ORIGINAL SUBMISSION

September 24, 2015

Dr. Paulette Gaynor
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Dear Dr. Gaynor:

Re: GRAS Exemption Claim N-Acetyl-D-neuraminic acid (NANA)

In accordance with proposed 21 CFR §170.36 [Notice of a claim for exemption based on a Generally Recognized as Safe (GRAS) determination] published in the *Federal Register* [62 FR 18938 (17 April 1997)], I am submitting one hard copy and one electronic copy (on CD), as the notifier [Glycom A/S, Diplomvej 373, DK-2800 Kgs. Lyngby, Denmark], a Notice of the determination, on the basis of scientific procedures, that N-Acetyl-D-neuraminic acid (NANA), produced by Glycom A/S, as defined in the enclosed documents, is GRAS under specific conditions of use in term infant formula and in food, and therefore, is exempt from the premarket approval requirements of the *Federal, Food, Drug and Cosmetic Act*. Information setting forth the basis for the GRAS determination, which includes detailed information on the notified substance and a summary of the basis for the GRAS determination, as well as a consensus opinion of an independent panel of experts in support of the safety of NANA under the intended conditions of use, also are enclosed for review by the agency.

The enclosed electronic files for the Notice entitled, "GRAS Exemption Claim N-Acetyl-D-neuraminic acid (NANA)" were scanned for viruses prior to submission and is thus certified as being virus-free using McAfee VirusScan 8.8.

Should you have any questions or concerns regarding this GRAS Notice, please do not hesitate to contact me at any point during the review process so that we may provide a response in a timely manner.

Sincerely,

(b) (6)

24 Sept 2015

Christoph H. Röhrig, Ph.D.
Senior Scientist and Regulatory Affairs Manager
Glycom A/S

September 24, 2015

Dr. Paulette Gaynor
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
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5100 Paint Branch Parkway
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(b) (6) 24 Sept 2015

Christoph H. Röhrig, Ph.D.
Senior Scientist and Regulatory Affairs Manager
Glycom A/S

GRAS Exemption Claim for
N-Acetyl-D-neuraminic acid (NANA)

Submitted to:

Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition (CFSAN)
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD
USA 20740-3835

Submitted by:

Glycom A/S
Diplomvej 373
DK-2800 Kgs. Lyngby
Denmark

September 25, 2015

GRAS Exemption Claim for N-Acetyl-D-neuraminic acid (NANA)

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I. GRAS EXEMPTION CLAIM

I.A Claim of Exemption From the Requirement for Premarket Approval Pursuant to Proposed 21 CFR §170.36(c)(1) [62 FR 18938 (17 April 1997)]

Glycom A/S hereby claims that the use of *N*-acetyl-D-neuraminic acid (NANA) in term infant formula and in foods, as described in Section I.D below, is exempt from the requirement of premarket approval of the *Federal Food, Drug, and Cosmetic Act* because we have determined that such uses are Generally Recognized as Safe (GRAS).

Signed,

(b) (6)

25 09 2015

Christoph H. Röhrig, Ph.D.
Senior Scientist and Regulatory Affairs Manager
Glycom A/S
christoph.roehrig@glycom.com

Date

I.B Name and Address of Notifier

Glycom A/S
Diplomvej 373
DK-2800 Kgs. Lyngby
Denmark
Tel: +45 4525 2247
Fax: +45 3841 1720

I.C Common Name(s) of the Notified Substance

N-Acetyl-D-neuraminic acid; sialic acid

I.D Conditions of Intended Use

I.D.1 Conditions of Intended Use in Infant Formula

N-acetyl-D-neuraminic acid (NANA) is intended for use in term infant formulas at a maximum use level of 50 mg/L (reconstituted formula). This maximum use level of NANA in term infant formulas is based on providing a similar level of NANA as that which occurs in milk from lactating women (see Section IV.B.1).

I.D.2 Conditions of Intended Use in Food

NANA also is intended for use in conventional food and beverage products across multiple categories as described in Table I.D.2-1.

Table I.D.2-1 Summary of the Individual Proposed Food Uses and Use Levels for NANA in the U.S.

Food Category	Proposed Food Uses	RACC[a]	Proposed Use Level (g/RACC)	Proposed Maximum Use Level (g/kg or g/L)[b]
Baked Goods and Baking Mixes	Breads and Baked Goods, Gluten-free	50 g	50 to 100	1,000 to 2,000
	Cereal Bars	40g	20	500
Beverages and Beverage Bases	Carbonated Beverages	240 mL	12	50
	Flavored and Enhanced Waters	240 mL	12	50
	Meal Replacement Drinks, for Weight Reduction	240 mL	50	200
	Sports, Isotonic, and Energy Drinks	240 mL	12	50
Coffee and Tea	Coffee	240 mL	50 to 100	200 to 400
	Tea	240 mL	50 to 100	200 to 400
Dairy Product Analogs	Beverage Whiteners	2 g	50	25,000 (25 g/kg)
	Imitation Milks	240 mL	12	50
	Non-Dairy Yogurt	225 g	50 to 100	200 to 400
Infant and Toddler Foods	Toddler Formulas	100 mL[c]	5	50
	Other Baby Foods for Infants and Young Children	7 to 170 g	5	50
	Other Drinks for Infants and Young Children	120 mL	0.4 to 9	50
Grain Products and Pastas	Meal Replacement Bars, for Weight Reduction	30g	1.5	50
Milk, Whole and Skim	Unflavored Pasteurized and Sterilized Milk[d]	240 mL	50	1,700
Milk Products	Buttermilk	240 mL	12	50
	Flavored Milk	240 mL	50 to 100	200 to 400
	Yogurt	225 g	12	50
Processed Fruits and Fruit Juices	Fruit Flavored Drinks and Ades	240 mL	12	50
	Fruit Juices and Nectars	240 mL	12	50
Processed Vegetables and Vegetable Juices	Vegetable Juices and Nectars	240 mL	12	50
Sugar Substitutes	Table Top Sweeteners	4 g	12	50

NANA = *N*-acetyl-d-neuraminic acid; RACC = Reference Amounts Customarily Consumed

[a] Serving sizes were based on Reference Amounts Customarily Consumed (RACC) per Eating Occasion in the U.S. CFR (21 CFR §101.12 - U.S. FDA, 2015a).

[b] The proposed maximum use level is presented on a g/kg basis for solids and on a g/L basis for liquids.

[c] RACC not available, 100 mL employed as an approximation.

[d] When the milk is fortified with NANA, it will then be classified as a milk product. The intake of the category 'Unflavored pasteurized and sterilized milks' was used here as a conservative proxy for the dietary pattern of the fortified milk drink product.

I.E Basis for the GRAS Determination

Pursuant to 21 CFR § 170.30 of the *Code of Federal Regulations* (CFR) (U.S. FDA, 2015b), *N*-acetyl-D-neuraminic acid has been determined by Glycom A/S to be GRAS through scientific procedures.

I.F Availability of Information

The data and information that serve as the basis for this GRAS Notification will be sent to the United States (U.S.) Food and Drug Administration (FDA) upon request, or will be available for review and copying at reasonable times at the offices of:

Glycom A/S
Diplomvej 373
DK-2800 Kgs. Lyngby
Denmark

Should the FDA have any questions or additional information requests regarding this notification, Glycom will supply these data and information.

II. DETAILED INFORMATION ON THE IDENTITY AND MANUFACTURING OF *N*-ACETYL-D-NEURAMINIC ACID (NANA)

II.A Identity

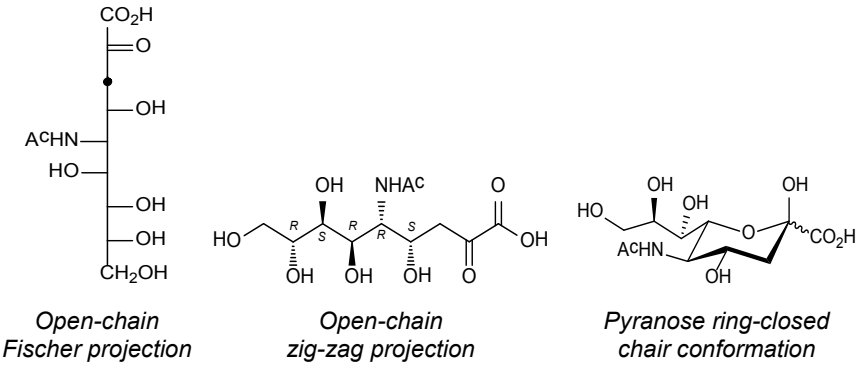
II.A.1 Chemical Identity

Common Name:	Sialic acid (dihydrate)
Common Abbreviation:	NANA; Neu5Ac, SA
IUPAC Name:	*N*-Acetyl-D-neuraminic acid (dihydrate) 5-Acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid (dihydrate)
Chemical Abstracts Service (CAS) Registry Number:	NANA: 131-48-6 NANA dihydrate: 50795-27-2

Chemical Formula: NANA: $C_{11}H_{19}NO_9$
NANA dihydrate: $C_{11}H_{23}NO_{10}$ ($C_{11}H_{19}NO_9$ *2 H_2O)

Molecular Weight: NANA: 309.3
NANA dihydrate: 345.3 (309.3 *36.0)

Structural Formula:



Open-chain Fischer projection | *Open-chain zig-zag projection* | *Pyranose ring-closed chair conformation*

The isolated crystalline product is predominantly in the crystal form that binds 2 molecules of water per molecule of NANA ("dihydrate"). Some crystal water may be lost during the drying process (accounted for in the specifications). The active ingredient is NANA.

II.A.2 Chemical and Physical Characteristics

Sialic acids comprise a family of over 60 nine-carbon acidic monosaccharides consisting of *N*- or *O*-substituted derivatives of D-neuraminic acid (reviewed by Angata and Varki, 2002; and Schauer, 2004), the most prominent of which is *N*-acetyl-D-neuraminic acid (NANA) (Figure II.A.2-1).

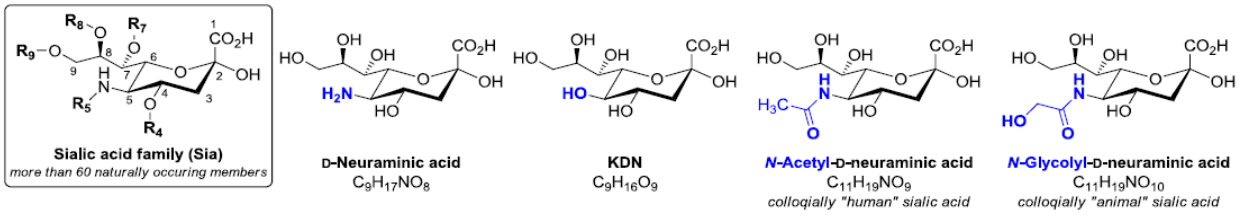


Figure II.A.2-1 *N*-Acetyl-D-neuraminic acid and related members of the sialic acid family (Adapted from Röhrig *et al*., 2015).

Sialic acids are natural monosaccharides widely found throughout higher animals (and some microorganisms), with the highest concentrations detected in mammalian milk and brain. However, the occurrence of sialic acid in the pure *N*-acetylated form is a characteristic feature of human milk; in most other mammalian milks, sialic acid occurs as a mixture with the *N*-glycolyl form, *N*-glycolylneuraminic acid (NGNA)].

NANA occurs in both the bound form as well as in the free form (*i.e.*, the free monosaccharide). The bound form of NANA consists of NANA linked to oligosaccharides *via* a glycosidic bond. These oligosaccharides in turn consist of either free oligosaccharides [*e.g.*, human milk oligosaccharides (HMOs)] or oligosaccharide chains of glycoconjugates (*e.g.*, glycoproteins and glycolipids) [*e.g.*, see Figure II.A.2-2]. Whilst human milk is known to contain high concentrations of bound NANA, where the monosaccharide is linked to HMOs, glycoproteins, and glycolipids known as gangliosides, it is important at this stage to emphasize that NANA also occurs in the free form in human milk, as well as in a number of other foods of animal origin, and it is this free form that is the subject of this dossier as Glycom's NANA ingredient is manufactured as a free monosaccharide. The literature documenting the presence of free NANA in human milk and in foods of animal origin is discussed in detail in Sections IV.B.1 and IV.B.2.

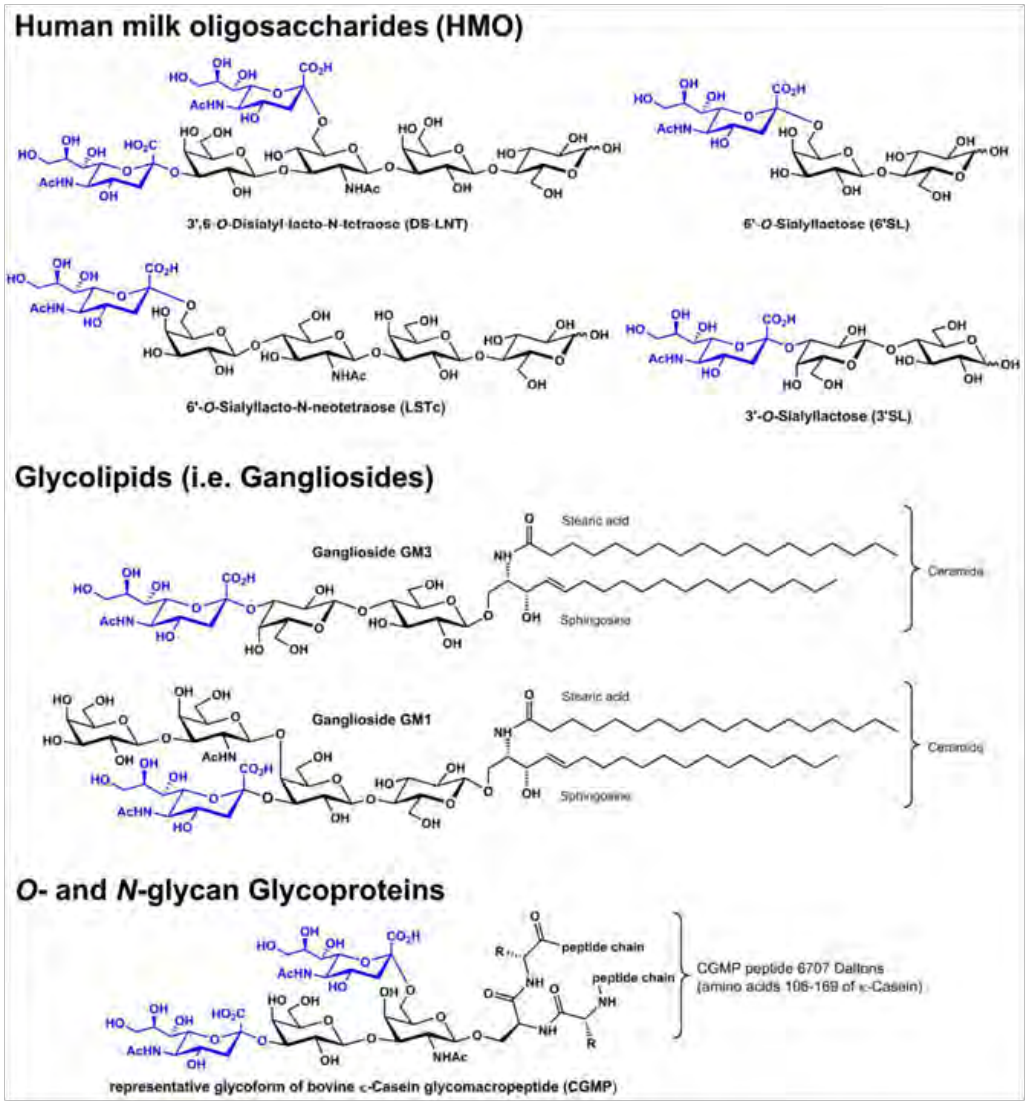


Figure IV.A.2-2 Prominent examples of biomolecular structures containing bound sialic acid (Adapted from Röhrig *et al*., 2015).

II.B Method of Manufacture

II.B.1 Raw Materials and Processing Aids

Crystalline NANA dihydrate is derived from anhydrous NANA. This starting material is produced from the coupling of *N*-acetylmannosamine and sodium pyruvate in an enzyme-catalyzed reaction in the first stage of the manufacturing process as described in Section II.B.2. *N*-Acetylmannosamine is produced by Glycom from D-fructose in a two-step process and the material is food grade quality (>98% pure). D-fructose and sodium pyruvate used during the production of *N*-acetylmannosamine meet the specifications established in the European Pharmacopeia, and therefore meet or exceed food grade quality standards.

Processing-aids used during the manufacture of NANA dihydrate from anhydrous NANA are safe and suitable food-grade materials and where applicable are used in accordance with appropriate federal regulations and/or determined to be GRAS for their respective food uses. The materials used in the manufacture of NANA dihydrate from anhydrous NANA are listed in Table II.B.1-1.

Table II.B.1-1 Starting Materials and Processing Aids Used in the Manufacture of NANA Dihydrate

Material	Function
Starting Materials	
N-acetylmannosamine	Starting material
Sodium pyruvate	Starting material
Anhydrous NANA	Starting material
NANA dihydrate	Seeding crystals
Processing Aids	
2-Propanol	To initiate crystallization and to wash NANA dihydrate crystals
Filters and Filter Aids	
Activated charcoal	To remove coloration and impurities
Sparkler filter	Filtration
Microfilter (0.2 µm)	Bacterial microfiltration

NANA = *N*-acetyl-D-neuraminic acid dihydrate

II.B.1.1 Enzyme

NANA is synthesized using an aldolase enzyme preparation [*N*-acetylneuraminate lyase; CAS: 9027-60-5; E.C.: 4.1.3.3. aldolase enzyme] obtained from a modified strain of a *Escherichia coli* K12-derivative. The enzyme catalyzes the coupling of N-acetylmannosamine and sodium pyruvate to produce NANA. The enzyme preparation is produced in-line with current Good Manufacturing processes using master and working cell banks and safe and suitable food production procedures. The enzyme preparation meets the general purity

specifications for enzyme preparations as described in the monograph “Enzyme Preparations” of the 6th (FCC, 2008) or current edition of the Food Chemicals Codex (FCC, 2014).

The enzyme preparation is used to produce the crude NANA (Stage 1 manufacturing) preparation, which is then subjected to extensive purification using multiple filtration and crystallization steps, which serve to effectively remove any residual protein or other carry-over products from the enzyme preparation. *N*-acetyl-D-neuraminic acid dihydrate is a high purity crystalline ingredient (>97%), and has been determined to be free of quantifiable protein as determined base on the results an enzyme specific ELISA (LOQ = 0.78 ppm) and Bradford protein analysis (LOQ = 0.01%).

