Without blood samples confirming the uptake of the DHA and ARA, it is not possible to conclude that DHA and ARA have no effect on neurodevelopment. Indeed, there were many other biochemical differences between the two formulas other than the DHA and ARA which would further confound the issue. Finally, these authors were even unable to demonstrate a difference between breast-fed and formula-fed infants with respect to the 18-month MDI. This, again, is contrary to most of the published literature including the author's own data of several years earlier (87).

A second publication, out of Gibson's laboratory (111) reported the visual and mental developmental data from an infant formula trial described in Table 2 (96). In this small trial, 68 full term infants were randomly assigned to either a standard formula group, a group given formulas supplemented 0.35% DHA from tuna oil, or a group given 0.34% DHA and 0.34% ARA from egg yolk phospholipid. The authors used the same commercial source of infant formula as in the Lucas study (110). Visual acuities were assessed at 16 and 34 weeks of age with a sweep

VEP, and mental acuities were assessed at 1 and 2 years of age using the Bayley Scales of Mental Development (Bayley I).

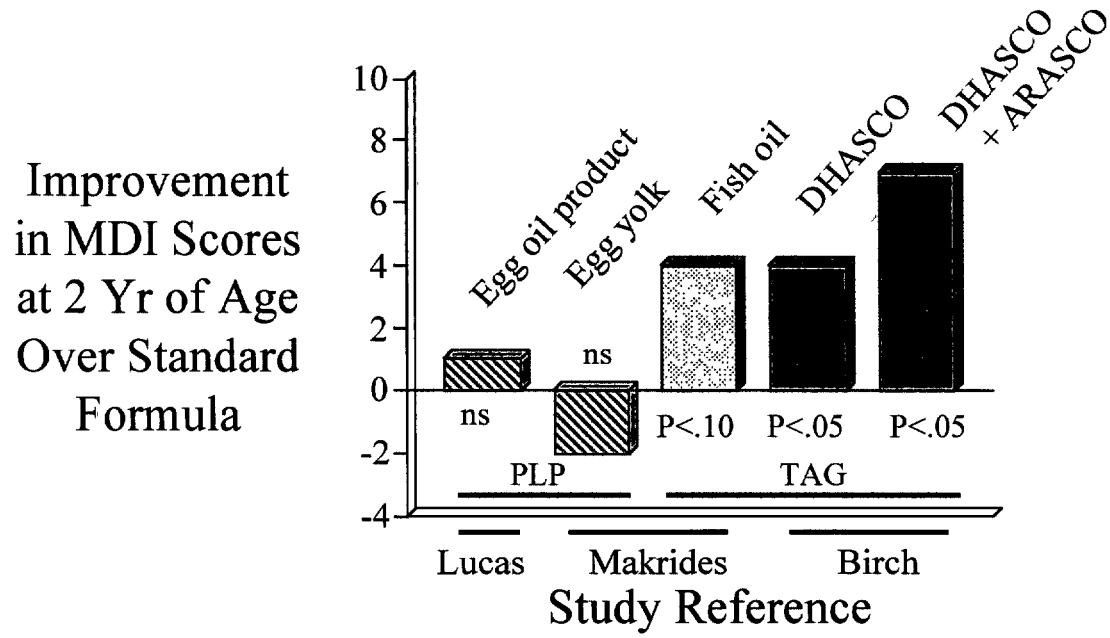

Figure 4. Change in cognitive development measured by the Bayley Mental Development Index in three independent studies using different sources of DHA and ARA supplementation compared to infants fed standard unsupplemented formulas. Lucas (110) and Makrides both used a special egg yolk composition from Nestle (PLP); Makrides (111) also used a triglyceride source (TAG) of DHA from tuna oil; Birch (106) used triglyceride forms (TAG) of DHA and ARA from Martek's DHASCO and ARASCO.

As reported in Table 2, no differences in visual development were observed between any of the formula-fed groups. However, breast-fed infants did demonstrate better visual acuity at 34 weeks of age compared to either of the formula-fed groups. Fully breast-fed infants also scored 6 points higher on the Bayley MDI at 1 year of age and 16 points higher at 2 years of age compared to standard formula-fed infants. Infants fed formulas supplemented with the DHA in triglyceride form (tuna oil) scored 4 points better than those fed standard formulas at both 1 and 2 years of age (Figure 4). With the small sample size, however, the difference between the two formula groups (+/- DHA) only reached a significance level of $P<0.10$. Those infants fed the egg yolk phospholipid based formula, however, actually scored 2 points worse than infants fed standard formulas at both 1 and 2 years of age.

The data of both the Lucas study (110) and the Makrides study (111) are presented in Figure 4 for comparison with the data generated from Birch *et al* (106). Both Lucas (110) and Birch (106) used the Bayley II scales which have been corrected for certain factors and are generally

considered to be more accurate than the old Bayley I scales used by Makrides (111). Although both Birch (106) and Makrides (111) found the same 4 point advantage in the DHA alone supplementation, the tighter standard deviation of the Birch study (106) allowed a statistical significance of $P<0.05$ whereas the Makrides data (111) only reached ($P<0.10$). These data suggest that there may be a significant influence of the form of supplementation. The egg yolk phospholipids of Lucas (110) and Makrides (111) do not seem to provide the benefits seen from the triglycerides forms of DHA and ARA (106, 110). Perhaps this is not surprising as most of the DHA and ARA delivered to infants from mother's milk is in the form of triglyceride, and it is well known that the route of metabolic processing of dietary phospholipids is different from that of dietary triglycerides. Given the differences in the sources of DHA and ARA, it is difficult to predict what, if any, relevance these two studies have on the efficacy of DHASCO and ARASCO in the infant diet.

Although these studies failed to find a significant benefit with DHA and ARA supplementation in the infant diet, they both concluded that DHA and ARA could be safely added to infant formula. The studies, therefore, are further evidence that DHA and ARA can be safely added to infant formulas.

2.5 Growth/Morbidity/Mortality Outcomes

A recent study by Diersen-Schade *et. al.* (112) demonstrated that DHASCO and ARASCO-fortified preterm infant formulas enhanced the growth of preterm infants compared to standard, unsupplemented preterm formulas (Figure 5). Not only was the growth rate enhanced, but by 57 weeks postmenstrual age, the DHASCO/ARASCO-supplemented formula-fed preterm infants attained a weight comparable to a full-term infant who was fed human milk. Another study with term infants fed DHA/ARA-supplemented formula similarly found improved growth in the supplemented infants compared to the standard formula controls (90).

To the best of our knowledge, only one study has investigated the effect of DHA/ARA supplemented formulas on one of the most serious issues of morbidity and mortality affecting preterm infants. Carlson *et. al.* (113) recently reported that there was a highly significant 83% reduction in the incidence of Necrotizing Enterocolitis (NEC) in preterm infants who were fed formulas supplemented with DHA/ARA (using egg yolk lipids) compared to those receiving standard preterm formulas. Preterm infants who receive breast milk also have a significantly lower incidence of NEC compared to formula-fed infants. In other words, preterm infants who are receiving enteral nutrition that does not contain DHA and ARA (*i.e.,* all formulas in the U.S. today) are at a much higher risk for NEC than otherwise. These data were consistent with an animal (rat) model of NEC established by Caplan and colleagues (114). In this case, animals supplemented with DHASCO and ARASCO as sources of DHA and ARA in a dietary formula also exhibited a similar, highly significant reduction of NEC when stressed, compared to pair matched animals receiving a standard formulation.

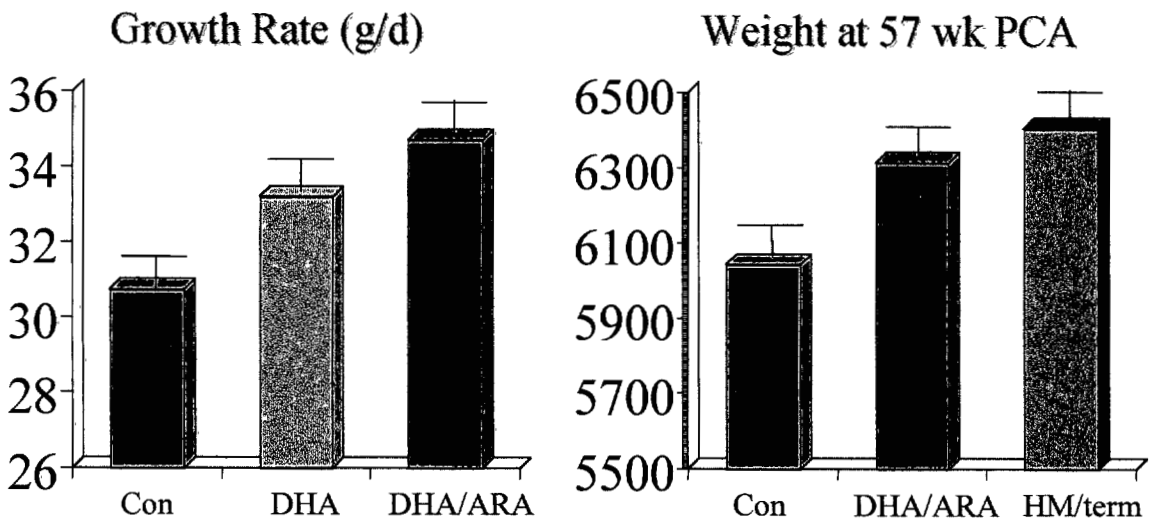

Figure 5. Growth rates and attained weights at 57 weeks postconceptual age (PCA) of preterm infants fed formulas enriched with DHASCO alone, or a combination of DHASCO and ARASCO. The attained weight of a full term infant fed human milk is shown for comparison. Data redrawn from Hansen *et al* (89).

2.6 Reports Inconsistent With the GRAS Status of DHA and ARA from Other Sources

We are unaware of any reports that are inconsistent with the GRAS status of ARASCO and DHASCO for use in infants, children or adults. However, there have been three reports in the literature noting potential negative findings in human infants when fed an infant formula using fish oil as a source of DHA. It is important to note that all fish oils contain EPA, a fatty acid expressly absent (by design) from DHASCO, and did not contain supplemental arachidonic acid. DHASCO and ARASCO also do not contain any omega-6 docospentaenoic acid (DPA), a fatty acid which antagonizes the absorption of DHA and is found in some microbial species such as *Schizochytrium* and *Thraustochytrium* (115). Although these findings involve materials that are not the subject of this GRAS affirmation, these reports are reviewed below.

2.6.1 Effects on growth

In one of the first clinical studies in which DHA supplementation was tested, a fish oil rich in EPA was used as a DHA source (0.3% EPA and 0.2% DHA). This formula was provided to preterm infants through nine months past their term date (94). No supplemental ARA was provided. Carlson and co-workers reported that infants given this formula exhibited an even greater decline in serum ARA compared to infants fed standard formulas, and they measured a reduced growth rate in those infants. Upon further analysis, the authors concluded that there was a significant correlation between the growth of the infants and their ARA status (116). Because EPA is a well-known antagonist of ARA metabolism, it was not surprising that providing an

EPA-rich fish oil to an infant would further compromise the infant's ARA status, already low because of the lack of dietary ARA in the formula, and negatively impact growth.

Subsequent studies indicated that ARA may affect infant growth through ARA-derived eicosanoids that elevate the levels of growth hormone (117). Two subsequent clinical studies (100, 118) which both used fish oil with a lower EPA content (formulas contained 0.04 - 0.07 wt % EPA and 0.2 wt % DHA) but no supplemental ARA, also reported reduced growth, albeit less pronounced than the earlier Carlson study with the higher EPA fish oils. In the case of Ryan and coworkers (100), the effect was limited to boys. A more recent study with term infants using fish oil supplemented formulas showed no effect on growth (107).

Of the 14 clinical trials with infants fed formulas containing DHASCO as a source of DHA (there is no EPA or omega-6 DPA in DHASCO), there has not been a single report of any negative impact on growth, even in groups where DHASCO was used without any additional ARA (see Appendix 2 for details). In fact, two studies have now reported that the combination of DHASCO and ARASCO actually improved growth of preterm (112) as well as full term infants (90) compared to infants fed control formulas.

These reports cited above suggest that EPA at any level is an undesirable component of infant formulas. This fact was also recognized by a recent expert workshop in Washington DC which not only recommended the addition of DHA and ARA to infant formulas, but established an upper limit (not to exceed) for EPA in infant formulas (109) because of this problem. One of the main reasons that DHASCO has been chosen over fish oil as a source of DHA in infant formulas sold outside of the United States, is that DHASCO contains no EPA.

2.6.2 Language development in infants

In another major study, language development at 14 months of age was reported to have been negatively affected in the group receiving a fish oil-supplemented formula (103). The authors subsequently reported that this difference was no longer present at three years of age (119). Furthermore, this negative effect was only associated with the fish oil (EPA-containing) formula without supplemental ARA since no such effect was observed in a parallel arm using a formula containing both DHA and ARA. In fact, there was a three-point improvement in the developmental quotients (DQ) of the DHA/ARA supplemented children (119) although the variability and sample size for this study was such that this difference did not reach a statistical significance of $P<0.5$.

There have been <u>no</u> negative reports on language development in the 14 trials undertaken using DHASCO/ARASCO supplemented formulas. On the contrary, it has recently been reported that at 18 months of age there is a seven point improvement in the developmental quotients of infants who received DHASCO/ARASCO supplemented formulas compared to those receiving standard formulas as assessed by the Bayley scales of development (106). Vocalization and language skills are important components of this test.

2.6.3 SIDS

The aforementioned trial which noted reduced growth in boys fed formulas supplemented with a fish oil, also reported more SIDS-related deaths in the group fed the fish oil (EPA-containing) supplemented formula with no ARA, compared to the control group (100). An independent safety committee, which included medical specialists with expertise in SIDS, carefully reviewed each case and concluded that none of the SIDS deaths were related to the dietary treatments. In support of this opinion, none of the other trials (more than 30 to date involving over 2,500 babies) where DHA and ARA supplemented formulas have been used, regardless of the sources, have noted any similar observation. Breastfeeding, which supplies DHA and ARA to the infant is associated with a reduced risk of SIDS (120, 121), not an increased risk. Furthermore, there have been no reports of frequencies of SIDS changing in countries where DHA and ARA supplemented formulas have been in use for several years (*e.g.,* Israel). There have been no reports of such adverse events in postmarket surveillance after launching DHASCO/ARASCO supplemented formulas (*cf.* Wyeth Ayerst Research, personal communication), nor have there been any concerns reported by physicians worldwide, monitoring an estimated 100,000 LBW infants who have been given DHASCO/ARASCO supplemented formulas.

2.6.4 Bleeding times

Although there have been no reports of excessive bleeding in controlled infant formula trials using fish oil-supplemented formulas, there are observations in children and adults of increased bleeding times when consuming fish oils (122-124). This, again, is entirely consistent with an effect of the eicosanoid precursor – EPA – contained in fish oil, downregulating the omega-6 eicosanoids PGE2 and TXA2, both of which are important in stopping bleeding in response to an injury. Because this is an EPA-mediated response, and neither DHASCO nor ARASCO contains EPA, this issue does not apply to these products. Nevertheless, bleeding times were monitored in a well-controlled, USDA-sponsored study in adults maintained in a metabolic ward and fed 15 g of DHASCO every day for a period of 90 days. At the end of the trial, as expected, there were no changes in platelet aggregation or prothrombin bleeding time in the DHASCO-treated subjects compared to the placebo controlled group (124). In a large well controlled clinical trial with full term infants given DHASCO/ARASCO supplemented formulas, bleeding times were specifically monitored and also found not to change relative to standard formula-fed infants (98).

2.7 Numerous Independent Expert Panels Have Recommended the Addition of ARA and DHA to Infant Formulas

Several Expert Panels have been convened over the last 10 years to assess whether infant formulas should contain long chain polyunsaturated fatty acids (*i.e.,* DHA and ARA). There has been general agreement among all of them regarding the importance of DHA and ARA in infant nutrition, but each panel approached the issue with different questions or objectives in mind. Such questions ranged from the allowance of such supplements to their absolute requirement in all infant formulas. The most recent Panel actually established what it considered to be Adequate Intake (AI) levels of DHA and ARA (*i.e.,* those levels required to support normal brain growth and development) for infants. The various Panels' recommendations are listed below.

2.7.1 European Society of Pediatric Gastroenterology and Nutrition (ESPGAN) (1991)

In an ESPGAN Committee Report commenting on the content and composition of lipids in infant formulas (125), the importance of long chain polyunsaturated fatty acids (LCP) in infant nutrition was recognized. It noted that both preterm and term infants fed standard formulas *"develop LCP-depletion of structural lipids"* and that animal studies showed that prolonged omega-3 deficiency *"reduces DHA content in the brain and retina and impairs development of visual acuity, and possibly also of discrimination learning"*. They concluded that *"during this period of life, LCP are therefore considered essential nutrients that should be supplied with the diet."* The Committee felt that *"enrichment* [of low birth weight infant formulas] *with metabolites of both linoleic acid* [*i.e.,* ARA] *and alpha linolenic acid* [*i.e.,* DHA] *approximating levels typical of human milk lipids (n-6 LCP* [ARA] *1%, n-3 LCP* [DHA] *0.5% of total fatty acids) is desirable"*. They went on to say that *"LCP supplementation to* [term] *infant...might also be of advantage, but further data on this question are required prior to a definite recommendation"*. Over the intervening years, such data have been generated.

2.7.2 British Nutrition Foundation (1992)

In 1992, a Task Force of Experts were invited by the Council of the British Nutrition Foundation to review the state of knowledge and make recommendations on the nutritional and physiological significance of unsaturated fatty acids (126). One section of that report relating specifically to unsaturated fatty acids in early development recognized the importance of the delivery of DHA and ARA to the growing infant *in utero* by transplacental transfer and, during nursing, via breast milk. Much of this recognition was based on *"evidence from animal studies, which suggested that retinal function and learning ability are permanently impaired if there is a failure in the accumulation of sufficient DHA during development"*. They concluded with recommendations that *"it would be prudent for DHA to be present in <u>all</u> infant formulae at the same level as it is present in human milk (i.e., 0.2% of total energy)"*. This corresponds to about 20 mg DHA/kg/day or about 50 mg DHASCO/kg/day. Furthermore, the Task Force recommended *"that preterm infant formulae should contain DHA at a level of 20 mg DHA/kg/day"*. Finally, the Task Force also recommended *"that infant formulae should contain preformed arachidonic acid in an attempt to replicate the fatty acid profile of human milk"*.

2.7.3 World Health Organization/Food and Agriculture Organization (1994)

In October of 1993, an Expert Consultation was established jointly between the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) to review the latest scientific evidence and make appropriate recommendations on the role of dietary fats in human nutrition in order to assist policy makers, health care professionals, the food industry, and consumers. The report, published the following year (45), includes a chapter on lipids in early development where they note that in addition to rodent studies, *"studies with nonhuman primates confirm that n-3 deficiency depresses the development of retinal function and visual acuity"*. Throughout the discussion, they underscore the need to maintain adequate DHA and ARA supplies to the infant, from the preconceptual preparation of the mother to the post-partum nursing of the infant. The Expert Consultation recommended that *"preterm formula should provide 60 mg of ARA, and 40 mg of DHA per kg body weight per day"*. For term infants, the

recommendation was for "*40 mg of ARA and 20 mg of DHA per kg per day*". This latter recommendation would correspond to 100 mg ARASCO/kg/day and 50 mg DHASCO/kg/day. The Consultation goes on to say that "*these levels were suggested to provide the greatest possible release of the full genetic potential of neural and visual development of the infants*".

2.7.4 International Society for the Study of Fatty Acids and Lipids (ISSFAL) (1994)

In June of 1994, a subcommittee of the Board of ISSFAL approved a series of recommendations for the essential fatty acid requirements for infant formulas. These followed the general tenet that "*human milk is the best and only proven source of fat and essential fatty acids in the infant diet*". ISSFAL recognized that "*preterm infants fed soy oil supplemented formulae have altered electroretinograms and delayed visual maturation relative to those fed DHA supplemented formulas or human milk. These changes are correlated to biochemical indices of low DHA status*". The report goes on to recommend that "*formulas for these* [preterm] *infants should provide 60-100 mg/kg/day as preformed arachidonic acid*", and "*should provide 35-75 mg...per kg body weight per day as DHA*".

Although these recommendations were detailed for preterm infants, the statement goes on to acknowledge that "*In general, the specific recommendations given above are applicable to term infants; however, the requirements for long chain polyunsaturates (AA and DHA) for term infants await the results of clinical trials that are now in progress*". Those studies have now been completed and they resulted in the new recommendations (see below) issued five years later in June of 1999 (109).

2.7.5 ISSFAL/NIH Workshop on the Essentially of and Recommended Dietary Intakes for Omega-6 and Omega-3 Fatty Acids (1999)

In April of 1999, an International workshop sponsored by NIH, The Center for Genetics, Nutrition and Health, and ISSFAL, and co-sponsored by several industry groups, was convened to make recommendations to the Board of Nutrition of the US National Academy of Sciences regarding the establishment of Dietary Reference Intakes (DRIs) for omega-6 and omega-3 fatty acids (109). This group thought it was of "*utmost importance*" to focus on the composition of infant formula and recommended that an adequate intake (AI) for an infant formula diet would include 0.50% of total fatty acids as ARA and 0.35% of total fatty acids as DHA. A typical fat content of an infant formula of 5.3 g/100 kcal would lead to a consumption of about 5.8 g fat/kg body wt/day. This corresponds to about 30 mg ARA/kg body wt/day and 20 mg DHA/kg body wt/day; values which closely approximate the WHO/FAO Expert Panel recommendations of 1995. The ISSFAL Workshop also recognized the need for additional DHA supplementation to the mother during the period of pregnancy and lactation so that adequate delivery of DHA to the infant can take place from the maternal source. Consequently, they also recommended that "*during pregnancy and lactation women must ensure a DHA intake of 300 mg/day*".

2.8 Two Panels Have Postponed their Decision on Whether DHA and ARA Supplementation Should Be Mandated in Infant Formulas

2.8.1 Life Sciences Research Organization (LSRO) (1998)

In September 1995, under contract with the FDA, the LSRO Panel began to evaluate the composition of infant formulas for healthy term (but not preterm) infants. Among other issues, the Panel reviewed the requirements of DHA and ARA for full term infants. Throughout the discussion of this issue, the Panel repeatedly recognized the need to maintain adequate DHA and ARA status in infants. (141) Although the Panel considered the evidence available to it to be "*insufficient to warrant a recommendation that DHA and ARA be added to full term infant formulas at this time*", it did recognize that the relevant clinical trials were ongoing and that this issue should be readdressed in the near future.

The LSRO Panel based its opinion on only four (11, 22, 75, 78, 92) of the ten term infant intervention studies listed in Table 2 where DHA/ARA supplemented formulas were used and functional outcomes were measured. Three of the four studies considered (11, 22, 75, 78, 92) clearly demonstrated a deficit in visual and/or neurological parameters, caused by standard formula feeding relative to human milk. In all three studies the deficits were overcome by the supplementation of DHA and ARA to the formulas. The fourth study (11) showed no difference in visual or neurological assessments between human milk-fed, standard formula-fed, or DHA/ARA supplemented formula-fed infants. This study has been criticized due to the fact that the DHA level in the human milk was very low, as was the enrichment level in the formula (0.12%). These levels would deliver only about 6-10 mg DHA/kg body wt/day to the infant (about one-third that recommended by the WHO/FAO Expert Panel).

The Panel's conclusion was surprising in light of the emphasis that it placed on elevating the red blood cell and brain levels of DHA in formula fed infants. The Panel recognized that formula fed infants have lower levels of DHA in the blood and brain than breast fed infants. The Panel further observed that visual deficiencies are associated with consumption of current formula preparations (141). The Panel suggested that the DHA status of the infant could be improved if enough precursor alpha linolenic acid (ALA) was added to the formulas, and therefore recommended that all term formulas contain a minimum of 1.75% of energy as ALA. "*This recommendation was based on the essentially of ALA as a precursor of the n-3 series of LCPUFAs*" (*i.e.,* DHA), underscoring the importance of maintaining an adequate DHA status. The LSRO Panel made this recommendation, apparently in an attempt to increase DHA levels in infants, in the absence of data demonstrating that an increase in ALA will translate into an increase in DHA levels. Indeed, the LSRO Panel specifically recognized that *"[i]t is evident from the review of studies to date that efforts to increase plasma and RBC membrane DHA levels of formula-fed infants to levels comparable to those observed in breast-fed infants, either by altering the LA or ALA content or the ratio of these two fatty acids, have not been successful"* (141). In fact, the clinical data clearly indicate that even when the ALA levels are increased to 4.5% (about 5-fold higher than breast milk), the DHA levels in the infants are still significantly lower than those of breast-fed babies (127). It is difficult to reconcile the LSRO recommendation to increase ALA levels for the purpose of increasing DHA levels when data clearly establish that such increases in DHA levels can best be obtained through supplementation of the infant diet with DHA, as in breast milk, and not through increases of ALA.

It also is interesting that the LSRO Panel made this recommendation since human milk contains very little ALA. Indeed, the Panel made the unprecedented recommendation that formula should contain no less than twice as much ALA as is found in breast milk (63). The recommendation is inconsistent with the intent of the Infant Formula Act, which was to have infant formula more closely match the gold standard of human breast milk. Despite their lack of a recommendation for the requirement of DHA and ARA in all term formulas, *"the Expert Panel strongly endorsed breast feeding as the preferred source of nutrients for infants and, for a number of nutrients* [not DHA and ARA], *used the amounts found in breast milk as a guide in establishing minimum and maximum levels"*. The Panel also recognized that the *"levels of DHA in the breast milk of women consuming a typical North American diet are generally found to be in the range of 0.2% - 0.4% of fatty acids"*, but levels as high as 1.4% have been reported elsewhere (55). These typical breast milk levels would provide about 10 to 25 mg DHA/kg body wt/day for an infant.

Although the LSRO Panel did not recommend the addition of DHA and ARA at this time, it has asked that this issue be reviewed again in the near future. This recommendation would allow future Panels to review the data and assess whether DHA and ARA should be added to infant formulas.

2.8.2 Health Canada (1988)

A working group assembled by Health Canada concluded that commercial formulas in Canada contain adequate amounts of linoleic and linolenic acids (128). However, they also recognized that *"it may be that ARA and DHA synthesis* [from these precursors] *is too slow to meet the tissue needs* [of the infant]". They further stated that *"clinical studies have attempted to determine the need for a dietary source or for the addition to infant formulas of ARA and DHA, but have yielded inconsistent results"*. However, they cite only two of the ten available studies (see Table 2) which analyzed functional outcomes. Finally, they did agree that *"it is reasonable to presume that infants not fed breast milk might benefit from dietary sources of ARA and DHA"*, but they did not recommend their addition until the safety of the sources of these fatty acids, not the fatty acids themselves, could be established.

2.9 Conclusions

A number of scientific and medical expert panels have critically discussed and evaluated the scientific evidence and concluded that the biochemical deficiency observed in the formula-fed infants can be eliminated by providing dietary sources of DHA and ARA. DHA and ARA are found in breast milk and are GRAS components on the basis of common use in foods. Nature apparently has included DHA and ARA in breast milk so that the developing infant has a readily available source of these PUFAs which are the building blocks of brain and retinal tissues, two tissues that undergo extensive development in the first year of the infant's life.

Well controlled clinical studies (Table 2) demonstrate that formula fed infants have significantly lower levels of DHA and ARA than breast fed infants. The studies also clearly show that infant formulas that are supplemented with DHASCO and ARASCO restore the infant DHA and ARA status to that of the breast fed infant. There are also numerous studies suggesting that the absence of these LCPUFAs in the infant diet is having a detrimental impact on the infant's development. The supplementation of infant formulas with DHA and ARA is in line with the general tenet of

providing infants with nutritive components that most closely match the formula that nature developed for nourishing infants (*i.e.,* breast milk).

3 PROPOSED USES FOR DHASCO AND ARASCO

Several Scientific Panels have addressed the issue of use levels of DHA and ARA in infant formulas (45, 48, 125, 126). The levels recommended by those Panels are given in Table 3 for pre-term and term infant formulas and range from 0.4% to 1.5% of total formula fat for ARA, and 0.35% to 1.1% of total formula fat for DHA. These values closely approximate levels of DHA and ARA found in human milks from around the world.

Perhaps most relevant, are the recommendations coming from the April 1999 expert panel assembled by NIH, The Center for Genetics, Nutrition and Health, and ISSFAL (and co-sponsored by several industry groups). This group of world-wide experts in lipid nutrition reviewed the latest data, including many studies not reviewed or discussed by the LSRO Panel. This Expert Panel concluded that an infant formula would need to contain at least 0.35% of lipid as DHA and 0.50% of lipid as ARA for an infant to receive an adequate intake (AI) of these components (109). The experts also identified the potential problems with EPA and recommended that there should be an upper limit (not to exceed) for EPA of not more than one quarter the level of DHA.

Table 3. Recommendations of five expert panels for the inclusion of ARA and DHA in infant formulas.

	ESPGAN	*BNF*	*ISSFAL*	*FAO/WHO*	*ISSFAL*
Year	1991	1992	1994	1994	1999
Preterm Formula					
*ARA (% of fat)**	1%	0.40 %	0.90 -1.5 %	0.90 %	0.50 %
DHA (% of fat)	0.5%	0.40 %	0.5-1.1 %	0.60 %	0.35 %
Term Formula					
ARA (% of fat)		0.40 %		0.70 %	0.50 %
DHA (% of fat)		0.40 %		0.35 %	0.35 %
DHA/EPA ratio			> 5 to 1	10 to 1	> 4 to 1

* Assumptions for the calculated values are 1) preterm infants consume 120 kcal/kg/d and term infants consume 110 kcal/kg/d, 2) fat comprises 50% of energy of the formula, and 3) 1 g fat has 9 kcal.

In the last 15 years, at least 32 well-controlled clinical trials have been completed using DHA/ARA supplemented formulas (see Table 2). All trials have used supplemental DHA at levels in preterm formulas from 0.15% to 0.78%, and for term formulas from 0.1% to 0.36% of total formula fat. Supplemental ARA has been used in most, but not all trials. In trials where supplemental ARA has been used, the concentration ranged from 0.10% to 1.1% of total fat in preterm formulas, and from 0.20% to 0.72% for term formulas. Of particular relevance to this submission are the 14 clinical studies in which DHASCO and ARASCO were used as the DHA and ARA sources for supplementation (see Appendix 2). In full term infant formula trials, dose levels ranged from 0.20 % to 0.36 % of total formula fat for DHA, and from 0.20 % to 0.72 % of total formula fat for ARA. These values clearly fall well within the normal range of mother's milk (Figure 1) and the Expert recommendations (Table 3).

Table 4. Commercial infant formulas containing DHASCO and ARASCO

Country	Company	Product Name	Term	Preterm
Argentina	Wyeth	SMA LBW		X
Australia	Wyeth	SMA LBW		X
		SMA Gold	X	
	Numico	Nenatal		X
		KariCare First	X	
Bahrain	Wyeth	SMA LBW		X
Bolivia	Wyeth	SMA LBW		X
Belgium	Numico	Nenatal		X
		Premilon		X
Chile	Wyeth	SMA LBW		X
China (P.R.C.)	Wyeth	SMA LBW		X
Colombia	Wyeth	SMA LBW		X
Cyprus	Wyeth	SMA LBW		X
Dominica	Wyeth	SMA LBW		X
Ecuador	Wyeth	SMA LBW		X
Egypt	Wyeth	SMA LBW		X
El Salvador	Wyeth	SMA LBW		X
Finland	Numico	Nenatal		X
France	Wyeth	Modilac LBW		X
Greece	Wyeth	SMA LBW		X
Guatemala	Wyeth	SMA LBW		X
Guyana	Wyeth	SMA LBW		X
Haiti	Wyeth	SMA LBW		X
Honduras	Wyeth	SMA LBW		X
Hong Kong	Wyeth	SMA LBW		X
		SMA Gold	X	
Hungary	Wyeth	SMA LBW		X
Iceland	Wyeth	SMA LBW		X
Indonesia	Wyeth	SMA LBW		X
Ireland	Wyeth	SMA LBW		X
Israel	Maabarot	Materna Stage One	X	
Jamaica	Wyeth	SMA LBW		X
Jordan	Wyeth	SMA LBW		X
Kenya	Wyeth	SMA LBW		X
Kuwait	Wyeth	SMA Gold	X	
		SMA LBW		X
Lebanon	Wyeth	SMA LBW		X
Malawi	Wyeth	SMA LBW		X
Malaysia	Wyeth	SMA LBW		X
Malta	Wyeth	SMA LBW		X
Mauritius	Wyeth	SMA LBW		X
Mexico	Wyeth	SMA LBW		X
Netherlands	Numico	Nenatal		X
New Zealand	Wyeth	SMA LBW		X
		SMA Gold	X	
	Numico	Nenatal		X
		KariCare First	X	
Nicaragua	Wyeth	SMA LBW		X
Oman	Wyeth	SMA LBW		X
Panama	Wyeth	SMA LBW		X
Peru	Wyeth	SMA LBW		X
Philippines	Wyeth	SMA LBW		X

Country	Company	Product Name	Term	Preterm
Portugal	Wyeth	SMA LBW		X
Qatar	Wyeth	SMA LBW		X
Russia	Wyeth	SMA LBW		X
Saudi Arabia	Wyeth	SMA LBW		X
Seychelles	Wyeth	SMA LBW		X
Singapore	Wyeth	SMA LBW		X
South Africa	Wyeth	SMA LBW		X
Spain	Novartis	Adapta PEG	X	
		Preadapta		X
	Numico	Nenatal		X
Switzerland	Numico	Preadapta		X
Taiwan	Wyeth	SMA LBW		X
Thailand	Wyeth	SMA LBW		X
Trinidad	Wyeth	SMA LBW		X
Turkey	Wyeth	SMA LBW		X
U.A.E.	Wyeth	SMA LBW		X
		SMA Gold	X	
United Kingdom	Wyeth	SMA LBW		X
Uruguay	Wyeth	SMA LBW		X
Venezuela	Wyeth	SMA LBW		X
Vietnam	Wyeth	SMA LBW		X
Zimbabwe	Wyeth	SMA LBW		X

In the past five years, commercial infant formulas containing supplemental DHA and ARA from DHASCO and ARASCO have been introduced around the world (Table 4). DHASCO/ARASCO-supplemented formulas are used more extensively around the world than formulas supplemented with any other source of DHA and ARA. The DHA and ARA levels in current commercial formulas for term infants range from 0.25% to 0.40% of formula fat for DHA and from 0.45% to 0.50% of formula fat for ARA. In addition, Wyeth/Ayerst has introduced a formula for low birth weight (LBW) infants. This formula is given under a doctor's strict supervision and it is estimated that over 100,000 infants have been given these formulas. Infants receiving these low birth weight formulas are under a doctor's vigilant supervision and no adverse effects that could be associated with DHASCO and ARASCO have been observed.

After reviewing all of the available data, the Expert Panel convened by Martek concluded that ARASCO and DHASCO are GRAS when used at dose levels for DHA and ARA at a level up to 1.0 % each of daily fat intake. This would correspond to 2.5% of daily fat intake as DHASCO and 2.5% of daily fat intake as ARASCO (DHASCO and ARASCO are only 40% by weight DHA and ARA). This level is two-fold higher than the level proposed in this notification for use in commercial infant formulas, that is, 1.25% of daily fat at a ratio of 1:1 to 1:2 (DHA:ARA).

Assuming human infants consume about 100 to 120 kcal/kg body wt/day, of which fat comprises about 50%, an infant will consume about 50 - 60 kcal/kg body wt/day of fat, or about 5.5 - 6.7 g of fat/kg body wt/day (1 g fat = 9 kcal). The DHASCO and ARASCO intake of 1.25% of daily fat for an infant would correspond to about 75 mg DHASCO/kg/day and 75 mg ARASCO/kg/day.

4 IDENTITY AND DESCRIPTION

ARASCO and DHASCO are macronutrient triglyceride oils produced in fungal and algal microorganisms respectively. These microorganisms have not been genetically engineered. They are grown in axenic liquid culture, harvested, dried, and the oils are extracted and processed using current Good Manufacturing Practices (CGMPs). All processing materials are of food grade quality or better. The extracted oils naturally contain between 40 and 55 percent arachidonic acid or docosahexaenoic acid. The harvested oils are standardized with high oleic sunflower oil (HOSO) to a standard 40% DHA or ARA content prior to being shipped worldwide for addition to infant formulas. The comprehensive chemical descriptions of the oils are described below.

4.1 Chemical Composition of DHASCO

DHASCO oil is triglyceride preparation that is enriched to 40% by weight in DHA. It is a mixture of an oil extracted from the marine microalgae *Crypthecodinium cohnii* and a high oleic sunflower oil. DHASCO is a free flowing, yellow-orange oil, which is predominantly triglyceride (>95%) with some diglyceride and nonsaponifiable material (<5%) as is typical for food-grade vegetable oils. The fatty acid composition of DHASCO is shown in Table 5.

Minor fatty acid components listed as "other" in Table 5 generally constitute about 1% of the total fatty acid composition. A recent report (129) indicated the presence of small amounts of C28:8 (n-3) in DHASCO oil, as well as fish oil. This fatty acid is the next expected omega-3 end product of the Sprecher biochemical pathway beyond DHA (130) and is one of the minor components (approximately 1%) of DHASCO and many fish oils. Compositional analyses of other components of the oils are also provided in Table 5. The levels of these latter components compare favorably with other commercial edible oils. In general, there are: (1) no residues of the extraction solvent (it is either absent or undetectable at a detection limit <0.3 ppm); (2) no detectable cyclic fatty acids or trans fatty acids; (3) no pesticide residues (negative for a standard panel of 74 pesticides); and (4) no heavy metals such as arsenic, mercury and lead (all below the 0.1 ppm detection limit).

Table 5. Chemical composition of four lots of DHASCO.

Specification	*Lot 1*	*Lot 2*	*Lot 3*	*Lot 4*
Fatty Acid Analysis				
C 8:0	0.35	0.13	0.18	<0.10
C 10:0	1.24	0.87	0.99	1.66
C 12:0	5.91	5.09	5.93	7.16
C 14:0	16.82	15.43	17.52	17.38
C 16:0	13.95	12.92	13.99	14.97
C 16:1	1.62	1.49	1.23	1.48
C 18:0	0.45	0.64	0.55	0.36
C 18:1	13.32	17.69	14.34	13.33
C 18:2	0.40	0.74	0.51	0.31
C 20:0	0.14	0.10	0.11	0.10
C 22:0	0.13	0.18	0.15	0.11
C 22:5 n-3	<0.10	<0.10	<0.10	<0.10
C 22:6 DHA	44.78	43.57	43.93	41.85
Others	0.42	1.02	0.46	1.25

Specification		Lot 1	Lot 2	Lot 3	Lot 4
Chemical Analysis	units				
DPA (omega-6)	area %	<0.1	<0.1	<0.1	<0.1
EPA	area %	<0.1	<0.1	<0.1	<0.1
Free fatty acid	%	0.16	0.14	0.22	0.16
Peroxide value	meq/kg	<0.10	<0.10	0.24	<0.10
Volatiles	%	<0.01	<0.01	<0.01	<0.01
Nonsaponifiables	%	1.85	1.55	1.36	1.79
Insoluables	%	<0.01	<0.01	<0.01	<0.01
Trans fats	%	<1.0	<1.0	<1.0	<1.0
Elemental Analysis					
Arsenic	ppm	<0.5	<0.5	<0.5	<0.5
Cadmium	ppm	<0.1	<0.1	<0.1	<0.1
Chromium	ppm	<0.1	<0.1	<0.1	<0.1
Copper	ppm	<0.02	<0.02	<0.02	<0.02
Iron	ppm	<0.02	<0.02	<0.02	<0.02
Lead	ppm	<0.1	<0.1	<0.1	<0.1
Manganese	ppm	<0.01	<0.01	<0.01	<0.01
Mercury	ppm	<0.04	<0.04	<0.04	<0.04
Molybdenum	ppm	<0.05	<0.05	<0.05	<0.05
Nickel	ppm	<0.1	<0.1	<0.1	<0.1
Phosphorous	ppm	<1	1	<1	1
Silicon	ppm	135	41	18	41
Sulfur	ppm	29	18	80	74

The 1.5% by weight nonsaponifiable fraction of DHASCO is made up primarily of sterols. Two independent laboratories have analyzed the sterols of this fraction and their results are shown in Table 6. These data agree with results previously published on the sterol fraction of *C. cohnii* (131-133). The principle component of these sterols is a 4-methyl sterol, dinosterol (Figure 5). 4-methyl sterols are found in the normal metabolic pathway of cholesterol biosynthesis in man (134-136) and have been identified in several common food sourcess, including fish and shellfish (137).

Table 6. Sterol content of DHASCO from two independent laboratories (Professor J. Weete, Auburn University, Alabama and Dr. J. Volkman, CSIRO Division of Oceanography, Australia) and from Withers (131).

			Percent of Total Sterols		
Sterol Fraction		*Common Name*	*Volkman*	*Weete*	*Withers (131)*
4α,23,24-trimethyl cholesta-22-en-3β-ol	C30:1	dinosterol	31.5	25.5	40
cholesta-5,7-dien-3β-ol	C27:2	dehydrocholesterol	9.6*		14
4α,24-dimethyl cholestan-3β-ol	C29:0		9.2		minor
4α,23,24-trimethyl cholesta-5,22-dien-3β-ol	C30:2	dehydrodinosterol	8.2		major
cholesta-7-en-3β-ol	C27:1	lathosterol	7.5		minor
4α,24-dimethyl cholesta-22-en-3β-ol	C29:1		6.4	6.2*	
4α,23,24-trimethyl cholesta-22-en-3β-ol	C30:1	dinosterone	6.0	8.8*	14
23,24-dimethyl cholesta-5-en-3β-ol	C29:1		4.6		
4α,23,24-trimethyl cholesta-24(28)-ene-3β-ol	C30:1		4.2	3.6*	
*cholesta-x,x-dien-3β-ol***	C27:2		3.6*	9.8	trace

Sterol Fraction		*Common Name*	*Percent of Total Sterols*		
			Volkman	*Weete*	*Withers (151)*
cholesta-5,24-dien-3β-ol	C27:2	desmosterol	2.4*	9.4	
cholesta-5-en-3β-ol	C27:1	cholesterol	1.7*	2.7	2
23 or 24-methyl cholesta-5,7-dien-3β-ol	C28:2		1.9*		trace
	C27:3		1.3		
a 5,7-dien sterol	C29:2			6.9*	trace
Total sterols (mg per g dry weight)			9.5		30

* Assignment based on known number of carbons and double bonds as well as number and/or placement of methyl groups, all of which are consistent with the cited chemical structure; ** The *x* refers to unassigned double bond placement.

