GR

ORIGINAL SUBMISSION

000002

1800 M Street, N.W.
Washington, D.C. 20036-5869
202-467-7000
Fax: 202-467-7176

Morgan, Lewis & Bockius LLP

2000 AUG 11 P 2:54

Sandra J.P. Dennis
(202)467-7009
sdennis@morganlewis.com

August 11, 2000

VIA HAND DELIVERY

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

Re: GRAS Notification

Dear Madam or Sir:

Enclosed, in triplicate, is a notification by Kao Corporation that diacylglycerol oil ("DAG") is generally recognized as safe (GRAS). Please note that materials marked "Confidential/Trade Secret Information" are deemed by Kao Corporation to be trade secret or confidential commercial information that is exempt from public disclosure pursuant to 21 C.F.R. §20.61. Also, appendices and references are not attached, but are available for your review upon request.

If you have any questions, please contact me at (202) 467-7009.

Sincerely,

Sandra J.P. Dennis

SJPD/dgl

1-WA:1458360.1

000002.001

August 10, 2000

GRAS STATUS OF DAG

Kao Corporation

TABLE OF CONTENTS

		Page
I.	GRAS Exemption Claim	1
	A. Claim of Exemption From the Requirement for Premarket Approval	2
	B. Name and Address of Notifier	2
	C. Name of GRAS Substance	3
	D. Conditions of Use	3
	E. Basis for GRAS Determination	3
	1. Description	4
	2. Summary of Safety Data	7
	3. Estimated Consumption	8
	4. General Recognition of Safety	9
	F. Availability of Information	10
II.	Identity and Specifications	11
	A. Chemical Identity	11
	B. Chemical Abstract Service (CAS) Registry Number	11
	C. Empirical formula	11
	D. Structural formula	12
	E. Molecular Weight	13
	F. Specifications for food grade materials	13
	G. Quantitative composition	15
	1. Analytical Methods	15
	2. Representative Lots	19
	3. Stability of DAG	20
	H. Physicochemical characteristics of DAG	21
III.	Manufacturing Process	23
	A. Reaction scheme	24
	B. Use of Immobilized Lipase Preparation in the Manufacture of DAG	26
	C. Refining Process for DAG	27
	D. Typical Product Characteristics	29
	E. List of Constituents Used in Manufacture of DAG and Recognition in FDA's Regulations	29
	F. Potential Contaminants and Human Toxicants	31
IV.	Estimated Consumption of DAG	33
V.	Self-Limiting Uses	41
VI.	Basis for GRAS Determination	41
	A. Information to Assess the Safety of DAG	41
	1. Overview	41
	2. Safety of DAG Components	41
	3. Metabolism of Triglycerides and DAG	46
	4. Toxicological Studies	52
	5. Clinical Studies	70
	6. Review of the Safety Aspects of DAG-Related Substances	96
	7. Summary and Discussion	101
	8. Conclusions	107

000004

		Page
B.	Reports or Investigations that May Appear Inconsistent	107
C.	Basis for Expert Consensus	108
VII.	Statement of Compliance with Good Laboratory and Clinical Practices	110

	Tab
GRAS Expert Panel Critical Evaluation of Diacylglycerol (DAG)	1
List of References	2

APPENDICES

		Volume
I.	Analytical Methods	2
II.	Examples of Kao's products related to DAG Table of typical composition of DAG Oil	3 3
III.	Test reports for analytic methods for DAG & DAG Oil	3
IV.	Content and Fatty Acid Composition of Diacylglycerols Animal Studies Clinical Studies Resin Residue	4
V.	Estimated Daily Intake of DAG By the U.S. Population from Proposed Food-Uses For DAG Oil	5
VI.	Expert Panel Qualifications and Evaluation of DAG	6

REFERENCES

Part I	7
Part II	8

I. GRAS Exemption Claim

A. Claim of Exemption From the Requirement for Premarket Approval Pursuant to Proposed 21 C.F.R. § 170.36 (c)(1)

Kao Corporation has determined that diacylglycerol oil ("DAG") is generally recognized as safe, and therefore, exempt from the requirement for premarket approval, for the specified uses described below. The basis for this finding is described in the sections below.

Signed,

Takuji Yasukawa

Director

Food Products Research Laboratory

Kao Corporation

000007

B. Name and Address of Notifier

Kao Corporation

1-3, Bunka 2-chome

Tokyo 131 8501, Japan

Contact: Takuji Yasukawa, Director

Food Products Research Laboratory

Telephone: 81-3-5630-7263

Facsimile: 81-3-5630-9329

Electronic Mail: 155039@mail.kao.co.jp

C. Name of GRAS Substance

The ingredient proposed by Kao for classification as Generally Recognized as Safe ("GRAS") is diacylglycerol oil. Kao has proposed a common or usual name of "DAG" for the substance. DAG is a name that is derived from the common or usual name for the randomized diacylglycerol in general fatty acids, i.e., Diacylglycerol, which is the main component of DAG. Other components of DAG include triacylglycerol and monoacylglycerol. The main constituent fatty acids of DAG, derived from general edible oils such as soybean oil, rapeseed oil, corn oil, and olive oil, are oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3).

D. Conditions of Use

1. Intended use of DAG

DAG is intended to be used as a replacement for vegetable oils used in the domestic preparation (*e.g.,* cooking) of foods, and in the commercial formulation of margarine for home use. These foods are identified in the appendix for fats and oils category of the United States Department of Agriculture ("USDA") 1989-91 Continuing Survey of Food Intakes by Individuals ("CSFII 1989") and have been used in calculating the Estimated Dietary Intake ("EDI") values for DAG.[1/] (U.S.D.A. 1996).

E. Basis for GRAS Determination

Pursuant to 21 C.F.R. § 170.30, Kao has determined that DAG is GRAS based upon a scientific procedures GRAS determination. Kao's determination is based on the views of experts who are qualified by scientific training and experience to evaluate the safety of DAG as a food ingredient.[2/] The safety of DAG is supported by a number of studies including acute, subacute, and chronic studies in rats, *in vitro* mutagenicity studies and clinical studies of the human tolerance and nutritional effects of DAG. In addition, the GRAS status of DAG is supported by

---

1/ It is our understanding that the CFSII 1989 continues to serve as the primary source for consumption analysis, rather than the 1996 CSF.

2/ The expert panel includes: Joseph Borzelleca, Ph.D; Walter Glinsmann, M.D.; Ian Munro, Ph.D; and David Kritchevsky, Ph.D. See GRAS Expert Panel Critical Evaluation of DAG, Tab 1; Also See Appendix VI for qualifications of expert panel.

numerous published studies regarding the safety of the components of DAG and related substances. This determination is further supported by additional unpublished data and information that corroborate these published studies. Further, the safety and stability of DAG was favorably evaluated by the Ministry of Health and Welfare of Japan, approved for use in foods for specified health use ("FOSHU"),[3/] and for use in cooking oil in Japan.

1. Description

DAG is an edible oil that is equivalent in taste and physicochemical properties to naturally occurring oils. Kao intends to use DAG, in combination with other GRAS substances, to market a product called "DAG Oil."[4/] The main constituent of DAG is a randomized diacylglycerol ("DG") that is manufactured through the esterification of fatty acids derived from natural edible plant oils with either monoacylglycerol or glycerol in the presence of lipase.[5/] DG and the other constituents of DAG and DAG oil have been shown to be safe in a number of published studies

---

3/ FOSHU is described in Appendix IV-4.

4/ DAG Oil consists of >99.8% DAG; it also contains emulsifiers and antioxidants used to stabilize DAG in the manufacturing process. The typical composition of DAG Oil is shown in Appendix II-2. The antioxidants and emulsifier that are used to manufacture and stabilize DAG Oil already have been affirmed as food additives or GRAS. The relevant components include: Ascorbyl palmitate (21 C.F.R. § 182.3149); Polyglycerol esters of fatty acids (21 C.F.R. § 172.854); and Tocopherol (21 C.F.R. § 184.1890).

5/ Lipozyme IM, GRAS Affirmation Petition (GRASP) 7G0323, filed by NOVO NORDISK. See also, A. Broadmeadow et al., 1994. An overview of the Safety Evaluation of *Rhizomucor miehei* Lipase Enzyme. Food Additives and Contaminants 11(1):105-119.

which have focused on, among other ingredients, natural oils and fats. Many of these oils and fats contain monoacylglycerol ("MG") and DG in much lower concentrations at levels of 1 to 10% by weight. (Goh and Timms, 1985, Gopalakrishnan *et al.*, 1987, Kamal-Eldin *et al.*, 1996, Perez-Camino *et al.*, 1995).[6/]

Below, Kao has set forth a brief summary of information and reports of past uses of diacylglycerols, as well as the history of use of DAG.

a. Prior Use of Diacylglycerols

DG, the main constituent of DAG, is present in the body (primarily as 1,2/2,3-DG) as a product of triacylglycerol metabolism and is approved by the U.S. Food and Drug Administration ("FDA") for use in food as a component of emulsifiers (mono- and diglycerides). In addition, DG is used in food as one of the ingredients of medium purity monoacylglycerol,[7/] which has been used as an emulsifier for food for many years and has been established as safe. Thus, DG has been extensively evaluated for its safety.

6/ A list of references, in alphabetical order, is attached at Tab 2.

7/ In Europe and the United States, DG is used in medium purity mono- and diglycerides.

Kao has manufactured products that contain DG in its production facilities in Japan for over fifteen years.[8/] All of Kao's DG products have been sold without any safety concerns in Japan for many years. These products have been used in Japan for a number of different uses, including home use cooking oils, which are used to stir-fry and deep-fry foods, and margarine for industrial use. The addition of DG to such foods improves cooking, taste and flavor because DG is more hydrophilic and soluble than natural triacylglycerol oil. Further, functional compounds such as emulsifiers and antioxidants can be more soluble in DG than other conventional oil.

Kao's DG products that are sold for industrial use are generally used as emulsifiers. As described in Appendix II-1, these industrial products have various uses and generally consist of about 40-55% MG and up to 40% DG and triglycerides ("TG"). Kao has marketed DG-related products such as cooking oil for home use and margarine for food industry use in Japan for several years, and has sold over 12,400 tons of these products in Japan over a fifteen year period.

b. Prior Use of DAG

Kao's conclusion that DAG is safe is supported by the marketing experience in Japan of products that contain DAG. Kao has had sales of over 9800 metric tons of DAG in Japan, over a period beginning in February 1999 through May 2000. During this marketing period, there have only

---

8/ The terms DG, diacylglyceride and diglyceride are synonymous with the term diacylglycerol, the scientific name for compounds with two fatty acids bound to one glycerin.

been 15 reports of consumer complaints received by Kao from DAG consumers. These complaints involved minor events such as light constipation, light diarrhea, slight heartburn and mild eczema; Kao believes they were not related to DAG consumption.

Based on the marketing experience of DAG in Japan, clinical studies conducted by Kao, published studies, and the conclusion by experts that DAG is safe, Kao has concluded that DAG is GRAS.

2. Summary of Safety Data for DAG

Section VI of this document presents the safety profile of DAG, an oil prepared from a mixture of 1,3- and 1,2-diacylglycerol (at a ratio of 7:3). The safety of DAG is supported by several acute, subacute, and chronic studies in rats; *in vitro* mutagenicity studies on diglyceride, and 10 clinical studies conducted to determine the human tolerance and nutritional effects of DAG. In particular, a chronic rat study (Soni *et al.*, 2000; submitted for publication) and 7 repeated dose clinical studies (Watanabe *et al.*, 1997d,e [unpublished]; Katsuragi *et al.*, 1999 [published]; Hasegawa, 2000 [unpublished]; Matsuyama, 2000 [unpublished]; Nagao *et al.*, 2000 [published]; and Teramoto *et al.*, 2000 [published]) were considered pivotal studies in determining the safety of DAG. In addition, there is a wealth of information in the literature on the metabolism and toxicology of DAG components and related substances, many of which the Joint FAO/WHO Expert Committee on Food Additives (JECFA) has evaluated. JECFA has concluded that "there

is no evidence that the presence of monoglycerides and diglycerides of food fats has any deleterious effect on cells or tissues" (JECFA, 1974). Furthermore, diacylglycerol (DG), the main component of DAG, occurs endogenously in the body (mainly as 1,2/2,3-DG) (Douglas *et al.*, 1953; Borgstrom *et al.*, 1957; Hamosh and Scow, 1973; Small, 1991), as a product of triglyceride metabolism and is approved by the US FDA for use in food as a component of emulsifiers (mono- and diglycerides) (21 C.F.R. § 184.1505 (2000)). Finally, the raw materials used in the production of DAG are of food grade quality and the immobilized lipase preparation has been established as safe in the published literature (Broadmeadow *et al.*, 1994). Also of note, DAG Oil is permitted for use in a cooking oil in Japan by the Ministry of Health and Welfare (May 20, 1998).

3. Estimated Consumption of DAG

DAG Oil is intended for use as a replacement for vegetable oils used in the domestic preparation (*e.g.*, cooking) of foods and in commercially-formulated margarine for home use. Assuming DAG Oil would replace all vegetable oils used in the domestic preparation of foods, and those used in formulating margarine products, the estimated mean all person (per capita) consumption of DAG for the total population was determined to be 3.30 g/day (0.06 g/kg body weight/day) using USDA CSFII 1989-91 food consumption data. The 90th percentile intake for the total population was determined to be 9.12 g/day (0.16 g/kg body weight/day). Mean users only consumption was slightly higher at 5.11 g/day (0.09 g/kg body weight/day) with the 90th

percentile for users only being 11.50 g/day (0.21 g/kg body weight/day). Considering that mean *per capita* fat consumption approximates 70 g/day, DAG may replace up to 5 % of the total fat intake. Estimated consumption is discussed in detail in section IV.

4. General Recognition of Safety

Pursuant to 21 C.F.R. § 170.30, general recognition of safety based upon scientific procedures shall ordinarily be based upon published studies and may be corroborated by unpublished studies and other data and information. The scientific data and information on which the safety assessment of DAG and its constituents is based – including several acute, subacute, and chronic studies in rats, *in vitro* mutagenicity studies, clinical studies conducted to assess the human tolerance and nutritional effects of DAG, and published studies regarding the components of DAG, are available in the published literature or are otherwise publicly available to experts qualified by training and experience to evaluate the safety of food and food additives. Thus, these data meet the common knowledge element required for all GRAS determinations. The published studies are corroborated by numerous additional unpublished human and animal studies of the components of DAG.

Kao concurs with the conclusions of the DAG Expert Panel and believes that other qualified and competent scientists reviewing the same publicly available data would reach the same scientific conclusion.

In sum, the data described in this notification demonstrate that DAG is GRAS under its intended conditions of use, and accordingly is exempt from the definition of food additive and the pre-market requirements of section 409 of the Federal Food, Drug, and Cosmetic Act (21 U.S.C. § 348).

F. Availability of Information

The data and information that serve as a basis for this GRAS determination are available for the FDA's review and copying at a reasonable time at the offices of:

Sandra J.P. Dennis, Esq.

Morgan, Lewis & Bockius

1800 M Street, N.W.

Washington, D.C. 20036

Telephone: (202) 467-7009

Fax: (202) 467-7176

Electronic Mail: sdennis@morganlewis.com

Alternatively, copies of data and information can be provided to FDA upon request, by contacting Ms. Dennis.

II. Identity and Specifications of DAG

Set forth below is a summary of the following information regarding DAG: chemical identity, chemical abstract number, empirical formula, structural formula, molecular weight, specifications for food grade materials, quantitative composition, physicochemical characteristics, and stability.

A. Chemical Identity

The primary component of DAG is DG (1,2-DG, 1,3-DG). Other components of DAG include triacylglycerol and monoacylglycerol. Component fatty acids are derived from general edible oils such as soybean oil, rapeseed oil, corn oil, and olive oil. Oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3) are the main constituent fatty acids of DAG.

B. Chemical Abstract Service (CAS) Registry Number

None of the DAG glycerides have been assigned a CAS Registry Number.

C. Empirical formula

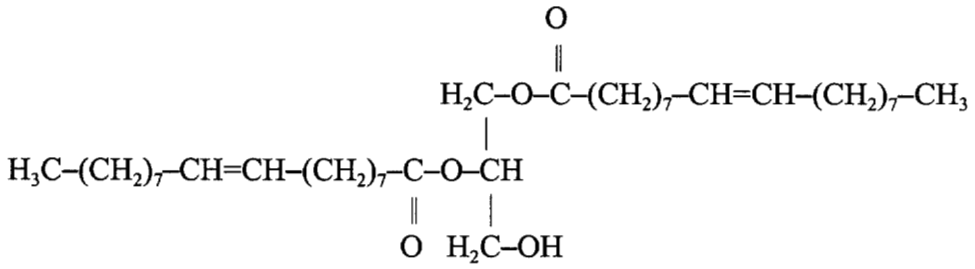
The empirical formulas of the primary DG components of DAG are as follows:

$C_{39}H_{72}O_5$:	diolein
$C_{39}H_{68}O_5$:	dilinolein
$C_{39}H_{70}O_5$:	linoleylolein
$C_{39}H_{68}O_5$:	linolenylolein
$C_{39}H_{66}O_5$:	linolenyllinlolein

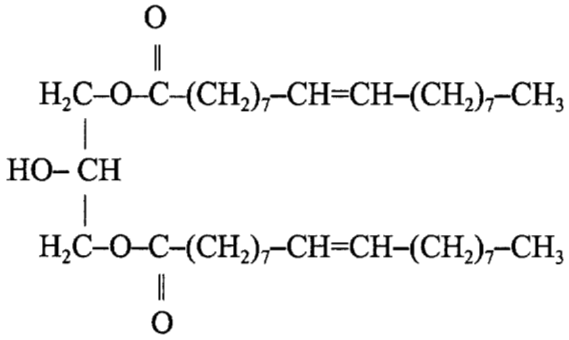
D. Structural Formula

DAG has a generalized structural formula as shown below.

- 1,2/2,3-DG



- 1,3-DG



The typical structure of oleic acid bound to glycerol is only described here as a representative fatty acid. In addition, only the typical structural formulas of DG (the main component of DAG) are shown here. As shown, two fatty acids are randomly distributed at the 2 and either the 1 or 3 position or at the 1 and 3 position of the glycerin structure.

Typical chromatographic, spectroscopic and spectrometric data of DAG are provided in Appendices I-1 and I-2. The method used for the analysis of this data is also provided in Appendix I-3.

E. Molecular Weight

The following are the molecular weights of primary components of DAG:

	Molecular Weight
diolein	620
dilinolein	616
linoleylolein	618
linlenylolein	616
linolenyllinolein	614

F. Specifications for Food Grade Materials

The specifications for DAG are as follows:

Clear pearl-yellow viscous liquid at room temperature; free from particulate matter; bland odor and moderate taste; free from foreign and rancid flavor; soluble in ethanol, benzene, chloroform and other chlorinated hydrocarbons; and insoluble in water.

Ester Distribution:

Diacylglycerol	At least 80%
Triacylglycerol	Not more than 20%
Monoacylglycerol	Not more than 5%

Fatty Acid Composition:

The following fatty acids are the main constituents of DAG:

Oleic acid (C18:1)	20-65% by weight
Linoleic acid (C18:2)	15-65% by weight
Linolenic acid (C18:3)	not more than 15% by weight
Saturated fatty acid (C16:0 and C 18:0)	not more than 10% by weight
Acid value	Not more than 0.5 mg KOH/g of sample
Moisture	Not more than 0.1% by weight
Residue on ignition	Not more than 0.5% by weight
Heavy metals (as Pb)	Not more than 10 ppm
Lead	Not more than 0.5 ppm
Arsenic (as As)	Not more than 2 ppm
Phytosterol (total amount of campestrol, stigmasterol, brassicasterol, and betasitosterol)	Not more than 0.05-1.2% by weight

The methods used to determine these specifications are listed below:

Specification items	Method	Location in Appendices
Ester Distribution	Kao's method	I-3
Fatty acid Composition	A.O.C.S.- Ce 1b-89	I-5
Acid Value	A.O.C.S.-Cd 3d-63	I-6
Moisture	A.O.C.S.- Ca 2e-84	I-7
Residue on Ignition	F.C.C. IV- Residue on Ignition	I-8
Heavy Metals (as Pb)	F.C.C. IV- Heavy Metals	I-9
Lead	F.C.C. IV- Lead Limit Test	I-10
Arsenic (as As)	F.C.C. IV- Arsenic	I-11
Phytosterol	Standard method issued by Japan Food Research Laboratories (JFRL)	I-18B

G. Quantitative Composition

1. Analytical Methods

Although the specifications for DAG were developed using American standard methods for the analysis of oils and fats, measurements in this GRAS notification generally were calculated using Japanese standard methods. The Japanese standard methods that were used to analyze the data are based on global standard methods. The measurement of one representative lot was calculated using both Japanese and American methods, and compared to one another. Using the different standards resulted in relatively minor differences between the two different sets of data.

The analytical methods used for these measurements are listed below. The code number of each analytical method is shown for Japanese standard methods. American standard methods are provided in the Appendices as listed below:

Analytical Item[9/]	Method	Appendices
Ester distribution	(J) JFRL method[*1]	
	(A) AOAC (Sixteen Edt.) - 965.35[*2]	I-4
Fatty acid composition	(J) JFRL method	
	(A) AOCS - Ce 1b-89[*3]	I-5
Acid Value	(J) JOCS - 2.4.1-83[*4]	
	(A) AOCS - Cd 3d-63	I-6
Moisture	(J) JOCS - 2.2.2.4-90	
	(A) AOCS - Ca 2e-84	I-7
Residue on ignition	(J) Standard method for food additives[*5]	
	(A) FCC IV - Residue on ignition[*6]	I-8
Heavy metals (as Pb)	(J) JFRL method	
	(A) FCC IV - Heavy Metals	I-9
Lead	(J) JOCS - 2.2.9.1 c-77	

9/ (J) represents Japanese standard methods or equivalent methods.
(A) represents American standard methods or equivalent methods.

