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Iain Weatherston, Ph.D.
Senior Regulatory Consultant
Pesticide Division

TECHNOLOGY SCIENCES GROUP INC

Dr. Robert Pooler
Program Manager,
USDA/AMS/TM/NOP
Room 4004 So. Ag. Stop 0268
1400 Independence Avenue SW.
WASHINGTON, D.C. 20250

April 17, 2009

Dear Dr. Pooler:

SUBJECT: Petition to include propylene glycol monolaurate on to 7 CFR § 205.

COMPANY: Otsuka Chemical Company Ltd., 3-2-27 Otedori, Chuo-Ku, Osaka, 540-0021 Japan.

CONTACT: Dr. Iain Weatherston, Technology Sciences Group Inc., 40-61 North 156th Drive, Goodyear, AZ 85395

As agent for, and on behalf of Otsuka Chemical Company Ltd., I submit for your review and acceptance this petition to include propylene glycol monolaurate on to 7 CFR § 205. The petition consists, in addition to this letter, four documents:

Document 1 The petition developed according to the instructions at 72 FR (11): 2168 - 2170.

Document 2 The Appendix containing the documentation being used to support the petition.

Document 3 This is a copy of the successful waiver request made to the U.S. EPA in regards to the toxicity and ecotoxicity of propylene glycol monolaurate as part of the data required for approval of the registration of Acaritouch.

Document 4 This is a copy of the successful waiver request made to the U.S. EPA in regards to the nature of the residue, the magnitude of the residue and the residue analytical method of propylene glycol monolaurate as part of the data required for approval of the registration of Acaritouch.

None of the documents submitted contains Confidential Business Information, and as discussed in our telephone conversation I am submitting two complete hard copies of the petition and associated documents.

If you have any questions, or require further information please do not hesitate to contact me at jazkatz@qwestoffice.net or by telephone at 623-535-4060.

Sincerely

Iain Weatherston
Regulatory Consultant to Otsuka Chemical Co., Ltd.

PETITION TO INCLUDE PROPYLENE GLYCOL MONOLAURATE ONTO 7 CFR § 205

DOCUMENT 1

APR 24 2009

A PETITION SUBMITTED TO

USDA/AMS/TM/NOP

BY

TECHNOLOGY SCIENCES GROUP INC.
4061 North 156th Drive
Goodyear, AZ 85395

ON BEHALF OF

OTSUKA CHEMICAL COMPANY LTD.
3-2-27 Otedori, Chuo-Ku
Osaka, 540-0021, Japan

THIS VOLUME CONTAINS NO CONFIDENTIAL BUSINESS INFORMATION

ALL COMMUNICATIONS REGARDING THIS PETITION SHOULD BE DIRECTED TO

Dr. Iain Weatherston
Technology Sciences Group Inc.
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Goodyear, AZ 85395

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PETITION TO INCLUDE PROPYLENE GLYCOL MONOLAURATE ON TO 7 CFR § 205

This petition is comprised of four documents and a cover letter. The first document is the actual petition as described in 7 CFR 105. The second document is the Appendix to the petition, and contains materials used in the petition. Document 3 and 4 are copies of the successful waiver rationales submitted to EPA in support of the registration of ACARITOUCH.

ITEM A. The petition is to have propylene glycol monolaurate [PGML] included the National List at §205.601, specifically §205.601 (e).

ITEM B.

1. Propylene glycol monolaurate [PGML],
Systematic names: 1,2-propanediol monolaurate, Dodecanoic acid, monoester with 1,2-propanediol and lauric acid, monoester with 1,2-propanediol
Synonyms: AI3-00986, E 2580, EINECS 248-315-4, Emalex PGML, Propylene glycol monodecanoate and Rikemal PL 100.

2. Manufacturer
Riken Vitamin Co., Ltd.
2-9-18, Misaki-cho, Chiyoda-ku, Tokyo, Japan

Telephone: +81-3-5275-5130

3. Propylene glycol monolaurate [PGML] is the **sole active ingredient** in a biochemical contact miticide called ACARITOUCH which is labeled for the control of tetranychid mites and other mites on fruits, vegetables and ornamentals. A full listing of crops is as follows:
Cucurbits, such as Acorn Squash, Butternut Squash, Cantaloupe, Masaba Melon, Crenshaw Melon, Crookneck Squash, Cucumbers, Gherkins, Honeydew Melon, Pumpkin, Scallop Squash, Watermelon and Zucchini.
Pome Fruits: such as Apples, Pears and Quince
Stone fruits: such as Apricots, Cherries, Nectarines, Peaches and Plums
Grapes: (wine, fresh market and raisin)
Nut Crops: such as Almonds, Walnuts and Pistachios
Hops (not registered for use on hops in California)
Small fruits/Berries: such as Blackberry, Blueberry, Currant, Loganberry, Raspberry and Strawberry
Fruiting Vegetables: such as Eggplant, peppers and tomatoes (not registered for this use on in California)
Tuberous and Corm Vegetables: such as Potatoes, Artichokes, Sweet Potatoes and Yams (not registered for this use on in California)
Cereal Grains: such as Corn, Millet, Popcorn and Sorghum (not registered for this use on in California)
Citrus: such as Grapefruit, Lemon, Lime and Oranges (not registered for this use on in California)
Cotton: (not registered for this use on in California)

Ornamentals: Field and Greenhouse such as bare root, container, bedding and flowering stock, field grown cut flowers, vegetable transplants, nursery and landscape potted flowering, shade and flowering trees, and woody ornamentals (not registered for this use on in California).

The function of the PGML is as a contact control agent for tetranychid and other mites. It acts by a non-toxic mode of action thought to operate by blocking the peritreme of the mites and solubilizing cuticle wax thereby causing death by suffocation and/or desiccation.

4. The application rate of ACARITOUCH is 12 - 25 oz of product [8.5 - 17.7 oz of PGML] per 100 gallons of water and usage is such that the minimum volume per acre is 50 gallons.

The product is not to be applied through any type of irrigation system. Half fill the spray tank with half of the calculated amount of water, add the ACARITOUCH, agitate the tank thoroughly and then add the remaining water. Mixing should be thorough to ensure adequate coverage. Use immediately if possible, but it must be used within 24 hours of mixing.

5. PGML is manufactured by Riken Vitamin Co., Ltd., according to a generalized scheme for producing propylene glycol fatty acid monoesters, whereby propylene glycol and in this particular case lauric acid (from coconut oil) are reacted in an esterification process in a 10,000 liter, stainless steel reaction vessel., the product is then subjected to molecular distillation (thin-film, high vacuum molecular still) and then filtered (stainless steel filter with 5 mμ filter) to give the desired product. Riken, claiming proprietary technology will not reveal further details. The typical yields in this process are PGML (>97%), propylene glycol dilaurate (<2.5%), unreacted propylene glycol (<0.2%) and unreacted lauric acid (<0.3%).

6. In the Appendix, there are two letters from OMRI relative to the application for certification of ACARITOUCH an end-use product containing PGML.
In a letter dated January 29, 2008, in reference to PGML (Riken PL-100), OMRI asks for " .... complete description (preferably including a flow chart) of the manufacturing process of this ingredient. Include ingredient amounts, sequence and duration of events, temperature changes, reactions, and all steps taken to assure that prohibited substances are not intentionally or accidently in the product and methods to verify that the product is not contaminated. Include description of any composting, digestion, fermentation, extraction or processes and any methods used for removing extractants or growth media from final product.."

A letter dated June 9, 2008 which was the final response from OMRI and which states in part "The Review Panel determined that Acaritouch was prohibited because of the presence of synthetic substances that are not on the 2004 EPA List 4: Rikemal PL-100 (propylene glycol mono laurate) and Actor M-1. Substances used in a pesticide must be either non-synthetic or be on the EPA

List 4 as of 2004. Otsuka Chemical Co. Ltd., can petition the NOP to have the prohibited substance considered for use in organic production ...... Should your petition be successful, OMRI will re-open the Acaritouch application file upon your request."

Otsuka have reformulated Acaritouch and replaced Actor M-1 with corn oil CAS# 8001-30-7 which is both exempt from the requirement of a tolerance at 40 CFR 180..950 [c][1] and originally on EPA Inerts List 4A.

7. Environmental Protection Agency
Although the EPA has not registered PGML as a " technical active ingredient" they have registered two end-use products containing this material as an active ingredient.

On May 18, 2000, Toagosei the original registrant of ACARITOUCH, petitioned the EPA to classify PGML as a biochemical pest control agent based on its safety, GRAS status, widespread use as a food additive (in bakery products and ice cream, etc.) and the non-toxic mode of action when used as a pest control agent. On August 3, 2000 EPA agreed to classify ACARITOUCH as a biochemical-like pesticide eligible for review with the Biopesticide & Pollution Prevention Division using a reduced set of data requirements.

[In Dr. Schneider's letter there is a statement that monoacylglycerols may affect plasma membranes, note that PGML is not a monoacylglycerol.]

These two products are (a) VWX-42 Technology Propylene Glycol Monolaurate which was registered to then 3M Company on September 30, 2003 and has registration number 10350-67 [this product is used as a broad spectrum antimicrobial agent to control fungi and bacteria that cause decay of post-harvest fruits and vegetables. and (b) ACARITOUCH which was originally registered on October 12, 2004 to Toagosei Chemical Co. Ltd. This registration was transferred to Otsuka Chemical Co. LTD., the current registrant on December 12, 2005 with registration number 11581-3. The Otsuka product is currently registered in California, Oregon and Arizona.

PGML is exempt from the requirement of a tolerance at 40 CFR 180.1250 in or on all food commodities.

EPA has determined, based on the weight of evidence of data available to them there is no evidence that PGML and other monoesters of propylene glycol will effect the endocrine system and none is expected since they are natural components of vertebrate systems.

All of the above is confirmed in the Appendix by copies of correspondence to and from the Agency, printouts from the NPIRS Public and the NPIRS databases, the EPA Fact Sheet for Fatty Acid Monoesters with Glycerol and Propylene

Glycol. and tolerance related citations from the Code of Federal Regulations and the Federal Register.

Food & Drug Administration
PGML has GRAS status at 21 CFR 172.856 as a multipurpose direct food additive since the fatty acid is in compliance with 21 CFR 172.860. It is also permitted for use as an indirect food additive (adhesive component) at 21 CFR 175.105

California Department of Pesticide Regulation
The product Acaritouch is registered as a miticide in California and the CDPR memorandum of evaluation is included in the Appendix.

CODEX
There are no CODEX MRL values for PGML.

Miscellaneous
1. Admitted as a food additive in Japan in 1961 (Official Regulation of Food Additives, 6th Edition., Food Chemicals #8, 1996, p 219.
2. ADI in Japan 25 mg/kg/day
3. In CODEX list of food additives with INS # 477
4. Complies with the European Pharmacopoeia Monograph for Propylene Glycol Monolaurate Type II
5. Complies with the United States Pharmacopoeia/ National Formulary Monograph for Propylene Glycol Monolaurate Type II

8. Chemical Abstracts Services and other identifying numbers
PGML has CAS# 27194-74-7 and has INS (International Numbering System of Codex Food additives of 477). Product labels are in the Appendix, relative to Item B 3 and B 8.

9. The physical./chemical characteristics of PGML are as follows:

Color	very pale yellow
Physical State	liquid
Odor	weakly irritative peculiar odor
pH	5.9 [1% aqueous solution]
UV/Visible spectrum	λ_{max} 210 nm [ε 64.78]
Melting point	8.3 °C
Boiling point	246.6 °C
Specific Gravity	0.92 g/ml (25 °C)
Water solubility	4 mg/L
Solubility in organic solvents	> 1kg/L for hexane, heptane, xylene, toluene, dichloromethane, acetone, methanol, ethanol and ethyl acetate.
Vapor pressure	0.162 Pa/°C at 25 °C

As stated previously, when used as the active ingredient in ACARITOUCH the PGML does not cause its effect by a toxic mode of action but is thought to operate by blocking the peritreme of the mites and solubilizing cuticle wax thereby causing death by suffocation and/or desiccation.

Toxicology information for PGML (studies carried out by Riken Vitamin Co., Ltd., waivers of specific data requirements prepared by Drs Beth Mileson and Iain Weatherston). The waivers comprise Documents 3 and 4 of this petition.

Acute oral LD_{50} (mice)	=	40,000 mg/kg
Acute dermal LD_{50}	=	waiver granted by EPA*
Acute inhalation LD_{50}	=	waiver granted by EPA*
Primary eye irritation (rabbit)	=	minimally irritating (EPA toxicity category IV)
Primary skin irritation (rabbit)	=	slightly irritating (EPA toxicity category IV)
Skin sensitization (guinea pig)	=	not a sensitizer **
Genotoxicity studies	=	waiver granted by EPA*
Immune response	=	waiver granted by EPA*
90 day oral toxicity	=	waiver granted by EPA*
Teratology	=	waiver granted by EPA*
Ecotoxicity and Environmental Persistence		
Avian acute oral toxicity	=	waiver granted by EPA*
Avian dietary toxicity	=	waiver granted by EPA*
Acute toxicity to *Daphnia spp*	=	EC at 24 hours = 0.85 mg/L, at 48 hours = 0.52 mg/L. NOEC at 24 and 48 hours was 0.39 and 0.18 mg/L respectively, and the lowest 100% inhibition concentration was 1.55 mg/L (48 hrs)
Acute toxicity to juvenile Carp	=	24 hrs LC_{50} 6.22 mg/L, NOEC = 4.16 mg/L 48 hrs LC_{50} 5.25 mg/L, NOEC = 3.88 mg/L 72 hrs LC_{50} 5.25 mg/L, NOEC = 3.88 mg/L 96 hrs LC_{50} 5.20 mg/L, NOEC = 3.80 mg/L
Honey bee	=	No harmful effects on adult honey bees caused by acute contact with PGML at 25μg/bee (LD50 could not be determined)
Nature of residue	=	waiver granted by EPA**
Magnitude of residue	=	waiver granted by EPA**

* Document 3 in the submission
** Document 4 of the submission

10. Safety Information

The MSDS of Riken PL-100 (PGML) and the MSDS of USP PGML are to be found in the Appendix.

A copy of the final report on the safety assessment of Propylene Glycol (PG) Dicaprylate, PG Dicaprylate-Dicaprate, PG Dicocoate, PG Dipelargonate, PG Isostearate, PG Laurate, Pg Myristate, PG Oleate, PG Oleate SE, PG Dioleate PG Dicaprate, PG Diisostearate and PG Dilaurate is also to be found in the Appendix.

11. Research Information

PGML uses are primarily limited to use as a food additive, pharmaceutical or cosmetic excipient and in two cases as a pesticide. Most of the research documentation is already contained in the Appendix or in Documents 3 and 4. Database searches in the medical, biomedical, cosmetic and agricultural fields turned up research data which would neither support or deny the inclusion of PGML of the National List.
PGML belongs to the class of compounds known as propylene glycol mono aliphatic esters which form part of a category in the EPA's HPV Challenge Program. The test plan for the glycol esters category of aliphatic esters chemicals is include in the Appendix as an overview of the research .
Included in the Appendix is a statement signed by Otsuka that commercial availability does not depend on geographic location or local market conditions. The letter refers not to PGML which is sold on the open market but to ACARITOUCH which is the pesticide product containing PGML, and as can be seen the product is registered and sold in the U.S.A., Japan and the Kingdom of Morocco with active registration applications ongoing in Mexico, Philippines, Taiwan and Sri Lanka. Field trials as a first step in the registration process have been initiated in the Dominican Republic, Guatemala, Costa Rica, Colombia, Equador and Thailand.

12. Petition Justification Statement

The vast majority of miticides used in agriculture are conventional chemicals and not only do they kill the target mites they also have deleterious effects on predators and parasites of the pest mites., the environment and their residues pose a certain risk. Therefore more benign miticides were sought. PGML is a novel miticide being developed as part of a strategy of the Japanese Ministry of Education, Culture, Science and Technology to introduce into pest control strategies compounds used as foods or food additives, such compounds having been verified as safe for the environment and for human consumption.

Hundreds of different foods and food additives were screened against adult female two-spotted spider mites and it was demonstrated that propylene glycol fatty acid esters showed significant efficacy. PGML was one of the esters showing the highest activity and the formulation of this material was optimized to give the highest miticidal activity at low application rates.

Currently, farnesol, nerolidol, geraniol and citronellol both as natural extractives and as "natural identical pheromones" are formulated into an EPA registered product called Biomite where they act as "arresting pheromones" causing mites to remain stationary where they desiccate or become prey.

The beneficial effects of PGML on the environment include [a] its minimal effect on predacious mites, honey bees, predatory bugs and parasitic wasps. A 1999 study by the Japan Crop Protection Association indicates that the direct and residual effect of PGML formulations on the eggs and female adults predatory

mites is minimal. PGML formulations are also widely used as a "cleaner" of adult and nymph spired mites before and after the introduction of predacious mites such as *Phytoseiulus persimilis* and *Amblyseius califiornicus.* Also since the PGML product does not have any efficacy against spider mite eggs the synergy between its use with the use of predacious mites is excellent. [b] the use of PGML leads to significantly less phytotoxicity that soaps and oils. [c] PGML is also effective against powdery mildew further reducing the use of oils and concomitant phytotoxicity [d] the amount of PGML used per acre is up to 14 x less than with soaps and oils. [e] PGML is rapidly hydrolyzed to propylene glycol and lauric acid by soil microorganisms, propylene glycol and lauric acid themselves are also bioavailable to, and quickly metabolized by microorganisms ensuring that there is no accumulation of the PGML or its metabolites in the environment or farm ecosystem.

The beneficial effects pf PGML to human health when used as a miticide is that {a] it is an approved food additive, [b] it is widely used as a forming agent in the food industry, [c] it is prepared from two GRAS (generally regarded as safe) compounds propylene glycol and lauric acid from coconut oil. The metabolism of PGML both in animals and plants is the same as fats and oils.

13. Confidential Business Information Statement.
No information contained in this petition (Document 1) or the supporting documents (Documents 2, 3 and 4 is deemed to be Confidential Business Information.

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13. Confidential Business Information Statement.
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APR 24 2009

PETITION TO INCLUDE PROPYLENE GLYCOL MONOLAURATE

ONTO 7 CFR § 205

APPENDIX

DOCUMENT 2

TECHNOLOGY SCIENCES GROUP INC.
4061 North 156th Drive
Goodyear, AZ 85395

ON BEHALF OF

OTSUKA CHEMICAL COMPANY LTD.
3-2-27 Otedori, Chuo-Ku
Osaka, 540-0021, Japan

THIS VOLUME CONTAINS NO CONFIDENTIAL BUSINESS INFORMATION

ALL COMMUNICATIONS REGARDING THIS DOCUMENT SHOULD BE DIRECTED TO

Dr. Iain Weatherston
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4061 North 156th Drive
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Mobile 623-217-9013

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DOCUMENTATION IN REGARDS TO ITEM B2

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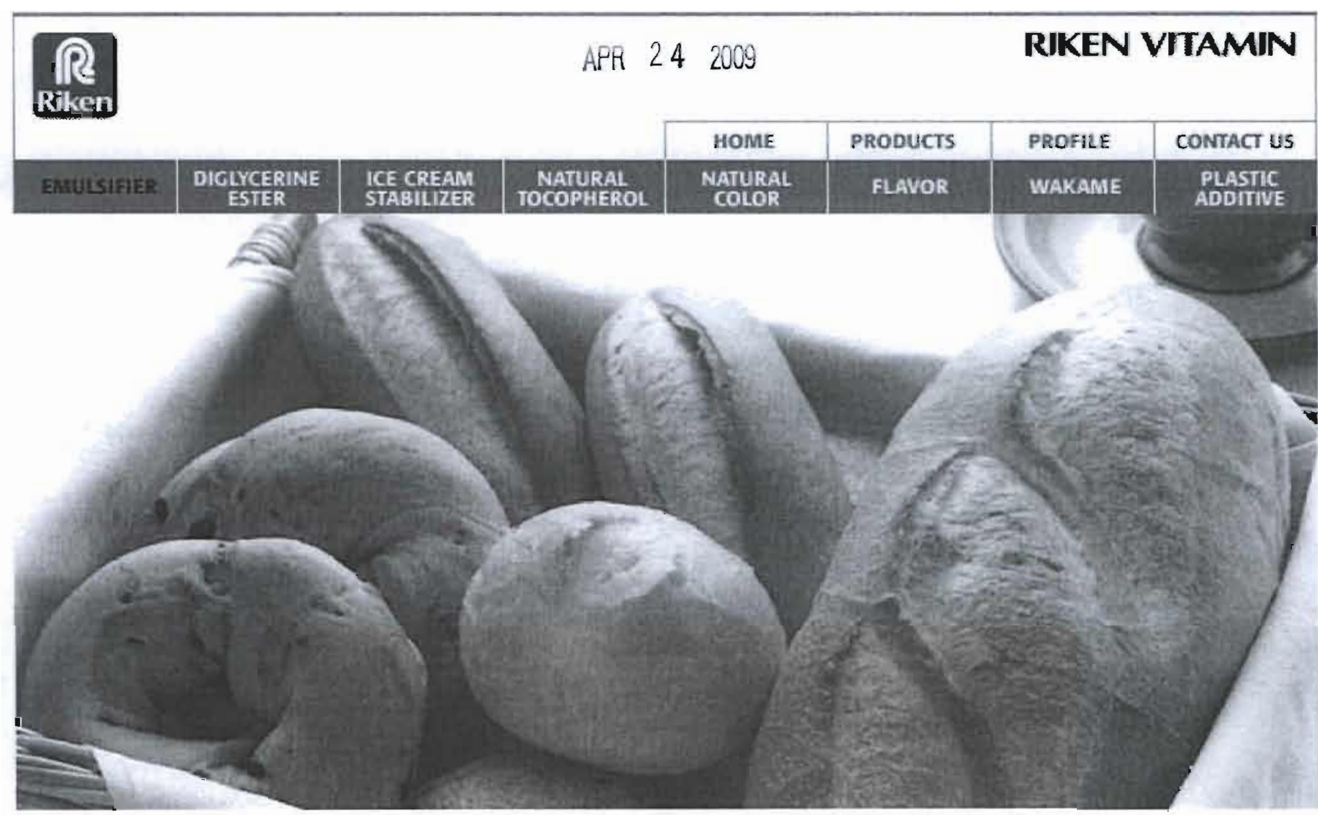

EMULSIFIER

Food is a complicated mixture of carbohydrate protein, oil and fat, water, and air, as well as a variety of other minute components such as minerals, vitamins, and flavors. Food processing subjects this mixture to a wide range of thermal treatments, such as baking, boiling, steaming, freezing; and mechanical treatments, such as kneading, mixing, extruding etc, all of which futher complicate the structure of the food. The desired result of these treatments is good tasting food. For this reason, products with inproved flaver, texture and quality are always in demand. The challenge is to bring together the deiverse and often conflicting natural components of food into a consistent and pleasing blend. Each component of food (carbohydrate, protein, oil and fat, water, air, etc.) has its own unique properties which sometimes come into conflict with other components, for example, oil and water. Thus a third substance is neccessary to increase the compatibility of the contact surfaces of the two components, that is, to serve as an interface between two mutually exclusive materials, The substance which is used to improve the quality of food and its processing is called a food emulsifier.

Made from Pure Vegitable Oil
New Rikemal Series

Specification

- Specialities
  - for Bread
  - for Cake
  - for Tofu
  - for Noodle
  - for Ice Cream
  - Lecithin & Others

- Distilled monoglycerides (DMG)
- Mono- & diglycerides (MG)
- Acetic acid esters of monogrlycerides (AMGt)
- Organic acid esters of monoglycerides
- Sorbitan estels of fatty acids (SOE)
- Propylene glycol esters of fatty acids (PGME)
- Polyglycerol esters of fatty acids (PGE)
- Legal Status

Function

- Basic
- Application

Kind of Emulsifier

Corporate Profile

Company name: Riken Vitamin Co., Ltd.
Date of incorporation: 27, Aug, 1949
Capitalized at: ¥2.537 billion
President: Yoshiyasu Sakai
Number of Employees: 1000

Head Office:

2-9-18 Misakicho, Chiyoda-ku, Tokyo, Japan
Fax 03-3237-1087 Phone 03-5275-5130

Branch Offices:

Tokyo, Osaka

Factories:

Soka, Chiba, Tokyo, Kyoto, Osaka

Subsidiaries:

- Riken Food Co., Ltd.
- Kenseido Co., Ltd.
- Eiken Shoji Co., Ltd.
- Sanny Housou Co.,Ltd.
- Shinken Sangyo Co., Ltd.
- Shinwa Food Co.,Ltd.
- Tianjin Rikevita Food Co., Ltd.
- Rikevita Fine Chemical & Food Industry (Shanghai) Co., Ltd
- Qingdao Fusheng Food Stuff Co., Ltd.
- Dalian Riken Maruha Foodstuffs Co., Ltd.
- Dalian Riken Food Co., Ltd.
- Rikevita Asia Co., Ltd
- Riken Food(Dalian) Co., Ltd.
- Rikevita (Malaysia) SDN BHD
- Rikevita (Singapore) Pte Ltd.
- Riken Vitamin Europa GmbH
- Riken Vitamin USA Inc.
- Guymon Extracts Inc.

Sales:

Industries:

- Consumer and commercial foods
- Processed food ingredients
- Food chemicals for industrial uses
- Vitamin production and sales

History:

1949	Riken Vitamin Oil Co., Ltd. established
1953	Tokyo Factory starts operation
1959	Osaka Factory starts operation
1961	Riken Vitamin Oil Co., Ltd. listed on the Tokyo Stock Exchange's second section
1968	Soka Factory starts operation
1973	Chiba Factory starts operation
1980	Company renamed Riken Vitamin Co., Ltd.
1985	Kyoto Factory starts operation
1991	Rikevita (Malaysia) SDN BHD established
1993	Tianjin Rikevita Food Co., Ltd. established
1994	Rikevita (Singapore) Pte Ltd. established
1995	Qingdao Fusheng Food Stuff Co., Ltd. established
1996	Affiliated with Eiken Shoji Co., Ltd.
1999	Application Centers established at Soka Factory, Japan, at Tianjin Rikevita Food Co., Ltd.
2000	Presentation Center established
2002	Rikevita(Malaysia) SDN.BHD. expanded
2003	Riken Vitamin Europa GmbH established
2004	Guymon Extracts Inc. established Riken Vitamin USA Inc. established
2005	Rikevita Fine Chemical & Food Industry (Shanghai) Co., Ltd established
2006	Rikevita Asia Co., Ltd established Riken Food(Dalian) Co., Ltd. Established

- '04.4 ~ '05.3
  ¥ 64.3 billion (consolidated: 73.7 billion)
- '05.4 ~ '06.3
  ¥ 66.2 billion (consolidated: 79.0 billion)
- '06.4 ~ '07.3
  ¥ 65.2 billion (consolidated: 80.8 billion)

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DOCUMENTATION IN REGARDS TO ITEM B3

APR 24 2009

ACARITOUCH®

Environmental-Friendly Novel Contact Miticide

APR 24 2009

ACTIVE INGREDIENT:

Propyleneglycol fatty acid monoester 70%

- **Non-toxic mode of action**
  - Suffocation by blocking the spiracles
  - Repeated sprays are available
  - Suited for resistance management
- **Lower input**
  - Effective at comparatively lower dosage than other contact miticides such as oils and soaps
  - Not sticky
- **Control also Powdery Mildew**
- **Human-friendly**
  - A.I. is registered as a food additive
  - Safe and easy to use
  - PHI of 1 day
- **Environmental-friendly**
  - Minimal toxic against beneficial insects and bees
  - Intended for use in an integrated pest management (IPM) program to control mites

Otsuka Chemical Co., Ltd.
3-2-27, Ote-dori, Chuo-ku
Osaka 540-0021, Japan

Mode of Action

ACARITOUCH is a miticide with a non-toxic mode of action, blocking the spiracles and de-waxing the cuticle, which causes death by suffocation and desiccation.
ACARITOUCH is effective against from larva to adult mites.

Effective against

ACARITOUCH is effective against tetranychid and other phytophagous mites including...

- Two-spotted spider mite (*Tetranichus urticae*)
- Kanzawa spider mite (*Tetranychus kanzawai*)
- Pacific mite (*Tetranichus pacificus*)
- Strawberry spider mite (*Tetranychus atlanticus*)
- Citrus red mite (*Panonychus citri*)
- European red mite (*Panonychus ulmi*)
- Brown almond mite (*Bryobia rubrioculus*)

T. urticae

T. kanzawai

P. citri

For better control

Disadvantages of ACARITOUCH are...

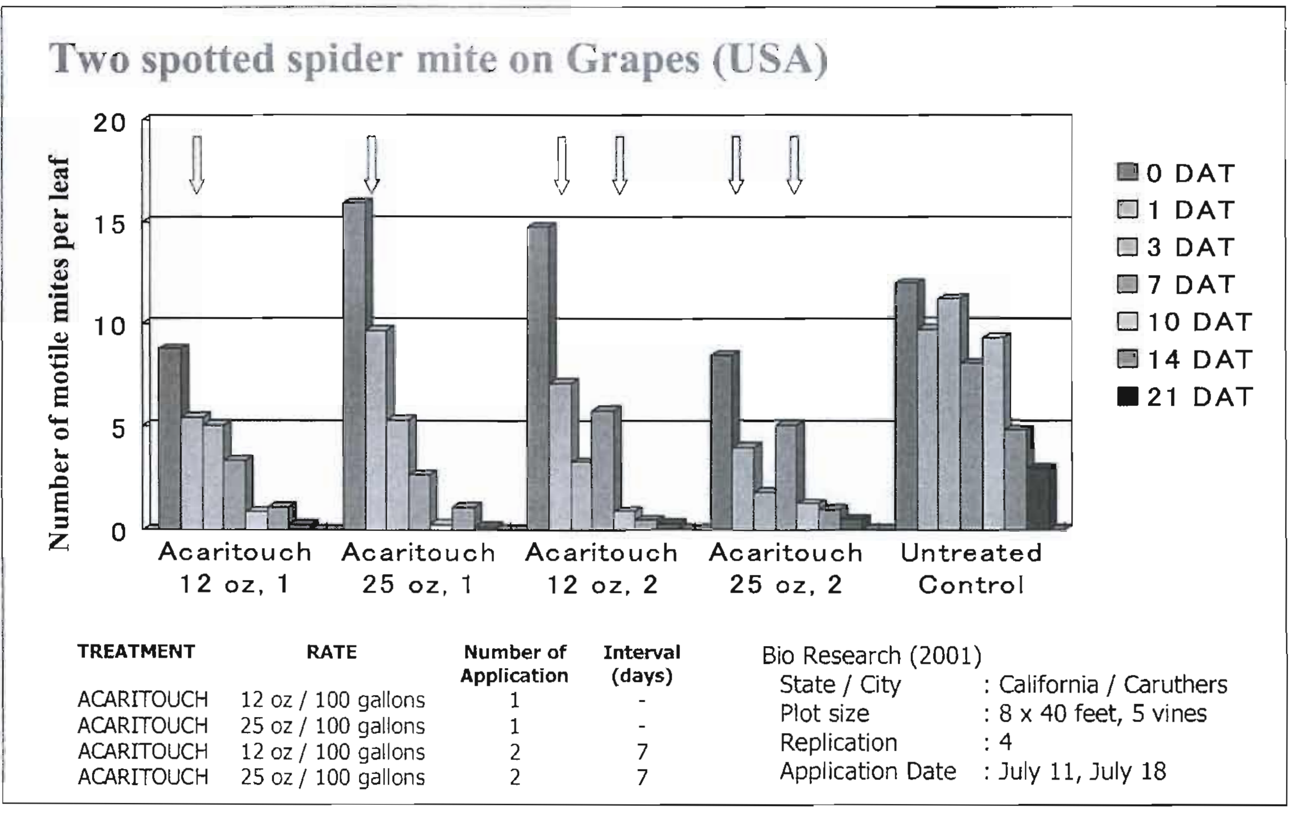
- Lack of ovicidal activity
- Lack of residual activity

So the instructions to achieve a better control are ...

- Apply ACARITOUCH as soon as mites are identified on the plants, or when environmental conditions favor mite outbreaks. It is necessary to spray thoroughly over foliage and fruits/vegetables in order to assure a complete coverage of mites and to achieve a maximum level of control.
- At first sign of mite infestation, apply in sufficient water to thoroughly cover plant with spray. Make two applications 7 days apart to suppress and control mites present on the plants and those newly emerged. If temperature is such that mite egg hatch is accelerated, make the inter-treatment interval 3-5 days.
- Integrated pest management (IPM) is one of the solutions to improve its utility. For example, quick reduction of the population of adult and larval mites by ACARITOUCH and subsequent introduction of commercial predatory mites or application of ovicide may contribute to an effective control of mites.

Experimental Results

Two spotted spider mite on Grapes (USA)

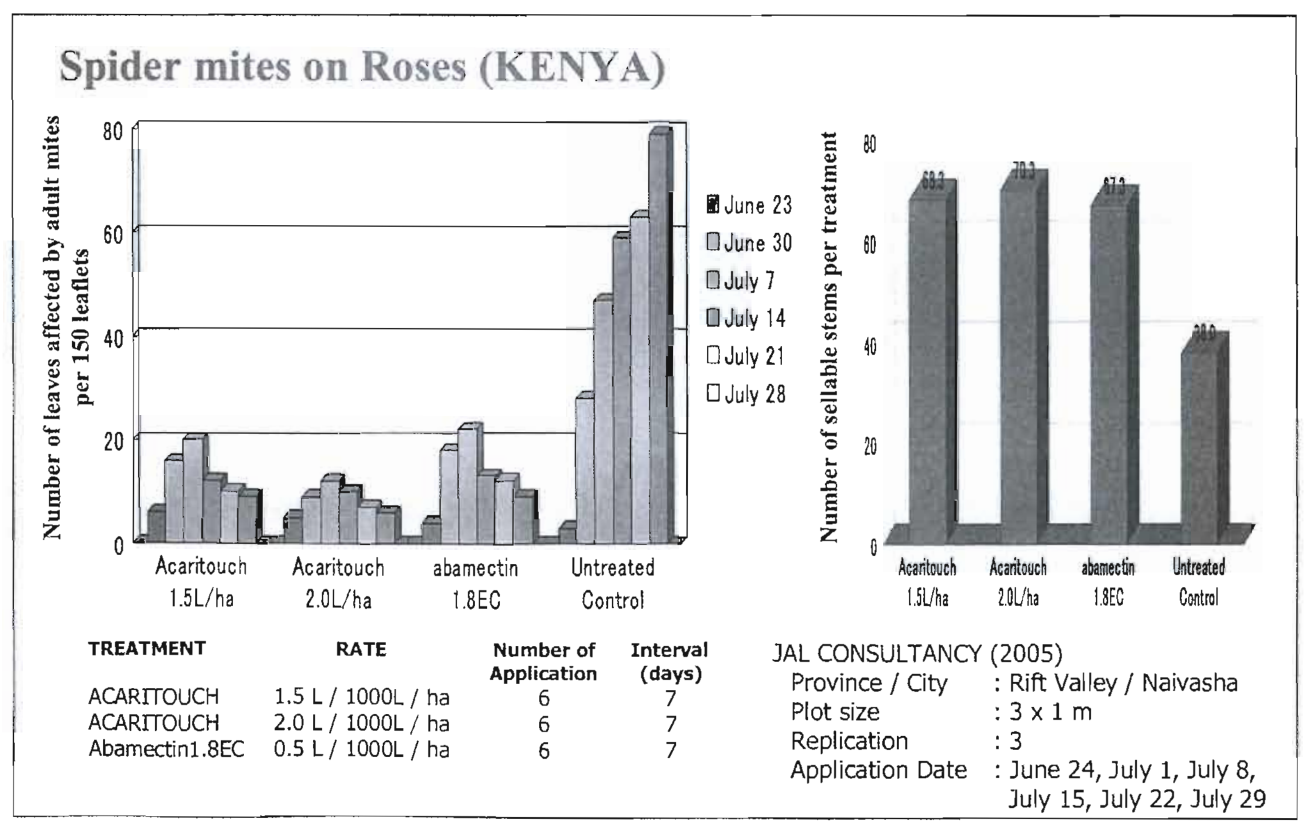


TREATMENT	RATE	Number of Application	Interval (days)
ACARITOUCH	12 oz / 100 gallons	1	-
ACARITOUCH	25 oz / 100 gallons	1	-
ACARITOUCH	12 oz / 100 gallons	2	7
ACARITOUCH	25 oz / 100 gallons	2	7

Bio Research (2001)

- State / City : California / Caruthers
- Plot size : 8 x 40 feet, 5 vines
- Replication : 4
- Application Date : July 11, July 18

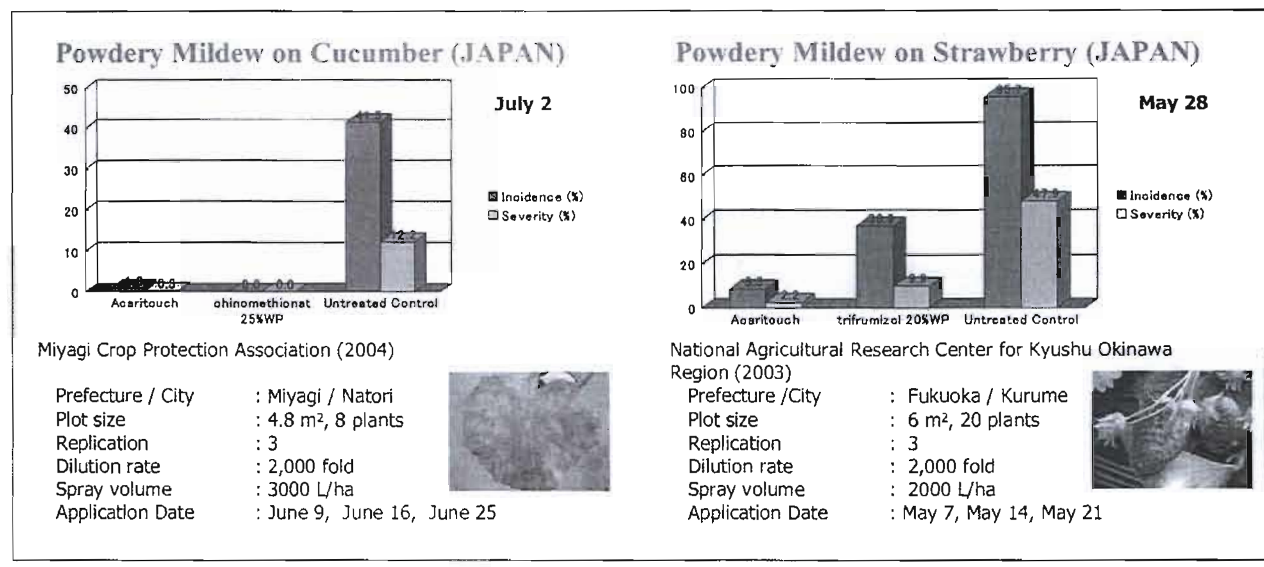
Spider mites on Roses (KENYA)



TREATMENT	RATE	Number of Application	Interval (days)
ACARITOUCH	1.5 L / 1000L / ha	6	7
ACARITOUCH	2.0 L / 1000L / ha	6	7
Abamectin1.8EC	0.5 L / 1000L / ha	6	7

JAL CONSULTANCY (2005)

- Province / City : Rift Valley / Naivasha
- Plot size : 3 x 1 m
- Replication : 3
- Application Date : June 24, July 1, July 8, July 15, July 22, July 29



Recommendations

(Based on the US label)

CROPS
Cucurbits, such as Acorn Squash, Butternut Squash, Cantaloupe, Casaba Melon, Crenshaw Melon, Crookneck Squash, Cucumbers, Gherkin, Honeydew Melon, Pumpkin, Scallop Squash, Watermelon, and Zucchini.
Pome Fruits, such as Apples, Pears, and Quince
Stone Fruits, such as Apricots, Cherries, Nectarines, Peachs, and Plums
Grapes (wine, fresh market, raisin)
Nut Crops, such as Almonds, Walnuts, and Pistachios
Hops
Small Fruits and Berries, such as Blackberry, Blueberry, Currant, Loganberry, Raspberry, Strawberry
Fruiting Vegetables, such as Eggplant, Peppers, and Tomatoes
Tuberous and Corm Vegetables, such as Potatoes, Artichokes, Sweet Potatoes, and Yams
Cereal Grains, such as Corn, Millet, Popcorn, and Sorghum
Citrus, such as Grapefruit, Lemon, Lime, and Oranges
Cotton
Ornamentals-Field and Greenhouse, such as bare root, container, bedding, and flowering stock, field grown cut flowers, vegetable transplants, nursery and landscape, potted flowering, shade and flowering trees, and woody ornamentals

APPLICATION RATES: Mix 12 to 25 fl. oz. of ACARITOUCH per 100 gallons of water. Apply sufficient spray volume to ensure thorough coverage of crop.

ACARITOUCH®

Hard hitting mite control

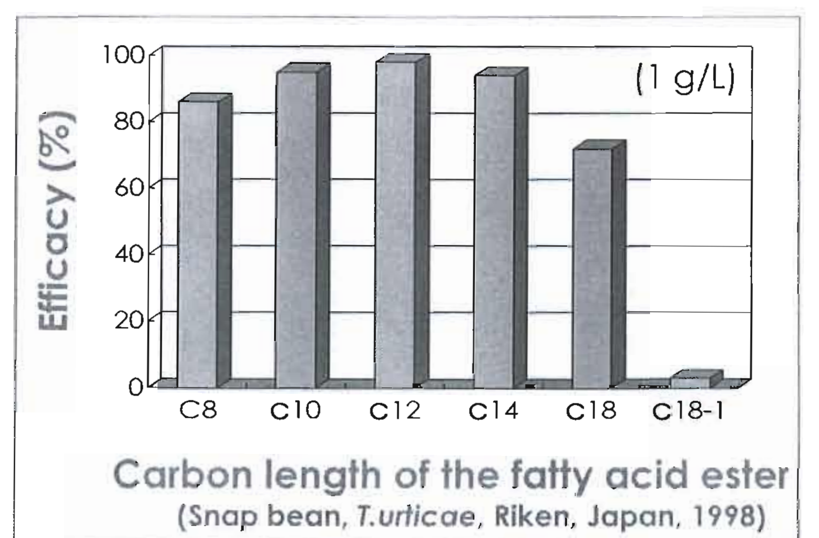
with a soft touch on the environment

Otsuka Chemical Co., Ltd.
AgriTechno Division

Introduction

ACARITOUCH® is a novel miticide which had originally been developed by Dr. Arimoto of RIKEN INSTITUTE and TOA GOSEI CO., LTD.. They had focused into the compounds being used for foods or food additives as their developmental policy of creating new pesticides because these compounds had been verified as safe to human beings and the environment.

They screened hundreds of different foods and food additives against adult female two-spotted spider mites and found that propylene glycol fatty acid esters showed significant efficacy. They continued the research and optimized the formulation in order to present the highest miticidal activity at the lowest input.



(Snap bean, *T.urticae*, Riken, Japan, 1998)

ACARITOUCH® was introduced to the Japanese vegetables and fruits fields in 2001 and has been gaining market share as its effective usages are developed and known. It would be greatly appreciated if you would find new usages of this product.

General Information

ACTIVE INGREDIENT:

Propyleneglycol fatty acid monoester 70 %

TOXICITY: (70%EC)

Acute Toxicity : LD_{50} > 5,000 mg/kg (mice)
Eye irritation : Not irritative
Skin irritation : Slightly irritative
Skin sensitization : Not sensitizing

REGISTRATION:

Japan : # 20609
United States : # 11581-3
Morocco : # E 01-04-001

Characteristics

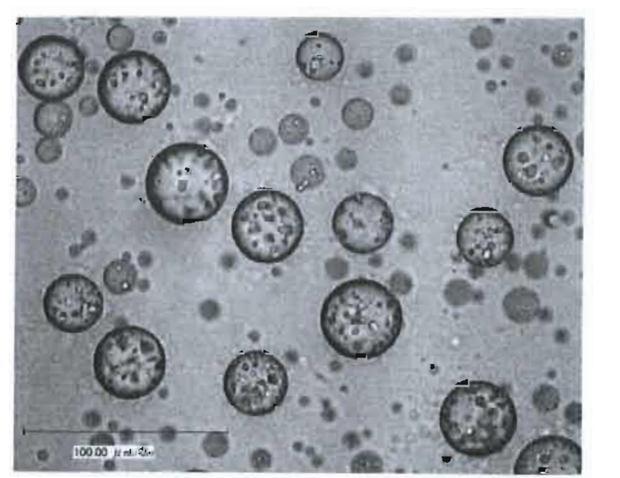
- Non-toxic mode of action
  - Suffocation by blocking the spiracles
  - Not likely to develop resistance
  - Can be used for repeated sprays
  - Suited for resistance management
- Efficacy and Usability
  - Rapid reduction of mite populations
  - Certain contact efficacy against powdery mildew
  - No stain
- Human-friendly
  - A.I. is registered as a food additive
  - PHI of 1 day
- Environmental-friendly
  - Low affect on beneficial insects and bees
  - Easily degraded to water and carbon dioxide in the environment

Mode of Action

When **ACARITOUCH®** is put into water, the diluted solution becomes pale milky emulsion. This is because relatively large-sized emulsion droplets have been formed to wrap propyleneglycol fatty acid monoester, the active ingredient, and have been uniformly dispersed in the emulsion.

Propyleneglycol fatty acid monoester exists in the droplet while the ingredient has not been diluted by water.

Emulsion droplets
in ACARITOUCH solution



Particle size : 30 ~50 μm

General range of droplet or particle size in spray liquid

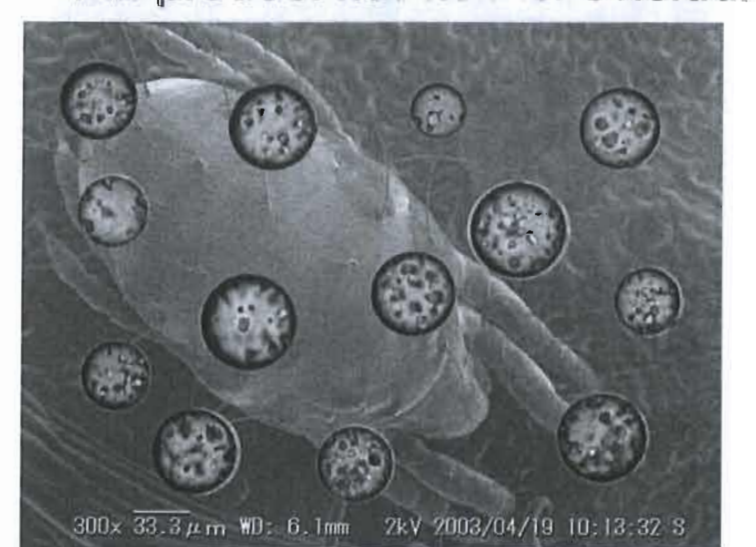
Formulation	Diameter			
EC	0.1	-	5	μm
EW	0.1	-	5	μm
ME		-	0.1	μm
MC	1	-	60	μm
WP	5	-	20	μm
SC	0.5	-	10	μm
Milk	0.1	-	10	μm
KALIGREEN	10	-	30	μm
ACARITOUCH	30	-	50	μm

Mode of Action

Propyleneglycol fatty acid monoester has a strong affinity to the surface of the spider mite cuticle. It is supposed that the undiluted ingredient in the emulsion droplets (approximately 30-50 μm diameter) directly reach the spiracles after adhering to and spreading on the cuticle, which causes death by suffocation of the mite. This is why ACARITOUCH® has good efficacy at comparatively low rates. At the same time, this means the coverage is critical.

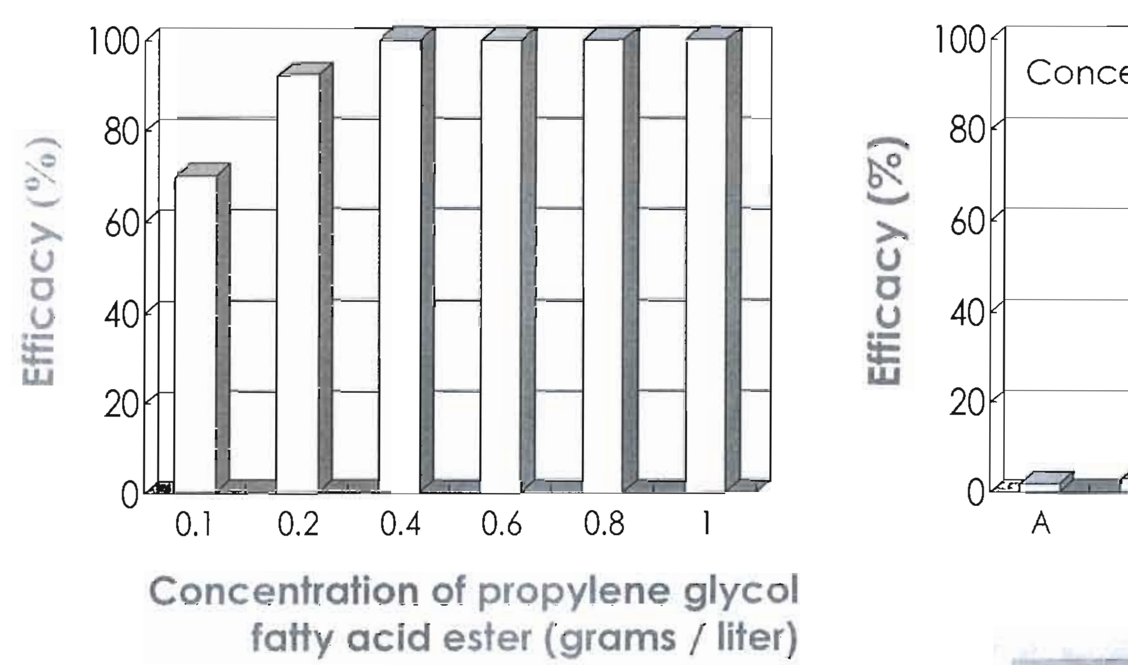
ACARITOUCH® is effective against larva and adult mites. When the spray solution has dried, it is no longer active.

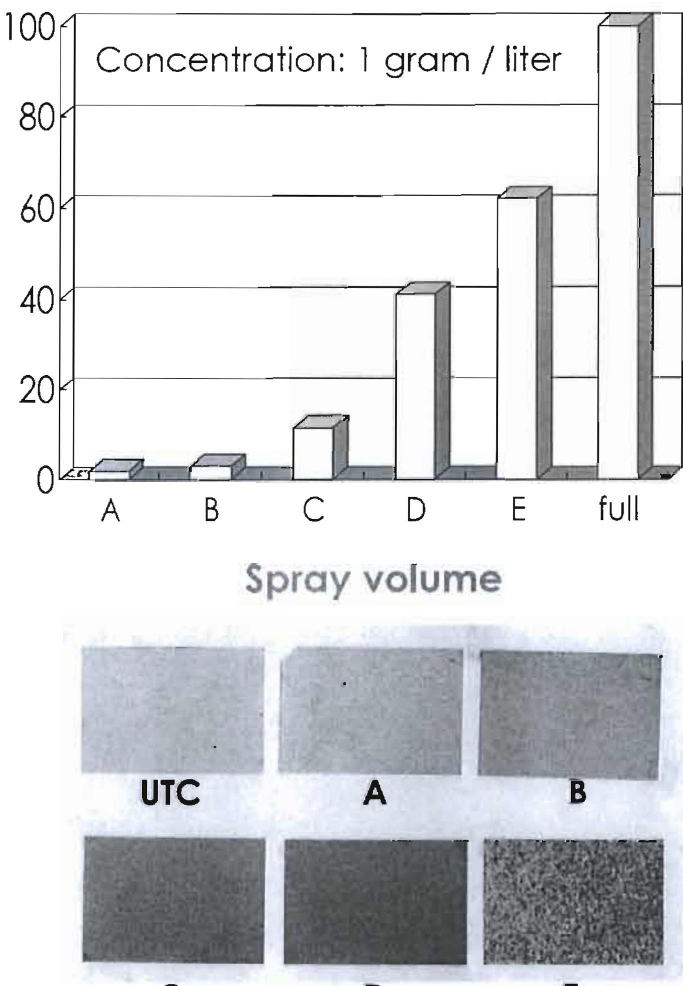
This product has neither ovicidal or residual activity.



Importance of Coverage

Otsuka Chemical Co., Ltd.
AgriTechno Division





Coverage is far more important than the dilution rate.

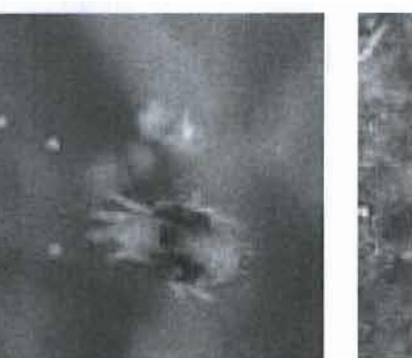
Snap bean, *T. urticae*, Riken, Japan, 1998

Nov 4, 2008

Effective against

ACARITOUCH® is effective against tetranychid and other phytophagous mites including…

- Two-spotted spider mite (*Tetranychus urticae*)
- Kanzawa spider mite (*Tetranychus kanzawai*)
- Pacific mite (*Tetranychus pacificus*)
- Strawberry spider mite (*Tetranychus atlanticus*)
- Citrus red mite (*Panonychus citri*)
- European red mite (*Panonychus ulmi*)
- Brown almond mite (*Bryobia rubrioculus*)



T. urticae

T. kanzawai

P. citri

Effect on Natural Enemies

1. Predatory mite (*Phytoseiulus persimilis*)

Phytoseiulus persimilis
SPIDEX® by Arysta LifeScience

Effect on eggs

Conc. (g/L)	Number of eggs applied	Number of adult mites *1		Survival rate (%)
		Alive	Missing	
1.0	55	53	2	96.4

*1) 5 days after the application

Effect on adult female mites (direct spray)

Conc. (g/L)	Number of mites applied	Number of mites *1			Mortality (%)	Egg production *2	Reproduced mites *3
		Alive	Dead	Others			
1.0	29	23	0	6	0	35	35

*1) 24 hrs after the application *2) Laid eggs in the 24 hrs *3) 5 days after the application

Effect on adult female mites (release 2 hrs after spray)

Conc. (g/L)	Number of mites applied	Number of mites *1			Mortality (%)	Egg production *2	Reproduced mites *3
		Alive	Dead	Others			
1.0	20	17	0	3	0	35	35

*1) 24hrs after the application *2) Laid eggs in the 24hrs *3) 5 days after the application

Effect on Natural Enemies

2. Predatory mite (*Amblyseius cucumeris*)

Effect on adult female mites (direct spray)

Conc. (g/L)	Number of mites applied	Number of mites [*1] Alive	Dead	Mortality (%)
0 [*2]	30	30	0	0
0.5	30	26	4	10.0
1.0	30	27	3	13.3
dimethoate [*3]	30	0	30	100

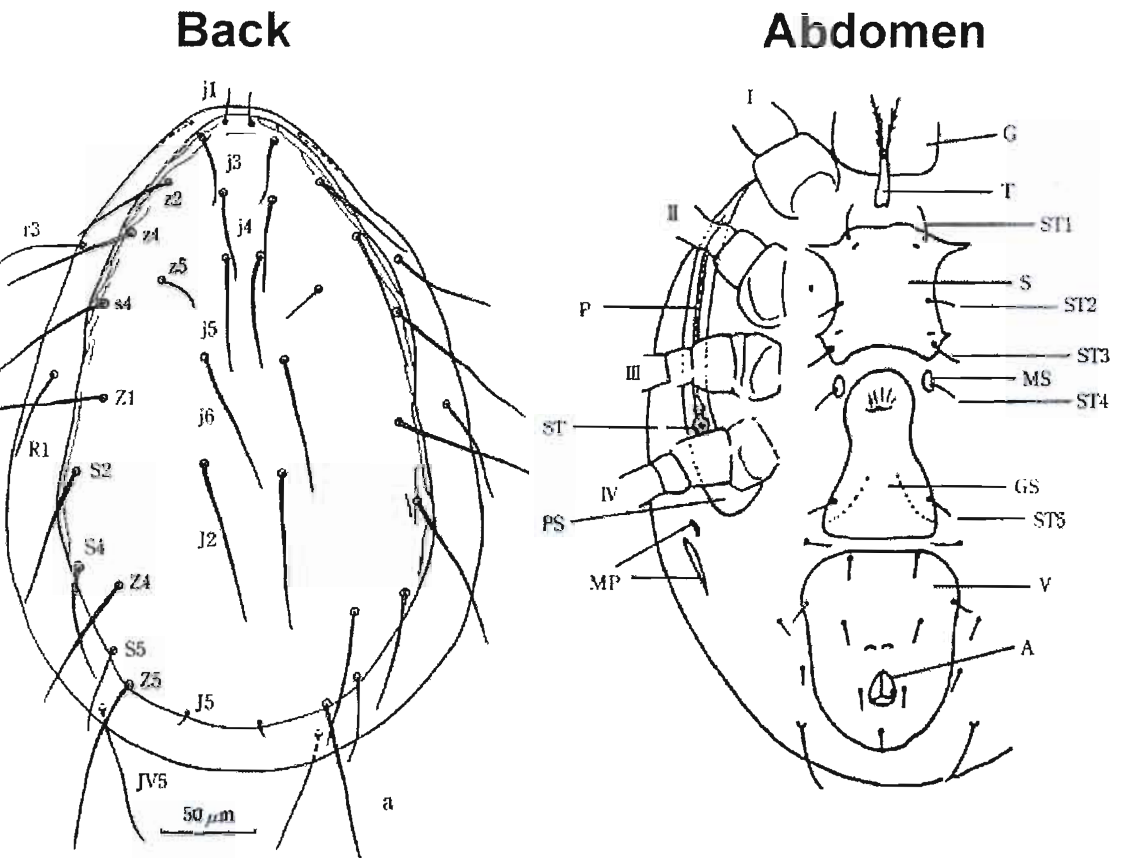
*1) 24 hrs after the application
*2) water
*3) 43%EC: 1.0 ml/L

Amblyseius cucumeris
CUCUMERIS® by Arysta LifeScience

Otsuka Chemical Co., Ltd.

AgriTechno Division

Position of spiracles of Predatory Mites (Phytoseiidae, Order: Mesostigmata)



Amblyseius womersleyi

（Ehara, 1959；江原，1980；いずれも改変）

（江原，1975を改変）

Cf. *Tetranychidae* (Order: *Prostigmata*)

Effect on Natural Enemies

3. Predatory bug (*Orius strigicollis*)

Effect on adult bugs (direct spray)

Conc. (g/L)	Number of bugs applied	Number of bugs *1 Alive	Dead	Mortality (%)
0 *2	30	30	0	0
0.5	30	29	1	3.3
1.0	30	6	24	80
dimethoate *3	30	0	30	100

*1) 24 hrs after the application *2) water *3) 43%EC: 1.0 ml/L

Orius strigicollis
TAIRIKU® by Arysta LifeScience

Effect on larval bugs (direct spray)

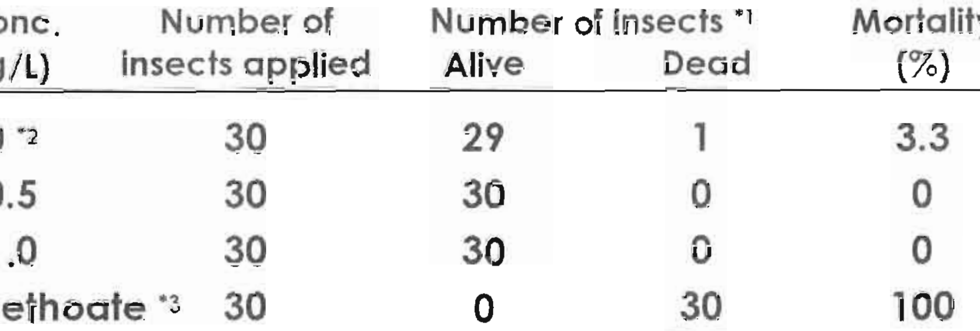
Conc. (g/L)	Number of bugs applied	Number of bugs *1 Alive	Dead	Mortality (%)
0 *2	30	30	0	0
0.5	30	30	0	0
1.0	30	30	0	0
dimethoate *3	30	0	30	100

*1) 24 hrs after the application *2) water *3) 43%EC: 1.0 ml/L

Effect on Natural Enemies

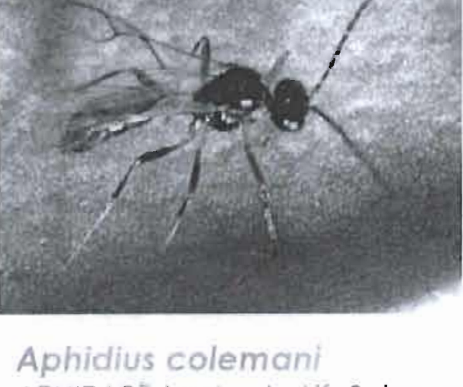
4. Parasitic insect (*Aphidius colemani*)

Effect on adults (direct spray)



Conc. (g/L)	Number of insects applied	Number of insects *1 Alive	Dead	Mortality (%)
0 *2	30	29	1	3.3
0.5	30	30	0	0
1.0	30	30	0	0
dimethoate *3	30	0	30	100

*1) 24 hrs after the application *2) water *3) 43%EC: 1.0 ml/L



Aphidius colemani
APHIPAR® by Arysta LifeScience

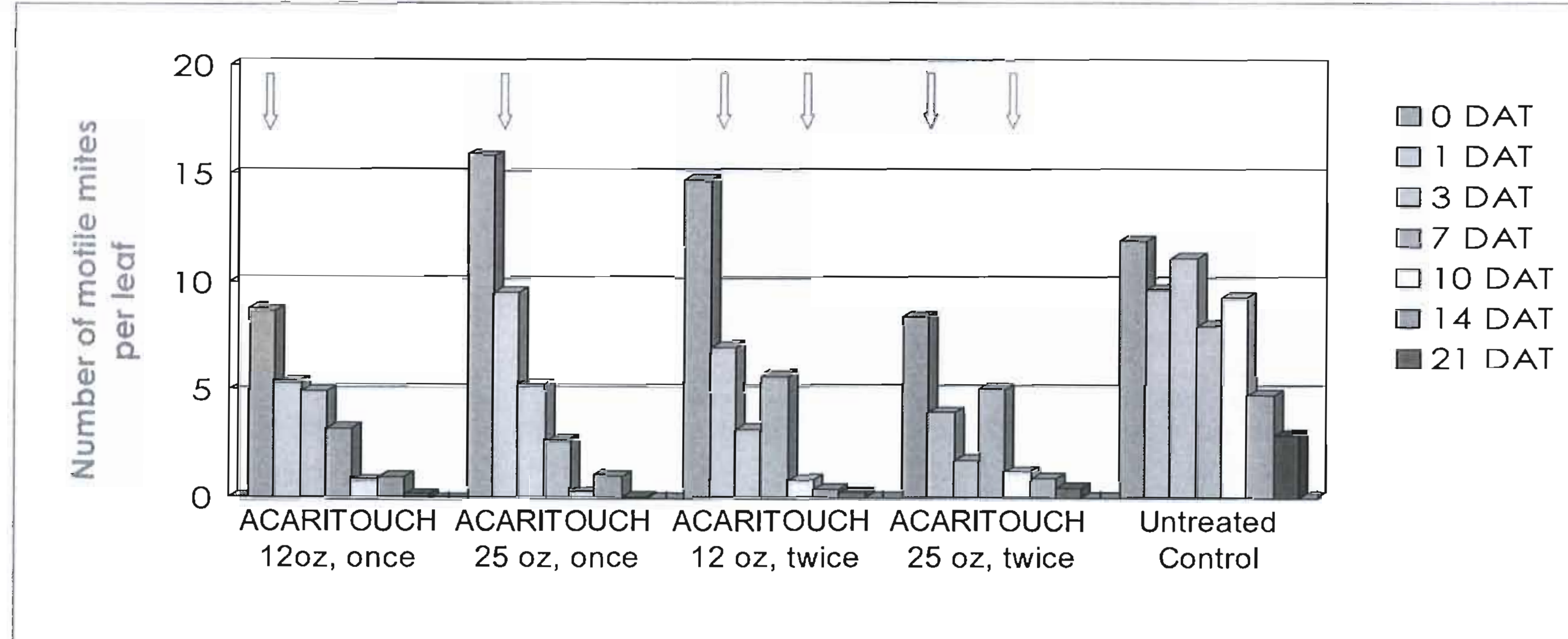
Effect on aphid mummies (direct spray)

Conc. (g/L)	Number of mummies applied	Number of *1 emerged insects	Emerged rate (%)
0 *2	80	76	95.0
0.5	80	77	96.3
1.0	80	74	92.5

*1) 8 days after the application *2) water

Trial Results

Two-spotted spider mite on Grapes (USA)

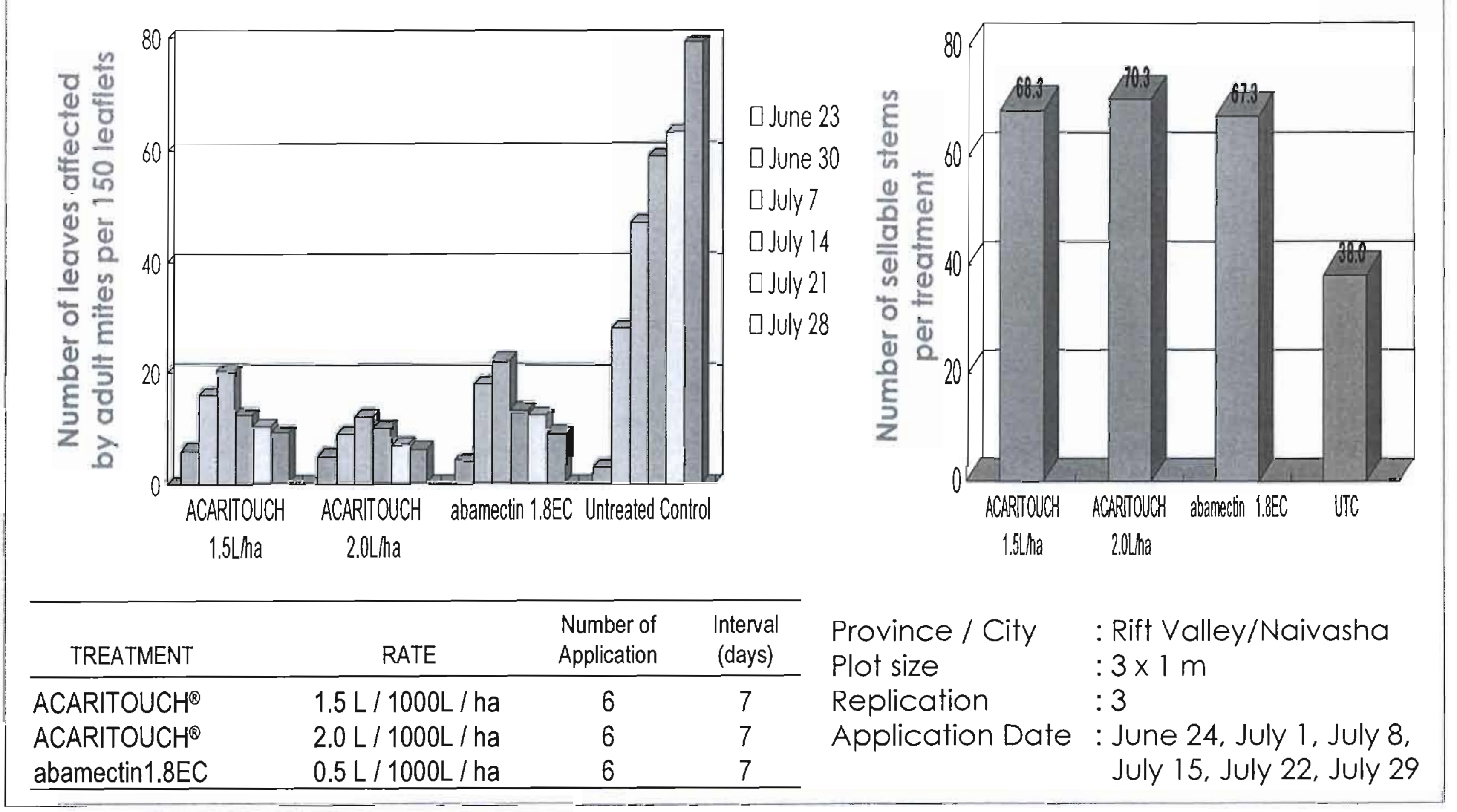


TREATMENT	RATE	Number. of Application	Interval (days)
ACARITOUCH®	12 oz / 100 gallons	1	-
ACARITOUCH®	25 oz / 100 gallons	1	-
ACARITOUCH®	12 oz / 100 gallons	2	7
ACARITOUCH®	25 oz / 100 gallons	2	7

State / City : California/Caruthers
Plot size : 8 x 40 feet, 5 vines
Replication : 4
Application Date : July 11, July 18

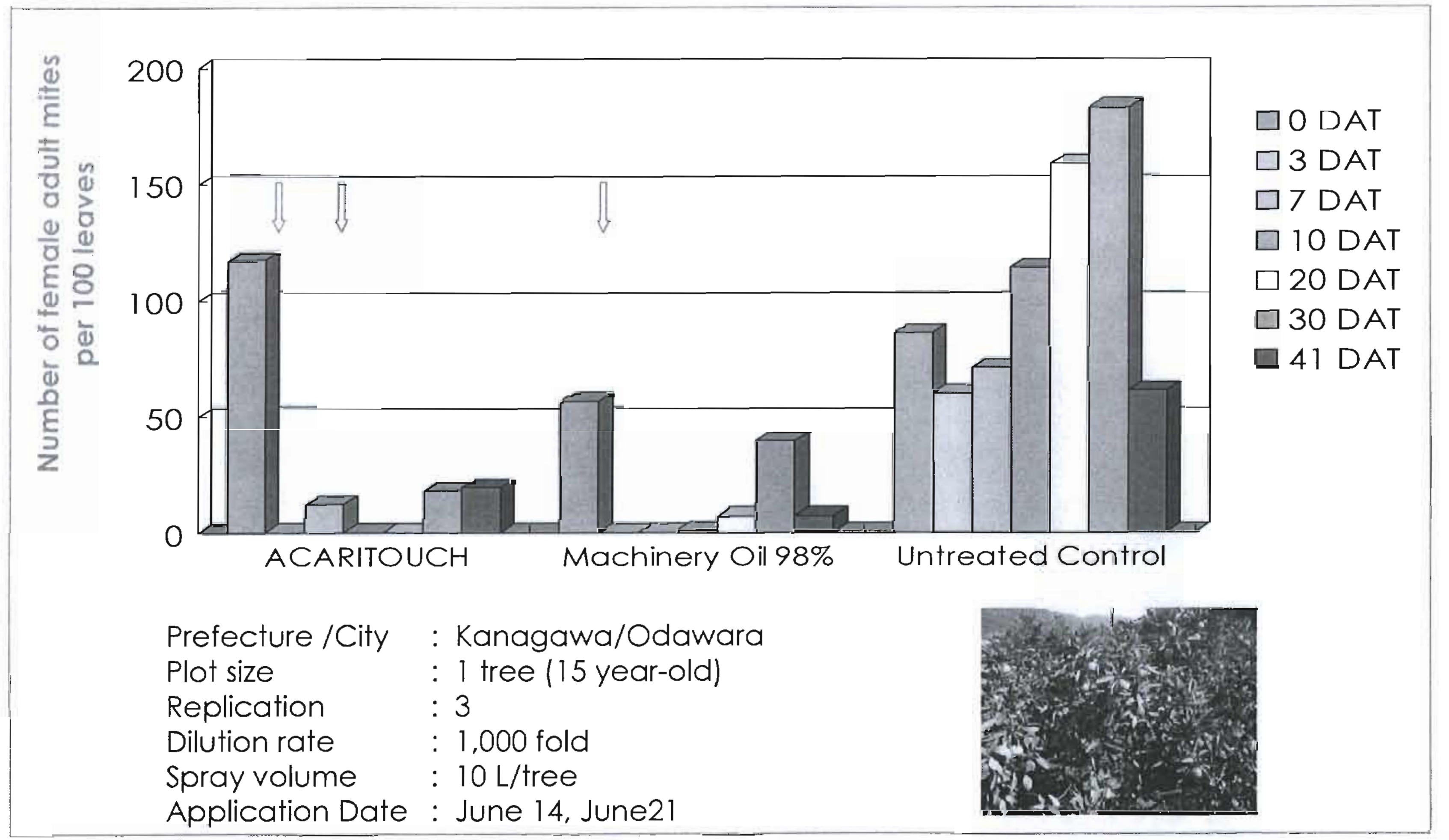
Trial Results

Spider mites on Roses (KENYA)



TREATMENT	RATE	Number of Application	Interval (days)
ACARITOUCH®	1.5 L / 1000L / ha	6	7
ACARITOUCH®	2.0 L / 1000L / ha	6	7
abamectin1.8EC	0.5 L / 1000L / ha	6	7

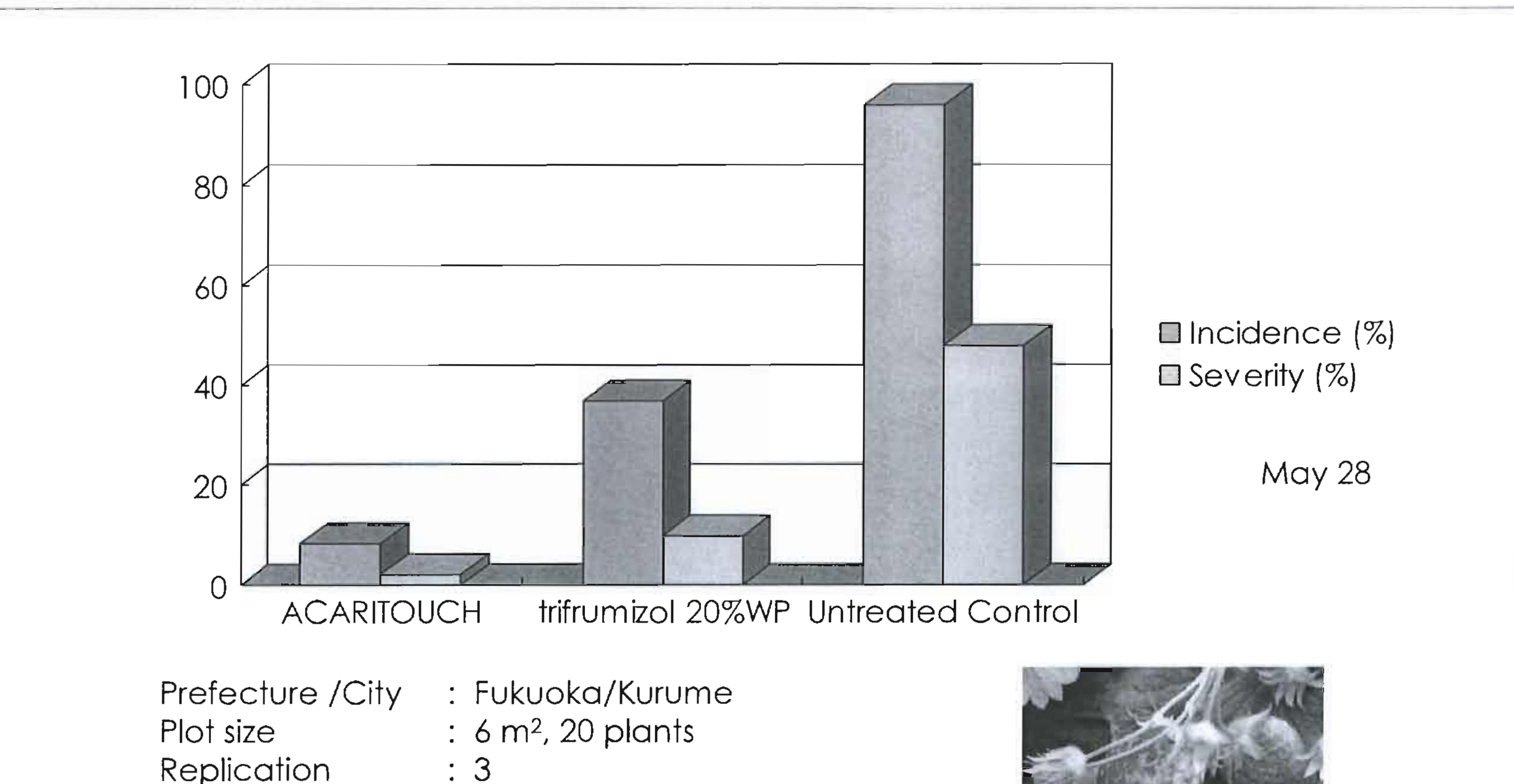
Province / City : Rift Valley/Naivasha
Plot size : 3 x 1 m
Replication : 3
Application Date : June 24, July 1, July 8, July 15, July 22, July 29


Otsuka Chemical Co., Ltd.
AgriTechno Division
Trial Results
Citrus red mites (*Panonychus citri*) on Mandarin Orange (JAPAN)
Number of female adult mites per 100 leaves
200
150
100
50
0
ACARITOUCH
Machinery Oil 98%
Untreated Control
0 DAT
3 DAT
7 DAT
10 DAT
20 DAT
30 DAT
41 DAT
Prefecture /City : Kanagawa/Odawara
Plot size : 1 tree (15 year-old)
Replication : 3
Dilution rate : 1,000 fold
Spray volume : 10 L/tree
Application Date : June 14, June21
Kanagawa Agricultural Technology Center Nebukawa Branch (1998)
Nov 4, 2008

Otsuka Chemical Co., Ltd.
AgriTechno Division

Trial Results

Powdery Mildew on Strawberry (JAPAN)



Prefecture /City	:	Fukuoka/Kurume
Plot size	:	6 m^2, 20 plants
Replication	:	3
Dilution rate	:	2,000 fold
Spray volume	:	2000 L/ha
Application Date	:	May 7, May 14, May 21

National Agricultural Research Center for Kyushu Okinawa Region (2003)

For an effective control

- Apply ACARITOUCH® as soon as mites are identified on the plants, or when environmental conditions favor mite outbreaks. Thorough spray coverage of foliage and fruit/vegetables and complete coverage of mites is necessary to achieve the maximum level of control.

- At first sign of mite infestation, apply in sufficient water to thoroughly cover plant with spray. Make two applications 7 days apart to suppress and control mites present on the plants and those newly emerged. If temperatures are such that mite egg hatch is accelerated, make the treatment interval 3-5 days.

- Integrated pest management (IPM) is one of the solutions to improve its utility. For example, quick reduction of the population of adult and larval mites by ACARITOUCH® and subsequent introduction of commercial predatory mites or application of ovicide may contribute to an effective control of mites.

Recommendations

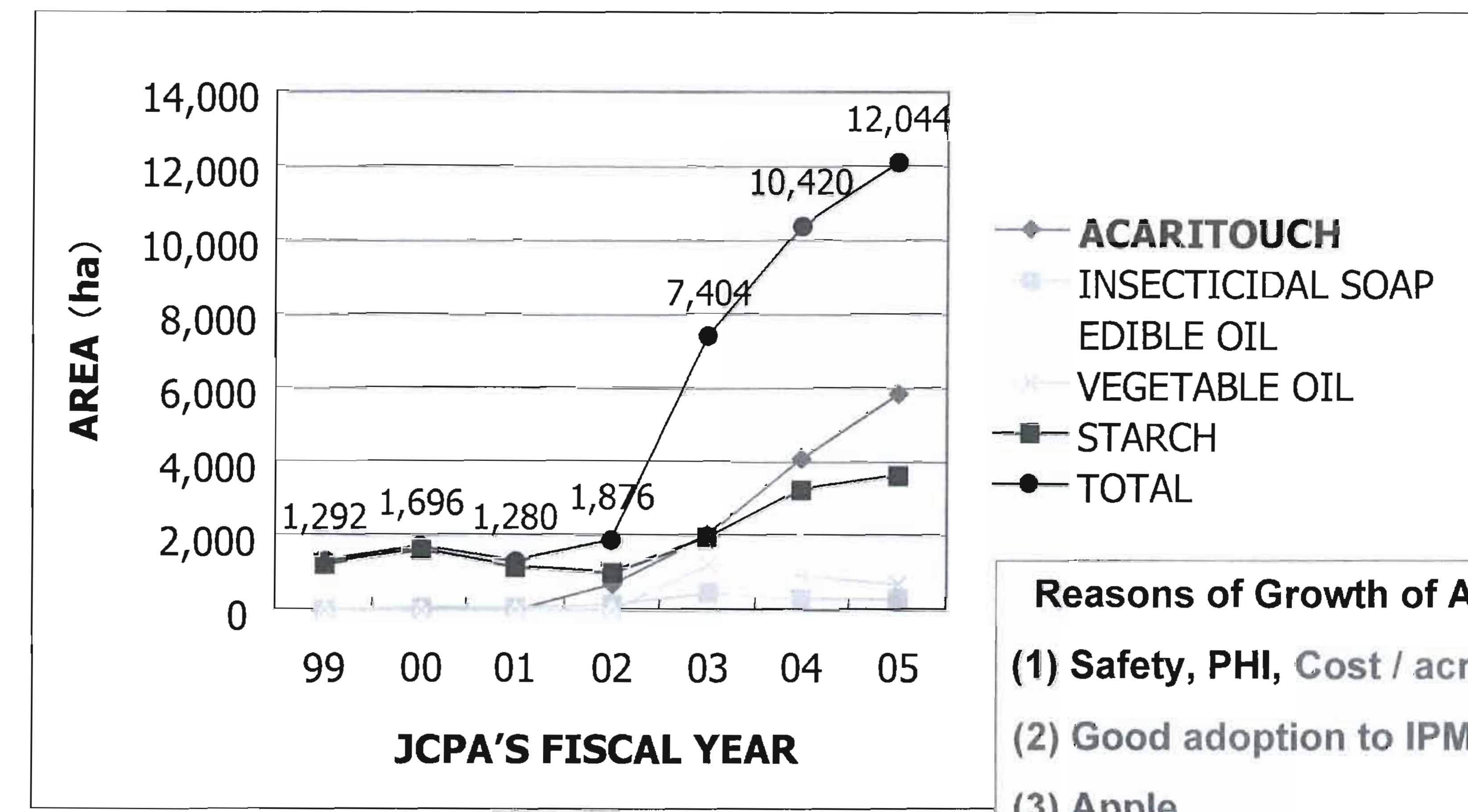
(Based on the Japanese label)

CROPS	TARGET PESTS	DILUTION RATE	WATER VOLUME (L/ha)	PHI (day)	APPLICATION NUMBERS
Vegetables	Spider Mites	1000 – 2000	1000 – 4000	1	–
	Powdery Mildew	2000			
Fruit trees	Spider Mites	1000 – 2000	2000 – 7000	1	–

* Dilution rate: 1000 ….. 0.10% solution (1.0 L FP/1000L of water)

2000…… 0.05% solution (0.5 L FP/1000L of water)

Evolution of Spiracle Blockers in Japan



Reasons of Growth of AT

(1) Safety, PHI, Cost / acre

(2) Good adoption to IPM

(3) Apple

IPM Calendar for Strawberry (1)

Otsuka Chemical Co., Ltd.
AgriTechno Division

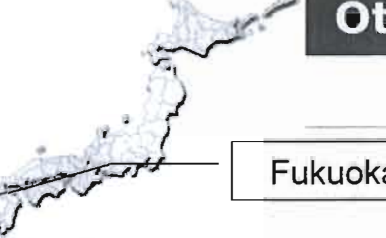
Miyagi

	Crop Stage/Management	Mite Control
Aug	nursery	(miticides which are less toxic to predatory mites)
Sep	transplanting	
		emamectin benzoate EC
Oct		milbemectin WP
	introduction of honeybees	
	beginning of blooming	ACARITOUCH EC + *Amblyseius californicus*
Nov	beginning of harvest	
Dec		ACARITOUCH EC + *Amblyseius californicus*
Jan		(cyflumetofen SC or bifenazate SC)
Feb		
Mar		
Apr		*Phytoseiulus persimilis*
May		(cyflumetofen SC or bifenazate SC)
Jun	end of harvest	

Amblyseius californicus:
Act relatively high temp.
Can be set well in place
Strong against hunger
Eat also pollen and thrips
Eat not so much = Slow
→ As a long lasting miticide

Phytoseiulus persimilis:
Act relatively low temp.
Can not be set well in place
Weak against hunger
Eat only mites
Eat a lot of mites = Fast
→ As a fast acting miticide

IPM Calendar for Strawberry (2)



	Crop Stage/Management	Mite Control	
Aug	nursery	(miticides which are less toxic to predatory mites)	
Sep			
	transplanting		
Oct			
		milbemectin WP	
	introduction of honeybee		
Nov	beginning of harvest	*Amblyseius californicus*	Assistant: ACARITOUCH EC or bifenazate EC or hexythiazox WP
Dec		*Phytoseiulus persimilis*	
Jan		*Phytoseiulus persimilis*	
Feb		*Phytoseiulus persimilis*	
Mar			
Apr			
May	end of harvest		

IPM Calendar for Eggplant

Otsuka Chemical Co., Ltd.

AgriTechno Division

Kochi

<table>
<tr><th></th><th>Sep
Transplanting</th><th>Oct</th><th>Nov</th><th>Dec</th><th>Jan</th><th>Feb</th><th>Mar</th><th>Apr</th><th>May</th><th>Jun
End</th></tr>
<tr><td></td><td></td><td>Introduction of Natural Enemies</td><td colspan="8">Applications of Insecticides and Miticides</td></tr>
<tr><td>Mites</td><td>emamectin benzoate EC</td><td></td><td colspan="8">ACARITOUCH EC, bifenazate EC, hexythiazox WP, etoxazole SC, fenbutatin oxide WP</td></tr>
<tr><td>Thrips</td><td></td><td>Pirate bug
(Orius strigicollis)</td><td colspan="8">emamectin benzoate EC, chlorfenapyr SC, pyriproxyfen EC, pyridalyl SC, B. bassiana (Mycotrol)</td></tr>
<tr><td>Aphids</td><td></td><td>Parasitic Wasp
(Aphidius colemani)</td><td colspan="8">pymetrozine WP, V. lecanii</td></tr>
<tr><td>Leafminers</td><td></td><td>Parasitic Wasp
(Dacnusa sibirica)</td><td colspan="8">cyromazine L</td></tr>
<tr><td>Whiteflies</td><td></td><td>Parasitic Wasp
(Eretmocerus eremicus)</td><td colspan="8">buprofezin WP, sodium oleate L, hydroxy propyl starch L, B. bassiana, V. lecanii, P. fumosoroseus</td></tr>
<tr><td>Worms</td><td></td><td></td><td colspan="8">BT WP, chromafenozide SC, methoxyfenozide SC, indoxacarb-MP SC</td></tr>
</table>

Spray Calendar for Apple

Akita

	Crop Stage	Insecticides/Miticides
Apr		machinery oil
		flufenoxuron EC
May	beginning of blooming	
	petal fall	organophosphates
Jun		dinotefuran WSG
Jul		chlorpyrifos DF
		cyhalothrin WP + etoxazole SC
Aug		chlorfenapyr SC
		bifenthrin WP + milbemectin EC
Sep		ACARITOUCH EC
Oct		
Nov	harvest	

- Quick cleanup of remaining mites
- Resistance management

Phytotoxicity

- In case of citrus, please take account of concentration and temperature, especially in the early stages of fruits.
- In case of Asian pears, avoid using in early stages of fruits.
- In strawberries, there were some phytotoxicity cases on fruits in Japan in the condition when the sprayed solution could not get dry easily for hours, for example, at the points of contact with mulch-film in green houses in winter at high concentration.
- In case of ornamentals, phytotoxicity trials to each variety have not been conducted. Therefore, please examine it in advance locally, especially for roses.

Thank you

DOCUMENTATION IN REGARDS TO ITEM B4

ACARITOUCH®

A novel contact miticide for the suppression and control of tetranychid mites and other mites on fruits, vegetables and ornamental plants

ACTIVE INGREDIENT:
Propyleneglycol monolaurate................. 70.81%
OTHER INGREDIENTS................................29.19%
TOTAL: 100.00%

KEEP OUT OF REACH OF CHILDREN

CAUTION

APR 24 2009

See First Aid Statements on Back Panel

Net Contents: 1 gallon (5 gallons)

Manufactured by:
Otsuka Chemical Co. Ltd.
3-2-17 Otedori, Chuo-Ku
Osaka 540-0021, Japan

EPA Reg. No.: 11581-3
EPA Establishment No.11581-JP-1
U.S. Patent No. 6,294,578

PRECAUTIONARY STATEMENTS
HAZARD TO HUMANS AND DOMESTIC ANIMALS

CAUTION. Harmful if swallowed, absorbed through skin or inhaled. Causes moderate eye irritation. Avoid contact with skin, eyes or clothing. Avoid breathing spray mist. Wash thoroughly with soap and water after handling. Remove contaminated clothing and wash clothing before reuse

FIRST AID	
If swallowed	· Call a poison control center or doctor immediately for treatment advice. · Have person sip a glass of water if able to swallow. · Do not induce vomiting unless told to do so by the poison control center or doctor. · Do not give anything to an unconscious person.
If in eyes	· Hold eye open and rinse slowly and gently with water for 15-20 minutes. · Remove contact lenses, if present, after the first 5 minutes, then continue rinsing eye. · Call a poison control center or doctor for treatment advice.
If on skin or clothing	· Take off contaminated clothing. · Rinse skin immediately with plenty of water for 15-20 minutes. · Call a poison control center or doctor for treatment advice.
If inhaled	· Move person to fresh air. · If person is not breathing, call 911 or an ambulance, then give artificial respiration, preferably by mouth-to-mouth, if possible. · Call a poison control center or doctor for further treatment advice.
Have the product container or label with you when calling a poison control center or doctor, or going for treatment. You may also contact the National Pesticide Information Center at 1-800-858-7378 for emergency medical advice.	

PERSONAL PROTECTIVE EQUIPMENT

Applicators and other handlers who may be exposed to the dilution through application or other tasks must wear long-sleeved shirt and long pants, waterproof gloves and shoes plus socks. Discard clothing and other absorbent materials that have been drenched or heavily contaminated with the product's concentrate. Do not reuse them. Follow manufacturer's instructions for cleaning/maintaining PPE. If no such instruction for washables, use detergent and hot water. Keep and wash PPE separately from other laundry.

USER SAFETY RECOMMENDATIONS

Users should:

- Wash hands before eating, drinking, chewing gum, using tobacco or using toilet.
- Remove clothing immediately if pesticide gets inside. Then wash thoroughly and put on clean clothing.
- Remove PPE immediately after handling this product. Wash the outside of gloves before removing. As soon as possible, wash thoroughly and change into clean clothing.

ENVIRONMENTAL HAZARDS

This product is highly toxic to aquatic invertebrates, toxic to fish and aquatic plants. Do not apply directly to water, or to areas where surface water is present or to intertidal areas below the mean high water mark. Do not contaminate water by cleaning of equipment, washwaters or rinsate. Do not discharge effluent containing this product into lakes, streams, ponds, estuaries, oceans, or other waters unless in accordance with the requirements of a National Pollutant Discharge Elimination System (NPDES) permit and the permitting authority has been notified prior to discharge. Do not discharge effluent containing this product to sewer systems without previously notifying the local sewage treatment plant authority. For guidance contact your State Water Board or Regional Office of the EPA.

PHYSICAL OR CHEMICAL HAZARD

Combustible. Do not store near heat or open flame.

DIRECTIONS FOR USE

It is a violation of Federal law to use this product in a manner inconsistent with its labeling. Do not apply this product in a way that will contact workers or other persons, either directly or through drift. Only protected handlers may be in the area during application. For any requirements specific to your State or Tribe, consult the agency responsible for pesticide regulation.

AGRICULTURAL USE REQUIREMENTS

Use this product only in accordance with its labeling and with the Worker Protection Standard, 40 CFR 170. This Standard contains requirements for the protection of agricultural workers on farms, forests, nurseries, and greenhouses, and handlers of agricultural pesticides. It contains requirements for training, decontamination, notification, and emergency assistance. It also contains specific instructions and exceptions pertaining to the statements on this label about personal protective equipment (PPE), notification to workers, and restricted-entry intervals. The requirements in this box only apply to uses of this product that are covered by the Worker Protection Standard.

Do not enter or allow worker entry into treated areas during the restricted entry interval (REI) of 4 hours or until sprays have dried, whichever is longer.

PPE required for early entry to treated areas that is permitted under the Worker Protection Standard and that involves contact with anything that has been treated such as plants, soil or water is:

- Long-sleeved shirt and long pants
- Waterproof gloves
- Shoes plus socks

GENERAL INFORMATION

ACARITOUCH is a novel miticide for the suppression and control of tetranychid and other phytophagous mites including Two-spotted spider mites, Pacific spider mites, Citrus red mites, Strawberry spider mite, Brown almond mite and European red mite. ACARITOUCH is a miticide with a non-toxic mode of action blocking the spiracles and de-waxing the cuticle causing death by suffocation and desiccation. This product may be used alone, tank-mixed with or in rotation with other miticides. ACARITOUCH is intended for use in an integrated pest management (IPM) program to control mites.

READ ALL DIRECTIONS FOR USE BEFORE APPLYING THIS PRODUCT

COMPATIBILITY

ACARITOUCH has been found to be compatible with most commonly used pesticides and

fertilizers. However, a compatibility test is recommended before using this product in a tank mix with other products. To test for compatibility mix a small amount of each product, in the recommended proportion, in a small jar to ensure that the mixture will be suitable.

ACARITOUCH has been evaluated for phytotoxicity on a variety of trees and plants under normal field conditions. However, since testing on all varieties of all trees and field crops is not feasible, testing a small portion of the area to be treated for phytotoxicity is recommended prior to treating the entire area. Further, all possible combinations or sequences of pesticide sprays, including fertilizers, adjuvants and other pesticides have not been tested, thus testing for phytotoxicity of spray mixtures is also recommended.

MIXING

Fill spray tank with half the recommended water, add ACARITOUCH. Agitate the mixture thoroughly and then fill the tank with the remaining water. Thorough mixing is necessary for uniform coverage. For tank mixes, add the other products to the tank mix according the directions for use on those product labels. Typically, the least soluble products will be added first followed by the most soluble products. Agitate mixture thoroughly and use immediately. Use within 24 hours.

Do not apply this product through any type of irrigation system.

APPLICATION INSTRUCTIONS

Apply ACARITOUCH as soon as mites are identified on the plants, or when environmental conditions favor mite outbreaks. Thorough spray coverage of foliage and fruit/vegetables and complete coverage of mites is necessary to achieve the maximum level of control.

At first sign of mite infestation, apply ACARITOUCH in sufficient water to thoroughly cover plant with spray. Make two applications 7 days apart to suppress and control mites present on the plants and those newly emerged. If temperatures are such that mite egg hatch is accelerated, make the inter-treatment interval 3-5 days. When applied in rotation with other miticides, apply ACARITOUCH up to 8 times during the season. Apply ACARITOUCH up to the day before harvest.

APPLICATION RATES

Mix 12 to 25 ounces of ACARITOUCH per 100 gallons of water. Apply sufficient spray volume to ensure thorough coverage of crop. In general, adequate coverage requires a minimum of 50 gallons of total spray volume per acre depending on the crop. For additional information on crop coverage and sprayer settings, contact your local ACARITOUCH representative.

CROPS

Cucurbits. Such as Acorn Squash, Butternut Squash, Cantaloupe, Casaba Melon, Crenshaw Melon, Crookneck Squash, Cucumbers, Gherkin, Honeydew Melon, Pumpkin, Scallop Squash, Watermelon, and Zucchini.

Pome Fruits. Such as Apples, Pears, and Quince

Stone Fruits. Such as Apricots, Cherries, Nectarines, Peaches, and Plums

Grapes (wine, fresh market, raisin)

Nut Crops. Such as Almonds, Walnuts, and Pistachios,

Hops [not registered for use in California]

Small Fruits and Berries. Such as Blackberry, Blueberry, Currant, Loganberry, Raspberry, and Strawberry

Fruiting Vegetables. Such as Eggplant, Peppers, and Tomatoes [not registered for use in California]

Tuberous and Corm Vegetables. Such as Potatoes, Artichokes, Sweet Potatoes, and Yams. [not registered for use in California]

Cereal Grains. Such as Corn, Millet, Popcorn, and Sorghum. [not registered for use in California]

Citrus. Such as Grapefruit, Lemon, Lime, and Oranges [not registered for use in California]

Cotton. [not registered for use in California]

Ornamentals – Field and Greenhouse. Such as bare root, container, bedding, and flowering stock, field grown cut flowers, vegetable transplants, nursery and landscape, potted flowering, shade and flowering trees, and woody ornamentals. [not registered for use in California]

STORAGE AND DISPOSAL

Do not contaminate water, food or feed by storage and disposal.

Pesticide Storage: Store in a cool, dry area until used. Store in original container only.

Pesticide Disposal: Wastes resulting from the use of this product may be disposed of on site or at an approved waste disposal facility.

Container Disposal: Triple rinse (or equivalent). Then offer for recycling or reconditioning, or puncture and dispose of in a sanitary landfill, or incineration, or, if allowed by State and local

authorities, by burning. If burned, stay out of smoke.

WARRANTY STATEMENT

OTSUKA warrants that this product conforms to the description on this label and is reasonably fit for the purposes stated on this label when used in accordance with the directions on this label under normal conditions of use.

OTSUKA MAKES NO WARRANTIES OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE NOR ANY OTHER EXPRESS OR IMPLIED WARRANTY EXCEPT AS STATED ABOVE.

If this product is defective, buyer's exclusive remedy shall be the replacement of the product, or if replacement is impractical, refund of the purchase price. In no case will OTSUKA be liable for incidental, consequential or special damages resulting from handling, storage or use of this product.

ACARITOUCH®

KEEP OUT OF REACH OF CHILDREN

CAUTION

See inside booklet for Precautionary Statements

A novel contact miticide for the suppression and control of tetranychid mites on fruits, vegetables and ornamental plants

EPA Reg. No.: 11581-3
EPA Establishment No. 48498-CA-1

U.S. Patent No. 6,294,578

ACTIVE INGREDIENT:	
Propyleneglycol monolaurate	70.81%
OTHER INGREDIENTS:	29.19%
TOTAL:..................................	100.00%

NET CONTENTS: 1 GALLON

AD051908
102354

Manufactured by:
Otsuka Chemical Co. Ltd.
3-2-27 Ote-dori, Chuo-ku,
Osaka 540-0021, Japan

PRECAUTIONARY STATEMENTS

HAZARD TO HUMANS AND DOMESTIC ANIMALS

CAUTION Harmful if swallowed, absorbed through skin or inhaled. Causes moderate eye irritation. Avoid contact with skin, eyes or clothing. Avoid breathing spray mist. Wash thoroughly with soap and water after handling. Remove contaminated clothing and wash clothing before reuse.

FIRST AID	
If swallowed	• Call a poison control center or doctor immediately for treatment advice. • Have person sip a glass of water if able to swallow. • Do not induce vomiting unless told to do so by the poison control center or doctor. • Do not give anything to an unconscious person.
If in eyes	• Hold eye open and rinse slowly and gently with water for 15-20 minutes. • Remove contact lenses, if present, after the first 5 minutes, then continue rinsing eye. • Call a poison control center or doctor for treatment advice.
If on skin or clothing	• Take off contaminated clothing. • Rinse skin immediately with plenty of water for 15-20 minutes. • Call a poison control center or doctor for treatment advice.
If inhaled	• Move person to fresh air. • If person is not breathing, call 911 or an ambulance, then give artificial respiration, preferably by mouth-to-mouth, if possible. • Call a poison control center or doctor for further treatment advice.
Have the product container or label with you when calling a poison control center or doctor, or going for treatment. You may also contact the National Pesticide Information Center at 1-800-858-7378 for emergency medical advice.	

PERSONAL PROTECTIVE EQUIPMENT (PPE)

Applicators and other handlers who may be exposed to the dilution through application or other tasks must wear long-sleeved shirt and long pants, waterproof gloves and shoes plus socks. Discard clothing and other absorbent materials that have been drenched or heavily contaminated with the product's concentrate. Do not reuse them. Follow manufacturer's instructions for cleaning/maintaining PPE. If no such instruction for washables, use detergent and hot water. Keep and wash PPE separately from other laundry.

USER SAFETY RECOMMENDATIONS

Users should:

- Wash hands before eating, drinking, chewing gum, using tobacco or using toilet.
- Remove clothing immediately if pesticide gets inside. Then wash thoroughly and put on clean clothing.
- Remove PPE immediately after handling this product. Wash the outside of gloves before removing. As soon as possible, wash thoroughly and change into clean clothing.

ENVIRONMENTAL HAZARDS

This product is highly toxic to aquatic invertebrates, toxic to fish and aquatic plants. Do not apply directly to water, or to areas where surface water is present or to intertidal areas below the mean high water mark. Do not contaminate water by cleaning of equipment, washwaters or rinsate. Do not discharge effluent containing this product into lakes, streams, ponds, estuaries, oceans or other waters unless in accordance with the requirements of a National Pollutant Discharge Elimination System (NPDES) permit and the permitting authority has been notified prior to discharge. Do not discharge effluent containing this product to sewer systems without previously notifying the local sewage treatment plant authority. For guidance contact your State Water Board or Regional Office of the EPA.

DIRECTIONS FOR USE

It is a violation of Federal law to use this product in a manner inconsistent with its labeling. Do not apply this product in a way that will contact workers or other persons, either directly or through drift. Only protected handlers may be in the area during application. For any requirements specific to your State or Tribe, consult the agency responsible for pesticide regulation.

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Fill spray tank with half the recommended water, add ACARITOUCH. Agitate the mixture thoroughly and then fill the tank with the remaining water. Thorough mixing is necessary for uniform coverage. For tank mixes, add the other products to the tank mix according the directions for use on those product labels. Typically, the least soluble products will be added first followed by the most soluble products. Agitate mixture thoroughly and use immediately. Use within 24 hours.

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CROPS	
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Pome Fruits	Such as Apples, Pears, and Quince
Stone Fruits	Such as Apricots, Cherries, Nectarines, Peaches, and Plums
Grapes	(wine, fresh market, raisin)
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Ornamentals – Field and Greenhouse	Such as bare root, container, bedding, and flowering stock, field grown cut flowers, vegetable transplants, nursery and landscape, potted flowering, shade and flowering trees, and woody ornamentals [not registered for use in California]

STORAGE AND DISPOSAL

Do not contaminate water, food or feed by storage and disposal.

Pesticide Storage: Store in a cool, dry area until used. Store in original container only.

Pesticide Disposal: Wastes resulting from the use of this product may be disposed of on site or at an approved waste disposal facility.

Container Disposal: Nonrefillable container. Do not reuse or refill this container. [Metal]: Triple rinse (or equivalent) promptly after emptying. Triple rinse as follows: Empty remaining contents into application equipment or a mix tank. Fill container with 1/4 full of water and recap. Shake 10 seconds. Pour rinsate into application equipment or mix tank or store rinsate for later use or disposal. Drain for 10 seconds after flow begins to drip. Repeat this procedure two more times. Then offer for recycling or reconditioning, or puncture and dispose of in a sanitary landfill, or by other procedures approved by State and local authorities. [Plastic]: Triple rinse (or equivalent) promptly after emptying. Triple rinse as follows: Empty remaining contents into application equipment or a mix tank. Fill container with 1/4 full of water and recap. Shake 10 seconds. Pour rinsate into application equipment or mix tank or store rinsate for later use or disposal. Drain for 10 seconds after flow begins to drip. Repeat this procedure two more times. Then offer for recycling or reconditioning, or puncture and dispose of in a sanitary landfill, or incineration, or, if allowed by State and local authorities, by burning, or by other procedures approved of by state and local authorities.

WARRANTY STATEMENT

OTSUKA warrants that this product conforms to the description on this label and is reasonably fit for the purposes stated on this label when used in accordance with the directions on this label under normal conditions of use.

OTSUKA MAKES NO WARRANTIES OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE NOR ANY OTHER EXPRESS OR IMPLIED WARRANTY EXCEPT AS STATED ABOVE.

If this product is defective, buyer's exclusive remedy shall be the replacement of the product, or if replacement is impractical, refund of the purchase price. To the extent allowed by applicable law, OTSUKA will not be liable for incidental, consequential or special damages resulting from handling, storage or use of this product.

DOCUMENTATION IN REGARDS TO ITEM B5

RIKEN VITAMIN CO.,LTD.

Tokyo, Aug. 22, 2008

PRODUCT INFORMATION

Product : RIKEMAL PL – 100

Substance : Propylene Glycol Esters of Fatty Acids

Manufacturing Process :

Propylene Glycol (Synthetic) + Fatty Acid (Coconut) → Reaction → Molecular Distillation → Filtration

→ Weighing/Packing → Product

Riken Vitamin Co.,Ltd.
Quality Assurance Dept.

M. Yamazaki

Manager ; M. Yamazaki

2-9-18, Misaki-cho, Chiyoda-ku, Tokyo 101-8370, Japan Tel . 81-3-5275-5130 Fax : 81-3-3237-1087

DOCUMENTATION IN REGARDS TO ITEM B6

P.O. Box 11558, Eugene, Oregon 97440-3758 USA
541.343.7600 • fax 541.343.8971
info@omri.org

January 29, 2008

Ms. Heather R. Bjornson
Otsuka Chemical Co., LTD
1150 18th Street NW #1000
Washington, DC 20036

APR 24 2009

Dear Ms. Heather R. Bjornson:

Thank you for submitting your application for Acaritouch to OMRI for inclusion in the *OMRI Products List*. We appreciate your application. We are committed to providing timely, courteous, and accurate service.