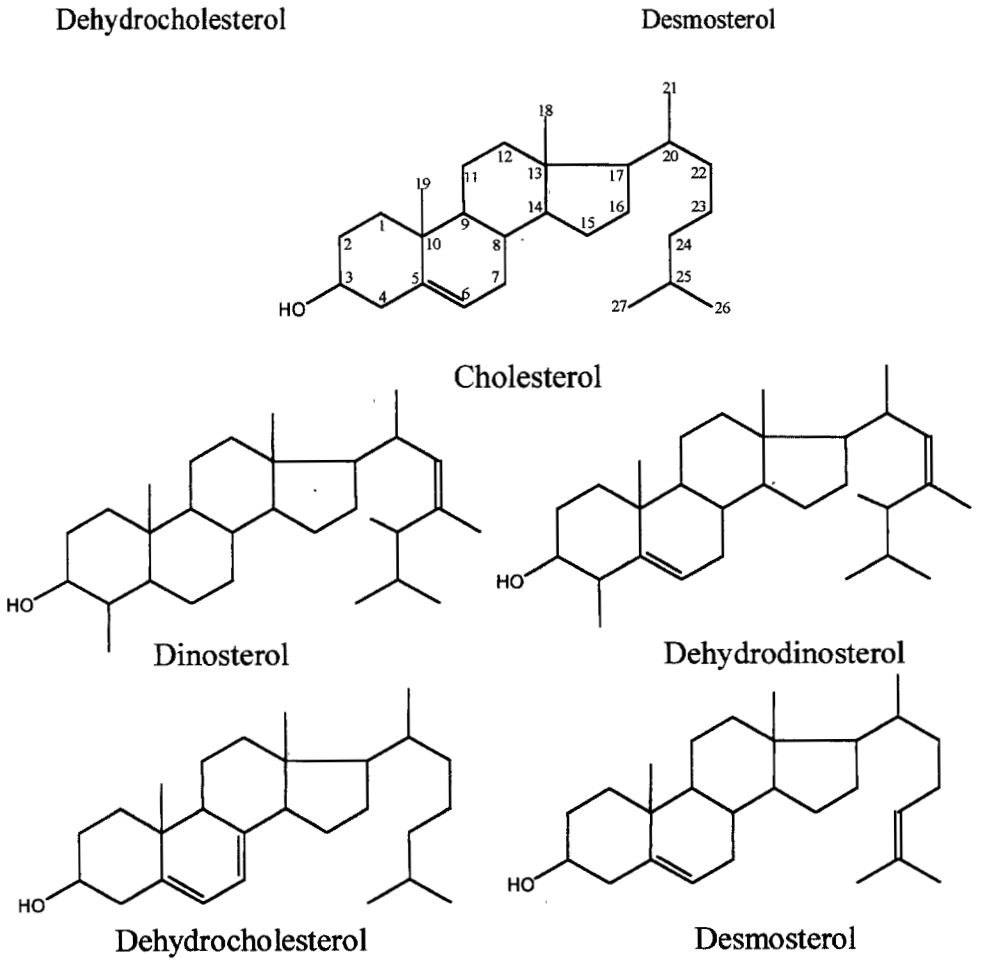

Figure 5. Structures of common sterols in DHASCO and ARASCO.

There is no *a priori* reason to believe that the sterols in DHASCO have any significant biological activity. The sterols would be expected to feed into the normal cholesterol metabolic pathways in humans. It is also important to note that at the proposed GRAS level for DHASCO of 1.25% of daily fat intake, the contribution of the sterols from the algal oil to the diet is very small relative to other phytosterols found in infant formulas (from the vegetable oil) or cholesterol and

phytosterols provided by human milk. A dose level of 75 mg DHASCO/kg body wt/day would correspond to less than 1.5 mg total algal sterols/kg body wt/day. A typical infant formula would deliver about 33.5 mg of phytosterols/kg body wt/day (6700 mg fat/kg body wt/day and 0.5% sterols), and human milk delivers about 30 mg of cholesterol/kg body wt/day (400 mg cholesterol per 100 g fat) to an infant (63). If mothers are consuming a phytosterol-rich diet, the amount of phytosterol in the breast milk can also be as high as the cholesterol levels (138).

Triglycerides are the predominant component of any natural fat or oil and are present as a family of compounds wherein the various fatty acids may be found attached to any of the three positions on the glycerol backbone (see Figure 1). Corn oil, for example, is made up of at least 21 individual triglycerides (139). In some cases, certain fatty acids are found preferentially in either the sn-1, sn-2 or sn-3 positions on the glycerol backbone. In human milk, for example, palmitic acid is commonly found on the sn-2 position, whereas in vegetable oils found in infant formulas, palmitic acid is almost never found on the sn-2 position (140). Despite these discrepancies, the oils in infant formula are generally hydrolyzed and the fatty acids are taken up efficiently by the infant gut (see Section 7.1 for a description of absorption, distribution, metabolism and excretion of dietary fats and oils).

It is desirable to match as closely as possible the positional placement of DHA in human milk triglycerides. We could find only two references relating to the positional specificity of DHA and ARA in human milk triglyceride and they both indicate that about 50-60% of the DHA is found on the sn-2 position (27, 141). Myher *et. al.* (142) have reported that approximately 45% of the DHA found in DHASCO is also located on the sn-2 position. Since the triglyceride structure of DHASCO is quite similar to human milk with respect to the positional specificity of DHA, there would be no reason to believe that the digestion and absorption of DHA from DHASCO should be any different than the DHA from human milk fats.

Carnielli *et al.* (28) have shown that the absorption of DHA from DHASCO in human infants more closely approximates the absorption of DHA in human milk than when DHA is in the form of a phospholipid. Many clinical studies have also shown that blood levels of DHA increase to levels matching those of the breast-fed infant when infants are fed DHASCO-supplemented formulas (see Appendices 2 and 3).

4.2 Chemical Composition of ARASCO

ARASCO oil is a macronutrient triglyceride preparation that is enriched to 40% by weight in ARA. It is made up of a mixture of an oil extracted from the soil fungus *Mortierella alpina* and a commercial grade of high oleic sunflower oil. It is a free flowing, yellow oil, which is predominantly triglyceride (>95%) with some diglyceride and nonsaponifiable material (<5%) as is typical for food-grade vegetable oils. The fatty acid composition of ARASCO is shown in Table 7. Compositional analyses of the other components of ARASCO are also provided in Table 7. These latter components are found at levels similar to those in other commercial edible oils. In general, there are: (1) no residues of the extraction solvent (it is either absent or undetectable at a detection limit <0.3 ppm); (2) no detectable cyclic fatty acids or trans fatty acids; (3) no pesticide residues (negative for a standard panel of 74 pesticides); and (4) no heavy metals such as arsenic, mercury and lead (all below the 0.1 ppm detection limit).

Table 7. Analytical Specification of four lots of ARASCO.

Specification		*Lot 1*	*Lot 2*	*Lot 3*	*Lot 4*
Fatty Acid Analysis					
C 14:0		0.58	0.34	0.39	0.44
C 16:0		9.59	7.32	7.17	8.45
C 17:0		0.42	0.38	0.33	0.41
C 18:0		10.2	9.13	7.60	9.24
C 18:1 n-9		15.95	20.82	23.35	18.62
C 18:1 n-7		0.14	0.28	0.32	0.37
C 18:2 n-6		7.62	6.77	5.56	7.16
C 18:3 GLA		2.99	2.82	2.45	2.81
C 20:0		0.96	0.93	0.83	0.92
C 20:1 n-9		0.40	0.35	0.36	0.50
C 20:2 n-6		0.62	0.57	0.60	0.72
C 20:3 DGLA		2.57	2.03	1.82	1.43
C 20:4 ARA		42.69	42.96	44.26	43.14
C 22:0		2.02	2.02	1.98	2.00
C 24:0		1.92	1.91	2.04	1.85
C 24:1 n-9		0.11	0.13	0.23	0.20
Others		1.22	1.24	0.65	0.78
Chemical Analysis	units				
DPA (omega-6)	area %	<0.10	<0.10	0.10	<0.10
EPA	area %	<0.10	<0.10	0.16	<0.10
Free fatty acid	%	0.18	0.11	0.10	0.27
Peroxide value	meq/kg	1.2	0.41	0.12	1.51
Volatiles	%	<0.01	0.03	<0.01	0.02
Nonsaponifiables	%	1.73	1.36	1.69	1.18
Insoluables	%	<0.01	<0.01	<0.01	<0.01
Trans fats	%	<1.0	<1.0	<1.0	<1.0
Elemental Analysis					
Arsenic	ppm	<0.5	<0.5	<0.5	<0.5
Cadmium	ppm	<0.1	<0.1	<0.1	<0.1
Chromium	ppm	<0.1	<0.1	<0.1	<0.1
Copper	ppm	<0.02	<0.02	<0.02	<0.02
Iron	ppm	<0.02	<0.02	<0.02	<0.02
Lead	ppm	<0.1	<0.1	<0.1	<0.1
Manganese	ppm	<0.01	<0.01	<0.01	<0.01
Mercury	ppm	<0.04	<0.04	<0.04	<0.04
Molybdenum	ppm	<0.05	<0.05	<0.05	<0.05
Nickel	ppm	<0.1	<0.1	<0.1	<0.1
Phosphorous	ppm	<1	<1	<1	<1
Silicon	ppm	310	280	280	350
Sulfur	ppm	5	3	6	4

About 1.5% by weight of ARASCO is a nonsaponifiable fraction which is composed primarily of sterols. Two independent laboratories have analyzed the sterols of this fraction and their results are presented in Table 8. These data are in agreement with published results on the sterol fraction of *M. alpina* (143).

The principle component of these sterols is desmosterol (Figure 5). Desmosterol is found in the normal metabolic pathway of cholesterol biosynthesis in man (134) and is commonly found in

several other food sources including animal fat, vegetable oils, and human milk (63). Shimizu (143) has reported the presence of a novel cyclopropyl-containing sterol, 24,25-methylenecholest-5-en beta-ol, in *M. alpina* at a level of 22% of the total sterols. This compound was not detected in the chromatograms of the Martek ARASCO samples by either of the two independent analytical laboratories. It is, therefore, possible that this compound was either misidentified by Shimizu (143), or that it was a real component unique to the strain 1S-4 developed by these Japanese workers.

Table 8. Sterol content of ARASCO from two independent laboratories (Professor J. Weete, Auburn University, Alabama and Dr. J. Volkman, CSIRO Division of Oceanography, Australia) and from Shimizu (143).

Sterol Fraction		*Common Name*	Percent of Total of Sterols		
			Volkman	*Weete*	*Shimizu (143)*
cholesta-5,24-dien-3β-ol	C27:2	desmosterol	67.3	66.4	58
24-methyl cholesta-5,24(25 or 28)-dien-3β-ol	C28:2		14.0	13.4*	11
24-methyl cholesta-5,25-dien-3β-ol	C28:2		12.3**	9.7***	9
	C28:2		2.1	3.6	
4α,4β,14-trimethyl-8,24-dien-3β-ol	C30:2	lanosterol	1.1	2.7	
cholesta-5,25-dien-3β-ol	C27:2		2.0	1.0	
24,25-methylene cholesta-5-en-3β-ol	C28:1		nd	nd	22
Total sterols (mg per g)			7.9		5.3

* Identified as $C28\Delta^{5,x}$; ** Identified as C28:2; *** Identified as $C28\Delta^{5,x}$

At the proposed GRAS level for ARASCO of 1.25% of daily fat intake, the contribution of the desmosterol from the fungal oil to the diet is very small relative to other phytosterols, cholesterol, and even desmosterol provided by human milk or infant formulas. At a dose level of 75 mg ARASCO/kg body wt/day, this would correspond to about 0.5 mg desmosterol/kg body wt/day. A typical infant formula would deliver about 33.5 mg of phytosterols/kg body wt/day (6700 mg fat/kg body wt/day and 0.5% sterols), and human milk delivers about 30 mg cholesterol/kg body wt/day (400 mg cholesterol per 100 g fat) to an infant (63). Desmosterol is the second most abundant sterol in human milk (63) and it is present at levels of about 10% that of cholesterol (*i.e.,* about 3-4 mg/kg body wt/day delivery to the baby; nearly ten times greater than the quantity of desmosterol from ARASCO). If mothers are consuming a phytosterol-rich diet, the amount of phytosterol in the breast milk can be as high as the cholesterol levels (138).

As with DHASCO, it would be most desirable for the ARA in ARASCO to have a positional placement on the triglyceride backbone that closely matches the position of this fatty acid in the triglycerides found in human milk. Two references have reported that about 45% of the ARA in human milk triglyceride is located on the sn-2 position (27, 141). Myher *et. al.* (142) have reported that approximately 29% of the ARA found in ARASCO is also located on the sn-2 position. Since the triglyceride structure of ARASCO is quite similar to human milk with respect to the positional specificity of ARA, there would be no reason to believe that the digestion and absorption of ARA from ARASCO should be any different than ARA from human milk fats. In

fact, studies have demonstrated that blood levels of ARA increase to levels matching those of breast-fed babies when those infants are fed ARASCO-supplemented formulas (see Appendix 2).

5 MANUFACTURING PROCESSES

5.1 DHASCO

5.1.1 Natural History of *C. Cohnii*

Crypthecodinium cohnii is a member of the Dinophyta (dinoflagellates). This is a distinct phylum of unicellular eucaryotic microalgae comprising an estimated 2,000 species (144). The Dinophyta comprise an extremely important and vastly abundant group of primary producers in both freshwater and marine environments. Among the eukaryotic algae they are second only to the diatoms as primary producers in coastal waters (144). As such, they represent a primary link in the food chain of all marine and freshwater animals. In tropical and subtropical marine environments some species of Dinophyta have been found in a symbiotic relationship with many invertebrates, especially corals, and serve a critical role as the major primary producers in reef ecosystems (144). In these associations the host organism is able to swallow the alga and incorporate it into its own tissue without harming it. The algal cell then produces sugars, which are utilized by the host. Some organisms rely almost exclusively on the food provided by their endosymbiotic algal cells.

Most species of the Dinophyta are photosynthetic, but there are also several heterotrophic species (144). Heterotrophic Dinophyta feed on diatoms and other protists and, as a result, they can also be the dominant grazers in certain environments.

A small number of photosynthetic species of Dinophyta are known to produce a group of closely related toxins (145). These toxins are passed through the food chain via zooplankton and can contaminate fish and bivalves. However, these toxin producing species are few in numbers, and there are no known heterotrophic species of the Dinophyta which are toxin producers. There are also no described cases of any of the Dinophyta species, or any other algae, being pathogenic to man or other mammals.

The genus *Crypthecodinium* is monospecfic (*i.e.,* containing a single species, *C. cohnii*). While containing the vegetative and nuclear characteristics in common with all other species of the Dinophyta, *C. cohnii* is further characterized by being incapable of photosynthesis. Thus, it is an obligate heterotroph. The natural history of this organism has been extensively studied for many years, and isolates *of C. cohnii* have been collected from all around the world (146). *C. cohnii* is frequently isolated from the surface of seaweeds (147). Because this species is easy to cultivate in the laboratory, it has become one of the best studied of all the algae, particularly with regard to ultrastructure, biochemistry and genetics. *C. cohnii* produces DHA but with no detectable EPA or omega-6 DPA, both of which may be contraindicated in infant nutrition.

Crypthecodinium cohnii, like other algae, reproduces primarily by asexual cell division (mitosis) and is haploid in the vegetative stage (147). This organism is capable of sexual reproduction in the laboratory, and with the exception of having a single division meiosis, has normal genetic

behavior (147). Genetic studies indicate that *C. cohnii* exhibits genetic stability consistent with other eukaryotic species used in fermentative production of foods and food additives (146-149); that is, there is very little genetic drift in these algae. In the many reports of *C. cohnii* in culture over the last 30 years, there has never been any indication that *C. cohnii* produces any toxin, nor is it related to any toxin-producing species (150). More broadly speaking, there are no known heterotrophic Dinophyta species, which are either toxigenic or pathogenic (145).

5.1.2 DHASCO Fermentation Process

The *C. cohnii* strain used for the production of DHASCO is proprietary to Martek Biosciences Corporation (US Patents 5,397,591, 5,407,957 and 5,492,938) and the process has been published (151). The strain originated from the University of Texas culture collection and has been selected for rapid growth and high levels of production of the specific oil. The specific production strain of *C. cohnii* has been deposited with American Type Culture Collection (ATCC # 40750) under the obligations of the U.S. patent relating to its use. Master seed banks of the production strain are maintained under liquid nitrogen conditions at Martek and at ATCC. Working seed stock, prepared from this master seed bank, is also maintained cryogenically.

On initiation of a production run, an individual ampoule from a working seed is used to inoculate a shake flask. The medium used to grow *C. cohnii* from shake flask to production scale contains dextrose, a nitrogen source, and certain salts required for optimal growth. All ingredients used in the fermentation process are of food grade quality or better, and the medium is prepared according to Standard Operating Procedures. When the culture reaches a determined cell density and the fatty acid content reaches a determined percentage, the culture is harvested by centrifugation and spray dried. The dried biomass is flushed with nitrogen prior to transport to the oil processing facility or to a frozen storage facility.

The quality of all raw materials used in the fermentation is verified from the manufacturer's Certificate of Analysis and by Martek's policy of procuring ingredients only from verified reputable vendors of products of food grade quality or better. In order to maintain kosher certification, Martek also ensures that ingredients are procured from recognized suppliers of products meeting strict kosher guidelines. Martek products are certified kosher by the Orthodox Union (OU). This process has also been reviewed by the B.D.Z. Eda Charedith organization of Israel and has been certified as Halal by the Islamic Food and Nutrition Council of America (IFANCA).

5.1.3 Extraction and Purification of DHASCO

The DHASCO oil is extracted from the algal biomass and processed using methods and procedures that have been well established in the edible oils industry. These processes include refining, bleaching and deodorizing the oil. In order to protect this DHA-rich oil from oxidation, the Martek process uses the lowest effective temperatures and shortest times for each process step, and the oil is continuously protected from oxygen by nitrogen blanketing or vacuum. The oil is packaged in either nitrogen-purged containers and stored frozen, or vacuum packaged. Following analytical release testing, a Certificate of Analysis is generated and included with each shipment.

5.1.4 Process Controls

All processes were set up using a Hazard Analysis Critical Control Point (HACCP) procedure. They are documented according to current Good Manufacturing Practices (CGMP) and the identified critical control points (CCPs) are monitored. Significant process related data points are recorded and plotted on control charts. These data points are regularly monitored and reviewed to ensure that they are within Martek's recognized standard processing parameters. Batch records are maintained and reviewed on a regular basis by Quality Control personnel to ensure that they are complete and accurate representations of the production process. Quality Assurance personnel monitor the production records to ensure that batch process changes have been properly authorized, documented, and recorded in the records for each batch. The Food and Drug Administration (FDA) has inspected the manufacturing plant in Winchester, Kentucky, at least on an annual basis since 1995.

In-process monitoring of the product for bacteriological contamination is done on a continuous basis throughout the fermentation process. Sterility checks are performed daily by plating sterile samples of the fermented broth. The plates are cultured and held for 96 hours. During this time the plates are checked daily for signs of contamination. Laboratory personnel and production operators also routinely visually inspect samples from the fermentor under the microscope. Each harvested batch is isolated until the plates made from the harvest time samples have been held for 96 hours and examined.

5.2 ARASCO

5.2.1 Natural History of *M. Alpina*

ARASCO, the ARA enriched single cell oil, is synthesized by a common soil fungus, *Mortierella alpina*. Fungi are eukaryotic, nonphotosynthetic organisms that have a vegetative structure known as a mycelium (a multinucleate mass of cytoplasm enclosed within a rigid, multi-branched system of tubes or hyphae). Asexual reproduction occurs by the formation of spores, which are pinched off at the tips of the hyphae. A mycelium normally arises by the germination and outgrowth of a single reproductive cell, or spore. The fungi comprise three major groups: the Phycomycetes, the Ascomycetes and the Basidiomycetes (152).

All Phycomycetes share two properties that readily distinguish them from the remaining classes of fungi. Firstly, their asexual spores are always endogenous, and secondly, their mycelium shows no cross walls except in regions where a specialized cell is formed from a hyphal tip (nonseptate mycelium). The Phycomycetes include a group known as the terrestrial Phycomycetes, which are inhabitants of soil. *Mortierella alpina* is one of the most common species of the soil inhabiting phycomycetes. It can be easily isolated from the soil and has been isolated from soils all over the world (152). Like all fungi, *Mortierella alpina* may be found associated with common root crops and may, therefore, be directly in the food chain of many mammals.

Mortierella species have been well studied for many years in isolated laboratory culture. The morphology, biochemistry and physiology are well documented in more than 25 references in the last 20 years. *Mortierella alpina* has been described in Japanese publications and patents as a

potential source of arachidonic acid and as a consequence, it has been more recently the subject of many intensive laboratory investigations (153-156). In none of these recent studies, nor in any earlier studies, has there been any report of pathogenicity or toxigenicity to humans or animals (157, 158) by *M. alpina.*

Only a few of the thousands of known species of fungi cause human diseases. In a clinical setting involving an already diseased or immunocompromized host, any fungus that grows at 37°C could be considered an opportunistic pathogen (159, 160). In 1966, *Mortierella alpina* was reported to be isolated from liver lesions of a calf in New Zealand (159) as a secondary infection. However, this report has been questioned as to whether this fungus was indeed *Mortierella alpina,* or some other species of *Mortierella.* (158).

Although some fungal species have been reported to produce mycotoxins, the mycotoxin-producing fungi belong to the class of Deuteromycota, which differ clearly from the Zygomycetous fungi to which *Mortierella* belongs (161). There has never been any report of mycotoxin production from *M. alpina* or any other of the many species of the genus *Mortierella* .

5.2.2 ARASCO Fermentation Process

The *M. alpina* strain used for production of ARASCO originates from ATCC (ATCC # 32222) and has been selected for rapid growth and high levels of production of the specific oil. The overall process has recently been described in the literature (162). Master seed banks of the production strain are maintained under liquid nitrogen conditions at Martek, Gist-brocades (who manufacturers ARASCO under contract for Martek), and at the ATCC. The working seed stock prepared from this master seed bank is also maintained cryogenically.

Upon initiation of a production run, an individual ampoule from a working seed is used to inoculate a shake flask. The medium used to grow *M. alpina* from the shake flask to production scale contains dextrose, a nitrogen source and certain salts required for optimal growth. The cultures are transferred to successively larger vessels based on certain criteria. Temperature, pH, air flow, pressure, agitation, dextrose concentration, and dissolved oxygen are all monitored and controlled.

The culture is harvested based on measurements of pH, dextrose concentration, fat content of the biomass, and arachidonic acid content of the fat. The culture is harvested by centrifugation, and the biomass is then vacuum dried. The dried biomass is flushed with nitrogen prior to being shipped to the oil processing facility or the frozen storage facility.

All ingredients used in production are of food grade quality or better. This is verified from the manufacturers Certificate of Analysis and by Martek's policy of procuring ingredients only from verified reputable vendors of food grade quality products. In order to maintain kosher certification, Martek also ensures that ingredients are only procured from recognized suppliers of products meeting strict kosher guidelines. Martek products are certified kosher by the Orthodox Union (OU). This process has also been reviewed by the B.D.Z. Eda Charedith organization of Israel and has been certified as Halal by the Islamic Food and Nutrition Council of America (IFANCA).

5.2.3 Extraction and Purification of ARASCO

The ARASCO oil is extracted from the fungal biomass and processed using methods and procedures that have been well established in the edible oils industry. These processes include refining, bleaching and deodorizing the oil. In order to protect this ARA-rich oil from oxidation, the Martek process uses the lowest effective temperatures and shortest times for each process step, and the oil is continuously protected from oxygen by nitrogen blanketing or vacuum. The oil is packaged in either nitrogen-purged containers and stored frozen, or vacuum packaged. Following analytical release testing, a Certificate of Analysis is generated and included with each shipment.

5.2.4 Production of *M. ALPINA* by Gist-Brocades (now DSM)

In a partnership agreement with Gist Brocades (GB), Martek is also supplied with crude ARASCO for further processing in its plant. The fermentation process employed by GB is fundamentally similar to that described in Section **5.2.2**. The manufacturing process is performed in accordance with CGMP and kosher requirements. The dried biomass is extracted with hexane and the crude ARASCO oil is produced upon removal of the residual solvent by evaporation. This crude oil is delivered to Martek's oil processing facility for final purification. Incoming crude oil has to meet certain quality specifications before being accepted by Martek.

5.2.5 Process Controls

All processes are documented according to CGMP with CCPs. Laboratory personnel use laboratory notebooks to record the results of lab tests and sterility checks, whereas production personnel record the continuous batch monitoring results in the batch records themselves, according to CGMP. Significant process-related data points are recorded and plotted on control charts. These data points are regularly monitored and reviewed to ensure that they are within Martek's recognized standard processing parameters.

Batch records are maintained and reviewed on a regular basis by Quality Control personnel to ensure that they are complete and accurate representations of the production process. Quality Assurance personnel monitor the production records to ensure that batch process changes have been properly authorized, documented, and recorded in the records for each batch. The FDA has inspected the manufacturing plant in Winchester, Kentucky at least on an annual basis since 1995.

Working seed from the master seed culture is shipped to the manufacturing plant in Winchester, Kentucky in one-ml cryopreservation vials, on an as needed basis, from the Martek laboratories in Columbia, MD. Each shipment of frozen seed is accompanied by a Certificate of Analysis, which provides product identification and other analytical information from laboratory notebook records maintained in Maryland. At the manufacturing plant in Kentucky, frozen seed is maintained in liquid nitrogen until thawed for use. The thawing of a seed culture vial and the initiation of the seed train is documented in laboratory notebooks maintained at the Kentucky plant.

In-process monitoring of the product for bacteriological contamination is done on a continuous basis throughout the fermentation process. Sterility checks are performed daily by plating sterile samples of the fermentation broth. The plates are cultured and held for 96 hours. During this time

the plates are checked daily for signs of contamination. Laboratory personnel and production operators also routinely visually inspect samples of the broth under the microscope. Each harvested batch is isolated until the plates made from the harvest time samples have been held for 96 hours and examined.

6 SPECIFICATIONS

The specifications for DHASCO andARASCO are shown in Figures 6 and 7 respectively. Current production lots of DHASCO and ARASCO are standardized to approximately 40% DHA and ARA, respectively, prior to packaging. This is accomplished by diluting the final deodorized product with high oleic sunflower oil (usually less than 10% dilution).

DHASCO®

General Characteristics

Description:	Vegetable oil from microalgae, containing 40% docosahexaenoic a(
Appearance:	Free flowing yellow to orange liquid oil (triglyceride)
Odor:	Characteristic
Antioxidants:	Ascorbyl palmitate (0.025%), and tocopherols (0.025%)

Chemical Characteristics

Free Fatty Acid:	< 0.4%
Peroxide Value	< 5 meq/kg
Non-Saponifiables	< 3.5%

Fatty Acid Composition:	Area %
10:0	0-2
12:0	0-6
14:0	10-20
16:0	10-20
16:1	0-2
18:0	0-2
18:1	10-30
18:2	0-5
22:5 (DPA omega-6)	0-1
22.6 (DHA)	**40-45**
24:1	0-2
others	0-3

Elemental Composition:

Phosphorous:	< 10 ppm
Iron:	< 0.5 ppm
Copper	< 0.1 ppm
Arsenic:	< 0.5 ppm
Lead:	< 0.2 ppm
Mercury:	< 0.2 ppm

Figure 6. Specification for DHASCO®, DHA single cell oil from *C. cohnii.*

Due to the final production process used for the DHASCO and ARASCO oils (a high vacuum deodorization step at over 200 degrees C) the volatile solvents used in processing are no longer present in the final product at significant levels. However, for verification purposes, several lots

of oil produced at Martek's Kentucky plant were tested for residual hexane used in both the DHASCO and ARASCO processes. Samples were stored frozen in polyethylene bottles prior to analysis. None of the samples showed detectable amounts of this solvent (detection limit < 0.3 ppm).

ARASCO®

General Characteristics

Description:	Vegetable oil from fungi, containing 40% arachidonic acid
Appearance:	Free flowing yellow liquid oil (triglyceride)
Odor:	Characteristic
Antioxidants:	Ascorbyl palmitate (0.025%), and tocopherols (0.025%)

Chemical Characteristics

Free Fatty Acid:	< 0.4%
Peroxide Value	< 5 meq/kg
Non-Saponifiables	< 3.5%

Fatty Acid Composition:	Area %
14:0	0-2
16:0	3-15
16:1	0-2
18:0	5-20
18:1	5-38
18:2	4-15
18:3 (GLA)	1-5
20:0	0-1
20:3	1-5
20:4 (ARA)	**38-44**
22:0	0-3
22:5 (DPA omega 6)	0-1
24:0	0-3
others	0-3

Elemental Composition:

Phosphorous:	< 10 ppm
Iron:	< 0.5 ppm
Copper	< 0.1 ppm
Arsenic:	< 0.5 ppm
Lead:	< 0.2 ppm
Mercury:	< 0.2 ppm

Figure 7. Specification for ARASCO®, ARA single cell oil from *M. alpina*.

7 SAFETY STUDIES

7.1 Absorption, Distribution, Metabolism, and Excretion (ADME)

DHASCO and ARASCO are triglyceride oils, and the ADME of triglyceride oils is well known. Regardless of the source of fat or oil, triglycerides represent the principal source of dietary lipid for humans. A general review of the ADME of fats and oils can be found in standard biochemistry textbooks (134, 163). The main points are presented below:

7.1.1 Generally

7.1.1.1 Absorption

Ingested triglycerides are generally emulsified in the stomach and upper intestine (duodenum) where they are mixed with bile salts and enzymatically hydrolyzed by pancreatic lipase. The pancreatic lipase has a high degree of specificity for the sn-1 and sn-3 positions of the triglyceride, and the digested triglycerides are generally absorbed by the intestinal mucosa either as free fatty acids or as the sn-2 monoglyceride. Infants also have significant levels of lingual, gastric, and bile salt stimulated lipases, all of which are less specific for the positions on the triglyceride molecule.

7.1.1.2 Distribution

The intestinal mucosal cells absorb the free fatty acids and sn-2 monoglycerides, and "retailor" these components into new triglycerides and phospholipids. Triglycerides accumulate in large 1 mm droplets containing about 88% triglyceride, 8% phospholipid, 3% cholesterol ester, and 1-2% protein (apolipoprotein B-48) which are ejected from the gut mucosal cells by exocytosis as chylomicrons (CMs). These CMs are too large to cross the basement membranes of capillaries, so they enter the large-pored lacteal (lymph) vessels and leave the intestine with lymph via the thoracic duct before being dumped into venous circulation. The CMs are then tagged with Apolipoprotein E (ApoE) and Apolipoprotein C (ApoC) following an interaction with circulating HDL. This makes the CMs vulnerable to attack by vascular lipoprotein lipase, which hydrolyses the triglyceride, releasing individual fatty acids to the tissues for metabolism. CM remnants are resorbed by the liver, via the ApoE receptor. Excess fat and ApoB-100 (+ApoE and ApoC) results in VLDL synthesis in liver. VLDL is also attacked by lipoprotein lipase, providing free fatty acids to the tissues.

7.1.1.3 Metabolism

Fatty acids are building blocks for new membranes as well as energy rich fuel sources for cellular metabolism. In adipose cells, for example, the free fatty acids produced by lipoprotein lipase are retailored to triglycerides and stored as fat (triglyceride) for later use. Most of the fatty acids in tissues throughout the body, however, are oxidized in mitochondria by the well-known ß-oxidation pathway to produce 2-carbon substrates for the Kreb's cycle. The Kreb's metabolic cycle produces CO_2 and reducing power in the form of NADPH. NADPH is then oxidized in the mitochondria to produce ATP, releasing two electrons (added to O_2 to make H_2O). The ATP produced in this way provides energy for all cellular functions.

7.1.1.4 Excretion

The lipids consumed during a meal are virtually all metabolized and excreted as carbon dioxide and water. In cases of malabsorption due to certain pathologies (*e.g.*, pancreatic insufficiency, short bowel, etc.), the triglycerides may not even enter the body and are excreted in the stools.

7.1.2 ADME of DHASCO and ARASCO

DHA and ARA from DHASCO and ARASCO are absorbed in a manner similar to other fatty acids from other dietary fats and oils. A number of human studies have demonstrated the bioavailability of the DHA and ARA from DHASCO and ARASCO in normal adults (1, 164-167) as well as in patients with various metabolic disorders (168-170). Bioavailability is generally

observed by the elevation of levels of DHA and ARA in red blood cells and/or serum phospholipids after dietary intake of the triglycerides DHASCO and/or ARASCO. Makrides *et. al.* (1) also have demonstrated bioavailability of DHA from dietary DHASCO in lactating women by the elevation of DHA in their breast milk lipids in a linear, dose dependent fashion with respect to their dietary intake level.

Both DHA and ARA are found circulating in the blood either as constituent parts of the erythrocyte membranes (*i.e.,* as membrane phospholipids), or in a non-cellular form as either triglyceride or serum phospholipids. In a recent study with human infants and using the metabolic balance method, Carnielli *et. al.* (28) demonstrated that about 80% of the DHA and ARA is absorbed when provided in a formula in the form of DHASCO and ARASCO. This value was identical to the absorption rates of ARA and DHA from the triglyceride in human milk. Starting with a 13C-labeled DHASCO, Sauerwald *et. al.* (171) have also shown that DHA is taken up from the gut, transported through the vasculature, and ultimately appears in the breast milk of lactating women at rates that were not different from any other fatty acids. These data are similar to those reported by Croset and coworkers (172) who also used 13C-labeled DHASCO to study the metabolic fate of the DHA in both rats and humans.

DHA and ARA are distributed throughout the body, but are found in higher concentrations in specific tissues such as brain, eye, testes, and heart. Studies in both rats (173, 174) and mice (175) have indicated that the former three tissues are resistant to changes in fatty acid composition in mature animals, even when provided with very large doses of DHA or ARA. Other tissues such as liver, red muscle, cardiac muscle, and adipose can vary in DHA and ARA content over a wide range, depending on the circulating levels of these fatty acids. This characteristic, however, is not unique to DHA and ARA, but is similar for other fatty acids as well.

7.2 Metabolism and Biotransformation

The metabolism of DHA and ARA is somewhat different from other dietary fats. Whereas most 16- and 18-carbon dietary fats are catabolized completely to CO2, H2O and energy, the catabolic rate of ARA, and particularly DHA, is very slow under most circumstances. Furthermore, free ARA can act as a precursor for lipoxygenase or cyclooxygenase reactions producing various omega-6 eicosanoids (*e.g.*, thromboxane A2, prostaglandin E2, *etc.*). One might expect that the very slow catabolic rate of DHA would lead to its accumulation in the body. Such an accumulation is reflected in the elevation of tissue contents of DHA when the body mass is not changing (*i.e.,* in adults). In a growing infant, on the other hand, the body mass is also increasing at a rapid rate, so even the maintenance of tissue DHA and ARA levels requires constant input of these components from the diet. The problem of declining tissue DHA and ARA levels in infants fed formulas without supplemental DHA and ARA is well known and was previously discussed.

The biosynthesis of long chain polyunsaturated fatty acids (LC-PUFAs -- greater than 18 carbons in length) involves both the sequential addition of two-carbon units to an 18-carbon precursor, and the activity of Δ5- and Δ6-desaturases (Figure 8). The desaturation steps are thought to be rate limiting in this process. The 18-carbon, essential fatty acids precursors to ARA and DHA are linoleic acid (LA) and alpha linolenic acid (ALA), respectively. The last steps in the biosynthesis of DHA have recently been demonstrated to be quite unique. A 24-carbon, hexaene precursor (C24:6(Δ6, 9, 12, 15, 18, 21)) is transported into the peroxisome and, through one step of beta

oxidation, the 22-carbon DHA (C22:6(Δ4, 7, 10, 13, 16, 19)) is formed (176). A similar process results in the formation of C22:5 (4,7,10,13,16), omega-6 DPA, which may compete with DHA matabolism or uptake into cells.

Different mammalian species have different abilities to form DHA from the essential fatty acid precursor ALA (177). Mammals that rely strictly on plant material for sustenance (*e.g., Muscus*) have no access to preformed DHA in the diet, and are generally quite efficient at the conversion of ALA to DHA. On the other hand, some animals that are obligate carnivores (*e.g.,,* felines) have no ability to convert ALA to DHA and rely completely on preformed DHA in the diet for brain development (178). Humans have some ability to convert ALA to DHA, but only at a very slow rate. This makes it difficult to find an accurate model for assessing the neurological consequences of the presence or absence of preformed DHA in the diet. Most clinical investigators have, therefore, analyzed the consequences of total omega-3, or omega-6, deficiencies for extrapolating to the human condition.

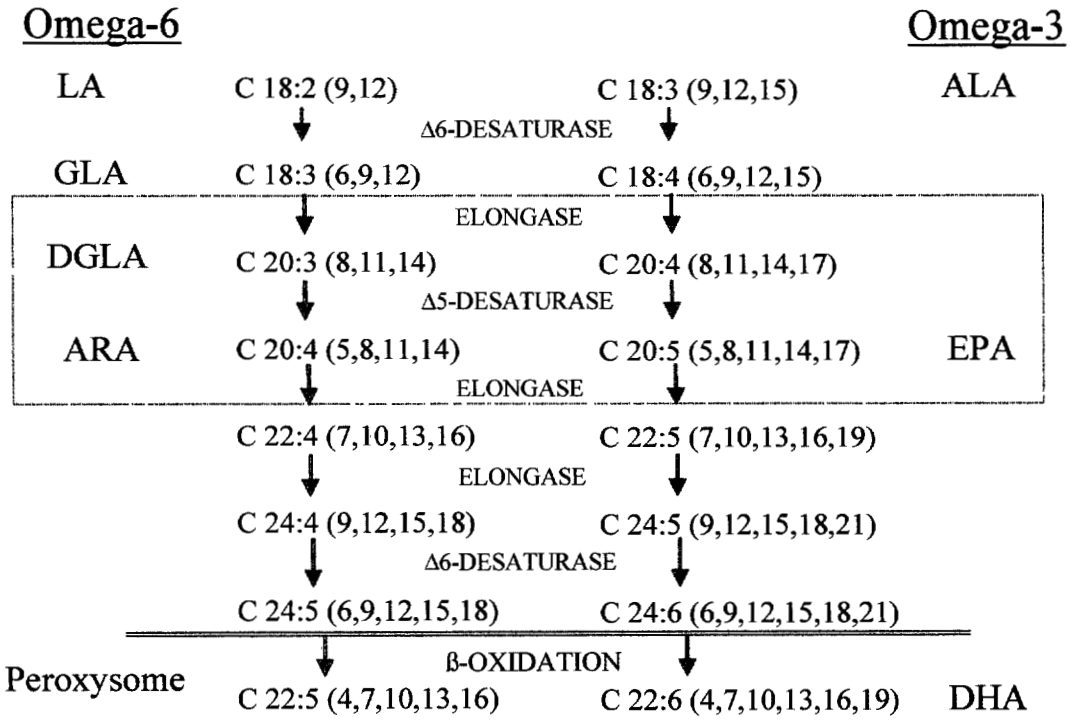

Figure 8. The omega-6 and omega-3 fatty acid biosynthetic pathways.

Few measurements have been made in man to assess abilities to elongate and desaturate ALA to DHA, or LA to ARA *in vivo*. Studies looking at the effect of dietary limitations (or excesses) of omega-6 and/or omega-3 fatty acids can provide a partial answer. Only through the use of stable isotope-labeled precursor fatty acids, however, can accurate measurements of elongation and desaturation be made. Unfortunately, there have been very few of the latter studies (179-184).

With particular reference to infants, however, the data seem clear and consistent. Neither preterm nor full term infants have the ability to elongate and desaturate dietary precursors to the extent needed to match the tissue accretion rates of babies receiving preformed DHA and ARA delivered in their mother's milk. This conclusion comes from three basic observations:

1) in every study (34 reported herein) where the blood lipids of a breast-fed baby are compared to a standard formula fed baby, the circulating DHA and ARA levels are significantly lower (typically 50% lower) in the formula-fed infants in spite of excesses of the precursors LA and ALA in the formula (see Table 1);

2) studies looking at DHA accretion in the brain tissues of infants *post mortum* have demonstrated that the DHA contents of the brains of standard formula-fed infants were much lower than those of breast-fed infants (185, 186); and

3) using stable isotope precursors, researchers demonstrated that the fractional synthetic rate for DHA in a human infant is far too slow to account for the required accumulation of DHA in the brain (179). In this case, the required accumulation is established by the gold standard of breast-fed babies.

The above three observations clearly indicate that LA and ALA are not adequate precursors for the ARA and DHA demands of a newborn infant. Such a conclusion is further supported by the studies which have found that increases in the levels of ALA and in the infant formula do not translate to significant increases in circulating levels of DHA. In fact, even the LSRO Panel specifically concluded in its 1998 report that *"[i]t is evident from the review of studies to date that efforts to increase plasma and RBC membrane DHA levels of formula-fed infants to levels comparable to those observed in breast-fed infants, either by altering the LA or ALA content or the ratio of these two fatty acids, have not been successful"* (141).

Furthermore, unless preformed ARA and DHA are added to an infant formula, one cannot expect to achieve normal rates of DHA and ARA accretion in the brain and eye tissues of that infant. Although we cannot definitively say that a slow rate of DHA accretion in the brain of an infant will have long term subtle neurological consequences, several recent well-controlled studies support this possibility. Consequently, it seems prudent to provide a nutritional source that matches the DHA and ARA accretion rates of the normal breast-fed infant.