	(A) FCC IV - Lead Limit Test	I-10
Arsenic	(J) JOCS - 2.2.9.6-77	
	(A) FCC IV - Arsenic	I-11
Saponification value	(J) JOCS - 2.4.3.1.-71	
	(A) AOCS - Cd 3-25	I-12
Iodine value	(J) JOCS - 2.4.5.1.-71	
	(A) AOCS - Cd 1-25	I-13
Peroxide value	(J) JOCS - 2.4.12-86	
	(A) AOCS - Cd 8-53	I-14
Hydroxyl value	(J) JOCS - 2.4.9.2-83	
	(A) AOCS - Cd 13-60	I-15
Free glycerine	(J) JFRL method	
	(A) AOCS - Ca 14-56	I-16
Free fatty acids	(J) JOCS - 2.4.1-83	
	(A) AOCS - Ca 5a-40	I-17
Unsaponifiable matter	(J) JOCS - 2.4.10-71	
	(A) AOCS - Ca 6a-40	I-18
Phytosterol	Standard method issued by JFRL	I-18B
Color	(J) JOCS - 2.3.1.1b-71	
	(A) AOCS - Cc 13e-92	I-19

*1: These measurements were derived based on the methods of Japan Food Research Laboratories ("JFRL") which conducted measurements of representative lots. JFRL is a specialized agency that analyzes food in Japan. The methods used by JFRL in its analysis of the ester distribution corresponds to Japanese standard methods.

*2: Derived from methods used by the Association of Official Analytical Chemists ("AOAC").

*3: Derived from methods used by the American Oil Chemists' Society ("AOCS").

*4: Derived from methods used by the Japanese Oil Chemists' Society ("JOCS"). JOCS methods are Japanese standard methods for the analysis of oils, fats and derivatives.

*5: Derived from methods for food additives issued by the Ministry of Health and Welfare of Japan under the Specifications and Standards of Foods, Additives, and the Japanese Standard for Food Additives. Because a Japanese standard method for residue upon ignition of fats and oils does not exist, Kao substituted this method as a standard method.

*6: Derived from methods used by the Food Chemicals Codex.

2. Representative Lots

Each lot of DAG used in testing was manufactured using the method described below in further detail in Section III: Manufacturing Process. The manufacturing process for DAG involves esterification of fatty acids derived from edible plant oils and either glycerol or monoacylglycerols in the presence of immobilized lipase Lipozyme IM.

Analytical data for five lots are provided in Appendix I-23. Lots 1030, 1032, and 1034 were analyzed in June and July 1998 and certified in October 1998. Lots 1157 and 1174 were analyzed in January 2000 and certified in February 2000.

The analysis was conducted using Japanese standard methods for all five lots. Additionally lot number 1034, was analyzed using the American standard method as well.

Both sets of analytical results (American and Japanese methods) showed that the methods used yielded products that met proposed specifications, and there were no significant differences observed between the results using the Japanese and American analytical methods.

There were, however, differences in the monoglyceride content of DAG that were observed during the analysis of ester distribution between Japanese (JFRL method) and American (AOAC method) standard methods due to the different analytical process used in each method. The JFRL method uses Gas Chromatography ("GC") with capillary columns in the analytical process, and is equivalent to Kao's method used for the measurement of DAG's specifications. By contrast, the AOAC method is based on a fractionation analysis of each glyceride using several

solvents with different polarity and open columns. As such, the analytical result of MG content by JFRL method was about 1.2%. In contrast, the analytic result using the AOAC method was about 4%. Nonetheless, both analytic values meet the proposed specifications for DAG.

In addition to the analytical results for the specifications for DAG, results from a number of other tests which have been used to characterize fats and oils also are reported in Appendix I-23. Like the analytical results of the DAG specifications, there were no significant differences between these measurements that were derived using either the Japanese or American standard methods.

In addition to MG, DG, and TG, DAG also contains trace amounts of free glycerol, free fatty acids and tocopherol as unsaponifiable matter. The level of unsaponifiable matters in finished DAG (less than 0.2%), is less than the reported values for cocoa butter (0.4%) and refined olive oil (0.9%) (Swern, Ed., 1979).

3. Stability of DAG

The properties of DAG are similar to that of other edible fats and oils. DAG is generally stable under normal atmospheric pressure and at room temperature. Indeed, under ordinary conditions, DAG is relatively inert and shows little activity. When a closed bottle is filled with DAG in the absence of oxygen, it is quite stable. If DAG is exposed to the air, however, oxidation reactions will take place gradually.

To test the stability of DAG, Kao studied DAG which had been stored in a tank under a nitrogen atmosphere at room temperature for two weeks, as well as the stability of DAG following storage

under a nitrogen atmosphere at room temperature in the dark for four weeks. In each instance, the substance was evaluated and compared to the specifications of DAG that had been analyzed immediately after the manufacturing process. The composition of DAG remained almost unchanged under the storage conditions. Each analytic value met the normal specifications of DAG even after 4 weeks storage. Standard items, specification limits of DAG, analytic values and analytic methods are shown in Appendix I-24. The taste and outward appearance of DAG also were evaluated using sensory methods. These data are also shown in Appendix I-24.

H. Physicochemical Characteristics of DAG

The physicochemical characteristics of DAG were measured and compared with rapeseed oil. Rapeseed oil is one of the most popular edible oils in Japan and it has an almost identical fatty acid composition to that of DAG. (See measurement and methods shown below; see also appendix I-25 for analytical data.) Moreover, the physicochemical properties of DAG are similar to rapeseed oil. Kao has thus concluded that DAG can be used as a replacement for rapeseed oil, as well as other general edible oils.

Measurement item[10/]	Method	Appendices
Specific gravity (20° C)	(J) JOCS - 2.3.2-71	
	(A) AOCS - Cc 10a-25	I-20
Viscosity (20° C)	(J) JOCS - 2.3.9.5-71	
	(A) AOCS - Ja 10-87	I-21
Smoke Point	(J) JOCS - 2.3.10	
	(A) AOCS - Cc 9a-48	I-22
Flash Point	(J) JOCS - 2.3.10	I-22
	(A) ACOS - Cc 9a-48	

10/ These measurements were taken using only Japanese standard methods. The processes and measurements are not significantly different from American standard methods. Consequently, only the code number of each analytic method is shown for Japanese standard methods; the American standard method that corresponds to the Japanese method and different points between both the methods are shown in each reference.

CONFIDENTIAL/TRADE SECRET INFORMATION: DO NOT DISCLOSE

III. Manufacturing Process

DAG Oil is produced through the addition of emulsifiers and antioxidants to DAG. DAG is manufactured by the esterification of fatty acids that are derived from natural edible plant oils and either monoacylglycerol or glycerol and Lipozyme IM. In its final form DAG is composed of more than 80% diacylglycerol, less than 20% TG, less than 5% MG, and trace amounts of antioxidants and emulsifier.

DAG will be manufactured in accordance with the Food Hygiene Act of Japan. Kao has received certification for the manufacture of food from the prefectural governor of Ibaraki where Kao's Kashima Plant is located. Kao also has fully considered the effects on the environment, and has received an ISO14001 certification. The manufacturing processes for DAG is described in this section below.

CONFIDENTIAL/TRADE SECRET INFORMATION: DO NOT DISCLOSE

Pages 30 - 38 have been removed. (b)(4)

IV. Estimated Consumption of DAG

Plant and vegetable-based oils and fats naturally contain triacylglycerol (TG), and minor amounts of diacylglycerol (DG) and monoacylglycerol (MG). Oils (*e.g.,* soybean, cottonseed, palm, corn, sunflower, coconut, rice bran, peanut, sesame, olive, and rapeseed) and fats (*e.g.*, cacao butter, tallow, lard, milk fat) consumed as a part of the human diet are comprised of 1 to 15 % diacyglycerols (DG) by weight (Sleeter, 1981; Abdel-Nabey *et al.*, 1992; Mariani and Fedeli, 1985; George and Arumughan, 1992; Arroyo *et al.*, 1996; Goh and Timms, 1985; Siew, 1995; Sanchez-Muniz *et al.*, 1993; Cuesta *et al.*, 1993; Gopalakrishnan *et al.*, 1987; Inoue and Noguchi, 1967; Bhattacharyya and Bhattacharyya, 1989; Kamal-Eldin and Appelqvist, 1994; Catalano *et al.*, 1994; Giovanna, 1996; Modi *et al.*, 1996; Fincke, 1982; Mariani *et al.*, 1990; Perez-Camino *et al.*, 1996; D'Alonzo *et al.*, 1982; Brühl, 1997).

Both DG and MG (*ie.,* mono- and diglycerides) are generally recognized as safe (GRAS) for use as ingredients in foods (21 C.F.R. § 184.1505), and have found extensive use in a wide variety of food applications (*e.g.*, baked goods, carbonated beverages, confections, jams, jellies, dairy products, coffee whiteners, and whipped toppings) (Informatics Inc., 1973; FASEB, 1975; Akoh, 1998). Based on 1987 production data in the United States (NAS, 1989), the combined intake of DG and MG from their use as food additives was determined to be 3.61 g/person/day, assuming an effective response rate of 60% for industry survey participants, and a 10% exposure rate for individual consumers within a total population of 240 million people. Earlier estimates for

intake of DG and MG from food additive sources were reported to be between 1 and 10 g/person/day (FASEB, 1975).

Based on the taste and physico-chemical profile exhibited by DAG oil, Kao is proposing its use as a replacement for vegetable oils used in the domestic preparation (*e.g.*, cooking) of foods and in the commercial formulation of fat-based foods, such as margarine.

Food consumption data obtained from the United States Department of Agriculture (USDA) 1989-91 Continuing Survey of Food Intakes by Individuals (CSFII) (USDA, 1996) and information pertaining to the proposed use of DAG oil in foods were used to establish estimates for the intake of DAG (all-person and per-user) by demographic group and by the total population. Estimates for the intake of DAG were based on the proposed use of DAG oil as a replacement for vegetable-based table and cooking oils consumed in the home, and as an ingredient in formulating commercially-prepared margarine and margarine-like spreads.

To ensure all estimates for the intake of DAG were conservative, it was assumed that DAG oil would replace 100% of the vegetable-based oils used in the domestic preparation of all proposed food-uses. The amount of vegetable oil present in each proposed food-use was determined based on a review of raw ingredient data included in the standard recipe files for the USDA CSF II 1989-1991 consumption survey (USDA, 1996) and the USDA Nutrient Database for Standard

Reference (Release 12) (USDA, 1998). In addition, other published sources of formulation data were reviewed as required (Latondress, 1981; Chrysam, 1996; Hastert, 1996; Sipos and Szuhaj, 1996; O'Brien, 1998). The proposed food-uses and ranges for the level of use of DAG oil are summarized in Table 4.1.

Table 4.1 Summary of The Individual Proposed Food Uses and Use-Levels for DAG Oil In The U.S.

Food Category	Proposed Food Use	Use-Levels for DAG Oil (%)
Fats and Oils		
	Edible oils (plant or vegetable-based)	100
	Margarine and margarine-like spreads (regular, stick/tub, blends, reduced-calorie)	12.1 - 64.4

The intake of DAG was established for each of the individual proposed food uses of DAG oil, as well as, for the total intake from all proposed food uses in the U.S. The mean all-person intake, mean per-user intake, 90th percentile intake, and percent consuming were reported on both a per person and per kilogram body weight basis for the following population groups:

Infants, ages 0 to 2;
Children, ages 3 to 11;
Female teenagers, ages 12 to 19;
Male teenagers, ages 12 to 19;
Female adults, ages 20 and up;
Male adults, ages 20 and up; and,
Total population (all population and gender groups combined).

A more detailed discussion of the individual proposed food uses and use levels for DAG oil, adjustment factors employed, the USDA 1989-91 CSFII consumption data, population groups, and intake analysis is provided in a consumption report accompanying this notification. See Appendix V.

The mean all-person and all-user daily intakes of DAG by the total population from all proposed food uses for DAG oil in the United States by population group are summarized in Tables 4.2-1 and 4.2-2. Based on the USDA CSF II 1989-1991 consumption survey data, only 49% of the infant population (aged 0 - 2) are consumers of foods in which DAG oil is proposed for use. Approximately 65% of all individuals within the total population were reported to be consumers of foods formulated with DAG oil.

Table 4.2-1 Summary of Estimated Daily Per Person DAG Consumption From All Proposed Food Uses For DAG Oil In The U.S. By Population Group

Group	Age Group (Years)	% Users	All-Person Consumption		All-Users Consumption	
			Mean (g)	90th Percentile (g)	Mean (g)	90th Percentile (g)
Infant	0-2	49.3	1.19	3.69	2.41	6.33
Child	3-11	64.7	2.76	7.67	4.27	9.47
Female Teenager	12-19	62.1	2.81	7.92	4.53	9.94
Male Teenager	12-19	65.2	4.71	13.30	7.23	15.80
Female Adult	20 and Up	65.8	2.93	7.92	4.46	9.91

Table 4.2-1 Summary of Estimated Daily Per Person DAG Consumption From All Proposed Food Uses For DAG Oil In The U.S. By Population Group

Group	Age Group (Years)	% Users	All-Person Consumption		All-Users Consumption	
			Mean (g)	90th Percentile (g)	Mean (g)	90th Percentile (g)
Male Adult	20 and Up	65.8	4.13	11.10	6.27	13.40
Total Population	All Ages	64.6	3.30	9.12	5.11	11.50

On an all-user basis, the estimated mean intake of DAG by the total population from all proposed food uses of DAG oil in the United States was determined to be 5.11 g/person/day or 0.09 g/kg body weight/day. The heavy consumer (90th percentile) per-user intake of DAG by the total population was 11.50 g/person/day or 0.21 g/kg body weight/day. Male teenagers were subject to the largest per-user intake of DAG from all proposed food-uses for DAG oil, exhibiting mean and heavy consumer (90th percentile) intakes of 7.23 and 15.80 g/person/day, respectively. However, on a body weight basis, infants and children exhibit the highest intakes of DAG, up to 0.42 g/kg body weight/day, as a result of their higher food or energy requirements.

Table 4.2-2 Summary of Estimated Daily Per Kilogram Body Weight DAG Consumption From All Proposed Food Uses For DAG Oil In The U.S. By Population Group

Group	Age Group (Years)	% Users	All-Person Consumption		All-Users Consumption	
			Mean (g/kg)	90th Percentile (g/kg)	Mean (g/kg)	90th Percentile (g/kg)
Infant	0-2	49.3	0.09	0.29	0.19	0.42
Child	3-11	64.7	0.11	0.30	0.17	0.38
Female Teenager	12-19	62.1	0.05	0.16	0.08	0.20
Male Teenager	12-19	65.2	0.08	0.21	0.12	0.32
Female Adult	20 and Up	65.8	0.04	0.13	0.07	0.16
Male Adult	20 and Up	65.8	0.05	0.14	0.08	0.16
Total Population	All Ages	64.6	0.06	0.16	0.09	0.21

Estimates for the daily intake of DAG from all proposed food uses of DAG oil are considered 'worst case' since a number of conservative assumptions were used in their development. Intake estimates for DAG would be considered overestimates, since DAG oil would not necessarily be used at the maximum proposed use level, consumers and food manufacturers would not be expected to completely substitute DAG oil for other edible vegetable oils in the preparation and formulation of food, and 3-day food consumption data are known to overestimate the chronic intake of food ingredients or nutrients by individuals (Anderson, 1988).

The impact of the length of dietary surveys on the user consumption of different types of food products has recently been studied (Institute of European Food Studies, 1998). In this multi-country study, the consumption of 32 food types including different types of fat sources was examined in 1,000 males and females aged 10 to 18 years in a food diary study for 1, 3, 5, 7, 10, and 14 days. In general, the mean consumption of users decreased over the length of the study, depending on the food type (see Table 4.3-1).

Table 4.3-1 Effect of Food Consumption Survey Length on User Consumption of Different Food Types

Food Product	Users 3-Day Consumption		Users 14-Day Consumption		Ratio of 3-Day/14-Day Consumption	
	Mean (g/day)	90th Percentile (g/day)	Mean (g/day)	90th Percentile (g/day)	Mean	90th Percentile
Butter/ Margarine	15	31	13	28	1.2	1.1
Mayonnaise	10	20	4	9	2.5	2.2
Chicken	53	94	24	50	2.2	1.9
Pasta	110	240	69	189	1.6	1.4
Fish	46	86	18	37	2.6	2.3
Average					2.0	1.8

Although the consumption of fat sources, such as table fats, which are considered "staple" food products, does not change significantly following 3-days of collection of food consumption data, consumption of other fat sources, such as mayonnaise or fat involved in food preparation (e.g., for chicken, fish or pasta), decreases. As such, the impact that survey length has on the consumption of individual food products by users varies given their frequency of use. For foods consumed on a daily basis, a 3-day survey likely approximates long-term user consumption. However, for foods consumed less frequently, longer time periods would be required to establish long-term user consumption. Therefore, current estimates for the mean and 90th percentile users projected intake of DAG from the use of DAG oil during the domestic preparation (*e.g.,* cooking) of foods, determined using 3-day food consumption data, may substantially overestimate the chronic consumption of DAG.

V. Self Limiting Uses

To the best of Kao's knowledge, there is no level of self-limiting use.

VI. Basis for GRAS Determination

A. Information to Assess the Safety of DAG

1. Overview

The following comprehensive review provides a well-documented basis from which a safety evaluation of DAG can be conducted. The safety of DAG components is presented in Section 2 below, the metabolism of DAG relative to triglyceride metabolism is discussed in Section 3, the toxicology data are reviewed in Section 4, and the clinical studies in Section 5. Section 6 presents a review of the safety of DAG-related substances.

2. Safety of DAG Components

The manufacturing process for DAG is described in detail in Section III of this report. Briefly, DAG is a randomized DG manufactured through the esterification of fatty acids derived from natural edible plant oils with either monoacylglycerol (MG) or glycerol in the presence of an immobilized lipase preparation (Lipozyme IM). The safety aspects of these primary constituents of DAG are discussed below.

2.1 Diacylglycerol

Quantitative analyses of 5 representative batches of DAG indicate that DAG is comprised of approximately 87% DG (Appendix I-23). DG is a component of mono- and diglycerides which are approved for use in foods (21 C.F.R. § 184.1505). The safety aspects of these GRAS compounds are reviewed in Section 6 below. In addition to DG intake from these sources, DG is also consumed in the diet naturally. DG is contained in edible oils such as soybean, cottonseed, palm, corn, sunflower, coconut, rice bran, peanut, sesame, olive, rapeseed, cacao butter, tallow, lard, and milk fat at concentrations ranging from approximately 1 to 15% by weight (Sleeter, 1981; Abdel-Nabey *et al.*, 1992; Mariani and Fedeli, 1985; George and Arumughan, 1992; Arroyo *et al.*, 1996; Goh and Timms, 1985; Siew, 1995; Sanchez-Muniz *et al.*, 1993; Cuesta *et al.*, 1993; Gopalakrishnan *et al.*, 1987; Inoue ad Noguchi, 1967; Bhattacharyya and Bhattacharyya, 1989; Kamal-Eldin and Appelqvist, 1994; Catalano *et al.*, 1994; Giovanna, 1996; Modi *et al.*, 1996; Fincke, 1982; Mariani *et al.*, 1990; Perez-Camino *et al.*, 1996; D'Alonzo *et al.*, 1982; Brühl, 1997). In addition to intake through the diet, DG is also present in the body as a product of normal triglyceride metabolism (see Section 3 below).

2.2 Immobilized Lipase (Lipozyme IM)

Lipozyme IM is an immobilized lipase from *Rhizomucor miehei* which is produced by *Aspergillus oryzae* through insertion of *R. miehei* cDNA encoding the precursor of the lipase. The immobilized lipase allows for the modification of lipids through interesterification of

triglycerides and is used in the manufacture of DAG. The immobilized lipase preparation consists of the purified enzyme adsorbed onto an ion exchange resin (Duolite 568). Covered by a filed GRAS-Affirmation petition (GRASP 7G0323, 54 Fed. Reg. 9565) accepted for filing by FDA in 1989 and amended in 1992, this immobilized lipase preparation is permissible for use as a direct human food ingredient. The safety of Lipozyme IM has been reviewed in the literature and is discussed below (Broadmeadow *et al.*, 1994).

2.2.1 Production Strain and Lipase

The safety of the *R. miehei* lipase enzyme produced by *A. orzyae* has been investigated in *in vitro* mutagenicity studies and subacute and subchronic toxicity studies reported by Broadmeadow *et al.* (1994). The *R. miehei* lipase enzyme used in these studies was produced in the same manner as commercial preparations.

In an Ames test, sterile deionized water, the solvent used in the study, was employed as a negative control and the positive control compounds included *N*-ethyl-*N*'-nitro-*N*-nitrosoquanidine, 9-aminoacridine, 2-nitrofluorene, and 2-aminoanthracene. Two experiments were conducted. In the first experiment concentrations of 0, 0.1, 0.33, 1.0, 3.3, and 10 mg enzyme preparation/ml were tested in 4 strains of *Salmonella typhimurium* (strains TA 1535, TA 1537, TA 98 and TA 100) with and without metabolic activation by a liver S-9 fraction from Aroclor-induced Sprague-Dawley rats. The 2nd experiment tested these same concentrations in

the absence of metabolic activation and used lower concentrations of 0, 0.01, 0.033, 0.1, 0.33, 1.0 mg enzyme preparation/ml for treatments in the presence of metabolic activation (and for TA1535 treatments in the presence of metabolic activation). The enzyme preparation was reported to show no mutagenic activity when tested (Broadmeadow *et al.*, 1994).

