March 20, 2006

National Organic Standards Board
c/o Robert Pooler, Agricultural and Marketing Specialist
USDA/AMS/TM/NOP, Room 2510-So., Ag Stop 0268
P.O. Box 96456
Washington, D.C. 20090-6456
Phone: 202-720-3252
Fax: 202-205-7808

Dear Mr. Robert Pooler,

Please find enclosed duplicate copies of our petition to have short-chain fructooligosaccharides (scFOS®) included on the National List of Allowed Substances. If you have any questions or need additional information please contact me directly. My contact information can be found below.

Sincerely,

Luke R. Kazmierski
Quality Assurance and Regulatory Affairs Specialist
GTC Nutrition
600 Corporate Circle, Suite H
Golden, CO 80401
Phone: 303-216-2489 ext. 242
Fax: 303-216-2477
E-mail: lkazmierski@gtcnutrition.com

GTC NUTRITION

SPECIALTY INGREDIENTS

Petition for Inclusion on the National List of Allowed Substances

CBI Deleted Copy

600 Corporate Circle ● Suite H ● Golden, CO 80401 ● 800-522-4682 ● www.gtcnutrition.com
A business unit of Corn Products International, Inc.

Table of Contents

Petition for Inclusion on the National List of Allowed Substances

EVALUATION CRITERIA FOR SUBSTANCES ADDED TO THE NATIONAL LIST

Category 1. Adverse impacts on humans or the environment?

Substance: Short-Chain Fructooligosaccharides (scFOS®)

Question	Yes	No	N/A[1]	Documentation (TAP; petition; regulatory agency; other)
1. Are there adverse effects on environment from manufacture, use, or disposal? [§205.600 b.2]		X		There is no toxicity or environmental persistence, as the substance is a short-chain, water soluble carbohydrate, consisting of only glucose and fructose units. It is environmentally harmless. See MSDS (Appendix 6, Tab 7)
2. Is there environmental contamination during manufacture, use, misuse, or disposal? [§6518 m.3]		X		There is no toxicity or environmental persistence, as the substance is a short-chain, water soluble carbohydrate, consisting of only glucose and fructose units. It is environmentally harmless. See MSDS (Appendix 6, Tab 7)
3. Is the substance harmful to the environment? [§6517c(1)(A)(i);6517(c)(2)(A) i]		X		There is no toxicity or environmental persistence, as the substance is a short-chain, water soluble carbohydrate, consisting of only glucose and fructose units. It is environmentally harmless. See MSDS (Appendix 6, Tab 7)
4. Does the substance contain List 1, 2, or 3 inerts? [§6517 c (1)(B)(ii); 205.601(m)2]		X		There is no toxicity or environmental persistence, as the product is a short-chain, water soluble carbohydrate, consisting of only glucose and fructose units. See MSDS (Appendix 6, Tab 7)
5. Is there potential for detrimental chemical interaction with other materials used? [§6518 m.1]		X		This substance is intended as an ingredient in food and feed products. The substance is GRAS recognized (Appendix 2, Tab 3). See MSDS (Appendix 6, Tab 7)
6. Are there adverse biological and chemical interactions in agro-ecosystem? [§6518 m.5]			X	This substance is intended as an ingredient in food and feed products and is produced from an agricultural source.
7. Are there detrimental physiological effects on soil organisms, crops, or livestock? [§6518 m.5]			X	This substance is intended as an ingredient in food and feed products and is produced from an agricultural source.
8. Is there a toxic or other adverse action of the material or its breakdown products? [§6518 m.2]		X		There is no toxicity or environmental persistence, as the product is a short-chain, water soluble carbohydrate, consisting of only glucose and fructose units. It is environmentally harmless. See MSDS (Appendix 6, Tab 7)
9. Is there undesirable persistence or concentration of the material or breakdown products in environment?[§6518 m.2]		X		There is no toxicity or environmental persistence, as the product is a short-chain, water soluble carbohydrate, consisting of only glucose and fructose units. It is environmentally harmless. See MSDS (Appendix 6, Tab 7)
10. Is there any harmful effect on human health?		X		Studies have shown that this substance has no potential to cause a harmful effect on human health (Appendix 8, Tab

[§6517 c (1)(A)(i) ; 6517 c(2)(A)i; §6518 m.4]				9). See MSDS (Appendix 6, Tab 7)
11. Is there an adverse effect on human health as defined by applicable Federal regulations? [205.600 b.3]		X		Studies have shown that this substance has no potential to cause a harmful effect on human health (Appendix 8, Tab 9). The substance is GRAS recognized (Appendix 2, Tab 3). See MSDS (Appendix 6, Tab 7)
12. Is the substance GRAS when used according to FDA's good manufacturing practices? [§205.600 b.5]	X			GRAS Notice Number: GRN 000044 (Appendix 2, Tab 3)
13. Does the substance contain residues of heavy metals or other contaminants in excess of FDA tolerances? [§205.600 b.5]		X		The substance is GRAS recognized (Appendix 2, Tab 3) and does not exceed FDA tolerances.

[1]If the substance under review is for crops or livestock production, all of the questions from 205.600 (b) are N/A—not applicable.

Category 2. Is the Substance Essential for Organic Production?

Substance: Short-Chain Fructooligosaccharides (scFOS®)

Question	Yes	No	N/A[1]	Documentation (TAP; petition; regulatory agency; other)
1. Is the substance formulated or manufactured by a chemical process? [6502 (21)]		X		The substance is produced by a natural fermentation process. A flow chart marked as CBI is attached (Appendix 1, Tab 2).
2. Is the substance formulated or manufactured by a process that chemically changes a substance extracted from naturally occurring plant, animal, or mineral, sources? [6502 (21)]	X			The substance is produced by a natural fermentation process. Sucrose is converted to short-chains of glucose molecules containing two, three or four fructose molecules. A flow chart marked as CBI is attached (Appendix 1, Tab 2).
3. Is the substance created by naturally occurring biological processes? [6502 (21)]	X			The substance is produced by a natural fermentation process. A flow chart marked as CBI is attached (Appendix 1, Tab 2).
4. Is there a natural source of the substance? [§205.600 b.1]	X			This substance does occur in nature and is distributed in very small quantities in a wide variety of fruits, vegetables and grains. See studies in Appendix 8, Tab 9.
5. Is there an organic substitute? [§205.600 b.1]		X		Even though the substance occurs in very small quantities in a wide variety of fruits, vegetables and grains there are currently no organic equivalents of this substance available.
6. Is the substance essential for handling of organically produced agricultural products? [§205.600 b.6]			X	As a non-digestible carbohydrate, this substance is used as a prebiotic fiber inclusion in food and feed processing and as a selective source of energy by probiotic bacteria in the guts of humans and animals. This action favorably affects the growth and activity of probiotic bacteria for the benefit of health. Studies have shown the numerous health benefits when this substance is consumed (Appendix 7, Tab 8).
7. Is there a wholly natural substitute product? [§6517 c (1)(A)(ii)]			X	The substance is produced by a natural fermentation process and is considered natural.
8. Is the substance used in handling, not synthetic, but not organically produced? [§6517 c (1)(B)(iii)]	X			As a non-digestible carbohydrate, it is used as a prebiotic fiber inclusion in food and feed processing and as a selective source of energy by probiotic bacteria in the guts of humans and animals. This action favorably affects the growth and activity of probiotic bacteria for the benefit of health. Studies have shown the numerous human health benefits when this substance is consumed (Appendix 7, Tab 8).
9. Is there any alternative substances? [§6518 m.6]		X		The substance occurs in very small quantities in a wide variety of fruits, vegetables and grains. However, there are currently no equivalents of this substance available.
10. Is there another practice that would make the substance unnecessary? [§6518 m.6]			X	This substance is intended as an ingredient in food and feed products and is produced from an agricultural source.

[1] If the substance under review is for crops or livestock production, all of the questions from 205.600 (b) are N/A—not applicable.

Category 3. Is the substance compatible with organic production practices?

Substance: Short-Chain Fructooligosaccharides (scFOS®)

Question	Yes	No	N/A[1]	Documentation (TAP; petition; regulatory agency; other)
1. Is the substance compatible with organic handling? [§205.600 b.2]	X			This substance is intended as an ingredient in food and feed products and is produced from an agricultural source. Studies have shown the numerous human health benefits when consuming this substance (Appendix 7, Tab 8).
2. Is the substance consistent with organic farming and handling? [§6517 c (1)(A)(iii); 6517 c (2)(A)(ii)]	X			This substance is intended as an ingredient in food and feed products and is produced from an agricultural source. Studies have shown the numerous human health benefits when consuming this substance (Appendix 7, Tab 8).
3. Is the substance compatible with a system of sustainable agriculture? [§6518 m.7]	X			This substance is produced from an agricultural source.
4. Is the nutritional quality of the food maintained with the substance? [§205.600 b.3]	X			This substance improves the nutritional quality of foods and feeds in which it is added.
5. Is the primary use as a preservative? [§205.600 b.4]		X		This substance is intended as an ingredient in food and feed products with no preservative effect.
6. Is the primary use to recreate or improve flavors, colors, textures, or nutritive values lost in processing (except when required by law, e.g., vitamin D in milk)? [205.600 b.4]		X		This substance is intended as an ingredient in food and feed products and is produced from an agricultural source. As a non-digestible carbohydrate, this substance is used as a prebiotic fiber inclusion in food and feed processing and as a selective source of energy by probiotic bacteria in the guts of humans and animals. This action favorably affects the growth and activity of probiotic bacteria for the benefit of health.
7. Is the substance used in production, and does it contain an active synthetic ingredient in the following categories: a. copper and sulfur compounds;			X	This substance is intended as an ingredient in food and feed products and is produced from an agricultural source that is not synthetic consisting of only glucose and fructose units. As a non-digestible carbohydrate, this substance is used as a prebiotic fiber inclusion in food and feed processing and as a selective source of energy by probiotic bacteria in the guts of humans and animals. This action favorably affects the growth and activity of probiotic bacteria for the benefit of health.
b. toxins derived from bacteria;			X	
c. pheromones, soaps, horticultural oils, fish emulsions, treated seed, vitamins and minerals?			X	
d. livestock parasiticides and medicines?			X	
e. production aids including netting, tree wraps and seals, insect traps, sticky barriers, row covers, and equipment cleaners?			X	

[1]If the substance under review is for crops or livestock production, all of the questions from 205.600 (b) are N/A—not applicable.

NOSB RECOMMENDED DECISION

Form NOPLIST2. Full Board Transmittal to NOP

For NOSB Meeting: _____ | Substance: **Short-Chain Fructooligosaccharides**

A. Evaluation Criteria (Documentation attached; committee recommendation attached)

	Criteria Satisfied?
1. Impact on humans and environment	Yes ☐ No ☐ (see B below)
2. Availability criteria	Yes ☐ No ☐ (see B below)
3. Compatibility & consistency	Yes ☐ No ☐ (see B below)

B. Substance fails criteria?

Criteria category: _____

Comments:

C. Proposed Annotation: _____

Basis for annotation:

To meet criteria above: ____ Criteria: _____

Other regulatory criteria: ____ Citation: _____

D. Final Board Action & Vote: Motion by: _____ Second: _____

Vote:

Yes: _____

No: _____

Abstain: _____

Agricultural		Nonagricultural		Crops	
Synthetic		Not synthetic		Livestock	
Allowed[1]		Prohibited[2]		Handling	
No restriction		Deferred4		Rejected[3]	

1—substance voted to be added as "allowed" on National List

Annotation: _____

2—substance to be added to "prohibited" paragraph of National List

Describe why a prohibited substance: _____

3—substance was rejected by vote for amending National List

Describe why material was rejected: _____

4-substance was recommended to be deferred

Describe why deferred; if any follow-up is needed. If follow-up needed, who conducts follow-up. _____

E. Approved by NOSB Chair to transmit to NOP:

_____ _____

Kevin R. O'Reil, NOSB Chair Date

F. NOP Action: Include in FR to amend National List: ☐

Return to NOSB ☐ Reason: _____

_____ _____

Richard H. Mathews, Program Manager Date

NOSB COMMITTEE RECOMMENDATION

Form NOPLIST1. Committee Transmittal to NOSB

For NOSB Meeting: _____ | Substance: **Short-Chain Fructooligosaccharides**

Committee: Crops ☐ Livestock ☐ Handling ☐

A. Evaluation Criteria (Documentation attached; committee recommendation attached)

	Criteria Satisfied?
4. Impact on humans and environment	Yes ☐ No ☐ (see B below)
5. Availability criteria	Yes ☐ No ☐ (see B below)
6. Compatibility & consistency	Yes ☐ No ☐ (see B below)

B. Substance fails criteria?

Criteria category: _____

Comments:

C. Proposed Annotation: _____

Basis for annotation:

To meet criteria above: ____ Criteria: _____

Other regulatory criteria: ____ Citation: _____

D. Recommended Committee Action & Vote: Motion by: _____

Seconded: _____

Vote:

Yes: _____

No: _____

Abstain: _____

Agricultural		Nonagricultural		Crops	
Synthetic		Not synthetic		Livestock	
Allowed[1]		Prohibited[2]		Handling	
No restriction		Deferred4		Rejected[3]	

1—substance voted to be added as "allowed" on National List

Annotation: _____

2—substance to be added to "prohibited" paragraph of National List

Describe why a prohibited substance: _____

3—substance was rejected by vote for amending National List

Describe why material was rejected: _____

4-substance was recommended to be deferred

Describe why deferred; if follow-up is needed. If follow-up needed, who will follow up _____

E. Approved by Committee Chair to transmit to NOSB:

_____ _____

Committee Chair Date

Item A:

Category: § 205.606 Nonorganically produced agricultural products allowed as ingredients in or on processed products labeled as "organic" or "made with organic (specified ingredients or food group(s))."

Item B:

1. Common name of substance:

Short-chain fructooligosaccharides (scFOS®)

2. Manufacturer's information:

GTC Nutrition Company
600 Corporate Circle, Suite H
Golden, Colorado 80401
(303) 216-2489
Contact for manufacturers:

Meiji Food Material Co., Ltd.
4-16, Kyobashi 2-Chome, Chuo-Ku,
Tokyo 104-0031 Japan

Béghin Meiji
Rue du Petit Versailles
BP 08
59239 Thumeries
France

Casco Inc.
401 The West Mall, 2nd Floor
Etobicoke, Ontario M9C 5P7

3. Intended or current use:

Ingredient in food and feed products

4. Handling activity:

scFOS® are a soluble prebiotic fiber inclusion. The product is usually added to other dry ingredients.

5. Source and manufacturing procedures:

The raw material is sucrose derived from an agricultural source. The scFOS® are produced by a proprietary fermentation process using an *Aspergillus japonicus* enzyme. A flow chart marked as CBI is attached (Appendix 1, Tab 2).

6. Summary of previous regulatory reviews.

GRAS Letter (Appendix 2, Tab3)
Health Canada Letter (Appendix 3, Tab 4)
European Commission Letter (Appendix 4, Tab 5)

7. Information regarding regulatory registrations.

List of international regulatory/claim status (Appendix 5, Tab 6)

8. CAS number.

308066-66-2

9. Chemical properties and mode of action.

A) The substance is a mixture of beta-linked fructose chains (2-4), bound to a terminal glucose. As a non-digestible carbohydrate, it is used as a prebiotic fiber inclusion in food and feed processing and as a selective source of energy by probiotic bacteria in the guts of humans and animals. This action favorably affects the growth and activity of probiotic bacteria for the benefit of health.

B) There is no toxicity or environmental persistence, as the product is a short-chain, water soluble carbohydrate, consisting of only glucose and fructose units. It is environmentally harmless.

C) This type of product does not have a significant effect on the human environment, due to the biological methods of production, the simple carbohydrate nature of the product, and the small production volume.

D) Effects on human health are attached (Appendix 7, Tab 8). Generally, the product is used for the improvement of human and animal health through the selective feeding of probiotic gut microflora. The product has been in use for over 20 years in Japan, where it is recognized as a food for specified health use (FOSHU). In addition, the product has been widely used in Europe and has been included in medical foods in the United States for at least 8 years.

E) Effects on soil organisms, crops, or livestock include the reduction of *Escherichia coli* species in Bovine species and a reduction in *Salmonella* species

in swine and poultry. The product has been used in animal husbandry for commercial animal production for over a decade in Japan. The product is not directly used in crops or as a soil amendment. However, short-chain fructooligosaccharides do occur in nature. They are distributed in very small quantities in a wide variety of fruits, vegetables, and grains. However, the concentration of scFOS® in the plant kingdom is too low to be viable for commercial production from these materials. The scFOS® are produced by fermentation of sucrose using a non-GMO enzyme isolated from a food-grade Aspergillus fungus and is chemically identical to that found in plants.

10. Safety information.

MSDS attached (Appendix 6, Tab 7).
No substance report from the National Institute of Environmental Health Studies available.
Independent toxicology review attached.

11. Research reviews provided in Appendix 8, Tab 9.

These pertain to health benefits. There are no known reviews on the subject of the National list.

12. Petition justification statement.

The product falls under the category of § 205.606 which encompasses nonorganically produced agricultural products allowed as ingredients in or on processed products labeled as "organic" or "made with organic (specified ingredients or food group(s))." There are currently no organic equivalents of the product available. Efforts are being made by GTC Nutrition to certify scFOS® as organic. The manufacturing process is being reviewed in order to determine the steps needed to obtain the organic certification for scFOS®. The product is not synthetic, being efficiently produced from natural biological processes. Therefore, scFOS® should be included on the National List, as it provides a valuable source of prebiotic soluble fiber that specifically feeds the probiotic microflora in the guts of humans and animals. Even organically produced food and feed are frequently processed and stripped of vital nutrients, such as the unique scFOS® found in fruits and vegetables. Pure scFOS® are easily incorporated into a wide variety of foods, feeds and beverages and leads to interesting, documented health benefits, at low inclusion rates. Current human consumption of scFOS® are thought to be in the range of 100 mg per day in the modern diet. This number represents a deficit in this specific fiber, which was previously thought to exist in the diet at approximately 20 grams per day.

13. Commercial Confidential Information Statement.

The diagram of the manufacturing process of scFOS® is considered Confidential Business Information (CBI). This diagram is located in Appendix 1, Tab 2.

CBI Deleted

MANUFACTURING PROCESS

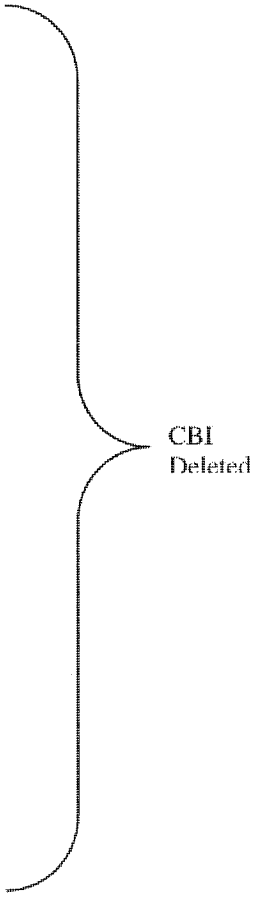
Fructooligosaccharides are produced on a commercial scale by a natural fermentation from a mixture of sucrose using an enzyme, derived from *Aspergillus japonicus*. The sucrose is combined with the enzyme and heated. The enzyme then changes the glucose/fructose of sucrose molecules to yield:

Glucose - fructose - fructose	GF2
Glucose - fructose - fructose - fructose	GF3
Glucose - fructose - fructose - fructose - fructose	GF4

Once the enzyme reaction is completed, the product is filtered, concentrated and packaged.

Processing inputs:
Sucrose, water, enzyme, HCl or NaOH (pH control), active carbon.

Diagram of Process:



DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
Washington, DC 20204

Paula Karabell
GTC Nutrition Company
14252 W. 44th Ave., Unit F
Golden, CO 80403

NOV 22 2000

Re: GRAS Notice No. GRN 000044

Dear Ms. Karabell:

The Food and Drug Administration (FDA) is responding to the notice, dated May 1, 2000, that Environ Corporation submitted on your behalf in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS)). The Office of Premarket Approval (OPA) received the notice on May 2, 2000 and designated it as GRAS Notice No. GRN 000044. In a letter of August 31, 2000, you informed FDA of the intent of GTC Nutrition Company (GTC Nutrition) to act as its own agent.

The subject of your notice is fructooligosaccharide. Your notice informs FDA of the view of GTC Nutrition that fructooligosaccharide is GRAS, through scientific procedures, for use as a bulking agent as listed in Table 1. Your notice includes the findings of a panel of individuals who evaluated the data and information that are the basis for GTC Nutrition's GRAS determination. GTC Nutrition considers these individuals to be qualified by scientific training and experience to evaluate the safety of substances added to food.

Table 1
Intended Use of Fructooligosaccharide

Food	Level Of Use
Acidophilis milk	2 per cent
Bars	4.6-13.6 per cent
Baby foods	0.1-3.6 per cent
Beverages	1.2 per cent
Biscuits	3.6 per cent
Cakes	1.6-3.6 per cent
Confectionery	5.0 per cent
Cookies	2.5-3.3 per cent

Food	Level Of Use
Crackers	1.7-3.3 per cent
Flavored and unflavored milks	0.4 per cent
Hard candy	6.7 per cent
Ice cream	1.5 per cent
Jams and jellies	0.9 per cent
Muffins	3.6 per cent
Ready-to-eat cereals	3.3-15.4 per cent
Sorbet and sherbet	1.4 per cent
Soup	0.4 per cent
Yogurt	1.3 per cent

Your notice describes published information pertaining to fructooligosaccharide's chemical identity and its natural occurrence. Fructooligosaccharide is a mixture composed of fructose (F) chains with a terminal glucose (G) unit. The number of fructose units varies from two to four. The first fructose unit that is attached to glucose is joined by an alpha 1-1' glycosidic linkage. The remaining fructose units are joined to the first fructose unit in a chain by beta 2-1 glycosidic linkages.

Because the combination of the terminal glucose and the first attached fructose is chemically identical to the moiety known as sucrose, another way to describe each oligosaccharide in the fructooligosaccharide mixture is as a terminal sucrose unit to which one to three additional fructose units are attached. In fact, the starting point in the manufacture of fructooligosaccharide is sucrose. Fructooligosaccharide is manufactured from sucrose syrup by the action of the fungal enzyme beta-fructofuranosidase. The enzyme acts as an invertase on sucrose, yielding fructose and glucose. The enzyme also acts as a fructosyltransferase between sucrose and a fructofuranosyl-sucrose molecule (i.e., a molecule comprised of fructose chains with a terminal glucose), yielding GF_2, GF_3, and GF_4. Your notice includes published information pertaining to the source organism from which the beta-fructofuranosidase is derived. Your notice also provides information on specifications for fructooligosaccharide, including a lead specification of less than 0.2 parts per million.

Fructooligosaccharide exists naturally in plants, and is consumed by humans as a component of the commonly consumed foods onions, bananas, lettuce, and wheat (in rough and bran forms). You estimate that background exposure to fructooligosaccharide from its consumption as a component of various foods ranges from approximately 145 to 250 milligrams per person per day at the 90th percentile consumption level. You also estimate that dietary exposure to fructooligosaccharide from its intended use as a bulking agent would range from approximately 3.1 to 12.8 grams per person per day at the 90th percentile consumption level.