7.3 Retroconversion of DHA into EPA.

Retroconversion is an enzymatic process whereby long-chain fatty acids are converted to their related shorter-chain precursor fatty acids through the incremental removal of two-carbon units from the molecule. This process is similar to beta-oxidation, but in this case, the reaction stops after the removal of two or four carbons. The two-carbon units are removed from the carboxyl end of the fatty acid leaving the methyl end (omega) intact, so only fatty acids from the same family are produced by this process. Thus, 22:4n-6 is thought to be retroconverted into ARA (20:4n-6) and erucic acid (22:1n-9) into 20:1n-9.

The retroconversion of DHA into EPA, however, is more complicated. It involves the removal of a double bond at the Δ4 position in addition to the removal of a two-carbon unit (*i.e.,* C22:6 is

converted to C20:5). Consequently, there is some controversy as to whether the elevation of EPA levels seen during supplementation of DHA is really due to the retroconversion of preformed DHA into EPA, or simply due to a feedback inhibition of DHA biosynthesis at a point beyond EPA, thereby resulting in a build up of EPA as an intermediate in the pathway.

Whether the biochemical mechanism involves retroconversion or feedback inhibition, clinical data have established that large oral doses of DHA consumed by humans and animals lead to an increase in circulating EPA levels (164, 165, 167, 173, 187, 188). In experiments feeding [13C]-labeled DHA, Brossard et al. (189) reported that labeled EPA and DPA(n-3) were synthesized starting from labeled DHA. Although this seems to support the hypothesis of retroconversion, these authors calculated that the conversion rate to EPA in humans receiving normal dietary levels of DHA was vanishingly small (only about 1.4%). In contrast, they also reported that rodents in the same experiment exhibited a much higher intrinsic rate of DHA retroconversion to EPA (maximum of 9%). The human clinical data available to date further indicates that the apparent retroconversion rate of DHA into EPA appears to be dramatically dependent on the amount of DHA in the diet. Holub and colleagues, for example, calculated that at very high chronic oral doses in humans (>1.2 g DHA/day), the apparent retroconversion rate could be as high as 12% (165, 187).

High levels of EPA may perturb the ARA balance in infants, and in one early clinical study using a high EPA fish oil supplementation, the high EPA levels resulted in slower growth in preterm infants (116). Thus, the possibility of retroconversion of DHA into EPA could be of concern. For this reason, only DHA sources with minimal levels of EPA (preferably no EPA) have been recommended for the supplementation of infant formulas. Once again, however, we should look to human milk as the gold standard. DHA is normally provided to a growing infant in the form of human milk so if retroconversion was taking place in infants, it is the natural process. Nevertheless, several clinical studies with preterm and full term infants have been completed and provide us with the empirical answer. The very low levels of DHA used in supplemented infant formulas (< 1.0 % of fatty acids) or in mother's milk, do not result in any significant increases in EPA in the plasma or red blood cells when the supplement used in the formulas was a mixture of DHASCO and ARASCO (29, 38, 78, 190).

In conclusion, there is no evidence for over elevation of serum EPA levels when infants are fed formulas supplemented with both DHASCO and ARASCO providing DHA and ARA levels comparable to those in human milk.

7.4 Nonsaponifiable Fraction

The nonsaponifiable fractions of DHASCO and ARASCO represent only a small fraction of the overall mass of the oils (*i.e.,* typically no more than 1.5% of the total oil mass), and are a mixture of several minor components. Like most nonsaponifiable fractions from vegetable oils, these components are predominantly sterols. The sterols from the nonsaponifiable fractions of DHASCO and ARASCO have been fractionated and analyzed by two independent laboratories, and the results are provided in Tables 6 and 8. The values given in these tables are in general agreement with values reported in the literature.

Some of these sterols, such as desmosterol, are already known to be in an infant's diet via its mother's milk (63). 4-Methyl sterols are normal intermediates in cholesterol biosynthesis, and humans have the enzymatic capability to convert 4-methyl sterols into 4-desmethyl sterols like cholesterol (135, 136). Microalgae such as *C. cohnii* are commonly found in the food web of filter feeders like mollusks (*e.g.*, oysters and clams), crustaceans (*e.g.*, shrimp and lobster), and certain fish, and therefore, these sterols can also be found in such animals. As a result, these sterols have been in the human food chain, both directly and indirectly, ever since man was eating marine foods.

Because there have been no literature reports on the effects of consumption of such 4-methyl sterols directly, a feeding study was undertaken using a high concentration of the nonsaponifiable fraction of *C. cohnii* in rats. This study compared the effects of consuming a high concentration of the nonsaponifiable fraction from DHASCO with the nonsaponifiable fraction of soybean oil, which contains predominantly sitosterol (191). Although sitosterol is known to inhibit the re-uptake of cholesterol from the gut, the nonsaponifiable fraction of DHASCO did not show a similar effect. Furthermore, when a very large portion (0.5% by weight) of the diet of rats was comprised of this nonsaponifiable fraction, there was no adverse effect on growth, general physiology, or the gross organ morphology in the recipient animals. The study concluded that there were "*no toxicological effects on the rats of consuming up to 0.5% of their diet in the DHASCO nonsaponifiables*". This dose would be equivalent to giving the rat a diet containing about 50% by weight pure DHASCO.

7.5 Toxicological Studies (Non-Human)

A large number of safety tests have been conducted on ARASCO and DHASCO oils by several different organizations. As a result, there is a large degree of redundancy in the standard toxicological assessments. This redundancy allows for an unprecedented and extremely valuable assessment of these oils. The studies include *in vitro* mutagenicity and genotoxicity assays, and a variety of controlled animal studies, such as acute, subchronic, developmental and multigenerational reproductive studies. Because these components represent macronutrients, all studies included appropriate positive as well as negative controls. All studies reported herein were conducted in Good Laboratory Practice (GLP)-compliant laboratories following guidelines outlined by the FDA in *Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Foods*, commonly referred to as Redbook I, or from their draft Redbook II. The audited summaries from each toxicology study appear in Appendix 5. Only the significant findings will be presented here. These data have also been reported in a number of publications and summarized recently by Kyle and Arterburn (192).

At the outset, it is important to recognize that DHASCO and ARASCO are macronutrients, not micronutrients, vitamins, or drugs. Safety testing of macronutrients poses several unique problems (193). For example, it is often difficult to distinguish whether an observed response is related to a toxicological effect of the test material, or due to a dietary deficiency of some other component caused by the use of such large amounts of the test macronutrient in the diet. Clearly, one cannot achieve safety margins of greater than 50-fold for a macronutrient that comprises more than 2% of the diet. Finally, when a macronutrient test material is added to the diet at a very high dose, one must carefully distinguish a truly toxicological response due to the test material from a normal physiological response resulting from the high dietary load of that particular

macronutrient. For example, serum cholesterol is elevated in a human who changes their diet from one with 25% of dietary calories as fat to one with 45% of dietary calories as fat. Is this a toxicological response to the particular fat used, or a normal physiological response to increasing total dietary fat? With this caveat, the toxicological studies completed on DHASCO and ARASCO are summarized below.

7.5.1 Acute Toxicity Studies

Five independent studies have shown that very high acute oral doses (up to 20 grams of DHASCO or ARASCO/kg body wt) did not have any major toxicological consequences in rats (Table 9). Soft stools were typically noted in the first day, but all animals gained weight during the two-week post-dosing period. Soft stools are a normal and expected consequence of a large, single dose of any fatty substance, and it was not considered to be an adverse event. Because no animals died, acute oral LD50 values could not be determined, but based on the lack of toxicity in the tests, they were greater than 20 g/kg body wt/day for each oil. In addition to the DHASCO/ARASCO studies, six acute oral dose studies using either whole biomass, delipidated biomass (residual material after removal of the oils), or a microencapsulated oil powder at doses from 4-10 g/kg also failed to show any significant toxicological finding (Table 9).

Table 9. Acute toxicity studies with DHASCO and ARASCO and various biomass forms in Rats.

Test Material	*Sponsor/ Study Date*	*Dose (g/kg bw)*	*Mortality (male female)*		*LD50*	*Reference*
DHASCO	Martek	20	0/5	0/5	>20 g/kg bw	(173)
DHASCO	Martek	20	0/5	0/5	>20 g/kg bw	(194)
C. cohnii algal biomass	Martek	7	0/5	0/5	>7 g/kg bw	(195)
C. cohnii biomass	Martek	7	0/5	0/5	>7 g/kg bw	(196)
Delipidated C. cohnii biomass	Martek	10	0/5	0/5	>10 g/kg bw	(197)
Delipidated algal biomass	Martek	6	0/5	0/5	>6 g/kg bw	(198)
ARASCO	Martek	20	0/5	0/5	>20 g/kg bw	(173)
ARASCO	Martek	20	0/5	0/5	>20 g/kg bw	(199)
ARASCO	Gist-brocades	18.2	0/5	0/5	>18.2 g/kg bw	(200)
Fungal biomass	Martek	5	0/5	0/5	>5 g/kg bw	(201)
Delipidated fungal biomass	Martek	4	0/5	0/5	>4 g/kg bw	(202)
Microencapsulated ARASCO/DHASCO blend	Martek	5	0/5	0/5	>5 g/kg bw	(203)

7.5.2 Sub-Chronic (28-63 Day) Toxicity Studies

In six independent studies, DHASCO and/or ARASCO was administered by gavage, or as a dietary admixture, daily for 28 or 63 days using doses ranging from 0.025 to 9.4 grams of test material /kg body wt/day (Table 10). The No Adverse Effect Levels (NOAEL) were determined to be the highest doses tested for each individual oil during the specific study (up to 2.5 g oil/kg body wt/day for ARASCO and 1.25 g/kg body wt/day for DHASCO) and for the combined oils (up to 9 g/kg body wt/day). At the highest dose levels there were some recurrent findings (see section 7.5.9), but these were not considered to be adverse effects of the test material because they

did not occur consistently and were not accompanied by changes in histology or clinical chemistry. The differences in organ weights were considered to be normal physiological responses to high doses of polyunsaturated fatty acids and, in some cases, due to the use of artificial diets. An additional subchronic 28-day study was undertaken with the whole *C. cohnii* biomass at a dose level of 5 g/kg body wt/d and, once again, there were no test material related toxicological findings. For the biomass, the NOAEL was therefore found to be 5 g biomass/kg body wt/d in rats.

Table 10. Sub-chronic (28 to 63 day) oral safety studies with DHASCO and ARASCO in Rats.

Test Material	*Sponsor*	*Highest Dose (g/kg bw/day)*	*Details*	*Conclusions*	*Reference*
ARASCO/DHASCO 1.5:1 blend	Wyeth Ayerst	1.2	63 days in the diet; 10 rats/sex/dose	Not toxic	(204)
DHASCO	Martek	1.25	28 days by gavage; 5-10 rats/sex/dose	No toxic	(173)
ARASCO	Martek	2.5	28 days by gavage; 5-10 rats/sex/dose	Not toxic	(173)
ARASCO	Gist-brocades	3.0	28 days by gavage; 10 rats/sex/dose	Not toxic	(200)
ARASCO/DHASCO 2:1 blend	Martek	3.75	28 days by gavage; 5-10 rats/sex/dose	Not toxic	(173)
ARASCO/DHASCO 2:1 blend	Mead Johnson	9.1/9.4	28 days in the diet; 10 rats/sex/dose	Not toxic	(205)
Algal biomass	Martek	5	28 days in diet, 5-10 rats/sex/dose	Not toxic	(206)

7.5.3 Sub-Chronic (90-Day) Studies

In six independent subchronic 90-day studies, DHASCO and/or ARASCO were administered by gavage or as a dietary admixture at doses up to 8.9 grams of test material/kg body wt/day to 20 rats per sex per dosing group. The NOAELs as determined following a critical evaluation of the data by the study site toxicologists, were the highest doses tested for each material in all studies (up to 2.5 grams of ARASCO and 1.25 grams DHASCO/kg body wt/day and up to 8.9 g/kg body wt/day for combined oils) except for the Gist-brocades study (see Table 11) (174, 188, 207). A NOAEL of 8.9 gm/kg body wt/day (about 6 grams ARASCO and 3 grams of DHASCO) represents at least a 80-fold excess of this macronutrient from the maximum intended use level of ARASCO, and a 40-fold excess from the maximum intended use level of DHASCO as recognized by this GRAS Notification (*i.e.,* 75 mg/kg body wt/day). At the highest dose levels, there were some recurrent findings (see section 7.5.9) that were not considered to be adverse because they were not consistent or accompanied by changes in histology or clinical chemistry. These "findings" were considered normal physiological responses to high doses of polyunsaturated fatty acids and/or to be due to the use of artificial diets in the feeding studies. Some of these studies also involved detailed assessments of neurotoxicity (188, 208) as outlined by the FDA Redbook II Guidelines, and other studies also involved an *in utero* supplementation phase (207). One additional 90-sub-chronic study with a full neurotoxicological assessment was also completed on the intact *C. cohnii* biomass. As in the other studies, there were no test material related

toxicological findings at the highest dose used and the NOAEL for was determined to be 5.8 g biomass/kg body wt/day (Table 11)

Table 11. Sub-chronic 90-day toxicity studies with DHASCO and ARASCO in Rats.

Test Material	*Sponsor*	*Highest Dose (g/kg bw/d)*	*Details*	*Findings*	*Reference*
DHASCO	Martek	1.25	gavage, full neurotox assessment	Not toxic	(188)
DHASCO/ARASCO blend	Numico	2.0	in diet	Not toxic	(209)
ARASCO	Martek	2.5	gavage, full neurotox assessment	Not toxic	(174)
DHASCO/ARASCO 1.5:1 blend	Wyeth Ayerst	2.5	in diet	Not toxic	(204)
ARASCO	Gist-brocades	4.9	in diet, *in utero* phase, full neurotox assessment	Not toxic	(210)
DHASCO/ARASCO 2:1 blend	Mead Johnson	8.9	in diet, *in utero* phase, full neurotox assessment	Not toxic	(207)
Algal biomass	Martek	5.8	in diet, full neurotox assessment	Not toxic	(211)

7.5.4 Chronic Toxicity/Carcinogenicity Studies

Based on *in vitro* toxicological information (see Section 7.5.6) and knowledge of the macronutrient nature of the test material, it was determined that chronic toxicity/carcinogenicity studies were not necessary for this nutrient.

7.5.5 Reproductive/Developmental Toxicology Studies

Two developmental toxicology studies were conducted with DHASCO and ARASCO administered by gavage at dose levels of 1.25 and 2.5 g/kg body wt/day respectively (Table 12). There were no adverse developmental effects at the doses tested as determined by the independent study site toxicologists setting the NOAEL at those highest doses. Developmental and reproductive (multigenerational) studies were also completed using the biomass at dose levels of 4.3 and 8.0 g/kg body wt/day (5% by weight of diet) (Table 12). In both cases there were no test material related toxicologically significant findings at the highest doses used and the NOAELs were determined to be 4.3 and 8.0 g biomass/kg body wt/day respectively.

Table 12. Developmental and reproductive toxicity studies with DHASCO and ARASCO in Rats.

Test Material	*Study Type*	*Sponsor*	*Highest Dose (g/kg bw/d)*	*Details*	*Findings*	*Reference*
DHASCO	Developmental	Martek	1.25	gavage 25/sex/dose	Not toxic	(212)
ARASCO	Developmental	Martek	2.5	gavage 25/sex/dose	Not toxic	(212)
Algal biomass	Developmental	Martek	4.3	in diet 20/sex/dose	Not toxic	(213)
Algal biomass	Reproductive	Martek	8	in diet 20/sex/dose	Not toxic	(214)

7.5.6 Genotoxicity Studies – *In Vitro*

The test materials were determined to be neither mutagenic, clastogenic, nor genotoxic in six independent *in vitro* mutagenicity tests (Table 13) when tested with and without *in vitro* metabolic activation using rat liver microsomal S9 fractions. As done previously, the algal biomass was also tested in three independent *in vitro* tests and also found to be nonmutagenic, nonclastogenic or nongenotoxic (Table 13)

Table 13. *In vitro* genotoxicity studies with DHASCO and ARASCO.

Test Material	*Test*	*Doses*	*Findings*	*Reference*
DHASCO	AMES	100-5,000 ug/plate	Not mutagenic	(215, 216)
DHASCO	Forward mutation	750-5,000 ug/ml	Not mutagenic	(215, 216)
DHASCO	Chromosomal aberration	1250-5,000 ug/ml	Not clastigenic	(28, 29)
ARASCO	AMES	100-5,000 ug/plate	Not mutagenic	(215, 216)
ARASCO	Forward mutation	750-5,000 ug/ml	Not mutagenic	(215, 216)
ARASCO	Chromosomal aberration	1250-5,000 ug/ml	Not clastogenic	(215, 216)
Algal biomass	AMES	33-5,000 ug/plate	Not mutagenic	(217)
Algal biomass	Forward Mutation	30-1,000 ug/ml	Not mutagenic	(218)
Algal biomass	Chromosomal Aberration	50-1,000 ug/ml	Not clastogenic	(219)

7.5.7 Special Studies

Tests for potential dinoflagellate toxins were conducted even though there is no indication from historical data that this species produces any toxins. DHASCO was given by intraperitoneal injection to mice at doses as high as 10% of the animal's body weight (100 g/kg body wt) without any toxicological activity. Analytical and biochemical tests for specific dinoflagellate-related toxins in the DHASCO oil were all negative. In addition, no endotoxins were identified in studies using the complete biomass as a test material when provided intraperitoneal injections of up to 1.25 g/kg body wt.

7.5.8 Studies with Whole Algal Biomass

In addition to studies on the oil product produced using the production process outlined in Sections 5 and 6, a further level of safety testing was undertaken using the intact, unprocessed biomass directly as a food component. In such an instance, all available potential toxicants would be imposed on the animals. When fed in doses as large as 7 g/kg body wt/day, the biomass showed no acute toxicity (Table 9). At a sub-chronic dose of 5 g/kg bw/day for 28 days, the biomass likewise showed no toxic indications (Table 10).

More extensive developmental and multigenerational toxicology tests were also undertaken with the intact biomass (Tables 11 and 12). At a dose of 8 g biomass/kg body wt/day in the diets of rats, there were no dose-related toxicological findings in reproductive parameters, over three

generations. Since these products are intended for use in the perinatal period, additional developmental toxicity testing was completed at doses of 4.3 g biomass/kg body wt/day and there were again no test material related toxicological findings at that dose. Finally, the intact biomass also revealed no mutagentic or clastogenic effects using *in vitro* assays (Table 13). In conclusion, not only does the processed oil show no toxicological activities of concern, but the parent biomass itself is equally free of toxicological findings.

7.5.9 Recurrent Findings – Relative Organ Weights

Out of thirteen 4 sub-chronic toxicity studies completed on these oils, five indicated a statistically significant increase in relative liver weights at the highest doses of ARASCO or ARASCO/DHASCO blends (Table 14). These findings were not consistently observed across the studies, however. More importantly, changes in liver weights were not accompanied by changes in liver histopathology or abnormally high levels of liver enzymes in the serum. The independent study pathologists separately, and unanimously, concluded that these were not adverse toxicological findings.

Because this phenomenon might be considered an important toxicological finding, a detailed, simultaneous evaluation of the thirteen sub-chronic studies was undertaken to gain a better understanding of the relevance and consistency of this finding. A careful simultaneous evaluation of the liver-related clinical chemistries in all studies did not reveal any consistent dose-dependent effects. All trials contained both low fat and high fat controls, although the choice of control fat source varied (corn oil, soybean oil, canola oil, and high oleic sunflower oil). The high fat control was necessary to distinguish physiological responses to a high fat diet from specific test material related phenomena. (The total fat load in these studies were generally two to three times the normal level found in standard rat chow.) In addition, synthetic diets were used in some studies while others used standard chow, some groups mixed the oils directly into the diet, and others provided the oils by gavage at a specific dose based on animal weight. As a result, the trials represented a broad spectrum of designs, although the numbers of animals of each sex and multiple-dose approaches were generally consistent with FDA Redbook guidelines.

Although some studies reported a statistically significant increase in the liver weights relative to body weights compared to high fat controls, none of the mean relative liver weights were outside the historical normal range (shaded bar in Figure 9). Study pathologists also noted a slight to moderate vacuolization (accumulation of lipid) in some, but not all, of the high dose treatment groups, but the incidence of this finding was not different than the high fat control group and was attributed to the high fat diet or the use of synthetic diets with high fat and carbohydrate, as has been previously reported in the literature (220-223). No other histopathological changes (*i.e.*, necrosis , *etc.*) were observed consistently in any of the groups, and there were no consistent changes in clinical chemistry that would suggest toxicity. Three studies reported a decrease in albumin levels and/or total protein levels, but this finding was not consistent across studies, nor did the changes parallel increases in liver weights within or across studies.

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4 Excluding Wyeth 63-day and Numico 90-day studies for which detailed data was not available.

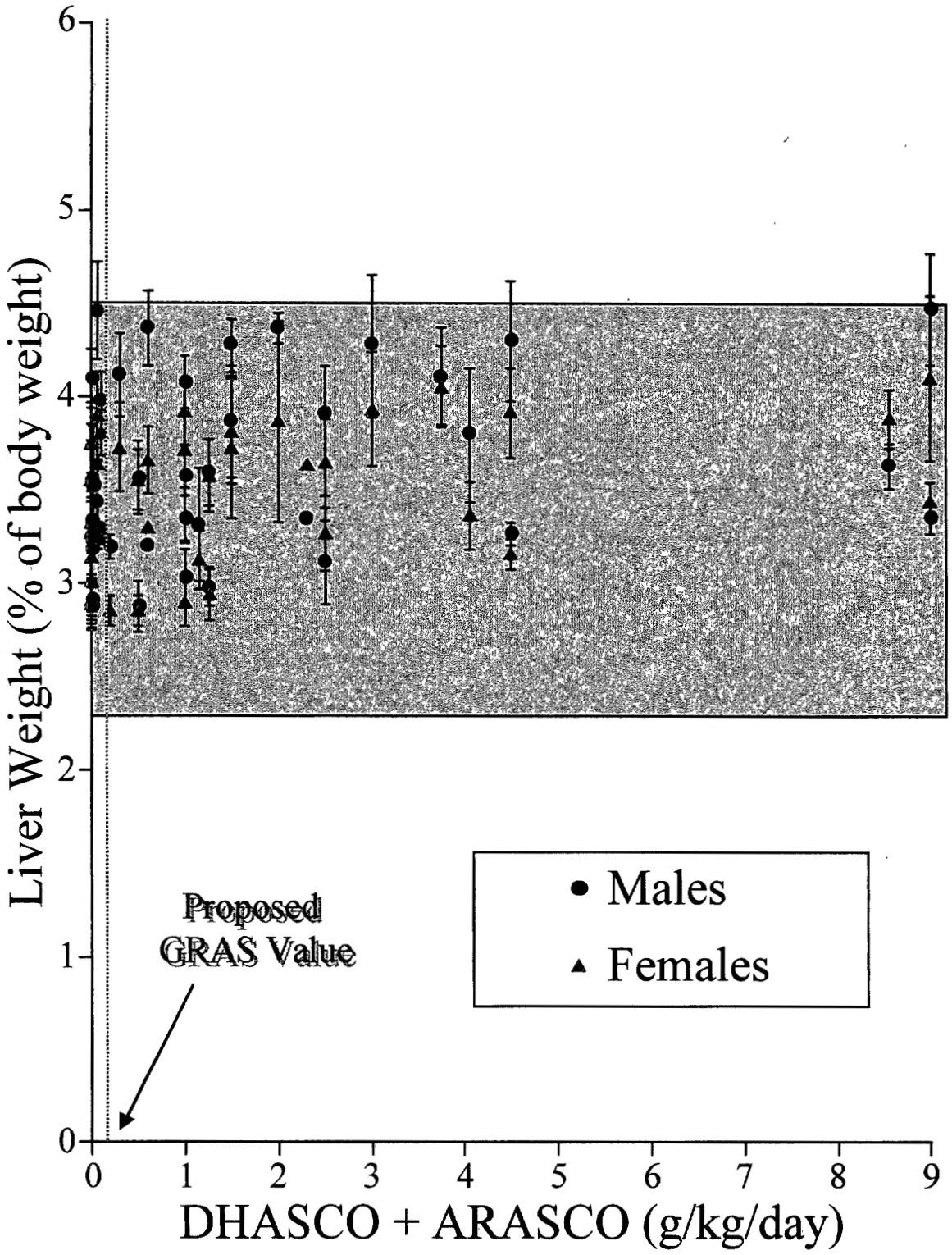

Figure 9. Changes in relative liver weights in rats with chronic consumption of DHASCO and ARASCO. Shaded area represents the historical control range.

Table 14. The Effect of Dietary Supplementation With DHASCO and/or ARASCO at various doses (mg/kg/day) on the Relative Liver Weights (% of body weight) of Male and Female Rats*.

Males

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F*	GB28A	GB28fo	GB28Afo	GB90A	GB90Afo
0				2.87	2.87	3.22	3.32	3.35				3.53	3.53
H	3.55	3.55	3.55	2.9	2.9	3.23	3.1	3.23	4.08	4.08	4.08	3.33	3.33
25	3.54												
50		3.42								4.45			
100								3.27	3.98				
200												3.2	
300										4.11			
500	3.56				2.89								
600								3.21	4.37				
1000		3.59		3.03						4.08		3.35	
1150							3.3						
1250	3.61				2.98								
1500			3.88							4.3			
1600						3.39							
2000									4.36				
2300								3.37					
2500		3.89		3.12									
3000									4.25		4.28		
3750			4.11										
4050							3.76						
4500						3.69					4.47		
4900												3.27	
8550							3.64						
9100													3.36
9250						3.53							

Females

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F*	GB28A	GB28fo	GB28Afo	GB90A	GB90Afo
0				2.89	2.89	3.53	3.25	3.64				3.15	3.15
H	3.56	3.56	3.56	2.89	2.89	3.5	3.03	3.31	3.74	3.74	3.74	2.9	2.9
25	3.58												
50		3.63								3.9			
100								3.24	3.81				
200												2.87	
300										3.73			
500	3.56				2.86								
600								3.31	3.66				
1000		3.73		2.91						3.92		2.91	
1150							3.14						
1250	3.58				2.99								
1500			3.73							3.81			
1600						3.65							
2000									3.89				
2300								3.34					
2500		3.64		2.4									
3000									3.94		3.92		
3750			4.06										
4050							3.56						
4500						3.72					4.09		
4900												3.75	
8550							3.89						
9100													3.45
9250						3.87							

* Values in yellow represent the results of 13 separate toxicological studies; Highlighted values are statistically different from controls. Tox study labels indicate sponsor (MK, Martek; MJ, Mead Johnson; W, Wyeth/Ayerst; GB, Gist-brocades), duration (28 or 90 days), and test material (D, DHASCO; A, ARASCO; F, DHASCO+ARASCO; fo, fish oil). H, high fat control.

A comprehensive survey of the published literature revealed that significant increases in relative liver weights from high doses of long chain polyunsaturated fatty acids has been well established

in rats (224-230), mice (231, 232), Guinea pigs (233), and rabbits (234). Most of these studies used various fish oils, including Menhaden oil which has already been affirmed by the FDA as GRAS for many food uses. The salient features of these studies are provided in Table 15. Regardless of the source of the PUFA, this table indicates that there is a consistent 20-40% increase in relative liver weights in response to the feeding of test fish oils at levels of 3-5% of the diet as LC-PUFA (sum of EPA + DHA). In this respect, DHASCO and ARASCO are no different than the fish oils

Table 15. Effect of dietary fish oils on Liver Weights of Rats. Highlighted values represent significant differences relative to controls.

Species	*Study Reference*	*Test Oil*	Control Oil	*Duration (wks)*	*Dose (wt % of diet)*		*% Increase Liver Wt*	
					Test Oil	*LC-PUFA*	*Absolute*	*Relative*
Rats, female	(235)	Menhaden	Corn	8	17	3.5	32	22
		Cod liver	Corn	8	19	3.2	47	41
		Menhaden	Lard	5	17	3.5	18	11
		Sardine	Lard	5	17	5.1	16	14
		Cod liver	Lard	5	19	3.2	36	48
		Menhaden	Lard	10	17	3.5	7	27
		Sardine	Lard	10	17	5.1	16	29
		Cod liver	Lard	10	19	3.2	36	48
	(236)	Menhaden	Lard	5	17	3.5	45	33
		Sardine	Lard	5	17	5.1	37	22
		Cod liver	Lard	5	19	3.2	29	33
		Menhaden	Lard	10	17	3.5	32	25
		Sardine	Lard	10	17	5.1	47	38
		Cod liver	Lard	10	19	3.2	29	38
Rats, male	(224)	Menhaden	Safflower	10	20	3.4	33	nr
	(225)	Omega	Coconut/	3	1.4	0.1	23	5
		Pharma	Safflower/	3	2.8	0.2	25	8
		fish oil	tallow	3	5.6	0.3	51	18
	(226)	Menhaden	Corn	10	24	4.8	20	nr
	(237)	MaxEPA	Safflower	2	15	6	2	9
	(238)	EPA-EE	Palmitic	12	1.3	1.2	13	4
		DHA-EE	Palmitic	12	1.3	1.3	29	14
	(229)	Fish oil	Corn	4	17	4.4	18	17
Rats, male, lean	(230)	Menhaden	Coconut	10	18	3.6	13	13
			Safflower	10	18	3.6	18	17
Rats, male, obese		Menhaden	Coconut	10	18	3.6	3	25
			Safflower	10	18	3.6	-4	13
Mice, female	(231)	DHA-EE	Safflower	8	1	1	nr	-32
Mice, male		DHA-EE		8	1	1	nr	87
	(232)	Salmon	Low fat	0.4	10	5.3	nr	25
			chow	2			nr	25
				3			nr	25
Guinea pigs, male	(239)	Menhaden	Corn	4.5	10	2.0	nr	17
		Seal		4.5	10	1.3	nr	38
		Shark liver		4.5	10	2.0	nr	40
Rabbits, female	(234)	Menhaden	nr	129	14	4.0	18	27
				129	7	2.0	11	15
				129	0.7	0.2	-8	-2

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Table 16 presents a summary of the results of the toxicology studies performed on DHASCO and ARASCO, in a manner that they can be compared directly to the literature values in Table 15. In most cases, the doses of DHASCO and ARASCO used were lower than those for the fish oil, and there were no significant increases in relative liver weights at these low levels. Responses to DHASCO and ARASCO diets were similar to those reported for the fish oils when the doses used were similar such as in the Mead Johnson studies (Table 15). Thus, we conclude that the increase in relative liver weights seen in some studies where very high levels of DHASCO or ARASCO were used, is consistent with a well established effect of the PUFAs themselves, and are not due to some unknown component unique to these oils.

Table 16. Effect of dietary DHASCO and ARASCO on Liver Weights of Rats. Highlighted values represent significant differences relative to controls.

Study References	*Test Oil(s)*	*Control Oil*	Duration (wks)	*Dose (wt % of diet)*		*% Increase in Liver Wt*			
						Absolute		*Relative*	
				Test Oil	*LC-PUFA*	*M*	*F*	*M*	*F*
(173)	DHASCO	HOSO	4	1.1	0.4	4	1	2	0
	ARASCO	HOSO	4	2.3	1.1	13	3	10	2
	Formulaid	HOSO	4	3.3	1.5	18	14	16	14
(174)	ARASCO	HOSO	13	3.3	1.8	3	10	9	12
(188)	DHASCO	HOSO	13	1.7	0.9	1	0	4	3
(205)	Formulaid	Canola	4	6.0	2.6	11	0	14	6
				12	5.3	7	7	9	11
(207)	Formulaid	Canola	13	6	2.8	30	23	21	11
				12	5.6	17	36	17	28
Wyeth study, unpublished	Formulaid	Soy	13	3.3	1.0	8	2	4	12
Gist-brocades study, unpublished	ARASCO	Corn	4	3.5	1.2	4	-2	4	5
	ARASCO + FO			5.7	1.8	7	2	9	10
Gist-brocades study, unpublished	ARASCO	Corn	13	7.5	3.0	2	8	-2	9
	ARASCO + FO			13.0	4.4	2	19	-2	19

There are several hypotheses in the literature to explain the effect of high doses of LC-PUFAs, regardless of source, on liver weights. Polyunsaturated fatty acids are well known to down-regulate lipogenesis (fat biosynthesis), thereby slightly decreasing the total body weight without affecting lean body mass. This is often difficult to detect in a growing animal and, in fact, no significant change in growth was seen as a consequence of the treatment oils in any of the trials. If there was a reduction in total body fat as a result of the PUFAs in the diet, then other organs should also show an increase relative to body weight. Organ to organ weight ratios, therefore, are generally accepted to be a better measure of specific changes in an organ under these circumstances.

Liver:brain weight ratios in these studies reveal that there is no longer an observable effect of dose on liver weights in twelve of the thirteen studies (Table 17). The hypothesis that the change in relative liver weights is due to a reduced lipogenesis and body fat content would be consistent with the lack of histological or clinical chemical evidence for any liver toxicity. Literature reports also note that PUFAs are generally metabolized in the liver and the increased liver size in response to high doses of PUFAs, simply represents a natural hypertrophy of this organ to handle the increased metabolic load imposed upon it by the high doses of PUFA.

Table 17. The Effect of Dietary Supplementation with DHASCO and/or ARASCO at various doses (mg/kg/day) on the Liver/Brain wt Ratio in Male and Female Rats*.

Males

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W 90F	GB28A	GB28fo	GB28A fo	GB90A	GB90A fo
0				7 03	7 03	5.97	8 86	9 61				7 9	7 9
H	5 86	5 86	5 86	7 44	7 44	6 21	8 8	8.68	6 25	6 25	6 25	7 09	7 09
25	5 75												
50		5 36								7 18			
100								9 79	6 35				
200												6 89	
300										6 38			
500	5 97				7 46								
600								8 89	6 94				
1000		5 72		7 6						6 82		7 37	
1150							9 27						
1250	5 99				7 5								
1500			6 51							6 51			
1600						6 3							
2000									6 92				
2300								9 38					
2500		6 55		7 54									
3000									6 6		6 63		
3750			6 62										
4050							11 32						
4500						6 92					6 58		
4900												7 03	
8550							10 31						
9100													7 19
9250						6 71							

Females

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Afo	GB90A	GB90Afo
0				4.17	4.17	4.56	5.3	5.32				4.4	4.4
0	4.47	4.47	4.47	4.28	4.28	4.96	4.93	5.47	4.42	4.42	4.42	4.15	4.15
25	4.32												
50		4.25								4.31			
100								5.77	4.37				
200												4.03	
300										4.05			
500	4.42				4.37								
600								5.63	4.2				
1000		4.77		4.19						4.52		4.34	
1150							4.99						
1250	4.4				4.34								
1500			4.63							4.45			
1600						4.53							
2000									4.29				
2300								6					
2500		4.53		4.66									
3000									4.28		4.52		
3750			5.08										
4050							5.96						
4500						4.69					4.46		
4900												4.44	
8550							6.6						
9100													4.9
9250						5.07							

* Values in yellow represent the results of 13 separate toxicological studies; Highlighted values are statistically different from controls. Tox study labels indicate sponsor (MK, Martek; MJ, Mead Johnson; W, Wyeth/Ayerst; GB, Gist-brocades), duration (28 or 90 days), and test material (D, DHASCO; A, ARASCO; F, DHASCO+ARASCO; fo, fish oil). H, high fat control.

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No significant increases were observed in any of the other organs except spleen. Like the liver, relative spleen weights were increased in only some of the studies, and the increased spleen weights were found only in the high dose groups. The spleen weight data are shown in Table 18 and Figure 10. Once again, the spleen weight changes were all well within the historical normal values, and there were no consistent dose-related responses. Furthermore, there were no significant changes in any of the studies when comparing spleen/brain weight ratios. Because there are no associated histopathology or alterations in clinical chemistry, all the pathologists reportede that these findings were not adverse events. In many of the studies listed in Table 15, the authors also reported an increase in relative spleen weight in addition to the increases in relative liver weight. Again, the observation is made when using equivalent doses of PUFA, regardless of the source of that PUFA, establishing that this is a PUFA-related phenomenon, and is independent of the carrier oil itself.

Clinical studies further demonstrate that the modest increases in liver and spleen weight are of no toxicological significance. Wyeth-Ayerst conducted a large, multi-center safety study using ARASCO and DHASCO in preterm infant formula that showed no effects on growth, or any serological marker of liver or spleen function (98). These clinical studies showed no differences between formula-fed groups (with and without DHASCO and ARASCO) for liver function markers such as serum protein, albumin, ALT, AST, ALK P, bilirubin, triglycerides, BUN, or other routine analytes. Nor did these studies show any differences in markers for spleen function such as hemoglobin, mean cell hemoglobin, mean cell hemoglobin concentration, mean cell volume, hematocrit, basophils, eosinophils, white blood cells, lymphocytes, monocytes, neutrophils, platelets or red blood cells.

In conclusion, the administration of high doses of ARASCO, DHASCO or fish oil (more than 2 g/kg/day) to rats in a subchronic fashion can modestly increase liver and spleen weights relative to body weights. This effect largely takes place within a few weeks of administration of the high levels of the PUFAs. Regardless of the source of PUFA (*i.e.,* DHASCO, ARASCO, or fish oil) the magnitude of the response was similar when using similar levels of PUFA, and consistent with other reports in the literature for a wide variety of different fish oils and animal models. Thus, the relative liver and spleen weight changes appear to be a generalized LC-PUFA effect, and is not specific to either of the single cell oils.

The safety of DHASCO and ARASCO when used as sources of DHA and ARA in infant formulas is further supported by the controlled conditions under which the formula will be manufactured. Infant formulas are manufactured under strict GMP controls. These strict controls will ensure that the infant formula has a consistent formulation, including ARA and DHA levels.

The calculated safety margins for DHASCO and ARASCO should reflect the safety relative to the sources of the oils rather than effects relative to PUFAs. The various independent toxicologists who conducted the safety studies have concluded that the NOAEL for DHASCO and ARASCO was the highest doses used in these studies. The Expert Panel convened by Martek to review the available data reached a similar conclusion. These levels represent a 40- to 80-fold excess over the intended use levels and are use levels for this macronutrient that would not be physically attainable in any sustained fashion.

Table 18. The Effect of Dietary Supplementation With DHASCO and/or ARASCO at various doses (mg/kg/day) on the Relative Spleen Weights (% of body weight) of Male and Female Rats*.

Males

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Afo	GB90A	GB90Afo
0				0 156	0 156	xx	0.175	xx				0 152	0.152
H	xx	xx	xx	0 155	0 155	xx	0.157	xx	0 187	0 187	0 187	0 159	0 159
25	xx												
50		xx								0 182			
100								xx	0 196				
200												0 15	
300										0 182			
500	xx				0 152								
600								xx	0 175				
1000		xx		0.178						0 195		0 152	
1150							0 165						
1250	xx				0 16								
1500			xx							0 201			
1600						xx							
2000									0 21				
2300								xx					
2500		xx		0.19									
3000									0.215		0 199		
3750			xx										
4050							0.201						
4500						xx					0 199		
4900												0 168	
8550							0.195						
9100													0.177
9250						xx							

Females

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Afo	GB90A	GB90Afo
0				0 19	0 19	xx	0 192	xx				0 192	0 192
H	xx	xx	xx	0.199	0 199	xx	0.165	xx	0 206	0.206	0.206	0.182	0 182
25	xx												
50		xx								0 207			
100								xx	0 223				
200												0 18	
300										0 214			
500	xx				0 192								
600								xx	0.226				
1000		xx		0 233						0 217		0 181	
1150							0 187						
1250	xx				0 198								
1500			xx							0 222			
1600						xx							
2000									0 233				
2300								xx					
2500		xx		0 231									
3000									0 226		0 224		
3750			xx										
4050							0.194						
4500						xx					0.248		
4900												0.203	
8550							0.244						
9100													0.214
9250						xx							

* Values in yellow represent the results of 13 separate toxicological studies; Highlighted values are statistically different from controls. Tox study labels indicate sponsor (MK, Martek; MJ, Mead Johnson; W, Wyeth/Ayerst; GB, Gist-brocades), duration (28 or 90 days), and test material (D, DHASCO; A, ARASCO; F, DHASCO+ARASCO; fo, fish oil). H, high fat control.

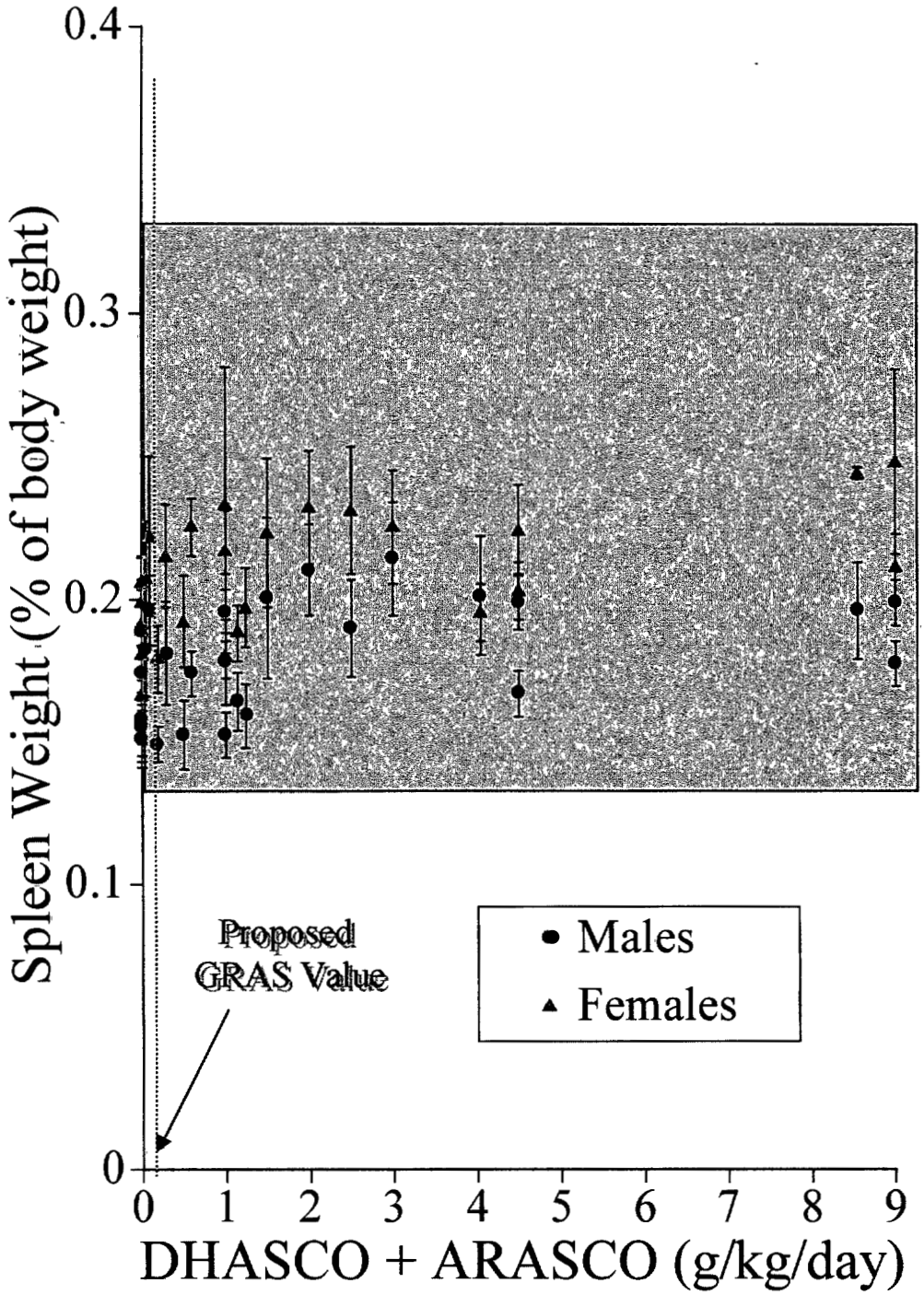

Figure 10. Changes in relative spleen weights in rats with chronic consumption of DHASCO and ARASCO. Shaded area represents the historical control range.