Unfortunately, some of the information specified in the OMRI checklists and/or forms was not included in the materials sent to us. Before we can complete the review of Acaritouch, more information is needed in accordance with OMRI's policies and standards. Specifically, the following information is required:

For the final product Acaritouch:

1. Please provide EPA Confidential Statements of Formula (CSF Form 8570-4) for the basic formulation and all alternate formulations currently on file with EPA under the same EPA Reg. Number and a written declaration that no other formulations than those submitted to OMRI exist under the same EPA Reg. Number. Company name and product name on at least one CSF for the final product must match the company and product names on the product label.
2. If the formulation contains one or more Technical Grade Active Ingredient(s), provide CSFs for the basic and all alternate formulations of each TGAI and a written declaration that no other formulations than those submitted to OMRI exist under the same EPA Reg. Number. Company name and product name on at least one of the TGAI's CSFs must match those declared on the CSF of the final product that the TGAI is used in.
3. Describe any and all other materials or products handled at the manufacturing site or state that site use is exclusive to Acaritouch.
4. If Acaritouch contains products of microbial processes, and any prohibited substances are used in the growth medium, declare whether any trace of those substances remains in the final product and document the methods used to remove them.
5. If Acaritouch contains products of microbial processes, provide an independent verifiable lab analysis to document the Salmonella level of the finished product using standard method 9260.D with a detection limit below 3MPN/4g reported as MPN/4g.
6. If Acaritouch contains products of microbial processes, provide independent verifiable lab analysis to document the fecal coliform level of the finished product using an EPA recognized method of testing with a detection limit below 1,000MPN/g and reported as MPN/g.
7. If Acaritouch contains products of microbial processes, declare if there is a program in place to address pathogenic organisms and other contaminants.

occ-1122

P.O. Box 11558, Eugene, Oregon 97440-3758 USA
541.343.7600 • fax 541.343.8971
info@omri.org

For the ingredient Actor M-1:

8. Provide complete descriptions (preferably including a flow chart) of the manufacturing process for this ingredient. Include ingredient amounts, sequence and duration of events, temperature changes, reactions, and all steps taken to assure that prohibited substances are not intentionally or accidentally in the product and methods to verify that the product is not contaminated. Include description of any composting, digestion, fermentation, extraction or other processes and any methods used for removing extractants or growth media from final product.

For the ingredient Rikemal PL-100:

9. Provide complete descriptions (preferably including a flow chart) of the manufacturing process for this ingredient. Include ingredient amounts, sequence and duration of events, temperature changes, reactions, and all steps taken to assure that prohibited substances are not intentionally or accidentally in the product and methods to verify that the product is not contaminated. Include description of any composting, digestion, fermentation, extraction or other processes and any methods used for removing extractants or growth media from final product.

Please send the requested information to OMRI, P.O. Box 11558, Eugene, OR 97440-3758 by **February 29, 2008.** Information may be faxed to 541-343-8971 up to the deadline if the original is also postmarked the same day or earlier. All confidential information submitted to OMRI must be marked as "CONFIDENTIAL" on every page as described in the *OMRI Policy Manual.*

Please refer to the OMRI Policy Manual §2.3 for more information about deadlines or to request an extension. In order to receive a deadline extension, you need to request one in writing before the deadline. All requests must be accompanied with a valid reason for the extension and an estimate of the additional time required. Please note that any extension is at OMRI's discretion and that requests will not be automatically granted. Failure to meet the deadline or properly request an extension will end the review process and result in forfeiture of the review fee. Also, if you wish to be assigned a product review coordinator other than the one whose signature appears below, please make a written request for the change.

While we hope that you are able to complete the file by providing the documentation requested in this letter, OMRI's staff or Review Panel may require additional information after evaluating your response or any other documentation on file. In particular, if the information requested is inconsistent with documentation previously submitted, please explain any discrepancies.

I will be your reviewer throughout the review process. Please contact me with any questions. I look forward to your reply.

Sincerely,

Angela Hobson

Angela Hobson

Product Review Coordinator

occ-1122

P.O. Box 11558, Eugene, Oregon 97440-3758 USA
541.343.7600 • fax 541.343.8971
info@omri.org

June 9, 2008

Ms. Heather R. Bjornson
Otsuka Chemical Co., LTD
1150 18th Street NW #1000
Washington, DC 20036

Dear Ms. Heather R. Bjornson:

The OMRI Review Panel has reviewed Otsuka Chemical Co., LTD's product, Acaritouch, and has recommended that it be *Prohibited* for use in organic production. This decision indicates that Acaritouch does not comply with *OMRI Standards Manual* or the *OMRI Policy Manual,* which are based on the requirements of the USDA National Organic Program (NOP) Rule (7 CFR Part 205).

The Review Panel determined that Acaritouch was prohibited because of the presence of synthetic substances that are not on the 2004 EPA List 4: Rikemal PL-100 (propylene glycol monolaurate) and Actor M-1. Substances used in a pesticide must be either non-synthetic or be on EPA List 4 as of 2004.

Otsuka Chemical Co., LTD can petition the NOP to have the prohibited substance considered for use in organic production. For information on the petition procedure, see the NOP website at www.ams.usda.gov/nop/NationalList/ListHome.html. Should your petition be successful, OMRI will re-open the Acaritouch application file upon your request. You may also choose to reformulate your product to remove any prohibited substances, and submit a new product application and fee to OMRI for the review of a reformulated product at a later date.

Prohibited listings are circulated to subscribing certifiers and provided to other OMRI subscribers when requested. Please be aware that organic certification agents retain the right to make final certification decisions concerning use of products in organic production. These certifiers may choose not to recognize OMRI's recommendation. OMRI is not responsible for any losses that may occur as a result of the OMRI Prohibited Status of Acaritouch.

This letter serves as OMRI's Final Response Letter to Otsuka Chemical Co., LTD regarding the status of Acaritouch. If Otsuka Chemical Co., LTD wishes to rebut or appeal this decision, please refer to the "Decision Rebuttals, Appeals and Mediation" section of the *OMRI Policy Manual.* All confidential information submitted to OMRI must be marked as "CONFIDENTIAL" on every page as described in the *OMRI Policy Manual.*

Thank you for your participation in the Review Program of the Organic Materials Review Institute.

Sincerely,

Angela Hobson

Angela Hobson
Review Program Coordinator

occ-1122

Technology Sciences Group Inc.
Arizona: Registration Division
4061 North 156th Drive
Goodyear, AZ 85338
Phone: (623) 535-4060
FAX (623) 535-4061
E-Mail: jazkatz@uswest.net

Iain Weatherston, Ph.D.
Senior Regulatory Consultant
Pesticide Division

Dr. Sheryl Reilly
Branch Chief, Biochemicals Branch
Biopesticide and Pollution Prevention Division [7511C]
U.S. Environmental Protection Agency
Crystal Mall Building #2, 9th Floor
1921 Jefferson Davis Highway,
Arlington, VA 22202

May 18, 2000

SUBJECT: Request for biochemical pest control agent classification for propyleneglycol monolaurate [CAS# 27194-74-7]

COMPANY: Toagosei Co. Ltd.
1-14-1, Nishi Shimashi,
Minato-ku
Tokyo 105-8419
Japan
[EPA Company Number 70231]

CONTACT: Iain Weatherston, Ph.D.
Technology Sciences Group, Inc.
4061 north 156th Drive
Goodyear, AZ 85338
Tel: 623-535-4060
Fax: 623-535-4061
E-mail: jazkatz@uswest.net or iweatherston@tsgusa.com

Dear Sheryl:
As agent for, and on behalf of Toagosei I wish to submit for the review of the Biopesticide Classification Committee the enclosed rationale to have propyleneglycol monolaurate [PGML][CAS# 27194-74-7] classified as a biochemical pest control agent and have the subsequent application for registration of an end-use product containing PGML, and petition for exemption from the requirement of a tolerance reviewed in BPPD.

Toagosei is proposing to register an end-use product called ACARITOUCH which will be labeled for control of tetranychid mites on a variety of crops including cotton, grapes, strawberries, pome fruits, nuts, tomatoes and cucurbits. ACARITOUCH which contains PGML as its sole active ingredient controls the target pests by a non-toxic mode of action, namely by coating the insect, blocking the spiracles and causing death by suffocation.

Toagosei believes that the rationale detailed on the enclosure is sufficient for the committee to classify PGML as a biochemical pest control agent and therefore, also

at this time, requests a pre-application meeting. Once the date of the meeting has been confirmed I will forward to you a list of attendees representing Toagosei, a meeting agenda and if it is necessary, a request to have specific Agency personnel at the meeting. This will be followed by, at least a week before the meeting submission of a background document describing the product including a mock up label and a list of proposed registration requirements.

At this time I would like to propose that the meeting be held any afternoon of the week of June 26 - 30, 2000, on the day which is most convenient for your schedule.

I wish to thank you for your assistance in this matter.

Sincerely yours,

Iain Weatherston

enclosure:

cc: William Schneider [Chairman, Biochemicals Classification Committee]
Akira Motegi [Toagosei Co. Ltd.]
Kaoru Kasuga {Nichimen Corporation]
Yukio Tachibana [Nichimen America Inc.]

RATIONALE DOCUMENT

COMPOUND

NAME: Propyleneglycol mono laurate CAS# 27194-74-7

SYNONYMS:
Propyleneglycol monofattyacid ester
Dodecanoic acid, monoester with 1,2-propane diol
Lauric acid, monoester with propane-1,2-diol
Propylene glycol monododecanoate
Emalex PGML
Rikemal PL 100

OTHER CAS#:
1322-87-8
10108-22-2

MOLECULAR FORMULA: $C_{15}H_{30}O_3$

MOLECULAR WEIGHT: 258

BOILING POINT: 246.6°C

SOLUBILITY:
4 mg/l -water
1,000 gram/l hexane, heptane, xylene, toluene, methylene chloride

REGULATORY STATUS:

- Admitted as a food additive in Japan in 1961 [Official regulation of Food Additives 6th Edition, 1962, also Food Chemicals #8, 1996, page 219] for use in bakery products and ice-creams.
- ADI in Japan 25 mg/kg/day [first reference above]
- PGML has GRAS status at 21 CFR 172.856 as a multipurpose food additive since the acid is in compliance with 21 CFR 172.860.

SAFETY OF PGML
TOXICITY

- LD_{50} [mice] > 40,000 mg/kg [safety toxic study report H-98190] Acute oral toxicity of propylene glycol fatty acid monoester, Nippon Experimental Medical Research Co., Ltd., 1998]
- No lesions were found on the kidneys of rats fed on a diet of 60% propylene glycol fatty acid monoester for 40 days [S. Lepkovsky et al., Biochemical Journal, 108: 431-438, 1935]

- There were no histological differences in the tissues of rats fed for 13 weeks on a diet containing 0 1, 5, 3.36 and 7.52% propylene glycol fatty acid monoester. [J.D. Brander WHO Food Additive Series, No. 5,276 Toxicological evaluation of some food additives including anticaking agents, antimicrobials, antioxidants, emulsifiers and thickening agents - 1973]

METABOLISM

- PGML is easily and quickly hydrolyzed by organisms and soil to propylene glycol and lauric acid.
- Propylene glycol has GRAS status at 21 CFR 582. 4666 as an emulsifier, at 21 CFR 582.1666 as a general purpose food additive [except in cat food] and is affirmed as a specific GRAS substance at 21 CFR 184.1666. It is used as a non-toxic antifreeze in breweries and dairies, as a solvent in pharmaceutical preparations and as an inhibitor of fermentation and mold growth [Merck Index 9th .Edition, page 1017] Propylene glycol can be manufactured from sorbitol, a component in many berries and fruits [Merck Index 9th Edition, 1127]. The lowest published oral toxic dose [human child] is 79 grams/kg [J. Pediatrics, 93: 515, 1978]; the oral LD_{50} [rat] = 20 grams/kg [Toxicology & Applied Pharmacology, 45: 363, 1978; the dermal LD_{50} [rat]= 20.8 grams/kg [NPIRI Raw materials handbook Volume 1, Organic Solvents 1074] This value is also the lowest lethal avian oral dose [quail] [Ecotoxicology & Environmental Safety. 6:149, 1982. For a review of the toxicity, metabolism and biochemistry of propylene glycol see Ruddick, 1972. Toxicol. Appl. Pharmacol. 21: 102.
- Lauric acid has been isolated from many plant and animal fats, and as the free acid from coconut oil, other nut oils and seed oils. Lauric acid is included in the multipurpose additive GRAS status at 21 CFR 172.860. The toxicology profile to be found at http://siri.org/msds/tox/f/q73/q749.html includes oral LD_{50} [rat] = 12 gram/kg; it is a mild irritant in both the primary eye and primary skin irritation tests. It is widely used in the manufacture of detergents, soaps and shampoos.

The above information is indicative that PGML does not pose significant hazards to humans, other mammals or the environment.

NATURAL OCCURRENCE

PGML does not occur naturally but is manufactured by the transesterification of propylene glycol and lauric fatty acid ester derived from an edible oil or fat. There are various precedents of compounds not of natural occurrence being accepted for review and approval by BPPD *eg* the synthetic Mediterranean fruit fly attractant, trimedlure; the systemic fungicide, mono- and di- potassium salts of phosphorous acid; the repellent, 3-[N-butyl-N-acetyl]-aminopropionic acid, ethyl ester, and the insecticide sucrose octanoate fatty acid esters.

APPLICATION RATES

The specific gravity of ACARITOUCH is 0.92 gm/ml @ 20°C. The standard application rate will be 12.5 - 25 fl.oz./acre in 100 gallons of water.
12.5 - 25 fl.oz/acre equates to 369 - 738 ml/acre which equals 344 - 688 grams of Acaritouch/acre. At the nominal concentration of 73% for the PGML, this gives an application rate of 251 - 502 grams of PGML per acre or 0.067 - 0.134% [w/w].
It is proposed that the product will be used with a minimum of two sprays at least 7 days apart.

MODE OF ACTION

The action of ACARITOUCH is non-toxic, when the PGML is formulated as an emulsifiable concentrate and diluted for use, the inner side of the emulsion droplets have a high concentration of PGML which can cover the body of the target insect or mite and block the spiracles and solubilize [de-wax] the cuticle thereby causing death by physical, non-toxic modes of action of suffocation and desiccation.

SUMMARY

PGML is an affirmed GRAS material that does not pose any significant hazards to man or the environment, it is used at low application rates, has a non-toxic mode of action and although not naturally occurring warrants classification as a biochemical pesticide or a "biochemical-like" pesticide warranting a reduced data set and review by BPPD.

DOCUMENTATION IN REGARDS TO ITEM B7

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

08/03/2000

OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

Technology Sciences Group, Inc.
4061 North 156th Drive
Goodyear, AZ 85038

for

Toadosei Co. Ltd.
1-14-1, Nishi Shimashi, Minato-ku,
Tokyo 105-8419 Japan

APR 24 2009

Dear Iain Weatherston,

The Biochemical Classification Committee has determined that your product, Propylene glycol monolaurate, is not a Biochemical Pesticide but is eligible for review in the Biopesticide and Pollution Prevention Division (BPPD) using a reduced data set akin to that used for biochemical pesticides.

Similar chemicals have been recently approved to be reviewed in BPPD. There was some evidence that the monoacylglycerolas affected the plasma membrane. This activity is negated by substances in food such as proteins, e.g. serum albumin, starch, cholesterol, lecithin, saponin, calcium ions, magnesium ions, and charcoal. This may be an explanation for the lack of reported dietary toxicity for these compounds in the human diet.

This product is being used for a non-toxic mode of action so the committee could consider classifying this new use as a biochemical pesticide use. However, Propylene glycol monolaurate is not actually naturally-occurring, nor is it substantially similar and functionally identical to a naturally-occuring substance so this product does not technically fit the definition of a biochemical pesticide. The committee recommends this product be reviewed in BPPD using a data set akin to that used for biochemical pesticides. The assessment may require a comparison of the exposure in the diet of the GRAS uses to the residues on the RAC's. If there is a significant additional exposure due to the pesticidal use, additional data may be required.

Janet Andersen, Director, Biopesticides and Pollution Prevention Division, has approved your product for review by BPPD. Please contact Sheryl Reilly, acting Biochemical Pesticide Branch Chief (703-308-8265), for further information in registering your product.

Sincerely,

William R. Schneider, Ph.D., Chair
Biochemical Classification Committee
Biopesticides & Pollution Prevention Division (7511C)

Internet Address (URL) • http://www.epa.gov
Recycled/Recyclable • Printed with Vegetable Oil Based Inks on Recycled Paper (Minimum 25% Postconsumer)

Company Information

PC Code: 11288
Chemical Name: Dodecanoic acid, monoester with 1,2-propanediol
Number of Active Registrants: 2

Represents a link to a registrant list for this chemical.

Firm Number: 10350
3M
3M CENTER, BLDG 220-6E-03
ST. PAUL MN 55144
651/733-3461

Display: Active Products Only All Products Get Product Info

Firm Number: 11581
OTSUKA CHEMICAL COMPANY, LTD.
3-2-27 OTEDORI
CHU0-KU, OSAKA, 540-0021

Display: Active Products Only All Products Get Product Info

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Product Report

Firm Number: 10350
3M
3M CENTER, BLDG 220-6E-03
ST. PAUL MN 55144
651/733-3461

Number of Selected Products: 1

Represents a link to view USEPA/OPP Label Images (tiff format).
Represents a link to additional chemical information.

VWX-42 TECHNOLOGY PROPYLENE GLYCOL MONOLAURATE
Registration Number: 10350-67

Status: Active
Approval Date: 09-30-03
Product Manager: Patricia Moe (703)308-8713

Percent	Active Ingredient
75.8500	Dodecanoic acid, monoester with 1,2-propanediol (11288)

Contact NPIRS for more detailed product info available through custom searching or subscription

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First Aid

If in eyes:	Hold eye open and rinse slowly and gently with water for 15-20 minutes. Remove contact lenses, if present, after the first 5 minutes, then continue rinsing eye. Call a poison control center or doctor for treatment advice.

Have the product container or label with you when calling a poison control center or doctor, or going for treatment.

Directions for Use

It is a violation of Federal law to use this product in a manner inconsistent with its labeling.

Propylene Glycol Monolaurate is a broad spectrum antimicrobial agent. It may be used to control fungi or bacteria that cause decay of post-harvest fruits and vegetables.

Propylene Glycol Monolaurate is intended for formulation into end use products for post-harvest treatment of crops and foods. Formulators must obtain EPA registration for their end-use products.

WARRANTY STATEMENT

ACCEPTED
SEP 30 2003
[illegible] Insecticides, Fungicide, and Rodenticide Act, as amended, for the pesticide registered under EPA Reg. No. 10350-67

(DRAFT 7/10/2003)

VWX-42 Technology
Propylene Glycol Monolaurate

KEEP OUT OF REACH OF CHILDREN

CAUTION

Active Ingredient:	Wt. %
Propylene glycol monolaurate	75.85%
Other Ingredients	24.15%
Total	100.00%

Weight per gallon: 7.55 lbs.

St. Paul, MN 55144-1000

EPA Reg. No. 10350-XX
EPA Est. No.

Lot. Number:________

Net Volume: ______ gal.

Precautionary Statements

Hazards to Humans and Domestic Animals
CAUTION: Causes moderate eye irritation. Avoid contact with eyes or clothing. Wash thoroughly with soap and water after handling. Prolonged or frequently repeated skin contact may cause allergic reactions in some individuals.

Environmental Hazards

Do not contaminate water when cleaning equipment or disposing of equipment wash waters. Do not discharge effluent containing this product into lakes, streams, ponds, estuaries, oceans, or other waters unless in accordance with the requirements of a National Pollutant Discharge Elimination System (NPDES) permit and the permitting authority has been notified in writing prior to discharge. Do not discharge effluent containing this product to sewer systems without previously notifying the local sewage treatment plant authority. For guidance, contact your State Water Board or Regional Office of the EPA.

Storage and Disposal

Do not contaminate water, food, or feed by storage and disposal. Store product in a cool, dry area out of direct sunlight. Store in original container only. Wastes resulting from the use of this product may be disposed of on site, or at an approved waste disposal facility. Triple rinse container, then offer for recycling, or puncture and dispose of in a sanitary landfill or incinerator. Or, if allowed by state and local authorities, dispose of container by burning.

3/3

Product Report

Firm Number: 11581
OTSUKA CHEMICAL COMPANY, LTD.
3-2-27 OTEDORI
CHU0-KU, OSAKA, 540-0021

Number of Selected Products: 1

Represents a link to view USEPA/OPP Label Images (tiff format).
Represents a link to additional chemical information.

ACARITOUCH
Registration Number: 11581-3

Status: Active
Approval Date: 10-12-04
Product Manager: Patricia Moe (703)308-8713

Percent	Active Ingredient
70.8100	Dodecanoic acid, monoester with 1,2-propanediol (11288)

Contact NPIRS for more detailed product info available through custom searching or subscription

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grapefru

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Federal Registration Data

```
Date of Search: 11/01/08
Time of Search: 11:34

Number of Products Selected: 1

Federal Data:   Federally ACTIVE Registrations Only

EPA Registration Data:
11581-3
```

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ACARITOUCH
 EPA REG NO: 11581-3                    NAME STATUS: PRIMARY NAME

  PRODUCT STATUS: ACTIVE
    TRANSFERRED FROM: 70231-2           TRANSFER DATE: 12-20-05
             COMPANY: TOAGOSEI CO, LTD
                      1-14-1. NISHI SHIMBASHI
                      MINATO-KU, TOKYO 105-8419

    REGISTRANT:  OTSUKA CHEMICAL COMPANY, LTD.
                 3-2-27 OTEDORI
                 CHUO-KU, OSAKA, 540-0021

    FORMULATION: SOLUBLE CONCENTRATE
    SIGNAL WORD: CAUTION

    TYPES: BIOCHEMICAL PESTICIDE
           MITICIDE

    PERCENT  ACTIVE INGREDIENT
    70.8100  Dodecanoic acid, monoester with 1,2-propanediol (11288)

    STATES REG: OR-08 CA-08 AZ-08

    ORIGINAL APPROVAL DATE:  10-12-04
    MOST RECENT ACCEPT DATE: ** NA **    USES LAST UPDATED BY EPA: ** NA **
**** END OF DATA FOR THIS PRODUCT ****

*** END OF OUTPUT ***
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Fatty Acid Monoesters with Glycerol or Propanediol Fact Sheet (011288)

Related Information

- Regulating Biopesticides
- Active Ingredient Index

Information related to this page:

- Factsheet
- Federal Register Notices
- Products
- Registrants

Issued: 10/20/04

ACTIVE INGREDIENT NAME OPP CHEMICAL CODE (CAS NO.) (alternate chemical name)

- Glycerol monocaprylate (octanoic acid monoester with glycerol), 011292 (CAS No. 26402-26-6)
- Glycerol monocaprate (decanoic acid monoester with glycerol), 011291 (CAS No. 26402-22-2)
- Glycerol monolaurate (dodecanoic acid monoester with glycerol), 011290 (CAS No. 27215-38-9)
- Propylene glycol monocaprylate (octanoic acid monoester with 1,2-propanediol), 082074 (CAS No. 68332-79-6)
- Propylene glycol monocaprate (decanoic acid monoester with 1,2-propanediol), 011289 (CAS No. 68795-69-7)
- Propylene glycol monolaurate (dodecanoic acid monoester with 1,2-propanediol), 011288 (CAS No. 27194-74-7)

On This Page

I. Description of the Active Ingredient
II. Use Sites, Target Pests, and Application Methods
III. Assessing Risks to Human Health
IV. Assessing Risks to the Environment
V. Regulatory Information
VI. Registrant Information
VII. Additional Contact Information

Summary

These active ingredients may be used on food and feed crops both before harvest to protect against mites, and after harvest to prevent microbial damage during storage. These chemicals and their breakdown products are approved for food use by the FDA. No harm is expected from use of these substances in pesticide products as long as users follow label instructions.

I. Description of the Active Ingredient

This fact sheet summarizes information about six chemicals with similar structures. Three of the chemicals consist of glycerol monoesters containing specified fatty acids, and the other three chemicals consist of propylene glycol monoesters containing the same specified fatty acids (C8, C10, and C12 straight chain saturated fatty acids). These chemicals control mites on growing crops. They also control microbes that can

cause decay on stored food; researchers suggest that these monoesters act by disrupting microbial membranes.

Glycerol fatty acid esters are found in all living organisms, including plants and humans, whereas propylene glycol esters are manufactured. However, the six substances behave the same in various toxicity tests, and are metabolized by living organisms using the same biochemical pathways. The six substances and their breakdown products are approved by the United States FDA for use in food and feed.

II. Use Sites, Target Pests, and Application Methods

- **Use Sites:** Major use sites are expected to be both
  1. in the field,
  2. on food and feed in indoor storage facilities

- **Target pests:**
  1. Mites in the field;
  2. Fungi, yeast, gram positive and gram negative bacteria, and lipid-coated viruses that cause decay in stored crops.

- **Application Methods:**
  1. In the field, the first end-use miticide product is sprayed at a concentration of up to 25 oz/100 gal of water, with a minimum application of 50 gal/acre.
  2. After the crop is harvested and ready for storage, the first fungicidal/biocidal product will be applied as a liquid containing less than 1% by weight of active ingredient.

III. Assessing Risks to Human Health

Whether a substance poses a risk to humans or other organisms depends on two factors: how toxic the substance is, and how much of it an organism is exposed to. Therefore, the EPA considers toxicity data and exposure data in deciding whether to approve a pesticide for use. In studies using laboratory animals, the fatty acid monoesters showed no adverse effects except for mild eye irritation for both the glycerol and the propylene glycol monoesters and dermal sensitization for the propylene glycol monocaprylate. Therefore, special precautions were put on some of the propylene glycol monoester labels to warn users that the product might cause an allergic response: An example of a precautionary statement is: "Prolonged or frequently repeated skin contact may cause allergic reactions in some individuals exposed to this product."

IV. Assessing Risks to the Environment

Adverse effects on birds and higher organisms are expected to be low due to the low mammalian toxicity found in animal testing and the ability of most organisms to metabolize these substances. Testing has demonstrated, however, that the miticide is moderately toxic to fish and algae and very toxic to aquatic invertebrates, although harmless to honey bees. The label on the first registered miticide product must specifically warn users not to apply the product to bodies of water or to contaminate bodies of water during application, cleaning, or disposal.

V. Regulatory Information

The six fatty acid monoesters were initially registered (licensed for sale) as manufacturing use products in September 2003 by 3M. Two end products were registered as of October 2004:

June 2004. 3M registered an end product to prevent microbial growth on stored potatoes. Product (Reg # 10351-61) contains propylene glycol monocaprylate as its major active ingredient.

October 2004. Toagosei Co. Ltd, Japan, registered an end product to control mites on food crops and ornamental plants. The product (Reg # 70231-2) contains propylene glycol monolaurate as the active ingredient.

VI. Registrant Information

Iain Weatherston, Ph.D. (U.S. contact for Toagosei, Co., which is the registrant.)
Senior Regulatory Consultant
Pesticide Division
Technology Sciences Group, Inc.,
4061 North 156th Drive
Goodyear, AZ 85338
e-mail: jazkatz@qwest.net
ph: 623-535-4060 (fax 623-535-4061)
Toagosei Co., Ltd.
1-14-1, Nishi Shimbashi
Minato-Ku, Tokyo 105-8419, Japan

VII. Additional Contact Information

Ombudsman, Biopesticides and Pollution Prevention Division (7511P)
Office of Pesticide Programs
Environmental Protection Agency
1200 Pennsylvania Avenue, NW
Washington, D.C. 20460

§ 180.1244 Ammonium bicarbonate; exemption from the requirement of a tolerance.

An exemption from the requirement of tolerance is established for residues of ammonium bicarbonate used in or on all food commodities when used in accordance with good agricultural practices.

[69 FR 13745, Mar. 24, 2004]

§ 180.1245 Rhamnolipid biosurfactant; exemption from the requirement of a tolerance.

An exemption from the requirement of a tolerance is established for residues of rhamnolipid biosurfactant when used in accordance with good agricultural practices as a fungicide in or on all food commodities.

[69 FR 16800, Mar. 31, 2004]

§ 180.1246 Yeast Extract Hydrolysate from *Saccharomyces cerevisiae:* exemption from the requirement of a tolerance.

This regulation establishes an exemption from the requirement of a tolerance for residues of the biochemical pesticide Yeast Extract Hydrolysate from *Saccharomyces cerevisiae* on all food commodities when applied/used for the management of plant diseases.

[69 FR 9958, Mar. 3, 2004]

§ 180.1248 Exemption of citronellol from the requirement of a tolerance.

An exemption from the requirement of a tolerance is established for residues of the biochemical pesticide citronellol in or on all food commodities.

[69 FR 23146, Apr. 28, 2004]

§ 180.1249 Hygromycin B phosphotransferase (APH4) marker protein and the genetic material necessary for its production in all plants; exemption from the requirement of a tolerance.

Hygromycin B phosphotransferase (APH4) and the genetic material necessary for its production in all plants are exempt from the requirement of a tolerance when used as a plant-incorporated protectant inert ingredient in cotton. "Genetic material necessary for its production" means the genetic material which comprise genetic material encoding the APH4 protein and its regulatory regions. "Regulatory regions" are the genetic material that control the expression of the genetic material encoding the APH4 protein, such as promoters, terminators, and enhancers.

[69 FR 18278, Apr. 7, 2004]

§ 180.1250 C8, C10, and C12 fatty acid monoesters of glycerol and propylene glycol; exemption from the requirement of a tolerance.

The C8, C10, and C12 straight-chain fatty acid monoesters of glycerol (glycerol monocaprylate, glycerol monocaprate, and glycerol monolaurate) and propylene glycol (propylene glycol monocaprylate, propylene glycol monocaprate, and propylene glycol monolaurate) are exempt from the requirement of a tolerance in or on all food commodities when used in accordance with approved label rates and good agricultural practice.

[69 FR 34944, June 23, 2004]

§ 180.1251 Geraniol; exemption from the requirement of a tolerance.

An exemption from the requirement of a tolerance is established for residues of the biochemical pesticide geraniol in or on all food commodities.

[69 FR 23151, Apr. 28, 2004]

§ 180.1252 Phosphomannose isomerase and the genetic material necessary for its production in all plants; exemption from the requirement of a tolerance.

Phosphomannose isomerase (PMI) protein and the genetic material necessary for its production in plants are exempt from the requirement of a tolerance when used as plant-incorporated protectant inert ingredients in plant commodities. Genetic material necessary for its production means the genetic material which comprise genetic material encoding the PMI protein and its regulatory regions. Regulatory regions are the genetic material, such as promoters, terminators, and enhancers,

ALASKA—CARBON MONOXIDE

Designated area	Designation		Classification	
	Date [1]	Type	Date [1]	Type
Anchorage Area:				
Anchorage Election District (part) Anchorage nonattainment area boundary.	July 23, 2004	Attainment.		
The Anchorage Nonattainment Area is contained within the boundary described as follows: Beginning at a point on the centerline of the New Seward Highway five hundred (500) feet of the centerline of O'Malley Road; thence, Westerly along a line five hundred (500) south of and parallel to the centerline of O'Malley Road and its westerly extension thereof to a point on the mean high tide line of the Turnagain Arm; thence, Northeasterly along the mean high tide line to a point five hundred (500) feet west of the southerly extension of the centerline of Sand Lake Road; thence, Northerly along a line five hundred (500) feet west of and parallel to the southerly extension of the centerline of Sand Lake Road to a point on the southerly boundary of the International Airport property; thence, Westerly along said property line of the International Airport to an angle point in said property line; thence, Easterly, along said property line and its easterly extension thereof to a point five hundred (500) feet west of the southerly extension of the centerline of Wisconsin Street; thence, Northerly along said line to a point on the mean high tide line of the Knik Arm; thence, Northeasterly along the mean high tide line to a point on a line parallel and five hundred (500) feet north of the centerline of Thompson Street and the westerly extension thereof; thence, Easterly along said line to a point five hundred (55) feet east of Boniface Parkway; thence, Southerly along a line five hundred (500) feet east of and parallel to the centerline of Boniface Parkway to a point five hundred (500) feet north of the Glenn Highway; thence, Easterly and northeasterly along a line five hundred (500) feet north of and parallel to the centerline of the Glenn Highway to a point five hundred (500) feet east of the northerly extension of the centerline of Muldoon Road; thence, Southerly along a line five hundred (500) feet east of and parallel to the centerline of Muldoon Road and continuing southwesterly on a line of curvature five hundred (500) feet southeasterly of the centerline of curvature where Muldoon Road becomes Tudor Road to a point five hundred (500) south of the centerline of Tudor Road; thence, Westerly along a line five hundred (500) feet south of the centerline of Tudor Road to a point five hundred (500) feet east of the centerline to Lake Otis Parkway; thence, Westerly along a line five hundred (500) feet south of the centerline of O'Malley Road, ending at the centerline of the New Seward Highway, which is the point of the beginning.				

* * * * * * *

[1] This date is November 15, 1990 unless otherwise noted.

* * * * *

[FR Doc. 04–14216 Filed 6–22–04; 8:45 am]

BILLING CODE 6560–50–P

ENVIRONMENTAL PROTECTION AGENCY

40 CFR Part 180

[OPP–2003–0379; FRL–7352–6]

C8, C10, and C12 Straight-Chain Fatty Acid Monoesters of Glycerol and Propylene Glycol; Exemption from the Requirement of a Tolerance

AGENCY: Environmental Protection Agency (EPA).

ACTION: Final rule.

SUMMARY: This regulation establishes an exemption from the requirement of a tolerance for residues of the C8, C10, and C12 straight-chain fatty acid monoesters of glycerol and propylene glycol on all raw agricultural commodities and food when applied/used in accordance with good agricultural practices. 3M Corporation submitted a petition to EPA under the Federal Food, Drug, and Cosmetic Act (FFDCA), as amended by the Food Quality Protection Act of 1996 (FQPA), requesting an exemption from the requirement of a tolerance. This regulation eliminates the need to establish a maximum permissible level for residues of C8, C10, and C12 straight-chain fatty acid monoesters of glycerol and propylene glycol.

DATES: This regulation is effective June 23, 2004. Objections and requests for hearings, must be received on or before August 23, 2004.

ADDRESSES: To submit a written objection or hearing request follow the detailed instructions provided in Unit VIII. of the **SUPPLEMENTARY INFORMATION**. EPA has established a docket for this action under Docket ID number OPP–2003–0379. All documents in the docket are listed in the EDOCKET index at *http://www.epa.gov/edocket*. Although listed in the index, some information is not publicly available, i.e., confidential

business information (CBI) or other information whose disclosure is restricted by statute. Certain other material, such as copyrighted material, is not placed on the Internet and will be publicly available only in hard copy form. Publicly available docket materials are available either electronically in EDOCKET or in hard copy at the Public Information and Records Integrity Branch (PIRIB), Rm. 119, Crystal Mall #2, 1801 S. Bell St., Arlington, VA. This docket facility is open from 8:30 a.m. to 4 p.m., Monday through Friday, excluding legal holidays. The docket telephone number is (703) 305–5805.

FOR FURTHER INFORMATION CONTACT: Carol E. Frazer, Biopesticides and Pollution Prevention Division (7511C), Environmental Protection Agency, 1200 Pennsylvania Ave., NW., Washington, DC 20460–0001; telephone number: (703) 308–8810; e-mail address: *frazer.carol@epa.gov.*

SUPPLEMENTARY INFORMATION:

I. General Information

A. Does this Action Apply to Me?

You may be potentially affected by this action if you are an agricultural producer, food manufacturer, or pesticide manufacturer. Potentially affected entities may include, but are not limited to:

- Crop production (NAICS 111), e.g., farmer.
- Animal production (NAICS 112), e.g., rancher.
- Food manufacturing (NAICS 311), e.g., restaurant.
- Pesticide manufacturing (NAICS 32532).

This listing is not intended to be exhaustive, but rather provides a guide for readers regarding entities likely to be affected by this action. Other types of entities not listed in this unit could also be affected. The North American Industrial Classification System (NAICS) codes have been provided to assist you and others in determining whether this action might apply to certain entities. If you have any questions regarding the applicability of this action to a particular entity, consult the person listed under **FOR FURTHER INFORMATION CONTACT**.

B. How Can I Access Electronic Copies of this Document and Other Related Information?

In addition to using EDOCKET (*http://www.epa.gov/edocket/*), you may access this **Federal Register** document electronically through the EPA Internet under the "**Federal Register**" listings at *http://www.epa.gov/fedrgstr/*. A frequently updated electronic version of 40 CFR part 180 is available at E-CFR Beta Site Two at *http://www.gpoaccess.gov/ecfr/.*

II. Background and Statutory Findings

In the **Federal Register** of December 12, 2001 (66 FR 64251) (FRL–6809–8), EPA issued a notice pursuant to section 408(d)(3) of the FFDCA, 21 U.S.C. 346a(d)(3), announcing the filing of a pesticide tolerance petition (PP 1F6314) by 3M Corporation, 3M Center, St. Paul, MN 55144–1000. This notice included a summary of the petition prepared by the petitioner 3M Corporation. There were no comments received in response to the notice of filing.

The petition requested that 40 CFR part 180 be amended by establishing an exemption from the requirement of a tolerance for residues of C8, C10, and C12 straight-chain fatty acid monoesters of glycerol and propylene glycol.

Section 408(c)(2)(A)(i) of the FFDCA allows EPA to establish an exemption from the requirement for a tolerance (the legal limit for a pesticide chemical residue in or on a food) only if EPA determines that the exemption is "safe." Section 408(c)(2)(A)(ii) of the FFDCA defines "safe" to mean that "there is a reasonable certainty that no harm will result from aggregate exposure to the pesticide chemical residue, including all anticipated dietary exposures and all other exposures for which there is reliable information." This includes exposure through drinking water and in residential settings, but does not include occupational exposure. Pursuant to section 408(c)(2)(B), in establishing or maintaining in effect an exemption from the requirement of a tolerance, EPA must take into account the factors set forth in section 408(b)(2)(C), which require EPA to give special consideration to exposure of infants and children to the pesticide chemical residue in establishing a tolerance and to "ensure that there is a reasonable certainty that no harm will result to infants and children from aggregate exposure to the pesticide chemical residue. . . . " Additionally, section 408(b)(2)(D) of the FFDCA requires that the Agency consider "available information concerning the cumulative effects of a particular pesticide's residues" and "other substances that have a common mechanism of toxicity."

EPA performs a number of analyses to determine the risks from aggregate exposure to pesticide residues. First, EPA determines the toxicity of pesticides. Second, EPA examines exposure to the pesticide through food, drinking water, and through other exposures that occur as a result of pesticide use in residential settings.

III. Toxicological Profile

Consistent with section 408(b)(2)(D) of the FFDCA, EPA has reviewed the available scientific data and other relevant information in support of this action and considered its validity, completeness, and reliability and the relationship of this information to human risk. EPA has also considered available information concerning the variability of the sensitivities of major identifiable subgroups of consumers, including infants and children.

The fatty acid monoesters of glycerol and propylene glycol are six closely-related monoesters of C8, C10, and C12 straight-chain fatty acids. There are three glycerol monoesters (glycerol monocaprylate, glycerol monocaprate, and glycerol monolaurate), and three propylene glycol monoesters (propylene glycol monocaprylate, propylene glycol monocaprate, and propylene glycol monolaurate).

In vertebrate organisms (including humans), glycerol fatty acid monoesters are formed naturally as part of the metabolism of triglycerides. They also occur naturally in vegetable oils (e.g., coconut and palm oils) and in saw palmetto leaves and berries. Glycerol fatty acid monoesters are, in addition, used as direct food additives. Propylene glycol fatty acid monoesters, also used as direct food additives, are naturally metabolized in vertebrate systems in an identical manner to the glycerol fatty acid monoesters.

Toxicity studies supporting this tolerance exemption are referenced below. More detailed analyses of these studies can be found in the specific Agency review of the studies (Ref. 1). Additional information relevant to toxicity also has been published and is cited in Ref. 2.

Acute toxicity studies were generated to support EPA registration of the C8, C10, and C12 straight-chain fatty acid monoesters of glycerol and propylene glycol as biochemical pesticides. In all studies, EPA limit doses were used, and the test compounds were found to be non-toxic at the limit dose, but all tests were not conducted on each of the six active ingredients. Instead, a full acute toxicity test battery (6 studies) was generated for the C8 propylene glycol monoester (propylene glycol monocaprylate) and for the C12 glycerol ester (glycerol monolaurate), thereby bounding the chemical structures of all six active ingredients. In addition, because all six active ingredients are known to be identical with respect to acute toxicity and metabolism, a 90–day

rat oral toxicity study was conducted on propylene glycol monocaprylate only. The registrant requested and was granted waivers from toxicity testing for the additional monoesters (Ref. 3), since the metabolism and toxicity of the active ingredients have been well-documented for many years in the scientific literature. This represented all six active ingredients.

1. *Acute oral toxicity for glycerol monolaurate (OPPTS Harmonized Guideline 870.1100; 152–10; MRID 45405505): Non-toxic.* Fasted rats (three male and three female) received a single oral gavage of glycerol monolaurate formulated in corn oil and administered at a dose level of 5,000 milligrams/kilogram of body weight (mg/kg bwt). All rats survived and gained weight throughout the study with the exception of one female with a slight weight loss on the final day. Piloerection and increased salivation were observed in all rats within minutes of dosing. Normal salivation resumed shortly after dosing and piloerection resolved by day 3 in males and day 4 in females. No abnormalities were revealed in any rats at the macroscopic examination at study termination on day 15. The acute oral lethal dose $(LD)_{50}$ for rats was >5,000 mg/kg. Classification: Acceptable; Toxicity Category IV (Ref. 4).

2. *Acute oral toxicity for propylene glycol monocaprylate (OPPTS Harmonized Guideline 870.1100; 152–10; MRID 45428501): Non-toxic.* Fasted rats (three males and three females) received a single oral gavage of propylene glycol monocaprylate administered at a dose of 5,000 mg/kg bwt. All rats survived and gained weight throughout the study. Piloerection (all rats) and increased salivation (one female only) were evident within a few minutes of dosing, with piloerection persisting for the remainder of day 1. Piloerection was resolved by day 2 in females and by day 4 in males. No abnormalities were revealed in any animal at the macroscopic examination at study termination on day 15. The acute oral LD_{50} for rats was >5,000 mg/kg. Classification: Acceptable; Toxicity Category IV (Ref. 5).

3. *Acute dermal toxicity for glycerol monolaurate (OPPTS Harmonized Guideline 870.1200; 152–11; MRID 45428501): Non-toxic.* Ten rats (five males and five females) received a single topical application of glycerol monolaurate formulated in corn oil and administered at a dose of 5,000 mg/kg bwt. All rats survived and had normal weight gains throughout the study, with the exception of two females with low or no weight gain during week 1. No clinical signs of reaction to treatment were observed in any animal throughout the study, and no macroscopic abnormalities were observed in any animal at study termination on day 15. The acute dermal LD_{50} for rats was >5,000 mg/kg. Classification: Acceptable; Toxicity Category IV (Ref. 6).

4. *Acute dermal toxicity for propylene glycol monocaprylate (OPPTS Harmonized Guideline 870.1200; 152–11; MRID 45428503): Non-toxic.* Ten rats (five males and five females) received a single topical application of propylene glycol monocaprylate at a dose of 5,000 mg/kg bwt. All rats survived and gained weight, with the exception of one female with a slight weight loss during week 2. No macroscopic abnormalities were observed in any animal at study termination on day 15. The acute dermal LD_{50} for rats was >5,000 mg/kg. Classification: Acceptable; Toxicity Category IV (Ref. 7).

5. *Acute inhalation for glycerol monolaurate (OPPTS Harmonized Guideline 870.1300; 152–12; MRID 45405506): Harmless by inhalation.* In all instances, the aerosol generator was blocked following the start of generation. The waxiness of glycerol monolaurate made it impossible to generate aerosols. Because respirable particles cannot be produced from such low-melting waxy materials, the test substance is considered harmless by the inhalation route of exposure under normal handling conditions. Classification: Acceptable; Toxicity Category IV (Ref. 8).

6. *Acute inhalation for propylene glycol monocaprylate (OPPTS Harmonized Guideline 870.1300; 152–12; MRID 45405507): Non-toxic.* Ten rats (five males and five females) were exposed for 4 hours to a droplet aerosol generated from propylene glycol monocaprylate at a target concentration of 5 mg/liter (L). Another group (five males and five females), exposed to clean dry air only, were controls. The mass median aerodynamic (MMAD) was 2.0 microns and was within the ideal range (1 micron to 4 microns) for an acute inhalation study. Approximately 88% of the particles were considered a respirable size (less than 7 microns in aerodynamic diameter). The lethal concentration $(LC)_{50}$ (4–hour inhalation) for propylene glycol monocaprylate was >4.92 mg/L (4,920 ppm) in air. EPA's limit dose for this test is 2 mg/L. Classification: Acceptable; Toxicity Category IV (Ref. 9).

7. *Eye irritation for glycerol monolaurate (OPPTS Harmonized Guideline 870.2400, 152–13, MRID 45405508): Slight irritant.* Each of three rabbits was administered a single ocular dose of 0.1 milliliter (mL) (mean weight 60 mg) of glycerol monolaurate and observed for up to 7 days after instillation. The instillation in one animal elicited a corneal lesion and iritis (both Grade 1) 48 hours post-dose. All rabbits exhibited transient conjunctival inflammation (up to Grade 3). Resolution was complete in two instances within approximately 72 hours of dosing and, in one animal, 7 days after dosing. Glycerol monolaurate is considered a slight eye irritant. Classification: Acceptable; Toxicity Category III (Ref. 10).

8. *Eye irritation for propylene glycol monocaprylate (OPPTS Harmonized Guideline 870.2400, 152–13, MRID 45405509): Slight irritant.* Three rabbits were each administered a single ocular dose of 0.1 mL of propylene glycol monocaprylate and observed for up to 7 days after instillation. The test substance elicited a transient, slight to well-defined conjunctival irritation in two rabbits. Propylene glycol monocaprylate is not considered a major ocular irritant. Classification: Acceptable; Toxicity Category III (Ref. 11).

9. *Skin irritation for glycerol monolaurate (OPPTS Harmonized Guideline 870.2500, 152–14, MRID 45405510): Non-Irritant.* Each of three rabbits was administered a single dermal dose of 0.5 g of glycerol monolaurate under semi-occlusive conditions for 4 hours and observed for up to 7 days. The test material produced transient slight erythema in 2 animals that resolved by 72 hours; the third animal had well-defined erythema at 48 hours that resolved by day 7. Glycerol monolaurate is not considered a dermal irritant. Classification: Acceptable; Toxicity Category IV (Ref. 12).

10. *Skin irritation for propylene glycol monocaprylate (OPPTS Harmonized Guideline 870.2500, 152–14, MRID 45405511): Non-irritant.* Each of three rabbits was administered a single dermal dose of 0.5 mL of propylene glycol monocaprylate under semi-occlusive conditions for 4 hours and observed for up to 11 days. The test substance produced only slight erythema in all animals. Propylene glycol monocaprylate is not considered a dermal irritant. Classification: Acceptable; Toxicity Category IV (Ref. 13).

11. *Skin sensitization for glycerol monolaurate (OPPTS Harmonized Guideline 870.2600, 152–15, MRID 45428504): Non-sensitizer.* Guinea pigs (10 test and 5 control) were dosed by intradermal injection and topical

application. Based on the results of a preliminary study, and in compliance with regulatory guidelines, the following dose levels were selected:

Intradermal injection: 2.5% w/v (weight/volume) in sterile water.

Topical application: 10% w/v in sterile water.

Challenge applications: 0.5% and 1% w/v in sterile water.

Following the first challenge application, negative responses were observed in six test animals, inconclusive responses in three animals and a positive response was observed in the remaining test animal. A second challenge was conducted to clarify these reactions. Following the second challenge application, glycerol monolaurate did not produce dermal reactions in any test or control animal. Glycerol monolaurate is not thought to cause skin sensitization. The sensitivity of the guinea pig strain used by the laboratory is checked periodically with a weak/moderate sensitizer - hexyl cinnamic aldehyde (HCA). In this study, HCA produced evidence of skin sensitization (delayed contact hypersensitivity) in 9 of the 10 animals, thus confirming the sensitivity and reliability of the experimental technique. Classification: Acceptable. (Ref. 14)

12. *Skin sensitization for propylene glycol monocaprylate (OPPTS Harmonized Guideline 870.2600, 152–15, MRID 45448201): Potential sensitizer.* The guinea pigs (10 test and 5 control) were dosed by intradermal injection and topical application. Based on the results of a preliminary study and in compliance with the regulatory guidelines, the following dose levels were selected:

Intradermal injection: 0.5% v/v in sterile water.

Topical application: as supplied.

Challenge application: 25% and 50% v/v in sterile water.

In this study, propylene glycol monocaprylate produced evidence of skin sensitization (delayed contact hypersensitivity) in all of the test animals. Propylene glycol monocaprylate may cause skin sensitization in humans. Propylene glycol itself is known to cause allergic reactions in patients receiving medical treatments containing this substance. The sensitivity of the guinea pig strain used is checked periodically by the laboratory with a weak to moderate sensitizer-HCA. In this study, HCA produced evidence of skin sensitization (delayed contact hypersensitivity) in 9 of the 10 animals, thus confirming the sensitivity and reliability of the experimental technique. This risk, however, is mitigated as long as the products are used according to the precautionary statements on the label, which advise washing thoroughly with soap and water after handling and that prolonged or frequently repeated skin contact may cause allergic reactions in some individuals. Classification: Acceptable (Ref. 15).

13. *28–Day oral for propylene glycol monocaprylate (OPPTS Harmonized Guideline 870.3050, MRID 45441101): Non-toxic.* The effects of propylene glycol monocaprylate (T–7475.8) were assessed in rats (groups of five males and five females) by oral gavage administration once a day for 4 weeks, employing dose levels of 0, 500, 750, or 1,000 mg/kg/day. Doses up to 1,000 mg/kg/day were well tolerated with the only effects noted being higher protein and albumin values and a higher lung and liver weight, all in females. In the absence of histopathological examination, the toxicological importance of these findings is unclear. However, it was considered that 1,000 mg/kg/day was well tolerated and that it would be suitable for use as a high dose level in the subsequent 13–week toxicity study. Classification: Acceptable (Ref. 16).

14. *13–Week oral for propylene glycol monocaprylate (OPPTS Harmonized Guideline 870.3100 and 870.7800, MRID 45428505): Non-toxic.* The systemic toxicity of propylene glycol monocaprylate (T–7475.8) was assessed in groups of rats (20 males and 20 females per group) by oral gavage administration at 0, 100, 500, or 1,000 mg/kg/day dose levels for 13 weeks. There were no unscheduled deaths in any of the groups and clinical observation, neurotoxicity, metabolic parameters, and organ histopathology indicated no changes of toxicological significance. It was concluded that a dosage of 1,000 mg/kg/day was considered to be a no observable adverse effect level (NOAEL) for either sex. Classification: Acceptable (Ref. 17).

15. *Genotoxicity.* Fatty acid monoesters of glycerol and propylene glycol in vertebrate systems are immediately metabolized to polyols and free fatty acids. Upon ingestion these compounds become indistinguishable from those in living systems. Polyols and free fatty acids in living systems are not genotoxic. Hence, waivers were requested and granted for all genotoxicity testing requirements on the basis that conducting such tests would not be of value to EPA in its evaluation of risks. The fatty acid monoesters of glycerol and propylene glycol are already known not to be genotoxic from a metabolic standpoint.

16. *Reproductive and developmental toxicity.* On their metabolic basis, fatty acid monoesters of glycerol and propylene glycol and their natural breakdown products are known not to be reproductive or developmental toxicants. Waivers therefore were requested and granted for all such testing requirements on the basis that conducting such tests would not be of value to EPA in its evaluation of risks (for both the registration action and this tolerance exemption action).

17. *Scientific literature on toxicity and metabolism.* Basic toxicity testing on mono- and diacylglycerols and saturated fatty acids was conducted in the 1930–1960 period and included intermediate-term and long-term studies. Less work has been published on propylene glycol saturated fatty acid esters, but the available data are adequate to demonstrate equivalence between propylene glycol esters and acylglycerols. Comprehensive reviews of these chemicals prepared by a number of sources including the Food and Drug Administration (FDA) and the Food and Agricultural Organization of the United Nations (FAO) and the World Health Organization (WHO) are available through the Joint FAO/WHO Expert Committee on Food Additives (JECFA). The no observed adverse effect levels (NOAELs) for monoacylglycerols, regardless of the saturated fatty acid, are similar. Rats can be fed from 10–15% in the diet for a lifetime without ill effects, dose levels corresponding to 5 g/kg bwt/day. Rats fed propylene glycol monosuccinate and monostearate at levels up to 10% of the diet for 6 months showed no evidence of gross or histological pathology attributable to treatment. Dogs fed at the same levels for 6 months showed no signs of toxicity.

The fatty acid moiety in monoacylglycerols is of no consequence because vertebrate systems are capable of metabolizing each of the acids in the range of C8 to C18 with equal facility. In fact, oxidation of fatty acids is a primary source of energy in vertebrate systems. Fatty acids are supplied in the diet in the form of triacylglycerols (fats) which are hydrolyzed by pancreatic lipase enzymes to form free fatty acids, glycerol and monoacylglycerols. The glycerol monoester active ingredients are indistinguishable from the natural acylglycerols and fatty acids found in the intestine following ingestion of fats. Specificity of the pancreatic lipase enzyme is independent of the nature of the fatty acid. It is also not stereospecific in its action and glycerol esters and propylene glycol esters are hydrolyzed by it with equal facility.

Studies with 14C-labeled propylene glycol show that it is readily absorbed from the gastrointestinal tract and rapidly converted in the liver to 14C-glycogen or 14CO2. Similarly, when 14C-glycerol is administered to rats, radiolabel appears in expired CO2, blood glucose, liver glycogen, liver fat and liver phosphatides within 15 minutes. Within 6 hours, 40% of the label is contained in expired CO2 and the remainder is distributed through the test animal. Very small amounts are excreted.

FDA has looked at metabolism of propylene glycol mono- and distearates as model compounds to represent propylene glycol fatty acids. In studies on radiolabeled propylene glycol distearate the rate-limiting factor in the metabolism was found to be hydrolysis of the ester, which is complete in about 3 hours. In 5 hours, 94% of the propylene glycol is absorbed and 94% of the absorbed material is found in expired CO2 in 72 hours. The fatty acid portion of the ester is absorbed and metabolized more slowly than the propylene glycol. Only 51% of the stearic acid label was expired as CO2 in the same period.

In addition, there is a long history of consumption by humans of fatty acids and their monoesters in food and the Agency knows of no instance where these have been associated with any toxic effects related to the consumption of food. Due to this knowledge of fatty acid monoesters' presence and function in the human system (Ref. 2) and the recent acute testing, EPA believes the fatty acid monoesters are unlikely to be carcinogenic or have other long-term toxic effects.

The data from the toxicity studies (Ref. 1) and the additional information from the scientific literature submitted by the registrant (Ref. 2) are sufficient to support the current waiver requests, and to demonstrate that no substantial risks to human health are expected from the use of glycerol or propylene glycol fatty acid monoesters, when used in accordance with good agricultural practices and in accordance with all relevant labeling.

IV. Aggregate Exposures

In examining aggregate exposure, section 408 of the FFDCA directs EPA to consider available information concerning exposures from the pesticide residue in food and all other non-occupational exposures, including drinking water from ground water or surface water and exposure through pesticide use in gardens, lawns, or buildings (residential and other indoor uses).

A. Dietary Exposure

Aggregate dietary exposure estimates were generated using EPA's Dietary Exposure Potential Model (DEPM) customarily used by the agency. The model is designed to generate dietary exposure estimates by combining food consumption and residue data. In this case, food consumption data came from the 10th National Food Consumption Survey conducted during the 3–year period of 1994–1996 by the Agricultural Research Service of the U.S. Department of Agriculture. These data are also known as the Continuing Survey of Food Intake by Individuals, 1994–1996 (CSFII 1994–1996).

1. *Food.* Food residue estimates were generated for use in the DEPM analysis to simulate broad use of the fatty acid monoesters of glycerol and propylene glycol. Specifically, residue estimates were constructed for all food commodities corresponding to 18 raw agricultural commodities (RACs) for which residue data were generated for the following major food groups: Fruits; vegetables; beverages; and infant food. In keeping with the worst case nature of the analysis, residue data for a tested commodity was used also for similar commodities not tested (e.g., spinach values were used for other delicate greens; kale values were used for other heavy greens such as collard; peach values were used for apricots). It was also assumed residue levels are not changed by cooking and that fruit and vegetable mixtures contain 50% of one or more RAC, unless the composition of the mixture is specified. Total dietary exposure estimates were generated using the model for the U.S. population and 20 subpopulations, including non-nursing infants and children. The subpopulation groups were defined by age, gender, geographic location, ethnicity and income level. All calculations represented residue levels assuming treatment of 100% of every commodity consumed in the U.S. for which residue estimates could be generated, another severe worst-case assumption. The model produced data tables containing the consumption of each food, its assumed residue level and the calculated exposure from that consumption in µg/kg-bwt/day for each of the subpopulations. For all subpopulation groups, the commodity that contributed in the analysis the most to exposure was cooked green beans. This result reflects the fact that green beans absorbed an unexpectedly large amount of treatment solution in the experimental procedure used to generate RAC residue estimates. Based upon the worst-case data and assumptions described above, the model calculated the highest exposure of 0.5 mg/kg bwt/day for non-nursing infants. Dietary exposure for the total U.S. population was less than 0.2 mg/kg bwt/day. These levels are below the FDA approved dosage for addition to prepared foods, and the highest dose accepted as a chronic NOAEL for either sex was 5,000 times higher (Ref. 17).

2. *Drinking water exposure.* All anticipated or proposed uses of glycerol and propylene glycol fatty acid monoesters will be indoors and the compounds are not soluble in water. Hence, drinking water is not a feasible route of exposure.

B. Other Non-Occupational Exposure

Glycerol fatty acid monoesters are natural components of dietary fats and natural breakdown products from metabolism of fat (triacylglycerol) in all living systems. Additionally, fatty acid esters of both glycerol and propylene glycol occur as direct food additives.

1. *Dermal exposure.* Results of the acute dermal toxicity studies for glycerol monolaurate and propylene glycol monocaprylate indicated no toxicity (Toxicity Category IV) at the maximum dose tested (5,000 mg/kg) with no significant dermal irritation (Toxicity Category IV). Based on these results, the anticipated risks from dermal exposure are minimal. Dermal sensitization may occur with the propylene glycol monoesters as the caprylate is a potential sensitizer. This risk, however, is mitigated as long as the products are used according to the precautionary statements on the label, which advise washing thoroughly with soap and water after handling and that prolonged or frequently repeated skin contact may cause allergic reactions in some individuals.

2. *Inhalation exposure.* Because the inhalation toxicity study for propylene glycol monocaprylate showed no toxicity (Toxicity Category IV), and the glycerol fatty acid monoesters are waxy solids at room temperature (not present as respirable particles), the risks anticipated for this route of exposure are minimal.

V. Cumulative Effects

Section 408(b)(2)(D)(v) of FFDCA requires the Agency, when considering whether to establish, modify, or revoke a tolerance, to consider "available information" concerning the cumulative effects of a particular pesticide's residues and "other substances that have a common mechanism of toxicity." These considerations include the possible cumulative effects of such residues on infants and children.

In assessing their cumulative effects, the fatty acid monoesters of glycerol and propylene glycol are members of a much larger class of compounds that are toxicologically and metabolically equivalent. All vertebrate systems deal with this class of compounds as food rather than toxicants. Glycerol fatty acid monoesters are natural components in dietary fats and natural breakdown products from metabolism of fat (triacylglycerol) in all living systems. Fatty acid esters of propylene glycol also occur as direct food additives in the human diet in substantial quantities. The use of fatty acid monoesters of glycerol and propylene glycol as pesticides will contribute a negligible amount (total U.S. population worst case estimate less than 0.2 mg/kg/day) to the existing cumulative exposure to the class of compounds when compared to natural levels of such compounds and their metabolites in tissue and foods (50–100 g/day in humans for glycerol esters), and to the levels permitted in food as direct additives (grams per day). Accordingly, exposure to these monoesters as a result of their label directed use as pesticides on raw agricultural food or feed commodities will result in a negligible increase in the cumulative exposure to this class of compounds over the present exposure, occurring as a result of daily consumption by the human population of this class of compounds from both naturally occurring sources and processed foods.

VI. Determination of Safety for U.S. Population, Infants and Children

1. *U.S. population.* It is doubtful harm will result from aggregate exposure to residues of the fatty acid monoesters of glycerol or propylene glycol in the U.S. population. This includes all anticipated dietary exposures and all other exposures for which there is reliable information. The Agency has arrived at this conclusion based on the very low levels of mammalian toxicity (no toxicity at the maximum doses tested, Toxicity Category IV) associated with the fatty acid monoesters of glycerol and propylene glycol and the long history of their consumption.

2. *Infants and children.* FFDCA section 408 provides that EPA shall apply an additional tenfold margin of exposure (safety) for infants and children in the case of threshold effects to account for prenatal and postnatal toxicity and the completeness of the data base unless EPA determines a different margin of exposure will be safe for infants and children. Margins of exposure (safety) are often referred to as uncertainty factors. The registrant used the NOAEL of 1,000 mg/kg bwt/day determined in the 90–day oral toxicity study in rats to calculate an estimated exposure of the active ingredients to the U.S. population of 0.13 mg/kg bwt/day and to non-nursing infants of 0.44 mg/kg bwt/day. The corresponding margins of exposure were calculated to be 7,690 for the U.S. population and 2,270 for non-nursing infants (Ref. 2).

In this instance, based on all the available information, the Agency concludes that the C8, C10, and C12 monoesters of glycerol and propylene glycol are virtually non-toxic to mammals, including infants and children. Further, the provisions of consumption patterns, special susceptibility, and cumulative effects do not apply. Since no toxic endpoints have been identified, any hazard is impossible to determine. As a result, EPA has not used a margin of exposure approach to assess the safety of the C8, C10, and C12 monoesters of glycerol and propylene glycol. Based on their abundance in nature and long history of use by humans without deleterious effects, there is reasonable certainty that no harm will result from aggregate exposure to the U.S. population, including infants and children, to residues of these glycerol and propylene glycol straight-chain fatty acid monoesters. This includes all anticipated dietary exposures and all other exposures for which there are reliable information. Thus, the Agency has determined that the additional margin of safety is not necessary to protect infants and children and that not adding any additional margin of safety will be safe for infants and children.

VII. Other Considerations

A. Endocrine Disruptors

EPA is required under the FFDCA, as amended by FQPA, to develop a screening program to determine whether certain substances (including all pesticide active and other ingredients) may have an effect in humans that is similar to an effect produced by a naturally-occurring estrogen, or other such endocrine effects as the Administrator may designate. Following the recommendations of its Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), EPA determined that there was scientific basis for including, as part of the program, the androgen- and thyroid hormone systems, in addition to the estrogen hormone system. EPA also adopted EDSTAC's recommendation that the Program include evaluations of potential effects in wildlife. For pesticide chemicals, EPA will use FIFRA and, to the extent that effects in wildlife may help determine whether a substance may have an effect in humans, FFDCA authority to require the wildlife evaluations. As the science develops and resources allow, screening of additional hormone systems may be added to the Endocrine Disruptor Screening Program (EDSP).

Based on the weight of the evidence of available data, no endocrine system-related effects are identified for the C8, C10, or C12 fatty acid monoesters of glycerol or propylene glycol and none is expected since they are natural components of vertebrate systems. Thus, there is no impact via endocrine-related effects on the Agency's safety finding set forth in this Final Rule for C8, C10, or C12 fatty acid monoesters of glycerol or propylene glycol.

B. Analytical Method(s)

The Agency proposes to establish an exemption from the requirement of a tolerance for the C8, C10, and C12 straight-chain fatty acid monoesters of glycerol and propylene glycol without any numerical limitation, based on their lack of mammalian toxicity. Their use will create only minuscule exposures (<1 mg/kg bwt/day) when compared to the natural levels of such compounds in living tissue and in foods (50–100 grams (g)/day), and compared to the levels permitted in food as direct additives (g/day). Based on this, the Agency has concluded that an analytical method is not required for enforcement purposes for the fatty acid monoesters of glycerol or propylene glycol.

C. Codex Maximum Residue Level

There are no CODEX values for the C8, C10, and C12 straight-chain saturated fatty acid monoesters of glycerol or propylene glycol.

D. Conclusions

Based on the toxicology data submitted and other information available to the Agency, there is reasonable certainty no harm will result to the U.S. population, including infants and children, from aggregate exposure of residues of the C8, C10, and C12 straight-chain fatty acid monoesters of glycerol or propylene glycol when the product is used in accordance with good agricultural practices and in accordance with all relevant labeling. This includes all anticipated dietary exposures and all other exposures about which there is reliable information. As a result, EPA is establishing an exemption from tolerance requirements pursuant to FFDCA 408(c) and (d) for residues of the C8, C10, and C12 straight-chain fatty acid monoesters of glycerol and

propylene glycol in or on all food commodities.

VIII. Objections and Hearing Requests

Under section 408(g) of the FFDCA, as amended by the FQPA, any person may file an objection to any aspect of this regulation and may also request a hearing on those objections. The EPA procedural regulations which govern the submission of objections and requests for hearings appear in 40 CFR part 178. Although the procedures in those regulations require some modification to reflect the amendments made to the FFDCA by the FQPA, EPA will continue to use those procedures, with appropriate adjustments, until the necessary modifications can be made. The new section 408(g) of the FFDCA provides essentially the same process for persons to "object" to a regulation for an exemption from the requirement of a tolerance issued by EPA under new section 408(d) of the FFDCA, as was provided in the old sections 408 and 409 of the FFDCA. However, the period for filing objections is now 60 days, rather than 30 days.

A. What Do I Need to Do to File an Objection or Request a Hearing?

You must file your objection or request a hearing on this regulation in accordance with the instructions provided in this unit and in 40 CFR part 178. To ensure proper receipt by EPA, you must identify docket ID number OPP–2003–0379 in the subject line on the first page of your submission. All requests must be in writing, and must be mailed or delivered to the Hearing Clerk on or before August 23, 2004.

1. *Filing the request.* Your objection must specify the specific provisions in the regulation that you object to, and the grounds for the objections (40 CFR 178.25). If a hearing is requested, the objections must include a statement of the factual issues(s) on which a hearing is requested, the requestor's contentions on such issues, and a summary of any evidence relied upon by the objector (40 CFR 178.27). Information submitted in connection with an objection or hearing request may be claimed confidential by marking any part or all of that information as CBI. Information so marked will not be disclosed except in accordance with procedures set forth in 40 CFR part 2. A copy of the information that does not contain CBI must be submitted for inclusion in the public record. Information not marked confidential may be disclosed publicly by EPA without prior notice.

Mail your written request to: Office of the Hearing Clerk (1900C), Environmental Protection Agency, 1200 Pennsylvania Ave., NW., Washington, DC 20460–0001. You may also deliver your request to the Office of the Hearing Clerk in Rm. 104, Crystal Mall #2, 1801 S. Bell St., Arlington, VA. The Office of the Hearing Clerk is open from 8 a.m. to 4 p.m., Monday through Friday, excluding legal holidays. The telephone number for the Office of the Hearing Clerk is (703) 603–0061.

2. *Tolerance fee payment.* If you file an objection or request a hearing, you must also pay the fee prescribed by 40 CFR 180.33(i) or request a waiver of that fee pursuant to 40 CFR 180.33(m). You must mail the fee to: EPA Headquarters Accounting Operations Branch, Office of Pesticide Programs, P.O. Box 360277M, Pittsburgh, PA 15251. Please identify the fee submission by labeling it "Tolerance Petition Fees."

EPA is authorized to waive any fee requirement "when in the judgement of the Administrator such a waiver or refund is equitable and not contrary to the purpose of this subsection." For additional information regarding the waiver of these fees, you may contact James Tompkins by phone at (703) 305–5697, by e-mail at *tompkins.jim@epa.gov*, or by mailing a request for information to Mr. Tompkins at Registration Division (7505C), Office of Pesticide Programs, Environmental Protection Agency, 1200 Pennsylvania Ave., NW., Washington, DC 20460–0001.

If you would like to request a waiver of the tolerance objection fees, you must mail your request for such a waiver to: James Hollins, Information Resources and Services Division (7502C), Office of Pesticide Programs, Environmental Protection Agency, 1200 Pennsylvania Ave., NW., Washington, DC 20460–0001.

3. *Copies for the Docket.* In addition to filing an objection or hearing request with the Hearing Clerk as described in Unit VIII.A., you should also send a copy of your request to the PIRIB for its inclusion in the official record that is described in Unit I.B.1. Mail your copies, identified by docket ID number OPP–2004–0379, to: Public Information and Records Integrity Branch, Information Resources and Services Division (7502C), Office of Pesticide Programs, Environmental Protection Agency, 1200 Pennsylvania Ave., NW., Washington, DC 20460–0001. In person or by courier, bring a copy to the location of the PIRIB described in Unit I.B.1. You may also send an electronic copy of your request via e-mail to: *opp-docket@epa.gov.* Please use an ASCII file format and avoid the use of special characters and any form of encryption. Copies of electronic objections and hearing requests will also be accepted on disks in WordPerfect 6.1/8.0 or ASCII file format. Do not include any CBI in your electronic copy. You may also submit an electronic copy of your request at many Federal Depository Libraries.

B. When Will the Agency Grant a Request for a Hearing?

A request for a hearing will be granted if the Administrator determines that the material submitted shows the following: There is a genuine and substantial issue of fact; there is a reasonable possibility that available evidence identified by the requestor would, if established resolve one or more of such issues in favor of the requestor, taking into account uncontested claims or facts to the contrary; and resolution of the factual issues(s) in the manner sought by the requestor would be adequate to justify the action requested (40 CFR 178.32).

IX. References

1. USEPA. Science Review in Support of the Registration of the Technical Grade Active Ingredient (TGAI) Product, VMX–42 Technology Propylene Glycol Monocaprylate, memo from Jones, Russell S., Ph.D., to Carol E. Frazer, Ph.D., April 4, 2003.

2. Dubeck, J.B., S.M. Price, and A.P. Jovanovich. Petition Proposing an Exemption from Tolerance for Pesticide Residues in or on Raw Agricultural Commodities and Processed Food. 3M, St. Paul, MN. April 13, 2001.

3. Jovanovich, A.P., Ph.D., MBA. Application for Pesticide Registration VWX–42 Technology. Request for Waivers. May 2, 2001.

4. Blanchard, Emma L., B.Sc. (Hons.). Acute oral toxicity study. MRID 45405505.

5. Coleman, David G., B.Sc. (Hons.). Acute oral toxicity study. MRID 45428501.

6. Coleman, David G., B.Sc. (Hons.). Acute dermal toxicity study. MRID 45428502.

7. Coleman, David G., B.Sc. (Hons.). Acute dermal toxicity study. MRID 45428503.

8. Paul, Graham R., B.Sc. (Hons.), M.Sc. Biol., M.I. Biol. Acute inhalation toxicity study. MRID 45405506.

9. Paul, Graham R., B.Sc. (Hons.), M.Sc. Biol., M.I. Biol. Acute inhalation toxicity study. MRID 45405507.

10. Blanchard, Emma L., B.Sc. (Hons.). Primary eye irritation study. MRID 45405508.

11. Blanchard, Emma L., B.Sc. (Hons.). Primary eye irritation study. MRID 45405509.

12. Blanchard, Emma L., B.Sc. (Hons.). Primary dermal irritation study. MRID 45405510.

13. Blanchard, Emma L., B.Sc. (Hons.). Primary dermal irritation study. MRID 45405511.

14. Coleman, David G., B.Sc. (Hons.). Dermal sensitization study. MRID 45428504.

15. Coleman, David G., B.Sc. (Hons.). Dermal Sensitization study. MRID 45448201.

16. Bottomley, Sarah M., B.Sc. (Hons.), M.Sc., C.Biol., M.I.Biol. 28–Day oral toxicity study in rats. MRID 45441101.

17. Bottomley, Sarah M., B.Sc. (Hons.), M.Sc., C.Biol., M.I.Biol. 90–Day oral toxicity study in rats. MRID 45428505.

X. Statutory and Executive Order Reviews

This final rule establishes an exemption from the tolerance requirement under section 408(d) of the FFDCA in response to a petition submitted to the Agency. The Office of Management and Budget (OMB) has exempted these types of actions from review under Executive Order 12866, entitled *Regulatory Planning and Review* (58 FR 51735, October 4, 1993). Because this rule has been exempted from review under Executive Order 12866 due to its lack of significance, this rule is not subject to Executive Order 13211, *Actions Concerning Regulations That Significantly Affect Energy Supply, Distribution, or Use* (66 FR 28355, May 22, 2001). This final rule does not contain any information collections subject to OMB approval under the Paperwork Reduction Act (PRA), 44 U.S.C. 3501 *et seq.*, or impose any enforceable duty or contain any unfunded mandate as described under Title II of the Unfunded Mandates Reform Act of 1995 (UMRA) (Public Law 104–4). Nor does it require any special considerations under Executive Order 12898, entitled *Federal Actions to Address Environmental Justice in Minority Populations and Low-Income Populations* (59 FR 7629, February 16, 1994); or OMB review or any Agency action under Executive Order 13045, entitled *Protection of Children from Environmental Health Risks and Safety Risks* (62 FR 19885, April 23, 1997). This action does not involve any technical standards that would require Agency consideration of voluntary consensus standards pursuant to section 12(d) of the National Technology Transfer and Advancement Act of 1995 (NTTAA), Public Law 104–113, section 12(d) (15 U.S.C. 272 note). Since tolerances and exemptions that are established on the basis of a petition under section 408(d) of the FFDCA, such as the exemption in this final rule, do not require the issuance of a proposed rule, the requirements of the Regulatory Flexibility Act (RFA) (5 U.S.C. 601 *et seq.*) do not apply. In addition, the Agency has determined that this action will not have a substantial direct effect on States, on the relationship between the national government and the States, or on the distribution of power and responsibilities among the various levels of government, as specified in Executive Order 13132, entitled *Federalism* (64 FR 43255, August 10, 1999). Executive Order 13132 requires EPA to develop an accountable process to ensure "meaningful and timely input by State and local officials in the development of regulatory policies that have federalism implications." "Policies that have federalism implications " is defined in the Executive Order to include regulations that have "substantial direct effects on the States, on the relationship between the national government and the States, or on the distribution of power and responsibilities among the various levels of government." This final rule directly regulates growers, food processors, food handlers and food retailers, not States. This action does not alter the relationships or distribution of power and responsibilities established by Congress in the preemption provisions of section 408(n)(4) of the FFDCA. For these same reasons, the Agency has determined that this rule does not have any "tribal implications" as described in Executive Order 13175, entitled *Consultation and Coordination with Indian Tribal Governments* (65 FR 67249, November 6, 2000). Executive Order 13175, requires EPA to develop an accountable process to ensure "meaningful and timely input by tribal officials in the development of regulatory policies that have tribal implications." "Policies that have tribal implications" is defined in the Executive Order to include regulations that have "substantial direct effects on one or more Indian tribes, on the relationship between the Federal Government and the Indian tribes, or on the distribution of power and responsibilities between the Federal Government and Indian tribes." This rule will not have substantial direct effects on tribal governments, on the relationship between the Federal Government and Indian tribes, or on the distribution of power and responsibilities between the Federal Government and Indian tribes, as specified in Executive Order 13175. Thus, Executive Order 13175 does not apply to this rule.

XI. Congressional Review Act

The Congressional Review Act, 5 U.S.C. 801 *et seq.*, as added by the Small Business Regulatory Enforcement Fairness Act of 1996, generally provides that before a rule may take effect, the agency promulgating the rule must submit a rule report, which includes a copy of the rule, to each House of the Congress and to the Comptroller General of the United States. EPA will submit a report containing this rule and other required information to the U.S. Senate, the U.S. House of Representatives, and the Comptroller General of the United States prior to publication of this final rule in the **Federal Register**. This final rule is not a "major rule " as defined by 5 U.S.C. 804(2).

List of Subjects in 40 CFR Part 180

Environmental protection, Administrative practice and procedure, Agricultural commodities, Pesticides and pests, Reporting and recordkeeping requirements.

Dated: June 10, 2004.

James Jones,

Director, Office of Pesticide Programs.

■ Therefore, 40 CFR chapter I is amended as follows:

PART 180—[AMENDED]

■ 1. The authority citation for part 180 continues to read as follows:

Authority: 21 U.S.C. 321(q), 346a and 371.

■ 2. Section 180.1250 is added to subpart D to read as follows:

§ 180.1250 C8, C10, and C12 fatty acid monoesters of glycerol and propylene glycol; exemption from the requirement of a tolerance.

The C8, C10, and C12 straight-chain fatty acid monoesters of glycerol (glycerol monocaprylate, glycerol monocaprate, and glycerol monolaurate) and propylene glycol (propylene glycol monocaprylate, propylene glycol monocaprate, and propylene glycol monolaurate) are exempt from the requirement of a tolerance in or on all food commodities when used in accordance with approved label rates and good agricultural practice.

[FR Doc. 04–14222 Filed 6–22–04; 8:45 am]

BILLING CODE 6560–50–S

§ 172.856 Propylene glycol mono- and diesters of fats and fatty acids.

Propylene glycol mono- and diesters of fats and fatty acids may be safely used in food, subject to the following prescribed conditions:

(a) They are produced from edible fats and/or fatty acids in compliance with § 172.860 and/or oleic acid derived from tall oil fatty acids in compliance with § 172.862.

(b) They are used in food in amounts not in excess of that reasonably required to produce their intended effect.

§ 172.858 Propylene glycol alginate.

The food additive propylene glycol alginate (CAS Reg. No. 9005–37–2) may be used as an emulsifier, flavoring adjuvant, formulation aid, stabilizer, surfactant, or thickener in foods in accordance with the following prescribed conditions:

(a) The additive meets the specifications of the Food Chemicals Codex, 3d Ed. (1981), p. 256, which is incorporated by reference (Copies are available from the National Academy Press, 2101 Constitution Ave. NW., Washington, DC 20418, or available for inspection at the National Archives and Records Administration (NARA). For information on the availability of this material at NARA, call 202–741–6030, or go to: *http://www.archives.gov/federal_register/code_of_federal_regulations/ibr_locations.html.*), and the additional specification that it shall have up to 85 percent of the carboxylic acid groups esterified with the remaining groups either free or neutralized.

(b) The additive is used or intended for use in the following foods as defined in § 170.3(n) of this chapter, when standards of identity established under section 401 of the act do not preclude such use:

(1) As a stabilizer in frozen dairy desserts, in fruit and water ices, and in confections and frostings at a level not to exceed 0.5 percent by weight of the finished product.

(2) As an emulsifier, flavoring adjuvant, stabilizer, or thickener in baked goods at a level not to exceed 0.5 percent by weight of the finished product.

(3) As an emulsifier, stabilizer, or thickener in cheeses at a level not to exceed 0.9 percent by weight of the finished product.

(4) As an emulsifier, stabilizer, or thickener in fats and oils at a level not to exceed 1.1 percent by weight of the finished product.

(5) As an emulsifier, stabilizer, or thickener in gelatins and puddings at a level not to exceed 0.6 percent by weight of the finished product.

(6) As a stabilizer or thickener in gravies and in sweet sauces at a level not to exceed 0.5 percent by weight of the finished product.

(7) As a stabilizer in jams and jellies at a level not to exceed 0.4 percent by weight of the finished product.

(8) As an emulsifier, stabilizer, or thickener in condiments and relishes at a level not to exceed 0.6 percent by weight of the finished product.

(9) As a flavoring adjunct or adjuvant in seasonings and flavors at a level not to exceed 1.7 percent by weight of the finished product.

(10) As an emulsifier, flavoring adjuvant, formulation aid, stabilizer or thickener, or surface active agent in other foods, where applicable, at a level not to exceed 0.3 percent by weight of the finished product.

(c) To ensure safe use of the additive, the label of the food additive container shall bear, in addition to the other information required by the act:

(1) The name of the additive, "propylene glycol alginate" or "propylene glycol ester of alginic acid".

(2) Adequate directions for use.

[47 FR 29950, July 9, 1982]

§ 172.859 Sucrose fatty acid esters.

Sucrose fatty acid esters identified in this section may be safely used in accordance with the following prescribed conditions:

(a) Sucrose fatty acid esters are the mono-, di-, and tri-esters of sucrose with fatty acids and are derived from sucrose and edible tallow or hydrogenated edible tallow or edible vegetable oils. The only solvents which may be used in the preparation of sucrose fatty acid esters are those generally recognized as safe in food or regulated for such use by an appropriate section in this part. Ethyl acetate or methyl ethyl ketone or dimethyl sulfoxide and isobutyl alcohol (2-methyl-1-

PART 175—INDIRECT FOOD ADDITIVES: ADHESIVES AND COMPONENTS OF COATINGS

Subpart A [Reserved]

Subpart B—Substances for Use Only as Components of Adhesives

Sec.
175.105 Adhesives.
175.125 Pressure-sensitive adhesives.

Subpart C—Substances for Use as Components of Coatings

175.210 Acrylate ester copolymer coating.
175.230 Hot-melt strippable food coatings.
175.250 Paraffin (synthetic).
175.260 Partial phosphoric acid esters of polyester resins.
175.270 Poly(vinyl fluoride) resins.
175.300 Resinous and polymeric coatings.
175.320 Resinous and polymeric coatings for polyolefin films.
175.350 Vinyl acetate/crotonic acid copolymer.
175.360 Vinylidene chloride copolymer coatings for nylon film.
175.365 Vinylidene chloride copolymer coatings for polycarbonate film.
175.380 Xylene-formaldehyde resins condensed with 4,4′-isopropylidenediphenol-epichlorohydrin epoxy resins.
175.390 Zinc-silicon dioxide matrix coatings.

AUTHORITY: 21 U.S.C. 321, 342, 348, 379e.

SOURCE: 42 FR 14534, Mar. 15, 1977, unless otherwise noted.

EDITORIAL NOTE: Nomenclature changes to part 175 appear at 61 FR 14482, Apr. 2, 1996, 66 FR 56035, Nov. 6, 2001, and 70 FR 72074, Dec. 1, 2005.

Subpart A [Reserved]

Subpart B—Substances for Use Only as Components of Adhesives

§ 175.105 Adhesives.

(a) Adhesives may be safely used as components of articles intended for use in packaging, transporting, or holding food in accordance with the following prescribed conditions:

(1) The adhesive is prepared from one or more of the optional substances named in paragraph (c) of this section, subject to any prescribed limitations.

(2) The adhesive is either separated from the food by a functional barrier or used subject to the following additional limitations:

(i) *In dry foods.* The quantity of adhesive that contacts packaged dry food shall not exceed the limits of good manufacturing practice.

(ii) *In fatty and aqueous foods.* (*a*) The quantity of adhesive that contacts packaged fatty and aqueous foods shall not exceed the trace amount at seams and at the edge exposure between packaging laminates that may occur within the limits of good manufacturing practice.

(*b*) Under normal conditions of use the packaging seams or laminates will remain firmly bonded without visible separation.

(b) To assure safe usage of adhesives, the label of the finished adhesive container shall bear the statement "food-packaging adhesive".

(c) Subject to any limitation prescribed in this section and in any other regulation promulgated under section 409 of the Act which prescribes safe conditions of use for substances that may be employed as constituents of adhesives, the optional substances used in the formulation of adhesives may include the following:

(1) Substances generally recognized as safe for use in food or food packaging.

(2) Substances permitted for use in adhesives by prior sanction or approval and employed under the specific conditions of use prescribed by such sanction or approval.

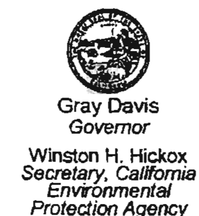
(3) Flavoring substances permitted for use in food by regulations in this part, provided that such flavoring substances are volatilized from the adhesives during the packaging fabrication process.

(4) Color additives approved for use in food.

(5) Substances permitted for use in adhesives by other regulations in this subchapter and substances named in this subparagraph: *Provided, however,* That any substance named in this paragraph and covered by a specific regulation in this subchapter, must meet any specifications in such regulation.

Substances	Limitations
Ethyl hydrogen polysiloxane.	
Ethyl phenyl polysiloxane.	
Methyl ethyl polysiloxane.	
Methyl hydrogen polysiloxane.	
Methyl phenyl polysiloxane.	
Phenyl hydrogen polysiloxane.	
Polysorbate 60.	
Polysorbate 80.	
Polysorbate 20 (polyoxyethylene (20) sorbitan monolaurate).	
Polysorbate 40 (polyoxyethylene (20) sorbitan monopalmitate).	
Poly[styrene-co-disodium maleate-co-α-(*p*-nonyl-phenyl)-*omega*-(*p*-vinyl-benzyl)poly(oxyethylene)] terpolymer.	
Polytetrafluoroethylene.	
Polyurethane resins produced by: (1) reacting diisocyanates with one or more of the polyols or polyesters named in this paragraph, or (2) reacting the chloroformate derivatives of one or more of the polyols or polyesters named in this paragraph with one or more of the polyamines named in this paragraph, or (3) reacting toluene diisocyanate or 4,4' methylenebis(cyclohexylisocyanate) (CAS Reg. No. 5124–30–1) with: (i) one or more of the polyols or polyesters named in this paragraph and with either *N*-methyldiethanolamine (CAS Reg. No. 105–59–9) and dimethyl sulfate (CAS Reg. No. 77–78–1) or dimethylolpropionic acid (CAS Reg. No. 4767–03–7) and triethylamine (CAS Reg. No. 121–44–8), or (ii) a fumaric acid-modified polypropylene glycol or fumaric acid-modified tripropylene glycol), triethylamine (CAS Reg. No. 107–15–3), and ethylenediamine (CAS Reg. No. 121–44–8), or (4) reacting *meta*-tetramethylxylene diisocyanate (CAS Reg. No. 2778–42–9) with one or more of the polyols and polyesters listed in this paragraph and with dimethylolpropionic acid (CAS Reg. No. 4767–03–7) and triethylamine (CAS Reg. No. 121–44–8), *N*-methyldiethanolamine (CAS Reg. No. 105–59–9), 2–dimethylaminoethanol (CAS Reg. No. 108–01–0), 2–dimethylamino–2–methyl–1–propanol (CAS Reg. No. 7005–47–2), and/or 2–amino–2–methyl–1–propanol (CAS Reg. No. 124–68–5).	
Polyvinyl alcohol modified so as to contain not more than 3 weight percent of comonomer units derived from 1-alkenes having 12 to 20 carbon atoms.	
Polyvinyl butyral.	
Polyvinyl formal.	
Potassium ferricyanide	For use only as polymerization-control agent.
Potassium *N*-methyldithiocarbamate.	
Potassium pentachlorophenate	For use as preservative only.
Potassium permanganate.	
Potassium persulfate.	
Potassium phosphates (mono-, di-, tribasic).	
Potassium tripolyphosphate.	
α, α', α''-1,2,3-Propanetriyltris [*omega*-(2,3-epoxypropoxy) poly (oxypropylene) (24 moles)].	
β-Propiolactone.	
Propyl alcohol (propanol).	
Propylene carbonate.	
Propylene glycol and *p*-*p*'-isopropylidenediphenol diether.	
Propylene glycol dibenzoate (CAS Reg. No. 19224–26–1)	For use as a plasticizer at levels not to exceed 20 percent by weight of the finished adhesive.
Propylene glycol esters of coconut fatty acids.	
Propylene glycol monolaurate.	
Propylene glycol monomethyl ether.	
Propylene glycol monostearate.	
α, α', α''-[Propylidynatris (methylene)] tris [*omega*-hydroxypoly (oxypropylene) (1.5 moles minimum)], minimum molecular weight 400.	
Quaternary ammonium chloride (hexadecyl, octadecyl derivative)	For use as preservative only.
Rosin (wood, gum, and tall oil rosin), rosin dimers, decarboxylated rosin (including rosin oil, disproportionated rosin, and these substances as modified by one or more of the following reactants:.	
Alkyl (C_1-C_9) phenolformaldehyde.	
Ammonia.	
Ammonium caseinate-*p*-Cyclohexylphenolformaldehyde.	
Diethylene glycol.	
Dipentaerythritol.	
Ethylene glycol.	
Formaldehyde.	
Fumaric acid.	
Glycerin.	
Hydrogen.	

Department of Pesticide Regulation



Paul E. Helliker
Director

Gray Davis
Governor

Winston H. Hickox
Secretary, California Environmental Protection Agency

MEMORANDUM

TO: Gary Sprock, Pesticide Use Specialist

FROM: Worker Health and Safety Branch

DATE: November 26, 2003

SUBJECT: PESTICIDE PRODUCT LABEL EVALUATION

PRODUCT NAME	: Acaritouch
ACTIVE INGREDIENT(S)	: (70.81%) PROPYLENEGLYCOL MONOLAURATE
I.D. NUMBER	: MC-200113-N
DOCUMENT NUMBER	: -----
EPA REGISTRATION NO.	: 70231-XX
COMPANY NAME	: Toagosei Co., Ltd.

REGISTRATION TYPE: Section 3

LABEL AMENDMENT: Yes ☐ No ☒

JUSTIFICATION/ADDITIONAL DATA REQUIRED: Yes ☐ No ☒

PROPOSED REGISTRATION ACTION: Register

U.S. EPA RED ISSUED FOR THIS A.I.: Yes ☐ No ☒

SUMMARY OF REGISTRATION REQUEST:
Toagosei Co., Ltd. is requesting a Section 3 Registration for the subject product, Acaritouch, EPA Reg. No. 70231 –XXX. It contains a new active ingredient (AI), propyleneglycol monolaurate. This proposed product is concurrently being reviewed by the California Department of Pesticide Regulation (DPR) and the U.S. Envionmental Protection Agency. The proposed product is an aqueous concentrate formulated contact acaricide/miticide for the control of tetranychid mites on vegetables (e.g., cucurbits, peppers including spice peppers, tomatoes, eggplant, tomatillo), fruit and nuts, and berries including grapes. It is only for agriculture use in the field, greenhouse, and nursery, and may be applied via ground and aerial equipment. The proposed labeling does not allow application via chemigation. The label's narration shows a restricted-entry interval (REI) of 4 hours.

The Medial Toxicology (MT) Branch reported that no acute dermal and acute inhalation toxicity data were submitted for the technical. Additionally, MT stated that the acute dermal and inhalation studies reported for the the formulated product were adequate to characterize these potential hazards of the AI (Moore, 2003b). MT determined that the AI and subject product

were Toxicity Category IV for acute oral, acute dermal, acute inhalation, eye and skin irritation, and is not a dermal sensitizer (Moore, 2003a; 2003b).

BASIS FOR PROPOSED ACTION:

Worker Heath and Safety (WHS) is recommending registration of the subject product for the following reasons:

1. The proposed label's narration is consistent with 40 CFR 156 sections 62 (Toxicity category), 64 (signal word), 66 (Child hazard warning), 68 (First aid statement), 70 (Precautionary statements for human hazards), 208 (restricted-entry statements), and 212 (PPEs); and PR Notice 2001-1 (First aid statements).
2. Based on the criteria (PR Notices 95-3, 97-3) used for classifying an AI as a low/reduced risk pesticide and the MT hazards characterization, the WHS Branch recommends that this AI, propyleneglycol monolaurate, be characterized as a low/reduced risk pesticide. Therefore, the reduced REI from 12 hr to 4 hr, as shown on the proposed product's label, adequately complies with the criteria shown in PR Notice 95-3.

The proposed subject product is consistent with the WHS guidelines for pesticide exposure and safety.

ADDITIONAL COMMENTS:

None

REFERENCES:

40 CFR (Code of Federal Regulations), Title 40. July 2002. Part 152 (Pesticide Registration and Classification Procedures) section 25 (Exemptions for pesticides of a character not requiring FIFRA regulation); Part 156 (Labeling Requirements for Pesticides and Devices), sections 62 (Toxicity Category), 64 (signal word), 66 (Child hazard warning), 68 (First aid statement), 70 (Precautionary statements for human hazards), 208 (Restricted-entry statements), and 212 (Personal protective equipment statements). Washington, D.C.: U. S. Government Printing Office, Office of the Federal Register National Archives and Records Administration.

Moore, T. 2003a. Medical Toxicology Branch. Dermal Sensitization Potential of Propyleneglycol Monolaurate. Memorandum to Joshua Johnson, Worker Health and Safety Branch, DPR. September 4, 2003. ID No. MC-200113-N. Sacramento, CA: California Department of Pesticide Regulation, California Environmental Protection Agency.

Moore, T. 2003b. Medical Toxicology Branch. Product Registration Recommendation Sheet, July 11, 2003. ID No. MC-200113-N. Sacramento, CA: California Department of Pesticide Regulation, California Environmental Protection Agency.

PR Notice 95-3 (Pesticide Registration Notice 1995-3). June 7, 1995. Reduction of Worker Protection Standard (WPS) interim restricted entry intervals (REIs) for certain low risk pesticides. Washington, D.C.: Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency.

PR Notice 2001-1 (Pesticide Registration Notice 2001-1). January 2, 2001. First Aid Statements on Pesticide Product Labels. Washington, D.C.: Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency.

Joshua L. Johnson
(Associate Environmental Research Scientist)

Joseph P. Frank, D.Sc.
(Senior Toxicologist)

State of California **Department of Pesticide Regulation**

EVALUATION REPORT – PESTICIDE

Date: November 20, 2003

Pest and Disease Protection – **Arun K. Sen**

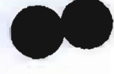
Product Name	:	AcariTouch
I.D. No.	:	MC 200113 N
Applicant	:	Toagosei Co. Ltd.
EPA Reg. No.	:	70231-
Document No.	:	52943-0007, 0008
Active Ingredient	:	Propyleneglycol Monolaurate 70.81%
Use	:	Miticide
Registration Action	:	Section 3, New Active Ingredient
Area of Review	:	Product Efficacy
Registration Specialist	:	Gary Sprock

[ ] Data/Information Support Registration

[X] Data/Information Support Conditional Registration

[ ] Data/Information Do Not Support Registration

[ ] No Registration Action Required



Summary:

Toagosei Co., Ltd requests California concurrent registration of the proposed new active ingredient for the control and suppression of tetranychid mites and other phytophagous mites on various cucurbits, pome fruits, stone fruits, grapes, nut crops, small fruits and berries, and fruiting vegetables. It is to be used as spray application only, and is prohibited for use through any type of irrigation system. AcariTouch has a non-toxic mode of action, blocking the spiracles and de-waxing the cuticles causing death by suffocation and desiccation. It can be used alone, tank-mixed with or in rotation with other miticides.

AcariTouch has been identified to be compatible with most insecticides and fertilizers. Two applications 7 days apart should be made to suppress and control mites present on the plants and those newly emerged. AcariTouch should be used as 12 to 25 ounces in 100 gallons of water and thorough coverage is essential.

The registrant has submitted efficacy data to support this registration. The tests were set up in a randomized complete block design with four to six replicates of two trees each. Different rates of the product were compared with untreated control and grower standards. Pre-treatment counts were taken, followed by weekly evaluation and mite counts (leaf samples were taken from the field to the lab). Adults, immature (larva, protonymph, and deutonymph), eggs and predator mite counts were taken from each replicated trial. Counts were taken from day 1 through day 21. Percent control from the AcariTouch plots were 79% by day 3 compared to 0 for untreated control plots and 51% for grower standard plots. Raw data was analyzed using LSD, CV and Duncan's new MRT (p = 0.5).

The mite numbers gradually decreased in all sprayed plots over the first seven days of the test. Two applications of AcariTouch at the rate of 25 fl.oz./100 gallon provided the best control. The lower dose of AcariTouch also provided significant control reducing the mite population in the single application by day 7. The lower rate provided 40% mite control. Submitted data indicates adequate control of the mite population on all the crops where the product was tested.

No data for fruiting vegetables has been submitted.

Conclusion:

A conditional registration for year is recommended for the registrant to develop efficacy studies on fruiting vegetables. The label recommended rates and application methods should be used when conducting the trials. Trials should be conducted in California.

Arun K. Sen, Ph. D.
Senior Environmental Research Scientist

State of California
Department of Pesticide Regulation

EVALUATION REPORT - PESTICIDE

Date: September 24, 2003

Fish and Wildlife – Jon Shelgren

Product Name	: **ACARITOUCH®**
I.D. No.	: MC 200113-N
Applicant	: TOAGOSEI CO., LTD.
EPA Reg. No.	: 70231-
Document No.	: 52943-0003 to –oo06
Active Ingredient	: Propyleneglycol monolaurate
Use	: Miticide
Registration Action	: Section 3 Registration
Area of Review	: Fish and Wildlife Hazard

Registration Specialist : Gary Sprock

[X] Data/Information Support Registration

[ ] Data/Information Support Conditional Registration

[ ] Data/Information Do Not Support Registration

[ ] No Registration Action Required

Summary: TOAGOSEI CO., LTD. requests California registration for their product **ACARITOUCH®**, containing the new active ingredient Propyleneglycol monolaurate, for use on fruits, vegetables and ornamental plants to control tetranychid mites and other mites.

The first study evaluated is entitled "Acute Toxicity of Propylene Glycol Monolaurate [RIKEMAL PL-100] to an Aquatic Invertebrate [*Daphnia magna*]" This study was conducted by The Japan Clinical Laboratories, Inc. Bioassay Division of Hyogo-ken, Japan according to Good Laboratory Practice Standards.
Twenty juvenile daphnids per test concentration (5 per replicate) were exposed to mean measured test concentrations of 0.18, 0.28, 0.39, 0.64, 0.92 and 1.55 mg/l for 48 hours under semi-static conditions. A dilution water control and an adjuvant control were also utilized.
No mortalities or immobilizations occurred in the 0.40 mg/l test concentration group and no sublethal effects were reported. However, at 48 hours, 100% immobilization occurred in the highest test concentration group.
The final 48 hour EC_{50} value for *Daphnia magna* was determined to be 0.52 mg a.i./l. and the NOEC was 0.40 mg a.i./L.

The second study evaluated is entitled "Acute Toxicity of Propylene Glycol Monolaurate [RIKEMAL PL-100] to Juvenile Carp [*Cyprinus carpio*]." This study was conducted by The Japan Clinical Laboratories, Inc. Bioassay Division of Hyogo-ken, Japan according to Good Laboratory Practice Standards.
Seventy juvenile carp (*Cyprinus carpio*), ten per test concentration, were exposed to nominal test concentrations of 0 (control), 2.38, 4.29, 7.72, 13.89 and 25 mg/mL for 96 hours under semi-static conditions. A dilution water control and an adjuvant control were also utilized.
First mortalities (8) occurred at 24 hours in the 13.89 mg/mL test concentration and total

mortality occurred in the 25 mg/mL test concentration at 24 hours. No sublethal effects (other than one lethargic fish in the 13.89 mg/mL test group) were reported.
The final 96 hour LC_{50} value was determined to be 5.20 mg/L while the NOEC was 3.80 mg/L.

The third study evaluated is entitled "Acute Oral Toxicity of ACARITOUCH [RM-131A] to Honeybees [A*pis mellifera L.*]." This study was conducted by Tamagawa University Honeybee Science Research Center of Tokyo, Japan according to Good Laboratory Practice Standards. Twenty worker honeybees per replicate (3) were allowed to feed on sucrose solutions containing 1/1000, 1/500 and 1/100 RM 131A of the total volume for four hours when all solutions were completely consumed. At eight hours, no behavioral abnormalities or mortalites due to acute toxicity were observed. Control group mortality paralleled treatment groups over time (20 days). No acute toxicity value was provided.

The fourth study evaluated is entitled "Acute Contact toxicity of ACARITOUCH [RM 131a] to Honeybees [A*pis mellifera L.*]." This study was conducted by Tamagawa University Honeybee Science Research Center of Tokyo, Japan according to Good Laboratory Practice Standards. Twenty worker honeybees per replicate (3) were sprayed by a chromatography sprayer with solutions containing 1000X, 500X and 100X RM 131A and observed at 3, 6, 24 and 48 hours after initiation of the test.
After 48 hours, the bee mortality was reported to be 88% in the 100X group, 57% in the 500X group and 8% in the 100X group.
No 48 hour LD_{50} value was provided.

NOTE: Waivers for the Avian acute toxicity study and the Avian dietary toxicity study are hereby granted

ENVIRONMENTAL FATE CONSIDERATIONS:
No Environmental fate studies were submitted.

John H. Shelgren
Senior Environmental Research Scientist

State of California

Memorandum

To : Joshua Johnson, Assoc. Environmental Research Scientist
Worker Health & Safety Branch

Date : 9/4/03

Place : Sacramento

From : **Department of Pesticide Regulation** - Thomas Moore, Staff Toxicologist
Medical Toxicology Branch

Subject : Dermal Sensitization Potential of Propyleneglycol Monolaurate

Company: Toagosei Co. Ltd.
ID#: MC 200113 N
Active Ingredient(s): Propyleneglycol Monolaurate
Chemical Code #: 5856
SB 950 #: New A.I.
EPA #: 70231-

The registrant did not submit a dermal sensitization study for the technical grade active ingredient. However, a dermal sensitization study was submitted for the formulated product which contains 70% of the active ingredient. The study results indicated that the product is not a dermal sensitizer. It is the decision of the Medical Toxicology Branch that the active ingredient alone should not be considered a dermal senitizer as well.

D52943>M200113

Leung

Summary and WHS Tracking Information for Registration Data Package # 200113

Active Ingredient: PROPYLENEGLYCOL MONOLAURATE

Product: Acariitouch

Applicant: TOAGOSEI CO., LTD.

Registration Type: New Active Ingredients

Decision: Other **Primary Reviewer:** Josh Johnson

Date Received: 07/30/03 **Date Out:** **Length of stay in WHS:** 7 days (to date)

Comments: Please reroute to Med Tox for review of FR 66(234) for the sensitization caterization of the AI (i.e., the AI is a sensitizer or is not a sensitizer in the animal model) . WH&S need this info in memo form to determine the REI (40 CFR 156.212; PR Notice 95-3) of the subject product and to determine if the AI can be classified as a low/reduced risk pesticide (PR Notices 97-3 & 95-3).

Internal Package Tracking

Staff Name	Role	Date In	DateOut	Duration	Comments
Josh Johnson	Primary Reviewer	07/30/03	08/06/03	7 days	WH&S needs Med Tox memo indicating the AI's sensitization potential.

Please reroute to Med Tox — thanks per Josh

TO: Gary Sprock, Senior Pesticide Use Specialist
Pesticide Registration Branch

FROM: Medical Toxicology Branch

Date: 7/11/03

PRODUCT REGISTRATION RECOMMENDATION SHEET

Formulated Product Name: Acaritouch
Chemical Code #: 5856
EPA Reg. #: 70231-
Document #: 52943-0002, -0005
Company Name: Toagosei Co., Ltd.

ID #: MC 200113 N
SB 950 #: New A.I.

RECOMMENDATION:

Submitted for a Section 3 registration as a biochemical miticide.

The data reviewed are adequate for a complete toxicological evaluation.

The product label adequately identifies the acute toxicity hazards indicated by the data reviewed.

Registration is recommended.

Thomas Moore, Ph.D.
Staff Toxicologist

7-16-03
Date

Peter Leung, Ph.D.
Senior Toxicologist

7/16/03
Date

Joyce Gee, Ph.D.
Senior Toxicologist

7/16/03
Date

TO--File: Registration
Branch: Registration
FROM--Medical Toxicology

Senior Pesticide Use Specialist: Gary Sprock

DATA PACKAGE SUMMARY AND RECOMMENDATION SHEET

Active Ingredient: Propyleneglycol Monolaurate
Formulated Product Name: Acaritouch
Formulation: A.I. 70.81%, inert ingredients 29.19%
Chemical Code #: 5856
EPA Reg. #: 70231-
Document #: 52943-0002, -0005
Company Name: Toagosei Co., Ltd.

ID #: MC 200113 N
SB 950 #: New A.I.

SUMMARY ("One-liners" from each study worksheet, significant information not mentioned in worksheets, other pertinent information for ongoing review or registration. Attach additional sheets if needed):

The registrant has submitted the subject product for a Section registration as a new biochemical active ingredient for use on fruits, vegetables and ornamental plants.

Propyleneglycol Monolaurate (Rikemal PL-100) **Acute Toxicity Categories**

Acute Oral LD50	IV
Acute Dermal LD50	Study not submitted
Acute Inhalation LC50	Study not submitted
Eye Irritation	IV
Dermal Irritation	IV
Dermal Sensitization	Study not submitted.

Propyleneglycol Monolaurate (Rikemal PL-100) **Acute Toxicity Studies**

Acute Oral LD50
52943-0002; 204242; "Acute Oral Toxicity of Propylene Glycol Monolaurate [Rikemal PL-100] in Mice"; (M. Shirai; Nippon Experimental Medical Research Institute Co., Ltd., Agatsuma-gun, Gunma-ken, Japan; Study No. H-98190; 7/7/98); Five Crj:CD-1(ICR) mice/sex/group were dosed orally by gavage with 0 (water) or 40000 mg/kg of Propyleneglycol Monolaurate (Rikemal PL-100) (lot no. W120901, purity not reported). No deaths resulted from the treatment. Clinical signs for the treated animals included watery diarrhea and soiled perineal area. Treated animals exhibited a loss of body weight through day 3 and reduced body weight gain on day 7. No treatment-related lesions were noted in the necropsy examination. LD50 (M/F) > 40000 mg/kg; Toxicity Category IV; **Study acceptable.** (Moore, 7/9/03)

Acute Dermal LD50
Study not submitted.

Acute Inhalation LC50
Study not submitted.