Based on published studies, which were conducted with fructooligosaccharide or related oligosaccharides, you conclude that fructooligosaccharide is virtually unabsorbed and undigested by endogenous enzymes, although a very small amount is hydrolyzed by stomach acid and absorbed into the body as fructose and glucose. About 89 percent of the undigested fructooligosaccharide passes unchanged into the colon where it is fermented by microflora into gases and short-chain carboxylic acids (predominantly acetic acid, while propionic and butyric acids are generated in smaller amounts).

Your notice describes additional studies conducted with fructooligosaccharide. The published animal studies include acute studies in rats and mice, 6-week feeding studies in rats, a teratogenicity study in rats, and a chronic bioassay in rats. The animal studies also include an unpublished 90-day feeding study in rats. Additional published studies include mutagenicity studies, studies describing physiological or systemic effects of fructooligosaccharide, and human studies.

Labeling issues

Section 403(a) of the Federal Food, Drug, and Cosmetic Act (FFDCA) provides that a food is misbranded if its labeling is false or misleading in any particular. Section 403(r) of the FFDCA lays out the statutory framework for a health claim. In describing the intended use of fructooligosaccharide and in describing the information that GTC Nutrition relies on to conclude that fructooligosaccharide is GRAS under the conditions of its intended use, GTC Nutrition raises a number of issues under these labeling provisions of the FFDCA. These issues include (1) physiological effects of fructooligosaccharide that GTC Nutrition views as "beneficial;" (2) the caloric value of fructooligosaccharide; and (3) the classification of fructooligosaccharide as "soluble" fiber. These issues are the purview of the Office of Nutritional Products, Labeling, and Dietary Supplements (ONPLDS) in the Center for Food Safety and Applied Nutrition (CFSAN). OPA neither consulted with ONPLDS on these labeling issues nor evaluated the information in your notice to determine whether it would support any claims made about fructooligosaccharide on the label or in labeling.

OPA did consult with ONPLDS regarding the common or usual name that you provided under proposed 21 CFR 170.36(c)(1)(ii) (i.e., "short-chain fructooligosaccharide"). ONPLDS advises that fructooligosaccharides vary in chain length from 2 to 10 monomers and are also referred to as oligofructose or fructosugar, and that the term "oligosaccharides" itself refers to short chain lengths with 2-10 monomers while longer chain lengths of above 10 monomers are referred to as "polysaccharides." Moreover, sugars composed of 2-5 monomers are associated with specific terms such as di-, tri-, tetra-, and penta-saccharides. ONPLDS found no literature reference that specifically defines or distinguishes fructooligosaccharides as short-chain, medium-chain, or long-chain. For these reasons, in this letter, OPA uses the term "fructooligosaccharide," rather than the term "short-chain fructooligosaccharide," to refer to the ingredient that is the subject of your notice. If you have any questions about the common or usual name that would be used to identify fructooligosaccharide in the ingredient statement of food products that would be marketed in the United States, OPA suggests that you contact the Division of Standards and Labeling Regulations, ONPLDS, CFSAN, HFS-820, 200 C Street S.W., Washington, DC 20204. You can reach this division by telephone at (202)205-4168.

Conclusions

Based on the information provided by GTC Nutrition, as well as other information available to FDA, the agency has no questions at this time regarding GTC Nutrition's conclusion that fructooligosaccharide is GRAS under the intended conditions of use. The agency has not, however, made its own determination regarding the GRAS status of the subject use of fructooligosaccharide. As always, it is the continuing responsibility of GTC Nutrition to ensure that food ingredients that the firm markets are safe, and are otherwise in compliance with all applicable legal and regulatory requirements.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter, as well as a copy of the information in your notice that conforms to the information in proposed 21 CFR 170.36(c)(1), is available for public review and copying on OPA's homepage on the Internet (at http://vm.cfsan.fda.gov/~lrd/foodadd.html).

Sincerely,

Alan M. Rulis, Ph.D.
Director
Office of Premarket Approval
Center for Food Safety
and Applied Nutrition

Health Canada
Santé Canada

Health Protection Branch
Direction générale de la protection de la santé

Bureau of Nutritional Sciences
Food Directorate
Sir F.G. Banting Research Centre
2203A
Tunney's Pasture
Ottawa, Ontario
K1A 0L2

May 9, 1995

Ms. Paula Karabell
Golden Technologies
1400 West 122nd Avenue
Westminster, Colorado
80234 U.S.A.

Dear Ms. Karabell,

In response to your telephone request, this is to inform you that we reviewed the use of fructooligosaccharides (FOS), brand name Nutraflora, made by Golden Technologies, as a food ingredient in meal replacements and other food products.

We have concluded that we would have no objection to the inclusion of Nutraflora FOS as an ingredient in meal replacements or to foods in general at levels that would not be likely to induce diarrhea. In this context, we note that the one study that tested tolerance, per se, (Hata and Nakajima, 1985) has been widely misquoted as demonstrating a lack of inducement of diarrhea at up to 44 and 49 g per day for men and women respectively. These values were actually the amounts of a FOS mixture which was only 56% FOS and therefore, risk of diarrhea may begin at intakes closer to 20 g of FOS per day depending on body weight. This can be verified by noting that the maximum non-effective dose was given as 0.30 g FOS/kg body weight for men and 0.40 g/kg for women.

Please note that FOS is not considered dietary fibre and therefore, the declaration of dietary fibre on a label of a food containing it may not include this ingredient as a source of fibre nor can a claim for dietary fibre be made for this ingredient.

If you have any questions, please do not hesitate to contact us again.

Yours truly,

Nora S. Lee

Nora S. Lee
Nutrition Evaluation Division

Canada

EUROPEAN COMMISSION
DIRECTORATE-GENERAL XXIV
CONSUMER POLICY AND PUBLIC HEALTH
Directorate B - Scientific opinions on health matters
Unit B2 - Management of scientific committees I

Brussels, 6 May 1997
DG XXIV/B/2/PW/II/svi D(97)

FAX

To:	Mr Y Le Bail Collet - Eridiana Béghin-Say, Vilvoorde R&D Center	**Telephone:**	
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From:	P J Wagstaffe DG XXIV/B/2 RP11 - 3/44	**Telephone:**	(+32-2)295.74.64
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Number of pages:	11		
Subject:	**Opinion on actilight - a fructo oligosaccharide (FOS) (expressed on 21 March 1997)**		

Message:

Please find enclosed an advanced copy of a letter regarding the above mentioned subject. The original will follow through the mail.

With kind regards.

P J Wagstaffe

Rue de la Loi 200, B-1049 Bruxelles/Wetstraat 200, B-1049 Brussel - Belgium - Office: RP11 - 3/44.
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EUROPEAN COMMISSION
DIRECTORATE-GENERAL XXIV
CONSUMER POLICY AND PUBLIC HEALTH
Directorate B - Scientific opinions on health matters
Unit B2 - Management of scientific committees I

ADVANCED COPY

Brussels,
DG XXIV/B/2/PW/LJ/svi D(97)

Mr Y Le Bail Collet
Eridania Béghin-Say
Vilvoorde R & D Center
Havenstraat 84
1800 Brussels

Dear Mr Le Bail Collet,

In response to your enquiry I inform you that the Scientific Committee for Food reached a conclusion in its consideration of questions relating to the use of Actilight (fructo oligo saccharide - FOS). The minutes of the meeting record the following position:

"At its 105th Meeting the Committee concluded that, on the basis of the additional information and other recent publications on digestion, excretion and energy value in healthy human beings, there is now sufficient evidence to show that there is no reason to object to the use of Actilight as a foodstuff as far as these aspects are concerned provided that its laxative action was kept in mind. The Committee had however recommended that the draft opinion should include a section addressing the toxicological evaluation made by the Additives Working Group and endorsed by the Committee at the 83rd Meeting. The Rapporteur appointed for the toxicological evaluation presented the revised opinion.

The Secretariat confirmed that the Standing Committee for Foodstuffs had indicated that it would consider fructo-oligosaccharides as a food ingredient and not as a food additive. It was therefore agreed that references to the ADI were inappropriate and they were deleted. As concerns the toxicological evaluation, the Committee concluded as follows

"A complementary file provided by the petitioner presented a more comprehensive analysis of toxicological studies previously submitted. In addition, the petitioner presented new data on sub-chronic toxicity (90-day oral rat study), embryotoxicity in the rat, and a study on colon carcinogenesis after treatment with FOS and a chemical inducer of carcinogenesis. This information showed that FOS has no significant effects, other than gastrointestinal symptoms at doses from 5 - 40 times higher than the no-effect level for laxative effects in humans".

The Committee adopted the opinion after editorial amendment (Annex IV)."

The full text of the opinion is enclosed.

The opinion will be published in due course in the Food Science and Techniques Series through the Office of Official Publications in Luxembourg.

Yours sincerely,

P J Wagstaffe

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EUROPEAN COMMISSION
DIRECTORATE-GENERAL III
INDUSTRY
Industrial affairs III: Consumer goods industries
Foodstuffs - Legislation and scientific and technical aspects

ANNEX IV TO DOCUMENT
III/5157/97

SCIENTIFIC COMMITTEE FOR FOOD

CS/ADD/EDUL/145-FINAL
March 1997

OPINION

ON

ACTILIGHT - A FRUCTO OLIGOSACCHARIDE (FOS)

(expressed on 21 March 1997)

Rue de la Loi 200, B-1049 Brussels, Belgium
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DRAFT OPINION ON ACTILIGHT - A FRUCTO OLIGOSACCHARIDE (FOS)
(expressed on 21 March 1997)

Terms of Reference

To give an opinion on the safety of Actilight (FOS) in accordance with its proposed use as a food ingredient.

Background

The Commission was asked by the petitioner to evaluate Actilight in the context of food additive use in 1988. The Committee concluded at its 83rd Meeting (10 April 92) as follows : "The Committee endorsed the conclusions of the Additives Working Group. Although the Committee had no concern about exposure (estimated at around 2 g/day) through the consumption of normal items of the diet in which fructo-oligosaccharides occur naturally, it noted that even from single, typical portion sizes of foods to which Actilight had been added, intakes approached those at which gastro-intestinal effects in humans had been reported. Furthermore, in feeding studies with experimental animals numerous effects had been seen, in some cases at all dose levels. The Committee concluded that for these reasons, "Actilight" could not be considered acceptable for addition to food at the levels requested".

The petitioner responded in February 1995 by providing additional information which it believes addresses the concerns expressed by the Committee. The legal status of Actilight was clarified by the Standing Committee on Food who indicated in June 1995 that it it would classify fructo oligosaccaride as a food ingredient (and not as a novel food ingredient nor as an additive). At its 100th meeting (7-8 March 1996), it was therefore decided to examine the above mentioned additional information and more recent publications on digestion, excretion and energy value of FOS in healthy humans, and on the effects of chronic consumption of FOS by healthy subjects, in the light of the previous reservations of the Committee related specifically to digestibility.

A complementary file provided by the petitioner presented a more comprehensive analysis of toxicological studies previously submitted. In addition, the petitioner presented new data on sub-chronic toxicity (90-day oral rat study), embryotoxicity in the rat, and a study on colon carcinogenesis after treatment with FOS and a chemical inducer of carcinogenesis. This information showed that FOS has no significant effects, other than gastrointestinal symptoms at dose from 5 - 40 times higher than the no-effect level for laxative effects in humans.

Digestion, absorption and metabolic fate of FOS

Actilight fructo oligosaccharides (FOS) result from the action on sucrose of a fructosyl furanosidase present in *Aspergillus niger*. Sucrose plays the dual role of

fructose donor and fructose acceptor. The first reaction on two sucrose molecules results in kestose (glucose-fructose-fructose ; GF2). The same enzyme acts on kestose to produce nystose (GF3) and on nystose to produce fructosyl-nystose (GF4). Bonds between fructose units are *b* (1-2) [1].

There is no enzyme present in the small intestine that can specifically hydrolyze the (2-1)-*b*-glycosic linkages found in FOS. Consequently, FOS are not hydrolyzed nor absorbed in the small intestine but are totally fermented in the colon. Their main nutritional properties and their value in human nutrition are just related to their inability to be hydrolyzed in the small intestine and to their capacity to reach the colon and to be fermented by the microflora [1].

Studies in rats showed that FOS are not hydrolyzed by salivary and pancreatic amylases, and that few or none are hydrolyzed by intestinal brush border enzymes [2]. Moreover, FOS recovery from rat small intestine was approximately the same as that of an unabsorbable marker [3]. Long-term ingestion of FOS did not cause induction or suppression of the rat intestinal brush border enzymes [2]. FOS did not influence the transmural potential difference of everted sacs prepared from rat jejunum [4]. In addition, when injected intravenously to rats, FOS are rapidly excreted in urine without having undergone any degradation, suggesting that FOS are not used as an energy source in the body [2]. On the other hand, *in vitro* incubation of [U-^{14}C] FOS with the caecal content of rats showed that most of the label appeared in short-chain fatty acids (SCFAs) ; FOS fed to normal rats showed rapid fermentation, whereas germ-free animals delayed excretion of the label for many hours with substantial amounts appearing in feces [5]. Compared with other undigestible sugars (e.g. cellulose, pectins or lactulose), the fermentation of FOS produced higher percentages of propionic and butyric acid, which may be relevant to predicting their metabolic effects *in vivo* [6].

In humans, no change in blood glucose was noted after oral ingestion of FOS [7]. Breath-hydrogen studies have also shown that FOS are fermentable, resulting in an amount of hydrogen in breath similar to that excreted after ingestion of an identical load of lactulose [8], suggesting again that FOS are not digested in the human small intestine. This was recently confirmed by *in vitro* and *in vivo* studies [9]. Only sucrose was hydrolyzed during *in vitro* incubations with homogenates from duodenal mucosa, whereas the constituents oligosaccharides of FOS were not hydrolyzed at all. *In vivo*, the fate of FOS in the human gastrointestinal tract was evaluated in six healthy volunteers over an 11-d period ; after an equilibration phase, 20.1 g FOS/d was given in three identical postprandial doses : distal ileal output of FOS and their constituent components were determined by intestinal aspiration after a single meal, and the amounts of FOS excreted in stools and urine were also measured ; most of ingested FOS, 89 ± 8.3 % ($\bar{x}$ ± SEM), was not absorbed in the small intestine, and none was excreted in stools, indicating that the portion reaching the colon was completely fermented by colonic flora ; a small fraction of ingested FOS (0.12 ± 0.04 %) was recovered in urine ; the mean estimated energy value of FOS was 9.5 kJ, i.e. 2.4 kcal/g [9].

Digestive tolerance

The digestive tolerance to undigestible sugars depends on the amount ingested, on the presence of factors that reduce their osmotic load in the small intestine, and on the degree of adaptation of the colonic microflora to ferment these sugars. The importance of the osmotic effect of undigestible sugars is determined by the concentration of sugar leaving the stomach. This obviously depends on the amount of undigestible sugar ingested but also on factors reputed to slow down gastric emptying, such as the energy content of the meal, the solid content, and the viscosity. The worst conditions for testing the digestive tolerance to undigestible sugars are encountered after fasting, when sugars are tested in a single liquid load, and when the microflora of the subject has not been adapted by chronic sugar ingestion. All these factors must therefore be taken into account when comparing digestive tolerance thresholds of different undigestible sugars [1].

Animal studies

The influence of chronic intake of FOS on growth and intestinal function was investigated in rats by Tokunaga *et al.* [10]. Male Wistar rats, initially weighing 40-50 g each, were fed *ad libitum* for 6-8 weeks ; the only variable in the experimental diets was the carbolydrate source (corn starch only, corn starch partially replaced by 10 or 20 % FOS, or by 20 % glucomannan) ; daily food intake was similar in all four groups of six rats each. After feeding rats on these diets for 6 weeks, the body weight gain of the group receiving the 20 % FOS diet was significantly lower compared to the control group ; in animals on the 10 % diet, no significant decrease in body weight gain was observed ; a remarkable suppression of body weight gain was also observed in animals consuming the 20 % glucomannan diet (a kind of dietary fiber). The smaller body weight gain was interpreted as a consequence of an incomplete utilization of FOS as an energy source.

The feeding of 10 % and 20 % FOS diets produced a significant increase in both wet weight and the ratio of caecum to colon weights : a greater effect was observed in the caecum that in the colon of animals fed on the 20 % FOS diet, as in the case of animals fed on 20 % glucomannan diet. A similar enlargment of the caecum and colon has been observed in rats fed with dietary fibers such as pectin, cellulose, guar gum and wheat bran.

The faecal wet weight increased significantly in animals fed on either the 10 % or the 20 % FOS diet ($p < 0.01$), although the range was considerable. The gastrointestinal transit time was about 28 h, 21 h and 14 h in the control, 10 % and 20 % FOS diets, respectively, in inverse correlation to the faecal wet weight. The concentration of volatile acids (SCFAs) per gram of wet feces also greatly increased in animals fed FOS or glucomannan compared with the control group, but the profile of SCFAs were different, indicating that the effects on intestinal microflora also differ.

Finally, it was pointed out by the authours that rats developed diarrhoea after starting FOS feeding. This stopped within 2 to 3 weeks, differing with individuals rats. However, FOS intake of rats in the present study was more 10 g per kg bw in

the early period of feeding. Another study, quoted by Tokunaga *et al.*, but only published as an abstract, has shown previously that single-dose intake of FOS at less than 0.8 g per kg bw does not produce diarrhoea in males, but that it does at above this level, and that females are more resistant to diarrhoea than males [10].

Human studies

The gastrointestinal tolerance of the mixture of oligosaccharides consisting of glucose linked to a series of 2, 3 or 4 fructose molecules was evaluated in three studies.

The Japanese study

A first estimation of the maximum non-effective of FOS in humans and of the 50 % laxative effective dose (the amount of FOS which causes diarrhoea in 50 % of people, ED_{50}) was performed in 85 healthy Japanese volunteers (51 men, 34 women) by Hata & Nakajima [11]. To test the effect on the digestive tract using diarrhoea as index, a FOS mixture (56 % FOS, 12 % sucrose, 29 % glucose, 3 % fructose) given in six different dosages (0.21, 0.27, 0.40, 0.53, 0.67 and 0.8 g/kg bw) in 180 ml of water after lunch was compared with a sucrose mixture (68 % sucrose, 29 % glucose, 3 % fructose). At the administration of 0.2 to 0.3 g/kg bw standard dose, diarrhoea did not occur in both men and women. The maximum non-effective dose of FOS on diarrhoea (expressed as pure FOS) was 0.3 g/kg (approximately 44 g of the FOS mixture) for men, and 0.4 g/kg (approximately 49 g of the FOS mixture) for women ; in comparison, the maximum non-effective dose of sorbitol for men was 0.15 g/kg. The ED_{50} of FOS was 0.78 g/kg for men and 0.84 g/kg for women (ED_{50} for sorbitol : 0.5 g/kg) ; incidence of diarrhoea due to doses above the maximum non-effective doe was higher in men than in women by approximately 10 %. Effects of 0.27 g FOS/kg bw on the abdominal symptoms and the macroscopic aspects of stools were not influenced by differences in the form of intake (FOS mixture *vs* purified FOS, with or without mixing with food), sex and age. At the same dosage (14 g/d for women and 17 g/d for men), no significant difference was noted between FOS and sucrose [11].

The US study

Clinical tolerance to regular consumption of FOS also was studied by Stone-Dorshow and Levitt [8]. In volunteers receiving a constant daily amount of FOS (5 g three times a day with meals), they showed that gaseous symptoms, such as flatulence, bloating and abdominal discomfort were significantly more severe in subjects ingesting the FOS than in control subjects ingesting sucrose. Moreover, symptoms did not improve after a 12-day period on FOS. However, at this daily dose, with the exception of flatulence, symptoms were rated absent or mild and no subject experienced diarrhoea.

The French study

The French study, performed on healthy volunteers (6 females, 8 males, aged from 21-37 years), was a double-blind, randomized cross-over study. The tolerance to FOS was compared with that to sucrose ; both sugars as hard candies (2.5 g of sugar each) were consumed occasionally and regularly. In the first period, FOS consumption was occasional, i.e. one dose of FOS and sucrose were tested at random on tuesdays and thursdays of each week ; to avoid any adaptation, subsequent ingestions of FOS thus were separated by at least 5 days ; the starting dose was 10 g ; sugar doses were increased by 10 g until diarrhoea and/or a symptom graded 3 occurs, or when volunteers did not want to ingest more candies.

In the second period, volunteers were asked to consume the same sugar (either FOS or sucrose) every day according to an increasing schedule lasting at most 18 days ; in the same manner, they consumed the other sugar after a washout period of 15 days ; according to the schedule, subjects should reach the threshold found in the first period (diarrhoea and or grade 3 symptom) on the 15th day ; on days 16, 17 and 18, they should ingest this dose plus 10, 20 and 30 g, respectively ; as in the first period, subjects were asked to stop sugar ingestion if diarrhoea and/or a grade 3 symptom occured.

At each dose, the mean scores for each symptom (flatus, borborygmi, bloating, cramps, diarrhoea) experienced with FOS and sucrose were compared ; the 50 % effective dose (ED_{50}) was determined graphically. H_2 excretion in breath was measured on the 15th day of FOS and sucrose consumption. Fifteen days after the conclusion of the study, breath H_2 excretion was again assessed before and after FOS ingestion, to evaluate if H_2 excretion was higher in these conditions than H_2 excretion measured for the same daily dose during chronic consumption of FOS.

Symptomatic responses and laxatives thresholds were roughly similar during the occasional and regular consumption of FOS. The threshold FOS dose was reached in all subjects : 13 experienced diarrhoea and one severe abdominal pain in the first period ; during the second one, 9 had diarrhoea, 1 complained of severe flatus and 4 did not want to take more candies. In both studies, approximately 10 % of the volunteers complained of flatus and borborygmi with 10 g/d ; for excess flatus, the first dose at which the severity of the symptom was significantly higher with FOS than with sucrose was 30 g ; for borborygmi, bloating and abdominal cramps, this dose was 40 g ; the mean threshold and laxative doses were approximately 50-60 g, as ED_{50} for diarrhoea. The mean volume H_2 excreted in breath was not significantly higher during the occasional consumption of FOS than during chronic consumption (185 ± 103 *vs* 120 ± 104 ml/16 h ; p = 0.16) ; H_2 excretion was 45 ± 18 ml/16 h during the sucrose consumption.

The authors concluded that chronic consumption of FOS initiated cautiously with subsequent gradual increase did not improve tolerance, nor reduce excretion of hydrogen. Their results confirm that adaptive response of the microbial flora may be different with various unabsorbable sugars [12,13].

Conclusions

Metabolic studies on Actilight (a mixture of oligosaccharides consisting of glucose linked to 2, 3 or 4 fructose units) have shown that FOS are poorly absorbed in the human small intestine, but completely fermented by colonic flora.

Diarrhoea has been reported in rats fed a 10 % or 20 % FOS diet (more than 10 g/kg bw in the early period of feeding) but laxative effect of FOS stopped within 2-3 weeks. However, the fecal wet weight and the concentration of SCFAs per gram of wet feces remained higher than in control rats at the beginning of the 6th week of 10

or 20 %. FOS diet ; gastrointestinal transit time was inversely correlated with the fecal wet weight. In animals on the 10 % FOS diet, no significant decrease in body weight gain was observed ; the body weight gain of the group receiving the 20 % FOS diet was significantly lower, compared to the control group.