In a mammalian cell mutation assay in L5178Y mouse lymphoma cells, 2 experiments were conducted. Concentrations of up to 5,000 µg enzyme preparation/ml were tested in the first experiment, and concentrations of up to 5,000 and 1,000 µg enzyme preparation/ml in the presence and absence of metabolic activation, respectively, were utilized in the second. Appropriate positive and negative controls were employed. Based on the data obtained from these experiments, the lipase was reported to be non-mutagenic (Broadmeadow *et al.*, 1994). The mutagenic potential of the lipase also was investigated in a chromosome aberration assay using cultured human lymphocytes at concentrations of up to 5,000 µg/ml. The lipase enzyme also was reported to test negative in this study (Broadmeadow *et al.*, 1994).

The effects of continuous exposure to the lipase was investigated in 2 studies in Sprague-Dawley rats. In a 4-week study, 10 rats/sex/group were fed a diet containing 2,500, 10,000, or 40,000 mg lipase/kg diet (approximately 250, 957, and 3,633 mg lipase/kg body weight/day). In the 13-week study, concentrations of 1,600, 8,000, or 40,000 mg lipase/kg diet (approximately 121, 600, and 2,892 mg lipase/kg body weight/day) were fed to groups of 20 rats/sex. Potential indices of

toxicity that were tested included food and water intake, body weight, clinical chemistry, urinalysis, organ weights, and histopathological examinations. The few effects reported in each study (reduced body weight, slight changes in a few hematological parameters, hyperkeratosis and aconthosis of the stomach, and chronic myocarditis) were limited to the high concentrations tested (8,000, 10,000 and 40,000 mg/kg diet) and were attributed to a reduced food intake (food conversion ratios were unaffected) and aging by the study authors.

On the basis of these studies, the *R. miehei* lipase enzyme was concluded by the study authors to be generally recognized as safe (Broadmeadow *et al.*, 1994).

2.2.2 Ion-exchange resin

Duolite 568, the phenol formaldehyde based ion-exchange resin functionalized with triethylenetetramine, is used for immobilization of the lipase in DAG production. Duolite phenolic resins have been approved for the treatment of foods in aqueous media in the US since 1964 (21 C.F.R. § 173.25 (2000); 29 Fed. Reg. 9708 (1964)).

Duolite 568 has been reported to release extractable material (residues) of an innocuous nature only under forced conditions, using either severe extraction conditions or elevated temperatures (Jensen and Eigtved, 1990). Three batches of DAG produced using the immobilized lipase and 1 batch produced without the immobilized lipase were tested for residues of the Duolite 568 resin.

A sample from each batch was heated from 50 to 400^{0}C and mass spectra on the collected extracts were measured as a function of temperature. Samples also were subjected to extreme extraction conditions (acetic acid extraction at 60^{0}C for 3 hours followed by heating as described above) before mass spectra analysis, treated with sodium sulfate, acetylacetone and acetic acid ammonium for UV detection of formaldehyde or analyzed by the Kjeldahl method for the detection of nitrogen. Extract levels were reported to range from < 5 to 200 ppm and included nitrogen (< 100 to 200 ppm) and formaldehyde (< 5ppm). For details see Appendix IV-15.

3. Metabolism of Triglycerides and DAG

DAG, comprised predominantly of the mono- and diglyceride component 1,3-DG, is considered to share the same metabolic pathways of partial glycerides and of triglycerides. Given the long history of safe use of DG in the human diet, the available human and animal metabolic data on mono- di- and triglycerides and on diolein (a diacylglycerol of similar composition to DAG; 1,3-DG and 1,2-DG at a ratio of 7:3; see below), and the abundance of metabolic and nutritional data available on DAG in human clinical studies, the conduct of a metabolism study in experimental animals using DAG or of hydrolysis studies in the gut were not considered necessary. As a preface to the discussion of the metabolism of DAG, it is of value to review the general pathways of fat metabolism to provide background and perspective on the DAG metabolic data.

3.1 General Overview of Fat Metabolism

Triglycerides (fats) undergo digestion in the gastrointestinal lumen where they are broken down by pancreatic lipase to form mono- and diglycerides and the accompanying fatty acids (Grundy, 1996). 2-Monoglyceride is the main product of triglyceride digestion, with 1-monoglyceride formed to a lesser extent (Mattson and Volpenhein, 1964; Grundy, 1996). These partial glycerides and fatty acids are then absorbed into the endoplasmic reticulum of intestinal epithelial cells for re-synthesis into triglycerides. The re-synthesized triglycerides are then assembled into lipoproteins known as chylomicrons and secreted initially into the lymphatic system and then into the plasma (Mattson and Volpenhein, 1964; Kayden *et al.*,1967; Small, 1991). Lipoproteins are lipid-rich particles containing apolipoproteins, polar lipids (phospholipids and unesterified cholesterol) and a non-polar lipid core consisting of triglycerides and cholesterol esters (Grundy, 1996). Chylomicron triglycerides are not taken up by the liver. Instead, they are metabolized in extrahepatic tissues where lipoprotein lipase, which is activated by apolipoprotein C-II, hydrolyzes the triglycerides releasing fatty acids that are taken up by adipose and muscle cells for re-esterification to triglycerides (tissue lipids) or oxidized as a fuel to CO_2 through β-oxidation to acetyl-CoA (Small, 1991; Grundy, 1996).

Alternatively, monoglycerides produced from triglyceride digestion may undergo complete hydrolysis within the intestinal lumen and be cleaved to glycerol and free fatty acids (Mattson and Volpenhein, 1964; Small, 1991). 2-Monoglycerides are rapidly esterified to triglycerides as

described above; however, the esterification of 1-monoglycerides is much slower such that they are more likely to undergo hydrolysis to glycerol and free fatty acids (Mattson and Volpenhein, 1964; Small, 1991). As determined in a rat study using the diacylglycerol dioleoilglycerol (Diolein)[12/], the digestive product of DAG, which is rich in 1,3-DG, is predominantly 1-monoglyceride, not 2-monoglyceride, and therefore, is likely to be completely hydrolyzed rather than utilized as a substrate for re-synthesis of triglyceride (Watanabe *et al.*, 1997a). The glycerol resulting from the hydrolysis of 1-monoglyceride is then either used in the synthesis of triglycerides *via* the glycerol 3-phosphate pathway or converted directly into glucose. In the liver, surplus triglycerides from free fatty acids are secreted into the circulation as very low density lipoproteins (VLDL). Oxidation of free fatty acids leads to the formation of carbon dioxide and water and partial oxidation leads to the formation of ketone bodies (*eg.*, β-hydroxybutyric acid) which are transported to extrahepatic tissues to act as a fuel source (Grundy, 1996).

3.2 Metabolism of DAG

DAG metabolism does not differ significantly from general fat metabolism as described above. The metabolism of the primary digestive product of DAG, 1-monoglyceride, is largely completely hydrolyzed in the intestinal lumen to glycerol and free fatty acids as opposed to being

---

12/ Diolein is a diacylglycerol that, like DAG, is comprised of 1,3-DG and 1,2-DG at a ratio of 7:3 and synthesized from oleic acid and glycerol (Watanabe *et al.*, 1997a).

absorbed and esterified to chylomicron triglyceride (Mattson and Volpenhein, 1964; Small, 1991; Watanabe *et al.*, 1997a).

Experiments conducted in male rats with the diacylglycerol diolein indicated an increase in serum fatty acids (oleic acid) in the portal blood following consumption (Watanabe *et al.*, 1997a). This result indicates that free fatty acids formed from the main digestion product 1-monoglyceride are largely absorbed into the liver for subsequent β-oxidation. This conclusion is also supported by the findings of the single-dose clinical studies discussed in Section 5.1 where a significant decrease in the elevation of triglycerides following DAG *versus* triacylglycerol (TG) oil ingestion was reported. Given that re-synthesized triglycerides are transported through the lymph as chylomicron (Mattson and Volpenhein, 1964; Kayden *et al.*,1967; Small, 1991), the formation of chylomicron would be expected to be reduced following DAG consumption. This is in fact supported by the reported decrease in triglyceride in the chylomicron lipoprotein fraction following DAG *versus* TG oil intake (see Section 5.1 of this report). In the liver, the absorbed fatty acids are metabolized by β-oxidation and then activated by esterification with CoA to form acyl CoA esters. Through a series of enzymatic reactions, acetyl CoA is then formed which enters the tricarboxylic acid cycle (Grundy, 1996). In this regard, DAG serves efficiently as an energy source rather than a substrate of triglyceride re-synthesis. Under the following conditions (*i.e.*, carbohydrate restriction and reduced oxaloacetate formulation) energy

production from fatty acid oxidation can vary such that metabolism can switch to the production of ketone bodies.

That the described metabolic processes are active for DAG is substantiated by findings from studies conducted by Hara *et al.* (1993) and Murata *et al.* (1994). In these studies, dietary DG, which is similar in composition to DAG, was reported to have a serum TG-lowering effect related to an impairment of chylomicron assembly and subsequent release into the blood through the lymph. As indicated, DG metabolism involves β-oxidation of the fatty acids derived from DG digestion, as opposed to triglyceride resynthesis, as evidenced by an increase in the activity of the enzymes responsible for the β-oxidation of fatty acids in the liver (Murata *et al.*, 1997).

It is important to recognize that there is variability in the absorption of fatty acids that may be present in preparations of mono- and diglycerides, as well as from the metabolism of DAG. In an evaluation of the health aspects of glycerides by the Federation of American Societies for Experimental Biology (FASEB, 1975), the absorption of saturated long-chain fatty acids, such as stearic and palmitic acid, was reported to be lower than that of unsaturated fatty acids and to cause poor growth when fed in the diet to weanling mice (Tove, 1964). Poor growth was prevented by the addition of oleate or linoleate (unsaturated fatty acids) to the diet. The fatty acid composition of DAG includes the well-absorbed unsaturated fatty acids, oleic acid (20-65%), linoleic acid (15-65%) and linolenic acid (≤15%). Only small amounts of the saturated

fatty acids (palmitic and/or stearic acid) are used in the manufacture of DAG (≤10%). The presence of the unsaturated fatty acids in DAG indicates the utility of DAG metabolic products for energy production as compared to triglyceride re-synthesis.

Taguchi *et al.* (2000; unpublished; Appendix IV-6B) compared the energy value and digestibility (bioavailability) of a DG oil and a TG oil of comparable fatty acid composition. The energy value of each test oil was determined by a bomb calorimeter. To measure digestibility of the test oils, 2 groups of 8 male rats were fed either 20g DG or TG oil/100g diet for 15 days. Food intake was recorded daily, body weights were measured every 3 to 4 days and feces from the last 3 days of the study period were collected and pooled for analysis (fecal mass and lipid content determination). Energy values for the DG and TG oils were 38.9 and 39.6 kJ/g, respectively. No significant differences occurred in food consumption, body weight, fat intake, dried fecal mass, fat excretion, fecal fat content or fat absorption coefficients between the 2 treatment groups. Absorption of the DG and TG oils was calculated to be 96.3±0.4% and 96.3±0.3%, respectively.

In conclusion, the metabolism of DAG is comparable to that of partial glycerides (mono- and diglycerides) and triglycerides. Depending upon their composition, and the overall diet composition, they are partially or completely hydrolyzed in the intestinal lumen by lipases and the resulting products are absorbed for re-esterification to triglycerides and/or oxidized as a source of fuel to varying degrees. However, for DAG, absorption, β-oxidation in the liver, and

utilization as an energy source is favored. Although energy production from fatty acid oxidation can vary depending upon a number of conditions, such as oxaloacetate availability, and lead to reduced energy output from β-oxidation and ketosis, there is no evidence that the proposed use levels of DAG would result in this condition. Furthermore, DG-rich oils have comparable energy values and digestibility coefficients to TG oils of comparable fatty acid composition.

4. Toxicological Studies

DAG has been well studied in a number of toxicological studies. The studies encompass a range of investigations, including acute, subacute, and chronic studies. Mutagenicity studies using Kao diglyceride also have been conducted. The results of these studies indicate that DAG is well tolerated, without mutagenic potential, or treatment-related toxic effects. Collectively, these studies support the safety of DAG for its intended use in foods. The ester distribution and fatty acid composition of DAG used in each study described below is provided in Appendix IV-5A. A summary of the animal toxicological studies conducted using DAG and DAG Oil in rats is provided in Table 4.0.1 and each study is described in detail in the following sections.

Table 4.0.1 Oral Toxicity Studies Conducted Using DAG and DAG Oil in Rats

Test Substance	Study Duration	Concentration [%] / Dose (g/kg body weight/day)	Results	Reference
DAG	acute	15	acute oral LD_{50} > 15 g/kg	Ishida, 1996a
DAG Oil	acute	15	acute oral LD_{50} >15 g/kg	Ishida, 1996b
DAG	4 weeks	0.2, 1.0, 5.0	NOAEL[1]:	Serabian, 1991
		0.14, 0.72, 3.48	3.48 g/kg body weight/day	
DAG Oil	105 weeks	2.6, 5.3	NOAEL:	Soni *et al.*, 2000[2]
		♂0.89/♀1.18 ♂ 1.77/♀2.35	2.35 g/kg body weight/day	

[1] no-observed-adverse-effect level
[2] submitted for publication

4.1 Acute Studies

The acute toxicity of DAG and DAG Oil has been evaluated in 2 well-conducted studies using Crj:CD (SD) SPF rats (Ishida, 1996a,b, unpublished; Appendix IV-6 and Appendix IV-6A). In the first study, a group of ten 6-week-old rats (5 males and 5 females) received a single 15 g/kg body weight dose of DAG or control product (rapeseed triglyceride) by oral intubation following an over-night fast (approximately 16 hours). Animals were observed for clinical signs and mortality for 6 hours after administration and then daily for 14 days. Body weights were measured on days 0 (immediately before administration), 1, 2, 3, 7, 10 and 14. After the 14-day observation period, animals were killed and the following organs and tissues of the cranial, thoracic and abdominal cavities were examined for possible abnormalities: brain, pituitary,

thyroid, salivary gland, thymus, heart, lung, liver, spleen, kidney, adrenal, stomach, small intestine, large intestine, pancreas, gonads, urinary bladder, and lymph nodes.

No deaths were reported in this study indicating that the acute oral LD_{50} for DAG is in excess of 15 g/kg body weight. Given that the maximum dose level required for testing in acute toxicity studies in rodents is 2 g/kg body weight (OECD, 1993), investigations at higher doses were not warranted. Clinical signs of toxicity in this study were limited to diarrhea and discoloration of the lower abdomen as a result of the diarrhea in both control and treated animals 1 to 6 hours post-dosing. These observations were not reported on days 1 to 14. These effects are probably physiological responses to the administration of a high quantity of oil (1.5 ml/100 g body weight) and not of toxicological significance. Notably, there were no effects on body weight or body weight gain in either males or females of either group. Also, no gross pathological findings were reported following necropsy.

In the second study, 2 groups of rats (5/sex/group) received an oral dose of 15 g/kg body weight of either DAG Oil or rapeseed oil (control). Parameters measured were identical to the previously described study. Diarrhea and discoloration of the lower abdomen were reported in both control and treated animals but were limited to 1 to 6 hours post-dosing only, and, again were considered to be physiological responses to the administration of a high quantity of oil. Consistent with the findings discussed above, no deaths and no differences in body weights or

weight gains were reported. Similarly, there were no abnormal findings reported in macroscopic examinations conducted upon necropsy.

Both of the studies on DAG and DAG Oil were conducted according to the Japanese Ministry of Health and Welfare Good Laboratory Practice (GLP) Standards.

4.2 Subacute Studies

The subacute toxicity of DAG was tested in a 4-week GLP-compliant dietary study using Crl:CD® BR rats (Serabian, 1991, unpublished; Appendix IV-7). In this study, 3 treatment groups and 1 control group received fat-free Purina® Basal Purified Diet. Diets were set to contain 10% fat. In the controls, the 10% dietary fat source was 5% rapeseed oil + 5% corn oil. In the DAG treatment groups, the dietary fat source was either 0.2% DAG/9.8% corn oil, 1% DAG/9% corn oil, or 5% DAG/5% corn oil (equivalent to approximately 0.144, 0.722, and 3.48 g DAG/kg body weight/day, respectively). This information is summarized in Table 4.2.1. A second control group (control group A in Table 1) received Purina® Certified Rodent Chow®. To achieve the protocol-specified 10% total dietary fat requirement, an appropriate amount of corn oil, rapeseed oil or DAG was added as a "premix" (corn oil alone or corn oil plus rapeseed oil or DAG) to the base feed for each test group (Table 1). Prior to initiation of treatment, peroxide values for premix samples for control group B and the high- and low-dose groups were tested to establish their stability. Premixes were then added to the base feeds and, after an 8-day storage

period, the fat was extracted and stability (peroxide value) was again tested for each of these groups. The fatty acid composition of the extracted fat was also tested for the high- and low-dose groups to determine the concentration of the test article. Fatty acid composition also was tested weekly throughout the study for each DAG-treated group for concentration verification. Gravimetric analysis to determine the total fat content of test diets was performed prior to treatment for the control groups and high- and low-dose groups, and then weekly for all groups throughout the study.

During the course of the study, the criteria evaluated for toxicological effects of DAG included survival (twice daily observations), clinical signs (daily), weekly physical examinations, food consumption and body weight measurements (pretreatment and weeks 1-4), ophthalmoscopic examinations (pretreatment and week 4), clinical pathology (clinical hematology, clinical chemistry, and urinalysis) (week 4), gross pathological examination (week 4), organ weight measurement and microscopic examination of all appropriate tissues (upon necropsy).

Table 4.2.1 Subacute Toxicity of DAG: Composition of Diets

Group and number of animals	Base feed	Source of 10% dietary fat
Control A (10/sex)	Purina® Certified Rodent Chow®	4.3% (in feed) + 5.7% corn oil
Control B (10/sex)	Purina® Basal Purified Diet (fat free)	5.0% rapeseed oil + 5.0% corn oil
Low dose (10/sex)	Purina® Basal Purified Diet (fat free)	0.2% DAG + 9.8% corn oil (approximately 0.144 g DAG/kg body weight/day)
Mid dose (10/sex)	Purina® Basal Purified Diet (fat free)	1.0% DAG + 9.0% corn oil (approximately 0.722 g DAG/kg body weight/day)
High dose (10/sex)	Purina® Basal Purified Diet (fat free)	5.0% DAG + 5.0% corn oil (approximately 3.48 g DAG/kg body weight/day)

Day 8 peroxide values from stability analyses performed on the fat extracted from the diets of control group B and the low- and high-dose groups were reported to be higher than Day 0 values (8.26, 4.26 and 11.8 meq/kg extracted fat *versus* 1.77, 2.76, and 4.24 meq/kg premix for Day 8 and Day 0, respectively). The significance of these elevated peroxide values, as a measure of rancidity, is not known. Day 0 peroxide values were determined from premix samples *versus* extracted fat from mixed feed on Day 8 and thus the two cannot be definitively correlated. Also, despite the finding of a significant decrease in mean food consumption in the low- and high-dose male groups compared to control group A during the study (weeks 1 to 4), mean food consumption in these DAG-treated groups was reported not to be significantly different from control group B. No statistically significant differences were reported in mean food consumption

among female groups (see discussion below). Pre-treatment gravimetric analyses indicated that the tested mixes were within ±6% of the target value of 10% fat and fatty acid composition determinations were reported to be within ±3% of target concentrations (0.2 and 5% test article for low- and high-dose groups, respectively). Total fat content and test article concentration for the mixes tested weekly during the study were all reported to be within ±10% of target values (10% fat and 0.2, 1 and 5 % test article for low- mid- and high-dose groups, respectively).

All animals survived to the end of the study and clinical observations did not reveal any indications of toxicity. Mean food consumption was significantly decreased in males of the low- and high-dose groups for weeks 1 through 4 compared to control group A; however, these values were comparable to mean values for group B. This finding was not considered to be related to the test substance as the effect was not dose-responsive and no significant differences were reported in females at any dose. Furthermore, there were no statistically significant differences in mean body weight among treated and control groups in either sex. Mean food consumption was decreased in all groups (treated and non-treated) in week 4 compared to weeks 1-3 and was considered to be due to the over-night fast required for all animals prior to blood collection for clinical pathology at the completion of the study (week 4).

Upon termination of the study, no ophthalmoscopic abnormalities were reported and evaluation of clinical pathology data (hematology, clinical chemistry and urinalysis) and gross pathology

findings revealed no treatment-related toxicological effects. A significant decrease ($p \leq 0.05$) in blood urea nitrogen (BUN) was reported in female control group B and all treated female groups compared to female control group A (10±2.8, 9±4.1, 10±3.2, 9±3.3 mg/dl *versus* 14±5.1 mg/dl, respectively). The author considered this effect to be incidental as the differences were of low magnitude and none of these values exceeded the normal range for BUN concentrations in rats (approximately 15 to 21 mg/dl) (Harkness and Wagner, 1989).

A significant increase ($p \leq 0.05$) in mean absolute mandibular salivary gland weight, but not in the relative weight, was reported in the low-dose female group compared to control groups. This effect was not treatment-related since no differences were reported at higher doses in females or in males. Evaluation of mean relative organ weight data identified a significant decrease ($p \leq 0.05$) in mean kidney weights in all treated female groups and in liver weights of all treated male groups in comparison to control group A; however, mean values for all treated groups were comparable to control group B values. This effect is likely due to a slightly higher than normal kidney and liver weight value in female and male group A controls, respectively. For example, in this strain of rat, the average kidney-to-terminal body weight ratio (%) for females aged 12 weeks is approximately 0.683 ± 0.043% (Meingassner and Schmook, 1990) and the mean relative kidney weight of control group A, at approximately 11 weeks of age, was much greater, at 0.907 ± 0.098%.

Histopathological findings from microscopic examination of tissues from both male and female high-dose groups did not reveal any treatment-related histomorphological alterations. Any microscopic changes reported (*e.g.* lymphoid peribronchial/perivascular infiltration, chronic liver inflammation) were considered consistent with those commonly occurring spontaneously in this strain of rat and were reported to have occurred in at least one of the two control groups studied concurrently. This conclusion is supported by the lack of adverse clinical pathology findings.