Eye Irritation
52943-0005; 204323; "Acute Eye Irritation Study of Propylene Glycol Monolaurate [Rikemal PL-100] in Rabbits"; (J. Kuhn; Stillmeadow, Inc., Sugar Land, TX; Study No. 6660-1; 3/5/02); The eyes of 6 New Zealand White rabbits were treated by ocular instillation with 0.1 ml/eye of Propyleneglycol Monolaurate (Rikemal PL-100) (lot no. A041701, purity: 98.9%). Neither corneal opacity nor iritis were evident during the 3 day observation period. No conjunctival redness,

chemosis nor discharge were noted at 24 hours post-dose. Toxicity Category IV; **Study acceptable.** (Moore, 7/9/03)

Dermal Irritation

52943-0005; 204324; "Acute Dermal Irritation Study of Propylene Glycol Monolaurate [Rikemal PL-100] in Rabbits"; (J.O. Kuhn; Stillmeadow, Inc., Sugar Land, TX; Study No. 6661-01; 3/7/02); The skin of 6 New Zealand White rabbits was treated with 0.5 ml/site, one site/animal, of Propyleneglycol Monolaurate (Rikemal PL-100) (lot no. A041701, purity: 98.9%) for 4 hours under an occlusive wrap. Erythema, grade 1 (4/6), was evident at 1 and 24 hours post-exposure, persisting with grade 1 (3/6) at 48 and 72 hours, grade 1 (1/6) at 7 days, clearing by 10 days. No edema was noted throughout the observation period. Toxicity Category IV; **Study acceptable.** (Moore, 7/10/03)

Dermal Sensitization

Study not submitted.

Acaritouch (RM-131A Emulsion) **Acute Toxicity Categories**

Acute Oral LD50	IV
Acute Dermal LD50	IV
Acute Inhalation LC50	IV
Eye Irritation	IV
Dermal Irritation	IV
Dermal Sensitization	Not a sensitizer

Acaritouch (RM-131A Emulsion) **Acute Toxicity Studies**

Acute Oral LD50

52943-0005; 204325; "Acute Oral Toxicity Study of Acaritouch [RM-131A Emulsion] in Mice"; (M. Shirai; Nippon Experimental Medical Research Institute Co., Ltd., Agatsuma-gun, Gunma-ken, Japan; Study No. H-98583; 12/22/98); Five Crj:CD-1(ICR) mice/sex/group were dosed orally by gavage with 0 (water) or 5000 mg/kg of Acaritouch (RM-131A Emulsion) (lot no. 980629, a.i.: 70%). No deaths resulted from the treatment. Clinical signs for the treated animals included watery diarrhea and soiled perineal area. No treatment-related lesions were noted in the necropsy examination. LD50 (M/F) > 5000 mg/kg; Toxicity Category IV; **Study acceptable.** (Moore, 7/10/03)

Acute Dermal LD50

52943-0005; 204326; "Acute Dermal Toxicity Study of Acaritouch in Rabbits"; (J.O. Kuhn; Stillmeadow, Inc., Sugar Land, TX; Study No. 6662-01; 3/7/02); The skin of 5 rabbits/sex was treated with 5050 mg/kg of Acaritouch (lot no. 011012, a.i.: 71.87%) for 24 hours under an occlusive wrap. No deaths resulted from the treatment. Very slight to well-defined erythema and very slight edema, atonia and desquamation were noted at the site of application up through day 4. Otherwise, no treatment-related effects were evident. In the necropsy examination, discolored lungs and/or kidneys were noted for 3 males and 3 females. LD50 (M/F) > 5050 mg/kg; Toxicity Category IV; **Study acceptable.** (Moore, 7/10/03)

Acute Inhalation LC50

52943-0005; 204327; "Acute Inhalation Toxicity Study of Acaritouch in Rats"; (L. Carter; Stillmeadow, Inc., Sugar Land, TX; Study No. 6663-01; 3/7/02); Five Sprague-Dawley rats/sex were exposed nose-only to 2.23 mg/l (analytical) of Acaritouch (lot no. 011012, a.i.: 71.87%) for 4 hours. The mean MMAD (GSD) was 2.4 (3.9) um. No deaths resulted from exposure. Clinical signs included decreased activity and a respiratory chirp. All signs had been resolved by day 5. Mottled lungs were noted for one male and two females in the necropsy examination. LC50 (M/F) > 2.23 mg/l, Toxicity Category IV; **Study acceptable.** (Moore, 7/10/03)

Eye Irritation

52943-0005; 204328; "Primary Eye Irritation Study of Acaritouch [RM-131A Emulsion] in Rabbits"; (K. Suzuki; Nippon Experimental Research Institute Co., Ltd., Agatsuma-gun, Gunma-ken, Japan; Study No. H-98416; 11/27, 98); The eyes of 9 Japanese White rabbits were treated by ocular instillation with 0.1 ml/eye of Acaritouch [RM-131A Emulsion] (lot no. 980629, a.i.: 70%). The eyes of 3 animals were washed with physiological saline 3 minutes after dosing. For both groups, no signs of irritation were evident over the 72 hour observation period. Toxicity Category IV; **Study acceptable.** (Moore, 7/11/03)

Dermal Irritation

52943-0005; 204329; "Primary Skin Irritation Study of Acaritouch [RM-131A Emulsion] in Rabbits"; (K. Suzuki; Nippon Experimental Research Institute Co., Ltd., Agatsuma-gun, Gunma-ken, Japan; Study No. H-98415; 11/27/98); The skin of 6 Japanese White rabbits was treated with 0.5 ml/site, one site/animal, of Acaritouch [RM-131A Emulsion] (lot no. 980629, a.i.: 70%) for 4 hours under an occlusive wrap. Erythema, grade 1 (5/6), was evident at 1 and 24 hours post-exposure, grade 1 (4/6) at 48 hours, grade 1 (3/6) at 72 hours, grade 1 (1/6) at 4 days and clearing in the last animal on day 5. Edema, grades 2 (1/6) and 1 (1/6), was noted at 1 hour post-exposure, clearing by 24 hours. Toxicity Category IV; **Study acceptable.** (Moore, 7/11/03)

Dermal Sensitization

52943-0005; 204330; "Skin Sensitization Study of Acaritouch [RM-131A Emulsion] in Guinea Pigs"; (K. Suzuki; Nippon Experimental Research Institute Co., Ltd., Agatsuma-gun, Gunma-ken, Japan; Study No. H-98417; 11/27/98); The skin of 20 female Hartley guinea pigs was treated with 0.2 ml/site, one site/animal, of Acaritouch [RM-131A Emulsion] (lot no. 980629, a.i.: 70%) for 6 hours, once per week for 3 weeks in the induction phase. After a two week interlude, these animals were challenged with 0.2 ml/site of a 50% preparation of the test material in injectable water for 6 hours. A control group of 10 animals was treated with the diluted test material in the same manner. No dermal irritation was evident for the challenged or the control groups at 24 or 48 hours post-exposure. The positive control was functional. The test material is not a dermal sensitizer in accordance with the Buehler assay. **Study acceptable.** (Moore, 7/11/03)

CONCLUSIONS: Are data adequate to support registration?

The Propyleneglycol Monolaurate (Rikemal PL-100) acute oral toxicity and primary eye and dermal irritation studies are acceptable. The acute dermal and inhalation toxicity studies were not submitted on the technical grade material. However, the acute dermal and inhalation toxicity studies on the formulated product (70% a.i.) are considered to adequately characterize the potential dermal and inhalation toxicity hazards of the active ingredient.

The Acaritouch (RM-131A Emulsion) acute oral, dermal and inhalation toxicity, primary eye and dermal irritaition and dermal sensitization studies are acceptable.

Propyleneglycol monolaurate is a fatty acid ester of propylene glycol. Hydrolysis of the molecule results in the fatty acid moiety and propylene glycol. Laurate is ubiquitous in living organisms. Propylene glycol is oxidized in the liver to form lactic acid. The active ingredient is a food additive generally recognized as safe by the FDA. The World Health Organization has established an Acceptable Daily Intake for propyleneglycol monolaurate. Fatty esters of propylene glycol have been used as emulsifiers and stabilizers in foods and in pharmaceutical formulations. With this understanding, additional toxicity studies are not required at this time.

RECOMMENDATIONS: What type of registration action is being requested? In case of ongoing registration, register or do not register? What other specific studies or data are requested?

Submitted for a Section 3 registration as a biochemical miticide.

The data reviewed are adequate for a complete toxicological evaluation.

The product label adequately identifies the acute toxicity hazards indicated by the data reviewed.

Registration is recommended.

Thomas Moore, Ph.D. — 7-16-03 Date
Staff Toxicologist

Peter Leung, Ph.D. — 7/16/03 Date
Senior Toxicologist

Joyce Gee, Ph.D. — 7/16/03 Date
Senior Toxicologist

State of California
Department of Pesticide Regulation

EVALUATION REPORT - PESTICIDE Date: July 8, 2003

Plant Physiology - John Heaton

Product Name	: Acaritouch
I.D. No.	: 200113-N
Applicant	: Toagosei Co., Ltd.
EPA Reg. No.	: 70231-
Document No.	: 52943-007, 008
Active Ingredient	: Propyleneglycol Monolaurate (70.81%)
Use	: Miticide
Registration Action	: Section 3 – New Product Registration
Area of Review	: Phytotoxicity

Registration Specialist : G. Sprock

[ ] Data/Information Support Registration
[√] Data/Information Support Conditional Registration
[ ] Data/Information Do Not Support Registration
[ ] No Registration Action Required

Summary:
Toagosei Co., Ltd. is requesting California Section 3 registration for a new product called Acaritouch. This product is formulated as an aqueous concentrate containing 70.81% propyleneglycol monolaurate (PGML) a commonly used emulsifier in bakery and ice cream products as well as in hand creams. PGML has a fatty acid ester 12 carbons in length attached to the propylene glycol molecule. Acaritouch is intended for use in an integrated pest program (IPM) to control mites. The proposed label provides instructions for applications to cucurbits, pome fruits, stone fruits, grapes, nut crops, small fruits and berries, and fruiting vegetables. Application rates recommended on the label are 12 to 25 ounces of formulated product (0.53 to 1.10 lbs. a.i.) per 100 gallons of water.

Documents 52943-007 and 52943-008 contain results from seventeen efficacy trials conducted at various locations in California. Applications of 12 and 27 oz per 100 gallons of water were made to species listed on the proposed label including, apples, pears, cantaloupes, grapes, nectarines, peaches, almonds, walnuts and strawberries. No phytotoxicity was observed as a result of any of the treatments with Acaritouch.

Conclusion:
Toagosei Co., Ltd provided observations for phytotoxicity on at least one species in each of the crop groups listed on their label, except for the group fruiting vegetables. The lack of phytotoxicity on the any of the species tested when the maximum rates of Acaritouch were used, is adequate to support a conditional registration. Data must be provided to support the maximum recommended rates on fruiting vegetables. Trials should include tomatoes, peppers and two types of beans. If any plant types are found to be sensitive to Acaritouch they should be listed on the label as sensitive plants. A conditional registration can be granted for 18 months, during which time the registrant must submit the requested phytotoxicity data.

John Heaton
Associate Environmental Research Scientist

Don E. Koehler
Senior Environmental Research Scientist

TO: Gary Sprock, Senior Pesticide Use Specialist
Pesticide Registration Branch

FROM: Medical Toxicology Branch

Date: 7/9/03

PRODUCT REGISTRATION RECOMMENDATION SHEET

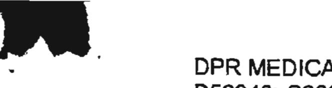
Formulated Product Name: Propyleneglycol Monolaurate (Rikemal PL-100)
Chemical Code #: 5856
ID #: 200112 E
EPA Reg. #: 70231-
SB 950 #: NA
Document #: 52943-0002
Company Name: Riken Vitamin Co. Ltd

RECOMMENDATION:

Submitted as additional data.

Staff Toxicologist

7-9-03
Date



CONCLUSIONS: Are data adequate to support registration?

The acute oral toxicity study is acceptable.

RECOMMENDATIONS: What type of registration action is being requested? In case of ongoing registration, register or do not register? What other specific studies or data are requested?

Submitted as additional data.

Staff Toxicologist

7-5-03
Date

DOCUMENTATION IN REGARDS TO ITEM B10

MATERIAL SAFETY DATA SHEET

1. PRODUCT AND COMPANY INFOMATION

Product Name : RIKEMAL PL-100
General Use : nonionic surfactant
Product Description : nonionic surfactant
MSDS Number : OUSA-151

APR 24 2009

MANUFACTURER
Company Name : RIKEN VITAMIN CO., LTD.
Address : 2-9-18, Misaki-cho, Chiyoda-ku, Tokyo, Japan
Telephone No. : +81-3-5275-5130 (Sales Department)

EMERGENCY TELEPHONE NUMBER :
+81-3-5275-5130 (Sales Department)

2. COMPOSITION / INFORMATION ON INGREDIENTS

Component	Wt %	CAS Registry #
Propylene glycol monolaurate	100	27194-74-7

OSHA HAZARDOUS INGREDIENTS(29 CFR1910. 1200):
Propylene glycol monolaurate is not hazardous ingredients.

3. HAZARDS IDENTIFICATION

EMERGENCY OVERVIEW :
Appearance : Light yellow liquid under 50-70℃ with a slightly characteristic odor.
A build-up of hazardous electrostatic charges could cause a flash fire or explosion when contents are emptied into a flammable atmosphere.
Environmental : This product is of no toxicity.
Health : Temporary irritation to mucous membranes may result from excessive dust.
Disposal : Sweep or shovel spilled material and place into a sealable container.
Dispose in accordance with local, state and federal regulations.
Incineration is recommended.

POTENTIAL HEALTH EFFECTS:

Eye : This is not expected to cause eye irritation.

Skin : This product is not expected to cause skin irritation or allergic reactions.

Swallowing : Small amounts, if swallowed, are not expected to cause injury.

Inhalation : This product is not expected to cause respiratory irritation.

POTENTIAL ENVIRONMENTAL EFFECTS:

4. FIRST AID MEASURES

First Aid for eye : Following eye contact, flash eyes with plenty of water for several minutes. Get medical attention if irritation occurs.

First Aid for skin : Following skin contact, wipe away excess material with a dry towel. Then wash affected areas with plenty of water and soap, if available, for several minutes. Get medical attention if irritation occurs.

First Aid for inhalation : If inhaled, remove from area to fresh air. Get medical attention if irritation develops, or if breathing becomes difficult.

First Aid for swallowing : If swallowed, give at least 3-4 glasses of water, but do not Induce vomiting.
Do not give anything by mouth to an unconscious person.

PROTECTION TO FIRST-AIDERS:

5. FIRE FIGHTING MEASURES

Flash Point : : 352 ° F (178℃)

Flash Point Method Used : Cleveland Open Cup Method

Extinguishing Media : Carbon dioxide, dry chemical, foam, sand, water mist.

Fire Fighting Instructions : Use self-contained breathing apparatus.

6. ACCIDENTAL RELEASE MEASURES

LAND SPILL : Sweep or vacuum and place in sealable container for disposal.

WATER SPILL: Flash residue with water. Dispose of in accordance with local regulations, preferably by incineration.

7. HANDLING AND STORAGE

Handling : Avoid continuous or repetitive breathing of dust. Use only with adequate ventilation. Wash after handling and before eating, drinking.

Storage : Store in well-closed containers and keep at ambient temperature. Keep away from heat and flame.

Shelf life : 9 months after date of manufacture.

8. EXPOSURE CONTROLS / PERSONAL PROTECTION

Engineering Controls : Work in ventilated areas.

General Protection : Handle in accordance with good occupation hygiene and safety practices.

Eye / Face Protection : Wear safety glasses if eye contact is possible.

Skin Protection : Wear impervious gloves as a standard handling procedure.

9. PHYSICAL AND CHEMICAL PROPERTIES

Appearance : Light yellow liquid under 50-70℃.

Odor : A slightly characteristic odor

Boiling Point : Not Applicable

Evaporation Rate: Not Applicable

Flash Point : 352 ° F (178℃)

Melting Point : Not Applicable

Specific Gravity : 0.876 at 80℃ (H_2O=1)

pH : 5.9 (1%)

Solubility in water : Practically insoluble in water.

10. STABILITY AND REACTIVITY

CONDITION TO AVOID : Stable under normal conditions.

STABILITY : Stable

MATERIAL TO AVOID : Strong oxidizing agents, strong acids, strong bases.

HAZARDOUS REACTIONS / DECOMPOSITION PRODUCTS :

Thermal decomposition and burning may produce carbon dioxide, carbon monoxide, and low molecular weight organic species.

11. TOXICOLOGICAL INFORMATION

No toxicological data are available for this product.

In the body food fasts undergo digestion and they are as a first step broken down to Propylene glycol monolaurate.

Acute Toxicity:
(Oral,Dermal,Inhalation)
Eye Irritation: This is not expected to cause eye irritation
Skin Irritation: This product is not expected to cause skin irritation or allergic reactions.
Sensitization:
Mutagenicity:

12. ECOLOGICAL INFORMATION

No ecological data are available for this product.

ECOTOXICITY: No data available
MOBILITY: No data available
PERSISTENCE AND DEGRADABILITY: No data available
BIOACCUMULATIVE POTENTIAL: No data available

13. DISPOSAL CONSIDERATIONS

Disposal considerations : Incinerate is a chemical incinerator equipped with an after-burner and scrubber. Follow all federal, state and local regulations.

14. TRANSPORT INFORMATION

This product is not regulated by any means of transport.

LAND TRANSPORT
ADR,RID
Class:
Packing Group(PG):
UN Number:
Proper Shipping Name:

SEA TRANSPORT
IMDG
Class:
Packing Group(PG):
UN Number:

Proper Shipping Name:
Marine Pollutant:

AIR TRANSPORT
ICAO/IATA:
Class:
Packing Group(PG):
UN Number:
Proper Shipping Name:

15. REGULATORY INFORMATION

Label information : Not classified as dangerous.

16. OTHER INFORMATION

This information is furnished without warranty, express or implied, except that it is accurate to the best knowledge of RIKEN VITAMIN CO.,LTD.

It relates only to the specific material designated herein, and does not relate to use in combination with any other material or process. RIKEN VITAMIN CO.,LTD. assumes no legal responsibility for use or reliance upon this information.

Material Safety Data Sheet

12601 Twinbrook Parkway,
Rockville, MD 20852 USA

Phone Calls: 301-816-8129
8 a.m. to 5 p.m. EST Mon. - Fri.

ATTENTION !

USP Reference Standards are sold for chemical test and assay purposes only, and NOT for human consumption. The information contained herein is applicable solely to the chemical substance when used as a USP Reference Standard and does not necessarily relate to any other use of the substance described, (i.e. at different concentrations, in drug dosage forms, or in bulk quantities). USP Reference Standards are intended for use by persons having technical skill and at their own discretion and risk. This information has been developed by USP staff from sources considered reliable but has not been independently verified by the USP. Therefore, the USP Convention cannot guarantee the accuracy of the information in these sources nor should the statements contained herein be considered an official expression. NO REPRESENTATION OR WARRANTY, EXPRESS OR IMPLIED, INCLUDING THE WARRANTIES OF MERCHANTABILITY AND FITNESS FOR A PARTICULAR PURPOSE is made with respect to the information contained herein.

PROPYLENE GLYCOL MONOLAURATE TYPE II

Catalog Number: 1576796 **Revision Date:** December 19, 2007

SECTION 1 - PRODUCT AND COMPANY IDENTIFICATION

Common Name: Propylene Glycol Monolaurate Type II

Manufacturer: U. S. Pharmacopeia

Responsible Party: Reference Standards Technical Services

Mailing Address: 12601 Twinbrook Parkway, Rockville, MD 20852 USA

Phone: 301-816-8129

Hours: 8 a.m. to 5 p.m. EST Mon. - Fri.

Product Use: USP Reference Standards and Authentic Substances are used for chemical tests and assays in analytical, clinical, pharmaceutical, and research laboratories.

SECTION 2 - HAZARD INFORMATION

EMERGENCY OVERVIEW - Irritant.

Adverse Effects: Possible allergic reaction to material if inhaled, ingested or in contact with skin.

Overdose Effects: n/f

Acute: Eye, skin, gastrointestinal and/or respiratory tract irritation.

Chronic: Possible hypersensitization.

Medical Conditions Aggravated by Exposure: Hypersensitivity to material.

Cross Sensitivity: n/f

Target Organs: n/f

For additional information on toxicity, see Section 11.

SECTION 3 - COMPOSITION/INFORMATION ON INGREDIENTS

Common Name: Propylene Glycol Monolaurate Type II

Formula: C12H24O2 . xC3H8)2

Synonym: n/f

Chemical Name: Propylene glycol mono and diesters of lauric acid

CAS: 37321-62-3

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n/f = not found

RTECS Number: JR3575000

Chemical Family: Propylene glycol with fatty acid esters

Therapeutic Category: Pharmaceutical excipient, solubilizing agent

Composition: Pure Material

SECTION 4 - FIRST AID MEASURES

Inhalation: May cause irritation. Remove to fresh air.

Eye: Causes irritation. Avoid contact. Flush with copious quantities of water for at least 15 minutes.

Skin: Causes irritation. Avoid contact. Flush with copious quantities of soap and water.

Ingestion: May cause irritation. Flush out mouth with water.

General First Aid Procedures: Remove from exposure. Remove contaminated clothing. Persons developing serious hypersensitivity (anaphylactic) reactions must receive immediate medical attention. If person is not breathing give artificial respiration. If breathing is difficult give oxygen. Obtain medical attention.

Note to Physicians

Overdose Treatment: For current information about the treatment of overdose, consult a certified Regional Poison Control Center by calling the number listed in your local telephone directory.

SECTION 5 - FIREFIGHTING MEASURES

Extinguisher Media: Water spray, dry chemical, carbon dioxide or foam as appropriate for surrounding fire and materials.

Fire and Explosion Hazards: This material is assumed to be combustible.

Firefighting Procedures: As with all fires, evacuate personnel to a safe area. Firefighters should use self-contained breathing equipment and protective clothing.

SECTION 6 - ACCIDENTAL RELEASE MEASURES

Spill Response: Wear approved respiratory protection, chemically compatible gloves and protective clothing. Wipe up spillage or collect spillage using a high efficiency vacuum cleaner. Avoid breathing vapor. Place spillage in appropriately-labelled container for disposal. Wash spill site.

SECTION 7 - HANDLING AND STORAGE

Handling: As a general rule, when handling USP Reference Standards avoid all contact and inhalation of dust, mists, and/or vapors associated with the material. Wash thoroughly after handling.

Storage: Store in tight container as defined in the USP-NF. This material should be handled and stored per label instructions to ensure product integrity.

SECTION 8 - EXPOSURE CONTROL / PERSONAL PROTECTION

Engineering Controls: Engineering controls such as exhaust ventilation are recommended.

Respiratory Protection: Use a NIOSH approved respirator, if it is determined to be necessary by an industrial hygiene survey involving air monitoring.

Gloves: Chemically compatible

Eye Protection: Safety glasses or goggles

Protective Clothing: Protect exposed skin.

Exposure Limits: n/f

SECTION 9 - PHYSICAL AND CHEMICAL PROPERTIES

Properties as indicated on the MSDS are general and not necessarily specific to the USP Reference Standard Lot provided.

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n/f = not found

Appearance and Odor: Oily liquid; faint odor.

Odor Threshold: n/f

pH: n/f

Melting Range: n/f

Boiling Point: > 150° C

Flash Point: > 150° C

Autoignition Temperature: n/f

Evaporation Rate: n/f

Upper Flammability Limit: n/f

Lower Flammability Limit: n/f

Vapor Pressure: n/f

Vapor Density: n/f

Specific Gravity: 0.9165 - 0.9260 at 20° C

Solubility in Water: Insoluble

Fat Solubility: n/f

Other Solubility: Very soluble in ethanol, in chloroform, in methylene chloride, and in n-hexane.

Partition Coefficient: n-octanol/water: n/f

Percent Volatile: n/f

Reactivity in Water: n/f

Explosive Properties: n/f

Oxidizing Properties: n/f

Formula: C12H24O2 . xC3H8)2

Molecular Weight: n/f

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SECTION 10 - STABILITY AND REACTIVITY

Conditions to Avoid: Avoid exposure to air, heat, and moisture

Incompatibilities: Strong acids, oxidizing agents.

Decomposition Products: n/f

Stable? Yes **Hazardous Polymerization?** No

SECTION 11 - TOXICOLOGICAL PROPERTIES

Oral Rat: LD50: >2003 mg/kg

Oral Mouse: n/f

Other Toxicity Data: n/f

Irritancy Data: Rabbit/Skin: Moderate
Rabbit/Eye: Slight

Corrosivity: n/f

Sensitization Data: n/f

Listed as a Carcinogen by: **NTP:** No **IARC:** No **OSHA:** No

Other Carcinogenicity Data: n/f

Mutagenicity Data: Not mutagenic in the Ames test.

Reproductive and Developmental Effects: n/f

SECTION 12 - ECOLOGICAL INFORMATION

Ecological Information: n/f

SECTION 13 - DISPOSAL CONSIDERATIONS

Disposal: Dispose of waste in accordance with all applicable Federal, State and local laws.

SECTION 14 -TRANSPORT INFORMATION

Shipping Name: n/f

Class: n/f

UN Number: n/f

Packing Group: n/f

Additional Transport Information: n/f

SECTION 15 - REGULATORY INFORMATION

U.S. Regulatory Information: n/f

International Regulatory Information: EINECS # 253-462-2

SECTION 16 - OTHER INFORMATION

Revision: 19-Dec-07

Previous Revision Date: 23-Aug-07

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n/f = not found

Final Report on the Safety Assessment of Propylene Glycol (PG) Dicaprylate, PG Dicaprylate/Dicaprate, PG Dicocoate, PG Dipelargonate, PG Isostearate, PG Laurate, PG Myristate, PG Oleate, PG Oleate SE, PG Dioleate, PG Dicaprate, PG Diisostearate, and PG Dilaurate[1]

The Propylene Glycol Dicaprylate family of ingredients includes several esters and diesters of Propylene Glycol and fatty acids. These ingredients are used in cosmetic formulations as skin conditioning agents, viscosity increasing agents, and surfactants. Two skin irritation studies (minimal to no irritation) and a comedogenicity study (insignificant comedogen) on Propylene Glycol Dicaprylate/Dicaprate and a skin irritation study (slight) and an acute oral toxicity study (nontoxic) on Propylene Glycol Laurate were available. Available data were also found indicating that Propylene Glycol Dicaprylate/Dicaprate and Propylene Glycol Dipelargonate may enhance the skin penetration of other chemicals. Because of the ability of these Polyethylene Glycol esters and diesters to enhance penetration of other agents, it was recommended that care be taken in using these and other Polyethylene Glycol esters and diesters in cosmetic products. Previous Cosmetic Ingredient Review safety assessments of related ingredients, including Polyethylene Glycol, Polyethylene Glycol Stearate, Coconut Oils and Acids, Isostearic Acid, Lauric Acid, Myristic Acid, Oleic Acid, and Caprylic/Capric Triglyceride, were summarized. Included were mutagenicity, chronic toxicity, and skin irritation and sensitization data. Based in part on the limited data available on the ingredients included in the report, but more so on the previous reviews of chemically similar moieties, it was concluded that Propylene Glycol Dicaprylate, Propylene Glycol Dicaprylate/Dicaprate, Propylene Glycol Dicocoate, Propylene Glycol Dipelargonate, Propylene Glycol Isostearate, Propylene Glycol Laurate, Propylene Glycol Myristate, Propylene Glycol Oleate, Propylene Glycol Oleate SE, Propylene Glycol Dioleate, Propylene Glycol Dicaprate, Propylene Glycol Diisostearate, and Propylene Glycol Dilaurate are safe for use as cosmetic ingredients in the present practices of use.

The safety of the following Propylene Glycol esters and diesters in cosmetic products is reviewed: Propylene Glycol Dicaprylate; Propylene Glycol Dicaprylate/Dicaprate; Propylene Glycol Dicocoate; Propylene Glycol Dipelargonate; Propylene Glycol Isostearate; Propylene Glycol Laurate; Propylene Glycol Myristate; Propylene Glycol Oleate; Propylene Glycol Oleate SE (self-emulsifying); Propylene Glycol Dioleate; Propylene Glycol Dicaprate; Propylene Glycol Diisostearate; and Propylene Glycol Dilaurate.

With the exceptions of two skin irritation studies and a comedogenicity study on Propylene Glycol Dicaprylate/Dicaprate and a skin irritation study and acute oral toxicity study on Propylene Glycol Laurate, no other studies on the toxicity of the Propylene Glycol esters or diesters included in this review have been found.

However, the Cosmetic Ingredient Review (CIR) Expert Panel has issued Final Reports on the safety of Propylene Glycol, Propylene Glycol Stearate, Propylene Glycol Stearate SE, and other chemical moieties of the Propylene Glycol esters and diesters included in the present review, and determined that the data included in these Final Reports are sufficient for evaluating the safety of the thirteen Propylene Glycol esters and diesters that are mentioned above.

Therefore, data from the following CIR Final Reports were considered: Propylene Glycol (Andersen 1994); Propylene Glycol Stearate and Propylene Glycol Stearate SE (Elder 1983a); Caprylic/Capric Triglyceride (Elder 1980); Coconut Acid and Coconut Oil (Elder 1986); Isostearic Acid (Elder 1983b); and Lauric Acid, Myristic Acid, and Oleic Acid (Elder 1987). The results of studies from these safety assessments are included in the report summary.

CHEMISTRY

Chemical and Physical Properties

Properties of the following Propylene Glycol esters and diesters are summarized in Table 1: Propylene Glycol Dicaprylate; Propylene Glycol Dicaprate; Propylene Glycol Dicaprylate/Dicaprate; Propylene Glycol Dipelargonate; Propylene Glycol Laurate; Propylene Glycol Dilaurate; Propylene Glycol Oleate; and Propylene Glycol Dioleate. Properties of Propylene Glycol Dicaprylate/Dicaprate and Propylene Glycol Laurate, submitted to the Cosmetic Ingredient Review by a chemical supplier, are included in Table 2.

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[1]Reviewed by the Cosmetic Ingredient Review Expert Panel. Wilbur Johnson, Senior Scientific Analyst prepared this report. Address correspondence to him at 1101 17th Street, NW, Suite 310, Washington, DC 20036, USA.

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TABLE 1
Properties of Propylene Glycol Esters and Diesters

Property	Value/description	Reference
	Propylene Glycol Dicaprylate	
Formula weight	328.49	STN International 1995
Boiling point @ 0.050 torr	108°C	STN International 1995
Density @ 60°C	0.891	Patwardhan, Thapar, and Subrahmanyam 1974
Molar volume @ 60°C	368	Patwardhan, Thapar, and Subrahmanyam 1974
Refractive index @ 60°C	1.422	Patwardhan, Thapar, and Subrahmanyam 1974
Molar refraction @ 60°C	93.26	Patwardhan, Thapar, and Subrahmanyam 1974
Viscosity (centipoises) @ 40°C	4.08	Patwardhan, Thapar, and Subrahmanyam 1974
Viscosity @ 50°C	3.69	Patwardhan, Thapar, and Subrahmanyam 1974
Viscosity @ 60°C	3.33	Patwardhan, Thapar, and Subrahmanyam 1974
	Propylene Glycol Dicaprylate/Dicaprate	
Form	Clear, odorless, oily liquid	Nikitakis and McEwen 1990
Identification	Positive: close match to a standard IR spectrum with no indication of foreign materials	Nikitakis and McEwen 1990
Specific Gravity @ 25°/25°C	0.912 to 0.922	Nikitakis and McEwen 1990
Solubility	Soluble in most organic solvents; insoluble in water	Nikitakis and McEwen 1990
Acid value	0.1 maximum	Nikitakis and McEwen 1990
Iodine value	1.0 maximum	Nikitakis and McEwen 1990
Saponification value	315 to 335	Nikitakis and McEwen 1990
Moisture	0.1% maximum	Nikitakis and McEwen 1990
	Propylene Glycol Diperlargonate	
Formula weight	356.54	STN International 1995
Form	Odorless, colorless liquid	Nikitakis and McEwen 1990
Identification	Positive: close match to a standard IR spectrum with no indication of foreign materials	Nikitakis and McEwen 1990
Specific gravity @ 25°/25°C	0.895 to 0.935	Nikitakis and McEwen 1990
Density @ 80°C	0.873 g/cm^3	Lewis and Subrahmanyam 1983
Dynamic viscosity @ 80°C	0.022 g/cm·s	STN International 1995
Refractive index @ 80°C	1.417 (λ = 589 nm)	Lewis and Subrahmanyam 1983
Solubility	Soluble in common organic solvents; insoluble in water	Nikitakis and McEwen 1990
Acid value	0.5 maximum	Nikitakis and McEwen 1990
Ester value	310 to 325	Nikitakis and McEwen 1990
Iodine value	1.0 maximum	Nikitakis and McEwen 1990
	Propylene Glycol Laurate	
Formula weight	258.40	STN International 1995
Form	Light, yellow liquid with mild, fatty odor	Nikitakis and McEwen 1990
Identification	Positive: close match to a standard IR spectrum with no indication of foreign materials	Nikitakis and McEwen 1990
Specific gravity @ 25°/25°C	0.905 to 0.915	Nikitakis and McEwen 1990
Refractive index @ 25°/25°C	1.440 to 1.446	Nikitakis and McEwen 1990

TABLE 1
Properties of Propylene Glycol Esters and Diesters *(Continued)*

Property	Value/description	Reference
Solubility	Soluble in organic solvents; insoluble in propylene glycol or water	Nikitakis and McEwen 1990
Boiling point @ 0.6 torr	138 to 141°C	STN International 1995
Acid value	3.0 to 5.0	Nikitakis and McEwen 1990
Saponification value	230 to 250	Nikitakis and McEwen 1990
Iodine value	2.0 maximum	Nikitakis and McEwen 1990
	Propylene Glycol Dilaurate	
Form	Liquid	STN International 1995
Formula weight	440.71	STN International 1995
Density @ 30°C	0.897	STN International 1995
Refractive index @ 30°C	1.444 (λ = 589 nm)	STN International 1995
Melting point	21.9°C	STN International 1995
	35.0°C (in acetone)	STN International 1995
Boiling point @ 0.04 torr	196°C	STN International 1995
	Propylene Glycol Oleate	
Form	Liquid	STN International 1995
Molecular weight	340	STN International 1995
	Propylene Glycol Dioleate	
Formula weight	605	STN International 1995
	Propylene Glycol Dicaprate	
Formula weight	384.60	STN International 1995
Melting point	24.2 to 25.2°C (in acetone)	STN International 1995
	24.2 to 25.2°C (in petroleum ether and ethyl acetate)	STN International 1995
Refractive index	1.4276 (70°C; λ = 589 nm)	STN International 1995

TABLE 2
Chemical and physical properties of Propylene Glycol Dicaprylate/Dicaprate, and Propylene Glycol Laurate (Stepan Company 1996)

Property	Propylene Glycol Dicaprylate/Dicaprate	Propylene Glycol Laurate
Form	Liquid at 75°F	Liquid
Appearance	Slightly yellow color; free of suspended matter	Off-white color
Odor	Bland	Typical, mild fatty
Type	—	Nonionic
Melting point	—	8 to 12°C (specification)
Flash point (closed cup)	—	370°F
Solubility	Soluble in alcohol containing up to 20% water	Insoluble in water; soluble in isopropyl alcohol, mineral oil, and vegetable oil (peanut)
Moisture	0.05%	—
Free fatty acid (as oleic)	0.03%	—
Acid value	—	3.0 max (specification)
Iodine value	0.1	1.0 max (specification)
Saponification value	326	231–241 (specification)
Hydroxyl value	1.0	—

Information on the chemical and physical properties of Propylene Glycol Dicocoate, Propylene Glycol Isostearate, Propylene Glycol Myristate, Propylene Glycol Oleate SE, and Propylene Glycol Diisostearate were not identified in the published literature.

Propylene Glycol Dicaprylate

Propylene Glycol Dicaprylate (CAS Nos. 7384-98-7 and 56519-70-1) is the diester of propylene glycol and caprylic acid that conforms generally to the formula shown in Figure 1 (Wenninger and McEwen 1997). Other names for this chemical

$C(H_2C)_6H_3C(=O)-OCH_2CH(CH_3)O-C(=O)(CH_2)_6CH_3$

Propylene Glycol Dicaprylate

$C(H_2C)_8H_3C(=O)-OCH_2CH(CH_3)O-C(=O)(CH_2)_8CH_3$

Propylene Glycol Dicaprate

$R-C(=O)-OCH_2CH(CH_3)O-C(=O)-R$

where RCO represents the fatty acids derived from coconut oil

Propylene Glycol Dicocoate

$C(H_2C)_7H_3C(=O)-OCH_2CH(CH_3)O-C(=O)(CH_2)_7CH_3$

Propylene Glycol Diperlargonate

$H_3C-(CH_2)_X-CH(CH_3)(CH_2)_Y-C(=O)-OCH_2CH(OH)CH_3$

Propylene Glycol Isostearate

$C(H_2C)_{10}H_3C(=O)-OCH_2CH(CH_3)-OH$

Propylene Glycol Laurate

$H_3C-(CH_2)_X-CH(CH_3)(CH_2)_Y-C(=O)-OCH_2CH(CH_3)O-C(=O)-(CH_2)_YCH(CH_3)-(CH_2)_X-CH_3$

Propylene Glycol Diisostearate

$H_3C-(CH_2)_{12}C(=O)-OCH_2CH(CH_3)-OH$

Proplene Glycol Myristate

$C(H_2C)_{10}H_3C(=O)-OCH_2CH(CH_3)O-C(=O)(CH_2)_{10}CH_3$

Propylene Glycol Dilaurate

$H_3C-(CH_2)_7CH=CH(CH_2)_7C(=O)-OCH_2CH(CH_3)-OH$

Propylene Glycol Oleate

$H_3C-(CH_2)_7CH=CH(CH_2)_7C(=O)-O-CH_2-CH(CH_3)-O-C(=O)(CH_2)_7CH=CH(CH_2)_7-CH_3$

Propylene Glycol Dioleate

FIGURE 1

Chemical formulas for esters and diesters of Propylene Glycol and fatty acids.

are as follows: Octanoic Acid, 1-Methyl-1,2-Ethanediyl Ester; 1,2-Dicaprylin; 1-Methyl-1,2-Ethanediyl Octanoate; 1,2-Propanediol Dioctanoate; 1,2-Propyleneglycol Dioctanoate; and Propylene Di(Octanoate) (Wenninger and McEwen 1997; Chemline 1995; The Scientific and Technical Information Network [STN] International 1995).

Propylene Glycol Dicaprate

Propylene Glycol Dicaprate (CAS No. 56519-72-3) is the diester of propylene glycol and capric acid that conforms to the formula shown in Figure 1 (Wenninger and McEwen 1997). Other names for this chemical are as follows: Decanoic Acid, 1,3-Propanediyl Ester; n-Decanoic Acid, 1,3-Propanediyl Ester; Decanoic Acid, Trimethylene Ester (Wenninger and McEwen 1997; Chemline 1995); and 1,3-Bis-decanoyloxy-propane; 1,3-Bis-decanoyloxy-propan; and Decanoic Acid, 3-Decanoyloxy-propyl ester (STN International 1995).

Propylene Glycol Dicaprylate/Dicaprate

Propylene Glycol Dicaprylate/Dicaprate (CAS Nos. 58748-27-9; 9062-04-8; and 68988-72-7) is a mixture of the propylene glycol diesters of caprylic and capric acids (Wenninger and McEwen 1997). The structures of Propylene Glycol Dicaprylate and Propylene Glycol Dicaprate appear on the preceding page. Propylene Glycol Dicaprylate/Dicaprate is also defined as the propylene glycol diester of short chain, predominantly naturally derived C_8–C_{10} fatty acids (Nikitakis and McEwen 1990). It is soluble in alcohol containing up to 20% water and its viscosity is usually low (Stepan Company 1996). Other names for this mixture include: Decanoic Acid, 1-Methyl-1,2-Ethanediyl Ester mixed with 1-Methyl-1,2-Ethanediyl Dioctanoate; Decanoic Acid, Mixed Diesters with Octanoic Acid and Propylene Glycol; Caprylic, Capric Acid, Propylene Glycol Diester; Propylene Glycol Dicaprate-Caprate; and Propylene Glycol, Caprylate Caprate Diester (Wenninger and McEwen 1997; Chemline 1995). Propylene Glycol Dicaprylate/Dicaprate has also been defined as the propylene glycol diester of saturated vegetable acids (C_8–C_{10} chain length) that contains 65 to 80% caprylic acid and 15 to 30% capric acid (Mahjour et al. 1993).

Propylene Glycol Dicocoate

Propylene Glycol Dicocoate (CAS No. 68953-19-5) is the diester of propylene glycol and coconut acid that conforms generally to the formula shown in Figure 1 (Wenninger and McEwen 1997). The RCO group represents the fatty acids derived from coconut oil. Other names for this chemical are as follows: Coconut Fatty Acids, 1-Methyl-1,2-Ethanediyl Ester; Propylene Glycol Dicoconate; Propylene Glycol Diester Coconut Acids; and Fatty Acids, Coco, 1-Methyl-1,2-Ethanediyl Esters (Wenninger and McEwen 1997; Chemline 1995).

Propylene Glycol Dipelargonate

Propylene Glycol Dipelargonate (CAS No. 41395-83-9) is the diester of propylene glycol and pelargonic acid that conforms generally to the formula shown in Figure 1 (Wenninger and McEwen 1997). Other names for this chemical include Nonanoic Acid, 1-Methyl-1,2-Ethanediyl Ester; 1-Methyl-1,2-Ethanediyl Nonanoate; and Propylene Dinonanoate (Wenninger and McEwen 1997; Chemline 1995; STN International 1995).

Propylene Glycol Isostearate

Propylene Glycol Isostearate (CAS Nos. 32057-15-1 and 68171-38-0) is the ester of propylene glycol and isostearic acid (Wenninger and McEwen 1997) with the formula shown in Figure 1 (Sciarra, Iannacone, and Mores 1976). The sum of any combination of x and y values in the structure is 14 ($x + y = 14$). Other names for this chemical include Isooctadecanoic Acid, Monoester with 1,2-Propanediol and Propylene Glycol Monoisostearate (Wenninger and McEwen 1997).

Two commercially available forms of Propylene Glycol Isostearate were identified in the published literature. Propylene Glycol Monoisostearate consists of 52% monoester and 48% diester. Propylene Glycol Monoisostearate (90%) consists of 90% monoester and 10% diester (Sciarra, Iannacone, and Mores 1976).

Propylene Glycol Diisostearate

Propylene Glycol Diisostearate (CAS No. 68958-54-3) is the diester of propylene glycol that conforms generally to the formula shown in Figure 1 (Wenninger and McEwen 1997). Isooctadecanoic Acid, 1,3-Propanediyl Ester is another name for Propylene Glycol Diisostearate (Wenninger and McEwen 1997).

Propylene Glycol Laurate

Propylene Glycol Laurate (CAS No. 142-55-2) is the ester of propylene glycol and lauric acid that conforms generally to the formula shown in Figure 1 (Wenninger and McEwen 1997). It is a mixture of lauric acid esters of 1,2-propylene glycol in which the monoester predominates (Nikitakis and McEwen 1990). More recent information indicates the following composition of Propylene Glycol Laurate: free Propylene Glycol (0%), monoester (43.52%), and diester (56.48%) (Stepan Company 1996). Other names for Propylene Glycol Laurate are as follows: Dodecanoic Acid, 2-Hydroxypropyl Ester; Dodecanoic Acid, Monoester with 1,2-Propanediol; 2-Hydroxypropyl Dodecanoate; Propylene Glycol Monolaurate; Lauric Acid, 2-Hydroxypropyl Ester; and 2-Hydroxypropyl Laurate (Wenninger and McEwen 1997; Chemline 1995; STN International 1995).

Propylene Glycol Dilaurate

Propylene Glycol Dilaurate (CAS No. 22788-19-8) is the diester of propylene glycol and lauric acid that conforms generally to the formula shown in Figure 1 (Wenninger and McEwen 1997). Other names for this chemical include Dodecanoic Acid, 1-Methyl-1,2-Ethanediyl Ester; Lauric Acid, Propylene Ester; and 1,2-Bis-Lauroyloxy-Propane (Wenninger and McEwen 1997; Chemline 1995; STN International 1995).

Propylene Glycol Myristate

Propylene Glycol Myristate (CAS No. 29059-24-3) is the ester of propylene glycol and myristic acid that conforms generally to the formula shown in Figure 1 (Wenninger and McEwen 1997). Other names for this chemical are as follows: Propylene Glycol Monomyristate; Tetradecanoic Acid, Monoester with 1,2-Propanediol; and Myristic Acid, Monoester with 1,2-Propanediol (Wenninger and McEwen 1997; Chemline 1995).

Propylene Glycol Oleate

Propylene Glycol Oleate (CAS Nos. 27213-39-4 and 1330-80-9) is the ester of propylene glycol and oleic acid that conforms generally to the formula shown in Figure 1 (Wenninger and McEwen 1997). Other names for this chemical include 9-Octadecanoic Acid, Monoester with 1,2-Propanediol; Oleic Acid, Monoester with 1,2-Propanediol; and Propylene Glycol Monooleate (Wenninger and McEwen 1997; Chemline 1995; STN International 1995).

Propylene Glycol Oleate SE

Propylene Glycol Oleate SE (Self-Emulsifying) is a self-emulsifying grade of Propylene Glycol Oleate (q.v.) that contains some sodium and/or potassium oleate (Wenninger and McEwen 1997).

Propylene Glycol Dioleate

Propylene Glycol Dioleate (CAS No. 105-62-4) is the diester of propylene glycol and oleic acid that conforms to the formula shown in Figure 1 (Wenninger and McEwen 1993). Other names for Propylene Glycol Dioleate are as follows: 1-Methyl-1,2-Ethanediyl 9-Octadecenoate; 9-Octadecenoic Acid, 1-Methyl-1,2-Ethanediyl Ester; 9-Octadecenoic Acid, 1,3-Propanediyl Ester; and 9-Octadecenoic Acid (Z)-, 1-Methyl-1,2-Ethanediyl Ester (Wenninger and McEwen 1993; Chemline 1995), 1,2-Bis-Oleoyloxy-Propane; 1,2-Bis-Oleoyloxy-Propan; 1-Methyl-1,2-Ethanediyl Dioleate; and Octadec-9- Enoic Acid 1-Methyl-2-Octadec-9-Enoyloxy-Ethyl Ester (STN International 1995).

Methods of Production

Propylene Glycol Dicaprylate

Propylene Glycol Dicaprylate is a product of the reaction of propane-1,2-diol and octanoyl chloride with pyridine (% yield = 45% at 12 hours) (STN International 1995).

Propylene Glycol Dicaprylate/Dicaprate

Propylene Glycol Dicaprylate/Dicaprate is produced via the combination of Propylene Glycol with capric and caprylic acids. The mixture is heated to temperatures high enough to cause esterification. Water of reaction is removed to drive the reaction to completion and to obtain the low hydroxyl specification. The product is then fully refined and deodorized (Stepan Company 1996).

Propylene Glycol Dicaprate

Propylene Glycol Dicaprate is a product of the reaction of decanoic acid with propane-1,3-diol (temperature = 180°C) (STN International 1995).

Propylene Glycol Dipelargonate

Propylene Glycol Dipelargonate may be prepared by reacting nonanoyl chloride and $C_{12}H_{24}O_3$ with pyridine (solvent = $CHCl_3$) at 15°C or room temperature (STN International 1995).

Propylene Glycol Laurate

In the production of Propylene Glycol Laurate, Propylene Glycol and lauric acid are charged to the reactor, and a nitrogen sparge is initiated. The reactor is heated to approximately 225°C, and the water of reaction is removed. Vacuum is applied to remove unreacted propylene glycol when water evolution ceases. After specifications have been met, the finished product is cooled and drummed (Stepan Company 1996).

Propylene Glycol Dilaurate

Propylene Glycol Dilaurate may be prepared using either of the three methods: (1) reacting lauric acid and propylene oxide with the reagent, potassium hydroxide at 160°C; (2) reacting lauroyl chloride and propylene glycol with pyridine; and (3) reacting dodecanoic acid and propane-1,2-diol with the reagent, 0.2 M phosphate buffer (pH 7, solvent = water) at 45°C; % yield = 18.4% at 18 hours (STN International 1995).

Propylene Glycol Oleate

Propylene Glycol Oleate has been produced via the acylation of propylene glycol with oleic anhydride (*Pseudomonas* lipase catalyst; 160 to 180°C) (Shaw and Lo 1994).

Propylene Glycol Dioleate

Propylene Glycol Dioleate is a product of the reaction of propylene glycol with oleic acid chloride (STN International 1995).

The methods for production of the following esters and diesters were not identified in the published literature: Propylene Glycol Dicocoate; Propylene Glycol Myristate; Propylene Glycol Oleate SE; Propylene Glycol Isostearate; and Propylene Glycol Diisostearate.

Reactivity

Propylene Glycol Oleate

Propylene Glycol Oleate is stable under normal temperatures and pressures. It may burn, but does not ignite readily. Thermal decomposition products may include toxic oxides of carbon (STN International 1995). Propylene Glycol Oleate is incompatible with strong oxidizers (fire and explosion hazard). Under normal temperatures and pressures, hazardous polymerization has not been reported (STN International 1995).

Analytical Methods

Propylene Glycol Dicaprylate

Propylene Glycol Dicaprylate has been analyzed by infrared (IR) and nuclear magnetic resonance (NMR) spectroscopy (STN International 1995) and mass spectrometry (Le Tellier and Nawar 1975).

Propylene Glycol Dipelargonate

Propylene Glycol Dipelargonate has also been analyzed by mass spectrometry (Le Tellier and Nawar 1975).

Propylene Glycol Oleate

Propylene Glycol Oleate has been analyzed by gas chromatography–flame ionization detection (Shaw and Lo 1994).

Information on analytical methods for the following Propylene Glycol esters and diesters were not identified in the published literature: Propylene Glycol Dicaprylate/Dicaprate; Propylene Glycol Dicaprate; Propylene Glycol Laurate; Propylene Glycol Dilaurate; Propylene Glycol Dioleate; Propylene Glycol Dicocoate; Propylene Glycol Myristate; Propylene Glycol Oleate SE; Propylene Glycol Isostearate; and Propylene Glycol Diisostearate.

Impurities

Information on the presence of impurities (e.g., potential pyridine residues) in the Propylene Glycol esters and diesters that are being reviewed was not identified in the published literature.

USE

Purpose in Cosmetics

Skin-conditioning agent–occlusive and viscosity increasing agent–nonaqueous are the intended cosmetic uses for the following propylene glycol diesters: Propylene Glycol Dicaprylate, Propylene Glycol Dicaprate, Propylene Glycol Dicocoate, Propylene Glycol Dipelargonate, Propylene Glycol Diisostearate, Propylene Glycol Dilaurate, and Propylene Glycol Dioleate. Propylene Glycol Dicaprylate/Dicaprate is intended for use as a skin-conditioning agent–occlusive. Propylene Glycol Oleate SE is used as a surfactant-emulsifying agent (Wenninger and McEwen 1997).

Other propylene glycol esters, listed as follows, are intended for use as skin-conditioning agent–emollients and surfactant-emulsifying agents: Propylene Glycol Isostearate, Propylene Glycol Laurate, Propylene Glycol Myristate, and Propylene Glycol Oleate (Wenninger and McEwen 1997).

Scope and Extent of Use in Cosmetics

The product formulation data submitted to the Food and Drug Administration in 1996 (FDA 1996) included the following use frequencies for Propylene Glycol esters and diesters: Propylene Glycol Dicaprylate (1 product); Propylene Glycol Dicaprylate/Dicaprate (202 products); Propylene Glycol Oleate (6 products); Propylene Glycol Myristate (11 products); Propylene Glycol Isostearate (22 products); Propylene Glycol Dipelargonate (82 products); and Propylene Glycol Laurate (87 products). Product formulation data on Propylene Glycol esters and diesters are summarized in Table 3 (FDA 1996).

The following Propylene Glycol ester/diesters reviewed in the present report were not reported to FDA as being used in cosmetic products: Propylene Glycol Dicocoate, Propylene Glycol Oleate SE, Propylene Glycol Dioleate, Propylene Glycol Dicaprate, Propylene Glycol Diisostearate, and Propylene Glycol Dilaurate.

Concentration of use values are no longer reported to FDA by the cosmetics industry (FDA 1992). Data on Propylene Glycol esters and diesters were received from industry (CTFA 1995) and are included in Table 4.

Cosmetic products containing Propylene Glycol esters and diesters are applied to the skin (eyelids and lips included), hair, and nails and may come in contact with the ocular, nasal, and oral mucosae. These products may be used on a daily basis, and have the potential for being applied frequently over a period of several years.

International Use

With the exceptions of Propylene Glycol Dilaurate, Propylene Glycol Myristate, and Propylene Glycol Oleate SE, the Propylene Glycol esters that are being reviewed in the present report are also listed in the *CTFA List of Japanese Cosmetic Ingredients*. Ingredients that are used in cosmetic products marketed in Japan appear on this list. The inclusion of any ingredient on the *CTFA List of Japanese Cosmetic Ingredients* does not guarantee either that the ingredient is safe for use as a cosmetic ingredient, or that the use of the substance as a cosmetic ingredient complies with the laws and regulations governing such use in Japan. Neither Propylene Glycol Dilaurate, Propylene Glycol Myristate, nor Propylene Glycol Oleate is prohibited from use in cosmetics manufactured in or imported into Japan (Rempe and Santucci 1992).

None of the Propylene Glycol esters or diesters reviewed in the present report is included among the substances listed as prohibited from use in cosmetic products that are marketed in the European Union (Dupuis 1994).

Noncosmetic Use

Propylene Glycol esters have been used as emulsifiers in foods and pharmaceuticals (Rosen 1978). FDA has determined that Propylene Glycol mono- and diesters of fats and fatty acids can be used safely in food, provided that (1) they are produced from edible fats and/or fatty acids in compliance with 21 CFR (Code of Federal Regulations) 172.860 and/or oleic acid derived from tall oil fatty acids in compliance with 21 CFR 172.862, and (2) they are used in food in amounts not in excess of that reasonably required to produce their intended effect (21 CFR 172.856). Propylene Glycol mono- and diesters of fats and fatty acids also

TABLE 3
Product formulation data on Propylene Glycol Dicaprylate and Propylene Glycol Dicaprylate/Dicaprate (FDA 1996)

Product category	Total no. of formulations in category	Total no. containing ingredient
Propylene Glycol Dicaprylate		
Moisturizing skin care preparations	942	1
1996 totals		**1**
Propylene Glycol Dicaprylate/Dicaprate		
Baby lotions, oils, powders, and creams	64	1
Eye shadows	588	4
Eye lotions	22	2
Eye makeup removers	95	5
Other eye makeup preparations	136	3
Other fragrance preparations	195	12
Blushers (all types)	277	6
Face powders	313	1
Foundations	355	27
Lipsticks	997	24
Makeup bases	154	2
Rouges	30	1
Other makeup preparations	157	1
Cuticle softeners	26	1
Nail polish and enamel removers	36	1
Other manicuring preparations	83	2
Aftershave lotions	268	2
Other shaving preparation products	63	1
Cleansing preparations (cold creams, cleansing lotions, liquids, and pads)	820	10
Face and neck skin care preparations (excluding shaving preparations)	300	5
Body and hand skin care preparations (excluding shaving preparations)	1012	15
Foot powders and sprays	33	1
Moisturizing skin care preparations	942	52
Night skin care preparations	226	3
Paste masks (mud packs)	300	2
Other skin care preparations	810	7
Suntan gels, creams, and liquids	196	6
Indoor tanning preparations	67	3
Other suntan preparations	68	2
1996 totals		**202**
Propylene Glycol Oleate		
Other eye makeup preparations	136	1
Foundations	355	1
Makeup bases	154	1
Body and hand (excluding shaving preparations)	1012	2
Foot powders and sprays	33	1
1996 totals		**6**

TABLE 3
Product formulation data on Propylene Glycol Dicaprylate and Propylene Glycol Dicaprylate/Dicaprate (FDA 1996) *(Continued)*

Product category	Total no. of formulations in category	Total no. containing ingredient
Propylene Glycol Myristate		
Lipstick	997	1
Other makeup preparations	157	1
Face and neck skin care preparations (excluding shaving preparations)	300	1
Body and hand skin care preparations (excluding shaving preparations)	1012	3
Other skin care preparations	810	3
Suntan gels, creams, and liquids	196	2
1996 totals		**11**
Propylene Glycol Isostearate		
Eye makeup remover	95	1
Foundations	355	2
Lipstick	997	1
Other makeup preparations	157	1
Shaving cream (aerosol, brushless, and lather)	158	6
Other shaving preparations products	63	7
Cleansing preparations (cold creams, cleansing lotions, liquids, and pads)	820	1
Moisturizing skin care preparations	942	1
Other skin care preparations	810	1
Suntan gels, creams, and liquids	196	1
1996 totals		**22**
Propylene Glycol Dipelargonate		
Baby lotions, oils, powders, and creams	64	1
Bath oils, tablets, and salts	147	1
Eye makeup remover	95	1
Other eye makeup preparations	136	1
Powders (dusting and talcum, excluding aftershave talc)	307	1
Other fragrance preparations	195	4
Blushers (all types)	277	4
Face powders	313	1
Foundations	355	26
Lipstick	997	8
Makeup bases	154	1
Other makeup preparations	157	2
Other personal cleanliness products	339	1
Aftershave lotion	268	1
Preshave lotions (all types)	20	1
Cleansing preparations (cold creams, cleansing lotions, liquids, and pads)	820	5
Body and hand skin care preparations (excluding shaving preparations)	1012	4
Moisturizing skin care preparations	942	9
Paste masks (mud packs)	300	1
Other skin care preparations	810	9
1996 totals		**82**

(Continued on next page)

TABLE 3
Product formulation data on Propylene Glycol Dicaprylate and Propylene Glycol Dicaprylate/Dicaprate (FDA 1996) *(Continued)*

Product category	Total no. of formulations in category	Total no. containing ingredient
Propylene Glycol Laurate		
Eye lotion	22	2
Mascara	218	2
Other eye makeup preparations	136	2
Cologne and toilet waters	834	6
Perfumes	286	3
Other fragrance preparations	195	2
Hair conditioners	715	1
Shampoos (noncoloring)	972	2
Tonics, dressings, and other hair grooming aids	604	3
Other hair preparations	395	1
Blushers (all types)	277	3
Foundations	355	9
Lipstick	997	7
Makeup bases	154	4
Makeup fixatives	11	3
Other makeup preparations	157	3
Cuticle softeners	26	1
Other personal cleanliness products	339	1
Aftershave lotion	268	1
Other shaving preparations products	63	2
Cleansing skin care preparations (cold creams, cleansing lotions, liquids, and pads)	820	6
Body and hand skin care preparations (excluding shaving preparations)	1012	9
Moisturizing skin care preparations	942	4
Paste masks (mud packs)	300	1
Other skin care preparations	810	4
Suntan gels, creams, and liquids	196	3
Indoor tanning preparations	67	1
Other suntan preparations	68	1
1996 totals		**87**

can be used as components of the food-contact surface of paper and paperboard, provided that the food-contact surface of the paper or paperboard complies with the prescribed limitations for extractives (21 CFR 176.170).

Defoaming agents containing Propylene Glycol mono- and diesters of fats and fatty acids can be used safely in processed foods in accordance with the provisions included in the preceding paragraph (21 CFR 173.340). Additionally, defoaming agents containing Propylene Glycol esters can be used safely in the manufacture of paper and paperboard intended for use in packaging, transporting, or holding food (21 CFR 176.210).

Reaction products resulting from the reaction of Propylene Glycol with certain fats, oils, fatty acids, and fatty alcohols are among the substances that are used in the production of, or are added to, textiles and textile fibers. Such textiles and textile fibers can be used safely as articles or components of articles intended for use in producing, manufacturing, packing, processing, preparing, treating, packaging, transporting, or holding food (21 CFR 177.2800). Similarly, esters resulting from the reaction of reconstituted oils (from triglycerides or fatty acids derived from certain oils) with Propylene Glycol are ingredients of resinous and polymeric coatings. Coatings of this composition may be used safely as the food-contact surface or articles intended for use in producing, manufacturing, packing, processing, preparing, treating, packaging, transporting, or holding food (21 CFR 175.300).

TABLE 4
Concentration of use data (CTFA 1995)

Ingredient	Product type	Use concentrations
Propylene Glycol Dicaprylate/Dicaprate	Blush	45%
	Makeup	24%
	Sun block stick	24%
	Moisturizer	16%
	Lip products	10%
	Eyeshadow	7%
	Eyeliner	19%
	Makeup remover	27%
	Antiperspirant	33%
	Aftershave balm	7%
Propylene Glycol Dipelargonate	Body oils	up to 8%
	Moisturizer	4%
	Aftershave balm	4%
	Other hair preparations	33.7960%
	Blushers (all types)	5%
	Foundations	9.30%
	Nail polish and enamel removers	5%
	Face and neck skin care	5%
	Moisturizing skin care	3%
	Night skin care	1%
Propylene Glycol Diester	Eye shadow	23.5%
	Blushers (all types)	51.730%
	Face powders	15%
	Foundations	30.640%
	Cuticle softeners	3.5%
	Nail creams and lotions	21.6%
	Other manicuring preparations	8%
	Face and skin care	1%
	Moisturizing skin care	2.5%
	Night skin care	1%
Propylene Glycol Isostearate	Shave gel	1.4%
Propylene Glycol Laurate	Hairdress	22%
	Tanning gel moisturizer	6%
	Shampoo	1.25%
	Lash gel	1%
	Eye shadow	1.3%
	Lipstick	9%

BIOLOGICAL PROPERTIES

Absorption, Distribution, Metabolism, and Excretion

Pharmacokinetic studies on the Propylene Glycol esters and diesters reviewed in the present report were not identified in the published literature.

Skin Penetration Enhancement

Propylene Glycol Dicaprylate/Dicaprate

Propylene Glycol Dicaprylate/Dicaprate reportedly enhanced the in vitro skin permeation rate of several transdermal drug candidates across human and hairless mouse skin, making it a

potential candidate for use in marketed transdermal products. The investigators suggested that these findings can indicate a nondiscriminative enhancement effect that resulted from a reduction in the stratum corneum diffusional resistance. This reduction could have been caused by an increase in the stratum corneum lipid fluidity or by extraction of the stratum corneum's lipids by Propylene Glycol Dicaprylate/Dicaprate (Mahjour et al. 1993).

Propylene Glycol Dipelargonate

Similarly, in another study, a saturated solution of Propylene Glycol Dipelargonate in propylene glycol promoted the percutaneous penetration of drugs across excised human skin in vitro. The investigators suggested that this property can be linked to the comparatively low polarity of Propylene Glycol Dipelargonate, enabling it to penetrate into the stratum corneum and interact with the lipid bilayers, thus, increasing their fluidity or forming fluid-like channels (Bonina et al. 1993). The results of an in vitro embryotoxicity study suggest that the skin penetration enhancement property of propylene glycol esters and diesters noted above can be due to the presence of propylene glycol. In this study, the exposure of $B_6D_2F_1$ mouse zygotes to $\geq$2.5 M propylene glycol for 2 to 7 minutes altered both intracellular pH and developmental potential. In that these effects were independent of volume changes noted in zygotes, and, therefore, intracellular propylene glycol concentrations, the researchers postulated that the toxicity of propylene glycol is mediated by direct alteration of the cell membrane (Damien, Luciano, and Peluso 1989).

TOXICOLOGY

Regarding all Propylene Glycol esters and diesters reviewed in this report, the following types of studies were not identified in the published literature: subchronic toxicity, mutagenicity, carcinogenicity, reproductive and developmental toxicity, and skin sensitization/photosensitization.

Acute Oral Toxicity

Propylene Glycol Laurate

In a study involving rats (number and strain not stated), the oral LD_{50} for Propylene Glycol Laurate was greater than 34.6 g/kg. The test substance was classified as practically nontoxic (Stepan Company 1996).

Antitumor Activity

Propylene Glycol Myristate

The antitumor activity of Propylene Glycol Myristate in vivo was evaluated using 20 ddY mice (5 weeks old; 18 to 22 g). Following the intraperitoneal implantation of one-million tumor cells, the test substance (in 0.86% NaCl solution or suspension) was administered once daily for 5 consecutive days. The final test concentrations administered were expressed as 0.5 mg/10^7 tumor cells (10 mice) and 2.5 mg/10^7 tumor cells (10 mice). After 7 days, tumor growth, body weight gain, and life span up to 30 days were evaluated. Ten control mice received injections of untreated tumor cells. Tumors were not observed in any of the 10 mice treated with the higher concentration (average survival time > 30 days). At the lower concentration (10 mice), there were no cytotoxic effects on tumor cells. Tumors were observed in each mouse after 7 days, and the average survival time was 21.6 days. The average survival time for the 10 control mice (100% tumor incidence) was 18.8 days (Kato et al. 1971).

In a similar study, the antitumor activity of Propylene Glycol Myristate in vivo was evaluated using four 5-week-old ddY mice. Following the intraperitoneal injection of one million Ehrlich ascites tumor cells, the test substance (in saline solution or suspension) was administered (5 and 20 mg/mouse/day, 2 mice per dose) once daily for 5 consecutive days. Two untreated mice that were injected with tumor cells served as controls. Tumor growth and body weight gain were evaluated after 7 days. Compared to untreated controls, Propylene Glycol Myristate inhibited tumor growth and prolonged the life span of treated mice. No tumor growth was observed in mice dosed with 5 and 20 mg/day and the survival time for both groups was >30 days. The life span of control mice (marked tumor growth) was 16 days (Kato et al. 1969; 1970).

Propylene Glycol Oleate

Propylene Glycol Oleate had no antitumor activity in the preceding study by Kato et al. (1969; 1970). Tumor growth was marked at doses of 5 mg/mouse/day (2 mice) and 20 mg/mouse/day (2 mice), and the life span of treated mice (both doses) was 16 days (Kato et al. 1970).

Skin Irritation

Propylene Glycol Dicaprylate/Dicaprate

The skin irritation potential of Propylene Glycol Dicaprylate/Dicaprate was evaluated using six rabbits (strain not stated). Patches (type not stated) were applied for 24 hours. An average skin irritation score of 0.5 (maximum score possible = 8) was reported, and the test substance was classified as minimally irritating to the skin (Stepan Company 1996).

Propylene Glycol Laurate

The skin irritation potential of Propylene Glycol Laurate was also evaluated in a study involving rabbits (number and strain not stated). The experimental procedure was not included. An average skin irritation score of 0.8 (maximum score possible = 8) was reported, and the test substance was classified as slightly irritating to the skin (Stepan Company 1996).

Comedogenicity

Propylene Glycol Laurate

The comedogenicity of Propylene Glycol Laurate was evaluated in a 14-day study using three rabbits (strain not stated).

Details concerning the experimental protocol were not included. An average comedogenicity score of 0.7 (maximum score possible = 3) was reported, and the test substance was classified as an insignificant comedogen (Stepan Company 1996).

CLINICAL ASSESSMENT OF SAFETY

Skin Irritation

Propylene Glycol Dicaprylate/Dicaprate

The skin irritation potential of a 95% ethanol:Propylene Glycol Dicaprylate/Dicaprate mixture (20:80) was evaluated using three volunteers (two females, one male). Four patches (2.5 cm^2 surface area), each containing 50 $\mu l/cm^2$ of the mixture, were applied for 24 hours to the lower dorsal left forearm of each female and to both arms of the male subject. At the end of the contact period and 24 hours later, test sites were examined grossly for signs of either erythema or edema. Skin irritation was not observed in either of the three subjects tested. Similar results were reported when a fourth subject (male) was tested with a 95% ethanol:Propylene Glycol Dicaprylate/Dicaprate mixture (40:60) according to the same procedure (Mahjour et al. 1993).

SUMMARY

Propylene Glycol Esters and Diesters

The limited information on chemical properties of Propylene Glycol esters and diesters indicates that, generally, these ingredients are soluble in most organic solvents. Methods of production that have been reported for some of the esters and diesters included in this review are as follows: Propylene Glycol Oleate is produced via the acylation of propylene glycol with oleic anhydride, and the dioleate is a product of the reaction of propylene glycol with oleic acid chloride. Propylene Glycol Dicaprate is a product of the reaction of decanoic acid with propane-1,3-diol. Similarly, Propylene Glycol Dicaprylate is produced by reacting propane-1,2-diol and octanoyl chloride with pyridine. Pyridine is also used in the production of Propylene Glycol Dipelargonate and Propylene Glycol Dilaurate. Propylene Glycol is a product of the reaction of nonanoyl chloride and $C_{12}H_{24}O_3$ with pyridine, and, Propylene Glycol Dilaurate, a product of the reaction of lauroyl chloride and propylene glycol with pyridine.

Cosmetic uses of Propylene Glycol esters and diesters include skin-conditioning agent–occlusive, viscosity increasing agent–nonaqueous, skin conditioning agent–emollients, and surfactant-emulsifying agents. These ingredients are used widely in a variety of rinse-off and leave-on cosmetics products. Data submitted to CIR by the cosmetics industry in 1995 indicated that Propylene Glycol diesters were used at concentrations up to 51.7%, and, Propylene Glycol esters, at concentrations up to 22%.

Propylene Glycol Dicaprylate/Dicaprate and Propylene Glycol Dipelargonate promoted the percutaneous penetration of drugs across excised human skin/hairless mouse skin in vitro.

Propylene Glycol Laurate was classified as practically nontoxic (LD_{50} > 34.6 g/kg) when administered orally to rats.

In two skin irritation studies involving rabbits, Propylene Glycol Dicaprylate/Dicaprate and Propylene Glycol Laurate were classified as minimally irritating and slightly irritating, respectively. Propylene Glycol Dicaprylate/Dicaprate was also classified as an insignificant comedogen in rabbits.

Antitumor activity (in vivo) in ddY mice was observed following the intraperitoneal injection of Propylene Glycol Myristate, but not Propylene Glycol Oleate. Skin irritation was not observed in either of the three subjects patch tested with a 95% ethanol:Propylene Glycol Dicaprylate/Dicaprate mixture (20:80). Patches were removed at 24 hours postapplication. Similar results were reported for a fourth subject patch tested with a 95% ethanol:Propylene Glycol Dicaprylate/Dicaprate mixture (40:60).

Propylene Glycol, Esters, and Fatty Acids

Propylene Glycol

Propylene Glycol (PG) was relatively harmless (LD_{50} = 21 g/kg) in acute oral toxicity studies involving rats.

Test substance–related lesions were not observed in rats that were fed diets containing 50,000 ppm PG (2.5 g/kg/day) for 15 weeks or in rats that were fed PG concentrations up to 50,000 ppm in the diet for 2 years. Similar results were reported in a study in which dogs were fed 2 or 5 g/kg PG in the diet for approximately 103 weeks. In another subchronic study, dogs received 5% PG in drinking water for 5 to 9 months. The results of tests for hepatic and renal impairment were negative.

PG did not induce corneal damage in the Draize test and was classified as a slight ocular irritant in another ocular irritation study.

In a 24-hour skin irritation test involving nude mice, no reactions to 10% PG were observed. Hypertrophy, dermal inflammation, and proliferation were observed at a concentration of 50% PG.

Draize test results indicated that PG was, at most, a mild skin irritant when applied for 24 hours to abraded and intact skin of rabbits. When PG was applied to the skin of guinea pigs and rabbits (guinea pigs and rabbits lack sweat glands) for 48 hours using open and closed patches, no reactions were observed. The results of 48-hour and 21-day open and closed patch tests involving Gottingen swine (no sweat glands) indicated no reactions to PG.

Results were negative for 100% PG in a mouse external ear swelling sensitization test. The results of a guinea pig maximization test, open epicutaneous test, and chamber (Finn chamber) test indicated no sensitization reactions to 70% PG. In another maximization test, PG was classified as a potentially weak sensitizer. The results of six other guinea pig sensitization tests indicated that PG was not an allergen.

PG was not teratogenic in female CD-1 mice when administered at a concentration of 10,000 ppm on days 8 to 12 of gestation. Malformations were observed in 5 of 226 living fetuses from female mice injected subcutaneously with PG (dose =

0.1 ml/g body weight on day 9, 10, or 11 of gestation). Three fetuses with malformations were noted among 1026 living fetuses from the untreated control group of pregnant mice.

In a continuous breeding reproduction study, no significant differences were observed between control and experimental groups of albino mice with respect to the following: mating index, fertility index, mean number of live pups per litter, proportion of pups born alive, and sex of pups born alive.

Embryonic development was reduced in cultures of mouse zygotes exposed to 3.0 M PG and inhibited completely in cultures exposed to 6.0 M PG for 20 min.

In the Ames test, PG was not mutagenic in strains TA 1535, TA 1537, TA 1538, TA 98, and TA 100 of *Salmonella typhimurium* with and without metabolic activation. PG caused a dose-dependent increase in the frequency of sister chromatid exchanges (SCEs) in a Chinese hamster cell line, and was classified as a weak inducer of SCEs. In another study, PG was not mutagenic when tested in a sister chromatid exchange assay involving human cultured fibroblasts and a cultured Chinese hamster cell line both with and without metabolic activation. PG also was not mutagenic in additional in vitro tests: chromosomal aberrations, mitotic recombination, base pair substitution, micronucleus test, reverse mutation, and DNA damage.

PG disturbed the proliferation of urinary bladder epithelial cells from the rat, having reduced DNA production in tetraploid cells 1 and 2 months after the rats were injected subcutaneously. This effect was not observed at 3 months.

The results were negative when PG was tested in the hamster embryo cell transformation bioassay. In a 2-year feeding study involving CD strain rats, PG was not carcinogenic when concentrations up to 50,000 ppm were administered in the diet. In a life-time dermal carcinogenicity study, three groups of Swiss mice received dermal applications of 10, 50, and 100% PG, respectively. The tumor incidence in each of the three groups did not differ from that noted in the negative control group; skin tumors were not observed.

PG induced skin irritation and sensitization reactions in normal subjects and in patients. In these studies, test concentrations ranged from 2 to 100% PG. Reactions were observed at concentrations as low as 10% PG in predictive tests, and as low as 2% in provocative tests.

PG also increased the allergic responses in 43 patients patch tested with 50 μg of 1% nickel sulfate solution.

Propylene Glycol Stearate and Propylene Glycol Stearate SE

In rats, the acute oral toxicity of Propylene Glycol Stearate (PGS) was approximately 25.8 g/kg. The raw ingredient produced no significant dermal toxicity, skin irritation, or ocular irritation in acute tests with rabbits. Subchronic animal studies produced no evidence of oral or dermal toxicity. In a 6-month feeding study, no signs of toxicity were observed in dogs or rats fed a mixture containing 17% PGS; the mixture was incorporated into the diet at a concentration of 10%. PGS was negative in in vitro microbial assays for mutagenicity.

Although Propylene Glycol Stearate Self-Emulsifying (PGS-SE) has not been tested as extensively as PGS, it produced no apparent significantly different results in any of the animal tests. The acute oral LD_{50} in rats was estimated as greater than 32 g/kg. The ingredient produced no significant skin or ocular irritation in Draize rabbit irritation tests, and it was not a sensitizer in a guinea pig sensitization test. No other subchronic or chronic studies were available.

In clinical studies, PGS produced no significant skin irritation at concentrations up to 55% in 24 h single insult skin patch tests. A 28-day controlled use test on a product containing 2.5% PGS demonstrated no cumulative irritation with normal product use, but mild to moderate irritation with a challenge skin patch; the offending ingredient was not identified. Several skin sensitization tests on product formulations containing 1.5% to 2.5% PGS produced no evidence of sensitization reactions in a total subject population of 4084. Two photo-contact allergenicity tests on product formulations containing 1.5% PGS were negative.

No clinical data were available for PGS-SE. However, the chemical components of PGS-SE that distinguish it from PGS have been considered previously to be safe, and the information generally applicable to PGS is considered applicable to PGS-SE.

Caprylic/Capric Triglyceride

Caprylic/Capric Triglyceride has very low toxicity in humans and animals, as indicated by results of tests involving oral ingestion, intraperitoneal and intramuscular injection, skin and eye irritation tests, skin sensitization, percutaneous toxicity and, finally, by two-generation feeding studies.

The safety assessment of this ingredient rests on the information at hand and on the considerable usage at various concentrations in a variety of cosmetic and other consumer products. Additional biological assessments might reasonably be recommended to include studies on photosensitization.

Coconut Acid and Related Compounds

The results of oral toxicity studies indicate that Coconut Oil and Hydrogenated Coconut Oil are relatively nontoxic by ingestion. Administered as a single 5 g/kg dose to rats, neither compound caused deaths over a 7-day observation period. In a 90-day subchronic feeding study of diets containing 25% Coconut Oil, rats had slight fatty change of the liver but no other pathological changes. The results of a chronic study in which mice were fed, for a lifetime, diets supplemented with 15% Hydrogenated Coconut Oil indicated no effect on lifespans of the test animals.

Hydrogenated Coconut Oil was nontoxic when applied dermally. A single 3 g/kg dose applied to guinea pigs caused no deaths during a 7-day observation period. It was nonirritating to the skin in three single-insult occlusive patch tests. A primary irritation index of 0.11/8.0, indicating minimal irritation, was reported in a fourth study. Hydrogenated Coconut Oil was not a sensitizer in guinea pigs when applied to the skin in a modified Buehler test.

Coconut Oil did not cause skin irritation when applied to rabbit skin in a 24-hour single-insult occlusive patch test. It was nonsensitizing to the skin in a Magnusson-Kligman maximization test.

Coconut Acid caused minimal irritation in rabbits when assayed in a 24-hour single-insult occlusive patch test. Primary irritation indices of 0.13/4.0 and 0.17/4.0 were reported for 10% Coconut Acid in corn oil and undiluted Coconut Acid, respectively. These scores were indicative of minimal skin irritation.

Results of several studies suggest that the eye irritation potential of Coconut Oil and Hydrogenated Coconut Oil is low. Coconut Oil in Draize eye tests scored a maximum of 2/110, indicating minimum irritation. Hydrogenated Coconut Oil was assayed in ten Draize eye tests. In nine tests, ocular irritation ($\leq$2/110) was minimal, and in one test it was mild (6/110).

No mutagenicity data are available on any of the Coconut Oil ingredients. Coconut Oil was reported less effective than polyunsaturated fat as a tumor promoter for mammary tumors in rats induced by 7,12-dimethylbenz(a)anthracene.

Clinical assessment of cosmetic products containing Coconut Oil has used a variety of assays. Bar soaps containing 13% Coconut Oil, when tested using standard Draize procedures, produced very minimal skin reactions. In a 2-week normal use test, bar soaps caused no unusual irritation response. The results of soap chamber tests of bar soaps were minimal irritation in one study and mild irritation in another. No phototoxicity or photosensitivity was produced by these same bar soap formulations. A tanning butter containing 2.5% Coconut Oil did not cause erythematous reactions in a six-week repeat insult predictive patch test.

Lipstick containing 10% Hydrogenated Coconut Oil was tested using Schwartz-Peck prophetic patch test procedures. There was no evidence of primary irritation after a single patch application and no indication of sensitization in retests performed 14 days later.

Isostearic Acid

In rats, the acute oral LD_{50} for Isostearic Acid was estimated at greater than 32 ml/kg. The pure ingredient produced no significant skin or eye irritation in Draize rabbit eye irritation tests, whereas variable degrees of irritation were produced by product formulations containing Isostearic Acid. A product formulation both with and without 2.5% Isostearic Acid was tested in a rabbit ear comedogenicity assay. The formulation without Isostearic Acid was irritating but did not produce comedones; however, the formulation with Isostearic Acid was both irritating and comedogenic.

In clinical studies, 100 subjects had no signs of irritation after application of a 24-hour single-insult skin patch with undiluted Isostearic Acid, and product formulations containing up to 4% Isostearic Acid produced, at most, minimal irritation when similarly tested usintg a total of 221 subjects. In another study, 35% Isostearic Acid in mineral oil was neither an irritant nor a sensitizer in 168 subjects. A subset population of 25 individuals from this study group, when tested in a similar manner but exposed to UVA + UVB, gave no indication that Isostearic Acid is a photosensitizer. Isostearic Acid at 10% in mineral oil was similarly not irritating nor sensitizing to 103 subjects. Product formulations containing 2.5 to 2.85% Isostearic Acid produced no evidence of contact sensitization when tested in repeated-insult patch tests on a total of 333 subjects.

Lauric, Myristic, and Oleic Acids and Related Compounds

Little acute toxicity was observed when Oleic, Lauric, Palmitic, Myristic, or Stearic Acid, or cosmetic formulations containing these fatty acids at concentrations of 2.2 to 13% were given to rats orally at doses of 15 to 19 g/kg body weight.

In subchronic oral toxicity studies, Oleic, Palmitic, Myristic, and Stearic Acids were fed to rats at doses ranging from 5 to 50%. Thrombosis, aortic atherosclerosis, anorexia, and mortality were observed. In a subchronic study, no signs of toxicity were observed in chicks fed 5% dietary Stearic and Oleic Acids. Rats fed 15% Oleic Acid (in diet) in a chronic study had normal growth and general health, but reproductive capacity of female rats was impaired.

Results from topical application of Oleic Acid (at concentrations from 50% Oleic Acid to commercial grade Oleic Acid) to the skin of mice, rabbits, and guinea pigs ranged from no toxicity to signs of erythema, hyperkeratosis, and hyperplasia. Intradermal administration to guinea pigs of 25% Oleic Acid to commercial grade Oleic Acid resulted in local inflammation and necrosis. A formulation containing 2.2% Palmitic Acid was considered nontoxic to rabbits. A topically applied dose of 5 g/kg commercial grade Stearic Acid was not toxic to rabbits. Intradermal administration of 10 to 100 mM Stearic Acid was not toxic to rabbits. Intradermal administration of 10 to 100 mM Stearic Acid to guinea pigs and rabbits resulted in mild erythema and slight induration.

Eighteen mmol% concentrations of the fatty acids topically applied to the skin of the external ear canals of albino rabbits for 6 weeks produced a range of responses, varying from no irritation with Stearic Acid to slight irritation with Myristic and Palmitic Acids to erythema, desquamation, and persistent follicular keratosis with Oleic and Lauric Acids. Slight local edema and no deaths were observed among New Zealand white rabbits after 4 weeks of topical administration of product formulations containing 2.0% Stearic Acid.

In 13-week dermal toxicity studies, two cosmetic product formulations containing, at most, 5% Stearic Acid produced moderate skin irritation in rats receiving 4.0 ml/kg and 227 mg/kg doses. All other physiological parameters were normal.

In single-insult occlusive patch tests for primary irritation, commercial grades of all five fatty acids (Oleic, Stearic, Myristic, Lauric, and Palmitic), at doses of 35 to 65% in vehicles (Stearic Acid only) and at 1 to 13% in cosmetic product formulations (other fatty acids), produced none to moderate erythema and slight, if any, edema in the skin of rabbits. Slight increases in

irritation were observed in the short-term repeated insult patch tests (daily for 3 to 14 days) of Oleic and Myristic Acids.

In maximization studies with two cosmetic product formulations containing 5.08% Oleic Acid and 1.0% Stearic Acid, slight reactions to challenge patches were observed. These formulations were considered weak, grade 1, sensitizers. In another maximization study, after intradermal induction and booster injections of a formulation containing 3.5% Stearic Acid, reactions to topical challenge applications of the formulation were few and minimal in intensity.

Skin lotion formulations containing 2.8% Stearic Acid were not photosensitizing to the skin of Hartley guinea pigs.

Oleic Acid and its UVA-induced peroxides were associated with increased comedone formation on the treated ears of two species of rabbits.

In ocular irritation studies, the fatty acids alone and at concentrations ranging from 1 to 19.4% in cosmetic product formulations produced no to minimal irritation after single and multiple (daily, 14-day) instillations into the eyes of albino rabbits. Irritation was primarily in the form of very slight conjunctival erythema. A single instillation of Lauric Acid also produced corneal opacity and iritis.

Although Oleic and Lauric Acids induced mitotic aneuploidy in in vitro mutagenicity tests, both have been noted as inhibitors of mutagenicity produced by positive controls, such as *N*-nitrosopyrrolidine and sodium azide, in other tests. Stearic Acid was inactive in aneuploidy induction tests and in the Ames test, and it did not inhibit mutagenicity, as did Oleic and Lauric Acids. No increase in mitotic crossing-over events was induced by Oleic, Lauric, or Stearic Acids. Oleic Acid did not increase the number of sister chromatid exchanges over background.

In carcinogenicity studies, no malignant tumors were induced by repeated subcutaneous injections of 1 to 16.5 mg Oleic Acid in two species of mice. Intestinal and gastric tumors were found in mice receiving dietary Oleic Acid at daily concentrations up to 200 mg/mouse. Repeated subcutaneous injections of 25 and 50 mg Lauric Acid into mice were not carcinogenic. Low incidences of carcinomas, sarcomas, and lymphomas were observed in mice receiving single or repeated subcutaneous injections of 25 and 50 mg Palmitic and up to 82 mg Stearic Acid. Stearic Acid fed to mice in dietary doses of up to 50 g/kg/day was not carcinogenic.

In clinical primary and cumulative irritation studies, Oleic, Myristic, and Stearic Acids at concentrations of 100% or 40 to 50% in mineral oil were nonirritating. Mild to intense erythema in single-insult occlusive patch tests, soap chamber tests, and 21-day cumulative irritation studies were produced by cosmetic product formulatipns containing 2 to 93% Oleic, Palmitic, Myristic, or Stearic Acid and were generally not related to the fatty acid concentrations in the formulations.

In clinical repeated-insult patch tests (open, occlusive, and semiocclusive), maximization tests, and prophetic patch tests with cosmetic product formulations containing Oleic, Lauric, Palmitic, and Stearic Acids at concentrations ranging from <1 to 13%, no primary or cumulative irritation or sensitization was reported. A few subjects (<5% of approximately 4000 subjects tested) reacted to a few, isolated induction patches. Slight, if any, reactions were observed after challenge patching at original or adjacent sites on the upper backs or forearms of some subjects (≲2%). Intensity of observed reactions to the formulations was not directly related to the concentrations of the fatty acid ingredients.

Cosmetic product formulations containing 1 to 13% Oleic, Palmitic, or Stearic Acid produced no photosensitization in human subjects. Slight reactions to a few induction patches were observed.

No treatment-related ocular irritation was observed in female subjects, some of whom wore contact lenses, involved in two 3-week exaggerated-use studies of mascara formulations containing 2 and 3% Oleic Acid. These formulations were used in combination with other eye area cosmetics.

DISCUSSION

With the exceptions of two skin irritation studies and a comedogenicity study on Propylene Glycol Dicaprylate/Dicaprate and a skin irritation study and acute oral toxicity study on Propylene Glycol Laurate, no other studies on the toxicity of the Propylene Glycol esters or diesters included in this review have been found. However, the CIR Expert Panel has issued Final Reports on the safety of Propylene Glycol, Propylene Glycol Stearate, and other chemical moieties of the Propylene Glycol esters and diesters included in the present review and, because of chemical similarities, determined that the data included in these Final Reports are sufficient for evaluating the safety of the following thirteen Propylene Glycol esters and diesters: Propylene Glycol Dicaprylate; Propylene Glycol Dicaprylate/Dicaprate; Propylene Glycol Dicocoate; Propylene Glycol Dipelargonate; Propylene Glycol Isostearate; Propylene Glycol Laurate; Propylene Glycol Myristate; Propylene Glycol Oleate; Propylene Glycol Oleate SE (self-emulsifying); Propylene Glycol Dioleate; Propylene Glycol Dicaprate; Propylene Glycol Diisostearate; and Propylene Glycol Dilaurate.

Accordingly, data from the following CIR Final Reports were considered in the present safety assessment: Propylene Glycol (Andersen 1994); Propylene Glycol Stearate and Propylene Glycol Stearate SE (Elder 1983a); Caprylic/Capric Triglyceride (Elder 1980); Coconut Acid (Elder 1986); Isostearic Acid (Elder 1983b); and Lauric Acid, Myristic Acid, and Oleic Acid (Elder 1987). The CIR Expert Panel concluded that Propylene Glycol is safe at concentrations up to 50%, and that the remaining ingredients are safe in the present practices of use. Except for Caprylic/Capric Triglyceride, most of these ingredients can be easily identified (by name) as components of one or more of the 13 Propylene Glycol esters and diesters reviewed in this report. The Caprylic/Capric moiety of Caprylic/Capric Triglyceride is also similar to the dipelargonate moiety of Propylene Glycol Dipelargonate. Propylene Glycol Dipelargonate is the

diester of propylene glycol and pelargonic acid ($C_9H_{18}O_2$), and pelargonic acid is similar to caprylic acid ($C_8H_{16}O_2$) and capric acid ($C_{10}H_{20}O_2$).

The more crucial studies that were used in arriving at the safe as used ingredient conclusions in the CIR Final Reports noted above are as follows: Propylene Glycol Stearate (mutagenicity, chronic toxicity, and skin sensitization); Caprylic/Capric Triglyceride (reproductive toxicity, chronic toxicity, and skin sensitization); Coconut Acid (chronic toxicity, tumor promotion, and skin sensitization, phototoxicity, and photosensitization); Isostearic Acid (skin sensitization, photosensitization, and phototoxicity); and Lauric Acid, Myristic Acid, and Oleic Acid (reproductive toxicity, carcinogenicity, and skin sensitization and photosensitization). The 50% concentration limit on Propylene Glycol is based on the CIR Expert Panel's assessment of the skin irritation potential of this cosmetic ingredient. In consideration of this limitation relative to the review of Propylene Glycol esters and diesters, the Panel noted that use concentrations of these ingredients should not be limited, even though certain Propylene Glycol diesters are used in cosmetics at concentrations as high as 51.7%. This decision is based on data from a chemical supplier indicating that Propylene Glycol Laurate does not contain any free Propylene Glycol, and the assumption that this is true of other Propylene Glycol esters and diesters.

The Expert Panel recognizes that, reportedly, Propylene Glycol Dicaprylate/Dicaprate and Propylene Glycol Dipelargonate can enhance the skin penetration of other chemicals, and recommends that care should be exercised in using these and other Propylene Glycol esters and diesters in cosmetic products.

CONCLUSION

Based on the available animal and clinical data included in this report and data from CIR Final Reports on chemically similar cosmetic ingredients/ingredient moieties (Propylene Glycol, Propylene Glycol Stearate, Propylene Glycol Stearate SE, Caprylic/Capric Triglyceride, Coconut Acid, Isostearic Acid, Lauric Acid, Myristic Acid, and Oleic Acid) that are referenced in the report discussion, the CIR Expert Panel concludes that Propylene Glycol Dicaprylate, Propylene Glycol Dicaprylate/Dicaprate, Propylene Glycol Dicocoate, Propylene Glycol Dipelargonate, Propylene Glycol Isostearate, Propylene Glycol Laurate, Propylene Glycol Myristate, Propylene Glycol Oleate, Propylene Glycol Oleate SE, Propylene Glycol Dioleate, Propylene Glycol Dicaprate, Propylene Glycol Diisostearate, and Propylene Glycol Dilaurate are safe as cosmetic ingredients in the present practices of use.

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HIGH PRODUCTION VOLUME (HPV)

CHEMICAL CHALLENGE PROGRAM

TEST PLAN

FOR THE GLYCOL ESTERS CATEGORY OF THE ALIPHATIC ESTERS CHEMICALS

Prepared by:

American Chemistry Council's
Aliphatic Esters Panel

December 24, 2003

(With corrected text and pagination)

EXECUTIVE SUMMARY

The American Chemistry Council's (ACC) Aliphatic Esters Panel (Panel) hereby submits the revised test plan for the "glycol esters" category of the "aliphatic esters" chemicals, under the High Production Volume (HPV) Chemical Challenge Program. The Panel has used existing available public and company data in conjunction with scientific judgment/analysis to characterize the Screening Information Data Set (SIDS) of human health, environmental fate and effects, and physicochemical property endpoints for the glycol esters category.

This test plan addresses the nine HPV glycol esters chemicals listed in Table 1A. The distinguishing feature of this category of chemicals is that they are ester derivatives of ethylene glycol and propylene glycol (the alcohol portion of the ester molecule). Fatty acids (C6-C18) make up the carboxylic acid portion of the ester molecule and include the naturally occurring fatty acids, oleic and stearic acids. The HPV glycol esters cover the C20-C41 carbon number range. The commonalities of the ethylene glycol or propylene glycol substructure and the fatty acids (e.g., C6-10 fatty acids, oleic, stearic and isostearic acids) are the main reason for grouping these HPV glycol esters together. The glycol esters in this category find commercial use as lubricants, cosmetic ingredients, emulsifiers or solvents.

The chemical and structural similarities of the glycol esters listed in Table 1A justify grouping these nine HPV chemicals collectively together under the glycol esters category of the aliphatic esters. They have close commonalties in their physicochemical properties, chemical characteristics and biological/toxicological activities as a result of the structural glycol ester similarities in their molecules. Grouping these glycol esters together also represents a rational structural approach: (1) to systematically compare existing data; (2) to justify read-across assessments for structurally related or analogous glycol esters, and (3) to develop a stepwise strategy test plan for the glycol esters substances based on their ester group type. The glycol esters as an ester group type are structurally differentiated from other aliphatic ester types such as diacid esters, polyol esters, and sorbitan esters.

There was published information for five structurally analogous surrogate glycol esters, which provided useful supplementary data to help bridge the toxicity data for the HPV glycol esters. The five structurally analogous surrogate glycol esters are: [1] heptanoic acid, ester with 2,2,4-trimethyl-1,3-pentanediol (CAS 71839-38-8); [2] triethylene glycol, diheptanoate (CAS 7434-40-4); [3] propylene glycol, monostearate (CAS 1323-39-3); [4] propylene glycol, dilaurate (CAS 22788-19-8]; and [5] propylene glycol, diisostearate (CAS 68958-54-3). It should be pointed out that the propylene glycol stearates, oleates and laurates as well as polyethylene glycol (PEG) fatty acid esters [which are commonly used in many cosmetics] are very structurally similar to many of the HPV glycol esters substances and have low degrees of toxicity. (For example, it is noteworthy that propylene glycol stearate has been used in many pharmaceutical applications and is "Generally Recognized as Safe" (GRAS) in food applications.) Thus, the surrogate glycol esters provided useful toxicity information for read-across assessments of the HPV glycol esters.

Measured physicochemical property data were available for the HPV and surrogate glycol esters. Computer estimation models were used to calculate physicochemical property and environmental fate data for the glycol esters. The calculated data were obtained using the EPIWIN and EQC (Level III) models that the EPA has cited for use in the HPV Chemical Challenge Program. Use of

the calculated and experimental values for HPV substances and for the surrogate glycol esters provided the information on the physicochemical and environmental fate properties of the chemicals in the glycol esters category to satisfy HPV program requirements. No additional testing for physicochemical and environmental fate properties is proposed.

Aquatic toxicity and biodegradation data exist for both the HPV glycol esters and the structurally analogous surrogate glycol esters to sufficiently allow for read-across assessments of the HPV substances and for bridging data. In addition, there are published data which indicate that the constituent free ethylene and propylene glycols and free fatty acids, generated from enzymatic ester cleavage of the parent glycol esters, are expected to be extensively biodegraded and to have low degrees of aquatic toxicity. No further aquatic toxicity and biodegradation testing is proposed for glycol esters category of the aliphatic esters.

There were sufficient existing toxicity data for the HPV and structurally related surrogate glycol esters to make hazard assessments for mammalian health effects (SIDS data endpoints) for the HPV glycol esters substances. Given the similar chemical and structural features between the HPV and surrogate glycol esters (including the structurally analogous polyethylene glycol or propylene glycol fatty acid esters), it was justifiable to utilize the available existing data to make read-across assessments on potential toxicity and to bridge toxicity data for the HPV substances. No additional mammalian toxicity testing is proposed for substances in the glycol esters category. This resourceful use of existing data will help minimize the use of animals for testing while assessing the potential hazards in the glycol esters category of the aliphatic esters. Taken into consideration in the assessment were the published health safety assessments for thirteen propylene glycol fatty acid esters [Andersen, 1999a] as well as the multigeneration feeding studies for several polyethylene glycol fatty acid esters [Oser *et al.* (1956b), Elder (1983b)]. A technical discussion was provided to address the reproductive/developmental potential of the HPV glycol esters, based on the published data that have been reported for related polyethylene glycol monostearates.

The following member companies of the American Chemistry Council's Aliphatic Esters Panel are sponsoring the Glycol Esters category:

LIST OF MEMBER COMPANIES

BASF Corporation

Cognis Corporation

C.P. Hall Company

Crompton Corporation

E.I. duPont de Nemours & Company, Inc.

Goldschmidt Chemical Corporation

Inolex Chemical Company

Kaufman Holdings Corporation

Quaker Chemical Company

Stepan Company

Uniqema

TABLE OF CONTENTS

Executive Summary ii

List of Member Companies iv

Table of Contents v

1.0 Introduction 1

2.0 Description of the Glycol Esters Category 2

3.0 Description of Available Public and Company Data 8

3.1 Physicochemical Properties Data 8
3.2 Environmental Fate and Biodegradation Data 9
3.3 Aquatic Toxicity Data 9
3.4 Mammalian Toxicity Data 9

4.0 Evaluation of Existing Data 10
4.1 Physicochemical Properties Data 10
4.2 Environmental Fate and Biodegradation Data 11
4.3 Aquatic Toxicity Data 12
4.4. Mammalian Toxicity Data 14
A) Acute Mammalian Toxicity 14
B) Mutagenicity and Genotoxicity 15
C) Repeated-Dose Toxicity 16
D) Reproductive/Developmental Toxicity 17

5.0 Test Plan Summary 21

6.0 References 24
7.0 List of Tables and Figures 29

Table 1A List of Individual Substances in the Glycol Esters Category 1
Table 1B Organization of the Nine HPV Glycol Esters according to Total Carbon Number 3
Table 1C Organization of Nine HPV Glycol Esters and Five Surrogate Glycol Esters According to Total Carbon Number for Use in HPV Data Assessment and Testing Rationale 4
Table 2 Summary Table of Physicochemical and Environmental Fate Data for the Glycol Esters 30
Table 3 Summary Table of Mammalian Health Effects, Ecotoxicity and Biodegradation Data for the Glycol Esters 31
Table 4 Assessment Plan for Substances in Glycol Esters Category under the HPV Program 21

Figure 1 Chemical Structure of the Glycol Esters Listed in Table 1A 5
Figure 2 Chemical Structure of Surrogate Glycol Esters 7

Appendix - Robust Summaries for Glycol Esters

Part I. HPV Substances in the Glycol Esters Category Test Plan
Part II. Surrogate Glycol Esters

TEST PLAN FOR THE GLYCOL ESTERS CATEGORY OF THE ALIPHATIC ESTERS

1.0 INTRODUCTION

The American Chemistry Council's (ACC) Aliphatic Esters Panel (Panel) has committed voluntarily to develop a Screening Information Data Set (SIDS) (i.e., physicochemical data, environmental fate and effects, and human health effects) for the "glycol esters" category of aliphatic esters chemicals, listed under the High Production Volume (HPV) Chemical Challenge Program. This test plan sets forth how the Aliphatic Esters Panel intends to address the testing information for the nine glycol esters listed in Table 1A (organized by CAS Numbers in ascending order).

The Panel added one chemical, Hexanoic acid, 2-ethyl, diester with triethylene glycol (94-28-0), to the original glycol esters group of chemicals. The other chemicals in this test plan were originally part of a larger test plan submitted on December 20, 2001. As a result of comments, the Panel has revised the original test plan for these chemicals, and the revised approach follows below.

The chemical structures of the glycol esters are given in Figure 1. The test plan identifies the CAS numbers used to characterize the SIDS endpoints for the glycol esters in this category, describes the chemical and structural features/similarities of the glycol esters, identifies existing data of adequate quality for substances in the glycol esters category and provides the Panel's rationale for applying the available SIDS data to characterize the hazards of the category members. The primary objective of this effort is to identify and to characterize the physicochemical properties, mammalian health and environmental fate and effects for the glycol esters category of the aliphatic esters consistent with the EPA HPV Program.

The data from this HPV category will be used to inform the public about the potential health effects of the glycol esters category of the aliphatic esters. Developing a data matrix with reliable studies and applying justifiable read-across assessments will help provide a sufficiently robust data set to characterize the endpoints in the HPV Chemical Challenge Program. This approach to the resourceful use of existing data will help minimize the use of animals for testing while assessing the potential hazards in the glycol esters category of the aliphatic esters.

Table 1A: List of Individual Substances in the Glycol Esters Category
(by ascending CAS Numbers and designated TSCA HPV chemical name)

Chemical Name (designated TSCA HPV chemical name)	**CAS Number**
Hexanoic acid, 2-ethyl-, diester with triethyl ene glycol *	94-28-0
Oleic acid, propylene ester	105-62-4
Stearic acid, 2-hydroxyethyl ester	111-60-4
Stearic acid, ethylene ester	627-83-8
Hexanoic acid, 2-ethyl-, diester with tetraethyl ene glycol	18268-70-7
9-Octadecenoic acid (Z)-, 2,2-dimethyl-1,3-propanediyl ester	42222-50-4
9-Octadecenoic acid (Z)-, ester with 2,2-dimethyl-1,3-propanediol	67989-24-6
Decanoic acid, mixed diesters with octanoic acid and triethylene glycol	68583-52-8
Heptanoic acid, oxybis(2,1-ethanediyloxy-2,1-ethanediyl) ester	70729-68-9

*This chemcial was added to glycol esters category.

2.0 DESCRIPTION OF THE GLYCOL ESTERS CATEGORY

Nine CAS Numbers are used to describe the glycol esters in this HPV category of the aliphatic esters (Table 1A). The glycol esters category of the HPV aliphatic esters is comprised of aliphatic esters derived from a monocarboxylic acid (e.g., C6-C10 fatty acids, oleic, stearic and isostearic acids) and a dihydroxy alcohol (glycol or diol such as ethylene glycol, polyethylene glycol, propylene glycol, 2,2-dimethyl-1,3-propanediol). These esters are often referred to as "glycol or diol esters" or as "alkylidene or alkanediyl esters."

The rationale for grouping the glycol or diol esters is that they represent structurally similar ethylene/propylene glycol esters in which the hydroxyl groups in the glycol are functionalized with fatty acids as ester derivatives. Esterification of the glycol with fatty acids such as stearic and oleic acid can provide glycol diesters in the 38 to 41 carbon number range, which typically make them relatively non-volatile and high boiling liquids with limited water solubility and with sufficient polar characteristics to make them useful as lubricants and solvents. In the case of the tri- and tetraethylene glycol diesters, the ether linkage in the polyalkylene portion of the glycol also imparts additional polar character to these glycol esters (Reck, 1999).

Metabolism of the HPV glycol esters in animals would be expected to occur initially via enzymatic hydrolysis leading to the corresponding free fatty acids and free glycol alcohols (e.g., ethylene glycol, propylene glycol, 2,2-dimethyl-1,3-propanediol, polyethylene glycol) [Long *et al.* (1958b); Elder (1982; 1983a); Andersen (1999a)]. These free fatty acids and glycols can be further metabolized or conjugated (e.g., glucuronides, sulfates, etc.) to polar products that are excreted in the urine [Long *et al.* (1958a); Bisesi (2001); Cragg (2001a,b); Bevan (2001b); Thurman (1992)]. The fatty acids, especially the natural occurring ones such as stearic and oleic acids, have low degrees of toxicity [Cragg (2001a,b); Elder (1986, 1987); Chow (1999)]. The toxicity of the alkylidene or alkanediyl glycols has been extensively reviewed, especially in case of ethylene glycol [ATSDR (1997); Cavender (2001); Andersen (1999b)] and propylene glycol [Andersen (1994); Hardman *et al.* (2001); NTIS (1973)].

Metabolic hydrolytic reactions of esters have been extensively reviewed in the literature [Testa and Mayer (2003); Bisesi (2001); Buchwald (2001); Parkinson (2001); Heyman (1982); Long *et al.* 1958a,b)]. It is beyond the scope of this test plan to discuss or review this topic in more detail except to mention its contribution in the general metabolism scheme for ester linkages.

Organization of HPV Glycol Esters and Surrogate Glycol Esters

Due to the number of substances in this category, it is useful to organize the nine HPV glycol esters on the basis of total carbon numbers rather than in the order of their CAS numbers as in Table 1A. Hence, Table 1B below has been organized in that manner.

Table 1B. Organization of the Nine HPV Glycol Esters according to Total Carbon Number in the Glycol Ester

Individual Glycol Ester (organized according to total carbon number) Chemical Name (designated TSCA HPV names)	CAS Number	Carbon Number in Acid	Carbon Number in dihydroxy alcohol	Total Carbons in Glycol Ester	MW
Stearic acid, 2-hydroxyethyl ester	111-60-4	C18	C2	C20	329
Hexanoic acid, 2-ethyl-, diester with triethylene glycol	94-28-0	C8	C6	C22	403
Heptanoic acid, oxybis(2,1-ethanediyloxy-2,1-ethanediyl) ester	70729-68-9	C7	C8	C22	419
9-Octadecenoic acid (Z)-, ester with 2,2-dimethyl-1,3-propanediol	67989-24-6	C18	C5	C23	368
Decanoic acid, mixed diesters with octanoic acid and triethylene glycol (use average of C9*)	68583-52-8	C9 *	C6	C24	431
Hexanoic acid, 2-ethyl-, diester with tetraethylene glycol	18268-70-7	C8	C8	C24	447
Stearic acid, ethylene ester	627-83-8	C18	C2	C38	595
Oleic acid, propylene ester	105-62-4	C18	C3	C39	605
9-Octadecenoic acid (Z)-, 2,2-dimethyl-1,3-propanediyl ester	42222-50-4	C18	C5	C41	633

* An average of C9 carbon atoms was used for the fatty acid in the mixed diester of decanoic and octanoic acids of triethylene glycol (CAS 68583-52-8)

There are relevant published or unpublished toxicity data that also exist for five structurally homologous or analogous glycol esters (denoted as "surrogate glycol esters") which provide very useful read-across information to help bridge the toxicity data for the HPV substances.