In humans, the maximum non-effective dose of FOS (expressed as pure FOS) on diarrhoea was 0.3 g/kg bw (approximately 24 g/day) for men and 0.4 g/kg bw (approximately 28 g/day) for women. At the administration of 0.2-0.3 g/kg bw, diarrhoea did not occur in both men and women.

With 15 g (5 g three times a day with meals) gaseous symptoms, such as flatulence, bloating and abdominal disconfort were significantly more severe in subjects ingesting the FOS than in control subjects ingesting sucrose. At this daily dose, with the exception of flatulence, symptoms were rated absent or mild and no subject experienced diarrhoea.

The amount of FOS which causes diarrhoea in 50 % of healthy adult volunteers (ED_{50}) was estimated in two different studies to 0.8 g/kg bw, i.e. approximately 50-60 g/day. Gastrointestinal symptoms did not improve after a 12 to 15 day period on FOS. In comparison the maximum non-effective dose of sorbitol and ED_{50} for sorbitol were lower (0.15 g/kg bw and 0.5 g/kg, respectively) than those for FOS.

In accordance with its previous opinion on polyols (16th series), the Committee concluded that, although laxation may be observed at high intakes (more than 30 g/day) a consumption of the order of 20 g a day of FOS is unlikely to cause more undesirable laxative symptoms than isomalt, lactitol, maltitol, mannitol, sorbitol and xylitol. The Committee has no objection to the use of Actilight provided the limitation due to its laxative action are kept in mind.

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Regulatory Status/Claims
3/2006

Australia/New Zealand
Therapeutic Good Administration Listing
ANZFA - Approved
Fiber - Accepted
Bifidogenic Properties - Any statements must comply with FSANZ Clause 1 of Standard 1.1.3.

Argentina
Food Ingredient - Approved 1999
Fiber - Approved 1999

Belgium
Food ingredient - Approved 1991
Fiber - Approved 1991
Bifidogenic Properties - Not subject to prior approval

Brazil
Food ingredient - Approved 2002
Fiber - Approved 2002
Bifidogenic Properties - Accepted, must comply with Brazilian Health Authority (ANVISA) standards

Canada
Food ingredient - Approved 1995
Fiber - Pending issue
Bifidogenic properties - Pending issue

Chile
Food Ingredient - Not subject to prior approval
Fiber - Pending
Bifidogenic properties - Not subject to prior approval

Denmark
Food Ingredient - Accepted
Fiber - Accepted

Finland
Food Ingredient - Accepted
Bifidogenic Properties - Accepted, must comply with the report of the National Food Administration "Medicinal & Health claims in the marketing of food stuffs"

France

Food Ingredient - Approved 1995
Fiber - Approved 1995
Bifidogenic properties (2.5g/day) - Approved 1997

Germany

Food Ingredient - Accepted
Fiber - Accepted
Bifidogenic Properties - Accepted

Italy

Food Ingredient - Accepted
Fiber - Approved 1993
Bifidogenic Properties - Not subject to prior approval

Japan

Foods for Specified Health Use (FOSHU) 1991
FOSHU: Food approved by the Ministry of Health and Welfare as effective for preservation of health by adding certain active ingredients or removing undesirable ones. These foods are designed to be effective for the maintenance and improvement of health by incorporating them into one's diet. Since the legislation of FOSHU in 1991, short chain fructooligosaccharides has been approved and marketed in Japan. In 1998, scFOS was an ingredient in 11 out of 47 products containing oligosaccharides.

Mexico

Food Ingredient - Accepted
Fiber - Accepted
Bifidogenic Properties - Not subject to prior approval

Netherlands

Food Ingredient - Approved 1995
Fiber - AOAC Method being considered
Bifidogenic Properties - Not subject to prior approval

Peru

Food Ingredient - Accepted
Fiber - Accepted
Bifidogenic Properties - Not subject to prior approval

Spain

Fiber - Accepted
Bifidogenic Properties - Not subject to prior approval

Sweden

Food Ingredient - Accepted
Fiber - AOAC Method
Bifidogenic Properties - Pending

Switzerland

Food Ingredient - Approved 1995
Fiber - Approved 1995

United Kingdom

Food Ingredient - Accepted
Bifidogenic Properties - Not subject to prior approval

United States

Dietary Supplement - Approved 1994
Self-Affirmed as GRAS in yogurt (1994), medical foods and poultry (1990)
FDA GRAS Affirmation Notification - No Questions Letter, November 2000
Dietary Fiber Definition - **
Bifidogenic Properties - Not subject to prior approval

**The Institutes of Medicine (IOM) has proposed a dietary fiber definition which includes scFOS as a dietary fiber. This definition is applied by most companies in advance of FDA adoption.

GTC NUTRITION

SPECIALTY INGREDIENTS

Material Safety Data Sheet

Product: NutraFlora® P-95
Short-Chain Fructooligosaccharides (scFOS®)

MSDS No: GTC / N1
Date: October 2004

National Paint and Coatings Association

Hazardous Material Identification System

HEALTH HAZARD	0 – Minimal
FLAMMABILITY HAZARD	0 – Minimal
REACTIVITY HAZARD	0 – Minimal
PERSONAL PROTECTION	SEE SECTION VIII

SECTION I. MATERIAL IDENTIFICATION

Trade/Material Name: NUTRAFLORA® P-95

Chemical Name: Fructooligosaccharides Powder

CAS: Fructooligosaccharides 308066-66-2

Manufacturer: GTC Nutrition
600 Corporate Circle, Suite H
Golden, CO 80401

Phone: Emergency: (303) 216-2489
Other Calls: (303) 216-2489

SECTION II. INGREDIENTS AND HAZARDS

Ingredient Name:	Percent:	Exposure Limits:
Glucose, Fructose, Sucrose	< 5	Not Established
Fructooligosaccharides	> 95	Not Established
GF2: 1-Kestose	35 ± 6	Not Established
GF3: Nystose	50 ± 6	Not Established
GF4: 1-Fructofuranosyl Nystose	10 ± 4	Not Established

SECTION III. PHYSICAL DATA

Appearance & Odor:	White Powder, Odorless
Boiling Point:	N/A
Vapor Pressure:	N/A
Water Solubility (%):	Soluble in water
Vapor Density (air=1):	N/A
pH:	N/A
Evaporation Rate:	N/A
Specific Gravity (H_2O=1)	N/A
% Volatile by Volume:	N/A

SECTION IV. FIRE AND EXPLOSION DATA

Flash Point (Method): N/A

Limits: LEL %: N/A UEL %: N/A

Extinguishing Media: N/A

Unusual Fire or Explosion Hazards: None

Special Fire-Fighting Procedures: None

SECTION V. REACTIVITY DATA

Material is stable

Hazardous polymerization cannot occur

Chemical Incompatibilities: None

Conditions to Avoid: None

Hazardous Decomposition Products: None

SECTION VI. HEALTH HAZARD INFORMATION

Acute Effects: N/A

Chronic Effect (s): None

First Aid:

- Eye Contact: Flush with water
- Skin Contact: Flush with water
- Inhalation: Remove to fresh air

SECTION VII. SPILL, LEAK AND DISPOSAL PROCEDURES

Spill / Leak Procedures: Vacuum / Sweep spillage

Waste Management / Disposal: Dispose of waste product in accordance with applicable Local, County, State and Federal regulations

SECTION VIII. SPECIAL PROTECTION INFORMATION

Personal Protective Equipment:

- Goggles: Not generally required, safety glasses are good practice
- Gloves: Not generally required, otherwise rubber or leather gloves

Respirator: None

Workplace Considerations:

Ventilation: None

SECTION IX. SPECIAL PRECAUTIONS

Other Precautions: None

While GTC Nutrition believes that the data contained herein are factual and the opinions expressed are those of qualified experts regarding the results of the tests conducted, the data are not to be taken as a warranty or representation for which GTC Nutrition assumes legal responsibility. They are offered solely for your consideration, investigation and verification. Any use of these data and information must be determined by the user to be in accordance with applicable Federal, State and Local laws and regulations.

Review

Bioscience Microflora Vol. 21 (1), 43–54, 2002

Application of Fructooligosaccharides to Medical Foods as a Fermentable Dietary Fiber

Keith A. Garleb,* Maureen K. Snowden, Bryan W. Wolf and JoMay Chow

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Presented at International Symposium on Fructooligosaccharides, held in Tokyo, July 3, 2001. Received for publication, August 31, 2001

Fructooligosaccharides are an ideal source of fermentable fiber for medical foods. Typically, medical foods are liquid, many of which are fed to patients through a tube. Liquid medical foods that are fed through a tube must be low in viscosity. Fructooligosaccharides are soluble and will not clog feeding tubes and do not significantly increase the viscosity of the product. Rationale for the use of fructooligosaccharides in medical foods includes: normalization of bowel function, maintenance of large bowel integrity, restoration of colonization resistance, alteration in route of nitrogen excretion, and improvement in calcium absorption. Normalization of bowel function refers to the treatment or prevention of constipation or diarrhea in patients receiving a medical food. Fructooligosaccharides, through anaerobic fermentation by colonic bacteria and the production of short chain fatty acids, may be useful in preventing large bowel atrophy or treating distal ulcerative colitis. Fructooligosaccharides, by selectively supporting the growth of bifidobacteria or producing an environment in the colon (e.g., increased short chain fatty acid concentration or decreased pH) that is not conducive to the growth of certain pathogenic organisms, may help restore colonization resistance. Anaerobic fermentation of fructooligosaccharides, leading to the bacterial cell growth and a reduction is colonic pH, may shift nitrogen excretion from the urinary to the fecal route. Improvements in calcium absorption may occur through mechanisms involving short chain fatty acid absorption and a reduction in large bowel pH. Overall, compatibility with liquid products and numerous physiological benefits to the patient justify the use of fructooligosaccharides in medical foods.

Key words: fructooligosaccharides; fermentable fiber; medical foods; bowel function; colonization resistance

INTRODUCTION

Numerous health benefits are associated with the consumption of dietary fiber. In 1987, the Federation of American Societies for Experimental Biology recommended a dietary fiber intake of 10–13 g/1000 kcals *(25)*. Also, based on investigational experience with a variety of self-selected and experimental diets, the Panel estimated that the dietary fiber in the recommended diet would comprise approximately 70–75% insoluble fibers and 25–30% soluble fibers. Most individuals can meet this recommendation by incorporating grains, fruits, and vegetables into their diet. However, certain individuals must use a medical food to meet their dietary needs. A medical food is defined by the U.S. Food and Drug Administration *(19)* as "a food that is formulated to be consumed or administered enterally under the supervision of a physician and is intended for the specific dietary management of a disease or condition for which distinctive nutritional requirements, based on recognized scientific principles, are established by medical evaluation." Medical foods are typically liquid; patients may require that the product be administered via an enteral feeding tube. Internal diameter of an enteral feeding tube, for obvious reasons, is small. If a medical food is fed without the aid of a pump, its viscosity must be kept below 0.1 pascal seconds. Unfortunately, dietary fiber, particularly soluble dietary fiber, has a tendency to increase the viscosity of liquid medical foods. Also, insoluble fibers settle to the bottom of the container, increasing the risk of tube clogging. Hence, it was imperative that sources of fiber or fiber-like material be identified that provided physiological benefits but did not compromise the physical stability and feeding characteristics of the medical food. Nondigestible oligosaccharides are soluble and fermentable and represent an ideal source of dietary fiber for use in medical foods.

An oligosaccharide is a carbohydrate consisting of a small number (from 2 to 4 or as many as 10, according to various authorities) of monosaccharides *(24)*. Many types of nondigestible oligosaccharides are produced commercially from various sources of food materials (Table 1). Xylooligosaccharides (XOS) are prepared

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Table 1. Nondigestible oligosaccharides.

Oligosaccharide	Source/ origin	Reducing sugar
Short chain fructooligosaccharides	sucrose	no
Frutan-based oligosaccharides	inulin	yes
Xylooligosaccharides	xylan	yes
Soybean oligosaccharides	soybeans	no
Galactooligosaccharides	lactose	yes
Lactulose	lactose	yes

through the enzymatic hydrolysis of the xylan from corn, sugar cane, or cottonseed. Xylobiose, the main component of XOS, is relatively abundant in bamboo shoots *(53* cited from *31)*. XOS is a reducing sugar, thus making it susceptible to the formation of Maillard products during liquid nutritional manufacturing. The Maillard reaction includes all reactions involved when an aldehyde (or ketone) and an amino group are heated together *(1)*. This non-enzymatic browning reaction forms linkages that are not hydrolyzed during digestion resulting in the loss of amino acid availability. Any oligosaccharide that contains reducing sugars would be susceptible to the formation of Maillard products with free α-NH_2 groups and especially the ε-NH_2 group of lysine.

Soybean oligosaccharides are extracted from soybean whey, a by-product of soy protein concentrate production. Soybean oligosaccharides contain primarily sucrose and the nondigestible oligosaccharides raffinose and stachyose. The safety of soybeans and its components is considered unquestionable in view of their long history of use in common foods.

Galactooligosaccharides, also known as transgalactosylated oligosaccharides (TOS), are produced from lactose through the transgalactosylation reaction of *Aspergillus oryzae* β-galactosidase. TOS has the general formula Gal-$(Gal)_n$-Glc where Gal denotes a galactose residue, Glc denotes a glucose residue, and n denotes an integer of 1 to 4 *(95)*. TOS or TOS-like oligosaccharides are found in human milk. TOS is a reducing sugar and is susceptible to the Maillard reaction.

Lactulose is a synthetic disaccharide (4-*O*-β-D-galactopyranosyl-D-fructofuranose) produced through the alkali isomerization of lactose. Lactulose is used in the treatment of patients with hepatic encephalopathy *(67)*.

Fructan-based oligosaccharides are also referred to as oligofructose in the scientific literature *(77, 78)*. Fructan-based oligosaccharides are produced by the enzymatic hydrolysis of inulin, a storage carbohydrate found in high concentrations in Jerusalem artichokes and chicory. The hydrolysis results in a wide array of oligosaccharides such as 1-kestose, nystose and 1^F-β-fructofuranosyl nystose as well as oligosaccharides containing only fructose. Because fructan polymers have a reducing end, they are susceptible to Maillard product formation.

Short chain fructooligosaccharides (eg., scFOS, Neosugar, Nutraflora™, Meioligo®) are enzymatically synthesized (β-fructosyltransferase) from sucrose and have been isolated from such foodstuffs as onions, wheat, barley, bananas, tomatoes, garlic, and artichokes *(15, 92, 96)*. The isolation and development of fructooligosaccharides was first reported in the Japanese literature in 1983 *(46* cited from *45)*. Although fructooligosaccharides can be extracted from a variety of plants, they can also be produced by adding *Aspergillus niger* fructosyltransferase to sucrose *(47)*. Short chain FOS (scFOS) consists of the following fructooligosaccharides: 1-kestose, nystose and 1^F-β-fructofuranosyl nystose. Basically, short chain fructooligosaccharides are a sucrose molecule linked to a sequence of 1 to 3 fructose molecules via a (2-1)-β glycosidic bond to the fructose unit of sucrose. Short chain FOS is a non-reducing sugar and will not undergo the Maillard reaction. The Ross Products Division of Abbott Laboratories incorporates scFOS into several medical foods. The application of scFOS to medical foods as a fermentable dietary fiber is discussed below.

MICROFLORA DISTRIBUTION AND FERMENTATION OF FRUCTOOLIGOSACCHARIDES

The physiological effects of scFOS are directly related to the fact that the oligosaccharides are not digested in the upper gastrointestinal tract but remain intact as they enter the large bowel where they are fermented by the indigenous microflora. A number of studies have shown that scFOS are not hydrolyzed by the digestive enzymes of vertebrates. Hydrolysis of scFOS could not be demonstrated during *in vitro* incubations with either human jejunal homogenates *(118)* or human salivary enzymes *(46)*. Furthermore, scFOS were shown to be indigestible in rats *(74)* and failed to elicit an insulin response when fed to humans *(46)*. While mammalian enzymes are unable to degrade scFOS, there exists within the gastrointestinal tract a large and diverse microflora population capable of utilizing scFOS as an energy source.

The population distribution of bacteria differs in the various parts of the gastrointestinal tract. In general,

the mouth, stomach, and most of the small intestine are dominated by aerobes or facultative anaerobes since these locations are not anaerobic, and population densities are relatively low (10^3 to 10^8 bacteria per gram). Population density rises markedly at the ileum reaching close to 10^{11} per gram from the cecum to the rectum. In the colon, strict anaerobes outnumber aerobic organisms by a factor of 1,000:1, and the predominant genera include bacteroides, eubacteria, peptococci, and bifidobacteria *(29)*.

Fermentation, the process by which anaerobic organisms break down dietary and other substrates to obtain energy for growth and the maintenance of cellular function, is an important component of large bowel activity *(23)*. More than 70% of the energy from carbohydrate fermentation is conserved as short chain fatty acids (SCFA) and other fermentation products (methane, CO_2, and H_2); some of these fermentation end-products can be absorbed and utilized by the host *(91)*. The remaining 30% of the energy is used by bacteria to support growth (synthesis of monomers and polymerization) and non-growth-related (maintenance of ion gradients, nutrient transport, motility) functions.

The three predominant SCFA, acetate, propionate, and butyrate, account for 83% of the SCFA produced during fermentation *(84)*, and they are produced in an approximate ratio of 60:20:20 *(22)*. Theoretically, the complete fermentation of 1 gram of carbohydrate should lead to the formation of 10 mmol of SCFA *(21)*. Daily production of SCFA in the human colon has been estimated to be greater than 300 mmol/day, yet fecal excretion is only about 10 mmol/day *(50)*. Most of the SCFA produced during fermentation are absorbed from the large bowel. Using a dialysis bag technique, McNeil et al. *(65)* estimated absorption rates of SCFA from the rectum to be 8.9 μmol/cm^2/hr (5.2, 1.8. and 1.9 μmol/cm^2/hr for acetate, propionate and butyrate, respectively).

SCFA serve as a source of energy for the host. Acetate, the most abundant SCFA, is primarily used as a fuel for host tissues. It is the only SCFA found at appreciable levels in the peripheral blood where it may be oxidized in muscle and adipose tissue. Propionate may be oxidized by the colonocytes as a source of energy but is believed to be primarily used by the liver as a substrate for gluconeogenesis *(39)*. Butyrate is preferentially oxidized by colonocytes as a source of energy. Roediger *(79)* found that more than 70% of the oxygen consumed by colonocytes from the ascending and descending colon was due to butyrate oxidation in isolated human colonocytes.

PHYSIOLOGICAL EFFECTS

As mentioned previously, the structural characteristics (i.e. nondigestible in the upper gastrointestinal tract, non-reducing sugar, and highly soluble) of scFOS make for an ideal fermentable fiber source in medical foods. Potential physiological benefits of scFOS for patients include positive effects on bowel function, large bowel integrity, prebiotic and colonization resistance, nitrogen excretion and calcium absorption.

Bowel Function

Of all the physiological benefits of dietary fiber, its effect on bowel habits is the most noted. The effect of dietary fiber can best be described as a "normalization" of bowel function and has been used for the treatment and prevention of both constipation *(52)* and diarrhea *(119)*. Human studies confirm that the consumption of scFOS can affect stool consistency and subsequently constipation. Hata and Nakajima *(43)* administered varying single doses of scFOS to 80 healthy adults (51 males and 29 females; ages 20 to 59) and determined that the dosage resulting in 50% of the subjects experiencing diarrhea (a watery stool during the first defecation following ingestion of scFOS) was approximately 0.8 g scFOS/kg body weight. Subsequent studies conducted by Briet et al. *(12)* in which healthy human adults received acute or chronic dosing with scFOS generated similar results. Tokunaga et al. *(100)* showed that healthy volunteers had softer stools and increased frequency of bowel movements when they consumed scFOS for two weeks (up to 5 g/day) than during baseline or after a week washout period. The administration of scFOS has been reported to relieve moderate cases of constipation; *(46, 94)* however, severe constipation was not alleviated *(94)*.

Fructooligosaccharides may promote laxation via a mechanism similar to that of other nondigestible carbohydrates *(49, 18)* such as lactulose. Based on available evidence from rats *(16, 46, 97)* and from humans *(3, 12, 18, 48, 93)*, the laxation effect of fructooligosaccharides is dose-dependent and is the result of a combination of factors similar to those attributed to lactulose *(6)*: 1) increased bacterial growth, 2) gas production, and 3) increased intraluminal osmolarity and/or decreased stool pH.

Fructooligosaccharides, through anaerobic fermentation and production of short chain fatty acids, may play a positive role in the alleviation of diarrhea. Wolf et al. *(116)* compared the fermentability of several nondigestible oligosaccharides. Short chain fructooli-

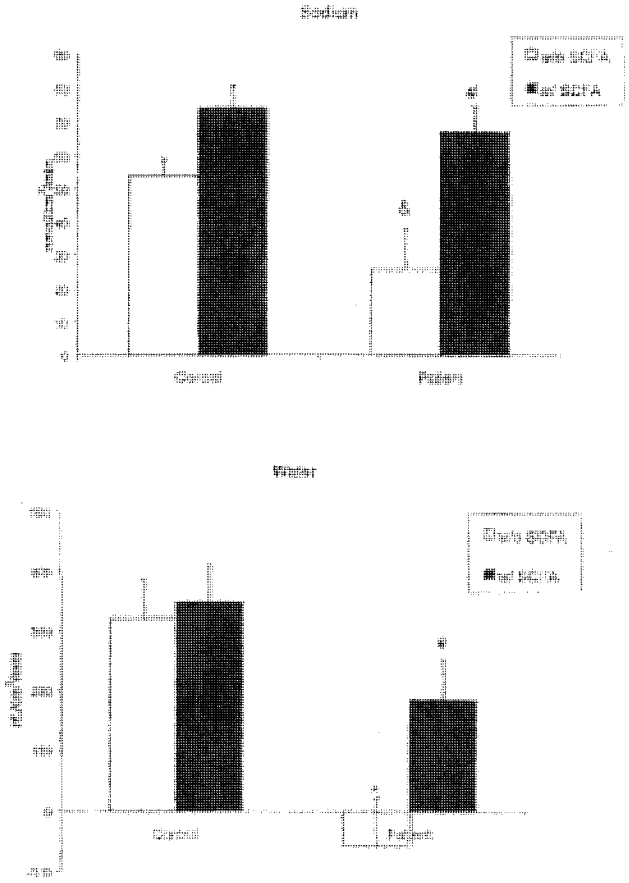

Fig. 1. Net movement of sodium and water in the rectum of control subjects ($n = 9$) and patients with acute diarrhea ($n = 17$).
All values are mean±SEM. W/o SCFA contained 140 mmol/l sodium choride while w/ SCFA contained a mixture of short chain fatty acids (sodium, acetate, propionate, butyrate, and chloride; 140, 60, 40, 20 and 20 mmol/l, respectively). &, $p < 0.05$ compared with control; #, $p < 0.01$ compared with w/o SCFA patients; *, $p < 0.01$ compared with control. Modified from Ramakrishna and Mathan *(75)*.

gosaccharides, xylooligosaccharides, and hydrolyzed inulin were fermented with human fecal inoculum *in vitro*. Fermentation rates of all oligosaccharides were rapid, being essentially complete by 6 hours for the scFOS and hydrolyzed inulin, and by 12 hours for the xylooligosaccharides. A similar study evaluating the fermentability of soy fiber, gum arabic, fructooligosaccharides and lactulose was conducted by Garleb et al. *(32)*. Fructooligosaccharides and lactulose were fermented much more rapidly ($p < 0.01$) than either gum arabic or soy fiber. Furthermore, the fermentation of scFOS was essentially complete by 6 hours and by 12 hours for lactulose.

