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Original Article

Maltosyltrehalose Syrup: Bacterial reverse mutation test, and 90-day feeding and 90-day repeated oral dose toxicity studies in rats

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ABSTRACT — Maltosyltrehalose syrup (TG4 syrup) is an enzymatically derived starch hydrolysate consisting of only glucose. The main components are maltotetraose (G4) and maltosyltrehalose (TG4). G4 is a common component of essentially all starch hydrolysates, and consists of only four glucose molecules with α-1,4 linkages. TG4 also consists of four glucose units, but the terminal glycosidic bond at the reducing end of the glucose chain is inverted, which results in the final bond being α-1,1. The two terminal glucose units form a trehalose disaccharide molecule, attached to a maltose molecule. The presence of the trehalose moiety in the TG4 molecule results in unique functional and technical properties from what is found in other α-1,4 linked oligosaccharides. This paper presents results of standardized toxicity studies of TG4 syrup, including an *in vitro* bacterial mutagenicity test, a 90-day oral feeding study in rats, and a 90-day oral toxicity (gavage) study in rats. Treatment with TG4 syrup resulted in no microbial mutagenic activity or growth inhibition in either *Salmonella typhimurium* or *Escherichia coli*, even at the maximum dose of 5,000 μg/plate. The NOAEL in the 90-day oral feeding study was calculated as 10% of TG4 syrup in the diet, which was equal to 6,818 and 7,464 mg/kg/day (dwb) in male and female rats, respectively. The 90-day oral gavage toxicity study had a NOAEL of 5,000 mg/kg/day (dwb) in male and female rats. Taken together these data showed that TG4 syrup was safe for use in these studies, suggesting it is safe for consumption by humans.

Key words: Maltosyltrehalose Syrup (TG4 Syrup), Maltosyltrehalose (TG4), Maltotetraose (G4), Mutagenicity, No Observed Adverse Effect Level (NOAEL)

INTRODUCTION

Trehalose is a multi-functional disaccharide that can be used for increasing various quality measures of food products. Based on the results of various safety studies, trehalose has been evaluated to be safe for food use by essentially all national and international food organizations such as JECFA, US FDA GRAS, EU Novel Food, Health Canadian Novel Food, FSANZ Novel Food, etc. (Higashiyama and Richards, 2012). It is used in a wide variety of food products in many countries throughout the world due to its low sweetness, and/or functionalities such as a cryoprotectant for freeze-dried foods, suppression of moisture absorption, suppression of starch retrogradation, masking of unpleasant odor/taste, good crystallinity, and high glass transition temperature (Higashiyama and Richards, 2012). However, the crystallinity properties of trehalose can be an issue during storage in foods

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containing high concentrations of trehalose. To solve this problem, Hayashibara Co., Ltd. developed maltosyltrehalose syrup (TG4 syrup), which is composed of glycosyltrehaloses, maltooligosaccharides and glucose (Maruta *et al*., 1998).

TG4 syrup is manufactured from the hydrolysis and transglycosylation of starch by enzymatic reactions. This process is similar to that of conventional starch syrups such as glucose syrup, corn syrup, starch hydrolysate, etc., except for the additional use of the enzyme, (1->4)-α-D-glucan 1-α-D-glucosylmutase (maltooliogosyltrehalose synthase [MTSase]; EC 5.4.99.15). This enzyme results in a higher concentration of transglycosylation in TG4 syrup. Since the mid-1990s MTSase has primarily been used for the production of trehalose (Maruta *et al*., 1995). The main components of TG4 syrup are maltotetraose (G4; CAS RN® 34612-38-9; approximately 15% dry weight basis [dwb]) and maltosyltrehalose (TG4; CAS RN® 25545-20-4; ≥ 50.0% dwb), for which the product is named. G4 is a simple tetraglucose molecule with the glucose molecules being bound by α-1,4 linkages. G4 is common in glucose syrup, corn syrup, maltodextrin, and dextrin, which are listed as GRAS substances in the US Code of Federal Regulations (US FDA, 21CFR §168.120; US FDA, §184.1865; US FDA, §184.1444; US FDA, §184.1277; US FDA, 2019a, 2019b, 2019c, 2019d, respectively). All these starch-based products have long histories of use as existing food ingredients and/or additives in thousands of food products in the US and worldwide. It is well established that α-1,4-glycosidic linkages of glucose molecules are quickly and completely digested by α-amylase (EC 3.2.1.1) and α-glucosidase (EC 3.2.1.20) to individual glucose units in the small intestine, and the glucose is completely absorbed into the body (Fig. 1; Dahlqvist and Borgstrom, 1961; Ravich and Bayless, 1983; Whistler and BeMiller, 1997; Dhital *et al*., 2013).

TG4 consists of 4 glucose units in which the terminal glycosidic bond at the terminal reducing end of the glucose chain has been inverted by MTSase, which results in the final bond between the 3rd and 4th glucose molecules being an α-1,1 linkage instead of the standard α-1,4. The structure of the 2 terminal glucoses is the same as that of trehalose. Due to the intramolecular trehalose structure of TG4, TG4 syrup effectively suppresses trehalose crystallization in food to a greater extent than conventional starch syrup (Takemori, 2012). Additionally TG4 syrup has unique functionalities because of the combination of the trehalose and conventional starch syrup moieties. These include lower dextrose equivalent, reduced Maillard reaction, higher glass transition temperature, and masking unpleasant tastes and odors. These functionalities can be beneficial in many foods (Takemori, 2012). Conversely, it may not be useful or could even be disadvantageous in other food systems, meaning that TG4 syrup is not applicable for replacement of all uses of starch-based hydrolytic ingredients. The α-1,4-glycosidic linkages of glycosyltrehaloses are completely hydrolyzed by α-amylase and α-glucosidase to glucose and trehalose in the small intestine (Fig. 2). The trehalose moiety is further enzymatically hydrolyzed in the small intestine into 2 glucose molecules by a trehalose-specific disaccharidase, trehala-