7.5.10 Recurrent Finding—Blood Chemistry

As with liver and spleen weights, some of these studies also noted statistically significant changes in certain blood parameters measured. Appendix 5 contains charts which plot the results from the 13 sub-chronic studies and identifies those values that are found to be statistically significant. A review of these charts reveals that although there are a few reported statistically significant effects, these effects are not dose-related, they are not seen consistently across comparable studies, and the observations are not consistently observed in both genders. Due to these and other factors, the pathologists in these studies concluded that these observations were not of toxicological significance. The Expert Panel convened by Martek to review the data reached a similar conclusion.

Notably, there are minimal changes in mean HCT and APTT among high dose animals compared to high fat control animals in one of the thirteen studies. In this study, however, serologic measures (including white blood cell, red blood cell, and platelets) for the high dose treatment animals were nearly identical to those of the low fat controls. Furthermore, the APTT values of the high dose females were nearly identical to those for female rats in the low fat control group. These results suggest that the statistically significant differences between high fat controls and high dose treatment groups in this one study were of no physiological consequence.

Data collected from clinical studies further demonstrates that the minor differences noted in some of the blood chemistry measurements should not be considered of toxicological significance. As discussed in section 7.5.9, the clinical studies of Wyeth-Ayerst (98) showed no significant differences between formula-fed groups (with and without DHASCO and ARASCO) with respect to serum protein, albumin, ALT, AST, ALK P, bilirubin, triglycerides, BUN, hemoglobin, mean cell hemoglobin, mean cell hemoglobin concentration, mean cell volume, hematocrit, basophils, eosinophils, white blood cells, lymphocytes, monocytes, neutrophils, platelets, red blood cells and other routine analytes.

The only blood chemistry markers in the thirteen rat studies that reached statistical significance was cholesterol and triglycerides. Significant reductions in cholesterol levels were seen in the one high dose supplementation study which consisted of ARASCO and fish oil. This observation was not unexpected because cholesterol lowering by fish oil is a well observed phenomenon (240). The observation was not consistent across studies that used DHASCO rather than fish oils. A reduction of serum triglycerides was noted in the highest dose groups of three of the thirteen studies. Again, this observation is consistent with literature reports for the effect of high dose LC-PUFA supplementation (especially fish oil) and is attributed to the PUFA not the test materials DHASCO or ARASCO.

A thorough review of the blood chemistry data, therefore, shows that the effects that have been observed are not considered of toxicological significance. Such a finding is supported by the pathologists who reviewed the original study results and the Expert Panel convened by Martek to review the available data. The absence of any changes in blood chemistry parameters, with the exception of cholesterol which can be attributed to the fish oil and triglycerides, which is a well known effect of high dose LC-PUFA supplementation, further corroborates the position that the observed effects are of no toxicological significance.

7.6 PUFA Potential for Oxidation

All polyunsaturated fatty acids are prone to oxygen radical attack. Such an interaction results in lipid peroxides that can cause severe injury to biological membranes. Nevertheless, the best nutritional source for a human infant is its mother's breast milk which effectively delivers DHA and ARA during early growth and development. Lipophilic antioxidants such as carotenoids or tocopherols exhibit a remarkable ability to prevent oxidative damage to biological membranes. In many cases, these lipophilic antioxidants act in concert with hydrophilic antioxidants such as ascorbic acid and glutathione. One antioxidant of major importance in the protection of oxidative damage within the cells is a large protein known as superoxide dismutase (SOD).

Infants, particularly preterm infants, are notoriously susceptible to metabolic disruptions caused by oxidative attack. Such pathologies as necrotizing enterocolitis (NEC), bronchopulmonary dysplasia (BPD), and retinopathy of prematurity (ROP) are all aggravated by oxygen in preterm infants. Since human milk contains DHA and ARA, albeit at relatively low concentrations, one might expect *a priori* that human milk feedings might exacerbate the oxidation problem. On the contrary, however, it has long been known that these conditions generally occur at a lower frequency in infants who are receiving human milk (*i.e.,* with DHA and ARA) compared to those on artificial formulas. Recent biochemical evidence from several laboratories helps us to understand this phenomenon. It has been shown that when LC-PUFAs are incorporated into cellular membranes, there is a stimulation of the production of SOD (241, 242). Thus, the antioxidant status of an infant fed human milk may actually be better than that of a formula-fed infant because of the stimulation of the SOD by the DHA and ARA in the milk.

The above biochemical evidence explains why eliminating DHA and ARA from the diet of infants, particularly preterm infants, may put them at higher risk for oxidation dependent pathologies such as NEC, BPD and ROP. This has been recently confirmed in an animal model for NEC by Caplan *et al.* (114). Those authors demonstrated that the incidence of NEC was significantly lower in rats fed formulas containing DHA and ARA (using the DHASCO and ARASCO as sources) than in rats fed formulas containing no DHA or ARA. This has also been confirmed in human infants by Carlson and colleagues (113) using egg yolk as a source of DHA and ARA. In her controlled clinical study, infants who received standard preterm infant formula had an incidence of NEC of 18%, whereas the incidence was reduced to only 3% if the babies were given a formula that was supplemented with DHA and ARA. About 4,000 babies die each year in the United States as a result of NEC (243). Extrapolating Carlson's data to all U.S. babies, the inclusion of DHA and ARA into the formulas could substantially reduce the morbidity and mortality associated with this disease.

We conclude, therefore, that if DHASCO and ARASCO are provided to infants at the GRAS levels (*i.e.,* DHA and ARA up to 0.5% of total milk fat lipids), no oxidative injury would be expected. In fact, based on laboratory and clinical evidence, it is quite likely that the addition of DHA and ARA to formulas at these levels (similar to breast milk) may well reduce the incidence of oxidative damage in predisposed infants because of the stimulation of intracellular antioxidants such as SOD.

8 PRECLINICAL STUDIES.

There have been a number of reports involving the supplementation of the diets of various mammalian species including mice, rats, pigs, cats, dogs, monkeys and baboons with DHASCO and/or ARASCO. We are also aware of ongoing or unpublished studies with hamsters, cows, horses, and chickens. Some of these studies are tabulated and summarized in Appendix 1. Dosages used ranged from levels normally expected to be used as a dietary supplement, to doses wherein the total fat intake of the animal was comprised solely of DHASCO or ARASCO. None of these studies were designed as toxicology studies such as those described in Section 7, but rather, each of the investigators studied the effects of enriching tissue levels of DHA and/or ARA on a particular physiological response in a selected animal model. DHASCO and/or ARASCO are now commonly used as the dietary source of DHA and/or ARA by researchers because they are the most concentrated and purest sources available for use in such studies. These studies, in total, represent a very large experience base of dietary treatment of many different mammalian species with DHASCO and ARASCO, and there have been no suggestions from these reports of any toxicological or safety issues with these oils.

9 CLINICAL STUDIES

9.1 INFANT STUDIES

ARASCO and/or DHASCO oils have been the subject of at least 14 well-controlled clinical intervention trials involving approximately 1,500 term or preterm infants, with over 700 of the infants receiving DHASCO and/or ARASCO oils (29, 38, 69, 77, 78, 89, 90, 244). These trials are summarized in Appendix 2. All of the trials analyzed blood lipids (either erythrocyte or plasma phospholipids) and growth as primary endpoints. In all cases, the supplementation of DHASCO and ARASCO resulted in an improvement of the circulating DHA and ARA status of the infant equivalent to that of a breast-fed infant and no study had any adverse effect on infant growth.

One trial (38) was of particular interest in this respect because it involved the comparison of various doses of DHASCO and ARASCO to optimize the blood lipids of the infants. Other functional endpoints were also studied in a few cases. In contrast to preliminary reports of a reduced growth rate when using a fish oil (*i.e.,* EPA-containing) formula (100, 245), none of these studies reported reduced growth. In fact, two studies using DHASCO/ARASCO supplemented formulas report significantly increased growth in the supplemented formula-fed infants (90, 112). As discussed in section 2.7, reduced growth rate in the fish oil trials may have been due to EPA, which is always present in fish oils, and/or the lack of ARA in the formulas.

Significant improvements in visual and mental acuity have also been reported when infants were fed DHASCO/ARASCO supplemented formulas. Visual acuity improvements were reported to be equivalent to one line in an eye chart at one year of age (78). Mental acuity improvements in the same study were seven IQ points at 18 months of age, as determined by a Bayley MDI assessment (106). In one preterm study (89) where visual acuity was assessed but no significant improvements were observed, the authors explain that the lack of a statistically significant response was likely due to the short duration of the supplementation (*ca.* only 4 weeks during the

in-hospital stay). Another study did an extensive analysis of potential adverse events and the only statistically significant observations were that the DHASCO/ARASCO- supplemented formula-fed infants experienced less anemia and less nervousness or irritability (72, 244). Although these would generally be categorized as beneficial aspects of the supplemented formula, the incidence levels were low and these were not primary endpoints of the study.

The data indicate that infants fed standard formula had significantly altered blood biochemistry and a deficiency in visual and neurological functional assessments compared to breast-fed controls. *Post mortem* examination of the brains of babies that had died of accidental causes demonstrated brain DHA deficiencies in formula-fed infants compared to breast-fed infants (80, 246). Although the DHASCO/ARASCO-supplemented, formula-fed babies had significantly better functional outcomes than the standard formula-fed infants, they were never better than the breast-fed infants. Thus, re-supplying the DHA and ARA via the formula (using DHASCO and ARASCO) at the levels provided in breast milk is sufficient to overcome these deficiencies and the problems or biochemical abnormalities caused by feeding infants with a standard formula.

9.2 Other Clinical Studies

There have been at least 29 well-controlled, clinical intervention trials reported with adults, adolescents, or children wherein DHASCO and/or ARASCO have been used at various dose levels and for various periods of time (over 30 publications). These trials and relevant publications are summarized in Appendix 3 (note that some of these trials have led to multiple publications pertaining to various aspects of the trial).

The studies by Nelson (53, 136) are of particular import because they were undertaken under the highly controlled environment of a metabolic ward by the United States Department of Agriculture. Furthermore, these studies used particularly high levels of supplementation – 3 g ARASCO per day (*ca.* 5% of daily fat) in the first trial (247), and 15 g DHASCO per day (*ca.* 25% of daily fat) in the second trial (248). A vast amount of data were gathered on the subjects (10 publications to date from these two studies). It was clear that the levels of DHA and ARA were significantly elevated in these subjects (*i.e.,* the material was bioavailable) and there were no reported adverse responses to the treatments. The other studies listed in Appendix 3 represent a cross section of healthy men, women and children of all ages, women who were pregnant or nursing, individuals with certain dietary restrictions (*i.e.,* vegetarians), and individuals with pre-existing metabolic disorders, including hyperlipidemia, retinitis pigmentosa, long chain hydroxyacylCoA dehydrogenase deficiency, ADHD, *etc.* The Nelson studies, in combination with the other studies using lower doses, provide additional evidence that the dietary supplementation of adults with DHASCO and/or ARASCO at the levels of 2-3 grams of oil per day would be generally recognized as safe by experts in the field.

10 COMMERCIAL USE OF DHASCO AND ARASCO

Since 1995, infant formulas containing DHASCO and ARASCO have been commercially produced and marketed around the world. At the present date, preterm formulas supplemented with DHASCO and ARASCO are available in at least 64 countries (Table 4). Full term formulas containing DHASCO and ARASCO are available in seven countries around the world. Although

many of these countries did not require major safety evaluations and marketing approvals before commercialization, other countries, such as the UK, France and The Netherlands, did require an extensive evaluation before official approval to market the products was granted.

We estimate that over 500,000 babies have now been fed formulas containing DHASCO and ARASCO, and there have been no reported adverse events associated with these products. This includes about 1,500 babies involved in 14 published and unpublished controlled clinical trials where even minor adverse events would have been carefully scrutinized (see Appendix 2). One company, Wyeth/Ayerst, has been following their product launches with careful postmarket surveillance. Their specialty low birth weight formula (LBW liquid and powder) is presently on the market in 56 countries around the world including Mexico, Australia, France, and the United Kingdom (Table 4). It has been on the market in some of these countries since 1997. This formula is provided under the careful supervision of doctors and the Company conservatively estimates that over 100,000 babies of most races, cultures and both sexes have consumed the DHASCO/ARASCO supplemented formula and there has not been a single adverse event reported by the physicians.

Since 1997, DHASCO has also been commercially produced and marketed as a dietary supplement for adults (including women who are pregnant or lactating) in the United States under the tradename Neuromins®. Neuromins is a soft gelatin encapsulated form of the triglyceride DHASCO and is marketed by several different distributors in the US (Table 19). To date, the Company has sold over 35 million capsules for consumption. This product is primarily marketed in the United States, but it is now also available in Canada, Europe and Southeast Asia. We estimate that well over 500,000 individuals have consumed this product. To date there have been no adverse events reported to the Company with reference to the use of this product. This includes the nearly 30 well-controlled clinical trials where the appearance of any adverse events were carefully monitored (see Appendix 3).

The large numbers of individuals (infants, children and adults) who have consumed the commercial products containing DHASCO and/or ARASCO with no reported adverse effects provides additional support for the establishment of GRAS status for this product at the normal use levels.

Table 19. Commercial Products in the United States Containing DHASCO.

Manufacturer	*Trade Name*	*Product Form*
Martek	Neuromins	500 mg soft gel (100 mg DHA)
	Neuromins PL	500 mg soft gel (200 mg DHA)
	Neuromins for Kids	250 mg soft gel (100 mg DHA)
Solgar	Neuromins	500 mg soft gel (100 mg DHA)
Source Naturals	Neuromins	500 mg soft gel (100 mg DHA)
	Neuromins	500 mg soft gel (200 mg DHA)
	Focus Child	Chewable vitamin
Nature's Way	Neuromins	500 mg soft gel (100 mg DHA)
Solaray	Neuromins	500 mg soft gel (100 mg DHA)
BioDynamax	Neuromins	500 mg soft gel (100 mg DHA)
Whole Foods	Neuromins	500 mg soft gel (100 mg DHA)
Vitamin Shoppe	Neuromins	500 mg soft gel (100 mg DHA)
Your Life	Neuromins	500 mg soft gel (100 mg DHA)

Puritan's Pride	Neuromins	500 mg soft gel (100 mg DHA)
Omega Nutrition	Neuromins	500 mg soft gel (100 mg DHA)
Natrol	Neuromins	500 mg soft gel (100 mg DHA)
Safeway Select	Neuromins	500 mg soft gel (100 mg DHA)
Royal Body Care	EyeQ	500 mg soft gel (100 mg DHA)
KAL	Neuromins	Liquid Drop
Healthcom	UltraCare for Kids	Drink mix
Great Circles	Recovery	Bars and Drinks

11 CONCLUSIONS

DHA and ARA are GRAS macronutrients that are found naturally in breast milk and are important building blocks for brain and retinal tissue. The data clearly demonstrate a functional need for DHA and ARA in the infant diet and many reputable, independent scientific bodies have called for the formulation of infant formulas with DHA and ARA to meet this need. Based upon a critical evaluation and analysis of the information available on DHASCO and ARASCO, as summarized herein, data establish that DHASCO and ARASCO are generally recognized as safe on the basis of scientific procedures when used as sources of DHA and ARA in infant formula. The proposed use level for DHASCO or ARASCO is at a maximum level of 1.25% of dietary fat (or 75 mg DHASCO (or ARASCO) per kg body wt per day) and at a ratio of 1:1 to 1:2 (DHA:ARA).

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APPENDIX 1

PRECLINICAL STUDIES USING DHASCO AND/OR ARASCO[5]
(12 different animal species/49 publications)

Author	Ref	Animal	Dose	General Finding
Abedin, et al. (1999)	1	rat	0.9% ARA + 0.6% DHA (% of total FA) or 2.7% ARA + 1.8% DHA	Tissue FA levels reflected dietary FA levels, with the brain and retina being more resistant to change compared to the liver and heart. Dietary DHA was more effective than dietary ALA in increasing tissue DHA levels. There were no significant. changes in plasma levels of thromboxane A2 metabolite.
Arterburn, et al. (1999; submitted)	2	rat	0.5 or 1.25 g DHASCO/kg/d or 1.0 or 2.5 g ARASCO/kg/d by oral gavage	ARASCO and DHASCO did not cause malformations or adverse developmental effects in the fetuses.
Arterburn, et al. (1999; in press)	3	rat	0.5 or 1.25 g DHASCO/kg/d by oral gavage	DHA from DHASCO was bioavailable, and there were no adverse effects.
Atkinson, et al. (1997a)	4	rat	10% DHASCO in diet (wt)	Dietary DHA lowered plasma cholesterol and triglycerides and enhanced bone marrow cellularity, suggesting that DHA supplementation might be useful in adjuvant chemotherapy.
Atkinson, et al. (1997b)	5	rat	10% DHASCO in diet (wt)	Dietary DHA slowed tumor growth, prevented hyperlipidemia, enhanced bone marrow cellularity, and promoted intestinal growth compared with a moderate-fat n-6 rich diet.
Boswell, et al. (1996)	6	rat	0.5, 1.0 or 2.5 g ARASCO/kg/d; 0.025, 0.5 or 1.25 g DHASCO/kg/d; 1.5 & 3.75 g Formulaid/kg/d	DHA and ARA were bioavailable. There were no adverse effects.
Bowen, et al. (1997; abstract)	7	rat	0.7% DHA + 1.0% ARA	Feeding DHA & ARA in the diet increased the level of DHA in GM1 gangliosides without changing the level of ARA.
Boyle, et al. (1998)	8	rat	2% of total fat as n-3 LCPUFA from a mixture of DHASCO & menhaden oil with a DHA/EPA ratio of 2.3:1.0 + either 1,2, or 5% ARA from ARASCO	DHA & ARA were bioavailable. Tissue n-3 LCPUFA levels were reciprocally related to n-6 levels. The brain was relatively resistant to changes in FA composition compared with the liver and plasma. The use of ARA was more effective in supporting n-6 status than the use of 18:2n-6.
Burns, et al. (1999)	9	rat	Females: 5.7-15.3 g ARASCO/DHASCO blend/kg/d; Males: 4.8-5.7g ARASCO/DHASCO blend/kg/d	There were no adverse effects at dietary levels in excess of those expected in infant formulas.

5 Abbreviations used: ALA, alpha-linolenic acid; CF, control formula; FA, fatty acid; HDL, high density lipoprotein; LA, linoleic acid; RBC, red blood cell; LCPUFA, long chain polyunsaturated fatty acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI - phosphatidylinositol; PL, phospholipid; Pnd–post natal day; PS, phosphatidylserine; P/S, polyunsaturated/saturated; RP, retinitis pigmentosa; SHR, spontaneous hypertensive rat; VLCPUFA, very long chain polyunsaturated fatty acid.

Author	Ref	Animal	Dose	General Finding
Champoux, et al. (1998; abstract)	10	rhesus monkey	2.5% of fat as ARASCO + 2.5% of fat as DHASCO in formula	Plasma DHA and ARA levels were comparable to those of mother-raised monkeys. Supplemented monkeys exhibited higher mean scores on the orienting and motor maturity tests. There were no differences between groups in the state control or activity tests.
Connolly & Rose (1998; abstract)	11	mouse	20% total fat diet with 5% as DHASCO	Mice fed a DHA-containing diet developed fewer tumors than those fed a LA-containing diet; when present, the tumor growth rates were suppressed. The incidence of lung metastasis was reduced as was the total metastatic volume.
Rose & Connolly (1999; in press)	12	mouse	20% total fat diet with 5% or 10% as DHASCO	Mice fed DHASCO had a reduction in tumor growth rate, final tumor weights, and tumor prostaglandin E2 concentrations.
Craig-Schmidt & Huang (1995; abstract)	13	piglet	CF with fat containing either 1.75% DHASCO, 2.25% ARASCO, or 2% DHASCO plus 2.5% ARASCO	The addition of DHASCO and ARASCO to the formula increased levels of DHA & ARA in lung PL.
Craig-Schmidt & Mozaffar (1995; abstract)	14	piglet	CF with fat containing either 1.75% DHASCO, 2.25% ARASCO, or 2% DHASCO plus 2.5% ARASCO	Dietary DHA supplementation increased accumulation of DHA in the brain without affecting ARA accretion in the brain. ARA supplementation did not result in greater accretion of brain PL ARA nor was DHA accretion in brain influenced by ARA addition.
Craig-Schmidt & Ping (1996; abstract)	15	piglet	CF with fat containing either 1.75% DHASCO, 2.25% ARASCO, or 2% DHASCO plus 2.5% ARASCO	DHA supplementation alone or in combination with ARA increased DHA levels in the testes while decreasing the levels of 22:4n-6 and 22:5n-6 without significantly altering the sum of DHA and n-3 FAs.
Craig-Schmidt et al. (1995; abstract)	16	piglet	CF with fat containing either 1.75% DHASCO, 2.25% ARASCO, or 2% DHASCO plus 2.5% ARASCO	Supplementation with DHA, ARA, or both resulted in increases in the respective FAs in the heart PLs. DHA suppressed ARA in PE, but not PC; addition of ARA did not suppress DHA in either PC or PE.
Craig-Schmidt et al. (1996; abstract)	17	piglet	CF with fat containing either 1.75% DHASCO, 2.25% ARASCO, or 2% DHASCO plus 2.5% ARASCO	Dietary supplementation with DHA, ARA, or both resulted in increases in the respective FAs in the kidney PLs.
Craig-Schmidt et al. (1996; abstract)	18	piglet	CF with fat containing either 1.75% DHASCO, 2 25% ARASCO, or 2% DHASCO plus 2.5% ARASCO	Dietary supplementation with DHA or ARA alone resulted in increases in the respective FAs in the liver PLs, however, DHA alone significantly decreased ARA. Supplementation with DHA and ARA together increased DHA levels while maintaining ARA levels.
Craig-Schmidt, et al. (1996)	19	piglet	CF with fat containing either 1.75% DHASCO, 2.25% ARASCO, or 2% DHASCO + 2.5% ARASCO	The addition of DHA, ARA, or both resulted in increases in the added FAs relative to the CF in the PC fraction of the retina. Retina PE was less responsive to dietary supplementation.
Engler, et al. (1998; abstract)	20	rat (SHR)	2% DHASCO in the diet (wt)	DHA supplementation reduced systolic blood pressure, plasma cholesterol, and HDL levels, but did not affect aortic contractile or vasorelaxant responses.

Author	Ref	Animal	Dose	General Finding
Engler, et al. (1999)	21	rat (SHR)	2% DHASCO in the diet (wt)	DHA supplementation significantly reduced systolic blood pressure, plasma aldosterone levels, plasma cholesterol, and HDL levels. Plasma levels of corticosterone were 18% lower in the DHA fed animals, but this was not statistically significant. Adrenal glomerulosa cells from the DHA-fed rats produced less aldosterone in vitro in response to angiotensin II, ACTH, and potassium. There were no significant differences in angiotensin receptor subtype, number or affinity. Production af ARA epoxides by renal microsomes was significantly lower in the DHA-fed rats. Renal cortical mRNA levels of CYP4A genes and formation of 10- & 2-HETE did not differ.
Engler, et al. (1999; submitted)	22	rat (SHR)	2% DHASCO in the diet (wt)	The DHA diet markedly increased the levels of DHA in the aorta, renal artery, plasma, liver, heart, kidney, and lung. The levels of EPA were also increased while there was a concomitant reduction in ARA and 22:4n-6.
Freedman, et al. (1998; abstract)	23	mouse (cystic fibrosis transmembrane conductor regulator(CFTR) (-/-))	100 mg DHASCO/d	Supplementation with 40 mg DHA/d corrected the lipid imbalance in lung and pancreas and reversed the villus hypertrophy and pancreatic ductal dilatation.
Haubner, et al. (1998)	24	rat	Maternal diets containing 3% of total FAs as DHA	Length of gestation, maternal weight gains and pup weights on pnd 3 did not differ between diet groups. The FA composition of the dam milk and pup brainstems reflected maternal diet. Auditory electromyelographic reflexes were not different. Auditory brainstem conduction times on pnd 24 were longer and the time of appearance of the auditory startle reflex was later in the pups of dams fed DHA.
Haubner, et al. (1999)	25	rat	Maternal diets containing 0.3%, 0.7%, or 3% of total FAs as DHA	There was a significant positive correlation between the myelin content of DHA and the latency of the acoustic startle reflex and a significant negative correlation between the myelin content of DHA and the myelin content of cholesterol.
Huang & Craig-Schmidt (1996)	26	piglet	CF with fat containing either 1.75% DHASCO, 2.25% ARASCO, or 2% DHASCO + 2.5% ARASCO	Dietary ARA elevated ARA accumulation in lung PE & PC, but not PI, and resulted in greater prostacyclin and thromboxane production. Dietary DHA elevated DHA accumulation in lung PE, PC, & PI. Significant suppression of eicosanoid production was observed with supplementation with both ARA and DHA compared with supplementation with ARA alone.
Hui-Min, et al. (1999)	27	baboon	4.4 mg [U-13C]-DHA as nonesterified FA from [U-13C]-DHASCO	Preformed DHA was more effective than ALA as a source for DHA accretion in the brain and retina.
Jolly, et al. (1997)	28	mouse	2.5% of fat as ARASCO (This was a control diet to see if the effects observed were due to EPA and /or DHA substitution, or to the reduction in LA which accompanied both of those diets.)	ARA had no effect on concanavalin A-induced T-lymphocyte proliferation in splenocyte cultures compared to the safflower oil control. The kinetics of mitogen-induced diacyl-sn-glycerol and ceramide production did not differ significantly between the ARA and control groups. Low dose, short term dietary exposure to highly purified EPA or DHA ethyl esters from fish oil appeared to suppress mitogen-induced T-lymphocyte proliferation by inhibiting IL-2 secretion, with accompanying reductions in the production of diacyl glycerol and ceramide.

Author	Ref	Animal	Dose	General Finding
Jolly, et al. (1998)	29	mouse	2.5% of fat as ARASCO (This was a control diet to see if the effects observed were due to EPA and /or DHA substitution, or to the reduction in LA which accompanied both of those diets.)	EPA and DHA ethyl esters from fish oil did not affect IL-2 mRNA expression but suppressed IL-2 receptor alpha mRNA levels.
Jumpsen, et al. (1997a)	30	rat	CF with fat containing either 0.8% DHA, 1.0% DHA, 1.2% ARA, or 1.2% ARA + 0.7% DHA	Individual brain regions and cell types varied in amount and rate of ARA and DHA accretion, and alteration of brain PI & PS fatty acid composition reflected the fatty acid composition of the diet. When animals were fed increased dietary ALA, an increase in DHA level was observed in PI, but not in PS. Accretion of DHA was enhanced when DHA was supplied in the diet.
Jumpsen, et al. (1997b)	31	rat	CF with fat containing either 0.8% DHA, 1.0% DHA, 1.2% ARA, or 1.2% ARA + 0.7% DHA	Individual brain regions and cell types varied in amount and rate of ARA and DHA accretion, and alteration of brain PE & PC FA composition reflected the FA composition of the diet.
Kelley & Lepine (1998; abstract)	32	kitten	Milk replacer containing DHA and ARA at levels similar to feline mother's milk	Serum FA profiles reflected dietary FA profiles.
Kelley, et al. (1998; abstract)	33	puppy	Milk replacer containing DHA + ARA at levels similar to canine mother's milk	The diet containing DHA and ARA enhanced structural tissue growth indicating an improved nutritional status.
Koskelo, et al. (1997)	34	rat	1.0 g & 2.5 g ARASCO/kg/d	ARA from ARASCO was bioavailable, and there were no adverse effects.
Kritchevsky, et al. (1999; in press)	35	rat	0.5% algal sterol in diet	There were no adverse effects on weight gain or organ weight and no effect on serum or liver lipids.
Lepine, et al. (1998; abstract)	36	kitten	Milk replacer containing DHA + ARA at levels similar to feline mother's milk	Kittens who received milk replacer with FA profile similar to feline milk were comparable in growth and body composition to kittens who received feline milk.
Nagy, et al. (1997;submitted)	37	rat	26% of calories as DHASCO	Long term feeding of a high fat diet containing DHA, but no omega-6 fats, impaired basal glucose disposal and disrupted normal hormonal regulation of glucose uptake by the adipocyte compared to a low fat corn oil containing diet.
Reinhart, et al. (1998; abstract)	38	puppy	Milk replacer containing DHA + ARA at levels similar to canine mother's milk	Serum FA profiles reflected dietary FA profiles.
Stockard, et al. (1997; abstract)	39	rat	Maternal diets containing 2%, 4%, or 6% of total FAs as DHA	Maternal and pup weights and weight gains were not different among diet groups. The FA composition of dam milk and pup tissues reflected maternal diet. Auditory brainstem conduction times were longer in pups of dams fed the higher levels of DHA.
Stockard, et al (1999; abstract)	40	rat	Maternal diets containing 0.3%, 0.7%, or 3% of total FAs as DHA	Distortion product otoacoustic emissions were improved in the DHA-supplemented groups compared to the control group.
Streekstra, et al. (1996; abstract)	41	rat	18.2g ARASCO/kg acutely; 3.0g ARASCO/kg/d for 28 days; 4.9 g ARASCO/kg/d in utero & 90 days.	There was no acute toxicity and no subchronic toxicity for 28 days. The no observed adverse effect level (NOAEL) was greater than or equal to 1.0g/kg/d for 90-day subchronic study.

Author	Ref	Animal	Dose	General Finding
Suh, et al. (1996)	42	rat	CF with fat containing either 0.7% DHA, 1.0% ARA, or 1.0% ARA + 0.7% DHA	The rod outer segment membrane FA profile reflected the dietary FA profile. Dietary FA influenced the VLCPUFA composition of the PC.
Suh, et al. (1997; abstract)	43	rat	CF with fat containing either 0.6% DHA, 1.0% ARA, or 1.0% ARA + 0.6% DHA	Feeding DHA & ARA in the diet increased the levels of DHA in PC and PS in the retina and further increased the levels of n-3 and n-6 VLCPUFA respectively in PC. The highest rhodopsin content occurred in the retina of animals fed diets containing ARA and/or DHA.
Wainwright, et al. (1997)	44	mouse	Diets containing 6% total fat with the following: 1) no DHA/no ARA; 2) 9.2% DHA + 21.2% ARA; 3) 9 2% DHA + no ARA; 4) 35% DHA + 5.8% ARA; or 5) 35.2% DHA + no ARA	Brain FA composition reflected the diet FA composition. There were no effects on the ability of the mice to learn the place of the hidden platform in the Morris water maze, nor were there effects on activity in the spatial open field. However, in both the cued and the place learning, the mice fed the low n-6:n-3 diet swam more slowly, unless ARA substituted partially for LA as the source of n-6 FA.
Ward, et al. (1998)	45	mouse	Rat milk substitutes in which the fat comprised 10% LA and 1% LNA and, using a 3x3 factorial design, one of three levels of both ARA and DHA (0, 0.4, and 2.4% FAs).	Brain and RBC PE and PS/PI fatty acid compositions reflected dietary composition. DHA levels in the brain correlated more closely with those of the RBCs than did ARA levels.
Wibert, et al. (1997)	46	rat	Females: 7.8-13.1 g ARASCO/DHASCO blend/kg/d; Males: 6.9-12.6 g ARASCO/DHASCO blend/kg/d	There were no adverse effects.
Yeh & Yeh (1995; abstract)	47	rat	Rat milk substitute with 0, 0.25, 0.5, or 1.0% of DHA as total fatty acids	DHA supplementation enhanced accretion of DHA in brain PLs, however, the ARA/DHA ratios were lowered with or without a concomitant reduction in ARA levels.
Yeh & Yeh (1996; abstract)	48	rat	Rat milk formula containing: 1) no DHA/no ARA; 2) 0 5% ARA + 0.3% DHA; 3) 1.0% ARA + 0.6% DHA	Co-supplementation with DHA & ARA at both doses increased plasma, RBC, brain, and liver phospholipid DHA and ARA levels. The high dose decreased the ARA/DHA ratios in plasma, RBC's, brain and liver PLs, however, the low dose did not decrease the ratio in RBCs or brain PLs.
Yeh, et al. (1999)	49	rat	Rat milk formula supplemented with ARA at 0, 0.5, or 1.0% of total FAs, or supplemented with DHA at 0,0 5, or 1.0% of total FAs, or co-supplemented with ARA and DHA at levels of 0:0, 0.5:0.3, or 1.0:0.6% of the fat blend.	Supplementation with DHA & ARA had no effect on pulmonary surfactant.
Ongoing/Unpublished Studies:				
Principal Investigator		Animal	Dose	General Finding or Objective
Anderson (unpublished)		dog	1 g DHASCO/d	Ongoing;The study will determine the effect of DHA in a dog RP model
Birch (unpublished)		mouse	2.5% DHASCO in diet	Ongoing; The study will determine the effect of DHA in a mouse RP model.

Author	Ref	Animal	Dose	General Finding
Bougnoux (unpublished)		rat	8% DHASCO in diet	Ongoing; The study will determine the effect of dietary DHA on anthracyclin-incuded cardiotoxicity and will determine the effect of dietary DHA on the response rate of metastatic mammary tumors to epirubicin.
Clandinin, et al. (unpublished)		rat	Diets containing 20% fat with either a high or low P/S ratio containing 12.5% DHASCO	When the diet P/S ratio was high and contained 5% DHA, there was less DHA and more LA in the adipocyte membranes, and there was less insulin bound and less glucose transported across the adipocyte membrane. The opposite was true when the diet had a low P/S ratio.
Connor (unpublished)		laying hen	ARASCO & DHASCO	DHA levels in the eggs increased almost 4X while ARA remained constant.
Connor (unpublished)		hamster	Dietary ARASCO (5% ARA in the fat	ARA from ARASCO was bioavailable and had no effect on cholesterol levels.
Denkins (unpublished		cat	10% fat diet with 0.2% DHASCO	There was 87% more DHA in the retinas of kittens in the supplemented group. PUFA levels were also higher in the brains of the supplemented group. When the kittens were exposed to alcohol, the PUFA levels were actually higher in the retina and brain in the unsupplemented group, possibly due to over-compensation since these animals had been taken off of alcohol for several weeks before the measurements were done.
Doerr (unpublished)		broiler chicken	5% ARASCO biomass in diet	There was no effect on growth of the chickens.
German (unpublished)		rat	5% of calories as ARASCO in diet	The study will examine the immunomodulation of Kupffer cells by ARA.
Lippiello (unpublished)		rabbit	2% ARASCO in diet	Although not statistically significant, the data indicated that tissues from animals fed ARASCO alone or in combination with Cosamin (glucosamine) had decreases in the amount of degenerative area in arthritic joints.
McBride, Wood, & Holub (unpublished)		cow	9,18, & 36 g DHA/d from algal biomeal	Ongoing; The study will determine whether dietary DHA will increase DHA levels in cows' milk.
McCarthy (unpublished)		horse	11 g DHASCO/d	DHA in semen increased by 50%. There was no effect on sperm motility.
Olsson (unpublished)		rat	DHASCO & ARASCO (0.5% of the diet)	PUFAs had a protective effect on the livers of animals exposed to alcohol.
Pardini (unpublished)		mouse	DHASCO	Ongoing; The study will determine the effect of dietary DHA on mammary tumor growth and responsiveness to chemotherapy with cyclophosphamide and mitomycin C.
Sudhir, K. (unpublished)		rat	2% DHASCO in the diet	Dietary DHA in rats did not reduce the myocardial infarct size compared to rats fed the control diet.
Toney (unpublished)		laying hen	5% ARASCO biomass in diet	There were no differences in food consumption by the hens, the number of eggs laid, or the weight of the eggs. The ARA content of the eggs doubled after 7 days and remained at that level for the 21 days of feeding. DHA levels dropped by 20%.
Zicker (unpublished)		dog	22mg DHA/kg/d	Ongoing; The study will compare the effects of DHA, DHA + EPA, and ALA on serum and RBC FA composition, electroretinograms, and behavior of service dogs.

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APPENDIX 2

CLINICAL STUDIES WITH INFANTS USING DHASCO AND/OR ARASCO 6 (14 trials/1,478 babies)

Author/Principal Investigator: Location	*Ref*	*Dose*	*Duration*	*Subjects (n)*	*Outcome*
Pre-Term Infants					
Carnielli, et al. (1994) (abs.), Carnielli, et al. (1996) (abs.); Europe (Numico)	1 2	1) CF, 2) SF with 0.75 ARA from ARASCO + 0.6% DHA from DHASCO, 3) human milk (HM)	(not given)	n=60	Plasma PL of SF group similar to HM group; no SD between groups in growth; visual acuity and neurodevelopment outcomes pending.
Carnielli, et al. (1998); Sophia Children's Hospital, Rotterdam, The Netherlands (Numico)	3	1) CF, 2) SF with 0.24% DHA + 0.35 ARA from PL, 3) SF with 0.84% ARA from ARASCO + 0.64% DHA from DHASCO, 4) HM	up to 5 weeks of age	n=46	triglyceride form of DHA and ARA (ARASCO+DHASCO) absorbed as efficiently as DHA and ARA in HM.
Clandinin et al. (1997); Children's Health Center, Edmonton, Alberta, Canada (Wyeth)	4	1) CF, 2) Low SF with 0.32% ARA from ARASCO + 0.24% DHA from DHASCO, 3) Medium SF with 0.49% ARA from ARASCO + 0.35% DHA from DHASCO, 4) High SF with 1.1% ARA from ARASCO + 0.76 DHA from DHASCO, 5) HM	4-6 weeks	n=91	Dose ranging trial established that plasma PL of Low SF and Medium SF groups similar to HM group, while plasma PL of High SF group higher than HM group; no difference in growth or clinical parameters between formula groups.
Damli, et al. (1996) (abs.); Europe (Humana)	5	1) CF, 2) SF with 0.3% ARA from ARASCO + 0.2% DHA from DHASCO, 3) HM	3 months	n=57	No difference in visual acuity; significantly higher Bayley Mental Development in SF group.
Foreman-van Drongelen et al. (1996); The Netherlands (Numico)	6	1) CF, 2) SF with 0.61% ARA from ARASCO + 0.3% DHA from DHASCO, 3) HM	approx. 22 weeks	n=43	Plasma PL and RBC of SF group higher than CF group; no difference between groups in growth or clinical events.
Gross et al. (1997) (abs.), Vanderhoof et al. (1997) (abs.); Vanderhoof et al (1999) Multi-center trial-USA & Canada (Wyeth)	7 8 9	1) CF, 2) SF with 0.6% ARA from ARASCO + 0.4% DHA from DHASCO, 3) HM	from full GI feeds to 40 weeks post conceptual age	n=191	No difference in growth, serum chemistries or GI symptoms between formula groups; Plasma PL of SF group similar to HM group.

6 Abbreviations used in this table: CF, control formula; HM, human milk; MDI, mental development index; PL – phospholipid; RBC, red blood cell; SF, supplemented formula; VEP –visual evoked potential.

Author/Principal Investigator: Location	Ref	Dose	Duration	Subjects (n)	Outcome
Hansen, et al. (1997) (abs.), Diersen-Schade, et al. (1998); Multi-center trial-North America (Mead Johnson)	10 11	1) CF, 2) DHA SF with 0.34% DHA from DHASCO, 3) DHA/ARA SF with 0.6% ARA from ARASCO + 0.33% DHA from DHASCO, 4) HM	approx. 28 d	n=284	ARA and/or DHA SF is safe as measured by growth and absence of adverse events; growth in the DHA/ARA SF group was better than the CF group; no difference in visual acuity between groups.
Koletzko (ongoing); Multi-center trial-Europe (Humana)		1) CF, 2) SF with fish oil + borage oil, 3) SF with fish oil + egg phospholipid, 4) SF with DHASCO/ ARASCO 1:1, 5) SF with DHASCO/ ARASCO 1:2, 6) HM	28 d	n=75	Ongoing. Evaluating endogenous synthesis of LCPUFAs and cholesterol and physiological responses to different LCPUFA doses and sources.
Monsolis et al, 1997 (abs.); Spain (Novartis)	12	1) control formula (CF); 2) supplemented formula (SF) with ARASCO:DHASCO 2:1	8 weeks	n=45	No differences between groups in growth or tolerance of formula; plasma RBC of SF group maintained while CF group declined.
Full Term Infants					
Birch, et al. (1998) Retina Foundation of the Southwest, Dallas, TX (Mead Johnson)	13	1) CF; 2) SF with 0.35% DHA; 3) SF with 0.35% DHA + 0.72% ARA; 4) HM	4 months	n=108	All groups had similar growth rates and tolerated all diets well. DHA or DHA + ARA SF groups had better sweep VEP acuity at 6, 17, and 52 weeks of age. DHA SF groups had better visual acuity, equivalent to "one line on an eye chart" Blood lipids and visual acuity of SF groups similar to those of HM group. Blood lipids and visual acuity of CF group significantly lower than all groups.. Recent analysis (unpublished) indicates a 6 IQ point advantage in DHA/ARA SF group by Bayley MDI at 18 months of age.
Carlson, et al. (1999; abstract) Multi-center trial-USA & Canada (Mead Johnson)	14	1) CF, 2) SF with 0.3% DHA from fish oil + 0 6% ARA from ARASCO; 3) SF with 0.3% DHA from DHASCO + 0.6% ARA from ARASCO	(not stated)	n=322	ARA and DHA SF had no adverse effects on growth or development. Infants fed DHA/ARA SF gained weight more rapidly and weighed more than the CF group through 12 months of age. Bayley Scales of Mental and Psychomotor Development scores were highest in the DHASCO/ARASCO SF group at 12 months, but did not reach statistical significance. No differences in visual acuity.