Short chain fatty acids can improve bowel function by facilitating water absorption. The absorption of 100 mmol SCFA is associated with the absorption of 360 ml water *(17)*. Ramakrishna and Mathan *(75)* found that fecal output of SCFA in patients with acute diarrhea was low on the first day of illness, but increased over the next five days as the patients condition improved. Furthermore, they demonstrated that luminal SCFA could restore net water and sodium reabsorption in the rectum of patients with acute diarrhea (Fig. 1). *In vivo* perfusion studies in healthy subjects have shown secretion of salt and water in the ascending colon in response to enteral feeding *(9, 10)*. Bowling et al. *(8)* investigated the effect of SCFA on colonic fluid secretion induced by enteral feeding. The researchers found that SCFA infusion into the cecum of healthy subjects reversed the fluid secretion seen in the ascending colon during enteral feeding and theorized that these findings could have implications for the management of diarrhea related to enteral feedings.

Large Bowel Integrity

Fructooligosaccharides, through the production of SCFA, may be useful in maintaining large bowel integrity or serving as adjunctive therapy for the treatment of inflammatory bowel disease. The feeding of dietary fiber has been associated with the stimulation of colonic cell proliferation. The stimulatory effect has been attributed to the SCFA produced during fiber fermentation. SCFA may stimulate proliferation directly or indirectly by mediating blood flow or pH. This phenomenon has been observed in both the large and small bowel. For example, Aghdassi et al. *(2)* found that reducing colonic fermentation resulted in reduced intestinal adaptation and nutritional recovery in rats with massive small bowel resection. Moreover, others have observed stimulation of small bowel mucosal proliferation with SCFA supplementation *(58, 85)*. Younes et al. *(117)* administered to rats a fiber-free diet or diets containing 7.5% fiber as oat fiber, gum arabic, scFOS, or XOS. Cecal wall weight was significantly greater ($p < 0.05$) for the fermentable substrates (that is, gum arabic, scFOS and XOS) compared with the poorly fermented oat fiber and the fiber-free control. Howard et al. *(51)* fed supplemental (3 g/l) scFOS in a liquid diet to neonatal pigs and observed increased cell proliferation in the proximal and distal colonic epithelial mucosa (Fig. 2). From these animal experiments it can be concluded that fermentable substrates may play a key role in the maintenance of large bowel integrity and function.

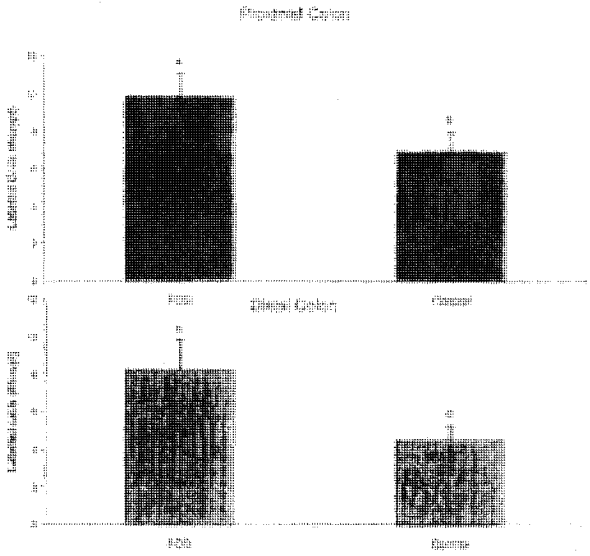

Fig. 2. Proximal and distal mucosa growth in neonatal pigs fed diets containing 0 or 3 grams scFOS per liter.
All values are mean ± SEM. Within colonic section, bars with unlike superscrip letters differ ($p < 0.01$). Modified from Howard et al. *(51)*.

Increasing interest has been generated in the use of enemas/irrigation solutions containing buffered, physiologic levels of SCFAs for the treatment of diversion colitis and ulcerative colitis. Diversion colitis is an inflammatory process arising in segments of the colorectum at various intervals after surgical diversion of the fecal stream. The endoscopic appearance is similar to those of active Crohn's disease and ulcerative colitis *(36)*. The cause of this condition is not known, but one mechanism has been postulated; a nutritional deficiency of the colonic epithelium, specifically due to the absence of SCFAs normally present in colonic contents *(57, 81)*. Harig et al. *(42)* tested this hypothesis by assessing whether a SCFA irrigation could ameliorate inflammation in four patients with diversion colitis. After 2–3 weeks of therapy, macroscopic and histological resolution of inflammation was evident.

An impaired utilization of SCFA also has been implicated in ulcerative colitis which suggests that diminished intracellular energy production may be important in the inflammatory process *(80)*. It has been demonstrated that fecal water from patients with ulcerative colitis contains reduced concentrations of SCFA as well as markedly increased lactate and low pH *(105, 106)*. In a study by Breuer et al. *(11)* the effect of large bowel irrigation with SCFA in patients with ulcerative colitis was studied. It was found that 9 out of 10 patients completing the study were judged to be at least much improved and showed a significant change in mean disease activity index score and mucosal histology score. Senagore et al. *(90)* confirmed the results of Breuer et al. *(11)* demonstrating an 80% response rate in patients with idiopathic proctosigmoiditis. This study indicates that administering a solution of SCFA similar to Harig et al. *(42)* for six weeks was equally efficacious to corticosteroid or 5-aminosalicylate for the treatment of proctosigmoiditis at a significant cost savings. Scheppach et al. *(86)* investigated the use of butyrate enemas alone rather than the SCFA mixture to treat ten patients with distal ulcerative colitis in a placebo-controlled, single-blind, randomized trial. The authors concluded that butyrate as an end product of bacterial fermentation in the large bowel markedly improved disease activity index and histological parameters suggesting that the effect of a SCFA mixture on the inflamed mucosa in ulcerative colitis is largely attributable to its butyrate moiety.

It is unlikely that SCFA added directly to an enteral product would reach the large bowel. Medical foods can take advantage of the positive effect of SCFA by providing fermentable fiber. For example, Rolandelli et al. *(82)* demonstrated a benefit of a fermentable fiber (pectin) in the treatment of experimental colitis. Also, Grisham et al. *(38)* evaluated the effect of enteral diets containing fish oil or fermentable substrates such as scFOS or XOS on chronic colitis induced with peptidoglycan polysaccharide in rats. Histological and biochemical markers of inflammation were improved. Recently, Seidner et al. *(89)* assessed the efficacy of a novel medical food supplemented with fish oil, scFOS, gum arabic, and antioxidants on reducing corticosteroid use in adults with mild to moderate ulcerative colitis. Patients given this formula had a significantly greater rate of reduction in the daily dose of prednisone over 6 months as compared to controls receiving a sucrose based placebo.

Prebiotics and Colonization Resistance

Fructooligosaccharides are a prebiotic: a nondigestible food ingredient that improves host health by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon *(35)*. *In vitro* experiments indicate that scFOS are utilized by several beneficial species of gastrointestinal bacteria, but not utilized by potentially pathogenic species. Using pure cultures, Hidaka et al. *(46)* showed that bacteroides and bifidobacteria grew to high optical densities and produced large amounts of organic acid when scFOS were provided as an energy source. However, potential patho-

gens such as *Escherichia coli* and *Clostridium perfringens* did not. Similar results were noted by Wang and Gibson *(108)* who showed that oligofructose or inulin selectively enhanced the growth of bifidobacteria in fecal slurries with little change in the numbers of either clostridia or coliforms.

Enrichment for bifidobacteria in human feces has also been demonstrated *in vivo*. When scFOS were fed to elderly Japanese patients, bifidobacteria numbers in feces increased approximately 10-fold *(66)*. The magnitude of the increase depended upon the number of bifidobacteria that were initially present; individuals with high initial counts of bifidobacteria showed little, if any, increase in bifidobacteria in response to treatment with scFOS. Individuals who initially had low numbers (less than 10^8 cfu/g stool) showed two to four log increases in counts *(46)*. In healthy adult volunteers, Williams et al. *(110)* reported an increase in fecal bifidobacteria numbers with the consumption of 4 g FOS/day. A study conducted by Garleb et al. *(33)* further documented the bifidogenic capacity of scFOS. In this study, healthy subjects were fed a low residue polymeric formula (LRPF), LRPF + 5 g FOS/*l* (~15 g/day), or LRPF + 10 g FOS/*l* (~31 g/day). The scFOS had a significant impact on fecal bifidobacteria levels. On day 14 a greater number ($p < 0.001$) of bifidobacteria were detected in the feces of subjects consuming formulas containing scFOS compared with those subjects consuming the LRPF without scFOS.

Colonization Resistance

Indigenous bacteria confer colonization resistance to the host. In the gastrointestinal tract, this phenomenon refers to the ability of the indigenous microflora to prevent the colonization, overgrowth and/or translocation of potentially pathogenic microorganisms. This concept was first described by Van der Waaij et al. *(104)* who showed that indigenous anaerobic organisms prevented colonization by opportunistic pathogens. Colonization resistance has been attributed to several mechanisms: production of inhibitory compounds such as SCFA, H_2S, and bacteriocins *(30, 40, 83)*; reduction of pH and low oxidation-reduction potential *(44)*; and competition for substrates *(40, 111)*. Many researchers *(13, 104, 109)* believe that the anaerobic flora play a primary role in colonization resistance and that the resistance involves the mucosal immune system *(103)*.

An excellent example of colonization resistance is the relationship between indigenous bacterial microflora and *Clostridium difficile*. *C. difficile*, a spore forming obligate anaerobe, is the leading known cause of nosocomial diarrheal infections *(37, 63)*. Although this organism is a component of the normal intestinal flora of about 3 to 5% of healthy adults, it can be detected in the stools of up to 15 to 20% of hospitalized adults *(26)*. Infections due to *C. difficile* are responsible for all cases of pseudomembranous colitis (PMC) and for up to 20% of cases of antibiotic-associated diarrhea without colitis *(54, 64)*.

When established in the colon, pathogenic strains of *C. difficile* produce exotoxins (toxin A and toxin B) that are the cause of diarrhea and colitis *(7, 98)*. Toxin A causes fluid secretion, mucosal damage, and intestinal inflammation when injected into rodent intestine *(101)*. Toxin B is a more potent cytotoxin in tissue culture than toxin A, but it is not enterotoxic in animals *(54)*.

Use of almost any antibiotic can cause *C. difficile* infection, but broad-spectrum antibiotics with activity against enteric bacteria are the most frequent agents. Clindamycin is notorious for its propensity to induce the disease *(99)*. In current practice, however, broad-spectrum penicillins and cephalosporins are the most common culprits, reflecting their widespread use *(69)*. Other factors including chemotherapy *(4)*, dietary changes, anesthesia, surgery of the intestinal tract, uremia, and various nonantibiotic medications have been known to precipitate PMC. PMC may occur during the period immediately and almost always within 6 weeks after the use of antibiotics has been discontinued.

All of these causative factors suggest that *C. difficile* infections are associated with the disruption of the normal bowel microbiota. Wilson et al. *(113)* noted that *C. difficile* could not colonize hamsters in the presence of an undisturbed colonic microflora, yet *C. difficile* rapidly attained a large population size when introduced into antibiotic-treated animals. Likewise, *C. difficile* was able to achieve a population of over 10^8 CFU per cecum when inoculated into gnotobiotic mice, but colonization was suppressed to undetectable levels by intestinal flora of conventionally-colonized hamsters and mice *(112)*.

The standard treatment for *C. difficile*-associated disease is administration of the antibiotics vancomycin or metronidazole. Standard antibiotic therapy is effective in 80% of patients with *C. difficile*-associated disease, but the remaining 20% experience further episodes of diarrhea or colitis during the permissive period after the antibiotic has been discontinued *(5, 28, 107)*. Once patients have had one recurrence, they may experience repeated episodes of the disease over several years *(27, 56)*.

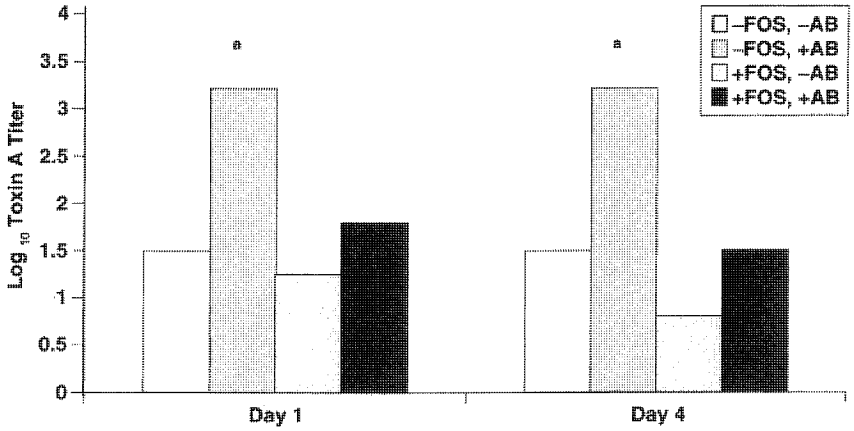

Fig. 3. The effects of diet (with (+FOS) and without (–FOS) scFOS) and antibiotic (with (+AB) and without (–AB) antibiotic) treatment on fecal toxin A titers on days 1 and 4 post *C. difficile* challenge.
A toxin titer of 0.7 was assigned to animals that were below the limits of assay detection (1.0).
[a]Designates a statistically significant ($p < 0.05$) increase in response to antibiotic treatment within diet and day. Source: Gaskins et al. *(34)*

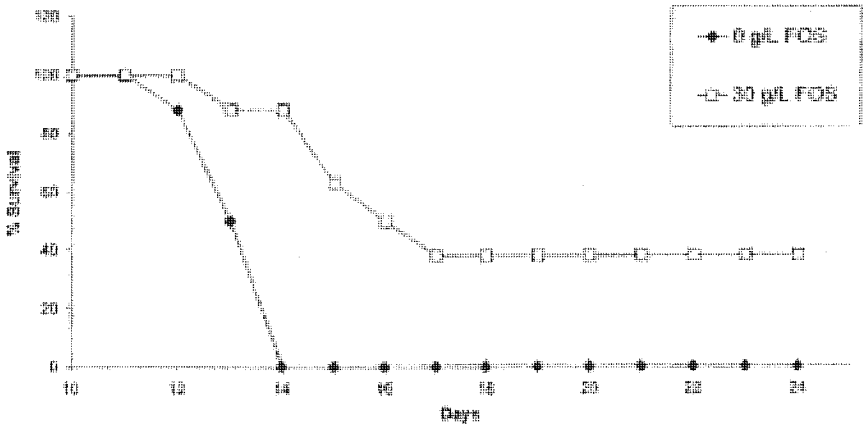

Fig. 4. Survival curves for ciprofloxacin treated hamsters ($n = 8$ per treatment) receiving either 0 g or 30 g scFOS per liter of drinking water.
Modified from Wolf et al. *(115)*.

Given the causative role of antibiotics in the onset of *C. difficile* infection, alternative therapies might offer more effective strategies for the prevention or treatment of *C. difficile* infection. Promising results have been obtained by restoring normal gut flora. Using a hamster model, Wilson et al. *(114)* found that administration (oral plus rectal) of normal cecal homogenates decreased the numbers of viable *C. difficile* and prevented cecitis in antibiotic-challenged animals. Schwan et al. *(87, 88)* effectively treated recurrent *C. difficile* infection by giving enemas with fecal contents from healthy adult humans. While direct inoculation with gastrointestinal microflora appears effective, its acceptability in a clinical setting is questionable. A better approach may be to use fermentable fibers such as scFOS to restore the gastrointestinal microflora and environment.

In an *in vitro* model, May et al. *(61)* also showed that fermentable fiber (resulting in increased SCFA concentrations and decreased pH) effectively inhibited the growth of *C. difficile* and toxin A production. The *in vitro* system further demonstrated that pH levels less than 6.0 and/or a SCFA concentration greater than 100 mM could markedly suppress the growth of *C. difficile* and the production of toxin A. In addition, May et al. *(62)* found that dietary supplementation with oligosac-

charides (scFOS or XOS) suppressed the growth of *C. difficile*, protected cecal epithelial tissue in mice, and reduced the incidence of diarrhea in *C. difficile*-challenged mice. Gaskins et al. *(34)* demonstrated that the administration of scFOS to cefoxitin treated mice inoculated with *C. difficile* produced toxin A titer in feces significantly lower ($p < 0.05$) than animals not receiving scFOS and similar to mice not compromised with antibiotics (Fig. 3). Wolf et al. *(115)* demonstrated that dietary supplementation with scFOS increased survival time in a hamster model of *C. difficile*-colitis (Fig. 4). It is interesting to note that the benefit due to the supplementation of scFOS in this study was above any effects of fiber contained in the basal diet. In consideration of the research conducted in this area, the addition of scFOS to a medical food may be beneficial for patients at risk of *C. difficile* infection in long-term care institutions and hospital wards.

Nitrogen Excretion and Calcium Absorption

The addition of fiber to a diet can potentially alter total body nitrogen metabolism by enhancing bacterial metabolism and thereby increasing the incorporation of nitrogen into fecal bacteria. This in turn increases fecal nitrogen excretion and subsequently reduces urinary nitrogen excretion *(14, 55)*. For example, lactulose has been reported to depress ammonia absorption from the large bowel *(20)* and to enhance fecal nitrogen excretion *(68)*. In addition, inulin has been shown to enhance urea capture by the rat cecum *(76)* and promote fecal nitrogen excretion, particularly when the level of protein in the diet is moderate *(60)*. Younes et al. *(117)* demonstrated in rats that fermentable fibers such as gum arabic, xylooligosaccharides and scFOS can increase fecal nitrogen excretion at the expense of urinary nitrogen excretion. Such data indicate a potential benefit for nondigestible oligosaccharide therapy in patients with renal insufficiency or chronic renal disease.

Considerable efforts are underway to encourage all people, women in particular, to increase their calcium consumption for improved bone health. Ingredients that improve mineral absorption also could be beneficial. Fermentable fibers such as inulin *(59)*, hydrolyzed guar gum *(41)* and scFOS *(71–73)* have been shown to enhance calcium absorption. This enhanced absorption occurs in the large bowel *(70, 72 73)* through a variety of mechanisms involving SCFA *(102)*, pH, and calbindin-D9K *(70)*.

CONCLUSION

Fructooligosaccharides represent an ideal source of fermentable fiber for use in medical foods. Fructooligosaccharides are highly soluble but low viscosity fibers that will not compromise the tube feeding characteristics of a medical food. *In vitro*, animal, and in several instances, human research has been conducted to identify and support the potential physiological benefits of fructooligosaccharides. Clinical research to support the use of fructooligosaccharides in several patient populations is underway.

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Review

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Enhancement of Gut Immune Functions by Short-Chain Fructooligosaccharides and Reduction of Colon Cancer Risk

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Short-chain fructooligosaccharides occur in a number of edible plants, such as chicory, onions, asparagus, wheat... They are produced industrially from sucrose. They are a group of linear fructose oligomers with a degree of polymerisation ranging from 1 up to 5 (oligosaccharides). Short-chain fructooligosaccharides to a large extent escape digestion in the human upper intestine and reach the colon where they are totally fermented mostly to lactate, short chain fatty acids (acetate, propionate and butyrate), and gas. Butyrate is the most interesting of the short chain fatty acids (SCFA) since, it regulates cell growth and differentiation of colonocyte. In addition to this trophic effect, butyrate stimulates the immunogenicity of the cancerous cells. Short-chain fructooligosaccharides also stimulate bifidobacterial growth. The colonic microflora has a considerable influence on the immune system of the host. The intestinal mucosa, play an important role in the immune system too, it is the largest immunological organ of the body containing. The gut-associated lymphoid tissue (GALT) plays a key role according to its singular interface situation in the body and constitutes an important line of defence which is confronted with a large range of antigenic or immunomodulating substances. Recent founding in animal models clearly demonstrate that pre and probiotic may exert beneficial effects on gut health by enhancing GALT responses directly or indirectly by the mediation of butyrate and lactic bacteria. GALT may play a pivotal role in the rejection of nascent colon tumours. Intestinal microflora modulates the GALT responses and recent founding in animal models clearly demonstrate that pre and probiotic may exert beneficial effects on gut health by enhancing GALT responses directly or indirectly by the mediation of butyrate. The demonstration of the potential health benefits of sc-FOS on reduction risk of colon cancer is an active field of research in human nutrition. The sc-FOS, in animal models, reduce colon tumour development by enhancing both colon butyrate concentrations and local immune system effectors. The objective of this review is to discuss the critical role of GALT and its effectors, associated to butyrate, on colorectal cancer prevention. Both target functions have shown to be enhanced by sc-FOS.

Key words: short-chain fructooligosaccharides; immune functions; bifidobacteria; prebiotic; colon cancer

INTRODUCTION

Short-chain fructooligosaccharides (sc-FOS) are a group of linear Glucosyl $\alpha(1 \quad 2)(\text{fructosyl})_n\beta(2 \quad 1)$ fructose polymers with a degree of polymerisation (DP) ranging from 1 up to 5 (oligosaccharides). They have aroused interest in the past decade, mostly because of their nutritional properties. Sc-FOS to a large extent escape digestion in the human upper intestine and reach the colon where they are totally fermented and stimulate bifidobacterial growth. The prebiotic effect of sc-FOS is dose-dependent. It is associated with a decrease of faecal pH and an increase of production of organic acids (lactic acid, short chain fatty acids). Butyrate is the most interesting of the short chain fatty acids (SCFA) since, it regulates cell growth and differentiation of colonocyte. In addition to this trophic effect, butyrate stimulates the immunogenicity (sensitivity to the immune response) of the cancerous cells. The colonic microflora has a considerable influence on the immune system of the host.

The intestinal mucosa also, play an important role in the immune system, it is the largest immunological organ of the body containing. The gut-associated lymphoid tissue (GALT) constitutes an important line of defence. Recent studies suggest that it may play a pivotal role in the rejection of nascent tumours. The demonstration of the potential health benefits of sc-FOS on reduction risk of colon cancer is an active field of research in human nutrition. The sc-FOS, in animal models, reduce colon tumour development by enhancing both colon butyrate concentrations and local immune system effectors. The objective of this review is to discuss the critical role of GALT and its effectors, associated to butyrate, on colorectal cancer prevention. Both target functions have shown to be enhanced by sc-FOS.

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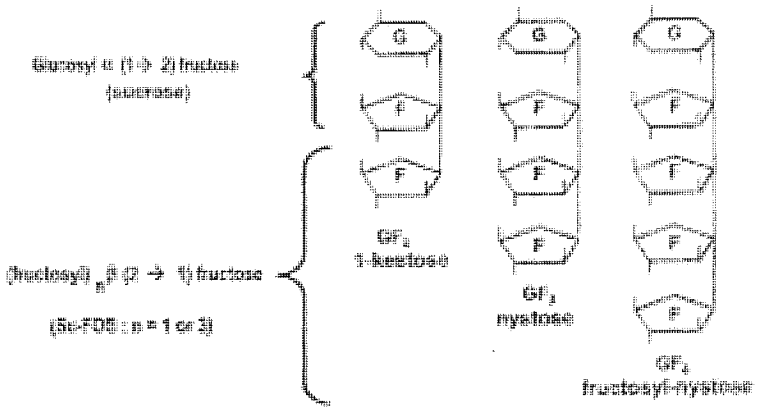

Fig. 1. Short-chain fructooligosaccharides structure of sc-FOS.