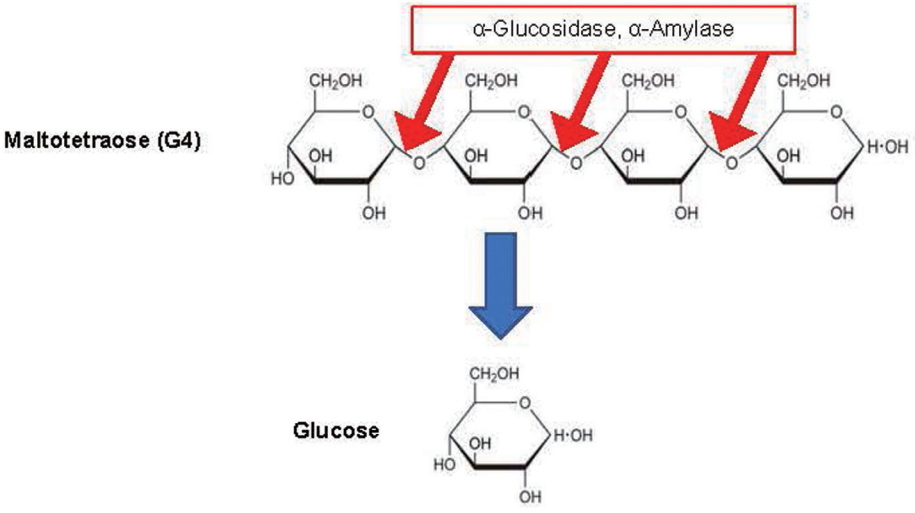

Fig. 1. Diagram of Digestion of G4 in TG4 Syrup.

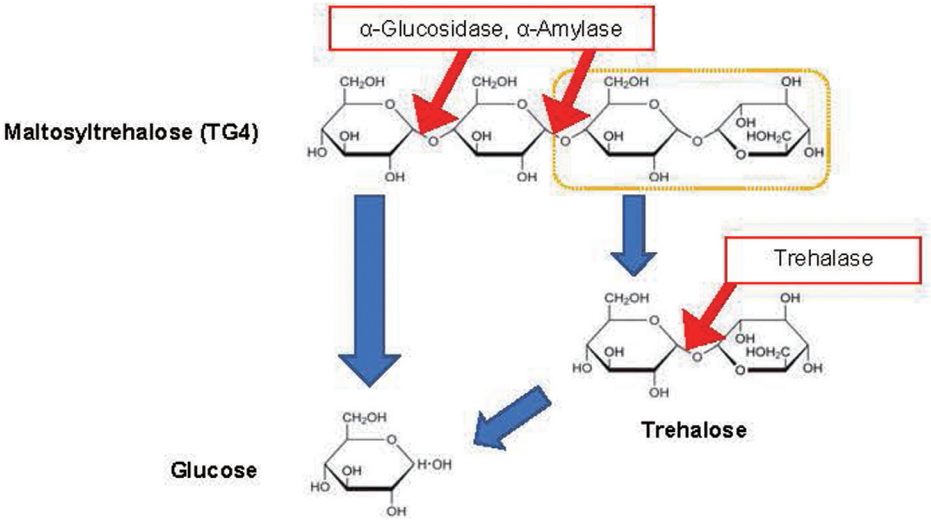

Fig. 2. Diagram of Digestion of TG4 in TG4 Syrup.

se (EC 3.2.1.28). The glucose is subsequently absorbed and metabolized (Fig. 2; Whistler and BeMiller, 1997; Richards *et al.*, 2002; Takemori, 2012).

It is believed that the chemical composition consisting of only glucose, and α-1,4 and α-1,1 structural linkages of TG4 syrup strongly argues its safety for human consumption (Irving *et al*., 1975; Richards *et al.*, 2002). However, to further insure the safety of TG4 syrup, *in vitro* and animal studies have been undertaken to directly address the question of safe ingestion of TG4 syrup as an ingredient in the human diet. It is believed that the available scientific evidence substantiates the conclusion that TG4 syrup is safe to use as an ingredient in food.

MATERIALS AND METHODS

Test substance

All test material (TG4 syrup) used for the studies were prepared from starch by enzymatic reactions by Hayashibara Co., Ltd. (Okayama, Japan), using α-amylases (EC 3.2.1.1), isoamylase (EC 3.2.1.68), glucan 1,4-α-maltotetraohydrolase (EC 3.2.1.60) and MTSase (EC 5.4.99.15) (Maruta *et al*., 1998). All the enzymes used are listed in Japan's List of Existing Food Additives, and monographed in Japan's Specifications and Standards for Food Additives in Japan (MOH, 1996; MHLW, 2018, respectively).

The test material used for the *in vitro* mutagenicity test was from TG4 syrup lot 020611 (54.8% TG4 dwb). The test substance for the 90-day oral feeding study in rats consisted of TG4 syrup (lot 020907), which contained 52.0% TG4 (dwb). The 90-day gavage toxicity study in rats was performed using commercial lot 6C25481. This lot had a TG4 content of 53.9% (dwb). G4 comprises approximately 15% (dwb) of TG4 Syrup. It also contains approximately 4.5% glucose, maltose (7.1%) and maltotriose (9.8%), for a total of about 36.3% of molecules with exclusively α-1,4 linkages, except glucose. In addition to TG4, TG4 Syrup also contains glucosyltrehalose (TG3) molecules at about 3.5%, making a total of about 56% with terminal α-1,1 bonds. Finally there are approximately 7.7% of other saccharides that are in TG4 Syrup. These include larger molecules, like those that are produced by most all other starch syrup and maltodextrin production processes (Hobbs, 2009). Together these three types of saccharides constitute 99.9% of the dry weight of the TG4 Syrup, and only include glucose molecules. Test substances were kept at room temperature in closed containers out of direct sunlight.