The five structurally analogous surrogate glycol esters are:

- Heptanoic acid, ester with 2,2,4-trimethyl-1,3-pentanediol (CAS 71839-38-8)
- Triethylene glycol, diheptanoate (CAS 7434-40-4)
- Propylene glycol, monostearate (CAS 1323-39-3)
- Propylene glycol, dilaurate (CAS 22788-19-8)
- Propylene glycol, diisostearate (CAS 68958-54-3)

Incorporation of these five surrogate glycol esters into Table 1B leads to Table 1C below, which is useful in the overall HPV data review and test plan evaluation and provides reasonable justification (based on total carbon number, structural or MW similarities, etc.) to support read-across health effects and environmental fate/toxicity assessments.

Table 1C. Organization of the Nine HPV Glycol Esters and Five Surrogate Glycol Esters According to Total Carbon Number for Use in HPV Data Assessment and Testing Rationale**

Individual Glycol Ester (organized according to total carbon number) Chemical Name (designated TSCA HPV names)	CAS Number	Carbon Number in Acid	Carbon Number in dihydroxy alcohol	Total Carbons in Glycol Ester	MW
Heptanoic acid, ester with 2,2,4-trimethyl-1,3-pentanediol **	71839-38-8	C7	C8	C15	258
Stearic acid, 2-hydroxyethyl ester	111-60-4	C18	C2	C20	329
Triethylene glycol, diheptanoate **	7434-40-4	C7	C6	C20	375
Propylene glycol, monostearate **	1323-39-3	C18	C3	C21	343
Hexanoic acid, 2-ethyl-, diester with triethylene glycol	94-28-0	C8	C6	C22	403
Heptanoic acid, oxybis(2,1-ethanediyloxy-2,1-ethanediyl) ester	70729-68-9	C7	C8	C22	419
9-Octadecenoic acid (Z)-, ester with 2,2-dimethyl-1,3-propanediol	67989-24-6	C18	C5	C23	368
Decanoic acid, mixed diesters with octanoic acid and triethylene glycol	68583-52-8	C9	C6	C24	431
Hexanoic acid, 2-ethyl-, diester with tetraethylene glycol	18268-70-7	C8	C8	C24	447
Propylene glycol dilaurate**	22788-19-8	C12	C3	C27	441
Stearic acid, ethylene ester	627-83-8	C18	C2	C38	595
Oleic acid, propylene ester	105-62-4	C18	C3	C39	605
Propylene glycol diisostearate**	68958-54-3	C18	C3	C39	609
9-Octadecenoic acid (Z)-, 2,2-dimethyl-1,3-propanediyl ester	42222-50-4	C18	C5	C41	633

** The five surrogate glycol esters (highlighted or shaded) are not part of the present HPV glycol esters category test plan. They are included in this matrix table since existing toxicity data for these materials can be used for read-across assessment or for bridging data to the HPV glycol esters category members based on chemical /structural similarities.

Figure 1. Chemical Structure of the Glycol Esters Listed in Table 1A

The structures of the HPV glycol esters are given in the order listed in Table 1A, which is organized according to ascending CAS Numbers. The chemical structure depicted for each HPV substance is consistent with the designated CAS Number and is considered representative of the commercial product evaluated.

Hexanoic acid, 2-ethyl-, diester with triethylene glycol (CAS 94-28-0)

Oleic acid, propylene ester (CAS 105-62-4)

Stearic acid, 2-hydroxyethyl ester (CAS 111-60-4)

Stearic acid, ethylene ester (CAS 627-83-8)

Hexanoic acid, 2-ethyl-, diester with tetraethylene glycol (CAS 18268-70-7)

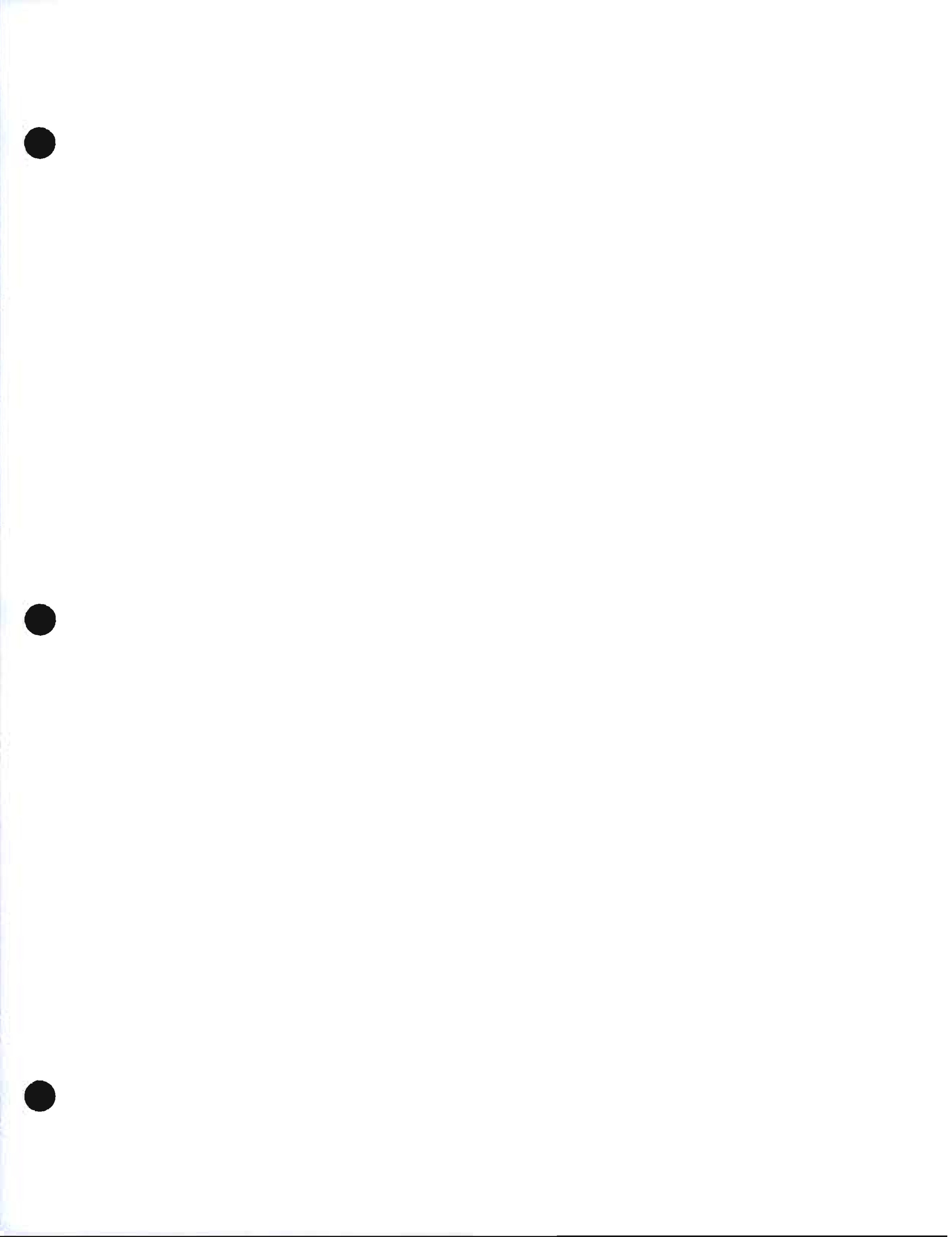

Figure 2. Chemical Structure of Surrogate Glycol Esters

Heptanoic acid, ester with 2,2,4-trimethyl-1,3-pentanediol (CAS 71839-38-8)

OH O O

Triethylene glycol, diheptanoate (CAS 7434-40-4)

O O O O O O

Propylene glycol, monostearate (CAS 1323-39-3)

O O OH

Propylene glycol, dilaurate (CAS 22788-19-8)

O O O O

Propylene glycol, diisostearate (CAS 68958-54-3)

O O O O

3.0 DESCRIPTION OF AVAILABLE PUBLIC AND COMPANY DATA

A review of the literature and confidential company data was conducted on the physicochemical properties, mammalian toxicity endpoints, and environmental fate and effects for the nine glycol esters using CAS numbers and chemical names. Searches included the following sources: MEDLINE and TOXLINE databases; the TSCATS database for relevant unpublished studies on these chemicals; and standard handbooks and databases (e.g., Sax, CRC Handbook of Chemistry and Physics, IUCLID, Merck Index, and other references) for physicochemical properties.

The reports were selected for review based on the following criteria: relevant SIDS endpoint, relevant CAS number, final report of company study (TSCATS), peer reviewed journal, or comprehensive reviews. Safety assessment reviews for various ethylene glycol or propylene glycol fatty acid esters and related polyethylene glycol (PEG) fatty acid esters have been carried out by the Cosmetic Ingredient Review Expert Panel in the Journal of the American College of Toxicology [Elder (1982, 1983a, 1983b); Andersen (1994)] and in the International Journal of Toxicology [Andersen (1999a); Andersen (2000)]. Five surrogate glycol esters that were chemically or structurally-related or analogous to the HPV glycol esters were also reviewed to identify available published or unpublished data to help bridge data for environmental fate, aquatic toxicity or mammalian toxicity.

3.1 Physicochemical Properties Data

Physicochemical data [i.e., melting point, boiling point, vapor pressure, water solubility and octanol-water partition coefficient] for the HPV glycol esters and surrogate glycol esters were obtained from the searches and sources described above. In addition to available experimental and measured data, calculated physicochemical values were also incorporated into a summary table for all these physical and chemical properties. There are a number of reasons for this approach:

- The EPA guidance (www.epa.gov/chmrtk/robsumgd.htm) allows inclusion of calculated values in the robust summaries for physicochemical elements.
- A complete set of physical property data was a prerequisite to calculate fugacity or the chemical distribution in the environment (see below)
- Physicochemical properties data had yet to be developed for some of the glycol esters.

The physicochemical properties were also modeled using the Syracuse Research Corp./EPA computer program EPIWIN, a modeling package that includes a number of algorithms developed for the EPA [EPIWIN (1999); US EPA (1999b)]. EPIWIN is the program used and advocated by the EPA. Because the model is a structure-property model, a specific discreet structure is required. EPIWIN contains a CAS number database, which contains the structures for a large number of chemicals. For mixtures, a single representative structure is contained in the database, and in this test plan these surrogate chemical structures were accepted for further modeling.

3.2 Environmental Fate and Biodegradability Data

Environmental fate data including biodegradability, photodegradation, stability in water (i.e., hydrolysis) and fugacity (chemical distribution in the environment) data were primarily obtained through the literature, from unpublished confidential company data, or from modeling [e.g., EPIWIN, EQC (Level III) - Mackay et al. (1996)]. When relevant studies (particularly biodegradability endpoints) were identified, the study reports were reviewed, robust summaries were prepared and the reliability of the data was assessed. The method of Klimisch *et al.* (1997) was utilized to evaluate the data quality of the studies.

3.3 Aquatic Toxicity Data

Existing data for aquatic toxicity studies (e.g., fish, invertebrate and algae) for the HPV and surrogate glycol esters were obtained primarily from the literature or from unpublished confidential proprietary studies. When relevant studies were identified, the study reports were reviewed, robust summaries were prepared and the reliability of the data was assessed. The method of Klimisch *et al.* (1997) was utilized to evaluate the data quality of the aquatic toxicity studies.

3.4 Mammalian Toxicity Data

The existing data for the mammalian toxicity endpoints for the HPV and surrogate glycol esters were reviewed using the literature searches to identify the most relevant studies for the substances in the glycol esters category. For some substances, there may have been no relevant studies identified in the searches. For the HPV glycol esters that contained relevant data, the available studies were reviewed using the criteria outlined in the EPA's methods for determining the data quality and adequacy of the existing data and the reliability ranking method of Klimisch *et al.* (1997). Relevant studies that were available for the mammalian toxicity endpoints are summarized in the HPV test plan and presented in greater detail in the robust summaries in the Appendix.

Studies that were selected for the robust summaries generally represented the most relevant or reliable data for a particular SIDS endpoint. Published studies from the general literature as well as from a number of unpublished confidential company reports were obtained and summarized. Some of the reported studies (particularly older acute data) could not be summarized because of limited experimental details to assess their quality (i.e., not assignable, Klimisch reliability code 4) or only were reported as LD_{50} values from secondary sources. These studies were included in the summary data table and may be included in the robust summaries with reference to the secondary literature source.

4.0 EVALUATION OF EXISTING DATA

The nine HPV substances in Table 1A were grouped together under the glycol esters category of aliphatic esters because of the presence of the diol or glycol functionality that was common to all the HPV glycol esters. The HPV substances were fatty acid (i.e., C6-18 fatty acids which include the naturally occurring fatty acids, stearic and oleic acids) ester derivatives of ethylene glycols (EG), propylene glycols (PG) or polyethylene glycols (PEG). In addition to the existing data for the HPV substances, there were read-across data for five surrogate glycol esters not on the HPV list in this category. Because of their structural similarities, these five surrogate glycol esters provided useful data for bridging toxicity information for structurally analogous HPV glycol esters in regards to mammalian toxicity, aquatic toxicity and biodegradability endpoints.

The five surrogate glycol esters were:

- Heptanoic acid, ester with 2,2,4-trimethyl-1,3-pentanediol (CAS 71839-38-8)
- Triethylene glycol, diheptanoate (CAS 7434-40-4)
- Propylene glycol, monostearate (CAS 1323-39-3)
- Propylene glycol, dilaurate (CAS 22788-19-8)
- Propylene glycol, diisostearate (CAS 68958-54-3)

The existing data for the HPV glycol esters and for the surrogate glycol esters have been reviewed. Discussion will be provided in this section regarding the available data for SIDS toxicity endpoints, an assessment and summary of the data, and comments on HPV test plan as to whether the existing data are adequate and whether further testing is needed or planned. The order of discussion of endpoints will be: (1) physicochemical properties; (2) environmental fate and biodegradability; (3) aquatic toxicity; and (4) mammalian health effects.

4.1 Physicochemical Properties Data

Summary of Physicochemical Properties Data

The physicochemical properties for the HPV glycol esters and surrogate glycol esters are summarized in Table 2. EPIWIN was used to calculate the physicochemical properties for the nine HPV glycol esters as well as for the five surrogate glycol esters. The experimental data and calculated (EPIWIN) data for the physicochemical properties of the glycol esters are summarized in Table 2.

Data Assessment and Test Plan for Physicochemical Properties

In general, the glycol monoesters with shorter carbon-number fatty acids (C6-C7) were predicted to be more water-soluble and less lipophilic than the corresponding glycol monoesters containing long-chain fatty acids such as stearic and oleic acids. The glycol diesters were predicted to be more lipophilic and less water-soluble than the corresponding glycol monoesters [e.g., ethylene glycol distearate (CAS 627-83-8) *versus* its monostearate (CAS 111-60-4); 2,2-dimethyl-1,3-propanediol dioleate (CAS 42222-50-4) *versus* its monooleate (CAS 67989-24-6)]. In addition, the glycol diesters have higher boiling points than the corresponding monoesters.

Polyethylene glycol (or polyoxyethylene) esters, that contain more than one repeating ethylene glycol unit, generally showed greater water solubility than the corresponding monoethylene glycol esters, owing to the increased polarity of multiple ether linkages; this was consistent with what would be expected (Reck, 1999). The greater degree of ether linkage was also consistent with the lower lipophilicity (log P) values predicted by EPIWIN.

Most of the glycol esters on the HPV list have molecular weights of greater than 300, have high boiling points (>400 °C) and showed very low water solubility and high lipophilic characteristics (log P >4 or 5). The glycol distearates and dioleates had total carbon numbers above C38 and high predicted boiling points (>550 °C) (Table 2).

The five surrogate glycol esters were selected because they were structurally similar to the nine HPV glycol esters. These surrogate glycol esters were examined and their experimental and calculated (EPIWIN) data were used to help assess the physicochemical properties expected for the HPV glycol esters.

Based on the summarized data in Table 2, there are sufficient physicochemical data to characterize the substances in the glycol esters category and no additional testing is proposed.

4.2 <u>Environmental Fate and Biodegradability Data</u>

Summary of Environmental Fate and Biodegradability Data

The environmental fate and biodegradability data relevant to the glycol esters category are summarized in Table 2 and Table 3, respectively. Biodegradation testing has been carried out for two HPV glycol esters [heptanoic acid, oxybis(2,1-ethanediyloxy-2,1-ethanediyl) ester (CAS 70729-68-9) and 9-octadecenoic acid (Z)-, ester with 2,2-dimethyl-1,3-propanediol (CAS 67989-24-6)] and for two surrogate glycol esters [heptanoic acid, ester with 2,2,4-trimethyl-1,3-pentanediol (CAS 71839-38-8) and triethylene glycol, diheptanoate (CAS 7434-40-4)].

Other environmental fate endpoints such as photodegradation, stability in water (hydrolysis), and chemical distribution (transport) in the environment (fugacity modeling) have been calculated for the glycol esters using EPIWIN. Calculated hydrolysis half-lives and atmospheric photodegradation rates for the glycol esters using EPIWIN are summarized in Table 2.

Chemical distribution of the glycol esters in the environment has been determined using the EQC (Level III) fugacity-based multimedia model [Mackay *et al.* (1996)]. The calculated values for the transport (or distribution) in the soil, air, water and sediment environmental compartments are summarized in Table 2.

Data Assessment and Test Plan for Environmental Fate and Biodegradability

Biodegradation studies with two HPV and two surrogate glycol esters have been reported. The extent of biodegradation has been reported to range from 65% to 98% in 28 days for the four glycol esters. These results indicate that the glycol esters are rapidly and extensively biodegraded. The tested substances covered the C15-C23 carbon range for the glycol esters. Glycol esters above C30 appear to be mainly comprised of the glycol diesters such as the dioleates and distearates, and several of the HPV substances are simply to be diester homologs of the corresponding monooleate or monostearate esters. These diesters are expected to be microbi-

ally metabolized in the environment to the corresponding monoesters, some of which have been reported to be extensively biodegraded. For example, the glycol monooleate ester, 9-octadecenoic acid (Z)-, ester with 2,2-dimethyl-1,3-propanediol (CAS 67989-24-6) has been demonstrated to be readily biodegradable in the OECD 301B modified Sturm test. Since 9-octadecenoic acid (Z)-, 2,2-dimethyl-1,3-propanediyl (di)ester (CAS 4222-50-4) is the corresponding diester derivative of the above glycol monooleate (i.e., CAS 67989-24-6), it is not unexpected that the dioleate would be extensively biodegraded as well. This is based on the premise that the dioleate would be expected to be microbially metabolized (hydrolyzed) to the monooleate, which has already been found to be readily biodegradable.

Biodegradation or enzymatic (microbial) breakdown of the glycol esters would be expected to ultimately lead to the free glycol such as propylene glycol, ethylene glycol, polyethylene glycol and to the corresponding free fatty acids, including stearic acid and oleic acid. There are adequate biodegradability data in the scientific literature to support the premise that these constituent components, namely, the free glycols and the free fatty acids, would be expected to be rapidly and extensively biodegraded in aerobic systems (e.g., sewage, activated sludge, wastewater) in the environment [Swisher (1987); Vershueren (1996); IUCLID (1996); ASTDR (1997); Howard (1990)].

Based on the above discussion and the existing data for the four glycol esters and published biodegradability data for constituent free glycols (e.g., ethylene glycol, propylene glycol and polyethylene glycol) and free fatty acids (e.g., stearic acid, oleic acid), it is expected that the HPV glycol esters would be rapidly and extensively biodegraded in the environment. Further biodegradation testing for substances in this group is not proposed at the present time given that sufficient existing data are available to assess the biodegradability potential of the structurally related HPV glycol esters substances.

In addition, hydrolysis half-lives and atmospheric photodegradation rates have been calculated using EPIWIN. Environmental distribution was determined using the EQC (Level III) model (Mackay, et al. 1996). The distribution between the environmental compartments for glycol esters in this category appears to be influenced by lipophilicity or water solubility. For lipophilic glycol esters with calculated log Pow >7, the EQC (Level III) model predicted a greater chemical distribution in the sediment and soil compartment (see Table 2). Conversely, for glycol esters with greater water solubility characteristics [e.g., heptanoic acid, ester with 2,2,4-trimethyl-1,3-pentanediol (CAS 71839-38-8); triethylene glycol, diheptanoate (CAS 7434-40-4); heptanoic acid, oxybis(2,1-ethanediyloxy-2,1-ethanediyl) ester (CAS 70729-68-9)], the EQC Level III model predicted greater distribution in the water compartment, in comparison to other very water-insoluble glycol esters (Table 2). Based on the calculated data for these environmental fate endpoints in Table 2, sufficient data exist and that no additional testing is proposed for the substances in the glycol esters category.

4.3 Aquatic Toxicity Data

Summary of Aquatic Toxicity Data

Twelve acute aquatic toxicity studies (e.g., fish, invertebrates, algae) relevant to the glycol esters category are summarized in Table 3. Aquatic toxicity testing have been reported for three HPV glycol esters [hexanoic acid, 2-ethyl-, diester with triethylene glycol (CAS 94-28-0); heptanoic acid, oxybis(2,1-ethanediyloxy-2,1-ethanediyl) ester (CAS 70729-68-9); 9-

octadecenoic acid (Z)-, ester with 2,2-dimethyl-1,3-propanediol (CAS 67989-24-6)] and for two structurally analogous surrogate glycol ester [heptanoic acid, ester with 2,2,4-trimethyl-1,3-pentanediol (CAS 71839-38-8); triethylene glycol diheptanoate (CAS 7434-40-4)]. The existing acute aquatic toxicity data for these structurally related glycol esters provides sufficient information to help assess the potential aquatic toxicity for the substances in the glycol esters category.

Data Assessment and Test Plan for Aquatic Toxicity

Acute aquatic toxicity data have been reported for the HPV and surrogate glycol esters. Three HPV substances [hexanoic acid, 2-ethyl-, diester with triethylene glycol (CAS 94-28-0); heptanoic acid, oxybis(2,1-ethanediyloxy-2,1-ethanediyl) ester (CAS 70729-68-9); and 9-octadecenoic acid (Z)-, ester with 2,2-dimethyl-1,3-propanediol (CAS 67989-24-6)] have been tested. Hexanoic acid, 2-ethyl-, diester with triethylene glycol (CAS 94-28-0) was shown to have LC50 and EC50 values of > 97 mg/L in both fish and daphnids. Heptanoic acid, oxybis(2,1-ethanediyloxy-2,1-ethanediyl) ester (CAS 70729-68-9) was shown to have LC50 and EC50 values of 720-4800 mg/L and 3800 mg/L, in fish and daphnids, respectively. It also had an EC50 value of 25 mg/L in algae. On the other hand, 9-octadecenoic acid (Z)-, ester with 2,2-dimethyl-1,3-propanediol (CAS 67989-24-6) was reported to have an EC50 of ~2000 mg/L in daphnia. The two surrogate glycol esters, heptanoic acid, ester with 2,2,4-trimethyl-1,3-pentanediol (CAS 71839-38-8) and triethylene glycol diheptanoate (CAS 7434-40-4), have also been tested in three aquatic species (fish, invertebrates, algae) and the results are summarized in Table 3. The available data for the tested HPV and surrogate glycol esters indicate that acute aquatic toxicity would not be expected at the water solubility limit or water saturation levels (WSL) of the tested glycol ester materials.

While the higher molecular weight glycol esters (>C38, MW ~600) have not been evaluated, they are probably expected to have low degrees of aquatic toxicity due to their very low water solubility. It should be noted that several substances on the HPV list are simply the diester analogs of the corresponding HPV ethylene or propylene glycol monoesters. One example is that stearic acid, ethylene ester (CAS 627-83-8) is simply the diester analog of stearic acid, 2-hydroxyethyl ester (CAS 111-60-4). Another example is that 9-octadecenoic acid (Z)-, 2,2-dimethyl-1,3-propanediyl (di)ester (CAS 4222-50-4) is simply the diester analog of 9-octadecenoic acid (Z)-, ester with 2,2-dimethyl-1,3-propanediol (CAS 67989-24-6). Interestingly, the latter HPV substance (i.e., monooleate ester: CAS 67989-24-6) has been tested and has been shown not to be acutely toxic to fish (LC50 ~2000 ppm). Therefore, the dioleate HPV substance (CAS 4222-50-4), would be expected to show a similar low degree of aquatic toxicity because of its very low water solubility and the fact that it can be enzymatically hydrolyzed to the monooleate substance, which already has been tested and found not to be acutely toxic. It is of interest to note that ethylene glycol and propylene glycol are not acutely toxic to aquatic organisms [Verschueren (1996); ATSDR (1997); IUCLID (1996)]. In addition, free fatty acids (e.g., stearic and oleic acids) that may be generated from enzymatic metabolism of the glycol esters are expected to have low degrees of aquatic toxicity [Verschueren (1996); IUCLID (1996)]

Based on the above findings/discussion and on the structural similarities between the tested HPV and surrogate glycol esters, the existing aquatic toxicity data for the glycol esters, free glycols and free fatty acids, is sufficient to address the potential aquatic toxicity of the HPV substances in glycol esters category and, therefore, no further aquatic testing is proposed.

The acute aquatic studies followed generally accepted test guidelines in which water solutions or water accommodated fractions (WAFs) were generated for poorly water-soluble lubricant test materials at nominal loading rates and then evaluated for toxicity. However, the ACC Panel believes that in cases where the LC50 or EC50 values (based on nominal loading rates to generate the test solutions or WAFs) clearly exceeded the water solubility of the glycol esters and appear exceedingly improbable (e.g., 3800 mg/L, 4800 mg/L, >1000, >2000 mg/L), it would be more appropriate to note (as in Table 3) that the toxicity endpoint (LC50 or EC50 value) greatly exceeded the maximum water solubility limit (WSL) of the test material. For very water insoluble test materials such as for the glycol esters (Table 2), the existing data suggest that aquatic toxicity would not be expected at or close to the maximum water solubility limit (WSL) or at water saturated levels, typical of water solutions or WAFs generated using high nominal loading rates.

4.4 Mammalian Toxicity Data

A) Acute Mammalian Toxicity

Summary of Available Acute Oral Toxicity Data

Acute oral toxicity data relevant to the glycol esters category are summarized in Table 3 and have been reported for five of the nine HPV substances as well as for the surrogate glycol esters. Overall, the acute oral LD_{50} values for these substances was greater than the 2000 mg/kg, indicating a very low order of toxicity for the glycol esters. It should be mentioned that acute dermal toxicity studies have also been carried out and reported for the various propylene glycol fatty acid esters and polyethylene glycol fatty acid esters, particularly those used in cosmetic applications [see reviews by Elder (1982; 1983a; 1983b); Andersen (1994; 1999a; 2000)] but will not be discussed in any depth here. Overall, the glycol fatty acids exhibit very low degrees of acute oral and dermal toxicity.

Data Assessment and Test Plan for Acute Mammalian Toxicity

Adequate acute oral toxicity studies have been located for five of the nine HPV glycol esters and for the structurally analogous surrogate glycol esters. There were no deaths when the HPV glycol esters and the surrogate glycol esters were administered at oral doses of >2000 mg/kg in rodents. The reported oral rat LD50 values ranged from > 2g/kg to >34.6 g/kg for the HPV substances and the surrogate glycol esters tested (Table 3). Hence, the existing data (covering C15-C39 carbon number range) consistently demonstrated a very low order of acute oral toxicity for the glycol esters and, overall, covered the carbon number range for the HPV substances (C20-C41). No additional acute toxicity testing is proposed for HPV substances in the glycol esters category.

As mentioned above, the ethylene glycol fatty acid esters, propylene glycol fatty acid esters and the PEG fatty acid esters have been extensively studied and their health safety evaluated [Andersen (1999a); Elder (1982; 1983a)]. For example, propylene glycol monostearate (CAS 1323-39-3) has an acute oral LD50 of 25.8 g/kg in rats. In addition, propylene glycol stearate is extensively used in many pharmaceuticals and is considered as "Generally Recognized as Safe" GRAS) for food applications [Elder (1983a); Andersen (1999a)]. Numerous other propylene glycol fatty acid monoesters and diesters as well as polyethylene glycol (PEG) fatty acid monoesters and diesters,

have been demonstrated also to have very low degrees of acute oral and dermal toxicity [Andersen (1999a,c); Elder (1983b)].

B) Mutagenicity and Genotoxicity

Summary of Mutagenicity and Genotoxicity Data

A summary of the mutagenicity and genotoxicity data for the HPV substances in the glycol esters category and structurally analogous surrogate substances is presented in Table 3. Bacterial gene mutation assays have been conducted with one HPV glycol ester [heptanoic acid, oxybis(2,1-ethanediyloxy-2,1-ethanediyl) ester, (CAS 70729-68-9)] and three surrogate glycol esters [heptanoic acid, ester with 2,2,4-trimethyl-1,3-pentanediol (CAS 71839-38-8); triethylene glycol diheptanoate (CAS 7434-40-4); and propylene glycol monostearate (CAS 1323-39-3)]. In addition, the HPV substance, heptanoic acid, oxybis[2,1-ethanediyloxy-2,1-ethanediyl] ester (CAS 70729-68-9) has been evaluated for mutagenicity in the Chinese hamster ovary cell forward mutation assay on the HGPRT locus. The surrogate glycol ester, heptanoic acid, ester with 2,2,4-trimethyl-1,3-pentanediol (CAS 71839-38-8), has been evaluated for *in vitro* genotoxicity using human peripheral lymphocytes.

Mutation Assay

One HPV substance, heptanoic acid, oxybis[2,1-ethanediyloxy-2,1-ethanediyl] ester (CAS 70729-68-9), has been shown to be negative for mutagenic activity in the Ames assay and in the Chinese ovary cell forward mutation assay on the HGPRT locus, with and without metabolic activation. In addition, three surrogate glycol esters [heptanoic acid, ester with 2,2,4-trimethyl-1,3-pentanediol (CAS 71839-38-8); triethylene glycol diheptanoate (CAS 7434-40-4); and propylene glycol monostearate (CAS 1323-39-3)] have been evaluated in the bacterial reverse mutation test. All three surrogate glycol esters were shown to be negative for mutagenic activity, with and without metabolic activation.

Chromosomal Aberration Genotoxicity Assay

The surrogate glycol ester, heptanoic acid, ester with 2,2,4-trimethyl-1,3-pentanediol (CAS 71839-38-8), has been evaluated in the *in vitro* cytogenetics test using human peripheral lymphocytes. The results were negative for chromosomal aberrations. The genotoxicity data for this surrogate suggest that glycol esters are not likely to cause chromosomal aberrations. This is consistent with the chemistry of the glycol esters, which does not suggest the likelihood that these substances, or their constituent glycols or fatty acids, are electrophilic or reactive in nature. Therefore, the likelihood that the glycol esters may cause chromosomal aberration is expected to be very low.

Data Assessment and Test Plan for Mutagenicity and Genotoxicity

Due to the close structural and chemical similarities (i.e., glycol and fatty acids constituents) between the three surrogate glycol esters and the HPV glycol esters, read-across assessment for mutagenic toxicity is justifiable. Based on the existing data for the four glycol esters that were tested, the HPV glycol esters would not be expected to be mutagenic, with or without metabolic activation. These findings indicate that the glycol esters are not expected to cause point mutations. The existing data are sufficient to address the mutagenic potential for members of the glycol esters category and therefore, no further mutagenicity testing is proposed.

One surrogate glycol ester substance has been evaluated for mammalian cell genotoxicity and the negative findings, with and without metabolic activation, suggest that members of the glycol esters category and related structural analogs are not expected to be genotoxic. This is consistent with the chemistry of the glycol esters which does not suggest the likelihood that these substances, or their constituent glycols or fatty acids, are electrophilic or inherently reactive in nature. The available data adequately address the genotoxicity potential of the HPV substances in the glycol esters category and, therefore, no additional testing for genetic toxicity (e.g., chromosomal aberration) is proposed at this time.

In addition, the existing data for ethylene glycol, propylene glycol and natural fatty acids, do not indicate that these constituents are mutagenic or genotoxic [see reviews by ATSDR (1997); Andersen (1999a,b), Andersen (1994); WHO (2003); Elder (1986; 1987)].

C) Repeated-Dose Toxicity

Summary of Repeated-Dose Toxicity Data

Repeated-dose oral toxicity studies have been reported for two substances in the HPV glycol esters category [hexanoic acid, 2-ethyl-, diester with triethylene glycol (CAS 94-28-0); heptanoic acid, oxybis (2,1-ethanediyloxy-2,1-ethanediyl) ester (CAS 70729-68-9)] and for two surrogate glycol esters [propylene glycol monostearate (CAS 1323-39-3); heptanoic acid, ester with 2,2,4-trimethyl-1,3-pentanediol (CAS 71839-38-8)]. The results are summarized in Table 3. It should be noted that repeated-dose dermal toxicity studies have also been carried out for various propylene glycol fatty acid esters and polyethylene glycol esters [see reviews by Elder (1982; 1983a; 1983b); Andersen (1994; 1999a; 2000)].

Repeated-Dose Oral Toxicity

In a 12-day rat oral feeding study with 0.1% or 1% in the diet, the HPV substance, hexanoic acid, 2-ethyl-, diester with triethylene glycol (CAS 94-28-0), did not cause any systemic toxicity. No adverse effects were observed with respect to food consumption, body weight gain, behavior, hematology, clinical chemistry, liver or kidney weight, gross or microscopic examination of the organs. Subchronic studies have also been carried out with another HPV substance, heptanoic acid, oxybis[2,1-ethanediyloxy-2,1-ethanediyl] ester (CAS 70729-68-9). In 28-day oral gavage studies in rats, the NOAEL was determined to be 1000 mg/kg for this material. No signs of toxicity were observed and no treatment-related changes in hematology and clinical chemistry were reported.

Two surrogate glycol esters have also been evaluated in repeated dose toxicity studies. Propylene glycol monostearate (CAS 1323-39-3), which was administered for 13 weeks at dietary concentrations of 0, 1.5%, 3.36% and 7.52%, showed no signs of toxicity in rats. Elder (1983a) in his review of these studies noted that the dosed groups showed no significant differences from the control group with respect to growth, relative organ weights, histopathology, blood chemistry, hematocrit, hemoglobin, white cell count, white cell differential count, clotting time, or urinalyses. Similarly, in 6-month oral studies, no signs of toxicity, gross or histological pathology were observed in rats and dogs fed diets containing up to 1.7% propylene glycol stearate (Elder, 1983a). For the surrogate glycol ester, heptanoic acid, ester with 2,2,4-trimethyl-1,3-pentanediol (CAS 71839-38-8), doses up to 1000 mg/kg/day were well tolerated in rats that were orally gavaged daily with the test material for 28 days. Signs of

toxicity were minor, reversible or sex/species specific. Increased liver weights observed at 1000 mg/kg/day regimen were believed to be associated with adaptive changes associated with metabolism (e.g., enzyme induction) and were considered not toxic as such. Hyaline droplet formation observed in the male kidneys was believed to be a sex/species condition specific to only male rats, which has little relevance to humans.

Data Assessment and Test Plan for Repeated-Dose Toxicity

Sufficient read-across data for the two surrogate esters, especially the data reported for propylene glycol monostearate (CAS 1323-39-3) as well as the data for two HPV substances, suggest that members of the glycol esters category would be expected to exhibit a low order of toxicity following repeated oral administration. Additional support data that glycol esters are likely to have low orders of repeated-dose toxicity are based on a number of feeding studies conducted in rats, dogs, mice, rabbits and monkeys for PEG-8 stearate [polyethylene glycol stearate ester in which there are 8 repeating oxyethylene units in the polyethylene glycol (PEG-8) giving total of 34 carbon atoms in the PEG ester structure] (Elder 1983b). The Cosmetic Ingredient Review expert panel has reviewed these studies and has reported that PEG-8 stearate produced no significant changes in growth mortality rates, histopathological observations or hemotology values in long-term feeding studies in rats (i.e., 8-week feeding study at 2% in diet; 9-week feeding study at 4% in diet and 2-year 3-generation feeding studies at 4% in the diet)(Elder, 1983b). Repeated-dose toxicity studies carried out with PEG-40 stearate and PEG-100 stearate also have been reported to demonstrate low degrees of toxicity [Elder (1983b)].

Since the HPV glycol esters and surrogate glycol esters are structurally analogous and have essentially similar constituent ethylene glycol or propylene glycol substructures and fatty acids, the available repeated-dose oral toxicity data are considered adequate for read-across assessment and for bridging data. The propylene glycol stearate repeated-dose toxicity data would be justified for read-across assessment of the lower carbon range glycol monoesters (covering the C20-C24 range of the HPV substances) while the PEG-8 stearate repeated-dose toxicity data would be justified for read-across assessment of the higher carbon range HPV glycol esters (i.e., C38-C41). Therefore, no further testing for repeated dose toxicity is proposed.

Overall, the ACC Aliphatic Esters panel believes that extensive subchronic toxicity data exist for numerous propylene glycol fatty acid esters and polyethylene glycol fatty acid esters to adequately address the repeated-dose toxicity of the HPV glycol esters [see reviews by Elder (1982; 1983a,b); Andersen (1994; 1999a,c; 2000; 2001)].

D) Reproductive/Developmental Toxicity

Summary of Reproductive/Developmental Toxicity Data

No adequate studies were located regarding reproductive and developmental effects in animals exposed to members of the HPV glycol esters category. Since members of this category are ester derivatives (e.g., C6-C18 fatty acids) of mainly, ethylene glycols or propylene glycols, chemically related glycol esters (e.g., polyethylene glycol fatty acid esters) were reviewed to identify available useful data to address potential reproductive/developmental effects.

Reproductive Toxicity

Although no adequate reproductive toxicity studies were located on members of the glycol esters category, numerous regulatory bodies have determined that these substances do not pose a reproductive hazard. These hazard and/or risk assessments are based on the fact that glycol esters would be metabolized (hydrolyzed) *in vivo* to the corresponding fatty acids and free glycol alcohols (e.g., ethylene glycol, propylene glycol) [WHO (2003)]. The free fatty acids and glycols can undergo further metabolism or conjugation to polar products that are either excreted or can be used as nutrients. In most cases, the parent fatty acids derived from the glycol esters are comprised of natural fatty acids that are typical of those (e.g., oleic, stearic acid) found in edible oils and fats.

For example, the FDA has determined that propylene glycol mono- and diesters of fats and fatty acids can be used safely in food, provided they are produced from edible fats and used in amounts not in excess of the reasonably required to produce their intended effect. The FDA and FAO/WHO Expert Committee on Food Additives have approved propylene glycol stearate (PGS), a mixture of mono- and diesters of stearic acid and propylene glycol, as a food additive. PGS is considered to be Generally Recognized as Safe (GRAS) for food use and has been approved for a variety of uses in the pharmaceutical industry and personal care (cosmetic) industry. Studies have demonstrated PGS to be readily hydrolyzed *in vivo*, and the resulting propylene glycol (PG) and stearic acid constituents enter their respective metabolic pathways. Propylene glycol distearate (PGDS) is similarly metabolized to PGS, PG and stearic acid in the gut. The mechanism, by which PG is utilized, by oxidation to lactic acid, has been thoroughly described in the literature (Elder, 1983a). The metabolism of stearic acid, a fatty acid constituent found in vegetable oils and in the fats and oils of animals, has also been thoroughly described in the literature.

Although reproductive studies have not been reported for PGS, a continuous breeding reproduction study in mice has been conducted by Morrissey *et al.* (1989) to evaluate the reproductive and developmental effects of its corresponding glycol alcohol, namely, propylene glycol (PG). Three experimental groups of mice were administered either 1.0% PG (daily dose of 1.82 g/kg), 2.5% PG (daily dose of 4.8 g/kg), or 5.0% PG (daily dose of 10.10 g/kg) during a 7-day pre-mating period in feed and water. The mice were than randomly grouped as mating pairs, cohabitated, and treated continuously for 98 days. There were no significant differences between control and experimental groups of mice with respect to mating index, mean number of live pups per litter, proportion of pups born alive, and sex of pups born alive. In addition, PG had no significant effects on gonads in rats given doses of 0%, 0.625%, 1.25%, 2.5% and 5% PG in a long-term feeding study (2-years). On the basis of these data, propylene glycol esters would not be expected to demonstrate reproductive effects.

Additional supporting data that glycol esters are unlikely to be reproductive toxicants are based on a multiple generation feeding of polyethylene glycol-8 stearate [PEG-8 stearate; a "related chemical" in which there are 8 repeating oxyethylene units in polyethylene glycol molecule giving a total of 34 carbon atoms in the PEG ester structure] in rats (Elder, 1983b). Animals receiving 4% PEG-8 stearate in their diet for three successive generations did not affect growth or fecundity. In another three-generation study in rats receiving diets containing 5%, 10%, or 20% PEG-8 stearate, reproduction and lactation responses were no different from controls at the 5% dose level. Newborn litter survival times were diminished most likely due to maternal neglect at the 10% and 20% dose levels. The overall level of reproductive performance (e.g., greater mortality rate of nurslings, impairment of lactation efficiency) was lower in animals fed the 20% PEG-8 stearate diet [Oser *et al.* (1956b); Elder (1983b)].

Results from these studies showed a low order of reproductive/developmental toxicity. PEG stearates (including PEG-8 stearate) have been approved by the FDA for use in the bakery and pharmaceutical industries.

Although adequate reproductive and developmental studies have not been reported for ethylene glycol stearates or other ethylene glycol fatty acid esters, numerous studies have been conducted to evaluate reproductive and developmental effects of the parent glycol alcohol, namely, ethylene glycol (EG). EG itself is considered to have a relatively low order of toxicity; however, it is oxidized to more toxic metabolites such as glycolic acid, glycolaldehyde, glyoxalic acid, and oxalic acid. Accumulation of these C2 acid products leads to metabolic acidosis which is the underlying cause of EG systemic toxicity. The Cosmetic Ingredient Review (CIR) expert panel has reviewed these reproductive and developmental studies [Andersen, 1999b] as have many regulatory organizations. The Lowest Observed Adverse Effect Levels (LOAELs) and No Observed Adverse Effect Levels (NOAELs) for EG have been reported [see review by Andersen (1999b)]. The CIR report [Andersen, 1999b], have concluded along with other investigators, "... that normal human uses of EG would result in negligible plasma concentrations of EG that were well below the threshold limits for reproductive and developmental toxicity."

Developmental Toxicity/Teratogenicity

Although no adequate developmental toxicity studies were located on members of the glycol esters category, numerous regulatory bodies have determined that these substances do not pose a reproductive/developmental hazard. This is based on the previously discussed reproductive effects of related substances in the section above.

In addition to the above discussion, two developmental studies were located in the literature for propylene glycol (PG). PG was found not to be teratogenic in female mice given single oral doses of 10,000 ppm PG during gestation days 8-12. Fertility rates and all other parameters measured in mice given PG were not significantly different from controls (Kavlock, et al. 1987). However, Nomura (1977) observed malformations in 5 of 226 living fetuses from female mice injected subcutaneously with PG (0.01 ml/g body weight on day 9, 10, 11 of gestation). The water control group (0.01 ml/g body weight on day 9, 10, 11 of gestation) only had one (1) malformation of 320 living fetuses. The incidence of malformations in historical untreated controls was 3 fetuses of 1,026 living fetuses. From these findings, it appears unlikely that glycol esters, as a category would pose developmental toxicity concerns.

Although NOAELs and LOAELs have been reported for EG, exposure to EG would result in negligible plasma concentrations of ethylene glycol that were well below the threshold limits for reproductive and developmental toxicity [Andersen, 1999b].

Data Assessment and Test Plan for Reproductive/Developmental Toxicity

No adequate data were available on the reproductive or developmental toxicity of members of the HPV glycol esters category. It is unlikely that the glycol esters would cause reproductive and developmental effects based on their structural characteristics and *in vivo* metabolic processes. In addition, repeated-dose toxicity studies with glycol esters and related substances have not been reported to adversely affect the reproductive organs.

The PEG esters are not considered to produce reproductive or developmental toxicity in sub-chronic or chronic toxicity studies. These available reproductive/developmental toxicity data, in conjunction with reproductive/developmental data for ethylene glycol, propylene glycol, propylene glycol stearate, PEG-8 stearate and natural fatty acids support no additional reproductive/developmental toxicity testing for the HPV glycol esters.

5.0 TEST PLAN SUMMARY

The American Chemistry Council's Aliphatic Esters Panel believes that sufficient health effects and toxicity data exist for the glycol esters category of the aliphatic esters [taking into account data available for structurally related and analogous surrogate glycol esters] to substantially characterize the mammalian health effects, aquatic toxicity and biodegradation endpoints for the members of this category under the HPV program (Table 4). No additional toxicity tests are proposed for the glycol esters category of the aliphatic esters. This approach to the resourceful use of existing data will help minimize the use of animals for testing and at the same time assess the potential hazards in the glycol esters category of the aliphatic esters.

Table 4. Assessment Plan for Substances in the Glycol Esters Category under the HPV Program

Glycol Ester	Total Carbon No. MW	Mammalian Health Effects						Ecotoxicity - Biodegradability			
		Acute	Repeat dose	Genetic tox (mutation)	Genetic tox (chrom ab)	Reprod	Develop	Acute fish	Acute daphnia	Algal	Biodeg
Heptanoic acid, ester with 2,2,4-trimethyl-1,3-pentanediol *	C15 258	□□	□□	□□	□□			□□	□□	□□	□□
Stearic acid, 2-hydroxyethyl ester	C20 329	□□	R	R	R	TD	TD	R	R	R	R
Triethylene glycol, diheptanoate *	C20 375			□□				□□	□□	□□	□□
Propylene glycol, monostearate *	C21 343	□□	□□	□□							
Hexanoic acid, 2-ethyl-, diester with triethylene glycol	C22 403	□□	□□	R	R	TD	TD	□□	□□	R	R
Heptanoic acid, oxybis (2,1-ethanediyloxy-2,1-ethanediyl) ester	C22 419	□□	□□	□□	R	TD	TD	□□	□□	□□	□□
9-Octadecenoic acid (Z)-, ester with 2,2-dimethyl-1,3-propanediol	C23 368	□□	R	R	R	TD	TD	□□	R	R	□□
Decanoic acid, mixed diesters with octanoic acid and triethylene glycol	C24 431	R	R	R	R	TD	TD	R	R	R	R
Hexanoic acid, 2-ethyl-, diester with tetraethylene glycol	C24 447	R	R	R	R	TD	TD	R	R	R	R
Propylene glycol dilaurate*	C27 441	□□									
Stearic acid, ethylene ester	C38 595	□□	R	R	R	TD	TD	R	R	R	R
Oleic acid, propylene ester	C39 605	R	R	R	R	TD	TD	R	R	R	R
Propylene glycol diisostearate*	C39 609	□□									
9-Octadecenoic acid (Z)-, 2,2-dimethyl-1,3-propanediyl ester	C41 633	R	R	R	R	TD	TD	R	R	R	R

* Shaded (highlighted) areas denote surrogate glycol ester substances - their data are included in table to help bridge data for structurally analogous HPV glycol esters.

Abbreviations in table:
□ = adequate existing data available,
R = read-across data from structurally analogous glycol esters
-- denotes that no data for specific toxicity endpoint heading available for this surrogate glycol ester

TD = Technical discussion on reproductive/developmental toxicity potential for the glycol esters [see Section 4.4 (D)]

Adequate experimental and calculated data for physicochemical properties (i.e., melting point, boiling point, vapor pressure, water solubility and octanol-water partition coefficient) exist for the glycol esters and surrogates in this category. No further testing is proposed for these endpoints for the glycol esters category of the aliphatic esters.

In addition, there are adequate calculated and experimental data for environmental fate endpoints such as photodegradation, hydrolysis, biodegradability (see below) and chemical distribution in the environment (via fugacity modeling) for the glycol esters in this category. No further testing is proposed for these endpoints for the glycol esters category.

Aquatic toxicity and biodegradation data exist for both the HPV glycol esters and the structurally analogous surrogate glycol esters to sufficiently allow for read-across assessments for the HPV substances and for bridging data. In addition, there are published data which indicate that the constituent free ethylene and propylene glycols and free fatty acids, generated from enzymatic ester cleavage of the parent glycol esters, are expected to be extensively biodegraded and to have low degrees of aquatic toxicity. No further aquatic toxicity and biodegradation testing are proposed for glycol esters category of the aliphatic esters.

There were existing toxicity data for the HPV and structurally related surrogate glycol esters to sufficiently make hazard assessments for mammalian health effects (SIDS data endpoints) for the HPV glycol esters substances. Given the similar chemical and structural features between the HPV and surrogate glycol esters (including the structurally analogous polyethylene or propylene glycol fatty acid esters), it was justifiable to utilize the available existing data to make read-across assessments on potential toxicity and to bridge toxicity data for the HPV substances. No additional mammalian toxicity testing is proposed for substances in the glycol esters category. A technical discussion was provided to address the reproductive/developmental potential of the HPV glycol esters, based on the published data that have been reported for related polyethylene glycol monostearates.

It should be noted that the propylene glycol (PG) stearates, oleates and laurates as well as polyethylene glycol (PEG) fatty acid esters (which are commonly used as nonionic surfactants, emulsifiers, emollients in many cosmetic applications) are very structurally similar to many of the HPV glycol esters substances and have low degrees of toxicity. It is important to point out that there is a large body of existing toxicity and health safety data for these structurally related PG and PEG fatty acid esters. It should be noted that propylene glycol stearate has been used in many pharmaceutical applications and is "Generally Recognized as Safe" (GRAS) in food applications. Thus, the surrogate glycol esters provided useful toxicity information for read-across assessments of the HPV glycol esters. Taken into consideration in the assessment were the published health safety assessments for thirteen propylene glycol fatty acid esters [Andersen, 1999a] as well as the multi-generation feeding studies for several polyethylene glycol fatty acid esters [Oser *et al.* (1956b), Elder (1983b)].

Robust summaries of existing health effects, environmental fate and effects, and physicochemical properties data are attached in the Appendix. Summaries of other environmental fate endpoints are also included. Existing data for the HPV and structurally analogous surrogate glycol esters are either included in robust summaries or are referenced in the Appendix should they have been reviewed or summarized elsewhere (such as existing SIDS, HPV test plans, other peer reviews) in the literature/public domain. This test plan is expected to provide adequate information to substantially characterize the mammalian health effects, physicochemical properties and environmental

fate and effects (including aquatic toxicity, biodegradability) endpoints for the glycol esters category of the aliphatic esters under the HPV Chemical Challenge Program.

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7.0 List of Tables and Figures

Page

Table 1A List of Individual Substances in the Glycol esters Category 1

Table 1B Organization of the Nine HPV Glycol Esters according to Total Carbon NumberList of Individual Substances in the Glycol esters Category 3

Table 1C Organization of Nine HPV Glycol Esters and Five Surrogate Glycol Esters According to Total Carbon Number for Use in HPV Data Assessment and Testing Rationale 4

Table 2 Summary Table of Physicochemical Properties and Environmental Fate Data for the Glycol esters 29

Table 3 Summary Table of Mammalian Health Effects, Ecotoxicity and Biodegradation Data for the Glycol Esters 30

Table 4 Assessment Plan for Substances in the Glycol Esters Category under the HPV Program 20

Figure 1 Chemical Structure of the Glycol Esters Listed in Table 1A 5

Figure 2 Chemical Structure of Surrogate Glycol Esters 7

Table 2. Summary Table of Physicochemical Properties and Environmental Fate Data for the Glycol Esters

Total Carbon Number in Ester	MW	CAS Number	Chemical Name	MP* (°C)	BP** (°C)	Vapor Pressure (mm Hg@25°C)	Octanol-Water Partition Coefficient (log Pow)	Water Solubility (mg/L @25°C)	Photo-degradation Half-life (days)	Hydrolysis Half-life (yrs)	Chemical Distribution (Transport) within Environmental Compartments- Fugacity Model			
											Soil %	Air %	Water %	Sediment %
15	258	71839-38-8	Heptanoic acid, ester with 2,2,4-trimethyl-1,3-pentanediol	-50 75 c	>300 322 c	2.8 E-05 Pa at 25 C 1.08 E-05 c	>6.3 4.6 c	2.7 7.84 c	0.50 c	10.6 c	58.7 c	1.1 c	32.2 c	7.9 c
20	329	111-60-4	Stearic acid, 2-hydroxyethyl ester	60.5 138 c	189-191 (3 mm Hg) 404c	6.58 E-08 c	7.26 c	0.01711 c	0.39 c	7.7 c	31.0 c	0.5 c	7.5 c	61 c
20	375	7434-40-4	Triethylene glycol, diheptanoate	-24 54 c	decomp >250 394 c	6.29 E-06 c	4.77 c	30 0.3732 c	0.25 c	0.81 c	67.4 c	0.0 c	24.3 c	8.3 c
21	343	1323-39-3	Propylene glycol, monostearate	132 c	405 c	1.12 E-08 c	7.67 c	0.0062 c	0.34 c	7.7 c	31.3 c	0.4 c	7.1 c	61.2 c
22	403	94-28-0	Hexanoic acid, 2-ethyl-, diester with triethylene glycol	< -40 48 c	344 403 c	4.22 E-06 c	5.60 c	0.04851 c	0.24 c	30.8 c	54.7 c	0.0 c	15.8 c	29.5 c
22	419	70729-68-9	Heptanoic acid, oxybis(2,1-ethanediyloxy-2,1-ethanediyl) ester	94 c	429 c	3.39 E-07 c	2.86 4.49 c	0.3419 c	0.19 c	0.81 c	69.5 c	0.0 c	25.7 c	4.8 c
23	368	67989-24-6	9-Octadecenoic acid (Z)-, ester with 2,2-dimethyl-1,3-propanediol (Monoester)	157 c	431 c	1.01 E-09 c	8.40 c	0.0010 c	0.07 c	6.5 c	28.8 c	0.1 c	7.3 c	63.9 c
24	431	68583-52-8	Decanoic acid, mixed diesters with octanoic acid and triethylene glycol	96 c	441 c	1.74 E-07 c	6.73 c	0.0035 c	0.22 c	1.1 c	42 c	0.0 c	7.3 c	50.7 c
24	447	18268-70-7	Hexanoic acid, 2-ethyl-, diester with tetraethylene glycol	89 c	439 c	2.28 E-07 c	5.33 c	0.0441 c	0.18 c	30.8 c	59.7 c	0.0 c	19.1 c	21.2 c
27	441	22788-19-8	Propylene glycol dilaurate	75 c	444 c	2.31 E-07 c	10.64 c	1.38 E-06 c	0.34 c	5.9 c	30.1 c	0.5 c	7.0 c	62.4 c
38	595	627-83-8	Stearic acid, ethylene ester (Diester)	79 212 c	241 (20 mm Hg) 579 c	8.01 E-11 c	16.12 c	2.97 E-12 c	0.23 c	1.8 c	30.6 c	0.3 c	7.0 c	62.1 c
39	605	105-62-4	Oleic acid, propylene ester	197 c	591 c	2.0 E-12 c	16.11 c	2.61 E-12 c	0.04 c	0.73 c	27.6 c	0.0 c	3.5 c	68.9 c
39	609	68958-54-3	Propylene glycol diisostearate	175 c	569 c	1.29 E-11 c	16.39 c	1.41 E-12 c	0.22 c	5.9 c	30.4 c	0.1 c	2.3 c	67.2 c
41	633	42222-50-4	9-Octadecenoic acid (Z)-, 2,2-dimethyl-1,3-propanediyl ester (Diester)	234 c	609 c	2.38 E-13 c	17.05 c	2.67 E-13 c	0.03 c	3.3 c	27.6 c	0.0 c	3.5 c	68.9 c

Highlighted row denotes substance that was not on the HPV list for the Glycol Esters category but that was included in table to facilitate group evaluation or to bridge data due to their chemical/structural similarities as glycol esters.

c = calculated data using EPWIN; all other values in table are derived from measurements or data obtained from company reports, documents, MSDS, reference handbooks, secondary literature sources.

* = Note: Mixtures are expected to have melting points below those of pure components. Modeled data may not accurately reflect melting points for these substances.

** = Some boiling points may have been determined under reduced pressure and some values may have been extrapolated to one atmosphere

Table 3. Summary Table of Mammalian Health Effects, Ecotoxicity and Biodegradation Data for the Glycol Esters

				Mammalian Health Effects						Ecotoxicity and Biodegradation			
Total Carbon Number in Ester	MW	CAS Number	Chemical Name	Acute Oral LD50	Repeated Dose Toxicity	Genetic Tox (Point/Gene Mutation)	Genetic Tox (Chrom. Aber.)	Reproductive Toxicity	Developmental Toxicity/ Teratogenicity	Acute Fish LC50 or LL50	Daphnia EC50 or EL50	Algae EC50 or EL50	Biodegradation %
15	258	71839-38-8	Heptanoic acid, ester with 2,2,4-trimethyl-1,3-pentanediol	>2 g/kg	28-Day Oral Gavage (rat) Doses up to 1000 mg/kg were well-tolerated. NOAEL was 180 mg/kg	Negative (Ames)	Negative for chromosomal aberration (human peripheral lymphocytes)	Repeated-dose oral toxicity study has not been shown to adversely affect reproductive organs.		> 1000 mg/L Aquatic tox not expected at WSL*	> 2000 mg/L Aquatic tox not expected at WSL*	> 2000 mg/L Aquatic tox not expected at WSL*	87.3% in 28 days OECD 301B
20	329	111-60-4	Stearic acid, 2-hydroxyethyl ester	> 5 g/kg >10 g/kg >21.3 g/kg									
20	375	7434-40-4	Triethylene glycol, diheptanoate (a)			Negative (Ames)				> WSL* (ca. 30 mg/L) >1000 mg/L with emulsifier Aquatic tox not expected at WSL*	9.1 mg/L	559-712 mg/L Aquatic tox not expected at WSL*	65% in 28 days OECD 301D
21	343	1323-39-3	Propylene glycol, monostearate (b)	25.8 g/kg	6-Month Oral Study at 1.7% in diet. No signs of toxicity in rats and dogs. 13-Week Oral Study at 1.5, 3.36 and 7.52% in diet. No signs of systemic toxicity in rats.	Negative (Ames)		Repeated-dose oral toxicity study has not been shown to adversely affect reproductive organs.					
22	403	94-28-0	Hexanoic acid, 2-ethyl-, diester with triethylene glycol	12.5 g/kg 31.37 g/kg	12-Day Oral Study at 0.1% or 1% in diet No adverse effects					> 100 ul/L (~97 mg/L) Aquatic tox not expected at WSL *	> 100 ul/L (~97 mg/L) Aquatic tox not expected at WSL *		
22	419	70729-68-9	Heptanoic acid, oxybis(2,1-ethanediyloxy-2,1-ethanediyl) ester	> 2 g/kg > 25 g/kg 24-25 g/kg	28-Day Oral NOAEL 1000 mg/kg	Negative (Ames, CHO HGPRT locus)				720 mg/L 4800 mg/L Aquatic tox not expected at WSL *	3800 mg/L Aquatic tox not expected at WSL *	25 mg/L Aquatic tox not expected at WSL *	98% in 28 days OECD 301E
23	368	67989-24-6	9-Octadecenoic acid (Z)-, ester with 2,2-dimethyl-1,3-propanediol (Monoester)	> 10 ml/kg						~2000 mg/L Aquatic tox not expected at WSL *			Readily Biodeg. 73% in 28 days OECD 301B
24	431	68583-52-8	Decanoic acid, mixed diesters with octanoic acid and triethylene glycol										
24	447	18268-70-7	Hexanoic acid, 2-ethyl-, diester with tetraethylene glycol										
27	441	22788-19-8	Propylene glycol dilaurate (b)	> 34.6 g/kg (c)									
38	595	627-83-8	Stearic acid, ethylene ester (Diester)	> 5 g/kg, > 10 g/kg > 16 g/kg									
39	605	105-62-4	Oleic acid, propylene ester (Diester)										
39	609	68958-54-3	Propylene glycol diisostearate (b)	25.8 g/kg (c)									
41	633	42222-50-4	9-Octadecenoic acid (Z)-, 2,2-dimethyl-1,3-propanediyl ester (Diester)										

Highlighted row denotes read-across data from surrogate glycol esters that were included in the table in order to help facilitate category evaluation or+A2 to help bridge data for HPV glycol esters due to their chemical/structural similarities.

a) Data for triethylene glycol diheptanoate based on European Chemical Bureau IUCLID toxicity data set for CAS No. 7434-40-4

b) Data for various ethylene or propylene glycol esters and their diesters were obtained from several references including: Andersen FA, Internat. J Toxicol. 18 (Suppl. 2): 35-52 (1999)

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c) Read across for the oral LD50 for the propylene glycol dilaurate was based on the LD50 for propylene glycol monolaurate; the diester was considered to be similar or less toxic than the corresponding monoester similarly, the oral LD50 for propylene glycol diisostearate was estimated based on the oral toxicity data for the corresponding monostearate

* WSL = Water solubility limit or water saturation level. Actual experimental LC50 or EC50 value (nominal loading rate) was many times greater than water solubility limit (WSL) of the chemical. Therefore, aquatic toxicity would not be expected at the maximum water solubility limit or water saturated levels (WSL) of test material based on findings at nominal loading rate or water accommodated fractions (WAF)

Otsuka Chemical Co., Ltd.
3-2-27, Ote-dori, Chuo-ku, Osaka 540-0021, Japan
TEL 06-6943-7711 FAX 06-6946-0860

April 3, 2009

Dear Sir/Madam,

Statement

We hereby declare that Acaritouch is registered and sold in Japan, and the Kingdom of Morocco besides the United States. Currently registrations are being pursued in Mexico, Philippines, Taiwan and Sri Lanka, and field trials have been initiated in Dominican Republic, Guatemala, Costa Rica, Colombia, Ecuador and Thailand. All Acaritouch is manufactured in Japan and exported to these countries.

Tomoyuki Baba

General Manager,
Overseas Group
AgriTechno Division
Otsuka Chemical Co. Ltd.

PETITION TO INCLUDE PROPYLENE GLYCOL MONOLAURATE ONTO 7 CFR § 205

DOCUMENT 3

APR 24 2009

A PETITION SUBMITTED TO

USDA/AMS/TM/NOP

BY

TECHNOLOGY SCIENCES GROUP INC.
4061 North 156th Drive
Goodyear, AZ 85395

ON BEHALF OF

OTSUKA CHEMICAL COMPANY LTD.
3-2-27 Otedori, Chuo-Ku
Osaka, 540-0021, Japan

THIS VOLUME CONTAINS NO CONFIDENTIAL BUSINESS INFORMATION

ALL COMMUNICATIONS REGARDING THIS PETITION SHOULD BE DIRECTED TO

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INTRODUCTION

ACARITOUCH is an emulsifiable concentrate containing propylene glycol monolaurate as an active ingredient at a nominal concentration of 70.81%.

ACARITOUCH is a novel contact miticide which operates by a non-toxic mode of action (suffocation) and is labeled for control of tetranychid mites on fruit, vegetables and ornamental plants.

The label use directions for ACARITOUCH range from 170.4 grams of PGML in 50 gallons of water per acre to 340.8 grams of PGML in 50 gallons of water per acre.

PGML is included in a tolerance exemption petition [PP 1F6314] filed in December 2001 [66 FR 239, 64251-64257 (December 12, 2001)] by the 3M Company.

The specific waivers requested in this document are in reference to toxicology and ecotoxicology guidelines for the active ingredient PGML.

SPECIFIC WAIVERS

OPPTS 870.1200 Acute Dermal Toxicity

Toagosei Co., Ltd., requests a waiver of the requirement for an acute dermal toxicity study of the active ingredient in Acaritouch, propylene glycol monolaurate, based on the fact that propylene glycol monolaurate is not toxic by the dermal route, as has been demonstrated by its development as an excipient for dermal delivery of pharmaceutical compounds (Irion et al., 1995; Gatefosse, 2002).

Propylene glycol monolaurate is one of a number of fatty acid esters of propylene glycol that are similar in structure, each having a fatty acid of variable length attached to the 3-carbon propylene glycol backbone. Propylene glycol monolaurate has a fatty acid ester 12 carbons in length attached to the propylene glycol molecule, propylene glycol monostearate has a fatty acid ester of 18 carbons, and propylene glycol monocaprylate has a fatty acid ester 8 carbons in length. These compounds are used as emulsifiers and stabilizers in cosmetics and in pharmaceutical formulations (Gatefossé, 2002). Dermal exposure to fatty acid esters of propylene glycol is common, for example propylene glycol monostearate is used in hand creams (e.g., Lubriderm Skin Renewal Hand Cream), and in topical and vaginal steroid creams (e.g., Novasone and Premarin).

Propylene glycol monolaurate and other related compounds have been used as excipients to enhance dermal transfer of drugs, and in hand and body creams sold over the counter. These uses indicate that the fatty acid esters of propylene glycol are not toxic by the dermal route, and consequently a dermal toxicity study is not necessary to evaluate the safety of dermal exposures to propylene glycol monolaurate.

OPPTS 870.1300 Acute Inhalation Toxicity

Toagosei Co., Ltd., requests a waiver of the requirement for an acute inhalation toxicity study of the active ingredient in Acaritouch, propylene glycol monolaurate, based on the lack of inhalation toxicity of the end use product, and the lack of toxicity of propylene glycol in inhalation studies. Acaritouch contains more than 70 % propylene glycol monolaurate.

The end-use product, Acaritouch was not toxic by inhalation, as all 10 animals studied survived the inhalation limit test at an exposure of 2.23 mg/l (Carter, 2002 - see Volume 70231-E-8 of this application). The only in-life signs of toxicity were decreased activity and respiratory chirp in males and females, and red crusty eyes in 2 of 5 males. Animals recovered from these effects by day 5. At necropsy, 2 females and one male exhibited mottled lungs.

The potential inhalation toxicity of propylene glycol, the portion of the propylene glycol monolaurate molecule that is not naturally occurring, has been evaluated in monkeys and rats (Robertson et al., 1947; Suber et al., 1989). Exposure of monkeys to

saturated vapor of propylene glycol for 8 to 18 months produced no evidence of adverse effects (Robertson et al., 1947). A more recent study was conducted on rats, exposed by inhalation to a high dose of 2.2 mg/l propylene glycol, and a mid dose of 1 mg/l, for 90 days (Suber et al., 1989). The major adverse effects observed in this study were nasal hemorrhage and ocular discharge that the authors ascribed to dehydration of the nares and eyes. The lack of a lethal effect of inhalation of 2.2 mg/l of propylene glycol on rats exposed for 90 days suggests that this portion of the propylene glycol monolaurate molecule is not acutely toxic.

Data from the inhalation toxicity study demonstrate that Acaritouch, containing 70.81 % propylene glycol monolaurate, is not acutely toxic. Propylene glycol is the primary metabolite of propylene glycol monolaurate that is not naturally occurring, which could present a concern if it were toxic by inhalation. Data from published studies demonstrate that propylene glycol is not acutely toxic by the inhalation route of exposure. Data from the acute Acaritouch inhalation study and the subchronic inhalation studies conducted using propylene glycol, allay any potential concern from inhalation exposure to propylene glycol monolaurate. Thus, an acute inhalation study of propylene glycol monolaurate is not necessary in order to demonstrate the safety of inhalation exposure to propylene glycol monolaurate.

OPPTS 870.5100 Genotoxicity Studies

Toagosei Co., Ltd., requests a waiver of the requirement for a study on genotoxicity of the active ingredient in Acaritouch, propylene glycol monolaurate, based on the fact that propylene glycol monolaurate is readily metabolized in the body to naturally occurring products that are normal components of lipid and carbohydrate metabolism. These metabolites are not genotoxic. Propylene glycol monolaurate is a food additive generally recognized as safe (GRAS) by the U.S. Food and Drug Administration (FDA; 21 CFR 172.856). The World Health Organization (WHO) evaluated the fatty acid esters of propylene glycol (including propylene glycol monolaurate) in the 1970s and established an Acceptable Daily Intake (ADI; WHO, 1974). Consequently, people have been exposed to propylene glycol monolaurate and other fatty acid esters of propylene glycol via the diet for many years.

Propylene glycol monolaurate is one of a number of fatty acid esters of propylene glycol approved for food use in the U.S. and around the world. The fatty acid esters of propylene glycol are all similar in structure, each having a fatty acid ester of variable length attached to the 3-carbon propylene glycol backbone. Propylene glycol monolaurate has a fatty acid ester 12 carbons in length attached to the propylene glycol molecule, propylene glycol monostearate has a fatty acid ester of 18 carbons, and propylene glycol monocaprylate has a fatty acid ester 8 carbons in length. The nutritive value and potential toxicity of fatty acid esters of propylene glycol have been studied for over 65 years (e.g., Lepkovsky et al., 1935). These compounds are often used as emulsifiers and stabilizers in foods such as ice cream, and in pharmaceutical liquid and capsule formulations (Gorman, 2001; Gatefossé, 2002).

The safety of food additives can be projected based on metabolic and biochemical studies that show the biochemical effects and metabolic fate of the compound in question is the same as compounds known to be nontoxic, according to the evaluation procedure used by the WHO (JECFA, 1966; WHO, 1974). The fatty acid esters of propylene glycol, including propylene glycol monolaurate, are hydrolyzed in the GI tract by pancreatic lipase to yield free fatty acids and propylene glycol (WHO, 1974; King et al., 1970; King et al., 1971). Neither of these metabolites is genotoxic. These free fatty acids produced by hydrolysis are indistinguishable from fatty acids from natural sources, which are a major source of energy in the body. Hydrolysis of propylene glycol monolaurate produces lauric acid, the principal fatty acid found in coconut milk. Lauric acid is metabolized in the body through beta-oxidation. Propylene glycol, the other product of the hydrolysis reaction, is of low toxicity and the pathways and products of propylene glycol metabolism have been well characterized (Ruddick, 1972; Snyder and Andrews, 1996). Propylene glycol is oxidized in the liver by alcohol dehydrogenase to form lactic acid and is further metabolized to pyruvic acid, a normal constituent of carbohydrate metabolism.

A study to evaluate the potential genotoxicity of propylene glycol monolaurate is not warranted based on the rapid hydrolysis in the GI tract and metabolism to non-genotoxic compounds that are common energy sources in the body. Humans have been exposed to fatty acid esters of propylene glycol, including propylene glycol monolaurate, in the diet for decades and no concerns related to genotoxicity have arisen.

OPPTS 870.3500 Immune Response

Toagosei Co., Ltd., requests a waiver of the requirement for an immune response toxicity study of the active ingredient in Acaritouch, propylene glycol monolaurate based on the fact that propylene glycol monolaurate is readily metabolized in the body to naturally occurring products that are normal components of lipid and carbohydrate metabolism. These metabolites are compounds that humans consume in the diet daily, and do not cause adverse effects on the immune system. Propylene glycol monolaurate is a food additive generally recognized as safe (GRAS) by the U.S. Food and Drug Administration (FDA; 21 CFR 172.856). The World Health Organization (WHO) evaluated the fatty acid esters of propylene glycol (including propylene glycol monolaurate) in the 1970s and established an Acceptable Daily Intake (ADI; WHO, 1974). Consequently, people have been exposed to propylene glycol monolaurate and other fatty acid esters of propylene glycol via the diet for decades.

Propylene glycol monolaurate is one of a number of fatty acid esters of propylene glycol approved for food use in the U.S. and around the world. The fatty acid esters of propylene glycol are all similar in structure, each having a fatty acid ester of variable length attached to the 3-carbon propylene glycol backbone. Propylene glycol monolaurate has a fatty acid ester 12 carbons in length attached to the propylene glycol molecule, propylene glycol monostearate has a fatty acid ester of 18 carbons,

and propylene glycol monocaprylate has a fatty acid ester 8 carbons in length. The nutritive value and potential toxicity of fatty acid esters of propylene glycol have been studied for over 65 years (e.g., Lepkovsky et al., 1935). These compounds are often used as emulsifiers and stabilizers in foods such as ice cream, and in pharmaceutical liquid and capsule formulations (Gorman, 2001; Gatefossé, 2002).

The safety of food additives can be projected based on metabolic and biochemical studies that show the biochemical effects and metabolic fate of the compound in question is the same as compounds known to be nontoxic, according to the evaluation procedure used by the WHO (JECFA, 1966; WHO, 1974). The fatty acid esters of propylene glycol, including propylene glycol monolaurate, are hydrolyzed in the GI tract by pancreatic lipase to yield free fatty acids and propylene glycol (WHO, 1974; King et al., 1970; King et al., 1971). These free fatty acids produced by hydrolysis are indistinguishable from fatty acids from natural sources, which are a major source of energy in the body. Hydrolysis of propylene glycol monolaurate produces lauric acid, the principal fatty acid found in coconut milk. Lauric acid is metabolized in the body through beta-oxidation. Propylene glycol, the other product of the hydrolysis reaction, is of low toxicity and the pathways and products of propylene glycol metabolism have been well characterized (Ruddick, 1972; Snyder and Andrews, 1996). Propylene glycol is absorbed from the GI tract and oxidized in the liver by alcohol dehydrogenase to form lactic acid. Lactic acid is further metabolized to pyruvic acid, a normal constituent of carbohydrate metabolism. The WHO and Food and Agriculture Organization (FAO) derived an ADI for fatty acid esters of propylene glycol of 25 mg/kg/day, based on the propylene glycol portion of the molecules.

There is no justification for conducting a study to evaluate the potential immunotoxicity of propylene glycol monolaurate, given the long history of use in foods and lack of toxicity of the metabolites. The safety of oral exposure to propylene glycol monolaurate has been demonstrated by the long history of use as a food additive and the clear understanding of the metabolic pathways that result in hydrolysis and oxidation of propylene glycol monolaurate into natural components of fat and carbohydrate metabolism.

OPPTS 870.3100 90-Day Oral Toxicity

Toagosei Co., Ltd., requests a waiver of the requirement for a 90-day feeding study (of the active ingredient in Acaritouch, propylene glycol monolaurate, based on the fact that propylene glycol monolaurate is readily and fully metabolized in the body to naturally occurring products that are normal components of lipid and carbohydrate metabolism. These metabolites are compounds that humans consume in the diet daily. Propylene glycol monolaurate is a food additive generally recognized as safe (GRAS) by the U.S. Food and Drug Administration (FDA; 21CFR172.856). The World Health Organization (WHO) evaluated the fatty acid esters of propylene glycol (including propylene glycol monolaurate) in the 1970s and established an Acceptable Daily Intake (ADI; WHO, 1974). Consequently, people have been exposed to propylene glycol

monolaurate and other fatty acid esters of propylene glycol via the diet for decades.

Propylene glycol monolaurate is one of a number of fatty acid esters of propylene glycol approved for food use in the U.S. and around the world. The fatty acid esters of propylene glycol are all similar in structure, each having a fatty acid ester of variable length attached to the 3-carbon propylene glycol backbone. Propylene glycol monolaurate has a fatty acid ester 12 carbons in length attached to the propylene glycol molecule, propylene glycol monostearate has a fatty acid ester of 18 carbons, and propylene glycol monocaprylate has a fatty acid ester 8 carbons in length. The nutritive value and potential toxicity of fatty acid esters of propylene glycol have been studied for over 65 years (e.g., Lepkovsky et al., 1935). These compounds are often used as emulsifiers and stabilizers in foods such as ice cream, and in pharmaceutical liquid and capsule formulations (Gorman, 2001; Gatefossé, 2002).

The safety of food additives can be projected based on metabolic and biochemical studies that show the biochemical effects and metabolic fate of the compound in question is the same as compounds known to be nontoxic, according to the evaluation procedure used by the WHO (JECFA, 1966; WHO, 1974). The fatty acid esters of propylene glycol, including propylene glycol monolaurate, are hydrolyzed in the GI tract by pancreatic lipase to yield free fatty acids and propylene glycol (WHO, 1974; King et al., 1970; King et al., 1971). These free fatty acids produced by hydrolysis are indistinguishable from fatty acids from natural sources, which are a major source of energy in the body. Hydrolysis of propylene glycol monolaurate produces lauric acid, the principal fatty acid found in coconut milk. Lauric acid is metabolized in the body through beta-oxidation. Propylene glycol, the other product of the hydrolysis reaction, is of low toxicity and the pathways and products of propylene glycol metabolism have been well characterized (Ruddick, 1972; Snyder and Andrews, 1996). Propylene glycol is absorbed from the GI tract and oxidized in the liver by alcohol dehydrogenase to form lactic acid. Lactic acid is further metabolized to pyruvic acid, a normal constituent of carbohydrate metabolism. The WHO and Food and Agriculture Organization (FAO) derived an ADI for fatty acid esters of propylene glycol of 25 mg/kg/day, based on the propylene glycol portion of the molecules (WHO, 1974).

There is no justification for conducting a study to characterize the effects of propylene glycol monolaurate on laboratory animals in a 90-day feeding study. The safety of oral exposure to propylene glycol monolaurate has been demonstrated by the long history of use as a food additive and the clear understanding of the metabolic pathways that result in hydrolysis and oxidation of propylene glycol monolaurate into natural components of fat and carbohydrate metabolism.

OPPTS 870.3700 Teratology

Toagosei Co., LTD. requests a waiver of the requirement for a teratogenicity study of the active ingredient in Acaritouch, propylene glycol monolaurate based on the fact that propylene glycol monolaurate is readily metabolized in the body to naturally occurring

products that are normal components of lipid and carbohydrate metabolism. These metabolites are compounds that humans consume in the diet daily, and are not teratogenic. Propylene glycol monolaurate is a food additive generally recognized as safe (GRAS) by the U.S. Food and Drug Administration (FDA; 21 CFR 172.856). The World Health Organization (WHO) evaluated the fatty acid esters of propylene glycol (including propylene glycol monolaurate) in the 1970s and established an Acceptable Daily Intake (ADI; WHO, 1974). Consequently, people have been exposed to propylene glycol monolaurate and other fatty acid esters of propylene glycol via the diet for decades.

Propylene glycol monolaurate is one of a number of fatty acid esters of propylene glycol approved for food use in the U.S. and around the world. The fatty acid esters of propylene glycol are all similar in structure, each having a fatty acid ester of variable length attached to the 3-carbon propylene glycol backbone. Propylene glycol monolaurate has a fatty acid ester 12 carbons in length attached to the propylene glycol molecule, propylene glycol monostearate has a fatty acid ester of 18 carbons, and propylene glycol monocaprylate has a fatty acid ester 8 carbons in length. The nutritive value and potential toxicity of fatty acid esters of propylene glycol have been studied for over 65 years (e.g., Lepkovsky et al., 1935). These compounds are often used as emulsifiers and stabilizers in foods such as ice cream, and in pharmaceutical liquid and capsule formulations (Gorman, 2001; Gatefossé, 2002). The safety of food additives can be projected based on metabolic and biochemical studies that show the biochemical effects and metabolic fate of the compound in question is the same as compounds known to be nontoxic, according to the evaluation procedure used by the WHO (JECFA, 1966; WHO, 1974). The fatty acid esters of propylene glycol, including propylene glycol monolaurate, are hydrolyzed in the GI tract by pancreatic lipase to yield free fatty acids and propylene glycol (WHO, 1974; King et al., 1970; King et al., 1971). These free fatty acids produced by hydrolysis are indistinguishable from fatty acids from natural sources, which are a major source of energy in the body. Hydrolysis of propylene glycol monolaurate produces lauric acid, the principal fatty acid found in coconut milk. Lauric acid is metabolized in the body through beta-oxidation. Propylene glycol, the other product of the hydrolysis reaction, is of low toxicity and the pathways and products of propylene glycol metabolism have been well characterized (Snyder and Anderson, 1996; Ruddick, 1972). Propylene glycol is absorbed from the GI tract and oxidized in the liver by alcohol dehydrogenase to form lactic acid. Lactic acid is further metabolized to pyruvic acid, a normal constituent of carbohydrate metabolism. The WHO and Food and Agriculture Organization (FAO) derived an ADI for fatty acid esters of propylene glycol of 25 mg/kg/day, based on the propylene glycol portion of the molecules.

There is no justification for conducting a study to evaluate the potential teratogenicity of propylene glycol monolaurate, given the long history of use and lack of teratogenicity of the metabolites. The safety of oral exposure to propylene glycol monolaurate has been demonstrated by the long history of use as a food additive and the clear understanding

of the metabolic pathways that result in hydrolysis and oxidation of propylene glycol monolaurate into natural components of fat and carbohydrate metabolism.

OPPTS 850.2100 Avian Acute Oral

Toagosei Co., Ltd., requests a waiver of the requirement for acute oral avian toxicity study of the active ingredient in Acaritouch, propylene glycol monolaurate based on the fact that birds are able to metabolize propylene glycol monolaurate to naturally occurring products via the same metabolic pathways that are active in mammals. These metabolites are normal components of lipid and carbohydrate metabolism. The fatty acid esters of propylene glycol, including propylene glycol monolaurate, are hydrolyzed in the GI tract of vertebrates by pancreatic lipase to yield free fatty acids and propylene glycol (WHO, 1974). Pancreatic lipase is produced in birds for digestion of dietary triglycerides, just as it is in mammals. Pancreatic lipase activity has been measured in pine warblers, chickens, turkeys and other birds (Levey et al., 1999; Sayari et al, 2000). As in mammals, the free fatty acids produced in birds by hydrolysis of propylene glycol monolaurate are indistinguishable from fatty acids from natural sources, which are a major source of energy. Propylene glycol, the other product of the hydrolysis reaction catalyzed by pancreatic lipase, is of low toxicity to birds. In fact, propylene glycol is an accepted vehicle for suspension of the test article administered to birds in the acute oral avian toxicity study (OPPTS 850.2100). Propylene glycol is absorbed from the GI tract into the portal circulation and oxidized in the liver by alcohol dehydrogenase to form lactic acid in birds as well as mammals (Ruddick, 1972; Snyder and Andrews, 1996; Hjelmqvist et al, 1995). Lactic acid is further metabolized to pyruvic acid, a normal constituent of carbohydrate metabolism.

The safety of propylene glycol monolaurate consumption has been demonstrated for mammals based on the clear understanding of the hydrolysis and oxidation into natural components of fat and carbohydrate metabolism. These same metabolic pathways are present in the bird digestive system and liver. In addition, one of the breakdown products, propylene glycol is an acceptable vehicle for dosing the subjects in the acute oral avian toxicity study, and so can be considered nontoxic to birds. There is no justification for conducting a study to characterize the effects on birds of acute oral exposure to propylene glycol monolaurate.

OPPTS 850.2200 Avian Dietary Toxicity

Toagosei Co., LTD. requests a waiver of the requirement for an avian dietary toxicity study of the active ingredient in Acaritouch, propylene glycol monolaurate based on the fact that birds are able to fully metabolize propylene glycol monolaurate to naturally occurring products via the same metabolic pathways that are active in mammals. These metabolites are normal components of lipid and carbohydrate metabolism.

The fatty acid esters of propylene glycol, including propylene glycol monolaurate, are hydrolyzed in the GI tract of vertebrates by pancreatic lipase to yield free fatty acids

and propylene glycol (WHO, 1974). Pancreatic lipase is produced in birds for digestion of dietary triglycerides, just as it is in mammals. Pancreatic lipase activity has been measured in pine warblers, chickens, turkeys and other birds (Levey et al., 1999; Sayari et al, 2000). As in mammals, the free fatty acids produced in birds by hydrolysis of propylene glycol monolaurate are indistinguishable from fatty acids from natural sources, which are a major source of energy. Propylene glycol, the other product of the hydrolysis reaction catalyzed by pancreatic lipase, is of low toxicity to birds. In fact, propylene glycol is an accepted vehicle for suspension of the test article administered to birds in the acute oral avian toxicity study (OPPTS 850.2100). Propylene glycol is absorbed from the GI tract into the portal circulation and oxidized in the liver by alcohol dehydrogenase to form lactic acid in birds as well as mammals (Ruddick, 1972; Snyder and Andrews, 1996; Hjelmqvist et al, 1995). Lactic acid is further metabolized to pyruvic acid, a normal constituent of carbohydrate metabolism.

The safety of propylene glycol monolaurate consumption has been demonstrated for mammals based on the clear understanding of the hydrolysis and oxidation into natural components of fat and carbohydrate metabolism. These same metabolic pathways are present in the bird digestive system and liver. In addition, one of the hydrolysis products, propylene glycol, is an acceptable vehicle for dosing the subjects in the acute avian toxicity study, and so can be considered nontoxic to birds. There is no justification for conducting a study to characterize the effects on birds of dietary exposure to propylene glycol monolaurate.

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APPENDIX

- Bioavailability enhancement
- Taste-masking
- Sustained release
- Topical formulations
- Suppository formulations

Enhancing Pharmaceutical Performance

As an expert in lipids, Gattefossé develops and manufactures functional pharmaceutical excipients, for oral, topical and rectal dosage forms. Recently introduced, a line of coated active ingredients completes our product range.
To customers seeking formulation exerpertise on a contract basis, Gattefossé also offers innovative drug delivery systems and technologies for bioavailability enchancement, taste-masking or sustained-release of drugs and customized topical or suppository formulations.

[All our products ?] [All about Formulation Development Partnership ?]

Excipients for microemulsions

Gattefossé provides all the components and expertise to design microemulsions.

- Surfactant — **Labrasol®** (Caprylocaproyl macrogol-8 glycerides)
- Cosurfactant — **Plurol® oleique** (Polyglyceryl-6 dioleate)
- Specialized oils — **Capryol® PGMC** (Propylene glycol monocaprylate)
  Capryol® 90 (Propylene glycol monocaprylate)
  Labrafil® M 1944 CS (Oleoyl macrogol-6 glycerides)
  Lauroglycol® FCC (Propylene glycol laurate)
  Lauroglycol® 90 (Propylene glycol monolaurate)
- Solubilizer — **Transcutol® P** (Diethylene glycol monoethyl ether)

BACK

TOP

Lauroglycol® 90

Solubilizing agent for liquid and capsule formulations

- **Chemical description :**

Propylene glycol monolaurate, containing 90% monoesters.

- **Physical characteristics :**

Appearance : oily liquid
Odour : faint
HLB value : 5

- **Applications :**

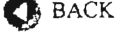
Since Lauroglycol® 90 is a monoester of C12 fatty acids, it is a good solubilizer of poorly-soluble drugs, used in liquid or capsule formulations.
It is also a cosurfactant in microemulsion formulations and a penetration enhancer often used in transdermal formulations.

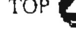
- **Regulatory status :**

Lauroglycol® 90 has a worldwide food additive status.

USA : 21 CFR § 172.856 "Propylene glycol mono-and diesters of fats and fatty acids"
Europe : E 477
Japan : Japanese Standards of Food Additives "Propylene glycol esters of fatty acids"

US Drug Master File n°7818





Interim Market Authorization

GOVERNMENT NOTICES

DEPARTMENT OF HEALTH

FOOD AND DRUGS ACT

Food and Drug Regulations - Amendments

Interim Marketing Authorization

Provision currently exists in Table IV to section B. 16.100 of the *Food and Drug Regulations* for the use of propylene glycol monostearate and sorbitan tristearate as emulsifiers in a wide variety of foods. Health Canada has received a submission to permit the use of propylene glycol monostearate in ice cream mix at a maximum level of use of 0.35 percent and of sorbitan tristearate in ice cream mix and unstandardized ice cream desserts at a maximum level of use of 0.035 percent. Evaluation of available data supports the safety and effectiveness of these new uses of these substances.

The new uses of propylene glycol monostearate and sorbitan tristearate will benefit the consumer through a greater availability of quality ice cream products. It will also benefit the industry by facilitating their production.

Therefore, it is the intention of Health Canada to recommend that the *Food and Drug Regulations* be amended to permit the use of propylene glycol monostearate in ice cream mix at a maximum level of use of 0.35 percent and of sorbitan tristearate in ice cream mix and unstandardized ice cream desserts at a maximum level of use of 0.035 percent.

As a means to improve the responsiveness of the regulatory system, an Interim Marketing Authorization (IMA) is being issued to permit the immediate use of propylene glycol monostearate and sorbitan tristearate, as indicated above, while the regulatory process is undertaken to formally amend the Regulations.

Date: December 21, 2001
DIANE GORMAN
Assistant Deputy Minister
Health Products and Food Branch

For more information, please contact: Gary_Trivett@hc-sc.gc.ca

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Biochemistry

The vertebrate alcohol dehydrogenase system: Variable class II type form elucidates separate stages of enzymogenesis

(class II alcohol dehydrogenase/ostrich enzyme/variable protein/protein family)

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ABSTRACT A mixed-class alcohol dehydrogenase has been characterized from avian liver. Its functional properties resemble the classical class I type enzyme in livers of humans and animals by exhibiting low K_m and k_{cat} values with alcohols (K_m = 0.7 mM with ethanol) and low K_i values with 4-methylpyrazole (4 μM). These values are markedly different from corresponding parameters of class II and III enzymes. In contrast, the primary structure of this avian liver alcohol dehydrogenase reveals an overall relationship closer to class II and to some extent class III (69 and 65% residue identities, respectively) than to class I or the other classes of the human alcohol dehydrogenases (52–61%), the presence of an insertion (four positions in a segment close to position 120) as in class II but in no other class of the human enzymes, and the presence of several active site residues considered typical of the class II enzyme. Hence, the avian enzyme has mixed-class properties, being functionally similar to class I, yet structurally similar to class II, with which it also clusters in phylogenetic trees of characterized vertebrate alcohol dehydrogenases. Comparisons reveal that the class II enzyme is ~25% more variable than the "variable" class I enzyme, which itself is more variable than the "constant" class III enzyme. The overall extreme variability, the activity unexpected for a class II enzyme, and the unusual chromatographic behavior may explain why the class II enzyme has previously not been found outside mammals. The properties define a consistent pattern with apparently repeated generation of novel enzyme activities after separate gene duplications.

Known alcohol dehydrogenase complexity has increased rapidly during the last few years. The zinc-containing liver enzyme first characterized is now recognized to form part of a system of separate forms or classes (1), with different activities and corresponding gene multiplicity in humans and most vertebrates (2). Presently, at least six classes have been described, of which five have been structurally characterized from human origin (cf. ref. 3). Properties and relationships have been best established for three classes (4, 5), the classical liver enzyme with considerable ethanol activity (class I), the ubiquitous class III enzyme with a major glutathione-dependent formaldehyde dehydrogenase activity (6), and the stomach-expressed class IV enzyme with the highest ethanol activity (7). In molecular terms, these classes have ~60% residue identity. Between species, class I structures are fairly variable, class III is more constant, and class IV is intermediate (4, 5). Class III appears to be the ancestral type, present in prokaryotes (8) and invertebrates (4). An evolutionary tree based on a series of gene duplications starts to be discernible (9). Tentatively, the class III/I duplication, with the subsequent emergence of class I activity, has been traced to early vertebrate times. This dating has been deduced from estimates of molecular changes (10), the apparent absence of class I in lower life forms (4), and the presence of an enzyme in bony fish that still exhibits mixed properties, structurally closest to class III but functionally of class I type, presumably reflecting the original enzymogenesis (11).

This emerging knowledge reveals many details that need to be established. One of particular interest is the late origin of the vertebrate enzyme multiplicity, which is noteworthy since enzyme multiplicity is present also in forms of much earlier origin, such as yeasts and plants. This suggests the possibility of functional convergence in separate lines, with repeated emergence of ethanol activity (2). Unresolved questions also occur for the other human and mammalian enzyme classes, classes II, V, and VI, of which little is known. They have not been defined or even detected in submammalian forms, although considerable structural divergence between the forms is seen in humans, and evolutionary trees suggest distant origins (9).

During recent screening of nonmammalian sources, we detected another alcohol dehydrogenase form* in ostrich liver. The present analysis reveals it to be structurally of class II derivation vs. the human enzymes, while functionally it is of class I activity type. It evidently represents an ancestral duplication other than the class III/I duplication previously traced in bony fish (11). Furthermore, it suggests a distinct pattern with which also the class II enzyme is consistent in a system that eventually will define the original functions of the alcohol dehydrogenase classes.

MATERIALS AND METHODS

Protein Purification. Alcohol dehydrogenase from ostrich liver, obtained from Kolmården Zoological Park, Sweden, was purified by ion-exchange chromatography on DEAE-Sepharose, affinity chromatography on AMP-Sepharose, and exclusion chromatography on Sephadex G-100, essentially as described for the class I enzyme from this source (12). The ethanol class I alcohol dehydrogenase of this liver binds to DEAE-Sepharose (12), but, unexpectedly, we found that much ethanol dehydrogenase activity did not bind. We therefore applied the flow-through fraction to AMP-Sepharose for purification. After elution of the class III activity with a gradient of 0–0.5 mM NAD^+ in Tris·HCl (pH 8.0), we continued with a second gradient to 10 mM NAD^+. To increase yields, the column was then eluted with a gradient of 0–2 M KCl in the same buffer. The fraction obtained was desalted by exclusion chromatography and submitted to enzymatic and structural characterization.

Enzymatic Characterization. Activities were tested (6, 13) with ethanol, pentanol, octanol, cyclohexanol, and formaldehyde/glutathione by monitoring NAD^+ reduction spectrophotometrically at 340 nm. K_m and k_{cat} values and the K_i for 4-methylpyrazole were determined at pH 10.0 in 0.1 M glycine·NaOH buffer (13). Constants were calculated with

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*The sequence reported in this paper has been deposited in the Swiss-Prot data base (accession no. P80468).

the program ENZYME (14), and k_{cat} values are given per subunit.

Structural Analysis. The protein was carboxymethylated by reduction with dithiothreitol and treatment with iodo-[^{14}C]acetate (12). It was then cleaved in separate batches with Lys-specific, Glu-specific, or Asp-specific proteases, with chymotrypsin, or with CNBr, and the resulting peptide digests were fractionated by reverse-phase HPLC (12). Amino acid compositions were determined with a Pharmacia LKB Alpha Plus analyzer after acid hydrolysis with 6 M HCl/0.5% phenol for 24 h at 110°C in evacuated tubes. Amino acid sequences of peptides were determined in Applied Biosystems model 477A and MilliGen Prosequencers 6600 and 6625 instruments with on-line analyzers. The N-terminal amino acid sequence and blocking group were determined by mass spectrometry (15).

The primary structure obtained was correlated with the three-dimensional model deduced for human class II alcohol dehydrogenase (16). Sequence relationships were expressed quantitatively in terms of a phylogenetic tree constructed with the program CLUSTAL W (17).

RESULTS

Protein Purification and Enzymatic Properties. Alcohol dehydrogenase purification from liver usually involves a DEAE ion-exchange chromatography step, an affinity step, and an exclusion chromatography step (4, 10, 12, 18). By using this protocol to purify alcohol dehydrogenases from ostrich liver, we noticed that recovery of total alcohol dehydrogenase activity was low at the intermediate AMP-Sepharose step (37%) after completion of the gradient of NAD^+ from 0–10 mM [in 50 mM Tris·HCl (pH 8.0)]. We therefore continued elution with a salt gradient, 0–2 M KCl, in the same buffer. This produced a second fraction of alcohol dehydrogenase that was eluted at about 1 M KCl and increased the total activity recovered to acceptable levels (68%). This second fraction was desalted by the usual exclusion chromatography step and characterized.

In enzymatic screenings, this alcohol dehydrogenase behaved as a class I enzyme, demonstrating considerable activity to ordinary alcohols, including ethanol. The presence of two other alcohol dehydrogenases during purification was also confirmed. The class III alcohol dehydrogenase previously not reported from this species but expected to be present from its occurrence in other vertebrates (2, 6, 11) was detectable by its glutathione-dependent formaldehyde dehydrogenase activity. The class III enzyme was present in the same fraction as the present enzyme during the first purification step but was separated from it by earlier elution from AMP-Sepharose. Similarly, the class I enzyme, known by structural and enzymatic properties (12), had separated during the first purification step by absorbing to DEAE. K_m and k_{cat} values for the present enzyme with most alcohols were low. The ones with ethanol and the K_i value with the class-distinguishing inhibitor 4-methylpyrazole were in the range of those of the human class I enzymes (Table 1). Values with ethanol and 4-methylpyrazole, the substrate and inhibitor often used to distinguish the properties of the classes (7, 13, 16, 19), are considerably higher for the characterized class II form (from humans) and very much higher for all class III forms known (Table 1).

In conclusion, the purification and enzymatic characterization suggest the presence of a third type of alcohol dehydrogenase in ostrich liver, in addition to the established class I and III types of mammals and birds. Its unexpected behavior during purification, requiring high salt for elution in the affinity step, may explain why it has not been previously observed.

Structural Characterization and Relationships. The present alcohol dehydrogenase was homogeneous after the final exclusion chromatography step, as judged by SDS/PAGE and subsequent protein staining, showing an apparent 40-kDa band, similar to that of other vertebrate alcohol dehydrogenases. The protein was [^{14}C]carboxymethylated and subjected to five types of cleavage in separate batches to generate peptides for structural analysis. The fractions obtained produced overlapping peptides covering all regions of the molecule, yielding a continuous amino acid sequence of 379 residues (Fig. 1). The N terminus is blocked by an acetyl group as in all other characterized vertebrate alcohol dehydrogenases and was established by mass spectrometry of an N-terminal peptide (15). The C terminus was established as Phe-379 by identical ends of peptides from three digests (Fig. 1) with different cleavage specificity (Lys-specific protease, Asp-specific protease, and CNBr).

Table 1. Enzymatic properties of the present enzyme form, compared with those of the characterized human enzymes

	Ostrich	Human				
Compound	II	Iβ_1	Iγ_1	II	III	IV
Substrate						
Ethanol	0.71	1.2	1.1	120	NS	11
	36	35	140	470		590
Pentanol	0.025	0.15	0.16	0.09	22	0.08
	22	38	370	480	240	560
Octanol	0.003	0.013	0.010	0.007	1.2	0.01
	16	25	160	500	220	460
Cyclohexanol	3.4	14.5	0.042	210	NS	140
	13	14	130	35		310
Inhibitor						
4-Methylpyrazole	4.0	0.3 ($\beta_1\gamma_1$)		400	≫50,000	10

K_m values (mM) are the upper value in each pair and k_{cat} values (min^{-1}) are the lower value for aliphatic alcohols and cyclohexanol measured at pH 10. NS, nonsaturable. K_i values (μM) for the class I inhibitor 4-methylpyrazole in the presence of ethanol as substrate are shown. Values for the ostrich class II were determined herein; remaining values are from the literature (7, 13, 16, 19).

The structure immediately revealed features distinct from those of class I, in contrast to its class I enzymatic properties. Thus, the enzyme is 5 residues longer than the class I form, and alignment of sequences (also shown in Fig. 1) reveals that these residues correspond to insertions in the class I enzyme sequence, one close to position 60 and four in a segment close to position 120. All these positions of internal differences coincide with those known for the human class II enzyme. Overall residue identities also relate the form to the structure of class II, with an identity toward the human enzyme of 69%. Lower values were obtained toward all other alcohol dehydrogenase classes [61% for human class I γ, 65% for human class III, 58% for human class IV, 58% for human class V, and 52% for deer-mouse class VI enzymes (20)]. In addition, several residue positions characteristic of class distinctions are related to those of the human class II type enzyme. Residues at class I positions 57 and 93 [of importance in class III distinctions (19)], residues at class I positions 110, 309, and 318, and the residues at class II positions 115 and 128 are as in the human class II enzyme and distinguish it from the class I enzyme (Fig. 1). Furthermore, the structure clusters with the human and rat class II structures in a phylogenetic tree constructed with the program CLUSTAL W (17) (Fig. 2) and values from bootstrap analysis (22) are statistically significant. In separate phylogenetic trees constructed for the N-terminal and C-terminal halves of the structures, the structure also clusters with the class II enzymes.

The structure determined and the alignments with known forms of the enzyme establish the present enzyme as an alcohol dehydrogenase that is related to the class II form, reported in humans (26) and also in rats (21). Overall size, insertions, residue identities, and actual residue types show class II relationships, although the residue types also show some

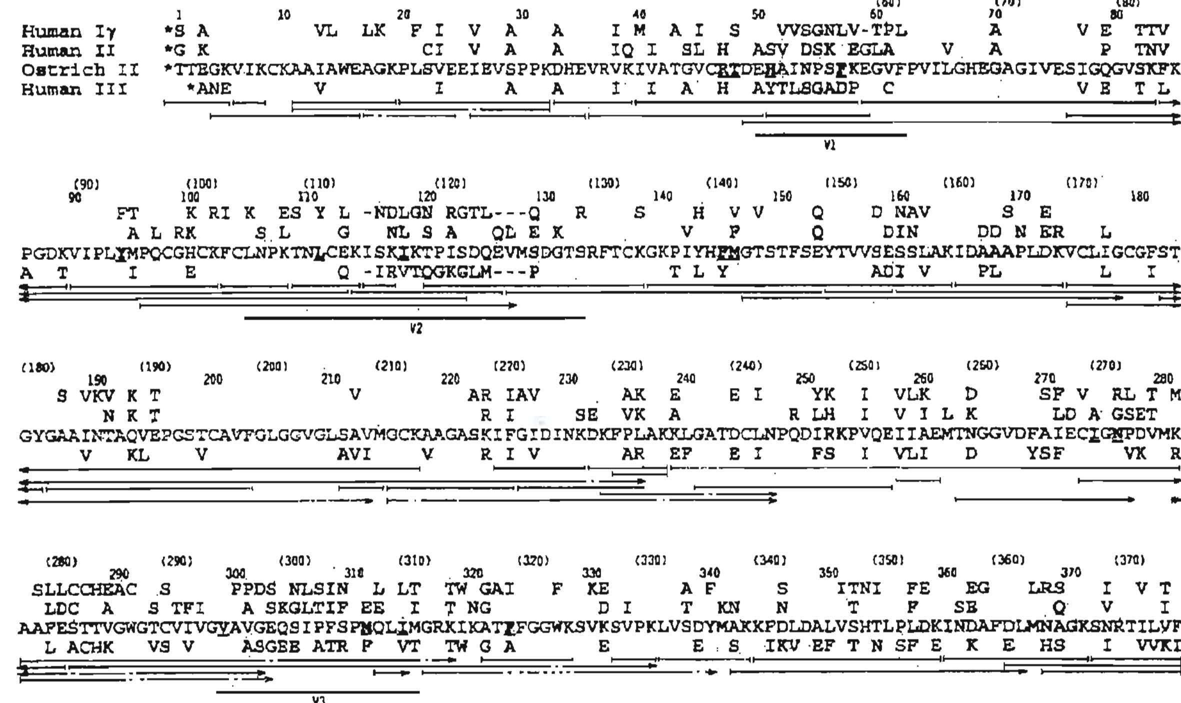

FIG. 1. Primary structure of the class II type alcohol dehydrogenase from avian liver. The 379-residue protein chain determined is represented by the continuous sequence designated Ostrich II. For comparison, residue deviations at equivalent positions in the human class I–III enzymes are indicated. Empty positions, residue identities with the ostrich II structure; dashes, gaps. Residues in boldface type and underlined are functional residues further outlined in Table 2. Asterisks show acylated N termini. Thin lines below the sequences show all peptides now analyzed (dashed parts indicating tentative identification in individual sequencer degradations). Thick lines indicate three segments variable within class I (4) and herein shown to be variable also within class II. Numbers above the sequences give residue positions in the enzyme, and numbers in parentheses give those of the class I enzymes (human and horse) for reference. The two numbering systems deviate from position 60 and still more from position 115, because of the extra residues in class II (16) vs. class I.

class I characteristics compatible with the enzymatic activity. It may be concluded that the present enzyme constitutes a form with mixed properties, enzymatically related to class I and structurally related to class II.

DISCUSSION

Active-Site Structure–Function Relationships. Computer graphics model building has established (16) that the class II human enzyme is compatible with an overall fold closely related to that of the crystallographically determined class I conformations (23, 24). Subsequently, an even more distantly related enzyme (the major cod liver alcohol dehydrogenase with 45% residue differences) has also been established by modeling (18) and actual x-ray crystallography (S. Ramaswamy, M. El-Ahmad, O. Danielsson, H.J., and H. Eklund, unpublished data) to be compatible with the same overall fold. Hence, the identity level and class II relationship in the present case make it meaningful to correlate functional residues of the enzyme with those of other alcohol dehydrogenases based on the positional assignments. Alignment of 11 substrate-interacting and 5 coenzyme-interacting critical positions (Table 2) reveals both conservation and divergence, in agreement with the enzymatically and structurally mixed properties. In particular, coenzyme-interacting residues are largely conserved in relation to those of class I, as they are in alcohol dehydrogenases in general, but some differences exist, all seen before in other alcohol dehydrogenases. Differences are larger with the substrate-binding site; relationships are closest to those of the class II enzyme (Table 2), corroborating the overall structural assignment. However, positions 116, 141, and 306 have residues differing from those of the human class II form. Unexpectedly, however, two of these residues, although different, are not of class I type, suggesting that enzymatic properties in the present enzyme are defined not only by known interactions in the substrate pockets but also by additional interactions that probably include those at the coenzyme-binding site, which is similar to that of the class I enzyme (Table 2).

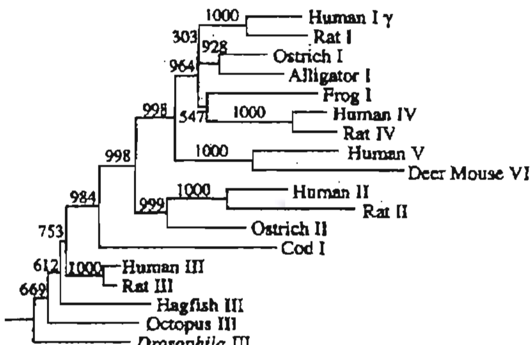

FIG. 2. Phylogenetic tree, relating the present enzyme to class II and the known vertebrate alcohol dehydrogenase classes to each other. Sequence data were from data banks except for the rat II (21) and the present (Fig. 1) structures. In the case of classes I and III, additional forms are known but not included since they are related to those shown and do not influence overall patterns. *Paracoccus* alcohol dehydrogenase was used as the outgroup structure. Numbers indicate results from bootstrap analysis [1000 bootstrap replicates (22)].