II.B.2 Manufacturing Process

Anhydrous NANA is manufactured in compliance with current Good Manufacturing Practice (cGMP) and the principles of Hazard Analysis and Critical Control Points (HACCP). All batches are controlled for conformity to established strict specifications by certificate of analysis. The overall manufacturing process for NANA is briefly outlined in Figure II.B.2-1. The manufacturing process can be broadly divided into 2 stages. In Stage 1, *N*-acetylmannosamine and sodium pyruvate are coupled using an isolated and purified enzyme to produce the starting material anhydrous NANA. In Stage 2, anhydrous NANA is subjected to processing, purification, and a re-crystallization reaction that generates the final crystalline NANA dihydrate product. NANA is manufactured in a pathway where key intermediates are isolated in purified form and analytically characterized. Specifications are set according to HACCP principles, and conformity is monitored by certificates of analysis at each key-stage of the production process.

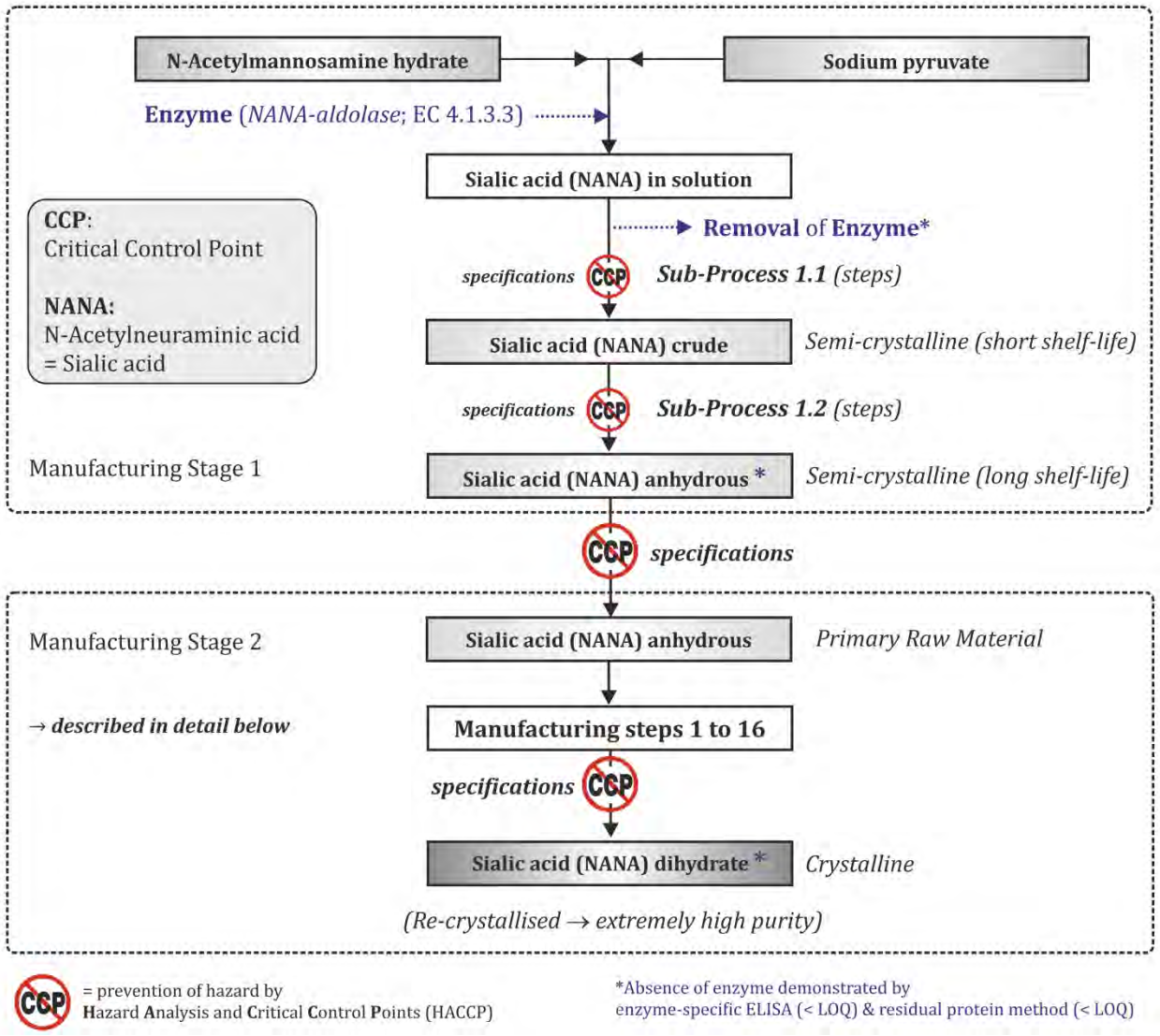


Figure II.B.2-1 Schematic Overview of the Manufacturing Process for NANA

Manufacturing Stage 1: Precursor Formation

A schematic overview of Stage 1 of the manufacturing process is depicted in Figure II.B.2-2.

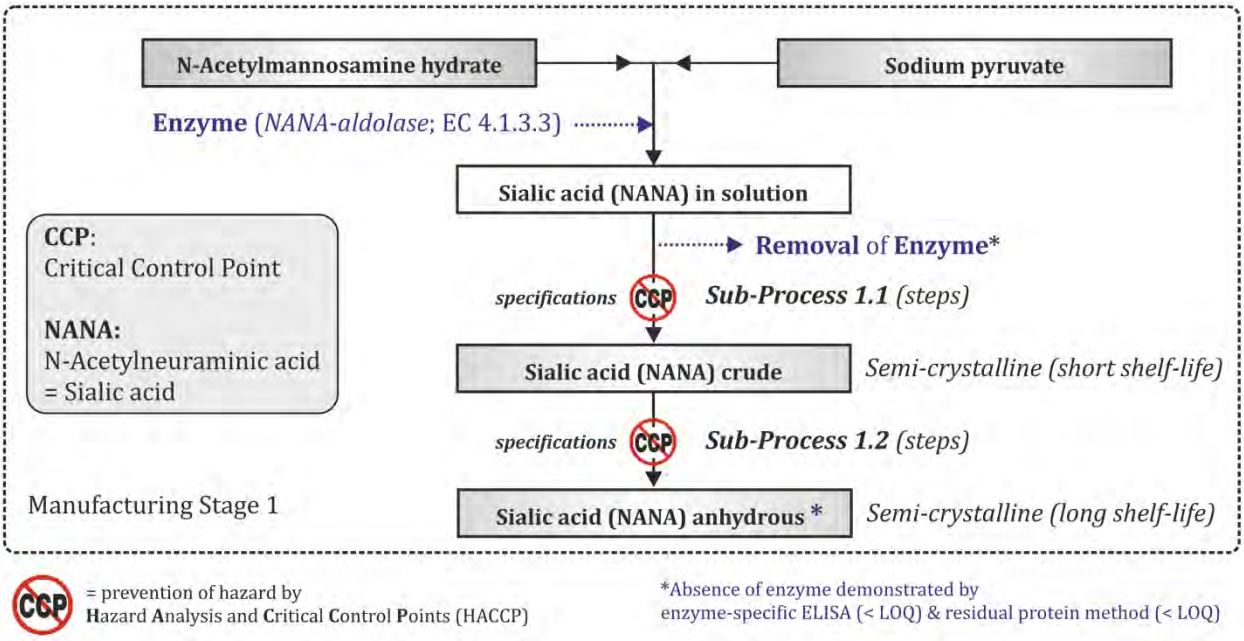


Figure II.B.2-2 Schematic Overview of Manufacturing Stage 1: NANA Precursor Formation

Stage 1 of the manufacturing process consists of coupling *N*-acetylmannosamine and sodium pyruvate, through an aldolase enzyme-catalyzed aldol condensation reaction to produce NANA in solution. The product of the enzymatic reaction is then subjected to a series of processing steps, including removal of the enzyme, to produce crude NANA, which in turn is furnished into the starting material, anhydrous NANA, following a second series of processing steps. All precursors and intermediates produced in Stage 1 are single, well-characterized, and pure compounds. A more detailed description of Stage 1 is available upon request.

The Stage 1 process for the final intermediate (starting material), anhydrous NANA, offers full control on all process parameters under HACCP, where starting material and intermediate specifications are set, certificates of analysis are issued, and batch release procedures are followed. It provides a single, well-characterized final intermediate, and thus it is possible at this point of the process to set strict specifications under cGMP and HACCP. All batches of the final intermediate are controlled for conformity to the established strict specifications by certificate of analysis, ensuring that only batches of high purity starting material enter Manufacturing Stage 2. Due to the crystalline nature of the final intermediate, re-crystallization may be applied as a corrective action until specifications are met, following which the material is released to enter Stage 2 of the manufacturing process. Thus, the certificate of analysis for the final intermediate

serves as the key critical control point for Stage 2 of the manufacturing process and corrective actions are in place. It is important to note that the specifications for impurities of the final intermediate are set at this stage in the process to the levels also required for the final NANA dihydrate product, and the subsequent process is designed and optimized to ensure that these levels are kept throughout all downstream steps.

Manufacturing Stage 2: Crystallization to Produce NANA

A general overview of Stage 2 of the manufacturing process is depicted in Figure II.B.2-3, while further details are provided in Figure II.B.2-4.

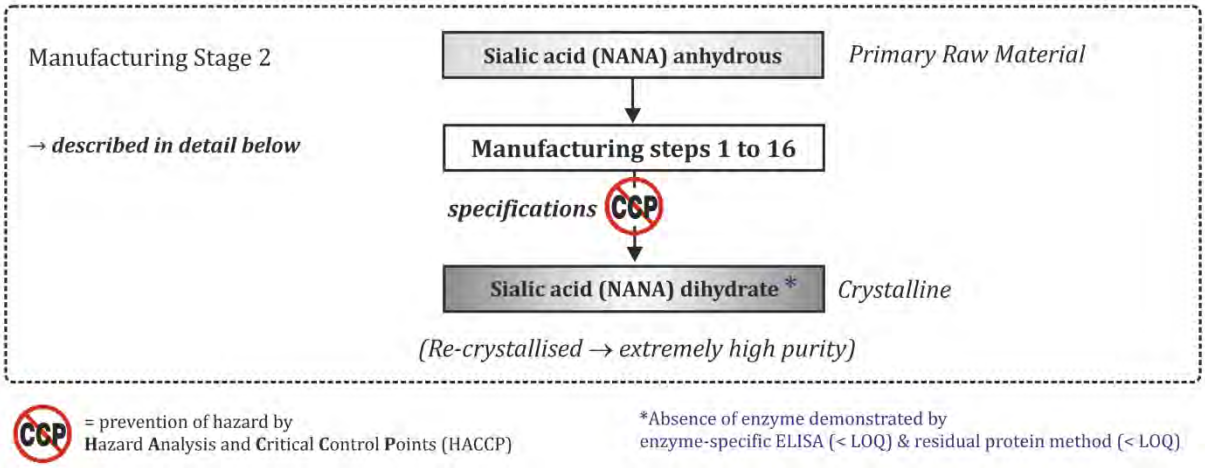


Figure II.B.2-3 General Overview of Manufacturing Stage 2: Crystallization to Produce NANA

STEP	PROCESSING AIDS / STARTING MATERIAL		OPERATION	COMMENTS	IPC / ANALYTICS
1	Sterile water	→	**Filling stainless steel reactor**	*Water specifications*	n/a
2	n/a		**Warming to set temperature**	n/a	n/a
3	NANA anhydrous (starting material)	→	**Dissolving NANA anhydrous**	*Starting material specifications*	n/a
4	Activated charcoal	→	**Addition of activated charcoal**	*Activated charcoal specifications*	n/a
5	n/a		**Stirring for set time and temperature**	n/a	n/a
6	Sparkler filter		**Filtration from charcoal**	*Sparkler filter specifications*	n/a
7	Microfilter	→	**Bacterial microfiltration into crystallization reactor**	*Filter specifications (0.2 µm)*	n/a
8	2-Propanol	→	**Addition of 2-Propanol**	*2-Propanol specifications*	n/a
9	n/a		**Start of cooling process**	n/a	n/a
10	NANA seeding crystals	→	**Seeding crystals are added at set temperature**	*Starting material specifications*	n/a
11	n/a		**Continued stirring at set temperature**	n/a	Endpoint of crystallization
12			**Filtration by centrifuge**	n/a	n/a
13	2-Propanol	→	**2-Propanol wash of crystals**	*2-Propanol specifications*	n/a
14	n/a		**Drying**	n/a	**Karl-Fischer** (Water content) **GC** (Residual solvents)
15	n/a		**Crystalline NANA dihydrate**	*Product specifications (including microbiological and trace elements)*	**Certificate of Analysis**
16	n/a		**Packaging**	*Packing material specifications*	n/a
17	n/a		**Release of batches**	n/a	n/a

GC = gas chromatography; HPLC = high-performance liquid chromatography; IPC = in-process control; n/a = not applicable; NANA = *N*-acetyl-D-neuraminic acid

Figure II.B.2-4 Details of Manufacturing Stage 2: Crystallization to Produce NANA

Stage 2 of the manufacturing process consists of the following detailed steps:

(1) Filling the stainless steel reactor with sterile water;
(2) Warming the reactor to a set temperature;
(3) Dissolving anhydrous NANA under gentle stirring until a homogeneous solution is obtained;
(4) Treatment with activated charcoal to remove any coloration and impurities;
(5) Stirring the solution for a set time and temperature;
(6) Filtering the solution from the activated charcoal with the use of a sparkler filter;
(7) Bacterial microfiltration of the solution into the closed-system stainless steel crystallization vessel;
(8) Addition of 2-propanol to the solution to initiate crystallization;
(9) Cooling the solution;
(10) Addition of NANA seeding crystals to the solution at a set temperature;
(11) Stirring is continued and the process is monitored by in-process control for crystallization;
(12) Filtration by centrifugation;
(13) Washing the crystals with 2-propanol (to remove water allowing for fast drying process);
(14) Drying the crystals during which the process is monitored by in-process control for water content and residual solvents;
(15) Collection of samples for full analysis to allow confirmation of conformity with specifications;
(16) Packaging, according to set requirements, which completes the manufacturing procedure; and
(17) Issuing of certificates of analysis from collected samples for the release of manufactured batches.

As discussed, the synthetically produced NANA is identical to NANA present in human milk. Through the manufacturing process, there has been no modification to the molecular structure of synthetic NANA from that of the NANA that is present in human milk.

II.B.3 Quality Control

II.B.3.1 Quality Control of the Production Process

Due to the precursors, intermediates, and the final product being single, well-characterized, and pure compounds, the whole production process can be chemically followed in detail by a range of analytical techniques. These techniques are applied either as in-process controls or at batch release (by certificate of analysis) to allow full control of the production process. A HACCP plan is in place.

Manufacturing Stage 1 is controlled by a HACCP plan which includes specifications for starting materials, precursors, and intermediates. Master operating instructions are followed,

in-process controls are applied, and isolated intermediates are controlled by certificates of analysis and batch release routines.

Manufacturing Stage 2 is likewise controlled by a HACCP plan, including specifications for starting materials/processing aids, equipment, and packaging materials. It comprises a number of in-process controls to minimize the amount of potential impurities and contaminants to the level technically possible as described below.

II.B.3.2 Quality Control of Potential Inherent Impurities and External Contaminants

As mentioned, the HACCP plan for Manufacturing Stage 2 comprises a number of in-process controls to minimize the amount of potential impurities and contaminants to the level technically possible. In-process controls occur in the following steps of the process:

(11) In-process control for the endpoint of crystallization;

(14) In-process control for water content and residual solvents to determine end of drying period; and

(15) A final product certificate of analysis is issued for release of batches.

To note, a key critical control point of the process in terms of potential impurities and contaminants is the crystallization of the starting material, anhydrous NANA, before Manufacturing Stage 2 and its batch release procedures. The crystallization step is one that is of the standard for pharmaceuticals and is applied in order to obtain a high purity product or intermediate. Upon change of temperature, anhydrous NANA, separates from the liquid solution by crystallization (a suspension is formed), and essentially any of the potential impurities or contaminants from the previous processing steps remain in solution. The liquid solution[1] is filtered off from the suspended crystalline anhydrous NANA, which does not pass through the filter. The crystalline anhydrous NANA product is then washed with 2-propanol. The established specifications and batch release procedure for anhydrous NANA ensure that only batches of high purity starting material enter Manufacturing Stage 2. Impurities which may potentially remain in the product at the end of Manufacturing Stage 2 are covered by the final product specifications and in more detail by the additional internal product specifications.

[1] The solution that is removed by filtration from the crystallized product is called the ”mother liquor”. It contains potential impurities and traces of product.

II.C Specifications and Batch Analyses

II.C.1 Specifications

Appropriate food-grade specifications have been established for NANA dihydrate (Table II.C.1-1 below). All methods of analysis are nationally or internationally recognized or have been validated by Glycom or a third party laboratory.

Table II.C.1-1 Product Specification for NANA Dihydrate

Parameter	Specification	Method
Appearance	Crystalline powder	Visual
Color	White to off white	Visual
Identification	RT (main component) corresponds RT (standard) ± 3%	Glycom method HPLC-003-002
Assay by HPLC (additional water and solvent free dihydrate)	Min. 97.0 %	Glycom method HPLC-003-002
pH (20°C, 5% suspension)	1.7 to 2.5	WBSE-77:2012
Water (dihydrate calculates to 10.4%)	Max. 12.5 %	Karl-Fischer (EP 2.5.32)
Ash, sulfated	Max. 0.2 %	EP 6.7 04/2010:20414
Acetic acid (as free acid and/or sodium acetate)	Max. 0.5 %	MSZ EN ISO 10304-1:2009
Residual solvents	Max. 0.1 % singly Max. 0.3 % in combination	EP GC 2.4.24
Residual proteins[a]	Max. 0.01 %[c]	Glycom method UV-001 (Bradford)
Lead	Max. 0.1 mg/kg	EPA 6010C:2007
Microbiological specifications		
Salmonella	Absent in 25 g	MSZ-EN-ISO 6579:2006
Aerobic mesophilic total count	Max. 500 CFU/g	MSZ-EN-ISO 4833:2003
Enterobacteriaceae	Absent in 10 g	MSZ-ISO 21528-2:2007
Cronobacter (Enterobacter) sakazakii	Absent in 10 g	ISO-TS 22964:2006
Listeria monocytogenes	Absent in 25 g	MSZ-EN-ISO 11290-1:1996, 1998/A1:2005
Bacillus cereus	Max. 50 CFU/g	MSZ-EN-ISO 7932:2005
Yeasts	Max. 10 CFU/g	MSZ-ISO 7954:1999
Molds	Max. 10 CFU/g	MSZ-ISO 7954:1999
Residual endotoxins[a]	Max. 10 EU/mg	LAL Kinetic chromogenic assay (EP 2.6.14)

CFU = colony forming units; EP = European Pharmacopeia; EU = endotoxin units; GC = gas chromatography; HPLC = high performance liquid chromatography; ISO = international organization for standardization; LAL = *Limulus* amebocyte lysate; NANA = *N*-acetyl-D-neuraminic acid ; RT = retention time

[a] Specifications for residual proteins and endotoxins are included as a quality control measure to demonstrate that potential impurities originating from the enzyme preparation are effectively excluded during the purification procedures.