Current specifications for TG4 syrup manufactured by Hayashibara Co., Ltd. include: TG4 not less than 50.0% (dwb); glucose not more than 6.0% (dwb); solid content not less than 72.0%; ash not more than 0.05%; pH 3.5 to 6.5; color of solution not more than 0.100; turbidity of solution not more than 0.050; total aerobic microbial count not more than 300 CFU/g; coliform organisms negative in 0.1 g.

Bacterial reverse mutation test of TG4 syrup

TG4 syrup was examined for mutagenicity in a

standardized bacterial assay (Guidelines under the Japanese Industrial Safety and Health Law; GLP under the Japanese Industrial Safety and Health Law) using *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537, and the *Escherichia coli* strain WP2 *uvrA*$^-$. The experiments were conducted in the presence and absence of S-9 mix. The negative control and dilution medium used was sterilized distilled water, and the positive controls were 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2), sodium azide (NaN_3), 9-aminoacridine hydrochloride (9-AA), *N*-ethyl-*N*'-nitro-*N*-nitrosoguanidine (ENNG) and 2-aminoanthracene (2-AA). All positive control substances except NaN_3 (water), were diluted in DMSO.

In the first experiment (dose-finding test), 6 doses ranging from 5 to 5,000 µg/plate were graded at a common ratio of 4. The dose-finding test also included examination of microbial growth inhibition and precipitation of the TG4. In the second experiment (main test), 5 doses ranging from 313 to 5,000 µg/plate were graded at the common ratio of 2 based on the results of the dose-finding test. The final concentrations were formulated by dilution.

The reverse mutation test (main test) was done by the pre-incubation method. For one concentration, 0.1 mL of the test substance solution, 0.1 mL of each bacterial suspension and 0.5 mL of either 0.1 mol/L sodium phosphate buffer (pH 7.4) for the assay without metabolic activation, or S-9 mix for the assay with metabolic activation were mixed in a sterilized test tube and incubated with gentle shaking at 37°C for 20 min. After pre-incubation, 2 mL of molten top agar was added to this mixture and poured onto a minimal glucose agar plate. When the agar overlay solidified, these plates were incubated at 37°C for 48 hours. The presence of the background lawn was observed using a stereoscopic microscope and microbial growth was confirmed. Revertant colonies were counted manually or with an automatic colony counter. Two plates per dose were used for each assay, and the test was performed twice.

The test substance was judged to be mutagenic when the mean number of revertant colonies was more than double that of the negative control value, and when it showed a significant dose-dependent increase in a reproducible manner. No statistical methods were used for data analysis.

90-Day oral feeding study of TG4 syrup in rats

A 90-day oral feeding study of TG4 syrup in 7-week old Wistar albino rats was based on the Organization for Economic Cooperation and Development (OECD) Guidelines for Testing of Chemicals, Guideline #408, adopted September 21, 1998. The protocol was reviewed by the Institutional Animal Care and Use Committee of MB Research Laboratories, USA, and found to be in compliance with acceptable standards of animal welfare and humane care. Animals were equilibrated to the test conditions for 15 days before being randomly assigned to either the control or treatment group. The animals were housed individually in suspended stainless steel wire bottom cages. Fresh water was provided *ad libitum*, the room was kept at 20.0 to 25.6°C, 18 to 78% humidity, with a 12-hr light and dark cycle. The test diet was prepared fresh every two weeks, and samples were frozen and retained for analysis of homogeneity, stability and test article concentration. Feed consumption was measured weekly, and body weights were recorded immediately before treatment, weekly during treatment and at study termination. Calculation of the mean dose of the test article consumed (mg/kg/day) was performed weekly.

The study consisted of two groups of 10 male (241 to 290 g) and 10 female (167 to 211 g) rats each. Groups were given either TG4 syrup mixed in feed at a 10% concentration (dwb) for 91 consecutive days, or control feed with no test article.

All animals were observed once daily and observations were recorded for toxicity and pharmacological effects including evaluations of skin, fur, eyes, mucous membranes, respiratory and circulatory effects, autonomic effects, central nervous system effects, changes in motor activity, gait and posture, reaction to stimulus and any other abnormal signs. The absence of abnormal signs was also documented. All animals were observed twice daily for mortality. Immediately prior to study initiation and once per week during the study, a detailed clinical examination of each animal was made. Observations included changes in skin, fur, eyes, mucous membranes, occurrence of secretions or excretions and autonomic activity. Additionally, changes in the level of activity, gait, posture, strength and response to handling were evaluated, and the presence of clonic or tonic movements and stereotypical and abnormal behaviors were noted. Prior to the start of the study and one day prior to study termination, the eyes of all animals were examined. During the last 2 weeks of the study, a Functional Observational Battery (FOB) was conducted on each animal. This neurobehavioral examination was intended as a rapid, gross evaluation of functions sub-served by the central and peripheral nervous systems. Four (4) main categories were included with a number of separate evaluations for each category. The 4 main categories were: Sensory Functions (orientation/sensory responsiveness); Muscle Functioning

(posture); Centrally-Controlled Motor functions (locomotion/patterned movement), and Sensory-Motor Functions (integrated movement).