Enzyme Evolution and Emergence of Novel Forms. The present structure can be incorporated into a phylogenetic tree

Table 2. Important residues at substrate and coenzyme-binding pockets as determined for the class I alcohol dehydrogenases crystallographically analyzed (23, 24), the human class II and III enzymes modeled (16), and the present enzyme

	Substrate-binding cleft															
	Inner				Middle				Outer			Coenzyme-binding site				
Source	48	93	140	141	57	116	294	318	110	306	309	47	48	51	269	271
Ostrich II	Thr	Tyr	Phe	Met	Phe	Ile	Val	Phe	Leu	Met	Ile	Arg	Thr	His	Ile	Asn
Human Iβ_1	Thr	Phe	Phe	Leu	Leu	Leu	Val	Val	Tyr	Met	Leu	Arg	Thr	His	Ile	Arg
Human Iγ_1	Ser	Phe	Phe	Val	Leu	Leu	Val	Ile	Tyr	Met	Leu	Arg	Ser	His	Ile	Arg
Human II	Thr	Tyr	Phe	Phe	Phe	Leu	Val	Phe	Leu	Glu	Ile	His	Thr	Ser	Ala	Gly
Human III	Thr	Tyr	Tyr	Met	Asp	Val	Val	Ala	Leu	Phe	Val	His	Thr	Tyr	Ile	Asn

Replacements at the various amino acid positions indicated relative to the present enzyme are underlined, and positional numbers refer to class I. Additional positions in coenzyme interactions but conserved between the human classes (16) are not listed. Inner, middle, and outer refer to the relative positions in the substrate-binding cleft.

relating the characterized six classes of the enzyme (Fig. 2). Importantly, the present form clusters with class II, reflecting the separate duplicatory origins of class I (11) and II (this work). Exact branching is still premature for final judgement since few structures are known, but separate groupings of the classes are apparent, distinguishing the class concept. The duplication giving rise to class II was previously enigmatic, since no class II structure had been established outside mammals. We now see that such structures occur, compatible with early duplication and with the overall pattern of the evolution of the enzyme system.

The analysis reveals that the structural divergence within class II is extensive, even more extensive than that for the class I protein, previously concluded to be the "variable" type of the classes then characterized (4). Thus, all the classes apparently have quite distinct evolutionary patterns. Class III is "constant," differing very little between separate mammalian lines; class I is "variable," differing ~3-fold more; and class IV is intermediate [the human/rat variants differing by 6%, 18% (25), and 13% (5), respectively]. We see that class II is particularly variable, being 25% more variable than class I for the human/ostrich forms. In total, therefore, the speed of evolutionary change is now seen to vary up to 4-fold between the relatively constant class III enzyme of ancient origin (4) and the class II enzyme of later emergence. Apart from distinguishing the separate classes, this shows a system of highly different rates of evolutionary change although all folds and enzyme activities are related.

Within the subunits, separate patterns of class-distinguishing segment variability have been observed (4). In particular, three functional segments of variability distinguish class I from class III in a manner atypical of species differences in general. Checking all regions in class II, we find that the atypical pattern of class I also applies to class II. The three regions outlined in class I (4) are variable also in class II (V1–V3, Fig. 1). In particular, V1 and V3 are highly variable within class II, with only a few residues conserved between the species variants (Fig. 1). The variable segments set up a pattern of extensive exchanges at the center of the dimer (Fig. 3), involving both subunit interactions and the entrance to the active site. Hence, it may be concluded that the patterns constitute general phenomena, distinguishing evolving enzymes from constant ones by an atypical vs. a typical pattern, affecting functional and nonfunctional segments, respectively. It appears significant that the overall most variable class II enzyme also is the alcohol dehydrogenase with the largest variation at the active site (V1) and the dimer interaction area (V3) (Fig. 3).

The finding of an alcohol dehydrogenase with mixed-class properties is of interest. Previously, this has been encountered in the major cod liver enzyme, where it was interpreted to reflect enzymogenesis of class I properties from the class III ancestral form (11). We now see that mixed-class properties also relate to class II. The present enzyme has functional properties of class I, overall structural properties of class II, and root relationships with class III. Although branchings are thus far based on few known structures, and additional classes

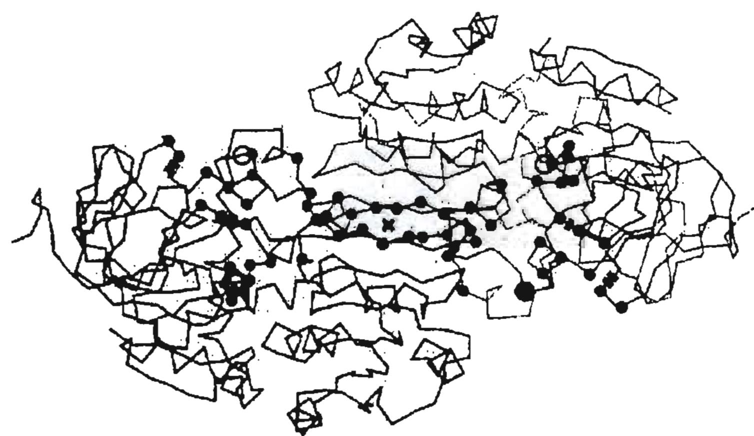

FIG. 3. Structural assignment of residue replacements in variable segments of the alcohol dehydrogenase dimer. The dimer symmetry axis is perpendicular to the paper and passes through the cross at the center. Solid circles, C_α positions of replacements within class II between the human and ostrich enzymes in three variable segments, V1–V3; bars between C_α positions, replacements corresponding to gaps in class I; open circles, positions of the zinc atoms. These segments, outlined in Fig. 1 and defined in ref. 4, show the dense representation of replacements along subunit-interacting segments in the dimer and part of the entrance to the active site. The conformation shown is that of the crystallographically determined human class I alcohol dehydrogenase (24), obtained from the Brookhaven Protein Data Bank, and actual replacements are as given in Fig. 1.

or mixed forms may still be encountered altering details regarding gene numbers, duplicatory orders, or class assignments, the delineation of the alcohol dehydrogenase system illustrates successive duplications and subsequent evolution of novel enzyme activities. The enzyme activities emerge with changes affecting the active site, even when the general relationships still reflect the ancestral origin from another class.

The mixed-class forms constitute a nomenclature problem. Previously the enzyme with mixed-class properties in cod liver was called class I from its functional assignment, because no other class I enzyme was detected in that species. The present mixed-class enzyme is intuitively regarded as class II from its structural properties because another class I enzyme is already known in this species (12). Although clarifying the distinctions, this alternative naming of enzymes after function or structure is inconsistent, but reconsideration may best be postponed until further forms, origins, and properties have been established.

Finally, the assignment of each class into separate groups of distinct evolutionary patterns is concluded to reflect correspondingly distinct metabolic roles. Class III is the most constant form of distant ancestral origin and is involved in cell defense and detoxication (see refs. 2 and 6). The form characterized herein reveals extensive differences in class variability yet shows a constant pattern in each class, once established. These class patterns and functional differences will be useful in defining the metabolic role(s) of all forms, including of the human alcohol dehydrogenase complex with its still further isozyme gene multiplicity.

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Effect of PGML Excipient Mixture in a Transdermal System on the in Vitro Transport of Estradiol Across Skin

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Purpose. To measure the effect of a combination of excipients from a silicone based pressure sensitive adhesive (PSA) on drug transport across skin.
Methods. Partitioning of propylene glycol monolaurate (PG-ML) from silicone PSA and a solution formulation into the stratum corneum (SC) was measured using radiolabeled PG-ML. Transport of a model drug, estradiol, as well as PG-ML across skin were measured in vitro using heat separated epidermis from human cadaver skin.
Results. The PG-ML partitioning into SC showed a saturation and was independent of the formulation. The local dielectric of the lipid bilayers of the SC showed an increase as a result of PG-ML uptake, as determined by the decrease in fluorescence lifetime of a lipophilic probe incorporated into the SC. However, there was no alteration of lipid packing in SC.
Conclusions. The PG-ML and estradiol transport showed a good correlation over 3 days, suggesting that the two species are co-transported across the epidermis.

KEY WORDS: transdermal drug delivery; pressure sensitive adhesive; transport enhancement; fluorescence spectroscopy; estradiol.

INTRODUCTION

Transdermal drug delivery has become an attractive alternative in controlled drug delivery (1). The stratum corneum (SC) of the epidermis provides the major barrier to percutaneous penetration of drugs (2,3). This diffusional barrier is often overcome with the aid of excipients in the transdermal formulations (4,5). These excipients are thought to act as solubilizers, fluidizers, plasticizers or vehicles. The excipient could increase the solubility of the drug in the adhesive, thereby increasing the concentration in the delivery system in situations where the drug is present in a powdered form in the adhesive. It could partition into the skin and perturb the highly organized lipid bilayers of the SC, increasing the permeability of the skin (4). The excipient could act as a plasticizer in the adhesive, increasing the diffusion of the drug in the adhesive. It could also act as a solvent or as a vehicle, facilitating the transport of the drug across skin. Excipients are used individually or in combination in transdermal systems to provide any one or a combination of the above effects resulting in an enhanced delivery of drugs across skin (4,6).

One of the first steps in the development of a transdermal drug delivery system is to perform a formulation feasibility and optimization study from a solution formulation before exploring a suitable pressure sensitive adhesive (PSA) platform. Quite often, the transdermal flux of the drug observed from a PSA matrix formulation is less than from a solution formulation. The literature is replete with reports from solution formulations on enhancer effects (4-6). However, there are only few studies reported from solid matrix formulations (7).

In the present investigation, we have measured the uptake of an excipient, propylene glycol monolaurate, into skin and its transport across skin from a silicone based PSA and a liquid formulation. Propylene glycol monolaurate, a fatty acid ester, is used to enhance the transport of drugs from transdermal systems (8,9). The transport of a model drug, estradiol, was measured to determine if there was any correlation between the transport of drug and excipient from a given formulation. The objective of the present study is to understand the effect of a combination of excipients from a silicone based PSA on skin. Building on our earlier work (10), we have used a frequency-domain fluorescence spectroscopic technique to determine the effect of these excipients on the lipid order of the SC.

MATERIALS AND METHODS

A mixture (PGML) of propylene glycol monolaurate (PG-ML, 52.9% by weight), propylene glycol dilaurate (PG-DL, 32.7%), methyl laurate (ML, 9.2%), and propylene glycol (PG, 5.2%) obtained from Gattefosse (France) was used without further purification. The composition of this mixture was determined by gas chromatography using a HP-5890 gas chromatograph with a Restek RTX-35 capillary column and a FID detector. Radiolabeled PG-ML was synthesized using PG and 1-^{14}C lauric acid (American Radiolabeled Chemicals, Inc., St. Louis, Missouri). One mCi (3.66 mg) of ^{14}C lauric acid was added to a mixture of 2 mg of unlabeled lauric acid and 5.36 mg of PG and the resulting reaction mixture of lauric acid and PG (1:2.5, mole ratio) in hexane was mixed with 0.3M Boron Trifluoride (BF_3) ethyl etherate catalyst in diethyl ether and the reaction mixture was maintained at 70°C for 6h under nitrogen. The excess catalyst was removed by drying under a stream of nitrogen and the ^{14}C PG-ML was isolated from the reaction mixture by preparative thin layer chromatography. The reaction mixture was dissolved in chloroform, applied on a 1 mm thick silica gel plate (Alltech Associates), and eluted in hexane:ethyl ether:acetic acid (69:30:1, by volume). Commercial PGML mixture was used as the standard to identify the different bands. The PG-DL eluted the furthest on the plate, followed by lauric acid and PG-ML eluted about one-third the distance from the origin. The ^{14}C PG-ML fraction from this plate was purified on a second plate using a polar solvent system (5% methanol in chloroform). In this solvent system, the ^{14}C PG-ML moved one-half the distance from the origin, while the lauric acid (which was a cross-contamination from the first fraction) was retained close to the origin. The final yield of PG-ML was 0.73 mg or ~ 10%.

Two different donor formulations were used in the up-

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take and transport study. Neat PGML liquid spiked with 4µCi of ^{14}C PG-ML was used as a solution formulation. A silicone PSA formulation was made by adding a known amount of PGML to a silicone solution in Freon (non end capped, 58% solids from Dow Corning) to a final composition of 14% by dry weight. The solution was spiked with 2µCi of ^{14}C PG-ML. The liquid adhesive was stirred for 2h before casting the film at a thickness of 5 mil. A polyester film was used as the backing and as the release liner for the patches. The specific activity of ^{14}C PG-ML in the final formulations were 25.5 µCi/g of PG-ML in the neat PGML liquid and 24.9 µCi/g of PG-ML in the silicone formulation.

Skin uptake and transport studies were performed using Franz cells across a diffusional area of 0.785 cm^2. The estradiol transport studies were done using multiple pieces of skin from different donors, while the rest of the experiments were done using three pieces of skin from the same donor. The patches with the backing material were applied on to pieces of heat separated human epidermis obtained from human cadaver skin, and the receiver fluid was analyzed for ^{14}C PG-ML by liquid scintillation counting, at intervals of 24h for 3 days. The amount of ^{14}C PG-ML left in the patch and in epidermis were also analyzed at each time point. After the flux studies, the epidermis was removed from the diffusion cell, and the adhesive was peeled away. The patches and the backing were extracted in methanol:methylene chloride (1:1, by volume) overnight and analyzed for ^{14}C PG-ML. The epidermis was digested in 1 ml of Solvable (0.5M sodium hydroxide, NEN Research Products) overnight and then counted. In one of the time points, i.e., the third day sample, the SC was separated from viable epidermis by trypsin digestion. The epidermis was placed dermal side down on a filter paper soaked in 1% trypsin for 4 to 6h at room temperature and then at 4°C overnight. The partially digested tissue was agitated in a fresh 0.5% trypsin solution for 1h resulting in a translucent sheet of SC, which was rinsed in distilled water, digested in Solvable and counted for PG-ML. The trypsin digests along with the filter paper were counted for PG-ML in the viable layers. Day 3 samples were analyzed for ^{14}C PG-ML in the SC as representative samples. In the samples where neat PGML was the donor, the epidermis was rinsed three times in 3 ml portions of unlabeled PGML to remove any PG-ML from the epidermis surface before digesting in Solvable. In a separate experiment, SC was separated from pieces of epidermis by trypsin digestion as described above. The SC pieces of known area were then equilibrated with PGML liquid mixture in a scintillation vial. The PGML mixtures was spiked with ^{14}C PG-ML to a specific activity of 15 µCi/g of PG-ML. After 72h equilibration, the pieces of SC were rinsed three times in unlabeled PGML to remove any ^{14}C PG-ML from the surface, and the tissue was treated with trypsin identical to the method described above. After rinsing in distilled water, the SC pieces were digested in Solvable and analyzed for ^{14}C PG-ML. The trypsin fractions along with the filter paper and the water rinses were pooled and counted for ^{14}C PG-ML as a check for the amount of PG-ML that could have leached out during the different experimental steps. In the estradiol transport experiments, the drug was present at 5% by weight in the silicone PSA formulation and was present as a saturated solution in the PGML liquid formulation. The estradiol in the receiver fluid was analyzed by reverse-phase HPLC using a C-18 column.

For fluorescence experiments, the epidermis was treated similar to the transport studies, but the donor formulation did not contain any radiolabeled PG-ML or estradiol. The epidermis was equilibrated for 18h in a side-by-side diffusion cell, with a donor solution of a fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene (DPH) in water. The aqueous solution of DPH was prepared by adding 5µl of 2mM DPH solution in ethanol to 1.5 ml of water corresponding to a final concentration of 6.7µM. After pretreatment with DPH, the epidermis was treated with the PGML liquid and the silicone PSA formulations separately, as described above in the uptake studies. At the end of the treatment period (24h to 72h), the epidermis was removed from the diffusion cell, mounted on an aluminum block and placed in the sample holder for spectral acquisition. The samples in which the epidermis was treated with the silicone-based formulations, the spectral acquisition was performed with the adhesive on the epidermis. All fluorescence measurements were performed using an SLM-Aminco MHF 4850 spectrofluorimeter. Fluorescence lifetimes and anisotropy were measured using the phase modulated 325 nm line of a HeCd laser as described elsewhere (10 - 12).

RESULTS

Skin Uptake Data. The uptake of PG-ML into epidermis showed tissue saturation after day 2 from the silicone but not from the PGML donor, as seen in Fig 1. The distribution of PG-ML between the SC and the viable layers of epidermis is shown in Table I. The amount of PG-ML per cm^2 of SC was comparable from both the silicone and the PGML formulations, suggesting tissue saturation. The amount of PG-ML per unit area of the viable layers of the epidermis was three-fold higher from the PGML formulation compared to the silicone. The amount of ^{14}C PG-ML lost from the SC during trypsin treatment and water rinses was 11.5 ± 2.1% of the total ^{14}C PG-ML in the SC, which is within the standard deviation of the SC-uptake data shown in Table I. The data in Table I are not corrected for this PG-ML loss.

Transport Data. The transport data for PG-ML across epidermis is shown in Fig 2. The amount of PG-ML transported across epidermis gradually increased with time up to 3 days from both silicone and PGML liquid formulations. The amount of PG-ML transported across epidermis from the PGML mixture was six-fold higher than that from the silicone PSA formulation. Estradiol transport across epidermis from these two formulations is shown in Fig 3. The estradiol transport from the PGML liquid formulation was about 15 times that from the silicone PSA formulation. The drug transport across the epidermis from these two formulations showed a good correlation with the PG-ML transport over a period of 3 days, as seen in Fig 4.

Fluorescence Data. Table II shows changes in lifetime and limiting anisotropy for DPH in epidermis treated with different formulations. The lifetime decreased after 3 days of exposure to PGML from both formulations. The decrease in lifetime was observed even after 24h exposure to PGML from the neat liquid and was twice that from the PSA-treated epidermis. The limiting anisotropy showed no decrease after

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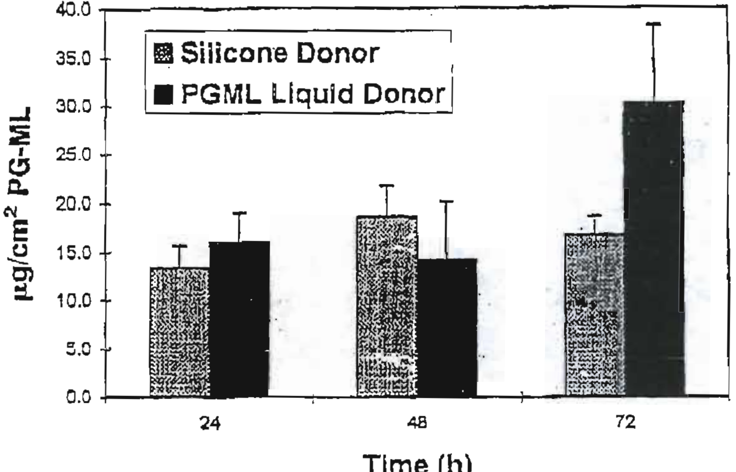

Fig. 1. Amount of propylene glycol monolaurate partitioning into the epidermis from silicone PSA and PGML liquid formulation ($n = 3$).

treatment with the PGML liquid formulation. The limiting anisotropy measurements in samples treated with the silicone formulations did not yield any useful information due to interference from the overlying silicone adhesive. Spectral data from an earlier study (10) using oleic acid-containing formulation showed large drops in lifetime and limiting anisotropy and are included in Table II for comparison.

DISCUSSION

We concentrated on following the fate of the major component, viz. PG-ML, as representative of the behavior of the PGML mixture. Among the different components, PG is polar while the other components are lipophilic. However, PG constitutes only 9% of the mixture, which translates into 1.25% in the silicone formulation containing 14% of PGML. Comparatively, PG-ML constitutes 53% of the PGML mixture and represents 7.4% of the PSA system. PG is known to act as a cosolvent, showing a synergy with other penetration enhancers in terms of flux enhancement (4,8,13). This synergy is observed only at very high PG concentrations, when the PG is used as the vehicle or solvent (4,8). Therefore, PG transport may not be relevant to drug transport at low concentrations of PG as in the present formulations. Thus, PG-ML is a useful representative molecule to monitor the fate of mixture of lipophilic excipients in the PSA matrix. PG-ML is freely miscible in PGML mixture, while the 14% PGML-containing silicone did not show any signs of phase separation when examined by light microscopy. Thus, it is reasonable to assume that the PG-ML was present below unit activity in both formulations. However, there was much higher amount of PG-ML in the PGML liquid formulation, than in the silicone. The PG-ML partitioning into epidermis at 72h was higher from neat PGML mixture than from the PSA, which could be explained in terms of the larger driving force resulting from a higher concentration gradient and therefore higher thermodynamic activity of PG-ML in neat PGML as compared to the silicone formulation.

The uptake of PG-ML by SC, the barrier layer of epidermis, is more important in terms of flux enhancement than the uptake by epidermis. The saturation of SC suggests that the effect of PG-ML on lipid bilayers of the SC should also be saturated. This was further confirmed by the biophysical investigation of the effect of these two formulations on the

Table I. Distribution of PG-ML in the epidermis

Formulation	Stratum corneum (µg/cm²)	Viable epidermis (µg/cm²)	Ratio[a]	Stratum corneum (% Total)
Silicone	9.12 ± 1.02	7.58 ± 2.35	12.03	54.6 ± 10.0
PGML	7.81 ± 2.52	22.35 ± 5.55	3.49	25.9 ± 1.9

[a] The ratio of PG-ML in SC to viable epidermis was calculated after normalizing for unit thickness of the different layers.

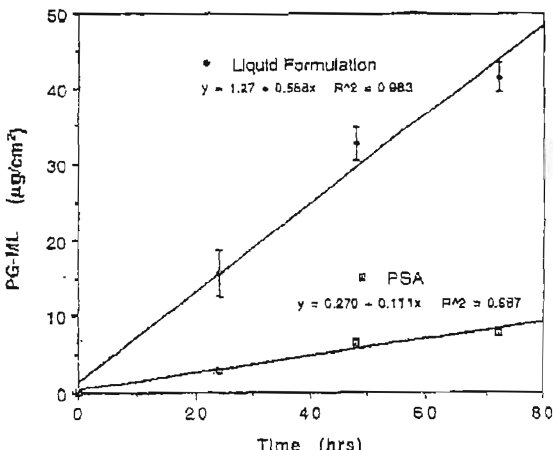

Fig. 2. Transport of propylene glycol monolaurate across human epidermis from silicone PSA (□) and PGML liquid (◆) formulations ($n = 3$).

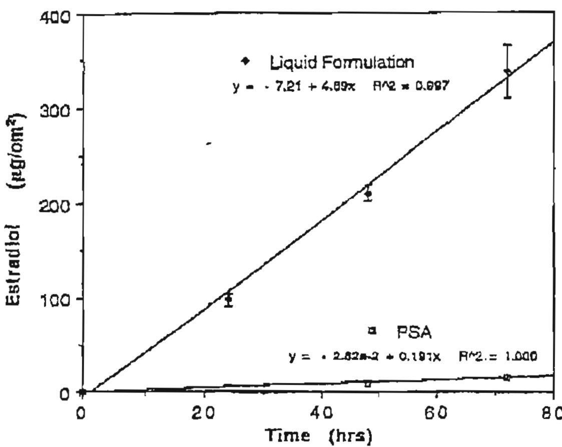

Fig. 3. Transport of estradiol across human epidermis from silicone PSA (□) and PGML liquid (♦) formulations (n = 12).

SC lipids. Changes in fluorescence lifetime of DPH is a measure of changes in the local dielectric of the medium (10). Thus the decrease in fluorescence lifetime, shown in Table II, indicated that the local dielectric in SC bilayers increased upon treatment with PGML and this effect was observed in both formulations. The larger drop of about 2.3 nsec in lifetime in the epidermis treated with liquid PGML formulation, as compared to about 1.1 nsec drop in the epidermis treated with silicone formulation suggests that the dielectric of lipid bilayers increased more with the application of solution formulation. This could be due to increased transport of PG-ML across SC as seen in Fig 2. This is purely speculative at this point as we have not investigated large number formulations to generalize this observation. The reason for the decrease in the lifetime in epidermis treated with the placebo patch is not clear, although the occluded condition from such treatment could increase hydration and therefore the dielectric of the lipid bilayers. Nevertheless, the perturbation resulting from the partitioning of PG-ML into SC is clearly evident from both the solution and the silicone formulations.

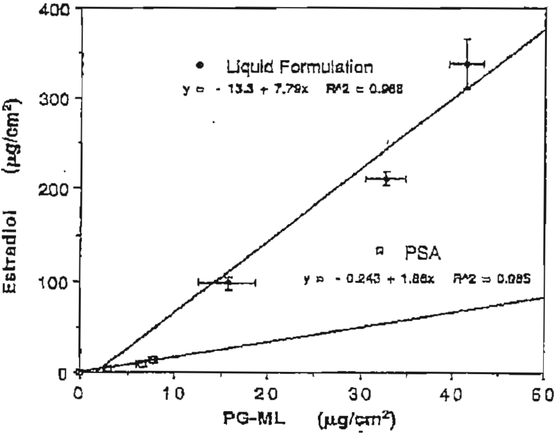

Fig. 4. Correlation of transport of propylene glycol monolaurate and estradiol over 72h, from silicone PSA (□) and PGML liquid (♦) formulations.

Table II. Fluorescence Parameters of DPH in Epidermis Treated with Different Formulations

Formulation	Equilibration Time (h)	$\Delta\tau$ ± 0.2 nsec	r_∞ ± 0.005
Water	24	0	0
Placebo silicone patch	72	−0.5	—
Silicone (14% PGML)	72	−1.1	—
Liquid PGML	24	−2.3	0
5% OA in 40% ethanol	24	−2.2	−0.1[a]

[a] From ref (10); $\Delta\tau$, r_∞ – changes in fluorescence lifetime and limiting anisotropy compared to the control formulation (water).

From a thermodynamic consideration, if the SC uptake of PG-ML reached saturation, then the two phases, i.e. the SC and the donor phase must be at the same, unit activity at equilibrium. But PG-ML is freely miscible in the PGML mixture and makes up only 52.9% of the mixture and therefore is not at unit activity. Thus the saturation of PG-ML uptake that is observed in this study is due to a volume constraint of the intercellular lipid matrix of the SC and is not a thermodynamic equilibrium. In other words, the PG-ML does not reach its solubility limit in the lipid bilayers. This is further supported by the calculated value of the Hildebrand solubility parameter (δ) for PG-ML, which is around 9.06 H which compares well with the estimated δ of 9.7 H for SC (14). Thus, the lipophilic PG-ML would be expected to be freely miscible with the nonpolar lipids of the SC. However, the lipid bilayers accommodate only a small number of exogenous molecules such as PG-ML in their packed configuration in the tissue, as measured in this study.

The distribution ratio of PG-ML per unit area per unit thickness of the different layers is shown in Table I. The SC consists of 10 to 15% by weight of lipids (15), while the viable layers are predominantly aqueous. The SC is about 15 µm thick while the viable epidermis is about 150 µm thick. Thus it is not surprising that the SC is able to accommodate more PG-ML (which is lipophilic) per unit volume compared to the viable epidermis. While the amount of PG-ML partitioning into SC is independent of the formulation, the relative ratio is dependent on the formulation.

The partitioning of PG-ML did not show any effect on the anisotropy values. The limiting anisotropy is a measure of the lipid packing order in the SC and other bilayer membranes (12,16). Thus the anisotropy data in Table II suggest that the PG-ML does not perturb the epidermis lipid packing to the same extent as the oleic acid. It is interesting to compare the mole ratio of PG-ML to lipid in SC to that of oleic acid. Assuming the mass of SC to be 1mg/cm² (based on the average weight determined for pieces of porcine SC in our laboratory), assuming an average molecular weight of 600 for the SC lipids (17), and based on the average amount of PG-ML partitioning into SC (Table I), we can calculate a mole ratio of 1:51 (PG-ML:SC lipid). The amount of oleic acid partitioning into SC was shown to be 3:100 (oleic acid:SC lipid) in an earlier study (10). Although the amount of PG-ML uptake is significant and is about 67% of the oleic acid uptake, the perturbation in terms of lipid packing in the SC

bilayers is almost completely absent for the PG-ML. However, there is a significant increase in estradiol flux from PGML-containing PSA formulation compared to the PSA formulation without PGML, as shown in Fig 5. Also, there was a strong correlation between estradiol transport and PG-ML transport, as shown in Fig 4. The drug loading is a key factor in this comparison. Estradiol was present above its solubility in silicone. It was present as a saturated solution in PGML liquid formulation. Thus, the drug was at unit activity in both formulations. However, more PG-ML and therefore more drug was transported across epidermis from the liquid formulation. This is because of the higher driving force from the solution formulation for PG-ML. Perturbation of lipid packing in the barrier layer by PG-ML does not seem to play a major role in flux enhancement. The increased PG-ML transport could facilitate increased transport of estradiol by co-transporting with it or by increasing the solubility of the drug in skin.

In conclusion, PG-ML from a mixture of mono-, di-, and methyl-laurate and PG in PGML seem to perturb the SC lipids minimally. A strong correlation seen between the transport of PG-ML and estradiol suggests that the two species are co-transported across the epidermis. There is a tissue saturation effect in the uptake of PG-ML that is independent of the formulation. The increased transport of estradiol and PG-ML from a solution formulation could be explained in terms of a higher concentration gradient compared to a silicone formulation. The challenge to the formulation scientist trying to achieve higher drug flux by the use of excipients is the limit imposed by the ability of the PSA matrix to accommodate a given amount of excipient, without compromising adhesion and other bulk properties of the PSA.

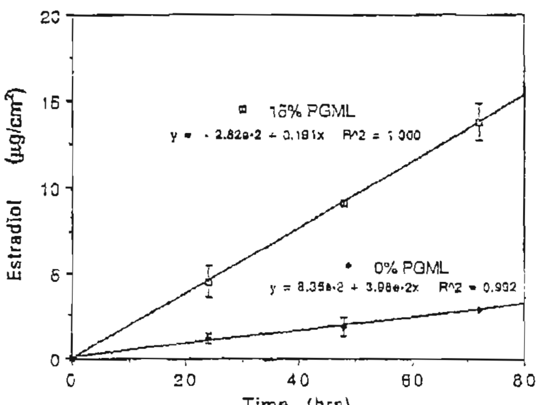

Fig. 5. Transport of estradiol across human epidermis from silicone PSA formulation containing 0% (♦) and 15% (⊡) PGML by weight (n = 12).

ACKNOWLEDGMENTS

The authors would like to thank Professor Donald Downing, University of Iowa, for his helpful suggestions in the synthesis of radiolabeled propylene glycol monolaurate, and Dr. Kathy Farinas at Cygnus for stimulating discussions on thermodynamic equilibrium conditions.

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Home

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TOXICOLOGICAL EVALUATION OF SOME
ANTIMICROBIALS, ANTIOXIDANTS, EMULSIFIERS,
STABILIZERS, FLOUR-TREATMENT AGENTS, ACIDS AND BASES

The content of this document is the result of the deliberations of the Joint FAO/WHO Expert Committee on Food Additives which met at Rome, 13-20 December, 1965[1] Geneva, 11-18 October, 1966[2]

[1] Ninth Report of the Joint FAO/WHO Expert Committee on Food Additives, FAO Nutrition Meetings Report Series, 1966 No. 40; Wld Hlth Org. techn. Rep. Ser., 1966, 339

[2] Tenth Report of the Joint FAO/WHO Expert Committee on Food Additives, FAO Nutrition Meetings Report Series, 1967, in press;

Food and Agriculture Organization of the United Nations
World Health Organization
1967

PROPYLENE GLYCOL ESTERS OF FATTY ACIDS

Chemical description — Mixtures of propylene glycol mono- and di-esters of fatty acids of food fats.

Structural formula

CH_3-CH-OR
    |
    CH_2-OR

Where R or R' represents the fatty acid moiety and R or R' is hydrogen in the mono-esters.

Definition — Propylene glycol esters of fatty acids are mixtures of the esters of these fatty acids with propylene glycol. They are mainly the mono-esters with some di-esters and the commercial products will contain mono- and diglycerides when fats are used for

	transesterification with propylene glycol.
Description	Propylene glycol esters are white to yellowish white beads, or flakes having a bland odour and taste.
Uses	As emulsifier. The content of propylene glycol in the commercial products must be indicated on the label.

Biological Data

Biochemical aspects

Pancreatic lipase hydrolysed 70 per cent. of propylene glycol monostearate *in vitro* at 40° in 15 hours (Balls & Matlack, 1938). Similarly, steapsin hydrolysed 70 per cent. of propylene glycol distearate (PGDS) *in vitro* at 30° in 18 hours (Long et al., 1958). The absorption, metabolism and hydrolysis of PGDS was studied in rats using isotopically labelled compounds, and found to be similar to those of the glyceryl stearate esters (Long at al., 1958a; 1958b).

Acute toxicity

No data are available.

Short-term studies

Rat. Six 21-day old rats were fed for 40 days a diet containing 60 per cent. propylene glycol ester. The animals showed no adverse effect on body-weight gain. On histological examination of the kidneys no lesions were observed (Lepkovsky et al., 1935).

Long-term studies

No data are available.

Comments

There is evidence that the propylene glycol esters of fatty acids are hydrolyzed to propylene and fatty acids. Evaluation is based on the content of propylene glycol, for which an acceptable daily intake has been established (FAO/WHO, 1964).

Evaluation

Estimate of acceptable daily intakes for man

	mg/kg body-weight[1]
Unconditional acceptance	0-20
Conditional acceptance	20-60

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[1] Calculated as propylene glycol; see the Seventh Report of the Joint FAO/WHO Expert Committee on Food Additives (FAO/WHO, 1964).

See Also:
- Toxicological Abbreviations
- Propylene glycol esters of fatty acids (WHO Food Additives Series 5)
- Propylene glycol esters of fatty acids (JECFA Evaluation)

TOXICOLOGY AND APPLIED PHARMACOLOGY 17, 519–528 (1970)

Metabolism of Stearoyl Propylene Glycol Hydrogen Succinate[1]

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Received September 22, 1969

Metabolism of Stearoyl Propylene Glycol Hydrogen Succinate. KING, W. R., MICHAEL, W. R., and COOTS, R. H. (1970). *Toxicol. Appl. Pharmacol.* 17, 519–528. Metabolism of stearoyl propylene glycol hydrogen succinate (SPGHSu) has been studied *in vivo* in the rat and to a limited extent in man, and *in vitro* with pancreatic enzymes. Both 1-^{14}C-stearic acid-labeled and 1-^{14}C-succinic acid-labeled SPGHSu were used. Data from rat metabolism studies were compared with those obtained when either 1-^{14}C-stearic acid-labeled soybean oil, or free 1-^{14}C-succinic acid was fed. The data show that SPGHSu is efficiently metabolized by the animal body.

Hydrolysis of SPGHSu takes place to a large extent prior to absorption. About 86% of the stearic acid moiety was absorbed via the thoracic duct, while more than 90% of the succinic acid moiety was absorbed by another pathway. Respiratory CO_2 was the major end product of catabolism, and no intact SPGHSu was deposited in the animal body. The metabolic fate of the stearic acid moiety of SPGHSu was similar to that of stearic acid in 1-^{14}C-stearic acid-labeled soybean oil. The rate of hydrolysis of the succinic acid–propylene glycol bond in SPGHSu was such that some propylene glycol hydrogen succinate (PGHSu) was excreted in the urine.

During digestion *in vitro* of SPGHSu, both stearic acid and succinic acid were liberated, indicating that the pancreatic enzymes were capable of attacking either end of the molecule. Propylene glycol monostearate and PGHSu were also shown to be products of the digestion.

The tenth report of the Joint FAO/WHO Expert Committee on Food Additives (*World Health Organ.*, 1967) emphasized the importance of metabolic and biochemical studies in the investigation of the biological effects of a food additive. The report indicated that safety-in-use could be satisfactorily projected on this basis alone. Thus, one could extrapolate to the safety of a compound from studies which show that its metabolic fate is identical to that of a material whose safety has been established. As an example, Long *et al.* (1958) were able to demonstrate that propylene glycol stearate esters undergo enzymatic hydrolysis, absorption and metabolism in the body in a manner similar to the naturally occurring stearate glycerides. The data being reported are from metabolism studies that were done to help establish the safety of stearoyl propylene glycol hydrogen succinate (SPGHSu), the principal constituent of a shortening emulsifier known commercially as Succistearin. The data establish that this compound is metabolized by normal metabolic pathways.

[1] This paper was presented in part at the Fifth Annual Meeting of the Society of Toxicology, Inc., Williamsburg, Virginia, March 14–16, 1966.

METHODS

Both 1-^{14}C-stearic acid-labeled and 1-^{14}C-succinic acid-labeled SPGHSu were synthesized. Stearate 1-^{14}C-labeled and nonlabeled propylene glycol monostearate (PGMS) were prepared by the method of Martin and Lutton (1965) for isomerically mixed PGMS. The unlabeled PGMS was reacted with ^{14}C-labeled succinic anhydride which was prepared by metathesis of labeled acid (1-^{14}C-succinic acid) with acetic anhydride in the presence of a catalytic amount of 70% perchloric acid. The product was crystallized from toluene at 10°C. Similarly stearate-labeled PGMS was reacted with unlabeled succinic anhydride. The reaction of PGMS with succinic anhydride used equivalent amounts of the reactants in 5 parts of xylene with refluxing for 6 hr. The products (in xylene) were water washed and the xylene was evaporated. The residue was then crystallized from 10 volumes of hexane at −18°C and finally from 10 volumes of petroleum ether (b.p. 30–60°C) at 10°C. The SPGHSu products were recovered in 65–70% yield and in high purity as indicated by thin-layer chromatography (TLC) and chemical analysis. Analytical values for 1-^{14}C-stearic acid-labeled SPGHSu (S*PGHSu) and 1-^{14}C-succinic acid-labeled SPGHSu (SPGHSu*) were as follows, respectively: acid value, 126 and 127 (theoretical 127); saponification value, 377 and 375 (theoretical 381); percent carbon, 67.9 and 67.8 (theoretical 67.9); and percent hydrogen, 10.6 and 10.6 (theoretical 10.5). The structural formula of SPGHSu is shown in Fig. 1.

$$
\begin{array}{l}
CH_2-O-\overset{O}{\overset{\|}{C}}-R \\
| \\
CH-O-\overset{O}{\overset{\|}{C}}-R \\
| \\
CH_3
\end{array}
$$

FIG. 1. Structural formula of stearoyl propylene glycol hydrogen succinate (SPGHSu). One R is $C_{17}H_{35}$; the other is $CH_2—CH_2—COOH$.

Animals, dosage, and route of administration. Male, Sprague-Dawley rats[2] (200–250 g) were fed the labeled compounds at a concentration of 1% in liquid diets in a manner similar to that described by Coots (1964a). Preparation of the liquid diet was described by Coots (1964a); composition of the diet may be seen in Table 1. Each animal received approximately 5.5 g of diet containing 5–15 μCi of ^{14}C. The precise amount of diet and radioactivity fed was determined for each animal. Data from S*PGHSu studies were compared with those reported by Coots (1964a) in which 1-^{14}C-stearic acid-labeled soybean oil (1-^{14}C-stearic-SBO) was fed, while data from SPGHSu* studies were compared with those obtained when free 1-^{14}C-succinic acid was fed as a control in the present study. All animals were housed individually in stainless steel cages for the duration of the study. The total number of animals fed each compound is shown in the appropriate tables.

Catabolism studies. The experimental procedure was identical to that described by Coots (1964a). Distribution of radioactivity among collected respiratory CO_2, feces, urine, gastrointestinal tract contents, and carcass fractions was determined for each animal at the end of the 51-hr experimental period.

In these, and all studies, glass devices designed to separate urine and feces were

[2] Sprague Dawley, Inc., Madison, Wisconsin.

TABLE 1

COMPOSITION OF LIQUID DIET CONTAINING STEAROYL PROPYLENE GLYCOL HYDROGEN SUCCINATE (SPGHSu)

Component	Weight (%)
Casein	18.5
Sucrose	16.2
Vitamin Mix (in sucrose)[a]	3.6
Salt Mix USP XIV	1.7
Water	32.6
Soybean oil	26.4
Radioactive SPGHSu	1.0

[a] Contained the following in mg/g: 0.06 menadione, 0.08 thiamine, 0.20 riboflavin, 0.40 niacin, 0.40 calcium pantothenate, 0.005 folic acid, 0.08 pyridoxine, 0.003 B_{12}, 60 choline chloride, 40 inositol, 2 ascorbic acid, 2 *p*-aminobenzoic acid, 0.005 biotin.

attached to the metabolism cages and the excreta were collected as single fractions in glass vessels.

Absorption and simultaneous catabolism-absorption studies. Each animal was provided with a thoracic duct cannula by a procedure similar to that of Bollman *et al.* (1948). The day after surgery, each radioactive compound was fed to 4 or 5 animals. Two rats from each group were selected for the simultaneous catabolism-absorption portion of the study and were placed immediately into individual metabolism chambers designed for the collection of lymph, respiratory CO_2, feces, and urine. Each of these constituents was collected as a single fraction during the 51-hour experimental period. The remaining 2 or 3 animals in each group were used for lymph collection in the absorption study. The experimental procedure was similar to that described by Coots (1964a) but with the following exceptions: (1) lymph was collected in a single fraction; and (2) lipids were extracted from freeze-dried lymph using chloroform–methanol (2:1, v/v). Methods described by Hirsch and Ahrens (1958) and Carroll (1963) were employed to separate the lipids into neutral lipid and phospholipid fractions. Distribution of ^{14}C in these fractions was determined using the Packard Tri-Carb liquid scintillation spectrometer. TLC, using silica gel G, was carried out on the lipids and selected segments from the developed chromatograms were radioassayed to determine the distribution of radioactivity among the various lipid constituents, and to determine whether any intact SPGHSu was present. Solvent systems found to give satisfactory separation of the various constituents (sterol esters, free sterols, triglyceride, diglycerides, free fatty acids, and monoglycerides) were: petroleum ether–ethyl ether–acetic acid (90:10:5, v/v), petroleum ether–ethyl ether–pyridine (20:80:2, v/v), and benzene–tetrahydrofuran–pyridine (90:10:2, v/v).

Digestion, in vitro. Both stearic acid-labeled and succinic acid-labeled SPGHSu were subjected to enzymatic digestion *in vitro*. The following procedure was employed: to 50 mg of SPGHSu was added 0.9 ml of Tris buffer (pH 8) and 0.03 ml of a 45% solution of $CaCl_2$. After high frequency sonication, 2 ml of fresh pancreatic juice plus bile

(obtained by cannulating the common pancreatic-bile duct of the rat) was added, and the mixture was shaken in a Dubnoff-type incubator at 38°C for 6 hr. The digestion w. stopped by adjusting the pH to about 2 using 50% HCl. The acidified mixture was then extracted with ethyl ether, and the distribution of radioactivity between the ether and aqueous phases was determined. TLC and radioassay procedures were used to determine the distribution of ^{14}C among the products of digestion. A two-dimensional system was used to separate free stearic acid, PGMS, and SPGHSu. In the benzene-tetrahydrofuran–pyridine (90:10:1, v/v) solvent system, the R_f value of PGMS was 0.8, while free stearic acid and SPGHSu remained near the origin at an R_f of 0.2–0. The plate was then turned on its side and developed with petroleum ether–ethyl eth. acetic acid (90:10:1, v/v) to obtain a separation between free stearic acid and SPGHSu. It was necessary to esterify the digestion products of SPGHSu* in order to distinguish between free succinic acid and the partial hydrolytic product propylene glycol hydrogen succinate (PGHSu).

Urine studies (rat). Urine collected during metabolism studies from animals fed the succinate-labeled compound (SPGHSu*) was subjected to various treatments in an effort to identify the nature of the ^{14}C in the urine. Selected segments from thin-la r chromatograms of untreated, hydrolyzed, and methylated urine were radioassayed. Hydrolysis was accomplished by refluxing the urine for 6 hr in 3 N HCl, while urine was methylated in the following manner: samples were freeze-dried, the residue was suspended in tetrahydrofuran, and methyl esters were prepared using ethereal diazomethane. The TLC solvent system was ethyl ether–petroleum ether–acetic acid (90:10:5, v/v). Gas-liquid chromatographic analyses of the methyl esters were obtained using an Aerograph Model A700 unit equipped with a Barber-Coleman recorder. Separation of the urine constituents was obtained using a 10-ft, ¼-inch (o.d.) column packed w 60/80-mesh Gas-chrom P[3] containing 15% by weight EGSSX.[3] Column temperature was 195°C, and the helium gas pressure was maintained at 30 psi, resulting in a gas flow rate of about 100 ml/min.

Urine studies (human). Urine was collected from two human subjects over a 48-hr period following the consumption of 10 g of high purity, unlabeled SPGHSu. The subjects were nonfasted males, 28 and 35 years of age and weighed ~75 and 80 kg, respectively. The entire urine collection from each subject was combined to represent a single 48-hr fraction. The urine was acidified to pH 1–2 using 50% HCl and then extracted with diethyl ether. This procedure was capable of recovering 60–75% of added PGHSu from urine. The ether extracts were esterified using ethereal diazomethane. After removal of solvent, the residue was suspended in 3–5 ml of methanol prior to gas-liquid chromatography (GLC). Urine to serve as controls was taken from the two subjects prior to administration of SPGHSu and carried through the same procedure. While most of the studies with human urine were carried out using the GLC conditions described above, additional studies were conducted under conditions designed to vary the retention time of the PGHSu standard. An 8-ft, ¼-inch (o.d.) column packed with 80/100-mesh acid-washed Chromosorb W[3] containing 15% by weight SE-30[3] was maintained at 175°C; another 8-ft, 3/16-inch (o.d.) column was packed with 60/80 -mesh acid-washed Chromosorb W containing 10% by weight Apiezon.[3] Temperature in this column was maintained at 180°C.

[3] Manufactured by Applied Science, State College, Pennsylvania.

Catabolism Studies

Figure 2 shows tl ing the ^{14}C-labeled from all animals fed compounds appear ^{14}C from the steari appeared in the CC SPGHSu*.

% of Fed Radioactivity (cumulative) 100 90 80 70 60 50 40 30 20 10 0

FIG. 2. Rate and exte of S*PGHSu, 1-^{14}C-st activity resides in this

DISPOSITION OF ^{14}C

Fraction
CO_2
Feces
Gastrointestinal trac contents
Urine
Carcass
Total
Number of animals

ª Standard error of

Table 2 shows tl experimental perio that fed. Absorptio by the small amour

RESULTS

Catabolism Studies

Figure 2 shows the rate of appearance of radioactivity in respiratory CO_2 after feeding the ^{14}C-labeled compounds. Each point on the curves is the average of the values from all animals fed the particular compound. Radioactivity from succinic acid-labeled compounds appeared in the CO_2 at a much faster rate and to a greater extent than did ^{14}C from the stearic acid-labeled materials. Radioactivity from free ^{14}C-succinic acid appeared in the CO_2 at a faster rate in the early hours of the study than did ^{14}C from SPGHSu*.

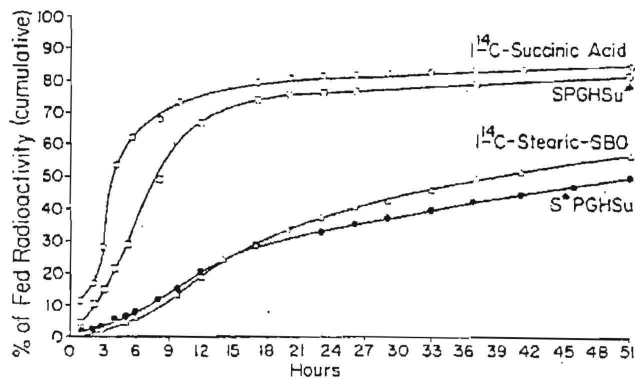

FIG. 2. Rate and extent of appearance of ^{14}C in the respiratory CO_2 of rats after feeding a single dose of S*PGHSu, 1-^{14}C-stearic-SBO, SPGHSu*, or 1-^{14}C-succinic acid. Asterisk (*) indicates that ^{14}C activity resides in this moiety.

TABLE 2

DISPOSITION OF ^{14}C BY THE RAT 51-HOURS AFTER FEEDING S*PGHSu, 1-^{14}C-STEARIC-SBO, SPGHSu*, AND 1-^{14}C-SUCCINIC ACID

Fraction	Percent of fed ^{14}C			
	S*PGHSu	1-^{14}C-Stearic-SBO	SPGHSu*	1-^{14}C-Succinic acid
CO_2	47.8 ± 2.4[a]	57.0 ± 0.9	77.0 ± 0.8	82.7 ± 1.0
Feces	1.5 ± 0.5	2.4 ± 0.3	1.0 ± 0.1	0.5 ± 0.1
Gastrointestinal tract contents	1.4 ± 0.4	1.3 ± 0.5	0.3 ± 0	0.3 ± 0
Urine	1.1 ± 0.4	0.5 ± 0.3	6.3 ± 0.8	1.6 ± 0.1
Carcass	45.1 ± 2.6	39.0 ± 1.4	10.4 ± 0.2	7.0 ± 0.5
Total	96.9	100.2	95.0	92.1
Number of animals	8	4	4	3

[a] Standard error of the mean.

Table 2 shows the disposition of administered radioactivity at the end of the 51-hr experimental period. Total recovery of radioactivity ranged from about 92 to 100% of that fed. Absorption of the ^{14}C portion of each compound was excellent, as evidenced by the small amounts of radioactivity present in the feces and gastrointestinal contents.

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Page 38 of 127

In general, the distribution of ^{14}C from SPGHSu labeled in either the stearic acid o- succinic acid moiety was similar to that of the appropriate control. The only notab exception was an increased urinary excretion of radioactivity by animals fed the succi-nate-labeled compound, SPGHSu*. This finding will be discussed in detail.

Differences in distribution of administered radioactivity between stearic acid-labeled and succinic acid-labeled SPGHSu are obvious. When the label was in the succinate portion of the compound (SPGHSu*), total excretion of radioactivity as $^{14}CO_2$ was 77% of that fed, compared to a total $^{14}CO_2$ excretion of about 48% when the stearic acid moiety (S*PGHSu) was radioactive. Concomitantly, a larger portion of the stearic a moiety remained in the carcass than of the succinic acid portion of the molecule. analysis of the carcass lipids, which were recovered by the procedure of Olivecrona (1962), did not reveal the presence of any unhydrolyzed SPGHSu.

Absorption and Simultaneous Catabolism-Absorption Studies

Data from the two portions of this study have been combined and are presented in Table 3. Since all fractions were collected in the simultaneous catabolism-absorption studies, it was possible to determine total recovery of the administered ^{14}C. Recov values represent data obtained from those animals taking part in the simultaneous catabolism-absorption portion of the study. A simultaneous catabolism-absorption study was not conducted when 1-^{14}C-stearic-SBO was fed.

When the stearate labeled compounds (S*PGHSu and 1-^{14}C-stearic-SBO) were administered, 83–86% of the radioactivity was recovered in the lymph. In addition, about 14% of the ^{14}C from stearic acid-labeled SPGHSu was absorbed by a pathway other than the thoracic duct, and most of this radioactivity (10% of that fed) was then excreted as $^{14}CO_2$. It can be assumed that metabolism of 1-^{14}C-stearic-SBO occurred much the same manner. In absorption studies where respiratory CO_2 and urine were not collected, about 13% of the fed ^{14}C was missing (Table 3). Urinary excretion of radio-activity by animals fed 1-^{14}C-stearic-SBO has been shown to be less than 1% (Table 2). Therefore (by difference) about 12% of the administered ^{14}C from cannulated animals fed the 1-^{14}C-stearic-SBO must have been catabolized. Blomstrand (1954) and Coots (1964b) have shown that animals provided with thoracic duct cannulae may excrete as much as 10% of the administered radioactivity in the respiratory CO_2 after being f long-chain fatty acids.

Data from animals fed free 1-^{14}C-succinic acid and the succinate-labeled compound (SPGHSu*) reveal that most of the labeled moiety was absorbed by a pathway other than the thoracic duct (presumably the portal vein) and was then catabolized to CO_2. In addition, about 6% of the total ^{14}C from SPGHSu* was excreted in the urine (Table 3), thus confirming the observation made in the intact animal (Table 2). Gastrointestinal tract contents plus feces data again show that the total absorption of the ^{14}C portion of each compound was excellent.

Lipids recovered from the lymph of animals fed the ^{14}C-stearic acid-labeled com-pounds contained 97–99% of the total lymph ^{14}C. Neutral lipids accounted for 91–93% of this lipid radioactivity, while the remaining 7–9% was in the phospholipid fraction. Triglycerides were the major neutral lipid constituent. There was no evidence of any unhydrolyzed SPGHSu in the lymph lipids. Because of the very small amounts of radio-activity in the lymph of animals fed SPGHSu* or free 1-^{14}C-succinic acid, it was difficult

TABLE 3

TABLE 3

DISPOSITION OF ^{14}C IN THORACIC DUCT-CANNULATED RATS 51-HOURS AFTER FEEDING S*PGHSu, 1-^{14}C-STEARIC-SBO, SPGHSu*, AND 1-^{14}C SUCCINIC ACID

Fraction	Percent of fed ^{14}C			
	S*PGHSu	1-^{14}C Stearic-SBO	SPGHSu*	1-^{14}C-Succinic acid
CO_2	10.2 (11.0 and 9.4)	—	68.4 (70.4 and 66.4)	77.5 (78 and 77)
Lymph	86.2 ± 1.8[a]	83.0 ± 3.1	5.5 ± 0.5	2.6 ± 0.3
Gastrointestinal tract contents + feces	1.4 ± 0.7	3.2 ± 0.3	0.7 ± 0.3	0.5 ± 0.3
Urine	0.8 (0.8 and 0.8)	—	6.2 (6.8 and 5.6)	2.0 (1.4 and 2.6)
Carcass	3.1 ± 0.5	0.9 ± 0	7.9 ± 0.7	5.3 ± 0.2
Total recovery[b]	103.0	—	88.9	87.3
Number of animals	5	4	5	4

[a] Standard error of the mean.
[b] Total recovery based only on animals taking part in simultaneous study.

and impractical to obtain a complete distribution of the lymph ^{14}C. However, less than 50% of the total lymph radioactivity was present as lipid material. Neutral lipids contained 77–88% of the lipid ^{14}C while the remainder of the activity was found in the phospholipid fraction.

Digestion in vitro

Distribution of radioactivity following enzymatic digestion *in vitro* of the stearate-labeled compound (S*PGHSu) showed that 44% of the total ^{14}C remained as intact S*PGHSu, while 53% was present as free stearic acid, and 3% as propylene glycol mon stearate (PGMS). After the succinate-labeled compound (SPGHSu*) was carried through this digestion procedure, 46% of the radioactivity was present as intact SPGHSu*, 40% as free succinic acid, and 14% as the partial hydrolytic product PGHSu. Therefore, enzymes of pancreatic juice hydrolyzed both the stearate and succinate linkages in SPGHSu. The percentage of the SPGHSu molecule which was completely hydrolyzed was calculated and found to be 38%. In addition, 17% of the molecule was partially hydrolyzed to form either PGMS or PGHSu. It may be concluded that further digestion would have occurred had the incubation been continued for more than 6 h. In other studies, the digestive enzymes were also shown to be capable of breaking down PGMS and PGHSu to yield free stearic and free succinic acids.

Urine Studies (Rat)

Thin-layer chromatography of untreated and methylated urine, followed by radioassay of the plate scrapings revealed that about 5% of the urine ^{14}C from animals fed the succinate-labeled compound (SPGHSu*) corresponded to the partial hydrolytic product PGHSu. This was about 0.3% of the administered ^{14}C. Distribution of the remaining urine radioactivity was difficult to obtain because of its very polar nature. Under the TLC conditions employed, the ^{14}C was found to be spread or "streaked" from the point of application to an R_f of about 0.4 regardless of whether the urine was untreated or hydrolyzed.

Gas-liquid chromatographic analysis of methyl esters obtained from the urine revealed trace amounts of a material having a retention time of 9–10 min, corresponding exactly to that of the PGHSu standard. Such material was not present in the urine of animals fed free 1-^{14}C-succinic acid. Similar studies were not conducted when the stearate-labeled compound (S*PGHSu) was fed.

Urine Studies (Human)

When methyl esters obtained from the urine of human subjects fed high purity, unlabeled SPGHSu were subjected to GLC, material corresponding to the partial hydrolytic product PGHSu was shown to be present. Retention times of the PGHSu standard were as follows: about 3 min when the stationary phase contained SE-30; about 4 min in the Apiezon column; and just over 9 min using the column containing EGSSX. On all three columns, material with a retention time corresponding to that of the PGHSu standard was present in the urine of the test subjects, while no such material was present in the control urine. To further establish that the peak in question did represent PGHSu, trace amounts of the PGHSu standard were added to the urine extracts. The resulting

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GLC tracings revealed an increased height of the peak previously identified as representing PGHSu in the urine of the test subjects. When control urine was treated in the same manner, the added PGHSu was observed as a new peak having a retention time identical to that of the PGHSu standard and corresponding exactly to the peak in question in the urine of the test subjects.

By comparing the GLC tracings of the urine extracts with those of known PGHSu standards, it was possible to estimate the minimum total excretion of the partial hydrolytic product by each test subject. Minimum excretion over the 48-hr period by one subject was about 11 mg, while about 6 mg of PGHSu was excreted by the second subject. On a weight basis this was about 0.1 % of the total SPGHSu consumed.

DISCUSSION

Data from metabolism studies clearly show that absorption of administered radioactivity was excellent for each compound investigated. Hydrolysis of SPGHSu occurred to a large extent prior to absorption; the thoracic lymph duct was the major pathway of absorption for the stearic acid moiety, while absorption of the more polar, water-soluble succinic acid moiety occurred by a pathway other than the thoracic duct, presumably the portal vein.

In catabolism studies (intact animals), 48–57 % of the radioactivity from the stearate-labeled compounds appeared in the respiratory CO_2 over the 51-hr experimental period (Fig. 2). Most of the remaining ^{14}C was found in the carcass, as shown in Table 2. This was not surprising as long-chain fatty acids are known to be stored in depot fat. No unhydrolyzed SPGHSu was found in the carcass lipids.

In catabolism studies involving the succinic acid-labeled compounds, 77–83 % of the administered ^{14}C was excreted in the respiratory CO_2 (Table 2). The catabolism of both the succinic acid moiety of SPGHSu and free succinic acid was quite rapid, as shown in Fig. 2. The fact that radioactivity from free ^{14}C-succinic acid appeared in the CO_2 at a faster rate in the early hours of the study than did ^{14}C from SPGHSu* was reasonable. The succinic acid portion of SPGHSu had to be released by hydrolysis before it became available for absorption and catabolism, while free 1-^{14}C-succinic acid could be rapidly absorbed and carried to the liver for immediate catabolism. In addition, about 6 % of the radioactivity arising from the succinic acid-labeled portion of the compound was excreted in the urine. If complete hydrolysis of the succinate-labeled compound (SPGHSu*) had taken place prior to absorption, free ^{14}C-succinic acid would have been the only radioactive digestion product formed. Urinary excretion of ^{14}C would then have been similar for both the succinic acid portion of SPGHSu* and for free 1-^{14}C-succinic acid. The simultaneous absorption-catabolism data (Table 3) showed that urine ^{14}C arose from material absorbed by a nonthoracic duct pathway, and that part of this material was not free ^{14}C-succinic acid, but some other water-soluble material. Once PGHSu was shown to be a product of digestion, *in vitro* of SPGHSu, it also seemed reasonable that some of this partial hydrolytic material might have been formed *in vivo* and excreted as such. PGHSu is a low molecular weight, water-soluble, anionic compound which could be absorbed via the portal system and easily excreted by the kidney. The finding of PGHSu in the urine of animals fed the succinic acid-labeled compound confirmed that this was the case. Thus, the increased urinary excretion of ^{14}C by animals

fed the succinic acid-labeled compound, and the metabolic difference between the succinic acid moiety of SPGHSu and free ^{14}C-succinic acid are explained. Feeding studies by King *et al.* (1970) showed that the formation and excretion of PGHSu was not deleterious to the animal.

After PGHSu was shown to be a urinary metabolite of SPGHSu in the rat, it became a matter of interest whether or not a similar metabolic process would occur in man. Data obtained from the human studies indicated that about 0.1% of the consumed SPGHSu was partially hydrolyzed to PGHSu and excreted in this form. This then confirmed that the same pathway was available in man as in the rat for the metabolism of SPGHSu.

ACKNOWLEDGMENTS

The authors wish to express their gratitude to Mr. D. D. Dearwester for expert technical assistance and to Dr. J. B. Martin and Mr. H. W. Lampe for preparation of the test materials and radioassay.

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Feeding of Succistearin to Rats and Dogs

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Feeding of Succistearin to Rats and Dogs. KING, W. R., MICHAEL, W. R., and COOTS, R. H. (1971). *Toxicol. Appl. Pharmacol.* 18, 26–34. Feeding of Succistearin to rats and dogs for periods up to 6 months, and at dietary levels as great as 10% did not cause any deleterious effects. All animals in each study appeared to be normal and in excellent health for the duration of the feeding period, and there was no evidence of either gross or microscopic pathology which could be attributed to the shortening emulsifier.

Absorption of dietary fatty acids by rats fed Succistearin in the 90-day study decreased as the level of Succistearin in the diet was increased. This resulted in a decreased utilization of feed by these animals. It was established that the reduced absorption of fatty acids was a reflection of the high level of stearic acid present in Succistearin.

Even though group average values were within established ranges, a few scattered, statistically significant group differences between test and control animals were observed in blood, urine, and organ weight data. The differences were usually quite small, did not establish trends or patterns indicative of a dose-related response, and were considered to be of no special biological significance. None of the parameters studied revealed indications of toxicity related to the feeding of Succistearin.

Succistearin is a shortening emulsifier which is prepared by reacting edible grade succinic anhydride with a mixture of propylene glycol monostearate and monostearin.[1] Composition of Succistearin used in these studies is shown in Table 1. The potential usefulness of this material as an emulsifier in shortening prompted an evaluation of its safety as a

TABLE 1

COMPOSITION OF SUCCISTEARIN

Constituent	%
Stearoyl propylene glycol hydrogen succinate (SPGHSu)	50.5
Triglycerides + propylene glycol distearate	12.0
Diglycerides + free fatty acids	6.3
Monoglycerides	3.6
Propylene glycol monostearate (PGMS)	17.3
(Propylene glycol hydrogen succinate)$_2$ PGHSu	2.1
Succinic acid, succinic anhydride, and succinated polyols	8.2

[1] The reaction products are hereafter referred to as Succistearin; details of the synthesis are described in U.S. Patent 3,375,262 issued 3/21/68.

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DIETS FO

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Succistearin[c]
Soybean oil
Stearic acid
Succinic acid
Total
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shown in Table 2.

component of foods. A 90-day feeding experiment in rats served as the basis for 6-month feeding studies in both rats and dogs. Succistearin was fed at levels as great as 10% of the total diet. Data clearly show the material to be innocuous, even when fed at the high level.

Metabolic studies have been conducted with stearoyl propylene glycol hydrogen succinate (SPGHSu), which is the principal constituent of Succistearin. Results of these studies, reported separately (King *et al.*, 1970), established that SPGHSu is readily utilized by the animal and that it is metabolized by normal metabolic pathways.

METHODS

Rats (*90-day study*). Fifty male and 50 female weanling rats of the Sprague-Dawley strain were distributed into 5 groups of 10 males and 10 females each on the basis of litter and body weight. The rats were housed in individual cages, and, except during periods of urine collection, food and water were offered ad libitum.

TABLE 2

DIETS FOR 90-DAY AND 6-MONTH SUCCISTEARIN FEEDING STUDIES IN RATS

Ingredient	Diet group				
	1 Soybean oil control (%)	2 Succistearin at 2.5% (%)	3 Succistearin at 5.0% (%)	4 Succistearin at 10% (%)	5 Free acids (%)
Ground pellets	71.5	71.5	71.5	71.5	71.5
Casein	5.5	5.5	5.5	5.5	5.5
Salt Mix USP XIV	1.0	1.0	1.0	1.0	1.0
H_2O Sol. Vit. Mix[a]	1.0	1.0	1.0	1.0	1.0
Fat Sol. Vit. Mix[b]	1.0	1.0	1.0	1.0	1.0
Succistearin[c]	—	2.5	5.0	10.0	—
Soybean oil	20.0	17.5	15.0	10.0	15.5
Stearic acid	—	—	—	—	3.4
Succinic acid	—	—	—	—	1.1
Total	100.0	100.0	100.0	100.0	100.0
Cal/g	4.89	4.85	4.80	4.71	4.83

[a] Furnished the following in mg/kg of diet: menadione, 0.6; thiamine, 0.8; riboflavin, 1.0; niacin, 4.0; folic acid, 0.5; Ca pantothenate, 4.0; pyridoxine, 0.8; inositol, 4000; *p*-aminobenzoic acid, 20.0; biotin, 0.005; cyanocobalamin, 30.0; ascorbic acid, 20.0; and choline chloride, 600.
[b] Furnished the following IU per kg of diet: 6000 vitamin A; 1600 vitamin D_2; and 50 vitamin E as *d*-α-tocopheryl acetate.
[c] Caloric value: 7.6 cal/g (calculated).

The basal diet consisted of a mixture of ground pellets (Purina Lab Chow), inorganic salts, vitamins, casein, and fat. The diets were approximately isocaloric and the total fat content of each was 21%, exclusive of fat contained in the ground pellets. Soybean oil was used to adjust the fat content of each diet to the proper level. Diet compositions are shown in Table 2. Group 1 (serving as the major control) was fed the basal diet in which

soybean oil (SBO) was the dietary fat. Succistearin was fed to groups 2–4 at dietary levels of 2.5, 5.0, and 10%, respectively. Group 5 was fed a diet which contained free stearic acid and free succinic acid at levels corresponding to the total stearic and succinic acid content of Succistearin fed at the 5% level (group 3). This group was included as a positive control to determine what might happen if rapid and complete hydrolysis of Succistearin occurred prior to absorption.

Food consumption and body weights were measured on a weekly basis. Regular observations were made throughout the study on the general appearance, condition, and behavior of each animal. During the third and ninth weeks, 8 males and 8 females from each group were placed in individual metabolism cages for urine collection. Animals to be used during each collection period were selected at the beginning of the study. The animals were allowed one day to become conditioned to the new environment, after which food was removed and urine was collected under toluene over a 16-hr period. The general appearance of the urine was noted, volume and pH measurements were made, and samples were assayed for total nitrogen using a semimicro Kjeldahl method. Specific gravity was also determined on urine collected during the ninth week.

Feces were collected over a 5-day period from each animal during the fourth and tenth weeks of the study and were dried in a vacuum oven at 80°C for 16 hr. Samples were ground to a powder, saponified with 2% alcoholic KOH, acidified to pH 4.5 with 6 N HCl, and the total long-chain fatty acids were extracted with petroleum ether. From the weight of these acids and the weight of fatty acids consumed in the diet, the percentage of dietary fatty acids absorbed was calculated.

Blood was taken from the tails of all animals in each dietary group during the fifth and eleventh weeks of the study, and red cell count, white cell count, differential white cell counts, and hemoglobin content were determined. In addition, a microhematocrit was performed on blood taken during the eleventh week of the study using the Adams Autocrit centrifuge.[2] The Coulter counter (Model F)[3] was used to determine total red and white blood cell counts, and Giemsa stain[4] was applied to blood films for leukocyte differential counts.

At the end of the 90-day feeding period, the animals were killed by decapitation. A gross autopsy was performed on each animal, and the following organs were removed and weighed: brain, heart, liver, lungs, spleen, kidneys, adrenals, and testes or ovaries. Sections from each of these organs along with portions of the stomach, small intestine, urinary bladder, esophagus, trachea, thyroid, and perirenal and epididymal fat were taken for histologic examination. Tissues were fixed in Bouin solution, mounted in paraffin, and stained with hematoxylin and eosin.

Data from each phase of the study were analyzed statistically to determine whether there were significant differences between animals fed Succistearin and those fed the SBO control diet.

Rats (6-month study). The design of this study was similar to the 90-day study, with the following exceptions: (1) groups 2 and 5 (low level of Succistearin and free acids) were omitted; (2) 24-hr urine collections were made during weeks 13 and 26, and in addition to the analyses noted above, urine was examined for the presence of propylene

[2] Clay-Adams, Inc., New York, New York.
[3] Coulter Electronics, Inc., Hialeah, Florida.
[4] Matheson, Coleman and Bell, Norwood, Ohio.

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glycol hydrogen succinate (PGHSu) according to the procedure reported separately (King *et al.*, 1970); (3) fat absorption was not measured; and (4) blood was taken from the tail during the eleventh week of the study and from severed neck vessels at necropsy.

Dogs (6-month study). Twelve, young adult (6–8 months of age), purebred beagle dogs obtained from the American Animal Industries of Indianapolis, Indiana, were distributed into three groups of four animals each. Each group contained two males and two females. The animals were housed in individual cages, and except during periods of urine collection, food and water were offered ad libitum. Diets were approximately isocaloric, with the total added fat (SBO or SBO + Succistearin) amounting to 15% of the diet. Group 1 served as the control and was fed Wayne Dog Meal to which was added SBO and casein. Groups 2 and 3 were fed a ration similar to the control, but with Succistearin replacing a portion of the added SBO. Diet compositions are shown in Table 3.

TABLE 3
DIETS FOR 6-MONTH SUCCISTEARIN FEEDING STUDY IN DOGS

Ingredient	Diet group: 1 Soybean oil control (%)	2 Succistearin at 5% (%)	3 Succistearin at 10% (%)
Wayne Dog Meal[a]	82.0	82.0	82.0
Casein	3.0	3.0	3.0
Soybean oil	15.0	10.0	5.0
Succistearin[b]	—	5.0	10.0
Total	100.0	100.0	100.0
Cal/g	4.99	4.90	4.81

[a] Caloric value: 4.23 cal/g.
[b] Caloric value: 7.6 cal/g (calculated).

Food consumption and body weights were measured on a weekly basis, and daily observations of each dog were made throughout the study. Urine was collected from each animal during weeks 4, 13, and 26 of the study; samples were assayed for specific gravity, pH, total nitrogen, and the presence of PGHSu.

Blood was taken from the cephalic vein prior to the study and during weeks 4, 13, and 26 of the study. The following determinations were made: red cell count, white cell count, differential white cell counts, hemoglobin, and hematocrit.

At the end of the 6-month feeding period, the animals were killed by iv administration of an overdose of thiamylal sodium (Surital[5]). After exsanguination, a gross autopsy was performed, and selected organs were removed and weighed. Organs and tissues taken for histology were the same as those in the 90-day rat feeding study.

Data from animals fed the Succistearin diets were compared with the control values to determine whether there were appreciable differences. Because of the small number of animals in each group, however, data were not treated statistically.

[5] Parke, Davis & Co., Detroit, Michigan.

RESULTS AND DISCUSSION

Rat Feeding Studies

No significant abnormalities of behavior or appearance were noted in either the 90-day or 6-month feeding study. All animals appeared to be active and in excellent health, and no deaths occurred prior to necropsy. Group average values for initial body weight, gain in body weight, cumulative food consumption, and feed efficiency are shown in Table 4. Values significantly different from the SBO-fed controls are designated. In the 90-day study, males fed Succistearin at the high dietary level (10%) consumed significantly more food than did the SBO-fed control animals. In addition, several feed efficiency differences were noted; with one exception these occurred in groups 4 and 5, the 10% Succistearin and free acids groups. The decreased feed efficiencies are related to total fatty acid absorption, which will be discussed later. The poor efficiency experienced in females fed Succistearin at the low level (2.5%) was not considered to be a function of the test material since the value for females fed Succistearin at the 5% level was not significantly different from the SBO control value. It should be noted that at the end of the 6-month feeding period, feed efficiency values were much lower than in the 90-day study and there were no statistically significant differences between animals fed the Succistearin and SBO control diets ($p > 0.05$). The difference in feed efficiencies between the 90-day and 6-month studies probably reflects a decreased demand on the diets once the period of rapid growth was passed. The only significant difference between test and control animals in the 6-month study was a slightly decreased growth by males fed Succistearin at the 10% dietary level.

Urine collected from all animals during each collection period was clear and pale yellow with no visible sediment. Urine data are not reported because all group average values were within normal ranges, and there were no apparent differences between test and control animals. Trace amounts of PGHSu were shown to be present in the urine of animals fed Succistearin in the 6-month study. It is not known whether the urinary PGHSu represented dietary or metabolic material because the test material contained about 2% of PGHSu (Table 1). However, metabolic studies (King *et al.*, 1970) using succinic acid-1-^{14}C-labeled SPGHSu showed that PGHSu can be a product of metabolism of SPGHSu.

Table 5 shows the total fatty acids (TFA) absorption data obtained during the 90-day study. It is apparent that as the dietary level of Succistearin was increased, TFA absorption decreased. This was anticipated because metabolic work involving SPGHSu had shown that hydrolysis of the molecule occurred to an appreciable extent prior to absorption, and that free stearic acid was a major product of hydrolysis. Absorbability of free stearic acid is known to be poor (Carroll, 1958). Absorption of fatty acids by animals fed the diet containing free acids also was significantly less than corresponding control values, thus confirming the poor absorption of free stearic acid. The decreased TFA absorption in the 10% Succistearin and free acids groups represented a substantial caloric loss and helped to explain the poor feed efficiency values observed in the 90-day study. This loss of calories was not reflected in the 6-month feed efficiency values, probably because demands on the diets had been reduced as a result of decreased growth rates. It should be noted, however, that both feed efficiency and TFA absorption data indicate that digestion and absorption of the 5% Succistearin diet was more similar to that of the SBO control than to that of the free acids diet.

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TABLE 4

FOOD CONSUMPTION, INITIAL BODY WEIGHT, BODY WEIGHT GAIN AND FEED EFFICIENCY VALUES FROM THE 90-DAY AND 6-MONTH SUCCISTEARIN FEEDING STUDIES IN RATS

Group	Initial body weight (g)		Cumulative gain in body weight (g)		Cumulative food consumption (g)		Cumulative feed efficiency[a]	
	Male	Female	Male	Female	Male	Female	Male	Female
90-Day Study								
SBO Control	71	67	383	196	1540	1096	24.8	17.9
2.5% Succistearin	71	67	380	191	1505	1136	25.2	16.8[b]
5.0% Succistearin	71	67	388	207	1553	1146	25.0	18.1
10% Succistearin	71	67	379	198	1655[b]	1179	22.9[b]	16.8[b]
Free Acids	71	67	374	185	1581	1104	23.6[b]	16.8[b]
Six-Month Study								
SBO Control	63	61	487	242	3078	2400	15.8	10.1
5.0% Succistearin	63	61	505	254	3106	2462	16.3	10.3
10% Succistearin	63	61	462[b]	253	3034	2415	15.2	10.5

[a] Grams of weight gained per 100 g of diet consumed.
[b] Significantly different from the SBO Control ($P < 0.05$).

TABLE 5
TOTAL FATTY ACID (TFA) ABSORPTION DATA FROM THE 90-DAY SUCCISTEARIN FEEDING STUDY IN RATS

Group	TFA absorption (% of fed)			
	Males		Females	
	4th wk	10th wk	4th wk	10th wk
SBO Control	88.4	92.4	92.8	93.2
2.5% Succistearin	85.9[a]	89.6[a]	91.4	92.2
5.0% Succistearin	83.6[a]	87.5[a]	88.3[a]	91.7
10% Succistearin	80.3[a]	84.9[a]	86.3[a]	88.7[a]
Free acids	73.0[a]	74.5[a]	78.0[a]	77.9[a]

[a] Significantly different from the SBO Control ($p < 0.05$).

All hematologic values were within the normal ranges reported in the literature (Burns and DeLannoy, Jr., 1966; Altman and Ditmer, eds., 1964), and there were no indications of any blood disorders. Differences between test and control values were minimal and did not establish any trend or patterns indicative of a toxic response. Therefore, blood data from the rat feeding studies are not included in this report.

The total leukocyte counts obtained from terminal blood for all groups in the 6-month study were appreciably less than corresponding values obtained either earlier in the 6-month study or in the 90-day study. This difference is believed to be a reflection of the anatomical source of the blood since it has been reported by Quimby and Goff (1952) that peripheral blood contains an increased number of leukocytes as compared to heart blood of rats. Terminal blood in the 6-month study was taken from the severed neck vessels, whereas all previous blood had been taken from the tail. Similar differences have been observed in other studies in this laboratory.

Gross autopsies, conducted at the end of each study, revealed no apparent pathologic changes. Microscopic examination of the various organs and tissues did not reveal any changes or lesions which could be interpreted as evidence of toxicity.

Organ weights, both absolute and as a percentage of the final body weight, and organ/brain weight comparisons appeared to be normal for animals of this age and species. A few scattered, statistically significant ($P < 0.05$) differences were observed between the SBO control animals and those fed the Succistearin and free acids diets. In no case, however, did the differences establish a trend or pattern that was indicative of a test-related response.

Dog Feeding Study

All dogs remained in apparent good health and showed normal behavior throughout the study. Table 6 shows average values for initial body weight, growth, food consumption, and feed efficiency. While most group differences were small, it may be seen that the Succistearin diets were utilized less efficiently than the SBO control diet. This again probably reflects a decreased absorption of the stearic acid content of the test material. It is evident that dogs of this age were not in a period of rapid growth. Therefore, parameters such as growth, food consumption, and feed efficiency are not so meaningful as in studies where younger animals are used.

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TABLE 6

6-MONTH SUCCISTEARIN FEEDING STUDY IN DOGS BODY WEIGHT GAIN, FOOD CONSUMPTION, AND FEED EFFICIENCY

Group	Initial weight (kg)		Growth (kg)		Food consumption (kg)		Feed efficiency[a]	
	Males	Females	Males	Females	Males	Females	Males	Females
SBO Control	7.71	6.80	2.96	1.37	35.5	31.1	8.3	4.4
5% Succistearin	8.62	6.58	2.96	0.91	45.6	31.6	6.5	2.9
10% Succistearin	8.85	7.71	1.36	1.36	43.3	36.2	3.1	3.8

[a] Grams of weight gained per 100 g of diet consumed.

Urine obtained from animals during the three collection periods appeared to be normal with regard to color, clarity, and sediment. The analytical data revealed no consistent differences between the test and control animals. As in the 6-month study with rats, PGHSu was demonstrated in the urine of the test animals. Because small amounts of the compound were present in the Succistearin, it cannot be said if the urinary PGHSu represented dietary or metabolic material.

Blood values recorded in this study generally fall within the ranges reported in the literature (Robinson and Ziegler, 1968) and showed no more than the normal amount of variation for animals of this age and species. There was no evidence of anemia or other blood disorder.

No gross pathologic changes were observed at the time of necropsy, and microscopic examination of the various organs and tissues did not reveal any changes or lesions which could be interpreted as a toxic response. Differences in organ weights, organ/body weight, and organ/brain weight values were small and, because of their random nature, probably reflected normal biological variation.

ACKNOWLEDGMENTS

The authors wish to express their gratitude to Messrs. D. D. Dearwester and F. B. Knapke for expert technical assistance in this work.

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THE NUTRITIVE VALUE OF THE FATTY ACIDS OF LARD AND SOME OF THEIR ESTERS*

BY SAMUEL LEPKOVSKY, ROY A. OUER, AND HERBERT M. EVANS

(From the Institute of Experimental Biology, University of California, Berkeley)

(Received for publication, November 16, 1934)

The increasing importance of studies of the biochemical behavior of fats seems certain to lead to the progressive decline of the use of natural fats in nutrition research because of their complexity. The saturated fatty acids and the unsaturated fatty acids as well as the non-saponifiable matter are now known to possess different biological activities, and the natural fats must be saponified to separate these components. After saponification, the fatty acids may be fed as such, resynthesized into the glyceride, or esterified with some other alcohol such as methyl or ethyl alcohol, propylene glycol, ethylene glycol, or diethylene glycol.

Information bearing on the nutritive value of these esters as compared with fatty acids or with one another is entirely too meager. Only very recently Cox (1) has shown that the ethyl esters of lard are definitely inferior to the natural lard. The lard and its ethyl esters constituted 55 per cent of his diet. Takahashi (2) fed stearic, palmitic, lauric, and oleic acids at levels of 10 per cent and compared them with their glycerides. In all cases the glycerides were superior to the fatty acids, even when the fatty acids were fortified with the glycerol necessary for the corresponding glycerides. Why the glyceride should be superior to the fatty acids plus the glycerol when these are the compounds formed in the intestine upon hydrolysis of the glyceride remains unexplained. Evans and Lepkovsky (3) found better growth with the glyceride of stearic acid than with the ethyl ester, but the ethyl ester of palmitic acid produced somewhat better growth than the glyceride. In these

* Aided by grants from the Research Board and the College of Agriculture of the University of California, and the Rockefeller Foundation.

studies the fats were fed as 25 per cent of the diet. From the foregoing work it would appear that in general the glycerol ester is superior to the fatty acids or their ethyl esters. In this study the growth produced by lard, the lard fatty acids, or their ethyl esters was reinvestigated at the levels of 25 per cent and 60 per cent of the diet. The work was extended to include the synthetic glyceride, the methyl ester, and the esters of ethylene glycol, propylene glycol, and diethylene glycol. All conditions were kept strictly constant, so that the only variable studied was the character of the fat employed.

EXPERIMENTAL

Lard fatty acids were prepared by saponification with about 30 per cent alcoholic potassium hydroxide or with 40 to 50 per cent aqueous potassium hydroxide. The fatty acids were liberated with sulfuric acid, dried, and distilled in a high vacuum at a temperature ranging from 165–190°. Some of the fatty acids distilled at as high as 230°, but biological tests seemed to show no inferiority over those distilled at temperatures under 200°.

The methyl esters were prepared by refluxing lard for 24 hours with 1.5 volumes of absolute methyl alcohol containing 2 to 2.5 per cent dry hydrochloric acid. The esters were salted out with brine, washed with water, and taken up in ether, and then dried with anhydrous sodium sulfate, the ether removed, and the methyl esters distilled at 170–185°.

The ethyl esters were prepared similarly, 2 volumes of absolute alcohol containing 7 per cent concentrated sulfuric acid being used. The glyceride and various glycol esters were prepared by heating the fatty acids and alcohol at 210–230° with dry CO_2 bubbling constantly through the mixture, washing with water, and drying.

The esters were solid at room temperature with the exception of the methyl and ethyl esters, which were liquid. All of the compounds were colorless except diethylene glycol, which was light yellow, and propylene glycol, which was light brown.

Groups of six rats 21 days old were put on the experimental diets containing 25 or 60 parts of one of the fatty compounds. The composition of the diets used is shown in Table I.

Results with 25 Per Cent Fat—The growth curves of this series are shown in Chart I. Little difference exists between the syn-

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thetic glyceride, lard, free fatty acids, and their ethyl and methyl esters. The ester of ethylene glycol and the low fat diet are inferior.

Results with 60 Per Cent Fat—A second lot of the same substances was prepared and with the exception of the methyl and ethyl esters fed as 60 parts of the diet. The results of this series are shown in Chart II. At this high level of fat, the diets containing the synthetic glyceride and the lard are the best. The

TABLE I

Composition of Diets Given in Parts by Weight

	Diet No.																
	680	681	682	683	684	685	686	687	689	690	691	694	695	696	697	718	719
Casein* (commercial)	25	35	35	35	35	35	35	35	40	40	40	40	40	40	40	40	40
Sugar "	75	46	46	46	46	46	46	46									
Salt Mixture 185 (4)	4	4	4	4	4	4	4	4	5	5	5	5	5	5	5	5	5
Lard		25							60								
Glyceride					25							60					
Fatty acid			25							60							
" " and glycerol				25							60						
Methyl ester							25							60			
Ethyl "								25							60		
Ethylene glycol ester						25							60				
Diethylene glycol ester																	60
Propylene glycol ester																60	

These diets were supplemented by 2 drops of cod liver oil and 1 gm. of brewers' yeast given daily except Sunday. (The brewers' yeast was generously supplied through the courtesy of the president, R. M. Allen, of the Vitamin Food Company, Inc., New York.)

* The casein was generously donated by the Golden State Company, Ltd.

diets with free fatty acids, with or without the glycerol, are definitely inferior and the ester of ethylene glycol the poorest.

Another series of feeding tests at the 60 per cent level was made which consisted of the lard fatty acid esters of glycerol, ethylene glycol, propylene glycol, diethylene glycol, ethyl alcohol, and methyl alcohol. The esters of ethyl and methyl alcohol were liquid and the diets containing them were fed in small quantities in small glass dishes, and complete consumption of all the con-

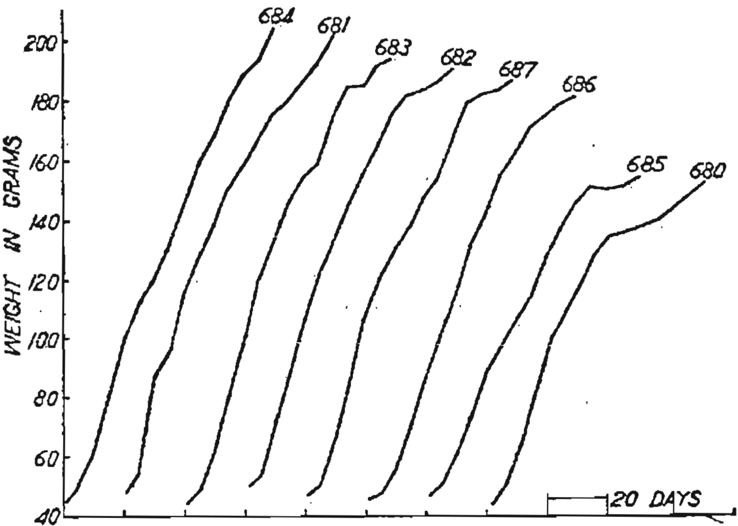


CHART I. Composite growth curves of six female rats each, showing the growth obtained when diets containing 25 per cent of lard fatty acids or their esters were fed. Diet 684, glycerol ester; Diet 681, lard; Diet 683, fatty acid and glycerol; Diet 682, fatty acids; Diet 687, ethyl esters; Diet 686, methyl esters; Diet 685, ethylene glycol esters; Diet 680, low fat controls.

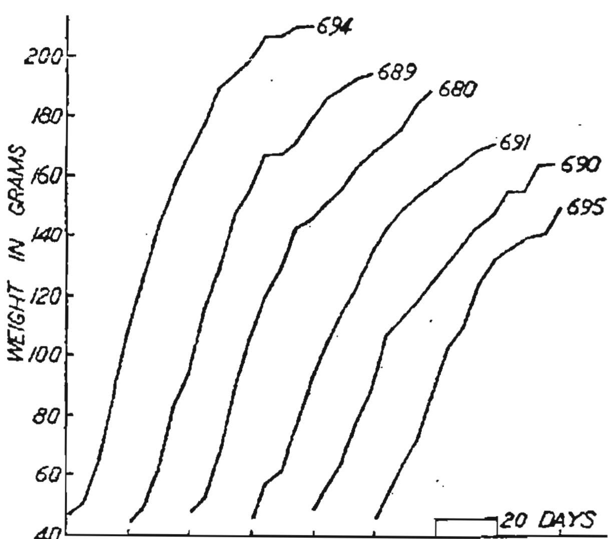


CHART II. Composite growth curves of six female rats each, showing the growth obtained when diets containing 60 per cent of lard fatty acids or their esters were fed. Diet 694, glycerol ester; Diet 689, lard; Diet 680, low fat controls; Diet 691, fatty acid and glycerol; Diet 690, fatty acids; Diet 695, ethylene glycol ester.

stituents was assured before additional diet was measured out. The results are shown in Chart III.

The glyceride (Diet 694) again proved the best, and the propylene glycol esters (Diet 716) definitely inferior. The results with the ethylene glycol esters (Diet 695) differed from those illustrated in Chart II. The mortality at the beginning was very great, five rats dying during the early part of the experiment. Six additional rats were added to the group, of which three died. The four survivors grew very poorly. The animals on the diethylene glycol

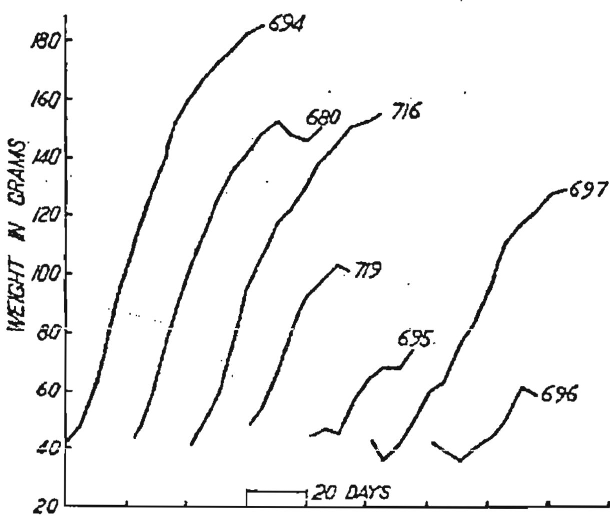
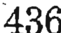


CHART III. Composite growth curves of female rats, showing the growth obtained with 60 per cent of different esters of lard fatty acids. Diet 694, glycerol ester (six rats); Diet 680, low fat controls (six rats); Diet 716, propylene glycol ester (six rats); Diet 719, diethylene glycol ester (six rats); Diet 695, ethylene glycol ester (four rats); Diet 697, ethyl ester (three rats); Diet 696, methyl ester (three rats).

esters (Diet 719) did fairly well at the beginning of the experiment, but after about 3 weeks on the diet they began to die, apparently from kidney damage. The rats receiving ethyl esters (Diet 697) did poorly at the beginning of the experiment, three out of six rats dying. Those that survived grew fairly well. The mortality among the rats receiving the methyl esters (Diet 696) was great and they never grew well at any time. The superiority of the glyceride (Diet 694) over other esters is marked.



Pathology[1]

When the experiment had been in progress 40 days, postmortem examination was made of two animals from each group. The only lesions observed grossly were in the kidneys of the animals fed with esters of ethylene glycol and diethylene glycol. These abnormal kidneys presented a striking appearance. They were definitely enlarged and considerably heavier than normal. They were milky white in color or mottled with white, the surface being studded with nodules and pits.

Microscopic sections showed marked dilatation of some of the collecting and some of the convoluted tubules. This was apparently the result of plugging of the collecting tubules by a yellowish crystalline deposit in the lumen, with a consequent hydronephrosis of the upper reaches of the tubular system. The epithelium of the collecting tubules was flattened. In the convoluted tubules the epithelial cells appeared to be normal in some portions of the sections. In other places they were clear and unstained as if the normal cytoplasm had been replaced by a substance which had dissolved out during fixation and staining, and the nuclei were flattened and pressed to one side as in fat cells. (Frozen sections stained with Sudan III failed, however, to show an excess of fat in the tubules.) In certain areas the convoluted tubules appeared to have completely degenerated and to have been replaced by a bluish substance resembling calcium or a calcium soap. In contrast to the marked abnormality of the tubules, most of the glomeruli appeared to have suffered little damage.

Microscopic sections of the kidneys of animals fed with the ester of propylene glycol did not show the characteristic lesions seen in the animals fed with esters of ethylene and diethylene glycol. No deposits were observed in the lumen of the tubules; the tubules were not noticeably dilated and the tubular epithelium appeared to be essentially normal.

[1] The authors wish to express their thanks for the cooperation of Professor Charles L. Connor of the Department of Pathology, University of California, and Professor Thomas A. Addis of the Department of Medicine, Stanford University, who kindly examined sections of the kidneys of these animals.

DISCUSSION

Of the different forms of lard fatty acids studied in this communication, that which occurs in nature, the glycerol ester, is the best, in spite of exposures to temperatures of 200–230° for 6 to 8 hours. The free lard fatty acids yield good results also, but definitely poorer than the glyceride at the 60 per cent level. No improvements resulted if free glycerol was added in amounts equal to that present in the glyceride. Since the glyceride is hydrolyzed in the intestinal tract to fatty acids and glycerol, it is difficult to understand the inferiority due to direct feeding of the glycerol and free fatty acids.

Of the other polyhydric alcohols used in esterification, the propylene glycol ester seems the best. Pharmacologically (5–7) it is apparently non-toxic, and should therefore be as good as the glyceride. This, however, was not the case. Ethylene glycol, however, is toxic (5–7), and the inferior growth and high mortality in the rats fed lard fatty acid esters of ethylene glycol may be explained on this basis. The rats fed the esters of diethylene glycol did so poorly as to suggest that the diethylene glycol is also toxic.

SUMMARY

1. Lard was saponified and its fatty acids collected, distilled, and esterified with glycerol to form "synthetic" lard; this was fully as satisfactory for the normal growth of rats as the untreated lard whether fed as 25 or 60 per cent of the diet.

2. The free fatty acids with and without glycerol additions were good sources of energy when fed as 25 per cent of the diet, but somewhat inferior to the glyceride at the 60 per cent level.

3. The methyl and ethyl esters were also good when fed as 25 per cent of the diet. When the methyl esters were fed as 60 per cent of the diet, growth was very poor. When the ethyl esters were fed as 60 per cent of the diet, there was an initial mortality but the survivors grew fairly well.

4. Of the esters of the dihydric alcohols, propylene glycol was the best. Ethylene glycol and diethylene glycol were both poor and histologically the kidneys of these animals showed grave lesions.

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An Experimental Test of Dietary Enzyme Modulation in Pine Warblers *Dendroica pinus*

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ABSTRACT

Modulation of gut function is important in an ecological and evolutionary context because it likely determines what food items an animal can and cannot eat. We examined how diet affects activity of digestive enzymes in an omnivorous bird, the pine warbler (*Dendroica pinus*). Pine warblers were fed insect-based, fruit-based, and seed-based diets for ~54 d. We then measured activity of amylase, maltase, sucrase, aminopeptidase-N, trypsin, chymotrypsin, carboxypeptidase A, carboxypeptidase B, pancreatic lipase, and carboxyl ester lipase. We predicted that carbohydrase activities would be highest in birds fed the diet highest in carbohydrates (fruit based), protease activities would be highest in those fed the diet highest in protein (insect based), and lipase activities would be highest in those fed the diets highest in lipid (insect based and seed based). Also, we predicted that pine warblers would exhibit greater dietary modulation of enzyme activity than reported for a less omnivorous congener, the yellow-rumped warbler (*Dendroica coronata*). All predictions were upheld, supporting the hypothesis that pine warblers modulate the activity of digestive enzymes in proportion to demand from substrates in the diet.

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Introduction

Birds often switch diets, especially between plant and animal matter (Martin et al. 1951). These switches take place on a continuum of temporal scales, from meal-to-meal differences in composition to seasonal shifts in diet brought about by changes in resource abundance. A central issue in studies of foraging ecology and digestive physiology is to what extent digestive features allow or constrain such shifts in diet (Karasov 1990). More generally, why does a given bird eat what it does and, conversely, why does it not eat the vast majority of plant and animal matter it encounters? Studies of behavior and external morphology have offered many insights into diet selection (Stephens and Krebs 1986); we focus on the role of internal morphology and physiology.

Morphological and physiological traits that change during a shift in diet are important in helping a bird maximize its rate of energy gain (Karasov 1996). Changes are commonly reported in gut morphology (Walsberg and Thompson 1990; Brugger 1991; Piersma et al. 1993; Novoa et al. 1996; Witmer 1998), although in most cases it remains unclear how these alterations help birds adapt to new diets. Modulation of nutrient uptake across the brush-border membrane of the intestine has also been reported with a shift in diet (Caviedes-Vidal and Karasov 1996; Afik et al. 1997), but the patterns of modulation are unclear and generally do not parallel those predicted or found in other vertebrates (Buddington et al. 1991; Karasov 1992, 1996). Retention time of food in the gut is another digestive trait that varies with diet. In some cases, variation in retention time does not affect digestive efficiency (Levey and Martínez del Rio 1999); in other cases it apparently does (Levey and Karasov 1992; Afik and Karasov 1995). Taken together, these studies show the complexity of digestive function—that, although changes in digestive processing frequently accompany a change in diet, it remains difficult to predict which changes will take place in a given situation.

We focus on dietary modulation of digestive enzymes, which for two reasons provide a good study system. First, each enzyme's role in digestion is fairly specific because each acts on a particular class of substrate. This type of interaction between a feature of the digestive system and digestion contrasts with most cases reviewed in the preceding paragraph. Changes in gut morphology or retention time, for example, affect all substrates simultaneously and to different extents, which makes the functional significance of their modulation difficult to understand. Enzymes, though, allow us to pose simplistic yet de-

fensible predictions about their dietary regulation (discussed later). Second, it has been known for approximately 100 yr that mammalian digestive enzymes respond to changes in diet (Pavlov 1902). However, the ecological and evolutionary significance of these responses remains unclear because the vast majority of such studies have been performed on laboratory mammals and motivated by clinical applications.

We know of only three studies of dietary modulation of digestive enzymes in nondomesticated birds (Afik et al. 1995; Martínez del Rio et al. 1995; Sabat et al. 1998). Our study is a companion to that of Afik et al. (1995), which focused on yellow-rumped warblers (*Dendroica coronata*). We used an almost identical protocol on a closely related species, the pine warbler (*Dendroica pinus*). In contrast to yellow-rumped warblers, which switch between diets of primarily insects and diets of primarily fruits (references in Afik et al. 1995), pine warblers commonly switch among insects, fruits, and seeds; they even include sprouts, suet, and tree sap in their diet (Martin et al. 1951; Morse 1967; Register 1995). Both species are highly insectivorous during the summer breeding season and then adopt generalized diets in the fall and winter, when insects are relatively scarce. Details of these diet shifts are provided in Martin et al. (1951). The more general diet of pine warblers, which even scavenge out of dumpsters (Leatherman 1993), allows a comparison between their ability to modulate enzyme activity and the ability of yellow-rumped warblers to do so (see prediction 4).

We placed the birds on three nutritionally disparate diets: fruit based, insect based, and seed based. We then measured activity of the following intestinal enzymes and pancreatic enzymes, which together are responsible for digestion of the major macromolecules in food—carbohydrates, protein, starch, and triglycerides.

Amylase (EC 3.2.1.1; EC numbers are from a hierarchical classification scheme of the International Enzyme Commission; Bairoch 1993) is secreted by the pancreas and hydrolyzes α1,4-glucosidic bonds in large oligosaccharides, resulting in maltose and small oligosaccharides (Robyt and French 1970). These compounds are then hydrolyzed by disaccharidases located on microvilli of enterocytes to monosaccharides, which are absorbed. We assayed two disaccharidases, maltase (EC 3.2.1.20) and sucrase (EC 3.2.1.48). Maltase is a key indicator of a bird's ability to digest complex soluble carbohydrates because maltose is a dominant by-product of their hydrolysis by amylase (Alpers 1987). Likewise, we measured sucrase activity because it is correlated with an animal's ability to assimilate complex carbohydrates (Martínez del Rio 1990; Malcarney et al. 1994). Trypsin (EC 3.4.4.4) and chymotrypsin (EC 3.4.4.5) are the predominant pancreatic proteases. Trypsin cleaves peptide bonds associated with basic amino acids, whereas chymotrypsin cleaves bonds associated with aromatic amino acids (Brannon 1990). Oligopeptides are then hydrolyzed by a large variety of peptidases in the brush-border membrane. We chose to measure activity of aminopeptidase-N (EC 3.4.11.2, also known as leucine-aminopeptidase and amino-oligopeptidase; Vonk and Western 1984), a dipeptidase with broad specificity that accounts for almost all the peptidase activity of the brush-border membrane (Maroux et al. 1973).

We also measured activity of the pancreatic exopeptidases, carboxypeptidase A (EC 3.4.2.1) and carboxypeptidase B (EC 3.4.2.2), which hydrolyze the peptide bond adjacent to the C-terminal end of polypeptide chains. Carboxypeptidase A preferentially cleaves C-terminal L-amino acids that are either aromatic or contain a branched side chain, while carboxypeptidase B catalyzes hydrolysis of the basic amino acids, lysine, arginine, and ornithine from the C-terminal position. In humans, carboxypeptidases make up greater than 30% of the mass of exocrine pancreatic proteins (Gorelick and Jamieson 1987). Finally, we measured pancreatic lipase activity. In most vertebrates, dietary triglyceride is hydrolyzed by pancreatic lipase (EC 3.1.1.3), nonspecific carboxyl ester lipase (EC 3.1.1.1), or by a combination of the two. Pancreatic lipase is highly efficient at hydrolyzing the fatty acids from the 1 and 3 positions of triglycerides, while carboxyl ester lipase can hydrolyze all three fatty acids, as well as sterol esters and wax esters (Place 1992). The avian exocrine pancreas secretes at least these two lipolytic enzymes (Place 1992). We did not measure a common enzyme of pancreatic juice in mammals, phospholipase A_2, because in birds it accounts for <3% of the digestive activity found in mammals (Bosc-Dierne et al. 1984).

We tested four predictions, all based on the general premise of optimal evolutionary design of gut function (Diamond 1991; Karasov 1996). In the case of our study, this premise leads to the expectation that the activity of a given enzyme will be modulated in direct proportion to the level of its substrate in the diet; any excess activity would be wasteful, and insufficient activity would reduce digestive performance. Thus, on the basis of nutritional compositions of our diets (see "Material and Methods"), we predicted (1) carbohydrase activities would be highest in birds fed the fruit diet, (2) proteinase activities would be highest in birds fed the insect diet, (3) lipase activities would be highest in birds fed the seed and insect diets, and (4) more enzymes would show significant modulation in pine warblers than Afik et al. (1995) found in yellow-rumped warblers. As discussed by Garland and Adolph (1994), the last prediction is severely limited, as are all two-species comparisons. We note however, that the comparison is enhanced by restricting it within a genus and by predicting directional differences for several traits (Garland and Adolph 1994).

Material and Methods

Pine warblers were captured near Gainesville, Florida, and held individually in 0.5 × 0.5 × 0.5–m cages under a constant light cycle (14L : 10D) and temperature (21°C). For several weeks, we maintained them on live meal worms (*Tenebrio molitor*), a

formulated banana-based diet (Denslow et al. 1987), and water, all provided ad lib. Within 1 wk, body masses stabilized to within 5% of that at time of capture. We then acclimated birds to a 1 : 1 : 1 homogenized mixture of our three test diets. These diets were a banana-based diet (Denslow et al. 1987), a mealworm-based diet (210 g mealworms, 500 g water, 4.5 g agar, 0.75 g vitamin and mineral supplement), and a sunflower seed–based diet (65 g raw husked seeds, 500 g water, 4.5 g agar, 0.75 g vitamin and mineral supplement). We hereafter refer to these diets as the fruit, insect, and seed diets, respectively (see exceptions in the next two paragraphs). These diets are identical to those used by Afik et al. (1995), who provide details on their preparation, storage, and nutritional composition.

Our original goal was to maintain equal numbers of pine warblers on each of the three diets. Unfortunately, birds fed exclusively the fruit and seed diets lost body mass, even after several weeks of gradual acclimation onto those diets. Because pine warblers always include at least some insects in their diet (Martin et al. 1951), we decided to supplement fruit and seed diets with the insect diet. Our revised goal was to maintain birds on seed and fruit diets with the minimum proportion of added insect diet necessary for them to maintain body mass.

By systematically varying the proportion of insect diet homogenized into the fruit and seed diets and closely monitoring the warblers' body masses, we settled on composite diets of 70% seed diet : 30% insect diet and 80% fruit diet : 20% insect diet. Hereafter, these will be referred to as seed and fruit diets, respectively.

Composite diets were a necessary compromise. Still, the diets we used varied approximately sixfold in carbohydrate content, twofold in protein content, and fourfold in lipid content (Table 1). The fruit diet contained mostly carbohydrate (60% dry mass), the insect diet mostly protein (53%), and the seed diet mostly lipid (41%; Table 1).

Excluding acclimation periods, which lasted approximately 2 wk, we held birds on the above diets for 54 ± 2 consecutive days. Body masses were recorded each morning before birds had eaten. Occasionally, birds would appear in poor health (i.e., erected feathers), or their body mass would be >15% below that at time of capture. Rather than risk these birds' death, we supplemented their diet with 1 g/d of a 2 : 1 : 1 mixture of lean ground beef, cooked eggs (with shell), and cooked carrots, until their condition improved, which was almost always within 1–3 d. We do not know if this occasional protein supplementation influenced enzyme activity at the end of the feeding trials. If it did, it would almost certainly obscure rather than enhance differences in enzyme activity among diets.

We started with 27 individuals. Because of four deaths (two probably diet related, one accidental, one mysterious), our final sample sizes of birds on fruit, insect, and seed diets were seven, eight, and eight, respectively. These totals include several individuals from a shorter-term pilot study. Sample sizes for the pancreatic enzymes were reduced by one bird for the insect and seed diets because of lost tissue (i.e., $n = 7$ for all diets).

Table 1: Nutritional composition (dry mass) of three diets fed to pine warblers

	Carbohydrate (% dry mass)	Protein (% dry mass)	Lipid (% dry mass)
Fruit diet	60	23	10
Insect diet	10	53	32
Seed diet	15	31	41

Note. Values were calculated from data in Afik et al. (1995). Fruit and seed diets contained 20%–30% of the insect diet. Most carbohydrate in the fruit diet consisted of sucrose and monosaccharides, and most of the carbohydrate in the insect diet consisted of glycogen.

After the birds were anesthetized, we removed the digestive tracts and immediately placed them in ice-cold Ringers solution, bubbled with 95% O_2 : 5% CO_2. Within 3–5 min, we separated the pancreas from the intestine. The pancreas was then blotted on tissue paper for 2–3 s, placed in a tared cryovial, weighed, and placed in liquid nitrogen. For each bird, three 0.3–0.5-cm sleeves of intestine were likewise blotted, weighed, and frozen. One sleeve came from the most proximal portion of the duodenum, one came from the midpoint of the intestine, and the third came from the distal portion of the intestine, immediately proximal to the cecae.

We conducted intestinal enzyme assays in homogenates (1) because the enrichment of brush-border membrane preparations differs significantly among batches and (2) because brush-border membrane preparations tend to have low and variable yields (Martínez del Rio et al. 1995 and references therein). These two factors increase the variability of estimates of intestinal enzyme activities from brush-border membrane preparations and thus hinder intra- and interspecific comparisons.