II.C.2 Product Analysis

Batch analyses for 5 lots of NANA demonstrating compliance with food grade specifications and consistency of the manufacturing process are presented in Table II.C.2-1 below.

Table II.C.2-1 Batch Analyses for NANA Dihydrate

Parameter	Specification	Batch Results				
		L12058K	**L3A075K**	**L3A076K**	**L3B014K**	**L3B015K**
Appearance	Crystalline powder	Crystalline powder	Crystalline powder	Crystalline powder	Crystalline powder	Crystalline powder
Color	White to off-white	White	White	White	White	White
Identification	RT (main component) corresponds RT (standard) ± 3%	Complies	Complies	Complies	Complies	Complies
Assay by HPLC (additional water and solvent free dihydrate) (%)	Min. 97.0	98.6	98.9	99.0	98.5	99.2
pH (20°C, 5% solution)	1.7 to 2.5	2.1	1.84	1.83	1.83	1.84
Water (%)	Max. 12.5	10.6	10.9	10.6	10.6	10.5
Ash, sulfated (%) (as free acid and/or sodium acetate)	Max. 0.2	0.03	0.11	0.01	0.04	0.01
Acetic acid (as free acid and/or sodium acetate) (%)	Max. 0.5	< 0.01	< 0.2	< 0.2	< 0.2	< 0.2
Residual solvents[a] Singly (%) Combination (%)	 Max. 0.1 Max. 0.3	 Complies Complies	 Complies Complies	 Complies Complies	 Complies Complies	 Complies Complies
Residual proteins[b] (%)	Max. 0.01[c]	< 0.01 (nd)[c]	< 0.01 (nd)[c]	< 0.01 (nd)[c]	< 0.01 (nd)[c]	< 0.01 (nd)[c]
Lead (mg/kg)	Max. 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Microbiological Specifications						
Salmonella	Absent in 25 g	Complies	Complies	Complies	Complies	Complies
Aerobic mesophilic total count (CFU/g)	Max. 500	< 10	< 10	< 10	< 10	< 10
Enterobacteriaceae	Absent in 10 g	Complies	Complies	Complies	Complies	Complies
Cronobacter (*Enterobacter*) *sakazakii*	Absent in 10 g	Complies	Complies	Complies	Complies	Complies
Listeria monocytogenes	Absent in 25 g	n/a	Complies	Complies	Complies	Complies
Bacillus cereus (CFU/g)	Max. 50	n/a	< 10	< 10	< 10	< 10
Yeasts (CFU/g)	Max. 10	n/a	< 10	< 10	< 10	< 10

Table II.C.2-1 Batch Analyses for NANA Dihydrate						
Parameter	**Specification**	**Batch Results**				
		L12058K	**L3A075K**	**L3A076K**	**L3B014K**	**L3B015K**
Molds (CFU/g)	Max. 10	n/a	< 10	< 10	< 10	< 10
Residual endotoxins[b] (EU/mg)	Max. 10	< LOR[d]	< LOR[d]	< LOR[d]	< LOR[d]	< LOR[d]

CFU = colony forming units; EU = endotoxin units; LOR = Level of Reporting; n/a = not applicable; nd = not detected; NANA = *N*-acetyl-D-neuraminic acid ; RT = retention time

[a] Class 3 solvent with low toxic potential - ICH Harmonized tripartite guideline impurities: Guideline for residual solvents Q3C(R5) (ICH, 2011).

[b] Specifications for residual proteins and endotoxins are included as a quality control measure to demonstrate that potential impurities originating from the enzyme preparation are effectively excluded during the purification procedures.

[c] Limit of quantitation (LOQ) = 0.01 %.

[d] LOR = 0.01 EU/mg

II.C.3 Other Qualitative Analyses

NANA manufactured by Glycom, is qualitatively identical to free NANA present in human breast milk. The first correct molecular structure of NANA was reported by (Kuhn and Baschang, 1962; Kuhn and Brossmer, 1962). To note, a high number of inaccurate structural assignments for NANA have been reported in the literature prior to these publications as summarized by Williams *et al*. (1957). The correct structure proposed by Kuhn has been confirmed by x-ray crystallography (Flippen, 1973). In addition, since then, a number of publications have further reported on the structure characterization of NANA by ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) techniques (Brown *et al.*, 1975; Dabrowski *et al.*, 1979; Friebolin *et al.*, 1980, 1981; Ogura *et al.*, 1984; Klepach *et al.*, 2008a,b). Based on x-ray crystallography, ^{1}H- and ^{13}C-NMR, and mass spectrometry (MS) data, Glycom's has demonstrated that NANA is chemically and structurally fully identical to the naturally occurring free NANA that is present in human milk.

II.D Stability

II.D.1 Bulk Stability

Long-Term Real-Time Stability Study

A 5-year long-term stability study on crystalline NANA from a single batch (Batch L12058K) is ongoing. Three-year interim stability data is currently available (Table II.D.1-1, II.D.1-2). In this study, samples of the batch were packed into polyethylene bags as the primary packaging material, which was then packed into polyethylene/aluminum/polyester triple layer foil bags as the secondary packaging material; 27 3-g samples were packed for chemical analysis and 18 60-g samples were packed for microbiological testing. Packages were sealed and stored

under 1 of the following 3 storage conditions: 25°C/60% relative humidity, 5°C, or -20°C. Chemical analysis and microbiological testing were performed at regular intervals on one sample per time point. NANA was analyzed by high-performance liquid chromatography (HPLC) and water content was analyzed by Karl Fischer titration. Degradation products were measured by high-performance anion exchange chromatography (HPAEC) at the 36-month time point only. No significant change in assay value was observed under any of the storage conditions for up to 36 months of storage. Similarly, no significant change in the water content was observed under any of the storage conditions throughout the 36 months of storage, indicating that the packaging is appropriate in protecting NANA from water absorption. Microbiological purity also was maintained for up to 36 months under all storage conditions. Values for all measured parameters remained within the established specifications for NANA under all storage conditions. Analysis for degradation products revealed 3 unidentified carbohydrate-type degradation products that were detected at 36 months of storage. The levels of these degradation products increased with increasing storage temperature; however, values remained within the established internal specifications for NANA (maximum of 0.4% for each individual degradation product and maximum of 1.0% for the total amount of degradation products). The results of this study indicate that NANA is stable with no significant degradation when stored at a temperature of 25°C at 60% relative humidity, 5°C, or -20°C for periods of up to 36 months. The current shelf-life of crystalline NANA stored at 25°C at 60% relative humidity, 5°C, -20°C has thus been established as 36 months until longer term data is available.

Table II.D.1-1 Real-Time Stability Study on NANA Stored at 25°C and 60% Relative Humidity (Batch L12058K)								
Parameter Tested	**Analytical Data[a]**							
	Initial	**3 Months**	**6 Months**	**9 Months**	**12 Months**	**18 Months**	**24 Months**	**36 Months**
Chemical Analysis								
Color	White	Not documented	Not documented	White	White	White	White	White
Description	Crystalline powder	Not documented	Not documented	Crystalline powder	Crystalline powder	Crystalline powder	Crystalline powder	Crystalline powder
Assay by HPLC (additional water and solvent free dihydrate) (%)	98.6	99.2	98.7	99.2	98.7	98.3	99.8	99.2
Water (w/w %)	10.6	10.4	10.6	11.0	10.7	10.6	10.6	10.3
Microbiological Analysis								
Salmonella (CFU/25 g)	Negative	n/a	Negative	n/a	Negative	n/a	Negative	Negative
Aerobic mesophilic total count (CFU/g)	< 10	n/a	< 10	n/a	< 10	n/a	< 10	< 10
Enterobacteriaceae (CFU/10 g)	Negative	n/a	Negative	n/a	Negative	n/a	Negative	Negative
Cronobacter (*Enterobacter*) *sakazakii* (CFU/10 g)	Negative	n/a	Negative	n/a	Negative	n/a	Negative	Negative

CFU = colony forming units; n/a = not applicable; NANA = *N*-acetyl-D-neuraminic acid
[a] Chemical analysis was conducted on samples separate from those subjected to microbiological testing.

Table II.D.1-2 Real-Time Stability Study on NANA Stored at 5°C (Batch L12058K)								
Parameter Tested	**Analytical Data[a]**							
	Initial	**3 Months**	**6 Months**	**9 Months**	**12 Months**	**18 Months**	**24 Months**	**36 Months**
Chemical Analysis								
Color	White	Not documented	Not documented	White	White	White	White	White
Description	Crystalline powder	Not documented	Not documented	Crystalline powder	Crystalline powder	Crystalline powder	Crystalline powder	Crystalline powder
Assay by HPLC (additional water and solvent free dihydrate) (%)	98.6	99.4	99.9	99.0	98.6	98.7	99.5	99.0
Water (w/w %)	10.6	10.4	10.5	11.0	10.6	10.6	10.7	10.3
Microbiological Analysis								
Salmonella (CFU/25 g)	Negative	n/a	Negative	n/a	Negative	n/a	Negative	Negative
Aerobic mesophilic total count (CFU/g)	< 10	n/a	< 10	n/a	< 10	n/a	< 10	< 10
Enterobacteriaceae (CFU/10 g)	Negative	n/a	Negative	n/a	Negative	n/a	Negative	Negative
Cronobacter (*Enterobacter*) *sakazakii* (CFU/10 g)	Negative	n/a	Negative	n/a	Negative	n/a	Negative	Negative

CFU = colony forming units; n/a = not applicable; NANA = *N*-acetyl-D-neuraminic acid
[a] Chemical analysis was conducted on samples separate from those subjected to microbiological testing.

Accelerated Study Data

A 24-month accelerated stability study was performed on crystalline NANA from a single batch (Batch L12058K). In this study, samples of the batch were packed as in the long-term real-time stability study described above; 7 3-g samples were packed for chemical analysis and 3 60-g samples were packed for microbiological testing. Packages were sealed and stored at 40°C and 75% relative humidity in a climatic chamber. Chemical analysis and microbiological testing were performed at regular intervals on one sample per time point. NANA was analyzed by HPLC and water content was analyzed by Karl Fischer titration. Degradation products were measured by HPAEC at the 24-month time point only. The analytical data available to date are presented in Table II.D.1-3, including the initial analytical data for the batch. No significant change in assay value was observed under the accelerated storage conditions for up to 24 months of storage. Similarly, no significant change in the water content was observed throughout the 24 months of storage, indicating that the packaging is appropriate in protecting NANA from water absorption. Microbiological purity also was maintained throughout the 24 months of storage. Values for NANA and water content remained within the established specifications for NANA. The only change observed was the discoloration of the samples from white to brownish-white throughout the course of the study, the cause for which could not be determined. Analysis for degradation products revealed 3 unidentified carbohydrate-type degradation products that were detected at 24 months of storage; only 2 of the degradation products were identical to those detected in the real-time stability study. Values for the degradation products remained within the established internal specifications for NANA (maximum of 0.4% for each individual degradation product and maximum of 1.0% for the total amount of degradation products). These results indicate that NANA is stable with no significant degradation when stored under accelerated conditions (40°C and 75% relative humidity) for periods of up to 24 months. However, considering the discoloration observed under these conditions during the 24 months of storage, the shelf-life of crystalline NANA is established based on the ongoing long-term real-time stability study.

Table II.D.1-3 Accelerated Stability Study on NANA Stored at 40°C and 75% Relative Humidity (Batch L12058K)									
Parameter Tested	**Analytical Data[a]**								
	Initial	**1 Month**	**2 Months**	**3 Months**	**6 Months**	**9 Months**	**12 Months**	**18 Months**	**24 Months**
Chemical Analysis									
Color	White	Not documented	Not documented	Not documented	Not documented	Yellow (jonquil)	Light tan (light tawny)	Light tan (light tawny)	Brownish-white
Description	Crystalline powder	Not documented	Not documented	Not documented	Not documented	Crystalline powder	Crystalline powder	Crystalline powder	Crystalline powder
Assay by HPLC (additional water and solvent free dihydrate) (%)	98.6	99.3	99.4	99.1	99.3	100.0	99.7	99.3	99.6
Water (w/w %)	10.6	Not determined	Not determined	10.5	10.6	11.4	10.7	10.7	10.5
Microbiological Analysis									
Salmonella (CFU/25 g)	Negative	n/a	n/a	n/a	Negative	n/a	Negative	n/a	Negative
Aerobic mesophilic total count (CFU/g)	< 10	n/a	n/a	n/a	< 10	n/a	< 10	n/a	< 10
Enterobacteriaceae (CFU/10 g)	Negative	n/a	n/a	n/a	Negative	n/a	Negative	n/a	Negative
Cronobacter (*Enterobacter*) *sakazakii* (CFU/10 g)	Negative	n/a	n/a	n/a	Negative	n/a	Negative	n/a	Negative

CFU = colony forming units; HPLC = high-performance liquid chromatography; n/a = not applicable; NANA = *N*-acetyl-D-neuraminic acid
[a] Chemical analysis was conducted on samples separate from those subjected to microbiological testing.

II.D.2 Stability under the Intended Conditions of Use

Stability in Powdered Infant Formula

The stability of NANA in a representative powdered infant formula is currently under evaluation in an ongoing 3-year study. The infant formula powder used in the study is a whey-based formula also containing skimmed milk powder, vegetable oil, lactose, a vitamin/mineral premix, and lecithin at concentrations intended for full nutritional support of infants from birth to 6 months of age. The composition of the infant formula powder was representative of commercial infant formulas on the market. The caloric density of the infant formula powder was 518 kcal/100 g.

The infant formula powder batches were produced at pilot scale using a commercial infant formula production process consisting of a wet blending-spray-drying process. Twenty-five (25)-kg batches of the infant formula powder were prepared. NANA from a single batch (Batch L12058K) was added to one of the infant formula powder batches at a target concentration of 0.2 g/100 g (dry matter). One of the batches consisted of the control infant formula with no added NANA. The ingredients were blended together with water (pre-heated to 60°C) at a temperature of 60°C. Following dissolution of the ingredients, the mixtures were pasteurized at a temperature of 75°C for 15 seconds. Subsequent steps consisted of homogenization, cooling to 60°C, and spray drying to produce a powdered product. Batches were packaged into 2 L plastic bags, which were vacuum packed/heat sealed. Infant formula samples were then collected, blended to ensure homogeneity, and 400 g samples packaged in aluminum milk powder cans, which were then sealed and filled with nitrogen gas. Cans were then stored at 5°C, 25°C/60% relative humidity, 30°C/65% relative humidity, or 40°C/75% relative humidity (1 can per time and temperature point for each of the control and NANA infant formula powders) under standard storage conditions according to the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH). Measurement for NANA content was scheduled for the 90, 180, 360, 540, and 720-day time points. While the study is currently ongoing, data is available for the 90-day time point. NANA was analyzed in duplicate for each NANA sample and singly for each control sample by high performance anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD). The analytical results are presented in Table II.D.2-1. The presence of carbohydrate-type degradation products was not assessed.

Table II.D.2-1 Stability of NANA (Batch L12058K) in Powdered Infant Formula				
Days of Storage	**NANA Content of Control Infant Formula (g/100 g)**	**NANA Content of Infant Formula with Added NANA (g/100 g)**		
		Replicate 1	**Replicate 2**	**Mean**
Initial Concentration				
0 – post production	<2	227	229	228
0 – post canning	<2	225	232	229
5°C				
90	<2	224	231	228
25°C/60% Relative Humidity				
90	<2	223	223	223
30°C/65% Relative Humidity				
90	<2	211	218	215
40°C/75% Relative Humidity				
90	<2	228	226	227

NANA = *N*-acetyl-D-neuraminic acid
[a] Method uncertainty = 7 mg/100 g

Stability in Other Food Applications

The stability of NANA in other food applications was assessed using representative food products, such as yogurts, ready-to-drink flavored milk, citrus fruit drinks, and cereal bars in stability studies. The NANA content in all food matrices is measured by HPLC with fluorescent detection. All stability studies were conducted using formulations representative of commercial food products on the market and under typical processing (including pasteurization of yogurt, ready-to drink chocolate-flavored milk, and a citrus fruit drink) and typical storage conditions (*e.g.*, temperature and shelf-life) for such products. All test samples were analyzed for NANA content in duplicate and the results provided in Tables II.D.2-2 to II.D.2-5.

The analytical data demonstrate that there was no significant loss of NANA in yogurt, ready-to drink chocolate-flavored milk, a citrus fruit drink, or a cereal bar at each time point tested when compared to the initial NANA concentration. In each study, the apparent difference in NANA content between each time point was determined to fall within the limits of the method uncertainty value. Testing in ready-to drink chocolate-flavored milk and a citrus fruit drink also demonstrated no loss of NANA between pre-processing and the start of the stability study; pasteurization did not affect the stability of NANA in these foods. The analytical data therefore demonstrate that NANA is stable when added to yoghurt, citrus fruit drinks, and ready-to-drink chocolate-flavored milk following typical processing conditions and when stored at 4°C over the shelf-life of these foods. The analytical data further demonstrate that NANA is stable when added to cereal bars following storage under ambient conditions over a 3-month period.

Table II.D.2-2 Stability of NANA (Batch L12058K) in Yogurt Following Pasteurization/Processing and Storage at 4°C				
Sample Number	**Time Point (days)**	**NANA Concentration (mg/kg)**		**Average NANA Concentration (mg/kg)**
		Sample Replicate 1	**Sample Replicate 2**	
NANA Added to Yogurt Base				
P14-01226-69	0	382	388	385
P14-01226-72	10	375	378	377
P14-01226-75	21	392	388	390
NANA Added to Fruit Preparation				
P14-01226-70	0	377	376	377
P14-01226-73	10	339	336	338
P14-01226-76	21	372	379	376

NANA = *N*-acetyl-D-neuraminic acid

Table II.D.2-3 Stability of NANA (Batch L12058K) in Ready-to-Drink Chocolate-Flavored Milk Following Pasteurization and Storage at 4°C				
Sample Number	**Time Point (days)**	**NANA Concentration (mg/kg)**		**Average NANA Concentration (mg/kg)**
		Sample Replicate 1	**Sample Replicate 2**	
P14-01226-47	Pre-processing	141	134	138
P14-01226-49	0	122	116	119
P14-01226-51	7	129	129	129
P14-01226-53	14	132	128	130

NANA = *N*-acetyl-D-neuraminic acid

Table II.D.2-4 Stability of NANA (Batch L12058K) in Orange Juice Following Pasteurization and Storage at 4°C				
Sample	**Time Point (days)**	**NANA Concentration (mg/L)**		**Average NANA Concentration (mg/L)**
		Sample Replicate 1	**Sample Replicate 2**	
P14-01226-39	Pre-processing	76	83	80
P14-01226-41	0	67	74	71
P14-01226-43	14	74	76	75
P14-01226-45	28	65	69	67

NANA = *N*-acetyl-D-neuraminic acid

Table II.D.2-5 Stability of NANA (Batch L12058K) in Cereal Bars Following Storage at Ambient Temperature				
Sample Number	**Time Point (months)**	**NANA Concentration (mg/kg)**		**Average NANA Concentration (mg/kg)**
		Sample Replicate 1	**Sample Replicate 2**	
P14-01226-63	0	483	464	473
P14-01226-65	1	505	438	472
P14-01226-67	3	509	477	493

NANA = *N*-acetyl-D-neuraminic acid

III. SELF-LIMITING LEVELS OF USE

No known self-limiting levels of use are associated with food uses of NANA.