Author/Principal Investigator: Location	Ref	Dose	Duration	Subjects (n)	Outcome
Gibson,et al. (1997a; abstract) Flinders Medical Center, Australia (Wyeth Nutritionals)	15	1) CF; 2) SF1 (0.2% ARA/0.2% DHA); 3) SF2 (0.32% ARA/0.2% DHA); 4) SF3 (0.4% ARA/0.25% DHA); 5) HM	6 weeks	n=113	The formulas containing ARASCO and DHASCO (SF2 & 3) resulted in plasma ARA and DHA levels similar to those in the HM group, while supporting normal growth during the first 6 weeks of life.
Gibson, et al. (1997b; abstract) Flinders Medical Center, Australia	16	1) CF; 2) SF with 0.3% DHA; 3) SF with 0.3% DHA + 0.3% ARA	16 weeks	n=67	RBC PL DHA levels in the SF groups exceeded the published range in breast-fed infants. When ARA was absent from the formula, RBC ARA levels were below values seen in CF infants. The VEP acuity scores were not significantly different between dietary groups, and all were within the normal expected values. There were no growth differences between any of the groups
Weizman (unpublished) Saroka Medical Center, Israel (Maabarot Products)		1) SF (0.45% DHA+ 0.45% ARA of 3.6% fat); 2) CF (Materna Plus Meshuperet)	3 months	n=20	A presentation at the Annual Meeting of the Israeli Pediatric Society concluded that locally produced SF is safe and clinically efficient. No differences were observed in developmental assessments at 3 months. No effects on growth.

Appendix 2 - References for Term and Preterm Infant Studies with DHASCO and/or ARASCO

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APPENDIX 3

CLINICAL STUDIES WITH ADULTS USING DHASCO AND/OR ARASCO (29 separate trials/1,410 subjects/ 33 publications)7

Author	Ref.	Dose	Duration	Total No. of Subjects	General Finding
Publications:					
Agren, et al. (1996)	1	Unsupplemented, 4 g DHASCO/d, 4 g fish oil/d, or fish diet providing 0.38 g EPA + 0.67 g DHA/d	14 weeks	healthy adult males; n=55	Fasting plasma TG. levels decreased by 17%. Total-CHOL did not change, but the HDL2/HDL3 ratio increased more than 50%. Postprandial total and chylomicron TG. were also lowered by 16% and 20%, respectively. No significant changes in weight or body mass index (BMI) during the study.
Agren, et al. (1997)	2	Unsupplemented, 4 g DHASCO/d, 4 g fish oil/d, or fish diet providing 0.38 g EPA + 0.67 g DHA/d	14 weeks	healthy adult males; n=55	DHASCO did not inhibit in vitro platelet aggregation, nor did it affect hemostatic factors.
Conquer and Holub (1996)	3	4.5 g DHASCO/d or placebo	42 days	healthy vegetarians; n=24	DHASCO supplementation markedly enhanced the DHA status of serum and platelets, provided for the formation of substantial EPA, and lowered the total-CHOL. and LDL:HDL ratio in vegetarians.
Conquer and Holub (1997)	5	4.5 g DHASCO/d	42 days	healthy vegetarians & omnivores; n=20	DHA levels in serum and platelet PL increased significantly in both vegetarians and omnivores. EPA levels increased, while ARA and 22:5n-6 and 22:5n-3 levels decreased. There was no significant difference between omnivores and vegetarians in retroconversion of DHA to EPA.

7 Abbreviations used in this table: ALA, alpha-linolenic acid; CHOL, cholesterol; ERG, electroretinogram; FA, fatty acid; HDL, high density lipoprotein (cholesterol); LA, linoleic acid; LCHADD, long chain 3 hydroxyacyl CoA dehydrogenase deficiency; LCPUFA, long chain polyunsaturated fatty acid; LDL, low density lipoprotein (cholesterol); PL, phospholipid; RBC, red blood cell; TOVA, test of variability of atttention; TG, triglyceride; VEP- visual evoked potential.

Author	Ref.	Dose	Duration	Total No. of Subjects	General Finding
Conquer and Holub (1998)	4	2 g DHASCO/d, 4 g DHASCO/d, or placebo	42 days	healthy men and women of Asian Indian background; n=19	There were no effects on serum lipid/lipoprotein parameters or on blood pressure in Asian Indians. DHA levels increased in the serum PLs Circulating DHA:non-essential FA levels also increased.
Davidson, et al. (1997)	6	3 g DHASCO/d, 6 g DHASCO/d, or placebo	6 weeks	subjects with Type IIB hyperlipidemia; n=26	In subjects with Type IIB hyperlipidemia, the DHA content of the plasma PLs increased in a dose-dependent manner. Serum TG decreased by 17 to 21%. HDL increased equally (+6%) in both dose groups. LDL increased by 13.6% in the high dose group. No significant change in Total-CHOL. DHASCO was well tolerated.
Emken, et al. (1997)	7	3 g ARASCO/d or basal diet with low ARA	50 days	healthy adult males; n=4	Conversión of 20:3n-6 to ARA was stimulated 7-8 fold by ARASCO supplementation. Neither absorption nor retroconversion appeared to be influenced by diet.
Emken, et al. (1998)	8	3 g ARASCO/d or basal diet with low ARA	50 days	healthy adult males; n=6	Dietary ARA did not affect uptake of LA[d2] in plasma lipid classes over the study period. Plasma total lipid concentration of 20:3n-6[d2] and ARA[d2] were lower in samples from the ARASCO-supplemented group.
Ferretti, et al. (1997)	9	3 g ARASCO/d or basal diet with low ARA	50 days	healthy adult males; n=10	The metabolites of thromboxane and PGI2 increased on the high-ARA diet.
Ferretti, et al. (1998)	10	15 g DHASCO/d or basal diet with low DHA	90 days	healthy adult males; n=11	Mean excretion of 11-dehydrothromboxane B2 was significantly reduced in men consuming a high DHA diet, while the mean excretion of 2,3-dino-6-oxo-prostaglandin F1a was not significantly changed. Body weights were not significantly changed during the study.
Fidler, et al. (1999; submitted)	11	0.5 g DHASCO/d or placebo for 2 weeks. At 6 weeks post-partum, a single dose of 2mg/kg 13C-DHASCO was given.	2 weeks	lactating women; n=10	Oxidation of 13C-labeled DHASCO in the supplemented and placebo group was similar. Maximal 13C-enrichment was reached earlier in the group receiving 13C-DHASCO. The cumulative 13C-recovery in breath was higher in the DHASCO vs. the linoleic acid group until 10 hours after tracer application and comparable thereafter.
Gibson, et al. (1997)	12	Maternal diets were supplemented with 0, 0.5, 1.0, 2.0, or 3.0 g DHASCO/d	12 weeks	Nursing mothers & their infants; n=52	All infants grew well and were appropriate weight, length, and head circumference at birth, 12 week, and one and two years of age. Breast milk DHA was related to infant plasma PL and RBC in a saturable curvilinear manner. The rise in plasma PL and RBC DHA was approximated by a fall in total n-6 PUFAs. DHA did not effect the development of VEP acuity, but DHA was positively related with Bayley's mental development index at one year of age.

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Author	Ref.	Dose	Duration	Total No. of Subjects	General Finding
Innis and Hansen (1996)	13	Subjects were given 28.8 g fat/d containing either 0 ARA/0 DHA; 0.8g ARA/0.6g DHA; 2.2 g ARA/1.7 g DHA, or 3.6 g ARA/2.9 g DHA	14 days	healthy adult males; n=24	No clinically significant dose-related effects were seen on physical examination or from routine laboratory tests. There was a dose-dependent, but non-significant increase in plasma Total-CHOL and a decrease in percentage TG.
Kelley, et al. (1997)	14	3 g ARASCO/d or basal diet with low ARA	50 days	healthy adult males; n=10	There were no adverse effects on the immune response. Dietary ARA did not influence peripheral blood mononuclear cell proliferation in response to phytohemagglutinin, Concanavalin A, pokeweed, measles/mumps/rubella, and influenza vaccines prior to immunization, and natural killer cell activity. The post-immunization proliferation in response to influenza vaccine was higher in the ARA-supplemented group. The number of circulating granulocytes was higher in the subjects fed the high dose ARASCO.
Kelley, et al. (1998a)	15	15 g DHASCO/d or basal diet with low DHA	90 days	healthy adult males; n=11	Proliferation of peripheral blood mononuclear cells cultured with phytohemagglutinin or concanavalin A, the delayed hypersensitivity skin response, number of T cells producing IL2, the ratio between the helper/suppressor T cells in circulation, and serum concentrations of immunoglobulin G, C3, and IL2 receptor were not changed with dietary DHA. Dietary DHA did, however, cause a decrease in the number of circulation granulocytes. There were no increases in infections or other health problems.
Kelley, et al. (1998b)	16	3 g ARASCO/d or basal diet with low ARA	50 days	healthy adult males; n=10	ARA supplementation caused significant increases in the in vitro secretion of LTB4 and PGE2 but had no effect on immune cell functions. There were no adverse health effects.
Locke, Hoffman, et al. (1997)	17	1 g DHASCO/d or placebo	3 years	patients with X-linked retinitis pigmentosa; n=46	This paper describes the background and rationale for the study as well as the study protocol. The study is ongoing, and the results have not been published.
Makrides, et al. (1996)	18	0, 0.5, 1.0, 2.0, or 3.0 g DHASCO/d	79 days	Nursing mothers; n=52	Breast milk DHA levels increased in a dose-dependent manner. Maternal plasma PL and RBC DHA levels increased and were also strongly associated with dietary dose of DHA. Increasing maternal dietary doses of DHA did not affect breast milk ARA levels or antioxidant status.
Martinez (1996)	19	0.4-1.8 g ARASCO/d	6-12 months	patients with disorders of peroxisomal biogenesis; n=5	ARASCO supplementation maintained RBC ARA levels during DHA-ethyl ester supplementation in patients with disorders of peroxisomal biogenesis. In some patients, muscular tone seemed to improve further after introducing ARASCO supplements.

Author	Ref.	Dose	Duration	Total No. of Subjects	General Finding
Nelson, et al. (1997a)	20	15 g DHASCO/d or basal diet with low DHA	90 days	healthy adult males; n=10	There was a 3-fold increase in platelet DHA content, and the EPA levels also increased significantly. There were no effects on platelet aggregation, prothrombin time, activated partial thromboplastin time, antithrombin-III levels, or *in vivo* bleeding times.
Nelson, et al. (1997b)	21	15 g DHASCO/d or basal diet with low DHA	90 days	healthy adult males; n=10	DHA levels increased in plasma as well as adipose tissue. EPA levels also increased in plasma, but not adipose tissue. The plasma lipid fractions were similar in their DHA enrichment. Plasma Total-CHOL, LDL, and apolipoproteins were unchanged. HDL was increased. Plasma TG levels were significantly reduced. Apo-E was significantly increased.
Nelson, et al. (1997c)	22	3 g ARASCO/d or basal diet with low ARA	50 days	healthy adult males; n=10	This paper describes the background and rationale and design of the study. Plasma PL and cholesterol ester ARA levels increased markedly, while the plasma triglyceride levels increased slightly. Adipose tissue ARA levels did not increase. Blood lipid levels showed no change nor did the apoproteins and lipoproteins. There were no changes in platelet aggregation or bleeding times. Most indices of the immune response were not affected. The detailed results are published in the same journal issue in 5 separate papers and in two papers by Emken (1997) and Kelley (1997).
Nelson, et al. (1997d)	23	3 g ARASCO/d or basal diet with low ARA	50 days	healthy adult males; n=10	Platelets exhibited only small changes in their ARA content during the ARASCO supplementation period. There were no significant differences in platelet aggregation, prothrombin time, partial thromboplastin time, antithrombin III levels, or *in vivo* bleeding times before and after supplementation.
Nelson, et al. (1997e)	24	3 g ARASCO/d or basal diet with low ARA	50 days	healthy adult males; n=10	Dietary ARA had no statistically significant effect on blood cholesterol levels, lipoprotein distribution, or apoprotein levels, nor did it affect adipose tissue fatty acid composition. Plasma total FA composition was markedly enriched with ARA, with different degrees of enrichment in the plasma lipid fractions, cholesterol esters, TG, free FA, and PL. The 22:4n-6 levels in the plasma PL fraction also increased with dietary ARA. The total RBC FA composition was also enriched with ARA which mainly replaced LA acid. There were no adverse health effects.
Vidgren, et al. (1997)	25	Unsupplemented, 4 g DHASCO/d, 4 g fish oil/d, or fish diet providing 0.38 g EPA + 0.67 g DHA/d	14 weeks	healthy adult males; n=52	The levels of n-3 FAs (DHA and EPA) were increased and those of n-6 FAs decreased in plasma lipid fractions and in platelets and RBC membranes. DHA was preferentially incorporated into PL and TG, and there was very little uptake in cholesterol ester.

Abstracts:

Author	Ref.	Dose	Duration	Total No. of Subjects	General Finding
Emken et al. (1999; abstract)	26	15 g DHASCO/d or basal diet with low DHA	90 days	healthy adult males; n=6	Dietary DHA did not affect absorption of deuterated 18:1n-9, LA, or ALA. Chylomicron TG clearance was enhanced by 30%. No effect was observed on acyltransferase selectivity or on uptake and clearance of the deuterated FAs. The concentrations of most deuterated n-6 and n-3 LCPUFAs were reduced.
Gillingham, et al. (1997; abstract)	27	65 mg DHA/d to children weighing less than 20 kg and 130 mg DHA/d to children weighting over 20 kg. (Microencapsulated powder)	6 months	children with LCHADD; n=2	Plasma PL and RBC DHA levels have been elevated to high normal. Preliminary results after 6 months of supplementation indicate a mild but significant improvement in visual acuity as measured by VEP. The study is ongoing with additional children.
Jensen, et al. (1996; abstract)	28	Maternal supplementation with 0.5 g DHASCO/d, high DHA eggs, or high DHA fish oil, or no supplementation	6 weeks	nursing mothers and their infants; n=24	DHA supplementation of lactating women increased the DHA content of their milk and increased the DHA content of the recipient infant's plasma PL.
Jensen, et al. (1998; abstract	29	Maternal supplementation with 0.5 g DHASCO/d or placebo	120 days	nursing mothers and their infants; n=55	DHA supplementation of lactating women significantly increased the DHA content of their milk. The DHA levels in the plasma PL of the recipient infants' blood was also significantly increased, however, the levels of ARA, 22:4n-6, and 22:5n-6 were significantly decreased. There were no differences between groups in VEP latency, VEP amplitudes, sweep VEP acuity, or Teller Card acuity at 120 or 240 days Visual function measures did not correlate with milk or infant plasma PL DHA levels at 120 days. There were no statistically significant differences in weight, length, head circumference or skinfold thickness between groups at either 120 or 240 days.
Jensen, et al. (1999; abstract)	30	Maternal supplementation with 0.5 g DHASCO/d or placebo	120 days	nursing mothers and their infants; n=89	There were no differences in visual function, visual-motor problem-solving ability or language development of the infants, however the gross motor development of the DHA-supplemented infants improved. DHA supplementation did not affect the incidence of maternal depression, however it did improve maternal concentration (less cognitive interference).

Author	Ref.	Dose	Duration	Total No. of Subjects	General Finding
Otto, et al. (1997; abstract)	31	Fish oil supplements with either 300 mg DHA + 60 mg EPA or 600 mg DHA + 120 mg EPA, or DHASCO supplements with either 300 mg DHA, 600 mg DHA, or 600 mg DHA + 250 mg ARA from ARASCO in non-pregnant women, and 600 mg DHA + 250 mg ARA in pregnant women	4 weeks	healthy non-pregnant women, n=84 and healthy pregnant women, n=12	Plasma PL and RBC DHA levels were increased in both healthy non-pregnant women as well as pregnant women. The n-6 LCPUFA levels were generally reduced, except when ARA was included in the supplementation.
Sauerwald & Koletzko (abstract)	32	1 g DHASCO/d or placebo. After 2 weeks of supplementation, a single dose of 2mg/kg 13C-DHASCO was given.	2 weeks	lactating women; n=10	Dietary DHA supplementation prevented a decline of DHA in human milk. Transfer of DHA from the diet into human milk was comparable to that of palmitic and oleic acids.
Van Maldergem (1995; abstract)	33	100-250 mg DHASCO/kg/d	1-2 years	patients with generalized peroxisomal disorders; n=4	After supplementation of patients with generalized peroxisomal disorders for 1-2 years, RBC membrane and plasma PL concentrations of DHA were normalized in all patients and slight improvement of the clinical condition and ERG maximal amplitude was seen in 2 out of 4 patients. No definitive significant change in the course of the disease was observed in these patients.
Voigt, et al. (1998; abstract	34	Maternal supplementation with 0.5 g DHASCO/d or placebo	120 days	nursing mothers and their infants; n=41	The DHA content of the plasma PL of infants from DHA-supplemented mothers was significantly higher at 4 months of age than that of infants from placebo-supplemented mothers. There were no statistically significant differences at one year of age in any neurodevelopmental measure between infants whose mothers were supplemented with DHA versus those whose mothers were given placebo.
Voigt, et al. (1999; abstract)	35	0.75 g DHASCO/d or placebo	4 months	children, ages 6-12 years, with AD/HD; n=46	Plasma PL DHA levels increased over 2.5 fold while ARA and 22:5n-3 levels decreased. Both groups (DHA & placebo) of children improved by TOVA, Color Trails, and Achenbach scores over the course of the study, but there were no significant differences between the groups. Further data analysis is ongoing.
Unpublished:					
Benton (unpublished)		1 g DHASCO/d or placebo	50 days	healthy female university students; n=70 for each study	Attention and short-term memory were improved with DHA supplementation in the first study. However, the results were not replicated in the second study.

Author	Ref.	Dose	Duration	Total No. of Subjects	General Finding
Carnielli (unpublished)		~0.6 g ARASCO/kg/d & ~2 g DHASCO/kg/d	1 month -1 year	patients with peroxisomal disorders; n=15	There was improvement in psychomotor function in some patients
Conquer, et al. (unpublished)		placebo, 1 g DHASCO/d or 2 g DHASCO/d	3 months	Asthenozoospermic males, n=28 and unsupplemented normozoospermic males, n=37	Asthenozoospermic males had lower levels of DHA in seminal fluid and sperm compared to normozoospermic males, but serum DHA levels were equivalent between the groups. Sperm motility correlated with sperm DHA levels. In the supplemented asthenozoospermic males, DHA levels increased in serum and seminal fluid in a dose dependent fashion. However, DHA levels in sperm did not respond to dietary DHA. There was no effect of DHA on sperm motility.
Hibbeln, Kaye, & Halmi (unpublished)		0.5 g ARASCO/d and 1.5 g DHASCO/d or placebo	6 months	women ages 16-35 years with diagnosed anorexia nervosa; n=40	Ongoing. The study will determine the effects of essential FAs on anorexia nervosa.
Howe (unpublished)		5 g DHASCO/d or placebo	12 weeks	male and female adults with treated or untreated hypertension; n=36	DHASCO had a mild antihypertensive effect on systolic blood pressure.
Marangell (unpublished)		5 g DHASCO/d or placebo	6 weeks	patients with mild to moderate unipolar clinical depression; n=40	Ongoing. The study will determine the effects of DHA on unipolar depression.
Marra (unpublished)		4 g ARASCO/d or placebo	8 weeks	patients with liver cirrhosis and low plasma ARA; n=30	The pilot study in which patients with liver cirrhosis were supplemented with 1 g ARASCO/d showed increased platelet aggregation with concomitant increases in levels of ARA. A larger study using higher levels of ARASCO is ongoing.
Raymond (unpublished)		400 mg each ARA and DHA per day (microencapsulated powder) or placebo	1 year	children ages 3-36 months with peroxisomal disorders; n=60 for placebo-controlled study; (n=9+ in open label study)	The open label, dose-ranging study showed that DHA levels were normalized in patients with adrenoleukodystrophy (ALD) and infantile Refsum's disease with DHASCO/ARASCO more effectively than with ethyl esters. The double-blind, placebo-controlled study is ongoing and will measure visual and neurological outcomes.
Russell, et al. (unpublished)		2 g DHASCO/d (+/- lutein), lutein alone, or placebo	4 months	women ages 60-80 years; n=60	Ongoing. The study will determine the effect of DHA and lutein, alone or combined, on dark adaptation and macular pigment as well as on cognitive functioning in elderly women.

Author	Ref.	Dose	Duration	Total No. of Subjects	General Finding
Schaefer (unpublished)		3 g DHASCO/d or placebo	3 months	normolipidemic men and women ages 18-49 years; n=18	Serum DHA was increased while ARA was decreased. Plasma TG were lowered Immunocompetence was not significantly affected. No adverse effects were reported.
Swearingen & Hess (unpublished)		1 g DHASCO/d or placebo	6 weeks	healthy adult males, ages 18 -24 year; n=30	Ongoing; The study will determine the effect of DHA on contrast sensitivity, glare and photostress recovery in healthy adult males.

Appendix 3 - Adult Clinical Trials Using DHASCO and/or ARASCO

PUBLICATIONS

1. Ågren, J.J., O. Hänninen, A. Julkunen, L. Fogelholm, H. Maliranta, U. Schwab, O. Pynnönen, and M. Uusitupa (1996) Fish diet, fish oil and docosahexaenoic acid rich oil lower fasting and postprandial plasma lipid levels. *European Journal of Clinical Nutrition* 50:765-771.

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3. Conquer, Julie A. and Bruce J. Holub (1996) Supplementation with an algae source of docosahexaenoic acid increases n-3 status and alters risk factors for heart disease in vegetarian subjects. *Journal of Nutrition* 126: 3032-3039.

4. Conquer, Julie A. and Bruce J. Holub (1997) Dietary docosahexaenoic acid as a source of eicosapentaenoic acid in vegetarians and omnivores. *Lipids* 32: 341-345.

5. Conquer, Julie A. and Bruce J. Holub (1998) Effect of supplementation with different doses of DHA on the levels of circulating DHA as non-esterified fatty acid in subjects of Asian Indian background. *Journal of Lipid Research* 39: 286-292.

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10. Ferretti, Aldo, Gary J. Nelson, Perla C. Schmidt, Giovanni Bartolini, Darshan S. Kelley, and Vincent P. Flanagan (1998) Dietary docosahexaenoic acid reduces the thromboxane/prostacyclin synthetic ratio in humans. *Journal of Nutritional Biochemistry* 9: 88-92.

11. Fidler, N., T. Sauerwald, H. Demmelmair, A. Pohl, B. Koletzko (1999?) Oxidation of an oil rich in docosahexaenoic acid compared to linoleic acid in lactating women. (submitted?)

12. Gibson, R.A., M.A. Neumann, and M. Makrides (1997) Effect of increasing breast milk docosahexaenoic acid on plasma and erythrocyte phospholipid fatty acids and neural indices of exclusively breast fed infants. *European Journal of Clinical Nutrition* 51: 578-584.

13. Innis S. and J. Hansen (1996) Plasma fatty acid responses, metabolic effects, and safety of microalgal and fungal oils rich in arachidonic and docosahexaenoic acids in healthy adults. *American Journal of Clinical Nutrition* 64: 159-167.

14. Kelley, Darshan S., Peter C. Taylor, Gary J. Nelson, Perla C. Schmidt, Bruce E. Mackey, and David Kyle (1997) Effects of dietary arachidonic acid on human immune response. *Lipids* 32 (4): 449-456.

15. Kelley, D.S., P.C. Taylor, G.J. Nelson, and B.E. Mackey (1998a) Dietary docosahexaenoic acid and immunocompetence in young healthy men. *Lipids* 33(6): 559-566.

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Appendix 4

APPENDIX 4

QUESTIONS RAISED BY THE FDA IN AN EARLIER SUBMISSION

In response to a GRAS Notification petition (GRN 000007), the Office of Premarket Approval of the FDA responded on March 4, 1999, that the aforementioned notice did not provide a sufficient basis for a determination that the subject use of ARASCO and DHASCO is GRAS. On December 1, 1999, Wyeth/Ayerst responded with a written statement that resolved to their satisfaction all of the points raised in the FDA letter of March 4, 1999. That response letter is included herein this Appendix 4. After careful evaluation of all the avaiable data both published and unpublished, the Panel concurs with Wyeth/Ayerst and also believes that all of the issues raised in that letter have been resolved and thoroughly discussed in both the present document as well as in the response of Dec 1, 1999 by Wyeth/Ayerst. With the resolution of these concerns of the FDA, this Panel of qualified by scientific and/or medical experts with relevant international experience to evaluate the safety of food and food ingredients believes that, using scientific procedures, the single cell oils DHASCO and ARASCO, are Generally Recognized as Safe (GRAS) for consumption by infants and children at dose levels of up to 2.5% each of total dietary fat.

WYETH NUTRITIONALS INTERNATIONAL
REGULATORY AFFAIRS

Linda Kahl, Ph.D.
Office of Premarket Approval
Center for Food Safety and Applied Nutrition
Food & Drug Administration
1110 Vermont Avenue, N.W.
Washington, DC 20204

December 1, 1999

Via Fax 202-418-3131
(Hard copy follows)

Re: GRN 000007

Dear Dr. Kahl:

At our last meeting, Wyeth agreed to provide FDA with a textual review of the data that was provided in our original Generally Recognized As Safe (GRAS) Notice and that was again reviewed at subsequent meetings earlier this year. We are pleased to provide that information (attached). To put this submission in context, it may be useful to review activities to date.

On August 27, 1998 Wyeth Nutritionals filed a GRAS Notice according to FDA proposed regulations, (designated GRN 000007 by the Agency). That Notice pertained to Wyeth's assertion that certain levels and ratios of ARASCO (Arachidonic [ARA] acid-rich triglyceride), and DHASCO (Docosahexaenoic [DHA] acid-rich triglyceride) are GRAS for use in infant formula as set forth in our filing. Our submission was prompted by the rationale to provide human milk levels of ARA and DHA in infant formula as recommended by a growing body of published data.

Our Notice included in its basis for GRAS determination, both short and long term animal studies from rats and other species, data from extensive clinical evaluations on the oils, government and other independent expert safety reviews of our formula containing the oils, authoritative recommendations for inclusion of ARA and DHA into infant formula and a summary of the safe use of ARASCO and DHASCO in Wyeth formulas in numerous countries worldwide for about 2 years.

Clinical data included non-problematic use of the oils at the upper range cited in the Notice in a more sensitive infant population (low birthweight). Independent safety determinations by the U.K., France and The Netherlands were provided. Historical safe use included information about other manufacturers and their non-problematic use of ARASCO and DHASCO in their formulas as well as Wyeth's own experience in this regard. Authoritative recommendations for the use of ARA and DHA in infant nutrition at the levels proposed in GRN 000007 included those of the World Health Organization.

150 N.RADNOR-CHESTER ROAD, ST. DAVIDS, PA. 19087 U.S.A.
Telephone #: (610) 902-4781
Facsimile # (610) 964-5962
E-Mail maggior @war.wyeth.com

On March 4th 1999 FDA responded to our Notice. FDA noted a No Observable Effect Level (NOEL) which was many fold higher than the highest levels Wyeth cited as GRAS in GRN 000007. FDA did not conclude that the ARASCO and DHASCO as proposed for use were unsafe. However, the Agency did state that the FDA had certain questions about some animal study findings and, accordingly, did not have a sufficient basis (at the time) to agree with Wyeth's GRAS determination.

The Agency made the March 4th letter publicly available shortly after it was mailed to Wyeth and in advance of the receipt of Wyeth's explanations to FDA questions and concerns. We believe the end result of this decision has been to leave an unclear regulatory record that would not seem to be in either FDA or Wyeth's best interests.

Accordingly, Wyeth entered into a cooperative series of FDA meetings and supplemental data submissions over the next several months in attempts to mutually explore the data and thoroughly review all possible safety issues. Our goal was to identify legitimate concerns and address them to our own and to the Agency's satisfaction and to consider possible regulatory avenues leading to clarification and resolution of the regulatory record.

In this context, senior Wyeth research personnel and an outside expert toxicologist met with FDA on May 24, 1999. A systematic and comprehensive review of every Agency question in the March 4th letter took place at that meeting. Wyeth used all available data from seven rat toxicology studies in this review.

Because of the uniqueness of this regulatory situation, Wyeth's presentation and discussion suggested the need for basic principles of toxicology to be applied to the total data. Our independent expert toxicologist also recommended this course. In particular, the following principles were referenced to establish that a finding has potential toxicological relevance and to help in understanding the large amount of safety data involved:
-The observation should be dose-related,
-The observation should be seen consistently across comparable studies,
-The observation should occur in both genders, and
-Multiple lines of evidence should point to the same conclusion.

Wyeth expressed the opinion that the last point may be the most substantive in relation to the resolution of FDA's main questions. Specifically, sporadic differences in rat organ weights questioned by FDA are not accompanied by corroborating differences in clinical chemistry/hematology results or by pathological differences in histology. Thus, Wyeth's interpretation was that the observed sporadic differences are a physiologic response of no toxicological relevance. Wyeth emphasized that the application of these basic principles of toxicological evaluation to the results of the seven rat toxicology studies leads to the conclusion that there are no findings of toxicological significance related to the use of ARASCO and DHASCO in the data set.

Wyeth provided a notebook containing extensive data during this meeting which summarized the safety data that was reviewed and discussed. That notebook included written responses to all questions in FDA's March 4th letter as well as further written statements of interpretation of toxicological study data by experts whose specialties are directly relevant to the particular findings. These reviewers, each of whom is highly qualified by scientific and/or medical training and experience to evaluate safety, confirmed Wyeth's assessments. Additional data from a study in neonatal piglets, and extensive data from a large, multi-center growth and safety study in low birth weight infants were also presented to FDA.

Our May 24th meeting concluded with an understanding that all of FDA's concerns had been addressed and that the Agency would review the materials presented and meet again with Wyeth to discuss any remaining questions[1].

The follow up meeting took place on June 30, 1999. FDA advised that the Agency still had questions with the data and that a text to explain the voluminous data presented on May 24th would be a useful aid in the Agency's review. Additionally, FDA recommended that Wyeth integrate the summary and interpretations of data according to organ system.

Wyeth noted at this June meeting that there are numerous additional clinical studies wherein ARASCO and DHASCO used in accordance with the conditions set forth in Wyeth's GRAS notice have been shown to support healthy growth and development of infants. And, that in none of these studies have adverse effects related to ARASCO and DHASCO been observed.

The purpose of this letter then is to continue our cooperative efforts to provide FDA further information to assist with the Agency's decision-making process as regards resolution of the GRN 000007 record. Thus, we herein provide the requested text and integrated summary of the data and information presented and discussed earlier. Wyeth is confident that the data which led to the safety assessment in multiple countries before using ARASCO and DHASCO in our infant formulas outside the United States is compelling and growing further still and should now provide a basis for FDA agreement.

As regards the Agency's own determination process, Wyeth fully appreciates that the proposed GRAS Notice process has some provisions that may be further refined. Based on our experience with GRN 000007, we would be pleased to accept FDA's encouragement to discuss the process itself. Meanwhile, the attached text does point out one substantive Agency review aspect of GRN 000007 that is relevant to the technical issues at hand. We believe that the finding of *an* effect of a substance, without characterizing the nature of that effect, is not an appropriate criterion by which to address safety. The reasons for this opinion are more completely discussed in the attached text. We also expect that the information in the attached text will resolve any concerns that FDA has about the technical components of the GRAS Notice.

Wyeth feels that ARASCO and DHASCO may now be the most studied infant formula ingredients in history. These studies have occurred over almost 10 years and have involved numerous international governments, scientific and medical authorities, health care professionals, Journal Review Boards, Institutional Review Boards and others,- none of whom has raised a safety concern with the use of these oils as delineated in GRN 000007.

In this context, and in the context of our continued and expanding non-problematic international marketing of infant formula supplemented with ARASCO and DHASCO according to the provisions of GRN 000007, Wyeth has absolutely no reason to question the safety of these oils in any way.

Accordingly, Wyeth maintains its determination that ARASCO and DHASCO are safe for use in infant formula when used in accordance with the conditions set forth in GRN 000007. We trust that the FDA will now also have sufficient information to agree that Wyeth has provided a *reasonable certainty of no harm under intended conditions of use* for ARASCO and DHASCO in infant formula.

Please let me know if there are questions or comments.

Sincerely Yours,

WYETH NUTRITIONALS

Raymond J. Maggio
Director, Regulatory Affairs

cc: G. Pauli, L. Tarantino, A. Rulis
M.Donnella, Esq.

[1] Subsequent to the May 24th meeting, the detailed clinical data provided have been published in a peer-reviewed journal. (Vanderhoof, JA, et al. 1999, Evaluation of a long-chain polyunsaturated fatty acid supplemented formula on growth, tolerance, and plasma lipids in preterm infants up to 48 weeks postconceptional age. Journal of Pediatric Gastroenterology & Nutrition. 29:318-326,- copy enclosed.) This study was specifically undertaken to document safety. The results are consistent with Wyeth's conclusions about safety drawn from the preclinical data, and supersede all preclinical data pertinent to safety.

I. FDA's Concerns

FDA indicated in its letter of March 4, 1999, that it had three basic areas of concern.

A. First, that those effects that were observed in some studies might have toxicological significance. FDA also recognized, however, that the effects seen in some studies might not have any toxicological significance, in particular mentioning "physiological effects or adaptation."

Wyeth showed that there was virtually no consistent finding among the studies for any of the effects that FDA referenced. Because FDA emphasized changes in liver and spleen weights, Wyeth carefully considered the occurrences of statistically significant differences in weights of these organs. Wyeth showed that when the various lines of evidence were considered collectively, such as histopathology and blood levels of tissue enzymes and of metabolic markers of organ function, the conclusion obtained is that there is no toxicological significance of the effects. Wyeth also provided evidence that the same effects were observed in numerous animal studies on oils rich in long chain polyunsaturated fatty acids (LCPs), obtained from sources other than from single cell sources, showing that the effects were not source-related, but were expected physiological adaptations.

B. Second, FDA expressed concern that at the target exposure, the estimated dietary exposure in infants is 8 to 20-fold lower than the level at which no effects, the NOEL, were observed in the rat toxicology data (or 5-14 fold lower than the maximum dietary intake).

Wyeth noted that because the effects that were seen were of no toxicological concern, there was more assurance of safety than FDA's estimate of 8-20-fold above any effect. Also, given the large number of statistical comparisons reported in the toxicology studies, some provision should be made for the finding of a statistically significant difference by chance. One provision to guard against over reliance on the finding of a statistically significant difference is to see whether or not the finding is consistently detected among various studies. The absence of such consistency of findings in the rat toxicology data suggests that the NOEL, determined from some occurrence of a statistically significant difference, may not be a reliable guide to a real effect. Additionally, Wyeth believes that a NOEL is not a reliable guide for an assurance of safety, because it is silent on the nature of an effect (i.e. the effect could reflect a positive outcome).

Wyeth emphasized in its GRAS notice that there were numerous conservatisms built into the specified conditions of use that it considers GRAS. Among these conservatisms are:

- the extraordinary quality control used in infant formula manufacture that assures exposures are precisely defined per unit of energy intake,
- the upper limit on total amount of the fatty acids DHA and AA in the infant formula (which corresponds to the levels in LBW formulations used in hospital),
- the fact that the upper limit in LBW formulations (the level determined GRAS in Wyeth's notice) is twice the upper limit in formulas for term infants (the primary population base for exposure estimates),

- the fixed composition of the food matrix, fat blend and processing in which the oils are used
- the narrow range of total fat per unit energy in infant formula, and
- the acceptable ratio of the two fatty acids (DHA and AA) to each other.

C. The third concern of the agency was that Wyeth did not explain why such effects in rats are not of concern in human infants, particularly preterm and young infants.

Wyeth finds that the rat toxicology data do not suggest any safety concern in the rat, and thus, there would be none predicted for the human. Wyeth did include in its GRAS notice summaries from its large, multi-center clinical safety study conducted in preterm infants. These results support the conclusion that ARASCO and DHASCO, used in the manner described in the GRAS notice, are safe. In the presentation of May 24 Wyeth provided more detailed safety data from that same clinical study that show no differences in any measures related to safety among infants fed formula with the oils of interest and those infants receiving formula not supplemented with the oils. Included among the endpoints measured were all standard serologic measures relevant to the organ systems that were of possible concern. In addition, a careful collection and analysis of all adverse events demonstrated no overall significant differences between infants fed formulas with and without LCPs. These data are now published in a peer-reviewed journal (Vanderhoof, J, et al., 1999, Journal of Gastroenterology and Pediatric Nutrition 29:318-326) and thus, clinical data are publicly available that demonstrate safety and that supercede all preclinical data pertinent to safety.

II. Response to FDA's concerns

In order to more completely explain the basis of Wyeth's determination that ARASCO and DHASCO are safe, in accordance with the specific conditions of use set forth in Wyeth's notice GRN 000007, the total available rat toxicology data were summarized. The description of the total data and the interpretations that derive from the data were presented at the May 24, 1999 meeting, and are restated below in response to the same three "concerns" expressed in FDA's March 4, 1999, letter.

The seven rat toxicological studies described in Wyeth's presentation of May 24, 1999, are referred to by Roman numerals as study I (Wyeth, 9 week); study II (Wyeth, 13 week), study III (Mead Johnson, 4 week study, published as Wibert et al., 1997), study IV (Mead Johnson, 13 week study published as Burns et al., 1999); study V (Martek, 4 week study published as Boswell et al., 1996); study VI (Martek, 3 month study published as Koskelo, et al., 1997) and study VII (Gist-brocades, 13 week study, in press).

In the discussion of the endpoints of concern to FDA, Wyeth stressed that general scientific principles of toxicology need be kept in mind:

- The observation should be dose-related,
- The observation should be seen consistently across comparable studies,
- The observation should occur in both genders, and
- Multiple lines of evidence should point to the same conclusion.

A. There may be some toxicological significance of the effects observed.

1. FDA was concerned about increases in liver weight or increases in relative liver weight.

a. There was no consistent effect of treatment article on liver weight. (The term "liver weight" in this document refers to the absolute weight of the liver. Discussion of weights of liver or spleen relative to body weight or relative to brain weight use the term "relative weight.")

Data from 6 rat studies (I, II, IV, V, VI, VII) are available, as well as the text of an additional published study (study III) that reported no effect on liver weights. Four studies with data on liver weights were subchronic (13 week) studies. Two of these studies (II, VI) with dosage levels up to 2.5 g oil/kg body weight (17 fold over the expected clinical exposure) showed no effects at all. Two shorter studies also found no effect at highest doses of 1.2 g/kg body weight (I) or 9.1 g/kg body weight (III), and a third (V) found no effect at a highest dose of 3.75 g/kg body weight in females, but a significant effect was seen in males.

The only liver weight "effect" that was seen in more than one study (i.e. a possible consistent effect) was an increase in the liver weights of F_1 females after gestational, lactational and 90 days oral exposure (IV, VII) at a dose approximately 59 and 64 times the expected clinical dose (146 mg/kg) on a g/kg body weight basis. In neither of these studies was an effect observed in F_1 males, nor was an effect seen in females or males in a shorter study (III) after a comparable dose. Because the "effect" occurred only in studies that included gestational exposure, the findings are confounded and may not be directly relevant to the intended use described in Wyeth's notice.

Increase in absolute liver weight is not as useful an indicator of changes in liver size as relative liver weights. Liver to body weight is often used as a comparison measure, but body weight is more susceptible to change than is liver weight in diet studies because of altered food consumption. Thus, an animal might consume less food due to sensory characteristics of the diet, resulting in a reduced body weight, but no loss in liver weight (an increase in liver/body weight ratio). Liver to brain weight ratios are the most useful, since brain weight is highly protected and not prone to dietary intake differences. When liver to brain weight ratios are considered, most of the statistically significant differences described above are lost.

If there was a toxic principle in the materials tested that affected liver weight, one would expect consistent increases in liver weight or in relative liver weight in similarly dosed animals in different studies.

b. Effects did not occur in both genders.

In those three studies out of seven studies, where some effect on liver weight was seen, one short term study found a statistically significant increase in the high dose male group but no occurrence among females (V); one long term study found a significant increase among high dose females but no occurrence among males (VII). One study found an increase in liver weight in males at a mid dose but no effect among males at a higher dose, and an effect among females at the mid and high doses (IV).

If there was a toxic principle in the materials tested, increased liver weights would be expected to be seen in both sexes, across all studies and in a dose-related manner.

c. Histopathology and clinical chemistry.

In each study where there was no observed change in liver weights, there was evidence of no treatment-related microscopic findings (I, II, VI) or findings that were not different in treatment animals than in controls (III). In two of the three studies (IV, V, VII) where there was a statistically significant difference in liver weight reported, no microscopic changes were detected. Burns et al. (IV) reported that "No test material-related microscopic changes were found," and "There was no morphologic correlation with the increased liver and spleen weights in the mid- and high-dose groups of both sexes." Boswell et al. (V) report, "The liver weight changes were mild, less than 20%, and there were no abnormal microscopic findings in the livers." In only one study (VII) in only female rats there was a finding of hepatocellular vacuolation. This single occurrence of a non-pathognomonic finding in the rats dosed at 59 times the expected clinical dose, but not corroborated by two other studies with comparable doses, clearly stands out as an anomalous finding of unknown importance. Wyeth provided an assessment of this finding, written by a renowned expert in the area, Dr Rodger Haggitt that "lipid was a harmless, incidental finding. It is reasonable to think that the treatment resulted in a physiological adaptation in order to metabolize the extreme LCP burden." (See **e.** Interpretation, below).