On Day 91, after animals were fasted overnight, whole blood was collected under anesthesia, and slides for differential hematology were prepared. Hematology included hematocrit (HCT), hemoglobin concentration (HGB), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), platelet count (PLT), prothrombin time (PT), red blood cell count (RBC), and white blood cell count (WBC), including a differential count. Collected blood was processed for serum and plasma to test clinical chemistry. The analyses included alanine aminotransferase (ALT), albumin (Alb), alkaline phosphatase (ALP), aspartate aminotransferase (AST), blood urea nitrogen (BUN), calcium, chloride, creatinine, γ-glutamyl transpeptidase (γ-GTP), globulin, glucose (fasting), magnesium, phosphorous, potassium, sodium, sorbitol dehydrogenase, total bilirubin, total cholesterol, total protein, and triglyceride analyses. On Day 92, all animals were sacrificed and underwent a gross necropsy, which included examination of the external surfaces of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents. The heart, liver, spleen, kidneys, adrenals, testes, ovaries, epididymides, brain, thymus, and uterus were trimmed and weighed wet as soon as possible after dissection.

The following tissues and organs were preserved in 10% neutral buffered formalin and histologically examined. These are listed by body system: salivary glands, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, liver, pancreas; brain (multiple sections), sciatic nerve, spinal cord (3 levels), pituitary; adrenals, thyroid, parathyroids; trachea, lungs; aorta (thoracic), heart, bone marrow (sternal), spleen, thymus, lymph nodes (cervical, mesenteric); kidneys, urinary bladder, prostate, testes, bulbourethral gland, epididymides, seminal vesicles, uterus, ovaries, mammary gland; all gross lesions and masses, skin and ear tag area.

All non-discrete data from clinical chemistry, hematology, organ weights, body weights, FOB (including total score and the scores of the four main categories), organ/body weight ratios and food consumption were tabulated with means and standard deviations, and an Analysis of Variance (ANOVA) was performed to identify statistically different groups. Parametric data were analyzed using ANOVA techniques with the Tukey-Kramer post hoc test. Non-parametric data were analyzed using Kruskal-Wallis analysis of variance with Dunn's post hoc test.

90-Day repeated oral dose (gavage) toxicity study of TG4 syrup in rats

A 90-day gavage toxicity study was performed on rats using escalating doses of TG4 syrup to evaluate the repeated oral dose toxicity of TG4 syrup. The study was conducted according to OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM(98)17, and the test protocol complied with OECD Guidelines for Testing of Chemicals, Guideline #408, adopted September 21, 1998. Further the study was in compliance of the appropriate Act on the Welfare and Management of Animals (Act No. 105, October 1973, and Act No. 46, May 2014), and Ministry of the Environment Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain (Notifications No. 88, April 2006, and No. 84, August 2013). The study was reviewed and approved by the Institutional Animal Care and Use Committee of the Public Interest Incorporated Foundation Biosafety Research Center, Japan.

Five-week old Crl:CD(SD) [SPF] rats were quarantined and acclimated for 7 days. The condition of the animals was observed daily, body weights were measured twice a day, and ophthalmological examinations were performed. There were 3 animals with congenital eye defects, which excluded them from randomization. After quarantine/acclimation, 40 male (164 to 195 g) and 40 female (163 to 182 g) rats were stratified based on body weight, and randomly assigned on the day that treatment was initiated (Day 1) to one of 4 groups. Each dose group was comprised of 10 males and 10 females. The weight variation of the assigned animals was within ± 20% of the mean weight of the males/females. The animals were individually housed in wire-mesh suspended cages. The conditions of the room in which they were kept included a temperature of 22.9 to 23.0°C, 46 to 63% humidity, air exchange of not less than 12 times per hour, and the light/dark cycle was 12 hr. The rats had *ad libitum* access to a standard sterilized pellet diet, except when fasted. *Ad libitum* access to tap water was by automatic water dispensing units, or water bottles when urine was collected.

TG4 syrup doses (0, 1,000, 3,000 or 5,000 mg/kg/day on dwb) were administered daily by gavage for 90 consecutive days (Day 1 [Initiation of dosing] to Day 90), and necropsy was performed on Day 91. The treatment or control dose was calculated for each animal from the last body weight measurement, which was taken every 7 days. The control and dilution media were water for injection. The dosing volume was 1.0 mL/100 g. The dosing solutions were tested for stability, contamination, and consistency to their nominal concentrations (100.0 ± 10.0%). All preparation batches met stability, contamination and nom-

inal concentration requirements. Examples of concentration consistency of the three doses from the first and final preparations were 99.1, 98.9 and 97.5%, and 99.9, 98.9 and 98.0%, respectively. Treatment and control doses were aliquoted for daily use.