IV. DETAILED SUMMARY OF THE BASIS FOR GLYCOM'S GRAS DETERMINATION

Glycom's determination that NANA is GRAS under the conditions of intended use in infant formula, toddler and baby foods and select conventional food and beverage products as described herein is based on scientific procedures. To obtain necessary information relevant to the safety of NANA comprehensive searches of the published scientific literature were conducted, through April 2015, using the electronic search tool, DIALOG, with several databases, including MEDLINE®, ToxFile, AGRICOLA, AGRIS, BIOSIS Toxline®, FOODLINE®: Science, Food Science and Technology Abstracts, CAB Abstracts, BIOSIS Previews®, NTIS, and EMBASE. Published studies were manually selected from search hits based on their relevance to the safety of NANA as a food ingredient and included consideration of all relevant studies, both favorable and unfavorable.

The proposed maximum use level of NANA in term infant formulas is 50 mg/L and is based on providing a similar level of free NANA as that which occurs in human milk. Concentrations of free NANA in milk samples obtained from lactating women across a variety of demographic types typically ranges between 10 to 60 mg/L [see Section IV.B.1]. Highest concentrations of NANA are consistently reported from 'early' human milk samples (20 to 60 mg/L), with levels decreasing to between 10 to 20 mg/L over the course of 3 to 4 months post-birth. NANA also is present in significant quantities bound to disaccharides (*e.g.*, 3'-siallylactose; 6'siallylactose) and oligosaccharides. Concentrations of total NANA from acid hydrolyzed milk sample range from 322 mg/L to 1,782 mg/L in term and colostrum milk samples, with highest levels reported for colostrum and 'early' milk. The bioavailability of bound NANA is not known; however, hydrolysis of bound NANA by acid hydrolysis and by microbial and intestinal neuraminidases is expected to at least partially release NANA bound to saccharides. Accordingly concentrations

of free NANA that have been measured in human milk samples are expected to greatly underestimate the total quantity of NANA that is ultimately available for absorption by the infant.

The estimated daily intakes of NANA from intended uses in term infant formulas and conventional food products were calculated for infants and toddlers using consumption data provided by the U.S. National Center for Health Statistics' (NCHS) 2009-2010 National Health and Nutrition Examination Surveys (NHANES) (CDC, 2011; USDA, 2012) [see Section IV.A]. The estimated mean and 90th percentile daily intakes of NANA in infants ages 0 to 6 months were determined to be 7.3 and 11.8 mg/kg body weight/day, respectively. In infants ages 7 to 12 months, the estimated mean and 90th percentile intakes of NANA were 6.0 and 9.2 mg/kg body weight/day, respectively. In toddlers 1 to 3 years of age, the estimated mean and 90th percentile intakes of NANA were 4.0 and 6.6 mg/kg body weight/day, respectively. Of note, in infants ages 0 to 6 months, the consumption of infant formulas contributed 87.2% to the estimated intakes of NANA, while the consumption of baby foods for infants and young children contributed 10.9%. No contribution of toddler formulas to the estimated intakes of NANA in this infant age group occurred. In infants ages 7 to 12 months, the consumption of infant formulas contributed 45.8% to the estimated intakes of NANA, while the consumption of baby foods for infants and young children contributed 22.4%. Dietary exposures for the general population of potential consumers of NANA products also were estimated. The greatest estimated mean and 90th percentile intake of NANA among population groups ≥4 years of age were determined to be 1.8 and 3.2 mg/kg body weight/day, respectively, which occurred in children.

The background dietary intakes of free NANA from early human milk were calculated using mean concentrations of NANA reported for human milk samples. On a mg/kg basis, dietary intakes of NANA in newborn infants ranged from 3.5 to 11 mg/kg body weight/day. In older infants (*e.g.*, up to 4 months), consumption of 'mature' human milk resulting in estimated intakes of 1.5 to 7.8 mg/kg body weight/day. Considering that Glycom's NANA is chemically and structurally identical to the NANA present in human milk, the background dietary range of free NANA intakes from human milk in infants serves as the safe range of Glycom's NANA intakes for infants. In addition, considering that infants are a susceptible population group, it was also concluded that background dietary intakes of NANA by infants would be similarly safe on an equivalent mg/kg basis for older population groups. The background exposure to free NANA therefore serves as the safe reference range for all population groups.

The results of published studies on the metabolic fate of NANA in mice and rats demonstrate that NANA is almost completely absorbed following oral administration with the bulk of the dose excreted unchanged in urine [see Section IV.B.5]. Small amounts of NANA are distributed to the major organs, particularly the liver, brain, and heart, where the compound may be metabolized by *N*-acetylneuraminate lyase to acetylmannosamine and acetic acid. Pyruvic acid also may be formed from the action of *N*-acetylneuraminate lyase together with other enzymes. It is expected that any unmetabolized free NANA would be incorporated into the

oligosaccharide chains that make up glycolipids, glycoproteins, and HMOs through the endogenous pathway for NANA and that the NANA metabolite, acetylmannosamine, would enter the salvage pathway for NANA, whereby NANA metabolites are converted to NANA.

NANA manufactured by Glycom has been evaluated in a standard battery of animal toxicity and mutagenicity/genotoxicity evaluations conducted under current Good Laboratory Practice and in consideration of OECD guidelines for the toxicity testing of chemicals [See Section IV.B.6]. These studies included a 13-week oral toxicity study in Crl:CD® Sprague-Dawley rats preceded by an *in-utero* exposure phase to evaluate the effects of NANA on female reproduction and on the general growth and development of rats following in utero and lactational exposure. The *in-utero* and lactational phases were then followed by a 13-week dietary toxicity study conducted with the F_1 generation offspring to assess subchronic toxicity. No evidence of maternal toxicity adverse reproductive or developmental effects were observed following administration of NANA to dams at doses up to 1,895 mg/kg body weight/day, the highest dose tested. In the subchronic toxicity study conducted on the F1 generation a NOAEL of 974 mg/kg body weight/day, the highest dose tested. NANA produced negative results in a bacterial reverse mutation assay in the presence and absence of metabolic activation using *Salmonella* Typhimurium TA98, TA100, TA1535 and TA 1537 and *E. coli* WP2 *uvr*A tester strains. NANA also was negative in an *in vitro* mammalian cell micronucleus test conducted in human peripheral blood lymphocytes.

Glycom has therefore concluded, using scientific procedures, that NANA dihdyrate, meeting appropriate food-grade specifications and manufactured according to cGMP, is safe and suitable, and generally recognized as safe and suitable for use in infant formula and all other intended food uses as discussed herein. A summary of all generally available information pertinent to the safety of NANA is presented below.

IV.A Probable Consumption

IV.A.1 Estimated Daily Intake of NANA from Use in Infant Formula (Infants and Toddlers)

Estimates for the daily intake of NANA from its use in term infant formulas were calculated based on a maximum use level of 50 mg/L as indicated in Section I.D.1 in conjunction with infant formula consumption data included in the U.S. National Center for Health Statistics' (NCHS) 2009-2010 National Health and Nutrition Examination Surveys (NHANES) (CDC, 2011; USDA, 2012). The population groups consuming infant formulas consisted of infants (0 to 6 and 7 to 12 month age groups) and toddlers (1 to 3 years), with infants ages 0 to 6 months identified as the largest consumer group as expected. For toddlers, only 1.1% of respondents were identified as potential consumers of NANA from infant formula.

Estimates for the daily intake of NANA from its use in term infant formulas are summarized in Table IV.A.1-1 on a per person basis by population group. Table IV.A.1-2 presents these data on a per kilogram body weight basis.

From the use of NANA in infant formulas only, the estimated all-user mean and 90th percentile intakes of NANA were greatest in infants ages 0 to 6 months and were determined to be 43.7 and 64.1 mg/person/day, respectively. On a body weight basis, these intakes were determined to be 7.1 and 11.6 mg/kg body weight/day, respectively. In infants 7 to 12 months of age, the estimated mean and 90th percentile intakes of NANA from infant formulas were determined to be at 34.7 and 54.9 mg/person/day, respectively. On a body weight basis, these intakes were determined to be 3.8 and 6.1 mg/kg body weight/day, respectively. In toddlers 1 to 3 years of age, the estimated mean and 90th percentile all-user intakes of NANA from infant formulas were determined to be 22.5 and 29.3 mg/person/day, respectively. On a body weight basis, these intakes were determined to be 1.9 and 2.4 mg/kg body weight/day, respectively.

Table IV.A.1-1 Summary of the Estimated Daily Intake of NANA from Term Infant Formula in the U.S. by Infant and Toddler Population Groups (2009-2010 NHANES Data)

Population Group	Age Group	All-Person Consumption (mg/day)		All-Users Consumption (mg/day)			
		Mean	90th Percentile	% Users	n	Mean	90th Percentile
Infants	0 to 6 months	33.0	61.0	75.5	173	43.7	64.1
Infants	7 to 12 months	25.4	51.9	73.2	117	34.7	54.9
Toddlers	1 to 3 years	0.3[a]	na	1.1	7	22.5[a]	29.3[a]

na = not available; NANA = *N*-acetyl-D-neuraminic acid
[a] Indicates an intake estimate that may not be statistically reliable, as the sample size does not meet the minimum reporting requirements.

Table IV.A.1-2 Summary of the Estimated Daily Per Kilogram Body Weight Intake of NANA from Term Infant Formulas in the U.S. by Infant and Toddler Children Population Groups (2009-2010 NHANES Data)

Population Group	Age Group	All-Person Consumption (mg/kg body weight/day)		All-Users Consumption (mg/kg body weight/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Infants	0 to 6 months	5.3	10.8	75.5	173	7.1	11.6
Infants	7 to 12 months	2.8	5.4	72.8	116	3.8	6.1
Toddlers	1 to 3 years	<0.1[a]	na	1.2	7	1.9[a]	2.4[a]

na = not available; NANA = *N*-acetyl-D-neuraminic acid
[a] Indicates an intake estimate that may not be statistically reliable, as the sample size does not meet the minimum reporting requirements.

IV.A.2 Estimated Daily Intake of NANA from Use in Infant Formula and in Food Combined (Infants and Toddlers)

NANA is intended for use in term infant formulas and in foods, including baby foods. Considering that older infants begin consuming baby food and other foods while still consuming infant formula, the estimated daily intakes of NANA from its use in term infant formulas combined with its use in foods were calculated in order to determine the total daily intake of the ingredient from all sources in the population groups identified as consumers of infant formula (*i.e.*, infants and toddlers). Estimates for the total daily intake of NANA in these population groups from its use in term infant formulas combined with its use in all other intended food uses are summarized in Table IV.A.2-1 on a per person basis by population group. Table IV.A.2-2 presents these data on a per kilogram body weight basis.

For the infant population group ages 0 to 6 months, 80.5% of respondents were identified as potential consumers of NANA, while in the other infant and toddler population groups, the percentage of consumers exceeded 99%. As a result of the high percentage of users identified within these population groups, the intake estimates for the all-person and all-user categories were similar; therefore, only the all-user results are discussed in detail.

The estimated all-user mean and 90th percentile intakes of NANA in infants ages 0 to 6 months were determined to be 46.5 and 67.1 mg/person/day, respectively. On a body weight basis, these intakes were determined to be 7.3 and 11.8 mg/kg body weight/day, respectively. In infants 7 to 12 months of age, the estimated mean and 90th percentile intakes of NANA were determined to be at 55.5 and 79.2 mg/person/day, respectively, and on a body weight basis, 6.0 and 9.2 mg/kg body weight/day, respectively. In toddlers 1 to 3 years of age, the estimated mean and 90th percentile all-user intakes of NANA were determined to be 52.3 and 87.9 mg/person/day, respectively, and on a body weight basis, 4.0 and 6.6 mg/kg body weight/day, respectively.

Of note, the greatest contributors to the estimated daily intakes of NANA in the infant population groups were use in infant formulas and use in baby foods for infants and young children. In infants ages 0 to 6 months, use in infant formulas contributed 87.2% to the estimated intakes of NANA, while use in baby foods for infants and young children contributed 10.9%. No contribution of toddler formulas to the estimated intakes of NANA in this infant age group occurred. In infants ages 7 to 12 months, use in infant formulas contributed 45.8% to the estimated intakes of NANA, while use in baby foods for infants and young children contributed 22.4%. The contribution of toddler formulas to the estimated intakes of NANA in this infant age group was 1.1%.

Table IV.A.2-1 Summary of the Estimated Daily Intake of NANA from Term Infant Formulas and from all Proposed Food Uses in the U.S. Infant and Toddler Population Groups (2009-2010 NHANES Data)

Population Group	Age Group	All-Person Consumption (mg/day)		All-Users Consumption (mg/day)			
		Mean	90th Percentile	% Users	n	Mean	90th Percentile
Infants	0 to 6 months	37.8	67.1	81.3	181	46.5	67.1
Infants	7 to 12 months	55.5	79.2	100.0	148	55.5	79.2
Toddlers	1 to 3 years	52.2	87.3	99.7	644	52.3	87.9

NANA = *N*-acetyl-D-neuraminic acid

Table IV.A.2-2 Summary of the Estimated Daily Per Kilogram Body Weight Intake of NANA from Term Infant Formulas and from all Proposed Food Uses in the U.S. Infant and Toddler Population Groups (2009-2010 NHANES Data)

Population Group	Age Group	All-Person Consumption (mg/kg bw/day)		All-Users Consumption (mg/kg bw/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Infants	0 to 6 months	6.0	11.4	81.3	181	7.3	11.8
Infants	7 to 12 months	6.0	9.2	100.0	147	6.0	9.2
Toddlers	1 to 3 years	4.0	6.6	99.9	637	4.0	6.6

bw = body weight; NANA = *N*-acetyl-D-neuraminic acid

IV.A.3 Estimated Daily Intake of NANA from Use in Foods (Ages ≥4 Years)

For U.S. population groups ≥4 years of age, the estimated total intakes of NANA (mg/person/day) from all proposed food uses are summarized in Table IV.A.3-1. The data are presented on a per kilogram body weight basis (mg/kg body weight/day) in Table IV.A.3-2. The percentage of users was high among all age groups evaluated in the current intake assessment; at least 97% of the individual population groups comprised users of those food products in which NANA is currently proposed for use. As a result of the high percentage of users identified within all population groups, the intake estimates for the all-person and all-user categories were similar; therefore, only the all-user results are discussed in detail.

For the total U.S. population, estimated mean and 90th percentile all-user intakes of NANA were 65.3 mg/person/day (1.2 mg/kg body weight/day) and 130.9 mg/person/day (2.6 mg/kg body weight/day), respectively.

Among the individual population groups, female adults were determined to have the greatest estimated mean and 90th percentile all-user intakes of NANA on an absolute basis at 71.8 and 154.3 mg/person/day, respectively. Similar absolute estimated daily intakes of NANA were calculated for all other adult population groups. Within women of child-bearing age, who

represent a target demographic for many of the proposed uses of NANA, the mean intake of NANA was estimated to be 65.8 mg/person/day with the 90th percentile level of intakes reaching 138.0 mg/person/day. When compared to the larger sample of women between the ages of 19 and 64 years, women of child-bearing age were observed to have slightly lower estimates for the intake of NANA. Children were determined to have the lowest estimated mean and 90th percentile all-user intakes at 47.9 and 81.8 mg/person/day, respectively.

Table IV.A.3-1 Summary of the Estimated Daily Intake of NANA from Proposed Food-Uses in the U.S. by Population Group (2009-2010 NHANES Data)

Population Group	Age Group (Years)	All-Person Consumption (mg/day)		All-Users Consumption (mg/day)			
		Mean	90th Percentile	% Users	n	Mean	90th Percentile
Children	4-10	47.7	81.7	99.7	1,092	47.9	81.8
Female Teenagers	11 to 18	52.2	93.5	99.7	551	52.3	93.5
Male Teenagers	11 to 18	62.0	109.1	99.4	571	62.4	109.1
Female Adults of child bearing age	19 to 40	64.3	132.3	97.7	977	65.8	138.0
Female Adults	19 to 64	70.5	151.1	98.2	2,042	71.8	154.3
Male Adults	19 to 64	70.0	139.1	97.6	1,822	71.7	140.0
Elderly	65 and up	58.6	133.8	97.0	1,185	60.4	135.6
Total Population	All Ages	64.0	128.8	98.1	8,236	65.3	130.9

NANA = *N*-acetyl-D-neuraminic acid

On a body weight basis, children were determined to have the greatest mean and 90th percentile all-user NANA intakes at 1.8 and 3.2 mg/kg body weight/day, respectively. Male adults and the elderly were identified as having the lowest mean all-user intakes at 0.8 mg/kg body weight/day, and male adults had the lowest 90th percentile intake at 1.6 mg/kg body weight/day. Similar estimated daily intakes of NANA on a body weight basis were calculated for all other adult population groups.

Table IV.A.3-2 Summary of the Estimated Daily Per Kilogram Body Weight Intake of NANA from Proposed Food-Uses in the U.S. by Population Group (2009-2010 NHANES Data)							
Population Group	**Age Group (Years)**	**All-Person Consumption (mg/kg body weight/day)**		**All-Users Consumption (mg/kg body weight/day)**			
		Mean	**90th Percentile**	**%**	**n**	**Mean**	**90th Percentile**
Children	4-10	1.8	3.2	99.7	1,087	1.8	3.2
Female Teenagers	11 to 18	0.9	1.7	99.7	543	0.9	1.7
Male Teenagers	11 to 18	1.0	2.0	99.4	567	1.0	2.0
Female Adults of child bearing age	19 to 40	0.9	1.8	97.7	971	0.9	1.9
Female Adults	19 to 64	1.0	2.1	98.3	2,031	1.0	2.2
Male Adults	19 to 64	0.8	1.6	97.6	1,810	0.8	1.6
Elderly	65 and up	0.8	1.8	97.0	1,169	0.8	1.9
Total Population	All Ages	1.2	2.6	98.1	8,172	1.2	2.6

NANA = *N*-acetyl-D-neuraminic acid

IV.B Information to Support the Safety of NANA

IV.B.1 Background Dietary Intake of NANA from Human Breast Milk

NANA is an endogenously produced monosaccharide widely found throughout higher animals (and some microorganisms). It is present in mammalian milk, with the highest concentrations found in human milk (Puente and Hueso, 1993; Puente *et al.*, 1994, 1996; Martín-Sosa *et al.*, 2003; Goto *et al.*, 2010; Lacomba *et al.*, 2010; German *et al.*, 2012; Urashima *et al.*, 2013). Moreover, the occurrence of NANA in the pure *N*-acetylated form is a characteristic feature of human milk (while in almost all other mammalian milks, NANA occurs as a mixture with the *N*-glycolyl form, NGNA). NANA occurs in human milk predominantly bound at the terminal end of free oligosaccharides, which are known as HMOs, and also occurs as a component of glycoproteins and gangliosides (Rueda *et al.*, 1995; Puente *et al.*, 1996). Importantly, NANA also has been found to occur in the free (unbound) form in human milk (Sabharwal *et al.*, 1991; Hayakawa *et al.*, 1993; Thurl *et al.*, 1996; Wang *et al.*, 2001; Wiederschain and Newburg, 2001; Martín-Sosa *et al.*, 2004; Oriquat *et al.*, 2011; Galeotti *et al.*, 2012).