Not only is there no suggestion of pathology in morphology, but there is consistent evidence across all studies, and including all groups that had increased liver weights, that there was no change in serum measures of liver function or liver integrity. The absence of effect on histopathology and on clinical chemistry greatly strengthens the conclusion that the reported findings of increased liver weights, inconsistent as they are, are not pathological.

The principles of toxicology allow the conclusion that even at the highest doses used, as high as 64 times greater than the expected clinical dose for preterm infants and over 120 times the expected clinical dose for term infants on a g/kg body weight basis, there is no evidence of any adverse effect. In the cases where some effects on liver weight were observed, there is morphological and biochemical evidence that the effects are not pathological.

d. Additional evidence.

Wyeth noted that data from neonatal piglets fed formula supplemented with ARASCO and DHASCO at expected human dose levels had growth, liver weights and histopathology that was not different from unsupplemented animals.

Wyeth also presented the complete clinical data from its large, multi-center safety study of ARASCO and DHASCO in preterm infant formula that showed no effects on growth, or any serological marker of liver function or integrity. There were no differences between formula-fed groups (with and without ARASCO and DHASCO) for serum protein, albumin, ALT, AST, ALK P, bilirubin, triglycerides, BUN, or other routine

analytes. Only cholesterol was statistically different, slightly higher in the supplemented group than in the unsupplemented group, as is typically true for breast-fed versus formula-fed infants.

e. Interpretation.

Wyeth interprets the occasional increase in liver weights to be a physiological response to a high LCP stress, not a toxicological finding. There are numerous reasons that support this interpretation.

- The magnitude of the effects was due in part to changes in body weight. The liver weight increases are much less pronounced when reported with respect to brain weight. Indeed, most occurrences of statistically significance differences in liver weights are no longer significant when expressed relative to brain weight.

- There is evidence of similar effects in rat studies from closely related materials (fish oils) not obtained from the same source. Wyeth listed results from studies of feeding high doses of oils from at least 4 species of fish where there was an increase in organ weights, corroborated by internationally recognized expert Dr R.G. Ackman.

- The effects that were reported may have been in part because of the nature of the control oil and in part because of the synthetic diets used. The choice of canola oil as the control treatment in one study that reported changes in liver weights (IV) may not have been ideal, as the load of total PUFAs (ARA, DHA and C18 polyunsaturates) was less than in treatment groups. Canola is low in C18:2 and C18:3 relative to either corn or soy oils that are routinely used in rat diets for studies of this type. Indeed, in that study (study IV) there are many occurrences where endpoints for the high fat control group were different from those for the low fat control group, and were in the opposite direction as changes in the test-material groups. Thus, the change in high fat control group contributed to the total difference and finding of statistical significance between high fat control and test groups. Wyeth provided data that shows synthetic diets alone (without any test substance) result in increased occurrence of fatty liver. Fatty changes in organs were considered to be non-pathological changes in the view of R.W. Perry, DVM, Ph.D., Wyeth's Associate Director of Pathology, and Rodger Haggitt, MD, Professor of Pathology, University of Washington School of Medicine (an internationally recognized authority in the area of gastrointestinal pathology).

- The effects were primarily from one study that included a gestational exposure, a study design that introduces a confounding factor.

- FDA has determined that changes in organ weights can be a physiological response to the high dose treatments used in safety evaluations, as evidenced by FDA rule on sucralose (FR Volume 63, Number 64 pp 16417-16433, April 3, 1998).

f. Conclusion regarding liver weights.

Wyeth finds uniform evidence of no effect on liver weights after doses as high as 2.5 g/kg body weight. This NOEL is 17 times the expected clinical dose, higher than the NOEL determined by FDA. There is no consistent result of increased liver weight after higher levels. Among the toxicological studies, there were occurrences of no significant changes seen after 3.75 g/kg body weight (26 times clinical dose for preterm, 52 times for term infants, study V females), and 4.5 g/kg body weight (31 times expected clinical dose for preterm and 62 times for term infants, study III) and 9.4 g/kg body weight (64 times expected clinical dose for preterm and 128 times for term infants, study III, males in studies IV and VII). There is evidence that even at the highest doses where some occurrences of increased liver weight were observed (studies IV and VII among females), there is no associated pathology, shown in multiple studies by the absence of abnormal histopathology; in every case by normal serum levels of liver enzymes. The magnitude of difference between the expected use level and safe level is remarkable especially in comparison to some other nutrients in infant formula, e.g. vitamins A and D.

Evidence corroborating safety (with respect to measures relevant to liver) was obtained in piglets. Evidence that established safety was obtained in preterm infants. Specifically, in the clinical study, no significant differences were observed between formulas with or without LCPs in serum albumin, alanine aminotransferase, alkaline phosphatase, asparate aminotransferase, total bilirubin or direct bilirubin.

2. FDA was concerned about increases in spleen weight and relative spleen weight.

a. Spleen weight.

Three long term studies (IV, VI and VII) reported data. In 2 of these studies (IV and VII) there was an increase in spleen weights at the highest dose and the increase was seen in both sexes. In one study (IV) there was also an increase in spleen weights in both males and females at the mid dose, compared to the spleen weight of high fat controls that was reduced about 10% relative to low fat controls. Relative spleen weight, expressed as either spleen weight to body weight, or spleen weight to brain weight, was also increased in the mid and high dose groups in one study (IV), relative to high fat controls.

In study VII, the magnitude of change in spleen weight in the high fat controls from that of low fat controls, in both males and females, was the same magnitude as of the increase in spleen weight of the group treated with 8.5 g/kg compared to low fat controls. Thus, the statistically significant differences detected between treatment and high fat groups were markedly dependent on changes in the control animals. Similarly, in study IV, the high fat control animals had lower spleen weights and lower relative spleen

weights than low fat controls. In fact, among males the relative spleen weight of the high fat diet controls was statistically significantly different (lower) from that of low fat controls. Among females (study IV) the relative spleen weight of the mid dose group was statistically significant different from the high fat control (which is low compared to the low fat control), but is numerically identical to that of the low fat control. A reasonable interpretation of these data is that the mid dose ARASCO/DHASCO treatment neutralized the depression of relative spleen weight observed in high fat controls. Clearly, the results of statistical analysis in a study where changes are observed among controls must be interpreted carefully.

The effect on spleen weight, however, does not suggest an adverse effect. Wyeth provided a statement by Dr. Kevin Fritsche, a noted expert in the field of LCPs and immune system function in this regard. Dr. Fritsche has conducted more than 2 dozen feeding trials with LCPs. He writes, "In several of our feeding trials we have noted that mice and rats fed diets enriched in n-3 PUFA have had significantly heavier spleens (15-35%) and that these spleens contained significantly more immune cells (10-50%)." Dr. Fritsche observes these results whether the test material is any of a number of LCP- or n-3 rich oils, including sardine oil, cod liver oil, menhaden oil or flaxseed oil. These results support the conclusion that the increased spleen weights in ARASCO and DHASCO studies are not a function of the source of the oils, but are an expected finding after feeding *any* source of LCPs. Dr. Fritsche also notes that the increased spleen weights occur only after feeding high doses, about 5 g LCP/kg body weight (or 5-10% of dietary energy), that corresponds to the findings in Burns et al. (IV).

Importantly, Dr. Fritsche also writes that several possibilities for the increase in spleen weights can be ruled out. "First, this increase in spleen weight does not appear to be a general toxic property of the diets. Gross examination of the spleen did not indicate edema or other gross abnormality. Weight gain and final body weight were normal in fish oil fed mice/rats in all our studies."

b. Histopathology.

There is consistent microscopic evidence of no damage to spleen following LCP treatment assessments. Burns et al. (IV) reported that "No test material-related microscopic changes were found," and "There was no morphologic correlation with the increased liver and spleen weights in the mid- and high-dose groups of both sexes." No abnormal histopathological findings were reported in studies III, V or VI, and there were no histopathological differences for spleen among treatment groups in Wyeth's studies (studies I and study II).

c. Clinical chemistry/hematology.

- There are minimal changes in mean HCT, and APTT among high dose animals compared to high fat control animals in one study (IV). However, in this study serologic measures (including WBC, reticulocyte, RBC, and platelets) for high dose treatment animals were nearly identical to those of the low fat controls. Furthermore, the APTT values of the high dose females were nearly identical

to those for female rats in the low fat control group. These results suggest that the statistically significant differences between high fat controls and high dose treatment groups were of no physiological consequence.

- There were consistently no changes at any dose in all studies for WBC (excepting a spurious finding in the mid-dose males (IV)). Similarly, there were consistently no differences for treatment groups in any study for reticulocytes, RBCs, or PLT, although there were differences between the high fat and low fat or chow controls.

d. Additional information.

Wyeth also presented data from a study in piglets that found no differences in spleen to body weight between ARASCO/DHASCO supplemented animals and sow fed controls. No treatment-related histopathological abnormalities were found.

Wyeth presented clinical chemistry data from its large safety study that showed no effect of ARASCO/DHASCO at intended levels of use on spleen-related measures of hemoglobin, mean cell hemoglobin, mean cell hemoglobin concentration, mean cell volume, hematocrit, basophils, eosinophils, WBCs, lymphocytes, monocytes, neutrophils, platelets, or RBCs.

Finally, Wyeth presented clinical evidence of normal immune function in ARASCO/DHASCO supplemented infants. Levels of IL-10, the CD4/CD8 ratio, and two measures of T cell maturity (CD45RO+ and CD45RA+) were not different between LCP supplemented infants and breast-fed controls, whereas the non-supplemented group was in each case different. These results suggest that at the intended supplementation level, the immune system may be benefitted by LCP supplementation to become not different than that of breast-fed infants.

e. Conclusions about spleen weights.

Wyeth finds that the data on spleen weights do not suggest any adverse effect related to treatment. Because control treatments themselves produced effects, the statistical analyses are difficult to interpret. In most cases it appears that the high fat control resulted in an effect of similar or greater magnitude than the treatment. The comparability of relative spleen weights of low fat control and high dose treatment groups suggests that there is no physiological significance of the statistically significant differences between treatment and high fat control groups. The findings of normal histpathology and the consistent evidence of unchanged serologic data for spleen-relevant measures provides strong evidence that increased spleen weights are not adverse toxicological findings. Furthermore, the evidence in piglets and preterm human infants supports the conclusion of no adverse effects at intended use levels. The evidence in humans is that there is a positive effect (normalization to the outcome of breast fed infants) on immune system function at the intended use levels.

B. The estimated dietary exposure in comparison to a NOEL.

FDA expressed concern that at the target exposure, the estimated dietary exposure in infants is 8 to 20 fold lower than the level at which no effects, the NOEL, were observed in the rat toxicology data (or 5-14 fold lower than the maximum dietary intake).

On the face of the matter, it is difficult to understand why in this particular instance FDA feels uncomfortable with a 8-20 fold difference between expected exposure and any observed effect, given that much less difference is observed for many nutrients in infant formula.

The particular margin between expected exposures and a NOEL is not related to assurance of safety, because the NOEL is silent on the nature of the effect. Indeed, any beneficial substance added to food, including infant formula, would be expected to have no NOEL, because the substance is added precisely to have an effect. In fact, FDA has relied on the demonstration of an effect in order to permit the addition of a substance to infant formula, e.g., sodium selenite and effects on blood selenium and glutathione peroxidase response. Thus, the use of NOEL to assess safety of a biologically active nutrient is inappropriate. Reliance on a NOEL in a safety assessment leads to the illogical conclusion that no beneficial ingredient can be added to food, because it cannot simultaneously have a beneficial effect and be safe.

It also is relevant that FDA has affirmed (Federal Register 62:30751) that menhaden oil (a closely related material by virtue of its content of LCPs) is GRAS for use in numerous foods, up to an exposure of 3.0 g/d. FDA did not state a NOEL in that final rule, but described numerous physiological differences after exposures above 3.0 g/d. That is, a NOEL was the same as the GRAS affirmed level.

FDA may have determined a NOEL rather than a NOAEL in responding to Wyeth's GRAS notice, in order to avoid a comprehensive assessment of the nature of the effect. In the GRAS notice proposed rule FDA wrote, "FDA does not intend to conduct its own detailed evaluation of the data that the notifier relies upon to support a determination that a use of a substance is GRAS" (Federal Register 62:18938 at 18941). The determination of a NOAEL requires the type of detailed evaluation that FDA proposed to avoid by the GRAS notice procedure.

Thus, the determination by FDA of a NOEL cannot (and remain consistent with previous decisions) and should not (according to scientific procedures) determine whether or not FDA agrees that a manufacturer has a satisfactory basis for its conclusion that a substance is GRAS. Wyeth, and the authors of the published scientific investigations cited above, have used general toxicological principles to reach the conclusion that the sporatic changes in organ weights were of no toxicological concern. Wyeth suggests that the appropriate parameter to use in consideration of toxicity is a NOAEL, rather than a NOEL. Wyeth interprets the available rat, piglet and human data to indicate that a NOAEL is greater than 64 fold higher than the expected clinical dose for preterm infants, and more than 128 fold higher than the expected clinical dose for term infants.

C. The third concern of the agency was that Wyeth did not explain why such effects in rats are not of concern in human infants, particularly preterm and young infants.

Wyeth presented the conclusions from its large, multi-center safety study in preterm infants in its GRAS notice. At the May 24 meeting Wyeth provided the complete data that show no significant differences in growth, or any standard safety measures between preterm infants fed supplemented formula and those fed unsupplemented formula. Additionally, there were no differences in reported adverse events between groups. It must be stressed that the study was designed for the purpose of establishing safety, and was powered accordingly. Wyeth believes these data alone are sufficient to demonstrate safety of the materials used in accordance with the specifications of the GRAS notice. The full manuscript from this study has recently been published. These clinical data are the primary basis for Wyeth's conclusion that the oils are safe as specified in Wyeth's GRAS notice. The publication of the clinical safety data adds substantially to the general recognition component of the GRAS notice (although Wyeth maintains that the notice as initially filed demonstrated the generality of the recognition).

In materials provided to FDA at the May 24, 1999 meeting, Wyeth included a comprehensive Cochrane review (Simmer, 1997) that concluded that LCPs (without specifying source) were safe for use in infant formula. There is international recognition that the Cochrane reviews meet the most rigorous standards in the review of clinical studies. NIH sponsors, in part, the Cochrane Neonatal Collaborative Review Group, and writes, "NICHD has placed a high priority on making the work of this collaboration available because of the importance of relaying this information to health care professionals and to the public."

There are numerous additional clinical studies published since the Cochrane review that have evaluated the effects on infants of formulas *that contain ARASCO and DHASCO in accordance with the conditions of use specified in Wyeth's GRAS notice.* While many of these studies were not designed for the purpose of assessment of safety (except Vanderhoof et al. 1999 that was specifically designed and conducted for the purpose of assessment of safety), it is noteworthy that in every study, growth of the infants fed the supplemented formula was the same as or better than the control infants. FDA has proposed to recognize normal physical growth as the sole outcome measure to demonstrate the quality of a new infant formula (Federal Register 61:36154). Each of the small studies (under 30 infants per group) found normal growth when ARASCO and DHASCO were supplemented to the infant formula (Birch et al., 1998; Clandinin et al., 1997; Carnielli et al., 1998; Boehm et al., 1996). Each of the larger studies that were specifically powered to detect differences in growth (Diersen-Schade et al., 1998; Vanderhoof et al., 1999; Carlson, 1999 abst) also found normal growth. None of these studies have reported any adverse effects related to LCPs. (Copies of papers not previously submitted to FDA are attached).

Wyeth pointed out to FDA that the conclusions about safety of the oils reached by the large number of scientific and medical investigators who conducted the clinical studies, not to mention the institutional review boards who determined the clinical studies were safe to conduct, add to the basis of Wyeth's determination that ARASCO and DHASCO, are Generally Recognized as Safe for use in infant formula.

III. Conclusion

Wyeth believes that the data and other information provided to FDA provides an unarguable basis for Wyeth's determination that ARASCO and DHASCO oils, used in accordance with the conditions of use set forth in Wyeth's notice GRN 00007, are safe for use in infant formula. There is abundant evidence obtained through scientific procedures, including preclinical studies, clinical studies, compositional and analytical studies, actual safe use experience for more than two years in over 65 countries, evidence obtained through general scientific principles, independent assessment by experts in toxicology, and various additional information and methods, to support the conclusion that the oils are safe. Conversely, there is no evidence that contradicts the conclusion that ARASCO and DHASCO oils, used in accordance with the conditions set forth in Wyeth's notice GRN 00007, are safe for use in infant formula. We believe that the data presented far exceed the extent of safety data for nutrients already required by law to be in infant formula. We believe that the basis of Wyeth's determination fully meets the legal standard of reasonable certainty of no harm.

Pages 000218 - 000226 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

Appendix 5

APPENDIX 5

AUDITED EXECUTIVE SUMMARIES OF TOXICOLOGY STUDIES

1. Mutagenesis (AMES) DHASCO (02/23/94)
2. Microsome reverse mutation DHASCO (01/04/97)
3. Microsome reverse mutation ARASCO (13/05/97)
4. Mutagenesis (AMES) ARASCO (02/23/94)
5. Lymphoma forward mutation DHASCO (06/17/94)
6. Lymphoma forward mutation DHASCO biomass (05/20/97)
7. Lymphoma forward mutation ARASCO (06/17/94)
8. Chromosomal aberrations in CHO cells DHASCO (06/15/94)
9. Chromosomal aberrations in CHO cells DHASCO biomass (02/28/97)
10 Chromosomal aberrations in CHO cells ARASCO (05/23/94)
11. Acute oral toxicity DHASCO (12/18/90)
12. Acute oral toxicity DHASCO (02/28/97)
13. Acute oral toxicity DHASCO biomass (10/24/95)
14. Acute oral toxicity DHASCO biomass (03/15/96)
15. Acute oral toxicity DHASCO extracted biomass (12/18/90)
16. Acute oral toxicity DHASCO extracted biomass (07/03/97)
17. Acute oral toxicity ARASCO (01/17/92)
18. Acute oral toxicity ARASCO (08/27/93)
19. Acute oral toxicity ARASCO biomass (07/03/97)
20. Acute oral toxicity ARASCO extracted biomass (07/03/97)
21. Acute oral toxicity microencapsulated DHASCO/ARASCO (03/04/97)
22. 4-week ARASCO; 4-week DHASCO; and 4-week DHASCO+ARASCO (06/29/94)
23. 4-week DHASCO biomass final report
24. 3-month DHASCO and 3-month ARASCO (07/17/95)
25. Developmental toxicology DHASCO and ARASCO (07/16/95)
26. 4-week ARASCO; 4-week DHASCO; and 4-week DHASCO+ARASCO (03/96)
27. 13-week ARASCO and 13-week ARASCO + DHASCO (05/97)
28. 13-week ARASCO + DHASCO (05/97)
29: Additional Publications:
    - Mead Johnson 4-week study (ARASCO + DHASCO)
    - Mead Johnson 13-week study (ARASCO + DHASCO)
    - Gist-brocades 4-week study (ARASCO and ARASCO+fish oil)
    - Summation of studies by Wyeth/Ayerst

COVANCE
THE DEVELOPMENT SERVICES COMPANY

MUTAGENICITY TEST WITH

DHASCO

IN THE *SALMONELLA - ESCHERICHIA COLI*/MAMMALIAN-MICROSOME REVERSE MUTATION ASSAY

FINAL REPORT

AUTHOR

Timothy E. Lawlor, M.A.

PERFORMING LABORATORY

Covance Laboratories Inc. (Covance)
9200 Leesburg Pike
Vienna, Virginia 22182

LABORATORY PROJECT ID

Covance Study No.: 18247-0-409

SUBMITTED TO

Martek Biosciences Corporation
6480 Dobbin Road
Columbia MD, 21045

STUDY COMPLETION DATE

April 1, 1997

000229

SUMMARY

A. Introduction

At the request of Martek Biosciences Corporation, Covance investigated **DHASCO** for mutagenic activity in the *Salmonella - Escherichia coli*/Mammalian-Microsome Reverse Mutation Assay. This assay evaluated the test article and/or its metabolites for their ability to induce reverse mutations at the histidine locus in the genome of specific *Salmonella typhimurium* tester strains and at the tryptophan locus in an *Escherichia coli* tester strain both in the presence and absence of an exogenous metabolic activation system of mammalian microsomal enzymes derived from Aroclor™-induced rat liver (S9).

The doses tested in the mutagenicity assay were selected based on the results of a dose rangefinding study using tester strains TA100 and WP2*uvr*A and ten doses of test article ranging from 5,000 to 6.67 µg per plate, one plate per dose, both in the presence and absence of S9 mix.

The tester strains used in the mutagenicity assay were *Salmonella typhimurium* tester strains TA98, TA100, TA1535, TA1537, and *Escherichia coli* tester strain WP2*uvr*A. The assay was conducted with six doses of test article in both the presence and absence of S9 mix along with concurrent vehicle and positive controls using three plates per dose. The doses tested were 5,000, 1,000, 500, 250, 100, and 50.0 µg per plate in both the presence and absence of S9 mix.

B. Conclusions

The results of the *Salmonella - Escherichia coli*/Mammalian-Microsome Reverse Mutation Assay indicate that under the conditions of this study, Martek Biosciences Corporation's test article, **DHASCO**, did not cause a positive increase in the number of revertants per plate of any of the tester strains either in the presence or absence of microsomal enzymes prepared from Aroclor™-induced rat liver (S9).

HAZLETON
WASHINGTON
9200 LEESBURG PIKE
VIENNA, VA 22182-1699

MUTAGENICITY TEST ON

RBD-DHASCO

IN THE SALMONELLA/MAMMALIAN-MICROSOME REVERSE MUTATION ASSAY (AMES TEST)

FINAL REPORT

AUTHOR

Timothy E. Lawlor, M.A.

PERFORMING LABORATORY

Hazleton Washington, Inc.
9200 Leesburg Pike
Vienna, Virginia 22182

LABORATORY PROJECT ID

HWA Study No.: 16016-0-401

SUBMITTED TO

Martek Biosciences Corporation
6480 Dobbin Road
Columbia, Maryland 21045

STUDY COMPLETION DATE

February 23, 1994

SUMMARY

A. Introduction

At the request of Martek Biosciences Corporation, Hazleton Washington, Inc. investigated RBD-DHASCO for mutagenic activity in the Salmonella/Mammalian-Microsome Reverse Mutation Assay (Ames Test). This assay evaluates the test article and/or its metabolites for their ability to induce reverse mutations at the histidine locus in the genome of specific Salmonella typhimurium tester strains both in the presence and absence of an exogenous metabolic activation system of mammalian microsomal enzymes derived from Aroclor™-induced rat liver (S9).

The doses tested in the mutagenicity assay were selected based on the results of a dose rangefinding study using tester strain TA100 and ten doses of test article ranging from 5,000 to 6.67 μg per plate, one plate per dose, both in the presence and absence of S9.

The tester strains used in the mutagenicity assay were TA98, TA100, TA1535, TA1537 and TA1538. The assay was conducted both in the presence and absence of S9 mix using six doses of test article and three plates per dose. The following six doses of the test article were tested: 5,000, 3,330, 1,000, 667, 333, and 100 μg per plate.

B. Conclusions

The results of the Salmonella/Mammalian-Microsome Reverse Mutation Assay (Ames Test) indicate that under the conditions of this study, Martek Biosciences Corporation's test article, RBD-DHASCO, did not cause a positive increase in the number of histidine revertants per plate of any of the tester strains either in the presence or absence of microsomal enzymes prepared from Aroclor™-induced rat liver (S9).

COVANCE
THE DEVELOPMENT SERVICES COMPANY

MUTAGENICITY TEST WITH

Spray-dried DHASCO Biomass

IN THE *SALMONELLA - ESCHERICHIA COLI*/MAMMALIAN-MICROSOME REVERSE MUTATION ASSAY

FINAL REPORT

AUTHOR

Timothy E. Lawlor, M.A.

PERFORMING LABORATORY

Covance Laboratories Inc. (Covance)
9200 Leesburg Pike
Vienna, Virginia 22182

LABORATORY PROJECT ID

Covance Study No.: 18203-0-409

SUBMITTED TO

Martek Biosciences Corporation
6480 Dobbin Rd.
Columbia, MD 21045

STUDY COMPLETION DATE

May 13, 1997

SUMMARY

A. Introduction

At the request of Martek Biosciences Corporation, Covance investigated **Spray-dried DHASCO Biomass** for mutagenic activity in the *Salmonella - Escherichia coli*/Mammalian-Microsome Reverse Mutation Assay. This assay evaluated the test article and/or its metabolites for their ability to induce reverse mutations at the histidine locus in the genome of specific *Salmonella typhimurium* tester strains and at the tryptophan locus in an *Escherichia coli* tester strain both in the presence and absence of an exogenous metabolic activation system of mammalian microsomal enzymes derived from Aroclor™-induced rat liver (S9).

The doses tested in the mutagenicity assay were selected based on the results of a dose rangefinding study using tester strains TA100 and WP2*uvr*A and ten doses of test article ranging from 5,000 to 6.67 µg per plate, one plate per dose, both in the presence and absence of S9 mix.

The tester strains used in the mutagenicity assay were *Salmonella typhimurium* tester strains TA98, TA100, TA1535, TA1537, and *Escherichia coli* tester strain WP2*uvr*A. The assay was conducted with six doses of test article in both the presence and absence of S9 mix along with concurrent vehicle and positive controls using three plates per dose. The doses tested were 5,000, 3,330, 1,000, 333, 100, and 33.3 µg per plate in both the presence and absence of S9 mix.

B. Conclusions

The results of the *Salmonella - Escherichia coli*/Mammalian-Microsome Reverse Mutation Assay indicate that under the conditions of this study, Martek Biosciences Corporation's test article, **Spray-dried DHASCO Biomass**, did not cause a positive increase in the number of revertants per plate of any of the tester strains in either the presence or absence of microsomal enzymes prepared from Aroclor™-induced rat liver (S9).

HAZLETON
WASHINGTON
9200 LEESBURG PIKE
VIENNA, VA 22182-1699

MUTAGENICITY TEST ON

RBD-ARASCO

IN THE SALMONELLA/MAMMALIAN-MICROSOME REVERSE MUTATION ASSAY (AMES TEST)

FINAL REPORT

AUTHOR

Timothy E. Lawlor, M.A.

PERFORMING LABORATORY

Hazleton Washington, Inc.
9200 Leesburg Pike
Vienna, Virginia 22182

LABORATORY PROJECT ID

HWA Study No.: 16015-0-401

SUBMITTED TO

Martek Biosciences Corporation
6480 Dobbin Road
Columbia, Maryland 21045

STUDY COMPLETION DATE

February 23, 1994

000235

SUMMARY

A. Introduction

At the request of Martek Biosciences Corporation, Hazleton Washington, Inc. investigated RBD-ARASCO for mutagenic activity in the Salmonella/Mammalian-Microsome Reverse Mutation Assay (Ames Test). This assay evaluates the test article and/or its metabolites for their ability to induce reverse mutations at the histidine locus in the genome of specific Salmonella typhimurium tester strains both in the presence and absence of an exogenous metabolic activation system of mammalian microsomal enzymes derived from Aroclor™-induced rat liver (S9).

The doses tested in the mutagenicity assay were selected based on the results of a dose rangefinding study using tester strain TA100 and ten doses of test article ranging from 5,000 to 6.67 μg per plate, one plate per dose, both in the presence and absence of S9.

The tester strains used in the mutagenicity assay were TA98, TA100, TA1535, TA1537 and TA1538. The assay was conducted both in the presence and absence of S9 mix using six doses of test article and three plates per dose. The following six doses of the test article were tested: 5,000, 3,330, 1,000, 667, 333, and 100 μg per plate.

B. Conclusions

The results of the Salmonella/Mammalian-Microsome Reverse Mutation Assay (Ames Test) indicate that under the conditions of this study, Martek Biosciences Corporation's test article, RBD-ARASCO, did not cause a positive increase in the number of histidine revertants per plate of any of the tester strains either in the presence or absence of microsomal enzymes prepared from Aroclor™-induced rat liver (S9).

HAZLETON
WASHINGTON
9200 LEESBURG PIKE
VIENNA, VA 22182-1699

MUTAGENICITY TEST ON

RBD-DHASCO

IN THE L5178Y TK+/- MOUSE LYMPHOMA FORWARD MUTATION ASSAY

FINAL REPORT

AUTHOR

MARIA A. CIFONE, PH.D.

PERFORMING LABORATORY

HAZLETON WASHINGTON, INC.
9200 LEESBURG PIKE
VIENNA, VIRGINIA 22182

LABORATORY PROJECT ID

HWA STUDY NO.: 16141-0-431

SUBMITTED TO

MARTEK BIOSCIENCES CORPORATION
6480 DOBBIN ROAD
COLUMBIA, MARYLAND 21045

STUDY COMPLETION DATE

JUNE 17, 1994

000237

ABSTRACT

The objective of this in vitro assay was to evaluate the ability of RBD-DHASCO to induce forward mutations at the thymidine kinase (TK) locus in the mouse lymphoma L5178Y cell line. The test material formed a slightly viscous dark amber solution in DMSO at approximately 500 mg/ml. In the preliminary cytotoxicity assay, cells were exposed to the test material at concentrations up to 1000 µg/ml for four hours in the presence and absence of rat liver S9 metabolic activation. The test material was weakly to nontoxic without activation and, at most, moderately toxic with activation. The mutation assays were initiated with treatments up to 5000 µg/ml in an attempt to obtain moderate and high cytotoxicities. In medium, the test material was soluble up to 1000 µg/ml and slightly cloudy from 1000 µg/ml to 2000 µg/ml; treatments from 3000 µg/ml to 5000 µg/ml were cloudy and contained oily droplets.

A single mutation assay was performed for both nonactivation and S9 metabolic activation conditions. Nine treatments from 125 µg/ml to 5000 µg/ml were initiated with and without activation. At most, moderate cytotoxicities were induced. Higher concentrations were not analyzed because insoluble doses were included and 5000 µg/ml is the testing limit in this assay. Treatments from 125 µg/ml to 500 µg/ml were terminated because sufficient nontoxic doses were available for analysis. Under nonactivation and activation conditions, none of the six assayed treatments induced a mutant frequency that exceeded the minimum criterion for a positive response and no dose-related trend was observed. The test material, RBD-DHASCO, was therefore evaluated as negative for inducing forward mutations at the TK locus in L5178Y mouse lymphoma cells under the nonactivation and S9 metabolic activation conditions used in this study.

COVANCE
THE DEVELOPMENT SERVICES COMPANY

MUTAGENICITY TEST ON

Spray-Dried DHASCO Biomass

IN THE L5178Y TK $^{+/-}$ MOUSE LYMPHOMA FORWARD MUTATION ASSAY

FINAL REPORT

AUTHOR

Maria A. Cifone, Ph.D.

PERFORMING LABORATORY

Covance Laboratories Inc. (Covance)
9200 Leesburg Pike
Vienna, Virginia 22182

LABORATORY PROJECT IDENTIFICATION

Covance Study No.: 18203-0-431

SUBMITTED TO

Martek Bioscience Corporation
6480 Dobbin Road
Columbia, MD 21045

STUDY COMPLETION DATE

May 20, 1997

000239

ABSTRACT

The objective of this *in vitro* assay was to evaluate the ability of Spray-dried DHASCO Biomass to induce forward mutations at the thymidine kinase (TK) locus in the mouse lymphoma L5178Y cell line. The test article was insoluble is water, dimethylsulfoxide (DMSO), ethanol and acetone. The best suspension formed in DMSO and DMSO was chosen as the vehicle. The test article formed a suspension in DMSO at concentrations as low as 0.195 mg/ml .

In the preliminary dose range finding assay, cells were exposed to the test article for four hours in the presence and absence of rat liver S9 metabolic activation. Treatments from 1.95 µg/ml to 1000 µg/ml were initiated. The test article was soluble in culture medium at the time of dosing at concentrations from 1.95 µg/ml to 62.5 µg/ml. The test article was noncytotoxic at all concentrations tested. The mutation assays were initiated with treatments up to 1000 µg/ml. Higher concentrations were not tested because of the insoluble nature of the test article.

A single trial of the nonactivation and the S9 metabolic activation mutation assays was performed. Seven treatments from 15.6 µg/ml to 1000 µg/ml were initiated and six doses were cloned for mutant analysis. No cytotoxicity was observed under either activation condition.

None of the six analyzed treatments with or without metabolic activation induced a mutant frequency that exceeded the minimum criterion for a positive response. Spray-dried DHASCO Biomass was therefore evaluated as negative with and without metabolic activation at the TK locus in L5178Y mouse lymphoma cells under the conditions used in this study.

HAZLETON
WASHINGTON
9200 LEESBURG PIKE
VIENNA, VA 22182-1699

MUTAGENICITY TEST ON

RBD-ARASCO

IN THE L5178Y TK+/- MOUSE LYMPHOMA FORWARD MUTATION ASSAY

FINAL REPORT

AUTHOR

MARIA A. CIFONE, PH.D.

PERFORMING LABORATORY

HAZLETON WASHINGTON, INC.
9200 LEESBURG PIKE
VIENNA, VIRGINIA 22182

LABORATORY PROJECT ID

HWA STUDY NO.: 16140-0-431

SUBMITTED TO

MARTEK BIOSCIENCES CORPORATION
6480 DOBBIN ROAD
COLUMBIA, MARYLAND 21045

STUDY COMPLETION DATE

JUNE 17, 1994

000241

ABSTRACT

The objective of this in vitro assay was to evaluate the ability of RBD-ARASCO to induce forward mutations at the thymidine kinase (TK) locus in the mouse lymphoma L5178Y cell line. The test material was soluble in dimethylsulfoxide (DMSO) at 200 mg/ml and was cloudy from 300 mg/ml to 500 mg/ml. In the preliminary cytotoxicity assay, cells were exposed to the test material at concentrations up to 1000 µg/ml for four hours in the presence and absence of rat liver S9 metabolic activation. In the preliminary cytotoxicity assay, the test material was nontoxic and soluble up to 1000 µg/ml with and without S9 metabolic activation. The mutation assays were initiated with treatments up to 4990 µg/ml in an attempt to obtain cytotoxicity. Oily droplets were observed in treatments above 2000 µg/ml.

A single mutation assay was performed for both nonactivation and S9 metabolic activation conditions. Nine treatments from 125 µg/ml to 4990 µg/ml were initiated with and without activation. At most, moderate cytotoxicities were induced. Higher concentrations were not analyzed because insoluble doses were included and 5000 µg/ml is the testing limit in this assay. Treatments from 125 µg/ml to 500 µg/ml were terminated because sufficient nontoxic doses were available for analysis. Under nonactivation and activation conditions, none of the six assayed treatments induced a mutant frequency that exceeded the minimum criterion for a positive response and no dose-related trend was observed. The test material, RBD-ARASCO, was therefore evaluated as negative for inducing forward mutations at the TK locus in L5178Y mouse lymphoma cells under the nonactivation and S9 metabolic activation conditions used in this study.

HAZLETON
WASHINGTON
9200 LEESBURG PIKE
VIENNA, VA 22182-1699

MUTAGENICITY TEST ON

RBD-DHASCO

MEASURING CHROMOSOMAL ABERRATIONS IN
CHINESE HAMSTER OVARY (CHO) CELLS

FINAL REPORT

AUTHOR

Hemalatha Murli, Ph.D.

PERFORMING LABORATORY

Hazleton Washington Inc.
9200 Leesburg Pike
Vienna, Virginia 22182

LABORATORY PROJECT ID

HWA Study No.: 16141-0-437

SUBMITTED TO

Martek Biosciences Corporation
6480 Dobbin Road
Columbia, Maryland 21045

STUDY COMPLETION DATE

June 15, 1994

000243

HAZLETON
WASHINGTON

ABSTRACT

The objective of this in vitro assay was to evaluate the ability of RBD-DHASCO to induce chromosomal aberrations in Chinese hamster ovary (CHO) cells with and without metabolic activation.

The test article was solubilized in dimethylsulfoxide at a stock concentration of 501 mg/ml for the rangefinding assay. Concentrations of 0.167, 0.501, 1.67, 5.01, 16.7, 50.1, 167, 501, 1670, and 5010 µg/ml were tested in rangefinding assays with and without metabolic activation. Slight cytotoxicity was observed in the cultures dosed with 501, 1670, and 5010 µg/ml, and slight cell cycle delay was observed in the cultures dosed with 501 and 1670 µg/ml from the nonactivation assay. No evidence of cytotoxicity and no appreciable cell cycle delay were observed in the cultures analyzed with metabolic activation.

Based on these data, replicate cultures of CHO cells were incubated with target concentrations of 500, 1250, 2500, 3750, and 5000 µg/ml with 10.0 and 20 hour harvests in the aberrations assays without metabolic activation. Target concentrations of 500, 1250, 2500, 3750, and 5000 µg/ml were tested with a 10.0 hour harvest in the aberrations assays with metabolic activation. Cultures treated with target concentrations of 1250, 2500, 3750, and 5000 µg/ml from the assays with and without metabolic activation were analyzed for chromosomal aberrations. No significant increase in cells with chromosomal aberrations was observed at the concentrations analyzed.

The test article, RBD-DHASCO, was considered negative for inducing chromosomal aberrations in Chinese hamster ovary cells under both nonactivation and activation conditions of this assay.

MUTAGENICITY TEST ON

SPRAY-DRIED DHASCO BIOMASS

CHROMOSOMAL ABERRATIONS IN CHINESE HAMSTER OVARY (CHO) CELLS WITH AND WITHOUT EXOGENOUS METABOLIC ACTIVATION

FINAL REPORT

AUTHOR

Hemalatha Murli, Ph.D.

PERFORMING LABORATORY

Corning Hazleton Inc. (CHV)
9200 Leesburg Pike
Vienna, Virginia 22182

LABORATORY PROJECT IDENTIFICATION

CHV Study No.: 18203-0-437

SUBMITTED TO

Martek Biosciences Corporation
6480 Dobbin Road
Columbia, Maryland 21045

STUDY COMPLETION DATE

February 28, 1997

000245

ABSTRACT

The objective of this in vitro assay was to evaluate the ability of Spray-dried DHASCO Biomass to induce chromosomal aberrations in Chinese hamster ovary (CHO) cells with and without metabolic activation.

For the dose rangefinding assays with and without metabolic activation, the test article was suspended in dimethylsulfoxide at a concentration of 99.6 mg/ml. Concentrations of 0.0332, 0.0996, 0.332, 0.996, 3.32, 9.96, 33.2, 99.6, 332, and 996 µg/ml were tested in the dose rangefinding assays. All dosing was achieved using a dosing volume of 1% (10.0 µl/ml). Slight to no cytotoxicity and slight to no cell cycle dalay was observed in the cultures analyzed.

Based on the data from the dose rangefinding assay, replicate cultures of CHO cells were treated with 50.0, 150, 250, 500, and 1000 µg/ml for 17.8 hours without metabolic activation and 3 hours with metabolic activation. All cultures were harvested at 20.0 hours from initiation of treatment. Cultures treated with 50.0, 250, 500, and 1000 µg/ml were analyzed for chromosomal aberrations. No significant increase in cells with chromosomal aberrations, polyploidy, and endoreduplication was observed in the cultures analyzed.

The test article, Spray-dried DHASCO Biomass, was considered negative for inducing chromosomal aberrations in CHO cells with and without metabolic activation.

HAZLETON
WASHINGTON
9200 LEESBURG PIKE
VIENNA, VA 22182-1699

MUTAGENICITY TEST ON

RBD-ARASCO

MEASURING CHROMOSOMAL ABERRATIONS IN
CHINESE HAMSTER OVARY (CHO) CELLS

FINAL REPORT

AUTHOR

Hemalatha Murli, Ph.D.

PERFORMING LABORATORY

Hazleton Washington Inc.
9200 Leesburg Pike
Vienna, Virginia 22182

LABORATORY PROJECT ID

HWA Study No.: 16140-0-437

SUBMITTED TO

Martek Biosciences, Corporation
6480 Dobbin Road
Columbia, Maryland 21045

STUDY COMPLETION DATE

May 23, 1994

000247

ABSTRACT

The objective of this in vitro assay was to evaluate the ability of RBD-ARASCO to induce chromosomal aberrations in Chinese hamster ovary (CHO) cells with and without metabolic activation.

The test article was solubilized in dimethylsulfoxide at a stock concentration of 500 mg/ml for the rangefinding assay. Concentrations of 0.167, 0.500, 1.67, 5.00, 16.7, 50.0, 167, 500, 1670, and 5000 µg/ml were tested in rangefinding assays with and without metabolic activation. No evidence of cytotoxicity and no appreciable cell cycle delay were observed in the cultures analyzed.

Based on these data, replicate cultures of CHO cells were incubated with 501, 1260, 2510, 3760, and 5010 µg/ml with 10.0 hour harvests in the aberrations assays with and without metabolic activation. Cultures treated with 1260, 2510, 3760, and 5010 µg/ml from the assays with and without metabolic activation were analyzed for chromosomal aberrations. No significant increase in cells with chromosomal aberrations was observed at the concentrations analyzed.

The test article, RBD-ARASCO, was considered negative for inducing chromosomal aberrations in Chinese hamster ovary cells under both nonactivation and activation conditions of this assay.

HAZLETON
WISCONSIN
3301 KINSMAN BOULEVARD
POST OFFICE BOX 7545
MADISON, WISCONSIN 53707

a CORNING Laboratory Services Company

Sponsor:

Martek Corporation
Columbia, Maryland

FINAL REPORT

Study Title:

Acute Oral Toxicity Study of DHASCO (Oil) in Rats

Author:

Steven M. Glaza

Study Completion Date:

December 18, 1990

Performing Laboratory:

Hazleton Wisconsin, Inc.
3301 Kinsman Boulevard
Madison, Wisconsin 53704

Laboratory Project Identification:

HWI 00900012

000249

United States • Germany • Japan • France • United Kingdom
Phone 608-241-4471 Fax 608-241-7227 Telex 703956 HAZRAL MDS UD

SUMMARY OF RESULTS

Test Animal: Albino Rats - Crl:CD®BR
Source: Charles River Laboratories, Inc., Portage MI
Date Animals Received: 09/10/90

Temperature and Humidity of Animal Room: 65 to 78°F;
27 to 50% Relative Humidity

Start Date (In-life): 09/21/90 End Date (In-life): 10/05/90

Estimated Oral LD_{50} : Male - Greater than 20.00 g/kg of Body Weight
Female - Greater than 20.00 g/kg of Body Weight

Sex	Dose Level (g/kg)	Average Body Weight (g) Initial	Day 7	Terminal	Mortality (Number Dead/Number Dosed)
Male	20.00	238	319	359	0/5
Female	20.00	213	249	259	0/5

Clinical Signs
(Number of Animals Affected)

Observation	Hour 1.0	2.5	4.0	Day 1	2	3	4	5	6	7	8	9	10	11	12	13	14
Males																	
Appeared normal	2	0	0	0	0	5	5	5	5	5	5	5	5	5	5	5	5
Soft stool	3	5	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dark-stained urogenital area	0	0	0	5	5	0	0	0	0	0	0	0	0	0	0	0	0
Females																	
Appeared normal	4	1	0	0	0	5	5	5	5	5	5	5	5	5	5	5	5
Soft stool	1	4	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dark-stained urogenital area	0	0	0	5	5	0	0	0	0	0	0	0	0	0	0	0	0

DISCUSSION

The acute oral toxicity of DHASCO (Oil) was evaluated in male and female albino rats when administered at a level of 20.00 g/kg of body weight. The estimated LD_{50} for males and females was determined to be greater than 20.00 g/kg. All animals exhibited body weight gain. Clinical signs of toxicity included soft stool and dark-stained urogenital area. All animals returned to a normal appearance within 3 days of test material administration. The gross necropsy examination of the animals at termination revealed no visible lesions.