The general condition of the animals was observed twice a day (before and after dosing) during the administration period. On the day of the scheduled necropsy, observation was performed once before the animals were sacrificed. A FOB was conducted weekly to examine the behavioral, physiological and neuronal functions in detail. All animals were subjected to detailed observations once during the quarantine period, and once a week thereafter. The tests for sensorimotor function, grip strength and locomotor activity were additionally conducted during the 13th week of administration. The animals were weighed before the start of dosing, once a week on Days 1 (before grouping) to 85 and on Day 90. In addition, body weight gain from Days 1 to 90 was calculated. The animals to be necropsied were weighed on the necropsy day (Day 91). The amounts of food supplied were weighed on the days of body weight measurement, and the remaining food was weighed on the days of the next body weight measurement for all animals. From the differences of amounts of the food, the mean daily food consumption (g/day) was calculated for individual animals. Hematological and blood chemical samples were collected on Days 91 and 92. Hematological variables assayed were activated partial thromboplastin time, fibrinogen, HCT, HGB, MCH, MCHC, MCV, PLT, PT, RBC, reticulocyte count, reticulocyte ratio, and WBC, including a differential count. Blood chemistries included ALT, Alb concentration, Alb ratio, albumin/globulin ratio, ALP, α_1-globulin concentration, α_1-globulin ratio, α_2-globulin concentration, α_2-globulin ratio, AST, β-globulin concentration, β-globulin ratio, BUN, calcium, chloride, creatinine, γ-globulin concentration, γ-globulin ratio, γ-GTP, glucose, inorganic phosphorus, potassium, sodium, total bilirubin, total protein, total cholesterol, and triglyceride. On Days 85 and 86, fresh urine (within 3 hr) after urination were evaluated for pH, occult blood, ketone bodies, glucose, protein, bilirubin, and urobilinogen. Pooled urine (24 hr) was collected for urinary volume, color, osmotic pressure, sediment, sodium concentration, potassium concentration, chloride concentration, total sodium excretion, total potassium excretion, and total chloride excretion. The residue from the supernatant was strained and examined for erythrocytes, casts, leukocytes, fat globules, squamous cells, mucous threads, transitional epithelial cells, crystals, and renal tubular epithelial cells. Ophthalmological examinations were conducted during the quarantine period (Days -3 and -2) and Day 86. The pathological examinations consisted of organ weight measurement (heart, liver, spleen, kidneys, adrenal glands, prostate (including urethra), testes, ovaries, epididymides, brain, pituitary gland, thymus, uterus, thyroid (including parathyroid), seminal vesicle (including coagulating glands), lungs (including bronchi), salivary glands (sublingual glands and mandibular glands), and macroscopic examination (necropsy) of the organs. Histopathological examinations, by body system were: salivary glands (sublingual and mandibular), tongue, esophagus, stomach, duodenum, jejunum, ileum (including Peyer's patch), cecum, colon, rectum, liver, pancreas; brain, sciatic nerve, spinal cord (cervical, thoracic and lumbar) pituitary; adrenals, thyroid, parathyroid; trachea, lungs (including bronchi); aorta, heart, bone marrow (sternum and femur), spleen, thymus, lymph nodes (mesenteric and cervical); kidneys, urinary bladder, prostate (including urethra), testes, epididymides, seminal vesicles (including coagulating glands), uterus, vagina, ovaries, oviducts, mammary glands; eyes (including optic nerve), harderian glands, Zymbal's glands; sternum, femur, skeletal muscle (femoral region), and skin. The data were analyzed by Dunnett's multiple comparison test to assess the statistical significance of differences between the control group and each test substance-treated group (Yoshida, 1988). When the Bartlett's test showed heteroscedasticity, the data were analyzed by the Steel's test to assess the statistical significance of difference between the control group and each test substance-treated group (Steel, 1959). Counting FOB data (number of defecation/urination) were analyzed by Steel's test.

RESULTS

Bacterial reverse mutation test of TG4 syrup

In both the dose-finding and in the main reverse mutation tests the number of revertant colonies induced by TG4 syrup at all doses, even the highest dose (5,000 μg/plate), was less than twice that in the corresponding control value for all test strains, both in the presence and absence of the S-9 mix. Table 1 shows the number of revertants at the 5,000 μg/plate concentration of both the dose-finding and the main tests which demonstrates the consistency of the negative results. No microbial growth inhibition or test substance precipitation was observed on the agar plates. The negative and positive controls were within the normal range of the laboratory values and confirm the tests were performed correctly.

90-Day oral feeding study of TG4 syrup in rats

All animals survived the 90-day feeding study. Daily

and weekly detailed observations showed 1 male in the control group developed alopecia on both front limbs at Day 41, which continued until the end of the study. A second male from the control group had chromodacryorrhea on Day 82 of the study. Eight (8) males and all females in the 10% TG4 syrup group appeared normal throughout the observation period. One animal had soiling of the anogenital area on Day 54, and a second had localized alopecia (forelimbs) on Days 69 through 92. The alopecia in both the control and treatment animals was attributed to the design of the feeders rather than to any effect of the test article. Additionally, one animal in the TG4 syrup group developed aggressive behavior at one observation time. This abnormal clinical observation was sporadic, not seen in both sexes, not statistically significant and judged not treatment related.

Instances of individual animal weight loss were noted in some animals of both groups, particularly females. A statistically significant increase ($p \leq 0.05$) in female mean body weights was noted between the control (266 g) and test article (280 g) groups on Day 36. There were no other significant differences in mean body weights. Instances of statistically significant differences ($p \leq 0.05$) in mean food consumption were noted between the control and test article group at various times during the study (males; weeks 3 & 5: females; weeks 2, 3, 5 & 9). In all instances the mean food consumption of the animals receiving the test article were significantly greater ($p \leq 0.05$) than the controls. The combined calculated mean daily doses of male and female groups ranged from 5,705 to 9,938 mg/kg/day and averaged 7,141 ± 1,165 mg/kg/day (6,818 and 7,464 mg/kg/day in male and female rats, respectively) over the course of the study.

There were no statistically significant differences ($p \leq 0.05$) in FOB between mean individual variables or total scores of control and TG4 syrup groups. Seventeen (17) of 20 animals in the control group appeared normal at necropsy. Smaller than normal adrenals were noted in one animal; a darker than normal thymus in a second animal; and alopecia of the forelimb in third animal. The alopecia was related to the feeders. Thirteen (13) of 20 animals in TG4 Syrup group appeared normal at necropsy. Herniated livers were noted in 3 animals. Smaller than normal adrenals were noted in two animals, one of which also exhibited nodules on the left kidney. The thymus was mottled and darker than normal in one animal, and localized alopecia of the forelimbs was noted in another animal. The observations at necropsy were sporadic, not seen in both sexes and judged as not treatment associated.

There were no statistically significant differences

Table 1. Revertant colonies in the 5,000 µg/plate samples with and without the S-9 mix from the dose-finding and main tests.