The NANA content of human milk has been reported in several publications from various independent research groups (Table IV.B.1-1). The concentration of NANA (total, bound, and free) in human milk is dependent on the lactation period, with the highest levels reported in colostrum and decreased levels reported thereafter. In addition, pre-term milk contains significantly higher levels of NANA than full-term milk (Wang *et al.*, 2001; Oriquat *et al.*, 2011). The average total NANA content (free and bound) of human milk is high, with levels ranging between 900 and 1,800 mg/L in early milk (colostrum, transition milk, and first month milk)

(Hayakawa *et al.*, 1993; Wang *et al.*, 2001; Martín-Sosa *et al.*, 2004; Oriquat *et al.*, 2011) and between 300 and 800 mg/L in mature milk (>1 month milk) (Wang *et al.*, 2001; Martín-Sosa *et al.*, 2004; Oriquat *et al.*, 2011; Qiao *et al*., 2013). The highest average level of total NANA detected in human milk was reported to occur in pre-term colostrum at a concentration of 1,782 mg/L (Wang *et al.*, 2001). See Table IV.B.1-1 for a summary of these data.

Table IV.B.1-1 Average NANA Concentrations in Human Milk

Publication	Sample No.	Location	Method	Time of Milk Collection	Total NANA (mg/L)	Free NANA[a] (mg/L)
Hayakawa *et al.* (1993)	N = 3	Japan	HPLC-FLD[b]	Not reported	1,131	54 ± 20
Thurl *et al.* (1996)	N = 1	Germany	HPAEC-PED[b]	Not reported	-	23
Wiederschain and Newburg (2001)	N = 4	USA	GC-MS	2 to 9 month postpartum	-	8 (0.5 - 30)
Wang *et al.* (2001)	N = 20 (term) N = 14 (pre-term)	Australia	Colorimetric	Colostrum – Term Colostrum – Pre-term	1,559 1,782	43 ± 3 59 ± 6
				Transition milk – Term Transition milk – Pre-term	1,070 1,321	28 ± 3 34 ± 6
				Full-term at 1 month Pre-term at 1 month	612 792	22 ± 1 31 ± 3
				Full Term at 3 months Pre-term at 3 months	322 394	9 ± 0.3 9 ± 3
Martín-Sosa *et al.* (2004)	N = 12	Spain	Colorimetric	Colostrum Transition milk Mature Milk	1,277 911 526	35 ± 3.7 22 ± 2.4 16 ± 1.4
Oriquat *et al.* (2011)	N = 20 (term) N = 15 (pre-term)	Egypt	Colorimetric	Term – Colostrum Pre-term – Colostrum	1,017 1,234	43 59
				Full Term at 3 months Pre-term at 3 months	- -	8 9
Qiao *et al*. (2013)	N = 90	China	HPLC-FLD	Mature milk at day 40	714	16.96 ± 4.38

GC-MS = Gas Chromatography Mass Spectrometry; HPLC-FLD = High Performance Liquid Chromatography-Fluorescence Detector; HPAEC = High-performance Anion Exchange Chromatography at high pH coupled with pulsed electrochemical detection; NANA = *N*-acetyl-D-neuraminic acid

[a] Reported as average concentration ± standard deviation ranges are reported in parentheses.

[b] NANA standards used during analyses

As mentioned, the majority of the total NANA content in human milk is bound to HMOs, and therefore, the amount of free NANA in human milk makes up only a fraction of the total content. The average levels of free NANA in human milk typically range between approximately 20 and 60 mg/L in early milk (Wang *et al.*, 2001; Martín-Sosa *et al.*, 2004) and between 10 and 50 mg/L in mature milk (Hayakawa *et al.*, 1993; Thurl *et al.*, 1996; Wang *et al.*, 2001; Wiederschain and Newburg, 2001; Martín-Sosa *et al.*, 2004; Qiao *et al*., 2013).

The intakes of total and free NANA from early and mature human milk in infants were calculated using the above data. Based on the total level of NANA present in early human milk of 900 to 1,800 mg/L and on an a 3.4-kg newborn infant (WHO, 2015) drinking 600 mL of breast milk per day during the first month (Hester *et al.*, 2012), the intake of total NANA from early human milk is calculated to be approximately 160 to 320 mg/kg body weight/day in young infants. In addition, based on the total level of NANA present in mature human milk of 300 to 800 mg/L and on a 6.5-kg infant consuming 1 L of breast milk per day (Davies *et al.*, 1994; Hester *et al.*, 2012), the intake of total NANA from mature human milk is calculated to be approximately 45 to 125 mg/kg body weight/day.

Likewise, based on the level of free NANA present in early human milk of 20 to 60 mg/L, the intake of free NANA from early human milk is calculated to be approximately 3.5 to 11 mg/kg body weight/day in young infants. In addition, based on the level of free NANA present in mature human milk of 10 to 50 mg/L, the intake of free NANA from mature human milk is calculated to be approximately 1.5 to 7.8 mg/kg body weight/day. Additional amounts of free NANA also would be released from HMOs during passage through the infant gastrointestinal tract, owing to the limited acid stability of the glycosidic bond of NANA (Sonnenburg *et al.*, 2002). Consistent with this, studies have shown that the level of total NANA is reduced in human milk samples following simulated gastrointestinal digestion (Lacomba *et al*., 2011) and that the ratio of free to bound NANA is strongly shifted towards the free form in the feces of breast-fed infants when compared to the initial ratio in breast milk (Sabharwal *et al*., 1991). While the release of free NANA from acidic cleavage may be limited in infants due to the weakly acidic gastric pH of infants (typically between pH 3 and 5.5) (Maffei and Nóbrega, 1975; Armand *et al*., 1996), gastric hydrolysis will nonetheless contribute to dietary exposures of free NANA from human milk. Free sialic acid also may be released by enzymatic hydrolysis through the action of neuraminidases (sialidases) which have been detected in human milk, human intestinal mucosa, and gut commensal bacterial cultures (reviewed in Röhrig *et al*., 2015). Nöhle and Schauer (1981) reported that half of an oral dose of sialyllactose (a conjugated form of sialic acid) is hydrolyzed to fee sialic acid by intestinal sialidase in mice and rats.

Considering that Glycom's NANA is chemically and structurally identical to the NANA present in human milk, the background dietary range of free NANA intakes from human milk in infants serves as the safe range of Glycom's NANA intakes for infants. In addition, considering that infants are a susceptible population group from a safety perspective (SCF, 1998), it may be concluded that this background dietary range of free NANA intakes in infants also would be safe for older population groups. This background exposure to free NANA therefore serves as the safe reference range of Glycom's NANA intakes for all population groups.

IV.B.2 Background Dietary Intake of NANA from Food

A low background dietary exposure to free NANA currently exists for infants also as a result of infant formula consumption. In this regard, free NANA has been detected and quantified in

food-grade D-lactose, a nutritional ingredient added to infant formula. Free NANA concentrations occurring in the ingredient, however, are low at 20 to 50 mg/kg D-lactose (Spichtig *et al.*, 2011). D-Lactose is added to infant formula at a level of approximately 65 g/L, resulting in free NANA levels of approximately 1.3 to 3.3 mg/L in current infant formulae. Therefore, there currently exists a low background exposure to exogenous free NANA in infants, which is associated with the safe consumption of infant formula.

A low background dietary exposure to free NANA also exists in humans as a result of its presence in edible animal tissues and other foods of animal origin (Röhrig *et al*., 2015). Fish eggs (caviar), fish, red meat, and dairy products contain high levels of sialic acids, mainly in the form of glycoconjugates of NANA and NGNA (Tangvoranuntakul *et al.*, 2003; Bardor *et al.*, 2005; Martín *et al.*, 2007; Lacomba *et al.*, 2010, 2011; Sørensen, 2010; Spichtig *et al.*, 2010; Taylor *et al.*, 2010; Hurum and Rohrer, 2012; Chen *et al.*, 2014; Samraj *et al.*, 2015). Until recently, quantitative data on the presence of the free monosaccharide in foods was very limited. Data was only available for porcine (Schoop *et al.*, 1969), ovine (Jourdian *et al.*, 1971), and bovine (Schauer, 1970) submaxillary glands. Based on these data, it is estimated that free NANA is present in these animal tissues at concentrations of 50 to 100 µg per gram (50 to 100 mg/kg) of tissue. However, recent quantitative data has become available on the free NANA content of a wide array of animal tissues (Samraj *et al*., 2015). According to these data, the highest levels of free NANA occur in fish eggs (caviar) from whitefish and salmon, with reported concentrations of 459 and 149 mg/kg, respectively. Fish tissues have the next highest reported levels of free NANA, for example 117 mg/kg in mahi-mahi, 106 mg/kg in wild salmon, and 100 mg/kg in tilapia. Free NANA levels in other fish and seafood range from 4.6 to 45 mg/kg. In red meats (from cows, bison, lambs, pigs, and goats) and milk and milk products (cheese), free NANA concentrations range from 10 to 39 mg/kg. Lower levels of free NANA occur in poultry meat (approximately 7 mg/kg) and chicken eggs (0.2 to 0.6 mg/kg). For a summary of published studies characterizing the NANA content of food, the reader is directed to the review by Röhrig *et al*. (2015).

Using data obtained from the literature regarding the concentrations of free NANA in foods (Röhrig *et al*., 2015) combined with dietary intake data from the NHANES 2011-2012 dataset, background dietary exposure to free NANA was calculated for the U.S. population. Tables IV.B.2-1 and IV.B.2-2 present the estimated intakes of free NANA from the diet of the U.S. population on an absolute and body weight basis, respectively. Both mean and 90th percentile figures were calculated. Data is presented for the total population and by age and gender groups, for all-persons (all individuals in the survey database) and all-users (only individuals who reported consumption of foods with analytical data for levels of free NANA).

Based on the range of foodstuffs for which there was data available on the concentration of free NANA, the percent users was high, with greater than 72.8% of all individuals in the survey identified as consumers of products containing free NANA. On this basis, the figures for all-

persons and all-users are very similar; consequently only all-user results will be discussed in detail below.

The total population mean and 90th percentile intakes were calculated to be 7.0 and 15.7 mg/person/day. Of the individual population groups, infants were determined to have the greatest mean and 90th percentile all-user intakes at 11.2 and 21.9 mg/person/day, respectively, while female adults had the lowest mean and 90th percentile all-user intakes at 5.2 and 11.8 mg/day.

Table IV.B.2-1 Summary of the Estimated Daily Intake of Sialic Acid from Background Dietary Sources in the United States by Population Group (NHANES 2011-2012 Data)

Population Group	Age Group (Years)	All-Person Consumption (mg/day)		All-Users Consumption (mg/day)			
		Mean	90th Percentile	% Users	n	Mean	90th Percentile
Infants	0 to 2	8.1	20.3	72.8	480	11.2	21.9
Children	3 to 11	10.1	18.5	99.7	1,517	10.1	18.6
Female Teenagers	12 to 19	6.4	14.4	98.3	521	6.5	14.4
Male Teenagers	12 to 19	9.9	19.5	98.6	507	10.1	19.5
Female Adults	20 and up	5.1	11.6	98.5	2,176	5.2	11.8
Male Adults	20 and up	6.9	15.2	98.0	2,053	7.0	15.3
Total Population	All Ages	6.8	15.5	97.5	7,254	7.0	15.7

NANA = *N*-acetyl-d-neuraminic acid

Considering intakes on a body weight basis, infants were identified as having the highest mean and 90th percentile all-user intakes of any population group at 0.91 and 1.70 mg/kg body weight/day, respectively. Female adults had the lowest mean and 90th percentile all-user intakes at 0.07 and 0.170 mg/kg body weight/day, respectively.

Table IV.B.2-2 Summary of the Estimated Daily Per Kilogram Body Weight Intake of Sialic Acid from Background Dietary Sources in the United States by Population Group (NHANES 2011-2012 Data)							
Population Group	**Age Group (Years)**	**All-Person Consumption (mg/kg bw/day)**		**All-Users Consumption (mg/kg bw/day)**			
		Mean	**90th Percentile**	**% Users**	**n**	**Mean**	**90th Percentile**
Infants	0 to 2	0.66	1.62	72.7	477	0.91	1.70
Children	3 to 11	0.41	0.82	99.7	1,517	0.41	0.82
Female Teenagers	12 to 19	0.11	0.28	98.3	510	0.12	0.28
Male Teenagers	12 to 19	0.15	0.32	98.6	504	0.15	0.32
Female Adults	20 and up	0.07	0.16	98.5	2,153	0.07	0.17
Male Adults	20 and up	0.08	0.18	98.0	2,035	0.08	0.18
Total Population	All Ages	0.15	0.34	97.5	7,196	0.15	0.35

bw = body weight; NANA = *N*-acetyl-D-neuraminic acid

Thus, based on the presence of NANA in animal tissues and products thereof, there currently exists a safe low background exposure to exogenous free NANA in the general human population from foods of animal origin. Additional amounts of free NANA also are released from glycoconjugates of NANA upon dietary exposure during passage through the gastrointestinal tract, owing to the limited acid stability of the glycosidic bond of NANA (Ruano *et al.*, 1999; Sonnenburg *et al.*, 2002). This release of free NANA in the gastrointestinal tract would therefore add to the dietary exposure to free NANA from animal tissues.

IV.B.3 Comparison of Dietary Intake of NANA from Intended Food Uses to Background Dietary Intakes

Comparison of Estimated Intake of NANA from Use in Infant Formula to Background Dietary Intake of NANA from Human Breast Milk (Infants and Toddlers)

The maximum use level of NANA of 50 mg/L in term infant formulas is based on providing a similar level of free NANA as that which occurs in human milk, which ranges from 20 to 60 mg/L in early human milk and 10 to 50 mg/L in mature human milk. Heavy consumer (90th percentile) intake of NANA from its use in term infant formulas was estimated to be 11.6 mg/kg body weight/day in infants ages 0 to 6 months, 6.1 mg/kg body weight/day in infants ages 7 to 12 months, and 2.4 mg/kg body weight/day in toddlers ages 1 to 3 years. In comparison, the background dietary intake of free NANA from early human milk ranges from 3.5 to 11 mg/kg body weight/day in young infants and that from mature human milk ranges from 1.5 to 7.8 mg/kg body weight/day. The estimated intakes of NANA from its use in term infant formulas in infants and toddlers are therefore within the background dietary range of free NANA intake from human milk in infants, which is considered to be the safe reference range of Glycom's NANA intakes for all population groups as previously noted.

The safety of the use of NANA in term infant formulas is therefore supported by the fact that NANA manufactured by Glycom is chemically and structurally identical to NANA present in human milk and that the estimated intakes of NANA from its use in term infant formulas is within the safe reference range of Glycom's NANA intakes for infants and older population groups.

Furthermore, considering that term infant formulas are consumed in (complete or partial) substitution for breast milk, the addition of NANA to term infant formulas would not add to the background dietary intake of free NANA from breast milk.

Comparison of Estimated Intake of NANA from Use in Infant Formula and in Food Combined to Background Dietary Intake of NANA from Human Breast Milk (Infants and Toddlers)

For the population groups identified as consuming infant formulas (*i.e.*, infants and toddlers), the estimated daily intakes of NANA from its use in term infant formulas combined with its use in foods were calculated in order to determine the total daily intake of the ingredient from all sources in these population groups. The resulting heavy consumer (90^{th} percentile) intake of NANA from its use in term infant formulas combined with its use in foods was estimated to be 11.8 mg/kg body weight/day in infants ages 0 to 6 months, 9.2 mg/kg body weight/day in infants ages 7 to 12 months, and 6.6 mg/kg body weight/day in toddlers ages 1 to 3 years. The use of NANA in foods therefore increases the NANA intakes in these population groups from the intakes from use in infant formula alone. These combined NANA intakes, however, remain within the background exposure to free NANA from human milk in infants (3.5 to 11 mg/kg body weight/day from early human milk and 1.5 to 7.8 mg/kg body weight/day from mature human milk), supporting the safety of the use of the ingredient in term infant formulas combined with its use in foods.

Comparison of Estimated Daily Intake of NANA from Use in Food to Background Dietary Intake of NANA from Human Breast Milk (Ages ≥4 Years)

The greatest estimated heavy consumer intake of NANA among population groups ≥4 years of age was determined to be 3.2 mg/kg body weight/day, which occurred in children. In comparison, the background exposure to free NANA in infants ranges from 3.5 to 11 mg/kg body weight/day from early human milk and 1.5 to 7.8 mg/kg body weight/day from mature human milk. The intake of NANA in population groups ≥4 years of age from its use in food is therefore within the background dietary range of free NANA intake from human milk in infants on a mg per kg body weight basis, which is considered to be the safe reference range of Glycom's NANA intakes for all population groups.

The safety of the use of NANA in food is therefore supported by the fact that NANA manufactured by Glycom is chemically and structurally identical to NANA present in human milk and that the estimated intakes of NANA in population groups ≥4 years of age from its use in food is within the safe reference range of NANA intakes from human milk in infants.

IV.B.4 Nutritive Role and Case-of-Need

Sialic acids are produced endogenously in all vertebrates, with the most predominant form in mammals being NGNA, except in humans where the prevalent form is NANA. NANA is an important structural component of cell surface oligosaccharide chains of glycolipids (notably, gangliosides) and glycoproteins that are bound to the cell membrane. Although NANA is found ubiquitously throughout the human body, the highest levels occur in the brain (Klenk *et al.*, 1941; Papadopoulos, 1960; Wang *et al.*, 1998) and in breast milk (Carlson, 1985). As discussed, NANA exists in human milk mainly in the bound form at the terminal end of free HMOs, but also occurs in the free form (Sabharwal *et al.*, 1991; Hayakawa *et al.*, 1993; Thurl *et al.*, 1996; Wang *et al.*, 2001; Wiederschain and Newburg, 2001; Martín-Sosa *et al.*, 2004; Oriquat *et al.*, 2011; Galeotti *et al.*, 2012).