SIGNATURE

12-18-90
Date

Steven M. Glaza
Study Director
Acute Toxicology

REFERENCE

1. NIH Publication No. 86-23 (revised 1985).

Corning Hazleton Inc.
P.O. Box 7545
Madison, WI 53707-7545
Deliveries: 3301 Kinsman Blvd., Madison, WI 53704
608.241.4471 608.241.7227 Fax

CORNING Hazleton

Sponsor:

Martek Biosciences Corporation
Columbia, Maryland

FINAL REPORT

Study Title:

Acute Oral Toxicity Study of High S DHASCO in Rats

Author:

Steven M. Glaza

Study Completion Date:

February 28, 1997

Performing Laboratory:

Corning Hazleton Inc.
3301 Kinsman Boulevard
Madison, Wisconsin 53704

Laboratory Project Identification:

CHW 61204198

Statistical Analyses

No statistical analyses were required by the protocol.

Location of Raw Data, Records, and Final Report

The raw data, records, and an original signed copy of the final report will be retained in the archives of CHW in accordance with CHW SOP.

RESULTS

Mortality

A summary of the survival rate is in Table 1. No mortality was observed during the study. The estimated oral LD_{50} values for male and female rats were determined to be greater than 20,000 mg/kg of body weight.

Body Weights

Individual and mean body weights and body weight gains are in Table 2. All animals exhibited body weight gain throughout the study.

Clinical Signs

Individual clinical signs are in Table 3. Clinical signs included red-stained face, soft stool, oily discharge from rectum, wet/yellow stained urogenital area, yellow stained abdominal area, oily haircoat urogenital/abdominal area, and general oily haircoat. All animals returned to a normal appearance by Day 13 after treatment.

Pathology

Individual gross necropsy findings are in Table 4. A summary report by the study pathologist is on Page 12. There were no test material related lesions observed at necropsy.

DISCUSSION

The acute oral toxicity of High S DHASCO was evaluated in male and female rats when administered as a single gavage dose at a level of 20,000 mg/kg of body weight. The estimated oral LD_{50} values for male and female rats were determined to be greater than 20,000 mg/kg. The clinical signs observed during this study are attributed to the excretion/leakage of the test material from the rectum of each animal. All animals returned to a normal appearance by Day 13 after treatment. All animals exhibited body weight gain during the study. The gross necropsy examinations at termination did not reveal any test material-related lesions.

SIGNATURE

Steven M. Glaza
Study Director
Acute Studies

2-28-97
Date

000254

Corning Hazleton Inc.
P.O. Box 7545
Madison, WI 53707-7545
Deliveries: 3301 Kinsman Blvd., Madison, WI 53704
608.241.4471 608.241.7227 Fax

CORNING Hazleton

Sponsor:

Martek Biosciences Corporation
Columbia, Maryland

FINAL REPORT

Study Title:

Acute Oral Toxicity Study of MK8841 Biomass in Rats

Author:

Steven M. Glaza

Study Completion Date:

October 24, 1995

Performing Laboratory:

Corning Hazleton Inc.
3301 Kinsman Boulevard
Madison, Wisconsin 53704

Laboratory Project Identification:

CHW 50803601

Reason for Route of Administration

Historically, the oral route has been the route of choice for administering a known amount of test material.

Observations

Clinical observations and mortality checks were conducted at approximately 1, 2.5, and 4 hours after test material administration. Additional clinical observations and twice a day mortality checks (morning and afternoon) were conducted daily thereafter for 14 days.

Body weights were determined before test material administration (Day 0), at Day 7, and at termination of the experimental phase (Day 14).

Pathology

At termination of the in-life phase, all animals were euthanized, subjected to an abbreviated gross necropsy examination, and any abnormalities were recorded. After necropsy, the animals were discarded and no tissues were saved.

Statistical Analyses

No statistical analyses were required by the protocol.

Location of Raw Data, Records, and Final Report

The raw data, records, and an original signed copy of the final report will be retained in the archives of CHW in accordance with CHW SOP.

RESULTS

Mortality

A summary of the survival rate is in Table 1. No mortality was observed during the study. The estimated oral LD_{50} for male and female rats was determined to be greater than 7,000 mg/kg of body weight.

Body Weights

Individual and mean body weights and body weight gains are in Table 2. All animals exhibited body weight gain throughout the study.

Clinical Signs

Individual clinical signs are in Table 3. All animals appeared normal throughout the study.

Pathology

Individual gross necropsy pathology findings are in Table 4. A summary report by the study pathologist is on Page 10. There were no lesions observed at necropsy.

DISCUSSION

The acute oral toxicity of MK8841 Biomass was evaluated in male and female rats when administered as a single gavage dose at a level of 7,000 mg/kg of body weight. The estimated oral LD_{50} for male and female rats was determined to be greater than 7,000 mg/kg. All animals appeared normal and exhibited body weight gain throughout the study. The gross necropsy at termination revealed no visible lesions.

SIGNATURE

Steven M. Glaza
Study Director
Acute Studies

10-24-95
Date

Corning Hazleton Inc.
P.O. Box 7545
Madison, WI 53707-7545
Deliveries: 3301 Kinsman Blvd., Madison, WI 53704
608.241.4471 608.241.7227 Fax

CORNING Hazleton

Sponsor:

Martek Biosciences Corporation
Columbia, Maryland

FINAL REPORT

Study Title:

Acute Oral Toxicity Study of MK8841 Biomass in Rats

Author:

Steven M. Glaza

Study Completion Date:

March 15, 1996

Performing Laboratory:

Corning Hazleton Inc.
3301 Kinsman Boulevard
Madison, Wisconsin 53704

Laboratory Project Identification:

CHW 60100881

Reason for Route of Administration

Historically, the oral route has been the route of choice for administering a known amount of test material.

Observations

Clinical observations were conducted at approximately 1, 2.5, and 4 hours after test material administration and daily thereafter for 14 days. Mortality checks were conducted twice a day (morning and afternoon) for 13 days after test material administration and again the morning of Day 14.

Body weights were determined before test material administration (Day 0), at Day 7, and at termination of the in-life phase (Day 14).

Pathology

At termination of the in-life phase, all animals were euthanized, subjected to an abbreviated gross necropsy examination, and any abnormalities were recorded. After necropsy, the animals were discarded and no tissues were saved.

Statistical Analyses

No statistical analyses were required by the protocol.

Location of Raw Data, Records, and Final Report

The raw data, records, and an original signed copy of the final report will be retained in the archives of CHW in accordance with CHW SOP.

RESULTS

Mortality

A summary of the survival rate is in Table 1. No mortality was observed during the study. The estimated oral LD_{50} for male and female rats was determined to be greater than 7,000 mg/kg of body weight.

Body Weights

Individual and mean body weights and body weight gains are in Table 2. All animals exhibited body weight gain throughout the study.

Clinical Signs

Individual clinical signs are in Table 3. All animals appeared normal throughout the study.

Pathology

Individual gross necropsy pathology findings are in Table 4. A summary report by the study pathologist is on Page 10. There were no lesions observed at necropsy.

DISCUSSION

The acute oral toxicity of MK8841 Biomass was evaluated in male and female rats when administered as a single gavage dose at a level of 7,000 mg/kg of body weight. The estimated oral LD_{50} for male and female rats was determined to be greater than 7,000 mg/kg. All animals appeared normal and exhibited body weight gain throughout the study. The gross necropsy at termination revealed no visible lesions.

SIGNATURE

3-15-96
Date

Steven M. Glaza
Study Director
Acute Studies

HAZLETON
WISCONSIN
3301 KINSMAN BOULEVARD
POST OFFICE BOX 7545
MADISON, WISCONSIN 53707

a CORNING Laboratory Services Company

Sponsor:

Martek Corporation
Columbia, Maryland

FINAL REPORT

Study Title:

Acute Oral Toxicity Study of CEBAC (Powder) in Rats

Author:

Steven M. Glaza

Study Completion Date:

December 18, 1990

Performing Laboratory:

Hazleton Wisconsin, Inc.
3301 Kinsman Boulevard
Madison, Wisconsin 53704

Laboratory Project Identification:

HWI 00900013

United States • Germany • Japan • France • United Kingdom
Phone 608-241-4471 Fax 608-241-7227 Telex 703956 HAZRAL MDS UD

SUMMARY OF RESULTS

Test Animal: Albino Rats - Crl:CD®BR
Source: Charles River Laboratories, Inc., Portage MI
Date Animals Received: 09/10/90

Temperature and Humidity of Animal Room: 65 to 78°F;
27 to 50% Relative Humidity

Start Date (In-life): 09/21/90 End Date (In-life): 10/05/90

Estimated Oral LD_{50} : Male - Greater than 10.00 g/kg of Body Weight
Female - Greater than 10.00 g/kg of Body Weight

Sex	Dose Level (g/kg)	Average Body Weight (g) Initial	Day 7	Terminal	Mortality (Number Dead/Number Dosed)
Male	10.00	224	317	359	0/5
Female	10.00	216	264	275	0/5

Clinical Signs
(Number of Animals Affected)

Observation	Hour 1.0	2.5	4.0	Day 1	2	3	4	5	6	7	8	9	10	11	12	13	14
Males																	
Appeared normal	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Females																	
Appeared normal	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

DISCUSSION

The acute oral toxicity of CEBAC (Powder) was evaluated in male and female albino rats when administered at a level of 10.00 g/kg of body weight. The estimated LD_{50} for males and females was determined to be greater than 10.00 g/kg. All animals appeared normal and exhibited body weight gain throughout the study. The gross necropsy examination of the animals revealed no test material-related lesions.

SIGNATURE

12-18-90

Steven M. Glaza
Study Director
Acute Toxicology

Date

REFERENCE

1. NIH Publication No. 86-23 (revised 1985).

Covance Laboratories Inc.
P.O. Box 7545
Madison, WI 53707-7545
Deliveries: 3301 Kinsman Blvd., Madison, WI 53704
Tel: 608/241-4471 Fax: 608/241-7227

COVANCE
THE DEVELOPMENT SERVICES COMPANY

Sponsor:

Martek Biosciences Corporation
Columbia, Maryland

FINAL REPORT

Study Title:

Acute Oral Toxicity Study of Delipidated Algae Biomass (Refined Biomeal) in Rats

Author:

Steven M. Glaza

Study Completion Date:

July 3, 1997

Performing Laboratory:

Covance Laboratories Inc.
3301 Kinsman Boulevard
Madison, Wisconsin 53704

Laboratory Project Identification:

Covance 70401788

Pathology

At termination of the in-life phase, all animals were euthanized, subjected to an abbreviated gross necropsy examination, and any abnormalities were recorded. After necropsy, the animals were discarded and no tissues were saved.

Statistical Analyses

No statistical analyses were required by the protocol.

Location of Raw Data, Records, and Final Report

The raw data, records, and an original signed copy of the final report will be retained in the archives of Covance in accordance with Covance SOP.

RESULTS

Mortality

A summary of the survival rate is in Table 1. No mortality was observed during the study. The estimated oral LD_{50} values for male and female rats were determined to be greater than 6,000 mg/kg of body weight.

Body Weights

Individual and mean body weights and body weight gains are in Table 2. All animals exhibited body weight gain throughout the study with the exception of one female which exhibited a weight loss of 12 g during the second week.

Clinical Signs

Individual clinical signs are in Table 3. All animals appeared normal throughout the study.

Pathology

Individual gross necropsy findings are in Table 4. A summary report by the study pathologist is on Page 12. There were no test material-related lesions observed at the gross necropsy examinations conducted at termination.

DISCUSSION

The acute oral toxicity of Delipidated Algae Biomass (Refined Biomeal) was evaluated in male and female rats when administered as a single gavage dose at a level of 6,000 mg/kg of body weight. No mortality was observed during the study. The estimated oral LD_{50} values for male and female rats were determined to be greater than 6,000 mg/kg. All animals appeared normal and exhibited body weight gain during the study with the exception of one female which exhibited a slight weight loss of 12 g during the second week. This weight loss is not considered to be meaningful. The gross necropsy examinations at termination did not reveal any test material-related lesions.

SIGNATURE

7-3-97

Date

Steven M. Glaza
Study Director
Acute Studies

000266

HAZLETON
WISCONSIN
3301 KINSMAN BOULEVARD
MADISON, WISCONSIN 53704

a CORNING Laboratory Services Company

Sponsor:

Martek Corporation
Columbia, Maryland

FINAL REPORT

Study Title:

Acute Oral Toxicity Study of ARASCO in Rats

Author:

Steven M. Glaza

Study Completion Date:

January 17, 1992

Performing Laboratory:

Hazleton Wisconsin, Inc.
3301 Kinsman Boulevard
Madison, Wisconsin 53704

Laboratory Project Identification:

HWI 11101042

United States • Germany • Japan • France • United Kingdom
Phone 608-241-4471 Fax 608-241-7227

HAZLETON
WISCONSIN
3301 KINSMAN BOULEVARD
MADISON, WISCONSIN 53704

SUMMARY OF RESULTS

Test Animal: Albino Rats - Crl:CD®BR
Source: Charles River Laboratories, Inc., Portage, MI
Date Animals Received: 11/18/91

Environmental Conditions: 21 to 25°C;
36 to 43% Relative Humidity

Start Date (In-life): 11/26/91 End Date (In-life): 12/10/91

Estimated Oral LD_{50} : Male - Greater than 20,000 mg/kg of Body Weight
Female - Greater than 20,000 mg/kg of Body Weight

Sex	Dose Level (g/kg)	Average Body Weights (g) Day 0	Day 7	Day 14	Mortality (Number Dead/Number Dosed)
Male	20,000	292	346	363	0/5
Female	20,000	236	275	290	0/5

Clinical Signs
(Number of Animals Affected)

Observation	Hour 1.0	2.5	4.0	Day 1	2	3	4	5	6	7	8	9	10	11	12	13	14
Males																	
Appeared normal	2	0	0	0	5	5	5	5	5	5	5	5	5	5	5	5	5
Soft stool - oily	3	5	5	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Oily hair coat	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0
Females																	
Appeared normal	4	2	0	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Soft stool - oily	1	3	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0

United States • Germany • Japan • France • United Kingdom
Phone 608-241-4471 Fax 608-241-7227

HAZLETON
WISCONSIN
3301 KINSMAN BOULEVARD
MADISON WISCONSIN 53704

DISCUSSION

The acute oral toxicity of ARASCO was evaluated in male and female albino rats when administered as a single gavage dose at a level of 20,000 mg/kg of body weight. The estimated oral LD_{50} for males and females was determined to be greater than 20,000 mg/kg. Clinical signs of toxicity included oily soft stool and oily hair coat. All animals exhibited body weight gain throughout the study. The gross necropsy at termination revealed no visible lesions.

SIGNATURE

Steven M. Glaza
Study Director
Acute Toxicology

1-17-92
Date

REFERENCE

1. NIH Publication No. 86-23 (revised 1985).

United States • Germany • Japan • France • United Kingdom
Phone 608 241-4471 Fax 608-241-7227

HAZLETON
WISCONSIN
POST OFFICE BOX 7545
MADISON WI 53707-7545

Sponsor:

Martek Corporation
Columbia, Maryland

FINAL REPORT

Study Title:

Acute Oral Toxicity Study of Hi-ARASCO in Rats (EPA Guidelines)

Author:

Steven M. Glaza

Study Completion Date:

August 27, 1993

Performing Laboratory:

Hazleton Wisconsin, Inc.
3301 Kinsman Boulevard
Madison, Wisconsin 53704

Laboratory Project Identification:

HWI 30601749

SUMMARY OF RESULTS

Test Animal: Albino Rats - Crl:CD®(BR)
Source: Charles River Laboratories, Inc., Portage, MI
Date Animals Received: 06/07 and 06/21/93

Environmental Conditions: 18 to 23°C;
55 to 84% Relative Humidity

Experimental Start Date: 07/01/93 Experimental Termination Date: 08/27/93

Estimated Oral LD_{50} : Male - Greater than 20,000 mg/kg of Body Weight
Female - Greater than 20,000 mg/kg of Body Weight

Individual Body Weights

Animal Number	Body Weight (g) Day 0	Day 7	Day 14
Males (20,000 mg/kg)			
C17496	245	316	354
C17497	252	342	391
C17498	250	327	381
C17499	252	323	366
C17500	251	329	390
Mean	250	327	376
Females (20,000 mg/kg)			
C16237	214	246	260
C16256	226	260	272
C17563	223	257	272
C16249	224	257	276
C17565	216	241	257
Mean	221	252	267

DISCUSSION

The acute oral toxicity of Hi-ARASCO was evaluated in male and female rats when administered as a single gavage dose at a level of 20,000 mg/kg of body weight. Based on the observed mortality, the estimated oral LD_{50} for male and female rats was determined to be greater than 20,000 mg/kg. Clinical signs of toxicity included watery/soft stool, red-stained face, and wet/yellow-stained urogenital areas. All animals returned to a normal appearance by Day 4. All animals exhibited body weight gain throughout the study. The gross necropsy of the animals revealed no test material-related lesions. Page 12 contains a pathology report by the report pathologist.

SIGNATURE

Steven M. Glaza
Study Director
Acute Toxicology

8-27-93
Date

REFERENCES

1. "Acute Oral Toxicity," *Health Effects Test Guidelines*, 40 CFR 798.1175 (1989).

2. NIH Publication No. 86-23 (revised 1985).

ovance Laboratories Inc.
O. Box 7545
adison, WI 53707-7545
eliveries: 3301 Kinsman Blvd., Madison, WI 53704
l: 608/241-4471 Fax: 608/241-7227

COVANCE.
THE DEVELOPMENT SERVICES COMPANY

Sponsor:

Martek Biosciences Corporation
Columbia, Maryland

FINAL REPORT

Study Title:

Acute Oral Toxicity Study of Fungal Biomass in Rats

Author:

Steven M. Glaza

Study Completion Date:

July 3, 1997

Performing Laboratory:

Covance Laboratories Inc.
3301 Kinsman Boulevard
Madison, Wisconsin 53704

Laboratory Project Identification:

Covance 70403367

Pathology

At termination of the in-life phase, all animals were euthanized, subjected to an abbreviated gross necropsy examination, and any abnormalities were recorded. After necropsy, the animals were discarded and no tissues were saved.

Statistical Analyses

No statistical analyses were required by the protocol.

Location of Raw Data, Records, and Final Report

The raw data, records, and an original signed copy of the final report will be retained in the archives of Covance in accordance with Covance SOP.

RESULTS

Mortality

A summary of the survival rate is in Table 1. No mortality was observed during the study. The estimated oral LD_{50} values for male and female rats were determined to be greater than 5,000 mg/kg of body weight.

Body Weights

Individual and mean body weights and body weight gains are in Table 2. All animals exhibited body weight gain throughout the study with the exception of two females which exhibited insignificant weight losses of 4-9 g during the second week.

Clinical Signs

Individual clinical signs are in Table 3. All animals appeared normal throughout the study with the exception of one female and four males which exhibited non-formed feces (soft stool) on the day of treatment. Three of the males also exhibited dark stained urogenital areas. All animals returned to normal appearance by Day 2 after treatment.

Pathology

Individual gross necropsy findings are in Table 4. A summary report by the study pathologist is on Page 12. There were no gross lesions observed at the necropsy examinations conducted at termination.

DISCUSSION

The acute oral toxicity of Fungal Biomass was evaluated in male and female rats when administered as a single gavage dose at a level of 5,000 mg/kg of body weight. No mortality was observed. The estimated oral LD_{50} values for male and female rats were determined to be greater than 5,000 mg/kg. All animals appeared normal throughout the study with the exception of one female and four males which exhibited non-formed feces (soft stool) on the day of treatment. Three of the males also exhibited dark stained urogenital areas. All animals returned to normal appearance by Day 2 after treatment. All animals exhibited body weight gain during the study with the exception of two females which had insignificant weight losses during the second week. The gross necropsy examinations at termination did not reveal any lesions

SIGNATURE

Steven M. Glaza
Study Director
Acute Studies

7-3-97
Date

000276

ovance Laboratories Inc.
O. Box 7545
ladison, WI 53707-7545
eliveries: 3301 Kinsman Blvd., Madison, WI 53704
el: 608/241-4471 Fax: 608/241-7227

COVANCE
THE DEVELOPMENT SERVICES COMPANY

Sponsor:

Martek Biosciences Corporation
Columbia, Maryland

FINAL REPORT

Study Title:

Acute Oral Toxicity Study of Delipidated Fungal Biomass (Refined Biomeal) in Rats

Author:

Steven M. Glaza

Study Completion Date:

July 3, 1997

Performing Laboratory:

Covance Laboratories Inc.
3301 Kinsman Boulevard
Madison, Wisconsin 53704

Laboratory Project Identification:

Covance 70401789

Body weights were determined before test material administration (Day 0), at Day 7, and at termination of the in-life phase (Day 14).

Pathology

At termination of the in-life phase, all animals were euthanized, subjected to an abbreviated gross necropsy examination, and any abnormalities were recorded. After necropsy, the animals were discarded and no tissues were saved.

Statistical Analyses

No statistical analyses were required by the protocol.

Location of Raw Data, Records, and Final Report

The raw data, records, and an original signed copy of the final report will be retained in the archives of Covance in accordance with Covance SOP.

RESULTS

Mortality

A summary of the survival rate is in Table 1. No mortality was observed during the study. The estimated oral LD_{50} values for male and female rats were determined to be greater than 4,000 mg/kg of body weight.

Body Weights

Individual and mean body weights and body weight gains are in Table 2. All animals exhibited body weight gain throughout the study with the exception of one female which exhibited an insignificant weight loss of 5 g during the second week.

Clinical Signs

Individual clinical signs are in Table 3. All animals appeared normal throughout the study with the exception of two males which exhibited soft stool on the day of treatment. In addition, two females exhibited general alopecia on Days 5 through 10. All animals returned to a normal appearance by Day 11 after treatment.

Pathology

Individual gross necropsy findings are in Table 4. A summary report by the study pathologist is on Page 13. At necropsy, the axillary and deep cervical lymph nodes in one male were observed to be enlarged. These were considered incidental findings and unrelated to the test material. There were no visible lesions in any of the remaining animals.

DISCUSSION

The acute oral toxicity of Delipidated Fungal Biomass (Refined Biomeal) was evaluated in male and female rats when administered as a single gavage dose at a level of 4,000 mg/kg of body weight. No mortality was observed during the study. The estimated oral LD_{50} values for male and female rats were determined to be greater than 4,000 mg/kg. All animals appeared normal throughout the study with the exception of two males which exhibited soft stool on the day of treatment. In addition, two females exhibited general alopecia on Days 5 through 10. All animals returned to a normal appearance by Day 11 after treatment. All animals exhibited body weight gain during the study with the exception of one female which exhibited an insignificant weight loss during the second week. The gross necropsy examinations at termination did not reveal any test material-related lesions.

orning Hazleton Inc.
O. Box 7545
Iadison, WI 53707-7545
eliveries: 3301 Kinsman Blvd., Madison, WI 53704
08.241.4471 608.241.7227 Fax

CORNING Hazleton

Sponsor:

Martek Biosciences Corporation
Columbia, Maryland

FINAL REPORT

Study Title:

Acute Oral Toxicity Study of MICROENCAPSULATED FORMULAID® in Rats

Author:

Steven M. Glaza

Study Completion Date:

March 14, 1997

Performing Laboratory:

Corning Hazleton Inc.
3301 Kinsman Boulevard
Madison, Wisconsin 53704

Laboratory Project Identification:

CHW 70101732

Pathology

At termination of the in-life phase, all animals were euthanized, subjected to an abbreviated gross necropsy examination, and any abnormalities were recorded. After necropsy, the animals were discarded and no tissues were saved.

Statistical Analyses

No statistical analyses were required by the protocol.

Location of Raw Data, Records, and Final Report

The raw data, records, and an original signed copy of the final report will be retained in the archives of CHW in accordance with CHW SOP.

RESULTS

Mortality

A summary of the survival rate is in Table 1. No mortality was observed during the study. The estimated oral LD_{50} values for male and female rats were determined to be greater than 5,000 mg/kg of body weight.

Body Weights

Individual and mean body weights and body weight gains are in Table 2. All animals exhibited body weight gain throughout the study with the exception of one female which exhibited a weight loss of 18 g during the second week of the study.

Clinical Signs

Individual clinical signs are in Table 3. All animals appeared normal throughout the study.

Pathology

Individual gross necropsy findings are in Table 4. A summary report by the study pathologist is on Page 12. There were no lesions observed at necropsy.

DISCUSSION

The acute oral toxicity of MICROENCAPSULATED FORMULAID® was evaluated in male and female rats when administered as a single gavage dose at a level of 5,000 mg/kg of body weight. The estimated oral LD_{50} values for male and female rats were determined to be greater than 5,000 mg/kg. All animals appeared normal throughout the study. All animals exhibited body weight gain during the study with the exception of one female which exhibited a weight loss of 18 g during the second week of the study. The gross necropsy examinations at termination did not reveal any lesions.

SIGNATURE

3-14-97

Date

Steven M. Glaza
Study Director
Acute Studies

000282

HAZLETON
WISCONSIN
POST OFFICE BOX 7545
MADISON WI 53707-7545

a CORNING Company

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Martek Corporation
Columbia, Maryland

FINAL REPORT

Study Title:

4-Week Oral Gavage Toxicity Study with ARASCO, DHASCO, and Formulaid in Rats

Author:

Kevin D. Williams, PhD, DABT

Study Completion Date:

June 29, 1994

Performing Laboratory:

Hazleton Wisconsin, Inc.
3301 Kinsman Boulevard
Madison, Wisconsin 53704

Laboratory Project Identification:

HWI 6539-100

Phone 608-241-4471 Fax 608-241-7227
EXPRESS-MAIL DELIVERY 3301 KINSMAN BLVD MADISON, WI 53704

ABSTRACT

The purpose of the study was to assess the short-term toxicity of oils with various relative amounts of very-long-chain, unsaturated fatty acids when administered orally to rats for at least 4 weeks.

Male and female Crl:CD®(SD)BR VAF/Plus® rats were assigned at random to nine groups [10/sex/group in Groups 1 (3,750 mg/kg High Oleic Sunflower Oil), 4 (2,500 mg/kg ARASCO), 7 (1,250 mg/kg DHASCO), and 9 (3,750 mg/kg Formulaid) and five/sex/group in Groups 2 (50 mg/kg ARASCO), 3 (1,000 mg/kg ARASCO), 5 (25 mg/kg DHASCO), 6 (500 mg/kg DHASCO), and 8 (1,500 mg/kg Formulaid)]. Each group received dose preparations containing the carrier, test materials, or a combination of both at a dose volume of 4.17 mL/kg.

Food and water were provided *ad libitum*. The animals were observed twice daily (a.m. and p.m.) for mortality and moribundity. At least once each week, each animal was removed from its cage and examined for abnormalities and signs of toxicity. Body weight and food consumption data were collected weekly for 4 weeks. Blood samples were collected for hematology and clinical chemistry tests from five animals/sex/group during Week 5. Blood samples were also collected from five animals/sex before initiation of treatment and during Weeks 2 and 5; serum was harvested and sent to the Sponsor for possible future analyses. During Week 5, the animals were anesthetized, weighed, exsanguinated, and necropsied. At necropsy, macroscopic observations were recorded, selected organs were weighed, and selected tissues were collected and preserved. The brain, heart, liver (representative sample), and right testis were collected from five animals/sex/group, frozen in liquid nitrogen, and stored in a freezer set to maintain -70°C ±10° until packed on dry ice and shipped to the Sponsor. Microscopic examinations were done on tissues from five animals/sex/group from Groups given 3,750 mg/kg HOSO (control), 2,500 mg/kg ARASCO, 1,250 mg/kg DHASCO, and 3,750 mg/kg Formulaid.

There were no test material-related antemortem observations noted. No significant differences in body weights, cumulative body weight gains, or food consumption were noted in any test material-treated group compared with the control group (Group 1).

Slight differences in total protein in females given 1,000 mg/kg ARASCO or 1,250 mg/kg DHASCO and albumin in females given 1,000 mg/kg ARASCO, 2,500 mg/kg ARASCO, or 1,250 mg/kg DHASCO when compared to the control group (Group 1) were sporadic and exhibited no dose relationship and were, therefore, not considered test material-related. Several animals, including control animals, exhibited abnormally high serum potassium concentrations. These results were considered spurious and likely resulted from an excessively deep plain of anesthesia before blood collection.

There were no test material-related changes for terminal body weights, absolute organ weights, organ-to-body weight percentages, or organ-to-brain weight ratios. The only significant organ weight finding was higher liver weights for males given 3,750 mg/kg Formulaid compared with those of the controls and with those of males given 1,250 mg/kg DHASCO.

Oral administration of ARASCO, DHASCO, or Formulaid to rats for at least 4 weeks had no apparent toxic effect on body weights, food consumption, or clinical or anatomical pathology results.

Final Report

4-Week Dietary Toxicity Study
with MK 8841 in Rats

PREPARED FOR:
Martek Biosciences Corporation

STUDY NUMBER:
CHW 6539-107

000286

COVANCE
THE DEVELOPMENT SERVICES COMPANY

ABSTRACT

The purpose of this study was to assess the toxicity of the test material, MK 8841, when fed to rats for at least 4 weeks.

Male and female Crl:CD®(SD)BR VAF/Plus® rats were assigned to four groups. Each group received diets containing the test material at dose levels of 0 (control group; received basal diet only), 1%, 2%, and 5% w/w. There were 10 animals/sex in the control and high-dose groups (Groups 1 and 4) and five animals/sex in the low- and mid-dose groups (Groups 2 and 3); five animals/sex in the control and high-dose groups were used for special tissue collection.

Food and water were provided *ad libitum*. The animals were observed twice daily (a.m. and p.m.) for mortality and moribundity. At least once each week, each animal was removed from its cage and examined for abnormalities and signs of toxicity. Body weights were recorded on the first day of treatment (Day 1) and weekly thereafter. Food consumption data were calculated on a weekly basis; freshly dished diets were offered every third or fourth day. Ophthalmic examinations were done for all animals before initiation of treatment and before the scheduled sacrifice for control and high-dose animals. Blood samples were collected from five animals/sex before initiation of treatment (Day -1) and from five animals/sex/group during Week 5. The samples were kept at room temperature and allowed to clot, then serum was harvested and stored in a freezer set to maintain -20°C ±10°. The serum samples were packed on dry ice and shipped to the Sponsor for possible future analysis. During Week 5, five animals/sex/group were bled for hematology and clinical chemistry tests. The animals were then anesthetized, weighed, exsanguinated, and necropsied. At necropsy, macroscopic observations were recorded, selected organs were weighed, and selected tissues were collected and preserved. Microscopic examinations were done on tissues collected from the control and high-dose animals. The brain, heart, liver (representative sample), skeletal muscle (representative sample), and omental fat from the remaining five animals/sex in the control and high-dose groups were collected, weighed, frozen in liquid nitrogen, and stored in a freezer set to maintain -70°C ±10°. Samples were packed on dry ice and shipped to the Sponsor for possible future analysis.

The test material (MK 8841), basal diet, and prepared diets were analyzed for concentrations of docosahexanoic acid [DHA (a major constituent of MK 8841)]. The results showed that the prepared diets were homogeneous, were mixed at the appropriate concentrations, and were stable under the conditions of use in this study.

All animals survived to the scheduled sacrifice on Day 32 (Week 5). There were no apparent adverse test material-related antemortem observations noted for any animal at the weekly examinations. There were no differences in body weights, body weight gains, or food consumption that were attributed to the test material. Dietary administration of MK 8841 was associated with mildly lower total cholesterol and high-density lipoprotein cholesterol for females receiving the 5% diet. There were no correlative anatomical pathology findings, and the differences for these parameters were not considered adverse. There were no test material-related changes in terminal body weights, absolute or relative organ weights, or macroscopic or microscopic findings.

Based on the results of this study, no adverse effects were noted in male and female Crl:CD®(SD)BR rats that were fed diets containing as much as 5% w/w MK 8841 for at least 4 weeks. The 5% w/w diet concentrations of MK 8841 contained approximately 0.4% DHA, a level that was eight to ten times that of the basal diet.

PHARMACO :: LSR

STUDY NO. 94-2352

SUBCHRONIC (3-MONTH) COMBINED NEUROTOXICITY AND TOXICITY STUDY OF ARASCO™ AND DHASCO™ IN THE RAT VIA ORAL GAVAGE ADMINISTRATION

Final Report

VOLUME I OF III

Submitted to: Martek Biosciences Corp.
6480 Dobbin Road
Columbia, MD 21045

Attn: Kim Boswell

Date: 17 August 1995

000289

PHARMACO LSR, TOXICOLOGY SERVICES WORLDWIDE • METTLERS ROAD • P.O. BOX 2360 • EAST MILLSTONE, NEW JERSEY 08875-2360 • U.S.A. • TEL: 908 873 2550 • FAX: 908 873 3992 AND EYE • SUFFOLK • IP23 7PX • ENGLAND • TEL: 011 44 1 379 644122 • FAX: 011 44 1 379 651165

STUDY NO. 94-2352

SUBCHRONIC (3-MONTH) COMBINED NEUROTOXICITY AND TOXICITY STUDY OF ARASCO™ AND DHASCO™ IN THE RAT VIA ORAL GAVAGE ADMINISTRATION

ABSTRACT

This study, conducted for Martek Biosciences Corp., was designed to assess the toxicity of ARASCO™ and DHASCO™ when administered orally, via oral gavage, to 160 Sprague-Dawley CD® rats (20/sex/group) at dose levels of 1.0 and 2.5 g/kg/day of ARASCO™ and 0.5 and 1.25 g/kg/day of DHASCO™ for a period of 90 days. Two groups of control animals (20/sex) received untreated standard laboratory diet or the vehicle at the same dose volume as administered to the treated animals.

Physical observations, ophthalmoscopic examinations, body weight, food consumption measurements and neurobehavioral assessments were performed on all animals pretest and at selected intervals during the treatment period. Clinical chemistry and hematology parameters were evaluated on all surviving animals at study termination.

After at least 90 days of treatment, all survivors were sacrificed, selected organs were weighed and organ/body weight and organ/brain weight ratios calculated. Complete macroscopic postmortem examinations and histopathological evaluation of selected tissues were conducted on all animals in Groups II (vehicle control), IV (high-dose ARASCO™) and VI (high-dose DHASCO™).

Mean serum alkaline phosphatase values for males and females receiving both doses of DHASCO™ were elevated, relative to control values, at study termination. No other changes in serum enzymes were seen and no morphologic changes were seen upon microscopic examination. These differences may represent metabolic changes associated with DHASCO™ administration but were not considered to be a toxic effect.

No effects were seen in mortality or in the physical observations, body weights and food consumption values, organ weights, neurobehavioral evaluations, terminal organ and body weights and macroscopic and microscopic evaluations.

Based on the absence of toxic effects in all treated groups the no observable adverse effect level (NOAEL) was 2.5 g/kg/day for ARASCO™ and 1.25 g/kg/day for DHASCO™ when administered via oral gavage to rats for 90 days under the conditions of this study .

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HAZLETON
WISCONSIN
POST OFFICE BOX 7545
MADISON, WI 53707 7545

a CORNING Company

Sponsor:

Martek Biosciences Corporation
Columbia, Maryland

FINAL REPORT

Study Title:

Developmental Toxicity Study with ARASCO and DHASCO in Rats

Author:

Susan M. Henwood, MS, DABT

Study Completion Date:

August 16, 1995

Performing Laboratory:

Hazleton Wisconsin, Inc.
3301 Kinsman Boulevard
Madison, Wisconsin 53704

Laboratory Project Identification:

HWI 6539-103

Phone 608-241-4471 Fax 608-241-7227
EXPRESS-MAIL DELIVERY 3301 KINSMAN BLVD MADISON, WI 53704

ABSTRACT

The purpose of this study was to assess the developmental toxicity, including teratogenic potential, of ARASCO and DHASCO when administered by oral gavage to pregnant rats during the period of organogenesis (Days 6 through 15 of gestation).

Mated female Crl:CD® (SD) BR VAF/Plus® rats were assigned at random to five groups of 25 animals/group. Four groups were administered the test material in the carrier (high oleic sunflower oil) as a single daily dose. Low- and high-dose concentrations of ARASCO and DHASCO were prepared at 1,000 and 2,500 and 500 and 1,250 mg/kg, respectively, in the appropriate concentration of high-oleic sunflower oil to achieve a total dose of 2,500 mg/kg. Doses were administered at a volume of 2.78 mL/kg of body weight on Days 6 through 15 of gestation. Animals in Group 1 received the carrier according to the same dosing regimen as the test material groups. The animals were observed twice daily for mortality and moribundity and for indications of toxic effects. Detailed clinical observations were done and body weights were recorded on Days 0 and 6 through 20 of gestation. Individual food consumption data were recorded during Days 0 to 6, 6 to 9, 9 to 12, 12 to 16, and 16 to 20 of gestation. Cesarean sections and necropsies were done on Day 20 of gestation, selected maternal tissues were collected and held for possible histological examination, and the fetuses were removed and examined externally. Fetuses from all groups were processed for soft tissue, and skeletal examinations, fetuses from the control and high dose groups were examined for external and soft tissue abnormalities.

All animals survived to Gestation Day 20. There were no test material-related clinical observations or effects on mean body weights, body weight changes, gravid uterine weights, and food consumption.

There were no test material-related necropsy findings for the maternal animals. There were no significant differences in mean preimplantation loss, postimplantation loss, percent live fetuses (male, female, and total), resorptions (early, late, and total) or sex ratio for any test material-treated groups. Fetal external, soft tissue, or skeletal abnormalities were present in control and treated groups in a nondose-related pattern and were not considered toxicologically adverse effects.

Administration of ARASCO and DHASCO to Crl:CD® (SD) BR VAF/Plus® rats during organogenesis of the conceptus did not produce any adverse developmental effects at any of the dose levels tested in this study.

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Wyeth Nutritionals International

Re: Notice of A Claim For Exemption From Premarket Approval; Fermentation Oils For Use In Infant Formula

000308

infant intakes). The estimated LD_{50} for males and females was determined to be greater than 20.0 g/kg, the highest dose tested. The gross necropsy at termination revealed no visible lesions (Ref. 97).

4-Week Oral Gavage Toxicity Study with ARASCO, DHASCO, and Formulaid (ARASCO and DHASCO) in Rats (Wyeth GTR# 26205; sponsored by Martek Biosciences)

ARASCO and DHASCO oils were administered by oral gavage to male and female rats for four weeks. The study groups were: (Group 1) 3,750 mg/kg High Oleic Sunflower Oil, (Group 2) 50 mg/kg ARASCO, (Group 3) 1,000 mg/kg ARASCO, (Group 4) 2,500 mg/kg ARASCO, (Group 5) 25 mg/kg DHASCO, (Group 6) 500 mg/kg DHASCO, (Group 7) 1,250 mg/kg DHASCO, (Group 8) 1,500 mg/kg "Formulaid" ("Formulaid" is a Martek trademark for a 2:1 • mixture of ARASCO:DHASCO), (Group 9) 3,750 mg/kg Formulaid. These levels of exposure range between approximately 0.6 to 28 times 50th percentile infant intakes.

At dose levels of up to 2,500 mg/kg ARASCO, or up to 1,250 mg/kg DHASCO, or up to 3,750 mg/kg Formulaid (the highest doses tested) there was no evidence of toxicity as based on antemortem observations, body weight, body weight gain, or clinical or anatomical pathology (Ref. 97).

Nine Week Oral (Diet) Safety Study in Rats (Wyeth GTR# 24227; conducted in Wyeth-Ayerst Research's safety testing laboratories)

ARASCO and DHASCO oils were administered orally in the diet to male and female rats (15/sex/group) continuously for nine consecutive weeks. The study groups were: (Group I; chow control) Certified Rodent Chow #5002 Meal, (Group II; synthetic diet control) Soybean Oil Basal Diet, (Group III) 2.9% ARASCO, 2.0% DHASCO, (Group IV) 23.2% ARASCO, 16.0% DHASCO. The diets of the animals in Groups II, III, and IV were standard synthetic diets. Percentages are expressed as a percent of the fat blend (50 grams per kg) used in standard synthetic diets. Total fat in the diets was kept constant.

No treatment-related changes in mortality, clinical observations or ophthalmology occurred. There were no biologically significant treatment related organ weight changes. The 23.2% ARASCO, 16.0% DHASCO level (the highest dose tested) was considered the no toxicologic effect level (NTEL). The levels of exposure in this study ranged between approximately 1.45 to 11.6 times 50th percentile infant intakes.

Subchronic (3-Month) Combined Neurotoxicity and Toxicity Study of ARASCO and DHASCO in the Rat Via Oral Gavage Administration (Wyeth GTR# 26206; sponsored by Martek Biosciences)

This study was conducted to assess the toxicity of ARASCO and DHASCO when administered separately via oral gavage to male and female rats (20/sex/group) at dose levels of 1.0 and 2.5 g/kg/day of ARASCO and 0.5 and 1.25 g/kg/day of DHASCO for 90 days. Two groups of control animals (20/sex) received untreated standard laboratory diet or the vehicle (high oleic sunflower oil) at the same dose volume as administered to the treated animals.