In summary, no overt signs of toxicity were reported in rats fed DAG at concentrations of 0.2, 1.0, or 5.0% (w/w) in the diet for at least 28 days. In addition, 5.0% rapeseed in the diet was well tolerated by rats without signs of toxicity.

4.3 Chronic Studies

The chronic toxicity of DAG Oil was investigated in a feeding study using groups of 60 male and 60 female Crj:CD (SD) strain SPF rats fed DAG Oil in the diet for 105 weeks (Soni *et al.*, 2000; submitted for publication; Appendix IV-8). As outlined in Table 4.3.1, 4 groups of 60 rats/sex were fed a low fat basal feed (L-Fat; 1.7% fat) containing DAG Oil, 5.3% in L-fat (high-dose group), DAG Oil at 2.65 % plus edible oil composed of rapeseed, corn, high linoleic safflower and high oleic safflower oils at 2.65% (low-dose group), an edible oil composed of rapeseed, corn, high linoleic safflower and high oleic safflower oils at 5.3 % (control group 1), or an edible oil composed of rapeseed and soybean oils at 5.3% (control group 2). Accordingly, each test

diet contained a total of 7% fat (5.3% test or control articles + 1.7% fat from the L-fat base feed) which is consistent with the recommended 70 g/kg fat content for the American Institute of Nutrition (AIN) Rodent Diet AIN-93G (Reeves *et al.*, 1993).

Table 4.3.1 Test Groups Studied in a Chronic Crj:CD (SD) Rat Feeding Study

Test group[a] and number of animals	Dose (% of test article in low-fat feed)
High-dose (60/sex)	5.3 % DAG Oil (approximately 1.77 [males] and 2.35 [females] g/kg body weight/day)
Low-dose (60/sex)	2.65 % DAG Oil + 2.65 % edible oil composed of rapeseed, corn, high linoleic safflower and high oleic safflower oils (equivalent fatty acid composition to DAG Oil) (approximately 0.89 [males] and 1.18 [females] g/kg body weight/day)
Control 1 (60/sex)	5.3 % edible oil composed of rapeseed, corn, high linoleic safflower and high oleic safflower oils (equivalent fatty acid composition to DAG Oil)
Control 2 (60/sex)	5.3 % edible oil composed of rapeseed and soybean oils

[a] In the study report for this study (Appendix IV-8), the high-dose DAG group is referred to as KA-1, the low-dose as ½ KA-1, control 1 as KA-M1 and control group 2 as KA-C1.

Animals that died during the study and animals killed at the 30th and 77th week were necropsied and examined. The remainder of the rats were killed at week 105 and necropsied. The parameters evaluated throughout the duration of the study are summarized in Table 4.3.2. Survival data are summarized in Table 4.3.3.

Table 4.3.2 Parameters Evaluated in a Chronic Study Using Crj:CD(SD) Rats Fed DAG Oil

Parameters	Number of animals tested	Time of measurement	
		Pre-treatment to Week 30	**Week 31 to 105**
general signs	all surviving	once daily	once daily
food consumption	all surviving	weekly	24 consecutive hours once every 2 weeks
body weight	all surviving	weekly	once every 2 weeks
clinical hematology	10/sex/group	on day of necropsy (week 30)	on day of necropsy (week 77)
clinical chemistry	10/sex/group	on day of necropsy (week 30)	on day of necropsy (week 77)
clinical urinalysis	10/sex/group	during week prior to necropsy (week 30), 3-hour fasted urine, 21-hour (consecutive) non-fasted urine and total 24 hour urine was examined	during week prior to necropsy (week 77), 3-hour fasted urine, 21-hour (consecutive) non-fasted urine and total 24-hour urine was examined
gross pathology	10-18/sex/group and all dead animals	upon death or necropsy (week 30)	upon death or necropsy (week 77 and 105)
organ weights	10-18/sex/group	upon necropsy (week 30)	upon necropsy (week 77 and 105)
histopathology	10-18/sex/group and all dead animals	upon death or necropsy (week 30)	upon death or necropsy (week 77 and 105 (females only))

Table 4.3.3 Survival of Male and Female Rats in Each Group at 105 Weeks of Administration[1]

Group	High-dose	Low-dose	Control 1	Control 2
Male	11/40	16/40	12/40	12/40
Female	12/40	10/40	18/40	9/40

[1] Values represent number of rats surviving out of a total of 40 rats that remained following the 30 and 77-week interim sacrifices.

By the end of the study (105th week of administration), 28 animals in each male control group, and 22 and 31 in each female control group (control groups 1 and 2, respectively) were reported to have died (from a total of 60 per group). Twenty-nine males and females each from the high-dose group and 24 males and 30 females of the low-dose group also died during the study. No significant differences in cumulative survival rate were seen in either sex among the test groups and no treatment-related clinical signs of toxicity were apparent.

Food consumption (g/kg body weight/day) in high-dose females significantly differed from controls, either increasing or decreasing at various time points, on a transient basis and was significantly lower or tended to be lower in the low-dose female group from days 127 to 499 compared to control group 1 and from day 50 on compared to control group 2. Due to the transient nature of this effect and the lack of effect on body weight, these differences were not considered to be of toxicological significance. Food consumption also significantly differed

from controls on an intermittent basis in the high- and low-dose male groups which also was not considered to be of toxicological significance given the transient nature of the effect. High-dose male food consumption values were significantly lower or tended to be lower than control group 2 from day 155 of administration on with no corresponding effect on body weights. Food consumption in the low-dose male group was reported to be significantly lower or tended to be lower than control group 1 from days 127 to 527 and from day 78 on for control group 2; however, male body weights in this low-dose group were only significantly lower than those of control group 2 and the effect was limited to days 148-162. These differences in body weight are not considered to be a toxicological response to DAG Oil treatment as the effect was not dose-dependent. Also, no differences in body weight values were reported on any other days among this group nor in any other group (males or females) on any day. Food efficiencies in the high- and low-dose groups (male and females) were generally comparable to controls with significant differences from controls occurring only on a sporadic and infrequent basis.

Marked or moderate occult blood in the urine was reported in 4 out of 10 males in both the high- and low-dose groups after 77 weeks of exposure, with animals in control groups presenting either no or slight occult blood (2 males in control group 2 presented with slight occult blood). The clinical significance of this finding is unknown; however, it may indicate urinary tract infections in these rats which is known to be common in older rats. Slight to mild nephropathy was reported in 6 out of 10 animals in each of these groups (high- and low-dose male); however, no

lesions were reported in the bladder and there was no accompanying decrease in hematocrit or hemoglobin values reported to indicate anemia. In addition, no significant findings were reported in females. The results of hematological analyses showed statistically-significant changes in several parameters (*eg.* activated partial thromboplastin time (APTT), prothrombin time (PT), mean corpuscular hemoglobin concentration (MCHC), eosinophilic hemogram values and platelet counts), however, these differences were all considered to be incidental as they were either limited to one sex, one collection period, or lacked dose-response or were not supported by any other changes in related clinical hematology parameters.

As with the hematological findings, the few reported significant findings in clinical chemistry parameters (*eg.* alanine aminotransferase (GPT), insulin, aspartate aminotransferase (GOT), alkaline phosphatase (ALP), high-density lipoprotein cholesterol (HDL-CHO), sodium ion and lactate dehydrogenase concentrations) also were considered to be of an incidental nature.

At 30 weeks of administration, absolute thyroid and heart weights in the low-dose male group were reported to be significantly lower than both control groups and absolute thyroid and kidney, and relative liver, weights were significantly lower than control group 2; however, no differences were reported in the high-dose male group or in the relative weights of the thyroid or kidneys in either of the male DAG Oil-treated groups. As a result, these changes were not considered to be treatment related. Also, no significant differences were reported in any of these organ weights at

77 weeks. Similarly, a reported increase in absolute pituitary weight in the high-dose female group compared to control groups 1 and 2, and an increase in relative pituitary weight compared to control group 1 at 30, but not 77 weeks of treatment, was not considered toxicologically significant.

The few statistically significant differences in organ weights in the high-dose male and female or low-dose female groups at 77 and 105 weeks (*eg.* absolute and relative brain, relative spleen, absolute thyroid, and absolute and relative heart weights) also were not considered to be toxicologically significant or treatment-related as they were either limited to one sex, one collection period, or lacked dose-response.

Notably, there were no abnormal findings upon examination at necropsy that could be attributed to exposure to DAG Oil treatment. In addition, there were no significant differences in the incidence of tumors among all groups at the 77-week termination (a low incidence of pituitary tumors was reported in all groups and mammary tumors were reported in all female groups, including controls). Non-neoplastic findings at 30, 77 and 105 weeks of administration and of all dead animals were limited to only a few animals, were often present in both control and treated animals, and were considered to be common observations in laboratory rats of this age and strain. Among decedent female rats, slight to moderate necrosis of the liver and slight to marked

extramedullary hematopoiesis of the spleen was reported in all groups with slightly higher incidences in the high-dose group.

Following terminal kill, necropsy, and histopathological evaluation, the study pathologist reported that the incidence of benign mammary gland tumors in high-dose female rats was significantly elevated compared to control group 2, but not compared to control group 1 (Appendix IV-8B). The study pathologist also reported that these tumors occurred without clear dose-dependency and that the incidence values were all within historical control ranges. Based on these data, the study author concluded that the increased incidence of benign mammary gland tumors in the high-dose females was not biologically significant.

In the original study report much of the histopathology terminology used to describe mammary gland lesions was significantly different from terminology commonly used in North America. Moreover, one of the terms used, "acinar adenosis", was rather poorly defined as to whether it represented a hyperplastic or neoplastic lesion. To standardize the terminology, the mammary gland slides from the females of all groups, including controls, were read by a reviewing pathologist, Dr. William C. Hall from Pathology Associates International (Appendix IV-8C). The results of this review are presented in Table 4.3.4 below.

Table 4.3.4 Summary of Proliferative Lesions in Mammary Gland in Early Death and Final Kill Rats

Lesion	Group			
	High-dose n=41	Low dose n=40	Control 1 n=41	Control 2 n=40
hyperplasia	0	2	4	0
fibroadenoma	10	8	12	9
fibroadenoma, multiple	8	8	8	6
adenoma	5	2	2	1
adenoma, multiple	1	1	0	0
adenoma/fibroadenoma, multiple	2	0	0	0
adenocarcinoma	7	7	9	4
adenocarcinoma, multiple	2	1	1	0
fibrosarcoma	0	2	1	0
total benign epithelial mammary gland tumors	26	19	22	16
total malignant epithelial mammary gland tumors	9	8	10	4
total rats with either benign or malignant epithelial mammary gland tumors	31*	23	26	18

* $p<0.05$ compared with control group 2, but not with control group 1.

There was a significant increase in the number of high-dose females with either a benign or malignant epithelial mammary gland neoplasms compared to control group 2 but not control group 1. Given that the normal spontaneous incidence of fibroadenoma, adenoma, and adenocarcinoma, in the mammary gland of female Sprague-Dawley rat is in the range of 24-68%, 0-12%, and 0-32%, respectively, (van Zweiten *et al.*, 1994) and noting that these tumors occur at

variable incidence, as evidenced by the differences in the 2 control groups of this study (*i.e.*, combined tumor incidence of 26 in control group 1 *versus* a combined tumor incidence of 18 in control group 2), the reported increase in total numbers of rats with a benign or malignant mammary gland tumor in the high-dose group compared to control group 2 was considered by the reviewing pathologist to be of no toxicological significance. The study pathologist concurred with these conclusions (Appendix IV-8D).

No other statistically or biologically significant differences were reported in early death or final kill females or in early death males. Histopathological examination of male tissues was not conducted at the final sacrifice. In summary, the 2-year chronic rat toxicity study revealed no toxicologically significant or treatment-related effects of DAG Oil consumption at levels of up to 5.3% in the diet.

4.4 Mutagenicity Studies

The mutagenic potential of DAG was investigated using diglyceride in an *in vitro* reverse mutation Ames assay (Jones, 1992; unpublished; Appendix IV-5). In a preliminary study, concentrations of up to 5,000 µg diglyceride/plate were tested for toxicity, as indicated by a substantial reduction in revertant colony counts or an absence of a complete background bacterial lawn. Since diglyceride was reported to be non-toxic by the study authors, 5,000 µg diglyceride/plate was chosen as the highest concentration to be used in the mutation tests.

Ethanol, the solvent used in the study, was employed as a negative control in the main study. Positive control compounds included *N*-ethyl-*N*'-nitro-*N*-nitrosoguanidine, 9-aminoacridine, 2-nitrofluorene, and 2-aminoanthracene. In the Ames tests, diglyceride at concentrations of 0, 312.5, 625, 1,250, 2,500 and 5,000 µg/plate showed no mutagenic activity when tested in 4 histidine-dependent auxotrophic mutants of *Salmonella typhimurium* (strains TA 1535, TA 1537, TA 98 and TA 100) or in a tryptophan-dependent mutant of *Escherichia coli* (WP2 *uvr*A), with and without metabolic activation by a liver S-9 fraction from Aroclor-induced rats. Results from the positive control compounds tested concurrently with diglyceride demonstrated the sensitivity of the assay and the metabolizing activity of the liver preparations.

The mutagenicity assays produced negative responses and used concentrations which were sufficiently high, according to OECD guidelines, for mutagenicity testing. Based on the results of these tests, it is concluded that DAG is devoid of mutagenic activity. Given the lack of mutagenic activity reported in these tests, no need for further evaluation through conduction of *in vivo* mutagenicity studies was considered necessary.

5. Clinical Studies

The clinical investigations conducted for DAG Oil involved a series of single-dose and repeat-dose, short and long duration studies which examined the human tolerance and nutritional effects

of DAG Oil. In total, 10 clinical studies were conducted[13/]; 4 published studies and 6 unpublished studies which support the findings of the published studies. In all cases, the clinical studies showed DAG Oil to be well tolerated and not to cause any toxicologically relevant effects, even following high-dose exposure (up to 0.782 g/kg body weight)[14/].

The ester distribution and fatty acid composition of DAG used in each clinical study described below is provided in Appendix IV-5A.

In addition to the single- and repeat-dose clinical studies described below, there are currently 11 ongoing clinical studies being conducted on DAG in adult men and women to investigate the potential beneficial effects of DAG (see Table 5.0.1). The repeated dose studies range in duration from 2 to 6 months with DAG consumed either *ad libitum* or at a daily dose of 10-40 g/day in either a food or as a cooking oil. The study of 110 subjects is being conducted in the

---

13/ In a number of the clinical studies, each clinical study parameter tested over time was analyzed statistically as a "percent variation" (*i.e.* percent of the baseline value), as indicated in the individual study reports (Appendices IV-9 to IV-14 only). The data from each of these studies were then re-analyzed using a more conservative statistical method in which the differences between the 2 treatment groups were compared with respect to the change from baseline at each time point (see Appendix IV-9A for the clinical studies statistical report). The results of these statistical analyses follow each study report in their respective appendices (Appendices IV-9 to IV-14).

14/ Serum thymol and zinc sulfate turbidity tests (TTT and ZTT, respectively) (reported indicators of liver function and fat content) were conducted in a number of the clinical studies; however, they were not considered to be appropriate, standard tests and thus their findings are not reported here.

U.S.; all other studies are being conducted in Japan. To date, no adverse effects have been reported in any of these studies.

Table 5.0.1 Summary of Ongoing DAG Clinical Studies

Study Type/Purpose	Number of Subjects	Exposure Period	Dose	Parameters
Double-blind, cross-over Investigating the suppression of postprandial remnant elevation	6 ♂	acute (DAG and TG)	51.4-57.7 g	anthropometric measurements, hematology, clinical chemistry
Double-blind, cross-over Comparison to TG on gastric emptying time	5♂/1♀	acute (DAG and TG)	20-35 g	scintigraphy imaging
Single-blind, parallel Comparison to TG oil for adverse events	200♀	6 months (DAG and TG)	*ad libitum*	questionnaire
Double-blind, parallel Investigating effects of DAG on body composition and body fat distribution	110 (number/sex not available)	6 months (DAG and TG)	*20-40 g/day*	anthropometric measurements, hematology, clinical chemistry
Monadic Long-term DAG intake	27♂/7♀	6 months	*ad libitum*	anthropometric measurements, hematology, clinical chemistry
Double-blind, parallel Investigating effects of DAG on hyperlipidemic subjects	50 (number/sex not available)	3 months (DAG and TG)	10 g/day	anthropometric measurements, hematology, clinical chemistry
Monadic	19♂/11♀	2 months	10 g/day	anthropometric measurements, hematology, clinical chemistry
Single-blind, parallel Investigating effects of DAG on elderly subjects (>50 yrs.) with hyperlipidemia	40 (number/sex not available)	3 months (DAG and TG)	10 g/day	anthropometric measurements, hematology, clinical chemistry

Study Type/Purpose	Number of Subjects	Exposure Period	Dose	Parameters
Double-blind, parallel Investigating effects of DAG on subjects with diabetes mellitus	7♂/9♀	3 months (DAG and TG)	approx. 10 g/day	anthropometric measurements, hematology, clinical chemistry
Monadic Investigating effects of DAG on subjects undergoing hemodialysis treatment	8♂/5♀	5 months	*ad libitum*	anthropometric measurements, hematology, clinical chemistry, computed tomography scans
Monadic Investigating effects of DAG in an LPL-gene deficient male	1♂	4 months	approx. 20 g/day	anthropometric measurements, hematology, clinical chemistry

5.1 Single-dose Studies

A total of 3 short-term, single-dose studies were conducted with DAG Oil at doses of approximately 0.17, 0.33 or 0.73 g/kg body weight (Watanabe *et al.* 1996,1997 b, c; Appendices IV-14, IV-11, and IV-9, respectively). A summary of the single dose clinical studies conducted using DAG Oil is provided in Table 5.1.1.

Table 5.1.1 Summary of DAG Oil Single Dose Clinical Studies, as Compared to the Estimated Intake of DAG in Humans

Number of Subjects	Exposure Period	Estimated Intake (g/kg body weight/day)	Ratio of Clinical Dose/Human Intake [1,2,3]	Reference
17 ♂ adults	single dose	0.73	3.48	Watanabe *et al.*, 1997b[4]
10 ♂ adults	single dose	0.33	1.57	Watanabe *et al.*, 1996
13 ♂ adults	single dose	0.17	0.81	Watanabe *et al.*, 1997c

[1]based on all-users consumption (90th percentile) of 0.21 g/kg body weight/day
[2]ratio of clinical dose/estimated daily human intake
[3]highest dose used in all calculations
[4]published

In all three studies, DAG Oil was administered by oral ingestion of an emulsified drink containing 10% lipid. TG, prepared to contain the same fatty acid composition as DAG Oil, was used as a control in each study. Table 5.1.2 presents the comparable fatty acid composition of each test article used in the Watanabe *et al.* (1997c) study as an example of the fatty acid composition of the test articles used in these 3 studies.

In a published study by Watanabe *et al.* (1997b; Appendix IV-9) (double-blind), 17 healthy men aged 26 to 47 ingested DAG Oil and TG, separately, with a 7-day interval between each ingestion, in an emulsified beverage containing 10% lipid at an amount equivalent to approximately 0.73 g/kg body weight. Following a standardized evening meal on the day preceding the study, subjects were required to fast until the completion of the study. Blood

samples were taken prior to ingestion (baseline values) and at 2-hour intervals post-ingestion for up to 8 hours for clinical chemistry analysis.

Table 5.1.2 Fatty Acid Composition of DAG Oil and TG Used in the Watanabe *et al.* (1997c) Study

Fatty acids	Percentage of each fatty acid (%)	
	DAG Oil	TG
C14	0.07	ND
C16	5.84	6.17
C16:1	0.17	0.26
C18	3.27	2.26
C18:1	44.07	45.52
C18:2	37.42	34.4
C18:3	7.06	10.14
C20	0.62	0.36
C20:1	0.8	0.82
C22	0.15	ND
C22:1	0.07	ND

ND = not detected
TG = triacylglycerol

Overall, DAG Oil and TG produced similar changes in clinical chemistry parameters over time. Serum TG levels increased over the first 4 to 6 hours following ingestion in both groups and then returned to near baseline levels by 8 hours post-ingestion. The increase in serum TG

concentration from baseline was significantly lower following treatment with DAG Oil in comparison to TG treatment at 6 hours post-treatment ($p < 0.05$). Serum phospholipid level changes also were significantly lower following exposure to DAG Oil *versus* TG at 4 and 6 hours post-ingestion ($p<0.01$ and $p<0.05$, respectively). No significant differences in serum free fatty acid, β-lipoprotein, ketone (as 3-hydroxybutyric acid), insulin, cholesterol or blood glucose concentrations were reported. The reduction in serum phospholipid following DAG Oil *versus* TG ingestion indicated a reduced lipoprotein content which was further substantiated by a slight reduction, at all time points, in the change from baseline of apolipoprotein C II and C III levels, key players in the biosynthesis of body fat from lipid-originating fatty acids. These findings (*e.g.* significant reductions in serum TG and phospholipid, and slight reductions in apolipoproteins C II and C III) suggest a beneficial effect in consuming DAG Oil over TG (*i.e.* reduced fat production) and its primary use as an energy source as opposed to resynthesizing triglycerides.