SHORT-CHAIN FRUCTOOLIGOSACCHARIDES: ORIGIN AND NUTRITIONAL FACTS

By definition, oligosaccharides have a DP lower than 9. Short-chain fructooligosaccharides (sc-FOS) are a group of linear glucosyl α(1 2)(fructosyl)$_n$$\beta$(2 1) fructose polymers with a degree of polymerisation (DP) ranging from 1 up to 5 (Fig. 1).

Short-chain fructooligosaccharides occur in a number of plants such as onions, Jerusalem artichokes, asparagus, wheat, rye, and garlic *(8)*. Pure sc-FOS are produced on a commercial scale from sucrose using a food grade fungal fructosyltransferase, (ACTILIGHT® (Béghin Meiji, France) or MEIOLIGO® (Meiji Seika Kaisha, Japan) or NUTRAFLORA (GTC, USA).

Sc-FOS to a large extent, escape digestion in the human upper intestine *(23, 24)* and reach the colon where they are totally fermented, mostly to lactate, short chain fatty acids (acetate, propionate and butyrate). Compared with other fermentable products, like cellulose, pectin or lactulose, the fermentation of FOS produces higher percentages of propionic and butyric acid *(2, 18)*. The most important property of sc-FOS is their ability to stimulate SCFA production and bifidobacterial growth.

Unlike other undigestible sugars, such as lactose or lactulose which are hydrolysed by a wide variety of gut bacteria, sc-FOS are only fermented *in vitro* by a limited range of micro-organisms that include most species of bifidobacteria (except *Bifidobacterium bifidum*) *(11, 20, 22)*. Indeed, bifidobacteria have relatively high amounts of β-fructosidase, which is selective for the β-(2,1) glycosidic bonds present in sc-FOS. After sc-FOS hydrolysis, fructose serves as an efficient growth substrate for the bifidus pathway of hexose fermentation, which is almost exclusively carried out by bifidobacteria *(33)*.

Numerous studies in humans showed that sc-FOS ingestion led to an increase of faecal bifidobacteria *(3, 6, 11, 12, 20–22, 30, 32, 39, 40, 43)* have been conducted in healthy subjects using a "control" group and a double or single blind design. The main characteristics and results of the studies conducted in humans are summarised in Table 1. Lastly, Bouhnik et al. *(4)* observed a significant correlation between the dose of sc-FOS ingested and the faecal bifidobacteria counts at the end of the 7 day period.

BIFIDOBACTERIA AND IMMUNE RESPONSES

It has been reported that bifidobacteria exert various effects on immune system related function, such as, mitogenic activity *(14)*, adjuvant activity *(15, 37)*, promotion of macrophages *(14, 35)*, stimulation of antibody production *(41, 44, 45)* and antitumour effects *(15, 36)*. Lee et al. *(17)* tested the immunopontentiating activity (i.e. to stimulate the proliferative response of murine immune cells) of twenty seven micro-organisms *in vitro*. They showed that bifidobacteria strains have a higher immunopotentiating activity than do *Lactobacillus casei* or *L. acidophilus*. *Bifidobacterium adolescentis* M100-4, originally derived from human intestinal microflora, had the strongest mitogenic activity on splenocytes and Peyer's patches cells. This activity was shown to be dose dependent and was increased after disruption of the cells by sonication, indicating the existence of an intra soluble immunopotentiator.

The intestinal mucosa plays, also, a important role in immunologic response of the body. The gut-associated lymphoid tissue (GALT) constitutes an impor-

Table 1. Main characteristics and results of clinical studies conducted in healthy subjects on the prebiotic effects of short-chain FOS ACTILIGHT®.

Healthy Subjects (n)	Age (yr)	Sc-FOS daily ingestion (g)	Duration (Day)	Bifidobacteria count in stools log CFU/g (mean ± SEM)		Statistical significance (p)	Authors
				Before	After		
6	—	6	30	9.6	9.8	NS	Mitsuoka, et al. *(21)*
23	73 ± 9	8	14	8.8 ± 1.1	9.7 ± 0.5	< 0.005	Mitsuoka, et al. *(22)*
27	36.8 ± 9	1	14	9.8 ± 0.6	10.2 ± 0.4	< 0.05	
(9 × 3)	25.2 ± 3.3	3	14	9.9 ± 0.6	10.4 ± 0.4	< 0.05	Tokunaga, et al. *(40)*
		5	14	9.7 ± 0.6	10.3 ± 0.4	< 0.01	
38	—	8	14	5.2 ± 0.9	6.2 ± 0.6	< 0.01	Rochat, et al. *(30)*
10	20–40	4	14	8.3 ± 1.8	9.4 ± 2.3	< 0.05	William, et al. *(43)*
10	22–39	12.5	12	7.9 ± 0.5	9.1 ± 0.3	< 0.01	Bouhnik, et al. *(3)*
		2.5	8	8.0 ± 1.1	8.2 ± 1.1	NS	
32	29.6	5	8	8.1 ± 0.8	9.1 ± 0.4	< 0.05	Bouhnik, et al. *(4)*
(8 × 4)		10	8	8.0 ± 1.3	9.5 ± 0.3	< 0.02	
		20	8	8.2 ± 0.9	9.5 ± 0.6	< 0.002	

NS: not significant.

tant line of defence.

THE GUT-ASSOCIATED LYMPHOID TISSUE (GALT)

The intestinal mucosa is the largest immunological organ of the body containing over 10^6 lymphocytes/g tissue. Its represent the major part of the body's contact. About 60% of the total immunoglobulin produced daily is secreted into the gastrointestinal tract. The structure of the GALT, the intestinal immune cell distribution among the Peyer's patches, epithelium and lamina propia have been reviewed by Brandtzaeg et al. *(5)*. The GALT-T lymphocytes are not homogenous. There are classified as CD4+ helper/inducer cells and CD8+ suppressor/cytotoxic cells generating different cytokines profiles which distinct yet unproven functions. The majority of intra-epithelial T-cells are CD8+, contrasting with the lamina propia where CD4+ are predominant. The lamina propia is also endowed with lymphocytes belonging to the B-cell lineage. These are mainly memory cells and plasmocytes which 70–90% of them are Ig-A producing cells.

An immune response initiated in the GALT can affect immune responses at other mucosal surfaces. The lymphocytes activated within the Peyer's patches disseminate via mesenteric lymph nodes, thoracic duct and the bloodstream back to the lamina propia, and traffic between other secretory tissues including the respiratory tract and the lacrymal, salivary and mammary glands. The digestive flora is the major antigenic stimulus responsible for the migratory pathway and maturation of precursor lymphoid cell present in the Peyers's patches. Recent studies suggest that it may play a pivotal role in the rejection of nascent tumours, and consequently in the reduction of risk of colon cancer.

BUTYRATE—GALT AND COLON CANCER

Butyrate is also known to have preventive effects on colon cancer and adenoma development *(38)*. In the colon, it comes from bacterial fermentation, and constitutes a good substrate for colonocytes. Butyrate oxydation has been shown to make up for more than 70% of the oxygen consumption by the human colonic tissue *(31)*, indicating that butyrate is the prime energy substrate of the colonocyte. It is not only an energy source for colonocytes. Sodium butyrate (NaB) exerts an antiproliferative activity on many cell types. It is an inducer of differentiation of colon carcinoma cell lines. It also has been observed to induce gene expression, to influence the rate of gene expression through its effects on post translational modifications, to induce apoptosis and to reverse the resistance of colonic cancer cells to programmed cell death *(38, 41)*.

Perrin et al. (personnal communication) have characterize the modification of the phenotype of PROb rat colon adenocarcinoma cells when NaB treated. They focused their study on surface oligosaccharides that could play a role in their tumorigenicity. Blood-group H antigens, formed by the addition of fucose on type

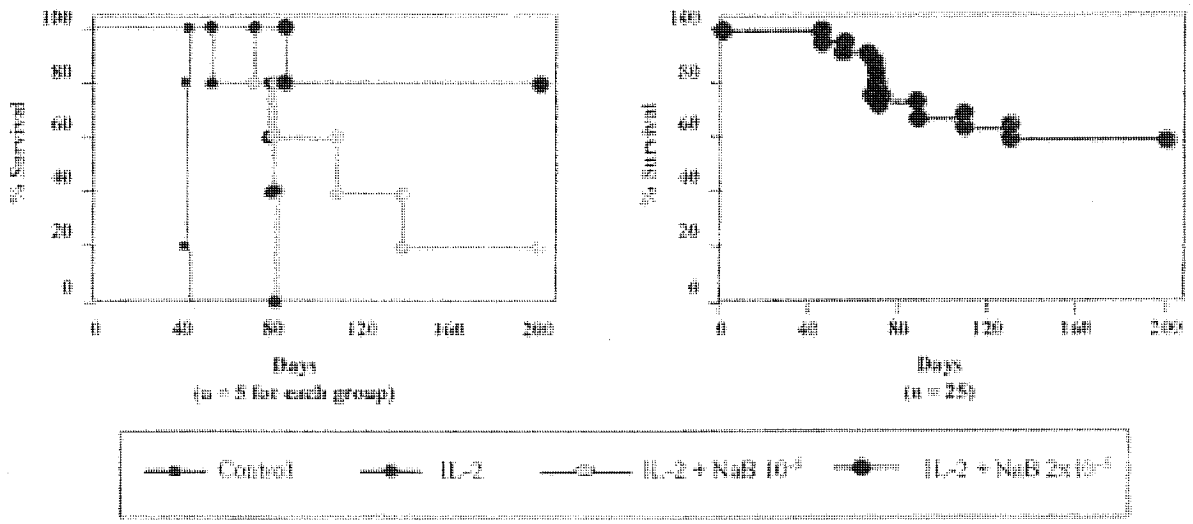

Fig. 2. Effects of butyric acid on experimental carcinogenesis: percentage of survival of rat traited with interleukin 2 (IL-2) and sodium butyrate (NaB), alone or in combination *(25)*.

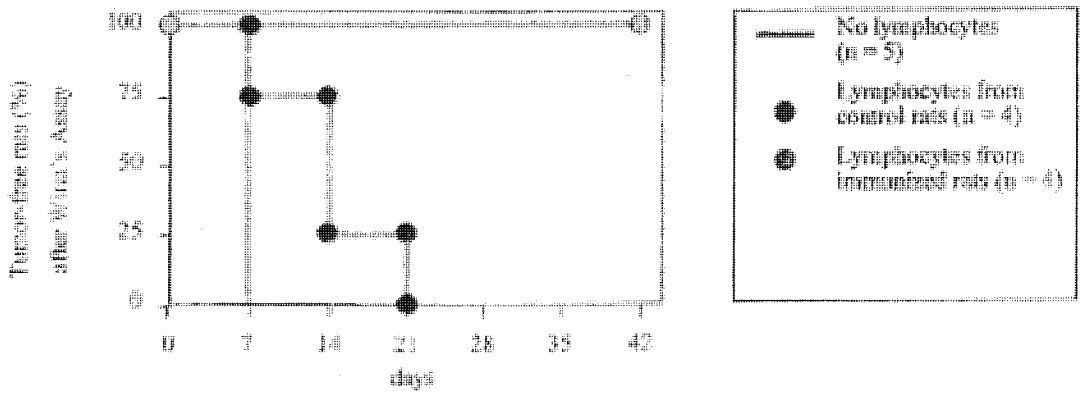

Fig. 3. Effects of butyric acid on experimental carcinogenesis: effects of subcutaneous injection of 10^6 PROb cells alone or mixed with lymphocytes extracted from naive rats or from immunized rats *(25)*.

1,3 precursors, were less expressed on butyrate-treated PROb cells, while α(2 3) linked sialic acids were enhanced. This phenotype was maintained after sodium butyrate withdrawal, whereas cell growth inhibition was lost. The decrease of H1,3 antigens would be related to the lower activity of α(2 3) fucosyltransferase(s) and competition between fucoses and sialic acids precursors, borne by CD44v. When subcutaneously grafted, NaB-treated PROb cells induced significantly small tumours. That could result from a more efficient host response, attributable to the phenotype the cancer cells acquired with transient *in vitro* NaB treatment, since the lower level of H 1,3 antigens was maintained in growing tumours.

Butyrate stimulates the immunogenicity of the cancer cells *(25)*. The phenotype of the weakly immunogenic rat colon cancer PROb cells was modified with sodium butyrate. After a 4-days *in vitro* sodium butyrate treatment, the lymphokine-activated killer cell sensitivity, the expression of Major Histocompatibility Complex class I, and the intercellular adhesion molecule 1 of PROb cells, were increased in a dose-dependent manner.

Perrin et al. *(25)* tested the efficiency of interleukin 2 (IL-2) and sodium butyrate (NaB), alone or in combination, against experimental widespread carcinomatosis induced in rats by intraperitoneal injection of 2×10^6 PROb colon carcinoma cells. IL-2/butyrate combination resulted in cases of complete cure of carcinomatosis with specific protection against PROb cells (Fig. 2 and 3). IL-2 secretion probably leads to activation of non-specific effectors such as natural killer and/or lym-

phokine-activated killer cells resulting in a rapid clearance of otherwise immunogenic sodium butyrate-treated cells. The complete regression of tumour masses may be attributed, to butyrate-induced decrease of tumorigenicity and increase of immunogenicity of the cancer cells.

So, it may be advantageous to provide indigestible carbohydrates as an indirect source of butyrate to the large bowel.

SHORT CHAIN FRUCTOOLIGOSACHARIDES AND COLON CANCER

Classically defined as non-starch polysaccharides, fibre now include other sources of fermentable substrate for microflora, such oligosaccharides, resistant starch *(10)*. Dietary fibres have been proposed as protective agents against colon cancer but results of both epidemiological and experimental studied are debatable and prevention programme have been limited to general lifestyle guidelines *(42)*. These conflicting results may relate to the heterogeneity of the fibre and basal diet, feeding protocol, chosen biomarker, and/or stage of colon carcinogenesis. Among fibres, carbohydrates producing large amounts of butyrate appear to be greatest interest as butyrate is an energy yielding substrate for colonocytes, affects cellular function, is an antineoplastic agent *in vitro*, and has been implicated in the protective effect of fibre in rodents *(19, 34)*. It has been hypothesised that protection against colon cancer may be restricted to butyrate producing fibres.

To investigate the effects of different type of fibres, rats *(26)* and *Min* mice *(28)* have been used: chemically induced and spontaneous cancer models. The relevance of animal models, as compared to human colon tumour studies depends on the criteria considered *(29)*. Azoxymethane (AOM) induced tumours are similar to human tumours in many histological, biochemical, immunological and cellular aspects; but many of the tumours do not follow the adenoma to carcinoma progression, frequently arising *de novo* from flat mucosa. This model permits the investigation of early stages of carcinogenesis, the end-point being a consensual precancerous marker, the aberrant crypt foci. The mouse model (*Min* mice) is a model for both familial adenomatous polyposis and sporadic colon cancer. The *Min* mice are heterozygous for a non-sense mutation of the *Apc* gene, the murine homologue of *APC*. The *Min* mouse model adenomas are pertinent by their genetic origin, but they are more frequent in the small bowel than in the colon, as opposed to the human situation. These studies provided data on later stages of colon cancerogenesis, and the end-point was the number of detectable tumours.

A two part randomised blinded study in rats, mimicking a prospective study in humans, was performed using a low fibre control diet (CD) and three high fibre diets: starch free wheat bran (WB) type III resistant starch (RS) and short-chain fructooligosaccharides (sc-FOS) *(9)*. Using a randomised block design, 96 inbred rats were fed for 16, 30 or 44 days to determine the period of adaptation to the diet, fermentation profiles, and effects on the colon, including mucosal proliferation on day 44. Subsequently, 36 rats fed the same diets for 44 days were injected with azoxymethane and checked for aberrant crypt foci 30 days after. After fermentation had stabilised (44 days), only RS and sc-FOS produced large amounts of butyrate, with a trophic effect in the large intestine. No difference in the mucosal proliferation between the diets was noted at this time. In the subsequent experiment one month later, fewer aberrant crypt foci were present in rats fed high butyrate producing diets (RS, $p = 0.022$. sc-FOS = 0.043) (Fig. 4). Similar effects on a reduction of the number of aberrant crypt foci in CF1 mice treated with AOM and fed diets containing sc-FOS and exogenous bifidobacteria, were published by Koo and Rao *(16)*. Campbell et al. *(7)* evaluated in rats the effects of selected indigestible oligosaccharides on caecal and faecal SCFA concentration, pH, total large bowel wet weight and wall weight, and concentrations of intestinal microbiota. The duration of the study was 14 days. The sc-FOS containing diet resulted in higher caecal butyrate concentrations compared with the control, or with the cellulose or xylooligosaccharide containing diets.

The same fibre diets have been tested in *Min* mice aged 6 or 7 weeks ($n = 40$) *(28)*. Each group was fed *ad libidum* for 42 days either the control low-fibre diet (CD) or one of the three high-fibre diet (WB, RS, sc-FOS). Gut tumours and small intestine lymphoid nodules were counted (Fig. 5). Neither WB nor RS modified the number of tumours. However, sc-FOS dramatically reduced the incidence of colon tumours and concomitantly developed GALT. Interestingly, the sc-FOS effect was limited to the colon; they were no significant differences between diets in the number of tumours found in the small intestine suggesting strongly that events specific to the colon were involved. A bifidogenic effect probably occurred in the experiment because Howard et al. *(13)* demonstrated that dietary supplementation with the same sc-FOS enhanced the population of bifidobacteria in mouse colon as soon as 14 days.

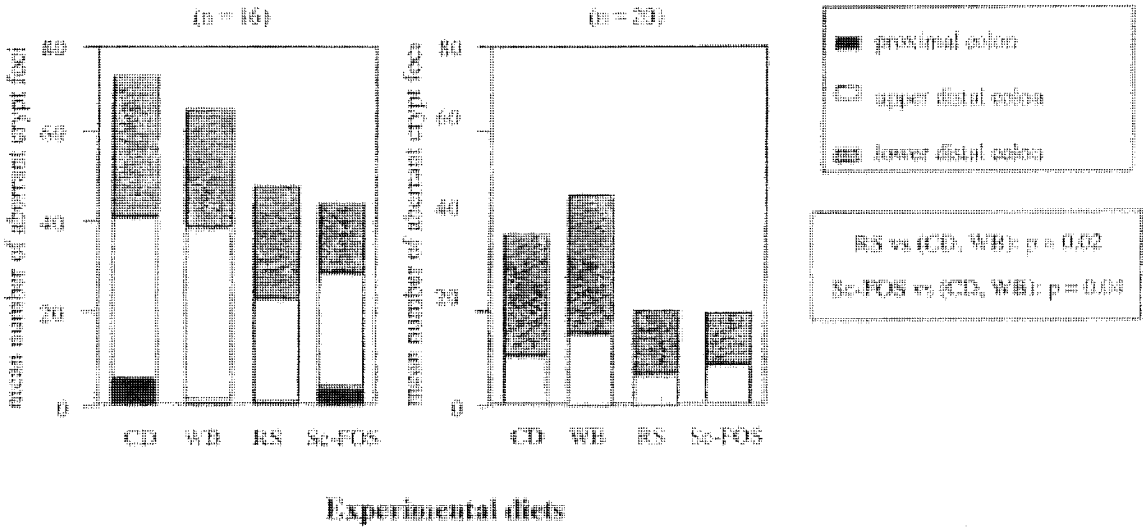

Fig. 4. Effects of short chain fructooligosaccharides on experimental carcinogenesis: mean number of aberrant crypt foci per rat fed with control low-fibre diet (CD), wheat bran (WB), resistant starch (RS) or short chain fructooligosaccharides (sc-FOS) *(26)*.

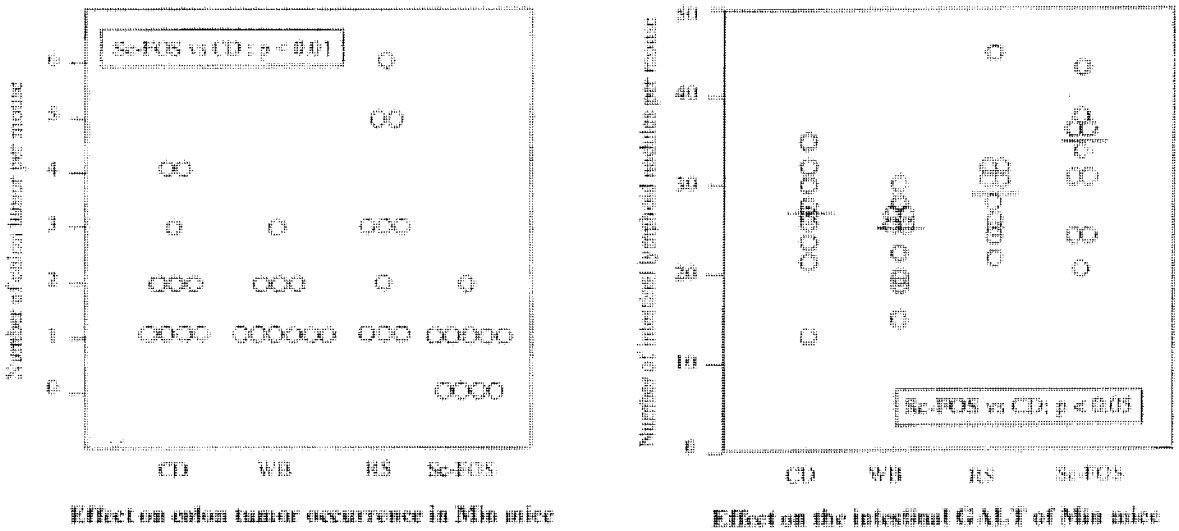

Fig. 5. Effects of short-chain fructooligosaccharides on experimental carcinogenesis: gut tumours and small intestine lymphoid nodules in *Min* mice ($n = 40$) fed *ad libidum* for 42 days with control low-fibre diet (CD), wheat bran (WB), resistant starch (RS) or short-chain fructooligosaccharides (sc-FOS) *(28)*.

To obtain an insight into the GALT response to changes in the colonic ecosystem, the authors looked at the lymphoid tissue of the small intestine. The examination of the colon for that purpose was impossible because treatment of this tissue, to study colon tumours, made impossible to accurately evaluate colon lymphoid follicles. A significantly higher number ($p < 0.05$) of macroscopically detectable lymphoid nodules were noted in the small intestine with the sc-FOS diet. This suggests that immune system may play a role in inhibiting tumour formation by eliminating cells that express antigens if they are immunogenic enough to allow the expansion of immune cell specific for these antigens.

To investigate whether T cell status may influence colon tumour formation in *Min* mice fed a sc-FOS diet, Pierre et al. *(27)* have chosen to immunodeplete mice with antibodies against target T cells (CD4+ and CD8+), rather than NK cells, which do not affect the incidence

of intestinal neoplasia in *Min* mice *(9)*. *Min* mice depleted of CD4+ and CD8+ lymphocytes developed twice as many tumours as immunocompetent mice.