Without (-) or with (+) S-9 mix	Concentration of TG4 syrup (µg/plate)	Dose-finding study, mean number of revertant colonies per plate				
		Base-pair changes			Frameshift changes	
		TA 100	TA 1535	WP2 *uvrA*$^-$	TA 98	TA 1537
(-) S-9 mix	0 (control)	94	6	22	16	8
	5,000	79	7	20	14	8
(+) S-9 mix	0 (control)	100	6	17	21	7
	5,000	115	8	12	17	6
Positive control (-) S-9 mix (name; concentration µg/plate)		475 (AF-2*; 0.01)	420 (NaN_3*; 0.5)	712 (ENNG*; 2)	537 (AF-2*; 0.1)	241 (9-AA*; 80)
Positive control (+) S-9 mix (name; concentration µg/plate)		699 (2-AA*; 1)	294 (2-AA*; 2)	764 (2-AA*; 10)	435 (2-AA*; 0.5)	135 (2-AA*; 2)

Without (-) or with (+) S-9 mix	Concentration of TG4 syrup (µg/plate)	Main test study, mean number of revertant colonies per plate				
		Base-pair changes			Frameshift changes	
		TA 100	TA 1535	WP2 *uvrA*$^-$	TA 98	TA 1537
(-) S-9 mix	0 (control)	96	8	22	20	7
	5,000	80	7	17	21	8
(+) S-9 mix	0 (control)	91	10	20	26	8
	5,000	99	4	20	23	6
Positive control (-) S-9 mix (name; concentration µg/plate)		389 (AF-2*; 0.01)	343 (NaN_3*; 0.5)	627 (ENNG*; 2)	530 (AF-2*; 0.1)	146 (9-AA*; 80)
Positive control (+) S-9 mix (name; concentration µg/plate)		553 (2-AA*; 1)	271 (2-AA*; 2)	695 (2-AA*; 10)	352 (2-AA*; 0.5)	163 (2-AA*; 2)

* AF-2, 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide; NaN_3, Sodium azide; ENNG, *N*-ethyl-*N*'-nitro-*N*-nitrosoguanidine; 9-AA, 9-aminoacridine hydrochloride; 2-AA, 2-aminoanthracene.

($p \leq 0.05$) in any mean hematological variables between control and TG4 Syrup groups. In males, the mean sodium concentration (146.40 meq/L) of the test animals was significantly less ($p \leq 0.05$) than the control (147.50 meq/L). There were no other significant differences in mean clinical chemistries between male or female controls and the animals receiving the test article. The differences are considered to be biologically insignificant given the lack of corroborating histological and related chemical data.

There were no significant ($p \leq 0.05$) differences in mean organ weights or mean organ/body weight ratios between controls or animals receiving the test article. Ophthalmological examination revealed no evidence of compound-related ocular disease in any animal. Histopathological evaluation of specified tissues from the control and test article animals revealed no treatment-related microscopic changes. The microscopic changes observed were typical of those that occur spontaneously in Wistar albino laboratory rats of this age and type. The incidence and degree of severity were not influenced by administration of the test article.

90-Day repeated oral dose (gavage) toxicity study of TG4 syrup in rats

There were no deaths or treatment-related clinical signs in any of the dose groups in either sex. Trauma to the neck of one male rat from Days 15 to 21 in the 1,000 mg/kg/day group, and from Days 57 to 81 in one female in the 3,000 mg/kg/day group were observed. However, because there were no abnormal findings in other animals it was not considered to be attributable to the test substance treatment, rather spontaneous occurrences. There were no noteworthy abnormalities on each experimental day in regard to response to removal from cage, condition on hand-held observation, behavior in the open-field or sensorimotor reactivity. Furthermore, there were no significant differences in any of the quantitative data between the control group and each of the test substance-treated groups.

No significant differences were observed in the body weights of the animals of either sex between the control group and each of the test substance-treated groups during the administration period. Further, there were no significant differences between the control and test substance-treated groups in the overall body weight gains from Days 1 to 90. In males the mean daily food consumption from Days 36 to 43 and from Days 50 to 90 of the 5,000 mg/kg/day group were significantly lower ($p \leq 0.05$; $p \leq 0.01$ Days 85 to 90) than those of the control group. In the females, the mean daily food consumption from Days 8 to 15 and from Days 22 to 29 of the 5,000 mg/kg/day group were also significantly lower ($p \leq 0.05$) than those in the control group.

Hematological comparison of the control values of male animals to the treated groups showed only a significantly greater MCV ($p \leq 0.05$) in the 5,000 mg/kg/day group. In the female groups, there were no significant differences in any parameters between the control group and test substance-treated groups. When blood chemistry was examined the AST and ALT levels in the females in the 3,000 mg/kg/day and the 5,000 mg/kg/day groups versus control were significantly less ($p \leq 0.05$; $p \leq 0.05$ and 0.01, respectively). However, these were very minor changes and the enzyme activity-decreasing changes were judged not to be toxicologically significant. In males, there were no significant differences in any variables between the control group and each test substance-treated group.

The number of animals with protein-positive (more than 30 mg/dL) urine at Day 85 was increased in the males of the 3,000 and 5,000 mg/kg/day groups, and in the females of the 5,000 mg/kg/day group. The number of males with ketone body-positive urine was increased slightly in the 3,000 mg/kg/day group. Moreover, the number of animals with urobilinogen-positive urine increased slightly in the males of the 3,000 mg/kg/day group, and in the females of 5,000 mg/kg/day group. However, these changes were considered to be incidental because there was no dose-relationship (Table 2).

No ophthalmological findings related to test substance-treatment were observed in the 5,000 mg/kg/day group in either sex. All the observed findings were judged to be spontaneous in consideration of their types, grades, and incidences. In males, the relative weight of the kidneys in the 5,000 mg/kg/day group was less than that in the control group. Moreover, the absolute and relative weight of the thymus in the 1,000 mg/kg/day group were greater than those in the control group. However, the greater thymus weight was considered to be incidental because there was no dose-relationship. In females, the absolute weight of the ovaries in the 1,000 and 3,000 mg/kg/day groups and the relative weight of the ovaries in the 3,000 mg/kg/day group were greater than those in the control group. These changes were also considered to be incidental because there was no dose-relationship. There were no gross necropsy or histopathological findings related to test substance-treatment in either sex. All gross lesions observed were sporadic, focal and are often observed in this strain of rats. The incidences of the noted histopathological changes in the 5,000 mg/kg/day group are presented in Table 3. All were comparable to those in the control group, and these changes are often observed in this

Table 2. Urinalysis variables where differences were observed with TG4 syrup treatment groups.