A second double-blind study (Watanabe *et al.*, 1996, unpublished; Appendix IV-11) was conducted to investigate the reduction in serum TG level found following ingestion of DAG Oil *versus* TG in the previous study. Ten healthy men, aged 27 to 48 years, ingested approximately 0.33 g/kg body weight of either DAG Oil or TG (prepared with a comparable fatty acid composition to DAG Oil). Blood samples were collected at 4 and 6 hours post-ingestion for hematological, clinical chemistry, and lipoprotein analysis. No differences were reported in the

hematological parameters tested or in any serum metabolic indices with the exception of TG, HDL cholesterol, and β-lipoprotein content and white blood cell counts. Consistent with the findings of the first study where a higher dose was investigated (0.73 *versus* 0.33 g DAG Oil/kg body weight), the change from baseline in serum TG concentration was significantly lower ($p<0.05$) following DAG Oil *versus* TG ingestion at 4 and 6 hours post-ingestion. In comparison to TG intake, the change from baseline concentrations of serum high-density lipoprotein (HDL) cholesterol and β-lipoprotein were significantly lower 4 hours following ingestion of DAG Oil. At 6 hours post-ingestion, white blood cells were significantly greater in the DAG-treated group in comparison to the TG-treated group, but counts were still within the normal range for a healthy adult population.

Upon analysis of each lipoprotein fraction (chylomicron, VLDL, low density lipoprotein (LDL), and HDL), fatty acid composition differed within the chylomicron and HDL fractions 4 hours following lipid ingestion and 4 and 6 hours post-ingestion in the VLDL fraction. Cholesterol and TG concentrations also were significantly lower in the chylomicron lipoprotein fraction 4 hours after DAG Oil *versus* TG ingestion. These findings support those of the previous study which indicated a decrease in lipoprotein content following DAG Oil *versus* TG ingestion, and suggest a DAG Oil-associated reduction in the formation of chylomicron.

For each test group in the final single-dose study (Watanabe *et al.*, 1997c, unpublished; Appendix IV-14), 13 healthy men (aged 27 to 42 years) ingested a single dose of DAG Oil or TG at a dose of approximately 0.17g/kg body weight following a 12-hour overnight fast. Analysis of serum metabolic indices and lipoproteins was conducted from blood samples collected 2, 4, and 6 hours after lipid ingestion. Supporting the findings of the studies using 0.73 and 0.33 g DAG Oil/kg body weight, the change in serum TG concentrations again were significantly lower following DAG Oil *versus* TG intake 6 hours after ingestion. Additional, statistically significant findings reported in the DAG Oil- *versus* TG-treated group included a greater increase from the baseline total/esterified cholesterol ratio at all time points tested and in serum phospholipid at 2 hours post-ingestion and a decrease in the ketone body 3-hydroxybutyric acid 2 hours after treatment in the DAG Oil treated group. The change from baseline concentration of total ketone also differed significantly between the 2 treatment groups with concentrations decreasing in the DAG Oil treatment group and increasing in the TG group 2 hours after administration. With the exception of a decrease in the TG content of the VLDL lipoprotein fraction in the DAG Oil-treated group in comparison to the TG group at 4 hours, no significant differences in TG or cholesterol levels were reported upon analysis of each lipoprotein fractions.

Collectively, the results of these single-dose studies indicate a significant reduction in serum TG concentration following DAG Oil ingestion in comparison to the intake of an equal amount of

TG. DAG Oil, consumed at levels of up to 0.73 kg/kg body weight/day, was reported to be well tolerated and without adverse effects.

5.2 Repeat-dose Studies

Seven studies have been conducted to investigate the possible effects of repeated ingestion (1 to 12 months) of DAG Oil. A summary of the repeated dose clinical studies conducted using DAG Oil is provided in Table 5.2.1. The first 3 of these studies were conducted in healthy men aged 28 to 49 years (Appendices IV-10, IV-12 and IV-13). In all 3 of these studies, a period of controlled diet preceded the onset of the study and fat intake was controlled throughout the course of each study. Test articles in these 3 studies were administered at a constant amount daily (g/day).

Table 5.2.1 Summary of DAG Oil Repeated Dose Clinical Studies, as Compared to the Estimated Intake of DAG in Humans

Number of Subjects Consuming DAG	Exposure Period	Estimated Intake (g/kg body weight/day)	Ratio of Clinical Dose/Human Intake [1,2,3]	Reference
8 ♂ adults	1 month	0.52 - 0.78 (44g/day)	3.71	Watanabe *et al.*, 1997d
15 ♂ adults	3 month	0.23 - 0.40 (20g/day)	1.90	Watanabe *et al.*, 1997e
19 ♂ adults	4 month	0.11 - 0.17 (10g/day)	0.81	Nagao *et al.*, 2000[4]
114 adults (95♂/19♀)	12 month	NA (*ad libitum*)	NA	Katsuragi *et al.*, 1999[4]
10 adults (7♂/3♀)	3 month	NA (*ad libitum*)	NA	Teramoto *et al.*, 2000[4]
28 ♀ (mean age of 20 years)	2 month	0.26 - 0.45	2.14	Hasegawa, 2000
13 children 9 ♀/4 ♂ (7-17 years)	5 month	0.08 - 0.40	1.90	Matsuyama, 2000

[1]based on all-users consumption (90th percentile) of 0.21 g/kg body weight/day
[2]ratio of clinical dose/estimated daily human intake
[3]highest dose used in all calculations
[4]published
NA= not available

In the first study (Watanabe *et al.,* 1997d, unpublished; Appendix IV-10), 8 men per group fasted over-night before receiving 44 g of DAG Oil or TG in a controlled breakfast diet (brioche, pancakes and mayonnaise) daily in a double-blind cross-over study, for 1 month each. Fat intake for lunches and dinners was limited to 36 grams such that consumption of the test oils exceeded 50% of the total daily fat intake (1.33 g/kg body weight). The physical condition of each subject was monitored daily for general symptoms such as diarrhea and stomach discomfort. Body

weight, body mass index (BMI), waist and hip size, and waist/hip ratios were measured before treatment with DAG Oil and TG, and at the end of the study. Hematological and clinical chemistry analyses also were performed both before test oil administration and upon completion of the study (week 4).

No differences were reported in the physical condition of the test subjects nor were any adverse symptoms or illnesses reported for either treatment. In addition, no differences were reported in mean body weights, BMI, waist or hip size or waist/hip ratios between the two groups and no statistically significant differences between DAG Oil- and TG-treated groups were reported in hematological or clinical chemistry analyses.

Included in this study was the determination of changes in liver, visceral and subcutaneous fat content following the intake of TG or DAG Oil. Results from these measurements indicated a significant difference between the two test groups in liver fat content as calculated from the ratio of liver and spleen computed tomography (CT) scan values, arising from decreased values in 4 out of 8 subjects in the DAG Oil-treated group. The liver fat content of the remaining 4 subjects in this group was reported not to have changed from the baseline values. This finding, although somewhat variable, supports the results of the single-dose studies which indicated a decrease in triglyceride biosynthesis in DAG Oil-treated humans on the basis of slight reductions in

apolipoprotein C II and C III levels and alterations in chylomicron formation. The small magnitude of change in these parameters supports the safety of use of DAG.

In the second study (Watanabe *et al.*, 1997e, unpublished; Appendix IV-12), 27 healthy men received 20 g of DAG Oil (n=15) or TG (n=12) daily in the form of an emulsified beverage and mayonnaise, for 3 months (double-blind, placebo-controlled, parallel study). Clinical pathology and hematological analyses were conducted before ingestion and then monthly to the completion of the study (3 months). Physical examinations by a specialist also were undertaken. No significant differences between the 2 test groups were identified in any of the study parameters. Overall, the metabolic changes following DAG Oil consumption were reported to be comparable to that of TG consumption. Also included in this study was an investigation of the potential effect of DAG on the absorption of fat-soluble vitamins present in the diet in comparison to TG (Appendix IV-12A). Monthly serum vitamin A, E and D (25-hydroxyvitamin D; 1-alpha-dihydroxyvitamin D; 24,25-dihydroxyvitamin D) levels were determined from fasting blood samples during the study period.

Vitamin A concentrations tended to be slightly reduced from initial values at all time points in both groups over the course of the study but the change did not reach statistical significance. Vitamin E concentrations were significantly lower than initial values at week 4 and 8 in both groups but returned to pretreatment values by week 12. 25-hydroxyvitamin D values increased

slightly over the first 2 months in the DAG-treated group, without reaching statistical significance, and then returned to initial values at 3 months. A similar increase occurred in the TG-group which reached statistical significance at month 2 and then returned to initial values at month 3. Serum concentrations of 1-alpha, 25-dihydroxyvitamin D significantly increased from initial values at week 8 in the DAG group and weeks 8 and 12 in the TG group. 24,25-dihydroxyvitamin D, which is not an active form, significantly decreased over the course of the study in both groups. Given the above findings, and the fact that no significant difference occurred between the 2 groups for any of the vitamin measurements, it can be concluded that DAG does not affect the absorption of fat soluble vitamins present in the diet.

A third study (double-blind, parallel), published by Nagao e*t al.* (2000; Appendix IV-13) and conducted in a manner similar to the studies described above, investigated the effects of daily ingestion of 10 g DAG Oil or TG in 2 groups of 19 healthy men who were mildly to moderately obese (as determined from BMI calculations of 21.7 to 27.7). The study was conducted for a period of 4 months. A significantly greater reduction in body weight at 8, 12, and 16 weeks of exposure and in the BMI at 12 and 16 weeks, was reported in the DAG Oil-treated group. Changes in a number of nutritional parameters also were reported. Waist circumference and hip/waist ratios were significantly more reduced in the DAG Oil group compared to the TG group at 16 and 8 weeks of exposure, respectively. Reductions in total and visceral fat from baseline values were significantly greater in the DAG Oil group following 4, 12, and 16, but not

8, weeks of exposure in comparison to TG exposure. Reductions in subcutaneous fat also were significantly greater in the DAG Oil group at 12 and 16 weeks of exposure. The change from baseline in the liver CT/spleen CT ratio in the DAG Oil group was significantly increased compared to the TG group at 8 and 12 weeks of exposure. Changes in leukocyte counts in the DAG Oil group at 4, 12, and 16 weeks were significantly different than the TG group; leukocyte counts in the DAG Oil group at each of these time points were lower than baseline counts whereas the corresponding values in the TG group were higher than baseline counts. These changes in leukocyte counts are not considered to be biologically significant given that the counts were within the normal range for the general population and due to the large inter- and intra-individual variation in the values that existed in both treatment groups. Total ketone, acetoacetic acid and 3-HBA levels were generally increased over the course of the study in the DAG group in comparison to initial values, but the changes were small and no significant differences occurred between the 2 treatment groups despite slight reductions in the levels of these ketone bodies in the TG group. Additional differences between the 2 treatment groups also were reported in glucose and HDL concentrations (slight decreases from initial values in the DAG group compared to increases from initial values in the TG group); however, these findings also were not considered biologically significant or treatment-related as they were limited to a short-term consumption of DAG Oil and did not persist beyond 4 weeks of exposure.

In a later published study, a total of 114 adults (95 males and 19 females) with an average age of 39 years were administered DAG in the diet for 12 months (Katsuragi *et al.*, 1999; Appendix IV-14A). The males in this study had, on average, a larger BMI than the Japanese standard value for this age group (24.7 *versus* 23) and 26% of the study population had a serum triglyceride level exceeding the standard level set by the Japan Atherosclerosis Society (150 mg/dl). Blood samples and body measurements were collected prior to the start of the study and every 3 months thereafter and included body weight, body mass index, waist circumference, hip circumference, skin-fold thickness[13/], serum lipid levels (triglycerides, total cholesterol, and LDL- and HDL-cholesterol, free fatty acids, phospholipids, esterified cholseterol, free cholesterol), liver function indices (GOT, GPT, γ-GTP, total bile acid), white and red blood cell counts, hemoglobin, hematocrit, amylase and plasminogen activator inhibitor type-1 (PAI-1) levels.

DAG, in the form of a cooking oil (80% DAG), was used in place of all other edible oils for all breakfasts and dinners at the subject's homes, and lunches were requested to be consumed in the Kao Corporation cafeteria where the oil was used, when appropriate, in the preparation of the food. Alcohol consumption was prohibited on the day prior to blood sampling and dinners were completed by 9 pm with no breakfast being eaten on the day of sampling. No other dietary restrictions were imposed.

13/ Sum of the upper arm and the back.

Total energy intake during the study was 2,005 kcal/day which was 8.4% higher than the initial value and fat intake was slightly increased from the initial value at 3, 9, and 12 months. Body weight tended to decrease over the course of the study and was significantly lower than initial values in males, but not females, by the end of the study. Hip circumference was significantly lower than initial values at 3, 6 and 12 months. Waist circumference and skin-fold measurements also decreased during the study and were significantly different from pre-treatment values at 6, 9 and 12 months and 3, 6, 9 and 12 months, respectively.

In individuals with an initial serum triglyceride level below 150 mg/dl, no significant changes occurred in serum TG levels except for a significant increase from the pre-treatment value at 6 months. This was considered to be an incidental finding given that no significant increase in serum TG was noted at 9 months and that serum triglyceride levels tended to decrease in individuals with initial values that exceeded 150 mg/dl. Among study subjects with an initial serum triglyceride level between 150 to 200 mg/dl or exceeding 200 mg/dl, serum TG levels decreased over the course of study and were significantly lower than the initial value at 6 months and 3, 6 and 9 months, respectively.

Total cholesterol levels remained relatively unchanged over the course of the study while serum HDL- and LDL-cholesterol levels increased and decreased, respectively. These changes reached statistical significance at 6, 9 and 12 months for HDL-cholesterol and 6 and 9 months for LDL-

cholesterol[14/] and PAI-1 levels were significantly reduced at 6, 9 and 12 months in comparison to values at 3 months. These study findings support those of the other clinical studies in indicating a positive effect of DAG consumption on HDL- and LDL-cholesterol levels and in lowering serum triglyceride levels. Slight changes occurred in a number of the clinical chemistry parameters that reached statistical significance at various time points during the study (*i.e.*, phospholipids, esterified cholesterol, free cholesterol, total bile acid, GOT, γ-GTP); however, the changes were considered to be incidental since they were small, values were within the normal ranges for each parameter, and the changes were not time-responsive. A significant increase in GPT and amylase occurred in the middle of the test period but the increases were small in magnitude and disappeared by the end of the test period. Significant differences from initial values also occurred periodically in red blood cell counts and hemoglobin and hematocrit values that again were small and still within the normal healthy ranges for each parameter.

In a study investigating the effects of DAG intake on serum lipids in hyperlipidemic adults receiving dialysis treatment, 7 males and 3 female adults received DAG as a replacement for cooking oil for a period of 3 months (Teramoto, 2000; published; Appendix IV-14B). Fasting blood samples, prior to hemodialysis treatment, were collected before DAG treatment, monthly during the DAG treatment (3 months) and then monthly for 3 months during a wash-out period.

14/ LDL cholesterol level was calculated using the Friedewald equation and applied only to subjects whose serum TG level was below 350 mg/dl

Total, visceral, and subcutaneous fat areas were measured by CT scans before and at the end of the DAG treatment period, and then 3 months after DAG treatment. The average intake of DAG during the study was approximately 9 g/day.

Total and visceral fat areas were significantly decreased by the end of the DAG treatment period in comparison to initial values and then returned to pre-treatment values by the end of the wash-out period. Similar changes, although not statistically significant, occurred in subcutaneous fat area values and the visceral/subcutaneous fat ratio by the end of the DAG treatment period and the wash-out period. Serum TG levels increased initially then returned to basal levels by the end of the treatment period while serum HDL and VLDL levels tended to increase and decrease, respectively, over the course of the treatment period, without statistical significance, before returning to near basal levels by the end of the wash-out period. Serum free fatty acid levels tended to increase significantly during the treatment period and remained higher than basal levels during the wash-out period. Lipoprotein(a) levels tended to decrease slightly during the treatment period while no changes were noted in total or HDL-cholesterol levels during the study.

The nutritional effects of DAG in children with hyperlipidemia and/or obesity also has been investigated (Matsuyama, 2000; unpublished; Appendix IV-14C). Prior to the start of the study, a diet content survey was conducted and fasting blood samples, body size measurements and CT-

scans were obtained for each of the 13 volunteers. Four male and 9 female children, aged 7 to 17 years, received DAG, *ad libitum*, in the form of a cooking oil for 5 months. Guardians of the participants were instructed to provide a similar diet content to the children during the study as before its commencement. Fasting blood samples and body measurements (weight, height, waist, hip) were collected each month. Body measurements were used to calculate obesity level, body mass index and Rohrer's index[15/] for each individual. Blood samples and CT-scans were used to test for changes in the following parameters: serum leptin, serum PAI-1, glucose, glycoalbumin, insulin, creatinine, serum lipids (phospholipids, total ketone bodies, acetoacetic acid, 3-hydroxybutyric acid, total cholesterol, LDL-cholesterol, HDL-cholesterol, free cholesterol, serum triglycerides, total/esterified cholesterol ratio, and free fatty acids) and visceral, subcutaneous and total fat area.

The average consumption of DAG in the study, for all 13 volunteers, was 11.3 g DAG/person/day or 0.2 g DAG/kg body weight/day. Among the 13 individuals, 6 were aged 7 to 11 years (children) and consumed, on average, 0.27 g DAG/kg body weight/day during the study. The remaining individuals, aged 13 to 17 (teenagers), consumed an average of 0.15 g DAG/kg body weight/day during the study.

15/ Rohrer's index = body weight (kg) ÷ height $(m)^3$ x10

Height increased significantly over the course of the study and a statistically significant increase in body weight from initial values occurred at 2, 4 and 5 months. Overall, body weight values increased in 9 of the 13 individuals, remained unchanged in 1 and decreased slightly (from 65.5 to 64.0 kg and from 77 to 76.5 kg) in the remaining 2 individuals. The changes in body height and weight also were compared to Japanese standard growth curves. Eleven of the 13 children had similar or higher growth curves than the Japanese standards but 2 females, aged 11 and 13, had lower curves. The height of the 13-year-old female remained relatively unchanged over the course of the study; however, she had already reached the average height for a 17-year-old female upon initiation of the study. Therefore, her growth curve was not considered to be abnormal. The 11-year-old grew 1.5 cm during the course of the study and thus, despite being lower than the standard growth curve for her age, her lower rate of change in height was not considered to be of biological significance. Furthermore, each of these individuals consumed an average of 8 g DAG/day in comparison to other study subjects that consumed up to 14.7 g DAG/day without adverse effect.

Waist measurements were significantly increased at 3, 4 and 5 months as were hip measurements at 3 and 4 months. Upon calculation of waist/hip ratios, a significant increase in this ratio occurred in month 3 only. Obesity level, calculated based on standard body weights and heights for Japanese children, generally increased over the course of the study and was significantly increased from initial values at 2, 4 and 5 months. Body mass index remained relatively

unchanged over the course of the study but was significantly increased from initial values at the end of the study (month 5). No significant changes occurred in Rohrer's index values. CT-scan images revealed a significant decrease in total and visceral fat area at 3 and 5 months and a significant decrease in subcutaneous fat area at 3 months.

No biologically or statistically significant differences were reported in the levels of various serum lipids tested, with the exception of a significant decrease in serum triglyceride level in month 4 and a significant increase in HDL-cholesterol in months 4 and 5. No significant changes were reported in serum glycoalbumin, insulin or creatinine. Serum glucose showed a tendency to decrease over the first few months and significantly decreased from the initial value at month 2. Significant decreases from initial values also occurred in serum PAI-1 and leptin levels at 2 and 2, 3, and 4 months, respectively.

The study findings add further support to the safety of DAG consumption. No adverse effect occurred in children consuming DAG in the diet at exposure levels ranging from 5.3 to 14.7 g DAG/person/day or 0.08 to 0.4 g DAG/kg body weight/day. The positive changes in HDL-cholesterol and serum triglyceride levels also add further support to the potential benefits of DAG consumption.

The nutritional effects of DAG also were investigated in a 2 month double-blind parallel study in 56 healthy females (Hasegawa, 2000; unpublished; Appendix IV-14D). Two groups of 28 women, with an average age of 20 years, were administered either 20 g DAG or TG in bread, cookies, soup or shortbread for 8 weeks. Total daily intake of fats and oils was restricted to approximately 60 g/day and all food items, except test oils, were the same between the 2 groups during the week. Weekend meals consisted of recommended food items. The test articles were of comparable fatty acid composition. Pre-treatment measures included body size determinations (weight, height, waist, and hip measurements), blood samples (serum triglyceride, total cholesterol, HDL-cholesterol, free fatty acid, total ketone body, acetoacetic acid, 3-hydroxybutyric acid, fasting blood sugar, insulin, GPT, GOT, and γ-GTP) and CT-scans for visceral and subcutaneous body fat determinations. Body size measurements and blood samples were collected again at 4 and 8 weeks for comparative analyses and CT-scans were performed again at 8 weeks. Alcohol consumption was prohibited on the days prior to blood sampling and dinner was completed by 9 pm with no breakfast being eaten on the day of sampling.

Mean total energy intake was approximately 1,900 kcal/day and mean total fat intake, including test oils, was 58 g/day, in each group, without significant difference between the 2 test groups. The average consumption of DAG in the study was 0.36 g/kg body weight/day and ranged from 0.26 to 0.45 g/kg body weight/day. Average TG consumption was 0.36 g/kg body weight/day and ranged from 0.30 to 0.42 g/kg body weight/day.

No significant changes occurred in body weight, waist or hip measurements or in the waist/hip ratio over the course of the study. Visceral fat area decreased significantly in both groups at 8 weeks in comparison to initial values, without significant difference between the two groups. Subcutaneous fat area decreased by 8 weeks; however the decrease did not reach statistical significance in either group and there was no significant difference between the 2 groups. Serum triglyceride levels increased over the course of the study in both groups and levels at 8 weeks were significantly increased from initial values but did not differ significantly between the 2 groups. Total cholesterol levels differed significantly between the 2 groups prior to the administration of the test articles but not thereafter. Total cholesterol levels decreased over the course of the study in both groups with decreases in the DAG group significantly differing from initial values at both 4 and 8 weeks. HDL-cholesterol decreased, without statistical significance, in both groups by 8 weeks but no significant difference occurred between the 2 groups. Free fatty acid levels were significantly increased in both groups at week 4 and 8 and were significantly different between the 2 groups at week 8. Total ketone, acetoacetic acid and 3-hydroxybutyric acid levels generally increased over the course of the study in both test groups and were significantly different from initial values at various time points in one or both of the groups but did not differ significantly between the 2 groups. Insulin levels significantly increased from initial values in both groups at week 4 and 8 but did not differ significantly between the 2 groups. Fasting blood sugar and serum GOT, GPT, γ-GTP did not change significantly during the test period.