NANA is not considered an essential nutrient as mammalian species can synthesize NANA from simple sugars and phosphoenolpyruvate (Carlson, 1985). However, the metabolic capacity of the neonate to support optimal accretion of NANA in brain gangliosides and proteins is unclear as analyses of tissue samples of frontal cortex derived from infants who died from sudden infant death syndrome have reported higher brain ganglioside-bound and protein-bound NANA levels in infants fed human milk relative to levels observed in formula-fed infants (Wang *et al.*, 2003; Figure IV.B.4-1).

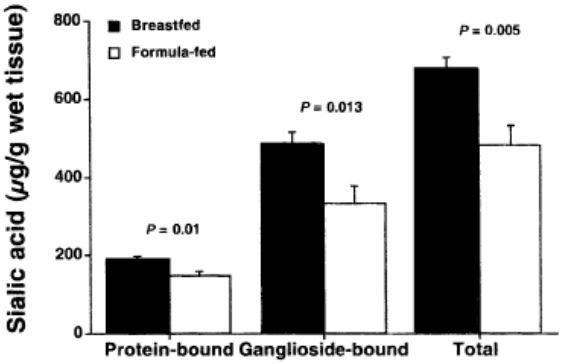


Figure IV.B.4-1 Comparison of brain sialic acid concentrations in frontal cortex tissues obtained from breast-fed *vs*. formula fed infants that died from sudden infant death syndrome. Adapted from Wang *et al*. (2003).

Humans and rats have 2 major periods of brain ganglioside concentration increase. The first occurs during the third intrauterine trimester and other begins shortly after birth (Carlson, 1985). Consistent with the ontogenic timing of brain development and corresponding increased metabolic demand for NANA immediately after birth to replace maternal sources and maintain optimal support of brain ganglioside accretion, the highest concentrations of NANA in human milk are observed during the early phases of lactation (Carlson, 1985; Figure IV.B.4-2).

Although the oral bioavailability of NANA (bound and free) in human infants is not known, studies in juvenile rats have suggested that up to 10% of NANA in brain gangliosides and glycoproteins can originate from bound sources provided in the diet (Carlson and House, 1986).

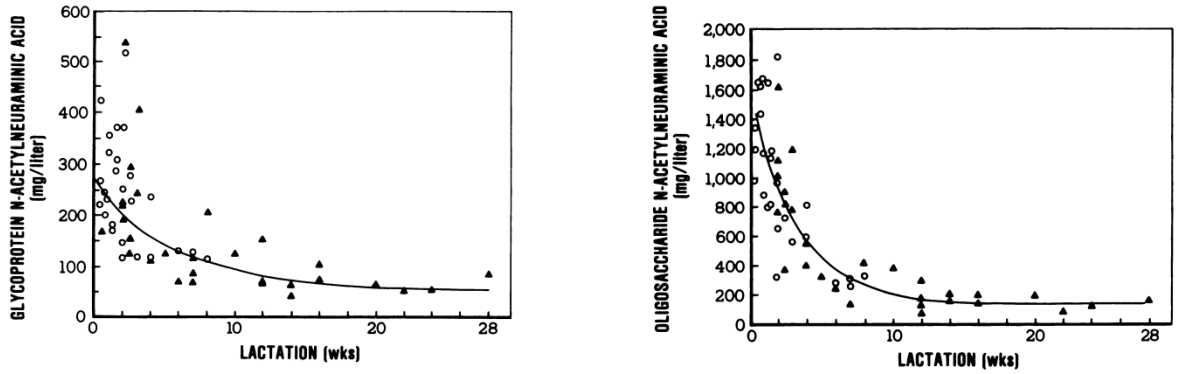


Figure IV.B.4-2 Concentrations of glycoprotein and oligosaccahride NANA in human milk over 28 weeks of lactation. Adapted from Carlson (1985).

The above observations suggest that NANA may be conditionally essential during early infancy and that maintenance of optimal nutritional status of NANA in the infant may require exogenous dietary sources. The AAP recommends that infants are provided with sufficient amounts of all nutrients to achieve postnatal rates of growth and nutrient accretion that approximate those of a normal fetus during the same period (AAP, 1998). The addition of NANA to infant formula is therefore consistent with efforts to produce formulas that are compositionally similar to human milk.

In addition to information supporting the role of NANA in infant nutrition, NANA also may be required for optimal nutrition in adults as advancing age is associated with decrease levels of brain gangliosides and sialoglycoproteins (Svennerholm *et al.*, 1997).

IV.B.5 Metabolic Fate

Pharmacokinetic studies on orally administered NANA were identified in the scientific literature. These studies were carried out in mice and rats and were conducted on a NANA product prepared by enzymatic methods (Witt *et al.*, 1979; Nöhle and Schauer, 1981). In each study, NANA was orally administered to animals *via* gavage as a radiolabeled product. In the study by Witt *et al.* (1979), 3-day-old Sprague-Dawley pups were administered 0.1 mL of a 0.7% *N*-acetyl-[^{14}C]neuraminic acid solution, and radioactivity was measured in blood, organs (heart, liver, brain, kidneys, spleen, and lungs), and urine for periods of up to 6 hours post-administration. Radioactivity from the radiolabeled NANA product was detected in plasma within 30 minutes of administration, with peak levels attained at 1.5 hours. Plasma concentrations declined rapidly during the next 4 hours. The radiolabeled product(s) were

distributed mainly to the heart, liver, and brain (maximum levels of 0.86, 3.9, and 2.8% of administered dose, respectively), but also distributed to other major organs, including the kidneys, spleen, and lungs. Distribution of radioactivity to organs followed a pattern of uptake similar to that observed in plasma with levels detected in the kidneys and carcass at 30 minutes post-administration and in other organs at 1 hour. Radioactivity levels in organs were reduced by 3 hours post-administration. No sex-dependent variations in uptake or distribution were observed. The radiolabeled product(s) were rapidly excreted in the urine, with radioactivity detected in urine as early as 1 hour post-administration. Urinary levels increased linearly with time thereafter. It was determined that the majority of the administered dose of radioactivity was absorbed from the gastrointestinal tract and that 70% of the administered dose was excreted in the urine within 6 hours, while 30% of the administered dose was retained in the body.

Nöhle and Schauer (1981) evaluated the kinetics of NANA following oral and intravenous administration of double-labeled *N*-acetyl-D-[2-^{14}C,9^{3}H] neuraminic acid to 20-day-old C57 mice. Radioactivity was measured in blood, organs (liver, brain, kidneys, and spleen), urine, and exhaled air for periods of up to 72 hours. The authors reported that 95% of the administered dose entered the intestine within 15 minutes, and 90% of the radioactivity was absorbed from the gastrointestinal tract within 6 hours. ^{3}H- and ^{14}C-radioactivity were detected in plasma within 1 hour of administration, with peak levels of ^{3}H-radioactivity in plasma and organs (10% of administered dose) attained at 3 to 4 hours, while peak levels of ^{14}C-radioactivity in plasma and organs (3% of administered dose) were attained at 2 hours. As reported by Witt *et al.* (1979), the radiolabeled product(s) were distributed mainly to the liver and brain, but also distributed to the kidneys and spleen (maximum ^{3}H-radioactivity levels of 3, 2.9, 0.8, and 0.3% of administered dose, respectively). Significant hydrolysis of NANA by lyase's in tissues was observed. The amount of ^{3}H-radioactivity detected in organs was greater than that of ^{14}C-radioactivity. Hydrolysis products (pyruvate and N-acetyl-D-[6-^{3}H]mannosamine) were metabolized by tissues and up to 30% of the administered radioactivity could be detected as $^{14}CO_2$ in exhaled air. Equimolar ratios of ^{3}H- and ^{14}C-radioactivity were excreted in urine and purity analyses of the urinary products using high voltage paper electrophoresis suggested that NANA was absorbed and excreted intact. Consistent with the results of Witt *et al.* (1979), radiolabeled products were rapidly excreted in the urine, with radioactivity detected in urine as early as 1 hour post-administration. The amount excreted in urine varied among animals; some animals excreted 90% of the dose administered within 1 hour post-administration and others excreted 30% after 6 hours. By 24 hours, only 0.5% of the administered ^{3}H-radioactivity and 0.2% of the administered ^{14}C-radioactivity remained in the body (blood and tissues). In addition, the investigators detected *N*-acetylneuraminate lyase activity in liver, kidney, spleen, brain, and intestinal tissues following incubation with NANA *in vitro*. The metabolites detected in all tissues assessed consisted of *N*-acetylmannosamine and acetic acid. Radioactive pyruvic acid also was detected in kidney tissues as a result of other enzyme activity. The investigators therefore concluded that NANA is likely metabolized by *N*-acetylneuraminate lyase in organs following distribution.

Together, the results of the studies by Witt *et al.* (1979) and Nöhle and Schauer (1981) suggest that NANA may be absorbed intact and distributed to the major organs, particularly the liver, brain, and heart. NANA is partially metabolized by *N*-acetylneuraminate lyase in tissues and utilized as a carbon source for cell metabolism. Excretion products include expired CO_2 and significant amounts of NANA appear to be excreted unchanged in urine. It is expected that any unmetabolized free NANA would be incorporated into the oligosaccharide chains that make up glycolipids, glycoproteins, and free HMOs through the endogenous pathway for NANA (Röhrig *et al*., 2015). It is also anticipated that the hydrolysis product acetylmannosamine, would enter the salvage pathway for NANA, whereby NANA metabolites are converted to NANA (Röhrig *et al*., 2015).

IV.B.6 Toxicological Studies

IV.B.6.1 Repeat-Dose

The toxicity of Glycom's NANA ingredient was investigated in a subchronic (13-week) oral toxicity study preceded by an *in-utero* exposure phase (Choi *et al.*, 2014). The objective of the study was to investigate the effects of NANA on female reproduction and on the general growth and development of rats following *in-utero* and lactational exposure. The *in-utero* and lactational phases were followed by a 13-week dietary toxicity study conducted with the F_1 (first generation) offspring to assess for systemic toxicity. The study design was consistent with those previously used for the safety assessment of other infant formula nutrients associated with neural development, including docosahexaenoic acid (DHA) and arachidonic acid (ARA) (Burns *et al.*, 1999; Hempenius *et al.*, 2000; Lina *et al.*, 2006; Casterton *et al.*, 2009; Fedorova-Dahms *et al.*, 2011). The study was conducted in compliance with the Organisation for Economic Co-operation and Development (OECD) Principles of Good Laboratory Practices (GLP), and the 13-week dietary toxicity phase in the F_1 generation was conducted according to OECD Guideline Test No. 408 (OECD, 1998), and in consideration of U.S. FDA Redbook Guideline for Subchronic Toxicity Studies with Rodents (U.S. FDA, 2003), and the International Conference on Harmonisation of Technical Requirements of Pharmaceuticals for Human Use (ICH) Guideline M3(R2) (ICH, 2009).

Male and female (nulliparous and non-pregnant) CD® [Crl:CD®(SD)] rats were randomized into 4 groups of 26 animals/sex/group; these animals comprised the parental (P) animals. P females were provided diets (ground, certified chow; Meal Lab Diet Certified Rodent Diet #5002 from PMI Nutrition International) containing NANA (98.6% purity) at dietary concentrations of 0.5 (low-dose), 1.0 (mid-dose), or 2.0 (high-dose) % for 28 days prior to mating and throughout the mating, gestation, and lactation periods up until weaning of the first generation (F_1) animals on post-natal day (PND) 21. Animals, including F_1 pups after weaning, were housed individually in suspended, stainless steel, wire-mesh cages, unless otherwise noted. P males received the test diet only during the mating period and were euthanized after

mating. During mating, P males and females were paired in a 1:1 ratio for 7 days. The day on which positive evidence of mating was observed was designated as gestation day (GD) 0, and the day on which all F_1 pups from a litter were delivered was designated as PND 0. On GD 20, pregnant females were individually housed in plastic cages (with wood chip bedding) and were allowed to deliver their litters naturally. Dams were housed individually with their litters until weaning on PND 21. On PND 0, 8 live pups (4 males and 4 females) from each litter were randomly selected for inclusion in the study, and on PND 4, pups were weighed and litters were culled to include only the selected pups. At weaning on PND 21, 1 male and/or 1 female from each litter were randomly selected to continue on the 13-week (91-day) dietary toxicity phase of the study. The control and high-dose F_1 groups each consisted of 30 animals/sex and the low- and mid-dose groups each consisted of 20 animals/sex. A 4-week (28-day) recovery period was included for which 10 animals/sex were selected from each the control and high-dose groups. P females were necropsied on PND 22.

The following female reproductive parameters were recorded: number of females paired, mated, and pregnant; female mating, fertility, and fecundity indices; and number of females with confirmed mating day. For each litter, the total number of pups born, number of stillborn pups, number of live pups, number of male and female pups, individual body weights, and any gross abnormalities were recorded. The gestation index, stillborn index, and pup sex ratio also were calculated. Following parturition, the incidence of dead pups was recorded and pup survival indices (viability and lactation indices) were calculated. In addition, the following parameters were recorded at regular intervals: number of male and female pups, pup sex ratio, body weight, and observations of external examinations. The numbers of implantations were recorded upon necropsy of P females.

All animals were observed for mortality and morbidity twice daily. A detailed clinical examination was performed weekly, and body weight and food consumption were measured weekly for P females during the pre-mating, gestation, and lactation periods and for F_1 animals during the 13-week administration period and recovery period. Body weight changes, food efficiency, and test article consumption also were assessed for P females and F_1 animals. Opthalmoscopic examinations were performed on F_1 animals shortly after weaning and prior to necropsy. F_1 animals (10/sex/group) also were subjected to a functional observational battery (FOB) test in the last week of the 13-week administration period. Hematology, coagulation, clinical chemistry, and urinalysis were performed on samples from 10 F_1 males and 10 F_1 females per group in the last week of the administration period and on samples from 5 F_1 males and 5 F_1 females per recovery group at the end of the recovery period. A detailed macroscopic examination was conducted on all animals at necropsy, except for P males. Terminal body weights and organ weights were recorded for all animals. Histopathological examination of all organs and tissues was conducted for 10 F_1 males and 10 F_1 females of the control and high-dose groups at the end of the administration period.

Observations in P Females

There were no mortalities observed in P females. In addition, no clinical observations attributable to NANA were noted. There was a slight, yet statistically significantly, lower mean body weight observed at PND 14 in the low-dose group as compared to the control group, which was accompanied by a significantly lower body weight gain during the PND 7 to 14 interval. There were no other statistically significant changes in body weights observed throughout the administration period in any dose group. Transient statistically significant differences in body weight gains were observed in all dose groups. These differences in body weight gains were incidental in nature, were not dose-dependent, and had no effect on the overall body weights of dams administered NANA compared to control dams throughout the administration period. Mean food consumption was slightly, yet statistically significantly, lower in the mid- and high-dose groups in the second week of the pre-mating period and in the low-dose group in the second week of gestation. In addition, a statistically significant increase in cumulative food efficiency was observed in the low-dose group during the lactation period. These changes in food consumption and cumulative food efficiency were transient and slight with no dose-dependency or remarkable overall effects. The statistically significant findings were therefore considered to be incidental. The compound consumption rate for all periods combined (pre-mating, gestation, and lactation) for P females was 472, 946, and 1,895 mg/kg body weight/day in the 0.5, 1.0, and 2.0% NANA dietary groups, respectively. No compound-related macroscopic findings were observed. Furthermore, no statistically significant effects on reproductive performance or fertility parameters were observed between dams administered NANA and control dams. Parturition and litter parameters also were unaffected by dietary supplementation with NANA.

Observations in F_1 Pups

No compound-related signs of toxicity or gross external abnormalities were noted in F_1 offspring on PND 0. Shortly following birth, a slight increased incidence of clinical observations in the F_1 pups of NANA administered dams was noted when compared to F_1 pups of control dams. These included a decrease in activity and cold skin upon touch in 2 males of the mid-dose group and 4 males of the high-dose group; of these, 1 was found dead and 2 were missing (likely due to maternal cannibalism). These observations are common findings in the rat during the first four days of parturition, and therefore, were not considered to be compound-related. All other clinical observations were incidental in nature.

During the pre-weaning period, the mean body weight of male pups of the high-dose group was slightly lower (by 4 to 8%) than that of the control group, with statistical significance noted for PND 7 and 21. Growth rates, however, were similar among groups throughout the pre-weaning period, and therefore, pup growth has not considered to be adversely affected by maternal NANA administration to any extent. No statistically significant differences in mean body weight were noted for female pups in the NANA groups compared to the control group.

13-Week Dietary Toxicity Study in F_1 Animals

During the 13-week administration period in F_1 animals, the average compound consumption in males was 247, 493, and 974 mg/kg body weight/day at the 0.5, 1.0, and 2.0% NANA dietary concentrations, respectively. For females, the average compound consumption was 318, 646, and 1,246 mg/kg body weight/day at the 0.5, 1.0, and 2.0% NANA dietary concentrations, respectively.

No mortality was observed in F_1 animals during the 13-week administration period. Several clinical signs were observed in 3 to 5 males of the high-dose group during the last 3 weeks of the study, including aggressive behavior, malocclusion, and black material around the eyes. Two of these animals presented with several clinical signs, one of which was euthanized moribund (on day 74) due to a non-compound related-self-induced injury to the palate. The other male continued on the study and exhibited a lower body weight when compared to the other males of the high-dose group. Clinical signs in this male began to subside during the last few weeks of the study. The clinical signs noted in males could not be attributed to NANA as no consistent pattern was observed. No notable clinical signs were observed in females throughout the 13-week administration period or in any animals of the recovery group. Furthermore, no compound-related ophthalmoscopic findings were noted.

Although a few statistically significant changes were observed in the FOB tests and locomotor activity assessment, the changes were incidental in nature and not dose-dependent. It was therefore concluded that dietary administration of NANA had no adverse effects on neurobehavioral responses.

As a result of having reduced body weights in the pre-weaning period, males of the high-dose group entered the 13-week administration period with slight, yet statistically significant, lower body weights relative to control males, leading to lower body weights throughout the 13-week administration and recovery periods. The growth rates, however, were similar among all male groups and the mean body weight of high-dose males remained greater than 90% of that of the male control group throughout the course of the study. The lower body weights in high-dose males were therefore not considered as an adverse effect or toxicologically relevant. A slight, yet statistically significant, lower food consumption accompanied the lower body weights to the same degree in high-dose males, but was considered to be a response to the lower body weights of these animals and not a compound-related effect. There were no statistically significant differences in mean body weight or food consumption between NANA-administered females and control females. Additionally, no compound-related effects on food efficiency were observed.