Sex	Dose (mg/kg/day)	Number of animals	Protein (mg/dL)				Ketone bodies				Urobilinogen (E.U./dL)[1]	
			-	+/-	30	100	-	+/-	1+	2+	0.1	1.0
Male	0	10	7	2	1	0	8	1	1	0	10	0
	1,000	10	5	4	1	0	9	1	0	0	10	0
	3,000	10	0	1	6	3	0	2	7	1	6	4
	5,000	10	0	6	3	1	4	3	3	0	10	0
Female	0	10	9	0	0	1	9	0	1	0	9	1
	1,000	10	8	1	1	0	9	1	0	0	9	1
	3,000	10	6	3	1	0	8	2	0	0	9	1
	5,000	10	1	5	4	0	6	4	0	0	6	4

[1]Ehrlich units/dL.

Table 3. Histopathological findings in rats consuming 5,000 mg/kg/day TG4 or control for 90 days.

Sex		Male animals		Female animals	
Dose (mg/kg/day)		0	5,000	0	5,000
No. of animals necropsied		10	10	10	10
Organ/tissue	Findings				
Heart	Cardiomyopathy	2	3	0	0
Thymus	Hemorrhage	1	1	0	0
Lungs	Macrophage aggregation	0	1	0	2
Glandular stomach	Cyst	0	0	1	2
Pancreas (exocrine)	Inflammatory change	0	1	0	0
Liver	Hepatocyte fatty change	4	4	3	4
	Microgranuloma	6	5	5	4
	Hepatodiaphragmatic nodule	0	0	1	0
Kidneys	Hyaline cast	1	1	0	0
	Cyst	1	0	0	0
	Mineralization	3	1	4	4
	Tubule regeneration	3	1	0	1
	Inflammatory change	2	0	0	1
Testes	Seminiferous tubule atrophy	1	1	na[1]	na[1]
Epidiymides	Decreased sperm	0	1	na[1]	na[1]
Prostate	Inflammatory change	7	5	na[1]	na[1]
Uterus	Lumen dilatation	na[1]	na[1]	1	0
Vagina	Cyst	na[1]	na[1]	1	0
Harderian glands	Inflammatory change	0	1	0	0

[1]Not applicable

strain. Therefore, the observed findings in the gross and histopathological examination of the animals were considered to be spontaneously occurring lesions.

DISCUSSION

Hayashibara Co., Ltd. developed maltosyltrehalose syrup (TG4 syrup), which is composed of glycosyltrehaloses, maltooligosaccharides and glucose, to suppress trehalose recrystallization in food with high concentrations of trehalose. In addition to this technical function, TG4 syrup can be used in a variety of foods as a multi-functional syrup due to similar unique functionalities to trehalose. The dextrose equivalent of TG4 syrup is approximately 19 (dwb) and equal to approximately 14 on a product basis (28% water). The viscosity, refractive index, osmotic pressure, freezing-point depression and water activity of TG4 syrup resemble conventional starch syrup with a similar molecular weight distribution (Takemori, 2012). The sweetness is approximately 27% of a 5% sucrose solution. Comparing TG4 syrup to conventional starch syrups, it has relatively lower hygroscopicity, a relative

higher glass transition temperature, and a lower Maillard reaction due to the intramolecular trehalose structure (no reducing end) (Takemori, 2012). With these unique functionalities, TG4 syrup has been used in various food products in Japan to improve the quality of a variety of foods.

The data presented herein demonstrates that TG4 syrup prepared by the described enzymatic method showed no ability to cause revertant mutations in the presence or absence of the S-9 mix in a standardized mutagenicity test, even at the highest concentration (5,000 μg/plate). The test substance also did not precipitate or result in inhibition of microbial growth. Based on the results, it was concluded that TG4 syrup is not mutagenic under the test conditions employed.

Oral administration of TG4 syrup for 91 days at a concentration of 10% (dwb) mixed in feed produced no mortality. Most animals appeared normal throughout the study. There were significant differences ($p \leq 0.05$) in body weights and food consumption between animals receiving the test article and controls. However, in both cases the animals receiving the test article either weighed more or consumed more than the control animals. The differences were transient and did not persist to the end of the study. There were no significant differences noted in FOB and hematological parameters. There was no evidence of TG4 syrup-related ocular disease noted in any animal. The one significantly different clinical chemistry, a mean sodium concentration in serum, was significantly less than the control, but was considered biologically insignificant given the lack of corroborating histological and related chemical data. Microscopic examination of animals receiving the 10% TG4 syrup in the diet, compared to the control group revealed no treatment-related changes. Based on these findings, the no observed adverse effect level (NOAEL) was 10% in the diet, which was equal to 6,818 mg/kg/day in male rats and 7,464 mg/kg/day in female rats. If this amount were consumed by a 60 kg human it would equal 409 g/day in a male and 448 g/day in a female for 91 days.