Overall, no adverse effects occurred in body measurements or blood parameters following an 8 week intake of up to 0.45 g DAG/kg body weight/day.

In conclusion, DAG Oil was reported to be well tolerated in the clinical trials and consumption resulted in the same general metabolic changes as TG. In a number of the studies, beneficial effects were noted on HDL- and LDL-cholesterol levels and, in particular, on serum triglyceride levels. The extent of DAG-induced alterations in serum lipids appears to be modified by diet composition.

It is well known that the intake of medium chain triglycerides (MCT), composed principally of 8 to 10 carbon atoms, results in an increase in ketone bodies due to the conversion of some of the acetyl-CoA derived from the oxidation of the medium chain fatty acids into acetoacetate and D-β-hydroxybutyrate (ketone bodies) in the liver which is perfused with these fatty acids following the digestion and metabolism of the parent triglyceride (Pi-Sunyer *et al.*, 1969; Greenberger *et al.*, 1968; Bougneres *et al.*, 1989). The increase is dependent upon the fatty acid composition of the triglyceride and is generally 5 to 8 times greater than that induced by general (long-chain) triglycerides, and approximately equal to the ketone body concentration characteristic of a fasted-state (Owen and Cahill, 1973). This rapid, short-term increase could present a possible health risk to diabetics. No significant alterations in ketone levels were reported in the single-dose clinical studies; however, acute results are not easily or directly comparable to the findings of

longer term studies which are more important in assessing safety and it is anticipated that the conditions of use of DAG will vary such that the energy output and ketones produced will also vary. In the repeated-dose clinical studies discussed in this report, DAG consumption was associated with an increase in ketone concentration; however, this increase was small, comparable to that induced by TG intake, and may have been influenced by over-night fasting prior to blood sampling. As a result, given the lack of significant chronic effects following long-term consumption of DAG, it is not expected that DAG would have any adverse effects in diabetics.

In the study conducted by Nagao *et al.* (2000), consumption of 10 g DAG Oil/day for 4 months was reported to cause a significant, time-responsive decrease in mean body weight and BMI in 19 adult men. In the study conducted by Katsuragi *et al.* (1999), a larger study population (109) was used which included both adult men and women. Body weights tended to decrease over the course of the 9 month exposure to DAG in this study; however, changes from pre-treatment values never reached statistical significance. Although a reduced body weight may not be considered to present a safety concern for adults, and may even be considered a beneficial effect for some, the potential disruption of the normal growth and development of children would not be a desirable effect. Two studies, one carried out using 13 moderately obese and/or hyperlipidemic children aged 7 to 17 years and another, stronger study, using 56 young women (average age of 20 years), support the safe use of DAG in children at the estimated exposure

level for this population group (0.38 g/kg body weight/day). Collectively these studies provide data supporting the safe use of DAG following a longer exposure period to DAG (up to 5 months) and/or higher exposure levels, than in the Nagao *et al.* (2000) study. In the children's study, no adverse effects occurred in body size measurements, including body weight and BMI, or blood parameters following exposures ranging from 0.08 to 0.4 g DAG/kg body weight/day and averaging 0.2 g DAG/kg body weight/day, for 5 months. Similarly, no adverse effects were noted in 20-year-old women consuming DAG for 2 months at exposure levels ranging from 0.26 to 0.45 g/kg body weight/day and averaging 0.36 g DAG/kg body weight/day. Notably, a large number of the women in the DAG treated group in this study consumed more than the mean intake of DAG for the study, up to 1.25 times the mean, without adverse effects. In conclusion, the available clinical data supports the safe use of DAG at the estimated levels of exposure from its intended use in foods in all population groups, including children.

6. Review of the Safety Aspects of DAG-Related Substances

DAG is comprised of at least 80% DG, no more than 20% TG and 5% MG, and trace amounts of antioxidants and emulsifier; therefore, its primary constituents are all components of mono- and diglycerides. Similar to DAG production, the mono- and diglycerides used in the food industry are synthesized largely by direct esterification of glycerin and fatty acids. Mono- and diglycerides are generally recognized as safe (GRAS) as direct human food ingredients and are approved for use in food as dough strengtheners, emulsifiers, flavoring substances and adjuvants,

formulation aids, lubricant and release agents, solvents and vehicles, stabilizers and thickeners, surface-active agents, surface-finishing agents and as texturizers (21 C.F.R. § 184.1505). In this regard, mono- and diglycerides are used in foods such as baked goods, carbonated beverages, confections, jam, jellies, dairy products, coffee whiteners, and whipped toppings (Informatics Inc., 1973; FASEB, 1975; Akoh, 1998). Given that DG is a component of the mono- and diglycerides used in these food products, the primary constituent of DAG has been consumed in the human diet for many years. Furthermore, as described in Section 3.1 of this report, DG occurs naturally in the body through the digestion of triglycerides.

Adding further to the body of evidence in support of the safety of DAG, the partial glycerides (mono- and diglycerides) have been evaluated by JECFA (1974) and FASEB (1975). Based on an analysis of the metabolism of mono- and diglycerides, JECFA concluded that "there is no evidence that the presence of monoglycerides and diglycerides of food fats has any deleterious effect on cells or tissues". In consideration of the variable digestibility of fatty acids that are present in mono- and diglyceride food additive preparations, JECFA reported findings that indicated that saturated long-chain fatty acids have a lower digestibility than unsaturated fatty acids if fed alone, or in large quantities, and that administration of numerous polyunsaturated fatty acids can cause a reduction in blood cholesterol levels (conversely, ingestion of saturated fatty acids increases blood cholesterol levels). Notably, the fatty acid composition of DAG includes the unsaturated, well-absorbed fatty acids, oleic acid (20-65%), linoleic acid (15-65%),

and linolenic acid (≤15%). Only small amounts of palmitic and/or stearic acid are used in the manufacture of DAG (≤10%).

Few toxicological studies on specific mono- or diglycerides have been reported. Due to the fact that most mono- and diglycerides are derived from glycerin and natural fatty acids, it is likely that toxicological investigation has been deemed unnecessary. Both JECFA and FASEB have reviewed the few toxicological studies that have been conducted in animals using partial glycerides. The studies reviewed by JECFA included a short-term (22-28 weeks) feeding study where hamsters were fed 5 or 15% glyceryl monostearate in the diet, and 4 long-term feeding studies (two 2-year studies, one 3-generation study and one of unspecified length) conducted in rats using concentrations of up to 25% glyceryl monostearate or a mixture of lauric acid glycerides in the diet. JECFA concluded that no significant histopathological changes were evident in any of these studies. Increased liver weights reported in a few studies using long-chain saturated fatty acids were considered by JECFA to be common in animals receiving a high fat diet and not to be of toxicological significance. FASEB reviewed additional studies conducted in dogs, rats and chick embryos to investigate the possible toxicological effects of diacetyl tartaric acid esters of mixed mono- and diglycerides, acetoglycerides, acetostearin, acetoolein, triacetin, glycerol lacto-fatty acid esters, succistearin, oxystearins and sulfoacetyl derivatives. In one of these studies, Ambrose *et al.* (1958a) reported decreased body weight and reproductive performance, and differences in organ weights (liver, testes and kidney) in rats fed

20% acetostearin in the diet compared to control rats. In another study, the reproductive performance of rats fed a diet containing 10% acetostearin was reduced, as indicated by a decrease in the number of litters, total number of pups, and total number weaned. The effect was considered due to a vitamin E deficiency despite normally adequate amounts being present in the acetostearin-diet (Ambrose *et al.* 1958b). Ambrose *et al.* (1958b) concluded that acetostearins may facilitate the destruction of vitamin E. In all other studies, no significant treatment-related toxicological effects were reported.

JECFA concluded that mono- and diglycerides have no acute or chronic toxic effects at "practicable" doses. Similarly, FASEB concluded that,

> There is no evidence in the available information on mono- and diglycerides of fat-forming fatty acids that demonstrates or suggests reasonable grounds to suspect a hazard to the public when they are used at levels that are now current or that might reasonably be expected in the future.

Based on the lack of adverse findings in animals studies and a consideration of the daily consumption of these partial glycerides in the human diet and their endogenous presence in the body through the digestion of triglycerides, JECFA allocated an acceptable daily intake (ADI) of "not limited" for mono- and diglycerides. ADI "not limited" is a term no longer used by JECFA

that has the same meaning as ADI "not specified". With respect to food additives, "not specified" is defined by JECFA as follows,

> A term applicable to a food substance of very low toxicity which, on the basis of the available data (chemical, biochemical, toxicological, and other), the total dietary intake of the substance arising from its use at the levels necessary to achieve the desired effect and from its acceptable background in food does not, in the opinion of JECFA, represent a hazard to health. For that reason, and for reasons stated in individual evaluations, the establishment of an acceptable daily intake expressed in numerical form is not deemed necessary (JECFA, 1996).

To illustrate how safety testing programs effectively provide the information required to make safety evaluations of emulsifier in fats and oils, Artman (1975) conducted a review of the studies carried out with mono- and diglycerides, similar to those conducted by JECFA and FASEB. In his review, Artman (1975) reported the findings of a number of toxicity studies conducted in rats which included one 10-week and one multi-generation feeding study in which rats were fed mono- and diglycerides at concentrations of up to 25% in the diet. In addition, an 11-month developmental study (15% mono- and diglycerides in the diet), and a 2-year feeding study using 100 rats (doses not specified) also were reviewed. Artman (1975) concluded that the few adverse effects that have been reported in animal studies, such as reduced body weight or body weight

gain (Ambrose *et al.*, 1958a; Tove, 1964), were reported to have occurred when high doses of partial glycerides containing saturated fatty acids were administered to the test species and, therefore, were likely to be the result of poor absorption.

Given that DG, the primary constituent of DAG, is a component of mono- and diglycerides, these safety evaluations provide further support to the safety of DAG for its intended use in food.

7. Summary and Discussion

There is a wealth of information which supports the safety of DAG for human consumption. The metabolism of DAG does not differ significantly from the well-characterized metabolism of mono- and diglycerides. The digestive product of DAG, 1-monoglyceride, is hydrolyzed to glycerol and free fatty acids (Mattson and Volpenhein, 1964; Small, 1991; Watanabe *et al.*, 1997a). The glycerol is then either utilized in the synthesis of triglycerides *via* the glycerol 3-phosphate pathway or converted directly into glucose. The fatty acids of DAG are considered to be absorbed into the portal blood for β-oxidation in the liver (Watanabe *et al.*, 1997a), thereby serving as a source of energy as opposed to being utilized for the re-synthesis of TG. This conclusion is supported by a significant reduction in TG elevation following DAG ingestion compared to TG ingestion reported in the single-dose clinical studies.

The safety of DAG has been demonstrated in toxicological studies in rats, including acute, subacute and chronic investigations from which there were no adverse toxicological findings that could be attributed to treatment with DAG. In the acute rat study, diarrhea was reported in both treated and control animals; however, this was considered to be a physiological, not toxicological, response to administration of a high quantity of oil. In addition, Kao diglyceride tested negative in the Ames test for mutagenic potential in 4 *Salmonella typhimurium* strains (TA 1535, TA 1537, TA 98 and TA 100) and a tryptophan-dependent mutant of *Escherichia coli* (WP2 *uvr*A), with and without metabolic activation.

A total of 10 clinical studies have been conducted to investigate the human tolerance and nutritional effects of DAG. To this end, DAG was found to be well tolerated, without toxicological effects, and to produce similar metabolic changes as TG (control). In particular, DAG intake resulted in a decrease in the temporary elevation of blood triglyceride which occurs following lipid ingestion. Watanabe *et al.* (1997a) considered this effect to be indicative of a decrease in triglyceride re-synthesis upon catabolism of DAG digestive products. Watanabe *et al.* (1997a) considered DAG metabolism to alternatively involve the β-oxidation of fatty acids in the liver for the provision of energy.

Important to the safety assessment of DAG is a consideration of potentially sensitive populations. Infants and children may respond differently from adults to exposures to substances

such as food additives as a result of being in a state of growth and development and/or due to differences in the toxicokinetics or toxicodynamics of substances (Ostergaard and Knudsen, 1998). ADIs, defined as "an estimate by JECFA of the amount of a food additive, expressed on a body weight basis, that can be ingested daily over a lifetime without appreciable health risk" (WHO, 1987), are applicable to children older than 12 years of age; however, the ADI allocated for mono- and diglycerides, which are comparable to DAG, is "not limited". On this basis and the fact that daily consumption estimates for these partial glycerides have been evaluated, on a per kg of body weight basis, for specific age groups designated as 0-5, 6-11 and 12-23 months and 2-65+ years (FASEB, 1975), the safety assessment data contained in this report are intended to support the consumption of DAG by adults and children aged 2 years or more. The development of digestive organs in infants is considered to be comparable to that of adults by approximately 20 months of age (Ostergaard and Knudsen, 1998), suggesting that infants older than 20 months could consume DAG in the diet safely. Since DAG is intended for home use as a cooking oil and as an ingredient in formulating commercially-prepared margarine, infant exposure to DAG is expected to be minimal.

In the clinical studies discussed in this report, DAG consumption was associated with an increase in ketone concentration; however, this increase was comparable to that induced by TG intake and may have been influenced by over-night fasting prior to blood sampling. As a result, given the lack of significant chronic effects following long-term consumption of DAG, it is not expected

that DAG would have any adverse effects in diabetics. In conclusion, DAG metabolism is not expected to cause adverse health effects and is considered safe for use in the same manner as all other general edible oils.

Adding to the safety profile, are the high standards employed in the manufacturing of DAG. The raw materials utilized in its production are food grade materials permitted for use in the manufacture of foods and all are commonly and widely used in the food manufacturing industry. The safety of the immobilized lipase preparation used in the esterification process in manufacturing DAG has been evaluated in the literature and is considered safe for use in food manufacturing. Furthermore, tests conducted to determine the presence of any residues in the final product from the resin used to immobilize the lipase indicated that only very low levels of extractables (nitrogen and formaldehyde) were present.

As previously stated, the primary constituent of DAG is DG. DG is consumed in the diet as a minor component of oils and fats (*i.e.* DG is a component of mono- and diglycerides) and is also formed endogenously by the metabolism of TG during the digestion and absorption of oils and fats (Small, 1991; Mattson and Volpenhein, 1963). This endogenous formation has even been reported in infants with underdeveloped digestion and absorption functions (Olivecrona *et al.*, 1973; Hamosh *et al.*, 1978). DG is composed of several isomers (1,2/2,3-DG, 1,3-DG). Endogenous DG, derived from the digestion of TG in the duodenum, exists largely as 1,2/2,3-

DG (Small, 1991; Hamosh and Scow, 1973; Borgstrom *et al.*, 1957; Douglas *et al.*, 1953). Exogenous DG, derived from general edible oils and emulsifier (mono- and diglycerides) exists generally as a mixture of 1,2/2,3-DG and 1,3-DG at varying ratios (Siew 1995; Perez-Camino *et al.*, 1996; Koplivnjak *et al.*, 1995; Goto *et al.*, 1995; Yamane *et al.*, 1994). In the case of solid form DG, 1,3-DG is reported as being more stable than, 1,2/2,3-DG; therefore, mixtures of the two isoforms stored below their melting point undergo rapid isomerization to contain mostly 1,3-DG isoforms (Goto *et al.*, 1995; Groot, 1972; Yamane *et al.*, 1994). DG contained in commercial solid or plastic-like products such as mono- and diglycerides, including DAG, are expected to exist largely as 1,3-DG rather than 1,2/2,3-DG isoforms (Miraglia *et al.*, 1985). The various isomers of DG are not distinguished from one another and are reported to be metabolized by the same metabolic processes (FASEB, 1975). Further, the nutritional value of DG is considered to be equivalent to that of TG and MG (Harris and Sherman, 1954; Mattson *et al.*, 1951).

In addition to these DAG-specific indices of safety, it is worthwhile to consider the safety of substances related to DAG. Of particular relevance is the safety aspects of mono- and diglycerides, as the primary constituent of DAG, DG, is a component of these partial glycerides. A number of studies have been conducted in animals to determine the possible toxicological effects of mono- and diglyceride exposure and their carcinogenic and mutagenic potential. No adverse effects were reported that could be attributed to treatment (Fitzhugh *et al.*, 1960; Eagle

and Poling, 1956; Litton Bionetics Inc., 1975; Holtz *et al.*, 1996; Migas *et al.*, 1997; Gustavsson *et al.*, 1995). It is important to note that mono- and diglycerides have been given GRAS status (FDA, 1999a) and an ADI of "not limited" (JECFA, 1974) based on reviews of the toxicological data derived from animal studies that show no significant adverse effects. In addition, glyceryl behenate, a substance containing more than 77% di- and triglycerides (Parexel International Corp., 1986), has also been given GRAS status (21 C.F.R. § 1328 (2000)).

DAG has the same general physical properties as mono- and diglycerides, *i.e.*, all are insoluble in water and soluble in ethanol, chloroform, and benzene, and all are metabolized *via* the same general metabolic pathways. The mono- and diglycerides used in the food industry are synthesized largely by direct esterification of glycerin and fatty acids (FDA, 1999a), a method comparable to that of DAG production (*i.e.*, the esterification of fatty acids derived from natural edible plant oils and either MG or glycerol).

The safety and function of DAG was approved by the Ministry of Health and Welfare in Japan on May 20, 1998, and the cooking oil prepared with DAG Oil is permitted for sale as a "Food for Specified Health Use" (FOSHU). FOSHU is defined as a food which has had beneficial, effective ingredients added to help in the maintenance of a healthy body condition. The standards required of such foods include criteria stating that the food should be expected to contribute to the improvement of one's diet and the maintenance/enhancement of health, the food

or its constituents should be safe to eat, and there should be no significant loss in nutritive constituents of the food in comparison with those normally contained in similar types of foods. For details of FOSHU criteria see Appendix IV-4.

8. Conclusions

The data and information contained within this report support the conclusion that the intake of DAG would not be expected to cause adverse effects in humans under the conditions of its intended use in food. The available studies demonstrate that DAG digestion products are readily absorbed and metabolized to serve largely as a source of energy. Moreover, DAG components occur endogenously and naturally in the diet. Both animal toxicological studies and clinical studies have consistently demonstrated the safety of DAG, even when consumed on a daily basis in high amounts. Based on the entire safety package for DAG, it is concluded that DAG is safe for its intended use in food.

B. Reports or Investigations That May Appear Inconsistent

From February 1999 through May 2000, fifteen complaints have been received by Kao from DAG consumers, including slight heartburn, light constipation, light diarrhea, and minor eczema. Kao conducted follow-up on each complaint and observed that the effects were not serious. Further, Kao believes there was no correlation between these events and consumption of DAG.

Any inconsistent findings reported within the available studies conducted using DAG are discussed in detail in section VI A. (Information to Assess the Safety of DAG). None of these findings are inconsistent with the conclusion that DAG is GRAS.

C. Basis for Expert Consensus

An independent panel of recognized experts (Expert Panel), qualified by their scientific and/or medical training and relevant national and international experiences to evaluate the safety of food and food ingredients, was requested by the Kao Corporation to determine the GRAS status of DAG for use as a general edible oil. The members of the Expert Panel are:

- Joseph F. Borzelleca, PhD.
  Medical College of Virginia;
  Toxicology & Pharmacology, Inc.
- Walter H. Glinsman, M.D.
  Fellow, Georgetown University
  Center for Food and Nutrition Policy;
  Glinsmann, Inc.
- David Kritchevsky, PhD.
  Caspar Wistar Scholar
  The Wistar Institute
- Ian C. Munro, PhD., FRCPath.
  CANTOX HEALTH SCIENCES INTERNATIONAL

Their resumes appear in Appendix VI.

The Expert Panel independently and collectively critically evaluated available relevant human and experimental information on DAG and DAG Oil, and concluded that DAG, meeting appropriate food grade specifications and produced in accordance with current good manufacturing practices, is GRAS by scientific procedures when used as a cooking oil for home use and in commercially prepared margarine for home use. A copy of this opinion is attached at Tab 1.

VII. Statement of Compliance with Good Laboratory and Clinical Practices

All toxicity tests in section VI, excluding the long-term feeding study, were conducted in compliance with the following Good Laboratory Practices. A compliance statement is filed in each report. The tests are as follows:

- Mutagenesis test by Huntingdon Research Center Ltd. in England is in compliance with the Good Laboratory Practice in United States, Japan and United Kingdom (Appendix IV-5).

- Acute toxicity by Bozo Research Center Ltd. in Japan is in compliance with the Good Laboratory Practice in the United States and Japan (appendix IV-6, IV-6B).

- Four week subacute toxicity study by Hazleton Washington, Inc. is in compliance with Good Laboratory Practices in the United States and Japan. (Appendix IV-7).

The test methods used for the Long-Term Toxicity Study of KA-1 in Rats, by Hashima Laboratory, Nihon Bioresearch Inc., (Appendix IV-8) are not GLP. The long term study was originally conducted to assess the nutritional characteristics of DAG, testing which is not required to conform with GLP standards in Japan. Long-term intake and single dose

examinations of human consumption of DAG in Section II and IV were conducted in compliance with the Declaration of Helsinki, with ample consideration for the safety of subjects.

July 12, 2000

GRAS EXPERT PANEL CRITICAL EVALUATION OF DIACYLGLYCEROL (DAG)

INTRODUCTION

An independent panel of recognized experts (Expert Panel), qualified by their scientific and/or medical training and relevant national and international experiences to evaluate the safety of food and food ingredients, was requested by the Kao Corporation to determine the Generally Recognized As Safe (GRAS) status of diacylglycerol (DAG) for use as a general edible oil. The members of the Expert Panel and their resumes appear in Appendix 1.