To investigate the response of the tissue to sc-FOS at the effector molecule level, Bassonga et al. *(1)* assessed the expression of cytokines present in the colon. They chosen to study the mRNAs since certain cytokines (e.g. IL-15) are frequently not translated or secreted by resting cells. They used a multiprobe ribonuclease protection assay to study the expression of selected cytokines in the colon of C57BL/6 and *Min* mice fed low fibre diet (CD) or a sc-FOS enriched diet (sc-FOS).

Five cytokines were consistently detected regardless of the animals or diet (IL-4, IL-5, IL-13, IL-15 and IFN γ). IL-4, IL-5 and IL-13 were expressed at low but comparable levels and were not sensitive to the diet. IL-10, IL-9, IL-6 and IL-2, were not detected. The IL-15 mRNA was frequently highly expressed in both *Min* groups but a level significantly higher ($p = 0.01$) in the *Min* group fed sc-FOS as compared to the *Min* group fed CD. IFN γ mRNA, when detected, showed the same pattern of expression as IL-15. The fact that IFN γ appears to be modulated in the same way as IL-15 support the hypothesis that IL-15 could be secreted in an active form, since IFN γ, a cytokine produced by activated T-cells and which stimulates cytotoxic activity, is a target of active IL-15.

CONCLUSION

Short-chain fructooligosaccharides have aroused interest in the past decade, mostly because of their nutritional properties. To a large extent, sc-FOS escape digestion in the human upper intestine and reach the colon where they are totally fermented, mostly to lactate and short chain fatty acids (acetate, propionate and butyrate). The most important property of sc-FOS is their ability to specifically stimulate bifidobacterial growth and to induce butyrate production. Recent studies have shown that these both effects with the stimulation of the activity of some compound of the GALT play an important role in the colon cancer prevention.

The demonstration of the health benefits of sc-FOS and the stimulation of immune rejection of nascent tumours offers new perspective for the prevention of colon cancer and opportunities for better understanding of GALT to colon cancer.

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seen in the problem area of south Texas was introduced experimentally by administration of C pedunculata to calves. The intent of the study was to reproduce photosensitization, using this suspect plant, and not to produce lethality. However, there was a judgmental error in dosing the first calf with this previously untested plant material. Death was attributed to the massive release of toxic components from the necrotic dermis. Consequently, the total dose given during the second trial was reduced from 40 g of dried plant/kg of body weight to an acceptable nonlethal, but responsive dosage of 6.6 g/kg. This lower dose (16.7% of the original) was sufficient to induce signs of mild photosensitization without severe discomfort to the animal. Clinical signs and gross lesions were identical to those described in field cases of south Texas. Microscopic examination of tissues from the one heifer revealed findings also compatible with those previously reported in field cases (1).

Clinical signs during the summer trial took about twice as long to appear as those in the winter trial (96 vs 48 hr). This was attributed to the reduced dosage. The increased sunlight intensity during the summer feeding trial probably helped facilitate reproduction of the typical clinical signs at this lower dosage. Although the source of the phototoxin cannot be traced to either the green or dead leaf by the results of this study, experiments with Candida albicans and mice indicate that phototoxicity is associated only with dead portions of the plant (2). This suggests that, given adequate sunlight, a dosage of 0.4 g/kg/d of the dead leaf material for 4 days could induce photosensitization in cattle since mild signs were induced in this study after 4 days of treatment at 1.7 g/kg/d, using plant material containing only 25% dead leaf material.

The corneal opacities that developed in the high-dose calf are characteristic of primary photosensitization caused by furocoumarins (3,4). It is speculated that the corneal lesions may be caused by the presence of the phototoxic agent in aqueous humor or lacrimal fluid. Another possible explanation for these lesions may be irritation of the cornea by sloughed flakes of desiccated epithelium from adjacent eyelid margins. It is thought the phototoxic agent may be a mycotoxin or a phytoalexin produced by the plant in response to a microbial infection; however, the isolation and characterization of the compound in C pedunculata has not been completed.

The results of these dosing trials lend support for C pedunculata as an etiologic agent associated with epizootics of photosensitization in cattle of south Texas. Current efforts by a multidisciplinary task force are focused on isolation and characterization of the photosensitizing compound, and development of methods to control the plant on pasture and rangeland.

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Fructooligosaccharides: A Review

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ABSTRACT. Fructooligosaccharides are naturally occurring compounds that have been reported in a variety of plants. Neosugar is a fructooligosaccharide mixture of 1'-(1-β-fructofuranosyl)-sucrose polymers which is produced on a commercial scale from sucrose using a fungal fructosyltransferase. The resulting product is 0.4 to 0.6 times as sweet as sugar and is resistant to digestion by mammalian alpha-amylase, sucrase and maltase. Although Neosugar is non-digestible in humans, it is selectively utilized by bifidobacteria. Neosugar has been examined extensively in human and animal studies which indicate a lack of toxicity, carcinogenicity and genotoxic effects. Neosugar is used as a feed additive for poultry and swine in Japan and has been approved in foods as a raw material. Additional studies in progress in the US suggest that it could provide an economic alternative as an additive to poultry and swine feed.

HISTORICAL BACKGROUND

Neosugar, a mixture of fructooligosaccharides developed originally as a low-calorie, non-nutritive, sweetening agent in Japan, is currently employed as a feed additive in poultry and livestock in Japan (1). Fructooligosaccharides are naturally occurring

compounds that have been reported in a variety of plants such as onion (2), asparagus root (3) tubers of Jerusalem artichoke (4), wheat (5) and triticale (6). Edible plants that contain fructooligosaccharides listed in Table 1 (7). The development isolation of neosugar was first reported in the Japanese literature in 1983 (8).

CHEMISTRY AND UTILITY

Neosugar is a fructooligosaccharide mixture of 1^F-(1-β-fructofuranosyl)$_{n-1}$-sucrose oligomers in which n may vary from 2 to 4 (9). Basically they are sucrose molecules (glucose-fructose disaccharides) to which 1, 2 or 3 additional fructose units have been added by β(2→1) glycosidic linkage to fructose units of sucrose. These components are abbreviated as GF_2 (1-kestose), GF_3 (nystose) and GF_4 (1^F-β-fructofuranosylnystose) (8,9) and are illustrated in Fig 1 (3).

The presence of 3 fructooligosaccharides in edible plants was confirmed via their isolation from purified oligosaccharide fractions of Asparagus officinalis L. These were derived from the partial acid hydrolysates and products of β-fructofuranosidase action and confirmed by gas-liquid chromotography to be 1^F(1-β-fructofuranosyl)$_n$-sucrose [n=1 (neokestose), 2 and 3]; $1^F,n_6{}^G$-di-β-fructofuranosyl sucrose; and a new pentasaccharide 1^F(1-β-fructofuranosyl)$_2$-6^G-β-fructofuranosyl sucrose (3).

Number of these sugars have been isolated from the brans of triticale, wheat and rye (6). In order of magnitude, the sugars isolated from triticale, wheat and rye respectively were from triticale bran: sucrose, raffinose, kestose, nystose and fructosyl raffinose; from wheat bran: sucrose, raffinose, fructosyl raffinose, nystose and kestose; and from rye bran: sucrose, kestose, nystose, raffinose and fructosyl raffinose. Table 2 lists the distribution of total sugars, nystose and neokestose (%) in triticale brans from different crop years and in wheat and rye brans (6).

Table 1. Some Edible Plants Containing Fructooligosaccharides (7)

Fructooligosaccharides content	Plant family: plants
Relatively high*	Liliaceae: onion, welsh onion, garlic, echalote Asteraceae: burdock, artichoke Gramineae: wheat, barley, rye Musaceae: banana
Relatively low*	Liliaceae: asparagus, Chinese chive Asteraceae: lettuce, chichory Others: honey

*Determined by TLC spots

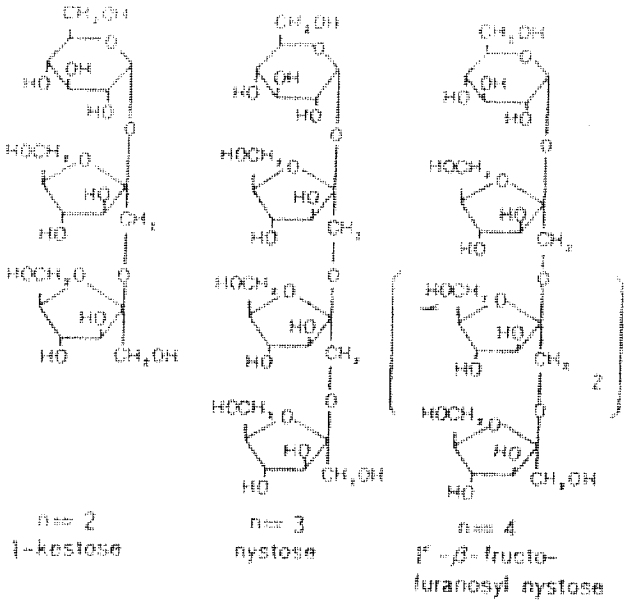

Figure 1 Chemical structure of fructooligosaccharides.

Neokestose and 1-kestose are also present in Italian rye grass and tall oat grass (Gramineae) (10), stem of oats (Gramineae) (10), sap of sugar maple (Aceraceae) (11), in onion (10, 12, 13), leek (13) and Welsh onion (13).

Coors Biotech Products Company has suggested that "fructooligosaccharides" are present at levels of 0.3% in bananas and 2.7% in onions, but it is not known whether these sugars are identical to neosugar (15). Gough (15) has calculated that assuming the sugars in bananas and onions were identified as neosugar, then the daily consumption of neosugar would be as follows: from bananas= 1.42% x 1500 g food/person/day = 21.3 g bananas/day x 0.3% neosugar/banana = 0.064 g/person/day; from onions= 0.832 x 1500 g food/person/day = 12.4 g onions/day x 2.8% neosugar/onion = 0.349 g/person/day; and the total neosugar consumption from bananas and onions is 0.064 g plus 0.349 g = 0.413 g/person/day.

Neosugar can also be produced by the action of the fungal enzyme β-fructofuranosidase (naturally produced by Aspergillus niger) (16-18). In the laboratory, the neosugar precursor compound, neosugar G, can be pur-

Table 2. Distribution of Total Sugars, Nystose and Neokestose (%) in Triticale Brans from Different Crop Years and in Wheat and Rye Brans.

	Triticale			Wheat	Rye
	1970	1971	1973		
Nystose	0.34	0.28	0.25	0.16	0.30
Neokestose	0.65	0.69	0.75	trace	1.39
Total Sugars	5.62	5.30	6.06	5.84	7.46

ified into neosugar by the removal of mono- and disaccharides via a resin treatment (8).

Although the intensity of sweetness of neosugar is about 0.4-0.6 fold that of sugar and, as noted earlier, it was developed as a potential low-calorie non-nutritive sweetener, (19) it has never been employed for that purpose (9). It is used as a feed additive in poultry and livestock in Japan. When fed to weanling pigs, neosugar is reported to accelerate weight gain and improve the feed conversion rate (20). Application of neosugar G to the feed is usually carried out by mixing 0.25-0.5% (solid basis) of the material to the diet. In the US fructooligosaccharides are being examined in male broilers (21) and in swine. Preliminary studies (21) have indicated that dietary fructooligosaccharides increase feed efficiency in four pen-reared male broilers.

BIOCHEMISTRY

Neosugar is resistant to digestion by mammalian alpha-amylase, sucrase and maltase. For example, in vitro and in vivo studies of neosugar in the rat showed that GF_2 (1-kestotose) and GF_3 (nystose) (Fig 1) were not hydrolyzed by pancreatic or mucosal duodenum homogenates. Additionally, an isolated and purified sucrose-isomaltose complex from rat intestinal mucosa had no effect on GF_2 or GF_3. When young rats were maintained on a 30% Neosugar diet (containing a mixture of 28% GF_2, 60% GF_3 and 12% GF_4) for 6 weeks no detectable activity of the jejunal mucosa toward pure GF_2 or GF_3 was detected, while a 30 to 40% decline in mucosal hydrolytic activity toward sucrose and maltose was also noted (9). Thus, these results would indicate that neosugar is scarcely hydrolyzed by the digestive enzymes of the gastrointestinal tract and pancreas and suggests that neosugar is not utilized as an energy source in vivo. The non-digestibility of fructooligosaccharides was confirmed in humans in clinical studies by the sugar tolerance test (22,23) While most fructooligosaccharides ingested pass through the body unabsorbed, the principal utilization pathway for the remainder is via metabolism by intestinal microorganisms to form carbon dioxide and organic acids. The effects of fructooligosaccharides on intestinal flora and human health were recently reported by Hidaka et al (8) and showed that these substances were selectively utilized by bifidobacteria. The clinical studies showed that neosugar administration improved the intestinal flora, with subsequent relief of constipation, improved blood lipids in hyperlipidemia and suppressed the production of intestinal putrefactive substances (8). The relationship between fructooligosaccharide intake and intestinal symptoms has been studied in human volunteers in Japan (24). This study reported the calculation of the maximum non-effective dose and 50% effective dose in relation to the digestive tract in dose-effect experiments; the differences in effect when pure neosugar powder was ingested compared to that when added to food prior to ingestion; and the comparison of the transitory laxative effect with sorbitol. The maximum effective dose in relation to fructooligosaccharide induced diarrhea was 0.30 g/kg or 44 g of the mixture for men and for women, 0.40 g/kg or approximately 29 g of the fructooligosaccharide mixture. For sorbitol, the values for men were 0.15 g/kg or approximately 9 g. The 50% effective dose for men and women were 0.78 g/kg or 115 g of the fructooligosaccharide mixture and 0.84 g/kg or 102 g, respectively. The values for sorbitol for the 50% effective dose for men were 0.5 g/kg and approximately 9 g.

The effects of fructooligosaccharides on blood glucose and serum lipids in diabetic subjects were reported by Yamashita et al (25). Daily intake of 8.0 g/day of fructooligosaccharides for 14 days significantly reduced mean fasting blood glucose levels by 15 mg/dl, mean serum total cholesterol levels by 15 mg/dl and LDL-cholesterol levels by 17 mg/dl in 18 diabetic subjects, while 10 control diabetic subjects, who were given 8.0 g/day of sucrose, showed no significant changes. The levels of serum HDL-cholesterol, triglycerides or free fatty acids were not significantly affected by either fructooligosaccharides or sucrose. Although the authors suggested that fructooligosaccharides could be generally utilized as a sweetener or as an adjunct to the dietary therapy of diabetic subjects with various beneficial effects on glucose or lipid metabolism, it was also cautioned that more studies are necessary to assess the effects of daily intake of these substances over a longer time period (25).

TOXICITY

Neosugar did not exhibit any genotoxic potential when tested in a battery of three assays: microbial reverse mutation assays in Salmonella typhimurium (Ames assay) and E coli WP2 uvrA; the L5178 Y mouse lymphoma TK mammalian cell mutation assay; and an assay for the induction of unscheduled DNA synthesis (UDS) in human epithelial cells (Hela S3). Each assay was conducted at a wide range of dose levels, both with and without a microsomal metabolic activation system from Aroclor induced rat liver (25).

The carcinogenicity and chronic toxicity of neosugar was elaborated in Fischer 344 rats (26). No dose-related effects on survival, growth hematology, blood chemistry, organ weights or non-neoplastic lesions were noted following treatment of male and female rats fed diets containing 5,000 20,000 or 50,000 ppm neosugar for 104 weeks. The incidence of spontaneous tumors was comparable between control and neosugar treatment groups of both sexes except for pituitary adenomas in male rats. However, because of the high and variable background incidence of this tumor and equivocal dose-response trend, the incidence of pituitary adenomas in male rats was not considered treatment related (26).

Since neosugar is a mixture of simple oligosaccharides comprised of glucose and fructose, such oligosaccharides or their metabolites would appear to possess no genotoxic, carcinogenic or chronic toxic potential.

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CORRECTION

The paper entitled "Evaluation Study of the Booklet 'Preschoolers and Poisons'", that was published in the Continuing Education/Reviews section of the October 1987 (volume 29, number 5:401-404) issue of Veterinary and Human Toxicology, should have been published in the Scientific Reports section as a refereed and peer-reviewed and accepted publication. We apologize to the authors for this editorial error.

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Toxicological Evaluation of Neosugar: Genotoxicity, Carcinogenicity, and Chronic Toxicity

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ABSTRACT

Neosugar, a fructooligosaccharide mixture, was tested for genotoxicity in three assays: (1) microbial reverse mutation assays in *Salmonella typhimurium* (Ames assay) and *Escherichia coli* WP2 *uvr*A, (2) the L5178Y mouse lymphoma TK$^{+/-}$ mammalian cell mutation assay, and (3) an assay for the induction of unscheduled DNA synthesis (UDS) in human epithelioid cells (HeLa S3). Each assay was conducted at a wide range of dose levels, both with and without metabolic activation. Test results gave no indication that neosugar possessed any genotoxic potential. The carcinogenicity and chronic toxicity of neosugar were examined in Fischer 344 rats. Rats were fed diets containing 0, 8000, 20,000, or 50,000 ppm neosugar for 104 weeks. No dose-related effects on survival, growth, hematology, blood chemistry, organ weights, or nonneoplastic lesions were observed. The incidence of rare and spontaneous tumors was comparable between control and neosugar treatment groups, with the exception of pituitary adenomas in male rats. In light of the background incidence of this tumor and an equivocal dose–response trend, it is unlikely that neosugar treatment is related to the incidence of pituitary adenomas in male rats. The results of this study indicate that neosugar is nonmutagenic and that rats are not adversely affected by chronic neosugar exposure.

INTRODUCTION

Neosugar is a fructooligosaccharide mixture of 1^F-(1-β-fructofuranosyl)$_n$ $_1$-sucrose oligomers in which n may vary from 2 to 4. That is, it consists of sucrose molecules (glucose–fructose disaccharides) to which one, two, or three additional fructose units have been added by β(2→1) linkage to the fructose unit of sucrose. These components are abbreviated as GF_3, GF_4, and GF_5, and their chemical structures are shown in Figure 1. Similar or identical fructooligosaccharides are found in a variety of plants, including Jerusalem artichoke,[1] asparagus root,[2]

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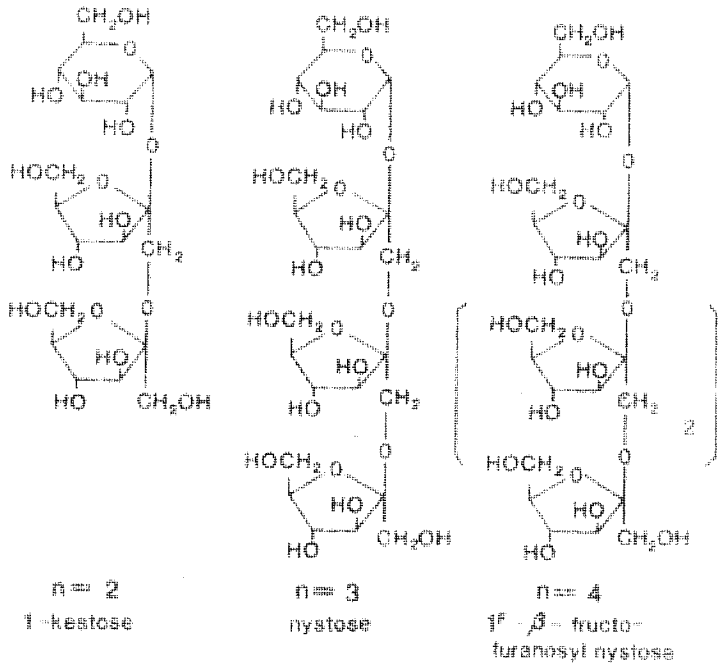

FIG. 1. Structure of major components of neosugar

onion,[8] wheat,[4,5] rye,[5] and triticale.[5,6] These oligomers are resistant to hydrolysis by mammalian α-amylase, sucrase, and maltase.[1,8] They are sweet tasting, however, and, therefore, have potential as low-calorie sweeteners.[9] When fed to weanling pigs, neosugar is reported to accelerate weight gain,[10] and it is used as a feed additive in poultry and livestock in Japan.

The present genotoxicity and chronic rat studies were conducted as part of an investigation to examine the safety of neosugar. The genotoxicity studies were conducted by Huntingdon Research Center, Huntingdon England, and the chronic rat study was conducted by the Biosafety Research Center, Foods, Drugs and Pesticides (An-Pyo Center), Shizuoka-ken (437-12), Japan.

MATERIALS AND METHODS

Mutagenicity study

Test material. Neosugar, a clear viscous liquid, was obtained from Meiji Seika Kaisha Ltd., Pharmaceuticals Division, Tokyo 104, Japan. Neosugar was dissolved in distilled water for testing.

Microbial gene mutation assay. Salmonella typhimurium strains TA 1535, TA 1537, TA 1538, TA 98, and TA 100 were obtained from Dr. Bruce Ames, University of California, Berkeley, CA, and *Escherichia coli* WP2 *uvr*A was obtained from the National Collection of Industrial Bacteria, Aberdeen, Scotland. The bacteria were stored at −80°C and grown in Oxoid nutrient broth at 37°C for 18 hr before use in mutation assays.

Neosugar was tested in a standard plate incorporation assay at 0, 50, 150, 500, 1500, and 5000

μg/plate in each tester strain, with and without metabolic activation, using procedures complying with OECD and Japanese Ministry of Health and Welfare guidelines. Three test plates per strain per treatment condition were used. Appropriate negative and positive controls were used with each strain. The positive controls used in the absence of metabolic activation were *N*-ethyl-*N'*-*nitro*-*N*-nitrosoguanidine with TA 1535, TA 100, and *E. coli* WP2 *uvr*A, 2-nitrofluorene with TA 98 and TA 1538, and 9-aminoacridine with TA 1537. With metabolic activation, 2-aminoanthracene was used with all strains.

Mammalian cell mutation assay. Mouse lymphoma L5178Y cells (3.7.2c) were obtained from Dr. J. Cole, Sussex University. The cells, which are heterozygous at the thymidine kinase locus ($TK^{+/-}$) were grown routinely as suspensions in roller culture in sodium bicarbonate-buffered RPMI 1640 medium supplemented with sodium pyruvate (110 μg/ml), pluronic F68 (1 mg/ml), gentamicin (50 μg/ml), and 10% heat-inactivated horse serum, in an atmosphere of 5% CO_2 in air. For cloning, the serum content of the medium was doubled to 20%, the pluronic content reduced to 0.2 mg/ml, and 0.3% Noble agar was incorporated. Chemical treatment of the cells took place in hepes-buffered RPMI 1640 medium without added serum, pluronic, or sodium pyruvate.

Preliminary toxicity tests were conducted by treating cells in suspension with neosugar at 50, 100, 250, 1000, 2500, and 5000 μg/ml at 37°C for 3 hr. The highest dose used represented the maximum dose used routinely in this assay to avoid confounding physical effects. The cells were then washed and resuspended in normal growth medium and cultured at 37°C. Cell population growth was monitored at 24 and 48 hr using a Coulter counter.