Few statistically significant changes were observed in the hematology and clinical chemistry data of NANA-administered groups compared to the control groups at the end of the administration period. These changes consisted of a decrease in platelet counts in males of the

high-dose group, an increase in leukocyte counts in both males and females of the mid-dose group, and a decrease in creatinine levels in females of the mid-dose group. The changes observed at the mid-dose were not observed at the high-dose (*i.e.*, were not dose-dependent), and therefore, were considered to be incidental and not compound-related. Moreover, the decrease in platelet counts observed in males of the high-dose group was primarily attributed to a low platelet count in 1 male, which may have been a result of the occurrence of *situs invertus* in this animal detected at necropsy. The decrease in platelet counts was thus considered not to be biologically significant as platelet counts of all other high-dose males were comparable to those of the control males. Furthermore, none of the changes observed were associated with histopathological findings. No statistically significant effects on coagulation parameters and no statistically significant changes or notable differences in urinalysis parameters were observed at the end of the administration period between the NANA groups and the control groups.

A statistically significant decrease in kidney weights relative to body weight was observed in mid-dose females and a statistically significant decrease in absolute and relative ovary weights was observed in high-dose females when compared to control females. All organ weight changes were small in magnitude and were non-dose-dependent, and were therefore, considered to be normal biological variations. Moreover, changes in organ weights were not associated with histopathological findings. No other statistically significant differences in organ weights relative to body weight were observed. Furthermore, no compound-related macroscopic or histopathological findings were observed in other organs as well.

Based on the results of the above study, NANA was without maternal toxicity or compound-related adverse effects on female reproduction and general growth and development of offspring at maternal dietary concentrations of up to 2%, providing doses of up to 1,895 mg/kg body weight/day, the highest dose tested, in CD® [Crl:CD®(SD)] rats. In addition, no compound-related adverse effects were observed during the 13-week administration period in F_1 animals at doses of up to 974 mg/kg body weight/day in males and 1,246 mg/kg body weight/day in females. The NOAEL for the subchronic toxicity of NANA was determined to be to 974 mg/kg body weight/day in male CD® [Crl:CD®(SD)] rats and 1,246 mg/kg body weight/day in female CD® [Crl:CD®(SD)] rats, the highest doses tested. The overall NOAEL for the oral toxicity of NANA in rats was determined to be 974 mg/kg body weight/day.

IV.B.6.2 Other Oral Studies

Three other animal studies involving oral administration of NANA were identified in the scientific literature. Although these studies were of short-term exposure and were not specifically designed to assess for toxicity and are therefore limited for the purpose of a safety assessment, the results demonstrate that there were no adverse effects on endpoints related to general toxicity following oral exposure.

In one study, 8-week-old male Sprague-Dawley rats were randomly assigned to receive either a control diet or supplemented diets, including a diet supplemented with 1% NANA (6 animals/group), for 2 weeks (Sakai *et al.*, 2006). Based on an average body weight of 382.5 g and an average total food intake of 437 g (27.3 g/day) over the 2-week feeding period, the intake of NANA was calculated to be 714 mg/kg body weight/day in animals fed the NANA supplemented diet. No statistically significant differences in final body weight, total body weight gain, total food intake, food efficiency, or absolute brain weight were observed between the NANA supplemented group and the control group. In addition, no statistically significant differences in learning behavior (assessed *via* the T-maze learning test and the Morris-swimming maze test) were observed between groups.

In another study, 3-day-old male domestic piglets (Landrace/Large White cross) were randomly allocated to receive either a control pig milk replacer or a NANA supplemented milk replacer containing 300, 635, or 830 mg/L of NANA for a period of 36 days (Wang *et al.*, 2007). These concentrations represented an approximate intake of 40, 85, 180, and 240 mg/kg body weight/day of NANA, respectively. No statistically significant differences in body weight or body weight gain were observed between the NANA supplemented groups and the control group throughout the course of the study. In addition, no compound-related adverse effects on learning or memory performance (using a radial maze and visual cues) were observed.

Lastly, Hiratsuka *et al.* (2013) investigated the effects of maternal dietary NANA supplementation in n-3 fatty acid-deficient dams on the learning abilities of F_1 offspring after weaning. In this study, 9-week-old female Wistar rats were fed a diet deficient or adequate in n-3 fatty acids for 3 weeks until mating at 12 weeks. During pregnancy and lactation, half of the dams on the diet deficient in n-3 fatty acids continued on this diet (control group), while the other half were fed the same diet but were supplemented with 1% NANA in drinking water. The dams fed the n-3 fatty acid-adequate diet were not supplemented with NANA. After weaning, F_1 animals were fed the same diet as their dams for 25 or 26 days, but were not supplemented with NANA. Average maternal body weights before parturition and after weaning were not significantly different between groups. The final body weights of F_1 animals also were not significantly different between groups. Water intake of dams in the NANA supplemented group was significantly reduced compared to the other 2 groups. No adverse effects on memory performance (assessed in the novel object-recognition test) were observed in the F_1 animals from dams supplemented with NANA; the results of this test were similar among the F_1 animals from dams supplemented with NANA and the F_1 animals from dams fed the n-3 fatty acid adequate diet.

IV.B.6.3 Mutagenicity and Genotoxicity Studies

The potential mutagenicity of Glycom's NANA (98.6% purity) was assessed in a bacterial reverse mutation test using *Salmonella typhimurium* test strains TA98, TA100, TA1535, and TA1537, and *Escherichia coli* WP2 *uvr*A in the absence and presence of metabolic activation

(S9), using the plate-incorporation and pre-incubation methods (Choi *et al.*, 2014). The study was conducted in compliance with the OECD principles of GLP and in accordance with the ICH Guidelines for genotoxicity testing (ICH, 1996, 1997) and OECD Guideline Test No. 471 (OECD, 1997). The water vehicle served as a negative control for all strains. One of the following compounds was used as a positive control in assays conducted in the absence of S9: 2-nitroflurorene (TA98), sodium azide (TA100 and TA 1535), 9-aminoacridine (TA1537), and methyl methanesulfonate (WP2 *uvr*A). For assays conducted in the presence of S9, 2-aminoathracene was used as the positive control. Based on the results of a preliminary test, the following NANA concentrations were selected for the main test: 0, 50, 150, 500, 1,500, and 5,000 µg/plate. NANA did not induce cytotoxicity at any of the concentrations tested in either the preliminary test or the main test. In addition, no precipitation of the test article was observed at any concentration in either test. Treatment with NANA did not result in significant increases in the number of revertants compared with the negative control at any concentration in both experiments either in the presence of absence of S9. In contrast, positive control agents substantially induced the number of revertant colonies compared to the negative control. Thus, NANA was determined to be non-mutagenic in the Ames test at concentrations of up to 5,000 µg/plate.

The genotoxic potential of NANA (98.6% purity) was further investigated in an *in vitro* mammalian cell micronucleus test conducted in human peripheral blood lymphocytes (Choi *et al.*, 2014). The test was conducted in compliance with the OECD principles of GLP and in accordance with OECD Test No. 487 (OECD, 2010) using the cytokinesis-block method as described by Fenech and Morley (1986). Human peripheral blood lymphocytes were obtained from a healthy, non-smoking 23-year-old male with no history of radiotherapy, viral infection, or drug administration. The water vehicle served as a negative control. Based on the results of a preliminary test, the following NANA concentrations were selected for the main test: 150, 300, 600, 1,180, 1,690, 2,420, and 3,450 µg/mL. In the absence of S9, 0.4 µg/mL mitomycin C and 20 ng/mL vinblastine were used as positive controls in the 4-hour treatment group, while 0.4 µg/mL mitomycin C and 10 ng/mL vinblastine were used in the 24-hour treatment group. In the presence of S9 (4-hour treatment group), 10 µg/mL cyclophosphamide was used as the positive control. NANA did not induce substantial cytotoxicity (≥ 55 ± 5%) in human peripheral blood lymphocytes compared to the negative control at any concentration or under any of the exposure conditions in the preliminary test or main test. The three highest concentrations of NANA (1,690, 2,420, and 3,450 µg/mL) tested in the main test were selected for micronucleus examination. There were no statistically significant differences observed in the percentage of micronucleated cells at any of the NANA concentrations analyzed compared to the negative control. In contrast, treatment with the positive control agents resulted in a significant increase in the percentage of micronucleated cells compared to the negative control. NANA was therefore determined to be non-genotoxic in human peripheral blood cells.

IV.B.7 Safety of Enzyme

NANA is synthesized using an aldolase enzyme preparation (N-acetylneuraminate lyase) obtained from a modified strain of *Escherichia coli* BL21 overexpressing the *N*-acetylneuraminate lyase gene (nanA) from *E. coli*. The enzyme catalyzes the reversible aldol cleavage of N-acetylneuraminic acid to form pyruvate and N-acetylmannosamine (ManNAc) *via* a Schiff base intermediate. The safety of the host organism (*E. coli* BL21) for use in the production of food grade enzyme preparations and pharmaceuticals has been the subject of previous comprehensive reviews by the FDA, and by other Qualified Experts (Ferrer-Miralles *et al*., 2009; U.S. FDA, 2014). Therefore, data and information supporting the determination that that *E. coli* BL21 is a non-pathogenic and non-toxigenic host, and therefore generally recognized as safe and suitable for use in the production of food enzymes is incorporated by reference to GRAS notice 485 (U.S. FDA, 2014).

The N-acetylneuraminate lyase gene (nanA) used for expression of the enzyme is of *E. coli* origin. A bioinformatics search of the complete amino acid sequence of the enzyme was conducted using the National Institute of Medicine BLAST (Basic Local Alignment Search Tool) search tool. Alignments of 99 to 100% against N-acetylneuraminate lyase proteins originating from *E. coli* and other *Enterobacteriaceae* species were identified indicating that the enzyme is a common protein widely expressed by *Enterobacteriaceae* species. No homologous matches to putative toxins or pathogenicity factors were apparent. As members of *Enterobacteriaceae*, including *E. coli*, are ubiquitous human commensal microorganisms in the lower gastrointestinal tract of humans, there is a history of safe exposure to this enzyme.

In addition to general BLAST searches, an allergenicity search was conducted using the Allergen Online database. The Allergen Online database version 15 (Updated January 12, 2015) was used to conduct a preliminary screen of the complete N-acetylneuraminate lyase protein sequence for relevant matches against putative allergens. This database is maintained by the Food Allergy Research and Resource Program of the University of Nebraska. A FASTA3 overall search of Allergen Online was conducted using default settings (E cutoff = 1 and maximum alignments of 20). No sequences with E value <1.0 were identified. An 80 amino acid sliding window (segments 1-80, 2-81, 3-82, *etc*.) also was used to scan the amino acid sequence of the protein against the allergen database using FASTA3 to search for matches of 35% identity or more. This 35% identity for 80 amino acid segments is a suggested guideline proposed by Codex for evaluating proteins in genetically modified crops (Codex, 2003; Goodman *et al*., 2008).

The results of the FASTA3 alignments of all possible 80 amino acid segments of the enzyme against all putative allergen sequences in the database were all less than the 35% threshold over 80 amino acids. Based on the information demonstrating the widespread history of exposure to the enzyme from *Enterobacteriaceae* sp. residing in the gastrointestinal tract of all humans combined with the findings from the bioinformatics assessment it can be concluded

that *N*-acetylneuraminate lyase does not represent an allergenic or toxicity risk. More importantly the enzyme preparation is effectively removed during manufacturing using sequential filtration and crystallization steps and protein residues are absent from finished product at levels above the limit of quantification using enzyme specific ELISA and protein analyses methods.

IV.C Expert Panel Evaluation

Glycom A/S has determined that NANA is GRAS for use in infant formula and in food as described in Section I.D on the basis of scientific procedures. This GRAS determination is based on data generally available in the public domain pertaining to the safety of NANA, as discussed herein, and on consensus among a panel of experts (the Expert Panel) who are qualified by scientific training and experience to evaluate the safety of infant formula ingredients and food ingredients. The Expert Panel consisted of the following qualified scientific experts: Dr. Joseph F. Borzelleca (Virginia Commonwealth University School of Medicine), Dr. Ronald Kleinman (Mass General Hospital for Children), Dr. Robert J. Nicolosi (University of Massachusetts Lowell), and Dr. John A. Thomas (Indiana University School of Medicine).

The Expert Panel, convened by Glycom A/S, independently and critically evaluated all data and information presented herein, and also concluded that NANA was GRAS for use in infant formula and in food as described in Section I.D based on scientific procedures. A summary of data and information reviewed by the Expert Panel, and evaluation of such data as it pertains to the proposed GRAS uses of NANA is presented in Appendix A.

IV.D Conclusion

Based on the above data and information presented herein, Glycom A/S has concluded that the intended uses of NANA in infant formula and in food, as described in Section I.D, is GRAS based on scientific procedures. General recognition of Glycom A/S's GRAS determination is supported by the unanimous consensus rendered by an independent Panel of Experts, qualified by experience and scientific training, to evaluate the use of NANA in infant formula and in food, who similarly concluded that the intended use of NANA in infant formula and in food as described herein is GRAS.

NANA therefore may be marketed for its intended purpose in the U.S. without the promulgation of a food additive regulation under Title 21, Section 170.3 of the Code of Federal Regulations.

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Appendix A

Expert Panel Consensus Statement Concerning the Generally Recognized as Safe (GRAS) Status of the Proposed Infant Formula and Food Uses of *N*-acetyl-d-neuraminic acid (NANA, Sialic Acid)

Expert Panel Consensus Statement Concerning the Generally Recognized as Safe (GRAS) Status of the Proposed Infant Formula and Food Uses of Human-identical Milk Monosaccharide *N*-Acetyl-D-neuraminic acid (NANA, Sialic Acid)

April 17, 2015

INTRODUCTION

Glycom A/S (Glycom) convened a panel of independent scientists (the "Expert Panel"), qualified by their scientific training and relevant national and international experience to evaluate the safety of food ingredients, to conduct a critical and comprehensive evaluation of the available pertinent data and information on the human-identical milk monosaccharide *N*-acetyl-D-neuraminic acid dihydrate (NANA), and to determine whether the proposed uses of NANA in food production would be Generally Recognized as Safe (GRAS) based on scientific procedures.

The Expert Panel consisted of the below-signed qualified scientific experts: Dr. Joseph F. Borzelleca (Virginia Commonwealth University School of Medicine), Dr. Ronald Kleinman (Mass General Hospital for Children), Dr. Robert J. Nicolosi (University of Massachusetts Lowell), and Dr. John A. Thomas (Indiana University School of Medicine).

The Expert Panel, independently and collectively, critically evaluated a comprehensive package of scientific information and data compiled from the literature. This information was presented in a dossier provided by Glycom [Documentation Supporting the Evaluation of the Human-identical Milk Monosaccharide *N*-Acetyl-D-neuraminic Acid (NANA) as Generally Recognized as Safe (GRAS) for Use in Infant Formula and in Food (April 2015)], which included an evaluation of all available scientific data and information, both favorable and unfavorable, relevant to the safety of the intended food uses of Glycom's NANA ingredient. This information was prepared in part from a comprehensive search of the scientific literature performed by Glycom through March 2015 and included information characterizing the identity and purity of the ingredient, manufacture of the ingredient, product specifications, supporting analytical data, intended conditions of use, estimated exposure under the intended uses, information on the history of safe consumption from human breast milk and from food, and information on the safety of Glycom's NANA ingredient. In addition, the Expert Panel evaluated other information deemed appropriate or necessary.

Following its independent critical evaluation, the Expert Panel unanimously concluded that the intended uses described herein of Glycom's NANA ingredient, meeting appropriate food-grade specifications, and manufactured consistent with current Good Manufacturing Practice (cGMP),

are GRAS based on scientific procedures. A summary of the basis for the Expert Panel's conclusion is presented below.

SUMMARY AND BASIS FOR GRAS

The human-identical milk monosaccharide (HiMM) *N*-acetyl-D-neuraminic acid dihydrate (NANA or Neu5Ac), commonly known as sialic acid, is intended to be used as an ingredient in term infant formulas (0 to 12 months) and in a variety of other foods. NANA belongs to a family of over 60 nine-carbon acidic monosaccharides consisting of *N*- or *O*-substituted derivatives of D-neuraminic acid collectively known as sialic acids (reviewed by Angata and Varki, 2002; and Schauer, 2004). NANA specifically is a nine-carbon acidic monosaccharide consisting of an *N*-acetyl substituted derivative of D-neuraminic acid (5-amino-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid). Sialic acids are natural monosaccharides widely found in animals, with the highest concentrations detected in mammalian milk and brain. However, the occurrence of NANA in the absence of other sialic acids is a characteristic feature of human milk; in most other mammalian milks, sialic acid occurs as a mixture with the *N*-glycolyl form, *N*-glycolylneuraminic acid (NGNA)]. NANA occurs in both the bound form as well as in the free form (*i.e.*, the free monosaccharide). The bound form of NANA consists of NANA linked to oligosaccharides *via* a glycosidic bond. These oligosaccharides in turn consist of either free oligosaccharides [*e.g.*, human milk oligosaccharides (HMOs)] or oligosaccharide chains of glycoconjugates (*e.g.*, glycoproteins and glycolipids). In human milk, NANA is present in high concentrations bound to HMOs, glycoproteins, and glycolipids known as gangliosides; however, NANA also occurs in the free (unbound) form in human milk, as well as in a number of other foods of animal origin [*e.g.*, fish eggs (caviar), fish, red meat, dairy products, poultry, and chicken eggs], and it is this free form that is the subject of the GRAS determination as Glycom's NANA ingredient is manufactured as a free monosaccharide.

The molecular structure of NANA has been characterized and is documented in the literature (Kuhn and Baschang, 1962; Kuhn and Brossmer, 1962; Flippen, 1973; Brown *et al.*, 1975; Dabrowski *et al.*, 1979; Friebolin *et al.*, 1980, 1981; Ogura *et al.*, 1984; Klepach *et al.*, 2008a,b). Glycom's NANA has been analytically determined by x-ray crystallography, ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR), and mass spectrometry (MS) to be chemically and structurally identical to the NANA that is present in human milk and is, therefore, referred to as a HiMM. NANA, therefore, has an established long history of safe consumption as a normal component of human milk in infants on the basis that the NANA manufactured by Glycom is chemically identical to NANA present in human milk.

The Expert Panel critically reviewed details of the manufacturing process for Glycom's NANA that is produced through a chemical process that is highly controlled and compliant with cGMP and the principles of Hazard Analysis and Critical Control Points (HACCP). Appropriate control measures and analytical testing are implemented throughout manufacture to ensure production

of a high quality ingredient. The manufacturing process can be broadly divided into two stages. Stage 1 consists of manufacturing the starting material, anhydrous NANA, used to produce the final ingredient NANA. In this stage, anhydrous NANA is produced from the enzymatic coupling of *N*-acetylmannosamine and sodium pyruvate. The *N*-acetylmannosamine source material is produced by Glycom from European Pharmacopeia-grade D-fructose and is of high purity and quality. The sodium pyruvate source material used also is of high purity (>98%) and quality, meeting the specifications established in the European Pharmacopeia. The product of the enzymatic reaction is then subjected to downstream processing to produce crude NANA, which in turn is further processed into anhydrous NANA. The production process for anhydrous NANA is tightly controlled under a HACCP plan with critical control points in place at key steps. All precursors and intermediates are single, well-characterized, and pure compounds and isolated intermediates are analyzed at critical control points to ensure conformance to established strict specifications. Certificates of analysis for these intermediates are then issued and batch release procedures are followed prior to use in the production of anhydrous NANA. The manufactured anhydrous NANA also is a single, well-characterized, and pure compound that is produced in crystalline form. All batches of anhydrous NANA are controlled for conformity to established strict specifications by certificate of analysis, which ensures production of a high quality and high purity material that is acceptable for use in the production of food ingredients/food.