A comprehensive search of the scientific literature was conducted by Kao Corporation in October 1998 and was made available to each member of the Expert Panel, who identified pertinent articles. These were then made available to all members of the Expert Panel. In addition, the Expert Panel considered data submitted by Kao Corporation and other materials deemed appropriate or necessary. The Expert Panel considered manufacturing methods, product specifications, including batch analyses, proposed conditions of use, anticipated exposure, and information relating to the safety of DAG and related materials. All materials were independently and critically evaluated by each member of the Expert Panel to determine whether the information available was adequate to meet the statutory requirements of safety as a GRAS food ingredient based on scientific principles. The members conferred by telephone. The Expert Panel then met in Washington, DC, with representatives of the Kao Corporation and discussed their independent and collective critical evaluation. In response to these discussions, additional data was generated to evaluate the potential impacts of DAG exposure on health. The Expert Panel then met in executive session, prepared the following report and unanimously agreed to the conclusions presented below.

BACKGROUND

Diacylglycerol (DAG) is a randomized diacylglycerol of fatty acids. Constituent fatty acids are derived from general edible oils such as soybean oil, rapeseed oil, corn oil and olive oil. The major components of DAG and their molecular weights include diolein, 620; dilinolein, 616; linoleylolein, 618; linolenylolein, 616; and linolenyllinolein, 614. The ester distribution is: diacylglycerol, at least 80%; triacylglycerol, not more than 20%; and monoacylglycerol, not more than 5%. The fatty acid composition is oleic, 25-65%; linoleic, 15-65%; and linolenic, not more than 15%. The physical-chemical characteristics of DAG are similar to other major edible oils. The proposed food grade specifications appear in Appendix 2.

MANUFACTURING AND SPECIFICATIONS

DAG is manufactured by the esterification of fatty acids derived from natural edible plant oils and either monoacylglycerol or glycerol using lipase (the subject of a 1989 GRAS affirmation petition) in appropriate production facilities and current good manufacturing practices. The procedures are commonly used for other food grade oils and the materials involved are all food grade. The process is described in detail in Appendix 3. Specifications and batch analyses appear in Appendix 4.

FUNCTIONALITY

DAG is considered a raw material; the finished product contains small amounts of antioxidants and emulsifier and will be identified as DAG Oil for use as general edible oil.

Materials added to DAG include ascorbyl palmitate, tocopherol and polyglycerol esters of fatty acids and these are affirmed food additives or GRAS substances. The typical composition of DAG Oil appears in Appendix 5.

CURRENT AND PROPOSED USES

DAG Oil was approved for use as a cooking oil in Japan by the Ministry of Health and Welfare (May 20, 1998) and is permitted for use in foods for specified health uses (FOSHU). It is the most widely used oil in Japan and over 6,000 tons were produced in 1999. Through May 2000, fifteen complaints were received by Kao from consumers. The complaints were common conditions such as slight heartburn, mild diarrhea, mild constipation and slight eczema. The Expert Panel believes these minor events were not likely to be causally related to DAG Oil consumption.

Diacylglycerol is the main component of DAG Oil. It occurs endogenously in the body and is approved for food use in the U.S. as a component of emulsifiers (21 CFR §184.1505). It is widely consumed on a daily basis.

Although DAG Oil can be used as a replacement for any general edible oil, it is being proposed for use as a cooking oil for home use and for use in commercially prepared margarine for home use.

EXPOSURE

The average total fat intake in the U.S. is 74 grams per capita per day (U.S.D.A., 1996). DAG consumption was estimated using the 3-day U.S.D.A. Continuing Survey of Food Intake by Individuals of 1989-91. The mean estimated consumption for users of DAG Oil in the U.S. is 5.11 grams per day for the total population; 6.27 grams per day for male adults; and 4.27 grams per day for children 3-11 years of age. The 90th percentile consumption for the total population is 11.5 grams/day. The daily 90th percentile consumption for users is the highest, on a body weight

(bw) basis [0.38 grams per kilogram (g/kg)], in the 3 to11 year age group. Although infants consume the largest amount of fat per kg body weight, they are less likely to replace current dietary fat sources with DAG Oil. The quantitatively largest per-user intake occurs in male teenagers. Exposure data is shown in Appendix 6. The Expert Panel noted that these 3-day exposure estimates were likely overestimates for chronic consumption, based on comparison data from the 1998 Institute of European Food Studies which examined consumption pattern relative to days of sampling, longer sampling periods being associated with lower estimates.

ABSORPTION, BIOTRANSFORMATION, DISTRIBUTION AND ELIMINATION

Monoglycerides (MG), diglycerides (DG) and triglycerides (TG) of fatty acids share common metabolic pathways. DAG contains 80% DG. The fate of DAG is very similar to that of other DG. The extensive database on glycerides, especially DG, can be used to assess the biological fate of DAG.

DAG is a mixture of 1,3-DG in which the constituent fatty acids are distributed at both the 1 and 3 positions of glycerine and 1,2-DG in which the constituent fatty acids are distributed at both the 1 and 2 positions of glycerine. The ratio of 1,3-DG to 1,2-DG is 7:3. DAG is converted in the rat to 1 or 3-MG and then to glycerol and fatty acids (which are absorbed as such). Only a limited amount of 2-MG is formed. As such, DAG may serve largely as an energy source rather than as a substrate for TG resynthesis. DAG intake has been shown to depress TG resynthesis, chylomicron formation and the release of chylomicra from lymph to blood. This results in a lowering of serum TG levels and lipoprotein synthesis and a depression of the accumulation of fatty acids in the body.

The caloric value of DAG is 9 kilocalories per gram (kcal/g).

SAFETY OF DAG

Acute toxicity

A study designed to evaluate the acute oral toxicity of DAG and DAG Oil was conducted in male and female SD rats, 6 weeks of age. The rats received 15,000 milligrams per kilogram (mg/kg) bw DAG or 15,000 mg/kg bw DAG Oil by stomach tube. Diarrhea and perianal staining were observed but there were no deaths. The acute oral LD50 is greater than 15,000 mg/kg/bw.

Repeated dosing study (4-week subacute oral toxicity study)

A study to evaluate the subacute oral toxicity of DAG was conducted in male and female Crl:CD BR rats. DAG was presented as a dietary admixture at concentrations of 0.2, 1.0 or 5.0% for 28 consecutive days. There were no overt signs of toxicity in any group nor was there any mortality. There were no compound-related dose-dependent adverse effects on any of the parameters evaluated. Relative kidney weights in treated females and relative liver weights in treated males were significantly lower than in 1 control group (fed Purina® Certified Rodent

Chow), but not the 2nd control group (fed fat-free Purina® Basal Purified Diet), used in the study. Therefore, these findings were considered to be incidental. There were no adverse histopathologic findings. The No Observed Adverse Effect Level (NOAEL) for this study was 5%, the highest concentration fed.

Long-term toxicity study

This 105-week study with 60 male and 60 female Crj:CD (SD) rats per group, was designed to evaluate the nutritional physiology and potential toxicity of orally administered DAG Oil at 2.65% or 5.3% of the diet. Two control groups received a diet of 5.3% TG Oils composed of either a mixture of fatty acids equivalent to DAG Oil or a combination of rapeseed and soybean oils. Mortality at the 77th week was 28 animals in the DAG Oil groups and 34 animals in the control groups, averaging 9% overall.

Food consumption and body weight gains varied among the groups tested. There were no significant compound-related adverse effects on any of the parameters evaluated including behavior, hematology, clinical chemistry, urinalysis, necropsy findings, organ weights and ratios and histopathological findings at 30, 77 and 105 weeks. There were no signs of toxicity at the concentrations tested. The NOAEL for this study is the highest concentration tested, 5.3%.

Mutagenicity

The mutagenic potential of Kao DG was evaluated in Salmonella typhimurium strains TA 1535, TA 1537, TA 98 and TA 100 and in Escherichia coli WP2 uvrA. There was no evidence of mutagenic activity at any concentration (up to 5000 ug/plate) with and without metabolic activation (S9 fraction). No toxicity was evident at this level.

The Expert Panel was not aware of available information on the reproductive, developmental, or allergenic potential of DAG.

Nutritional studies

Animal

DAG has been shown to lower the serum level of triacylglycerol by decreasing the resynthesis of TG and to depress the accumulation of fatty acids. Chylomicron formation is also reduced.

Human

A total of 10 single-dose, repeat dose, short and long duration studies were performed; 4 have been published. The Expert Panel is aware that other clinical studies are ongoing. The Expert Panel has reviewed the results of studies that were completed as of June 15, 2000, and which are discussed below.

Single-dose studies

Doses of DAG Oil or a preparation of TG with the same fatty acid composition at approximately 0.17, 0.33 or 0.73 grams per kilogram (g/kg) bw were administered orally as a 10% emulsion after a standard overnight fast. Ten to 17 healthy men were involved in each study. In the high dose study lasting 8 hours, relative to TG administration, consumption of DAG Oil was associated with a transient lowering of serum TG, a reduction in serum phospholipids and a slight reduction of apolipoprotein C II and C III levels which reflect some diminution of lipoprotein synthesis. There were no differences between groups in serum free fatty acids, beta-lipoprotein, 3-hydroxybutyric acid, insulin, cholesterol or blood glucose. In the 6-hour mid-range study, there was no effect of DAG Oil on hematological parameters except for higher white blood cell counts that remained within the normal range. Clinical chemistries fell within the normal range. There was an effect of DAG Oil to lower circulating TG and beta-lipoprotein levels. There was a transient decline in the high-density lipoprotein fraction and changes in the fatty acid composition of lipoproteins which supports the view that DAG Oil, compared with TG Oil, is associated with the reduction of chylomicron formation. In the low-dose group, DAG Oil again lowered TG levels. Thus the results of single dose studies are compatible with an acute effect of DAG Oil to lower serum TG levels. No acute adverse effects were demonstrated.

Repeat-dose studies

Seven studies have been conducted to test for effects of repeated ingestion over 1 to 12-month periods. In three studies, a period of controlled diet preceded the studies, which lasted from one to three months and employed doses of 44, 20, and 10 grams DAG Oil daily, respectively. This resulted in various exposure ranges to DAG Oil or an equivalent amount of TG, the total range of exposure being from 0.782 to 0.165 g/kg bw per day. In all cases, the test substance was given once per day in a single meal. In the high exposure group a double-blind crossover design was used. With the other lower dose studies, a parallel design was employed. With the higher dose given to 8 subjects for one month, there were no statistical differences in hematological or clinical chemistry values, basal metabolic rates, waist or hip size or waist/hip ratios. There were no reported adverse events. In the 3-month mid-range study (15 DAG Oil and 12 TG subjects), physical examinations, clinical pathology, and hematology were within normal limits. In the low-dose study, 2 groups of 19 moderately obese men were tested for three months. An extensive battery of clinical chemistries was measured. Exposure to DAG Oil significantly reduced body weight, subcutaneous fat and visceral fat, as estimated by computed tomography. There was a relative decline in leukocyte counts in the DAG Oil group compared to the TG group; however, large intra-and inter group variations were present and the Expert Panel did not consider this an adverse reaction to DAG Oil. Transient differences (decreases) in blood glucose and HDL-cholesterol levels were judged not to be significant because they did not persist beyond 4 weeks of exposure.

A subsequent study involved 114 adults in which DAG Oil was used in a home and work setting as a cooking oil for 12 months. The male population had on average a higher body mass index than the Japanese standard value and a quarter of the population also had serum TG levels above 150 milligrams per deciliter (mg/dl). Testing for body morphometrics, serum lipid levels, blood

cell counts, liver function tests and levels of hemoglobin, hematocrit, amylase, and plasminogen activator inhibitor type-1 (PAI-1) was conducted every three months. (Elevated levels of PAI-1 are associated with obesity and bear a relationship to a number of health conditions related to coronary artery disease, body fat distribution, and TG and HDL cholesterol metabolism.) Body weight tended to decrease over the course of the study but only reached statistical significance in males by the end of the study. Waist and hip circumference and skin fold measure declined and TG levels were significantly lowered in individuals with levels above 150 mg/dl. PAI-1 levels declined as did LDL-cholesterol levels. HDL-cholesterol levels increased. Slight changes occurred in liver function tests, but test values remained within normal limits and were not time-responsive.

Another study lasting 5 months involved 4 male and 9 female children, ages 7 to 17, with hyperlipidemia and/or obesity. They received DAG Oil ad libitum and had widely different exposures ranging from 0.08 to 0.4 g DAG Oil/kg body weight/day. Fasting blood samples and body measurements, as well as serum leptin, serum PAI-1, glucose, glycoalbumen, insulin, creatinine, serum lipids, ketone bodies, total cholesterol levels and fractions, serum triglycerides and visceral, subcutaneous, and total fat areas were measured. There were no adverse events or any significant change in body weight. There was a significant decrease in serum TG in month 4 and a significant decrease in HDL-cholesterol in months 4 and 5. Of importance to the present safety evaluation of DAG Oil was the observation that growth occurred in a normal manner in 11 of the 13 children. Of the remaining 2 children, one had already achieved her growth spurt and there was no reason to ascribe any growth retardation mechanism to the second child. Thus, the Expert Panel concluded that under normal conditions of use, DAG Oil did not present a potential problem to energy production that might adversely affect growth.

Of particular importance to documenting safety at higher exposures in the range of the 90th percentile intake are the results of a double blind parallel study in 56 healthy females with an average age of 20 years, over a 2-month period. Exposure to DAG ranged from 0.26 to 0.45 g/kg bw per day. There were no significant differences between the DAG Oil group and the TG control in terms of clinical chemistries. Insulin levels, fasting blood sugar, liver function tests, ketogenesis and body fat distribution were not significantly different between the groups. No adverse effects were seen at any level. In the Expert Panel's opinion, these data strongly support the conclusion that daily exposure to DAG Oil at the proposed level of use is safe within the standard of representing a reasonable certainty of no harm to consumers.

Remaining data and an additional study also support the safety of use of DAG Oil as proposed. They further document the effects of DAG Oil in additional populations, including patients undergoing renal dialysis. They show a lack of an effect of DAG Oil on the absorption of the fat-soluble vitamins, A, E, and D, although, with a fully absorbed lipid product, such an effect would not be anticipated.

When evaluating the above human studies, the Expert Panel focused on the effects of dose (g/kg bw/day) and assumed that use of DAG Oil as a cooking oil and a component of margarine would occur over the course of a day and would be accompanied by the ingestion of other fats containing TG. Furthermore, the Expert Panel assumed that under actual conditions of use,

DAG will tend to exist largely as the stable 1,3-DG isoform rather than the 1,2-DG isoform. As noted above, the metabolism of 1,3 diglycerides leads to the formation of glycerol and the free fatty acids whereas triglycerides are hydrolyzed to 2-acylglycerol and fatty acids. The absorption of 2-acyl glycerol from TG provides substrate for TG resynthesis. With increasing use of DAG, a limited supply of 2-acyl glycerol for TG resynthesis could occur and the plasma free fatty acid level can increase. Such an increase can have many effects. It can provide substrate for energy production in many tissues and inhibit a number of enzymes, including some in the citric acid cycle. Energy production from fatty acids largely takes place via beta-oxidation in the mitochondria. However, depending on a number of conditions, such as the availability of oxaloacetate, energy production from fatty acid oxidation can vary and metabolism can switch to the production of acetoacetate and 3-hydroxybutyrate, producing ketosis.

Thus, in examining the increased use of DAG as a substitute for triglyceride oils, a salient question for the Expert Panel became: At what level of exposure to DAG, as opposed to TG, does metabolism become altered, and, if it is altered as described above, are there adverse health consequences? While under certain conditions of DAG consumption, the possibility of decreased energy production and ketosis exists, the Expert Panel found no evidence that currently proposed levels of use would result in such a condition or would adversely impact health. Furthermore, there is evidence that increasing dietary DG results in a potentially benefical effect of lowering chylomicron formation and TG resynthesis in favor of the conversion of dietary fatty acids to the production of energy primarily via the mechanism of mitochondrial oxidation. Overall, the Expert Panel concluded that the human clinical data further lends support to the opinion that the proposed uses of DAG meet the GRAS safety standard based on scientific procedures.

SUMMARY

The Expert Panel concurs with the findings of the SCOGS of the LSRO/FASEB (1975): "Although mono- and diglycerides of edible fat-forming fatty acids are found naturally, those that are used as food additives are prepared synthetically. Mono-, di- and triglycerides are metabolized by the same mechanisms. The biological effects of glycerides are either those of the entire molecule or of the metabolic products, fatty acids and glycerin. Triglyceride fats are a major source of calories in the diet of many people. Mono- and diglycerides are minor components of natural fats. They are intermediate metabolic products of ingested triglycerides. There is no evidence that the mono- and diglycerides of edible fat-forming fatty acids behave differently from triglycerides upon ingestion." The Expert Panel would add that, in the present case of DAG use, this statement requires clarification. As noted, DAG contains predominantly 1,3 DG as compared 1,2 DG. Depending on the relative dose effects between dietary TG and DAG, the availability of 2- MG, and other factors, such as dietary carbohydrate restriction, there may be significant changes in the rates of TG resynthesis, energy produced from fatty acid oxidation, or ketogenesis to an extent that there could theoretically be an adverse effect on health. However, under the current projected conditions of use within a normal diet, DAG used as a general cooking oil and in margarine, no adverse impact on lipid metabolism and health is anticipated, based on the evidence reviewed above.

MG and DG have been used in foods in the US since 1911. Other glycerides have been introduced since then. Glycerides are approved for use as emulsifying agents, miscellaneous and/or general purpose food additives, and as synergists and solubilizers for antioxidants in oils and fats.

DG are a normal constituent of the diet and are present naturally in edible oils and as added ingredients in other foods. Although it has been reported that the daily per capita intake of mono- and DG in the U.S. is in the range of 1-10 g, the maximal intake from all sources has been estimated to be 6-7 g.

Available animal data support the lack of toxicity of DG. The only effect reported following the administration of very large doses (15,000 mg/kg bw) was diarrhea.

The safety of DAG is based on a long history of safe use of DG in the food supply as well as a significant amount of safety data available on DAG and DAG Oil. The Joint FAO/WHO Expert Committee on Food Additives has evaluated available information on DG and has allocated an Acceptable Daily Intake of "not specified"; that is, the establishment of an acceptable daily intake expressed in numerical form is not deemed necessary on the basis of the available data, the total dietary intake of the substance from its use at levels necessary to achieve the desired effect and from its background level in food.

CONCLUSIONS

The Expert Panel independently and collectively critically evaluated available relevant human and experimental information on DAG and DAG Oil, and concludes that DAG meeting appropriate food grade specifications and produced in accordance with current good manufacturing practices is Generally Recognized As Safe (GRAS) by scientific procedures when used as a cooking oil for home use and in commercially prepared margarine for home use.

Joseph F. Borzelleca, Ph.D.
Medical College of Virginia
Toxicology & Pharmacology, Inc.

12 July 2000
Date

Walter H. Glinsmann, M.D.
Fellow, Georgetown University
Center for Food and Nutrition Policy
Glinsmann, Inc.

July 12 00
Date

David Kritchevsky, Ph.D.
Caspar Wistar Scholar
The Wistar Institute

13 July 00
Date

Ian C. Munro, Ph.D., FRCPath.
CANTOX
HEALTH SCIENCES
INTERNATIONAL

July 17, 2000
Date

REFERENCES

Abdel-Nabey, A.A., Shehata, A.A.Y., Ragab, M.H., and Rossell, J.B. 1992. Glycerides of cottonseed oils from Egyptian and other varieties. La Rivista Italiana Delle Sostanze Grasse 69:443-447.

Akoh, C.C. 1998. Fat replacers. Food Technol 52:47-53.

Ambrose, A.M., Robbins, D.J., and Cox Jr., A.J. 1958a. Studies on the long-term feeding of acetoglycerides to rats. Food Res 23:536-549.

Ambrose, A.M., Robbins, D.J., and DeEds, F. 1958b. Effect of acetostearin on reproduction in albino rats. Food Res 23:550-553.

Anderson, S.A. 1988. Estimation of Exposure To Substances In The Food Supply. Life Sciences Research Office, Federation of American Societies For Experimental Biology, Bethesda, Maryland.

Arroyo, R., Sanchez-Muniz, F.J., Cuesta, C., Buguillo, F.J., and Sanchez-Montero, J.M. 1996. Hydrolysis of used frying palm olein and sunflower oil catalyzed by porcine pancreatic lipase. Lipids 31:1133-1139.

Artman, N.R. 1974. Safety of emulsifiers in fats and oils. J Am Oil Chem Soc 52(1):49-52.

Baur, F.J. 1973. Analytical methodology for emulsifiers used in fatty foods: a review. J Am Oil Chem Soc 50:83-85.

Bhattacharyya, S., and Bhattacharyya, D.K. 1989. Biorefining of high acid rice bran oil. J Am Oil Chem Soc 66:1809-1811.

Borgstrom, B., Dahlqvist, A., Lundg, G., and Sjovall, J. 1957. Studies of intestinal digestion and absorption in the human. J Clin Invest 36:1521-1536.

Bougneres, P.F., Castano, L., Rocchicciolo, F., Gia, H.P., Leluyer, B., and Ferre, P. 1989. Medium-chain fatty acids increase glucose production in normal and low birth weight newborns. Am J Physiol 256:E692-E697.

Broadmeadow, A., Clare, C. and de Boer, A.S. 1994. An overview of the safety evaluation of the *Rhizomucor miehei* lipase enzyme. Food Additives and Contaminants 11(1):105-119.

Brühl, L. 1997. Determination of free fatty acids and diacylglycerols in native vegetable oils. Fett/Lipid 99:428-432.

Catalano, M., De Leonarbis, T., and Comes, S. 1994. Diacylglycerols in the evaluation of virgin olive oil quality. Grasas y Aceites 45:380-384.