Mutagenicity was tested by treating populations of 12×10^6 cells for 3 hr at 37°C with neosugar at 2000, 3000, 4000, and 5000 μg/ml in the presence and absence of an Aroclor-induced rat liver microsomal metabolic activation system. After treatment in serum-free medium, the cells were washed and grown for 48 hr in normal growth medium to allow expression of any induced mutations, then cloned in soft agar to test for viability (600 cells/plate) and mutations (10^6 cells/plate, plus trifluorothymidine at 4 μg/ml). The procedures used are based on those of Clive and Spector[13] and Amacher et al.[11,14]

Unscheduled DNA synthesis (UDS) assay. UDS was examined using the procedures of Martin et al.[15,16] and complying with OECD guidelines. Briefly, HeLa S3 epithelioid cells, originally derived from a human cervical carcinoma, were obtained from Flow Laboratories, Ltd., and cultured in Eagle's Minimum Essential Medium (EMEM) with Earle's salts and gentamicin at 50 μg/ml, plus 15% fetal calf serum. For the UDS assay, cells were grown to confluence on 22 mm coverslips in multiwell tissue culture dishes in normal EMEM. The medium was then replaced with arginine-deficient medium containing 2.5% dialyzed fetal calf serum and kept at 37°C for an additional 72 hr to suppress normal scheduled DNA replication. ^{3}H-Thymidine (20 Ci/mmole) was then added to a final concentration of 5 μCi/ml, together with 100 μl of an appropriate concentration of the test chemical to give a final concentration of 25, 50, 100, 200, 400, 800, 1600, 3200, 6400, 12,800, 25,600, or 51,200 μg/ml. Each dose level was tested with and without metabolic activation. Appropriate negative (water and dimethylsulfoxide) and positive (4-nitroquinoline-1-oxide and 2-aminoanthracene) controls also were tested.[17] After treatment for 3 hr, the coverslips and cells were removed, washed, fixed, stained in orcein, mounted on slides, and processed for autoradiography using Kodak AR-10 stripping film. After application of the photographic emulsion to the coverslips, they were kept in the dark for 13 days to expose the emulsion. The autoradiographs were then developed, fixed, rinsed, and air dried.

UDS was quantitated by counting silver grains overlying 100 non-S-phase cells and over equal areas of cytoplasm (background counts).

Metabolic activation system. For all three tests, a metabolic activation system was used, consisting of liver microsomal fraction (S-9) from Charles River CD rats, 6–8 weeks old, given a single intraperitoneal injection of Aroclor 1254 at 500 mg/kg body weight 5 days before killing. The livers were removed and homogenized in three times their weight of ice-cold 0.15 *M* KCl. The homoge-

nate was centrifuged at 900 *g* for 10 min, and the supernatant (S-9 fraction) was collected and stored at −70°C. The S-9 fraction was mixed with appropriate cofactors immediately before use. In all cases, the functioning of the S-9 mix was tested by including positive control materials that required metabolic activation to express a genotoxic effect. For the microbial tests, the S-9 mix contained 10% vol/vol S-9 fraction, 8 m*M* $MgCl_2$, 33 m*M* KCl, 100 m*M* sodium phosphate buffer (pH 7.4), 5 m*M* glucose-6-phosphate, 4 m*M* NADPH, and 4 m*M* NADH; 0.5 ml of this mixture was added to 2 ml of top agar with 0.1 ml bacterial suspension and 0.1 ml test solution.

For the mouse lymphoma assay, the S-9 fraction was added to a threefold larger volume of ice-cold RPMI 1640 medium containing isocitric acid at 15 mg/ml and NADP at 8 mg/ml immediately before use, and 8 ml of that mixture was added to 12 ml of cell suspension with 0.2 ml of test solution.

For the UDS assay, S-9 mix was prepared by mixing 4 ml of S-9 fraction with 2 ml of 0.15 *M* KCl, 0.36 g glucose-6-phosphate, 0.05 g NADP, and 4 ml distilled water. Aliquots of 0.1 ml of that mixture were added to each 2 ml of medium in the tissue culture dishes containing cells.

Chronic animal study

Animals and diet. Male and female specific pathogen-free Fischer 344 (F344) rats (4 weeks of age) were obtained from Charles River Japan, Inc. The animals were housed singly in wire mesh cages. A 12-hr light–dark cycle was provided. The temperature was maintained at 22–24°C, and relative humidity was 50–60%. Test animals received a modified NIH open formula rat and mouse diet (Oriental Yeast Co., Tokyo, Japan) containing neosugar. Control animals received the same diet without neosugar. Food and water were provided ad libitum. Neosugar powder containing greater than 95% fructooligosaccharides was obtained from Mie Kariyo Co., Mie, Japan. The oligosaccharide ratio (GF_2:GF_3:GF_4) of the test material was 37:51:12.

Experimental design. After 1 week of acclimation, groups of 50 rats of each sex began receiving neosugar at concentrations of 0, 8000, 20,000, or 50,000 ppm in their diet. Because of the low toxicity of neosugar, the selection of the highest exposure concentration was based on the maximum volume of neosugar that could be incorporated as a food additive. The experiment was terminated after 104 weeks of treatment. Animals were observed at least twice daily. Body weights were determined weekly for the first 26 weeks and biweekly thereafter. Food consumption was determined weekly for all animals throughout the study. Food efficiency and neosugar intake were calculated from body weight and food consumption data.

At the termination of the study, surviving animals were anesthetized with ether, and blood samples were collected from the abdominal aorta. Hematology measurements were performed on all samples using a Coulter Counter Model S[P] (Coulter Electronics, USA). Blood smears fixed with methanol and stained with Wright-Giemsa were used for differential leukocyte counts and morphological evaluation. Blood chemistry measurements using serum were performed using a Centrifichem System 400 (Union Carbide Co., USA) except for Na, K, and Cl, which were measured with an automatic electrolysis analyzer (Toa Electronics, Tokyo, Japan). Brain, adrenals, heart, spleen, lungs, testes, liver, ovaries, and kidney weights were recorded for all animals surviving the length of the experiment. All animals, including those dying spontaneously or killed in a moribund condition, were necropsied. Tissues were fixed in 10% neutral buffer formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Statistical evaluation. A two-tailed Yates corrected Chi-square analysis was used to determine the significance of differences in survival and differences in the incidence of nonneoplastic lesions. When pairwise comparisons of nonneoplastic lesions revealed a significant difference between treatment and control groups, logistic regression analysis was performed to test for dose-related trends.[15] One-way analysis of variance (ANOVA) was used to assess the significance of differences in body weight, food consumption, food efficiency, organ weight, hematology, and blood

chemistry. Where significant effects were found, the Student-Newman-Keuls (SNK) multiple range test for multiple comparisons among means[19] was performed to determine where differences among the group means were located.

A one-tailed Yates corrected Chi-square test was used to determine the significance of differences in the incidence of tumors.[20] Logistic regression and Cochran-Armitage Chi-square[20] tests for trend were used to determine significant dose-related trends. A significance level of $P \leq 0.05$ was used in all analyses.

RESULTS

Mutagenicity study

Microbial gene mutation assay. Two independent trials were conducted, both with and without metabolic activation. No signs of toxicity were seen at any of the doses of neosugar tested, and there was no increase in mutants per plate in any bacterial strain either with or without metabolic activation. The positive control chemicals produced the expected clear increases in mutation frequency. The results of the first trial are shown in Table 1. The second trial gave very similar results.

Mammalian cell mutation assay. Two independent trials were conducted both in the presence and in the absence of metabolic activation (Table 2). In both trials, no significant toxicity at any dose level was detected, as measured by cell population growth after treatment or colony-forming ability. Also, no increase in mutation frequency was seen in either trial, either with or without metabolic activation. As with the microbial assays, the positive control chemicals produced the expected clear increases in mutation frequency.

UDS assay. Two independent trials were conducted, both with and without metabolic activation (Table 3). In the first trial, a statistically significant ($P < 0.05$, ANOVA) increase in net nuclear grains occurred at a concentration of neosugar of 1600 μg/ml in the absence of metabolic activation. There was no indication of a dose-related trend in grain counts, however, and the increase was not reproduced in the second trial. In neither trial with metabolic activation was there any significant effect on net nuclear grain counts.

Chronic animal study

Survival. Survival curves for male and female rats are shown in Figure 2. At the termination of the experiment, survival of male rats administered 0, 8000, 20,000 and 50,000 ppm neosugar was 88%, 72%, 68%, and 84%, respectively. The survival rates of the male 8000 and 50,000 ppm groups were comparable to the control group, but mortality in the 20,000 ppm group was significantly higher than in controls. The survival of female rats administered 0, 8000, 20,000, and 50,000 ppm neosugar was 82%, 74%, 74%, and 88%, respectively. All female neosugar-treated groups had survival rates comparable to controls.

Growth, food efficiency, and chemical intake. Growth curves for male and female rats are shown in Figure 3. Mean body weights of all male and female neosugar treatment groups were comparable to their respective controls. Overall food consumption by neosugar-treated male rats was comparable to the control group. When overall food consumption data for females were analyzed by ANOVA, significant variation among groups was found; however, subsequent pairwise group comparisons by the SNK test procedure showed no significant intergroup differences involving the control group. Overall food efficiencies by neosugar-treated male and female groups were comparable to their control groups.

Neosugar intake by the 8000, 20,000, and 50,000 ppm groups was calculated to be approxi

TABLE 1. RESULTS OF MICROBIAL GENE MUTATION ASSAY WITH NEOSUGAR (TRIAL 1)

Material	Test concentration (μg/plate)	With or without S-9	Reverse mutation (No. of colonies/plate)[a]					
			Base pair exchange type			Frame shift type		
			TA 100	TA 1535	WP2 uvrA	TA 98	TA 1537	TA 1538
Solvent control		–	119	20	46	27	14	11
Neosugar	5000	–	130	23	57	18	9	6
	1500	–	118	19	47	23	16	9
	500	–	106	33	45	24	11	12
	150	–	119	21	48	21	11	12
	50	–	121	20	42	21	12	10
Solvent control		+	116	23	53	23	17	16
Neosugar	5000	+	118	20	53	21	13	9
	1500	+	115	19	43	22	17	17
	500	+	99	19	49	22	12	11
	150	+	105	16	52	27	15	13
	50	+	128	16	51	23	13	14
Positive controls								
ENNG[b]	2	–	–	–	1337	–	–	–
	3	–	399	–	–	–	–	–
	4	–	–	737	–	–	–	–
NF	1	–	–	–	–	52	–	–
	2	–	–	–	–	–	–	38
9 AC	80	–	–	–	–	–	1231	–
AA	0.5	+	245	–	–	115	–	71
	2	+	–	89	–	–	83	–
	80	+	–	–	265	–	–	–

[a]Values are the mean of 3 plates.
[b]ENNG, N-ethyl-N'-nitro-N-nitrosoguanidine; 9 AC, 9-aminoacridine; AA, 2-aminoanthracene; NF, 2-nitrofluorene.

TABLE 2. SUMMARY OF RESULTS OF MOUSE LYMPHOMA TK^{+} MUTATION ASSAY WITH NEOSUGAR

	Without metabolic activation				*With metabolic activation*			
Concentration of neosugar (μg/ml)	*% Survival*		*Mutant frequency (× 10^{-6})*		*% Survival*		*Mutant frequency (× 10^{-6})*	
	Trial 1	*Trial 2*	*Trial 1*	*Trial 2*	*Trial 1*	*Trial 2*	*Trial 1*	*Trial 2*
0	100	100	48	72	100	100	85	57
2000	69	134	49	58	157	97	71	63
3000	77	114	53	76	143	73	76	90
4000	64	124	44	65	111	98	89	54
5000	64	112	63	67	120	87	73	78
Positive control[a]	35	53	489	478	18	28	228	239

[a]Ethyl methane sulfonate (500 μg/ml) without metabolic activation; cyclophosphamide 15 μg/ml) with activation.

mately 341, 854, and 2170 mg/kg/day, respectively, for male rats and 419, 1045, and 2664 mg/kg/day, respectively, for female rats.

Hematology and blood chemistry. Hematology results are presented in Table 4. Neosugar exposure had no significant effect on any of these parameters. In addition, neosugar treatment had no effect on differential leukocyte counts (data not shown).

Blood chemistry results are presented in Table 5. Male rats fed neosugar showed a slight but significant elevation of Na and Cl. Male rats fed 20,000 ppm neosugar had slightly elevated levels of blood glucose and creatinine. The male 50,000 ppm group had slightly decreased creatinine levels. All other parameters for male neosugar-treated rats were similar to control values. In females, all blood chemistry parameters were similar to controls except for a slight elevation of uric acid in the 8000 and 20,000 ppm groups.

Organ weights. Organ weights are shown in Table 6. Neosugar treatment had no effect on organ weights. Organ/body weight ratios also were similar among groups except for adrenal weight ratios of females. However, pairwise comparisons showed no significant differences involving the control group.

Nonneoplastic lesions. As might be expected in aging rats, many types of nonneoplastic lesions were observed in all groups, including controls. Numerous lesions were present in liver, kidneys, adrenals, and lymph nodes (Table 7). Other age-related lesions that occurred in the majority of animals were hyaline bodies in brain tissue, pigment deposits in the spleen, and atrophy of the thymus. Stomach fibrosis, seminal vesicle atrophy, and decreased spermatogenesis were common in males, and pituitary and adrenal angiectasis were extremely common in females.

Nonneoplastic lesions were classified as slight, moderate, or marked. The majority of lesions were slight. Exceptions were chronic nephropathy in males, where 20-40% of these lesions were moderate in severity. In females, renal calcium deposits and protein casts and pigment deposits in the spleen were of moderate or marked severity in 15-40% of animals with these lesions.

Neosugar treatment did not affect the severity of these lesions except for renal protein casts in male rats. Renal casts of moderate severity were found in 0, 4, 7, and 6 rats in the male control,

TABLE 3. SUMMARY OF RESULTS OF UDS ASSAY WITH NEOSUGAR

	Without metabolic activation				*With metabolic activation*			
Concentration of neosugar (μg/ml)	*Net grains/ 100 nuclei*		*% Nuclei with >3 net grains*		*Net grains/ 100 nuclei*		*% Nuclei with >3 net grains*	
	Trial 1	*Trial 2*	*Trial 1*	*Trial 2*	*Trial 1*	*Trial 2*	*Trial 1*	*Trial 2*
0 (water)	−2	12	0.6	0	4	30	0.9	0.6
0 (DMSO)	5	18	0.6	0.4	13	8	0.5	1
25	14	16	0	0	18	17	2	1
50	15	23	0	0.5	26	24	1.5	1
100	5	27	0	0.5	10	25	0.5	0
200	−2	5	0.5	0.5	7	0	1	0
400	−2	17	0.5	2	14	8	2.5	0
800	−16	17	0	3	17	44	1	1.5
1600	24[a]	8	1	0	12	5	1.5	0
3200	6	27	1	1	12	1	1	0
6400	7	42	0	1.5	14	25	0	1
12,800	10	33	0.5	1	2	7	0	0
25,600	−3	25	0	0.5	12	22	0.5	0
51,200	−9	21	0	0.5	−19	15	0.5	0
Positive control[b]								
x	321	529	41	67.5	32	31	1	0
2x	1051	845	75.5	82	40	26	3	1.5
4x	1185	853	92	81.5	53	58	2.5	5.5
8x	1646	1237	95.5	95	50	88	1.5	8
16x	1732	1351	97.5	99	40	71	3	7.5

[a]Significantly greater than control ($P < 0.05$, one way analysis of variance).

[b]4-Nitroquinoline 1-oxide at 0.02, 0.04, 0.08, 0.16, and 0.32 μg/ml without metabolic activation; 2-aminoanthracene at 2.5, 5, 10, 20, and 40 μg/ml with activation.

8000, 20,000, and 50,000 ppm groups, respectively. One male in the 20,000 ppm group had renal casts of marked severity.

The incidence of the majority of nonneoplastic lesions was similar in all groups whether fed control diet or neosugar. However, pairwise comparisons revealed that some lesions occurred in greater or lesser incidence in neosugar groups than in controls. These lesions are shown in Table 8. It is apparent from the incidence data that simple pairwise comparisons alone were inadequate for determining a cause–effect relationship between neosugar treatment and the occurrence of a particular lesion. Therefore, additional intergroup comparisons were made, which included a quantitative dose response trend analysis, comparison of the severity of lesions, and consideration of the background incidence of these lesions in historical controls.

Logistic regression is a parametric statistical test that analyzes for dose-related trends. Table 8 presents the results of logistic regression analysis of the nonneoplastic lesions data. Positive and

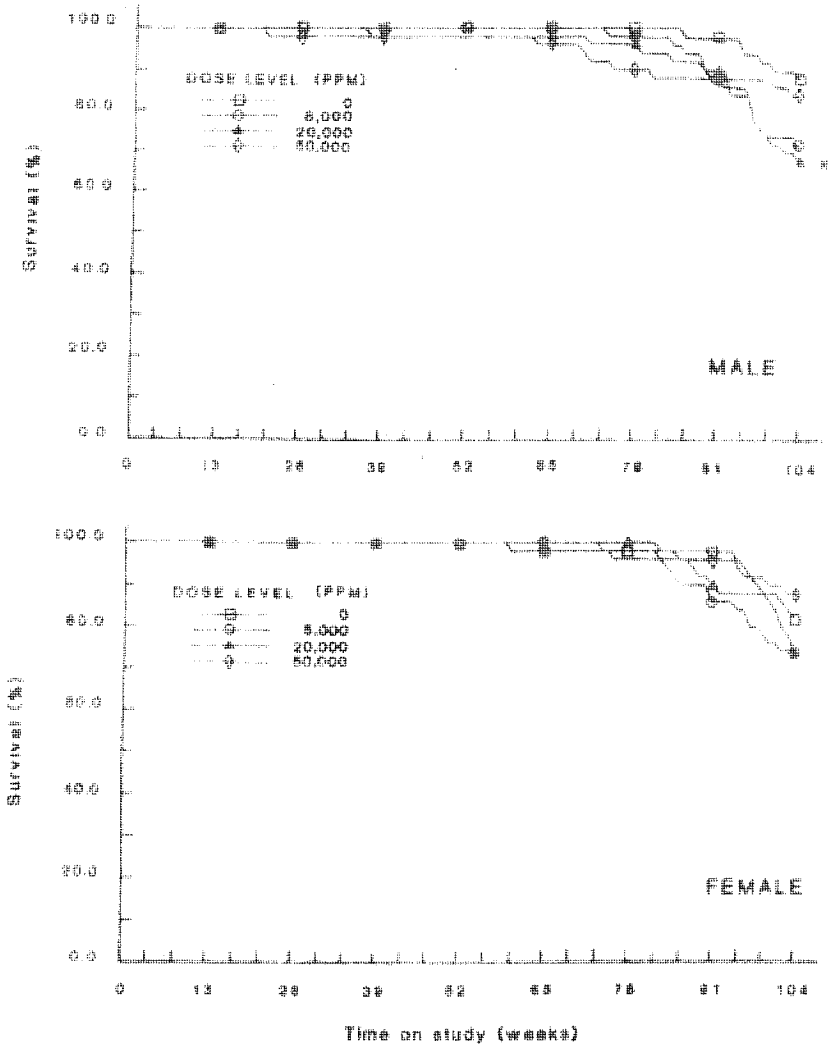

FIG. 2. Survival curves for male and female rats administered neosugar in the diet. *Significantly different from control, $P \leq 0.05$.

negative trends are denoted by the sign of the dose coefficient and Student's t value. Of the 17 lesions with significant findings by pairwise comparisons, only 3 were common to both males and females, and for 2 of the 3 (lymph node granulation and stomach fibrosis), opposite trends were observed. However, as indicated by the P values, no significant positive or negative dose-related trends were detected by logistic regression for any of the lesions in either sex.

A borderline positive trend ($P = 0.07$) was noted for lymph node granulation and prostatic atrophy in males. In females, only two lesions, adrenal angiectasis and adrenal hyperplasia, showed a positive (although nonsignificant) dose-related trend. The severity of these particular lesions in males and females was not affected by neosugar treatment. Furthermore, their incidence in neo-

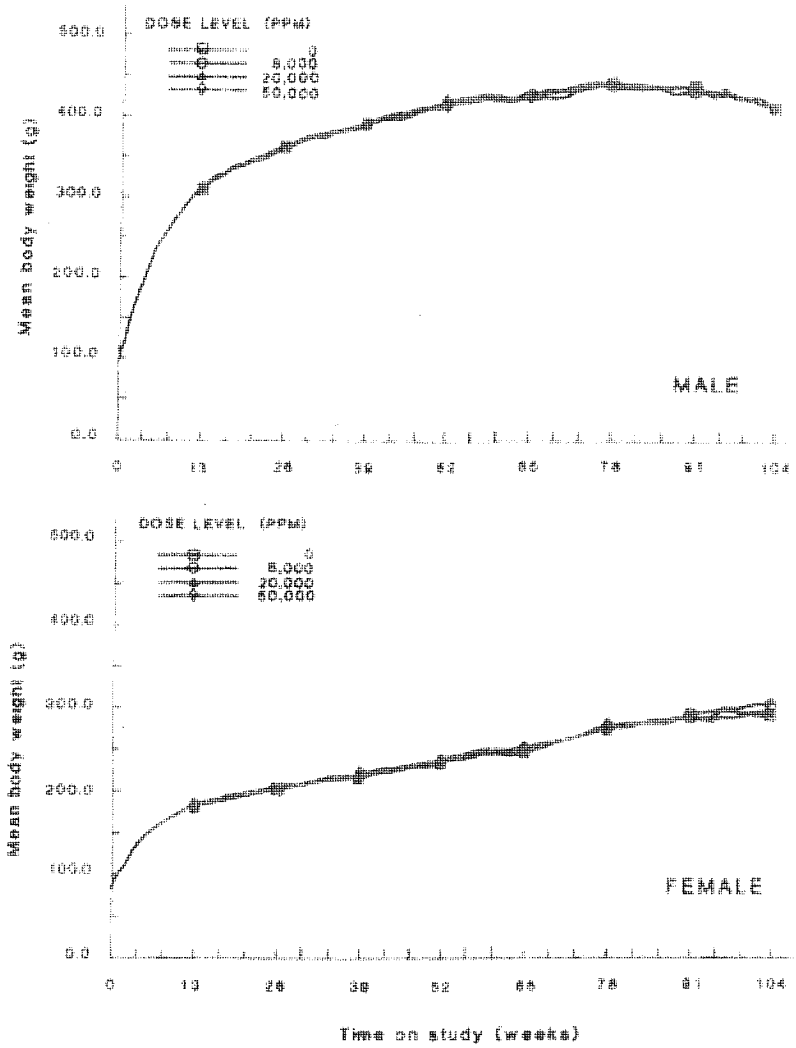

FIG. 3. Growth curves for male and female rats administered neosugar in the diet.

sugar groups was within the historical control range (see Discussion). With regard to lymph node granulation, opposite dose-related trends were noted in males and females.