Pancreatic Enzymes

Protein Extraction and Assay. Whole pancreases were powdered with a mortar and pestle in liquid nitrogen. The powder (~50 mg wet mass) was placed into two tared cryovials and kept frozen at −70°C until extraction. One set (for amylase and lipase activity measurements) of powdered pancreas was weighed and then extracted with 10 vol of 10 mM sodium acetate (pH = 4.8) containing 0.9% (w/v) NaCl, 0.2% (w/v) Triton X-100, 3 mM sodium taurocholate, 2 mM hydrocinnaminic acid, and 1 mM benzamidine, at 4°C for 15 min. The extract was sonicated (Branson Model 460) on ice for 1 min at 50% power and 50% duty cycle with a microtip. The sonicated extract was centrifuged at 20,000 *g* for 20 min at 4°C. The supernatant was then removed and either assayed within 12 h or frozen at −20°C until assayed. The second set (for trypsin and chymotrypsin activity measurements) of powders was treated identically, ex-

cept that the two protease inhibitors (hydrocinnaminic acid and benzamidine) were omitted from the extraction buffer.

Total pancreatic protein in each extract, used to standardize enzymatic activities, was determined with the bicinchoninic acid assay (Pierce Chemical, Rockford, Ill.), adapted for use in microtiter plates. Absorbance at 590 nm was recorded by using a microplate reader (Vmax; Molecular Devices, Menlo Park, Calif.) with bovine serum albumin as a primary standard. All assays were performed in duplicate, with a mean coefficient of variation of 5.7%.

Amylase Activity Assay. Amylase activity was determined at pH 7.0 by using 4,6 ethylidene (G_7)-*p*-nitrophenyl (G_1-α-D-maltoheptaside; ET-G_7PNP) as a substrate, followed by α-glucosidase release of *p*-nitrophenol. Absorbance at 405 nm was recorded continuously for 10 min at 30°C by means of a microplate reader. All assays were performed in duplicate. To correct for rates at 40°C (avian body temperature), identical assays were performed on randomly selected samples by using a Hewlett Packard diode array spectrophotometer (Model 8452A) with water-jacketed cuvette holders at 30°C and then 40°C. The absorbance was recorded every second for 10 min, and the rate of change was determined by regression of the linear portion of the product versus time curve. From these assays we determined the Q_{10} of amylase was 2.09 ± 0.18 ($n = 13$). This factor was used to correct the rates determined at 30°C to normal avian body temperature.

Lipase Activity Assay. The best way to measure lipase activity is with a pH-stat with the nonphysiological substrate, tributyrin (Borgström and Erlanson 1973). Tributyrin, a short chain triglyceride, is hydrolyzed up to 10 times faster than long chain triglycerides, yields completely ionized water-soluble products at alkaline pH, and does not require an emulsifying agent during assays. However, tributyrin can also be hydrolyzed by nonspecific esterases. Thus, to establish that hydrolytic enzymes in the pancreatic extracts were true lipases, olive oil, a long chain triglyceride, was used also. Because long chain fatty acids (pk ~6.8) are not completely ionized at a pH below 9, the lipolytic activity is underestimated when using a pH-stat with long chain triglycerides.

Titrametric assays of lipolytic activity (both bile dependent and bile independent) were performed by using a 0.5% (w/v) gum arabic-stabilized emulsion of tributyrin and/or olive oil. Temperature was maintained at 41°C and pH at 8.0, by using a pH-Stat (TTT80 Radiometer) with 0.02 N NaOH. Just before use, the tributyrin or olive oil emulsions were sonicated for 3 min at a power setting of 30 in a Branson sonicator. The assay volume was 10 mL containing 500 μL of substrate emulsion in 2 mM Tris-maleate (pH = 8.0), 150 mM NaCl, 1 mM $CaCl_2$, and 0.02% mM sodium azide. The reaction mixture was first incubated without enzyme for several minutes to record the nonenzymatic base uptake. Reactions were done in duplicate with and without addition of a common bile salt in birds, sodium taurocholate (5 mM). The addition of bile salt fully activates both the bile-dependent carboxyl-ester lipase activity and the colipase-pancreatic lipase activity. Preliminary studies found that maximal activation by taurocholate occurred at bile salt concentrations above 2.5 mM.

An independent measure of bile salt–dependent lipase activity (carboxyl ester lipase) was determined spectrophotometrically by using a microplate reader at 30°C by hydrolysis of 4-nitrophenyl acetate (4-NPA) in the presence of taurocholate. No correction to avian body temperature was made. Each assay (0.2 mL) contained 0.15 M Tris-HCl (pH = 8.5), 15 mM sodium taurocholate and 0.35 mM 4-NPA. The reaction was measured by increase in absorption at 405 nm. The extinction coefficient used for nitrophenol at 405 nm and pH = 8.5 was 19,800 L/(M × cm).

Trypsinogen, Chymotrypsinogen, and Procarboxypeptidase Activation. Trypsinogen was activated to trypsin by using porcine intestine enterokinase (320 U/mg). Preliminary studies indicated that activation for 15 min at room temperature with 0.5 U of enterokinase (5 U/mL in cold distilled water) in 40 mM succinate buffer (pH 5.6) was sufficient to completely activate avian trypsinogen. After 15 min, the activation was stopped by addition of 1.1 vol of 40 mM HCl containing 5 mM $CaCl_2$ and placement on ice. Trypsin activity was determined spectrophotometrically within 8 h.

Chymotrypsinogen was converted to chymotrypsin in the presence of bovine pancreatic TPCK trypsin (11,700 U/mg). Preliminary studies indicated that activation for 15 min at room temperature with 10 μg/mL trypsin in 40 mM succinate buffer (pH = 5.6) was sufficient to completely activate avian chymotrypsinogen. After 15 min, the activation was stopped by addition of 1.1 vol of 40 mM HCl containing 5 mM $CaCl_2$ and placement on ice. Chymotrypsin activity was determined spectrophotometrically within 8 h. A similar activation protocol was used for carboxypeptidases A and B.

Trypsin, Chymotrypsin, and Carboxypeptidase A and B Activity Assays. In the trypsin assay, on the basis of the method of Hummel (1959), the rate of hydrolysis of *p*-toluenesulfonyl-L-arginine methyl ester (TAME) was measured by the increase in absorbancy at 247 nm. One unit of activity is equal to 1 μM of TAME per minute at 41°C and pH = 8.1 in the presence of 1 mM $CaCl_2$. A molar extinction coefficient of 540 L/(M × cm) at 247 nm for TAME was used.

The procedure to determine chymotrypsin activity was based on the rate of hydrolysis of benzyol-L-tyrosine ethyl ester (BTEE) by determining the change in absorbancy at 256 nm. One unit of activity is equal to 1 μM of BTEE per minute at 41°C and pH = 7.8 in the presence of 1 mM $CaCl_2$. A molar extinction coefficient of 965 L/(M × cm) for BTEE was used.

The procedure used to determine carboxypeptidase activities

was based on the rate of hydrolysis of hippuryl-L-phenylalanine (HP) in the case of carboxypeptidase A or hippuryl-L-arginine (HA) in the case of carboxypeptidase B by determining the change in absorbency at 254 nm. One unit of activity is equal to 1 μM of HP or HA per minute at 41°C and pH = 7.5 in the presence of 0.5 M NaCl. A molar extinction coefficient of 0.36 L/(M × cm) for HA and HP was used.

All assays were measured on the Hewlett Packard diode array spectrophotometer. The absorbance was recorded every second for 10 min, and the rate of change was determined by regression of the linear portion of the product versus time curve.

Intestinal Enzymes

Disaccharidases. Small intestines were thawed at 5°C and homogenized (30 s, OMNI 5000 homogenizer at setting 6, in 9 vol of 350 mM mannitol in 1 mM Hepes/KOH, pH = 7.5). Disaccharidase activities were measured according to Dahlqvist (1964) as modified by Martínez del Rio et al. (1995). Briefly, tissue homogenates (100 μL) diluted with 350 mM mannitol in 1 mM Hepes/KOH were incubated at 40°C with 100 μL of 56 mM sugar (sucrose or maltose) solutions in 0.1 M maleate/NaOH buffer, pH = 6.5. After 10–20 min incubations, reactions were stopped by adding 3 mL of a stop/developing glucose-trinder (one bottle of glucose-trinder 500 reagent in 250 mL 1.0 M TRIS/HCL, pH = 7.0, plus 250 mL of 0.5 M NaH_2PO_4/Na_2HPO_4, pH = 7.0). After 18 min at 20°C, absorbance of the resulting solution was measured at 505 nm with a Beckman DU-64 spectrophotometer. In our preparation, disaccharide hydrolyses were linear even after 30 min. The Michaelis constants (K_m ± SD) for maltase and sucrase in our preparations were 2.17 ± 0.07 mM and 12.03 ± 0.81 mM, respectively. Optimum pH's for these two enzymes were 5.5 and 6.0, respectively. We used slightly different pH's in our assays, but in all cases our reported activities should be within 5%–10% of activity at the optima.

Aminopeptidase-N. Aminopeptidase-N assays were conducted after Roncari and Zuber (1969) and by using L-alanine-*p*-nitroanilide as the substrate. Briefly, 100 μL of homogenate diluted with mannitol/KOH buffer was mixed with 1 mL of a prewarmed (40°C) assay mix (2.04 mM L-alanine-*p*-nitroanilide in 0.2 M NaH_2PO_4/Na_2HPO_4, pH = 7.0). The reaction was incubated at 40°C and stopped after 10 min with 3 mL of ice-cold 2 N acetic acid. Absorbance was measured at 384 nm. The hydrolysis of L-alanine-*p*-nitroanilide was linear for up to 20 min in our preparations. The K_m and pH optimum for aminopeptidase-N in our preparation were 9.63 ± 0.35 mM and 7.5, respectively.

Standardization. On the basis of absorbance standards constructed for glucose and *p*-nitroanilide, we calculated total (summed) and standardized intestinal activities (Martínez del Rio 1990; Biviano et al. 1993). Thus, data on enzyme activities are presented as total hydrolytic activity (μmol/min), activity per unit intestinal wet mass (μmol/[min × g wet tissue]), and activity per mg of protein (μmol /[min × mg protein]). Protein was measured by using the Bradford method, as modified by Biorad. Martínez del Rio et al. (1995) provide justification for our choice of standardization.

Source of Chemicals

Grade A sodium salts of taurocholate were purchased from Calbiochem (La Jolla, Calif.). Porcine intestine enterokinase (320 U/mg), bovine pancreatic trypsin (Type XIII), chymotrypsinogen (Type II), BTEE, TAME, HP, HA, ET-G_7PNP, amylase activity kit, glucose-trinder reagent, purified olive oil, tributyrin, gum arabic, Triton X-100, and 4-NPA were purchased from Sigma Chemical (St. Louis). Hydrocinnaminic acid and benzamidine were purchased from Aldrich Chemical Company (Milwaukee). All other chemicals were purchased from Fisher Scientific (Pittsburgh). Whatman 3-MM chromatographic filter paper was obtained from VWR Scientific (Philadelphia). All solvents were either pesticide or HPLC grade. All other chemicals were reagent grade, unless specified otherwise.

Results

The three experimental diets had no significant effect on body mass ($\overline{X}$ = 11.7 ± 1.1 g, $F_{2,16}$ = 0.62, P > 0.05) but had a highly significant effect on intestinal mass ($F_{2,16}$ = 5.50, P < 0.02; Table 2) and length ($F_{2,16}$ = 4.69, P < 0.04; Table 2). Pancreatic mass did not vary among diets ($\overline{X}$ = 0.031 ± 0.005 g, 0.030 ± 0.005 g, and 0.034 ± 0.002 g for birds fed fruit, insect, and seed diets, respectively; $F_{2,18}$ = 0.33, P = 0.09), nor did pancreatic protein mass ($\overline{X}$ = 0.038 ± 0.016, 0.042 ± 0.014, and 0.054 ± 0.017 mg protein/mg wet mass for birds on fruit, insect, and seed diets, respectively; P = 0.18). Thus, any changes observed in enzyme specific activities (i.e., U/mg protein or U/mg wet mass) indicate increases in the enzyme rather than simply decreases in water content of tissue.

Pancreatic Enzymes

Amylase activity differed significantly among birds fed the three diets ($F_{2,18}$ = 3.74, P = 0.04). Birds eating seed and fruit diets had nearly identical amylase activities, which were significantly greater than those of birds eating the insect diet (P's < 0.040, Fisher's PLSD post hoc tests; Fig. 1*a*). Trypsin and chymotrypsin activities showed the opposite pattern; they were significantly higher in birds fed the insect diet than in birds fed both seed and fruit diets ($F_{2,18}$ = 3.94 and 5.21 for overall variation in trypsin and chymotrypsin activity, respectively; P's < 0.04; post hoc tests; Fig. 1*b*, 1*c*). Trypsin and chymotrypsin

Table 2: Comparison of dietary variables in pine warblers fed three laboratory diets

	Fruit	Insects	Seeds	F	P
Body mass (g)	11.6 ± 1.1[a]	11.4 ± 1.4[a]	12.1 ± .6[a]	.62	.55
Pancreas mass (g)	.031 ± .005	.030 ± .005	.034 ± .002	.33	.09
Intestinal mass (g)	.84 ± .15[a]	.52 ± .16[b]	.62 ± .18[b]	5.50	.02
Intestinal length (cm)	15.0 ± 1.2[a]	12.4 ± 1.3[a,b]	13.7 ± 1.4[b]	4.71	.03
Sucrase (μmol/min)	2.19 ± .34[a]	.88 ± .20[b]	.80 ± .20[b]	9.35	.003
Maltase (μmol/min)	17.3 ± 7.2[a]	6.45 ± 1.07[b]	5.88 ± .93[b]	4.12	.04
Aminopeptidase-N (μmol/min)	3.69 ± 1.74	3.78 ± .57	3.14 ± .91	.42	.66

Note. Values are means ± SD. For variables with significant variation among diets, post hoc tests were completed. Means with the same superscript letters do not differ significantly (<0.05, Tukey's Multiple Comparisons).

activities in birds maintained on fruit and seed diets were nearly identical.

Lipase activity on tributyrin in the presence of taurocholate differed significantly among dietary groups ($F_{2,18} = 3.62$, $P = 0.048$). Birds maintained on insect and seed diets displayed similar activities, in both cases significantly greater than those of birds maintained on the fruit diet (P's < 0.05, Fisher's PLSD post hoc tests; Fig. 1*d*). Lipase activity with olive oil in the presence of taurocholate showed exactly the same pattern (P's < 0.02, post hoc tests), except that the overall F value was only marginally significant ($F_{2,18} = 6.93$, $P = 0.06$). Lipase activity on tributyrin was significantly higher (8.0 ± 0.9-fold; paired t-test, $P < 0.0001$) than on olive oil.

In the absence of taurocholate, lipase activity again differed significantly among dietary groups for both tributyrin ($F_{2,18} = 4.21$, $P = 0.03$) and olive oil ($F_{2,18} = 12.80$, $P < 0.01$). Likewise, Fisher's PLSD post hoc tests revealed the same pattern of variation among diets described in the presence of taurocholate, except that lipase activity with tributyrin did not differ significantly between birds fed fruit and seed diets. As expected, lipase activity in the absence of the bile salt taurocholate was significantly lower (1.5 ± 0.1-fold; paired t-test, $P < 0.0001$) than in its presence.

Carboxyl ester lipase varied significantly among diets ($F_{2,18} = 3.66$, $P = 0.046$). Its activity was significantly higher in birds feeding on the insect diet than in birds feeding on fruit and seed diets (P's < 0.04, post hoc tests).

In contrast to researchers conducting studies in mammals, we were not able to detect carboxypeptidase activity (A or B) in the native or trypsin-activated pancreatic extracts.

Intestinal Enzymes

Hydrolytic activities standardized by intestinal wet mass (g) were linearly correlated with activities standardized by mg of protein ($r > 0.89$ for all enzyme activities). The conversion factor to transform intestinal wet mass into milligrams of protein is 0.11 mg protein/g. In the results that follow, we standardize by wet mass exclusively.

Effects of Diet

Diet had a highly significant effect on activities of sucrase, maltase, and aminopeptidase-N ($F_{2,12} = 7.5$, $F_{2,12} = 10.2$, and $F_{2,12} = 5.5$; $P = 0.008$, $P = 0.003$, and $P = 0.04$, respectively; repeated measures ANOVA). Likewise, position along the intestine explained a significant portion of variation in activities of sucrase, maltase, and aminopeptidase-N ($F_{2,24} = 25.3$, $F_{2,24} = 52.3$, and $F_{2,24} = 108.0$, respectively; P's < 0.0001). Diet and position did not show significant interactions for the expression of sucrase and maltase ($F_{4,24}$'s > 2.0, P's > 0.10, repeated measures ANOVA), which suggests that the relative distribution of these enzymes does not vary with diet (Fig. 2). The lack of a significant interaction between diet and intestinal position allowed us to compare among intestinal positions averaged for diet and among diets averaged among positions. For post hoc tests, we used Tukey's HST multiple comparisons, modified for a repeated measures design (Winer et al. 1991). Sucrase and maltase expression were indistinguishably high (P's > 0.01, Tukey's tests) in the first two sections of the intestine and declined significantly in the most distal section (P's < 0.05, Tukey's tests; Fig. 2). Birds fed the fruit diet had significantly higher sucrase and maltase levels than birds fed insect or seed diets (P's < 0.05, Tukey's tests). The levels of sucrase and maltase did not differ significantly among birds fed on seed and insect diets (P's > 0.10, Tukey's tests; Fig. 2).

The pattern exhibited by aminopeptidase-N was more complex. Diet and position did show a significant interaction ($F_{4,24} = 4.04$, $P < 0.01$), which suggests that the relative distri-

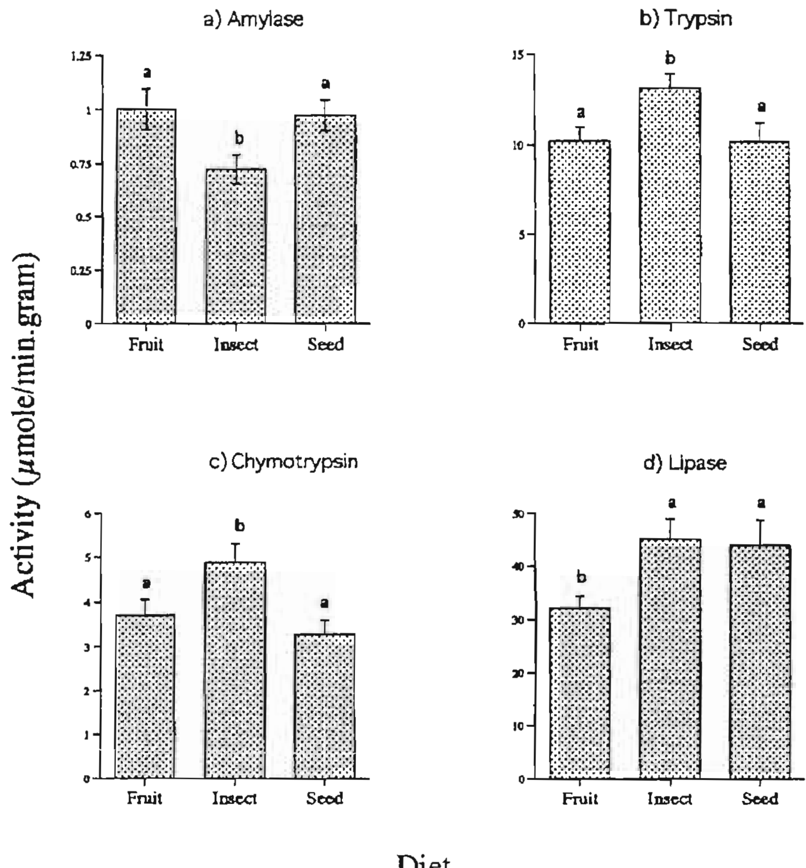

Figure 1. Activity of pancreatic enzymes in pine warblers (*Dendroica pinus*) fed three diets (± SE). Within each plot, bars not sharing a common letter at their top are significantly different ($P < 0.05$; Fisher's PLSD post hoc tests). Pancreatic lipase activity was measured in the presence of taurocholate, a common bile salt.

bution of aminopeptidase-N along the intestine varied with diet (Fig. 2). Birds fed the insect and seed diets showed a similar pattern: activity was high in the first two sections and declined in the distal portion of the intestine. Birds fed the fruit diet showed low levels of aminopeptidase-N in the proximal intestine, high levels in the medial section, and low levels distally. Tukey's tests revealed significant differences in aminopeptidase-N activity only in the proximal section of the intestine. In this intestinal section, only birds fed the insect diet exhibited significantly higher activities than those fed fruit. Birds fed the seed diet did not differ significantly from birds fed the insect and fruit diets.

Summed sucrase and maltase activities differed significantly among birds fed the three diets ($F_{2,14} = 9.35$ and 4.21, respectively, P's < 0.05; Table 2). Birds fed the fruit diet exhibited significantly higher sucrase and maltase activities than birds fed the insect and seed diets (Table 2). Summed aminopeptidase-N activity did not differ significantly among birds fed any of the three diets ($F_{2,14} = 0.59$, $P > 0.05$; Table 2).

When hydrolytic activities were standardized by intestinal mass, diet had a significant effect in all cases ($F_{2,14}$'s > 4.0, P's < 0.04). Summed activity of hydrolases depended on both tissue mass-specific activity and on the mass of intestinal tissue. Thus, summed aminopeptidase-N did not differ among diets because the two diets with the lower mass-specific aminopeptidase-N activities exhibited larger intestinal masses. When the effect of intestinal tissue mass was removed by expressing activity on a mass-specific basis, aminopeptidase-N exhibited a significant effect in the predicted direction—birds fed the insect diet showed higher activity than those fed the seed and fruit diets. Mass-specific disaccharidase activities were also as predicted—activities in birds fed fruit were higher than those in birds fed seeds and insects. The trends shown by a comparison of hydrolytic activities standardized by intestinal mass are also

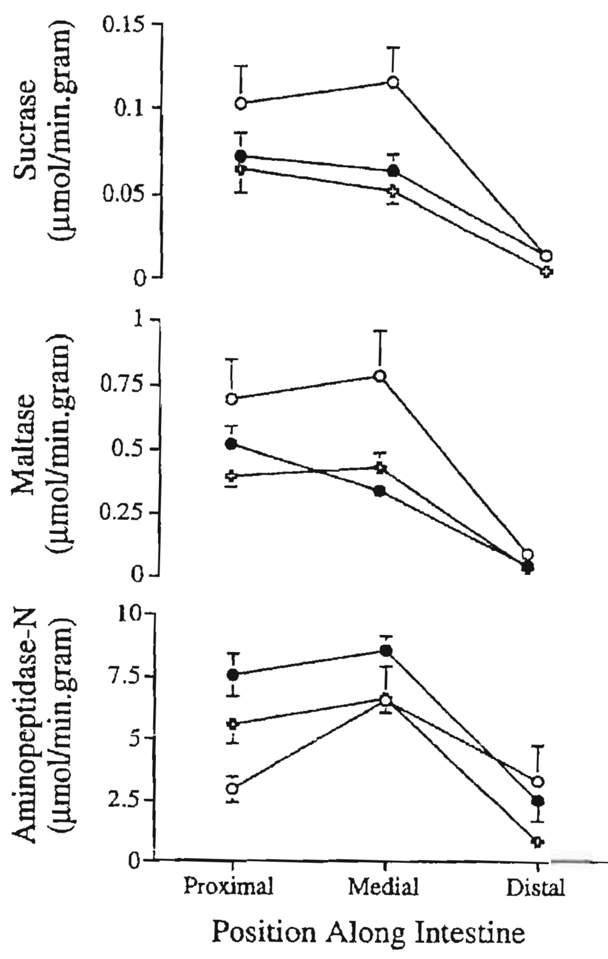

Figure 2. Variation in intestinal hydrolases as a function of diet and position along the intestine in pine warblers. Open circles indicate birds fed a fruit-based diet, closed circles indicate birds fed an insect-based diet, and crosses indicate birds fed a seed-based diet. Bars are standard errors.

in the predicted directions—disaccharidases were highest in birds fed fruit, and aminopeptidase-N was highest in birds fed insects (Table 2). Sucrase and maltase activity were tightly correlated ($r = 0.81$, $P < 0.001$; Fig. 3), but aminopeptidase-N activity was not significantly correlated with either sucrase or maltase activity (r's < 0.3, P's > 0.2; Fig. 3).

Discussion

The results of this study supported, in general, each of our four predictions. We found amylase, sucrase, and maltase activities highest in birds fed the fruit diet, regardless of how we calculated activities. As predicted, trypsin, chymotrypsin, and aminopeptidase-N activities were highest in birds fed the insect diet, although higher activities of aminopeptidase-N were restricted to the proximal part of the intestine. We found lipase activity to be higher in birds fed the seed and insect diets, and carboxyl ester lipase activity was highest in birds fed the insect diet. Finally, as predicted, pine warblers displayed a greater ability to modulate enzymatic activity than did yellow-rumped warblers, which showed in a similar experiment no modulation of intestinal carbohydrase activity (Afik et al. 1995; pancreatic enzymes were not assayed in yellow-rumped warblers).

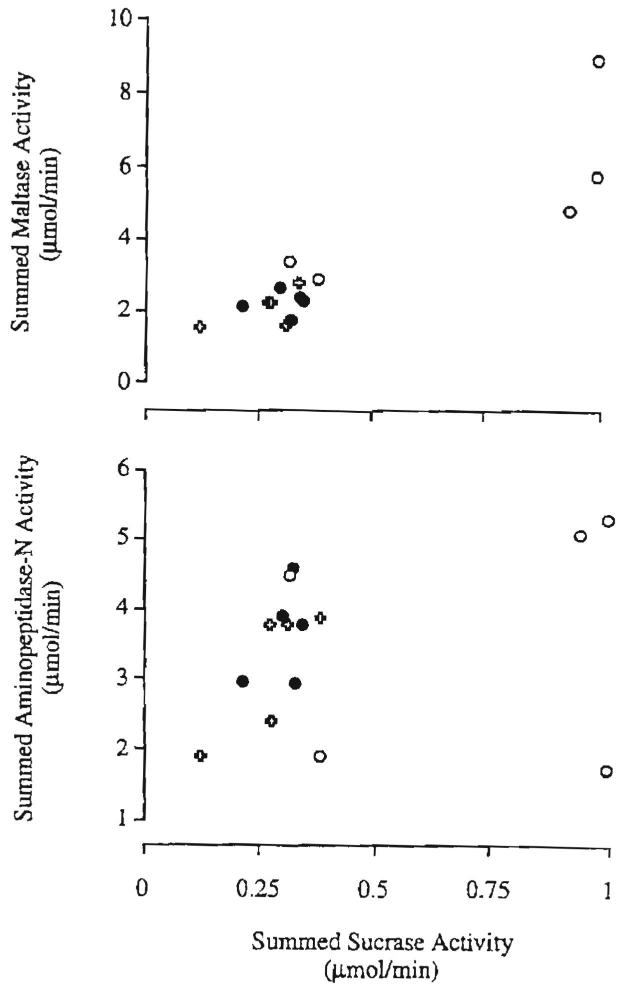

Figure 3. Relationship between intestinal sucrase activity and maltase and aminopeptidase-N activities, standardized by intestinal mass, in pine warblers fed three artificial diets. Open circles are individuals fed a fruit-based diet, closed circles are individuals fed an insect-based diet, and crosses are individuals fed a seed-based diet. Note that the correlation between sucrase and maltase activities is statistically significant ($r = 0.81$, $P < 0.05$), whereas the correlation between sucrase and aminopeptidase-N activity is not ($r = 0.27$, $P > 0.05$). The relationship between intestinal sucrase (Y) and intestinal maltase activity is $Y = 2.5 + 5.9X$.

Taken together, these results support the hypothesis that animals possess the ability to modulate digestive enzyme activities in proportion to their demand—relative concentrations of carbohydrates, proteins, and lipids in the diet appear to regulate differential production of carbohydrases, proteases, and lipases, respectively (Buddington et al. 1991; Karasov 1992). Our results also suggest that species with the greatest propensity to switch between disparate types of diets may show the greatest ability to modulate enzymatic activity. We emphasize, however, that the comparison of our results with those of Afik et al. (1995) constitute a preliminary test of this hypothesis because the comparison involves only two species. However, the test is strengthened because the two species are within the same genus and we predicted the multiple differences we found between them (Garland and Adolph 1994).

Arguing against the hypothesis is a study of two emberizid finches (rufous-collared sparrow, *Zonotrichia capensis*, and common diuca finch, *Diuca diuca*), one of which consumes more insects than the other (Sabat et al. 1998). Captive individuals were fed either a high-carbohydrate, low-protein diet or a carbohydrate-free, high-protein diet. After 20 d, activities of maltase, sucrase, and aminopeptidase-N were significantly higher in birds fed the carbohydrate-free, high-protein diet. This pattern of enzyme modulation was "remarkably similar" in both species, despite the fact that one has a much broader natural diet than the other (Sabat et al. 1998).

Only a handful of other studies have examined modulation of digestive enzymes in birds, and of these, most have focused on poultry (Imondi and Bird 1967; Lei and Slinger 1970; Hulan and Bird 1972; Siddons 1972; Sell et al. 1989; Biviano et al. 1993). Chickens and turkeys, like mammals, have the ability to regulate activity of intestinal enzymes in accordance with dietary demands (reviewed in Karasov 1996). Among nondomesticated species, this ability is much less clear-cut. European starlings and yellow-rumped warblers showed no change in maltase or sucrase activity when fed diets high in starch or sucrose but did increase aminopeptidase-N activity when fed diets high in protein (Afik et al. 1995; Martínez del Rio et al. 1995). Rufous-collared sparrows and diuca finches showed increases in aminopeptidase-N activity on a high-protein, no-carbohydrate diet but did not show increased maltase or sucrase activities on a low-protein, high-carbohydrate diet (Sabat et al. 1998). In this narrow context, pine warblers appear exceptional; they displayed significant modulation in all eight enzymes we assayed. Furthermore, these enzymes were disparate in terms of their substrates and sites of synthesis.

Why do some species of wild birds appear to have a much greater ability than other species to modulate enzymatic activity? In addition to the hypothesis linking omnivory with digestive plasticity mentioned previously, we offer four nonmutually exclusive explanations. First, the observed differences among species may be artifacts of different experimental designs or enzyme assays. We doubt this explanation because all trials and assays have thus far been done by an overlapping group of collaborators; our goals and methodologies have been similar. There is, however, one obvious difference between this study and those of Afik et al. (1995) and Martínez del Rio et al. (1995) that could account for their different results: our trials lasted ~54 d, whereas trials on starlings and yellow-rumped warblers lasted 20 d and 7 d, respectively. Thus, lack of modulation in starlings and yellow-rumped warblers may have been because of insufficient time for modulation to occur. Afik et al. (1995) discount this possibility, noting that induction of intestinal enzymes typically occurs within 1–4 d in poultry and mammals (Cezard et al. 1983; Goda et al. 1983; Raul et al. 1987; Brannon 1990; Biviano et al. 1993). Nonetheless, differences between dietary groups in experiments of this kind are a result not only of upward regulation in some treatments but downward regulation in others. This is noteworthy because downward regulation appears to require more time than upward regulation (Karasov 1996). In the only study testing downward regulation of enzymes in birds, Biviano et al. (1993) found that it was incomplete after 8 d. Perhaps pine warblers displayed obvious differences in enzyme activity among dietary groups because they were held on diets for considerably longer than 8 d, allowing sufficient time for divergence through both upward and downward regulation.

Second, modulation would not be necessary in a species if constitutive levels of enzymes were sufficient for thorough and efficient hydrolysis of any potential diet. In this situation, upward modulation would not provide any added benefits, and downward modulation would compromise digestive performance. Evolutionary theory argues against such a scenario, positing that modulation in enzyme activity should be common among generalist feeders. Diamond (1991) argued that maintaining a full complement of membrane-bound enzymes would be wasteful in terms of metabolic energy and ineffectively used space along the crowded surface of apical enterocytes. Thus, specific enzymes or nutrient transporters should be synthesized and maintained only when needed. This optimality approach to gut function is appealing but currently lacks general support in studies of birds (Levey and Karasov 1992; Caviedes-Vidal and Karasov 1996; Karasov et al. 1996; Levey and Cipollini 1996; Afik et al. 1997; Sabat et al. 1998).

Third, differences in inducibility of digestive enzymes may be explained by differences in the temporal scale at which animals shift diets relative to the scale at which enzymes can be induced. Sabat et al. (1998) predict that inducibility should be found in animals in which enzymes can be expressed at timescales much shorter than those that characterize diet shifts. We speculate that the inducibility exhibited by the carbohydrases of chickens, turkeys, and pine warblers are the result of these animals having natural diets that are relatively stable and exhibiting only long-term diet shifts.

Fourth, substrate-induced regulation of digestive enzymes may be phylogenetically determined. Just as some avian lineages

show consistently low activity of intestinal enzymes (Karasov and Levey 1990; Martínez del Rio 1990), some lineages may have fixed levels of activity. This explanation, however, fails to account for the observed difference in the regulatory ability between pine and yellow-rumped warblers, which are closely related (this study and Afik et al. 1995).

Magnitude of Modulation

Thus far we have focused on the ability to modulate enzyme activity. We now examine the magnitude of modulation. These comparisons are on the basis of summed activity (μM/min) for intestinal enzymes and activity standardized to tissue mass for pancreatic enzymes (μM/[min $\times$ mg]).

Sucrase and maltase activities were most variable among diets, differing by factors of 2.7 and 2.6, respectively, between diets with highest and lowest activities. This magnitude of modulation is appreciably greater than typically reported for sucrase and maltase in other bird species (factors typically range from 1.4 to 1.7; Sell et al. 1989; Biviano et al. 1993; Martínez del Rio et al. 1995; Sabat et al. 1998). Amylase activity varied by a factor of 1.4 among diets, which is comparable to values of 1.2–1.4 found by Hulan and Bird (1972). The large differences in disaccharidase activities we observed among diets were the result of both diet effects on activity per unit of tissue mass and of increases in intestinal mass (see Table 2).

Proteinase activities in pine warblers varied among diets by factors of 1.2–1.5, a degree of modulation slightly lower than that reported for chicks (2.0–2.2; Imondi and Bird 1967), yellow-rumped warblers (1.7; Afik et al. 1995), starlings (1.7; Martínez del Rio et al. 1995), and two species of emberizid finch (1.7 for each; Sabat et al. 1998). Finally, lipase activity varied by 1.6-fold. In chickens fed different diets, lipase activity varied slightly (1.1-fold) but significantly (Hulan and Bird 1972).

The activities of sucrase and maltase exhibited by pine warblers were lower than those found by Afik et al. (1995) in yellow-rumped warblers but similar to those expected from allometric equations derived for birds (Martínez del Rio 1990). Comparing measurements in the midregion of the intestine, sucrase had approximately one-third the activity and maltase approximately one-fifth the activity found in yellow-rumped warblers, which in turn had low activities of these enzymes compared to most other birds (Afik et al. 1995). Aminopeptidase-N activity in pine warblers was similar to that in yellow-rumped warblers, starlings, and two species of finch (Afik et al. 1995; Martínez del Rio et al. 1995; Sabat et al. 1998; note that the activity reported in Afik et al. 1995 for yellow-rumped warblers is tenfold too high because a dilution factor was omitted, C. Martínez del Rio, personal observation). Activity of trypsin was approximately one-third and activity of chymotrypsin was one-twentieth that in chickens (Hulan and Bird 1972). These differences may be attributable to measurement of pancreatic juice in chickens and pancreatic tissue in pine warblers.

Enzyme Kinetics

Our value of K_m^* for sucrase is 35% lower than that reported for yellow-rumped warblers (Afik et al. 1995), yet well within the range of values reported for other birds (Siddons 1969; Martínez del Rio 1990; Martínez del Rio et al. 1995). Our value of K_m^* for maltase is 36% higher, and for aminopeptidase-N is 56% higher than that reported for yellow-rumped warblers, but again neither is unusually high or low, compared to other species (Siddons 1969; Afik et al. 1995; Martínez del Rio et al. 1995).

Effect of Intestinal Position on Activity

Pine warblers displayed an unusual spatial pattern of enzymatic activity that was consistent for all three intestinal enzymes we examined. Activities tended to increase or remain unchanged between the proximal and medial portions of the intestine (Fig. 2). In only one of nine cases (maltase activity in birds fed the insect diet) was there a clear decrease in activity along this region. This general pattern contrasts sharply with that reported for 10 other bird species, in which activities of intestinal enzymes clearly decreased from proximal to medial sections of the intestine (Martínez del Rio 1990; Biviano et al. 1993; Afik et al. 1995; Martínez del Rio et al. 1995). Only aminopeptidase-N activity in yellow-rumped warblers displays a pattern similar to that in pine warblers (Afik et al. 1995). As in other species, pine warblers show little enzymatic activity in the most distal portion of their intestine.

Conclusion

Pine warblers display a wider range of substrate-induced regulation of digestive enzymes than any nondomesticated bird tested thus far. Their ability to modulate enzymatic activity appears adaptively tuned to a diet that switches between insects, fruits, and seeds. These findings therefore support the hypothesis that digestive plasticity has evolved in response to an omnivorous diet (Karasov and Diamond 1988; Buddington et al. 1991). Yet, curiously, pine warblers cannot subsist as well on pure fruit or pure seed diets as can a close relative, the yellow-rumped warbler, which apparently cannot regulate carbohydrase activities (Afik et al. 1995). An obvious explanation for this difference is that carbohydrase activities in yellow-rumped warblers, although static, are much higher than in pine warblers, even after those in pine warblers have been induced. It remains puzzling why yellow-rumped warblers maintain relatively high levels of intestinal carbohydrases (compared with pine warblers) and why pine warblers cannot match those levels, given that they can modulate enzymatic activity and that

their diet is probably more diverse than that of yellow-rumped warblers. These results underscore the elusive nature of the link between diet breadth and lability of digestive processes in birds.

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Page 71 of 127

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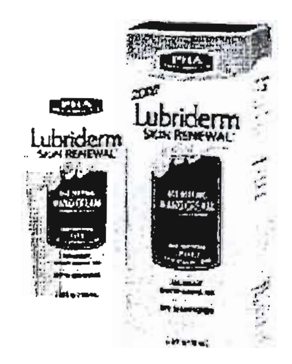

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NOVASONE

Mometasone furoate

Consumer Medicine Information **9 June 1998**

What is in this leaflet

This leaflet answers some common questions about Novasone.

It does not contain all the available information.

It does not take the place of talking to your doctor or pharmacist.

All medicines have risks and benefits. Your doctor has weighed the risks of you using Novasone against the benefits it is expected to have for you.

If you have any concerns about this medicine, ask your doctor or pharmacist.

Keep this leaflet with your medicine.

You may need to read it again.

What Novasone is used for

The name of your medicine is Novasone. It contains the active ingredient called mometasone furoate.

It is a type of cortisone and belongs to the group of medicines called corticosteroids. Novasone is classified as a high potency topical corticosteroid.

There are three forms of Novasone: cream, ointment and lotion.

Novasone is used on the skin to relieve the redness, swelling, itching and discomfort of many skin problems such as:

- psoriasis
- eczema
- other types of dermatitis

Novasone Lotion is also used on the scalp and hairy parts of the body.

Your doctor, however, may have prescribed Novasone for another purpose.

Ask you doctor if you have any questions about why Novasone has been prescribed for you.

This medicine is available only with a doctor's prescription.

Before you use Novasone

When you must not use it

Do not use Novasone if you have had an allergic reaction to:

- **mometasone furoate**
- **any other corticosteroid**
- **any of the ingredients listed at the end of this leaflet.**

Do not use Novasone if you have:

- **a viral skin infection (such as cold sores, shingles or chicken pox)**
- **a fungal skin infection (such as thrush, tinea or ringworm)**
- **tuberculosis of the skin**
- **acne rosacea**
- **inflammation around the mouth**
- **skin conditions with ulcers**

unless your doctor tells you.

Ask your doctor to be sure you do not have any of these conditions.

Do not use Novasone after the expiry date.

A use-by date is shown on the tube and carton of Novasone Cream and Ointment and on the bottle of Novasone Lotion.

Do not use Novasone if the packaging shows signs of tampering.

Before you start to use it

Tell your doctor or pharmacist if you have allergies to:

- any other medicines
- any other substances, such as foods, dyes or preservatives.

Tell you doctor if:

- **you are pregnant or breast feeding.**

Your doctor will tell you if you can use Novasone during pregnancy or while you are breast feeding.

- **you have any other medical conditions, especially if you have an infection.**

Using other medicines

Tell your doctor or pharmacist if you are using other creams, ointments or lotions or taking any other medicines. This includes any that you buy without a prescription from a pharmacy, supermarket or health food shop.

How to use Novasone

How to use it

Apply a thin film of Novasone Cream or Ointment or a few drops of Novasone Lotion to the affected skin or scalp once daily. Massage gently until it disappears.

It is important to use Novasone exactly as your doctor has told you. If you use it less often than you should, it may not work as well and your skin problem may not improve. Using it more often than you should may not improve your skin problem any faster and may cause or increase side effects.

How long to use it

Do not use Novasone for more than four weeks at a time unless your doctor tells you.

If you forget to use it

If you forget to use Novasone, use it as soon as you remember and then go back to your normal time for applying Novasone. Do not try to make up for the amount you missed by using more than you would normally.

If you swallow it

Telephone your doctor or Poisons Information Centre (Phone 13 11 26) or go to the accident and emergency centre at your nearest hospital immediately if anyone swallows Novasone.

Keep the telephone numbers of these places handy.

While you are using Novasone

Things you must do

Tell all doctors and pharmacists who are treating you that you are using Novasone.

Tell your doctor if you feel that Novasone is not helping your condition or if your skin condition worsens or seems infected.

Tell your doctor if, for any reason, you have not used Novasone exactly as prescribed.

Otherwise, your doctor may think that it was not effective and change your treatment unnecessarily.

Things you must not do

Do not use Novasone under dressings or on large areas of skin unless your doctor tells you.

Do not use plastic pants or tight fitting nappies if Novasone is to be used on the nappy area of young children.

Do not use Novasone in or near the eyes.

Do not give Novasone to anyone else even if their symptoms seem similar to yours.

Do not use Novasone to treat other conditions unless your doctor tells you.

Your doctor has prescribed Novasone specially for you and your condition. If you use it for another condition, it may not work or make the condition worse.

Things to be careful of

Do not use large amounts for a long time.

If you use large amounts for a long time, the chance of absorption through the skin and the chance of side effects increases.

Only use Novasone on skin areas that rub together such as under the arm or in the groin area if your doctor tells you.

Only use Novasone on the face if your doctor tells you. If improvement does not occur within one week, tell your doctor.

Side Effects

Tell your doctor if you do not feel well while you are using Novasone.

Novasone helps most people with skin problems but it may have unwanted side effects in a few people.

Novasone is generally well tolerated. However, unwanted effects that have been reported by some people using Novasone include:

- itching
- burning
- tingling/stinging
- thinning of the skin
- appearance of small blood vessels on the surface on the skin
- stretch marks or streaks on the skin
- acne/pimples/lumps on the skin/blisters containing pus
- redness
- boils/abscesses
- dermatitis
- increased size of affected area/worsening of disease
- numbness
- dry skin
- inflamed hair roots

Novasone may cause other side effects.

If you have any other side effects, check with your doctor.

Do not be alarmed by this list of possible side effects. You may not experience any of them.

After using Novasone

Storage

Keep Novasone in a cool dry place. Novasone Cream and Lotion should be stored where the temperature stays below 25°C and the Ointment where the temperature stays below 30°C.

Do not refrigerate Novasone.

Do not leave it in the car or on window sills.

Heat and dampness can destroy some medicines.

Keep Novasone where children cannot reach it. Keep the medicine away from pets.

A locked cupboard at least one-and-a-half metres above the ground is a good place to store medicines.

Disposal

If your doctor tells you to stop using Novasone or it has passed its expiry date, ask your pharmacist what to do with any that is left over.

Product Description

What it looks like

Novasone Cream is a white to off-white cream. It is packed in 15 g and 45 g tubes.

Novasone Ointment is a white to off-white ointment. It is packed in 15 g and 45 g tubes.

Novasone Lotion is a colourless to light yellow lotion. It is packed in 30 mL bottles.

Ingredients

Novasone Cream contains:

- mometasone furoate 1 mg/g
- soft white paraffin
- hexylene glycol
- aluminium starch octenylsuccinate
- propylene glycol monostearate
- stearyl alcohol
- ceteareth-20
- white beeswax
- purified water
- titanium dioxide
- phosphoric

Novasone Cream does not contain preservative.

Novasone Ointment contains:

- mometasone furoate 1 mg/g
- soft white paraffin
- hexylene glycol
- white beeswax
- purified water
- propylene glycol monostearate
- phosphoric acid

Novasone Ointment does not contain preservative.

Novasone Lotion contains:

- mometasone furoate 1 mg/g
- isopropyl alcohol
- propylene glycol
- hydroxypropylcellulose
- sodium phosphate monobasic
- phosphoric acid
- purified water

Novasone Lotion does not contain preservative.

Manufacturer

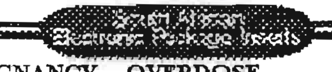
Schering-Plough Pty Ltd
11 Gibbon Road
Baulkham Hills NSW 2153
AUSTRALIA

Australian Registration Numbers

Cream - AUST R 58612
Ointment - AUST R 58613
Lotion - AUST R 58614

Date of Preparation

9 June 1998



INDICATIONS CONTRA-INDICATIONS DOSAGE SIDE-EFFECTS PREGNANCY OVERDOSE
IDENTIFICATION PATIENT INFORMATION

PREMARIN* CREAM 0,625 mg

SCHEDULING STATUS:
S1

PROPRIETARY NAME
(and dosage form):

PREMARIN* CREAM 0,625 mg

COMPOSITION:
PREMARIN Cream - each gram contains 0,625 mg **Conjugated Oestrogens** USP. (Also contains cetyl esters wax, cetyl alcohol, white wax, glyceryl monostearate, propylene glycol monostearate, methyl stearate, sodium lauryl sulphate, glycerin, mineral oil and 1% benzyl alcohol as the preservative.

PHARMACOLOGICAL CLASSIFICATION:
Category A, 21.8.1 Oestrogens.

PHARMACOLOGICAL ACTION:
PREMARIN Cream provides the local oestrogenic activity of topical oestrogen.

INDICATIONS:
PREMARIN Cream is indicated for the treatment of postmenopausal and senile vulvovaginitis, atrophic vaginitis, pruritus vulvae caused by atrophic changes in the vulval epithelium, dyspareunia associated with an atrophic vaginal epithelium, and for use prior to plastic pelvic surgery in menopausal cases.

CONTRA-INDICATIONS:
Not for use during pregnancy.
PREMARIN Cream is contra-indicated in patients hypersensitive to its ingredients and in patients with known or suspected cancer of the breast, or oestrogen-dependent neoplasia such as endometrial carcinoma.

DOSAGE AND DIRECTIONS FOR USE:
Usual dosage:
Intravaginally or topically, 1 to 2 g daily, depending on the severity of the condition. Do not exceed 4 g per day. In postmenopausal vaginal surgery, therapy is suggested 10 days before and 10 days following intervention. PREMARIN Cream should be administered cyclically (e.g. 3 weeks on and 1 week off).

SIDE-EFFECTS AND SPECIAL PRECAUTIONS:
Hypersensitivity reactions, systemic effects such as breast tenderness and, rarely, withdrawal bleeding.
Diagnostic measures should be taken to eliminate the possibility of neoplasia.
An increased incidence of endometrial uterine carcinoma related to the continuous use of oestrogens in the postmenopausal period has been reported.
The use of PREMARIN Cream does not preclude the use of appropriate antimicrobial therapy in the presence of trichomonal, monilial, or bacterial infection.
Because of salt and water retention associated with oestrogenic anabolic activity, oestrogens should be used with caution in patients with epilepsy, migraine, asthma, cardiac, or renal disease.
Certain liver and endocrine function tests may be affected by oestrogen administration.
Due to the possibility of systemic absorption, the following must be noted:
Other side-effects include venous thromboembolism and pulmonary embolism.
In some epidemiological studies, women on estrogen replacement therapy, given alone or in combination with a progestin, have been reported to have an increased risk of thrombophlebitis, and/or thromboembolic disease, although the evidence is conflicting. The physician should be aware of the possibility of thrombotic disorders (including thrombophlebitis, retinal thrombosis, cerebral embolism, and pulmonary embolism) during estrogen replacement therapy and alert to their earliest manifestations. Should any of these occur or be suspected, estrogen replacement therapy should be discontinued immediately. Patients who have risk factors for thrombotic disorders should be kept under careful observation.

KNOWN SYMPTOMS OF OVERDOSAGE AND PARTICULARS OF ITS TREATMENT:

Overdosage may result in systemic hyperoestrogenic effects such as abnormal or excessive uterine bleeding, oedema, breast tenderness, or reactivation of endometriosis.

IDENTIFICATION:
PREMARIN Cream is a white cream containing 0,625 mg Conjugated Oestrogens USP per gram, in a nonliquefying base. PREMARIN (Conjugated Oestrogens USP) is a mixture of oestrogens, obtained exclusively from natural sources, occurring as the sodium salts of water-soluble oestrogen sulphates blended to represent the average composition of material derived from pregnant mares' urine. It contains oestrone, equilin and 17 alpha-dihydroequilin, together with smaller amounts of 17alpha -oestradiol, equilenin, and 17 alpha-dihydroequilenin as salts of their sulphate esters.

PRESENTATION:
Each pack contains a 42,5 g tube with one calibrated applicator.

STORAGE INSTRUCTIONS:
Store in a cool dry place below 25°C.
Keep out of the reach of children.
For external use only.

REFERENCE NUMBER:
G3019 (Act 101/1965)

NAME AND BUSINESS ADDRESS OF APPLICANT:
WYETH SOUTH AFRICA (PTY) LTD
Thornhill Office Park
94 Bekker Road
Vorna Valley Ext 60
Midrand, 1685

DATE OF PUBLICATION OF THIS PACKAGE INSERT:
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Toagosei Co., Ltd.
70231-E
Page 79 of 127

TESTS FOR THE CHRONIC TOXICITY OF PROPYLENE GLYCOL AND TRIETHYLENE GLYCOL ON MONKEYS AND RATS BY VAPOR INHALATION AND ORAL ADMINISTRATION

O. H. ROBERTSON, CLAYTON G. LOOSLI, THEODORE T. PUCK, HENRY WISE, HENRY M. LEMON, AND WILLIAM LESTER, JR.

From the Department of Medicine, the Douglas Smith Foundation for Medical Research and the Bartlett Memorial Fund of the University of Chicago and the Commission on Air-Borne Infections, U. S. Army Epidemiological Board

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The finding that vapors of certain glycols were highly germicidal for air-borne bacteria and viruses (1-3) presented the possibility of employing these compounds for disinfection of atmospheres occupied by human beings. Of the two glycols studied most extensively, namely, propylene and triethylene, the former was known to be essentially non-toxic (4-7) and while acute toxicity tests on triethylene glycol had likewise shown that very large doses are required to kill animals (8-10) the effect of prolonged administration of this glycol had not been determined. Moreover, not withstanding the known inocuousness of propylene glycol when administered by mouth or injected intravenously, it was felt that in the absence of adequate tests we could not assume that the inhalation of this glycol would be without deleterious effect on the lungs. Preliminary intratracheal injections in the rat of as little as 0.25 cc. of undiluted propylene glycol caused marked pulmonary irritation, acute edema and later fibrosis and abscess formation.

The very low vapor pressures of propylene glycol and triethylene glycol at room temperature (table 2) as well as the fact that both glycols are very soluble in the body fluids, made it seem unlikely that in the amounts present as vapor in the air, enough glycol could accumulate to cause irritation of the respiratory tract. Nevertheless it was only as a result of prolonged exposure of animals to atmospheres saturated first with propylene glycol and later with triethylene glycol that this possibility was definitely excluded. A brief report on this work was made several years ago when the study was still under way (11). The present communication embodies the results of the completed investigation.

MATERIALS AND METHODS

Characteristics of Glycols Employed. The glycols employed in these toxicity tests were purified materials which met with the specifications listed in table 1.[1]

Vaporization of Glycols for Inhalation Experiments. Various methods for the vaporization of the glycols were employed, during the course of these experiments

[1] The glycols used in this study were supplied through the kindness of Mr. D. B. Williams and Mr. E. Fogle of the Carbide and Carbon Chemicals Corporation. This company has placed on the market a purified triethylene glycol labelled "Air Sterilization Grade."

since as time went on refinements in techniques were developed which made possible more careful control of the experimental conditions. In the first test of propylene glycol vapor on rats, a DeVilbiss atomizer #180, operated by air under 300 mm. pressure was used to introduce a very fine spray of propylene glycol into the chamber. The droplets produced were sufficiently small so that enough glycol evaporated to maintain a constantly saturated atmosphere. The air pressure supply was regulated automatically by clockwork so that glycol was

TABLE 1

Purity specifications for propylene and triethylene glycols intended for use in aerial disinfection

	PROPYLENE GLYCOL	TRIETHYLENE GLYCOL
Specific gravity	1.0370 to 1.0400 at $\frac{20°}{20}$C.	1.1220 to 1.1270 at $\frac{20°}{20}$C.
Boiling point	Range between 180–210°C., with 90% of the material boiling below 195°C.	275–310°C.
Refractive index	1.4316–1.4335 N_D^{20}	1.4549–1.4565 N_D^{20}
Acidity	Equivalent to not more than 0.093 mg. KOH per gm. sample	Equivalent to not more than 0.19 mg. KOH per gm. sample
Solubility	Completely miscible with water at 20°C., and leaving no insoluble residue	Completely miscible with water at 20°C., and leaving no insoluble matter
Color	Not darker than 15 on the platinum cobalt scale (12)	Not darker than 30 on platinum cobalt scale (12)
Ash	Not more than 0.010% by weight	Not more than 0.010% by weight
Analysis	By periodate oxidation (13) to give at least 95.0% propylene glycol by weight	Hydroxyl value of 22.25–23.00 by means of acetic anhydride-pyridine test
Odor	None	Slight, or none
LD_{50} when administered by stomach tube in 50% aqueous solution	In rats, 22 cc. per kilogram body weight*	In rats, 15–18.0 gm. per kilogram body weight†

* Similar values were found by previous workers (4, 5).
† Similar values were reported by earlier workers (8, 10).

sprayed for 30 minutes out of every hour. This method was eventually abandoned, however, because it was found to be very wasteful of glycol. In its stead, a regulated drip of liquid glycol onto the bare surface of an electric hot plate at about 225°C. was at first substituted. However, this arrangement was also found unsatisfactory as it produced extensive decomposition of the glycol (vide infra). An arrangement which worked fairly well for propylene glycol consisted in a sleeve of glass cloth sewed around the blade of a knife-type electric heater so as to cover it completely. The heater was mounted in a horizontal position above a dish of glycol, into which one end of the glass cloth wicking dipped. Glycol was drawn up into the wick by capillary attraction, and on reaching the

zone near the heater, was vaporized. The current through the heater was regulated so that the decomposition temperature of the glycol was not exceeded. A fan placed behind the vaporizer distributed the vapor throughout the chamber.

The vaporization of triethylene glycol in these tests was accomplished by a slightly different method, because this compound is more sensitive to thermal decomposition. A satisfactory system for producing atmospheres continuously saturated or supersaturated with triethylene glycol was achieved by placing a shallow dish of the liquid on a hot-plate which was regulated to keep the temperature of the glycol at 100°C. A constant level device attached to a larger reservoir prevented loss of volume of the liquid in the dish, with consequent rise in temperature. A small electric fan behind the vaporizer, dispersed the vapor throughout the test chamber. In the final test where the concentration of triethylene glycol vapor was kept constant by means of a glycostat (21) this same principle of vaporization was employed, except that the vaporizer was enclosed in a metal cylinder at the top of which a magnetically-controlled butterfly valve was arranged. The glycostat, responding to the actual concentration of glycol vapor in the air, automatically adjusted the butterfly valve so as continuously to maintain the desired vapor concentration. This last method, which operated continuously for ten months provided the most precise and convenient means for producing a controlled vapor concentration, over long periods of time.

A summary of the atmospheric conditions which prevailed in the tests which will be described is presented in table 2. In considering the concentrations of each glycol vapor attained in the air, cognizance must be taken of the variation in the saturation point with relative humidity (15). In all the experiments where the glycol level is listed as "continuous supersaturation," so much excess vapor was introduced that the room was constantly filled with a dense fog of condensed glycol droplets. This condition would represent an amount of glycol greatly in excess of that regarded as desireable for aerial disinfection under conditions of human habitation (16).

Propylene glycol

Inhalation of Vapor by Rats. The first tests on the effect of inhaling propylene glycol vapor were made on white rats. An initial colony of 30 animals weighing 80 to 90 grams at the beginning of the test were divided into two groups—20 test and 10 controls. The animals were placed in identical chambers 5′ long, 3′ high and 2′6″ deep with 5–6 individuals in a cage. The number of rats in each group was increased by birth of young. Breeding was controlled to produce about equal populations in the two groups. The temperature in the chambers was maintained between 75 and 80°F. by means of a fan blowing on a small radiator through which flowed a controlled stream of tap water. By using large trays of $CaCl_2$ it was found possible to prevent the relative humidity exceeding a range of 45 to 65 per cent.

The rats were fed on a dry meal diet consisting of corn meal, linseed meal, casein, powdered alfalfa, powdered brewers yeast, $CaCO_3$ and NaCl. Observations during life were made on gain in weigh, color of coat, possible effects on

conjunctiva, number of young born and general condition. As shown in table 2 these animals were maintained constantly in an atmosphere supersaturated with propylene glycol vapor.

Growth Rate. The growth curves of the two sets of rats shown in figure 1, were made by averaging the weights of individual animals observed during equivalent periods of time. The weights of the young born in the chambers were added to the growth curve when they reached the value at which their parents began the experiment, e.g. 80 to 90 grams. The weights of only the male rats (20 in the control and 19 in the test group) were plotted since the females varied so much with the birth of young. The animals in the glycol chamber gained weight more rapidly than did the control rats and the weights of the former group at 12

TABLE 2

Atmospheric conditions prevailing in the glycol vapor inhalation experiments

EXPERIMENT	ANIMALS	ROOM TEMP.	RH	CONCENTRATION OF GLYCOL REQUIRED FOR SATURATION	ACTUAL GLYCOL CONCENTRATION
		°F.	%	mg./liter	
Propylene glycol	Rats	75–80	45–65	0.17 –0.35*	Continuous supersaturation
Propylene glycol	Monkeys	78–82	50–60	0.23 –0.35*	About 60% saturation (.10–.22 mg./l.)
Propylene glycol	Monkeys	77–82	50–60	0.23 –0.35*	Continuous supersaturation
Triethylene glycol	Rats	77–82	45–65	0.0025– .005†	Continuous supersaturation
Triethylene glycol	Monkeys	78–82	50–60	0.0031– .0046†	Continuous supersaturation
Triethylene glycol	Monkeys	78–82	50–60	0.0031– .0046†	65–75% saturation (0.002–0.003 mg./l.)

* Calculated on the basis of Raoult's law, using experimental data of Puck and Wise (14) for vapor pressures of pure propylene glycol.

† From experimental data of Wise and Puck (15).

months were about 50 per cent greater than the latter. We have no explanation to offer for this difference since, except for the presence of the glycol vapor, all conditions were approximately the same in each group. There was no essential change in the weights of the rats of either group after twelve months.

The rats in the glycol atmosphere bred just as regularly and produced just as large litters as did the control animals. The young of the two groups were indistinguishable in appearance and weight-gain. No conjunctival irritation was observed. At one period, shortly after the hot plate vaporization was substituted for the atomization method of dispersal, a yellowish discoloration of the coats of the rats in the glycol chamber was observed. This was found to be due to decomposition of the glycol by an excessively high temperature (220°C.) of the surface of the hot plate. When the method of vaporization was changed so that the temperature of the glycol remained below 95°C., the yellow tint of the

Toagosei Co., Ltd.
70231-E
Page 81 of 127

coat disappeared. All the rats, both glycol-exposed and normal, appeared to be in good condition at the time they were sacrificed. No deaths occurred.

Examination at Autopsy. After intervals of time from 3 to 18 months as shown in table 3, the rats were killedwith an intra-peritoneal injection of pentobarbital sodium. Urine aspirated from the bladder showed no abnormal findings

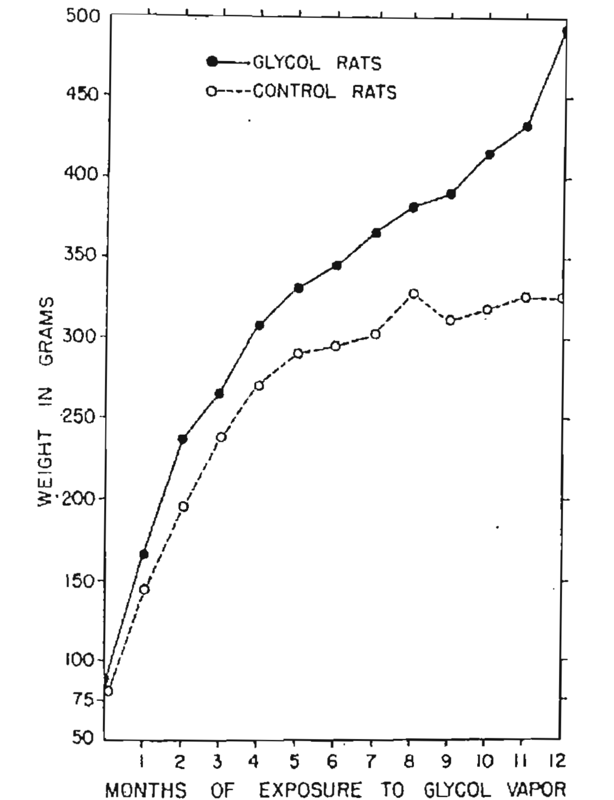


Fig. 1. Comparative Growth Rates of Rats Kept Continuously in an Atmosphere Saturated with Propylene Glycol Vapor and a Similar Group of Control Animals

Male rats only included in the graph; nineteen in the test and twenty in the control groups. Rats about seven weeks old when experiment begun.

in either test or control animals. The lungs were fixed with Zenker-formol by the method previously described (17) which consists in clamping off the aorta, next compression of the ventricles in order to fill the pulmonary vessels, followed by application of a tight suture around the base of the heart. The fixing fluid is then allowed to run into the trachea under slight pressure. One section through

each lobe of the lungs was taken as well as sections of liver, kidney and spleen. Maximow's hemotoxylin-eosin-azure stain was employed.

Lungs. The gross appearance of the lungs in both the glycol exposed and control rats was normal except in certain instances in which consolidation of a part or a whole lobe was present. On microscopic examination of such regions, evidence of a localized infectious process was found. This varied from a very small area of intra-alveolar accumulation of polymorphonuclear leucocytes to the involvement of a whole lobe. In some cases the lesion was in the process of resolution as shown by the macrophagic nature of the exudate. Such lesions were with a single exception found only in rats which had been kept in the chambers for 8 months or longer[3] and occurred in 25 per cent of the control animals (table 4). In contrast only two lesions of a similar nature were found in the 26 rats kept in a propylene glycol atmosphere for 8 to 18 months.

The most common change in the lungs was a pervascular and peribronchial accumulation of round cells which first began to appear after the end of $4\frac{1}{2}$ to 5

TABLE 3

Exposure of rats to propylene glycol vapor

	Number of months of exposure to test conditions														Total number of rats
	3	4	5	6	7	8	9	10	11	12	13	15	17	18	
Number of rats killed in each chamber at successive intervals															
Glycol	1	4	5	2	1	3	3	2	0	3	8	3	0	4	39
Control	2	3	2	4	2	0	5	2	1	3	0	4	3	7	38

months residence in the chambers. As time went on the size of these "collars" of cells increased and in certain animals became most pronounced. This change occurred with equal frequency in both control and test animals. Figure 2 shows perivascular collars of moderate degree in a 5 months-old control rat. Examination of rats from other sources has revealed the same appearance of the lungs which is most pronounced in old animals. Except for these cellular changes which presumably indicate the occurrence of chronic irritation of some kind and the occasional focal pneumonitis, the lungs of both groups of rats presented a normal appearance.

Other Organs. Examination both gross and histological of the kidneys, liver and spleen revealed no pathological changes. No concretions were found in the bladder or calices of the kidneys except in one instance, that of a control rat which exhibited an oblong stone, 1 cm. long and 0.6 cm. in diameter in the bladder.

Inhalation of Propylene Glycol Vapor by Monkeys. It seemed advisable also to test the effect of inhalation of glycol vapors on monkeys since their upright

[3] In only one out of 51 rats, 3 to 7 months old, was a pulmonary lesion found.

TABLE 4

Number of pulmonary lesions occurring in rats both exposed and unexposed to atmospheres containing propylene or triethylene glycol vapors

LENGTH OF TIME OBSERVED	RATS USED FOR	TOTAL NUMBER OF RATS	NUMBER SHOWING PULMONARY LESIONS	PER CENT TOTAL SHOWING PULMONARY LESIONS
months				
8-18	Inhalation of propylene glycol	26	2	8
	Controls on inhalation of propylene glycol	25	6	24
	Inhalation of triethylene glycol*	34	8	24
	Controls on inhalation or ingestion of triethylene glycol	44	12	27
3-7	Inhalation of propylene glycol	13	0	0
	Inhalation of triethyleue glycol	25	1	4
	Controls on inhalation or ingestion of glycols	13	0	0

* This group included animals considerably older than those in the propylene glycol group and it is probable that lung lesions had occurred in some before the experiment began.

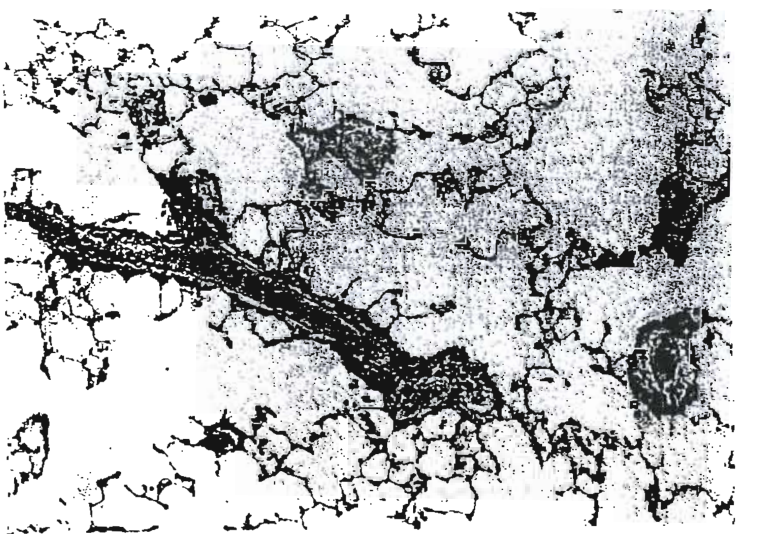


Fig. 2. Photograph of the Lung of a Control Rat Five Months Old

Each blood vessel is surrounded by a collar of cells (mononuclear) which appears to be almost continuous along the vessel wall. The remainder of the lung architecture is normal. Magnification × 125.

position would provide the optimum opportunity for the condensed glycol to remain in the lungs. The lack of deleterious effect found in rats could conceivably be attributed to the more effective elimination of this material from their lungs due to the horizontal position of the air passages (18).

Forty-five Macacus Rhesus monkeys were secured from the tuberculosis-free colony of Columbia University at San Juan, Puerto Rico.[3] Twenty-nine were exposed to propylene glycol vapor and sixteen employed as controls.

Three insulated air-conditioned chambers 9 x 6.5 x 8 feet high were constructed for the experiment in which temperatures of 78 to 82°F. and relative humidities of 50 to 60 per cent were constantly maintained. The air flow through each chamber amounted to 13 complete changes of air per hour. The cages made of heavy 2" mesh steel wire were 36 x 28 x 39 inches high. Since the monkeys were small, three to four were able to live comfortably in a single cage. The two glycol

TABLE 5

Exposure of monkeys to propylene glycol vapor

	NUMBER OF MONTHS OF EXPOSURE TO TEST CONDITIONS											TOTAL NUMBER OF MONKEYS
	1	2	3	4	5	6	7	9	11	12	13	
Number of monkeys autopsied in each chamber*												
Glycol	1			6	3	7	2	1	2	1	6	29
Control	2		1		8	1	4					16

* Monkeys died or were sacrificed at termination of exposure times indicated in table.

chambers contained 14 and 15 monkeys respectively and the control 16. The diet consisted of oranges, apples, potatoes, bananas, carrots and bread to which was added egg-nogs containing vitamin B complex and cod liver oil. No ultraviolet radiation was employed.

Propylene glycol vapor was dispersed in two of the chambers as shown in table 2. Supersaturation of the air with glycol vapor was present constantly in one chamber while in the other a concentration of about 60 per cent saturation was maintained.

The lengths of time the monkeys were kept in the test and control chambers is shown in table 5. An x-ray of the lungs was made shortly after the monkeys arrived. They also were tuberculin-tested by means of the intracutaneous injection of O.T. 1–1000 into the soft tissue lateral to the eye. All x-rays and tuberculin tests were negative. These two procedures were repeated at the end of the experiment. The animals were weighed at monthly intervals and observations made on the texture and color of hair and skin, condition of eyes, appetite, activity and any abnormal signs or symptoms. Complete blood counts were made at the beginning of the experiment and again just before they were sacri-

[3] These monkeys were supplied to us through the kindness of Dr. Earl T. Engle, Professor of Anatomy, Columbia University Medical School.

Toagosei Co., Ltd.
70231-E
Page 82 of 127

ficed. Tests for the ability of the kidneys to concentrate urine were conducted at the end of the period of observation.

Within the first seven months, three of the sixteen monkeys in the control chamber and seven of the 29 animals in the glycol chamber died. Eight other monkeys were sacrificed at the time they were very ill in order to obtain antimortem tissues. The distribution of these deaths and infections is shown in table 6.

The weights of the two groups of animals are shown in figure 3. Final weights of sick monkeys just before being sacrificed are not included. Unfortunately, the weight gains in the two groups of monkeys could not be compared for longer than five months due to an insufficient number of remaining controls. Except for the period from five to eight months when the monkeys in the glycol chambers lost weight (found to be due to insufficient food)[4] these animals gained weight at a normal rate until they were sacrificed at twelve months.

TABLE 6

Occurrence of disease and death in control and propylene glycol-exposed monkeys

	NUMBER SUCCUMBING TO INFECTION FROM					TOTAL NUMBER DEATHS	NUMBER REMAINING WELL UNTIL SACRIFICED
	Nematode infection	Bacterial infection	Dysentery	Malnutrition	Cause unknown and injury		
Glycol group (29 animals)							
Died	2	2 oral	1	1	1 cause unknown	7	
Sacrificed when very ill	3	2 oral	1			6	16
Control group (16 animals)							
Died	2				1 cause unknown	3	
Sacrificed when very ill	5	1 oral			1 injury	7	6

The results of blood counts and hemoglobin determinations are shown in table 7. Upon arrival in the laboratory the monkeys were all found to be suffering from a considerable degree of anemia. That this was probably not due entirely to the round worm infection (described below) is shown by the fact that on a liberal diet containing ample vitamins the blood picture had improved markedly by the time these animals were sacrificed. Restoration of the higher level occurred within three to four months and thereafter the blood picture remained essentially unchanged. The blood of the monkeys maintained in the glycol atmosphere showed a slightly greater increase in red blood cells and a distinctly higher hemoglobin content than did the control animals.[5]

[4] At about the 6th month the quantity of food was reduced to the amount which the animals would entirely consume. This was found to be mistaken economy since restoration of an excess supply of food following the 8th month weighing resulted in a rapid and continuous gain in weight.

[5] We wish to record our indebtedness to Dr. Leon O. Jacobson, Department of Medicine, University of Chicago, and his laboratory staff for carrying out these red counts and hemoglobin determinations.

Tests for possible impairment of kidney function were made by determining their ability to concentrate the urine during a period of 24 hours in which both

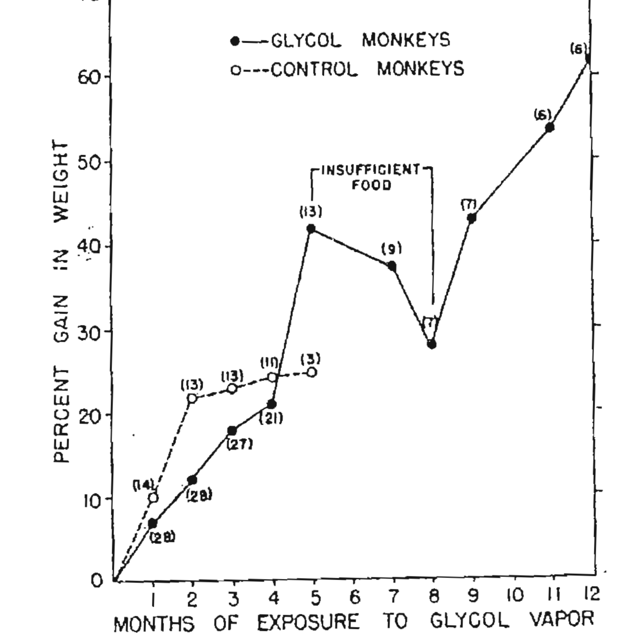


FIG. 3. COMPARATIVE GROWTH RATES OF MONKEYS KEPT CONTINUOUSLY IN AN ATMOSPHERE SATURATED WITH PROPYLENE GLYCOL VAPOR AND A GROUP OF CONTROLS

Average initial weights of monkeys in glycol group 2959 grams and in control group 2459 grams. The figures in parenthesis indicate number of animals in each weighing.

TABLE 7

Blood counts and hemoglobin determinations of monkeys exposed to propylene glycol vapor

	TIME OF DETERMINATIONS	RED BLOOD CELLS	WHITE BLOOD CELLS	HEMOGLOBIN	DIFFERENTIAL				
					Polynuclears	Lymphocytes	Monocytes	Eosinophiles	Basophiles
Glycol group	Initial	2.58	15.9	11.9	58	27	11	6	1
	Final	5.18	22.3	13.4	71	18	7	9	1
Control group	Initial	2.61	18.8	11.6	44	29	17	7	1
	Final	4.77	26.1	11.9	67	17	10	1	1

water and food were withheld. Both groups of monkeys concentrated their urine equally well under these conditions. Microscopic examination of the

urine showed occasional hyaline casts, a few white blood cells and sometimes a slight trace of albumin. No differences were found between the controls and test monkeys.

In addition to those monkeys sacrificed at a time when they were very ill, certain well ones in both control and test groups were killed from time to time. All these are included in the figures of table 5. The animals which remained well showed no change except that certain ones in the glycol chambers developed brownish discoloration of the face after a few months. This cleared up following a week or two's sojourn outside the chamber and was considered to be due to the drying effect of the glycol on the skin. Their coats remained glossy and they were very active.

Examination at Autopsy. Two pathological conditions were found in practically all the monkeys both test and control: (1) infection with Aesophagostomum apiostomum, a parasitic nematode closely related to the hookworm, and (2) infestation of the lungs with the lung mite Pneumonyssus griffithi. On opening the abdomen, small chocolate colored cysts, 3–5 mm. in diameter, enclosing the larval worms, were scattered throughout the omentum and along the wall of the bowel. These varied from a few to many scores. Adhesions were frequently present, and were most extensive in animals dying of the disease where they occasionally caused obstruction of the common duct, or interference with the portal circulation as evidenced by ascites, or partial intestinal obstruction. Adult worms were found in the intestinal contents. The monkeys with severe infection showed malnutrition and sometimes marked anemia.

The lungs of almost all the animals showed numerous small whitish nodules, 2–5 mm. in diameter, both on the surface and throughout the lung. On microscopic examination they were found to consist of a central cavity surrounded by a dense cellular wall which was composed mostly of macrophages though in places numerous polymorphonuclear leucocytes were present (fig. 4). In some of the cavities the mite was present as shown in figure 5. No evidence of tuberculosis was found nor were any other pathological changes detected in the lungs of either the glycol-exposed or the normal monkeys. The lungs were fixed by the method employed in the preparation of the rats' lungs. Two sections were made through each of the principal five lobes of the lungs and one through the small subcardiac lobe—eleven sections in all.

With the above exceptions the gross appearance of the organs of the thoracic and abdominal cavities was normal. Microscopic examination of the liver, kidneys, spleen, mesenteric glands, adrenals and in certain cases stomach, intestines and testes showed no differences between test and control monkeys. No stones were found in the kidneys or bladder. In the animals which died from disease or were killed when very ill (table 6) certain other changes characteristic of the disease process were found, e.g. hemorrhagic bowel wall in those dying of dysentery.

The tests on exposing monkeys and rats continuously to atmospheres saturated with propylene glycol vapor for periods of 12 to 18 months may be summarized by the statement that no deleterious effects either functional or organic could be

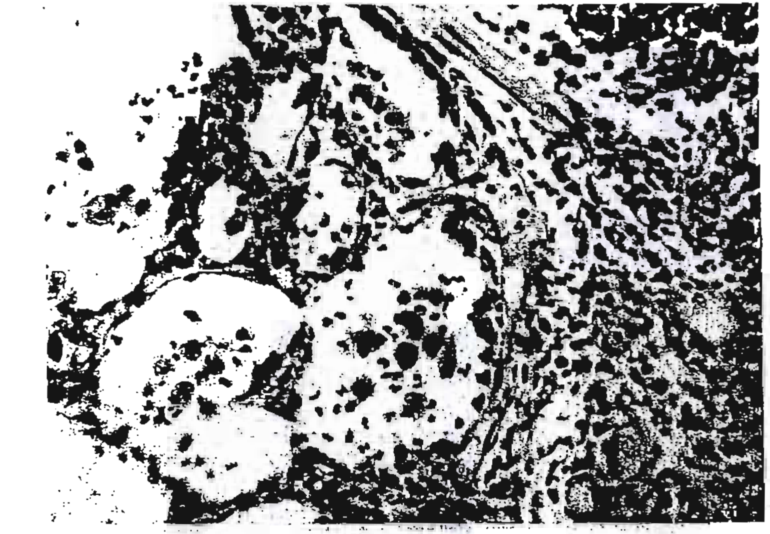


Fig. 4. High Power View of a Portion of the Wall of the Lung Mite Cyst Which Shows a Dense Accumulation of Mononuclear and Polymorphonuclear Leucocytes

In the adjacent alveoli macrophages and polymorphonuclear leucocytes are present. In places the septal cells are increased in number. Magnification ×480.

Fig. 5. Photograph of a Lung Mite (Pneumonyssus griffithi) Cyst Showing a Longitudinal Section of the Parasite Lying in the Lower Portion of the Cavity

The head is toward the left hand side. Beyond the head is a collection of exudate cells which partly surround a fragment of another mite. Magnification ×82.

Toagosei Co., Ltd.
70231-E
Page 84 of 127

Toagosei Co., Ltd.
70231-E
Page 85 of 127

attributed to this treatment. Actually both the rats and monkeys kept in the glycol atmosphere appeared to do somewhat better than the control animals in respect to gain in weight, incidence of pulmonary infection and increase in red blood cells and hemoglobin.

Triethylene glycol

Inhalation of Vapor by Rats. Thirty-six rats ranging from 100 to 350 grams in weight, two-thirds of which were males, were placed in a chamber containing glycol vapor and 6 rats in another which served as the control. The populations were increased during the experiment by the birth of young to 60 in the vapor and 14 in the control groups (table 8). As shown in table 2 the rats in the glycol group were maintained continuously in an atmosphere supersaturated with triethylene glycol vapor.

TABLE 8

Rats exposed to triethylene glycol vapor and given the glycol orally

	Number of months exposure to test conditions												Total number of animals
	½	3	4	5	6	7	8	9	10	11	12	13	
Number of rats killed in each group at successive intervals Vapor	0	10	10	0	5	0	26	1	0	0	5	3	60
Oral 35×*	0	0	0	2	0	0	4	0	0	0	0	2	8
80×	1	0	1	2	0	0	3	0	0	0	0	2	9 45
700×	0	4	0	2	0	1	3	0	3	0	3	12	28
Control	0	0	0	2	0	0	3	0	0	0	5	4	14

* 35×, 80× and 700× = the calculated dosage in terms of the maximum amount of triethylene glycol vapor a rat could inhale in 24 hours in an atmosphere saturated with this substance.

Growth Rates. Since the rats employed in this test varied in age from six weeks to six months at the beginning of the experiment it was not possible to plot the growth curve in the same manner as that exhibited for the propylene glycol rats which were all initially of the same age. Each dot on the graph (fig. 6) represents the average weight of a group of rats consisting of from four to 14 animals. Certain of the 2-3 months-old rats shown in the graph were carried through to the termination of the test (13 months). The older rats were added to the graph at their respective ages when started in the test. There were 36 animals exposed to glycol and 46 controls.[4] Only male rats are included in the graph. It will be noted that for the first seven months there was essentially no

[4] For purposes of the weight chart the male control rats of the propylene glycol experiment plus half a dozen normal rats kept under usual room conditions were combined with the triethylene glycol control animals. All the rats were fed the same diet.

difference in the growth rates of the two groups. After this age, however, the rats in the glycol atmosphere continued to grow while the weights of those in the control group remained almost stationary. The animals in the two groups appeared in excellent condition. The young born in the glycol atmosphere seemed to be normal in every way, and gained weight just as rapidly as did the controls of the same age.

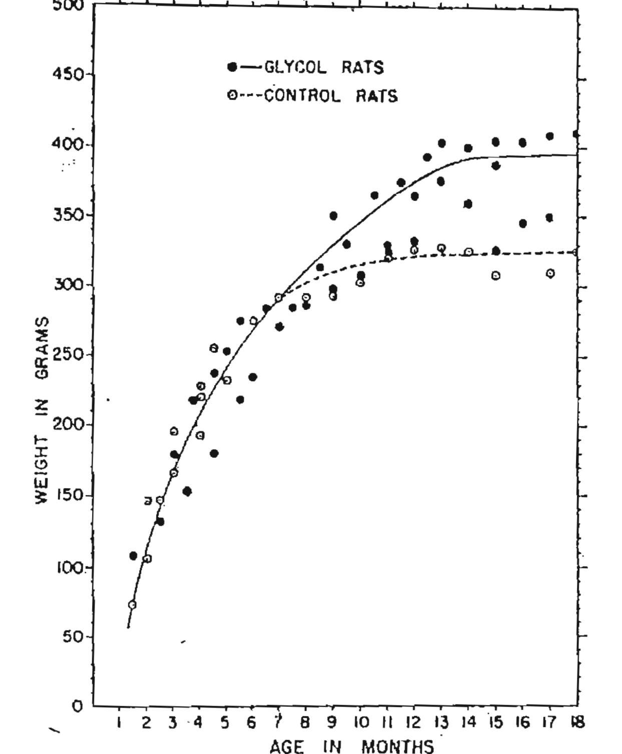


Fig. 6. Comparative Growth Rates of Rats Kept Continuously in an Atmosphere Saturated with Triethylene Glycol Vapor and a Similar Group of Control Rats
Male rats only included on the graph

Blood Examination. Complete blood counts and hemoglobin determinations were made on each rat at the end of the time of residence in the vapor and control chambers and are exhibited in table 9. After 8-13 months continuous exposure to a fog of triethylene glycol vapor, rats showed no differences in their blood picture from that of the control animals.

Ingestion of Triethylene Glycol by Mouth. Four groups of eight rats each weighing between 200 and 300 grams were employed as shown in table 8. The

glycol was added to the drinking water in amounts calculated in terms of multiples of the maximum quantity of glycol a rat could inhale in vapor form if kept in a saturated atmosphere for 24 hours. This quantity is approximately 0.004 cc. of glycol per kilogram of rat per day.[7] Preliminary tests showed that individual rats of the same weight drank about the same quantity of water each day. The quantity of glycol solution consumed in each cage of two rats was measured at the end of each week. This data is exhibited in table 10. The dosage indicated for each group represents the amount of glycol actually consumed which is somewhat less than that expected from the initial calculations. Since it seemed desirable to secure the most information about the largest dose of glycol, the rats on 700× (i.e. ingesting approximately 700 times the quantity inhaled in 26

TABLE 9

Blood counts of rats after exposure to triethylene glycol vapor and oral ingestion of the glycol

	Length of exposure	Red blood cells	White blood cells	Differential count				
				Polynuclears	Lymphocytes	Monocytes	Eosinophiles	Basophiles
	mo.	*millions*	*thousands*					
Glycol vapor (57 rats)	6–13	7.61	15.2	32	56	5	6	>0.5
Controls—vapor (17 rats)	6–13	7.51	12.1	28	66	3	2.5	0.5
Oral ingestion dose								
(7 rats) ×35	5–13	5.87	14.5	35	56	6.4	2.6	0
(8 rats) ×80	5–13	6.11	11.5	28	62	7.2	2	0.5
(24 rats) ×700	5–13	6.14	12.0	35	60	2	3	0
Controls—oral (9 rats)	12–13	5.62	12.1	32.5	56.5	5.5	5.5	0

TABLE 10

Amounts of triethylene glycol ingested per day per rat in the different groups exhibited in table 8

	Dosage groups of rats			Controls
	35×	80×	700×	
Total fluid per month	708 cc.	784 cc.	712 cc.	836 cc.
Total glycol per month	0.85 cc.	1.96 cc.	16 cc.	0
Glycol per day	0.028 cc.	0.065 cc.	0.533 cc.	0
Glycol per day per kilograms of rat	0.14 cc.	0.32 cc.	2.66 cc.	0
Average weight, in grams, at				
Beginning of experiment	264	292	246	260
End of experiment	334	363	328	305

[7] These calculations were only approximate as they were based on the actual measurement of the tidal air in 18 gram mice which was 0.25 cc. (19). The rats tidal air was estimated to be ten times this figure and the 24 hour air volume was calculated on the basis of 75 respirations per minute. The average glycol concentration corresponds to 0.004 mg./l.

hours) were, in the latter half of the experiment allowed to breed. They had frequent large litters which increased this population from 8 to 28.

All these animals remained in good condition. The weight gains of the four groups of animals were approximately the same (table 10). Examination of the blood at the end of the test period showed no essential differences between the glycol-fed and the control rats (table 9). Microscopic examination of the urine during life revealed no abnormalities.

Examination at Autopsy. The post mortem findings in rats exposed to triethylene glycol vapor and drinking the glycol were essentially the same as those of the rats exposed to propylene glycol; i.e. there were no pathological changes except focal areas of pneumonitis. As shown in table 4 these lesions were present in approximately the same percentage of test animals and controls.

Inhalation of Triethylene Glycol Vapor by Monkeys. First Test—Supersaturated Glycol Atmospheres. Twenty-five monkeys from the Puerto Rico colony were divided into two groups. Seventeen were maintained in a fog of triethylene glycol, eight were kept in one of the other chambers as controls. The atmospheric conditions and concentration of glycol vapor are given in table 2. The glycol supersaturation was sufficiently high to produce a dense fog which caused continuous condensation on all surfaces inside the chamber. The other conditions under which they were maintained were the same as those described for the monkeys exposed to propylene glycol vapor. X-rays of the lungs and tuberculin tests carried out at the inception of the experiment were all negative. The number of months the animals were kept in the test is shown in table 11.

As table 12 shows there was a considerable morbidity and mortality from the same causes which affected the former group of monkeys. The animals in the control group gained weight somewhat more rapidly than did those in the glycol atmosphere (fig. 7) and at the end of eight months had shown a weight gain equivalent to 66 per cent of their initial weight while those in the glycol had gained only 44 per cent. Even by the end of 13 months the glycol monkeys had gained not quite 60 per cent of their initial weight.

Another difference between the glycol-exposed and the control group was the appearance of a browning of the skin of the face which occurred in the former animals after 3 to 4 months in the glycol atmosphere. This was of the same nature as that observed in the case of the monkeys exposed to propylene glycol vapor, and disappeared within a few days after removal to glycol-free air. Some of the animals exposed to triethylene glycol vapor also showed crusting of the ears with thickening of the lateral edge of the ear and actual loss of tissue in certain instances. Microscopic sections of these tissues revealed the presence of numerous cysts containing an unidentified ectoparasite. Such lesions were only very occasionally seen in the control monkeys and were not nearly as pronounced. It is possible that the effect on the skin caused by the supersaturated glycol atmosphere rendered the integument of the ears more susceptible to this form of parasitic invasion. Furthermore, the fact that these monkeys were observed to scratch and pull at their ears constantly, suggested the possibilty of the implantation of the parasite by this means.

Toagosei Co., Ltd.
70231-E
Page 86 of 127

In other respects, however, the findings on monkeys in the glycol atmosphere were essentially the same as those of the control animals. The red blood counts and hemoglobin determinations showed a decline in both groups of animals to about the same degree (table 13, Test no. 1). This was probably caused by the presence of nematode infection. No significant differences in the total number of white blood cells or differential were found. Repeated examination of the urine showed no albumin, sugar or microscopic elements in most of the animals and a slight trace of albumin, an occasional granular and hyaline cast or a few white

TABLE 11

Exposure of monkeys to triethylene glycol vapor test number 1

	Number of months of exposure to test conditions										Total number of monkeys
	1	2	3	4	5	6	7	8	9	12	
Number of monkeys autopsied in each chamber[a]											
Glycol	4		2	3		3				5	17
Control					2	1	1	4			8

[a] Monkeys died or were sacrificed at termination of exposure times indicated in table.

TABLE 12

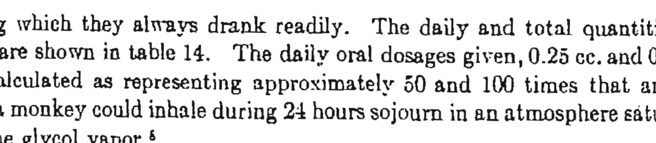
Occurrence of disease and death in control monkeys and those exposed to triethylene glycol vapor test no. 1

	Number succumbing to infection from				Total number deaths	Number remaining well till sacrificed
	Nematode infection	Bacterial infection	Dysentery	Injury or cause unknown		
Glycol group (17 animals)						
Died		2		2 injury	4	
Sacrificed when very ill	2		1		3	10
Control group (8 animals)						
Died	1				1	
Sacrificed when very ill	4				4	3

blood cells in some of them. Both groups of animals showed equal ability to concentrate urine from a specific gravity of 1.010 to 1.030 or 1.040. These tests were conducted soon after the beginning of the experiment and again just before the animal was sacrificed.

Except for these animals which died or were killed because they were very ill (table 12) the monkeys in both groups were very active, ate well and had smooth glossy coats.

Oral Ingestion of Triethylene Glycol by Monkeys. Eight monkeys from the same shipment as those used for exposure to triethylene glycol vapor in Test no. 1 plus two others (older ones) were given the glycol orally by adding it to



egg nog which they always drank readily. The daily and total quantities ingested are shown in table 14. The daily oral dosages given, 0.25 cc. and 0.5 cc., were calculated as representing approximately 50 and 100 times that amount which a monkey could inhale during 24 hours sojourn in an atmosphere saturated with the glycol vapor.[5]

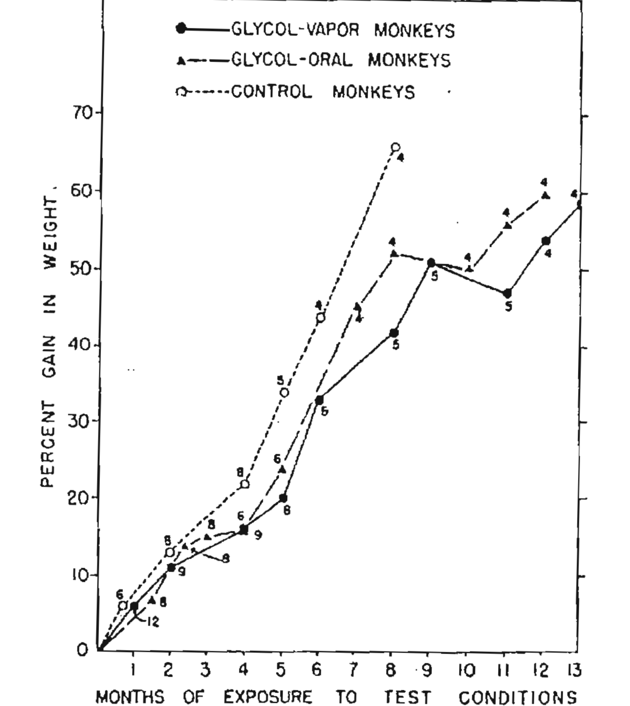


FIG. 7. COMPARATIVE GROWTH RATES OF MONKEYS KEPT CONTINUOUSLY IN AN ATMOSPHERE SATURATED WITH TRIETHYLENE GLYCOL VAPOR (TEST NO. 1), MONKEYS INGESTING THE GLYCOL AND A CONTROL GROUP

Average weights of the different groups at the beginning of the experiment were: glycol vapor 2276 grams, glycol ingestion 2036; control 1973. Figures in parenthesis indicate number of animals in each weighing.

The weight gain of these animals shown in figure 7 was approximately the same as that of the monkeys in the glycol atmosphere and a little less than that of the control group. These animals all remained healthy and one gave birth to a live

[5] The tidal air of the monkey was calculated from that of the human being in relation to weight. In this case approximately 1/8 of the tidal air of human beings or 25 cc. per respiration. Calculations were based on 30 respirations per minute and a concentration of glycol of 0.004 mg./l. This gave a 24 hour total of about 0.0045 mg. of triethylene glycol.

normal-appearing infant at the end of four months during which time she had ingested 64.5 cc. of triethylene glycol. The baby monkey lived for two months and seemed to do well but was found dead one morning. Autopsy revealed no cause for death.

Examinations of the blood and urine of these monkeys yielded practically identical results with the controls except that the monkeys taking the glycol by mouth showed less anemia at the end of the test period (table 13).

TABLE 13

Blood counts and hemoglobin determinations at beginning and end of exposure of monkeys to triethylene glycol vapor and oral ingestion

Monkeys exposed	Red blood cells (millions)		White blood cells (thousands)		Hemoglobin (gm.)		White blood counts									
							Polynuclears		Lymphocytes		Monocytes		Eosinophiles		Basophiles	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final
Test no. 1																
Glycol vapor (fog)......	5.20	4.30	19.70	18.32	11.9	11.5	48.23	73.59	40.71	20.0	7.28	5.71	6.0	1.66	1.33	1.0
Control................	6.00	4.30	23.95	19.58	12.3	11.1	54.85	71.85	35.28	23.42	3.16	2.80	5.71	1.50	2.0	1.0
Oral ingestion............	5.27	4.99	20.36	20.37	11.3	12.6	65.25	79.25	23.50	13.25	5.75	5.62	3.0	2.0	1.25	1.60
Test no. 2																
Glycol vapor (no fog)..	4.45	6.00	23.26	—	14.3	97%*	44.0	—	53.60	—	—	—	2.33	—	—	—
Control................	5.39	5.90	27.52	—	14	87%*	53.71	—	46.0	—	—	—	2.0	—	—	—

* These determinations made on Dare Hemoglobinometer.

TABLE 14

Monkeys taking triethylene glycol orally

Number of animals	Dose of TEG* daily	Duration of ingestion	Total amount of TEG ingested
	cc.	*mo.*	*cc.*
2	0.5	3	45
2	0.5	5½	83
4	2 = 0.25 2 = 0.5	12	2 = 91 2 = 183
2	0.5	14½	220

* Triethylene glycol.

Autopsy. Since the findings at autopsy of all three groups of monkeys, i.e. those exposed to the glycol vapor, those drinking glycol and the controls, were essentially the same, they will be described together. With the exception of two animals in the oral-ingestion group which were obtained from another source, the monkeys all showed lung mites and varying degrees of round worm infestation. The two animals above mentioned showed neither of these parasites.

Examination, both gross and microscopic, of the different organs—lungs, liver, kidneys, spleen, bone marrow, revealed no differences between the control and the test animals. Gomeri's (20) phosphotase stain of the kidneys showed a heavy precipitation along the secretory portion of the tubules indicating good functional activity. The bone marrow was very cellular, appeared normal and exhibited both the myelogenous and erythroid series. No evidence of tuberculosis or any sign of chronic irritation (except for that due to the lung mites) was found in the lungs. The other organs, spleen, liver, stomach and intestines were likewise normal except for the presence of nematode cysts in the intestinal walls and mesentery.

In animals dying during the course of the experiment for causes indicated in table 12, pathology peculiar to the particular infection was found, which in no way could be ascribed to any toxic effect of glycol since such lesions were present in the controls as well as in the test animals.

Inhalation of Triethylene Glycol Vapor by Monkeys. Second Test—Glycol Maintained Below Saturation with Glycostat. Since the monkeys kept in an atmosphere supersaturated with triethylene glycol vapor did not gain weight quite as well as did the control animals and also showed certain effects on the skin, such as drying and discoloration, it was felt that a second test should be conducted under conditions approximating more nearly those which would be desirable for the use of glycol vapor in human habitations. The lessened gain in weight might have been due to a greater but undetectable degree of nematode infection in the test group, but the fact that the monkeys ingesting glycol also failed to grow as rapidly suggested that glycol was probably the important factor. Since no pathology was detectable in animals drinking 100 times as much glycol as they could possibly ingest from exposure to vapor alone, it seemed likely that the lesser weight gain in these animals might be attributable to a depressant action of the glycol on the appetite. In the case of those living in the fog, a similar effect might be ascribed to an unfavorable environment.

At the time the first test was carried out we had available no means of controlling the concentration of glycol vapor in the atmosphere. The only way of insuring the presence of a bactericidal concentration was to maintain a slight fog. The subsequent development of the glycostat (21) made it possible to carry out another experiment in which monkeys could be kept in an atmosphere free from fog but at the same time containing bactericidal concentrations of triethylene glycol.

The monkeys, obtained from a source other than that of the two previous tests, were free from peritoneal nematodes, lung mites and tuberculosis (with one exception). The group was composed of sixteen animals about two years old, half of which was used for the test and half for controls. The triethylene glycol was dispersed as in the previous experiment except that the output of the vaporizer was controlled by a glycostat which was set to maintain the concentration of the glycol vapor in the air from 65 to 75 per cent saturation (see table 2). No fog was visible at any time. All other conditions were kept the same as described in the previous experiments. These monkeys were kept under test conditions for ten months.

Within a month it was observed that one monkey in the glycol group failed to

Toagosei Co., Ltd.
70231-E
Page 88 of 127

gain weight. During the succeeding month it became ill and died. Autopsy revealed widespread pulmonary tuberculosis. In spite of the fact that the tuberculin tests on the monkeys shortly after reaching the laboratory were all negative, it seems probable that the infection was acquired before arrival in Chicago since x-rays of the lungs of the caretaker and all other persons who had contact with the monkeys were normal. The cage mate of this monkey remained well, gained weight and showed no evidence of pulmonary involvement by x-ray. No other illnesses developed in either group of animals.

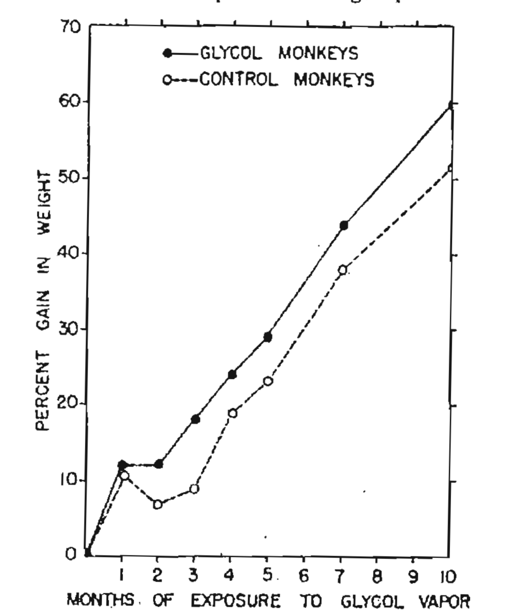


FIG. 8. COMPARATIVE GROWTH RATES OF MONKEYS KEPT CONTINUOUSLY IN AN ATMOSPHERE OF TRIETHYLENE GLYCOL VAPOR MAINTAINED BELOW THE SATURATION LEVEL (TEST NO. 2), AND A GROUP OF CONTROLS

Average initial weight of the seven monkeys in glycol group 2516 grams. The eight control animals averaged 2630 grams.

The growth rates charted in figure 8 show a progressive and satisfactory gain in weight of both test and control monkeys. After the first month the animals in the glycol showed a slight but consistently greater weight gain than did the controls. This result gives some support to the inference that the less rapid growth of the monkeys in the first triethylene glycol experiment was due to a diminished food intake.

In contrast to the course of the blood counts in the previous triethylene glycol experiment (first test) the numbers of red blood cells and amount of hemoglobin increased during the ten months of the study—about equally in both glycol-exposed and control animals (table 13, Test no. 2). None of the monkeys in the

glycol atmosphere showed any discoloration of the skin of the face or any change in the color or texture of the ears. They were all lively, exhibited glossy coats and gained weight about equally well with the exception of one of the test animals which showed a relatively small increase in weight.

Autopsy. Since this second experiment with triethylene glycol was conducted primarily for observations on weight-gain and skin effects, it was not considered necessary to do post-mortem examinations in all the monkeys in view of the completely negative findings in respect to any deleterious effect of the glycol in animals of the first test group. Three animals were sacrificed, two from the glycol group and one control. The internal organs were entirely normal with the exception of one of the test animals which exhibited a localized area of chronic gastritis at the pyloric end of the stomach. This was the single monkey which had shown an unsatisfactory weight gain from the beginning of the experiment.

In summarizing the above described tests on the toxicity of triethylene glycol for monkeys and rats it may be stated that among the large number of animals studied no pathological changes, ascribable to effect of the glycol were detected. The only disturbances which could be related to the presence of triethylene glycol vapor in the air were drying of the skin and slight interference with nutrition. That these two effects were due to an excess of glycol vapor (fog) was indicated by their absence in the second experiment in which the concentration of glycol was kept below saturation by means of the glycostat.

DISCUSSION

Two studies on the prolonged administration of propylene glycol have been reported, one by Seidenfelt and Hanzlik (7) the other by Morris, Nelson and Calvery (22). The animals tested—rats—either drank water containing 10 per cent propylene glycol or ingested the glycol incorporated in the food in quantities of 2.5 to 4.9 per cent of the total food weight. In the latter experiment the animals showed no ill effects after two years on such a regime. The quantities of glycol administered to these animals daily was several hundred times the amounts estimated to have been inhaled by the rats in our study. The only new information contributed by the present investigation of the toxicity of propylene glycol is the demonstration that the continuous residence of monkeys and rats for a year or more in an atmosphere supersaturated with the vapor of this glycol was without deleterious effect on the lungs or functional activity of the body as a whole. In fact the animals in the glycol atmosphere seemed to thrive somewhat better than the control groups as judged by weight gain, and increase in red blood cells and hemoglobin content.

On the other hand there are no data available on long term toxicity studies with triethylene glycol. Tests carried out previously have all been of an acute nature except those of Lauter and Vler (9). These authors found that young rats drinking water containing 3 per cent triethylene glycol showed no ill effect after 30 days. Adult rats given water containing 5 per cent of the glycol died—although the young rats survived. While the maximum concentration of glycol in

Toagosei Co., Ltd.
70231-E
Page 90 of 127

the water of the present experiment on oral toxicity was only a little over 2 per cent, the administration was continued for 13 months without detectable disturbance of the rats' physiology. Our monkeys ingested in proportion to their weight only one seventh the amount taken by the rats on the maximum dosage, but even this was approximately 100 times the quantity they could possibly take into the body through inhalation.

Since the absorption of triethylene glycol from inhalation of atmospheres containing this vapor represents such a minute fraction (less than 1/1000th of the toxic dose for rats) and since the experiments showed no evidence of either generalized pulmonary irritation, which one would expect from a toxic vapor, or of more localized disturbances, if glycol accumulated in the lungs, the particular value of the present investigation lies in the demonstration of the tolerance of these animals for prolonged exposure to glycol-containing atmospheres. Such information is of particular value in the case of monkeys since the responses of this animal to changes in environmental conditions approach much more nearly those of the human being than do the reaction of the common laboratory animals.

The question may of course be raised concerning the relative toxicity of triethylene glycol for animals and men. Such information as we have indicates that the effects of glycols on the tissues is much the same for the different animal species. Geiling and Cannon's (23) study of diethylene glycol, following the fatalities from elixir sulphanilamide, showed that the fatal dose for rats, rabbits and dogs was within the same range as that for humans. It should be pointed out that triethylene glycol is much less toxic for animals than is diethylene glycol. The comparative toxicities of the various glycols have been extensively reviewed by Van Oettingen (24).

While the outcome of our studies provided a high degree of assurance that exposure of human beings to triethylene glycol vapor could be safely undertaken, clinical tests of the effectiveness of triethylene glycol for aerial disinfection and the control of air-borne infections have contributed direct evidence of the inocuousness of this vapor in the air. In the studies of Harris and Stokes (25) Hamburger and associates (26) (16) and Bigg and Jennings (27) in which groups of individuals were exposed for periods ranging from several weeks to over a month at a time to atmospheres either partially or completely saturated with triethylene glycol vapor, no untoward effects of the glycol were observed. Loosli and co-workers (28) were able to detect no disturbance of the skin or other organs of very young infants kept continuously for 5-6 months in an atmosphere containing bactericidal concentrations of this glycol. Other observations (not reported in the literature) on large groups of industrial workers exposed during working hours to atmospheres partially saturated with triethylene glycol for many years have not recorded a single instance of ill effects attributable to this glycol vapor.

SUMMARY

With a view to determining the safety of employing the vapors of propylene glycol and triethylene glycol in atmospheres inhabited by human beings, monkeys and rats were exposed continuously to high concentrations of these vapors for

periods of 12 to 18 months. Equal numbers of control animals were maintained under physically similar conditions. Long term tests of the effects on ingesting triethylene glycol were also carried out. The doses administered represented 50 to 700 times the amount of glycol the animal could absorb by breathing air saturated with the glycol.