No significant differences were observed between groups in mortality or in physical observations, body weights and food consumption values, organ weights, neurobehavioral evaluations, terminal organ and body weights, or macroscopic and microscopic evaluations. Based on the absence of toxic effects in all treated groups, the no observable adverse effect level (NOAEL) was 2.5 g/kg/day for ARASCO and 1.25 g/kg/day for DHASCO when administered via oral gavage to rats for 90 days under the conditions of this study. The levels of exposure in this study ranged between approximately 9-28 times estimated infant 50th percentile infant intakes. The results of this study have been published (Ref. 108).

Developmental Toxicity Study with ARASCO and DHASCO in Rats (Wyeth GTR# 26207; sponsored by Martek Biosciences)

This study assessed the developmental toxicity, including teratogenic potential, of ARASCO and DHASCO when administered by oral gavage to pregnant rats during the period of organogenesis (Days 6 through 15 of gestation). Four groups were administered the test material in a carrier (high oleic sunflower oil) as a single daily dose.

Analysis of Dinoflagellate Extract (DHASCO - Oil and Spray-Dried Biomass) (Wyeth GTR# 26219; sponsored by Wyeth; conducted in Dr. Daniel Baden's laboratory)

In order to both confirm that the final processed product is free of toxins as well as to confirm that the production organism is incapable of producing such toxins, we have had both crude biomass as well as the processed product (RBD DHASCO) analyzed for known dinoflagellate toxins by standard procedures. A series of analyses were conducted on dinoflagellate *(Crypthecodinium cohnii)* extract (DHASCO oil and spray dried biomass). The analyses of the DHASCO oil and spray-dried biomass included an intraperitoneal mouse bioassay (non-specific for lethal substances), a radioimmunoassay (specific for brevetoxin and ciguatoxin structures), a synaptosome binding assay (specific for Site 5 Toxins, including brevetoxin and ciguatoxin structures), an HPLC analysis with ADAM derivatizing reagent (specific for okadaic acid and derivatives), a microtiter plate protein phosphatase inhibition assay (specific for okadaic acid and mycrocystin), a high performance liquid chromatography assay (for saxitoxin and derivatives), and an enzyme-linked immunoassay (RidaScreen assay for saxitoxin). In addition, capillary electrophoresis (specific for saxitoxin and derivatives, used as a check on the HPLC and ELISA assays) was conducted on the DHASCO spray-dried biomass. These analyses were generally conducted using published, peer-reviewed methods (Refs. 93, 94, 95, 101, 111, 114, 122, and 123), but were not conducted under GLP conditions.

The bioassay used (the intraperitoneal injection of mice) is an AOAC-recognized method and is a non-specific and sensitive test capable of detecting the presence of a wide variety of dinoflagellate toxins; the largest injected dose in this bioassay exceeded 10% of the animal's bodyweights. The analytical techniques used (HPLC, ELISA, radioimmunoassay, and capillary electrophoresis) are relatively specific for a given class of toxins. The biochemical assays (the phosphatase inhibition and synaptosome binding assays) are specific for given functional classes of dinoflagellate toxins, but should also be able to detect functionally-related molecules (i.e., those with related biochemical activities). All tests were negative (at the limits of detection) and the materials were considered non-toxic at the levels tested.

Appendix 6

APPENDIX 6

SPREADSHEET ANALYSIS FORMS FROM 13 TOXICOLOGICAL STUDIES ASSESSING CLINICAL CHEMISTRY AND HEMATOLOGY

1. Summary of clinical chemistry data in spreadsheets from 13 major toxicology studies (28-90 days duration)
2. Summary of hematoloty data in spreadsheets from 13 major toxicology studies (28-90 days duration)

Alkaline Phosphatase (Iu/L)

males

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Afo	GB90A	GB90Afo
0				134	134	146	93	206.4				134	134
0	xx	xx	xx	116	116	151	112	187.3	209	209	209	227	227
25	xx												
50		xx								216			
100								179.3	204				
200												222	
300										209			
500	xx				172								
600								170.3	217				
1000		xx		133						212		192	
1150							100						
1250	xx				166								
1500			xx							204			
1600						150							
2000									199				
2300								152.1					
2500		xx		122									
3000									187		209		
3750			xx										
4050							111						
4500						183					188		
4900												179	
8550							110						
9100													248
9250						150							

000314

Alkaline Phosphatase (Iu/L)

females

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Afo	GB90A	GB90Afo
0				59	59	94	55	146.8				130	130
0	xx	xx	xx	63	63	78	71	127.7	161	161	161	199	199
25	xx												
50		xx								167			
100								121.6	143				
200												200	
300										146			
500	xx				87								
600								141.9	152				
1000		xx		65						176		186	
1150							61						
1250	xx				81								
1500			xx							145			
1600						100							
2000									133				
2300								133.4					
2500		xx		61									
3000									151		139		
3750			xx										
4050							80						
4500						112					160		
4900												145	
8550							64						
9100													172
9250						102							

000315

Aspartate Aminotransferase (Iu/L)

males

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Afo	GB90A	GB90Afo
0				86	86	65	124	128.4				73	73
0	119	119	119	82	82	71	167	127.2	50	50	50	72	72
25	115												
50		113								50			
100								109.8	47				
200												70	
300										50			
500	100				89								
600								114.7	54				
1000		116		79						52		68	
1150							153						
1250	119				84								
1500			126							52			
1600						85							
2000									52				
2300								135.3					
2500		120		92									
3000									54		52		
3750			118										
4050							167						
4500						70					55		
4900												69	
8550							109						
9100													79
9250						79							

Aspartate Aminotransferase (Iu/L)

Females

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Afo	GB90A	GB90Afo
0				89	89	61	102	104.7				69	69
0	104	104	104	98	98	63	98	102.6	51	51	51	64	64
25	106												
50		96								54			
100								108.9	56				
200												61	
300										53			
500	101				83								
600								100.1	49				
1000		92		80						50		62	
1150							92						
1250	111				74								
1500			95							48			
1600						62							
2000									60				
2300								105.2					
2500		85		88									
3000									56		52		
3750			99										
4050							99						
4500						63					61		
4900												63	
8550							101						
9100													67
9250						72							

Alanine aminotransferase (Iu/L)

males

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Afo	GB90A	GB90Afo
0				45	45	27	53	56.7				30	30
0	32	32	32	36	36	25	134	41.5	30	30	30	33	33
25	30												
50		30								30			
100								39.8	28				
200												31	
300										31			
500	26				45								
600								39.5	33				
1000		26		39						34		28	
1150							94						
1250	31				39								
1500			29							39			
1600						31							
2000									30				
2300								46.9					
2500		34		42									
3000									34		31		
3750			35										
4050							69						
4500						29					32		
4900												29	
8550							34						
9100													31
9250						25							

000318

Alanine Aminotransferase (Iu/L)

Females

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Afo	GB90A	GB90Afo	
0				45	45	20	28	58.3				41	41	
0	29	29	29	46	46	18	34	45.1	31	31	31	30	30	
25	25													
50		26								36				
100								40.2	35					
200												28		
300										33				
500	28				40									
600								35.7	28					
1000		27		43						33		29		
1150							29							
1250	28				36									
1500			26							32				
1600						17								
2000									34					
2300								45.9						
2500		28		48										
3000									32		29			
3750			33											
4050							29							
4500						18					36			
4900												27		
8550							38							
9100													27	
9250						26								

000319

Gamma glutamyltransferase (Iu/L)

males

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Afo	GB90A	GB90Afo
0				0	0	0	0	xx				0	0
0	0	0	0	0	0	0	0	xx	0	0	0	0	0
25	0												
50		0								0			
100								xx	0				
200												0	
300										0			
500	0				0								
600								xx	0				
1000		0		0						0		0	
1150							0						
1250	0				0								
1500			0							0			
1600						0							
2000									0				
2300								xx					
2500		0		0									
3000									0		0		
3750			0										
4050							0						
4500						0					0		
4900												0	
8550							0						
9100													0
9250						1							

000320

Gamma Glutamyltransferase (Iu/L)

females

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Afo	GB90A	GB90Afo
0				0	0	0	0	xx				0	0
0	0	0	0	0	0	0	0	xx	0	0	0	0	0
25	0												
50		0								0			
100								xx	0				
200												0	
300										0			
500	0				1								
600								xx	0				
1000		0		0						0		0	
1150							0						
1250	0				1								
1500			0							0			
1600						1							
2000									0				
2300								xx					
2500		0		0									
3000									0		0		
3750			0										
4050							0						
4500						0					0		
4900												0.1	
8550							0						
9100													0
9250						0							

000321

Total Protein (g/dL)

males

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Afo	GB90A	GB90Afo
0				6.8	6.8	6.6	6.8	6.99				6.7	6.7
0	5.8	5.8	5.8	6.7	6.7	6.6	6.7	7.04	6.3	6.3	6.3	6.6	6.6
25	5.9												
50		5.9								6.4			
100								7.3	6.3				
200												6.4	
300										6.3			
500	5.9				6.5								
600								7.2	6.4				
1000		5.9		6.7						6.2		6.6	
1150							6.6						
1250	5.8				6.6								
1500			5.8							6.4			
1600						6.7							
2000									6.4				
2300								7.13					
2500		5.9		6.4									
3000									6.3		6.3		
3750			5.9										
4050							6.7						
4500						6.7					6.3		
4900												6.6	
8550							6.5						
9100													6.5
9250						6.1							

Total Protein (g/dL)

Females

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Afo	GB90A	GB90Afo
0				7.4	7.4	7.1	7.1	7.37				6.4	6.4
0	6.4	6.4	6.4	7.3	7.3	7.5	7.3	7.55	6.2	6.2	6.2	6.4	6.4
25	5.9												
50		5.9								6.2			
100								7.62	6.1				
200												6.4	
300										6.2			
500	6.1				6.9								
600								7.8	6.2				
1000		5.8		7						6.2		6.4	
1150							7.1						
1250	5.7				7.4								
1500			6.1							6.3			
1600						7							
2000									6.2				
2300								7.51					
2500		5.9		7									
3000									6.2		6.2		
3750			6										
4050							7						
4500						7.1					6.1		
4900												6.5	
8550							7						
9100													6.6
9250						7							

000323

Albumin (g/dL)

males

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Afo	GB90A	GB90Afo
0				4.3	4.3	4.4	4.6	3.7				3.5	3.5
0	4.3	4.3	4.3	4.4	4.4	4.5	4.6	3.75	3.5	3.5	3.5	3.6	3.6
25	4.3												
50		4.2								3.6			
100								3.9	3.6				
200												3.5	
300										3.6			
500	4.4				4.3								
600								3.75	3.6				
1000		4.3		4.3						3.5		3.5	
1150							4.4						
1250	4.3				4.4								
1500			4.3							3.7			
1600						4.4							
2000									3.6				
2300								3.71					
2500		4.3		4.1									
3000									3.5		3.6		
3750			4.3										
4050							4.2						
4500						4.5					3.6		
4900												3.5	
8550							3.9						
9100													3.6
9250						4							

0003324

Albumin (g/dL)

females

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Afo	GB90A	GB90Afo
0				5.3	5.3	5.1	5	4.16				3.6	3.6
0	4.7	4.7	4.7	5.1	5.1	5.4	5.4	4.34	3.6	3.6	3.6	3.7	3.7
25	4.4												
50		4.4								3.6			
100								4.34	3.6				
200												3.7	
300										3.6			
500	4.6				4.8								
600								4.52	3.6				
1000		4.2		4.8						3.7		3.7	
1150							5.1						
1250	4.3				5.2								
1500			4.5							3.7			
1600						5							
2000									3.6				
2300								4.26					
2500		4.3		4.9									
3000									3.5		3.6		
3750			4.5										
4050							5						
4500						5.2					3.6		
4900												3.7	
8550							4.8						
9100													3.9
9250						4.8							

000325

Globulin (g/dL)

Females

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Afo	GB90A	GB90Afo
0				2.4	2.4	2.2	2.2	3.29				xx	xx
0	1.5	1.5	1.5	2.3	2.3	2.2	2.1	3.29	xx	xx	xx	xx	xx
25	1.6												
50		1.6								xx			
100								3.4	xx				
200												xx	
300										xx			
500	1.5				2.2								
600								3.45	xx				
1000		1.6		2.5						xx		xx	
1150							2.2						
1250	1.5				2.2								
1500			1.5							xx			
1600						2.3							
2000									xx				
2300								3.42					
2500		1.6		2.3									
3000									xx		xx		
3750			1.6										
4050							2.5						
4500						2.2					xx		
4900												x	
8550							2.7						
9100													xx
9250						2.1							

000326

Globulin (g/dL)

Females

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Afo	GB90A	GB90Afo
0				2.2	2.2	2	2	3.21				xx	xx
0	1.7	1.7	1.7	2.2	2.2	2.1	1.9	3.21	xx	xx	xx	xx	xx
25	1.5												
50		1.6								xx			
100								3.28	xx				
200												xx	
300										xx			
500	1.5				2.1								
600								3.28	xx				
1000		1.6		2.1						xx		xx	
1150							2						
1250	1.4				2.2								
1500			1.5							xx			
1600						2							
2000									xx				
2300								3.25					
2500		1.6		2.2									
3000									xx		xx		
3750			1.4										
4050							2.1						
4500						1.9					xx		
4900												xx	
8550							2.2						
9100													xx
9250						2.2							

000327

A/G Ratio (albumin/globulin)

males

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Afo	GB90A	GB90Afo
0				1.8	1.8	2	2.1	1.14				1.13	1.13
0	xx	xx	xx	1.9	1.9	2	2.2	1.15	1.3	1.3	1.3	1.19	1.19
25	xx												
50		xx								1.28			
100								1.14	1.3				
200												1.17	
300										1.3			
500	xx				2								
600								1.11	1.29				
1000		xx		1.7						1.33		1.16	
1150							2						
1250	xx				2.1								
1500			xx							1.36			
1600						1.9							
2000									1.29				
2300								1.1					
2500		xx		1.8									
3000									1.28		1.3		
3750			xx										
4050							1.7						
4500						2					1.34		
4900												1.13	
8550							1.5						
9100													1.21
9250						1.9							

003028

A/G Ratio (albumin/globulin)

females

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Afo	GB90A	GB90Afo
0				2.5	2.5	2.5	2.5	1.31				1.33	1.33
0	xx	xx	xx	2.4	2.4	2.7	2.9	1.36	1.41	1.41	1.41	1.39	1.39
25	xx												
50		xx								1.37			
100								1.32	1.43				
200												1.38	
300										1.4			
500	xx				2.3								
600								1.39	1.39				
1000		xx		2.3						1.45		1.39	
1150							2.6						
1250	xx				2.5								
1500			xx							1.45			
1600						2.5							
2000									1.4				
2300								1.34					
2500		xx		2.3									
3000									1.35		1.38		
3750			xx										
4050							2.4						
4500						2.7					1.4		
4900												1.32	
8550							2.2						
9100													1.4
9250						2.1							

000329

Total Bilirubin (mg/dL)

males

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A**	GB28fo**	GB28Afo	GB90A**	GB90Afo**
0				0.1	0.1	0.1	0.2	0.19				1.6	1.6
0	0.1	0.1	0.1	0.2	0.2	0.1	0.2	0.26	1	1	1	2	2
25	0.1												
50		0.1								0.9			
100								0.23	0.9				
200												1.6	
300										0.7			
500	0.1				0.1								
600								0.24	0.9				
1000		0.1		0.1						0.8		1.7	
1150							0.2						
1250	0.1				0.1								
1500			0.1							0.8			
1600						0.1							
2000									0.9				
2300								0.23					
2500		0.1		0.1									
3000									0.9		0.8		
3750			0.1										
4050							0.2						
4500						0.1					0.7		
4900												1.4	
8550							0.2						
9100													1.6
9250						0.1							

**Units in GB studies for total bilirubin are umol/L

000330

Total Bilirubin (mg/dL)

Females

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A**	GB28fo**	GB28Afo	GB90A**	GB90Afo**
0				0.2	0.2	0.1	0.2	0.17				6.3	6.3
0	0.1	0.1	0.1	0.2	0.2	0.1	0.2	0.26	1	1	1	1.3	1.3
25	0												
50		0.1								0.6			
100								0.23	1.5				
200												2.3	
300										0.7			
500	0.1				0.1								
600								0.25	0.5				
1000		0		0.1						0.7		1.8	
1150							0.2						
1250	0.1				0.1								
1500			0							0.7			
1600						0.1							
2000									0.7				
2300								0.21					
2500		0		0.2									
3000									0.7		0.6		
3750			0										
4050							0.2						
4500						0.1					0.7		
4900												1.3	
8550							0.2						
9100													1.5
9250						0.1							

**Units for total bilirubin for GB studies are umol/L

Creatinine (mg/dL)

males

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A**	GB28fo**	GB28Afo	GB90A**	GB90Afo**
0				0.3	0.3	0.5	0.3	0.79				28	28
0	0.6	0.6	0.6	0.3	0.3	0.5	0.3	0.74	21	21	21	27	27
25	0.6												
50		0.6								23			
100								0.72	26				
200												29	
300										24			
500	0.6				0.3								
600								0.74	23				
1000		0.6		0.3						25		30	
1150							0.3						
1250	0.5				0.3								
1500			0.6							25			
1600						0.5							
2000									22				
2300								0.8					
2500		0.6		0.4									
3000									22		22		
3750			0.6										
4050							0.3						
4500						0.5					21		
4900												31	
8550							0.4						
9100													32
9250						0.5							

**Units for GB studies for creatinine are umol/L

000332

Creatinine (mg/dL)

Females

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A**	GB28fo**	GB28Afo	GB90A**	GB90Afo**
0				0.4	0.4	0.6	0.4	0.86				29	29
0	0.6	0.6	0.6	0.4	0.4	0.5	0.4	0.88	22	22	22	30	30
25	0.6												
50		0.6								21			
100								0.9	26				
200												33	
300										21			
500	0.6				0.4								
600								0.89	24				
1000		0.6		0.4						23		35	
1150							0.4						
1250	0.6				0.4								
1500			0.6							24			
1600						0.5							
2000									24				
2300								0.86					
2500		0.6		0.4									
3000									23		20		
3750			0.6										
4050							0.4						
4500						0.5					21		
4900												37	
8550							0.4						
9100													34
9250						0.6							

**Units for creatinine for GB studies are umol/L

000333

Total Cholesterol (mg/dL)

males

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A**	GB28fo**	GB28Afo	GB90A**	GB90Afo**
0				xx	xx	xx	xx	80.6				1.95	1.95
0	64	64	64	xx	xx	xx	xx	91.7	1.52	1.52	1.52	2.02	2.02
25	53												
50		70								1.66			
100								105.8	1.54				
200												2.1	
300										1.64			
500	57				xx								
600								95.4	1.68				
1000		65		xx						1.32		2.21	
1150							xx						
1250	50				xx								
1500			66							1.32			
1600						xx							
2000									1.53				
2300								80.8					
2500		60		xx									
3000									1.57		1.36		
3750			58										
4050							xx						
4500						xx					1.47		
4900												1.82	
8550							xx						
9100													1.37
9250						xx							

**Units for GB studies for cholesterol are mmol/L

000334

Total Cholesterol (mg/dL)

Females

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A**	GB28fo**	GB28Afo	GB90A**	GB90Afo**
0				xx	xx	xx	xx	68.5				1.77	1.77
0	58	58	58	xx	xx	xx	xx	97.3	1.63	1.63	1.63	1.76	1.76
25	70												
50		65								1.64			
100								98.1	1.58				
200												1.68	
300										1.54			
500	65				xx								
600								98.6	1.45				
1000		53		xx						1.43		1.87	
1150							xx						
1250	51				xx								
1500			62							1.36			
1600						xx							
2000									1.53				
2300								99					
2500		52		xx									
3000									1.73		1.37		
3750			56										
4050							xx						
4500						xx					1.43		
4900												1.48	
8550							xx						
9100													1.39
9250						xx							

**Units for cholesterol for GB studies are mmol/L

000335

Triglycerides (mg/dL)

males

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A**	GB28fo**	GB28Afo	GB90A**	GB90Afo**
0				xx	xx	xx	xx	128.5				2.73	2.73
0	31	31	31	xx	xx	xx	xx	137.2	0.96	0.96	0.96	1.98	1.98
25	40												
50		43								1.22			
100								134.7	0.72				
200												1.51	
300										0.89			
500	46				xx								
600								102.6	1.01				
1000		44		xx						0.87		1.58	
1150							xx						
1250	29				xx								
1500			41							0.74			
1600						xx							
2000									0.62				
2300								108.3					
2500		40		xx									
3000									0.62		0.54		
3750			76										
4050							xx						
4500						xx					0.67		
4900												1	
8550							xx						
9100													1
9250						xx							

**Units for GB study for Triglycerides are mmol/L

000336

Triglycerides (mg/dL)

Females

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A**	GB28fo**	GB28Afo	GB90A**	GB90Afo**
0				xx	xx	xx	xx	115.4				1.42	1.42
0	38	38	38	xx	xx	xx	xx	111	0.49	0.49	0.49	0.8	0.8
25	31												
50		29								0.47			
100								89.4	0.49				
200												0.85	
300										0.55			
500	26				xx								
600								88.9	0.51				
1000		25		xx						0.43		0.89	
1150							xx						
1250	23				xx								
1500			34							0.43			
1600						xx							
2000									0.45				
2300								55.4					
2500		24		xx									
3000									0.41		0.4		
3750			38										
4050							xx						
4500						xx					0.43		
4900												0.72	
8550							xx						
9100													0.58
9250						xx							

**Units for triglycerides for GB studies are mmol/L

000337

HDL Cholesterol (mg/dL)

males

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Afo	GB90A	GB90Afo
0				xx	xx	xx	xx	57.11				xx	xx
0	50	50	50	xx	xx	xx	xx	63.5	xx	xx	xx	xx	xx
25	37												
50		53								xx			
100								71.1	xx				
200												xx	
300										xx			
500	42				xx								
600								67.5	xx				
1000		46		xx						xx		xx	
1150							xx						
1250	37				xx								
1500			49							xx			
1600						xx							
2000									xx				
2300								61.33					
2500		44		xx									
3000									xx		xx		
3750			42										
4050							xx						
4500						xx					xx		
4900												xx	
8550							xx						
9100													xx
9250						xx							

000338

HDL Cholesterol (mg/dL)

Females

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Afo	GB90A	GB90Afo
0				xx	xx	xx	xx	50.44				xx	xx
0	49	49	49	xx	xx	xx	xx	67.4	xx	xx	xx	xx	xx
25	57												
50		54								xx			
100								73.7	xx				
200												xx	
300										xx			
500	53				xx								
600								66.4	xx				
1000		43		xx						xx		xx	
1150							xx						
1250	39				xx								
1500			50							xx			
1600						xx							
2000									xx				
2300								82.3					
2500		42		xx									
3000									xx		xx		
3750			45										
4050							xx						
4500						xx					xx		
4900												xx	
8550							xx						
9100													xx
9250						xx							

000339

LDL Cholesterol (mg/dL)

males

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Afo	GB90A	GB90Afo
0				xx	xx	xx	xx	26.78				xx	xx
0	8	8	8	xx	xx	xx	xx	28.2	xx	xx	xx	xx	xx
25	8												
50		8								xx			
100								34.7	xx				
200												xx	
300										xx			
500	8				xx								
600								27.9	xx				
1000		10		xx						xx		xx	
1150							xx						
1250	8				xx								
1500			9							xx			
1600						xx							
2000									xx				
2300								20.33					
2500		8		xx									
3000									xx		xx		
3750			3										
4050							xx						
4500						xx					xx		
4900												xx	
8550							xx						
9100													xx
9250						xx							

000340

LDL Cholesterol (mg/dL)

Females

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Afo	GB90A	GB90Afo
0				xx	xx	xx	xx	17.11				xx	xx
0	3	3	3	xx	xx	xx	xx	29.9	xx	xx	xx	xx	xx
25	6												
50		6								xx			
100								24.4	xx				
200												xx	
300										xx			
500	6				xx								
600								32.2	xx				
1000		5		xx						xx		xx	
1150							xx						
1250	7				xx								
1500			5							xx			
1600						xx							
2000									xx				
2300								16.7					
2500		5		xx									
3000									xx		xx		
3750			4										
4050							xx						
4500						xx					xx		
4900												xx	
8550							xx						
9100													xx
9250						xx							

000341

Prothrombin Time (sec)

males

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Afo	GB90A	GB90Afo
0				10.4	10.4	10.2	9.8	14.37				41.8	41.8
0	16.8	16.8	16.8	10.2	10.2	10.3	9.6	14.42	39.1	39.1	39.1	39.3	39.3
25	16.6												
50		16.1								39.7			
100								14.69	39.8				
200												38.8	
300										39.2			
500	16.9				10.4								
600								14.57	41				
1000		16.5		10.5						40.4		39.3	
1150							9.8						
1250	17.6				10.4								
1500			17.5							40.2			
1600						10.3							
2000									38.4				
2300								15.42					
2500		16.3		11.1									
3000									40.2		39		
3750			17.6										
4050							10.2						
4500						10.4					38.2		
4900												39.8	
8550							10.2						
9100													39.8
9250						10.8							

Prothrombin Time (sec)

Females

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Afo	GB90A	GB90Afo
0				9.4	9.4	9.5	9.6	14.31				37.2	37.2
0	15.6	15.6	15.6	9.4	9.4	9.6	9.4	13.33	37.5	37.5	37.5	34.9	34.9
25	16.5												
50		16.4								37.2			
100								13.66	39.4				
200												36	
300										37.4			
500	16				9.7								
600								13.41	36				
1000		16.6		9.4						37.8		34.6	
1150							9.5						
1250	16.5				9.9								
1500			16.8							37.6			
1600						10							
2000									42.2				
2300								14.46					
2500		16.3		9.6									
3000									36.8		38.1		
3750			16										
4050							9.6						
4500						10					37.8		
4900												34.7	
8550							9.5						
9100													34.7
9250						10.3							

000343

males

Activated Partial Thromboplastin Time (sec)

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Afo	GB90A	GB90Afo
0				29	29	21.1	23.7	17.09				xx	xx
0	xx	xx	xx	29	29	23.2	23.8	15.18	xx	xx	xx	xx	xx
25	xx												
50		xx								xx			
100								15.96	xx				
200												xx	
300										xx			
500	xx				25.5								
600								15.04	xx				
1000		xx		30.1						xx		xx	
1150							20.8						
1250	xx				24.9								
1500			xx							xx			
1600						18.9							
2000									xx				
2300								15.11					
2500		xx		27.7									
3000									xx		xx		
3750			xx										
4050							21.1						
4500						19.9					xx		
4900												xx	
8550							19.7						
9100													xx
9250						20.1							

000344

Activated Partial Thromboplastin Time (sec)

Females

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Afo	GB90A	GB90Afo
0				22.2	22.2	19.2	18.6	14.23				xx	xx
0	xx	xx	xx	22	22	20.5	21.7	14.31	xx	xx	xx	xx	xx
25	xx												
50		xx								xx			
100								12.96	xx				
200												xx	
300										xx			
500	xx				20.2								
600								14.43	xx				
1000		xx		19.6						xx		xx	
1150							20.3						
1250	xx				20.9								
1500			xx							xx			
1600						21.3							
2000									xx				
2300								13.91					
2500		xx		22.4									
3000									xx		xx		
3750			xx										
4050							18.3						
4500						20.3					xx		
4900												xx	
8550							17.6						
9100													xx
9250						19.1							

000345

Phosphate (mg/dL)

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A**	GB28fo**	GB28Afo	GB90A**	GB90Afo**
0				8.4	8.4	8.8	7.9	6.35				1.78	1.78
0	10.8	10.8	10.8	8.5	8.5	10.2	7.7	5.92	1.95	1.95	1.95	1.63	1.63
25	10.2												
50		10.8								1.97			
100								6.18	2.08				
200												1.72	
300										1.99			
500	10.2				9.1								
600								6.32	2				
1000		10.8		8.3						1.97		1.61	
1150							7.4						
1250	10.2				9								
1500			10.7							1.93			
1600						10.6							
2000									1.94				
2300								6.35					
2500		11.8		8.9									
3000									1.93		2		
3750			10										
4050							7.6						
4500						10.5					2.12		
4900												1.68	
8550							8.2						
9100													1.83
9250						9.6							

**Units for Phos in GB studies are mmol/L

Phosphat (mg/dL)

Females

Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A**	GB28fo**	GB28Afo	GB90A**	GB90Afo**
0				7.9	7.9	8.9	7.5	5				1.68	1.68
0	10.1	10.1	10.1	7.3	7.3	8.5	7	5.52	1.97	1.97	1.97	1.3	1.3
25	9.6												
50		9.7								1.55			
100								5.04	1.69				
200												1.39	
300										1.91			
500	10.1				8.1								
600								5.45	1.79				
1000		9.8		7.7						1.75		1.41	
1150							7						
1250	9.2				8								
1500			10							1.88			
1600						9.2							
2000									1.8				
2300								5.77					
2500		9.2		7.8									
3000									1.62		1.91		
3750			8.5										
4050							8.4						
4500						8.6					1.87		
4900												1.33	
8550							8.8						
9100													1.34
9250						9.3							
	**Units for Phos in GB studies are mmol/L												

White Blood Cells

Male

WBC	E3/uL							Th/cmm	10E9/L			10E9/L	
Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Af	GB90A	GB90Af
0				12.45	12.45	13.80	13.37	11.07				9.30	9.30
0	10.10	10.10	10.10	12.60	12.60	14.22	14.13	10.64	9.60	9.60	9.60	9.60	9.60
25	9.90												
50		10.10								9.50			
100								12.12	9.21				
200												8.70	
300										10.30			
500	11.20				13.32								
600								11.68	8.70				
1000		10.00		13.44						9.20		9.70	
1150							14.30						
1250	9.90				12.03								
1500			12.40							11.00			
1600						15.10							
2000									10.70				
2300								13.27					
2500		11.00		14.36									
3000									9.10		10.20		
3750			8.70										
4050							17.85						
4500						14.99					9.80		
4900												9.50	
8550							17.16						
9100													8.70
9250						12.98							

Female

WBC	E3/uL								10E9/L				
Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Af	GB90A	GB90Af
0				7.75	7.75	8.27	7.97	7.56				6.20	6.20
0	6.50	6.50	6.50	7.97	7.97	9.39	9.21	7.29	7.90	7.90	7.90	5.90	5.90
25	6.80												
50		7.20								5.80			
100								7.68	8.90				
200												5.90	
300										8.50			
500	8.10				7.38								
600								6.91	8.70				
1000		8.00		7.99						8.60		7.00	
1150							9.34						
1250	6.90				8.47								
1500			8.00							8.10			
1600						9.39							
2000									7.90				
2300								10.64					
2500		6.60		9.52									
3000									9.20		7.80		
3750			8.30										
4050							10.08						
4500						8.12					8.40		
4900												7.30	
8550							12.76						
9100													6.80
9250						13.43							

Red Blood Cells

Male

RBC	E6/uL							m/cmm	10E12/L				
Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Afc	GB90A	GB90Afc
0				8.69	8.69	8.00	8.35	8.56				7.96	7.96
0	7.40	7.40	7.40	8.67	8.67	7.88	8.60	8.45	7.39	7.39	7.39	7.86	7.86
25	7.58												
50		7.78								7.49			
100								8.43	7.36				
200												7.88	
300										7.45			
500	7.38				8.60								
600								8.61	7.20				
1000		7.50		8.53						7.23		8.00	
1150							8.36						
1250	7.64				8.48								
1500			7.78							7.26			
1600						8.16							
2000									7.31				
2300								8.19					
2500		7.96		8.69									
3000									7.04		7.42		
3750			7.20										
4050							8.29						
4500						8.41					7.28		
4900												7.71	
8550							8.24						
9100													7.67
9250						7.69							

Female

RBC	E6/uL								10E12/L				
Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Afc	GB90A	GB90Afc
0				8.12	8.12	7.92	8.41	7.79				7.16	7.16
0	7.52	7.52	7.52	7.77	7.77	7.80	8.69	8.09	7.31	7.31	7.31	7.16	7.16
25	7.60												
50		7.35								7.31			
100								7.96	7.30				
200												7.16	
300										7.11			
500	7.38				7.97								
600								7.92	6.98				
1000		7.48		7.90						7.21		7.22	
1150							8.26						
1250	7.20				7.81								
1500			7.46							7.34			
1600						7.70							
2000									7.43				
2300								7.53					
2500		7.20		8.07									
3000									7.50		7.24		
3750			7.20										
4050							8.36						
4500						7.78					7.33		
4900												6.87	
8550							8.27						
9100													7.04
9250						7.62							

Hemoglobin B (HGB)

Male

HGB	G/DL								mmol/L			mmol/L	
Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Af	GB90A	GB90Af
0				15.20	15.20	15.20	15.20	15.76				9.40	9.40
0	15.00	15.00	15.00	15.40	15.40	15.20	15.30	15.90	9.20	9.20	9.20	9.20	9.20
25	15.00												
50		15.10								9.50			
100								15.98	9.20				
200												9.00	
300										9.30			
500	15.10				14.90								
600								15.61	9.10				
1000		14.90		15.00						9.10		9.20	
1150							15.00						
1250	15.10				15.10								
1500			15.30							9.50			
1600						15.10							
2000									9.20				
2300								15.64					
2500		15.60		14.90									
3000									8.90		9.30		
3750			14.50										
4050							14.20						
4500						15.80					9.30		
4900												9.00	
8550							14.10						
9100													8.90
9250						14.50							

Female

HGB	g/dL								mmol/L				
Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Af	GB90A	GB90Af
0				15.10	15.10	15.10	15.40	15.06				9.00	9.00
0	15.10	15.10	15.10	14.80	14.80	15.20	15.90	15.62	9.20	9.20	9.20	8.70	8.70
25	15.30												
50		14.70								9.30			
100								15.39	9.30				
200												8.70	
300										9.10			
500	15.10				15.00								
600								15.79	8.90				
1000		14.90		14.90						9.20		8.90	
1150							15.20						
1250	14.40				14.90								
1500			15.50							9.40			
1600						14.90							
2000									9.30				
2300								15.51					
2500		14.90		15.00									
3000									9.50		9.20		
3750			14.90										
4050							15.30						
4500						15.00					9.40		
4900												8.50	
8550							15.20						
9100													8.50
9250						15.00							

Heamatocrit

Male

HCT	%												
Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Af	GB90A	GB90Af
0				45.30	45.30	44.00	45.90	37.52				xx	xx
0	43.30	43.30	43.30	46.30	46.30	44.10	45.80	37.43	xx	xx	xx	xx	xx
25	44.80												
50		44.60								xx			
100								36.86	xx				
200												xx	
300										xx			
500	44.00				44.90								
600								35.85	xx				
1000		43.80		44.80						xx		xx	
1150							45.50						
1250	44.90				45.40								
1500			44.90							xx			
1600						43.80							
2000									xx				
2300								35.97					
2500		45.40		44.20									
3000									xx		xx		
3750			41.80										
4050							42.70						
4500						45.70					xx		
4900												xx	
8550							42.00						
9100													xx
9250						42.60							

Female

HCT	%												
Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Af	GB90A	GB90Af
0				45.00	45.00	42.80	45.80	36.39				xx	xx
0	43.50	43.50	43.50	43.70	43.70	43.40	47.50	36.12	xx	xx	xx	xx	xx
25	44.30												
50		42.10								xx			
100								35.64	xx				
200												xx	
300										xx			
500	43.30				44.70								
600								36.73	xx				
1000		42.90		43.60						xx		xx	
1150							45.10						
1250	41.50				44.50								
1500			44.00							xx			
1600						42.20							
2000									xx				
2300								35.50					
2500		41.50		44.30									
3000									xx		xx		
3750			41.80										
4050							44.60						
4500						42.40					xx		
4900												xx	
8550							44.50						
9100													xx
9250						41.80							

Mean Corpuscular Volume (MCV)

Male

MCV	fL							cu/mcr	fL				
Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Af	GB90A	GB90Af
0				52.2	52.2	55.0	54.9	43.9				52.9	52.9
0	59.0	59.0	59.0	53.4	53.4	56.0	53.3	44.2	56.9	56.9	56.9	52.9	52.9
25	59.0												
50		57.0								57.3			
100								43.7	57.0				
200												51.1	
300										56.4			
500	60.0				52.2								
600								41.8	57.2				
1000		58.0		52.5						58.5		51.0	
1150							54.5						
1250	59.0				53.6								
1500			58.0							56.3			
1600						53.8							
2000									56.8				
2300								44.0					
2500		57.0		50.9									
3000									56.5		55.5		
3750			58.0										
4050							51.6						
4500						54.4					56.5		
4900												51.4	
8550							50.9						
9100													51.4
9250						55.3							

Female

MCV	fL												
Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Af	GB90A	GB90Af
0				55.3	55.3	54.1	54.5	46.7				56.2	56.2
0	58.0	58.0	58.0	56.3	56.3	55.7	54.7	44.7	56.0	56.0	56.0	54.3	54.3
25	58.0												
50		57.0								55.6			
100								44.8	56.7				
200												54.2	
300										56.8			
500	59.0				56.1								
600								46.7	56.9				
1000		57.0		55.2						57.2		54.9	
1150							54.6						
1250	58.0				57.0								
1500			59.0							57.3			
1600						54.9							
2000									54.5				
2300								47.2					
2500		58.0		54.9									
3000									54.2		54.9		
3750			58.0										
4050							53.4						
4500						54.5					55.1		
4900												54.8	
8550							53.8						
9100													52.9
9250						54.8							

Mean Corpuscular Heamoglobin (MCH)

Male

MCH	PG								fmol			fmol	
Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Af	GB90A	GB90Af
0				17.40	17.40	19.00	18.20	18.43				1.17	1.17
0	20.30	20.30	20.30	17.80	17.80	19.30	17.80	18.82	1.24	1.24	1.24	1.17	1.17
25	19.90												
50		19.40								1.26			
100								18.98	1.25				
200												1.14	
300										1.25			
500	20.50				17.30								
600								18.25	1.27				
1000		19.80		17.60						1.26		1.15	
1150							17.90						
1250	19.80				17.80								
1500			19.60							1.24			
1600						18.60							
2000									1.26				
2300								19.17					
2500		19.60		17.20									
3000									1.27		1.25		
3750			20.10										
4050							17.10						
4500						18.70					1.28		
4900												1.17	
8550							17.10						
9100													1.17
9250						18.80							

Female

MCH	PG								fmol				
Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Af	GB90A	GB90Af
0				18.60	18.60	19.00	18.20	19.35				1.26	1.26
0	20.10	20.10	20.10	19.00	19.00	19.50	18.40	19.34	1.26	1.26	1.26	1.21	1.21
25	20.10												
50		20.00								1.27			
100								19.33	1.27				
200												1.22	
300										1.28			
500	20.40				18.90								
600								19.98	1.27				
1000		20.00		18.90						1.28		1.23	
1150							18.40						
1250	20.00				19.10								
1500			20.80							1.28			
1600						19.30							
2000									1.25				
2300								20.62					
2500		20.70		18.70									
3000									1.27		1.28		
3750			20.70										
4050							18.30						
4500						19.30					1.28		
4900												1.24	
8550							18.40						
9100													1.22
9250						19.70							

Mean Corpuscular Heamatocrit (MCHC)

Male

MCHC	%			g/dL					mmol/L			mmol/L	
Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Af	GB90A	GB90Af
0				33.50	33.50	34.60	33.20	42.03				22.20	22.20
0	34.60	34.60	34.60	33.20	33.20	34.40	33.40	42.52	21.90	21.90	21.90	22.10	22.10
25	33.60												
50		33.90								22.10			
100								43.38	22.00				
200												22.40	
300										22.20			
500	34.40				33.00								
600								43.51	22.10				
1000		34.00		33.50						21.60		22.50	
1150							32.90						
1250	33.70				33.30								
1500			34.00							22.10			
1600						34.50							
2000									22.10				
2300								43.49					
2500		34.40		33.70									
3000									22.50		22.50		
3750			34.60										
4050							33.10						
4500						34.50					22.60		
4900												22.70	
8550							33.50						
9100													22.70
9250						33.90							

Female

MCHC	%			g/dL					mmol/L				
Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Af	GB90A	GB90Af
0				33.70	33.70	35.20	33.50	41.40				22.40	22.40
0	34.80	34.80	34.80	33.80	33.80	35.00	33.50	43.28	22.60	22.60	22.60	22.40	22.40
25	34.40												
50		35.00								22.80			
100								43.16	22.40				
200												22.50	
300										22.60			
500	34.80				33.70								
600								43.03	22.40				
1000		34.90		34.20						22.40		22.50	
1150							33.60						
1250	34.60				33.60								
1500			35.40							22.30			
1600						35.20							
2000									23.00				
2300								43.69					
2500		35.90		33.90									
3000									23.40		23.20		
3750			35.70										
4050							34.20						
4500						35.40					23.30		
4900												22.60	
8550							34.10						
9100													23.00
9250						36.00							

(PLT)

Male

PLT	E3/uL							Th/cmm					
Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Af	GB90A	GB90Af
0				902	902	710	652	654.9				xx	xx
0	1104	1104	1104	813	813	676	661	574.1	xx	xx	xx	xx	xx
25	1000												
50		1046								xx			
100								590.1	xx				
200												xx	
300										xx			
500	994				796								
600								697	xx				
1000		1076		711						xx		xx	
1150							622						
1250	1146				831								
1500			1234							xx			
1600						801							
2000									xx				
2300								595.6					
2500		1144		733									
3000									xx		xx		
3750			1088										
4050							720						
4500						638					xx		
4900												xx	
8550							741						
9100													xx
9250						504							

Female

PLT	E3/uL												
Dose	MK28D	MK28A	MK28F	MK90A	MK90D	MJ28F	MJ90F	W90F	GB28A	GB28fo	GB28Af	GB90A	GB90Af
0				843	843	628	647	545.5				xx	xx
0	962	962	962	744	744	814	601	555.1	xx	xx	xx	xx	xx
25	982												
50		975								xx			
100								592.5	xx				
200												xx	
300										xx			
500	1058				832								
600								563.3	xx				
1000		906		704						xx		xx	
1150							553						
1250	1000				795								
1500			1032							xx			
1600						813							
2000									xx				
2300								611.2					
2500		856		761									
3000									xx		xx		
3750			868										
4050							611						
4500						768					xx		
4900												xx	
8550							606						
9100													xx
9250						539							

SUBMISSION END