Table 8 shows the incidence of the nonneoplastic lesions in historical control F-344 rats from this laboratory. Although most of these lesions were quite common, there were also wide variations in occurrence in historical controls. Despite the wide historical range, the incidence of some lesions in the neosugar study controls was outside the historical control range. The incidence of prostatic atrophy in concurrent controls was 10%, whereas the historical mean was 58%, and the range was 15–100%. Similarly, the incidence of dilated gastric glands and lymph node granulation in concurrent male controls were at the lower extreme of the historical ranges. Consequently, lesions that were significantly increased in neosugar groups as compared by pairwise comparison to concurrent

TABLE 4. HEMATOLOGY RESULTS OF MALE AND FEMALE RATS ADMINISTERED NEOSUGAR IN THE DIET FOR 2 YEARS[a]

Dietary level (ppm)	*HCT*[b] *(%)*	*HGB (g/dl)*	*RBC (× 10⁶/mm³)*	*MCV (μm³)*	*MCH (pg)*	*MCHC (%)*	*PLT (× 10³/mm³)*	*WBC (× 10³/mm³)*
Males								
0	46.0 ± 9.1	16.5 ± 3.2	8.95 ± 1.99	51.9 ± 4.8	18.7 ± 1.9	35.9 ± 0.8	811 ± 176	5.6 ± 2.3
8000	49.2 ± 6.3	17.6 ± 2.1	9.62 ± 1.39	51.2 ± 2.3	18.4 ± 1.0	35.8 ± 0.6	770 ± 154	5.2 ± 1.8
20,000	46.2 ± 7.5	16.5 ± 2.6	8.94 ± 1.79	52.5 ± 6.9	18.8 ± 2.6	35.8 ± 0.6	807 ± 176	5.4 ± 1.7
50,000	47.8 ± 6.6	17.2 ± 2.3	9.41 ± 1.41	50.8 ± 1.1	18.3 ± 0.5	36.0 ± 0.5	835 ± 150	5.1 ± 2.8
Females								
0	45.5 ± 7.8	16.0 ± 2.6	8.05 ± 1.83	57.9 ± 8.0	20.5 ± 3.1	35.3 ± 0.7	742 ± 308	4.6 ± 4.9
8000	47.3 ± 7.0	16.5 ± 2.3	8.51 ± 1.40	55.9 ± 3.0	19.6 ± 1.2	35.1 ± 0.8	738 ± 95	3.5 ± 2.3
20,000	44.1 ± 6.3	15.5 ± 2.1	7.94 ± 1.40	56.0 ± 3.7	19.7 ± 1.4	35.1 ± 0.6	832 ± 264	3.7 ± 1.8
50,000	44.9 ± 5.6	15.9 ± 1.9	8.14 ± 1.25	56.0 ± 7.1	19.9 ± 2.7	35.4 ± 0.4	735 ± 135	4.9 ± 8.1

[a]Values represent mean ± SD, $n = 34–44$.

[b]HCT, hematocrit; HGB, hemoglobin; RBC, red blood cell count; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, platelet count; WBC, white blood cell count.

TABLE 5. BLOOD CHEMISTRY RESULTS OF MALE AND FEMALE RATS ADMINISTERED NEOSUGAR IN THE DIET FOR 2 YEARS[a]

	Dietary level (ppm)							
	Males				*Females*			
	0	*5000*	*20,000*	*50,000*	*0*	*5000*	*20,000*	*50,000*
Na (mEq/L)	146 ± 1	147 ± 1	148 ± 2[c]	149 ± 2[c]	148 ± 1	148 ± 2	148 ± 1	148 ± 2
K (mEq/L)	5.1 ± 0.3	5.1 ± 0.4	5.1 ± 0.3	5.5 ± 0.5	4.9 ± 0.4	5.1 ± 0.4	5.0 ± 0.6	4.6 ± 0.5
Cl (mEq/L)	106 ± 1	107 ± 1[c]	109 ± 2[c]	108 ± 1[c]	107 ± 2	107[c] ± 2	106 ± 2	107 ± 2
Ca (mg/dl)	9.8 ± 0.6	9.8 ± 0.5	9.5 ± 0.9	9.8 ± 0.3	10.0 ± 0.6	10.4 ± 0.9	10.4 ± 0.9	10.8 ± 0.8
IP[b] (mg/dl)	5.0 ± 0.8	4.1 ± 0.4	4.4 ± 0.8	4.5 ± 0.8	4.8 ± 0.9	4.0 ± 0.5	4.8 ± 1.1	4.6 ± 0.6
Glu (mg/dl)	84 ± 25	86 ± 24	107 ± 10[c]	76 ± 22	112 ± 17	111 ± 10	107 ± 34	121 ± 16
BUN (mg/dl)	26 ± 6	26 ± 6	32 ± 7	29 ± 5	15 ± 2	14 ± 1	13 ± 2	15 ± 4
UA (mg/dl)	1.4 ± 0.4	1.4 ± 0.4	1.1 ± 0.3	1.3 ± 0.4	0.8 ± 0.3	1.1 ± 0.2[c]	1.3 ± 0.5[c]	0.7 ± 0.2

Creat (mg/dl)	0.59 ± 0.05	0.65 ± 0.04	0.73 ± 0.12*	0.46 ± 0.11*	0.53 ± 0.05	0.54 ± 0.04	0.46 ± 0.12	0.51 ± 0.06
T-Chol (mg/dl)	131 ± 39	135 ± 33	138 ± 7	129 ± 40	121 ± 38	110 ± 19	114 ± 38	95 ± 18
Alb (g/dl)	2.7 ± 0.3	2.8 ± 0.2	2.6 ± 0.3	2.6 ± 0.3	2.2 ± 0.2	2.3 ± 0.3	2.3 ± 0.2	2.3 ± 0.1
T-Bil (mg/dl)	0.45 ± 0.06	0.47 ± 0.04	0.45 ± 0.12	0.44 ± 0.09	0.48 ± 0.11	0.41 ± 0.04	0.47 ± 0.07	0.47 ± 0.06
Alk-P (IU/L)	101 ± 49	116 ± 20	140 ± 33	90 ± 24	91 ± 31	90 ± 40	92 ± 32	81 ± 18
SGOT (IU/L)	104 ± 16	109 ± 47	105 ± 78	134 ± 58	68 ± 24	95 ± 43	98 ± 36	86 ± 24
SGPT (IU/L)	42 ± 15	41 ± 9	48 ± 16	47 ± 17	42 ± 16	50 ± 19	45 ± 16	41 ± 11
LDH (IU/L)	122 ± 32	195 ± 128	217 ± 146	186 ± 44	56 ± 17	104 ± 52	128 ± 42	236 ± 182

*Values represent the mean ± SD, $n = 10$.

TP, anorganic phosphorus; Glu, glucose; BUN, blood urea nitrogen; UA, uric acid; Creat, creatinine; T-Chol, total cholesterol; Alb, albumin; T-Bil, total bilirubin; Alk-P, alkaline phosphatase; SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamic pyruvic transaminase; LDH, lactate hydrogenase.

Significantly different from control, $P < 0.05$.

TABLE 6. ORGAN WEIGHTS OF MALE AND FEMALE RATS ADMINISTERED NEOSUGAR IN THE DIET FOR 2 YEARS[a]

Dietary level (ppm)	*Brain*	*Heart*	*Lungs*	*Liver*	*Kidneys*	*Spleen*	*Adrenals*	*Testes*	*Ovaries*
Males									
0	2.20 ± 0.05	1.18 ± 0.11	1.44 ± 0.17	11.64 ± 2.84	3.14 ± 0.46	1.42 ± 1.63	0.082 ± 0.053	5.51 ± 2.75	
8000	2.20 ± 0.05	1.18 ± 0.08	1.43 ± 0.14	10.46 ± 1.16	2.98 ± 0.22	1.20 ± 0.96	0.075 ± 0.024	5.49 ± 1.93	
20,000	2.19 ± 0.06	1.20 ± 0.07	1.47 ± 0.28	11.66 ± 3.39	3.06 ± 0.43	1.32 ± 0.81	0.113 ± 0.205	4.95 ± 1.56	
50,000	2.19 ± 0.06	1.16 ± 0.07	1.42 ± 0.13	10.89 ± 1.00	3.13 ± 0.53	1.10 ± 0.75	0.080 ± 0.020	5.11 ± 2.12	
Females									
0	1.98 ± 0.05	0.94 ± 0.11	1.11 ± 0.39	7.74 ± 1.79	2.21 ± 0.40	1.57 ± 3.46	0.071 ± 0.013		0.064 ± 0.016
8000	1.99 ± 0.04	0.93 ± 0.09	1.05 ± 0.19	7.40 ± 1.04	2.16 ± 0.19	0.79 ± 1.45	0.069 ± 0.005		0.069 ± 0.013
20,000	1.99 ± 0.05	0.93 ± 0.08	1.07 ± 0.24	7.57 ± 1.49	2.13 ± 0.28	1.39 ± 2.59	0.077 ± 0.030		0.069 ± 0.014
50,000	2.00 ± 0.05	0.93 ± 0.10	1.10 ± 0.25	7.49 ± 1.39	2.14 ± 0.16	1.22 ± 2.69	0.076 ± 0.010		0.068 ± 0.015

[a]Values represent the mean ± SD, $n = 34$–44; weights are expressed in grams.

TABLE 7. INCIDENCE OF COMMONLY OCCURRING NONNEOPLASTIC LESIONS IN MALE AND FEMALE RATS ADMINISTERED NEOSUCHAR IN THE DIET FOR 2 YEARS

	Male (50 rats/group)				*Female (50 rats/group)*			
	0 (ppm)	*8000 (ppm)*	*20,000 (ppm)*	*50,000 (ppm)*	*0 (ppm)*	*8000 (ppm)*	*20,000 (ppm)*	*50,000 (ppm)*
Liver								
Fatty change	30 (60%)	27 (54%)	19 (38%)	35 (70%)	44 (88%)	40 (80%)	37 (74%)	40 (80%)
Granulation	15 (30%)	21 (42%)	26 (52%)	27 (54%)	29 (58%)	27 (54%)	24 (48%)	31 (62%)
Fibrosis	22 (44%)	20 (40%)	20 (40%)	26 (52%)	0 (0%)	0 (0%)	2 (4%)	0 (0%)
Hyperplasia of bile ducts	47 (94%)	48 (96%)	47 (94%)	45 (90%)	10 (20%)	12 (24%)	11 (22%)	12 (24%)
Necrosis	0 (0%)	1 (2%)	1 (2%)	2 (4%)	12 (24%)	11 (22%)	9 (18%)	10 (20%)
Kidney								
Basophilic change	47 (94%)	43 (86%)	43 (86%)	46 (92%)	23 (46%)	26 (52%)	24 (48%)	21 (42%)
Deposit of pigment	48 (96%)	43 (86%)	45 (90%)	47 (94%)	44 (88%)	45 (90%)	42 (84%)	42 (84%)
Lymphocytic infiltration	42 (84%)	39 (58%)	39 (78%)	31 (62%)	5 (10%)	4 (8%)	4 (8%)	0 (0%)
Protein cast	49 (98%)	48 (96%)	49 (98%)	48 (96%)	44 (88%)	43 (86%)	37 (74%)	37 (74%)
Chronic nephropathy	33 (66%)	27 (54%)	39 (78%)	33 (66%)	16 (32%)	9 (18%)	5 (10%)	4 (8%)
Glomerulosclerosis	39 (78%)	35 (70%)	44 (88%)	40 (80%)	26 (52%)	21 (42%)	17 (34%)	9 (18%)
Hyaline droplet	1 (2%)	0 (0%)	3 (6%)	0 (0%)	21 (42%)	21 (42%)	14 (28%)	14 (28%)
Lymph nodes								
Granulation	1 (2%)	8 (16%)	12 (24%)	16 (32%)	10 (20%)	5 (10%)	5 (10%)	3 (6%)
Plasma cell increase	21 (42%)	27 (54%)	33 (66%)	29 (58%)	22 (44%)	15 (30%)	19 (38%)	19 (38%)
Deposit of pigment	2 (4%)	0 (0%)	0 (0%)	1 (2%)	16 (32%)	16 (32%)	19 (38%)	17 (34%)
Sinus histiocytosis	1 (2%)	0 (0%)	0 (0%)	0 (0%)	19 (38%)	9 (18%)	10 (20%)	9 (18%)
Adrenal								
Angiectasis	1 (2%)	2 (4%)	2 (4%)	8 (16%)	20 (40%)	26 (52%)	25 (50%)	32 (64%)
Vacuolic change	17 (34%)	17 (34%)	16 (32%)	15 (30%)	34 (68%)	42 (84%)	37 (74%)	37 (74%)
Hyperplasia	13 (26%)	14 (28%)	26 (52%)	22 (44%)	0 (0%)	1 (2%)	7 (14%)	4 (8%)

TABLE 8. INCIDENCE OF SIGNIFICANT NONNEOPLASTIC LESIONS IN HISTORICAL CONTROL AND NEOSUGAR STUDY RATS AND LOGISTIC REGRESSION ANALYSIS OF NEOSUGAR STUDY DATA

	Neosugar study incidence (%)[a]				*Logistic regression analysis*			*Historical control incidence (%)*[b]	
	0 (ppm)	*8000 (ppm)*	*20,000 (ppm)*	*50,000 (ppm)*	*Dose coefficient (× 10⁻⁴)*	*Student's t*	*P value*	*Mean*	*Range*
Males									
Lymph node granulation	3	16[c]	34[c]	32[c]	3.3017	3.54	0.07	16	(6–40)
Lymph node plasma cell increase	42	54	66[c]	58	1.0270	1.25	0.31	35	2–53
Liver granulation	30	42	52[c]	54[c]	1.7055	2.25	0.15	15	0–38
Liver fatty changes	60	52	38[c]	70	1.0329	1.36	0.31	61	29–82
Prostatic atrophy	10	42[c]	48[c]	52[c]	3.7417	3.50	0.07	58	15–100
Adrenal hyperplasia[a]	36	28	52[c]	44	1.5754	2.06	0.18	3	0–19
Dilated gastric glands	4	14	20[c]	30[c]	2.6774	2.87	0.10	31	0–79
Stomach fibrosis	70	74	90[c]	82	1.4815	1.50	0.27	23	0–74
Kidney infarct	0	6	16[c]	10	2.1169	1.64	0.24	3	0–17
Kidney lymphocytic infiltration	84	58[c]	78	62[c]	−1.2196	−1.52	0.27	37	0–86
Brain hyaline bodies	84	80	68	62[c]	−2.1676	−2.63	0.12	69	31–90
Heart fibrosis	42	10[c]	12[c]	22	−1.1098	−1.16	0.36	37	4–79
Females									
Lymph node sinus histiocytosis	38	18[c]	20	18[c]	−1.6147	−1.70	0.23	11	0–36
Lymph node granulation	20	10	10	4[c]	−3.4264	−2.14	0.17	16	0–68
Adrenal angiectasis	40	32	50	54[c]	1.6960	2.21	0.16	37	0–79
Adrenal hyperplasia[a]	0	2	14[c]	8	3.5822	1.73	0.23	6	0–17
Pituitary angiectasis	76	60	72	46[c]	−2.1673	−2.80	0.11	51	16–78
Stomach fibrosis	12	40[c]	52[c]	16	−0.6052	−0.73	0.54	18	0–80
Chronic nephropathy	32	18	10[c]	8[c]	−3.6171	−2.76	0.11	8	0–30
Glomerulosclerosis	52	42	34	18[c]	−3.1184	−3.49	0.07	39	18–56

[a] *n* = 50 for each group.
[b] *n* = 330 males, *n* = 334 females.
[c] Medullary cell hyperplasia.
[d] Significantly different from control, $P \leq 0.05$, by pairwise comparison.

control groups and that had borderline dose-response trends (lymph node granulation and prostatic atrophy in males) were within the historical control range.

Similarly, the incidence of adrenal angiectasis and adrenal hyperplasia in historical female controls was highly variable, and the incidence of these lesions in neosugar-treated females was within the historical control range.

If neosugar treatment were responsible for the occurrence of a particular lesion, the severity of the lesion would be expected to increase with increasing dose. Most of the lesions listed in Table 8 were slight in severity in control as well as neosugar groups. Neosugar treatment had no effect on severity of any of the lesions where significant differences in incidence were observed by pairwise comparisons, which further supports the conclusion that the observed lesions were not related to neosugar treatment.

Tumor incidence. Tumors that occurred in greater than 5% incidence in any group are shown in Table 9. Pituitary adenomas, pheochromocytomas, thyroid C-cell adenomas, Langerhans' islet adenomas, and leukemias occurred commonly in control groups of both sexes. Interstitial cell tumors of the testis were extremely common in control male rats, with an incidence greater than 80%. In the female control group, mammary gland fibroadenomas and endometrial stromal polyps were common. All of these tumors are considered to be spontaneous in F-344 rats.[31,32]

Neosugar treatment did not increase the incidence of rare tumors in male or female rats. The incidence of spontaneous tumors in neosugar-treated animals was comparable to their incidence in concurrent controls, with the exception of pituitary adenomas. In male rats, the incidence of pituitary adenomas for the 0, 8000, 20,000, and 50,000 ppm dose groups was 20%, 26%, 38%, and 44%, respectively. The average background rate of pituitary adenomas in control male F-344 rats in this laboratory is 31%, with a range of 17–49%. Thus, the incidence of this tumor in male rats used in this experiment was within the historical range. However, pairwise comparisons between neosugar treatment groups and the concurrent control group showed the 20,000 and 50,000 ppm dose groups to have a significantly higher incidence of pituitary adenomas. To test for dose-related trends, two widely accepted trend tests were performed on these data. The Cochran-Armitage Chi-square test indicated a dose-response trend ($P = 0.007$), whereas logistic regression analysis showed no such trend ($P = 0.51$).

In female rats, the incidence of pituitary adenomas for the 0, 8000, 20,000, and 50,000 ppm dose groups was 48%, 39%, 38%, and 28%, respectively. The incidence of pituitary adenomas in females showed an apparent negative trend (opposite to that seen in males), but the difference between the high-dose group and control was not significant using a two-tailed Chi square test ($P = 0.06$). The background rate in control female F-344 rats ranges from 24% to 49%, with a mean incidence of 40%.

DISCUSSION

The results of the mutagenicity study demonstrate that, over a wide range of dose levels, neosugar does not cause gene mutations in bacteria or mammalian cells in culture and does not induce UDS in mammalian cells in culture either in the absence or in the presence of a functional, Aroclor-induced, rat liver microsomal metabolic activation system. Because neosugar was not appreciably toxic to the cells, it was necessary to impose an arbitrary maximum testing dose in these experiments rather than to test at doses up to and including toxic levels as is normal practice in mutagenicity testing.

In the chronic rat study, survival of both sexes was unaffected by neosugar treatment. The only significant finding was a decreased rate of survival in the male 20,000 ppm dose group. This is not believed to be treatment related, since it was an isolated occurrence and no dose-response relationship was evident. Body weight gain, food intake, hematology, and organ weights of both sexes were likewise unaffected by neosugar treatment.

TABLE 9. INCIDENCE OF NEOPLASMS IN MALE AND FEMALE RATS ADMINISTERED NEOSUGAR IN THE DIET FOR 2 YEARS

	Male (50 rats/group)				*Female (50 rats/group)*			
	0 (ppm)	*8000 (ppm)*	*20,000 (ppm)*	*50,000 (ppm)*	*0 (ppm)*	*8000 (ppm)*	*20,000 (ppm)*	*50,000 (ppm)*
Skin								
Keratoacanthoma	3 (6%)	2 (4%)	2 (4%)	3 (6%)	2 (4%)	0 (0%)	0 (0%)	2 (4%)
Subcutaneous tissue								
Fibroma	0 (0%)	3 (6%)	1 (2%)	2 (4%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Pituitary								
Adenoma	10 (20%)	13 (26%)	19 (38%)[a]	22 (44%)[b]	24 (48%)	19 (38%)	19 (38%)	14 (28%)
Adrenal								
Pheochromocytoma	6 (12%)	5 (10%)	7 (14%)	6 (12%)	1 (2%)	1 (2%)	4 (8%)	2 (4%)
Thyroid								
C-cell adenoma	3 (6%)	6 (12%)	5 (10%)	3 (6%)	3 (6%)	0 (0%)	3 (6%)	6 (12%)
Pancreatic islets								
Langerhans' islet adenoma	6 (12%)	5 (10%)	9 (18%)	6 (12%)	1 (2%)	0 (0%)	4 (8%)	0 (0%)
Testis								
Interstitial cell tumor	41 (82%)	41 (82%)	37 (74%)	40 (80%)	—	—	—	—
Mammary gland								
Fibroadenoma	0 (0%)	0 (0%)	1 (2%)	0 (0%)	7 (14%)	4 (8%)	3 (6%)	5 (10%)
Uterus								
Endometrial stromal polyp	—	—	—	—	8 (16%)	10 (20%)	11 (22%)	10 (20%)
Spleen								
Leukemia	2 (4%)	4 (8%)	6 (12%)	4 (8%)	4 (8%)	7 (14%)	12 (24%)	7 (14%)

[a]Significantly different from control, $P = 0.04$.
[b]Significantly different from control, $P = 0.01$.

Nonneoplastic lesions were extremely common in all rats. Sporadic increases and decreases in the incidence of some lesions were found by pairwise comparisons between control and neosugar treatment groups. If neosugar treatment were responsible for these significant findings, a dose-response relationship with regard to incidence and severity would be expected. Logistic regression analysis of the incidence data showed no significant positive or negative dose-related trends. In addition, the severity of the lesions with significant findings was not increased by neosugar treatment. Any decrease in severity would be undetectable, since almost all lesions were very mild. It should be noted that of the 17 lesions with significant findings, only 3 were common to both males and females, and for 2 of the 3 (lymph node granulation and stomach fibrosis), opposite trends were observed. No biological basis for a sex difference was apparent. A survey of the historical control incidence of these lesions showed that these lesions are common and highly variable in aging F-344 rats. Consideration of all of these factors leads to the conclusion that neosugar treatment did not affect the incidence of nonneoplastic lesions.

A slight but significant increase in blood Na and Cl was observed in male neosugar-treated rats, which is suggestive of mild renal impairment. However, other indicators of renal function, including K, Ca, phosphorus, BUN, uric acid, creatinine, and albumin, were not affected by neosugar treatment.

Neosugar did not increase the incidence of rare tumors in male or female rats, and pituitary adenoma was the only spontaneous tumor that was significantly increased in a neosugar treatment group. The increased incidence of pituitary adenomas occurred only in male rats. Pituitary adenoma is one of the most frequently occurring spontaneous tumors in male and female F-344 rats based on the historical incidence in this laboratory as well as National Cancer Institute and National Toxicology Program databases.[35,36] The background incidence also is highly variable, ranging from 20% to 50% in this laboratory. Although the incidence of this tumor in this experiment was well within the historical range for all male rats, the incidence in the two highest dose groups was significantly greater than the incidence in concurrent controls. However, the significance of a dose-related trend was equivocal in that one trend test showed a significant trend, whereas another test did not. If males are compared to females, a similar but opposite dose-response trend is noted. This dichotomy has no apparent biological basis. If male and female pituitary adenoma incidences are combined, no significant across-dose group differences are found. All of these observations point toward the conclusion that the higher incidence of pituitary adenomas in neosugar-treated male rats is a chance artifact. Such chance artifacts can arise when large numbers of statistical comparisons are made. In this study, 54 comparisons were made, and one to three significant results would be expected by chance alone at the significance levels of 0.01 and 0.05, respectively. It is unlikely, therefore, that neosugar was responsible for the occurrence of pituitary adenomas in male rats.

The results of this study indicate that neosugar is not mutagenic and does not produce chronic toxicity in rats. This lack of toxic activity is expected because, as described in the Introduction, neosugar is a mixture of simple oligosaccharides comprised of glucose and fructose. There is no reason to believe that such oligosaccharides or their metabolites would have genotoxic, carcinogenic, or chronic toxic potential.

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