Comparative observations on the growth rates, blood counts, urine examinations, kidney function tests, fertility and general condition of the test and control groups, exhibited no essential differences between them with the exception that the rats in the glycol atmospheres exhibited consistently higher weight gains. Some drying of the skin of the monkeys' faces occurred after several months continuous exposure to a heavy fog of triethylene glycol. However, when the vapor concentration was maintained just below saturation by means of the glycostat this effect did not occur.

Examination at autopsy likewise failed to reveal any differences between the animals kept in glycolized air and those living in the ordinary room atmosphere. Extensive histological study of the lungs was made to ascertain whether the glycol had produced any generalized or local irritation. None was found. The kidneys, liver, spleen and bone marrow also were normal.

The results of these experiments in conjunction with the absence of any observed ill effects in patients exposed to both triethylene glycol and propylene glycol vapors for months at a time, provide assurance that air containing these vapors in amounts up to the saturation point is completely harmless.

Acknowledgements. We wish to express our appreciation to Mr. John Daniels for the preparation of several thousand microscopical sections the excellence of which greatly facilitated the histological study and to Dr. Eleanor M. Humphries of the Department of Pathology of the University of Chicago for examining many histological sections especially of the kidney and liver.

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70231-E
Page 91 of 127

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THE EFFECT OF DI-ISOPROPYL FLUOROPHOSPHATE ON NEUROMUSCULAR TRANSMISSION[1]

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The principal pharmacologic actions of di-isopropyl fluorophosphate (DFP) have been attributed to its ability to inactivate cholinesterase, (1, 2). The acute administration of DFP resulted in fasciculations and weakness of skeletal muscle in addition to parasympathomimetic effects. Modell et al. (2) noted that large doses of DFP administered intravenously to atropinized cats resulted in the development of profound muscular weakness which lasted for 1 to 2 weeks or longer. The gastrocnemii of such poisoned cats were unable to sustain a tetanus induced by nerve stimulation with an inductorium. Koelle and Gilman (3) reported the development of fasciculations of the skeletal muscles and hind leg weakness in dogs chronically poisoned with DFP. Harvey et al. (4) have studied the effect of DFP on neuromuscular transmission in normal and myasthenic man. The intra-arterial injection of DFP in normal man caused numerous spontaneous fasciculations and a pronounced weakness of muscles in the injected region. Electromyograms showed the second of 2 successive stimuli to be reduced in amplitude. In contrast, patients with myasthenia gravis showed a localized increase in muscle strength and a return to normal of the usual electromyographic defect. Riker and Wescoe (5) reported that the close intra-arterial injection of DFP in the cat gastrocnemius preparation caused unorganized contractions after a latent period of 2 to 5 minutes.

The present report is concerned with the effect of DFP on neuromuscular transmission in the intact cat. The effects of DFP were compared with those of 2 other anticholinesterases, prostigmine and physostigmine.

METHODS. Adult cats were used, weighing between 2.7 and 4.5 kg. A transection of the spinal cord at level L-1 was performed on all animals under preliminary ether anesthesia. The gastrocnemius muscle was freed by elevating the Achilles tendon with the posterior portion of the calcaneum. Blood supply to the muscle was carefully preserved, while other branches of the tibial vessels were ligated. Utilizing a holder described by Wolff and Cattell (6), the lower end of the femur was fixed by means of a steel pin and the tendon was wired vertically to a heavy isometric lever. The popliteal artery was exposed. Intra-arterial injections were made with a #26 needle, during which time the artery was occluded from above. All doses were dissolved in a volume of 0.1 cc./kg. of distilled water. The intact sciatic nerve was stimulated with enclosed silver electrodes which were fixed in situ. Maximal break shocks were delivered from an inductorium by an interruptor, at a constant frequency of 1 in 12 seconds. Drying of the muscle was retarded by the repeated application of mineral oil to its surface.

[1] The work described in this paper was done under contract between the Medical Division, Chemical Corps, U. S. Army and Cornell University Medical College. Under the terms of the contract, the Chemical Corps neither restricts nor is responsible for the opinions or conclusions of the authors.

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Toxicology, Metabolism, and Biochemistry of 1,2-Propanediol

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Toxicology, Metabolism, and Biochemistry of 1,2-Propanediol. RUDDICK, JOSEPH A. (1972). *Toxicol. Appl. Pharmacol.* 21, 102–111. The toxicity of 1,2-propanediol is reviewed, and a table of the LD50 values for the rat, rabbit, mouse, guinea pig, and dog is presented. Metabolic and chronic studies demonstrate that 1,2-propanediol can be used as a substitute for carbohydrate in the diet of rats and young chicks. The glycol is primarily oxidized to lactate or pyruvate.

1,2-propanediol (1,2-PD, PG) is a widely used compound with diverse applications. It is used as a solvent for flavoring material in baking and candy production as well as for inks for printings on food wrappers (Curme and Johnston, 1953). The glycol is a preservative which prevents fermentation and mold growth in syrups (Heine *et al.*, 1950; Gray and Soa, 1956; Selenka, 1963; Rae, 1948, 1951). 1,2-Propanediol is a good humectant to keep packaged foods moist and crisp. It has been extensively employed in the pharmaceutical industry as a solvent for drugs, as a stabilizer for vitamins, and in pastes for medicinal purposes (Heine *et al.*, 1950). 1,2-PD is introduced as a preservative, humectant, spreader, and emollient in cosmetics. The glycol was used for therapeutic treatment of ketosis in cattle (Johnson 1954).

The chemical and biochemical aspects of 1,2-propanediol were reviewed by Curme and Johnston (1953) and Miller and Bazzano (1965), respectively. However, for those employing 1,2-propanediol in biological systems, information of a wider spectrum is required. This paper reviews the information on the toxicity, metabolism, and biochemistry of 1,2-propanediol.

TOXICITY OF 1,2-PROPANEDIOL

To present the LD50 values of 1,2-PD, it was necessary, especially with the investigations before Laug *et al.* (1939), to choose that percent mortality which most closely approximated a LD50. Variations were noted, but there was agreement among the investigators in some instances. Where there were differences, they were attributable to the selection of the number of animals used, the doses selected, and the percent mortality chosen as the designated minimum fatal dose (MFD), minimum lethal dose (MLD) or fatal limiting dose (Laug *et al.*, 1939). Laug *et al.* (1939) discussed these points very well and pointed out that there were only two important factors; the number of animals used and the doses selected. Since the investigators employed a sufficient

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total number of animals, only the number of animals used in a dose group as well as the percent mortality for that dose group were shown in Table 1.

Although described and synthesized by Charles Wurtz in 1859 (Heine *et al.*, 1950), 1,2-propanediol (propylene glycol, 1,2-dihydroxy propane was apparently introduced in 1932 as a replacement solvent for the more toxic ethylene glycol (Hanzlik *et al.*, 1939; Mulinos *et al.*, 1943; Gessner *et al.*, 1961; Gershoff and Andrews, 1962; Coen and Weiss, 1966) for agents used for the treatment of syphilis and neurosyphilis (Seidenfeld and Hanzlik, 1932).

TABLE 1

LD50 OF 1,2-PROPANEDIOL

Species	Route	LD50[a]	Reference
Rat	po	30.0 (5) (40%)[b]	Weatherby and Haag (1938)
		21.0 (10)	Laug *et al.* (1939)
		28.0 (5)	Thomas *et al.* (1949)
		28.8 (?)	Merck Index (1969)
	im	15.0 (5) (60%)	Braun and Cartland (1936)
		13.0 (10) (40%)	Seidenfeld and Hanzlik (1932)
		20.0 (5)	Thomas *et al.* (1949)
		14.0 (5) (60%)	Weatherby and Haag (1938)
	sc	22.0 (5) (60%)	Braun and Cartland (1936)
		25.0 (5) (80%)	Weatherby and Haag (1938)
		28.0 (5)	Thomas *et al.* (1949)
	iv	7.0 (5) (80%)	Weatherby and Haag (1938)
		15.0 (10) (40%)	Seidenfeld and Hanzlik (1932)
	ip	13.0 (5)	Thomas *et al.* (1949)
Mouse	po	23.9 (20)	Laug *et al.* (1939)
	iv	4.8 (?)	Lehmann and Flury (1943)
	ip	10.9 (10)	Davis and Jenner (1959)
Rabbit	po	18.0 (9) (33%)	Braun and Cartland (1936)
		19.0 (7) (86%)	Braun and Cartland (1936)
	im	6.0 (3) (33%)	Seidenfeld and Hanzlik (1932)
	iv	5.0 (3) (66%)	Seidenfeld and Hanzlik (1932)
		7.0 (5) (80%)	Weatherby and Haag (1938)
Guinea pig	po	18.9 (10)	Laug *et al.* (1939)
Dog	po	20.0 (?)	Laug *et al.* (1939)

[a] Expressed as mg per kg body weight. Number of animals in dose group is given in parentheses.
[b] Percent selected to represent LD50.

Toxicity studies by Seidenfeld and Hanzlik (1932) identified an iv LD50 of 13.0 ml/kg and 6.0 ml/kg for rats and rabbits, respectively. Intramuscular administration showed a LD50 of 15.0 ml/kg for rats and 5.0 ml/kg for rabbits.

Weatherby and Haag (1938), in an extensive study, paid closer attention to iv injections and route of administration and confirmed the above values for im injections to rats (14.0 ml/kg) and iv administration to rabbits (7.0 ml/kg). They suggested a LD50 value for iv injections to rats of 7.0 ml/kg based on more precise injections into the vein. It was also reported that the po LD50 for rats was 30.0 ml/kg, while sc it was 25.0 ml/kg. The study by Braun and Cartland (1936) showed the following LD50 values for

rats; im, 13.0 ml/kg; sc, 22.0 ml/kg. Oral dosing in rabbits gave 2 values which showed wide variation, 18.0 and 19.0 ml/kg, probably due to the number of animals in each dose group (Table 1) (Braun and Cartland, 1936).

With the work of Laug *et al.* (1939), the standard LD50 appeared replacing such vague terms as MFD and MLD. They showed the following po LD50 values for the rat, mouse, and guinea pig: 21.0, 23.9, and 18.9 ml/kg, respectively. Thomas *et al.* (1949) reported doses of 28.0 ml/kg for po, 20.0 ml/kg for im, 28.0 ml/kg for sc, and 13.0 ml/kg for ip LD50 of 1,2-propanediol to rats. The Merck Index stated that the po LD50 for the rat was 28.8 ml/kg (Merck Index, 1968).

Lehmann and Flury (1943) showed an iv LD50 of 4.8 ml/kg for mice. The ip dose killing 50% of the mice was reported as 10.9 ml/kg (Davis and Jenner, 1959). The use of 1,2-PD as a carrier solvent in experiments with mice was contraindicated (Lampe and Easterday, 1953), since it was decided that the glycol produced unfavorable results in this species.

Chronic toxicity data showed that the glycol produced no deleterious effects when given in small amounts. Rats that drank a 10% glycol solution in lieu of water demonstrated no pathologic changes in the kidneys, heart, spleen, or liver after 24 wk (Seidenfeld and Hanzlik, 1932). Weatherby and Haag (1938) observed that at this same concentration the rate of growth was slower for the first 10 days but returned to normal thereafter for their 100-day study.

Daily po dosing of 8.0 ml/kg to rabbits for 50 days showed no demonstrable cumulative effects (Braun and Cartland, 1936).

Substitution of the carbohydrate portion of a diet in rats was tested by Hanzlik *et al.* (1939). Equal parts of carbohydrate and the glycol resulted in a loss of body weight in the tested animals after 5.5 mo. A 3/4 replacement of the carbohydrate in the diet resulted in death after 14 wk, whereas complete replacement by the glycol caused death within 4 wk. Loss of weight and mortality was attributed to rejection of the experimental diet by the rats. Drinking water containing up to 10% 1,2-propanediol produced no adverse effects during the course of a 24-wk experiment which confirmed Seidenfeld and Hanzlik (1932).

Bayley *et al.* (1967) showed that 8% of the dietary corn starch for young chickens could be replaced by 1,2-propanediol without detriment. Their experiment started with day-old chicks and ended when the birds were 26 days old.

The observance of hematuria in rats after a sublethal dose of PG suggested a possible hemolytic effect (Braun and Cartland, 1936). In vitro studies of aqueous solutions of the glycol have subsequently confirmed that hemolysis did occur (Cadwallader, 1963). The hemolysis in vitro is prevented in preparations of 1–30% 1,2-propanediol prepared with 0.9% sodium chloride.

While employing 1,2-propanediol in equal proportions with distilled water to eliminate erythrocytes for leukocyte counts, Mallery and Randolph (1944) and Randolph and Mallery (1944) observed that erythrocytes passed through three stages. First, the test tubes possessed a turbid phase, followed by a transparent phase and, finally, a redevelopment of turbidity due to precipitate formation. Microscopic observations revealed that the final turbidity was due to agglutination of red blood cells. The erythrocytes eventually hemolyzed. The hemolysis occurred in concentrations greater than 30% 1,2-propanediol in water.

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The hemolytic effect of 1,2-propanediol could be eliminated in vivo by preparation of not greater than 30% of the glycol with 0.9% sodium chloride or water. Brittain and D'Aray (1962) have demonstrated in the rabbit that iv injections of 12.5, 25.0, or 50.0% 1,2-propanediol in normal saline did not affect red blood cells, hemoglobin concentration, or total white blood cell counts.

1,2-PD was observed to have a variable toxicity for fertile chicken eggs, depending on the method of injection. Application of 0.05 ml/egg of 1,2-propanediol to the air sac membrane from 0 to 7 days of incubation showed an increasing percent mortality from day 2 (18%), peaking to 90% on day 4, then dropping after day 4 to 35% on day 7 (Gebhardt, 1968). Injection into the yolk sac with the same volume of 1,2-PD gave 45% mortality prior to incubation, and 14% mortality on the first day of incubation followed by values to day 4 which were within the range of controls (Gebhardt, 1968).

No system or organ has been established as the target of the lethal effect of high doses. Histologic investigations failed to show damage to the kidneys, heart, spleen, or liver in rats and rabbits (Seidenfeld and Hanzlik, 1932; Weatherby and Haag, 1938; Newman *et al.*, 1940). Laug *et al.* (1939) noted that shortly after administration of large doses, animals showed signs of loss of equilibrium, marked depression, analgesia, coma, and finally death. Microscopic observation revealed nuclear pycnosis, vacuolar degeneration of the cytoplasm, and protein debris or loose casts in the cortical tubules of the kidney. The liver showed slight congestion and hyperemia with no fatty changes. The ependymal cells of the central nervous system, not mentioned in these investigations, were severely damaged in chick embryos (Ruddick and LaHam, 1972).

Selenka (1963) attributed the toxicity of 1,2-propanediol in bacteria to inhibition of a specific enzyme. The enzyme was not specified.

The low toxicity in adult animals makes 1,2-propanediol acceptable as a solvent in experiments and for pharmaceuticals. Daily quantities for 24 wk of 12.8 ml/kg body weight of absolute 1,2-propanediol produced no pathologic changes in kidneys, heart, spleen, and liver in rats and rabbits (Seidenfeld and Hanzlik, 1932). This is equivalent to 895.2 ml (or 1.8 lb) daily for a 70 kg man. There appear to be no data on the daily amount consumed by man. It is highly improbable that man would approach a daily amount of 1.8 lb unless he consumed pure 1,2-propanediol daily.

METABOLISM OF 1,2-PROPANEDIOL

The intermediate oxidation products of 1,2-propanediol, lactic and pyruvic acids, suggested use as an energy source and a glycogenic capability for the glycol (Hanzlik *et al.*, 1939). 1,2-Propanediol has served as a nutrient for dogs, cattle, rats, and chickens.

Dogs force fed 8.0 ml/kg and 12.0 ml/kg of a 50% aqueous solution of 1,2-propanediol showed concentrations in the blood of 9.0 mg/ml in 4 hr and 13.0 mg/ml in 2 hr (Lehman and Newman, 1937). Recovery of 12–45% of the unchanged administered dose in the urine indicated not only that the compound was eliminated by the kidney, but also that a large proportion can be metabolized. Lehman and Newman (1937) pointed out that high blood levels of the glycol failed to cause a corresponding increase in the rate of its metabolism. That is, the enzymatic systems involved were saturated and a maximum conversion rate had been achieved.

Waldo and Schultz (1960) demonstrated an increase in blood sugars in steers fed 1,2-propanediol.

Perfusion experiments with cat livers have shown that 1,2-propanediol administration alters the glycogen levels (Newman *et al.*, 1940). During the 2-hr infusion period, a toxic concentration (500–600 mg/100 ml) increased the glycogen content, and a nontoxic concentration (100–200 mg/100 ml) maintained a constant level of glycogen in the liver while the levels in the controls decreased. The observed elevation in blood lactate accompanied by a decrease in oxygen consumption during 1,2-propanediol perfusion was attributed to the oxidation of 1,2-propanediol to lactate. If 1,2-propanediol and dextrose are compared in the production of 1 calorie of energy, less oxygen is required by 1,2-propanediol for oxidation, accounting for the decreased oxygen consumption. Insulin added to the perfusion fluid prevented the increase in the blood lactate, and stimulated glycol oxidation. It was suggested that insulin stimulated the removal of lactic acid to shift the chemical reaction toward 1,2-propanediol oxidation.

Thus 1,2-propanediol does contribute to glycogen formation and can be used as a nutrient as 10% PG in the drinking water of rats (Hanzlik *et al.*, 1939), or as an 8% replacement of the corn starch for young chickens (Bayley *et al.*, 1967).

BIOCHEMICAL STUDIES

The oxidation of 1,2-propanediol to lactic acid or pyruvic acid appears to follow 1 of 2 pathways depending on whether the substrate is the free glycol or phosphorylated glycol (Rudney, 1954b; Miller and Bazzano, 1965). Free glycol takes the following route: lactaldehyde, methylglyoxal, and lactic acid (Eq. 1), while the phosphorylated glycol passes through acetol phosphate, lactaldehyde phosphate, and lactyl phosphate to lactic acid (Eq. 2). The evidence to be presented conforms to that stated above, but 1,2-propanediol conversion to propionaldehyde, 1 and 2 carbon units, or the corresponding deoxaldehyde also results.

$$\underset{\text{1,2-PD}}{CH_3CH(OH)CH_2OH} \xrightleftharpoons{-2H^+} \underset{\text{lactaldehyde}}{CH_3CH(OH)CHO} \xrightarrow{-2H^+} \underset{\text{methylglyoxal}}{CH_3C(=O)CHO} \xrightarrow{+H_2O} \underset{\text{lactic acid}}{CH_3CH(OH)COOH} \quad (1)$$

$$\underset{\text{1,2-PD phosphate}}{CH_3CH(OH)CH_2OPO_3H_2} \xrightleftharpoons{-H^+} \underset{\text{acetol phosphate}}{CH_3C(=O)CH_2OPO_3H_2} \xrightarrow{+H_2O} \underset{\text{lactaldehyde phosphate}}{CH_3CH(OH)CH(OH)OPO_3H_2} \xrightarrow{-2H}$$

$$\underset{\text{lactyl phosphate}}{CH_3CH(OH)C(=O)OPO_3H_2} \xrightarrow{-PO_3} \underset{\text{lactic acid}}{CH_3CH(OH)COOH} \quad (2)$$

Rudney (1954a, b) isolated the naturally occurring phosphorylated glycol from rat liver, and Linberg (Miller and Bazzano, 1965) extracted this glycol from sea urchin eggs and beef brain. By isotope dilution, Rudney (1954a) determined that the level of

propanediol phosphate represents 1–2% of the total acid-soluble phosphorus in the rat liver.

1,2-propanediol or its phosphorylated derivative was a suggested (Rudney 1950; Sakami, 1950a, b; Sakami and Welch, 1950) intermediate in the metabolism of acetone. The end products were "formate," "acetate," or a 3-carbon (pyruvate, lactate) precursor of the tricarboxylic acid cycle. A radioactive label on the methyl carbons of acetone was transferred to the β-carbon of serine and the methyl groups of choline and methionine (Sakami, 1950a, b). The analysis of liver glycogen from rats showed that the extracted glucose was labeled in carbons 3 and 4. The pattern of label suggested that a portion of the labeled acetone may have been converted to lactate. The activity in carbons 1 and 6 of glucose was much greater than in carbons 2 and 5, further suggesting direct conversion of acetone to 3-carbon intermediates (Eq. 3).

$$\underset{\text{acetone}}{CH_3C(=O)CH_3} \underset{}{\overset{+O}{\rightleftharpoons}} \underset{\text{acetol}}{CH_3C(=O)CH_2OH} \overset{+2H}{\rightleftharpoons} \underset{\text{1,2-PD}}{CH_3CH(OH)CH_2OH} \rightarrow \begin{cases} \text{"formate" and "acetate"} \\ \text{3-carbon product} \rightarrow \text{glucose} \end{cases} \quad (3)$$

Sakami and Lafaye (1951) labeled the carbonyl group of acetone with ^{14}C to determine whether the glycol was the intermediate and that the acetone was not metabolized by carboxylation to acetoacetone or cleaved to "acetate" and "formate" to give the observed end product labeling. They found that glucose possessed more activity in the 2 and 5 carbons than in the 1 and 6 carbons, confirming that a direct oxidation to a 3-carbon intermediate was more likely. 1,2-Propanediol in a lactating cow was converted in the liver to glucose by pyruvate carboxylation (Emery *et al.*, 1967).

The 2 possibilities for 1- and 2-carbon units or a 3-carbon product (lactate or pyruvate) in the metabolism of acetone was observed for 2 microorganisms which use 1,2-propanediol as the carbon source (Taylor, 1960). A pseudomonad type oxidized 1,2-propanediol to acetol, which then was metabolized to a 1-carbon and a 2-carbon unit, while a diphtheroid type attacked the primary alcohol in the following manner:

$$\underset{\text{1,2-PD}}{CH_3CH(OH)CH_2OH} \xrightarrow{-2H} \underset{\text{lactaldehyde}}{CH_3CH(OH)CHO} \xrightarrow{H_2O} \underset{\text{lactate}}{CH_3CH(OH)COOH} + H_2 \xrightarrow{-2H} \underset{\text{pyruvate}}{CH_3C(=O)COOH}$$

Goepfert (1940) observed that 1,2-propanediol was dehydrogenated to acetol by the mold *Fusarium lini* Bolley (Eq. 3).

1,2-Propanediol 1-phosphate stimulated keto acid production (presumably pyruvate) and oxygen consumption in rat liver homogenates, whereas 1,2-propanediol did not (Miller *et al.*, 1953). A schematic breakdown of the phosphorylated glycol was presented. In the presence of myogen-A and DPN, the phosphorylated glycol was oxidized to acetol phosphate (Eq. 4). This step was also confirmed by Huff and Rudney (1959).

$$\underset{\text{1,2-PD phosphate}}{CH_3CH(OH)CH_2OPO_3H_2} + DPN^+ \longrightarrow \underset{\text{acetol phosphate}}{CH_3C(=O)CH_2OPO_3H_2} + DPNH + H^+ \quad (4)$$

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Page 98 of 127

The acetol phosphate proceeds to lactaldehyde phosphate with the addition of triose isomerase (Eq. 5).

$$\underset{\text{acetol phosphate}}{CH_3\overset{O}{\overset{\|}{C}}CH_2OPO_3H_2} \xrightarrow{\text{isomerase}} \underset{\text{lactaldehyde phosphate}}{CH_3\underset{OH}{CH}-\underset{OH}{CH}OPO_3H_2} \qquad (5)$$

The lactaldehyde phosphate was further oxidized by D-glyceraldehyde-3-phosphate dehydrogenase to lactyl phosphate (Eq. 6):

$$\underset{\text{lactaldehyde phosphate}}{CH_3\underset{OH}{CH}-\underset{OH}{CH}OPO_3H_2} + DPN^+ \longrightarrow \underset{\text{lactyl phosphate}}{CH_3\underset{OH}{CH}\overset{O}{\overset{\|}{C}}OPO_3H_2} + DPNH + H^+ \qquad (6)$$

Sellinger and Miller (1959) corrected the reaction in Eq. 4 where α-glycerol phosphate dehydrogenase present in the rabbit muscle extract was implicated for the reduction of acetol phosphate to 1,2-propanediol 1-phosphate. The conversion was actually due to a specific enzyme, 1,2-propanediol-1-phosphate dehydrogenase, in the original mixture. They have also shown that rat liver and kidney homogenates form acetol phosphate from acetol and ATP by means of an enzyme distinct from glycerokinase. The naturally occurring phosphorylated glycol is formed as follows:

$$\underset{\text{acetol}}{CH_3\underset{O}{\underset{\|}{C}}CH_2OH} + ATP \xrightarrow{\text{acetol kinase}} \underset{\text{acetol phosphate}}{CH_3\underset{O}{\underset{\|}{C}}CH_2OPO_3H_2} + ADP$$

$$\underset{\text{acetol phosphate}}{CH_3\underset{O}{\underset{\|}{C}}CH_2OPO_3H_2} + DPNH + H^+ \rightleftharpoons \underset{\text{1,2-PD phosphate}}{CH_3\underset{OH}{CH}CH_2OPO_3H_2} + DPN^+$$

The biochemical conversion between 1,2-propanediol and pyruvate was further illustrated by Groth and Lepage (1954). Working with normal and neoplastic rat tissue under anaerobic conditions and using a fluoride block, they demonstrated that pyruvate can be converted to 1,2-propanediol 1-phosphate. Also, bakers' yeast metabolized 1,2-propanediol and 1,2-propanediol 1-phosphate either through acetol or acetol phosphate to lactic acid (Huggins and Miller, 1956).

Acetol and DL-lactaldehyde, 2 proposed intermediates of the propanediol-lactate pathway, are glycogenic in fasting mice (Shull and Miller, 1960); one of the intermediate products has been identified as 1,2-propanediol 1-phosphate (Shu-Mei *et al.*, 1964).

The metabolism of 1,2-propanediol does not always produce lactic acid which subsequently leads to pyruvic acid. With a cell-free extract of *Aerobacter aerogenes* and a cobamide coenzyme, Abeles and Lee (1961) and Lee and Abeles (1963) showed that an isolated dioldehydrase oxidized 1,2-propanediol to propionaldehyde.

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$$\underset{\text{1,2-PD}}{CH_3\underset{OH}{\underset{|}{C}}HCH_2OH} \xrightarrow[\text{cobamide}]{\text{dioldehydrase}} \underset{\text{propionaldehyde}}{CH_3CH_2CHO} + H_2$$

In this reaction, the hydrogen from the C-1 of 1,2-propanediol is transferred to the adenosyl moiety at the C-5′ position of the cobamide, and from this position to the C-2 position of the 1,2-propanediol (Frey and Abeles, 1966; and Frey *et al.*, 1967).

An enzymatic system from a different strain of *Aerobacter aerogenes* converts 1,2-diols into the corresponding deoxyaldehydes (Pawelkiewicz and Zagalak, 1964).

Velle and Engel (1964) have isolated an enzyme from bovine placenta and seminal vesicles which is stereospecific for the D form of 1,2-propanediol. The oxidation product is lactaldehyde.

Baer *et al.* (1968) have demonstrated by in vitro studies that phospholipids, which are analogs of α-lecithins and α-cephalins, can be synthesized from 1,2-propanediol. The D-propyleneglycol-α-phosphorylcholine is very hemolytic whereas the D-propyleneglycol-α-phosphorylethanolamine is not.

Thus, the biochemical oxidation of 1,2-propanediol appears to follow 2 pathways (Rudney, 1954b; Miller and Bazzano, 1965). The free glycol is oxidized through lactaldehyde, methylglyoxal, and lactic acid, whereas the phosphorylated glycol passes through acetol phosphate, lactaldehyde phosphate, and lactyl phosphate to lactic acid. 1,2-Propanediol oxidation is not restricted to lactate or pyruvate formation. The corresponding deoxyaldehyde, as well as propionaldehyde, lactaldehyde, phospholipids, formate, and acetate can be formed.

In summary, 1,2-propanediol with its diverse applications possesses a low toxicity. Acute and chronic studies showed that PG can be employed within limits as a solvent in toxicology. The hemolysis of red blood cells following iv injections can be eliminated for in vivo work by preparations of up to 30% 1,2-PD in 0.9% sodium chloride. Biochemically, oxidation of 1,2-propanediol is mainly to lactic or pyruvic acid through 1 of 2 pathways depending on whether the glycol is present as the free or phosphorylated form. Once present as lactate or pyruvate, 1,2-PD can provide energy by further oxidation through the tricarboxylic acid cycle or contribute to glycogen formation through the glycolytic pathway.

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Characterization of turkey pancreatic lipase

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Abstract — Turkey pancreatic lipase (TPL) was purified from delipidated pancreases. Pure TPL (glycerol ester hydrolase, EC 3.1.1.3) was obtained after ammonium sulfate fractionation, Sephacryl S-200 gel filtration, anion exchange chromatography (DEAE-Sepharose) and size exclusion column using high performance liquid chromatography system (HPLC). The pure lipase, which is not a glycoprotein, was presented as a monomer having a molecular mass of about 45 kDa. The lipase activity was maximal at pH 8.5 and 37 °C. TPL hydrolyses the long chains triacylglycerols more efficiently than the short ones. A specific activity of 4300 U/mg was measured on triolein as substrate at 37 °C and at pH 8.5 in the presence of colipase and 4 mM NaTDC. This enzyme presents the interfacial activation when using tripropionin as substrate. TPL was inactivated when the enzyme was incubated at 65 °C or at pH less than 5. Natural detergent (NaTDC), synthetic detergent (Tween-20) or amphipatic protein (β-lactoglobulin A) act as potent inhibitors of TPL activity. To restore the lipase activity inhibited by NaTDC, colipase should be added to the hydrolysis system. When lipase is inhibited by synthetic detergent or protein, simultaneous addition of colipase and NaTDC was required to restore the TPL activity. The first 22 N-terminal amino acid residues were sequenced. This sequence was similar to those of mammal's pancreatic lipases. The biochemical properties of pancreatic lipase isolated from bird are similar to those of mammals. © 2000 Société française de biochimie et biologie moléculaire / Éditions scientifiques et médicales Elsevier SAS

pancreatic lipase / colipase / amphiphiles / inhibition / reactivation

1. Introduction

The hydrolysis of dietary triacylglycerol in mammals is catalyzed by the major lipases of the digestive tract: preduodenal and pancreatic lipases. In man, the first step of hydrolysis of dietary lipids begins in the stomach and is catalyzed by the acid-stable lipase present in gastric juice [1].

In 1981, the first amino acid sequence of porcine pancreatic lipase (PPL) was established by De Caro et al. [2]. Over the past few years, the amino acid sequences of some pancreatic lipases have been deduced from the corresponding cDNA in the case of several species, including man [3, 4], dog [5], guinea pig [6, 7], rat [8, 9], coypu [10, 11], and rabbit [12]. On the basis of the primary structure comparison, the pancreatic lipase family can be subdivided into three subgroups: i) classical pancreatic lipases; ii) pancreatic lipase-related protein 1 (PLRP1); and iii) pancreatic lipase-related protein 2 (PLRP2) [4, 10]. It is well known that in the guinea pig [6], the coypu [10], and the rat [9], PLRP2 displays different kinetic properties from those of the classical lipases.

Other kinetic studies performed with emulsion of triacylglycerols as lipase substrate have shown that other amphiphiles such as synthetic detergents [13, 14] or proteins [15, 16] are also inhibitors of pancreatic lipase.

The aim of this study is to compare some biochemical properties of a bird pancreatic lipase with mammal's pancreatic lipases. To allow this comparison, we proposed to purify to homogeneity TPL in order to study the effect of amphiphiles and the kinetics properties of the pure enzyme. The N-terminal sequence of TPL was determined and compared to that of mammals.

2. Materials and methods

2.1. Materials

Tributyrin (99%; puriss), benzamidine and 4-methyl morpholine were from Fluka (Buchs, Switzerland); tripropionin (99%, GC) was from Janssen Chimica (Geel, Belgium), sodium taurodeoxycholate (NaTDC) and triacetin (99%; puriss) were from Sigma Chemical (St. Louis, USA); gum arabic was from Mayaud Baker LTD (Dagen-

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* Correspondence and reprints

Abbreviations: DrPL, dromedary pancreatic lipase; HPL, human pancreatic lipase; PPL, porcine pancreatic lipase; TPL, turkey pancreatic lipase; BSA, bovine serum albumin; CMC, critical micellar concentration; GA, gum arabic; NaDC, sodium deoxycholate; NaTDC, sodium taurodeoxycholate; OO, olive oil; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TC_2, triacetin; TC_3, tripropionin; TC_4, tributyrin; TX-100, Triton X-100.

ham, UK); acrylamide and electrophoresis grade, were from BDH (Poole, UK); marker proteins and supports of chromatography used for TPL purification: Sephacryl S-200 and DEAE-Sepharose gel were from Pharmacia (Uppsala, Sweden); PVDF membrane was purchased from Applied Biosystems (Roissy, France); trans blot cell apparatus was from Bio-Rad (Paris, France); HPLC column protein Pak 300 SW was from Waters (St. Quentin, France). All chemicals used on HPLC column were of analytical grade from Merck (Darmstadt, Germany); pH-stat was from Metrohm (Switzerland).

2.2. *Pancreas collections*

Pancreases from different species were collected immediately after death from the local slaughterhouse of Sfax or Sidi Salem (Tunisia). A stock of turkey pancreases was kept at –20 °C before delipidation.

2.3. *Delipidation of turkey pancreases*

After decongelation, the turkey pancreases were cut into small pieces (1–2 cm^3) and delipidated according to the method described previously [17].

After delipidation, 20 g of delipidated powder of turkey pancreases were obtained from 100 g of fresh tissue.

2.4. *Enzymes and proteins*

Bovine serum albumin fraction IV (BSA) and β-lactoglobulin A were from Sigma (St. Louis, USA). Crude porcine colipase was obtained after homogenization at pH 3.0 of delipidated powder of porcine pancreases (1 g/10 mL) (Laboratoire Industriel de Biologie, France) followed by a centrifugation at 10 000 rpm for 10 min.

The supernatant, which contains all the colipase but no lipase activity, was adjusted to pH 6.0. After centrifugation at 10 000 rpm for 10 min, colipase sample was stored at –20 °C. The specific activity measured under standard conditions [18] is 350 U/mg. In some cases, pure porcine pancreatic colipase from Boehringer (Mannheim, France) was used (specific activity under standard conditions is 10 000 U/mg). No significant difference was observed when pure TPL activity was measured in the presence of crude or pure porcine colipase (data not shown).

2.5. *Lipase and colipase activity determination*

The lipase activity was measured titrimetrically at pH 8.5 and 37 °C with a pH-stat, under the standard assay conditions described previously, using tributyrin (0.25 mL) or liprocil (0.25 mL) in 30 mL of 2.5 mM Tris-HCl, pH 8.5, 100 mM NaCl, 5 mM $CaCl_2$ [19] or olive oil emulsion [18] as substrate. Some lipase assays were performed in the presence of NaTDC, colipase, TX-100 or β-lactoglobulin A.

Lipase activity was also measured using TC_2 or TC_3 as substrate according to Ferrato et al. [20]. One lipase unit corresponds to 1 µmol of fatty acid liberated per min.

The colipase activity was measured at pH 8.5 and 37 °C using a pH-stat. Assays were performed with olive oil emulsion as substrate in the presence of 6 mM NaTDC. The level of colipase of all pancreases was measured using an homogenate prepared in the same conditions described by Rathelot et al. [18]. One colipase unit is the amount of cofactor that increases bile salt inhibited pancreatic lipase activity by one enzyme unit [18].

2.6. *Determination of protein concentration*

Protein concentration was determined as described by Bradford et al. [21].

2.7. *Analytical methods*

Analytical polyacrylamide gel electrophoresis of proteins in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed by the method of Laemmli [22]. Samples for sequencing were electroblotted according to Bergman and Jörnvall [23]. Protein transfer was performed during 1 h at 1 mA/cm^2 at room temperature.

2.8. *Sugar content*

Total sugar of the purified TPL was measured by anthrone-sulfuric acid method using glucose as a standard [24].

2.9. *Amino acid sequencing*

The N-terminal of TPL was sequenced by automated Edman's degradation, using an Applied Biosystems 470A protein sequencer equipped with PTH 120A Analyser [25]. The sequence has kindly been determined by Dr. Reinbolt (IBMC, UPR 9002 du CNRS, Strasbourg, France).

3. Results and discussion

3.1. *The level of pancreatic lipase and colipase in some animals*

In order to compare the level of TPL and colipase activities with other species, we measured, under the same conditions, the rate of hydrolysis of olive oil emulsion or tributyrin by bovine, chicken, dromedary, and sheep pancreatic lipases. Results reported in *table I* show a large variation in pancreatic lipases and colipases levels between species. For all species, except turkey, the TC_4 was hydrolyzed more efficiently than the olive oil. The ratio short/long chains is about 2. In the case of turkey, the

Table I. Pancreatic lipase and colipase levels in some animals.

Species	*Lipase activity (U/g of fresh pancreases)*		*Colipase activity (U/g of fresh pancreases)*
	Olive oil	*TC_4*	
Turkey	14 600 ± 2 400	5 700 ± 274	3 000 ± 316
Chicken	3 350 ± 1 313	7 375 ± 2358	2 375 ± 530
Bovine	2 180 ± 790	3 940 ± 1 392	1 080 ± 421
Sheep	2 620 ± 1 602	5 300 ± 908	1 600 ± 300
Dromedary	581 ± 73	1 051 ± 135	517 ± 85

The determination of the lipase and colipase content of pancreases from all species was performed in an homogenate prepared in a Waring Blendor (2 × 30 s) with 10 mL of 10 mM Tris-HCl, pH 8, 2 mM benzamidine per gram of fresh tissue. After centrifugation at 10 000 rpm for 15 min, the amount of enzyme was estimated on an aliquot of the supernatant using olive oil emulsion or tributyrin (0.25 mL of tributyrin in 30 mL of 2.5 mM Tris-HCl, pH 8.5, 100 mM NaCl, 5 mM $CaCl_2$) as substrate in the presence of a molar excess of colipase (colipase to lipase molar ratio = 5) and 4 mM NaTDC. The lipase and colipase activities were measured trimetrically at pH 8.5 and 37 °C using a pH-stat. One lipase unit corresponds to 1 µmol of fatty acid liberated per min. One colipase unit corresponds to the amount of cofactor that increases bile salt inhibited pancreatic lipase activity by one enzyme unit. For each species, the activities represent the average ± standard error mean of five assays obtained from five different pancreases.

ratio short/long chains is about 0.5. When olive oil emulsion was used as substrate, TPL presented the highest level and about 16 000 U/g of fresh turkey pancreases were detected. The maximal ratio in lipase units per gram of different pancreases was observed between turkey and dromedary, more than 27-fold. *Table I* shows that the ratio lipase/colipase is about 1.5 for chicken, bovine, sheep and dromedary. This ratio reaches 5 in the case of turkey.

3.2. *Purification of TPL*

5 g of delipidated powder of turkey pancreases were suspended in 55 mL of buffer A: 25 mM Tris-HCl, pH 8.2, 2 mM benzamidine, 25 mM NaCl, and mixed mechanically twice for 30 s at 4 °C using the Waring Blendor system. The mixture was then stirred with a magnetic bar for 60 min at 4 °C, and then centrifuged for 20 min at 10 000 rpm.

3.2.1. *Ammonium sulfate precipitation*

The supernatant (50 mL) was brought to 60% saturation with solid ammonium sulfate (19.5 g) under stirring conditions and maintained for 60 min at 4 °C. After centrifugation (30 min at 10 000 rpm), the precipitate was resuspended in 15 mL of buffer A. Insoluble material was removed by centrifugation for 10 min at 10 000 rpm.

3.2.2. *Filtration on Sephacryl S-200*

The supernatant (15 mL) was loaded on a column (3 × 100 cm) of gel filtration Sephacryl S-200 equilibrated with buffer A. Elution of lipase was performed with buffer A at 40 mL/h. The fractions containing the lipase activity (eluted at 1.4 void volumes) were pooled.

3.2.3. *Anion exchange chromatography*

The pooled fractions of Sephacryl S-200 column were added to the DEAE-Sepharose anion exchanger equilibrated in buffer A. The column (1.6 × 20 cm) was rinsed with 400 mL of buffer A. No lipase activity was detected in the washing flow. Proteins were eluted by a linear NaCl gradient (500 mL of 25 to 250 mM in buffer A). The protein elution profile obtained is shown in *figure 1A*. TPL activity was eluted between 80 and 100 mM NaCl. The fractions containing the lipase activity were pooled and lyophilized.

The results of SDS-PAGE analysis of the pooled fraction of this DEAE-Sepharose chromatography are given in *figure 1B* and showed that TPL was contaminated by two proteins with molecular masses of 25 and 30 kDa. To remove these contaminants, the lyophilized proteins were resuspended in 5 mL phosphate buffer, pH 7. 100 µg of this enzyme solution was loaded on size exclusion HPLC column Protein Pak 300 SW (7.8 mm × 30 cm) equilibrated in phosphate buffer.

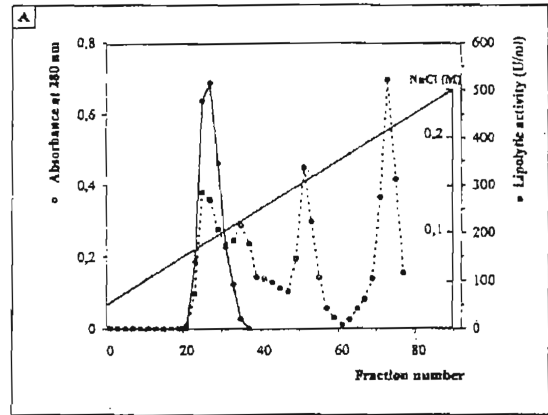
The lipase emerged 12 min after injection. Elution was performed with phosphate buffer at 0.5 mL/min. The fractions containing the TPL activity were pooled and analyzed on SDS-PAGE (*figure 1B*).

This figure shows that only one band was revealed for TPL with a molecular mass of about 45 kDa. The molecular mass of TPL estimated by gel filtration on HPLC column Protein Pak 300 SW (7.8 mm × 30 cm) was 44 kDa (data not shown).

These results (SDS-PAGE and gel filtration) suggested that TPL was a monomeric protein like all pancreatic lipases described so far [26].

The presence of sugar in TPL was tested. Our results indicate that the TPL, like bovine and ovine pancreatic lipases, is not a glycoprotein (data not shown). It differs from the two porcine lipases, which are glycoproteins [26].

The purification flow sheet is given in *table II* which shows that the specific activity of TPL reaches 4300 U/mg



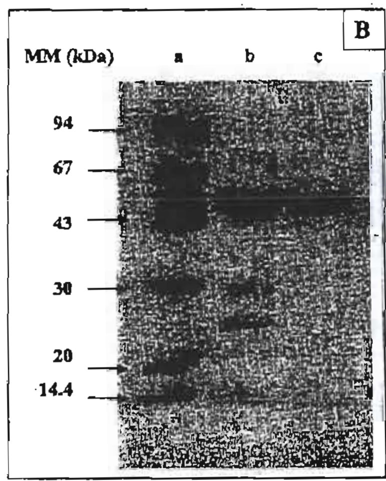


Figure 1. A. Chromatography of TPL on DEAE-Sepharose. The column (1.6 × 20 cm) was equilibrated with 25 mM Tris-HCl, pH 8.2, 2 mM benzamidine, 25 mM NaCl; a linear salt concentration gradient (25–250 mM NaCl) was applied to the column; gradient chamber 250 mL; fraction 3 mL; flow rate, 25 mL/h. The substrate was olive oil emulsion. **B.** SDS-PAGE (13%). Lane a, molecular mass markers (Pharmacia); lane b, characterization of the TPL obtained after DEAE-Sepharose chromatography (15 μg); lane c, 12 μg of purified TPL. The gel was stained with Coomassie blue.

using gum arabic emulsified olive oil as substrate in the presence of colipase and 4 mM NaTDC at pH 8.5 and 37 °C. Under the same conditions, specific activities of 109, 900, 1600 and 2600 U/mg were obtained when TC_2, TC_3, TC_4 and liprocil were used as substrates respectively. When TC_2 and TC_3 were used as substrate, the TPL activities were measured at pH 7 and 37 °C [21]. result shows that, in contrast to all mammal [26] chicken pancreatic lipases [27, 28], TPL hydrolyses efficiently the long- than the short-chain triacylglyc The ratio of TPL activity long/short chains is about

3.3. *Characterization of TPL*

3.3.1. *Activation of TPL by interface*

As has been recently shown by Ferrato et al. among the short chain triacylglycerols tested as subst (TC_2, TC_3, TC_4), TC_3 is the best system to check interfacial activation of pancreatic lipases.

In this study we have selected the TC_3 to evaluate presence of interfacial activation phenomenon in TP

The hydrolysis rate of TC_3 emulsified in 0.33% GA 0.15 M NaCl (*figure 2A*) or in 0.33% GA (*figure 2B*) function of substrate concentration shows that TPL hy lyzed very slowly the TC_3 when it is in monomeric s however, up to the solubility limit of TC_3 (12 mM) TPL activity increased dramatically to reach 900 U/m 24 mM of TC_3.

This result indicates that TPL, as PPL [20, 29], pres the interfacial activation phenomenon.

3.3.2. *Effects of pH and temperature on activity and stabilit TPL*

The maximal activity of TPL was measured at pH and 37 °C. Like all the mammal's pancreatic lipases, T was found to be stable between pH 7 and pH 9, and to l its activity at pH > 9 or pH < 5.

When the enzyme was incubated at a temperat higher than 50 °C, TPL was inactivated after few minu like all mammal's pancreatic lipases [26].

3.3.3. *Effects of bile salts on TPL activity*

The effect of varying concentrations of NaTDC on rate of hydrolysis of olive oil emulsion (*figure 3A*) tributyrin (*figure 3B*)by TPL is presented in *figure 3*. T figure shows that bile salt acts as in vitro inhibitor of T activity when TC_4 or olive oil emulsion is used substrate. In the two systems, inhibition is reversed addition of colipase even at bile salt concentrations larg exceeding their CMC [30, 31].

The effect of varying concentrations of synthetic det gents like Tween-20 (non-ionic detergent) or amphiphi proteins like β-lactoglobulin A on the rate of hydrolysis olive oil emulsion by TPL shows that the activity of T decreases rapidly at a given amphiphilic concentrati threshold.

The cofactor failed to counteract the inhibition in large range of inhibitor concentration in contrast to th observed in the case of bile salt (data not shown).

Comparable results were obtained using ionic or zw terionic detergents (data not shown). Thus the inhibiti of TPL can not be related to the charge or the structure the synthetic detergents.

Table II. Flow sheet of the TPL purification.

Purification step	*Total activity[a] (units)*	*Protein[b] (mg)*	*Specific activity (U/mg)*	*Activity recovery (%)*	*Purification factor*
Extract of TPL (pH 8.2)	120 000	3 529	34	–	1
$(NH_4)_2SO_4$ precipitation	95 000	208	456.7	79	13.43
S-200 chromatography	40 500	15.2	2 664	33.75	78.3
DEAE-Sepharose chromatography	28 000	7.4	3 783	23.3	111.2
Filtration on HPLC	17 500	4	4 364	14.58	128.3

[a]1 unit: 1 µmol of fatty acid released per min using olive oil emulsion as substrate in the presence of 4 mM NaTDC and in the presence of a molar excess of colipase (colipase to lipase molar ratio = 5).
[b]Proteins were estimated by Bradford method [27]. The experiments were conducted three times.

The addition of increasing concentrations of NaTDC to the reaction system containing TPL, colipase (colipase to lipase molar ratio 5), and inhibitory concentration of tween 20 (0.6 mM) or β-lactoglobulin A (12 µM) fully restores lipolysis (data not shown). Then bile salt shows the ability to activate lipolysis in the presence of various inhibitory amphiphiles.

These experiments confirm those described by Gargouri et al. [13, 32] which have showed that inhibition of pancreatic lipase activity by amphiphiles such as proteins or detergents appears to be a general phenomenon related to a desorption of lipase from its substrate, occurs after a change in interfacial quality.

3.3.4. N-terminal sequence of TPL

The NH_2-terminal sequencing of the blotted TPL allowed the identification of 22 residues, S-E-V-X-Y-D-R-V-G-X-F-T-D-D-I-P-W-S-G-T-A-E (where residue X was not identified). *Table III* shows the N-terminal sequence of TPL, together with those of DrPL [33],

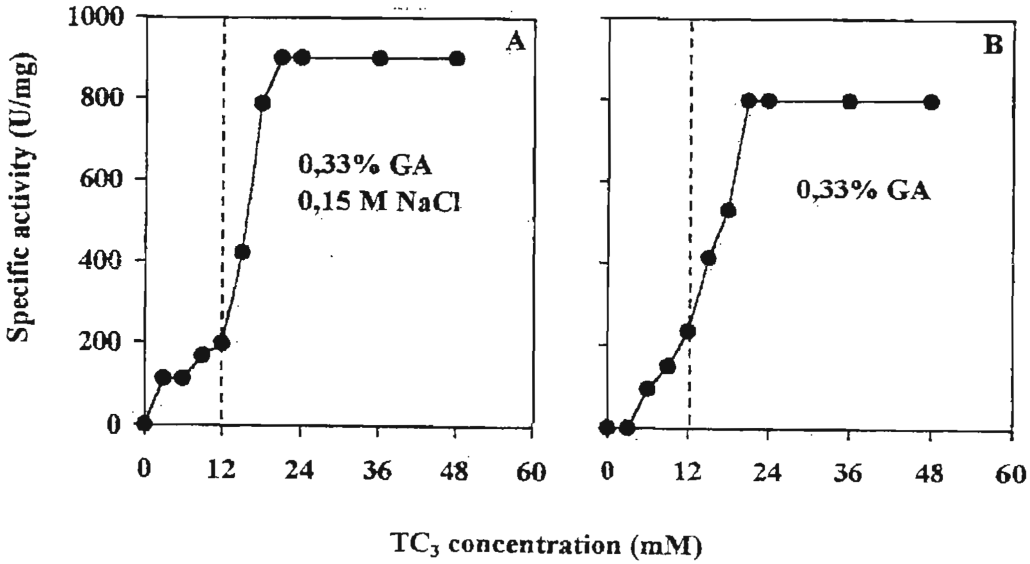
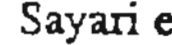
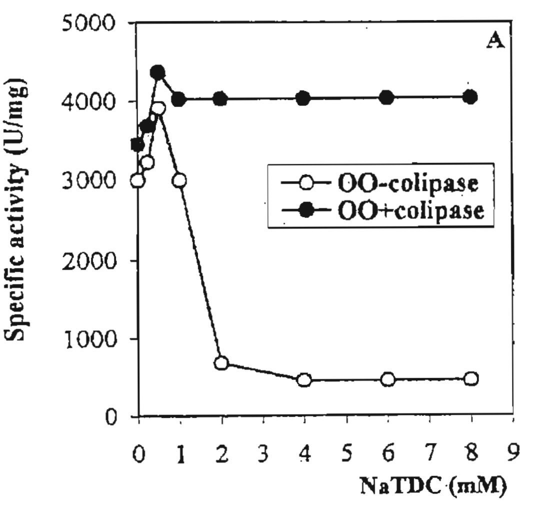


Figure 2. Hydrolysis rate of TC_3 by TPL as function of substrate concentration. The TC_3 solutions were systematically prepared by mixing (3 × 30 s in a warring blender) a given amount of TC_3 in 30 mL of 0.33% GA and 0.15 M NaCl (A), or in 30 mL of 0.33% GA (B). The release of propionic acid was recorded continuously at pH 7 and 37 °C using a pH-stat. The CMC of TC_3 (12 mM) is indicated by vertical dotted lines.





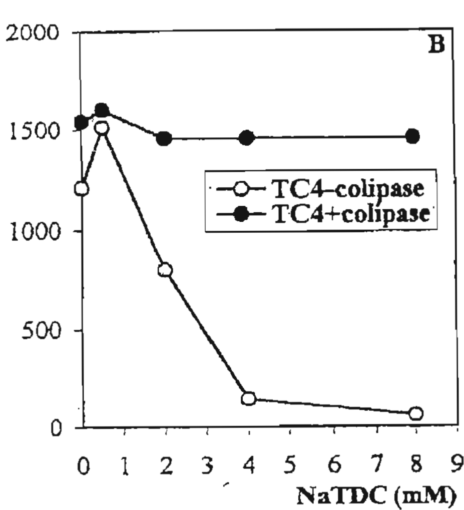


Figure 3. Effect of increasing concentration of bile salt. NaTDC on the rate of hydrolysis of olive oil emulsion (**A**) or tributyrin by TPL. Lipolytic activity was measured at pH 8.5 and 37 °C in the absence or in the presence of a molar excess of colipase (colip to lipase molar ratio = 5).

HPL [3], and PPL [2]. TPL was found to exhibit a high degree of homology with the other mammalian pancreatic lipases.

4. Conclusion

TPL was isolated to electrophoretic purity from delipidated pancreases. The pure enzyme is not a glycoprotein. It is a monomer with a molecular mass of 45 kDa. TPL hydrolyses the long chains more efficiently than the short chain triacylglycerols. It is inactivated at 60 °C and it is not stable at pH less than 5. TPL presents the interfacial activation phenomenon. Natural detergents (NaTDC, NaDC) act as strong inhibitors of TPL activity and colipase reverses this inhibition. Other amphiphiles (synthetic detergents or proteins) are inhibitors of TPL activity. In contrast to that observed in the case of bile salt, colipase failed to counteract the inhibition in a large range of inhibitor concentration. Reactivation of TPL by colipase in presence of inhibitory concentration of proteins or synthetic detergents requires the presence of bile salt.

The sequence of the N-terminal part shows that TPL highly similar to other known mammalian pancrea lipases. Thus no significant difference was observed wh the biochemical properties of the TPL were compared those of mammals.

Acknowledgments

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Table III. Sequence comparison of TPL with HPL, PPL and DrPL.

TPL	[1]S E V - Y D R V G [10]- F T D D I P W S G [20]T A E	This study
HPL	K E V C Y E R L G C F S D D S P W S G I T E	[3]
PPL	S E V C F P R L G C F S D D A P W A G I V Q	[2]
DrPL	T E V C F E R L G C F R D D A P W A G I	[33]

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Page 108 of 127

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SUBCHRONIC NOSE-ONLY INHALATION STUDY OF PROPYLENE GLYCOL IN SPRAGUE–DAWLEY RATS*

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Abstract—Groups of nineteen Sprague–Dawley rats of each sex were exposed by a nose-only inhalation to 0.0, 0.16, 1.0 or 2.2 mg propylene glycol/litre air, for 6 hr/day, 5 days/wk for 90 days. There were no significant differences in respiratory rates, minute volumes or tidal volumes between any of the groups during aerosol exposure. The uniformity of respiratory parameters between dose groups implied that the delivered doses were proportional to the exposure concentrations. The mean terminal body weights were not significantly different from controls for any group of male animals. The mean body weights of the females exposed to 2.2 mg/litre were significantly less than those of female controls from day 50 onwards. This effect, in female rats, was consistent with a decrease in feed consumption for the high-exposure female rats beginning on study day 43. Statistically significant differences between the treated and control groups in certain haematological parameters, serum enzyme activities, other serum chemistry parameters and organ weights did not show clear dose relationships. There was a significant increase in the number of goblet cells or an increase in the mucin content of the existing goblet cells in the nasal passages of the medium- and high-exposure animals. Exposure to the above concentrations of propylene glycol caused nasal haemorrhage and ocular discharge in a high proportion of animals, possibly as a result of dehydration of the nares and eyes.

INTRODUCTION

Propylene glycol or 1,2-propanediol is prepared from glycerol or propylene and is Generally Recognized as Safe (GRAS) as a food ingredient (Code of Federal Regulations Chapter 21, Section 184.1666). Propylene glycol is used as a surfactant, humectant, solvent for flavourings, an emulsifier and as a plasticizing agent (FASEB-SCOGS Report, 1973; Griffin and Lynch, 1983).

The oral LD_{50} of propylene glycol has been reported to be 20 g/kg body weight in the rat (*RTECS*, 1985). When administered in drinking-water for 140 days, rats died after 69 days' exposure to concentrations of 25 or 50% (Seidenfeld and Hanzlik, 1932). However, no adverse effects were reported, even on histopathology, in Sprague–Dawley rats given 10.0% or less propylene glycol in the drinking-water for 140 days. No effects were reported on plasma concentrations of total phospholipids, cholesterol, triglycerides, free fatty acids or on liver concentrations of phospholipids, triglycerides and gangliosides when male Sprague–Dawley rats were given 4.13 g propylene glycol/kg body weight by gastric intubation for 30 days (Hoenig and Werner, 1980). Total hepatic cholesterol was reportedly increased by 7%.

Propylene glycol was not mutagenic in *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 without metabolic activation (Pfeiffer and Dunkelberg, 1980). Propylene glycol did not produce dominant lethal effects after ip injections of 10 mg/kg body weight in male mice (Kennedy *et al.*, 1975), did not transform hamster embryo cells *in vitro* (Pienta, 1980), and did not produce chromosomal breaks, sister chromatid exchanges or micronuclei formation in mammalian cells (Sasaki *et al.*, 1980).

Morris *et al.* (1942) reported slight hepatic changes, including diffuse centrilobular atrophy, bile-duct proliferation and/or fatty degeneration, but no renal changes in rats fed up to 49,000 ppm propylene glycol in the diet. Gaunt *et al.* (1972) reported no adverse effects in any tissue in male and female CD rats fed 6250, 12,500 or 500,000 ppm propylene glycol in the diet for 2 yr. Male and female beagle dogs fed 2 g propylene glycol/kg body weight in the diet for 2 yr were unaffected; however, at 5.0 g/kg body weight, both sexes gained weight and females showed increased erythrocyte haemolysis and increased serum bilirubin compared with male dogs and control females (Weil *et al.*, 1971). No increase in dermal or systemic tumours was noted when propylene glycol was applied to the skin of female Swiss mice, undiluted (0.2 ml), as a 50% solution in acetone, or as a 10% solution in acetone, over their lifetime (Stenback and Shubik, 1974).

Rats fed 10, 20 or 30% propylene glycol in the diet consumed less feed, grew slower, produced smaller litters and weaned fewer young than controls, but no adverse effects were reported on reproductive capacity through six generations fed 2.5, 5.0 or 7.5% propylene glycol in the diet (Guerrant *et al.*, 1947). Pregnant CD-1 mice, Wistar rats, and golden hamsters given up to 1.6 g propylene glycol/kg body

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weight in water by gastric intubation for 10 consecutive days, 10 days and 5 days, respectively, did not show any effects on nidation, maternal survival, foetal survival, or skeletal abnormalities (Food and Drug Research Labs, 1973). Rabbits given up to 1.2 g propylene glycol/kg body weight in water by gastric intubation for 13 days did not exhibit effects on nidation, foetal survival or skeletal abnormalities (Food and Drug Research Labs, 1973).

Rats and monkeys exposed to atmospheres saturated with propylene glycol (up to 0.35 mg/litre) for 12 to 18 months did not show any adverse effects on growth rate, fertility, kidney function or blood counts (Robertson *et al.*, 1947). Rats, however, exhibited higher weight gains in these atmospheres (Robertson *et al.*, 1947). Inhalation of a 10% propylene glycol solution (no data on particle size) by rabbits for 20 min has been reported to alter ciliated cells and act as a secretory stimulus for goblet cells in the trachea (Konradova *et al.*, 1978). Following inhalation of this 10% propylene glycol solution for 2 hr, the alteration of ciliated cells was more pronounced and the number of degenerated mucous-discharging goblet cells had increased by 60% in the rabbits' tracheal epithelium.

The present 90-day nose-only inhalation study was intended to determine the effects, if any, of propylene glycol on the respiratory tract of the laboratory rat under the conditions used. These data were intended to provide for comparison with reported effects on the goblet cells in the tracheal epithelium of rabbits (Konradova *et al.*, 1978).

MATERIALS AND METHODS

Materials. Commercial grade propylene glycol, USP, was obtained from Dow Chemical SA, Geneva, Switzerland. The purity of the material was greater than 99% as confirmed by gas chromatography with flame-ionization detection. Butanediol was used as an internal standard on a Fractovap Model 2300 Carlo Erba gas chromatograph (Peabody, MA, USA) with a 250°C injector temperature. The test article was stored at 20 to 23 C and 50% relative humidity.

Animals and exposure. Young, healthy, adult (3-month-old) Sprague–Dawley rats (IFFA-CREDO, L'Arbresle, France) were housed, two per cage, (Macrolon Type III cages) with dust-free hardwood bedding. The male rats weighed 355–365 g and the females, 255–265 g when 3 months old. When not being exposed, animals were allowed access to pelleted feed (Special Diet Services Rat and Mouse No. 1, Shell Diet Services, England) and tap-water *ad lib.* Animal room controls were set to maintain a temperature of 22 ± 3°C, 30–70% relative humidity and a 12-hr light/dark cycle. Animals were identified by neck tag after random assignment to control and treated groups. After a 10-day quarantine period, the rats were divided into four groups of 19 males and 19 females each. Rats were exposed for 5 days/wk, 6 hr/day for 13 wk by nose-only inhalation to mean target aerosol concentrations of 0.1, 1.0 or 2.2 mg propylene glycol/litre air. Rats in a fourth group (controls) were exposed to humidified, filtered room air for the duration of the study.

Observations and pathology. Animals were observed daily for evidence of toxicity; diet consumption and body weights were recorded weekly. At the end of the 90-day exposure period, rats were exsanguinated by transecting the ventral aorta after anaesthesia induced by inhalation of 70% CO_2 in air. All animals received a complete autopsy. Organ weights were recorded for the lungs, thymus, spleen, liver, heart, kidneys, adrenals, ovary, urinary bladder, uterus, testes, prostate and brain. Thirty-nine organs and tissues were placed in 10% buffered formalin. All organs were fixed by immersion into the formalin, except the lungs which were fixed by instillation of 10% buffered formalin through the trachea under a pressure of 25 cm water using a medical infusion set. This method of lung fixation provides a uniform expansion of the bronchial tree and alveoli for group comparisons. The respiratory tracts, including nasal passages, lungs, trachea and larynx, of all groups were examined by light microscopy. The respiratory tract tissue was stained with hemalum–phloxin–saffron (HPS), a trichromic stain, and alcian blue–periodic acid Schiff (PAS), a stain for polysaccharides and mucous (particularly in goblet cells) before microscopic evaluation. Tissues were stained using an automatic slide stainer (Fisher Histomatic™ Slide Stainer, Model 172).

Generation of exposure atmosphere. To produce the concentrations of respirable aerosol needed, Battelle-designed nebulizers were used. The nebulizers (Hospitek, Farmingdale, NY) use the Wright jet baffle principle, with a double orifice system (diameter 0.6 mm) and a hemisphere baffle. They were operated with filtered compressed air at subsonic velocities. One nebulizer supplied the high-concentration chamber (2.2 mg/propylene glycol/litre air). The other nebulizer supplied the medium-concentration chamber (1.0 mg/litre), and then, following serial dilution with air (AIR-VAC TD 190H/TD 320H), the low-concentration chamber (0.1 mg/litre). The concentration of propylene glycol in the nebulizers was maintained by a constant-pressure reservoir feed system. Dilution air was supplied and monitored through calibrated rotameters. The nebulized aerosols were delivered to Battelle nose-only exposure chambers (Cannon *et al.*, 1983). The exposure chamber was designed using a dynamically equivalent water-flow system using a half-scale model to optimize the fluid-dynamic characteristics of the system, and thus the variability of the aerosol concentration in the chamber was less than 5%. The internal active volume of the chamber for exposing 38 animals by nose-only was 1.0 litre. The resulting time for the proper exposure at an animal port to reach 99% of its ultimate value was less than 60 sec for this chamber. The system is also unique in that it eliminates the problem of depletion of aerosol at lower chamber positions by animals in the higher positions because it supplies 'fresh' aerosol to each animal. This design ensures uniform exposure of all animals in the system, and ensures that the air exhaled by one animal does not reach any other animal in the chamber.

The aerosol generation system operated at flow-rates of between 35 and 50 litre/min to the exposure and control chambers. Because the exposure system

provides uniform individual aerosol supply to each animal, the air flow-rate to each animal tube was on average 1 to 1.5 litre/min (38 of 40 available animal ports were used for each group).

Analysis of exposure atmospheres. The aerosol concentrations were measured once per day by samples taken at animal ports with two gas washbottles (Aver & Co. AG, cat. No. 30852) in series, each containing 100 ml distilled water. Samples were routinely taken for about 10 min for the high, 30 min for the medium and 120 min for the low concentration. The air flow-rates for sample collection were nominally 2 litre/min. During sampling, volumes were measured by calibrated dry gasmeters (Compagnie des Compteurs, model G 1.6, No. 160 525). Also, sampling flow-rates were obtained from calibrated rotameters. After sampling, the contents of each washbottle were poured into a 150-ml volumetric flask, the sampling bottle was rinsed with distilled water and the flask was brought to volume. Samples were analysed daily for propylene glycol concentration by gas chromatography. The particle concentration was monitored continuously throughout exposures for the high-concentration group. A dilution system (10:1) in connection with a Mini-RAM (GCA Corp., Bedford, MA) aerosol monitor was used. The Mini-RAM was calibrated against gravimetric measurements. The particle size distributions of the propylene glycol aerosol were determined once a week for each exposure group using a Mercer seven-stage cascade impactor (Model 02-100; IN-TOX Products, Albuquerque, NM). Isokinetic samples were taken at a flow-rate of 1 litre/min directly from an animal port for each exposure group. Impactor stages were weighed using a Mettler microbalance (Model AJ, Mettler, Zurich, Switzerland) with a sensitivity of 0.1 μg. The effective cutoff aerodynamic diameter (ECAD) values for the seven stages were: 0.325, 0.715, 1.06, 1.60, 2.13, 3.0 and 4.6 μm. Particle size distribution parameters (Raabe, 1971) were determined from the weight of material collected on each stage of the cascade impactor. The data were fitted using a probit model and the mass median aerodynamic diameter (MMAD) and geometric standard deviation were determined.

Respiratory physiology. Respiratory rates and tidal volumes were measured in four rats/group/sex on study day 7, and repeated on the same animals on study days 42 and 84. Four animals were measured simultaneously for 15 min preceding exposure and for the first 30 min of exposure while in exposure/plethysmograph tubes (Coggins *et al.*, 1981). Pressure changes from the pneumotachographs (Fleisch No. 4 Metabo, Epalinges, Switzerland) connected to the exposure/plethysmograph tubes were converted to electrical signals by pressure transducers (Statham No. 270 Statham, Hato Ray, PR, USA). The signals were amplified (HP8805B carrier amplifiers, Hewlett-Packard, Cupertino, CA) and then recorded on a four-channel instrumentation tape recorder (HP 3960) at 15/16 ips. The tape was replayed later to data files in a HP 1000 computer using analogue to digital conversion (HP 5610A). Data were analysed with the HP 1000 using a series of programs developed by Battelle (Geneva, Switzerland).

Haematology and clinical chemistry. Clinical chemistry and haematology measurements on all of the animals were made before starting exposures and before autopsy. The pre-study measurements were carried out over 4 days, 36 or 40 animals, which comprised equal numbers of males and females, were retro-orbitally bled each day after mild carbon dioxide anaesthesia. About 0.3 ml blood was collected in an EDTA tube for haematology studies, and the remainder (at least 1.5 ml) was collected in two tubes for clinical chemistry measurements. The measurements at the end of the study were carried out over 3 days. About 50 animals (approximately equal numbers of males and females from each treatment group) were retro-orbitally bled on each day of killing. The blood samples were randomized before the clinical measurements were made. Unstable serum components were measured first (e.g. sorbitol dehydrogenase). The other measurements were conducted on the same day with the exception of cholesterol, triglycerides, phospholipids, serum urea nitrogen and albumin. For these five components an aliquot of serum was frozen at -20°C until the time of measurement (within 1 month). A Cobas-Roche BIO Centrifugal Analyzer (Roche Diagnostics, Nutley, NJ, USA) was used for clinical chemistry measurements. The results were stored in the internal memory of the analyser and transferred in batches to computer files (HP 1000) at the end of each day. Determinations were made of the serum concentrations of, glucose, urea nitrogen, total protein, albumin, total bilirubin, total cholesterol, calcium, inorganic phosphate, chloride, alkaline phosphatase, γ-glutamyl-transferase, aspartate aminotransferase, alanine aminotransferase, serum creatinine, 5'-nucleotidase, sorbitol dehydrogenase, sodium, potassium, creatine phosphokinase, triglycerides and total phospholipids. A TOA Sysmex (TOA Medical Electronics, Co., Ltd; Microcellcounter CC 180M) was used to analyse the following haematology measurements on whole blood: haematocrit, haemoglobin concentration, red blood cell count, white blood cell count, mean red blood cell volume, mean red blood cell corpuscular haemoglobin, mean corpuscular haemoglobin concentration and platelets.

Statistics. Statistical analyses were conducted by one-way analysis of variance, Bartlett's tests for homogeneity. Dunnett's test for significance or a modified *t*-test (Gad and Weil, 1982).

RESULTS

The daily aerosol exposure concentrations were 0.16 ± 0.04, 1.01 ± 0.11 and 2.18 ± 0.31 mg propylene glycol/litre air (mean $\pm$ SD; n = 65) which compares favourably with the target concentrations of 0.1, 1.0 and 2.2 mg propylene glycol/litre air, respectively. The MMADs of the diluted aerosol were less than 2.22 and 1.96 μm for the medium- and high-concentration groups, respectively. The mean geometric diameter for the low-concentration group was not obtainable, possibly due to evaporation which occurred with large quantities of dilution air. No measurements were made of the particle size of the undiluted aerosol. The geometric standard deviations were 1.44 and 1.57, respectively for the medium- and high-dose groups.

Table 1. Summary of frequency of clinical signs of nasal haemorrhages (%) in Sprague-Dawley rats exposed to propylene glycol by nose-only inhalation for 90 days

Exposure concn (mg propylene glycol/litre air)	Percentage of animals showing signs of haemorrhage*		
	1	2†	13†
Males			
0.16	3.0	69.9	65.8
1.0	18.8	69.2	100.0
2.20	12.8	71.4	94.7
Females			
0	0.0	2.3	0.0
0.16	0.0	65.1	0.0
1.0	0.0	63.9	97.4
2.20	5.3	68.4	93.6

*There were 19 animals per group except in the low-dose female group which comprised 18 rats.
†No significant difference between the values as assessed at weekly intervals from week 2 to 13.

There were no significant differences in respiratory rates, tidal volumes or minute volumes between the control group and any of the treatment groups, nor did respiratory rates within groups decrease as the animals became acclimatized to the nose-only exposure conditions.

A treatment-related effect was nasal haemorrhaging which began during the second week of exposure and persisted throughout the study; recovery from clinical signs of nasal haemorrhaging occurred during the non-exposure weekend periods (Table 1). This haemorrhaging was reduced to less than 4% in the low-exposure female group after the fourth week of exposure. From week 2 to 13, the average incidences of nasal haemorrhaging in males were less than 1% in controls, 64% in the low-exposure group, 74% in the medium-exposure group and 75% in the high-exposure group. In females, the average incidences were less than 1% in controls, 14% in the low-exposure group and 71% in the medium- and high-exposure groups. Similar trends were observed for ocular discharge, with incidences of 16% in low-exposure males, 40% in medium- and high-exposure males, and 5% in controls. There was generally less ocular discharge in females, who had incidences of 8% in controls, 14% in the low-exposure group, 28% in the medium-exposure group and 35% in the high-exposure group.

The high-exposure female rats showed a significant reduction in body weight, approximately 5–7%, starting on day 50 and continuing until the end of the study (Fig. 1). The medium-exposure females also had reduced body weights beginning on study day 64 which continued until the end of the study. There were no significant differences between the body weights of control and treated males; however, a trend was established by day 56: the control animals were heaviest, the low-exposure group were lighter than the medium-exposure group and the high-exposure group were the lightest. The differences be tween the control and high-exposure mean body weights were only 4% for males and 6% for females on the final day of the study. The sudden drop in body weight (Fig. 1) for the final day of the study was due to a 6-hr fast, the animals were weighed after the final exposure. On previous weighings, the animals were weighed before they were placed in the nose-only chambers.

The reduction in the females' body weights during the study was correlated with a significant reduction in feed consumption beginning on study day 43 for the high-exposure females and on study day 50 for the medium-exposure females (Fig. 2). The mean feed consumption was also significantly reduced on study day 50 for the low-exposure females. High-exposure male rats had significantly reduced feed consumption on study days 57, 64 and 88. There were no trends or significant differences from the controls in feed consumption for the low-exposure or medium-exposure male rats.

Pre-study measurements of selected haematology components revealed statistically significant

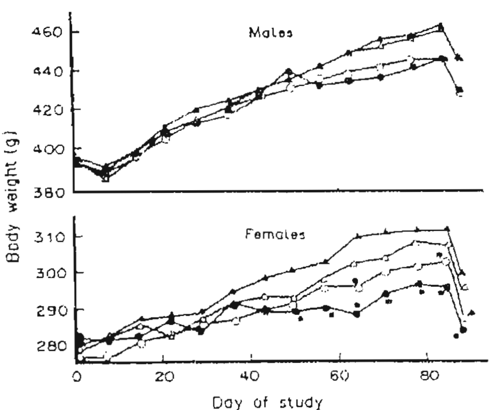

Fig. 1. Mean body weights of male and female Sprague Dawley rats exposed for 90 days by nose-only inhalation to propylene glycol at concentrations of 0 [control] (▲), 0.16 (△), 1.0 (○) or 2.2 (●) mg propylene glycol/litre air. There were 19 animals per group except in the low-dose female group which comprised 18 rats. Asterisks indicate values that differ significantly (Dunnett's test) from that for the corresponding control group (* $P < 0.05$).

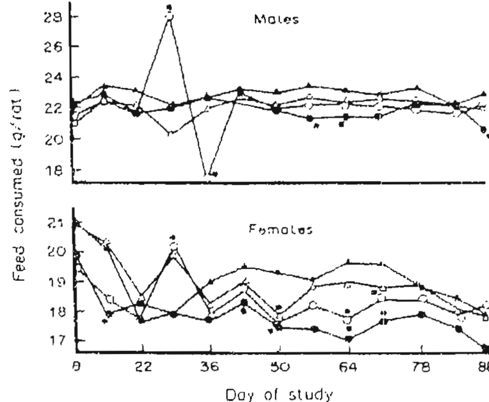

Fig. 2. Mean feed consumption in male and female Sprague-Dawley rats exposed for 90 days by nose-only inhalation to propylene glycol at concentrations of 0 [control] (▲), 0.16 (△), 1.0 (○) or 2.2 (●) mg propylene glycol/litre air. There were 19 animals per group except in the low-dose female group which comprised 18 rats. Asterisks indicate values that differ significantly (Dunnett's test) from that for the corresponding control group (* $P < 0.05$).

Table 2. Erythrocyte profile of male and female Sprague–Dawley rats exposed to propylene glycol by nose-only inhalation for 90 days

Exposure concn (mg propylene glycol/litre air)	Red blood cell count ($\times 10^6$/mm^3)	Haematocrit (%)	Haemoglobin concentration (g/dl)	Mean erythrocyte volume (μm^3)	Mean corpuscular haemoglobin (pg)	Mean corpuscular haemoglobin concn (g/dl)	Platelets ($\times 10^3$/mm^3)
				Males			
0	9.14 ± 0.34	45.55 ± 2.01	16.29 ± 0.78	49.84 ± 1.95	17.82 ± 0.72	35.78 ± 1.39	1281.26 ± 202.57
0.16	9.21 ± 0.33	45.98 ± 2.54	16.25 ± 0.58	49.79 ± 1.69	17.65 ± 0.53	35.58 ± 1.30	1250.84 ± 215.10
1.0	9.05 ± 0.29	45.99 ± 2.36	16.01 ± 0.52	50.79 ± 1.72	17.70 ± 0.69	34.86 ± 1.45	1274.53 ± 150.60
2.20	8.98 ± 0.34	45.41 ± 2.51	15.85 ± 0.74	50.47 ± 2.01	17.66 ± 0.56	34.95 ± 1.31	1176.21 ± 251.11
				Females			
0	8.49 ± 0.44	44.67 ± 2.42	15.61 ± 0.75	52.63 ± 1.95	18.38 ± 0.54	34.98 ± 0.94	1095.16 ± 135.06
0.16	8.65 ± 0.36	45.41 ± 2.11	15.68 ± 0.42	52.53 ± 1.74	18.16 ± 0.60	34.58 ± 1.24	1083.53 ± 104.51
1.0	8.57 ± 0.39	44.67 ± 1.93	15.52 ± 0.60	52.11 ± 0.99	18.11 ± 0.36	34.75 ± 0.40	1142.95 ± 129.15
2.20	8.54 ± 0.36	45.51 ± 2.43	15.45 ± 0.71	53.16 ± 1.50	18.08 ± 0.71	33.99 ± 1.16**	1067.79 ± 129.60

Values are means ± SD for groups of 19 rats, except in the low-exposure female group which comprised 18 rats; those values marked with asterisks differ significantly from the corresponding control value (**$P \leqslant 0.01$)

($P \leqslant 0.05$) differences between animals assigned to treated groups and control groups. However, the changes showed no consistent trends with respect to treatment group or sex and were not considered to be biologically significant.

There were no statistically significant changes in pre-study concentrations of serum enzymes, serum chemistries or serum electrolytes in the male rats. In the pre-study only serum phosphate was significantly increased ($P \leqslant 0.05$) in females of the high-exposure group (2.84 ± 0.48 mmol/litre) compared with the control group (2.49 ± 0.40 mmol/litre).

The only statistically significant change in the erythrocytic profile at the end of the study occurred in the high-exposure females (Table 2). The mean corpuscular haemoglobin concentration was reduced when compared with that for the female control group. There were significant decreases in white blood cell count, banded neutrophil count and lymphocyte count in medium- and high-exposure females (Table 3). High-exposure male rats also showed a significantly reduced banded neutrophil count which was not considered relevant to propylene glycol exposure.

There were statistically significant decreases in serum sorbitol dehydrogenase and γ-glutamyl transferase (Table 4) in the medium- and high-exposure male rats, respectively. Sporadic statistically significant changes also were reported (Table 5) for serum glucose, albumin and creatinine in medium-exposure males and for serum protein, albumin and cholesterol in high-exposure males. In the female rats, serum protein was elevated only in the medium-exposure animals. There were no changes in serum electrolytes (Table 6) in males of any group but serum chloride was elevated in the low-exposure females. As propylene glycol levels increased, decreasing trends in serum aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and γ-glutamyl transferase (Table 4), serum protein, albumin, cholesterol (Table 5) and inorganic phosphate (Table 6) were observed in male rats, but were not observed in the female rats.

Absolute organ weights (Table 7) were significantly decreased for the lungs of high-exposure females, spleens of low- and high-exposure males, livers of medium- and high-exposure males, kidneys of high- and medium-exposure males and females. Lung weights were significantly increased in low-exposure males. When organ weights were expressed relative to terminal body weights or brain weights (Table 8), high-exposure male spleen weights were significantly decreased and low-exposure male lung weights remained increased. There were no significant changes in absolute weights of adrenal glands, testes, brain, thymus, heart, prostate, uterus, urinary bladder or ovaries nor in these organ weights when expressed relative to body or brain weight.

There were no treatment-related changes in gross pathology when the animals were killed. Microscopic evaluation of the nasal cavity revealed a thickening of the respiratory epithelium, noted as an increase in the

Table 3. Leucocyte profile of male and female Sprague–Dawley rats exposed to propylene glycol by nose-only inhalation for 90 days†

Exposure concn (mg propylene glycol/litre air)	White blood cell count ($\times 10^3$/mm^3)	Segmented neutrophils ($\times 10^3$)	Band neutrophils (cells/mm^3)	Lymphocytes ($\times 10^3$)	Monocytes (cells/mm^3)	Eosinophils (cells/mm^3)
			Males			
0	12.25 ± 1.82	1.67 ± 0.61	245 ± 179	9.80 ± 1.53	492 ± 286	44.0 ± 68.0
0.16	10.46 ± 1.80*	1.54 ± 0.43	253 ± 195	8.16 ± 1.66*	431 ± 287	78.3 ± 84.2
1.0	11.77 ± 2.23	1.74 ± 0.48	181 ± 121	9.40 ± 2.11	372 ± 193	71.3 ± 87.8
2.20	12.09 ± 2.57	1.97 ± 0.57	122 ± 98*	9.56 ± 2.30	405 ± 150	35.2 ± 80.0
			Females			
0	9.72 ± 2.56	0.93 ± 0.55	215 ± 233	8.12 ± 2.23	406 ± 257	66.4 ± 121.3
0.16	9.02 ± 2.38	1.17 ± 0.39	102 ± 70	7.29 ± 2.04	387 ± 230	59.6 ± 81.5
1.0	7.43 ± 2.80*	1.10 ± 0.38	86 ± 80*	5.94 ± 2.44**	228 ± 167*	70.1 ± 73.1
2.20	7.37 ± 1.64**	1.21 ± 0.35	64 ± 73*	5.77 ± 1.31**	297 ± 151	32.7 ± 65.1

†No basophils, mast cells, myelocytes or blast cells were observed.

Values are means ± SD for groups of 19 rats, except in the low-exposure female group which comprised 18 rats; those values marked with asterisks differ significantly (Dunnett's test) from the corresponding control value (*$P \leqslant 0.05$, **$P \leqslant 0.01$)

Table 4 Serum enzyme profiles of male and female Sprague–Dawley rats exposed to propylene glycol by nose-only inhalation for 90 days

Exposure concn (mg propylene glycol/litre air)	AST (IU/litre)	ALT (IU/litre)	CPK (IU/litre)	ALP (IU/litre)	5'NT (IU/litre)	SDH (IU/litre)	GGT (IU/litre)
			Males				
0	78.68 ± 87.59	56.21 ± 62.88	100.68 ± 49.17	207.63 ± 42.95	1.29 ± 0.92	11.69 ± 3.57	1.06 ± 1.38
0.16	55.16 ± 27.21	36.47 ± 15.88	94.00 ± 119.64	201.89 ± 48.63	1.62 ± 3.02	11.13 ± 4.60	0.74 ± 1.03
1.0	52.42 ± 13.52	34.63 ± 6.43	77.42 ± 39.32	197.37 ± 63.18	1.59 ± 1.14	9.53 ± 2.44*	0.44 ± 0.56
2.20	49.68 ± 7.26	31.32 ± 6.02	89.79 ± 56.90	183.16 ± 41.05	1.23 ± 1.29	9.99 ± 2.73	0.32 ± 0.44*
			Females				
0	50.74 ± 7.35	34.32 ± 8.77	71.95 ± 36.71	104.37 ± 45.51	3.88 ± 1.95	10.84 ± 2.32	0.43 ± 0.60
0.16	50.44 ± 6.70	30.56 ± 6.46	93.12 ± 74.25	100.24 ± 36.25	3.46 ± 1.94	10.59 ± 2.77	0.72 ± 0.87
1.0	56.74 ± 18.42	35.74 ± 15.09	59.42 ± 25.71	120.32 ± 55.21	3.09 ± 1.55	11.52 ± 5.46	0.73 ± 0.70
2.20	49.16 ± 6.44	33.68 ± 8.04	66.79 ± 42.75	107.11 ± 30.21	3.25 ± 1.47	9.60 ± 1.39	0.97 ± 2.62

ALP = alkaline phosphatase ALT = alanine aminotransferase AST = aspartate aminotransferase
CPK = creatine phosphokinase GGT = γ-glutamyl transferase 5'NT = 5'nucleotidase SDH = sorbitol dehydrogenase
Values are means ± SD for groups of 19 rats, except in the low-exposure female group which comprised 18 rats; those values marked with asterisks differ significantly (Dunnett's test) from the corresponding control value (*$P \leq 0.05$).

Table 5. Serum chemistry profiles of male and female Sprague–Dawley rats exposed to propylene glycol by nose-only inhalation for 90 days

Exposure concn (mg propylene glycol/litre air)	Glucose (mmol/litre)	BUN (mmol/litre)	Total protein (g/litre)	Albumin (g/litre)	Total bilirubin (mmol/litre)	Serum creatine (mmol/litre)	Cholesterol (mmol/litre)	Triglycerides (mmol/litre)
				Male				
0	5.93 ± 1.75	6.93 ± 1.34	72.00 ± 3.22	29.95 ± 1.55	3.42 ± 0.54	78.58 ± 9.45	3.21 ± 0.74	1.01 ± 0.29
0.16	5.37 ± 1.51	6.78 ± 1.03	70.25 ± 3.61	29.56 ± 1.02	3.49 ± 0.60	73.57 ± 7.44	2.93 ± 0.65	1.01 ± 0.30
1.0	4.94 ± 0.53*	6.89 ± 1.44	69.88 ± 3.48	28.68 ± 2.10*	3.42 ± 0.55	73.33 ± 4.20*	2.77 ± 0.51	0.97 ± 0.25
2.2	6.43 ± 2.17	7.07 ± 0.94	68.03 ± 3.14**	28.48 ± 1.57**	3.16 ± 0.41	75.70 ± 6.27	2.70 ± 0.48*	0.96 ± 0.30
				Female				
0	5.02 ± 1.02	6.22 ± 0.87	72.79 ± 3.19	33.38 ± 1.92	3.95 ± 0.68	74.07 ± 6.75	3.98 ± 0.94	1.03 ± 0.42
0.16	5.65 ± 1.30	6.58 ± 1.38	75.51 ± 3.50	34.80 ± 2.36	4.18 ± 0.77	79.14 ± 6.89	4.39 ± 0.73	0.99 ± 0.30
1.0	5.09 ± 0.62	6.42 ± 1.08	76.86 ± 4.38**	34.62 ± 1.84	4.17 ± 0.59	77.67 ± 6.62	4.28 ± 1.04	1.10 ± 0.58
2.2	5.24 ± 0.96	6.58 ± 1.36	75.23 ± 3.92	34.26 ± 2.40	4.10 ± 0.54	76.81 ± 5.09	4.24 ± 0.98	1.06 ± 0.42

BUN = blood (serum) urea nitrogen
Values are means ± SD for groups of 19 rats, except in the low-exposure female group which comprised 18 rats; those values marked with asterisks differ significantly (Dunnett's test) from the corresponding control group (*$P \leq 0.05$, **$P \leq 0.01$).

Table 6. Serum electrolyte concentrations of male and female Sprague–Dawley rats exposed to propylene glycol by nose-only inhalation for 90 days

Exposure concn (mg propylene glycol/litre air)	Phosphorus (mmol/litre)	Sodium (mmol/litre)	Potassium (mmol/litre)	Chloride (mmol/litre)	Phosphate (mmol/litre)	Calcium (mmol/litre)
			Males			
0	2.35 ± 0.38	146.21 ± 4.54	6.01 ± 0.56	101.70 ± 3.87	2.86 ± 0.44	2.75 ± 0.16
0.16	2.28 ± 0.41	146.32 ± 4.30	6.19 ± 0.37	102.72 ± 4.12	2.84 ± 0.53	2.75 ± 0.18
1.0	2.12 ± 0.28	144.95 ± 3.46	6.08 ± 0.47	102.43 ± 3.98	2.78 ± 0.30	2.70 ± 0.15
2.2	2.10 ± 0.28	144.42 ± 3.15	5.92 ± 0.36	103.88 ± 4.72	2.88 ± 0.33	2.69 ± 0.16
			Females			
0	3.16 ± 0.59	145.53 ± 2.22	5.75 ± 0.41	102.09 ± 4.64	2.62 ± 0.33	2.85 ± 0.15
0.16	3.51 ± 0.61	144.17 ± 3.40	5.73 ± 0.31	106.26 ± 4.86*	2.84 ± 0.50	2.88 ± 0.17
1.0	3.44 ± 0.59	144.58 ± 3.36	5.95 ± 0.53	103.80 ± 3.98	2.55 ± 0.36	2.86 ± 0.17
2.2	3.43 ± 0.66	145.21 ± 3.17	5.71 ± 0.45	104.60 ± 3.95	2.62 ± 0.27	2.79 ± 0.22

Values are means ± SD for groups of 19 rats, except in the low-exposure female group which comprised 18 rats; those values marked with asterisks differ significantly (Dunnett's test) from the corresponding control value (*$P \leq 0.05$)

Table 7 Absolute organ weights of male and female Sprague–Dawley rats exposed to propylene glycol for 90 days by nose-only inhalation

Exposure concn (mg propylene glycol/litre air)	Lung (g)	Spleen (g)	Liver (g)	Kidneys (g)
		Males		
0	1.86 ± 0.17	0.74 ± 0.08	10.82 ± 1.50	2.64 ± 0.37
0.16	2.02 ± 0.23*	0.67 ± 0.08*	10.37 ± 1.08	2.52 ± 0.16
1.0	1.85 ± 0.21	0.73 ± 0.13	9.89 ± 0.98*	2.38 ± 0.25*
2.2	1.88 ± 0.17	0.62 ± 0.05**	9.81 ± 1.03*	2.39 ± 0.20*
		Females		
0	1.82 ± 0.24	0.57 ± 0.07	6.85 ± 0.67	1.84 ± 0.22
0.16	1.78 ± 0.23	0.58 ± 0.08	6.93 ± 0.55	1.76 ± 0.20
1.0	1.82 ± 0.17	0.55 ± 0.06	6.83 ± 0.42	1.67 ± 0.09**
2.2	1.62 ± 0.14**	0.54 ± 0.07	6.53 ± 0.49	1.68 ± 0.19*

Values are means ± SD for groups of 19 rats, except in the low-exposure female group which comprised 18 rats; those values marked with asterisks differ significantly from the corresponding control value (*$P \leq 0.05$, **$P \leq 0.01$)

Plate 1 Nasal turbinates from a control Sprague–Dawley rat (a) and a Sprague–Dawley rat exposed to 2.0 mg propylene glycol/litre air (b). Note the abundance of dark goblet cells in the nasal epithelium overlying the turbinates in the high-dose group. Tissues were stained with alcian blue–periodic acid Schiff stain for polysaccharides and mucus. (× 160)

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Toagosei Co., Ltd.
70231-E
Page 117 of 127

Table 8. Organ weights relative to body weight, or to brain weight, of rats exposed to propylene glycol by nose-only inhalation for 90 days

Exposure concn (mg propylene glycol/litre air)	Weight relative to body weight (g/100 g body weight): Lung (g)	Spleen (g)	Liver (g)	Kidneys (g)	Weight relative to brain weight (g/100 g brain weight): Lung (g)	Spleen (g)	Liver (g)	Kidneys (g)
Males								
0	0.43 ± 0.04	0.17 ± 0.02	2.49 ± 0.31	0.61 ± 0.08	89.84 ± 7.69	35.24 ± 3.90	523.11 ± 75.20	127.64 ± 18.57
0.16	0.47 ± 0.05*	0.16 ± 0.02	2.40 ± 0.17	0.59 ± 0.05	97.77 ± 11.01*	32.40 ± 3.69*	502.33 ± 58.48	122.20 ± 9.78
1.0	0.44 ± 0.04	0.17 ± 0.03	2.35 ± 0.15	0.57 ± 0.05	88.45 ± 8.38	34.62 ± 5.57	473.00 ± 49.08*	113.76 ± 13.33*
2.2	0.44 ± 0.05	0.15 ± 0.02**	2.30 ± 0.29	0.56 ± 0.06	92.62 ± 7.98	30.35 ± 2.70**	482.86 ± 50.35	112.70 ± 11.24
Females								
0	0.63 ± 0.07	0.20 ± 0.02	2.37 ± 0.23	0.64 ± 0.07	91.26 ± 12.00	28.57 ± 4.12	344.36 ± 47.01	91.98 ± 11.34
0.16	0.61 ± 0.08	0.20 ± 0.02	2.40 ± 0.24	0.61 ± 0.08	92.09 ± 12.62	30.24 ± 3.91	358.90 ± 28.49	91.37 ± 10.36
1.0	0.65 ± 0.07	0.20 ± 0.02	2.43 ± 0.14	0.60 ± 0.03*	93.70 ± 11.45	28.14 ± 2.85	350.86 ± 30.04	85.92 ± 6.50
2.2	0.59 ± 0.05	0.19 ± 0.03	2.38 ± 0.16	0.61 ± 0.06	83.30 ± 6.52*	27.39 ± 3.34	335.97 ± 32.57	86.34 ± 9.82

Values are means ± SD for groups of 19 rats, except in the low-exposure female group which comprised 18 rats; those values marked with asterisks differ significantly from the corresponding control value (* $P \leq 0.05$; ** $P < 0.01$).

numbers of goblet cells or as an increase in the mucin content of the goblet cells in the medium- and high-exposure male and female rats (Table 9). The increased number of goblet cells or increased mucin content of the goblet cells was observed in the posterior portion of the nasal cavity, lining of the septum, the lateral walls, the anterior turbinates and in selected cases the ethmoid turbinates. The subjective judgement of the proliferation of goblet cells in the posterior nasal cavity was accompanied by an increased volume of mucus in the individual goblet cells (Plate 1). There were no histological changes in the trachea, lungs or larynx.

DISCUSSION

Minute volume, tidal volume and respiratory rates were not significantly altered when Sprague-Dawley rats were exposed to 0.16, 1.01 or 2.2 mg propylene glycol/litre air following a 13-wk, nose-only inhalation study. This lack of changes demonstrated that the animals adapted to the exposure concentrations and conditions. This also suggests that the delivered doses were proportional to the exposure concentrations.

The observation of nasal haemorrhaging which appeared during the second week of exposure suggests that propylene glycol can act as a dehydrating agent on peripheral tissues. The frequency of nasal haemorrhaging remained constant throughout the study and was highest (65 to 75%) in the medium- and high-concentration groups. During the non-exposure weekend periods, the nasal haemorrhaging did not occur, and, in some cases, it disappeared completely following a weekend recovery period.

Among the high-exposure female rats, the concentration of propylene glycol was sufficient to produce a 5–7% reduction in body weight. This weight reduction correlated with the observed reduction in feed consumption. There was no trend towards reduced feed consumption among male rats, but reduced consumption on selected days for the high-exposure male rats was seen. These data demonstrate that the female animals were exposed to a sufficiently high concentration of propylene glycol to cause a significant reduction in body weight. These exposure concentrations would probably have been sufficient to produce a toxic effect on the organ systems examined if propylene glycol were responsible for any specific organ toxicity.

Pre-study measurements of clinical pathological parameters including clinical chemistry and haematology did not reveal consistent differences related to treatment group or sex. Animals were assigned to groups by randomization based on body weight. At the end of the study there were inconsistent changes in selected haematological and clinical chemical parameters within and between treatment groups and sexes. These changes (e.g. decreased enzyme activities) did not show clear relationships with propylene glycol exposure concentrations.

Inconsistent but statistically significant changes were also observed with absolute organ weights. These changes were not considered biologically significant when the weights for all of the

Table 9. Altered goblet cell scores in nasal turbinates of rats exposed to propylene glycol by nose-only inhalation for 90 days

Goblet cell score†	Propylene glycol concn (mg/litre air)	No. of animals affected*							
		Males				Females			
		0	0.16	1.00	2.20	0	0.16	1.00	2.20
1		19	17	2	—	19	17	—	—
2		—	2	2	1	—	1	6	2
3		—	—	12	13	—	—	12	12
4		—	—	3	5	·	—	1	5

*There were 19 rats/group except in the low-exposure female group which comprised 18 rats.
†Goblet cell score: 1 = normal; 2 = slight number; 3 = moderate increase; 4 = marked increase observed by alcian blue–PAS staining.

treatment groups were compared and when the gross histological findings were taken into account.

The changes in clinical, gross pathological, histopathological or organ-weight variables were not life-threatening to the rats exposed to propylene glycol by the inhalation route at the concentrations administered. The changes observed in organ weights and clinical pathology parameters did not indicate a toxic effect on any single organ system or blood component.

An increase in the number of goblet cells or an increase in the mucin content of the goblet cells present was observed in the nasal turbinates of both male and female rats. These changes appeared to be exposure related and were probably due to the physical effects of propylene glycol. This increase in goblet cell numbers or increase in the mucin content of existing goblet cells is contrary to the report of Konradova et al. (1978). The current subchronic study in rats did not produce an increase in the number of degenerated, exhausted goblet cells like that seen by Konradova et al. (1978) in rabbits inhaling a 10% propylene glycol solution for up to 2 hr. Within the limits of the present study it is not possible to determine whether the increase in PAS-AB in goblet cells represents a true increase in mucin cells or an increase in mucin production in previously quiescent cells.

This study supports previous studies indicating that propylene glycol is not a systemic toxin when administered by inhalation (Robertson et al., 1947) or by other routes (Gaunt et al., 1972; Hoenig, 1980; Seidenfeld and Hanzlik, 1932; Weil et al., 1971). The histological changes observed were probably the result of physical irritation of propylene glycol upon the nasal epithelium in the rat. The toxicity observed in Sprague–Dawley rats following 90-day inhalation of propylene glycol at concentrations of up to 2.2 mg per litre air was not life-threatening and does allow the assessment of a no-observed-effect exposure level (the no-effect level is 1.0 mg/litre). The previous report (Konradova et al., 1978), of cilia-cell alteration and increased numbers of exhausted, degenerated goblet cells in rabbit tracheal epithelium when propylene glycol was inhaled, was not verified. In fact, this study reports an increase in the number of goblet cells or an increase in the mucin content of the goblet cells in the Sprague–Dawley rat's nasal epithelium. The earlier study (Konradova et al., 1978) used a different exposure regimen and a different species, and did not document exposure conditions, size of particles or any other methodological information. Our study confirms that propylene glycol would not cause adverse health effects when exposures are based upon these no-observed-effect levels.

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CHAPTER 24

TOXIC EFFECTS OF SOLVENTS AND VAPORS

Robert Snyder and Larry S. Andrews EXCERPT

Nearly everyone is exposed to solvents. The utility of these fluids as solubilizers, dispersants, or diluents leads to the manufacture and use of billions of pounds each year. Occupational exposures can involve applications ranging from a secretary using correction fluid to a gas station attendant pumping gasoline. A refinery worker may be exposed to solvents on the job and upon returning home may paint a room, change the oil in the family car, or glue together an item in need of repair, thereby extending his exposure to solvents. Although the solvents, which are usually mixtures, have different trade names, they frequently contain similar chemicals. Clearly, exposure should not be equated with toxicity. The fundamental principle of toxicology, which is the dose-response relationship, requires that there be (1) exposure and (2) a toxic effect. Nevertheless, the potential for toxicological interaction increases as exposure increases, and exposure to mixtures leads to the possibility of unpredictable additivity, synergism, or potentiation of effects. In the long run we must learn to understand the interactive effects of solvents because the exposure of human populations in the environment is not usually to a single chemical. Until we have developed that needed body of knowledge we must make use of the database available to us, which is the toxicology of individual solvents and the relationship between the structures of solvents and their toxicity within chemical classes.

THE PROPERTIES OF SOLVENTS

Exposure

Many solvents exhibit appreciable volatility under conditions of use, and consequently the worker, or people who use products containing solvents in the home, may be exposed to solvent vapors. The potential hazard posed as a result of exposure to a solvent is a function of the dose-response relationship. The dose, ideally, is the concentration of the toxic form of the chemical at its physiological receptor. Because that information is usually not available, the next best estimate of dose is the blood level of the chemical, and sometimes, by extrapolation, we can derive an accurate estimate of body levels from the concentration in the urine. Concentrations in physiological fluids, such as blood and urine, are often expressed as units of weight/volume (w/v), for example, mg/mL, mg/L and so on. Concentrations of gas mixtures given in w/v units depend on both temperature and pressure, as mass depends on the number of molecules present, but the volume occupied by that mass depends on temperature and pressure and is described by the ideal gas law ($PV = nRT$). For regulatory purposes, and in many experimental situations, vapor concentrations are expressed as parts of vapor per million parts of contaminated air (ppm) by volume at a specified temperature and pressure or as mg solvent/m^3 air. Given that respiratory rates and volumes are fairly consistent among people, mg/m^3 provides a means of readily estimating exposure to a solvent over a period of time. Examples of methods for interconverting ppm and mg/m^3 are shown in the appendix to this chapter.

The volatility of solvents indicates that a major route of exposure will be by the respiratory system. Once vapors enter the lungs they may readily diffuse across a large surface area of respiratory membranes and enter the bloodstream. The ability of solvent vapors to enter the bloodstream and their rate of membrane transport depend upon their lipid solubility, since lipoprotein cell membranes must be traversed. Many solvents are very lipid soluble and will enter the blood with ease. Because diffusion occurs from relatively high concentrations in lung air to low concentrations in blood and tissues, the driving force for the movement is the vapor concentration in inspired air.

Concerns for solvent exposures include both acute and chronic effects. Any situation in which a person may be harmed by exposure to high levels of vapor should be controlled by adequate techniques of safety engineering. In the event of an unexpected release of vapor, emergency operating procedures will require the availability of emergency respiratory equipment and rapid evacuation of the premises. Safety engineering procedures are designed around acceptable exposure standards established by regulatory agencies to protect against both acute and chronic effects of chemicals. Standard setting for solvent exposures often includes recommendations for short-term exposure limits (STEL) to protect against acute toxicity. The establishment of 15-min STEL values for volatile solvents is aimed at determining an air level of vapor below which workers could perform for 15 min without loss of consciousness or loss of the ability to perform the tasks expected of them. The need to remain in an area where there is an unacceptable level of solvent vapor may relate to the need to stabilize a dangerous situation which might otherwise lead to greater danger to the exposed individual or to others in danger of exposure. There are, therefore, two issues. One is loss of consciousness; the second is the potential impairment of ability to perform essential emergency procedures.

In this discussion volatile solvents will initially be examined as if they were anesthetic agents. Organic vapors may produce depression of the central nervous system and, in theory, are capable of producing anesthesia. Anesthetic potency, that is, the dose of each solvent vapor necessary to produce anesthesia, varies widely. As the dose of vapor increases, the relative concentration of oxygen in the inspired air may decrease, with the effect that the vapor acts as an asphyxiant. A number of the more potent vapors, however, have been used to produce surgical anesthesia.

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70231-E
Page 121 of 127

viduals who had ingested 250 to 1000 mL of ethylene glycol antifreeze. Gastric lavage was not undertaken until admission to the hospital, some 6 to 9 h after the antifreeze was ingested; thus large quantities were presumably absorbed into the general circulation. Both patients were treated with ethanol infusion, which resulted in a prompt disappearance of oxaluria, and adequate urinary output was maintained. These individuals made uneventful recoveries from these rather massive ingestions of ethylene glycol.

The value of ethanol administration in treating methanol poisoning has also been demonstrated (Jacobsen and McMartin, 1986). Administration of 4-methylpyrazole, a noncompetitive inhibitor of alcohol dehydrogenase, is also used in treating ethylene glycol and methanol poisoning. Control of blood pH by the administration of sodium bicarbonate and the use of hemodialysis to remove the parent compound and metabolites are important additional means of treating poisoned individuals (Jacobsen and McMartin, 1986; Wiener and Richardson, 1988; Baud et al., 1988).

Lamb and coworkers (1985) administered ethylene glycol to male and female mice in the drinking water and pairs of animals were mated. After 14 weeks a second generation was selected and evaluated in a similar manner. Ethylene glycol (1.0 percent in drinking water) caused a decrease in the number of litters per fertile pair, in the number of pups per litter, and in live pup weight. These effects were observed without any concurrent effects on body weight, water consumption, or clinical signs of toxicity in the parents. Examination of offspring revealed a pattern of skeletal defects in treated mice including the skull, sternebrae, ribs, and vertebrae. The developmental toxicity of ethylene glycol was confirmed using a conventional teratology protocol in rats and mice (Price et al., 1985).

Diethylene Glycol ($HOCH_2CH_2OCH_2CH_2OH$)

Diethylene glycol is used in the lacquer industry, in cosmetics, in permanent antifreeze formulations, in lubricants, as a softening agent, and as a plasticizer. It presents little hazard during industrial handling at ordinary temperatures. Where mists are generated or where operations are carried out at high temperatures, industrial hygiene control methods should be followed to eliminate repeated prolonged inhalation. The major hazard from diethylene glycol occurs following the ingestion of relatively large single doses. Impetus for the study of the toxicity of diethylene glycol was provided by 105 fatalities among 353 people who ingested a solution of sulfanilamide in an aqueous mixture containing 72 percent diethylene glycol (Ruprecht and Nelson, 1937; Smyth, 1952). The symptoms included nausea, dizziness, and pain in the kidney region. This was followed in a few days by oliguria and anuria with death resulting from uremic poisoning. Based on these data it has been estimated that the single oral dose lethal for humans is approximately 1 mL/kg. Following an episode of poisoning known as the elixir of sulfanilamide incident, the Food, Drug and Cosmetics Act was subsequently amended in 1938 to require that marketed drugs be proved safe and effective.

A long-term rat-feeding study by Fitzhugh and Nelson (1946) showed that 1 percent diethylene glycol in the diet over a 2-year period resulted in slight growth depression, a few calcium oxalate bladder stones, minimal kidney dam and occasional liver damage. At the 4 percent dietary l there was increased mortality, a marked depression of growth rate, bladder stones, severe kidney damage, and erate liver damage. In addition, bladder tumors appeared quently. The authors concluded that bladder tumors n developed in the experimental rats without the precedin concurrent presence of a foreign body. They suggest diethylene glycol is not a primary carcinogen, but when fed in very high concentrations it does result in the forma of calcium oxalate bladder stones and subsequent rare b der tumors. The toxic effects seen following exposur diethylene glycol are consistent with its metabolic conver to ethylene glycol and subsequent acidosis and oxalate cr formation.