Stage 2 of the manufacturing process consists of purifying the anhydrous NANA starting material by treatment with activated charcoal and subsequent filtration using a sparkler filter to remove coloration and any potential impurities. Microfiltration using a 0.2 µm filter is applied to ensure absence of microbiological contamination in the NANA solution. The NANA solution is then subjected to a crystallization step (with the aid of 2-propanol), and the crystals washed and dried to generate the final NANA dihydrate crystalline powdered product. The manufacturing process for NANA is tightly controlled under a HACCP plan with critical control points in place at key steps where in-process controls are applied to minimize the amount of potential impurities and contaminants to the level technically possible. All processing aids used in the manufacture of NANA from anhydrous NANA are food-grade materials permitted for use as processing aids and all filters and filter aids are those commonly used by the food industry in the purification of food ingredients and are permitted for use in the U.S. for such purposes.

Glycom have established appropriate food-grade specifications for NANA. The specifications for NANA include parameters relating to physical properties, purity, water, ash content, lead, and microbiological contaminants. The assay minimum for NANA is 97%. Specifications also have been established for acetic acid and residual solvents to control for their use/production during manufacturing and carryover of solvents produced from starting materials. While Glycom's NANA ingredient is currently manufactured using chemical processes, specifications for residual proteins and microbial endotoxins have been established to provide a control for impurities that might stem from the use of starting materials produced from fermentation and to

provide further reassurance that Glycom's NANA ingredient does not contain residual proteins or microbial endotoxins. All analytical methods are nationally or internationally recognized or have been validated by Glycom. The Expert Panel reviewed the results from 5 batches of NANA and confirm that the data demonstrate that the manufacturing process produces a consistent material in conformance with the product specifications.

The current shelf-life of crystalline NANA has been established by Glycom as 36 months when stored under ambient conditions (25°C at 60% relative humidity), 5°C, or -20°C based on the results of an ongoing 5-year real-time stability study. The Expert Panel reviewed the analytical results of the real-time stability study available to date, which confirm that NANA is stable for 36 months, with no significant change in the assay value or in water content, when stored under these conditions. A 24-month accelerated stability study also was conducted, the results of which demonstrate that NANA is stable for up to 24 months, but displays discoloration (the cause for which could not be determined), under accelerated conditions (40°C, 75% relative humidity). The shelf-life of NANA is therefore established as 36 months based on the results of the real-time stability study. The Expert Panel also reviewed the results of the stability studies conducted in food matrices representative of the conditions of use of NANA in infant formula and other food and beverage products. NANA has been demonstrated to be stable in powdered infant formula for 90 days in an ongoing 3-year study when stored at 5°C, 25°C/60% relative humidity, 30°C/65% relative humidity, or 40°C/75% relative humidity. NANA was further demonstrated to be stable in yogurt, ready-to-drink flavored milk, and a citrus fruit drink following typical processing conditions (including pasteurization) and storage over a period of 21, 14, and 28 days, respectively, when stored under refrigeration (4°C). The analytical data also demonstrated that NANA is stable when added to cereal bars and following storage under ambient conditions over a 3-month period. Therefore, NANA has been sufficiently demonstrated to be stable in infant formula and representative food and beverages under the conditions of these studies.

NANA is intended for use in term infant formulas and in a variety of other foods and beverages from selected food categories, including infant and toddler foods (toddler formulas, other baby foods for infants and young children, and other drinks for young children), baked goods and baking mixes, beverages and beverage bases, coffee and tea, dairy product analogs, grain products and pastas, milk (whole and skim), milk products, processed fruits and fruit juices, processed vegetables and vegetable juices, and sugar substitutes.

The maximum level of use of NANA in term infant formulas is 50 mg/L and is based on providing a similar level of free NANA as that which occurs in human milk, which ranges from 20 to 60 mg/L in early human milk (Wang *et al.*, 2001; Martín-Sosa *et al.*, 2004) and 10 to 50 mg/L in mature human milk (Hayakawa *et al.*, 1993; Thurl *et al.*, 1996; Wang *et al.*, 2001; Wiederschain and Newburg, 2001; Martín-Sosa *et al.*, 2004; Qiao *et al.*, 2013).

The maximum use level in toddler formulas and other drinks for young children also is 50 mg/L and that in other baby foods for infants and young children is 50 mg/kg. The maximum intended use level in most other foods range from 50 to 500 mg/kg or mg/L, while the maximum intended use level in breads and baked goods and meal replacement bars is 1,000 to 2,000, and 1,700 mg/kg, respectively, and that in beverage whiteners and table top sweeteners is 25 and 12.5 g/kg, respectively.

An estimate of the dietary exposure to NANA in the United States (U.S.) population was performed using individual proposed food uses and use levels in conjunction with survey data obtained from the U.S. National Center for Health Statistics' (NCHS) National Health and Nutrition Examination Surveys (NHANES) 2009-2010 (CDC, 2011; USDA, 2012). The estimated daily intake of NANA in infants from the use of NANA in infant formulas only was calculated as part of the dietary exposure assessment and reviewed to determine the estimated daily intake of NANA from this use alone in the target population group (*i.e.*, infants). Infants ages 0 to 6 months were determined to have the greatest all-user estimated 90th percentile intake of NANA from infant formulas only. This intake was determined to be 64.1mg NANA/person/day on an absolute basis and 11.6 mg/kg body weight/day on a body weight basis. In infants ages 7 to 12 months, the use of NANA in infant formulas only resulted in an all-user estimated 90th percentile intake of 54.9 mg NANA/person/day on an absolute basis and 6.1 mg/kg body weight/day on a body weight basis. Infant formula also was determined to be consumed by a small percentage (1.1%) of toddlers 1 to 3 years of age. In this age group, the use of NANA in infant formulas only resulted in an all-user estimated 90th percentile intake of 29.3 mg NANA/person/day on an absolute basis and 2.4 mg/kg body weight/day on a body weight basis.

The estimated daily intakes of NANA from its use in term infant formulas combined with its use in foods also were calculated for infants and toddlers in order to determine the total daily intake of the ingredient from all sources in the population groups identified as consumers of infant formula. In infants 0 to 6 months of age, the all-user estimated 90th percentile intake of NANA from all sources was determined to be 67.1 mg/person/day on an absolute basis and 11.8 mg/kg body weight/day on a body weight basis. The greatest contributor to the estimated daily intake of NANA in this infant age group was the use in infant formula at a contribution level of 87.2%, while the use in baby foods for infants and young children contributed 10.9%. No contribution of toddler formulas to the estimated intakes of NANA in this infant age group occurred. In infants 7 to 12 months of age, the all-user estimated 90th percentile intake of NANA from all sources was determined to be 79.2 mg/person/day on an absolute basis and 9.2 mg/kg body weight/day on a body weight basis. The greatest contributor to the estimated daily intake of NANA in this infant age group was the use in infant formulas at a contribution level of 45.8%, while use in baby foods for infants and young children contributed 22.4%. The contribution of toddler formulas to the estimated intakes of NANA in this infant age group was 1.1%. In toddlers ages 1 to 3 years, the all-user estimated 90th percentile intake of NANA from all

sources was determined to be 87.9 mg/person/day on an absolute basis and 6.6 mg/kg body weight/day on a body weight basis.

The estimated daily intake of NANA in all other population groups from all food uses of NANA also was calculated as part of the above dietary exposure assessment. From this assessment, the greatest estimated 90th percentile all-user daily intake of NANA from all uses on a body weight basis was determined to be in children at 3.2 mg/kg body weight/day (81.8 mg/person/day on an absolute basis). The estimated 90th percentile all-user daily intakes of NANA were relatively similar on a body weight basis among all other population groups, ranging from 1.6 to 2.2 mg/kg body weight/day.

The above estimated daily intakes of NANA were compared to the intake of free NANA from human milk in infants. As mentioned, the level of free NANA present in early human milk is 20 to 60 mg/L and that in mature human milk is 10 to 50 mg/L. Based on the level of free NANA in early breast milk and on a 3.4-kg newborn infant (WHO, 2013) drinking 600 mL of breast milk per day during the first month (Hester *et al.*, 2012), the intake of free NANA from early breast milk was calculated to be 3.5 to 11 mg/kg body weight/day in young infants. Likewise, based on the level of free NANA in mature breast milk and on a 6.5-kg infant drinking approximately 1 L of mature human milk per day (Davies *et al.*, 1994; Hester *et al.*, 2012), the intake of free NANA from mature human milk was calculated to be 1.5 to 7.8 mg/kg body weight/day. Evidence from the literature suggests that additional amounts of free NANA also would be released from HMOs during passage through the infant gastrointestinal tract, owing to the limited acid stability of the glycosidic bond of NANA (Sabharwal *et al*., 1991; Sonnenburg *et al.*, 2002; Lacomba *et al*., 2011). Considering that Glycom's NANA is chemically and structurally identical to the NANA present in human milk, this background dietary exposure to free NANA from human milk serves as the safe range of Glycom's NANA intakes for infants, as well as for older population groups given the comparative sensitivity and susceptibility of infants from a safety perspective.

In comparison to the intakes of NANA from human milk, the greatest estimated 90th percentile daily intake of Glycom's NANA in infants from its use in infant formula alone was calculated to be 11.6 mg/kg body weight/day, which was determined to occur in infants ages 0 to 6 months. The estimated intake of NANA from infant formula only is therefore well within the safe range of NANA intake from human milk in infants. When all food uses, including infant formula, were considered, the greatest estimated 90th percentile daily intake of NANA in infants was determined to be 11.8 mg/kg body weight/day, which was determined to occur in infants ages 0 to 6 months. The estimated intake of NANA from all intended food uses, including infant formula, is therefore also within the safe range of NANA intake from human milk in the infants. Therefore, the estimated intake of NANA in infants from the consumption of infant formula and all other foods and beverages lies within the safe intake levels of NANA from human milk. The estimated intakes of NANA from all food uses in all other population groups also lie within the

safe intake levels of NANA from human milk in infants, which serve as the safe reference intakes.

The Expert Panel further noted that a low background dietary exposure to free NANA also exists as a result of its presence in edible animal tissues and other foods of animal origin. While such foods contain high levels of sialic acids mainly in the form of glycoconjugates of NANA and NGNA (Tangvoranuntakul *et al.*, 2003; Bardor *et al.*, 2005; Martín *et al.*, 2007; Lacomba *et al.*, 2010, 2011; Sørensen, 2010; Spichtig *et al.*, 2010; Taylor *et al.*, 2010; Hurum and Rohrer, 2012; Chen *et al.*, 2014; Samraj *et al.*, 2015), recent quantitative data indicates the presence of free NANA in a wide array of animal tissues, including fish eggs, fish, red meats, milk and milk products, poultry meat, and chicken eggs (Samraj *et al*., 2015). Using data obtained from the literature regarding the concentrations of free NANA in foods combined with dietary intake data from the NHANES 2011-2012 dataset, background dietary exposure to free NANA was calculated for the U.S. population. On a body weight basis, the 90th percentile background exposure to free NANA was determined to range from 0.17 mg/kg body weight/day in female adults to 1.70 mg/kg body weight/day in infants. Thus, a safe low background exposure to exogenous free NANA exists in the general human population from foods of animal origin.

The Expert Panel critically evaluated published data and information characterizing the safety of NANA. The data included information pertaining to the metabolic fate of NANA, which indicates that NANA is almost completely absorbed following oral administration with the bulk of the dose excreted unchanged in urine (Witt *et al.*, 1979; Nöhle and Schauer, 1981). Small amounts of NANA are distributed to major organs, such as the liver, brain, and heart, where the compound may be metabolized by *N*-acetylneuraminate lyase to acetylmannosamine and acetic acid. Pyruvic acid also may be formed from the action of *N*-acetylneuraminate lyase together with other enzymes. It is expected that any unmetabolized free NANA would be incorporated into the oligosaccharide chains that make up glycolipids, glycoproteins, and HMOs through the endogenous pathway for NANA and that the NANA metabolite, acetylmannosamine, would enter the salvage pathway for NANA, whereby NANA metabolites are converted to NANA.

The Expert Panel critically evaluated the toxicological data on Glycom's NANA, including a subchronic (13-week) oral toxicity study preceded by an *in-utero* exposure phase conducted in CD® [Crl:CD®(SD)] rats (Choi *et al.*, 2014). NANA was provided in the diet at dietary concentrations of 0.5 (low-dose), 1.0 (mid-dose), or 2.0 (high-dose) %. The experimental design of the study was such that parental (P) females were administered NANA for 28 days prior to mating, as well as throughout the mating, gestation, and lactation periods up until weaning of the first generation (F_1) animals on post-natal day (PND) 21. P females were necropsied and examined on PND 22. The dosing schedule therefore allowed for *in utero* and lactation exposure of F_1 animals prior to commencement of the 90-day study in the F_1 generation to determine any effects of NANA on the general growth and development of the animals. Female reproductive performance/fertility, as well as parturition and F_1 litter

observations (*e.g.*, gestation length, number of liveborn and stillborn pups/litter, total implantation scars/litter, sex ratio, pup viability and lactation indices), also were assessed to determine any effects of NANA on these parameters. At weaning on PND 21, one male and/or one female from each F_1 litter were then randomly selected to continue on the 13-week dietary toxicity phase of the study. The control and high-dose F_1 groups each consisted of 30 animals/sex and the low- and mid-dose groups each consisted of 20 animals/sex. A 4-week (28-day) recovery period was included for which 10 animals/sex were selected from each the control and high-dose groups.

The design of this study is consistent with the protocol used in the safety assessment of infant formula ingredients that are associated with neural development, including docosahexaenoic acid (DHA) and arachidonic acid (ARA) (Burns *et al.*, 1999; Hempenius *et al.*, 2000; Lina *et al.*, 2006; Casterton *et al.*, 2009; Fedorova-Dahms *et al.*, 2011), and has gained acceptance by regulatory bodies in the U.S. and in the European Union (EU). The study was conducted in compliance with the Organisation for Economic Co-operation and Development (OECD) Principles of Good Laboratory Practices (GLP), and the 13-week dietary toxicity phase of the overall study was conducted according to OECD Testing Guideline No. 408 (OECD, 1998), the U.S. FDA Redbook Guideline for Subchronic Toxicity Studies with Rodents (U.S. FDA, 2003), and the International Conference on Harmonisation of Technical Requirements of Pharmaceuticals for Human Use (ICH) Guideline M3(R2) (ICH, 2009).

The results of the study demonstrated no compound-related maternal toxicity or adverse effects on female reproduction or on the general growth and development of offspring at maternal dietary concentrations of up to 2%, providing doses of up to 1,895 mg/kg body weight/day, in CD® [Crl:CD®(SD)] rats. In addition, no compound-related adverse effects were observed during the 13-week administration period in F_1 animals at doses of up to 974 mg/kg body weight/day in males and 1,246 mg/kg body weight/day in females. The no-observed-adverse-effect level (NOAEL) for the oral toxicity of NANA was determined to be to 974 mg/kg body weight/day in male CD® [Crl:CD®(SD)] rats and 1,246 mg/kg body weight/day in female CD® [Crl:CD®(SD)] rats, the highest doses tested. The overall NOAEL for the oral toxicity of NANA in rats was determined to be 974 mg/kg body weight/day. Based on outcomes from this study, the Expert Panel concluded that the oral toxicity of NANA is low and without adverse effects on the safety endpoints assessed at the doses administered.

Three other animal studies involving oral administration of NANA identified in the scientific literature were reviewed by the Expert Panel. All three studies were of short-term duration (Sakai *et al.*, 2006; Wang *et al.*, 2007; Hiratsuka *et al.*, 2013) and were not considered sufficiently appropriate to assess for toxicity of NANA. While the results of these studies demonstrate that there were no adverse effects on endpoints related to general toxicity following oral exposure to NANA, the studies are limited for the purpose of a safety assessment and are

not considered pivotal to the safety assessment of NANA (as they were of short-term duration and not sufficiently appropriate in design).

The toxicological data on Glycom's NANA also included a bacterial reverse mutation assay using *Salmonella typhimurium* test strains TA98, TA100, TA1535, and TA1537, and *Escherichia coli* WP2 *uvr*A in the absence and presence of metabolic activation (S9), using the plate-incorporation and pre-incubation methods (Choi *et al*., 2014). The test was conducted at NANA concentrations of up to 5,000 µg/plate. An *in vitro* mammalian cell micronucleus test conducted in human peripheral blood lymphocytes under short-term (4 hours with and without S9) and continuous (24 hours without S9) treatment conditions using the cytokinesis-block method also has been performed (Choi *et al*., 2014). This test was conducted at NANA concentrations of up to 3,450 µg/mL. Both tests were conducted in compliance with the OECD principles of GLP and according to OECD Testing Guideline No. 471 and 487, respectively (OECD, 1997, 2010). All test results reported indicate that NANA is non-mutagenic/non-genotoxic.

The Expert Panel noted that the safety of Glycom's NANA for use in term infant formulas and in foods is supported by the substance being chemically identical to NANA present in human milk and by the safe intake of NANA from human milk by infants at levels greater than those estimated from its addition to infant formula and to food, as well as by the available scientific literature.

CONCLUSION

We, the members of the Expert Panel, have independently and collectively critically evaluated the information summarized above and conclude that the intended use as an ingredient in term infant formula and in food of Glycom A/S's *N*-acetyl-D-neuraminic acid dihydrate (NANA) ingredient, meeting appropriate food-grade specifications and manufactured consistent with current Good Manufacturing Practice, is safe and suitable.

We, the members of the Expert Panel, have independently and collectively critically evaluated the information summarized above and conclude that the intended use as an ingredient in term infant formula and in food of Glycom A/S's *N*-acetyl-D-neuraminic acid dihydrate (NANA), meeting appropriate food-grade specifications and manufactured consistent with current Good Manufacturing Practice, is Generally Recognized as Safe (GRAS), based on scientific procedures.

It is our opinion that other qualified experts would concur with these conclusions.

(b) (6)

22 April 2015

Professor Joseph F. Borzelleca, Ph.D.
Virginia Commonwealth University School of Medicine

Date

(b) (6)

30 April 2015

Ronald E. Kleinman, M.D.
Mass General Hospital for Children

Date

(b) (6)

04 May 2015

Professor Robert J. Nicolosi, Ph.D.
University of Massachusetts Lowell

Date

(b) (6)

28 April 2015

Professor John A. Thomas, Ph.D.
Indiana University School of Medicine

Date

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