Chrysam, M.M. 1996. Margarines and spreads. In: Hui, Y.H. (Ed.) Bailey's Industrial Oil and Fat Products (5th Ed.) John Wiley and Sons, New York, NY, Toronto, ON.

Cuesta, C., Sanchez-Muniz, F.J., Lopez-Varela, S., Garrido-Polonio, M., and Garcia Diz, L. 1993. Thermoxidation in a sunflower oil used in 75 deep fat frying of potatoes. Effects of its inclusion in rat-diet upon growth and food intake. Grasas y Aceites 44:263-269.

D'Alonzo, R.P., Kozarek, W.J., and Wade, R.L. 1982. Glyceride composition of processed fats and oils as determined by glass capillary gas chromatography. J Am Oil Chem Soc 59:292-295.

Douglas Jr., G.J., Reinauer, A.J., Brooks, W.C., and Pratt, J.H. 1953. The effect on digestion and absorption of excluding the pancreatic juice from the intestine. Gastroenterology 23:452-459.

Eagle, E., and Poling, C.E. 1956. The oral toxicity and pathology of polyoxyethylene derivatives in rats and hamsters. J Food Sci 21:348-361.

FASEB. 1975. Evaluation of the health aspects of glycerine and glycerides as food ingredients. FASEB (Federation of American Societies for Experimental Biology), U.S. Department of Commerce, National Technical Information Service, PB-254 536.

FDA. 29 Federal Register 9708 (1964).

FDA. 54 Federal Register 9565 (1989).

Fincke, A. 1982. Untersuchungen zum diglyceridgehalt von kakaobutter. Gordian 82:246-249.

Fitzhugh, O.G., Schouboe, P.J., and Nelson, A.A. 1960. Oral toxicities of lauric acid and certain lauric acid derivatives. Toxicol and Appl Phamcol 2:59-67.

Geeraert, E., and Sandra, P. 1987. Capillary GC of triglycerides in fats and oils using a high temperature phenylmethylsilicone stationary phase: Part II. The analysis of chocolate fats. J Am Oil Chem Soc 64:100-101.

George, S., and Arumughan, C. 1992. Lipid profile of process streams of palm oil mill. J Am Oil Chem Soc 69:283-287.

Giovanna, V. 1996. Improved quantitative ^{13}C nuclear magnetic resonance criteria for determination of grades of virgin olive oil the normal ranges for diglycerides in olive oil. J Am Oil Chem Soc 73:1201-1203.

Goh, E.M., and Timms, R.E. 1985. Determination of mono- and diglycerides in palm oil, olein and stearin. J Am Oil Chem Soc 62:730-734.

Gopalakrishnan, N., Narayanan, C.S., Mathew, A.G., and Arumughan, C. 1987. Lipid composition of coconut cake oil. J Am Oil Chem Soc 64:539-541.

Goto, M., Honda, K., Di, L., and Small, D.M. 1995. Crystal structure of a mixed chain diacylglycerol, 1-stearoyl-3oleyl-glycerol. J Lipid Res 36:2185-2190.

Greenberger, N.J., Tzagournis, M., and Graves, T.M. 1968. Stimulation of insulin secretion in man by medium chain triacylglycerides. Metabolism 17:796-801.

Groot, D. 1972. Acyl migration solid phase isomerization of 1,2-diglycerides to 1,3-isomers. Lipids 7(9):626-628.

Grundy, S. 1996. Dietary Fat. In: Ziegler, E.E., and Filer, L.J. (Eds.) Present Knowledge in Nutrition. Seventh Edition. ILSI Press, Washington, DC, pp. 44-57.

Gustavsson, J., Parpal, S., and Stralfors, P. 1995. Uptake and metabolism of long-chain 1,2-diacylglycerols by rat adipocytes and h4lle hepatoma cells. Exp Cell Res 221:443-447.

Hamosh, M., and Scow, R.O. 1973. Lingual lipase and its role in the digestion of dietary lipid. J Clin Invest 52:88-95.

Hamosh, M., Sivasubramanian, K.N., Salzman-Mann, C., and Hamosh, P. 1978. Fat digestion in the stomach of premature infants. J Pediatrics 93:674-679.

Hansen, T.T., and Eigtved, P. 1985. Proceedings of World Conference on Emerging Technologies in the Fats and Oils Industry 365.

Hara, K., Onizawa, K., Honda, H., Otsuji, K., Ide, T., and Murata, M. 1993. Dietary Diacylglycerol-dependent reduction in serum triacylglycerol concentration in rats. Ann Nutr Metab 37:185-191.

Harkness, J.E., and Wagner, J.E. 1989. The Biology and Medicine of Rabbits and Rodents. Third Edition. Lea & Febiger, Philadelphia, PA, pp. 47-54.

Harris, R.S., and Sherman, H. 1954. Comparison of the nutritive values of mono-, di-, and triglycerides by a modified pair-feeding technique. Food Res 19:257-262.

Hastert, R.C. 1996. Hydrogenation. In: Hui, Y.H. (Ed.) Bailey's Industrial Oil and Fat Products (5th Ed.) John Wiley and Sons, New York, NY, Toronto, ON.

Holt, P.R., Moss, S.F., Whelam, R., Guss, J., Gilman, J., and Lipkin, M. 1996. Fecal and rectal mucosal diacylglycerol concentrations and epithelial proliferative kinetics. Cancer Epid Biomarker Prev 5:937-940.

Informatics, Inc. 1973. GRAS (generally recognized as safe) food ingredients - Glycerine and Glycerides. Prepared for Food and Drug Administration, February 1973, PB-221 227.

Inglett, J.S., and Inglett, G.E. 1982. Dairy and dairy-type foods. In: Inglett, J.S., and Inglett, G.E. (Eds.) Fabricated Foods. AVI Publishing Company, Inc., Westport, CT, (AVI Food Products Formulary Series) Vol. 4:51-56.

Inoue, H., and Noguchi, T. 1967. The influences of hydrolysis on partial glycerides composition in rice bran oils. Yukagaku 16:361-363.

Institute of European Food Studies, 1998. Influence of Survey Duration on the Estimation of Food Chemical Intakes.

JECFA. 1974. Toxicological Evaluation of Some Food Additives Including Anticaking Agents, Antimicrobials, Antioxidants, Emulsifiers and Thickening Agents. (17th JECFA Session, June 25-July 4, 1973, Geneva, Switz.) Food and Agriculture Organization of the United Nations (FAO), Rome, Italy, (FAO Nutrition Meetings Report Series, No. 53A. WHO Technical Report Series, No. 539. WHO Food Additive Series) No. 5, pp. 238-240.

JECFA. 1996. Summary of the Evaluations Performed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). Revision 1. ILSI Press, Washington, DC.

Jensen, B.F., and Eigtved, P. 1990. Safety aspects of microbial enzyme technology, exemplified by the safety assessment of an immobilized lipase preparation, Lipozyme™. Food Biotechnol 4(2):699-725.

Kamal-Eldin, A., and Appelqvist, L.A. 1994. Variation in fatty acid composition of the different acyl lipids in seed oil from four sesamum species. J Am Oil Chem Soc 71:135-139.

Katsuragi, Y., Toi, T., and Yasukawa, T. 1999. Effects of dietary diacylglycerol on obesity and hyperlipidemia. J Japanese Society of Human Dry Dock 14(3):12-16.

Kayden, H.J., Senior, J.R., and Mattson, F.H. 1967. The mono-glyceride pathway of fat absorption in man. J Clin Invest 46:1695-1703.

Koprivnjak, O., Bertacco, G., Boschelle, O., and Conte, L.S. 1995. Determination of diglycerides in virgin olive oil, salami and cheese by solid phase extraction. Prehrambeno-Tehnol Biotehnol Rev 33(2-3):97-101.

Latondress, E.G. 1981. Formulation of products from soybean oil. J Am Oil Chem Assoc 58(3):185-187.

Litton Bionetics, Incorporated. 1975. Mutagenic evaluation of compound FDA 71-59, mono- and di-glycerides monosodium phosphate derivates. Prepared for Food and Drug Administration, 30 May 1975, PB-245 505.

Macrae, A.R. 1983. Lipase-catalyzed interesterification of oils and fats. J Am Oil Chem Soc 60: 291-292.

Mariani, C., and Fedeli, E. 1985. Partial glycerides of vegetable oils. La Rivista Italiana Delle Sostanze Grasse 57:129-132.

Mariani, C., Contarini, G., Zucchetti, and Toppino, P.M. 1990. Significance of minor components of milk fat. J High Resol Chromatogr 13:356-360.

Mattson, F.H., Baur, F.J., and Beck, L.W. 1951. The comparative nutritive value of mono-, di-, and triglycerides. J Am Oil Chem Soc 28:386-390.

Mattson, F.H., and Volpenhein, R.A. 1964. The digestion and absorption of triglycerides. J Biol Chem 239:2772-2777.

Meignassner, J.G., and Schmook, F.P. 1990. Reference Paper: Reference Values for Crl:CD® (SD) BR Rats. Sandoz Research Institute, Vienna, Austria. Charles River Laboratories, Wilmington, MA, pp. 7-11.

Migas, I., Chuang, M., Sasaki, T., and Severson, D.L. 1997. Diacylglycerol metabolism in SM-3 smooth muscle cells. Can J Physiol Pharmacol 75:1249-1256.

Miraglia, M., Tassi Micco, C., Benedetti, E., Onori, R. 1985. Mono- and diglycerides present in fats used in baked products I. Riv Ital Sos Grasse 62(3):133-136.

Modi, G., Simiani, G., and Tracchi, S. 1996. Di and triacylglycerols in Tuscan extra virgin olive oils. Boll Chim Igien 47:127-129.

Murata, M., Hara, K., and Ide, T. 1994. Alternation by diacylglycerols of the transport and fatty acid composition of lymph chylomicrons in rats. Biosci Biotech Biochem 58(8):1416-1419.

Murata, M., Ide, T., and Hara, K. 1997. Reciprocal responses to dietary diacylglycerol of hepatic enzymes of fatty acid synthesis and oxidation in the rat. British J Nutr 77:107-121.

Nagao, T., Watanabe, H., Goto, N., Onizawa, K., Taguchi, H., Matsuo, N., Yasukawa, T., Tsushima, R., Shimasaki, H., and Itakura, H. 2000. Dietary Diacylglycerol Suppresses Accumulation of Body Fat Compared to Triacylglycerol in Men in a Double-Blind Controlled Trial. J Nutr 130: 792-797. Originally published in: Watanabe, H., Nagao, T., Goto, N., Fukushima,Y., Onizawa, K., Taguchi, H., Ohmachi, T., Yasukawa, T., Naito, S., Shimasaki, H., and Itakura, H. 1998. Long-Term Effects of Dietary Diacylglycerols on Body Fat Metabolism in Man. J. Japan Oil Chemists Society 47(4):369-376.

National Academy of Sciences, 1989. 1987 Poundage and Technical Effects Update of Substances Added to Food, Committee on Food Additives Survey Data, Food and Nutrition Board, National Research Council, National Academy of Sciences, Washington, DC.

O'Brien, R.D. 1998. Margarine. In: O'Brien, R.D. (Ed.) Fats and Oils: Formulating and Processing for Applications. pp. 437-458.

OECD. 1987. OECD Guidelines for the testing of Chemicals. Organization for Economic Co-Operation and Development (OECD), Paris, France, Vol.2.

Olivecrona, T., Hernell, O., Egelrud, T., Billstron, A., Helander, H., Samuelson, and Fredrikzon, B. 1973. Studies on the Gastric Lipolysis of Milk Lipids in Suckling Rats and in Human Infants. In: Galli, C., Jacini, G., and Pecile, A. (Eds). Dietary Lipids and Postnatal Development. Raven Press, New York, pp. 77-89.

Ostergaard, G., and Knudsen, I. 1998. The applicability of the ADI (Acceptable Daily Intake) for food additives to infants and children. Food Addit Contam 15:63-74.

Owen, O.E., and Cahill, G.F., Jr. 1973. Metabolic effect of exogenous glucocorticoids in fasted man. J Clin Invest 52:2596-2605.

Parexel International Corporation. 1986. Petition for GRAS Affirmation of Glyceryl Behenate. Parexel International Corporation, Cambridge, Massachusetts.

Perez-Camino, M.C., Moreda, W., and Cert, A. 1996. Determination of diacylglycerol isomers in vegetable oils by solid-phase extraction followed by gas chromatography on a polar phase. J Chromatogr A 721:305-314.

Pi-Sunyer, F.X., Hashim, S.A., and Van Itallie, T.B. 1969. Insulin and ketone responses to ingestion of medium and long-chain triacylglycerides in man. Diabetes 18:96-100.

Reeves, P.G., Nielsen, F.H., and Fahey, G.C. 1993. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition Ad Hoc Committee on the Reformulation of the AIN-76A rodent diet. J Nutrition 123:1939-1951.

Sanzhez-Muniz, F.J., Cuesta, C., and Garrido-Polonio, C. 1993. Sunflower oil used for frying: combination of column, gas and high-performance size-exclusion chromatography for its evaluation. J Am Oil Chem Soc 70:235-240.

Siew, W.L. 1995. Diglyceride content and composition as indicators of palm oil quality. J Sci Food Agric 69:73-79.

Singleton, J.A., and Pattee, H.E. 1987. Characterization of peanut oil triacylglycerols by HPLC, GLC and EIMS. J Am Oil Chem Soc 64: 534.

Sipos, E.F., and Szuhaj, B.F. 1996. Soybean oil. In: Hui, Y.H. (Ed.) Bailey's Industrial Oil and Fat Products (5th Ed.) John Wiley and Sons, New York, NY, Toronto, ON.

Sleeter, R.T. 1981. Effects of processing on quality of soybean oil. J Am Oil Chem Soc 58:239-247.

Small, D.M. 1991. The effects of glyceride structure on absorption and metabolism. Annu Rev Nutr 11:413-434.

Soni, M.G., Kimura, H., and Burdock, G.A. In press. Chronic Studies of Diacylglycerol Oil in Rats.

Sontag, N.O.V. 1982. In: Swern, D. (Ed.) Bailey's Industrial Oil and Fat Products (24th Ed.) John Wiley and Sons, New York, NY, Toronto, ON.

Swern, D., (Ed). 1979. Bailey's Industrial Oil and Fat Products (4th Ed.) John Wiley and Sons, New York, NY.

Taguchi, H., Nagao, T., Watanabe, H., Onizawa, K., Matsuo, N., Tokimitsu, I., and Itakura, H. In press. Energy value and digestibility of dietary diacylglycerol are similar to those of dietary triacylglycerol.

Teramoto, T., Nagao, T., Watanabe, H., Itoh, K., Omata, Y., Hurukawa, T., Shimoda, K., and Hoshino, M. 2000. Effects of diacylglycerol diet on lipid metabolism in the patient with hemodialysis. 21(3):35-38.

Tove, S.B. 1964. Toxicity of saturated fat. J Nutra 84:237-243.

U.K. Published Patent Application 2 159 527 A.

U.S. Department of Agriculture (USDA). 1996. Nationwide Food Consumption Survey: 1989-91 Continuing Survey of Food Intakes by Individuals (CSFII), and Diet and Health Knowledge Survey (DHKS). Springfield, VA: National Technical Information Service.

U.S. Department of Agriculture (USDA). 1998. Nutrient Database for Standard Reference (Release 12). Nutrition Monitoring Division, Human Nutrition Information Service, U.S. Department of Agriculture, Hyattsville, MD.

U.S. Patent 4,275,081.

U.S. Patent 4,472,503.

van Zweiten, M.J., HogenEsch, H. Majka, J.A., and Boorman, G.A. 1994. Nonneoplastic and Neoplastic Lesions of the Mammary Gland. In: Mohr, U., Dungworth, D.L., and Capen, C.C. (Eds.) Pathobiology of the Aging Rat. Volume 2. ILSI Press, Washington, DC, pp.459-475.

Watanabe, H., Onizawa, K., Taguchi, H., Kobori, M., Chiba, H., Naito, S., Matsuo, N., Yasukawa, T., Hattori, M., and Shimaski, H. 1997a. Nutritional characterization of diacylglycerols in rats. J Japan Oil Chem Soc 46(3):301-308.

WHO, 1987. Principles for the safety assessment of food additives and contaminants in food. Environmental Health Criteria No. 70. WHO (World Health Organization, Geneva).

Yamane, T., Kang, S.T., Kawahara, and Koizumi, Y. 1994. High-yield diacylglycerol formation by solid-phase enzymatic glycerolysis of hydrogenated beef tallow. J Am Oil Chem Soc 71(3):339-342.

SUBMISSION END

1800 M Street, N.W.
Washington, D.C. 20036-5869
Tel. 202-467-7000
Fax: 202-467-7176

FAX MESSAGE

MORGAN LEWIS E-MAIL ADDRESSES HAVE CHANGED
THE MORGAN LEWIS E-MAIL DOMAIN NAME HAS BEEN CHANGED TO "MORGANLEWIS.COM." MOST E-MAIL ADDRESSES ARE NOW FIRST INITIAL AND LAST NAME @MORGANLEWIS.COM. FOR INDIVIDUAL E-MAIL ADDRESSES, PLEASE REFER TO WWW.MORGANLEWIS.COM, OR SEND AN E-MAIL TO INFO@MORGANLEWIS.COM. PLEASE UPDATE YOUR RECORDS ACCORDINGLY. TOLL FREE HOTLINE FOR TECHNICAL PROBLEMS: 877-963-4652

Morgan, Lewis & Bockius LLP
COUNSELORS AT LAW

AM

Send to:

(1) Name: Kathleen McAveney, Ph.D. FAX Number: (202) 418-3131

Firm: Food and Drug Administration Telephone Number: (202) 418-3097

(2) Name: Takuji Yasukawa FAX Number: 011-81-299-93-8449

Firm: Kao Corporation Telephone Number: 011-81-299-93-8440

From:

Name: Sandra J.P. Dennis Floor: 6N Operator Sending:

Telephone Number: (202) 467-7009 Time Sent: Date Sent: September 13, 2000

Number of Pages (INCLUDING COVER PAGE): 2

Note:

THE INFORMATION CONTAINED IN THIS FAX MESSAGE IS INTENDED ONLY FOR THE PERSONAL AND CONFIDENTIAL USE OF THE RECIPIENT(S) NAMED ABOVE. THIS MESSAGE MAY BE AN ATTORNEY-CLIENT COMMUNICATION AND AS SUCH IS PRIVILEGED AND CONFIDENTIAL. IF THE READER OF THIS MESSAGE IS NOT THE INTENDED RECIPIENT OR AN AGENT RESPONSIBLE FOR DELIVERING IT TO THE INTENDED RECIPIENT, YOU ARE HEREBY NOTIFIED THAT YOU HAVE RECEIVED THIS DOCUMENT IN ERROR AND THAT ANY REVIEW, DISSEMINATION, DISTRIBUTION, OR COPYING OF THIS MESSAGE IS STRICTLY PROHIBITED. IF YOU HAVE RECEIVED THIS COMMUNICATION IN ERROR, PLEASE NOTIFY US IMMEDIATELY BY TELEPHONE, AND RETURN THE ORIGINAL MESSAGE TO US BY MAIL. THANK YOU.

Comments:

1800 M Street, N.W.
Washington, D.C. 20036-5869
202-467-7000
Fax: 202-467-7176

Morgan, Lewis & Bockius LLP
COUNSELORS AT LAW

Sandra J.P. Dennis
(202) 467-7009
sdennis@morganlewis.com

September 13, 2000

VIA FACSIMILE

Kathleen McAveney, Ph.D.
Consumer Safety Officer
Office of Premarket Approval (HFS-206)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
200 C Street, SW
Washington, DC 20204

Re: GRAS Notice (GRN) No. 000056 (Kao Corporation Diacylglycerol Oil)

Dear Dr. McAveney:

Pursuant to our telephone discussions, this will confirm that page 23 of the above-referenced GRAS Notice does not include confidential/trade secret information, and may be disclosed.

Please contact me at (202) 467-7009 if you have any questions.

Sincerely,

Sandra J.P. Dennis

cc: Takuji Yasukawa
Kao Corporation

000141

BLANK PAGE INSERTED FOR CORRECT PAGINATION

AM

McAveney, Kathleen

From:
Sent: Wednesday, October 25, 2000 6:15 PM
To: McAveney, Kathleen
Subject: GRAS Notification for DAG Oil--second email try!

Kathleen--

Thanks for your voicemail message, responding to my questions about the review. Sorry the email didn't go through (I think I gave you the right address)--anyway, now you'll have my email.

I have one additional question regarding the conclusion that a DAG product cannot be called "margarine". (I was so pleased with the oil decision that I didn't think to ask this earlier.) I'd like to know the reason that a DAG product would not fit under the margarine standard of identity. I'm not challenging this--I just want to understand the reason, so I can explain it to my client. If you don't know, could you ask the OMPLADS contact, perhaps for a cite to the particular part of the regulation (21 CFR 166.10) that is an issue?

Thanks again.
--Sandi Dennis

****
Please note that the Morgan Lewis e-mail domain has been changed to "morganlewis.com". In most cases, the person's e-mail address is their first initial and their full last name plus @morganlewis.com. To locate the e-mail address of a specific individual at Morgan Lewis, please refer to the firm's web site, now located at http://www.morganlewis.com, or send an e-mail to info@morganlewis.com. Please update your address book and bookmarks accordingly.

If you are experiencing technical problems, please call our toll free hot line at 877-963-4652.

The information contained in this e-mail message is intended only for the personal and confidential use of the recipient(s) named above. This message may be an attorney-client communication and as such is privileged and confidential. If the reader of this message is not the intended recipient or an agent responsible for delivering it to the intended recipient, you are hereby notified that you have received this document in error and that any review, dissemination, distribution, or copying of this message is strictly prohibited. If you have received this communication in error, please notify us immediately by e-mail, and delete the original message.
****

000150