Propylene Glycol (1,2-Propanediol, $CH_3CHOHCH_2OH$)

Propylene glycol, in sharp distinction to ethylene a diethylene glycol, has a low order of toxicity. Propylene gl is used in human and pet foods, cosmetics, and pharmace cals with no apparent adverse effects. Other major uses propylene glycol include antifreeze formulations, heat changers, and hydraulic fluids. Propylene glycol has a very l order of acute toxicity. The acute oral LD_{50}s of propyl glycol in rats, rabbits, and dogs are approximately 30, 18, 19 g/kg body weight, respectively (Ruddick, 1972). Sympto of acute propylene glycol intoxication in animals are those CNS depression or narcosis. No system or organ has be established as a target for the acute oral lethal effects of pr pylene glycol. In contrast to ethylene glycol, propylene glyco vapors do not appear to be irritating. Neither OSHA n ACGIH have established exposure limits for propylene glyco vapors.

Because of propylene glycol's use in foods and pharma ceuticals, extensive toxicity data are available. Robertson an coworkers (1947) exposed monkeys and rats to atmospher saturated with propylene glycol vapor and found no advers effects in animals after periods of 12 to 18 months. No adverse effects were noted and there were no increases in tumor incidences in rats fed diets containing up to 5 percent propylene glycol for 2 years (Robertson et al., 1947; Gaunt et al., 1972). Furthermore, propylene glycol is used as a carbohydrate source without any adverse effects when fed to dogs at a concentration of 8 percent in the diet for 2 years. This dietary concentration equates to approximately 2 g propylene glycol per kg body weight per day (Weil et al., 1971). Propylene glycol is not mutagenic or teratogenic, nor did propylene glycol adversely affect reproduction when fed at a 7.5 percent dietary concentration to rats for three generations (FDA, 1973, 1974, 1977).

The explanation for the low toxicity of propylene glycol lies in its metabolism. Propylene glycol, in contrast to ethylene glycol, is metabolized by alcohol dehydrogenase to lactic acid and further to pyruvic acid (Ruddick, 1972). These acids are normal constituents of carbohydrate metabolism and are further broken down to carbon dioxide and water. Propylene glycol, like ethanol, has been reported to be an effective antidote for ethylene glycol poisoning (Holman et al., 1979).

Based on a review of existing health effects data for propylene glycol, a select committee of experts convened by the FDA reaffirmed the "generally recognized as safe" (GRAS) status of propylene glycol (FDA, 1977).

GLYCOL ETHERS

Glycol ethers (Fig. 24-6) find extensive use in industry as solvents in the manufacture of lacquers, varnishes, resins, printing inks, textile dyes, anti-icing additives in brake fluids, and as gasoline additives. In consumer products they are found in latex paints, cleaners, and other household products. Structurally, the glycol ethers are categorized as ethylene glycol ethers, or monopropylene, dipropylene, or tripropylene glycol ethers. The ether function may be bound to methyl, ethyl, n-propyl, n-butyl, or t-butyl groups. In some cases the alcohol groups are bound in the form of their acetate esters. Because of the ease and rapidity of ester hydrolysis in vivo there is no reason to assume that the toxicities of the esters differ from those of the unesterified glycols.

The glycol ethers, as a class of materials, are not acutely hazardous by the oral route. The rabbit appears to be more sensitive than the rat with regard to acute oral toxicity. Tanii and coworkers (1992) studied correlations between acute in vivo and in vitro toxicity and log P (where P is the n-octanol-water partition coefficient) among glycol ethers. Data collected included toxicity to neuroblastoma and glial cells in vitro expressed as ED_{50}, LD_{50} in mice pretreated with corn oil or with CCl_4, and log P. The data were analyzed by multiple regression analysis and revealed that acute toxicity was probably a function of the parent compound rather than of metabolites and that relative hydrophobicity played a significant role in acute toxicity.

Glycol ethers, particularly the ethylene series, are well-absorbed from the skin. Sabourin and colleagues (1992) reported that 20 to 25 percent of the applied dose of methoxyethanol, ethoxyethanol, or butoxyethanol was absorbed from the shaved skin of F344/N rats regardless of the dose. Indeed, the dermal LD_{50} to oral LD_{50} ratio is approximately 1 for ethylene glycol ethers. High vapor concentrations of the ethylene series are lethal, but saturation levels or levels approaching saturation of the propylene series are not lethal to rodents.

Ethylene Glycol Ethers
R—OCH_2CH_2OH R = CH_3—EM
CH_3CH_2—EE
$CH_3CH_2CH_2CH_2$—EB

Metabolite
R—OCH_2COOH Alkoxy acetic acid

Propylene Glycol Ethers
R—O—$CH_2CH(CH_3)OH$ R = CH_3—PM
CH_3CH_2—PE
$CH_3CH_2CH_2CH_2$—PB

Metabolite
HO—$CH_2CH(CH_3)OH$ Propylene glycol

Figure 24–6. Glycol ethers and their metabolites.

It has been known for many years (Wiley et al., 1938; Nagano et al., 1979) that ethylene glycol ethers cause reproductive toxicity. Mice given large amounts of ethylene glycol monomethyl ether (EM), ethylene glycol monoethyl ether (EE), or their respective acetic acid esters for 5 weeks displayed testicular atrophy. Ahmed and coworkers (1994), using whole body autoradiography, demonstrated that treating mice with labeled EE led to accumulation of radioactivity not only in the liver, kidney, and bladder but also in the epididymis and bone marrow. Rabbits and rats exposed to EM vapor exhibited degeneration of the testicular germinal epithelium. At the end of the exposure period male rats were mated to unexposed females and found to be infertile (Miller et al., 1983a; Rao et al., 1983). A second mating at 13 weeks postexposure revealed a partial recovery of fertility. The dose-response relationship demonstrated that the rabbit is the more sensitive of the two species.

Fetotoxic and teratogenic responses were observed in pregnant rabbits and rats exposed by inhalation to either EM or EE (Nelson et al., 1984; Andrew et al., 1981) or to EE applied dermally. The site most commonly affected was the cardiovascular system, but skeletal and other malformations also were observed.

Ethylene glycol monobutyl ether (EB), in contrast to EM and EE, exerts its primary effect upon the red blood cell. Rats exposed to high vapor concentrations (Dodd et al., 1983) or to EB applied to the skin (Bartnik et al., 1987) exhibited a marked degree of hemolysis. Bartnik and coworkers (1987) measured the degree of absorption of EB from the skin and reported that approximately 25 percent of the applied material was absorbed from unoccluded skin within 48 h. EB appears to be an exception to the rule that the inhalation route of exposure is quantitatively more important than skin contact.

Treatment of pregnant mice and rabbits with ethylene glycol diethyl ether (George et al., 1992) resulted in embryo/fetal toxicity. Litters were smaller and malformations increased with increasing doses. Similar results were observed (Schwetz et al., 1992) using diethylene glycol dimethyl ether and triethylene glycol dimethyl ether.

The monopropylene, dipropylene, and tripropylene glycol ethers do not share the potent reproductive toxicity exhibited by the ethylene glycol ethers regardless of whether the route of administration is oral, dermal, or by inhalation (Miller et al., 1984). Whereas some embryo or fetal toxicity may have been observed at the highest doses, no dose produced birth defects, testicular atrophy, or damage to blood or thymic tissues. In a National Toxicology Program bioassay (NTP, 1986) mice were given PM in drinking water at several doses over 14 weeks in a two-generation study of reproductive toxicity. In both generations no effects were observed on fertility, litters per pair, live pups per litter, or sex ratio. Examination of sperm revealed no effects on motility, density, or abnormal sperm frequency. Aberrant observations included decreased pup body weights in the first generation and decreased weights of the right epididymis and prostate gland in the second generation. The latter effects were not seen in both generations and their

physiological significance has yet to be determined. Nevertheless it is clear that the propylene glycol ethers exhibit considerably less reproductive toxicity than the ethylene glycol ethers.

These differences in reproductive toxicity between EM and propylene glycol monomethyl ether (PM) are explained by the differences in metabolism of the two materials. Miller and colleagues (1983b) studied the metabolism of orally administered EM or PM, radiolabeled in the glycol carbons, in rats. Most of the administered PM is metabolized, via propylene glycol, to $^{14}CO_2$. In contrast, methoxyacetic acid is the major metabolite of EM. Liver alcohol dehydrogenase can oxidize EM to methoxyacetaldehyde, which can then be oxidized to methoxyacetic acid. It is of interest that methoxyacetaldehyde (Chiewchanwit and Au, 1994) induced mutations in the bacterial *gpt* gene inserted into an autosome of CHO-AS52 cells but not in the *hprt* gene on the X chromosome of CHO-K1-BH4 cells. In both cell lines and in human lymphocytes in culture, methoxyacetaldehyde caused both sister chromatid exchanges and chromosome aberrations. Methoxyacetic acid has been shown to produce the same toxic effects as EM in male rats (Miller et al., 1982; Moss et al., 1985; Sleet et al., 1988).

Administration of 4-methylpyrazole, an inhibitor of alcohol dehydrogenase, significantly reduced EM-induced embryotoxicity, underscoring the importance of this enzyme in the metabolic activation of EM. Because EE, EB, and ethylene glycol monoisopropyl ether (EIP) are metabolized to analogous metabolites (ethoxyacetic, butoxyacetic, and isopropoxyacetic acids, respectively), alkoxyacids are also considered to be toxic metabolites (Jonsson et al., 1982).

Although the primary alcohol function on glycol ethers is easily oxidized by liver alcohol dehydrogenase, propylene glycol ethers have a secondary alcohol function and are relatively poorer substrates for alcohol dehydrogenase (von Wartburg, 1964). They undergo microsomal *o*-dealkylation to propylene glycol, a material that is not a reproductive toxin. However, propylene glycol ethers may exist in either of two isomeric forms termed the alpha and beta forms:

Major isomer	Minor isomer
$CH_3{-}O{-}CH_2{-}CH(OH){-}CH_3$	$HO{-}CH_2{-}CH(O{-}CH_3){-}CH_3$
1-methoxy-2-propanol	2-methoxy-1-propanol
secondary alcohol	primary alcohol
α isomer	β isomer

Merkle and colleagues (1987) reported that the acetic acid ester of 2-methoxy-1 propranol was capable of inducing malformations in rabbits. Miller and coworkers (1986) showed that the beta isomer of PM is indeed metabolized to methoxypropionic acid. Since commercial PM is more than 95 percent of the alpha-isomer, there is no occupational concern for adverse effects on reproduction from exposure to PM vapors.

Mebus and coworkers (1989) suggested that simple physiological substrates such as serine, acetate, sarcosine, and glycine, given concomitantly with EM, ameliorated developmental toxicity and testicular damage in rats. It is postulated that the toxic metabolite of EM may interfere with the availability of one-carbon units for incorporation into purine and pyrimidine bases. Substrates such as sarcosine or acetate can provide additional one-carbon units needed during differentiation of the developing embryo or for maturation of pachytene spermatocytes. Another protective mechanism was described by Burhan and Chapin (1990), who demonstrated that calcium channel blockers protected against EM-induced testicular toxicity.

An addition to the well-studied reproductive toxicity associated with ethylene glycol ethers is the observation that these compounds can also produce hemolytic anemia. These compounds are taken up into rat erythrocytes over time, the cells swell, and they then hemolyze (Burhan, 1989). Structure-activity studies revealed that in order of effectiveness butoxyacetic acid > propoxyacetic acid > ethoxyacetic acid > methoxyacetic acid. Hemolysis appears to parallel decreases in red cell ATP. The susceptibility of human erythrocytes is less than that of rat erythrocytes (Burhan, 1989).

CARBON DISULFIDE

Carbon disulfide (CS_2) is primarily used in the production of regenerated rayon and cellophane and in the manufacture of carbon tetrachloride. It is also used as a solvent for many applications (Timmerman, 1985). A recent estimate of CS_2 production worldwide was one million metric tons.

Adverse effects resulting from prolonged human exposure to high levels of CS_2 have been extensively reported and documented. These include organic brain damage, peripheral nervous system decrements, neurobehavioral dysfunction, ocular and auditory effects, and atherosclerosis. Hearing loss to high frequency tones is a common feature of CS_2 intoxication (Zenk, 1970). Excellent reviews of these effects are available (Coppack et al., 1981; Beauchamp et al., 1983). Recent studies have also implicated CS_2 in alimentary and endocrine defects (Peplonska, 1994). CS_2 toxicity is reviewed in Chap. 16.

Exposure to CS_2 has been called a contributing factor in coronary heart disease (Tiller et al., 1968). This effect has been confirmed by Finnish epidemiologists studying an occupationally exposed cohort using a 10-year follow-up plan. While advanced age and hypertension were predominant factors in determining coronary heart disease, exposure to CS_2 alone contributed a statistically significant relative risk (Tolonen et al., 1975, 1979). Occupational CS_2 exposure can be an important contributing factor to the development of coronary heart disease and this issue should continue to be monitored in future epidemiological studies.

Hoffmann and coworkers (Hoffmann and Muller, 1990; Hoffman and Klapperstuck, 1994) examined the effects of acute and subacute exposure to CS_2 in rats. Using the urethane-anesthetized rat model, they found that CS_2 (1.66 or 3.32 mmol/kg) enhanced the hypertensive effect and decreased the inotropic effects of epinephrine and norepinephrine on the heart. They concluded that CS_2 caused a delay in atrioventricular conduction time.

Home

Summary of Evaluations Performed by the Joint FAO/WHO Expert Committee on Food Additives

PROPYLENE GLYCOL ESTERS OF FATTY ACIDS	
INS:	477
Synonyms:	PROPANE-1,2-DIOL ESTERS OF FATTY ACIDS
Functional class:	EMULSIFIER
Latest evaluation:	1973
ADI:	0-25 mg/kg bw
Comments:	As propylene glycol
Report:	NMRS 53/TRS 539-JECFA 17/21
Specifications:	COMPENDIUM ADDENDUM 5/FNP 52 Add.5/135 (1997)
Tox monograph:	FAS 5/NMRS 53A-JECFA 17/275
Previous status:	1996, COMPENDIUM ADDENDUM 4/FNP 52 Add.4/123. R,T 1973, FNP 4-JECFA 17/288 (1978), R; COMPENDIUM/1225 1969, NMRS 46/TRS 445-JECFA 13/15, FAS 70.37/NMRS 46B-JECFA 13/57. 0-20 (AS PROPYLENE GLYCOL). FU. R 1966, NMRS 43/TRS 373-JECFA 10/26. 0-20 (CONDITIONAL, 20-60; AS PROPYLENE GLYCOL). CO 1965, FAS 67.29/NMRS 40A,B,C-JECFA 9/102, FAS 67.29/NMRS 40A,B,C-JECFA 9/102. N

12 Nov 01

Home

Toxicological evaluation of some food additives including anticaking agents, antimicrobials, antioxidants, emulsifiers and thickening agents

WHO FOOD ADDITIVES SERIES NO. 5

The evaluations contained in this publication were prepared by the Joint FAO/WHO Expert Committee on Food Additives which met in Geneva, 25 June - 4 July 1973[1]

World Health Organization
Geneva
1974

[1] Seventeenth Report of the Joint FAO/WHO Expert Committee on Food Additives, Wld Hlth Org. techn. Rep. Ser., 1974, No. 539; FAO Nutrition Meetings Report Series, 1974, No. 53.

PROPYLENE GLYCOL ESTERS OF FATTY ACIDS

Explanation

These substances have been evaluated for acceptable daily intake by the Joint FAO/WHO Expert Committee on Food Additives (see Annex 1, Ref. No. 13) in 1966.

Since the previous evaluation, additional data have become available and are summarized and discussed in the following monograph. The previously published monograph has been expanded and is reproduced in its entirety below.

BIOLOGICAL DATA

BIOCHEMICAL ASPECTS

Pancreatic lipase hydrolyzed 70% of propylene glycol monostearate *in vitro* at 40° in 15 hours (Balls & Matlack, 1938). Similarly, steapsin hydrolized 70% of propylene glycol distearate (PGDS) *in vitro* at 30° in 18 hours (Long et al., 1958). The absorption, metabolism and hydrolysis of PGDS was studied in rats using isotopically labelled compounds, and found to be similar to those of the glyceryl stearate esters (Long et al., 1958a, 1958b).

Metabolic studies were carried out with ^{14}C-stearyl and ^{14}C-succinate labelled stearyl propylene glycol hydrogen succinate. The substance was hydrolyzed *in vitro* by rat pancreatic juice and

bile to yield stearic acid, propylene glycol monostearate, succinic acid, propylene glycol mono hydrogen succinate, and propylene glycol. After oral administration to rats, the proportions of radioactivity appearing in expired CO_2 corresponded closely to those obtained when ^{14}C-stearyl soybean oil or ^{14}C-succinic acid were administered. Likewise, the proportions in urine, faeces and the carcass were similar. A small part of the radioactivity in urine was as propylene glycol hydrogen (^{14}C)-succinate. The substance was also found in the urine of two men (28 and 35 years of age) who took 10 g of non-radioactive stearyl propylene glycol over a 48 hour period: the amount of the partially hydrolyzed material recovered corresponded to about 0.1% of that administered (King et al., 1970).

TOXICOLOGICAL STUDIES

Acute toxicity

Oral toxicities studies were performed in the rat for propylene glycol diacetate. It was shown that propylene glycol diacetate possesses an LD_{50} of 13.53 g/kg (Smyth et al., 1941).

Short-term studies

Rat

Six 21-day-old rats were fed for 40 days a diet containing 60% propylene glycol ester. The animals showed no adverse effect on body weight gain. On histological examination of the kidneys no lesions were observed (Lepkovsky et al., 1935).

Rats in groups of 48 were fed for 13 weeks on diets containing 0, 1.5, 3.36 and 7.52% of propylene glycol monostearate with mono- and diglycerides added to bring the total fat to 7.52%. There were no differences between the groups in respect of growth, relative organ weight (adrenals, gonads, heart, kidneys, liver, spleen, brain), histology, blood glucose, BUN, plasma cholesterol, plasma glutamate-pyruvate transaminase, haemoglobin, haematocrit, white cell count, white cell differential counts, clotting time or urinary analyses (Brandner, 1973).

A preparation containing 50% of propylene glycol esterified with stearic and succinic acids (stearyl propylene glycol hydrogen succinate), 17% of propylene glycol monostearate and lesser amounts of other propylene glycol derivatives ("Succistearin") was incorporated in diets at 2.5, 5 and 10% levels and fed to rats (10 per group) for six months. It was reported that there was no evidence of gross or histological pathology attributable to the substance (King et al., 1971).

Dogs

A preparation named Succistearin was fed at levels of 5 and 10% in the diet to groups of four dogs for six months. There were no signs of toxicity (King et al., 1971).

Long-term studies

No data are available.

Comments:

There is evidence that the propylene glycol esters of fatty acids are hydrolyzed to propylene glycol and fatty acids. Evaluation is based on the contents of propylene glycol, for which an acceptable daily intake has been established.

EVALUATION

Estimate of acceptable daily intake for man

0-25 mg/kg bw.*

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Smyth, H. F. jr, Seaton, J. & Fisher, L. J. (1941) Ind. Hyg. Tox., 23, 259-268

* Calculated as propylene glycol.

See Also:
Toxicological Abbreviations
Propylene glycol esters of fatty acids (FAO Nutrition Meetings Report Series 40abc)
Propylene glycol esters of fatty acids (JECFA Evaluation)

PETITION TO INCLUDE PROPYLENE GLYCOL MONOLAURATE ONTO 7 CFR § 205

APR 24 2009

DOCUMENT 4

A PETITION SUBMITTED TO

USDA/AMS/TM/NOP

BY

TECHNOLOGY SCIENCES GROUP INC.
4061 North 156th Drive
Goodyear, AZ 85395

ON BEHALF OF

OTSUKA CHEMICAL COMPANY LTD.
3-2-27 Otedori, Chuo-Ku
Osaka, 540-0021, Japan

THIS VOLUME CONTAINS NO CONFIDENTIAL BUSINESS INFORMATION

ALL COMMUNICATIONS REGARDING THIS PETITION SHOULD BE DIRECTED TO

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INTRODUCTION

These waivers are being submitted post-approval of the registration of Acaritouch at the request of the EPA transmitted by e-mail by Dr. Carol Frazer to Dr. Weatherston on March 23, 2005. The data requirements for which waivers are requested are, nature of residue [OPPTS 860.1300], residue analytical method [OPPTS 860.1340] and magnitude of residue [OPPTS 860.1500].

By way of background, in the application for registration of Acaritouch, Toagosei used the selective method of data support [40 CFR 152.90]. Toagosei did not list these three residue requirements in its selective data matrix, and stated to EPA its belief that Toagosei was not required to address these data requirements. Inasmuch as the EPA approved Toagosei's application without requiring Toagosei to address these data requirements it appears that EPA agreed with Toagosei's position even although no formal written waiver requests were submitted addressing these three data requirements.

The Agency has now advised that its regulations at 40 CFR 158.45 (a)(2) only allow the granting of a waiver on receipt of a formal written request from the applicant. Because EPA apparently granted waivers of these three data requirements without a formal written request from Toagosei, Toagosei is now submitting these formal waiver requests to rectify this omission.

Rationale for a request for waivers from the requirements for Nature of the Residue studies (OPPTS 860.1300)

Toagosei Co., Ltd. requests waivers from the requirements for Nature of the Residue studies for the active ingredient, propylene glycol monolaurate, based on the facts that: 1) propylene glycol monolaurate is readily and fully metabolized in animals to naturally occurring products that are normal components of lipid and carbohydrate metabolism, 2) propylene glycol monolaurate may be readily metabolized in plants to naturally occurring products that are normal components of lipid metabolism and other known metabolites, 3) propylene glycol monolaurate is an FDA approved direct food additive, and 4) propylene glycol monolaurate has been evaluated for safety as a food additive by the WHO.

The World Health Organization has summarized the metabolism of the fatty acid esters of propylene glycol in mammals (JECFA, 1966, WHO, 1974). The fatty acid esters of propylene glycol, including propylene glycol monolaurate, are hydrolyzed in the GI tract by pancreatic lipase to yield free fatty acids and propylene glycol (WHO, 1974; King et al., 1970; King et al., 1971). These free fatty acids produced by hydrolysis are indistinguishable from fatty acids from natural sources, which are a major source of energy in the body. Hydrolysis of propylene glycol monolaurate produces lauric acid, the principal fatty acid found in coconut milk. Lauric acid is metabolized in the body through beta-oxidation. Propylene glycol, the other product of the hydrolysis reaction, is of low toxicity and the pathways and products of propylene glycol metabolism have been well characterized (Ruddick, 1972; Snyder and Andrews, 1996). Propylene glycol is absorbed from the GI tract and oxidized in the liver by alcohol dehydrogenase to form lactic acid. Lactic acid is further metabolized to pyruvic acid, a normal constituent of carbohydrate metabolism. Thus the qualitative metabolic fate of propylene glycol monolaurate in mammals including livestock is understood.

Plants have the capacity to store and utilize energy in the form of triacylglycerol, and the lipases involved in metabolism of glycerol esters in plants have been well characterized (e.g., Eastmond, 2004; Thorneycroft, et al., 2001). In plants as in animals, lipases breakdown triglycerides into fatty acids that undergo beta-oxidation. These lipases would be expected to metabolize the propylene glycol fatty acid esters if incorporated into the plant just as the mammalian lipases are able to. The resulting action of plant lipase on propylene glycol monolaurate is production of the free fatty acid monolaurate that may undergo beta-oxidation just as the naturally occurring fatty acids do. Propylene glycol, the other product of the hydrolysis reaction is an approved inert ingredient on the U.S. EPA 4B list of ingredients that have sufficient data to substantiate they can be used safely in pesticide products. Propylene glycol is also an approved food additive (e.g. 21 CFR 169.175). Thus the qualitative metabolic fate of propylene glycol monolaurate in plants is understood.

Propylene glycol monolaurate is a food additive generally recognized as safe (GRAS) by the U.S. Food and Drug Administration (FDA; 21 CFR 172.856). Propylene glycol monolaurate is a multipurpose additive permitted for direct addition to food in amounts required to produce its intended effect, for example as shortening in breads (21CFR 136.110). The WHO and Food and Agriculture Organization (FAO) derived an acceptable daily intake (ADI) for fatty acid esters of propylene glycol of 25 mg/kg/day.

Toagosei Co., Ltd. requests waivers from the requirements for Nature of the Residue studies for propylene glycol monolaurate. The metabolic fate of propylene glycol monolaurate in animals has been described and the metabolic fate propylene glycol monolaurate in plants is understood adequately. Propylene glycol monolaurate is a food additive approved by the U.S. FDA and evaluated for safety by the WHO. Sufficient information is available in the scientific literature to determine that the metabolic products of propylene glycol monolaurate in livestock and plants are known, and additional studies to identify the nature of the residues in livestock and plants are not necessary.

Request for a waiver from the requirement of a Residue Analytical Method study (OPPTS 860.1340)

Toagosei Co., Ltd. requests a waiver from the requirements for a Residue Analytical Method study for the active ingredient, propylene glycol monolaurate, based on the facts that: 1) propylene glycol monolaurate is not toxic by the oral route, 2) propylene glycol monolaurate is readily metabolized to naturally occurring products that are normal components of lipid and carbohydrate metabolism, 3) propylene glycol monolaurate is an FDA approved direct food additive, and 4) propylene glycol monolaurate has been evaluated for safety by the WHO.

Propylene glycol monolaurate is not toxic by the oral route. A single large oral dose of 40,000 mg/kg propylene glycol monolaurate administered to mice resulted in no deaths during a 14-day observation period (MRID # 45852403). Clinical signs of watery diarrhea and soiled perineal region observed in treated mice were resolved in both sexes by day 3, and decreases in body weight observed in treated mice resolved for female mice by day 3 and for male mice by the end of the study.

The World Health Organization has summarized the metabolism of the fatty acid esters of propylene glycol in mammals, and the metabolites are normal components of lipid and carbohydrate metabolism (JECFA, 1966, WHO, 1974). The fatty acid esters of propylene glycol, including propylene glycol monolaurate, are hydrolyzed in the GI tract by pancreatic lipase to yield free fatty acids and propylene glycol (WHO, 1974; King et al., 1970; King et al., 1971). These free fatty acids produced by hydrolysis are indistinguishable from fatty acids from natural sources, which are a major source of energy in the body. Hydrolysis of propylene glycol monolaurate produces lauric acid, the principal fatty acid found in coconut milk. Lauric acid is metabolized in the body through beta-oxidation. Propylene glycol, the other product of the hydrolysis reaction, is of low toxicity and the pathways and products of propylene glycol metabolism have been well characterized (Ruddick, 1972; Snyder and Andrews, 1996). Propylene glycol is absorbed from the GI tract and oxidized in the liver by alcohol dehydrogenase to form lactic acid. Lactic acid is further metabolized to pyruvic acid, a normal constituent of carbohydrate metabolism. Thus metabolites of propylene glycol monolaurate are normal components of lipid and carbohydrate metabolism.

Propylene glycol monolaurate is a food additive GRAS by the U.S. FDA (21 CFR 172.856). Propylene glycol monolaurate is a multipurpose additive permitted for direct addition to food in amounts required to produce its intended effect, for example as shortening in breads (21CFR 136.110). The WHO and FAO derived an ADI for fatty acid esters of propylene glycol of 25 mg/kg/day (WHO, 1974). The evaluations conducted by the FDA and the WHO demonstrate that there is a long history of human consumption of monoesters of fatty acids in foods with no apparent adverse impacts.

Toagosei Co., Ltd. requests a waiver from the requirement of a Residue Analytical Method for propylene glycol monolaurate. A recent acute toxicity study demonstrated that propylene glycol monolaurate is not toxic by the oral route. The metabolism of propylene glycol monolaurate has been described and the metabolites are normal components of lipid and carbohydrate metabolism. Propylene glycol monolaurate is an approved food additive considered GRAS by the U.S. FDA and evaluated for safety by the WHO. The facts that propylene glycol monolaurate is an approved food additive with a long history of use in the U.S. and throughout the world with no indication of adverse effects, and that it is metabolized to normal components of lipid and carbohydrate metabolism suggest that an analytical method to characterize the residues in crops and livestock is not necessary.

Request for a waiver from the requirement of a Magnitude of the Residue study (OPPTS 860.1500)

Toagosei Co., Ltd. requests a waiver from the requirement of a Magnitude of the Residue study for Acaritouch, containing the active ingredient propylene glycol monolaurate, based on the facts that: 1) Acaritouch and propylene glycol monolaurate are not toxic by the oral route, 2) propylene glycol monolaurate is readily metabolized to naturally occurring products that are normal components of lipid and carbohydrate metabolism, 3) propylene glycol monolaurate is an FDA approved direct food additive, and 4) propylene glycol monolaurate has been evaluated for safety by the WHO.

Acaritouch and propylene glycol monolaurate are not toxic by the oral route. A single oral dose of Acaritouch containing the active ingredient propylene glycol monolaurate was not toxic to mice following a single oral dose of 5,000 mg/kg, and the reported LD50 was greater than 5,000 mg/kg (MRID # 45852406). A single oral dose of 40,000 mg/kg propylene glycol monolaurate administered to mice resulted in no deaths during a 14-day observation period (MRID # 45852403). Clinical signs of watery diarrhea and soiled perineal region observed in treated mice were resolved in both sexes by day 3, and decreases in body weight observed in treated mice resolved for female mice by day 3 and for male mice by the end of the study.

The World Health Organization has summarized the metabolism of the fatty acid esters of propylene glycol in mammals, and the metabolites are normal components of lipid and carbohydrate metabolism (JECFA, 1966, WHO, 1974). The fatty acid esters of propylene glycol, including propylene glycol monolaurate, are hydrolyzed in the GI tract by pancreatic lipase to yield free fatty acids and propylene glycol (WHO, 1974; King et al., 1970; King et al., 1971). These free fatty acids produced by hydrolysis are indistinguishable from fatty acids from natural sources, which are a major source of energy in the body. Hydrolysis of propylene glycol monolaurate produces lauric acid, the principal fatty acid found in coconut milk. Lauric acid is metabolized in the body through beta-oxidation. Propylene glycol, the other product of the hydrolysis reaction, is of low toxicity and the pathways and products of propylene glycol metabolism have been well characterized (Ruddick, 1972; Snyder and Andrews, 1996). Propylene glycol is absorbed from the GI tract and oxidized in the liver by alcohol dehydrogenase to form lactic acid. Lactic acid is further metabolized to pyruvic acid, a normal constituent of carbohydrate metabolism. Thus metabolites of propylene glycol monolaurate, the active ingredient in Acaritouch, are normal components of lipid and carbohydrate metabolism.

Propylene glycol monolaurate is a food additive approved by the U.S. FDA (21 CFR 172.856). Propylene glycol monolaurate is a multipurpose additive

permitted for direct addition to food in amounts required to produce its intended effect, for example as shortening in breads (21CFR 136.110). The WHO and FAO derived an ADI for fatty acid esters of propylene glycol of 25 mg/kg/day (WHO, 1974). These evaluations demonstrate that there is a long history of human consumption of monoesters of fatty acids in foods.

Toagosei Co., Ltd. requests a waiver from the requirement of a Magnitude of the Residue study for Acaritouch, containing the active ingredient propylene glycol monolaurate. Recent acute toxicity studies have demonstrated that Acaritouch and propylene glycol monolaurate are not toxic by the oral route. The metabolism of propylene glycol monolaurate has been described and the metabolites are normal components of lipid and carbohydrate metabolism. Propylene glycol monolaurate is an approved food additive considered GRAS by the U.S. FDA and evaluated for safety by the WHO. The facts that propylene glycol monolaurate is an approved food additive with a long history of use in the U.S. and throughout the world with no indication of adverse effects, and that it is metabolized to normal components of lipid and carbohydrate metabolism suggest that studies to determine the magnitude of residues in crops and livestock are not necessary.

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WHO 1974. Toxicological evaluation of some food additives including anticaking agents, antimicrobials, antioxidants, emulsifiers and thickening agents. Propylene glycol esters of fatty acids. Food Additives Series No 5, World Health Organization, Geneva.

APPENDIX

This appendix contains copies of two publications used in the preparation of the wiavers; these publications are:

Eastmond PJ, 2004 Cloning and characterization of the acid lipase from castor beans. J. Biological Chemistry, 279 (44): 45540 - 45545.

Thorneycroft D., Sherson SM Smith SM, 2001. Using gene knockouts to investigate plant metabolism. J. Experimental Botany, 52(361): 1593 - 1601.

All other citations given in the references have previously been submitted to the Agency either as studies identified by MRID number, or in the appendix of the previous request for waivers volume, Volume 70231-E-17.

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Cloning and Characterization of the Acid Lipase from Castor Beans*

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Published, JBC Papers in Press, August 19, 2004, DOI 10.1074/jbc.M408686200

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Castor bean endosperm contains a well known acid lipase activity that is associated with the oil body membrane. In order to identify this enzyme, proteomic analysis was performed on purified oil bodies. A ~60-kDa protein was identified (RcOBL1), which shares homology with a lipase from the filamentous fungus *Rhizomucor miehei*. RcOBL1 contains features that are characteristic of an α/β-hydrolase, such as a putative catalytic triad (SDH) and a conserved pentapeptide (GXSXG) surrounding the nucleophilic serine residue. RcOBL1 was expressed heterologously in *Escherichia coli* and shown to hydrolyze triolein at an acid pH (optima ~4.5). RcOBL1 can hydrolyze a range of triacylglycerols but is not active on phospholipids. The activity is sensitive to the serine reagent diethyl *p*-nitrophenyl phosphate, indicating that RcOBL1 is a serine esterase. Antibodies raised against RcOBL1 were used to show that the protein is restricted to the endosperm where it is associated with the surface of oil bodies. This is the first evidence for the molecular identity of an oil body-associated lipase from plants. Sequence comparisons reveal that families of OBL1-like proteins are present in many species, and it is likely that they play an important role in regulating lipolysis.

Many eukaryotic organisms store (and transport) chemical energy in the form of triacylglycerol (TAG),[1] which is contained in small (0.2–2 μm) oil bodies surrounded by a phospholipid monolayer (1, 2). In plants oil bodies have been found in various tissues (1, 2). They are particularly abundant in many seeds whereupon germination the TAG is broken down, and the carbon skeletons are used to fuel post-germinative growth (1, 2). The initial step in this metabolic process is catalyzed by lipase (EC 3.1.1.3), which hydrolyzes TAG at the oil/water interface to yield free fatty acids and glycerol (3). The free fatty acids are then transferred to the glyoxysome and activated to acyl-CoAs for subsequent catabolism by β-oxidation. Much of the acetyl-CoA produced is ultimately converted to sugars by the glyoxylate cycle and gluconeogenesis. These latter pathways have been relatively well studied in plants, and many of the genes concerned have been cloned and characterized (4). In contrast, remarkably little is known about the process of lipolysis despite its fundamental importance.

Lipase activities have been studied at the biochemical level in a variety of seeds (3). In most, the activities are only detectable upon germination and increase concomitantly with the disappearance of TAG (3). These lipase activities are usually membrane-associated and can be found in the oil body, glyoxysome, or microsomal fractions of seed extracts, depending upon the species (3). Because lipases are interfacial enzymes, those that reside on the surface of the oil body might logically be expected to play a role in TAG breakdown. However, studies using electron microscopy have indicated that oil bodies are in close proximity with other organelles (particularly glyoxysomes), and it has long been hypothesized that an association between them may be required to facilitate fatty acid release and transfer (5, 6). Lipases have been purified to apparent homogeneity from the seeds of several plants such as maize (*Zea maize*) (7), castor (*Ricinus communis*) (8, 9), and ironweed (*Vernonia galamensis*) (10). However, most surprisingly none of the genes that encode these enzymes have been cloned and characterized. Consequently, many questions remain concerning the mechanism and regulation of lipolysis.

Probably the best studied lipase from plants is the castor bean acid lipase, which was first discovered over a century ago (11). The enzyme is unusual in that it is extremely active in mature seeds (prior to germination) and has a low pH optima of ~4.2. Nevertheless, it is associated with the oil body membrane (12), and its catalytic properties are quite well defined (13, 14) and broadly similar to lipases from mammals and fungi. Fuchs *et al.* (9) were able to partially solubilize the acid lipase from oil body membranes and purified a ~58-kDa protein using chromatographic methods. Subsequently, Altaf *et al.* (15) raised an antibody against a major oil body membrane protein of similar molecular weight and showed that it could immunoprecipitate acid lipase activity. The polypeptide composition of oil body membranes is relatively simple (1, 2), and here a proteomic approach has been used to identify, clone, and characterize the gene. The castor acid lipase exhibits homology with several fungal lipases, and orthologs are present in other plant species, defining a new family of putative oil body-associated lipases.

EXPERIMENTAL PROCEDURES

Plant and Chemical Sources—Castor beans (*R. communis* var. *gibsonii*) were supplied by Chiltern Seeds (Ulverston, Cumbria, UK). The beans were soaked in running water for 1 day and germinated in the dark on moist vermiculite at 30 °C. All reagents were obtained from Sigma, except for glycerol tri[1-^{14}C]oleate that was from Amersham Biosciences. Antibodies raised against the ~60-kDa band (RcOBL1) from castor bean oil body membranes (15) were provided by Dr. Mustak A. Kaderbhai, at the Institute of Biological Sciences, University of Wales, Aberystwyth, UK.

Preparation of Oil Body Membranes—Oil bodies were isolated from the endosperm of soaked castor beans by floatation centrifugation using the method of Hills *et al.* (16). For proteomic analysis, peripheral

* This work was supported by the Biotechnology and Biological Sciences Research Council through David Phillips Research Fellowship 87/JF/16985 (to P. J. E.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY360218, AY360219, AY360220, and AY360221.

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[1] The abbreviations used are: TAG, triacylglycerol; MALDI, matrix-assisted laser desorption/ionization; CID, collision-induced dissociation; IPTG, isopropyl-β-D-thiogalactopyranoside; MBP, maltose-binding protein; OBL, oil body lipase; RACE, rapid amplification of cDNA ends; EST, expressed sequence tag; RT, reverse transcription; MS, mass spectrometry.

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proteins were removed from the oil bodies by washing them sequentially with 2 M $NaCl_2$ and 9 M urea according to Millichip *et al.* (17). The oil body membranes were delipidated by extraction with diethyl ether (16) and solubilized in SDS-loading buffer by heating at 70 °C for 10 min. The polypeptides were separated on a 10–20% (v/v) SDS-PAGE gradient gel from Bio-Rad as described by Laemmli (18), and the gel was stained using 0.1% (w/v) Coomassie Brilliant Blue R-250 in methanol:acetic acid:water (4:1:5, v/v/v).

For proteolytic treatments purified, urea washed, oil bodies were incubated in 20 mM Tris/HCl (pH 8) containing 10 μg ml^{-1} of trypsin for 2 h at 37 °C. The oil body fraction was separated by centrifugation and washed, and the peptides were analyzed by SDS-PAGE using a 10–20% (v/v) gradient gel.

Tryptic Digestion and Peptide Analysis—In-gel tryptic digestion was performed (19), and the peptides were applied to the matrix-assisted laser desorption/ionization (MALDI) target plate. Positive ion MALDI mass spectra were obtained using an Applied Biosystems 4700 Proteomics Analyzer (CTS version, Applied Biosystems) in reflectron mode with an accelerating voltage of 20 kV. MS spectra were acquired with a total of 1000 laser pulses over a mass range of *m/z* 800–4000. Final mass spectra were the summation of 20 sub-spectra, each acquired with 50 laser pulses and internally calibrated using the tryptic peptides at *m/z* 842.509 and 2211.104. Monoisotopic masses were obtained from centroids of raw, unsmoothed data.

For collision-induced dissociation (CID)-MS/MS, a source 1 accelerating voltage of 8 kV, collision energy of 1 kV, and a source 2 accelerating voltage of 15 kV were used. Air was used as the collision gas at the "medium" pressure setting of the instrument with a recharge threshold of 9.9×10^{-7} torr, which produced a source 2 pressure of about 1×10^{-6} torr. The precursor mass window was set to ±10 "Da," and the metastable suppressor was enabled. The default calibration was used for MS/MS spectra.

Mass spectral data obtained in batch mode were submitted to data base searching by using a locally running copy of the Mascot program (20) (Matrix Science Ltd., version 1.7). Batch-acquired MS and MS/MS spectral data were submitted to a combined peptide mass fingerprint and MS/MS ion search through the Applied Biosystems GPS Explorer software interface (version 1.0) to Mascot. Search criteria included the following: maximum missed cleavages, 1; variable modifications, oxidation (M); peptide tolerance, 25 ppm; MS/MS tolerance, 0.2 Da. Peptide sequence tags were generated from CID-MS/MS spectra by manual interpretation or by using a *de novo* sequencing program supplied by Applied Biosystems.

Subcellular Fractionation, Western Blotting, and Immunocytochemistry—The endosperm from soaked beans was homogenized and fractionated according to the method of Hills and Beevers (21). Western blot analysis (22) was carried out on the fractions using anti-RcOBL1 antiserum (15). Endosperm tissue was also prepared for electron microscopy, and immunocytochemistry was carried out according to the methods of Schmid *et al.* (23). Rabbit polyclonal anti-RcOBL1 (15) and goat anti-rabbit IgG conjugated to 10 nm gold were used at 1:1000 and 1:20, respectively.

RNA Extraction, cDNA Synthesis, and PCR—Total RNA from various tissues was isolated using the RNeasy kit from Qiagen Ltd. (Crawley, West Sussex, UK). The synthesis of single-stranded cDNA was carried out using SuperScript™ II RNase H⁻ reverse transcriptase from Invitrogen (Paisley, UK). Degenerate primers (5′-ttgatagtirtyagyttyaga and 5′-ctgtccraatgtrtaiarctt) corresponding to peptide sequence tags (DANLIVISFR and LLNVYTFGQPR) were designed and used to amplify a fragment of the *RcOBL1* cDNA by using PCR. The following gene specific primers were used to obtain full-length cDNA sequences by 3′- and 5′-RACE, using the SMART™ RACE cDNA amplification kit from Clontech: *RcOBL1* (5′-gaccacttggtatgggcatatgatgg and 5′-catgtcattgcagtaaaccaccctga), *RcOLE1* (5′-ttggcgacttctttgaacttggaagctact and 5′-aaatgccatcatagcaaggccaataacaac), *RcOLE2* (5′-cgatggcctttgaaccaagacaaatctat and 5′-gtattgttacaggaaagcagccaccaggag), *RcACT* (5′-cgttctctccttgtatgccagtggtc and 5′-gagctgctcttggcagtctcaagttc). The same primers were also used to detect gene expression via RT-PCR.

Expression of RcOBL1 and Determination of Lipase Activity—A truncated version of the RcOBL1 cDNA that lacks the hydrophobic N terminus was amplified using primers 5′-gaattcgtgtcgcaccaggcagacgaagtgatttca and 5′- tctagactagtaaccttgtgccatcattttcagag and cloned into the pCR 2.1-TOPO vector from Invitrogen (Paisley, UK). The insert was then excised by using EcoRI and XbaI and cloned into the pMAL-c2E vector from New England Biolabs (Hitchin, Hertfordshire, UK). The cMBP fusion protein was expressed in BL21-Codon Plus-RIL cells from Stratagene (La Jolla, CA). The cells were cultured at 30 °C and induced using 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The culture was centrifuged at 700 × *g* for 10 min, and the pellet was resuspended in 200 mM sodium acetate (pH 4.2) plus 2 mM dithiothreitol. The cells were lysed by sonication and the cell debris removed by centrifugation at 21,000 × *g* for 10 min. Western blot analysis (22) was carried out on the supernatant by using anti-RcOBL1 antiserum (15). Protein content was determined as described by Bradford (24) using bovine serum albumin as a standard.

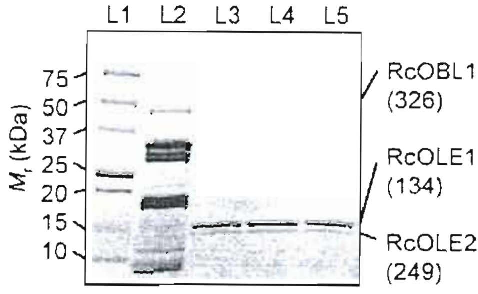

FIG. 1. **Analysis of oil body membrane polypeptides from castor bean endosperm.** Polypeptides were separated on a 10–20% (v/v) SDS-PAGE gradient gel. Molecular weight (M_r) markers, *lane 1*; crude extract, *lane 2*; oil bodies purified by floatation centrifugation, *lane 3*; $NaCl_2$-washed oil bodies, *lane 4*; and urea washed oil bodies, *lane 5*. The ~60, ~16, and ~14-kDa bands were subjected to tryptic digestion, and the peptides were analyzed by MALDI and CID-MS/MS. The corresponding cDNAs were cloned, and the Mascot (20) total ion scores from combined peptide mass fingerprint and MS/MS ion searches are shown in *parentheses*. Scores greater than 25 are statistically significant ($p < 0.05$).

Assays were performed on the supernatant by using an emulsified substrate essentially according to the method of Fuchs *et al.* (9). Reactions were carried out at 30 °C in a 100-μl reaction mixture consisting of 200 mM sodium acetate (pH 4.5), 2 mM DTT, and substrate. The substrates were emulsified in 5% (w/v) gum arabic using sonication (9), and 10 μl was added to the assay mixture. Reactions using [^{14}C]triolein as the substrate were stopped by the addition of 1 ml of chloroform: methanol:heptane (1.25:41:1, v/v/v) and 72 μl of 0.2 M NaOH, 150 mM $NaCl_2$ plus lipid carrier (50 μg of oleic acid, mono-, di-, and trioleoylglycerol). The reactions were vortexed and centrifuged for 5 min at 10,000 × *g*. 0.4 ml of the upper phase was removed and subjected to liquid scintillation counting. Alternatively to analyze the products, the assays were stopped with 1 ml of chloroform:methanol (2:1, v/v), and the total lipids were extracted according to Folch *et al.* (25), and the distribution of ^{14}C in acylglycerols and free fatty acids was determined by thin layer chromatography (26).

For assays using various nonradiolabeled substrates, the reactions were conducted in essentially the same manner as described above, but they were stopped with 200 μl of isopropyl alcohol and dried, and the pellet was resuspended in water. Free fatty acid content was measured with the NEFA colorimetric kit (Wako Chemicals, Neuss, Germany) according to the manufacturer's instructions. The liberation of *p*-nitrophenol from *p*-nitrophenyl esters was monitored spectophotometrically at 405 nm (9). The release of CoA from oleoyl-CoA was also monitored colorimetrically at 412 nm via detection of the complex formed following the addition of 1 mM 5,5′-dithiobis(2-nitrobenzoate) (26).

RESULTS

Analysis of Oil Body Membrane Proteins—Castor beans contain a well defined acid lipase activity, which is associated with the oil body membrane (12–15). To identify this enzyme, oil bodies were isolated from the endosperm of castor beans by floatation centrifugation (16) and stripped of peripheral proteins by washing sequentially with 2 M $NaCl_2$ and then 9 M urea (17). The polypeptides that are associated with the oil body membrane were solubilized in SDS loading buffer and separated by SDS-PAGE (Fig. 1). Three major bands of ~60, ~16 and ~14 kDa were visible. The bands were excised and subjected to tryptic digestion, and the resulting peptides were analyzed by MALDI-MS and CID tandem MS. The combined data were used to query the NCBI nonredundant and EST data bases with Mascot software (20). The ~60-kDa band matched most closely to an *Arabidopsis thaliana* lipase-like protein

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>RcOBL1
MDDAGKITSTSHLIVSPDEGTFLDLFKHIVLSDLGSGAKFFRASDQRVPATAAYYSRWPVSVFICKILQL   70
FQMPAAMLGHLTDFLLNFYYQNHGFLGILRNIFLIRLKIPKRGEADFISTIGYLDSRMDLHGTPMVSHQA  140
DEVISNADNPSLKEGHNSKIKGALGNRSLMDLCIMASKLAYENTKVVERVVAEHWKMHFVADYGGMNYFQ  210
DARNTHAFIFCDKPKDANLIVISFRGTGPFSIPNWCTDFDFSLVGLGDAGSVHVGFLEAMGLGHRNSISS  280
FETSINTKSPGSITELRKESEMAPDHLVWAYDGVYFLAASTLKGLLKDHKNAKFVVTGHSLGGALAILFT  350
CILEIQQETEVLDRLLNVYTFGQPRIGNYNLGYFMQNRLNFPERRYFRVVYCNDMVPRVPFDDVFFTFEH  420
FGTCIYYDSRFFGYFTKEEPSRNPFGIENAISAHITAWWELWRSFILNHVYGAEYKETWESRMFRILGLF  490
LPGVAAHSPVNYVNSVRLGRELAIPLMSLKMMAQGY                                  526

>RcOLE1
MADRPQPHQVQVHRYDPTTGYKGQQKGPSASKVLAVLTFLPVGGGLLSLSGITLTNTLIGMAIATPLFIL   70
FGPIILPAAVVIGLAMMAFMVAGALGLSGLTSQSWALKYFREGTAMPESLDQAKKRMQDMAGYVGMKTKE  140
VGQDIQRKAQEGK                                                         153

>RcOLE2
MAEHQQSPVVSHRPRVNQLVKAGTAATAGSSLLFLSGLTLTGTVIALALATPLMVLFSPVLLPAVIIISL   70
IGAGFLTSGGFGFGAILVLSWIYRYVTGKQPPGAESLDQARLKLAGKAREMKDRAEQFGQHVTGQQTS  138
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FIG. 2. **The amino acid sequences of RcOBL1, RcOLE1 and RcOLE2.** In RcOBL1 the conserved lipase motif (PS00120: (LIV)*X*(LIVFY)(LIVMST)G(HYWV)SXG(GSTAC)) surrounding the catalytic serine residue is shown in *italics*, and the putative catalytic triad (SDH) is shown in **boldface**. In RcOLE1 and RcOLE2, the regions predicted to be hydrophobic (28) are *underlined*, and the three conserved proline residues that define the "proline knot" motif (36) are also shown in **boldface**.

(At3g14360). The ~16- and ~14-kDa bands matched two castor oleosin ESTs (GenBank™ accession numbers T14916 and T14903, respectively). Full-length cDNAs were obtained for each oleosin by 3'- and 5'-RACE. The sequences were designated *RcOLE1* and *RcOLE2* and submitted to GenBank™. The total ion scores for the ~16-kDa band and the ~14-kDa band, when matched to RcOLE1 and RcOLE2, were 134 and 249, respectively (25 = $p < 0.05$) (Fig. 1).

Cloning of Oil Body Lipase 1 (OBL1)—In order to clone a cDNA encoding the ~60-kDa lipase-like protein, peptide sequences tags were interpreted from the CID-MS/MS spectra. Degenerate primers were designed, based on two peptide sequences (DANLIVISFR and LLNVYTFGQPR), and a cDNA fragment was amplified by RT-PCR. Gene-specific primers were then used to obtain a full-length cDNA sequence by 3'- and 5'-RACE. This sequence was designated Oil Body Lipase 1 (OBL1) and submitted to the GenBank™ data base. The cDNA is 1870 bp and contains a 1578-bp putative open reading frame. The deduced RcOBL1 protein is 525 amino acids (Fig. 2), has a calculated molecular mass of 59.6 kDa and a predicted pI of 6.68. The total ion score for the ~60-kDa band, when matched to RcOBL1, was 326 (25 = $p < 0.05$), and both peptide sequences tags were present (Fig. 1).

A similarity search revealed that the C-terminal region of RcOBL1 (amino acid residues ~220 to ~410) shares a low level of identity (31–28%) with the well characterized lipases from the filamentous fungi *Rhizomucor miehei*, *Rhizopus oryzae*, and *Thermomyces lanuginosa*. Lipases from different organisms can vary considerably in their primary amino acid sequences. However, all lipases are "serine esterases" and exhibit a common structural feature called an α/β fold that consists of a central β-sheet composed of parallel strands that are linked via α-helices (27). The nucleophilic serine forms part of a catalytic triad (S(DE)H) and is located in a sharp turn (nucleophilic elbow). A conserved signature surrounds the serine residue (PROSITE accession number PS00120: (LIV)*X*(LIVFY)(LIVMST)G(HYWV)SXG(GSTAC)). The RcOBL1 amino acid sequence (Fig. 2) contains both a putative catalytic triad (Ser-340, Asp-404, and His-497) and all but the first residue of the conserved signature (FVVTGHSLGG).

Association with the Oil Body—Acid lipase activity has been shown previously (15) to be associated exclusively with the oil body fraction from castor bean endosperm homogenates. Antibodies raised against the ~60-kDa band from purified castor bean oil body membranes (15) were used to investigate the subcellular localization of RcOBL1 in endosperm tissue from soaked beans. Fractionation of a homogenate was performed by centrifugation (21). The proteins from the oil pad, the 10,000 × *g* pellet, the 100,000 × *g* pellet, and the 100,000 × *g* supernatant were then separated by SDS-PAGE, and the presence of RcOBL1 was determined by Western blotting. RcOBL1 was only detected in the oil pad (Fig. 3*A*). To investigate the localization of RcOBL1 in intact tissue, immunogold labeling was performed on EM sections. Gold particles were found to be associated with the surface of oil bodies (Fig. 3*B*).

Oleosins (such as RcOLE1 and RcOLE2; Fig. 2) are anchored to the oil body by a single central hydrophobic domain consisting of ~70 amino acids, which is flanked by more hydrophilic regions (1, 2). Hydropathicity predictions using a variety of standard algorithms suggest that RcOBL1 is relatively amphipathic but does not contain a hydrophobic region as prominent as that found in oleosins. Of the algorithms that were used, the Hopp and Woods scale (28) (with a window size of 17 amino acids) predicted the longest hydrophobic stretch of amino acids in RcOBL1, situated near the N terminus (Fig. 3*C*).

Although the N terminus of RcOBL1 does not show significant sequence homology to oleosins, the prediction of a relatively hydrophobic region suggests that it might play a role in anchorage. To address this, purified urea washed oil bodies were subjected to proteolytic treatment using trypsin (29). The oil bodies were recovered by centrifugation and washed, and the remaining peptides were separated by SDS-PAGE (Fig. 3*D*). A major band of ~8-kDa and a minor ~6-kDa band were visible following proteolysis. The bands were further digested and analyzed using MALDI and CID-MS/MS. The ~8-kDa band contained peptide sequences tags from the hydrophobic regions of RcOLE1 and RcOLE2, and the ~6-kDa band contained tags from the predicted N-terminal hydrophobic region of RcOBL1 (Fig. 3*D*).

Determination of Enzyme Activity—To characterize further *RcOBL1*, the protein was expressed heterologously in *Escherichia coli*. Acid lipase activity from purified castor oil body membranes is poorly solubilized in detergents, and the protein has a strong tendency to aggregate (9, 15). Therefore, to maximize the likelihood that the recombinant protein would be soluble, a truncated version (lacking the first 135 amino acids) was produced, fused to maltose-binding protein (cMBP). The ~86-kDa fusion protein was detected by Western blotting, in soluble extracts from *E. coli* harboring the expression vector,

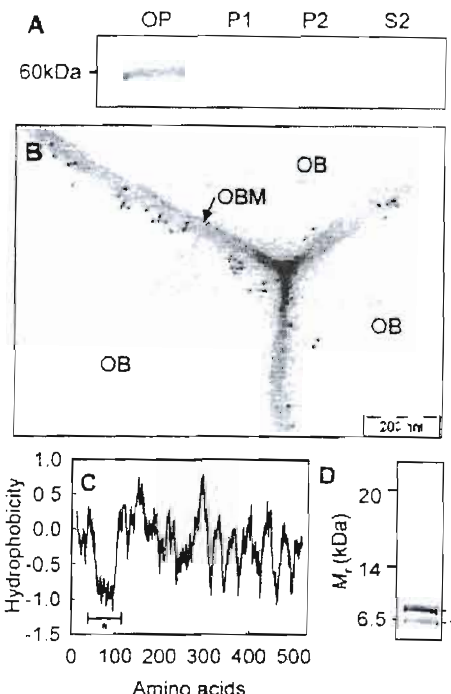

FIG. 3. **Association of RcOBL1 with the oil body.** *A*, subcellular fractionation of homogenized endosperm tissue. Protein was separated by SDS-PAGE, Western-blotted, and probed with antibodies against RcOBL1. *OP* is oil pad; *P1* is 10,000 × *g* pellet; *P2* is 100,000 × *g* pellet; and *S2* is 100,000 × *g* supernatant. *B*, EM of castor seed endosperm. Sections were labeled with 10 nm gold particles using RcOBL1 antibodies. *Bar* = 200 nm. *OB* is oil body, and *OBM* is oil body membrane. *C*, a hydropathicity plot of RcOBL1 using the Hopp and Woods scale (28), with a window size of 17 amino acids. *D*, protease treatment of purified urea-washed oil bodies using trypsin. After repeated washes the peptides retained by the oil bodies were solubilized, separated by SDS-PAGE, and analyzed using MALDI and CID-MS/MS. The ~6-kDa band contains peptide sequence tags from the region marked in *C*.

following induction by IPTG (Fig. 4*A*).

To investigate whether this protein had lipase activity, the capacity of extracts to hydrolyze [^{14}C]triolein emulsified with gum arabic was measured (9). No activity was detected in extracts from cells that were expressing MBP alone (data not shown). However, in those that contained the cMBP-RcOBL1 vector, induction by IPTG resulted in a >10-fold increase in acid lipase activity (Fig. 4*B*). The rate of hydrolysis was proportional to the amount of protein (data not shown) and also to the amount of emulsified substrate, up to ~18 mg ml^{-1} (equivalent to ~20 mM) (Fig. 4*C*). When the pH of the assay medium was varied, the optimum was found to be between 4 and 4.5 (Fig. 4*D*). Essentially no activity was detected above pH 6.

The activity of cMBP-RcOBL1 was also tested against a variety of other substrates (Table I). cMBP-RcOBL1 exhibited the greatest activity on TAGs containing short to medium chain saturated fatty acids, but it was also active on those with long chain saturated and unsaturated fatty acids (Table I). Castor oil, which contains the unusual hydroxylated fatty acid ricinoleic acid, is also hydrolyzed (Table I). Analysis of the products of [^{14}C]triolein hydrolysis after a 10-min incubation revealed that very little 1,3-dioleoylglycerol is produced *versus* mono-oleoylglycerol and 1,2(2,3)-dioleoyl-*sn*-glycerol (data not shown). In addition to TAGs, the enzyme is also capable of hydrolyzing artificial *p*-nitrophenyl fatty acid esters, but it has essentially no activity on oleoyl-CoA or a representative phospholipid (1,2-dioleoyl-3-phosphatidylcholine) (Table I).

The effect of a variety of known inhibitors and cofactors of lipases was investigated by using *p*-nitrophenyl butyrate as a

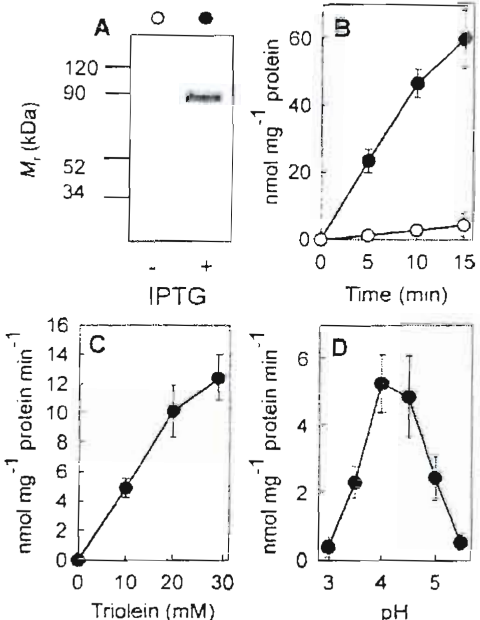

FIG. 4 **Expression of a cMBP-RcOBL1 fusion protein in *E. coli* and determination of acid lipase activity.** *A*, Western blot of soluble extracts from induced (●) (+0.4 mM IPTG) and uninduced (○) cells probed using RcOBL1 antibodies. *B*, a time course of [^{14}C]triolein hydrolysis at pH 4.5. *C*, the effect of the amount of emulsified substrate on the rate of hydrolysis. *D*, the effect of pH on the rate of hydrolysis. For assays the values are the mean ± S.E. of measurements from four separate incubations. The standard amount of substrate used was 9 mg ml^{-1} (~10 mM), except in *C* where the amount of substrate was varied. The rate of hydrolysis is consistently ~5 nmol mg total protein^{-1} min^{-1} at pH 4.5 using 10 mM triolein.

TABLE I
Substrate specificity of lipase activity from extracts of E. coli expressing cMBP-RcOBL1 fusion protein

The values are the mean ± S.E. of measurements from four separate incubations. All rates were determined as the difference between the activity from induced and uninduced cultures. Reactions contained 200 mM sodium acetate (pH 4.5), 2 mM dithiothreitol, cell extract and were initiated by the addition of substrate. The final concentration used was equivalent to 10 mM for all substrates with the exception of *p*-nitrophenyl esters and oleoyl-CoA, which were 100 μM. ND indicates not detected.

Substrate	Activity
	nmol mg protein^{-1} min^{-1}
Triolein	5.4 ± 0.3
Trilinolein	5.8 ± 0.5
Triricinolein	7.3 ± 0.2
Tripalmitin	3.4 ± 0.3
Trilaurin	8.9 ± 1.7
Tricaprin	21.4 ± 2.6
p-Nitrophenyl palmitate	2.8 ± 0.7
p-Nitrophenyl laurate	4.1 ± 0.6
p-Nitrophenyl butyrate	24.2 ± 3.9
Oleoyl-CoA	ND
1,2-Dioleoyl-3-phosphatidylcholine	ND

convenient substrate (Table II). The activity was sensitive to preincubation with a 100-fold molar excess of the classical serine esterase inhibitor diethyl-*p*-nitrophenyl phosphate. The thiol-directed reagent *p*-chloromercuribenzoic acid and $HgCl_2$ also blocked hydrolysis. The addition of $NaCl_2$ did not stimulate activity. Of the divalent cations tested, Ca^{2+} enhanced activity slightly. The inclusion of EDTA leads to a small decrease in activity.

Gene Expression and Protein Levels—RT-PCR was used to show that *RcOBL1* transcripts are present in the endosperm of mature seed but that the level declines prior to germination

TABLE II
The effect of inhibitors and cofactors on lipases activity from extracts of E. coli expressing cMBP-RcOBL1 fusion protein

Assays were performed using 100 μM *p*-nitrophenyl butyrate as the substrate in a basic reaction mixture consisting of 200 mM sodium acetate (pH 4.5) plus cell extract. Rates were determined as the difference between the activity from induced and uninduced cultures. Effectors were preincubated with cell extract for 30 min before the addition of the substrate. The values are the mean ± S.E. of measurements from four separate incubations. Activity with the addition of no effectors was 22.5 ± 1.9 nmol mg protein^{-1} min^{-1}. ND indicates not detected.

Effector	Activity
	% control
None	100
Diethyl-*p*-nitrophenyl phosphate (10 mM)	ND
p-Chloromercuribenzoic acid (1 mM)	ND
$HgCl_2$ (0.1 mM)	17 ± 2
$NaCl_2$ (150 mM)	103 ± 9
$CaCl_2$ (2 mM)	123 ± 7
$MgCl_2$ (2 mM)	106 ± 11
$MnCl_2$ (2 mM)	109 ± 5
EDTA (10 mM)	83 ± 7
BSA (1 mg/ml)	102 ± 4
Boiling extract for 15 min	ND

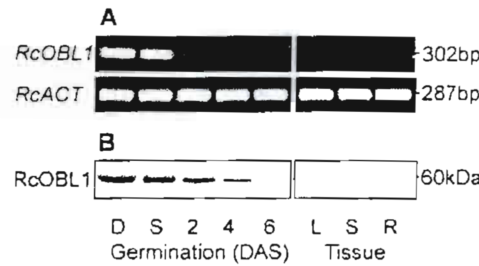

FIG. 5. **Developmental and tissue-specific distribution of RcOBL1.** *A*, RT-PCR analysis of the expression of *RcOBL1*. Expression levels are relative to *RcACT*, which was used as a constitutive control. *B*, Western blot analysis of RcOBL1. Lanes were loaded with 10 μg of total protein. *D* is dry seed endosperm; *S* is soaked seed endosperm; *DAS* is days after soaking; *R* is root; *S* is stem; and *L* is leaf. The data shown are representative of three replicate experiments performed on separate RNA or protein extracts.

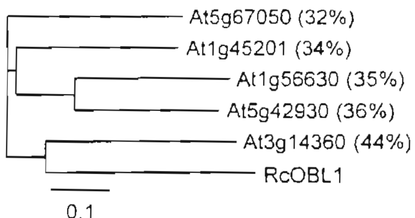

FIG. 6. ***Arabidopsis* proteins that have homology to RcOBL1.** A phylogenetic analysis was performed using ClustalW (43) and displayed with TreeView version 1.6.6. Sequences were obtained from the TIGR *Arabidopsis* data base. The existing annotations of At3g14360, At5g42930, and At1g56630 were modified based on alignments with RcOBL1. The percentage identity shared with RcOBL1 is shown in *parentheses*.

(Fig. 5*A*), which occurs ~2 days after soaking. The expression of *RcOBL1* was not detected in the leaves, stems, or roots of mature plants (Fig. 5*A*). Western blots of total protein also show that RcOBL1 is only detected in the endosperm, but the protein persists in this tissue for several days following germination, before it is eventually lost (Fig. 5*B*).

Identification of RcOBL1 Orthologs—An examination of the *Arabidopsis* genome sequence revealed that there are more than 20 lipase-like proteins with significant homology to RcOBL1. In the majority of cases the sequence similarity is limited to the lipase catalytic domain near the C terminus (particularly surrounding the nucleophilic elbow region). However, five lipase-like proteins displayed significant similarity over the whole length of RcOBL1, including the relatively hydrophobic N-terminal region (Fig. 6). A search of all available higher plant sequences suggests that there are RcOBL1 orthologs present in a taxonomically diverse set of species including soybean (*Glycine max*), tomato (*Lycopersicon esculentum*), potato (*Solanum tuberosum*), lettuce (*Lactuca sativa*), peach (*Prunus persicarice*), rice (*Oryza sativa*), maize (*Zea mays*), wheat (*Triticum aestivum*), and loblolly pine (*Pinus taeda*).

DISCUSSION

Many germinating oilseeds have been reported to contain lipase activity associated with the oil body membrane (3). It has been suggested that this activity is likely to initiate the breakdown of the TAG, releasing carbon skeletons to drive post-germinative growth (3). The genes that encode these enzymes have yet to be defined. Perhaps the best studied example of an oil body-associated lipase is the acid lipase from castor beans, which has been investigated by many researchers since its discovery over a century ago (9, 11–15). In this report the castor acid lipase has been cloned and characterized. Genes that encode acyl-hydrolases have been identified previously in plants (see for example Refs. 30 and 31). However, this is the first study to reveal the molecular identity of an oil body-associated lipase with relatively well studied TAG hydrolase activity (9, 11–15).

The castor acid lipase (RcOBL1) is homologous to lipases from several filamentous fungi and exhibits the characteristic features of an α/β-hydrolase. At the level of the primary amino acid sequence, these features include a putative catalytic triad (SDH) with a conserved motif (GXSXG) surrounding the nucleophilic serine residue (27). Overexpression of the catalytic domain in *E. coli* confirms that the protein can hydrolyze an emulsion of triolein and therefore fulfills the definition of a lipase (32). Furthermore, the activity is sensitive to the classical serine reagent diethyl-*p*-nitrophenyl phosphate, providing experimental evidence that RcOBL1 is a serine esterase (26). The enzyme is also inhibited by *p*-chloromercuribenzoic acid and mercuric ions, which react with sulfhydryl groups.

The catalytic properties of the castor bean acid lipase from purified oil body membranes have been defined previously in some detail (9, 11–15). In these preparations RcOBL1 is likely to be the most abundant enzyme (Fig. 1). The most distinctive feature of the acid lipase is its sharp pH optima of ~4.5 and its inactivity above pH 6 (13). The acid lipase is known to be capable of hydrolyzing a variety of natural and artificial esters (9, 11–15). When using triolein emulsified in gum arabic as a substrate, the rate of hydrolysis increases linearly with amount, up to at least ~18 mg ml^{-1} (9). The acid lipase exhibits apparent typo-selectivity toward TAGs containing short chain and medium chain saturated fatty acids but is also active on a variety of long chain saturated and unsaturated substrates (13). This includes the "unusual" hydroxylated fatty acid ricinoleic acid that is the principal fatty acid in castor oil (13). The enzyme has been reported to have some regio-selectivity toward fatty acyl groups at the *sn*-1 and *sn*-3 positions (33). However, in prolonged incubations it is capable of the complete hydrolysis of TAG (13). It has been proposed that acyl migration, promoted by the relatively acidic conditions of the assay, could account for this apparent discrepancy (33). Finally, the acid lipase is not active on phospholipids (13). All the characteristics of the acid lipase activity from purified oil bodies that are listed above are also evident in recombinant cMBP-RcOBL1.

Previous studies (9, 11–15) have reported that acid lipase activity is associated with castor oil body membranes. Here immunological evidence for the localization of the protein has

been provided both from subcellular fractionation experiments and from gold labeling of EM sections. Other proteins that have been identified from plant oil bodies include oleosin, caleosin, steroleosin, a putative glycosylphosphatidylinositol-anchored protein, and a putative aquaporin (34). However, the mechanism by which proteins are targeted and anchored to the oil body has only been studied in detail for oleosins.

Oleosins are the major protein constituent of oil body membranes, and their primary function is believed to be preventing coalescence by coating the oil body surface (1, 2). Oleosins are initially targeted to the endoplasmic reticulum by the signal recognition particle-mediated pathway (35), and the central hydrophobic domain, consisting of ~70 amino acids, is required for both endoplasmic reticulum trafficking and subsequent anchorage to the oil body (36). A model has been proposed in which this domain forms a "hair pin" structure that inserts through the phospholipid monolayer into the TAG matrix and consists of two antiparallel β-sheets connected by a proline-rich turn (proline knot) (1). However, some experimental evidence has led to the development of alternative models, and the precise structure of oleosins is currently unresolved (37, 38). The proline knot motif is unnecessary for oleosin endoplasmic reticulum membrane integration, but it is required for oil body targeting (36).

Hydropathicity analysis using standard algorithms indicates that RcOBL1 is a relatively amphipathic protein, like oleosins. Its amphipathic nature can also be demonstrated experimentally using the method of Jolivet *et al.* (34). RcOBL1 partitions entirely at the interface between the aqueous and organic phases when purified oil body membranes are extracted with 6:3 (v/v) chloroform:methanol (data not shown). It is possible that the N terminus of RcOBL1 might play a role in anchorage. It is predicted to contain a relatively long stretch of hydrophobic amino acids by some hydropathicity algorithms, such as the Hopp and Woods scale (28), and this same region is retained on purified oil bodies following proteolytic treatment. However, the hydrophobicity of the region is not as pronounced as that characterizing oleosins, and it also appears to contain no proline knot motif. Targeting, trafficking, and attachment of the acid lipase to the oil body may differ substantially from that of oleosins and will require further study.

The physiological function of the castor acid lipase is uncertain. No other seed has been reported to contain so much lipase activity (3). This activity clearly reflects the unusually high abundance of the protein (Fig. 1). The acid lipase is effectively inactive at physiological (neutral) pH and is most abundant prior to germination when no TAG breakdown is occurring (39). These data appear inconsistent with a role for the enzyme in TAG mobilization during post-germinative growth (39). It is possible that the acid lipase functions as an emulsifier and/or storage protein like oleosin, but this explanation cannot account for its catalytic activity. Alternatively, the acid lipase might play a role in defense against predation. The potato tuber storage protein patatin exhibits acyl hydrolase activity (40), and this activity has been shown to be insecticidal (41).

Because lipase catalyzes the initial step in TAG breakdown, it is a logical target for the developmental and metabolic regulation of this important process that governs early seedling growth (3, 4). Lipases from animals are known to play a central role in regulating carbon metabolism (42). In most seeds lipase activity exhibits a neutral or alkaline pH optima and is only detectable upon germination, increasing concomitantly with the onset of TAG breakdown (3). In fact, castor beans have been reported to contain a second oil body-associated lipase with precisely these characteristics (16, 39). The neutral lipase is ~40-fold less active than the acid lipase, and therefore probably far less abundant. However, its activity is sufficient to account for the *in vivo* rate of lipolysis (39). In light of the discovery that many plants possess families of RcOBL1 orthologs, it is possible that some encode the enzymes that are responsible for TAG breakdown following germination. Hence discovering the molecular identity of the castor acid lipase may prove a crucial first step to better understand the mechanism and regulation of lipolysis in germinated seeds.

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REVIEW ARTICLE

Using gene knockouts to investigate plant metabolism

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Abstract

***Arabidopsis* functional genomics resources now make the isolation of knockout mutants in any gene of choice both realistic and increasingly straightforward. Coupled with the completion of the genome sequence, this reverse genetics approach provides a platform facilitating dramatic progress in our understanding of fundamental aspects of plant metabolism. Recent experience shows that knockouts of genes encoding enzymes of primary metabolism can produce mutants with clear and sometimes unexpected phenotypes. They can provide new information about old pathways. Specific functions for individual members of multigene families can be revealed. Knockouts of enzymes of undefined function can lead to the discovery of those functions, and the analysis of enzymes which have previously never been studied at the biochemical level offers the potential to reveal new pathways of plant metabolism. Furthermore, the mutants isolated provide the starting point for genetic modification experiments to determine exactly how metabolism fuels growth and development, so providing a rational basis for the future modification of plant productivity.**

Key words: *Arabidopsis thaliana*, insertional mutagenesis, gene knockouts, reverse genetics, plant metabolism, plant growth.

Introduction

Molecular genetic approaches to plant metabolism

Molecular genetic approaches to the study of plant metabolism can be traced back to the isolation of the first cDNA encoding a plant enzyme (Bedbrook *et al.*, 1980), the use of the *Agrobacterium* Ti plasmid to introduce foreign DNA into plant cells (Hernalsteens *et al.*, 1980) and the establishment of routine plant transformation systems (Bevan, 1984; Horsch *et al.*, 1985). It became possible to express foreign genes in plants and potentially to overexpress plant genes using cDNAs linked to strong promoters, with the aim of modifying metabolism. However, the discovery of the antisense phenomenon of plant gene silencing (van der Krol *et al.*, 1988; Smith *et al.*, 1988), and subsequently co-suppression (Napoli *et al.*, 1990; van der Krol *et al.*, 1990), provided the most powerful and widely-used methods for investigating the roles of specific enzymes in metabolism and plant growth. The antisense or co-supression of gene expression, collectively known as post-transcriptional gene silencing (PTGS), has been particularly versatile and powerful in studies of plant metabolism. With such molecular tools in place, plant metabolism became accessible to investigation and manipulation through genetic modification and dramatic progress was made in subsequent years (Stitt and Sonnewald, 1995; Herbers and Sonnewald, 1996), particularly in studies of solanaceous species (Frommer and Sonnewald, 1995).

Limitations of gene-silencing approaches

PTGS approaches suffer from a number of shortcomings (Bourque, 1995). Firstly, the effectiveness of gene silencing is variable and not controllable, leading to failure of many experiments. It is only recently that the structural features of transgenes required to achieve effective silencing have been discovered (Smith *et al.*, 2000). Failure to obtain effective gene silencing may also be explained by a requirement for a particular enzyme during plant transformation and regeneration from culture since the process of making such a transgenic plant would select against effective silencing. A further disadvantage of gene silencing is that its effectiveness may vary between cells or organs, depending upon the gene promoter

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employed. Even the commonly used CaMV 35S promoter is expressed at very different levels in different cell types (Jefferson *et al.*, 1987; Tang *et al.*, 1999; Wilkinson *et al.*, 1997). PTGS can also be variable or unstable when comparing similar cells of the same tissue (van der Krol *et al.*, 1988; Bourque, 1995) or when comparing different organs on the same plant (Palauqui *et al.*, 1997; Wolters and Visser, 2000). With such potential variablity or instability, there is no opportunity to select mutants in which a phenotype caused by gene silencing is corrected by a mutation in another gene. A further limitation is that such gene silencing methods will potentially target all homologous genes, whether alleles, members of a family of closely-related genes or transgene introduced by re-transformation. The major disadvantage of PTGS is that it is never possible to eliminate an enzyme completely. There are several examples in which the amount or activity of an enzyme has been reduced to an almost undetectable level, without apparent phenotypic or metabolic effect (Gottlob-McHugh *et al.*, 1992; Hajirezaei *et al.*, 1994; Takaha *et al.*, 1998). In such cases firm conclusions are difficult to reach, since it can always be argued that the small amount of residual enzyme is sufficient to fulfil the required role. Even with the newly-developed approach of intron-spliced hairpin formation which gives much more effective gene silencing (Smith *et al.*, 2000), complete gene inactivation in all cells cannot be attained.

The power of gene knockouts generated by insertional mutagenesis

Transposon and T-DNA mutagenesis is now the prefered option for gene silencing in *Arabidopsis* (McKinney *et al.*, 1995; Krysan *et al.*, 1996, 1999) and increasingly for some other species (e.g. maize and *Antirrhinum*). With the creation of large populations of plants containing such mutagens (Table 1), and gene identification reaching an advanced stage through completion of the *Arabidopsis* genome sequence (The Arabidopsis Genome Initiative, 2000), a reverse-genetics screening approach is realistic and effective. Insertion of a transposon or T-DNA into a structural gene (whether into an exon or an intron) will usually disrupt gene expression completely and give a null mutation (see below). This is commonly referred to as a 'knockout'. In these cases one can have complete confidence that a particular enzyme or enzyme isoform is totally absent from all cells throughout a homozygous plant. Unlike PTGS approaches, insertional mutagenesis allows the recovery of mutations in essential genes, as the plants can be maintained in the heterozygous state. A further advantage is that insertional mutagenesis will target individual genes within a family of closely-related genes, so that functions of individual members can be investigated (see below).

In the case of T-DNA and some transposon insertions there is no realistic possibility of reversion to wild type. Such mutants can be used to select second-site suppressors of specific phenotypes, and mutations can be combined through crossing of different mutants to obtain double or multiple mutations. Transgenic reconstruction of null mutants can also be used to generate a set of plants with varying levels of gene expression from zero to more than 100% that of wild type, so that metabolic control analysis (ap Rees and Hill, 1994) can potentially be carried out. Null mutants can be used as the starting point for gene replacement such that an enzyme can

Table 1. *Resources available for reverse genetic screening of* Arabidopsis thaliana

Resource	Type of insertion	Ecotype	Population	Insert no.	Availability	Contact details
(A) Tagged lines						
Feldmann	T-DNA	Wassilewskija	10 500	1 to 3	DNA, Seed	NASC[a] ABRC[b]
INRA Versailles	T-DNA	Wassilewskija	9800	1 to 3	DNA, Seed	NASC[a] ABRC[b]
Weigel	T-DNA	Columbia	22 670	1 to 3	DNA, Seed	NASC[a] ABRC[b]
Jack	T-DNA	Columbia	11 300	1 to 3	DNA, Seed	NASC[a] ABRC[b]
SLAT	*En/Spm* Transposon	Columbia	48 000	1 to 3	DNA, Seed	NASC[a] ABRC[b]
ZIGIA	*En/Spm* Transposon	Columbia	11 000	Average 6	Screening service	Zigia project[c]
UWBC	T-DNA	Wassilewskija	133 440	1 to 3	Screening service	UWBC[d]
(B) Databases[e]						
SINS[f]	*En/Spm* Transposon	Columbia	1200	1 to 3	Database, Seed	Sainsbury Lab[f]
IMA[g]	*Ac/Ds* Transposon	Landsberg	500	Not reported	Database, Seed	NASC[a] ABRC[b]

[a]NASC (Nottingham Arabidopsis Stock Centre): http://nasc.nott.ac.uk

[b]ABRC (Arabidopsis Biological Resource Centre): http://www.biosci.ohio-state.edu/~plantbio/Facilities/abrc/ABRCHOME.HTM

[c]ZIGIA project (Max Planck Institute for Plant Breeding): http://www.mpiz-koeln.mpg.de/~zigia/index.php3

[d]UWBC (University of Madison-Wisconsin Biotechnology Centre, Arabidopsis Knockout Facility): http://www.biotech.wisc.edu/Arabidopsis/

[e]NASC has established InsertWatch, a free reverse genetics service (http://www.nasc.nott.ac.uk/insertwatch). Users enter a sequence of interest and InsertWatch searches against sequenced inserts in the currently available databases. The user is informed by e-mail when an insert sequence matching the query appears in a database.

[f]SINS (Sequenced INsertion Sites) database currently comprises 1200 sequenced insertion sites from the SLAT lines; see http://www.jic.bbsrc.ac.uk/Sainsbury-Lab/jonathan-jones/jjhome.htm

[g]IMA (Institute of Molecular Agrobiology, Singapore) Ds insertion lines. The insertion site sequence database and corresponding seeds are available from NASC[a]

be expressed under the control of a modified or novel promoter, thus giving expression which is inducible or with different spatial or temporal characteristics compared to the original wild-type gene. The subcellular location of an enzyme can effectively be changed by transforming a null mutant with a gene directing transport of the particular enzyme to a different compartment. Genes which encode an enzyme with amino acid sequence modifications can be introduced in order to study post-translational modification of specific enzymes, such as protein transport mechanisms, assembly, phosphorylation and allosteric regulation. At some point in the future such experiments will be carried out most effectively with homologous recombination so that the expression of the modified gene is as similar as possible to the original wild-type allele, but this technology is not yet sufficiently well developed in plants (Mengiste and Paszkowski, 1999).

Disadvantages of the knockout approach

The principal limitation is that it is only possible to investigate enzymes that are known about and for which the corresponding genes have been identified. Up to 40% of *Arabidopsis* genes of known or predicted function encode enzymes of metabolism or transport, amounting to about 7000 genes (The Arabidopsis Genome Initiative, 2000). However, there may be another 3000 such genes among those of unknown function. A reverse-genetics approach to discovering which of the 7500 genes with no predicted function are involved in metabolism, and the function of these genes, would require a massive systematic programme of mutant isolation and metabolite profiling, with only a limited probabilitiy of discovering gene function. Clearly then, forward genetic screening (also using metabolite profiling) will continue to be very important for the discovery of genes encoding enzymes of metabolism, as will traditional biochemical approaches. Even utilizing numerous different approaches, the function of many genes will remain unknown for many years to come. It is a sobering thought that of the 4300 *Escherichia coli* genes, about one-third are still of unknown function. A further consideration is that while there is currently a good probability of finding an insert in a target gene, there will be some genes for which insertions will not be found easily (Martinssen, 2000). In such cases, it may be necessary to target the locus by activation of a transposon from a neighbouring (closely-linked) launch pad, or to use the intron-spliced hairpin silencing method of PTGS (Smith *et al.*, 2000). A further major disadvantage of current technology is that a knockout of any enzyme which is essential for gamete formation and function will not be recovered (also a problem for PTGS methods). There is no reliable estimate for the number of genes essential for gamete function, but it could be very significant. Presumably genes encoding enzymes of fundamental importance, such as those of respiratory metabolism, will be required for gamete function. A further complication common to all gene-silencing methods, is that pleiotropic effects such as altered source–sink relationships, may confound functional analysis of the phenotype. Such pleiotropic effects may subsequently be dissected by engineering organ-specific gene expression. At the other extreme, many knockout mutations may be without phenotype because of gene redundancy. It is apparent that *Arabidopsis* was once tetraploid, and while much of this redundant genetic information has subsequently been lost, duplications of much of the genome remain. Furthermore, there are numerous examples of other gene duplication events, with the result that 70% of *Arabidopsis* genes are present in more than one copy (The Arabidopsis Genome Initiative, 2000; Martinssen, 2000). However, divergence of gene sequence and function since gene or genome duplication will effectively increase the proportion of single copy genes.

The reverse-genetics knockout method

Principles

The principle of the reverse-genetics gene knockout approach relies on the general assumption that insertion of transposons and of T-DNA by *Agrobacterium*, occur at random sites within the plant genome, such that all structural genes are potential targets for insertional mutagenesis. Given a large enough population of transgenic plants, there is a measurable probability that an individual gene will be disrupted. There are two ways to find insertions in a target gene: firstly by analysis of DNA from pools of such plants, and secondly by asking if any of the currently sequenced insertion sites includes that target.

The method for PCR-based screening of DNA pools has been described previously (Krysan *et al.*, 1996, 1999). Essentially it uses combinations of gene-specific and T-DNA or transposon-specific primers to detect an insertion in the target gene. In practice, many non-specific PCR products can be obtained, so Southern blotting and hybridization are necessary to detect the desired fragment. The use of 'nested' primers provides supporting evidence for the presence of an insertion in the target gene. DNA sequence analysis of putative junction fragments is then essential, because an appreciable number of 'false-positives' pass the first two selection criteria. Having found the desired product it is necessary to screen successively smaller pools until an individual plant is identified. Sometimes the trail 'goes cold' and no mutant is found. A variation on this approach is that in which transposon or T-DNA junction fragments are amplified

by inverse PCR of pooled DNAs, and then loaded onto a membrane for hybridization with gene-specific probes. The main resources available for such screening methods are summarized in Table 1. The service provided by the University of Wisconsin is particularly easy to use and has a high success rate. There are now increasing numbers of projects which aim systematically to sequence insertion sites, so that mutant isolation will simply involve interrogating databases and then requesting seed. Such databases currently exist (Table 1) but are still small.

Initial characterization of a mutant

Having isolated an individual knockout mutant plant, it is first necessary to establish if it is homozygous or heterozygous for the insertion. A pair of gene-specific primers spanning the insertion site will give a predictable PCR product from the single wild-type allele in a heterozygous plant, but give no product (or a larger than expected product) from a homozygous plant. If the plant genome contains only a single insert, marker gene inheritance in the subsequent generation could be used to confirm the genotype. However, many populations of transformed *Arabidopsis* plants contain, on average, more than one T-DNA insertion site per genome, while lines carrying transposons will probably contain multiple independent insertions (Table 1).

To establish if a particular T-DNA insertion mutant contains multiple insertion sites, it is initially helpful to carry out segregation analysis using the selectable phenotypic markers associated with the T-DNA (usually kanamycin, hygromycin or phosphinothricin resistance). It must be remembered, however, that deletions of T-DNA sequences can occur and may result in no, or a non-functional, marker being associated with a particular insert. Southern blotting using a T-DNA probe is also useful for gaining further insight into the number of insertions present. As the left border region of the T-DNA often remain intact, a probe derived from this region is particularly useful. Again, one must be aware that small T-DNA fragments may be missed with this approach. Furthermore, independent mutations not detectable by the methods described above, may have been introduced into the plant by one of several different mechanisms (for example, somaclonal variation or transposon footprinting). Thus, it is always essential to establish the link between target gene insert and phenotype (see below).

If it is clear that there is more than one insert present in the genome, attempts should be made to separate the desired mutation from all others by backcrossing the mutant to wild type. Assuming that the inserts are not linked, F_2 individuals in which the additional T-DNA locus/loci has segregated away from that in the target gene can be identified by Southern blotting and checking the marker gene segregation ratio in the F_3. However, if the insertion sites are tightly linked, it may not be possible to identify rare recombinants. In this case one would again proceed by establishing the link between genotype and phenotype.

Establishing the link between genotype and phenotype

One way to establish the link between genotype and phenotype is to isolate two or more independent mutants and show that they have the same phenotype (Eastmond *et al.*, 2000*a*; Gottwald *et al.*, 2000). The probability that both mutants have a second T-DNA or other mutation disrupting the same non-target gene, is so low as to be negligible. Similar arguments and requirements apply to mutants created by transposon mutagenesis. However, in this case, an alternative route to confirming the link between insert and phenotype can come from examples in which the transposon excises and the wild-type phenotype is re-established (Eastmond *et al.*, 2000*a*). This approach cannot always be relied upon, however, as many excisions will leave a footprint which results in a stable mutation rather than a revertant. Even analysis of independent mutants or revertants does not provide conclusive proof that a particular mutant phenotype is the result of silencing the target gene because, potentially, such insertions could reproducibly disrupt expression of a neighbouring gene(s). This could be checked by Northern analysis of immediate neighbours, but would require transcriptome analysis for a complete picture. A further means of confirming the link between genotype and phenotype is to complement the mutant by transformation with a wild-type cDNA or gene (transgenic complementation). A genomic clone is preferable since it is more likely to reproduce the correct pattern and level of gene expression, and so restore wild-type phenotype, whereas use of the CaMV 35S promoter is likely to lead to ectopic or overexpression. As a general rule, one of the above three methods (multiple independent mutants, revertants or transgenic complementation) is the minimum necessary to establish the link between genotype and phenotype.

Once a mutant has been found, it is necessary to show that the target gene transcript is missing, which can be achieved using Northern blotting, or RT-PCR for increased sensitivity. A full-length cDNA is recommended for use as the probe to reveal any partial or aberrant transcripts that may be produced. The absence of protein product or enzyme activity should also be established. Finally, it is important to show whether knocking out one particular enzyme has any pleiotropic effects on the synthesis or activity of related enzymes, so that any phenotypes observed can be attributed to the mutation in the target gene. This may require assaying a range of

enzymes (Eastmond *et al.*, 2000*a*; Critchley *et al.*, 2001), and will increasingly involve transcriptome analysis on DNA microarrays, or proteome analysis.

Discoveries in primary metabolism from the study of knockouts

Lipid metabolism and gluconeogenesis

Many seeds, including *Arabidopsis*, store triacylglycerol (TAG) in lipid bodies and this serves as a carbon source for heterotrophic growth of the seedling. During seed germination and seedling growth this TAG is hydrolysed by lipases and the resultant fatty acids subjected to β-oxidation in the peroxiosme, producing acetyl CoA for conversion into succinate by the glyoxylate cycle (Fig. 1). Succinate is transferred to the mitochondrion for conversion to malate and oxaloacetate, which provides the substrate for gluconeogenesis in the cytosol. 3-ketoacyl CoA thiolase is a key enzyme of fatty acid β-oxidation, and is encoded by at least three genes in *Arabidopsis*. Remarkably, a knockout of one of these peroxisomal thiolases prevents seedling growth due to a block in β-oxidation of TAG-derived fatty acids (Hayashi *et al.*, 1998; Germain *et al.*, 2001). This observation demonstrates quite clearly that such fatty acids cannot be metabolized via mitochondrial β-oxidation and, furthermore, that TAG metabolism is essential to support *Arabidopsis* seedling growth. Seedling growth can be recovered by supplying exogenous sugar, showing that the TAG is simply providing a carbon and energy supply. Glyoxylate cycle enzymes and PEP carboxykinase are synthesized normally in the thiolase mutant (Germain *et al.*, 2001) showing firstly that fatty acid metabolism is not required to generate a signal to activate expression of their genes, as previously hypothesized (Graham *et al.*, 1992) and, secondly, that gluconeogenesis is apparently still functional. Therefore other gluconeogenic carbon sources are not in sufficient supply in the seed to support seedling growth.

Study of a promoter-trapped acyl CoA oxidase gene in *Arabidopsis* has revealed that this particular enzyme acts preferentially on medium-chain substrates, and is active in seedlings (Eastmond *et al.*, 2000*b*). Although the T-DNA insertion eliminates 95% of the medium-chain acyl CoA oxidase activity, TAG metabolism and seedling growth are not affected in the mutant. Acyl CoA oxidases are encoded by at least four genes in *Arabidopsis*, and although the other three act preferentially on long-chain and short-chain substrates, apparently one or more can substitute for the medium-chain enzyme *in vivo*. A mutant in which a T-DNA interupts a gene encoding a peroxisomal multifunctional protein has been isolated as a result of a forward screen for inflorescence development mutants (Richmond and Bleecker, 1999). This

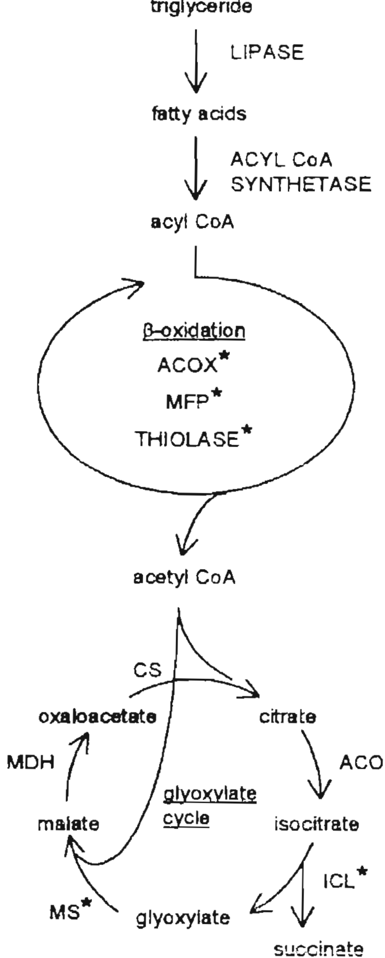

Fig. 1. Mutants in triglyceride utilization. Enzymes for which mutants have been isolated are indicated with an asterisk (*). Mutants of the three principal enzymes of the peroxisomal β-oxidation pathway have been isolated, although each enzyme is encoded by several genes, most of which have not yet been mutated. Both isocitrate lyase (ICL) and malate synthase (MS) are encoded by single genes in *Arabidopsis*, and knockouts of each have been isolated. Mutants of other glyoxylate cycle enzymes remain to be identified. Note that the glyoxylate cycle is depicted in simplified form (Mettler and Beevers, 1980; Hayashi *et al.*, 1995). Key: ACOX, acyl CoA oxidase; MFP, multifunctional protein; thiolase, 3-ketoacyl CoA thiolase; CS, citrate synthase; ACO, aconitase; MDH, malate dehydrogenase.

mutant clearly shows the importance of fatty acid β-oxidation in reproductive growth. Consistent with this observation, a thiolase knockout also shows modified inflorescence development (S Footitt, JH Bryce, SM Smith, unpublished results). A reverse genetics approach should now lead to determination of the functions of all isoforms of β-oxidation enzymes throughout growth and development.

In *Arabidopsis*, the key glyoxylate cycle enzymes isocitrate lyase (ICL) and malate synthase (MS) are encoded by single-copy genes. Knockouts of both have been isolated in order to confirm the metabolic role of

the glyoxylate cycle and also to determine how important this pathway is to the growth of seedlings (Eastmond *et al.*, 2000*a*; Germain *et al.*, 2000). The results establish that gluconeogenesis from acetate is drastically reduced in *icl* mutants, in keeping with our understanding of glyoxylate cycle function. Mutant seedling growth is compromised, but if sufficient light is available for photosynthesis, or if exogenous sugar is provided, seedlings readily grow and develop. Remarkably, even in the absence of the glyoxylate cycle, TAG is still metabolized, particularly if seedling growth is enhanced by exogenous sucrose. The lipid is apparently respired, showing that the carbon can be transferred to the mitochondrion even in the absence of the glyoxylate cycle, and therefore not as succinate. It could potentially be transferred from the peroxisome as either acetate or citrate (isocitrate is not synthesized in the peroxisome because aconitase is cytosolic: Hayashi *et al.*, 1995). If citrate is synthesized in the peroxisome, a supply of oxalaoacetate must be maintained, which effectively means that the TCA cycle operates collectively between three cellular compartments (Fig. 2). It is not known if significant transfer of these substrates takes place in the presence of a functional glyoxylate cycle. A knockout of peroxisomal citrate synthase would establish if acetate can be transported to the mitochondrion. The results discussed here therefore provide very important new information about the metabolic capabilities of peroxisomes and mitochondria in lipid metabolism and raise new questions to be addressed.

Starch metabolism

Starch is of critical importance in plant metabolism and yet the details of its synthesis and breakdown are still to be deduced. This aspect of metabolism is directly amenable to analysis through knockout mutants since there is a limited number of enzymes, most of which have probably been identified. Insertions in 11 such genes were screened and knockouts were found for seven (D Thorneycroft, unpublished results). This illustrates the recent success rate in such screening, but also shows that knockouts of all enzymes of starch metabolism is a realistic goal for the near future.

D-enzyme (1,4-α-D-glucan: 1,4-α-D-glucan: 4-α-glucanotransferase) is found in a range of plants, but it has not been possible until now to examine its function *in planta*. Despite eliminating up to 98% of D-enzyme activity using an antisense approach in potato, no effect on starch metabolism was observed (Takaha *et al.*, 1998). An *Arabidopsis* knockout mutant has now been isolated and shown to have impaired starch metabolism

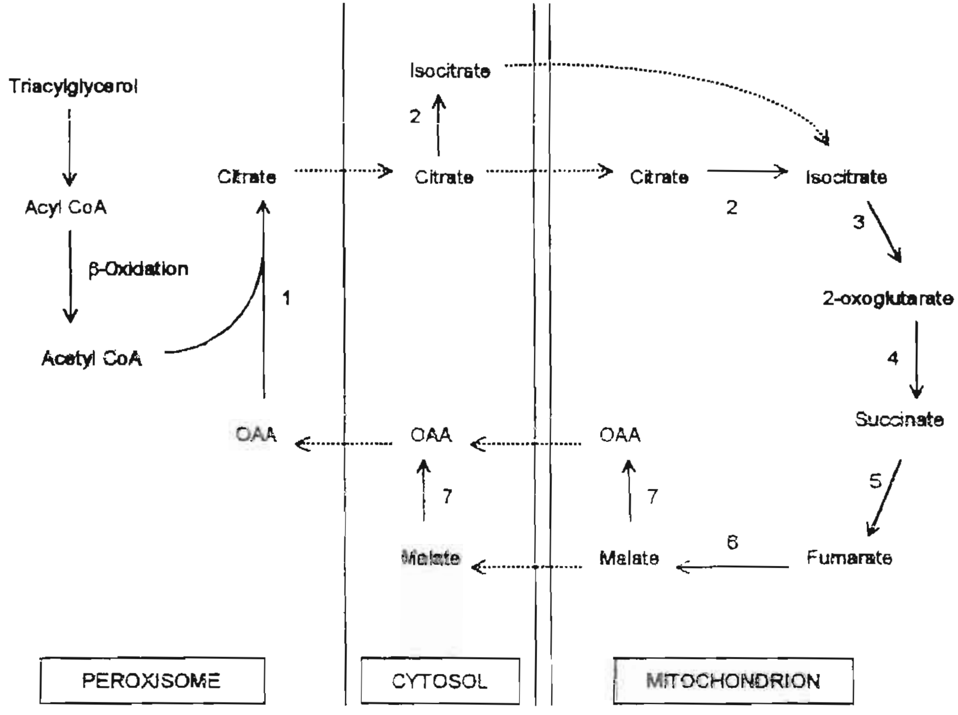

Fig. 2. Possible co-operation between peroxisome, cytosol and mitochondrion in operation of the TCA cycle, suggested by studies of an isocitrate lyase knockout. Citrate is exported to the cytosol where aconitase is located (Hayashi *et al.*, 1995). Either citrate or isocitrate could be imported into the mitochondrion for oxidation, and either malate or OAA could be exported. Solid arrows show metabolic conversions, while broken arrows show metabolite transport. 1, Citrate synthase; 2, aconitase; 3, isocitrate dehydrogenase; 4, 2-oxoglutarate dehydrogenase; 5, succinate dehydrogenase; 6, fumarase; 7, malate dehydrogenase.

(Critchley *et al.*, 2001). Detailed examination of the mutant revealed a very large accumulation of maltotriose in leaves in the dark, demonstrating that D-enzyme is required for malto-oligosaccharide metabolism. The conclusion from these studies is that leaf starch is degraded in the dark by amylases and starch phosphorylase to maltotriose but no further, and that the glucanotransferase activity of D-enzyme serves to convert maltotriose into larger malto-oligosaccharides upon which these enzymes can act (Fig. 3).

Not only can such studies provide information about mechanisms of starch metabolism, but they can also provide insight into metabolic pathways into which starch can feed. For example, it has been postulated that phosphorolytic starch breakdown at night is likely to lead to export of carbon from the chloroplast as triose phosphate, which can in turn fuel respiration, whereas amylolytic breakdown may lead to the export of glucose, which can preferentially fuel sucrose synthesis (Stitt *et al.*, 1985). A newly-isolated mutant of plastidial starch phosphorylase now provides the opportunity to test directly the function of the phosphorolytic pathway of starch breakdown in leaves (D Thorneycroft, SC Zeeman, AM Smith, SM Smith, unpublished results).

Some enzymes of α-glucan metabolism occur in the cytosol of leaf cells, but it is not known if they participate in metabolism of starch or of other substrates (Duwenig *et al.*, 1997; Tacke *et al.*, 1991). There is a cytosolic form of D-enzyme (Okita *et al.*, 1979), and a recently isolated knockout mutant (D Thorneycroft, unpublished results) shows a profound retardation of growth (Fig. 4). This mutant demonstrates the importance of cytosolic α-glucan metabolism and provides the means to uncover potentially novel aspects of cytosolic carbohydrate metabolism in plants.

Starch
Endoamylases
G
Soluble glucans (>G5)
Amylases
Phosphorylase
G
G1P
G
D-enzyme
G4
G3
Amylases
G

Fig. 3. Function of D-enzyme in starch breakdown in *Arabidopsis* leaves. Endoamylases are likely responsible for release of soluble glucans from the starch granule. Starch phopsphorylase can degrade such glucans to glucose-1-phosphate (G1P), but will not act on substrates smaller than maltopentose (G5). Endo- and exo-amylases can apparently degrade glucans to maltotriose (G3) in the dark, but no further. D-enzyme converts G3 into glucose (G) and larger glucans for further attack by amylases and phosphorylase.

Sugar transport

Sugars provide the energy and carbon currencies of the plant, but also act as signalling molecules to regulate gene expression, growth and development (Jang and Sheen, 1997; Smeekens and Rook, 1997). Both sucrose transporters and hexose transporters play key roles in source–sink relationships, and are encoded by multigene families (Lalonde *et al.*, 1999; The Arabidopsis Genome Initiative, 2000). The only realistic way to determine the function of each sugar transporter is through the isolation of knockouts of individual genes. A dramatic demonstration of the importance of the phloem-specific SUC2 sucrose transporter was provided earlier (Gottwald *et al.*, 2000). Knockouts of this gene produce viable plants, but they show retarded growth and sterility. Sucrose is not transported efficiently from shoot to root or inflorescence, and source leaves contain a great excess of starch. These observations demonstrate unequivocally the importance of apoplastic loading of sucrose into sieve elements in *Arabidopsis*.

In another study, a mutant lacking hexose transporter AtSTP1 (Sherson *et al.*, 2000) has no obvious morphological or growth phenotype, but has significantly impaired hexose uptake in germinating seeds and seedlings, and shows altered responses to exogenous sugars.

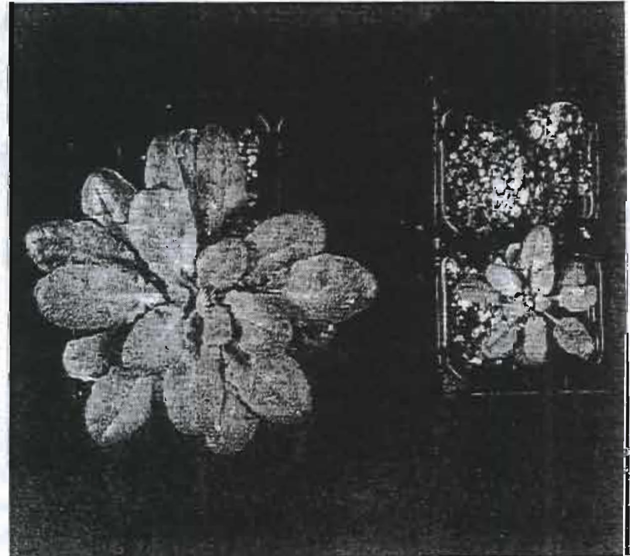

Fig. 4. Impaired growth of a mutant lacking a putative cytosolic D-enzyme. Wild type (left) and mutant (right) are shown after 8 weeks growth in vermiculite in short days.

This mutant provides the first opportunity to investigate the *in planta* characteristics of such a transporter. While phenotypes should not necessarily be expected in knockouts of individual members of sugar transporter gene families, these results show that they can potentially be revealed by appropriate experiments. Furthermore, creating lines with multiple knockouts by crossing individual mutants is expected to result in more profound phenotypes.

Future perspectives

The examples discussed here show that the knockout approach to plant metabolism is enormously powerful, and in its infancy. More mutants will rapidly become available and the means to identify knockouts of target genes will become easier. It is also possible to look forward to large collections of activation-tagged lines (Walden *et al.*, 1994) in which there is the possibility for overexpression of target enzymes with their correct spatial and temporal patterns of expression.

Some unexpected and exciting findings can be anticipated from the analysis of such mutants. For example, knockout of a plastidial phosphoenolpyruvate transporter results in compromised aromatic amino acid synthesis, underexpression of nuclear-encoded photosynthetic proteins, and mesophyll-specific cell defects causing reticulate leaves (Streatfield *et al.*, 1999). Answers to questions that have occupied plant biochemists for many years are now within reach. For example, there has been a long-standing controversy concerning the role of mitochondria in β-oxidation (Masterson and Wood, 2000), which can finally be resolved. The function of cytosolic enzymes of α-glucan metabolism can now be determined. Other issues that can be addressed are the controls of glycolysis and gluconeogenic production of sucrose, the functional redundancy of cytosolic and plastidial pentose phosphate pathway enzymes, and the roles of sugar transporters, sucrose synthase and invertases in determining sink activity. The integration of carbon and nitrogen metabolism is amenable to detailed study. There are also novel areas of metabolism that have not been studied. For example, the discovery in *Arabidopsis* of citramalate (Fiehn *et al.*, 2000), a potential precursor of pyruvate and acetate, suggests a novel aspect of carbon metabolism. If the net is spread wider to include secondary metabolism, the functions of the many cytochrome P450 enzymes and glutathione transferases are obvious targets for study. Finally, a cautionary note: it must be remembered that *Arabidopsis* is not typical of all plants and that it will not help in the understanding of mycorrhizal interactions, nitrogen fixation, C_4 and CAM photosynthesis, tuber metabolism, and so on. Equivalent studies in other species are therefore essential.

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