In the 90-day toxicity study where TG4 syrup was administered by gavage at a maximum dose of 5,000 mg/kg/day (dwb), there were no deaths of either sex. Furthermore, there were no adverse effects judged as related to the test substance treatment on clinical signs, FOB, body weight, body weight gain, blood chemistry, ophthalmology, necropsy or histopathological examination. The mean daily food consumption of males and females of the 5,000 mg/kg/day groups was significantly lower than that in the control group at a number of sampling times during the study. However, the toxicological significance of this change was judged to be low because there was no significant difference in body weight during the same periods compared with the control group. Hematological results showed that the MCV in the males of the 5,000 mg/kg/day group was significantly greater than that in the control group. Again, this change was judged not to be clinically significant because there were no toxicologically meaningful changes related to RBC, HCT or HGB. Urinalysis showed that the number of animals with protein-positive urine was increased in the males of the 3,000 and 5,000 mg/kg/day groups, and in the females of the 5,000 mg/kg/day group. The toxicological significance of this change was also judged to be minor because there were neither increases in BUN or creatinine levels. Additionally no treatment-related adverse effects on the kidneys were seen during histopathological examination. The relative kidney weight in the males of the 5,000 mg/kg/day group was significantly lower than that in the control group. This change was considered to be incidental because there were no findings related to administration of the test substance in the kidneys of the same group on macroscopic and histopathological examination. Under the conditions of this study, the NOAEL of TG4 syrup was concluded to be 5,000 mg/kg/day (the maximum dose) in both male and female rats, and would equal 300 g/day in a 60 kg human for 90 days.

Supporting the claim of safety for consumption by humans is the fact that TG4 syrup is allowed for use in food and already sold in Japan, Taiwan and Hong Kong. Hayashibara Co., Ltd. developed TG4 syrup in 1998 (Maruta *et al*., 1998), and it has been commercially marketed as a food ingredient in Japan since 2003. Hayashibara Co., Ltd. is not aware of any untoward effects that have been reported by individuals involved in the manufacture and handling of TG4 syrup, individuals using TG4 syrup as an ingredient in a final food product, or consumers that have ingested the various final food products that contain TG4 syrup.

As complementary safety data for TG4 syrup, additional safety studies were performed to investigate the safety of hydrogenated TG4 syrup (HTG4S). This product has also been dehydrated for use in some of the safety studies (HTG4S powder). The HTG4S used for these tests was made from TG4 syrup by the same hydrogenation process as that used for the production of many polyols (Kato and Moskowitz, 2001). HTG4S powder was made by dehydration of HTG4S. On a dry basis, HTG4S and HTG4S powder contain essentially the same number of trehalose end moieties (TG4, TG3, etc.) as TG4 syrup; however, all the molecules with reducing ends (G1, G2, G3, G4, etc.), without the trehalose moiety, are hydrogenated. The trade name of HTG4S is Tornare™ in Japan, Korea and Tai-

wan, and MG-60 in other countries. HTG4S has a CAS RN®s of 738602-93-2. In Australia it is categorized as "starch, hydrolyzed, hydrogenated" with a CAS RN® of 2088952-59-2. The two main classes of constituents, TG4 and hydrogenated starch hydrolysate (HSH) have CAS RN®s of 25545-20-4, and 68425-17-2, respectively.

The safety of HTG4S has been reviewed as a chemical substance by regulatory agencies and registered/listed for use as a cosmetic ingredient (Australian DOH, 2017; Chinese MEE, 2019). It is also allowed for use in many countries where cosmetic ingredients are not required to be approved for use. The safety data includes a bacterial reverse mutation test, chromosomal aberration tests in cultured mammalian cells, an acute oral toxicity study in rats, and a 28-day oral toxicity (gavage) study in rats. All studies were conducted under appropriate OECD and other national guidelines. In the bacterial reverse mutation test HTG4S powder (5.0% water; 54.2% TG4 dwb) was used in a pre-experiment, and two independent experiments at doses up to 5,000 μg/plate (2,570 μg/plate as TG4) with and without liver microsomal activation (S-9 mix). The bacterial strains consisted of *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, and TA 100, and *Escherichia coli* strain WP2 *uvr*A$^-$. The HTG4S did not induce gene mutations by base pair changes or frameshifts in any bacterial strain, with or without S-9 mix (unpublished data). HTG4S (27.5% water; 54.3% TG4 dwb) did not result in chromosomal aberrations in CHL/IU cells, with or without S-9 mix or treatment time at 5,000 μg/mL HTG4S (1,970 μg/mL as TG4) (unpublished data). In the acute oral toxicity study two sets of female rats were given HTG4S (27.5% water; 54.3% TG4 dwb) by oral gavage. All animals survived, no clinical signs were noted, body weights were within the normal range, and no macroscopic findings were observed. The LD_{50} of HTG4S after a single oral dose was greater than 2,759 mg/kg (1,086 mg/kg as TG4) (unpublished data). In the 28-day oral toxicity (gavage) study, 5 SPF-bred Wistar rats per sex per group were administered 50, 200 or 1,000 mg/kg of HTG4S powder (5.0% water; 54.2% TG4 dwb) dissolved in distilled water, or a control of distilled water for 28 days. All animals survived, and no clinical signs, functional observations, food consumption or weight gains, hematology, clinical chemistry or histopathology were noted as clinically relevant. The NOAEL of HTG4S powder was concluded to be 1,000 mg/kg/day (515 mg/kg as TG4) (unpublished data).

The preceding experiments demonstrate that the TG4 fraction of HTG4S and HTG4S powder, which is essentially the concentration found in TG4 syrup, as well as the hydrogenated oligoglucosides have no untoward safety effects when tested in standard model systems for food safety. These studies, taken together with the TG4 syrup studies and the multiple years of experience with human consumption in Japan, Taiwan and Hong Kong, demonstrate that TG4 syrup presents little, if any, risk of toxicity when consumed in the diet. Therefore, it is concluded that TG4 syrup can be considered generally recognized as safe when used as a food ingredient in accordance with Good Manufacturing Practices.

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Conflict of interest---- All authors, except A.B. Richards, are employees of Hayashibara Co., Ltd. Dr. Richards is an independent technical and regulatory advisor for Hayashibara Co., Ltd. The cost of the *in vitro* and animal studies reported in this publication were paid by Hayashibara Co., Ltd. to the commercial testing laboratories listed in the Acknowledgements.

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